

Bio Prospecting of Multipotent Bacterial Biosurfactant for the Remediation of Hydrocarbons

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Abstract : Oil pollution is an environmental problem of increasing importance. Hydrocarbon-degrading microorganisms, adapted to grow and thrive in oil-containing environments, have an important role in the biological treatment of this pollution. Bioremediation is an efficient tool practiced now a day for the removal of contaminants from the contaminated soil and water. In the present study, an attempt has been made to investigate the bioremediation of petroleum hydrocarbons using bio surfactants produced by Streptomycetes. Microbes such as bacteria and fungi were isolated from the effluent and oil contaminated soil. They were identified by phenotypic methods and we found out two potential bacteria namely *Pseudomonas fluorescens*, *Pseudomonas* sps, fungi *Aspergillus fumigatus*, and a Streptomycete *Rothia muscilagenosa*. Nature of biosurfactant produced by all the four chosen organisms were characterized and it was found to be glycolipopetides and Lipopetide biosurfactant. Efficiency of bioremediation process was superior by the biosurfactant produced from *Rothia muscilagenosa*, isolated from oil contaminated soil. Alkane monooxygenase (alkB) gene expression study was carried out and the gene was expressed in the selected Streptomycete, *Rothia muscilagenosa*. Based on the present study it can be concluded that the biosurfactant produced from *Rothia muscilagenosa* has the potential to remediate oil pollution.

Index Terms— Bioremediation, biosurfactants, Petroleum Hydrocarbons, soil, Alkane monooxygenase

INTRODUCTION

The process of bioremediation, defined as the use of microorganisms to decontaminate or eliminate contaminants remaining to their varied metabolic competences is a developing technique for the elimination and degradation of numerous environmental contaminants comprising the yields of petroleum industry (1). Apart from this, bioremediation technology is understood to be noninvasive and comparatively economical (2). Biodegradation by indigenous populations of microbes signifies one of the primary devices by which petroleum and other hydrocarbon contaminants can be detached from the environment (3) and is economical than other remediation tools (4).

The achievement of oil spill bioremediation is influenced by the ability to begin and sustain circumstances that errand improved oil biodegradation proportions in the polluted environment. Several technical review articles have discussed about many aspects that impact the rate of oil biodegradation (5-6). The most vital necessity is the occurrence of microbes with the suitable metabolic competences. If the suitable microbes are existing, then optimal rates of growth and hydrocarbon biodegradation can be sustained by confirming that suitable concentrations of substrates and other external factors are present and that the pH is must be neutral. This research has focused on the search for efficient indigenous oil degrading bacteria present in oil spilled soil and their ability to produce biosurfactants.

MATERIALS AND METHODS

Production of biosurfactant from the indigenous bacteria: (7)

Petroleum contaminated soil samples were collected from around Mambaakam, Chennai, petrol station like Indian oil, Bharat petrol station. Heterotrophic bacteria were isolated by pour plate method and morphologically distinct colonies were inoculated in 1% Glycerol containing Mineral salt broth and incubated at 28°C for 2 days under agitation at 120 rpm. Solvent extraction procedure was employed to extract biosurfactant. Solvents Chloroform:Methanol (2:1) was used and the cell free extract was transferred in separating funnel and the contents were agitated for 30 minutes and kept under static condition for overnight and the middle layer was collected for further characterization.

SCREENING WITH 2,6-DICHLOROPHENOL INDOPHENOL:

Bacterial cultures were transferred from Actinomycetes agar slants to Bushnell Haas broth and it was incubated for 24 h at 37 °C at 180 rpm. A mixture of 0.5% 2,6-dichlorophenol indophenol (2,6-DCPIP), 0.1% Tween 80 and 2% (v/v) Petrol was then introduced into the tubes. The experiment was monitored daily for colour change from blue to colourless. Colour change was observed after 7 days of incubation at 28 °C under agitation conditions and the liquid medium was filtered to separate the biomass. The filtrate was centrifuged at 8000 rpm for 15 min. The supernatant was then analysed at 609 nm using the ultra violet-visible (UV-VIS)

spectrophotometer. The percentage of biodegradation was subsequently estimated as follows:

% of degradation = $1 - \frac{\text{absorbance of treated sample}}{\text{Absorbance of control}} \times 100$

Absorbance of control $\times 100$

DETERMINATION OF DEGRADATION OF PETROL OF THE EFFECTS OF ROTHIA MUSCILANGENOSA BY FTIR:

RESULTS AND DISCUSSION:

Pollution by petroleum hydrocarbon is of major concern. At present various microorganisms were used for bioremediation of Petroleum hydrocarbons from soil. The aim of present work was to bioremediate the petroleum hydrocarbons present in the soil using biosurfactants produced by bacteria, streptomycetes & fungi to compare the efficiency of those organisms as well as to find out a better solution for this persisting issue.

ISOLATION OF BIOSURFACTANT PRODUCING BACTERIA:

The oil contaminated soil sample was collected from Indian oil, Bharat petrol station and was used for the isolation of microbes for the bioremediation of petroleum waste. Bacteria isolated were identified by phenotypic methods.

PRODUCTION OF BIOSURFACTANT:

There were four organisms chosen for further study namely, *Pseudomonas fluorescens*, *Pseudomonas* sps, *Aspergillus fumigatus* and *Rothia muscilagenosa* and used to produce Biosurfactant. Mineral Salt Medium was prepared and sterilized in autoclave 15 lbs for 20 mins. The culture was inoculated and kept in rotary shaker for 4-5 days at 37°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 was 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 contains 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. Figure number: 17-20 shows the intermittent layer of biosurfactants

Yield of the biosurfactant produced by four chosen strains were estimated. There was not much difference in quantity. In our study the production of biosurfactant was only 5.6 g/l because we provided glycerol as a carbon source.

Oil spreading technique:

Efficiency of the biosurfactant in terms of oil displacement was higher in *Pseudomonas fluorescens* than the other three candidates. All the strains were streaked on blood agar plates. All the four strains showed positive results for haemolytic activity and formation of a clear zone around the colonies. *Pseudomonas fluorescens* showed a β haemolysis where as other three was an alpha haemolytic.

Carbohydrate estimation:

The standard curve of carbohydrate and the amount of carbohydrate content in the biosurfactant is shown. Based on the result of the carbohydrate estimation we could able to confirm that the biosurfactants of *Pseudomonas* sp, has possessed little trace quantity of carbohydrate in its structure.

Presences of reducing sugars were estimated by DNS method and reducing sugars were present in the biosurfactant produced from *Pseudomonas fluorescens* and *Pseudomonas* sps. But it was absent in the biosurfactant produced from *Aspergillus fumigatus* and *Rothia muscilagenosa*. Amount of reducing sugars present in *Pseudomonas fluorescens* was found to be greater than the other strain.

Elizabeth Rani Juneius, C. & Jayasundari, (2016) reported that the biosurfactant Characterization of biosurfactant revealed that they are phospholipopeptides. FTIR was used to confirm the presence of peptides and GC-MS analysis for methanolic suspension of biosurfactant revealed the presence of hexadecanoic acid methyl ester and octadecanoic acid methyl ester. 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. In conclusion the biosurfactant produced by the bacteria *B. licheniformis* OSB 1 is a lipopeptide with an excellent bioremediating potentials which can also be used for commercial applications.

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. Four different types of samples are analysed, and results were noted.

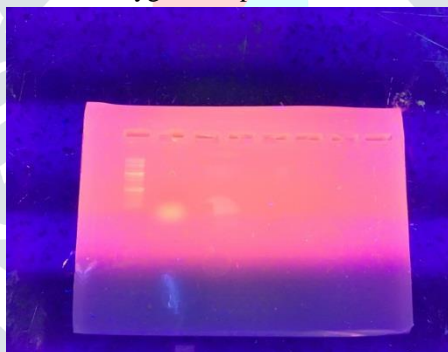
Based on the results derived from the spectrogram of FTIR and other estimations such as reducing sugars, protein, SDS – PAGE,

we could able to infer that the biosurfactant produced by *Pseudomonas fluorescens* and *Pseudomonas* sps were found to be glycolipopetides and the biosurfactants produced by *Rothia muscilagenosa* and *Aspergillus fumigatus* were found to be lipopeptides. Thavasi et al., 2011 reported that the biochemical composition of the biosurfactant is a mixture of lipid and protein with a combination of 49.8:50.2% respectively. FTIR analysis of the biosurfactant showed that, wave numbers 3,422 and 3,246 cm^{-1} for N–H bonds indicated the presence of amine groups. C–H bonds of the CH₃, CH₂ and CH groups observed at wave numbers 2962, 2923, 2863, 1481 and 1425 cm^{-1} confirmed the presence of alkanes. The wave number 1,650 cm^{-1} (amide I bond) indicated the presence of peptide groups. The wave number 1,066 cm^{-1} indicated the presence of C–O bonds. The above information from the respective wave numbers confirmed the lipopeptide nature of the biosurfactant. The mass spectrometric analysis of the biosurfactant also complements the biochemical and FTIR results that, the peaks observed at $m/z = 1076.2, 1347.3, 1348.4$ and $326.5, 413.3, 29.3$ indicated the presence of protein and lipid moieties.

Hydrocarbons in the soil ecosystems are biodegraded by bacteria, yeast, and fungi. The stated efficacy of biodegradation reached from 6% (11) to 82% (30) for soil fungi, 0.13% (29) to 50% for soil bacteria, and 0.003% to 100% (12) for marine bacteria. Several researchers have described that mixed consortium with overall comprehensive enzymatic capabilities are needed to destroy complex mixtures of hydrocarbons such as crude oil in soil (13), fresh aquatic system (14), and oceanic environments (15,16).

Among the other microbes, bacteria are detected as the most dynamic organisms in petroleum degradation, and they are the primary degraders of oil in environment (17,18). Numerous bacteria are identified to feed absolutely on hydrocarbons (19). As per the report of Floodgate there were 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi which were isolated from marine environment. *Acinetobacter* sp. was found to be accomplished of exploiting n-alkanes of chain length C₁₀–C₄₀ as a single source of carbon. Bacterial genus, such as, *Gordonia*, *Brevibacterium*, *Aeromicrobium*, *Dietzia*, *Burkholderia*, and *Mycobacterium* isolated from petroleum polluted soil substantiated to be the prospective organisms for hydrocarbon degradation(20). The degradation of poly-aromatic hydrocarbons by *Sphingomonas* was reported by Daugulis and McCracken (20). Our research has insighted that the actinomycete *Rothia muscilagenosa* could able to degrade petrol and use the hydrocarbon to produce bio-surfactant also.

PCR amplified product of ALKB gene of alkane monooxygenase expressed in *Rothia muscilagenosa*



Lane 1- 500 bp ladders

Lane 2- an unknown sample

Figure number: 31 Degradation of Petroleum waste by *Rothia muscilagenosa* – a) Day 1, b) Day30

Control showed characteristic bands at 3293,3030, 2917 to 22874 , 2281.87, 1650, 1460, 1376, 1231, 794 indicating presence of phenol or amines alkanes or carboxylic acids or esters or aromatic rings. Absence of characteristic peak in the region of 1610 to 2042 in extracted top layer sample indicates absence of ester linkage. This results gives the evidence of degradation petrol by the streptomycete *Rothia mucilagenosa*. The collective effects of biosurfactants and other physiological mechanisms of the bacteria could bring necessary changes in the structure of hydrocarbons and through which it can contribute in the bioremediation process.

CONCLUSION

Based on the above study it can be concluded that the Petroleum hydrocarbons can be effectively degraded using biosurfactants. All the four strains were able to produce biosurfactant with almost similar characteristics. *Rothia muscilagenosa* produces lipopeptide type of biosurfactant. *Rothia muscilagenosa* is able to degrade oil, but it takes lot of time to degrade completely. So the study of *Rothia muscilagenosa* oil degradation needed further process. FTIR is used to characterize the biosurfactants and the evidence for the change in the chemical structure of control petrol and the degraded petrol. The previous studies revealed the advantages of using biosurfactant to treat petroleum hydrocarbons and it shows greater efficiency. The present study also supports the same. It is concluded that the biosurfactant produced by the strain *Rothia muscilagenosa* is a glycolipopetides with an excellent bioremediating potentials which can also be used for commercial applications.

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