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Isolation and characterization of biosurfactant producing microorganisms from marine of Gujarat and their role in bioremediation

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Abstract

Petroleum based hydrocarbon degrading and biosurfactant producing bacterial strains were isolated from Alang, ship breaking yard of Bhavnagar, Gujarat. Three strains were identified as Pseudomonas sp, Pseudomonas aeruginosa and Serratia marcescens on the basis of 16S rDNA sequencing analysis and Gene Bank submission data. The hemolytic activity, methylene blue agar plate method, drop collapsing test as well as oil displacement test were performed to elucidate biosurfactant producing activity of the bacterial strains. Foaming percentages of the crude biosurfactant were found to be 30.8% ± 0.58 to 43.8 % ± 0.43. All the strains were found to be efficiently degrading petroleum based oils and edible oils. Emulsification index against different hydrocarbons showed their diverse application in bioremediation. FTIR analysis revealed the presence of lipid and peptide in the isolated biosurfactants.

Keywords: Biosurfactant, Emulsification, Alang, Marine of Gujarat, Bioremediation.

Introduction

Oil spill during exploration, transportation, refining and other anthropogenic reasons poses a continuous threat for the environment[1]. Most of the hydrocarbons are insoluble in water and the effect of hydrocarbons especially polycyclic aromatic hydrocarbon (PAHs) on marine life may be everlasting. Physiochemical and biological methods are generally applied for remediation in the contaminated areas. The conventional physicochemical methods can rapidly remove majority of the oil contaminants, but while performing toxic derivatives may produce. Therefore considerable attention is being given to the biological alternatives[2,3]. One such biological method is microbial degradation of hydrocarbons which has expanded significantly due to its non-oncogenic, non-explosive, wide spread and sustainable in nature as compared to other conventional methods. Biodegradation has been found most effective in removal of petroleum hydrocarbons from the marine environment and reinstate the oil contaminated ecosystem[4]. Various microorganisms have the capacity to use these hydrocarbons as their source of energy and oxidize the petroleum hydrocarbons[5]. Thus by degrading these hydrocarbons microbes eventually makes the ecosystem well-operative[6]. In addition, by producing various secondary metabolites such as biosurfactants, fatty acids, alcohols and solvents in-situ, these microorganisms solubilize the hydrocarbon fractions, reduce the critical micelle concentration (CMC), interfacial tension, surface tension and improve the remediation[7].

A large variety of microorganisms are known to synthesize biosurfactant, among them bacteria and yeast are predominant[7]. These microorganisms when grown on hydrocarbon substrate as carbon source, synthesize extensive range of chemicals with surface activity such as glycolipids, ornithine lipid, lipopeptides, phospholipids and others[8]. Such chemicals are seemingly synthesized to emulsify the hydrocarbon pollutant and enable its transport into the cell. Many scientists have reported extracellular excretions of biosurfactants from different microorganisams[9-11].

Therefore discovery of new biosurfactant producing microorganisms and characterization of the optimum condition for biosurfactant production are vital for effective bioremediation of soils, ground water, marine life contaminated with hydrocarbons.

In this paper we aim to isolate and characterize biosurfactant producing microorganisms from heavily contaminated marine of Alang, Gujarat. Further characterizations of biosurfactants have also been performed to elucidate their role in bioremediation.

Materials and methods

Isolation, screening and identification of oil degrading microorganism: Marine water samples were collected from Alang (21°24'04.5"N 72°11'11.4"E) ship breaking yard, Bhavnagar, Gujarat. The shipyards at Alang recycle approximately half of all ships salvaged around the world[12]. It is considered the world's largest graveyard of ships[13]. The yards are located on the Gulf of Khambat, 50 kilometers southeast of Bhavnagar. Water samples were collected from two different areas of shipyard and transported via sterile polypropylene bottles.

Isolation and enumeration of bacteria: Marine water samples were inoculated in Mineral Salt Medium (MSM) with 1% crude oil (v/v) for 72 hours at 37°C in orbital shaker[14] and subsequently plated on MSM agar. After incubation morphologically distinct colonies were selected for further studies.

Characterization of microorganisms: Bacterial Genomic DNA was isolated using the Insta Gene TM Matrix Genomic DNA isolation kit and PCR amplification of 16s rDNA gene was performed by following set of primers;

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

The 16s rDNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences[15]. The resulting aligned sequences were cured using the program Gblocks 0.91b. Finally, the program PhyML 3.0 a LRT was used for phylogeny analysis and HKY85 as substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering[16].

Optimization of Growth condition and production of Biosurfactant: 100 ml freshly prepared MSM broths supplemented with 1% crude oil (v/v) were inoculated with isolated colonies and incubated in orbital shaker incubator at 37˚C for 5days. The optimum concentration of salt for each strain was determined and maintained as described by Lakshmi M.B. et al[17]. After 5 days cultures were centrifuged at 4000 rpm for 20 minutes at 4˚C. Supernatants were collected in sterile screw cap bottle and used for the primary screening for the presence of biosurfactant.

Detection and characterization of Biosurfactant: Blue-Agar plate method: Selected colonies were streaked on Blue plate agar containing the cationic surfactant cetyl-trimethyl-ammonium bromide (CTAB) and the basic dye methylene blue and incubated at 37^0C for 7-8 days[18]. Positive colonies (Dark blue colonies) were selected.

Blood hemolysis test: Blood agar hemolysis method was used to screen biosurfactant producing strain[19]. The method was based on the fact that biosufactants were able to hemolysis the red blood cell present in blood. The selected isolates were inoculated on blood agar plates incubated for 24-48 hours at 37˚C. The zone of hemolysis indicated the production of biosurfactant.

Oil spreading technique: 10 µl of oil was added to the surface of the plate to form a thin oil layer and 10 µl of cell free media was gently placed on the centre of the oil layer[20]. The presence of biosurfactant would displace the oil and a clear zone would form. The diameter of the clearing zone on the oil surface would be visualized and measured after 30 seconds.

Drop Collapse Assay: 10 microliters of cell free media was placed on a oil coated surface to see whether the drop spread or collapsed as described by Lee H. et al, 1991[21].

Foaming: Hundred milliliters of the cell free media was shaken for foam formation and the percentage foam formation was determined by the following formula[22]

Foaming (%) = Height of the foam layer / total height) X 100

Determination of emulsification index (E_{24}) of the biosurfactant: The emulsification index of the Biosurfactant was determined by adding 2 ml of crude, kerosene, petrol, diesel, coconut, sunflower and mustered oil individually to the same amount of selected cell free culture followed by vortexing for 2 minutes. These were then allowed to stand for 24hr at room temperature. The E_{24} was determined by the following formula[23].

$$E_{24} = \frac{h_{emulsion}}{h_{total}} \times 100\%$$

Phenol: sulfuric acid method: 1ml collected supernatants were mixed with 1ml of 5% phenol and 5ml of concentrated H_2SO_4. Presence of biosurfactant in supernatant was confirmed by the change in color[24].

Extraction of Biosurfactant: The cultures in MSM with 1% crude oil (v/v) were incubated at 25°C for 7 days. centrifugation were carried at 13000 rpm, 4°C for 30 minutes and 6M HCl was added to the supernatant (pH 2). Equal volumes of ethyl acetate were added and left overnight in shaker. The presence of white colored precipitate at the interface confirmed production of biosurfactant[25].

Characterization of Biosurfactant: Thin Layer Chromatography: The detection was performed with the crude extract by TLC (pre-coated silica gel of standard 20×20 Kiesel-gel 60 F254 Merck plates). Chloroform: methanol: acetic acid (65:15:2, v/v/v) solvent was used and spots were detected by iodine vapor.

Characterization of biosurfactant by Fourier Transform Infrared analysis (FTIR): Fourier transform infrared spectroscopy (FTIR) is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture. FTIR spectroscopy was carried out using IR Prestige-21 Fourier Transform Infrared spectrophotometer (Samadzku, Japan) to determine the chemical nature of the biosurfactant[26-28]. The

molecular characterization was performed using lyophilized sample of biosurfactant with 4 cm^{-1} resolution yielding IR traces over the range of 400–4000 cm^{-1}.

Results and discussion

Enumeration of sample: Occurrence of biosurfactant-producing bacteria in hydrocarbon-polluted environments was reported by many researchers[29-31], considering which we selected Alang ship breaking yard, Bhavnagar, Gujarat, the world's largest graveyard of ships for sampling. Three morphologically distinct colonies were selected from the MSM agar plate. The strains were named as BC-05, BC-08 and BC-09 respectively.

Gram staining and biochemical characterization: The results of the gram staining and biochemical tests were reported in Table-1. The growth of microorganism on specific media, results of gram staining and biochemical tests indicated that the organisms were *Pseudomonas sp* and *Serratia sp.* Further confirmation was done by 16S rDNA sequencing data.

Table-1: Microbial and Biochemical characteristics of bacteria isolated from Alang marine region.

Test	Strain-BC-05	Strain-BC-08	Strain-BC-09
Gram Staining	Gram -Ve	Gram -Ve	Gram -Ve
Shape	Rods	Rods	Rods
Motility	+	+	+
Capsule	+	-	-
Spore	-	-	-
Flagella	Multiple	Single	Single
Catalase	+	+	+
Oxidase	-	+	+
Methylene blue Reduction test	-	-	-
Voges–Proskauer test	+	-	-
Indole	-	-	-
Citrate	+	+	+
Urease	-	-	-
Nitrate Reduction	+	+	+
Gas (From Nitrate)	+	+	+

16s rDNA sequence analysis and characterization: The sequences were studied using BLAST in NCBI database to find out maximum percentage homology with known bacterial DNA sequences. Sequences were submitted to NCBI Gene Bank database and the accession number KY655226, KY655227, KY655228 for bacterial strains SUB2440060B5, SUB2440060B8, SUB2440060 B9 respectively were successfully obtained. BC-05 (SUB2440060 B5) showed 99% homology with *Serratia marcescens* while BC-08(SUB2440060 B8) had 99% homology with *Pseudomonas sp.* and BC-09 (SUB2440060 B9) had 99% homology with *Pseudomonas aeruginosa* (Figure-1a,b,c).

Production of Biosurfactant: The three isolates were further screened for biosurfactant activities by blue agar test, hemolytic test, drop collapsing, oil displacement method, foaming percentage, emulsification index and phenol–sulphuric acid method as reported by Satpute et al.[32] that more than one screening methods should be included in the primary screening as to identify potential biosurfactant producers (Table-2). Dark blue zone in the methylene blue agar plate supplemented with CTAB confirmed the presence of anionic biosurfactant for all three isolates. The results on blood agar media supported the alpha hemolysis which was similar to the work done by Mulligan et al.[19] and Mulligan et al.[33]. All these three isolates showed positive result in oil displacement assay, but *Pseudomonas aeruginosa and Pseudomonas sp* showed a better zone formation when compared to *Serratia marcescens.* The flat appearance of the drops in microtitre plate confirmed the positive result for drop collapse test for all the isolates as suggested by Jain et al.[34]. Foaming percentage of the cell free medium (crude biosurfactant) was determined to be 30.8±0.58% to 43.8±0.43%. The observed colour change in phenol: sulphuric acid assay further confirmed biosurfactant production.

Table-2: Screening results of isolates for production of biosurfactant.

Test	Hemolytic Activity	Methylene Blue Agar method	Oil Dis-placement	Forming Capacity (%)
BC-05	+	+	+	33.3 ±0.58
BC-08	+	+	+	30.8±0.58
BC-09	+	+	+	43.8±0.43

Emulsification Index: Crude biosurfactant was recovered from the cell free media from each isolates and its emulsification index (E_{24}) was evaluated against crude oil, kerosene, petroleum, diesel, coconut oil, sunflower oil and mustered oil. Emulsification index of the isolated biosurfactant from *Pseudomonas aeruginosa* strains were in the order of crude oil > diesel > petrol > kerosene whereas in *Serratia marcescens*it was crude oil > petrol > diesel > kerosene. Among the edible oils E_{24} index was in order of Sunflower > Coconut > Mustered oil. Our study indicated that the biosurfactant produced by *P. aeruginosa and Serratiamarcescens* have the ability to emulsify different hydrocarbons and demonstrated the applicability of these strains against diverse hydrocarbon contamination as also referred by Aparna et al.[35] and Thavasi R.[11] (Figure-2a, 2b).

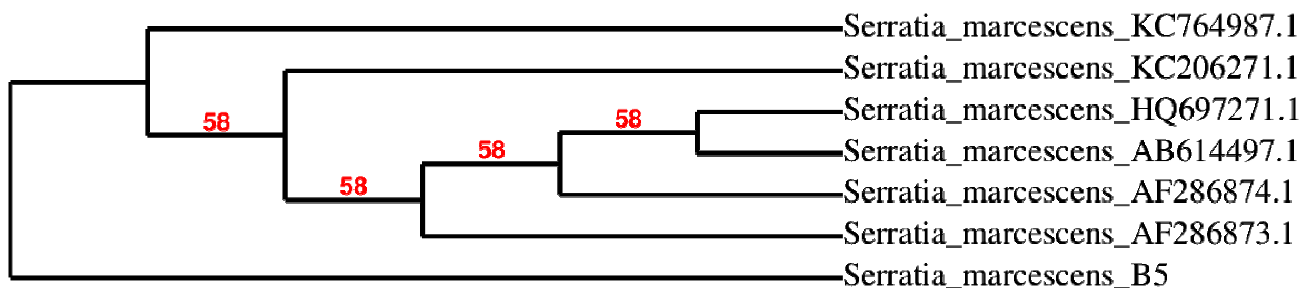

Figure-1a: Phylogenic tree of strain Serratia_marcescens_BC-05.

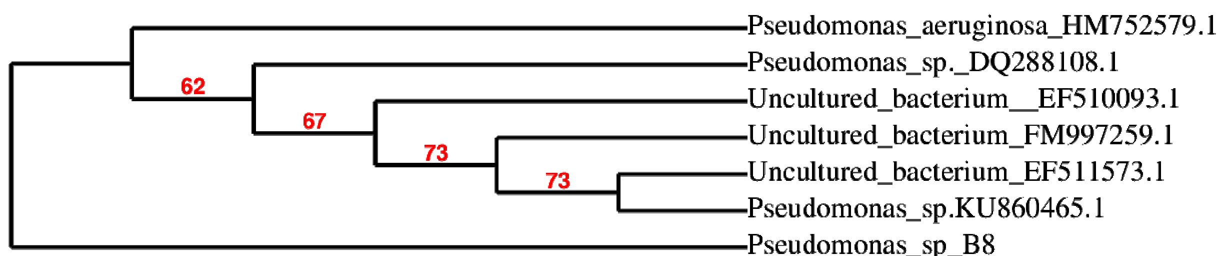

Figure-1b: Phylogenic tree of strain Psedomonas_sp_BC-08.

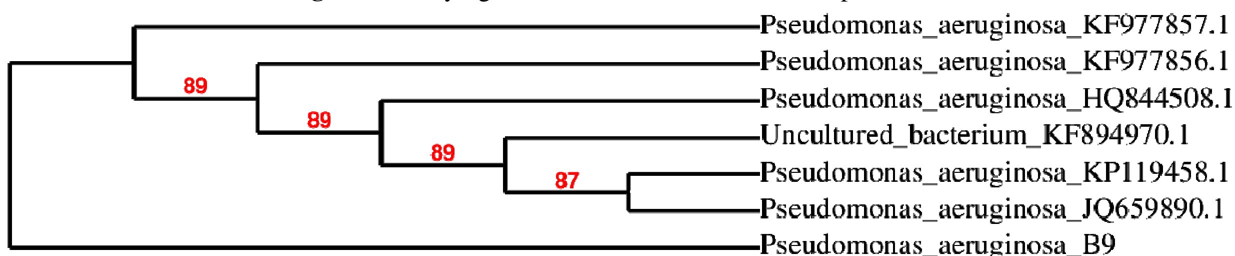

Figure-1c: Phylogenic tree of strain Pseudomonas_aeruginosa_BC-09.

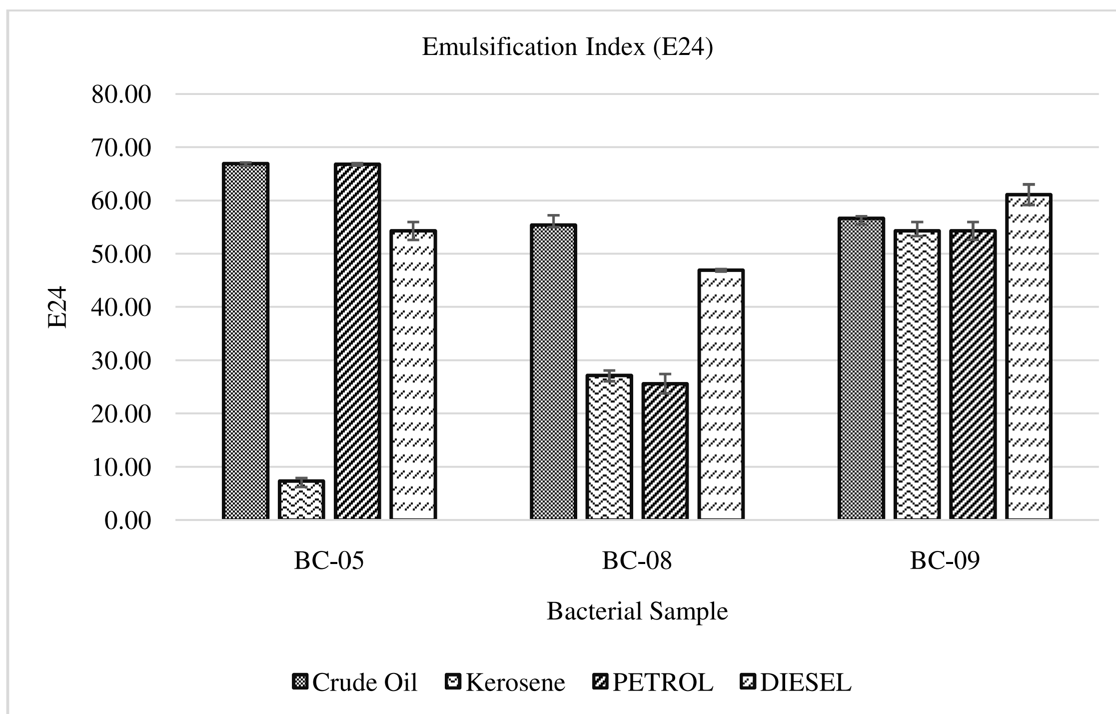

Figure-2a: E $_{24}$ index of the cell free extract against different petroleum hydrocarbons.

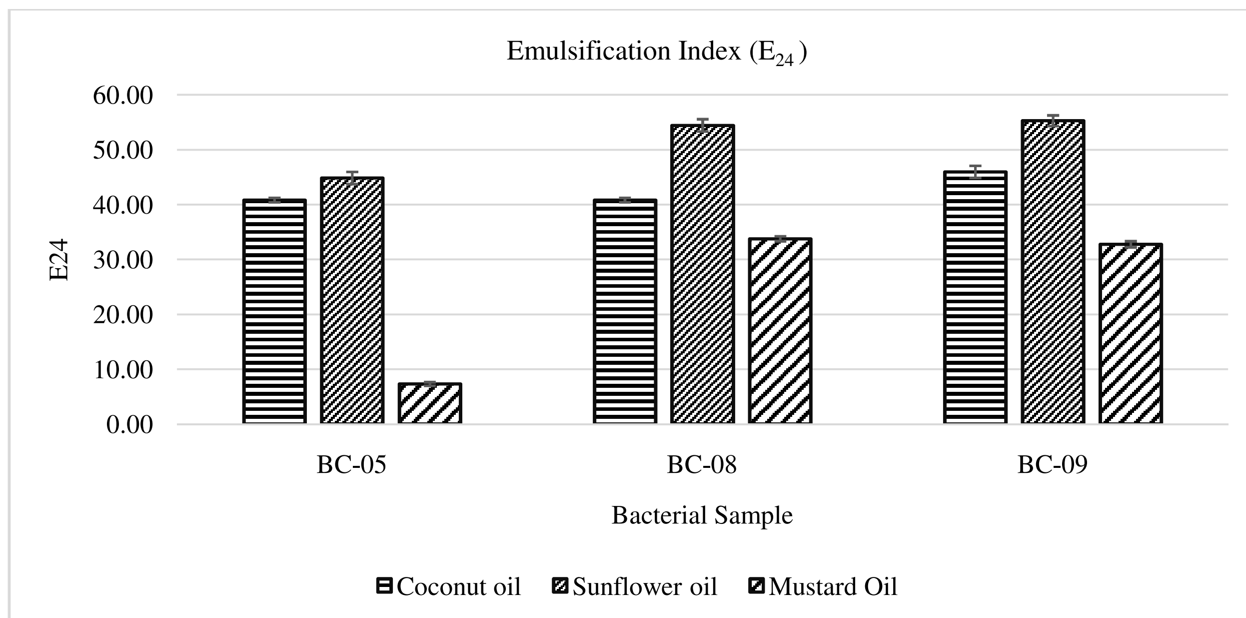

Figure-2b: E $_{24}$ index of the cell free extract against different edible oil.

Characterization of biosurfactant: Thin Layer Chromatography: TLC analysis suggested that the partially purified biosurfactant produced by strain BC-05, BC-08, BC-09 were lipid in nature.

Biosurfactants analysis by FTIR: FTIR for Strain BC- 05 (*Serratia marcescens*): In FTIR analysis (Figure-3a), the peak at 532cm^{-1} revealed the presence of C–Br bond. Peaks at 940 cm^{-1} and 1650cm^{-1} indicated N-H bend. The presence of C-N stretch in the sample was due to the peak at 1080cm^{-1}. An absorption band at 1459cm^{-1} showed stretching mode of the R-CH indicating the presence of alkenes.

The stretch 1289cm^{-1} and 1548 cm^{-1} denoted as the N–O symmetric stretch. The transmittance around at 3278cm^{-1} referred to the aliphatic chain of the C–H and C=C-H group. An intense stretching peak 2421cm^{-1} indicated the presence of O-H stretch. The availability of all these functional groups firmly substantiated that the biosurfactant is a conjugate of lipid and protein.

FTIR for Strain BC-08 (*Pseudomonas sp.*): The FTIR analysis (Figure-3b) revealed C-Br bond at 530 cm^{-1} peak. The peak at 942cm^{-1} and 1647cm^{-1} indicated the presence of N-H bend. The presence of C-N stretch in sample was demonstrated by the peak at 1075 cm^{-1}. Also, an absorption band at 1459 cm^{-1}, 2863 cm^{-1} and 2926 cm^{-1} showed stretching mode of the R-CH indicating the presence of alkenes. The stretch 1553 cm^{-1} denoted as the N–O asymmetric stretch.

The transmittance around at 1402 cm^{-1} referred to the C-C stretch. An intense stretching peak 3421 cm^{-1} and 2401 cm^{-1} indicated the presence of O-H stretch. The availability of all these functional groups indicated the presence of alkyl chain (fatty acid) and peptide.

FTIR for Strain Bc-09 (*Pseudomonas aeruginosa*): The FTIR analysis (Figure-3c) revealed that the peak at 535 cm^{-1} was due to C–Br bond. The peaks at 1642 cm^{-1} revealed the presence of N-H bend. The peaks at 1082 cm^{-1} and 1291 cm^{-1}showedthe presence of C-N stretch in the sample. An absorption band at 1406 cm^{-1}, 1455 cm^{-1} and 1542 cm^{-1} signified the stretching mode of the R-CH indicating the presence of alkenes.

The transmittance around at 1402 cm^{-1} referred to the C-C stretch. An intense stretching peak 2414 cm^{-1} and 939 cm^{-1} indicated the presence of O-H stretch. Altogether the presence of these functional groups depicted aliphatic chain (lipid) and peptides.

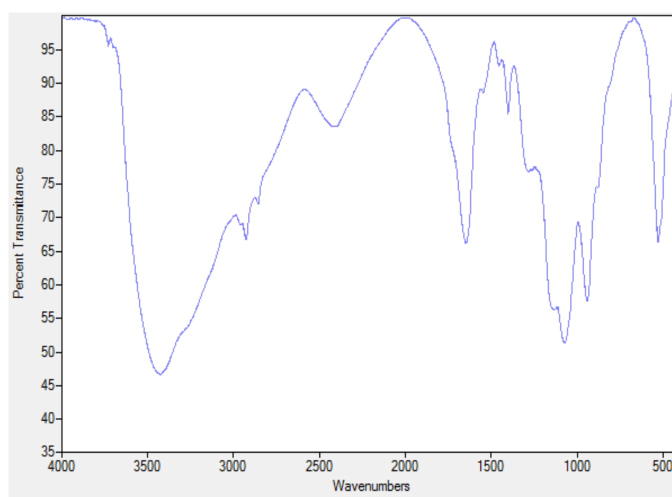

Figure-3a: FT-IR spectrograph of BC- 05 Biosurfactant.

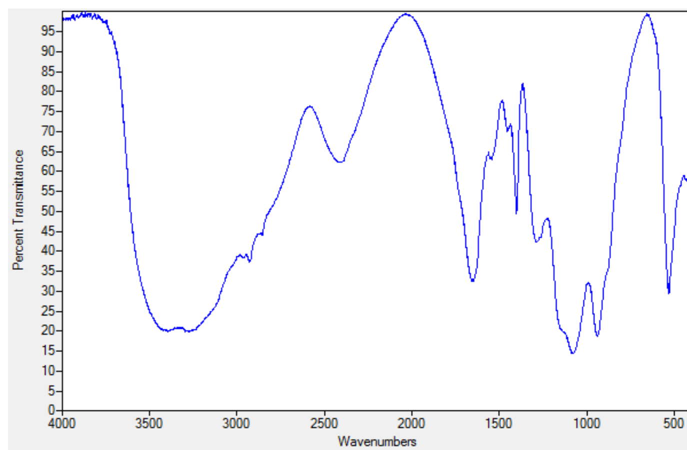

Figure-3b: FT-IR spectrograph of BC-08 Biosurfactant.

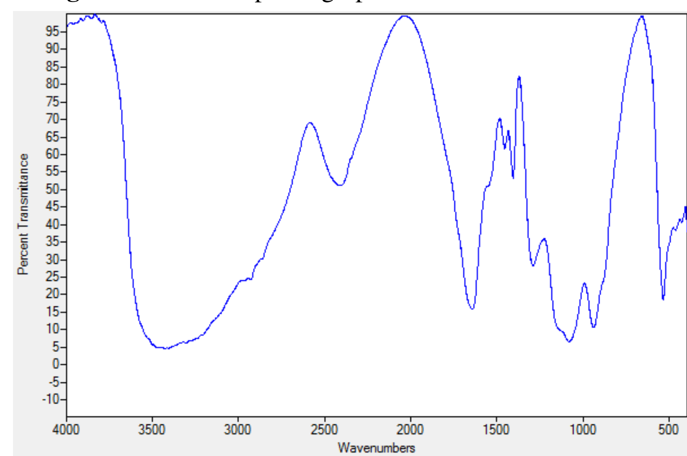

Figure-3c: FT-IR spectrograph of BC-09 Biosurfactant.

Conclusion

The current work shows successful isolation and characterization of hydrocarbon degrading bacteria *Pseudomonas sp, Pseudomona saeruginosa and Serratia marcescens* from Alang, ship breaking yard of Gujarat. Production of biosurfactant from isolates showed emulsification of petroleum hydrocarbon as well as other edible oils which may attract its future diverse application as potential tool for bioremediation. Further characterization by FTIR revealed the presence of Lipid as well as peptide. Research on structural characterization, gene regulation of biosurfactant production and cost of production are in progress.

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