# BIOSURFACTANT MEDIATED DEGRADATION OF PHENANTHRENE

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR THE DEGREE OF

MASTER OF SCIENCE IN LIFE SCIENCE

BY

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**May 11, 2015**

## CERTIFICATE

This is to certify that the project report entitled **"Biosurfactant mediated degradation of phenanthrene"** submitted by **Ms. Navyanita Patnaik** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in **LIFE SCIENCE** is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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DEDICATED TO MY BELOVED PARENTS AND
MY BROTHER

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.

## DECLARATION

I hereby declare that the thesis entitled **"Biosurfactant mediated degradation of phenanthrene"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Surajit Das, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge no part of this thesis has been submitted for any other degree or publication in any form.

Date: 11.05.2015 **NAVYANITA PATNAIK**

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**LIST OF SYMBOLS AND ABBREVIATIONS USED**

| | |
|---|---|
| g | Gram |
| mg | Milli gram |
| ng | Nano gram |
| h | Hour |
| l | Litre |
| µl | Micro litre |
| º | Degree |
| ml | Millilitre |
| C | Centigrade |
| Min | Minute |
| $cm^{-1}$ | /Centimetre |
| LB | Luria Bertani |
| BHB | Bushnell Haas Broth |
| Sp. | Species |
| % | Percentage |
| +ve | Positive |
| -ve | Negative |
| PAHs | Polycyclic Aromatic Hydrocarbons |
| HOC | Hydrophobic Organic Contaminants |
| HIV | Human Immunodeficiency virus |
| CLP | Cyclic Lipopeptide |
| BS | Biosurfactant |
| EPS | Extracellular polymeric substances |
| PHE | Phenanthrene |
| MEL | Mannnosylerythritol lipid |
| no. | Number |

| | |
|---|---|
| v | Volume |
| w | Weight |
| CMC | Critical micelle concentration |
| O.D | Optical density |
| MEOR | Microbial enhanced oil recovery |
| TLC | Thin Layer Chromatography |
| AGlyMSM | Anthracene Supplemented Medium |
| GlyMSM | Glycerol medium |
| PNA | Poly Nuclear Aromatic Hydrocarbon |
| TFLP | Terminal Restriction Fragment Length Polymorphism |
| ATR-FTIR | Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy |
| XRD | X Ray Diffraction Crystallography |
| FESEM | Field emission Scanning Electron Microscopy |
| PCR | Polymerase Chain Reaction |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| PAST | Palenteological Statistics Software |
| rRNA | Ribosomal Ribonucleic Acid |
| HCl | Hydrochloric Acid |
| NaCl | Sodium Chloride |
| Rpm | Revolution per minute |
| PAST | Palenteological Statistics Software |
| $EC_{50}$ | Effective concentration decrease to 50% |

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

**Abstract**

Biosurfactant (BS) produced from a marine bacterium *Pseudomonas aeruginosa* JP-11 and a terrestrial bacterium *Bacillus subtilis* SJ301 was utilized for phenanthrene degradation in culture medium and soil consortium. Functional and structural characterization by ATR-FTIR spectroscopy and XRD analysis revealed the structure of crude BS from JP-11 and SJ301 as glycolipid and lipopeptide respectively. BS of both the isolates JP-11 and SJ301 could reduce the surface tension of medium to 33.446 ± 0.029 mN/m and 37.15 mN/m respectively, with BS JP-11 having the maximum emulsification index of 52.94%. BS of JP-11 supplemented in the culture medium of JP-11 degraded 54.62% of phenanthrene with respect to BS of SJ301 supplemented in culture medium of SJ301 which degraded 43.29% of phenanthrene. Maximum degradation of phenanthrene was carried out by *B. subtilis* SJ301 and consortium of *P. aeruginosa* JP-11 and *B. subtilis* SJ301 in the soil consortium with 85.73 and 86.15 % of phenanthrene degradation respectively. The major functional groups involed in BS-phenanthrene interaction was -OH and -NH stretch, S-H stretch and P=O stretch. Fluorescence spectroscopy study showed the presence of one and three binding sites in the fluorophore of JP-11 and SJ301 with binding constant ($K_b$) of 3.32 $M^{-1}$ and 4.08 $M^{-1}$ respectively. With an increase in phenanthrene concentration (20-100 mg/l), fluorescence quenching reaction occurred which was spontaneous at 298 K with the ΔG value of -2.89 and -3.40 kJ/K/mol for JP-11and SJ301 respectively. Zeta potential analysis showed an increase in negative charge with increasing phenanthrene concentration from 20 to 100 mg/l indicating stable BS-phenanthrene interaction. Microbial consortium present in sample 1, 9, 10 had similar microbial communities arising from a single node, whereas microbial consortium present in sample 2, 7, 5 belonged to a different node from the sample of 8, 6, 3 representing the community structure change in the soil consortium in presence of phenanthrene.

**Keywords:** Biosurfactant, bacteria, degradation, phenanthrene, soil consortium, Fluorescence spectroscopy

## 1. Introduction

Surfactants are amphiphilic molecules made up of hydrophilic and hydrophobic groups monitor properly at the interface between fluid phases of different polarity and hydrogen bonding. Surface active molecules are secreted extracellularly by various classes of microorganisms like bacteria, algae, fungi and are known as biosurfactants. Microorganisms use wide variety types of organic compounds as the source of carbon for energy and growth. In case of hydrocarbons (C*x*H*y*), the carbon source is not soluble. Hence these are used by the microorganisms. These microorganisms are diffused into the cell there by producing distinct substances, called the biosurfactants. Some bacteria and yeasts produce ionic surfactants that mix the CxHy organic compounds present in the culture medium for growth. These molecules have the ability to decrease surface and interfacial tension in both aqueous solutions and organic mixtures. The amount of biosurfactants secreted by microorganisms relies on the physical and chemical parameters like pH, temperature, pressure, nitrogen, carbon and trace elements respectively. Biosurfactants of varying microbial origin differ due to their chemical properties and molecular size. Biosurfactants play significant role to blend water insoluble substrates like hydrocarbons and allows its transport into the cell to trigger growth in microbial cells (Singh, 2012).

Biosurfactants are grouped mainly on accordance to their chemical composition and microbial origin. On the basis of molecular mass the biosurfactants are divided into low molecular mass molecules and high molecular mass molecules. The low molecular mass molecules have the potential to decrease surface tension efficiently. The large molecular-mass molecules are efficient emulsion stabilizing agents. The important classes of low molecular mass surfactants are lipopeptides, glycolipids and phospholipids and the large molecular mass biosurfactants include polymeric and particulate biosurfactants. The majority of the biosurfactants are anionic or neutral in nature. The hydrophobic groups are defined on the length of fatty acids or fatty acid derivatives. The hydrophilic groups present in biosurfactants can be carbohydrate, phosphate, amino acid, or cyclic peptide (Nitschke and Coast, 2007).

### 1.1. Classification of Biosurfactants:

The major classes of biosurfactants include:

- **Glycolipids:** These are comprised of common carbohydrate of long chain hydroxyl aliphatic acid. The glycolipids are grouped into Rhamnolipids (produced by *Pseudomonas aeruginosa*), Trehalolipids (produced by *Actinomycetes, Mycobacterium, Nocardia* and *Corynebacterium*), Sophorolipids (produced by different strains of yeast like *Torulopis bombicola* and *T. Petrophilum*).

  ***a) Rhamnolipids:*** Rhamnolipids are comprised of one or two molecules of rhamnose attached to one or two molecules of β-hydroxydecanoic acid. A glycosidic linkage is

present between the hydroxyl groups in one of the acids and reducing ends of rhamnose disaccharides. The hydroxyl group of the second acid is involved in ester formation. As one carboxylic group is free, rhamnolipids are anionic and acidic in nature having pH 4. When the lipid group of rhamnolipid is bonded to one or more rhamnose groups hence are known as monorhamnolipid (Type I) or dirhamnolipid (Type II) (Fig. 1) repectively. The production of rhamnolipid was first observed in *Pseudomonas aeruginosa* and was later studied in other *Pseudomnas* species.

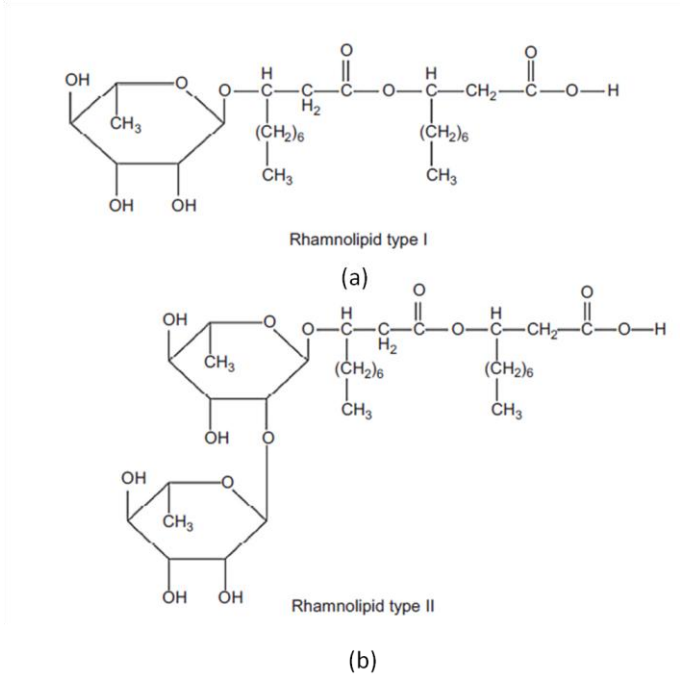



**Fig. 1.** Chemical structure of rhamnolipid **(a)** Monorhamnolipid **(b)** Dirhamnolipid

***b) Trehalolipids:*** Trehalolipids are broad group of glycolipids comprised of disaccharides linked at C-6 and C-6' position to mycolic acids, which are long chain α branched β branched hydroxy fatty acids (Fig. 2).They play an important role in industrial and environmental scenarios. They are also efficient therapeutic drugs.Trehalolipids are mainly produced by *Actinomycetes*, *Mycobacterium*, *Nocardia*, and *Cornybacterium* species.

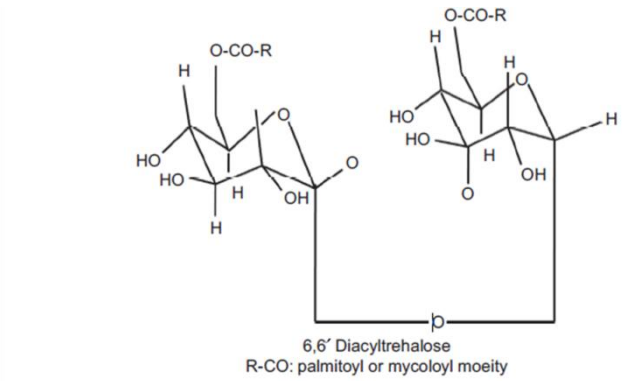



**Fig. 2.** Chemical structure of trehalolipid

***c) Sophorolipids:*** Sophorolipids are made up of hydrophilic part formed of sophorose (disaccharide ) containing two or more glucose molecules. They are attached by β - 1, 2 bond (Fig. 3). The hydrophobic group of amphiphilic molecule consist of terminal or sub terminal hydroxylated fatty acid.

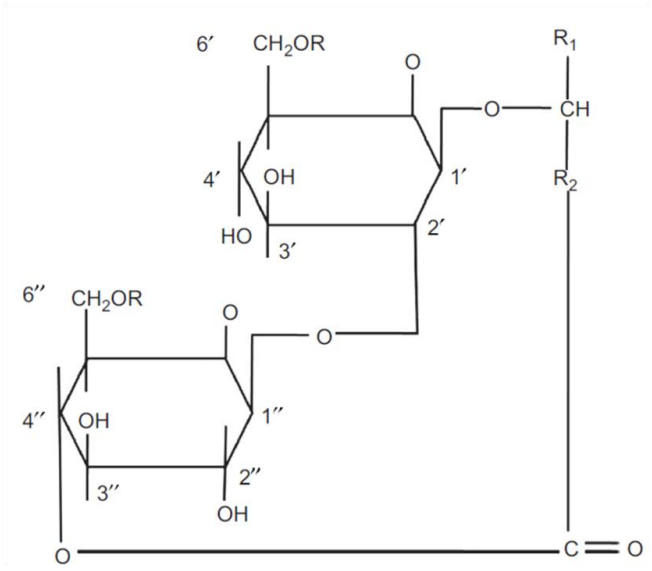



**Fig. 3.** Chemical structure of sophorolipid

- **Phospholipids, Fatty Acid and Natural Lipids:** Many bacteria and yeasts like *Thiobacillus thiooxidans*, *Aspergillus* sp., *Arthobacter*, *P. aeruginosa* produces large quantities of fatty acid and phospholipids during growth on n-alkanes as carbon source.
- **Peptides:** Many peptides antibiotics are amphiphilic in nature and exhibit surface active properties. Dipeptide antibiotics like gramicidin, lipopeptide antibiotics like polymyxins and cyclic lipopeptide are produced by *Bacillus brevis*, *B. polymyxa* and *B. subtilis*, respectively. These peptides are known to have surface active properties.
- **Lipopeptides:** Lipopeptides are short linear chains or cyclic structures of amino acids. They are linked to fatty acids by ester or amide bond . Lipopeptides are generally produced by various fungal species like *Aspergillus* and bacterial species like *Bacillus*, *Pseudomonas* and *Streptomyces.* The lipopeptide biosurfactants produced by *Bacillus* sp. are classified into three families of cyclic lipopeptide that are fengycin, iturin and surfactin.

  ***a) Surfactin:*** The surfactin family incorporates hepta peptide variants of esperin, lichenysin, pumilacidin and surfactin. The peptide group is linked to β hydroxyl fatty acids (Fig. 4).

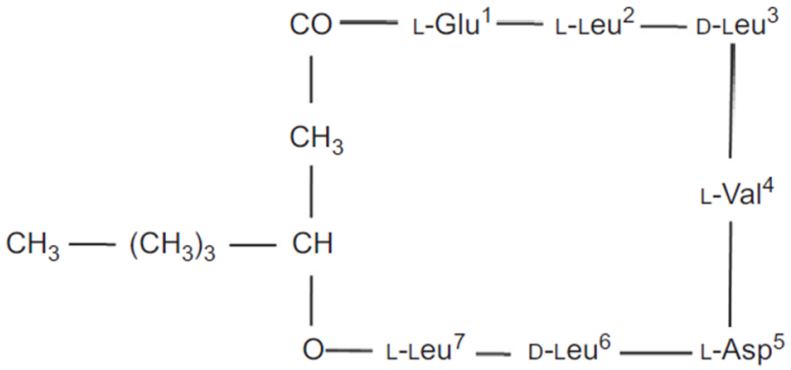



**Fig. 4.** Chemical structure of Surfactin

- **Polymeric biosurfactants:** The popularly known polymeric biosurfactant are emulsan, liposan, mannoprotein, polysaccharide-protein complexes. These complex are mainly produced by *Acinetobacter calcoacetius*, *Candida lipolytica*, *Saccharomyces cerevisiae*, *Schizonella malanogramma*, *Ustilago maydis* and *Pseudomonas* sp.
- **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 *Staphylococcus aureus*, Layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia* sp., gramicidin in *Bacillus brevis* spores and thin fimbriae in *A. calcoacetius* (Singh, 2012).

**1.2. Advantages of biosurfactants**

Biosurfactants have are more advantageous over chemical surfactants that are as follows:

- **Biodegradability:** The biological surfactants are easily degraded by microorganisms (Mohan et al., 2006).
- **Low toxicity:** Biosurfactant are less toxic than the chemical surfactants. It was also observed that biosurfactants show higher $EC_{50}$ (effective concentration to decrease 50% of test population) values than synthetic dispersants (Desai and Banat,1997)
- **Availability of raw materials:** Biosurfactants can be produced from cheap raw materials that are widely available in large quantities. The carbon source utilised by bacteria are obtained from hydrocarbons, carbohydrates, or lipids. (Kosaric. 2001).
- **Physical factors:** Many biosurfactants are not influenced by the environmental factors such as temperature, pH and ionic strength. Lichenysin produced by *Bacillus licheniformis* strain was not affected by temperature ranges of up to 50°C, a pH ranging between 4.5-9.0,NaCl concentration of 50 g/l and Ca concentration of 25g/l.
- **Surface and interface activity:** It was reported that a good surfactant can decrease surface tension of water from 75 to 35 mN/m and the interfacial tension water/hexadecane from 40 to 1mN/M (Mulligan, 2005). Surfactin has the capacity to lessen the surface tension of

water to 25 mN/m and the interfacial tension of water/hexadecane to <1mN/m (Krishnaswamy et al., 2008).

**1.3. Factors affecting biosurfactant production:**

The composition and emulsifying action of biosurfactant depends on the producer strain and the culture conditions. Thus, the nature of the carbon source, the nitrogen source as well as C: N ratio, nutritional limitations, chemical and physical parameters such as temperature, aeration, divalent cation and pH affect the quantity of biosurfactant production and the type of polymer produced (Ron and Rosenberg, 2001).

***Carbon sources:***

The quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate (Rahman and Gapke, 2008). Diesel, crude oil, glucose, sucrose, glycerol have been considered to be a good source of carbon for biosurfactant production (Desai and Banat, 1997).

***Nitrogen sources:***

Nitrogen is also important parameter in the production of biosurfactant as it is essential for microbial growth, protein and enzyme synthesis. Different nitrogen compounds used for the production of biosurfactant are urea, peptone, yeast extract, ammonium sulphate, ammonium nitrate, sodium nitrate, meat extract and malt extracts. Yeast extract is mostly used as a nitrogen source for biosurfactant production. The concentration of yeast extract requirement differs from organism to organism for biosurfactant production. Ammonium salts and urea are also preferred as nitrogen sources for biosurfactant production by *Arthrobacter paraffineus*. Ammonium nitrate favours maximum surfactant production in *P. aeruginosa* (Adamczak and Berdnaski, 2000)

***Environmental factors:***

These factors play an important role in determining the quantity and characteristics of the biosurfactant produced. In order to obtain large quantities of biosurfactants, it is always necessary to optimize the bioprocess as the product may be affected by changes in temperature, pH, aeration or agitation speed. Most biosurfactant productions are reported to be performed in a temperature range of 25-30$^{o}$C (Desai and Banat, 1997). pH 8.0 (natural pH of sea water) is reported to be the optimum pH for best biosurfactant production (Zinjarde and Pant, 2002).

***Aeration and Agitation:***

Aeration and agitation are the important factors that influence the production of biosurfactants. It facilitates the oxygen transfer from the gas phase to the aqueous phase. It may also be linked to the physiological function of microbial emulsifier. It has been suggested that the production of bioemulsifiers can enhance the solubilisation of water insoluble substrates and consequently facilitate nutrient transport to microorganisms.

***Salt concentration:***

Salt concentration of a particular medium also has an effect on the biosurfactant production as the cellular activities of microorganisms are affected by salt concentration.

**1.4. Applications of Biosurfactants:**

Biosurfactants from various microbial genera have wide range of applications in agriculture, medicine, petroleum and industries.

***Application in Agriculture:***

Biosurfactants enhance the solubility of bio-hazardous chemical compounds such as Poly Aromatic Hydrocarbons (PAHs). This increases the apparent solubility of Hydrophobic Organic Contaminants (HOC). It also help microbes to adsorb soil particles occupied by pollutants, hence decreases the diffusion path length between the site of absorption and site of uptake by the microorganisms These are used for hydrolyzing heavy soils to obtain good wet ability and to achieve uniform distribution of fertilizers in the soil. They also prevent the caking of certain fertilizer during storage and promote spreading and penetration of the toxicants in pesticides (Makkar and Rochne, 2003). Fengycins are also reported to possess antifungal activity and therefore may be employed in bio-control of plant diseases (Kachholz and Schlingmann, 1987).

***Application in Laundry:***

Biosurfactants being eco-friendly, are natural alternative of chemical surfactants, and are used in laundry detergents. Biosurfactants such as Cyclic Lipopeptides (CLP) are stable over a wide pH range (7.0- 12.0). On heating CLP at high temperatures, there is no loss of their surface-active property (Mukherjee, 2007). Hence, they form good emulsion with vegetable oils and illustrate excellent compatibility and stability with commercial laundry detergents considered in laundry detergents formulation (Das and Mukherjee, 2007).

***Application in medicine:***

Biosurfactants have wide range of applications in medicine (Das and Mukherjee, 2007) given as follows:

*Antimicrobial activity:*

The diverse structures of biosurfactants grant them the ability to display versatile performance. Due to its structure, biosurfactants exerts its toxicity on the cell membrane permeability bearing the similarity of a detergent like effect (Zhao et al., 2010). It has been reported that several biosurfactants have strong antibacterial, antifungal and antivirus activity (Gharei-Fathabad, 2011). These surfactants play an important role as anti-adhesive agents to pathogens making them useful for treating many diseases as well as its use as therapeutic and probiotic agent. A good example is biosurfactant produced by marine *B. circulans* that had a

potent antimicrobial activity against Gram-positive and Gram-negative bacteria and semi-pathogenic microbial strains including MDR strain (Mukherjee et al., 2009).

*Anti-cancer activity*:

Some extracellular glycolipids induce cell differentiation instead of cell proliferation in human promyelocytic leukemia cell line. Exposure of PC 12 cells to MEL enhanced the activity of acetylcholine esterase and interrupted the cell cycle at the G1 phase with resulting overgrowth of neuritis and partial cellular differentiation. This suggests that MEL induces neuronal differentiation in PC 12 cells and provides the ground work for the use of microbial extracellular glycolipids as novel reagents for the treatment of cancer cells (Krishnaswamy et al., 2008).

*Anti-adhesive agents:*

According to Rodrigues et al. (2011), biosurfactants inhibit the attachment of pathogenic organisms to solid surfaces or to infection sites. This demonstrated that pre-coating vinyl urethral catheter by running the surfactin solution through them before inoculation with media resulted in the decrease in the amount of biofilm formed by *Salmonella typhimurium*, *Salmonella enterica*, *E. coli* and *Proteus mirabilis*.

*Immunological adjuvants:*

Bacterial lipopeptides are non-toxic, non pyrogenic immunological adjuvants when mixed with typical antigens. An improvement in humoral immune response was observed when molecular mass antigens Iturin AL low and herbicolin (Gharaei-Fathabad, 2011) was introduced.

*Antiviral activity:*

Biosurfactants have antibiotic effects and inhibits the growth of human immunodeficiency virus in leucocytes (Desai and Banat, 1997; Krishnaswamy et al., 2008). Sophorolipids surfactants from *C. bombicola* and its structural analogues such as the sophorolipid diacetate ethyl ester proved as the most potent spermicidal and veridical agent.

***Application of biosurfactant in food processing industry*:**

Biosurfactants have been used for various food processing applications. They play an important role as food formulation ingredient and anti-adhesive agents. As food formulation ingredient, they promote the formation and stabilization of emulsion due to their ability to decrease the surface and interfacial tension. It is also used to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf-life of starch containing products, modify rheological properties of wheat dough and improve consistency and texture of fat based products (Krishnaswamy et al., 2008).

***Application of biosurfactants in cosmetic industry:***

The property of emulsification, foaming, water binding, spreading and wetting by the biosurfactant have effect on viscosity and product consistency. Biosurfactants have been proposed

to replace chemically synthesized surfactants. These surfactants are used as emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action, in insect repellents, antacids, bath products, acne pads, anti dandruff products, contact lens solutions, baby products, mascara, lipsticks, toothpaste and dentine (Gharaei-Fathabad, 2011) .

***Application of biosurfactants in environment cleaning:***

Biosurfactants are used in environment cleaning because some microorganisms like bacteria, yeasts use organic waste as carbon source for their metabolic activities and survival. Biosurfactants clean the oil contaminated sites by microbial enhanced oil recovery, contaminated soil sites by clean up combined and soil washing technology. Biosurfactants also clean the metal contaminated sites efficiently.

**1.5. Biosurfactants of marine origin:**

Biosurfactants/ surface active molecules producing microorganisms are found in both water (sea, fresh water, and groundwater) and land (soil, sediment, and sludge). They are also found in extreme environments like hyper saline sites and oil reservoirs. They survive at a wide range of temperatures, pH and salinity. Microorganisms produce biosurfactants to mediate solubilisation of hydrophobic compounds in their environment to be able to utilize them as substrates (Margesin and Schinner, 2001; Olivera et al., 2003; Floodgate, 1978). Various biosurfactants producing bacteria have been isolated and characterized from marine sites that are contaminated with oil, petroleum or their by-products. Marine microorganisms produce distinct types of biosurfactants like polymeric biosurfactants, glycolipids, lipopeptides, phospholipids, glycolipopeptide and fatty acids. Polymeric biosurfactants are produced by bacteria isolated from marine contaminated sites like *Acinetobacter*, *Pseudomonas*, *Halomonas*, *Myroides*, *Streptomyces*, *Antarctobacter*, *Marinobacter* sp. Glycolipids are produced by *Alcaligenes*, *Arthrobacter*, *Alcanivorax*, *Rhodococcus*, *Halomonas* sp. and lipopeptides are produced by Bacillus sp. *Bacillus vallismortis* JB201 was isolated from marine coastal sites contaminated with crude oil and its byproducts (Chakraborty et al., 2014).

**1.6. Biosurfactant mediated degradation:**

***Degradation of hydrocarbons:***

Large scale production and excessive use of hydrocarbons have led to environmental pollution with toxic and negative effects on flora and fauna. Hence, it has been the priority to clean up the environment. Biosurfactants play an important role in cleaning the pollutants present in the environment. The major organic pollutant categories include organic aqueous waste (pesticides), organic liquids (solvents from dry cleaning), oils (lubricating oils, automotive oils, hydraulic oils, fuel oils) and organic sludge/ solids (painting operations, tars from dyestuffs intermediates).

Biosurfactants improve remediation process of hydrocarbons by two mechanisms. The first mechanism includes the increase of substrate bioavailability for microorganisms and the second mechanism involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs, 2004). Biosurfactants increase the surface areas of insoluble compounds by reducing surface and interfacial tensions leading to increased mobility and bioavailability of hydrocarbons. Consequently, biosurfactant enhance biodegradation and enhances removal of hydrocarbons. Addition of biosurfactants can be expected to enhance hydrocarbon biodegradation by mobilization, solubilization or emulsification (Fig. 5). Mobilization occurs at concentrations below the biosurfactant critical micelle concentration (CMC). At this concentration, biosurfactants reduce the surface and interfacial tension between air/water and soil/water systems. Decrease in interfacial force, contact of biosurfactant with soil/oil system increases the contact angle and thus reduces the capillary force holding oil and soil together. Above the biosurfactant's CMC, solubilisation takes place. At these concentrations biosurfactant molecules associate to form micelles, which greatly increase the solubility of oil. The hydrophobic ends of biosurfactant molecules connect together inside the micelle while the hydrophilic ends are exposed to the aqueous phase on the outside. Consequently, the interior of a micelle creates an environment suitable for hydrophobic organic molecules. This process of incorporation of these molecules into a micelle is known as solubilisation. Emulsification is a process in which a liquid is formed known as an emulsion. It contains very small droplets of fat or oil suspended in a fluid, usually water. The high molecular weight biosurfactants are good emulsifying agents. They are often applied as an additive to stimulate bioremediation and removal of oil substances from environments (Płociniczak et al., 2011).

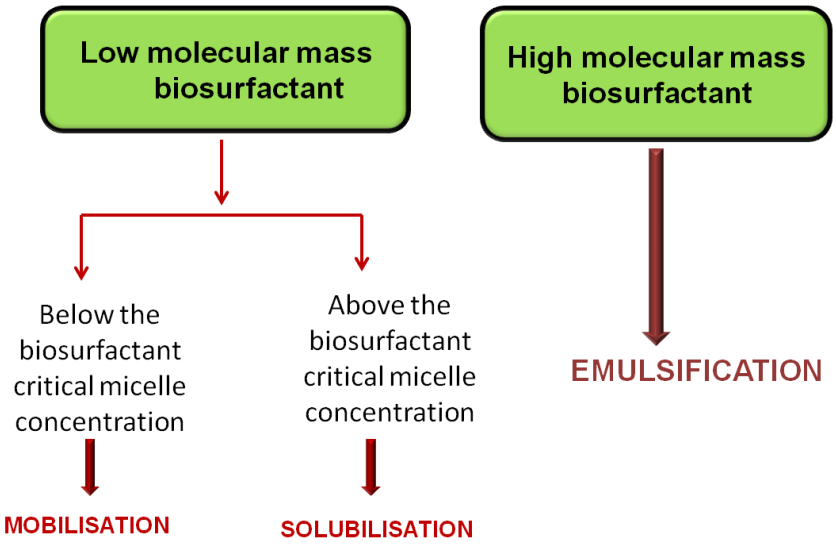



**Fig. 5.** Mechanisms of hydrocarbon removal by biosurfactants depending on their molecular mass and concentration (Rosenberg and Ron, 1999; Urum and Pekdemir, 2004).

***Degradation of Oil:***

Biosurfactants are useful in microbial enhanced oil recovery (MEOR). MEOR methods are used to recover oil remaining in reservoir. It is an important bioremediation process where microorganisms or their metabolites, including biosurfactants, biopolymers, biomass, acids, solvents, gases and also enzymes, are used to increase recovery of oil from depleted reservoirs. It is useful in cleaning oil in regions of the reservoir that are difficult to access and the oil is trapped in the pores by capillary pressure. Biosurfactants reduce interfacial tension between oil/water and oil/rock. Thus, this reduces the capillary forces preventing oil from moving through rock pores. Biosurfactants can also bind tightly to the oil-water interface and form emulsion (Fig. 6). This stabilizes the desorbed oil in water and allows removal of oil along with the injection water (Płociniczak et al., 2011).

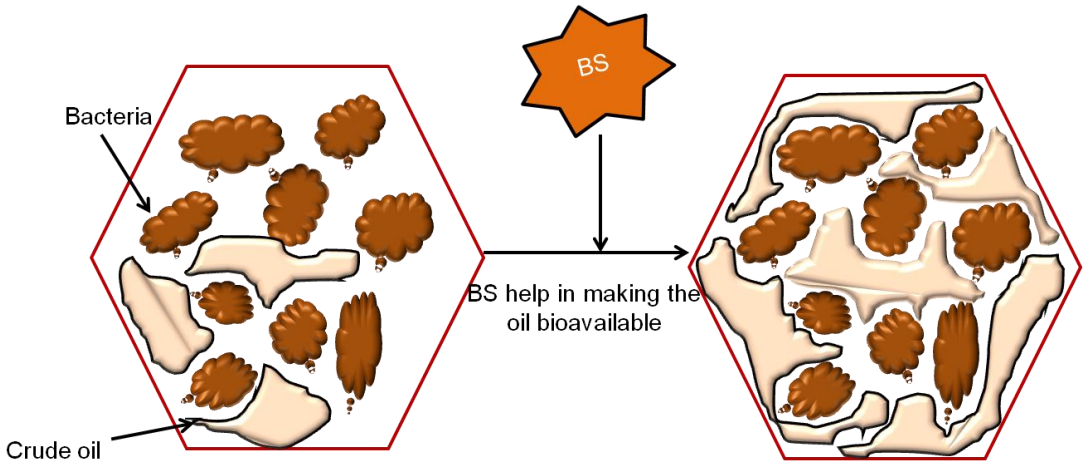



**Fig. 6.** Mechanism of enhanced oil recovery by biosurfactant

Polycyclic aromatic hydrocarbons (PAHs) are hydrocarbon containing only carbon and hydrogen. Incomplete combustion at high temperature (500-800$^{o}$C) or subjection of organic material at low temperature (100–300$^{o}$C) for long periods result in PAH production. They occur as colourless, white/pale yellow solids with low solubility in water, high melting and boiling points and low vapour pressure. With an increase in molecular weight, their solubility in water decreases; melting and boiling point increase and vapour pressure decreases. PAHs are accumulated in environment by two means natural and anthropogenic. Natural sources are forest and rangeland fires, oil seeps, volcanic eruptions and exudates from trees. Anthropogenic sources of PAH include burning of fossil fuel, coal tar, wood, garbage, refuse, used lubricating oil and oil filters, municipal solid waste incineration and petroleum spills and discharge. They are universally present contaminants which are toxic, mutagenic and carcinogenic.PAH are also made up of aromatic rings like naphthalene having two aromatic rings anthracene and phenanthrene having three aromatic rings. PAHs are neutral, non polar molecules. They are found in fossil fuels like coal, oil and in tar deposits. These are produced, when there is insufficient and incomplete

combustion of organic matter occurs like in engines and incinerators, when biomass burns in forest fires, etc. PAHs are carcinogenic and mutagenic (as well as teratogenic), in nature. They are considered as pollutants as they have potential adverse health impacts (Fig. 7).

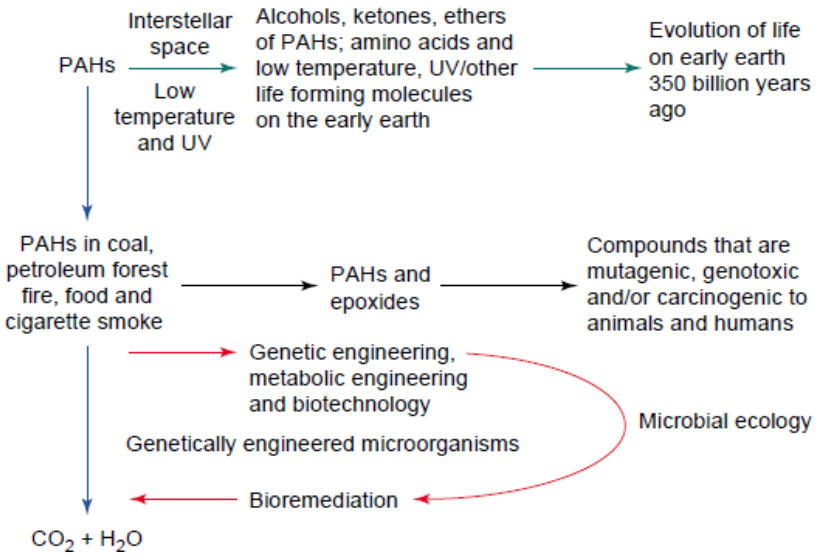



**Fig.7.** Fate, toxicity and remediation of polycyclic aromatic hydrocarbons (PAHs) from the environment (Dean et al., 2001)

Of several PAHs, phenanthrene is a polycyclic aromatic hydrocarbon made of three fused benzene rings (Fig. 8). The name phenanthrene is a composite of phenyl and anthracene. It is found in cigarette smoke and is a known irritant. Phenanthrene appears as a white powder having blue fluorescence. The compound with a phenanthrene skeleton and nitrogen at the 4 and 5 position is known as phenanthroline.

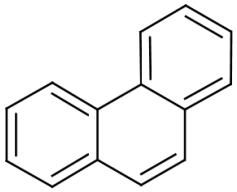


**Fig. 8.** Structure of phenanthrene

Phenanthrene is used to make dyes, plastics and pesticides, explosives and drugs. It has also been used to make bile acids, cholesterol and steroids. Phenanthrene enters our body by breathing contaminated air and reaches our lungs. Working in a hazardous waste site where PAHs are disposed, likely phenanthrene enters in breath with other PAHs. Exposure can also occur if skin comes into contact with contaminated soil or products like heavy oils, coal tar, roofing tar or creosote where PAHs have been found. As PAHs enter our body it spreads and targets fat tissues. The major target organs are kidneys, liver and fat. Therefore, this study marginalizes the characterization of marine and terrestrial biosurfactants, bioremediation of phenanthrene by the

isolates, their extracted biosurfactant and soil consortium based degradation studies helpful for the polluted contaminated sites.

## 2. Review of Literature

### 2.1. Microbial Degradation of PAHs:

PAH degradation by bacteria is initiated by the intracellular dioxygenases. The PAHs are taken up by the cells before degradation can take place. Bacteria oxidize PAHs to cis-dihydrodiols by addition of an oxygen molecule. The cis-dihydrodiols are further oxidized, then aromatic dihydroxy compounds (catechols) are channelled through the ortho- or meta cleavage pathways (Johansen et al., 2005).The biological degradation of PAHs can serve three different functions. (i) Assimilative biodegradation leading to production of carbon and energy for the degrading organism and mineralization of the compound or part of it. (ii) Intracellular detoxification processes aims to make the PAHs water-soluble as a pre-requisite for excretion of the compounds. Generally, it has been observed that intracellular oxidation and hydroxylation of PAHs in bacteria is an initial step preparing ring fission and carbon assimilation, whereas in fungi it is an initial step in detoxification (iii) Co-metabolism, where the degradation of PAHs takes place without generation of energy and carbon for the cell metabolism. Co-metabolism is defined as a non-specific enzymatic reaction, with a substrate competing with the structurally similar primary substrate for the enzyme's active site.

Phenanthrene has obtained significant concern because of their presence in all components of environment, resistance towards biodegradation, potential to bio-accumulate and carcinogenic activities. Phenanthrene is the major pollutant of air but a large amount of this deposit is found in soil (Haritash et al., 2009). They take part in various chemical processes in environment like volatilization, photo-oxidation, chemical oxidation, adsorption on soil particles, leaching and microbial degradation (Wild and Jones, 1995). The threats linked with the PAHs can be conquered by the use of prevalent methods which include removal, alteration, or isolation of the pollutant. Such techniques include excavation of contaminated soil and its incineration or containment. These technologies are expensive, and in most cases there is transfer of the pollutant from one phase to another. More over bioremediation is the tool to transform hazardous compounds into less hazardous/non hazardous forms with less addition of chemicals, energy, and time (Ward et al., 2003).To decrease the concentration of PAH, various chemical and biological process are adopted like adsorption, volatilization, photolysis, and chemical degradation, microbial degradation. Microbial degradation is the major degradation process (Yuan et al.2001). Microbes are known due to their catabolic activity in bioremediation, but they may also change microbial communities that are still unpredictable hence the microbial community is termed as a 'black box ' (Dua et al., 2002). The PAH-degrading microorganism can be algae, bacteria, and fungi. The biodegradation

of a pollutant and its rate depends on the environmental conditions, number and type of the microorganisms, nature and chemical structure of the chemical compound that is being targeted to degrade. Bacteria are the group of microorganisms that actively involved in the degradation of organic pollutants from contaminated sites. These bacterial species have biodegradation efficiency and are isolated from contaminated soil or sediments. Among the PAH in petrochemical waste, Benzo(a)pyrene is considered as the most carcinogenic and toxic (Ye et al.1995). It is difficult to decode the mechanisms controlling the biodegradation of PAH in complex media containing complex mixtures of substrates and heterogeneous microbial communities. One approach is to study the constituent bacteria contributing to the biotransformation and biodegradation processes in these complex systems.

By determining the PAH substrate range of individual organisms, for example, it may be possible to establish links between the metabolism of various substrates or identify those compounds for which more specialized metabolic capabilities are required for extensive degradation. The bacterial PAH-uptake remains saturated because PAH-dissolution is fast enough to keep up with the rising substrate consumption by the growing population. In this case, bacteria grow exponentially at their physiologically limited maximum rate. When the PAH consumption by the increasing population exceeds the PAH dissolution rate, the dissolved PAH concentration drops below saturation and exponential growth ceases. The bioavailability study of phenanthrene was studied by two bacterial strains of *Pseudomonas* sp. in presence of rhamnolipids. The study indicated that concentration of rhamnolipid above the critical micelle concentration liberated the phenanthrene from the soil (Dean et al., 2001). It was reported that the combined solubilization–biodegradation process was efficient in phenanthrene removal in two steps. In the solubilization step, it was observed there was higher removal efficacy below pH 6. This proved that the pH-dependent rhamnolipid structure showed different solubilizing capacity in this flushing process. In the biodegradation step, the phenanthrene content in the soil decreased notably. It suggested that remaining rhamnolipid did not notably inhibit the degradation and showed negligible toxicity. The cell density of the control at pH 7 and 8 had increased. This indicated that they could degrade and use phenanthrene as a carbon source. The study summarised that the removal efficacy was highest at pH 5 for flushing and at pH 7 for biodegradation. Though the highest degradation rate was measured at pH 7, a reasonable amount of phenanthrene was degraded at other pH except pH 4. This showed that the degradation of contaminants by specific species might not be influenced by application of the flushing process. In other words, residual biosurfactants present after the flushing process terminated seemed to be non-toxic to the phenanthrene degrader (Shin et al., 2006). Another novel phenanthrene degrading strain named as *Sphingomonas* sp. GF2B was isolated and identified from a farmland soil. Effects of a synthetic surfactant (Tween-80) and a

rhamnolipid biosurfactant on PHE degradation by *Sphingomonas* sp. GF2B were investigated at different concentrations of the surfactants. The results showed that the isolate was able to mineralize up to 83.6% of phenanthrene within 10 days without addition of surfactants whereas, the biosurfactant facilitated phenanthrene biodegradation, with up to 99.5% (Pei et al., 2010).

**2.2. Biosurfactant from marine bacteria in Bioremediation:**

The marine environment includes the wide majority of earth's surface which is a collection of a large number of microorganisms. The environmental functions of the biosurfactants produced by many such marine microorganisms have been observed earlier (Poremba et al., 1991; Schulz et al., 1991; Abraham et al., 1998). Biosurfactants of marine origin have wide structural diversity and higher surface activities. Hence there was increased bioavailability and microbial degradation of anthracene as a model of PAHs. It was reported that biosurfactant produced by a marine *B. circulans* strain efficiently solubilised anthracene. The biosurfactant efficiently caught the PAH molecules in its micellar frame work. This led to increase in solubility and bioavailability. This bacterial isolate could not use anthracene as the only source of carbon but it could consume anthracene in presence of a water-soluble carbon source like glycerol. It was proved from the increased growth and biosurfactant production by this isolate in presence of an anthracene supplemented medium (AGlyMSM) in contrast to a normal glycerol medium (GlyMSM). The biosurfactant produced in AGlyMSM was not as potent as an emulsifier in comparison to that produced from GlyMSM. The consumption of the bioavailable anthracene by this microorganism had a change in the biosurfactant production and characteristics of the biosurfactants. The production of a unique biosurfactant at Rf -0.5 utilizing anthracene as carbon source was proved by TLC. Hence, anthracene was digested and excreted in form of non-toxic biosurfactants there by influencing its bioremediation (Das et al., 2008).

**2.3. Biosurfactant and microbial consortia:**

It has been indicated that the majority of the previous studies on biosurfactant-mediated biodegradation were carried out along with the use of monocultures. Biodegradation was also carried out with mixed culture. Recently it has been proved that the use of consortia increased biodegradation efficiency as compared to monocultures (Kadali et al., 2012). The cooperation between the individual consortium members and the complementary effect of microbes on each other may result in notably increased growth and survival (Sampath et al., 2012). The effect of rhamnolipid on the biodegradation capacity of 218 bacterial consortia isolated from petroleum contaminated soil with respect to changes in cell surface properties was evaluated (Owsianiak et al., 2009). It was observed that the addition of biosurfactant increased the biodegradation efficacy for slow-degrading consortia. There was a significant decrease of biodegradation rate that occurred for fast degrading consortia. This phenomenon may potentially be explained by different

substrate uptake modes. The slow-degrading consortia most likely preferred uptake of hydrocarbons from the aqueous phase, therefore solubilization of hydrocarbons enhanced the biodegradation.

**2.4. Community Structure Determination from Soil Consortium:**

There is change in bacterial community structure during degradation of PAH in contaminated soils. It was stated that there was a change in the microbial community linked with fluoranthene or phenanthrene modification and consequent biodegradation (MacNaughton et al., 1999; Vinas et al., 2005; Gandolfi et al., 2010; Muckian et al., 2009). An important study was observed in the microbial community due to naphthalene degradation. It was observed that, both TRFLP and 16S rRNA gene clone library analysis uncovered a large change in microbial community structure with increasing degradation of anthracene (Piskonen *et al*. (2005). The detailed account phylogenetic framework of the bacterial community structure is an important factor to determine the microbial consortium involved in degradation. Proteobacteria was found in the soil/water system in community structure for PAH degradation in aged PAH contaminated soil (Chang et al., 2007; Cébron et al., 2009). Numerous different bacterial species were isolated from PAH-contaminated sites which belonged to *Sphingomonas* sp. (Pinyakong et al., 2000), *Paracoccus* (Teng et al., 2010), *Comamonas* (Goyal and Zylstra, 1996), *Pseudomonas* (Jacques et al., 2005; Santos et al., 2008), *Burkholderia* (Juhasz et al., 1997), *Janibacter* (Zhang et al., 2009), and *Sphingobium.* Supplementation of PAH-degraders is a common process in degradation of PAH (Guo et al., 1997; Piskonen et al., 2005). Based on the above study, community structure in the soil consortium supplemented with phenanthrene was determined.

**3. Objectives**

1. Extraction and characterization of biosurfactant from marine bacteria *Pseudomonas aeruginosa* JP-11 and terrestrial bacteria *Bacillus subtilis* SJ301.
2. Degradation kinetics of phenanthrene by biosurfactant in culture medium and in soil consortium.
3. Interaction study of phenanthrene and biosurfactant in different conditions.
4. Community structure determination in the soil consortium.

## 4. Materials and Methods

### 4.1. Extraction of Biosurfactant:

*Bacillus subtilis* SJ301 and *Pseudomonas aeruginosa* JP-11 were isolated from the Hindustan Petroleum Corporation Limited (HPCL), Andhra Pradesh, and Paradip, Odisha were used for the study. The isolates after 16S rRNA sequencing were submitted into NCBI GenBank with the accession number KF900213 and KC771235 respectively. The isolates were revived in Luria-Bertani (LB) medium and then inoculated in Bushnell Haas Mineral (BHM) medium supplemented with 1% glucose as sole carbon source in separate flasks. Marine sediment samples were processed with additional 1.5% NaCl. These flasks were incubated at 37°C for 7 days under shaking conditions at 120 rpm. After incubation, the supernatants were collected by centrifugation at 10,000 rpm for 20 min. They were acidified by 6N hydrochloric acid (HCl) and pH was maintained to 2. Equal volumes of Chloroform: methanol mixture in the ratio 2:1 was added to the supernatants. The mixtures were vortexed vigorously and incubated overnight at 4°C. A frothy interface layer was seen in the falcon tubes. This frothy layer was extracted carefully in separate falcon tube. The falcon was centrifuged at 10,000 rpm for 5 min in order to remove the chloroform methanol mixture. The supernatant was discarded and the pellet was dried in desiccators to obtain dried crude biosurfactant (Chakraborty et al., 2014).

### 4. 2. Characterization of Biosurfactant:

The extracted crude biosurfactants were characterized with the following techniques:

#### 4.2.1. Surface Tension:

Crude biosurfactant (1 mg/ml) in distilled water of volume 50 mL was taken for the measurement of surface tension with respect to distilled water. The CMC of the crude biosurfactants and cell-free supernatants were estimated by quantifying the surface tension by the duNouy method using a ring tensiometer (Fisher Scientific, Germany) as described by Cooper et al.,1987. The respective surface tension readings obtained from tensiometer were plotted. The differences in the surface tension with respect to distilled water were determined

#### 4.2.2. Emulsification Index:

Emulsification assay was carried out using Oil of Olive, Kerosene, SDS, Tween 80 Nitschke et al., 2006. 2ml of hydrocarbon was taken in a test tube to which 1ml of cell free supernatant obtained after centrifugation of the culture was added and was vortexed for 2 min to ensure homogenous mixing of both the liquids. The emulsification activity was observed after 24 h and it was calculated by using the formula:

$$\frac{Total\ height\ of\ emulsion}{Hieght\ of\ the\ aqueous\ layer} \times 100$$

The calculations were done for both bacterial isolates individually in triplicates and their emulsification index was compared.

**4.2.3. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR):**

ATR-FITR spectroscopy of both the extracted crude biosurfactant was carried out by ATR-FTIR spectrophotometer (Bruker, Germany) with a diamond ATR objective in an open atmosphere. The spectra were collected from 650 to 4000/cm. Background spectra of water were collected prior to the measurement and were subtracted from the samples.

**4.2.4. X- Ray Diffraction Crystallography:**

The crystalline structure of the biosurfactant samples were characterized by XRD (RIgaku Miniflex X-Ray diffractometer, Japan) scanned at a range from 10°-60° with a scanning rate of 10°/min.

**4.2.5. Zeta potential measurement:**

The mean size, size distribution and zeta potential measurements of the biosurfactant from both the isolates *B. subtilis* SJ301 and *P. aeruginosa* JP-11 were performed under two different pH conditions (2.5,5.3) by NanoZetasizer (Nano series ZS90, Malvern instruments Ltd, UK).

**4.2.6. Morphology of biosurfactants:**

Field Emission scanning electron microscopy (FESEM) was used to characterize the morphology of both the biosurfactants produced by *B. subtilis* SJ301 and *P. aeruginosa* JP-11. The samples were washed with ethanol and placed on carbon tapes coated with gold and then analyzed by FESEM (Nova NoanoSEM).

**4.3. Growth and Degradation Kinetics:**

**4.3.1. Growth in phenanthrene:**

The two bacterial isolates *B. subtilis* SJ301 and *P. aeruginosa* JP-11 were inoculated in LB medium for 24 h. After incubation, the bacterial cultures were centrifuged at 6000 rpm, 10 min at 4°C. The bacterial pellet ($O.D_{600}$=0.6) of both the isolates was inoculated in BHM medium supplemented with different concentration of phenanthrene (100-1000 mg/l) as the carbon source and incubated at 180 rpm, 37°C in shaker incubator (in dark) for 7 days. Absorbance was measured at 600 nm to determine the maximum phenanthrene concentration the isolates could grow upon.

After determining the maximum phenanthrene concentration, the bacterial pellet ($O.D_{600}$=0.6) of both the isolates was inoculated in BHM medium supplemented with the specific concentration of phenanthrene and incubated at 180 rpm, 37°C in shaker incubator (in dark) for 35 days. Absorbance was measured at 600 nm to determine the growth curve.

### 4.3.2. Degradation of Phenanthrene

The two bacterial isolates *B. subtilis* SJ301 and *P. aeruginosa* JP-11 were inoculated in LB medium for 24 h. After incubation, the bacterial cultures were centrifuged at 6000 rpm, 10 min at 4°C. Degradation experimental set ups were prepared for 6 conditions for 35 days with 500 mg/l of phenanthrene (Table 1). The bacterial pellet ($O.D_{600}$=0.6) was inoculated into each tube containing BHM medium supplemented with 100 mg/l of phenanthrene and incubated at 180 rpm, 37°C in shaker incubator (in dark). At every interval of 4 days, phenanthrene was extracted with equal volume of n-hexane. The tubes were vortexed for 5 minutes and absorbance was measured at 292 nm (Tao et al., 2007).

**Table 1.** Set up for phenanthrene degradation by the isolates *B. subtilis* SJ301 and *P. aeruginosa* JP-11

| Sets | Conditions | Phenanthrene conc. (mg/l) |
|---|---|---|
| 1 | BS SJ301(5mg/ml) | 100 |
| 2 | BS SJ301 (5mg/ml) + *B. subtilis* SJ301 | 100 |
| 3 | *B. subtilis* SJ301 | 100 |
| 4 | BS JP-11 (5mg/ml) | 100 |
| 5 | BS JP-11 (5mg/ml) + *P. aeruginosa* JP-11 | 100 |
| 6 | *P. aeruginosa* JP-11 | 100 |

### 4.3.3 Degradation of Phenanthrene by soil consortium:

Soil sample was collected from the outlet of drain in NIT Rourkela, Odisha. The soil was sieved and air dried. The experimental set up was prepared supplementing with 500 mg/l phenanthrene in 6 different conditions (Table 2). After 35 days, phenanthrene was extracted by n-hexane extraction. The tubes were vortexed for 5 minutes and absorbance was measured at 292 nm (Tao et al., 2007).

**Table 2.** Set up for phenanthrene degradation by soil consorium of *B. subtilis* SJ301 and *P. aeruginosa* JP-11

| Sets | Conditions | Phenanthrene conc. (mg/l) |
|---|---|---|
| 1 | 50 g soil + *P. aeruginosa* JP-11 | 500 |
| 2 | 50 g soil + *B. subtilis* SJ301 | 500 |
| 3 | 50 g soil + *P. aeruginosa* JP-11 + *B. subtilis* SJ301 | 500 |
| 4 | 50 g soil + BS JP-11 (5mg/ml) + BS SJ301 (5mg/ml) | 500 |
| 5 | 50 g soil + SDS (5 mg/ml) | 500 |
| 6 (Control) | 50 g autoclaved soil | 500 |

**4.4. Biomass and biosurfactant production:**

**4.4.1 Orcinol Method Quantification:**

Orcinol method was used to estimate the glycolipid concentration of the crude biosurfactants produced by *B. subtilis* SJ301 and *P. aeruginosa* JP-11. Orcinol reagent was prepared by adding 0.19 g of orcinol to 47 ml of distil water and 53 ml of concentrated sulphuric acid ($H_2SO_4$). From, the set up shown in Table 1, at an interval of 4 days, 2 ml of orcinol reagent was added to 200 µl of each culture. The samples were incubated at 80$^oC$ in water bath for 20 minutes. After the samples cooled down, absorbance was taken at 421 nm in UV spectrophotometer (Agilent technologies) (Kumar and et al).

**4.4.2 Bradford Method Quantification:**

Bradford method was used to estimate protein concentration of the crude biosurfactants produced by *B. subtilis* SJ301 and *P. aeruginosa* JP-11. Bradford reagent was prepared by adding 50 mg Coomasie Brilliant Blue G250 in mixture of 25 ml of 95% ethanol and 50 ml of 85% orthophosphoric acid and volume was made up to 500 ml by adding distilled water. From, the set up shown in Table 1, at an interval of 4 days, 5 ml of Bradford reagent was added to 200 µl of each culture. The samples were incubated in dark at room temperature for 20 minutes. The absorbance of samples was taken at 595 nm in UV spectrophotometer (Agilent technologies).

**4.5 Interaction of phenanthrene with biosurfactants:**

The interaction of biosurfactants (1mg/ml) from both the isolates *B. subtilis* SJ301 and *P. aeruginosa* JP-11 was treated with different concentrations of phenanthrene (20ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm) by the following techniques:

**4.5.1 ATR-FTIR:**

The interaction study of both the isolates *B. subtilis* SJ301 and *P. aeruginosa* JP-11 with phenanthrene was studied by ATR-FTIR spectrophotometer (Bruker, Germany) with a diamond ATR objective in an open atmosphere. The spectra were collected from 650 to 4000/cm.

**4.5.2 Zeta Potential measurement:**

Zeta potential of the samples was measured by by Nano Zetasizer (Nano series ZS90, Malvern instruments Ltd, UK).

**4.5.3 Field Emission scanning electron microscopy (FESEM):**

The samples were washed with ethanol and placed on carbon tapes coated with gold and then analyzed by FESEM (Nova NoanoSEM).

**4.5.4 Fluorescence Spectroscopy:**

The fluorescence spectra of the BS solution of both the isolates *B. subtilis* SJ301 and *P. aeruginosa* JP-11 were recorded with a fluorescence spectrophotometer (Horiba Jobin, USA). A 450W Xenon lamp was used as the excitation source. EEM spectra were collected every 5 nm

over an excitation range of 200–400 nm, with an emission range of 200–550nm by 2 nm. The excitation and emission slits were set to 5 and 5 nm of band-pass, respectively. Scan speed was 1200 nm/min. The fluorometer's response to a pure water blank solution was subtracted from the fluorescence spectra recorded for samples containing BS and phenanthrene at 298K.(Pan et al., 2010).

**4.6 Community structure analysis from soil consortium:**

Community structure analysis was studied by the following methods:

**4.6.1 Genomic DNA Extraction:**

Genomic DNA was extracted from the soil microcosm set ups after 35days cultures in triplicates following the protocol of Muyzer et al. (2003). The extracted DNA was quantified using Nanodrop biophotometer (Eppendorf, Germany).

**4.6.2 PCR-DGGE:**

The V3region of 16S rRNA gene was amplified by PCR using primers 341F-GC and 518R. PCR was performed in reaction mixtures composed of 50 µl of reaction having 5X buffer, 12.5 mM $Mgcl_2$, 10 mM dNTPs, 10 pM each forward and reverse primer, and approximately 10 ng of DNA extract. The amplification conditions in the thermo cycler were as follows: initial denaturation at 94°C for 5min, 94°C for 1min, followed by 20 cycles of 65°C for 1 min with decrease of 1°C and 72 °C for 3 mins and a final 10 mins extension step at 72°C and confirmed by 0.8% gel electrophoresis and viewed under UV illumination.

Denaturing gradient gel electrophoresis (DGGE) was performed using the DCode Universal Mutation Detection System (BioRad, USA). A gradient of 40–60% was prepared using urea and formamide as denaturants. After polymerization, 50 µl of the PCR product was loaded into each well. The samples were then run at a constant voltage of 60V, temperature of 60°C for16 h. Finally, the gel was carefully stained with ethidium bromide and viewed under Gel-Doc system. There were 10 samples, 6 from the different consortium, $7^{th}$ sample was the mixture of S1, S2, S3, $8^{th}$ sample was the mixture of S4, S5, S6, and 9th sample was the mixture of S1-S6.

**4.6.3 DGGE Band Imaging and Analysis:**

The DGGE gel images were analyzed using the Quantity-One software. The number and density of the bands in each lane were used to determine the abundance, diversity and evenness. The Shannon diversity index and evenness was computed using Paleontological Statistics (PAST) software (Bacosa and Inoue, 2015).

## 5. Results

### 5.1 Extraction of Biosurfactants:

Biosurfactants were extracted from two bacterial strains one terrestrial, *Bacillus subtilis* SJ301 and one marine, *Pseudomonas aeruginosa* JP-11 by acidification of cell free supernatant. The net amount of biosurfactant extracted were 115 mg and 294 mg from 250 ml culture of *P. aeruginosa* JP-11 and *B. subtilis* SJ301 respectively (Fig. 9).

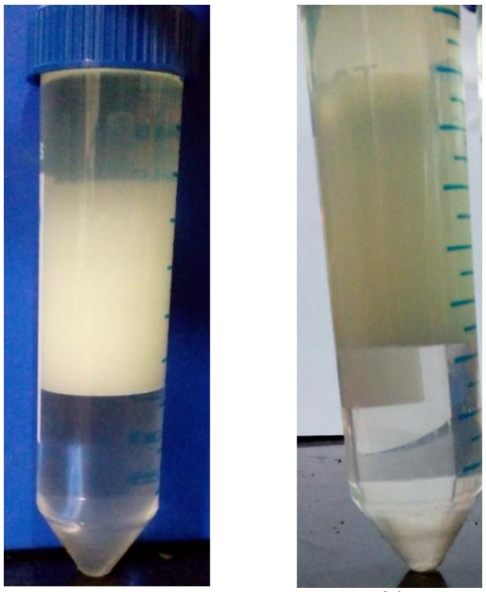



**Fig. 9.** The extracted crude biosurfactant from **a)** *P. aeruginosa* JP-11 **b)** *B. subtilis* SJ301

### 5.2. Structural and functional characterization of biosurfactants:

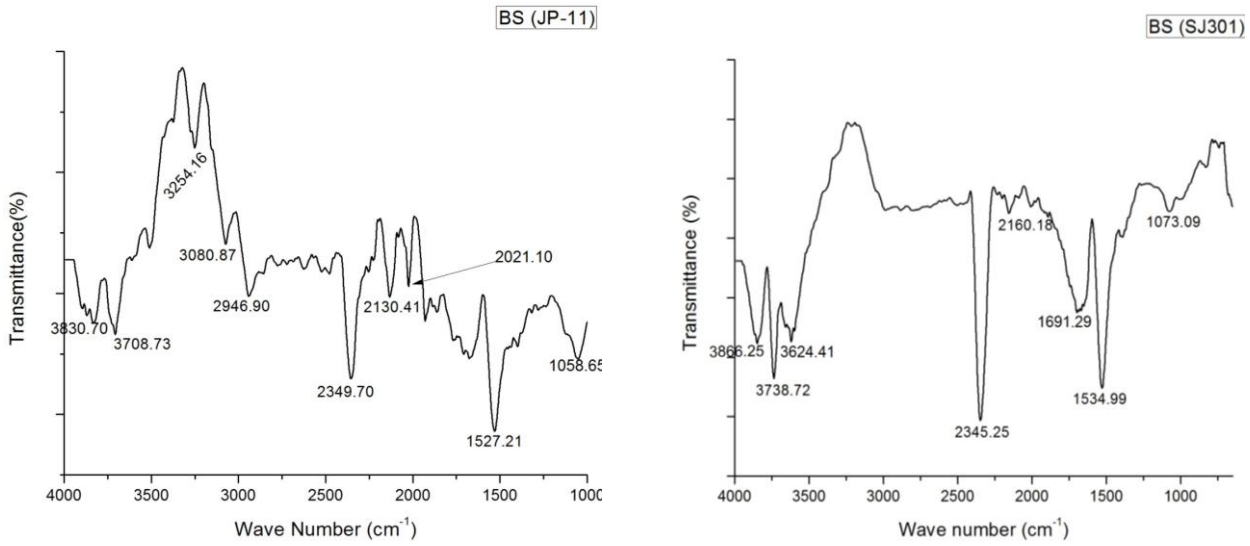
The structural and functional characterization of the crude biosurfactants from both the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301 has been represented below:

#### 5.2.1 FTIR Analysis:

ATR- FTIR determines the presence of different functional groups in the biosurfactant structure. The biosurfactant produced by bacterial isolate *P. aeruginosa* JP-11 showed peak at 3830.70 and 3708.73 $cm^{-1}$ determining the presence of O–H and N–H stretching vibrations. Peak at 3708.73 and 3254.16 $cm^{-1}$ depicted the presence of alcohol group. A peak at 3080.87 $cm^{-1}$, indicated the presence of primary and secondary amines. Another peak at 2946.90 $cm^{-1}$ revealed the presence of acid group and peak at 2349.70 $cm^{-1}$ signified the presence of sulphydryl group. Another peak observed at 2130.41 $cm^{-1}$ showed the presence of an alkylene group. Peaks at 2021.10 $cm^{-1}$ and 1527.21$cm^{-1}$ reflected the presence of nitro compounds. A peak at 1058.65 $cm^{-1}$ indicated the presence of an alkoxy group (Fig. 10a).

The biosurfactant produced by bacterial isolate *B. subtilis* SJ301 showed the presence of peak at 3866.25 and 3738.72 $cm^{-1}$ corresponding to the O-H and N-H stretching vibrations. Another peak observed at 3624.41 $cm^{-1}$ determined the presence of alcohols and phenols in the

surfactant. A peak at 2345.25 $cm^{-1}$ demonstrated the presence of sulphydryl group. Peak present at 2160.18 $cm^{-1}$ indicated the presence of alkynes. Peak at 1691.29 $cm^{-1}$ refers to the C-O stretching mode with carbonyls (unsaturated aldehydes, ketones) functional group. Peak at 1534.99 $cm^{-1}$ showed the presence of asymmetric N-O stretch which is present in nitro compounds. A peak at 1073.09 $cm^{-1}$ indicated the presence of alkoxy groups like alcohols, carboxylic acid, esters and ethers associated with phosphate and polysaccharide moieties (Fig. 10b).



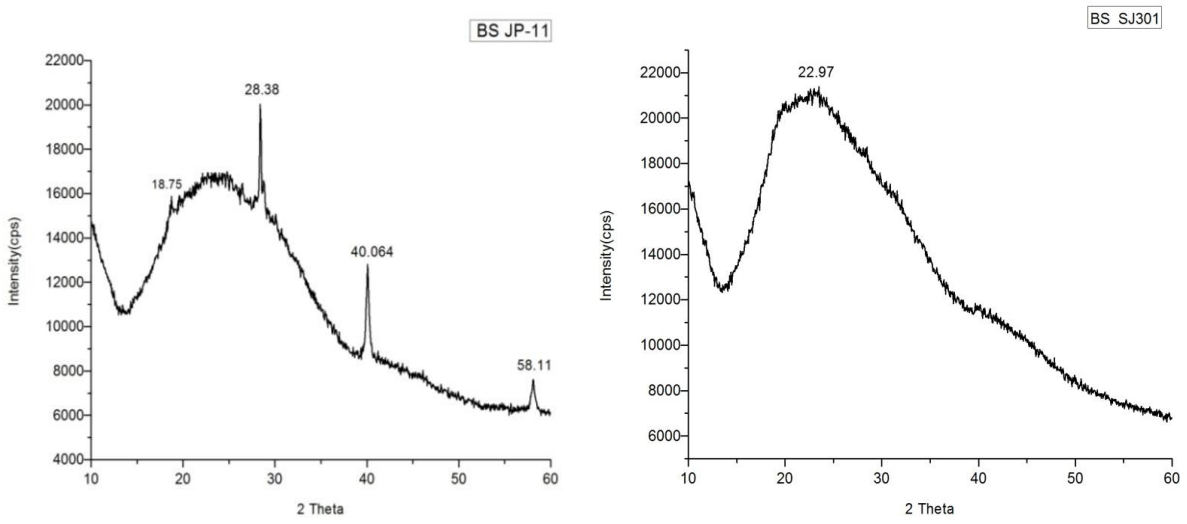

**a)** **b)**

**Fig. 10.** ATR-FTIR spectra of the crude biosurfactant from the isolate **a)** *Pseudomonas aeruginosa* JP-11, **b)** *Bacillus subtilis* SJ301

**5.2.2 XRD Analysis:**

The biosurfactant produced by bacterial isolate *P. aeruginosa* JP-11 showed peaks at 2θ = 58.2, 40.1,28.3 with d spacing values at 4.73, 3.14, 2.24,1.58 respectively.

The biosurfactant produced by bacterial isolate *B. subtilis* SJ301 showed peaks at 2θ = 22.97 with d spacing at 3.25 (Fig. 11).




**a)** **b)**

**Fig. 11.** XRD analysis of the crude biosurfactant from the isolate, **a)** *P. aeruginosa* JP-11, b) *B. subtilis* SJ301

### 5.2.3. Zeta potential measurement:

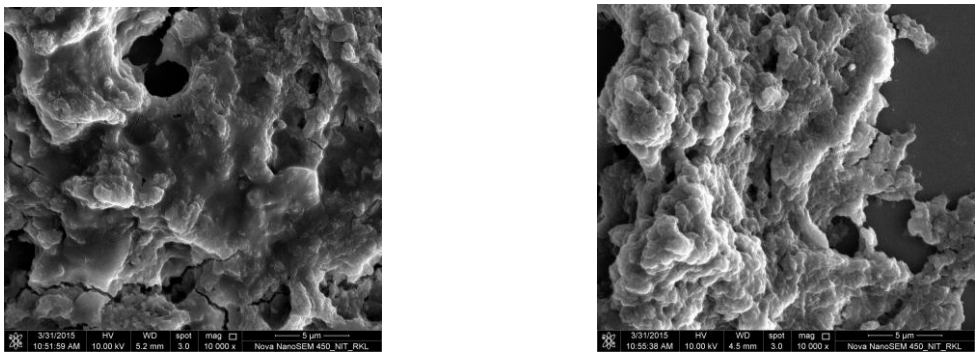
The surface charge of biosurfactants of *P. aeruginosa* JP-11 and *B. subtilis* SJ301 were -0.553 and -0.0165 mV at pH 2, whereas it was 1.85 and 0.242 mV at pH 6 (Table 3).

**Table 3.** Zeta potential measurement and particle size analysis of the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301

| Sample | pH | Zeta potential (mV) | Size ( nm) |
|---|---|---|---|
| BS JP-11 | 2.5 | -0.553 | 163.6 |
| BS JP-11 | 6 | 1.85 | 297.1 |
| BS SJ301 | 2.5 | -0.0165 | 957.2 |
| BS SJ301 | 6 | 0.242 | 716.5 |

### 5.2.4. Field emission scanning electron microscopy (FESEM):

The FESEM micrographs of the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301 showed the morphology of the crude biosurfactants (Fig. 12).

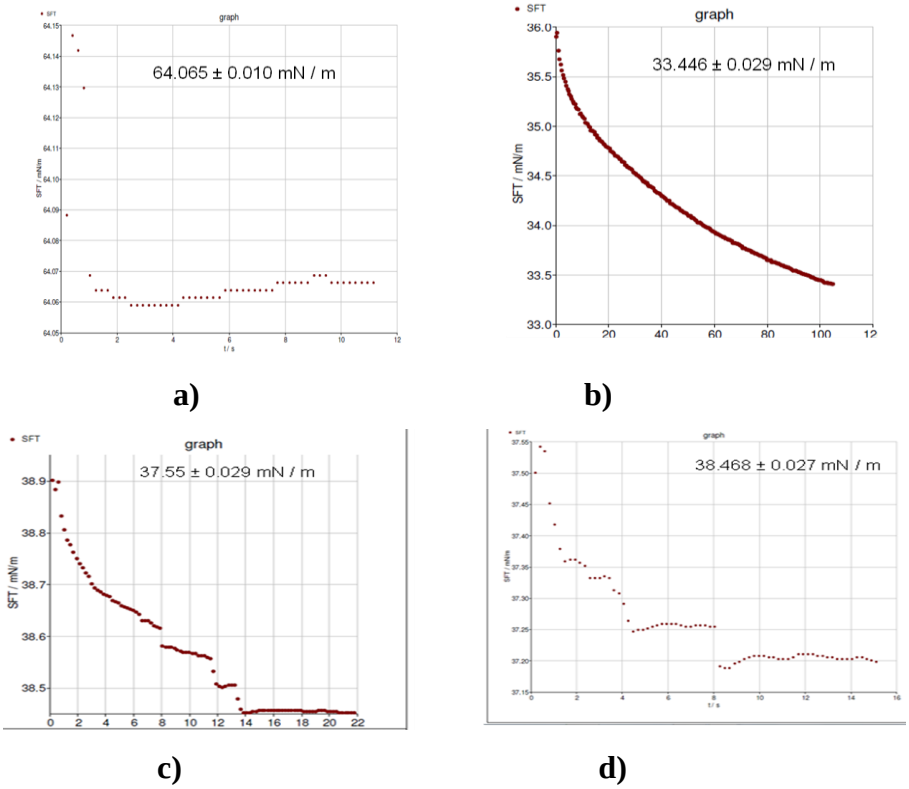


**a)** **b)**

**Fig. 12.** FESEM image of crude biosurfactant of the isolates **a)** *P. aeruginosa* JP-11, **b)** *B. subtilis* SJ301

### 5.2.5. Chemical Characterisation of biosurfactants:

The surface tension values of distilled water, BS JP-11, BS SJ301 and SDS were 64.065 ± 0.010 mN/m, 33.446 ± 0.029 mN/m, 37.15 mN/m and 38.468 ± 0.027 mN/m respectively (Fig. 13). BS JP-11 produced by *P. aeruginosa* JP-11 was found to have the lowest surface tension than the other sets. Lower the surface tension, higher is the surfactant nature. Hence, BS JP-11 proved to be a potential biosurfactant than produced by *B. subtilis* SJ301.




**a)** **b)**

**c)** **d)**

**Fig. 13.** Surface tension measurement of the crude biosurfactants of **a)** distilled water, **b)** BS JP-11, **c)** BS SJ301, **d)** SDS.

### 5.2.4 Emulsification Index:

It determines the capacity of the biosurfactant to form emulsion (Table 4).

**Table 4.** Emulsification Index

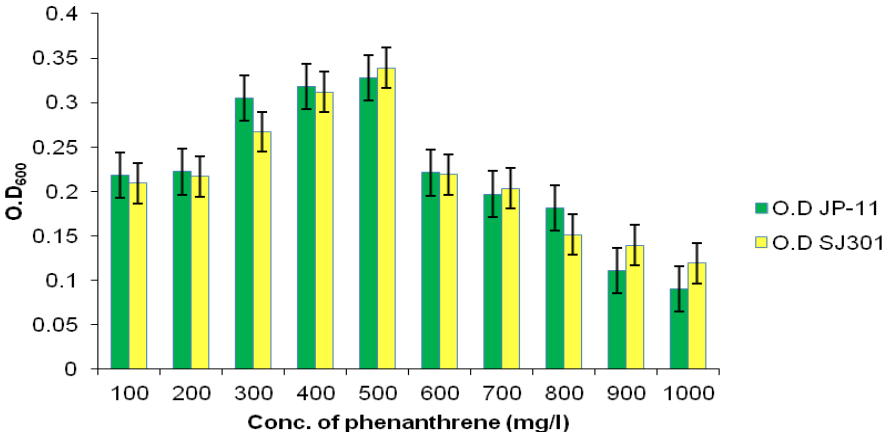
| Bacterial Culture | Control (Tween 80+ Kerosene) | Control (Tween 80+Oil of Olive) | Culture + kerosene | Culture + Oil of olive |
|---|---|---|---|---|
| *P. aeruginosa* JP-11 | 100% | 100% | 55.89% | 52.94% |
| *B. subtilis* SJ301 | 100% | 100% | 58.82% | 55.86% |

It was evident that emulsification index of *Bacillus subtilis* SJ301 was more than *Pseudomonas aeruginosa* JP-11. Emulsion formed by *Bacillus subtilis* SJ301 with kerosene was higher than emulsion with oil of olive.

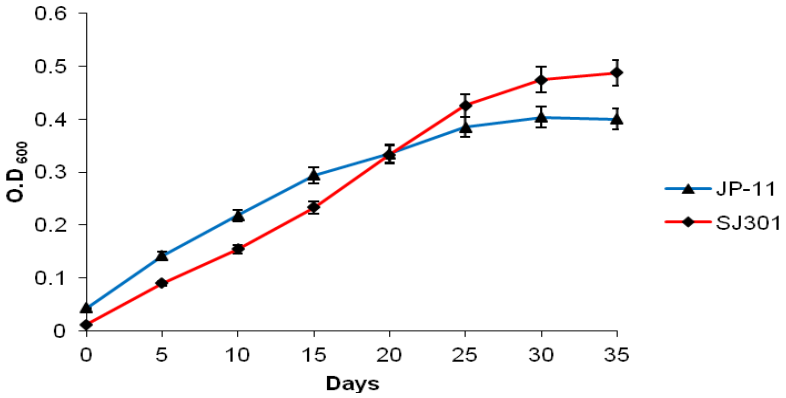
### 5.3. Degradation Kinetics of Phenanthrene

#### 5.3.1. Growth curve in Phenanthrene:

It was demonstrated that the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301 could grow at a range of 100-500 mg/l with 500 mg/l as the optimum concentration. The growth curve of both the isolates was shown in the figure (Fig. 14).




**a)**




**b)**

**Fig. 14.** Growth curve of the isolates *P. aeruginosa* JP-11 and *B.subtilis* SJ301, a) in different concentration of phenanthrene (100-1000 mg/l), b) in 500 mg/l of phenanthrene.

#### 5.3.2. Degradation of phenanthrene in culture medium:

Degradation of phenanthrene by the isolates demonstrated that after 35 d, 55.52+2.21 mg/l of phenanthrene was not degraded by the culture of *B. subtilis* SJ301 supplemented with BS of SJ301. Whereas, phenanthrene degradation was similar in case of *P. aeruginosa* JP-11 supplemented with BS and BS of JP-11, where residual phenanthrene concentration was 45.78±3.1 and 45.62±1.60 mg/l respectively (Table 5, Fig. 15).

**Table 5.** Degradation rate of phenanthrene in culture medium by the isolate *P. aeruginosa* JP-11 and *B.subtilis* SJ301

| Conditions | Residual phenanthrene (mg/l) in days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **0** | **5** | **10** | **15** | **20** | **25** | **30** | **35** |
| **BS- SJ301** | 96.98 ±1.16 | 91.75 ±0.60 | 87.25 ±1.18 | 84.84 ±1.94 | 80.34 ±1.34 | 73.27 ±1.15 | 67.12 ±2.31 | 62.93 ±1.37 |
| **BS-SJ301+ SJ301(culture)** | 98.81 ±0.31 | 92.79 ±1.15 | 89.36 ±3.64 | 82.56 ±0.66 | 78.01 ±2.73 | 72.53 ±1.82 | 60.26 ±1.06 | 55.52 ±2.21 |
| **SJ301(culture)** | 98.32 ±0.83 | 91.26 ±1.22 | 88.25 ±1.27 | 82.03 ±2.59 | 74.99 ±2.07 | 62.4 ±2.01 | 60.54 ±1.53 | 56.29 ±1.94 |
| **BS-JP-11** | 98.73 ±1.22 | 92.98 ±1.48 | 82.08 ±1.34 | 79.86 ±1.32 | 69.1 ±1.64 | 62.09 ±1.86 | 54.29 ±2.32 | 48.34 ±2.10 |
| **BS-JP-11+ JP-11(culture)** | 99.38 ±1.75 | 92.82 ±1.08 | 85.83 ±1.46 | 78.33 ±2.03 | 70.43 ±1.31 | 59.26 ±1.91 | 51.21 ±1.33 | 45.78 ±3.1 |
| **JP-11(Culture)** | 99.24 ±1.19 | 91.14 ±1.57 | 86.86 ±1.50 | 79.63 ±0.67 | 69.23 ±2.09 | 61.18 ±1.51 | 51.6 ±0.70 | 45.62 ±1.60 |
| Control | 99.16 ±0.52 | 97.72 ±0.72 | 97.3 ±1.74 | 96.81 ±0.43 | 96.5 ±0.83 | 95.59 ± 0.99 | 95.59 ±0.99 | 94.4 ±0.14 |

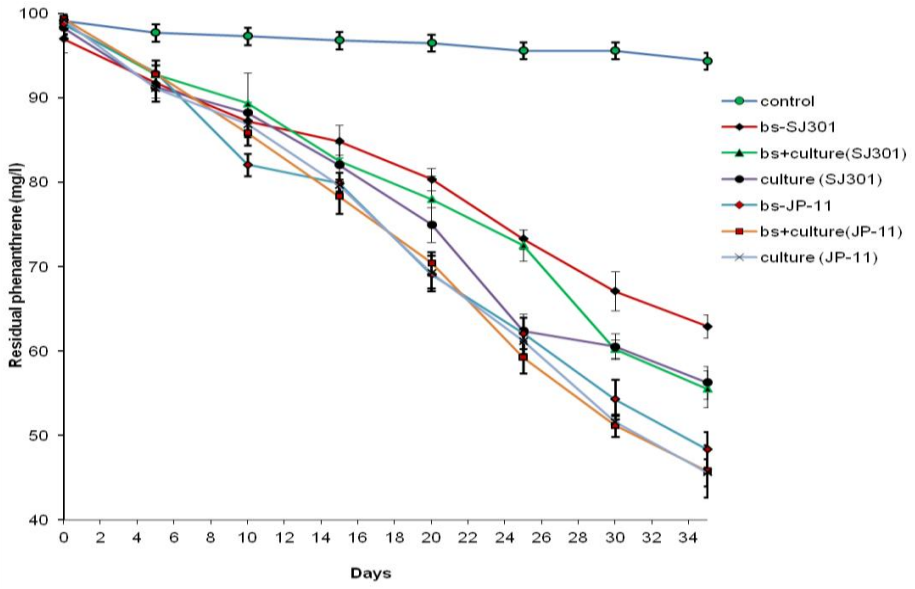
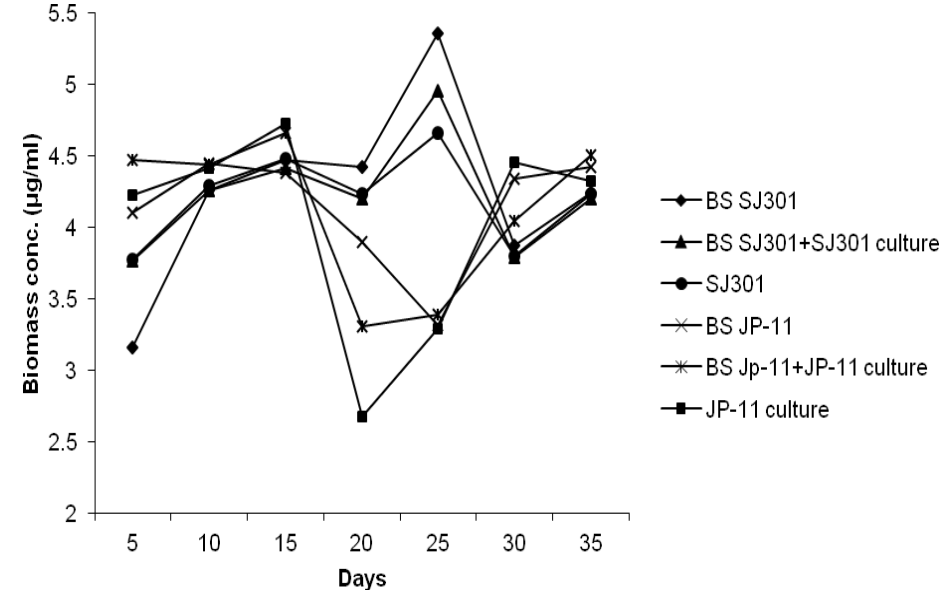


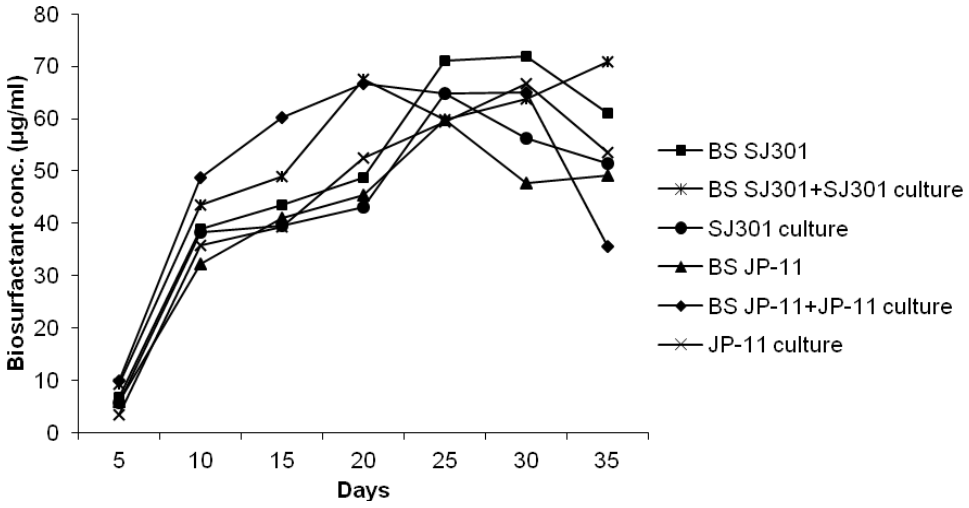

**Fig. 15.** Degradation kinetics of phenanthrene in different conditions by *P. aeruginosa* JP-11 and *B. subtilis* SJ301.

**5.3.3. Biomass and biosurfactant production:**

The graph showed the biomass production and concentration of biosurfactants produced by both the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301 (Fig. 16).




**a)**




**b)**

**Fig. 16. a)** Biomass production, b) biosurfactant production of both the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301 and their biosurfactant in different conditions.

**5.3.4. Degradation of phenanthrene in soil consortium:**

Consortium study of both the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301 in the presence and absence of biosurfactants showed that maximum degradation of phenanthrene was done by consortium culture of *P. aeruginosa* JP-11 and *B. subtilis* SJ301 followed by *B. subtilis* SJ301 culture with the percentage of phenanthrene degradation of 86.15 and 85.73 % respectively (Fig. 17). The least degradation was seen in control which contained autoclaved soil with no bacterial culture.

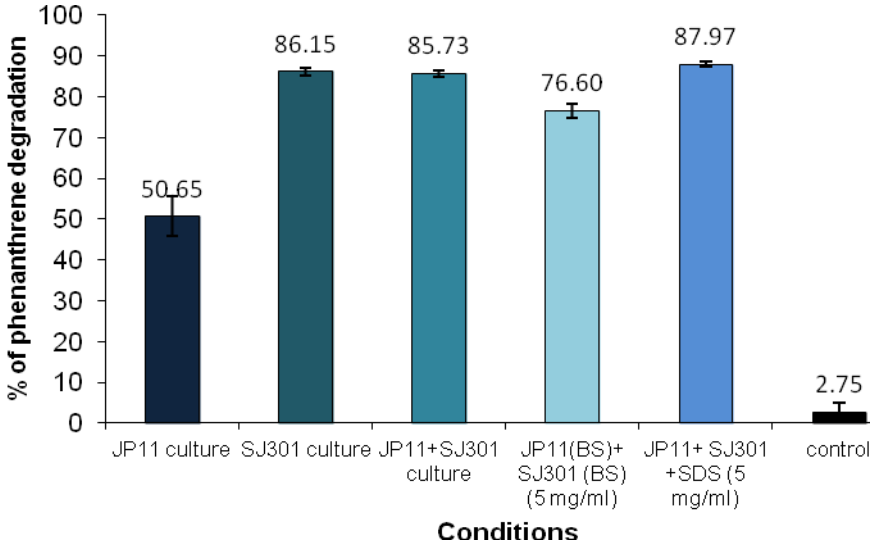



**Fig. 17.** Degradation of phenanthrene by soil consortium supplemented with *P. aeruginosa* JP-11 and *B. subtilis* SJ301

**5.4. Interaction study of phenanthrene with biosurfactants:**

Interaction study of biosurfactants by both the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301 studied in different concentration of phenanthrene (20, 40, 60, 80, 100 ppm) by ATR-FTIR spectroscopy, Zeta potential measurement, fluoroscence spectroscopy and Field Emission Scanning Electron Microscopy were discussed as following:

**5.4.1 ATR-FTIR Analysis:**

On supplementation with increasing concentration of phenanthrene with the biosurfactant produced by *P. aeruginosa* JP-11 there was shift in the peaks as shown in Table 6, 7 (Fig. 18).

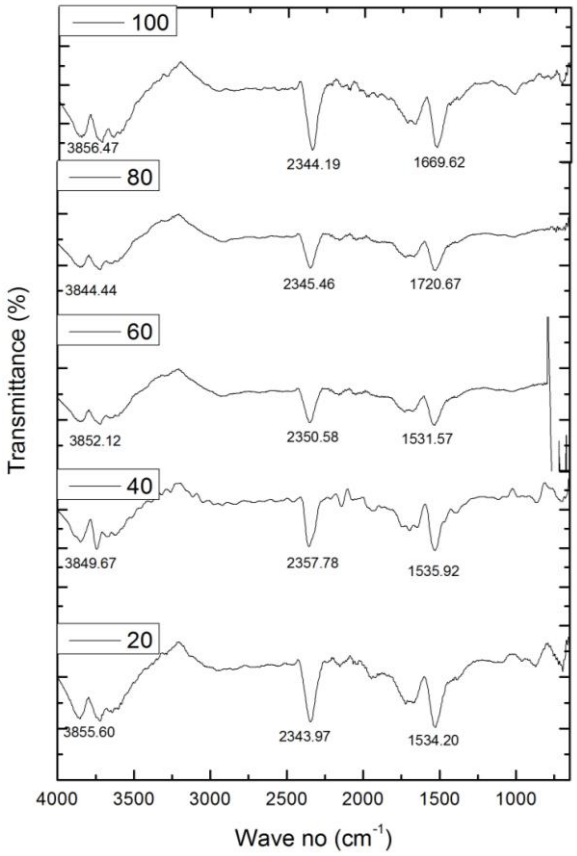
**Table 6**. Shift of wave numbers ($cm^{-1}$) of the functional groups in ATR-FTIR spectra of BS of *P. aeruginosa* JP-11 supplemented with different phenanthrene concentrations.

| **Functional group** | **Wave no ($cm^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | **BSJP-11** | **BSJP-11+20 mg/l phen** | **BSJP-11+40 mg/l phen** | **BSJP-11+60 mg/l phen** | **BSJP-11+80 mg/l phen** | **BSJP-11+100 mg/l phen** |
| -OH and -NH | 3830.70 | 3855.60 | 3849.67 | 3852.12 | 3844.44 | 3856.47 |
| stetch | 3708.73 | 3715.68 | 3746.18 | 3722.01 | 3720.65 | 3718.24 |
| -OH stretch | 3254.16 | - | - | - | - | 3641.94 |
| Primary secondary amines | 3080.87 | - | - | - | - | - |
| Carboxylic | 2946.90 | - | - | - | - | - |

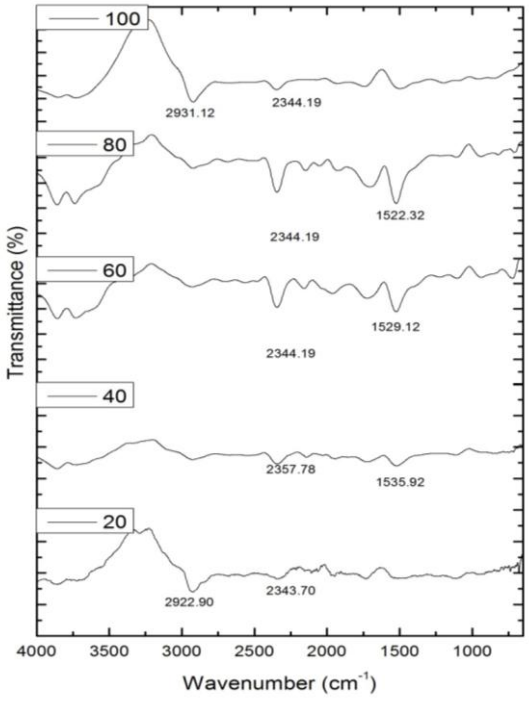
| | | | | | | |
|---|---|---|---|---|---|---|
| group | | | | | | |
| S-H stretch | 2349.70 | 2343.97 | 2357.78 | 2350.58 | 2345.46 | 2344.19 |
| Alkyl group | 2130.41, 2021.10 | - | 2137.21 | 2158.63 | - | - |
| Amide C=O stretch | - | 1701.66 | - | - | 1720.67 | 1669.62 |
| Nitro group | 1527.21 | 1534.20 | 1535.92 | 1531.57 | 1534.20 | 1529.12 |
| Phosphate (P=O stretch) | 1058.65 | - | - | - | | 1018.48 |
| Alkyl halide (Chlorine group) | - | 881.47 | 866.65 | - | - | - |
| Alkyl halide | - | 694.65 | - | - | - | 707.26 |

**Table 7.** Shift of wave numbers ($cm^{-1}$) of the functional groups in ATR-FTIR spectra of BS of *P. B. subtilis* SJ301 supplemented with different phenanthrene concentrations.

| **Functional group** | **Wave no ($cm^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | **BS SJ301** | **BSSJ301+ 20 mg/l phen** | **BS SJ301+40 mg/l phen** | **BS SJ301+60 mg/l phen** | **BS SJ301+80 mg/l phen** | **BS SJ301+100 mg/l phen** |
| -OH and -NH stetch | 3866.25 3738.72 3624.41 | 3861.36 - | 3849.67 3746.18 | 3856.47 3731.83 | 3856.47 3738.63 | 3849.67 3718.24 |
| Carboxylic group | - | 2922.90 | - | 2924.32 | 2931.12 | 2931.12 |
| S-H stretch | 2345.25 | 2343.70 | 2357.78 | 2344.19 | 2344.19 | 2344.19 |
| Alkyl group | 2160.18 | - | 2137.21 | 2157.60 | 2144.01 | - |
| Amide C=O stretch | 1691.29 | 1729.29 | | 1722.50, 1971.02 | 1702.11 | - |
| Nitro group | 1534.99 | - | 1535.92 | 1529.12 | 1522.32 | 1494.37 |
| Phosphate (P=O stretch) | 1073.09 | 1115.97 | - | 1108.37 | 942.19 | - |
| Alkyl halide (chlorine group) | - | - | 866.65 | - | - | - |
| Alkyl halide | - | - | 693.66 | - | - | - |




**a)**




**b)**

**Figure 18:** ATR-FTIR spectra of phenanthrene (20, 40, 60, 80, 100 mg/l) with interaction with BS of **a)** *P. aeruginosa* JP-11 and **b)** *B. subtilis* SJ301

The major functional groups involed in BS interaction was -OH and -NH stretch, S-H stretch, P=O stretch in both the isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301

**5.4.2 Fluorescence Spectroscopy:**

Fluorescence spectroscopy was used to investigate phenanthrene interaction with the biosurfactants of *P. aeruginosa* JP-11 and *B. subtilis* SJ301. With an increase in concentration of phenanthrene from 20 ppm to 100 ppm, fluorescence intensity decreased in both the isolates (Fig 19). The number of binding site available for binding phenanthrene and the binding constant was calculated using fluorescence spectroscopy. For understanding the fluorescence quenching of protein like fluorophore present in the BS of both isolates by phenanthrene fluorescence titration experiments were performed. The protein like fluorescence peak at 285/310 EX/EM was noticeably quenched by phenanthrene indicating that there was an interaction of phenanthrene with the protein like fluorophore known as fluorescence quenching. Stern-Volmer equation usually fits with the fluorescence quenching (Lakowicz, 2006).

$$F_0/F=1+ K[Q]=1+kq\tau_0[\text{Phenanthrene}] \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{eq.(1).}$$

In this equation, $F_0$ and F are the fluorescence intensities of the fluorophore in the absence and presence of quencher (phenanthrene) respectively. K is the Stern-Volmer quenching rate constant and kq the quenching rate constant of the biological macromolecule. $\tau_0$ is the average lifetime of the molecule which is equal to $10^{-8}$s for the biopolymer (Pan et al., 2010). A linear curve, $R^2 > 0.96$ and $R^2 > 0.92$ was obtained for *P. aeruginosa* JP-11 and *B. subtilis* SJ301 respectively and $F_0/F$ and [Phenanthrene] was obtained (Fig.).

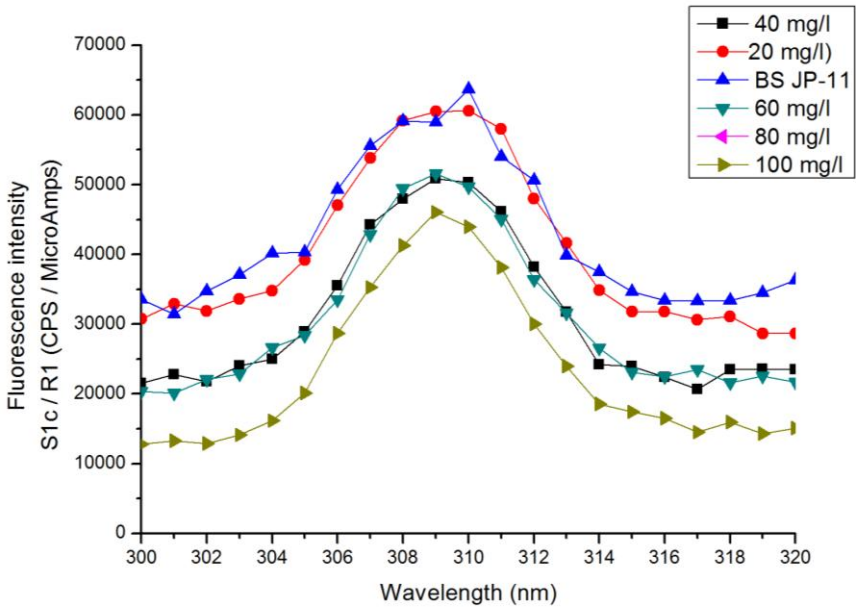
The modified Stern-Volmer equation is applied to the fluorescence quenching data to calculate binding site number and binding energy (Lakowicz, 2006).

$$\text{Log } (F_0 - F)/F = \text{Log } K_b + n \text{ log [Phenanthrene]} \quad \ldots\ldots\ldots\ldots\ldots \text{ eq.(2).}$$

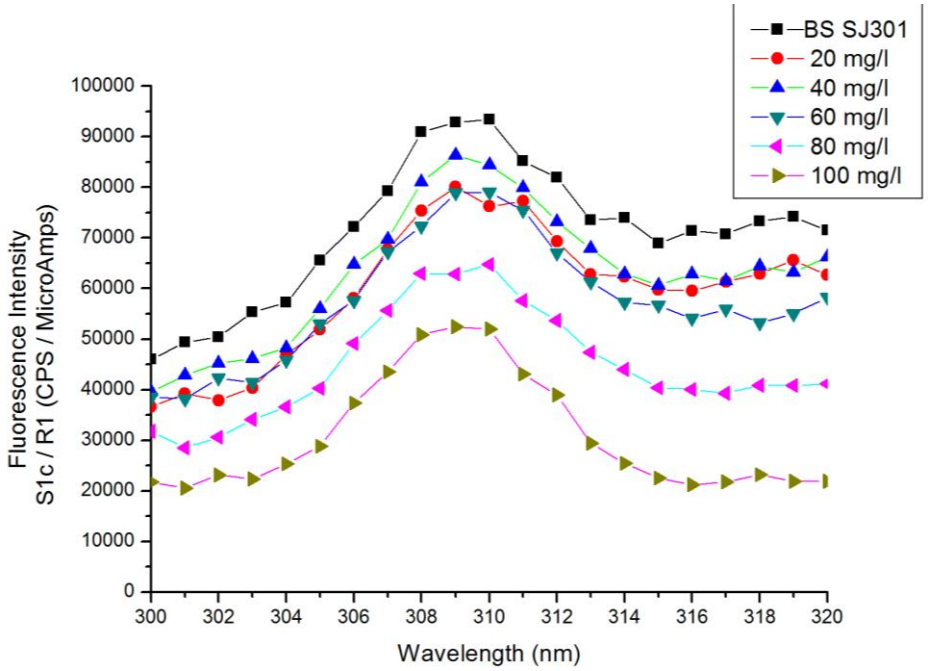
$K_b$ represents the binding constant for quencher-protein interaction and n is the number of binding sites present the fluorophore. The intercept of y axis of the plot log $(F_0 - F)/F$ against log [phenanthrene] gives the value of binding constant $K_b$ and the number of binding site n can be determined from the slope of the plot (Fig. 20). Only one binding site is present in the fluorophore of *P. aeruginosa* JP-11as the n value obtained from the eq (2) 0.595 and the value of binding constant $K_b$ is 3.32 $M^{-1}$. Similarly, there are 3 binding site present in the fluorophore of *B. subtilis* SJ301 as the n value obtained from the eq(2) 2.72 and the value of binding constant $K_b$ is 4.08 $M^{-1}$ .The spontaneity of the binding reactions was calculated using the thermodynamic Gibbs free energy equation

$$\Delta G= -RT\ln K_b \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{eq. (3).}$$

(R is the universal gas constant having value 0.008314kJ/K/mole and T is the room temperature in kelvin). The fluorescence quenching reaction takes place spontaneously at room temperature i.e. 298 K as the $\Delta G$ value calculated using eq (3) is -2.89 and -3.40 kJ/K/mol for *P. aeruginosa* JP-11and *B. subtilis* SJ301 respectively (Fig. 20).




**a)**




**b)**

**Fig. 19.** The exemplified fluorescence spectra of BS a) *P. aeruginosa* JP-11, b) *B. subtilis* SJ301 with increasing phenanthrene (20 ppm, 40ppm , 60 ppm, 80 ppm, 100 ppm) concentration at 293K.

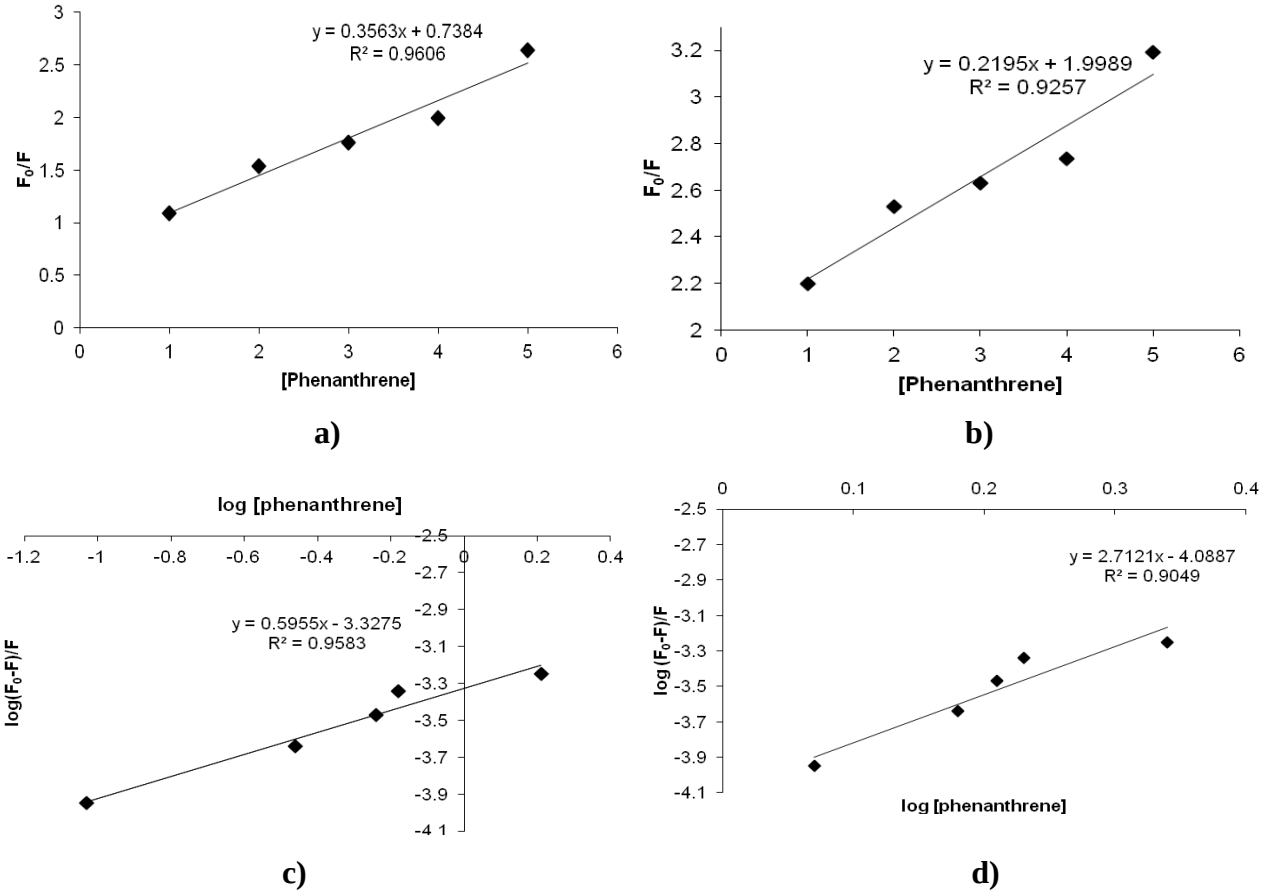
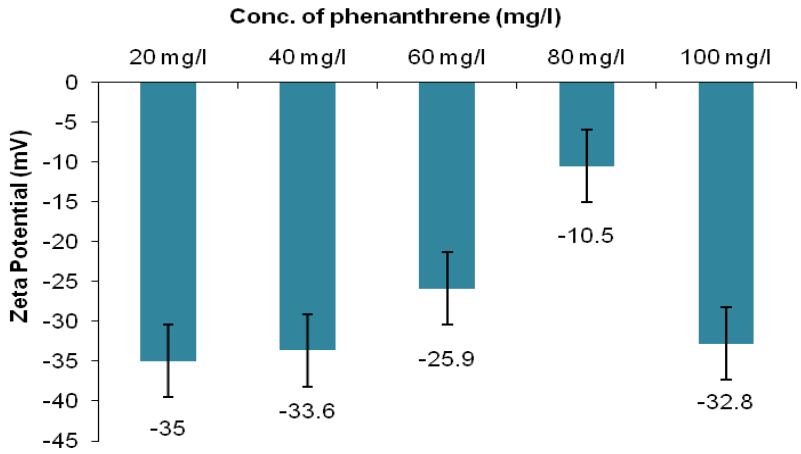
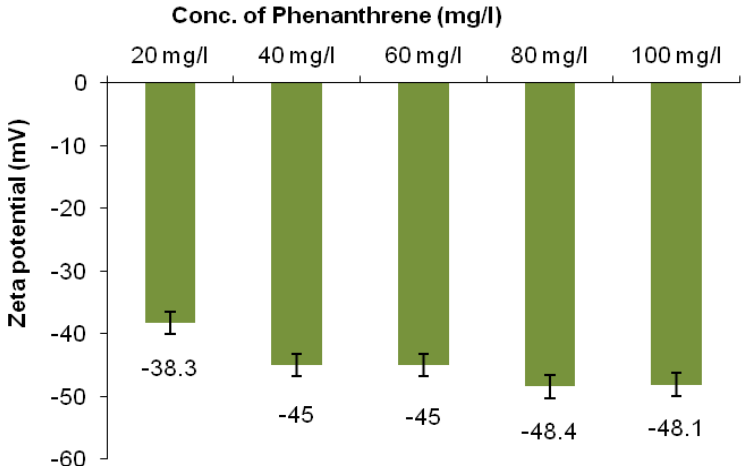



**Fig. 20.** Plot of $F_0/F$ vs phenanthrene concentration, **a)** *P. aeruginosa* JP-11, **b)** *B. subtilis* SJ301 and plot of log $(F_0-F)/F$ versus phenanthrene concentration, **c)** *P. aeruginosa* JP-11, **d)** *B. subtilis* SJ301.

### 5.4.3. Zeta potential:

Analysis of zeta potential helps to determine the behaviour of biosurfactants in solutions by giving the surface charge of the sample with different concentration of phenanthrene. The following Fig. 21 determined zeta-potential of BS JP-11 and BS SJ301 with increasing concentration of phenanthrene (20-100 mg/l).





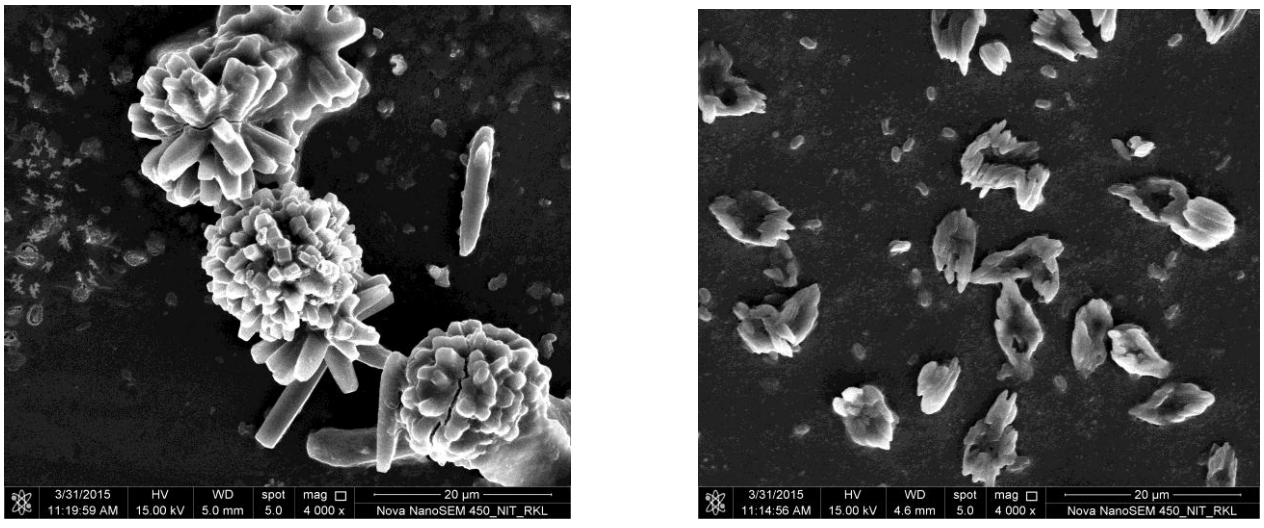

**b)**

**Fig. 21.** Interaction of 20 ppm, 40 ppm , 60 ppm, 80 ppm, 100 ppm of phenanthrene with BS of **a)** *P. aeruginosa* JP-11, **b)** *B. subtilis* SJ301.

In case of BS JP-11, there is a decrease in negative charge followed by increase. This indicates decrease in stable interaction with phenanthrene from 20 mg/l to 80 mg/l phenanthrene followed by an increase in 100 mg/l. In BS SJ301, there is an increase in negative charge with increasing phenanthrene concentration from 20 to 100 mg/l. This indicates stable BS-phenanthrene interaction with increasing phenanthrene concentration.

### 5.4.4 Field Emission Scanning Electron Microscope (FESEM):

The interaction of extracted biosufactants of the two isolates *P. aeruinosa* JP-11 and *B. subtilis* SJ301 with phenanthrene were seen under FESEM which showed the formation of micelles (Fig. 22).




**a)** **b)**

**Fig. 22.** FESEM image of crude biosurfactant of the isolates **a)** *P. aeruginosa* JP-11, **b)** *B. subtilis* SJ301, interacted with 500 mg/l of phenanthrene.

**5.6 Community Structure Study:**

**5.6.1 Genomic DNA Extraction:**

The bacterial cells were collected from soil sample under six different conditions. The figure 23 shows the gel containing different bands of DNA extracted from the six different conditions as mentioned in Table 2.

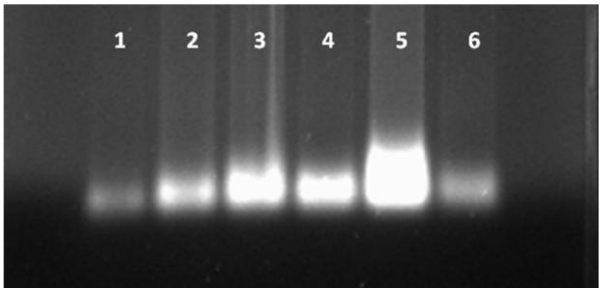



**Fig. 23.** Genomic DNA extracted from the soil consortium from the 6 different sets

The amount of genomic DNA obtained at six different conditions were measured by Nanodrop (Table 7).

**Table 8.** Quantity of genomic DNA obtained under six different conditions

| Conditions | Quantity Of DNA(ng/µl) |
|---|---|
| 1 | 373.6 |
| 2 | 831.8 |
| 3 | 713.5 |
| 4 | 725.4 |
| 5 | 1158.8 |
| 6 | 385.3 |

**5.6.2 Polymerase Chain Reaction (PCR) of the DNA extracted from the consortium by 16S-GC clamp primers:**

Bands were detected in 1.5% agarose gel (Fig. 24) showing amplification of 16S rRNA genes with the 16S GC clamp primers.

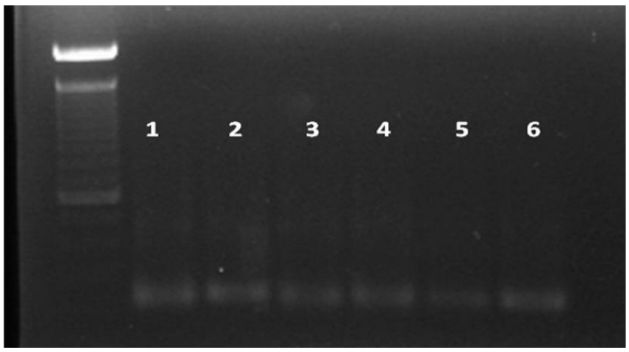



**Fig. 24.** Gel showing PCR amplification of the DNA

### 5.6.3 DGGE band imaging and analysis

The gel shows the community change in the DGGE gel (Fig. 25).

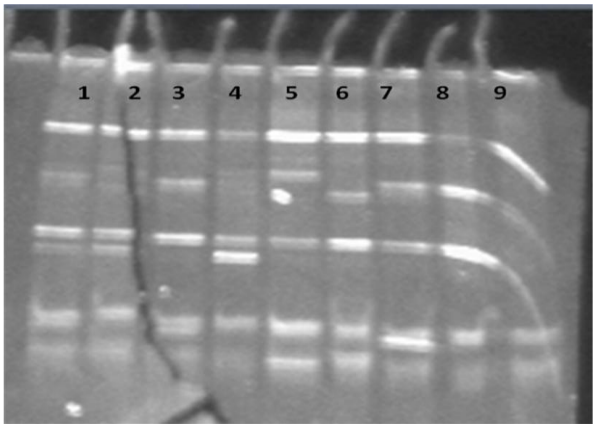



**Fig. 25.** DGGE profile of the 16S rRNA genes amplified from the microbial consortia

### 5.6.4 Phylogenetic tree construction:

Dendrograms was constructed from the DGGE lanes from the soil consortium grown in phenanthrene. Microbial consortium present in sample 1, 9, 10 have similar microbial communities arising from single node, whereas microbial consortium present in sample 2, 7, 5 belongs to different node. Microbial consortium present in sample 8, 6, 3 arise from different node. This shows the community structure change in the soil consortium.

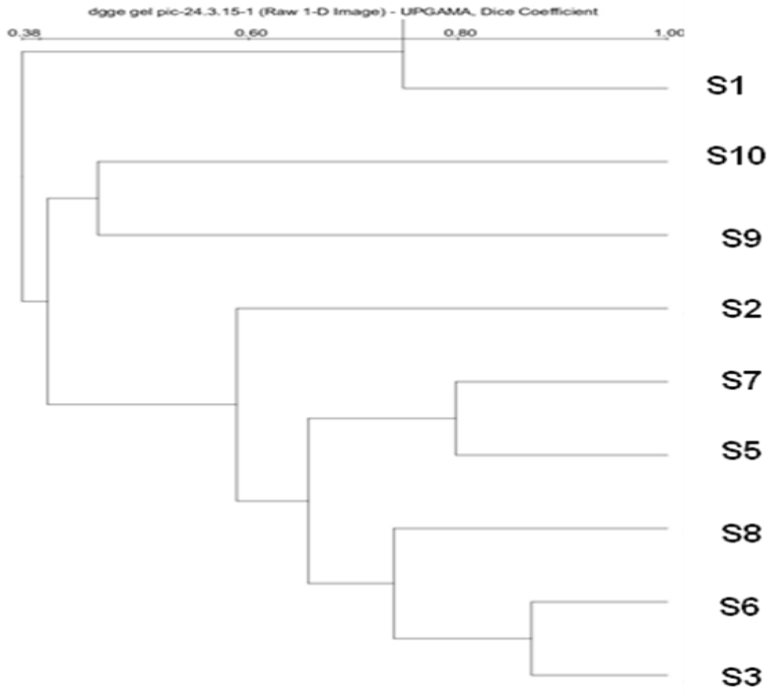



**Fig. 26.** Phylogenetic tree from the DGGE gel by Quantity One Software

## 6. Discussion

The present study imparts us with an approach to degrade phenanthrene, a poly aromatic hydrocarbon by biosurfactants produced by marine bacteria *P. aeruginosa* JP-11 and terrestrial bacteria *B. subtilis* SJ301. The bacterial isolates of marine and terrestrial origin are considered to be prospective agents for bioremediation of PAHs (Shin et al., 2006). Diverse types of aliphatic and aromatic hydrocarbons used for growth as sole source of carbon led to increased production of biosurfactants by the two bacterial isolates (Onwosi et al., 2012).

Diverse types of biosurfactants are derived from marine and terrestrial bacteria having huge application in bioremediation. In our study, we have given a detailed characterization of BS from marine and terrestrial bacteria as well as their application in phenanthrene degradation as single cultures and mixed microbial consortia. Zhang et al. (2009) reported similar study taking anthracene as a PAH model. The structural and chemical characterisation was done by ATR- FTIR analysis and X-Ray diffraction crystallography. ATR-FTIR analysis, demonstrated alkane stretch, carboxylic acid group, primary and secondary amines, nitro compounds and alcohol groups as the primary functional groups present in the crude biosurfactant in *P. aeruginosa* JP-11 with peaks ranging between 3830 to 1058 $cm^{-1}$ wave number. In addition to the above functional groups, peaks of esters, alkynes were also in *B. subtilis* SJ301 with peaks ranging from 3866 to1073 $cm^{-1}$. Singh et al. (2011) reported nearly similar peaks at different wave numbers ranging from 3428 to 843 $cm^{-1}$ showing presence of C═O and C–N vibration, sulfhydral group, C-O and C-N vibration and glycosidic linkage respectively. In X ray diffraction analysis, different peaks were obtained at 2θ values ranging between 58.2 to 28.3 with d spacing values at 4.73 to 1.58 respectively in *P. aeruginosa* JP-11 and *Bacillus subtilis* SJ301 showed peaks at 2θ values 22.97 with d spacing at 3.25. This disclosed the amorphous nature of BS in both the bacterial isolates *P. aeruginosa* JP-11 and *B. subtilis* SJ301. This study was similar to the XRD pattern of EPS obtained from *B. Licheniformis* (Singh et al., 2011). It illustrated the presence of several peaks within the range of 2θ=22.89 to 32.49. Chemical characterisation of the biosurfactants were analysed by surface tension and emulsification index. Benicasa et al. (2004) stated a range of surface tension between (25 mN/m to 60 mN/m) in different classes of biosurfactants. In our study, it was observed that BS from *P. aeruginosa* JP-11 had the least value of surface tension which was 33.446 ± 0.029 mN /m with respect to BS of *B. subtilis* SJ301 which had surface tension value of 37.55 ± 0.029 mN /m. This surface tension was analysed with comparison to distilled water whose surface tension value was determined to be 64.065 ± 0.010 mN /m. Hence, BS JP-11 proved to be an effective biosurfactant. Emulsifaction index of BS SJ301 was 58.82 % and 55.8% with kerosene and oil of olive repectively whereas, emulsification index by BS JP-11 was 55.8% and 52.94% with kerosene and oil of olive respectively. This study was in accordance with study by Singh et al.

(2011) where they got emulsification index of 32.14% and 3.9% in petrol, 38.43% and 10.2% in ground nut oil, 30.88% and 10.29% in toluene, 28.12% and 8.68% in mineral oil, 36.25% and 12.64% in Tween-80 and 33.94% and 11.67% in xylene after 30 min and 60 min, respectively.

Degradation kinetics of phenanthrene was studied with six set of conditions for time point of 35 days in two aspects. First aspect was stated to be the degradation of phenanthrene in culture medium and the second aspect was phenanthrene inoculated in soil of six different sub-conditions. The phenanthrene degradation in culture medium was highest in culture medium of JP-11 supplemented with BS of JP-11 having phenanthrene degradation of 53.62%. The least biodegradation of phenanthrene was observed in BS of SJ301 whose degradation was measured to be 34.05 % after 35 days. In the study by soil consortium, a higher rate of phenanthrene degradation was observed to be in phenanthrene inoculated in soil consortium supplemented with the culture of JP-11 and SJ301 where phenanthrene degradation was found to be 86.15%. Whereas, phenanthrene degradation was found to be 76.6% in soil supplemented with BS of JP-11 and BS of SJ301. Study by Oberbremer et al. (1990) had used a mixed soil population to determine hydrocarbon degradation in model oil and reported a statistically significant increment of hydrocarbon degradation when sophorose lipids were administered to a model system containing 10% soil and 1.35% hydrocarbon mixture of tetradecane, pentadecane, hexadecane, pristane, phenyldecane and naphthalene in mineral salt medium. In the absence of surfactant, 81% of the hydrocarbon mixture was degraded in 114 h while, in the presence of biosurfactant, up to 90% of the hydrocarbon mixture was degraded in 79 h. In another study, Noordman et al. (1998) used rhamnolipid biosurfactants for increased removal of phenanthrene from phenanthrene contaminated soil eluting it with an electrolyte solution containing rhamnolipid (500 mg/l). Rhamnolipids increased the removal of phenanthrene (2- to -5-fold shorter time for 50% recovery and 3.5-fold for 90% recovery) compared to controls. The enhanced removal of phenanthrene occurred mainly by micellar solubilisation.

In order to understand the mechanism of degradation of phenanthrene, the interaction of biosurfactant with phenanthrene was studied. The study of interaction of phenanthrene with the biosurfactant of *Bacillus* species was similar to the quenching study of phenanthrene by activated sludge (Pan et al., 2010). In our study we observed that only one binding site is present in the fluorophore of *P. aeruginosa* JP-11 as the n value obtained was 0.595 and the value of binding constant $K_b$ was 3.32 $M^{-1}$. Similarly, there are 3 binding site present in the fluorophore of *B. subtilis* SJ301, as the n value obtained was 2.72 and the value of binding constant $K_b$ was 4.08 $M^{-1}$. The interaction between the phenanthrene and biosurfactants was spontaneous and exothermic whereas reports by Pan et al. (2011) stated that EPS from aerobic activated sludge contained two fluorophores belonging to protein-like substances. Fluorescence of these two fluorophores was

clearly quenched by phenanthrene. The quenching constants ($\ln K_a$) and the binding constants ($\log K_b$) were in the range of 11.27–13.82 $M^{-1}$ and 6.11–8.98 $M^{-1}$, respectively. The interaction between the fluorophores in EPS and PHE was spontaneous and exothermic. The interaction of biosurfactant with phenanthrene was studied by fluorescence spectroscopy, zeta potential measurement and FESEM micrographs to reveal the mechanism between BS-phenanthrene interactions in both the isolates. There was shift in peaks of ATR-FTIR graph showing major binding groups as –OH, -NH, -SH and P=O stretch. FESEM micrographs demonstrated the structural morphology of both the isolates during BS-phenanthrene interaction showing micelle like structure formation. Zeta potential measurement also revealed the stable interaction of biosurfactant with increasing concentration of phenanthrene. Reports by Rodrigues et al. (2005), revealed that phenanthrene grown cells were slightly more negatively charged (-57.5±4.7 mV) than glucose grown cells (-26.8±3.3 mV) suggesting that PAH substrate induced modifications on the physical properties of bacterial surfaces.

It has been reported that there is change in bacterial community structure during biodegradation of PAHs (MacNaughton et al., 1999; Vinas et al., 2005; Gandolfi et al., 2010). It has been disclosed that there is a significant change of the microbial community associated with fluoranthene or phenanthrene modification following biodegradation. Piskonen et al. (2005) have depicted a collection in the microbial community as an outcome of naphthalene biodegradation. In this study, DGGE method was utilized to determine the community structure change in soil consortium in different conditions. DGGE was employed to study the microbial community involved in the degradation of phenanthrene. Bacosa and Inoue (2015) had employed DGGE in order to determine the microbial community structure during pyrene degradation in Tsunami sediments of Miyagi, Japan. Dendrograms based on the density of the DGGE bands were created to inspect the differences between the microbial communities in different conditions of soil consortium. Thus, the present work utilizes marine bacteria and terrestrial bacteria for biosurfactant production and their application in phenanthrene degradation in culture medium and in soil consortium also demonstrating the interaction mechanism and community structure change. This can be helpful for bioremediation of phenanthrene from toxic contaminated wastes through a biological approach.

## 7. Conclusion

The study gives an insight into phenanthrene bioremediation by biosurfactants produced by marine and terrestrial bacteria *P. aeruginosa* JP-11 and *B. subtilis* SJ301. Biosurfactants being amphipathic in nature tend to interact with the phase boundary between two hydrophobic phases in heterogeneous system enhancing bioavailability of hydrophobic compounds. This renders greater biodegradation potential for phenanthrene removing their toxicity from the environment. ATR-FTIR, fluorescence spectroscopy, zeta potential study depicts hydroxyl and amine bond interaction of phenanthrene with biosurfactants. The biosurfactant produced can be commercially produced in large quantity in future for bioremediation of toxic poly aromatic hydrocarbons at contaminated sites. Therefore, the present work showcases biosurfactant mediated biodegradation of phenanthrene helpful for lowering toxicity at contaminated sites. With the advent of new technologies, the biosurfactant produced by marine bacteria as well as terrestrial bacterial EPS can be further engineered for better solubilisation and degradation helping in bioremediation of industrial effluents heavily contaminated with poly aromatic hydrocarbons.

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