Restoration and Detoxification of Petroleum Contaminated Soil by Biosurfactant producing Plant Growth Promotory Bacteria for Growing Medicinal Plants and Studying the Stress Responses Based on Metabolomics

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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Under the Supervision of

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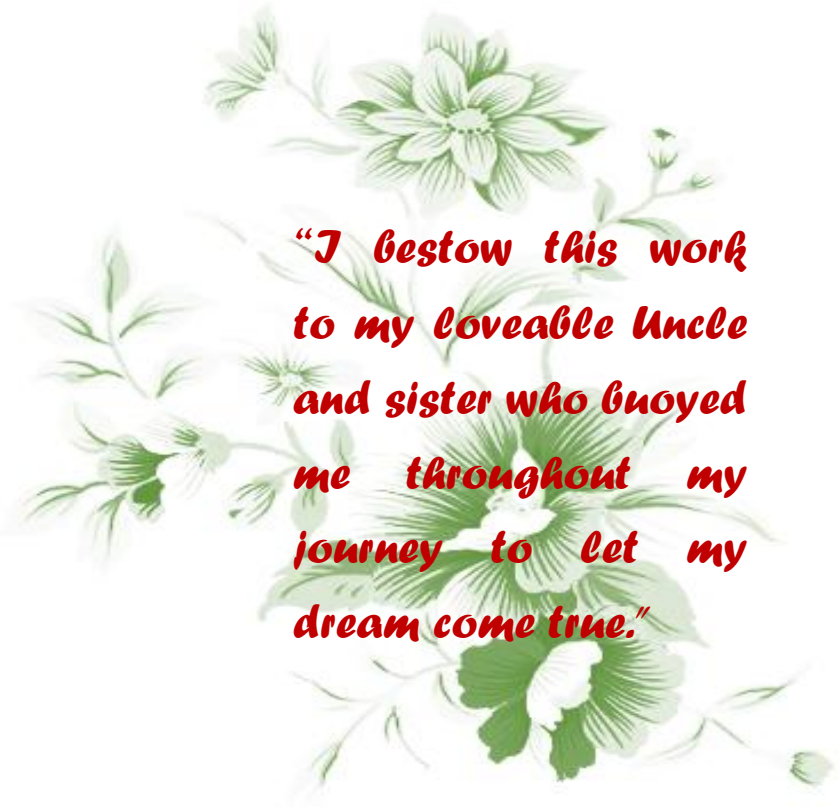
"I bestow this work
to my loveable Uncle
and sister who buoyed
me throughout my
journey to let my
dream come true."

CERTIFICATE

This is to certify that the thesis entitled "**Restoration and Detoxification of Petroleum Contaminated Soil by Biosurfactant producing Plant Growth Promotory Bacteria for Growing Medicinal Plants and Studying the Stress Responses Based on Metabolomics.**" submitted by "**Mr. Amar Jyoti Das**" is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university. The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (PhD) regulations – 1999 as amended in 2010 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date: / /2019 **Supervisor**

Head of the Department

DECLARATION

I, Amar Jyoti Das, hereby declare that the thesis work entitled "**Restoration and Detoxification of Petroleum Contaminated Soil by Biosurfactant producing Plant Growth Promotory Bacteria for Growing Medicinal Plants and Studying the Stress Responses Based on Metabolomics.**" is my own work carried out under the guidance of **Prof. Rajesh Kumar, Head, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, (A Central University) Vidya Vihar, Raebareli Road, Lucknow.** The matter embodied in this thesis is written by me and has not been submitted to any other university for the fulfillment of the requirement of any other Degree or Diploma. **"The thesis is essential free from all kinds of plagiarism"**

Place: **- Lucknow**

Date: …. /…. /….. (**Amar Jyoti Das**)

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Amar Jyoti Das

CONTENTS

सारांश

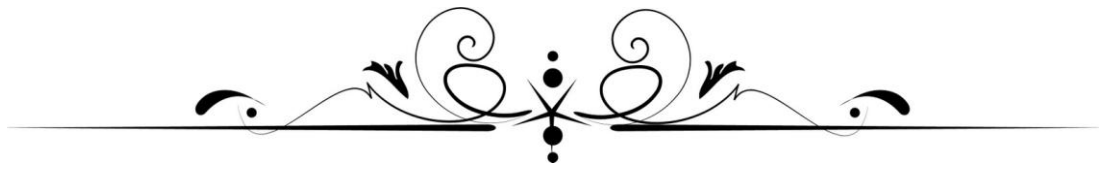
पेट्रोलियम तेल से दूषित मिट्टी को हाइड्रोकार्बन सहित विभिन्न दूषित पदार्थों द्वारा विषाक्तता के कारण कृषि उद्देश्यों के लिए सीधे उपयोग नहीं किया जा सकता है। तेल दूषित मिट्टी विषाक्तता को प्रेरित करती है जो पौधे के अंकुरण, वृद्धि और उत्पादकता को प्रभावित करती है। इसे कम करने के लिए, कई प्रस्तावित जैवोपचारण तकनीकों में से, एक दृष्टिकोण जो जैव पृष्ठसक्रियक उत्पादक संयंत्र विकास प्रोत्साहन जीवाणु (पीजीपी) का उपयोग करता है, को सबसे अधिक आशाजनक माना जाता है। वर्तमान अध्ययन में, *Pseudomonas azotoformans* AJ15, *Bacillus licheniformis* J1, *Bacillus safensis* J2 और *Pseudomonas azotoformans* N23 के रूप में पहचाने जाने वाले पीजीपी उपभेदों का रेत, खोई, गोमूत्र और जैव पृष्ठसक्रियक से उपचार का प्रभाव पेट्रोलियम प्रबंधन मे किया गया है। परिणाम बताते हैं कि पौधे का उपचार उपचारित मिट्टी से होता है, जिसमें जीवाणु के उपभेदों को अंकुरण, तने की लंबाई, जड़ की लंबाई, ताजे और सूखे वजन जैसे सभी मापदंडों के लिए उच्च मान व्यक्त किया जाता है। ये परिणाम मेटाबोलामिक्स अध्ययन द्वारा आगे पूरक थे जो बैक्टीरिया टीका और गैर-टीका उपचार में फाइटोकेमिकल सामग्री और प्रतिउपचायक गतिविधि की भिन्नता का संकेत देते हैं।

संक्षेप में, वर्तमान अध्ययन पेट्रोलियम दूषित जैव-उत्पादन का उपयोग करने के लिए एक पर्याप्त औचित्य प्रस्तुत करता है, जो जैव पृष्ठसक्रियक पीजीपी जीवाणुओ को पेट्रोलियम दूषित मिट्टी के विषहरण और मरम्मत करता है। एक अभिनव दृष्टिकोण के रूप में, आर्थिक रूप से महत्वपूर्ण फसलों को उगाने के लिए इसे और अधिक खोजा जा सकता है।

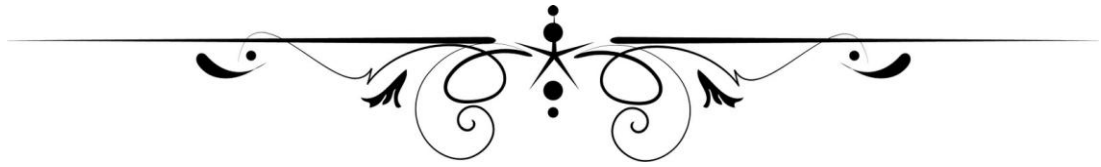
Abstract

Soil contaminated by petroleum oil cannot be directly utilized for agricultural purposes due to toxicity poses by various contaminant including hydrocarbons. Oil contaminated soil induces toxicity that affects germination, growth, and productivity of the plant. To mitigate it, out of several proposed bioremediation technologies, an approach that uses biosurfactant producing plant growth promotory bacteria (PGP) is considered to be most promising. In the present study the efficacy of treatment with, sand, bagasse, cow urine and biosurfactant producing PGP strains (identified as *Pseudomonas azotoformans* AJ15, *Bacillus licheniformis* J1, *Bacillus safensis* J2 and *Pseudomonas azotoformans* N23) in management of petroleum contaminated soil is explored. Results indicate that plant arose from the treated soil with the bacterial strains expressed high values for all the parameters studied namely germination, shoot length, root length, fresh and dry weight. These results were further complemented by the metabolomics studies that indicate the variation of phytochemical content and antioxidant activity in bacterial inoculated and non-inoculated treatments.

In a nutshell, the present study presents a substantial justification for utilizing petroleum degrading biosurfactant producing PGP microbes in restoration and detoxification of petroleum contaminated soils. As an innovative approach, this can further be explored for growing economically important crops.



Chapter 1



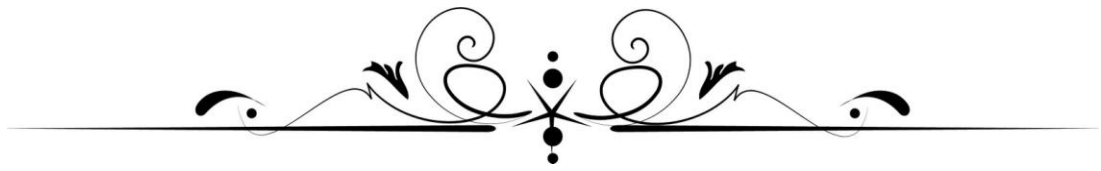
INTRODUCTION

Oil spills may occur for numerous reasons such as equipment failure, disasters, deliberate acts, or human error (Anderson and LaBelle, 2000). The places where crude oil is found are not always the places where it is refined and needed. During transportation and processing, sabotage and accidents lead to the release of these crude oils in the environment. In the refining process, the release of oil into refinery effluents as waste disposal, is although practically negligible and of a lower order of magnitude but it can affect the soil properties, microbial population and if the magnitude is large enough it can also seep into the groundwater. Soil contamination with petroleum and its products is an inevitable problem as it can affects many geographical regions to a variable extent (Banks et al., 2003; Chaineau et al., 2003; Hentati et al., 2013; Graj et al., 2013). In recent times, other novel strategies have attracted the attention of environmentalists and policy makers throughout the world depending on the local environmental law. Petroleum contaminated soil pollutes local groundwater, renders potable water unsafe, limits groundwater use, and causes ecological toxicity (Wang et al., 2008). The toxicity of petroleum hydrocarbon on soil organisms has been widely studied, but research regarding its ecotoxicity is still assaying behind (Cermak et al., 2010; Tang et al., 2011). Petroleum is a natural product resulting from the anaerobic conversion of biomass under high temperature and pressure. Their components are subject to relatively slow rate of biodegradation. Consequently, petroleum hydrocarbons are poorly degraded and thus become most widespread environmental contaminant (Margesin et al., 2000).Hence, to remediate it, various technologies have been proposed but the major limiting factor in bioremediation of such sites is the bioavailability of many fractions of the oil to

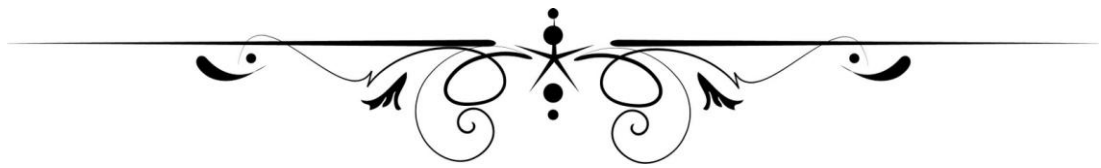
degradation. Since the scope of various bioremediation strategies is limited due to poor hydrocarbon accessibility and their low solubility. Use of biosurfactants which enhance bioavailability can be used for restoration of sites contaminated by petroleum hydrocarbons. Increasing population and industrialization has led to shrinkage of agricultural land, especially for medicinal plant cultivation. Petroleum contaminated sites can restore by detoxifying them using biosurfactant producing plant growth promoting bacteria that has the potentiality to degrade petroleum hydrocarbons. They are more habituated to peculiar such soil environment and can be effectively and commercially use for the management of petroleum contaminated wastelands for agriculture. Due to the cost effective and environment friendly nature of biosurfactants, they can be effectively and commercially use for this purpose. With this idea, this PhD thesis address five objectives mentioned below

- Isolation of bacterial isolates from petroleum contaminated soil, characterization and screening for biosurfactant production and plant growth promotory properties.
- Extraction and characterization of biosurfactants from the selected bacterial isolates.
- Soil collection from petroleum contaminated site for *in situ* experiment and its characterization for pollutant toxicity level.
- Exploring the potential of biosurfactant producing bacterial isolate for restoration and detoxification of the soil from the selected contaminated site.

- Comparison of soil detoxification process *in situ* under pot culture on medicinal plant and its active ingredient using antioxidant activity and stress response on metabolites using metabolomics.



Chapter 2



The majority of hydrocarbons found on earth occur naturally in crude oil, where decomposed organic matter provides an abundance of carbon and hydrogen which, when bonded, can catenate to form seemingly limitless chains. These hydrocarbons can be gases (e.g., methane and propane), liquids (e.g., hexane and benzene), waxes or low melting solids (e.g., paraffin wax and naphthalene), or polymers (e.g., polyethylene, polypropylene and polystyrene). Hydrocarbons existing on the planet based on their nature and properties are classified as saturated, unsaturated, cycloalkanes, and aromatic hydrocarbons. Saturated hydrocarbons (alkanes) are the basis of petroleum fuels and are found as either linear or branched species. These saturated hydrocarbons or petroleum hydrocarbonshave gained importance during the last century because of their use as a source of energy. The modern lifestyle and the world's economy today depend to a large extent on the availability of this "black gold" and its derivatives. Today the fuels derived from crude oil, supply more than half of the world's total supply of energy (OECD, 1998). Gasoline, kerosene, and diesel are used as fuel for cars, tractors, trucks, aircraft, and ships. Hexane [6C] is a widely used non-polar, non-aromatic solvent, as well as a significant fraction of common gasoline. The [6C] through [10C] alkanes, alkenes, and isomeric cycloalkanes are the top components of gasoline, naphtha, jet fuel, and specialized industrial solvent mixtures. With the progressive addition of carbon units, the simple non-ring-structured hydrocarbons have higher viscosities, lubricating indices, boiling points, solidification temperatures, and deeper color. At the opposite extreme from [1C] methane lies the heavy tars that remain as the lowest fractionin a crude oil refining retort. They are collected and widely utilized as roofing compounds, pavement composition, wood preservatives (the creosote series), and as extremely

high viscosity shear-resisting liquids. Heating oil and natural gas are used to heat homes and commercial buildings as well as to generate electricity. Besides this, crude oil products are the basic materials used in the manufacturing of synthetic fibers for clothing and in plastics, paints, fertilizers, insecticides, soaps, and synthetic rubber (Speight, 1999). This overdependence of the world on petroleum hydrocarbons to meet their energy andother requirement has resulted in more extraction and refinement of crude oils. However,there is another side to this usefulness, as crude oil prospecting is sometimes accompaniedwith spillage. These spillages are a source of environmental contamination.Oil spills may occur for numerous reasons such as equipment failure, disasters, deliberateacts, or human error (Anderson and LaBelle, 2000). The places where crude oil is foundare not always the places where it is refined and needed. Sabotage and accidents lead torelease of these crude oils in the environment. In the refining process, the release of oilinto refinery effluents as waste disposal, is although practically negligible and of a lowerorder of magnitude but it can affect the soil properties, microbial population and if themagnitude is large enough it can also seep into the groundwater. Discharge and washwaters from tankers and vessels are a kind of oil pollution that is fairly unnoticed but iscommon. Besides this, oil spills can take place near coastlines or in the open sea. Air andocean currents can also transport pollutants for thousands of kilometers; therefore, oilspills affect more than just isolated locations. In many spills involving tankers or offshoreoil wells, some of the oil spilled initially catches fire. When crude oil burns, the combustionresults in atmospheric emissions of gasses, which contribute to global warming (CO2)and acid rain (SO2, NOx), as well as large quantities of toxic ash. The toxic ash is made upof microscopic particles, which can travel for hundreds of kilometers. Humans inhalingthese particles may experience allergic reactions, which result in sore throats and breathingproblems. The less dense

(lighter) components of the spilled oil are more volatile andeventually evaporate into the atmosphere. The petroleum hydrocarbon then reacts withsunlight and oxygen to form greenhouse and acidic gases similar to those from the combustionof oil. The negative impacts of oil that burns or evaporates is more diffuse (spreadout) than that of oil which ends up on shore but still causes appreciable damage to thenatural environment. Kvenvolden et al. (2000) reported that the oil mixes with sedimentson the ocean floor and turns into a thick tar-like mass, which can destroy the habitat ofmany bottom-dwelling organisms. These tar-like clumps can also drift with tides andcurrents eventually washing up on beaches far away from the spill. If a spill occurs near acoastline, beached oil can leak into fresh groundwater reservoirs that often extend underbeaches, contaminating local wells.Although the pollution caused by oil spills in the air and oceans cannot be easily controlledusing biological means, the soil contaminated by these can be remediated usingdifferent techniques. Still, this is a new field which has attracted the attention of environmentalistsand policymakers throughout the world as environmental petroleum contaminationand its constituents is an inevitable problem that affects many geographical regionsto a variable extent depending on the local environmental law (Graj et al., 2013).Soil contamination with petroleum and its products is a serious worldwide concern (Banks et al., 2003; Chaineau et al., 2003; Hentati et al., 2013). Petroleum-contaminated soilpollutes local groundwater, renders potable water unsafe, limits groundwater use, andcauses ecological toxicity (Wang et al., 2008). The toxicity of petroleum hydrocarbon on soilorganisms has been widely studied, but research regarding its ecotoxicity is still assayingbehind (Cermak et al., 2010; Tang et al., 2011).

2.1 Aquatic Toxicity

Release of petroleum oil in water bodies accidentally alters the aquatic environment by affecting flora and fauna. Petroleum oil spills reduce density, growth, biomass and

photosynthetic rate of the marsh vegetations (Krebs and Tamer,1981). Oilspills in water often cause eutrophication and loss of biodiversityby affecting the dissolved oxygen content (Onwurahet al., 2007). Due to the consumption of oil-contaminatedseafood, the population of sea birds is decreasing day by day. Contaminated seafood causes abnormal metabolic functions such as immune suppression, anaemia, gastrointestinal inflammation in birds **(**Briggs et al., 1997). Fishes are more prone to oil pollution in water. Thehemorrhagic septicaemias, epidermal hyperplasia, lymphocytosis are someof the reported diseases in fishes that occur due to oil spills (Beeby, 1993). Davis (2002), demonstrated that oil spills in the northernGulf of Mexico has induces negative effect on the population of sperm whale as well astheir prey.

2.2 Terrestrial Toxicity

Petroleum oil pollution in soil pollution poses a risk of biomagnification. Petroleum in soil alters the physical, chemical and biological properties of soil (Onwurahet al., 2007). Oil spills create an anaerobic condition in soil i.e. anaerobiosis, resulting in the death of the plants due to non-availability of oxygen. Besides this, petroleum oil and its by-product inhibit the seedgermination and affect plant growth (Nogueira *et al*., 2011, Onwurah,1999, Kumar *et al*., 2015b).

2.3 Various Conventional Methods for Management of Petroleum Oil Hydrocarbon-Contaminated Soil

2.3.1 Soil Washing

Soil washing is an *ex situ* remedial method to treat soil contaminated with organic orinorganic compounds. In this method, solvents or mechanical processes are employed toscrub contaminant from the soil (Melanie Fortune—CHEE 484). Selection of solvents forwashing the soil depends on the basis of their ability to solubilize specific contaminants(Asante-Duah, 1996; Feng et al., 2001; Chu and

Chan, 2003; Urum et al., 2003; Khan et al.,2004). The soil-washing method separates fine soil (clay and silt) from coarse soil (Khanet al., 2004). Since hydrocarbon contaminants tend to bind and sorb to smaller soil particles,hence the soil-washing methods separate them from the larger ones and reducethe volume of the contaminated soil (Riser-Roberts, 1998; Khan et al., 2004). Further, thesmaller soil particles which contain the contaminants can be treated by other incinerationor bioremediation or disposedoff in accordance with federal regulations (Khan et al., 2004).

2.3.2 Landfarming

Landfarming is one of the successful methods for treating petroleum hydrocarbons(FRTR, 1999a; Khan et al., 2004). It has been practiced worldwide for over 100 years, andby the petroleum industry and refineries for more than 25 years (Riser-Roberts, 1998).Landfarming is a method, which reduces the concentration of petroleum constituentspresent in the soil through processes associated with bioremediation (Khan et al., 2004). Inthis method, excavated contaminated soil is spread in a thin layer not more than 1.5 m overthe ground surface of the treatment site and aerobic microbial activity is enhanced withinthe soil through aeration or by addition of nutrients, minerals, and water. Microbes, which have been selected for their success in breaking down hydrocarbons are often added to the soil to increase the degradation rate of adsorbed petroleum products (Riser-Roberts, 1998; USEPA, 1998a; Hejazi, 2002; Khan et al., 2004).

2.3.4 Thermal Desorption

Thermal desorption is a method where contaminated soil is excavated, screened andheated to release petroleum from the soil (USEPA, 1995; Khan et al., 2004). Generally,heating of the soils is done at 100–600°C range temperatures, so that those

contaminantswith boiling points in this range separate from the soil through vaporization (Khan et al.,2004). Further, the vaporized contaminants are collected and treated by some other means(Dermatas and Meng, 2003; Khan et al., 2004). Thermal desorption is an effective methodfor the treatment of hydrocarbon-contaminated soil, but its effect varies on the full rangeof organics (FRTR, 1999b; Khan et al., 2004).

2.3.5 Phytoremediation

Phytoremediation is a biological method to remove the contaminant from the environmentby using plants (Raskin, 1996; Ndimele et al., 2010). It is a broad term that has beenin use since 1991 to describe the use of plants to reduce the volume, mobility or toxicity ofcontaminants in soil and groundwater (McCutcheon and Schnoor, 2003). The phytoremediationmethod adopts various mechanisms to remove contaminants. Such mechanismsare phytoextraction, phytovolatilization, phytodegradation, rhizodegradation, rhizofiltration,phytostabilization, and hydraulic control Figure 1.3. Each of these mechanisms willhave an effect on the volume, mobility, or toxicity of contaminants, as the application of phytoremediation is intended to do (EPA, 2000).

2.3.5.1 Phytoextraction

Phytoextraction refers to the ability of plants to remove metals and other compounds fromthe subsurface and translocate them to the leaves or other plant tissues. The plants maythen need to be harvested and removed from the site (EPA, 2000).

2.3.5.2 Phytovolatilization

Phytovolatilization may also entail the diffusion of contaminants from the stems or another plantparts that the contaminant travels through before reaching the leaves.

2.3.5.3 Phytodegradation

When the phytodegradation mechanism is at work, contaminants are broken down afterthey have been taken up by the plant. As with phytoextraction and phytovolatilization,plant uptake generally occurs only when the contaminants' solubility and hydrophobicityfall into a certain acceptable range (EPA, 2000).

2.3.5.4 Rhizodegradation

Rhizodegradation refers to the breakdown of contaminants within the plant root zone orrhizosphere. Rhizodegradation is believed to be carried out by bacteria or other microorganisms whose numbers typically flourish in the rhizosphere. Studies have documented up to100 times as many microorganisms in rhizosphere soil as in soil outside the rhizosphere (McCutcheon, 2003). Microorganisms may be so prevalent in the rhizosphere because theplant exudes sugars, amino acids, enzymes, and other compounds that can stimulate bacterialgrowth (Lynch, 1990; Marilley and Aragno, 1999; García et al., 2001). The roots also provideadditional surface area for microbes to grow on and a pathway for oxygen transfer from the environment. The localized nature of rhizodegradation means that it is primarily usefulin contaminated soil, and it has been investigated and found to have at least some successin treating a wide variety of most organic chemicals, including petroleum hydrocarbons,polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, pesticides, polychlorinatedbiphenyls (PCBs), and benzene, toluene, ethylbenzene, and xylenes (BTEX) (EPA, 2000).

2.3.5.5 Rhizofiltration

In the rhizofiltration process, contaminants are taken up by the plant and removed fromthe site when the plant is harvested. However, in this case, the contaminant is removedfrom the dissolved phase and concentrated in the root system.

Rhizofiltration is typicallyexploited in groundwater (either *in situ* or extracted), surface water, or wastewater forremoval of metals or other inorganic compounds (EPA, 2000).

2.3.5.6 Phytostabilization

Phytostabilization takes advantage of the changes that the presence of the plant induces insoil chemistry and the environment. These changes in soil chemistry may induce adsorptionof contaminants onto the plant roots or soil or cause metals precipitation onto theplant root. The physical presence of the plants may also reduce contaminant mobility byreducing the potential for water and wind erosion. Phytostabilization has been successfulin addressing metals and other inorganic contaminants in soil and sediments (EPA, 2000).

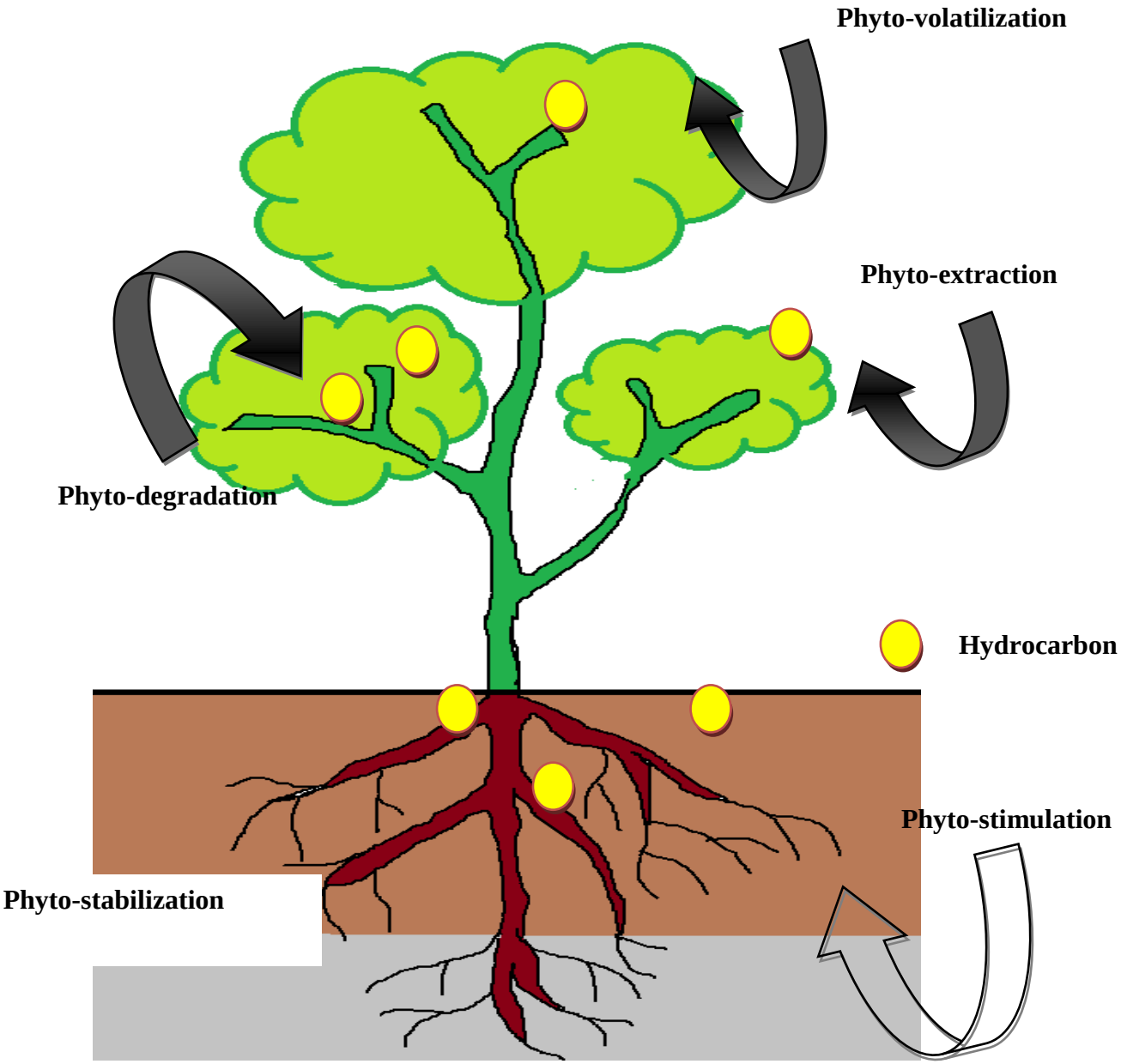


Fig. 2.1 Various Phytoremediation strategies (Source: Das et al. 2017)

2.3.6 Biopiles

Biopiles is a soil treatment method generally employed for treating petroleum-contaminatedsoil (Khan et al., 2004). This treatment involves the piling of contaminated soils(petroleum) into piles and then enhancing aerobic microbial

activity by aeration and theaddition of minerals, nutrients, and moisture (Filler et al., 2001; Khan et al., 2004). Most of the biopileshave an underground system through which air passes and have a coveringto prevent runoff, evaporation, and volatilization (Khan et al., 2004). This is a short-termtreatment method that lasts from a few weeks to a few months (Khan et al., 2004). Biopilestreat most petroleum products (USEPA, 1998b). Lighter petroleum products such as gasolinetend to be removed during aeration by evaporation and midrange products such asdiesel or kerosene have a greater tendency of biodegradation in this method as they containlower amounts of volatile components, whereas heating and lubricating has a lowerbiodegradation tendency as they consist of heavier compounds which do not evaporate(Chaineau et al., 2003; Khan et al., 2004).

2.3.7 Bioventing

In the bioventing technique, air is injected into the contaminated media at a rate designedto increase *in situ* biodegradation and reduce the off-gassing of volatilized contaminantsto the atmosphere (Khan et al., 2004). Baker and Moore (2000) have studied the optimizedperformance and effectiveness of *in situ* bioventing. Mihopoulos et al. (2002) and Dieleet al. (2002) have discussed numerical models and their applications in bioventing systemdesign and operation. This method is most successful in mid-weight petroleum productssuch as diesel because lighter products tend to volatilize quickly and the heavier productsgenerally take longer to biodegrade (Khan et al., 2004).

2.4 Microbial Degradation and Microbial Uptakeof Petroleum Hydrocarbons

Petroleum is a natural product resultingfrom the anaerobic conversion of biomass under high temperature and pressure. Althoughmost of its components are subject to biodegradation, this occurs at relatively slow rates.Moreover, petroleum

hydrocarbons are poorly degraded and have thus become the mostwidespread environmental contaminant (Margesin et al., 2000). The four classes of petroleumhydrocarbons (saturates, aromatics, asphaltenes, and resins; Sanchez et al., 2006) differin their susceptibility to microbial attack (bacteria and fungi). Bacteria and fungi generally.All the conventional methods mentioned above have some or the other limitation and

therefore a new field for degradation of petroleum hydrocarbons is gaining importancewhich employs microbes or their products for remediation of petroleum-contaminatedsites. Microbial degradation is one of the most promising mechanisms for removing petroleum hydrocarbon pollutants from the environment (Atlas, 1985; Lal and Khanna,1996). The microbial degradation and removal of contaminants in the soil occur throughtwo distinct but interrelated processes, biodegradation and microbial uptake. Microbialdegradation is the "microbially mediated chemical transformation of organic compounds,"whereas microbial uptake is the direct removal of the contaminant by adsorbingcompounds to the membrane surface or absorbing compounds through the membrane(Lyman et al., 1992). Both are correlated processes in which the contaminant is taken up mayeither be in original form or a biotransformed product. There are different types of microorganismsresponsible for biodegradation of petroleum hydrocarbon pollutants such asbacteria, fungi, and yeast, but bacteria are the most promising and active agents in petroleumdegradation (Jahangeer and Kumar, 2013).

2.4.1 Aerobic Degradation

The complete and rapid degradation of the petroleum hydrocarbon pollutant (organic pollutant)is brought about under aerobic conditions. Peroxidase and oxygenases are the mainenzymes which are involved in the intracellular attack of organic pollutants

and activationas well as the incorporation of oxygen in the enzyme key reaction. Organic pollutantsare converted step by step into intermediates of the central intermediary metabolism forexample, the tricarboxylic acid cycle . The end products of hydrocarbon mineralizationare CO2, H2O, and energy, although complete degradation does not always occur.Subsequent end products may be directly taken up by microbes and not degraded furtheror may be degraded to smaller, simpler, more stable intermediaries, and incorporated intothe soil as humus or soluble acids, ketones, and alcohols (Lyman et al., 1992). Biosynthesisof cell biomass occurs from the central precursor metabolites such as acetyl-CoA, succinate,pyruvate. Sugars required for various growth and biosyntheses are synthesized by aprocess termed gluco-neo-genesis (Das and Chandran, 2011).The potential of a particular petroleum hydrocarbon to be degraded, independent of soilproperties, depends on its chemical structure. The main considerations for biodegradationare the size of the contaminant and the type and geometry of its bonds. Some petroleumhydrocarbons have bonds that are difficult to assess or difficult to be broken down bymicrobes. Different molecular configurations also affect the degradability for example,linear alkanes are more readily degradable than branched alkanes (Riser-Roberts, 1998).The number of different microbes able to degrade a specific contaminant decreases as thecontaminant becomes more difficult to degrade. Also, not all microbes are able to directlytake up all the hydrocarbon contaminants. This results in variations of petroleum hydrocarbonsand microbial population composition over time with the most readily degradablehydrocarbons and associated microbes being replaced by less degradable hydrocarbonsand associated microbes. It should be noted that one type of microorganism is rarely ableto fully degrade any specific contaminant. The most effective remediation occurs with adiverse microbial population (Riser-Roberts, 1998).

2.4.2 Enzymatic Degradation

Cytochrome P450 alkane hydroxylases constitute a superfamily of ubiquitous heme-thiolatemonooxygenases which play an important role in the microbial degradation ofpetroleum oil hydrocarbon and other compounds. To initiate biodegradation of any hydrocarbon,enzyme systems are required to introduce oxygen in the substrate which basicallydepends on the chain length of the pollutant (Das and Chandran, 2011). Highereukaryotes generally contain several different P450 families that consist of a large numberof individual P450 forms that may contribute as an ensemble of isoforms to the metabolicconversion of a given substrate. In microorganisms, P450 multiplicity can only be seen ina few species (Zimmer et al., 1996). These cytochrome P450 enzymes had been predominatelyisolated from yeast species such as *Candida apicola, Candida tropicalis,* and *Candidamaltose* (Scheuer et al., 1998). Hence, the yeast species have the ability to use *n*-alkanes andother aliphatic hydrocarbons as a source of carbon and energy, which is governed by themultiple microsomal cytochrome P-450 (Das and Chandran, 2011).

2.5 Factors Affecting Biodegradation of Hydrocarbons

For successful biodegradation of the contamination of petroleum hydrocarbons properknowledge regarding the limiting factors which affect biodegradation is necessary.Limiting factors influence rates of the microbial degradation of petroleum pollutantswhich in turn depends on the quality and quantity of the pollutant mixture and physicaland chemical parameters of the affected ecosystem.

2.5.1 Temperature

Temperature plays a very important role in biodegradation of petroleum hydrocarbonsthrough its direct effect on the chemistry of the pollutants and on the diversity and physiologyof the microorganisms (Atlas, 1975; Das and Chandran,

2011). At low temperatures,the viscosity of the oil is increased, while the volatility of toxic low-molecular-weighthydrocarbons is reduced, delaying the onset of biodegradation (Atlas, 1975). Temperatureaffects the solubility of hydrocarbons to a great extent (Foght et al., 1996). Various researchfindings regarding the effect of temperature on biodegradation of hydrocarbons have beenreported at wide ranges of temperature so far. However, highest biodegradation rates generallyoccur in the range of 30–40°C in soil environments, 20–30°C in some freshwaterenvironments and 15–20°C in marine environments (Bartha and Bossert, 1984; Cooneyet al., 1985; Das and Chandran, 2011). There are many reports which demonstrate thebiodegradation efficiency of microbes in a mesophilic environment, but there are veryfew reports regarding biodegradation of hydrocarbons in a psychrophilic environmentas petroleum contamination is recognized as a significant threat to polar environments.(Yumoto et al., 2002; Delille et al., 2004; Pelletier et al., 2004; Okoh, 2006).

2.5.2 pH

An important factor which affects the activity of introduced bacteria in biodegradationof petroleum hydrocarbon is the pH of the soil or the environment. The pH of the soil ishighly variable, ranging from 2.5 in mine spoils to 11 in alkaline deserts. Most heterotrophicbacteria favor a pH of 7.0 but fungi prefer acidic conditions. Therefore, an extreme pHof soils would have a negative influence on the ability of microbial populations to degradehydrocarbons. Dibble and Bartha (1976) observed an optimal pH of 7.8, in the range 5.0–7.8for the mineralization of oily sludge in soil. Kastner et al. (1998) reported that the shiftof pH from 5.2 to 7.0 enables polyaromatic hydrocarbons degradation by *Sphingomonaspaucimobilis*. This led to the conclusion that the neutral pH of soil favors biodegradation

ofhydrocarbons and small pH shifts have dramatic negative effects on the degradation rate.

2.5.3 Nutrients

The nutrient status of the contaminated ecosystem has direct impacts on the biodegradation ofpetroleum hydrocarbon and microbial activity. Nitrogen, phosphorus, and iron play a vitalrole as the sole nutrient for biodegradation of hydrocarbon pollutants (Das and Chandran,2011). Nitrogen and phosphorus are necessary for cellular metabolism and can be foundin low concentrations in many soils (Mohn and Stewart, 2000). Therefore, the addition ofnutrients is necessary to enhance the biodegradation of oil pollutants (Choi et al., 2002;Kim et al., 2005; Das and Chandran, 2011). However, excessive nutrient concentrations caninhibit biodegradation activity (Chaillan et al., 2006; Das and Chandran, 2011).

2.6 Biosurfactant Assisted Bioremediation of Petroleum Hydrocarbons

Petroleum oil hydrocarbon degrading microorganisms play a vital role in the biologicaltreatment of petroleum oil contamination as they adapt to grow and thrive in oil-containingenvironments. One of the limiting factors in this process is the bioavailability ofmany fractions of the oil. This limitation can be overcome by the biosurfactants of diversechemical nature and molecular size produced by hydrocarbon-degrading microorganisms.Biosurfactants increase the surface area of hydrophobic water-insoluble substratesby increasing their bioavailability and enhancing the growth of bacteria and the rate ofbioremediation (Ron and Rosenberg, 2002).

2.6.1 Biosurfactants

Biosurfactants are heterogeneous group of surface active agents produced by microorganisms,which possess both hydrophobic and hydrophilic moieties that

reduce surface andinterfacial tensions by accumulating at the interface between two immiscible fluids suchas oil and water (Ilori et al., 2005; Mahmound et al., 2008; Muthusamy et al., 2008; Kiranet al., 2009; Obayori et al., 2009; Das and Chandran, 2011). Structurally, they contain a hydrophobicmoiety of unsaturated or saturated hydrocarbon chains of fatty acids and a hydrophilicmoiety, comprising an acid, peptide cations or anions, mono-, di-, or polysaccharides.Biosurfactants are generally grouped into two classes, one is low-molecular-weight surfaceactive agents called biosurfactants and other is high molecular weight surface active agentscalled bioemulsifiers (Karanth, 1999). They efficiently reduce surface and interfacial tensions(Karanth, 1999). These are the compounds with vast potential for use in the environment,and in petroleum, food, pharmaceutical and other industries as these are environmentallyfriendly, easily degradable, economical and stable at elevated temperatures, pH and saltconcentrations when compared with their chemical counterparts (Borjana et al., 2001).

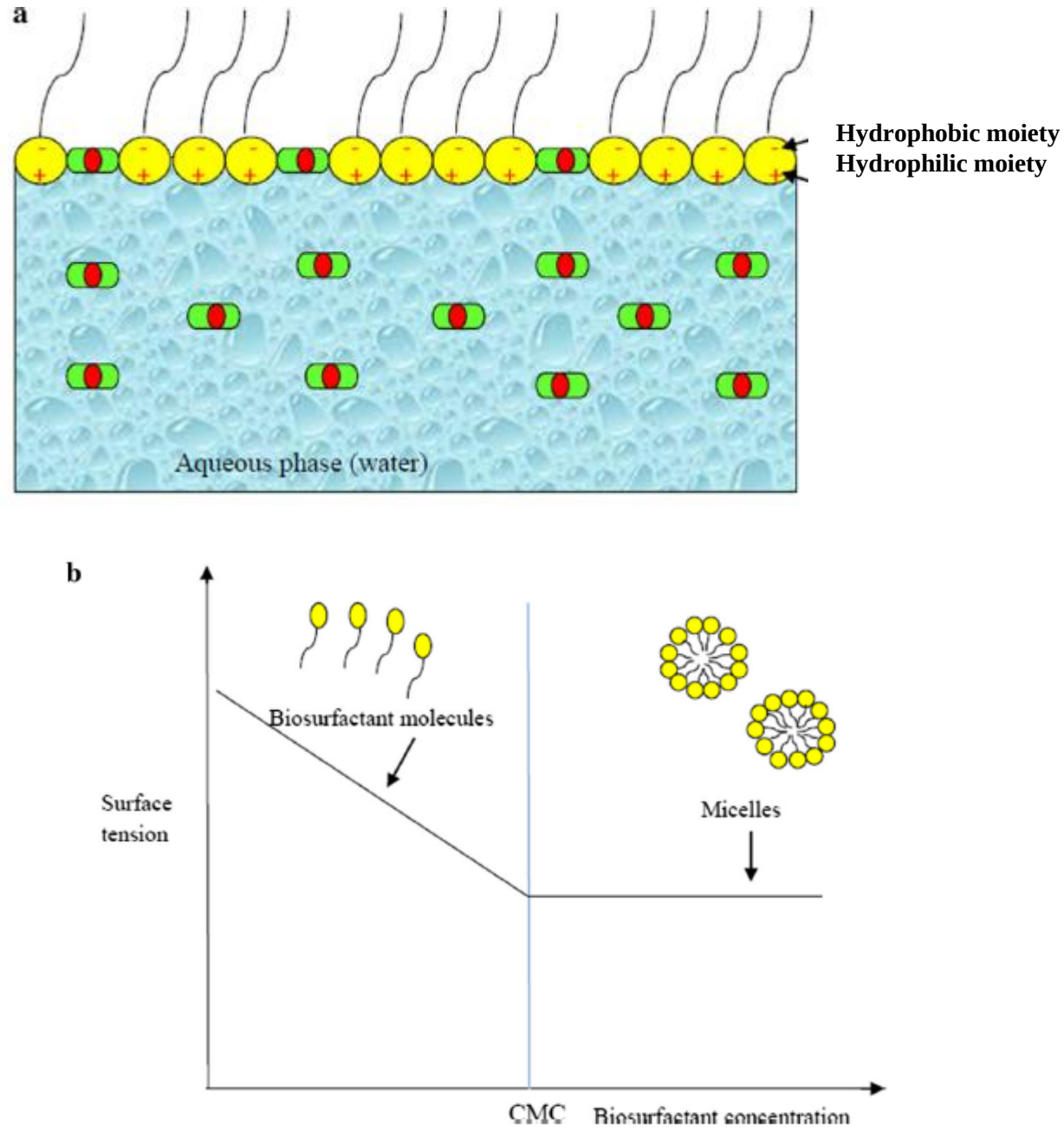


Fig. 2.2 The relationship between the concentration of biosurfactant, surface tension, and formation of micelles (Source: Das et al. 2017)

2.6.2 Types of Biosurfactants

The major classes of low-mass surfactants include glycolipids, lipopeptides and phospholipids,whereas high-mass surfactants include polymeric and particulate surfactants(Kappeli and Finnerty, 1979; Nitschke and Coast, 2007) as listed in Table 1.1.

Table 1.1 Types of biosurfactants and their applications

Biosurfactant		**Microorganism**	**Applications**	**References**
Groups	Class	*Pseudomonas aeruginosa, Pseudomonas sp., Burkholderia sp.*	Degradation and dispersion of different classes of hydrocarbons and their emulsification, removal of heavy metals from soil.	Geys et al.2014;PacwaPłociniczak et al. 2011; Ron and Rosenberg 2002
Glycolipids: Glycolipids are low molecular weight biosurfactants in which carbohydrates are attached to along-chain aliphatic acid.	Rhamnolipids Rhamnolipids are glycolipids that are composed of one or two L-rhamnose molecules coupled to mono or dimer of b-hydroxy fatty acids.			
	Trehalolipids: Trehalolipids are glycolipids that contain trehalose lipids as a hydrophilic moiety.	*Mycobacterium tuberculosis, Rhodococcuserythropolis, Arthrobacter sp., Nocardia sp., Corynebacterium sp.*	Enhancement in the bioavailability of hydrocarbons.	Shao et al.2011;PacwaPłociniczak et al. 2011
	Sophorolipids: Sophorolipids are glycolipids in which dimeric carbohydrate sophorose linked to a long-chain hydroxylfatty acid by glycosidic linkage.	*Torulopsisbombicola, Torulopsispetrophilum, Torulopsisapicola, Starmerellabombicola, Wickerhamielladomercqiae, Candida batistae.*	Recovery of hydrocarbons from dregs and muds (Microbial Enhanced Oil Recovery), heavy metal removal from sediments.	Kapadia Sanket and Yagnik et al.2013**;**Geys et al. 2014;PacwaPłociniczak et al. 2011

Lipopeptides: Lipopeptides are biosurfactants of low molecular weight in which consist of a lipid attached to a polypeptide chain.	**Surfactin** Surfactin are cyclic lipopeptides which of consist of a seven amino acid ring structure coupled to a fatty-acid chain via lactone linkage.	*Bacillus subtilis*	Enhancement of the biodegradation of hydrocarbons, removal of heavy metals.Antimycoplasmal activity, Anti-adhesive application,Antibacterial and anti-inflammatory application	Kapadia Sanket and Yagnik et al.2013;PacwaPłociniczak et al. 2011;Wang et al. 2008;Shaligram and Singhal,2010
	Lichenysin Lichenysin anionic cyclic lipoheptapeptide biosurfactants produced by *Bacillus licheniformis*.	*Bacillus licheniformis*	enhancement of oil recovery.	Nerurkar et al.2010;PacwaPłociniczak et al. 2011
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	bitumen recovery improvement.	PacwaPłociniczak et al. 2011
	Spiculisporic acid	*Penicillium spiculisporum*	metal ion sequestration from aqueous solution; preparation of new emulsion-type organogels, superfine microcapsules (vesicles or liposomes).	PacwaPłociniczak et al. 2011
	Phosphatidylethanolamine	*Acinetobacter sp., Rhodococcuserythropolis*	Increasing the tolerance of bacteria to heavy metals.	PacwaPłociniczak et al. 2011
	Emulsan	*Acinetobacter calcoaceticusRAG-1*	Stabilization of the hydrocarbon-in-water	PacwaPłociniczak et al. 2011

Polymeric biosurfactants			emulsions.	
	Alasan	*Acinetobacter radioresistensKA-53*	Stabilization of the hydrocarbon-in-water emulsions.	PacwaPłocinicz ak et al. 2011
	Biodispersan	*Acinetobacter calcoaceticusA2*	Dispersion of limestone in water.	PacwaPłocinicz ak et al. 2011
	Liposan	*Candida lipolytica*	Stabilization of hydrocarbon-in-water emulsions.	PacwaPłocinicz ak et al. 2011
	Mannoprotein	*Saccharomyces cerevisiae*	Stabilization of hydrocarbon-in-water emulsions	PacwaPłocinicz ak et al. 2011
Particulate biosurfactants		*Acinetobacter sp.*	Helps in the uptake of hydrocarbon such as alkane uptake.	Kapadia Sanket and Yagnik et al.2013

2.6.3 Properties of Biosurfactants

Low-molecular-mass biosurfactants are efficient in lowering surface and interfacial tensions,whereas high-molecular-mass biosurfactants are more effective in stabilizing oilin-water emulsions (Pacwa-Płociniczak et al., 2011; Geys et al., 2014). The biosurfactantsaccumulate at the interface between two immiscible fluids or between a fluid and a solid by reducing surface tension (Figure 2.2 A). The most active biosurfactants can lower thesurface tension of water from 72 dynes/cm to 25–30 dynes/cm and also the interfacialtension between water and *n*-hexadecane (Desai and Banat, 1997; Soberón-Chávez andMaier, 2011). Biosurfactant activity depends on the concentration of the surface-activecompounds until the critical micelle concentration (CMC) is obtained. At concentrationsabove the CMC, biosurfactants molecules associate to form micelles, bilayers and vesicles.Micelle formation enables biosurfactants to reduce the surface and interfacial tensionand increase the solubility and bioavailability of hydrophobic organic compounds(Whang et al., 2008; Figure 2.2 B). CMC is commonly used to measure the efficiency ofthe surfactant. Efficient surfactants have a low CMC, which means that less surfactantis required to decrease the surface tension (Desai and Banat, 1997). Micelle formationhas a significant role in micro-emulsion formation. Micro-emulsions are clear and stableliquid mixtures of water and oil domains separated by monolayer or aggregates of biosurfactants(Desai and Banat, 1997; Nguyen et al., 2008). Micro-emulsions are formed when one liquid phase is dispersed as droplets in another liquid phase, for example, oil dispersed in water (direct micro-emulsion) or water dispersed in oil (reverse microemulsion) (Desai and Banat, 1997). The effectiveness of microbial biosurfactants is determined by the carbon source used for its production, its charge, and its ability to change surface and interfacial tensions.

2.7 Factors Affecting Biosurfactant Production

2.7.1 Carbon Source

Two basic types of carbon sources, carbohydrates and hydrocarbons are used for the productionof biosurfactants. When microorganisms grow at the expense of water-immisciblesubstrates, a spontaneous release of biosurfactants is observed (Hisatsuka et al., 1971;Ito and Inoue, 1982) Carbohydrate substrates are used due to the relatively lower powerrequirement for dispersion and easy down streaming operations when compared with

hydrocarbons, whereas hydrocarbons generate more heat of reaction during cultivationwhich requires extensive cooling surfaces within the bioreactor system (Guerra-Santoset al., 1986; Shodhganga, 2014).

2.7.2 Nitrogen Source

Yeast extract is considered as the best nitrogen source for biosurfactant production by*Bacillus strains* isolated from the marine sediments of the Tamil Nadu (India) coastalarea (Gnanamani et al., 2010; Pacwa-Płociniczak et al., 2011). Apart from yeast andbeef extract, other nitrogen sources are often used for the production of biosurfactants.*Pseudomonas fluorescence* grown on olive oil as the carbon source proved to be amore efficient biosurfactant producer with ammonium nitrate as the nitrogen sourcewhen compared with ammonium chloride and sodium nitrate (Abouseoud et al., 2007;Pacwa-Płociniczak et al., 2011). Soniyamby et al. (2011) demonstrated that *Pseudomonassp*grown on a medium as sodium nitrate as the nitrogen source gives better yields ofbiosurfactant compared with ammonia and urea. According to Khopade et al. (2012)phenylalanine is a more efficient nitrogen source for the production of biosurfactants by*Nocardiopsis*sp. *B4* when used in combination with olive oil as the carbon source. Wuet al. (2008) demonstrated sodium nitrate as

the best nitrogen source among ammoniumnitrate, ammonium chloride, urea, and yeast extract for the production of biosurfactantsby *Pseudomonas aeruginosa* EM1.

2.7.3 Environmental Factors

Environmental factors such as temperature, pH, oxygen, and agitation affect the biosurfactant

production to a great extent. There are a few research studies which demonstratethe effect of temperature on biosurfactant production. Different organisms possess variedbiosurfactant production ability for example, *Pseudomonas aeruginosa* grown in a saltmedium shows increased rhamnolipid production at temperatures between 25 and 37°Cand at about 42°C, production decreases (Hon et al., 2005) while *Serratia marcescens* producesa lipopeptide type of biosurfactant at a temperature range up to 100°C and 12% NaClconcentration with a wide range of pH (Anyanwu et al., 2011). The agitation rate affects themass transfer efficiency of both oxygen and medium components and is considered crucialfor cell growth and biosurfactant production. With an increase in agitation rate from50 to 200 rpm, the growth rate of the aerobic strain of *Pseudomonas aeruginosa* increasesfrom 0.22 to 0.72/h. A little more research needs to be undertaken on these aspects of environmentaleffects on biosurfactant production (Maqsood and Jamal, 2011).

2.8 Enhancement of Petroleum Hydrocarbons Degradation by Biosurfactants

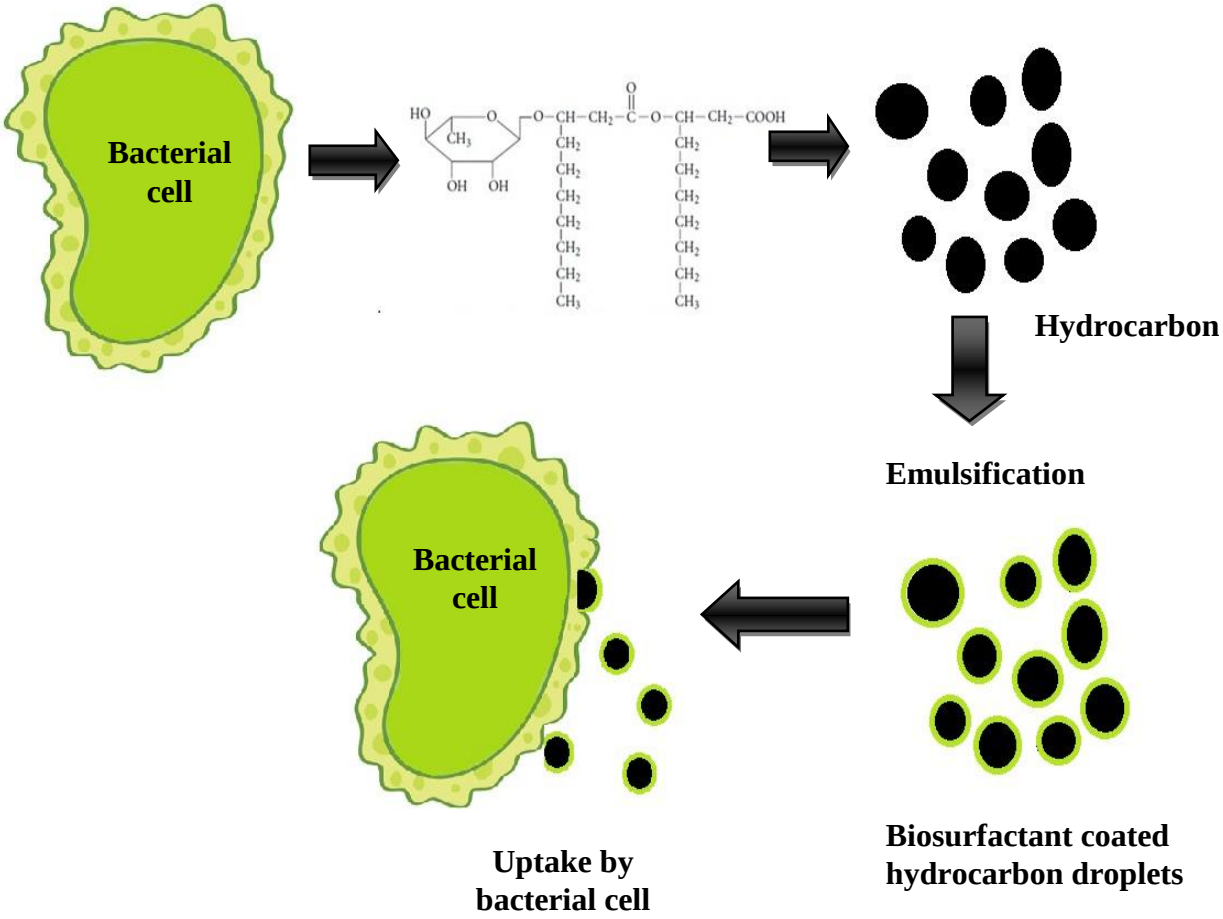
The use of biosurfactants to enhance the bioremediation of petroleum hydrocarbon-contaminated

environments are regarded as an effective method nowadays (Pacwa- Płociniczak et al., 2011). Biosurfactants can enhance hydrocarbon bioremediation by two mechanisms. The first includes the increase of substrate bioavailability for microorganisms, while the second involves interaction with the cell surface which

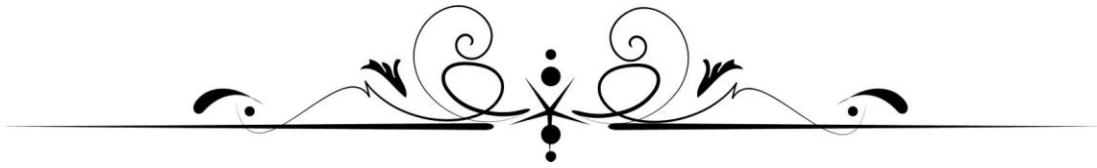
increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs, 2004; Pacwa-Płociniczak et al., 2011). Hydrocarbons are hydrophobic organic chemicals which exhibit limited solubility in water and tend to partition to the soil matrix. This partitioning can account for as much as 90%–95% or more of the total contaminant mass (Pacwa-Płociniczak et al., 2011). As a result, the hydrocarbon contaminants exhibit moderate to poor recovery by physicochemical treatments; limited bioavailability to microorganisms and limited availabilityto oxidative and reductive chemicals when applied to *in situ* and/or *ex-situ* applications.Due to their amphipathic/amphiphilic nature, biosurfactants enhance the emulsificationof hydrocarbons where they form micelles that accumulate at the interphase betweenliquids of different polarities such as water and oil**.** Hence, biosurfactants reduce surfacetension and facilitate hydrocarbon uptake. Biosurfactants are widely used in the remediationof petroleum hydrocarbon as they have the ability to stimulate growth on thehydrophobic surface and can increase the nutrient uptake of hydrophobic substrates.This leads to overcoming of the poor availability of hydrocarbon contaminants to microorganismsand thus increases the chances of biodegradation of hydrocarbons. Therefore,the addition of biosurfactants is expected to enhance biodegradation by mobilization,solubilization or emulsification. The mobilization mechanism occurs at concentrationsbelow the biosurfactants' CMC. At such concentrations, biosurfactants reduce the surfaceand interfacial tension between air/water and soil/water systems.

Due to the reduction of the interfacial force, contact of biosurfactants with soil/oil system increases the contactangle and reduces the capillary force holding oil and soil together. In turn, abovethe biosurfactant CMC, the solubilization process takes place.

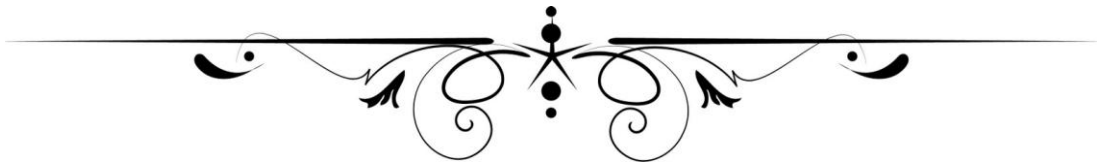
At these concentrations,biosurfactants molecules associate to form micelles, which dramatically increase thesolubility of oil (Déziel et al., 1996; Urum and Pekdemir, 2004; Nguyen et al., 2008; Nievaset al., 2008; Pacwa-Płociniczak et al., 2011). The hydrophobic ends connect together insidethe micelle, creating an environment compatible with hydrophobic organic molecules. Thisis known as solubilization. Emulsification is a process that forms a liquid, known as anemulsion, containing very small droplets of fat or oil suspended in a fluid, usually water.High molecular weight biosurfactants are efficient emulsifying agents. Cameotra andSingh (2009) reported the role of rhamnolipid biosurfactant synthesized by *Pseudomonasaeruginosa* in the uptake of *n*-alkane. They reported an exciting and new mechanism forhydrocarbon uptake involving the internalization of hydrocarbons inside the cell forsubsequent degradation. According to their mechanism, biosurfactants disperse hydrocarbonsinto microdroplets, increasing the availability of hydrocarbon to the bacterialcells. Thereafter, biosurfactant-coated hydrocarbon droplets are uptaken by the bacterialcells by adopting a similar mechanism to *pinocytosis* (a process through whichliquid droplets are ingested by living cells) as explained in Figure 2.2.There are reports where bacterial strains (PGPR) and the consortium producing a rhamnolipidclass of biosurfactant have been used for cultivation of medicinal plants such as*Withaniasonmifera*in hydrocarbon-contaminated soil without any toxicity in the activeingredient and reduction in the medicinal properties (Kumar et al., 2014). In the case of wheatand mustard rhizosphere, these biosurfactant producing bacterial strains in consortia havebeen used for enhancing petroleum hydrocarbon degradation in the rhizosphere (Kumaret al., 2013).



2.3 Mechanism for the uptake of petroleum hydrocarbons by rhamnolipid producing bacteria (Das et al. 2016)



Chapter 3



Chapter 3

The present work was planned for study under the following objective wise chapter:

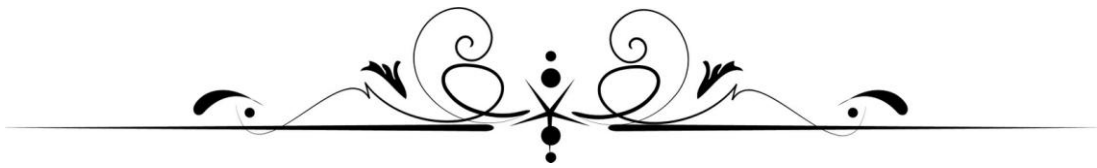
Chapter 3.1 Isolation of bacterial isolates from petroleum contaminated soil, characterization and screening for biosurfactant production and plant growth promotory properties.

Chapter 3.2 Extraction and characterization of biosurfactants from the selected bacterial isolates.

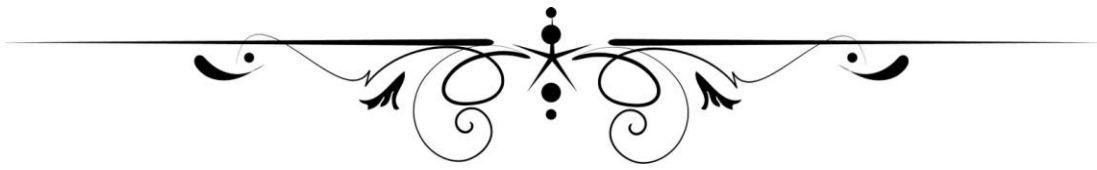
Chapter 3.3 Soilcollection from petroleum contaminated site for *in situ* experiment and its characterization for pollutant toxicity level.

Chapter 3.4 Exploring the potential of biosurfactant producing bacterial isolate for restoration and detoxification of the soil from the selected contaminated site.

Chapter 3.5 Comparison of soil detoxification process *in situ* under pot culture on medicinal plant and its active ingredient using antioxidant activity and stress response on metabolites using metabolomics.



Chapter 3.1



Chapter 3.1 Isolation of bacterial isolates from petroleum contaminated soil, characterization and screening for biosurfactant production and plant growth promotory properties

Contents:

3.1.1 Introduction

Petroleum refinery is an industrial plant which refines the crude oil into a useful product such as gasoline, petrol, diesel, kerosene, asphalt base, liquefied petroleum gas and heating oil (Gary and Handwerk, 1984). However in case of unwanted leakage of petroleum oil and its product might act as a persistent soil and water pollutant (Graj et al., 2013). Soil pollution by petroleum oil induces major changes in the physical and chemical properties of soil ensuing in an adverse effect on plant growth. Various microorganisms have acquired a mechanism to and grow in oil containing environment and play an immense role in treatment by degrading the pollutant (Pothuluri and Cerniglia, 1994; Jurelevicius et al., 2013; Pacwa-Płociniczak et al., 2016). Petroleum pollutants can be degraded by plants through biochemical reactions taking place within the plants and in the rhizosphere (Hrynkiewicz and Baum, 2011). The remediation of soils containing petroleum is possible with the use of plants and their rhizosphere processes (Mirsal, 2004; Hrynkiewicz and Baum, 2011). In this interaction, soil microorganism provides nutrients in the rhizosphere which leads to an increased microbial activity an degradation of toxic pollutants (Mirsal, 2004; Hrynkiewicz and Baum, 2011). Most of the soil bacteria with plant growth promoting traits can be well habituated to harsh soil conditions and enhance the remediation of disturbed soils directly and by plant growth promotion (Hrynkiewicz and Baum, 2011; Fomina et al., 2005; Wenzel, 2009). Apart, from PGP traits these microorganisms have biosurfactant producing efficiency. Certain microbial species such as *Pseudomonas* and *Bacillus* are accounted to excrete a various form of partially or totally extracellular biosurfactant that facilitates the uptake of hydrocarbons by reducing the surface

tension and enhance the removal of hydrocarbons from the oil contaminated soil (Bento et al., 2005; Franzetti et al., 2010).

Hence, the present chapter deals with isolation and characterization of biosurfactant producing plant growth promontory strains from petroleum contaminated soil.

3.1.2 Material and Methods

3.1.2.1 Sampling and isolation of biosurfactant producing plant growth promontory strains:

Sampling was done from petroleum contaminated sites of Lucknow, Uttar Pradesh and Guwahati, Assam, India **Table 3.1.1.** The strains were isolated in mineral salt medium (composition per litre K_2HPO_4: 1.0 g, $MgSO_4$: 0.5 g, $NaNO_3$: 2.5 g, KH_2PO_4: 6 g, $CaCl_2$: 0.01 g, $(NH_4)_2SO_4$: 1 g, $FeCl_3$: 0.1 g, $MnSO_4$: 0.005 g, agar: 15 g and Diesel and Petrol oil: 7 ml) through serial dilution method.Further, the strains were purified and stored at 4 °C. The inoculum was prepared by transferring a loopful of bacterial culture into 25 ml nutrient broth in 250 ml Erlenmeyer flask and incubated at 30 °C for 24 h. For screening the biosurfactant production ability and plant growth promoting traits, different tests were performed.

Table 3.1.1 Details of sampling sites

Sampling Sites	Location	No of samples
Lucknow, Uttar Pradesh, India	26°55' N latitude and 80° 59' E longitude	53
Guwahati, Assam, India	26° 11'N latitude and 91° 44'E longitude	47

3.1.2.2 Selection of potent bacteria

3.1.2.2.1 Screening of the bacterial strain for biosurfactant production

Isolated bacterial strains were tested for biosurfactant productionon minimal salt medium,(composition g/l $NaNO_3$: 2.5, $MgSO_4$: 0.5, $FeSO_4$: 0.01, KH_2PO_4: 1.0, Na_2HPO_4: 5.67, KCl: 0.1, $CaCl_2$: 0.1, $MnSO_4$: 0.002, NH_4NO_3: 0.39, dextrose: 15), incubated at 30°C for 72 hours with shaking at 160 rpm.

Foaming

Bacterial strains showing foaming in the medium as compared to negative controls were observed for reflected light which scattered like rainbow colour. Contents of the test tubes of minimal salt medium with the test strains (bacterial strains) showing foaming were subjected to centrifugation at 10,000 rpm for 20 minutes (4°C). Cell-free supernatant (crude biosurfactant) was used for further experiments.

Drop collapse test

Drop collapse test was performed by following the method of Bodour et al. (2003). Briefly, a thin coat of 10 W-40 oil was applied to each well of 96 microwell polystyrene plates and was equilibrated at 23°C for 24 h. 5 µl of supernatant aliquot was delivered into the center of each well. Beaded drops indicate a positive result, while collapse drop indicates positive results for the presence of biosurfactant.

Hemolytic assay

The hemolytic assay was performed by streaking strain onto a blood agar plate containing peptone:5g; beef extract: 3g, NaCl: 5g and agar: 15g. After autoclaving, 10% of sterile sheep blood wasadded. The plate was incubated for 24 h at 30º C and

visually inspected for clear zone around thecolony (Mulligan et al. 1984; Plaza et al. 2006).

3.1.2.2.2 Evaluation of plant growth promoting traits of the isolated strains

Qualitative and quantitative analysis of plant growth promoting (PGP) traits of the isolated strains were done by following the standard protocols. Plant growth promotory traits tested for the isolated strains were mentioned below.

Determination of phosphate solubilization:

For qualitative analysis of phosphate solubilization modified Pikovskaya agar was followed (Mehta and Nautiyal, 2001). In modified pikovskaya agar media bromophenol blue dye was added. Then after spot inoculated and incubated at 28 ± 2^{o}C and incubated for 72-96 hrs. After incubation, color zone in around the bacterial growth was observed. Quantitative analysis of phosphatesolubilisation of tricalcium phosphate in liquid medium was performed by following the method of King (1932). For quantitative analysis isolated bacterial strains were inoculated in tricalcium phosphate amended broth and incubated for 72-96 hrs at 30 °C in a shaker. After incubation, growth media were centrifuged at 15,000 rpm for 30 min. Then, after 1 ml of supernatant was mixed with 10 ml of chloro-molibidic acid and the volume was increased up to 45 ml employing distilled water. Then, 0.25 ml of choloro-stannous acid was added and further the volume was maintained up to 50 ml with distilled water. The absorbance was recorded at 600 nm and the amount of soluble phosphorus was demonstrated from the standard curve of KH_2PO_4.

Determination of siderophore production:

The siderophore production test was performed on Chrome Azural S (CAS) agar media by following the standard method of Schwyn and Neilands, (1987). Isolated bacterial strains were spot inoculated on CAS media and the plates were incubated for 72 to 96 hrs at 30^{o}C. After incubation period orange zone around bacterial growth considered as positive for siderophore production.

The quantitative analyses of siderophore production by the isolated strains were performed based on CAS shuttle assay (Schwyn and Nielands, 1987). In this method, strains were grown on nutrient broth at 30 ^{o}C for 48 hrs. After 48 hrs, culture the broth was centrifuged at 10,000 rpm for 10 minute and cell-free supernatant obtained i.e (0.5 ml) was mixed with CAS solution (0.5 ml). The absorbance of the mixture was recorded by spectrophotometer at absorbance 630 nm with reference to control (0.5ml un-inoculated nutrient broth + 0.5 ml CAS solution).

Production of indole acetic acid:

Production of Indole Acetic Acid (IAA) was demonstrated by following the method of Brick et al., (1991). In this method, isolated bacterial strains were inoculated in nutrient broth amended with 100µg/ml tryptophan and incubated for 72-96 hr at 30°C. After the incubation period, the culture broths were centrifuged and 3 to 4 drops of orthophosphoric acid were added along with Salkowski reagent and kept in dark for 30 min. Development of red to pink color indicates the production by the strains. For quantitative analysis, the absorbance of the mixture was recorded at 530 nm (Loper and Scroth, 1986).

ACC deaminase Activity:

Aminocyclopropane-1-carboxylate (ACC) deaminase production was determined by following the modified method of Li et al. (2011). Bacterial cultures grown in Luria Bertani broth were centrifuged at 8000 rpm for 5 min. The cell pellets obtained were washed with DF medium. Then inoculated in 2 ml DF-ACC medium and incubated for 24 hr at 28°C. Un-inoculated tubes serve as control. Then, 1 ml of this culture medium was centrifuged at 8000 rpm for 5 min and 100 µl of supernatant was diluted to 1 ml with liquid DF medium. 60 microliters of diluted supernatant were mixed with 120 µl of ninhydrin reagent in a tube and were kept on a water bath for 30 min till Ruhemann's purple color change. Further, the content of the tubes were measure absorbance at 570 nm with a spectrophotometer. In qualitative test, agar mineral salt agar medium containing ACC as nitrogen is used.

Hence, on the basis of PGP activity, 10 Strains were found as potential strains and use for further experiment.

3.1.2.3 Morphological characterization

Selected strains were characterized for determining the morphological characteristics. Cultural characteristics and Gram Staining of the strains were studied.

Cultural characteristics

Selected strains were cultured in Petri dishes on Nutrient Agar media for 48 hours at 28ºC and recorded for colony morphology.

Gram staining

For gram staining, a loop full of bacterial culture was smeared on a clean slide. Smear was stained with crystal violet (basic dye) for 1 minute, followed by washing under

running tap water, iodine (mordant dye) was added for 30 sec to form a crystal violet-iodine (CVI) complex, decolourised with ethanol (95%) and then counter stained with safranin for 30 seconds. The slide was observed under oil immersion lens of an inverted light microscope. In microscope shape and arrangement of cells were studied.

3.1.2.4 Biochemical characterization

For identification of the selected bacterial strains, biochemical tests were carried out (Bergey et al., 1994).The tests performed are as given below:

Hi-media IMViC test kit

Preparation of inoculum

Suspensions of the test strains were prepared in 2-3 ml of sterile saline. The density of the suspension was adjusted to a minimum of 0.1 optical densities at 620 nm.

Inoculation of the strip

- The kit was opened by peeling off the sealing tape by aseptically under laminar.
- Each well was inoculated with 50 µl of bacterial inoculum and incubated at 30°C for 24 to 48 hours.

Observations and Result

Results were determined after addition of the reagents as given below.

Indole Test: Well No. 1

- 1 to 2 drops of Kovac's reagent was added and the development of reddish pink colour within 10 seconds indicates positive reaction.
- Pale coloured indicates negative results negative.

Methyl Red Test: Well No. 2

- 1to 2 drops of Methyl Red reagent are added.
- If reagent remains red in colour indicates positive results.
- If reagent decolourises and becomes yellow then it indicates negative results.

Voges Proskaeur's Test: Well No. 3

- 2 to 3 drops of Baritt's reagent A and 1-2 drops of Baritt's reagent B are added.
- If pinkish red colour development within 5 to 10 minutes indicates a positive result.
- No change in colour indicates negative results.
- Result determination: Results were determined as per the chart is given below.

Table 3.1.2:Result interpretation chart for Hi-media IMViC kit

Test	Reagents to be added after incubation	Principle	Original colour of the medium	Positive reaction	Negative reaction
Indole	1-2 drops of Kovac's red reagent	Detects deamination of tryptophan	Colourless	Reddish pink	Colourless
Methyl red	1-2 drops of Methyl red reagent	Detects acid production	Colourless	Red	Yellow
Voges Proskauer's	1-2 drops of Barit reagent A and 1-2 drops of Baritt reagent B	Detects acetone production	Colourless	Pinkish red	Colourless/ slight copper
Citrate utilization	-	Detects capability of an organism to utilize citrate	Yellowish green	Blue	Yellowish green
Glucose	-	Glucose utilization	Red	Yellow	Red
Adonitol	-	Adonitol utilization	Red	Yellow	Red
Arabinose	-	Arabinose utilization	Red	Yellow	Red
Lactose	-	Lactose utilization	Red	Yellow	Red
Sorbitol	-	Sorbitol utilization	Red	Yellow	Red
Mannitol	-	Mannitol utilization	Red	Yellow	Red
Rhamnose	-	Rhamnose utilization	Red	Yellow	Red
Sucrose	-	Melibiose utilization	Red	Yellow	Red

3.1.2.5 Cell morphology characterization- Scanning electron microscopy (SEM) of bacteria:

In scanning electron microscopy analysis, bacterial cultures grown in nutrient broth were used. Bacterial cultures were inoculated in 1 ml glutaraldehyde (5%) in Eppendorf tube and kept at 4°C in the refrigerator for fixation of cells. After fixation, glutaraldehyde was removed and cells were washed with buffer saline. After then, 1 ml of ethanol (30%) was added and centrifuged at 3000 rpm for 10 minutes. The supernatant obtained was disposed off. The same process was repeated with 50, 70, 80, 90 and 100% of ethanol for dehydration purpose. Further, dehydrated cells suspended in 100 % ethanol, were mounted on stubs coated with carbon tape employing a micropipette. Then after mounted samples were dried and observed under the scanning electron microscope.

3.1.2.6 Molecular characterization of the bacterial strain

For molecular characterization, isolated DNA was electrophoresedin agarose gel. Further, PCR amplification of 16S rDNA was amplifiedby using universal primers 27F (AGAGTTTGATCMTGGCTCAG-20) and 1492R (GGTTACCTTGTTACGACTT-20). PCR products were viewed on1% (w/v) agarose gels, cleaned with PCR purification kit and eventuallywere processed to sequencing analysis. The evolutionary historyof the strain was inferred using the Neighbor-Joining method (Saitouand Nei, 1987).

3.1.3 Results and Discussion

Selected potent bacterial strains:

All the isolated bacterial strains were screened for biosurfactant production and plant growth promontory traits. Based on the results 10 strains were found to be potent and were employed for further studies**Table 3.1.1.**

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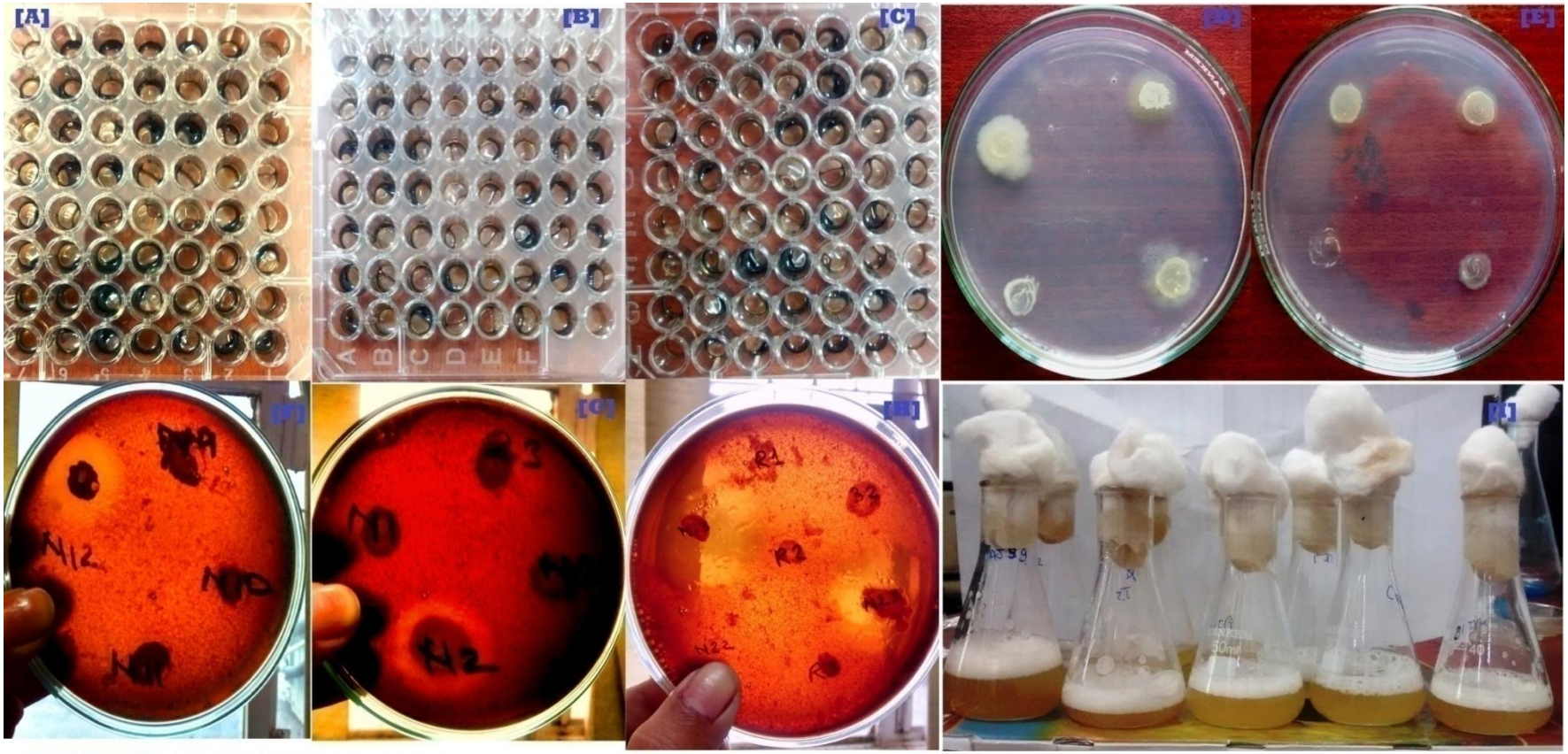
Table 3.1.1. Selection of potent strains based on biosurfactant production and plant growth promontory traits

Area of Isolation	strain	Biosurfactant Production Assay				Plant growth promoting activity		
		Haemolytic assay	Oil displaced agar assay	Drop collapse test	Foam test	Phosphate solubilisation	IAA Production	Siderophore production
Lucknow, Uttar Pradesh, India (26°55' N latitude and 80° 59' E longitude).	AJ1	+ve	-ve	-ve	+ve	+ve	-ve	-ve
	AJ2	-ve	-ve	+ve	+ve	-ve	-ve	-ve
	AJ3	-ve	-ve	-ve	+ve	-ve	-ve	-ve
	AJ7	-ve	-ve	+ve	+ve	+ve	-ve	-ve
	AJ8	+ve	-ve	-ve	+ve	-ve	-ve	-ve
	AJ9	-ve	+ve	+ve	+ve	+ve	-ve	-ve
	AJ11	+ve	-ve	-ve	+ve	-ve	-ve	+ve
	AJ15	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	AJ32	+ve	-ve	+ve	+ve	+ve	+ve	+ve
	AJ39	-ve	+ve	+ve	+ve	-ve	-ve	-ve
Guwahati, Assam, India (26° 11'N latitude and 91° 44'E longitude).	N12	+ve	+ve	+ve	+ve	-ve	+ve	-ve
	N13	+ve	+ve	+ve	+ve	-ve	+ve	+ve
	N23	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	L1	+ve	+ve	+ve	+ve	-ve	+ve	-ve
	L2	+ve	+ve	+ve	+ve	-ve	-ve	+ve
	J1	+ve	+ve	+ve	+ve	-ve	+ve	+ve
	J2	+ve	+ve	+ve	+ve	-ve	+ve	+ve
	J3	+ve	+ve	+ve	+ve	-ve	+ve	+ve
	J4	+ve	+ve	+ve	+ve	-ve	+ve	+ve
	J5	+ve	+ve	+ve	+ve	-ve	+ve	+ve

Highlights indicate potent strains based on biosurfactant production and plant growth promontory traits

Figure 3.1.1. Selection of potent strains based on biosurfactant production



[A],[B],[C]--DROP COLLAPSE TEST

[D],[E]------OIL DISPLACED AGAR ASSAY

[F],[G],[H]-HEAMOLYTIC ASSAY

[I]----------FOAM TEST

Morphological characterization of isolated bacteria

Cultural features of all the 10 selected isolated strains were mentioned in **Table 3.1.2.** Further, the selected strains were characterized for their morphology and gram reaction **Table 3.1.3.**

Table 3.1.2 Morphological Characterization of selected bacterial strain

Strain	Shape of the colony	Colony Margins	Surface Characteristics	Colour of colony
AJ15	Round	Smooth	Concentric	Light yellow
N12	Round	Smooth	Smooth	Light yellow
N13	Round	Smooth	Smooth	Green
N23	Irregular	Filamentous	Wrinkled	Light yellow
L1	Round	Smooth	Smooth	Yellowish green
J1	Irregular	Filamentous	Wrinkled	White
J2	Punctiform	Curled	Smooth	White
J3	Irregular	Lobate	Contoured	Light yellow
J4	Round	Smooth	Smooth	White
J5	Irregular	Curled	Contoured	Light yellow

Table 3.1.3 Gram staining of selected bacterial strain

Strain	Gram staining	Motility test
AJ15	-ve	Motile
N12	-ve	Motile
N13	-ve	Motile
N23	-ve	Motile
L1	+ve	Non-motile
J1	+ve	Motile
J2	+ve	Motile
J3	-ve	Motile
J4	+ve	Motile
J5	-ve	Motile

Biochemical Characterization of selected bacterial strain

All the selected bacterial strains were characterized biochemically. Results are depicted in **Table 3.1.4** and **Figure 3.1.2.** . For further confirmation about the isolates, molecular characterization was performed wherein 16S rRNA sequencing was undertaken for the bacterial isolates.

Figure 3.1.2 Biochemical Characterization of selected bacterial strain

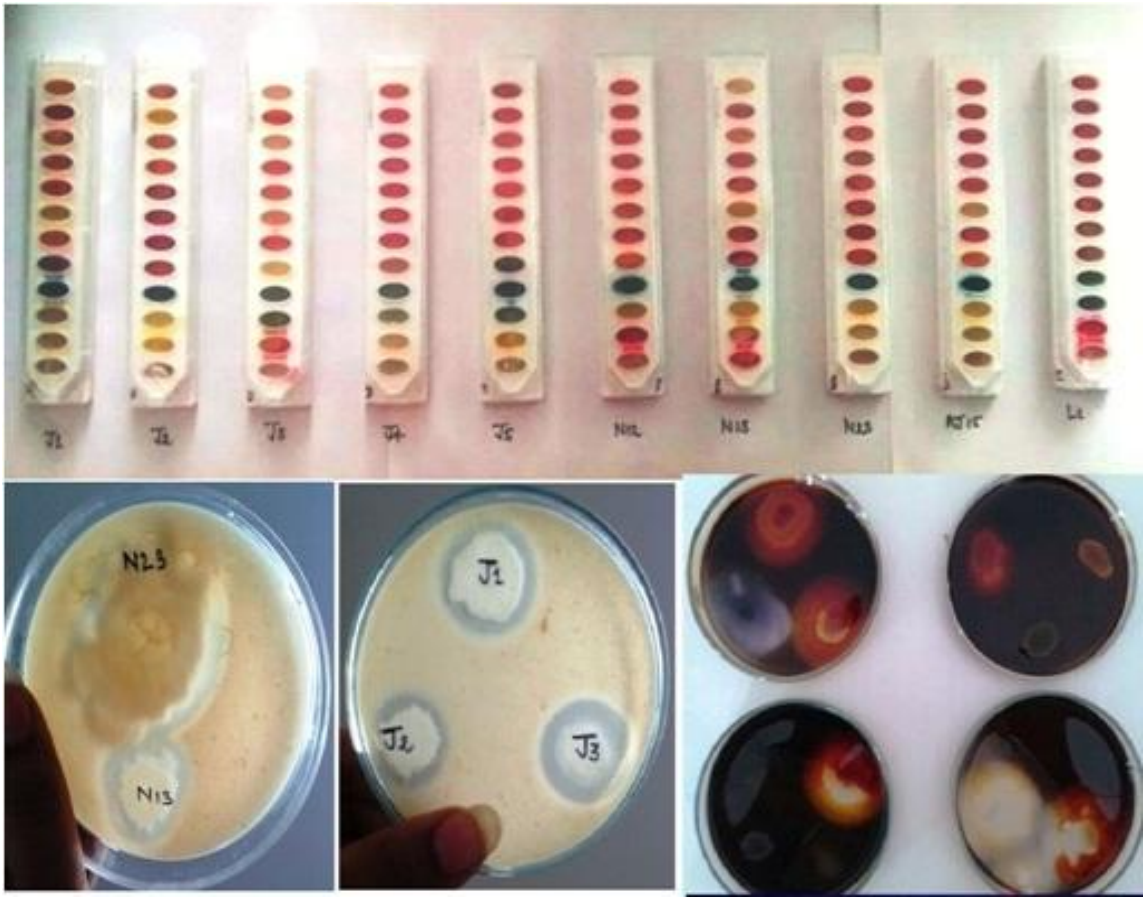


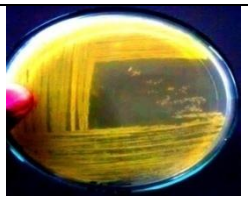
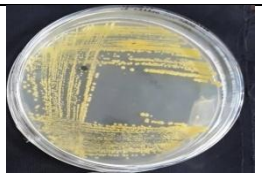
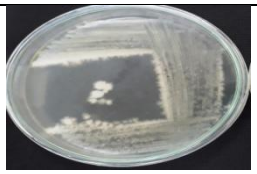
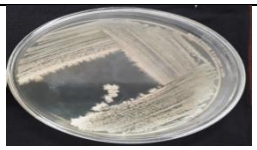
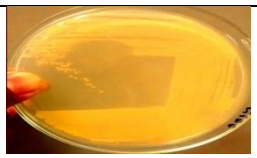
Figure 3.1.4 Biochemical Characterization of selected bacterial strain

Strain	Biochemical Characterization of selected bacterial strain											
	Indol test	Methyl red test	Voges Proskauers test	Citrate utilization test	Glucose	Adonitol	Arabinose	Lactose	Sorbitol	Mannitol	Rhamnose	Sucrose
AJ15	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
N12	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
N23	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
L1	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve-	-ve	-ve	-ve	-ve
J1	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
J2	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve
J3	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
J4	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
J5	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve

Selection of strains for further studies

Based on biosurfactant activity and good plant growth promontory activities mostly ACC deaminase activity, 5 bacterial strains were selected from the 10 potent isolates.

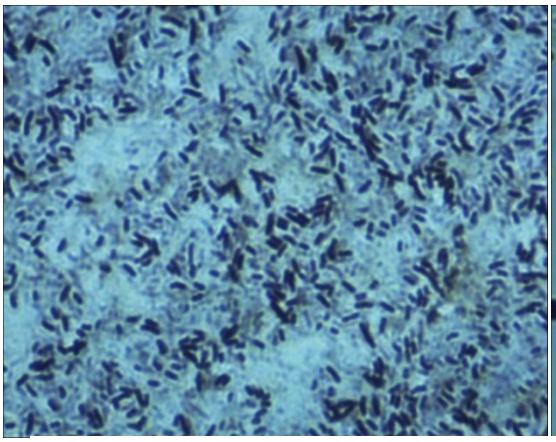
Table 3.1.5 Selected bacterial isolates and their morphological characteristics

S. No.	Name of isolates	Colony morphology	Gram's Characteristics	Photograph of colony characteristics
1.	**AJ15**	Green fluorescent, transparent and sticky	Gram-negative	
2.	**L1**	Yellowish green and smooth	Gram-positive	
3.	**J1**	White and wrinkled	Gram-positive	
4.	**J2**	White and smooth	Gram-positive	
5.	**N23**	Green fluorescent, transparent and sticky	Gram-negative	

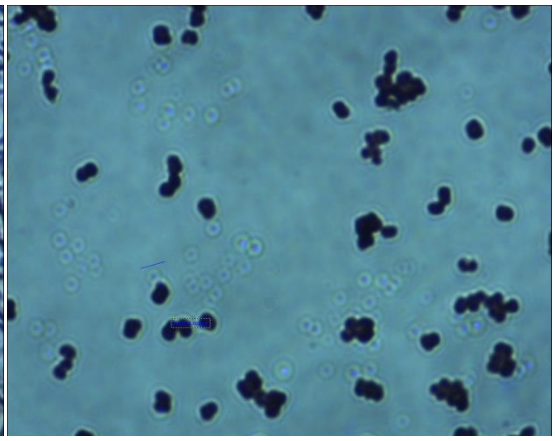
Microscopic images of the selected bacterial strain under a light microscope

The cell morphology of the 5 selected bacterial strains is depicted below:

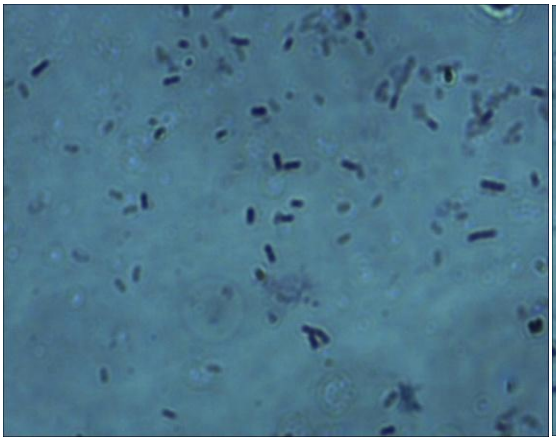
3.1. 3 Microscopic images of the bacterial strain under light microscope



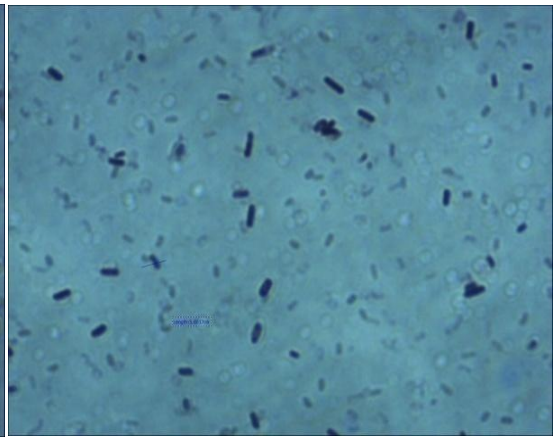
AJ15



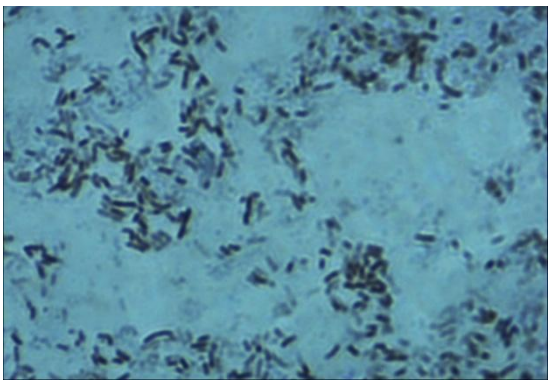
L1



J1



J2

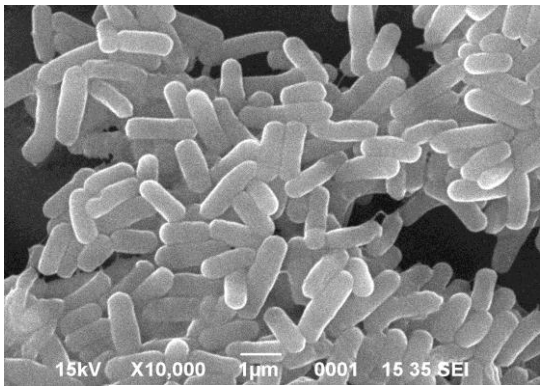


N23

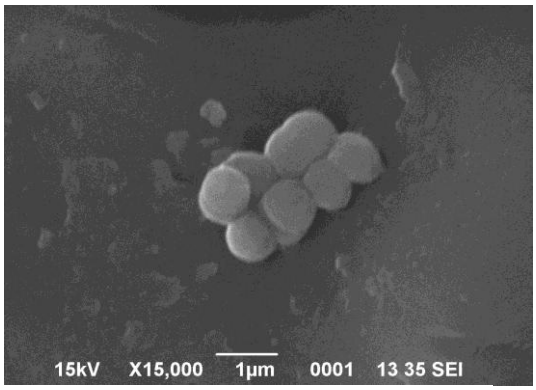
Microscopic images of the selected bacterial strain under an electron microscope

The cell bacterial strains'morphology of the 5 selected depicted below

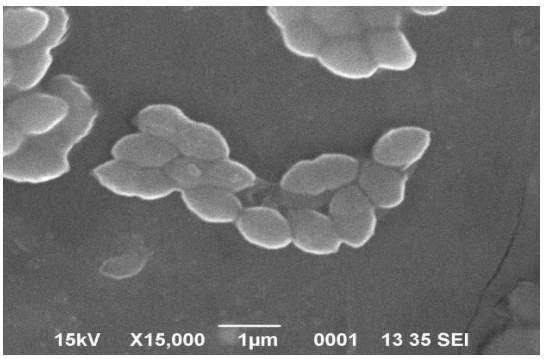
3.1.4 Microscopic images of the bacterial strain undera scanning electron microscope



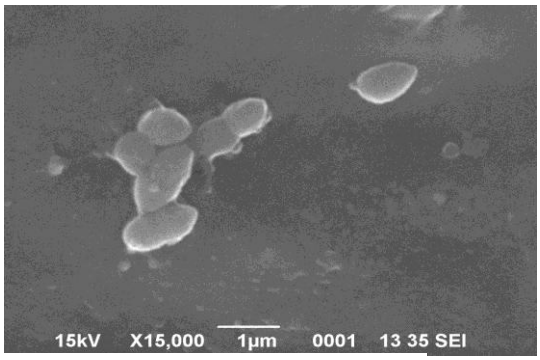
AJ15



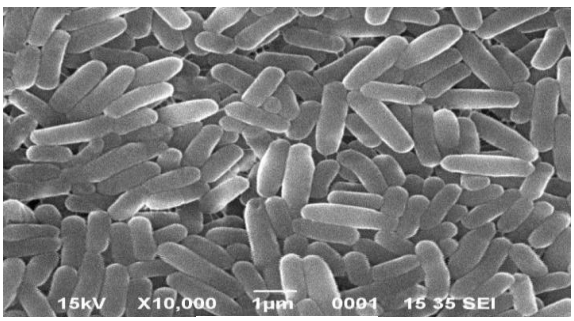
L1



J1



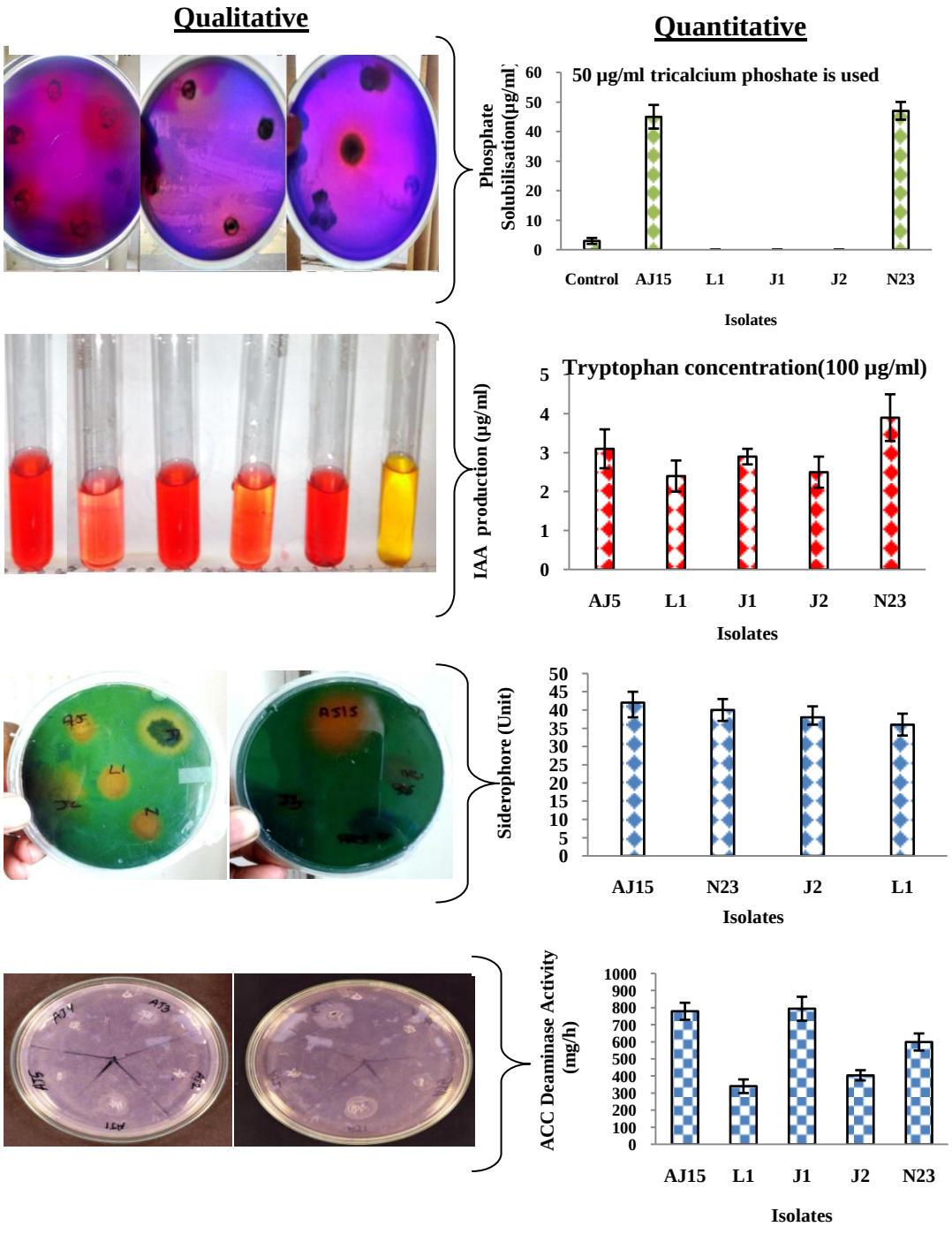
J2



N23

All the test strains were found positive for phosphate solubilization,siderophore and IAA production (Figure 3.1.5).

Figure 3.1.5. Plant Growth Promoting traits of the selected bacterial strain



Molecular Characterization of the selected isolates

For identification of isolates, 16S rRNA sequencing was carried out. 16S rRNA fragment was amplified using universal primers and sequenced. Based on 16S rRNA studies, isolated bacterial strains were found similar to *Pseudomonas azotoformans* (AJ15 and N23), *Micrococcus endophyticus* (L1), *Bacillus licheniformis* (J2) and *Bacillus safensis* (J2). Accession numbers allotted are KU671026, KT951267, KT951266, KU680817, KX865089 respectively.

Figure 3.1.6 a Phylogenetic tree of isolate *Pseudomonas azotoformans* AJ15

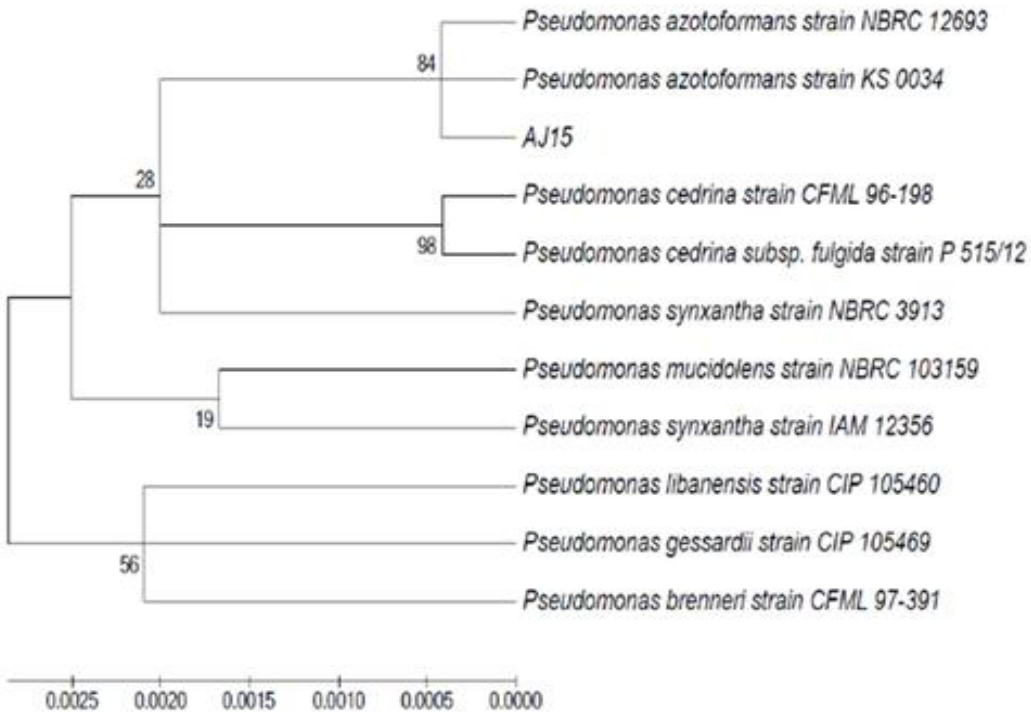


Figure 3.1.6b Phylogenetic tree of isolate *Micrococcus endophyticus* L1

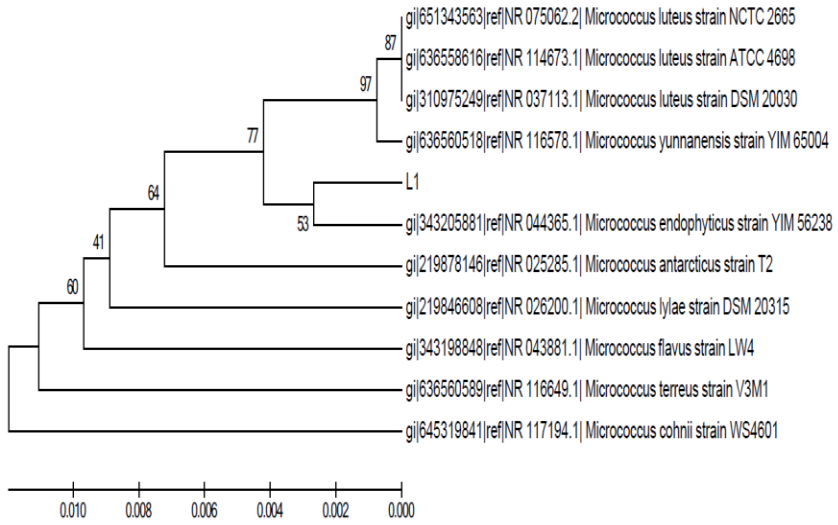


Figure 3.1.6c Phylogenetic tree of isolate *Bacillus licheniformis* J1

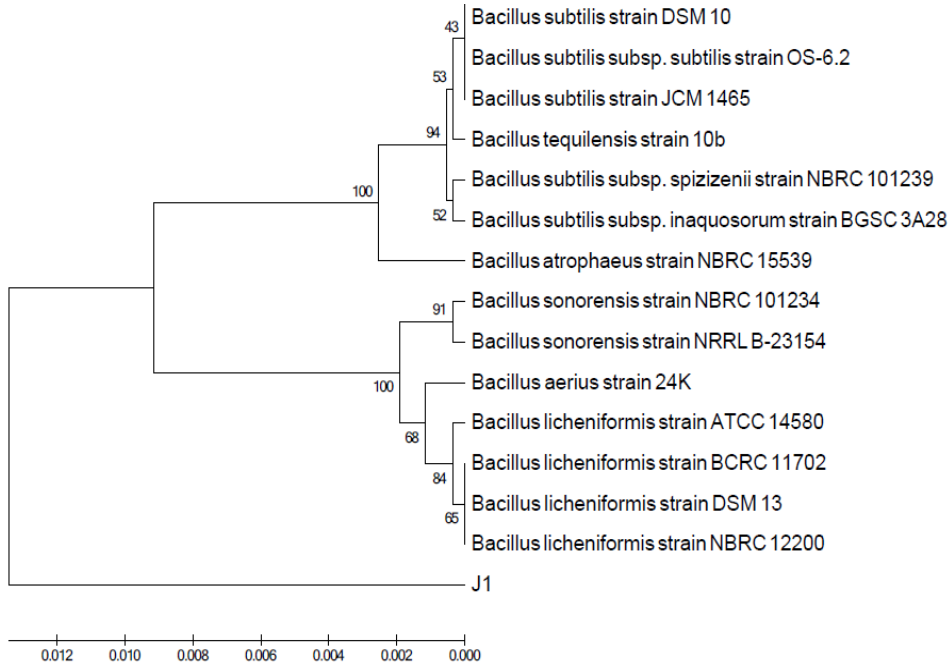


Figure 3.1.6 d Phylogenetic tree of isolate *Bacillus safensis* J2

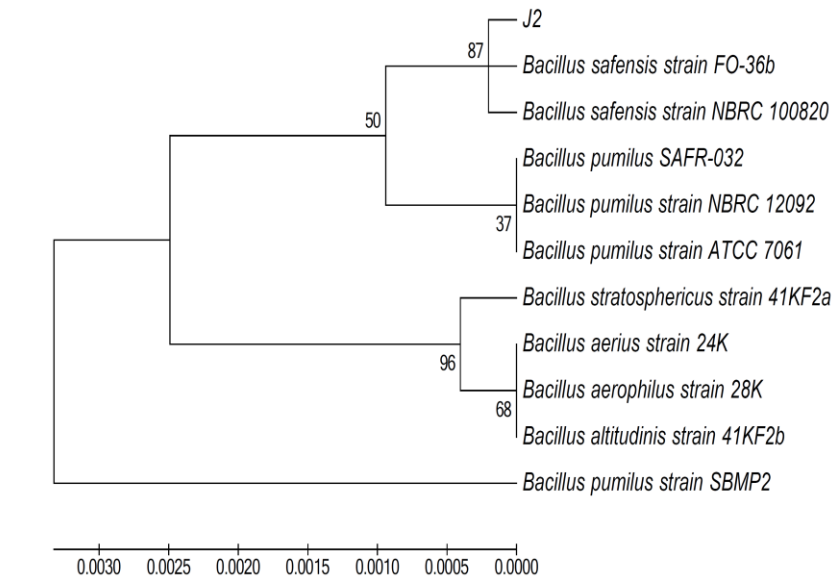


Figure 3.1.6 e Phylogenetic tree of isolate *Pseudomonas azotoformans* N23

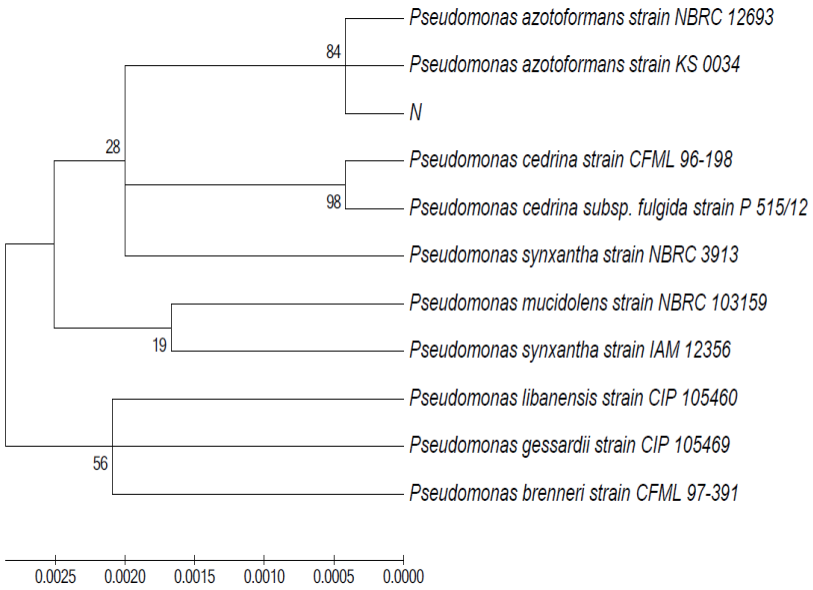


Figure 3.1.6 (a-e) Phylogenetic tree of the selected strains.

All the isolated bacterial strains were screened for biosurfactant production and plant growth promontory traits. Based on the results 10 strains shows both biosurfactant producing activity as well as plant growth promontory traits, but only 5 strains were

found to be potent and were employed for further studies based on ACC deaminase activity. From the selected 5 strains 2 strains were Gram-negative (AJ15 and N23) and 3 strains (L1, J1 and J2) were Gram-positive. Morphological and Biochemical studies indicate that the strains AJ15 and N23 might belong to *Pseudomonas* sps, L1 might be *Micrococcus* sps and J1 and J2 might be to *Bacillus* sps. Based on 16S rRNA studies, isolated bacterial strains were found similar to *Pseudomonas azotoformans* (AJ15 and N23), *Micrococcus endophyticus* (L1), *Bacillus licheniformis* (J2) and *Bacillus safensis* (J2) . The similarity index among thetest strains and with other strains is shown in Fig. Sequences of the test strains AJ15, L1, J1, J2 and N23 have beendeposited in the NCBI database and accession number are allotted.

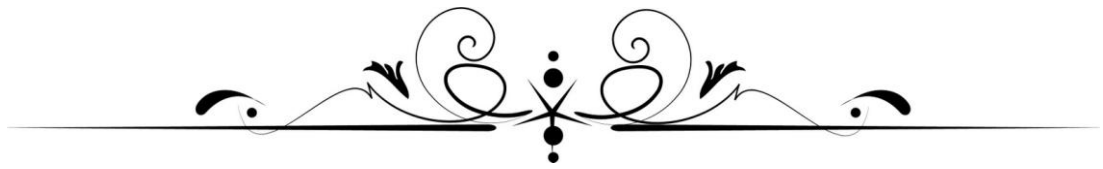
The selected strains produced biosurfactant on mineral salt medium (MSM). Each test strains produced foam with surfactants. All the strains shows positive results for drop collapse test as the drop spread and collapsed. This assay depends on the destabilization of liquiddroplets by biosurfactant. This was developed by Jain et al. (1991). In this method, drops of culture supernatant are kepton oil coated solid surface. If the liquid does not possess surfactants, then water moleculesare repelled from the hydrophobic surface, whereas liquid that containssurfactants collapse the oil droplet by reducing the interfacial tension betweenthe liquid drop and the hydrophobic surface.Furthermore, Bodour and Miller Maier modified the method in the year 1998. Hemolytic assay indicatesthat the strains produced biosurfactants as colorless transparent ring around the colonies were observed. The concept of hemolytic assay was developed by Mulligan et al. (1984) which is based on lysis of blood erythrocytes.

All the strain possess plant growth promoting traits. The strains AJ15 and N23 were found positive for phosphate solubilization.In qualitative analysis of phosphate reddish zone around the colonies indicate that the bacterial strain AJ15 has the ability to solubilise phosphate, whereas quantitative test for solubilization of tricalcium phosphate in liquid medium by the test strain showed that strain solubilize tricalcium phosphate. Qualitative and quantitative test for siderophore production on Chrome Azurol-S media were found to be positive for the strains AJ15, L1, J2 and N23 as indicated by change in colour of the media around the colonies.Quantitative estimation of Indole acetic acid (IAA) in the presence of 100 µg/ml concentrations of tryptophan indicates that the strain was able to produce good amount of IAA.

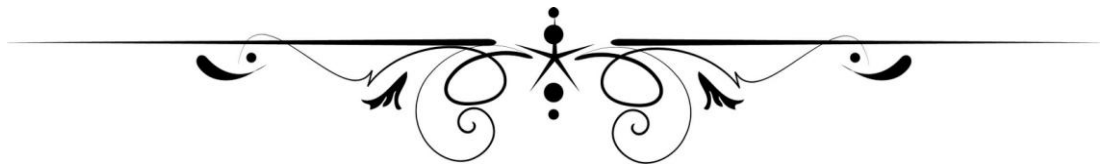
ACC deaminase activity is one of the important properties of microbes that enable them to survive in abiotic stress condition. In the present study all the 5 strains shows good ACC deaminase activity.

3.1.4 Conclusion

The present chapter demonstrates that bacterial strains isolated from petroleum contaminated sites are efficient biosurfactant producer along good plant growth promoting properties. Further, microorganism adapted to peculiar soil environment is need to isolate for studying the clues about the functional microorganisms and their metabolic function, So, that they can be effectively and commercially used for management and detoxification petroleum contaminated soil.



Chapter 3.2



Chapter 3.2 Extraction and characterization of biosurfactants from the selected bacterial isolates

Contents:

3.2.1 Introduction

Biosurfactants are amphiphilic molecules which consist of hydrophobic and hydrophilic moieties. They show a wide range of properties such as formation of microemulsions and micelles between two different phases, lowering of interfacial and surface tension of liquids. The hydrophobic moiety of a surfactant is stated as the "tail", whereas the hydrophilic part is defined as the "head" of the molecule. Surfactants are broadly classified as cationic, anionic, zwitterionic and non-ionic on the basis of ionic charge of the hydrophilic part. (Christofi and Ivshina, 2002; Franzetti et al.2010). Biosurfactant are of different type and each type belongs to particular group based on their chemical composition. Various groups of biosurfactant are glycolipids, lipopeptides, fatty acids, phospholipids and neutral lipids, polymeric biosurfactants and particulate biosurfactants (Pacwa-Płociniczak et al. 2011). Glycolipids are the lower molecular biosurfactant with carbohydrate and lipid. They are important class of glycoconjugates built with one or two sugar residues such as glucose and galactose attached to different lipid backbones. Glycolipids with characteristic hydrophilic head are widely found in cell membranes of animals, plants, fungi and bacteria in the form of diacylglycerolglycosides, glycosylceramides and sterylglycosides (Warnecke and Heinz, 2010; Cortés-Sánchez et al.2013). Glycolipids consist of different class like rhamnolipids, trehalolipids and sophorolipids. Lipopetides are biosurfactants of low molecular weight in which consist of a lipid attached to a polypeptide chain. They broadly consist of class surfactin, lichenysin and iturin. Both, the biosurfactants (glycolipids and lipopeptides) are used in various industrial, agricultural and environmental sectors due to lower toxicity and higher biodegradability as compared to their synthetic chemical surfactants (Rosenberg and

Ron, 1999; Kumar et al.2015). Hence, in the present chapter focus on biosurfactant production from the selected bacterial strains and their extraction characterization.

3.2.2 Materials and Methods

3.2.2.1 Biosurfactant production

For biosurfactant production selected bacterial strains were grown on minimal salt medium, (composition g/l $NaNO_3$: 2.5, $MgSO_4$: 0.5, $FeSO_4$: 0.01, KH_2PO_4: 1.0, Na_2HPO_4: 5.67, KCl: 0.1, $CaCl_2$: 0.1, $MnSO_4$: 0.002, NH_4NO_3: 0.39, dextrose: 15), incubated at 30°C for 72 hours with shaking at 150 rpm.

3.2.2.2 Extraction of biosurfactant

For extraction of biosurfactant, 72 h grown bacterial cultures in the substrate were centrifuged at 10,000 for 20 min at 4 °C temperature. Culture supernatant (crude biosurfactant) obtained was transferred to empty beakers and stored at 4 °C temperature. Further, 100 ml of crude biosurfactant was acidified with 1M HCl to obtain a pH of 2.0 and kept at 4 °C. The extraction was performed twice with an equal volume of diethyl ether by vigorously shaking using a separating funnel. The organic layer was collected and transferred to empty beakers and allowed to evaporate for 24 h and then dried in an oven at 60 °C for 1 h. Biosurfactant obtained was transferred to desiccator to remove traces of moisture (Pansiripat et al., 2010; George and Jayac, 2013; Gandhimathi et al., 2009; Das and Kumar, 2018).

3.2.2.3 Chemical analysis of the extracted biosurfactant

For analysis of carbohydrate three test were performed (Sawhney and Singh, 2000; Mahesh et al.2006)

Anthrone test: 2 ml of anthrone reagent was added to small amount of extracted biosurfacant and mixed thoroughly till the colour changed.

Iodine test: 1 ml of iodine solution was added to the small amount of the extracted biosurfactant and mixed gently. The colour formation was observed.

Bial'sTest:1ml Bial's reagent was added to small amount of biosurfactant and a test tube was allowed to stand for sometime in a boiling water bath. The color formation was observed.

For Analysis of Lipids two test were performed (Sawhney and Singh, 2000; Mahesh et al. 2006)

Solubility Test: For lipid analysis small amount of extracted biosurfactant was mixed with water, alcohol and chloroform in different test tube.

Saponification Test: Small amount of extracted biosurfactant was mixed with 2 ml of 2% NaOH solution shaken well and observed for formation of soap.

Detection of Glycolipids by phenol H_2SO_4 method

Glycolipids production was detected according to the method of Dubois et al. (1956). Briefly, 1 ml of 5 % phenol was added to small amount of crude biosurfactant. To above mixture, 4 ml of concentrated H_2SO_4 was added drop by drop. Colour change was observed; development of yellow to orange colour indicated the presence of glycolipids.

Detection of Rhamnolipids by CTAB method

Rhamnolipids production by the strains were detected by following the method of Siegmund and Wagner, 1991. Briefly,for detecting rhamnolipid production the strain was spot inoculated on the MSM agar plates (composition g/l Cetyltrimethyl ammonium bromide : 0.2g; Methylene Blue: 0.005g; Peptone:1.5g, $MgSO_4$: 0.5 g, K_2HPO_4: 1.0 g, $FeCl_3$: 0.1 g, KH_2PO_4: 1.0 g, $CaCl_2$: 0.01 g, $MnSO_4$: 0.005 g, Agar 15 g and Glycerol- 15 ml).After then the plates was incubated at 30° C for 7 days.

3.2.2.4 Characterization of the extracted biosurfactant

Determination of cell surface hydrophobicity

To determine the bacterial adhesion to hydrocarbons method proposed by Rosenberg et al. (1980) and Zhang and miller, 1994 was followed. Briefly, the bacterial cells were washed twice and resuspended in buffer salts Solution pH 7 (composition g/l KH_2PO_4: 7.5, K_2HPO_4: 18, $MgSO_4$: 0.2, urea: 1.8) to give an optical density at 400 nm of 1.0. Each, 1 ml of hexadecane and 4 ml of cell suspension were added into two test tubes and one of the test tube was mixed with 0.001 mM extracted biosurfactant. The test tubes were vortexed for 1 min and equilibrated for 1 h. The optical density of the bottom aqueous phase was then measured at 400 nm. Hydrophobicity is expressed as the percentage of adherence to hexadecane and calculated by: 100 x (1 - OD of the aqueous phase/OD of the cell suspension).

Emulsification index

Emulsification index E24 (%) was determined by addition of 5ml engine oil to 4ml of the test broth in a 15 ml graduated tube. The resulting mixture was vortexed for 2 min to enable proper mixing of engine oil and test broth. Tube was allowed to stand for 24 hours without any disturbance. After 24 hours, height of the emulsified layer was examined to calculate

emulsification index (Cooper and Goldenberg, 1987; Shavandi et al., 2011).

$$\text{Emulsification idex, E24(\%)} = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

Surface tension

For measuring surface tension 5 ml broth supernatant of 72 hours grown culture was taken in glass tube and then after the tube was submerged in water bath at a constant temperature of 28°C. Surface tension was measured on the basis of the height reached

by the 163 liquid when freely ascended through a capillary tube. Non- inoculated broth was taken as control and the surface tension was calculated according to the following formula (Munguia and Smith, 2001; Viramontes Ramos et al.2010).

$$\text{Surface tension } (\gamma) = \frac{\text{rhdg}}{2}$$

ϒ- Surface tension (mN/m); r - capillary radius (0.05 cm); d - Density (g/ml); g =gravity (980 cm/s_2); h - height of the liquid column (cm).

Oil displacement test

Oil displacement test was determined by following the method of Ohno et al. (1993). Briefly, 50 µl of engine oil was dropped on to the surface of 20 ml of distilled water in a 10 cm diameter petri dish. Then after 50 µl of isolated crude biosurfactant was added on to the surface of the engine oil layer in the petridish. The diameter of the clear zones was measured and calculated as the rate of diameter of the petridish.

3.2.2.5 Toxicity assessment of the extracted biosurfactant

Comparative toxicity assessment of the extracted biosurfactant with nonylphenol ethoxylate (chemical surfactant) was screened through seed germination inhibition assay and earthworm acute toxicity test.

Seed germination inhibition

Certified wheat seeds were surface sterilized with sodium hypochlorite. 10 sterilized seeds of wheat were placed in different Petri dish, followed by addition of 10 ml of extracted biosurfactants (concentrations: 100, 200, 300, 400 and 500 mg) and nonylphenol ethoxylate (concentrations: 100, 200, 300, 400 and 500 mg), plates were incubated for 7 days at 27 °C. For control experiments, 10 seeds of wheat placed in Petri dishes with 10 ml of distilled water for the same duration. Plates were incubated at 27 °C and examined for germination inhibition at every 24 h interval for after 7

days. The experiments were repeated thrice (U. S. Environmental Protection Agency, 1982).

Earthworm acute toxicity test

200 g garden soil was mixed with various concentrations (100, 200, 300, 400 and 500 mg) of biosurfactant and nonylphenol ethoxylate (chemical surfactant) was taken in different brown bottle for screening the earthworm acute toxicity test. Ten healthy earthworms were put into each brown bottle and kept for 7 days. After 7 days death percentage of the earthworms was determined by no response to acupuncture (Tang et al., 2011).

3.2.2.6 Biosurfactant stability studies

Stability of the extracted biosurfactant were studied under a wide range of pH, temperatures and salt concentration to demonstrate the effect on surface tension reducing capability and emulsification index of the biosurfactant. The analysis was done using crude biosurfactant. To analyze the effect of pH on surface tension and emulsification index, 10 ml of crude biosurfactant were adjusted at varying pH from 2 to 12 with 6 N NaOH and 6 N HCl solutions. The effect of temperature was estimated at different temperature of 4, 15, 30, 60 and 90 °C. The effect of salt concentration was checked by varying the concentration of sodium chloride (0% to 10 % w/v), added to 10 ml of crude biosurfactant.

3.2.2.7 FTIR characterization of the biosurfactant

The chemical compositions of the extracted biosurfactants were studied by using FTIR spectrometer. One milligram of the extracted biosurfactant was ground with 100 mg of KBr and transmission was recorded using FTIR model Thermo scientific Nicole 6700.

3.2.2.8 Liquid chromatography–mass spectrometry (LC–MS) of the extracted biosurfactants

For the LC–MS analysis, 50 mg of the extracted biosurfactant was dissolved in 5000 µL acetonitrile/HCOONH4 buffer in 40/60 (v/v) ratio and then filtered using a 0.20 µm syringe filter (Behrens et al., 2016). The filtered solution was further analyzed using LC-ESI-MS (Liquid chromatography–Mass spectrometry).

3.2.2.9 Utilization of agroindustrial waste for biosurfactant production under submerged fermentation (SmF) and its optimization

Agro-industrial wastes (sugarcane bagasse) used in the present study was collected from local potato chips making unit and sugar industry. Potato peels and sugarcane bagasse were washed three times with water and allowed to dry for 24 h at 70 °C. The dried bagasse were ground and passed through a sieve of 0.5mm to get a fine powder. Fine powders obtained were mixed in various concentrations in 1 L distilled water for the development of substrate for biosurfactant production along with optimized mineral salts (MS) (composition /liter: Na_2HPO_4 5.67 g, $MgSO_4$ 0.5 g, KH_2PO_4 1.0 g, $NaNO_3$ 2.5 g, $CaCl_2$ 0.1 g, $FeSO_4$ 0.01 g, KCl 0.1 g, $MnSO_4$ 0.002 g, NH_4NO_3 0.39 g) under submerged fermentation in Erlenmeyer flask. For determining the optimum condition of substrates for maximum biosurfactant production (yield), the developed substrates were monitored at a range of varying pH, temperature and agitation rate.

3.2.2.10 Optimization of biosurfactant productionby Response Surface Methodology using Box-Behnken Design

Biosurfactant production optimization was estimated by Response surface methodology employing different concentration of bagasse powder. RSM was employed for experimental design on the basis of Box Behnken design algorithm for the optimization of the major variables. The 4 variables designated as A, B, C and D

represents Bagasse powder, temperature, agitation and pH respectively. A total 29 experimental runs with different combinations of 4 factors were carried out. A second-order polynomial equation was fitted to correlate the relationship between independent variables and response 1 (biosurfactant production) by predicting the optimal point through the below mention equation.

$$Y_{pred} = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

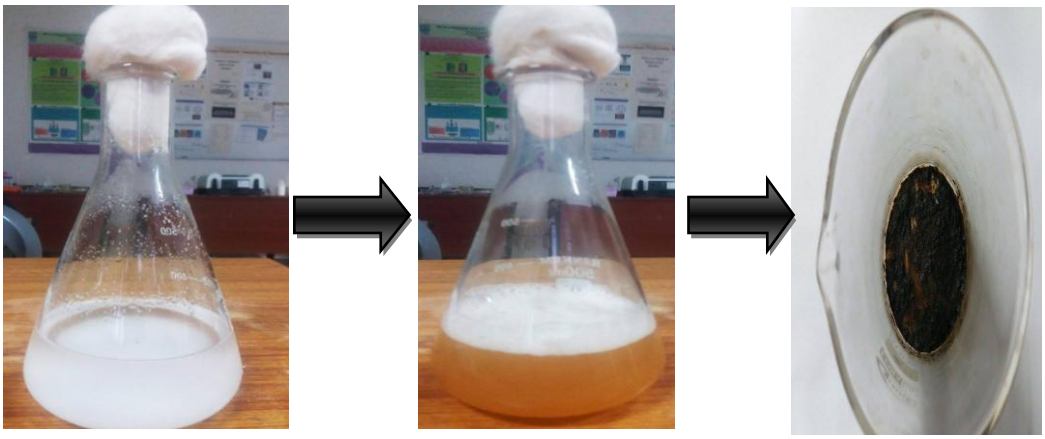
where Y_{pred} indicates the predicted response

x*i* and x*j* are input variables that influence the response Y

β_o is the constant, β_i is the *i*th linear coefficient, β_{ii} is the *i*th quadratic coefficient, β_{ij} is the *ij*th interaction coefficient.

3.2.3 Results and Discussion

The extracted biosurfactant from all the strains were stored for further studies.



Bacterial strain was tested for biosurfactant production minimal salt medium, MSM (composition g/l $NaNO_3$: 2.5, $MgSO_4$: 0.5, $FeSO_4$: 0.01, KH_2PO_4: 1.0, Na_2HPO_4: 5.67, KCl: 0.1, $CaCl_2$: 0.1, $MnSO_4$: 0.002, NH_4NO_3: 0.39, dextrose: 15)

Biosurfactant production at 72 hrs with foam

Extracted biosurfactant

Figure 3.2.1 Pictorial depiction biosurfactant production and extraction

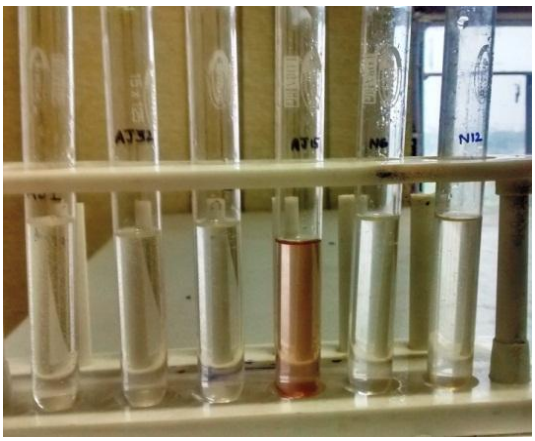
Chemical Analysis of the extracted biosurfactant

Chemical analysis results of the extracted biosurfactants from the strains are represented within Table 3.2.1. The strain AJ15 and N23 (*Pseudomonas azotoformans*) shows positive for anthrone test, barfoeds tests, iodine test, Bials test, saponification test and glycolipid test whereas strains J1(*Bacillus licheniformis*) and J2 (Bacillus safensis) shows negative results for anthrone test, barfoeds tests, iodine test, Bials test and glycolipid test , but both strains (J1 and J2) shows positive results for biuret test and saponification test. Of all the strains only strain the strain AJ15 and N23 shows positive for rhamnolipid test.

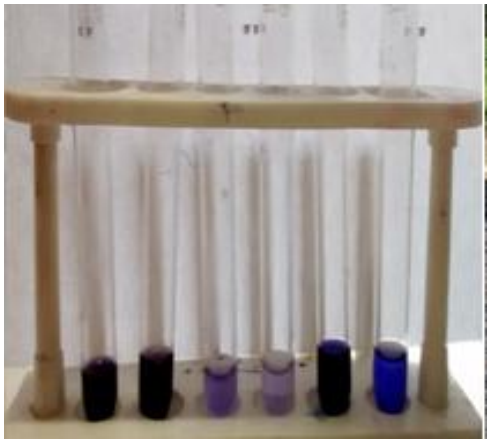
Table 3.2.1 Chemical Analysis of the extracted biosurfactant

Strain	Chemical analysis assay						
	Anthrone test	Iodine test	Solubility Test	CTAB test	Saponification Test	Phenol H_2SO_4 test	Biuret test
AJ15	Positive	Positive	Positive	Positive	Positive	Positive	Negative
L1	Positive	Positive	Positive	Negative	Positive	Negative	Negative
J1	Negative	Negative	Negative	Negative	Positive	Negative	Positive
J2	Negative	Negative	Negative	Negative	Positive	Negative	Positive
N23	Positive	Positive	Positive	Positive	Positive	Positive	Negative

Figure 3.2.2 Chemical Analysis of the extracted biosurfactant

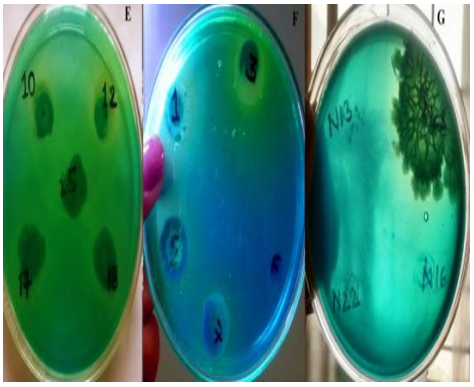


H_2SO_4 test

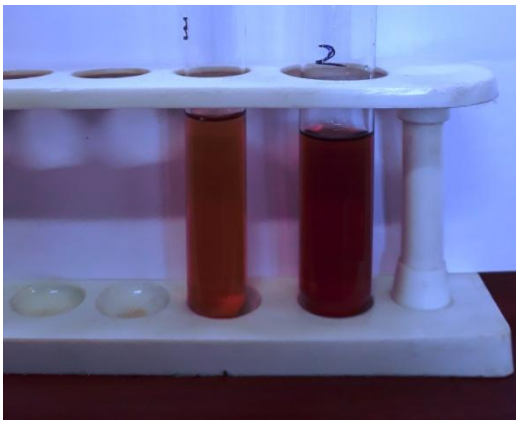


Biuret test

Saponification Test



CTAB Test



Iodine test

The change of colour in anthrone test to a bluish green that indicates the presence of carbohydrates in the extracted biosurfactant. A red precipitate formed in barfoeds tests and absence of blue or reddish brown complex in iodine test signifies the presence of monosaccharides carbohydrates and absence of polysaccharides respectively.The extracted biosurfactants were insoluble in water, whereas as soluble in alcohol and chloroform. In saponification test, NaOH used saponifies the extracted biosurfactanst by confirming the presence of lipid in the extracted biosurfactants. In phenol H_2SO_4 method, formation of light orange color indicated that the biosurfactant produced by AJ15 and N23 might be glycolipid group. In CTAB test dark blue halo zones around colonies of strain AJ15 and N23 signifies the production of rhamnolipids. Rhamnolipids consist of a polar head and non-polar tail group like synthetic surfactants and as a result, they combine with cationic cetyl-trimethyl ammonium bromide to form insoluble ion pairs in aqueous solution and precipitates as dark blue halo zones against a blue background (Siegmund and Wagner, 1991). And in biuret test dark blue colour of the solution indicates the lipopetide nature of biosurfactants reported in strain J1 and J2. Hence, the results of the chemical analysis revealed that the extracted biosurfactant from AJ15 and N23 might belong to glycolipid group and class rhamnolipid, whereas biosurfactant extracted from J1 and J2 might belong to lipopetide group.

Characterization of the extracted biosurfactant

Biosurfactants extracted from the selected bacterial are subjected to various experiments such as cell surface hydrophobicity, emulsification index, surface tension reduction and oil displacement assay. Results obtained from the experiments were depicted within Table 3.2.2.The results reveal that biosurfactant from each strains show good emulsification property, has efficiency in reducing surface tension, has ability to enhance cell hydrophobicity and has potential oil displacement activity.

Figure 3.2.3 Oil displacement area of the biosurfactants extracted

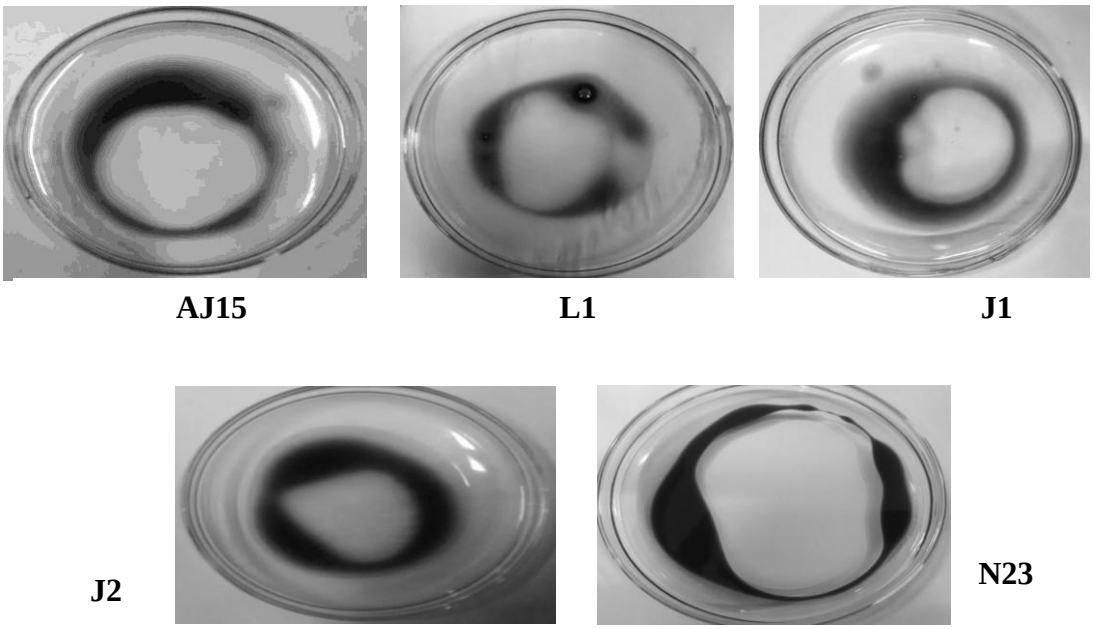


Table 1. Surface tension reduction (ST), emulsification activity (ET) , Oil Displacement area (ODA of the bacterial strains in MSM broth

Strains	72 hours		
	ET (%)	**ST (m/Nm)**	**ODA (cm)**
AJ15	62 ± 2.7	30 .5 ± 0	5.43 ± 0.51
L1	59 ± 4	33 ± 2	3.7 ± 0.4
J1	73.36 ± 5	29.5 ± 1	4.1 ± 0.2
J2	68.66 ± 4.01	34 ± 1.15	3.66 ± 0.6
N23	77 ± 3	29 ± 2	5.7 ± 0.5

Results analyzed by using single-factor ANOVA of variance and are mean of three replicates with standard deviation (Means ± S.D, n = 3). Optimized condition of growth: Temperature= **35 ° C**, pH of the substrates= **7,** agitation rate **=180 rpm**

Cell hydrophobicity is an important factor for predicting cell adhesion to surfaces (van Loosdrecht et al., 1987). Hence bacterial adherence to hydrocarbon (BATH) assay is employed for determining hydrophobicity of cell to hydrophobic substrates. The cell hydrophobicity of strains showed increase in adherence in presence of the extracted biosurfactants. The results reported were similar to those of Zhang and Miller (1994) who demonstrated that cell hydrophobicity can be induced in the presence biosurfactant. Cell hydrophobicity effectively increased the biodegradation of hydrophobic organic substrates (Rosenberg and Rosenberg, 1981). Hence; hydrophobicity of cell has immense importance in bioremediation and biodegradation of major organic pollutant. Emulsification capacity of a biosurfactant is of great importance as it can be employed for several applications such as bioremediation oil contaminated sites and for enhanced oil recovery. Emulsification index of biosurfactant produced by the strain AJ15 is 62.7%, L1 is 59%, J1 is 73.36%, J2 is 68.66% and N23 is 77% respectively. Surface tension of non-inoculated broth was 65.8 mN/m. After 72 h the surface tension of the broth were 30.5 mN/m for AJ15, 33 mN/m for L1, 29.5 mN/m for J1, 34 mN/m for J2 and 29 mN/m. These, indicates that biosurfactant produced by the strains decrease the surface tension of the broth.

Toxicity of extracted biosurfactant

In comparative toxicity assessment the extracted biosurfactant with nonylphenol ethoxylate (chemical surfactant) through seed germination inhibition and earthworm toxicity reveal that extracted biosurfactants are low toxic in nature Figure 3.2.4.

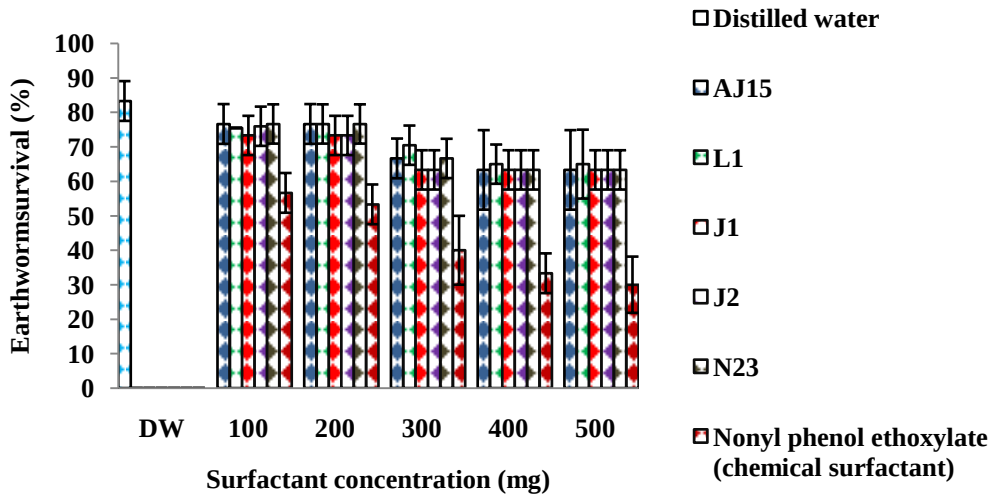


Figure 3.2.4 Toxic effect of surfactant (biosurfactant and chemical surfactant) earthworm survival.

Results analyzed by using single-factor ANOVA of variance and are mean of three replicates with standard deviation (Means ± S.D, n = 3). And differences were considered significant by determining the p-value (^{a} $p < 0.01$,^{b} $p < 0.05$, ^{c} Not significant).

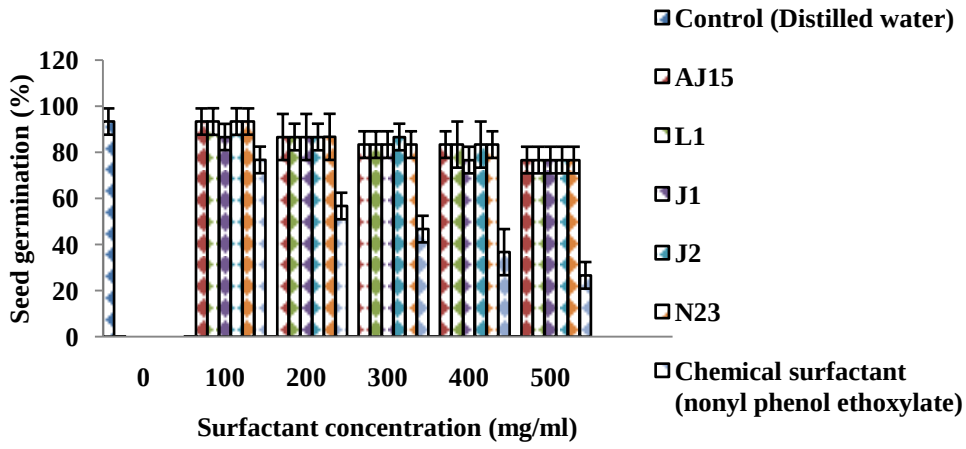


Figure 3.2.5 Toxic effect of surfactant (biosurfactant and chemical surfactant) on seed germination.

Results analyzed by using single-factor ANOVA of variance and are mean of three replicates with standard deviation (Means ± S.D, n = 3). And differences were considered significant by determining the p-value (^{a} $p < 0.01$,^{b} $p < 0.05$, ^{c} Not significant).

The non-toxic product is of basic importance for application within the environment domain. Hence, the toxicity of chemical substances can be assessed by eco-toxicity

bioassays that use living organism as bio-indicator tool for characterizing toxicity level and predicting the threshold limit of a chemical within a particular ecosystem (Fletcher, 1991; Nalini and Parthasarathi, 2014). Earthworms are sensitive to chemical substances present in soil that makes them a good bioindicators soil toxicity assessment. Acute toxicity bioassay is a short-term test for screening the effects of chemical substances on earthworm survivability (Handy, 2007). The literature on the effect of surfactants (biosurfactant and chemical surfactants) on earthworms is scarce. For this reason, we have revealed the effect of surfactants on earthworms. The investigation demonstrated that there is a great effect of chemical surfactant on earthworms. Figure 3.2.4 represents the earthworm survival rate in various concentrations of biosurfactant and chemical surfactant (nonyl phenol ethoxylate). Toxicity assessment of the extracted biosurfactant from the strains exhibited no inhibitory effect on seed germination and earthworm survival rate. The results of seed germination are shown in Figure 3.2.6. The results demonstrated that seed germination achieved in various concentration (100, 200, 300, 400 and 500 mg) of biosurfactant is better than nonylphenol ethoxylate (chemical surfactant) treated seeds. These might be due to lower toxicity and higher biodegradability of biologically produced surfactants (Franzetti et al., 2011).

Biosurfactant stability to Environmental stresses

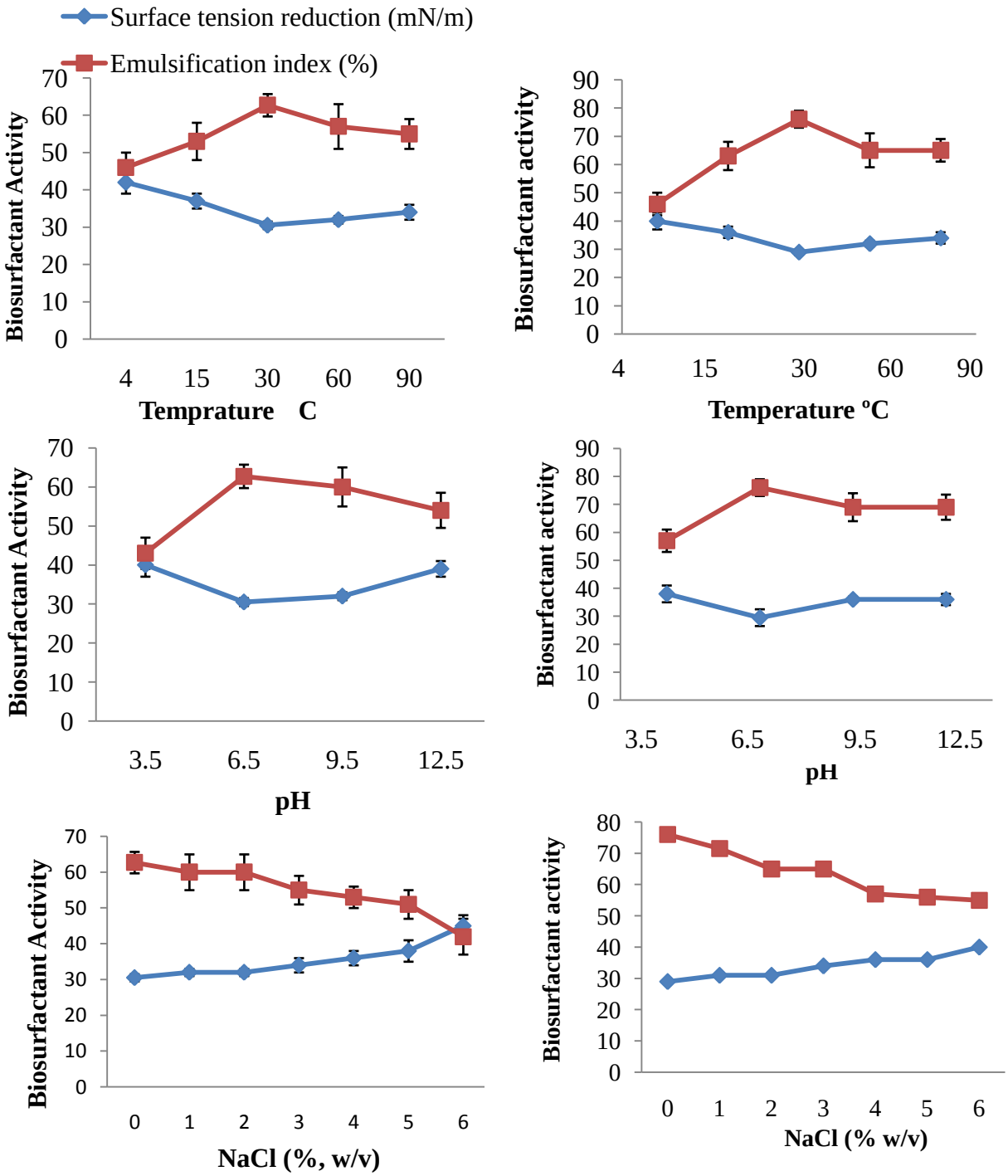
The effect of pH, temperatures and salt concentration on biosurfactant was studied demonstrate in the Figure 3.2.6 that represents the effect of pH; temperature and salinity on the stability of extracted biosurfactant. The temperature effect on biosurfactant showed that the extracted biosurfactants were quite stable at 30 °C. Heating of the crude biosurfactant up to 90 °C caused no significant effect on the emulsification and surface tension reducing the capability of the biosurfactants. Similar results of extreme stability of biosurfactant extracted from Pseudomonas sps were obtained by Aparna et al. (2012), Abdel-Mawgoud et al. (2008) and Kiran et al.

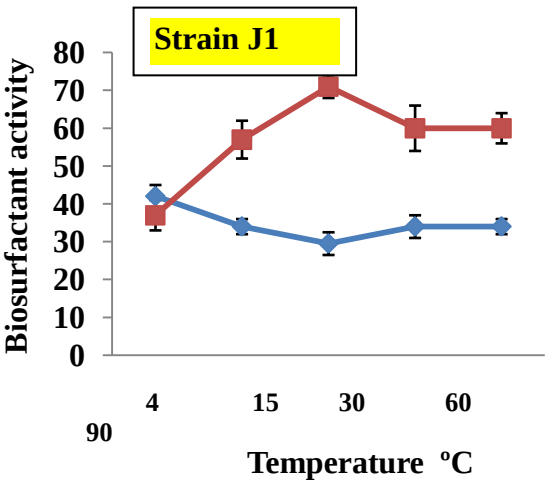
(2010). Studies on the effect of pH suggest that biosurfactants were stable at pH 7. Further, it was also confirmed that extracted biosurfactants remains stable in basic pH as compared to acidic pH. Effect of extreme pH in biosurfactant performance is due to result of structural alteration of the biosurfactant. Results are in accordance with those published by Raheb and Hajipour (2011). The effect of NaCl was investigated by increasing salt the concentrations from 1% to 10%. Very low effect of salinity was observed on biosurfactant stability till 5% concentration of NaCl, but increasing the concentration of the salt above 5% reduced activities of the all extracted biosurfactants except the biosurfactant from strain J2 that shows tendency to persist its activity at high salt concentration. Reduced activity of biosurfactant in high salinity might be due to the electrolytes present in NaCl affects the carboxylate and peptide groups of the biosurfactant. The interface between solution and air acquires negative charge which induces the repulsion between biosurfactant molecules. The negative charge is protected by the Na+ ions in an associate degree electrical double layer within the presence of NaCl, inflicting the formation of a compact monolayer that results in decrease performance of the biosurfactant (Aparna et al., 2012; Helvacı et al., 2004).

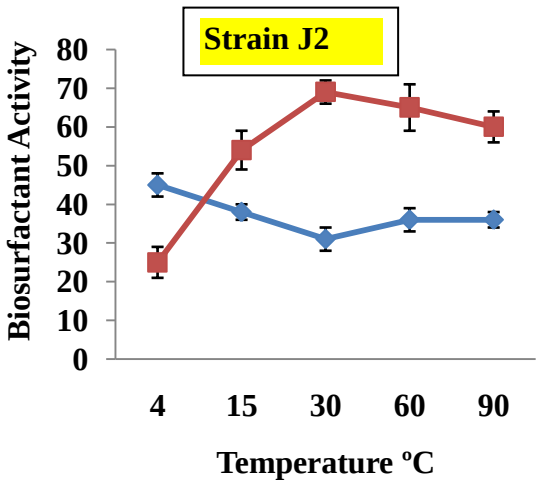
The results of strain J2 were similar to Pathak and Keharia (2014) who reported stablilty of biosurfactant over a 10 % (w/v) NaCl concentration.Hence, the present study indicates that the extracted biosurfactants can be employed in extreme environmental conditions.

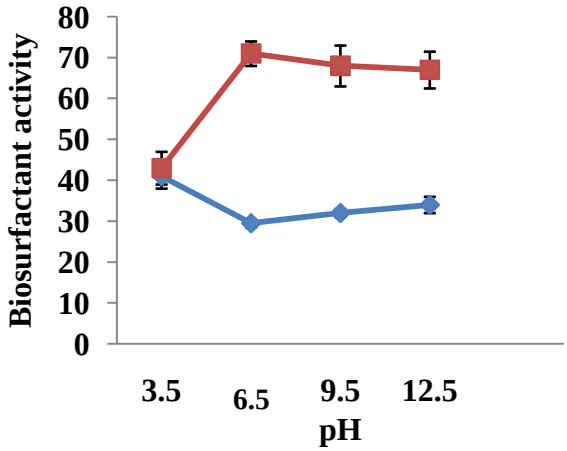
Note: The strain L1 (*Micrococcus endophyticus*) was not used for further studies due its extracted biosurfactant which shows complexity in structure as well as in biosurfactant activity. Moreover, the strain was not compatible with other strain AJ15, J1, J2 and N23.

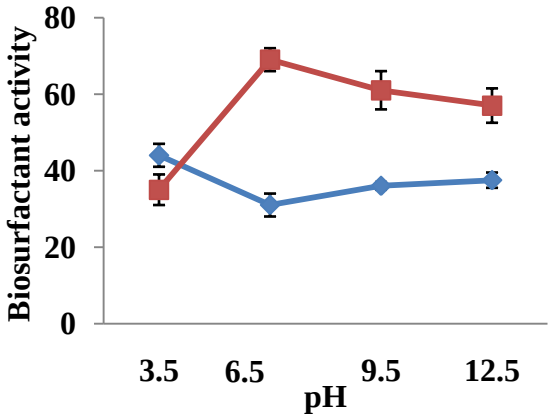
Figure 3.2.6 Effect of temperature, pH and salinity on biosurfactant performance **Strain AJ15** **Strain N23**

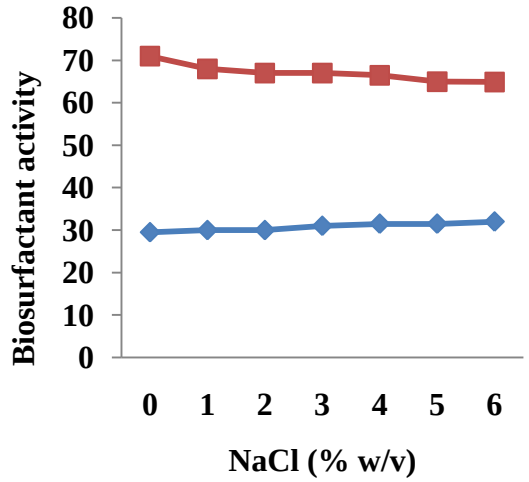


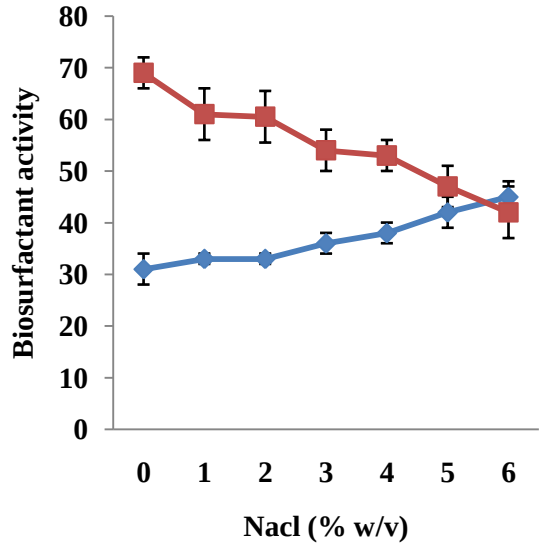

Strain J1
Biosurfactant activity
80
70
60
50
40
30
20
10
0
4
15
30
60
90
Temperature °C


Strain J2
Biosurfactant Activity
80
70
60
50
40
30
20
10
0
4
15
30
60
90
Temperature °C


Biosurfactant activity
80
70
60
50
40
30
20
10
0
3.5
6.5
9.5
12.5
pH


Biosurfactant activity
80
60
40
20
0
3.5
6.5
9.5
12.5
pH


Biosurfactant activity
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
NaCl (% w/v)


Biosurfactant activity
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Nacl (% w/v)

FTIR Characterization of the Biosurfactant

FTIR characterization of the extracted biosurfactant is represented in Figure 3.3.7 (a-d)

FTIR Characterization of the Biosurfactant extracted from the strain AJ15:

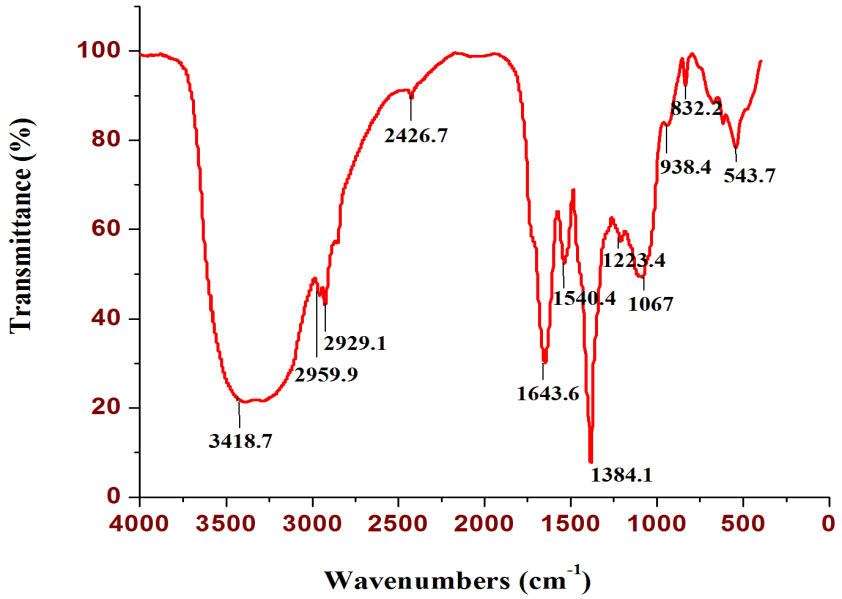


Figure 3.2.7a FTIR spectroscopy of extracted biosurfactant from *Pseudomonas azotoformans* AJ15

The FTIR spectroscopy of extracted biosurfactant from *Pseudomonas azotoformans* AJ15 shows various characteristic absorptions Figure 3.2.7a . The functional group reported in FTIR spectroscopy are C–H stretching (2959.9 cm^{-1} and 2929.1 cm^{-1}), C=O stretching (1643.6 cm^{-1}), C–H deformations (1384.1 cm^{-1}), C–O stretching (1223.4 cm^{-1} and 1067 cm^{-1}). The functional group reported in the present study is much similar to the result reported by Rahman et al. (2010) and Rikalovic et al. (2012). Hence, the extracted biosurfactant might belong to the rhamnolipid class.

FTIR Characterization of the Biosurfactant extracted from the strain J1:

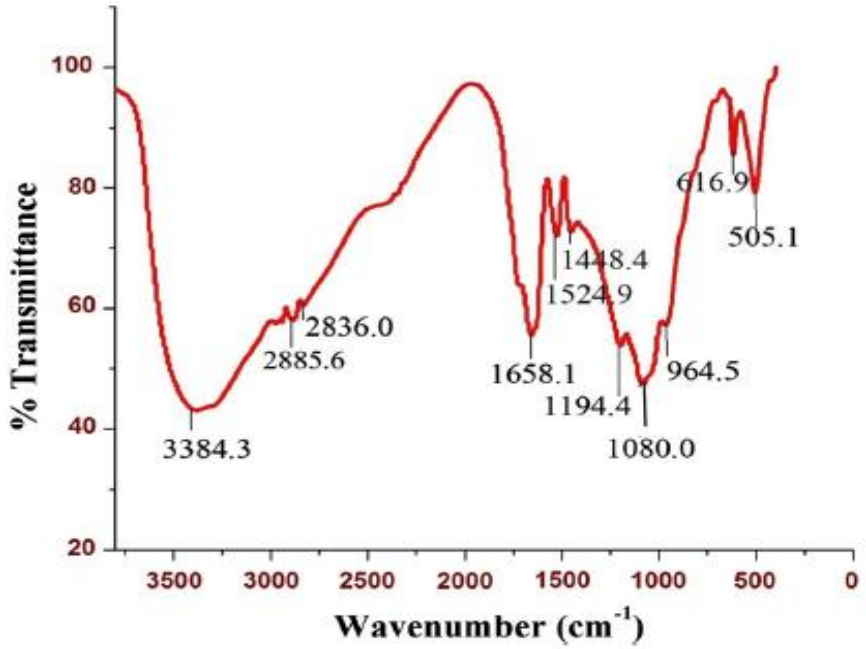


Figure 3.2.7b FTIR spectroscopy of extracted biosurfactant from *Bacillus licheniformis* J1

The FTIR spectroscopy of extracted biosurfactant from *Bacillus licheniformis* J1 is presented in Figure 3.2.7 b. Characteristic bands of peptides were observed at 3384.3 cm^{-1} (N–H stretching), 1658.1 cm^{-1} (C=O stretching), 1194.4 cm-1 (C–N), whereas aliphatic chains was observed at 2885.6 cm^{-1} and 2836.0 cm^{-1} (C–H stretching), indicating that the extracted biosurfactant belongs lipopeptide group. The characteristics bands reported is quite similar to the result reported by El-Sheshtawy et al. (2015).

FTIR Characterization of the Biosurfactant extracted from the strain J2:

The Fourier transform infrared spectroscopy of extracted biosurfactant from *Bacillus safeinsis* J2 is shown in **Figure Figure 3.2.7c.** Absorption bands reported in fourier transform infrared spectroscopy are N-H stretching (3398.9 cm^{-1}), C-H stretching (2936.4 cm^{-1}, 1460.4 cm^{-1}), stretching of N-H bond (1640.3 cm^{-1}). The functional group reported in the present study is much similar to the result reported by Jung et al. (2012). Hence, the extracted biosurfactant might belong to lipopetide group and surfactin class.

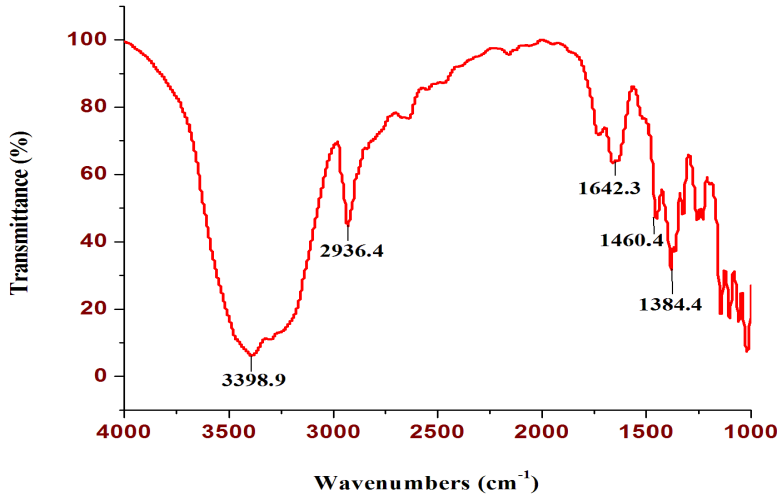


Figure 3.2.7c FTIR spectroscopy of extracted biosurfactant from *Bacillus safensis* J2

FTIR Characterization of the Biosurfactant extracted from the strain N23:

The FTIR spectroscopy of extracted biosurfactant from *Pseudomonas azotoformans* N23 shows various characteristic absorptions Figure 3.2.7d. The functional group reported in FTIR spectroscopy are C–H stretching (3340 cm^{-1}), C=O stretching (1640 cm^{-1}), C–H deformations (1370 cm^{-1}), C–O stretching (1220 cm^{-1} and 1050 cm^{-1}). The functional group reported in the present study is much similar to the result reported by Rahman et al. (2010), Das and Kumar, (2019) and Rikalovic et al. (2012). Hence, the extracted biosurfactant might belong to the rhamnolipid class.

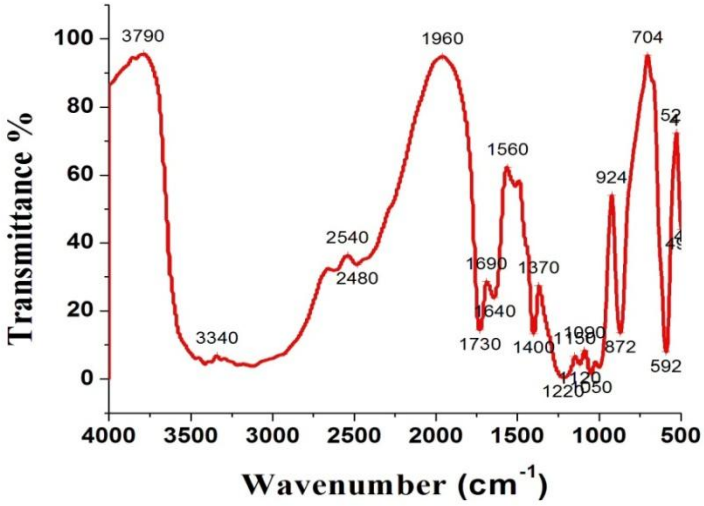


Figure 3.2.7d FTIR spectroscopy o biosurfactant from *Pseudomonas azotoformans* AJ15

Liquid chromatography–mass spectrometry (LC-MS) Characterization of the Biosurfactant:

The LC-MS characterization of the extracted biosurfactant from the bacterial strains were represented in Figure 3.2.8 (a,b,c and d).

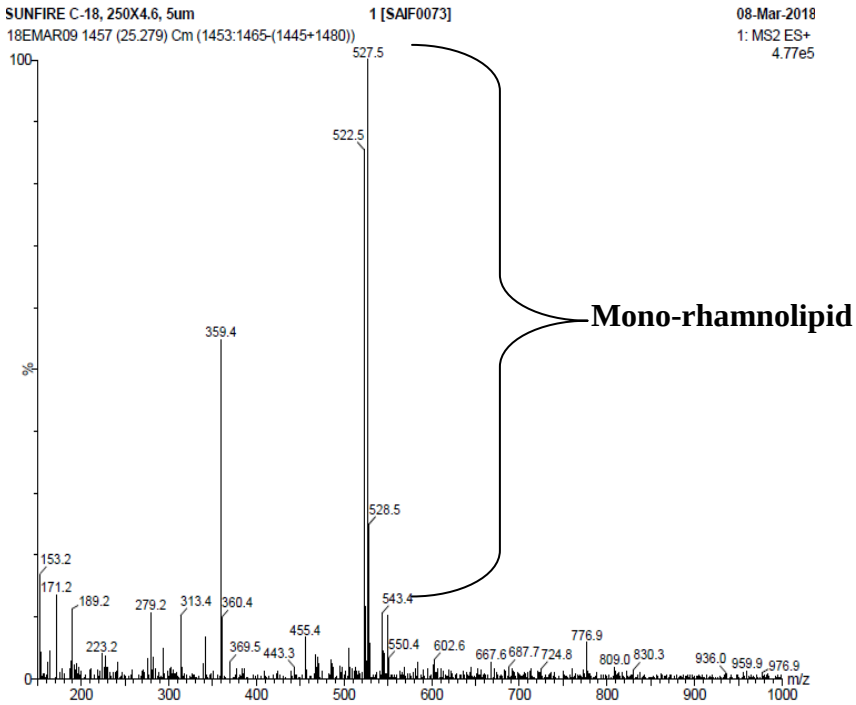


Figure 3.2.8 a LC-MS Characterization of the Biosurfactant from the strain AJ15

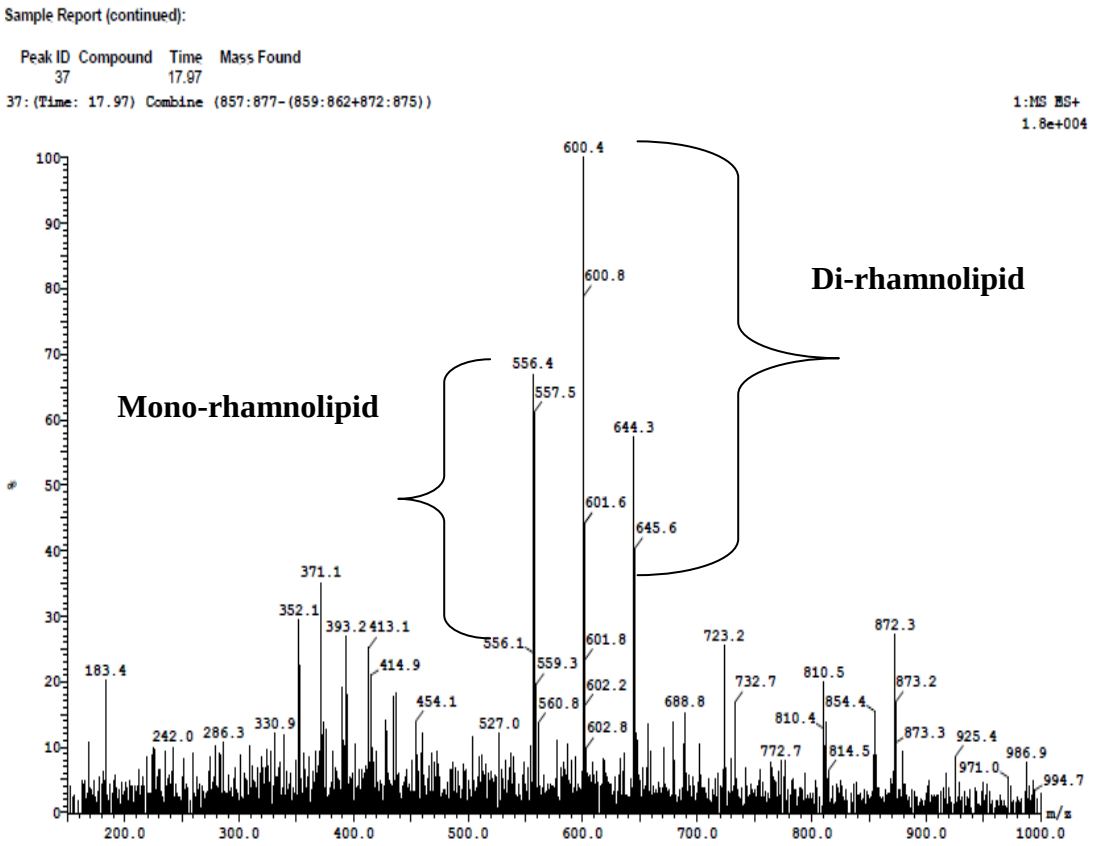


Figure 3.2.8 b LC-MS Characterization of the Biosurfactant from the strain AJ15

Mass spectra obtained from LC–MS analysis of the extracted biosurfactant confirm the presence of rhamnolipid congener in biosurfactant extracted from AJ15. The m/z peaks at 522.5, 527.5, and 528.5 respectively suggest that the extracted biosurfactant is mono-rhamnolipid Figure 3.2.8a. The m/z peaks at 527, 556, 556.4, 557.5 indicates the presence mono-rhamnolipid, while m/z peaks at 600.4,600.8,601.6,602.8,644. 3,645.6 indicates the presence of di-rhamnolipid in the extracted biosurfactant from the strain N23 Figure 3.2.8b.These findings were also complemented with the previously published report of Arora et al. (2016).

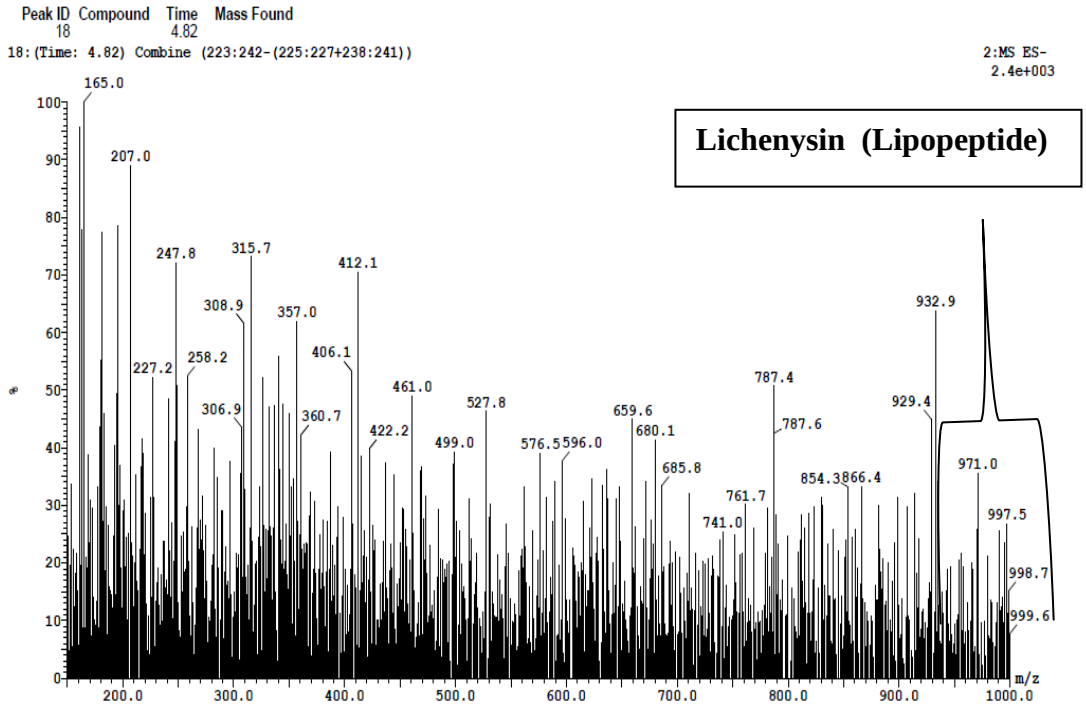


Figure 3.2.8c LC-MS Characterization of the Biosurfactant from the strain J1

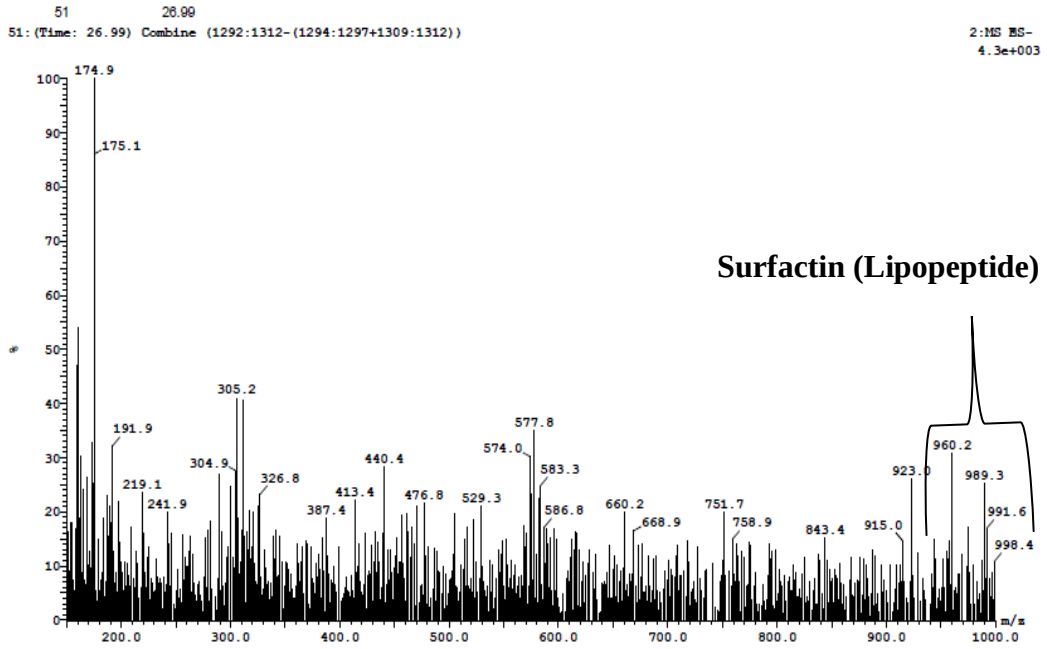


Figure 3.2.8d LC-MS Characterization of the Biosurfactant from the strain J2

Mass spectra obtained from LC–MS analysis of the extracted biosurfactant confirm the presence of lichenysin in biosurfactant extracted from J1. The m/z peaks at 971, 997.5, 998.7 and 99.6 respectively suggest that the extracted lichenysin Figure 3.2.8c. The m/z peaks at 960.2, 989.3, 991.6, 998.4 indicates the presence of surfactin in extracted biosurfactant from the strain J2 Figure 3.2.8d.These findings were also complemented with the previously published report of Ma et al.(2016) and Jasim et al (2016).

Utilization of agroindustrial waste for biosurfactant production undersubmerged fermentation (SmF) and its Modelling based optimization

According to the Box-Behnken Design (BBD) model, the optimum biosurfactant producion was estimated in various range of Bagasse concentration (g), Temperature i.e from 25 to 35º C, Agitation of 100 to 220 rpm and pH of 6 to 8. This model provides the exact optimum Bagasse concentration, temperature, agitation rate and pH required for maximum biosurfactant production.

Optimization of Biosurfactant production by strain by AJ15

The BBD model optimization was depicted in the below mentioned Table (3.2.3, 3.2.4 and 3.2.5) and Figure 3.2.9.

Table 3.2.3 Experimental design matrix of four levels of environmental factors with coded values using Box-Behnken Design with observed responses of biosurfactant

Run	Factor 1 A:Bagasse g	Factor 2 B:Temperature degree celcius	Factor 3 C:Agitation rpm	Factor 4 D:pH pH	Response 1 Biosurfactant production g
1	0.000	0.000	-1.000	-1.000	0.078
2	0.000	0.000	0.000	0.000	0.73
3	0.000	-1.000	0.000	1.000	0.17
4	1.000	0.000	1.000	0.000	0.27
5	0.000	1.000	1.000	0.000	0.25
6	-1.000	0.000	-1.000	0.000	0.4
7	1.000	1.000	0.000	0.000	0.89
8	0.000	-1.000	1.000	0.000	0.17
9	0.000	0.000	0.000	0.000	0.92
10	0.000	-1.000	-1.000	0.000	0.75
11	-1.000	0.000	1.000	0.000	0.08
12	0.000	1.000	0.000	-1.000	0.32
13	-1.000	-1.000	0.000	0.000	0.23
14	-1.000	1.000	0.000	0.000	0.61
15	1.000	0.000	-1.000	0.000	0.86
16	0.000	0.000	0.000	0.000	0.92
17	0.000	0.000	0.000	0.000	0.92
18	0.000	0.000	0.000	0.000	0.92
19	1.000	0.000	0.000	1.000	0.55
20	0.000	0.000	-1.000	1.000	0.29
21	0.000	0.000	1.000	-1.000	0.09
22	0.000	1.000	0.000	1.000	0.21
23	1.000	0.000	0.000	-1.000	0.3
24	0.000	0.000	1.000	1.000	0.18
25	1.000	-1.000	0.000	0.000	0.77
26	-1.000	0.000	0.000	1.000	0
27	-1.000	0.000	0.000	-1.000	0
28	0.000	1.000	-1.000	0.000	0.9
29	0.000	-1.000	0.000	-1.000	0.35

Table 3.2.4 Experimental design matrix of four levels of environmental factors with actual values using Box-Behnken Design with observed responses of biosurfactant

Run	Factor 1 A:Bagasse g	Factor 2 B:Temperature degree celcius	Factor 3 C:Agitation rpm	Factor 4 D:pH pH	Response 1 Biosurfactant production g
1	10	30	100	6	0.078
2	10	30	160	7	0.73
3	10	25	160	8	0.17
4	15	30	220	7	0.27
5	10	35	220	7	0.25
6	5	30	100	7	0.4
7	15	35	160	7	0.89
8	10	25	220	7	0.17
9	10	30	160	7	0.92
10	10	25	100	7	0.75
11	5	30	220	7	0.08
12	10	35	160	6	0.32
13	5	25	160	7	0.23
14	5	35	160	7	0.61
15	15	30	100	7	0.86
16	10	30	160	7	0.92
17	10	30	160	7	0.92
18	10	30	160	7	0.92
19	15	30	160	8	0.55
20	10	30	100	8	0.29
21	10	30	220	6	0.09
22	10	35	160	8	0.21
23	15	30	160	6	0.3
24	10	30	220	8	0.18
25	15	25	160	7	0.77
26	5	30	160	8	0
27	5	30	160	6	0
28	10	35	100	7	0.9
29	10	25	160	6	0.35

Figure 3.2.5 ANOVA for Quadratic mode of the factors for Biosurfactant production by the strain AJ15

Response 1: Biosurfactant production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.75	14	0.1962	9.06	< 0.0001	significant
A-Bagasse	0.4485	1	0.4485	20.72	0.0005	
B-Temperature	0.0456	1	0.0456	2.11	0.1686	
C-Agitation	0.4174	1	0.4174	19.28	0.0006	
D-pH	0.0057	1	0.0057	0.2642	0.6153	
AB	0.0169	1	0.0169	0.7805	0.3919	
AC	0.0182	1	0.0182	0.8417	0.3744	
AD	0.0156	1	0.0156	0.7216	0.4099	
BC	0.0012	1	0.0012	0.0566	0.8154	
BD	0.0012	1	0.0012	0.0566	0.8154	
CD	0.0037	1	0.0037	0.1719	0.6847	
A^2	0.2202	1	0.2202	10.17	0.0066	
B^2	0.0672	1	0.0672	3.10	0.1000	
C^2	0.4538	1	0.4538	20.96	0.0004	
D^2	1.54	1	1.54	71.05	< 0.0001	

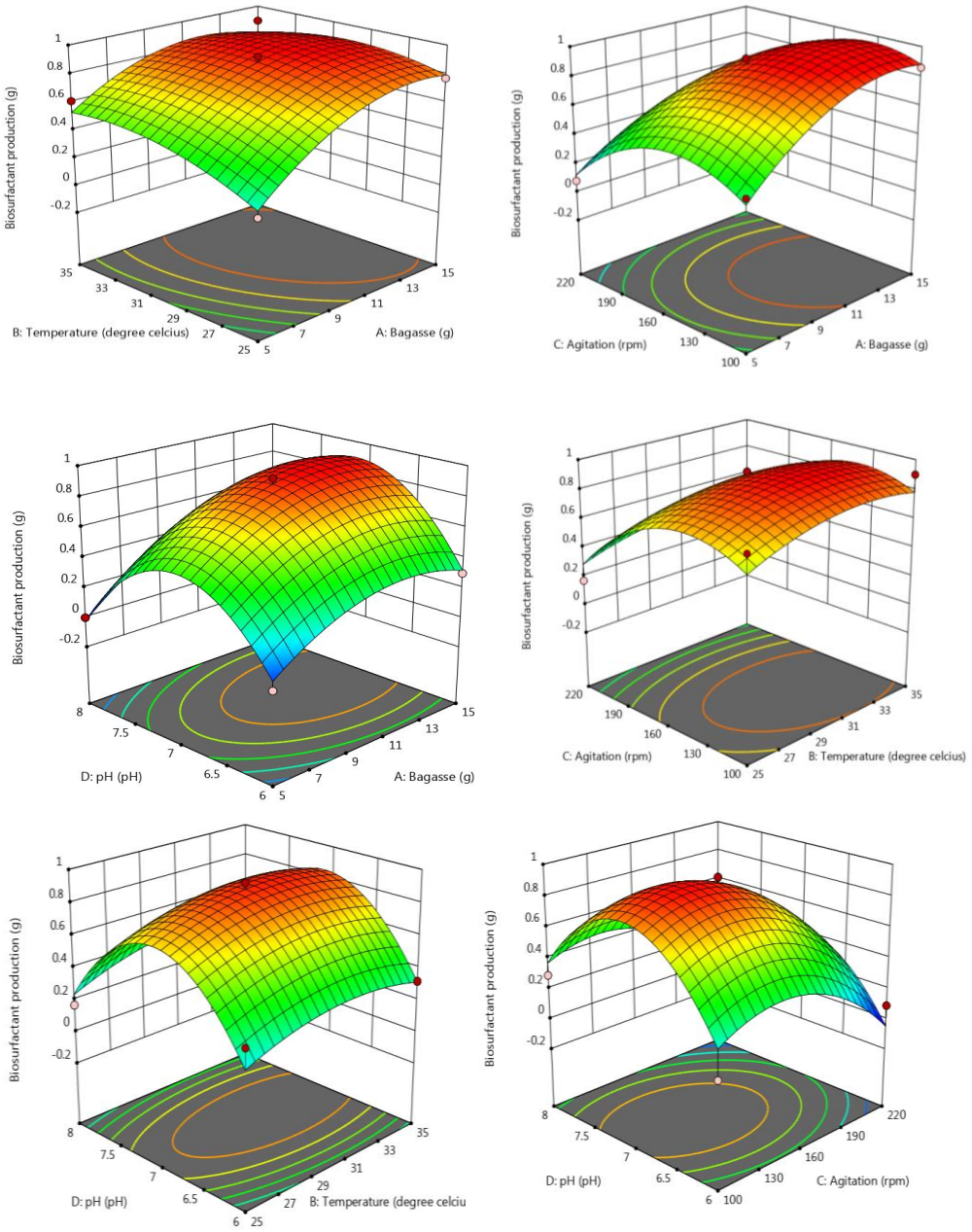


Figure 3.2.9. 3-D response surface plot depicting the interaction between Bagasse concentration (g), Temperature(degree celcius), Agitation rate (rpm) and pH for biosurfactant production (g) by the strain AJ15.

Optimization of Biosurfactant production by strain by J1

The BBD model optimization was depicted in the below mentioned Table (3.2.6, 3.2.7 and 3.2.8) and Figure 3.2.10.

Table 3.2.6. Experimental design matrix of four levels of environmental factors with actual values using Box-Behnken Design with observed responses of biosurfactant

Run	Factor 1 A:Bagasse g	Factor 2 B:Temperature degree celcius	Factor 3 C:Agitation rpm	Factor 4 D:pH pH	Response 1 Biosurfactant production g
1	0.000	0.000	-1.000	-1.000	0.2
2	0.000	1.000	0.000	-1.000	0.45
3	0.000	1.000	1.000	0.000	0.35
4	-1.000	-1.000	0.000	0.000	0.43
5	0.000	-1.000	1.000	0.000	0.24
6	0.000	0.000	1.000	-1.000	0.18
7	-1.000	1.000	0.000	0.000	0.52
8	-1.000	0.000	0.000	-1.000	0.1
9	-1.000	0.000	1.000	0.000	0.05
10	1.000	0.000	1.000	0.000	0.4
11	0.000	-1.000	0.000	1.000	0.33
12	0.000	0.000	0.000	0.000	0.79
13	1.000	1.000	0.000	0.000	0.9
14	1.000	0.000	0.000	-1.000	0.73
15	0.000	1.000	0.000	1.000	0.4
16	0.000	0.000	1.000	1.000	0.28
17	0.000	0.000	0.000	0.000	0.79
18	0.000	-1.000	0.000	-1.000	0.19
19	0.000	0.000	0.000	0.000	0.79
20	1.000	0.000	0.000	1.000	0.75
21	1.000	-1.000	0.000	0.000	0.83
22	-1.000	0.000	-1.000	0.000	0.41
23	1.000	0.000	-1.000	0.000	0.88
24	0.000	-1.000	-1.000	0.000	0.57
25	0.000	0.000	0.000	0.000	0.79
26	0.000	0.000	-1.000	1.000	0.41
27	0.000	1.000	-1.000	0.000	0.91
28	-1.000	0.000	0.000	1.000	0.08
29	0.000	0.000	0.000	0.000	0.79

Table 3.2.7 Experimental design matrix of four levels of environmental factors with actual values using Box-Behnken Design with observed responses of biosurfactant

Run	Factor 1 A:Bagasse g	Factor 2 B:Temperature degree celcius	Factor 3 C:Agitation rpm	Factor 4 D:pH pH	Response 1 Biosurfactant production g
1	10	30	100	6	0.2
2	10	35	160	6	0.45
3	10	35	220	7	0.35
4	5	25	160	7	0.43
5	10	25	220	7	0.24
6	10	30	220	6	0.18
7	5	35	160	7	0.52
8	5	30	160	6	0.1
9	5	30	220	7	0.05
10	15	30	220	7	0.4
11	10	25	160	8	0.33
12	10	30	160	7	0.79
13	15	35	160	7	0.9
14	15	30	160	6	0.73
15	10	35	160	8	0.4
16	10	30	220	8	0.28
17	10	30	160	7	0.79
18	10	25	160	6	0.19
19	10	30	160	7	0.79
20	15	30	160	8	0.75
21	15	25	160	7	0.83
22	5	30	100	7	0.41
23	15	30	100	7	0.88
24	10	25	100	7	0.57
25	10	30	160	7	0.79
26	10	30	100	8	0.41
27	10	35	100	7	0.91
28	5	30	160	8	0.08
29	10	30	160	7	0.79

Figure 3.2.8 ANOVA for Quadratic model of the factors for Biosurfactant production by the strain J1

Response 1: Biosurfactant production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.98	14	0.1412	10.76	< 0.0001	significant
A-Bagasse	0.7008	1	0.7008	53.38	< 0.0001	
B-Temperature	0.0736	1	0.0736	5.61	0.0328	
C-Agitation	0.2945	1	0.2945	22.43	0.0003	
D-pH	0.0133	1	0.0133	1.02	0.3307	
AB	0.0001	1	0.0001	0.0076	0.9317	
AC	0.0036	1	0.0036	0.2742	0.6087	
AD	0.0004	1	0.0004	0.0305	0.8639	
BC	0.0132	1	0.0132	1.01	0.3326	
BD	0.0090	1	0.0090	0.6874	0.4210	
CD	0.0030	1	0.0030	0.2304	0.6386	
A^2	0.0377	1	0.0377	2.87	0.1122	
B^2	0.0329	1	0.0329	2.51	0.1356	
C^2	0.3320	1	0.3320	25.29	0.0002	
D^2	0.6799	1	0.6799	51.78	< 0.0001	

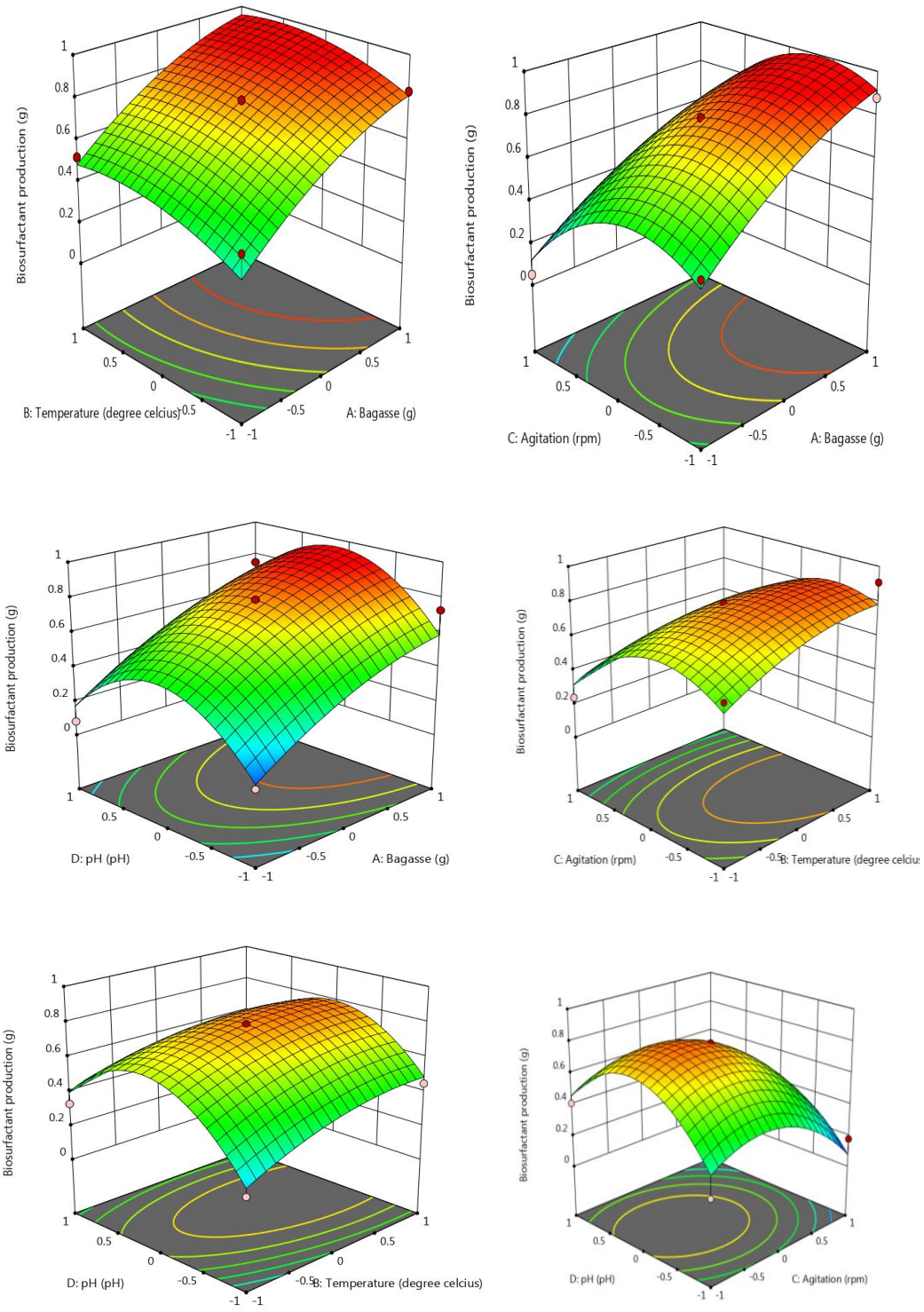


Figure. 3.2.10. 3-D response surface plot depicting the interaction between Bagasse concentration (g), Temperature(degree celcius), Agitation rate (rpm) and pH for biosurfactant production (g) by the strain J1.

Optimization of Biosurfactant production by strain by J2

The BBD model optimization was depicted in the below mentioned Table (3.2.9, 3.2.10 and 3.2.11) and Figure 3.2.11.

Table 3.2.9. Experimental design matrix of four levels of environmental factors with coded values using Box-Behnken Design with observed responses of biosurfactant

Run	Factor 1 A:Bagasse g	Factor 2 B:Temperature degree celcius	Factor 3 C:Agitation rpm	Factor 4 D:pH pH	Response 1 Biosurfactant production g
1	0.000	1.000	0.000	1.000	0.47
2	0.000	0.000	1.000	-1.000	0.15
3	0.000	0.000	1.000	1.000	0.24
4	0.000	0.000	0.000	0.000	0.59
5	1.000	-1.000	0.000	0.000	0.78
6	1.000	0.000	0.000	-1.000	0.39
7	0.000	0.000	-1.000	-1.000	0.21
8	1.000	0.000	1.000	0.000	0.46
9	-1.000	-1.000	0.000	0.000	0.45
10	0.000	1.000	-1.000	0.000	0.62
11	1.000	0.000	0.000	1.000	0.55
12	1.000	1.000	0.000	0.000	0.92
13	-1.000	0.000	1.000	0.000	0.08
14	-1.000	0.000	-1.000	0.000	0.39
15	0.000	0.000	0.000	0.000	0.59
16	0.000	-1.000	0.000	1.000	0.31
17	0.000	-1.000	-1.000	0.000	0.42
18	0.000	-1.000	1.000	0.000	0.19
19	-1.000	0.000	0.000	1.000	0
20	0.000	0.000	0.000	0.000	0.59
21	1.000	0.000	-1.000	0.000	0.85
22	-1.000	1.000	0.000	0.000	0.51
23	0.000	0.000	0.000	0.000	0.59
24	-1.000	0.000	0.000	-1.000	0
25	0.000	-1.000	0.000	-1.000	0.25
26	0.000	0.000	-1.000	1.000	0.3
27	0.000	0.000	0.000	0.000	0.59
28	0.000	1.000	1.000	0.000	0.2
29	0.000	1.000	0.000	-1.000	0.38

Table 3.2.10. Experimental design matrix of four levels of environmental factors with actual values using Box-Behnken Design with observed responses of biosurfactant

Run	Factor 1 A:Bagasse g	Factor 2 B:Temperature degree celcius	Factor 3 C:Agitation rpm	Factor 4 D:pH pH	Response 1 Biosurfactant production g
1	10	35	160	8	0.47
2	10	30	220	6	0.15
3	10	30	220	8	0.24
4	10	30	160	7	0.59
5	15	25	160	7	0.78
6	15	30	160	6	0.39
7	10	30	100	6	0.21
8	15	30	220	7	0.46
9	5	25	160	7	0.45
10	10	35	100	7	0.62
11	15	30	160	8	0.55
12	15	35	160	7	0.92
13	5	30	220	7	0.08
14	5	30	100	7	0.39
15	10	30	160	7	0.59
16	10	25	160	8	0.31
17	10	25	100	7	0.42
18	10	25	220	7	0.19
19	5	30	160	8	0
20	10	30	160	7	0.59
21	15	30	100	7	0.85
22	5	35	160	7	0.51
23	10	30	160	7	0.59
24	5	30	160	6	0
25	10	25	160	6	0.25
26	10	30	100	8	0.3
27	10	30	160	7	0.59
28	10	35	220	7	0.2
29	10	35	160	6	0.38

Figure 3.2.11.ANOVA for Quadratic model of the factors for Biosurfactant production by the strain J2

Response 1: Biosurfactant production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.41	14	0.1004	9.33	< 0.0001	significant
A-Bagasse	0.5292	1	0.5292	49.16	< 0.0001	
B-Temperature	0.0408	1	0.0408	3.79	0.0718	
C-Agitation	0.1801	1	0.1801	16.73	0.0011	
D-pH	0.0200	1	0.0200	1.86	0.1943	
AB	0.0016	1	0.0016	0.1486	0.7056	
AC	0.0016	1	0.0016	0.1486	0.7056	
AD	0.0064	1	0.0064	0.5945	0.4535	
BC	0.0090	1	0.0090	0.8384	0.3754	
BD	0.0002	1	0.0002	0.0209	0.8871	
CD	2.220E-16	1	2.220E-16	2.063E-14	1.0000	
A^2	0.0000	1	0.0000	0.0038	0.9519	
B^2	0.0010	1	0.0010	0.0941	0.7635	
C^2	0.1687	1	0.1687	15.67	0.0014	
D^2	0.4685	1	0.4685	43.52	< 0.0001	

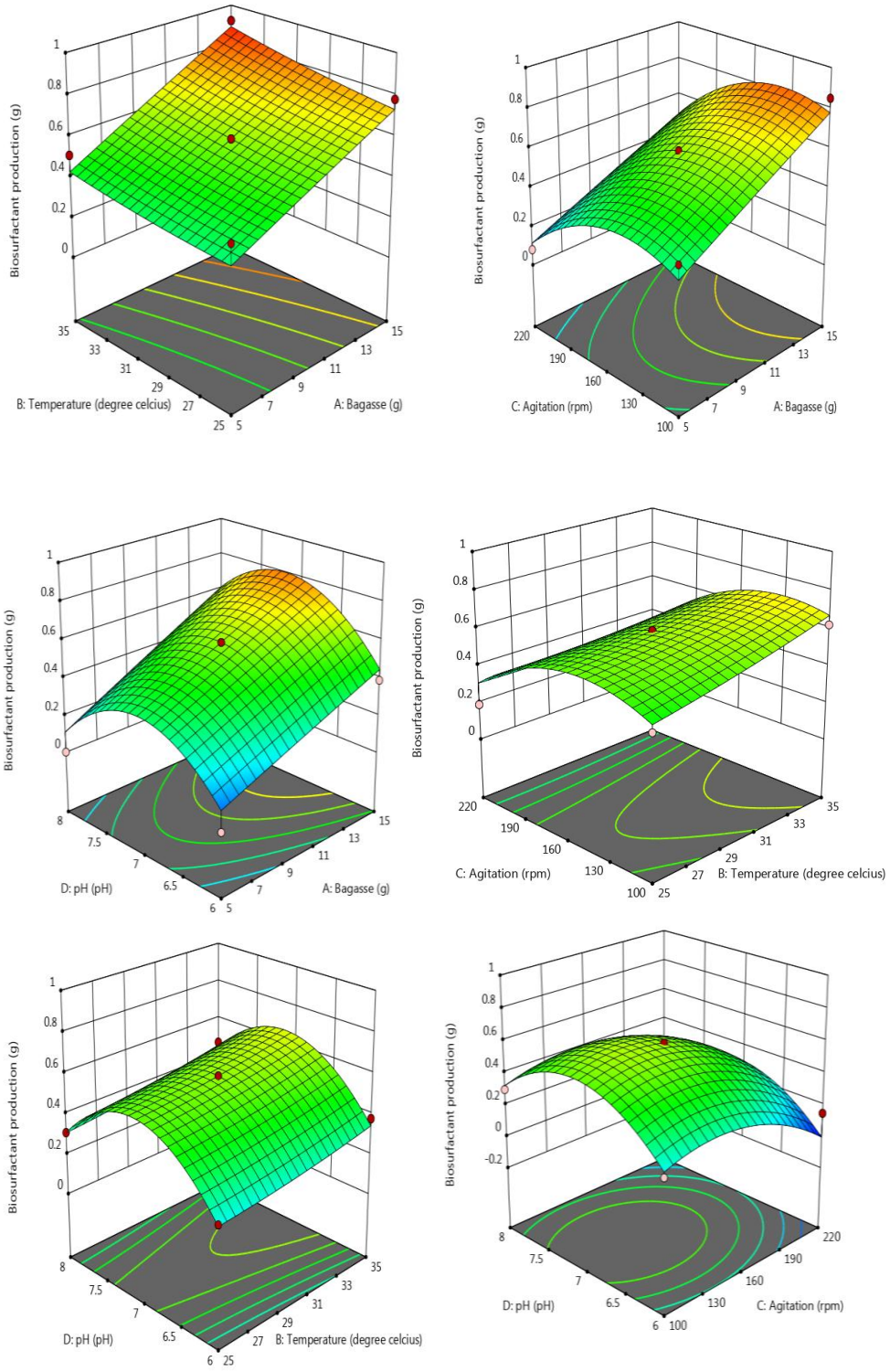


Figure. 3.2.11. 3-D response surface plot depicting the interaction between Bagasse concentration (g), Temperature(degree celcius), Agitation rate (rpm) and pH for biosurfactant production (g) by the strain J2.

Optimization of Biosurfactant production by strain by N23

The BBD model optimization was depicted in the below mentioned Table (3.2.12, 3.2.13 and 3.2.14 and Figure 3.2.12.

Table 3.2.12. Experimental design matrix of four levels of environmental factors with coded values using Box-Behnken Design with observed responses of biosurfactant

Run	Factor 1 A:Bagasse g	Factor 2 B:Temperature degree celcius	Factor 3 C:Agitation rpm	Factor 4 D:pH pH	Response 1 Biosurfactant production g
1	0.000	0.000	1.000	1.000	0.09
2	0.000	1.000	1.000	0.000	0.5
3	0.000	1.000	-1.000	0.000	0.79
4	-1.000	0.000	0.000	1.000	0
5	0.000	-1.000	1.000	0.000	0.35
6	0.000	0.000	0.000	0.000	0.98
7	0.000	0.000	1.000	-1.000	0.21
8	-1.000	-1.000	0.000	0.000	0.31
9	0.000	0.000	-1.000	-1.000	0.33
10	1.000	1.000	0.000	0.000	0.95
11	1.000	0.000	1.000	0.000	0.1
12	0.000	1.000	0.000	1.000	0.61
13	0.000	-1.000	-1.000	0.000	0.82
14	0.000	0.000	0.000	0.000	0.98
15	1.000	0.000	-1.000	0.000	0.87
16	0.000	0.000	0.000	0.000	0.98
17	0.000	0.000	0.000	0.000	0.98
18	0.000	0.000	-1.000	1.000	0.52
19	1.000	-1.000	0.000	0.000	0.79
20	0.000	1.000	0.000	-1.000	0.35
21	-1.000	1.000	0.000	0.000	0.61
22	0.000	-1.000	0.000	-1.000	0.34
23	-1.000	0.000	0.000	-1.000	0
24	0.000	0.000	0.000	0.000	0.98
25	-1.000	0.000	1.000	0.000	0
26	-1.000	0.000	-1.000	0.000	0.53
27	0.000	-1.000	0.000	1.000	0.5
28	1.000	0.000	0.000	1.000	0.56
29	1.000	0.000	0.000	-1.000	0.42

Table 3.2.13 Experimental design matrix of four levels of environmental factors with actual values using Box-Behnken Design with observed responses of biosurfactant

Run	Factor 1 A:Bagasse g	Factor 2 B:Temperature degree celcius	Factor 3 C:Agitation rpm	Factor 4 D:pH pH	Response 1 Biosurfactant production g
1	10	30	220	8	0.09
2	10	35	220	7	0.5
3	10	35	100	7	0.79
4	5	30	160	8	0
5	10	25	220	7	0.35
6	10	30	160	7	0.98
7	10	30	220	6	0.21
8	5	25	160	7	0.31
9	10	30	100	6	0.33
10	15	35	160	7	0.95
11	15	30	220	7	0.1
12	10	35	160	8	0.61
13	10	25	100	7	0.82
14	10	30	160	7	0.98
15	15	30	100	7	0.87
16	10	30	160	7	0.98
17	10	30	160	7	0.98
18	10	30	100	8	0.52
19	15	25	160	7	0.79
20	10	35	160	6	0.35
21	5	35	160	7	0.61
22	10	25	160	6	0.34
23	5	30	160	6	0
24	10	30	160	7	0.98
25	5	30	220	7	0
26	5	30	100	7	0.53
27	10	25	160	8	0.5
28	15	30	160	8	0.56
29	15	30	160	6	0.42

Figure 3.2.15 ANOVA for Quadratic model of the factors for Biosurfactant production by the strain N23

Response 1: Biosurfactant production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.90	14	0.2069	17.34	< 0.0001	significant
A-Bagasse	0.4181	1	0.4181	35.03	< 0.0001	
B-Temperature	0.0408	1	0.0408	3.42	0.0856	
C-Agitation	0.5677	1	0.5677	47.56	< 0.0001	
D-pH	0.0331	1	0.0331	2.77	0.1182	
AB	0.0049	1	0.0049	0.4106	0.5320	
AC	0.0144	1	0.0144	1.21	0.2906	
AD	0.0049	1	0.0049	0.4106	0.5320	
BC	0.0081	1	0.0081	0.6787	0.4239	
BD	0.0025	1	0.0025	0.2095	0.6542	
CD	0.0240	1	0.0240	2.01	0.1778	
A^2	0.5346	1	0.5346	44.79	< 0.0001	
B^2	0.0271	1	0.0271	2.27	0.1544	
C^2	0.5487	1	0.5487	45.97	< 0.0001	
D^2	1.25	1	1.25	104.42	< 0.0001	

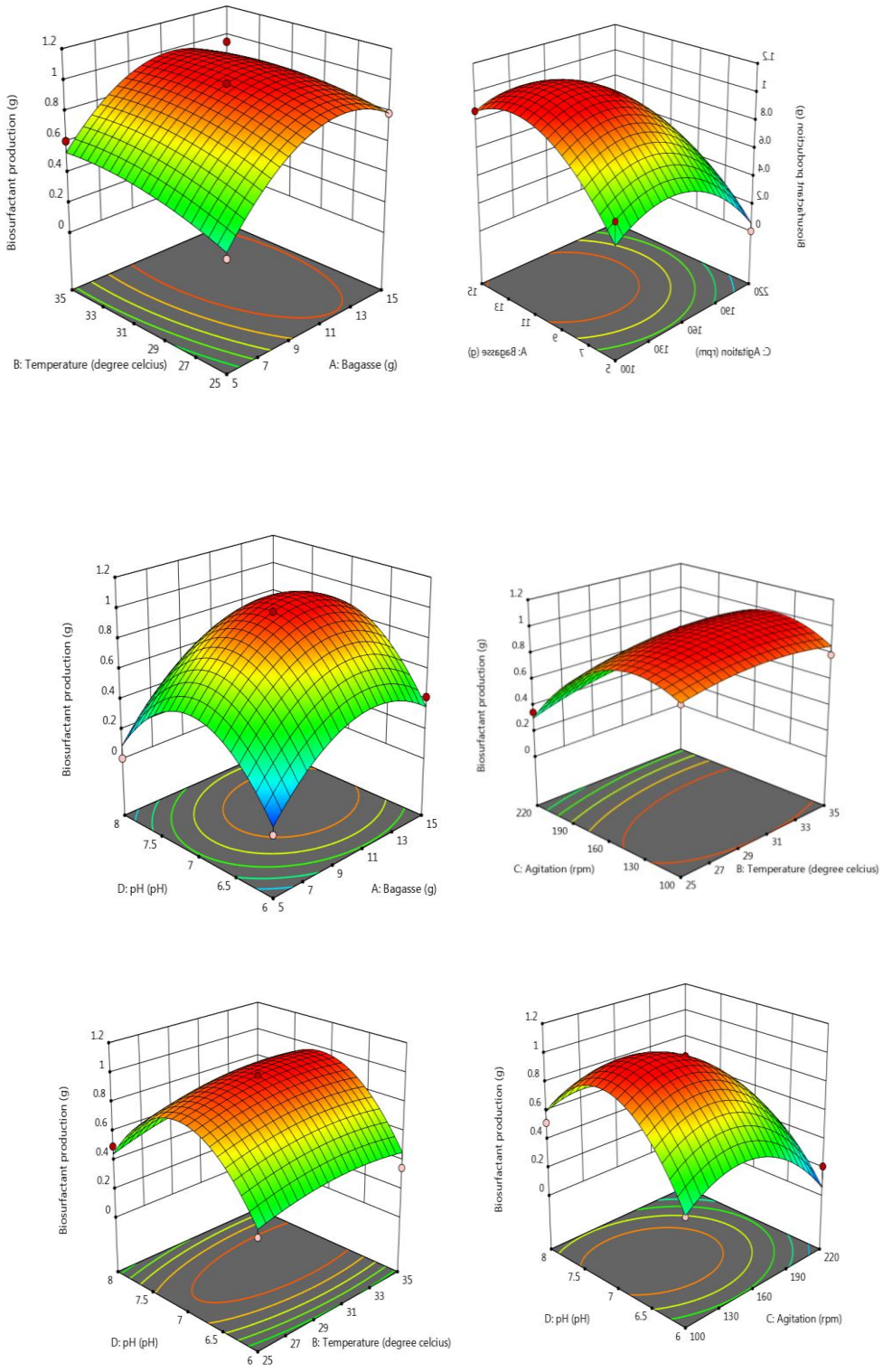


Figure 3.2.12.. 3-D response surface plot depicting the interaction between Bagasse concentration (g), Temperature(degree celcius), Agitation rate (rpm) and pH for biosurfactant production (g) by the strain N23

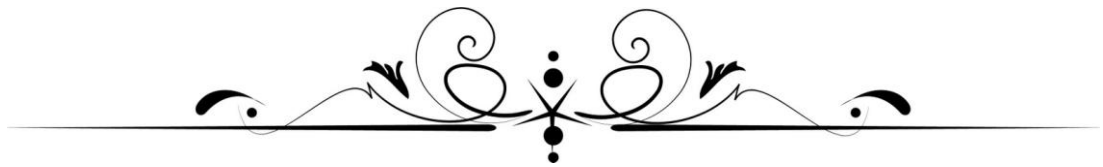
BBD design with various factors has been demonstrated within the Tables (3.2.3 to 3.2.15). The coded and format of four variables factors: Bagasse concentration (g) , Temperature (degree Celsius), Agitation (rpm) and pH has been presented along with responses biosurfactant production (g). To predict the maximum biosurfactant production by the strains variables factors were correlates along with response.

- The strain AJ15 produce maximum 0.92 g of biosurfactant at 30º C temperature, 7 pH , 160 rpm rate by utilizing 10 g bagasse for 72 hrs.
- The strain J1 produce maximum 0.91 g of biosurfactant at 35º C temperature, 7 pH , 100 rpm rate by utilizing 10 g bagasse for 72 hrs.
- The strain J2 produce maximum 0.92 g of biosurfactant at 35º C temperature, 7 pH , 160 rpm rate by utilizing 15 g bagasse for 72 hrs.
- The strain N23 produce maximum 0.98 g of biosurfactant at 30º C temperature, 7 pH , 160 rpm rate by utilizing 10 g bagasse for 72 hrs.

The BBD model shows that Biosurfactant production was maximum for all the strains at a temperature between 30 °C and 35 °C and this might due to the optimal temperature for growth of bacterial strain. The biosurfactant production is maximum between 100 to 160 rpm, but production rate decreases at 220 rpm. Increased agitation rate provides higher dissolved oxygen concentration in the medium which enhances the production of biosurfactant. At 220 rpm high foam was observed in all the medium that lead to reduction in the transfer of oxygen into the liquid medium resulting in low biosurfactant production rate (Wei et al., 2005; Moussa et al., 2014). The substrates were optimized at various pH ranging from 6 to 8, but highest production rate was reported at neutral pH of 7.

Conclusion

The present chapter clearly reveals that bacterial strains has the ability to utilize agroindustrial wastes account for biosurfactant production under submerged fermentation. Biosurfactants produced the by strains are non-toxic in nature and posses' excellent emulsification property. Notably, the biosurfactant was enabled to persist its activities over a varying range of pH, temperature, and salinity. The findings suggest that this approach could also be used and can be readily be implemented for fulfilling the enormous demand of surfactant.



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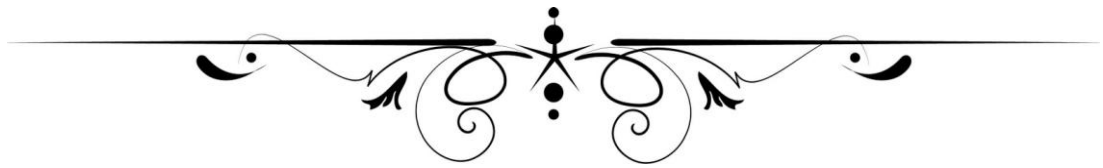


Chapter 3.3 Soil collection from petroleum contaminated site for in situ experiment and its characterization for pollutant toxicity level .

Contents:

3.3.1 Introduction

Petroleum is a natural product resulting from the anaerobic conversion of biomass under high temperature and pressure. Although most of its components are subject to biodegradation, this occurs at relatively slow rates. Moreover, petroleum hydrocarbons are poorly degraded and have thus become the most widespread environmental contaminant (Margesin et al., 2000). The four classes of petroleum hydrocarbons (saturates, aromatics, asphaltenes, and resins)(Kumar et al. 2015) differ in their susceptibility to microbial attack (bacteria and fungi). The toxicity of petroleum hydrocarbon on soil organisms has been widely studied, but research regarding its ecotoxicity is still assaying behind (Cermak et al., 2010; Tang et al., 2011). Petroleum hydrocarbons cause damage to plant through anaerobic and hydrostatic condition that interferes with soil plant water relation (Kuiper et al. 2004). Bona et al. (2011), Ahammed et al. (2012) demonstrated that accumulation of toxic petroleum compound by plant tissues results in decrease in their biomass and total size. Environmental contamination with petroleum and its constituent is an inevitable problem that affects many geographical regions to a variable extent depending on the local environmental law (Graj et al. 2013).

Hence, the present chapter depicts the third objective regarding soil collection from petroleum contaminated site and its characterization for detection of pollutants and their toxicity.

3.3.2 Materials and Methods

3.3.2.1 Soil collection and processing

Petroleum contaminated soil used in this study was collected from petroleum contaminated site of Lucknow, India (26°55' N latitude and 80° 59' E longitude). Soil samples were collected in sterile container and transported to Laboratory. The soils were vigorously mixed and sieved and stored at 4° C in a container for further experiments.

3.2.2.2 Preparation of soil Slurry

The soil slurry prepared was categorized into following:

S1= 10 g Contaminated soil + 25 ml distilled water

S2= 7.5 g Contaminated soil + 2.5 garden soil + 25 ml distilled water

S3= 5 Contaminated soil + 5 garden soil + 25 ml distilled water

S4=2.5 g Contaminated soil + 7.5 garden soil + 25 ml distilled water

S5=10 g garden soil+ 25 ml distilled water

3.2.2.3 Toxicity assessment of the collected soil

Seed germination inhibition

In seed germination experiment, certified mung (Phaseolus mungo L.) seeds were surface sterilized with sodium hypochlorite. Ten sterilized seeds of each were placed in different sterilized petri dish of uniform size lined with two Whatman No. 1 filter paper discs, followed by addition of 7 ml of soil slurry mentioned in the table. Plates were incubated at 27 ºC and examined for germination inhibition at every 24 h interval for after 7 days. The considered criterion of germination was the visible protrusion of radical from seed coat. Germinated seeds were counted .All the experiments were performed in triplicate.

Earthworm acute toxicity test

200 g soil slurry from each treatment was put in the brown bottle. Healthy earthworms of weight 400–500 mg were selected, washed with distilled water and dried with filter paper. Five earthworms were weighed as a group, and the average weight value was calculated. The earthworm was put into brown bottle soon after weighing, and the number of earthworm death was checked after 4 and 7 days. The basis for the death of earthworms was determined by no response to acupuncture. After the experiment, the earthworms were weighed, and average value was calculated. All the experiments were performed in triplicate (Tang et al. 2011).

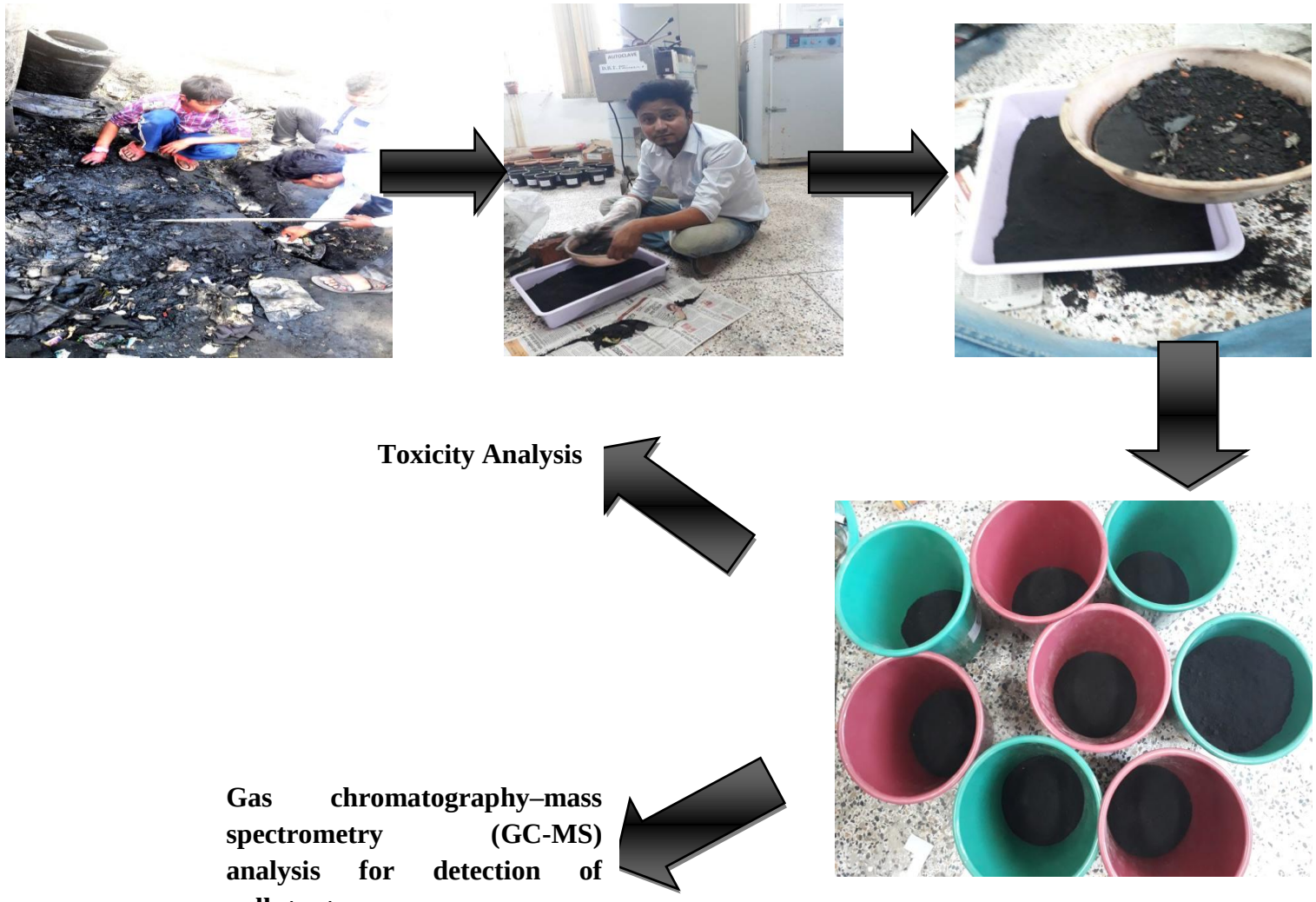
3.2.2.4 Detection of contaminate in the contaminated soil through GC-MS analysis

For detection of contaminate in the soil, the soil sample was first dried at 30°C and homogenized and pass through the 0.25-1 mm sieve. 5 g sample from the flask was put it in the thimble in soxhalet Apparatus and fill with the 150 ml solvent dichloromethane. The extraction process was started at 48°C for 24 hours after extraction dichloromethane was dried off and the sample was stored and derivatizes. For derivatization 50 µl N, O-Bis(trimethylsilyl)trifluoroacetamide and trimethyl chlorosilane (BSTFA+TMCS) was added which was followed by the addition 10µl of Pyridine. Samples were further heated at 60ºC for 30 minutes. For (BSTFA+TMCS), make 1 ml solution of 990µl of BSTFA and 10µl of TMCS (ratio of 99:1). Samples were transferred in GC vial and dried using nitrogen gas. Samples were finally dissolved in methanol before GC-MS analysis.

3.2.3 Results and Discussion

Soil collected from the petroleum contaminated site was dried and sieved with 2 mm sieve and autoclaved for 1 h at 121ºC for three successive days and stored for further use **Figure 3.3.1**

Figure 3.3.1 Pictorial view of collected petroleum contaminated soil and their processing for further use



Gas chromatography–mass spectrometry (GC-MS) analysis of the collected petroleum contaminated soil

Gas chromatography–mass spectrometry (GC-MS) analysis of the contaminated soil reveals the presence of hydrocarbon in the soil. The detected hydrocarbon chromatogram is shown in Figure 3.3.2, where corresponding compound are identified using (The National Institute of Standards and Technology) mass spectral library.Gas chromatography–mass spectrometry (GC-MS) analyses of the contaminated soil reveals that the contaminated soil contains 121 compounds which are mentioned in Table 3.3.1.

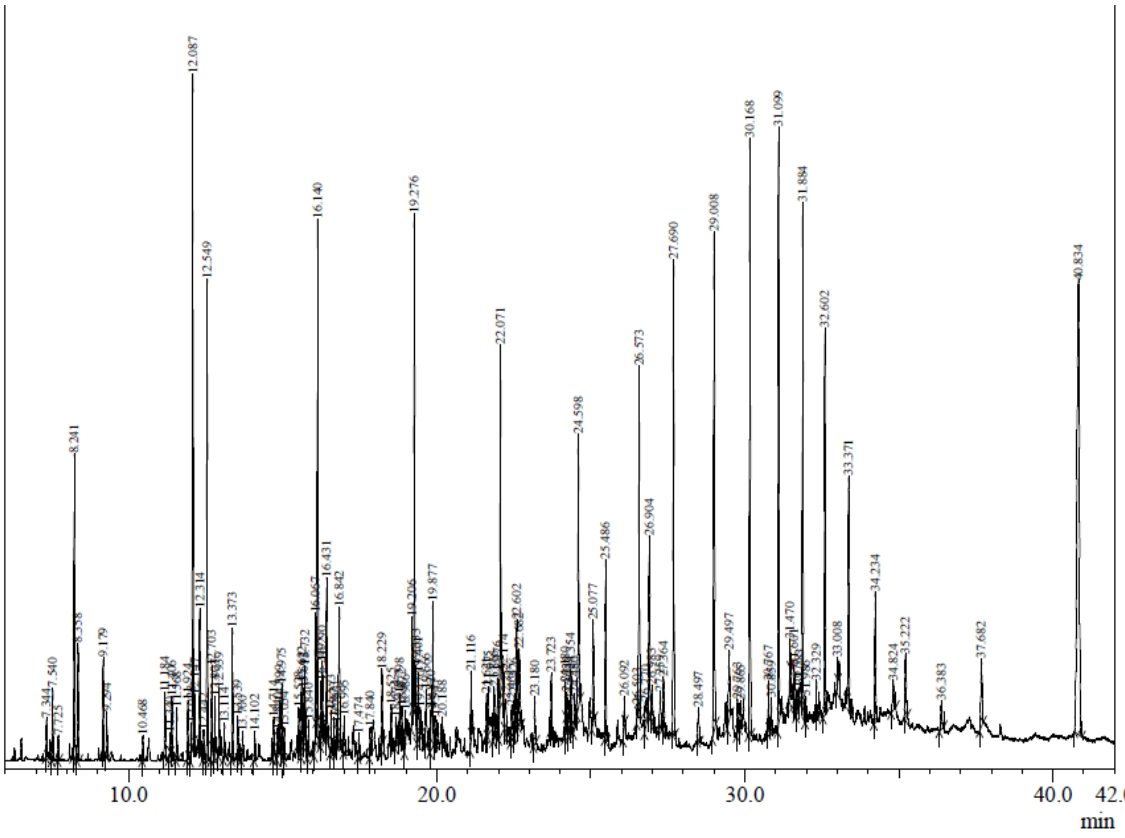


Figure 3.3.2. GC-MS chromatogram of the contaminated soil

Table 3.3.1. Organic compound identified in the contaminated soil

Peak#	R.Time	Area	Area%	Name
1	7.344	182747	0.25	UNDECANE, 5,6-DIMETHYL-
2	7.540	370380	0.50	Octane, 3,3-dimethyl-
3	7.725	125167	0.17	CYCLOHEXENE, 1-METHYL-4-(1-METHYLETHENYL)
4	8.241	1617891	2.18	Decane, 3,7-dimethyl-
5	8.358	611924	0.82	Decane, 3,7-dimethyl-
6	9.179	467748	0.63	Decane, 3,7-dimethyl-
7	9.294	231118	0.31	Decane, 3,7-dimethyl-
8	10.468	123658	0.17	TRIDECANE
9	11.184	330681	0.45	Tetradecane
10	11.340	93546	0.13	UNDECANE, 2,4-DIMETHYL-
11	11.406	286790	0.39	UNDECANE, 2,6-DIMETHYL-
12	11.568	250400	0.34	Dodecane, 4-methyl-
13	11.924	277291	0.37	Tetradecane
14	12.028	132017	0.18	Undecane, 3,8-dimethyl-
15	12.087	3151074	4.25	Benzene, 1,3-bis(1,1-dimethylethyl)-
16	12.147	253476	0.34	Dodecane, 4,6-dimethyl-
17	12.314	610597	0.82	Tetradecane, 5-methyl-
18	12.442	146097	0.20	Sulfurous acid, 2-ethylhexyl isohexyl ester
19	12.549	2339438	3.15	Hexadecane
20	12.703	389326	0.52	Nonane, 5-(2-methylpropyl)-
21	12.807	251729	0.34	Nonane, 5-(2-methylpropyl)-
22	12.939	294966	0.40	TETRADECANE
23	13.114	167115	0.23	DECANE, 2,3,7-TRIMETHYL-
24	13.373	674584	0.91	TETRADECANE
25	13.539	286562	0.39	TETRADECANE
26	13.700	205514	0.28	Decane, 3,7-dimethyl-
27	14.102	136885	0.18	PENTADECANE
28	14.714	179167	0.24	Tridecane
29	14.840	110667	0.15	3-OXO-1-CYCLOHEXENYL 2-METHYLPROPIONAT
30	14.899	189792	0.26	3,5-Dimethyldodecane
31	14.975	341160	0.46	Hexadecane, 1-iodo-
32	15.054	149691	0.20	Tetradecane, 4-methyl-
33	15.528	203406	0.27	Pentadecane, 2,6,10-trimethyl-
34	15.622	330749	0.45	Pentadecane, 2,6,10-trimethyl-
35	15.692	170503	0.23	2,6,10-Trimethyltridecane
36	15.732	271405	0.37	OCTADECANE
37	15.840	185585	0.25	Dodecane, 4,6-dimethyl-
38	16.067	584706	0.79	Heptadecane
39	16.140	2705387	3.65	Heptadecane
40	16.249	224814	0.30	Tetradecane
41	16.290	296943	0.40	Hexadecane
42	16.431	844659	1.14	Phenol, 3,5-bis(1,1-dimethylethyl)-

43	16.573	208236	0.28	Dodecyl nonyl ether
44	16.673	173954	0.23	Dodecane, 4,6-dimethyl-
45	16.763	76110	0.10	Octane, 4,5-diethyl-
46	16.842	865918	1.17	Hexadecane
47	16.995	201345	0.27	Sulfurous acid, hexyl octyl ester
48	17.474	123597	0.17	OCTADECANE
49	17.840	130162	0.18	Tridecane
50	18.229	356325	0.48	Heneicosane
51	18.525	208174	0.28	Pentadecane, 2,6,10-trimethyl-
52	18.674	137544	0.19	Dodecane, 2-methyl-
53	18.798	205263	0.28	Heneicosane
54	18.872	145885	0.20	Heneicosane
55	18.986	148375	0.20	Dodecane, 4,6-dimethyl-
56	19.206	448626	0.60	Heptadecane
57	19.276	2367508	3.19	Eicosane
58	19.333	247163	0.33	Eicosane
59	19.401	436565	0.59	Eicosane
60	19.470	103960	0.14	2,6,10-Trimethyltridecane
61	19.666	407521	0.55	DOCOSANE
62	19.820	155643	0.21	2H-PYRAN, 3,6-DIHYDRO-6-METHOXY-2-METHYL-, T
63	19.877	556627	0.75	Eicosane
64	19.947	165629	0.22	Hexadecane
65	20.188	118867	0.16	OCTADECANE
66	21.116	329118	0.44	Heneicosane
67	21.621	193507	0.26	Heneicosane
68	21.675	209404	0.28	Heneicosane
69	21.866	324290	0.44	Eicosane
70	21.976	293992	0.40	Eicosane
71	22.071	1745263	2.35	Eicosane
72	22.174	153289	0.21	Eicosane
73	22.252	357024	0.48	Heptadecane
74	22.433	246804	0.33	Nonyl tetradecyl ether
75	22.526	182103	0.25	Eicosane
76	22.602	553886	0.75	Eicosane
77	22.682	695027	0.94	OCTADECANOIC ACID
78	23.180	318113	0.43	Heneicosane
79	23.723	302853	0.41	PENTACOSANE
80	24.180	181529	0.24	Heneicosane
81	24.226	163719	0.22	Heneicosane
82	24.303	176746	0.24	Heneicosane
83	24.354	294886	0.40	Heneicosane
84	24.480	220612	0.30	Eicosane
85	24.598	1706652	2.30	Eicosane
86	25.077	610705	0.82	Eicosane
87	25.486	964439	1.30	Heneicosane
88	26.092	167283	0.23	Tetracosane, 1-iodo-

89	26.503	47476	0.06	(Z)-ISOBUTYL 7-(TRIMETHYLSILYL)-5-HEPTENOATE
90	26.573	1725464	2.33	Heneicosane
91	26.770	326016	0.44	3,5-Dimethyldodecane
92	26.904	1209586	1.63	Eicosane
93	26.983	286604	0.39	TETRACOSANE
94	27.259	223564	0.30	Eicosane
95	27.364	445668	0.60	Eicosane
96	27.690	2822336	3.80	Heneicosane
97	28.497	258749	0.35	Heneicosane
98	29.008	3456764	4.66	Heneicosane
99	29.497	649567	0.88	Tetratetracontane
100	29.763	182718	0.25	2-methyloctacosane
101	29.865	175855	0.24	2-methyloctacosane
102	30.168	3293359	4.44	PENTACOSANE
103	30.767	225500	0.30	2-methyloctacosane
104	30.859	155758	0.21	2-methyloctacosane
105	31.099	3023651	4.07	PENTACOSANE
106	31.470	192411	0.26	Tetracontane
107	31.601	177728	0.24	2-methyloctacosane
108	31.677	174633	0.24	Hexane, 2,2,3-trimethyl-
109	31.783	154723	0.21	2,6,10,15,19,23-HEXAMETHYLTETRACOSANE
110	31.884	2336304	3.15	PENTACOSANE
111	31.986	112874	0.15	Squalene
112	32.329	151872	0.20	2-methyloctacosane
113	32.602	1898465	2.56	.BETA.-D-MANNO-D-GLYCERO-.ALPHA.-D-GALACTO
114	33.008	122632	0.17	HEXATRIACONTANE
115	33.371	1208538	1.63	Tetratetracontane
116	34.234	873444	1.18	Tetratetracontane
117	34.824	271345	0.37	Tetrapentacontane
118	35.222	541944	0.73	Tetratriacontane
119	36.383	238728	0.32	Tetratriacontane
120	37.682	631200	0.85	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)
121	40.834	7428710	10.01	5,11,17,23-TETRATERT-BUTYLPENTACYCLO[19.3.1.1~

Toxicity Analysis

Toxicity report of contaminated soil was represented in Figure 3.2.3 and Figure 3.2.4. The results indicate that the contaminated soil is toxic in nature and not suitable for

cultivation for purpose. But, when the garden soil is mixed with the soil in various ratio then toxicity of contaminated soil reduce to a great extent.

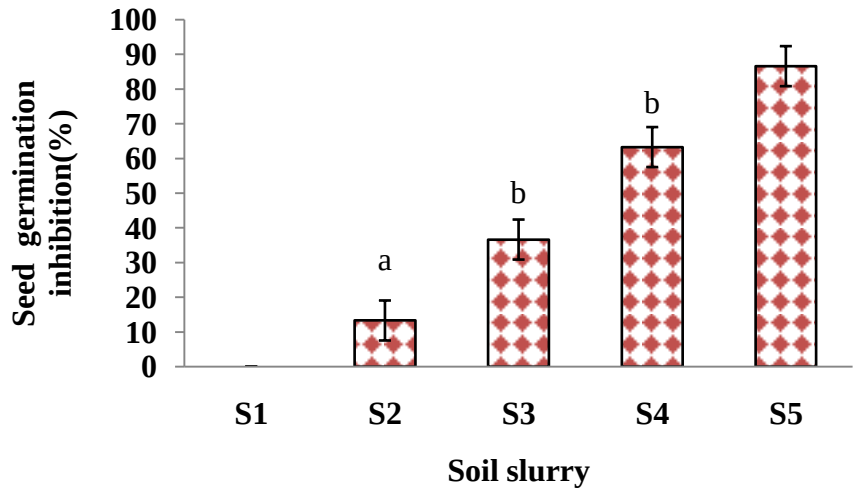


Figure 3.2.3 Effect of contaminated soil on seed germination

Results analyzed by using single-factor ANOVA of variance and are mean of three replicates with standard deviation (Means ± S.D, n = 3). And differences were considered significant by determining the p-value (^{a} $p < 0.01$,^{b} $p < 0.05$, ^{c} Not significant). Chemical properties of garden soil used [C organic (%) 1.4 ± 0.05, Ca (g /Kg) 3.5 ± 0.4, N (g /Kg) 1.19 ± 0.8, Na (g / Kg) 3.8 ± 0.1, P (g /Kg) 0.75 ± 0.05, K (g /Kg) 2.94 ± 0.4, Fe (g /Kg) 0.155 ± 0.03, Zn (g/ Kg) 0.0038 ± 0.02]

S1= 10 g Contaminated soil + 25 distilled waster
S2= 7.5 g Contaminated soil + 2.5 garden soil + 25 distilled waster
S3= g Contaminated soil + 5 garden soil + 25 distilled waster
S4=2.5 g Contaminated soil + 7.5 garden soil + 25 distilled waster
S5=10 g garden soil + 25 distilled waster

Plants are used as predictor for determining the ecotoxicity of soil, as they mainly depend on soil to germinate and grow, so any alterations in the seed development and physiological processes may reflect the presence of toxic substances in the soil. Germination studies are used to assess the primary and short-term toxicity effects (Banks and Schultz 2005). Different plant species may act differently in petroleum-contaminated soil (Banks and Schultz 2005; Plaza et al. 2005). In present study, pulse crop (mung) is evaluated to petroleum contamination. The study suggests that in S1

and S2 slurry the toxicity effect on germination and seedling growth is highest, while decrease in effect was observed in case of S3 and B4. This effect might be due to petroleum oil in the soil that creates an impermeable oily film layer around the seeds interfering water air relations resulting in adverse effect on germination. Similar effect on germination rate caused by petroleum contaminants in various commercially important plant species was also reported by Adam and Duncan (2002); Tang et al. (2011) and Das and Kumar, (2016).

Earthworms are extensively used soil organisms for assessment of soil toxicity. Earthworms are common inhabitants of soil that influence on soil chemical property, structure, nutrient and plant growth (Edwards and Bohlen 1996; Handy 2007). Earthworms are sensitive to soil pollutant that makes them good bioindicators for screening the soil toxicity. Acute toxicity test is a short-term test for measuring the effects of chemicals on survival of earthworm (Handy 2007).

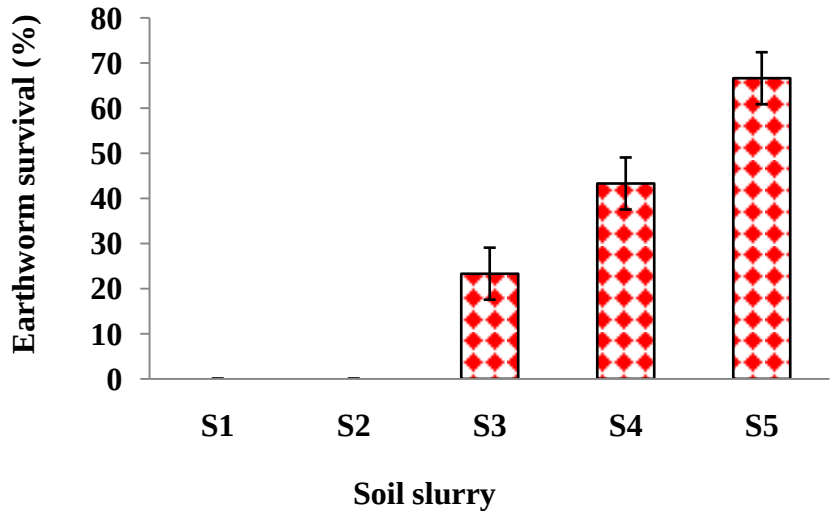


Figure 3.2.4 Effect of contaminated soil on earthworm survival

Results analyzed by using single-factor ANOVA of variance and are mean of three replicates with standard deviation (Means ± S.D, n = 3). And differences were considered significant by determining the p-value (^{a} $p < 0.01$,^{b} $p < 0.05$, ^{c} Not significant).

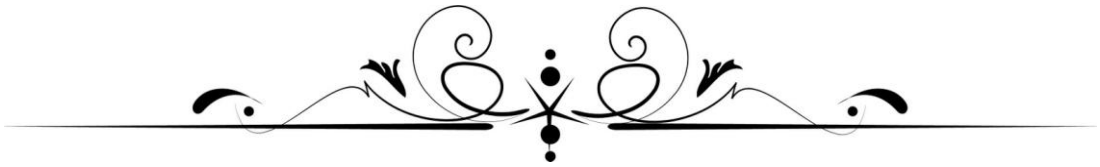
Chemical properties of garden soil used [C organic (%) 1.4 ± 0.05, Ca (g /Kg) 3.5 ± 0.4, N (g /Kg) 1.19 ± 0.8, Na (g / Kg) 3.8 ± 0.1, P (g /Kg) 0.75 ± 0.05, K (g /Kg) 2.94 ± 0.4, Fe (g /Kg) 0.155 ± 0.03, Zn (g/ Kg) 0.0038 ± 0.02]

S1= 10 g Contaminated soil + 25 distilled waster
S2= 7.5 g Contaminated soil + 2.5 garden soil + 25 distilled waster
S3= g Contaminated soil + 5 garden soil + 25 distilled waster
S4=2.5 g Contaminated soil + 7.5 garden soil + 25 distilled waster
S5=10 g garden soil + 25 distilled waster

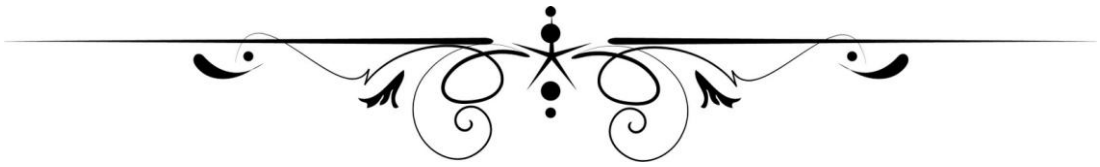
Figure 3.2.4 represents the earthworm survival rate in all the slurry. Apparently, no survival rates were reported in S1 and S2 slurry. The 100 % mortality rate in the slurry S1 and S2 indicates the nature of the soil. The weight of the earthworm decrease in all the slurry. Even in the S5 slurry (garden soil) decrease in earthworm weight was reported and this might be due to changes in soil nutrients and living conditions. There was swelling with ejecting coelomic fluid in the death earthworm of all the treatment. Similar results were reported by Ramadass et al. 2015 that when earthworms were exposed to used motor oil swelling, fragmentation and ejecting of coelomic fluid occur before death. Tang et al. 2011 observed that with higher oil concentration in soil cause death of the earthworm and the inhibition of body weight.

3.3.4 Conclusion

The toxicity studies revealed that oil contaminated soil containing various organic compounds and hydrocarbons are toxic in nature. Hence, development of management strategist for restoration of such soils without harming the environment is a matter of concern.



Chapter 3.4



Contents:

3.4.1 Introduction

Overdependence of the world on petroleum hydrocarbons to meet their energy and other requirement has resulted in more extraction and refinement of crude oils. However, there is another side to this usefulness, as crude oil prospecting is sometimes accompanied by spillage. These spillages are a source of environmental contamination. Although the pollution caused by oil spills in the air and oceans cannot be easily controlled using biological means, the soil contaminated by these can be remediated using different techniques. Still, this is a new field which has attracted the attention of environmentalists and policymakers throughout the world as environmental petroleum contamination and its constituents is an inevitable problem that affects many geographical regions to a variable extent depending on the local environmental law (Graj et al., 2013). Petroleum hydrocarbons bioremediation depends on the presence of microorganisms with the appropriate metabolic capabilities. Most of the bacteria frequently isolated from hydrocarbon-polluted sites belong to the genera *Pseudomonas, Sphingomonas,Acinetobacter, Alcaligenes, Micrococcus, Bacillus, Flavobacterium, Arthrobacter, Alcanivorax Mycobacterium, Rhodococcus,* and *Actinobacter* (Atlas, 1992; Kumar et al., 2015; Obayori et al., 2009). Optimal rates of hydrocarbon biodegradation by a microorganism can be sustained by the adequate concentration of nutrients, oxygen, and pH values (Perry, 1984; Atlas, 1992; Amund and Nwokoye, 1993). But, low solubility and high hydrophobic nature of petroleum hydrocarbon compounds make them highly unavailable to microorganisms. Release of biosurfactants is one of the strategies used by microorganisms to influence the uptake of petroleum hydrocarbon and hydrophobic compounds (Marin et al., 1996; Johnsen et al, 2005; Obayori et al., 2009).

Hence, the present chapter depicts the efficiency of biosurfactant producing bacterial isolate for restoration and detoxification of the soil collected from the contaminated site.

3.4.2 Materials and Methods

3.4.2.1 Experimental Setup for Biodegradation

For biodegradation experiment characterized soil mentioned in chapter 3.3 was used. Briefly, 10 g of contaminated soil was taken 500 ml Erlenmeyer flask and autoclaved. 250 ml of 24 hrs grown bacterial culture were added in the flask and incubated at rotary shaker for 3 months. At every 10 days interval, 2 g of sterilized bagasse powder was added to the each flasks for 90 days. After 90 days the content of the each flasks were processed for further analytical analysis (HPLC and GC-MS).

3.4.2.2 Preparation of the sample for HPLC analysis

For HPLC analysis of the treated sample, the stock solution of concentration 10 µl/ml was prepared by dissolving 10 µl standard in 0.5 ml HPLC-grade water followed by sonication for 10 minutes and the resulting volume was made up to 1 ml with the water. The same method was followed to prepare the sample solutions. The standard and sample solutions were filtered through 0.22µm PVDF-syringe filter and the mobile phase was degassed before the injection of the solutions.The mobile phase contains Methanol, the flow rate was adjusted to 1.0 ml/min, the column was thermostatically controlled at 40°C and the injection volume was kept at 20 µl. Isocratic elution was performed. Total analysis time per sample was 10 min. HPLC chromatograms were detected using a photodiode array UV detector at single wavelengths of 262 nm according to absorption maxima of analysed compounds. Each compound was identified by its retention time and by spiking with standards

under the same conditions. The quantification of the sample was done by the measurement of the integrated peak area.

3.4.2.3 Preparation of the sample for GC-MS analysis

Sample from each flask was first dried at 30°C and homogenized and pass through the 0.25-1 mm sieve. 5 g sample from from each treatment were taken in the thimble in soxhlet apparatus and fill with the 150 ml solvent dichloromethane. The extraction process was started for 24 hours at 48°C for. After extraction, dichloromethane was dried off and the sample was stored and derivatizes. For derivatization 50 µl N, O-Bis(trimethylsilyl)trifluoroacetamide and trimethyl chlorosilane (BSTFA+TMCS) and 10µl of Pyridine was also added. Samples were further heated at 60ºC for 30 minutes. 1 ml solution of 990µl of BSTFA mix with 10µl of TMCS in ratio of 99:1 leads to the formation of BSTFA+TMCS. Then the sample were transferred in GC vial and dried and finally dissolved in methanol before GC-MS analysis.

3.4.3 Results and Discussion

The HPLC chromatogram was represented Figure 3.4.1 (a,b,c and d).

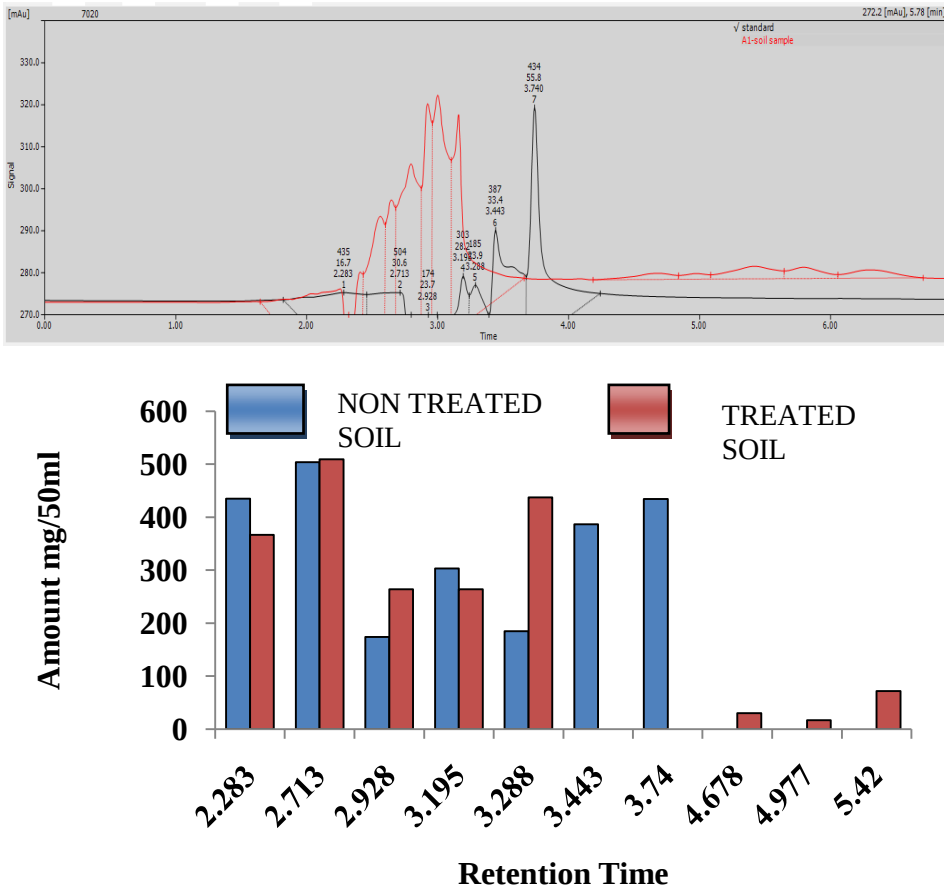
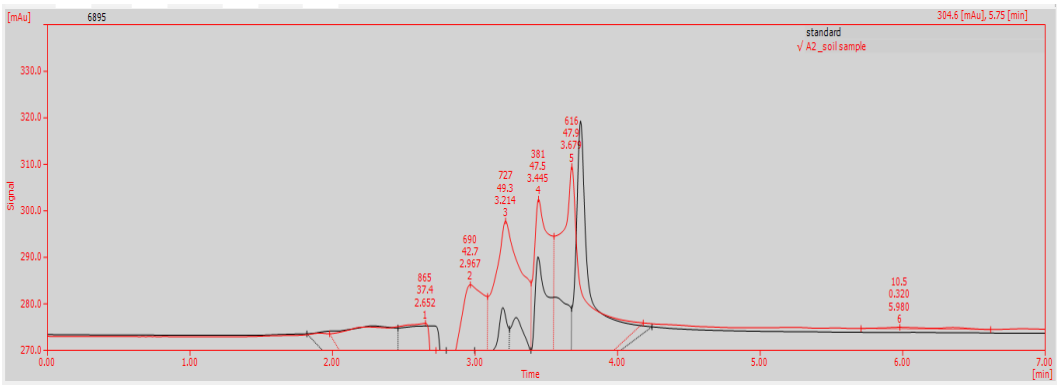


Figure 3.4.1a. HPLC chromatogram of the soil treated with *Pseudomonas azotoformans* AJ15

In the HPLC chromatogram of the soil treated with *Pseudomonas azotoformans* AJ15 shows the variation of the compounds in treated and non treated soil. The compound at retention time 2.283 of AJ15 treated soil is 15 % lesser than the non treated soil ,compound 2 at retention time 2.713 is almost in the same amount as of non-treated soil , compound at retention time 2.928 is 34% more in treated than the non -treated soil, compound 4 atretention time 3.195 is 12.98% lesser in treated soil than the non treated soil, compound 5 in retention time 58.12 % more than the non treated soil, two other compounds reported in the treated soil didn't show any match with the non treated soil .



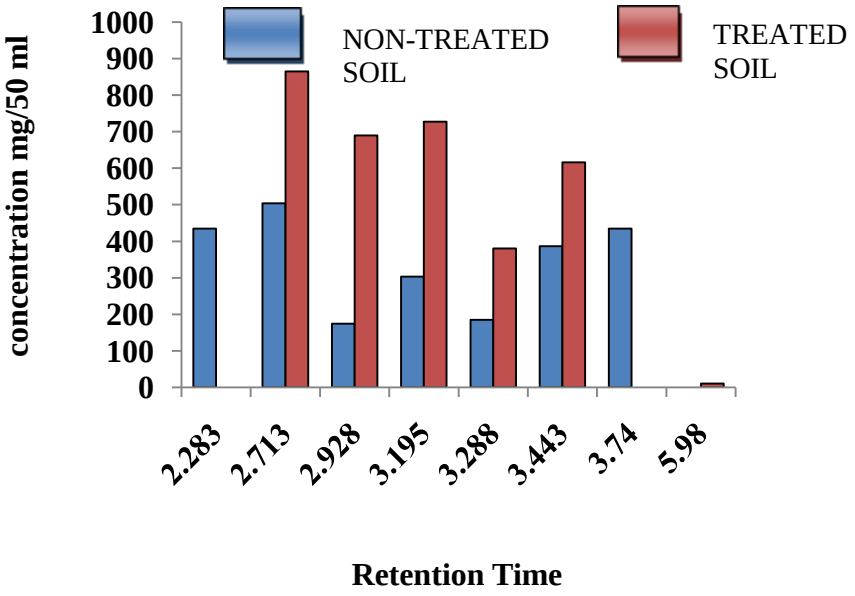
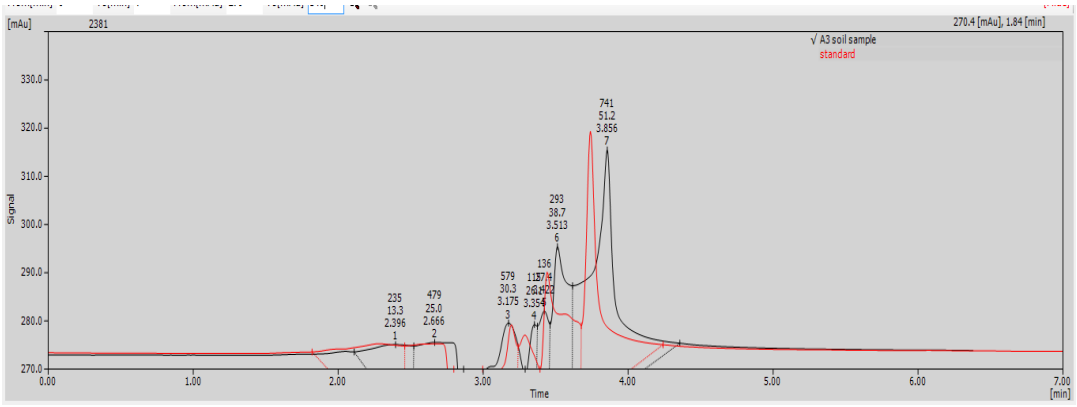


Figure 3.4.1b. HPLC chromatogram of the soil treated with *Bacillus licheniformis* J1

In HPLC chromatogram of treated soil with *Bacillus licheniformis* J1, it was revealed that a compound at retention time 2.283 of non treated doesn't show existence in the treated soil,compound at retention time 2.713 in treated is 35.41% more than the non treated, the compound at retention time 2.928 in treated soil is 58.28 % more than non-treated soil , the compound at retention time 3.195 is 51.38% more in treated than the non-treated, compound at retention time3.443 is 37.24 % more in treated than

the non treated, compound at retention time 3.74 in treated soil does show any resemblance with standard .



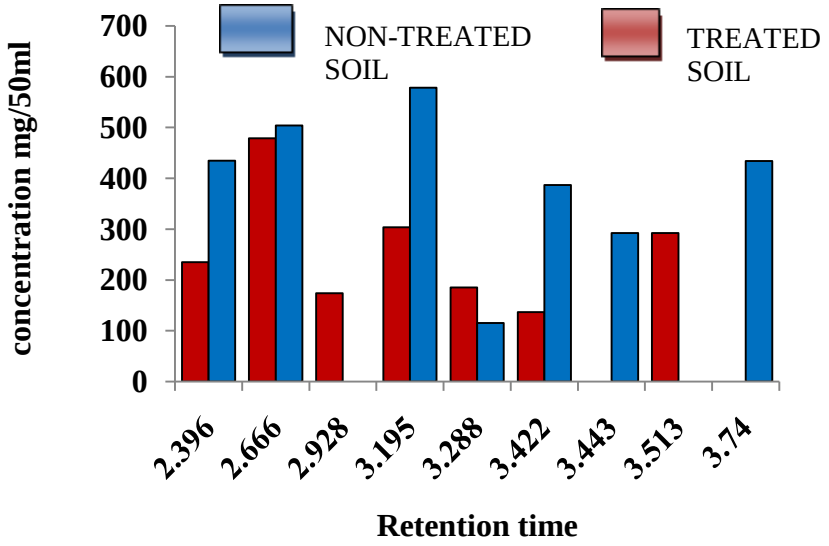


Figure 3.4.1c. HPLC chromatogram of the soil treated with *Bacillus safensis* J2

In HPLC chromatogram of the soil treated with *Bacillus safensis* J2 reveals that compound 1 at retention time 2.396 is 46.08 % lesser in treated soil than the non treated soil , compound 2 at retention time 2.666 is 14% lesser in treated than the non treated , compound 3 in treated didn't showed in any resemblance with non-treated soil, whereas compound 4 is 68 % greater than the non treated compound 5 is 24% less while compound 6 46% less. Rest of the compounds showed no match.

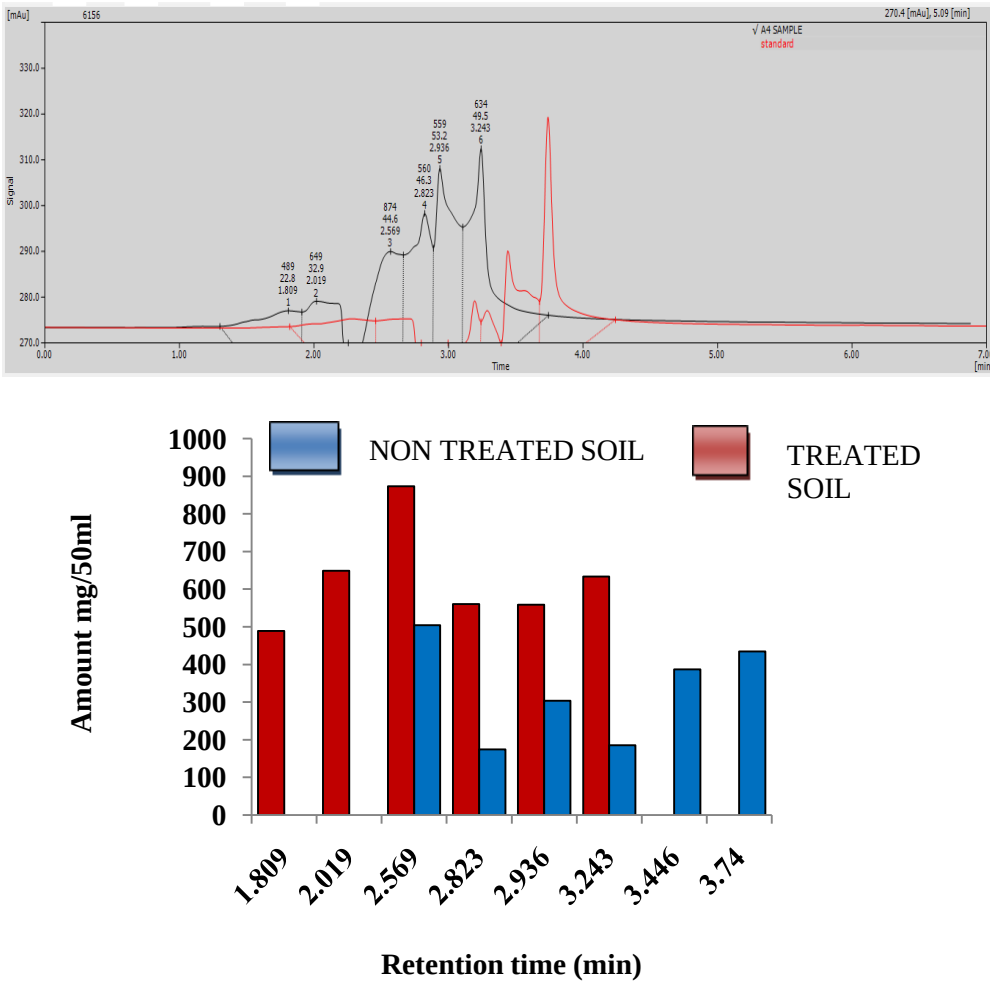


Figure 3.4.1d. HPLC chromatogram of the soil treated with *Pseudomonas azotoformans* N23

In HPLC chromatogram of the soil treated with *Pseudomonas azotoformans* N23 demonstrated thatcompound 1 and 2 are new compounds that do not exist in non treated soil. The compound 3 at retention time 2.569 is 38% than the non treated, compound at retention time 2.823 in treated was nearly 42 % more than the non treated whileretention time 2.936 and 3.243 also showed some variations from the treated. The compound at retention time 3.446 and 3.74 does not show any existence in treated soil.

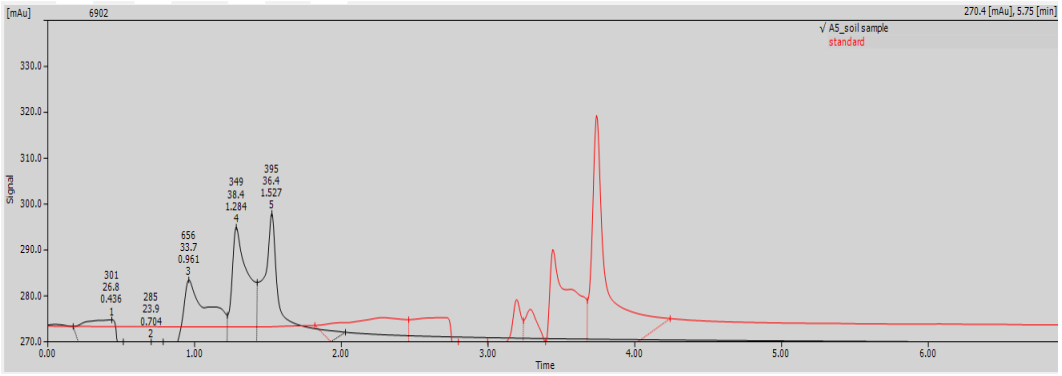


Figure 3.4.1e. HPLC chromatogram of the soil treated with bacterial Consortia

In HPLC analysis of the soil treated with bacterial consortia shows no relevances with the non treated soil.

In the result most of the compound present in the treated soil differ from the non-treated soil .This might be due to degradation of the compounds into some new metabolites. The result shows that consortia treatment is better as compared to the treated soil with bacterial strain AJ15, J1, J2 and N23. Thus, the result indicates that the bacterial consortium is more efficient as compared to the individual bacterial treatment.The metabolic compound reported after the treatments were further processed for GC-MS analysis.

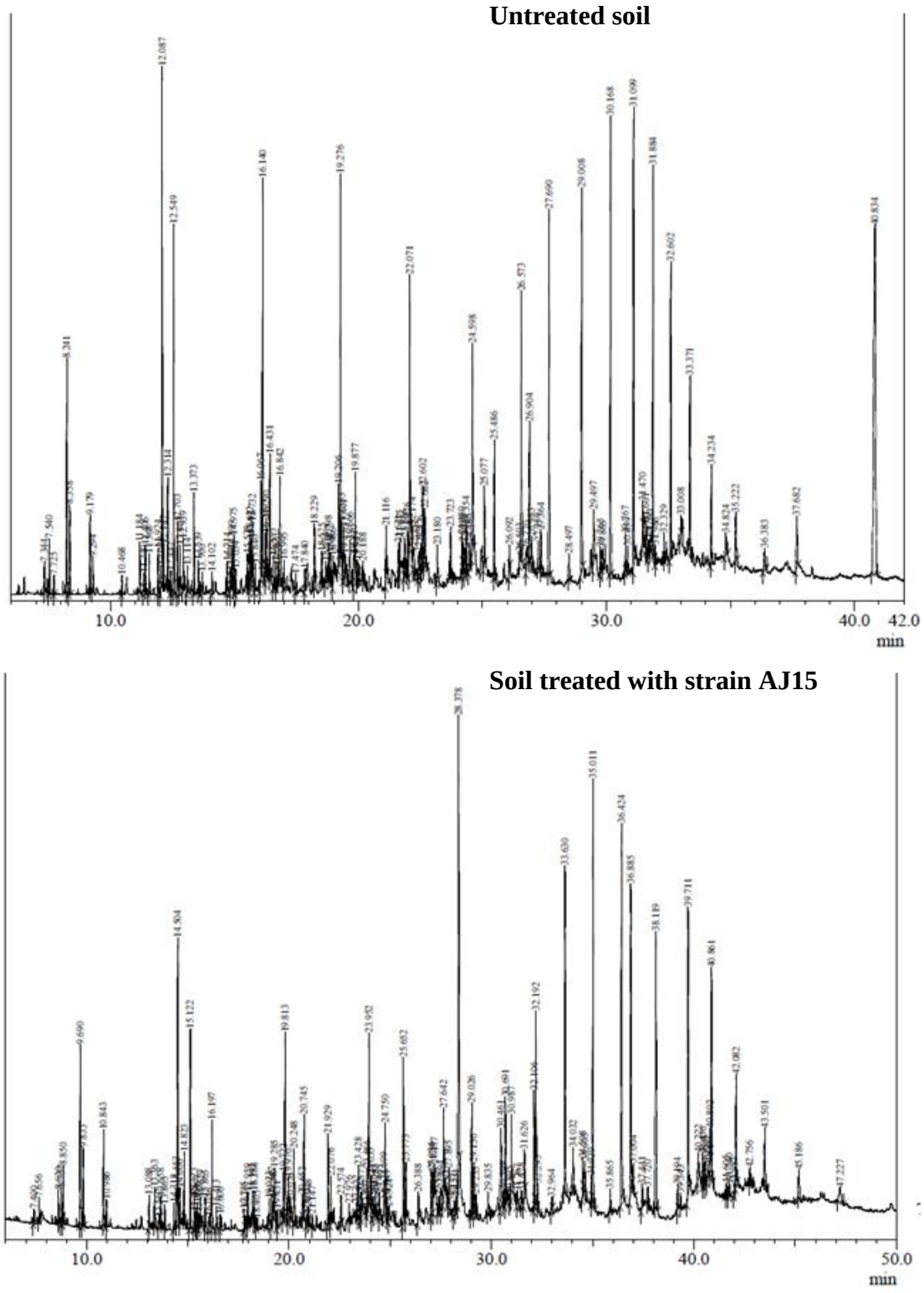


Figure 3.4.2a. GC-MS chromatogram of the soil treated with *Pseudomonas azotoformans* AJ15

GC-MS analysis of the soil treated with *Pseudomonas azotoformans* AJ15

The GC-MS results of the treated soil with strain AJ15 is depicted within the Table.

Table 3.4.1. Organic compounds identified in the strain AJ15 treated soil

Peak#	R.Time	Area	Area%	Name
1	7.391	131117	0.12	UNDECANE
2	7.650	204527	0.19	OCTANE, 2,4,6-TRIMETHYL-
3	8.613	288796	0.27	Decane, 2,5,6-trimethyl-
4	8.750	340962	0.32	Nonane, 2,5-dimethyl-
5	8.844	555625	0.52	Octane, 3,3-dimethyl-
6	9.683	1890178	1.77	Decane, 3,7-dimethyl-
7	9.826	796009	0.75	Decane, 3,7-dimethyl-
8	10.839	1019446	0.96	Decane, 3,7-dimethyl-
9	10.980	264384	0.25	Decane, 3,7-dimethyl-
10	13.083	315393	0.30	1-Methoxy-3-(2-hydroxyethyl)nonane
11	13.358	478573	0.45	HEPTADECANE
12	13.563	50955	0.05	4-Methyl-1,6-heptadien-4-ol
13	13.652	244018	0.23	Undecane, 2,6-dimethyl-
14	13.859	255907	0.24	Dodecane, 4-methyl-
15	14.314	355377	0.33	Tetradecane
16	14.450	139491	0.13	TETRADECANE
17	14.499	2704828	2.54	Benzene, 1,3-bis(1,1-dimethylethyl)-
18	14.600	437168	0.41	Dodecane, 4,6-dimethyl-
19	14.820	665527	0.62	NONADECANE
20	14.976	89524	0.08	Sulfurous acid, 2-ethylhexyl isohexyl ester
21	15.119	2043610	1.92	Dodecane, 4,6-dimethyl-
22	15.320	297549	0.28	Nonane, 5-(2-methylpropyl)-
23	15.383	134757	0.13	Decane, 2,3,5-trimethyl-
24	15.453	231339	0.22	Dodecane, 4,6-dimethyl-
25	15.519	132445	0.12	Dodecane, 4,6-dimethyl-
26	15.625	268263	0.25	Nonane, 5-(2-methylpropyl)-
27	15.863	331858	0.31	DECANE, 2,3,7-TRIMETHYL-
28	16.055	118488	0.11	Decane, 3,7-dimethyl-
29	16.195	1169485	1.10	Hexadecane
30	16.410	306059	0.29	Nonane, 5-(2-methylpropyl)-
31	16.604	236915	0.22	Undecane, 3,8-dimethyl-
32	17.780	298433	0.28	3-HEXADECENE, (Z)-
33	17.957	432120	0.41	PENTADECANE
34	18.117	118692	0.11	2,2,3-TRIMETHYL-CYCLOPROPANECARBOXYLIC AC
35	18.191	243615	0.23	Heptadecane, 2,6,10,15-tetramethyl-
36	18.285	370920	0.35	Heptadecane, 2,6,10,15-tetramethyl-
37	18.403	118219	0.11	DECANE, 2,3,4-TRIMETHYL-
38	19.021	226499	0.21	Nonadecane
39	19.145	364490	0.34	Heptadecane
40	19.237	85688	0.08	2,2'-Isopropylidenebis(tetrahydrofuran)
41	19.284	396575	0.37	Heptadecane

42	19.428	199935	0.19	Dodecane, 4,6-dimethyl-
43	19.573	497826	0.47	1-Tridecene
44	19.722	479398	0.45	Hexadecane
45	19.812	2393973	2.25	Heptadecane
46	19.967	333576	0.31	Pentadecane
47	20.013	66621	0.06	Nonane, 5-butyl-
48	20.075	363768	0.34	PENTADECANE
49	20.252	1059197	0.99	Phenol, 3,5-bis(1,1-dimethylethyl)-
50	20.652	214621	0.20	Hexadecane
51	20.744	1373524	1.29	Hexadecane
52	20.867	128218	0.12	Sulfurous acid, hexyl pentadecyl ester
53	20.949	130399	0.12	Dodecane, 2,6,11-trimethyl-
54	21.146	142699	0.13	Dodecane, 2,6,11-trimethyl-
55	21.927	1006163	0.94	9-Eicosene, (E)-
56	22.075	494967	0.46	PENTADECANE
57	22.575	294977	0.28	Heneicosane
58	22.976	190474	0.18	Decane, 3,8-dimethyl-
59	23.169	132533	0.12	HEXADECANE, 2,6,10,14-TETRAMETHYL-
60	23.430	756337	0.71	PENTADECANE
61	23.572	269534	0.25	Nonyl tetradecyl ether
62	23.866	397637	0.37	Heptadecane
63	23.952	2272481	2.13	Eicosane
64	24.034	280238	0.26	Eicosane
65	24.157	432499	0.41	Heneicosane
66	24.213	72052	0.07	Decane, 1-iodo-
67	24.286	248580	0.23	Eicosane
68	24.462	490963	0.46	2-Methylhexacosane
69	24.698	173208	0.16	TETRADECANE
70	24.750	839367	0.79	Eicosane
71	24.849	202073	0.19	Dodecane, 4,6-dimethyl-
72	24.920	105794	0.10	Nonane, 5-(2-methylpropyl)-
73	25.651	1880148	1.76	1-Heptadecene
74	25.772	539567	0.51	NONADECANE
75	26.387	299213	0.28	Heneicosane
76	27.031	1008429	0.95	8-Octadecanone
77	27.127	204372	0.19	Decane, 1-iodo-
78	27.176	379168	0.36	1-Hexadecanol
79	27.387	265021	0.25	Eicosane
80	27.493	466333	0.44	Heptadecane
81	27.642	998509	0.94	Eicosane
82	27.733	208787	0.20	OCTADECANE
83	27.847	49026	0.05	EICOSANE
84	27.897	347262	0.33	HEPTACOSANOIC ACID, METHYL ESTER
85	28.137	199440	0.19	2-methyloctacosane
86	28.252	158724	0.15	Tetratetracontane
87	28.378	8167646	7.66	Dibutyl phthalate
88	28.531	2046163	1.92	n-Hexadecanoic acid
89	29.025	1378661	1.29	1-Heneicosanol
90	29.128	558596	0.52	Heneicosane

91	29.232	279734	0.26	Acetic acid n-octadecyl ester
92	29.833	249431	0.23	Heneicosane
93	30.298	242538	0.23	10-Nonadecanone
94	30.462	1882443	1.77	Heptafluorobutyric acid, pentadecyl ester
95	30.603	232501	0.22	2-Methylhexacosane
96	30.694	1216388	1.14	Heneicosane
97	30.892	78408	0.07	2-Methylhexacosane
98	30.986	756085	0.71	Eicosane
99	31.267	140671	0.13	1-Octanol, 2-butyl-
100	31.479	305505	0.29	Eicosane
101	31.633	1214784	1.14	Eicosane
102	32.105	1334711	1.25	Behenic alcohol
103	32.191	2191762	2.06	Heneicosane
104	32.296	388508	0.36	Tricosyl acetate
105	33.630	3939700	3.70	Heneicosane
106	34.028	576370	0.54	Eicosane
107	34.507	475750	0.45	2,3-naphthalenedicarboxaldehyde, 5,6,7,8-tetrahydro-5,5,8,8
108	34.605	583074	0.55	OCTADECANE
109	34.936	271130	0.25	n-Tetracosanol-1
110	35.011	5081489	4.77	Heneicosane
111	35.866	281722	0.26	2-methyloctacosane
112	36.425	5790124	5.43	Heneicosane
113	36.887	5944359	5.58	1,2-BENZENEDICARBOXYLIC ACID
114	37.004	458112	0.43	OCTADECANE
115	37.443	354234	0.33	2-methyloctacosane
116	37.721	272593	0.26	Tetrapentacontane
117	38.020	98266	0.09	Cyclohexane, [6-cyclopentyl-3-(3-cyclopentylpropyl)hexyl]-
118	38.120	4656439	4.37	Heneicosane
119	39.199	344861	0.32	2-methyloctacosane
120	39.342	171058	0.16	2-methyloctacosane
121	39.709	3940027	3.70	Tetratetracontane
122	40.219	499611	0.47	TETRACOSANE
123	40.375	74023	0.07	Tetratetracontane
124	40.437	346922	0.33	Hexatriacontane
125	40.558	210448	0.20	2-methyloctacosane
126	40.688	155143	0.15	HEXADECANE, 2,6,10,14-TETRAMETHYL-
127	40.804	328870	0.31	Decanedioic acid, bis(2-ethylhexyl) ester
128	40.861	2138207	2.01	Tetratetracontane
129	40.985	134747	0.13	Squalene
130	41.605	184831	0.17	Tetratetracontane
131	41.739	209535	0.20	Tetratetracontane
132	41.987	79388	0.07	6,7-Dimethyl-9-oxo-1,5-diazabicyclo[5.2.0]non-5-ene
133	42.085	1602688	1.50	Hexatriacontane
134	42.754	262254	0.25	OCTADECANE
135	43.498	933682	0.88	Tetracontane
136	45.183	570925	0.54	Tetratetracontane
137	47.211	723789	0.68	Pentatriacontane

Compounds Degraded

When the compounds reported in strain AJ15 treated soil was compared with the untreated soil as mentioned in the Table 3.3.1 of Chapter 3.3, and then it was found that some of the compounds has been degraded by the bacterial strain. Such compound are mentioned below in Table 3.4.2.

Table 3.4.2. Organic compounds degraded by the strain AJ15 in the treatment

No.	**Compound Degraded**	No.	**Compound Degraded**
1	UNDECANE, 5,6-DIMETHYL-	14	DOCOSANE
2	CYCLOHEXANE, 1-METHYL-4-(1-METHYLETGENYL)	15	2H- PYRAN, 3,6-DIHYDRO-6-METHOXY-2-METHYL-,T
3	TRIDECANE	16	PENTACOSANE
4	UNDECANE,2,4-DIMETHYL-	17	Tetracosane, 1-iodo-
5	TETRADECANE,5-METHYL-	18	(Z)-ISOBUTYL 7-(TRIMETHYLSILYL)-5-HEPTENOATE
6	TRIDECANE	19	3,5-Dimetylidodecane
7	3-OXO-1-CYCLOHEXENYL 2-METHYLPROPIONAT	20	Tetratetracontane
8	3,5-DIMETHYLDODECANE	21	HEXANE, 2,2,3-TRIMETHYL-
9	Hexadecane, 1-iodo-	22	2,6,10,15,19,23-HEXAMETHYLTETRACOSANE
10	Tetradecane, 4-methyl-	23	PENTACOSANE
11	Pentadecane, 2,6,10-trimethyl-	24	BETA-D-MANNO-D-GLYCERO-ALPHA-D-GALACTO
12	2,6,10-Trimethyltridecane	25	Tetratriacontane
13	Phenol,2,4-bis(1,1-dimethylethyl)-, phosphate (3:1	26	5,11,17,23-tetratert-butylpentacyclo[19,3,1,1˜

New compounds reported

The degradation of the compounds leads to the formation of the new compounds. Such new compounds reported depicted within Table 3.4.3.

Table 3.4.3. New compounds reported in the strain AJ15 in the treatment

No.	**New compound reported**	No.	**New compound reported**
1	UNDECANE	20	Decane, 3.8 dimethyl-
2	OCTANE,2,4,6-TRIMETHYL	21	Hexadanecane, 2,6,10,14-TETRAMETHYL-
3	DECANE 2,5,6-TRIMETHYL-	22	Decane, 1 –iodo-
4	Nonane, 2,5-dimethyl-	23	2- Methylhexacosane
5	1-Methoxy-3-(2-hydroxyethyl)nonane	24	1-Heptadecane
6	4-Methyl-1,6-heptadien-4-ol	25	8-Octadecanone
7	NONADECANE	26	Decane, 1 –iodo-
8	3-HEXADECENE (Z)-	27	1-Hexadecanol
9	2,2,3-TRIMETHYL-CYCLOPROPANECARBOXYLIC AC	28	Acetic acid n-octadecyl ester
10	Heptadecane, 2.6.10.15-tetramethyl-	29	10-Nonadecanone
11	DECANE,2,3,4-TRIMETHYL-	30	Heptafluorobutyric acid, pentadecyl ester
12	2,2'-Isopropylidenebis(tetrahydrofuran	31	1-Octanol, 2-butyl-
13	1-Tridecene	32	Behenic alcohol
14	Nonane, 5-butyl-	33	Tricosyl acetate
15	Sulfurous acid, hexyl pentadecyl ester	34	2,3-napthalenedicarboxaldehyde, 5,6,7,8-tetrahydro-5,5,8,8
16	Dodecane, 2.6.11-trimethyl-	35	n-Tetracosanol-1
17	9-Eicosene, (E)-	36	1,2-BENZENEDICARBOXYLIC ACID
18	Cyclohexane, (6-cyclipentyl-3-(3-cyclopentypropyl) hexyl)-	37	HEXADECANE, 2,6,10,14-TETRAMETHYL-
19	Decanedioic acid, bis(2-ethylhexyl) ester	38	6,7-Dimethyl-9-oxo-1,5-diazabicyclo [5,2,0]non-5-ene

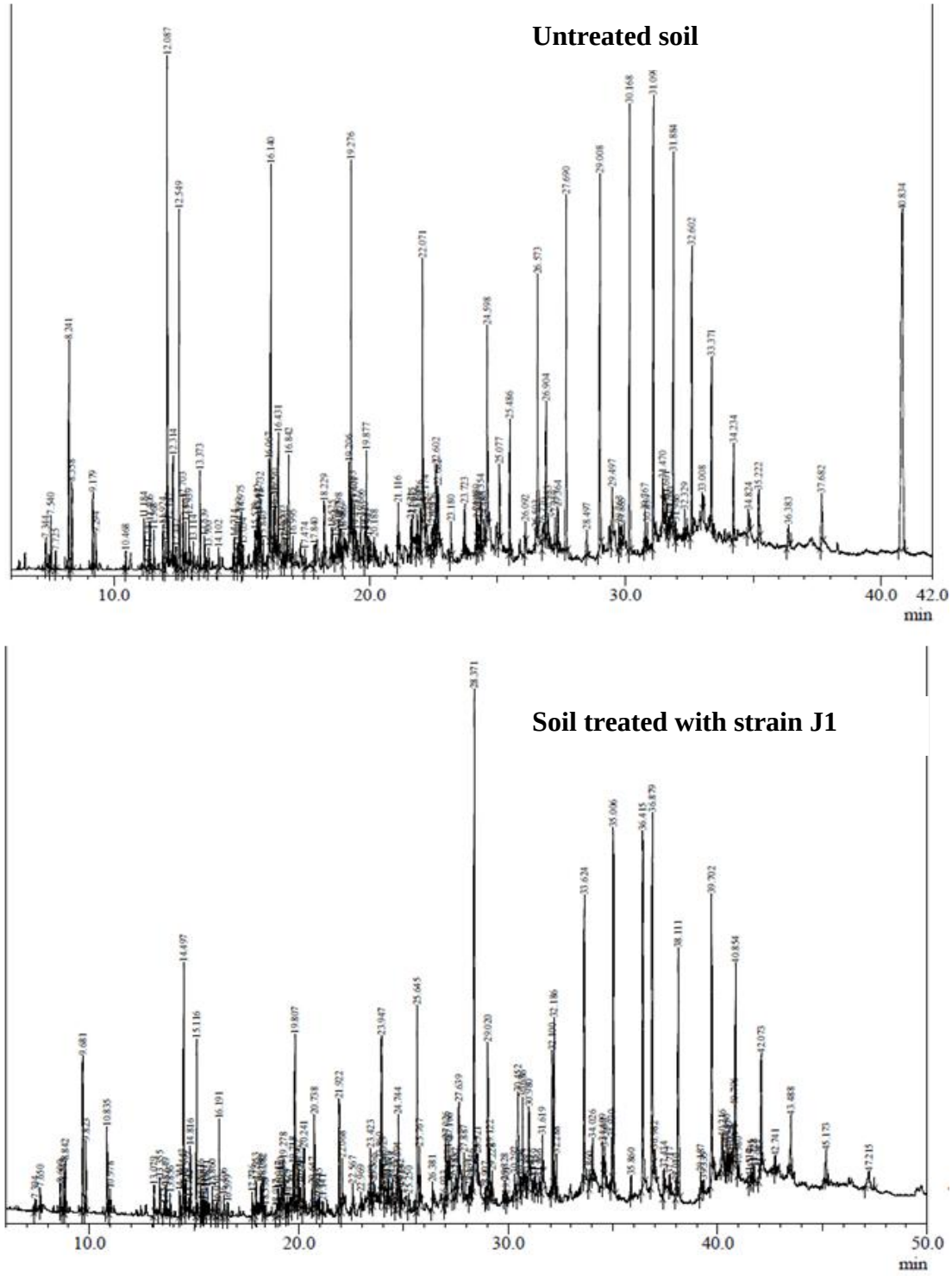


Figure 3.4.2b. GC-MS chromatogram of the soil treated with *Bacillus licheniformis* J1

GC-MS analysis of the soil treated with *Bacillus licheniformis* J1

The GC-MS results of the treated soil with strain J1 is depicted within the Table 3.4.4.

Table 3.4.4. Organic compounds identified in the strain J1 treated soil

Peak#	R.Time	Area	Area%	Name
1	7.394	142125	0.13	NONANE, 2-METHYL-
2	7.650	210240	0.19	OCTANE, 2,4,6-TRIMETHYL-
3	8.609	287605	0.26	NONANE, 2,5-DIMETHYL-
4	8.748	357182	0.32	Nonane, 2,5-dimethyl-
5	8.842	572184	0.51	Octane, 3,3-dimethyl-
6	9.681	1962978	1.76	Decane, 3,7-dimethyl-
7	9.823	816462	0.73	Decane, 3,7-dimethyl-
8	10.835	1034976	0.93	Decane, 3,7-dimethyl-
9	10.978	268396	0.24	Decane, 3,7-dimethyl-
10	13.079	315089	0.28	1,7-Octanediol
11	13.355	486921	0.44	Dodecane
12	13.563	46118	0.04	2,2'-BIFURAN,OCTAHYDRO-2,5-DIMETHYL-,[2.ALPH
13	13.649	229254	0.21	Undecane, 3,6-dimethyl-
14	13.856	255273	0.23	Dodecane, 4-methyl-
15	14.311	363327	0.33	Tetradecane
16	14.443	169815	0.15	Sulfurous acid, hexyl pentyl ester
17	14.497	2747548	2.46	Benzene, 1,3-bis(1,1-dimethylethyl)-
18	14.599	446093	0.40	Dodecane, 4,6-dimethyl-
19	14.762	92501	0.08	Sulfurous acid, 2-ethylhexyl pentyl ester
20	14.816	679943	0.61	NONADECANE
21	15.116	2113675	1.89	Nonane, 5-(2-methylpropyl)-
22	15.316	307770	0.28	Nonane, 5-(2-methylpropyl)-
23	15.380	135322	0.12	Nonane, 3,7-dimethyl-
24	15.451	245392	0.22	Dodecane, 4,6-dimethyl-
25	15.516	138425	0.12	Dodecane, 4,6-dimethyl-
26	15.621	278529	0.25	Nonane, 5-(2-methylpropyl)-
27	15.860	327306	0.29	DECANE, 2,3,7-TRIMETHYL-
28	16.050	120054	0.11	Nonane, 5-(2-methylpropyl)-
29	16.191	1199076	1.07	Hexadecane
30	16.406	311459	0.28	Nonane, 5-(2-methylpropyl)-
31	16.599	236937	0.21	Undecane, 3,8-dimethyl-
32	17.776	292599	0.26	3-HEXADECENE, (Z)-
33	17.953	463075	0.42	PENTADECANE
34	18.186	314768	0.28	Heptadecane, 2,6,10,15-tetramethyl-
35	18.282	377710	0.34	Heneicosane
36	18.398	121249	0.11	DECANE, 2,3,4-TRIMETHYL-
37	18.917	97816	0.09	METHANONE,(2-ETHENYL-3,4-

				DIHYDRO-2-METHYL
38	19.017	197690	0.18	2-Bromo dodecane
39	19.140	393611	0.35	Hexadecane
40	19.233	77721	0.07	2,2'-Isopropylidenebis(tetrahydrofuran)
41	19.278	415570	0.37	Heptadecane
42	19.423	205128	0.18	Dodecane, 4,6-dimethyl-
43	19.567	424129	0.38	1-DODECANOL
44	19.718	499515	0.45	Hexadecane
45	19.807	2482802	2.23	Heptadecane
46	19.962	354401	0.32	Heptadecane
47	20.007	87036	0.08	Nonane, 5-(2-methylpropyl)-
48	20.071	432321	0.39	PENTADECANE
49	20.241	992108	0.89	Phenol, 3,5-bis(1,1-dimethylethyl)-
50	20.647	234870	0.21	Hexadecane
51	20.738	1428531	1.28	Hexadecane
52	20.863	147809	0.13	Nonadecane
53	20.945	140044	0.13	Dodecane, 4,6-dimethyl-
54	21.141	130053	0.12	Dodecane, 4,6-dimethyl-
55	21.922	1541876	1.38	1-Pentadecene
56	22.068	550664	0.49	PENTADECANE
57	22.567	316417	0.28	Heneicosane
58	22.969	183255	0.16	Decane, 3,8-dimethyl-
59	23.423	699567	0.63	IRON, TRICARBONYL[N-(PHENYL-2-PYRIDINYLMET
60	23.493	112798	0.10	Heneicosane
61	23.566	282331	0.25	1-Decanol, 2-hexyl-
62	23.860	419818	0.38	Heptadecane
63	23.947	2411103	2.16	Eicosane
64	24.029	289518	0.26	Eicosane
65	24.152	427955	0.38	Heneicosane
66	24.203	89389	0.08	Decane, 1-iodo-
67	24.279	225843	0.20	Eicosane
68	24.458	483103	0.43	Eicosane
69	24.640	24245	0.02	Cyclohexane, 1,1,3-trimethyl-
70	24.694	198295	0.18	DECANE, 2,3,7-TRIMETHYL-
71	24.744	886486	0.79	Eicosane
72	24.842	205235	0.18	2,6,10-Trimethyltridecane
73	24.913	106248	0.10	Eicosane
74	25.250	250992	0.22	OCTADECANE
75	25.645	2809479	2.52	1-Heptadecene
76	25.767	667902	0.60	NONADECANE
77	26.381	303400	0.27	Heneicosane
78	26.933	104209	0.09	Cyclohexane, octyl-
79	27.026	1021726	0.92	8-Octadecanone
80	27.122	169934	0.15	Decane, 1-iodo-
81	27.166	400735	0.36	1-Hexadecanol
82	27.383	287357	0.26	Eicosane
83	27.487	496199	0.44	Heptadecane

84	27.639	998628	0.90	Eicosane
85	27.887	861878	0.77	TRIACONTANOIC ACID, METHYL ESTER
86	28.137	234109	0.21	CELIDONIOL, DEOXY-
87	28.246	193531	0.17	Eicosane
88	28.371	7780324	6.97	Dibutyl phthalate
89	28.521	269128	0.24	n-Hexadecanoic acid
90	28.907	133484	0.12	Undecane, 3-methylene-
91	29.020	2107334	1.89	1-Nonadecene
92	29.122	643255	0.58	Heneicosane
93	29.228	309092	0.28	1-Acetoxynonadecane
94	29.828	264583	0.24	Heneicosane
95	29.901	102238	0.09	Heneicosane
96	30.292	359416	0.32	10-Nonadecanone
97	30.452	2154587	1.93	1-NONADECENE
98	30.594	233249	0.21	2-Methylhexacosane
99	30.686	1256622	1.13	Heneicosane
100	30.887	90008	0.08	2-Methylhexacosane
101	30.980	905041	0.81	Eicosane
102	31.147	128771	0.12	Nonane, 5-methyl-5-propyl-
103	31.259	153406	0.14	1-Octanol, 2-butyl-
104	31.466	347730	0.31	Eicosane
105	31.619	673922	0.60	Decane, 1-iodo-
106	32.100	1774531	1.59	Behenic alcohol
107	32.186	2196802	1.97	Heneicosane
108	32.288	531572	0.48	Tricosyl acetate
109	33.624	4033752	3.62	Heneicosane
110	33.860	111669	0.10	Nonane, 5-(2-methylpropyl)-
111	34.026	768584	0.69	Eicosane
112	34.499	408203	0.37	n-Octanoic acid, ethyldimethylsilyl ester
113	34.600	508167	0.46	Eicosane
114	34.930	489814	0.44	n-Tetracosanol-1
115	35.006	5089894	4.56	Heneicosane
116	35.860	304603	0.27	2-methyloctacosane
117	36.415	5934798	5.32	Heneicosane
118	36.879	6300110	5.65	Bis(2-ethylhexyl) phthalate
119	36.982	484053	0.43	2-Methylhexacosane
120	37.434	404670	0.36	2-methyloctacosane
121	37.717	283966	0.25	Tetrapentacontane
122	38.010	164471	0.15	Cyclohexane, [6-cyclopentyl-3-(3-cyclopentylpropyl)hexyl]-
123	38.111	4667106	4.18	Heneicosane
124	39.187	341312	0.31	2-methyloctacosane
125	39.335	157805	0.14	2-methyloctacosane
126	39.702	4165769	3.73	Tetratetracontane
127	40.216	654765	0.59	Tetrapentacontane
128	40.367	86491	0.08	Tetratetracontane

129	40.430	373176	0.33	Hexatriacontane
130	40.547	222229	0.20	Tetratetracontane
131	40.676	145970	0.13	TETRACOSANE
132	40.796	363013	0.33	Decanedioic acid, bis(2-ethylhexyl) ester
133	40.854	2224607	1.99	Tetratetracontane
134	40.980	148092	0.13	Squalene
135	41.517	59104	0.05	Dodecanoic acid, 1,1-dimethylpropyl ester
136	41.595	168765	0.15	Tetratetracontane
137	41.728	224321	0.20	Tetratetracontane
138	41.983	89827	0.08	BUTANENITRILE, 4-HYDROXY-2-METHYLENE-
139	42.073	1777769	1.59	Tetracontane
140	42.741	275986	0.25	Tetrapentacontane
141	43.488	944913	0.85	Tetracontane
142	45.173	690787	0.62	Tetratetracontane
143	47.215	626624	0.56	Tetrapentacontane

Compounds Degraded

When the compounds reported in strain J1 treated soil was compared with the untreated soil as mentioned in the Table 3.3.1 of Chapter 3.3, and then it was found that some of the compounds has been degraded by the bacterial strain. Such compound are mentioned below

Table 3.4.5. Organic compounds degraded by the strain J1 in the treatment

No.	New compound reported	No.	New compound reported
1	NONANE, 2-METHYL	24	Sulfurous acid, hexyl pentyl ester
2	OCTANE, 2,4,6-TRIMETHYL	25	Sulfurous acid, 2-ethylhexyl pentyl ester
3	NONANE, 2,5-DIMETHYL	26	NONADECANE
4	Nonane, 2,5-dimethyl-	27	Nonane, 3,7-dimethyl-
5	1,7-Octanediol	28	3-HEXADECENE, (Z)-
6	Dodecane	29	Heptadecane, 2,6,10,15-tetramethyl
7	2,2'-BIFURAN, OCTAHYDRO-2,5-DIMETHYL-, [2.ALPHA	30	DECANE, 2,3,4-TRIMETHYL-
8	Undecane, 3,6-dimethyl-	31	METHANONE, (2-ETHENYL-3,4-

			DIHYDRO-2-METHYL
9	2-Bromo dodecane	32	2,2'-Isopropylidenebis(tetrahydrofuran
10	1-Pentadecene	33	1-DODECANOL
11	Decane, 3,8-dimethyl-	34	Cyclohexane, 1,1,3-trimethyl-
12	1-Heptadecene	35	NONADECANE
13	Decane, 1-iodo-	36	1-Hexadecanol
14	Cyclohexane, octyl-	37	8-Octadecanone
15	TRIACONTANOIC ACID, METHYL ESTER	38	CELIDONIOL, DEOXY
16	Dibutyl phthalate	39	n-Hexadecanoic acid
17	Undecane, 3-methylene	40	1-Nonadecene
18	1-Acetoxynonadecane	41	10-Nonadecanone
19	2-Methylhexacosane	42	Nonane, 5-methyl-5-propyl-
20	1-Octanol, 2-butyl-	43	Behenic alcohol
21	Tricosyl acetate	44	n-Octanoic acid, ethyldimethylsilyl ester
22	n-Tetracosanol-1	45	Bis(2-ethylhexyl) phthalate
23	2-Methylhexacosane	46	Cyclohexane, [6-cyclopentyl-3-(3-cyclopentylpropyl)hexyl]-

New compounds reported

The degradation of the compounds leads to the formation of the new compounds. Such new compounds reported depicted within **Table 3.4.6.**

Table 3.4.6. New compounds reported in the strain J1 in the treatment

No.	**New compound reported**	**No.**	**New compound reported**
1	NONANE, 2-METHYL	24	OCTANE, 2,4,6-TRIMETHYL
2	NONANE, 2,5-DIMETHYL	25	Nonane, 2,5-dimethyl-
3	1,7-Octanediol	26	Dodecane
4	2,2'-BIFURAN, OCTAHYDRO-2,5-DIMETHYL-, [2.ALPHA	27	Undecane, 3,6-dimethyl
5	Sulfurous acid, hexyl pentyl ester	28	Sulfurous acid, 2-ethylhexyl pentyl ester

6	NONADECANE	29	Nonane, 3,7-dimethyl-
7	3-HEXADECENE, (Z)-	30	Heptadecane, 2,6,10,15-tetramethyl-
8	DECANE, 2,3,4-TRIMETHYL-	31	METHANONE, (2-ETHENYL-3,4-DIHYDRO-2-METHYL
9	2-Bromo dodecane	32	2,2'-Isopropylidenebis(tetrahydrofuran)
10	1-DODECANOL	33	1-Pentadecene
11	Decane, 3,8-dimethyl-	34	Cyclohexane, 1,1,3-trimethyl-
12	1-Heptadecene	35	Decane, 1-iodo-
13	n-Hexadecanoic acid	36	1-Acetoxynonadecane
14	n-Octanoic acid, ethyldimethylsilyl ester	37	Nonane, 5-methyl-5-propyl-
15	Cyclohexane, octyl-	38	1-Hexadecanol
16	8-Octadecanone	39	TRIACONTANOIC ACID, METHYL ESTER
17	CELIDONIOL, DEOXY	40	Undecane, 3-methylene
18	Dibutyl phthalate	41	1-Nonadecene
19	10-Nonadecanone	42	2-Methylhexacosane
20	1-Octanol, 2-butyl-	43	Tricosyl acetate
21	Behenic alcohol	44	n-Tetracosanol-1
22	Bis(2-ethylhexyl) phthalate	45	2-Methylhexacosane
23	Cyclohexane, [6-cyclopentyl-3-(3-cyclopentylpropyl)hexyl]-	46	

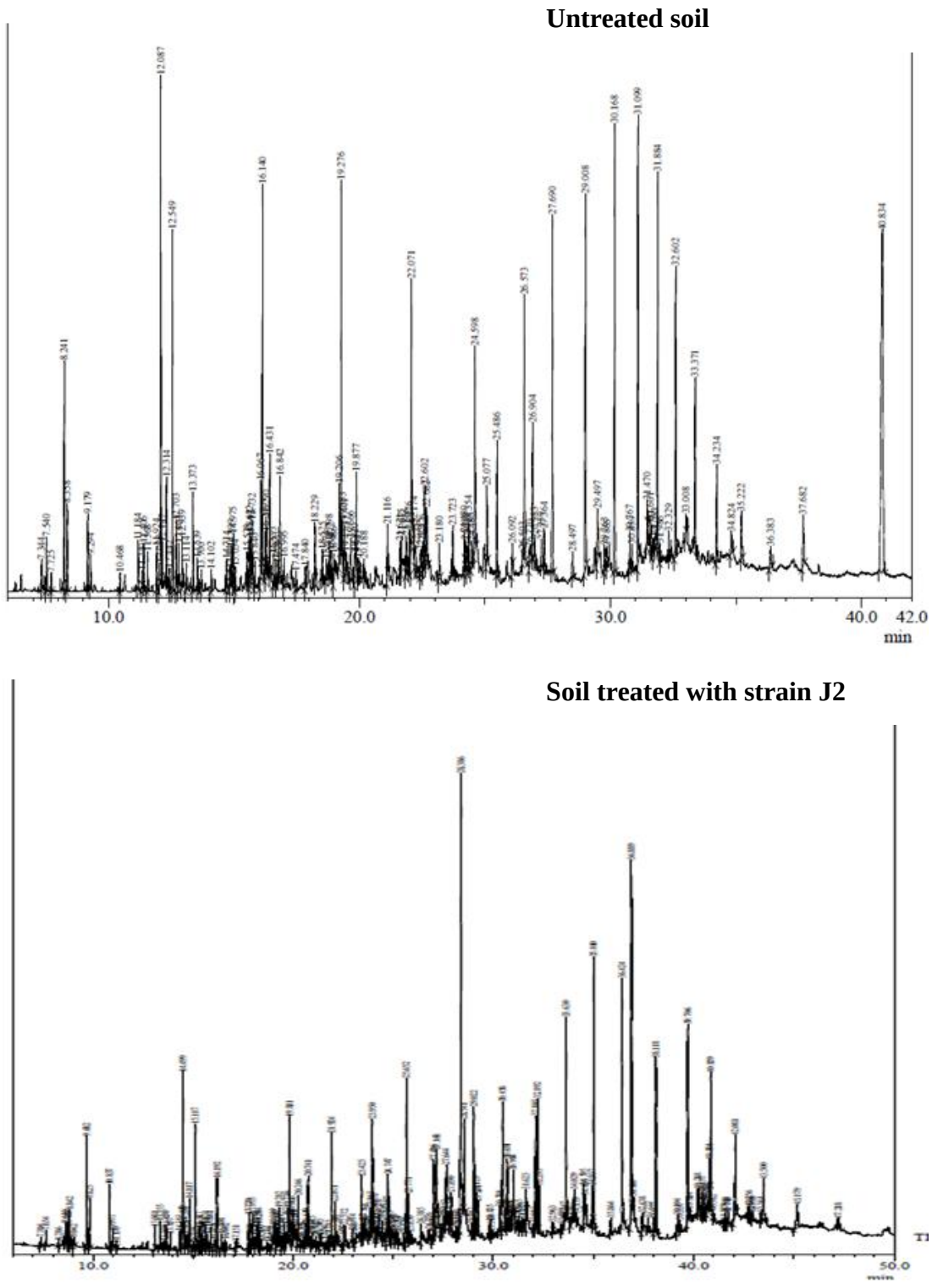


Figure 3.4.2c. GC-MS chromatogram of the soil treated with *Bacillus safensis* J2

Table 3.4.7. Organic compounds identified in the strain J2 treated soil

Peak#	R.Time	Area	Area%	Name
1	7.394	165614	0.10	NONANE, 2-METHYL-
2	7.654	257736	0.16	Octane, 2,4,6-trimethyl-
3	8.286	108705	0.07	TETRADECANE
4	8.610	361257	0.22	NONANE, 2,5-DIMETHYL-
5	8.748	429322	0.26	Nonane, 2,5-dimethyl-
6	8.842	702233	0.42	Octane, 3,3-dimethyl-
7	9.042	233731	0.14	(Z)-3,7-Dimethyl-2,7-octadien-1-ol, propanoate(ester)
8	9.682	2386011	1.44	Decane, 3,7-dimethyl-
9	9.825	993280	0.60	Decane, 3,7-dimethyl-
10	10.837	1302258	0.79	Decane, 3,7-dimethyl-
11	10.977	324492	0.20	Decane, 3,7-dimethyl-
12	11.170	76510	0.05	Octane, 2,3,6,7-tetramethyl-
13	13.081	395924	0.24	CYCLOOCTANE, (METHOXYMETHOXY)-
14	13.355	676012	0.41	Dodecane
15	13.566	100629	0.06	UNDECANE, 2,4-DIMETHYL-
16	13.650	361871	0.22	Undecane, 3,6-dimethyl-
17	13.857	341136	0.21	Dodecane, 4-methyl-
18	14.313	478457	0.29	Tridecane
19	14.450	171298	0.10	Nonane, 4,5-dimethyl-
20	14.499	3266215	1.97	Benzene, 1,3-bis(1,1-dimethylethyl)-
21	14.598	566959	0.34	Dodecane, 4,6-dimethyl-
22	14.764	173103	0.10	Heptane, 3,3,5-trimethyl-
23	14.817	853378	0.52	Tetradecane, 5-methyl-
24	14.974	114686	0.07	Sulfurous acid, 2-ethylhexyl isohexyl ester
25	15.117	2603039	1.57	Dodecane, 4,6-dimethyl-
26	15.317	377461	0.23	Nonane, 5-(2-methylpropyl)-
27	15.381	173395	0.10	DODECANE
28	15.452	304169	0.18	Dodecane, 4,6-dimethyl-
29	15.519	182818	0.11	Dodecane, 4,6-dimethyl-
30	15.624	349013	0.21	Dodecane, 4,6-dimethyl-
31	15.714	405489	0.24	Tridecane
32	15.861	425658	0.26	DECANE, 2,3,7-TRIMETHYL-
33	16.051	171850	0.10	Undecane, 3,8-dimethyl-
34	16.192	1520808	0.92	Hexadecane
35	16.408	398874	0.24	Nonane, 5-(2-methylpropyl)-
36	16.602	322394	0.19	Undecane, 3,8-dimethyl-
37	17.151	105006	0.06	Undecane, 3,8-dimethyl-
38	17.779	732495	0.44	3-HEXADECENE, (Z)-
39	17.868	118553	0.07	Tetradecane
40	17.955	722353	0.44	TETRADECANE
41	18.128	189778	0.11	1,2,4,5-TETRAETHYLCYCLOHEXANE

42	18.190	328687	0.20	PENTADECANE
43	18.284	524232	0.32	Heneicosane
44	18.400	153435	0.09	Tetradecane, 4-methyl-
45	18.714	108455	0.07	Decane, 2,3,5-trimethyl-
46	18.920	143545	0.09	4,7-DIOXODECANAL
47	19.019	338231	0.20	2-Methyltetracosane
48	19.142	491886	0.30	Hexadecane
49	19.237	118457	0.07	2,2'-Isopropylidenebis(tetrahydrofuran)
50	19.282	520976	0.31	Heptadecane
51	19.424	273276	0.17	Dodecane, 4,6-dimethyl-
52	19.565	462303	0.28	1-DODECANOL
53	19.720	655015	0.40	Hexadecane
54	19.811	3094689	1.87	Heptadecane
55	19.966	460407	0.28	Heptadecane
56	20.010	105480	0.06	Dodecane, 4,6-dimethyl-
57	20.073	286440	0.17	Dodecane
58	20.246	1409609	0.85	Phenol, 3,5-bis(1,1-dimethylethyl)-
59	20.376	180953	0.11	Dodecyl nonyl ether
60	20.521	143905	0.09	Dodecane, 4,6-dimethyl-
61	20.649	324906	0.20	Hexadecane
62	20.741	1832082	1.11	Heptadecane
63	20.879	245229	0.15	OCTADECANE
64	20.945	224572	0.14	Dodecane, 2,6,11-trimethyl-
65	21.142	203625	0.12	OCTADECANE
66	21.343	191425	0.12	TRICOSANE
67	21.576	107556	0.06	Undecane, 3,8-dimethyl-
68	21.783	157451	0.10	Cyclopropane, 1-methyl-1-(1-methylethyl)-2-nonyl-
69	21.924	2556573	1.54	1-Pentadecene
70	22.071	866504	0.52	PENTADECANE
71	22.203	521084	0.31	Nonyl tetradecyl ether
72	22.466	115062	0.07	Heneicosane, 11-(1-ethylpropyl)-
73	22.572	406919	0.25	Heneicosane
74	22.974	268328	0.16	Pentadecane, 2,6,10-trimethyl-
75	23.049	116422	0.07	2-Methyltetracosane
76	23.425	1375143	0.83	8-Pentadecanone
77	23.497	124304	0.08	HEXADECANE
78	23.568	430827	0.26	1-Decanol, 2-hexyl-
79	23.747	96091	0.06	Dodecane, 4,6-dimethyl-
80	23.807	43176	0.03	11-ETHYL-3-METHYL-5,9-DIOXA-7-PENTADECANOL
81	23.865	509464	0.31	Heptadecane
82	23.950	2965969	1.79	Eicosane
83	24.032	395108	0.24	Eicosane
84	24.153	533810	0.32	Heneicosane
85	24.213	69719	0.04	C33 BOTRYOCOCCANE (8)
86	24.278	441955	0.27	Ethanol, 2-(dodecyloxy)-
87	24.454	741537	0.45	2-Methylhexacosane

88	24.643	46058	0.03	2,4,6-TRIMETHYLBOROXIN #
89	24.697	242198	0.15	Hexadecane
90	24.747	1111411	0.67	Eicosane
91	24.845	258750	0.16	Eicosane
92	24.918	142747	0.09	5,5-Diethylheptadecane
93	25.120	644147	0.39	2-Methyltetracosane
94	25.251	341393	0.21	Eicosane
95	25.520	214874	0.13	Tridecane, 3-methylene-
96	25.652	3928927	2.37	1-Heptadecene
97	25.771	918226	0.55	Nonadecane
98	25.850	283360	0.17	1-Decanol, 2-hexyl-
99	26.385	371982	0.22	Eicosane
100	26.776	394060	0.24	1,2-BENZENEDICARBOXYLIC ACID, BIS(2-METHYLP
101	26.940	146804	0.09	Cyclohexane, octyl-
102	27.026	2108180	1.27	8-Octadecanone
103	27.168	2491367	1.50	1-Hexadecanol
104	27.385	349857	0.21	Eicosane
105	27.552	746093	0.45	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
106	27.644	1231924	0.74	Eicosane
107	27.734	233683	0.14	Hexadecane
108	27.890	1307647	0.79	TRIACONTANOIC ACID, METHYL ESTER
109	28.144	303517	0.18	Eicosane
110	28.253	202813	0.12	Eicosane
111	28.386	13575615	8.20	Dibutyl phthalate
112	28.541	2533582	1.53	n-Hexadecanoic acid
113	28.917	225179	0.14	Tridecane, 3-methylene-
114	29.022	2990565	1.81	1-Nonadecene
115	29.129	844842	0.51	Heneicosane
116	29.231	750297	0.45	1-Acetoxynonadecane
117	29.835	332749	0.20	Heneicosane
118	29.910	141725	0.09	Heneicosane
119	30.291	698081	0.42	10-Nonadecanone
120	30.456	4190022	2.53	1-OCTADECANOL
121	30.600	288799	0.17	Eicosane
122	30.691	1569624	0.95	Heneicosane
123	30.832	323440	0.20	Eicosane
124	30.891	140453	0.08	1-Decanol, 2-hexyl-
125	30.984	1068718	0.65	Tetratetracontane
126	31.162	173104	0.10	Eicosane
127	31.265	228536	0.14	Eicosyl octyl ether
128	31.473	312079	0.19	Eicosane
129	31.623	855622	0.52	Decane, 1-iodo-
130	32.013	163879	0.10	Tridecane, 3-methylene-
131	32.103	2428395	1.47	Behenic alcohol
132	32.192	2792642	1.69	Heneicosane

133	32.293	974373	0.59	Tricosyl acetate
134	32.963	178422	0.11	1,13-DIBROMOTRIDECANE
135	33.403	166001	0.10	CYCLOHEXANE, EICOSYL-
136	33.547	175115	0.11	1-METHYL-3,5-DIMETHOXY-1H-PYRAZOLE
137	33.630	5230512	3.16	Heneicosane
138	33.867	123478	0.07	HEXADECANE, 2,6,10,14-TETRAMETHYL-
139	34.029	942113	0.57	Eicosane
140	34.139	122548	0.07	2-Methylhexacosane
141	34.505	673818	0.41	2,3-naphthalenedicarboxaldehyde, 5,6,7,8-tetrahydro-5,5,8,8
142	34.603	608910	0.37	TETRACOSANE
143	34.937	681348	0.41	n-Tetracosanol-1
144	35.010	6094769	3.68	Heneicosane
145	35.864	377166	0.23	Eicosane
146	36.424	7343203	4.43	Heneicosane
147	36.889	10876195	6.57	Bis(2-ethylhexyl) phthalate
148	36.980	518138	0.31	1,54-DIBROMOTETRAPENTACONTANE
149	37.438	531085	0.32	Tetratetracontane
150	37.722	442525	0.27	Tetrapentacontane
151	38.027	268738	0.16	1-EICOSANOL
152	38.118	5756357	3.48	Heneicosane
153	39.194	480848	0.29	Tetratetracontane
154	39.337	228422	0.14	2-methyloctacosane
155	39.706	5120507	3.09	Tetratetracontane
156	39.814	328554	0.20	Pentatriacontane
157	40.218	1034830	0.62	Tetrapentacontane
158	40.369	110385	0.07	Tetratetracontane
159	40.437	473644	0.29	Hexatriacontane
160	40.493	45184	0.03	Cyclopropane, 1-methyl-1-(1-methylethyl)-2-nonyl-
161	40.553	248902	0.15	PENTACOSANE
162	40.684	227067	0.14	TETRACOSANE
163	40.801	586485	0.35	Decanedioic acid, bis(2-ethylhexyl) ester
164	40.859	2621416	1.58	Tetratetracontane
165	40.982	209810	0.13	Squalene
166	41.528	95286	0.06	Tetratetracontane
167	41.599	238153	0.14	Tetratetracontane
168	41.740	285260	0.17	Tetracontane
169	42.081	2652911	1.60	Tetracontane
170	42.756	311279	0.19	Tetrapentacontane
171	42.846	152668	0.09	Tetrapentacontane
172	42.938	216694	0.13	Hexatriacontane
173	43.361	149530	0.09	HEXADECANE, 2,6,10,14-TETRAMETHYL-

174	43.500	1514819	0.91	Tetracontane
175	45.179	1553861	0.94	Tetracontane
176	47.218	385844	0.23	Pentatriacontane

Compounds Degraded

When the compounds reported in strain J2 treated soil was compared with the untreated soil as mentioned in the Table 3.3.1 of Chapter 3.3, and then it was found that some of the compounds has been degraded by the bacterial strain. Such compound are mentioned below in **Table 3.4.8.**

Table 3.4.8. Organic compounds degraded by the strain J2 in the treatment

No.	**Compound Degraded**	No.	**Compound Degraded**
1	UNDECANE, 5,6-DIMETHYL-	9	Tetracosane, 1-iodo-
2	CYCLOHEXANE, 1-METHYL-4-(1-METHYLETGENYL)	10	(Z)-ISOBUTYL 7-(TRIMETHYLSILYL)-5-HEPTENOATE
3	3-OXO-1-CYCLOHEXENYL 2- METHYLPROPIONAT	11	3,5-Dimetylidodecane
4	3,5-DIMETHYLDODECANE	12	HEXANE, 2,2,3-TRIMETHYL-
5	Hexadecane, 1-iodo-	13	2,6,10,15,19,23-HEXAMETHYLTETRACOSANE PENTACOSANE
6	2,6,10-Trimethyltridecane	14	Tetratetracontane
7	DOCOSANE	15	5,11,17,23-tetratert-butylpentacyclo[19,3,1,1˜
8	2H- PYRAN, 3,6-DIHYDRO-6-METHOXY-2-METHYL-,T		

New compounds reported

The degradation of the compounds leads to the formation of the new compounds. Such new compounds reported depicted within

Table 3.4.9 New compounds reported in the strain J2 in the treatment

No.	**New compounds reported**	No.	**New compounds reported**
1	(Z)-3,7-Dimethyl-2,7-octadien-1-ol, propanoate(ester)	17	1,2-BENZENEDICARBOXYLIC ACID, BIS
2	CYCLOOOCTANE, (METHOXYMETHOXY)-	18	CYCLOHEXANE,OCTYL-
3	4,7-DIOXODECANAL	19	8-Octadecanone
4	2,2'-Isopropylidenebis(tetrahydrofuran	20	1-Hexadecanol
5	1-dodecanol	21	7,9-di.tert.butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
6	Cyclopropane, 10methyl-1-(1-methylethyl)-2-nonyl-	22	TRIACONTANOIC ACID, METHYL ESTER
7	1-Pentadecene	23	Dibutyl phthalate
8	Heneicosane, 11-(1-ethylpropyl)-	24	n-Hexadecanoic acid
9	1-decanol, 2-hexyl-	25	1-Nonadecene
10	11-ETHYL-3-METHYL-5,9-DIOXA-7-PENTADECANOL	26	1-Acetoxynonadecane
11	C33 BOTRYOCOCCANE (8)	27	10-Nonadecanone
12	Ethanol, 2-(dodecyloxy)-	28	1-octadecanol
13	2,4,4- trimethylboroxin	29	1-Decanol, 2-hexyl-
14	Tridecane, 3-methylene-	30	Eicosyl octyl ether
15	1-Decanol, 2-hexyl-	31	Tridecane, 3-methylene-
16	Behenic alcohol		

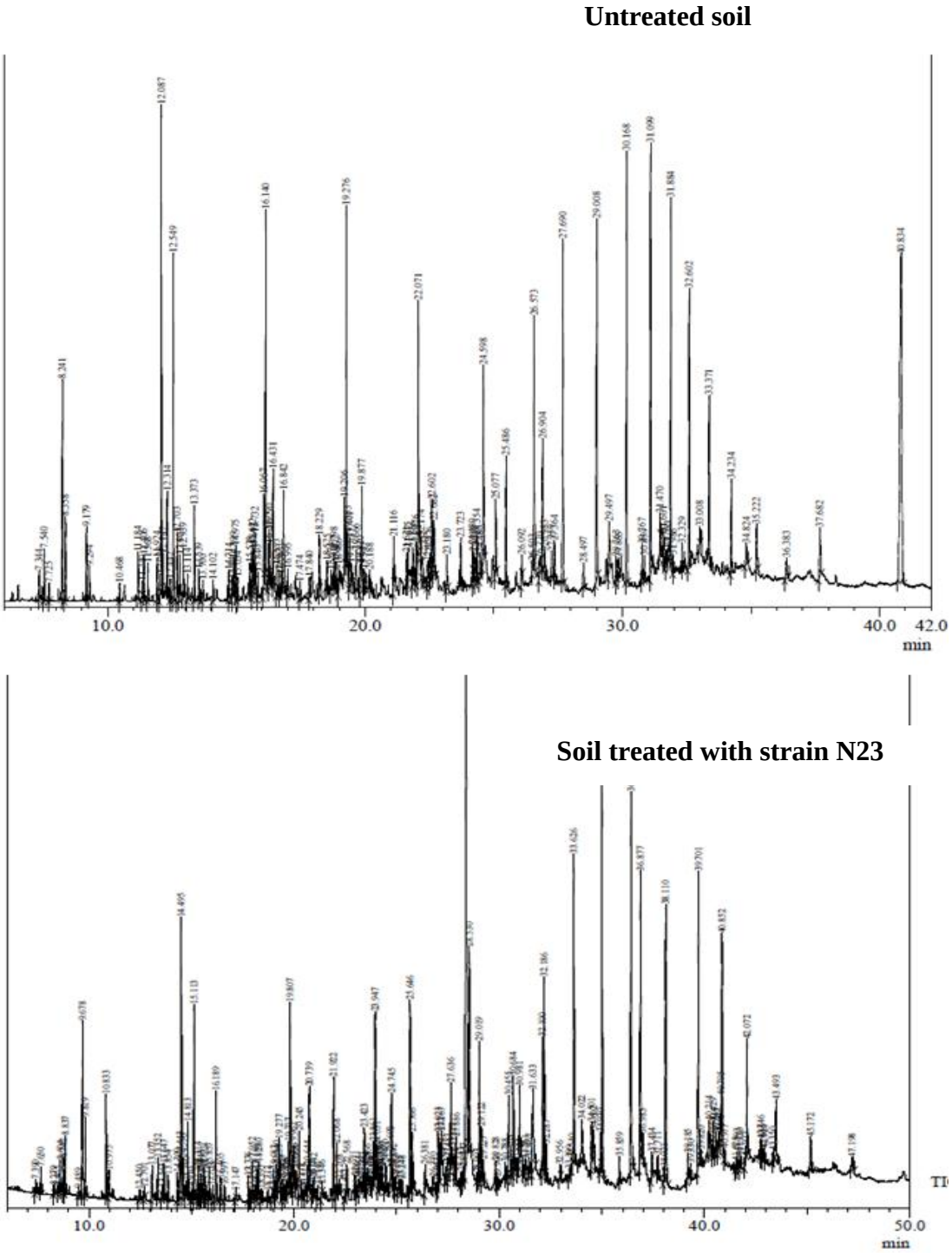


Figure 3.4.2d. GC-MS chromatogram of the soil treated with *Pseudomonas azotoformans* N23

GC-MS analysis of the soil treated with *Pseudomonasazotoformans* N23

The GC-MS results of the treated soil with strain N23 is depicted within the Table 3.4.10

Table 3.4.10. Organic compounds identified in the strain N23 treated soil

Peak#	R.Time	Area	Area%	Name
1	7.390	178021	0.12	NONANE, 2-METHYL-
2	7.650	268287	0.18	Octane, 2,4,6-trimethyl-
3	8.279	119486	0.08	UNDECANE
4	8.455	92486	0.06	HEPTANE, 3,3,6-TRIMETHYL-
5	8.604	366885	0.25	NONANE, 2,5-DIMETHYL-
6	8.742	442616	0.30	Nonane, 2,5-dimethyl-
7	8.837	735641	0.50	Octane, 3,3-dimethyl-
8	9.489	92082	0.06	Nonane, 5-(2-methylpropyl)-
9	9.678	2473341	1.68	Decane, 3,7-dimethyl-
10	9.819	1040280	0.71	Decane, 3,7-dimethyl-
11	10.833	1356109	0.92	Decane, 3,7-dimethyl-
12	10.975	344559	0.23	Decane, 3,7-dimethyl-
13	12.450	140085	0.10	Nonane, 5-(2-methylpropyl)-
14	12.701	189123	0.13	Undecane, 2,6-dimethyl-
15	13.077	406255	0.28	2-Hexadecanol
16	13.352	666676	0.45	Dodecane
17	13.563	113771	0.08	UNDECANE, 2,4-DIMETHYL-
18	13.647	386055	0.26	Undecane, 2,6-dimethyl-
19	13.854	357185	0.24	Dodecane, 4-methyl-
20	14.309	484418	0.33	Tetradecane
21	14.443	209077	0.14	TETRADECANE
22	14.495	3464366	2.35	Benzene, 1,3-bis(1,1-dimethylethyl)-
23	14.596	589495	0.40	Dodecane, 4,6-dimethyl-
24	14.760	171021	0.12	Sulfurous acid, 2-ethylhexyl pentyl ester
25	14.813	875065	0.59	Tetradecane, 5-methyl-
26	14.970	124808	0.08	Sulfurous acid, 2-ethylhexyl isohexyl ester
27	15.113	2714500	1.84	Dodecane, 4,6-dimethyl-
28	15.314	394456	0.27	Nonane, 5-butyl-
29	15.375	180594	0.12	Tetradecane
30	15.448	312226	0.21	Nonane, 5-(2-methylpropyl)-
31	15.515	180490	0.12	Dodecane, 4,6-dimethyl-
32	15.619	362734	0.25	Dodecane, 4,6-dimethyl-
33	15.710	302068	0.21	Hexadecane
34	15.857	442261	0.30	DECANE, 2,3,7-TRIMETHYL-
35	16.048	168925	0.11	Undecane, 3,8-dimethyl-
36	16.189	1559413	1.06	Hexadecane

37	16.405	417394	0.28	Nonane, 5-(2-methylpropyl)-
38	16.597	317000	0.22	Undecane, 3,8-dimethyl-
39	17.147	116939	0.08	Undecane, 3,8-dimethyl-
40	17.776	360606	0.24	3-HEXADECENE, (Z)-
41	17.865	124096	0.08	Sulfurous acid, 2-ethylhexyl hexyl ester
42	17.952	580871	0.39	PENTADECANE
43	18.117	140573	0.10	Undecane, 5-methyl-
44	18.186	350687	0.24	DECANE, 3,8-DIMETHYL-
45	18.280	524326	0.36	Heneicosane
46	18.397	161522	0.11	Tetradecane, 4-methyl-
47	18.712	112543	0.08	Decane, 2,3,5-trimethyl-
48	18.917	151582	0.10	CYCLOHEXANOL, 1,2-DIMETHYL-, CIS-
49	19.017	271791	0.18	Nonadecane
50	19.140	516013	0.35	Hexadecane
51	19.233	101002	0.07	4-Imidazolidinone, 2,2-dimethyl-3-[(1-methylethylidene)ami
52	19.277	508140	0.35	Heptadecane
53	19.423	281205	0.19	Dodecane, 4,6-dimethyl-
54	19.566	166610	0.11	Decyl octyl ether
55	19.626	124504	0.08	Dodecane, 2,6,11-trimethyl-
56	19.717	637450	0.43	Hexadecane
57	19.807	3170516	2.15	Heptadecane
58	19.962	458321	0.31	Pentadecane
59	20.006	92102	0.06	Nonane, 5-butyl-
60	20.068	230825	0.16	Tridecane
61	20.245	1263203	0.86	Phenol, 3,5-bis(1,1-dimethylethyl)-
62	20.375	140504	0.10	2-Bromotetradecane
63	20.515	112269	0.08	Dodecane, 4,6-dimethyl-
64	20.644	317602	0.22	Hexadecane
65	20.739	1815584	1.23	Hexadecane
66	20.875	214340	0.15	OCTADECANE
67	20.942	229388	0.16	Dodecane, 2,6,11-trimethyl-
68	21.141	173622	0.12	2,6,10-Trimethyltridecane
69	21.346	157756	0.11	NONADECANE
70	21.922	1815841	1.23	1-Pentadecene
71	22.068	742838	0.50	PENTADECANE
72	22.208	275618	0.19	Nonadecane
73	22.453	43029	0.03	CYCLOBUTANONE, 2,2,3,4-TETRAMETHYL-, TRANS-
74	22.568	397741	0.27	Heneicosane
75	22.971	242821	0.16	Decane, 3,8-dimethyl-
76	23.048	89829	0.06	2-Methyltetracosane
77	23.163	180287	0.12	TRIDECANE
78	23.322	152123	0.10	Heneicosane
79	23.423	911929	0.62	EICOSANE
80	23.494	156125	0.11	HEXADECANE

81	23.566	299432	0.20	Nonyl tetradecyl ether
82	23.747	121669	0.08	Dodecane, 4,6-dimethyl-
83	23.807	43753	0.03	11-ETHYL-3-METHYL-5,9-DIOXA-7-PENTADECANOL
84	23.861	507912	0.34	Heptadecane
85	23.947	3045198	2.07	Eicosane
86	24.031	373354	0.25	Eicosane
87	24.108	146954	0.10	Nonane, 5-methyl-5-propyl-
88	24.150	124841	0.08	Heneicosane
89	24.203	104494	0.07	Decane, 1-iodo-
90	24.280	261527	0.18	Eicosane
91	24.460	685264	0.47	2-Methylhexacosane
92	24.692	236360	0.16	Heptadecane
93	24.745	1063549	0.72	Eicosane
94	24.842	280549	0.19	OCTADECANE
95	24.916	134830	0.09	5,5-Diethylheptadecane
96	25.155	531704	0.36	Octadecane, 1-chloro-
97	25.248	302500	0.21	OCTADECANE
98	25.646	3166885	2.15	1-Heptadecene
99	25.766	794799	0.54	Nonadecane
100	26.381	370418	0.25	Heneicosane
101	26.770	360837	0.25	DIISOBUTYL BENZENE-1,2-DICARBOXYLATE
102	26.942	127551	0.09	n-Pentadecylcyclohexane
103	27.023	1111841	0.75	8-Octadecanone
104	27.122	245942	0.17	Decane, 1-iodo-
105	27.167	341155	0.23	1-Tetradecanol
106	27.377	333673	0.23	Eicosane
107	27.487	631468	0.43	Heptadecane
108	27.636	1238212	0.84	Eicosane
109	27.729	277641	0.19	Eicosane
110	27.772	97938	0.07	Hexadecane, 1-iodo-
111	27.886	984026	0.67	Tridecanoic acid, 4,8,12-trimethyl-, methyl ester
112	28.147	315731	0.21	Pentadecane, 8-hexyl-
113	28.247	222294	0.15	Eicosane
114	28.380	11409686	7.75	Dibutyl phthalate
115	28.530	3754121	2.55	n-Hexadecanoic acid
116	28.903	134366	0.09	1-Decen-3-one
117	29.019	2215106	1.50	1-Nonadecene
118	29.122	776187	0.53	Heneicosane
119	29.227	277623	0.19	1-Acetoxynonadecane
120	29.828	332889	0.23	Heneicosane
121	29.906	138829	0.09	2-methyloctacosane
122	30.288	281646	0.19	10-Nonadecanone
123	30.455	2203478	1.50	Heptafluorobutyric acid, hexadecyl ester
124	30.594	289305	0.20	Eicosane

125	30.684	1552304	1.05	Heneicosane
126	30.888	105703	0.07	2-Methylhexacosane
127	30.981	1006630	0.68	Eicosane
128	31.158	188713	0.13	HEXADECANE, 2,6,10,14-TETRAMETHYL-
129	31.261	205940	0.14	1-Octanol, 2-butyl-
130	31.467	325541	0.22	Eicosane
131	31.633	2613848	1.77	OCTADECANOIC ACID

Compounds Degraded

When the strain N23 treated soil was compared with the untreated soil as mentioned in the Table 3.3.1 of Chapter 3.3, and then it was found that some of the compounds has been degraded by the bacterial strain. Such compound are mentioned below in **Table 3.4.11**

Table 3.4.11. Organic compounds degraded by the strain N23 in the treatment

No.	Compound degraded	No.	Compound degrade
1	UNDECANE, 5,6-DIMETHYL-	15	3,5-Dimetylidodecane
2	CYCLOHEXANE, 1-METHYL-4-(1-METHYLETGENYL)	16	HEXANE, 2,2,3-TRIMETHYL-
3	3-OXO-1-CYCLOHEXENYL 2- METHYLPROPIONAT	17	2,6,10,15,19,23-HEXAMETHYLTETRACOSANE
4	3,5-DIMETHYLDODECANE	18	Squalene
5	Pentadecane, 2,6,10-trimethyl-	19	BETA-D-MANNO-D-GLYCERO-ALPHA-D-GALACTO
6	Dodecyl nonyl ether	20	Tetratriacontane
7	Octane, 4,5-diethyl-	21	Phenol,2,4-bis(1,1-dimethylethyl)-, phosphate (3:1
8	Sulfurous acid, hexyl octyl ester	22	5,11,17,23-tetratert-butylpentacyclo[19,3,1,1˜
9	Dodecane, 2-methyl-	23	
10	Docosane	24	
11	2H- PYRAN, 3,6-DIHYDRO-6-METHOXY-2-METHYL-,T		
12	PENTACOSANE		
13	TETRACOSANE, 1-IODO-		
14	(Z)-ISOBUTYL 7-(TRIMETHYLSILYL)-5-HEPTENOATE		

New compounds reported

When the compounds are degraded the new compounds were formed. Such new compounds reported depicted within **Table 3.4.12**

Table 3.4.12. New compounds reported in the strain N23 in the treatment

No.	New compound reported	No.	New compound reported
1	Tridecanoic acid, 4,8,12-trimethyl-, methyl ester	24	2-Bromotetradecane
2	NONANE, 2-METHYL-	25	Dodecane,2,6,11-trimethyl-
3	Octane,2,4,6-trimethyl-	26	1-pentadecane
4	UNDECANE	27	Nonadecane
5	HEPTANE,3,3,6-TRIMETHYL-	28	CYCLOBUTANONE,2,2,3,4-TETRAMETHYL-, TRANS-
6	NONANE,2,5-DIMETHYL-	29	DECANE,3,8-DIMETHYL-
7	2-Hexadecanol	30	2-Methyltetracosane
8	Dodecane	31	11-ETHYL-3-METHYL-5,9-DIOXA-7-PENTADEC
9	Sulfurous acid, 2-ethylexyl hexyl pentyl ester	32	Nonane, 5-methyl-5-propyl-
10	Nonane, 5-butyl-	33	Decane, 1-iodo-
11	Sulfurous acid, 2-ethylexyl hexyl hexyl ester	34	2-Methylhexacosane
12	Undecane,5-methyl-	35	5,5- Diethylheptadecane
13	DECANE,3,8-DIMETHYL-	36	Octadecane, 1 –chloro-
14	CYCLOHEXANOL,1,2-DIMETHYL-CIS-	37	1 -Heptadecene
15	Nonadecane	38	DIISOBUTYL BENZENE-1,2-DICARBOXYLATE
16	4-Imidazolidinone,2,2-dimethyl-3-[(1-methylethylidene)ami	39	N-Pentadecylcyclohexane
17	Decyl octyl ether	40	8-Octadecanone
18	Dodecane,2,6,11-trimethyl-	41	Decane, 1-iodo-
9	Nonane,5-butyl-	42	1- Tetradecanol
10	Pentaecane, 8-hexyl-	44	1-DECEN-3-ONE
11	Dibutyl phthalate	45	1-Nonadecene
12	n-Hexadecanoic acid	46	1-Acetoxynonadecane
13	10-Nonadecanone	47	Heptaflurobutyric acid, hexadecyl ester
14	2-Methylhexacosane	48	HEXADECANE, 2,6,10,14-TETRAMETHYL-
15	1-Octanol, 2-butyl-	49	Behenic alcohol
16	Tricosyl acetate	50	Octane, 1,1’-sulfonylbis-
17	Eicosane, 2-cyclohexyl-	51	XYLOPYRANOSIDE, METHYL,3-ACETATE 2,4-DIMET
18	2,3-naphthalenedicarboxaldehyde, 5,6,7,8-tetrahydro-5,5,8,8	52	n-Tetracosanol-1
19	Bis (2-ethylhexyl) phthalate	53	Cyclohexane, [6-cyclopentyl-3-(3-cyclopentylpropyl) hexyl]-
20	Hexacosane, 1-iodo-	54	Decanedioic acid, bis(2-ethylhexyl)ester
21	Decanedioic acid, bis (2-ethylhexyl)ester	55	2,6,10-DODECATRIEN-1-OL, 3,7,11-TRIMETHYL-
22	2,6,10-DODECATRIEN-1-OL,3,711-TRIMETHYL-	56	6,7-Dimethyl-9-oxo-1,5-diazabicyclo[5,2,0]non-5-ene
23	HEXADECANE, 2,6,10,14-TETRAMETHYL-	57	

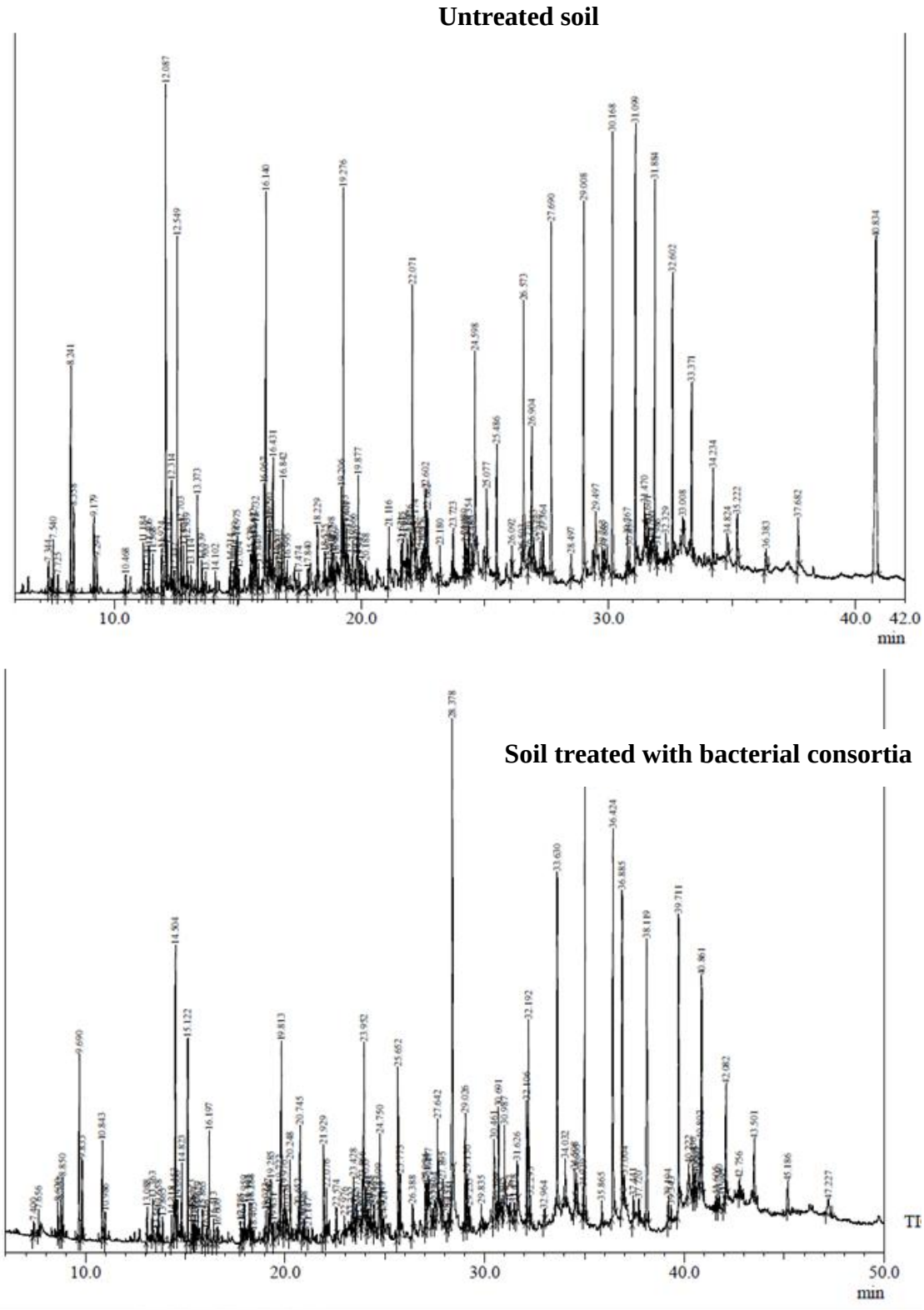


Figure 3.4.2b. GC-MS chromatogram of the soil treated with consortia

GC-MS analysis of the soil treated with bacterial consortia

The GC-MS results of the treated soil with strain N23 is depicted within the Table.3.4.13.

Table 3.4.13 Organic compound identified in the bacterial consortia treated soil

Peak#	R.Time	Area	Area%	Name
1	7.400	133445	0.14	NONANE, 2-METHYL-
2	7.656	199321	0.21	OCTANE, 2,4,6-TRIMETHYL-
3	8.620	273898	0.29	NONANE, 2,5-DIMETHYL-
4	8.757	334279	0.36	Nonane, 2,5-dimethyl-
5	8.850	532277	0.57	Octane, 3,3-dimethyl-
6	9.690	1845318	1.97	Decane, 3,7-dimethyl-
7	9.833	765343	0.82	Decane, 3,7-dimethyl-
8	10.843	957347	1.02	Decane, 3,7-dimethyl-
9	10.986	255229	0.27	Decane, 3,7-dimethyl-
10	13.088	297297	0.32	1,7-Octanediol
11	13.363	450662	0.48	Dodecane
12	13.567	53146	0.06	5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran
13	13.658	215610	0.23	Undecane, 3,6-dimethyl-
14	13.865	236818	0.25	Dodecane, 4-methyl-
15	14.318	344887	0.37	Tetradecane
16	14.453	144988	0.16	Sulfurous acid, hexyl pentyl ester
17	14.504	2576074	2.76	Benzene, 1,3-bis(1,1-dimethylethyl)-
18	14.606	407819	0.44	Dodecane, 4,6-dimethyl-
19	14.823	621623	0.66	NONADECANE
20	15.122	1971793	2.11	Dodecane, 4,6-dimethyl-
21	15.323	283529	0.30	Nonane, 5-(2-methylpropyl)-
22	15.386	126121	0.13	Undecane, 3,7-dimethyl-
23	15.457	225650	0.24	Dodecane, 4,6-dimethyl-
24	15.522	120605	0.13	Dodecane, 4,6-dimethyl-
25	15.629	256901	0.27	Nonane, 5-(2-methylpropyl)-
26	15.865	298746	0.32	DECANE, 2,3,7-TRIMETHYL-
27	16.056	107435	0.11	Undecane, 3,8-dimethyl-
28	16.197	1114197	1.19	Dodecane, 4,6-dimethyl-
29	16.413	292864	0.31	Undecane, 3,8-dimethyl-
30	16.606	219095	0.23	Undecane, 3,8-dimethyl-
31	17.785	214799	0.23	3-HEXADECENE, (Z)-
32	17.863	74472	0.08	Sulfurous acid, 2-ethylhexyl isohexyl ester
33	17.959	370551	0.40	PENTADECANE
34	18.192	303688	0.32	Heptadecane, 2,6,10,15-tetramethyl-
35	18.288	353371	0.38	Heptadecane, 2,6,10,15-

				tetramethyl-
36	18.405	107429	0.11	DECANE, 2,3,4-TRIMETHYL-
37	19.023	207351	0.22	2-Bromo dodecane
38	19.147	359104	0.38	Decane, 3,8-dimethyl-
39	19.240	74597	0.08	2,2'-Isopropylidenebis(tetrahydrofuran)
40	19.285	365371	0.39	Heptadecane
41	19.431	194594	0.21	Dodecane, 4,6-dimethyl-
42	19.722	464968	0.50	Hexadecane
43	19.813	2324710	2.49	Heptadecane
44	19.970	315872	0.34	Pentadecane
45	20.076	354307	0.38	PENTADECANE
46	20.248	1002224	1.07	Phenol, 3,5-bis(1,1-dimethylethyl)-
47	20.652	192251	0.21	Hexadecane
48	20.745	1319157	1.41	Heptadecane
49	20.863	142773	0.15	Oxalic acid, 6-ethyloct-3-yl heptyl ester
50	20.948	151290	0.16	Hexadecane
51	21.147	120427	0.13	Dodecane, 2,6,11-trimethyl-
52	21.929	999314	1.07	9-Eicosene, (E)-
53	22.076	494979	0.53	PENTADECANE
54	22.574	295085	0.32	Heneicosane
55	22.976	169879	0.18	Decane, 3,8-dimethyl-
56	23.322	112514	0.12	Eicosane, 1-iodo-
57	23.428	581056	0.62	Heneicosane
58	23.502	100341	0.11	HEXADECANE
59	23.573	279742	0.30	Nonyl tetradecyl ether
60	23.807	34163	0.04	R-(-)-2-OCTYL-N-OCTYL CARBONATE
61	23.866	352155	0.38	OCTADECANE
62	23.952	2214818	2.37	Eicosane
63	24.035	245775	0.26	Eicosane
64	24.154	412721	0.44	Heneicosane
65	24.210	62342	0.07	Decane, 1-iodo-
66	24.285	228255	0.24	Eicosane
67	24.465	459448	0.49	Eicosane
68	24.699	176123	0.19	OCTADECANE
69	24.750	818730	0.88	Eicosane
70	24.849	202516	0.22	Eicosane
71	24.921	104019	0.11	Eicosane
72	25.652	1882853	2.01	1-Heptadecene
73	25.773	555378	0.59	Heneicosane
74	26.388	287727	0.31	Heneicosane
75	27.034	715731	0.77	8-Octadecanone
76	27.128	223345	0.24	Decane, 1-iodo-
77	27.177	276337	0.30	1-Hexadecanol
78	27.387	254780	0.27	Eicosane
79	27.494	450982	0.48	Heptadecane

80	27.642	984625	1.05	Eicosane
81	27.847	45670	0.05	Decane, 2,8,8-trimethyl-
82	27.895	284560	0.30	Tridecanoic acid, 4,8,12-trimethyl-, methyl ester
83	28.137	196786	0.21	2-Methylhexacosane
84	28.251	148185	0.16	Eicosane
85	28.378	6182717	6.61	Dibutyl phthalate
86	29.026	1337675	1.43	1-Heneicosanol
87	29.130	567078	0.61	Heneicosane
88	29.235	178967	0.19	Acetic acid n-octadecyl ester
89	29.835	251234	0.27	Heneicosane
90	30.461	1563684	1.67	Heptafluorobutyric acid, hexadecyl ester
91	30.599	220970	0.24	2-methyloctacosane
92	30.691	1167607	1.25	Heneicosane
93	30.895	80232	0.09	2-Methylhexacosane
94	30.987	857290	0.92	Eicosane
95	31.267	131164	0.14	Dodecyl nonyl ether
96	31.478	208683	0.22	Eicosane
97	31.626	636254	0.68	Eicosane
98	32.106	1199892	1.28	Behenic alcohol
99	32.192	2048852	2.19	Heneicosane
100	32.295	269307	0.29	Hexacosyl acetate
101	32.964	131449	0.14	Octane, 1,1'-sulfonylbis-
102	33.630	3679444	3.94	Heneicosane
103	34.032	597355	0.64	Eicosane
104	34.508	350335	0.37	1-Triisopropylsilyloxypentane
105	34.605	529292	0.57	Tetratetracontane
106	34.939	227225	0.24	Behenic alcohol
107	35.011	4806374	5.14	Heneicosane
108	35.865	267978	0.29	2-methyloctacosane
109	36.424	5461478	5.84	Heneicosane
110	36.885	4369504	4.67	1,2-BENZENEDICARBOXYLIC ACID
111	37.004	461729	0.49	2-Methylhexacosane
112	37.441	341080	0.36	2-methyloctacosane
113	37.720	246669	0.26	TETRACOSANE
114	38.119	4352754	4.66	Heneicosane
115	39.194	302227	0.32	2-methyloctacosane
116	39.343	152985	0.16	2-methyloctacosane
117	39.711	3593858	3.84	Tetratetracontane
118	40.222	454932	0.49	Tetrapentacontane
119	40.436	321674	0.34	Tetratetracontane
120	40.555	186072	0.20	Heptadecane, 3-methyl-
121	40.680	210411	0.23	Hexadecane, 2-methyl-
122	40.802	276348	0.30	Decanedioic acid, bis(2-ethylhexyl) ester
123	40.861	2023238	2.16	Tetratetracontane

124	41.605	162075	0.17	2-Methylhexacosane
125	41.740	179728	0.19	2-methyloctacosane
126	41.987	95655	0.10	Cyclopentane, 1,1'-(1,4-butandiyl)bis-
127	42.082	1448632	1.55	Tetracontane
128	42.756	211994	0.23	Tetrapentacontane
129	43.501	822939	0.88	Tetracontane
130	45.186	485480	0.52	Tetratetracontane
131	47.227	514752	0.55	Pentatriacontane

Compounds Degraded

When the bacterial consortia treated soil was compared with the untreated soil as mentioned in the Table 3.3.1 of Chapter 3.3, and then it was found that some of the compound has been degraded by the bacterial consortia. Such compound are mentioned below in **Table 3.4.14**

Table 3.4.14. Organic compound degraded by the bacterial consortia in the treatment

No.	Compound degraded	No.	Compound degraded
1	UNDECANE, 5,6-DIMETHYL-	17	Tetradecane, 4-methyl-
2	CYCLOHEXANE, 1-METHYL-4-(1-METHYLETGENYL)	18	Pentadecane, 2,6,10-trimethyl-
3	TRIDECANE	19	2,6,10-Trimethyltridecane
4	UNDECANE,2,4-DIMETHYL-	20	OCTADECANE
5	Tetradecane, 5-methyl-	21	Octane,4,5-diethyl-
6	3-OXO-1-CYCLOHEXENYL 2-METHYLPROPIONAT	22	Pentadecane, 2,6,10-trimethyl-
7	3,5-Dimetylidodecane	23	Sulfurous acid, hexyl octyl ester
8	Hexadecane, 1-iodo-	24	Dodecane, 2-methyl-
9	BETA- D-MANNO-D-GLYCERO-ALPHA-D-GALACTO	25	HEXATRICONTANE
10	DODECANE,	26	2H- PYRAN, 3,6-DIHYDRO-6-METHOXY-2-METHYL-,T
11	Hexadecane,	27	OCTADECANOIC ACID
12	PENTACOSANE	28	Tetracosane, 1-iodo-
13	(Z)-ISOBUTYL 7-(TRIMETHYLSILYL)-5-HEPTENOATE	29	Hexane,2,2,3-trimethyl-
14	2,6,10,15,19,23-HEXAMETHYLTETRACOSANE	30	Squalene
15	PENTACOSANE	31	Tetratriacontane
16	Phenol,2,4-bis(1,1-dimethylethyl)-, phosphate	32	5,11,17,23-tetratert-butylpentacyclo[19,3,1,1˜

New compounds reported

When the compounds are degraded the new metabolic compounds were formed. Such new compounds reported depicted within **Table 3.4.15**

Table 3.4.15. New compounds reported in the bacterial consortia treatment

No.	New compound reported	No.	New compound reported
1	NONANE, 2-METHYL-	23	Oxalic acid, 6-ethyloct-3-yl heptyl ester
2	OCTANE, 2,4,6-TRIMETHYL-	24	Dodecane, 2,6,11-trimethyl-
3	NONANE, 2,5-DIMETHYL-	25	9-Eicosene,(E)-
4	1,7-Octanediol	26	Eicosane, 1-iodo-
5	Dodecane	27	R-(-)-2-OCTYL-N-OCTYL CARBONATE
6	5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran	28	Decane, 1-iodo-
7	Undecane, 3,6-dimethyl-	29	1-HEPTADECENE
8	Sulfurous acid, hexyl pentyl ester	30	8-Octadecanone
9	NONADECANE	31	Decane, 2,8,8-trimethyl
10	Undecane, 3,7-dimethyl-	32	Tridecanonic acid, 4,8,12-trimethyl-, methyl ester
11	Undecane, 3,8-dimethyl-	33	2-Methylhexacosane
12	3-HEXADECENE, (Z)-	34	Dibutyl phthalate
13	Heptadecane, 2,6,10,15-tetramethyl-	35	1- Heneicosanol
14	DECANE 2,3,4-TRIMETHYL-	36	Acetic acid n- octadecyl ester
15	2-Bromo dodecane	37	Heptafluorobutyric acid, hexadecyl ester
16	2,2'-Isopropylidenebis(tetrahydrofuran)	38	2-Methylhexacosane
17	Behenic alcohol	39	Hexacosyl acetate
18	Octane, 1,1'-sulfonylbis-	40	1-Triisopropylsilyoxypentane
19	1,2-BENZNENEDICARBOXYLIC ACID	41	2-Methylhexacosane
20	Heptadecane, 3-methyl-	42	Hexadecane,2-methyl
21	Decanedioic acid, bis(2-ethylhexyl) ester	43	2-Methylhexacosane
22	Cyclopentane, 1,1'-(1,4-butandiyl)bis-	44	Pentatriacontane

In GC-MS comparative study of the treated soil with non treated soil indicates the variation of some compounds in all the bacterial treatments as mentioned in the Tables.

- Table 3.4.1 demonstrates the compounds identified in the soil treated with the strain AJ15, Table 3.4.2 shows the compound degraded, whereas Table 3.4.3 demonstrates the new compound reported after the degradation.

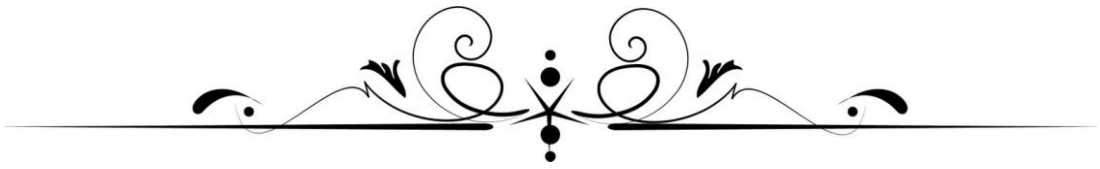
- Table 3.4.4 demonstrates the compounds identified in the soil treated with the strain J1, Table 3.4.5 shows the compound degraded , whereas Table 3.4.6 demonstrates the new compound reported after the degradation.
- Table 3.4.7 demonstrates the compounds identified in the soil treated with the strain J2 , Table 3.4.8 shows the compound degraded, whereas Table 3.4.9 demonstrates the new compound reported after the degradation.
- Table 3.4.10 demonstrates the compounds identified in the soil treated with the strain N23, Table 3.4.11 shows the compound degraded, whereas Table 3.4.12 demonstrates the new compound reported after the degradation.
- Table 3.4.13 demonstrates the compounds identified in the soil treated with bacterial consortia treated soil, Table 3.4.14 shows the compound degraded, whereas Table 3.4.15 demonstrates the new compound reported after the degradation.

Thus, it can be predicted that the bacterial strain selected in the present study has the ability to degrade organic compounds of petroleum contaminated soil. The study also focuses that the bacterial strain partially degrades the compounds in 90 days. But, for complete degradation of the compound the degradation time period needs to increase as most of the petroleum hydrocarbon are recalcitrant in nature.

3.4.4 Conclusion

The present study signifies that the microbes isolated from the petroleum hydrocarbons affected sites, which are more adapted to peculiar soil environment

and has the ability to degrade the contaminants of petroleum contaminated soil. Further, this microbes can be commercially and effectively used for bioremediation of hydrocarbons contaminated soils.



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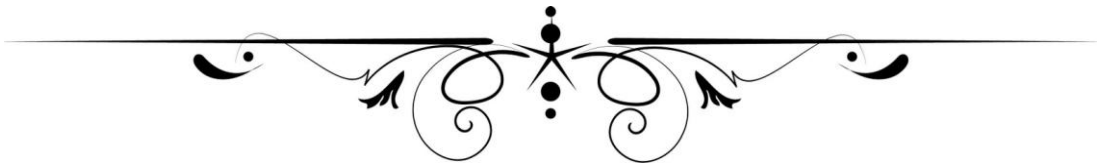


Chapter 3.5 Comparison of soil detoxification process in situ under pot culture on medicinal plant and its active ingredient using antioxidant activity and stress response on metabolites using metabolomics

Contents:

3.5.1 Introduction

Like other organisms, plants grow within a definite range of environmental conditions ideal for their growth and development. They often respond and adapt to the conditions that differ from the optimal, such as temperature, oxidative stress, dehydration, high salinity, organic contaminate and heavy metals and nutrient deficiency; these deviations are often responsible leads to abiotic stress in plants affecting plant growth and productivity.(Genga et al. 2011). Metabolomics is an important technology for determining the variation in functional genomics and systems biology. It plays a major role in functional annotation of understanding biotic and abiotic stress responses. Metabolomic studies in plant responses to biotic and abiotic stress are a key research topic in different laboratories worldwide. Through metabolomics study we can predict a model of the plant system based on its response to particular abiotic stress (Ghatak et al. 2018).

Hence, to our concern it's the first reported study dealing with metabolomics modeling for studying the abiotic stress due to petroleum contamination on *Withania somnifera.* The present chapter focuses a novel and effective technique for treating as well as managing petroleum contaminated soil. Further, the study deals metabolomics study to deciphers the systematic selection to determine of the treatment.

3.5.2 Materials and methods

3.5.2.1 Experimental design and treatment

The pot experiment was conducted in a green house for 6 months. The whole experiment was performed by utilizing the petroleum contaminated soil mentioned in the Chapter 3.3. Soil was dried and sieved with 2 mm sieve and autoclaved for 1 h at 121ºC for three successive days. After then various treatments were developed by mixing garden soil, sand, bagasse, cow urine and bacterial culture as per the design of the developed treatment.

3.5.2.2 Developed Treatments

Treatment 1 :(Both contaminated and garden soil were autoclaved soil and used in the treatment)

T11: 1kg Petroleum contaminated soil + 1kg garden soil + Consortium

T12: 1kg Petroleum contaminated soil + 1kg garden soil + Strain AJ15

T13: 1kg Petroleum contaminated soil + 1kg garden soil + Strain J1

T14: 1kg Petroleum contaminated soil + 1kg garden soil + Strain J2

T15: 1kg Petroleum contaminated soil + 1kg garden soil + Strain N23

T16: 1kg Petroleum contaminated soil + 1kg garden soil (control)

Treatment 2: (Both contaminated and garden soil were autoclaved soil and used in the treatment)

T21: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Consortium

T22: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain AJ15

T23: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain J1

T24: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain J2

T25: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain N23

T26: 1kg Petroleum contaminated soil + 250 g sand + 750 g garden soil (control)

Treatment 3: (Both contaminated and garden soil were autoclaved soil and used in the treatment)

T31: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Consortium + 15 ml cow urine

T32: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain AJ15+ 15 ml cow urine

T33: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain J1+ 15 ml cow urine

T34: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain J2+ 15 ml cow urine

T35: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain N23+ 15 ml cow urine

T36: 1kg Petroleum contaminated soil + 250 g sand + 750 g garden soil + 15 ml cow urine (control)

Treatment 4: (Both contaminated and garden soil were autoclaved soil and used in the treatment)

T41: 1kg Petroleum contaminated soil + 750 g garden soil + 235g sand + Consortium + 15 ml cow urine + 15 g sugarcane bagasse

T42: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain AJ15+ 15 ml cow urine + 15 g sugarcane bagasse

T43: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain J1+ 15 ml cow urine + 15 g sugarcane bagasse

T44: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain J2+ 15 ml cow urine + 15 g sugarcane bagasse

T45: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain N23+ 15 ml cow urine + 15 g sugarcane bagasse

T46: 1kg Petroleum contaminated soil + 250 g sand + 750 g garden soil + 15 ml cow urine + 15 g sugarcane bagasse (control)

Treatment 5: : (Both contaminated and garden soil were not autoclaved soil and used in the treatment)

T51: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Consortium + 15 ml cow urine + 15 g sugarcane bagasse

T52: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain AJ15+ 15 ml cow urine + 15 g sugarcane bagasse

T53: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain J1+ 15 ml cow urine + 15 g sugarcane bagasse

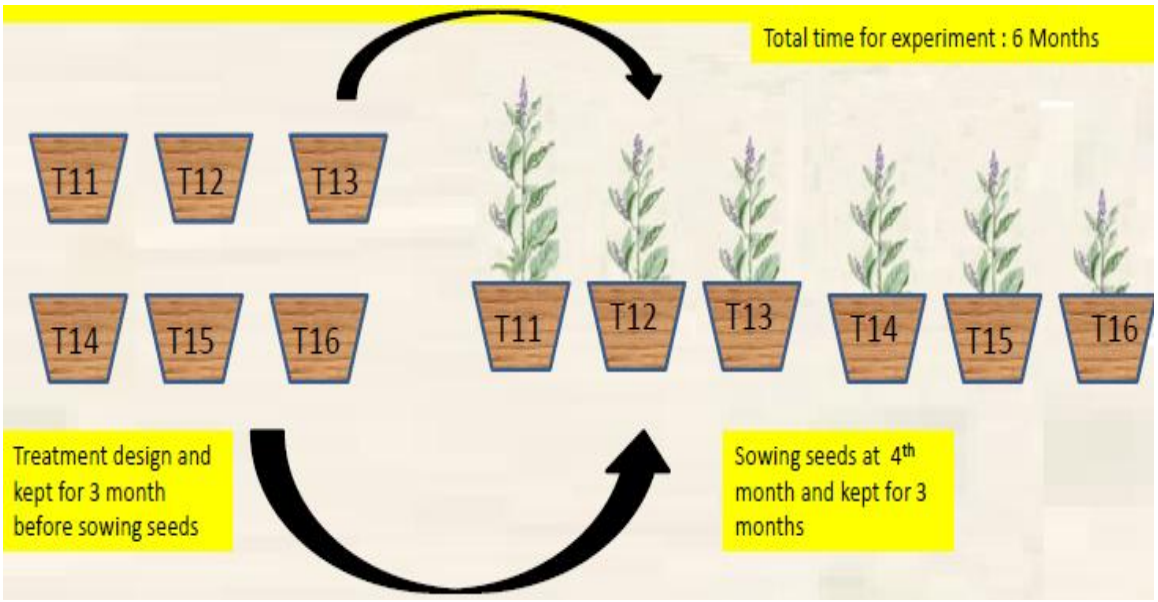
T54: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain J2+ 15 ml cow urine + 15 g sugarcane bagasse

T55: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain N23+ 15 ml cow urine + 15 g sugarcane bagasse

T56: 1kg Petroleum contaminated soil + 235g sand + 750 g garden soil + 15 ml cow urine + 15 g sugarcane bagasse (control)

The developed treatment pots were allowed to stabilize for 90 days. The seeds of *Withania somnifera* variety Poshita obtained from Central Institute of Medicinal and Aromatic Plants , Lucknow, India were washed with 0.1 % $HgCl_2$ followed by 8–10 times washings with distilled water. The treated seeds were sown in the pots after 90 days of the stabilization process. 15 seeds were sown in each pot.

Figure 3.5.1 . Graphical representation of the Experimental setup



Growth profiling to assess the effects of the treatments on *Withania somnifera*

For demonstrating the effects of the treatments percentage of germination, root/shoot length and fresh/dry weight were recorded after 90 days of seed sowing.

3.5.2.3 Effect of treatments on the Phyto-chemicals and antioxidant properties of the plants and prediction of stress response based on metabolomics

The chlorophyll and carotenoid content was performed according to Arnon's method, 1949. DPPH Free radical activity was determined using the procedure of Braca et al. (2001) and ferric reducing power activity was estimated according to the method of Oyaizu (1986). Phenylalanine ammonia-lyase (PAL) activity was determined by following the protocol of Dickerson et al.,1984. Peroxidases (POX) activity was demonstrated by following the method of Hammerschmidt et al., (1982).

3.5.2.4 Metabolomics analysis

For metabolomics analysis all the plant constituents and antioxidant activity data were arranged in excel sheet assigning each treatment a group. And each group contains 15 plants. Further data were analyzed through metabolomics modeling software MetaboAnalyst.

3.5.3 Results and Discussion

Germination

There was a delay in germination in all the cases and the germination percentage was low in all the treatment without bacterial strains (such as Treatment T16, T26, T36, T46 and T56).Nearly, 50-80 % of germination percentage was observed in all the bacterial treated treatments and 30-40% in treatments without bacterial strains. Adverse effects on the untreated soil T16 treatment is more than other untreated treatment (Treatment T26, T36, T46, T56 and T66). Adverse effect in T16 might be due to petroleum oil which has an adverse effect on the water air relations in the soil and creates an impermeable oily film layer around the seeds interfering the proper germination (Adam and Duncan, 2002; Ziołkowska and Wyszkowski, 2010; Hawrot-Paw et al., 2015). The low effect in the Treatment T26, T36, T46, T56 and T66 might be due to the sand which provides aeration in the soil. Kulakow et al. (2000) reported the legumes tested succumbed to hydrocarbons toxicity in absence of any growth promoting bacterial inoculums. Martín et al. (2009) reported 15% decreased in the germination of alfalfa seeds in presence of petroleum sludge when compared with control soil. Sangeetha and Thangadurai, (2014) found that high concentration of petroleum sludge retarded the germination rate of *Vigna unguiculata* seeds, which might be due to the toxic effect of the sludge. Similarly, Korade and Fulekar, (2009) noticed no germination in the loamy soil contaminated with hydrocarbons. Moreover,

low effect in other bacterial treatment might be due to degradation of petroleum hydrocarbons.

Figure. 3.5.1a. Effect of the treatments on plants in Treatment 1

Figure.3.5.2b. Effect of the treatments on plants in Treatment 2

Figure 3.5.2c. Effect of the treatments on plants in Treatment 3

Figure 3.5.2d. Effect of the treatments on plants in Treatment 4

Figure 3.5.2e. Effect of the treatments on plants in Treatment 5

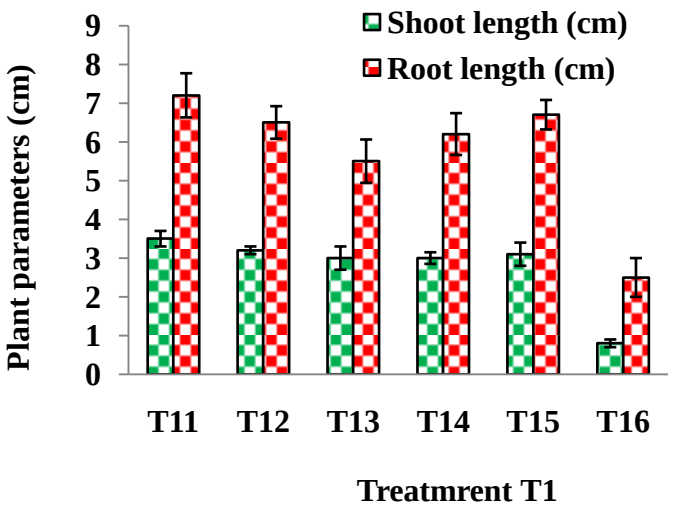
Figure 3.5.2 (a-e). Effect of the treatments on the plant growth

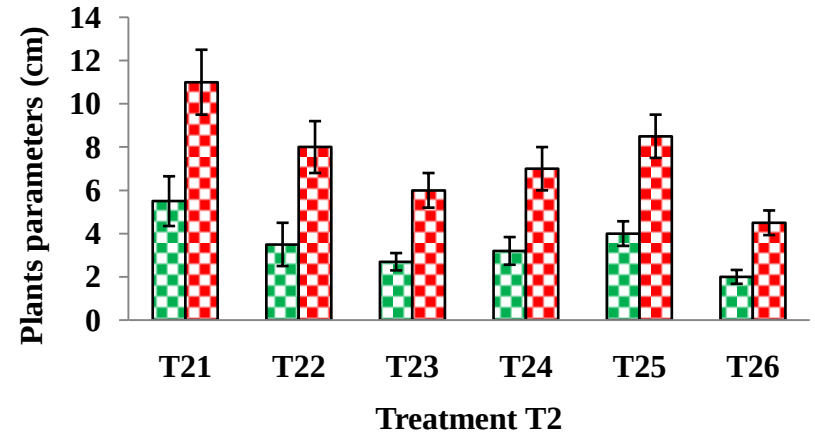
Effect of the treatments on the growth of plants

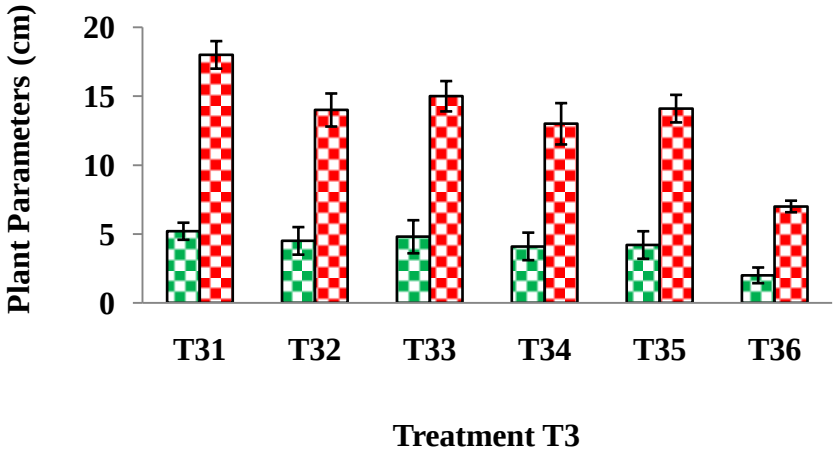
All the inoculated plants with individual strains (AJ15, J1, J2 and N23) showed increase in shoot length with the passage of time. However, the increase was less as compared to the plants treated with bacterial consortium (Treatments T11, T21, T31, T41 and T51) but better than the untreated plants (Treatments T16, T26, T36, T46 and T56). This increased in shoot length might due to PGPR traits and potentiality of the strain to degrade petroleum. Petroleum contamination cause distorts of carbon to nitrogen ratio and phosphorus, in the soil, and their scarcity makes some microorganisms fully use the energy contained in hydrocarbons. Hence, they degrade and utilized the petroleum and its

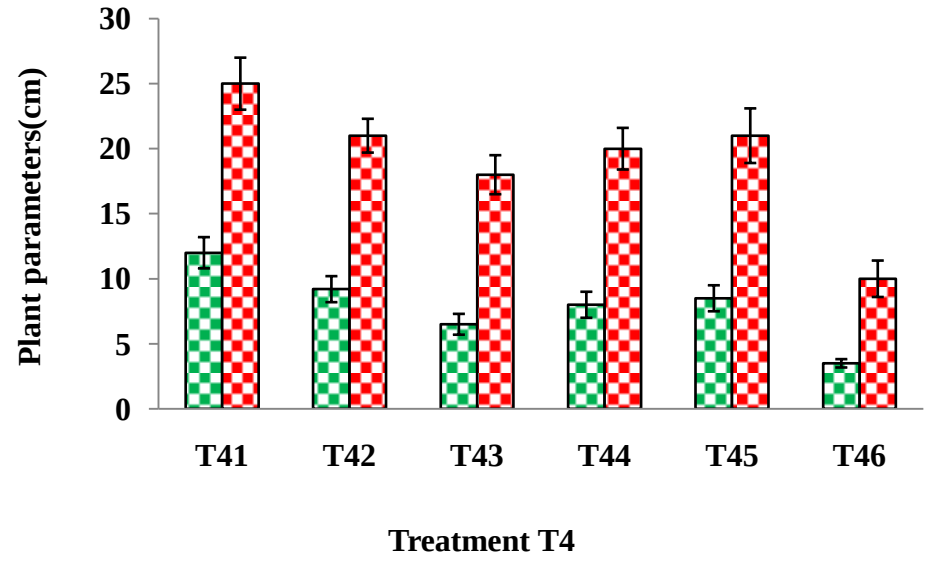
product in soil (Hawrot-Paw et al., 2015). But, the shoot length of treatment T36, T46 and T56 were much better than the treatment T16 and T26, this might due to the cow urine and bagasse employed in those treatments.

The treatments containing bacterial strains, cow urine, sand and bagasse depicts improved shoot and root length in all treatment, but plant raised in the Treatments without bacterial strains shows very poor shoot and root length. The poor root length might be due to anaerobiosis (Rowell, 1977). Petroleum contamination on soil reduces aeration by blocking air spaces between soil particles and creates a condition of anaerobiosis and leading to root stress (Smith et al., 1989; Shukry et al., 2013). Reduced in shoot length might be due to physical, chemical, and biological transformation of oil in soil (Lorestani et al., 2012; Baruah et al., 2014). Baruah et al., 2014 reported that the presence of crude oil in soil effect the morphology of the plant by causing many structural abnormalities such as reduction of leaf size and root length.




Plants parameters (cm)
14
12
10
8
6
4
2
0
T21
T22
T23
T24
T25
T26
Treatment T2


Plant Parameters (cm)
20
15
10
5
0
T31
T32
T33
T34
T35
T36
Treatment T3


Plant parameters(cm)
30
25
20
15
10
5
0
T41
T42
T43
T44
T45
T46
Treatment T4

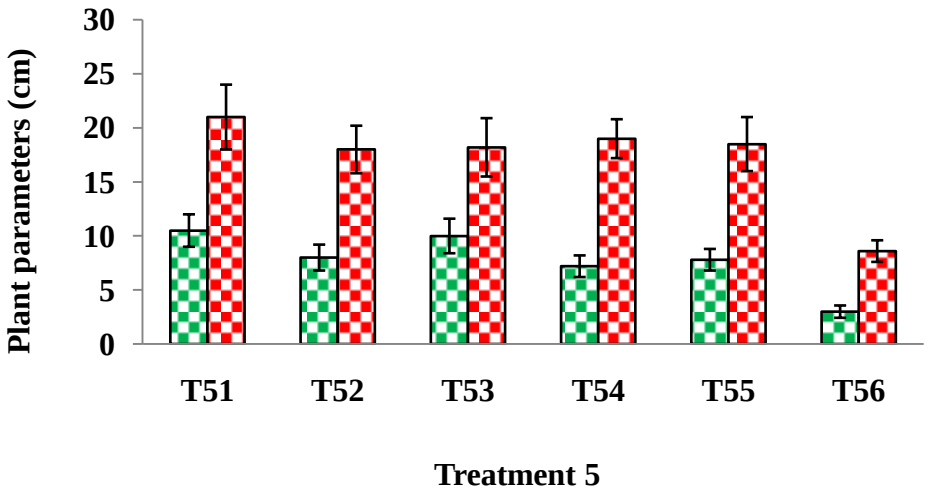
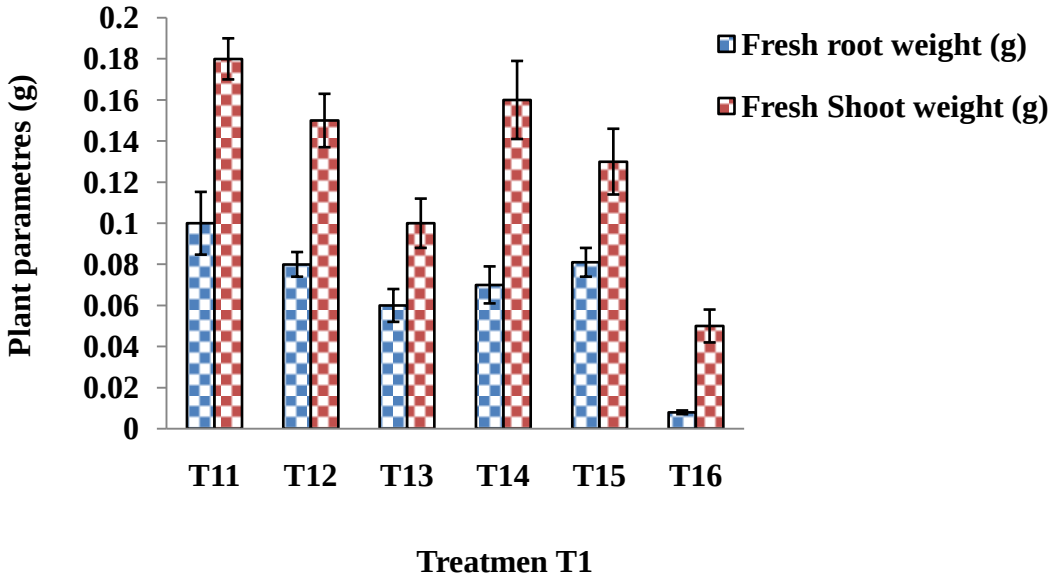
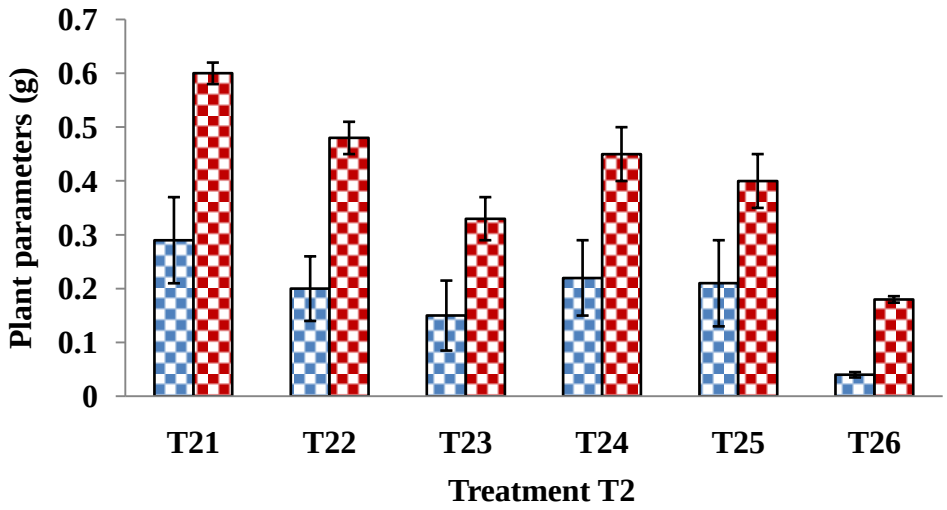


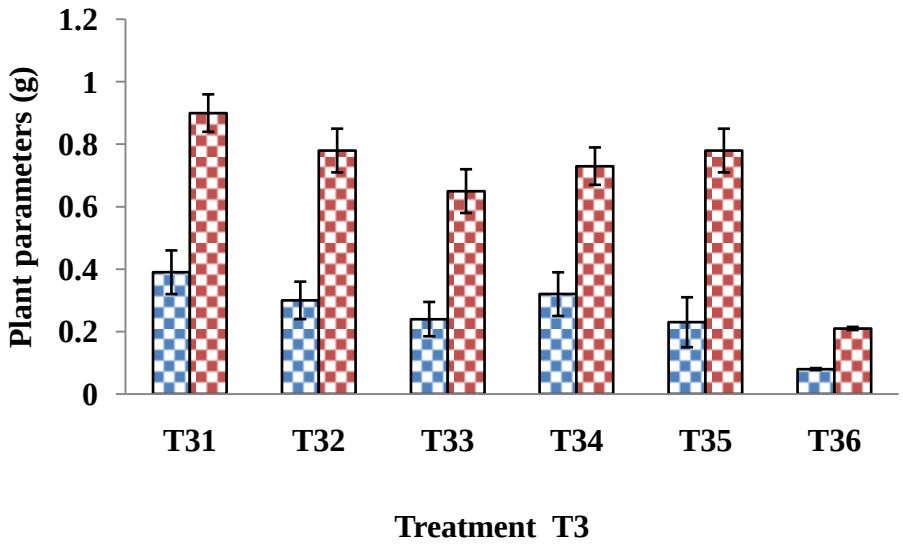
Figure 3.5.3 Effect of the treatments on the Shoot length and root length

Effect on fresh and dry weight of the plants

Fresh and dry weight of the plant is enhanced by the treatments. The results indicates higher mass in plants rose from bacterial strain containing treatments compared to the plants from the treatment without bacterial strains . This suggests that the effects of plant growth-promoting features (IAA, siderophore and solubilization of phosphate) by the strains AJ15, J1, J2 and N23. Various plant growth promoting bacteria (PGPR) are able to rapidly metabolize some readily available organic compounds such as total petroleum hydrocarbon present on soils. This leads to soil remediation and lower the petroleum toxicity to plants and then increase the plant tolerance to petroleum hydrocarbon stress. Thereafter, they vigorously promote plant growth, resulting in more rapid and massive biomass accumulation (Huang et al., 2001; Siciliano et al., 2003; Ajithkumar et al., 1998; Bashan and de Bashan, 2005).


Fresh root weight (g)
Fresh Shoot weight (g)
Plant parametres (g)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
T11
T12
T13
T14
T15
T16
Treatmen T1


Plant parameters (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
T21
T22
T23
T24
T25
T26
Treatment T2


Plant parameters (g)
1.2
1
0.8
0.6
0.4
0.2
0
T31
T32
T33
T34
T35
T36
Treatment T3

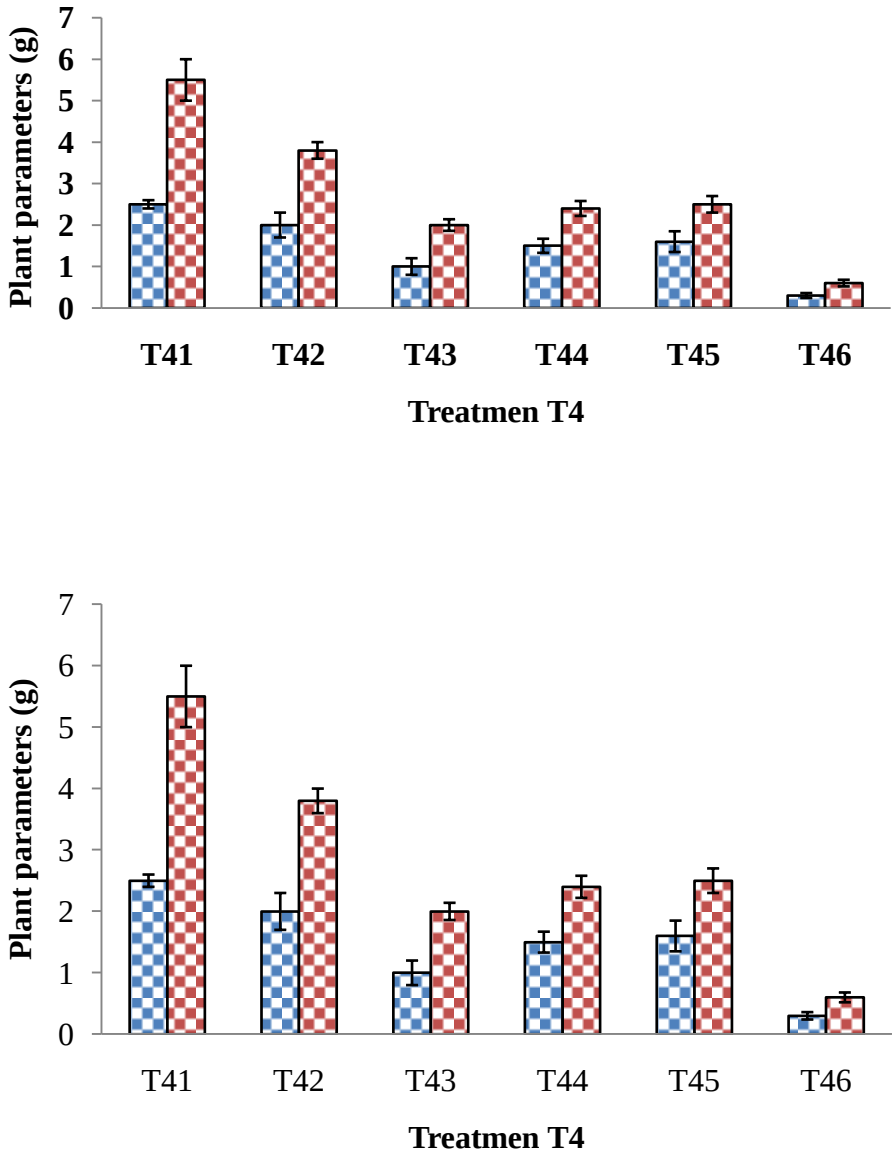


Figure 3.5.4 Effect of the treatments on the fresh shoot weight and fresh root weight

Metabolomics analysis of the plants

Score plot analysis:

Score plot of PCA and PLS-DA analyses of all the treatments were depicted within the Figure 3.5.5 (a-e). Score plot of PCA and PLS-DA demonstrates the divergence between treatments based on variation of phytochemical content and antioxidant activity.

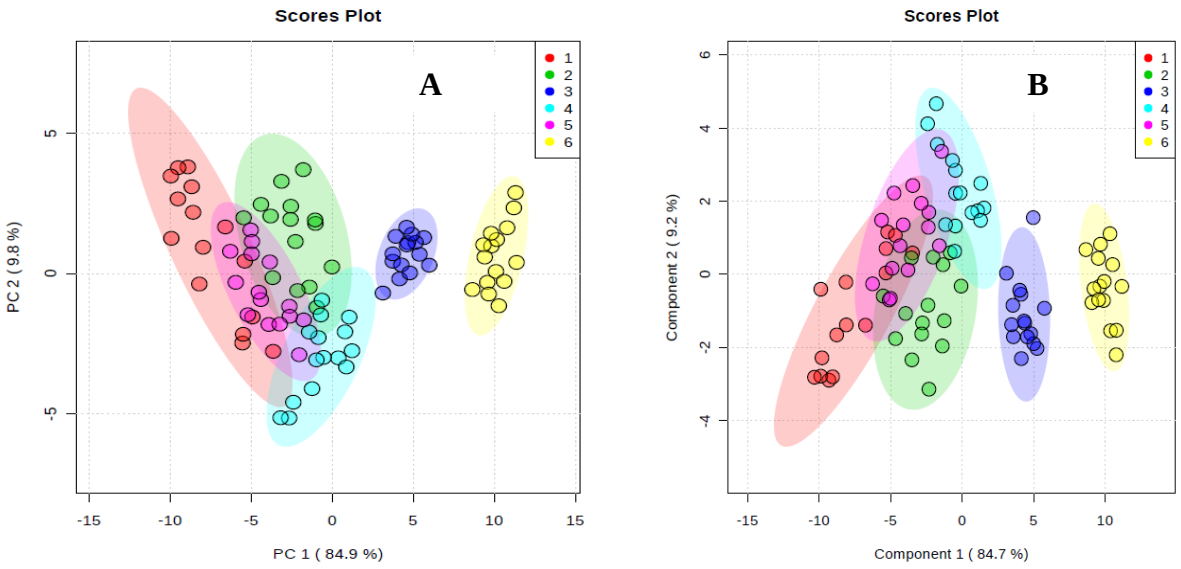


Figure 3.5.5a.Score plots of the component analysis of the plants of each treatment in Treatment 1 (n = 15). (A) Principal Component Analysis (PCA),(B) Partial Least Squares - Discriminant Analysis (PLS-DA). 1= T11 (n=15); 2=T12 (n=15); 3=T13 (n=15); 4=T14 (n=15); 5=T15 (n=15); 6=T16 (n=15)

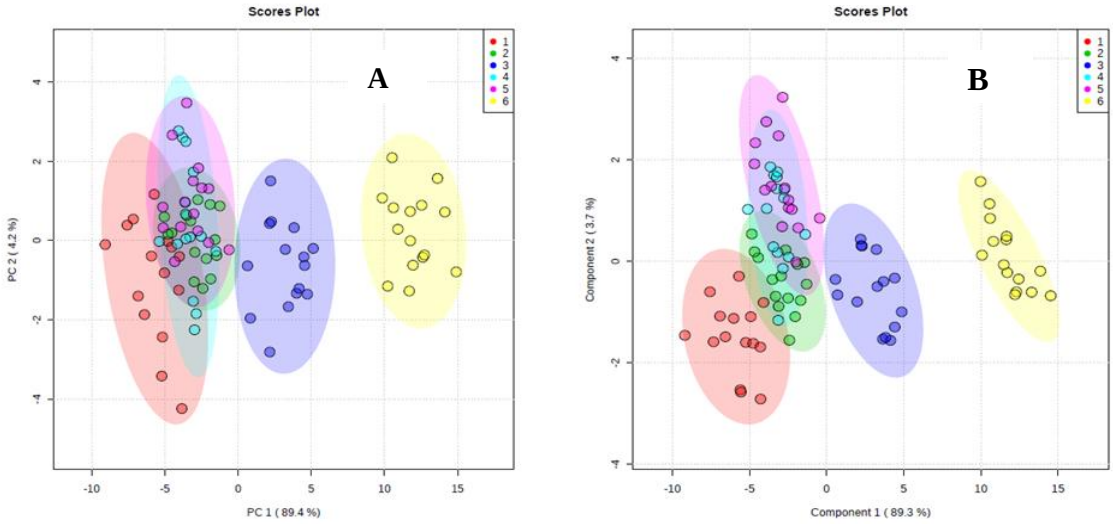


Figure 3.5.5b Score plots of the component analysis of the plants of each treatment in Treatment 2 (n = 15). (A) Principal Component Analysis (PCA),(B) Partial Least Squares - Discriminant Analysis (PLS-DA). 1= T21 (n=15); 2=T22 (n=15); 3=T23 (n=15); 4=T24 (n=15); 5=T25 (n=15); 6=T26 (n=15)

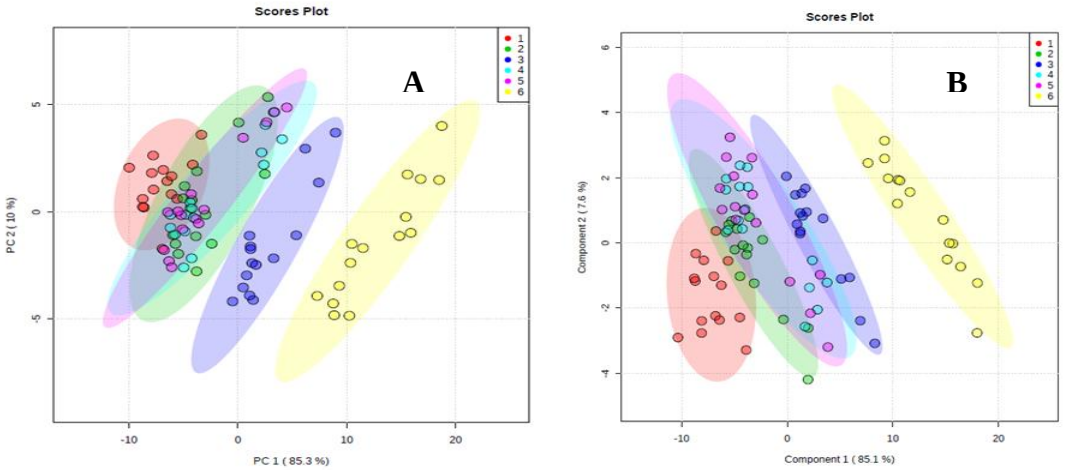


Figure 3.5.5c. Score plots of the component analysis of the plants of each treatment in Treatment 3 (n = 15). (A) Principal Component Analysis (PCA),(B) Partial Least Squares - Discriminant Analysis (PLS-DA). 1= T31 (n=15); 2=T32 (n=15); 3=T33 (n=15); 4=T34 (n=15); 5=T35 (n=15); 6=T36

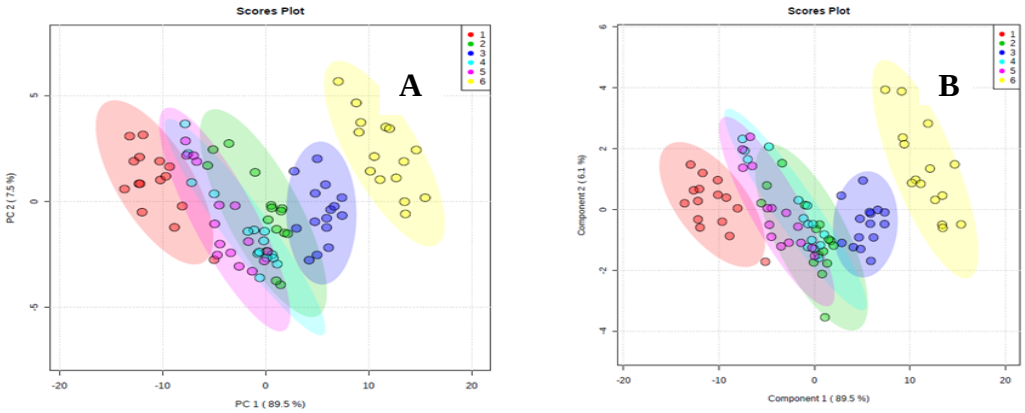


Figure 3.5.5d. Score plots of the component analysis of the plants of each treatment in Treatment 4 (n = 15). (A) Principal Component Analysis (PCA),(B) Partial Least Squares - Discriminant Analysis (PLS-DA). 1= T41 (n=15); 2=T42 (n=15); 3=T43 (n=15); 4=T44 (n=15); 5=T45 (n=15); 6=T46 (n=15)

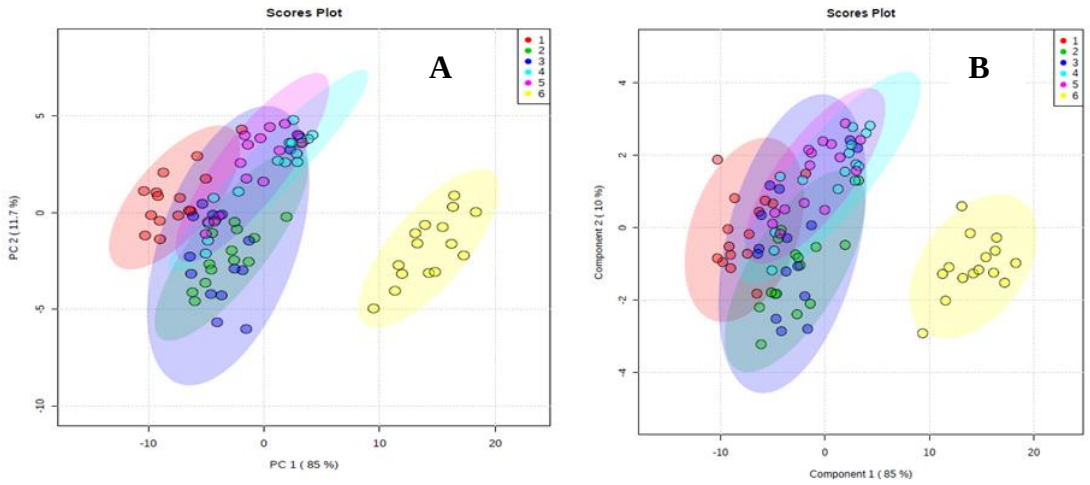


Figure 3.5.5e. Score plots of the component analysis of the plants of each treatment in Treatment 2 (n = 15). (A) Principal Component Analysis (PCA),(B) Partial Least Squares - Discriminant Analysis (PLS-DA). 1= T51 (n=15); 2=T52 (n=15); 3=T53 (n=15); 4=T54 (n=15); 5=T55 (n=15); 6=T56

Score plots of the component analysis of the plants of each treatment in represented in the Figures. Figure (A) represents score plot of principal component analysis (PCA), Whereas Figure(B) represents the score plot of partial least squares-discriminant analysis (PLS-DA) . In the Figure numbering denotes each treatments of the particular of designed Treatment:

1= T11, T21, T31, T41, T51

2=T12, T22, T32, T42, T52

3=T13, T23, T33, T43, T53

4=T14, T24, T34, T44, T54

5=T15, T25, T35, T45, T55

6=T16, T26, T36, T46, T56

Based on the score plot analysis it can be determine that the divergence in treatments (1= T11, T21, T31, T41, T51) ,(2=T12, T22, T32, T42, T52) , (4=T14, T24, T34, T44, T54), (5=T15, T25, T35, T45, T55, T56) shows quite similar results based on the variation of phytochemical content (chlorophyll a, chlorophyll b and total chlorophyll and carotenoid) and antioxidant activity (DPPH activity, ferric reducing power activity, phenylalanine ammonia-lyase and peroxidases concentration). But, the treatments (6=T16, T26, T36, T46, T56) score plot denotes that there is a great variation of phytochemical content and antioxidant activity which can be predicted on the basis of the divergence between the score plots. The score plot of the treatments (3=T13, T23, T33, T43, T53) is quite different from the score plots of other treatments. This signifies that the treatment is not as efficient as other treatments. Thus, based on score plot analysis it can be predicted that treatment (1= T11, T21, T31, T41, T51) is best. The order of the efficiency treatment is (1= T11, T21, T31, T41, T51) > (5=T15, T25, T35, T45, T55) > (2=T12, T22, T32, T42, T52)>(4=T14, T24, T34, T44, T54) >(3=T13, T23, T33, T43, T53)> (6=T16, T26, T36, T46, T56).

Loading plot:

Principal Component Analysis (PCA) loading plot demonstrated the phyto-chemical responsible for separation of the treatments from each other. Loading plot of each treatment is depicted in the Figure 3.5.6 (a-e).

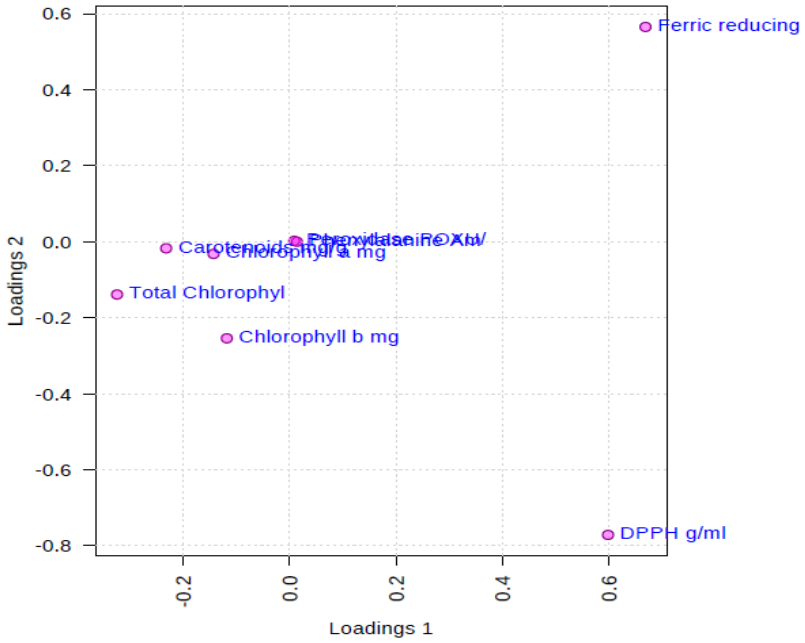


Figure 3.5.6a. Loading plots of the component analysis of the plants of each treatment in Treatment 1 based on Principal Component Analysis (PCA)

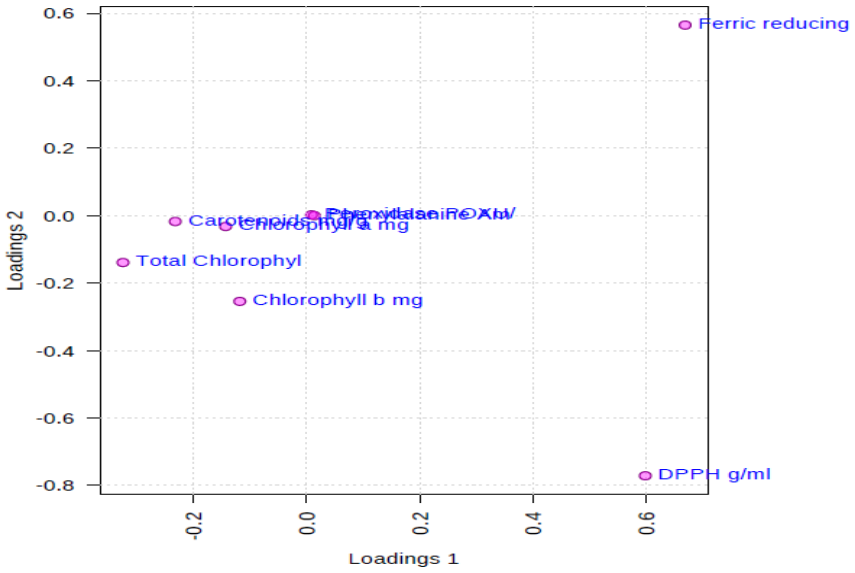


Figure 3.5.6b. Loading plots of the component analysis of the plants of each treatment in Treatment 2 based on Principal Component Analysis (PCA).

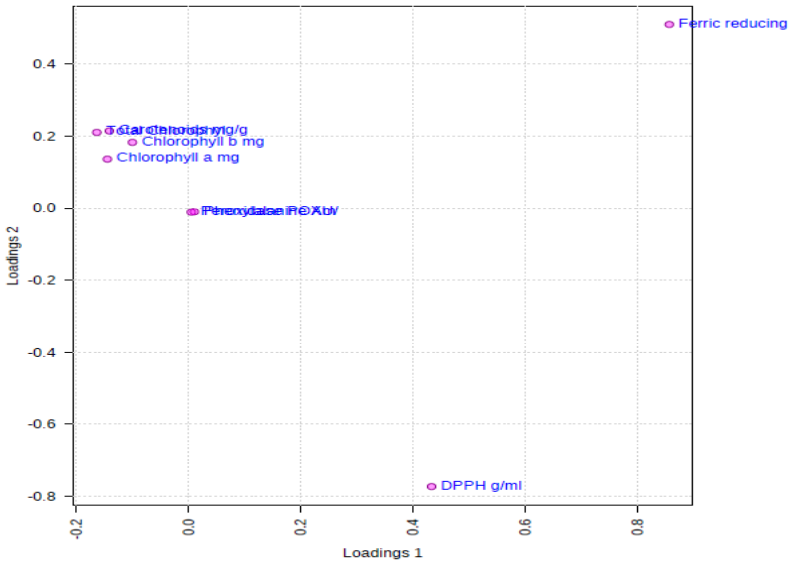


Figure 3.5.6c. Loading plots of the component analysis of the plants of each treatment in Treatment 3 based on Principal Component Analysis (PCA)

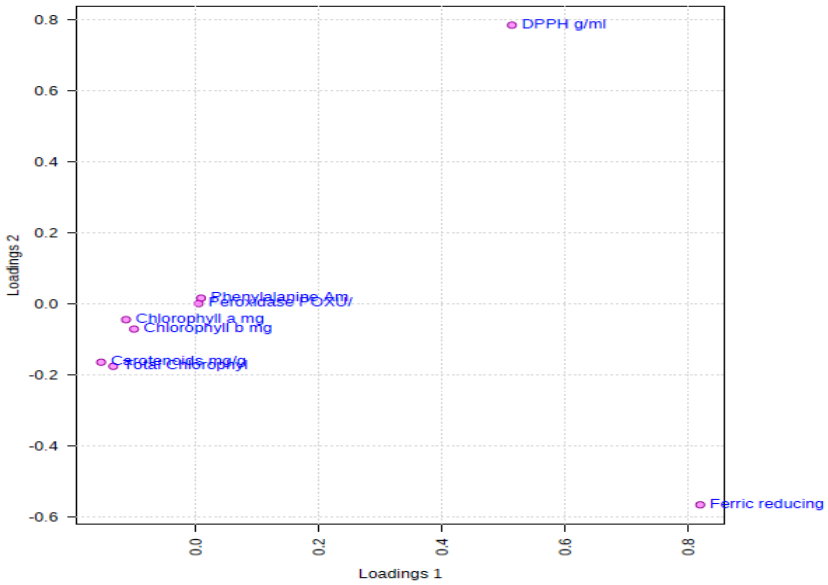


Figure 3.5.6d. Loading plots of the component analysis of the plants of each treatment in Treatment 4 based on Principal Component Analysis (PCA)

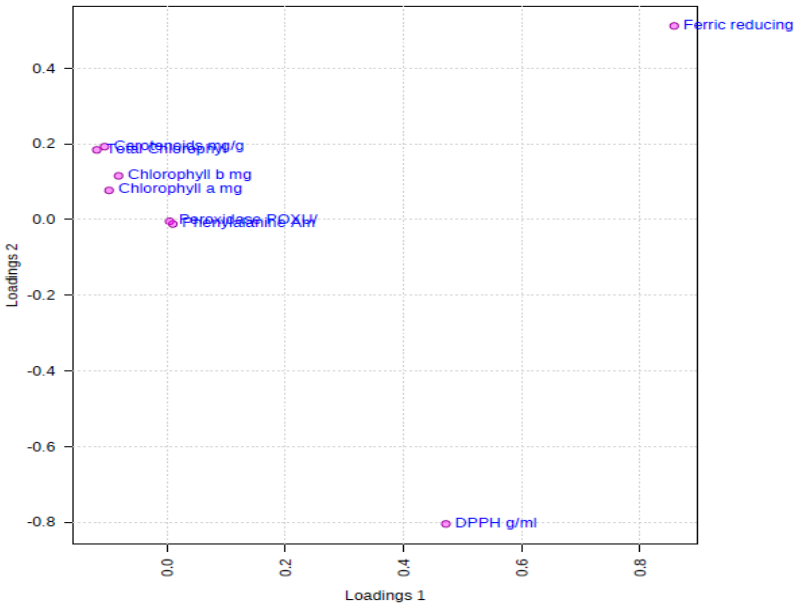


Figure 3.5.6 e. Loading plots of the component analysis of the plants of each treatment in Treatment 5 based on Principal Component Analysis (PCA)

Loading plots of the treatment based on Principal Component Analysis (PCA) demonstrate the phytochemical and antioxidant activities are responsible for the separation of group within the treatments.

- In Treatment 1 (T11, T12, T13, T14, T15 and T16) the major component responsible for separation of group within the treatment is ferric reducing activity and DPPH activity.
- In Treatment 2 (T21, T22, T23, T24, T25 and T26) the major component responsible for separation of group within the treatment is ferric reducing activity and DPPH activity.
- In Treatment 3 (T31, T32, T33, T34, T35 and T36) the major compou responsible for separation of group within the treatment is ferric reducing activity.

Figure 1. Imp plots of the component analysis of the plants of each treatment (n = 15)

- In Treatment 4 (T41, T42, T43, T44, T45 and T46) the major component responsible for separation of group within the treatment is ferric reducing activity.
- In Treatment 5 (T51, T52, T53, T54, T55 and T56) the major component responsible for separation of group within the treatment is ferric reducing activity.

Loading plot of the treatment denotes that ferric reducing activity and DPPH activity is the main component responsible for all the treatments. Hence, it can be state the stress on each treatment can be depicted based on antioxidant activity.

Metabolomics study (Score and loading plot) it can be demonstrate that the stress effect was more in treatment without bacterial strains. Chlorophyll plays a significant role in the plant physiology and its productivity. Growing plants on oil contaminated soil has a great impact on the synthesis of chlorophyll pigment (Baruah et al., 2014). The chlorophyll content was more is more in bacterial consortium treatment (T16, T26, T36, T46 and T56) and the treatment containing bacterial strain AJ15 (T12, T22, T32, T42 and T52), J2 (T14, T24, T34, T44, T54) and N23 (T15, T25, T35, T45 and T55) as compared to the treatment with strain J1. However, chlorophyll content in plants arose from non inoculated pots is very low. The decrease in the total chlorophyll content in the leaves of the *W. somnifera* in the different treatment may be due to the alkaline condition created through dissolution of chemicals present in the oil in the cell sap that responsible for chlorophyll degradation (Baruah et al., 2014). The literature on the effect of petroleum oil stress on carotenoid pigment is scarce. However, in the present study we reported the effect of petroleum contaminated soil on cartenoids pigment. Metabolomics investigation revealed that there is significant

effect petroleum contaminated soil on carotenoid content. Very low carotenoids content were reported in plants without bacterial strains (T16, T26, T36, T46 and T56), where as high carotenoid contents were reported bacterial consortium treatments (T11, T21, T31, T41 and T51).

There was a variation in the antioxidant properties like DPPH and ferric reducing activitiy among the treatments. The plants arose from bacterial consortium (T11, T21, T31, T41 and T51) containing pot showed decrease in absorbance of DPPH radical. This is due to the reaction between antioxidant molecules and DPPH radical, which results in the scavenging of the radical by hydrogen donation. Decrease in absorbance of the reaction mixture indicates higher free radical scavenging activity, whereas un-inoculated plants (T16, T26, T36, T46 and T56) depicts poor DPPH activity. Ferric reducing activity of *Withania somnifera* in various treatments shows varying results. Increase in absorbance indicates increase in reducing power. Reducing activity of bacterial consortium (T11, T21, T31, T41 and T51) is highest as compared to the other ttreatments containing bacterial strain AJ15 (T12, T22, T32, T42 and T52), J1 (T13, T23, T33, T43, T53), J2 (T14, T24, T34, T44, T54) and N23 (T15, T25, T35, T45 and T55), although better than treatments (T16, T26, T36, T46 and T56) where no bacterial strains were inoculated. The antioxidant activity and its consequence for the acclimatization of plants to climatic stresses has been reviewed often (Martia et al. 2009) although a very little is known regarding the effects of petroleum contamination on the growth and on the antioxidative activity in plant. Hence, present metabolomics study depicts DPPH and ferric reducing power activity as the major component in separation of the treatments.

The results of Treatment 5 (T51, T52, T53, T54 and T55) containing non-autoclave demonstrate quite different results from the autoclaved soil use in other treatment.

Score plot for all the other treatment containing strain J1 (T13, T23, T33 and T44) is quite different from the score plot obtained from T53 which shows divergence from T51, T52, T54 and T55. This indicates that the strain AJ15, J2, N23 and consortium (containing strain AJ15, J1, J2 and N23) can remediate the petroleum contaminated soil in both *insitu* and *exsitu* as they have the bioagumentation and biostimulation efficiency in both autoclaved and non-autoclaved soil, where as metabolomics results demonstrate that the strain J1 has good biostimulation activity in non-autoclaved soil.

Moreover, better results in Treatment 4 (T41, T42, T43, T44 and T45) and Treatment 5 (T51, T52, T53, T54 and T55) are due to addition of nutrients such as cow urine and bagasse. Bagasse favours the production of biosurfactant in the treatments by the bacterial strains as reported in chapter in 3.2, which results in reduction of various toxic compounds as biosurfactant enhance the degradation rate of hydrophobic petroleum hydrocarbons. Previously various researchers have demonstrated that the addition of biosurfactant in the oil contaminated soil treatment enhanced biodegradation rate of hydrocarbons as biosurfactants increases the bioavailability of hydrocarbons which promotes hydrocarbon degradation activity of microbes (Cameotra and Singh, 2008; Thavasi et al., 2011; Banat et al., 2014; Perfumo et al., 2010) . The present results were in accordance with previously published work of Tahseen et al.(2016) who investigated that addition of biosurfactant and nutrients enhance bacterial colonization and metabolic activity resulting in remediation of oil contaminated soil.

Effect of various components on the treatment

Sand

Petroleum and its product form an impermeable coating at the surface of the soil that prevents circulation of water in soil and also affects the gas exchange between the soil and air that cause roots of plants to suffocate. As results soil turns into anaerobic condition that affects the microbial population dynamics of the soil (Streche et al., 2018). So, addition of sand improves texture and quality of the soil because sand increases the macro pores percentage of the soil that provides better aeration into the soil. Hence, the treatments containing sand Treatment 2, 3, 4 and 5 have better results as compared to the treatment without sand.

Cow urine

Nutrients like nitrogen and phosphorus are important ingredients required for biodegradation of petroleum hydrocarbon. But, these nutrients become limiting factor affecting the biodegradation processes of petroleum hydrocarbons as carbon concentration significantly increased and the availability of nitrogen and phosphorus becomes the limited (Das et al., 2011; Atlas, 1985; Cooney et al. 1984). Hence, addition of cow urine may enhance the availability of nitrogen in the petroleum contaminated soil as cow urine contain rich nitrogen source of urea. Thus, addition of cow urine in the treatment enhances biodegradation rate and resulting in detoxification of the soil (Treatment 3, 4 and 5).

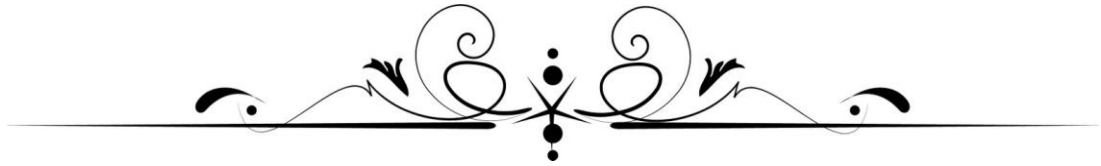
Bagasse

Sucrose is the important content of sugarcane juice (Mathur, 1975; Tewari & Irudayaraj, 2003; Tewari and Malik, 2007). In sugar processing, sugar cane juice is

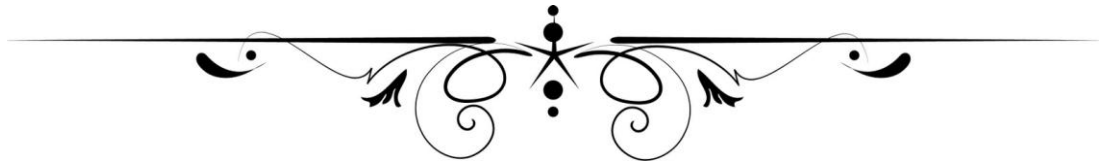
extracted by physically crushing of the stalk material. The stalk material left after juice extraction is called 'bagasse'. Sugarcane bagasse is the fibrous residue comprising of significant amount of sucrose. But, those bagasses are consider to be waste and burned at the sugar mill and paper mills as fuel. It is estimated 1to 5% of sucrose content can be found in waste bagasse (Tewari and Malik, 2007). So, adding bagasse in the treatment will increase the sugar content of the soil favouring the growth of microbes with particular metabolic activities. Hence, addition of bagasse in the Treatment 4 and 5 of the present study favours the growth of selected bacterial strains with biosurfactant producing ability. As, in the chapter 3.2 we explore the ability of selected bacterial strain (AJ15, J1 , J2 and N23) to utilize bagasse as carbon source for production of biourfactant.

3.5.6 Conclusion

The results signifies that employing the biosurfactant producing PGP bacteria with the potentiality to degrade petroleum can be effectively and commercially used for management and detoxification petroleum contaminated wastelands. It was further anticipated that the treatment developed employing sand, cow urine, bagasse and bacterial strains can be effectively and commercially used for management and detoxification petroleum contaminated wastelands for agriculture purpose. Further, metabolomics studies deciphers the systematic selection of bacterial strains based on bioaugmentation and biostimulation phenomenon.



Chapter 4



The conclusion obtained from the present is categorized into following points:

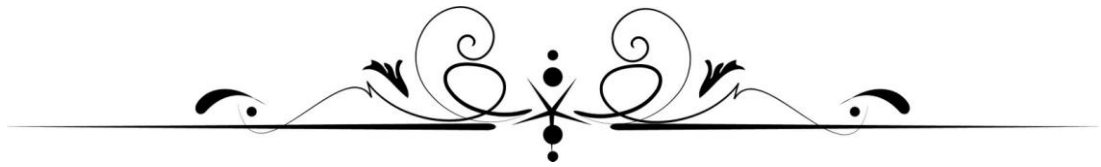
- Chapter 3.1 demonstrates that bacterial strains isolated were adapted to peculiar petroleum pollution stress environment. Hence, microbes of such environment are needed to isolate for studying the clues about the functional microorganisms and their novel metabolic function.

- Chapter 3.2 reveals that bacterial strains isolated from petroleum contaminated site has biosurfactants producing ability with PGP traits. Biosurfactant produced the by strains are non-toxic in nature and posses' excellent emulsification property. Notably, the biosurfactant was enabled to persist its activities over a varying range of pH, temperature, and salinity.

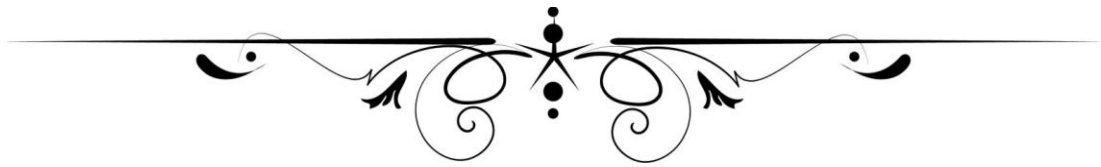
- Chapter 3.3 and 3.4 reveals that the bacterial strain selected in the present study has the ability to degrade organic compounds of petroleum contaminated soil. The study also focuses that the bacterial strain partially degrades the compounds in 90 days. But, for complete degradation of the compound the degradation time period needs to increase as most of the petroleum hydrocarbon are recalcitrant in nature.

- Chapter 3.5 signifies that employing the biosurfactant producing PGP bacteria with the potentiality to degrade petroleum can be effectively and commercially used for management and detoxification petroleum contaminated wastelands. It was further anticipated that the treatment developed employing sand, cow urine, bagasse and bacterial strains can be effectively and commercially used for management and detoxification

petroleum contaminated wastelands for agriculture purpose. Further, metabolomics studies deciphers the systematic selection of bacterial strains based on bioaugmentation and biostimulation phenomenon.



Chapter 5



With the advent of industrialization during last few decades, demand for fossil fuel (especially Petro-products) as an energy source has increased dramatically which lead to massive anthropological activities including increased rate of oil extraction/refinement. This has simultaneously resulted in contamination of important agricultural soils and thereby has become a prime concern for environmentalists and policymakers. The soil pollution by petroleum hydrocarbons is primarily responsible for the changes in physicochemical properties of soils and drop in the oxygen level that made the soil unfit for agricultural purposes. Hence, for sustainability and hoping for a better tomorrow for our successors, scientific remediation approaches must be of pressing need. In this regard, many technologies have been planned and tested; however, in general, most of them are either very expensive or result only in incomplete removal of contaminants. Notably, substantial attention has paid to the event and application of the latest biologically techniques, that is effective in remedy and cost, not harming the prevailing surroundings. Hence, use of biosurfactant producing bacterial strains is one of the recent eco-friendly techniques.

We have propelled this idea in the present study by working on 4 bacterial strains (selected potent strains) that were isolated from petroleum contaminated sites of Lucknow (26°55' N latitude and 80° 59' E longitude), Uttar Pradesh and Guwahati (26° 11'N latitude and 91° 44'E longitude) Assam, India. Based on morphological, biochemicaland on 16S rRNA studies, isolated bacterial strains were found similar to *Pseudomonas azotoformans* (AJ15 and N23), *Bacillus licheniformis* (J2) and *Bacillus safensis* (J2). Accession numbers allotted are KU671026, KT951266,

KU680817, KX865089 respectively. The selected strains produced biosurfactant on mineral saltmedium (MSM). Each test strains produced foam with surfactants. All the strains show positive results for drop collapse test and hemolytic assay indicates that the strains produced biosurfactants. In addition, all the strain posses plant growth promoting traits. Thes strains AJ15 and N23 were found positive for phosphate solubilization. In a qualitative analysis of phosphate reddish zone around the colonies indicates that the bacterial strains has the ability to solubilize phosphate, whereas quantitative test for solubilization of tricalcium phosphate in liquid medium by the test strain showed that strain solubilize tricalcium phosphate. Further, the qualitative, as well as quantitative test for siderophore production on Chrome Azurol-S media, were found to be positive for the strains AJ15, J2 and N23 as indicated by the change in color of the media around the colonies. Quantitative estimation of Indole acetic acid (IAA) in the presence of 100 µg/ml concentrations of tryptophan indicates that the strains were able to produce a good amount of IAA. All the 5 strains show good ACC deaminase activity which demonstrates their ability to survive in abiotic stress condition.

Biosurfactants extracted from the selected bacterial are subjected to various experiments such as cell surface hydrophobicity, emulsification index, surface tension reduction, and oil displacement assay. The cell hydrophobicity of the strains showed an increase in adherence in the presence of extracted biosurfactants. Emulsification index of biosurfactant produced by the strain AJ15 is 62.7%, J1 is 73.36%, J2 is 68.66% and N23 is 77% respectively. The surface tension of non-inoculated broth was 65.8 mN/m. After 72 h, the surface tension of the broth was 30.5 mN/m for AJ15, 29.5 mN/m for J1, 34 mN/m for J2 and 29 mN/m for N23. These indicate that biosurfactant produced by the strains has the a good surface

tension reduction ability. Toxicity assessment of the extracted biosurfactant from the strains exhibited no inhibitory effect on seed germination and earthworm survival rate. The effect of pH, temperatures and salt concentration on biosurfactant was also studied. The temperature effect on biosurfactant showed that the extracted biosurfactants were quite stable at 30 °C. Heating of the crude biosurfactant up to 90 °C caused no significant effect on the emulsification and surface tension. Studies on the effect of pH suggest that biosurfactants were stable at pH 7. Further, the obtained results have confirmed that extracted biosurfactants remains stable in basic pH as compared to acidic pH. The effect of NaCl was investigated by increasing salt the concentrations from 1% to 10%. Very low effect of salinity was observed on biosurfactant stability till 5% concentration of NaCl, but increasing the concentration of the salt above 5% reduced activities of the all extracted biosurfactants except the biosurfactant from strain J2 shows tendency to persist its activity at high salt concentration. FTIR spectroscopy and Liquid chromatography–mass spectrometry (LC-MS) characterization of the extracted biosurfactant reveal that the biosurfactant extracted from AJ15 is monorhamnolipid and from strain N23 is both mono-rhamnolipid and di-rhamnolipid nature, whereas, biosurfactant extracted from J1 is lichenysin and from J2 is surfactin. Agroindustrial waste (bagasse) was employed for biosurfactant production and was optimized through Box-Behnken Design (BBD) model with 4 variable factors (Bagasse concentration, temperature, agitation rate and pH).

- The strain AJ15 produce maximum 0.92 g of biosurfactant at 30º C temperature, pH 7, 160 rpm rate by utilizing 10 g bagasse for 72 hrs.
- The strain J1 produce maximum 0.91 g of biosurfactant at 35º C temperature, pH 7, 100 rpm rate by utilizing 10 g bagasse for 72 hrs.

- The strain J2 produce maximum 0.92 g of biosurfactant at 35º C temperature, pH 7, 160 rpm rate by utilizing 15 g bagasse for 72 hrs.
- The strain N23 produce maximum 0.98 g of biosurfactant at 30º C temperature, pH 7, 160 rpm rate by utilizing 10 g bagasse for 72 hrs.

Further, the study involves the collection of contaminated soil from contaminated site of Lucknow, Uttar Pradesh, India. Gas chromatography-mass spectrometry (GC-MS) analysis of the contaminated soil reveals the presence of hydrocarbon in the soil. The detected hydrocarbon chromatogram is identified using (The National Institute of Standards and Technology) mass spectral library which reveals that the contaminated soil comprises 121 organic compounds and hydrocarbon that were toxic in nature, as demonstrated by seed germination inhibitory test and earthworm survival test. Then, after the contaminated soil was subjected to bioremediation treatment within lab scale using the selected bacterial strains. HPLC and GC-MS study reveal that the bacterial strains have the ability to remediate the petroleum contaminated soil and also focuses that the bacterial strain partially degrades the compounds in 90 days. But, for complete degradation of compound the degradation time period needs to be increase as most of the petroleum hydrocarbon are recalcitrant in nature.

Through mixing contaminated soil with garden soil, sand, bagasse, cow urine and bacterial strains, various treatments were also developed.

Treatment 1 :(Both contaminated and garden soil were autoclaved soil and used in the treatment)

T11: 1kg Petroleum contaminated soil + 1kg garden soil + Consortium
T12: 1kg Petroleum contaminated soil + 1kg garden soil + Strain AJ15
T13: 1kg Petroleum contaminated soil + 1kg garden soil + Strain J1
T14: 1kg Petroleum contaminated soil + 1kg garden soil + Strain J2

T15: 1kg Petroleum contaminated soil + 1kg garden soil + Strain N23

T16: 1kg Petroleum contaminated soil + 1kg garden soil (control)

Treatment 2: (Both contaminated and garden soil were autoclaved soil and used in the treatment)

T21: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Consortium

T22: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain AJ15

T23: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain J1

T24: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain J2

T25: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain N23

T26: 1kg Petroleum contaminated soil + 250 g sand + 750 g garden soil (control)

Treatment 3: (Both contaminated and garden soil were autoclaved soil and used in the treatment)

T31: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Consortium + 15 ml cow urine

T32: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain AJ15+ 15 ml cow urine

T33: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain J1+ 15 ml cow urine

T34: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain J2+ 15 ml cow urine

T35: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain N23+ 15 ml cow urine

T36: 1kg Petroleum contaminated soil + 250 g sand + 750 g garden soil + 15 ml cow urine (control)

Treatment 4: (Both contaminated and garden soil were autoclaved soil and used in the treatment)

T41: 1kg Petroleum contaminated soil + 750 g garden soil + 235g sand + Consortium + 15 ml cow urine + 15 g sugarcane bagasse

T42: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain AJ15+ 15 ml cow urine + 15 g sugarcane bagasse

T43: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain J1+ 15 ml cow urine + 15 g sugarcane bagasse

T44: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain J2+ 15 ml cow urine + 15 g sugarcane bagasse

T45: 1kg Petroleum contaminated soil + 750 g garden soil + 250 g sand + Strain N23+ 15 ml cow urine + 15 g sugarcane bagasse

T46: 1kg Petroleum contaminated soil + 250 g sand + 750 g garden soil + 15 ml cow urine + 15 g sugarcane bagasse (control)

Treatment 5: :(Both contaminated and garden soil were not autoclaved soil and used in the treatment)

T51: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Consortium + 15 ml cow urine + 15 g sugarcane bagasse

T52: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain AJ15+ 15 ml cow urine + 15 g sugarcane bagasse

T53: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain J1+ 15 ml cow urine + 15 g sugarcane bagasse

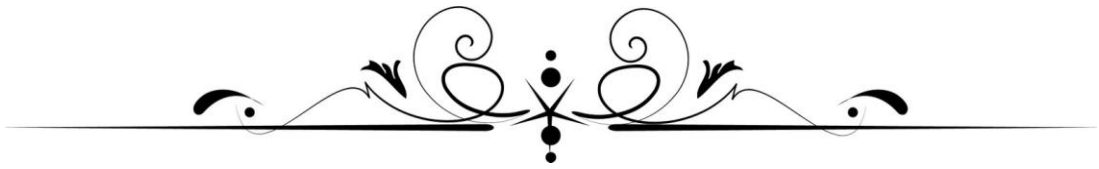
T54: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain J2+ 15 ml cow urine + 15 g sugarcane bagasse

T55: 1kg Petroleum contaminated soil + 750 g garden soil + 235 g sand + Strain N23+ 15 ml cow urine + 15 g sugarcane bagasse

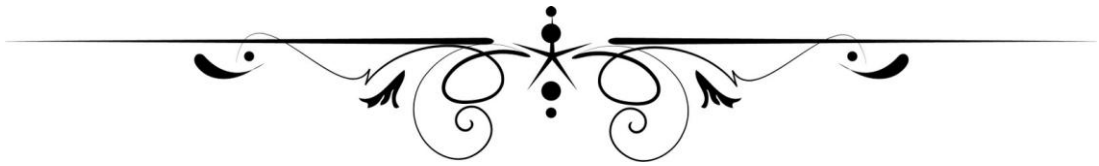
T56: 1kg Petroleum contaminated soil + 235g sand + 750 g garden soil + 15 ml cow urine + 15 g sugarcane bagasse (control)

The developed treatment pots were allowed to stabilize for 90 days. The seeds of *Withania somnifera* were sown in the pots after 90 days. The plant arose from a bacterial treated pot in various treatment expressed high values for all the parameters studied *viz.*germination, shoot length, root length, fresh and dry weight as compared

to the treatment (T16, T26, T36, T46 and T56) without bacterial strains.Further, metabolomics modeling based on the phytochemicals and antioxidant activity infers the variation in the stress effect of petroleum contaminated soil on the treatment with and without bacterial strains.



References



Abdel-Mawgoud, A. M., Aboulwafa, M. M., &Hassouna, N. A. H. (2008). Characterization of surfactin produced by Bacillus subtilis isolate BS5. Applied Biochemistry and Biotechnology, 150(3), 289-303.

Adam, G., & Duncan, H. (2002). Influence of diesel fuel on seed germination. Environmental pollution, 120(2), 363-370.Agency, PublicationEPA 510-B-95-007. Available at http://www.epa.gov/swerust1/cat/biopiles.

Ahammed, G. J., Wang, M. M., Zhou, Y. H., Xia, X. J., Mao, W. H., Shi, K., & Yu, J. Q. (2012). The growth, photosynthesis and antioxidant defense responses of five vegetable crops to phenanthrene stress. Ecotoxicology and environmental safety, 80, 132-139.

Ajithkumar, P. V., Gangadhara, K. P., Manilal, P., &Kunhi, A. A. M. (1998). Soil inoculation with Pseudomonas aeruginosa 3mT eliminates the inhibitory effect of 3-chloro-and 4-chlorobenzoate on tomato seed germination. Soil Biology and Biochemistry, 30(8-9), 1053-1059.

Amund, O. O., &Nwokoye, N. (1993). Hydrocarbon degradation potentials of yeast isolates from a polluted lagoon.

Anderson, C. M., & LaBelle, R. P. (2000). Update of comparative occurrence rates for offshore oil spills. Spill Science & Technology Bulletin, 6(5-6), 303-321.

Anderson, C. M., & LaBelle, R. P. (2000). Update of comparative occurrence rates for offshore oil spills. Spill Science & Technology Bulletin, 6(5-6), 303-321.

Anuradha S, N. (2010). Structural and molecular characteristics of lichenysin and its relationship with surface activity. Biosurfactants, 304-315.

Anyanwu, C. U., Obi, S. K. C., & Okolo, B. N. (2011). Lipopeptide biosurfactant production by Serratia marcescens NSK-1 strain isolated from petroleum-contaminated soil. Journal of Applied Sciences Research, (January), 79-87.

Aparna, A., Srinikethan, G., & Smitha, H. (2012). Production and characterization of biosurfactant produced by a novel Pseudomonas sp. 2B. Colloids and Surfaces B: Biointerfaces, 95, 23-29.

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant physiology, 24(1), 1

Arora, A., Cameotra, S. S., Kumar, R., Balomajumder, C., Singh, A. K., Santhakumari, B., ... &Laik, S. (2016). Biosurfactant as a promoter of methane hydrate formation: thermodynamic and kinetic studies. Scientific reports, 6, 20893.

Asante-Duah, D. K. (1996). Managing Contaminated Sites: Problem, Diagnosis and Development of Site Restoration.

Atlas, R. M. (1975). Effects of temperature and crude oil composition on petroleum biodegradation. Appl. Environ. Microbiol., 30(3), 396-403.

Atlas, R. M. (1985). Effects of hydrocarbons on microorganisms and petroleum biodegradation in arctic ecosystems. Petroleum effects in the arctic environment, 63-100.

Baker, R. S., & Moore, A. T. (2000). Optimizing the effectiveness of in situ bioventing. Pollution Engineering, 32(7), 44-44.

Banks MK, Schultz KE (2005) Comparison of plants for germination toxicity tests in petroleum-contaminated soils. Water, Air, Soil Poll 167(1):211–219

Banks, M. K., Mallede, H., & Rathbone, K. (2003). Rhizosphere microbial characterization in petroleum-contaminated soil. Soil and Sediment Contamination, 12(3), 371-385.

Banks, M. K., Mallede, H., & Rathbone, K. (2003). Rhizosphere microbial characterization in petroleum-contaminated soil. Soil and Sediment Contamination, 12(3), 371-385.

Banks, M. K., Mallede, H., & Rathbone, K. (2003). Rhizosphere microbial characterization in petroleum-contaminated soil. Soil and Sediment Contamination, 12(3), 371-385.

Barriuso, J., Solano, B. R., Lucas, J. A., Lobo, A. P., García-Villaraco, A., &Mañero, F. J. G. (2008). Ecology, genetic diversity and screening strategies of plant growth promoting rhizobacteria (PGPR). Journal of Plant nutrition, 1-17.

Bartha, R. and Bossert, I. (1984). The treatment and disposal of petroleum refinery wastes. In: Petroleum Microbiology (ed. ATLAS, R. M.). New York: Macmillan Publishing Co., pp. 1–61.

Bartlett, M. D., Briones, M. J., Neilson, R., Schmidt, O., Spurgeon, D., & Creamer, R. E. (2010). A critical review of current methods in earthworm ecology: from individuals to populations. European Journal of Soil Biology, 46(2), 67-73.

Baruah, P., Saikia, R. R., Baruah, P. P., & Deka, S. (2014). Effect of crude oil contamination on the chlorophyll content and morpho-anatomy of Cyperusbrevifolius (Rottb.) Hassk. Environmental Science and Pollution Research, 21(21), 12530-12538.

Bashan, Y., & de-Bashan, L. E. (2005). Fresh-weight measurements of roots provide inaccurate estimates of the effects of plant growth-promoting bacteria on root growth: a critical examination. Soil biology and biochemistry, 37(10), 1795-1804.

Beeby, A. (1993). Applying ecology. Chapman & Hall Ltd.

Bento, F. M., Camargo, F. A., Okeke, B. C., &Frankenberger, W. T. (2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresource technology, 96(9), 1049-1055.

Bergey DH, Holt JG, Krieg NR, Sneath PHA (1994). Bergey's Manual of Determinative Bacteriology, 9th ed., (Breed RS, Murray EGD and Smith NR, eds.) Williams and Wilkims, Baltimore.

Bodour, A. A., Drees, K. P., & Maier, R. M. (2003). Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Appl. Environ. Microbiol., 69(6), 3280-3287.

Bohlen, P. J., Groffman, P. M., Fahey, T. J., Fisk, M. C., Suárez, E., Pelletier, D. M., & Fahey, R. T. (2004). Ecosystem consequences of exotic earthworm invasion of north temperate forests. Ecosystems, 7(1), 1-12.

Bona, C., Rezende, I. M. D., Santos, G. D. O., & Souza, L. A. D. (2011). Effect of soil contaminated by diesel oil on the germination of seeds and the growth of Schinus terebinthifolius Raddi (Anacardiaceae) seedlings. Brazilian Archives of Biology and Technology, 54(6), 1379-1387

Braca, A., De Tommasi, N., Di Bari, L., Pizza, C., Politi, M., & Morelli, I. (2001). Antioxidant principles from bauhinia t arapotensis. Journal of natural products, 64(7), 892-895.

Bric, J. M., Bostock, R. M., & Silverstone, S. E. (1991). Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl. Environ. Microbiol., 57(2), 535-538.

Briggs, K. T., Gershwin, M. E., & Anderson, D. W. (1997). Consequences of petrochemical ingestion and stress on the immune system of seabirds. ICES Journal of Marine Science, 54(4), 718-725.

Cameotra, S. S., & Singh, P. (2009). Synthesis of rhamnolipid biosurfactant and mode of hexadecane uptake by Pseudomonas species. Microbial cell factories, 8(1), 16.

Cermak, J. H., Stephenson, G. L., Birkholz, D., Wang, Z., & Dixon, D. G. (2010). Toxicity of petroleum hydrocarbon distillates to soil organisms. Environmental toxicology and chemistry, 29(12), 2685-2694.

Chaillan, F., Chaineau, C. H., Point, V., Saliot, A., &Oudot, J. (2006). Factors inhibiting bioremediation of soil contaminated with weathered oils and drill cuttings. Environmental Pollution, 144(1), 255-265.

Chaineau, C. H., Yepremian, C., Vidalie, J. F., Ducreux, J., &Ballerini, D. (2003). Bioremediation of a crude oil-polluted soil: biodegradation, leaching and toxicity assessments. Water, Air, and Soil Pollution, 144(1-4), 419-440.

Choi, S. C., Kwon, K. K., Sohn, J. H., & Kim, S. J. (2002). Evaluation of fertilizer additions to stimulate oil biodegradation in sand seashore mesocosms. Journal of microbiology and biotechnology, 12(3), 431-436.

Chu, W., & Chan, K. H. (2003). The mechanism of the surfactant-aided soil washing system for hydrophobic and partial hydrophobic organics. Science of the Total Environment, 307(1-3), 83-92.

Cooney, J. J. (1984). The fate of petroleum pollutants in freshwater ecosystems.

Cooney, J. J., Silver, S. A., & Beck, E. A. (1985). Factors influencing hydrocarbon degradation in three freshwater lakes. Microbial ecology, 11(2), 127-137.

D'Apote, S. L. (1998, March). IEA biomass energy analysis and projections. In Proceedings of Biomass Energy Conference: Data, analysis and Trends, Paris: OECD (pp. 23-24).

Das, A. J., & Kumar, R. (2016). Bioremediation of petroleum contaminated soil to combat toxicity on Withaniasomnifera through seed priming with biosurfactant producing plant growth promoting rhizobacteria. Journal of environmental management, 174, 79-86.

Das, A. J., & Kumar, R. (2018). Bioslurry phase remediation of petroleum-contaminated soil using potato peels powder through biosurfactant producing Bacillus licheniformis J1. International journal of environmental science and technology, 15(3), 525-532.

Das, N., & Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnology res

Das, N., & Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnology research international, 2011.

Davis, R. W., Ortega-Ortiz, J. G., Ribic, C. A., Evans, W. E., Biggs, D. C., Ressler, P. H., ... &Würsig, B. (2002). Cetacean habitat in the northern oceanic Gulf of Mexico. Deep Sea Research Part I: Oceanographic Research Papers, 49(1), 121-142.

Dermatas, D., & Meng, X. (2003). Utilization of fly ash for stabilization/solidification of heavy metal contaminated soils. Engineering Geology, 70(3-4), 377-394.

Desai, J. D., & Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev., 61(1), 47-64.

Deziel, E., Paquette, G., Villemur, R., Lepine, F., &Bisaillon, J. (1996). Biosurfactant production by a soil Pseudomonas strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol., 62(6), 1908-1912

Dibble, J. T., & Bartha, R. (1976). Effect of iron on the biodegradation of petroleum in seawater. Appl. Environ. Microbiol., 31(4), 544-550.

Diele, F., Notarnicola, F., &Sgura, I. (2002). Uniform air velocity field for a bioventing system design: some numerical results. International Journal of Engineering Science, 40(11), 1199-1210.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical chemistry, 28(3), 350-356.

EPA. 2000. A Citizen's Guide to Phytoremediation. EPA 542-F-98-011. United States Environmental

Feng, D., Lorenzen, L., Aldrich, C., & Mare, P. W. (2001). Ex situ diesel contaminated soil washing with mechanical methods. Minerals Engineering, 14(9), 1093-1100.

Filler, D. M., Lindstrom, J. E., Braddock, J. F., Johnson, R. A., &Nickalaski, R. (2001). Integral biopile components for successful bioremediation in the Arctic. Cold Regions Science and Technology, 32(2-3), 143-156.

Foght, J. M., Westlake, D. W., Johnson, W. M., & Ridgway, H. F. (1996). Environmental gasoline-utilizing isolates and clinical isolates of *Pseudomonas aeruginosa* are taxonomically indistinguishable by chemotaxonomic and molecular techniques. Microbiology, 142(9), 2333-2340.

Fomina, M. A., Alexander, I. J., Colpaert, J. V., & Gadd, G. M. (2005). Solubilization of toxic metal minerals and metal tolerance of mycorrhizal fungi. Soil Biology and Biochemistry, 37(5), 851-866.

Franzetti, A., Gandolfi, I., Bestetti, G., & Banat, I. (2010). (Bio) surfactant and bioremediation, successes and failures. 145-156.

Franzetti, A., Gandolfi, I., Bestetti, G., Smyth, T. J., & Banat, I. M. (2010). Production and applications of trehalose lipid biosurfactants. European Journal of Lipid Science and Technology, 112(6), 617-627.

FRTR. 1999a. Thermal desorption. Federal Remediation Technologies Roundtable. USEPA, 401M

FRTR. 1999b. Landfarming. Federal Remediation Technologies Roundtable. USEPA, 401 M Street,

Ghatak, A., Chaturvedi, P., & Weckwerth, W. (2018). Metabolomics in plant stress physiology. In *Plant Genetics and Molecular Biology* (pp. 187-236). Springer, Cham.

Gandhimathi, R., Kiran, G. S., Hema, T. A., Selvin, J., Raviji, T. R., &Shanmughapriya, S. (2009). Production and characterization of lipopeptide biosurfactant by a sponge-associated marine actinomycetes Nocardiopsis alba MSA10. Bioprocess and biosystems engineering, 32(6), 825-835.

Genga, A., Mattana, M., Coraggio, I., Locatelli, F., Piffanelli, P., & Consonni, R. (2011). Plant Metabolomics: A characterisation of plant responses to abiotic stresses. In *Abiotic Stress in Plants-Mechanisms and Adaptations*. IntechOpen.

Geys, R., Soetaert, W., & Van Bogaert, I. (2014). Biotechnological opportunities in biosurfactant production. Current opinion in biotechnology, 30, 66-72.

Graj, W., Lisiecki, P., Szulc, A., Chrzanowski, Ł., &Wojtera-Kwiczor, J. (2013). Bioaugmentation with petroleum-degrading consortia has a selective growth-promoting impact on crop plants germinated in diesel oil-contaminated soil. Water, Air, & Soil Pollution, 224(9), 1676.

Handy, A. R. (2007). Acute Toxicity and Immunotoxicity Testing of Total Petroleum Hydrocarbons in Aquatic and Terrestrial Organisms. (Doctoral dissertation). Wright State University.

Hawrot-Paw, M., Wijatkowski, A., &Mikiciuk, M. (2015). Influence of diesel and biodiesel fuel-contaminated soil on microorganisms, growth and development of plants. Plant, Soil and Environment, 61(5), 189-194.

Helvacı, Ş. Ş., Peker, S., &Özdemir, G. (2004). Effect of electrolytes on the surface behavior of rhamnolipids R1 and R2. Colloids and Surfaces B: Biointerfaces, 35(3-4), 225-233.

Hentati, O., Lachhab, R., Ayadi, M., &Ksibi, M. (2013). Toxicity assessment for petroleum-contaminated soil using terrestrial invertebrates and plant bioassays. Environmental monitoring and assessment, 185(4), 2989-2998.

Hentati, O., Lachhab, R., Ayadi, M., &Ksibi, M. (2013). Toxicity assessment for petroleum-contaminated soil using terrestrial invertebrates and plant bioassays. Environmental monitoring and assessment, 185(4), 2989-2998.

Hisatsuka, K., Nakahara, T., & Yamada, K. (1972). Protein-like activator for n-alkane oxidation by *Pseudomonas aeruginosa* S7B1. Agricultural and Biological Chemistry, 36(8), 1361-1369.

Hrynkiewicz, K., & Baum, C. (2012). The potential of rhizosphere microorganisms to promote the plant growth in disturbed soils. In Environmental protection strategies for sustainable development (35-64). Springer, Dordrecht.

Huang, X. D., Glick, B. R., & Greenberg, B. M. (2000). Combining remediation techniques increases kinetics for removal of persistent organic contaminants from soil. In Environmental Toxicology and Risk Assessment: Science, Policy, and Standardization—Implications for Environmental Decisions: Tenth Volume. ASTM International.

Ilori, M. O., Amobi, C. J., &Odocha, A. C. (2005). Factors affecting biosurfactant production by oil degrading Aeromonas spp. isolated from a tropical environment. Chemosphere, 61(7), 985-992.

Inoue, S., & Ito, S. (1982). Sophorolipids from Torulopsisbombicola as microbial surfactants in alkane fermentations. Biotechnology Letters, 4(1), 3-8.

Jahangeer& Kumar, V. (2013). An overview on microbial degradation of petroleum hydrocarbon contaminants. Int J Eng Tech Res, 1(8), 34-37.

Jung, J., Yu, K. O., Ramzi, A. B., Choe, S. H., Kim, S. W., & Han, S. O. (2012). Improvement of surfactin production in *Bacillus subtilis* using synthetic wastewater by overexpression of specific extracellular signaling peptides, comX and phrC. Biotechnology and bioengineering, 109(9), 2349-2356.

Jurelevicius, D., Alvarez, V. M., Marques, J. M., de Sousa Lima, L. R. F., de Almeida Dias, F., & Seldin, L. (2013). Bacterial community response to petroleum hydrocarbon amendments in freshwater, marine, and hypersaline water-containing microcosms. Appl. Environ. Microbiol., 79(19), 5927-5935.

Kapadia, S. G., &Yagnik, B. N. (2013). Current trend and potential for microbial biosurfactants. Asian J. Exp. Biol. Sci, 4(1), 1-8.

Käppeli, O., & Finnerty, W. R. (1979). Partition of alkane by an extracellular vesicle derived from hexadecane-grown Acinetobacter. Journal of bacteriology, 140(2), 707-712.

Khan, F. I., Husain, T., & Hejazi, R. (2004). An overview and analysis of site remediation technologies. Journal of environmental management, 71(2), 95-122.

Khopade, A., Biao, R., Liu, X., Mahadik, K., Zhang, L., &Kokare, C. (2012). Production and stability studies of the biosurfactant isolated from marine Nocardiopsis sp. B4. Desalination, 285, 198-204.

Kim, S. J., Choi, D. H., Sim, D. S., & Oh, Y. S. (2005). Evaluation of bioremediation effectiveness on crude oil-contaminated sand. Chemosphere, 59(6), 845-852.

King JE (1932) The colorimetric determination of phosphorus Biochem J 26:292

Kiran, G. S., Thomas, T. A., Selvin, J., Sabarathnam, B., & Lipton, A. P. (2010). Optimization and characterization of a new lipopeptide biosurfactant produced by marine Brevibacteriumaureum MSA13 in solid state culture. Bioresource technology, 101(7), 2389-2396.

Korade, D. L., &Fulekar, M. H. (2009). Rhizosphere remediation of chlorpyrifos in mycorrhizospheric soil using ryegrass. *Journal of hazardous materials*, *172*(2-3), 1344-1350.

Krebs, C. T., & Tanner, C. E. (1981, March). Cost analysis of marsh restoration through sediment stripping and Spartina propagation. In International Oil Spill Conference (Vol. 1981, No. 1, pp. 386-390). American Petroleum Institute.

Kuiper, I., Lagendijk, E. L., Bloemberg, G. V., &Lugtenberg, B. J. (2004). Rhizoremediation: a beneficial plant-microbe interaction. Molecular plant-microbe interactions, 17(1), 6-15.

Kulakow, P. A., Schwab, A. P., & Banks, M. K. (2000). Screening plant species for growth on weathered, petroleum hydrocarbon-contaminated sediments. International Journal of Phytoremediation, 2(4), 297-317.

Kumar, R., Bharagava, R. N., Kumar, M., Singh, S. K., & Govind, K. (2013). Enhanced biodegradation of mobil oil hydrocarbons by biosurfactant producing bacterial consortium in wheat and mustard rhizosphere. J Pet Environ Biotechnol, 4(5).

Kumar, R., Das, A. J., & Lal, S. (2016). Petroleum hydrocarbon stress management in soil using microorganisms and their products. In Environmental waste management (pp. 539-564). CRC Press.

Kvenvolden, K. A., Rosenbauer, R. J., Hostettler, F. D., &Lorenson, T. D. (2000). Application of organic geochemistry to coastal tar residues from central California. International Geology Review, 42(1), 1-14.

Li, Z., Chang, S., Lin, L., Li, Y., & An, Q. (2011). A colorimetric assay of 1□aminocyclopropane□1□carboxylate (ACC) based on ninhydrin reaction for rapid screening of bacteria containing ACC deaminase. Letters in applied microbiology, 53(2), 178-185.

Loper, J. E., &Schroth, M. N. (1986). Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. Phytopathology, 76(4), 386-389.

Lorestani, B., Ghasemi, M., Cheraghi, M., &Yousefi, N. (2018). Survey the Effect of Oil Pollution on Morphological Characteristics in Faba Vulgaris and ViciaErvilia. Journal of Chemical Health Risks, 2(3).

Lyman, W. J., Reidy, P. J., Levy, B. S., & Fan, C. Y. (1992). Mobility and degradation of organic contaminants in subsurface environments.

Lynch, J. M. (1990). Microbial metabolites. The rhizosphere., 177-206.

Mahesh, N., Murugesh, S., & Srinivasan, V. M. (2010). Determination of the presence of biosurfactant produced by the bacteria present in the soil samples. research journal of microbiology, 5(10), 1031-1037.

Mahmound, A., Aziza, Y., Abdeltif, A., &Rachida, M. (2008). Biosurfactant production by Bacillus strain injected in the petroleum reservoirs. Journal of Industrial Microbiology & Biotechnology, 35(2), 1303-1306.

Maqsood, M. I., & Jamal, A. (2011). Factors affecting the rhamnolipid biosurfactant production. Pak. J. Biotechnol, 8(1), 1-5.

Margesin, R., Walder, G., & Schinner, F. (2000). The impact of hydrocarbon remediation (diesel oil and polycyclic aromatic hydrocarbons) on enzyme activities and microbial properties of soil. Acta Biotechnologica, 20(3□4), 313-333.

Marilley, L., &Aragno, M. (1999). Phylogenetic diversity of bacterial communities differing in degree of proximity of Loliumperenne and Trifoliumrepens roots. Applied Soil Ecology, 13(2), 127-136.

Marín, M., Pedregosa, A., Ríos, S., &Laborda, F. (1996). Study of factors influencing the degradation of heating oil by Acinetobacter calcoaceticus MM5. International biodeterioration & biodegradation, 38(2), 69-75.

Martí, M. C., Camejo, D., Fernández-García, N., Rellán-Álvarez, R., Marques, S., Sevilla, F., & Jiménez, A. (2009). Effect of oil refinery sludges on the growth and antioxidant system of alfalfa plants. Journal of Hazardous Materials, 171(1-3), 879-885.

McCutcheon, S. C., &Schnoor, J. L. (2004). Phytoremediation: transformation and control of contaminants(Vol. 121). John Wiley & Sons.

Mehta, S., &Nautiyal, C. S. (2001). An efficient method for qualitative screening of phosphate-solubilizing bacteria. Current microbiology, 43(1), 51-56.

Melanie Fortune-CHEE 484. 2014. Remediation Technologies - Soil Washing. Assignment.

Mihopoulos, P. G., Suidan, M. T., Sayles, G. D., &Kaskassian, S. (2002). Numerical modeling of oxygen exclusion experiments of anaerobic bioventing. Journal of Contaminant Hydrology, 58(3-4), 209-220.

Mirsal, I. (2004). Soil Pollution: Origin, Monitoring and Remediation. Springer, New York.

Mohn, W. W., & Stewart, G. R. (2000). Limiting factors for hydrocarbon biodegradation at low temperature in Arctic soils. Soil Biology and Biochemistry, 32(8-9), 1161-1172.

Moussa, T. A. A., Mohamed, M. S., &Samak, N. (2014). Production and characterization of di-rhamnolipid produced by Pseudomonas aeruginosa TMN. Brazilian Journal of Chemical Engineering, 31(4), 867-880.

Mulligan, C. N., & Gibbs, B. F. (2004). Types, production and applications of biosurfactants. Proceedings-Indian National Science Academy Part B, 70(1), 31-56.

Mulligan, C. N., Cooper, D. G., & Neufeld, R. J. (1984). Selection of microbes producing biosurfactants in media without hydrocarbons. Journal of fermentation technology, 62(4), 311-314.

Munguia, T., & Smith, C. A. (2001). Surface tension determination through capillary rise and laser diffraction patterns. Journal of Chemical Education, 78(3), 343.

Nalini, S., &Parthasarathi, R. (2014). Production and characterization of rhamnolipids produced by Serratia rubidaea SNAU02 under solid-state fermentation and its application as biocontrol agent. Bioresource technology, 173, 231-238.

Ndimele, P. E. (2010). A review on the phytoremediation of petroleum hydrocarbon. Pakistan Journal of Biological Sciences, 13(15), 715.

Nguyen, T. T., Youssef, N. H., McInerney, M. J., & Sabatini, D. A. (2008). Rhamnolipid biosurfactant mixtures for environmental remediation. Water research, 42(6-7), 1735-1743.

Nievas, M. L., Commendatore, M. G., Esteves, J. L., &Bucalá, V. (2008). Biodegradation pattern of hydrocarbons from a fuel oil-type complex residue by an emulsifier-producing microbial consortium. Journal of hazardous materials, 154(1-3), 96-104.

Nitschke, M., & Costa, S. G. V. A. O. (2007). Biosurfactants in food industry. Trends in Food Science & Technology, 18(5), 252-259.

Nogueira, L., Inckot, R. C., Santos, G. D. O., Souza, L. A. D., & Bona, C. (2011). Phytotoxicity of petroleum-contaminated soil and bioremediated soil on Allophylus edulis. Rodriguesia, 62(3), 459-466.

Obayori, O. S., Ilori, M. O., Adebusoye, S. A., Oyetibo, G. O., Omotayo, A. E., & Amund, O. O. (2009). Degradation of hydrocarbons and biosurfactant production by Pseudomonas sp. strain LP1. World Journal of Microbiology and Biotechnology, 25(9), 1615-1623.

Ohno, A., Ano, T., &Shoda, M. (1993). Production of the antifungal peptide antibiotic, iturin by Bacillus subtilis NB22 in solid state fermentation. Journal of Fermentation and Bioengineering, 75(1), 23-27.

Okoh, A. I. (2006). Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. Biotechnology and Molecular Biology Reviews, 1(2), 38-50.

Onwurah, I. N. E., Ogugua, V. N., Onyike, N. B., Ochonogor, A. E., &Otitoju, O. F. (2007). Crude oil spills in the environment, effects and some innovative clean-up biotechnologies.

Oyaizu M (1986) Studies on product of browning reaction prepared from glucose amine. Jpn J Nutr 44:755–762

Pacwa-Płociniczak, M., Płaza, G. A., Piotrowska-Seget, Z., &Cameotra, S. S. (2011). Environmental applications of biosurfactants: recent advances. International journal of molecular sciences, 12(1), 633-654.

Pacwa-Płociniczak, M., Płociniczak, T., Iwan, J., Żarska, M., Chorążewski, M., Dzida, M., &Piotrowska-Seget, Z. (2016). Isolation of hydrocarbon-degrading and biosurfactant-producing bacteria and assessment their plant growth-promoting traits. Journal of environmental management, 168, 175-184.

Pansiripat, S., Pornsunthorntawee, O., Rujiravanit, R., Kitiyanan, B., Somboonthanate, P., &Chavadej, S. (2010). Biosurfactant production by Pseudomonas aeruginosa SP4 using sequencing batch reactors: effect of oil-to-glucose ratio. Biochemical Engineering Journal, 49(2), 185-191.

Pathak, K. V., &Keharia, H. (2014). Application of extracellular lipopeptide biosurfactant produced by endophytic Bacillus subtilis K1 isolated from aerial roots of banyan (Ficusbenghalensis) in microbially enhanced oil recovery (MEOR). 3 Biotech, 4(1), 41-48.

Pelletier, E., Delille, D., & Delille, B. (2004). Crude oil bioremediation in sub-Antarctic intertidal sediments: chemistry and toxicity of oiled residues. Marine Environmental Research, 57(4), 311-327.

Perry, J. J. (1984). Microbial metabolism of cyclic alkanes. Petroleum microbiology, 61-98.Petroleum Microbiology. Macmillan, New York, pp. 553–578.

Plaza G, Naleez-jaweeki G, Ulfig K, Brigmon RL (2005) The application of bioassays as indicators of petroleum-contaminated soil remediation. Chemosphere 59:289–296.

Płaza, G. A., Zjawiony, I., & Banat, I. M. (2006). Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated and bioremediated soils. Journal of Petroleum Science and Engineering, 50(1), 71-77.

Pothuluri, J.V., Cerniglia, C.E. (1994). Microbial metabolism of polycyclic aromatic hydrocarbons. In: Chaudhhry GR (ed) Biological degradation and bioremediation of toxic chemicals. Dioscorides, Portland Ore. pp 92–124Protection Agency, p. 6. Available at: http//www.bugsatwork.com/XYCLONYX/EPA_GUIDES/

Raheb, J., &Hajipour, M. J. (2011). The stable rhamnolipid biosurfactant production in genetically engineered pseudomonas strain reduced energy consumption in biodesulfurization. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects, 33(22), 2113-2121.

Rahman, P. K., Pasirayi, G., Auger, V., & Ali, Z. (2010). Production of rhamnolipid biosurfactants by Pseudomonas aeruginosa DS10□129 in a microfluidic bioreactor. Biotechnology and applied biochemistry, 55(1), 45-52.

Ramadass, K., Megharaj, M., Venkateswarlu, K., & Naidu, R. (2015). Ecological implications of motor oil pollution: earthworm survival and soil health. Soil Biology and Biochemistry, 85, 72-81.

Raskin, I. (1996). Plant genetic engineering may help with environmental cleanup. Proceedings of the national academy of sciences of the United States of America, 93(8), 3164.

Rikalovic, M. G., GojgićCvijović, G., Vrvic, M. M., & Karadzic, I. (2012). Production and characterization of rhamnolipids from Pseudomonas aeruginosa san-ai. Journal of the Serbian Chemical Society, 77(1), 27-42.

Riser-Roberts, E. (1998). Remediation of petroleum contaminated soils: biological, physical, and chemical processes. CRC press.

Ron, E. Z., & Rosenberg, E. (2002). Biosurfactants and oil bioremediation. Current opinion in biotechnology, 13(3), 249-252.

Rosenberg, M., Gutnick, D., & Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell□surface hydrophobicity. FEMS microbiology letters, 9(1), 29-33.

Rowell, M. J. (1977). The effect of crude oil spills on soils. A Review of Literature. The Reclamation of Agricultural Soils after Oil Spills, Toogood, JA (Ed.). University of Alberta, Edmonton, 1-33.

S.W., Washington, DC, http://www.frtr.gov/matrix2/section4/4_15.html

Saitou, N., &Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution, 4(4), 406-425.

Sangeetha, J., &Thangadurai, D. (2014). Effect of biologically treated petroleum sludge on seed germination and seedling growth of Vigna unguiculata (L.)

Walp.(Fabaceae). Brazilian Archives of Biology and Technology, 57(3), 427-433.

Scheller, U., Zimmer, T., Becher, D., Schauer, F., &Schunck, W. H. (1998). Oxygenation cascade in conversion of n-alkanes to α, ω-dioic acids catalyzed by cytochrome P450 52A3. Journal of Biological Chemistry, 273(49), 32528-32534.

Schwyn, B., &Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. Analytical biochemistry, 160(1), 47-56.

Shaligram, N. S., & Singhal, R. S. (2010). Surfactin–a review on biosynthesis, fermentation, purification and applications. Food technology and biotechnology, 48(2), 119-134.

Shao, Z. (2011). Trehalolipids. In Biosurfactants (pp. 121-143). Springer, Berlin, Heidelberg.

Shodhganga.(2014).shodhganga.inflibnet.ac.in/bitstream/10603/9983/.../07_chapter%202.pdf

Shukry, W. M., Al-Hawas, G. H. S., Al-Moaikal, R. M. S., & El-Bendary, M. A. (2013). Effect of petroleum crude oil on mineral nutrient elements, soil properties and bacterial biomass of the rhizosphere of jojoba. International Journal of Environment and Climate Change, 103-118.

Siciliano, S. D., Germida, J. J., Banks, K., & Greer, C. W. (2003). Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial. Appl. Environ. Microbiol., 69(1), 483-489.

Siegmund, I., & Wagner, F. (1991). New method for detecting rhamnolipids excreted byPseudomonas species during growth on mineral agar. Biotechnology Techniques, 5(4), 265-268.

Smit, B., Stachowiak, M., &Volkenburgh, E. V. (1989). Cellular processes limiting leaf growth in plants under hypoxic root stress. *Journal of Experimental Botany*, *40*(1), 89-94.

Soberón-Chávez, G., & Maier, R. M. (2011). Biosurfactants: a general overview. In Biosurfactants (pp. 1-11). Springer, Berlin, Heidelberg.

Soniyamby, A. R., Praveesh, B. V., Hena, J. V., Kavithakumari, P., Lalitha, S., &Palaniswamy, M. (2011). Enhanced production of biosurfactant from isolated Pseudomonas sp growing on used edible oil. J Am Sci, 7, 50-52.

Speight, J. G. (Ed.). (1999). The desulfurization of heavy oils and residua. CRC Press.

Stavinoha, S., Chen, H., Walker, M., Zhang, B., &Fuhlbrigge, T. (2014, June). Challenges of robotics and automation in offshore oil&gas industry. In The 4th Annual IEEE International Conference on Cyber Technology in Automation, Control and Intelligent (pp. 557-562). IEEE.Street, S.W., Washington, DC, http://www.frtr.gov/matrix2/section4/4_29.html

Streche, C., Cocârță, D. M., Istrate, I. A., &Badea, A. A. (2018). Decontamination of petroleum-contaminated soils using the electrochemical technique: remediation degree and energy consumption. *Scientific reports*, *8*(1), 3272.

Tahseen R, Afzal M, Iqbal S, Shabir G, Khan QM, Khalid ZM, Banat IM (2016) Rhamnolipids and nutrients boost remediation of crude oil-contaminated soil by enhancing bacterial colonization and metabolic activities. Inter BiodeterBiodegrad 115: 192-198.

Tang, J., Wang, M., Wang, F., Sun, Q., & Zhou, Q. (2011). Eco-toxicity of petroleum hydrocarbon contaminated soil. Journal of Environmental Sciences, 23(5), 845-851.

Tuleva, B. K., Ivanov, G. R., &Christova, N. E. (2002). Biosurfactant production by a new Pseudomonas putida strain. ZeitschriftfürNaturforschung C, 57(3-4), 356-360.

Urum, K., &Pekdemir, T. (2004). Evaluation of biosurfactants for crude oil contaminated soil washing. Chemosphere, 57(9), 1139-1150.

Urum, K., Pekdemir, T., &Gopur, M. (2003). Optimum conditions for washing of crude oil-contaminated soil with biosurfactant solutions. Process Safety and Environmental Protection, 81(3), 203-209.

US EPA. (1995). How to evaluate alternative cleanup technologies for underground storage tank sites.

USEPA. 1998b. Biopiles. Office of the Underground Storage Tank, US Environmental Protection

Van Loosdrecht, M. C., Lyklema, J., Norde, W., Schraa, G., & Zehnder, A. J. (1987). The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol., 53(8), 1893-1897.

Viramontes-Ramos, S., Portillo-Ruiz, M. C., Ballinas-Casarrubias, M. D. L., Torres-Muñoz, J. V., Rivera-Chavira, B. E., &Nevárez-Moorillón, G. V. (2010). Selection of biosurfactan/bioemulsifier-producing bacteria from hydrocarbon-contaminated soil. Brazilian Journal of Microbiology, 41(3), 668-675.

Wang, D., Liu, Y., Lin, Z., Yang, Z., & Hao, C. (2008). Isolation and identification of surfactin producing Bacillus subtilis strain and its effect of surfactin on crude oil. Wei sheng wuxue bao= Acta microbiologicaSinica, 48(3), 304-311.

Wang, W. (1987). Root elongation method for toxicity testing of organic and inorganic pollutants. Environmental toxicology and chemistry, 6(5), 409-414.

Wei, Y. H., Chou, C. L., & Chang, J. S. (2005). Rhamnolipid production by indigenous Pseudomonas aeruginosa J4 originating from petrochemical wastewater. Biochemical Engineering Journal, 27(2), 146-154.

Wenzel, W. W. (2009). Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant and Soil, 321(1-2), 385-408.

Whang, L. M., Liu, P. W. G., Ma, C. C., & Cheng, S. S. (2008). Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. Journal of hazardous materials, 151(1), 155-163.

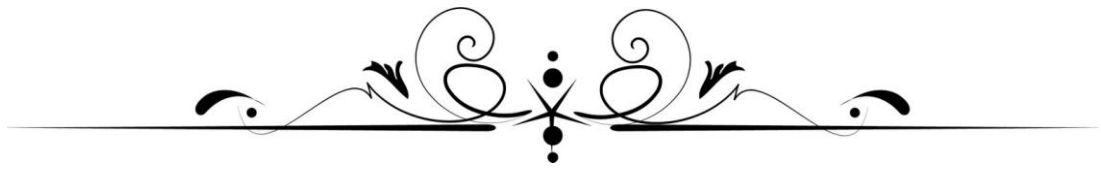
Wu, J. Y., Yeh, K. L., Lu, W. B., Lin, C. L., & Chang, J. S. (2008). Rhamnolipid production with indigenous Pseudomonas aeruginosa EM1 isolated from oil-contaminated site. Bioresource Technology, 99(5), 1157-1164.www.frtr.gov/matrix2/section4/4-19.html

Yumoto, I., Nakamura, A., Iwata, H., Kojima, K., Kusumoto, K., Nodasaka, Y., & Matsuyama, H. (2002). Dietziapsychralcaliphila sp. nov., a novel, facultatively psychrophilic alkaliphile that grows on hydrocarbons. International journal of systematic and evolutionary microbiology, 52(1), 85-90.

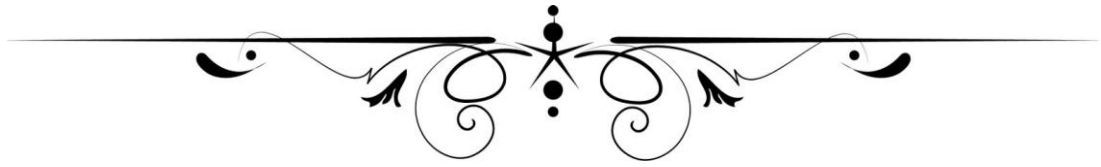
Zhang, Y., & Miller, R. M. (1994). Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Environ. Microbiol., 60(6), 2101-2106

Zhang, Y., & Miller, R. M. (1994). Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Environ. Microbiol., 60(6), 2101-2106.

Zimmer, T., Ohkuma, M., Ohta, A., Takagi, M., &Schunck, W. H. (1996). TheCYP52Multigene Family ofCandidamaltose Encodes Functionally Diversen-Alkane-Inducible Cytochromes P450. Biochemical and biophysical research communications, 224(3), 784-789.

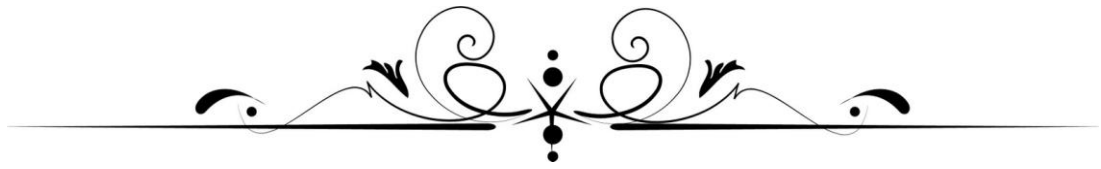


Biographical sketch

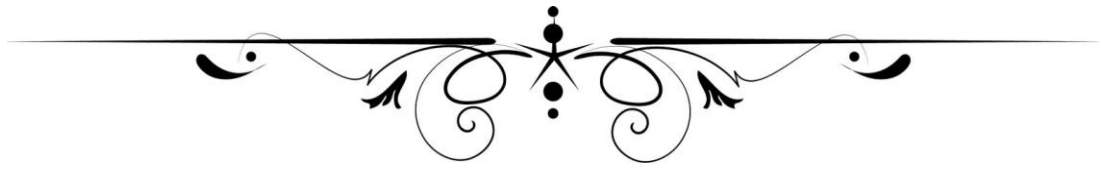


BIOGRAPHICAL SKETCH

Mr. Amar Jyoti Das was born in Tezpur, Assam India and completed his schooling from Don Bosco High School (10^{th} 2006) and Darrang college (12^{th} 2008), Tezpur. He received B.Sc. degree (2011) in Biotechnology, Zoology, Botany and Chemistry from Hemwati Nandan Bahuguna Garhwal (Central) University (HNBGU), Srinagar (Garhwal), Uttarakhand (UK), India and then moved to Babasaheb Bhimrao Ambedkar (Central) University, Lucknow (UP) India where completed M.Sc. degree (2013) in Environmental Microbiology. Because of his keen interest in environmental protection, he joined Babasaheb Bhimrao Ambedkar (Central) University, Lucknow (UP) India to further pursue doctoral research work in environmental microbiology at the Rhizopshere Biology Laboratory, Department of Microbiology (DEM). Presently, Mr. Das is engaged in developing eco-friendly cost-effective treatment solutions for restoration and detoxification of contaminated soil. His research interest includes Environmental Microbiology, Industrial Microbiology, plant-microbe interaction and abiotic stress management. He is a dual gold medalist in M. Sc Environmental Microbiology and has received DST Inspire (JRF & SRF) for pursuing Doctorate degree. He has qualified (2017 & 2018) National Eligibility Test (NET). He has published numerous research and review articles in reputed national and international journal and has presented numerous papers at conferences. He has been won several best presentation and best work award at various national and international conferences and symposiums. He is the author of two (02) books, *"Rhamnolipid Biosurfactant: Recent Trends in Production and Application* (by Springer Nature, Singapore) and *Antibiotic Resistant Bacteria: A Challenge to Modern Medicine* (by Springer Nature, Singapore). He is also serving as a reviewier *for World Journal of Microbiology and Biotechnology (Springer)*. He also holds the life memberships of the Asian PGPR Society of India.



Scientific Publications and Achievements



ACHIEVEMENTS

- **Have been awarded DST Inspire fellowship for pursuing Ph.D.**

- **Have received Anandaram Borah award for matric result.**

- **Have received award for best oral presentation: Amar Jyoti Das (2012)** Gahigaon wetland (Gahigaon Beel) IN biodiversity conservation of Nameri National PARK of Sonitpur district in Assam,North East India**. National Seminar on Wetland in Biodiversity Conservation. BBAU, Lucknow.**

- **Have received award for best oral presentation: Amar Jyoti Das** and Mohammad Athar (2012). Study of threatened plants of Assam and their impact on the traditional system and Biotechnological strategies for their Conservation. National Biotechnology Conference on Leading Ideas and Future Encampment, Ghaziabad.

- **Have received award for best oral presentation: Amar Jyoti Das,** Mohammad Athar, Jai Prakash, Manoj Kumar and Rajesh Kumar (2013).Mangifera indica Bark can be Best Bioindicator of Air Pollution. National Seminar on Environmental Issues and Challenges in the 21st Century, Bareilley.

- **Have received award for best poster presentation: Amar Jyoti Das** and Mohammad Athar (2012). Biocontrol activity of Plant growth promoting rhizobacteria (PGPR): A new vista in the field of Biotechnology for developing ecofriendly pesticides. National Biotechnology Conference on Leading Ideas and Future Encampment, Ghaziabad.

- **Have received award for best poster presentation:** Mohammad Athar, **Amar Jyoti Das,** Gyanendra Kumar, Durgesh Kumar (2013). A Great Menace to the outcome of Air pollution: Reduction in the size of suspended particulate matter. National Seminar on Environmental Issues and Challenges in the 21st Century, Bareilley.

- **Have received award for best oral presentation** .Rajesh Kumar and **Amar Jyoti Das** (2015). Rhamnolipid mediated synthesis of silver nanoparticles and exploring their antibacterial properties presented at Indo-US symposium on Recent trends in Nanobiotechnology organized by Uttarakhand Council for Biotechnology, Haldi, Uttarakhand on 10th March, 2015 at UCB Bhavan Haldi.

SCIENTIFIC PUBLICATIONS

BOOKS

1. Kumar, Rajesh, and **Amar Jyoti Das.** Rhamnolipid Biosurfactant: Recent Trends in Production and Application. Springer, 2018. ISBN 978-981-13-1289-2.
2. Sadhana Sagar, Shilpa Kaistha, **Amar Jyoti Das,** Rajesh Kumar. Antibiotic Resistant Bacteria: A Challenge to Modern Medicine Springer, 2019. (Accepted and on the verge of publication).

RESEARCH & REVIEW ARTICLES

1. **Das, Amar Jyoti,** and Rajesh Kumar. "Utilization of agro-industrial waste for biosurfactant production under submerged fermentation and its application in oil recovery from sand matrix." *Bioresource technology* 260 (2018): 233-240.

2. **Das, Amar Jyoti;** Ambust, S; Kumar, R (2018) 'Development of Biosurfactant Based Cosmetic Formulation of Toothpaste and Exploring its Efficacy'Adv Ind Biotechnol. 1: 005

3. **Das, Amar Jyoti**, and Rajesh Kumar. "Bioslurry phase remediation of petroleum-contaminated soil using potato peels powder through biosurfactant producing Bacillus licheniformis J1." *International Journal of Environmental Science and Technology* 15.3 (2018): 525-532.

4. Verma, Chhaya, **Amar Jyoti Das**, and Rajesh Kumar. "PGPR-assisted phytoremediation of cadmium: an advancement towards clean environment." *CURRENT SCIENCE* 113, no. 4 (2017): 715.

5. Goutam, Surya Pratap, Anil Kumar Yadav, and **Amar Jyoti Das**. "Coriander extract mediated green synthesis of zinc oxide nanoparticles and their structural, optical and antibacterial properties." *Journal of Nanoscience and Technology* (2017): 249-252.

6. **Amar Jyoti Das,** S Lal, R Kumar and C Verma (2016). Bacterial biosurfactants can be an ecofriendly and advanced technology for remediation of heavy metals and contaminated soils. Int. J. Environ. Sci.Technol. doi: 10.1007/s13762-016-1183-0.

7. Rajesh Kumar, **Amar Jyoti Das** (2016). Scenario of Intellectual Property Rights on microorganisms and Microbiological research: An imperative need to amend. International Journal of Intellectual Property Management. DOI: 10.1504/IJIPM.2016.079571.

8. **Amar Jyoti Das,** Rajesh Kumar, Surya Pratap Goutam and Sadhana Singh Sagar (2016). Sunlight Irradiation Induced Synthesis of Silver Nanoparticles usingGlycolipid Bio-surfactant and Exploring the Antibacterial Activity. J Bioengineering and Biomedical Sc. DOI: 10.4172/2155-9538.1000208(OMICS online.

9. **Amar Jyoti Das** and Rajesh Kumar (2016). Bioremediation of petroleum contaminated soil to combat toxicity on *Withania somnifera* through seed priming with biosurfactant producing plant growth promoting rhizobacteria. Journal of Environmental Management, Elsevier, 174: 79-86.

10. Rajesh Kumar, **Amar Jyoti Das** and Asha A Juwarkar (2015). Reclamation of petrol oil contaminated soil by rhamnolipids producing PGPR strains for growing *Withania somnifera* a medicinal shrub. World J Microbiol. Biotechnol. Online doi:10.1007/s11274-014-1782-1, 31:307-313 .

11. Rajesh Kumar and **Amar Jyoti Das** (2014). Climate change and its impact on land degradation: imperative need to focus. J Climatol Weather Forecasting 2:108. doi: http://dx.doi.org /10.4172/2332-2594.1000108 OMICS online.

12. Kumar R, **Das AJ**, Juwarkar AA (2014) Restoration of Petrol Contaminated Soil by PGPR Consortium Producing Rhamnolipids and Enhancement of Growth and Antioxidant activity of *Withania somnifera*. J Pet Environ Biotechnol S5:001. doi: 10.4172/2157-7463.S5-001 **(IF: 4.07, NAAS IF-8.1)**.

13. Athar Mohd and **Das Amar jyoti** (2014). Therapeutic nanoparticles: State-of-the-art of nanomedicine. Advanced Materials Reviews. 1(1), 25-37.

14. **Amar Jyoti Das (2013).** Moringa oleifera (Lamm.): A Plant with immense importance.Journal of Biologically Active Product from ;2(5):561-571

15. Sneha Navin, **Amar Jyoti Das,** Chayya Verma, Manoj Kumar and Rajesh Kumar (2013). Arsenic in the environment effectuates human health: an imperative need to focus. International Research Journal of Environmental Sciences 2(11): 101-105.

16. **Das Amar Jyoti**, Athar Mohammad, Kumar Manoj and Kumar Rajesh (2013). FTIR analysis for screening variation in antimicrobial activity of fresh and dried leaf extract of *Rhynchostylis retusa:* a threatened orchid species of Assam, India. International Research Journal of Pharmacy 4(7): 187-189**.**

17. **Amar Jyoti Das**, Manoj Kumar and Rajesh Kumar (2013). Plant growth promoting rhizobacteria (PGPR): An alternative of chemical fertilizer for sustainable, environment friendly agriculture. Res. J. Agri. Forestry Sc. 1(4): 21-23.

18. **Das AJ**, Kumar R, Mohammad A, Singh RD, Kumar M, Aqueel KM, Jai P. (2013). Ethno medicinal study of threatened plants of Sonitpur district in Assam, North East India. *Int. Res. J. Phar*. 4(1): 146-149.

19. Ratan Singh, **Amar Jyoti Das**, Sabita PokhreL, Preeti Gautam (2013).Biodegradation of pyrene by bacterial strain isolated from petroleum sludge collected from Barauni oil refinery, Begusari, Bihar (India). Journal of pure and applied Microbiology; 7(2):33-31**.**

20. **Das Amar Jyoti** (2012)**.** A study of antibacterial activity of ethanolic extracts and aqueous extracts of *Leucas longifolia* (Doron) leaves against *Eschreiashia coli.* International research journal of pharmacy;3(1):130-131.

21. **Das Amar Jyoti,** Athar Mohammad, Rawat Devendra Singh, Das Pranab Jyoti (2012).Ethno medicinal survey of medicinal plants used to cure wounds in Darikal gaon of Tezpur in Assam, North East India. International research journal of pharmacy;3(2):193-195.

22. **Amar Jyoti Das** and Mohammad Athar(2012). Variation of Antibiotic Sensitivity of *E. coli* Strain against Norfloxacin among Two Persons. Journal of pure and applied Microbiology; 6(1):387-393**.**

23. **Amar Jyoti Das**, Mohammad Athar, Sabita Pokhrel, Ratan Singh and Preeti Gautam (2012). Biotechnology tools:A new strategies for biodegradation of leather industry waste effluent. Journal of Biotechnolgy Biomaterial ; 2(6):264.

24. **Mohammad Athar and Amar Jyoti Das (2012).** Role of nanoparticles in the drug delivery system ;Journal of Biotechnolgy Biomaterial ; 2(6):232

25. **Sabita Pokhrel, Ratan Singh, Preeti Gautam and Amar Jyoti Das (2012). Bioremediation of paper and pulp mill effluents.** Journal of Biotechnolgy Biomaterial ; 2(6):264

26. Athar Mohd and **Das Amar jyoti** (2012). Current trends in drugs discoverey: Target identification to clinical development of the drugs. International research journal of pharmacy;3(4):23-28**.**

27. Athar Mohd and **Das Amar jyoti.** Superconductivity in organic materials: An interesting phenomenon. Research Journal of Chemical Science; 2(11):67-70.

28. **Amar Jyoti Das** (2011). Review on Nutritional, Medicinal and Pharmacological Properties of *Centella asiatica* (*Indian pennywort*). Journal of Biologically Active Product from Nature; 1 (4):216 – 228.

BOOK CHAPTERS

1. **Amar Jyoti Das,** Shweta Ambust, and Rajesh Kumar (2016). Management of Petroleum Industry Waste Through Biosurfactant-Producing Bacteria: A Step Toward Sustainable Environment. Bioremediation of Industrial Waste for Environmental Safety Volume I: Industrial waste and its management . Editors: **Saxena**, Gaurav, **Bharagava**, Ram Naresh (Eds.) ISBN 978-981-13-1891-7.

2. **Amar Jyoti Das,** Beenu Shastri, Shatrohan Lal and Rajesh Kumar (2016). Bioremediation of petroleum hydrocarbons and heavy metal contaminated sites by biosurfactants: an eco-friendly and sustainable technology *In* Bioremediation of industrial pollutants (*Eds)* Ram Naresh Bharagava and Gaurav Saxena, Write and Print Publications, New Delhi-15 Page no. 91-118, ISBN: 978-93-84649-60-9.

3. Rajesh Kumar, **Amar Jyoti Das** and Shatrohan Lal (2015). Petroleum hydrocarbon stress management in soil using microorganisms and their products. Edited book Environmental Stress Management (Ed.) Ram Chandra, CRC press, Taylor and Francis Boca Raton, Florida, USA. Page no. 525-550. **ISBN: 13:978-1-4987-2475-3.**

PAPER PRESENTED

1. **Amar Jyoti Das** and Rajesh Kumar (2017). Production of Biosurfactant from Agroindustrial waste and Development of Cost effective Technology for Restoration of petroleum contaminated wastelands". 58th Annual Conference of Association of Microbiologists of India (AMI-2017)& International Symposium on "Microbes for Sustainable Development: Scope & Applications" (MSDSA-2017) from November 16-19, 2017, Lucknow.

2. **Amar Jyoti Das** and Rajesh Kumar (2016). Remediation and Management of Petroleum Contaminated Soil for Growing Medicinal Plant through Glycolipid Producing Plant Growth Promoting Rhizobacteria. 57th Annual Conference of Association of Microbiologists of India (AMI-2015) and International Symposium on Microbes and Biosphere: What 's New What's Next organized by Department of Botany, Gauhati University, New Delhi from 24th Nov to 27th Nov, 2015.

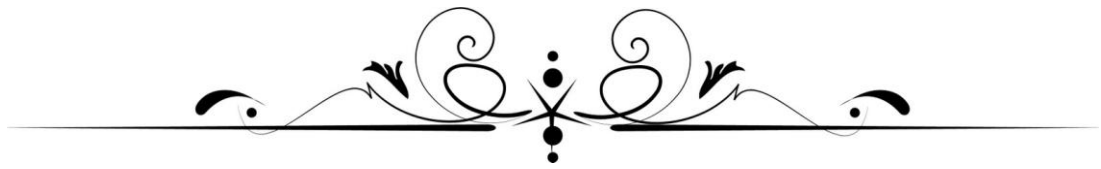
3. **Amar Jyoti Das** and Rajesh Kumar (2015). Development of Low Toxic and Biocompatible Biosurfactant Based Detergent: A Novel Approach. 56th Annual Conference of Association of Microbiologists of India (AMI-2015) and International Symposium on emerging discoveries in Microbiology organized by School of Life Sciences, Jawaharlal Nehru University, New Delhi from 7th Dec to 10th Dec, 2015.

4. Rajesh Kumar and **Amar Jyoti Das** (2015). Rhamnolipid mediated synthesis of silver nanoparticles and exploring their antibacterial properties presented at Indo-US symposium on Recent trends in Nanobiotechnology organized by Uttarakhand Council for Biotechnology, Haldi, Uttarakhand on 10th March, 2015 at UCB Bhavan Haldi. **(Best Paper Award)**.

5. **Das AJ;** Kumar R (2013*). Mangifera indica* bark can be a best indicator of air pollution monitoring. Presented at National seminar on Environmental issues

and challenges in the 21st century sponsored by DST, New Delhi and organized by Bareilly College, Bareilly, Feb 3-5, 2013, published in Abstract Souvenir. (**Best Oral presentation Award)**.

6. **Amar Jyoti Das** and Mohammad Athar (2012). Biocontrol activity of Plant growth promoting rhizobacteria (PGPR): A new vista in the field of Biotechnology for developing ecofriendly pesticides. National Biotechnology Conference on Leading Ideas and Future Encampment, Ghaziabad. **(Best Paper Award)**.



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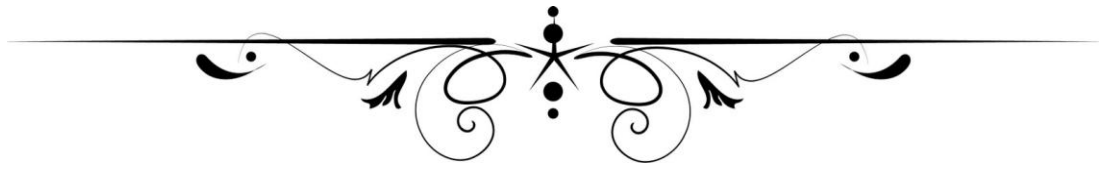

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Utilization of agro-industrial waste for biosurfactant production under submerged fermentation and its application in oil recovery from sand matrix

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ARTICLE INFO

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ABSTRACT

This study reports biosurfactant production by *Pseudomonas azotoformans* AJ15 under submerged fermentation *via* utilizing the agro-industrial wastes (bagasse and potato peels). The extracted biosurfactant was characterized for its classification (nature, group, and class) and stability against environmental stresses. Further, the biosurfactant was employed to explore its oil recovery efficiency from the sand matrix with 2000 ppm salt concentration. Results revealed that substrates developed by mixing both the agro-industrial wastes account for high yield of biosurfactant. The subsequent experimental studies demonstrated that the biosurfactant might belong to glycolipid group and rhamnolipid class. Moreover, the biosurfactant was stable at a high temperature of 90 °C and enable to persist its activity in the high salt concentration of 6% and varying pH. The biosurfactant was found to be effective in recovering up to 36.56% of trapped oil under saline condition.

1. Introduction

The issues related to the pollution and disposal of agro-industrial wastes have encouraged bioconversion of waste into industrially important products (Pandey et al., 2000). Hence, there has been increasing interest towards economical utilization of agro-industrial waste for rhamnolipid (biosurfactant) production (Reddy et al., 2016; Thavasi et al., 2011; Silva et al., 2014). Rhamnolipid biosurfactants are the heterogeneous group of biochemical molecules produced by microorganisms, which hydrophilic moiety consist of one or two rhamnose residues and a hydrophobic moiety of fatty acids. They posses properties such as surface and interfaces reducing ability, foam forming ability and various biological activities (Abdel-Mawgoud et al., 2011; Irfan-Maqsood and Seddiq-Shams 2014; Kumar et al., 2015; Irorere et al., 2017). Such biosurfactants are highly biodegradable, nontoxic, and have the ability to persist its activity at extreme pH, temperature and salinity. In recent time, rhamnolipid biosurfactant is used in environmental management, food, pharmaceutical and petroleum industries due to their environment-friendly and easily degradable nature (De et al., 2015; Rahman and Gakpe, 2008; Sachdev and Cameotra, 2013). In petroleum industries surfactants are used as demulsifiers such as nonyl phenol alkoxylates whose degradation leads to the formation of a more toxic persistent compound like alkyl phenol that affects both terrestrial and aquatic ecosystem (Franzetti et al., 2011). Thus, in seek of nontoxic and eco-friendly demulsifier, biosurfactant is gaining much attention in petroleum industries.

Hence, present study deals with the implication of agro-industrial wastes (sugarcane bagasse and potato peels) for biosurfactant production by *Pseudomonas azotoformans* AJ15 under submerged fermentation. Further, the study evaluates oil recovery from the sand matrix.

2. Materials and methods

2.1. Biosurfactant producing bacterial strain

In this present study, a biosurfactant producing microorganism, identified as *Pseudomonas azotoformans* AJ15 by morphological, biochemical and molecular analysis was used to perform the experiments. The strain was isolated from petroleum oil contaminated soil of Charbagh railway station Lucknow, Uttar Pradesh, India (26°55′ N latitude and 80° 59′ E longitude). Using serial dilution technique the bacterial strain was isolated in Mineral salt agar medium amended with petrol and diesel at 30 ° C in 48 hrs. For screening of biosurfactant production hemolytic assay was performed according to the methods of Mulligan et al. (1984), while drop collapse test was carried out by following the method of Bodour et al. (2003).

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2.2. Utilization of agroindustrial waste for biosurfactant production under submerged fermentation (SmF) and its optimization

Agro-industrial wastes (potato peels and sugarcane bagasse) used in the present study was collected from local potato chips making unit and sugar industry. Potato peels and sugarcane bagasse were washed three times with water and allowed to dry for 24 h at 70 °C. The dried peel and bagasse were ground and passed through a sieve of 0.5 mm to get a fine powder. Fine powders obtained were mixed in various concentrations in 1 L distilled water for the development of substrate for biosurfactant production along with optimized mineral salts (MS) (composition /liter: Na_2HPO_4 5.67 g, $MgSO_4$ 0.5 g, KH_2PO4 1.0 g, $NaNO_3$ 2.5 g, $CaCl_2$ 0.1 g, $FeSO_4$ 0.01 g, KCl 0.1 g, $MnSO_4$ 0.002 g, NH_4NO_3 0.39 g) under submerged fermentation in Erlenmeyer flask. For determining the optimum condition of substrates for maximum biosurfactant production (yield), the developed substrates were monitored at a range of varying pH, temperature and agitation rate. The pH of the substrate was adjusted at 6, 7 and 8 using 1 M NaOH and 1 M HCl solution, while the temperature of the incubator was adjusted at 25, 30, 35, 40 and 45 °C for demonstrating the optimum pH and temperature. For determining the optimum agitation rate, rpm's were set at 100, 140, 180, and 220 rpm.

2.3. Developed substrates

Developed substrates were categorized into 12 group based on their composition, such as **PP1** (5 g Potato peel powder + MS), **PP2** (10 g Potato peel powder + MS), **PP3** (15 g Potato peel powder + MS), **DX1** (5 g Dextrose + MS), **DX2** (10 g Dextrose + MS), **DX3** (15 g Dextrose + MS), **BP1** (5 g Bagasse powder + MS), **BP1** (10 g Bagasse powder + MS), **BP1** (15 g Bagasse powder + MS), **M1** (2.5 g Potato peel powder + 2.5 g Bagasse powder + MS), **M2** (5 g Potato peel powder + 5 g Bagasse powder + MS), **M3** (7.5 g Potato peel powder + 7.5 g Bagasse powder + MS).

2.4. Extraction of biosurfactant

For extraction of biosurfactant, 72 h grown bacterial culture of AJ15 in the substrate was centrifuged at 11,963*g* for 20 min at 4 °C temperature. Culture supernatant (crude biosurfactant) obtained was transferred to empty beakers and stored at 4 °C temperature. Further, 100 ml of crude biosurfactant was acidified with 1 M HCl to obtain a pH of 2.0 and kept at 4 °C. The extraction was performed twice with an equal volume of diethyl ether by vigorously shaking using a separating funnel. The organic layer was collected and transferred to empty beakers and allowed to evaporate for 24 h and then dried in an oven at 60 °C for 1 h. Biosurfactant obtained was transferred to desiccator to remove traces of moisture (Pansiripat et al., 2010; George and Jayac, 2013; Gandhimathi et al., 2009; Das and Kumar, 2018). Thereafter, it was subjected for characterization and for the liquid chromatography–mass spectrometry (LC–MS) analysis.

2.5. Characterization of the extracted biosurfactant

To determine the bacterial adhesion to hydrocarbons method developed by Rosenberg et al. (1980) and Zhang and Miller (1994) was followed. Surface tension reduction ability was calculated according to the procedure of Munguia and Smith (2001). Emulsification index E24 (%) was measured by following the method of Cooper and Goldenberg (1987). Oil displacement test was determined by following the method of Ohno et al. (1993). The ionic property of biosurfactant was determined by following the agar well diffusion method of Meylheuc et al. (2001).

2.6. Toxicity assessment of the extracted biosurfactant

Comparative toxicity assessment of the extracted biosurfactant with nonylphenol ethoxylate (chemical surfactant) was screened through seed germination inhibition assay and earthworm acute toxicity test.

2.6.1. Seed germination inhibition

Certified wheat seeds were surface sterilized with sodium hypochlorite. 10 sterilized seeds of wheat were placed in different Petri dish, followed by addition of 10 ml of extracted biosurfactant (concentrations: 100, 200, 300, 400 and 500 mg) and nonylphenol ethoxylate (concentrations: 100, 200, 300, 400 and 500 mg), plates were incubated for 7 days at 27 °C. For control experiments, 10 seeds of wheat placed in Petri dishes with 10 ml of distilled water for the same duration. Plates were incubated at 27 °C and examined for germination inhibition at every 24 h interval for after 7 days. The experiments were repeated thrice (U. S. Environmental Protection Agency, 1982).

2.6.2. Earthworm acute toxicity test

200 g garden soil was mixed with various concentrations (100, 200, 300, 400 and 500 mg) of biosurfactant and nonylphenol ethoxylate (chemical surfactant) was taken in different brown bottle for screening the earthworm acute toxicity test. Ten healthy earthworms were put into each brown bottle and kept for 7 days. After 7 days death percentage of the earthworms was determined by no response to acupuncture (Tang et al., 2011).

2.7. Chemical analysis of the extracted biosurfactant

For analysis of carbohydrate in the extracted biosurfactant, anthrone, iodine and bial's test were performed, whereas for lipid analysis, solubility and saponification test were performed by following the methods Sawhney and Singh (2000) and Mahesh et al. (2006). Glycolipids production was detected according to the method of Dubois et al. (1956), while rhamnolipids production was checked by utilizing the method of Siegmund and Wagner (1991).

2.8. Structural characterization of the biosurfactant

The chemical composition of biosurfactant was studied by using FTIR spectrometer. One milligram of the extracted biosurfactant was ground with 100 mg of KBr and transmission was recorded using FTIR model Thermo scientific Nicole 6700.

2.9. Liquid chromatography–mass spectrometry (LC–MS) of the biosurfactant

For the LC–MS analysis, 50 mg of the extracted biosurfactant was dissolved in 5000 µL acetonitrile/$HCOONH_4$ buffer in 40/60 (v/v) ratio and then filtered using a 0.20 µm syringe filter (Behrens et al., 2016). The filtered solution was further analyzed using LC-ESI-MS (Liquid chromatography–Mass spectrometry).

2.10. Biosurfactant stability studies

Stability of biosurfactant was studied under a wide range of pH, temperatures and salt concentration to demonstrate the effect on surface tension reducing capability and emulsification index of the biosurfactant. The analysis was done using crude biosurfactant. To analyze the effect of pH on surface tension and emulsification index, 10 ml of crude biosurfactant were adjusted at varying pH from 2 to 12 with 6 N NaOH and 6 N HCl solutions. The effect of temperature was estimated at different temperature of 4, 15, 30, 60 and 90 °C. The effect of salt concentration was checked by varying the concentration of sodium chloride (0% to 6% w/v), added to 10 ml of crude biosurfactant.

2.11. Determination of the Critical Micelle Concentration (CMC) of the biosurfactant

CMC was determined by measuring the surface tension by increasing the biosurfactant concentrations from 100 mg/L to 1000 mg/L.

2.12. Oil recovery from sand matrix

Sand pack technique described by Suthar et al. (2008) and Pathak and Keharia (2014) was followed to determine the oil recovery activity of the biosurfactant from the sand matrix. Briefly, 100 g of sand pretreated by washing with 1 N HCl and dried completely in a hot air oven at 100 °C for 12 h was packed in a 40 × 2.5 cm glass column. The brine solution (2000 ppm NaCl, w/v) was then passed through the column and pore volume was determined by measuring the volume required to make the sand matrix wet in a brine solution. To ensure the saturation three pore volumes of brine was passed through the column. After saturating column with brine solution, crude oil was passed through the column. Discharged of brine solutions from the column was collected and measured to calculate initial oil saturation, while the crude oil entered in the column. The oil saturated column was washed with brine solution until no further oil was discharged in the effluent. The oil retained after brine solution-wash was estimated on the amount of oil loaded and oil discharged in the effluent from the column. The activity was estimated by pouring 50 ml of crude biosurfactant and nonylphenol ethoxylate (commonly used a chemical surfactant in oil recovery) into the column. The amount of oil recovered in 24 h was calculated as described by Suthar et al. (2008) and Gudina et al. (2012).

$$Soi\ (\%) = \frac{OOIP}{PV} \times 100 \quad (1)$$

$$Swi\ (\%) = \frac{PV - OOIP}{PV} \times 100 \quad (2)$$

$$Sor\ (\%) = \frac{OOIP - Sorwf}{OOIP} \times 100 \quad (3)$$

$$OR\ (\%) = \frac{Sorsf}{OOIP - Sorwf} \times 100 \quad (4)$$

Original oil in place (OOIP, ml); pore volume (PV, ml); Initial oil saturation (Soi, %); initial water saturation (Swi, %); Oil recovered after water flooding (Sorwf, ml); Remaining oil saturation (Sor); Oil recovered after surfactant flooding (Sorsf, ml); Oil Recovery (OR, %).

2.13. Molecular characterization of the bacterial strain

For molecular characterization, isolated DNA was electrophoresed in agarose gel. Further, PCR amplification of 16S rDNA was amplified by using universal primers 27F (AGAGTTTGATCMTGGCTCAG-20) and 1492R (GGTTACCTTGTTACGACTT-20). PCR products were viewed on 1% (w/v) agarose gels, cleaned with PCR purification kit and eventually were processed to sequencing analysis. The evolutionary history of the strain was inferred using the Neighbor-Joining method (Saitou and Nei, 1987).

3. Result and discussion

3.1. Molecular characterization of the strain

The scanning electron microscopy analysis of the isolated bacterial strain AJ15 was found to be rod-shaped. Based on molecular characterization, the strain was found similar to *Pseudomonas azotoformans*. The similarity index of the strain and with other strain is shown in Fig. 1. Sequences of the test strain AJ15 have been deposited in the NCBI database and accession number allotted is KU671026.

3.2. Screening for biosurfactant production

The test microorganism *Pseudomonas azotoformans* AJ15 produced biosurfactant on mineral salt medium (MSM) and formed a foam with surfactants. In the hemolytic assay, transparent ring around the bacterial colony indicates that the strain produced biosurfactants as biosurfactant can cause lysis of blood erythrocyte (Mulligan, 1984). Drop collapse test was positive for surfactant production as the drops were spread and collapsed. Results of drop collapse test depend on the destabilization of liquid droplets by surfactants. Liquid drop spread and collapse due to reduced interfacial tension between the liquid and the hydrophobic surface (Bodour et al.2003). But, hemolytic and drop-collapse tests do not do much for biosurfactant identification, hence mass spectrometry study is required for biosurfactant identification (Claus and Van Bogaert, 2017).

3.3. Biosurfactant production and optimization in developed substrates

Increased in biosurfactant yield was observed in the substrates developed by mixing both the agroindustrial wastes Table 1. Highest biosurfactant yield with better activity was reported in developed substrate **M2** (5 g Potato peel powder + 5 g Bagasse powder + MS). The substrates with potato peel and bagasse (as carbon source) was account for high yield of biosurfactant as compared with dextrose. This indicates towards the cost-effective production of biosurfactant for the industrial level. Biosurfactant yield was reported to be maximum in all the substrate at a temperature between 30 °C and 40 °C and this might due to the optimal temperature for growth of bacterial strain. The biosurfactant production also increases with increasing agitation rate i.e 180 rpm, but production rate decreases at 220 rpm. Increased agitation rate provides higher dissolved oxygen concentration in the medium which enhances the production of biosurfactant. At 220 rpm high foam was observed in all the medium that lead to reduction in the transfer of oxygen into the liquid medium resulting in low biosurfactant production rate (Wei et al., 2005; Moussa et al., 2014). The substrates were optimized at various pH ranging from 6 to 8, but highest production rate was reported at neutral pH of 7.

3.4. Characterization of extracted biosurfactant

Cell hydrophobicity is an important factor for predicting cell adhesion to surfaces (van Loosdrecht et al., 1987). Hence bacterial adherence to hydrocarbon (BATH) assay is employed for determining hydrophobicity of cell to hydrophobic substrates. The cell hydrophobicity of strain AJ15 showed 24% adherence, whereas an increase in cell surface hydrophobicity i.e 29% adherence was observed in presence of 0.001 mM rhamnolipid. The results reported were similar to those of Zhang and Miller (1994) who demonstrated that cell hydrophobicity can be induced in the presence rhamnolipid biosurfactant. Cell hydrophobicity effectively increased the biodegradation of hydrophobic organic substrates (Rosenberg and Rosenberg, 1981). Hence; hydrophobicity of cell has immense importance in bioremediation and biodegradation of major organic pollutant. Emulsification capacity of a biosurfactant is of great importance as it can be employed for several applications such as bioremediation oil contaminated sites and for enhanced oil recovery. Emulsification index of biosurfactant produced by the strain AJ15 in **DX2** (10 g dextrose + MS) is 62.7%, whereas 62.23% emulsification index was reported from the biosurfactant produced in the developed substrate **M2** (5 g Potato peel powder + 5 g Bagasse powder + MS). Surface tension of non-inoculated broth **DX2** (10 g dextrose + MSM) was 65.8 mN/m and developed substrate **M2** (5 g Potato peel powder + 5 g Bagasse powder + MS) 65 mN/m. After 72 h the surface tension of both the broths (substrate) were 30.5 mN/m for **DX2** and 30.66 for **M2**, indicating that biosurfactant produced by the strain decrease the surface tension of the broth.

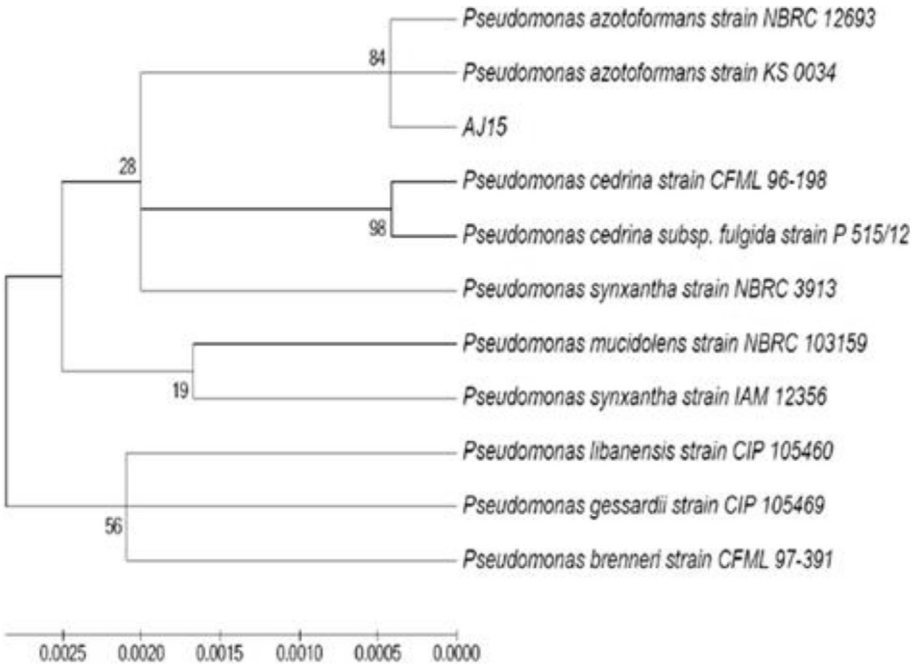

Fig. 1. 16S rRNA based phylogenetic tree of the strain.

Table 1
Effect of developed substrates on bacterial biomass (BB), surface tension reduction (ST), emulsification activity (ET), Oil Displacement area (ODA) and rhamnolipid yield (Rhm).

Composition of developed Substrates	72 h				
	BB (g/L)	Rhm (g/L)	ST (m/Nm)	ET (%)	ODA (cm)
PP1 (5 g Potato peel powder + MS)	2.5 ± 0.15	0.63 ± 0.055	39.33 ± 1.54	56.66 ± 4.16	4.16 ± 0.76
PP2 (10 g Potato peel powder + MS)	3.4 ± 0.3	0.90 ± 0.051	35.33 ± 2.30	60 ± 4.35	4.6 ± 0.52
PP3 (15 g Potato peel powder + MS)	3.86 ± 0.15	0.76 ± 0.045	36.66 ± 1.15	60.66 ± 3.51	4.86 ± 0.20
DX1 (5 g Dextrose + MS)	2.24 ± 0.21	0.75 ± 0.07	34.73 ± 0.461	57.66 ± 2.51	4.033 ± 0.25
DX2 (10 g Dextrose + MS)	2.76 ± 0.15	0.96 ± 0.057	30 .5 ± 0	62.7 ± 2.7	5.43 ± 0.51
DX3 (15 g Dextrose + MS)	3.5 ± 0.2	0.92 ± 0.026	31 ± 1	59.83 ± 1.75	4.26 ± 0.37
BP1 (5 g Bagasse powder + MS)	3.13 ± 0.21	0.63 ± 0.045	37.77 ± 1.15	55 ± 6.24	4.2 ± 0.264
BP2 (10 g Bagasse powder + MS)	3.50 ± 0.11	0.85 ± 0.055	33.33 ± 1.15	61 ± 4.58	4.66 ± 0.35
BP3 (15 g Bagasse powder + MS)	3.70 ± 0.27	0.97 ± 0.030	31.66 ± 0.57	61.60 ± 3.05	4.8 ± 0.45
M1 (2.5 g Potato peel powder + 2.5 g Bagasse powder + MS)	3.033 ± 0.50	0.60 ± 0.305	35.9 ± 0.36	53.66 ± 3.51	3.9 ± 0.1
M2 (5 g Potato peel powder + 5 g Bagasse powder + MS)	3.9 ± 0.21	1.16 ± 0.216	30.66 ± 0.28	62.23 ± 3.027	5.4 ± 0.3
M3 (7.5 g Potato peel powder + 7.5 g Bagasse powder + MS)	4.1 ± 0.26	1.036 ± 0.143	32.16 ± 1.04	58.66 ± 5.13	4.13 ± 0.321

Results analyzed by using single-factor ANOVA of variance and are mean of three replicates with standard deviation (Means ± S.D, n = 3).
All the developed substrates were the composition for 1 L.
MS (mineral salts) = (composition g/l $MnSO_4$: 0.002, $MgSO_4$ (anhydrous): 0.5 g, $NaNO_3$: 2.5 g, KH_2PO_4: 1.0 g , $CaCl_2$: 0.1 g, $FeSO_4$: 0.01 g, NH_4NO_3: 0.39, KCl: 0.1, Na_2HPO_4: 5.67).
Optimized condition of all substrates: Temperature = 35 °C, pH of the substrates = 7, agitation rate = 180 rpm.

3.5. Toxicity of extracted biosurfactant

The non-toxic product is of basic importance for application within the environment domain. Hence, the toxicity of chemical substances can be assessed by eco-toxicity bioassays that use living organism as bio-indicator tool for characterizing toxicity level and predicting the threshold limit of a chemical within a particular ecosystem (Fletcher, 1991; Nalini and Parthasarathi, 2014). Toxicity assessment of the extracted biosurfactant from strain AJ15 exhibited no inhibitory effect on seed germination and earthworm survival rate. The results of seed germination are shown in Fig. 2a. The results demonstrated that seed germination achieved in various concentration (100, 200, 300, 400 and 500 mg) of biosurfactant is better than nonylphenol ethoxylate (chemical surfactant) treated seeds. These might be due to lower toxicity and higher biodegradability of biologically produced surfactants (Franzetti et al., 2011). Earthworms are sensitive to chemical substances present in soil that makes them a good bioindicators soil toxicity assessment. Acute toxicity bioassay is a short-term test for screening the effects of chemical substances on earthworm survivability (Handy, 2007). The literature on the effect of surfactants (biosurfactant and chemical surfactants) on earthworms is scarce. For this reason, we have revealed the effect of surfactants on earthworms. The investigation demonstrated that there is a great effect of chemical surfactant on earthworms. Fig. 2b represents the earthworm survival rate in various concentrations of biosurfactant and chemical surfactant (nonyl phenol ethoxylate). Earthworm survival rate in control treatment (distilled water) was 83.33 ± 5.77%. Apparently, 76.66 ± 5.7%, 76.66 ± 5.77%, 66.66 ± 5.77%, 63.33 ± 11.54% and 63.33 ± 11.5% earthworm survival rates was reported in 100, 200, 300, 400 and 500 mg concentration of biosurfactant whereas, 56.66 ± 5.77%, 53.33 ± 6.7%, 40 ± 10%, 33.33 ± 5.7% and 30 ± 8.164% was observed in 100 , 200, 300, 400 and 500 mg

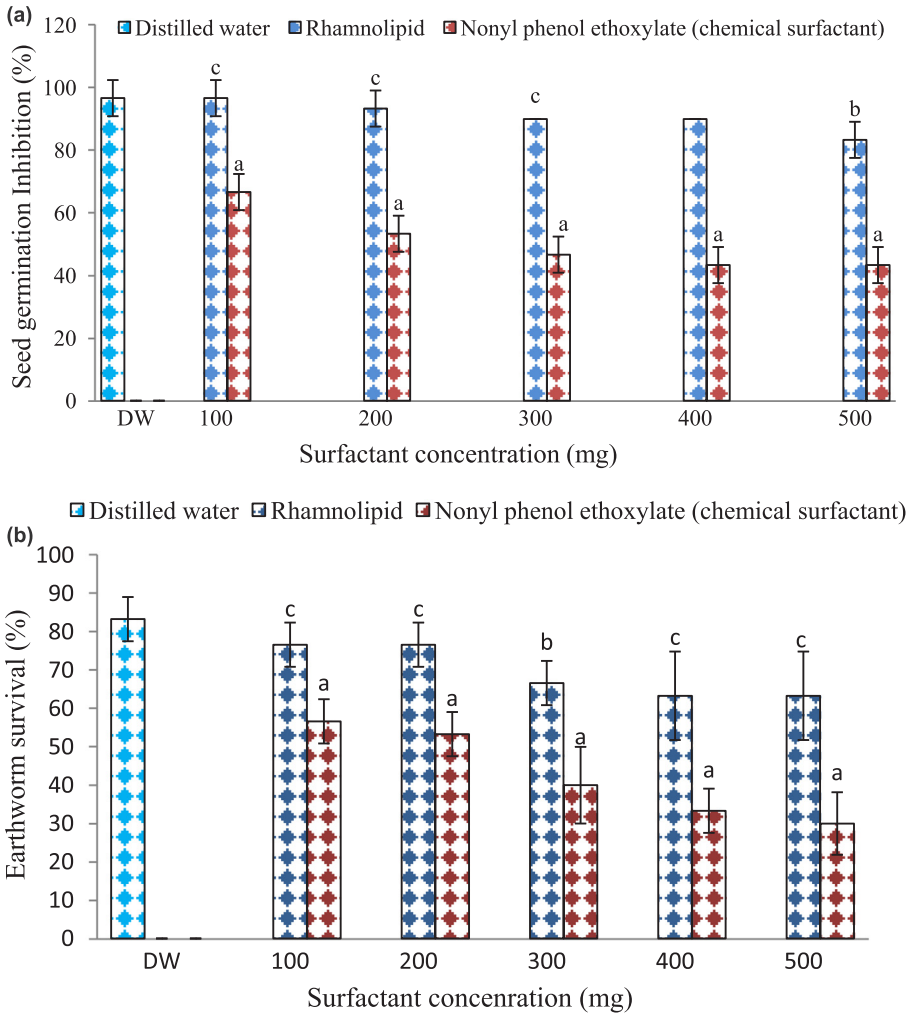

Fig. 2. (a). Effect of surfactant (biosurfactant and chemical surfactant) on seed germination. (b). Effect of surfactant (biosurfactant and chemical surfactant) on earthworm survival. Results analyzed by using single-factor ANOVA of variance and are mean of three replicates with standard deviation (Means ± S.D, n = 3). And differences were considered significant by determining the p-value ($^{a}p < 0.01$, $^{b}p < 0.05$, ^{c}Not significant).

concentration of chemical surfactant (nonyl phenol ethoxylate) respectively. Hence, the results indicate that biosurfactant is eco-friendly in nature and can be alternative to a toxic chemical surfactant that causes the hazardous effect on our environment (Franzetti et al., 2011).

3.6. Chemical analysis of the biosurfactant

The biosurfactant extracted showed a positive result for anthrone test for carbohydrates as the colour change was observed to a bluish green that indicates the presence of carbohydrates in the extracted biosurfactant. A red precipitate formed in barfoeds tests and absence of blue or reddish brown complex in iodine test signifies the presence of monosaccharides carbohydrates and absence of polysaccharides respectively. In Bial's test formation of the greenish blue complex indicates the presence of pentose sugar. The extracted biosurfactant was insoluble in water, whereas as soluble in alcohol and chloroform. In saponification test, NaOH used saponifies the extracted biosurfactant by confirming the presence of lipid in the extracted biosurfactant. In phenol H_2SO_4 method, formation of light orange color indicated that the extracted biosurfactant is glycolipid. In CTAB test dark blue halo zones around colonies of strain AJ15 signifies the production of rhamnolipids. Rhamnolipids consist of a polar head and non-polar tail group like synthetic surfactants and as a result, they combine with cationic cetyl-trimethyl ammonium bromide to form insoluble ion pairs in aqueous solution and precipitates as dark blue halo zones against a blue background (Siegmund and Wagner, 1991). Hence, the results of the chemical analysis revealed that the extracted biosurfactant might belong to glycolipid group and class rhamnolipid.

3.7. FTIR spectroscopy of the extracted biosurfactant

The FTIR spectroscopy of extracted biosurfactant from *Pseudomonas azotoformans* AJ15 shows various characteristic absorptions. The functional group reported in FTIR spectroscopy are C–H stretching (2959.9 cm^{-1} and 2929.1 cm^{-1}), C═O stretching (1643.6 cm^{-1}), C–H deformations (1384.1 cm^{-1}), C–O stretching (1223.4 cm^{-1} and 1067 cm^{-1}). The functional group reported in the present study is much similar to the result reported by Rahman et al. (2010) and Rikalovic et al. (2012). Hence, the extracted biosurfactant might belong to the rhamnolipid class.

3.8. Liquid chromatography–mass spectrometry (LC–MS) of the biosurfactant

Mass spectra obtained from LC–MS analysis of the extracted biosurfactant confirm the presence of rhamnolipid congener. The *m/z* peaks at 522.5, 527.5, and 528.5 with relative abundance of 87%, 98% and 24% respectively suggested that the extracted biosurfactant is mono-rhamnolipid. These findings were also complemented with the previously published report of Arora et al. (2016).

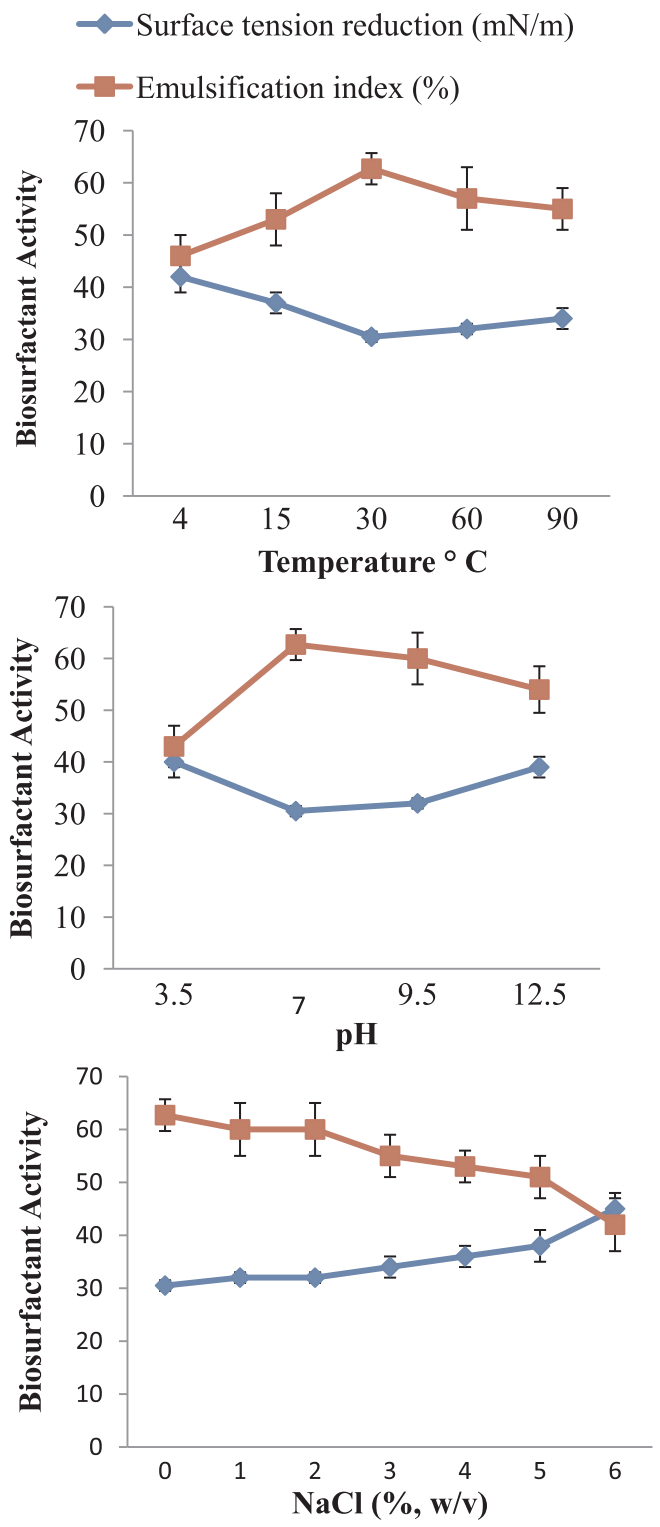

Fig. 3. Effect of temperature, pH and salinity on biosurfactant performance.

3.9. Biosurfactant stability

Fig. 3 represents the effect of pH; temperature and salinity on the stability of extracted biosurfactant. The temperature effect on biosurfactant showed that biosurfactant was quite stable at 30 °C with the surface tension of 30.5 mN/m and emulsification index of 62.7%. Heating of the crude biosurfactant up to 90 °C caused no significant effect on the emulsification and surface tension reducing the capability of the biosurfactant. The Surface tension of 34 mN/m and emulsification index of 55% was reported at 90 °C which indicates it can withstand extreme temperature. Similar results of extreme stability of biosurfactant extracted from *Pseudomonas sps* were obtained by Aparna et al. (2012), Abdel-Mawgoud et al. (2008) and Kiran et al. (2010). Studies on the effect of pH suggest that biosurfactant was stable at pH 7 with the surface tension of 30.5 mN/m and emulsification index. Further, it was also confirmed that extracted biosurfactant remains stable in basic pH as compared to acidic pH. Effect of extreme pH in biosurfactant performance is due to result of structural alteration of the biosurfactant. Results are in accordance with those published by Raheb and Hajipour (2011). The effect of NaCl was investigated by increasing salt the concentrations from 1% to 6%. Very low effect of salinity was observed on biosurfactant stability till 5% concentration of NaCl, but increasing the concentration of the salt above 5% reduced activities of the extracted biosurfactant. This might be due to the electrolytes present in NaCl affects the carboxylate groups of the biosurfactant (rhamnolipid). The interface between solution and air acquires negative charge (due to the ionized carboxylic acid groups at saline pH) which induces the repulsion between biosurfactant (rhamnolipid) molecules. The negative charge is protected by the Na^+ ions in an associate degree electrical double layer within the presence of NaCl, inflicting the formation of a compact monolayer that results in decrease performance of the biosurfactant (Aparna et al., 2012; Helvacı et al., 2004). Hence, the present study indicates that the extracted biosurfactant can be employed in extreme environmental conditions.

3.10. Critical Micelle Concentration (CMC)

The Critical Micelle Concentration (CMC) of the extracted biosurfactant produced by the bacterial strain for surface tension reduction and critical micelle concentration was depicted within Fig. 4.

3.11. Oil recovery from sand matrix with 2000 ppm salt concentration

Fig. 5 represents the results of the rhamnolipid (biosurfactant) and nonyl phenol ethoxylate (chemical surfactant) in the sand pack column. The value of OOIP, PV, Soi %, Swi %, Sorwf, Sor; Sorsf is given in Fig. 5. The oil entrenched column subjected to water flooding by using brine solution containing 2000 ppm sodium chloride (NaCl) concentration. About, 36.56 ± 1.28% additional oil was recovered by biosurfactant from the sand pack column whereas, 43.56 ± 4.2% oil was recovered by nonyl phenol ethoxylate. Oil recovery is traditionally divided into three phases: primary, secondary and tertiary process. Primary recovery involves natural pressure drive technique for oil recovery from the reservoir. After primary recovery can no longer produce oil at enough

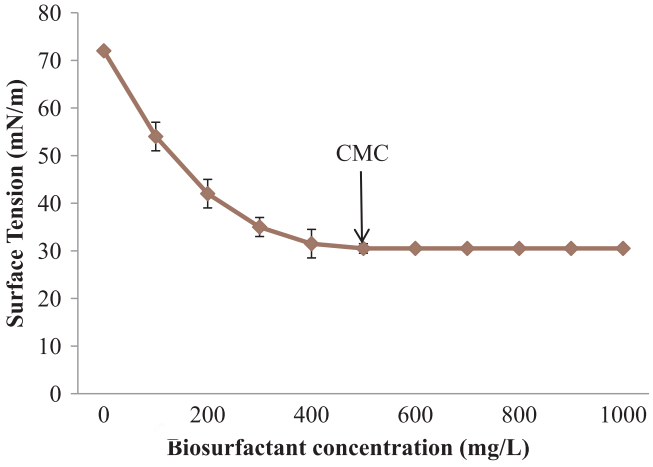

Fig. 4. The Critical Micelle Concentration (CMC) of the extracted biosurfactant from *Pseudomonas azotoformans* AJ15. Condition of the experiment: Temperature: room temperature; pH: 7.

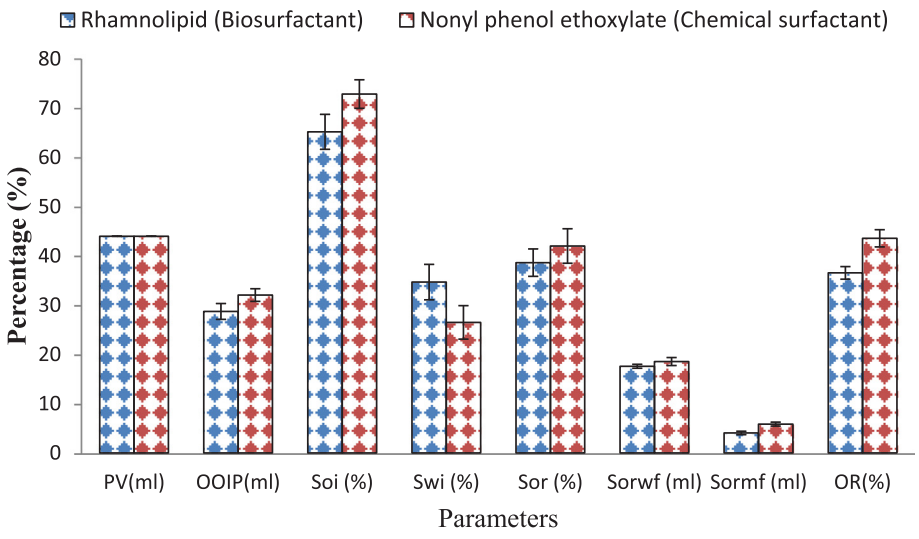

Fig. 5. Results obtained in sand matrix with high salt concentration for oil recovery using by rhamnolipid (biosurfactant) and Nonyl phenol ethoxylate (chemical surfactant). Original oil in place (OOIP, ml); pore volume (PV, ml); Initial oil saturation (Soi, %); initial water saturation (Swi, %); Oil recovered after water flooding (Sorwf, ml); Remaing oil saturation (Sor); Oil recovered after surfactant flooding (Sorsf, ml); Oil Recovery (OR, %).

rate, an additional method is employed in order to maintain reservoir pressure. The most common method of secondary recovery includes water flooding process. Injecting fresh water into a reservoir in water flooding process induces swelling of clay particles, resulting in severe reservoir damage and permeability reduction. Hence, brine containing sodium chloride is used in water flooding process. Tertiary recovery is often implemented for oil recoveries, which mainly include oil recovery using surfactants, but; surfactant activity is greatly affected by the salinity of the brine that is used in secondary recovery phase (Enge, 2014). Hence, in high salinity situation, the application of specifically developed surfactants with reasonable resistance towards high salt concentration is required. Hence, biosurfactant extracted in this study is found to be stable till 5% (5000 ppm) NaCl concentration, suggesting it can withstand high salinity and is suitable for application in oil reservoirs wells with high salinity for tertiary oil recovery. Apart, from it, the present study also demonstrated the biosurfactant can replace the toxic chemical surfactant.

4. Conclusion

Our finding clearly reveals that substrates developed by mixing agroindustrial wastes account for high yield of biosurfactant production by *Pseudomonas azotoformans* AJ15 under submerged fermentation. Biosurfactant produced is non-toxic in nature and posses' excellent emulsification property. Notably, the biosurfactant was enabled to persist its activities over a varying range of pH, temperature, and salinity. Oil recovery operation using the sand-matrix column demonstrated that the biosurfactant can be effectively used for remediation of petroleum contaminated saline soil or in enhanced oil recovery from saline oil wells. The findings suggest that this approach could also be used and can be readily be implemented for fulfilling the enormous demand of surfactant.

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Declarations of interest

The authors have read and approved the final manuscript and there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.03.093.

References

Abdel-Mawgoud, A.M., Aboulwafa, M.M., Hassouna, N.A.H., 2008. Characterization of surfactin produced by *Bacillus subtilis* isolate BS5. Appl. Biochem. Biotechnol. 150 (3), 289–303.

Abdel-Mawgoud, A., Hausmann, R., Lépine, F., Müller, M., Déziel, E., 2011. Rhamnolipids: detection, analysis, biosynthesis, genetic regulation, and bioengineering of production. In: Soberón-Chávez, G., (Ed.). Biosurfactants, vol 20. Microbiology monographs. Springer, Berlin Heidelberg. pp. 13–55.

Aparna, A., Srinikethan, G., Smitha, H., 2012. Production and characterization of biosurfactant produced by a novel *Pseudomonas* sp. 2B. Colloids Surf. B 95, 23–29.

Arora, A., Cameotra, S.S., Kumar, R., Balomajumder, C., Singh, A.K., Santhakumari, B., Kumar, P., Laik, S., 2016. Biosurfactant as a promoter of methane hydrate formation: thermodynamic and kinetic studies. Sci. Report 6, 1–13.

Behrens, B., Engelen, J., Tiso, T., Blank, L.M., Hayen, H., 2016. Characterization of rhamnolipids by liquid chromatography/mass spectrometry after solid-phase extraction. Analyt. Bioanalyt. Chem. 408 (10), 2505–2514.

Bodour, A.A., Drees, K.P., Maier, R.M., 2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid Southwestern soils. Appl. Environ. Microbiol. 69, 3280–3287.

Claus, S., Van Bogaert, I.N., 2017. Sophorolipid production by yeasts: a critical review of the literature and suggestions for future research. Appl. Microbiol. Biotechnol. 101 (21), 7811–7821.

Cooper, D.G., Goldenberg, B.G., 1987. Surface-active agents from two *Bacillus* species. Appl. Environ. Microbiol. 53, 224–229.

Das, A.J., Kumar, R., 2018. Bioslurry phase remediation of petroleum-contaminated soil using potato peels powder through biosurfactant producing *Bacillus licheniformis* J1. Int. J. Environ. Sci. Technol. 15, 525–532.

De, S., Malik, S., Ghosh, A., Saha, R., Saha, B., 2015. A review on natural surfactants. RSC Adv. 5, 65757–65767.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Calorimetric method for determination of sugars related substances. Anal. Chem. 28, 350.

Enge, I.B., 2014. The Effect of Brine Composition and Rock Type on Oil Recovery by the Use of Combined Low-Salinity Waterflooding and Surfactant Flooding: A Literature Review and Experimental Study (Master's thesis). Institutt for petroleumsteknologi og anvendt geofysikk.

Fletcher, J., 1991. A brief overview of plant toxicity testing. In: Goruch, J.W., Lower, W.R., Lewis, M.A., Wang, W. (Eds.), Plants for Toxicity Assessment. ASTM, Philadelphia, pp. 1–11.

Franzetti, A., Gandolfi, I., Bestetti, G., Banat, I.M., 2011. (Bio)surfactant and bioremediation, successes and failures. In: Płaza, G. (Ed.), Trends in Bioremediation and Phytoremediation. Research Signpost, Kerala, India, pp. 145–156.

Gandhimathi, R., Seghal, K.G., Hema, T.A., Selvin, J., Rajeetha, R.T., Shanmughapriya, S., 2009. Production and characterization of lipopeptide biosurfactant by a sponge-associated marine actino- mycetes Nocardiopsis alba MSA10. Bioproc. Biosyst. Eng. 32 (6), 825–835.

George, S., Jayac, K., 2013. Production and characterization of rhamnolipid biosurfactant from waste frying coconut oil using a novel *Pseudomonas aeruginosa* D. J. Appl. Microbiol. 114 (2), 373–383.

Gudina, E.J., Rodrigues, L.R., Teixeira, J.A., Pereira, J.F., Coutinho, J.A., Soares, L.P., Ribeiro, M.T., 2012. Microbial Enhnaced Oil Recovery by Bacillus subtilis strains under simulated reservoir conditions. Abu Dhabi International Petroleum Exhibition

& Conference, held in Abu Dhabi, UAE.
Handy, A.R., 2007. Acute Toxicity and Immunotoxicity Testing of Total Petroleum Hydrocarbons in Aquatic and Terrestrial Organisms (Doctoral dissertation). Wright State University.
Helvacı, Ş.Ş., Peker, S., Özdemir, G., 2004. Effect of electrolytes on the surface behavior of rhamnolipids R1 and R2. Colloids Surf. B 35 (3), 225–233.
Irfan-Maqsood, M., Seddiq-Shams, M., 2014. Rhamnolipid s:well-characterized glycolipids with potential broad applicability as biosurfactants. Ind. Biotechnol. 10, 285–291.
Irorere, V.U., Tripathi, L., Marchant, R., McClean, S., Banat, I.M., 2017. Microbial rhamnolipid production: a critical re-evaluation of published data and suggested future publication criteria. Appl. Microbiol. Biotechnol. 101 (10), 3941–3951.
Kiran, G.S., Thomas, T.A., Selvin, J., Sabarathnam, B., Lipton, A.P., 2010. Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. Bioresour. Technol. 101 (7), 2389–2396.
Kumar, R., Das, A.J., Lal, S., 2015. Petroleum hydrocarbon stress management in soil using microorganisms and their products. Waste Management Edited by Environmental Ram Chandra. CRC Press Taylor and Francis. ISBN.
Mahesh, N., Murgesh, S., Srinivasan, V.M., 2006. Determination of presence of biosurfactant produced by the bacteria present in the soil samples. Res. J. Microbiol. 1, 339–345.
Meylheuc, T., Van Oss, C.J., Bellon-Fontaine, M.N., 2001. Adsorption of biosurfactant on solid surfaces and consequences regarding the bioadhesion of Listeria monocytogenes LO28. J. Appl. Microbiol. 91, 822–832.
Moussa, T.A.A., Mohamed, M.S., Samak, N., 2014. Production and characterization of di-rhamnolipid produced by *Pseudomonas aeruginosa* TMN. Braz. J. Chem. Eng. 31 (4), 867–880.
Mulligan, C., Cooper, D., Neufeld, R., 1984. Selection of microbes producing biosurfactants in media without hydrocarbons. J. Fermen. Technol. 62, 311–314.
Munguia, T., Smith, C.A., 2001. Surface tension determination through capillary rise and laser diffraction patterns. J. Chem. Educ. 78, 343–344.
Nalini, S., Parthasarathi, R., 2014. Production and characterization of rhamnolipids produced by *Serratia rubidaea* SNAU02 under solid-state fermentation and its application as biocontrol agent. Bioresour. Technol. 173, 231–238.
Ohno, A., Ano, T., Shoda, N., 1993. Production of antifungal peptide antibioticsitur in by *Bacillus subtilis* NB22 in solid state fermentation. J. Fermen. Bioeng. 75, 23–27.
Pandey, A., Soccol, C.R., Nigam, P., Soccol, V.T., 2000. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. Bioresour. Technol. 74 (1), 69–80.
Pansiripat, S., Pornsunthorntawee, O., Rujiravanit, R., Kitiyanan, B., Somboonthanate, P., Chavadej, S., 2010. Biosurfactant production by *Pseudomonas aeruginosa* SP4 using sequencing batch reactors: effect of oil-to-glucose ratio. Biochem. Eng. J. 49 (2), 185–191.
Pathak, K.V., Keharia, H.K., 2014. Application of extracellular lipopeptide biosurfactant produced by endophytic *Bacillus subtilis* K1 isolated from aerial roots of banyan (Ficus benghalensis) in microbially enhanced oil recovery (MEOR). 3. Biotech 4, 41–48.
Raheb, J., Hajipour, M.J., 2011. The stable rhamnolipid biosurfactant production in genetically engineered *pseudomonas* strain reduced energy consumption in biodesulfurization. Energy Sour. Part A 33 (22), 2113–2121.
Rahman, P.K.S.M., Gakpe, E., 2008. Production, characterization and applications of biosurfactants – review. Biotech 7, 360–370.
Rahman, P.K.S.M., Pasirayi, G., Auger, V., Ali, Z., 2010. Production of rhamnolipid biosurfactants by *Pseudomonas aeruginosa* DS10-129 in a microfluidic bioreactor. Biotech. Appl. Biochem. 55 (1), 45–52.
Reddy, K.S., Khan, M.Y., Archana, K., Reddy, M.G., Hameeda, B., 2016. Utilization of mango kernel oil for the rhamnolipid production by *Pseudomonas aeruginosa* DR1 towards its application as biocontrol agent. Bioresour. Technol. 221, 291–299.
Rikalovic, M.G., Gojgic-cvijovic, G., Vrvic, M.M., Karadzic, I., 2012. Production and characterization of rhamnolipids from *Pseudomonas aeruginosa* san-ai. J. Serb. Chem. Soc. 77, 27–42.
Rosenberg, M., Rosenberg, E., 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. 148, 51–57.
Rosenberg, M., Gutnick, D., Rosenberg, E., 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9 (1), 29–33.
Sachdev, D.P., Cameotra, S.S., 2013. Biosurfactants in agriculture. Appl. Microbiol. Biotechnol. 97, 1005–1016.
Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Sawhney, S.K., Singh, R., 2000. Introductory Practical Biochemistry, vol. 1, 16–17.
Siegmund, I., Wagner, F., 1991. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. Biotechnol. Technol. 5, 265–268.
Silva, N.M.P., Rufino, R.D., Luna, J.M., Santos, V.A., Sarubbo, L.A., 2014. Screening of *Pseudomonas* species for biosurfactant production using low-cost substrates. Biocatal. Agric. Biotechnol. 3, 132–139.
Suthar, H., Hingurao, K., Desai, A., Nerurkar, A., 2008. Evaluation of bioemulsifier mediated microbial oil recovery using sand pack column. J. Microbiol. Meth. 2008 (75), 225–230.
Tang, J., Wang, M., Wang, F., Sun, Q., Zhou, Q., 2011. Eco-toxicity of petroleum hydrocarbon contaminated soil. J. Environ. Sci. 23 (5), 845–851.
Thavasi, R., Jayalakshmi, S., Banat, I.M., 2011. Application of biosurfactant produced from peanut oil cake by *Lactobacillus delbrueckii* in biodegradation of crude oil. Bioresour. Technol. 102 (3), 3366–3372.
U. S. Environmental Protection Agency, 1982. Seed germination/root elongation toxicity test. EG-12. Office of toxic substances. Washington, D.C.
Van Loosdrecht, M.C., Lyklema, J., Norde, W., Schraa, G., Zehnder, A.J.B., 1987. The role of the bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. 53, 1893–1897.
Wei, Y.H., Chou, C.L., Chang, J.S., 2005. Rhamnolipid production by indigenous Pseudomonas aeruginosa J4 originating from petrochemical wastewater. Biochem. Engineer. J. 27 (2), 146–154.
Zhang, Y., Miller, Y., 1994. Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Environ. Microbiol. 60 (6), 2101–2106.

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REVIEW

Bacterial biosurfactants can be an ecofriendly and advanced technology for remediation of heavy metals and co-contaminated soil

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Abstract Environmental pollution due to heavy metals has become a significant drawback as a result of their ecotoxicity. Hence, their remediation is of pressing concern. Many technologies are planned for their remediation; however, most of them are highly expensive and result in incomplete removal of contaminants. So, massive attention has paid to the event and application of the latest biologically techniques, that is effective in remedy and cost, not harming the prevailing surroundings. Hence, application of biosurfactant in heavy metal remediation is one among the recent ecofriendly technique. The present review critically highlights bacterial biosurfactants as a best alternative technique for heavy metals remediation. The review also emphasizes that bacterial biosurfactants can open up a new vista in remediation of metal-contaminated soil.

Keywords Heavy metal · Biosurfactant · Remediation · Soil washing · Soil flushing · Co-contaminated soil

Introduction

The fate of heavy metals is of immense environmental concern due to their persistent occurrence in nature and toxic properties. Heavy metals are electronegative elements with a density greater than 5 g/cm^3 (Duffus 2002). They are non-biodegradable in nature which is the main reason responsible for their prolonged persistent in the environment, and as a result, they pass from one level to another in the food chain causing many diseases and blocking the biological pathways (Tangahu et al. 2011). Comparably, accumulations of toxic heavy metals in soil and water bodies also have a detrimental effect on the ecosystem (Baeicii and Stotzky 1983; Sobolev and Begonia 2008). Hence, the presence of trace amount of heavy metals in the soils has been found to have serious hazardous effect. There are various techniques for remediation of heavy metal such as physical, chemical, biological and phytoremediation, but most of them are quite expensive and risky. So, large amount of attention has been paid on the development and implementation of new biologically techniques, which should be effective in remediation, easily available, not harm the existing environment, ecofriendliness and cost-effectiveness an alternative of conventional techniques, which are efficient at lower levels of contamination. Hence, application of biosurfactant in remediation of heavy metals is one of the recent ecofriendly techniques. Biosurfactants are diverse group of surface-active compound produced by microorganisms, which possess both hydrophilic and hydrophobic moieties. Structurally, they possess a hydrophobic moiety comprising of saturated or unsaturated fatty acids or hydrocarbon chains and a hydrophilic moiety of peptide cations or anions, mono-, di- or polysaccharides acid (Kiran et al. 2010; Muthusamy et al. 2008). Biosurfactants are potential compounds use in environment management, food industry, petroleum industry, pharmaceutical industry and other industries as these are environment friendly, easily degradable, economical and stable at elevated pH, temperatures and salt concentrations as compared to their

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chemical surfactants (Borjana et al. 2001; Kumar et al. Kumar et al. 2015a, b). The present review critically highlights bacterial biosurfactant and their various strategies for remediation of toxic metal and metal co-contaminated site. The review also emphasizes on both in situ and ex situ technology involved for remediation of heavy metal through biosurfactant.

Ecotoxicity of heavy metal

At present rapid industrialization and mining activities had resulted in heavy metal contamination of soil and water bodies. Heavy metal ions are naturally present in soil, but human activities such as application of pesticides containing metals, electroplating, mining, tanneries, manufacturing paints, batteries, metal pipes, ammunition and sewage sludge and disposal of wastes by various industries have abruptly increases the metal concentration in the environment (Meghraj and Daneshwar 2013). Heavy metals such as lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), arsenic (As), zinc (Zn), mercury (Hg) and nickel (Ni) have been reported as major environmental pollutants due to their terrestrial and aquatic toxicity (Table 1).

Various heavy metal remediation techniques and their limitations

Soil amendments technique

Soil amendments technique is quite used for removal of heavy metals, but it has some limitations (Peng et al. 2009). When organic materials amended in the soil, they tightly bind with heavy metal, causing difficulties in metal removal process. This process take more time, henceforth it affect the removal of metals. If nutrient availability in the soil is much higher with heavy metal, it can leach-out to surrounding water body and causes water pollution. Soil acidification, as well as soil physical degradation and attendant, increased the incidence of soil erosion (Kader 2012; Lege 2012; Sekar 2013). Besides all these limitations, high cost and amendments of inorganic fertilizers may also increase the heavy metal concentration (Guman 2011; Exma 2012). The limitation of the use of mineral fertilizers to improve soil fertility has consequently resulted in a shift of attention to the use of organic fertilizers for soil fertility improvement, especially the highly metal-contaminated soil (Ame 2012; Kader 2012). However, the use of organic fertilizer, too, has certain demerits of slow release and non-synchronization of nutrient release with periods of growth for most short-term crops, as well as being required in large quantities to sustain crop production, which may not readily be available to the small-scale farmers (Kiani et al. 2005; Peng et al. 2009; Guman 2011).

Sand capping

Sand capping or landfill technique is the most operant technique where a large quantity of inherent waste is above the water table. This technique is applied for giving an impermeable boundary to surface water invasion to the contaminated soil for the anticipation of further leaching of contaminants to the surrounding surface water or groundwater (Peng et al. 2009). With the usefulness, this technique also has some loopholes. It cannot impede the flow of groundwater along with contaminants without any external support. In several cases, sand caps are applied in collaboration with some vertical defenders to minimize the flat stream and movement of metal pollutants. This collaboration may cause the overflow of contaminants into surrounding soil and water body (Cornelissen et al. 2011). Through this technique, we can just diminish the exchange rate of metal in sediment, while their immobilization impact of heavy metal is less (Gome et al. 2013). This technique is just a break arrangement since the contamination remains on site resultant continuous monitoring and maintenance required for isolated barriers for long into the future, with all the related expenses and potential risk (Theofanis et al. 2001; Peng et al. 2009; Gome et al. 2013).

Phytoremediation

Phytoremediation is a very slow process. It generally depends on seasons and locations, hence highly affected by climate changes. Phytoremediation is a technique in which green living plants are use for the remediation of metals from soil. It is a green and ecofriendly technique with some limitations. This technique is limited to sites with small contamination concentration; the higher concentration of contaminant may check the plant growth. (Tangahu et al. 2011; Ali et al. 2013).

Electrochemical remediation

Electrochemical remediation technology (ECRTs) is a piece of a more extensive class of advanced technique known as direct current technology (DCTs). These techniques utilize an electric current in the treatment procedure to either activate or separate contaminants in soils. The reaction rate of ECRT techniques is conversely proportional to grain size, so ECRTs remediate quicker in silts and sediments than in sands and rock. The working

Table 1 Toxicity profile of heavy metals (Modified from Das et al. 2016)

Heavy metals	Toxicity on human beings	Toxicity on plants	References
Cadmium (Cd)	Causes renal dysfunction, obstructive lung disease, cadmium pneumonitis (resulting from inhaled dusts and fumes). It also causes chest pain, cough with foamy and bloody sputum and death of the lining of the lung tissues. Osteomalacia, osteoporosis and spontaneous fractures (bone defects), increment within the blood pressure and myocardic dysfunctions	Reduction in photosynthesis, nutrient and water uptake. Chlorosis, growth inhibition, browning of root tips and eventually death	McCluggage (1991), INECAR (2000), European Union (2002), Wójcik and Tukiendorf (2004), Mohanpuria et al. (2007)
Mercury (Hb)	Exerts neurotoxicological disorders, gingivitis, damage to the central nervous system and brain, psychological changes and congenital malformation	Induces physiological disorders in plants and induces leaf stomata to close and physical obstruction of water flow in plants	Ferner (2001), Lennetech (2004) Zhou et al. (2007), Zhang and Tyerman (1999)
Lead (Pb)	Affects the kidneys, gastrointestinal tract, reproductive system and joints. Inhibition in hemoglobin synthesis and also causes chronic or acute damage to nervous system	It causes adverse effect on growth, morphology and photosynthetic processes of plants also causes inhibition in the enzyme activity, water imbalance, alterations in membrane permeability and disturbance in mineral nutrition	Ogwuebgu and Muhanga (2005), McCluggage (1991), Ferner (2001), Sharma and Dubey (2005)
Arsenic (As)	It causes irritation to stomach, lung, intestine, skin and decreased formation of red blood corpuscle and white blood corpuscle. Very high concentrations of inorganic arsenic can cause infertility, brain damage and death and heart disruptions	Arsenic is non-essential and generally toxic to plants. Arsenate is an analog of phosphate and can disrupt at least some phosphate-dependent aspects of metabolism. Excess amount inhibits proliferation and root extension. Upon translocation to the shoot, it can adversely affect the plant growth by arresting expansion and biomass accumulation	Ogwuegbu and Ijioma (2003), Kantor (2006), Meharg and Macnair (1992), Meharg (1994)
Copper (Cu)	In humans, the copper is essentially needed, but their high doses lead to anemia, liver, kidney damage, stomach and intestinal irritation. During Wilson's disease, it affects greatly	Copper inhibits the growths and alteration of plasma membrane permeability. Its toxicity can also induce the deficiency of essential elements and leading to inhibition of the element-ion dependent reaction	Pandey and Madhuri (2014), Ouzounidou (1995)
Chromium (Cr)	Chronic exposure can damage kidney, circulatory, nerve tissues and liver. It also gets accumulated in the fishes and can cause toxicity while eating those fishes	Excess amount of chromium leads to inhibition of plant growth and chlorophyll biosynthesis, chlorosis in young leaves, nutrient imbalance and root injury	Pandey and Madhuri (2014), Chatterjee and Chatterjee (2000), Dixit et al. (2002), Sharma et al. (2003), Scoccianti et al. (2006), Vajpayee et al. (2000)
Zinc (Zn) Zinc is considered non-toxic, when taken orally	Zinc toxicity causes vomiting, diarrhea, bloody urine, icterus (yellow mucus membrane), liver, kidney failure and anemia. Its excess amount leads to system dysfunctions which result in impairment of growth and reproduction	Its toxicity retards both root and shoot of plant and leads to chlorosis, senescence. Excess amount of Zn can also give rise to manganese, copper and phosphorus deficiencies in plants	Fosmire (1990), INECAR (2000), Nolan (2003), Choi et al. (1996), Ebbs and Kochian (1997), Fontes and Cox (1998), Lee et al. (1996)
Selenium (Se) The Se is required in small amounts by humans and other animals but in excess amount harmful	Selenium toxicity fatigueness, irritability and damage liver tissue, nervous system, kidney and circulatory tissue. It also gets accumulated in the fishes and can cause toxicity while eating those fishes. Sometimes there may be a problem of hair and fingernail loss due to Se	An excess of selenium in plants can adversely affect the seed germination and growth	Pandey and Madhuri, (2014), Irwin et. al. (1997)

profundity of the technology is restricted by the accessibility of drilling to assemble the electrode (Yeung and Gu 2011; Peng et al. 2009). The main limitation of this technique is the requirement of energy, i.e., electric current that are not easily available and make it costly. Another important factor that limits the technique is the solubility of the contaminant in which the electrokinetic process is limited by desorption of contaminants from the soil matrix. When soil gets contaminated by toxic heavy metals, then these metals cannot easily dissolve and separate from soil samples by this technique. Organic compounds make tight coordination to soil particles; consequently, they cannot easily dissolve into ions so that by this technique we cannot easily remediate soil contaminated by organic compounds. This process is likewise less fruitful when the targeted ion concentration is low and non-target ions focus is high (Niroumand et al. 2012; Peng et al. 2009).

Flotation

Flotation is a process mainly based on separation technique in which the particles are grabs by bubbles and their accumulation in a foam layer. It can likewise be utilized to succeed the classification techniques with a specific end goal to clean the fines fraction. Along with good remediation efficiency, the technique also has some limitations. In the separation of small particles flotation causes problems. Factors like entrapment and entrainment lead to the reduction in efficiency (Cauwenberg et al. 1998; Peng et al. 2009). The entrapment problems occur when trapping fine hydrophilic particles by hydrophobic particles or aggregation of bubble particles. In flotation process, some metal sulfides are oxidized, released and subsequently redistributed in other fractions, like immediately precipitated iron oxides, which would reduce the degree of efficiency of flotation (Peng et al. 2009). However, the process is complex and has higher operating costs than other remediation techniques (Cauwenberg et al. 1998; Peng et al. 2009).

Why sustainable alternative technique for remediation of heavy metal is so important?

The increasing concentration of heavy metal in aquatic and terrestrial ecosystems due to anthropogenic activities is a wide spread and serious problem within the globe. For these reasons, several techniques such as mechanical, physical and chemical methods along with phytoremediation of heavy metals have been studied widely. Mechanical, physical and chemical methods are expensive techniques which lead to incomplete removal of contaminants, while phytoremediation technique has risk as plant matter can act as potential transfer of contaminants to the food chain leading to biomagnifications of heavy metals from one to trophic level to another (Shahid et al. 2014). Hence, biosurfactant-assisted remediation of heavy metal can be regarded as new biological and ecofriendly technique for removal of heavy metals. The main reasons that make biosurfactants promising alternative agents for remediation purposes are low toxicity, environmental biodegradability, compatibility, production from low-cost organic wastes and agro-based raw materials, active even at extremes of pH, salt concentration and temperature (Georgiou et al. 1992; Mukherjee et al. 2006).

The contributions of biosurfactant in remediation of heavy metal

Metals need sufficient time to stabilize in the long-term contamination; henceforth, their removal becomes more difficult. There are many reports from the research done in recent years which explain the successful contribution of biosurfactant in bioremediation of metal-contaminated soils. Tan et al. (1994) performed an experiment in which 5 mM solution of rhamnolipid produced by *Pseudomonas aeruginosa* strain ATCC9027 complexed 92% of cadmium. Frazer (2000) stated that cadmium (Cd) and lead (Pb) have more affinity toward rhamnolipids than any other soil components. Experiment was conducted with four different types of soils contaminated by Cd that was first treated with KNO (7 or 3.5 mM K^+) electrolyte solution and then further treated with a rhamnolipid solution (10 or 5 mM). Results demonstrated that between 15 and 36% of the Cd was removed by the electrolyte solution and an additional 8–54% of the Cd was removed by rhamnolipid solution. Thus, it is proved that rhamnolipid treatment was very effective (Torrens et al. 1998). Several studies have also shown that rhamnolipid produced by various strains of *Pseudomonas aeruginosa* is adept of complexing cationic metal species of Cd, Pb and Zn (Herman et al. 1995; Torrens et al. 1998; Juwarkar et al. 2007; Asci et al. 2008). Similarly, Juwarkar et al. (2008) conducted a column experiment to study the feasibility of dirhamnolipid produced by *Pseudomonas aeruginosa* strain BS2 for removal of metal from multimetal (Cd, Cr, Cu, Pb and Ni)-contaminated soil (Cd—430 ppm, Cr—940 ppm, Cu—480 ppm, Pb—900 ppm, Ni—880 ppm). During this study, metal-spiked soil washed in a glass column with a solution of 0.1% di-rhamnolipid resulted in a removal of Cr by 13-fold, Pb by nine to tenfold, Cu by 14-fold, Cd and Ni by 25-fold within 36 h. Das et al. (2009) demonstrated the possibility for removal of Pb and Cd from solutions by anionic marine biosurfactant. The ability of marine biosurfactant to remediate heavy metals through formation of insoluble precipitate proved as a beneficial technology for

removal of toxic metal from wastewater. Biosurfactant foam technology is another effective method for the remediation of heavy metal-contaminated soil. Wang and Mulligan (2009) performed an experiment to study the usefulness of rhamnolipid foam to chelate Ni and Cd from a sandy soil. After applying rhamnolipid foam, efficiency increases and chelates 68.1% of Ni and 73.2% of Cd, while the rhamnolipid solution removed 51% of Ni and 61.7% of Cd, respectively. Hence, the study demonstrated that rhamnolipid foam is a non-toxic and effective method of removing heavy metal from contaminated soils. Dahrazma and Mulligan (2007) demonstrated that removal rate of heavy metal increases (Cu and Ni) in the presence of 1% NaOH along with rhamnolipid solution. Most of the metals in environment usually present in the organic fraction, and hydroxide treatment solubilizes this fraction by increasing the metals availability for removal by a biosurfactant. Research conducted by Wang and Mulligan (2009) indicates that biosurfactants have provable application in remediating mine tailings or arsenic (As)-contaminated soil. The biosurfactant remediates As either through micelles formation or reducing the interfacial tension between mine tailings and arsenic. Gnanamani et al. (2010) remediate Cr(VI) by using biosurfactant produced by *Bacillus* sp strain MTCC 5514. The remediation occurred in two steps: firstly, reduction of Cr(VI) to Cr(III) through chromium reductase and further Cr(III) entrapment with biosurfactants. The first step converts the toxic state of Cr into low toxic state, and the second step prevents the bacterial cells from Cr(III) exposure. Both the steps keep strain active and also provide resistivity and tolerance toward Cr(VI) and Cr(III). Surfactin from *Bacillus subtilis* was also used to demonstrate its feasibility for the remediation of Zn, Cu and Cd heavy from a contaminated sediments and soil. And the results predict that after first and fifth washings of the soil, 6 and 25% of the zinc, 25 and 70% of the copper and 5 and 15% of the Cd could be remediated by 0.1% surfactin along with 1% NaOH, respectively (Mulligan et al. 1999). In a study, surfactin from *Bacillus subtilis*, sophorolipid from *Torulopsis bombicola* and rhamnolipids from *Pseudomonas aeruginosa* were screened using sediment with metal contaminated (3300 mg/kg zinc and 110 mg/kg copper). Washing the sediment with 4% sophorolipid remediate 60% of the zinc and 25% of the copper, washing with 0.5% rhamnolipid remediate, 18% of the zinc and 65% of the copper, while washing with surfactin was less effective with a removing rate of 6% of the zinc and 15% of the copper. Bioemulsans also to a great extent showed success in remediation of heavy metals. Franzetti et al. (2008) demonstrated that the bioemulsans were capable to remediate Cu, Cd, Pb, Zn, Ni, but their ability is lower than that of rhamnolipids.

Methods of management of heavy metal-contaminated soil through biosurfactant

Two methods, soil washing and soil flushing, are involved in remediation of metal-contaminated soil. Soil washing is an ex situ technique, where contaminated soil is excavated, put into the glass column and washed with biosurfactant solution. Consecutively, soil flushing is a in situ technique which involves drain pipes and trenches for introducing and collecting biosurfactant solution to and from the soil (Singh and Cameotra 2004).

Soil washing

Soil washing is an ex situ remedial method to treat soil contaminated with inorganic or organic compounds. In this method, solvents (biosurfactant) are employed to scrub contaminant from the soil (Melanie Fortune—CHEE 484). Various anionic, cationic and nonionic surfactants are used for soil washing. They remove the pollutants by reducing the interfacial tension resulting in mobilization of the pollutant. The mobilized pollutant can be recovered from the leachate (Khan et al. 2004; Kumar et al. 2015b). Selection of specific solvents for washing the soil depends on the basis of their ability to solubilize specific contaminants (Asante-Duah 1997; Chu and Chan 2003; Urum et al. 2003; Khan et al. 2004). Hence, biosurfactants are regarded as best due to their non-toxic and biodegradable nature (Fig. 1).

Soil flushing

Soil flushing is an ex situ technique involved in remediation of metal-contaminated soil. The surfactant flushing as an emerging technology first was described by Pankow and Cherry (1996). The soil flushing is an in situ method which employs the trenches and drain pipes for introducing and collecting biosurfactant solution to and from the soil (Herman et al. 1995; Singh and Cameotra 2004). Soil flushing involves an injection/recirculation process. In flushing technique heavy contaminated soil is placed in a huge cement mixer treated with biosurfactant, biosurfactant from metal complex flushed out, soil deposited back and biosurfactant–metal complex treated to precipitate out biosurfactant, leaving the metal (Lee and Zhai 2007). The bond formed between the negatively charged surfactant and positively charged metal is very strong that while flushing water through the soil removes the metal surfactant complex from the soil (Singh and Cameotra 2004). The flushing process starts with the drilling of injection and extraction wells into the ground where the metal contamination has been reported. The soil flushing injection pumps the (bio)surfactant solution into the injection wells.

The solution passes through the soil forming complexes with the contaminants (metals) as it moves toward the extraction wells. The extraction wells stockpile the flushing mixture solution of contaminants (metal) and surfactant. The contaminant mixture is then treated in a treatment system for retrieving the surfactant to remove the (metals) contaminants (Lee and Zhai 2007) (Fig. 2).

Benefits of using biosurfactant in metal remediation

Various researchers have shown that biosurfactants exhibit advantages (De et al. 2015) and the advantages of biosurfactants are as follows.

Low toxicity

Biosurfactant showed lower toxicity than the chemical surfactants. Kanga et al. (1997) showed that glycolipids produced by *Rhodococcus species* strain 413A were 50% low toxic than Tween 80 while evaluating naphthalene solubilization test.

Biodegradability

Due to biological origin and chemical nature, biosurfactant exhibits low toxicity, and these make them non-persistent and easily degraded by microorganism (De et al. 2015).

Biocompatibility

Biological origin of biosurfactant renders them inherent feature of biocompatibility, which allows their usage in agricultural fields, cosmetic industry, functional food additives and pharmaceuticals (De et al. 2015).

Production from inexpensive materials

Production of biosurfactants can be affordable by using inexpensive raw materials available in abundance, such as using agro-industrial and organic waste (De et al. 2015).

Biosurfactants and its properties

Biosurfactants are surface-active agents stable at extreme salinity, pH and temperature. They have startling properties such as complicated and bulky structures, liquid crystals forming potentiality, multiple chiral and diversified biological properties (Xie et al. 2005). They are non-toxic and biodegradable in nature as compared to synthetic surfactants. The most important properties that play an immense role in heavy metal remediation are their capacity to form micelle with metal. The non-covalent interactions occur through solvophobic effects of hydrophobic part of biosurfactant for self-aggregation resulting in structures such as vesicles and micelles. Vesicles are hollow spheres surrounded by bilayer of amphiphilic surfactants, whereas micelles subsist in

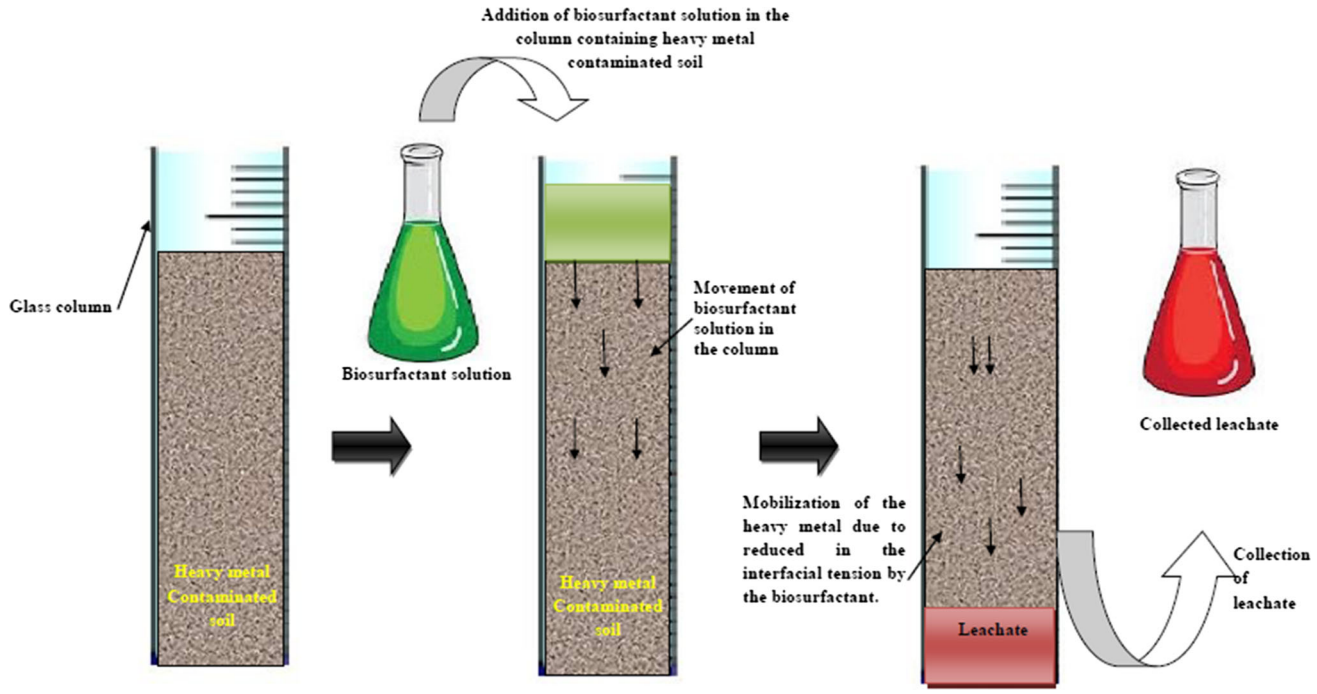

Fig. 1 Process of soil washing technique with biosurfactant

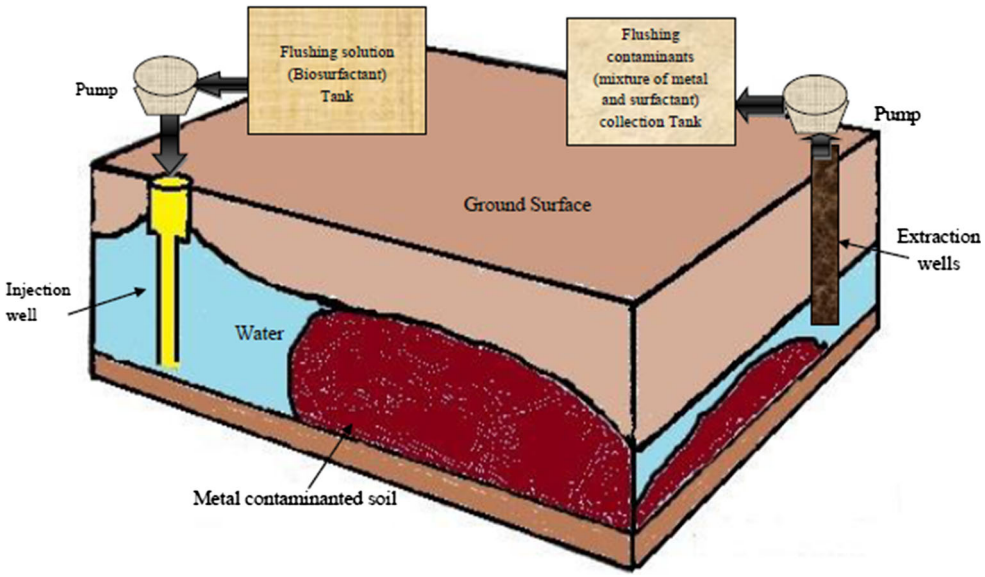

Fig. 2 Process of soil flushing technique with biosurfactant

various structures, suchlike cylindrical ellipsoidal and spherical form (Davies et al. 2006; Engberts and Kevelam 1996). Concentrations of biosurfactants above the critical micelle concentration (CMC) give rise to micelles. Micelle formation facilitates biosurfactants to reduce the interfacial and surface tension and increase the bioavailability and solubility of hydrophobic organic compounds (Kumar et al. 2015b; Whang et al. 2008) (Fig. 3a, b). Critical micelle concentration is generally employed to measure the efficacy of surfactant. Efficient biosurfactants have a low critical micelle concentration, which indicates that very low concentration of surfactant is needed to reduce the surface tension (Desai and Banat 1997). Micelle formation has an immense role in formation of micro-emulsion. Micro-emulsions are clear composite of water domains and oil scattered by monolayer of surfactants and are usually stable (Nguyen et al. 2008; Desai and Banat 1997). Micro-emulsions exist in two forms i.e reverse micro-emulsion and direct emulsion, when water phase is dispersed as droplets in oil phase is known as reverse micro-emulsion, while oil phase dispersed in water known as direct micro-emulsion (Desai and Banat 1997). Efficacy of biosurfactant depends on its molecular mass. High molecular mass biosurfactants are capable of stabilizing oil in water emulsions, while low molecular mass biosurfactants are more efficient in lowering interfacial and surface tensions (Kumar et al. 2015b; Geys et al. 2014; Pacwa-Płociniczak et al. 2011). The biosurfactants assemble at the interface between two immiscible fluids or between a solid and a fluid by lowering surface tension (Fig. 2). The most active biosurfactants have the capability for lowering the surface tension of water from 72 to 25–30 mM/m and also have the potentiality of lowering the interfacial tension between water and *n*-hexadecane (Soberón-Chávez and Maier 2011; Desai and Banat 1997).

Types of biosurfactants

Biosurfactants are generally categorized into two classes depending on the mass of the surfactant. The low mass biosurfactants include lipopeptides, glycolipids and phospholipids, while high mass biosurfactants include particulate and polymeric surfactants (Kumar et al. 2015b; Nitschke and Coast 2007; Kappeli and Finnerty 1979) as listed in Table 2.

Mechanisms involved behind the removal of metals by biosurfactant

Biosurfactants enhance desorption of heavy metals from solid phases by two ways (Miller 1995). First is complexation of free forms of metal residing in solution. This decreases the solution phase activity of the metal and, therefore, promotes desorption according to Le Chatelier's principle. Secondly, under reduced interfacial tension, biosurfactants accumulate at solid solution interface. This allows direct contact of biosurfactant to sorbed metal at solid solution. Miller (1995) also stated that biosurfactant structure size and charge affect the movement of biosurfactant–metal complexes through the soil. In addition, the structure size and charge affect the access of biosurfactants to soil pores and, therefore, the interaction of biosurfactant with sorbed metals. Soil

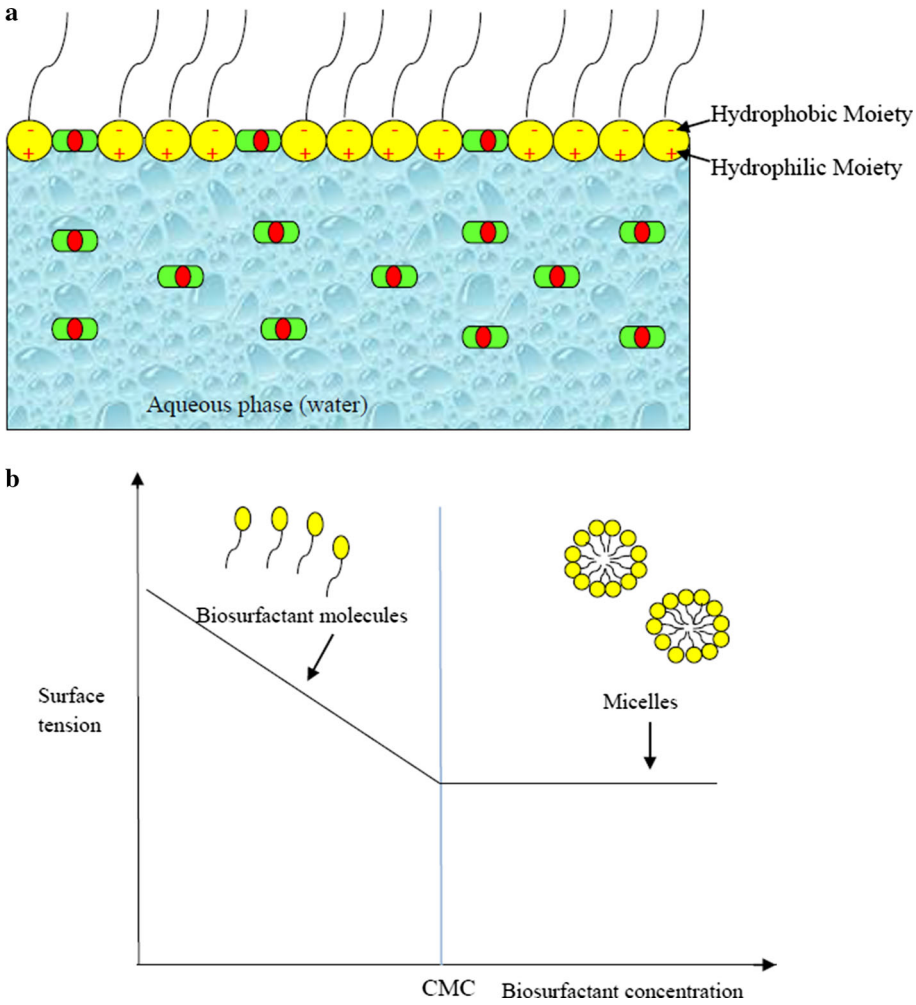

Fig. 3 **a** Biosurfactants accumulation at the interface between air and liquid. **b** Reduction of surface tension and formation of micelles with biosurfactant concentration (Modified from Wang et al. 2008; Pacwa-Polciniczak et al. 2011; Kumar et al. 2015b)

composition, soil pH, cation exchange capacity (CEC), particle size, time and type of contamination, etc., also play a major role in establishing the effectiveness of biosurfactant action (Mulligan et al. 2001a, b). Bioremediation of heavy metal-contaminated soil by biosurfactants is mainly based on their ability to form complexes with metals. Metal ions can be removed from soil surfaces also by the biosurfactant micelles (Mulligan and Gibbs 2004; Singh and Cameotra 2004; Mulligan 2005; Juwarkar et al. 2007; Asci et al. 2008). Micelles can bind metals which mobilize the metals in water as shown in Fig. 4.

Metal remediation in co-contaminated soils

Soil contaminated with both metals and organic chemicals and metals create challenges in terms of their remediation (Maier et al. 2001). Remediation of such sites is more challenging because metals are not biodegradable and hinder microbial activity by reducing its efficiency to degrade hydrocarbon (Shukla and Cameotra 2012). Malakul et al. (1998) first reported the beneficial role of surfactant in remediation of co-contaminated sites. They developed a model system comprising biodegradation of naphthalene by *Pseudomonas putida* in the presence of cadmium to demonstrate the metal toxicity associated with biodegradation of polyaromatic hydrocarbon in presence of surfactant-modified clays. They reported reduction in cadmium toxicity to *Pseudomonas putida* in surfactant-modified clay complex. Hence, the study depicts that the surfactant-modified clay adsorbent is an economical and efficient technology remediation of co-contaminated soil. But, most chemically synthesized surfactant showed toxicity. Hence, microorganisms producing biosurfactant could be better candidates for remediation of co-contaminated soil. Todd et al. (2000) explored the effectiveness of biosurfactant (rhamnolipid) in the remediation of naphthalene and cadmium co-contaminated site. They reported biosurfactant (rhamnolipid) and could reduce the cadmium toxicity to allow enhanced naphthalene degradation by *Burkholderia* sp. Rhamnolipid biosurfactant removed

Table 2 Types of biosurfactants (Modified from Kumar et al. 2015b; Pacwa-Polciniczak et al. 2011; Das et al. 2016)

Biosurfactant		Producing microorganism	References
Groups	Class		
Glycolipids: glycolipids are low molecular weight biosurfactants in which carbohydrates are attached to a long-chain aliphatic acid	Rhamnolipids: rhamnolipids are glycolipids that are composed of one or two L-rhamnose molecules coupled to a mono- or dimer of b-hydroxy fatty acids	*Pseudomonas aeruginosa, Pseudomonas* sp., *Burkholderia* sp.	Ron and Rosenberg (2002), Pacwa Płociniczak et al. (2011), Geys et al. (2014)
	Trehalolipids: trehalolipids are glycolipids that contain trehalose lipids as hydrophilic moiety	*Mycobacterium tuberculosis*, *Rhodococcus erythropolis*, *Arthrobacter* sp., *Nocardia* sp., *Corynebacterium* sp.	Płociniczak et al. (2011), Shao (2011))
	Sophorolipids: sophorolipids are glycolipids in which dimeric carbohydrate sophorose is linked to a long-chain hydroxyl fatty acid by glycosidic linkage	*Torulopsis bombicola, Torulopsis petrophilum, Torulopsis apicola*, *Starmerella bombicola, Wickerhamiella domercqiae, Candida batistae*	Płociniczak et al. (2011), Geys et al. (2014), Kapadia and Yagnik (2013)
Lipopeptides: lipopeptides are biosurfactants of low molecular weight in which consist of a lipid attached to a polypeptide chain	Surfactin: surfactin is cyclic lipopeptides which consist of a seven-amino-acid-ring structure coupled to a fatty-acid chain via lactone linkage	*Bacillus subtilis*	Wang et al. (2008), Płociniczak et al. (2011), Kapadia and Yagnik (2013)
	Lichenysin: lichenysin anionic cyclic lipoheptapeptide biosurfactants produced by *Bacillus licheniformis*	*Bacillus licheniformis*	Nerurkar (2010)
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	Płociniczak et al. (2011), Kapadia and Yagnik (2013)
	Spiculisporic acid	*Penicillium spiculisporum*	
	Phosphatidylethanolamine	*Acinetobacter* sp., *Rhodococcus erythropolis*	
Polymeric biosurfactants	Emulsan	*Acinetobacter calcoaceticus RAG-1*	
	Alasan	*Acinetobacter radioresistens KA-53*	
	Biodispersan	*Acinetobacter calcoaceticus A2*	
	Liposan	*Candida lipolytica*	
	Mannoprotein	*Saccharomyces cerevisiae*	
Particulate biosurfactants		*Acinetobacter* sp.	

cadmium toxicity when added at a tenfold greater concentration (890 μM), while reduced toxicity at an equimolar concentration (89 μM) had no effect at tenfold low concentration (8.9 μM). They proposed that the mechanism by which rhamnolipid biosurfactant reduces metal toxicity involves a combination of rhamnolipid complexation of cadmium and rhamnolipid interaction with the cell surface to alter cadmium uptake ensuing in enhanced rates of bioremediation. Mulligan et al. (1999b) demonstrated that rhamnolipid, surfactin, sophorolipids and all anionic biosurfactants were able to remove copper and zinc from a hydrocarbon-contaminated soil. Lima et al. (2011) stated that lipopeptide biosurfactants may be successfully used for simultaneous removal of heavy metal ions and organic pollutants. Advantage of biosurfactant in remediation of co-contaminated soil is that biosurfactants potentially can be produced in situ employing the organic contaminants and waste as substrates for their production by reducing the remediation cost (Singh and Cameotra 2004).

Conclusion

The application of biosurfactant in remediation heavy metal is of great interest, owing to their biodegradability and low toxicity. Moreover, biosurfactant production from low-cost renewable substrates (agro-industrial wastes) have create their production additional economically possible. The present review furnishes that the bacterial biosurfactants can open up a new vista for remediation of metals and metal co-contaminated soils. Therefore, research should be carried out in this field as very few researches indicate success story of bacterial biosurfactants in remediation of metals and metal co-contaminated sites. Future studies have to be carried out in this regard so as to extend

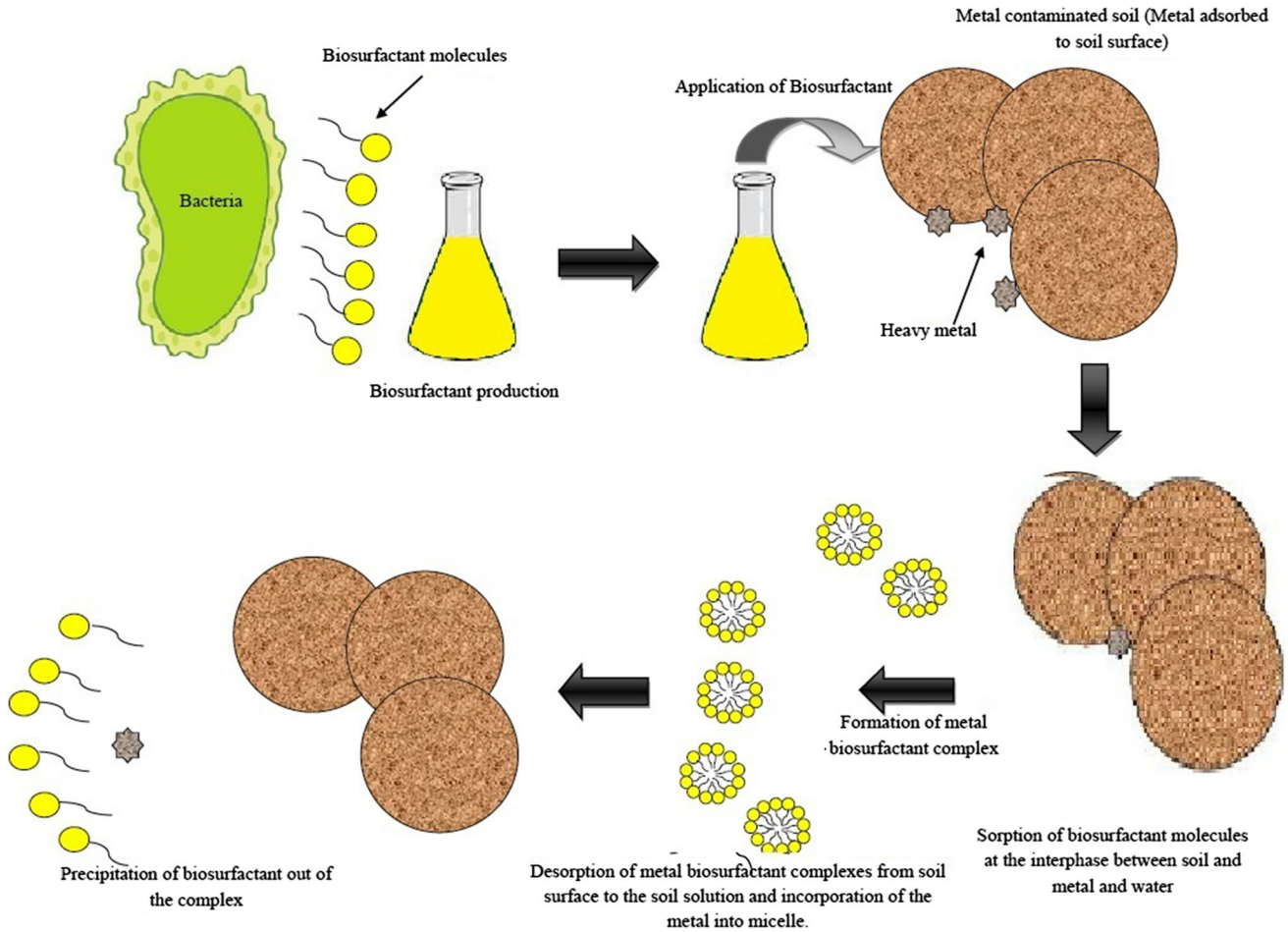

Fig. 4 Mechanism of biosurfactant activity in metal-contaminated soil

biosurfactant production together with the seek for new forms of biosurfactant and technology for its application on heavy metal remediation.

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References

Ali H, Khan E, Sajad MA (2013) Phytoremediation of heavy metals—concepts and applications. Chemosphere 91:869–881

Ame SP (2012) Influence of integrated application of organic and inorganic fertilizers on chemical properties of a degraded acid Alfisol in southwestern Nigeria. J Pure Appl Sci 51:822–828

Asante-Duah DK (1997) Managing contaminated sites. Wiley, New York

Aşçı Y, Nurbas M, Acikel YS (2008) A comparative study for the sorption of Cd(II) by soils with different clay contents and mineralogy and the recovery of Cd(II) using rhamnolipid biosurfactant. J Hazard Mater 154:663–673

Baeicii H, Stotzky G (1983) Toxicity of nickel to microbes: environmental aspects. Adv Appl Microbiol 29:195

Borjana KT, George RI, Nelly EC (2001) Biosurfactant production by a new *Pseudomonas putida* strain. Am J Sci Ind Res 57:356–360

Cauwenberg P, Verdonckt F, Maes A (1998) Flotation as a remediation technique for heavily polluted dredged material. 2. Characterisation of flotated fractions. Sci Total Environ 209(2):121–131

Chatterjee J, Chatterjee C (2000) Phytotoxicity of cobalt, chromium and copper in cauliflower. Environ Pollut 109:69–74

Choi JM, Pak CH, Lee CW (1996) Micronutrient toxicity in french marigold. J Plant Nutr 19:901–916

Chu W, Chan KH (2003) The mechanism of the surfactant-aided soil washing system for hydrophobic and partial hydrophobic organics. Sci Total Environ 307:83–92

Cornelissen G, Elmquist Kruså M, Breedveld GD, Eek E, Oen AM, Arp HPH, Raymond C et al (2011) Remediation of contaminated marine sediment using thin-layer capping with activated carbon a field experiment in Trondheim Harbor, Norway. Environ Sci Technol 45:6110–6116

Dahrazma B, Mulligan CN (2007) Investigation of the removal of heavy metals from sediments using rhamnolipid in a continuous flow configuration. Chemosphere 69:705–711

Das P, Mukherjee S, Sen R (2009) Biosurfactant of marine origin exhibiting heavy metal remediation properties. Bioresour Technol 100:4887–4890

Das AJ, Shastri B, Lal S, Kumar R (2016) Bioremediation of Petroleum Hydrocarbons and Heavy Metal Contaminated Sites by Biosurfactants: An Eco-friendly and Sustainable Technology. In: Bhargava RN, Saxena G (eds) Bioremediation of Industrial Pollutants, Chapter 3. Educationist Press (Write and PrintPublications)

Davies TS, Ketner AM, Raghavan SR (2006) Self-assembly of surfactant vesicles that transform into viscoelastic wormlike micelles upon heating. J Am Chem Soc 128:6669–6675

De S, Malik S, Ghosh A, Saha R, Saha B (2015) A review on natural surfactants. RSC Adv 5:65757–65767
Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol R 61:47–64
Dixit V, Pandey V, Shyam R (2002) Chromium ions inactivate electron transport and enhance superoxide generation in vivo in pea (*Pisum sativum* L.cv. Azad) root mitochondria. Plant Cell Environ 25:687–693
Duffus John H (2002) Heavy metals "a meaningless term? (IUPAC Technical Report)". Pure Appl Chem 74:793–807
Ebbs SD, Kochian LV (1997) Toxicity of zinc and copper to Brassica species: implications for phytoremediation. J Environ Qual 26:776–781
Engberts JBFN, Kevelam J (1996) Formation and stability of micelles and vesicles. Curr Opin Coll Inter Sci 1:779–789
European Union (2002) Heavy metals in wastes. European Commission on Environment. http://ec.europa.eu/environment/waste/studies/pdf/heavy_metalsreport.pdf
Exma LT (2012) Responses of an acid Alfisol and maize yield to wood ash based soil amendments. J Food Agric Res 31:42–427
Ferner DJ (2001) Toxicity, heavy metals. eMed J 2:1
Fontes RLS, Cox FR (1998) Zinc toxicity in soybean grown at high iron concentration in nutrient solution. J Plant Nutr 21:1723–1730
Fosmire GJ (1990) Zinc Toxicity. Am J. Clinical Nutrition 51(2):225–227
Franzetti A, Bestetti G, Caredda P, Colla-La P, Tamburini E (2008) Surface-active compounds and their role in the access to hydrocarbons in *Gordonia* strains. FEMS Microbiol Ecol 63:238–248
Frazer L (2000) Lipid lather removes metals. Environ Health Perspect 108:320–323
Georgiou G, Lin SC, Sharma MM (1992) Surface active compounds from microorganisms. Biotechnology 10:60–65
Geys R, Soetaert W, Bogaer IV (2014) Biotechnological opportunities in biosurfactant production. Curr Opin Biotechnol 30:66–72
Gnanamani A, Kavitha V, Radhakrishnan N, Rajakumar GS, Sekaran G, Mandal AB (2010) Microbial products (biosurfactant and extra cellular chromate reductase) of marine microorganism are the potential agents reduce the oxidative stress induced by toxic heavy metals. Colloid Surf B 79:334–339
Gome HI, Dias-Ferreira C, Ribeiro AB (2013) Overview of in situ and ex situ remediation technologies for PCB-contaminated soils and sediments and obstacles for full-scale application. Sci Total Environ 445:237–260
Guman SB (2011) Influence of poultry manure amended calcium ammonium nitrate on chemical properties of an Alfisol. Soil Sci Environ Qual 36:14–149
Herman DC, Artiola JF, Miller RM (1995) Removal of cadmium, lead, and zinc from soil by a rhamnolipid biosurfactant. Environ Sci Technol 29:2280–2285
INECAR (2000) Institute of environmental conservation and research position paper against mining in Rapu-Rapu, published by INECAR, Ateneo de Naga University, Philippines. www.adnu.edu.ph/Institutes/Inecar/pospaper1.asp
Irwin RJ, Van Mouwerik M, Stevens L, Seese MD, Basham W (1997) Environmental contaminants encyclopedia. Selenium entry. Nat Park Serv Suite 250, Fort Collons, Colorado
Juwarkar AA, Nair A, Dubey KV, Singh SK, Devotta S (2007) Biosurfactant technology for remediation of cadmium and lead contaminated soils. Chemosphere 68(10):1996–2002
Juwarkar AA, Dubey KV, Nair A, Singh SK (2008) Bioremediation of multi-metal contaminated soil using biosurfactant—a novel approach. Indian J Microbiol 48:142–146
Kader E (2012) Nitrogen and phosphorus uptake by maize as affected by organic matter quality, soil characteristics and land use history of soils from southwestern Nigeria. Soil Fertil Bull 18:222–227
Kanga SH, Bonner JS, Page CA, Mills MA, Autenrieth RL (1997) Solubilization of naphthalene from crude oil using biosurfactants. Environ Sci Tech 31:556–561
Kantor D (2006) Guillain-Barre syndrome, the medical encyclopedia, National Library of Medicine and National Institute of Health. www.nlm.nih.gov/medlineplus/
Kapadia SG, Yagnik BN (2013) Current trend and potential for microbial biosurfactants. Asian J Exp Biol Sci 4(1):1–8
Kappeli O, Finnerty WR (1979) Partition of alkane by an extracellular vesicle derived from hexadecane grown *Acinetobacter*. J Bacteriol 140:707–712
Khan FI, Husain T, Hejazi R (2004) An overview and analysis of site remediation technologies. J Environ Manag 71:95–122
Kiani MJ, Abbas KM, Rahim N (2005) Use of organic manure with mineral N-fertilizers to increase wheat yield at Rawakalot Azad. Arch Agron Soil Sci 51:299–309
Kiran G, Thomas TA, Selvin J, Sabarathnam B, Lipton AP (2010) Optimization and characterization of a new lipopeptide biosurfactant produced by marine *brevibacterium aureum* MSA13 in solid state culture. Bioresour Technol 101:2389–2396
Kumar R, Das AJ, Juwarkar A (2015a) Reclamation of petrol oil contaminated soil by rhamnolipids producing PGPR strains for growing *Withania somnifera* a medicinal shrub. World J Microbiol Biotechnol. doi:10.1007/s11274-014-1782-1
Kumar R, Das AJ, Lal S (2015b) Petroleum hydrocarbon stress management in soil using microorganisms and their products. Waste Management Edited by Environmental Ram Chandra. CRC press Taylor and Francis. ISBN 9781498724746 -CAT# K25742
Lee LS, Zhai X (2007) Indot guidance document for in situ soil flushing. FHWA/IN/JTRP-2006/28. Purdue University. pp.1-48
Lee CW, Choi JM, Pak CH (1996) Micronutrient toxicity in seed geranium 376 (Pelargonium×hortorum Baley). J. Am Soc Hortic Sci 121:77–82
Lege BN (2012) Soil chemical properties as affected by phosphorus and nitrogen—based manure and compost application. Soil Conserv 21:411–416
Lenntech water treatment and air purification (2004) Water treatment, published by Lenntech, Rotterdamseweg, Netherlands. www.excelwater.com/thp/filters/Water-379Purification.htm
Lima TMS, Procópio LC, Brandao FD, Carvalho AMX, Totola MR, Borges AC (2011) Biodegradability of bacterial surfactants. Biodegradation 22:585–592
Maier RM, Neilson JW, Artiola JF, Jordan FL, Glenn EP, Descher SM (2001) Remediation of metal-contaminated soil and sludge using biosurfactant technology. Int J Occup Med Environ Health 14:241–248
Malakul P, Srinivasan KR, Wang HS (1998) Metal toxicity reduction in naphthalene biodegradation by use of metal-chelating adsorbents. Appl Environ Microbiol 64:4610–4613
McCluggage D (1991) Heavy metal poisoning, NCS magazine published by The Bird Hospital, CO, USA. www.cockatiels.org/articles/Diseases/metals.html
Meghraj H, Daneshwar P (2013) Isolation and identification of heavy metals tolerant bacteria from industrial and agricultural areas in mauritius. Curr Res Microbiol Biotechnol 1:119–123
Meharg AA (1994) Integrated tolerance mechanism constitutive and adaptive plant responses to elevated metal concentrations in the environment. Plant Cell Environ 17:989–993
Meharg AA, Macnair MR (1992) Suppression of the high affinity phosphate uptake system: a mechanism of arsenate tolerance in *Holcus lanatus* L. J Exp Bot 43:519–524
Miller RM (1995) Biosurfactant facilitated remediation of contaminated soil. Environ Health Perspect 103:59–62
Mohanpuria P, Rana NK, Yadav SK (2007) Cadmium induced oxidative stress influence on glutathione metabolic genes of *Camellia sinensis* (L.) O Kuntze. Environ Toxicol 22:368–374

Mukherjee S, Das P, Sen R (2006) Towards commercial production of microbial surfactants. Trends Biotechnol 24:509–515
Mulligan CN (2005) Environmental applications for biosurfactants. Environ Pollut 133:183–198
Mulligan CN, Gibbs BF (2004) Types, production and applications of biosurfactants. Proc Indian Nat Sci Acad 1:31–55
Mulligan CN, Yong CN, Gibbs BF (1999) Removal of heavy metals from contaminated soil and sediments using the biosurfactant surfactin. J Soil Contam 8:231–254
Mulligan CN, Yong RN, Gibbs BF (2001a) Surfactant enhanced remediation of contaminated soil: a review. Eng Geol 60:371–380
Mulligan CN, Yong CN, Gibbs BF (2001b) Heavy metal removal from sediments by biosurfactants. J Hazard Mater 85:111–125
Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P (2008) Biosurfactants: properties, commercial production and application. Curr Sci 94(6):736–747
Nerurkar AS (2010) Structural and molecular characteristics of lichenysin and its relationship with surface activity. Adv Exp Med Biol 672:15
Nguyen TT, Youssef NH, McInerney MJ, Sabatini DA (2008) Rhamnolipid biosurfactant mixtures for environmental remediation. Water Res 42:1735–1743
Niroumand H, Nazir R, Kassim KA (2012) The performance of electrochemical remediation technologies in soil mechanics. Int J Electrochem Sci 7(6):5708–5715
Nitschke M, Coast SG (2007) Biosurfactants in food industry. Trends Food Sci Technol 18:252–259
Nolan K (2003) Copper toxicity syndrome. J Orthomol Psychiatry 12(4):270–282
Ogwuegbu MO, Ijioma MA (2003) Effects of certain heavy metals on the population due to mineral exploitation. International conference on scientific and environmental issues in the population. University of Ado Ekiti, Ekiti State, Nigerian, Environment and Sustainable Development in Nigeria, pp 8–10
Ogwuegbu MOC, Muhanga W (2005) Investigation of lead concentration in the blood of people in the copperbelt province of Zambia. J Environ 1:66–75
Ouzounidou G (1995) Effect of copper on germination and seedling growth of *Minuatia*, *Silene, Alyssum and Thlaspi*. Biol Plant 37:411–416
Pacwa-Płociniczak M, Płaza GA, Piotrowska-Seget Z, Cameotra SS (2011) Environmental applications of biosurfactants: recent advances. Int J Mol Sci 12:633–654
Pandey G, Madhuri S (2014) Heavy metals causing toxicity in animals and fishes. Res J Ani Vet Fish Sci 2(2):17–23
Pankow JF, Cherry JA (1996) Dense Chlorinated Solvents and other DNAPLs in Ground-Water. Waterloo Press, Portland
Peng JF, Song YH, Yuan P, Cui XY, Qiu GL (2009) The remediation of heavy metals contaminated sediment. J hazard Mater 161(2):633–640
Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Chem Biol 13:249–252
Scoccianti V, Crinelli R, Tirillini B, Mancinelli V, Speranza A (2006) Uptake and toxicity of Cr (Cr^{3+}) in celery seedlings. Chemosphere 64:1695–1703
Sekar MP (2013) Evaluation of the influence of liming on exchange acidity and grain yield of maize. Int J Agric Sci 62:231–238
Shahid M, Austruy A, Echevarria G, Arshad M, Sanaullah M, Aslam M, Nadeem M, Nasim W, Dumat C (2014) EDTA-enhanced phytoremediation of heavy metals a review. Soil Sediment Contam An Int J 23(4):389–416
Shao Z (2011) Trehalolipids. Microbiol Monogr 20:121–143
Sharma P, Dubey RS (2005) Lead toxicity in plants. Brazil J Plant Physiol 17:35–52
Sharma DC, Sharma CP, Tripathi RD (2003) Phytotoxic lesions of chromium in maize. Chemosphere 51:63–68
Shukla A, Cameotra SS (2012) Hydrocarbon pollution: effects on living organisms, remediation of contaminated environments, and effects of heavy metals co-contamination on bioremediation. In Romero-Zerón, L. (Ed.) Introduction to enhanced oil recovery (EOR) processes and bioremediation of oil-contaminated sites, ISBN 978-953-51-0629-6, doi:10.5772/48014, 185-206
Singh P, Cameotra SS (2004) Enhancement of metal bioremediation by use of microbial surfactants. Biochem Biophy Res Comm 319:291–297
Soberón-Chávez G, Maier RM (2011) Biosurfactants: A general overview. In: Soberón-Chávez G (ed) Biosurfactants. Springer, Berlin, Germany, pp 1–11
Sobolev DK, Begonia MFT (2008) Effects of heavy metal contamination upon soil microbes: lead-induced changes in general and denitrifying microbial communities as evidenced by molecular markers. Int J Environ Res Pub Health 5(5):450–456
Tan H, Champion JT, Artiola JF, Brusseau ML, Miller RM (1994) Complexation of cadmium by a rhamnolipid biosurfactant. Environ Sci Technol 28:2402–2406
Tangahu BV, Abdullah SRS, Basri H, Idris M, Anuar N, Mukhlisin M (2011) A review on heavy metals (As, Pb and Hg) uptake by plants through phytoremediation. Int J Chem Eng 2011:939161. doi:10.1155/2011/939161
Theofanis ZU, Astrid S, Lidia G, Calmano WG (2001) Contaminants in sediments: remobilisation and demobilization. Sci Total Environ 266:195–202
Todd RS, Andrea MC, Maier RM (2000) A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. Appl Environ Microbiol 66:4585–4588
Torrens JL, Herman DC, Miller RM (1998) Biosurfactant (Rhamnolipid) sorption and the impact on rhamnolipid-facilitated removal of cadmium from various soils under saturated flow conditions. Environ Sci Technol 32:776–781
Urum K, Pekdemir T, Gopur M (2003) Optimum conditions for washing of crude oil-contaminated soil with biosurfactant solutions. Process Saf Environ Protec Trans Inst Chem Eng Part B 81(3):203–209
Vajpayee P, Tripathi RD, Rai UN, Ali MB, Singh SN (2000) Chromium accumulation reduces chlorophyll biosynthesis, nitrate reductase activity and protein content in Nympaea alba L. Chemosphere 41:1075–1082
Wang S, Mulligan CN (2009) Arsenic mobilization from mine tailings in the presence of a biosurfactant. Appl Geochem 24:928–935
Wang D, Liu Y, Lin Z, Yang Z, Hao C (2008) Isolation and identification of surfactin producing Bacillus subtilis strain and its effect of surfactin on crude oil. Wei Sheng Wu Xue Bao 48(3):304–311
Whang LM, Liu PWG, Ma CC, Cheng SS (2008) Application of biosurfactant, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. J Hazard Mater 151:155–163
Wojcik M, Tukiendorf A (2004) Phytochelatin synthesis and cadmium localization in wild type of *Arabidopsis thaliana*. Plant Growth Regul 44:71–80
Xie YW, Li Y, Ye R (2005) Effect of alcohols on the phase behavior of microemulsions formed by a biosurfactant-rhamnolipid. J Disper Sci Technol 26:455–461
Yeung AT, Gu YY (2011) A review on techniques to enhance electrochemical remediation of contaminated soils. J Hazard Mater 195:11–29
Zhang WH, Tyerman SD (1999) Inhibition of water channels by $HgCl_2$ in intact wheat root cells. Plant Physiol 120:849–857
Zhou ZS, Huang SQ, Guo K, Mehta SK, Zhang PC, Yang ZM (2007) Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. J Inorg Biochem 101:1–9

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ORIGINAL PAPER

Bioslurry phase remediation of petroleum-contaminated soil using potato peels powder through biosurfactant producing *Bacillus licheniformis* J1

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Abstract Soil contamination due to petroleum oil has become significant ecological issue due to their toxicity. Thus, detoxification of petroleum-contaminated soil is of pressing concern. In this study, bench-scale bioslurry experiment was carried for remediation and detoxification of petroleum-contaminated soil. Potato peels powder was used as organic nutrient source in the slurry for biostimulation purpose, while biosurfactant producing *Bacillus licheniformis* strain J1 identified through molecular approach is used as inocula in the slurry treatment. The strain J1 has the capability to utilized petroleum as carbon source, but its efficiency increase in the presence of potato peels powder. Bioslurry phase experiment was categorized into four groups based on the treatment, such as B0 (soil + H_2O), B1 (soil + petroleum oil + H_2O), B2 (soil + petroleum oil + strain J1 + H_2O), B3 (soil + petroleum oil + potato peels powder + strain J1 + H_2O). After 90 days of treatment, the soils from each treatment were subjected to toxicity analysis using earth worm acute toxicity test and seed germination inhibition assay. The results suggest that in B1 treatment the toxicity effect on germination and seedling growth is highest, while decrease in effect was observed in case of B2 and B3 treatment. Results of earthworm acute toxicity test revealed that 30 ± 5% earthworm survival rates was reported in B1 treatment, whereas 71.6 ± 2.8 and 78.3 ± 2.8% was observed in B2 and B3 treatment, respectively. Hence, the result of the present study signifies that bioslurry phase treatment can be effectively and commercially used for detoxification petroleum-contaminated wastelands.

Keywords Remediation · Detoxification · Petroleum-contaminated soil · Biosurfactant · Bioslurry

Introduction

Soil contamination with petroleum oil and its product is a grave problem for the biotic components of the soil ecosystem (Ziółkowska and Wyszkowski 2010). Hence, environmental relevancy to petroleum oil contamination permits attention-grabbing findings for ecorisk assessments (Handy 2007). Several technologies are introduced for remedy of petroleum oil-contaminated soil; however, remediation through micro-organisms with the suitable metabolic capabilities is considered being most promising (Das and Chandran 2010; Kumar et al. 2015; Das and Kumar 2016). Optimal degradation of petroleum hydrocarbon can be maintained by the adequate concentration of nutrients, pH and oxygen; however, low solubility and high hydrophobic nature of hydrocarbon create them extremely inaccessible to micro-organisms (Atlas 1975; Amund and Nwokoye 1993; Perry 1984; Das and Kumar 2016). Thus, release of biosurfactants is one of the metabolic adaptabilities of micro-organisms for uptake of hydrophobic hydrocarbons (Johnsen et al. 2005; Marin et al. 1996; Ron and Rosenberg, 2002; Obayori et al. 2009; Das and Kumar 2016). Bioslurry is a newly used technique for remediation and detoxification of contaminated soil. Typically, bioslurry process

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involves the mixture of contaminated soil with nutrient, water and microbial inoculum. Organic carbon source is generally used in the form of nutrient for biostimulation purpose, whereas bacterial inoculum is used in the slurry for bioaugmentation (Robles-González et al. 2008; Torabifar et al. 2008). The ecotoxicity assessment of the petroleum-contaminated soil using earthworm acute toxicity test and seed germination inhibition bioassay provides information regarding the characterization strategy of ecological risk (Al-Mutairi et al. 2008; Tang et al. 2011). Hence, in the present study bench-scale bioslurry experiment was designed for remediation and detoxification of petroleum-contaminated soil by using potato peels powder and biosurfactant producing *Bacillus licheniformis* J1. Further, the study deals with toxicity assessment of the remediated soil.

Materials and methods

Isolation of biosurfactant producing bacterial strain

In this present study, a biosurfactant producing micro-organism J1 was used to perform the experiments. The strain was isolated from petroleum oil-contaminated soil of Guwahati, Assam, India (26 °11′ N latitude and 91 °44′ E longitude). The strain was isolated in mineral salt medium (composition per litre K_2HPO_4: 1.0 g, $MgSO_4$: 0.5 g, $NaNO_3$: 2.5 g, KH_2PO_4: 6 g, $CaCl_2$: 0.01 g, $(NH_4)_2SO_4$: 1 g, $FeCl_3$: 0.1 g, $MnSO_4$: 0.005 g, agar: 15 g and Diesel and Petrol oil: 7 ml). Further, the strain was purified and stored at 4 °C. The inoculum was prepared by transferring a loopful of bacterial culture into 25 ml nutrient broth in 250 ml Erlenmeyer flask and incubated at 30 °C for 24 h. This inoculum contained 10^8 cells/ml. For screening the biosurfactant production, different tests were performed. Hemolytic assay was performed by streaking strain onto blood agar plate containing 10% sterile sheep blood (Plaza et al. 2006; Mulligan et al. (1984). Drop collapse test was performed by following the method of Bodour et al. (2003).

Characterization of biosurfactant

Biosurfactant was characterized through several methods. Emulsification index E_{24} (%) was determined by following the method of Cooper and Goldenberg 1987. Oil displacement test was determined by following the method of Ohno et al. (1993). Surface tension was measured according to method of Viramontes Ramos et al. 2010.

Extraction and structural characterization of the biosurfactant

Seventy-two-hour grown bacterial culture in minimum salt medium (composition g/l $NaNO_3$: 2.5, $MgSO_4$: 0.5, $FeSO_4$: 0.01, KH_2PO_4: 1.0, Na_2HPO_4: 5.67, KCl: 0.1, $CaCl_2$: 0.1, $MnSO_4$: 0.002, NH_4NO_3: 0.39, dextrose: 15) with 150 rpm shaking on rotatory shaker was centrifuged at 10,000 rpm, 4 °C temperature for 20 min. The culture supernatant obtained was acidified to pH 2 by using 1 M HCl and stored at 4 °C. The extraction was performed over again with an equal volume of diethyl ether using a separating funnel. The organic layer formed was collected in an empty beaker and allowed to evaporate for a day and further dried in an oven (George and Jayachandran 2013; Gandhimathi et al. 2009; Pansiripat et al. 2010). For structural characterization, the extracted biosurfactant was studied by using FTIR spectrometer model Thermo Scientific Nicole 6700.

Utilization of petroleum oil in the absence and presence of potato peels powder

Potato peels powder used in the present study was prepared from the potato peels obtained from waste of household kitchen. Potato peels were washed five times and then dried at 70 °C for 24 h. The dried peels were ground and passed through 0.5-mm sieve to obtain a fine powder. To check the efficiency of the bacterial strain to utilized petroleum oil in the absence and presence of potato peels, 2% (v/v) petroleum oil was mixed with minimal salt medium. The incubation was carried out on shaker at room temperature for 72 h at 30 °C. Culture medium samples were drawn once at every 24 h for estimation of bacterial biomass and percentage of oil consumption through gravimetric analysis (Luna et al. 2009). A minimal salt medium with 2% (v/v) petroleum oil without bacteria is used as control. All the experiments were performed in triplicate.

Bioslurry phase remediation of petrol-contaminated soil under bench-scale laboratory studies

In the bioslurry study, 250 g of autoclave soil with chemical properties [C organic (%) 1.4 ± 0.05, N (g Kg-1) 1.19 ± 0.8, Ca (g Kg-1) 3.5 ± 0.4, Fe (g Kg-1) 0.155 ± 0.03, Na (g Kg-1) 3.8 ± 0.1, K (g Kg-1) 2.94 ± 0.4, P (g Kg-1) 0.75 ± 0.05, Zn (g Kg-1) 0.0038 ± 0.02] was spiked with petroleum oil and well mixed by hand stirring and kept at 4 °C for 24 h to induce the homogenous sorption of petroleum oil over the soil particles. For soil slurry preparation, the impregnated soil

was categorized into various treatments B0, B1, B2 and B3. The slurry treatments were mixed vertically on an orbital shaker at 120 rpm^{-1}. Treatments were incubated at room temperature for 90 days.

Treatments

B0 = 250 g of soil + 500 ml distilled H_2O.
B1 = 250 g of soil + 5% petroleum oil + 500 ml distilled H_2O.
B2 = 250 g of soil + 5% petroleum oil + strain J1 + 500 ml distilled H_2O.
B3 = 250 g of soil + 5% petroleum oil + 10 g potato peels powder + strain J1 + 500 ml distilled H_2O.

Toxicity test after bioslurry experiment

Seed germination inhibition

In seed germination experiment, certified mung (*Phaseolus mungo* L.), wheat (*Triticum aestivum*), maize (*Zea mays* L.), mustard (*Brassica juncea* L.) seeds were surface sterilized with sodium hypochlorite. Ten sterilized seeds of each were placed in different sterilized petri dish of uniform size lined with two Whatman No. 1 filter paper discs, followed by addition of 7 ml of slurry from the treatments. Plates were incubated at 27 °C and examined for germination inhibition at every 24 h interval for after 7 days. The considered criterion of germination was the visible protrusion of radical from seed coat. Germinated seeds were counted and removed from petri dishes at the first time count on each day till there was no further germination. All the experiments were performed in triplicate. Seed germination, root elongation and the germination index were determined as Tiquia et al. 1996.

Earthworm acute toxicity test

200 g soil slurry from each treatment was put in the brown bottle. Healthy earthworms of weight 400–500 mg were selected, washed with distilled water and dried with filter paper. Five earthworms were weighed as a group, and the average weight value was calculated. The earthworm was put into brown bottle soon after weighing, and the number of earthworm death was checked after 4 and 7 days. The basis for the death of earthworms was determined by no response to acupuncture. After the experiment, the earthworms were weighed, and average value was calculated. All the experiments were performed in triplicate (Tang et al. 2011).

Genotypic characterization of the bacterial strain

For genotypic characterization, isolated DNA was electrophoresed in agarose gel. Further, PCR amplification of 16S rDNA was amplified by using universal primers 27F (AGAGTTTGATCMTGGCTCAG-20) and 1492R (GGTT ACCTTGTTACGACTT-20) which generate 1461 bp product. PCR products were viewed on 1% (w/v) agarose gels, cleaned with PCR purification kit and eventually were processed to sequencing analysis.

Results and discussion

Characterization of the bacterial strain

Based on genotypic characterization, isolated bacterial strain J1 was found similar to *B. licheniformis*. The similarity index of the strain and with other strain is shown in Fig. 1. Sequences of the test strain J1 have been deposited in the NCBI database and accession number allotted to is KT951266.

Biosurfactant production and characterization

The test micro-organism *B. licheniformis* J1 produced biosurfactant on mineral salt medium (MSM) and formed foam with surfactants. Transparent ring around the bacterial colony in hemolytic test indicates that the strain produced biosurfactants as surfactant can cause lysis of blood erythrocyte (Mulligan 1984). Drop collapse test was positive for surfactant production as the drop collapsed. Results of drop collapse test depend on the destabilization of liquid droplets by surfactants. Liquid drop spreads and collapses due to reduced interfacial tension between the liquid and the hydrophobic surface (Bodour et al. 2003). Crude biosurfactant is potential candidate in the displacement of engine oil. Emulsification capacity of a biosurfactant is of immense importance as it can be used for remediation of oil-contaminated sites. Emulsification index of the biosurfactant produced by the stra in J1 is 75.36. Surface tension of noninoculated broth (MSM) was 65.8 mN/m, while inoculated broth was 29.5 mN/m. Hence, the surface tension results indicate that biosurfactant produced by the strain decreases the surface tension of the broth from 65.8 to 29.5 mN/m.

Structural characterization of the biosurfactant

The FTIR spectroscopy of extracted biosurfactant from *B. licheniformis* J1 is presented Fig. 2. Characteristic bands of peptides were observed at 3384.3 cm^{-1} (N–H stretching), 1658.1 cm^{-1} (C=O stretching), 1194.4 cm^{-1} (C–N),

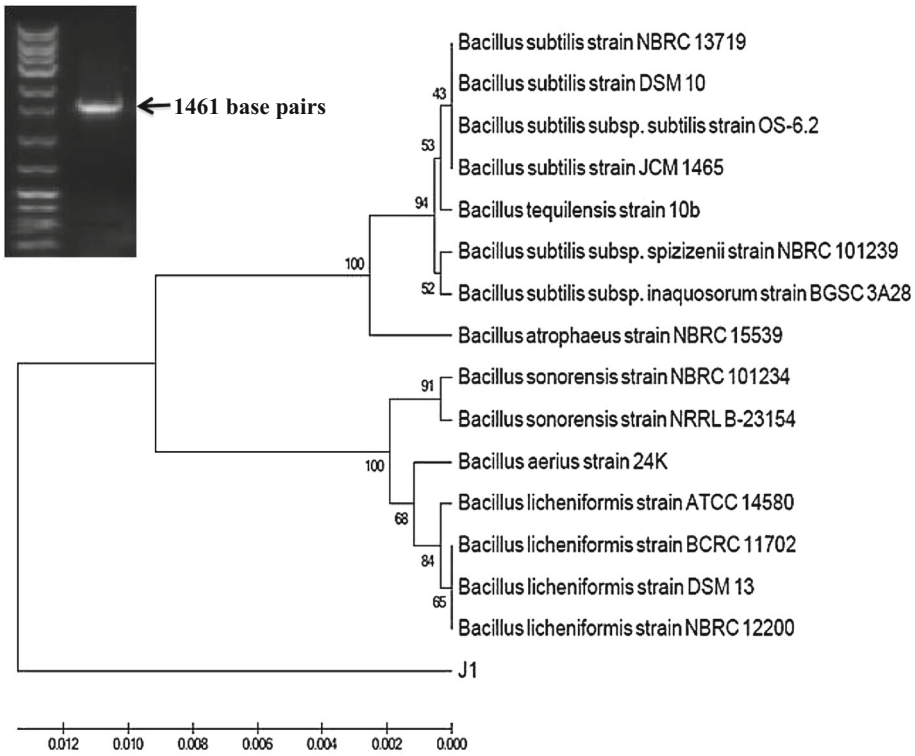

Fig. 1 Phylogenetic tree of the test strain

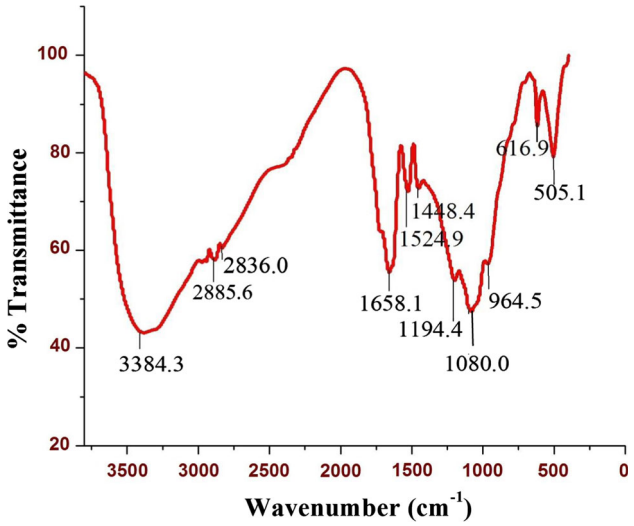

Fig. 2 FTIR spectroscopy of extracted biosurfactant from *B. licheniformis* J1

whereas aliphatic chains was observed at 2885.6 cm^{-1} and 2836.0 cm^{-1} (C–H stretching), indicating that the extracted biosurfactant belongs lipopeptide group. The characteristics bands reported is quite similar to the result reported by El-Sheshtawy et al. (2015).

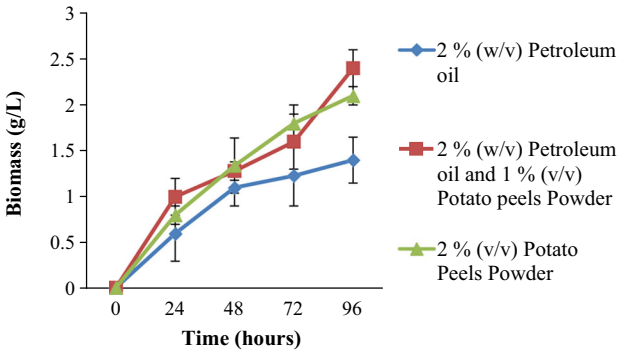

Fig. 3 Efficiency of the strain J1 to degrade and utilized petroleum as carbon source in the absence and presence of potato peels powder

Utilization of petroleum oil in the absence and presence of potato peels powder

The growth of *B. licheniformis* J1 with a 2% (v/v) concentration of petroleum oil in the presence and absence of potato peels powder is presented in Fig. 3. The culture was monitored at every 24 h interval; maximum bacterial biomass of 3.6 ± 0.6 g/L was observed in 2% petroleum oil along with 1% potato peels powder at 96 h, indicating that the potentiality of the strain to degrade and utilized petroleum oil increased in the presence of potato peels powder.

Toxicity of bioslurry phase remediated petrol-contaminated soil on seed germination inhibition

Plants are used as predictor for determining the ecotoxicity of soil, as they mainly depend on soil to germinate and grow, so any alterations in the seed development and physiological processes may reflect the presence of toxic substances in the soil. Germination studies are used to assess the primary and short-term toxicity effects (Banks & Schultz 2005). Different plant species may act differently in petroleum-contaminated soil (Banks and Schultz 2005; Plaza et al. 2005). In present study, cereal crop (wheat and maize), oil-yielding plant (mustard), pulse crop (mung) are evaluated to petroleum contamination. The study suggests that in B1 treatment the toxicity effect on germination and seedling growth is highest, while decrease in effect was observed in case of B2 and B3 treatment. This effect might be due to petroleum oil in the soil that creates an impermeable oily film layer around the seeds interfering water air relations resulting in adverse effect on germination. Similar effect on germination rate caused by petroleum contaminants in various commercially important plant species was also reported by Adam and Duncan (2002); Tang et al. (2011) and Das and Kumar, (2016). Figure 4 shows the inhibition of germination and relative germination in all the plants. The order of inhibition of germination and relative germination is B1 $\geq$ B2 $\geq$ B3. Figure 5 shows inhibition of root length of all the plants. The inhibition rate of root length is highest in B1 treatment, but in B2 and B3 treatment the inhibition is comparatively low. The inhibition of root length might be due to anaerobiosis process. Petroleum in soil reduces aeration by blocking the air spaces between soil particles and creates a condition of anaerobiosis and leading to root stress (Smith et al. 1989; Shukry et al. 2013; Rowell 1977). The low effect in B2 and B3 treatment might be due to remediation of the contaminants. The untreated soil has inhibitory effect on seed germination and seedling growth. But, after bioslurry phase bacterial treatment, the toxicity of the contaminated soil significantly reduced and showed improved seed germination, root length and germination index (Fig. 6). The results are in accordance with Baek et al. 2004; Cruz et al. 2013; D'Souza et al. 2011; Molina-barahona et al. 2005; Das and Kumar 2016 who found a decrease in toxicity in seedling after the bioremediation of the pollutant.

Earthworm acute toxicity test

Earthworms are extensively used soil organisms for assessment of soil toxicity. Earthworms are common inhabitants of soil that influence on soil chemical property, structure, nutrient and plant growth (Edwards and Bohlen 1996; Handy 2007). Earthworms are sensitive to soil

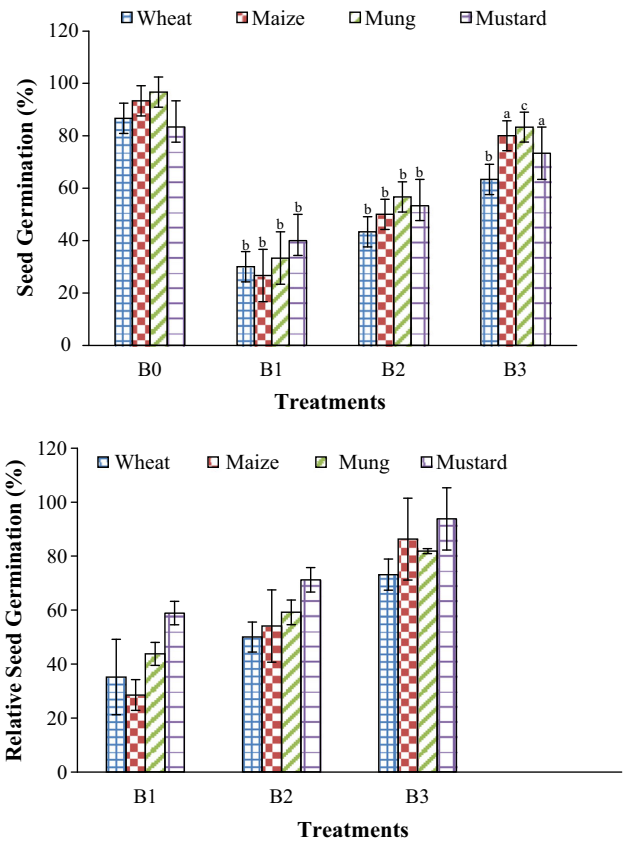

Fig. 4 Effect of petroleum-contaminated soil on germination and relative germination of seeds under various bioslurry treatments. Treatments results were by analysed single-factor analysis of variance (ANOVA) and are mean of three replicates with standard deviation (mean $\pm$ SD, $n = 3$). And differences were considered significant at $p < 0.01$ and $p < 0.05$ by comparing each treatment (B1, B2 and B3) with B0 treatment (**a** Not significant, **b** $p < 0.01$, **c** $p < 0.05$)

pollutant that makes them good bioindicators for screening the soil toxicity. Acute toxicity test is a short-term test for measuring the effects of chemicals on survival of earthworm (Hoffman et al. 1995; Handy 2007). Figure 7 represents the earthworm survival rate in all the treatments. Apparently, 30 $\pm$ 5% survival rates was reported in B1 treatment, whereas 71.6 $\pm$ 2.8% and 78.3 $\pm$ 2.8% was observed in B2 and B3 treatment, respectively. Figure 8 demonstrates the body weight of earthworms survived in various treatments. The weight of the earthworm decrease in all the treatment. Even in the control treatment decrease in earthworm weight was reported and this might be due to changes in soil nutrients and living conditions. There was swelling with ejecting coelomic fluid in the death earthworm of all the treatment. Similar results were reported by Ramadass et al. 2015 that when earthworms were exposed to used motor oil swelling, fragmentation and ejecting of coelomic fluid occur before death. Tang et al. 2011 observed that with higher oil concentration in soil cause death of the earthworm and the inhibition of body weight

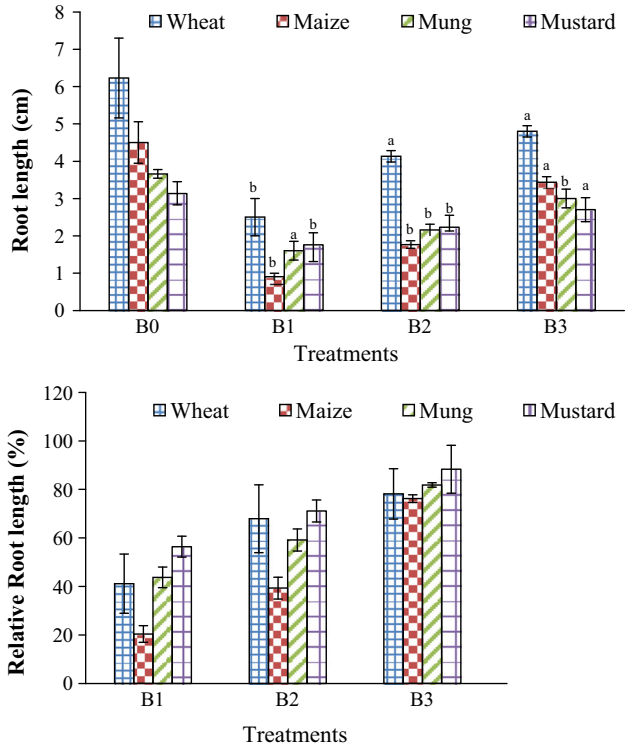

Fig. 5 Effect of petroleum-contaminated soil on root length and relative root length of seeds under various bioslurry treatments. Treatments results were by analysed single-factor ANOVA and are mean of three replicates with standard deviation (mean ± SD, $n = 3$). And differences were considered significant at $p < 0.01$ and $p < 0.05$ by comparing each treatment (B1, B2 and B3) with B0 treatment (**a** Not significant, **b** $p < 0.01$, **c** $p < 0.05$)

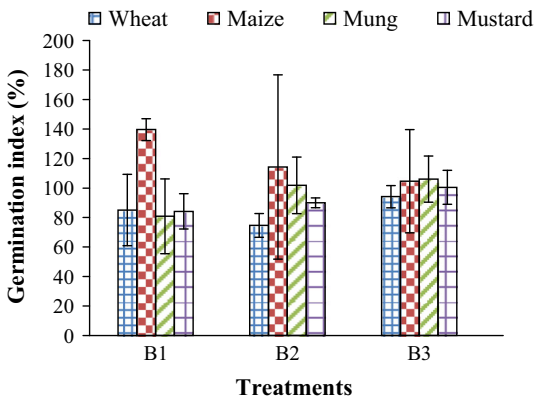

Fig. 6 Effect of petroleum-contaminated soil on germination index under various bioslurry treatments. Treatments results were analysed by single-factor ANOVA and are mean of three replicates with standard deviation (mean ± SD, $n = 3$). And differences were considered significant at $p < 0.01$ and $p < 0.05$ by comparing each treatment (B1, B2 and B3) with B0 treatment (**a** not significant, **b** $p < 0.01$, **c** $p < 0.05$)

increase. The literature regarding sensitivity of earthworms to petroleum-contaminated soil or their capability to survive in remediated soil is scarce (Salanitro et al. 1997;

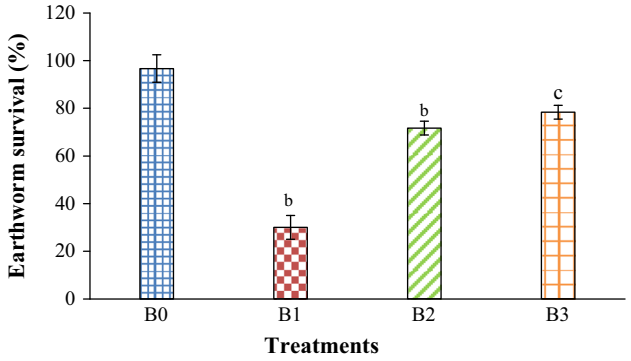

Fig. 7 Effect of petroleum-contaminated soil on earthworm survival under various bioslurry treatments. Treatments results were analysed by single-factor ANOVA and are mean of three replicates with standard deviation (mean ± SD, $n = 3$). And differences were considered significant at $p < 0.01$ and $p < 0.05$ by comparing each treatment (B1, B2 and B3) with B0 treatment (**a** not significant, **b** $p < 0.01$, **c** $p < 0.05$)

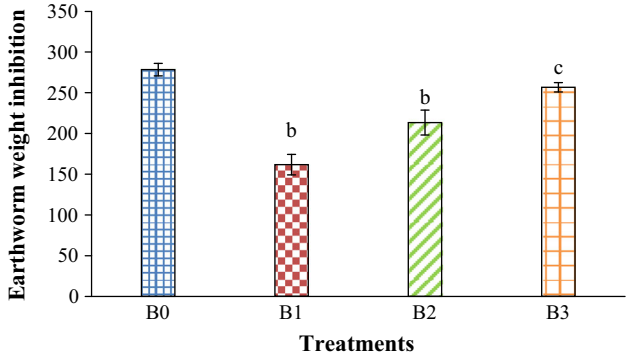

Fig. 8 Effect of petroleum-contaminated soil on earthworm body weight under various bioslurry treatments. Treatments results were analysed by single-factor ANOVA and are mean of three replicates with standard deviation (mean ± SD, $n = 3$). And differences were considered significant at $p < 0.01$ and $p < 0.05$ by comparing each treatment (B1, B2 and B3) with B0 treatment (**a** not significant, **b** $p < 0.01$, **c** $p < 0.05$)

Dorn and Salanitro 2000). The present study demonstrates that bioremediated soil (B2 and B3 treatment) has comparatively low effect on earthworm then untreated soil (B1 treatment). Previously, Frutos et al. 2012 demonstrated feasibility of land farming and slurry bioremediation for the treatment of petroleum hydrocarbon containing sludge. Efficiency was assessed by ecotoxicological assay, and after the treatment, decrease in acute toxicity was observed in earthworm. Hanna and Weaver (2002) investigated effect of crude oil on soil and bioremediated soil in two species of earthworms Lumbricus *terrestris* and *Eisenia fetida.* They observed that earthworms were capable to tolerate 0.5% concentrations of freshly added oil and 1% of oil in bioremediated soil.

Conclusion

In present study, bioslurry phase experiment was categorized into four groups based on the treatment, such as B0, B1, B2 and B3 treatment. Toxicity study revealed soil of B2 and B3 treatment exhibit low toxicity as compared to B1 treatment in both seed germination inhibition and earthworm acute toxicity test. Hence, the study signifies that bioslurry phase treatment using agroindustrial waste and indigenous biosurfactant producing bacteria with capability to utilized petroleum as carbon source can be effectively and commercially used for detoxification petroleum-contaminated wastelands.

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References

Adam G, Duncan H (2002) Influence of diesel fuel on seed germination. Environ Poll 120:363–370

Al-Mutairi N, Bufarsan A, Al-Rukaibi F (2008) Ecorisk evaluation and treatability potential of soils contaminated with petroleum hydrocarbon-based fuels. Chemosphere 74(1):142–148

Amund OO, Nwokoye N (1993) Hydrocarbon potentials of yeast isolates from a polluted Lagoon. J Sci Res Develop 1:65–68

Atlas RM (1975) Effects of temperature and crude oil composition on petroleum biodegradation. J Appl Microbiol 30:396–403

Baek KH, Kim HS, Oh HM, Yoon BD, Kim J, Lee IS (2004) Effects of crude oil, oil components, and bioremediation on plant growth. J Environ Sci Health, Part A 39(9):2465–2472

Banks MK, Schultz KE (2005) Comparison of plants for germination toxicity tests in petroleum-contaminated soils. Water, Air, Soil Poll 167(1):211–219

Bodour AA, Drees KP, Maier RM (2003) Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Appl Environ Microbiol 69:3280–3287

Cooper DG, Goldenberg BG (1987) Surface-active agents from two Bacillus species. Appl Environ Microbiol 53:224–229

Cruz JM, Lopes PRM, Montagnolli RN, Tamada IS, Silva NMMG, Bidoia ED (2013) Phytotoxicity of soil contaminated with petroleum derivatives and biodiesel. Ecotoxic Environ Conta 8(1):49–54

D'Souza R, Varun M, Paul MS (2011) Assessment of wheat bioassays as a monitoring system for phytoremediation of diesel. Biotechnol Bioinf Bioengineer 1:125–130

Das N, Chandran P (2010) Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotech Res Inter. Article ID 941810:13p. doi:10.4061/2011/941810

Das AJ, Kumar R (2016) Bioremediation of petroleum contaminated soil to combat toxicity on *Withania somnifera* through seed priming with biosurfactant producing plant growth promoting rhizobacteria. J Environ Manag 174:79–86

Dorn PB, Salanitro JP (2000) Temporal ecological assessment of oil contaminated soils before and after bioremediation. Chemosphere 40(4):419–426

Edwards CA, Bohlen PJ (1996) Biology and ecology of earthworms, 3rd edn. Chapman and Hall, Great Britain, p 426

El-Sheshtawy HS, Aiad I, Osman ME, Abo-ELnas AA, Kobisy AS (2015) Production of biosurfactant from *Bacillus licheniformis* for microbial enhanced oil recovery and inhibition the growth of sulfate reducing bacteria. Egyptian J Petro 24(2):155–162

Frutos FJ, García R, Pérez O, Escolano A, Rubio A, Gimeno MD, Fernandez G, Carbonell C, Perucha Laguna J (2012) Remediation trials for hydrocarbon-contaminated sludge from a soil washing process: evaluation of bioremediation technologies. J Hazard Mater 199:262–271

Gandhimathi R, Seghal Kiran G, Hema TA, Selvin J, Rajeetha Raviji T, Shanmughapriya S (2009) Production and characterization of lipopeptide biosurfactant by a sponge-associated marine actinomycetes Nocardiopsis alba MSA10. Bioproc Biosyst Eng 32(6):825–835

George S, Jayac K (2013) Production and characterization of rhamnolipid biosurfactant from waste frying coconut oil using a novel *Pseudomonas aeruginosa* D. J Appl Microbiol 114(2):373–383

Handy AR (2007) Acute toxicity and immunotoxicity testing of total petroleum hydrocarbons in aquatic and terrestrial Organisms (Doctoral dissertation, Wright State University)

Hanna SHS, Weaver RW (2002) Earthworm survival in oil contaminated soil. Plant Soil 240(1):127–132

Johnsen AR, Wick LY, Harms H (2005) Principles of microbial PAH degradation in soil. Environ Poll 133:71–84

Kumar R, Das AJ, Juwarkar AA (2015) Reclamation of petrol oil contaminated soil by rhamnolipids producing PGPR strains for growing Withania somnifera a medicinal shrub. World J Microbiol Biotechnol 31:307–313

Marin M, Pedregosa A, Rios S, Laborda F (1996) Study of factors influencing the degradation of heating oil by Acinetobacter calcoaceticus MM5. Inter Biodeter Biodeg 38:67–75

Molina-Barahona L, Vega-Loyo L, Guerrero M, Ramirez S, Romero I, Vega-Jarquín C, Albores A (2005) Ecotoxicological evaluation of diesel-contaminated soil before and after a bioremediation process. Environ Toxicol 20(1):100–109

Mulligan C, Cooper D, Neufeld R (1984) Selection of microbes producing biosurfactants in media without hydrocarbons. J Ferment Technol 62:311–314

Obayori OS, Ilori MO, Adebusoye SA, Oyetibo GO, Omotayo AE, Amund OO (2009) Degradation of hydrocarbons and biosurfactant production by Pseudomonas sp. strain LP1. World J Microbiol Biotechnol 25:1615–1623

Ohno A, AnoT Shoda N (1993) Production of antifungal peptide antibiotic, iturin by *Bacillus subtilis* NB22 in solid state fermentation. J Ferment Bioeng 75:23–27

Pansiripat S, Pornsunthorntawee O, Rujiravanit R, Kitiyanan B, Somboonthanate P, Chavadej S (2010) Biosurfactant production by Pseudomonas aeruginosa SP4 using sequencing batch reactors: Effect of oil-to-glucose ratio. Biochem Eng J 49(2):185–191

Perry JJ (1984) Microbial metabolism of cyclic alkanes. In: Atlas RM (ed) Petroleum microbiology. Macmillan, New York, pp 61–98

Plaza G, Naleez-jaweeki G, Ulfig K, Brigmon RL (2005) The application of bioassays as indicators of petroleum-contaminated soil remediation. Chemosphere 59:289–296

Plaza GA, Zjawiony I, Banat IM (2006) Use of different methods for detection thermophilic biosurfactant-producing bacteria from hydrocarbon contaminated and bioremediated soils. J Petrol Sci Engineer 50:71–77

Ramadass K, Megharaj M, Venkateswarlu K, Naidu R (2015) Ecological implications of motor oil pollution: earthworm survival and soil health. Soil Bio Biochem 85:72–81

Robles-González IV, Fava F, Poggi-Varaldo HM (2008) A review on slurry bioreactors for bioremediation of soils and sediments. Microb Cell Factor 7(1):5

Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Chem Biol 13:249–252

Rowell MJ (1977) The effects of crude oil spill on soils. In: Toogood JA (ed) A review of literature I, The reclamation of agricultural soils after oil spills, part I. Edmonton, Canada

Salanitro JP, Dorn PB, Huesemann MH, Moore KO, Rhodes IA, Rice Jackson LM (1997) Crude oil hydrocarbon bioremediation and soil ecotoxicity assessment. Environ Sci Technol 31(6):1769–1776

Shukry WM, Al-Hawas GHS, Al-Moaikal RMS, El-Bendary MA (2013) Effect of petroleum crude oil on mineral nutrient elements, soil properties and bacterial biomass of rhizosphere of jojoba. Br J Environ Clim Change 3:103–118

Smith B, Stachowisk M, Volkenburgh E (1989) Cellular processes limiting leaf growth in plants under hypoxic root stress. J Exp Bot 40:89–94

Tang J, Wang M, Wang F, Sun Q, Zhou Q (2011) Eco-toxicity of petroleum hydrocarbon contaminated soil. J Environ Sci 23(5):845–851

Tiquia SM, Tam NFY, Hodgkiss IJ (1996) Effects of composting on phytotoxicity of spent pig manure sawdust litter. Environ Poll 93:249–256

Torabifar B, Torabian A, Azimi AA, Vossoughi M (2008) Biological treatment of petroleum contaminated soils by soil slurry-sequencing batch reactors (SS-SBRs). WIT Transac Eco Environ 109:751–758

Viramontes-Ramos S, Portillo-Ruiz MC, Ballinas-Casarrubias MDL, Torres-Muñoz JV, Rivera-Chavira BE, Nevárez-Moorillón GV (2010) Selectionof biosurfactan/bioemulsifier producing bacteria from hydrocarbon-contaminated soil. Brazilian J Microbiol 41:668–675

Ziółkowska A, Wyszkowski M (2010) Toxicity of petroleum substances to microorganisms and plants. Ecol Chem Eng S 17:73–82

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Short communication

Bioremediation of petroleum contaminated soil to combat toxicity on *Withania somnifera* through seed priming with biosurfactant producing plant growth promoting rhizobacteria

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ABSTRACT

Soil contaminated by Petroleum oil cannot be utilized for agricultural purposes due to hydrocarbon toxicity. Oil contaminated soil induces toxicity affecting germination, growth and productivity. Several technologies have been proposed for bioremediation of oil contaminated sites, but remediation through biosurfactant producing plant growth promontory rhizobacteria (PGPR) is considered to be most promising methods. In the present study the efficacy of seed priming on growth and pigment of *Withania somnifera* under petroleum toxicity is explored. Seeds of *W. somnifera* were primed with biosurfactant producing *Pseudomonas* sp. AJ15 with plant growth promoting traits having potentiality to utilized petroleum as carbon source. Results indicates that plant arose from priming seeds under various petroleum concentration expressed high values for all the parameters studied namely germination, shoot length, root length, fresh and dry weight and pigments (chlorophyll and carotenoid) as compared to non primed seed. Hence, the present study signifies that petroleum degrarding biosurfactant producing PGPR could be further used for management and detoxification of petroleum contaminated soils for growing economically important crops.

1. Introduction

Petroleum refinery is an industrial plant which refined the crude oil into useful product such as gasoline, petrol, diesel, kerosene, asphalt base, liquefied petroleum gas and heating oil (Gary and Handwerk, 1984). However in case of an unwanted leakage of petroleum oil and its product might act as a persistent soil and water pollutant (Graj et al., 2013). Soil pollution by petroleum oil induces major changes in the physical and chemical properties of soil ensuing in adverse effect on plant growth. Petroleum oil and its constituents can decrease the availability of oxygen, water and nutrients in soil, which as a result may decline the seed germination rate and effect the plant growth (Nogueira et al., 2011). Several technologies have been introduced for remediation of oil contaminated sites, but bioremediation through microorganisms with the appropriate metabolic capabilities is most promising. Although, optimal rates of hydrocarbon biodegradation by microorganism can be maintained by the adequate concentration of oxygen, nutrients and pH values, but high hydrophobic nature and low solubility of petroleum hydrocarbon compounds make them highly unavailable to microorganisms (Atlas, 1975; Amund and Nwokoye, 1993; Perry, 1984), Hencerelease of biosurfactants is one of the strategies used by microorganisms to influence the uptake of petroleum hydrocarbon and hydrophobic compounds (Marin et al., 1996; Johnsen et al., 2005; Obayori et al., 2009; Ron and Rosenberg, 2002). Biosurfactants increases the surface area of hydrophobic water insoluble substrates and increase their bioavailability, thereby enhancing the growth of bacteria and rate of bioremediation. Certain microbial species such as fluorescent pseudomonads are accounted to excrete a various form of partially or totally extracellular biosurfactant that facilitates the uptake of hydrocarbons by reducing the surface tension and enhance the removal of hydrocarbons from the oil contaminated soil (Bento et al., 2005; Franzetti et al., 2010). Various microorganisms have acquired a mechanism to thrive and grow in oil containing environment and play an immense role in treatment by degrading the pollutant (Pothuluri and Cerniglia, 1994; Jurelevicius et al., 2013; Pacwa-Płociniczak et al., 2016). Petroleum pollutants can be degraded by plants

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through biochemical reactions taking place within the plants and in the rhizosphere (Hrynkiewicz and Baum, 2011). The remediation of soils containing petroleum is possible with the use of plants and their rhizosphere processes (Mirsal, 2004; Hrynkiewicz and Baum, 2011). In this interaction soil microorganism provides nutrients in the rhizosphere which leads to an increased microbial activity and degradation of toxic pollutants (Mirsal, 2004; Hrynkiewicz and Baum, 2011). Most of the soil bacteria with plant growth promoting traits can be well habituated to harsh soil conditions and enhance the remediation of disturbed soils directly and by plant growth promotion (Hrynkiewicz and Baum, 2011; Fomina et al., 2005; Wenzel, 2009). Fluorescent pseudomonad (versatile bacteria, gram-negative, motile, rod shaped and non-spore forming) have been reported to promote plant growth in rhizosphere directly by their plant growth promoting traits. These plant growth promoting traits can enhance various stages of plant growth development and combat abiotic stress (Kumar et al., 2013, 2015; Yang et al., 2008). *Withania somnifera* is a medicinal belonging to family Solanaceae, reportedly exhibit antipyretic, anti-inflammatory abortifacient, immunomodulatory and haematopoietic activity (Mishra et al., 2000). Leaves and roots of Indian *W. somnifera* content active components withaterin-A and withanolide-D, which serve as a source of drugs. But according to Red data book of threatened species *W. somnifera* is an endangered medicinal plant (Arumugam and Gopinath, 2013). So their cultivation is of urgent need, but due to scarcity of agricultural land no much attention has been given for their cultivation. Hence, managing the petroleum contaminated soil with biosurfactant producing bacterial strain for their cultivation could open a new vista for their conservation.

The present investigation was carried out for the management and detoxification of petroleum contaminated soil to reduce the toxic effect of petroleum on the growth and pigments of *W. somnifera* (economically important crop) through seed priming with biosurfactant producing plant growth promoting bacteria having the potentiality utilize petroleum as carbon source.

2. Materials and methods

2.1. Microorganism

In this present study, a biosurfactant producing microorganism, identified as *Pseudomonas* sp. AJ15, was selected to perform the experiments on the basis of its plant growth promoting traits and ability to degrade petroleum oil. This strain was isolated from petroleum oil contaminated soil and stored in our laboratory. The inoculum was prepared by transferring a loopful of bacterial culture into 25 ml nutrient medium (composition g/l beef extract-3 g, peptone-5 g, NaCl-5 g) in 250 ml Erlenmeyer flask and incubated at 30 °C for 24 h. This inoculum contained 10^8 cells/ml.

2.2. Screening of the bacterial strain for biosurfactant production

Bacterial strain was tested for biosurfactant production on 500 ml minimal salt medium, MSM (composition g/l MgSO4 (anhydrous) −0.5, $NaNO_3$ −2.5, KH_2PO_4 −1.0, $FeSO_4$ −0.01, KCl −0.1, Na_2HPO_4 −5.67, $CaCl_2$ −0.1,NH_4NO_3 −0.39, $MnSO_4$ −0.002, dextrose −15). Further the biosurfactant production was confirmed by various test. Drop collapse test was performed by following the method of Bodour et al., 2003. Haemolytic assay was performed by modifying the method of Plaza et al., 2006 and Mulligan et al., 1984. Emulsification index E_{24} (%) was determined by following the method of Cooper and Goldenberg (1987). Surface tension reducing ability was measured by following the method of Viramontes-Ramos et al. (2010). Oil displacement test of the of isolated crude biosurfactant was done by Ohno et al., 1993.

2.3. Screening the potentiality of the bacterial strain to utilized petroleum as carbon source

2% (v/v) petroleum oil was mixed with 100 ml minimal salt medium (MSM) to check the potentiality of the bacterial strain to utilized petroleum as carbon source for production of biosurfactant. The incubation was carried out on shaker for 72 h at 30 °C. Culture medium samples were drawn once at every 24 h for estimation of bacterial biomass, biosurfactant production. A minimal salt medium with dextrose as carbon source was used for comparative study. All the experiments were performed in triplicate.

2.4. Plant growth promoting traits

Phosphate solubilization activity was screened on NBRIP medium as per the method described by Nautiyal (1999) and Quantitative analysis of phosphate solubilization (tricalcium phosphate) in liquid medium was estimated by following the method of Jackson 1973. Siderophores production was assayed on the Chrome azurol S agar medium according to the method of Schwyn and Neilands (1987). Quantitative analysis of IAA was performed by following the method of Loper and Scroth (1986) at concentrations of 100 and 500 μg/ml of tryptophan.

2.5. Seed priming

Five certified seeds of *W. somnifera* were primed with 1 ml of 48 h grown bacterial cell suspensions of *Pseudomonas* sp. AJ15 by incubating at 22 °C for 4 h. During incubation, seeds were agitated at 150 rpm on a rotary shaker and were air dried overnight at 28 °C. After 2 days storage of the seeds at room temperature, to assure that each seed had between $\log 10^7$ CFU $seed^{-1}$, the number of bacteria per seed were assessed by grinding samples of ten seeds for 1 min in1 ml 0.85% NaCl using a sterilised mortar and pestle. Suspensions were serially diluted and plated on TSA (TSB supplemented with 1.6% agar and 100 mg/mL rifampicin). Plates were then incubated at 28° C in the dark for 48 h before CFUs were counted (Abuamsha et al. 2011).

2.6. Growth profiling to assess the stress effect

The effect of petroleum oil on the growth was conducted according to Sagar et al., 2012. The primed and non primed seeds (10 each) were sown in pots with 1 kg sterilized sandy clay soil with chemical properties [C organic (%) 1.4 ± 0.05, Ca (g Kg^{-1}) 3.5 ± 0.4, N (g Kg^{-1}) 1.19 ± 0.8, Na (g Kg^{-1}) 3.8 ± 0.1, P (g Kg^{-1}) 0.75 ± 0.05, K (g Kg^{-1}) 2.94 ± 0.4, Fe (g Kg^{-1}) 0.155 ± 0.03, Zn (g Kg^{-1}) 0.0038 ± 0.02] amended with 0.88% (10 ml), 2.2% (25 ml) and 4.4% (50 ml) of petroleum oil concentration. Each experimental set consisted of 3 pots. Pots were placed in a growth chamber at 30 °C with a 12 h dark–light cycle. Percentage germination, root/shoot length and fresh/dry weight were recorded after 30 days. Rhizospheric colonization for 5 representative plants from each set was determined by dilution plating of 1 g of rhizospheric soil at 20 day on TSA amended with rifampicin.

2.7. Effect on the pigments

Method of Arnon (1949) was followed to study the effect petroleum oil contamination on carotenoid and chlorophyll content. Briefly, 500 mg of fresh leaf was cut into small piece and homogenized with 10 ml of 80% acetone and centrifuged at 2500 rpm for 10 min at 4 °C.The extract obtained was mixed with 80% acetone

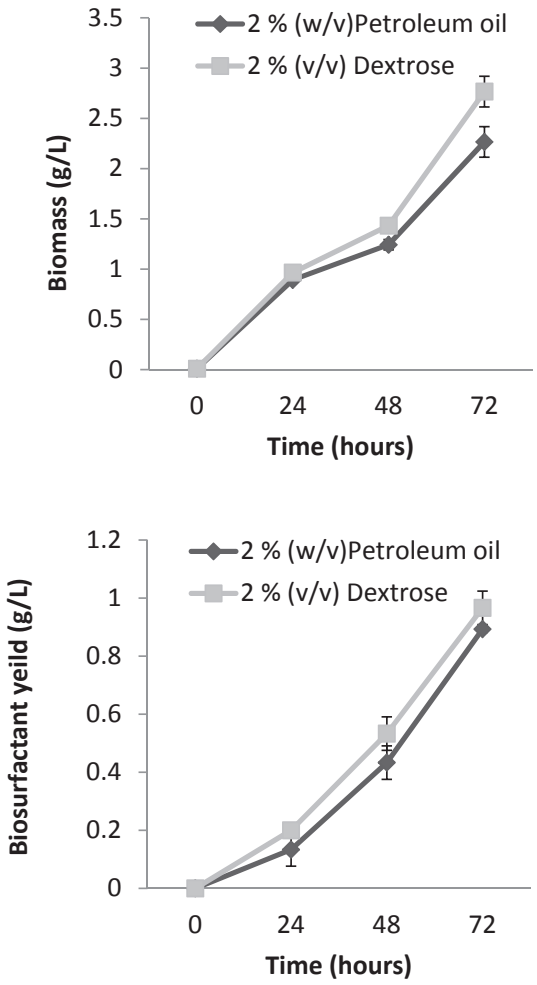


Fig. 1. Potentialities of the bacterial strain to degrade and utilized petroleum as carbon source for production of biosurfactant and its comparative analysis with dextrose.

and absorbance was noted at 645, 663 and 480 nm against 80% acetone as blank. Chlorophyll content in milligrams (mg) was estimated using the formula of Arnon (1949). The extract that was used for the chlorophyll estimation was also used for carotenoids estimation. Total carotenoids content was determined by the method of Kirk and Allen (1965).

2.8. Statistical analysis

Experimental results were analysed by using single-factor ANOVA analysis. Statistical data were expressed as means ± S.D and differences were considered significant at $p < 0.01$ and $p < 0.05$.

3. Results and discussion

3.1. Screening and characterization of biosurfactant

The test microorganism *Pseudomonas* sp. AJ15 produced biosurfactant on MSM and formed foam with surfactants. Drop collapse test was positive for surfactant production as the drop spread and collapsed, whereas clear zone in heamolytic assay indicates that the strain produced biosurfactants. Surfactant produced by the strain reduced surface tension up to 30.5 mN/m. Emulsification index of biosurfactant produced by the strain AJ15 is 62.7 for engine oil, whereas 67 for petroleum oil. Emulsification ability of surfactant indicates that strain has the ability to emulsify the petroleum engine oil. The emulsification property is very important as it can be used for several applications like oil recovery and bioremediation of oil spilled sites. During emulsification emulsion is formed in which one liquid phase is dispersed as microscopic droplets in some other continuous liquid phase (Desai and Banat, 1997).

3.2. Utilization of petroleum as carbon source for biosurfactant production

The growth kinetics and biosurfactant production by *Pseudomonas* sp. AJ15 with a 2% (v/v) concentration of petrol oil is represented in Fig. 1. The culture was monitored at every 24 h interval; maximum bacterial biomass of 2.266 ± 0.152 g/L and maximum biosurfactant yield of 0.89 ± 0.011 g/L was obtained at 72 h indicating the strain has the potentiality to degrade and utilized petroleum as carbon source for production of biosurfactant.

3.3. Plant growth promoting traits

In qualitative analysis of phosphate reddish zone around the colonies indicate that the bacterial strain AJ15 has the ability to solubilise phosphate, whereas quantitative test for solubilization of tricalcium phosphate in liquid medium by the test strain showed that strain solubilize tricalcium phosphate up to 91%. Siderophore production on Chrome Azurol-S agar (CAS agar) plates were found to be positive as indicated by change in colour of the media around the colonies. Quantitative estimation of Indole acetic acid (IAA) in the presence of 100 and 500 μg/ml concentrations of tryptophan indicates that the strain was able to produce good amount of IAA Table 1. Hence, *Pseudomonas* sp. AJ15 possess plant growth promoting traits such as phosphate solubilization, Indole Acetic Acid (IAA) and siderophore production which signifies that the strain has the tendency to promote plant growth in petroleum

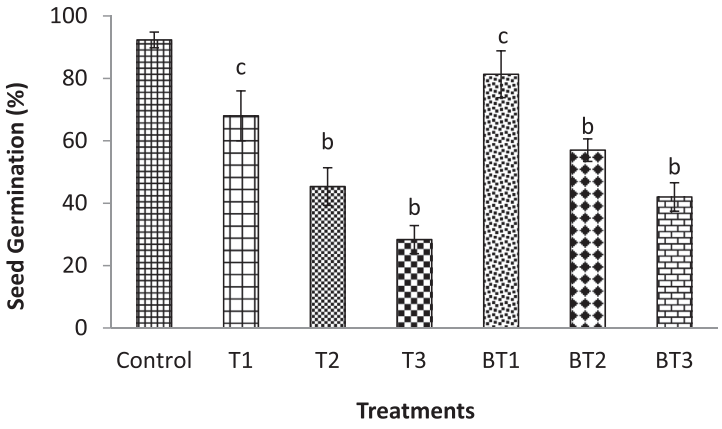
Table 1
Comparative study of IAA production by *Pseudomonas* sp. AJ15 with various fluorescent *Pseudomonas*.

Strain	Tryptophan concentration (μg/mL)	IAA production (μg/mL)	References
Fluorescent *Pseudomonas* Ps_7	150	5.90 ± 0.20	Ahmad et al., 2008
	500	18.07 ± 0.25	
Fluorescent *Pseudomonas* Ps_9	150	6.10 ± 0.20	Ahmad et al., 2008
	500	22.02 ± 0.20	
Pseudomonas fluorescens 1	200	12.82	Čolo et al., 2014
	500	12.93	
Pseudomonas fluorescens 2	200	7.68	Čolo et al., 2014
	500	11.0	
Pseudomonas sp. AJ15	100	3.1 ± 0.5	Present study
	500	16.3 ± 0.8	

contaminated soil.

3.4. Effect on germination

The germination percentages and speed of germination was significantly influenced by seed priming treatment. The results indicated the better performance of primed seed as compared to non primed seed under different petroleum oil concentration (10, 25 and 50 ml). About, 81.33%, 57% and 42% germination was observed at 10, 25 and 50 ml of petroleum oil through primed seeds, whereas, 68%, 45.33%, 28.3% germination occurred at 10, 25 and 50 ml of petroleum oil concentration by non primed seeds Fig. 2. This might be due to fuel (petrol or diesel) which has an adverse effect on the water air relations in the soil and creates an



Results were analyzed by single-factor ANOVA analysis of variance and are mean of three replicates with standard deviation (Means ± S.D, n = 3). And differences were considered significant at $p < 0.01$ and $p < 0.05$ by comparing T1 and BT1, T2 and BT2, T3 and BT3([a] $p < 0.01$,[b] $p < 0.05$, [c] Not significant). **Control,** non primed *Withania somnifera* seed + 1kg soil ;**T1**, non primed *Withania somnifera* seed + 1kg soil + 10 ml petroleum oil ; **T2**, non primed *Withania somnifera* seed + 1kg soil + 25 ml petroleum oil ; **T3,** non primed *Withania somnifera* seed + 1kg soil + 50 ml petroleum oil ; **BT1,** primed *Withania somnifera* seed + 1kg soil + 10 ml petroleum oil ; **BT2,** primed *Withania somnifera* seed + 1kg soil + 25 ml petroleum oil ; **BT3,** primed *Withania somnifera* seed + 1kg soil + 50 ml petroleum oil.

Fig. 2. Effect of seed priming on germination of *Withania somnifera* under different petroleum oil concentration.

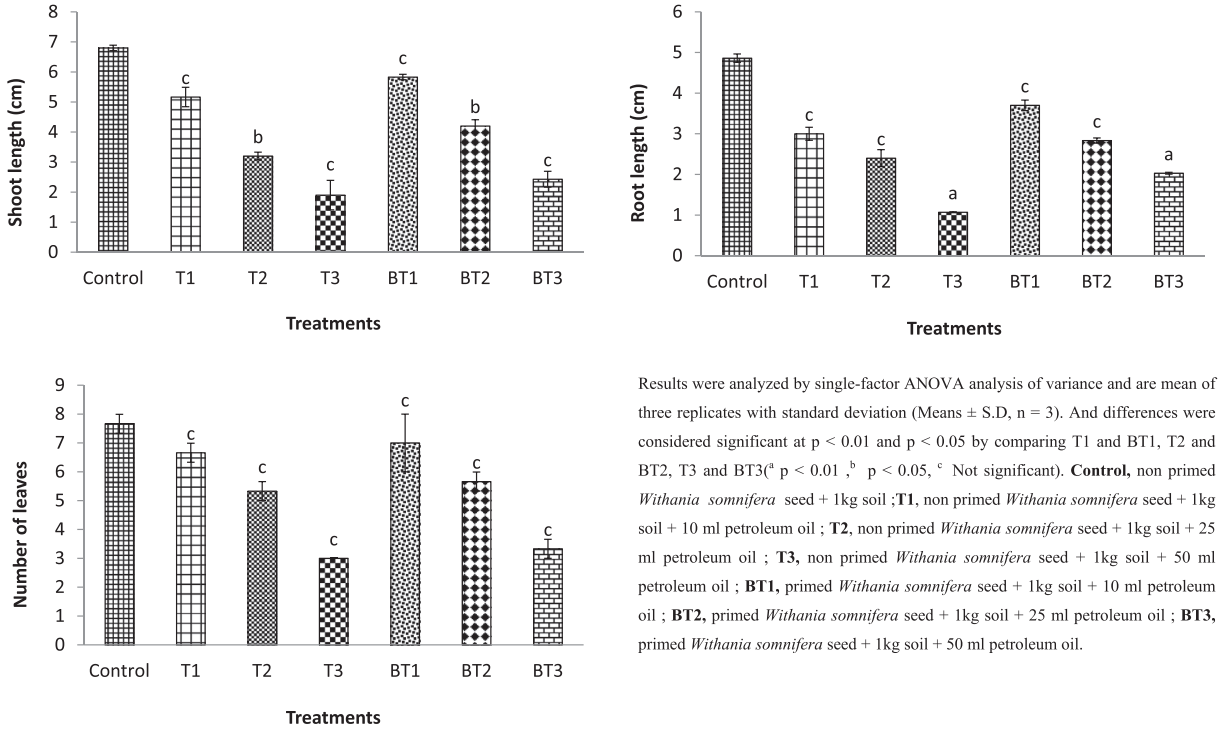


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Fig. 3. Effect of seed priming on growth parameters of *Withania somnifera* under different petroleum oil concentration.

impermeable oily film layer around the seeds interfering the proper germination (Adam and Duncan, 2002; Ziółkowska and Wyszkowski, 2010; Hawrot-Paw et al., 2015). Kulakow et al. (2000) reported the legumes tested succumbed to hydrocarbons toxicity in absence of any growth promoting bacterial inoculums. Martín et al. (2009) reported 15% decreased in the germination of alfalfa seeds in presence of petroleum sludge when compared with control soil. Sangeetha and Thangadurai, (2014) found that high concentration of petroleum sludge retarded the germination rate of *Vignaunguiculata* seeds, which might be due to the toxic effect of the sludge. Similarly, Korade and Fulekar, 2009 noticed no germination in the loamy soil contaminated with hydrocarbons.

3.5. Effect on growth

Seeds primed with *Pseudomonas* sp. AJ15 measured longer shoot and root in all the treatment as compared to non primed seeds Fig. 3. This might due to PGPR traits and potentiality of the *Pseudomonas* sp. AJ15 to degrade and utilized petroleum as carbon source. Petroleum contamination cause distorts of carbon to

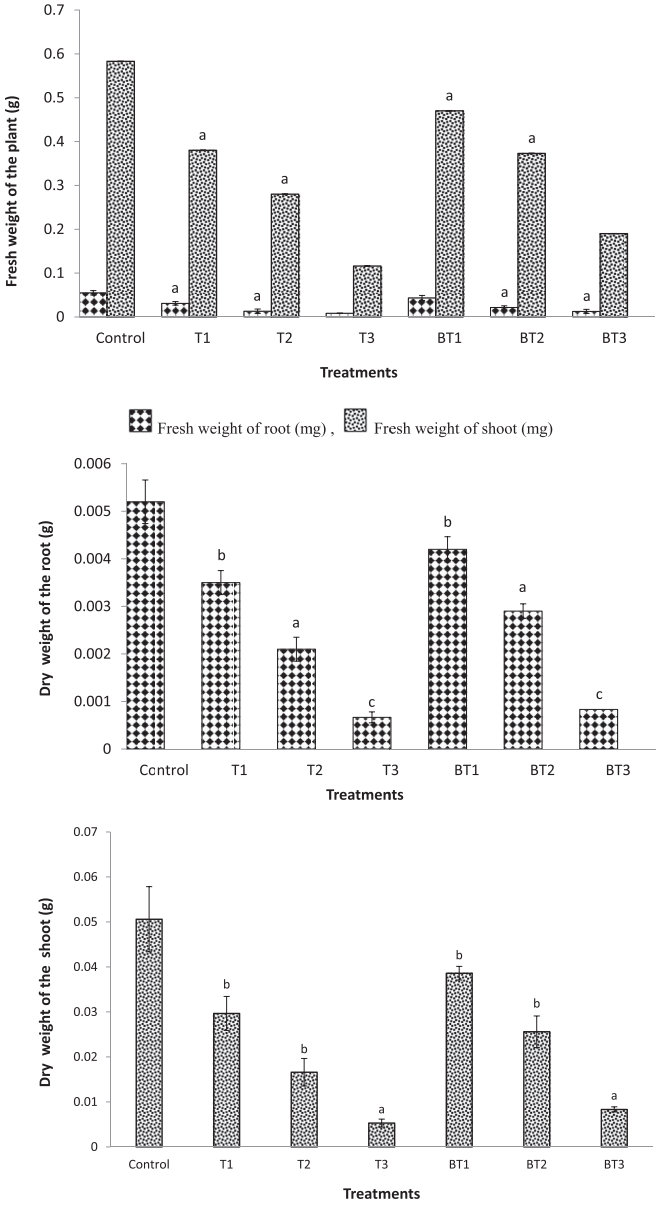

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Fig. 4. Effect of seed priming on fresh and dry weight of *Withania somnifera* under different petroleum oil concentration.

nitrogen ratio and phosphorus, in the soil, and their scarcity makes some microorganisms fully use the energy contained in hydrocarbons. Hence, they degrade and utilized the petroleum and its product in soil (Hawrot-Paw et al., 2015). Priming strategy improved the shoot and root length in all treatment, but plant raised from non primed seeds showed very poor shoot and root length Fig. 3. The poor root length might be due to anaerobiosis (Rowell, 1977). Petroleum contamination on soil reduces aeration by blocking air spaces between soil particles and creates a condition of anaerobiosis and leading to root stress (Smith et al., 1989; Shukry et al., 2013). Reduced in shoot length might be due to physical, chemical, and biological transformation of oil in soil (Lorestani et al., 2012; Baruah et al., 2014). Baruah et al., 2014 reported that the presence of crude oil in soil effect the morphology of the plant by causing many structural abnormalities such as reduction of leaf size and root length.

Kumar et al., 2013 reported that biosurfactant producing bacterial consortium is effective in reducing mobil oil stress on the plant growth parameters of wheat and mustard.

3.6. Effect on fresh and dry weight of the plants

Fresh and dry weight of the plant is enhanced by seed priming. The results indicated higher mass in plants rose from primed seed as compared to the plants from non primed seed under different petroleum oil concentration Fig. 4. This suggests that the effects of plant growth-promoting features (IAA, siderophore and solubilization of phosphate) by the strain AJ15. Various plant growth promoting bacteria (PGPR) are able to rapidly metabolize some readily available organic compounds such as total petroleum hydrocarbon present on soils. This leads to soil remediation and lower the petroleum toxicity to plants and then increase the plant tolerance to petroleum hydrocarbon stress. Thereafter, they vigorously promote plant growth, resulting in more rapid and massive biomass accumulation (Huang et al., 2001; Siciliano et al., 2003; Ajithkumar et al., 1998; Bashan and de Bashan, 2005).

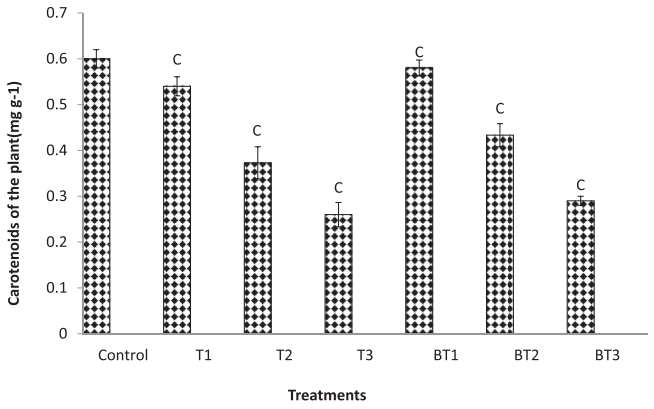

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Fig. 6. Effect of seed priming on carotenoids content of *Withania somnifera* under different petroleum oil concentration.

3.7. Effect on plant pigment

Chlorophyll plays a significant role in the plant physiology and its productivity. Growing plants on oil contaminated soil has a great impact on the synthesis of chlorophyll pigment (Baruah et al., 2014). In the present study, petroleum stress decreased the chlorophyll content of the plant as compared to control. However, seed priming increased the chlorophyll contents of the plant as compared to non primed seeds Fig. 5. Crude oil is a mixture of aromatic, aliphatic and high molecular weight organic compounds that inhibit the enzymes required for the synthesis of chlorophyll

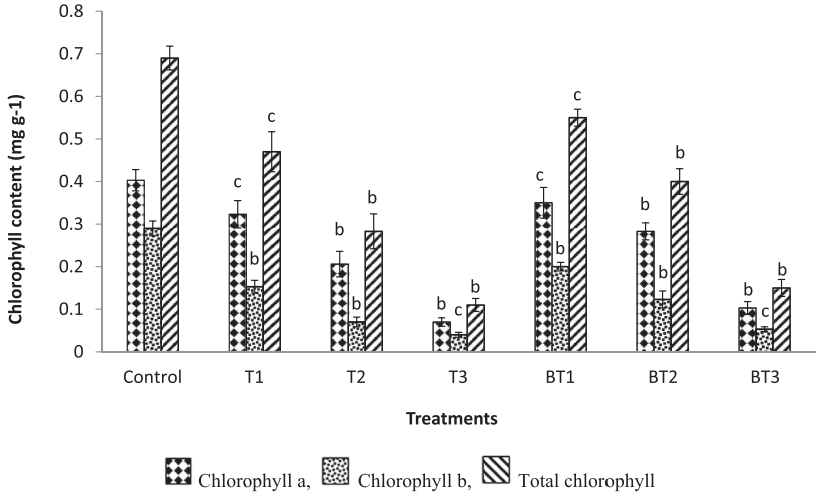

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Fig. 5. Effect of seed priming on chlorophyll content of *Withania somnifera* under different petroleum oil concentration.

(Baruah et al., 2014). Moreover, decrease in the total chlorophyll content in the leaves of the *W. somnifera* in the different treatment may be due to the alkaline condition created through dissolution of chemicals present in the oil in the cell sap that responsible for chlorophyll degradation (Baruah et al., 2014). The literature on the effect of petroleum oil stress on carotenoid pigment is scarce. However, in the present study we reported the effect of petroleum oil contamination on cartenoids pigment. Investigation revealed that there is a great effect of petroleum oil contamination on cartenoids content of the plant grown under various petroleum concentrations. Fig. 6 shows significant effect of petroleum oil and seed priming on cartenoid content. It is evident from the results that increasing concentration of petroleum profoundly affected cartenoid content of *W. somnifera*. However, the reduction values were higher in plant arose from non primed seed than plant from primed seed.

4. Conclusion

In the present study primed seeds of *W. somnifera* with *Pseudomonas* sp. AJ15 producing biosurfactant with plant growth promoting traits showed better response to petroleum toxicity or stress as compared to non primed seed. Hence, the study signifies that priming the seeds with biosurfactant producing PGPR bacteria with the potentiality to degrade and utilized petroleum as carbon source, which are more habituated to peculiar soil environment and can be effectively and commercially used for management and detoxification petroleum contaminated wastelands for agriculture purpose.

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References

Abuamsha, R., Salman, M., Ehlers, R.U., 2011. Improvement of seed bio-priming of oil seed rape (*Brassica napus ssp.oleifera*) with *Serratia plymuthica* and *Pseudomonas chlororaphis*. Biocontrol Sci.Technol. 21, 199–213.
Adam, G., Duncan, H., 2002. Influence of diesel fuel on seed germination. Environ. Pollut. 120, 363–370.
Ahmad, F., Ahmad, I., Khan, M.S., 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res. 163, 173–181.
Ajithkumar, P.V., Gangadhara, K.P., Manilal, P., Kunhi, A.A., 1998. Soil inoculation with Pseudomonas aeruginosa 3mT eliminates the inhibitory effect of 3-chloro- and 4-chlorobenzoate on tomato seed germination. Soil Biol. Chem. 30, 1053–1059.
Amund, O.O., Nwokoye, N., 1993. Hydrocarbon potentials of yeast isolates from a polluted Lagoon. J. Sci. Res. Dev. 1, 65–68.
Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts, polyphenol oxidase in Beta vulgaris. Plant Physiol. 2, 1–15.
Arumugam, A., Gopinath, K., 2013. *In vitro* regeneration of an endangered medicinal plant *Withania somnifera* using four different explants. Plant Tissue Cult. Biotechnol. 23, 79–85.
Atlas, R.M., 1975. Effects of temperature and crude oil composition on petroleum biodegradation. J. Appl. Microbiol. 30, 396–403.
Baruah, P., Saikia, R.R., Baruah, P.P., Deka, S., 2014. Effect of crude oil contamination on the chlorophyll content and morpho-anatomy of Cyperus brevifolius (Rottb.) Hassk. Environ. Sci. Pollut. Res. 21, 12530–12538.
Bashan, Y., de Bashan, L.E., 2005. Fresh-weight measurements of roots provide inaccurate estimates of the effects of plant growth promoting bacteria on root growth: a critical examination. Soil Biol. Biochem. 37, 1795–1804.
Bento, F.M., Camargo, F.A., Okeke, B.C., 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation,biostimulation and bioaugmentation. Biores. Technol. 96, 1049–1055.
Bodour, A.A., Drees, K.P., Maier, R.M., 2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Appl. Environ. Microbiol. 69, 3280–3287.
Colo, J., Hajnal-Jafari, T.I., Duric, S., Stamenov, D., Hamidovic, S., 2014. Plant growth promotion rhizobacteria in onion production. Pol. J. Microbiol. 63, 83–88.
Cooper, D.G., Goldenberg, B.G., 1987. Surface-active agents from two Bacillus species. Appl. Environ. Microbiol. 53, 224–229.
Desai, J.D., Banat, I.M., 1997. Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61, 47–64.
Fomina, M.A., Alexander, I.J., Colpaert, J.V., Gadd, G.M., 2005. Solubilization of toxic metal minerals and metal tolerance of mycorrhizal fungi. Soil Biol. Biochem. 37, 851–866.
Franzetti, A., Gandolfi, I., Bestetti, G., 2010. Production and applications of trehalose lipid biosurfactants. Eur. J. Lipid Sci. Technol. 112, 617–627.
Gary, J.H., Handwerk, G.E., 1984. Petroleum Refining Technology and Economics, second ed. Marcel Dekker, Inc. ISBN 0-8247-7150-8.
Graj, W., Lisiecki, P., Szulc, A., 2013. Bioaugmentation with petroleum-degrading consortia has a selective growth-promoting impact on crop plants germinated in diesel oil-contaminated soil. Water Air Soil Pollut. 224, 1676.
Hawrot-Paw, M., Wijatkowski, A., Mikiciuk, M., 2015. Influence of diesel and biodiesel fuel-contaminated soil on microorganisms, growth and development of plants. Plant Soil Environ. 61, 189–194.
Huang, X.D., Glick, B.R., Greenberg, M.B., 2001. Combining remediation technologies increases kinetics for removal of persistent organic contaminants from soil. In: Greenberg, B.M., Ruth, N.H., Roberts, H.M., Gensemer, W.R. (Eds.), Environ. Toxic. Risk Assess., 10, pp. 271–278.
Hrynkiewicz, K., Baum, C., 2011. The potential of rhizosphere microorganisms to promote the plant growth in disturbed soils. Environ. Protect. Strateg. Sustain. Dev. Strateg. Sustain. http://dx.doi.org/10.1007/978-94-007-1591-2_2.
Jackson, M.L., 1973. Soil Chemical Analysis. Prentice Hall of India Pvt. Ltd., New Delhi.
Johnsen, A.R., Wick, L.Y., Harms, H., 2005. Principles of microbial PAH degradation in soil. Environ. Pollut. 133, 71–84.
Jurelevicius, D., Alvarez, V.M., Marques, J.M., Sousa Lima, L.R.F.S., Dias, F.A., Seldin, L., 2013. Bacterial community response to petroleum hydrocarbon amendments in freshwater, marine, and hypersaline water-containing microcosms. Appl. Environ. Microbiol. 79, 5927–5935.
Kirk, J.T.O., Allen, R.L., 1965. Dependence of chloroplast pigment synthesis on protein synthesis: effect of actidione. Biochem. Biophy. Res. Commun. 21, 523–530.
Korade, D.L., Fulekar, M.H., 2009. Rhizosphere remediation of chlorpyrifos in mycorrhizospheric soil using ryegrass. J. Hazard. Mater. 172, 1344–1350.
Kulakow, P.A., Schwab, A.P., Banks, M.K., 2000. Screening plant species for growth on weathered, petroleum hydrocarbon-contaminated sediments. Int. J. Phytoremediat. 2, 297.
Kumar, R., Bharagava, R.N., Kumar, M., Singh, S.K., Govind, K., 2013. Enhanced biodegradation of mobil oil hydrocarbons by biosurfactant producing bacterial consortium in wheat and mustard rhizosphere. J. Pet. Environ. Biotechnol. 4, 2.
Kumar, R., Das, A.J., Juwarkar, A.A., 2015. Reclamation of petrol oil contaminated soil by rhamnolipids producing PGPR strains for growing Withania somnifera a medicinal shrub. World J. Microbiol. Biotechnol. 31, 307–313.
Loper, J.E., Scroth, M.N., 1986. Influence of bacterial sources on indole-3 acetic acid on root elongation of sugarbeet. Phytopath 76, 386–389.
Lorestani, B., Kolachi, N., Ghasemi, M., Cheraghi, M., Yousefi, N., 2012. Survey the effect of oil pollution on morphological characteristics in Faba vulgaris and Vicia eryilia. J. Chem. Health Risks 2, 5–8.
Marin, M., Pedregosa, A., Rios, S., Laborda, F., 1996. Study of factors influencing the degradation of heating oil by Acinetobactercalcoaceticus MM5. Int. Biodeterior. Biodegrad. 38, 67–75.
Martia, M.C., Camejoa, D., Garcia, N.V., 2009. Effect of oil refinery sludges on the growth and antioxidant system of alfalfa plants. J. Hazard. Mater. 171, 879–885.
Mirsal, I., 2004. Soil Pollution: Origin, Monitoring and Remediation. Springer, New York.
Mishra, L.C., Singh, B.B., Dagenais, S., 2000. Scientific basis for the therapeutic use of *Withania somnifera* (Aswagaandha), a review. Altern. Med. Rev. 5, 334–346.
Mulligan, C., Cooper, D., Neufeld, R., 1984. Selection of microbes producing biosurfactants in media without hydrocarbons. J. Ferment. Technol. 62, 311–314.
Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate solubilising microorganisms. FEMS Microbiol. Lett. 170, 265–270.
Nogueira, L., Rodrigues, A.C.F., Tridico, C.P., Fossa, C.E., de Almeida, E.A., 2011. Oxidative stress in Nile tilapia (*Oreochromis niloticus*) and armored catfish (*Pterygoplichthys anisitsi*) exposed to diesel oil. Environ. Monit. Asses. 180, 243–255.
Obayori, O.S., Ilori, M.O., Adebusoye, S.A., Oyetibo, G.O., Omotayo, A.E., Amund, O.O., 2009. Degradation of hydrocarbons and biosurfactant production by Pseudomonas sp. strain LP1. World J. Microbiol. Biotechnol. 25, 1615–1623.
Ohno, A., Ano, T., Shoda, N., 1993. Production of antifungal peptide antibioticsitur in by Bacillus subtilis NB22 in solid state fermentation. J. Ferment. Bioeng. 75, 23–27.
Pacwa-Płociniczak, M., Płociniczak, T., Iwan, J., Zarska, M., Chorazewski, M., Dzida, M., Piotrowska-Seget, Z., 2016. Isolation of hydrocarbon-degrading and biosurfactant-producing bacteria and assessment their plant growth-promoting traits. J. Environ. Manag. 168, 175–184.
Perry, J.J., 1984. Microbial metabolism of cyclic alkanes. In: Atlas, R.M. (Ed.), Petroleum Microbiology. Macmillan, New York, NY, USA, pp. 61–98.
Plaza, G.A., Zjawiony, I., Banat, I.M., 2006. Use of different methods for detection of thermophilicbiosurfactant-producing bacteria from hydrocarboncontaminated and bioremediated soils. J. Pet. Sci. Eng. 50, 71–77.
Pothuluri, J.V., Cerniglia, C.E. (Eds.), 1994. Microbial Metabolism of Polycyclic Aromatic Hydrocarbons in Biological Degradation and Bioremediation of Toxic Chemicals. Chapman & Hall, London, pp. 92–124.

Rowell, M.J., 1977. The effects of crude oil spill on soils. A review of literature I. In: Toogood, J.A. (Ed.), The Reclamation of Agricultural Soils after Oil Spills, Part I. Edmonton, Canada.
Ron, E.Z., Rosenberg, E., 2002. Biosurfactants and oil bioremediation. Curr. Opin. Chem. Biol. 13, 249–252.
Sagar, S., Dwivedi, A., Yadav, S., Tripathi, M., Kaistha, S.D., 2012. Hexavalent chromium reduction and plant growth promotion by *Staphylococcus arlettae* strain Cr11. Chemosphere 86, 847–852.
Sangeetha, J., Thangadurai, D., 2014. Effect of biologically treated petroleum sludge on seed germination and seedling growth of *Vigna unguiculata* (L.) Walp. (Fabaceae). Braz. Arch. Biol. Technol. 57, 427–433.
Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 47–56.
Shukry, W.M., Al-Hawas, G.H.S., Al-Moaikal, R.M.S., El-Bendary, M.A., 2013. Effect of petroleum crude oil on mineral nutrient elements, soil properties and bacterial biomass of rhizosphere of jojoba. Br. J. Environ. Clim. Change 3, 103–118.
Siciliano, S.D., Germida, J.J., Banks, K., Greer, C.W., 2003. Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial. Appl. Environ. Microbiol. 69, 483–489.
Smith, B., Stachowisk, M., Volkenburgh, E., 1989. Cellular processes limiting leaf growth in plants under hypoxic root stress. J. Exp. Bot. 40, 89–94.
Viramontes-Ramos, S., Portillo-Ruiz, M.C., Ballinas-Casarrubias, M.D.L., Torres-Muñoz, J.V., Rivera-Chavira, B.E., Nevárez-Moorillón, G.V., 2010. Selection of biosurfactan/bioemulsifier producing bacteria from hydrocarbon-contaminated soil. Braz. J. Microbiol. 41, 668–675.
Wenzel, W.W., 2009. Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant Soil 321, 385–408.
Yang, J., Kloepper, J.W., Ryu, C.M., 2008. Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci. 14 (1), 1–4.
Ziółkowska, A., Wyszkowski, M., 2010. Toxicity of petroleum substances to microorganisms and plants. Ecol. Chem. Eng. S 17, 73–82.

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ORIGINAL PAPER

Reclamation of petrol oil contaminated soil by rhamnolipids producing PGPR strains for growing *Withania somnifera* a medicinal shrub

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Abstract Soil contaminated by hydrocarbons, cannot be used for agricultural intents due to their toxic effect to the plants. Surfactants producing by plant growth promotory rhizobacteria (PGPR) can effectively rig the problem of petroleum hydrocarbon contamination and growth promotion on such contaminated soils. In the present study three *Pseudomonas* strains isolated from contaminated soil identified by 16S rRNA analysis were ascertained for PGPR as well as biosurfactants property. Biosurfactants produced by the strains were further characterized and essayed for rhamnolipids. Inoculation of the strains in petrol hydrocarbon contaminated soil and its interaction with *Withania somnifera* in presence of petrol oil hydrocarbons depict that the strains helped in growth promotion of *Withania somnifera* in petrol oil contaminated soil while rhamnolipids helped in lowering the toxicity of petrol oil. The study was found to be beneficial as the growth and antioxidant activity of *Withania sominfera* was enhanced. Hence the present study signifies that rhamnolipids producing PGPR strains could be a better measure for reclamation of petrol contaminated sites for growing medicinal plants.

Keywords Rhamnolipids · PGPR · *Withania somnifera* · Antioxidant activity · Reclamation

Introduction

Pollution of soil by petroleum hydrocarbons causes major changes in the physical and chemical properties of soil resulting in adverse effect on plant growth. The damage to plant is attributed to anaerobic and hydrostatic condition that interferes with soil plant water relation (Kuiper et al. 2004). Bona et al. (2011), Ahammed et al. (2012) demonstrated that accumulation of toxic petroleum compound by plant tissues results in decrease in their biomass and total size. Environmental contamination with petroleum and its constituent is an inevitable problem that affects many geographical regions to a variable extent depending on the local environmental law (Graj et al. 2013). Certain microorganisms have developed a mechanism to grow and thrive in oil containing environment and play an important role in treatment of this pollutant by degrading it (Pothuluri and Cerniglia 1994). One of the limiting factors in this process is the bioavailability of many fractions of oil. This limitation can be overcome by the biosurfactants of diverse chemical nature and molecular size produced by hydrocarbon degrading microorganisms. Biosurfactants are surface active materials increases the surface area of hydrophobic water insoluble substrates and increase their bioavailability, thereby enhancing the growth of bacteria and rate of bioremediation (Hou et al. 2001; Ron and Rosenberg 2002). Many strains of genus *Pseudomonas* possess the capability to promote plant growth. The

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bacteria inhabiting plant roots and influencing the plant growth positively by any mechanism are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). The PGPR improve plant growth either directly via production of plant growth regulators such as auxin and cytokinins or indirectly, through biological control of pathogen or introduction of host defense mechanisms (Glick 1995). Some of the important properties which these strains possess are phosphate solubilization, siderophore, indole acetic acid (IAA), ACC deaminase, Ammonia and HCN production etc. These properties of PGPR's can enhance various stages of plant growth development and combat abiotic stress (Yang et al. 2008). Various medicinal plants, which are threatened and of medicinal importance can be used for studying interaction with these PGPR strains in hydrocarbon contaminated wastelands.

Withania somnifera commonly known as Ashwagandha belonging to family Solanaceae, is a medicinal shrub found throughout the drier parts of Afghanistan, Egypt, India, Pakistan, Sri Lanka and South Africa (Dhanani et al. 2013). The shrub enhance defense against diseases and have antipyretic, anti-inflammatory and abortifacient properties (Ali el al. 1997). Due to its immense importance, the plant was selected for the present study to evaluate the effect of petrol oil contamination on its growth parameters and antioxidant properties. Further the study also deals with exploration of biosurfactant producing bacterial strains with PGPR property to reduce the stress created due to petrol oil contamination.

Materials and methods

Collection of soil sample and isolation of bacterial strains in pure form

Sub-surface soil sample was collected from oil contaminated sites of Pantnagar, Uttarakhand, India (29°N latitude and 79°E longitude). Using serial dilution technique, bacterial strains were isolated on King's B agar medium at 30 °C in 48 h. The strains were isolated in pure form from different samples.

Characterization of the bacterial strains

Bacterial strains isolated were characterized morphologically and biochemically and further screened for plant growth promotory properties and for biosurfactant production on minimal salt medium MSM (composition g/l $NaNO_3$-2.5, K_2HPO_4-1.0, KH_2PO_4-0.5, $MgSO_4$ (anhydrous)-0.5, KCl-0.1, $FeSO_4$-0.01, $CaCl_2$-0.1, Na_2HPO_4-5.67, $MnSO_4$-0.002, NH_4NO_3-0.39, dextrose-30, pH 7.0; dextrose autoclaved separately) incubated at 30 °C for 72 h with shaking at 150 rpm. Three potent bacterial strains named RK3, RK4 and RK7 were selected for present study on the basis of their property.

Plant growth promotory properties (PGPR)

Qualitative analysis of phosphate solubilization was screened on NBRIP medium according to the method described by Nautiyal (1999) and Quantitative analysis was performed as per the proposal of Bashan et al. (2013b), which suggest that selection of the metal phosphates candidates for potential Phosphate solubilizing bacteria (PSB) will depend on the type of soil (alkaline, acidic, or organic-rich) where the PSB will be used. Adding Calcium phosphate compounds for alkaline soils, Ferric phosphate and Alluminium phosphate compounds for acidic soils and phytates for soils rich in organic phosphate. For the quantitative estimation of phosphate solubilization NBRIP broth was amended with 5 g l^{-1} each of tricalcium phosphate and ferric phosphate (pH of petrol contaminated soil is 6–6.5) separately in two different set. One ml of each bacterial culture was added to each flask and incubated at 28 °C for 15 days. After the recommended day the contents of the each flask were filtered through Whatman filter paper No. 1. The sub samples were centrifuged and the supernatant was filtered. The filtrate was used to measure the soluble phosphate (Jackson 1973). Siderophores production was assayed on the Chrome azurol S agar medium as per the method of Schwyn and Neilands (1987). Quantitative analysis of IAA was done by following the method of Loper and Scroth (1986) at concentrations of 50 and 100 mg/ml of tryptophan.

Screening and charaterization of the biosurfactant produced by the strains

Biosurfactant produced by the bacterial strains was characterized by different methods:

Foaming

Bacterial strains showing foaming in the medium as compared to negative controls were observed for reflected light which scattered like rainbow colours. Contents of the test tubes of minimal salt medium with the test strains (bacterial strains) showing foaming were subjected to centrifugation at 10,000×*g* for 20 min (4 °C). Cell free supernatant (crude biosurfactant) was used for further experiments.

Surface tension

Cell free supernatant was subjected to surface tension determination using Du Nuoy Ring detachment method with Fischer Autotensiomat Model-21.

Emulsification index

Two ml of petrol engine oil hydrocarbons was taken in test tubes. They were then overlaid with 2 ml of crude biosurfactant. Height of oil, crude biosurfactant and total height of the column was recorded. The resulting mixture was vortexed for 2 min to enable proper mixing of petrol engine oil hydrocarbons and crude biosurfactant. Tubes were allowed to stand for 24 h without any disturbance. After 24 h, height of the emulsified layer was recorded to calculate emulsification index (Cooper and Goldenberg 1987). The equation used to determine the emulsion index E_{24} (%) is as follows: $E_{24}(\%) = \frac{H1}{H2} \times 100$ H1 = the height of emulsion layer, H2 = the height of total solution.

Detection of rhamnolipids by CTAB test

For screening of rhamnolipid production the isolates were spot inoculated on the plates composed of the mineral salts medium with the addition of 200 µg/ml cetyl-trimethyl ammonium bromide (CTAB; Himedia), 5 µg/ml methylene blue, and 1.5 % agar (Siegmund and Wagner 1991).

Experimental design and treatment

The pot experiment was conducted in a naturally ventilated climate controlled chamber made of polyethylene know as poly house at 20–24 °C for 90 days. The whole experiment was performed in presence of petrol engine oil hydrocarbons and replicated thrice with suitable control.

Culture treatment in pots

Experimental soil was dried and sieved with 2 mm sieve and autoclaved for 1 h at 121 °C for three successive days. 30 ml petrol engine oil hydrocarbons were mixed with 1.5 kg of sterilized soil in each pot. The mixture was allowed to stabilize and evaporate for 10 days. Fifty ml of 72 h grown culture in MSM on a rotary shaker (150 rpm) having bacterial population approx. 1×10^7 cells of the test strains were applied to respective pots. The inoculated pots were allowed to stabilize for 7 days. The seeds of *Withania somnifera* variety Jawahar-20 obtained from Medicinal plants Research and Development Centre, Pantnagar, India were washed with 0.1 % $HgCl_2$ followed by 8–10 times washings with distilled water. The treated seeds were sown in the pots after 7 days. Twenty seeds were sown in each pot just below the oil surfaces (2.5 cm).

Plant health parameter

The effect of different inoculated strains and the effect of petrol engine oil on plant health were monitored by taking the seedling germination percentage, plant height, root length, chlorophyll content and average yield per pot (Kulakow et al. 2000).

Dry weight determination and preparation of extract to assess effect of petrol oil contaminated soil on antioxidant properties

After 3 months plants were uprooted and fresh weight and dry weight of the plants were measured (Bashan and de Bashan 2005). For the preparation of the extract roots were washed with distilled water thrice, surface sterilized with 0.1 % mercuric chloride for 20 s, followed by three times repeated washing with sterile distilled water. Roots were then air dried for 4 weeks at room temperature and grounded to powder form, using an agate pestle and mortar.

Determination of antioxidant activities

DPPH Free radical activity was determined using the procedure of Braca et al. (2001) and the chelating of ferrous ions by the extract and standards was estimated using method reported by Decker and Welch (1990). Reducing power activity was estimated according to the method of Oyaizu (1986).

Molecular characterization of the bacterial strains

The PCR product (using universal primers) of the bacterial strains three in number was sequenced using 16 S rRNA sequencing. The sequences obtained were blasted using NCBI site and similarity coefficient was used to establish the genus and species based on the homology.

Statistical analysis

Experimental results were analyzed by using one-way-analysis of variance (ANOVA). Statistical analysis Data were expressed as mean ± SD and differences were considered significant at $p < 0.05$ and $p < 0.01$.

Results and discussion

Isolation and characterization of the bacterial strains

Three potent bacterial strains were isolated on King's B agar medium from soil sample by serial dilution method. All the isolated bacterial strains were Gram negative with RK3 and RK4 showing green fluorescence while RK7 showing yellow fluorescence on King's B agar. Based on 16S rRNA studies, isolated bacterial strains were found

similar to *Pseudomonas aeruginosa* (RK3 and RK4) and *Pseudomonas sp.* (RK7). The similarity index among the test strains and with other strains is shown in Fig. 1. Sequences of the test strains RK3, RK4 and RK7 have been deposited in the NCBI database and accession number allotted to them are HM771642, HM771643, HM 771645, respectively.

Table 1 Plant growth promotory properties of the test strains

Test strain	Accession no	Siderophore production	IAA production	Phosphate-solubilization
RK-3	HM771642	++	++	++
RK-4	HM771643	+	+	+
RK-7	HM771645	++	++	++

(+) moderate, (++) good, (−) negative results

Plant growth promoting activities

All the test strains were found positive for phosphate solubilization, siderophore and IAA production (Table 1). In qualitative analysis of phosphate clear zone around the colonies indicate that the strains has the ability to solubilise phosphate, whereas quantitative test for solubilization of tricalcium phosphate(TCP) in liquid medium by the test strains showed that strain RK3 and RK7 solubilize tricalcium phosphate up to 90 % and RK4 up to 75 %. The poor solubilization of ferric phosphate (Fe–P) was observed as compared to TCP. Both RK3 and RK7 solubilised Fe–P up to 65 % and RK4 up to 43 %. This is due to phosphates (P), mainly Fe–P, Al–P and Ca–P are even less soluble than TCP in water (Greaves 1922; Bashan et al. 2013a). TCP is use as a universal factor for isolating and evaluating phosphate-solubilizing bacteria (PSB), but is relatively weak and unreliable as a universal selection factor for isolating and testing a PSB for enhancing plant growth. The use of TCP usually yields many isolates supposedly PSB (Bashan et al. 2013b). When these isolates are further tested for direct contribution of phosphorus to the plants, only a very few truns to be true PSB, because soils greatly vary in pH and several chemical properties (Bashan et al. 2013b). Quantitative estimation of IAA in the presence of 50 and 100 mg/ml concentrations of tryptophan indicates that the strains were able to produced IAA. 3.9 ± 0.4 mg/ml by RK3, 4.6 ± 0.22 mg/ml by RK7 and 2.7 ± 0.7 mg/ml by RK4 IAA was produced at 50 mg/ml of tryptophan concentration, whereas 6.1 ± 0.5 mg/ml by RK4, 6.8 ± 0.3 mg/ml by RK7 and 4.91 ± 0.6 mg/ml by RK3 IAA was reported at 100 mg/ml of tryptophan concentration. Siderophore

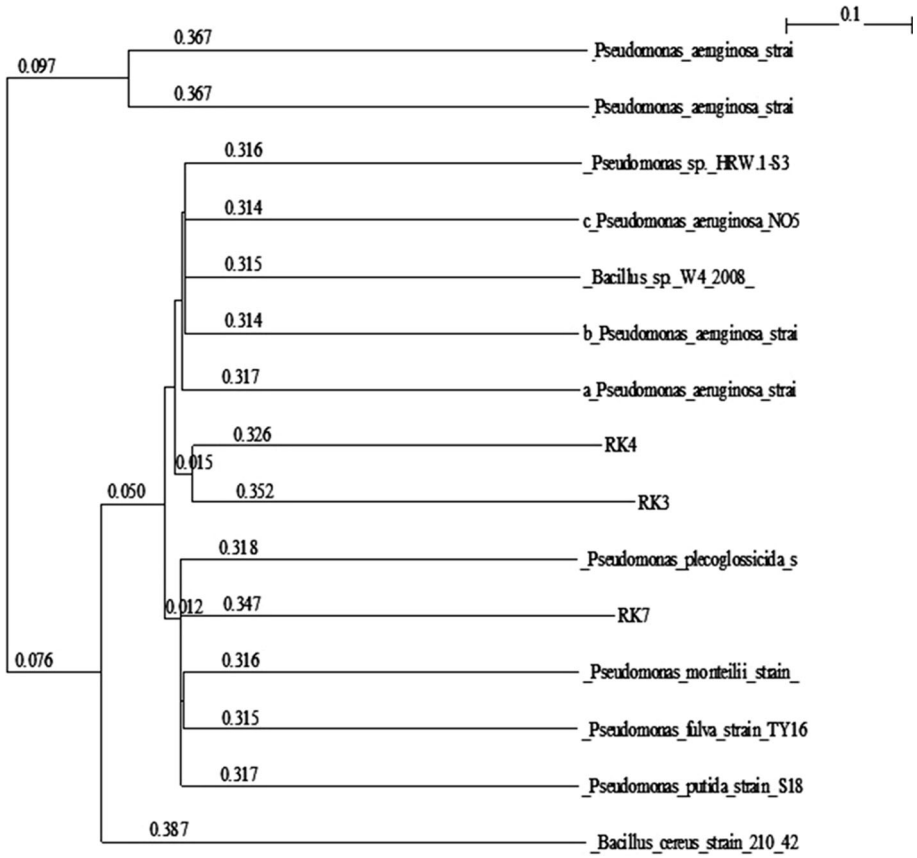

Fig. 1 16S rRNA-based phylogenetic tree of the strains

production on Chrome Azurol-S agar (CAS agar) plates were found to be positive as indicated by change in colour of the media around the colonies.

Characterization of biosurfactant produced by the test strains

All the test strains produced biosurfactant on mineral salt medium (MSM). Each test strains produced foam with surfactants, RK3 and RK4 giving better foaming as compared to RK7. Surfactant produced by the test strains reduced surface tension up to 29 dynes cm^{-1}. All the cultures were able to reduce surface tension with RK4 being the best culture in reducing surface tension while RK3 was comparable to RK4 in reducing surface tension (Table 2). Emulsification ability of surfactant produced by the bacterial strains RK3 and RK4 was better than RK7. Emulsification index of surfactant from RK3 and RK4 was 40.00 and 42.30 respectively (Table 2). The emulsification property is very important as it can be used for several applications like oil recovery and bioremediation of oil spilled sites.

Detection and quantification of rhamnolipids

In Cetyl-trimethyl Ammonium Bromide-methylene blue test, blue dark blue colonies were formed by the strains which indicates the production of rhamnolipids. Rhamnolipids consist of a polar head and non-polar tail group like synthetic surfactants and as a result they combine with cationic substances, like Cetyl-trimethyl Ammonium Bromide to form insoluble ion pairs in aqueous solution and precipitates as dark blue zones against a blue black ground (Siegmund and Wagner 1991; Tahzibi et al. 2004; Abouseoud et al. 2008). The amount of rhamnolipids produced by the strains RK3, RK4 and RK7 was estimated using the Orcinol method which indicates production occurred during the stationary phase of growth.

Germination

There was a delay in germination in all the cases and the germination percentage was low in presence of petrol engine oil hydrocarbons as compared to control (Table 3). However, as compared to C1 treatment (petrol engine oil only and no bacterial culture), germination percentage was more in the other treatments. Among the bacterial strains, plants in pots with bacterial strain RK7 showed better germination percentage than RK3 and RK4. The lower percentage of germination in C1 pots can be attributed to the toxicity of petrol engine oil hydrocarbons. Similar findings have been reported by Kulakow et al. (2000) where the legumes tested succumbed to hydrocarbons toxicity in absence of any growth promoting bacterial inoculums.

Table 2 Surface tension and emulsification index by crude surfactant after 72 h

Sr. No.	Test strain	Surface tension in dynes cm^{-1} with 6 cm ring	Emulsification index (E_{24}) for petrol engine oil hydrocarbons
1	Standard	72.80	–
2	RK3	30.50	40.00
3	RK4	29.00	42.30
5	RK7	44.00	34.61

Standard (distilled water)

Plant health parameters

Chlorophyll content was again better in RK7 culture treated plant as compared to the control (Table 3). The amount of siderophore, produced by the bacterial strain RK4 was less as compared to other test strains that might be the reason for low content of chlorophyll as iron deficiency inhibits both chloroplast development and chlorophyll biosynthesis under abiotic stress (Imsande 1998; Kulakow et al. 2000). All the inoculated plants showed increase in plant height with the passage of time. However, the increase was less as compared to the control pot plants but better than the C1 treated plants. The plants inoculated with test strain RK7 were the best in plant height as compared to other two bacterial strains which can be attributed to the production of IAA by the test strains (Table 3). Root length was also better in presence of all the three bacterial test strains while in case of C1, the root growth was very decelerated (Table 3). Dry weight determination indicates increase of biomass of the plants in pots treated with bacterial culture (Treatment RK3, RK4 and RK7) as compared to the C1 treatment pot where no bacterial culture was inoculated. This suggests that the effects of plant growth-promoting features (IAA, siderophore and solubilization of phosphate) by the strains.

Effect on antioxidant activity

There was a variation in the antioxidant properties like DPPH, metal chelating and reducing activities among the test strains. Control and RK7 inoculated plants showed decrease in absorbance of DPPH radical. This is due to the reaction between antioxidant molecules and DPPH radical, which results in the scavenging of the radical by hydrogen donation. Decrease in absorbance of the reaction mixture indicates higher free radical scavenging activity of control and RK7, whereas un-inoculated plant (C1) had poor DPPH activity as shown in (Table 4). Metal chelating capacity is important since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. The

Table 3 Health parameters of *Withania somnifera* in petrol engine oil contaminated soil day after germination (DAG), Results are mean of three replicates with standard deviation (Means ± S.D, n = 3) and differences were considered significant at $p < 0.05$ and $p < 0.01$

Plant health parameters		RK3	RK4	RK7	C1	Control
Germination percentage (%)	15 DAS	35 ± 0.5**	26.5 ± 0.11**	53 ± 0.4**	18 ± 0.91**	70 ± 0.66
	30 DAS	51.5 ± 1.5**	50 ± 0.8**	75 ± 1**	38 ± 1.6**	91.5 ± 0.97
Chlorophyll content index	45 DAG	0.19 ± 0.05	0.14 ± 0.043*	0.3 ± 0.02*	0.04 ± 0.043**	0.24 ± 0.026
	90 DAG	0.24 ± 0.04	0.17 ± 0.052	0.3 ± 0.01	0.038 ± 0.027*	0.33 ± 0.11
Plant height(cm) day after germination (DAG)	30 DAG	1.42 ± 0.20**	1.73 ± 0.20**	1.73 ± 0.5**	1.48 ± 0.21**	6.03 ± 0.12
	60 DAG	4.076 ± 0.24**	1.91 ± 006**	7.11 ± 0.62*	1.80 ± 0.30**	8.38 ± 0.49
	90 DAG	7.21 ± 0.61*	3.5 ± 0.5**	9.35 ± 0.52	2.17 ± 0.59**	11.62 ± 1.26
Root length (cm) day after germination(DAG)	30 DAG	1.65 ± 0.32**	1.4 ± 0.36**	1.76 ± 0.71**	1.25 ± 0.28**	5.54 ± 0.45
	60 DAG	4.47 ± 0.88*	1.71 ± 0.36**	5.03 ± 0.63*	1.26 ± 0.25**	7.45 ± 1.05
	90 DAG	6.45 ± 0.63**	2.83 ± 0.80**	9.62 ± 0.9**	1.64 ± 0.23**	12.79 ± 0.44

* $p < 0.05$, ** $p < 0.01$ (p values were determined by comparing the each treatment with Control)

RK3 Withania somnifera + culture RK 3 + petrol oil + soil, *RK4 Withania somnifera* + culture RK 4 + petrol oil + soil, *RK7 Withania somnifera* + culture RK 7 + petrol oil + soil, *C1 Withania somnifera* + no culture + petrol oil + soil, *Control Withania somnifera* + no culture + no petrol oil + soil

Table 4 Antioxidant Activity of *Withania somnifera* after 90 days of treatment, Results are mean of three replicates with standard deviation (Means ± S.D, n = 3) and differences were considered significant at $p < 0.05$ and $p < 0.01$

Antioxidant activity	RK3	RK4	RK 7	C1	Control
DPPH activity (μg ml^{-1})	80.96 ± 2.95	89.46 ± 2.15*	84 ± 1.05	95.93 ± 1.2**	81.26 ± 2.31
Metal chelating activity (μg ml^{-1})	41.78 ± 1.65**	41.93 ± 2.07**	39.9 ± 2.1	52.37 ± 1.48**	37.94 ± 1.66
Reducing power activity (μg ml^{-1})	62.16 ± 3.25*	61.96 ± 1.95**	64.2 ± 2.03*	55.1 ± 1.01**	71.5667 ± 2.28

* $p < 0.05$, ** $p < 0.01$ (p values were determined by comparing the each treatment with control)

RK3 Withania somnifera + culture RK 3 + petrol oil + soil, *RK4 Withania somnifera* + culture RK 4 + petrol oil + soil, *RK7 Withania somnifera* + culture RK 7 + petrol oil + soil, *C1 Withania somnifera* + no culture + petrol oil + soil, *Control Withania somnifera* + no culture + no petrol oil + soil

metal chelating activity of RK7 was nearly same as that of control (Table 4). These results indicated that RK7 bacteria have dual quality i.e. bioremediation activity and plant growth promotion activity, while treatment C1 showed very low metal chelating activity. Reducing power activity of the root extract of *Withania somnifera* in various treatments shows varying results. Increase in absorbance indicates increase in reducing power. Reducing activity of RK7 and control (only plant and no petrol engine oil hydrocarbons) is highest as compared to the other treatments while the other test strains had low reducing capability, although better than C1 plants (petrol engine oil hydrocarbons) where no bacterial strains were inoculated (Table 4). The antioxidant activity and its consequence for the acclimatization of plants to climatic stresses has been reviewed often (Martia et al. 2009) although a very little is known regarding the effects of petroleum contamination on the growth and on the antioxidative activity in plant. Hence, further studies are required to know the petroleum hydrocarbon stress on antioxidant activity of medicinal plants.

Petroleum cause pollution of local ground water and agricultural soil (Wang et al. 2008). Petrol oil has a toxic effect on the growth of the plants due to the hydrocarbons (Ahammed et al. 2012; Bona et al. 2011). In fact, the toxicity of petroleum hydrocarbon on soil organisms has been widely studied by the most of the researchers, still the effect of petroleum hydrocarbons in medicinal plant is assaying behind. Biosurfactants are biodegradable surface-active organic compounds with low toxicity and show better environmental compatibility and activity at extreme pH, temperatures and salinity and has a tendency to remediate petroleum hydrocarbon contaminated soil (Dibble and Bartha 1979; Desai and Banat 1997; Urum and Pekdemir 2004; Urum et al. 2006; Lai et al. 2009; Khopade et al. 2012). Rhamnolipids are best studied biosurfactants and have been identified predominantly from *Pseudomonas sps* (Geys et al. 2014). The strains (RK3, RK4 and RK7) produces rhamnolipids, but the strain RK7 showed more potential activity due to its plant growth promotory properties. The plant growth properties possessed by the strains helped in plant growth promotion of *Withania somnifera* in petrol engine oil contaminated soil while surfactant property of the strains helped in lowering the toxicity of hydrocarbons to the plant. The antioxidant activity of the

plants inoculated with test strain RK7 were equivalent to the control plants where no petrol engine oil hydrocarbons were present while other test strains (RK3 and RK4) inoculated plants of *Withania somnifera* had low antioxidant activity but still better than the plants where no bacterial culture was inoculated. So, microbes isolated from petroleum hydrocarbons affected sites, which are more adapted to peculiar soil environment and can be commercially and effectively used for raising medicinal plants like *Withania sominifera* in hydrocarbons contaminated soils. The study was found to be beneficial as there was no major drastic effect on the medicinal quality of *Withania sominfera* and the strains can be used for reclamation of hydrocarbons contaminated soils.

References

Abouseoud M, Maachi R, Amrane A (2008) Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. Desalination 223:143–151

Ahammed GJ, Wang MM, Zhou YH (2012) The growth, photosynthesis and antioxidant defense responses of five vegetable crops to phenanthrene stress. Ecotoxicol Environ Saf 80:132–139

Ali M, Shuaib M, Ansari SH (1997) Withanolides from the stem bark of *Withania somnifera*. Phytochemistry 44(6):1163–1168

Bashan Y, de Bashan LE (2005) Fresh-weight measurements of roots provide inaccurate estimates of the effects of plant growth-promoting bacteria on root growth: a critical examination. Soil Biol Biochem 37:1795–1804

Bashan Y, Kamnev AA, de Bashan LE (2013a) Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. Biol Fertil Soils 49:465–479

Bashan Y, Kamnev AA, de Bashan LE (2013b) A proposal for isolating and testing phosphate-solubilizing bacteria that enhance plant growth. Biol Fertil Soils 49:1–2

Bona C, Rezende I, Santos G, Souza L (2011) Effect of soil contaminated by diesel oil on the germination of seeds and the growth of *Schinus terebinthifoilus*. Brazilian archiv Biol Technol 54(6):1379–1387

Braca A, Tommasi ND, Bari LD, Pizza C, Politi M, Morelli I (2001) Antioxidant principles from *Bauhinia tarapotensis*. J Nat Prod 64(7):892–895

Cooper DG, Goldenberg BG (1987) Surface-active agents from two *Bacillus species*. Appl Environ Microbiol 53:224–229

Decker EA, Welch B (1990) Role of ferritin as a lipid oxidation catalyst in muscle food. J Agric Food Chem 38:674–677

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61(1):47–64

Dhanani T, Shah S, Gajbhiye NA, Kumar S (2013) Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*. Arabian J Chem. doi:10.1016/j.arabjc.2013.02.015

Dibble JT, Bartha R (1979) Effect of environmental parameters on the biodegradation of oil sludge. Appl Environ Microbiol 37(4): 729–739

Geys R, Soetaert W, Bogaert IV (2014) Biotechnological opportunities in biosurfactant production. Curr Opin Biotechnol 30:66–72

Glick BR (1995) The enhancement of plant growth by free living bacteria. Can J Microbiol 41:109–117

Graj W, Lisiecki P, Szulc A (2013) Bioaugmentation with petroleum-degrading consortia has a selective growth-promoting impact on crop plants germinated in diesel oil-contaminated soil. Water Air Soil Pollut 224:1676

Greaves JE (1922) Influence of salts on bacterial activities of soil. Bot Gaz 73:161–180

Hou FS, Milke MW, Leung DW, MacPherson DJ (2001) Variation in phytoremediation performance with diesel contaminated soil. Environ Technol 22:215–222

Imsande J (1998) Iron, sulfur and chlorophyll deficiencies: A need for an integrative approach in plant physiology. Physiol Plant 103:139–144

Jackson ML (1973) Soil chemical analysis. Prentice Hall of India Pvt. Ltd., New Delhi

Khopade A, Ren B, Liu XY, Mahadik K, Zhang L, Kokare C (2012) Production and characterization of biosurfactant from marine *Streptomyces species* B3. J Colloid Interface Sci 367:311–318

Kloepper JW, Schroth MN (1978) Plant growth-promoting rhizobacteria on radishes: proceedings of the 4th internatational conference on plant pathogenic bacteria, Station de Pathologie Vegetable et Phytobacteriologie, INRA, Angers, France, 879–882

Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJJ (2004) Rhizoremediation: a beneficial plant-microbe interaction. Mol Plant Microbe Interact 17:6–15

Kulakow PA, Schwab AP, Banks MK (2000) Screening plant species for growth on weathered, petroleum hydrocarbon-contaminated sediments. Int J Phytorem 2:297

Lai CC, Huang YC, Wei YH, Chang JS (2009) Biosurfactant-enhanced removal of total petroleum hydrocarbons from contaminated soil. J Hazard Mater 167(1–3):609–614

Loper JE, Scroth MN (1986) Influence of bacterial sources on indole-3 acetic acid on root elongation of sugarbeet. Phytopathol 76:386–389

Martia MC, Camejoa D, Garcia NV (2009) Effect of oil refinery sludges on the growth and antioxidant system of alfalfa plants. J Hazard Mater 171:879–885

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilising microorganisms. FEMS Microbiol Lett 170:265–270

Oyaizu M (1986) Studies on product of browning reaction prepared from glucose amine. Jpn J Nutr 44:755–762

Pothuluri JV, Cerniglia CE (1994) Microbial metabolism of polycyclic aromatic hydrocarbons. In: Chaudhary RG (ed) Biological degradation and bioremediation of toxic chemicals. Chapman & Hall, London, pp 92–124

Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Chem Biol 13:249–252

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Siegmund I, Wagner F (1991) New method for detecting rhamnolipids excreted by *Pseudomonas species* during growth on mineral agar. Biotechnol Tech 5:265–268

Tahzibi A, Kamal F, Assadi MM (2004) Improved production of rhamnolipds by a *Pseudomonas aeruginosa* mutant. Iran Biomed J 8:25–31

Urum K, Pekdemir T (2004) Evaluation of biosurfactants for crude oil contaminated soil washing. Chemosphere 57(9):1139–1150

Urum K, Grigson S, Pekdemir T, McMenamy S (2006) A comparison of the efficiency of different surfactants for removal of crude oil from contaminated soils. Chemosphere 62(9):1403–1410

Wang J, Zhang ZZ, Su YM, He W, He F, Song HG (2008) Phytoremediation of petroleum polluted soil. Petrol Sci 5(2):167

Yang J, Kloepper JW, Ryu CM (2008) Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci 14(1):1–4

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Preface

Biosurfactants are the secondary metabolites derived from plant, animal, and microorganisms. Biosurfactants of microbial origin are synthesized during the late stationary phase by the organisms and have diverse properties, which make them versatile for use in various fields. Rhamnolipids are one of them, which belong to the glycolipid group and were reported for the first time in the year 1946 from the bacterial strain *Pseudomonas aeruginosa*. Rhamnolipids comprise of three hydroxyl fatty acid unit linked to rhamnose unit by a β-glycosidic bond. The 3-hydroxyl fatty acids of rhamnolipids are linked to each other via an ester bond, whereas rhamnose units are linked together by O-glycosidic bonds. They are further categorized based on the number of rhamnose units and composition of fatty acid units.

Like chemical surfactants, rhamnolipids possess various physicochemical properties and characteristics such as emulsification, de-emulsification, detergency, wetting and foaming, and interfacial and surface tension reduction properties between liquids and solids. Due to their biological origin and consumer acceptability their market is increasing day by day.

This book highlights the recent developments on rhamnolipid biosurfactant, its properties, role in the natural environment, and various applications. Recently, rhamnolipids have shown promising applications in environment, agriculture, food, petroleum, and pharmaceutical industries due to their environment-friendly and degradable nature. The book emphasizes the various techniques that are utilized for the detection and isolation of rhamnolipid biosurfactant from microorganisms. It highlights the various aspects of the rhamnolipid biosurfactants including structural characteristics, production, and its application. The book presents the current knowledge and the latest advances in the function-based strategies to facilitate the exploration of novel biosurfactants. Authors wish to thank Springer for publishing this authored work.

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