



Microbiological and biotechnological approach for identification of biosurfactant producing bacteria

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Abstract

The present study is carried out by isolation, characterization of biosurfactant producing bacteria and their further activities. 12 bacterial species were isolated through serial dilution method from 4 different sites of Lucknow and Unnao district. The serial dilution method was performed in order to get pure and reduced number of bacterial colonies. 6 bacterial species out of 12 were detected of producing biosurfactant activity. These bacterial species were further morphologically and biochemically characterized through Bergey's manual. These bacterial species were tested for 7 days to oil degradation capacity by agar well diffusion method on small scale and flask method on large scale. The three types of oil samples were used for bio surfactant activities such as vegetable oil, used vegetable oil (cooked oil) and diesel. The best bio surfactant activities were obtained for all these bacterial species against used vegetable oil (cooked oil). The identified cultures were *Steptococcus spp.*, *M. luteus*, *S. aureus*, *Alcaligenes spp.*, *B. cereus*, *N. flavescens*.

Keywords: biosurfactant producing bacteria, vegetable oil, used vegetable oil, biosurfactant activity

Introduction

Life in our planet is sustained in a fragile biological balance; microorganisms play an important role on nutritional chains that are an important part of this biological balance. In recent years, there has been increasing interest in developing in situ techniques for remediation of oil-contaminated soil. Bioremediation is a waste management technique that involves the use of organisms to remove or neutralize pollutants from a contaminated site. According to the United States EPA, bioremediation is a "treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or non-toxic substances". Technologies can be generally classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site, while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, land farming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation. Bioremediation is popular and publicly acceptable due to its low costs and environmental safety. Biodegradation by naturally occurring populations (consortia) of microorganisms is a major mechanism for the degradation of petroleum contaminants from the environment. Microorganisms degrade these organic pollutants using their own enzymatic pathways; the end products of aerobic hydrocarbon mineralization are microbial biomass, CO₂ and water. Adapting several abilities, microorganisms have become an important influence on the ecological systems, making them necessary for superior organism's life in this planet. Ability of microorganisms to transform and degrade many types of pollutants in different

matrixes (soil, water, sediments and air) has been widely recognized during the last decades. Soil contamination with hydrocarbons cause extensive damage of local ecosystems since accumulation of pollutants in animals and plants tissues may cause progeny's death or mutation. In Mexico, an endless number of contaminated sites exist as a result of more than 60 years of oil petroleum activity; in recent years this problem has motivated researches to recover these contaminated sites [1]. Biosurfactant are heterogeneous group of surface active compounds which are produced by microorganisms, which either adhere to the cell surface or are excreted extracellular in the growth medium. These molecules reduce surface tension and Critical Micelle Dilution (CMD) in both aqueous solutions and hydrocarbon mixtures [2, 3]. The majority of known bio surfactants are synthesized by microorganisms grown on water immiscible hydrocarbons, but some have been produced on such water-soluble substrates as glucose, glycerol and ethanol. A characteristic feature of bio surfactants is a hydrophilic lipophilic balance (HLB) which specifies the portion of hydrophilic and hydrophobic constituents in surface-active substances. Chemically synthesized surfactants have been used in the oil industry for the removal of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment [4]. Oil pollution is an environmental problem of increasing importance. Hydrocarbon-degrading microorganisms, adapted to grow and thrive in oil-containing environments, have an important role in the biological treatment of this pollution. One of the limiting factors in this process is the bioavailability of many fractions of the oil. Microorganisms are equipped with

metabolic machinery to use petroleum as a carbon and energy sources [5, 6]. They are involved in degrading a considerable number of organic pollutants that are involved in oil including: aliphatic compounds, n-alkanes, diesel fuel and tetrachloroethylene, monoaromatic compounds, toluene, benzene, xylene, ethylbenzene and polycyclic aromatic hydrocarbons [7, 8]. In this present work oil degrading microbes were isolated and characterized from oil contaminated soil. The isolated microorganisms were further checked for its biosurfactant activity.

Materials and Methods

Sample Collection

Four soil samples were collected from petrol pumps and automobile shops of Lucknow and Unnao district in 2012.

Serial dilution

Serial dilution method was used on the principle that when soil samples along with bacterial colonies taken, the result obtained in the form of reduce number of colonies. The microbes are having importance in the industries for enzyme as well as antibiotic production.

Isolation of biosurfactant producing microbes from oil contaminated soil

All samples were collected and were serially diluted aseptically spread in already clean prepared culture plates of nutrient agar (NA). Samples were incubated for 24 hours at 28-30°C and healthy samples were selected for further process of pure culturing. Pure culture was obtained by sub culturing of healthy culture to get a single bacterial colony in nutrient agar media by streaking method. Nutrient agar broths were used of different isolates for further morphological, physiological and biochemical testing. All samples were analyzed by conventional techniques as described by Buchanan and Gibbons [9] and Carter and Cole [10]. After collection of samples, culture plates were incubated in BOD incubator at 28-30°C for 24 hours. After 24-48 h of incubation, the colonies that appeared visually dissimilar were chosen, counted and subculture to fresh nutrient agar and incubated at 37 °C for 24 h.

Pure culture preparation

Identification of microorganisms did not commence until it was evident that a pure culture had been obtained. A pure culture was maintained up to culture containing a single colony of an organism. A pure culture is usually derived from mixed culture by transferring a small sample in to a new sterile growth medium in such a manner as to disperse the individual cell across the surface medium or by thinning the individual cells so that when multiply each will form a discrete colony. The most common growth media for microorganisms are nutrient broths (liquid nutrient medium) or LB medium (Lysogeny broth), Basal media, Selective media, Differential media or Indicator media etc. were used to cultivate and purify the colonies.

Morphological and bio-chemical characterization of isolated bacterial culture

A culture characteristic such as the shape, color, size and

texture was carefully examined macroscopically. Some preliminary characters such as gram positive, gram negative and growth in broth media also helped to identify bacterial genus. The biochemical characterization of isolated oil degrading microbes was done according to Bergey's Manual including various test such as Oxidase test, Catalase test, Starch hydrolysis test, Mannitol salt agar test, Nitrate reduction test and glucose fermentation test were performed [11].

Gram Staining

It is useful stain for identifying and classifying bacteria in to two major groups: the gram positive and gram negative bacteria. The bacteria which retain blue or purple color (primary stain) are called positive and those that retain pink color of Safranin (counter Stain) are called gram negative bacteria.

Oxidase test

Oxidase test was used for the confirmation of desired bacteria. Cytochrome oxidase is an enzyme found in some bacteria that transfer electrons to oxygen. Presence of cytochrome oxidase can be detected through the use of an oxidase reagent (Gordon-McLeod reagent) which acts as an electron donor to cytochrome oxidase. If bacteria oxidize the reagent it turns dark purple due to formation of indophenol blue indicating positive reaction [12]. This test was applied for confirmatory test for *Micrococcus luteus* and *Neisseria flavescens*.

Catalase test

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H₂O₂.

The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor. Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor. Catalase test were used to differentiate aerotolerant strains of *Clostridium* (catalase negative), from *Bacillus* species (catalase positive).

Mannitol salt agar test

The essential feature of this test is the media. The media is a very high salt concentration which mainly *Staphylococcus* can tolerate and inhibits the growth of most other bacteria. It also contains mannitol which acts as fermentable sugar and a pH indicator dye. Mannitol fermenting colonies produce acid which react with the indicator dye forming a colored halo around the colonies. This test was applied for confirmatory test for *Staphylococcus aureus*.

Glucose fermentation test

This test was done to help differentiate species of the family Enterobacteriaceae. This test for the bacteria ability to ferment glucose and produce gas and/or an acid end product and the glucose broth were used for this study. A phenol red indicator

is added to indicate an acid end product. A Durham tube is added to indicate gas production. A positive result for acid is yellow after indicator is added (indicating glucose fermentation) and a positive result for gas is a bubble in the Durham tube. A completely negative result has no color change or reddish color and no bubble.

Endospore test

A differential staining technique (the Schaeffer-Fulton method) is used to distinguish between the vegetative cells and the endospores. A primary stain (malachite green) is used to stain the endospores. Because endospores resist staining, the malachite green will be forced into (*i.e.*, *malachite green permeate the spore wall*) the endospores by heating. In this technique heating acts as a mordant. There is no need of using any decolorizer in this spore staining as the primary dye malachite green bind relatively weakly to the cell wall and spore wall. In fact If washed well with water the dye come right out of cell wall however not from spore wall once the dye is locked in. Water is used to decolorize the vegetative cells. The vegetative cells should appear pink/red (*i.e.* color of counter stain), the vegetative cells that contain endospores should stain pink while the spores should be seen as green ellipses within the cells. Mature, free endospores should not be associated with the vegetative bacteria and should be seen as green ellipses.

Biosurfactant activity on small scale level

Biosurfactant activity on small scale was analyzed by agar well diffusion method in which oil sample was spread over nutrient agar plates and wells were made in which cultures were inoculated and incubated overnight. Nutrient agar media was prepared and autoclaved and petriplates were also autoclaved. After autoclaving, media was poured into the plates and oil samples; diesel, vegetable oil and vegetable oil were spread over different nutrient agar plates. Wells were made in the plates and the isolated bacterial culture broths were loaded into the wells which were then incubated at 37°C for overnight. Growth kinetics process was used to determine the time period at which the culture showed optimum activity.

Biosurfactant activity on large scale

Biosurfactant activity on large scale was checked by inoculating the isolated culture broths into the autoclaved 50 ml conical flasks containing 35ml nutrient broth for each of the isolated culture and 15ml of the oil sample. The bio surfactant activities were observed by degradation of the oil layer.

Results

12 bacterial species were isolated through serial dilution method from 4 different sites of Lucknow and Unnao district. The serial dilution method was performed in order to get pure and reduced number of bacterial colonies (figure. 1). 6 bacterial species out of 12 were detected of producing biosurfactant activity. These bacterial species were further morphologically and biochemically characterized through Bergey's manual. For morphological characterization the shape, color, texture, margin, opacity and elevation were analyzed and depicted in table 1 for all 4 sites samples (A, B, C and D). The shape regular except C₁ (circular), texture smooth and opacity opaque except B₄ and C₁ (translucent) were obtained in all 4 sites samples while the color off-white except A₄ (golden brown), B₃ (white) and C₁ (yellow), margin entire except A₂, A₃, C₂, C₃ and C₄ (lobate) and elevation elevated and flat were observed. The procedure of transfer of microorganisms from their parent growth source to a fresh one or from one medium to another to obtain pure culture is called sub culturing (figure. 2).

For biochemical characterization (Table 2) for catalase test the presence of bubbles on slide indicates the positive for this test (figure 5). For the carbohydrate test uses phenol red as an indicator. If the color change occurs from red to yellow then it is considered as positive test. This test differentiate aero tolerant strains of *Clostridium* (catalase negative), from *Bacillus* species (catalase positive). For the mannitol test uses phenol red as an indicator. If the color change occurs from red to yellow then it is considered as positive test and the growth on mannitol salt agar media indicates the presence of *S. aureus* (figure.7). For the glucose fermentation test uses bromothymol blue as an indicator. If the blue changes then it is considered as positive test. This test was done to help differentiate species of the family *Enterobacteriaceae* (figure.6). For oxidase test the occurrence of dark purple color indicates the presence of *M. luteus* and *N. flavescens* (figure. 8).

These bacterial species were tested for oil degradation capacity by agar well diffusion method in small scale depicted in table 3 (figure. 3) and flask method in large scale method depicted in table 4 (figure. 4). The three types of oil samples were used for bio surfactant activities (vegetable oil, used vegetable oil (cooked) and diesel. The best bio surfactant activities were obtained for all these bacterial species against used vegetable oil (cooked). The identified cultures were *Staphylococcus spp.*, *M. luteus*, *S. aureus*, *Alcaligenes spp.*, *B. cereus*, *N. flavescens*

Table 1: Morphological characteristics of isolated microorganisms from Lucknow and Unnao district

Bacterial Samples*	Shape	Colour	Texture	Margin	Opacity	Elevation
Culture A ₁	Regular	Off-white	Smooth	Entire	Opaque	Elevated
Culture A ₂	Regular	Off-white	Smooth	Lobate	Opaque	Flat
Culture A ₃	Regular	Off-white	Smooth	Lobate	Opaque	Flat
Culture B ₁	Regular	Golden brown	Smooth	Entire	Opaque	Elevated
Culture B ₂	Regular	Off-white	Smooth	Entire	Opaque	Elevated
Culture B ₃	Regular	Off-white	Smooth	Entire	Opaque	Elevated
Culture C ₁	Regular	White	Smooth	Entire	Opaque	Flat
Culture C ₂	Regular	Off-white	Smooth	Entire	Translucent	Flat
Culture C ₃	Circular	Yellow	Smooth	Entire	Translucent	Elevated

Culture D ₁	Regular	Off-white	Smooth	Lobate	Opaque	Flat
Culture D ₂	Regular	Off-white	Smooth	Lobate	Opaque	Flat
Culture D ₃	Regular	Off-white	Smooth	Lobate	Opaque	Flat

*A: Kathauta Petrol Pump, B: Unnao Petrol Pump, C: Vibhuti Khand Automobile Shop, D: Alambagh Automobile Shop

Table 2: Biochemical characteristics of isolated bacterial culture according to Bergey’s manual

Bacterial samples	Gram staining	Glucose Fermentation test	Catalase test	Mannitol test	Oxidase test	Endospore test
Culture A ₁	Negative, Cocci	Negative	-	-	-	-
Culture A ₂	Positive, Cocci	Negative	Negative	Negative	-	-
Culture A ₃	Positive, Cocci	Negative	Positive	Negative	-	-
Culture B ₁	Positive, Cocci	Negative	-	Positive	-	-
Culture B ₂	Negative, Cocci	Negative	Positive	-	-	-
Culture B ₃	Negative, Cocci	Negative	-	-	-	-
Culture C ₁	Positive, Rod	Negative	Positive	Negative	Positive	Positive
Culture C ₂	Negative, Rod	Negative	Negative	-	Positive	Negative
Culture C ₃	Positive, Cocci	Negative	Positive	Negative	-	-
Culture D ₁	Positive, Cocci	Negative	Positive	Positive	-	-
Culture D ₂	Positive, Cocci	Negative	Positive	Positive	-	-
Culture D ₃	Positive, Cocci	Negative	Positive	Negative	-	-

Table 3: Biosurfactant activity of isolated samples in small scale by agar well diffusion method

Sample	Vegetable oil	Used Vegetable oil	Diesel
Culture A ₁	medium	Very good	good
Culture A ₂	medium	Very good	good
Culture A ₃	medium	good	good
Culture B ₁	medium	Very good	medium
Culture B ₂	medium	Very good	medium
Culture B ₃	medium	Very good	medium
Culture C ₁	medium	Very good	good
Culture C ₂	medium	Very good	good
Culture C ₃	medium	Very good	good
Culture D ₁	medium	Very good	good
Culture D ₂	medium	good	good
Culture D ₃	good	good	good

Table 4: Biosurfactant activity of isolated samples in large scale by flask method

Sample	Vegetable oil	Used Vegetable oil	Diesel
Culture A ₁	0.6	1.1	0.8
Culture A ₂	0.5	1.3	0.9
Culture A ₃	0.6	1.0	0.8
Culture B ₁	0.6	1.2	0.6
Culture B ₂	0.7	1.4	0.7
Culture B ₃	0.6	1.2	0.6
Culture C ₁	0.8	1.1	0.9
Culture C ₂	0.7	1.1	0.8
Culture C ₃	0.6	1.3	0.9
Culture D ₁	0.7	1.1	0.9
Culture D ₂	0.6	1.0	1.0
Culture D ₃	0.9	0.9	0.9



Fig 1: Mixed bacterial species colonies in a mixed culture



Fig 2: Sub culturing methodology on agar plates of sample A, B, C and D



Fig 3: Biosurfactant activity on small scale by using agar well plates showing oil degrading capacity of isolated microorganisms

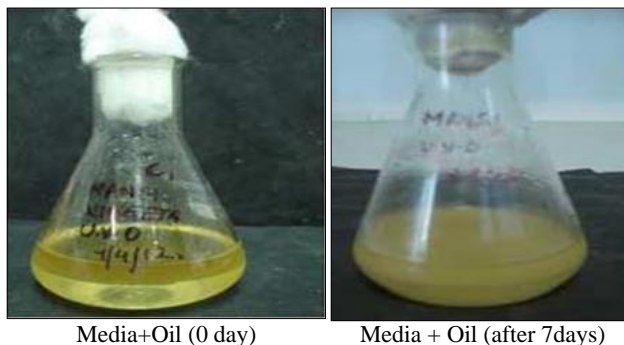


Fig 4: Biosurfactant activity on large scale by using flask method showing oil degrading capacity of isolated microorganisms

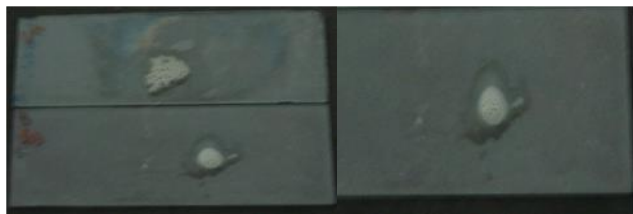


Figure 5: Result of Catalase test for Sample 1 and 2 Showed that bubbles indicating positive results.



Fig 6: Results of Carbohydrate fermentation test



Fig 7: Results of Mannitol test



Fig 8: Oxidase test result showing the positive result forming a blue stain when the culture grown in BHI agar media mixed with McLeod reagent

Discussion

Bioremediation is a process in which the environment can cleanup through microbes. There are so many types of microbes which used to eliminate contamination in seawater. The contamination can be in the form of oil or some chemicals. This type of contamination may lead to adverse effect on the organisms, animals as well as plants mainly found in sea water. Presently removal of these hydrocarbon contaminants is becoming a very serious problem. Currently accepted methods are safe disposal, incineration and burial in secure landfills. When the amounts of contaminants are large these methods can become prohibitively expensive. Some methods such as secure landfills, incinerator, pyrolysis and gasification are efficient but after treatment contaminants produce some by products which have negative impacts on the environment and as well as threat to public health^[13]. Thermal and chemical methods generally used to remove hydrocarbons from contaminated sites have limited effectiveness and can be expensive. In recent years bioremediation is the most emerging technology for treatment of petroleum contaminated sites. It is cost-effective and lead to degradation or complete mineralization of contaminants based on biodegradation into carbon dioxide, water, inorganic compounds. Biological agents like microorganisms or plants transform complex organic contaminants to other simpler organic compounds by owing to removal and degradation of many environmental pollutants^[14]. This process is accomplished by biological organisms, usually bacteria, fungi, and plants are involved in reduce or remove toxic pollutants. The principle of the biological remediation process is to utilize the petroleum hydrocarbons as a sole source of carbon and energy. The present study is carried out by isolation, production and characterization of oil degrading microbes from oil contaminated soil and also expression oil degrading microbes to non-oil degrading microbes. Humans are involved in the activities which are leading to the destruction of the environment. Industrialization has led to immense increase in the accumulation of waste products causing damage to the environment. Oil pollution is one of the worst types of pollution. Wastes from industries and oil refineries containing petroleum hydrocarbons, metals, radioactive materials and salts, have the potential to cause soil pollution preventing the growth of plants and disturbing the nature. The conventional techniques of remediation have some major disadvantages. The recent trend in environmental remediation is by the use of "Bioremediation" by "Bio stimulation". Since bioremediation is based on the process of natural attenuation it is considered to be more acceptable than other technologies. Microbial degradation is the major and ultimate natural mechanism by which one can clean up the petroleum hydrocarbon pollutants from the environment^[15-17]. The recognition of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reported by Jones *et al.*^[18]. Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6%^[19] to 82%^[20] for soil fungi, 0.13%^[19] to 50%^[20] for soil bacteria, and 0.003%^[21] to 100%^[22] for marine bacteria. Many scientists reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil^[23], fresh

water^[24], and marine environments^[25, 26].

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment^[27, 28]. Several bacteria are even known to feed exclusively on hydrocarbons^[29]. Floodgate^[26] listed 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi which were isolated from marine environment. A similar compilation by Bartha and Bossert^[23] included 22 genera of bacteria and 31 genera of fungi. In earlier days, the extent to which bacteria, yeast, and filamentous fungi participate in the biodegradation of petroleum hydrocarbons was the subject of limited study, but appeared to be a function of the ecosystem and local environmental conditions^[30]. Bioremediation technology is believed to be noninvasive and relatively cost-effective by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment and is cheaper than other remediation technologies.

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