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Isolation and Characterisation of Bio-Surfactant Producing Bacteria from Oil Contaminated Soil

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A B S T R A C T

Biosurfactants having the characteristics of amphipathic compounds. Biosurfactants are produced by several microorganisms which include *Acinetobacter spp., Bacillus spp., Candida antarctica, Pseudomonas aeruginosa.* Different screening methods, for example oil spreading assay, blood agar hemolysis, emulsification assay, foaming activity are used to select biosurfactant producing microbes. The main objective of this research work was to isolate and characterize the biosurfactant producing bacteria from oil contaminated soil. By using kerosene oil as the sole carbon source, bacterial strain was isolated and screened having the characteristics of biosurfactant properties. Number of techniques like oil spreading technique, blood hemolysis test, foaming activity, and emulsification activity were performed for the screening of the biosurfactant producing bacteria, and found positive oil spreading technique in bacterial strain B1 and B2. Strain B1 (52%) has high emulsifying activity, whereas in B2 it was around 42 %. Strain B1 showed complete breakdown of the hemoglobin of the red blood cells in the vicinity of bacterial colony i.e. β blood hemolysis test and in B2 it is lack of hemolysis i.e. γ hemolysis. It was observed that foaming stability in B2 strain is lesser than B2. Both the isolated bacterial strains B1 and B2 showed Gram positive, and they are in rod and circular shape respectively. From the result of various biochemical characterization and cell morphological characterization, the isolated strain was bacillus type bacteria.

Introduction

Surfactants are amphipathic molecules composed of hydrophobic and hydrophilic groups (Rosen and Kunjappu, 2007). According to their chemical structures, surfactants have the ability to reduce surface and interfacial tensions of two immiscible phases. Chemical synthetic surfactants have been commercially used in industries (Kosaric, 1993; Singh, 2007). For example, in the petroleum industry surfactants are widely applied to lower the interfacial tension of crude oil and matrix substances in Oil-Enhanced Recovery (OER). Sodium Dodecyl Sulfate (SDS), an anionic surfactant, is used as a preoperative skin cleaner, bacteriostatic agent, and also in medicated shampoo (Sekhon, 2013). Due to amphipathic nature, Biomolecules interact with the phase boundary in heterogeneous systems. The non-polar end is also known as "tail" and it is typically a hydrocarbon chain whereas the polar end is also

known as "head" appears in many varieties such as carbohydrates, amino acids or phosphates. Surfactants are widely used in various sectors like industrial, agricultural, food, cosmetics and pharmaceutical applications. Surfactants reduce the interfacial tension between two liquids or that between a liquid and a solid i.e. it is a surface-active compound. Biosurfactants are also surface-active compounds like chemical surfactants but unlike the chemical surfactants, biosurfactants are synthesized by microbes like bacteria, fungi and yeast. However, the disadvantages of synthetic surfactants have been reported such as environment pollution, animal and human toxicity (Mesquita *et al.,* 2011). Biosurfactants are natural surface-active compounds that are produced by many kinds of living organisms, especially microorganisms (Lourith *et al.,* 2009). Biosurfactants are important microbial products with properties applicable in a number of industries and processes. Being capable of lowering surface and interfacial tensions, biosurfactants are today thought to be efficient replacers of chemically synthesized surface-active agents. It has some superior properties, such as biodegradability, less toxicity, and their specificity, making these microbial products both attractive for particular industries and environmentally acceptable.

Surfactin is one of the most powerful biosurfactants. Surfactin was isolated from *Bacillus subtilis* in natural form. Purified surfactin was able to reduce surface tension of water from 72.0 mN/m to 27.0 mN/m (Yeh *et al.,* 2005). On the basis of their chemical structures, Biosurfactants are grouped into four groups; glycolipids, lipopeptides, phospholipids, and polymeric biosurfactants (Nitschke *et al.,* 2007).

Biosurfactants are suitable products for use in agricultural, environmental, pharmaceutical, biomedical, cosmetic and food applications. Biosurfactants are able to efficiently be used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil (Kosaric, 1998). Surfactant helps in decreasing surface strain and the interfacial pressure. Surfactin can reduce the surface tension of water to 25 mN m^{-1} and interfacial strain water/hexadecane to under 1 mN m^{-1}. *P. aeruginosa* produces rhamnolipids which reduce surface tension of water to 26 mN m^{-1} and interfacial strain of water/ hexadecane to esteem under 1 mN m^{-1}. Biosurfactants are more effective. The Critical Micelle Concentration of chemical surfactants are few times higher than biosurfactants, i.e., for maximal decline on surface strain, less surfactant is fundamental.

In a decade ago, extremophiles produced biosurfactants had widely commercial application. McInerny *et al.,* (1990) reported that lichenysin from *Bacillus licheniformis* was resistant to temperature up to 50 °C, pH in the vicinity of 4.5 and 9.0 and NaCl and Ca concentration up to 50 and 25 g L-1. Another biosurfactant produced by *Arthrobacter protophormiae* was observed to be both thermostable (30-100 °C) and pH (2 to 12) stable.

Microbial derived compounds can be easily degraded when compared to synthetic surfactants and appropriate for natural applications such as bioremediation/biosorption. Biosuractant can be taken in elective items due to the expansion of ecological concern. Biodegradable biosurfactants from marine microorganisms were concerned for the biosorption of ineffectively solvent polycyclic sweet-smelling hydrocarbons, phenanthrene contaminated in aquatic surfaces. The blossoms of marine algae, Cochlodinium have the ability to utilize the biodegradable biosurfactantsophorolipid with the removal of efficiency of 90% of every 30 min treatment. The higher toxicity of the chemical-derived surfactant was found to be 10 times lower than of rhamnolipids which displayed a LC50 against *Photobacterium phosphoreum* (Poremba *et al.,* 1991). The low biosurfactant, sophorolipids from *Candida bombicola* made them helpful in nourishment ventures due to low toxicity. Biosurfactants act as emulsifiers or de-emulsifiers. An emulsion can be illustrated as a heterogeneous framework, comprising one immiscible fluid scattered in another as beads, whose distance across

by and large surpasses 0.1 mm. Emulsions are classified into two types: oil-in-water and water-in-oil emulsions. Their stability nature is minimal which might be balanced out by added substances, for example, biosurfactants and can be kept up as steady emulsions for a considerable length of time to years.

Materials and Methods

Place of Work

The study was carried out in the department of Microbiology, School of Life Sciences, Rai University, Ahmedabad, Gujarat, India.

Collection of Sample

Soil samples were collected from different location of Petrochemical limited**,** Ahmedabad, Gujarat.

Isolation of Bacterial colonies

Biosurfactant producing bacteria was carried out by using the mineral salt medium (MSM) comprised in g/L: KH_2PO_4, $MgSO_4$, $FeSO_4$, $NaNO_3$, $CaCl_2$, $(NH_4)_2SO_4$ and pH was maintained at 7.4 ±0.2. The mineral salt medium was sterilized by autoclaving at 121^{o}C for 15 minutes. 5g of soil sample was weighed and inoculated in 100 ml of Mineral Salt Medium (MSM) with 3ml of kerosene oil added to the conical flask having capacity of 250 ml, as the carbon sources, and then it was incubated for 72 hours at 30 ℃ temperature.

After that, 1ml of incubated culture was streaked on the petri plates. By using the serial dilution technique, the samples were serially diluted up to 10^{-6} dilution. 1 ml of sample from serially diluted sample was transferred to nutrient agar for spreading the culture. The plate was inverted and incubated at 30° C, for 72 hours. After an incubation period, morphologically five distinct colonies were selected for further studies. The isolated strains were conserved in 30% (V/V) glycerol at -80^{o}C for further use.

Screening of biosurfactant producing bacteria

The isolated colonies were taken and tested for the confirmation of biosurfactant bacteria and screened the confirmation of biosurfactant bacteria by following two screening method: Primary Screening method and Secondary Screening Method.

Primary Screening Method

Oil spreading method (OSM)

The oil spreading test was also performed as described by Morikawa *et al.,* (1993). In this method, approximately 50 ml of distilled water was poured into a petri dish of 150 mm diameter, and then 10 µl of kerosene oil was spread on the water surface forming a thin layer. In the center of the oil layer, 10 µl cultured supernatant gently dropped. After 30 seconds the displaced diameter.

Foaming Activity

Isolated strains were grown separately in 250 mL Erlenmeyer flasks, each containing150 mL of nutrient broth medium. The flasks were incubated at 37℃ on a shaker incubator (150 rpm) for 72 h. As duration of foam stability, foam height and foam shape in the graduated cylinder we detected the foam activity.

Secondary Screening Method

Blood Haemolysis Test

After incubation for 36 h, the culture broth was spread on a blood agar plate and cultured in a constant incubator at 35 ℃ overnight. The yellow, transparent zones around the colonies showed the hemolysis of blood cells. No change in the color of the blood agar plates indicated the absence of hemolysis.

Emulsification Assay

The emulsification index (E_{24}) is used to

characterize the emulsifying activity of the biosurfactant. It was carried by adding 2ml kerosene oil in 1ml cell free supernatant which was obtained after the centrifugation, and then it was vortexed for 5 minutes to confirm regular mixing of both the liquids. After 24 hours, the emulsification activity was observed. The E_{24} is calculated according to Zhang *et al.*, as follows;

E_{24} = (Height of the emulsification layer/total height of mixture) × 100%

Characterisation of the Bacterial Strain

Physical Characterization

Gram Staining

Gram staining is a differential method that differentiate the bacteria in gram positive and gram negative on the basis of the chemical and physical properties of their cell walls by detecting peptidoglycan, in gram positive it is present as a thick layer. A Gram-positive result in a purple/blue color while a Gram-negative result in a pink/red color. In gram staining technique firstly we took the culture from pure culture by using a sterile loop then mix with water properly until just slightly turbid and made a smear on a glass slide. After that, the heat fixation process was done by using a spirit lamp and allowing cooling at room temperature. Then one or two drops of crystal solution was added and kept for 60 seconds and then washed by distilled water.

In the next step, gram's iodine solution was flooded on the slide for 60 seconds and again washed by tap water. Then put 1 to 2 drops of gram's decolourizer (95% alcohol and 5% acetone) on the smear and keep for 30 seconds and wash properly. After decolorizing step added counter stain (safranin) for 60 seconds and rinsed with distilled water and allowed to air dry. The slide was then observed under the light microscope at 100x objective.

Cell Morphology

The Gram stained cells were observed and the shape and color of the cells were determined.

Bio-Chemical Characterization of isolated bacteria

Characterization of isolated bacteria was done through number of biochemical test. These are:

Catalase Test

In this test it is to determine whether microbes are able to produce catalase enzymes or not, it is a protective enzyme i.e. able to degrade the dangerous chemical hydrogen peroxide. A pure culture, growth from the overnight was smeared on a glass slide and then added 3% hydrogen peroxide and observed. It shows positive results if rising bubbles are observed and if rising bubbles are not observed means the result will be negative.

Citrate Test

Citrate test means conversion of citrate to oxaloacetate and acetate. Bacteria are inoculated into a medium which contains sodium citrate and a pH indicator such as bromothymol blue. This medium contains inorganic ammonium salts, which are utilized as a source of nitrogen. Bacteria that can grow on this medium produce an enzyme, citrate permease which has the ability to decompose the citrate to oxaloacetate and acetate. Oxaloacetate is further broken down into pyruvate and carbon dioxide (CO2). When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The pH changes turn the bromothymol blue indicator in the medium from green to blue above pH 7.6. In this method, streaked the slant with an inoculum picked from the center of a well isolated colony. After that, it incubated at 35^{o}C for up to 4-7 days. After that, I observed the changes in color from green to blue. If color is changed from green to blue, it means positive result and if color is not changed, it means negative result.

Urease Test

In this method, prepared urea broth by dissolving 2.95 gram of urea powder in 150 ml of distilled

water. After that culture was inoculated in urea broth for 48 hours at 37 °C temperature and then observed the color, if color is changed from light yellow to pink then test is positive otherwise it is negative.

Methyl Red Test

Culture was inoculated in MR-VP broth for 3 days at temperature of 37 °C after the incubation added 2-3 drops of Methyl red and observed the color change that take place in few seconds. If color is changed from yellow to red then it will be positive and if there is no color change then it will be negative.

Nitrate Test

In this test the bacterial strain inoculated in nitrate broth and incubated at the optimal temperature (30 or37°C) for 48 hours then further added few drops of nitrate reagent and saw the reaction within the minute or less, if there is no evolve of N2 or nitrate gas then there is need to add a bit of powdered zinc. If it appears pink red color, it shows nitrate negative reduction and if there is no pink form it shows positive nitrate reduction.

Indole Test

In this test the old pure bacterial culture was inoculated in tryptone broth. After that, inoculated culture was incubated for 24 hours at 30 °C and then took 2ml media and added into the empty sterile test tube and added few drops of Kovacs reagent and agitated the tube for few minutes. Within few minute cherry red color form it means it is positive, and no color change means negative.

Gelatine Test

The pure culture was inoculated in nutrient gelatin media, and then it was incubated for 24 hours at 30 °C temperature. If media is liquefied then the result will be positive, otherwise the result will be negative.

Starch Hydrolysis

The pure culture streaked on starch agar plate and the inoculated plate was incubated for 24 hours at 30 -35 °C temperature. After that, iodine reagent was added to flood the growth. Presence of clear halo surrounding colonies shows the result will be positive.

Statistical Analysis

All the experiments were carried out three times and studied in triplicate. Results represent the mean ± standard deviation. One-way analysis of variance (ANOVA) with the least significant difference (LSD) test was conducted to determine the significant differences in hydrocarbon degradation efficacy of the bacterial strain at different time periods.

Results and Discussion

Isolation, Identification, screening and Characterisation of biosurfactant- producing bacteria

A total of 5 strains of bacteria (B1, B2, B3, B4 andB5) were isolated from the oil contaminated soil. (Fig 1). They were taken for further studies. All the isolated strains were screened for the confirmation of biosurfactant producing bacteria. Primary and Secondary tests were carried out for screening biosurfactant producers from these five isolates.

Primary screening methods include the oil spreading method (OSM) and foaming activity method (FAM). In oil spreading method, as biosurfactants have the ability of oil displacement, if there are biosurfactants produced, it will form an oil spreading circle on the oil film. Three isolates (B1 and B2) out of five strains showed positive results in the initial biosurfactant-production screening assay in table 1. Some studies have indicated that the foaming action of biosurfactants are related to their ability to reduce the surface tension of liquids; the lower surface tension, the stronger the foaming activity (Fig 2). Further Secondary screening tests were carried out of these five strains. In 1996, P.G. Carrillo *et al.*, found the blood agar plate could be used to screen

biosurfactant producing bacteria, and this method was widely applied to various biosurfactant producing bacteria screenings. Out of five B1 strains showed the positive result, other strains showed the negative result. (Fig 3). The hemolysis of biosurfactants may be because biosurfactant molecules and phospholipid bilayer on cell membranes form the mixed micelles, resulting in the fracture of cell membranes. Therefore, the stronger the hemolysis is, the higher the surface activity of biosurfactants. Therefore, this phenomenon indicated the formation of the excellent biosurfactant produced by B1. The oil displacement method and blood hemolysis test were also followed by Anandaraj & Thivakaran (2010); Priya & Usharani, (2009); Jaysree *et al.,* (2011); Tabatabaee *et al.,* (2005). Emulsifying activity is an important property for the performance of biosurfactants. (Fig 4). E_{24} is a parameter to measure the emulsifying ability. The E_{24} of B1 strain is the highest among the five strains (Table 2) which could reach up to 63.1% ± 1.07%. From the above results, it could be concluded that the strain B1 is the best biosurfactant producer from all the isolated strains. Furthermore, the strain B1 could surprisingly produce excessive foam, with the medium turning to black in the process of fermentation. Similar results have been reported during the biosurfactant production by strain *P. aeruginosa MR01*. From the Gram's staining, both the strains are found positive so for further characterization, different biochemical tests were performed to identify the species of strains.

Bergey's manual of determinative bacteriology was used for the Identification and characterization of isolated bacterial strains and on the basis of this manual the species was predicted as *Bacillus species* of bacteria. And it was observed that rod shaped bacteria (Bacillus type bacteria) are able to produce lipopeptides type biosurfactants. The strain was a Gram-positive and spore-forming bacterium whose morphological and biochemical properties were closely related to species of genus *Bacillus* (Figure 5). The strain was a Gram-positive and spore-forming bacterium whose morphological and biochemical properties were closely related to species of genus *Bacillus* (Table 3). Characterization of isolated bacteria was carried out by the physical characterisation (colony morphology and gram staining) and biochemical characterization (Catalase test, Citrate test, urease test, Methyl red test, Nitrate test, Indole test, gelatin Test, starch hydrolysis test). The result of the biochemical test was shown in table 4.

Table.1 Result of oil spreading technique.

Bacterial Strain	Diameter of clear zone (in cm)	Interpretation
B1	2.4	Positive
B2	3.9	Positive
B3	0	Negative
B4	0.8	Negative
B5	0	Negative

Table.2 E24 index of bacterial strain

Bacterial Strain	Emulsified layer (cm)	Total liquid layer (cm)	E24 (%)
B1	1.2	1.9	63.1 %
B2	1.0	1.8	58.8 %

Table.3 Result of cell and colony morphology

Strains	Gram Staining	Cell Shape	Colony Shape	Pigment (Colony)	Oxygen Requirement
B1	+ve	Rod shape Bacilli	Circular, dry, smooth, flat and irregular with lobate marines	Coloured	Aerobic
B2	+ve	Rod shape Bacilli	Circular, wet, smooth, Concave	Coloured	Aerobic

Table.4 Morphological and Biochemical Characteristics of Biosurfactant Producing Bacteria

Morphological and Biochemical Characteristics of Biosurfactant Producing Bacteria		
Name of Organism	B1 Strain	B2 strain
Gram Reaction	+	+
Shape	Rod	Rod
Arrangement	Circular, dry, smooth, flat and irregular with lobate marines	Circular, wet, smooth, Concave
Pigment	Coloured	Coloured
Oxygen Requirement	Aerobic	Aerobic
Catalase	+	+
Citrate	+	+
Urease	+	+
Methyl red	+	+
Nitrate test	+	-
Indole test	+	+
Gelatin	+	-
Starch hydrolysis	+	-
β–hemolysis	+	-

Fig.1 Isolated bacterial strain and colony on agar plate

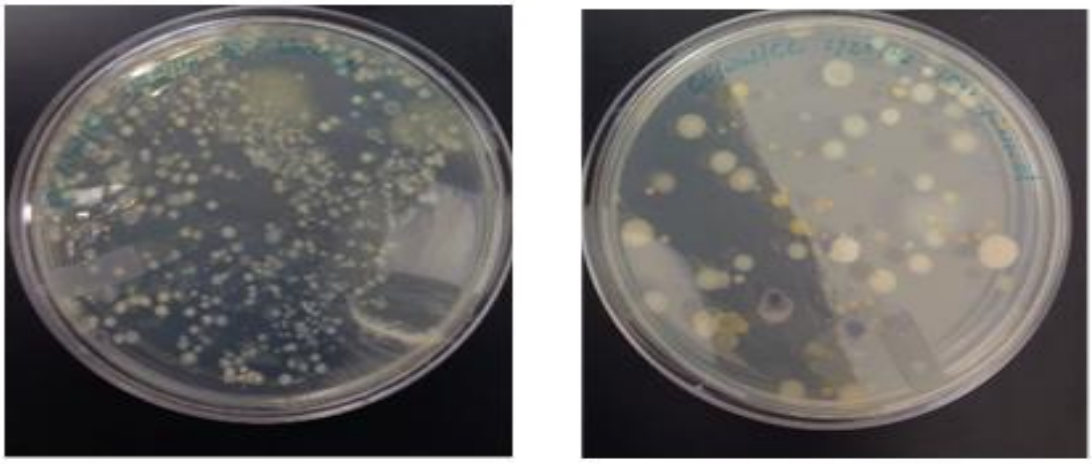


Fig.2 Foaming activity of both bacterial strain

Fig.3 Result of Blood Haemolysis Test

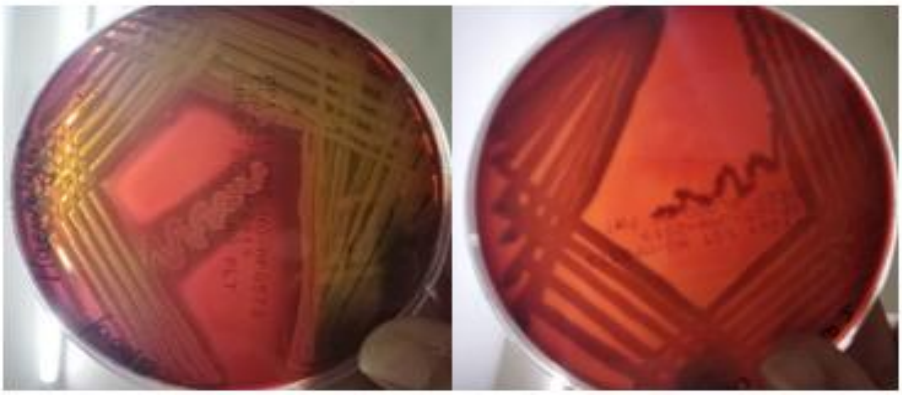


Fig.4 Result of Emulsification Activity

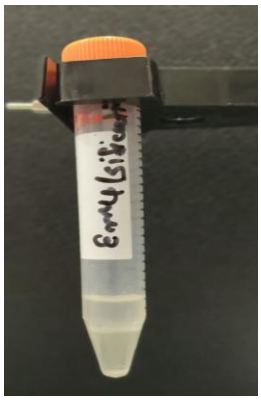
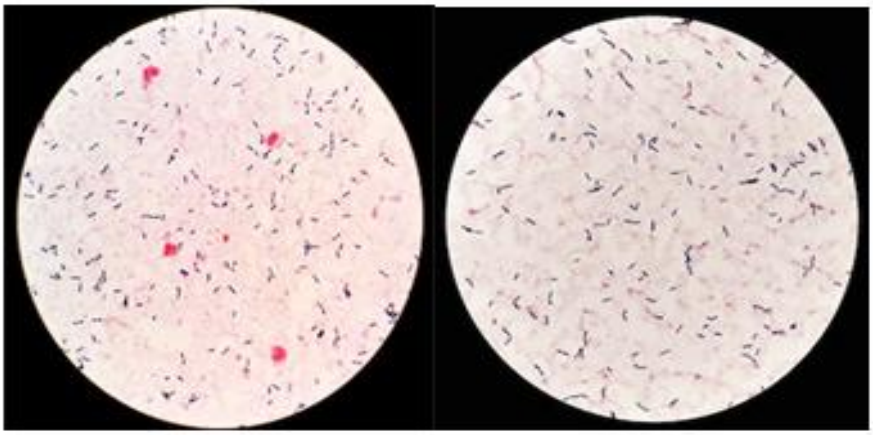


Fig.5 Results of Gram's Staining of Bacterial strain



Biosurfactants are produced extracellularly i.e. part of the cell membrane by bacteria, yeast and fungi, and it is one type of surfactant. Biosurfactants have a number of applications in various industries for example oil industry, foods, cosmetics, pharmacology and environmental technology because of their ability to stabilize emulsions. Because of their lower toxicity, high biodegradability and greater environmental compatibility, it has the best choice for commercial production as compared to chemically synthesized surfactants.

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