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Original Article

Efficacy of Indigenous Microbes for Removal of Oil Contaminated Soil by Producing Biosurfactant

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ABSTRACT

Bacteria with ability to produce biosurfactants have potential applications in environmental protection. They are surface active chemicals that can lower the surface tension between two liquids or a solid and a liquid. Microorganisms of several types manufacture them. **Objective:** To isolate, optimize, screen, and describe bacteria that produce biosurfactants from petroleum-contaminated soil. **Methods:** Isolates were named as ZMS1 and ZMS2, which were gram positive rods with mucoidy colonies and off white or colorless appearance, respectively. The isolation was carried out using initial screening methods including blood hemolytic assay, foaming activity, emulsification assay and oil spreading technique in kerosene supplemented media at culture conditions of pH7 and temperature 37ºC. **Results:** The results of these different tests showed the production of biosurfactant by bacteria. Stain removal efficiency of bacterial supernatant considered as a biosurfactant, was also tested following previously described method. Both isolates, ZMS1 and ZMS2 were producing biosurfactants with the capacity remove stains of blood and tea. Furthermore, to get the biosurfactant production using cheaper carbon source, potato peel extract and molasses extract were used as a carbon source in synthetic medium for the growth of ZMS1 and ZMS2. Despite the fact that the isolates produced biosurfactant, they were not purified or eluted. However, both the isolates ZMS1 and ZMS2 shown their significance in several biotechnological and industrial domains. **Conclusions:** Present study will be helpful in future and these microbes can be utilized for the remediation of oil polluted soil, which in turn can be proven in the improvement of soil fertility.

INTRODUCTION

Biosurfactants are surface-active, degradable organic chemicals of biological origin that have recently been utilized in numerous sectors [1]. Because of its industrial and therapeutic applications, bio surfactants have attracted a lot of attention in recent years [2]. The more specificity, easy preparation lower toxicity and widespread applicability of biosurfactants make it more pertinent. These compounds can also be used as emulsifiers, moistening agents, foaming agents, dispersing agents, detergents, and beneficial food items in a wide range of industries, including organic chemicals, petrochemicals, pharmaceuticals, beverages and foods, petroleum, biological control and management, and so on [3, 4]. Biosurfactants' pharmacological and immunological applications were further addressed by Cameotra and Makkar (2004). Biosurfactant fermentation nutrients have lately been available in a wide variety of agronomic, industrial byproducts, and material residues [5, 6]. So, there is potential for future microbial surfactants, which will rely heavily on the usage of abundant and inexpensive substrates, which can significantly enhance production [7-10]. The oils and fats are most commonly utilized in the food business, which generates a lot of frying oil waste. The problem of frying oil waste disposal is a major issue, which explains the growing interest in utilizing frying oil waste for microbial transformation [4]. Because of their beneficial

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effects, Biosurfactants produced by extremophilic bacteria i.e. halophilic and thermophilic bacteria gained much more attention for the isolation and characterization purposes [11-13]. Bio surfactants are the compounds of chemical origin. One of the established classes is glycolipids formed by Rhodococcus erythropolis that create the mono, di and trisaccarides that is used in oil spill clean-up process [14]. Another class of biosurfactants is Sophorolipids with many environments applications and is produced by Candida bombicola [15]. Pseudomonas aeruginosa produced another class of biosurfactants named Rhamolipids which have applications in bioremediation of oil contaminated sites [16]. However, phospholipids biosurfactants are reported to have applications in environmental management [17]. Biosurfactants have been useful in reducing the surface and interfacial tension [18]. Due to the production of biosurfactants, hydrocarbon degradation in the presence of microorganisms is enhanced [19]. Biosurfactant-producing microorganisms exist in nature and can be found in both water and soil. Moreover, these microorganisms also inhabit extreme environments such as extreme temperature and salinity [20]. Salinity and pH 4-10 can alter the action and solubility of biosurfactants [21]. Hydrocarbons are also produced by living cells in the form of natural oils and fats [22]. Scientists discovered that the oceans are not covered in an oily coating, which indicates that oil-degrading bacteria are active. Many bacteria have been found to feed exclusively on oil-degrading microorganisms [23]. As a result, biosurfactants are crucial in the bioremediation of hydrocarbon-polluted environments. In the biodegradation of hydrocarbons, biodegradative enzymes play a crucial role [24]. Numerous biosurfactants have also been demonstrated to have antimicrobial activity against bacteria, fungus, algae, and viruses. Biosurfactants have numerous advantages over their chemically manufactured equivalents. The presence of biosurfactant-producing bacteria is strongly influenced by the environment in which they dwell [25]. The current study aims to assess the potential of bacterial isolates from petroleum-polluted soil for biosurfactant synthesis for use in several biotechnological domains.

METHODS

Isolation of bacteria:

Petroleum contaminated soil was collected from Lahore and was used as a source of bacterial isolation. 10-fold dilution of soil sample was made and 10 µl of it was spread on nutrient agar plate enriched with 0.1% crude oil. Plates were then incubated at 37°C for 24 hours. After 24 hours, bacterial colonies were observed, of which selected colonies were streaked for purification.

Morphological and biochemical characterization of bacterial isolates:

Bacterial smear was prepared and then stained with gram stain. The color and shape of the stained cells were then observed under light microscope. For the biochemical characterization, different biochemical tests i.e. catalase test, citrate test, urease test, methyl red test, nitrate test and indole test and starch hydrolysis test were performed [26].

Screening of biosurfactant producing bacteria:

For the screening of biosurfactant producing bacteria, following tests were performed:

a) Biosurfactant screening:

The LB Broth medium was prepared with the addition of kerosene oil. The bacteria were inoculated and incubated for 24 hours at 37°C. The growth was measured at 600nm after incubation. The supernatant was utilized to determine biosurfactant after the culture was harvested at 2000 rpm for 5 minutes.

b) Oil spreading Assay:

A thin layer of kerosene oil was applied to the surface of the Petri plate. Then, on top of the oil layer, a 10µl bacterial culture was gently deposited. The presence of biosurfactant is shown by the clear zone. Oil displacement activity is another name for this. To measure the displaced diameter of oil, different inoculum sizes (5 µl, 10 µl, 25 µl, 50 µl, 100 µl and 200 µl) can be utilized.

c) Emulsification assay:

It was calculated using the E24 emulsification index. In 1 mL of cell-free supernatant, 2 mL of kerosene oil was added and vortexed for 5 minutes. After 24 hours, the emulsification activity was measured and computed using the formula:

E24 = (Total height of the emulsion layer/height of the aqueous layer) x 100

d) Foaming Activity:

In each flask, 100 ml of nutrient broth medium was prepared and inoculated with isolated bacteria. The flasks were then incubated for 72 hours at 37°C in a shaking incubator at 200 rpm. Foaming activity is divided into three categories: foam stability, foam height, and foam shape.

Blood Hemolysis Test:

The bacterial colony was streaked onto the agar plate using blood agar medium (including Peptone 5.0g/L, Beef extract 3.0g/L, Sodium chloride 3.0g/L, Agar 15.0g/L, Sterile defibrinated blood 10 ml). It was then incubated at 37°C for 24 hours. Blood hemolysis was measured as clear zones.

e) Stain removal efficiency test: [27]

10 mL supernatant was added to four beakers for stain removal to measure biosurfactant efficiency. The sterile bandage was divided into four equal parts. For 20 minutes,

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two of these pieces were stained with tea and the other two with blood. The stained pieces were then air dried before being placed in the appropriate beaker and incubated for 24 hours at 37°C. By rinsing cloth pieces with sterile water after 24 hours, the stain removal efficacy of biosurfactant was measured.

Biosurfactant Optimization Through the Use of Different Carbon Sources [28]

For the production of biosurfactants, Agro-industrial wastes i.e. molasses obtained from sugarcane and potato peel extracts (prepared by boiling method) were used.

Optimization of bio surfactant by using kerosene oil, potato peels extracts and molasses as carbon source.

Sterilized minimal medium (containing Glucose 40g/L, NH_4HPO_2 0.39g/L, Na_2HPO_4 5.67g/L, KH_2PO_4 4.08g/L, $FeSO_4.7H_2O$ 0.015g/L, $MnSO_4.H_2O$ 0.002g/L, $MgSO_4.7H_2O$g/L, $CaCl_2.2H_2O$ 0.001g/L, Agar 15g/L; pH: 6.8-6.9) was prepared and syringe filtered kerosene oil, potato peel extract and molasses were added into it. Colonies were streaked and cultured at 37 °C for 24 hours. The data were recorded after incubation.

Hydrophobicity assay:

Bacterial strains were cultured in L broth for 24 hours at 37 degrees Celsius. Following a 15-minute centrifugation at 5000 xg, the pellets were washed twice with phosphate buffer saline, and the optical densities of the bacteria were determined at 540 nm and adjusted to A540=1.0. For 30 seconds, 1 ml of bacterial solution was vortexed vigorously with 1 ml of each hydrocarbon (Xylene, chloroform, and toluene). The optical density of the aqueous phase was measured again after phase separation and compared to the initial value. The hydrophobicity was estimated using the equation below, as described in the protocol.

(A540 initial - A540 aqueous phase) / A540 initial x 100 = % Hydrophobicity

RESULTS

Isolation of bacteria:

The two bacterial isolates were found in oil-contaminated soil. ZMS1 and ZMS2 were assigned to the isolates. These isolates were characterized using Bergey's manual of systematic Bacteriology's cultural, morphological, and biochemical characteristics.

(a) Cultural Characteristics:

ZMS1 colonies were round, mucoid, with a smooth surface and irregular edges, whereas ZMS2 colonies were round, dry, flat, and concave. ZMS1 and ZMS2 colonies were white and colorless, respectively.

(b) Morphological and biochemical characterization:

Gram staining was used to determine the morphological characteristics of the isolates, and it was discovered that both isolates were Gram positive and rod-shaped bacteria. Different biochemical tests were used to select the strains for biochemical characterization.

Biosurfactant Screening, Production and Optimization With Different Carbon Sources

The isolated strains were tested for biosurfactant synthesis.

(a) Blood Hemolytic Assay:

Both bacterial strains tested negative for hemolysis when carbon was provided via kerosene, indicating that no zones had formed. Bacterial strains are tested for hemolytic activity using potato peel extracts showed negative results for both strains. While both bacterial strains using molasses also showed negative hemolytic activity.

(b) Emulsification Assay:

Carbon source	Bacterial Strain	Emulsified layer (cm)	Total liquid layer (cm)	E24 (%)
Kerosene oil	ZMS 1	1.00±0.057735	1.8 ±0.2848	55.5±6.799591
	ZMS 2	0.8±0.173205	1.9±0.21798	42.10±6.334386
Potato peel extract	ZMS1	1.1±0.145297	1.3±0.120185	84.6±3.48441
	ZMS2	0.6±0.202757	1.4±0.173205	42.8±4.801851
Molasses	ZMS1	1.0±0.11547	1.8±0.152753	55.4±5.512511
	ZMS2	0.4±0.088192	1.7 ±0.218581	23.5±2.107922

Table 1: Results of Emulsification Assay using medium containing different carbon sources

(c) Foaming Activity of bacterial strains using different carbon sources:

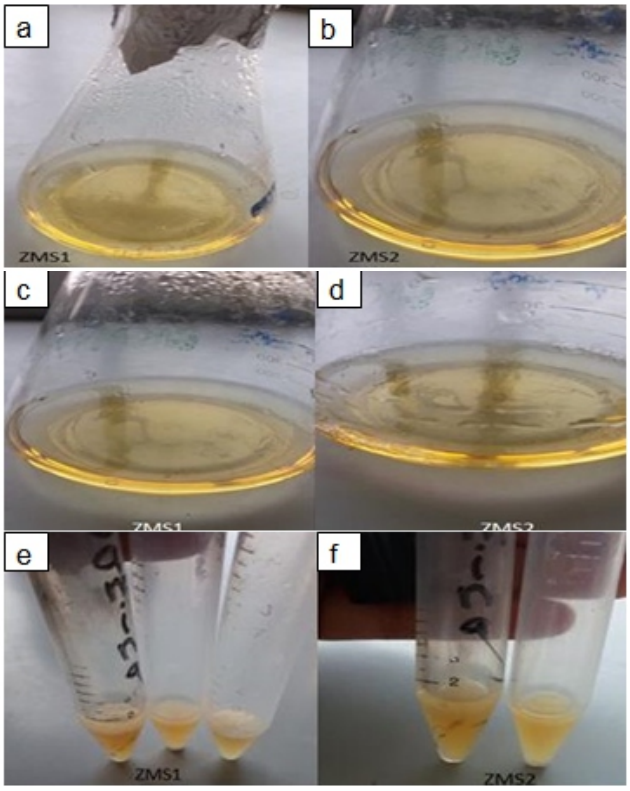

Figure 1: (a &b) Foaming activity of bacterial strains using kerosene as a Carbon source. ZMS1 strain showed visible foaming

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activity as compared to that of ZMS2 strain. (c & d) Bacterial strains using potato peel extract as carbon source. ZMS1 strain showed visible foaming activity as compared to that of ZMS2 strain. (e & f) Bacterial strains using molasses as a carbon source. ZMS2 strain showed more visible foaming activity as compared to ZMS1 strain

(c) Oil spreading technique:

When medium was supplemented with potato peel extract, molasses and kerosene oil, the ZMS1 strain showed the maximum diameter of clear zone of about 3.3, 3.1 and 3.0 cm respectively, while ZMS2 strain showed the maximum diameter of about 3.2, 3.3 and 3.3 respectively showing the positive results.

Quantity of cell free culture broth	Diameter of zone (potato peels)	Diameter of zone (molasses)	Diameter of zone (kerosene oil)
5 µl	1.0±0.120185	1.1±0.057735	1.2±0.152753
10 µl	1.8±0.128295	1.0±0.24037	1.6± 0.290593
25 µl	1.9±0.128295	2.1±0.088192	2.0±0.088192
50 µl	2.3±0.120185	2.9±0.185592	2.2±0.24801
100 µl	3.0±0.24037	3.0±0.2848	2.7±0.208167
200 µl	3.3±0.11547	3.1± 0.2848	3.0±0.057735

Table 2: Results of oil spreading assay for strain ZMS1

Quantity of cell free culture broth	Diameter of zone (potato peels)	Diameter of zone (molasses)	Diameter of zone (kerosene oil)
5 µl	1.6±0.176383	1.0±0.260342	1.5±0.11547
10 µl	2.00±0.152753	1.6±0.145297	1.9±0.260342
25 µl	2.2±0.120185	2.2±0.057735	2.1±0.057735
50 µl	2.9±0.260342	2.9±0.264575	2.2±0.152753
100 µl	3.1±0.145297	3.0±0.145297	3.0±0.145297
200 µl	3.2±0.208167	3.3±0.173205	3.3±0.202759

Table 3: Results of oil spreading assay for strain ZMS2

Optimization of biosurfactant producing bacteria by using potato peels & molasses as carbon source:

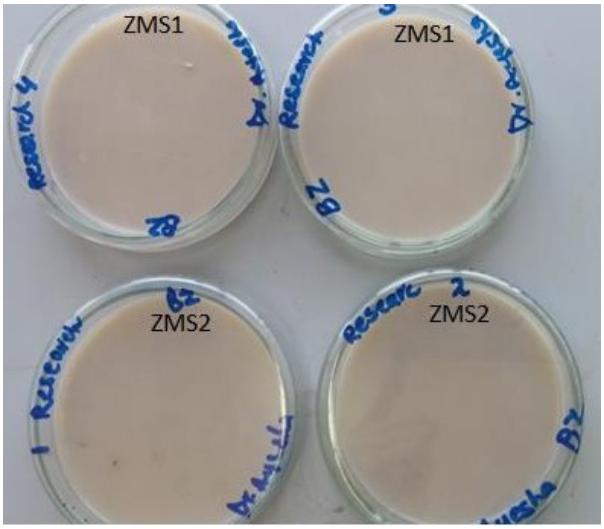

Figure 2: Results of bacterial strains by using molasses as carbon source

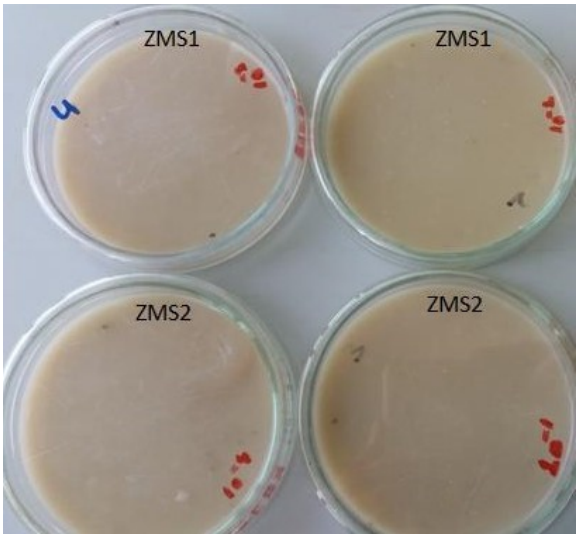

Figure 3: Results of bacterial strains by using potato peels as carbon source

Biosurfactant efficiency:

Both ZMS1 and ZMS2 strains showed positive results because stains of blood and tea disappeared after incubation of 72 hours.

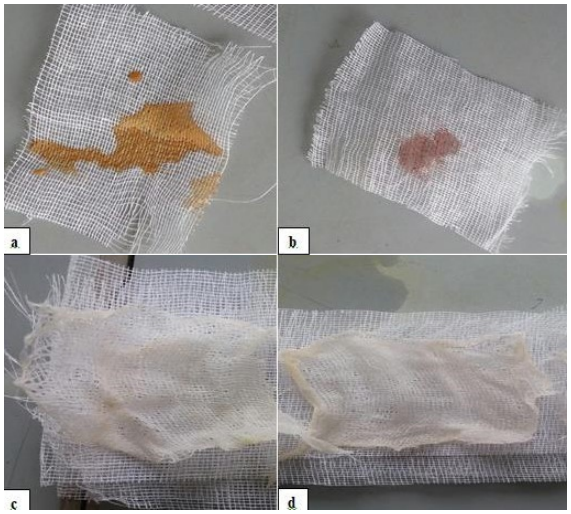

Figure 4: (a) Piece of bandage stained with tea. (b) Piece of bandage stained with blood. (c & d) Pieces of bandage after testing stain removal efficiency test.

Hydrophobicity assay:

Medium supplemented with hydrocarbons	Hydrophobicity % of ZMS1 strain	Hydrophobicity % of ZMS2 strain	Hydrophobicity % of ZMS1 strain	Hydrophobicity % of ZMS2 strain	Hydrophobicity % of ZMS1 strain	Hydrophobicity % of ZMS2 strain
Medium containing kerosene oil treated with chloroform	13.3 %	20.6 %	19.1 %	13.9 %	22.2 %	21.0 %
Medium containing kerosene oil treated with xylene	.33.3 %	.36.5 %	.31.0 %	.39.7 %	.33.1 %	.38.1 %
Medium containing kerosene oil treated with toluene	.46 %.	53 %	.54.7 %.	.56.9 %.	.55.5 %.	.57 %

Table 4: Results of Hydrophobicity assay of ZMS1 and ZMS2 strain (Using Kerosene Oil, potato peel extract and Molasses)

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DISCUSSION

The two bacterial strains were isolated and were named as ZMS1 and ZMS2. Both strains are Gram positive and have a rod and circular form, respectively. Both strains were evaluated for hemolytic activity, which some authors believe is suggestive of biosurfactant synthesis and can be used as a quick way to screen bacteria [29]. Biosurfactant generating microorganisms are regarded as a generous gift of nature due to their large-scale productivity, selectivity, performance under harsh conditions, potential uses in environmental protection, and diversity. The need for bioremediation in our environment has gradually increased interest in these bacteria [30]. Many studies described various strategies for screening biosurfactant generating microorganisms. Some prior studies described only two or three strategies, whereas numerous reports discussed six to eight ways for screening purposes. The most frequent procedures are the hemolytic assay, the emulsification assay, and the oil spreading technique. Oil spreading technique and drop collapse assay are two more reliable procedures for screening a large number of samples. These procedures only require a little amount of biosurfactant solution, about 5-10µl. The order of relevant methods for screening biosurfactant production includes oil spreading technique, emulsification assay, foaming activity, and drop collapse test, according to the findings of this study. Using kerosene oil as the sole carbon source, biosurfactant-producing bacterial strains were identified and screened from oil-contaminated soil. In 2012, Ghayyomi Jazeh and colleagues discovered 160 biosurfactant-producing bacteria in oil-contaminated soil, resulting in the discovery of 160 strains capable of creating biosurfactant. In this study, the oil spreading method, blood hemolysis test, foaming activity, and emulsification assay were used to look for bacteria that produce biosurfactants, and both bacterial strains ZMS1 and ZMS2 showed positive oil spreading technique. The emulsifying activity of strain ZMS1 was 55.2%, while it was 42.10% in strain ZMS2. The two categories of biosurfactants are low-molecular-mass molecules, which efficiently lower surface and interfacial tensions, and high-molecular-mass polymers, which are more effective as emulsion stabilising agents. Microbial growth and survival in the environment are dependent on biosurfactants. Bacillus subtilis, for example, requires surfactin production to form fruiting bodies [31]. Both strains indicated the negative hemolysis test because no zone formation observed. And the foaming stability in ZMS2 is higher than ZMS1. Several studies focused on high emulsifying abilities [32, 33]. Present study showed the significance of microbes isolated from oil spills and their ability to produce the biosurfactants. It is also useful for bioremediation of oil polluted soil.

CONCLUSIONS

It is concluded that the bacterial strains isolated from polluted soil were found to be capable of producing biosurfactants with varying responses to fabric stain removal and hydrophobicity. These strains can be used in the field to help bioremediate polluted soil and improve soil fertility.

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