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Effect of Addition of Biosurfactant Produced by *Pseudomonas sps*. on Biodegradation of Crude Oil

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***Abstract*—A total of sixteen microorganisms were isolated from from hydrocarbon contaminated region. All the isolates were screened for biosurfactant production and crude oil degradation. Among the sixteen isolates, a biosurfactant producing and hydrocarbon degrading bacterium 2B was selected for further studies. The isolate was identified as *Pseudomonas sps.* by analysis of 16S rRNA sequencing and biochemical analysis. The isolate produced biosurfactant during growth on glucose and crude oil containing medium. The biosurfactant produced lowered the surface tension of medium to 27 mN/m and formed a stable emulsion. The addition of 50mg of biosurfactant per 100 ml of the basal salt medium containing 1% crude oil significantly enhanced the crude oil degradation indicating that the organism utilized crude oil as carbon source. These findings further indicate that the biosurfactant produced by the isolate could be useful for bioremediation application.**

Keywords*—Biosurfactant; surface tension; emulsification activity; biodegradation; crude oil

I. INTRODUCTION

Petroleum exploitation, exploration, transportation, consumption, attendant spills and disposal often lead to release of hydrocarbon pollutants into the environment with serious ecological problems [1, 2, 21, 22]. Petroleum pollutants are not only toxic to biological components of the environment, some are indeed carcinogenic. Mechanical and chemical methods to reduce hydrocarbon pollution are often expensive, time consuming and not environment friendly [26]. Thus bioremediation remains the method of choice for total removal of hydrocarbon pollutants in the environment. A number of studies have reported the bioremediation of sites contaminated with petroleum hydrocarbons such as crude oil, diesel oil, and gasoline [3, 24].

In this era of green technology, biosurfactants are highly sought biomolecules for present and future applications as fine specialty chemicals, biological control agents, and new generation molecules for pharmaceutical, cosmetic and health care industries.The largest application of biosurfactant is the oil industry, for petroleum production and incorporation into oil formulations, oil spill bioremediation, removal of oil sludge from storage tanks and enhanced oil recovery [11].

Biosurfactants are polymers, totally or partially extra-cellular, with an amphipathic configuration, containing distinct polar and non polar moieties which allow them to form micelles that accumulate at interface between liquids of different polarities such as water and oil [11, 19]. This process is based upon the ability of biosurfactants to reduce surface tension, blocking the formation of hydrogen bridges and certain hydrophilic and hydrophobic interactions. They have several advantages over their synthetic counterparts, such as lower toxicity, higher biodegradability [12], better environmental compatibility [6], higher foaming [7], high selectivity and specific activity at extreme temperature, pH and salinity [14, 17, 18] and the ability to be synthesized from renewable feedstock [11]. These unique properties allow the use of biosurfactants and possible replacement of chemically-synthesized surfactants in a great number of industrial applications.

When micro organisms grow in environment rich in hydrocarbon, they undergo many adaptations. One such adaptation is biosurfactant production; it influences the uptake of hydrocarbons as substrates [16, 4]. The low solubility and high hydrophobicity of many hydrocarbon compounds make them highly unavailable to microorganisms. Biosurfactant production helps the hydrocarbon degrading bacterium to gain better access to their hydrophobic substrates as it brings about changes like reduction of surface tension of the environment around the bacterium, reduction of interfacial tension between bacterial cell wall and hydrocarbon molecules, membrane modifications like increasing the hydrophobicity of cell wall by reducing the lipopolysaccharide content of cell wall, enhancing the dispersion of hydrocarbon by encapsulation of the hydrocarbon into micelles, etc [23,25,11,28]. Many hydrocarbon utilizing bacteria and fungi possess emulsifying activities, due to whole cell or to extracellular surface active compounds [17].

In the present study, a biosurfactant producing and crude oil degrading organism, *Pseudomonas sps.* was isolated from hydrocarbon contaminated region. Since microbial growth on crude oil has been associated with the production

of surfactants, we were interested in finding out if crude oil metabolizing bacteria might be able to produce surfactants.

II. Materials and Methods

A. Enrichment and isolation of bacterium

A standard enrichment technique was used to isolate hydrocarbon-degrading microorganisms from soil sample obtained from a hydrocarbon contaminated site. A few grams of soil sample was transferred to 500 ml Erlenmeyer flask containing 100 ml of modified basal salt medium (BSM) [8] with 0.2% glucose and 1% (v/v) of crude oil as carbon sources. BSM containing KH_2PO_4 1, $Na_2HPO_4.12\ H_2O$ 6, NH_4NO_3 1, KCl, 0.06, $MgSO_4.7H_2O$ 0.406, glucose 2, pH 7 was used for the enrichment isolation of the biosurfactants producing microorganisms. Flasks were incubated at 32ºC on a rotary shaker (150 revolutions/minute) for 7 days. After 4 days, 1.0 ml of the culture was transferred to fresh media containing 1% crude oil and reincubated at 32ºC.

The isolates were further screened for biosurfactant production by drop collapse method and on Cetyl Tri Ammonium Bromide (CTAB) agar method.

B. Surface Tension Measurement

Surface tension of biosurfactant containing broth was measured using a stalagmometer at room temperature. Measurements were done in triplicate. Water was used as negative control and 1% Sodium Dodecyl Sulphate (SDS) was used as the positive control.

C. Extraction of Biosurfactant

Biosurfactant was extracted from the whole cell-free culture broth. The bacterial cells were removed by centrifugation at 9000 rpm at 4ºC for 30 minutes. The supernatant was adjusted to pH 2 using sulphuric acid, H_2SO_4 (1M) prior to biosurfactant extraction using equal volume of chloroform-methanol (2:1) mixture. The organic phase was separated and extracted. The solvent was evaporated to concentrate the crude biosurfactant. The crude biosurfactant was then dried at 60ºC to a constant weight prior to get the quantity of biosurfactant produced [20].

D. Emulsification Activity measurement

The emulsification index (E24) was measured using the method described by [18] to check the stability of the biosurfactant extracted. Biosurfactant activity was measured by adding 2 ml of crude oil to 2 ml of cell-free extract and vortexing at high speed for 2 minutes. Measurement was taken 24 hours later. Emulsions formed by the isolates were compared to those formed by a 1% (w/v) solution of the synthetic surfactant, Sodium Dodecyl Sulphate (SDS) in deionized water, as proposed by [15]. The emulsification activity was determined using the formula-

$$\text{Emulsification Index} = \frac{\text{Height of emulsion layer}}{\text{Height of liquid column}} \times 100$$

E. Effect of biosurfactant on degradation of crude oil

To study the effect of biosurfactant on crude oil degradation, the biosurfactants were extracted as described above. 50mg of biosurfactant was added to 100ml of BSM medium containing 1% crude oil. The flasks were incubated at 32^0C on a rotary shaker (150 revolutions/minute). The samples were drawn at regular time intervals to assess the degradation of crude oil. Similarly, the degradation of crude oil by synthetic surfactant, Sodium Dodecyl Sulphate (SDS) also was conducted.

F. Extraction of Residual Oil

The estimation of crude oil degradation was calculated by Gravimetric analysis. The residual crude oil was extracted in a preweighed beaker with dichloromethane. Extraction was repeated twice to ensure complete extraction. After extraction, dichloromethane was evaporated at 70ºC, the beaker was cooled down and weighed. The percentage degradation was calculated as per the method of [13] and it was as follows:

Weight of residual crude oil = Weight of beaker containing extracted crude oil – Weight of empty beaker

Amount of crude oil degraded = Weight of crude oil added in the media – Weight of residual crude oil

Percentage degradation = Amount of crude oil degraded/ amount of crude oil added in the media × 100

III. Results and Discussion

A. Enrichment and Isolation of Bacterium

A total of 16 organisms were isolated from a hydrocarbon contaminated region. Only one of the organisms, labelled as 2B, produced a halo on the CTAB media and showed positive reaction for drop collapse assay, which indicates the production of biosurfactant.

B. Biochemical Characterization and Identification of the Bacterium

The organism 2B was Gram negative bacteria, which produced green pigment. The biochemical characteristics of the organisms are presented in table 1. The alignment of the 16S rRNA gene sequences of 2B with sequences obtained by doing a Blast searching revealed 94% similarity to *Pseudomonas sps*. The isolate which produced biosurfactant was identified as *Pse*udomonas sps.

TABLE 1: BIOCHEMICAL CHARACTERISTICS OF THE ISOLATE 2B

Characteristics/ Test	**Result**
Motility test	Motile
Capsule	Present
Endospore staining	Absent
Starch hydrolysis test	Negative

Catalase test	Positive
Methyl red test	Negative
Voges – proskauer test	Negative
Indole test	Negative
Citrate utilization test	Positive
Urease test	Negative
H_2S production	Positive
Hydrolysis of gelatin	Negative
Oxidase test	Positive
Glucose fermentation	Positive
Sucrose fermentation	Negative
Lactose fermentation	Negative

C. *Surface Tension Measurement*

A good surfactant can lower surface tension of water from 72 to 35 mN/m. The initial surface tension of the supernatant of *Pseudomonas sps*. was 70 mN/m, which reduced to 27 mN/m respectively (Fig. 1). There was a positive correlation between the reduction of surface tension and population of microorganism. This shows that biosurfactant can increase the bioavailability of crude oil and biodegradation process. Similar results were obtained by Banat *et al*., 1991 [9].

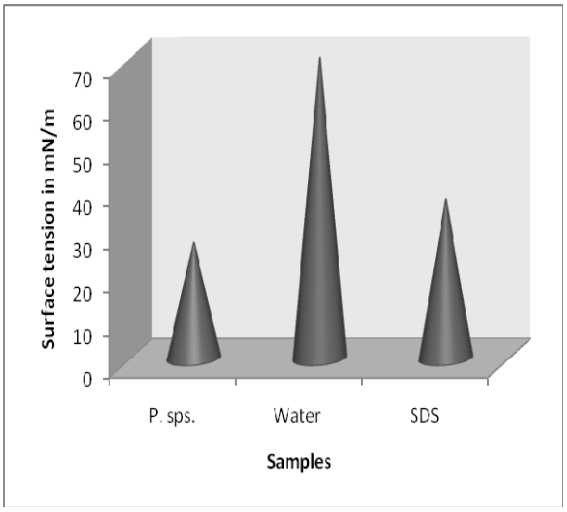

Figure 1: Comparison between the surface tension activities of the bacterial isolate, water and SDS.

A. *Emulsification Measurement:*

The emulsion formed by *Pseudomonas sps.* was stable with crude oil. The emulsion was stable for at least 30 days (Fig 2). The highest E24 value of 80 ± 1 was recorded on the 3rd day of incubation. The emulsion formed by the isolate was stable for 30 days. Most biosurfactants are specific and emulsify different substrates differently [18]. Formation of emulsion usually results from the dispersion of a liquid phase as microscopic droplets in another liquid continuous phase [11]. This property is especially useful for making oil/water emulsions for cosmetics and food. Emulsification enhances the biodegradation of hydrocarbons by increasing their bioavailability to the microorganisms involved in the process.

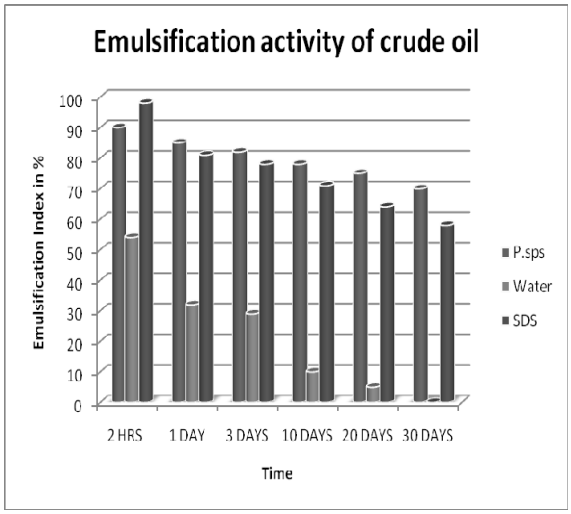

Figure 2: Comparison between the emulsification index of of the bacterial isolate, water and SDS.

B. *Characteristics of the Pseudomonas sps. Surfactant*

Biosurfactant was produced by *Pseudomonas sps.* culture mainly in stationary phase of growth. After 3 days of incubation in glucose containing medium, 3.4 g/L of crude surfactant was recovered where as 2.7 g/L of crude surfactant was extracted from crude oil containing medium. The concentrated material appeared yellow and had an oily consistency. The material readily caused drop collapse on an oily plate, suggesting that it possessed potent surfactant activity.

C. *Quantification of Crude Oil Biodegradation:*

Pseudomonas sps. showed a significant reduction in the crude oil concentration. The bacterium degraded around 52% of the crude oil by 7th day and 87% degradation of the crude oil was observed on 21st day as shown in the fig. 3. Similar results were reported by Okoh *et al.,* 2001 [3]. The degradation profile of crude oil was also studied using synthetic surfactant Sodium Dodecyl Sulphate (SDS), it was observed that 19% and 42% of crude oil was degraded on 7th and 21st day respectively. It can be concluded that biosurfactants could be used to enhance the degradation of crude oil instead of chemical surfactants. The advantage of using biosurfactants in the biodegradation of hydrocarbons is that they are extracellular, less toxic and easily degradable in the environment than their chemical counterparts. Hence, biosurfactants finds application in bioremediation studies of various hydrocarbons.

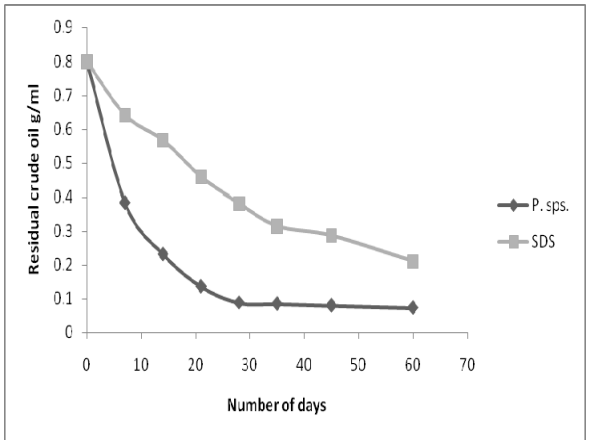

Figure 3: Comparison of the degradation profile of crude oil by the bacterial isolate *Pseudomonas sps.*

Biodegradation of crude oil by microorganisms appears to be the natural process by which the bulk of the polluting oil is used as an organic carbon source, causing the breakdown of petroleum components to lower molecular compounds or transformed into the other organic compounds such as biosurfactants [24]. During biodegradation, crude oil is used as an organic carbon source by a microbial process, resulting in the breakdown of crude oil components to low molecular weight compounds [3].

IV. CONCLUSIONS

Totally 16 microorganisms were screened for biosurfactant production and crude oil degradation. Only one bacterial isolate was capable of producing biosurfactant as well as degrading crude oil which was supplemented as a carbon source. It is evident from this investigation that isolated bacterium degraded crude oil and comparison of obtained results and existing statistics with similar studies revealed that isolated *Pseudomonas sps.* can be used for bioremediation of crude oil pollutant from the environment. Since growth of the bacterial isolate on crude oil has been associated with the production of biosurfactants, we can conclude that the crude oil metabolizing bacterium is able to secrete surfactants which further enhance the hydrocarbon degradation. Further understanding of the mechanism of the hydrocarbon degradation process by this organism will help in developing strategies for removing crude oil from polluted areas.

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