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Isolation and Characterization of Biosurfactant Producing Bacteria and their Potential Role in Oil Biodegradation

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Abstract

The present study is focused on the isolation and characterization of biosurfactant producing strains and the evaluation of their potential role in oil biodegradation. Four strains (Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis) were selected on the basis of 16S rRNA gene sequencing. The impact of oil biodegradation potential of bacterial strains at five mobil oil concentrations (1%, 1.5%, 2%, 2.5% and 3%) was estimated at various temperatures, pH, and incubation time intervals and in the presence of inhibitors including SDS and Chromium. At 1% and 1.5% oil concentration strains showed maximum oil degradation however the degradation capacity was decreased with an increase in oil concentration. At lower oil concentration bacterial strains showed maximum degradation in acidic medium while at higher concentration degradation was decreased. Maximum oil degradation was observed at 37°C. When the incubation time was increased, a positive trend of biodegradation was observed however at high concentration, the biodegradation ability decrease. All strains showed higher degree of inhibition in the presence of inhibitors. It was concluded that bacterial strains can effectively be employed in biodegradation of mobil oil and helpful in bioremediation at oil drilling sites as well as in aquifers.

Keywords: Biosurfactant; Bioremediation; Oil degradation; Biodegradability

Introduction

Surfactants, whether derived from chemical or biological origin, possess both hydrophilic (showing affinity to polar materials) and hydrophobic characteristics (having affinity to non-polar materials), which not only help in emulsion formation but also decrease the interfacial and surface tension. Bioemulsifier which are able to reduce surface tension are termed as biosurfactants. Biosurfactants are produced by using micro-organisms from a number of resources which are effective in modifying surface characteristics of substrate like modifying surface area, control in attachment and expulsion of micro-organisms. Surfactants also assist in increasing bioavailability of hydrophobic substrates. Bio-surfactants can effectively be employed in medical and industrial applications for the processes of emulsification and de-emulsification, pharmaceutical and cosmetic industry, oil recovery enhancement, metallurgical and mining industries, agrochemicals and in environmental management Rapid growth in the field of science and technology is responsible for industrialization in different regions of the world. Although industrial expansion is very important for developing nations however this expansion leads to serious environmental concerns [1]. It has been recognized that petroleum based hydrocarbons and their derivatives like mineral oil, diesel oil, engine oil, heavy oil residues are deteriorating planet health through polluting soil and aquatic life. Mobil oil is infact a mixture of different aromatic hydrocarbons, additives, cyclic alkanes and anticorrosive compounds that are difficult to degrade. Used mobil oil comprises of a higher proportion of heavy metals, carcinogenic compounds, PAHs (Polycyclic Aromatic Hydrocarbons) and toxic metals which impose serious threats not only to human beings but on vegetation as well. Different techniques and materials are employed for the degradation of hydrocarbons like chemical or biological methods. One of the famous techniques that is being used in various industries is the use of surfactant [2].

Nowadays researchers are trying to explore new dimensions in the field of remediation as chemical based surfactants are not suitable for remediation. Chemical surfactants are toxic in nature and not environment friendly. On the other hand, bioremediation appears to be a promising approach because it is found to be environment friendly. In bioremediation where micro-organisms help to degrade different kinds of pollutants seems to be the most attractive approach [3]. The hydrophobic compounds because of low solubility in water strongly adheres to soil particles and take more time to be released into water, hence time could be the important factor for bioremediation. Since diesel and mobil oil are not soluble in water, hence they are less available to the micro-organisms. Microbial organisms detoxify pollutants through different modes, including polymerization, transformation and mineralization [4]. Sometimes a single bacterial strain is sufficient for remediation, however, at times a group of bacterial strains called as consortium are used for bioremediation. More and more research is now focused on to explore different methods and raw materials for isolation of different kinds of bacterial strains which can effectively biodegrade different forms of hydrocarbon pollutants [5]. The main objective of this work is to isolate biosurfactant producing microscopic organisms from oil tainted conditions and explore their role in the biodegradation of Mobil oil.

Biochemical and physiological characterization was done for the identification of isolated strains. In this work the effect of oil degradation was observed at different factors, including different oil concentrations, varying pH, varying incubation times, varying temperatures and in the presence of inhibitors [6].

Materials and Methods

Sample collection

Different greasy soil samples were selected from three Toyota Auto workshops of Lahore, Pakistan. Temperature and pH of these samples were noted and samples were transferred to laboratory under sterile conditions for screening and isolation [7].

Isolation and purification of bacterial strains from samples

Soil samples were diluted up to 10-6 dilutions by using serial dilution method. Afterwards, Luria Bertani agar plates were prepared in triplicates and greasy soil samples were spreaded on it. Four pure cultures were obtained after sub-culturing [8].

These strains were characterized morphologically and biochemically *via* gram staining and several biochemical tests. The biochemical tests included catalase, oxidase, mannitol salt agar, citrate, eosin methylene blue agar, MR-VP test, hydrogen sulfide test, urease, triple sugar iron test, indole, motility etc. The strains which had the capability of producing biosurfactant were screened qualitatively [9].

Screening assay for biosurfactants producing bacteria

In this study different screening assays like, drop collapse assay and emulsification capacity assay were performed for the detection of biosurfactant producing bacteria [10].

Drop collapse assay

The drop collapse test was performed for the screening of biosurfactants as described by. The bacterial culture was inoculated in Luria Bertani broth media and incubated at 37°C for 24 hours. After incubation the bacterial culture was centrifuged for 5 minutes. The supernatant was collected in a petri plate and drop of oil was placed over it. After 1 minute the stability of the oil droplet was observed which confirmed that either the bacterial strains were biosurfactant producing or not [11].

Emulsification capacity assay

In this assay 2 ml of petrol and 2 ml of bacterial culture were taken in screw capped test tubes and vortexed at a very high speed for 2 minutes. To observe the emulsification layer, test tubes were placed at room temperature for 24 hours. After 24 hours the height of stable emulsion was measured. The emulsification index was calculated as shown in equation 1 [12].

$$E_{24} = \frac{height\ of\ emulsion\ formed}{Total\ height\ of\ solution} \times 100 \qquad (1)$$

Oil biodegradation assay

The biosurfactant producing strains were used to check the biodegradation of oil. Mobil oil was used as a carbon source [13]. The redox indicator DCPIP (2, 6 Dichlorophenol indophenol) was used for determining the oil degradation pattern. The color change of DCPIP from blue to colorless is a clear indication of biodegradation of oil. For the oil degradation assay, 1% stock solution of DCPIP was prepared. From stock solution, 10 µl indicators were used in oil biodegradation assay. Incubation was given at 37°C and the flasks were placed on the rotary shaker for five days [14]. Spectrophotometric analysis was performed at 750 nm to measure the absorbance of inoculated cultures along with DCPIP indicator and the selected mobil oil concentrations [15].

Optimization of different parameters in oil biodegradation assay

The impact of oil biodegradation potential of bacterial strains at 1%, 1.5%, 2%, 2.5% and 3% Mobil oil concentrations was estimated at various temperatures 28°C, 37°C, 42°C, various pH 3, 5, 7, 9, different incubation time intervals 24, 48, 72, 96 hrs, and in the presence of inhibitors including SDS and heavy metal [16].

16S rRNA gene sequencing

The strains were sent to Macrogen Korea, for 16SrRNA sequencing. After the sequences were obtained, they were modified to make a contig. Afterwards, the contig was used in NCBI Blast [17].

Results

Isolation and characterization of strains

Four strains were isolated from Auto workshop soil samples. Strains were selected on the basis of highest emulsification index and drop collapse assay for further study at different parameters i.e., (temperature, pH, incubation time interval, and in the presence of inhibitors) as seen in Figure 1.

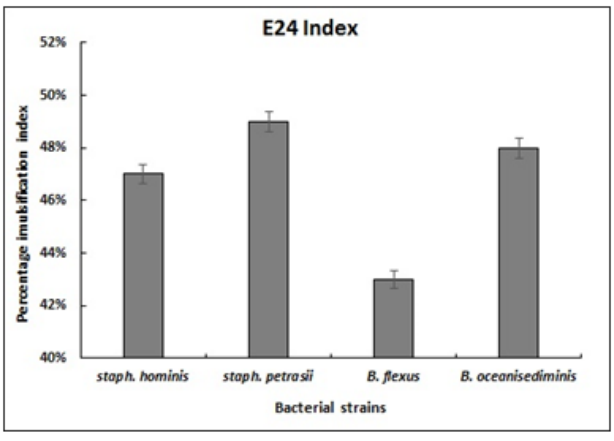

Figure 1: Emulsification index of four different bacterial strains (F1, F9, 20 and 32).

All of the strains had shown positive drop collapse assay as the drop of culture did not remain in its beaded form [18]. Strain F1, F9,

20 and 32 isolated from Toyota workshop soil. Two strains i.e. F1 and F9 had 99% homology to each other and their designated genera according to BLAST result is Staphylococcus, however strain 20 showed homology with Bacillus [19]. Strain 32 had 100% homology with Bacillus genus. Strain F1 was Staphylococcus hominis, strain F9 was Staphylococcus petrasii, strain 20 was Bacillus flexus and strain 32 was Bacillus oceanisediminis respectively [20].

Impact of different concentrations of oil on biodegradation potential

The oil biodegradation was analyzed at five different mobil oil concentrations (1%, 1.5%, 2%, 2.5% and 3%). The biosurfactant producing strains used in this study showed oil degradation as the bacterial strains reduced the color intensity of 2,6 Dichlorophenol Indophenol (DCPIP) as compared with the control [21]. All the bacterial strains showed maximum degradation from 82.81% to 82.18% at 1% and 1.5% oil concentration. Only Staphylococcus hominis showed less degradation behavior (4% and 8.81%) as compared to other bacterial strains where degradation was found to be 55% and 90% at 2% and 2.5% concentration of oil as shown in Figure 2. However, at 3% oil concentration the degradation was decreased from 8.33% to 1.19% [22].

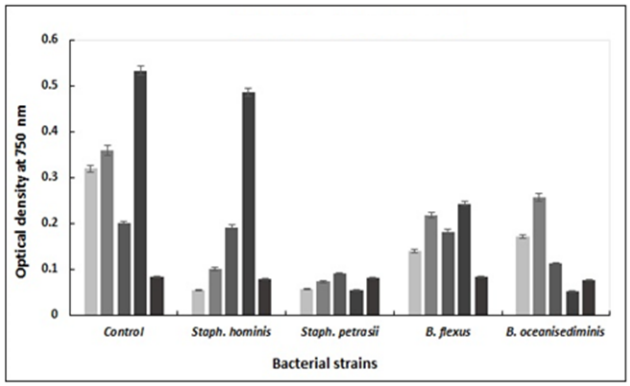

Figure 2: Impact of five different oil concentrations (1%, 1.5%, 2%, 2.5% and 3%) on biodegradation behavior by using four different bacterial strains (*Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis)* at 37°C after five days of incubation on rotary shaker at 240 rpm.

Impact of pH on oil biodegradation potential

In order to determine the impact of pH on oil biodegradation four levels of pH (3, 5, 7 and 9) were selected as bacterial strains show different behavior in acidic and alkaline medium. In acidic medium at 1% mobil oil concentration, the bacterial strains showed the highest biodegradation potential, as 76.63% oil was degraded, while this degradation rate was reduced to 34% at pH 5.

As the value of pH was increased from 7 to 9, the biodegradation rate in all four bacterial strains kept on decreasing and maximum degradation was observed to be 18% and 35%. Bacterial strains showed maximum degradation at pH 3 from (71.63% to 73.72%) and at pH 9 from (34.75% to 21.95%). When the oil concentration was maintained at 1.5%, the degradation was decreased from 39.26% to 12.39% at pH 5 and 7 respectively [23].

When 2% concentration of oil was used the pattern of degradation was almost same at all levels of pH as shown in Figure 3. However, as the concentration of oil was increased to 2.5%, the maximum and minimum oil degradation was 70.70% and 6.59%respectively. When the oil concentration was gradually increased up to 3%, the degradation potential was decreased from 52.88% to 9.81%. However, at 3% oil concentration the activity of bacterial strains was very slow for oil degradation (Figures 3-5) [24].

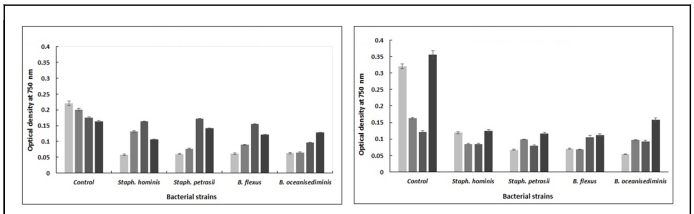

Figure 3: The impact of oil degradation on four levels of pH (3, 5, 7 and 9) by using four types of bacterial strains (*Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis*) on five different oil concentrations 1%, 1.5%.

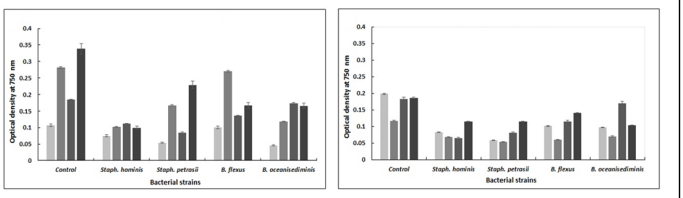

Figure 4: The impact of oil degradation on four levels of pH (3, 5, 7 and 9) by using four types of bacterial strains (*Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis*) on five different oil concentrations 2%, 2.5%.

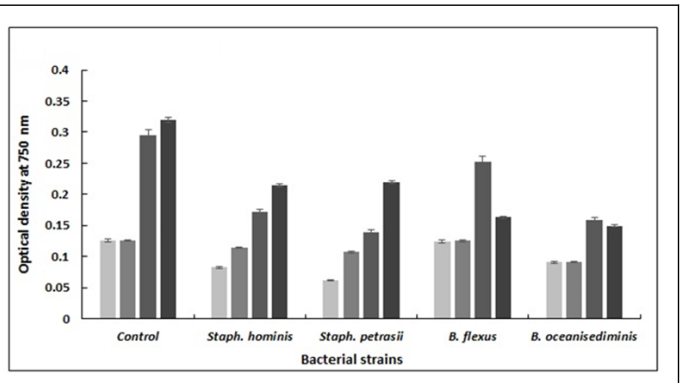

Figure 5: The impact of oil degradation on four levels of pH (3, 5, 7 and 9) by using four types of bacterial strains (*Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis*) on five different oil concentrations 3%.

Impact of temperature on oil biodegradation potential

The impact of temperature on oil degradation was observed by varying the temperature from 28°C to 37°C and finally to 42°C during the process at different mobil oil concentrations. At 1% and 1.5%mobil oil concentration the maximum degradation of 82.18% and 79.72% was observed while minimum degradation of (46.56% and 28.61%) was observed at 37°C (Figures 6-8).

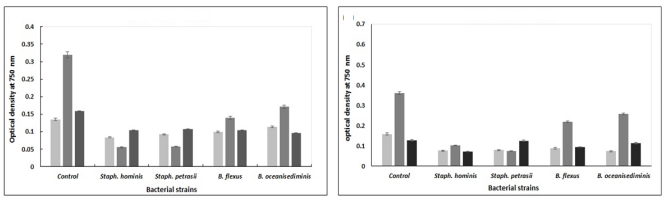

Figure 6: The effect of three levels of temperature (28°C, 37°C and 42°C) on oil degradation was observed by using different bacterial strains (*Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis*) on five different oil concentrations 1%, 1.5%.

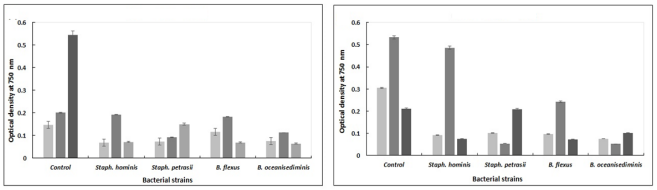

Figure 7: The effect of three levels of temperature (28°C, 37°C and 42°C) on oil degradation was observed by using different bacterial strains (*Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis*) on five different oil concentrations 2%, 2.5%.

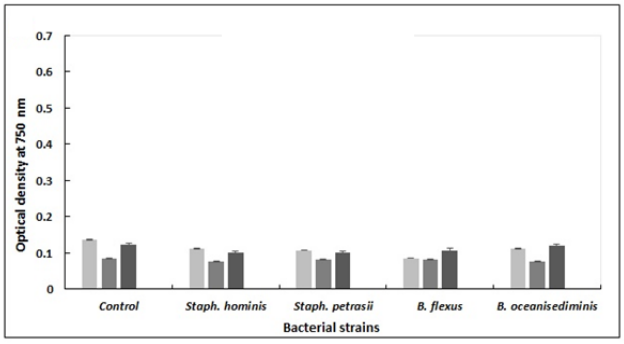

Figure 8: The effect of three levels of temperature (28°C, 37°C and 42°C) on oil degradation was observed by using different bacterial strains (*Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis*) on five different oil concentrations 3%.

When 2% mobil oil concentration was used, the rate of degradation kept on increasing with an increase of temperature and maximum degradation of 88.21% was observed at 42°C. When the oil concentration was increased to 2.5%, all bacterial strains showed higher degradation (70%, 66.57%, 69% and 75%) at 28°C. By increasing temperature up to 37°C and 42°C it was found that bacterial strains showed a mixed behavior of increase and decrease in degradation [27]. Here, the maximum degradation at both the temperatures was found to be 90% and 65%, while the minimum degradation was 9% and 1.42%. However, the majority of bacterial strains showed minimum degradation when the oil concentration was increased to 3%.

Impact of incubation time on oil biodegradation potential

In order to investigate the impact of incubation time on oil biodegradation four levels of time (24, 48, 72 and 96 hrs) were selected. It was found that as the time interval was increased all biosurfactant producing bacterial strains showed higher trend of biodegradation. This trend of biodegradation kept on increasing as the time interval was increased as shown in Figure 5. Significant biodegradation around 89.19% and 81% was observed when 1% and 1.5% concentration of mobil oil were used. When the higher concentration of mobil oil was used the rate of degradation decreased. However, when the concentration of oil was increased to 3% then maximum degradation was around 66% but further increase in time did not cause any further degradation (Figures 9-11) [28].

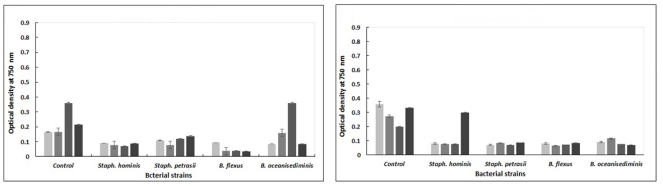

Figure 9: The impact of incubation time (24, 48, 72 and 96 hrs) on oil biodegradation was analyzed at five different levels of oil concentrations 1%, 1.5%.

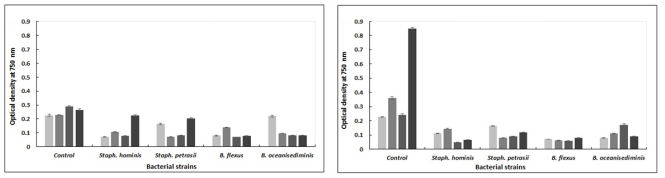

Figure 10: The impact of incubation time (24, 48, 72 and 96 hrs) on oil biodegradation was analyzed at five different levels of oil concentrations 2%, 2.5%.

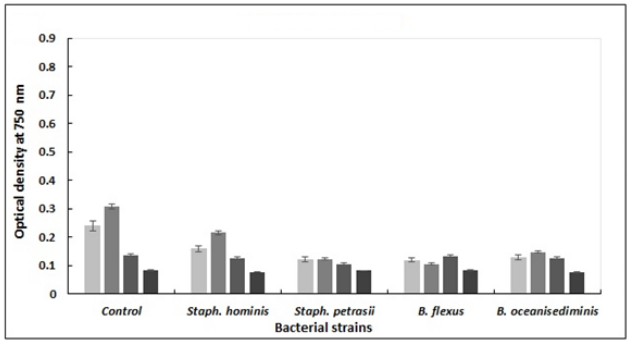

Figure 11: The impact of incubation time (24, 48, 72 and 96 hrs) on oil biodegradation was analyzed at five different levels of oil concentrations 3%.

Inhibition of biosurfactants producing ability by SDS

Sodium Dodecyl Sulfate (SDS) is also a biosurfactant but in this experiment it was used as an inhibitor against biosurfactant producing potential of selected bacteria. Two concentrations of SDS (1% and 2%) were selected for determining their inhibitiory role at different

concentrations of oil. When 1% SDS concentration was used the maximum inhibition of 3% to 11% was observed at 1% and 2% oil concentration. When the concentration of SDS was increased to 2% then maximum inhibition of 0.8% was observed at 3% oil concentration, while other concentrations of oil did not exhibit significant inhibition as shown in Figure 11.

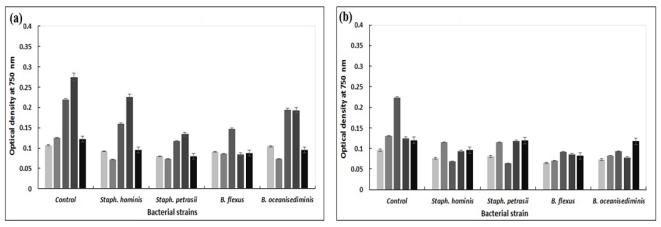

Figure 11: Inhibitory role of SDS with two concentrations. Note: A) 1% SDS concentration, B) 2% SDS concentration.

Inhibition of biosurfactants producing ability by heavy metals

Chromium is a toxic heavy metal which was used as an inhibitor against the biosurfactant producing bacteria. In this work 1000 µg/ml concentration of chromium was used against different concentrations of oil. All bacterial strains showed higher inhibition at 1% concentration of oil, however the maximum inhibition was around 0.75%, as can be seen in Figure 7. At 2.5% oil concentration no inhibition was observed, however, few bacterial strains showed inhibition at 1.5%, 2% and 3% oil concentrations (Figure 12).

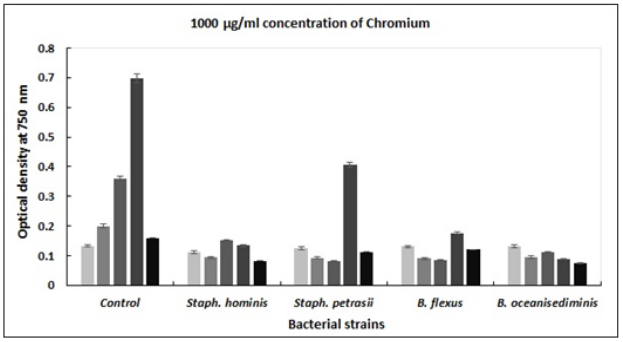

Figure 12: The inhibition capability of heavy metal Chromium was analyzed at five concentrations of mobil oil (1%, 1.5%, 2%, 2.5% and 3%). The Chromium concentration of 1000 µg/ml was taken as inhibitor against four bacterial strains (*Staphylococcus hominis, Staphylococcus petrasii, Bacillus flexus and Bacillus oceanisediminis*).

Discussion

A wide range of biosurfactants enables various microorganism to be effectively employed in diverse fields, like biotechnological and industrial applications for oil recovery enhancement, pharmaceutical and degradation of pollutants. As far as environmental applications are concerned, biosurfactants have been extensively used because of their distinct features like environment friendly, low toxicity, potential for improved biodegradation and increasing the solublility of less soluble compounds. In this work the main focus is on the isolation and characterization of biosurfactant producing bacteria and to find their capability in oil biodegradation. From the results it can be found that when the concentration of oil was increased, the resultant biodegradation activity was decreased. Similar results have also been reported by who found that there exists an inverse relation between concentration of oil and biodegradation. All bacterial strains showed maximum degradation at 1% and 1.5% oil concentrations. However, when the concentration of oil was increased the biodegradation ability decreased, as the same quantity of bacterial strains did not have sufficient ability to degrade such a high quantity of oil concentration or the higher proportion of oil components reduced biodegradation ability of bacterial strains because of possible toxic effects which decreased the viability of bacterial strains.

When the concentration of oil was increased to 2% to 3% there was no significant biodegradation shown by any isolate. The use of higher proportion of bacterial strains for biodegradation of oil at higher concentration may resolve this issue to some extent, as according to Chen, the activity of bacterial strains decreases with an increase in oil concentration. Not only the concentration of oil but the pH of system is also another important attribute that impacts the biodegradation behavior of bacterial strains. Four different values of pH were used for determining their impact on biodegradation activity of the biosurfactant producing strains. Micro-organisms helping in biodegradation of oil can survive more effectively at pH ranging from 4 to 9. The impact of pH on oil biodegradation can be seen. The bacterial strains in acidic medium showed higher biodegradation ability as compared with neutral pH and alkaline medium. Furthermore, as the pH of system was more acidic, it caused positive influence on biodegradation activity.

Temperature influences all life forms, from human beings to microorganisms. From Figure 3, the impact of different temperatures on the biodegradation behavior of microorganisms can be seen. It is clear from the Figure that at 1% and 1.5% oil concentrations, maximum biodegradation was found at 37°C, while there was no significant difference in biodegradation at 28°C and 42°C. When the concentration of oil was increased to 2% and 2.5% the behavior of bacterial strains was different as shown in Figure. Literature shows difference of opinion among the researchers on the most appropriate temperature for oil biodegradation. The suitable temperature for biodegradation has been reported to be 30°C, on average, temperature from 30°C to 40°C is more favorable for oil degradation. According to the researchers, an increase or decrease in temperature affect the various factors of Mobil oil such as oil viscosity, different behavior of alkane volatilization and decrease of water solubility. However, these are not only the parameters that explains different oil biodegradation potential of various strains, as the behavior of micro-organisms is a complex entity which needs further exploration and investigation. However, at higher concentration of oil, no significant biodegradation was observed.

In order to determine the impact of incubation time on the oil biodegradation behavior of our strains, four levels of time were selected. Figure 5 shows the impact of incubation time on oil biodegradation behavior. It is clear from the figure that as the incubation time was increased the bacterial strains showed higher biodegradation of oil. It was found that incubation time is directly proportional to the oil biodegradation activity of micro-organisms. At 1% oil concentration, when the incubation time was increased from 24 hours to 96 hours the resultant biodegradation activity also increased from 43% to 83.13%. When the oil concentration was increased to

2.5% the higher trend of biodegradation was observed as compared with 1 % oil concentration, the biodegradation was further increased with increase of incubation time and the maximum degradation from 65.19% to 89.01%. The similar behavior of oil degradation was observed with other oil concentrations and it was found that oil degradation was increased with an increase in incubation time. In present study all bacterial strains showed maximum biodegradation activity in the first 24 hrs. As the incubation time was further increased there was a gradual decrease in biodegradation.

The oil degradation depends on a number of factors that interact simultaneously for effective degradation. Surfactants, whether biological or chemical origin, have a dual behavior during the process of degradation of different pollutants. Sometimes the surfactants facilitate the process of degradation while on the other hand, they may inhibit the process of degradation. In this study, Sodium Dodecyl Sulfate (SDS) was used as surfactant which inhibit the process of degradation. When 1% concentration of SDS was used then bacterial strain B. oceanisediminis showed minimum oil degradation (2.02%). However, at 2.50% oil concentration this degradation reached upto 29.81%.

When the concentration of SDS was increased upto 2%almost similar inhibition was shown by the bacterial strains. Increasing the concentration of SDS also caused inhibition of biosurfactant producing ability of the bacteria. However, there was not significant difference in the inhibition at 2% SDS concentration. More or less similar thing has been explained by other researchers that surfactants may decrease or have no effect on oil degradation as other factors along with SDS concentration play their role in oil degradation. However, with rapid industrialization and development of science and technology, the environment is getting contaminated because of industrial wastes and byproducts like, the discharge of heavy metals in the environment. Heavy metals are present in the environment in small proportion, however, their proportion is increasing due to an increase in industrial activities.

These heavy metals can either inhibit or increase the degradation process. As already mentioned that different factors sometimes favor or retard the process of biodegradation because the behavior of microorganisms is a complex phenomenon that changes with any change in the environmental conditions. In this study chromium was selected for studying the inhibitory effect of heavy metals on biosurfactant producers. When 1000 µg/ml concentration of chromium was used the maximum inhibition was showed by bacterial strain at 1% oil concentration. At higher concentration of heavy metals, the micro-organisms show higher inhibition as they instantaneously stop their activities.

Conclusion

The bacterial strains at different oil concentrations and parameters showed different levels of biodegradation. The trend of higher degradation was observed at low oil concentrations while higher concentration of oil caused adverse effect on the microbial activity and viability. The biosurfactants behaved more effectively in acidic medium as compared with neutral and alkaline medium. Both the inhibitors, SDS and chromium, inhibited the oil biodegradation potential of the isolates. It was concluded that bacterial strains isolated from soil of Auto workshops can effectively be employed for successful oil biodegradation.

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