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Analysis of biodegradation pathway of crude oil by *Pseudomonas* sp. isolated from marine water sample

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ABSTRACT

Crude oil contamination in coastal areas leads to unmanageable extent of loss of biological life that negatively impacted on global economy. Intensified physical and chemical clean-up and prevention technologies have found temporary solutions. But present study had revealed that complete removal can be possible by bioremediation using biosurfactant producing marine bacteria. In the present study, a marine biosurfactant producing bacteria Pseudomonas sp. was isolated and biosurfactant activity of the isolate was recorded. In drop-collapse test, drop was collapsed within 10sec, emulsification activity was 0.11 OD, and emulsification index was 66.6% and 94% of supplemented crude oil was degraded in vitro by the isolate. The isolate degraded the crude oil under extremes of pH ranges between 4 and 9 and at salt concentrations from 1% to 15%. GC-MS analysis on degraded end product of crude oil showed complete mineralization of crude oil into low molecular weight compounds of octadecane, pentadecane, heptadecane, trideca -2,4,6,10-tetraene, 3,6 dimethoxy flourescene, nitrilomorphinan, dotriacontane, and hentriacontane. So, the bacterial isolate Pseudomonas sp. can be effectively utilized for bioremediation of crude oil in marine environment in a controlled way.

Keywords: Petrochemical pollution – biodegradation – GC-MS analysis – bacterial bioremediation.

INTRODUCTION

One of the major environmental problems in marine environment today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Oil spills from petrochemical industries are indirectly released into the water bodies which are hazardous to the environment as they damage the surrounding soil and water ecosystems [1]. Accidental release of petroleum products also is of particular concern in the environment. Leaks and accidental oil spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. Petroleum fuel spills are considered as the most frequent organic pollutant of environment and are classified as hazardous wastes due to their cytotoxic, mutagenic and carcinogenic effects on human [2, 3].
The amount of natural crude oil seepage was estimated to be 600,000 metric tonnes per year. Attention has been focused on oil spill in marine environments since the world's oceans are the largest and ultimate receptors of hydrocarbon pollutants. So, search for an efficient and effective method of crude oil removal from contaminated ecosystem has intensified in recent years.

Mechanical and chemical methods such as incineration or burial in secure landfills are used to remove hydrocarbons from contaminated sites become prohibitively expensive when amounts of contaminants are large. One promising method that has been commonly used is the biological degradation of oil by microbes [4], since it is cost-effective and will lead to complete mineralization.

Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6% to 82% for fungi, 0.13% to 50% for soil bacteria, and 0.003% to 100% for marine bacteria [5]. So bacteria are selected for mass culture and biodegradation since they are dominant hydrocarbon degraders in oceans. Moreover bacteria also have diverse metabolic pathways that are not present in fungi, which allow them to utilize most recalcitrant petroleum hydrocarbons.

One of the important limiting factors for bacterial oil degradation is the adaptability of the bacteria to grow at high salt environments, which will not support externally added bacteria. Fathepure has proposed that there are many explorations and production sites throughout the world that are contaminated with both oil and salt. This poses a problem for cleaning up those sites using bioremediation technologies since externally added bacteria will not survive in high salinity. On the other hand, though degradation of petroleum hydrocarbon compounds has been extensively studied, information on their degradation in crude oil contaminated marine environment is scanty.

The identification of key organism that plays a role in hydrocarbon pollutant degradation processes is essential to develop an optimal in situ bioremediation strategy. Current study aimed to isolate a biosurfactant producing, salt tolerant/requiring marine bacteria for bioremediation of oil in marine environment, so that it could well adapt in marine environment and degrade oil efficiently as it produce biosurfactant, since biosurfactant production enhances emulsification of crude oil and makes it easily available for microbial degradation.

MATERIALS AND METHODS

Isolation and identification of biosurfactant producing organism

Marine water sample was collected aseptically from Calicut coast of India, in a sterile container from 10 ft distance towards the sea from sea coast. Biosurfactant producing bacteria were isolated by enriching in minimal medium containing 2% crude oil as carbon source. The cultures were incubated on rotary shaker at 30°C for 48 h. Microscopic and biochemical tests were performed to identify the isolate.

Qualitative determination by drops collapse test (Irene Kuiper et al., 2004)

The bacterial isolate was inoculated in mineral salt medium and were incubated at room temperature for 24h. The cultures were then centrifuged at 5000 rpm for 20 min and cell free supernatant was used for the test. Uninoculated mineral salt medium was used as control. About 25 µl of cell free culture supernatants were pipetted out as droplet on paraffin coated glass slide. Collapse of drop was recorded within a minute.

Quantitative determination of biosurfactant activities

The selected isolates were grown in mineral salt medium for 24h at room temperature. The culture supernatant was centrifuged at 5000 rpm for 20 min and cell free supernatants were used for the tests. Exactly 2 ml of cell free broth was mixed with 2 ml of paraffin in a test tube. It was then vortexed for a minute using a cyclone mixer and left undisturbed. After one hour, the optical density of oil in water emulsion phase was measured at 610 nm [8]. The above set-up was left undisturbed for 24 h. After incubation, the height of emulsified layer was measured and emulsification index (E_{24}) was calculated using the formula,

$$E_{24} = \frac{\text{Height of emulsified layer (cm)}}{\text{Total height of liquid column (cm)}} \times 100$$

Influence of salt concentration

The effect of salinity on biosurfactant activity was determined by adding NaCl, concentrations ranging from 1% - 15% to the mineral salt medium. The cultures were incubated at 30°C on shaker at 150 rpm for 48 h. After incubation the optical density of the solution was measured at 610 nm [9] and emulsification index was measured.

Influence of pH on biosurfactant activity

The effects of pH on biosurfactant activities were studied by changing pH (4.2 -9.2) of mineral salt medium. The cultures were incubated at 30°C on shaker at 150 rpm for 48 h. After incubation the optical density of the solution was measured at 610 nm and emulsification index were measured.

Assay of biodegradation of crude oil

About 50 ml of marine water was mixed with 5ml of crude oil and inoculated with 0.5 ml of isolated bacterial inoculum. Uninoculated 50 ml of marine water sample was maintained as control. After 15 days of incubation, broths were centrifuged, 1ml of the sample extracted with methanol, organic layer was separated, allowed to air-dry and percentage of oil degradation was calculated using the formula,

$$\% \text{ of crude oil degradation} = \frac{\text{Weight of the tube with oil}}{\text{Weight of the tube}} X100$$

The metabolic end products produced from crude oil biodegradation of the sample was analyzed in gas chromatography mass spectrum analysis at a flow rate of 1ml/min at 250°C using helium gas as a carrier in the Shimadzu GC-MS instrument.

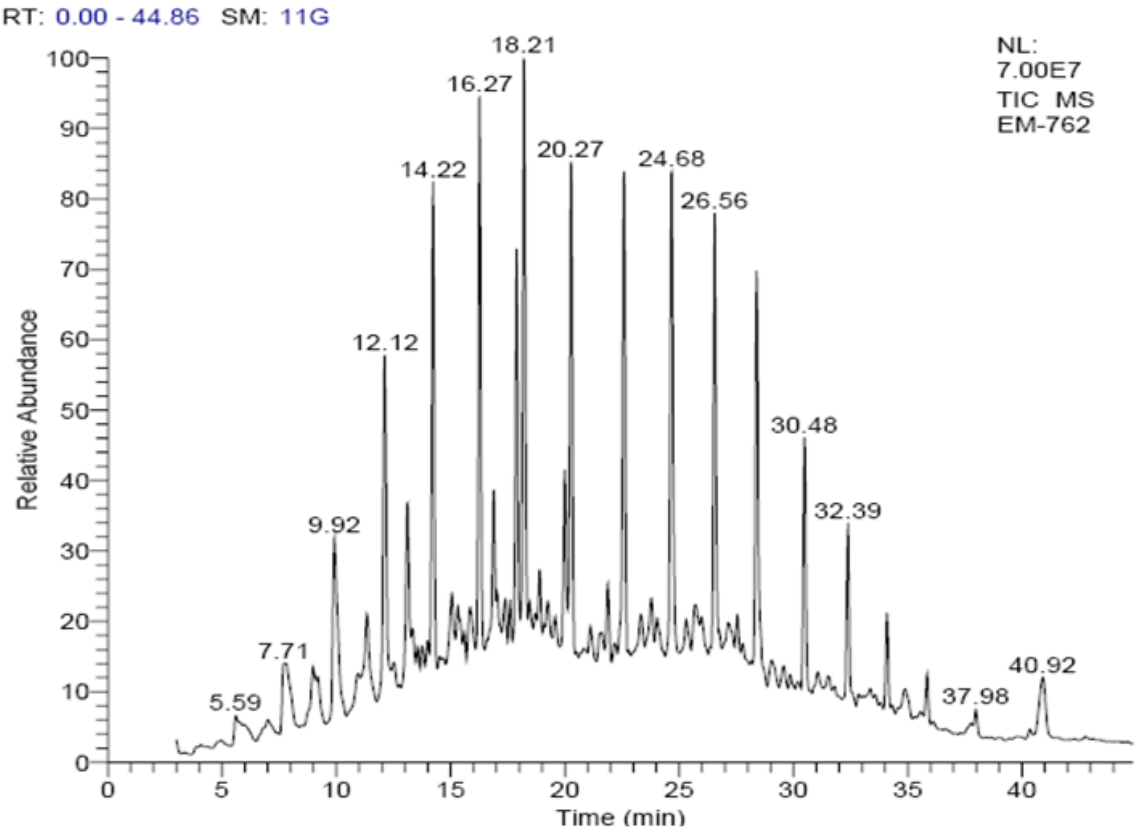
About 50 g of marine soil was mixed with 5ml of crude oil and inoculated with 0.5 ml inoculums of isolated bacteria. Uninoculated 50 g of soil sample was kept as a control. Samples were incubated for 15 day to study about biodegradation capacity of the bacteria. Visual observation of change in the colour of the soil was recorded.

RESULTS

Isolation of crude oil degrading bacteria

Marine water sample enriched in mineral salt medium had turned turbid, indicated the growth of biosurfactant producers in the sample (Plate - 1). Subculturing of inoculum yielded cream colour colonies with small, flat surface and was sub-cultured on nutrient agar plates (Plate - 2). The isolate was identified a Gram-positive, straight rod and by biochemical tests and microscopic techniques confirmed as *Pseudomonas* sp.

Graph 1: GC-MS spectrum of metabolic product produced from degradation of crude oil by *Pseudomonas* sp.



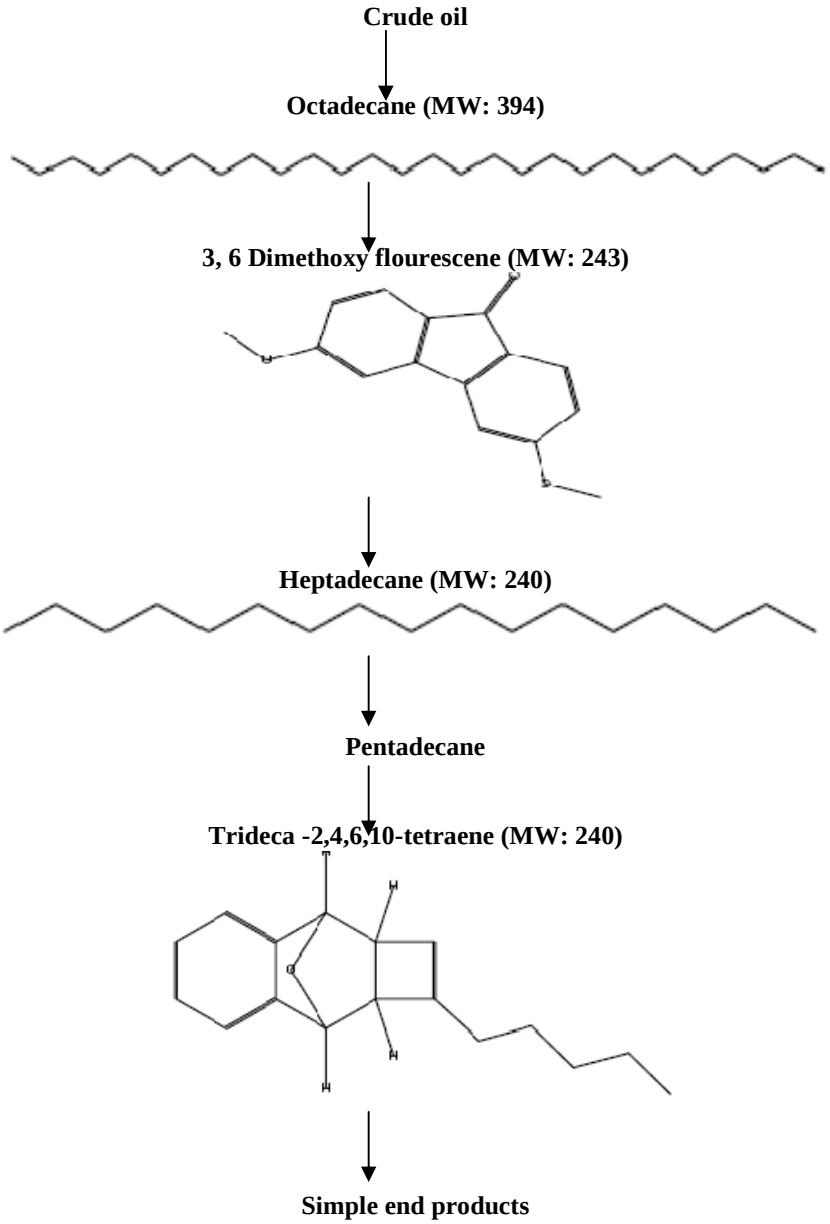
Determination of biosurfactant activities

The culture filtrate from bacterial isolate collapsed the drop within 10sec. Emulsification activity of isolate was recorded as 0.11 OD and emulsification index was recorded as 66. 6%.

Influence of physiological conditions

The biosurfactant activities were increased at increasing concentrations of salts. When salt concentrations were increased from 1% to 15 % emulsification activity gradually increased from 68% to 80% and emulsification index was increased from 0.03 to 0. 2 OD. So, the study recommends the application of marine bacterial isolate for bioremediation of oil in marine environment, which can withstand at higher salinity.

Graph 2. Schematic representation of possible degradation pathway of crude oil



When pH was changed from extreme acidic to alkaline conditions, biosurfactant activities was found unaffected by the isolate. But maximum activity recorded at pH 7. Emulsifications activities of the isolate were recorded as 56.2, 62.5, 87.5 and 56.2 per cent at pH of 4.2, 5.9, 7.6 and 9.2 respectively. So the study ensures that the isolate for bioremediation of oil in marine environment.

Degradation of crude oil

The per centage of crude oil degradation by the isolate was recorded as 94% and the following Graph 1 shows the metabolic intermediates produced during degradation of crude oil by the isolate.

Major peak compounds at the retention time of 12.12, 14. 22, 16. 27, 18. 21, 20. 27, 24. 68 and 26. 56 minutes were identified from the standard library compound as octadecane, pentadecane, heptadecane, trideca -2,4,6,10-tetraene, 3, 6 dimethoxy flourescene, nitrilomorphinan, dotriacontane, and hentriacontane. Possible degradation pathway of crude oil by the isolate is given in the graph 2. Soil bioremediation assay of crude oil showed visual colour change from black to light brown in the culture inoculated soil, and uninoculated soil colour remained unchanged (Plate - 3).

DISCUSSION

Most of the ports in the world are polluted with crude oil. Recent ship accident in August 2011, the result of a collision between two Panamanian fuel tankers has released 879 metric tonnes of oil in the coast of Mumbai. That caused destruction to marine life and incalculable loss. The numbers of oil spills from vessels in different ports are on the rise and accidents are bound to escalate in future.

The International Convention on Oil Pollution Preparedness Response and Cooperation (OPRC 90) Article 6, sets down a requirement for all operators of offshore installations, drilling rigs, terminals and ports to have in place an oil spill response system that will include contingency plans, pre-positioned response equipment, training and regular exercise, appropriate to perceived risk.

In view of the recent oil spill across the world, oil spill prevention, response and cleanup technologies have become extremely important and sought after. Immediate and complete removal of oil spill is quite necessary to avoid loss of biodiversity and cause of serious health issues to human.

Marine microbes have developed unique metabolic and physiological capabilities that not only ensure survival in marine habitats but also offer potential for production of metabolites, which would not be observed from terrestrial environment. The success of oil spill bioremediation depends on one's ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the contaminated environment.

Novel, indigenous strains of organisms are needed to be isolated and implemented for bioremediation. To satisfy this objective, in the present study mineral salt medium was used for the isolation of marine biosurfactant producing bacteria, because the MSM media contains the nutrients which influence the growth of biosurfactant producing microorganisms [10]. Then the inoculum was subcultured on nutrient agar medium. It supplies the nutrients for the growth of bacteria. The isolate was identified as *Pseudomon*as sp. Presence of biosurfactant producing *Pseudomon*as sp. in marine environment was previously reported by Akinde S. B, *et al*., in 2012 [11]. The researcher reported the presence of 53.5% of total heterotrophic bacterial in oily wastewater reservoir samples and among that species of Pseudomonas were clearly dominant in seawater and sediment of crude oil contaminated samples.

Drops collapsing test, emulsification activity, and emulsification index and oil degradation analysis tests were used to determine biosurfactant producing activity of bacteria [12]. In the drop-collapse test, the culture filtrate from bacterial isolate immediately (within 10 sec) collapsed the drop, emulsification activity was 0.11OD and emulsification index was 66.6%. Biosurfactant activities indirectly represent quantity of biosurfactant produced. Recent literature survey recorded the maximum emulsification activities of 0.08 - 0. 13OD and emulsification index was 60 – 86% [13, 14, 15]. Isolate also showed similar result that ensured the ability of the isolate to clean-up the oil polluted environment effectively.

In Arabian Sea, several metric tonnes of oil are released every year. Crude oil contains mutagenic, carcinogenic, growth inhibitory compounds, which can cause severe damage to aquatic and terrestrial environment. It is estimated that 0.08 -0.46% of the oil production is wasted into the environment, eventually causing pollution to waters and shores. Surface active agents, especially biosurfactant assists in the degradation of hydrocarbon pollutants by facilitating desorption from the soil and/or by dispersing in small droplets that are more easily attacked by microorganisms [16].

The current study aimed to degrade the crude oil using selected isolate. The per cent of crude oil degradation was recorded as 94. The success of oil spill bioremediation depends on one's ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the contaminated environment. Previous studies have proposed that loss of emulsifying activity at very acid condition as well as at high salt concentration [17].The isolated organism has degraded crude oil in extremes of pH and salt concentrations. Biosurfactant producing bacteria isolated from marine water were able to emulsify crude oil in marine water. GC-MS analysis on degraded end product of crude oil are octadecane, pentadecane, heptadecane, trideca -2,4,6,10-tetraene, 3,6 dimethoxy flourescene, nitrilomorphinan, dotriacontane, and hentriacontanes. Because molecular weights of these are lower than the crude oil. These compounds may be possible intermediates of crude oil degradation pathway. A similar study on degradation of hydrocarbons in marine environment reported the profile of unleaded gasoline in soil by differing levels of weathering, they have recorded that after 1 week, the more volatile compounds, dodecane and tetradecane were reduced by 46% and 44% respectively while naphthalene 1, 6, 7-trimethyl was reduced by less than 1% [18]. After 2 weeks, dodecane and tetradecane were reduced by 98% while naphthalene 1, 6, 7-trimethyl was reduced by 96% and after one month the more volatile compounds (dodecane and tetradecane) were undetected while the amount of naphthalene 1, 6, 7-trimethyl was further reduced by 96%. Similarly the isolate also effectively degraded the crude oil according to the proposed pathway in the marine environment.

Plate 1. Minimal media with marine water and control

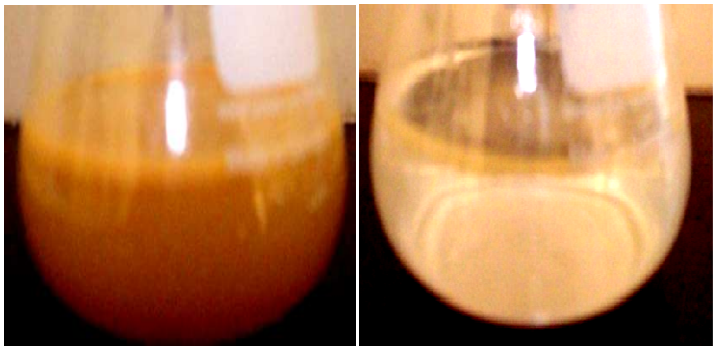


Plate 2. Isolated *Pseudomonas* sp.,

Plate 3. Marine soil samples after incubation

CONCLUSION

The marine water isolated bacteria *Pseudomonas* sp., can tolerate wide range of salt and pH, and hence can be applied for the degradation of oil spills in the marine environment. Soil bioremediation studies showed decrease in

the colour intensity of crude oil mixed soil samples because of the oil degradation capacity of bacteria. So the isolated species is recommended for bioremediation of crude oil in marine environment.

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