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Bioremediation of Coastal and Marine Pollution due to Crude Oil using a Microorganism *Bacillus subtilis*

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Abstract

Marine and coastal pollution has become a global concern in recent years due to the increase in intensity of contaminants in the marine environment. The release of crude oil in the marine environment during exploitation and transportation cause serious environmental pollution, owing to the presence of toxic organic compounds. Crude oil, which is the most predominant energy resource throughout the word is the complex mixtures of hydrocarbons including more than 70% of alkanes along with aromatics, naphthenes, and resins. The long chain alkanes present in the crude oil remains persistent due to its non-volatile nature and pose a major menace to terrestrial and marine ecosystems. Biodegradation has emerged as a potential and economical technology for the restoration of oil spilled environment. It provides efficient, economical and environment friendly solution for on-shore and off-shore oil spill remedies. The present study investigates the degradation of crude oil using a biosurfactant producing microorganism '*Bacillus subtilis'* to obtain maximum degradation. *Bacillus subtilis* isolated from polymer dump site, Chennai, India was used for the degradation of crude oil. Crude oil degradation and viscosity reduction was observed to be 80% and 60%, respectively, in 10 days. The high microbial adherence, surface tension reduction, emulsification activity, production of higher amount of biosurfactant, stability of the produced biosurfactant at extreme environment conditions, viscosity reduction and high rate of degradation indicates the potential of the microorganism for oil spill treatment.

Peer- Review under responsibility of organizing committee , IIT Madras , and International Steering Committee of APAC 2015

Keywords: Bacillus subtilis; Biodegradation; Biosurfactant; Crude oil; Marine pollution; Oil spill.

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1. Introduction

Crude oil is indispensable for world's economy and industrial growth. At present, the petroleum industry has entered into a period of modernization and transition. Due to this industrial development, pollution of seawater by crude oil has been increased and creating a serious issues (Bao et al., 2014). Marine pollution happens when unsustainable components entered into water body causing hostile organisms, diseases and eventually water becomes toxic and affects the marine and terrestrial ecosystem (Shahian et al., 2012). Major sources of marine pollution are spillovers from freight/ bulk ocean carriers. The oil spills generally during the production, transportation and storage in coastal area (Bao et al., 2014). The recent oil spills in the Gulf of Mexico, Montora, and the tanker collision in the Mumbai coast are intense examples.

Several studies have been carried out to address these issues. Microbial degradation of hydrocarbons has gained an attention due to its non-carcinogenic, non-combustible, wide spread and eco-friendly in nature as compared to other conventional methods. Biodegradation removes the petroleum hydrocarbons from the marine environment and restore the oil contaminated ecosystem (Al-Hadhrami, 1995). Generally, crude oil is a complex mixture of hydrocarbons, alkanes, aromatics, asphaltene, and resins. Various bacteria have the ability to use these hydrocarbons as their energy and carbon source and oxidize the petroleum hydrocarbons (Bao et al., 2014). Degradation is the main function of the microbes thereby making the ecosystem well-functioning (Wu et al., 2014). The variety of microbes is available in nature and ensures easy handling, performance and high operational safety for sustainable development and environmental compatibility. In addition, various metabolites such as biosurfactants, fatty acids, alcohols and solvents produced in-situ by microbes solubilize the paraffin fractions, reduces the critical micelle concentration (CMC), interfacial tension and surface tension, improve the remediation (Banat, 1995). Biosurfactants develops the ability of the microbial cells to grow on the hydrophobic (hydrocarbon) substrates and increase their bioavailability (Rosenberg, 1984).

The focus of this work is to study the performance of *Bacillus subtilis* to figure out the changes in the physico-chemical properties for the degradation of waxy crude oil. The potential of *Bacillus subtilis* for oil spill remediation is shown by analyzing the properties, such as, growth of the bacteria on waxy crude oil, interaction of bacteria and the crude oil, biosurfactant production, surface tension, emulsification activity, degradation and viscosity reduction of waxy crude oil.

2. Materials and methods

2.1. Waxy crude oil

The waxy crude oil was procured from Mehsana oil-field asset, Gujarat, India. Mass fractions of saturates, aromatics, resins and asphaltenes (SARA) in the waxy crude oil used in the experiments are given in Table 1.

Table 1. SARA distribution in crude oil

Parameter	Abundance % (w/w)
Saturates (S)	67.75±3.40
Aromatics (A)	22.66±1.10
Resin (R)	8.87±0.45
Asphaltene (A)	0.72±0.04

2.2. Microorganism and culture condition

Bacillus subtilis YB7 earlier isolated by Arutchelvi et al. (2009) from the polymer dump site, Chennai, India was used for the present work. An aliquot of 1 mL of inoculum was transferred into a production medium

along with 2 % (v/v) of waxy crude oil in a 250 mL conical flask and was incubated aerobically in a shaker (Orbitek, Scigenics Biotech, India) at a speed of 180 rpm. The incubation was performed at a room temperature. The production medium contained 0.3 g potassium dihydrogen phosphate, 0.6 g disodium hydrogen phosphate, 0.2 g ammonium chloride, 0.5 g sodium chloride, 0.8 g glucose, 0.01 g magnesium sulfate hepta hydrate and 200 mL distilled water. Chemicals with a purity of about 99% were used for the experiments. A flask without crude oil served as a control sample. The samples were collected from the flasks at regular time intervals for determining the growth, surface properties, and viscosity measurement and degradation analysis. Figure 1 shows the various equipments used to infer the biodegradation of waxy crude oil.

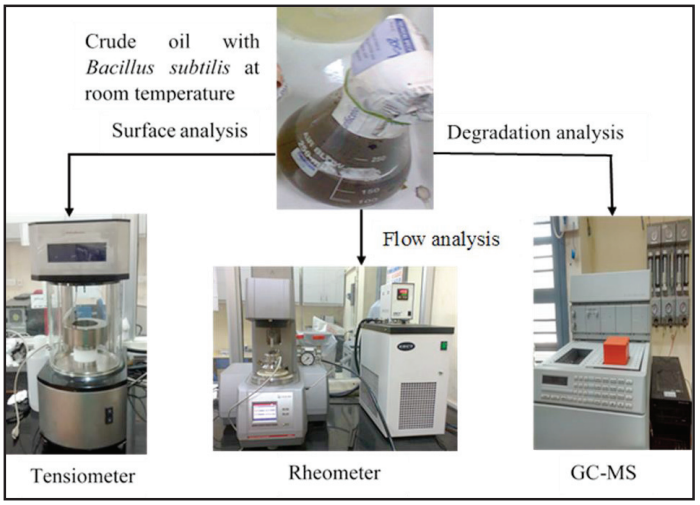

Fig. 1. Outline of the biodegradation of crude oil.

2.3. Growth analysis

The growth rate of microorganisms was measured using biomass dry weight and colony forming units (CFU). Biomass dry weight was measured by the procedure reported by Arjol et al. (2014) and CFU was estimated according to the method reported by Tehrani and Herfatmanesh (2015).

2.4. Extraction of Biosurfactant

The culture supernatant after discarding the biomass was by precipitated using 6 N HCl, and the biosurfactant was extracted using dichloromethane. The lower phase was collected, concentrated using a rotary vacuum evaporator (Buchi rota vapor, Switzerland), and weighed after drying.

2.5. Surface tension reduction

The bacterial cells were removed from the culture by centrifuging (Model 5804 R, Eppendorf, India) for 10 min at 10,000 x g and the biomass free culture supernatant was used for the measurement of surface tension using a tensiometer (model DAC11EA, Data physics, US). The instrument was calibrated against millipore water. Surface tension was determined with the aid of an optimally wettable platinum plate suspended from a precision scale. The platinum plate was oriented perpendicular to the interface of air, and culture supernatant for the attainment of equilibrium. The force exerted on the air–liquid interface is recorded and the equilibrium surface tension was observed using SCAT (Static contact angle tensiometer) software.

2.6. Emulsification Activity

Emulsification activity was measured according to the procedure reported by Cooper and Goldenberg (1987). Kerosene (2 ml) and equal volume of culture broth is taken in the flat bottomed test tube. The mixture was vortexed at high speed for 2 min using agitator and left undisturbed for 24 hour and the height of the emulsion layer and the total mixture was noted. Emulsification activity is given as the ratio of the height of the emulsion layer and the total height of the mixture.

2.7. Bacterial adherence to hydrocarbons (BATH) assay

The cell surface hydrophobicity (CSH) of the strains were determined according to a reported procedure reported by Ron and Rosenberg (2002). Bacterial cells were removed by centrifugation for 20 min at 12,000 × g and washed with phosphate buffer to separate the extracellular substances. The removed bacterial cell suspension (4 mL) was mixed with 1 mL of hexadecane in a test tube and vortexed (Spinix vortex shaker, Tarsons, India) for 5 min, and remained undisturbed for 30 min to permit the separation of phases. The optical density of the aqueous layer was measured at a wavelength of 600 nm using a UV spectrophotometer (Model V 550, Jasco, Germany). The CSH of the bacteria was calculated from the optical density of the aqueous phase before (a) and after (b) separation as given below,

$$CSH = [1 - \frac{a}{b}] \times 100\% \quad \textbf{(1)}$$

2.8. Viscosity measurement

Viscosity of waxy crude oil throughout the period of degradation was measured by a rheometer (Anton Parr Modular Compact Rheometer (MCR 52), Austria) fitted with double gap geometry (concentric cylinder system). The bacterial cells were centrifuged at room temperature for 10 min at 10000 x g and the bacteria free culture supernatant was tested for the viscosity reduction. The rheometer was attached with the water bath to study the variation of viscosity as a function of temperature.

2.9. Extraction of residual hydrocarbons

The degraded waxy crude oil from the culture supernatant was extracted using ethyl acetate and the solvent phase was evaporated to dryness using a rotary vacuum evaporator (R-210 rotavapor, Buchi, Switzerland). The dried extract was then concentrated using ethyl acetate. The concentrated extract was analyzed by Gas chromatography-mass spectroscopy (GC-MS) (JEOL, USA). The GC-MS instrument was functioned in the selective ion monitoring mode. The instrument was fitted with HP 5MS capillary column of medium polarity. Helium was used as a carrier gas at a flow rate of 1 mL/min. The sample (1 μL) was injected at the flow rate of 4 mL/min and the purge flow rate was 3 mL/min. The injector and interface temperatures were maintained at 220 and 350˚C, respectively.

3. Results and discussions

3.1. Bacterial growth

Growth of the bacteria is the primary requirement to carry out biodegradation. The microbial population directly accounts the production of biosurfactant accountable for aiding the hydrocarbon degradation. Figure 2 shows the growth of *Bacillus subtilis* on waxy crude oil at room temperature. The biomass dry weight reaches 2.11 g/L when the CFU reaches the maximum, and the steady state was achieved further. It is inferred that the growth

was increased upto day 12 and maintained till day 15 and decreased thereafter. CFU attains the maximum value of 28×10^6/mL of the bacterial culture.

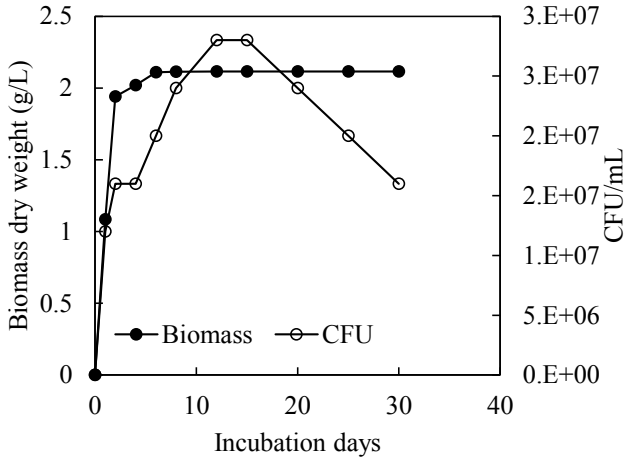

Fig. 2. Growth of *Bacillus subtilis* on waxy crude oil

3.2. Production and properties of biosurfactant

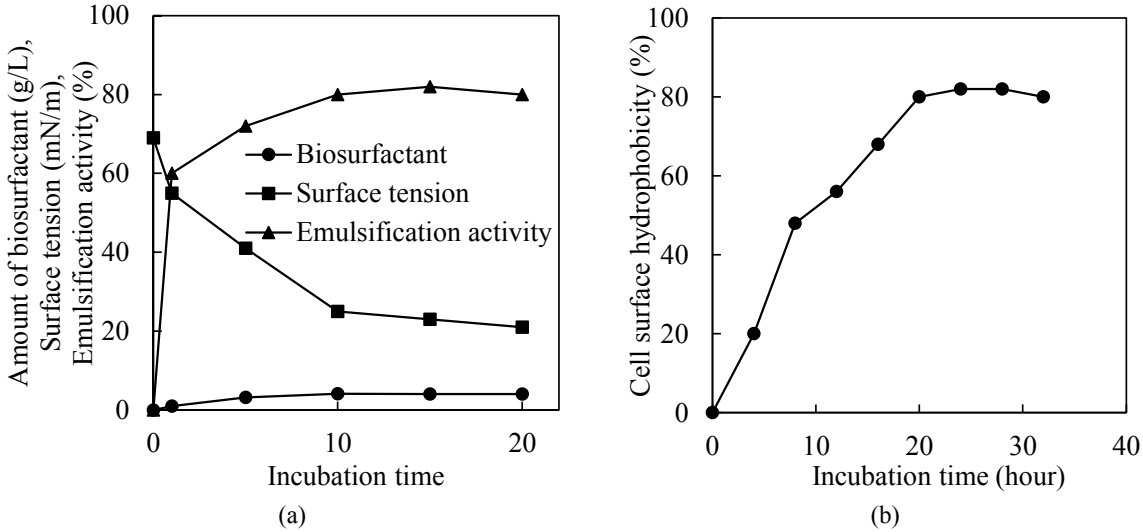

Fig. 3. (a) Production and properties of biosurfactant; (b) Bacterial adherence to waxy crude oil.

The use of biosurfactants has been observed to improve the crude oil degradation (Batista et al., 2007). Biosurfactants emulsifies hydrocarbons present in the crude oil, and increases the water solubility, decreases surface tension and increases the displacement of oily substances from the porous medium (Banat, 1995). Figure 3a shows the production of biosurfactant as a function of time. The biosurfactant production was observed to be 0.98 g/L in 1 day, and maximum 4.1 g/L in 10 days. The production of biosurfactants was also confirmed by the observed reduction in surface tension and increase in emulsification in waxy crude oil culture. Surface tension is reduced to 25 mN/m in 10 days and 21 mN/m in 20 days and there was no further change (Figure 3a).

Emulsification activity was conducted to check the interaction of biosurfactant with crude oil and other hydrocarbons. Typically, microbial degradation increases with increase in the emulsification activity since the latter provides better contact with the crude oil and water interface. In 1 day**,** 60% of emulsification was observed, while maximum 82% was achieved in 15 days (Figure 3a).

Variation of CSH with incubation period is shown in Figure 3b. The CSH was observed to be 82% in one day and subsequently reached the steady state. CSH is an indication of the potential of bacteria to grow on the hydrophobic substrates (Chakraborty et al., 2010). Biodegradation is usually hindered due to inadequate bioavailability of the hydrophobic substances to microorganisms. The biosurfactant produced by the bacteria behaves as a bond between bacteria and crude oil, and supports connection of the microbe to the hydrocarbon substrate, and increases CSH (Xia et al., 2013). The experimental results reveal that the biosurfactant produced in the presence of waxy crude oil can enhance the interaction between bacterial cell and crude oil, thereby speeding up the degradation.

3.3. Viscosity reduction and degradation analysis of waxy crude oil

Figure 4 shows the viscosity reduction of waxy crude oil during the degradation process by *Bacillus subtilis* at room temperature. It is observed that the treatment of waxy crude oil with the bacteria enhanced the fluidity of crude oil. The viscosity reduction is found to be around 25% in one day and reached about 60% in 8 days, and attained a steady state thereafter. The more reduction of viscosity was observed between days 4 to 8. This is due to the reason that the bacteria has attained the maximum growth during that period.

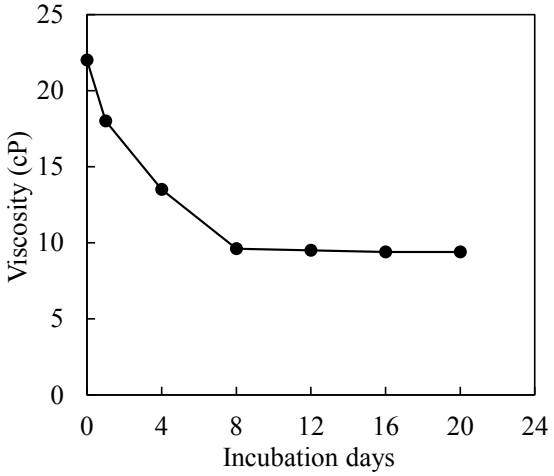

Fig. 4. Viscosity reduction of waxy crude oil throughout the degradation.

Figure 5 shows the waxy crude oil degradation and bacterial count as a function of time. It is observed that the degradation rate of crude oil increased along with the increase in incubation time. Degradation of waxy crude oil was observed to be 62% in 1 day and 80% after 10 days, and did not change thereafter. The degradation rate grew rapidly between day 1 and 10. However, the degradation rate did not increase after day 10. At day 10, the bacterial count has reached the maximum of 28×10^6 CFU/mL, and the same was maintained for a week and decreased thereafter. The degradation of crude oil follows the same trend as microbial growth. According to Bordenave et al. (2007) the individual bacteria could metabolize the crude oil only to a limited hydrocarbons and the complete biodegradation requires mixture of different bacterial groups. In contrast to this, the results reveal that the individual *Bacillus subtilis* could degrade wide range of hydrocarbons present in the crude oil.

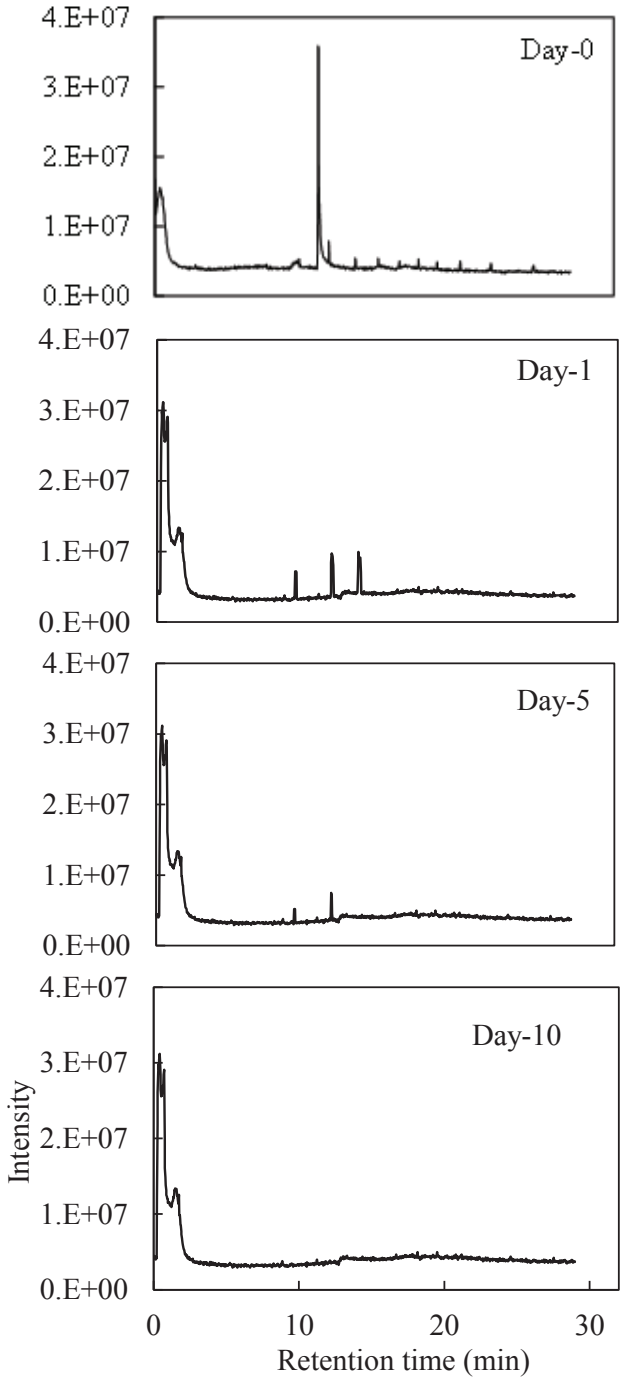

Fig. 5. Gas chromatograms before and after degradation of waxy crude oil using *Bacillus subtilis*.

4. Conclusion

With the knowledge of degradation of crude oil using bacteria in the laboratory scale experiments, it would be possible to develop approaches for using bacteria for the removal of hydrocarbons from polluted water. All of the results demonstrated that the bacteria selected in our study is favorable for the bioremediation of sites contaminated with petroleum hydrocarbons. The chemical composition of the crude oil before and after bacterial growth by GC-MS has proved that the components of the crude oil have been utilized as carbon and energy sources, and is a good indication for bioremediation of crude oil suitable for coastal and marine environment.

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Reference

Al-Hadhrami, M.N., Scott, L.H.M., Fisher, P.J., 1995. Bacterial survival and n-alkane degradation within Omani crude oil and a mousse. Mar. Pollut. Bull. 30, 403-408.

Arjol, I.M., Llacuna, J.L., Manresa, A., 2014. Yield and kinetic constants estimation in the production of hydroxy fatty acids from oleic acid in a bioreactor by *Pseudomonas aeruginosa* 42A2. Appl. Microbiol. Biotechnol. 98, 9609-9621.

Arutchelvi, J., Bhaduri, S., Uppara, P.V., Doble, M., 2009. Production and characterization of biosurfactant from *Bacillus subtilis* YB7. J. Appl. Sci. 9, 3151.

Banat, I.M., 1995. Biosurfactants production and use in microbial enhanced oil recovery and pollution remediation: A review. Bioresource Technol. 51, 1–12.

Bao, M., Pi, Y., Wang, L., Sun, P., Lia Y., Cao, L. 2014. Lipopeptide biosurfactant production bacteria *Acinetobacter* sp. D3-2 and its biodegradation of crude oil. Environ. Sci. Processes Impacts 2014, 1-7.

Batista, S.B., Mounteer, A., Amorim, F.R., Totola, M.R., 2006. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. Bioresour. Technol. 97, 868–875.

Bordenave, S., Goni-urriza, M.S., Caumette, P., Duran, R., 2007. Effects of waxy fuel oil on the bacterial community structure of a pristine microbial mat. Appl. Environ. Microbiol. 73, 6089–6097.

Chakraborty, S., Mukherji, S., Mukherji, S., 2010. Surface hydrophobicity of petroleum hydrocarbon degrading *Burkholderia* strains and their interactions with NAPLs and surfaces. Colloids Surf: B: Biointerfaces 78, 101-108.

Cooper, D.G., Goldenberg, B.G., 1987. Surface-active agents from two *Bacillus* species. Appl. Environ. Microbiol. 53, 224-229

Ron, E.Z., Rosenberg, E., 2002. Biosurfactants and oil bioremediation, Curr. Opin. Biotechnol. 13, 249–252.

Rosenberg, M., 1984. Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol. Lett. 22, 289–295.

Shahian, M.H., Emtiazi, G., Cappello, S., 2012. Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. Mar. Pollut. Bull. 64, 7–12.

Tehrani, D.M., Herfatmanesh, A., 2015. Biodegradation of aliphatic and aromatic fractions of waxy crude oil–contaminated soil: A pilot study. Biorem. J. 11, 71–76.

Wu, B., Lan, T., Lu D., Liu, Z., 2014. Ecological and enzymatic responses to petroleum contamination. Environ. Sci. Processes Impacts 16, 1501-1509.

Xia, W.J., Luo, Z.B., Dong, H.P., Yu, L., 2013. Studies of biosurfactant for microbial enhanced oil recovery by using bacteria isolated from the formation water of a petroleum reservoir. Petrol. Sci. Technol. 31, 2311-2317.