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STATISTICAL ANALYSIS OF HYDROCARBON DEGRADATION IN THE PRESENCE OF BIOSURFACTANT PRODUCED BY *BACILLUS THURINGIENSIS* AND *PROTEUS VULGARIS*

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ABSTRACT

The extensive applications of biosurfactant due to their diverse properties like specificity, low toxicity compared to chemical surfactants and stability in wide range of environmental conditions has increased its importance. In the present study, solubilization of anthracene by the biosurfactant produced by *Bacillus thuringiensis* and *Proteus vulgaris* was found to be high when compared to the chemical surfactant - SDS added with anthracene. Statistical analysis was used to confirm the studies performed for 24 hours in the presence of biosurfactant produced by *Bacillus thuringiensis* and *Proteus vulgaris.* Results of GC-MS analysis revealed that the solubilization of diesel by *Bacillus thuringiensis* and *Proteus vulgaris*.

KEYWORDS

Anthracene, Biosurfactant, Dissolution, *Bacillus thuringiensis*, *Proteus vulgaris*, Solubilization, GC-MS and Regression analysis.

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INTRODUCTON

Oil pollution is one of the environmental problems occurring in the natural environment due to the exploitation of the marine environment by man in different ways[1-2]. Report from had stated the reduction in the fresh and marine environment resources due to the oil spillages[3-5]. It was reported that the effects of crude oil such as, caused stress and resulting in reduced activity and suffocation and eventually leading to fish kill due to the higher concentration of WSF and crude oil[6-9]. Microorganisms utilize these hydrocarbons as carbon source for energy and metabolic activities

depending on the chemical structure of the petroleum mixture[10-11].

Microbiological degradation of hydrocarbons have been studied extensively both in aerobic and anaerobic environments with diverse microbial populations to remove pollutants from different contaminated sites in less time. Biodegradation by microorganisms are done by the mineralization of the pollutants leading to the formation of carbon dioxide, water and biomass[12-17]. Biosurfactants have already been known to be used as hydrocarbon dissolving agents and their applications as alternative to chemical surfactants have increased their popularity[18]. They are specifically active, biodegradable, non-toxic and effective at extreme environmental conditions like pH, temperature and salinity which have made it important for industrial applications also[19]. They have wide variety of applications because of its different properties like emulsification, wetting, solubilization etc[20]. The main objective of this chapter was to determine hydrocarbon degradation in the presence of biosurfactant produced by environmental bacterial isolates.

MATERIAL AND METHODS

Media preparation

Nutrient broth medium was composed of (g per 1000ml of distilled water); peptone (10); NaCl (5); meat extract (10). Medium pH was 7.2 ± 0.2 and the medium was autoclaved at 121^{o}C for 20 min at 15 lbs pressure.

Microorganism

The microorganism used in this study *Bacillus thuringiensis* and *Proteus vulgaris*. The sequence data were submitted in Gen Bank with the accession number of KJ372208 and KP289283 respectively. These strains had been qualitatively analyzed for surface active properties.

Inoculum

The strains that were maintained in agar slants were inoculated in a 10ml of nutrient broth. After 24 hours of growth, 1ml of inoculum was transferred to each of the experimental flask.

Biosurfactant extraction and purification

Bacterial culture broth was centrifuged at 10,000rpm, for 20 min at 4°C. The pellet was discarded and the cell-free broth was adjusted to pH 2 using 1N H_2SO_4[21]. It was maintained at 4 °C overnight, and then centrifuged at 10,000rpm for 10 min. The pellet was called as acid precipitate and weight was expressed as mg/ml (w/v). The acid precipitate was neutralized using 1N NaOH before the experiment

GC - MS

Sample was injected (1µl) into Perkin Elmer (Massachusetts, USA) gas chromatograph (GC) model Clarus 680 equipped with a capillary model Clarus 600 (EI) set to scan from 50 to 600Da. The capillary column was an Elite-5MS (30m x 250µm). The oven temperature was programmed from 60ºC to 300ºC at 10ºC / min. The temperature of the injector port was 250ºC and the detector transfer line temperature was 230ºC. The carrier gas was He at a flow rate of 1ml min-1 and a split ratio of 10:1. GC-MS analysis of partially purified biosurfactant in methanol from both the strains was also performed.

Solubilisation of crystalline anthracene

Willumsen and Karlson (1997) developed an assay based on the solubilisation of crystalline anthracene[22]. This screening method is based on the solubilisation of a highly hydrophobic, crystalline compound, anthracene by the biosurfactant[23]. Therefore, crystalline anthracene was added to the culture supernatant and incubated on a shaker at 25°C for 24 hrs. The concentration of the dissolved hydrophobic anthracene was measured photometrically at 354nm and correlated to the production of biosurfactant.

After centrifugation, supernatant of 72 hours incubated culture was collected. Three flasks were maintained for the experiment. Control flask contains culture supernatant with no addition of crystalline anthracene. Second flask was with culture supernatant and addition of crystalline anthracene and third flask was kept as positive control having culture supernatant with anthracene and chemical surfactant i.e. SDS. The different concentrations of crystalline anthracene used for the

study were 0.5, 1.0, 1.5 and 2.0g in 25ml of culture supernatant in second and third flasks.

Laboratory design

Acid precipitate of biosurfactant was obtained from each bacterial strain by using the Cooper method[21]. The acid precipitate was neutralized using 1M NaOH. Control was maintained with different amount of diesel added i.e. 2, 4, 6, 8, 10ml in separate conical flask and rest making up it to 100ml with distilled water. The test was added with the diesel (2, 4, 6, 8, 10ml) and additionally adding biosurfactant i.e. 10, 20, 30, 40 and 50ml and rest was made up to 100ml with distilled water and kept in a rotary shaker for 24 hours.

GC-MS analysis was performed for the confirmation of dissolution of oil into the distilled water for both control and test. After 24 hours of experiment, the distilled water was precipitated by using 1N HCL to pH 2 and then extracted with hexane.

Statistical analysis

24 hours degradation of diesel in the presence of biosurfactant produced by *Bacillus thuringiensis* and *Proteus vulgaris* was compared using regression statistical tool.

RESULTS AND DISCUSSION

Microorganisms have already been reported to be capable of degrading hydrocarbons like alkanes, monocyclic hydrocarbons, aromatic, resins and asphaltenes[12,24-26]. Biosurfactants by reducing surface tension and interfacial tension make the water insoluble part to be dissolved[27-28] and to be available to the microorganisms as carbon sources[29]. Polycyclic aromatic hydrocarbons (PAH) including naphthalene, anthracene, phenanthrene have already been studied for their ability to be degraded by the bacteria[23,30-32]. Solubilization of anthracene by the biosurfactant produced by *Bacillus thuringiensis* and *Proteus vulgaris* has been shown in Figure No.1 and Figure No.2, respectively.

According to the increasing amount of anthracene added to the biosurfactant, there was an increase in the O.D, which was higher than the chemical surfactant – SDS added with anthracene. In both the flasks i.e. one with anthracene added with biosurfactant produced by the microorganism and the other with chemical surfactant showed an increase in the O.D than the control flask which was with no addition of anthracene. In comparison to both the strains, *Proteus vulgaris* (1.1) showed increase in the O.D. than *Bacillus thuringiensis* (0.9) at 354nm. The increase in the O.D. of biosurfactant added with anthracene correlated to the biodegradation and solubilisation of anthracene in the presence of biosurfactant as explained by Garcia-Junco *et al*[33].

Laboratory design

Hydrocarbons are mainly nonaqueous soluble or water insoluble[34]. This limits its solubilization and interaction with microorganism that usually take up the substrates dissolved in water[35]. Biosurfactants produced by the microorganism is amphiphilic i.e. have hydrophobic and hydrophilic part which helps in the reduction of the surface tension and interfacial tension between two phases which helps in the solubilization. The addition of 20ml of partially purified biosurfactant with 30ml diesel was kept in the conical flask in an orbital shaker for 24 hours. This results in dissolution and solubilization of diesel in water which was confirmed by GC-MS analysis and also visible measurement of diesel. Barkay e*t al* reported the solubilization of PAH, phenanthrene (PHE), and fluoranthene (FLA) by alasan produced by *Acinetobacter radioresistens* and with increasing concentrations (50 to 500µg/ml) of alasan, there was an increased solubility of PHE and FLA and mineralization of PAH[36].

Control added with 30ml diesel without any biosurfactant showed very less solubilization of diesel (Figure No.3) and its mineralization in water. This suggests the occurrence of mineralization by mechanical way in natural environment. The GC chromatograms of the solubilization of 30ml of diesel by 20ml of biosurfactant from *Bacillus thuringiensis* and *Proteus vulgaris* were presented in Figure No.4 and Figure No.5, respectively. Addition of biosurfactant from *Bacillus thuringiensis* (Figure No.4) helped to produce a good solubilization of diesel compared to the

control. The increased height of the peak (Figure No.4 and Figure No.5) indicated the increased solubilisation of diesel by biosurfactant produced by both *Bacillus thuringiensis* and *Proteus vulgaris.*

Similar observation was explained by Rodrigues *et al* and they stated that biosurfactant helped in the solubilisation of water insoluble substrates[37]. Direct measurement indicated about 2mm decrease in the height of the flask added with biosurfactant from *Bacillus thuringiensis*. According to the number of peaks present in the chromatogram, the dissociation of hydrocarbon compounds into smaller compounds by the biosurfactant was understood and therefore diesel has been solubilized by the biosurfactant and also degraded by *Bacillus thuringiensis*.

The biosurfactant of *Proteus vulgaris* showed good solubilization compared to *Bacillus thuringiensis* and control (Figure No.5) but was very slow in the dissociation of the hydrocarbon compounds when compared to *Bacillus thuringiensis*. *Proteus vulgaris* might not be able to degrade as *Bacillus thuringiensis* even though there was an increase in the height of the peak because of solubilisation (Figure No.5). One mm decrease in the height of the flask added with biosurfactant from *Proteus vulgaris* was seen. This observation indicated that the biosurfactant produced by *Proteus vulgaris* with the addition of beef extract (1.5%) had increased the quality and quantity of biosurfactant.

But that might not be adequate for the dissociation of the hydrocarbon compounds present in diesel compared to *Bacillus thuringiensis*. Based on these results, it could be concluded that *Proteus vulgaris* might help in the bioavailability of carbon sources but was a gradual degrader of the hydrocarbons. The regression analysis of the biosurfactant, diesel and solubilization (in cm) by *Bacillus thuringiensis* and *Proteus vulgaris* has shown that association of different biosurfactant with diesel can bring about much difference in the solubilization rate in 24 hours.

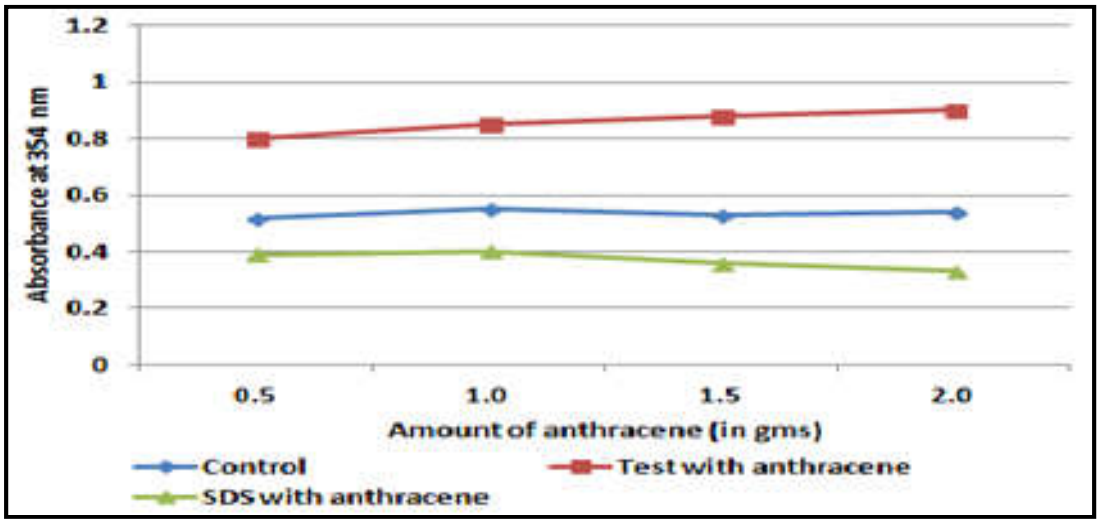

Figure No.1: Solubilization of anthracene in the presence of biosurfactant produced by *Bacillus thuringiensis*

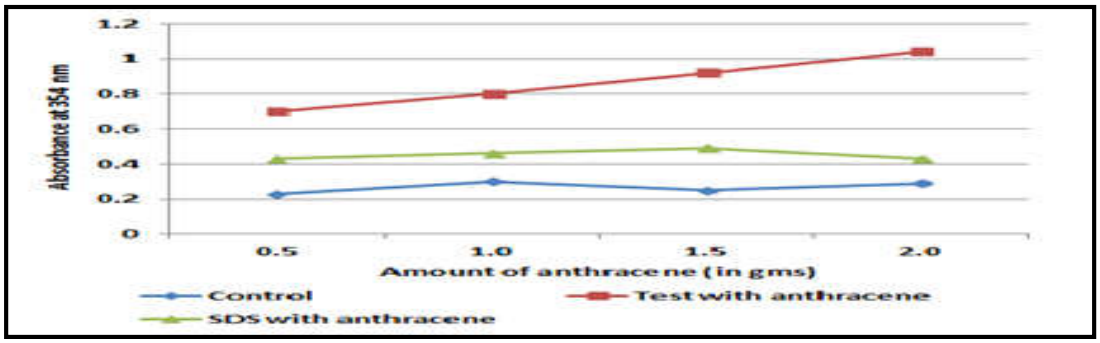

Figure No.2: Solubilization of anthracene in the presence of biosurfactant produced by *Proteus vulgaris*

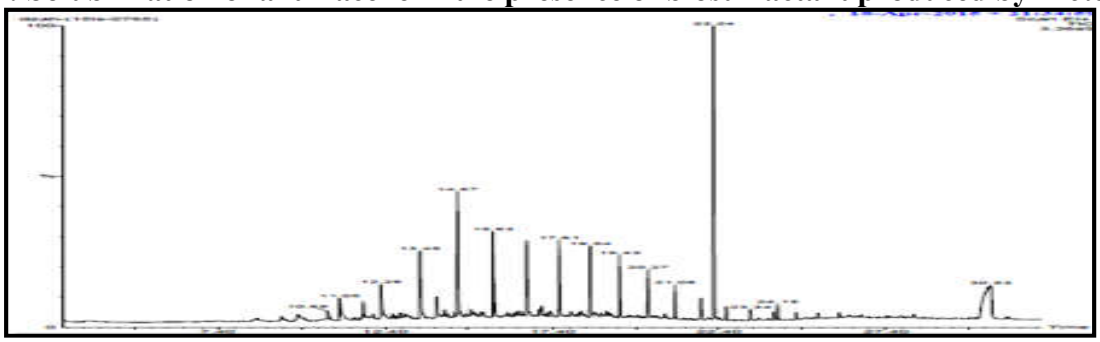

Figure No.3: GC-MS chromatogram of laboratory experiment conducted for 24 hours in control with no addition of biosurfactant

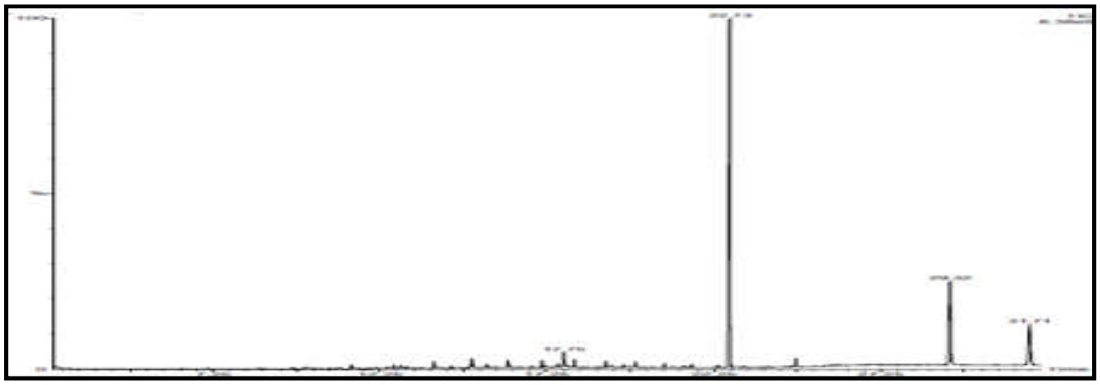

Figure No.4: GC-MS chromatogram of laboratory experiment after 24 hours of incubation in the presence of the biosurfactant produced by *Bacillus thuringiensis*

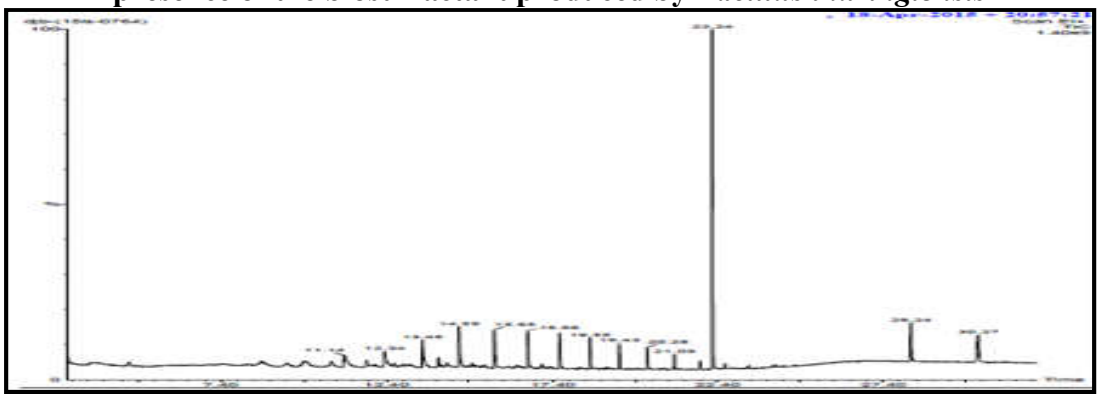

Figure No.5: GC-MS chromatogram of laboratory experiment after 24 hours of incubation by the biosurfactant produced by *Proteus vulgaris*

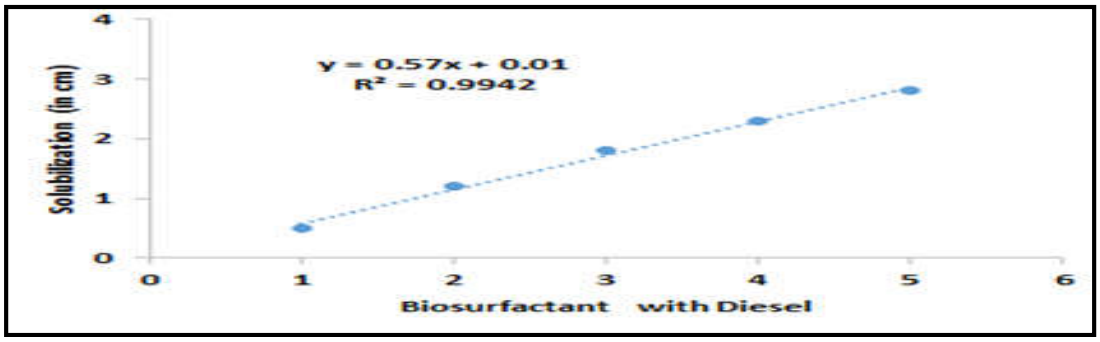

Figure No.6: Regression analysis of laboratory experiment after 24 hours by the biosurfactant produced by *Bacillus thuringiensis*

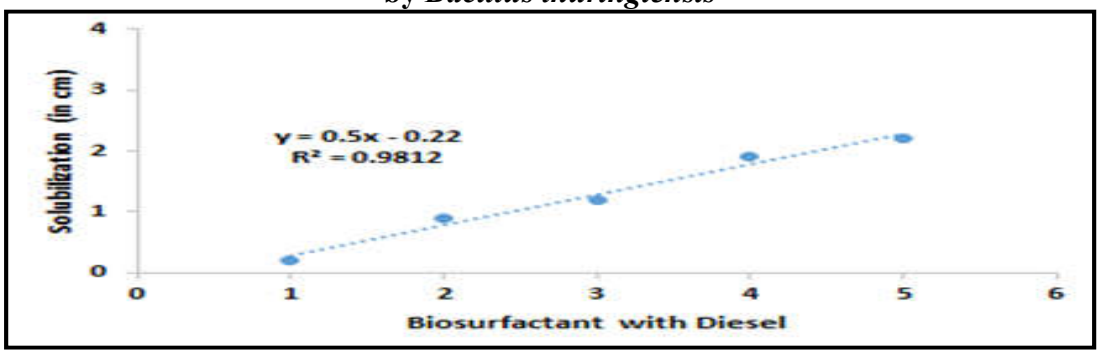

Figure No.7: Regression analysis of laboratory experiment after 24 hours by the biosurfactant produced by *Proteus Vulgaris*

CONCLUSION

Solubilization of anthracene by the cell supernatant having biosurfactant either from *Bacillus thuringiensis* and *Proteus vulgaris* was maximum compared to the chemical surfactant – (SDS) added with anthracene. Studies performed for 24 hours with the help of biosurfactant were found that the biosurfactants assisted in faster dissolution of oil by *Bacillus thuringiensis* and *Proteus vulgaris* into the water by decreasing the level of oil. GC-MS analysis and regression analysis of the laboratory experiment indicated that *Bacillus thuringiensis* and *Proteus vulgaris* were able to solubilize the diesel well, but *Bacillus thuringiensis* was found to be more efficient than *Proteus vulgaris*.

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CONFLICT OF INTEREST

We declare that we have no conflict of Interest.

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