Article

Biosurfactant mediated degradation of petroleum hydrocarbons by marine bacterial isolates

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Abstract
Petroleum hydrocarbons (PHCs) are one of the most hazardous pollutants affects human and animal health and alters the nature of the ecosystem. The aim of this study is to isolate the PHCs degrading bacteria and to assess its degrading potential. Used engine oil (UEO) was used as a sole carbon source for the growth of the bacteria isolated from marine soil sample. Biodegradation efficiency of bacterial isolates on UEO was determined by gravimetric analysis. Biosurfactant production by the PHC degrading isolate was assessed by drop collapse test, oil displacement assay and emulsification index measurement. The isolate was identified by morphological and biochemical characterization. Salt tolerance of the isolate on UEO biodegradation was studied by gravimetric analysis. Gravimetric analysis revealed that the isolates VITSK5 and VITSK6 showed 51.35% and 63.5% degradation of UEO respectively. The isolate VITSK6 showed comparatively higher biosurfactant production than VITSK5. VITSK5 showed increased UEO degrading efficiency with increase in salt concentration. Biofilm formation test showed that VITSK5 is a moderate biofilm producer and VITSK6 is a weak biofilm producer.

Keywords bioremediation; biosurfactant; marine bacteria; petroleum hydrocarbons; salinity.

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1 Introduction

PHCs based pollutants are generated in large scale due to extensive usage of petroleum products in our daily life. Used engine oil has high amount of polyaromatic hydrocarbons (PAHs) and pollutes the environment by accidental spillage and improper discharge (Bagherzadeh-Namazi et al., 2008). Bioremediation is an ecofriendly process, which uses microorganisms for cleaning pollutants. Petroleum products are frequently used as a primary energy resources of biological origin globally, mostly generated from crude oil. Accidental oil spills and leaks are a common problem that arises during the extraction, processing, transportation and storage of petroleum and its byproducts. These hydrocarbon contaminations pose a threat to plant health in

addition to having strong immunotoxic, carcinogenic, and mutagenic effects on human and animal health (Lin et al., 2009). Our ecosystem more often gets contaminated by the additional hydrocarbons found in oil, such as methyl tert-butyl ether and BTEX compounds (Hossain et al., 2022). The improper disposal of used engine oil into open environment is causing an increase in environmental pollution. This typically leads to extreme ecological disruption in both the biotic and abiotic environment (Lin et al.2009). Traditionally physical, chemical, and thermal techniques are used to clean up the oil-contaminated areas. But these methods are quite expensive and necessitate site rehabilitation. As a result, urgent reclamation of oil-polluted lands requires the promotion of environmentally sustainable techniques (Prathyusha et al., 2016).

Bioremediation uses living organisms to break down harmful chemicals and compounds into less toxic or harmless products It is a technique which involves the use of organisms to remove or degrade the pollutants from the polluted area. Bioremediation in a marine environment is challenging due to their high saline conditions. Isolation of microorganisms with potential to degrade petroleum hydrocarbons from marine environment helps in utilizing these microbes for biodegradation. Several families of marine bacteria, such as *Alkanivorax, Pseudomonas, Oceanospiralles* and *Marinobacter* species can consume and degrade petroleum-derived chemicals. *Bacillus anthracis*, *Bacillus cereus*, *Achromobacter* sp., *Aspergillus lentulus* and *Rhizopus arrhizus* contains PMO and AlkB genes responsible for degrading crude oil and hydrocarbon fractions (Mohammed et al., 2023) These microorganisms can degrade oil by producing biosurfactants and capable of degrading oil even at higher salt concentrations(Roy et al., 2014). *Lentzea albidocapillata* isolated from pesticide rich soil showed highest minerilisation of petroleum oil (Akinsanola et al. 2024).

Biosurfactants are surface- and interfacial-tension-reducing active substances that are generated by microbial cells internally or externally. In comparison to synthetic surfactants, microbial surfactants have a number of benefits including low toxicity, high biodegradability, and the ability to function at high salinity and pH levels. A variety of microbes that degrade petroleum hydrocarbons can produce biosurfactants and emulsifiers, which help scarce or insoluble substrates, become more soluble to use it as a source of carbon. It was reported that *Pseudomonas aeruginosa* SU-1utilizesUEOas a sole carbon source for their growth (Ganesan et al., 2022). More metals and carcinogenic PAHs are present in UEO and lubricating oils used in Car engines made up of additives and long-chain saturated hydrocarbons (base oil) and hence, UEO degradation remains to be a difficult process (Bagherzdeh-Namazi et al., 2008). The aim of this study is to isolate and to assess the UEO degrading potential of bacteria from marine soil samples.

2 Materials and Methods

2.1 Sample collection

Marine soil sample was collected from the coastal areas of Rameshwaram, Tamil Nadu, India. It was collected in a sterile air tight container and transported to the laboratory immediately and stored at 4^{o}C.

2.2 Used engine oil

Used engine oil was collected in a sterile screw-capped glass bottles from a vehicle service shops around Vellore, Tamil Nadu, India. It was subjected to tyndallization by placing it in a water bath at 80^{o}C for 3 days. Used engine oil was stored at 4^{o}C for further use.

Enrichment method was used for isolation of UEO degrading bacteria. Marine soil (5 g) and 1 ml of UEO was added to 100 ml of Bushnell Hass (BH) broth and incubated for 7 days at 150 rpm. 1 ml of culture was further added to 100 ml of BH broth and incubated at room temperature (RT) at 150 rpm for 7 days. Serial dilution on BH Agar was performed to isolate the microorganisms from the enrichment culture. For serial dilution, 1 ml of culture from the enrichment culture was transferred to test tube containing 9 ml of sterile distilled water and dilutions up to 10^{-6} were made. 100 µl of each dilution (10^{-4}, 10^{-5} and 10^{-6}) was spread onto

BH Agar plates and incubated 7 days at room temperature for the isolation of UEO degrading bacterial colonies (Singh and Lin, 2008).

2.3 Morphological and Biochemical characterization

Gram's staining; catalase test, oxidase test, indole test, MR-VP test, citrate utilization test and Nitrate reduction test were carried out for biochemical characterization of the isolate.

2.4 Quantification of biodegradation efficacy by gravimetric analysis

All the isolates were inoculated into 50 ml of BH broth containing 500 µl of UEO and incubated at room temperature and kept in an orbital shaker (150 rpm) for 7 days as reported earlier (Soumeya et al., 2022). After the incubation period, culture from the flasks were transferred into separating funnel and 50 ml of hexane was added. The solvent layer containing hexane was then filtered with 10 g of sodium sulphate anhydrous for removal of moisture. The filtered layer was then collected in a pre-weighed flask and allowed for evaporation of hexane. Then the combined weight of the evaporated filtered layer was measured. The degradation percentage of oil was measured using the formula given below

Degradation % = (Weight of control sample – Weight of test sample/ Weight of control sample) * 100

2.5 Biosurfactant screening

2.5.1 Oil displacement assay

Oil displacement assay was performed by adding 20 µl of UEO to a petri plate containing 20 ml distilled water. After addition of UEO, a thin layer of film was formed on the water surface and 10 µl of culture supernatant was added. Oil displacement activity of the biosurfactantwas indicated by the diameter of oil-free clearing zone. Distilled water was used as negative control (no clear zone formation or oil displacement). Triton X-100 was used as a positive control (Ibrahim, 2016).

2.5.2 Emulsification index measurement

Culture supernatant (2ml)and equal amount of the UEO in a test tube was vortexed for about 2 to 3 mins and kept at 37°C for 24 hours which was then measured for emulsification activity. *E%*was calculated by measuring emulsified layer's height and total height of the liquid column, using the formula provided below (Ilori et al., 2005)

Emulsification index (*E*%) = (Height of the emulsified layer / Total height of the liquid column) * 100

2.5.3 Drop collapse test

A drop of oil (15µl) was added on a clean glass slide containing culture supernatant (20 µl). The droplet was monitored to notice whether the droplet remained intact or collapsed. Triton X-100 was used as positive control and distilled water was used as a negative control. Presence of biosurfactant in the culture supernatant was indicated by the collapse of the drop measured as positive (Sohail and Jamil, 2020).

2.6 Effect of salinity on UEO biodegradation efficiency of the isolates

All the isolates were inoculated into 50 ml of BH broth containing 500 µl of UEO and incubated at RT and kept in an orbital shaker (150 rpm) for 7 days (Soumeya et al., 2022). Before sterilization, NaCl was added to BH broth in three different concentrations (0.5%, 1% & 1.5%). After the incubation period, culture from the flasks were transferred into a separating funnel 50 ml of hexane was added. The solvent layer containing hexane was then filtered with 10 g of anhydrous sodium sulphate for removal of moisture. The filtered layer was then collected in a pre-weighed flasks and allowed for evaporation of hexane. After that, combined weight of the evaporated filtered layer was measured. The degradation percentage of oil was measured using the formula given below

Degradation % = (Weight of control sample – Weight of test sample/ Weight of control sample) * 100

2.7 Biofilm formation test

Tube adherence test was performed to quantify biofilm formation. Overnight nutrient broth culture of isolates VITSK5 and VITSK6 was diluted to 1:100 dilution with distilled water. 100 µl from the dilution was added to 5 wells in 96 well plate and incubated overnight at RT. Culture was removed and the plate was washed with phosphate buffer. 1% crystal violet was added and incubated for 15 minutes followed by washing with phosphate buffer. Plate is inverted and allowed to air dry overnight. 30% acetic acid was added for solubilizing crystal violet and incubated for 15 minutes. OD was measured at 550nm in a plate reader with 30% acetic acid as blank (Eladawy et al., 2020).

Final OD = Average OD of test - Cut off value

3 Results

3.1 Isolation of bacteria by enrichment methods

Enrichment culture in BH broth was used for isolation of UEO degrading bacteria. After the incubation period, bacterial growth was observed in BHagar plates. Isolated colonies were streaked on nutrient agar plates and distinguished based on morphological characteristics. Two different isolates (named as VITSK5 and VITSK6) were selected based on UEO degradation. As the isolates were grown on the UEO, they used it as their sole carbon source for their growth, replication and survival.

3.2 Morphological and biochemical characteristics

Bacterial growth was assessed after 24 h of incubation for colony morphology and color. Gram's staining results indicated that both the isolates were gram negative, rod shaped. Biochemical test results of the two isolates are provided in Table 1.

Table 1 Biochemical tests for bacterial isolates VITSK5 and VITSK6.

Name of the biochemical test	VITSK5	VITSK6
Indole test	-	-
Methyl red test	-	-
VogesProskauer test	-	-
Citrate test	+	+
Nitrate reduction test	-	-
Urease test	-	-
Triple sugar iron test	+	+

+ Positive; - Negative.

3.3 Biodegradation efficiency by gravimetric analysis

Gravimetric analysis showed the percentage UEO degradation by the isolates. The isolate VITSK5 showed 51.35% and VITSK6 showed 63.5% degradation of UEO.

3.4 Biosurfactant screening

3.4.1 Oil displacement and Emulsification index measurement

The isolate VITSK5 showed 4.0 cm and VITSK6 showed 4.5cm zone of oil displacement. The formation of clear zone indicates the biosurfactant activity of the isolates. Emulsification index of VITSK5 and VITSK6 was measured to be 52.27% and 56.4% respectively.

3.4.2 Drop collapse test

Both the isolates VITSK5 and VITSK6 were assayed for biosurfactant activity using drop collapse test. Drop collapse activity was observed in both the isolates. The drop collapse test results are shown in Fig. 1. VITSK5 showed single positive and VITSK6 showed double positive while positive control Triton X 100 showed triple positive results.

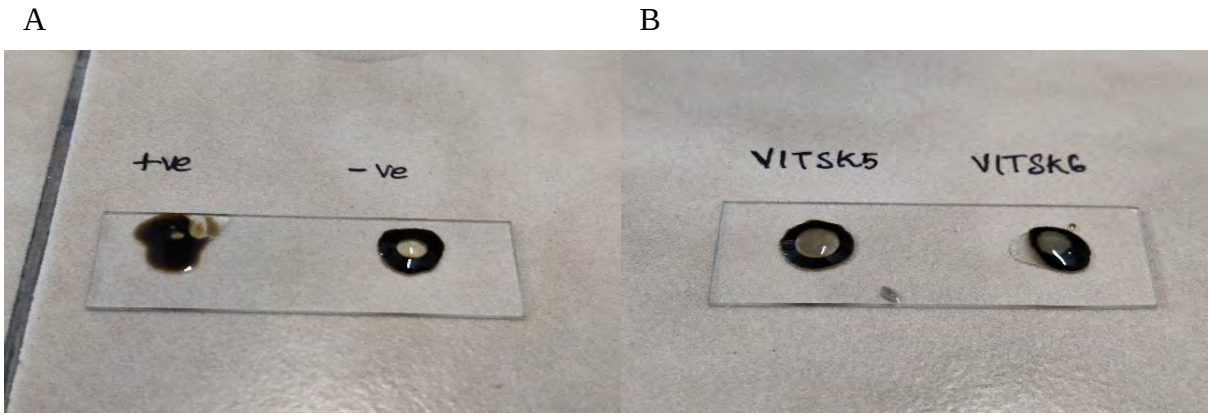

Fig. 1 Drop collapse test results of A) Triton X 100, distilled water and B) bacterial isolates VITSK5 and VITSK6.

3.5 Effect of salinity on degradation efficiency by gravimetric analysis

Salt tolerance by the isolates VITSK5 and VITSK6 on UEO degradation was measured by gravimetric analysis with different salt concentrations (0.5%, 1.0% and 1.5%). The isolates VITSK5 and VITSK6 showed the degradation of UEO by 62.84% and 61.71% respectively in the presence of 0.5% NaCl. At 1% NaCl concentration VITSK5 and VITSK6 showed the degradation percentage of 65.54% and 59.48% respectively. At 1.5% NaCl concentration VITSK5 and VITSK6 showed 73.06% and 58.0% degradation respectively. The degradation efficiency of VITSK5 and VITSK6 is given in Figure 2(A) and (B) respectively.

3.6 Biofilm formation test

Formation of biofilm was observed on wells after incubation with crystal violet. Absorbance (Optical density OD) values at 550 nm were used to arrive at OD_C and final OD values. Based on reference values, VITSK5 was found to be a moderate biofilm producer and VITSK6 was found to be a weak biofilm producer.

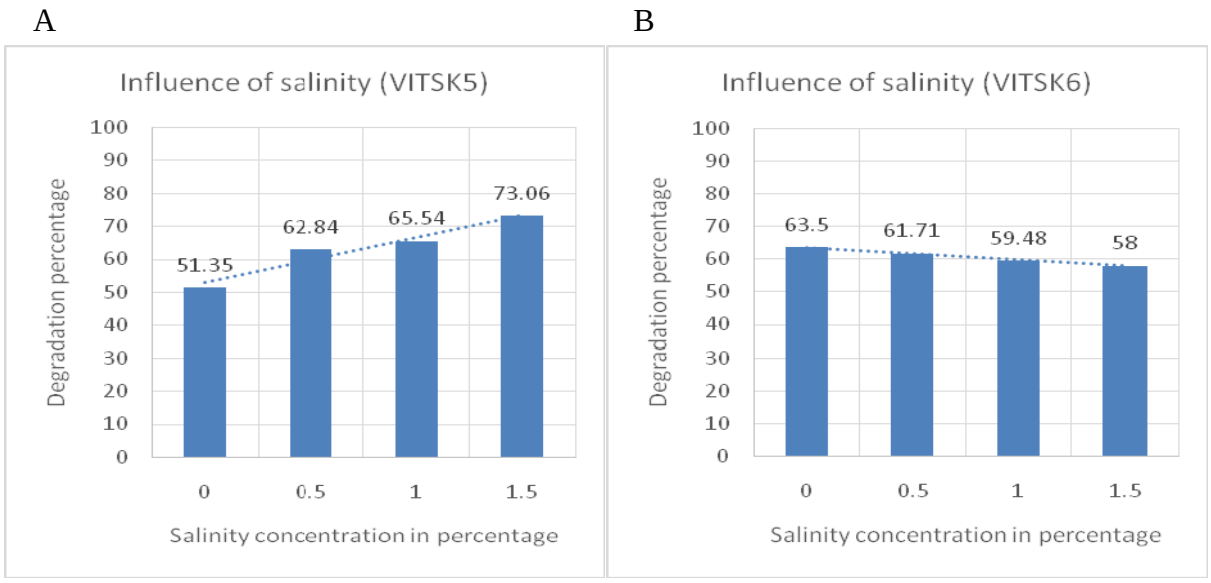

Fig. 2 Effect salt concentration on degradation of UEO by bacterial isolates A) VITSK5 and B) VITSK6.

4 Discussion

In this study, two bacterial strains (named as VITSK5 and VITSK6) were isolated from the enrichment culture (mixture of marine soil samples and UEO). UEO was used as sole carbon source to test their degradation efficiency. Bacterial biosurfactants are reported to be a green and effective method for bioremediation PHCs (Zahed et al., 2022). In a study by Hossain et al. (2022), petroleum hydrocarbon contaminated soil sample was used to isolate three bacterial isolates. They have reported to use used engine oil and diesel as sole carbon source to test the degradation efficiency of the isolates. Further, morphological and biochemical characterization were carried out to identify the isolates. Gravimetric analysis was carried out to examine the oil break down potential of the isolates. In this study the gravimetric analysis revealed that the isolates VITSK5 and VITSK6 degraded UEOby 51.35% and 63.5%, respectively after 7 days of incubation. A study by Idemudia et al. (2014) indicated that actinobacteria and other bacteria (*Nocardia* sp., *Gordonia* sp, *Micromonospora* sp, *Rhodococcus* sp.) can degrade used engine oil at the rate of 66.6 % and 59.4% after 5 days of incubation. Comparatively, VITSK5 has lower degradation efficiency and VITSK6 had higher degradation efficiency. Salt tolerance of the strains (VITSK5 and VITSK6) was tested with gravimetric analysis. Degradation efficiency was 62.84% and 61.71% for 0.5% NaCl, 65.54% and 59.48% for 1% NaCl and 73.06% and 58% for 1.5% NaCl for VITSK5 and VITSK6 respectively. Degradation efficiency of VITSK5 increased with increase in salt concentration indicating its tolerance to saline conditions while the degradation efficiency of VITSK6 decreased indicating the negative effect of salt on its degradation potential.

UEO is an immiscible pollutant and production of biosurfactant by bacteria enhances the biodegradation rate. For oil displacement assay, VITSK5 and VITSK6 showed a diameter of 4cm and 4.5cm of displaced circles respectively. In a previous study by Ibrahim (2016) the diameter of the displaced circles produced by two strains (HM-1 and HM-2) was 7cm and 6.5cm respectively. VITSK5 and VITSK6 had an emulsification index of 52.27% and 56.4% respectively. Sarubbo et al., (2006) have reported an emulsification index of 67% by *Candida glabrata* biosurfactant against n-hexadecane. Both VITSK5 and VITSK6 showed positive result for the drop collapse test. Four different strains (PWA, PWC, PWF and PWG) were studied for drop collapse assay. All the strains showed positive results for drop collapse test (Sohail and Jamil, 2020). Biosurfactant

screening results of the isolates indicated that further optimization of production conditions is required to improve the ability of bacteria to produce biosurfactants. Production of rhamnolipid-type biosurfactant by *P. aeruginosa* PTCC 1340 was already been reported (Gorbani et al., 2022). A biosurfactant produced by *Planococcus* sp. XW-1 capable of degrading (Guo et al., 2022) phenanthrene, pyrene, diesel oil, and crude oil. *Marinobacter* sp. has been reported to be very effective in biosurfactant production and degradation of 90 to 100% of low and medium molecular weight hydrocarbons (C35 to C50) (Al-Marri et al., 2023). Recently *Microbacterium barkeri* (SA9) and *Microbacterium deserti* (SA2, SA3, SA5, and SA7), isolated from hydrocarbon-contaminated soil has been shown to be effective in degrading PHCs (Hazaimeh et al., 2024). AchromobacteraegrifaciensS5 capable of degrading high molecular weight PAH Chrysene, isolated from crude oil contaminated sea water capable of 86% degradation after 7 days (Lazzem et al., 2024). Consortium of biosurfactant producing bacteria immobilised in biocomposite has been shown to be very effective in oil bioremediation (Purnomo et al., 2013). Degradation of organic pollutants by hydrocarbon degrading bacteria *Cyperus laevigatus* depends on the concentration of nutrients, surfactant, and aeration (NSA) (Hashmat et al., 2024). Both the ioslates VITSK5 and VITSK6were tested for biofilm formation, VITSK5 was found to be a moderate biofilm producer and VITSK6 was found to be a weak biofilm producer. The results of the study indicate that the isolates have the potential to degrade UEO, and capable producing biosurfactant and biofilm formation.

5 Conclusion

Bacteria VITSK5 and VITSK6 isolated from marine environments capable of degrading UEO. The UEO degrading ability of bacterial isolates were established by oil displacement assay, emulsification index measurement and drop collapse test. The isolate VITSK5 showed increased degradation with increasing salinity indicating their potential to degrade UEO under saline conditions as well. The study also revealed that both the isolates are capable of producing biosurfactant and biofilm. Based on the results of this study bacterial isolates from marine environment can be exploited for degradation of PHCs.

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