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Exploring the Indigenous Soil Microbes from Oil Refinery Site for Crude Oil Biodegradation Analysis

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This investigation focuses on exploring crude oil biodegradation by isolating microbes from oil-contaminated environment. Bacillus glycinifermentans, Bacillus subtilis subsp. stercoris, Enterobacter cloacae and Enterobacter ludwigii were identified as a result of their identification through Sanger's sequencing. For the biodegradation study, the isolated microbes were incubated for one week at 37°C, and their degradation characteristics were evaluated using Fourier Transform Infrared Spectroscopy (FT-IR) which indicated the absence of functional groups like C-H bending alkane and C-O stretching alkyl aryl ether. Gas Chromatography-Mass Spectrometry (GC-MS) analysis revealed the presence of 226 compounds in the untreated crude oil sample, whereas 26 of these compounds were degraded and heavy chain branched compounds were partially degraded to light chain compounds in the treated sample and overall showed 59% degradation activity at 7th day of incubation, showcasing the efficiency of the four strains in degrading crude oil compounds over the incubation period. This study concludes that the isolated microbes possess significant potential for degrading crude oil and can be effectively utilized in biodegradation experiments, as evidenced by the obtained results.

Keywords: Microbes, Crude oil, Biodegradation, FT-IR, GC-MS.

1. Introduction

Oil spills have a serious impact on the wellbeing of the environment and all living creatures, including people. The main causes of these are the leaks of refined goods, heavier fuels like bunker fuel used by big ships, and various kinds of oil or it's by products from drilling rigs, tankers, offshore platforms, wells, and so on. People all throughout the world have always found it difficult to remove these pollutants from the environment (Goswami et al. 2018). According to Zhang et al. (2019), the main sources of pollution from oil in the world's oceans include natural seeps, operating discharges from tankers, and the single largest oil supply that enters the ocean, which accounts for 45% of the total. The oil leaks during transportation not only endangered the lives of crew members, but also resulted in significant negative impacts on marine ecosystems (Chen et al. 2019). Only a small amount of attention has been paid to the sociocultural and psychological effects of marine oil spills, and this call for more research

to better understand how affected communities deal with the contamination and how to interact more effectively with them. One of the solutions focuses on biodegradation, which depends on the chemical makeup, physical state of the oil, temperature, the availability of nutrients, and the capacity to start redox processes, can happen either at the surface or within the water column. In Oil consistency, whether it is emulsified or dispersed, and the quantity of surface area accessible for microbial attachment define its chemical makeup (Zhang et al. 2019). Crude oil, which are natural, homogenous mixtures of hydrocarbons with a variety of chemical components, most notably alkanes with various chain lengths and branch points, cycloalkanes, mono and polycyclic aromatic hydrocarbons, pollute the ocean environment and are one of the major disasters. Some molecules include trace amounts of phosphorus, heavy metals like vanadium and nickel, as well as nitrogen, sulphur, and oxygen (McGenity et al. 2012). Over a million tons of crude oil, thought to pollute the seas annually, seriously and irreversibly damaging ecosystems and food webs all over the world (Neethu et al. 2019). These emissions can have disastrous effects on aquatic habitats on a physical, chemical, & biological level. Although biological methods are effective, they are directly impacted by things like the availability of the right microorganisms, the amount of oil present, and the nutrients bioavailability (Alabresm et al. 2018). In recent years, there has been a surge in the quest for effective methods to mitigate oil contamination in affected areas, driven by the sluggishness of traditional untreated oil spill clean-up processes. Harnessing a diverse array of microbes, particularly indigenous bacteria, has emerged as a promising approach to remediate hydrocarbon-contaminated sites. This endeavor falls under the purview of bioremediation, a process whereby microbial communities break down toxic substances, an endeavor that can be complemented by physical and chemical means (Latha et al. 2012). Yeast, fungus, and algae are just a few of the microbes that can be used in this process to help remove pollutants from the environment. It has long been known that bacteria in particular are skilled hydrocarbon-degrading organisms in ecosystems that are natural (Oyewole et al. 2019). Utilizing indigenous organisms for toxic waste removal offers a cost-effective and relatively safe alternative. The primary degradative pathways of hydrocarbons, catalysed by soil or water microbes, involve oxidative reactions that progressively convert larger hydrocarbon molecules into smaller intermediates (Banet et al. 2021). The success of bioremediation techniques deployed in hydrocarbon-polluted environments hinges significantly on the biodegrading prowess of native microbial populations or exogenous microbes introduced as inoculants, positioning bioremediation as a viable alternative for oil spill mitigation efforts (Vinothini et al. 2015). This research endeavor aims to scrutinize and evaluate the efficacy of isolated bacterial strains for crude oil degradation. Such analysis holds promise for informing subsequent bioremediation studies and advancing our understanding of microbial contributions to environmental clean-up efforts.

2. Materials and Methods

2.1. Isolation and Selection of Bacterial Culture

The crude oil-polluted soil sample was collected from an oil refinery site, in Chennai, Tamil Nadu (13°10’36” N 80°16’25” E). The sample was serial diluted and inoculated in LB-BH medium and kept in a shaking incubator overnight at 37°C at 100 rpm. After initial serial

dilution and growth observation, four strains were isolated and maintained as pure cultures (Neethu et al. 2019). To assess biodegradation, these isolates were used as a consortium, inoculated in LBBH medium with 2% crude oil, and incubated in a rotary shaker at 37°C in 150 rpm. Flask with LB-BH medium and crude oil served as a control for the comparative study.

2.2. Crude Oil Degradation Efficiency of Isolated Strains

The isolated bacterial strains were checked for their degradation potential. The identified strains were used as a consortium and cultured in the flask containing 100mL of LB-BH medium and 2% of crude oil was incubated at 37°C for one week in a rotary shaker at 100rpm. The non-inoculated medium with crude oil was used as a control for both experimental samples.

2.3. Qualitative Analysis of Experimental Sample

The qualitative analysis of crude oil degraded residues in a consortium of isolates was analysed using Fourier Transform – Infra Red spectroscopy (FT-IR), Gas Chromatography–Mass Spectroscopy (GC-MS) for crude oil degradation observation. For both FT-IR and GC-MS analysis, the content of the flasks containing degraded oil with microbes was centrifuged at 8000rpm for 15 minutes to obtain the cell precipitates. The filtrate was re-suspended in the ratio 1:1 Dichloromethane (DCM) solvent and again centrifuged at 5000rpm for 5mins. The degraded crude oil residues will get mixed with the added solvent and the remaining water content will settle up as supernatant. The homogenous solvent containing oil residues was analysed using FT-IR spectroscopy (Shimadzu, IRTRACER 100) and GC-MS (Shimadzu QP2010 PLUS) to elaborate on the changes and degradation in its chemical composition. For the GC-MS study (Neethu et al., 2019) the DB5 capillary column (30m × 0.25mm; 0.25µm of film thickness). Nitrogen gas was applied as carrier gas with a flow rate of 1mL/min. The column oven temperature was set at 50°C with a hold time of 1min and increased to 280°C with a ramp of 10°C/min with the final hold of 30min. Both the injector and the detector temperatures were set at 280°C. The degradation efficiency was calculated by the comparison of peak areas in the control and experimental samples.

3. Results and Discussions

3.1. Isolation and Identification of Bacteria

The study involved the isolation and selection of bacterial strains from a soil sample contaminated with crude oil. The collected soil sample was incubated in LB-BH medium overnight at 37°C and 100 rpm. After serial dilutions and growth observations, four bacterial strains (SH 1 - 4) were isolated and maintained as pure cultures. These strains were identified as Bacillus glycinifermentans, Bacillus subtilis subsp. Stercoris, Enterobacter cloacae, and Enterobacter ludwigii. Subsequently, the selected strains were used in degradation studies, focusing on their ability to utilize crude oil as the sole carbon source (Tian et al. 2018).

3.2. FT-IR Analysis of Crude Oil Degradation

In this study (Figures 1A and B) frequency range in the control sample at 4000 -3000cm^{-1} represents O-H, N-H stretching groups, and alcohol, aliphatic, primary and secondary amine,

are the compound classes were presented. In FT-IR results, are in comparative study with crude oil as control and the experimental sample with degraded crude oil residues. The variations in peak numbers were represented, a total of 18 peaks were shown in the control sample whereas the experimental sample contained 11 peaks. The crude oil and its derivatives contain carbon–hydrogen bonds giving rise to O-H stretching alcohol in the range 3257.77cm^{-1}, C–H stretching in the absorption range between 3200 to 2700 cm^{-1} of the IR spectrum such as vibrations in CH_2 groups at 2930 cm^{-1}, in CH_3 groups at 2960 cm^{-1} and in aromatic C–H bonds at 3010–3100 cm^{-1}. Experimental sample results showed C-Cl halo compounds, O-H bending alcohol, C-O aromatic ester, C=C bending alkene, C-Br, C-I stretching halo compounds, C-H bending alkane, C-O stretching alkyl aryl ether and C-O stretching tertiary alcohol were degraded vs. control. The biodegradation of benzene by free and immobilized bacteria utilizing graphene oxide was investigated by (Hossein Mohammadpour et al., 2020) under ideal circumstances. The study by FTIR of the Bacillus glycinifermentans GO 13T strain adhered to the graphene oxide surface could break down up to 77% of benzene after 24 hours. In (Naif Abdullah Al-Dhabi et al. 2020) Bacillus subtilis strain's biosurfactant IR spectra displayed strong absorption bands and the presence of CH_3 and CH_2, which suggests the existence of both aliphatic and alkyl chains in the biosurfactant. The biosurfactant sample showed N-H and C-H vibration was confirmed. The indigenous oil-degrading bacteria were isolated from sludge contaminated with engine oil (Sabina et al., 2014). The biodegradation of long and branched chains, and cyclic hydrocarbons found in used engine oil was demonstrated by the FT-IR analyses used to characterize the degraded metabolites in the anolyte of the microbial desalination cell (bio-electrochemical system). In the treated sample, the two most intense peaks in the untreated oil corresponding to C-H stretching in aliphatic molecules and C-N nitrile had less intensity. In (Karuppiah Prakash Shyam et al., 2021) the structural alterations of Poly Vinyl Alcohol (PVA) in the Enterobacter cloacae MBB8 culture supernatant during the PVA degradation were examined using an FTIR spectral analysis. The PVA concentration had decreased and the spectrum of PVA exhibits medium appearance of vinyl $C=CH_2$ and C=CH, and the CH_2 bending in PVA is noticed. Therefore, except for the peak that indicated the C=O group, the spectral characteristics of these compounds were said to be less visible in the IR spectrum.

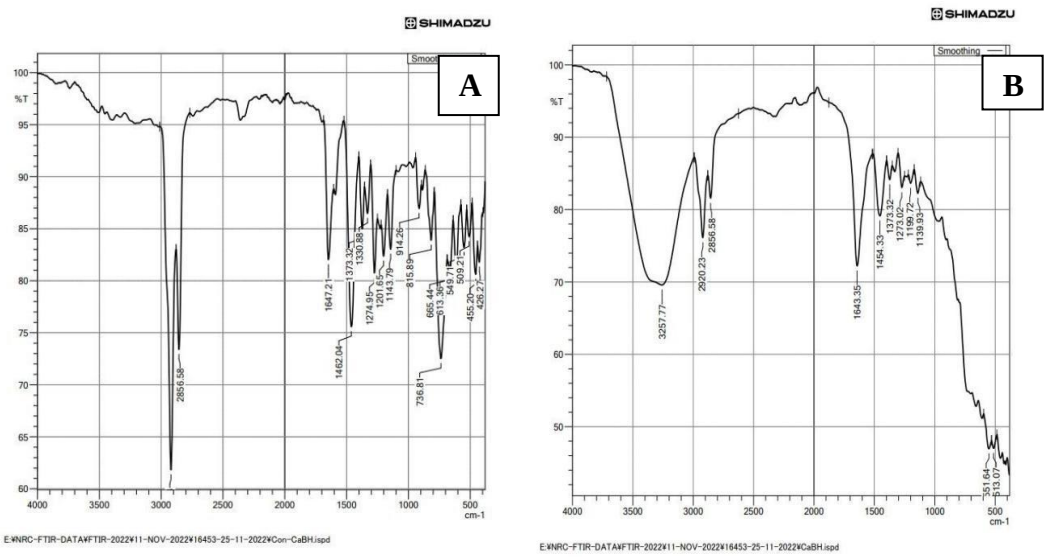

Figure 1: FTIR analysis of crude oil degradation. (A) Control - crude oil (B) Experimental sample

3.3. GC-MS Analysis of Crude Oil Degradation

In the FTIR technique, the functional groups of the unknown sample have been identified in both the control and experimental samples. As a result, the GCMS study characterises the presence of total petroleum hydrocarbons, aliphatic compounds, poly aromatic hydrocarbons and other complex compounds in crude oil. Hence it provides detailed qualitative results of microbial degraded crude oil residues compared with FT-IR analysis. Further, GC-MS results (Figures 2 and 3) conclude the presence of 226 compounds in the untreated crude oil while 26 of these peaks disappeared in the treated sample. 200 peaks were identified in the treated sample while the area of the other peaks was decreased, indicating the efficiency of four strains in degrading crude oil compounds. The compounds such as Benzene, 1-ethyl-4-methylcyclohexane, Cyclohexane, Octane, Nonane, Decane, Hexene, Undecane, Dodecane, Indene, Carbamic acid, phenyl-dodecyl ester, 1,1-Dimethyldecahydronaphthalene, 1-Sec-Butyl-4-Methylbenzene and other presented complex constituents were decreased or partially degraded in experimental sample vs. control. In the treated sample, the peak area of some of the chemical compounds also decreases and degrades when matched with the untreated sample. The efficiency of crude oil hydrocarbon degradation attained 59% degradation maintained for 7 days of incubation. The degradation may increase to high percentage relies and proportional to incubation of bacteria leads to well dissipation and degradation of crude oil components. GC-MS analysis demonstrated that the bacterial consortium was successful shown breaking down crude oil in the bioremediation studies. High-molecular-weight hydrocarbons were significantly reduced into simpler compounds. This proves the capability of the four isolated bacterial strains to degrade some of the chemical components present in crude oil. Additionally, the crude oil was more effectively dissolved in the medium and its concentration was reduced, which improved contact with the microorganisms and hastened decomposition. In (Ahmed et al., 2014) the degradation products of the engine oil were

indicated by peaks in the GC-MS spectra of ketones, alcohols, short-chain aliphatic hydrocarbon, and organic acids. The isolate E. cloacae E1 had the greatest emulsification index (E24%), which increased by 62%, decreased the medium's surface tension and led to the ideal circumstances for maximum degradation. In (Sugumar Ramasamy et al., 2016) the gas chromatogram of the diesel substrate revealed Enterobacter cloacae (KU923381), which has nearly degraded the hydrocarbons found in diesel fuel, including Tridecane, Hexadecane, Heptadecane, Heptacosane, Decahydro-Trans-Naphthalene, and Tert-Butyl-Benzene. The area of the principal peaks in the treated diesel substrate decreased, indicating that the main compounds had broken down, while new peaks that formed short-chain compounds were thought to be the breakdown products or metabolites. In (Ayşe EREN et al., 2022) the potential for petroleum hydrocarbon biodegradation of the Enterobacter ludwigii strain D8 isolated and developed in long and medium-chain hydrocarbons such as 1% decane, pentadecane, and squalene independently. The results of GCMS demonstrate that strain D8 degraded n-alkanes C11–C33 in 1% of crude oil and destroyed roughly 27% of the hydrocarbons in crude oil. In (Somayeh Kazemzadeha et al., 2020) Ten oil-degrading bacterial strains were identified as Bacillus spp., Bacillus pumilus, Rhizobium sp., Microbacterium oxydans, and Arthrobacter sp. after they were isolated from long-term petroleum-contaminated soil. Bacillus sp. strain X6 had the greatest biodegradation rate at over 50%, making it a suitable subject for more investigation, whereas Rhizobium sp. strain Z demonstrated the quickest growth and maximum adaptability in saline-alkaline environments. The crude oil GC-MS data identified C11 to C33 chain hydrocarbons and these investigated bacterial strains showed superior degradation outcomes towards C15–C19 chain hydrocarbons.

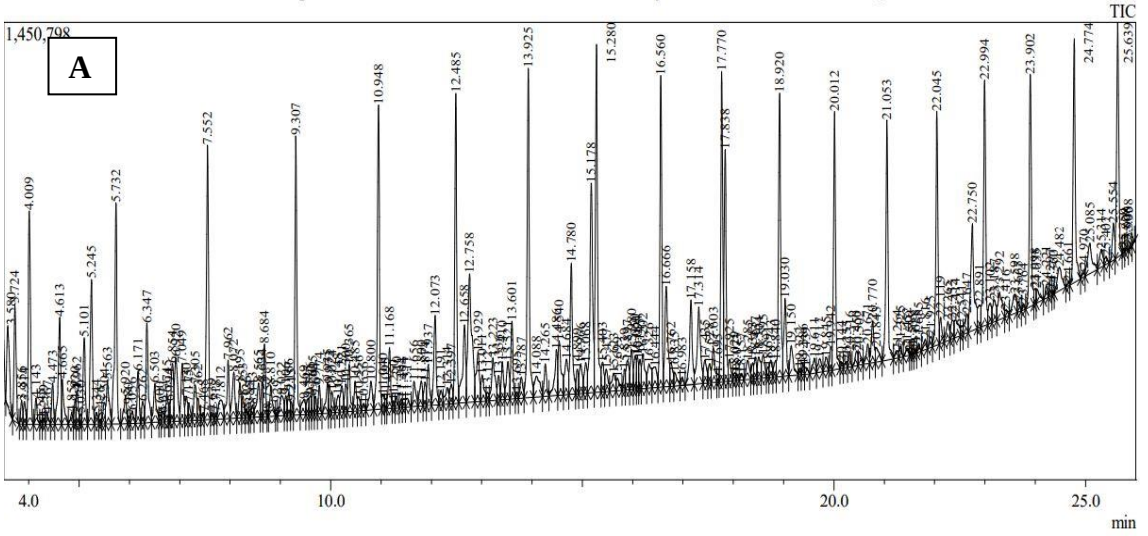

Figure 2: GC-MS analysis of crude oil degradation. (A) Control - crude oil

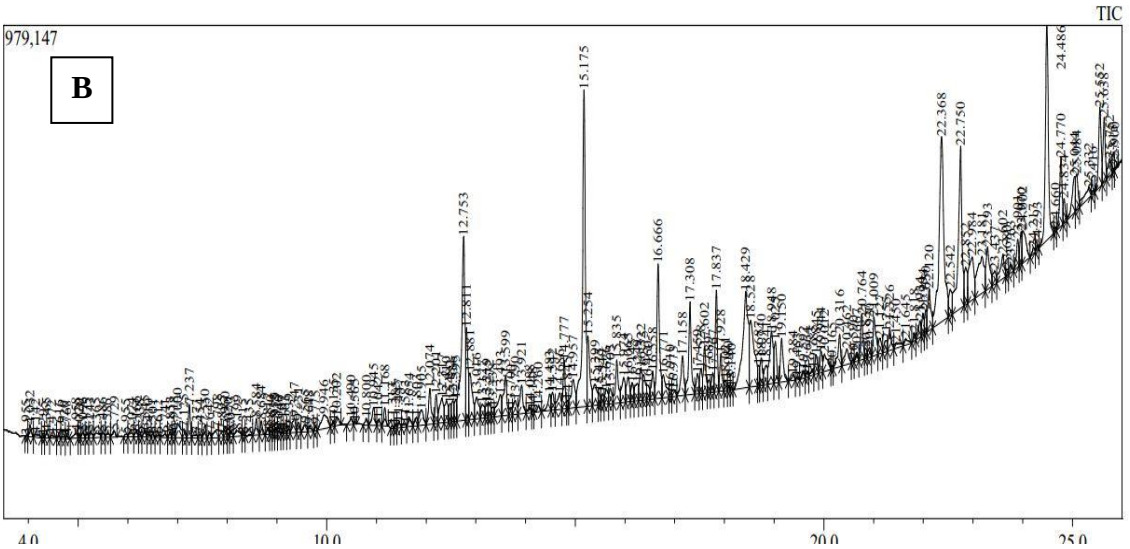

Figure 3: GC-MS analysis of crude oil degradation (B) Experimental sample

4. Conclusion

In conclusion, our investigation successfully isolated four bacterial strains with demonstrated biodegradation potential from a crude oil-contaminated site. Through identification, these strains were classified as Bacillus species and Enterobacter species. The individual strains, as well as the consortium, exhibited notable crude oil degradation activity, as validated by qualitative analysis such as FT-IR, and GC-MS. The findings affirm the capability of these four strains to degrade both short-chain and complex-chain compounds inherent in crude oil. This work was done in short span to check the degradation activity of isolated strains. If we extend the incubation time for the microbial degradation, we could expect the high possibilities in biodegradation activity. Also can perform the experiment with various parameters such as temperature, pH, nutrient availability and in different circumstances to exhibit the faster degradation. If there is any indulgence of nanoparticles in this experiment, the faster the degradation may be expected in further activity. This underscores the necessity for further research to enhance the degradation activity and ensure the stability of the isolated microbes. These future studies are crucial for advancing our understanding and potential applications in remediating oil-contaminated environments.

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Declaration Statement: The submitted work is original and not have been published elsewhere in any form or language (partially or in full), unless the new work concerns an expansion of

previous work. Results were presented clearly and no manipulation was done. All the authors mentioned have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors in the journal guidelines.

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