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Research Article

Isolation and Characterization of Hydrocarbon Degrading Bacteria Isolated from Oil Contaminated Soil

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ABSTRACT
This study investigates the physico-chemical properties of hydrocarbon-contaminated soil and the potential of isolated bacterial strains to degrade petroleum hydrocarbons. Soil samples were collected from Transport Nagar, Dehradun, and analyzed for pH, electrical conductivity, organic carbon content, moisture, and bulk density, etc. The results indicated significant differences between contaminated and uncontaminated soil properties. Out of 20 isolates, five isolates (DHCS2, DHCS6, DHCS9, DHCS11, and DHCS17) were screened as hydrocarbon degraders by spectrophotometric technique using DCPIP indicator. The percentage of degradation of five isolates were found to be 15.5%, 10.5%, 9.85%, 37.5%, and 15.5% respectively. DHCS11 was found to have highest degradation percentage (37.5%). The isolate DHCS11 was identified as Acromobacter sp., could be a potential candidate for the degradation of polycyclic aromatic hydrocarbons. These findings highlight the importance of microbial bioremediation in restoring contaminated soils.

Keywords: Biodegradation, DCPIP, Hydrocarbons, Gravimetric Method.

INTRODUCTION

Nature has been a potential source of oil-degrading agent for years. Recently, attentions have been made to oil pollution remediation processes. Soil pollution from petroleum is also one of the serious global problems. Everyday operations such as oil exploration, waste disposal (disposal of fuel and oil), and accidental spills cause serious environmental problems that lead to oxidative stress and alter the chemical composition of soils with low nutrient availability (Marín et al., 2020; Zhang et al., 2020). Petroleum has a negative impact on seed germination, reduces photosynthetic pigments, slows absorption, inhibits root growth, causes leaf defects, and causes cellular damage. Others include disruption of biological membranes, disruption of signaling of metabolic routes, and disruption of plant root structure (Jaiswal et al., 2021). Due to the high toxicity, carcinogenicity, mutagenicity, and teratogenicity of petroleum pollutants, their accumulation rate predominantly affects the entire human food chain. This indicates that petroleum contamination does not negatively affect plant growth but also affects people and the environment. Soil microorganisms in the contaminated soil utilize hydrocarbon as energy and carbon from organic matter. The term biodegradation has been defined as the biologically catalyzed reduction in the complexity of chemical compounds (Joutey et al., 2013). Organic substances such as these petroleum contaminants or products mentioned above are reduced into minor compounds by living microbes, reducing their defects. The environmental degradation of organic petroleum and other aromatic compounds is a complex task. The quantitative and qualitative characteristics are largely determined by the type and quantity of oil or petroleum products used (Urgun-Demirtas et al., 2008). Bioremediation techniques are categorized based on their location, with in-situ (on-site where the pollution occurred) and ex-situ (outside the pollution site) bioremediation being the two main classifications. In-situ bioremediation allows microorganisms to function efficiently, benefiting from the local environment without requiring an adaptation phase (Pino-Herrera et al., 2017). In contrast, ex-situ bioremediation methods primarily involve the physical removal of pollutants without direct microbial participation in the remediation process. Notably, in-situ bioremediation is preferred due to its cost-effectiveness compared to transporting contaminated soil off-site, and it is more effective for remediating large areas. This section provides an overview of recent bioremediation technologies and the operational factors that contribute to the success of each technology (Tekere et al., 2019; Adedeji et al., 2022). The susceptibility of hydrocarbons to microbial degradation can generally be ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Leahy and Colwell, 1990). Characterization of physio-chemical properties indicated the physical factors such as pH, electrical conductivity, bulk density, organic carbon and moisture of the soil. The determination of physio-chemical conditions for treating the contaminated soil with crude oil is revealed by physio-chemical parameters. This paper focuses on the isolation and biochemical characterization of hydrocarbon-degrading bacteria from contaminated soil, aiming to identify effective strains for bioremediation applications.

MATERIALS AND METHODS

2.1 Sample Collection and Preparation

Soil samples from hydrocarbon contaminated location i.e. vehicle maintenance Depot of Transport Nagar, Dehradun, Uttarakhand were used for this study. During collection, a clean stick was used to clear the debris away and 5-10 cm hole was dug. The soil sample was collected using sterile spatula and put in a sterile bag. The samples were transported to the laboratory within one hour for analysis.

2.2 Serial Dilution

A ten-fold serial dilution was carried out first by dissolving 1g of the test samples into 9ml of sterile water; the mixture was swirled clockwise and anti - clockwise to obtain a homogenous mixture. Subsequent dilution was made by transferring 1ml (mixture of soil sample in water) from the first bijou bottle to the second bijou bottle and in that order until the tenth bottle.

2.3 Inoculation

Mineral salt medium (MSM) was prepared using $NaNO_3$ (2.0 g/L), $CaCl_2.2H_2O$ (0.1 g/L), KCl (0.8 g/L), KH_2PO_4 (2.0 g/L), NaCl (0.8 g/L), FeSO4. $7H_2O$ (0.001 g/L), $Na_2HPO_4.12\ H_2O$ (2.0 g/L), $MgSO_4$ (0.2 g/L), and agar 20g, and additional trace elements (6) were combine. The pH of the mixture was set at 7.2. Aliquots (0.1 ml) of the 6^{th} and 8^{th} dilutions were inoculated in duplicate plates of freshly prepared sterile solid MSM media. After the inoculation, a sterilized L shaped spreader was used to spread the innoculum evenly on the surface of the media. Then the plates were inverted and incubated at the temperature of 28°C for 48-72 hours following the methods as described by (Holt et al., 1994) and analysed as per Bergey's manual of determinative bacteriology. Standard macroscopic and morphological characteristics with respect to colony formation were determined.

2.4 Morphological Characterization of Selected Three Bacteria

Microorganisms will differ in shape, size, colour and texture. They are complex and highly variable microbes and having four basic shapes: spherical (cocci), rod-shaped (bacilli), arc-shaped (vibrio), and spiral (spirochete). These traits are usually typical for a genus and are diagnostically useful.

2.5 Biochemical Characterization

To identify isolated bacteria and distinguish them from other rhizospheric bacteria, specific biochemical tests were conducted, including Gram's staining, gelatin hydrolysis, arginine dihydrolysis, starch hydrolysis, oxidase, catalase, ammonification, Methyl red and Voges- Proskauer test, urease, carbohydrate, indole test. The results were validated against Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

2.6. Screening of Hydrocarbon-Degrading Microbes

Isolated microbes were screened for their hydrocarbon-degrading ability by growing them in a BHM medium. The cultures were inoculated with bacterial cells and hydrocarbon sources, followed by the addition of 2,6-Dichlorophenol Indophenol (DCPIP). After a 5-day incubation at 37°C, decolorization of the medium was monitored, and absorbance was measured at 600 nm to assess degradation (Veerapagu et al., 2019).

2.7 Influence of Metal Ions on Microbial Growth

The effects of Mn and Cu on microbial growth were examined by inoculating screened bacteria in a nutritive medium containing various salts and hydrocarbon sources. The cultures were incubated, and microbial growth was evaluated by counting CFUs on meat-peptone agar plates after 4, 6, and 8 days (Gallego et al., 2001).

2.8 Influence of Metal Ions on Microbial Degradation Potential

Soil samples artificially contaminated with hydrocarbons were treated with Mn and Cu salts. Degradation was assessed using IR spectrophotometry after extracting hydrocarbons with chloroform and separating organic compounds via a chromatographic column filled with Aluminium oxide (Smreczak et al., 1999).

RESULTS & DISCUSSION

3.1 Physico-Chemical Properties of Normal and Hydrocarbon-Contaminated Soils

The physico-chemical properties of soil samples (SS1, SS2, SS3, SS4) revealed variations in pH, electrical conductivity (EC), total organic carbon, moisture content, and bulk density as depicted in table 1. All soil samples exhibited a near-neutral pH, ranging from 7.04 to 7.3, while SS4 had significantly lower EC (160 µs/cm) compared to the other samples, which ranged from 318 to 329 µs/cm. SS3 sample showed the highest organic carbon content (6.66%), while SS4 had the lowest (0.67%). Moisture varied across samples, with SS3 having the highest (18.83%) and SS4 the lowest (11.96%). SS2 exhibited the highest bulk density (0.97 g/ml), while SS1 had the lowest (0.66 g/ml). These variations indicate differences in soil quality and contamination levels.

Table 1: Physico- Chemical Properties of Normal Soil and Hydrocarbon Contaminated Soil

Parameters	Unit	Soil Sample SS1	Soil Sample SS2	Soil Sample SS3	Soil Sample SS4
pH	-	7.3 ± 0.03	7.3 ± 0.08	7.04 ± 0.02	7.15 ± 0.04
Electric conductivity (EC)	µs/cm	318 ± 0.01	329 ± 0.03	328 ± 0.21	160 ± 0.01
Total Organic Carbon	%	5.96 ± 0.33	5.33 ± 0.06	6.66 ± 0.06	0.67 ± 0.09
Moisture	%	17.01 ± 0.07	14.18 ± 0.03	18.83 ± 0.08	11.96 ± 0.02
Bulk Density	g/ml	0.66 ± 0.03	0.97 ± 0.14	0.85 ± 0.09	0.78 ± 0.01

3.2 Performance of Bacterial Isolates in Different Biochemical Tests

All 20 bacterial isolates exhibited consistent results across the biochemical tests (table 2; 3). Each isolate tested negative for Gram's staining, confirming their Gram-negative nature. They all showed positive results for arginine Di hydrolysis, catalase, oxidase, ammonification, and gelatin hydrolysis tests, indicating their enzymatic capabilities. However, all isolates were negative for starch hydrolysis. The

Isolates	Gram's straining	Arginine di-hydrolysis test	Starch hydrolysis test	Catalase test	Oxidase test	Gelatin hydrolysis test	Ammonification test
DHCS1	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS2	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS3	-ve	+ ve	-ve	+ ve	+ ve	_ ve	+ ve
DHCS4	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS5	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS6	-ve	+ ve	-ve	+ ve	+ ve	-ve	+ ve
DHCS7	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS8	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS9	-ve	+ ve	-ve	+ ve	+ ve	-ve	+ ve
DHCS10	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS11	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS12	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS13	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS14	-ve	+ ve	-ve	+ ve	+ ve	-ve	+ ve
DHCS15	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS16	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS17	- ve	+ ve	-ve	+ ve	+ ve	-ve	+ ve
DHCS18	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS19	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve
DHCS20	-ve	+ ve	-ve	+ ve	+ ve	-ve	+ ve

uniformity in these biochemical responses suggests that the isolates share similar metabolic profiles, which are characteristic of hydrocarbon-degrading bacteria.

Table 2: Performance of Bacterial Isolates in Different Biochemical Tests

Similarly, all 20 bacterial isolates were rod-shaped and showed diverse biochemical characteristics. Most isolates tested positive for HCN production, siderophore production, and phosphate solubilization, with only a few exceptions. The methyl red (MR) test was positive in isolates DHCS1, DHCS3, DHCS6, DHCS11, and DHCS16, while the urease test was predominantly negative, except for isolates DHCS3, DHCS4, DHCS5, DHCS9, DHCS14, and DHCS17. Indole production was seen in isolates DHCS1, DHCS6, and DHCS11. Regarding carbohydrate fermentation, most isolates could ferment dextrose and fructose, with lactose fermentation observed in only a few. These varied biochemical profiles suggest that the isolates have different metabolic capabilities, contributing to their potential application in bioremediation.

Table 3: Performance of Bacterial Isolates in Different Biochemical Tests

Isolates	Shape	HCN	Siderophore	Phosphate	MR	Urease	Indole test	Carbohydrate fermentation		
								Dextrose	Lactose	Fructose
DHCS1	Rod	+	+	+	+	-	+	+	-	+
DHCS2	Rod	+	+	+	-	-	-	-	-	+
DHCS3	Rod	-	-	-	+	+	-	+	-	-
DHCS4	Rod	+	+	+	-	+	-	+	-	+
DHCS5	Rod	+	+	+	-	+	-	-	+	-
DHCS6	Rod	-	+	+	+	-	+	+	+	+
DHCS7	Rod	+	+	+	-	-	-	-	+	-
DHCS8	Rod	-	+	-	-	-	-	+	+	-
DHCS9	Rod	-	+	+	-	+	-	+	-	+
DHCS10	Rod	+	+	+	-	-	-	+	+	+
DHCS11	Rod	+	+	+	+	-	+	+	+	+
DHCS12	Rod	-	+	-	-	-	-	+	+	-
DHCS13	Rod	+	+	+	-	-	-	-	+	-

DHCS14	Rod	-	+	-	-	-	-	+	-	+
DHCS15	Rod	+	+	+	-	-	-	+	+	+
DHCS16	Rod	-	+	+	+	-	+	+	-	+
DHCS17	Rod	-	+	+	-	+	-	+	-	+
DHCS18	Rod	+	+	+	-	+	-	-	+	-
DHCS19	Rod	-	+	+	-	-	-	-	-	+
DHCS20	Rod	+	+	+	-	-	-	+	-	-

3.3 Evaluation of Biodegradation Potential of Hydrocarbon Degrading Bacteria

Out of 20 isolates, five isolates (DHCS2, DHCS6, DHCS9, DHCS11, and DHCS17) were screened as hydrocarbon degraders by spectrophotometric technique using DCPIP indicator. The percentage of degradation of five isolates were found to be 15.5%, 10.5%, 9.85%, 37.5%, and 15.5% respectively (Fig. 1). DHCS11 was found to have highest degradation percentage (37.5%). Hence DHCS11 was screened for further studies.

3.4 Influence of Metals Ions on the Screened Microbial Growth

The CFU of hydrocarbon degrading bacteria were determined to observe the effect 100 mg/L of two different heavy metals (Mn and Cu) on bacterial growth Fig. 2 and 3, shows the change in microbial population of control and treated solution containing hydrocarbon and heavy metal Mn and Cu respectively.

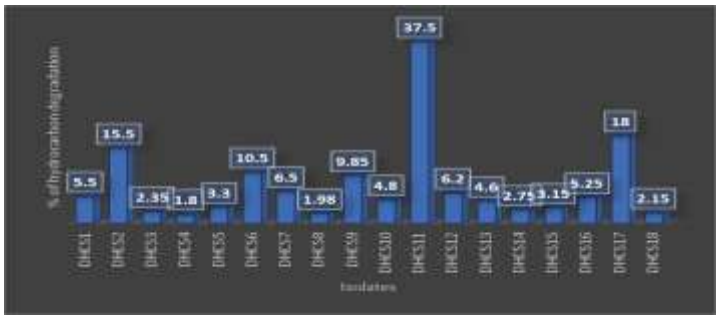

Fig.1: Hydrocarbon Degradation Percentage of Different Isolates

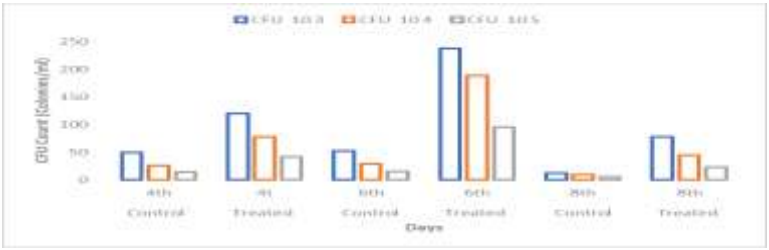

Fig 2: The Effect of 100 Mg/L of $Mnso_4$ on Screened Micro-Organism DHCS11

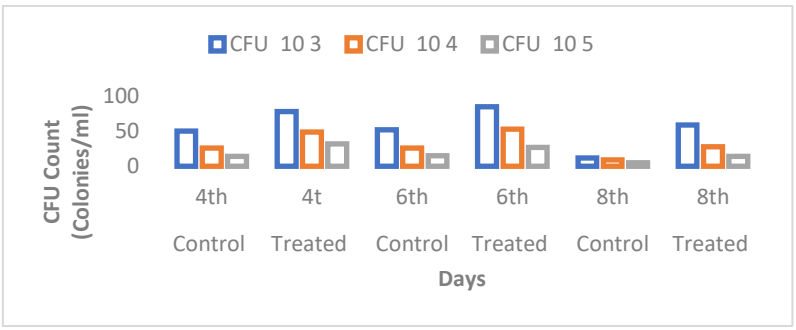

Fig 3: The Effect of 100 Mg/L of $Cuso_4$ on Screened Micro-Organism DHCS11

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3.5 Influence of Metal Ions on the Microbial Degradation Potential

The effect of metal ions on the degradation potential of DHCS11 was observed on 30th, 60th, 90th, and 120th day of inoculation in soil, the observed results show the continuous increase, and were the highest on 60th day and gradually decreases up to 120th day Fig. 4.

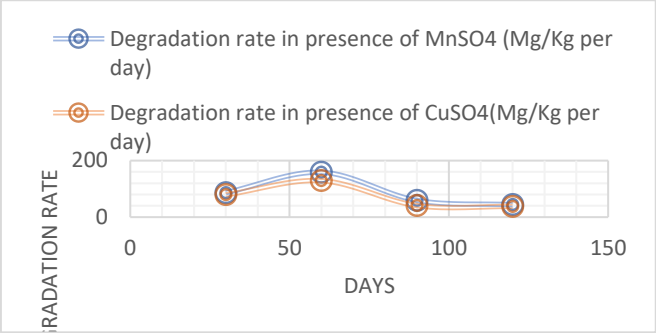

Fig 4: Influence of Mn and Cu on the Microbial Degradation Potential

DISCUSSION

The findings highlight the potential of indigenous bacteria in the bioremediation of hydrocarbon-contaminated sites. The ability of these microorganisms to adapt to extreme conditions and efficiently degrade hydrocarbons suggests their applicability in environmental cleanup efforts. Further studies on the metabolic pathways and enzyme activities involved in hydrocarbon degradation are necessary to enhance the bioremediation processes. Various researchers have reported the collection of oil soaked soil from different locations. These include one collected near from oil refinery of Tehran, Iran (Kebria et al., 2009); another from salt marshes and an estuary in Delaware and New Jersey (Cassel et al., 2015) and the collection of a gasoline-contaminated soil sample (Yerushalmi et al., 2006). Such soil samples contained crude oil while in present work diesel (a refined petroleum product) containing soil was used to isolate different bacteria capable of degrading naphthalene, paraffin, engine oil and toluene. The uncontaminated soil sample had a relatively increased pH value compared to contaminated soil. The reduction of pH levels in crude oil-contaminated soil was due to acids produced by microorganisms. The present study concluded that uncontaminated soil samples had lower organic values than contaminated ones. In the present study the maximum amount of organic carbon was determined in SS3 (6.66 ± 0.06) followed by SS1 (5.96 ± 0.35) and SS2 (5.33 ± 0.06) respectively and least in control i.e., 0.67 ± 0.09. the following is due to high rate of hydrocarbon decomposition as the organic carbon present in soil is directly proportional to the hydrocarbon contamination, therefore the increase in organic carbon indicated the rise in hydrocarbon in the soil, thus in present study SS3 sample is highly contaminated by the hydrocarbon. The same was also demonstrated by Wang et al, (2013) while studying the effect of crude oil contamination on soil properties of Momoge wetland of China and by Sharma and Vashishtha, (2021) when they study the effect of petroleum contamination on the refinery's peripheral area of Punjab. The high EC value in contaminated soil may be due to high metal ions concentration in the contaminated soil, same was found in the study of Onojake and Osuji, (2012); Brady et al., (2022); and Sharma and Vashishtha, (2021) according to them, carbonates and bicarbonates are the two important soils constitute, that maintain soil alkalinity but the estimation of these are found to be high in hydrocarbon contaminated soil.hydrocarbons can be degraded by microorganisms such as bacteria, fungi, and microalgae. However, bacteria were found to be the most active agents in hydrocarbon degradation, of spilled oil in an environment, similar results were also reported by Brooijmans et al., (2009). Several authors have studied (Shukor et. al., 2009; Habib et al., 2018) the biodegradation ability of bacteria to degrade petroleum hydrocarbon such as Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Berjerinckia, Corynebacterium, Enterobacter, Flavobacterium, Methylosinus, Mycobacterium, Mycococcus, Nitrosomonas, Nocardia, Penicillium, Pseudomonas, Rhizoctonia, Serratia, Trametes, and Xanthobacter (Bartha and Atlas, 1977; Bishnoi et al., 2024; Potentini and Rodriguez, 2006). According to Ward and Singh (2003) bacterial enrichment techniques for isolation of a bacterial culture capable of growing on specific hydrocarbons comprise of adding a sample of soil, sludge or other material containing a large population of bacteria to an aqueous medium containing hydrocarbon as the only or predominant carbon source. The isolated bacteria were found to be Gram-negative, rod-shaped bacteria. The methyl red test performed in lactose containing MacConkey's broth revealed gas formation in inverted Durham tubes and elevation of pH (methyl red in medium turned yellow) by isolated bacterium. This demonstrated that the isolated bacterium was a lactose fermentor. The Indole production test of isolated bacterium (Liquid) confirmed that the isolated bacterium was Indole negative as the cherry red colour ring was not formed on the meniscus of peptone containing medium upon addition of Kovac's reagent. Isolated bacterium was showing negative results for Indole test but fermenting lactose in MacConkey broth.

Out of 20 isolates, five isolates (DHCS2, DHCS6, DHCS9, DHCS11, and DHCS17) were screened as hydrocarbon degraders by spectrophotometric technique using DCPIP indicator. The percentage of degradation of five isolates were found to be 15.5%, 10.5%, 9.85%, 37.5%, and 15.5% respectively. DHCS11 was found to have highest degradation percentage (37.5%). The DHCS11 bacterial isolate that have maximum potential to degrade petroleum hydrocarbons were identified as Achromobacter spp. species. Literature review revealed very less information about this particular species; however several scientific reports were available for other species of same genus Achromobacter. Ujimaru et al. (1983) studied various conditions to produce tryptophanase by Achromobacter liquidum and for the conversion of L-serine and indole to L-tryptophan; Dees and Moss (1978) determined the cellular fatty acid composition and metabolic products of 12 reference strains of Achromobacter sp. and A. xylosoxidans by gas-liquid chromatography. Buckova et al. (2013), screened hydrocarbon degrading efficiency of bacterial isolates by DCPIP test and reported that 28 isolates able to degrade PAHs. Similarly Bidoia et al. (2010), reported that the degrading potential of bacterial cultures occurs in the complete reduction of DCPIP in 75 hrs. for mineral oil, 87 hrs. for used oil, 125 hrs. semi-synthetic oil, and 138 hrs. for synthetic oil. Similarly, Veerapago et al. (2019) isolated eleven bacteria investigated for hydrocarbon tolerance in Bushnell Haas broth containing 1% (w/v) crude oil as sole carbon source. Four bacterial isolates exhibited growth of > 1.0 OD screened for hydrocarbon degradation by DCPIP method. The isolate HDB5 showed 27.5% of biodegradation was identified as Pseudomonas spp. A potential technique for treating a variety of pollutants in soil and groundwater is bioremediation. The approach is economical, especially when used for procedures involving petroleum hydrocarbon pollution, and it is simple to use with other technologies for remediation. In present study, the highest number of isolated bacteria was found in the SS3 Sample (38.6 ± 0.19 x 103), followed by the SS1 (28.1 ± 0.36 x 103) and SS2 (21.4 ± 0.66 x 103) samples, in that order. Microorganisms have proven to be remarkably adaptable, allowing them to flourish in environments contaminated by hydrocarbons. Bekele et al. (2022) also shows that the gram-negative bacteria belonging to Achromobacter genera have high diesel degradation efficiency. It may be due to the fact that bacteria need manganese, an important micronutrient, for a variety of metabolic functions, such as electron transport chain reactions and enzyme activation. Similarly, Zukauskaite et al., (2008), studied the effect of different valences of Mn on the oil product degradation. However, there is no statistically significant difference between Cu and Mn micro inputs, thus it could be assumed that both Mn and Cu inputs the degradation somewhat equally as the microbial degradation capability of pollutants, organic compounds can be significantly impacted by metal ions. The activity of many microbial enzymes involved in breakdown processes depends on metal ions as cofactors.

CONCLUSION

Petroleum biodegradation is a significant transformation process that has a significant effect on the production of oil and gas economically. Anaerobic hydrocarbon-degrading bacteria are thought to be the cause of biodegradation in reservoir petroleum, according to evidence. In order to determine the bacterial strains' potential for bioremediation, the current study primarily focused on their isolation and identification from soils contaminated with hydrocarbons. This study successfully isolated and characterized hydrocarbon-degrading bacteria from contaminated soils. The identified strains, particularly those belonging to the Achromobacter spp., exhibit promising potential for bioremediation applications. Future research should focus on optimizing growth conditions and understanding the genetic basis of hydrocarbon degradation to improve bioremediation strategies.

CONFLICT OF INTEREST

The authors have no conflict of interest regarding this investigation

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