

 <p>ISSN NO. 2320-5407</p>	<p>Journal Homepage: www.journalijar.com</p> <h2 style="text-align: center;">INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)</h2> <p style="text-align: center;">Article DOI:10.21474/IJAR01/11911 DOI URL: http://dx.doi.org/10.21474/IJAR01/11911</p>	 <p>INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR) ISSN 2320-5407 Journal Homepage: http://www.journalijar.com Journal DOI:10.21474/IJAR01</p>
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RESEARCH ARTICLE

BIOREMEDIATION OF CRUDE OIL CONTAMINATED SOIL BY HYDROCARBON DEGRADING BACTERIA

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Manuscript Info

Manuscript History

Received: 25 August 2020

Final Accepted: 28 September 2020

Published: October 2020

Key words:-

Biodegradation, Crude Oil, Streptomycin, Hydrocarbon, Contamination

Abstract

Proficiency of hydrocarbon-degrading bacteria useful for eradicating soil pollution stimulated by seepage of petroleum and their derivative substances, therefore, sequestered different bacterial isolates were bear significant merits for utilizing hydrocarbons as an uncommon source of their growth and development. A colorimetric assay was performed at 595 nm wavelength which was subjected to the mineral salt broth with 1% oil (petrol, diesel, and burned out engine oil). This broth having minimum nutrients used by inoculated bacteria in which some isolates were adopted for taking a sufficient amount of either petrol, diesel, or burned-out engine oil as carbon source hence increases its turbidity that was measured in two days time interval up to twelve days of observation. We found that selected isolates were able to degrade burned out engine oil efficiently in contrast to petrol and diesel. At optical density 0.21 ± 0.13 , 0.19 ± 0.08 , 0.18 ± 0.09 , 0.17 ± 0.12 the result showed by isolates BI-7, BI-2, BI-4, BI-6 good competency to degrade burned out engine oil. On the other hand, maximum diesel and petrol utilized by BI-2 & BI-1 isolate at O.D. 0.09 ± 0.02 , 0.05 ± 0.02 was recorded. Most of the bacterial isolates resulted in a positive biochemical test. BI-1 & BI-7 resulted in positive starch hydrolysis test, whereas, all isolates resulted in positive urease test, simultaneously negative results for all isolates obtained when catalase and antibiotic sensitivity test against streptomycin were performed.

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Introduction:-

Pollution reflected the entrance of undesired substances in the ecosystem. The Petroleum components must be separated into four fractions: aromatic, saturated, resin, and as phaltene fractions analyzed through absorption chromatography (Harayama et al., 1999). Due to the high energy content hydrocarbons are the world's most commonly used primary energy and fuel resources. The anthropogeneous discharge of hydrocarbons into the environment is strongly connected exorbitant use of Petroleum products, which is extensively widespread all over the world (Winkelmann et al., 2009).

In the present scenario, the high demand and use of petroleum products are increasing everyday, which in turn increases the possibility of accidental spillage of petroleum hydrocarbons during transportation, natural seeps, or due to custom maintenance of infrastructure (Okoh, 2003). In industrialized and developing countries oil spills have become a global problem, the amount of natural crude oil seepage was expected to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year (Kvenvolden, 2013). Heavy metals and radionuclide both

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contaminants cause adverse effects on the chemical and biological nature of the soil and thus leads to soil degradation (Singh, 1997). Crude oil-polluted sites having different physiological characteristics as compared to the unpolluted area, Crude oil affects the hydraulic conductivity, total porosity, macroporosity, and bulk density of the soil (Abosedo, 2013). Spillage of crude oil alters the physicochemical properties of the soil, causing it impossible for the soil to produce at its optimal capacity as a result of the hardening of the soil (Ofoegbu et al., 2015). Soil pollution with petroleum hydrocarbons has emerged as a serious environmental and human health concern with a large percentage of burned oil discharged into the ecosystem without any treatment (Zhang et al., 2019). These hydrocarbon contaminations are mutagenic, carcinogenic, and potent immunotoxicants pretense (Liu et al., 2010), a serious threat to human and animal health and also hazardous to the health of plants (Vasudevan and Rajaram, 2001). Polycyclic aromatic hydrocarbon (PAH) is originated by incomplete combustion of organic materials such as petrol, oil, and petroleum refineries as well as motor vehicle exhaust. Most PAHs are mutagenic, carcinogenic, and teratogenic. Due to their lipophilic nature, it is easily absorbed by the gastrointestinal tract of animals and harms their physiology (Abdel et al., 2016). In-vitro and in-vivo studies suggest how oil contaminants enter the food chain and alter the physiology of biological component of the ecosystem because oil-derived substances originate genotoxicity and endocrine toxicity problems (Aguilera et al., 2010). Removal of oil seepage is an expansive process therefore a cost-effective technique is required for mitigating the excess of petroleum hydrocarbon from the environment (Islam and Rahman, 2017). The technologies which are commonly used for soil remediation include mechanical, burying, evaporation, dispersion, and washing. However, these technologies are expensive and can also lead to incomplete decomposition of contaminants (Alvarez et al., 1991). Most of the hydrocarbons are degraded primarily by bacteria and fungi so both types of environments temperature and oxygen and nutrient concentrations are important variables, in some aquatic environments pressure and salinity may also affect biodegradation rates, and moisture and pH may influence the biodegradation in soils. The hydrocarbon degradation rate increases due to Adaptation by prior exposure of the microbial community to hydrocarbons (Kumari et al., 2013). Adding 1% soil conditioner could considerably improve the soil conditions and offer microorganism adequate N, P, and K, which would support microbial growth and played a significant role in bioremediation of oil-contaminated soil. Although in contaminated soil, the microbial community could maintain metabolic activity and use petroleum as a carbon source (Roy et al., 2018). The indigenous microbes are good for decomposing low molecular compounds and saturated hydrocarbons, while the oil-degrading strains can effectively degrade high molecular weight aromatic compounds (Liu et al., 2020).

Application of Bioremediation technologies provides useful remedies to remove hydrocarbon polluted substances like xylene, toluene, crude oil, mineral oil, phenanthrene, fluoranthene, or pyrene as carbon source (Toledo et al., 2006). Microorganisms are exceptionally assorted and many of them have the potential to degrade and utilize complex organic contaminants such as petroleum hydrocarbon by using various types of degradative enzymes. Not only bacteria but cyanobacteria, some algae, and fungi can also degrade petroleum under various environmental conditions by chronological metabolism of the hydrocarbon compounds (Peixoto et al., 2011). Some bacterial isolates such as *Alcanivorax bor kumensis* SK2, *Rhodococcus erythropolis* HS4, and *Pseudomonas stutzeri* SDM are capable of oil degradation. All strains are grown on a microcosms system containing seawater and crude oil as a unique carbon source (Santisi et al., 2015). Strains of *corynebacterium*, *bacillus*, and *pseudomonas* species are much able to utilize crude oil from polluted soil samples (Kumar et al., 2008). Besides, bioremediation technology is supposed to be non-invasive and moderately cost-effective. Indigenous oil-consuming microorganisms, which can degrade organic compounds, play a significant role in the disappearance of oil from the soil. This microbiological bioremediation of the oil-polluted soils is claimed to be a competent, economic, and adaptable alternative to physiochemical treatments (Atlas, 1991; Bartha, 1986). The present study aimed to assess the biodegradation potential of hydrocarbon-degrading bacteria from the crude oil contaminated soil of Durg city, Chhattisgarh, India. Degradation studies were performed with different isolates at varying intervals of time to find out the competent hydrocarbon-degrading bacteria.

Materials and Methods:-

Collection of crude oil contaminated soil sample:

Samples of the crude oil and the petroleum polluted soil used in this study were collected from motor vehicle workshops. The Soil samples were collected randomly from 5-10 cm underneath the outside using a spatula and packed in a sterile poly bag.

Isolation of hydrocarbon-degrading bacteria:

Xenobiotic-degrading bacteria present in the collected soil samples were isolated by direct plating of dilutions of the samples on nutrient agar medium. 10 grams of prepared soil samples were suspended in 100 ml sterile distilled water, by vortexing, before being diluted up to 10^{-5} . The soil samples were prepared by filtering through a sterile 1 mm mesh screen to remove gravels and plant debris. Aliquots (1 ml) of the soil dilutions were placed on the Nutrient Agar medium, composition was (gm/l): Peptone, 5; Beef extract, 3; NaCl, 5; Agar, 15; pH, 7. The cultures were incubated at 37°C for 3 days, respectively, for adequate colony development. Distinct colonies were selected and purified by streaking method on nutrient agar media and preserve as stab cultures at 4°C.

Preliminary and Biochemical characterization of selected microorganisms:

The selected isolate was characterized and identified by culture (color, size, shape, margin, opacity, etc.) and cellular morphology i.e. gram staining and by the biochemical characterization, viz. starch hydrolysis, catalase production, urease production, and antibiotic-resistant test were also performed for isolated hydrocarbon consuming bacteria.

Starch hydrolysis test:

Isolated microorganism inoculated into starch agar medium with a composition (gm/l): meat extracts, 3.00; a peptic digest of animal tissue, 5.00; starch, soluble, 2.00; agar, 15.00, and final pH 7.2. Cultured plates incubated at 37°C for at least 48 hours. Plates were swamped with freshly prepared iodine solution and results were observed. The dark red colour indicated no hydrolysis, while a clear zone indicated hydrolysis. The plates were observed for starch hydrolysis as when iodine added, colour changes are dark red but the area which shows a positive result for amylase production showed a clear zone surrounding the microbial colonies.

Catalase test:

During aerobic respiration in the presence of oxygen, micro-organism produces H_2O_2 (hydrogen peroxide) which is lethal to the cell. Catalase breaks down hydrogen peroxide. So, a loopful culture of isolated bacteria was taken on a slide, and m culture was mixed by adding one drop of distilled water. The same amount of hydrogen peroxide was dropped on a slide, the slides were observed with bubble formation which showed a positive result.

Urease Test:

Urea agar medium was prepared with chemical constituents (gm/l): urea, 20.00; NaCl, 5.00; monopotassium phosphate, 2.00; peptone, 1.00; dextrose, 1.00; phenol red, 0.012; agar, 15.00. pH was adjusted 6.7 ± 0.2 at 25°C temperature and autoclaved at 121°C for 15 minutes and cooled for 50°C. It was mixed well and poured into a sterile test tube, i.e. Culture tubes, and allowed to solidify in a slanting position to form slopes and then incubated inoculated urea agar medium for 24-48 hrs at 37°C.

Antibiotic-Resistant test:

The test isolates were inoculated on the nutrient agar plates incorporated with filter-sterilized streptomycin at a rate of 0.1% and incubated for 24 hours at 37°C. The antibiotic-resistant was recorded as positive when the test colony appeared on the plates. A control plate was also maintained.

Evaluation of oil-degrading ability.

The degrading activities of each isolate were evaluated by using mineral salt broth (MSB) in which 1% of each hydrocarbon (petrol, diesel and Burned out engine oil) was added and incubated at room temperature for 12 days. The growth of the isolated bacterium was measured by taking the O.D. at 595 nm wavelength. This process was repeated at each 2-days interval for 12 days.

Result:-

The contaminated soil samples were enriched with the hydrocarbon-degrading bacteria, based on their abilities to grow on crude oil and/or individual hydrocarbons as their sole carbon source, eight bacterial isolates were used in this study (Figure 2 A, B&C).

Biochemical characterization of Bacterial Isolates

Out of 8 bacterial isolates, 4 (BI-2, 3, 4, and 5) were found gram-positive (Figure 3A), and the remaining 4 isolates (BI-1, 6, 7, and 8) were found gram-negative (Figure 3B). Bacterial isolates (BI-1, 2, 3, 4, 5, 6, 7, and 8) were found with a positive result for the urease test means all bacterial isolates were produced urease that degrades urea which was a supplement in urea agar media (Figure 4A). Total 7 out of 8 Bacterial isolates, BI-1 & BI-7 were found positive

when starch hydrolysis tests were performed with each isolate (Figure 4 B&C); whereas, in catalase and antibiotic-resistant tests (streptomycin), all bacterial isolates were found negative (Table 1).

Biodegradation Activity

Hydrocarbon utilization by purified Bacterial Isolates was determined by colorimetric assay at 595 nm wavelength (figure 1A, B&C). BI-1(0.06±0.06), BI-2(0.19±0.08), BI-3(0.15±0.01), BI-4(0.18±0.09), BI-5(0.13±0.08), BI-6(0.17±0.12), BI-7(0.21±0.13), and BI-8(0.13±0.08) showed hydrocarbon-degrading activity and possessed the best growth with burned-out engine oil as a sole source of carbon as compared to petrol and diesel (Table 2). Optical Density (OD) was found negligible in the case of petrol only (Table 3). It was found that BI-1(0.05±0.02) degraded hydrocarbon fairly but in the case of diesel, bacterial isolates(BI-1); (0.07±0.02), and BI-2(0.09±0.02)) degraded hydrocarbon moderately in contrast to other isolates (Table 4).

Observation:-

Table -1:- Showing morphological and biochemical characteristics of selected bacterial isolates.

S. No.	Characteristics	Bacterial Isolates							
		BI-1	BI-2	BI-3	BI-4	BI-5	BI-6	BI-7	BI-8
1.	Simple Staining	Round	Round	Round	Round	Round	Round	Round	Round
2.	Gram Staining	-	+	+	+	+	-	-	-
3.	Starch hydrolysis test	+	-	-	-	-	-	+	-
4.	Antibiotic resistant test (Streptomycin)	-	-	-	-	-	-	-	-
5.	Urease test	+	+	+	+	+	+	+	+
6.	Catalase test	-	-	-	-	-	-	-	-

+ = Positive, - = Negative

Table -2:- Showing burned out engine oil degradation activity by colorimetric assay.

S. No.	Day of Observation	OD of Bacterial Isolates							
		BI-1	BI-2	BI-3	BI-4	BI-5	BI-6	BI-7	BI-8
1.	Day 2	0.03	0.07	0.03	0.04	0.01	0.02	0.02	0.03
2.	Day 4	0.04	0.12	0.08	0.11	0.07	0.07	0.11	0.07
3.	Day 6	0.06	0.18	0.14	0.19	0.13	0.13	0.20	0.11
4.	Day 8	0.12	0.22	0.19	0.19	0.14	0.18	0.24	0.13
5.	Day 10	0.15	0.28	0.25	0.27	0.22	0.30	0.36	0.23
6.	Day 12	0.16	0.29	0.25	0.28	0.24	0.32	0.37	0.23
	X±D	0.06±0.06	0.19±0.08	0.15±0.01	0.18±0.09	0.13±0.08	0.17±0.12	0.21±0.13	0.13±0.08

OD = Optical Density, BI = Bacterial Isolates

Table -3:- Showing Petrol degradation activity by colorimetric assay.

S.No	Day of Observation	OD of Bacterial Isolates							
		BI-1	BI-2	BI-3	BI-4	BI-5	BI-6	BI-7	BI-8
1.	Day 2	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01
2.	Day 4	0.05	0.02	0.02	0.01	0.01	0.01	0.01	0.01
3.	Day 6	0.09	0.03	0.04	0.02	0.01	0.01	0.01	0.01
4.	Day 8	0.07	0.01	0.01	0.01	0.01	0.01	0.01	0.01
5.	Day 10	0.07	0.01	0.01	0.01	0.01	0.01	0.01	0.01
6.	Day 12	0.03	0.02	0.01	0.02	0.01	0.01	0.01	0.01
	X±D	0.05±0.02	0.01±0.01	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00

OD = Optical Density, BI = Bacterial Isolates

Table - 4:- Showing Diesel degradation activity by colorimeter assay.

S. No.	Day of Observation	O.D. of isolated Bacterial Isolates							
		BI-1	BI-2	BI-3	BI-4	BI-5	BI-6	BI-7	BI-8
1.	Day 2	0.06	0.06	0.04	0.01	0.02	0.01	0.01	0.02
2.	Day 4	0.06	0.08	0.04	0.05	0.01	0.01	0.01	0.02
3.	Day 6	0.06	0.11	0.04	0.10	0.01	0.02	0.02	0.03

4.	Day 8	0.06	0.11	0.02	0.09	0.02	0.07	0.01	0.02
5.	Day 10	0.06	0.08	0.02	0.07	0.05	0.07	0.01	0.02
6.	Day 12	0.13	0.11	0.08	0.06	0.15	0.05	0.03	0.06
	X±D	0.07±0.02	0.09±0.02	0.04±0.02	0.06±0.03	0.04±0.05	0.03±0.03	0.01±0.01	0.02±0.01

OD = Optical Density, BI = Bacterial Isolates.

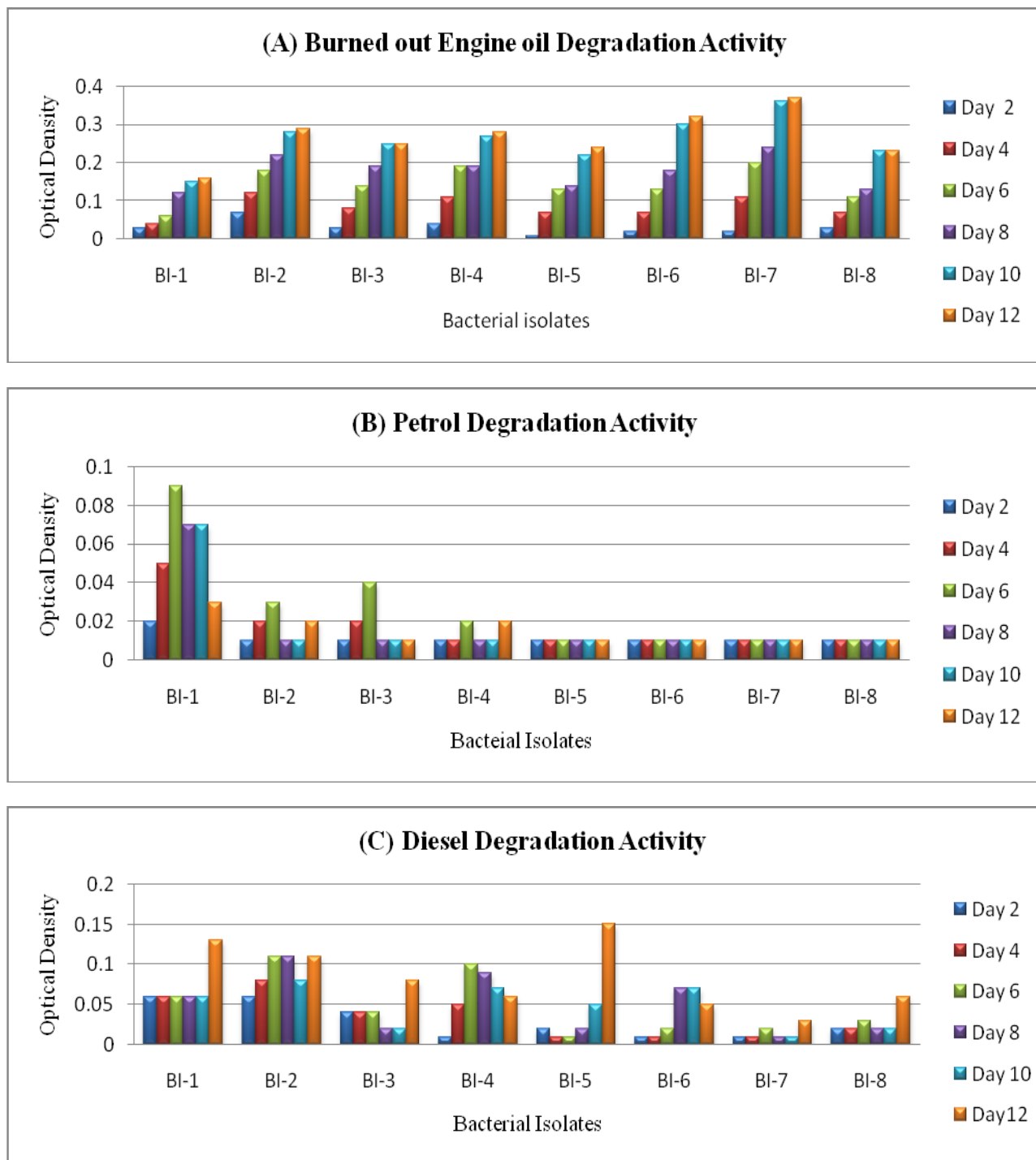


Fig. -1:- Showing degradation of Petroleum hydrocarbons in a liquid medium, (A) Burned out Engine oil, (B) Petrol, (C) Diesel.

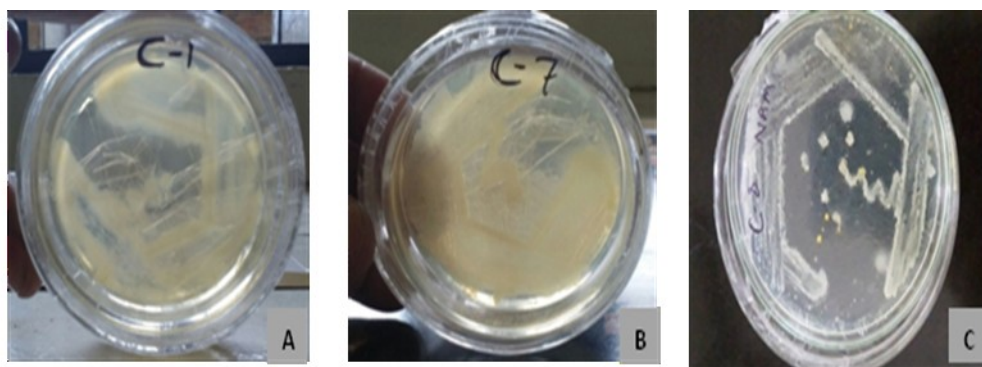


Fig. 2: Showing Selected Isolates in NAM (from Isolate 1, Isolate 7, Isolate 8).

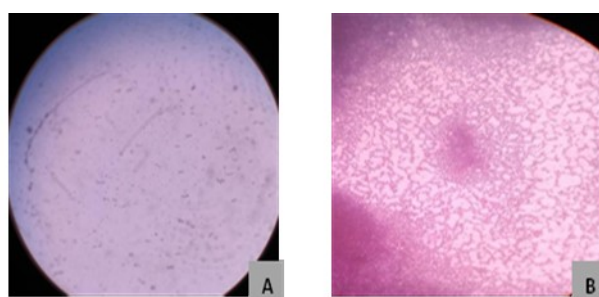


Fig. 3: Showing morphology of Bacterial Isolates- (A) Gram-positive, (B) Gram-negative.

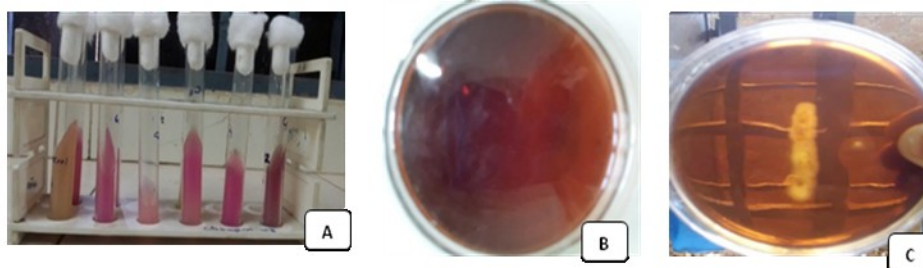


Fig. 4: Showing (A) Urease test: positive test, Starch Hydrolysis test of isolates (A) Control (C) Positive.

Discussion:-

Microbial bioremediation as well as bioaugmentation are the commonly used method for treating hydrocarbon contamination in both terrestrial and aquatic ecosystem. Delille et al., 2001, stated that as oil-degrading microbial communities are using carbon from hydrocarbons for their development, it should possible to establish a relationship between specific microbial populations and degradation indicators. In 2017, Islam and Rahman identified the *Pseudomonas* species as a prominent petrol degradative bacterium (58%), whereas, in the case of diesel and octane degradation, *Bacillus* species was found to be more effective, 79% and 54% respectively compared to the other isolates. In 2019 Abdulla et al. isolated 27 hydrocarbon-degrading bacterial isolates from 5 hydrocarbon contaminated sites.

The bacterial isolates possessed the ability to degrade a wide variety of hydrocarbons. The most competent among them was SD1 which degraded most tested hydrocarbon (98%) showing maximum growth at 3.3 gm /l of biomass concentration for 15 days incubation. In 2013, Kumari et al., isolated hydrocarbon-degrading bacteria from the contaminated soil with petrol and diesel oil. Petroleum hydrocarbon is an important energy source. Due to its demanding exploration, accidents resulting in oil spillson soil are frequent, which creates consequences for human

health and ecosystems. Based on morphological, and biochemical, characteristics bacteria were identified as *Bacillus thurigiensis*, *Bacillus pumilus*, and *Rhodococcus hoagii*, which were found as the potential for bioremediation activity. During in-vitro degradation assays, all three bacteria used petroleum hydrocarbons as the sole carbon source

In the present study, these bacterial isolates were purified from the crude oil contaminated soil sample. The hydrocarbons utilizing bacterial isolates were cultured first in a Nutrient agar medium. The observation showed that 4 (BI-2, BI-3, BI-4&BI-5) out of 8 isolates were gram-positive, and remaining 4 (BI-1, BI-6, BI-7 & BI-8) were gram-negative. Biochemical analysis like starch hydrolysis, urease production, and catalase test, and antibiotic sensitivity test was performed and most of the bacterial colonies have resulted in the positive biochemical test. BI- 1 & BI- 7 were found positive with the starch hydrolysis test, whereas, all isolates were found positive with the urease test. All isolates were obtained with a negative with catalase and antibiotic sensitivity test against streptomycin. The BI -7, BI-2; BI-4 & BI-6 out of the eight BI possessed the best growth with burned-out engine oil as a carbon source. The limitation of the present study was the non-characterization of bacterial strain at the molecular level which is essential for more scientific validation and application. Therefore, the use of microorganisms for biodegradation may be a strategy for a hydrocarbon polluted environment.

Conclusion:-

The hydrocarbon-degrading organisms are ubiquitous in the environment and they can be isolated from crude oil contaminated soil with petrol, diesel, and burned out engine oil. All selected isolates were able to degrade burned out engine oil in contrast to petrol and diesel. Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast, and microalgae, however; bacteria play the central role in hydrocarbon degradation. From the present study, it can be concluded that bioremediation is an effective, economical, and environmentally friendly treatment method in which microbes may be used to degrade hydrocarbons and suggested further study to characterize the bacterial strains on a genetic and molecular level.

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