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ISOLATION AND SCREENING OF HYDROCARBON DEGRADING BACTERIA FROM SLUDGE

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ABSTRACT

Petroleum hydrocarbons (PHs) are a large group of chemicals that cause great concern due to their diffusion in the environment, bioaccumulation potential, harmful effects, and resistance to environmental degradation. Soil and water pollution are closely linked to hydrocarbons from oil refineries, petrochemical industries, human activities, and other sources. The processes and factors that influence degradation should be more widely understood because the choice of bioremediation strategy depends on them. In the present study, the biodegradation behavior of petroleum hydrocarbon under different mitigation conditions has been investigated. N-Alkanes and poly-cyclic aromatic hydrocarbons (PAHs) degraded with NO^{3-}, Fe^{3+}, $SO4^{2-}$, or $HCO3^-$ as terminal electron acceptors (TEAs), link to four common degradation conditions (i.e., reduction of nitrate, ferric reduction, sulfate-reducing, and metanogenic conditions, respectively) in sediment. Rapid degradation rates are achieved under sulfate reduction conditions with a half life of 49.51 days in n-alkanes and 58.74 days of PAHs. Removal of petroleum hydrocarbon from tank bottom oil sludge is a major problem because of its properties. Bioremediation is considered for the release of hydrocarbon from the oil tank below the tank, the efficiency is low and time consuming due to the low yield of biocatalyst and biosurfactants. Biodegradation analysis of diesel / biodiesel compounds in liquid cultures by the petroleum degrading microbial consortium showed that in low biodiesel amendments (10%) the efficiency of the degradation compound after seven days was lower than in petroleum diesel fuel. We have considered the potential of

petroleum hydrocarbons biodegradation by 95 bacteria and 160 species of fungi separated from the previous petrochemical plant. Three species of the genetically tested GC-MS, Rhodococcus sp., Trichoderma tomentum, and Fusarium oxysporum, significantly damaged all four PAH compounds in the compound compared to control.

INTRODUCTION

In recent years, petroleum hydrocarbons have been released into the aquatic environment from anthropogenic activities such as the extraction, transport, or storage of petroleum-based products. contamination of petroleum hydrocarbons such as n- alkanes and polycyclic aromamatic hydrocarbons (PAHs), as they become a global concern due to their carcinogenicity or modification of aquatic and terrestrial organisms [2].

Petroleum hydrocarbons are a universal component of toxic pollutants collected from the environment due to a variety of anthropogenic activities.

In aquatic environments, petroleum hydrocarbons contribute to soil accumulation, low melting in water, and high concentration of soil particles. In the absence of oxygen, petroleum hydrocarbons can be biodegraded with NO^{3-}, Fe^{3+}, $SO4^{2-}$, or HCO^{3-} like other receptor electrons, associated with four common degradation conditions (i.e., nitrate reduction, ferric reduction, reduction of -sulfate.) in a sedimentary environment (compare the degraded performance of petroleum hydrocarbons with the use of different electron receivers.

[3]. Over the past two years, researchers have begun to Petroleum hydrocarbons (PHAs) are considered to be the main source of energy and materials for very different industries. Once locally it is used as a source of energy. Petroleum hydrocarbons are major environmental pollutants ranging from widespread production, offshore oil refining, offshore oil production, and spillage ping operations [5].

Bacteria, yeast, and fungi are major organisms used to pollute the environment polluted by petroleum hydrocarbons. Jones *et al*. and Pinholt *et al*. know that the hydrocarbons biodegradation capacity ranges from 6% to 82% in fungi, from 0.14% to 50% in natural bacteria, and from 0.002% to 100% in marine bacteria (https: // doi.org/10.1016/j. jece.2018.07.005).

That different levels of degradation of petroleum hydrocarbons have been identified in the nitrate-reducing <methanogenic <sulfate - sequence reduction. Confirmation of specific degradation of short chain alkane compared to long chain alkane is in the form of nitrate reduction. Our previous study recommended a special biodegradation

immersion of part of the total petroleum hydrocarbon (TPH) component with low carbon content in marine residues in nitrate reduction conditions, Although the fraction with high carbon content was significantly reduced in sulfate reduction conditions [1].

Soil pollution from petroleum hydrocarbons (diesel and fuel) is important, both in terms of health and the environment. They are highly hydrophobic by nature and penetration of oil into the soil makes the process of decomposition difficult, as hydrocarbons have a low melting point and adhere to soil particles, resulting in low bioavailability to microorganisms, which limits the rate of mass transfer to degradation [6].

The petroleum industry produces a large amount of sub-tank oil and ground oil waste, including both aliphatic and aromatic hydrocarbons,

Contents of Petroleum Hydrocarbon (TPH) and heavy metals such as nickel, chromium, zinc, manganese, cadmium, copper, and lead.

Environmental constraints and growth conditions such as temperature, pH, agitation, and ventilation of biosurfactant production and cell growth of organisms can significantly affect the rate of decomposition of microbial biodegradation of hydrocarbons.

Biosurfactants are amphipathic, surface-active compounds composed of

microorganism that reduce surface area and surface friction between the two liquids [11]. Biosurfactants emulsifiers form stable emulsions with water. These emulsifiers increase the surface area of hydrophobic moieties and increase their availability of bioavailability, thereby enhancing bacterial growth and biological repair. They also play an important role in the destruction of hydrocarbons and the removal of heavy metals Heavy metals such as arsenic, chromium, i Nickel, cadmium, and lead are present in petroleum refinery sludge [13].

Urban water pollution is a major source of water pollution and presents one of the biggest water quality challenges of the next century. Common pollutants observed in stormwater runoff include nutrients such as phosphorus and nitrogen, heavy metals, petroleum hydrocarbons, and bacteria. petroleum hydrocarbons are washed in immovable areas such as parking lots and roads with rainfall recorded at 0.2 to 277 mg / L. Petroleum hydrocarbons in such an environment are usually caused by leaks or spills of gasoline, gases, and fuel related to automotive use [4].

Biodegradation of petroleum hydrocarbons is an oxidative system proposed by enzymes that can lead to the digestion of hydrocarbon minerals, biodegradation decomposition has been observed in many areas, including under both aerobic and anaerobic conditions. Genetics that include

oxygenase code or other active enzymes specify the use of techniques such as quantitative polymerase chain reaction (qPCR).

Biodegradation plays a very important role in reducing the impact of petroleum hydrocarbons on polluted areas. After all these discarded pollutants are slightly separated under normal conditions due to certain symptoms. Petroleum hydrocarbons can be divided into 4 groups depending on the solubility of natural solvents such as aliphatic, aromatics, resin, and asphaltene. Aliphatic placed on the outer layer based on the visible model of petroleum, and aromatics and resin are responsible for the low availability of petroleum, especially part of asphaltene [12]. In addition, asphaltene is difficult to reduce due to its complex structure. Therefore, it is necessary to provide a biodegradation agent to build up the degradation of petroleum hydrocarbons in areas [13].

Widespread exploitation, transportation, and the use of crude oil have put public attention at the conclusion of petroleum hydrocarbon (PHC) compounds in the environment. Crude oil contains a complex mixture of thousands of chemicals, as well as aromatics such as polycyclic aromatic hydrocarbons (PAH) compounds. Polycyclic aromatic hydrocarbons (PHAs) are important for human health and the environment, after all, serious persistence

can adversely affect soil function, and threaten biodiversity [15].

Several technologies have been developed to improve PHC contaminated sites, including physical, chemical, and biological methods. The use of organic matter to improve polluted environments, also known as bioremediation, has attracted great interest in research over the past decade, as the ongoing and cost-effective variation of chemical treatments [17]. Biodegradation of petroleum hydrocarbons (PHCs) by natural bacteria and fungi is well known as many microorganisms are able to utilize hydrocarbons as both energy sources and carbon.

However, determining the biodegradation potential of microorganisms remains a challenge, after all major pollutants have been degraded much less than parts of the labile. In addition to abiotic substances that limit total degradation, a native microorganism that can completely tolerate and quickly destroy petroleum hydrocarbons (PHCs) may be present in very small quantities, or can only degrade certain compounds [10]. To gradually affect the decay of Petroleum hydrocarbons in the soil, the adjustment to nutrient concentration, pH, oxygen, and temperature (biostimulation), and microbial inoculation (bioaugmentation), can be used .

For bioaugmentation to be effective, injected microorganisms must tolerate

pollution, effectively reduce beneficial compounds, and thrive in the target area [9]. These microbial factors have been evaluated using culture-based materials, molecular mechanisms, and chemical analysis techniques such as gas chromatography-mass spectrometry (GC-MS). After all, some of these methods are important, colorimetric methods have also been used to quickly measure the biodegradation volume of microorganisms.

Recent studies have shown that compared with diesel fuel, biodiesel is more susceptible to bacteria. Some authors describe the synergistic effects of biodiesel in addition to fossil-based fats, suggesting that biodiesel affects biodegradation decay by co-metabolic modification of hydrocarbons [1, 2]. On the other hand, DeMello *et al*. (2007) noted that hydrocarbon biodegradation did not accelerate the presence of biodiesel. Prince *et al*. (2008) found that the biodegradation half-life of fatty acid methyl esters in the B20 blend was similar to that of alkanes. In addition, Taylor and Jones (2001) explained that biodiesel supplements increased the melting of coal-based hydrocarbons, but the rate of degradation was even higher when petroleum diesel supplements were used. UDeMello *et al*. (2007) previously tested and found that modification of biodiesel may result in the distribution of diesel fuel in water. Also,

the effect of rhamnolipids as a well-studied biosurfactant on the degradation of biodiesel blends and the stability of emulsion blends was investigated.

The presence of Petrochemicals in the area endangers human health due to its toxic, carcinogenic, mutagenic effect, and efficiency of accumulation in the food chain. Biodegradation, that is, the effective use of antimicrobials in reducing the risk of harmful chemicals, has become an interesting technology compared to the many available physio-chemicals, energy efficiency, labor requirements, final costs, and the use of chemical reagents to decompose petroleum [6].

Molds have a strong destructive effect on petroleum hydrocarbons. The ability of fungi to disperse pollutants is enhanced by harmful metals, metalloids, and radionuclides. They are known for performing chemical repairs and later improving the bioavailability of pollutants. The fungus can be used in conjunction with bacteria, other fungi, and plants such as endo or ectomycorrhizal degradation of hydrocarbon biodegradation.

Hydrocarbon biodegradation is highly concerned with nutrient availability, pH, air, temperature, light flow, and the number of active insects. These factors affect the number, composition, and function of the hydrocarbon-degrading microbial association in the area.

Modification of nutrients in the soil to improve the degradation of petroleum hydrocarbons by microorganisms has been determined, but there is a need for research work to determine the effect of light combined with nutrients on hydrocarbon depletion and biomass production **[8]**.

The dangers of oil pollution today are a simple one and have triggered natural and man-made disasters. And accidental pollution of the ecosystem, large amounts of oil spills created from refineries from the seawater separation system and the accumulation of crude oil in the buttocks of the crude oil storage tank poses a major problem because many of the basic treatment processes used to seal soil and groundwater. they are limited in their use which is more expensive, or perhaps less efficient.

Both local and regional therapies link the use of microbes to eradicate economic and technological corruption. Bacillus subtilis DM-04 and Pseudomonas aeruginosa M and NM, separated from the North-East India petroleum-contaminated soil sample (ONGC oil field) builds on a large number of hydrocarbons as a source of carbon and energy. this. species may be able to degrade hydrocarbon. These species have been able to produce biosurfactants from the rich hydrocarbons of the middle class.

MATERIALS AND METHODS

sample was collected from sludge collection pit, in a clean and dry sterile container. Petrol sample was also collected from the recognized petrol pump. nutrient agar medium contains the following: distilled water (1 L), trace element mixture (5 mL), NH_4NO_3 (1.0 g), $CaCl_2$ (0.02 g), Mg_2SO_4 (0.05 g), K_2HPO_4 (1.0 g)), and KH_2PO_4 (1.0 g). The combination of trace elements (pH 7.0) includes the following: $CaCl_2$ (2 mg / L), $FeCl3 \cdot 6H_2O$ (50 mg / L), $MnCl_2 \cdot 4H_2O$ (0.5 mg / L), $ZnSO4 \cdot 7H_2O$ (10 mg / L), and and $CuSO_4$ (0.5 mg / L). In solid medium, bacterial agar (20 g / L) was added to Nutrient agar medium. All media was automatically installed at 121 ° C for 30 minutes. Rotary shaker was used to incubate the liquid culture at 170 rpm, and for both solid and liquid cultures at 30 ° C.

Isolation and selection of engine-oil-degrading bacteria:-

A total of 10 g of contaminated soil samples were Mixed in sterile water in a 250-mL conical flask (100 mL). The flask was allowed to stand for 20 minutes after adequate shaking and blending, and the supernatant (10 mL) was extracted, added to nutrient agar medium (100 mL) containing 0.5 percent (w/v) petrol and engine oil (engine oil) as the only energy and carbon source, and incubated in a rotary shaker for 7 days at 30 °C and 170 rpm. The supernatant (10 mL) was then

extracted again and put to fresh medium containing 1.0 percent crude oil (w/v). a set to 20°C [16].

After incubation on LB agar slant for 24–48 hours, the morphological characteristics of the isolated colonies were examined using optical microscopy. Following that, in accordance with Bergey's Manual for Determinative Bacteriology and related investigations, a systematic study of the isolated strains was carried out based on physiological and biochemical features [15, 16].

Preparation of bacterial mixtures:-

We chose oil-degrading strains that were extremely efficient and had better environmental tolerances, as well as ones that we thought would give good results in the test. After that, we created various mixed bacterium agents and investigated their degrading effects on crude oil with a concentration of 1.5 percent (w/v). For the next tests, we chose agents with the best degrading effects [3].

Individual colonies were injected into LB liquid medium and cultured overnight in a shaker at 180 rpm and 30 °C. The bacterial solution was dispersed onto an LB plate using the dilution plate method, and the number of colonies was counted. This technique was repeated until a CFU/g concentration of greater than 108 was reached. To make the bioremediation reagent, the same amount of each strain was mixed together.

A total of 5% (v/v) of bacterial suspension was injected into 1.5 percent (w/v) crude oil medium and shaken for 14 days at 30°C and 180 rpm. A sterile media was used as a control. After incubation, the optical density of the culture at 600 nm was determined using UV-visible spectrophotometry (UV-9600, USA). To determine engene oil removal, the leftover crude oil was dissolved in dichloromethane (DCM) and the optical density of the oil extracts was measured at 230 nm [9].

RESULT:

Sample	Growth characteristics on EMB agar plate
1	Pale coloured colonies liquidous observed
2	Pink colour mucoid colonies observed
3	Pink coloured colonies
4	Pink colour colonies

sample	Growth characteristic on XLD agar
1	No growth
2	No growth
3	Red colour colonies were observed
4	Yellow opaque colonies observed

Sample	Motility	Gram's staining
1 E	Motile	-ve
2 P	Motile	-ve
3 K	Non motile	-ve
4 V	Non motile	-ve

Characteristics	E	P	K	V
Size	Small	Small	Medium	Medium
Shape	Circular	Round	Round	Circular
surface	Smooth	Smooth	Smooth	Smooth
elevation	Convert	Convert	Raised	Raised
Consistency	Mucoid	Moist	Viscous	Moist
opacity	translucent	translucent	Translucent	Translucent
pigmentation	Green	No pig.	No pig.	No pig.

| Sample	Growth characteristics on MacConkey's agar plate
E	Lactose nonfermented pale coloured colonies
P	Lactose fermented colonies
K	Lactose nonfermented colonies
V	Lactose non fermented colonies

- ## OD RESULTS:

sample	Days	Petrol		Oil	
		OD	OD rate (%)	OD	OD rate (%)
E	1	0.120	-	0.10	-
	2	0.130	8.3	0.113	10
	3	0.150	25	0.123	23
	4	0.168	40	0.132	32
	5	0.175	45	0.142	40
	6	0.179	49.16	0.147	47
	7	0.182	51.66	0.153	53
	8	0.189	57.5	0.160	60
	9	0.193	60.83	0.165	65
	10	0.197	64.16	0.169	69
	11	0.208	73.33	0.172	72
	12	0.212	76.66	0.178	78
	13	0.218	81.66	0.181	81.66
	14	0.223	85.83	0.186	86

sample	Day's	Petrol		Oil	
		OD	OD rate(%)	OD	OD rate
P	1	0.35	-	0.20	-
	2	0.406	16	0.216	8
	3	0.415	18.57	0.223	11.5
	4	0.423	20.85	0.260	30
	5	0.463	32.28	0.275	37.5
	6	0.486	38.85	0.291	45.5
	7	0.512	46.28	0.312	56
	8	0.523	49.42	0.316	58
	9	0.529	51.14	0.323	61.5
	10	0.536	53.14	0.327	63.5
	11	0.586	67.42	0.338	69
	12	0.598	70.85	0.349	74
	13	0.605	72.85	0.354	77
	14	0.619	76.85	0.362	81

Sample	Day's	petrol		Oil	
		OD	OD rate(%)	OD	OD rate(%)
k	1	0.14	-	0.11	-
	2	0.160	14	0.124	12.72
	3	0.175	25	0.135	22
	4	0.187	33.57	0.146	32.72
	5	0.192	37.14	0.153	39.09
	6	0.208	48.57	0.161	46.36
	7	0.212	51.42	0.172	56.36
	8	0.218	52.85	0.179	62.72
	9	0.221	57.85	0.183	66.36
	10	0.229	63.57	0.186	69.09
	11	0.238	70	0.196	78.18
	12	0.242	72.85	0.201	82.72
	13	0.257	83.57	0.205	86.36
	14	0.268	91.42	0.208	89.09

Sample	Day's	Petrol		Oil	
		OD	OD rate(%)	OD	OD rate(%)
V	1	0.30	-	0.10	-
	2	0.343	14.33	0.114	14
	3	0.360	20	0.123	23
	4	0.372	24	0.132	32
	5	0.392	30.66	0.143	46
	6	0.408	36	0.149	49
	7	0.413	37.66	0.156	56
	8	0.426	42	0.161	61
	9	0.436	45.33	0.167	67
	10	0.462	54	0.170	70
	11	0.483	61	0.179	79
	12	0.496	65	0.181	81
	13	0.514	71.33	0.184	84
	14	0.522	74	0.187	87

- **Degradation rate of petrol after 14 days (petrol)**
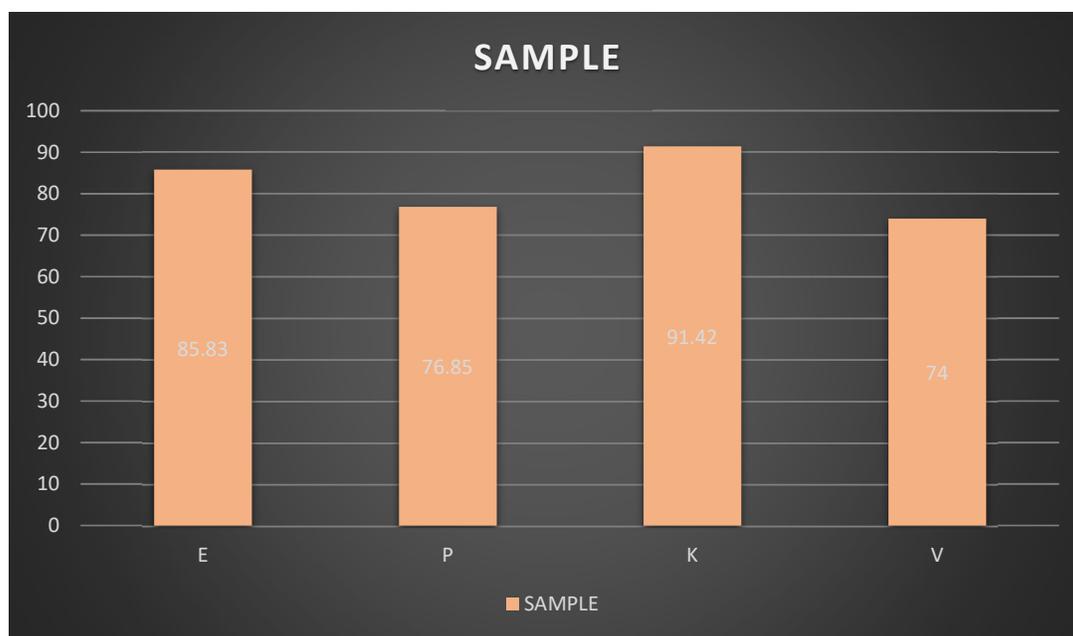
(x axis- sample, y axis – OD rate in %)
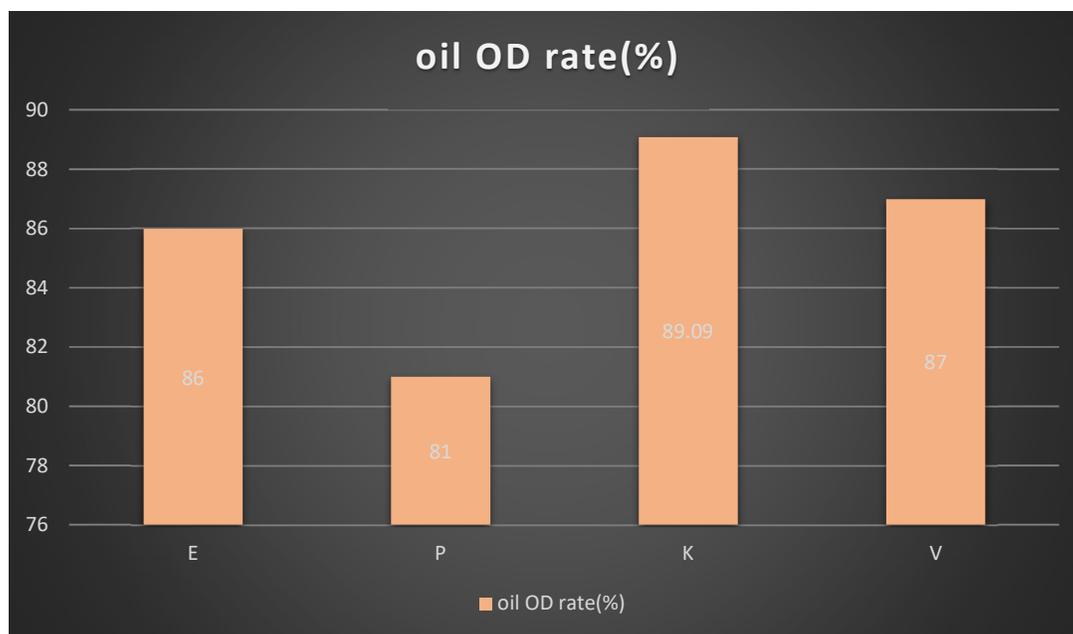
(X axis- sample, y axis- OD rate in%)

Biochemical Test:-

Test	Medium	Reagent	Appearance	Sample E		Sample P		Sample K		Sample V	
Indole test	1 % peptone	Kovac's R.	Red ring	-	-	-	-	-	-	+	+
Methyl Red	GPB	Methyl Red	Red colour	-	-	-	-	+	+	+	+
Voges Proskaver's	GPB	Alpha nepthol + 40% KOH	Cherry Red	-	-	+	+	-	-	-	-
Citrate Test	Simmon citrate slant	Bromothymol blue	Blue colour	+	+	+	-	-	-	V	+
Gelatin test	Nutrient gelatin	-	Liquefied after freezing	-	-	-	-	-	-	+	-
H2S production	2% peptone	Lead acetate paper	Blocking of lead acetate paper	v	-	-	-	-	-	-	+
Urea test	Urea broth	Phenol red	Pink colour	-		+		-		+	
Catalase test	Nutrient agar	H2O2	Effervesce	+	+	+	+	+	+	+	+
Oxidase test	Oxidase disc	Tetra methyl phospho phenyl dimethyl dichloride	Dark blue colour	+	+	+	+	+	+	+	+
sugar	glucose	Andrad's reagent	Acid + gas	-	-	+	+	+	+	+	+
	Lactose			-	-	+	-	v	-	+	+
	Sucrose			-	-	+	-	v	-	+	+
	Xylose			-	-	+	-	+	+	+	+
	Mamitol			+	-	+	+	+	-	-	+

DISCUSSION

Petroleum hydrocarbons are the most dangerous pollutants in the environment, and they will continue to be the most important energy and chemical source, as well as the most difficult organic

pollutants, in the future [11, 13]. Currently, a green alternative method based on the idea of petroleum hydrocarbon degradation by indigenous or exogenous microorganisms to bioremediate and treat pollutants safely is progressively evolving, which is both practical and required given current technological and societal conditions [12]. From the contaminated environment, various types of possible strains have been isolated [16, 18]. We identified four bacterial strains from oil/petrol-polluted for our study. By the conclusion of the 14-day incubation period, all of the samples had virtually completely decomposed. In 14 days, about 1gm of material can be degraded. The time it takes for a sample to degrade is determined by the kind of sample, culture, and incubation conditions. **Figure** shows that these strains digest 81–89.09% percent of engine oil, demonstrating that they have a substantial potential for degrading petroleum hydrocarbons in water. We can observe from *Proteus vulgaris* caused the most degradation of sample A, 89% in 14 days, and *Enterobacter aerogenes* caused the least, 81 %, and *Pseudomonas fluorescens* and *Shigella sonnei* caused the amount of degradation, 86 % and 87% respectively. Previous research has revealed that bacterial strains could be employed to break down petroleum compounds. *pseudomonas fluorescence*, for example,

degraded hydrocarbons at differing rates in liquid medium and soil after 14 days (85.83 percent and 86%, respectively [16]. *Proteus vulgaris* has the maximum number of degradation at the end of 14 days Nonetheless, a single bacterial strain's hydrocarbon breakdown rate is high, which could be due to the fact that some bacteria can only eliminate specific petroleum compounds. *Proteus vulgaris* has the largest amount of degradation at the end of 14 days. *Proteus vulgaris* degraded sample C the most (91.41% in 14 days) and *Shigella sonnei* degraded it the least (74% in 14 days), and the degradation rates of *Enterobacter aerogenes* and *Pseudomonas fluorescens* were the same (76.85%and 85.83%, respectively). We made consortium of all four cultures. Inoculated with 1gm of sludge sample, *Pseudomonas fluorescens + Enterobacter aerogenes + Shigella sonnei + Proteus vulgaris* showed 72 percent degradation in 10 days and 99 percent in 14 days. It is possible to prefer a consortium to a single culture.

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