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Isolation and Study of Biodegradation Capability of Hydrocarbanoclastic Bacteria by Gravimetric Analysis from Waste Engine Oil Impacted Soils of Auto Nagar, Guntur, Andhra Pradesh

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A B S T R A C T

Research was carried out on Isolation and Study of Biodegradation Capability of Hydrocarbanoclastic Bacteria from Waste Engine Oil Impacted Soils of Auto Nagar, Guntur, Andhra Pradesh. Soil samples are collected from the waste engine oil contaminated sites of Auto Nagar, Guntur, Andhra Pradesh. Soil samples was enriched with BH medium and bacterial cultures were isolated (GAS1, GAS2, GAS3, GAS4, GAS5) and purified using MS medium and tested their ability to degrade various hydrocarbons (Petrol, Diesel, Paraffin oil, Benzene, Toluene, Turbine oil, Heavy machinery oil) using DCPIP method. The isolates were found satisfactory in degrading hydrocarbons among the all isolate GAS3 was more potential in degrading provided all hydrocarbons. GAS3 was selected to study the degradation of various hydrocarbons using Gravimetric analysis results showed that high potential in degradation of hydrocarbons with 90% of Turbine oil, 88% of Paraffin oil, 85% heavy machinery oil, 70% of diesel, 59% of petrol, 38% of benzene, 30% of toluene respectively. Based on the morphology potential isolate was identified as gram-negative bacterium.

Introduction

Hydrocarbon degradation bacteria are microorganisms that have the ability to break down hydrocarbons, which are compounds composed of hydrogen and carbon atoms. These bacteria play a crucial role in the degradation of petroleum products, such as oil and gasoline, and other hydrocarbon-based pollutant. As a non-renewable strategic resource, oil plays a key role in ensuring international economic, social development and human welfare. Microorganisms which biodegrade the various components of petroleum hydrocarbons such as polynuclear aromatic hydrocarbons (PAHs), naphthalene, monoaromatic hydrocarbons such as toluene, or aliphatic hydrocarbons such as the n-alkanes, can be readily isolated from the environment, particularly from petroleum-contaminated sites (Lyle G., *et al*. 1997). The microorganisms can be obtained originally by enrichment culture procedures, where maximum specific growth rate or maximum final cell concentration can be used as the selection criterion. Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae [Riser -Robertz E 1992: Bundy JG *et al*.,2004]. However, bacteria play the central role in

hydrocarbon degradation. The present work was aimed at determining the hydrocarbon degrading microorganisms present in the oil contaminated sites from the Gujarat, India and to study the growth responses of the bacterial isolates to different hydrocarbons along with their characteristics.

In this context the study is extended based on the biodegradation of various hydrocarbons and industrial oils in order to find the indigenous bacterial cultures and the ability of degradation by gravimetric analysis.

Materials and Methods

Taking into these considerations the work was designed to study the following parameters.

Collection of soil sample

Soil samples (100 g) from surface soil (0-15 cm depth) were collected from 3 different locations from Autonagar, Guntur, Andhrapradesh (Gps coordinate Fig 1, 2,3 [Lat 16.32097° Long 80.477492°], [Lat 16.321838º Long 80.479333º] and [Lat 16.321232º Long 8o.483313º]. Samples were taken from 3-4 random locations per plot, mixed, and transferred into sterile plastic bags using a sterile spatula. Samples were stored in a refrigerator at 5ºC for experiment.

Enrichment of soil sample for hydrocarbon degrading bacteria

Media Preparation

Bushnell-Haas (BH) medium composition [Minimal salts medium [MSM]]

Compound	W/V
CaCl2	0.02 g/l
K2HPO4	1 g/l
KH2PO4	1 g/l
MgSO4	0.2 g/l
NH4NO3	1 g/l
FeCl3	0.05 g/l
pH	7.0 ± 0.2
Hydrocarbon (Diesel)	1g/l

Ingredients are dissolved in distilled water and 100 ml of medium was distributed in Erlenmeyer conical flasks and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After cooling added 3 grams of soil sample in each conical flask and 1% hydrocarbon incubated it for 7 -8 days on rotary shaker at 100 rpm. Enrichment was done periodically at weekly intervals.

Isolation of hydrocarbon degrading bacteria

Bushnell-Haas (BH) Agar medium composition [Minimal salts Agar medium [MSM]]

Compound	W/V
CaCl2	0.02 g/l
K2HPO4	1 g/l
KH2PO4	1 g/l
MgSO4	0.2 g/l
NH4NO3	1 g/l
FeCl3	0.05 g/l
pH	7.0 ± 0.2
Hydrocarbon (Diesel)	10g/l
Agar Agar	20 g

Ingredients are dissolved in distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes before pouring added 1% hydrocarbon and prepared MSM Agar plates. Inoculated the plates with 0.2ml of enriched culture and incubated for 72 hours at 37°C. After incubation based on the morphology five different colonies are identified and transferred into freshly prepared MSM Agar slants and incubated at 37°C for 48hrs and sub cultured periodically and stored in refrigerator. Morphologically colonies are identified and purified on MSM agar media with diesel were finally considered as pure cultures and the isolates was named as GAS1, GAS2, GAS3, GAS4 and GAS5.

Screening of the isolates for the degradation of various hydrocarbons by dcpip method

2,6 dichlorophenol Indophenol (dcpip test), DCPIP is a blue redox dye used to assist ability of microorganism to degrade hydrocarbon as change of colour from blue(oxidized) to colorless (reduced) indicating electron transfer during hydrocarbon oxidation it is artificial electron acceptor that is used to monitor redox reaction in microorganism that can degrade hydrocarbon and used as a (carbon) energy source during this process electrons are transferred from the hydrocarbon to electron acceptor such as dcpip when it accepts electron changes from blue oxidized state to colorless (reduced state) the loss of blue color indicates that microorganism are actively degrading hydrocarbons.

Preparation of Culture Medium and Inoculation with bacteria

Prepare Minimal salts Medium [MSM] from the stock add 2ml of MSM broth into a series of eight test tubes and added hydrocarbons 20 µl of Diesel, Petrol, Benzene, Toluene, Paraffin oil, Turbine oil, Heavy machinery oil into separate tubes as sole carbon source and 1 ml of test bacterial strain was inoculated the medium in such away experiment was setup for five isolates with a control (uninoculated medium with 2,6 dichlorophenol Indophenol to compare result).

Addition of 2,6 dichlorophenol indophenol indicator

2,6 dichloro phenol indophenol indicator 20 µl was added to all the test tubes and mixed well to ensure uniform distribution. Check the ability of microorganisms to use the substrate by observing the color change of DCPIP.

Oil spreading test or bio surfactant production test

Oil spreading technique was used to detect the oil displacement activity (ODA) of the isolates. The oil spreading test, used to assess the biosurfactant-producing capacity of hydrocarbon-degrading bacteria, involves observing the formation of a clear zone around a droplet of crude oil on water, with a larger zone indicating greater biosurfactant activity.

Steps

Seven Petri dishes was filled with a certain volume of distilled water. A small volume of hydrocarbon sources (petrol, diesel, benzene, toluene, paraffin oil, turbine oil, heavy machinery oil) is placed on the surface of the water. A sample of the bacterial culture supernatant (the liquid remaining after the bacteria have been removed) is added to the oil and water mixture. The diameter of the clear zone that forms around the oil droplet. A larger clear zone diameter indicates that the bacterial culture supernatant contains a higher concentration of biosurfactants, which are capable of spreading the oil. The oil spreading test and other biosurfactant screening methods are useful for identifying bacteria with the potential to degrade oil and other hydrocarbons. These bacteria can be used in bioremediation efforts to clean up oil spills and other hydrocarbon contamination.

Calculation of emulsification index of various hydrocarbons using Gas3

The Emulsification index (EI) is expressed as a percentage of the height of the emulsion layer to the total original height of oil in the tube. The result of a control assay is included to show no emulsification of the oil. The emulsification index (EI) assay, often represented as E24, is a test to determine the emulsifying ability of a biosurfactant by measuring the height of the stable emulsion layer after mixing oil and cell-free culture broth, expressed as a percentage of the total liquid height. The assay is used to screen for and quantify the ability of biosurfactants to emulsify (or stabilize the dispersion of) hydrocarbons, which are hydrophobic (water-repelling) substances. Biosurfactants, produced by certain microorganisms, can reduce the surface tension between oil and water, allowing them to mix and form a stable emulsion.

A known volume of a cell-free culture broth (containing potential biosurfactants) is mixed with an equal volume of an oil substrate (petrol, diesel, benzene, toluene, paraffin oil, turbine oil, heavy machinery oil). Take 2 ml of culture broth and take 2 ml of oil and shake the mixture for some time and leave it for separation of liquid and solid contents. The mixture is vortexed to create a suspension, and then allowed to stand for a specific time (usually 24 hours, hence the "E24" designation). After the incubation period, the height of the stable emulsion layer is measured, and the emulsification index (EI) is calculated.

Calculation:

The EI is calculated as: *(height of the emulsion layer / total height of the liquid column) x 100.*

A higher EI value indicates a greater ability of the biosurfactant to emulsify the oil substrate.

Gravimetric analysis of hydrocarbons using GAS3 (hydrocarbon degradation assay)

MSM medium incubated with GAS3 and various hydrocarbons for a period od of 10 days is used to study the degradation potential of hydrocarbons by gravimetric analysis. To this. Petroleum ether and acetone were taken

in the ratio 1:1 and was mixed with the experimental contents in a separating funnel. The mixture was shaken for about 45 minutes and then was left undisturbed for about 10 minutes. The upper solvent along with oil was separated from the lower extract. The solvent with the oil layer was then kept in the hot air oven at 50º C until the solvent gets evaporated. After the complete evaporation, the oil residue obtained was weighed and taken as the gravimetric value for further calculation. Analysis of hydrocarbon before and after treatment was done using this Gravimetric method (Saxena, 1990).

The percentage of oil degraded was determined from the following formula:

Percentage of oil degradation = Amount of crude oil degraded /Amount of oil added X 100

Amount of crude oil degraded = (Weight of crude oil added in the media)- (Weight of residual crude oil) Weight of Residual crude oil= (Weight of beaker containing extracted crude oil) – (Weight of empty beaker).

Study of morphology

The colony characteristics and cellular morphology of the isolate was identified by Gram staining.

Results and Discussion

1.Soil collection: soil collection sites are represented in figures along with GPS coordinates.

Fig. 1, 2, 3. Soil Samples Collected locations

Fig.4 Soil & Oil samples

Enrichment technique

Soil samples were enriched using MSM medium with 1% hydro carbon (diesel) for a period of one week and repeated the step for three weeks. Experimental setup is represented in Fig 5 & Fig 6.

Isolation of hydrocarbon degrading bacteria

After incubation based on the morphology five different colonies are identified and transferred into freshly prepared MSM Agar slants and incubated at 37°C for 48hrs and sub cultured periodically and stored in

refrigerator. Morphologically colonies are identified and purified on MSM agar media with diesel were finally considered as pure cultures and the isolates was named as GAS1, GAS2, GAS3, GAS4 and GAS5. Results are represented in fig 7.

Fig.5 & 6 Enrichment culture before and after incubation

Fig.7 Bacterial isolates

Screening of the isolates for the degradation of various hydrocarbons by DCPIP method

Screening of the isolates for the degradation of various hydrocarbons by DCPIP method for the five isolates named GAS1, GAS 2, GAS3, GAS4 & GAS5 with various hydro carbons including diesel, petrol, benzene, toluene, paraffin oil, turbine oil, heavy machinery oil. 2,6 dichlorophenol Indophenol (dcpip test), DCPIP is a blue redox dye used to assist ability of microorganism to degrade hydrocarbon as change of colour from blue(oxidized) to colourless (reduced) indicating electron transfer during hydrocarbon oxidation it is artificial electron acceptor that is used to monitor redox reaction in microorganism that can degrade hydrocarbon and used as a (carbon) energy source during this process electrons are transferred from the hydrocarbon to electron acceptor such as dcpip when it accepts electron changes from blue

oxidised state to colourless (reduced state) the loss of blue colour indicates that microorganism are actively degrading hydrocarbons. Results are represented in fig 8 (fig 8.1, fig 8.2, fig 8.3, fig 8.4, fig 8.5) and in Table 1, 2, 3, 4 &5 Among all the isolates analysis GAS 3 is potential inreducing various hydrocarbons in the short period of the time followed by GAS2, GAS5, GAS4 & GAS1.

Oil spreading test or bio surfacatant production test

Oil spreading technique was used to detect the oil displacement activity (ODA) of the isolates. The oil spreading test, used to assess the biosurfactant-producing capacity of hydrocarbon-degrading bacteria, involves observing the formation of a clear zone around a droplet of crude oil on water, with a larger zone indicating greater biosurfactant activity. The ability of biosurfactant production for GAS3 was analysed and showed clear zone around the droplet of all hydrocarbon oils on water. Results are represented in fig 9.

Calculation of Emulsification Index of Various Hydrocarbons using Gas3

After the incubation period, the height of the stable emulsion layer is measured, and the emulsification index (EI) is calculated. Results are represented fig 10 &11, Table 6, 7& 8.

Calculation: The EI is calculated as: (height of the emulsion layer / total height of the liquid column) x 100.

Study of Morphology of Isolates

The colony characteristics and cellular morphology of the isolated culture and their pigmentation, staining reagent by solvent methods and the isolates were identified. Results are represented in fig 15 and table 10.

Fig 8.1 DCPIP reduction for GAS 1 isolate at 15, 30 & 45 minutes

Fig 8.2.2 DCPIP reduction for gas 2 isolate at 15,30&45 minutes

Fig 8.3. DCPIP reduction for GAS 3 isolate at 15, 30 & 45 minutes

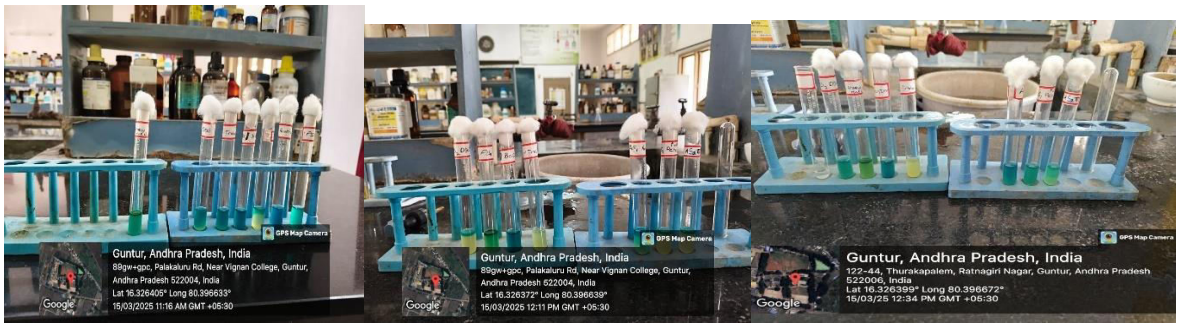

Fig 8.4. DCPIP reduction for GAS 4 isolate at 15, 30 & 45 Minutes

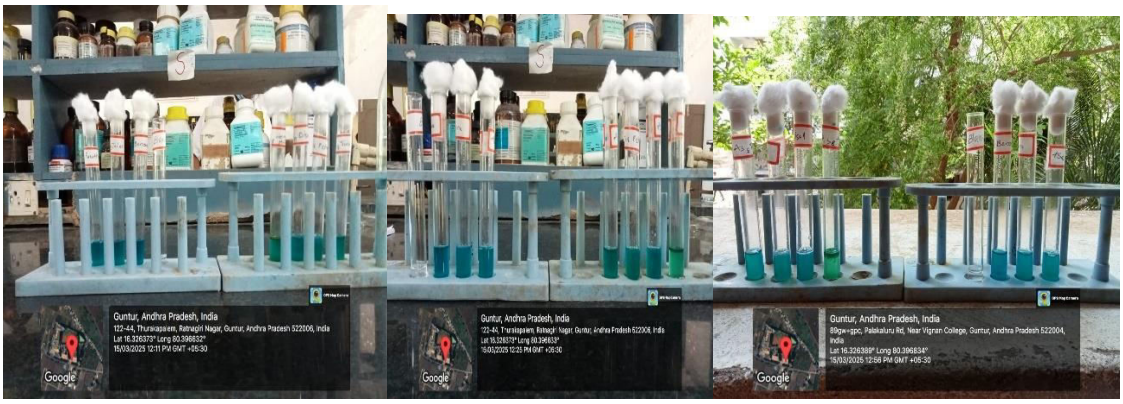

Fig 8.5 DCPIP reduction for GAS 5 isolate at 15, 30 & 45 minutes

Table.1 DCPIP analysis for (GAS 1 culture)

S.No.	Hydrocarbons	15 mins	30 mins	45 mins
1	Diesel	_	_	_
2	Petrol	_	_	_
3	Benzene	_	_	_
4	Toluene	_	_	_
5	Paraffin oil	_	_	_
6	Turbine ol	_	_	_
7	Heavy mechinary oil	+	+	+

(- = No reduction, + = Reduction, +++ = More discolouration)

Table.2 DCPIP analysis for (GAS 2 culture)

S.No.	Hydrocarbons	15 mins	30 mins	45 mins
1	Diesel	_	+	++
2	Petrol	_	_	_
3	Benzene	_	_	_
4	Toluene	_	+	++
5	Paraffin oil	+	++	+++
6	Turbine oil	+	++	+++
7	Heavy mechinary oil	+	++	+++

Table.3 DCPIP analysis for (GAS 3 culture)

S.No.	Hydrocarbons	15 mins	30 mins	45 mins
1	Diesel	_	_	+
2	Petrol	+	++	+++
3	Benzene	+	++	+++
4	Toulene	+	++	+++
5	Paraffin Oil	+	++	+++
6	Turbine oil	+	++	+++
7	Heavy Mechinary Oil	+	++	+++

Table.4 DCPIP analysis for (GAS 4 culture)

S.No.	Hydrocarbon	15 mins	30 mins	45 mins
1	Diesel	_	_	_
2	Petrol	_	_	_
3	Benzene	_	_	_
4	Toluene	_	_	_
5	Paraffin oil	_	_	_
6	Turbine oil	_	_	_
7	Heavy mechinary oil	+	++	+++

Table.5 DCPIP analysis for (GAS 5 culture)

S.No.	Hydrocarbons	15 mins	30 mins	45 mins
1	Diesel	–	–	–
2	Petrol	+	++	++
3	Benzene	–	–	–
4	Toluene	–	–	–
5	Paraffin oil	–	+	+
6	Turbine oil	-	-	-
7	Heavy mechinary oil	+	++	+++

Fig.9 Biosurfactants assay

Table.6 Initial Emulsification index

S.No.	Hydrocarbons	Emulsion Column (cm)	Total Liquid Column (cm)	EI
1	Petrol	1	2.5	40
2	Toluene	1	2.2	45.45
3	Benzene	1.3	2.8	46.42
4	Turbine oil	2	2.3	86.9
5	Paraffin oil	1.8	3.1	58.06
6	Diesel	1.2	2.7	44.44
7	Heavy machinery oil	1	2.3	43.47

Fig.10 Emulusification

Table.7 After incubation emulsification index

S.No.	Hydrocarbons	Emulsion Column (cm)	Total Liquid Column (cm)	Ei
1	Petrol	0.8	1.8	44.44
2	Toluene	0.6	1.6	37.5
3	Benzene	0.1	1.4	7.14
4	Turbine oil	0.8	2.1	38.09
5	Paraffin oil	1.3	2.3	56.52
6	Diesel	1	2	50
7	Heavy machinery oil	0.8	2	40

Table.8 Analysis of EI

S.No	Hydrocarbons	Initial Value	After Incubation
1	Petrol	40	44.44
2	Toluene	45.45	37.5
3	Benzene	46.42	7.14
4	Turbine oil	86.95	38.09
5	Paraffin oil	58.06	56.52
6	Diesel	44.44	50
7	Heavy machinery oil	34.78	40

Fig 11 Gravimetry

Table 9 Gravimetric analysis of Hydrocarbons Degradation by Gas 3 Isolate

S.No	Hydrocarbon (1%)	Weight of beaker (g)	Weight of beaker + residual oil (g)	Residual oil (wt of beaker(g) – wt of beaker + residual oil	Oil degraded (g)	% of oil degraded
1	Benzene	94.05	94.67	0.62	380	38%
2	Toluene	100.25	101.22	0.97	30	30%
3	Paraffin oil	95.36	95.48	0.12	880	88%
4	Diesel	93.56	93.86	0.30	700	70%
5	Petrol	99.59	100.00	0.41	590	59%
6	Turbine oil	94.85	94.95	0.10	900	90%
7	Heavy mechaniery oil	99.24	99.39	0.15	850	85%

Fig 12 Gram Staining of Gas3

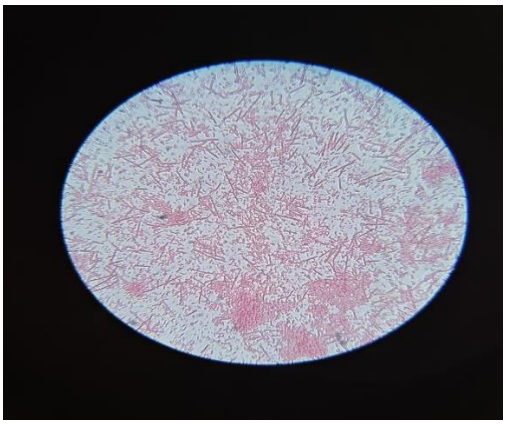

Table.10 Physical Charecterization of the Isolates

Tests	GAS1	GAS2	GAS3	GAS4	GAS5
Colony colour	Creamy white	Cream	Greenish white	white	Creamy white
Configuration	Waxy	mucoid	Metalic sheen	waxy	Metallic sheen
Elevation and margin	Convex circular	Smooth rasied and circular	Large opaque, irregular	Smooth round and opaque	Rasied circular
Gram`s reaction	Positive, rods	Positive, rods arranged in pairs	Negative, slightly curved rods	Positive, cocci	Positive, cocci pairs
Motility	Motile	motile	Motile	Non motile	Non motile
Spore staining	Yes	Yes	No	No	No

From the above analysis GAS 3 isolate degraded all the provided hydrocarbons efficiently and used them as sole source of carbon for the metabolism. Among all the hydrocarbons GAS3 isolate consumed 90% of Turbine oil, 88% of Paraffin oil, 85% heavy machinery oil, 70% of diesel, 59% of petrol, 38% of benzene, 30% of toluene respectively. Hydrocarbon degrading bacteria are abundantly and ubiquitously found in hydrocarbon-contaminated soils (Saadoun I, 2002) and can be isolated using BH medium. This is because those bacteria can easily adapt to the hydrocarbon-contaminated sites and use the contaminant as a source of carbon and energy for their metabolism and growth. The rate of hydrocarbon biodegradation is directly correlated to the availability of naturally existing potential hydrocarbon-degrading organisms in the contaminated environment (Das N. and Chandran P, 2011). The ability of various bacteria isolated from contaminated environments to produce biosurfactants and to biodegrade hydrocarbons was demonstrated. Generally, biodegradation of such polluted environments is carried out either through bio-augmentation procedure (where the contaminated environment is supplemented with the bacteria or bacterial consortium that has high capabilities to degrade the target pollutant) or through bio-stimulation (in which the environmental conditions of the indigenous bacteria is optimized to enhance biodegradation process) (M. Kanwal *et al*., 2022). Gao *et al*.,2013 also studied the degradation of polyaromatic hydrocarbons and differing length alkanes and its relation to biosurfactant production.

Results of biodegradation potentiality is comparable with Kumar and Manjunatha (Kumar PSV, Manjunatha BK., 2017) observed that degradation of engine oil and diesel by isolate CSN-1 was 58.21% and 69.64% and 52.45% and 63.50% by isolate OK6. P. aeruginosa and B. subtilis isolated from crude oil contaminated site exhibited maximum crude oil degrading ability 88.75% and 87.41% by gravimetric analysis [28] . It has been reported in several literatures that Pseudomonas sp. have potential to degrade many different PAHs (Bharti P, Irafan., 2011: Vinothini C., 2015). However in contrast it was reported that the mixed bacterial culture gave the maximum degradation percentage because there is no single strain of bacteria with the metabolic capacity to degrade all the components found within crude oil (Adebusoye SA., 2007). This research was established five potential isolates that have ability to degrade all the provided hydrocarbons among them Gas 3 have highest activity. For the future our study will be focused on species identification, the optimal conditions to degrade several industrial waste oils.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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