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The Diesel and Petrol Degrading Strain 'HC19' of Bacillus albus

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Abstract: ***A gram positive, rod shaped, aerobic bacterial strain HC19 was isolated from the soil of a local river bank in Mandi, Himachal Pradesh; which was tested for its ability for the bioremediation of diesel and petrol oil. The bacterial strain was observed to grow at 37 to 40 degree Celsius. The isolated colonies were further plated on growth media with different NaCl concentration which depicted that the species is halophilic in nature. The turbidity (cell density) of the strain was measured with UV spectrophotometer at 600nm; for which the HC19 strain was inoculated in test tubes consisting Nutrient broth media, different NaCl concentration (50mM, 100mM, 500mM and 1M) for different test tubes. The turbidity was measured for the strain in presence of crude oil and NaCl (500mM, 1M). This resulted in maximum degradation of diesel and petrol oil at salt concentration of 500mM. Identification tests were conducted by which the strain was concluded to belong to the Bacillus species as per the phenotypic reference of "Bergey's manual of Determinative Bacteriology". The bacterial sample was identified as Bacillus albus strain with the 16srna sequencing. The bacterial sequence of Bacillus albus with the property of bioremediation of diesel and petrol has been published in U.S National library of medicine, NCBI with the accession number as MK999906. The oil spills have become major contribution towards the risk of pollution and harm for the lives of various species. The cost for removing the oil spills is huge. Not only it disturbs the balance of environment but also affect the economy and social lives of people. The use of chemicals can be harsh and it takes about years for oil spills to clear up. So rather than using the harsh chemicals, the only alternative are to use the naturally present microbes.***

Keywords: HC19; Bacillus albus HC19; Oil spills solution; diesel and petrol oil degrading Bacillus species

1. Introduction

One of the major environmental as well as commercial catastrophes includes the Oil spills. The accidents involving oil rigs or ships; the soil and ocean water get contaminated with petroleum hydrocarbons, causing damages to the nature for decades to come. In addition this the oil spills damage beaches and wildlife habitats as well. When this spilled oil reaches the beach, it causes devastation to the human settlement on the beaches and mangrove forests etc. In conclusion, the problem disturbs an entire ecosystem for quite a long period of time. The problem is not only about the spillage but rather it takes about months for oil cleaning operations to bring the areas back to original state.

The total volume of oil lost to the environment recorded in 2018 was **approximately 116, 000 tonnes**, the majority of which can be attributed to the incident involving the MT SANCHI that occurred in the East China Sea. This annual quantity is the largest recorded in 24 years **(source: "The International Tanker Owners Pollution Federation Limited".** It's been decades since the research and development work for the solution of oil spills problem being carried on. The natural resources are not only exhausted by humans but rather they are harming the Mother Nature itself. The impacts of oil spills have also majorly contributed to the problem of Pollution.

Bioremediation is defined as a process of detoxifying or pollutants removal using microorganisms, owing to the unique metabolic capabilities for the degradation and removal of various environmental pollutants including the products of industries. In addition to this, bioremediation is relatively cheaper and do not invade with the environment (Aldrett, S., et al.).The microbial communities present in environment can degrade the hydrocarbon pollutants within hours and have higher biodegradation rates (Das, N., et al.). So the major aim of this experimental study is to isolate and then test the capability of the bacterial strain to degrade the crude oil contaminants.

Despite the presence of vast number of hydrocarbons in petroleum products, only a small number of the compounds are well characterized for their toxicity [AI Hawash et al.]. These crude oil hydrocarbons can lead to severe distinct levels of toxicity. The chemicals with high toxicity level present in crude oil damage any organ system in the human body like the nervous system, respiratory system, circulatory system, immune system, reproductive system, sensory system, endocrine system, liver, kidney, etc. and eventually cause range of diseases and disorders (Costello, 1979).

People who are living with pre- existing health concerns, new borns, young people and pregnant women easily get exposed to the effects of these hydrocarbons. A study was done by Singh et al. (2004) to determine the toxic level of various fuels on mice. The results of the experiment showed that the diesel exhaust had much more toxicity as compared to the forklift engine exhaust [AI Hawash et al.]. Another study was conducted by Kinawy (2009) revealed that (leaded or unleaded) the monoamine neurotransmitters level and other biochemical parameters in different areas of the rats' brains got weaken on inhalation of aromatics and oxygenated compounds of gasoline vapors [AI Hawash et al.].On the other hand, the crude oil remains pertinent over its release site due to which it can cause major issues for humans and other living species. Crude oil consist of C, N, S, O atoms in its various polar organic compounds which makes them a serious concern for the

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environment. These can directly enter the soil or aquatic ecosystem and can potentially contaminate them [AI Hawash et al.].

2. Materials and Methods

Collection of the soil sample;

The soil sample was collected from the Neri Khad, a river in Mandi, Himachal Pradesh situated in North India. The diesel and petrol that was used during the study was purchased from a local oil pump in Ludhiana, Punjab, North India.

Isolation and purification of bacterial colonies;

The soil was first diluted by mixing about 10g in 95ml of deionized water. This was further serially diluted from 10^{-1} through 10^{-5}grams of soil per mL{refer to figure 1.). To grow the bacterial colonies the spread plate method was used. The nutrient media (2.8g Agar, 1.3g Nutrient broth and 50mL water) was prepared and autoclaved. The media was poured into 3 petriplates labeled as K1, K2, K3 and left to solidify. With the help of a spreader, the serially diluted soil sample was spread on each petriplate. These plates were incubated for 24hrs at 37 degree Celsius (Wakefield et al.)

STEP	VOLUME OF DEIONIZED WATER	DILUTIONS
10g soil (w/v)	95ml	10^{-1}
1ml Solution 'A' (v/v)	9ml	10^{-2}
1ml Solution 'B' (v/v)	9ml	10^{-3}
1ml Solution 'C' (v/v)	9ml	10^{-4}
1ml Solution 'D' (v/v)	9ml	10^{-5}

Figure 1: Serial dilutions of soil sample

For the purification step, the streak plate method was used where the bacterial colonies were picked and streaked on each plate of Nutrient media. The incubation for 24 hrs was given at temperature 37 degree Celsius. The procedure of streak plate method was continued for weeks after which the growth of single cell colonies occurred on each plate. Hence, the purified bacterial colonies were obtained. The plate 'K3' was used for further studies. The cell colony from 'K3' was streaked on another Nutrient media and incubated at 37 degree Celsius strain was named as' HC19'.

Screening of 'HC19' isolates for halophilic nature

200ml of Nutrient media was prepared with 6g of Agar and 2.6g of Nutrient broth. Then, out of this 200ml nutrient media 50ml was poured in 4 different flasks. To these flasks, different concentration of NaCl was added and were labeled as same (refer to Fig. 2) The nutrient media of the flasks were poured in four different petriplates which were labeled as 50mM, 100mM, 500mM and 1M. The bacterial strain HC19 was streaked on each plate and incubated for 24hrs at 37 degree Celsius.

Volume of Nutrient Media (ml)	NaCl Concentration (mM)
50ml	50mM
50ml	100mM
50ml	500mM
50ml	1M

Figure 2: Different concentration of NaCl in different flasks

For further studies the turbidity of K3 isolate was measured for its turbidity. 200ml of Nutrient broth media was prepared with 4g of nutrient broth with deionized water. Out of the total volume, 20ml was poured into 4 test tubes with different NaCl concentration. The test tubes and the media were autoclaved. The sample bacterial strain HC19 was inoculated in first four test tubes and was labeled as A1, A2, A3 and A4. In rest of the four tubes, the sample of E.coli was inoculated and were labeled as E1, E2, E3 and E4 which were kept as reference with one test tube as a blank (refer to figure 3). These test tubes were incubated at 37 degree Celsius for 24hrs. The cell density of each test tube was measured with UV spectrophotometer at 600nm after 48 hrs.

TEST TUBE LABEL	NaCl concentration (mM and M)	Sample Bacteria
A1	50mM	HC19
A2	100mM	HC19
A3	500mM	HC19
A4	1M	HC19
E1	50mM	E.coli
E2	100mM	E.coli
E3	500mM	E.coli
E4	1M	E.coli
BLANK		

Figure 3: Test tubes with different NaCl concentration

Screening of 'HC19' strain isolate for the ability of diesel and petrol degradation in presence of NaCl;

100ml of Nutrient media was prepared with 2g of Agar and 1.3g of Nutrient broth. Then, out of the whole media 50ml was poured in 2 different flasks. To these flasks, 500mM and 1M concentrations of NaCl were added and labeled as 1 and 2. The Nutrient media 1 and 2 were poured in to two different petriplates which were labeled as 500mM D, 1M D and 500mM P, 1M P with the addition of 1ml diesel and petrol in both petriplates. These plates were left at 37 degree Celsius for 24 hrs. The cell density of isolate HC19 was measured in presence of NaCl. 200ml of Nutrient broth media was prepared with 4g of nutrient broth in deionized water. Out of the total volume, 20ml was poured into 2 test tubes with different NaCl concentrations (500mM, 1M) with one blank. The test tubes were autoclaved. The sample bacterial strain HC19 was inoculated in four test tubes with the addition of 1ml diesel and petrol and labeled as D1, D2 and P1, P2 (refer to fig. 4). These test tubes were incubated for 24 hrs

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at 37 degree Celsius. The turbidity was measured with U.V spectrophotometer at 600nm for each test tube after 24 hrs.

TEST TUBE LABEL	NaCl CONCENTRATION	OIL SAMPLE (1ml)
D1	500mM	Diesel
D2	1M	Diesel
P1	500mM	PETROL
P2	1M	PETROL

Figure 4: Test tubes with NaCl concentration and oil samples

Identification of the Bacterial Species;

For the identification of the bacterial species of HC19 strain various biochemical tests were run. (refer to Figure 4) (Brown et al.)

S.No.	Biochemical tests
1.	Amylase
2.	Protease
3.	Gelatin
4.	IMViC's a. Indole test b. Methyl red test c. Voges – Proskauer test d. Citrate agar test
5.	Motility
6.	Catalase
7.	Oxidase
8.	Nitrate reduction test
9.	Lipase test
10.	Carbohydrate fermentation test

Figure 5: List of biochemical tests run for the identification of bacterial species

16srna sequencing of Bacterial strain HC19;

The bacterial strain was sent to BIOKART INDIA PVT. LTD. Bangalore for the 16 srna sequencing of the sample.

3. Results and Discussion

Isolation and purification of bacterial colony:

The serially diluted soil sample was spread over the nutrient media and left at 37 degree Celsius for 24 hrs. After 24 hrs, different colonies were observed on the media petriplates. Out of these colonies, three were picked up and streaked on 3 different petriplates labeled as K1, K2 and K3. These colonies were purified for 3 weeks. Out of K1, K2 and K3, the' K3 ' plate was further studied and tested for its capability for diesel and petrol degradation. The strain of K3 plate was named as **"HC19".**

Screening of HC19 for its halophilic nature and oil degradation ability:

For testing the halophilic nature, four different Nutrient media were prepared with NaCl concentration as 50mM, 100mM, 500mM and 1M. The media was poured into 4 petriplates labeled as 50mM, 100mM, 500mM and 1M on which HC19 was streaked and E.coli was taken as the reference strain. The strain 'HC19' was streaked on to each petriplate and incubated for 24 hrs at 37 degree Celsius. After 24 hrs, bacterial colony was observed on each plate which made the halotolerant nature of the strain 'HC19' evident.

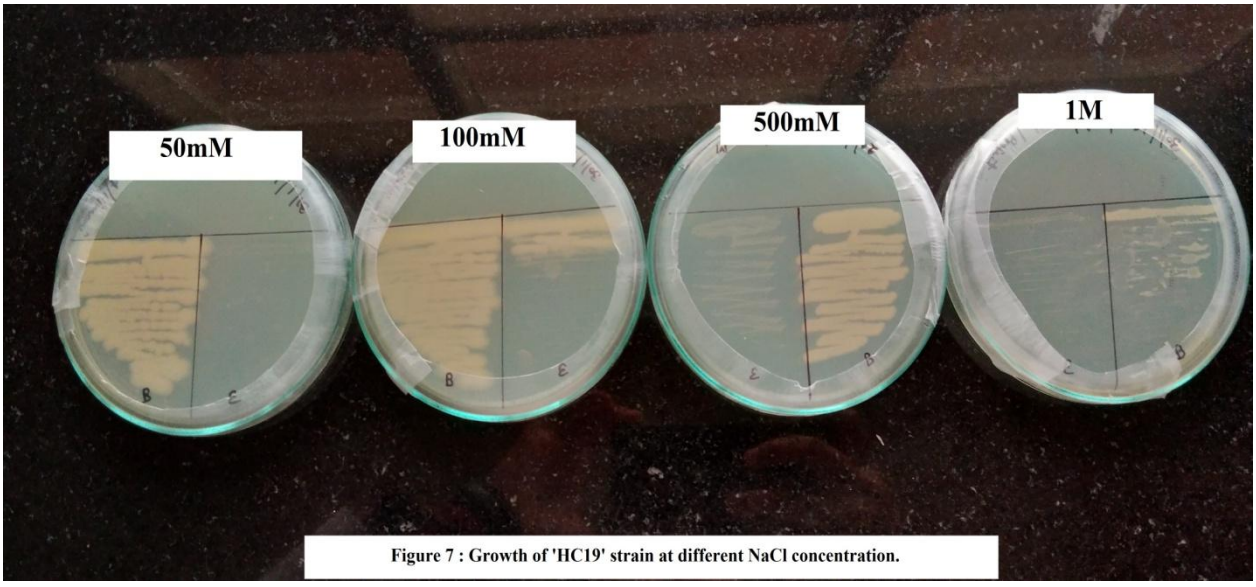

Figure 7 : Growth of 'HC19' strain at different NaCl concentration.

The turbidity or the cell density was measured for the same. The nutrient broth media was prepared in 200ml deionized water. 50 ml of media was poured into four different test tubes with NaCl concentration as 50mM, 100mM, 500mM and 1M.The Nutrient broth media flasks and test tubes were first autoclaved. Out of the the different flasks, 20ml of different NaCl concentration was poured into test tube and the strain 'HC19' was inoculated. The test tubes were incubated for 24 hrs at 37 degree Celsius. The cell density was measured after 24 hrs with U.V spectrophotometer at 600nm. The results are discussed in the figure 6:

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NaCl concentration	Cell density of 'HC19' strain	Cell density of E.coli
50mM	0, 416	0, 039
100mM	0, 729	0, 025
500mM	0, 912	0, 057
1M	0, 520	0, 018

Figure 6: Turbidity measure of 'HC19' strain in different NaCl concentration

The results show the maximum growth of the 'HC19' strain at 500mM of NaCl whereas that of E. coli is less. The turbidity measure calculated the cell density and made it evident that on increasing NaCl concentration the growth of 'HC19' strain increases.

The next step was to test the ability of the strain for the degradation of diesel and petrol in halophilic conditions. For this study, the strain was first grown on two plates with the addition of diesel labeled as 500mM D, 1M D and another two with petrol labeled as 500mM P, 1M P. The media flasks were autoclaved. After autoclaving, the media was poured into 4 different petriplates and diesel was added to 500mM D, 1M D whereas petrol in 500mM P, 1M P. The plates were incubated for 24 hrs at 37 degree Celsius. After incubating for 24 hrs the growth over the plates made the degradation of diesel and petrol evident in extreme saline conditions where the strain uses the oils as a major carbon source.

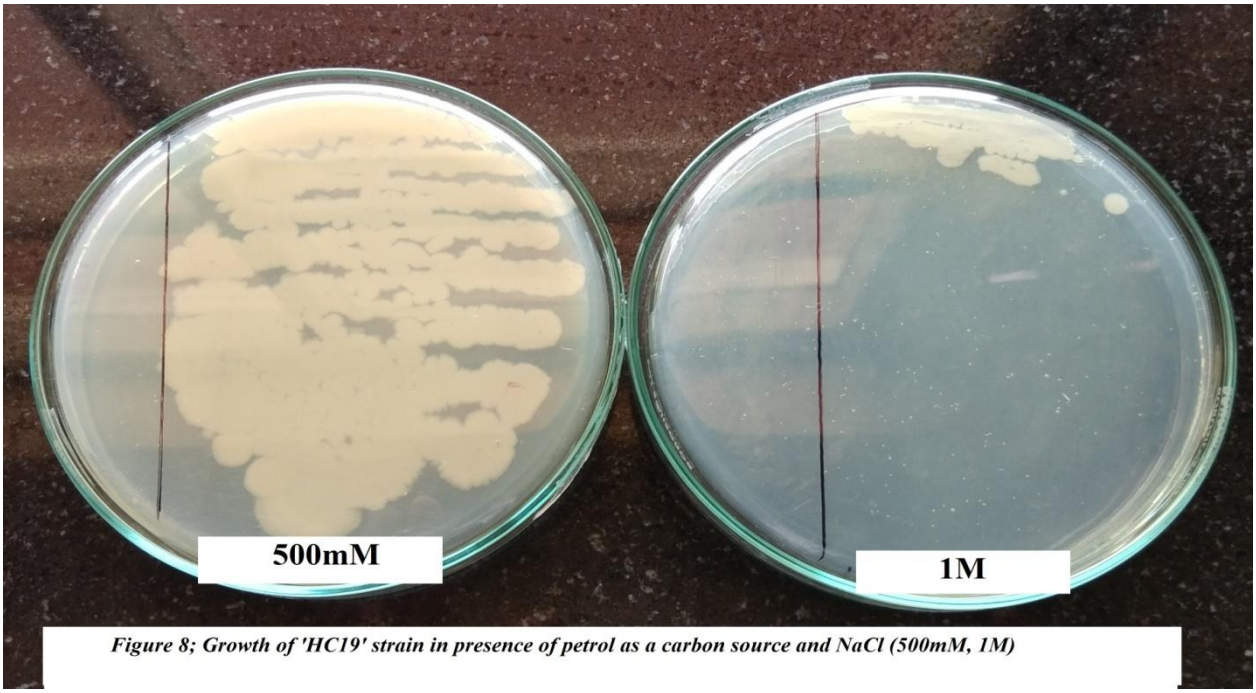

Figure 8; Growth of 'HC19' strain in presence of petrol as a carbon source and NaCl (500mM, 1M)

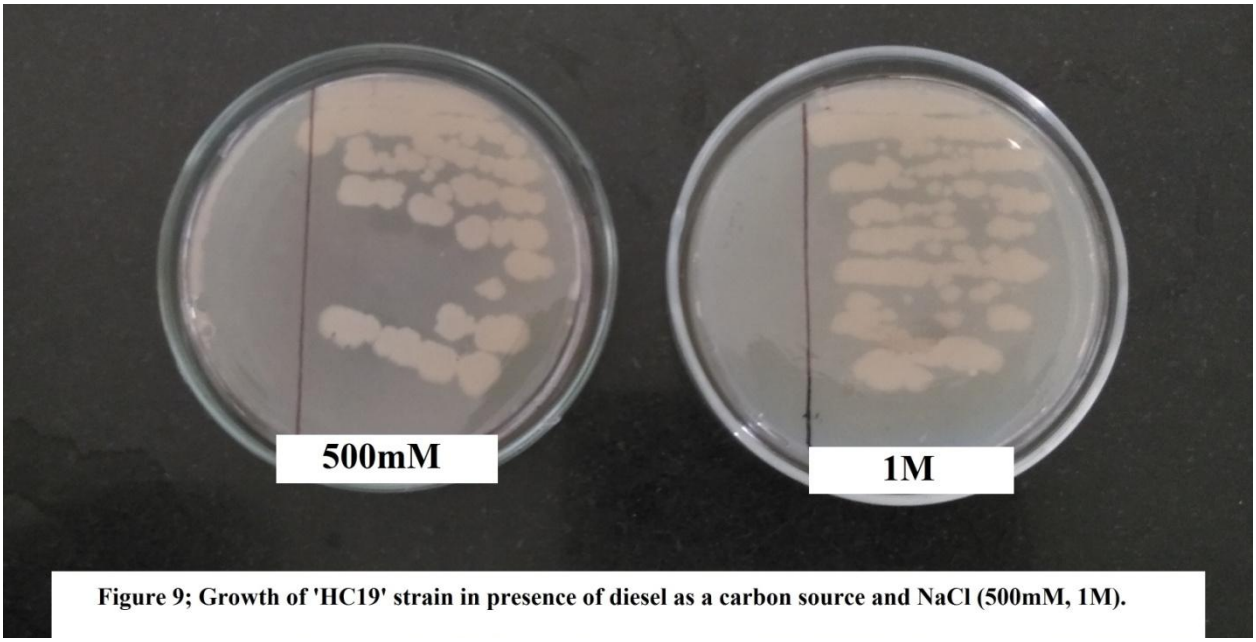

Figure 9; Growth of 'HC19' strain in presence of diesel as a carbon source and NaCl (500mM, 1M).

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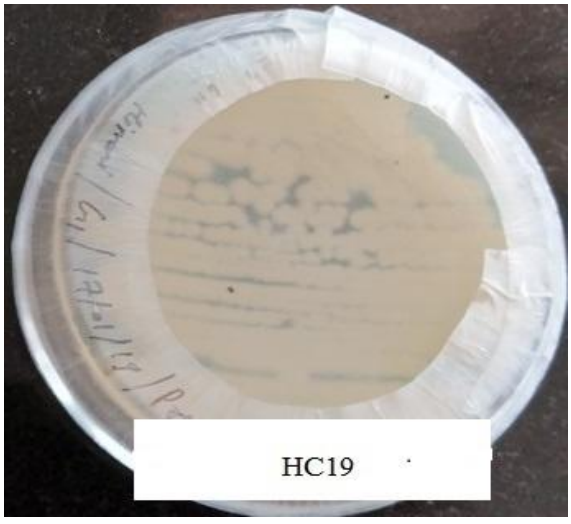
The turbidity of the strain was measured in presence of petrol and diesel at NaCl concentration 500mM and 1M. The Nutrient broth media with the addition of 500mM and 1M NaCl were prepared in four different flasks. These flasks were labeled as D1, D2 for diesel and P1, P2 for petrol with one blank. The nutrient broth media and the test tubes were autoclaved. 20ml media from each flask was poured into four different test tubes labeled as D1, D2 for diesel and P1, P2 for petrol. The diesel was added into D1 and D2 at 500mM and 1M. After that the HC19 strain was inoculated. Same procedure was followed in case of petrol also. The test tubes were incubated at 37 degree Celsius for 24 hrs. After 24 hrs, the test tubes were measured for the cell density with the U.V spectrophotometer at 600nm which showed the maximum degradation of Diesel and petrol at different NaCl concentration. The results are as discussed in figure 10. According

Test tube label	NaCl concentration	Cell density of HC19
D1	500mM	1, 094
D2	1M	0, 288
P1	500mM	0, 018
P2	1M	0, 180

Figure 10: Turbidity measure of 'HC19' strain in presence of diesel and petrol at NaCl concentration 500mM and 1M

Morphological and Biochemical characterization of 'HC19' strain:

The Bacterial strain 'HC19' appeared yellowish and bulky. The isolate stained gram's positive under the microscope.



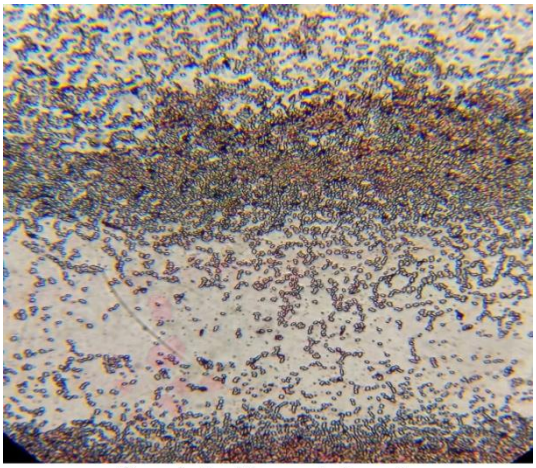


Figure 11: A. Yellowish and bulky 'HC19' strain. B. Gram positive, rod shaped HC19 strain

For the biochemical characterization of the bacterial strain, various tests were performed as mentioned in figure 12;

S.No.	Biochemical tests	Results
1.	Amylase	**+ve**
2.	Protease	**+ve**
3.	Gelatinase	**+ve**
4.	IMViC's a. Indole test b. Methyl red test c. Voges – Proskauer test d. Citrate agar test	 **a.-ve** **b.+ve** **c.-ve** **d.-ve**
5.	Motility	**+ve**
6.	Catalase	**-ve**
7.	Oxidase	**-ve**
8.	Nitrate reduction test	**+ve**
9.	Lipase test	**+ve**
10.	Carbohydrate fermentation test	+ve
11.	SIM agar test	+ve

Figure 12: Results of Biochemical characterization of 'HC19' strain

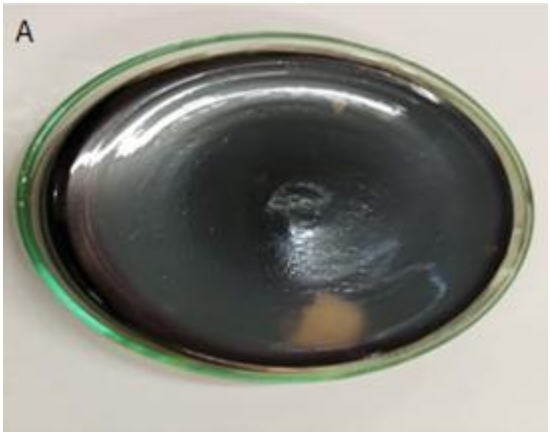


Figure 1:
Amylase test
Showing positive results for amylase test

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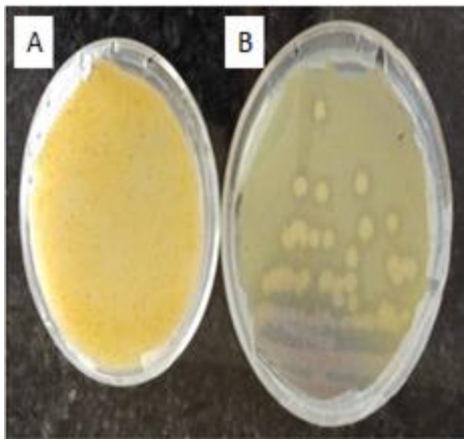

Figure 2:
A) Control
B) Showing positive results for protease test

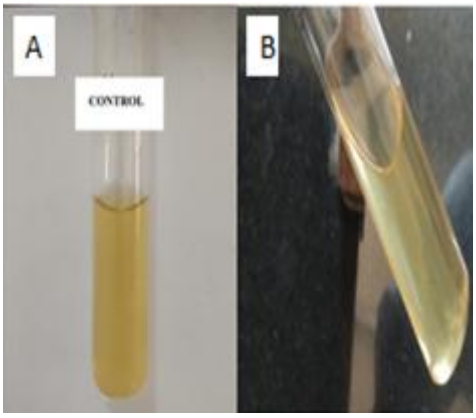

Figure 3:
A) Control
B) Positive results for Gelatinase liquification test

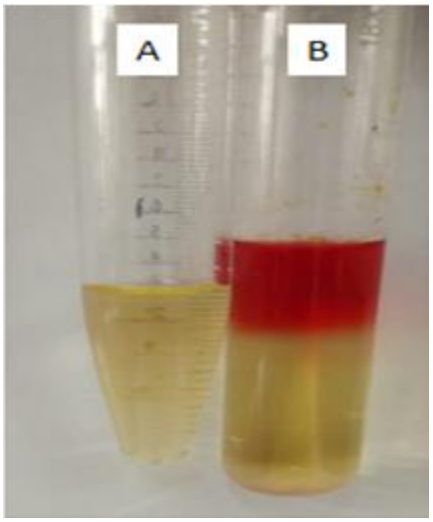

Figure 4:
A) Control
B) Positive results for methyl test

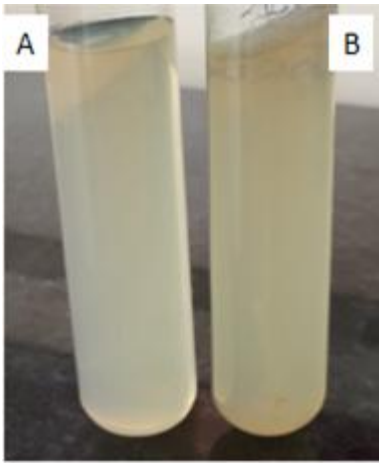

Figure 5:
A) Control
B) Positive results for motility test

Figure 6:
A) Positive results for Catalase test

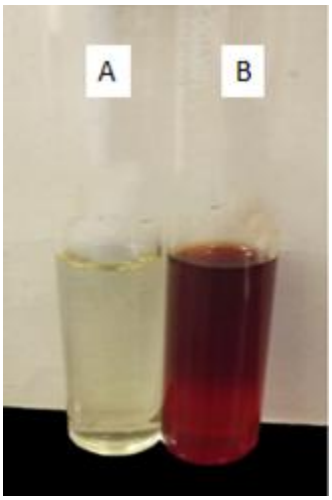

Figure 7:
A) Control
B) Positive results for nitrate reduction test

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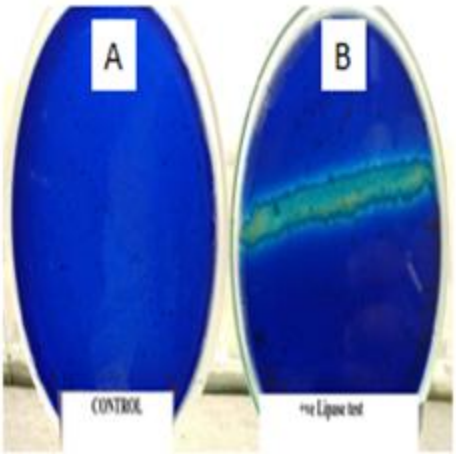

Figure 8:
A) Control
B) Positive results for Lipase test

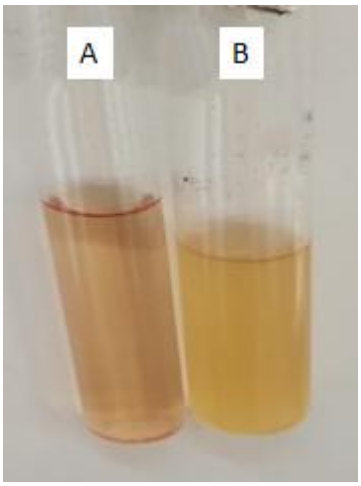

Figure 9:
A) Control
B) Positive results for carbohydrate fermentation test

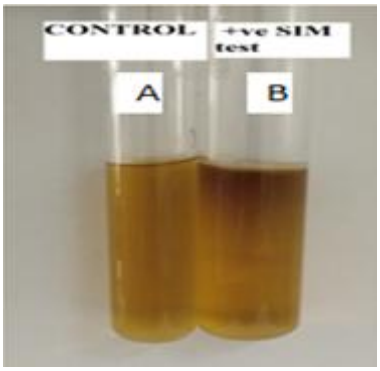

Figure 10:
A) Control
B) Positive results for SIM test

On the basis of the morphological and biochemical characterization criteria of 'Bergey's Manual of Systemic Bacteriology', the strain was identified to belong to the **Bacillus species**.

16 srna sequencing of 'HC19' strain:

The bacterial strain 'HC19' was sent to BIOKART INDIA PVT. LTD. for 16 srna sequencing. Genomic DNA was isolated from the sample provided. The ~1.3 kb/1.5kb, 16s-rDNA fragment was amplified using high–fidelity PCR polymerase. The PCR product was sequenced Bi-directionally. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors. The results showed 99.85% similarity to **Bacillus albus** strain **MCCC 1A02146** 16S ribosomal RNA. The sequence has been submitted to **NCBI** with the accession no. **MK999906.**

>MK999906.1 Bacillus albus strain HC19 16S ribosomal RNA gene, partial sequence
AGCTTGCTCAAATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGAT
AACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTT
CGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTT
CGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAA
TTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG
GGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCG
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GAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCACGAGAGTTTGTAACA

Figure 13: The aligned bacterial sequence of 'HC19' strain in NCBI

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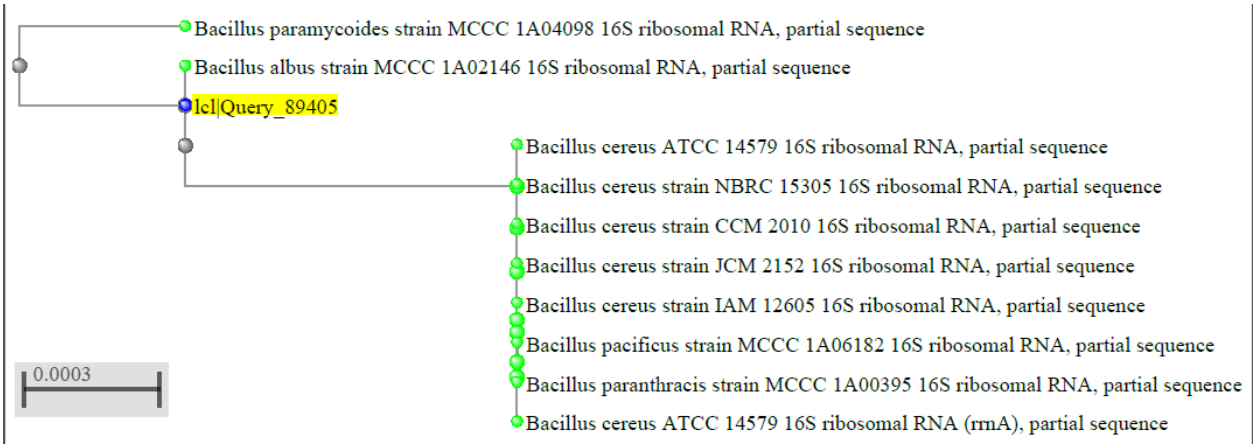

Figure 14: The Phylogenetic Tree of Bacterial strain 'HC19'b

4. Conclusion

The issue of oil spills has increased since past few years. The study conducted is an initiative to decrease the problem which majorly contributes to the pollution. The bacterial strain was isolated from soil and tested for its ability to degrade the diesel and oil in presence of saline or halophilic conditions. The results have shown 90% degradation of the oils in presence of NaCl. The bacteria is gram's positive according to the morphological characterization by gram staining. Apart from that, the biochemical characterization has been +ve for amylase, protease, gelatinase, Methyl red, Motility, Catalase, Nitrate reduction, Lipase, Carbohydrate fermentation and SIM agar test. So the Bacillus albus strain is a potential bioremediation microbial agent for diesel and petrol.

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