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Isolation, Identification and application of oil degrading bacteria for bio remediation of oil pollution

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Abstract

Bio remediation is the use of biological sources, especially bacteria and fungi for the clean up of the environment and prevention of pollution. Selection of oil degrading bacteria in the current investigatory research was solely for the purpose of oil spill and oil contamination cleaning from the environment which may include water bodies, land forms or any other natural habitat. These habitats especially the water bodies if untreated may cause death of many aquatic and terrestrial life's disturbing the ecological balance of nature. In the current work it was aimed to isolate and identify the oil consuming bacteria which can potentially convert the oil polymers into simple monomers for their metabolism and prevent the accumulation of these oils in the natural habitats. The source selected for the insolation was based on the highest chances of obtaining positive cells. Thus the study included the use of soil sample from an oil refinery near the laboratory. Bacteria were isolated from the source sample and screened for the presence of positive cultures capable of oil degradation using minimal salt agar media. Positive cultures were used for test oil degradation and assay. The bacteria showing positive for hydrocarbon degradation were identified using basic biochemical tests followed by sequencing of the 16s ribosomal RNA. Positive cultures thus obtained can be efficiently used in the bio-remediation of oil contamination in water bodies and other environment where they act as pollutants. The final confirmation of oil degradation was performed by inoculating the bacterial cells into BMH media supplemented with oil and estimating the reduction in oil content after 2 weeks volumetric ally. This can serve as a qualitative assay for the conformation of oil degrading cultures.

Keywords

Bioremediation, Hydrocarbon degradation, 16S Ribosomal RNA, Aquatic, Dissolved Oxygen

Introduction

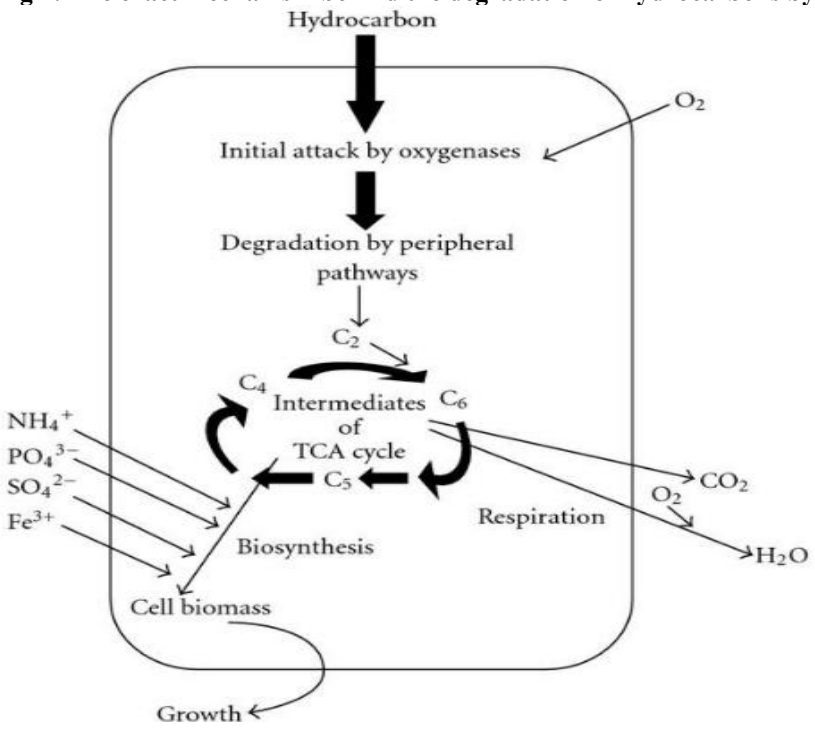
A constant sharp rise in the level of pollution by hydrocarbons is noticed with the development of science and technology. Increasing population may be an add on for this effect. The hydrocarbon based pollution especially the accumulation of oil in the water bodies is observed as a result of industrial effluents, ship accidents or by other accidental leakages into the water stream. This oil spill or leakage in the water causes the formation of a heavy dense layer of oil on the surface decreasing the dissolved oxygen levels of water. This causes a huge damage to the lives of the aquatic plants and animals underneath. The fact that some of the microbes feed on these hydrocarbons for their energy and metabolism is made use for the remediation of oil pollution.

The coast line of India is about 5500 km in main land and constitutes about 2000km in the offshore islands [8]. Being the major resource for trade and travel these water bodies are often used for the import and export of goods. One of the important goods being transported via these water bodies is oil and diesel. This increase the chances of creating oil spills in these regions disturbing the coastal life and aquatic ecosystem. Other causes of oil contamination in water bodies include discharge of oil from the refineries, ship accidents, oil tankers, storage tanks, grounding of ships, rupture of pipelines on sea beds etc. 1,300,000 tonnes of oil every year enters the environment, majority of the causes being natural petroleum seeps [6]. These activities create a very dense layer of oil floating on the water creating an impermeable layer for gases to pass through. This causes an obstruction for the movement of air and other essential gases in and out of the sea/ ocean. The dissolved oxygen concentration in the water falls gradually leading the the death of several aquatic organisms.This is a huge disturbance for the aquatic ecosystem and sea habitats, causing an irreversible destruction and imbalance to the biodiversity of that habitat.

This necessitates the development of environment friendly solutions in cleaning of these oil accumulations. Use of microbes as bioreactors in this cause is a wonderful and environment friendly approach. This facilitates not only the cleaning of the environment but also a development in the microbial research.

Bacteria capable of hydrocarbon degradation are ubiquitous in nature and can be made to utilize this polluting hydrocarbons as sole source of energy by making them devoid of any other simple carbon sources like glucose. This facilitates the mobilization of these oils and cleaning of the environment. Some of the microbes being known to possess the hydrocarbon degrading potential include Alcanivorax, Cycloclasticus , Thalassolituus, Bacillus subtilis, Pseudomonas aeruginosa, Pseudomonas putida, Marinobacter, Oceanospiralles etc,. This natural clean up of pollution by bacteria is on small scale and can be sufficient for cleaning basic leakage of oils at a low speed naturally. However in accidental cases of oil spills the amount of oil deposition overwhelms the capability of bacteria to clean it posing a challenge for the environmental safety.
Thus the culturing of these hydrocarbon degrading bacteria in bulk and using them for bioremediation is the task of the decade. This can utilize the natural efficacy of bacteria and also help in the clean up of the environment.

Fig 1: The exact mechanism behind the degradation of hydrocarbons by bacteria



The above picture shows various metabolic steps involved in the utilization of Hydrocarbons by bacteria [7].

Materials and Methods

Sample Collection and Processing

The sample selected for the study is collected from Sri Haritha Oil Mills, in Hayathnagar, Hyderabad. The reason for selecting this sample is a high probability of obtaining hydrocarbon degrading bacteria due to the presence of frequent and heavy oil pollution in the soil that generally spills during the oil refining and transportation process. Oil was collected using a sterile spatula into a clean and sterile container followed by sealing. The soil was dark brown and greasy in texture due to the presence of oils. The sample was bought to the laboratory for further processing.

Isolation of bacteria from the source sample
Into a 250 mL sterile conical flask with 100 mL sterile distilled water 10gms of the collected sample is added and allowed to soak with a constant shaking and blending for about 20 minutes. This was later allowed to stand still for the sedimentation of soil material for about 15minutes and the supernatant was extracted. This sample was added to a 100mL MSM media with the following composition:

All the elements are provided in grams per litre composition:
K2HPO4: 1.73, KH2PO4: 0.68, MgSO4.7H2O: 0.1 , NaCl: 4.0, FeSO4.7H2O: 0.03, NH4NO3: 1.0, CaCl2. 2H2O: 0.02, Oil: 1% which acts as a sole source of carbon for the growth of bacteria.

The media specified above is used for the inoculation but before inoculation it must be subjected for sterilization by autoclave at 121^0C for 30min. The media was cooled followed by inoculation with the above sample. The media is than incubated for 7days at 30^0C and 170 rpm in a rotatory shaker.
After 7days 10mL of the supernatant from this media was extracted and inoculated into a fresh medium containing 1% (w/v) oil. This sub culturing is repeated 3 times to increases the chances of getting pure hydrocarbon degrading bacteria and removal of other random strains. At the end of the 3rd sub culturing and incubation 10mL of the supernatant was extracted and subjected for serial dilution. The diluted sample of the count 10^{-7} can be used for further inoculation of MSM agar plate and incubated a 37^0C overnight. After incubation the plates can be screened for the presence of hydrocarbon (oil) degrading bacteria. Spread plate method was performed for bacterial inoculation.

Pure culture of Hydrocarbon degrading bacteria
The isolates obtained on the MSA agar plate are mixed cultures and needs to be sub cultured for obtaining pure cultures followed by their identification and preservation.

Luria–Bertani (LB) agar slants and plates were made to streak the single colonies of bacteria with different morphology obtained from the previous master plate. The colonies were streaked onto the plates and slants which can be used for further analysis and preservation respectively. All the plates and slants were incubated at 37^0C overnight.
After incubation all the colonies that are grown on the plates are subjected for secondary screening and identification.

Screening for Hydrocarbon degradation by bacteria
Based on the number of cultures to be tested for hydrocarbon (oil) degradation equal no of screening media plates were prepared. The media used for screening is the same Minimal Salt agar media with 1% oil as a sole carbon source [4]. The media was sterilized and poured onto the plates followed by solidification. All the bacterial cultures that are to be tested for hydrocarbon degradation are subjected for streaking onto these plates and incubated for 48hrs at 37^0C. Growth of the bacteria in the plate is an indication for positive cultures of hydrocarbon degradation [9]. Media being devoid of any other carbon source except the oil, only oil degrading bacteria can grow on the plates. All the positive cultures can further be subjected for identification and based on biochemical tests.

Identification of Bacteria
The bacteria showing positive growth on screening plates are subjected for identification. The initial step towards identification of any unknown bacterial species is Grams' Staining. To all the bacterial cultures whose growth was seen in the screening media, this staining is performed based on the protocol proposed by Christian Gram. All he slides were observed under 100x oil immersion objective to understand the morphology of the cells. The results are recorded based on which further biochemical tests can be performed.

Biochemical tests
All the biochemical tests like Catalase, oxidase, IMVIC etc are performed based on the result of Grams staining.

Molecular identification of Bacteria 16sr RNA sequencing

Biochemical tests can provide an idea about the shape and morphology of the cells but the exact identification with high end accuracy needs molecular level screening and identification based on genetic material. The prerequisite for the genetic level identification becomes the extraction of genomic DNA from the bacterial cells using the standard protocol [2]. The extracted DNA is known to posses more impurities which can be purified by ethanol washing. For the identification of bacteria there are some known standard primers called universal primers that target the 16s region of ribosomal RNA. Thus using these primers the extracted DNA can be amplified in PCR followed by sequencing of the sample.

Big dye terminator V3.1 kit was used for performing sequencing reaction [5]. Polymerase used here is the AmpliTac DNA Polymerase. Sequencing reaction mixture was made using 5X sequencing buffer 2µl, Big dye 1µl, 1µl $DMSO_4$ 50%. to this sequencing reaction mixture 2µl of primer and 2µl of sample DNA was added and subjected for sequencing. The results of sequencing can be subjected for insilico BLASTP analysis for he identification of he organism. This method of bacterial identification is highly accurate [3].

Assay for the hydrocarbon degradation by bacteria[10]

In order to confirm the quality and quantity of hydrocarbon degradation by bacteria, BHM media was prepared with the composition:

Magnesium sulphate :0.200gm/L, Calcium chloride: 0.020gm/L, Monopotassium phosphate : 1.000gm/L, Dipotassium phosphate: 1.000gm/L, Ammonium nitrate : 1.000gm/L, Ferric chloride: 0.050gm/L, Agar : 20.000gm/L, Final pH (at 25°C) 7.0±0.2

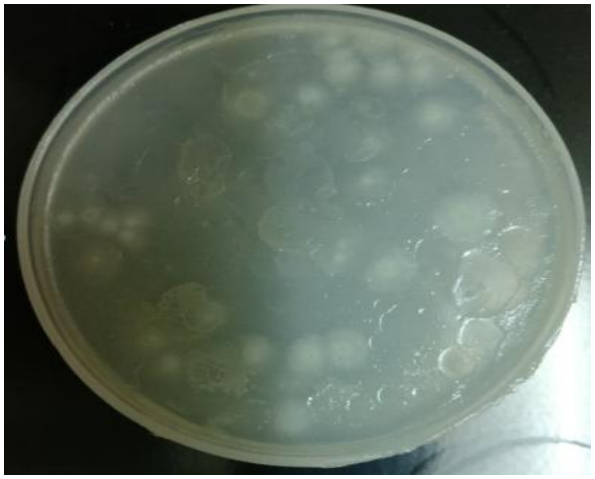
Above media is prepared and sterilized followed by its inoculation with the test bacteria. Additionally a predetermined amount of test oil is added to the medium prior to inoculation. The bacteria was allowed to grow and metabolize for 2 weeks. After two weeks the oil present in the medium is volumetrically measured by separation in a separating funnel and measuring the volume of left over oil. A decrease in the volume of oil added and an increase in the bacterial growth and turbidity of the medium is an indication of hydrocarbon degradation [1]. A blank can be used as a reference which includes all components in the medium except the bacteria.

Results and Discussion

Screening of the bacterial samples on MSA with oil as a substituent

All the bacteria growing on the screening medium (MSA+Oil) are assumed to be positive for oil degradation as shown in the figure.

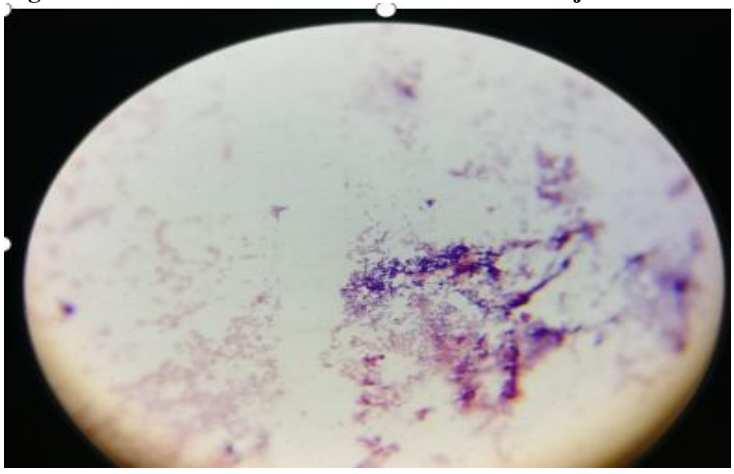
Fig 2: Growth of positive cultures on the screening media.



The above positive colonies can further be subjected for pure culturing and can be used for oil degradation.

Among all the colonies obtained only 1 was subjected for pure culture and identification due to the same colony morphology of all the cells. The pure cultured cells were later subjected for grams staining followed by microscopic identification.

Fig 3: Bacterial cells under 100X oil immersion objective



The microscopic observation of the cells under 100X oil immersion objective identified the bacteria to be gram positive rod shaped bacilli without prominent spores.

Table 1: Biochemical tests for the identification of Bacteria

S.No	Test	Observation
1	Grams staining	Gram positive rods
2	Acid Fast satin	Negetive
3	Catalase	Positive
4	Starch Hydrolysis	Positive

The above table shows that the bacteria are gram positive rods without spores and exhibit a positive result for catalase and starch test. The organism can be suspected as *Corynebactreium xerosis* which can be later confirmed by molecular tests.

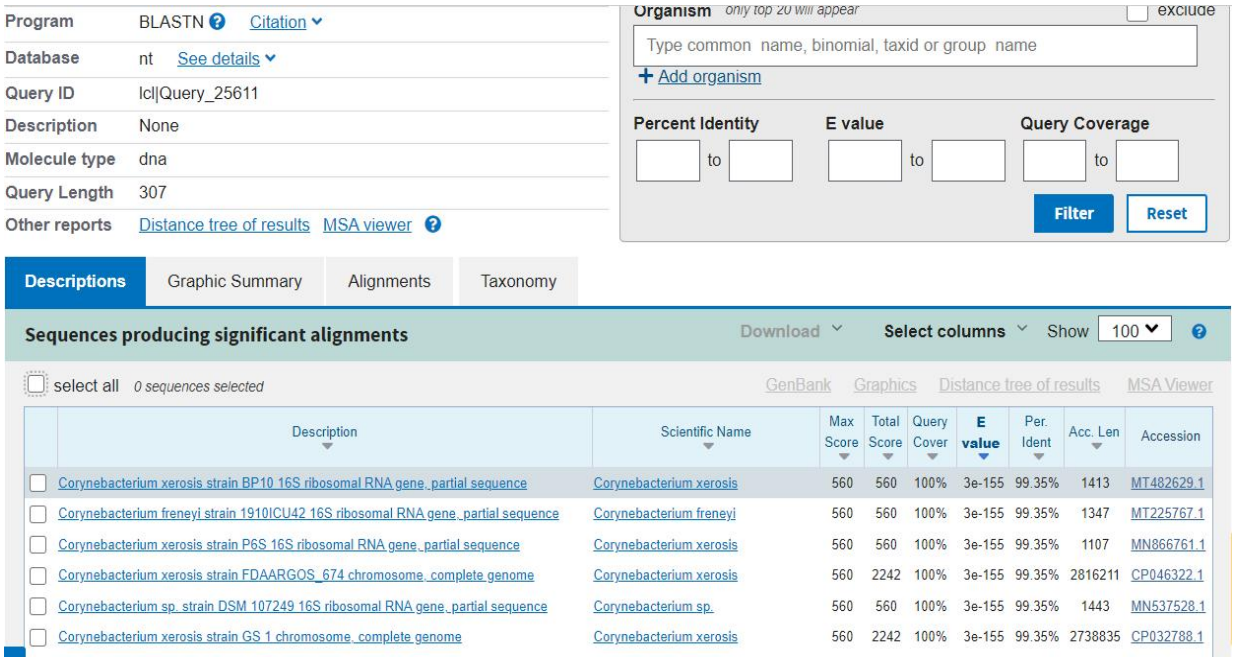
Molecular identification based on sequencing result and BLAST analysis

After performing DNA extraction and amplification using 16s rRNA universal primers the amplicons were subjected for sequencing and the results obtained are shown below:

GGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCATGGGTAGCGAA
CAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGGTGGGCGCTAGGTGTAGGGGTCTT
CCACGACTTCTGTGCCGTAGCTAACGCATTAAGCGCCCNGCCTGGGGAGTACGGCCGCAA
GGCTAAAACTCAAAGGAATTGACGGGGGCCNGCACAAGCGGCGGAGCATGTGGATTAAT
TCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATATACGGGACCGGGCCAGAGATGG
TCCTTCCCT

The sequence obtained was subjected for BLASTN analysis for the identification of the bacteria accurately. The results obtained in BLASTN are shown below:

Fig 4: BLAST result for the bacterial sequence obtained



Program	BLASTN Citation
Database	nt See details
Query ID	lcl|Query_25611
Description	None
Molecule type	dna
Query Length	307
Other reports	Distance tree of results MSA viewer

Organism only top 20 will appear — exclude
Type common name, binomial, taxid or group name
+ Add organism

Percent Identity: to — E value: to — Query Coverage: to
Filter Reset

Descriptions | Graphic Summary | Alignments | Taxonomy

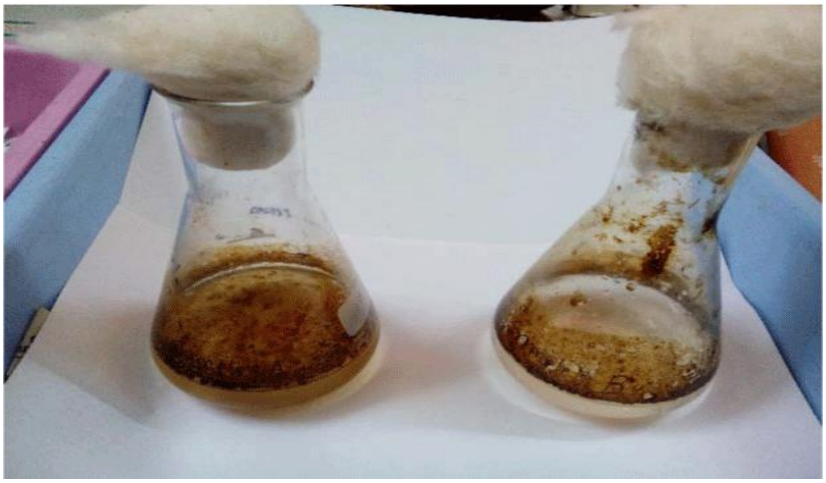
Sequences producing significant alignments — Download — Select columns — Show 100

select all 0 sequences selected — GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Corynebacterium xerosis strain BP10 16S ribosomal RNA gene, partial sequence	Corynebacterium xerosis	560	560	100%	3e-155	99.35%	1413	MT482629.1
Corynebacterium freneyi strain 1910ICU42 16S ribosomal RNA gene, partial sequence	Corynebacterium freneyi	560	560	100%	3e-155	99.35%	1347	MT225767.1
Corynebacterium xerosis strain P6S 16S ribosomal RNA gene, partial sequence	Corynebacterium xerosis	560	560	100%	3e-155	99.35%	1107	MN866761.1
Corynebacterium xerosis strain FDAARGOS_674 chromosome, complete genome	Corynebacterium xerosis	560	2242	100%	3e-155	99.35%	2816211	CP046322.1
Corynebacterium sp. strain DSM 107249 16S ribosomal RNA gene, partial sequence	Corynebacterium sp.	560	560	100%	3e-155	99.35%	1443	MN537528.1
Corynebacterium xerosis strain GS 1 chromosome, complete genome	Corynebacterium xerosis	560	2242	100%	3e-155	99.35%	2738835	CP032788.1

The above result indicates that the organism can be identified as corynebacterium xerosis of species BP10. This confirms the organism showing a positive hope for hydrocarbon degradation.

Fig 4: Laboratory test for hydrocarbon degradation by Corynebacterium xerosis



The above picture shows a control (right) and a test (left) broth containing the medium for growth and oil in it for testing.

Test media is inoculated with bacterium and the control was left un inoculated after sterilization. 10 ml of oil was added to both the medium and incubated for 2 weeks at 37^0C. During this incubation the media was extracted for every 3 days and the optical density was measured in a spectrophotometer at 660nm. The results revealed a gradual increase in the OD from 1.6 on day 1 to 1.9 , 2.6, 2.9, 3.2 and 3.6 respectively indicating a gradual increase in the bacterial growth.

Further at the end of 2 weeks of incubation media was subjected for extraction method to separate oil and the other aqueous portion. The oil collected was volumetrically measured. It was observed that oil got reduced from initial 10mL to final 9.2 mL in a period of 2 weeks. This indicates a positive hope for the use of corynebacterium in bioremediation process.

Conclusion

With the day to day increase in the population and technology there is a gradual and simultaneous raise in the levels of pollution. This pollution when focused towards water bodies, it is identified that the major source of pollution being oil spills occurring during national and international shipping of oils. This creates a need for the development of environment friendly technologies to clean up these spills and save the aquatic life. Thus a search for the suitable microbe that can act as bioreactor and clean the environment has to be undertaken. The current study involves the collection of soil sample from a region of high plastic burden and isolation of native bacteria present in the sample. The bacteria were allowed to grow on a screening media MSA that is devoid of any carbon source for the bacteria including only oil as the main carbon source in it. The inoculation of sample onto these plates allows the growth of only positive cultures. These positive cultures were subjected for molecular and biochemical identification which reveled the identity to be corynebacterium xerosis of speciesBP10. Further the bacteria were used to test their efficiency in oil degradation by inoculating them in the liquid broth of BHM (Bushnell Haas medium) along with oil. The results after 2 weeks revealed that the bacteria was efficient in reducing the oil from 10 mL to 9.2mL in a period of 2 weeks. Additional the OD values of the broth increased gradually from day 1 o day 14 when tested for every three days. This confirms the clear growth of the bacterial cells. Thus it can be developed and used for bioremediation of hydrocarbons.

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