

Isolation and Identification of Hydrocarbon Degrading Bacteria and Transformation of the Gene Responsible into Sensitive Bacteria

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Abstract:

Environmental pollution due to petroleum oil spillage is a major problem of the day. Bioremediation is an efficient method in oil spill treatment. Bacteria that are capable of degrading complex hydrocarbons into their simplest forms that can be degradable are used in Bioremediation protocol. In the present study, soil contaminated with petroleum was collected from petrol refilling station, to isolate bacteria capable of hydrocarbon degradation. Bushnell Haas media, with diesel as sole carbon source was used as screening media. The isolated bacteria, capable of hydrocarbon degradation was identified as *Corynebacterium xerosis* using the biochemical tests performed based on the Bergey's Manual of Determinative Bacteriology. The key enzyme involved in hydrocarbon degradation by *Corynebacterium xerosis* was identified as **protocatechuate 3,4-dioxygenase** using various data mining protocols. The protein sequence for protocatechuate 3,4-dioxygenase was retrieved from NCBI data base. The same sequence was used to design the primers for amplification. Amplicons for the gene that codes for protocatechuate 3,4-dioxygenase in *Corynebacteria*, were obtained using PCR. Soil sample from ground was collected again to isolate bacteria incapable of hydrocarbon degradation. The developed Amplicons were transformed into isolated non-hydrocarbon degrading bacteria. The same bacteria which were incapable of hydrocarbon degradation were able to degrade hydrocarbons after transformation more efficiently.

Key Words: Hydrocarbons, Bushnell Haas Media, *Corynebacteria xerosis*, Protocatechuate 3,4-dioxygenase

Introduction

Earth has good reservoirs of oils and gas beneath its surface. Crude oil can be extracted from the earth crust using techniques like Multilateral Drilling, Extended Reach Drilling, Complex Path Drilling, etc. Despite using the revolutionary and modernistic technology we have today, still we could see many oil spill accidents happening time to time. The spilled crude oil damages beaches, marshlands, fragile ecosystems, and also destroys wildlife Habitats and Breeding grounds.

Bioremediation is one of the most efficient technique, by which we can clean oil spills. Bioremediation is a treatment that uses naturally occurring organisms to breakdown hazardous substances into less toxic or non-toxic substances. Several microorganisms like *Pseudomonas* sp, *Corynebacterium* sp, *Rhodococcus* sp have the ability to utilize hydrocarbons as sole carbon source by converting them into simple carbons [1]. These microbes secrete enzymes like protocatechuate 3,4-dioxygenase, catechol 2,3-dioxygenase, etc [2]. These enzymes catalyze degradation of complex hydrocarbons into simple carbons. The hydrocarbon degrading micro-organisms can be isolated using BH media and can be identified by using Bergey's Manual of Determinative Bacteriology [13]. In the present study, we had isolated, identified the hydrocarbon degrading bacteria and transformed the gene responsible for hydrocarbon degradation into non-hydrocarbon degrading bacteria.

Materials and Methods:

3.1. Isolation of Bacteria:

Soil contaminated with petroleum, at petrol refilling station (Hyderabad) was collected to isolate bacteria capable of degrading hydrocarbons. The soil sample was collected in sterilized polythene cover and transported to laboratory under sterile condition. 1gm of collected soil was added to 10 ml sterilized distilled water, mixed well and subjected to serial dilution.

3.2. Master plate preparation:

Nutrient agar media was used for master plate preparation. In 250mL Erlenmeyer flask, 100mL of Nutrient agar media was prepared and taken into four Petri plates after sterilization in autoclave. Four Petri plates were inoculated with 100 μ L of inoculum with dilutions 10^{-4} , 10^{-7} , 10^{-8} and 10^{-9} respectively by spread plate method. Plates were incubated for 24-48 hours at 37°C in incubator.

3.3. Screening for Hydrocarbon Degrading Bacteria:

Bushnell Haas agar media was used as screening media. Composition of media for 1Lt of distilled water - 0.2gms of $MgSO_4 \cdot 7H_2O$, 0.02gms of $CaCl_2$, 1gm of KH_2PO_4 , 1gm of K_2HPO_4 , 1gm of NH_4NO_3 , 2 drops of 60% $FeCl_3$, 15gms of agar-agar and finally 1% of diesel[3]. In Bushnell Haas media,

diesel is the only source for carbon. So the bacteria which have the ability to use hydrocarbon as carbon source can only grow in Bushnell Haas media. In 250ml Erlenmeyer flask, 100mL Bushnell Haas agar media was prepared, sterilized in autoclave and transferred into four Petri plates. These plates were inoculated with well grown colonies from master plates by spread plate method and incubated for 72 hours at 37°C in incubator.

3.4. Pure Culture Preparation:

Nutrient agar media was used for preparation of pure culture. 25mL nutrient agar was prepared, sterilized in autoclave and transferred into Petri plates. A Bushnell Haas agar with a good number of colonies is selected, a colony from it was streaked on nutrient agar plate and incubated at 37°C for pure culture.

3.5. Identification of Bacteria:

Gram staining is performed to identify whether the bacteria are gram positive or gram negative. The cell wall of gram positive bacteria is made of thick layers of peptidoglycan whereas the cell wall of gram negative bacteria is made of few thin layers of peptidoglycan, but have an additional membrane called outer membrane, made of lipopolysaccharide. The ethanol used in gram staining, digests the lipids in outer member of gram negative bacteria. Due to which, gram negative bacteria will loss purple color and get pink color upon staining with safranin.

Further identification of bacteria was done by performing a series of biochemical tests using Bergey's Manual of Determinative Bacteriology.

By Acid Fast test, bacteria is separated into acid fast group and non-acid fast group. In this test, primary dye-carbol fuchsin, decolorizer-acid alcohol and counter stain-Methylene blue are used step by step.

The bacteria's ability to synthesis catalase enzyme, can be identified through Catalase test. It is performed by adding a drop of hydrogen peroxide on, a smear of bacteria prepared on glass slide. The bacteria's ability to utilize starch can be identified by Starch hydrolysis test. In this test, the bacteria is grown on starch agar overnight and starch utilization is checked by adding Iodine reagent.

3.6. Identification of Enzymes and Genes Involved in Hydrocarbon Degradation:

Several articles in NCBI PubMed were reviewed to find the enzymes responsible for hydrocarbon degradation in *Corynebacterium* sp[4,11,12]. The genes which codes for these enzymes and their sequence retrieval were done using NCBI Tools and data base.

Bacteria was screened for its ability to express the genes that codes for Protocatechuate 3,4-dioxygenase. The protein sequence of Protocatechuate 3,4-dioxygenase has been retrieved from NCBI Protein Data base[5].

The active domain of the protein was found by using SMART tool [7]. SMART, is a bioinformatics tool which identifies and gives the protein sequence for the active domain, when the whole protein sequence present in FASTA format is run on it.

Reverse Translate, is a Bioinformatics Tool from Sequence Manipulation Suite that converts the protein sequence present in FASTA format to nucleotide sequence. The protein sequence for active domain of protein was run in the tool to identify nucleotide sequence.

3.7. Primer Designing and Amplification of Gene Responsible for Hydrocarbon Degradation:

Primers for the nucleotide sequence of active domain were designed using Primer3 tool. Primer3 is a free online Bioinformatics Tool, used to design PCR primers for a particular nucleotide sequence. Hybridization probes and sequencing primers can also be designed using Primer3. Primers with desired characteristics can be designed by giving different input parameters to Primer3.

Starting primer and Ending primer designed in Primer3, were used in PCR to prepare amplicons of gene: protocatechuate 3,4-dioxygenase. The sequence of synthesized amplicons was found using auto sequencer. The amplicons sequence obtained, was run in BLAST to recheck its alignment with the gene of protocatechuate 3,4-dioxygenase present in genbank .

3.8. Transformation of Amplicons into Non-Hydrocarbon Degrading Bacteria:

Non-hydrocarbon degrading bacteria were isolated from soil and sub-cultures in nutrient agar plates. Colonies on nutrient agar were screened for their ability to degrade hydrocarbons using Bushnell Haas media.

Competent cells were prepared using 1.5mL overnight culture of non-hydrocarbon degrading bacteria, following standard procedure. In a vial, 2µL of prepared amplicons were added to the pellet of competent cells for transformation. Transformed cells were first incubated in 1mL of nutrient broth for 1 hour and then transferred onto nutrient agar plates for overnight growth. The overnight culture was screened for its ability to degrade hydrocarbon, on Bushnell Haas agar media.

4. Results and Discussions:

4.1. Isolation of Bacteria and Master Plate Preparation:

The bacterial master plate was obtained after plating with the dilutions from Serial Dilution tubes. The isolated colonies were obtained.

4.2. Screening of Hydrocarbon Degrading Bacteria:

Hydrocarbon degrading bacteria can be screened using Bushnell Haas media as shown above. The Bacteria which are truly efficient in utilizing the Diesel as the sole carbon source are isolated in the media. The same is shown in the Fig 1.

Fig 1: Screening media, with colonies of isolated bacteria.

Plate A of Bacteria

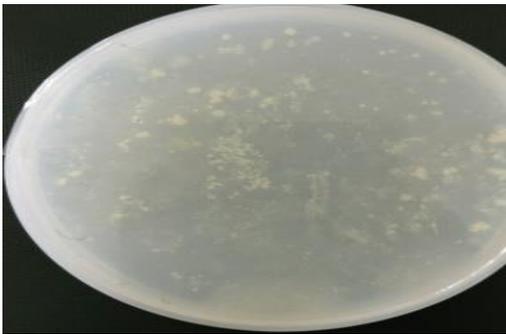


Plate B of Bacteria



Plate C of Bacteria

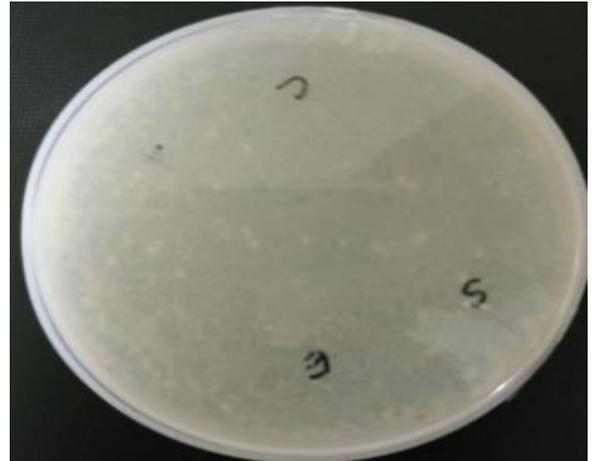
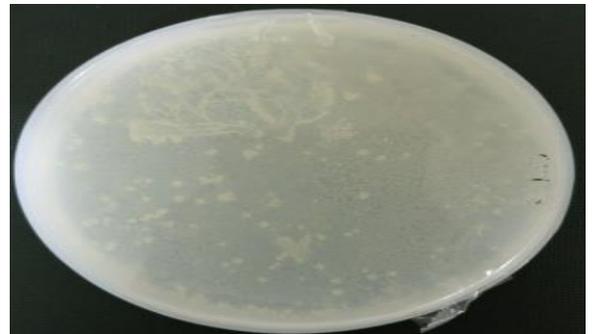


Plate D of Bacteria



4.3. Preparation of Pure Culture:

A Bushnell Haas Agar plate with good number of colonies was used for pure culture preparation. Nutrient agar was used for pure culture preparation. A colony from Bushnell Haas media was Streaked on solidified nutrient agar plate and incubated.

Fig 2: Pure Culture on Nutrient Agar



4.4. Identification of Isolated Bacteria:

Isolated bacteria was identified using Bergey's Manual of Determinative Bacteriology. A series of biochemical tests like Gram staining, Acid Fast test, Catalase test and Starch hydrolysis were performed. From Gram staining, the bacteria was

identified as non-sporulating, gram positive rod shaped bacteria. From the other biochemical tests, the bacteria was conformed as Corynebacteria xerosis.

Table 1. Biochemical characteristics of isolated bacteria

No	Test	Result
	Gram staining	+ve
	Acid Fast	-ve
	Catalase	+ve
	Starch hydrolysis	-ve

4.5. Identification of Enzyme and its Genes Involved in Hydrocarbon Degradation:

All the corynebacterium sp have the ability to utilize a large number of different hydrocarbons as the sole carbon source. They follow different biochemical pathways like Gentsiate pathway, Vannilate pathway, β-ketoadipate pathway, Putative hydroxyquinol pathway, etc.. for hydrocarbon degradation. In all these pathways the key enzymes involved are catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase, etc.[6] Protocatechuate 3,4-dioxygenase was significantly expressed in the presence of Protocatechuate, 4-hydroxybenzoate, and p-cresol as inducers.[6] Corynebacterium xerosis was screened for its ability to express the gene that codes protocatechuate 3,4-dioxygenase. The protein sequence for protocatechuate 3,4-dioxygenase in FASTA sequence was taken from NCBI protein data base[5].

Protein sequence:

MIDTGKNGEFRYEQSNIVDQNEAEFG
ITPSQTVGPYVHIGLTLEGAEHLVEPGSEGAV
SFTVSATDGNPDPIADAMFELWQADPEGIHN
SDLDPNRTAPATADGFRGLGRAMANTQGEA
TFTTLVPGFAFADEAPHFKVGVFARGMLERLY
TRAYLPDADLSTDPVLAVVPADRRDLLVAQK
TDDGFRFDITVQAEDNETPPFFGL

The active domain of this protein sequence was identified using SMART tool[8]. The sequence of active domain obtained:

PYVHIGLTLEGAEHLVEPGSEGAVSF
TVSATDGNPDPIADAMFELWQADPEGIHNSD
LDPNRTAPATADGFRGLGRAMANTQGEATF
TTLVPGFAFADEAPHFKVGVFARGMLERLYTRA
YLPDALSTDPVLAVVPADRRDLLVAQKTDD
GFRFDITVQA

The above sequence was run in Reverse translate tool[7] to get nucleotide sequence for the active domain. Nucleotide sequence obtained:

```
ccgtatgtcatattggcctgaccctggaaggcgcggaac
atctggtggaaccgggcagcgaaggcgcggtgagctttaccgtgagc
gcgaccgatggcaacggcgatccgattgcggatgcgatgtttgaactg
tggcagggcgatccggaaggcattcataacagcagctggatccgaa
ccgcaccgcgccggcgaccgcggatggctttcgcggcctgggccgc
gcgatggcgcaacaccaggcgaagcgaacattaccaccctgggtgcc
ggcgcgctttgctggatgaagcggcgatttaagtggcgctgtttgcg
cgcggcatgctggaacgctgtatacccgcgctatctgccggatgcg
gatctgagcaccgatccggtgctggcggtggtgccggcgatcgccg
cgatctgctggtggcgagaaaaccgatgatggctttcgtttgatatta
ccgtgcaggcg
```

4.6. Primer Designing and Amplification of Gene Responsible for Hydrocarbon Degradation:

Amplicons of the gene that codes for protocatechuate 3,4-dioxygenase were prepared using PCR. For amplicons preparation, PCR primers were developed using Primer3 tool[9]. The starting primer- "gcattttaagtggcgctgt" and ending primer- "aagccatcatcggtttctg" with melting temperature as 60.00°C and 60.07°C, with G-C% as 45 were run in PCR for amplicons preparation. The sequence of developed amplicons was identified using auto sequencer. The identified sequence gave 100% alignment score, when the amplicon sequence is run in BLAST.

Fig 4: Showing the Primer 3 result for gene: protocatechuate 3,4-dioxygenase.

Primer3 Output

```
No mispriming library specified
Using 1-based sequence positions
OLIGO      start len tm gc% any 3' seq
LEFT PRIMER 300 20 60.00 45.00 8.00 0.00 gcattttaagtggcgctgt
RIGHT PRIMER 455 20 60.07 45.00 5.00 1.00 aagccatcatcggtttctg
SEQUENCE SIZE: 480
INCLUDED REGION SIZE: 480

PRODUCT SIZE: 156, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00
```

Amlicon Sequence obtained after Sequencing:

```
Gcattttaagtggcgctgtttgctgcgcgcatgctggaa
gcctgtatacccgcgctatctgccggatgcggatctgagaccgat
ccggtgctggcggtggtgccggcgatcgccgatctgctggtggc
gcagaaaaccgatgatggctt
```

Fig 5: Showing the result of BLAST for Amplicons.

MULTISPECIES: protocatechuate 3,4-dioxygenase sub
[Corynebacterium]

Sequence ID: [WP_004567634.1](#) Length: 204 Number of Matche

» [See 15 more title\(s\)](#)

Range 1: 135 to 185 [GenPept](#) [Graphics](#)

▼ Next Match ▲ Previous

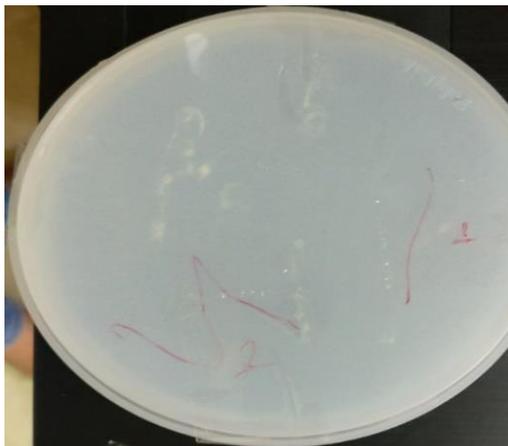
Score	Expect	Method	Identities	Positives	Gaps	Frame
104 bits(260)	6e-27	Compositional matrix adjust.	51/51(100%)	51/51(100%)	0/51(0%)	+2
Query 2	HFKVGVFARGMILERLYTRAYLPDADLSTDPVLAVVPADRRDLLVAQKTDG					154
Sbjct 135	HFKVGVFARGMILERLYTRAYLPDADLSTDPVLAVVPADRRDLLVAQKTDG					185

4.7. Transformation of Amplicons into Non-Hydrocarbon Degrading Bacteria:

Soil sample was again collected and transported to isolate non-hydrocarbon degrading bacteria. Collected soil was subjected to serial dilutions. Nutrient agar plates were inoculated with these dilutions by spread plate method. The colonies on nutrient agar are screened for their ability to degrade hydrocarbons using Bushnell Haas media. Competent cells of the bacteria which are incapable of hydrocarbon degradation were prepared using standard methods. 2µL of prepared amplicons were added to the pellet of competent cells in a vial. Transformed cell were incubated in 1mL nutrient broth for 1hour and then inoculated on nutrient agar plates for overnight growth. The transformed cells on were screened for their ability to degrade hydrocarbon using Bushnell Haas media. The transformation of gene: protocatechuate 3,4-dioxygenase into non-hydrocarbon degrading bacteria was successfully transformed. After transformation, the transformed cells showed good degradation of hydrocarbons.

Fig 6: Growth of sensitive bacteria before and after transformation

A) Before transformation



B) After Transformation



5. Conclusion:

Pollution due to petroleum oil spillage is increasing day by day. All eco systems are being affected by this, especially marine organisms and birds at sea shores are getting more effected. Wildlife habitat and breeding grounds are being destroyed by oil spillage in oceans. This challenge can be addressed to some extent by bioremediation, where the microorganisms are used to degrade complex hydrocarbon molecules into simple carbons. In the present study, bacteria capable of degrading hydrocarbons were isolated from soil contaminated with petroleum oil. The isolated hydrocarbon degrading bacteria were screened using Bushnell Haas media. The isolated bacteria were identified as *Corynebacterium xerosis* using Bergey's Manual of Determinative Bacteriology. Bacteria belonging to Corynebacterium species are good hydrocarbon degraders. Protocatechuate 3,4-dioxygenase is one of the enzymes secreted by Corynebacterium for hydrocarbon degradation. Amplicons of the gene coding for protocatechuate 3,4-dioxygenase were obtained using PCR. Non-hydrocarbon degrading bacteria are isolated from soil sample and sub-cultured in nutrient broth. The prepared amplicons are transformed into non-hydrocarbon degrading bacteria. The transformation was successful and the transformed bacteria showed good growth in Bushnell Haas media with diesel as the sole source of carbon. This indicates the success of transformation.

6. Reference:

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