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Isolation and Molecular Characterization of Hydrocarbon Degrading Bacteria from Industrial Effluents

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Abstract

Hydrocarbon contamination may happen in various ways such as accidents during fuel transportation by trucks and ships, leakage of oil from underground storage tanks, or during extraction and processing of oil. These contaminations can be treated by several methods including physical, chemical and biological treatment. During biological cleaning up, hydrocarbon-degrading bacteria emulsifying hydrocarbons by producing biosurfactants are used. Therefore, isolation and identification of biosurfactant producing and hydrocarbon degrading bacteria are pivotal for effective bioremediation of hydrocarbon contaminated surface waters. Hence, the aim of this study is to isolate and identify efficient bio surfactant producing and diesel oil degrading bacteria to remove spilled diesel oil from surface waters. The isolates D1 and was identified as *Bacillus cereus*. Isolates was further characterized for the presence of two novel catabolic genes (*alk*B and *C23*O), responsible for diesel oil degradation, the key enzymes (alkane monooxygenase and catechol 2,3 dioxygenase), encoded by these novel genes, and emulsifying ability of the biosurfactants produced by isolate through the use of several methods including DNA Extraction, Agarose Gel Electrophoresis And Polymerase Chain Reaction (PCR).

Keywords: Bacillus cereus, Hydrocarbon Contamination, Isolation Molecular Characterization, Industrial Effluents.

INTRODUCTION

Ecosystems have been changed recently with the growing influence of human activities. Thus, people have become

awaken of the need preserve ecosystems and additionally, evaluate the deterioration created by the contamination. Hydrocarbon contamination is one of the most important pollution source around the world. Due to oil spills, hydrocarbon pollution becomes a global problem especially in developing and industrialized countries. Petroleum is used as a conventional energy source even though it has significance as a global environmental pollutant. Crude oil is converted to gasoline and diesel oil in petroleum refineries. As a petroleum product, diesel oil contains aromatic hydrocarbon compounds as well as normal, branched and cyclic alkanes. Diesel oil can affect both surface and groundwater quality with the toxic compounds in it. Examples of oil spills such as the ones occurred in Panamanian Coast in 1986, in Exxon Valdez in 1989, and BP oil spill in the Gulf of Mexico in 2010 have attracted attention of the environmentalists, chemists, biotechnologists and engineers because of their effects on the environment and ecosystems.

HYDROCARBON POLLUTION IN SURFACE WATERS

Surface water pollution mainly occurs due to the hazardous substances coming into contact and physically mixing with water or dissolving in the water. Another part of the surface water contamination is considered as contaminated sediments due to their close relationship with water. Contamination of surface waters may occur in various ways such as direct discharge of hazardous materials thorough a pipe or channel, storm water runoff which may get contaminated when rain water contacts with contaminated soil and carries the contaminated soil particles. In addition, surface water can also be polluted due to contaminated groundwater's, sediments and air. Interests about the possibility for water and soil contamination by oil and oil byproducts gains importance since they are one of the major pollutants in the environment. Contamination can be as a result of various sources such as; accidents during fuel transportation by trucks and ships, leakage of oil from underground storage tanks, extraction and processing of oil. Also, byproducts of oil can serve as source of hydrocarbon contamination. Even though oil refineries and petrochemical plants are in favor of society, a great amount of solid oily waste is produced. This waste is categorized as hazardous since they are toxic, corrosive and flammable.

Because of these properties these waste cannot be recycled or reused.

Hydrocarbons are released into the air, water and soil as pollutants through different ways and act as common contaminants. In air, sources of hydrocarbons are mainl described as anthropogenic emissions to air from combustion of wood and organic matter, vehicle exhaust, production of coal tar, coke and asphalt, cigarette smoke; accidental discharges of petroleum hydrocarbons (PHCs) via gas and oil spills (e.g. U.S. Gulf Oil Spill in 2010) and natural releases such as volcanic eruptions and forest fires. In water, storm water runoff may include hydrocarbons due to asphalt, rubber tire wearing or vehicleexhaust in urban areas. Industrial areas with petroleum refineries, wood treatment plants etc. may also contain hydrocarbons. Source of hydrocarbons in soil is mostly atmospheric deposition. It is mainly caused by the deposition of vehicle exhaust, rubber tire or asphalt emissions. Also, land filling activities, application of compost or pesticides, disposal of sludge from wastewater treatment plants produce hydrocarbon emissions. Therefore, deposition of these emissions causes contamination of soil with hydrocarbons .

MICROORGANISM:

Hydrocarbon degradation bacteria isolated from 20 different sources in Hyderabad region. Among them *Bacillus cereus* isolated from industrial effluents of Uppal region, Hyderabad was showed good result so we used in this study. Stock cultures were maintained at 4^0 C by periodic transfer on nutrient agar slants.

MEDIA USED:

MEDIUM – 1:

COMPOSITION:

Component	(gm/lit)
$CaCl_2.2H_2O$	0.02
$FeSO_4.7H_2O$	0.02
Sucrose	20.0

KH_2PO_4	1.0
$(NH_4)_2SO_4$	2.0
$MgSO_4.7H_2O$	0.2

(To the above solution, 1 ml of trace element solution was added.

Composition of Trace element solution)

Component	gm / lit
$ZnSo_4.\ 7H_2O$	0.2
H_3BO_3	0.6
$MnCl_2.4H_2O$	0.06
$CoCl_2.6H_2O$	0.4
$CuSO_4.4H_2O$	0.02
$NaMoO_4.2H_2O$	0.06

MEDIUM – 2: (Nutrient broth)

Composition:

Component n	gm / lit
Peptone	5.0
NaCl	5.0
Beef extract	3.0
pH	7.0

MEDIUM – 3: (LURIA BROTH)

COMPOSITION:

Component	gm / lit
Tryptone	10.0
Yeast extract	5.0
NaCl	10.0
PH	7.0

STERILIZATION OF MEDIUM AND GLASS WARE:

Sugars and mineral salts were autoclaved separately at 121^0 C for 10 lbs/ $inch^2$ pressure, for 20 minutes respectively and mixed together after attaining the room temperature. The glass ware like conical flasks, pipettes, test tubes and measuring cylinders were autoclaved at 15 lbs / $inch^2$ pressure for 15 minutes.

INOCULUM PREPARATION:

Inoculum was prepared in 250 ml Conical flasks containing 50 ml sterile medium consisting nutrient broth with 10g / lit of sucrose and the pH was adjusted to 7.0. Flasks were incubated at 35 ± 0.5^0 C for over night on a rotary shaker at 250 rpm / min.

ISOLATION OF BACTERIA FROM SOIL POLLUTED WITH INDUSTRIAL EFFLUENT:

REQUIREMENTS:

1. Soil sample (collected from industrial effluent plant)

2. Sterile test tubes

3. Sterile Petri dishes

4.Distilled water

5. Pipettes

6. 70% Ethyl Alcohol

7. Spirit lamp or Bunsen burner

8.Absorbent cotton

REAGENTS:

(a) LB Agar composition (100ml)

Component	Quantity
Tryptone	1.0g
Sodium Chloride	1.0g
Yeast Extract	0.5g
Agar	1.0g

(b) LB Broth composition (100ml)

Component	Quantity
Tryptone	1.0g
Sodium Chloride	1.0g
Yeast Extract	0.5g

DNA EXTRACTION

Each isolate were grown in nutrient broth and total DNA of the diesel oil degrading isolates were extracted by using Roche – High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). Steps for DNA extraction were applied as described in the manual of the kit.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS OF THE CATABOLIC GENES FOR DIESEL OIL DEGRADATION

PCR-based detection of both catabolic genes namely *C23*O and *alk*B were performed. PCR was done by using T100 thermal cycler (Bio-Rad, USA). The PCR mixture in 25 µL contained 1.25 U of AmpliTag Gold, 5 µL of GeneAmp 10 x PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTP's, 10 pm μL^{-1} of each primer, and 1 µL template.

PCR ANALYSIS OF *C23O*

*C23*O gene fragments were amplified with the primers *C23*Of 5'-CGACCTGATC(AT)(CG)CATGACC GA-3', *C23* Or 5'-T(CT)AGGTCA(GT)(AC)ACGGTCA -3'. The PCR temperature program began with an initial 5-min denaturation step at 94°C, then 35 cycles of 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min; and a final 10-min extension step at 72°C. Amplification products were then analyzed by electrophoresis on a 1% agarose gel..

PCR ANALYSIS OF *ALK*B

*Alk*B gene fragments were amplified with the primers *alk*Bf 5'-AACATAACCGTGGCCATC AC-3', *alk*Br 5'- AAC ACCA CGC TGT ACATCCA - 3' as stated. The PCR temperature program began with an initial 5-min denaturation step at 94°C, then 35 cycles of 94°C for 45 sec, 55°C for . Amplification products were then analyzed by electrophoresis on a 1% agarose gel.

RESULTS

STREAK PLATE TECHNIQUE

Observation:

Colour less colonies were observed over the medium

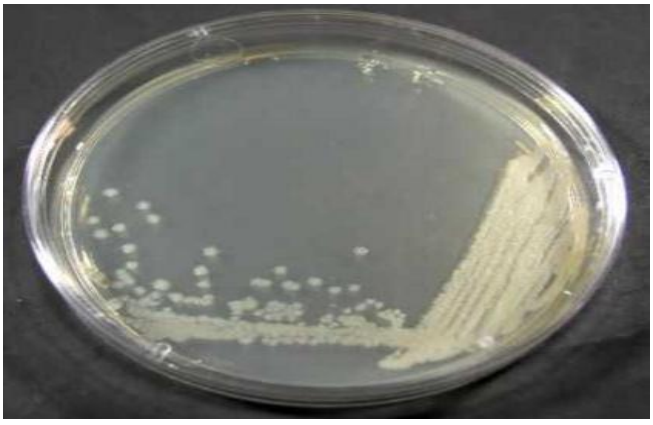


Figure 1: This *Bacillus cereus was* grown on Nutrient Agar medium by Streak plate technique.

GRAM STAINING

Observation:

On Gram staining violet colored rods were observed. Hence it is a Gram positive Bacterium.

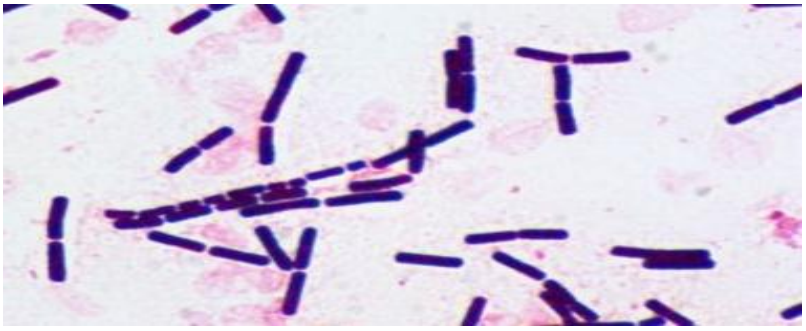


Figure 2: Gram positive Bacterium

Result:

From above observation it is said that is a Gram-positive bacterium

CATALASE ACTIVITY

Observation :

After 48 hours of incubation when four drops of hydrogen peroxide was added to the slants slow appearance of gas bubbles was observed .

Result :

After the addition of hydrogen peroxide gas bubbles were observed which is the Indication of positive test. Hence *Bacillus cereus* is positive for catalase

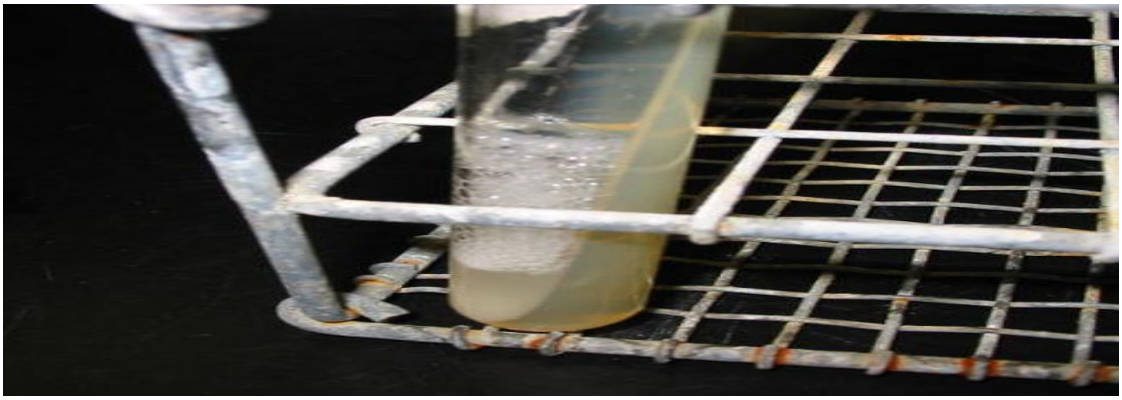


Figure (4.3) Catalase activity: – *B.cereus*

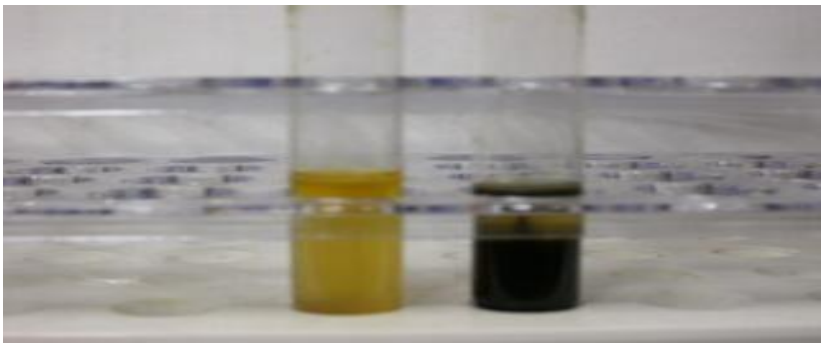
HYDROGEN SULPHIDE PRODUCTION TEST

OBSERVATION :

black coloration along the line of stab inoculation was not observed.

Result :

Black coloration along the line of stab inoculation was not observed. Hence the organism may be H_2S negative



A B

A: negative B: positive

Figure (4.4)Hydrogen sulphide production test

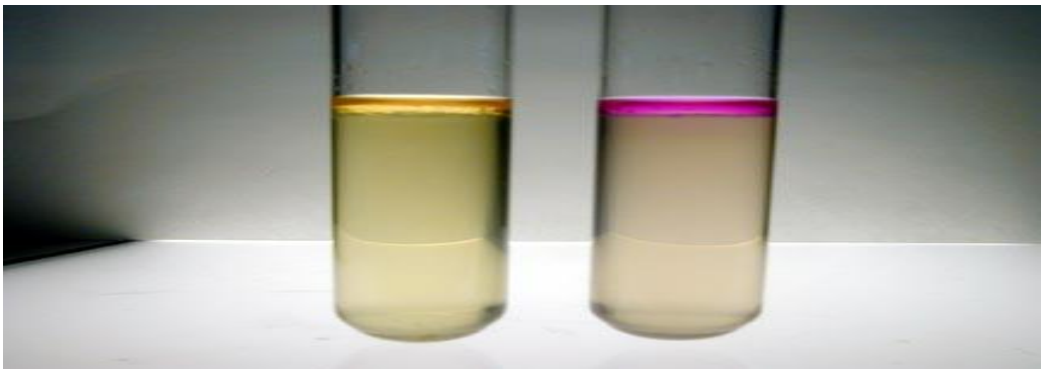
INDOLE PRODUCTION TEST

OBSERVATION :

Development of cherry (deep) red color in the top layer of the tube is not observed. Hence, *Bacillus cereus* an indole – negative bacterium.

Result :

As development of cherry red color is not observed in the top layer of the tube so *Bacillus cereus* is an indole-negative bacterium.



A B

A– *negative* **B – *positive***

Figure (4.5) cherry red color is not observed in the top layer of the tube

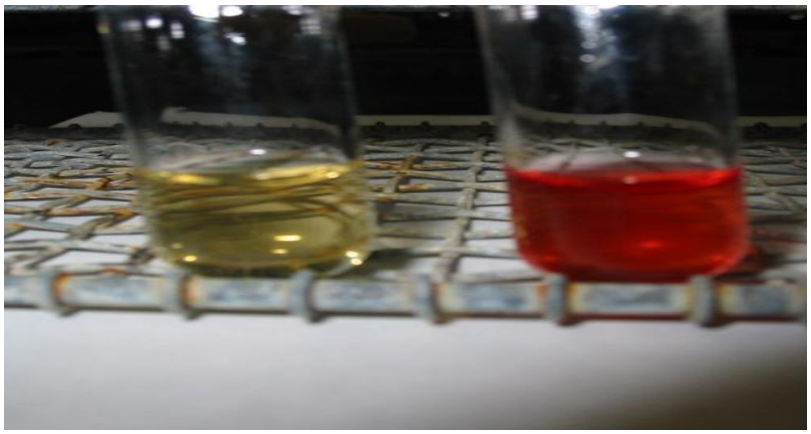
METHYL-RED AND VOGES-PROSKAUER TESTS

Observation :

The tubes in which methyl red was added pink color was observed, in the V-P test tubes when V-P reagents I & II were added red color observed.

Result :

As in the methyl red test red color is not observed hence, it is negative test. In the VP test, pink color is observed hence, it is positive test.



A- ***positive*** **B-** ***negative***

Figure (4.6) METHYL-RED AND VOGES-PROSKAUER TESTS

CITRATE UTILIZATION TEST

Observation :

After 48 hours of incubation it was observed that there is no change in color of the medium.

Result :

From the above observation it is said that *Bacillus cereus* is negative to this test.

A-positive B-negative

Figure (4.7) CITRATE UTILIZATION TEST

UREASE TEST

Observation:

After 48 hours of incubation it was observed that there is change in the medium..

Result:

From the above observation it is said that *Bacillus cereus* shows positive test. Its shows deep pink coloration of the medium thus showing positive reaction for the degradation of urea by means of the production of an enzyme urease.

A-positive B-negative

Figure (4.8) UREASE TEST

STARCH HYDROLYSIS TEST:

Observation :

After 24hrs incubation at 37^0C we have observed yellow color in the slant .

Result :

The bacteria is positive for this test.

CARBOHYDRATE FERMENTATION:

LACTOSE:

Observation:

After 24hrs incubation at 37^0C we have observed there is no bubbles in the duhram tube and observed pink color**.**

Result:

The bacteria will be positive for the acids production and negative for the gas formation**.**

4.10.2 GLUCOSE:

Observation:

After 24hrs incubation at 37^0C we have observed there is no bubbles in the duhram tube and observed pink color.

Result:

The bacteria will be positive for the acids production and negative for the gas formation.

IDENTIFICATION AND CHARACTERIZATION OF DIESEL OIL DEGRADING BACTERIA

Catabolic genes and catabolic enzymes responsible for diesel oil degradation were identified by using polymerase chain reaction (PCR) analysis respectively.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS OF THE CATABOLIC GENES FOR DIESEL OIL DEGRADATION

PCR analysis was conducted to check the presence of the both catabolic genes *C23O* and *alk* B for diesel oil degradation. Results of the analyses for *C23*O and *alk*B were given in the following parts. PCR analysis was applied isolate efficient in biosurfactant production. A standard curve was constructed for each gel amplification of *alk*B and *C23*O genes using the molecular weights of standard markers and the distance they migrated in the gel. The standard curve was used to calculate the molecular weight of each gene detected in the surface water isolates min, and 72°C for 1 min; and a final 10-min extension step at 72°C. Amplification products were then analyzed by electrophoresis on a 1% agarose gene.

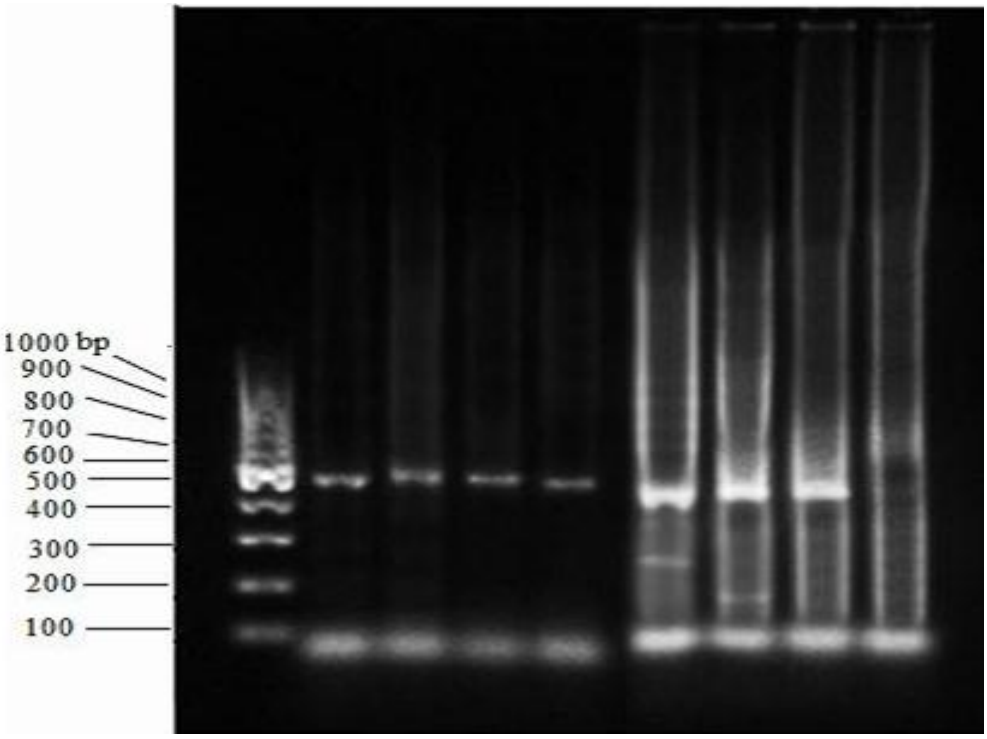


Figure (4.9) PCR FOR *alk*B gene

PCR Analysis of*C23O* :

Results of PCR analysis of *C23*O gene is given in Figure (4.8)

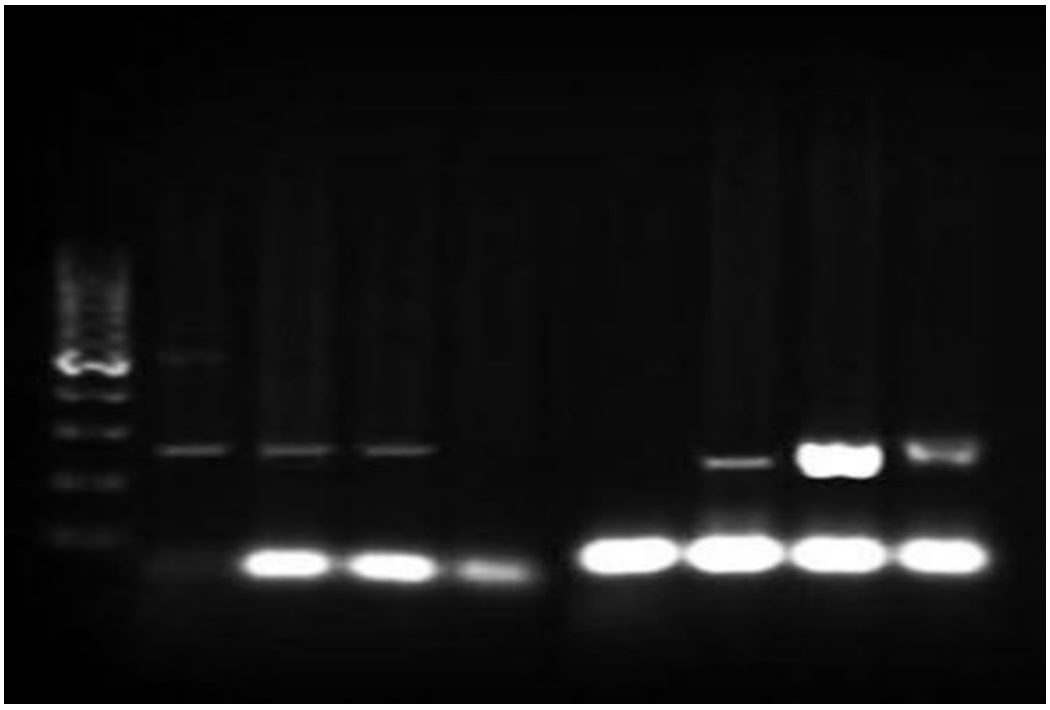


Figure (4.10) PCR FOR *C23*Ogene

DISCUSSION

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. Petroleum hydrocarbons can be

divided into four classes: the saturates, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides). Different factors influencing hydrocarbon degradation have been reported by Cooney et al. . One of the important factors that limit biodegradation of oil pollutants in the environment is their limited availability to microorganisms. Petroleum hydrocarbon compounds bind to soil components, and they are difficult to be removed or degraded . Hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes branched alkanes small aromatics cyclic alkanes. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all. Microbial degradation is the major and ultimate natural mechanism by which one can cleanup the petroleum hydrocarbon pollutants from the environment. The recognition of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reported by Jones et al. They studied the extensive biodegradation of alkyl aromatics in marine sediments which occurred prior to detectable biodegradation of n-alkane profile of the crude oil and the microorganisms, namely, Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus were found to be involved for alkylaromatic degradation. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye et al. Nine bacterial strains, namely, Pseudomonas fluorescens, P.aeruginosa, Bacillus subtilis, Bacillus sp., Alcaligenes sp., Acinetobacter lwoffi, Flavobacterium sp., Micrococcus roseus, and Corynebacteriumsp. were isolated from the polluted stream which could degrade crude oil. Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6% to 82% for soil fungi, 0.13% to 50% for soil bacteria, and 0.003% to 100% for marine bacteria. Many scientists reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil, fresh water and marine environments.

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment . Several bacteria are even known to feed exclusively on hydrocarbons . Floodgate listed 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi which were isolated from marine environment. A similar compilation by Bartha and Bossert included 22 genera of bacteria and 31 genera of fungi. In earlier days, the extent to which bacteria, yeast, and filamentous fungi participate in the biodegradation of petroleum hydrocarbons was the subject of limited study, but appeared to be a function of the ecosystem and local environmental conditions . Crude petroleum oil from petroleum contaminated soil from North East India was reported by Das and Mukherjee . Acinetobacter sp. was found to be capable of utilizing n-alkanes of chain length C_{10}–C_{40} as a sole source of carbon Fungal genera**,** namely, Amorphoteca, Neosartorya, T alaromyces, and Graphium and yeast genera, namely, Candida, Yarrowia, and Pichia were isolated from petroleum-contaminated soil and proved to be the potential organisms for hydrocarbon degradation. Sing also reported a group ofterrestrial fungi, namely,Aspergillus, Cephalosporium, and Pencillium which were also found to be the potential degrader of crude oil hydrocarbons. The yeast species, namely, Candida lipolytica,Rhodotorula mucilaginosa, Geotrichum sp, and Trichosporon mucoides isolated from contaminated water were noted to degrade petroleum compounds . Though algae and protozoa are the important members of the microbial community in both aquatic and terrestrial ecosystems, reports are scanty regarding their involvement in hydrocarbon biodegradation. Walker et al. isolated an alga, Prototheca zopfi which was capable of utilizing crude oil and a mixed hydrocarbon substrate and exhibited extensive degradation of n-alkanes and isoalkanes as well as aromatic hydrocarbons. Cerniglia et al. observed that nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms could oxidize naphthalene.Protozoa, by contrast, had not been shown to utilize hydrocarbon.

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