Identification and Molecular Characterization of Micro Organisms from Petroleum Soil

Chivukula L Gayitri

Abstract- The main objective of this study was experimentally to analyse the micro organisms isolated from soil samples from various areas and to find the isolated species by subjected them to polymerase chain reaction (PCR) and then elute the amplified product again using PCR. Then the eluted sample sent to sequencing to identify the bacteria which was present in the sample.

Keywords:- micro organisms, polymerase, bacteria.

I. MATERIALS AND METHODS

2.1. Collection of Soil Samples:

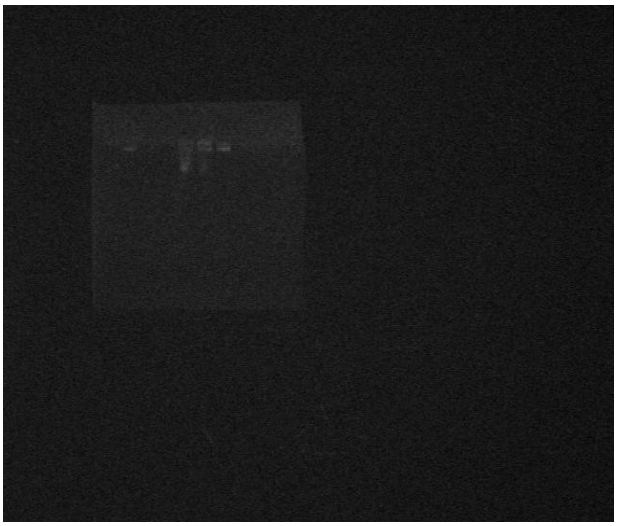
Samples were collected from petrol bunks and samples from soil surface (0-5 cm) and at a depth of approximately 20 cm (around the plants roots) were taken in sterilized polyethylene bags using sterilized the collected samples were taken to the laboratory under sterile conditions.

2.2. PETROLEUM SOIL:

ISOLATION OF SOIL DNA FROM PETROLEUM SOIL:

1. To isolate the DNA from soil samples two methods were followed. In first method collected soil samples were mixed with plant lysis buffer (PLB) and grind the soil gently and then the samples were added with lysozyme which is used to degrade the bacterial cellwall and pinch o charcoal and incubated at room temperature for 15min. Later these samples are added with proteinaseK and again incubated the samples at 60 degrees for 30min.Then centrifuge the samples for 5min at 10,000rpm.Collect the supernatent and discard the pellet. To the supernatant equal volumes of PBB is added and centrifuge them at 10,000rpm for 10min. Again collect the supernatent and is added with equal volumes of isopropanol and centrifuge them at 10,000rpm for 10min. Now discard the supernatent, to the pellet we have to add 70% ethanol and centrifuge the samples for 5min at 10,000rpm. Thus the samples are kept at room temperature for the complete evaporation of alcohol from the pellet. Then those samples are loaded in 1% agarose gel and run the samples for some time in agarose gel. Later observe the DNA bands under the

   UV- trans illuminator.



2.3. Petroleum Soil DNA PCR:

The purpose of PCR (Polymerase chain Reaction) is to make huge number of copies of a gene. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies.

Components of PCR:

I.	PE II buffer	- 2.5 µl
II.	Mgcl2	- 2 µl
III.	dNTP's	- 2 µl
IV.	Primers 1) Forward	- 1 µl
	2) Reverse	- 1µl
V.	Template	- 1 µl
VI.	Taq DNA polymerase	- 1.5 µl
VII.	Sterile water	- 14µl

2.4. CONDITIONS FOR POLYMERASE CHAIN REACTION (PCR):

1) DENATURATION: It was done at 94^{o}C. During the denaturation, the double strand melts open to single stranded DNA.

2) ANNEALING: It was done at 54^{o}C. . Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template.

3) EXTENSION: It was done at 72^{o}C. This is the ideal

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working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template.

2.5. PETROLEUMSOIL DNA PCR

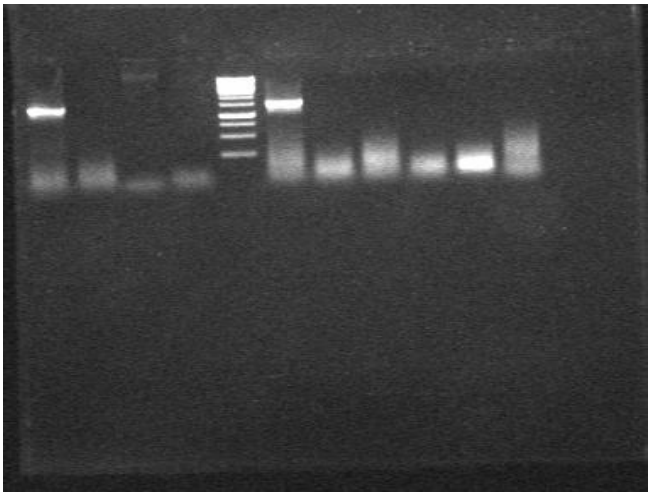

Fig: Petroleum Soil DNA PCR

Serial Dilution of Soil Samples:

The growth of bacteria will ranges from -3 to -7. For serial dilution take 1mg of soil sample in 1ml of water and mix it. Take some amount of sample from the first vial and into second vial and continue same for other vials upto seven**.**

Growth of Serial Diluted Soils:

Nutrient agar was prepared and the isolated bacterial species from nutrient broth were inoculated in nutrient agar and incubated at 37°C for 48 h. The developed colonies were counted in plates and the average number of colonies per all plates was determined. The number of total bacteria (CFU) per gram dry weight soil was determined. Individual colonies of bacteria which varied in shape and color were picked up and purified by streaking on nutrient agar. The bacterial isolates were kept on nutrient agar at 4°C and recultured every 4 weeks.

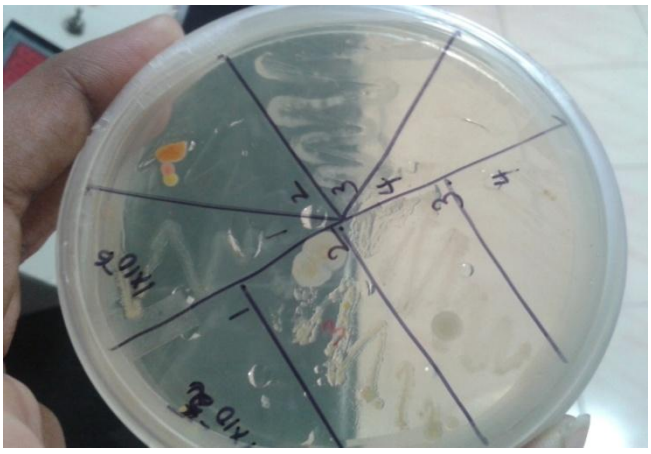

Fig: Streaking of Bacterial Colony

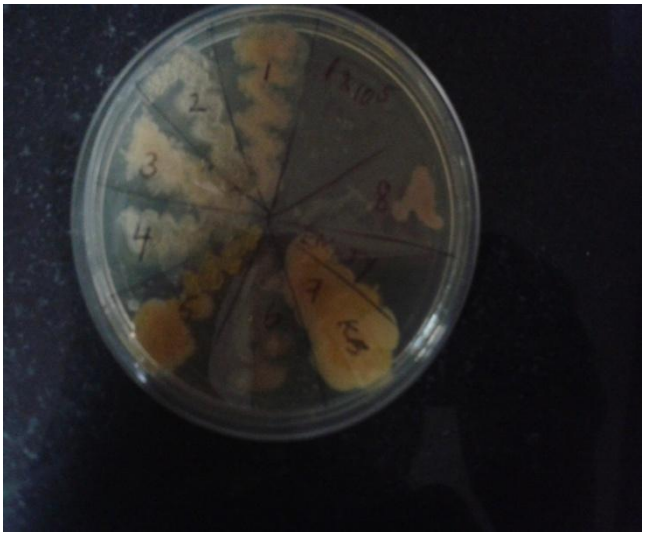

Fig: Spriding of Serially Diluted

Bio Chemical Test

By perfoming oxidase test the bacteria that are present in the pot soil are seemed to be PSEUDOMONAS. Because this test gave PESUDOMONAS positive result (the oxidase disc converted into blue color)

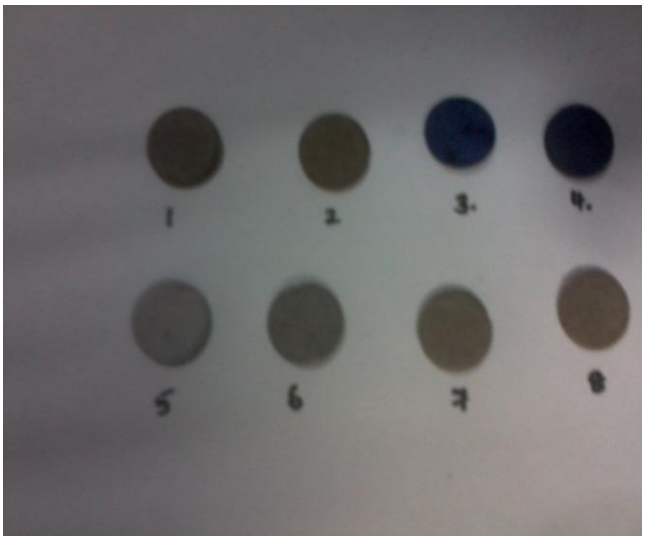

Fig: Bio Chemical Test

2.6. Gram Staining:

1. Prepare and heat fix the smear.
2. Stain the slide as follows
   a) Flood the crystal violet for one minute.
   b) Pour off excess dye and wash gently in tap water and drain the slide against a paper towel.
   c) Expose the smears to gram iodine for one minute by washing with iodine then adding more iodine and leaving it on the smear until the minute is over.
   d) Wash with tap water carefully but do not bolt.
   e) Wash with 95% alcohol for 30 sec.
   f) Wash with tap water at the end of 30 sec to stop the decolorization.
   g) Wash with tap water and drain.
   h) Counter stain with 0.25% saffranin for 30sec.
   i) Wash, drain, blot and examine under the microscope.

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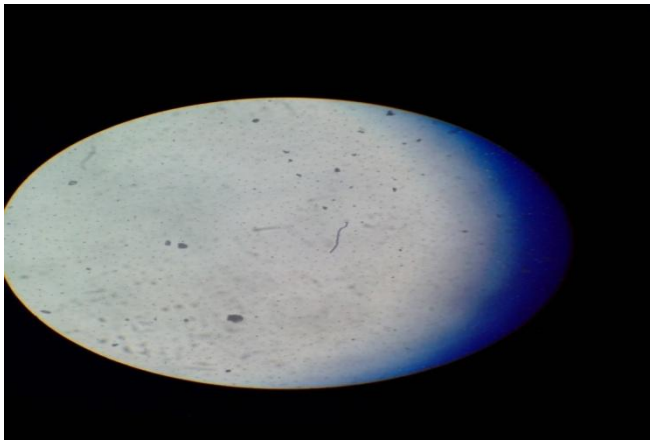


Fig: Gram Staining

2.7. Isolation of Bacteria from Petroleum Soil:

The bacteria were isolated from the collected samples by doing serial dilution and spreading the sample on LB (Luria Bertani) agar medium. From the numerous colonies obtained on the LB plates, pick the individual colonies and grow the culture. *DNA* was extracted from 1ml of bacterial culture. the culture was pellet by centrifuge at 12,000rpm for 2 min. the pellet was treated with lysis solution and proteinase k and incubated at 60°C for 30min. Nucleic acids were precipitated with isopropanol by centrifuging at 10,000 rpm for 10 min, washed with 500 ml of a 70% (v/v) ethanol solution and dissolved in 0.05 ml of a TE buffer. The purity and quantity of DNA were examined by recording its UV absorption spectrum and running on 1% agarose gel electrophoresis. The concentration of DNA was measured by spectrometer.

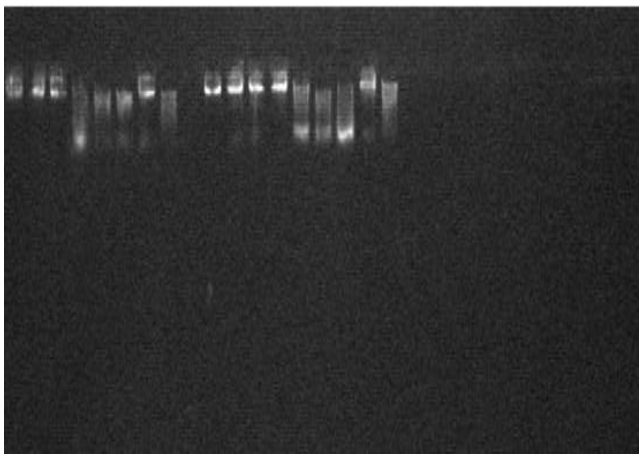


Fig: Isolation of Bacteria from Collected Samples

2.8. POLYMERASE CHAIN REACTION:

The purpose of PCR (Polymerase chain Reaction) is to make huge number of copies of a gene. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies.

Taq DNAPOLYMERASE: An enzyme that synthesizes new strands of DNA complementary to the target sequence.

PRIMERS: Short pieces of single stranded DNA that are complementary to target sequence.

dNTPs: Single units of bases A, T, G and C which are essentially building blocks for new DNA strands.

CONDITIONS FOR PCR:

1) DENATURATION
2) ANNEALING
3) EXTENSION

1) DENATURATION: It was done at 94^{o}C. During the denaturation, the double strand melts open to single stranded DNA.

2) ANNEALING: It was done at 54^{o}C. . Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template.

3) EXTENSION: It was done at 72^{o}C. This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template.

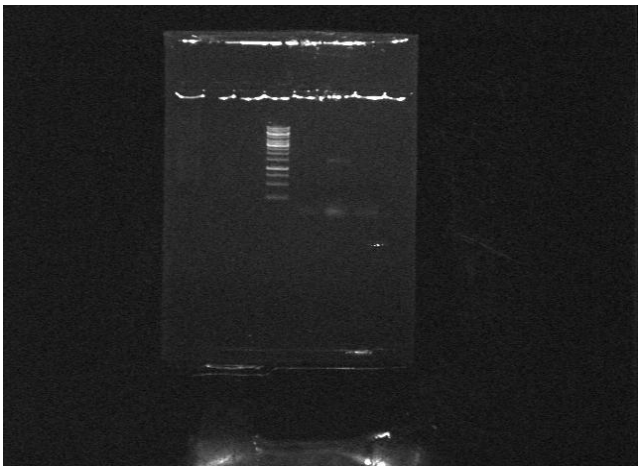


Fig: 4.27 PCR Petroleum Soil

2.9. DNA EXTRACTION FROM GEL

REAGENTS REQUIRED:

- Agarose gel slice contain DNA fragments
- GA buffer
- Wash buffer II
- Elution buffer/TE (here TE was used)

The DNA extraction from gel typically consist of four steps; gel dissociation, DNA binding, wash and DNA elution.

2.10. PROCEDURE

Gel Dissociation:

1) Excise the agarose gel slice containing relevant DNA fragments and remove any extra agarose to minimize

the size of the gel slice.

2) Transfer upto 300mg of the gel slice into a 1.5ml microcentrifuge tube.
3) Add 500µl of GA buffer to the sample and mix by vortexing.

DNA Binding:

1) Place a column in a 2ml collection tube.
2) Apply 800µl of the sample mixture from step 3 into the DF column.
3) Centrifuge at full speed (6500rpm) for 60 seconds.
4) Discard the flow through and place the DF column back in the 2ml collection tube.

Wash:

1) Add 600µl of wash buffer II into the DF column.
2) Centrifuge at full speed (12000rpm) for 60 seconds.
3) Discard the flow through and place DF column back in the 2ml collection tube. Transfer the dried DF column in a new 1.5ml microcentrifuge tube.
4) Add 20µl-50µl of elution buffer or TE into the centre of the column matrix.
5) Let stand for 2 minutes, until elution buffer or TE is absorbed by the matrix.
6) Centrifuge at full speed for 1 minute at 12000rpm to elute the purified DNA.
7) Check the obtained product in 1%agarose gel electrophoresis with ladder and check the purity of obtained product.
8) The obtained purified product is sent for sequencing to the isolated product result.

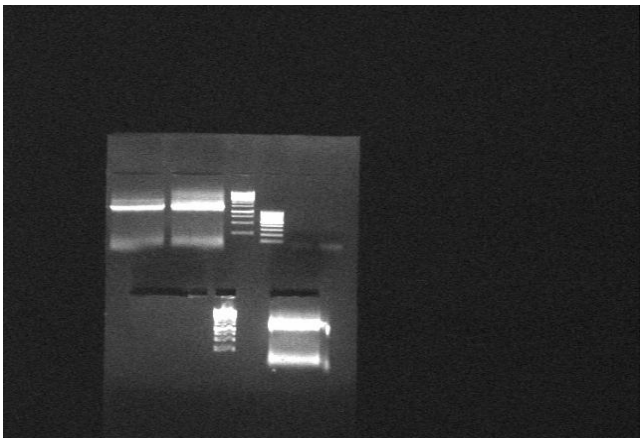


Fig: DNA Extraction from Gel

II. SEQUENCING

DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of all: the sequence of nucleotides. With this knowledge, for example, we can locate regulatory and gene sequences, make comparisons between homologous genes across species and identify mutations. Scientists recognized that this could potentially be a very powerful tool, and so there was competition to create a method that would sequence DNA. Then in 1974, two methods were independently developed by an American team and an English team to do exactly this. The Americans, lead by Maxam and Gilbert, used a chemical cleavage protocol, while the English, lead by Sanger, designed a procedure similar to the natural process of DNA replication. Even though both teams shared the 1980 Nobel Prize, Sangers method became the standard because of its practicality (Speed, 1992). Sangers method, which is also referred to as dideoxy sequencing or chain termination, is based on the use of dideoxynucleotides (ddNTPs) in addition to the normal nucleotides (NTPs) found in DNA. Dideoxynucleotides are essentially the same as nucleotides except they contain a hydrogen group on the 3 carbon instead of a hydroxyl group (OH). These modified nucleotides, when integrated into a sequence, prevent the addition of further nucleotides. (Speed, 1992).This occurs because a phosphodiester bond cannot form between the dideoxynucleotide and the next incoming nucleotide, and thus the DNA chain is terminated.

The Method

Before the DNA can be sequenced, it has to be denatured into single strands using heat. Next a primer is annealed to one of the template strands. This primer is specifically constructed so that its 3' end is located next to the DNA sequence of interest. Either this primer or one of the nucleotides should be radioactively or fluorescently labeled so that the final product can be detected on a gel (Russell, 2002). Once the primer is attached to the DNA, the solution is divided into four tubes labeled "G", "A", "T" and "C". Then reagents are added to these samples as follows:

"G" tube: all four dNTP's, ddGTP and DNA polymerase
"A" tube: all four dNTP's, ddATP and DNA polymerase
"T" tube: all four dNTP's, ddTTP and DNA polymerase
"C" tube: all four dNTP's, ddCTP and DNA polymerase

As shown above, all of the tubes contain a different ddNTP present, and each at about one-hundreth the concentration of the the normal precursors (Russell, 2002). As the DNA is synthesized, nucleotides are added on to the growing chain by the DNA polymerase. However, on occasion a dideoxynucleotide is incorporated into the chain in place of a normal nucleotide, which results in a chain. DNA sequencing refers to the methods and technologies that used to determine the orders of nucleotide bases in a DNA molecule, namely adenine (A), guanine (G), cytosine (C) and thymine (T). DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of all: the sequence of nucleotides. The knowledge of DNA sequences has formed the basis of basic biological researches and clinical genetic diagnosis. There are also numerous applied technology fields such as biotechnology, forensic science and biological systematics that are heavily dependent on the information generated through DNA sequencing. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of the human genome, in the Human Genome Project. Related projects, often by scientific collaboration across continents, have generated the complete DNA sequences the genomes of many animals, plants, and microorganisms. The Sanger method, also referred to as dideoxynucleotide sequencing or chain termination sequencing, is based on the use of dideoxynucleotide (ddNTP) in addition to the normal nucleotides (dNTP) found in DNA. Dideoxynucleotide are essentially the same as nucleotides except they contain a hydrogen group on the 3’ carbon instead of a

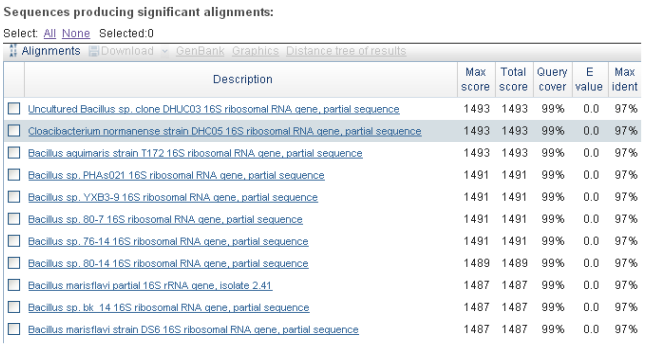
hydroxyl group (OH). These modified nucleotides, when integrated into a DNA sequence, prevent the addition of further nucleotides thus stop the elongation of the DNA chain. This occurs because a phosphodiester bond cannot form between the dideoxynucleotide and the next incoming nucleotide, and thus the DNA chain is terminated. Although the various reagents, equipment and methodological strategies for carrying out DNA sequencing have undergone constant evolution to improve the simplicity, speed and reliability of the process, the basic procedure which all modern gel-based enzymatic DNA sequencing utilizes has not changed over the decades since its invention. Following steps are the outlines of the procedures of the Sanger methods:

1. The region of DNA to be sequenced is amplified in some way and then denatured to produce single stranded DNA.
2. A sequencing primer is annealed to the single stranded DNA.
3. Dideoxynucleotide chain termination DNA sequencing then takes advantage of the fact that a growing chain of nucleotides, extending in the 5’ to 3’ direction, will terminate if, instead of a conventional deoxynucleotide, a 2’3’ dideoxynucleotide becomes incorporated. By performing four separate reactions, each containing a DNA polymerase and a small amount of one of the four dideoxynucleotides in addition to all four deoxynucleotides, four separate sets of chain-terminated fragments can be produced.
4. Following the replication/termination step, these chain terminated fragments will remain bound to the single stranded DNA molecule which has acted as a template. By heating these partially double stranded molecules and adding a denaturing agent such as formamide, the single stranded chain termination molecules can be released from their template and separated using high resolution denaturing gel electrophoresis.
5. The sequence of the original region of DNA is then finally deduced by examining the relative positions of the dideoxynucleotide chain termination products in the four lanes of the denaturing gel

II. RESULTS AND DISCUSSION

TYPE OF SOIL	Petroleum soil
OBSERVED BACTERIA	1. BACILLUS AQUIMARIS 2. STAPHYLOCOCCUS AUREUS

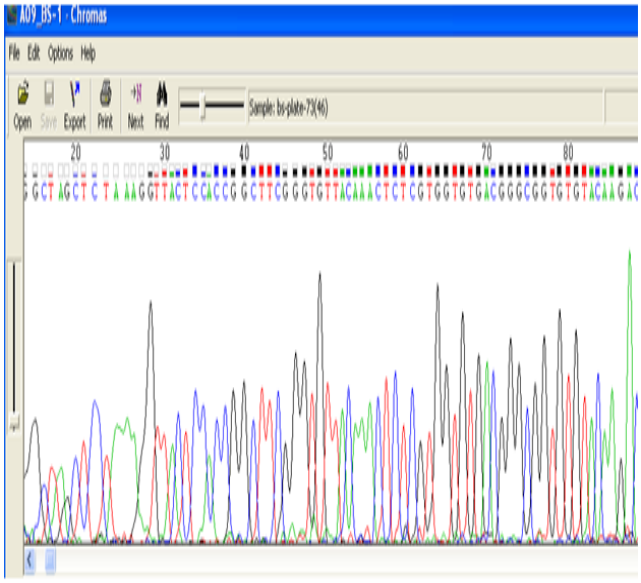
BACILLUS AQUIMARIS

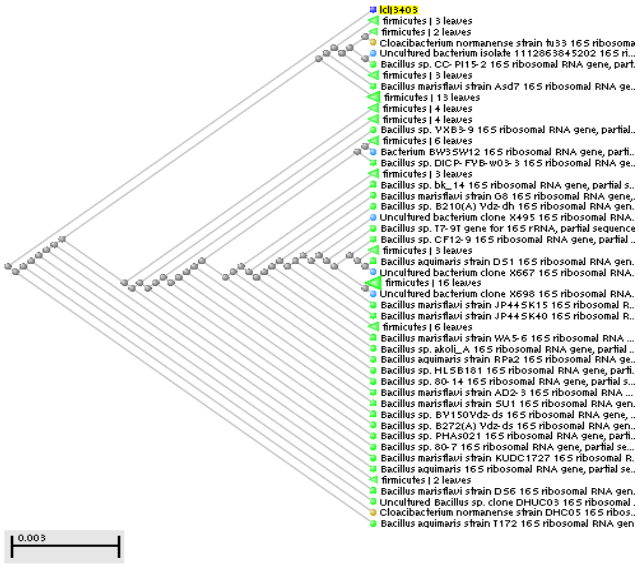

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Max ident
Uncultured Bacillus sp. clone DHUC03 16S ribosomal RNA gene, partial sequence	1493	1493	99%	0.0	97%
Cloacibacterium normanense strain DHC05 16S ribosomal RNA gene, partial sequence	1493	1493	99%	0.0	97%
Bacillus aquimaris strain T172 16S ribosomal RNA gene, partial sequence	1493	1493	99%	0.0	97%
Bacillus sp. PHAs021 16S ribosomal RNA gene, partial sequence	1491	1491	99%	0.0	97%
Bacillus sp. YXB3-9 16S ribosomal RNA gene, partial sequence	1491	1491	99%	0.0	97%
Bacillus sp. 80-7 16S ribosomal RNA gene, partial sequence	1491	1491	99%	0.0	97%
Bacillus sp. 76-14 16S ribosomal RNA gene, partial sequence	1491	1491	99%	0.0	97%
Bacillus sp. 80-14 16S ribosomal RNA gene, partial sequence	1489	1489	99%	0.0	97%
Bacillus marisflavi partial 16S rRNA gene, isolate 2.41	1487	1487	99%	0.0	97%
Bacillus sp. bk_14 16S ribosomal RNA gene, partial sequence	1487	1487	99%	0.0	97%
Bacillus marisflavi strain DS6 16S ribosomal RNA gene, partial sequence	1487	1487	99%	0.0	97%





STATHYLOCOCCUS AUREUS

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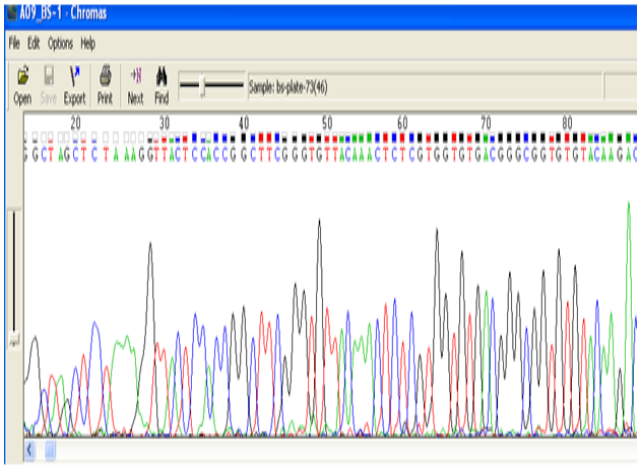
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Max ident
Staphylococcus sp. BGN1P-02d 16S ribosomal RNA gene, partial sequence	1635	1635	97%	0.0	98%
Staphylococcus haemolyticus strain BGN1L-01d 16S ribosomal RNA gene, partial sequence	1635	1635	97%	0.0	98%
Staphylococcus sp. M17 16S ribosomal RNA gene, partial sequence	1631	1631	98%	0.0	98%
Staphylococcus aureus subsp. aureus ST228 complete genome, isolate 18583	1631	8147	98%	0.0	98%
Staphylococcus aureus subsp. aureus ST228 complete genome, isolate 18412	1631	8147	98%	0.0	98%
Staphylococcus aureus subsp. aureus ST228 complete genome, isolate 18341	1631	8147	98%	0.0	98%
Staphylococcus aureus subsp. aureus ST228 complete genome, isolate 16125	1631	8140	98%	0.0	98%
Staphylococcus aureus subsp. aureus ST228 complete genome, isolate 16035	1631	8147	98%	0.0	98%
Staphylococcus aureus subsp. aureus ST228 complete genome, isolate 15532	1631	8147	98%	0.0	98%
Staphylococcus aureus subsp. aureus ST228 complete genome, isolate 10497	1631	8147	98%	0.0	98%
Staphylococcus aureus subsp. aureus ST228 complete genome, isolate 10388	1631	8147	98%	0.0	98%



REFERENCE

1. Yeates C, Gillings MR, Davison AD, Altavilla N, Veal DA. PCR amplification of crude microbial DNA extracted from soil. Letters in Applied Microbiology. 1997;25:303–307.
2. Atlas RM. Diversity of microbial communities. Advances in Microbial Ecology. 1984;7:1–47.