JOURNAL OF SCIENTIFIC RESEARCH IN ALLIED SCIENCES
ISSN NO. 2455-5800
Contents available at www.jusres.com

DETECTION AND IDENTIFICATION OF BACTERIA IN SOIL SAMPLES BY 16S rRNA GENE SEQUENCING

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ARTICLE INFO **ABSTRACT** **ORIGINAL RESEARCH ARTICLE**

Article History
Received: April 2022
Accepted: May 2022
Keywords:
Soil microbiome, Soil health, Metagenomics, 16S rRNA, and PCR.

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Soil is the lifeblood of the earth and is considered one of the largest reservoirs of microbial diversity. Examining the diversity of soil microbial communities is important because they play an important role in maintaining soil health by recycling nutrients and building soil structure and humus. However, cultural studies cannot provide an accurate estimate of diversity and untapped resources. Therefore, there is a need to investigate microbial diversity using culture-independent methods. The field of metagenomics contributes to the study of the genomes of different soils with new resource potential in their own habitats. In this research, we collected and examined three different soil samples from agriculture, parks and roads. Additionally, soil samples were inoculated into food broth media to promote the growth of top bacteria. Additionally, genomic DNA was extracted from cultured cells and then PCR was used to amplify the 16S rRNA gene. 16S rRNA gene sequencing is used as a tool to identify disease types and help distinguish between closely related diseases. The 16S rRNA gene is a highly conserved transcription factor of all forms of DNA-based life, making it well-suited as a target for DNA sequencing in samples containing thousands of different species. Universal PCR primers can be designed to target the conserved region of 16S, allowing the amplification of genes from many different organisms from a single sample. In our study, we detected three different diseases in three different samples

INTRODUCTION

Microorganisms dominate the world. Although most of us don't see them, bacteria are crucial to every aspect of human life, indeed to all life on Earth. All processes in the biosphere are affected by the apparent inability of organisms to change the world around them. The chemical cycles that transform the basic elements of life (carbon, nitrogen, oxygen, and sulfur) into bioavailable forms are mostly performed by and dependent on organisms. All plants and animals are closely associated with microbial communities that provide their hosts with essential nutrients, iron, and vitamins. Through fermentation and other natural processes, bacteria add or create value to many foods that are part of human consumption (Christopher J. 2017). We rely on microbes to treat toxins in the environment, both caused by toxins and man-made products (like oil and poisons). The bacteria found in the human stomach and mouth allow us to obtain energy from food that cannot be digested without them and protect us from disease-causing bacteria (Dudek et al. 2017). Soil, like other resources, is a mixture of

organic matter, minerals, gases, liquids, and living organisms that work together to support life. Microorganisms are an important component of soil biodiversity and an important part of soil ecosystems. Soil is a dynamic and diverse entity that lives between water and life on Earth. Soil is the best source of unknown organisms such as bacteria, algae and protozoa. Given that microbial populations are associated with geochemistry and greenhouse gas cycles, as well as climate and environmental change, it is important to understand how well we understand microbial communities. In the past, it was difficult to study microbes in their own environment. Microbiologists examine species one by one in the laboratory. Many organisms now appear to operate in multicellular, often multispecies, organized, sometimes even physically connected (e.g., biofilms) and often metabolically linked states. Genomics is a method that contributes greatly to the understanding of all living organisms by determining their DNA sequences to understand their evolution and potential. Genomics has also contributed to the development of microbiology, but traditional culture, like pure culture, has limitations in its ability to reveal microbial community dynamics. Microbiome research is rapidly expanding due to major advances in genome sequencing. The most commonly used method in connection with the study of the microbiome is metagenomics (Berendsen RL et al. 2012). Metagenomics refers to the sequencing of genes, mainly ribosomal RNA (rRNA) genes, which are conserved across taxa (Marchesi and Ravel, 2015; Handelsman J., 2005). This strategy, which requires sequencing of only a single gene, provides good results in identifying many diseases. Metagenomics refers to “shotgun” sequencing of microbial DNA without selecting specific genes (Bashir Y et al. 2014; Hernandez-Leon R et al. 2012). 16S rRNA gene sequencing is used as a tool to identify disease types and help distinguish between closely related diseases. The 16S rRNA gene is a highly conserved transcription factor of all forms of DNA-based life, making it well-suited as a target for DNA sequencing in samples containing thousands of different species (Nguyen N et al. 2016; Ramazzotti and Bacci 2018; Ranjan R et al. 2016). Universal PCR primers can be designed to target the conserved region of 16S, allowing the amplification of genes from many different organisms from a single sample. In our study, we detected three different diseases in three different samples. These study is well suited for phylogenetic analysis because they are widespread in populations, have regions that vary between different species, and are flanked by conserved regions that can be targeted by universal primers (Jia and Whalen, 2020; Singer E et al. 2016). In this research, we collected and examined three different soil samples from agriculture, parks and roads. Additionally, soil samples were inoculated into food broth media to promote the growth of top bacteria. Additionally, genomic DNA was extracted from cultured cells and then PCR was used to amplify the 16S rRNA gene.

MATERIALS AND METHODS

Collection and Serial Dilution of Soil Sample

Soil samples were collected from the industrial site. 1 gm of soil was inoculated in 10 ml of distilled sterile water. It is a method of diluting a stock sample (soil water) concentration decreased by the same quantity in each successive step.

Isolation and purification of Soil Bacteria

Samples of diluted water were cultured on NAM plates. A small amount of mixed culture is placed on the tip of an inoculation loop/needle and is streaked across the surface of the agar medium. The successive streaks ―thin out‖ the inoculum sufficiently and the micro-organisms are separated from each other. These plates are incubated to allow the growth of colonies. Such isolated colonies are picked up separately using sterile inoculating loop/needle and re-streaked onto fresh media to ensure purity. Further, cultured bacteria were inoculated in broth medium for DNA isolation.

Isolation of Genomic DNA from Broth Bacterial Culture

1ml bacterial cell culture was Centrifuge at 5000 rpm for 7 min then supernatant was discarded. 500µl TE buffer

was added to homogenize the solution and then 100µl 10% SDS Mix the solution by pipetting/vortex and incubated 30 min at 37°C. Added 100µl Potassium acetate and incubated for 30 min at 37°C Centrifuge at 12000 rpm for 10 min and collected the supernatant in fresh centrifuge tube. 500µl of chilled Isopropanol was added and incubated tube at -20°C for 15 to 20 minutes after that centrifuge at 13000 rpm for 10 min. Supernatant was discarded and add 500µl 70% ethanol Centrifuge at 12000 rpm for 7 min at 4°C (Chakravorty, S et al. 2007)

Quantity of DNA was checked by using UV-Spectrophotometer:

For quantitation, the optical density was measured at 260nm and 280nm wavelengths. The reading at 260nm allows calculations of the concentration of nucleic acid in the sample:

1 OD at 260nm for single stranded DNA=50µg/ml of ssRNA

The reading at 280nm gives the amount of protein in the sample. Pure preparations of RNA have OD260/OD280 values of 1.8 (Ramazzotti M, & Bacci G 2018). If there is contamination with protein or phenol, this ration will be significantly less than 2.0. Samples were diluted 200 times (5µl of DNA in 995µl of distilled water). The DNA concentrations were calculated accordingly:

OD260 x 50µg/ml x dilution factor (200)

Qualitative Analysis of Genomic DNA Using Gel Electrophoresis

Prepared 100 ml of 1.5% agarose gel by adding 1.5 gm of agarose to 100 ml in 1X TAE buffer in a conical flask. Heat the mixture on a microwave, swirling the conical flask, until the agarose dissolves completely. Allowed to cool down between 55-60°C. Added 2 µl ethidium bromide, mixed well and poured the gel solution into the gel tray. Allowed the gel to solidify for about 15 minutes at room temperature. Added 1µl of 6X gel with 3 µl of DNA and 2 µl water to make 1X dilution. Samples were loaded with 1 µl of pipette. Connected the power cord to the electrophoretic power supply according to the conventions: black-anode and red-cathode. Appled a voltage of 65-75 volts. Stoped the electrophoresis, when bromophenol blue crossed more than 2/3 of the length of 28 the gel. Visualized DNA bands on an UV trans-illuminator.

Primer Designing Using Oligonucleotide Properties Calculator

Universal Primers were used to for identification of the unknown bacteria. Primers are calculated in oligonucleotide properties calculator. Conditions for primer were: For Forward primer: GC content –50%; Tm – 51°C; Length – 20 For reverse primer: GC content – 45%; Tm – 49.7°C; Length – 20

Gene Name		**Sequence**
Universal primers	FORWARD (27F)	5‘-AGAGTTTGATCCTGGCTCAG-3‘
	REVERSE (1492R)	5‘-CGGTTACCTTGTTACGACTT-3‘

Screening of possible bacteria using16S rRNA primers in PCR

Reaction mixture was prepared and pours in the PCR tubes. Tubes were put in the thermo-cycler (Table 1).

Table 1: PCR reaction components concentration.

Components	Stock Concentration	Final concentration	Volume
dH_2O	-	-	13.67µl
Taqbuffer DNA	10X	1X	2µl 2µl
dNTPs	200ng/µl 10mM	100ng/µl 200µM	0.4µl 0.8µl
Primer(Forward) Primer	5pmol/µl 5pmol/µl	200nM	0.8µl
(Reverse)	3 unit/µl	200nM	0.33µl
Taqpolymerase		1 unit	
Total			20µl

Blast and Sequence Analysis Using Bioinformatics Tools

Eluted sample was sent to fro sequencing at Genescan Pvt Ltd, Delhi, India. Further obtained sequence was used in BLAST tool.

RESULTS AND DISCUSSIONS

Sample collection and serial dilution of samples:

A ten-fold serial dilution was done. Total 5 different samples were diluted and plated on Nutrient Agar Media for growth of the microorganisms. A decreasing number of colonies were grown with respect of dilution.

Isolation of DNA

The colonies were inoculated in the NAM broth for DNA extraction. DNA extraction was performed in overnight culture. Salt precipitation method was used for DNA extraction. The spool was seen when chilled isopropanol was added and the pellet was also seen in the bottom of the MCT.

Qualitative and Quantitative analysis of Extracted DNA by Gel electrophoresis and UV spectrophotometer

Total 4 colonies were picked for DNA extraction, which have shown a very good band on 1.2% agarose gel and a good quality ratio was obtained in UV spectrophotometer. Further, these DNA were used in PCR (Fig. 1).

UV spectrophotometer: DNA samples were diluted 200 times and analyzed at 260nm and 280 nm for quality check on spectrophotometer (Table 2). All samples are showing a good 260/280 ratio which is Spool of DNA around 1.8.

Table 2: Spectrophotometric analysis of isolated DNA

Sample	A_{260}	260/280	Concentration(ng/ul)
S1	0.09	1.89	900
S2	0.04	1.88	400
S3	0.05	1.82	500
S4	0.09	1.81	930

Molecular Identification of unknown bacteria in soil sample using 16SrRNA

The molecular identification of bacteria which was grown on NAM plates, was conducted by using universal primers. A sharp bands of (1512 bps) were observed in sample 3 and sample 4 while band was absent in sample 1 and sample 2. Universal primers were used which were designed by using Oligonucleotide Properties calculator (Fig 2).

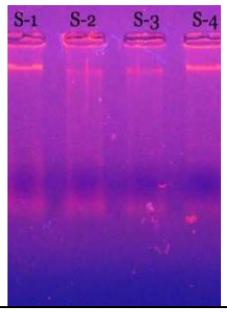

Figure 1: DNA run on 1.2% agarose Gel Electrophoresis

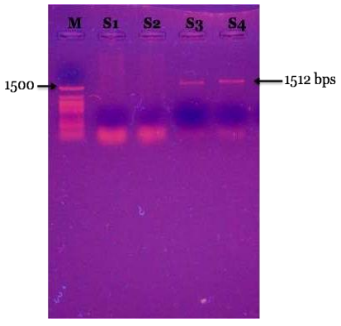

Figure 2: PCR products were run on 1.2% agarose gel, Lane 1: 100bps ladder, lane 2: sample 1, Lane 3: sample 2, Lane 4 Sample 3, Lane 5 Sample 4.

Sequencing of DNA and sequence was BLAST

The eluted DNA was sent for the sequencing to Genescan Pvt Ltd Delhi, India. The obtained sequence was used for the BLAST. The obtained sequence was aligned to *Bacillus subtilis.*

>NC_019948, from 6551844 to 6553355 (1512 bp); Bacillus subtilis BEST7613 DNA **AGAGTTTGATACTGGCTCAG**CGGCTACCTTGTTACGACTTCACCCCAATCAT
CTGTCCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGG
TGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGT
CGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAA
CCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCA
GGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTC
ACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGAC
AACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGG
ATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCG
CTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGT
CTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTA
AGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTAC
CAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTA
CAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTT
CACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTT
TCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAA
CCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGAAAACGCTTGCCACCTAC
GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTAC
CGTCAAGGTACCGCCCTGTTCGAACGGTACTTGTTCTTCCCTAACAACAGAGC
TTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTC
GTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTG
TCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGC
CTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGT
AAGTGGTAGCCGAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAACCATCCGGTATTAGCCCCG
GTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTAC
CCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCACCT
GTCCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCA
GATCAAACTCT**AAGTCGTAACAAGGTATCCG**

Shaded part indicates the position of primers. Further this sequence was used to study the relatedness among the species and a phylogenic tress was prepared. For this study, Oligonucleotide Properties Calculator software was used.

CONCLUSION

In our study, we used three different samples from agricultural, park and road side areas. In our hypothesis, we were assuming a vast diversity of microbes in studied three samples. We got an amplified DNA after PCR in all three samples using universal 16SrRNA primers. After sequencing, we obtained most common bacteria of soil *Bacillus subtilis.*

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