WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
Coden USA: WJPRAP Impact Factor 8.453
Volume 15, Issue 1, 754-771. Research Article ISSN 2277–7105

ISOLATION AND IDENTIFICATION OF SOIL BACTERIAL DIVERSITY IN FIRE WORKS AREA

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Article Received on 28 Nov. 2025,
Article Revised on 18 Dec. 2025,
Article Published on 01 Jan. 2026,

https://doi.org/10.5281/zenodo.18094531

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How to cite this Article: Fidha K., Prabhu V. (2026). ISOLATION AND IDENTIFICATION OF SOIL BACTERIAL DIVERSITY IN FIRE WORKS AREA. World Journal of Pharmaceutical Research, 15(1), 754–771.

ABSTRACT

The microbial diversity of soil and water samples collected from areas exposed to pyrotechnical chemicals in Sivakasi, Virudhunagar district (Tamil Nadu, India) was studied. Samples from cultivable land, waste areas, and urban locations within the same region were also analyzed for comparative purposes. The generic composition of the samples from the pyrotechnical exposure sites showed significant differences compared to the other samples. Results from 16S rDNA molecular sequencing indicated that *Pseudomonas* species were predominant in both the soil and water samples from the pyrotechnically exposed areas, followed by *Achromobacter* species in the soil and *Bacillus* and *Micrococcus* species (with *Micrococcus* accounting for 16.6%) in the water samples. Notably, Corynebacterium and *Micrococcus* were completely absent in the pyrochemical-exposed soil, and *Achromobacter* was missing in the exposed water samples, despite their presence in the other locations tested. This study clearly demonstrates that pollutants from pyrotechnical practices can significantly impact microbial biodiversity. It underscores the need for appropriate measures to control pollution levels and protect biodiversity.

KEYWORDS: Bacterial Diversity pyrotechnical chemicals, *Pseudomonas*, and *Achromobacter*.

INTRODUCTION

Plants are constantly attacked by a variety of pathogens, including bacteria, fungi and viruses. Plants have evolved a machinery to combat these pathogen attacks. Unlike humans, plants lack a proper immune system. However, plants have developed a capable molecular and chemical system to detect invading microorganisms and prevent them before they can cause any extensive damage to plants. A proper understanding of the plant defense mechanism is crucial for improving crop health and yield. Based on their lifestyles, plant pathogens can be divided into two major categories- Biotrophs and Necrotrophs. Most bacteria and viruses are considered as biotrophs, while fungi mostlyadopt necrotrophic life styles. Use of microbial inoculants or plant growth-promoting rhizobateria (PGPR) for the enhancement of sustainable agricultural production is becoming a more widely accepted practice in intensive agriculture in many parts of the world (Glaze brook. 2005).

The use of microorganisms as environmental friendly strategies to support crop production has great potential. A lot of attention has been given to microorganisms that confer disease suppression Antoszewski 2022 Certain organic compounds exuded act as chemical signals for microbes by stimulating colonization through Choudhary 2009.

Roots support plants physically by anchoring and chemically through the acquisition of nutrients and water from soil. The root architecture adopts the most suitable structure depending on plant needs and is influenced by various environmental stimuli Lucy *et al*., 2004 Many rhizobacteria can thus associate with plants and ideally promote plant growth and fitness and are referred to as plant growth promoting rhizobacteria (PGPR) Pieterse *et al*., 2012. Referred to as priming of induced systemic resistance (ISR) Majeed *et al*., 2015. Chemical fertilizers are generally used to supply essential nutrients to the soil–plant system throughout the world. However, the prices, availability, and the environmental concerns of chemical fertilizers especially the N fertilizers are real issues of today's agriculture.

Two routes of Rhizobium infection have been described for root-nodule formation in legume roots: entry via roothairs and via cracks. Root-hair entry occurs in most legumes, e.g. soybean and common bean (*Phaseolus vulgaris*). Crack entry occurs in few legumes: peanut and *Sesbania*. In peanut, root nodules develop only at the sites of lateral-root emergence (Uheda *et al*., 2001), the epidermis and cortex of the parent root are broken by emergence of the lateral root (reviewed in Boogerd and van Rossum, 1997). Uheda *et al*., 2001) visually demonstrated Rhizobium infection into the root through the intercellular gap created by

lateral-root emergence. Rhizobacteria that benefit plants by stimulating growth and suppressing disease are referred to as plant growth promoting rhizobacteria (Lugtenberg and Kamilova 2009).

Application of chemical fertilizers in slopping landscapes under high annual rainfall normally exist in the mountain ecosystem of the Hindu Kush Himalayan (HKH) region may not be effective because of surface runoff and leaching. Therefore, there is an urgent need to find alternative strategies that can ensure com- petitive crop yields, provide environmental safety, and protection while maintain long term ecological balance in agro-ecosystem. Use of microbial inoculants or plant growth- promoting rhizobateria (PGPR) for the enhancement of sustainable agricultural production is becoming a more widely accepted practice in intensive agriculture in many parts of the world Kumar *et al*., 2014. Plant growth-promoting rhizobacteria are free-living soil bac- teria that aggressively colonize the rhizosphere plant roots, and enhance the growth, and yield of plants when applied to seed or crops (Glick *et al*., 1995).

Groundnut is an invaluable source of protein, calories, essential fatty acids, vitamins, and minerals for human nutrition (Willett *et al*., 2019). Groundnut consumption is reported to be associated with several health benefits (Kris-Etherton *et al*., 2008; Sabate *et al*., 2010 and Guasch-Ferre *et al*., 2017).

Groundnut is a rich source of dietary protein with ability to meet up to 46% of recommended daily allowance; essential vitamins especially E, energy from its oils and fats, and dietary fiber. It is also a rich source of minerals such as K, Na, Ca, Mn, Fe, and Zn among others and a rich source of biologically active compounds (arginine, resveratrol, phytosterols, and flavonoids). Zinc in particular, is one of the limiting micronutrients especially among rural households in Africa affecting especially infants and young persons (Wessells and Brown, 2012).

Majeed *et al*., 2015 Plant growth-promoting rhizobacteria are free-living soil bacteria that aggressively colonize the rhizosphere plant roots, and enhance the growth, and yield of plants when applied to seed or crops Kumar *et al*., 2014. The plant growth promoting (PGP) effect of the PGPR is mostly explained by the release of metabolites directly stimulating growth. Several mechanisms have been postulated to explain how PGPR benefit the host plant. These

include: (a) the ability to produce plant growth regulators or phytohormones such as indole acetic acid (IAA), cytokinins, and gibberellins Glick 1995 and Marques *et al*., 2014.

The beneficial rhizobacteria associated with cereals has increased recently and several studies clearly demonstrated the positive and beneficial effects of PGPR on growth and yield of different crops especially wheat at different environment under variable ecological conditions Ozturk *et al*., 2003 and Mehnaz *et al*., 2010.

MATERIALS AND METHODS

Sample collection and Chemicals reagents

Nutrient Agar medium, Nutrient broth, was purchased from Himedia, India. Whatman filter paper No. 1, Gentamicin antibiotic solution, test samples, test tubes, beakers conical flaks, spirit lamp, double distilled water, petri-plates, Tris Hcl, Phenol chloroform, Methyl red, Kovac's reagent, KOH and Alpha napthol. Soil sample were collected from Sivakasi, Tamil Nadu. (Lattitude 9.4533° N, and Longitude 77.8024° E).

EnumerationSoil sample

Soil sample was used for the 0.1g of test sample was dissolved in 10ml of sterile double distilled water and 1 hours of incubation at room temperature. The filter the supernatant the serial dilutions from 10^1 to 10^8. The 10^8 dilution was used to bacterial isolation.

Nutrient Agar Medium

The medium was prepared by dissolving peptone- 0.25gm, NaCl- 0.25 gm, yeast extract- 0.1gm, beef extract- 0.05gm and agar powder- 0.875gm of the commercially available Nutrient Agar Medium (HiMedia) in 50ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten. The 10^{8th} dilution was plated on the nutrient agar medium by spread plate method and the plate was incubated at 37^o C for 24 hrs. After incubation, bacterial colonies were isolated and plated in to a fresh plate.

Nutrient broth

Nutrient broth was prepared by dissolving peptone- 0.25gm, NaCl- 0.25 gm, yeast extract- 0.1gm, beef extract- 0.05gm and agar powder- 0.875gm of the commercially available Nutrient Agar Medium (HiMedia) in 50ml of distilled water. The dissolved medium was

autoclaved at 15 lbs pressure at 121°C for 15 minutes. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

Screening and identification of microbes

The medium was prepared by dissolving peptone- 0.25gm, NaCl- 0.25 gm, yeast extract- 0.1gm, beef extract- 0.05gm and agar powder- 0.875gm of the commercially available Nutrient Agar Medium (HiMedia) in 50ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten. Five different colonies were isolated from 10^8 and dilution of test sample products. Five different colonies were streaked in a single petri plate.

Mass cultivation of microorganism

The nutrient broth was prepared by dissolving chemical composition of commercially available (HiMedia) in 50ml of distilled water. To identification of microbes single (S6-3) organisms. Therefore, a sterilize inoculation loop and loop full amount of microbes inoculation with nutrient broth. To allow with bacteriological incubator at 24 hours.

Gram staining

Procedure

A loop full of bacterial culture was spread in the glass slide. The slide was smeared in front of the flame. The slides were stained with crystal violet dye and kept it for 1 min and washed the slide in a distilled water. Gram's iodine was added and incubated for 1 minute, then rinsed with distilled water. The decolorizing agent was added and kept for 1 min and then safronin strain was added, after a minute it was washed using distilled water. The slides were observed under the Trinocular microscope the purple colors indicated gram positive bacteria and the pink color indicated gram negative organism.

Motility test - Hanging Drop Method

procedure

The motility test was performed by hanging drop method. The cover slip was taken where its edge was coated with Vaseline. The test samples were transferred to the cover slip which was placed over the cavity slide. The slide was viewed under 100X magnification and the organisms' characteristics being motile or non-motile were noted down.

IMVIC TEST (Physical chemical characterization)

Indole test

Inoculate the bacterium to be tested in tryphton broth. To allow incubated at 37 degree celcious for 24hrs. After Incubation add few drop of the kovacs reagent. Becomes the result formation of a red colour ring top of positive reaction (or) pink colour ring at the top positive reaction and yellow colour ring at the top negative reaction.

Methyl red test

Inoculate the bacterium to be tested in MRVP broth. To allow test tube inoculate at 37 degree celcious for 24hrs. After incubation add 5 drops of the methyl red reagent. Formation of red colour ring top of the positive reaction, formation of yellow colour ring at top negative reaction.

Voges proskauer test

Procedure

Inoculate the bacterium to be tested in MRVP broth. To allow Incubate at 37 degree celcious for 48hrs. After incubation add 0.6ml of alpha naphtholadd 0.2ml of 40% KOH to the broth. Allow tube to stand for 15 minutes. Formation of red colour ring positive reaction and formation of yellow colour ring negative reaction.

DNA isolation

DNA was isolated from the samples modified from the standardized salting-out procedure.

Stock solutions

0.5 M Tris HCl (pH-8.0)

Tris base - 3.028 g

Distilled water - 40 mL

pH adjusted to 8.0 using HCl and the volume was made to 50 mL, then autoclaved and stored at 4 °C.

0.5 M EDTA (pH-8.0)

EDTA - 9.31 g

Distilled water - 40 mL

The pH adjusted to 8.0, using NaOH was made up the volume to 50 mL, then autoclaved, and stored at 4 °C.

10 mM Tris HCl (pH-7.5)

Tris base	-	0.03 g
Distilled water	-	20 mL

The pH adjusted to 7.5 using HCl was made up the volume to 25 mL, then autoclaved, and stored at 4 °C.

TAE Buffer (50X – 1 Liter)

Trisbase	-	242 g
Glacial acetic acid	-	57.1 mL
EDTA	-	100 mL (0.5 M, pH- 8)

242 g of Trisbase, 57.1 mL of glacial acetic acid mixed. 100 mL of 0.5 M EDTA, (pH 8.0) added and made up with distilled water to 1liter.

Bromophenol blue

0.5% Bromophenol blue	-	500 mg

It was made up into final solution of 100 mL with double distilled water and then autoclaved and stored at 4 °C

Working Solutions

Solution 1

Tris-HCl	-	50 mM (pH 8.0)
EDTA	-	20 mM (pH 8.0)

Solution 2

Saturated NaCl solution	-	6 M
SDS	-	10 %

Proteinase K (20 mg/mL)

Proteinase K	-	10 mg
Autoclaved distilled water	-	500 µL

Proteinase K dissolved in distilled water and stored at -20 °C.

TE buffer

Stock 0.5 M Tris HCl (pH-8.0)	-	2.0 mL (10 mM)
Stock 0.5 M EDTA (pH-8.0)	-	0.2 mL (1 mM)

Made up the solution to 100 mL with distilled water, then autoclaved and stored at 4 °C.

Procedure

Small bacterial cultures samples were placed in 1.5 mL tubes separately and then added 500 µL of Solution1 and 10% of 10 µL SDS. Homogenize the sample with sterile homogenizer. And 5 µL of Proteinase K was added (20 mg/mL). The mixture was incubated at 55 °C for 2 hrs in water bath (with occasional mixing/quick vortex) for easy digestion. After complete digestion, the sample was kept on ice for 10 min. To this, 250 µL of Solution 2 was added (saturated NaCl) and inverted several times to mix. Subsequently, the samples were chilled on ice for 5 min. Then the samples were centrifuged at 8000 rpm for 15 min. After that, about 500 µL of clear supernatant were collected into a new-labeled 1.5 mL tubes. Then twice the volume of 100% molecular biology grade ethanol was added to precipitate the DNA. Then the samples were centrifuged at 11,000 rpm for 15 min. After that, the supernatant was removed and 500 µL of ice-cold 70% ethanol was added to the precipitate for washing. Sample was spun at 11000 rpm, for 5 min. Carefully, removed the supernatant, then pipetted out excess liquid and allowed to partially dry with lid-off at room temperature. Partially dried DNA was re-suspended in 100 µL of 1x TE buffer.

Quantification of DNA

Quantity of the extracted DNA was checked in UV spectrophotometer (SHIMADZHU, JAPAN) by taking the optical density (OD) at 260 nm and 280 nm. The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280). The value between 1.7 - 1.8 indicates good quality DNA without protein/RNA contamination. DNA quantification was done according to the following calculation: sample showing 1 OD at 260 nm is equivalent to 50 µg of DNA/mL. The OD of each DNA sample at 260 nm was measured and quantified accordingly.

DNA quality determination

The quality of DNA was checked on 0.8% agarose gels.

Casting of 0.8% agarose gel

The unit set according to manufacturer's instructions.

0.8% agarose gel

Agarose	-	0.24 g
1X TAE	-	30 mL

Procedure

0.24g of agarose powder was soaked in 30 mL of 1X TAE buffer and boiled until it formed into clear solution. Then it was allowed to cool, down to approximately 50 °C. Then added 1.5 µL of ethidium bromide and mixed well. It was poured in gel casting plate with already adjusted gel comb and kept at room temperature for 1/2 hrs for solidification. The gel was soaked in 1X TAE buffer in the electrophoresis tank. 3 µL of DNA with 3 µL of gel loading dye was loaded in the wells using micropipettes. It was run at 70 V for 15 to 20 min. The orange color (DNA) bands were observed in UV illuminator.

RESULTS AND DISCUSSION

The total heterotrophic bacterial population in soil samples from the fireworks area in Sivakasi showed significant findings. A similar trend was observed in the water samples. The total viable bacterial count was found to be higher in cultivable areas compared to wasteland, city, and fireworks areas. In fact, the highest total viable count was recorded in samples from the fireworks area, indicating a gradual increase in bacterial growth there.

This extreme variation can be attributed to the unfavorable conditions for microbial growth in both the fireworks and city areas. The increase in the number of bacterial colonies from the fireworks area to the cultivable lands can be explained by several factors. Sivakasi is known for its dry climate with limited rainfall, and it is also a highly industrialized region. The toxic and chemical effluents released in these areas may have significantly reduced the microbial population. The main factors that contribute to an increase in microbial populations include: a) the amount and type of available nutrients, b) the availability of moisture, and c) temperature and pH levels. In Sivakasi, these factors do not appear to be ideal. As previously mentioned, industrial effluents and toxic substances likely diminish the necessary nutrients for microbial growth. Conversely, samples taken from cultivable and wasteland areas exhibit a higher population of microbial species due to better availability of favorable conditions. Furthermore, 16S rDNA sequencing results indicated the presence of Pseudomonas species, confirmed by BLAST homology analysis.

Seasonal changes in climate parameters can have a significant impact on the growth of vegetation, which directly affects the microbial community. This trend may be due to the variations in the decomposition rate of litter and substances released through root exudates. It is also in accord with the trend observed in the alpha diversity indices shown in Figure 2. Soil moisture content plays a crucial role in plant growth and the microbial community by

influencing osmotic potential, nutrient and energy transport, and cellular metabolism. observed that increased availability of carbon sources and root growth during the growing season led to an increase in soil microbial biomass and activity. The soil microbial growth was limited during winter but increased during summer due to an increase in temperature.

The combustion of biomass and SOM during the fire resulted in the accumulation of ash on the soil surface. This ash accumulation plays a crucial role in altering the physical and chemical properties of the soil, ultimately influencing the dynamics of soil microbial communities. This increase was attributed to the deposition of ash during the fire event. However, over time, these values declined due to runoff, leaching, and the movement of nutrients into deeper soil layers through infiltration and percolation. Fire behavior directly impacts the extent of combustion and the maximum temperature reached during the fire event, which in turn influence the soil's physical, chemical, and biological properties. High-intensity fires result in the high volatilization of SOM and oxidation processes in the topsoil layer. The accumulation of ash affects soil microbial communities by increasing nutrient availability in the short term. Additionally, the darkening of the soil due to ash deposition reduces the albedo, resulting in an increase in soil surface temperature. observed higher values of diversity indices and pH immediately after fire, indicating more favorable soil conditions for stimulating bacterial populations. However, fire-induced changes in the functional diversity of microbial communities in RSC fields need to be further studied in the future.

Fig. 1: Sample collection.

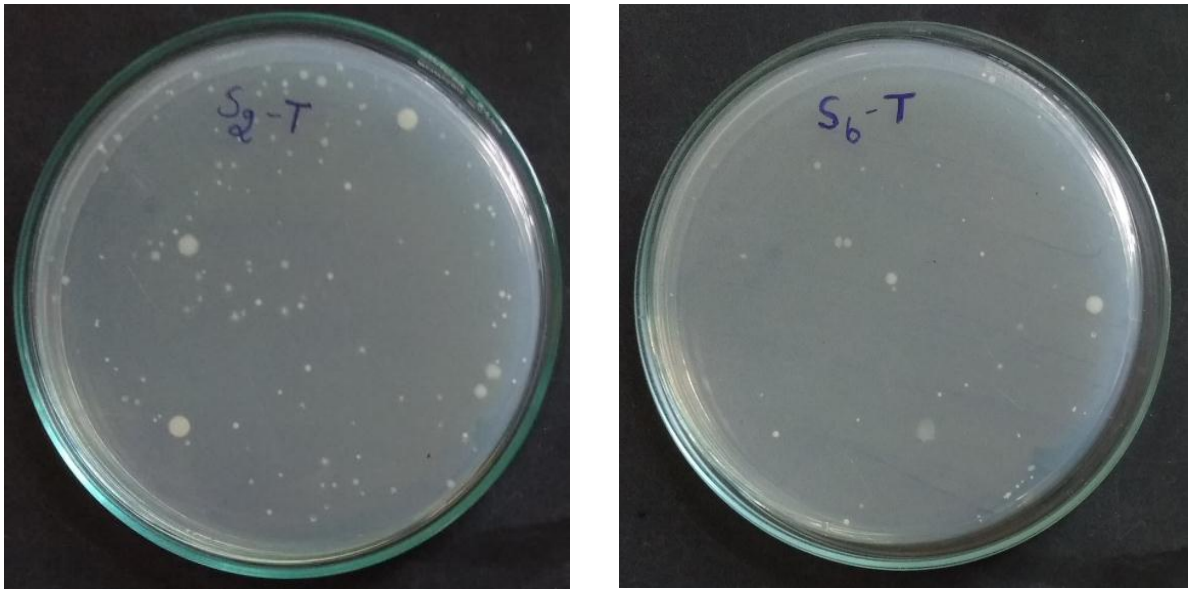


Fig. 2: Images for serial dilution with the spread plate technique using different concentration.

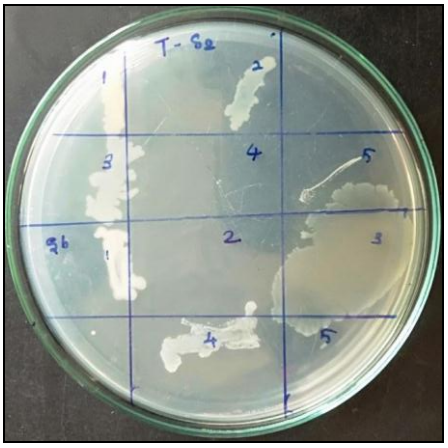
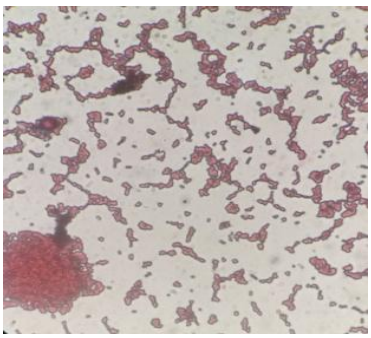
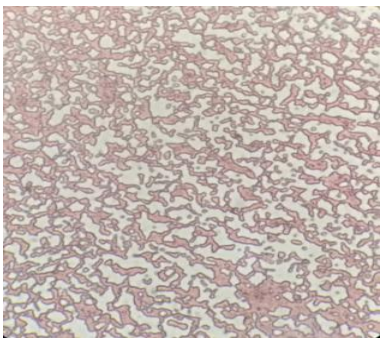


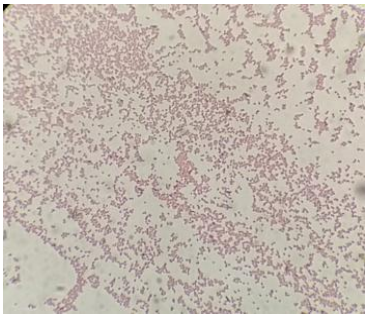
Fig. 3: Image for Screening and identification of microbes.



S2- 1- Gram negative- Rod chain- Motile

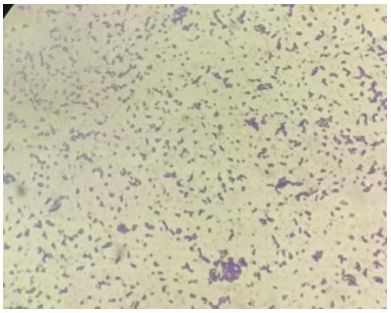


S2-2 Gram negative- Cocci chain- Motile

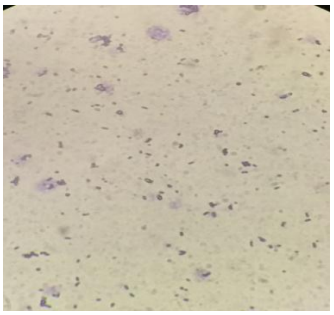


S2-3 Gram negative- Rod chain- Motile

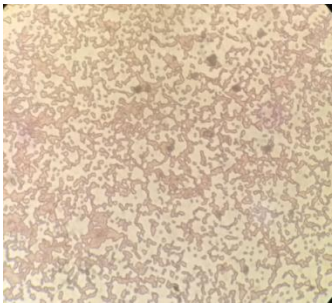
S2-5 Gram negative- cocci chain- Motile



S6-1 Gram positive small rod chain –motile



S6-3- Gram negative cocci chain –motile



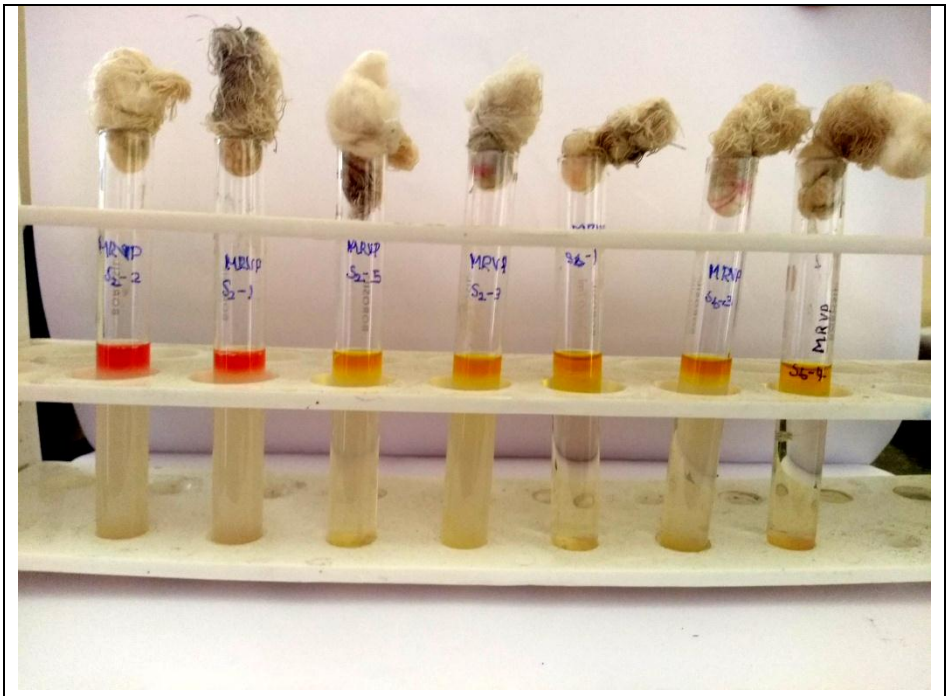
S6-4- Gram positive rod chain –motile

Fig. 4: Images for Gram staining.

Images for IMViC test

Indole test

Fig. 5: INDOLE TEST.



Methyl red and Voges proskauer
Fig. 6: Methyl Red And Voges Proskauer Test.

Table 1: Sequencing of the organisms.

S.No	Name of the organisms	Sequence
1	***Forward sequence***	AGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGG CGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTG GGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACG TCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCT ATCAGATGAGCCTAGGTCGGATTAGCTAGTAGGTGAGGT AATGGCTCACCTAGGCGACGATCCGTAACTGGTCTGAGA GGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG CGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCA GTAAGCTAATACCTTGCTGTTTTGACGTTACCGACAGAAT AAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC AGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG CGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCG GGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAG AGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGA AATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGT GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC GTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTT AGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAG TACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC AACGCGAAGAACCCTTACCAGGCCTTGACATGCAGAGAA CTTTCCAGAGATGG

2	**Reverse sequence**	CCCCCGAAGGTTAGACTAGCTACTTCTGGTGCAACCCACT CCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC GTATTCACCGCAACATTCTGATTTGCGATTACTAGCGATT CCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACT ACGATCGGTTTTGTGAGATTAGCTCCACCTCGCGGCTTGG CAACCCTCTGTACCGACCATTGTAGCACGTGTGTAGCCCA GGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTT CCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACC ATAACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTA CGGGACTTAACCCAACATCTCACGACACGAGCTGACGAC AGCCATGCAGCACCTGTGTCAGAGTTCCCGAAGGCACCA ATCCATCTCTGGAAAGTTCTCTGCATGTCAAGGCCTGGTA AGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCAC CGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCT TGCGGCCGTACTCCCCAGGCGGTCAACTTAATGCGTTAGC TGCGCCACTAAAATCTCAAGGATTCCAACGGCTAGTTGAC ATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTT TGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCC AGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTAC GCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACC ATACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGTTGA GCCCAGGGGCTTTTCACATCCAACTTAAACGAACCACCCT ACGCGCGCTTTTACGGCCCCAGTAAATTTCCGAATAAACG CCTTGCACCCTCTTGTATTACC

BLAST analysis result

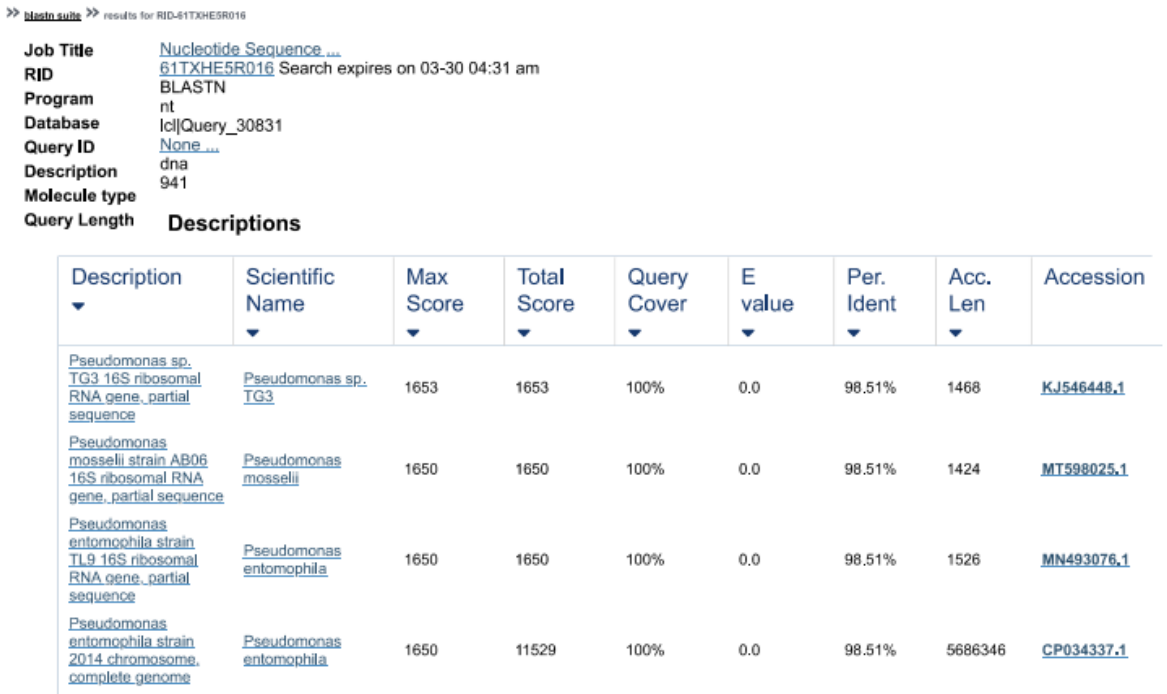
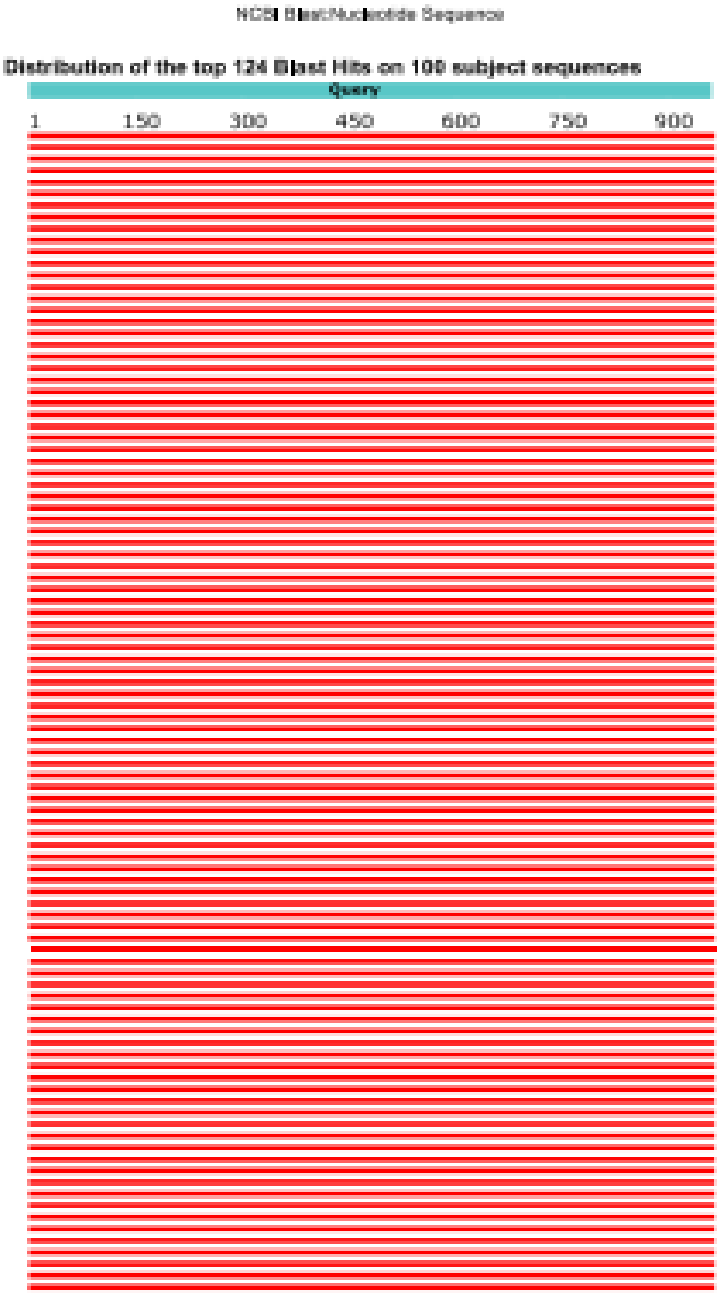


Figure 4.7: Blast analysis result.



CONCLUSION

The fireworks industry is a significant source of environmental pollution, releasing toxic chemicals into the air, water, and soil. These pollutants can have detrimental effects on soil microbial diversity, which plays a crucial role in ecosystem health and functioning.

Based on the available information, it is clear that biodiversity is declining at all levels and across various geographical areas. However, targeted response options, such as protecting soil, managing resources, and implementing pollution prevention programs, can reverse this

trend for specific habitats. In the current investigation, we found significant insights into the total heterotrophic bacterial density, generic composition, and physiological grouping in soils affected by fireworks. Given the severe impact of pollutants, it is crucial to monitor their concentrations in terrestrial environments. Therefore, this study strongly recommends the continuous monitoring of pollutants in terrestrial systems and the implementation of appropriate control measures to ensure the safety of terrestrial biodiversity and the overall ecosystem. To better understand the ecological roles and contributions of individual bacterial taxa to biogeochemical processes, it is essential to connect community composition to specific functions. While DNA-based analyses help reveal community dynamics and changes in microbial activity, RNA-based studies provide critical information about the active bacterial community and the processes occurring within ecosystems. RNA-based research in forest ecosystems has confirmed that many microbial taxa are transcriptionally active in forest litter and soil, highlighting the involvement of numerous species in important soil processes, as well as the significance of taxa that are present in low abundance.

ACKNOWLEDGEMENT

The authors would like to extend their sincere gratitude to their family for their unwavering support and Dr. V. Prabhu for his timely guidance and assistance, which were instrumental in providing the necessary resources and support to conduct this research.

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