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Isolation and culturing of microorganisms from soil samples, accompanied by morphological characterization.

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Abstract

Soil microbial communities exhibit immense diversity, leading to complex interactions among microbial species and functional groups that can influence ecosystem processes. Microorganisms are ubiquitous and thrive in highly competitive habitats. Many bacteria synthesize antibiotics as important secondary metabolites, which have significant commercial applications. The isolated bacterial strains were subjected to Gram and endospore staining, revealing that none of the isolates were endospore-forming. To evaluate their enzymatic capabilities, extracellular enzyme production was tested using starch and gelatin hydrolysis assays. Furthermore, various biochemical tests were performed to assess intracellular enzyme activities, including catalase, urease, Voges-Proskauer, hydrogen sulfide production, nitrate reduction, methyl red, phosphate solubilization, nitrogen fixation, citrate utilization, oxidase, motility, and triple sugar iron (TSI) tests. Most of the bacterial isolates showed positive results in these assays. A total of eight soil bacterial genera *Serratia, Bacillus, Pseudomonas, Micrococcus, Streptococcus, Staphylococcus, Proteus,* and *Alcaligenes* were successfully isolated and identified.

Keywords: Gram staining, Endospore staining, Triple Sugar Iron (TSI) tests, Soil bacteria, *Streptococcus, Staphylococcus, Proteus* and *Alcaligens*

INTRODUCTION

Soil microbial communities are so vastly diverse that complex interactions, which alter ecosystem functions, may occur among microbial species and functional groups. Bacteria and fungi play a significant role in global C and N cycling and are responsible for about 90% of all organic matter decomposition (Swift et al., 1979). Soil microbial communities are many times more diverse than their associated plant and animal communities (Whitman et al., 1998; Hawksworth, 2001), and less than 5% of all microbes have been identified and described (Schleifer, 2004). While it is broadly recognized that microbes are critical for driving soil nutrient cycling, the role of microbial community structure in determining ecosystem process rates is not well characterized. Soil microbial communities play several important ecological and physiological functions (e.g., soil organic matter decomposition and control of its cycle, regulation of mineral nutrient availability, atmospheric nitrogen fixation, formation of mycorrhiza, production of biologically active substances able to stimulate plant growth) ameliorating soil physical and chemical conditions and, consequently, soil habitability for plants. There is a growing interest in maintenance of agrosystem functionality (Adriano Sofo et al., 2014).

Most antibiotics were produced by screening soil microorganisms, but this limited resource of cultivable bacteria was overmined by the 1960s. We developed several methods to grow uncultured organisms by cultivation in situ or by using specific growth factors. Synthetic approaches to produce antibiotics have been unable to replace this platform. Uncultured bacteria make up approximately 99% of all species in external environments, and are an untapped source of new antibiotics. Teixobactin inhibits cell wall synthesis by binding to a highly conserved motif of lipid II (precursor of peptidoglycan) and lipid III (precursor of cell wall teichoic acid). The bacterial species such as *Staphylococcus aureus* or *Mycobacterium tuberculosis* resistant to teixobactin and due to this property teixobactin suggest a path towards developing antibiotics that are likely to avoid development of resistance (Ling LL et al., 2015).

Bacterial species are generally simple to isolate, cultivate, sustain, and enhance through strain improvement techniques. Soil samples were collected at two different ranges from logs during one complete growth cycle of *G. lucidum*. The changes in fungi and bacteria were investigated by using high-throughput sequencing and real-time PCR (Ahmad Zaheer et al., 2024). In this study, the isolated bacterial strains included *Bacillus lentus*, *Micrococcus roseus*, *Bacillus alvei*, *Enterobacter aerogenes*, and *Bacillus pumilus*. The antimicrobial properties of these isolates were evaluated against major opportunistic pathogens, including *Staphylococcus aureus* and various *Pseudomonas* species. While, Rejitha Nair (2012) conducted a study in which marine bacteria were isolated and screened for their potential to produce antibiotics active against common clinical pathogens, including *Pseudomonas* spp., *Escherichia coli*, *Bacillus subtilis*, and *Proteus* spp. Out of 36 isolates tested, four showed significant antibacterial activity against *Pseudomonas*, *Bacillus* , *Proteus*, *Klebsiella*, and *E. coli*. Notably, the isolate designated CW 602 produced antibiotics when co-cultivated with *Pseudomonas*, while CW 401 exhibited antibiotic production in the presence of *Bacillus* cells. Cell free extracts from both CW 602 and CW 401, grown alongside heat-killed *Pseudomonas* and *Bacillus* cells respectively, were extracted using ethyl acetate. The antimicrobial properties of these extracts were then evaluated using the disc diffusion assay.

While many members of the rhizosphere microbiome are beneficial to plant growth, also plant pathogenic microorganisms colonize the rhizosphere striving to break through the protective microbial shield and to overcome the innate plant defense mechanisms in order to cause disease (Rodrigo Mendes et al., 2023). Humans and microorganisms have coexisted since ancient times. While some bacteria establish a symbiotic relationship with humans serving as protective and stabilizing resident microbes pathogenic bacteria invade human tissues, causing diseases that can sometimes result in death. The fight against bacterial infections prompted the discovery and extensive use of antibiotics in the 1940s. While these drugs are effective in eliminating harmful bacteria, they can also disrupt beneficial microbial communities within the human body. As a result, their use requires careful consideration to balance therapeutic advantages with possible side effects. A detailed understanding of how antibiotics work, along with thorough characterization, is crucial to ensure their safe and effective application in medical treatment (Etebu et al., 2016).

Microorganisms are ubiquitous and thrive in highly competitive environments. Bacteria produce antibiotics as vital secondary metabolites, which have wide ranging commercial uses. The majority of antibiotics in use today originate from microbial sources. *Bacillus* species, frequently found in soil because of their capacity to form resilient endospores and synthesize potent antibiotics such as bacitracin, are known to suppress the growth of rival microorganisms (Abdulkadir et al., 2012).

Antibiotics are naturally produced chemical compounds that act as defense agents, capable of eliminating or inhibiting pathogenic microorganisms even at low concentrations (Donovick and Brown, 1965). The majority of antibiotics originate from bacteria, fungi, and molds, with some synthesized fully or partially through chemical processes. The biosynthesis method remains in use today for manufacturing various antibiotics, where the producing organisms are cultured under conditions that favor their growth, followed by extraction of the antibiotic compound. To enrich antibiotic-producing bacteria from soil samples, highly effective selective substrates were identified to formulate specialized growth media. This was accomplished by analyzing a collection of 74 soil bacteria comprising both eubacteria and actinomycetes based on their ability to produce antibacterial compounds and their responses to 43 different physiological and nutritional tests. Utilizing discriminant analysis, several isolation media containing these selective substrates successfully increased the yield of actinomycetes recovered from soil samples (Huck et al., 1991).

The new isolates were affiliated with seven phyla such as *Acidobacteria, Proteobacteria*, , *Firmicutes*, *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, and *Bacteroidetes* and analysis showed that NSE included more diverse components of low-molecular-weight organic substances than two conventional soil extracts made using distilled water (Taun Manh Nguyen et al., 2018). Cultivation of previously uncultured bacteria is expected to extend knowledge through the discovery of new phenotypic, physiological, and functional properties and even roles of unknown genes. Although these isolates were distributed over only four major phyla, the use of the newly developed technique resulted in the successful cultivation of 35 previously uncultured strains, whereas no such strains were successfully cultivated by the traditional method.

Furthermore, the study also found that the recovery of uncultured bacteria and novel isolates was related to sampling season, incubation period, and cultivation media. The use of soil collected in summer, a prolonged incubation period, and low-substrate modified media increased the recovery of uncultured and novel isolates (Dhiraj Kumar Chaudhary et al., 2019). Both metagenomics and single-cell sequencing can detect unknown genes from uncultured microbial strains in environments, and either method may find the significant potential metabolites and roles of these strains. However, such gene/genome-based techniques do not allow detailed investigations that are possible with cultures (Taun Manh Nguyen et al., 2018). The molecular analysis revealed that a diverse bacterial population was present in the soil sample. However, both the newly developed method and the traditional method recovered more than 400 isolates, which belonged to only four phyla: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes (Dhiraj Kumar Chaudhary et al., 2019).

MATERIAL AND METHODS

1. Soil Sample

To isolate bacteria, surface soil samples were collected in March 2023 from Vibhuti Khand, Gomti Nagar (Lucknow), an area renowned for its rich microbial diversity. About 5 grams of soil were carefully gathered using a sterile spatula and placed into a clean, dry, sterile polythene bag. For the preparation of a soil suspension, 1 gram of the collected soil was combined with 10 ml of distilled water (Figure 1).

2. Isolation of Microorganism through Serial Dilution

Serial dilution is a method employed to reduce the bacterial concentration to an appropriate level, either to suit the needs of a specific experimental protocol or to achieve a countable number of colonies for accurate enumeration when cultured on an agar medium (Figure 2). The steps involved in the serial dilution process are as follows:

I. In this procedure, 1 gram of soil was mixed with 10 ml of sterilized normal saline (NS) and thoroughly blended using a vortex to prepare a stock solution.
II. From the stock solution, 1.0 ml was transferred to a second test tube containing 9 ml of normal saline, which was then vortexed again. A series of serial dilutions were made from this mixture.
III. From the final three dilutions, 20 µl of each sample was taken and evenly spread on the surface of sterile petri dishes with solidified agar medium using the spread plate method.
IV. The petri dishes were incubated at 37°C for 24 hours.
V. After incubation, bacterial colonies were inspected, and those showing distinct growth patterns and morphologies were selected for further purification by streaking onto separate solidified agar plates.
VI. These new agar plates were then placed back into the incubator at 37°C for continued growth.

3. Morphological Analysis of soil bacteria

Bacteria are abundant and mostly free-living microorganisms, typically consisting of a single cell. As some of the earliest life forms on Earth, they inhabit almost every environment and are usually only a few micrometers in size. These organisms are capable of thriving in diverse habitats, such as soil, water, acidic hot springs, radioactive settings, and even deep below the Earth's surface. The study aims to investigate the morphological traits of bacteria, focusing on aspects like shape, size, colony margin, elevation, texture, and pigmentation (Figure 3; Table 2).

4. Streaking

A small amount of bacterial culture is spread across an agar plate in such a way that the sample is gradually diluted, allowing for the formation of separate, individual colonies. This classic method, which dates back to the time of Robert Koch (1878), was first demonstrated by Loeffler and Gaffky in Koch's laboratory. Streaking is done by five methods-

a. ***Quadrant Streaking-*** The agar plate is divided into four equal sections, each streaked sequentially. The first section, or 1st quadrant, holds the highest concentration of the bacterial sample. As the streaking progresses through each section, the sample becomes increasingly diluted. By the time the fourth quadrant is reached, the dilution is adequate to allow distinct, well-separated individual colonies to form.

b. ***Discontinuous streaking-*** In this method, the inoculating loop is sterilized after streaking each quadrant before proceeding to the next. This helps in reducing the bacterial load step by step, allowing better isolation of colonies.

c. ***Continuous streaking-*** when the bacterial sample is minimal or already diluted, used this method, involves streaking without sterilizing the loop between quadrants. As a result, by the final section, the bacterial cells are spaced far enough apart to form distinct colonies.

d. ***Zig-zag Streaking-*** This technique involves streaking in a back-and-forth pattern across the agar surface first from left to right, then right to left in a zig-zag manner. Bacterial growth is densest where the initial streak begins and becomes progressively lighter as the streak continues across the plate.

e. ***Radiant Streaking:*** In this approach, the inoculum is first applied along one edge of the Petri dish. From there, vertical streaks are made extending outward, followed by horizontal streaks intersecting them. This method is effective for obtaining isolated colonies and is especially suitable for pure cultures or diluted specimens.

5. Screening

In this study, screening is performed using the AST (Antimicrobial Susceptibility Testing) test by using Graham DR et al (1985). This laboratory technique is employed to determine whether microorganisms, especially bacteria, are sensitive or resistant to particular antimicrobial agents. The general procedure for AST is outlined below-

I. A 100 ml production medium was prepared.
II. 20 ml of the prepared medium was transferred into each of five separate flasks.
III. The media were sterilized by autoclaving at 15 psi and 121°C for 15 minutes, then allowed to cool to room temperature.
IV. Each flask was inoculated with a different microorganism, for a total of five.
V. The inoculated flasks were incubated for 72 hours.
VI. After incubation, the cultures were centrifuged at 10,000 rpm, and the supernatant was carefully collected for further use.
VII. **Preparation of AST Plate:** Each AST plate was prepared by adding 30 ml of Nutrient Agar Medium (NAM). The medium was sterilized by autoclaving at 15 psi and 121°C for 15 minutes. Once sterilized, the NAM was poured into sterile Petri dishes and left to solidify. The bottom of each plate was labeled with essential details such as the date, sample source, and the researcher's name or initials. Subsequently, 20 µL of an *E. coli* suspension was evenly spread across the agar surface using a sterile glass spreader to ensure uniform distributions.
VIII. **Placement of the Wells:** Using a sterile pipette tip or a well punch, create five wells in the Petri plate.

a. Adding the Samples: Carefully loaded 45ul of the sample from first flask into one of the wells using a pipette on the Petri plate. Repeat the same process for the second, third, fourth, and fifth sample, ensuring proper labelling for identification.

b. Incubation: Without inverting the plate to prevent the liquids placed in the wells from falling out and ruining the technique, incubate it at 37°C temperature. The incubation period can vary, but it is typically between 24 to 48 hours.

6. Biochemical characteristic of antibiotic producing microbes

a. Gram staining- This section outlines the Gram staining method, which is used to distinguish between Gram-positive and Gram-negative bacteria, as well as to examine their cell shape and arrangement by using Gram HC (1884) method. The procedure began with preparing a bacterial smear on a clean glass slide, which was then heat-fixed gently using a spirit lamp. The smear was stained with 2–3 drops of crystal violet and left for one minute. Afterward, excess dye was removed by rinsing the slide gently with tap water, and the slide was carefully dried with a paper towel. Following this, Gram's iodine was applied to the smear for one minute before being briefly rinsed off. Finally, decolorization was carried out using 95% ethyl alcohol for 30 seconds. Afterwards, a few drops of safranin were applied and allowed to sit for one minute. The slide was then rinsed once more with water to halt the decolorization process and left to air dry. Finally, the stained smear was observed under a microscope for analysis.

b. Endospore staining- Endospores have a tough outer layer composed of the protein keratin, which makes them highly resistant to staining according to the A.B. Schaeffer, M. Fulton (1993). To effectively stain these structures, the primary dye malachite green must be applied with heat to allow it to penetrate the protective spore coat. This staining method is used to differentiate endospore-forming bacteria from those that do not produce endospores and to determine the location of the spore within the cell. During the procedure, vegetative cells are decolorized with water, and a 0.5% safranin solution is used as a counterstain. As a result, endospores appear green, while vegetative cells stain red.

To begin the procedure, prepare a smear of the bacterial sample on a clean glass slide and heat-fix it. Apply 2–3 drops of malachite green and allow it to sit for one minute. Then, place the slide in a water bath maintained at 100°C for one hour to ensure thorough dye penetration. After removing the slide and allowing it to cool to room temperature, rinse it with distilled water. Next, apply 2–3 drops of safranin and let it sit for one minute. Rinse the slide again with distilled water and allow it to air dry. Finally, examine the smear under a microscope to detect the presence of endospores.

c. Starch hydrolysis test- The starch hydrolysis test assesses whether a microorganism can break down starch, which indicates the production of the enzyme amylase. This is evaluated in the laboratory by detecting the presence or absence of starch in the medium using iodine solution as an indicator according to the method of Leber Amy L. (2016). To conduct the test, starch agar medium is poured into sterile Petri plates, and the microorganism is streaked along the center of each plate. The plates are then incubated at 37°C for 48 hours. Following incubation, the surface of the agar is flooded with iodine solution using a dropper, left for one minute, and the excess iodine is then discarded.

d. Mannitol test- Microbial fermentation of a carbohydrate substrate produces acidic byproducts, which can be detected using a pH indicator dye. This experiment aims to determine whether a microorganism can ferment mannitol as its carbon source. To carry out the test, a fermentation medium containing mannitol is prepared and dispensed into fermentation tubes. The tubes are sterilized by autoclaving at 15 psi and 121°C for 15 minutes. Each tube is then labeled with the name of the microorganism to be tested. The mannitol broth is inoculated with the respective bacterial cultures, while one tube is left uninoculated to serve as a control. The inoculated tubes are incubated in an orbital shaker for 24 hours, and the control tube is stored at 4°C.

e. Methyl-red test- This test is performed by using AL Barry et al (1970) method and assesses an organism's ability to produce and maintain a stable acidic end product from glucose fermentation, one that is potent enough to surpass the medium's buffering capacity. It is helpful in differentiating between two primary groups of facultative anaerobic enteric bacteria based on their patterns of acid production. To conduct the test, Methyl Red (MR) broth is prepared and dispensed into test tubes, which are then sterilized by autoclaving at 15 psi and 121°C for 15 minutes. Each tube is labeled with the name of the microorganism to be tested and inoculated accordingly, with one tube left uninoculated as a control. The inoculated tubes are incubated in an orbital shaker for 24 hours, while the control tube is kept at 4°C. Following incubation, 5 drops of methyl red indicator are added to each tube to evaluate acid production.

f. Voges-proskauer test- Pyruvic acid, a key intermediate in glucose fermentation, is metabolized through various pathways depending on the bacterial enzymes present. One such pathway produces acetoin (also called acetyl methyl carbinol), a neutral end product. This test determines if a microorganism uses the 2,3-butanediol fermentation pathway during glucose metabolism. When acetoin is present, it is oxidized to diacetyl in the presence of α-naphthol, a strong alkali, and atmospheric oxygen.

To carry out the test, Voges-Proskauer (VP) broth is prepared by using the AL Barry (1967) method and distributed into test tubes (5.0 ml per tube), then sterilized using an autoclave and allowed to cool to room temperature. The broth is then inoculated with the test organisms, while one tube is kept uninoculated as a control. The inoculated tubes are incubated in an orbital shaker for 24 hours, and the control tube is stored at 4°C. After incubation, 12 drops of VP I reagent are added to each test tube and mixed well to ensure oxygen is introduced. After 15 minutes, 4 drops of VP II reagent are added, and the mixture is stirred again to promote aeration. A pink to red coloration at the surface within 30 minutes signifies a positive result.

g. Urease test- Some bacteria have the ability to hydrolyze urea, releasing ammonia, which serves as a nitrogen source. The test broth contains phenol red as a pH indicator; when ammonia is produced and the pH rises to 8.4, the medium changes color from orange to pink. This test is useful for distinguishing between bacteria that produce the enzyme urease and those that do not. Urea broth was prepared with the pH adjusted to 6.8, then distributed into test tubes and sterilized by autoclaving at 121°C for 15 minutes. After cooling to 50°C, the tubes were labeled with the names of the test organisms and incubated at 37°C for 24 to 48 hours. The tubes were then examined for color changes: a pink or cerise color indicates urease activity, while a yellow color shows a lack of urease production.

RESULT

This study aimed to isolate and purify bacterial species from soil samples and assess their capacity to produce and extract antimicrobial compounds. Soil samples were collected and processed using standard cultivation and isolation techniques to obtain bacterial strains. A total of eight distinct bacterial isolates were successfully obtained. These isolates were screened for antimicrobial production through antimicrobial susceptibility testing.

The isolates were cultured on Nutrient Agar Medium (NAM) plates and tested against various indicator microorganisms to evaluate their antimicrobial activity. Those showing significant antimicrobial effects were selected for further study and grown in production media supplemented with appropriate nutrients. The cultures were grown under optimal conditions and then centrifuged to separate the bacterial cells from the surrounding liquid. The liquid portion, containing antimicrobial compounds, was collected and subjected to purification using different techniques. The isolated antimicrobial agents were subsequently analyzed through a series of tests, such as Gram staining, spore staining, and the MR-VP test, to assess their chemical characteristics and their effectiveness against various pathogens. The findings confirmed the presence of multiple antimicrobial compounds exhibiting strong activity against both Gram-positive and Gram-negative bacteria.

1. Soil Sample Collection and Bacterial Strain Isolation: Soil samples were obtained from the area surrounding MRD Life Sciences, Vibhuti Khand, Lucknow. Around 5 grams of soil were collected using a sterile spatula and transferred into a clean, dry, sterile polythene bag. To create a soil suspension, 1 gram of the collected soil was mixed with 10 ml of sterile water (Figures 1 & 2). Serial dilutions were then carried out to isolate and identify the various bacterial species present in the sample. The bacterial count in the original soil sample was estimated by multiplying the number of colonies by their respective dilution factors. The dilution factor for each tube is calculated using a standard formula-

Dilution Factor = $V_{sample} / V_{sample} + V_{dilution\ blank}$

For a ten-fold dilution, where 1 ml of the sample is added to 9 ml of diluent, the dilution factor of that test tube will be—

Dilution factor = 1 ml/ 1 ml + 9 ml = 1/10 = 10^{-1}

The overall dilution is determined by multiplying the dilution factor of each individual tube together-

Total dilution factor = previous dilution × dilution of next tube.

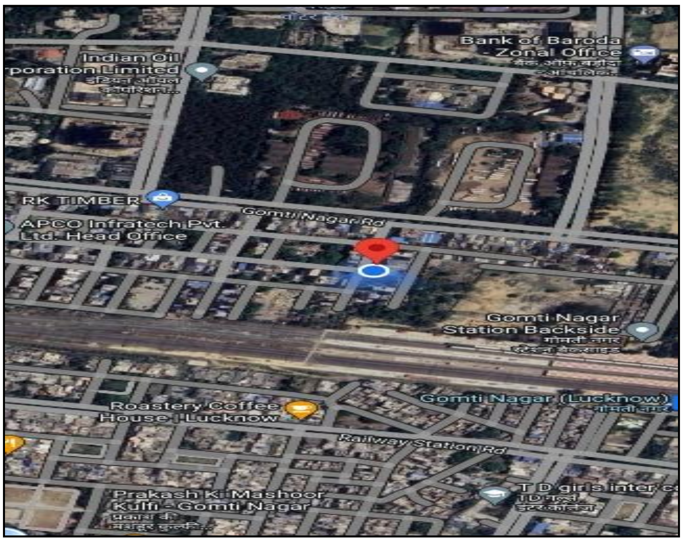

Figure 1- Map of Vibhuti Khand Lucknow area from where soil was collected.

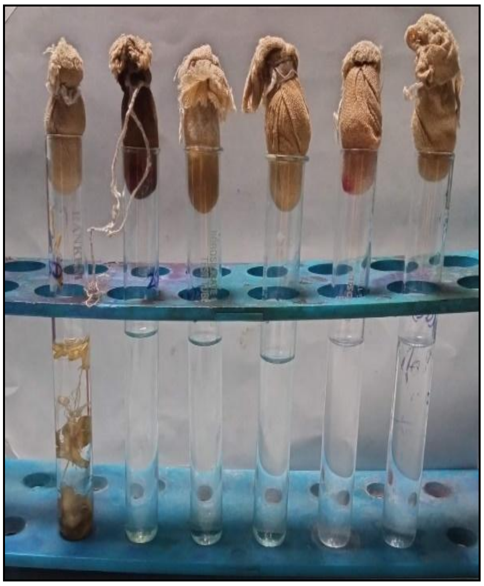

Figure 2- There were 6 test tubes serially diluted with water sample.

2. Colony morphology

The morphological characterization of soil-derived bacterial species was carried out using traditional macroscopic observations, focusing on attributes such as colony color, shape, structure, and elevation. The observed bacterial colonies were white with a semi-rough surface, exhibiting shapes that varied from circular to irregular. Several morphological features were recorded during the evaluation of colony appearance.

To determine extracellular enzyme production, the bacterial isolates were tested for their ability to hydrolyze starch and gelatin. Intracellular enzyme activity was assessed through a series of biochemical tests, including catalase, urease, Voges-Proskauer, hydrogen sulfide production, nitrate reduction, methyl red, phosphate solubilization, nitrogen fixation, citrate utilization, oxidase, motility, and the triple sugar iron (TSI) test.

Figure 3- Morphological analysis of bacteria colony grown.

a. ***Shape and Size:*** Bacteria exhibit a diverse range of shapes and sizes. Many species such as *Staphylococcus*, *Bacillus*, *Micrococcus* and *Streptococcus* are generally spherical, whereas others like *Proteus* and *Alcaligenes* display irregular forms. Certain isolates, particularly those belonging to the *Bacillus* genus, are rod-shaped. These differences in shape are largely determined by the composition of the bacterial cell wall and the cytoskeleton. Bacterial morphology plays a crucial role in functions such as nutrient uptake, surface attachment, movement through liquid environments, and evasion of predators. All the bacterial isolates observed were microscopic, typically ranging from 0.5 to 5.0 µm in length, and required a microscope for visualization (Table 1; Figure 3).

b. ***Margin:*** The margin refers to the edge or boundary of a bacterial colony. In soil microbes, the bacterial margins can vary and may include types such as *Micrococcus, Proteus, Alcaligens, Straptococcus, Pseudomonas* and *Seritia* species were possess entire margin.

Table 1- Colony morphology of recovered isolates of soil sample and this table presents the colony morphology of bacterial isolates recovered from the soil sample, detailing characteristics such as shape, size, color, surface texture, opacity, elevation, margin type, and the identified microorganism.

Shape	Size	Colour	Surface	Opacity	Elevation	Margin	Microorganism
Circular	Small	Dirty white	Smooth	Opaque	Raised	Entire	Staphylococcus sp.
Circular	Small	White	Dry	Opaque	Raised	Entire	Bacillus sp.
Circular	Small	Yellow	Smooth	Opaque	Flat	Entire	Micrococcus sp.
Circular	Small	White	Dry	Opaque	Raised	Entire	Bacillus sp.
Irregular	Small	Greyish	Smooth	Opaque	Raised	Entire	Proteus sp.
Irregular	Small	White	Smooth	Opaque	Raised	Entire	Alcaligenes sp.
Circular	Small	Yellow	Smooth	Opaque	Raised	Entire	Staphylococcus sp.
Circular	Small	Dirty white	Smooth	Opaque	Flat	Entire	Streptococcus sp.
Circular	Small	Yellow	Smooth	Opaque	Raised	Entire	Micrococcus sp.
Circular	Small	Light green	Wrinkled	Opaque	Raised	Entire	Pseudomonas sp.
Circular	Small	White	Dry	Opaque	Raised	Entire	Bacillus sp.
Circular	Small	Red	Smooth	Opaque	Raised	Entire	Serratia sp.

c. *Colony elevation:* It was the height of the vertical growth of the colony. In the morphological analysis of bacterial colony, the bacterial species such as *Micrococcus* and *Staphylococcus* shows flat colony elevation while the other soil bacteria such as *Proteus, Alcaligens, Straptococcus, Pseudomonas* and *Seritia* species were shows raised colony elevation (Figure 3; Table 1).
d. *Colony surface:* It was the consistency of the colony and in the investigations, there were smooth colony surface for *Staphylococcus, Micrococcus*, *Micrococcus, Proteus and* Serita while, wrinkled surface for *Pseudomonas* species and dry colony surface for *Bacillus* species were observed (Figure 3; Table 1).
e. *Colony pigmentation or colour:* The colour of the colony was referred to as pigmentation. The morphological analysis of soil bacteria was dirty white in colour such as *Staphylococcus* species, white colour such as *Bacillus*, *Alcaligens* species. The *Micrococcus* and *Staphylococcus* species colony was yellow; on the other hand, *Pseudomonas* species appear light green. Red colour colony of *Seritia* species while the *Proteus* specie of microorganism appears greyish in colour colony (Figure 3; Table 1).
f. *Colony transmission:* All isolates were opaque completely lacking in the light transmission from colonies.

3. Steaking

A loop of bacterial culture was streaked onto an agar plate using a technique designed to gradually dilute the sample with each streak, ultimately leading to the development of isolated colonies. This classic method, which originated during Robert Koch's time, was first demonstrated in his laboratory by Loeffler and Gaffky. To achieve bacterial purification, the streak plate method was employed, allowing selected bacterial cultures to be spread on Petri dishes to isolate pure colonies (Figure 4 and Figure 5 A and 5B).

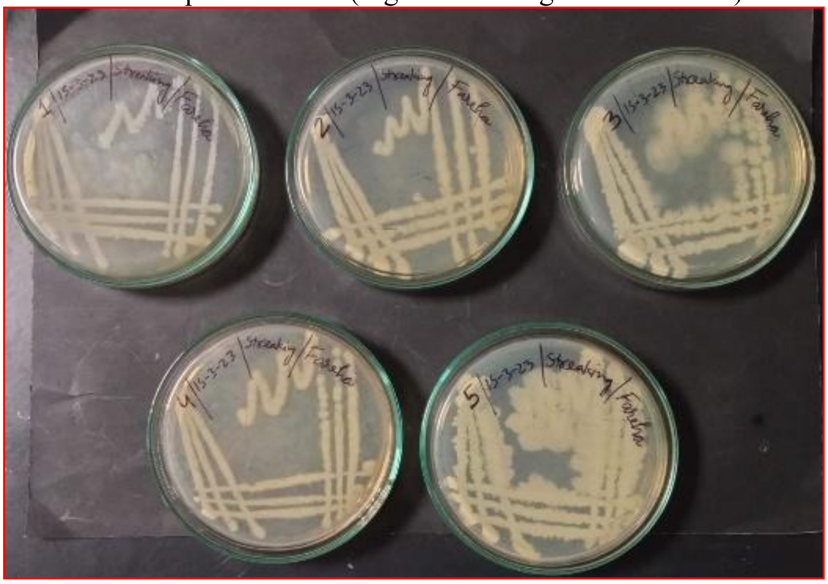

Figure 4- There were five pure bacteria culture isolated by quadrant streaking.

Figure 5- A and B: Production media for screening and AST screening.

Gram staining was carried out to differentiate between gram-positive and gram-negative bacterial species. The outcome showed that the bacteria were gram-positive and had a cocci (spherical) morphology. Furthermore, differential staining methods were applied to check for the presence of bacterial endospores. However, the endospore staining results were negative, suggesting that the bacteria did not produce endospores. (Figure 6 A and 6 B).

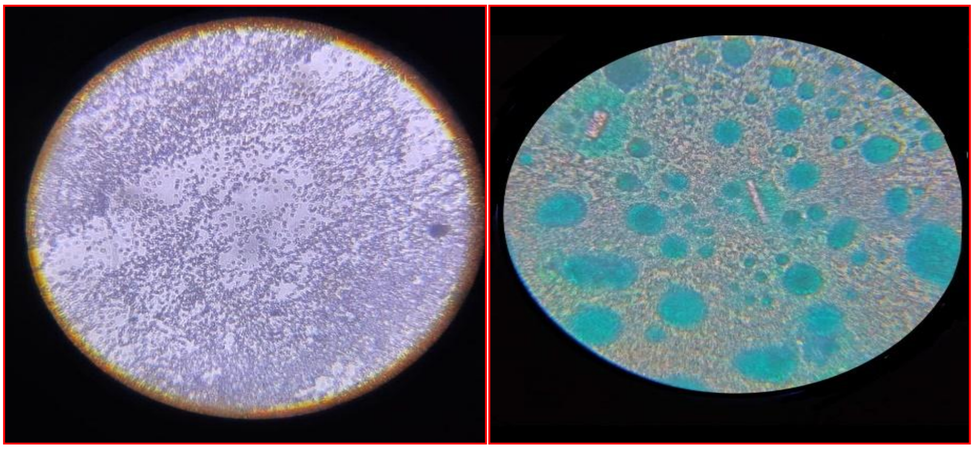

Figure 6: A) Gram-stained and B) Endospore staining of Bacterial culture.

The broth changed to yellow, signifying a positive reaction. In the Methyl Red (MR) test which helps distinguish between two primary groups of facultative anaerobic enteric bacteria based on their acid production a vivid red color developed, further indicating a positive result. (Figure 7-A, B).

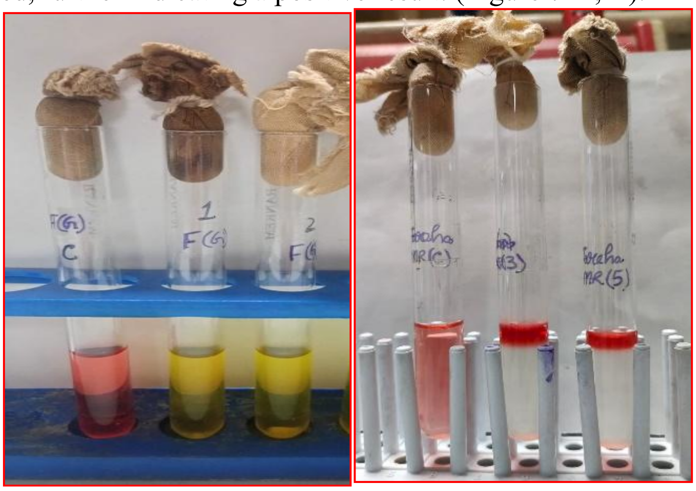

Figure 7-A) In manittol test the yellow colour show positive result and Figure 7-B) Methyl Red (MR) test the bright red colour obtained positive result.

To assess whether a microorganism utilizes the 2,3-butanediol fermentation pathway for glucose metabolism, the appearance of a pink-red color on the surface of the test medium indicates a positive result. In the urease test, which differentiates bacteria based on their ability to produce the enzyme urease, one culture caused the medium to turn pink signifying a positive result due to the breakdown of urea by urease. In comparison, another culture produced a yellow coloration, indicating a negative result and the absence of urease activity. (Figure 8-A, B).

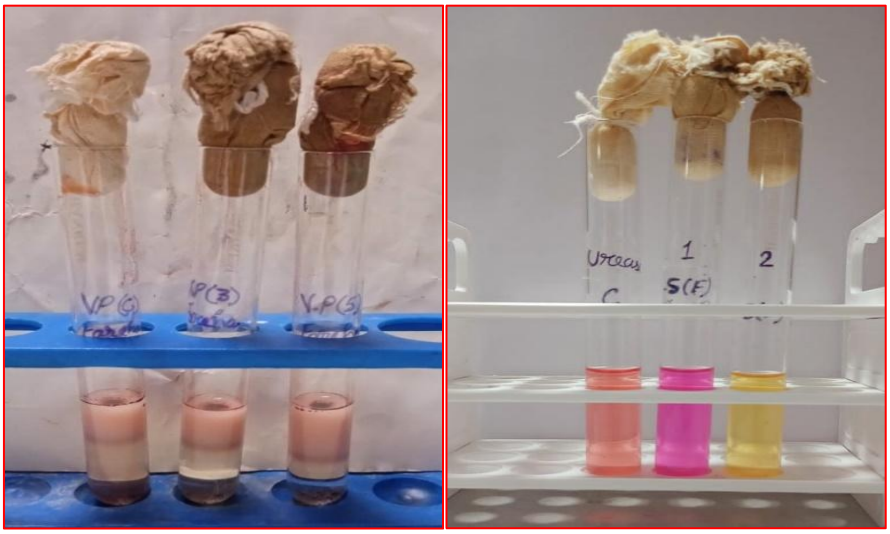

Figure 8A) Voges-proskuor test and 8B) Urease test.

The Starch Hydrolysis Test was performed to assess the ability of microorganisms to break down starch and produce the enzyme amylase. A bright red color observed in one test tube indicated a positive result, confirming amylase activity, while a light red coloration in another tube suggested a negative result (Figure 9-A, B). On agar plates, the formation of a clear zone surrounding the microbial growth line confirmed the hydrolysis of starch, further indicating the production of amylase.

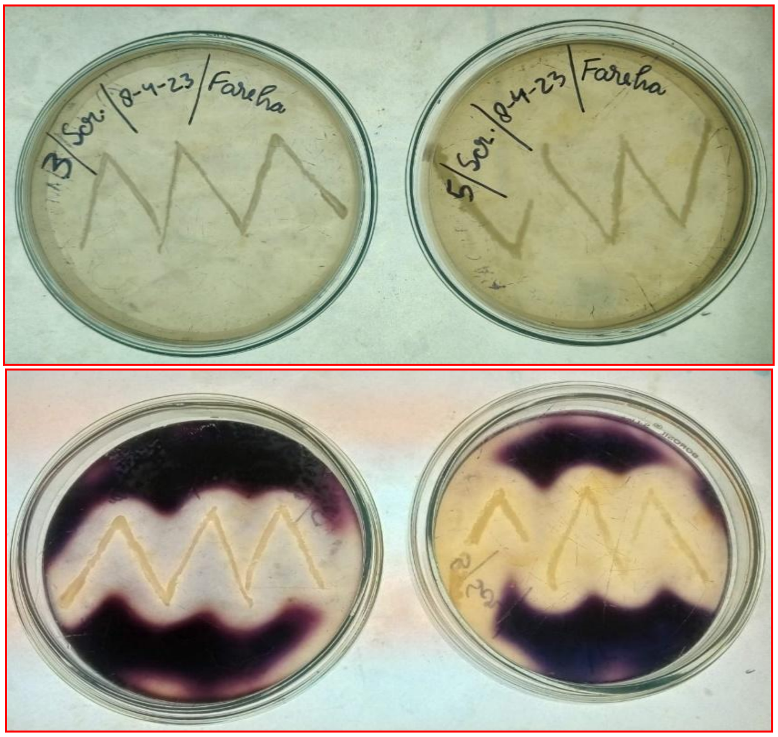

Figure 9 (a): Starch hydrolysis and 9 (b) Starch hydrolysis.

DISCUSSION

This research focused on isolating and identifying soil-dwelling bacteria with the potential to produce antimicrobial compounds, followed by the purification and detailed analysis of these bioactive substances. Soil samples were collected from a variety of natural environments, and bacterial strains were isolated using standard cultivation techniques. To enhance the production of antibiotics, fermentation was conducted under optimized conditions, carefully controlling factors such as temperature, pH, and nutrient availability. The antibacterial properties of the bacterial extracts were evaluated using agar diffusion assays, with comparisons drawn against standard antibiotics like tetracycline. Strains that exhibited strong inhibitory activity were selected for further study. *Pratylenchus vulnus* is involved in a disease of *Rosa noisettiana* 'Manetti' rose rootstock characterized by darkening of roots, death of feeder roots, and stunting of entire plants. The disease is more severe when plants are grown in silt loam soil than when they are grown in sandy loam soil. The nematodes reproduce best in silt loam soil at 20° C (G. S. Santo 1976).

The global incidence of emerging and reemerging infectious diseases (EIDs) continues to rise. Zoonotic infections, which spread from animals to humans through direct contact or indirectly via contaminated food, water, or environmental sources (Donna Behler McArthur, 2019), represent a significant portion of this threat. Despite their impact, vector-borne zoonotic diseases remain largely overlooked, particularly in resource-limited regions such as sub-Saharan Africa (Benjamin Mubemba et al., 2022). In light of the escalating issue of antibiotic resistance driven by the excessive use of current antimicrobial drugs, the search for new, safe, and effective antimicrobial agents has become increasingly urgent. This study explored the potential of soil microorganisms as valuable sources of novel antimicrobial compounds. Similarly, research by Taha Yasin Koc et al. (2024) demonstrated a cost-effective method for synthesizing piceatannol from resveratrol through regioselective hydroxylation by soil bacteria, highlighting the expanding interest in harnessing underexplored microbial ecosystems for biotechnological innovation.

The isolates were further examined using Gram staining (Figure 6A) and endospore staining techniques, which revealed that none of the strains formed endospores (Figure 6B). To assess their enzymatic activity, the isolates were tested for extracellular enzyme production using starch and gelatin hydrolysis assays. Additionally, a series of biochemical tests was conducted to evaluate intracellular enzyme functions. These tests included catalase, urease, Voges-Proskauer, hydrogen sulfide production, nitrate reduction, methyl red, phosphate solubilization, nitrogen fixation, citrate utilization, oxidase, motility, and triple sugar iron (TSI) tests. The majority of the bacterial isolates yielded positive results in these biochemical analyses.

A total of eight distinct bacterial species were isolated from the soil samples, belonging to the genera *Staphylococcus, Bacillus, Micrococcus, Proteus, Alcaligenes, Streptococcus, Serratia ,* and *Pseudomonas* (Table 2; Figures 4 and 5). Their classification was based on Gram staining results (Figure 6A) and the observation that none of the isolates formed endospores (Figure 6B). The isolates exhibited both extracellular and intracellular enzymatic activities, as confirmed through a range of hydrolytic and biochemical assays, with the majority showing strong enzyme production.

This study revealed that several bacterial strains isolated from soil exhibited significant antimicrobial activity against selected pathogenic organisms. Following purification and chemical characterization, a wide range of antimicrobial compounds with distinct structures and properties was identified. These findings underscore the tremendous potential of soil ecosystems as reservoirs of microbes capable of producing valuable antibiotics.
Infectious diseases continue to pose a serious global threat, a situation made worse by the growing problem of antimicrobial resistance. This highlights the critical need for new antimicrobial agents and effective methods to screen and evaluate them. Although commonly used techniques like well diffusion, disk diffusion, and broth dilution are standard in antimicrobial testing, they often face challenges with consistency and efficiency. To address these limitations, additional methods such as cross-streaking, poisoned food technique, co-culture assays, time-kill kinetics, resazurin assay, and bioautography are employed to assess antimicrobial activity more comprehensively. Advanced approaches like flow cytometry, impedance-based analysis, and bioluminescence offer faster, more sensitive detection and allow for detailed analysis of how antimicrobial compounds affect cells. However, the high cost and limited accessibility of these sophisticated tools can limit their use in certain research environments. This study offers a comprehensive review of various antimicrobial testing methods, explaining their principles, procedures, advantages, and drawbacks to aid in selecting suitable techniques and advancing the development of new antimicrobial agents.

Environmental changes also influence the distribution and mobility of antibiotic resistance genes (ARGs) across ecosystems and the influence of these changes and the appearance of malaria in the municipality of Jacunda Para using remote sensing and geographic information systems. The variables used to construct maps were distances from roads, farming areas, rivers, the dam, streams, and the urban area largely through the role of microbial generalists (Cintia Honorio Vasconcelos et al., 2006). Understanding these dynamics improves our ability to predict ARG spread and assess associated risks in environments influenced by human activity. Antibiotic resistance genes (ARGs), and Antibiotic resistant bacteria (ARB) in wastewater treatment plants

(WWTPs), and the reclamation of sewage sludge potentially threats human health and environmental safety (Jiaqi Wang et al., 2023). ARGs and antibiotic-resistant bacteria (ARB) are frequently detected in the vicinity of pharmaceutical manufacturing sites. It is crucial to implement strategies, such as integrating different networks, to control the spread of drug-resistant *Salmonella*. Novel technologies must be utilized to disinfect sewage and eliminate ARGs (Bihui Su et al., 2024). Interestingly, although non-antibiotic drugs represent a significant portion of pharmaceutical production, few comparative studies have examined soil ARG profiles and associated risks between antibiotic and non-antibiotic pharmaceutical production sites. Synthetic approaches to produce antibiotics have been unable to replace this platform. Uncultured bacteria make up approximately 99% of all species in external environments, and are an untapped source of new antibiotics. Teixobactin inhibits cell wall synthesis by binding to a highly conserved motif of lipid II (precursor of peptidoglycan) and lipid III (precursor of cell wall teichoic acid). We did not obtain any mutants of Staphylococcus aureus or Mycobacterium tuberculosis resistant to teixobactin. The properties of this compound suggest a path towards developing antibiotics that are likely to avoid development of resistance (Ling LL et al., 2015).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest of any kind.

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