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RESEARCH ARTICLE

Isolation, identification and characterization of bacteria from paddy and betel soils of chittar region, Tenkasi District.

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ABSTRACT

The study aimed to extract, identify, and characterize nitrifiers and other bacteria from paddy and betel soils. Soil samples were collected, and bacterial organisms were isolated using the serial dilution technique. The total number of bacterial colonies was determined using spread and pore plate procedures on Winogradsky Medium and Nutrient Agar Medium plates. A total of 10 bacterial species were obtained from the soil of a paddy field, including Bacillus, Corynebacterium, Micrococcus, Staphylococcus, Nitrosomonas, Nitrobacter, Azotobacter, Pseudomonas, Azospirillium, and E. coli. The bacterial isolates were characterized using Gram staining and biochemical tests. The bacteria found in paddy soil were described as having a variety of colony shapes, with rod-shaped, circular, oval-shaped, spore-forming, endospore-forming, capsulated, and non-capsulated gram-positive bacteria being the most common. The majority of the bacteria had the ability to generate spores and capsules. The bacteria exhibited negative acid-fast staining and were observed to be organized in a chain-like structure in both soil samples. Soil microorganisms have a significant impact on crop growth and output through natural genetic alteration.

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Introduction

Bacteria are unicellular, prokaryotic organisms that represent the most successful and resilient form of ancient life. Bacteria often exhibit three fundamental shapes: spherical, bacillus, and spiral. They can be located either as discrete cells or grouped together as clusters known as colonies (Young, 2006). Bacteria inhabit various environments such as soil, water, air, and even the most extreme regions of the earth, playing a crucial role in the operation and survival of these ecosystems (Gupta et al., 2017). Rice is a crucial staple crop, with over a billion individuals relying on its cultivation for their sustenance and more than 3.5 billion people depending on rice for over

20% of their daily caloric intake (Mohidem et al., 2022). Microorganisms in paddy fields engage in significant activities such as methanogenesis, methane oxidation, and biogeochemical cycles. These cycles encompass five primary processes: nitrogen fixation, nitrogen absorption, nitrogen mineralization, nitrification, and denitrification. Microorganisms, specifically bacteria, have significant involvement in the primary processes of carbon, nitrogen, and sulfur transformations (Shi et al., 2021; Wei et al., 2019).

Soil harbours a multitude of bacterial and fungal species. Soil microorganisms have a crucial function in carrying out numerous metabolic reactions in soil, including the breakdown of soil organic nitrogen and the decomposition of rice straw and compost that are added to the soil (Tang et al., 2021). These processes are essential for supporting rice production and preserving the fertility of paddy soil (Saeed et al., 2021). Nitrogen is a crucial component of the biogeochemical cycle that governs the conversion of nitrogen and nitrogen-containing chemicals in agricultural fields (Prosser et al., 2020). In well-oxygenated soils, bacteria and fungus are the main contributors, but in oxygen-deprived soils, bacteria are responsible for the majority of chemical and biological transformations (Naylor et al., 2022).

Piper betel refers to the foliage of the betel vine, commonly recognized as "Betel" in the English language. The likely point of origin of the betel vine is Malaysia, however it is currently farmed in India, Sri Lanka, Burma, Bangladesh, and Nepal. These plants have been recognized for their decorative qualities as well as their medicinal properties (Biswas et al., 2022). Plants are acknowledged for their fragrant qualities and their ability to provide medicinal substances. The indigenous populations of phosphate-solubilising bacteria and fungi were examined in several rhizospheric soil samples collected from betel vine plants (Piper betel L.) sources (Li et al., 2021). Additional phosphorus-solubilizing microorganisms included *Bacillus species, Streptomyces, Aspergillus fumigatus, Nocardia, actinomycetes,* and certain yeasts (Sabaridasan, 2012; Seshachala & Tallapragada, 2012).

Moreover, nitrification has been recognized as a two-step microbial reaction process involving two distinct functional groups: ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. This process involves the oxidation of ammonia to nitrate via nitrite (van Kessel et al., 2015). In bacteriological studies, the initial stage involves the isolation, purification, and identification of microorganisms. In order to obtain a pure bacterial culture from the isolated culture, a variety of mediums were employed (Bonnet et al., 2020). A pure culture is essential for studying the morphology, physiology, biochemical properties, and susceptibility of microorganisms. Multiple techniques, such as solid media, streak plate, or pour plate, can be employed to acquire uncontaminated cultures (Sanders, 2012).

Nitrogen is a vital ingredient for the growth and development of plants. Microbial breakdown in soils converts organic nitrogen into ammonia. Ammonium that is already present in the soil, whether it is provided as fertilizer or comes from precipitation, undergoes rapid oxidation to nitrate through the nitrification process. This process is carried out by specialized bacteria such as Nitrobacter, Nitrosomonas, and other bacteria (Grzyb et al., 2021; Rahimi et al., 2020).

The Tenkasi district is enriched by two prominent rivers, namely the Chittar and Anumanadhi, which contribute to the thriving agricultural sector in this region. The principal crops cultivated in the basin of river Chittar include paddy, chilli, cotton, sorghum, pigeon pea, black gram, green gram, bottle gourd, brinjal, ladies finger, pumpkin, bitter gourd, and Piper betel. The current study aimed to extract, identify, and characterize nitrifiers and other bacteria from paddy and betel soils.

Materials and Methods

Collection and processing of samples

Pristine soil samples were obtained from the agricultural areas of Melapavoor (8056’45.01’’N 77022’47.33’’E) and betel soil samples were

obtained from the fields of Ayikudy (8058'12.00''N and 77017'60.00''E) in the Tenkasi district. The samples were carefully collected using a sterile thermos flask and transported to the laboratory for subsequent analysis. Each test tube included 9 ml of sterile saline solution, into which about one gram of individual soil samples was added. The tubes were vigorously agitated, allowing the samples to separate into settled solids and discarded liquid supernatant. The remaining samples were then utilized for streaking.

Bacterial isolation

The samples underwent analysis, resulting in the isolation of two distinct types of isolates by the utilization of various mediums. The Winogradsky media is employed for the purpose of isolating Nitrobacter and Nitrosomonas spp, (Chidi Juliet Nwankwo et al., 2023). The media for isolation and culture of Nitrosomonas in Phase-I consisting of,

$(NH4)2SO4$ - 2.0 g
$K2HPO4$ - 1 g
$MgSO4.7H2O$ - 0.5 g
NaCl - 2.0 g
$FeSO4.7H2O$ - 0.4 g
$CaCO3$ - 0.01 g
Agar - 15.0 g
Distilled water - 1,000 ml

While Nitrobacter was isolated using medium B for nitrification in Phase-II consisting of,

$KNO2$ - 0.1 g
$Na2CO3$ - 1 g
NaCl - 0.5 g
$FeSO4.7H2O$ - 0.4 g
Agar - 5.0 g
Distilled water - 1,000 ml

The media were transferred into aseptic Petri dishes after chilling to approximately 45°C. The petridishes were subsequently inoculated and placed in an aerobic environment for 7 days at room temperature (28 ± 2°C) for both species. In addition, the process of identifying and describing distinct cultures of isolates was carried out following the methodology outlined (Singh et al., 2016).

Performing inoculation on Winogradsky media-A and media-B

The suspensions were subsequently inoculated onto hardened Winogradsky medium A and medium B using a sterilized inoculating needle through the four-way streaking procedure. The plates that were treated with the inoculum were thereafter placed in an incubator set at a temperature of 28 degrees Celsius for a duration of seven days. During this period, the plates were closely monitored for the emergence and growth of colonies (Prescott, 2002).

Quantitative assessment of living organisms

The viable count of bacteria was determined using the colony count method. The plates containing a range of 30 to 300 colonies were chosen for enumeration and quantified using the following equation as

Total bacteria per gram soil = (no of colonies × dilution factor) / (volume of sample (ml).

Bacterial identification

The identification of isolates was conducted by analyzing their physical and biochemical properties. The diminished level of ammonia and heightened levels of nitrite and nitrate indicate the proliferation and functioning of nitrifying bacteria (Koch et al., 2019).

Characterization

The properties of bacterial colonies and their morphology from both locations were assessed by assessing the well-isolated colonies on nutrient agar plates. The dimensions, coloration, shape, boundary, and height were examined as specified (Čepl et al., 2016).

Properties of staining

The morphology and disposition of bacteria were ascertained by techniques such as simple and negative staining, gram staining, capsule staining,

spore staining, and acid-fast staining (Ahern, 2018).

Assays of biological molecules

The study conducted various conventional biochemical assays, including catalase, oxidase, urease, nitrate reduction, ammonia consumption, and other tests (Koch et al., 2019).

Results and Discussion

The overall bacterial count was recorded in samples taken from both paddy and betel soils. The findings indicate the presence of variables inside bacterial colonies in both soil samples. The total number of bacterial colonies in all the samples was determined using the spread and pour plate procedures on Winogradsky Medium and Nutrient agar medium plates. The concentration of nitrifier bacteria in the soil, measured in colony forming units per gram (CFU/g), varied from 10^{-3} to 10^{-6} on Winogradsky Medium. The concentration of other bacteria, measured in CFU/g, ranged from 10^{-2} to 10^{-7} on Nutrient agar medium (Table 1 & 2). The bacterial counts of the paddy soil sample varied between 12 $\times 10^{-4}$ Colony Forming Units (CFU) per gram on Winogradsky Medium to 26.5×10^{-2} colony forming units per gram on Nutrient agar medium (Table 1). The bacterial counts of the betel soil sample varied between 9.0×10-4 colony forming units per gram when grown on Nutrient agar medium (Table 2).

Analyzed paddy soil samples for several types of gram-positive and gram-negative bacteria. The bacteria present in all 10 samples of paddy soil were identified and classified based on their biochemical characteristics as *Bacillus, Corynebacterium, Micrococcus,* and *Staphylococcus*. The soil samples are predominantly inhabited by *Nitrosomonas, Nitrobacter, Azotobacter, Pseudomonas, Azospirillium,* and *E. coli* species. Out of the 10 paddy soil samples, gram positive bacteria were identified from 4 samples, while gram negative bacteria were isolated from 6 samples. Four isolated gram-positive bacteria were recognized as Bacillus, Corynebacterium, Micrococcus, and Staphylococcus. Additionally, six isolated gram-negative bacteria were identified as *Nitrosomonas, Nitrobacter, Azotobacter, Pseudomonas, Azospirillium,* and *E. coli*. The isolates underwent gram staining to determine their bacterial classification as either gram-positive or gram-negative. Additionally, various biochemical tests were conducted on the isolates as respectively in Table 3.

Table 1. Enumeration of bacteria of Paddy soil samples

Sample	Medium	Dilution factor	Number of colonies
Paddy	Winogradsky Medium	10^{-1}	TNTC
		10^{-2}	TNTC
		10^{-3}	120
		10^{-4}	85
		10^{-5}	60
		10^{-6}	40
		10^{-7}	TLTC
Paddy	Nutrient Agar Medium	10^{-1}	TNTC
		10^{-2}	265
		10^{-3}	180
		10^{-4}	120
		10^{-5}	90
		10^{-6}	55
		10^{-7}	TLTC

Table 2. Enumeration of bacteria of betel soil samples

Sample	Medium	Dilution factor	Number of colonies
Betel	Nutrient Agar Medium	10^{-1}	TNTC
		10^{-2}	TNTC
		10^{-3}	90
		10^{-4}	75
		10^{-5}	50
		10^{-6}	TLTC

Table 3. Biochemical tests of bacteria from the paddy soils

Bacterial species	Catalse	Oxidase	Urease	Nitrate Reduction	Indole	Methyl Red	VP	Citrate	H_2S Production
Bacillus	+	-	-	+	-	-	+	+	+
Corynebacterium	+	+	-	+	-	+	-	+	+
Micrococcus	+	+	-	+	-	-	+	-	+
Staphylococcus	+	-	+	+	-	+	-	+	+
Nitrosomonas	+	+	+	-	-	-	-	-	+
Azotobacter	+		-	+	+	-	+	+	
Pseudomonas	+	+	-	+	-	-	-	+	-
Azospirillum	+	+	+	+	-	-	-	+	-
E. coli	+	-	-	+	+	+	-	-	-
Nitrobacter	+	-		+	-	-		-	-

Table 4. Biochemical tests of bacteria from the betel soils

Bacterial species	Catalse	Oxidase	Urease	Nitrate Reduction	Indole	Methyl Red	VP	Citrate	H_2S Production
Clostridium	-	-	+	-	-	+	-	+	+
Nitrosomonas	+	+	+	-	-	-	-		
Klebsilla	+	-	-	+	+	+	+	+	-
Nitrobacter	+	-		+	-	-		-	-
Pseudomonas	+	+	-	+	-	-	-	+	-
Micrococcus	+	+	-	+	-	-	+	-	+
Rhizobium	+	+	+	+	-	-	+	-	-

Table 5. Morphological characteristics of isolated bacteria of Paddy soil

Bacteria	Shape	Arrangement	Staining Characteristics		
			Gram Stain	Spore Stain	Capsule Stain
Bacillus	Rod	Chain	Gram positive	Endo spore forming	Capsulated
Corynebacterium	Club	V and Y shaped arrangements	Gram positive	Non-spore forming	Non capsulated
Micrococcus	Cocci	Irregular clusters	Gram positive	Non-spore forming	Non capsulated
Staphylococcus	Cocci	Pair and short chains	Gram positive	Non-spore forming	Capsulated
Nitrosomonas	Short rods	Rods	Gram negative	Non-spore forming	Capsulated
Nitrobacter	Rod	Rods	Gram negative	Spore forming	Capsulated
Azotobacter	Oval	Clusters	Gram negative	Non-spore forming	Capsulated
Pseudomonas	Rod	Slender	Gram negative	Non-spore forming	Capsulated
Azospirillium	Rod	Rods	Gram negative	Non-spore forming	Capsulated
E. coli	Rod	Rods	Gram negative	Non-spore forming	Capsulated

Table 6. Morphological characteristics of isolated bacteria of Betel soil

Bacteria	Shape	Arrangement	Staining Characteristics		
			Gram Stain	Spore Stain	Capsule Stain
Clostridium	Rod	Rods	Gram positive	Endo spore forming	Capsulated
Micrococcus	Cocci	Irregular clusters	Gram positive	Non-spore forming	Non capsulated
Nitrosomonas	Short Rods		Gram negative	Non-spore forming	Capsulated
Klebsilla	Rod	Short chains	Gram negative	Non-spore forming	Capsulated
Nitrobacter	Rod		Gram negative	Spore forming	Capsulated
Pseudomonas	Rod	Slender	Gram negative	Non-spore forming	Capsulated
Rhizobium	Rod	Single	Gram negative	Non-spore forming	Capsulated

The Gram staining analysis showed that Bacillus, *Corynebacterium, Micrococcus,* and *Staphylococcus* are classified as gram-positive bacteria, whereas *Nitrosomonas, Nitrobacter, Azotobacter, Pseudomonas, Azospirillium,* and *E. coli* are classified as gram-negative bacteria (Table 5). The morphological characteristics of bacteria isolated from paddy soil were reported to consist of a mixture of colony types, with a predominant presence of rod-shaped, circular, oval-shaped, spore-forming, endospore-forming, encapsulated, and non-encapsulated gram-positive bacteria (Table 5).

The betel soil samples were examined for various types of gram-positive and gram-negative bacteria. The bacteria included in all seven soil samples from betel plants were identified and classified based on their biochemical characteristics. The dominant species observed in the samples were *Clostridium, Micrococcus, Nitrosomonas, Nitrobacter, Klebsilla, Pseudomonas,* and *Rhizobium*. Among the 7 betel soil samples, gram positive bacteria were found in 2 samples, while gram negative bacteria were found in 5 samples. Two isolated gram-positive bacteria were recognized as *Clostridium* and *Micrococcus*, while five isolated gram-negative bacteria were identified as *Nitrosomonas, Nitrobacter, Klebsilla, Pseudomonas,* and *Rhizobium*. The isolates underwent gram staining to determine their bacterial classification as either gram-positive or gram-negative. Additionally, other biochemical tests were conducted to further characterize the isolates (Table 4).

The morphological features of bacteria isolated from betel soil were examined, revealing the presence of mixed colonies consisting of rod-shaped and cocci-shaped bacteria, both of which included both gram-positive and gram-negative species. All of the colonies exhibited spore formation and could be classified as either spore-forming, capsulated, or non-capsulated, as indicated in Table 6. The isolated bacteria were identified and characterized through various methods including morphological analysis, microscopic examination, and biochemical tests. These tests included assessing the shape, arrangement, colonies, growth, indole production, H_2S production, methyl red and voges proskauer test, oxidase test, citrate utilization test, catalase test, and growth at 37°C. The results of these tests are presented in Table 3 & 4.

The findings indicated that the isolates from paddy fields included of *Bacillus* sp, *Klebsiella oxytoca, Klebsiella pneumoniae, Pseudomonas* sp,

Aeromonas sp, and *Enterobacter cloacae*. The paddy field isolates showed the highest frequency of occurrence in *Bacillus* sp. followed by *Klebsiella pneumoniae, K. oxytoca,* and *Pseudomonas* sp. *Bacillus* sp. are commonly present in soil and have a significant impact on the nitrogen cycle, hence enhancing soil fertility (Guerrieri et al., 2020). The positive isolates in the nitrate reduction test shown their ability to utilize nitrate as a nitrogen source, whereas the positive isolates in the ammonia utilization test demonstrated their ability to utilize ammonia as a nitrogen source. The urease positive isolates demonstrated their ability to hydrolyze urea and liberate ammonia, which they may utilize as a growth substrate (Ingale & Phirke, 2017).

A comparative analysis was conducted on the results obtained from soil samples in Bangladesh (Chowdhury et al., 2013). Our reports closely resembled theirs. Evidence demonstrates that the bacterial colony found in various parts of Bangladesh is predominantly composed of *Bacillus* sp. Additionally, several other types of bacteria that are gram negative and capable of generating spores, such as *Enterobacter* spp., *Klebsiella* spp., Bacillus spp., and Azospirillum spp., were detected (Khan et al., 2008). The isolates were identified as *Bacillus, Pseudomonas, Streptomycis, Azotobacter,* and *Alcaligenes* based on their biochemical characterisation and carbohydrate fermentation (Oljira et al., 2018). A study by Kannan et al., (2018) found that many agricultural soil bacteria, including *E. coli, Micrococcus* sp, *Escherichia* sp, and *Staphylococcus* sp, were isolated.

The bacteria present in the six soil samples were identified and classified as *Staphylococcus* sp., *Streptococcus* sp., *Klebsiella pneumoniae*, *Enterobacter aerogenes, Shigella* sp., *Micrococcus* sp., *Bacillus anthracis, Bacillus subtilis*, *Cocci* sp., *Azomonas* sp., *Corynebacterium* sp., and *Rhizobium* sp. These species were found to be the most prevalent in the soil samples. The analyses detected the existence of multiple bacterial pathogens, such as *Klebsiella pneumoniae, Enterobacter aerogenes, Shigella* species, *Bacillus anthracis, Bacillus subtilis, Staphylococcus* species, *Streptococcus* species, *Corynebacterium, Micrococcus, Azomonas* species, *and Rhizobium* species (Kumar et al., 2021; Rashmi et al., 2017).

Conclusion

This research involved the collection and characterization of soil samples from the Chittar region in the Tenkasi district. This study involved the collection of soil samples from various locations in paddy fields and the subsequent characterization of different microorganisms present. Specifically, we identified and characterized *Bacillus, Corynebacterium, Micrococcus, Staphylococcus, Nitrosomonas, Nitrobacter, Azotobacter, Pseudomonas, Azospirillium,* and *E. coli*. Additionally, soil samples were collected from different sites of betel and the microorganisms such as *Clostridium, Micrococcus, Nitrosomonas, Nitrobacter, Klebsilla, Pseudomonas,* and *Rhizobium* were characterized. There is a scarcity of research conducted on the isolation, identification, and morphological characterization of soil bacteria in the study area. Obtaining accurate accounting of the diverse morphological forms of bacteria found in paddy and betel soils is crucial. All the isolates exhibited morphological and biochemical characteristics consistent with species belonging to the genera Nitrobacter, Nitrosomonas, gram-positive bacteria, and gram-negative bacteria. The paddy and betel ecosystem was regarded as the habitat for nitrogen-fixing microorganisms such as Nitrobacter, Nitrosomonas, and Rhizobium. Additionally, it proposes that aerobes and anaerobes play a crucial role in promoting plant growth through nitrogen fixation.

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