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Isolation and Characterization of soil bacteria from Barishal and Jhalokathi series of Bangladesh

Rashiduzzaman Emran[1], Md. Rayhan Kabir[2], Md. Abdur Rahman[2], Md. Kayes Mahmud[2], Md. Eshtiak Ahmed[2] and Mahmudul Hasan Chowdhury[2]

[1]Additional Agriculture Officer, Department of Agricultural Extension, Ministry of Agriculture, Bangladesh
[2]Scientific Officer, Soil Resource Development Institute, Ministry of Agriculture, Bangladesh

ABSTRACT

This study was conducted to isolate, quantify, and characterize bacterial populations from agricultural soils of Barishal and Jhalokathi regions of Bangladesh. Soil samples were collected from the surface layer (0–15 cm) under aseptic conditions and processed using standard microbiological techniques, including serial dilution, spread plate method, and staining procedures. The total viable bacterial count was found to be 7.6×10^7 CFU/g in Barishal soil and 9.4×10^7 CFU/g in Jhalokathi soil, indicating a high microbial load in both regions. A total of eight distinct bacterial colonies were isolated from Barishal soil, whereas six distinct colonies were obtained from Jhalokathi soil. Colony characteristics showed considerable variation in size, pigmentation (white, pink, and yellow), form (circular, irregular, rhizoid), margin (entire, lobate, serrate, undulate), and elevation (flat, raised, umbonate). Morphological and staining analyses revealed the presence of both rod-shaped and cocci bacteria with single and chain arrangements.

The isolates included both Gram-positive and Gram-negative bacteria, with several exhibiting spore-forming and capsule-forming abilities. However, all isolates were non-acid fast. The findings indicate that both soils harbor diverse and heterogeneous bacterial populations with significant physiological and morphological variability. This study provides valuable baseline information on soil bacterial diversity and contributes to the understanding of microbial ecology in Bangladesh agricultural soils.

KEY WORDS

CFU (Colony-Forming Units per gram); Colony characteristics; Gram staining; Microbial diversity; Soil bacteria; Spore-forming bacteria

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Introduction

Bacteria are unicellular, prokaryotic microorganisms that represent some of the earliest and most evolutionarily persistent forms of life on Earth. Due to their long evolutionary history, bacteria have developed remarkable adaptability, allowing them to survive and thrive under a wide range of environmental conditions. Structurally, bacterial cells are relatively simple, typically measuring between 0.5 and 2.0 µm in diameter. Despite this simplicity, they exhibit distinct morphological variations, primarily categorized into coccoid (spherical), bacillary (rod-shaped), and spiral forms. These cells may occur as solitary units or may associate with one another to form colonies, depending on environmental factors and nutrient availability [1]. Bacteria perform vital ecological functions in both terrestrial and aquatic ecosystems. They play a central role in maintaining ecosystem productivity by participating in organic matter decomposition and facilitating the recycling of essential nutrients such as carbon, nitrogen, and phosphorus [2]. Among all microbial groups, bacteria are the most abundantly distributed and ecologically versatile organisms. However, their limited morphological differentiation poses significant challenges for accurate identification and taxonomic classification. As a result, reliance solely on morphological characteristics is often insufficient. Therefore, systematic classification supported by culture-based methods and complementary analytical approaches is essential for gaining a comprehensive understanding of bacterial diversity. Such classifications frameworks help elucidate the ecological roles, physiological traits, and environmental interactions of various bacterial taxa within different ecosystems [3]. Bacteria are ubiquitous in nature and are found in virtually all habitats, including soil, water bodies, air, and even extreme environments such as high-salinity areas, acidic zones, and temperature extremes. Through their metabolic activities, bacteria contribute significantly to environmental stability and the regulation of biogeochemical cycles. In well-aerated soils, bacterial communities function synergistically with fungi to drive microbial processes and nutrient turnover. In contrast, under oxygen-limited or anaerobic soil conditions, bacteria dominate the microbial population and are responsible for the majority of chemical transformations and biological processes occurring within the soil system [4]. Bacteriological research fundamentally begins with the systematic isolation, purification, and identification of bacterial strains. Isolation techniques are applied to recover bacterial populations from well-defined sources, after which purification procedures are undertaken to remove unwanted microorganisms and obtain uniform cultures [5]. Establishing a pure bacterial culture is essential, as it provides a reliable foundation for comprehensive analyses of bacterial morphology, physiological functions, biochemical activities, and antimicrobial sensitivity. To separate pure cultures from heterogeneous microbial communities, both selective and non-selective growth media are routinely employed. Widely adopted approaches, such as growth on solid

***Correspondence:** Dr. Mahmudul Hasan Chowdhury, Scientific Officer, Soil Resource Development Institute, Ministry of Agriculture, Bangladesh, e-mail: lipuchowdhury@gmail.com

agar media, streak plate techniques, and pour plate methods, effectively facilitate the isolation of discrete bacterial colonies [6,7]. Multiple studies have reported that soils across various regions of Bangladesh are substantially affected by heavy metal contamination, leading to the selection and dominance of metal-tolerant and metal-resistant bacterial populations. These microorganisms demonstrate notable resilience and adaptability under harsh environmental conditions, thereby playing an important ecological role in contaminated habitats [8–11]. Indigenous soil bacteria in Bangladesh have been shown to provide several beneficial functions, including phosphate solubilization, assistance in environmental detoxification, and regulation of mineral cycling within soil systems [12–14]. In addition, these bacterial communities actively participate in critical biochemical processes such as nitrification, which is essential for maintaining soil fertility and nutrient availability [15–17]. Their presence further contributes to the remediation of metal-polluted soils, reduction of pathogenic microbial loads, and stabilization of acidic soil environments. Microbiological studies of soil ecosystems are vital for expanding scientific understanding and for developing effective solutions to environmental and agricultural challenges. The systematic and stepwise identification of soil bacterial populations is particularly important for elucidating the complexity and dynamic nature of soil microbial communities [13,18]. In this context, the present study emphasizes the isolation of soil bacteria, quantification of colony-forming units, and evaluation of colony characteristics. Moreover, key morphological features, including cellular shape, arrangement patterns, and staining responses, were analyzed to assess microbial abundance and diversity in soil samples.

Materials and Methods

Sample collection

Two fresh topsoil samples (Table 1) were aseptically obtained from agricultural fields of Bangladesh. Soil was collected specifically from the surface layer at a depth of 0–15 cm, as this zone is known to harbor the highest microbial activity and nutrient availability. Strict aseptic measures were maintained throughout the sampling process to prevent external contamination and to ensure the integrity of indigenous microbial populations. Following collection, the soil samples were immediately transferred to the laboratory using sterile thermos flasks to maintain stable temperature conditions and minimize microbial alteration during transport. Upon arrival, the samples were properly stored under controlled laboratory conditions until further microbiological and physicochemical analyses were conducted. This careful handling and preservation of soil samples ensured the reliability and reproducibility of subsequent experimental investigations.

Table 1. Related information of collected soil samples.

Sample no.	Series	Location	GPS reading	Physiographic unit	Cropping pattern
1	Barishal	Benerpota Satkhira	N-22°44′888″ E-89°86′361″	Ganges Tidal Floodplain	Aus-T. Amam
2	Jhalokathi	Mirjaganj Patuakhali	N-22°25′589″ E-90°13′631″	Ganges Tidal Floodplain	Aus-T. Amam

Isolation of bacteria

Bacterial isolation was conducted in accordance with recognized standard microbiological techniques reported in earlier studies.Initially, soil samples were homogenized by mixing a measured quantity of soil with physiological saline solution (distilled water containing 0.9% NaCl) to achieve an even suspension of microbial cells. This homogenized mixture was used as the source material for further microbiological procedures. Subsequently, the soil suspension was subjected to a stepwise serial dilution process to lower microbial concentration and enables the recovery of individual bacterial colonies. Samples from suitable dilution levels were aseptically transferred onto properly labeled Petri dishes and spread uniformly over the agar surface using the spread plate method. The inoculated plates were then incubated at 37°C for 24–48 hours to allow optimal bacterial growth and colony formation. After incubation, distinct and well-separated colonies were carefully selected and further purified through repeated streaking on fresh agar plates to obtain axenic cultures. This purification process was systematically carried out to remove mixed microbial populations and ensure culture consistency. All isolation and purification steps were performed in triplicate to ensure accuracy and reproducibility. The purified isolates were re-incubated at 37°C for an additional 24–48 hours to verify culture purity and growth stability [19,20].

Viable count

The viable bacterial population was determined using the standard colony count method. For accurate enumeration, agar plates containing 25 to 250 well-developed colonies were selected for analysis. The total number of viable bacteria present per gram of soil was calculated using the following formula:

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

Characterization

The morphological characteristics of bacterial colonies obtained from both sampling sites were carefully examined using well-isolated colonies cultivated on nutrient agar plates. A comprehensive assessment was performed, considering several key parameters including colony size, pigmentation, shape, margin configuration, and elevation (Figure 1). Observations were carried out systematically to ensure consistency and reproducibility, following the standardized descriptive criteria outlined by Dubey and Maheshwari (1998) [19]. These detailed morphological evaluations provide critical insights into the phenotypic diversity of bacterial populations present in the soil samples and form the basis for subsequent identification and classification studies.

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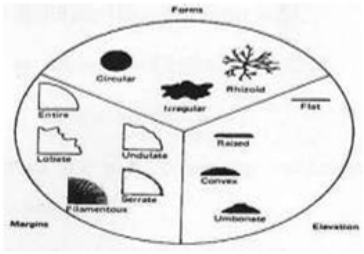

Figure 1. Colony characteristics of bacteria [21].

Staining characteristics

The morphology and spatial arrangement of bacterial isolates were systematically investigated through a series of established staining techniques, including simple staining, negative staining, Gram staining, capsule staining, spore staining, and acid-fast staining. Each method contributed complementary insights, collectively enabling a comprehensive characterization of bacterial cell shape, surface structures, and arrangement patterns. These observations are fundamental for precise phenotypic differentiation, accurate taxonomic classification, and a deeper understanding of the structural diversity present among the soil bacterial populations [21,22].

Simple staining

For the examination of bacterial morphology, smears were meticulously prepared on sterile glass slides and subjected to heat fixation to immobilize the cells and preserve their structural integrity. Crystal violet stain was then applied to the fixed smear and allowed to interact for 40–60 seconds to ensure adequate uptake by the bacterial cells. Following this, the slides were gently rinsed with tap water to remove any excess dye and subsequently air-dried. The stained smears were examined under a high-power microscope using oil immersion, enabling detailed observation of cellular shape, arrangement, and structural characteristics. This procedure provides essential information for the accurate phenotypic characterization and preliminary identification of bacterial isolates [21,22].

Negative staining

Negative staining was performed using a clean, dry glass slide, on which a small drop of nigrosin dye was placed at one end. A loopful of bacterial culture was carefully mixed with the dye, and the resulting suspension was spread across the slide using the edge of a second slide held at an approximately 30° angle to create a thin, uniform smear. The smear was allowed to air dry naturally, after which it was examined under a microscope using oil immersion. This approach enabled precise observation of bacterial cell morphology, including cellular shape and spatial arrangement, while avoiding structural distortions that can result from heat fixation. By preserving the native size and configuration of the cells, negative staining provides reliable information for phenotypic characterization of bacterial isolates [21,22].

Gram stain

For the purpose of Gram staining, bacterial smears were carefully prepared on sterile glass slides and subjected to heat fixation to immobilize the cells and preserve their native morphology. The fixed smears were initially stained with crystal violet for approximately one minute to allow sufficient uptake of the primary dye. Excess crystal violet was gently washed off with tap water. The smears were then treated with Gram's iodine for one minute, which functions as a mordant to form a stable dye-iodine complex within the cells, followed by another rinse to remove any unbound iodine. Decolorization was carried out by adding 95% ethyl alcohol dropwise until no further dye leached from the smear, after which the slides were rinsed again with tap water. To complete the staining process, the smears were counterstained with safranin for about 45 seconds, followed by a final rinse and air drying. The prepared slides were examined under oil immersion microscopy, providing clear differentiation between Gram-positive and Gram-negative bacteria while allowing detailed observation of cellular shape, size, and arrangement. This method ensures reliable phenotypic characterization and contributes to accurate taxonomic identification of the bacterial isolates [21,22].

Capsule stain

For the visualization of bacterial capsules, smears were prepared on sterile glass slides and allowed to air dry naturally, without the application of heat fixation, in order to preserve the original morphology of the cells and their surrounding capsules. Crystal violet stain was then applied to the dried smears and left for 5–7 minutes to ensure adequate penetration and staining of the bacterial cells. Excess dye was carefully washed off using a 20% copper sulfate solution, which serves both as a decolorizing agent and a background counterstain. The slides were subsequently air-dried and examined under oil immersion microscopy, enabling distinct observation of the bacterial cells and their capsules, which appear as clear halos surrounding the stained cells. This technique is essential for accurate identification of encapsulated bacterial strains and provides valuable insights into their structural characteristics [21,22].

Spore stain

For the purpose of endospore visualization, bacterial smears were initially prepared on sterile glass slides and subjected to heat fixation to immobilize the cells and preserve their structural integrity. Malachite green was applied to the fixed smear, and the slides were gently heated on a warm hot plate for 2–3 minutes to facilitate deep penetration of the dye into the resilient spore structures. Following heating, the slides were allowed to cool and were thoroughly rinsed with tap water to remove any excess stain. The smears were then counterstained with safranin for approximately 30 seconds and gently washed with water to provide contrast between vegetative cells and spores. After air drying, the slides were examined under oil immersion microscopy, which enabled the clear identification of endospores as green-stained structures embedded within red-stained vegetative cells. This staining procedure is critical for the accurate morphological characterization and identification of spore-forming bacterial species and provides valuable insights into their structural adaptations [21,22].

Acid fast stain

For the purpose of detecting and characterizing acid-fast bacteria, bacterial smears were carefully prepared on sterile glass slides and subjected to heat fixation to immobilize the cells while preserving their native structural features. The fixed smears were then treated with carbolfuchsin and placed on a warm hot plate for five minutes, allowing the dye to penetrate the robust, lipid-rich cell walls of acid-fast bacteria. After heating, the slides were removed, cooled, and gently rinsed with tap water to eliminate excess stain. Decolorization was carried

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out by the careful, dropwise application of acid-alcohol until no further carbolfuchsin was washed away, followed by a rinse with water to remove residual decolorizer. The smears were subsequently counterstained with methylene blue for approximately two minutes to provide contrast between acid-fast and non-acid-fast cells, followed by a gentle rinse and air drying. Examination under oil immersion microscopy enabled clear differentiation of acid-fast bacteria, which retained the red carbolfuchsin stain, from non-acid-fast cells, which appeared blue due to methylene blue staining. This method is critical for the accurate morphological assessment and reliable identification of acid-fast bacterial species, offering essential insights into their structural and diagnostic characteristics [21,22].

Statistical analysis

All experimental procedures, including bacterial isolation, viable count, and characterization, were conducted in triplicate to ensure the reliability and reproducibility of the results. The data obtained from colony-forming unit (CFU) counts were expressed as mean values. Descriptive statistical methods were applied to summarize the bacterial population data from the two soil samples. The total viable bacterial counts were calculated using standard formulae and presented in terms of CFU per gram of soil. Comparative observations between Barishal and Jhalokathi soils were interpreted based on differences in mean CFU values and colony characteristics.

Results and Discussion

The total bacterial population was observed from both Barishal and Jhalokathi soils, where successful isolation, purification, and characterization revealed considerable variability in bacterial colonies. The results demonstrated the presence of diverse bacterial communities in both soil samples. The colony count found 7.6×(10)^7 CFU/g soil and 9.4×(10)^7 CFU/g soil in Barishal and Jhalokathi soil respectively.

A total of eight distinct types of bacterial colonies were identified from Barishal soil, whereas six distinct colonies were observed from Jhalokathi soil. The bacterial colonies from Barishal soil exhibited variation in size ranging from pinpoint, small to moderate, while those from Jhalokathi soil ranged from small to large-sized colonies. This variation in colony size indicates differences in growth rate and adaptability of bacterial populations under different soil conditions.

In Barishal soil, the colonies were observed to be circular, irregular, and rhizoid in form; lobate, serrate, and entire in margin; and flat, raised, and umbonate in elevation (Table 2). Similarly, the colonies from Jhalokathi soil were circular and irregular in form; undulate, entire, and lobate in margin; and flat to raised in elevation (Table 3). The pigmentation of colonies in both soils varied from white, pink, and yellow, indicating metabolic diversity among the isolated bacteria.

Table 2. Colony characteristics of isolated bacteria of Barishal soil.

Colony no.	Size	Pigmentation	Form	Margin	Elevation
1	Moderate	Pink	Circular	Lobate	Raised
2	Moderate	White	Rhizoid	Serrate	Flat
3	Pinpoint	Yellow	Irregular	Lobate	Raised
4	Small	Pink	Irregular	Entire	Umbonate
5	Pinpoint	White	Circular	Entire	Raised
6	Moderate	White	Circular	Serrate	Umbonate
7	Small	Pink	Rhizoid	Entire	Flat
8	Small	Yellow	Irregular	Lobate	Flat

Table 3. Morphological characteristics of isolated bacteria of Barishal soil.

Colony no.	Shape	Arrangement	Gram stain	Spore stain	Capsule stain	Acid-fast stain
1	Round	Single	Gram Negative	Non-spore forming	Capsule forming	Non-acid fast
2	Rod	Chain	Gram Negative	Spore forming	Non-capsule forming	Non-acid fast
3	Rod	Chain	Gram Negative	Spore forming	Capsule forming	Non-acid fast
4	Rod	Single	Gram Negative	Non-spore forming	Non-capsule forming	Non-acid fast
5	Rod	Chain	Gram positive	Spore forming	Capsule forming	Non-acid fast
6	Rod	Single	Gram Negative	Non-spore forming	Capsule forming	Non-acid fast
7	Round	Single	Gram positive	Non-spore forming	Capsule forming	Non-acid fast
8	Rod	Chain	Gram Negative	Spore forming	Non-capsule forming	Non-acid fast

The morphological characteristics of bacteria isolated from Barishal soil revealed a mixed population consisting of both rod-shaped and round-shaped bacteria. These isolates included both Gram-positive and Gram-negative types, with a significant proportion showing spore-forming ability, particularly among rod-shaped bacteria (Table 4). Some isolates were capsule-forming, which may contribute to their survival under adverse environmental conditions. However, all isolates were non-acid fast. Similarly, bacterial isolates from Jhalokathi soil also showed mixed morphological characteristics, including both rod and cocci forms with single and chain arrangements. The isolates comprised both Gram-positive and Gram-negative bacteria, with several exhibiting spore-forming ability and capsule formation

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(Table 5). As in Barishal soil, all isolates were non-acid fast.

Overall, the results indicate that both Barishal and Jhalokathi soils harbor diverse and heterogeneous bacterial populations with varied morphological and physiological traits. This diversity plays a crucial role in maintaining soil health, nutrient cycling, and overall ecosystem functioning.

Table 4. Colony characteristics of isolated bacteria of Jhalokathi soil.

Colony no.	Size	Pigmentation	Form	Margin	Elevation
1	Large	Yellow	Irregular	Undulate	Flat
2	Small	White	Circular	Entire	Flat
3	Medium	Pink	Irregular	Lobate	Raised
4	Small	White	Irregular	Entire	Fla
5	Large	Pink	Circular	Lobate	Raised
6	Medium	Yellow	Irregular	Undulate	Flat

Table 5. Morphological characteristics of isolated bacteria of Jhalokathi soil.

Colony no.	Shape	Arrangement	Gram stain	Acid-fast stain	Spore stain	Capsule stain
1	Rod	Chain	Gram Negative	Non-acid fast	Non-spore forming	Capsule forming
2	Round	Single	Gram Positive	Non-acid fast	Spore forming	Non-capsule forming
3	Rod	Single	Gram Negative	Non-acid fast	Non-spore forming	Non-capsule forming
4	Rod	Chain	Gram Negative	Non-acid fast	Spore forming	Capsule forming
5	Round	Single	Gram Positive	Non-acid fast	Non-spore formin	Capsule forming
6	Rod	Chain	Gram Positive	Non-acid fast	Spore forming	Non-capsule forming

The result was compared with the previous study conducted by Chowdhury et al., from Bangladesh soil [23] . Our reports were very much similar to them. It proves that the bacterial colony from different regions of Bangladesh dominantly contains Bacillus sp. Many other gram negative, spore forming bacteria as Enterobacter spp., Klebsiella spp., Bacillus spp. and Azospirllum spp. are also identified [17]. Many reports from Bangladesh soil stated that there is abundant presence of different Bacillus Sp. which are mostly spore forming [24-27]. The spores of Bacilli can survive better in soils which are alkaline and can proceed with its life cycle. The sporulating bacterium of soil has few opportunities to complete its reproductive cycle; therefore, their extraordinary pathogenicity is one of the effective strategies to increase the probability of survival [28]. Although many new sporulating bacterial generations are neutralized by feeding on dead and decaying matter which helps them in survival and spreads into the soil. This process ensures the continuation of the species within the environmental density [29].

Limitation

This study provides important baseline information on the isolation and characterization of soil bacteria from Barishal and Jhalokathi regions; however, several limitations should be acknowledged. Firstly, the study relied primarily on culture-based techniques, which are known to recover only a small fraction of the total microbial diversity present in soil. A significant proportion of soil bacteria remain unculturable under standard laboratory conditions, which may lead to an underestimation of actual microbial diversity. Identification of bacterial isolates was based mainly on morphological and staining characteristics. Although these methods are useful for preliminary classification, they are insufficient for precise taxonomic identification. Advanced molecular techniques, such as 16S rRNA gene sequencing, were not employed, which limits the accuracy of species-level identification.

Conclusion

The present study successfully isolated, quantified, and characterized bacterial populations from Barishal and Jhalokathi agricultural soils, revealing significant diversity in both colony morphology and cellular characteristics. The variation in colony size, pigmentation, form, margin, and elevation reflects the heterogeneity of soil bacterial communities in these regions. Both soils exhibited the presence of Gram-positive and Gram-negative bacteria, including rod-shaped and cocci forms with different arrangements. A notable proportion of the isolates demonstrated spore-forming and capsule-forming abilities, which are key survival strategies under adverse environmental conditions. The absence of acid-fast bacteria suggests that the isolated populations are predominantly non-mycobacterial. The relatively high bacterial counts in both soils indicate active microbial ecosystems, which are essential for nutrient cycling, organic matter decomposition, and maintenance of soil fertility. The dominance of spore-forming bacteria suggests the prevalence of resilient groups such as Bacillus spp., which are known for their ecological importance and potential applications in agriculture, including biofertilizer development.

Overall, this study highlights the rich microbial diversity present in Bangladesh soils and underscores the importance of soil bacteria in sustaining agricultural productivity and environmental balance. Further molecular and biochemical studies are recommended to achieve precise identification and to explore their functional potential in sustainable agriculture.

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I hereby declare that I have used AI tools in the preparation of our manuscript. The use of such tools was limited to assistance (such as language improvement, structuring, or editing), and the core research work, data interpretation, and conclusions are

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entirely my/our original work. I take full responsibility for the accuracy, integrity, and authenticity of the submitted manuscript.

Disclosure Statement

No potential conflict of interest was reported by the author.

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