

ORIGINAL ARTICLE



Bacterial enumeration, diversity and characterization of Goainghat and Balaganj soils of Bangladesh

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ABSTRACT

Bacterial communities in soil are important in decomposition of organic matter, nutrient cycling and maintaining soil fertility. In the present study isolation, enumeration and morphological characterization of bacteria population in agricultural soils of Balaganj and Goainghat series, Sylhet division of Bangladesh was highlighted. Total viable counts obtained were 6.4×10^6 CFU/g soil in Balaganj and 6.0×10^6 CFU/g soil in Goainghat respectively. The number of effective bacterial diversity present in Balaganj was 7 colonies and 5 colonies were isolated from Goainghat. Colonies were found to be variable in size, shape, margin, elevation and color (circular, irregular, rhizoid; white, pink and yellow). In case of both Gram-positive and Gram-negative bacterial isolates, rod or cocci in shape arrange singly or chains were identified. Morphological Forms and their types: Spore formation, capsule formation, acid fast and Non-acid Fast Type. Both soils support a variety of bacterial communities which link to their fertility and nutrient cycling and are markers of their ecological significance in sustainable soil management.

KEY WORDS

Soil bacteria; Diversity; Gram stain; Spore; Capsule; Acid fast bacteria

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Introduction

Bacteria are unicellular, prokaryotic organisms that are thought to be one of the oldest and most constantly evolving life forms on earth. They have been around long enough to have evolved in nature at least 2 billion years – and are sufficiently versatile to thrive, grow and spread in remarkably diverse settings, from the common to the harsh. The cell structure of bacteria is relatively simple compared to eukaryotic cells and the diameter ranges from 0.5 – 2.0 μm [1,2]. Given this simplicity, they show a surprising diversity of shape that is characterized into 3 categories: cocci (spherical), bacilli (rod-like) and spiral shapes. Bacteria can be isolated as single cells or in groups called colonies, and the colony structures are largely determined by the environment, nutrients and interactions between the cells. Bacteria play key ecological roles, and are important in both terrestrial as well as aquatic ecosystems. They play an important role in ecosystem productivity by decomposing organic materials as well as recycling key nutrients, including carbon, nitrogen and phosphorus [3–7]. Bacteria are the most widely and amazingly distributed and versatile of all microorganisms, capable of living in different niches in the world. But the morphological variability in this group is allometrically constrained and poses problems for species recognition and classification. So, morphologically alone is not sufficient and trustworthy means of classification needs the combination of culture-based methods and analytic approaches. The combined view facilitates the understanding of the diversity, ecology, biology and environmental relations of bacteria [8,9]. Bacteria are ubiquitous, present in soils, freshwater, oceans ecosystems, as well as being present within the atmosphere and many extreme habitats (high salinity or acidity or both, high temperature etc.). Their metabolic activities have an important role in ecological balance and in biogeochemical cycles governor [3,4,6]. In well-drained soils, however, bacteria often work hand-in-hand with fungi to boost microbial activity and turnover.

Under anaerobic or oxygen limiting conditions, alternative bacteria predominate and control most of the biochemical steps which occur within soil. Isolation, purification and identification of bacteria from environmental samples is the initial step in their investigation [10,11]. Different bacterial populations are isolated using different methods, and then purified one or many times to remove contaminants and get pure cultures. All of these pure cultures are necessary for conducting in-depth studies on the morphology, physiology, biochemical characteristics and reaction to antimicrobial agents of bacteria. Large quantity of selective and non-selective media is used in these processes generally. Isolated bacterial colonies can be obtained by traditional microbiological methods such as streak plate and pour plate methods on solid media [2,12,13]. This is the continuation of previous study which revealed that the soils from various part of Bangladesh have toxic heavy metals and hence it is beneficial for the effective selection and development of a few metal tolerant or resistant bacterial community. These bacteria are ecologically important microorganisms in polluted soil, and have a remarkable environmental tolerance [14]. The soil bacteria of Bangladesh have also exhibited some beneficial activities like phosphate solubilization, pollution detoxification and soil cycle regulation [14,15]. They are also involved in important biochemical reactions like nitrification, which is essential for soil fertility and availability of nutrients [3–5,16]. They are also beneficial in improving soil remediation, decreasing pathogenic organisms and avoiding acid soil stabilization. Soil microbiological research, therefore, is of scientific interest and of relevance to agriculture and environmental management. To elucidate soil microbial community complexity, dynamics and functional diversity a systematic stepwise approach to isolate soil bacterial populations is essential [4,10,17].

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In the present study the soil bacteria are isolated and its CFUs (Colony forming units) is calculated and colony characteristics are detailed. In addition, key morphology features such as cell proportion, distribution and ability to stain were investigated to gain a deeper understanding of the microbial abundance, diversity and ecological significance in soil samples analyzed.

Materials and Methods

Sample collection

Two set of topsoil samples were aseptically collected from agricultural lands of Bangladesh (Table 1). Sampling was carried out only in the top (0–15 cm) soil layer that has a high nutrient

content and is known to be rich in microbes. To reduce the chances of contamination from other subsystems or introduction of foreign microbiomes to the soil, all aseptic and sterile techniques were used during sampling to preserve representative native soil microbiomes [11]. The samples were immediately placed in sterile thermos containers and brought to the laboratory for keeping the same temperature as at the site of collection and to prevent possible changes in the microbial composition during transport. Samples on arrival were kept at a controlled temperature in the laboratory till the necessary microbiological and physicochemical studies. This systematic and controlled procedure for collecting, transporting and storing samples is very useful for determining the precision of experimental results obtained from these soil samples.

Table 1. Related information on the collected soil samples.

No.	Soil Series	Location	GPS Reading	Taxonomy	AEZ	Physiographic unit	Cropping pattern
1	Goainghat	RARS Moulvibazar	N 24°24/ E 91°37/	Haplaquepts	22	Northern and Eastern Piedmont Plain	Aus-T. Amam
2	Balaganj	Akborpur Moulvibazar	N 24°24/ E 91°37/	Haplaquepts	29	Northern and Eastern Piedmont Plain	Citrus Fruits

Isolation of Bacteria

Isolation of microbes were done according to the standard microbiology procedures as mentioned in earlier literature [2,12,13]. First, a certain quantity of soil was mixed with the physiological saline solution (distilled water and 0.9% NaCl) to obtain a uniform soil microbial suspension. This suspension was used as the basis for further analyses. Serial dilution (in a stepwise fashion) of the prepared soil suspension for diminishing microbial load allowed for individual bacterial colony isolation [18]. Dilution aliquots were aseptically transferred onto sterile, pre-labeled Petri plates, and spread evenly on the surface of the petri plates with nutrient agar using the spread plate method. Inoculated plates were placed in an incubator at 37°C for 24–48 hours to promote optimum growth of bacteria which will form isolated colonies. Well separated and morphologically distinct colonies were carefully picked after incubation and repeatedly streaked on fresh agar media for purified (axenic) cultures. For this purification step, it was repeated several times to remove the mixed populations and to get a uniform culture, reliable for the purpose. To ensure that our results were as accurate and reproducible as possible, each experiment was repeated three times. Lastly, purified isolates were re-incubated at 37°C for another 24–48 hours to check purity and characteristically grown.

Viable count

The numbers of viable bacteria in soil sample were determined by the standard plate count technique [14]. Only the agar plates with 25–250 isolated colonies were chosen for counts that were likely to be accurate and valid. Next, the viable bacteria per gram of soil were calculated using a standard formula including both the dilution factor as well as volume of inoculum plated to agar surface.

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

Characterization

Bacterial colonies from the two sampling sites were morphologically described from well-isolated colonies on nutrient agar plate. Colony size, color (pigmentation), shape, margin and elevation were observed. The analysis was done following the standard descriptive criteria by Dubey and

Maheshwari in a consistent manner [14,15]. Such detailed morphology description provides information about the phenotypic heterogeneity of soil bacterial communities and serves as a main basis for their identification, classification and comparative studies [7,19].

Staining characteristics

A systematic investigation of the morphology and arrangement of the cells has been undertaken with different standard stains, such as simple stain, negative stain, gram stain, capsula stain, spore staining and acid-fast staining in the bacterial isolates [17]. Each staining method provides unique information, some of which overlaps while others complement each other, allowing us to finely observe the cell shape, structural features associated with topographical molecular distribution and spatial arrangement. These analyses are essential for focus identification of phenotypes, reliable taxonomical classification and information about the structure of the soil bacterial community.

Simple staining

To visualize the morphology of the bacteria, thin smears on clean, sterile glass slides were heat fixed (i.e. cells were immobilized and their structure maintained by heating it). Therefore, the fixed smears were stained for crystal violet for approximately 40–60 seconds (to ensure that the crystal violet is taken up by the bacterial cells). The slides were washed with running water to get rid of excess stain and allowed to dry. The smears were thereafter appropriately screened by high powered microscope in oil immersion to enable good evaluation of cellular morphology, arrangement and other structural features. These are the basic set of parameters necessary for accurate phenotypic description and initial identification of bacterial isolates all of which are important initial points for microbiological analysis.

Negative staining

A clean dry glass slide was marked with a small drop of negative stain nigrosin stain, 0.5cm from one end and negative staining was done. A loopful of the bacterial culture was mixed with the stain and with a second slide held at an angle of about 30° the mixture was spread over the slide to give thin, even

smears. The smear was prepared and air dried without heat. Then the slide was viewed at 100X oil immersion magnification which provided a better view of the bacterial morphology (cell shape, size and arrangement). This method does not use heat fixation to prevent any alteration of the structure and to preserve the natural size and morphology of the cells [20]. Therefore, negative staining is useful for accurate and reliable information for phenotypic characterization which assist in positive identification and classification of the bacterial isolates.

Gram stain

Bacterial smears were made on sterile glass slides, and the smears were heat fixed to maintain the structure. Fixed smears were stained with crystal violet for about 1 min, fixed with sufficient penetration of primary stain and rinsed gently with tap water to remove the excess stain. Then, Gram's iodine was used as a mordant for 1 minute and slides were washed of excess iodide which formed an insoluble crystal violet-iodine complex inside the cell. Each slide was then decolorized by adding 95% ethanol dropwise until the flow was clear, and washed immediately with water to prevent the decoloration. Safranin was then used to counterstain the smears for 45 seconds and the smears were washed and air dried. The slides were observed using an oil immersion microscope, allowing easy recognition of Gram-positive and Gram-negative bacteria, while the detailed morphology, size, and arrangement of the cells were observed. This method gives reliable phenotypic information which is crucial to correct taxonomic identification of bacterial isolates [14,21].

Capsule stain

To observe the bacterial capsules, smears were made on sterile glass slides and allowed to dry in the air without being heated as this ensures that the structure of the cells and the capsules surrounding them is not altered. The dried smears were then stained with crystal violet for 5–7 minutes to ensure adequate binding of the bacterial cells. For this 20% copper sulfate was used to eliminate the surplus stain, and served as a decolorant and background counterstain. The air-dried slides were removed from an air slide warmer and were then scanned at 780× using oil immersion microscopy; bacterial cells themselves were easily discernible from capsules which appeared as clear, unstained halos around the stained cells [22]. This is one of the key structural and phenotypic features which aids in the accurate determination of encapsulated bacterial strains and one technique used is forceps.

Spore stain

A smear was made on sterile glass slides and heat-fixed to incompatibly immobilize the cells and to provide structural integrity for badass observation of bacterial endospores. The fixed smears were stained with malachite green and the plates with slides were heated for 2–3 minutes on a warm hotplate for penetration of the dye into the highly resistant spore structures. Excess stain was removed from the slides after cooling by washing with tap water. Smears were then stained with safranin for 30 seconds, washed gently with distilled water to give contrast between the vegetative cells and the endospores. After air drying, slides were examined by oil immersion microscopy, the endospore being green stained inside the red stained vegetative cell. It is a necessary technique to characterize and identify the morphology and help to show the structural apparatuses and survival strategy of the spore-forming bacteria [23,24].

Acid fast stain

To immobilize the cell and preserve their normal morphology, smears were prepared on sterile glass slide and heat-fixed for the detection and description of acid-fast bacteria. The smears were fixed and stained with carbolfuchsin and left on a warm hot plate for 5 min to allow carbolfuchsin to permeate the thick lipid-rich cell wall present in organisms that are acid-fast. The slides were then cooled down, rinsed with tap water to remove excess stain. Then, decolorization was carried by drop wise addition of acid-alcohol until no dye could be removed and a rinse with water to remove the remaining decolorizer. The smears were then counter stained with methylene blue for around 2 min washed with distilled water and were air dried. It made a clear differentiation between acid fast and non-acid-fast cells. The latter were definitely visible under oil immersion microscopy as they appeared red after staining with carbolfuchsin while acid-fast bacteria kept the stain and appeared red, while non-acid-fast cells were stained mahine blue with methylene blue. Although this type of staining can provide more information on the structural properties and diagnostic features of acid fast bacteria, it is important for the proper morphological evaluation.

Statistical analysis

The bacterial isolation, determination of viable counts and characterization were done in triplicate to strengthen the accuracy, reliability and reproducibility of results. CFU level was determined and results were expressed in mean. The data for the bacterial population collected from two soil samples were summarized using univariate descriptive statistical analysis. Standard equations were used to calculate viable cell counts and presented per gram of soil. Comparison between the soils based on differences in CFU means and colony morphology and characteristics was done.

Results and Discussion

However, it was possible to isolate, purify and characterize the total bacterial population in Balagonj and Goainghat soil, in which distinct difference in distinct colonies was observed. The diversity analysis showed both soil samples to be colonized by a multitude of bacteria. Data revealed that the soil of Goainghat and Balagonj contained $6.0 \times (10)^6$ CFU/g soil and $6.4 \times (10)^6$ CFU/g soil respectively.

A total of five different bacterial colonies were isolated from the soil of Goainghat while seven colonies were obtained from soil of Balagonj, this shows a little difference in abundance of culturable bacteria in both the soil samples. However, the results showed that the colony sizes of different bacterial populations growing in Goainghat soil varied from small to large (i.e., growth dynamics may differ) and only a few were pinpoint but majority were large in Balagonj soil, suggesting that some bacterium population could grow better under respective environmental conditions than those living in Balagonj condition [3,6,7,15,25].

Table 2 indicated that the colonies of Goainghat soil were variable in shape with irregular and circular shape. Colony borders were noted as lobate, entire and undulate but an elevation was flat to raised. Isolates exhibited white, pink and yellow pigmentation signifying metabolic differences and phenotypic diversity. Moreover, in Balagonj soil (Table 3) colonies were found in different shapes such as, rhizoid, irregular and circular. Margins were observed to be whole, lacerate and lobate, elevations were from flat to raised and

umbonate [6,7,10]. The appearance of these colonies also showed some degree of pigmentation, such as white colonies,

pink and yellow, which is also a sign of the variation within the bacterial colonies.

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

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

Table 3. Colony characteristics of isolated bacteria of Balagonj soil.

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

Goainghat were morphologically characterised from soil isolates (Table 4) which showed diverse morphology and form arrangement such as rod-shaped, spherical and single or chain (cocci). Both of these isolates were gram-positive and gram-negative. Some of the colonies were found to produce spores and capsules, both of which are important survival strategies under harsh environmental conditions. Acid fast staining showed acid fast and non-acid fast bacteria. Likewise, the soil

bacteria of Balagonj region (Table 5) were mainly arranged in a single cell line and chains. These include Gram-positive bacteria, as well as Gram negative bacteria. There was significant production of spores and some even more recklessly formed capsules, enhancing the resistance (and swarming) of various conditions. The finding of acid-fast and non-acid fast bacteria was confirmed by acid fast staining.

Table 4. Morphological characteristics of isolated bacteria of Goainghat soil.

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

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

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

To conclude, the results reveal that the bacterial community of Goainghat and Balagonj soils is different and its distribution is heterogeneous and the bacteria are morphologically and physiologically diverse. Colony size, shape, pigmentation, margin elevation, cellular arrangement and staining status are the characteristics that reveal the high complexity of soil population and their local adaptation. In addition, the presence of spore-forming and encapsulated bacteria indicates their effective survival strategies in response to environmental stress, whilst both Gram-positive and Gram-negative as well as acid-fast and non-acid-fast bacteria indicates that they are much broader ecological versatility. This diversity is important to key processes such as soil health, nutrient cycling, and the continued functional capacity of ecosystems.

The present study results were compared to the results of Chowdhury et al who studied the bacteria of the soils of Bangladesh. The observations were in line with the findings of our work, namely, the presence of *Bacillus* species in soils throughout the country of various textures. More than *Bacillus*, the sequence surveillance picked out various Gram-negative, spore-forming bacteria: *Enterobacter* spp., *Klebsiella* spp. and *Azospirillum* spp. *Bacillus* species are ubiquitous in Bangladeshi soils as found in various studies and almost all of them are able to produce endospores resistant to extreme conditions. Spores allow *Bacillus* to thrive as a small number of vegetative cells, and complete their life cycles when the environmental conditions are if favorable. Sporulation is a very good strategy for survival – may increase persistence and versatility since sometimes soil environments may limit possibilities for sporulating bacteria to go through the full cycle of the reproductive system. Also, the decaying of decomposing organic matter aids in the emergence of new generations of bacteria, serving as nutrients and spore dispersal throughout the soil. This helps bacterial populations to persist in their niches for a long time [3-7].

Limitation

Mainly culture-dependent methods were applied, which in general can obtain only a small fraction of the whole microbial richness veiled in soil, since most of the bacteria stay uncultured under laboratory conditions. As a result, the real diversity of soil microorganisms might have been underestimated. Furthermore, the vocation of bacterial identification was founded primarily on morphological characteristics and staining methods. Though very helpful for first pass groupings, these methods do not allow species-level identification. Lack of molecular tools such as 16S rRNA gene sequencing has limited the taxonomic resolution and even accurate classification of bacteria.

Conclusion

Since the present study successfully isolated, purified and characterised diverse bacterial communities from Balagonj and Goainghat soils, it confirmed that these soils are populated by varied types of microorganisms. Circular, irregular, and rhizoid colonies with white, pink, and yellow pigmentation were observed in each soil. Microscopically, Gram-positive and Gram-negative microorganisms were observed, along with the presence of both rod-shaped and cocci bacteria, either singly or in chains. Observations included spore-forming, capsule-forming, acid-fast and non-acid-fast bacteria characteristic of a remarkable level of physiological and structural diversity. In general, the results indicate that both soils harbor a heterogeneous bacterial community performing essential steps in nutrient cycling and organic matter decomposition to

sustain soil fertility. The ecological role of soil bacteria in the sustainable nature of the soil is reflected by this diversity.

Disclosure Statement

No potential conflict of interest was reported by the author.

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