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Isolation and Identification of Culturable Cyanobacteria from Paddy Soils of the Intermediate Zone of Sri Lanka

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ABSTRACT

Cyanobacteria are an extremely diverse group of prokaryotes that inhabit various ecosystems. They are one of the major microbial groups found in the paddy soil microbial community and provide a valuable contribution to the soil fertility by fixing atmospheric nitrogen. Thus cyanobacteria are considered as potential bio fertilizers for paddy cultivation. In this study, nine cyanobacterial taxa from paddy soils (0-10 cm) of the Intermediate Zone of Sri Lanka were isolated and identified using culture based (BG 11 medium) morphological analysis. Both unicellular (*Aphanothece, Chroococcus, Johannesbaptistia, Microcystis*) and filamentous (*Anabaena, Pseudanabaena, Nostoc, Stigonema, Trichodesmium*) genera of cyanobacteria were identified. DNA extraction was done by modified Boom's method, with pre-treatment. Cyanobacterial cultures were maintained at 24 °C temperature and 2,000 lux light at 60 rpm. Abundance of two cyanobacterial genera (*Chroococcus* and *Microcystis*) were confirmed by molecular analysis using Polymarase Chain Reaction (PCR) with specific primers CYA359F and CYA781R(b) for unicellular cyanobacteria.

KEYWORDS: BG medium, Bio fertilizer, Cyanobacteria, Molecular analysis, Paddy soil

INTRODUCTION

Cyanobacteria commonly known as blue green algae or cyanophyta are a morphologically diverse group ranging from unicellular to colonial and filamentous forms. They are gram negative photoautotrophs having typical prokaryotic cell structure which lack membrane bound cell organelles but include simple granular bodies such as carboxysomes and pigments e.g. photosynthetic pigments such as chlorophyll a, chlorophyll b, phycobilin and phycocyanin (Wehr and Sheath, 2002). The latter pigment is responsible for the blue green color (cyano) of these organisms and the name "cyanobacteria". Some species have gas vesicles or aerotopes within cells or colonies. Some cyanobacteria have unique features like heterocysts and akinetes, which are specialized for nitrogen fixation and asexual reproduction respectively (Wehr and Sheath, 2002).

Cyanobacteria have been identified as one of the main components of the microbial community in rice soils (Prasanna *et al.*, 2009). Cyanobacterial colonies in paddy soils form bluish to greenish soil crusts. Abundance of cyanobacteria in paddy fields is due to the favorable environmental conditions including low temperature and high level of nutrients and water. In return, cyanobacteria provide essential growth factors for rice plants by nitrogen (N) fixation. Cyanobacteria play a major role in soil surface stabilization and increasing the water holding capacity. Soil pore structure is improved by the filamentous growth and adhesive substances secreted by them. In addition, cyanobacteria are known to excrete growth-promoting substances such as hormones, vitamins, amino acids and organic acids which are beneficial for plant growth by preventing weed growth, increasing soil phosphate level and decreasing soil salinity (Saadatnia and Riahi, 2009).

Nitrogen fixing cyanobacteria are a suitable candidate to be used as a bio fertilizer in rice fields (Roger and Ladha, 1992). However, field establishment of inoculants is less due to poor adaptation to varying environmental and soil conditions. Therefore, isolation of cyanobacterial strains which are already adapted to local conditions is very important as the first step towards producing a cyanobacterial bio fertilizer.

Current study aimed for the isolation and identification of culturable cyanobacteria in paddy soils of the intermediate zone of Sri Lanka using culture based techniques and molecular techniques.

MATERIALS AND METHODS

The study was carried out at the Department of Biotechnology, Faculty of Agriculture and Plantation Management (FAPM), Wayamba University of Sri Lanka from December 2015 to May 2016.

Study Site and Sample Collection

Soil samples were collected from rice fields at the Rice Research and Development Institute (RRDI), Bathalagoda, (7°31'27.02"N, 80°26'57.08" E; 116 m above mean sea level) located in the Intermediate Zone of Sri Lanka. The study site receives a mean annual

precipitation of 1100-1600 mm and the soil is classified as Low Humic Gley soils (Department of Agriculture, 2006) with clayey texture. Soil was characterized with neutral soil pH 6.17, EC 0.035 ds/m, total organic matter 0.42%, total-N 0.04%, exchangeable potassium (K) 11.17 mg/kg and available-phosphorous (P) 2.7 mg/kg (RRDI, unpublished data). Selected rice fields were cultivated with BG 358 variety and irrigation and fertilization were practiced according to the recommendation by the Department of Agriculture.

Soil samples were collected at 0-10 cm depth (n=3), one week after harvesting the crop, and immediately transported to the laboratory on ice and stored at 4 °C. Moisture content and pH of the soil were measured.

Sample Preparation and Initiation of Cyanobacterial Cultures

Sub samples of soil (10 g) were mixed well with 90 mL of water and the suspension was allowed to settle for half an hour (Lukesova, 1993). A serial dilution was carried out up to 10^{-3} and 0.5 mL aliquot of soil suspension was used to initiate cyanobacterial cultures on solidified growth media using spread plate method. Blue Green medium with nitrogen (BG 11) or without nitrogen (BG 11_0) was used (University of California Davis, 2015). The pH was adjusted to 7.5 and 1% of bacteriological agar was added for solid media. Plates were incubated at 24 °C and illuminated with fluorescent lights (2,000 lux) continuously.

Enumeration, Morphological Identification and Isolation of Culturable Cyanobacteria

After one week from culturing, number of colony forming units (CFU) in each replicate plate were counted using a colony counter. Purification and isolation of cyanobacterial colonies were done by transferring to new plates by streaking. Liquid cultures were prepared using isolated colonies and provided with same conditions and kept on a shaker at 60 rpm. Observations on morphological characteristics such as presence or absence of sheath, shape and size of vegetative cells, heterocysts, akinetes and branching pattern were made using a light microscope fitted with a digital camera (iVu 3100, Labomed, USA) and images were captured at 40X and 100X magnification. The culturable cyanobacteria were identified by following Komárek and Komarkova, 2002; Wehr and Sheath, 2002; Desikachary, 1959 and Green Water Laboratories, 2015.

Molecular Identification of Culturable Cyanobacteria

DNA Extraction

DNA of cyanobacterial cultures were extracted using the method described by Magana-arachchi and Wanigatunge, (2011) with modifications. Two unicellular cyanobacterial isolates were selected for molecular analysis. In addition, *Nostoc* standard culture obtained from the National Institute of Fundamental Studies (NIFS) was used as a representative of filamentous organisms. Pre-treatment by sequential heating (at 100 °C for 5 min) and freezing (at 0 °C for 5 min) was carried out to achieve lysis. Final pellet was re-dissolved in 50 µL of deionized water and stored at -20 °C.

PCR with Specific Primers

The DNA extracted from two selected cyanobacterial isolated cultures and standard *Nostoc* culture were subjected to PCR using cyanobacterial specific forward primer CYA359F (5' GGG GAA TTT TCC GCA ATG GG 3') and reverse primer CYA781R(a) (5' GAC TAC TGG GGT ATC TAA TCC CAT T 3') for filamentous cyanobacteria and CYA781R(b) (5' GAC TAC AGG GGT ATC TAA TCC CTT T 3') for unicellular cyanobacteria (Nübel *et al.*, 1997). Each reaction tube contained 0.5 µM forward primer and reverse primer a or b, 200 µM dNTPs, 1X PCR buffer, 1 mM of $MgCl_2$, 1 unit of Taq DNA polymerase (Promega), and template DNA (10 ng-30 ng) with a final volume of 20 µL.

The amplification cycle consisted of an initial denaturation of 10 min at 94 °C followed by 30 cycles of denaturation for 45 sec at 94 °C, annealing of 45 seconds at 52 °C, elongation for 1 min at 72 °C and final extension at 72 °C for 10 min. The resulting PCR products were electrophoresed in 1% agarose gel and visualized using UV trans illuminator (Spectroline, USA).

RESULTS AND DISCUSSION

The average pH value of the soil was 5.18 and average moisture content of the soil was 36.07%. Average number of colonies in BG 11_0 and BG 11 medium were 3.64×10^6 and 2.76×10^6 CFUs per g of dry soil respectively.

Morphological Identification

Nine taxa of cyanobacteria were identified (from colonies grown in both BG 11 and BG 11_0 media) based on morphological characteristics according to the taxonomic identification reported by Wehr and Sheath (2002), Komarek

and Komarkova (2002) and Green Water Laboratories (2015).

Unicellular Morphotypes

Aphanothece: Colonies are multicellular, spherical and cells arranged irregularly and densely. Cells enveloped within individual sheaths. Cells are widely oval to cylindrical pale greyish blue–green, to bright blue–green in color (Wehr and Sheath, 2002; Figure 1A).

Microcystis: Colonies of cells are arranged densely and irregularly and covered with colorless mucilage. Cells are spherical, blue-green in color. Cells are 0.8-0.9 µm in diameter and have no individual mucilaginous sheath (Wehr and Sheath, 2002; Figure 1B).

Chroococcus: Cells or groups of cells (mainly two to four cells), are irregularly arranged in colorless or yellowish mucilaginous envelopes. Cells are spherical or oval and 0.7-50 µm in diameter and the color vary from grey, blue-green, olive green, orange, or reddish violet (Wehr and Sheath, 2002; Figure 1C).

Johannesbaptistia: Cells are arranged in uni-seriate pseudo-filaments within wide, mucilaginous, tube-like strands, rounded at the ends. Cells are short discoid and pale grey-blue in color (Wehr and Sheath, 2002; Figure 1D).

Filamentous Morphotypes

Stigonema: The thallus is composed of true branched filaments. Sheaths are thin or thick, cells are barrel shaped, blue-green or olive green Heterocystes are solitary, and similar in form to vegetative cells. Akinetes are unknown (Wehr and Sheath, 2002; Figure 2A).

Nostoc: Filaments are typically coiled, forming irregular clusters, mucilaginous sheath yellow to brownish in color. Cells are barrel shaped or spherical, pale to bright blue–green or olive green in color. Heterocystes are barrel shaped or spherical. Akinetes are ellipsoidal, only slightly larger than vegetative cells (Wehr and Sheath, 2002; Figure 2B).

Anabaena: Arranged in free clusters or as solitary, filaments. Trichomes are straight, curved, or regularly coiled. Cells are spherical, ellipsoidal, or cylindrical and pale to bright blue–green or yellow–green in color. Heterocystes present and they occur in regular intervals along filament. Akinetes are spherical, ellipsoidal, and cylindrical and occur in groups of 2-5 (Wehr and Sheath, 2002; Figure 2C).

Trichodesmium: Colonies usually in parallel or radial arrangements that form fascicles or flocculent masses, joined by diffuse mucilage. Trichomes occur without individual sheaths; slightly motile (inconspicuous gliding), 6-22 µm wide, cylindrical or with slightly tapering ends (Wehr and Sheath, 2002; Figure 2D).

Pseudanabaena: Trichomes are solitary, straight or curved; they are usually short, consisting of a very few to several cells, usually with conspicuous constrictions at the cross walls, 1-3.5 µm wide. Trichomes lack firm sheath. Cells are usually cylindrical with rounded ends (Wehr and Sheath, 2002; Figure 2E).

DNA Extraction from Cyanobacterial Isolates

Extracted DNA from two isolated unicellular cultures: 1 and 2 (morphologically identified as *Chroococcus* and *Microcystis* respectively) and standard *Nostoc* culture were in good quality and quantity for PCR reaction (Figure 3). This proves that the modified protocol was successful for DNA extraction from both unicellular and filamentous cyanobacteria.

Molecular Identification of Cyanobacteria using Specific Primers

The PCR amplification of DNA extracted from cyanobacterial isolated culture 1 and 2 (*Chroococcus* and *Microcystis*) with cyanobacterial specific forward primer CYA 359F and reverse primer CYA781R(b) has resulted expected bands at 400 bp. (Figure 4, Lane 9, 10). Therefore, PCR protocol for unicellular types was successful. However, no band was observed for PCR of DNA from these samples using CYA781R(a) filamentous specific primer (Figure 4, Lane 4, 5). This proves that isolated culture 1 and 2 (*Chroococcus and Microcystis*) are belong to unicellular cyanobacterial group as observed *via* morphological characteristics.

Polymerase Chain Reaction results showed a band at 400 bp for *Nostoc* standard culture as well (Figure 4, Lane 3). Therefore, the developed PCR protocol can be successfully used to amplify DNA extracted from filamentous cyanobacterial types. However, isolated filamentous cultures could not be tested due to the slow growth of these cultures.

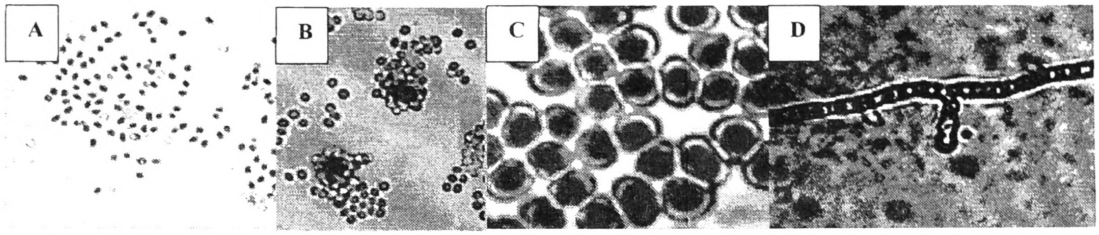

Figure 1. Unicellular cyanobacteria. *A: Aphanothece, B: Microcystis, C: Chroococcus D: Johannesbaptistia isolated and identified from the paddy soils of the Intermediate Zone*

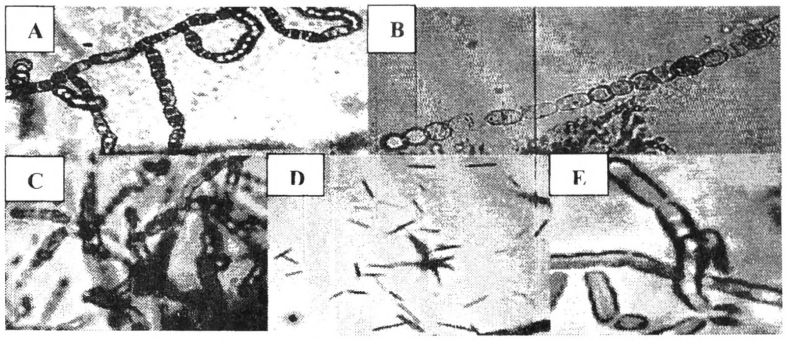

Figure 2. Filamentous Cyanobacteria. *A: Stigonema, B: Nostoc, C: Anabaena, D: Trichodesmium and E: Pseudanabaena isolated and identified from the paddy soils of the Intermediate Zone*

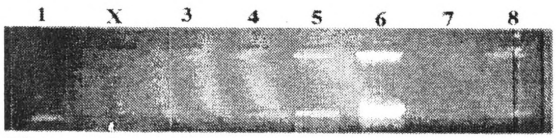

Figure 3. Agarose gel electrophoresis of cyanobacterial genomic DNA (15 µL). *Lane 1: λ DNA (50 ng), Lane 3 and 4: Standard Nostoc culture, Lane 5 and 6: Isolated culture 1, Lane 7 and 8: Isolated culture 2, X: Empty lane*

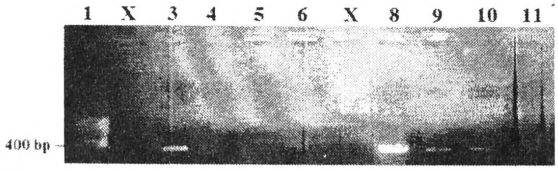

Figure 4. Agarose gel electrophoresis of PCR products from cyanobacterial DNA using specific primers . *Lane 1: 100 bp ladder, Lane 3: Standard Nostoc culture, Lane 4: Isolated culture 1, Lane 5: Isolated culture 2, Lane 6: Negative control, Lane 8: Standard Chroococcus culture, Lane 9: Isolated culture 1, Lane 10: Isolated culture 2 Lane 11: Negative control, Lane 3-6: PCR amplification with CYA781R(a), Lane 8-11: PCR amplification with CYA781R(b). X: Empty lane*

CONCLUSIONS

Several cyanobacterial taxa isolated from paddy soils of the Intermediate Zone were identified based on morphological characters. Four unicellular genera (*Aphanothece, Chroococcus, Johannesbaptistia, Microcystis*) and five filamentous genera (*Anabaena, Pseudanabaena, Nostoc, Stigonema, Trichodesmium*) were identified. Modified DNA extraction protocol was successful for the extraction of DNA from both types of cyanobacteria. Polymerase Chain Reaction with specific primers (CYA359F, CYA781R(a) and CYA781R(b) confirmed that isolated *Chroococcus* and *Microcystis* belong to unicellular cyanobacterial groups. Sequencing of DNA for further identification of the isolates is prospected.

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