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Evidence-based Analysis of a Novel Symbiotic and Epiphytic Cyanobacteria Associated with *Azolla* by Cyto and Molecular Taxonomy

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Abstract: *Azolla-Anabaena* symbiosis is one of the vital roles in the field of agriculture and development. Both the associates have mutualistic relationship and work together for carbon and nitrogen fixation to soil. The present study is to investigate the new entity in the relationship between the *Azolla* and Cyanobacteria. Endophytic and Epiphytic cyanobacterial associates were isolated from different *Azolla* sp. viz., *Azolla pinnata*, *Azolla caroliana* and *Azolla filliculoides*. The isolates were further characterized by conventional methods and molecular techniques. A novel entity, *Westiellopsis* was found in the association with *Azolla filiculoides* comparatively with previously reported. Furthermore, *Anabaena variabilis* precluded from *Azolla filiculoides* was produce red color pigmentation. The C-Phycoerythrin content of the isolated cyanobacterial symbiont was measured by absorption and fluorescence emission spectroscopy. The nutritional optimization was done showed two fold increased in pigment production than the control.

Key words: Symbiosis, *Azolla-Anabaena*, *Westiellopsis* sp. phycobiliproteins, 16S rDNA

INTRODUCTION

Cyanobacteria is a large group of photosynthetic prokaryotes of enormous environmental importance, being responsible for a large proportion of global CO_2 and N_2 fixation with the symbiotic association with wide variety of plants, fungi, sponges and protists. It is proved that can used for antibacterial (MubarakAli *et al*., 2008), decolorization of dyes, MubarakAli *et al*. (2011) and Adams *et al*. (2006) described about the cyanobacterial symbionts are often filamentous and fix the nitrogen in specialized cells known as heterocysts, enabling them to provide the host with fixed nitrogen and in the case of non-photosynthetic hosts, with fixed carbon. Gorelova (2006) presented the interactions between cyanobacteria and other components of biocenoses cover the whole spectrum interspecies interaction, from antagonism to mutualism. The *Azolla-Anabaena* symbiosis is a mutualistic association among the filamentous, heterogeneous, nitrogen fixing cyanobacterium and other cyanobacteria. Cyanobacteria are unique in the wide range of symbiotic associations, they form with eukaryotic hosts including plants, fungi, sponges, protists and other epiphytes (Bergman *et al*., 1985, 2007; Rai *et al*., 2000, 2002; MubarakAli *et al*., 2012a).

The cyanobacterial symbionts of plants all possess at least two essential characteristics the ability to differentiate heterocysts which are specialized nitrogen-fixing cells and hormogonia, short, gliding filaments that lack heterocysts and provide a means of dispersal. The hormogonia serve as the infective agents in most plant symbioses; some plants enhance their chance to infect by producing chemical signals that stimulate hormogonia formation and also chemo attractants that direct hormogonia into the plant tissue. Cyanobacteria is not restricted to the roots of plants but can infect thalli, stems and leaves. The major hosts are bryophytes, the angiosperm, *Gunnera* the aquatic fern *Azolla*, fungi, *Geosiphon* sp. and cycads. There was evidenced that some of the bacteria isolated from *A. pinnata* and *A. filiculoides* have been shown to contain lectins (Meeks *et al*., 1985; Meeks, 1988). The *Azolla*-Cyanobacterial symbiosis is a mutualistic association among the filamentous, heterogeneous, nitrogen fixing cyanobacterium, *Anabaena* and other cyanobacterial species (Serrano *et al*., 1999).

Cyanobacteria can be identified evolutionarily by studying the cellular and morphological characteristics (Suresh *et al*., 2012) and 16S rDNA gene sequence (Pandiaraj *et al*., 2012).

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The presentation investigation is aimed to study the interaction of cyanobacteria with isolated Azolla sp. namely, *Azolla pinnata*, *A. caroliniana*, *A. filliculoides* and to study the any novel isolates present in the association and to study the biotechnological potentials.

MATERIALS AND METHODS

Sample collection: Azolla fern such as *Azolla pinnata*, *A. caroliniana*, *A. filliculoides* were collected from three different places in Tamilnadu. The collected samples were surface sterilized three times using mercuric chloride (0.1%) and with sterilized distilled water. Surface sterilized water with mercuric chloride used separately and plated in BG11 culture media for the identification of Epiphytic organisms. The samples were crushed using mortar and pestle, crushed samples were cultured in BG11 culture media which was maintained at 24±1°C in alternative illumination (90 μ E m^{-1s-1}) Cyanobacterial isolates were identified using the taxonomic publications of Desikachary (1958).

Cytotaxonomy: Cellular and morphological features were studied for analysis of their phylogenic relationships conventionally using light and dark field microscope, stereo microscope and confocal microscope. Cross sections were made by the use of sharp knives, blades; teases out preparations were also made to examine the symbiotic cyanobacteria.

Molecular taxonomy: The total DNA was extracted by using phenol chloroform extraction procedure prescribed by Thajuddin *et al*. (2010) Milli Q water; 2x Genet Bio premix (contains dNTPs Mix, DNA polymerase, self indicator dye, Buffer); Forward primer (CYA 106 CGGACGGGTCAGTAACGCGTGA) Reverse primer (CYA781 GACTACAGGGGTATCTAATCCCTTT). PCR amplification was checked on 1.2 % Agarose gel with the help of 1 kb Finnzymes Ladder and the size of the amplified PCR product.

Pigment production: Among isolated strains were screened for pigment production. A strain *Anabaena variabilis* (NTVMK01) chosen for the further study. Where the different concentration of $MgSO_4$ were studied for the enhanced production of phycoerythrin pigment (MubarakAli, 2012). Briefly, BG 11 medium was mixed separately with different concentrations of $MgSO_4$ (1, 2, 3, 4, 5 and 6%) 75 mL amount of each concentration were dispensed into 100 mL conical flask, control medium contains with usual $MgSO_4$ concentration in BG11 medium.

Extraction of pigments: *Anabaena Variabilis* culture was added with PBS (pH 7.2) centrifuged at 5000 rpm for 5 min, pellet was taken again centrifuged, supernatant were removed, pellet which obtained further added with 5 mL milli pore water kept in freezer for overnight centrifuged again at 5000 rpm for 5 min using optimized protocol (Ilavarasi *et al*., 2011, 2012). Optical Density (OD) was measured for different wave lengths, respectively 562, 620 and 652 nm and the concentration of phycobilin was calculated using following equation:

$$PC\ (mg\ mL^{-1}) = \frac{[A620\text{-}0.0474(A652)]}{5.3}$$

$$APC\ (mg\ mL^{-1}) = \frac{[A652\text{-}0.208(A620)]}{5.09}$$

$$PE\ (mg\ mL^{-1}) = \frac{[A562\text{-}2.41(PC)\text{-}0.849(APC)]}{9.62}$$

RESULTS AND DISCUSSION

Evolutionary relationship between the symbiotic organisms clearly understood by the phenomenon symbiosis. This implies the central role of interactions in which individuality emerges through incorporation. *Azolla* collected from different places were showed morphological difference between each ferns. *Azolla filiculoides* was brownish green in color whereas *A. caroliniana* and *A. pinnata* were green in color but *Azolla pinnata* possess small leaves than *Azolla caroliniana*. It is indicates that the cellular interaction of *Azolla* and Cyanobacteria. A cross sectional image of *Azolla* contains the filaments of cyanobacteria (Fig. 1). Biodiversity of *Azolla* associated cyanobacterial species were documented and tabulated (Table 1). In which the cellular and morphological characteristics also clearly elaborated.

Totally 16 strains were isolated from the Azolla association, in which 6 of endosymbiotic *Anabaena* sp. (*Anabaena* sp. (NTVMK06), *Anabaena* sp. (NTVMKO1), *Anabaena* sp. (NTVMKO5), *Anabaena* sp. (NTVMK02), *Anabaena* sp. (NTVMK19), *Anabaena* sp. (NTVMK07) (Fig. 2) 6 from endosymbiotic and epiphytics region (*Anabaena* sp. (NTVMK08), *Anabaena* sp. (NTVMK09), *Westiellopsis* sp. (NTVMK03), *Lyngbya* sp. (NTVMK15), *Phormidium* sp. (NTVMK13), *Phormidium* sp. (NTVMK12) (Fig. 3) and 4 from epiphytic potion of *Azolla* (*Phormidium* sp. (NTVMK14), *Aphanocapsa* sp.

Table 1: Symbiotic and epiphytic cyanobacteria associated with *Azolla*

Organism	Dimension vegetative akinetes (BL)	TH	Shape of filaments	Hormogonia	AKLC	Mucilaginous envelope (sheath)	Gas vacuoles
Anabaena sp. (NTVMK06)	2-3 4-6 4-5 5-6	Oval	Irreg.coiled	A	P	ND	A
Anabaena sp. (NTVMKO1)	1-2 2-3 2-5 3-4	Conic	Long wavy	A	A	ND	A
Anabaena sp. (NTVMKO5)	2-3 3-5 3-6 4-6	Oval	Long wavy	A	P	ND	A
Anabaena sp. (NTVMK02)	1-2 1-2 2-5 2-5	Oval	Short wavy	A	P	Diffusive	A
Anabaena sp. (NTVMK19)	1-2 1-2 1-2 1-1.5	Oval	Long wavy	A	P	ND	A
Anabaena sp. (NTVMK07)	3-4 3-4 4-6 4-6	A	Long wavy	P	P	ND	A
Anabaena sp. (NTVMK08)	2-3 3-4 3-5 4-6	Oval	Long wavy	A	P	ND	A
Anabaena sp. (NTVMK09)	0.5-1 1-2 1-2 1-2	Oval	Short wavy	A	A	ND	A
Westiellopsis sp. (NTVMK03)	1-1.5 1-3 1-3 1-3	A	Long wavy	P	P	Enveloped	A
Lyngbya sp. (NTVMK15)	4-5 27-30	A	A	A	A	A	P
Phormidium sp. (NTVMK13)	2-3	A	A	A	A	P	A
Phormidium sp. (NTVMK12)	1-2	A	A	A	A	P	A
Phormidium sp. (NTVMK14)	1-2	A	A	A	A	P	A
Aphanocapsa sp. (*NTVMK16)*	2-3	A	A	A	A	Enveloped	A
Aphanocapsa sp. (NTVMK17)	4-7	A	A	A	A	Enveloped	A
Chroococcus sp. (NTVMK18)	25-50	A	A	A	A	Enveloped	A

S1: Sample 1, S2: Sample 2, S3: Sample 3, A: Absent, P: Present. ND: Not determined, Irreg.coiled: Irregularly coiled, B: Breath, L: length, AKLC: Akinetes like cells

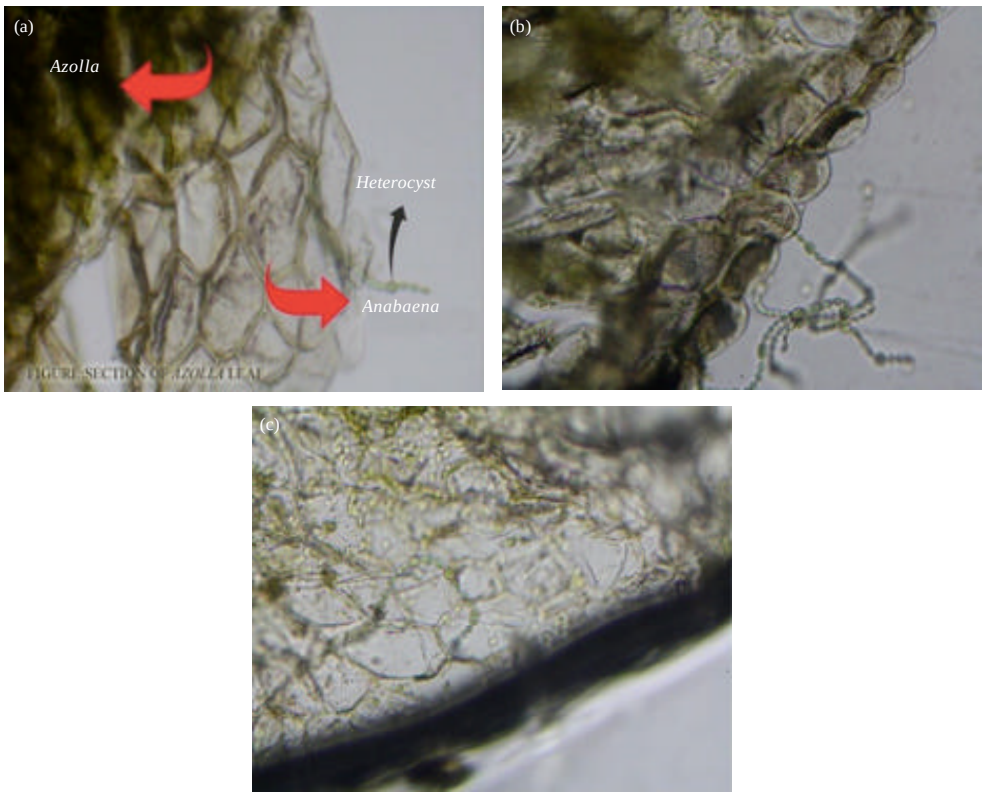

Fig. 1(a-c): *Azolla* leafs sections (a) S1, (b) S2, (c) S3, showing the presence of symbiotic cyanobacteria in the leaf cavity

(NTVMK16), *Aphanocapsa* sp. (NTVMK17), *Chroococcus* sp. (NTVMK18) (Fig. 4).

16S rRNA gene was amplified using cyanobacterial primers it was identified as ~600 bp, mentioned as *Anabaena variabilis* NTVMK 01, *Anabaena* sp. NTVMK 02 and *Westiellopsis* sp. NTVMK 03 respectively (Fig. 5).

It was screened for ptigment production for a strain, *Anabaena variabilis* (NTVMK01) among other isolates

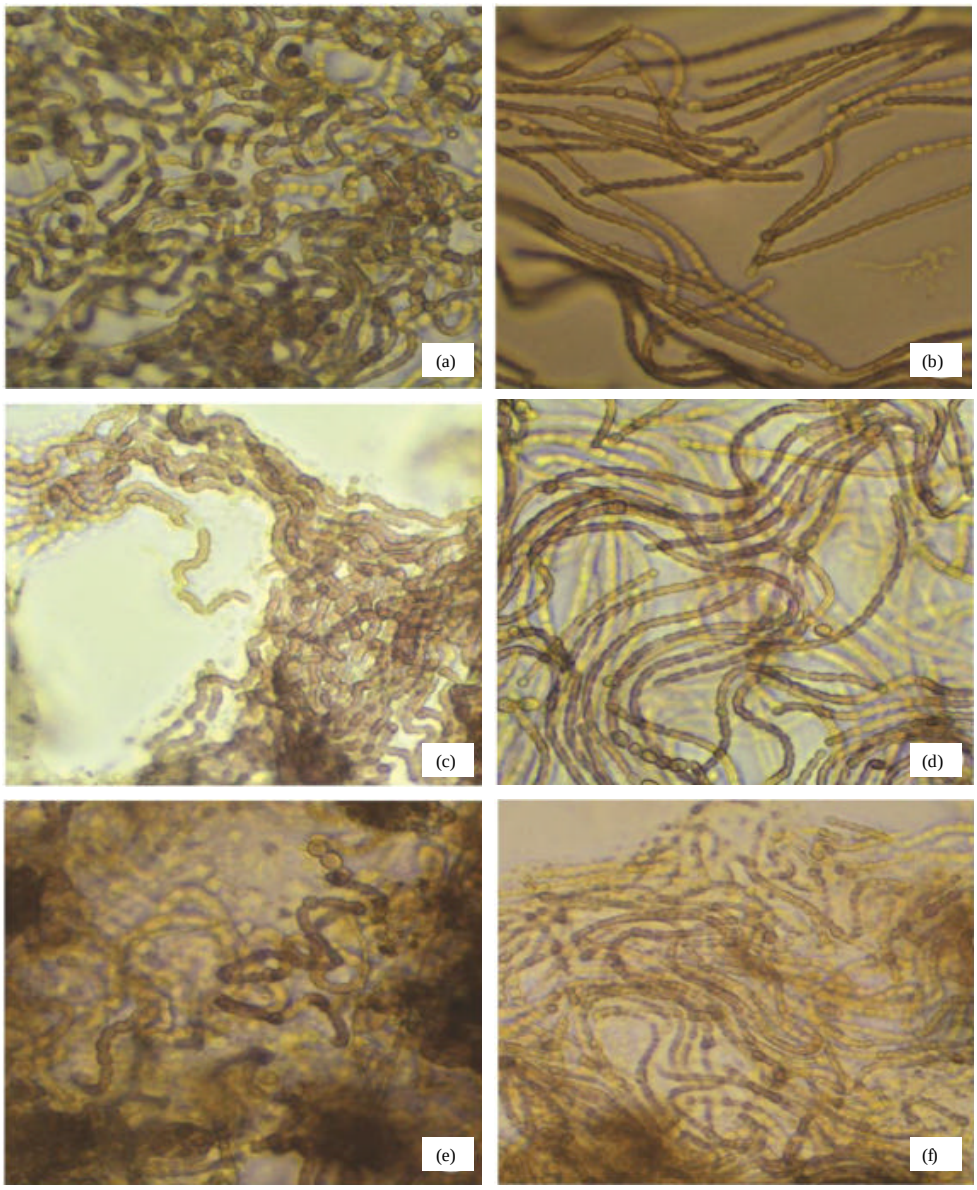

Fig. 2(a-f): Photomicrographs illustrating the endosymbiotic cyanobacteria isolated from *Azolla*. (a) *Anabaena* sp. NTVMK04, (b) *Anabaena variabilis* NTVMK01, (c) *Anabaena* sp. NTVMK05, (d) *Anabaena* sp. NTVMK06, (e) *Anabaena* sp. NTVMK07 and (f) *Anabaena* sp. NTVMK08

produced red colored pigment, Phycoerythrin (PE) which is responsible for the red color of the cells. BG11 medium can be optimized with different $MgSO_4$ concentration showed the maximum productivity of 0.1052 g mL^{-1} of Phycoerythrin with 22.5 mg mL^{-1} $MgSO_4$ concentrations in the culture medium (Table 2). This Phycoerythrin (PE) pigment mainly used for the food additive in recent food technology method to overcome the hazardous colorant used in commercial practice. Cyanobacteria also produce phycobilins and Phycoerythrin in normal condition and it can produce in larger amount under given optimized conditions. Chlorophyll a is can chelate the metal ion bonded to a large organic molecule, composed of carbon, hydrogen and other elements such as oxygen and nitrogen. Chlorophyll a has Magnesium as its central metal ion and the large organic molecule to which it bonds

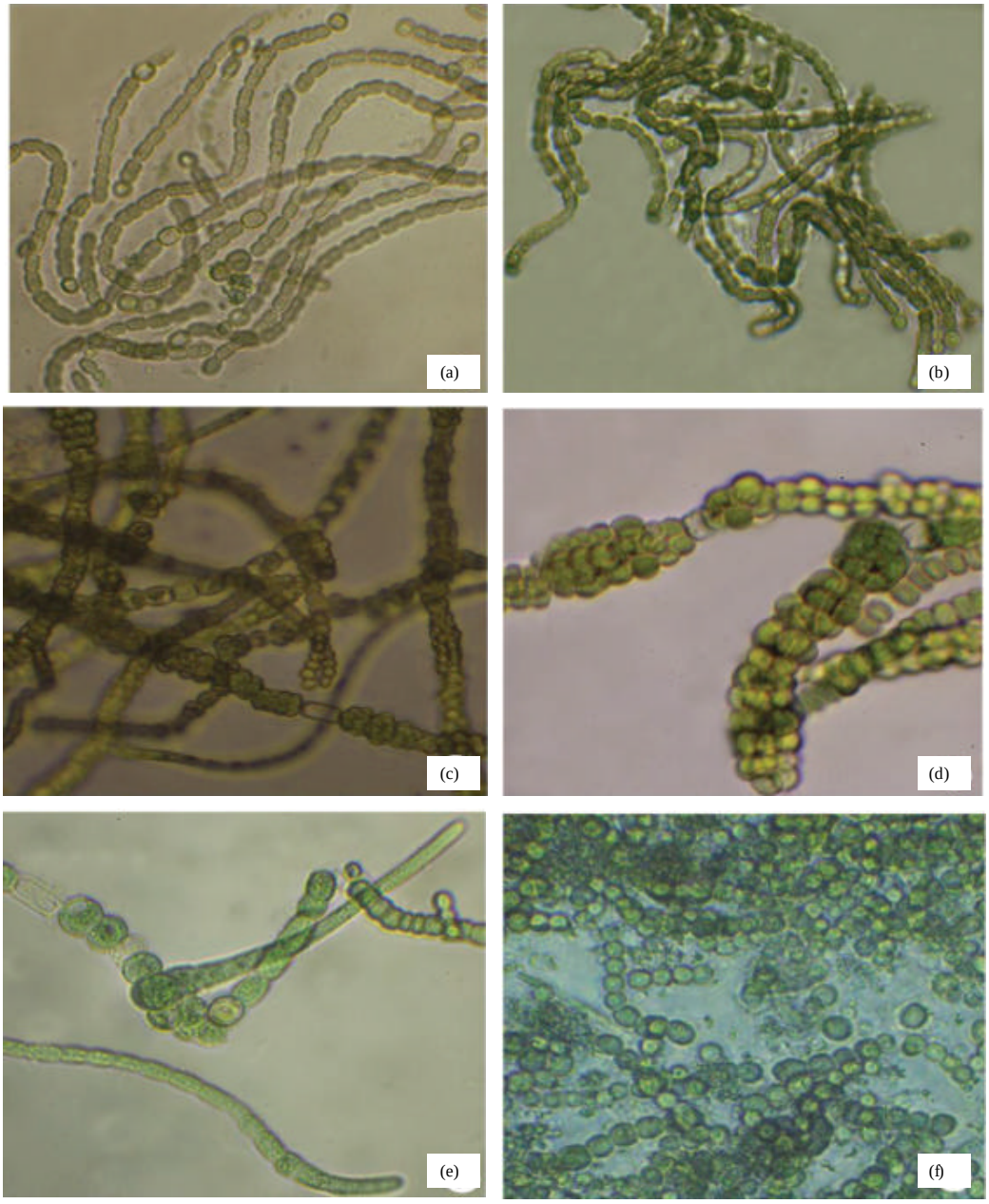

Fig. 3(a-f): Photomicrographs illustrating endosymbiotic and epiphytic cyanobacteria isolated from *Azolla*, (a) *Anabaena* sp. NTVMK06, (b) *Anabaena variabilis* NTVMK01, (c) *Westiellopsis* sp. NTVMK03, (d) *Westiellopsis* sp. NTVMK09, (e) *Westiellopsis* sp. NTVMK10 and (f) *Anabaena* sp. NTVMK07

Table 2: Effect of magnesium on production of phycobiliproteins from *Anabaena variabilis* (NTVMK01)

Concentration of $MgSO_4$ (mg mL^{-1})	Phycocyanin (g mL^{-1})	Allo phycocyanin (g mL^{-1})	Phycoerythrin (g mL^{-1})
7.5	0.05	0.03	0.07
15	0.0417	0.0179	0.0862
22.5	0.0505	0.0181	0.1052
30	0.042	0.0145	0.094
37.5	0.039	0.013	0.0814
45	0.0436	0.014	0.0774

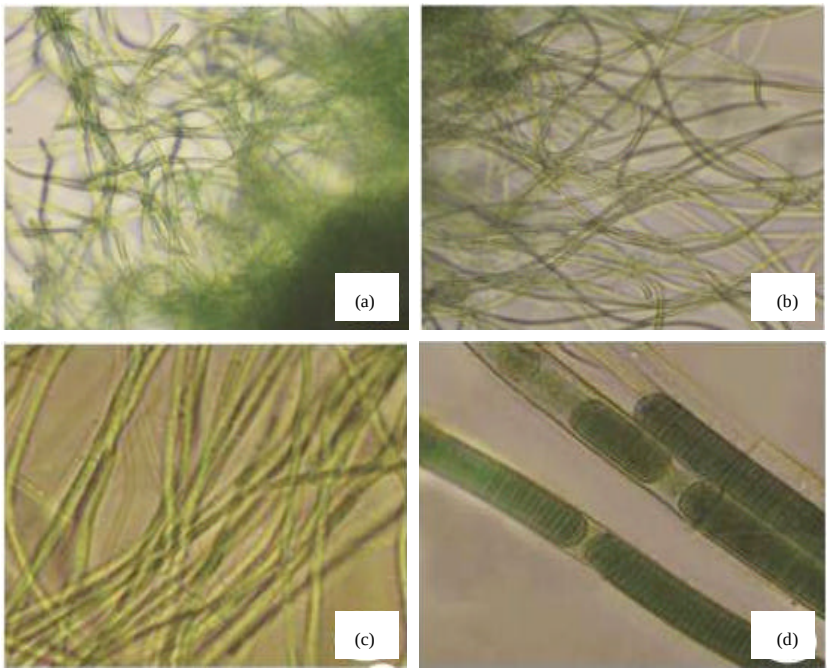

Fig. 4(a-d): Photomicrographs illustrating epiphytic cyanobacteria associated with *Azolla*, (a) *Phormidium* sp. NTVMK12, (b) *Phormidium* sp. NTVMK13, (c) *Phormidium* sp. NTVMK14 and (d) *Lyngbya* sp. NTVMK15

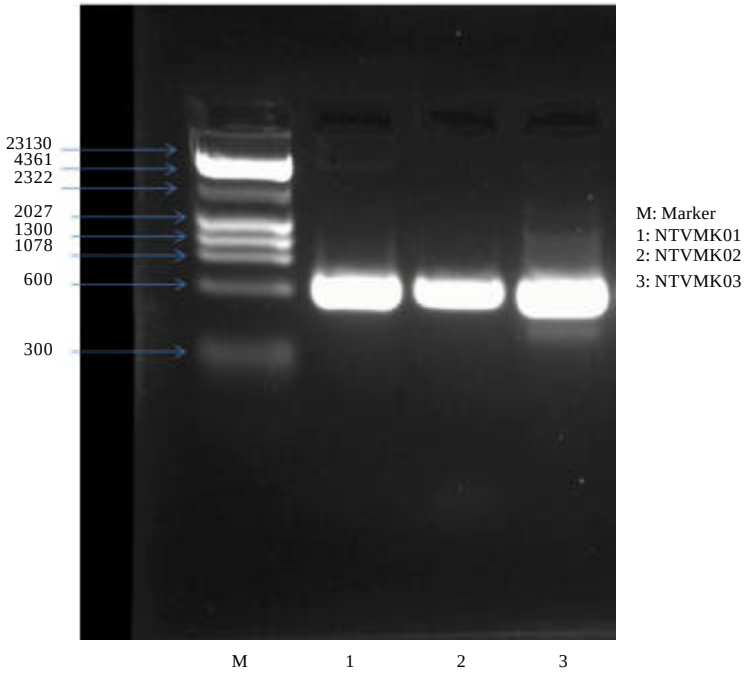

Fig. 5: Agarose gel electrophoresis (1.2%) showing amplified 16S rRNA gene of strains NTVMK01, NTVMK02, NTVMK03

is known as a porphyrin. The porphyrin contains four nitrogen atoms bonded to the magnesium ion in a square planar arrangement. Chlorophyll a occurs in a variety of forms. Magnesium is an important part of chlorophyll, a critical plant pigment important in photosynthesis. It is important in the production of ATP through its role as an enzyme cofactor.

It can be reported that Mg-ions not only restore, at least partly, the macrostructure of the thylakoid membranes but also enhance the light-harvesting and quenching capacity of the membranes. Interestingly, not only Mg^{2+} plays a major role in restoring the macro domain organization presented by Kannaiyan and Kumar (2006). A variety of fine chemicals such as pigments, vitamins and enzymes with varied applications can be obtained on a commercially viable scale from cyanobacteria. A number of cyanobacteria are rich in vitamins and many can excrete them into the surrounding environment. The carotenoids and phycobiliproteins, characteristic of cyanobacteria have high commercial value (Thajuddin *et al*., 2010). Elaborated about cyanobacteria are used as natural food colorants, as food additives to enhance the color of the flesh of Salmonid fish and to improve the health, fertility of cattle Feed grade *Phormidium valderianum* is an excellent source of phycocyanin, a blue natural colorant useful as a phycofluor in diagnostics and c-Phycoerythrin is used as capping agent for the synthesis of CdS nanoparticles (MubarakAli *et al*., 2012b). Structure and functions of the c-phycocyanin and c-phycoerythrin also studied computationally by sequencing the specific DNA sequences (MubarakAli *et al*., 2012c; Reehana *et al*., 2013). Symbiosis between Cyanobacteria and *Azolla* leads to the crop improvement in the fields in agriculture, this mainly because of the presence of Nitrogen fixing behavior. Molecular characterization and cross sectional analysis study shows the presence of Nitrogen fixing cyanobacteria in the *Azolla* as endosymbiont and also as ectosymbiont. The isolated endosymbionts produced phycoerythrin pigments, it can be study further for enhanced production of pigment from the Cyanobacteria *Anabaena variabilis* from *Azolla* fern for commercialization.

CONCLUSION

In the present study, totally 16 isolates were isolated from the *Azolla*. *Anabaena* sp. was the predominant in endosymbionts than other sites includes ectosymbionts and epiphytes. *Westiellopsis* sp. was found to be a novel entity to the *Azolla* in general and *Azolla filiculoides* in particular comparatively with previously reported. Furthermore, *Anabaena variabilis* precluded from *Azolla filiculoides* was produce red color pigmentation. The C-Phycoerythrin content of the isolated cyanobacterial symbiont was measured by maximum absorption. The nutritional optimization with $MgSO_4$ showed two fold increased in pigment production than the control. This preliminary data showed that due to environmental factors and other evolutionary importance chances the microbial interaction and their values. By this study a strain, Westiollopsis was found at *Azolla* at first time. The pigment productivity also higher than the control. The pigments from *Anabaena variabilis* from *Azolla* fern can be directly used for the commercialization.

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