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BIOCHEMICAL PROFILE AND ANTIMICROBIAL ACTIVITY OF A CYANOBACTERIUM, *SCYTONEMA TOLYPOTHRICHOIDES* ISOLATED FROM ACIDIC RICE FIELD SOIL OF CACHAR DISTRICT (ASSAM), INDIA

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ABSTRACT

Scytonema tolypothrichoides, a filamentous cyanobacterium, has been isolated from acidic rice field soil from Dholai area of Cachar district in Assam, North-East India. The growth rate determination and biochemical analysis of chlorophyll *a* (Chl-*a*), total carotenoid content (TCC), phycobiliproteins, total proteins (PRT), total carbohydrates (CHO), and exopolysaccharides (EPS) were carried out. The algal strain had a higher phycocyanin content than those of other pigments. The nitrogen fixation potential of the alga has also been ascertained. Antimicrobial studies against some selected microbial strains revealed moderate activity.

KEY WORDS: *Scytonema tolypothrichoides*, Rice field, Exopolysaccharides, Antimicrobial activity

INTRODUCTION

Cyanobacterial species occur in ecologically specific and geographically distinct habitats. Most species of cyanobacteria have specific ecological demands. Numerous species of the genera *Scytonema* are generally found to grow in ecologically distinct habitats of tropical ecosystem such as, lateritic soils, dripping rocks and aquatic bodies. The *Scytonema* group are the prominent components of microflora of tropical and subtropical soils, but their diversity and taxonomic classification are still less understood (Komárek *et al.,* 2013). They studied the phenotypic characteristics of fourteen morphotypes of *Scytonema* isolated from the microvegetation of lateritic, forest soils and stony substrates of South East Brazil. This heterocystous cyanobacterium has been found to enhance rice field soil fertility by fixing atmospheric nitrogen (Selvi and Sivakumar, 2012). *Scytonema javanicum* played a vital role in crust formation and maintenance of productivity (Metting 1981). The species, *Scytonema stuposum,* in particular, forms bluish soil crusts in rice fields suggesting their role in soil surface stabilization, moisture retention, providing a suitable habitat for growth of higher plants (Sethi *et al.,* 2012). Diversity of cyanobacteria belonging to *Scytonemataceae* family growing in alkaline rice field soils of Tamilnadu state, India has been documented by Madhumathi *et al.,* (2012). Hazarika *et al.,* (2012) reported five different species of *Scytonema* from the acidic rice fields of the upper Brahmaputra Valley(Assam), India. Species like *Scytonema bohneri, Scytonema hofmanni* and *Scytonema simplex* were found to be common in the rice fields of Bongaigaon district of Assam (Das and Sarma, 2010). Cyanobacteria from some rice field soil of Cachar district (Assam) are also documented (Rout and Dey, 1999). Several reviews (Geitler, 1932; Desikachary, 1959; Starmach, 1966; Bourrelly, 1970; and Komárek and Anagnostidis 1989) addressed the traditional taxonomic description of the genus *Scytonema* while a modern approach based on polyphasic characterization has recently gained popularity. The approach deals with various aspects

of their biochemistry such as chlorophyll-*a*, phycocyanin, phycoerithrin, carotenoids, proteins, carbohydrates, exopolysaccharides, fatty acids and DNA profiling as more reliable tools for characterization of cyanobacteria (Sfriso *et al*., 2014). Besides diversity and their role in improving soil fertility, cyanobacteria, as a source of pharmacologically active compounds, has attracted immense interest (Kumar *et al.,* 2010; Sethubathi and Prab , 2010; Battu *et al.,* 2011 and Mhadhebi *et al.,* 2012). Proteau *et al.,* (1993) reported scytonemin, a predominantly UV-A-photoprotective pigment from *Scytonema javanicum.* Kreitlow *et al.,* (1999) investigated the hydrophilic and lipophilic extracts of cyanobacterial strains for antibiotic activities. Cachar district located in the state of Assam, India is characterized by diverse habitats with potential for a wide range of cyanobacteria which remained virtually unexplored. Accordingly the present research focuses on the isolation and validation of one strain of *Scytonema* from acidic rice field soil of Dholai sub-division in the district through morphological and biochemical studies.

MATERIALS AND METHODS

Study site

Cachar district in Southern Assam lies between latitude 90.44°E and longitude 20.04° N encompassing an area of 3786 sq. km. The soil samples were collected from rice field of Dholai area (N24˚ 35'22.2" E 92˚ 50'57.4") having an average pH of 6.4 with a clay loam texture. Details of the geographical location of study sites have been mentioned in Figure1.

Isolation of the strains from soil samples

Dilution plate method (Lukešová, 1993) was followed for isolation of strain from 10g of rice field soil. 1mL aliquots of soil suspensions were spread on solidified (1.5%) BG-11$_0$ media (Rippka *et al.,* 1979). Plates were incubated at 24±1 °C for a 16:8 (light: dark) photoperiod under white fluorescent light having intensity of 2000-3000 lx. Morphological observations such as cell shape, length and breadth of intercalary cells and heterocysts were taken into account and taxonomically identified on the basis of their cell or colony morphology (Desikachary, 1959). Repeated measurements were obtained from different cells and heterocysts to ensure accuracy (Singh *et al.,* 2008).

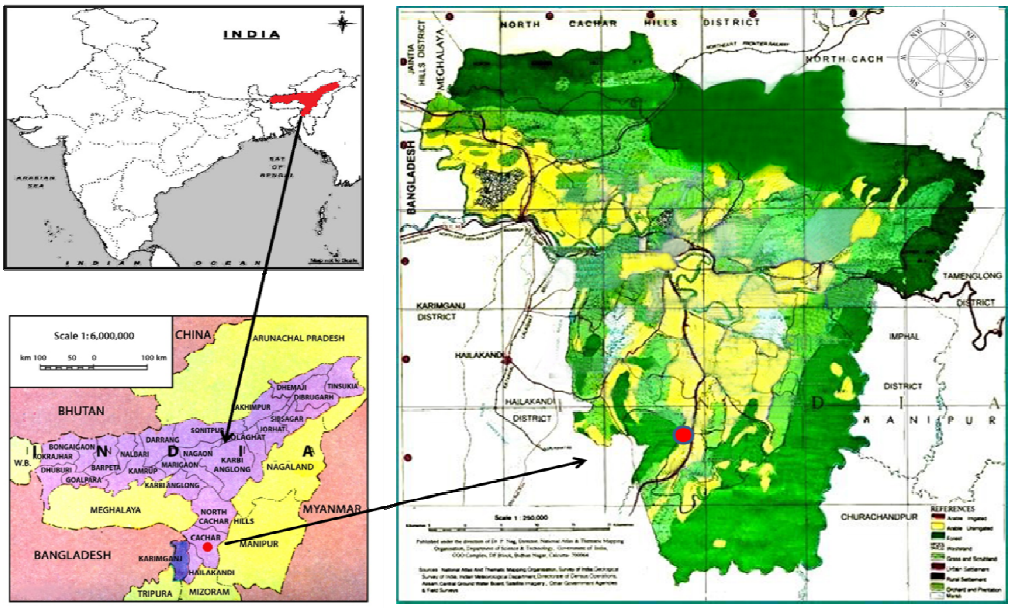


Figure 1

Map showing the location of study sites in Cachar district.

Cyanobacterial growth measurement

Cyanobacterial growth was monitored by the method of Sfriso *et al.,* (2014). A total of 8 culture tubes, containing 9 mL aliquots of sterile BG-11_0 liquid medium were inoculated with 1mL stock culture of the isolated strain. Each tube was incubated for 8 days on the culture rack, with a light exposition of 2000-3000 lx, in light/dark cycles of 16:8 hours at an average room temperature of 24 °C, with proper shaking. At 5 days time interval during growth, specific amount of culture was withdrawn from each tube and were used for quantification of chlorophyll-*a*(Chl-*a*), total carotenoid contents(TCC) and exopolysaccharides (EPS), total proteins(PRT) and total carbohydrates(CHO) having three biological replicates for analyses, with eight points to observe. The biochemical parameters, Chl-*a,* TCC and EPS were estimated on the same day of sample withdrawal while the remaining amount of culture samples were kept frozen for total protein and total carbohydrate quantification and analyzed together at the end of 40^{th} day. Chlorophyll *a* values were obtained according to Parsons and Strickland (1965). Specific growth rate and generation time were ascertained as per Levasseur *et al.*, (1993). Total carotenoid contents (TCC) were estimated following Parsons *et al.,* (1984). Phycobiliproteins of the dried biomass of *S. tolypothrichoides* were analyzed during the stationary growth phase and were extracted in 0.05M phosphate buffer (pH 6.8) by subjecting them to repeated freezing and thawing for 48h with a 24h interval (Moraes *et al.,* 2011) and evaluated according to Bennet and Bogorad (1973). The EPS fractions of exopolymeric substances were extracted by the method of Underwood *et al.,* (1995) and quantified as carbohydrate. Both total carbohydrates of the wet biomass and the carbohydrate (EPS fractions) were determined by phenol-sulfuric acid method (Dubois *et al.,* 1956). Total soluble protein was estimated by a modified Lowry method (Herbert *et al.,* 1971).

Acetylene reduction activity (ARA)

The gas chromatographic quantification of ethylene formed (acetylene reduction activity, ARA) was utilized as an index of nitrogen fixation, which was estimated by the method given by Prasanna *et al.,* (2003) in gas chromatograph (Bruker, model CDS 2.0). Commercially available standard ethylene was utilized for quantification and test tubes with an equivalent volume of water served as controls. The ARA values presented is means of triplicate measurements and is expressed as nmoles of ethylene per mg chlorophyll per hour.

Antimicrobial activity

A 100ml of cyanobacterial culture was harvested at stationary phase (after 35 days) by centrifugation at 5000 rpm. The aqueous supernatant was collected in vials and the pellet extracted with 10 ml of methanol. The culture supernatants and solvent extracts were stored at 4°C for further studies**.** Disc diffusion method was used to determine antibacterial activity (Nagi *et al.,* 2010). Sterile discs (6 mm, Himedia) were soaked in 500µl methanolic extracts and supernatant solution of cyanobacteria. The microbial strains, *Staphylococcus aureus* (KT68-07036)*, Escherichia coli* (KT68-21103)*,* were obtained from Bangalore Genei, Bangalore, India. The suspension of bacteria culture was prepared according to the MacFarland standard 0.5 and layered onto the Nutrient agar plates to produce the bacteria field. The sterile discs containing the extract and supernatant were placed on the bacteria field by a sterile forceps. Distilled water and methanol were used as negative control while commercial antibiotic disc of ciprofloxacin was used as a positive control. Finally, the plates were incubated at 37°C and the zone of inhibitions was observed after 24-48 hours. The extract showing positive activity in disc diffusion experiment was chosen for determination of their MIC (minimum inhibitory concentration) by using a tube dilution technique. These tests were carried out to determine the lowest concentration of methanolic extracts of the cyanobacterium that showed bacteriostatic effect.

Statistical analysis

Correlation coefficients between the biochemical attributes of the cyanobacterial strain was analyzed by determining their Spearman's correlation coefficients using SPSS(version 19)software.

RESULTS AND DISCUSSION

Morphology of Scytonema tolypothrichoides

The morphology and distinctive features of *Scytonema tolypothrichoides* was observed with initial dark-green thallus development. The bunche

later formed a thick brownish green mat around 1.5cm high, floating and suspended in the liquid media (Figure 2- a, b, c, d). The thallus was dark green with 11–15 µm broad filaments. The trichome is 11–12µm broad with quadrate cells, densely granulated, mostly longer than width while those at the apices are broader than than its length exhibiting a olive-yellowish colour. Heterocysts are intercalary, rectangular and varied in length (15–25µm) with breadth ranging from 11–15µm. Sheath was hyaline and later orange brown in colour. Filaments are repeatedly branched with false branches resembling the main filament (Figure 2-c, d). The strain is thus identified as *Scytonema tolypothrichoides* Kὺtzing ex Born. et Flah. after Desikachary (1959).

Biochemical estimations and antimicrobial activity

The growth curves (Figure 3,4,5,6,7) of *Scytonema tolypothrichoides* in terms of chlorophyll *a* (Chl-*a*), total carotenoid content (TCC), exopolysaccharides (EPS), total proteins (PRT) and total carbohydrate content (CHO) revealed that the strain had a longer exponential phase with its stationary phase starting on the 30th day. It showed a specific growth rate of 0.19 d^{-1} with a lower generation time (122.2 h^{-1}). During the mid stationary phase, highest content of chlorophyll *a*(4.94 mg L^{-1}), TCC(1.6 mg L^{-1}), exopolysaccharides (34.7 µg mL^{-1}), total proteins (143.8 µg mL^{-1}), total carbohydrate(275.2 µg mL^{-1}) and phycobiliprotein (51.4 mg g^{-1}) was recorded. The strain was found to produce relatively more phycocyanin (PC) than phycoerythrin (PE) and allophycocyanin (APC) (Figure 8, Table 1). The absorption spectrum of chlorophyll *a* during the stationary growth phase is presented in Fig.9. Similar results were obtained from the rice fields of Dima Hasao district in Assam, North-East India wherein two strains of *Nostoc commune* were characterized on the basis of their pigments and biochemical analysis in addition to molecular studies (Borah *et al.,* 2014). A steady increase in biochemical attributes, chlorophyll-*a* (Chl-*a*), total carotenoid contents (TCC) and exopolysaccharides (EPS), total proteins (PRT) and total carbohydrates (CHO) during the growth phase has been noticed.

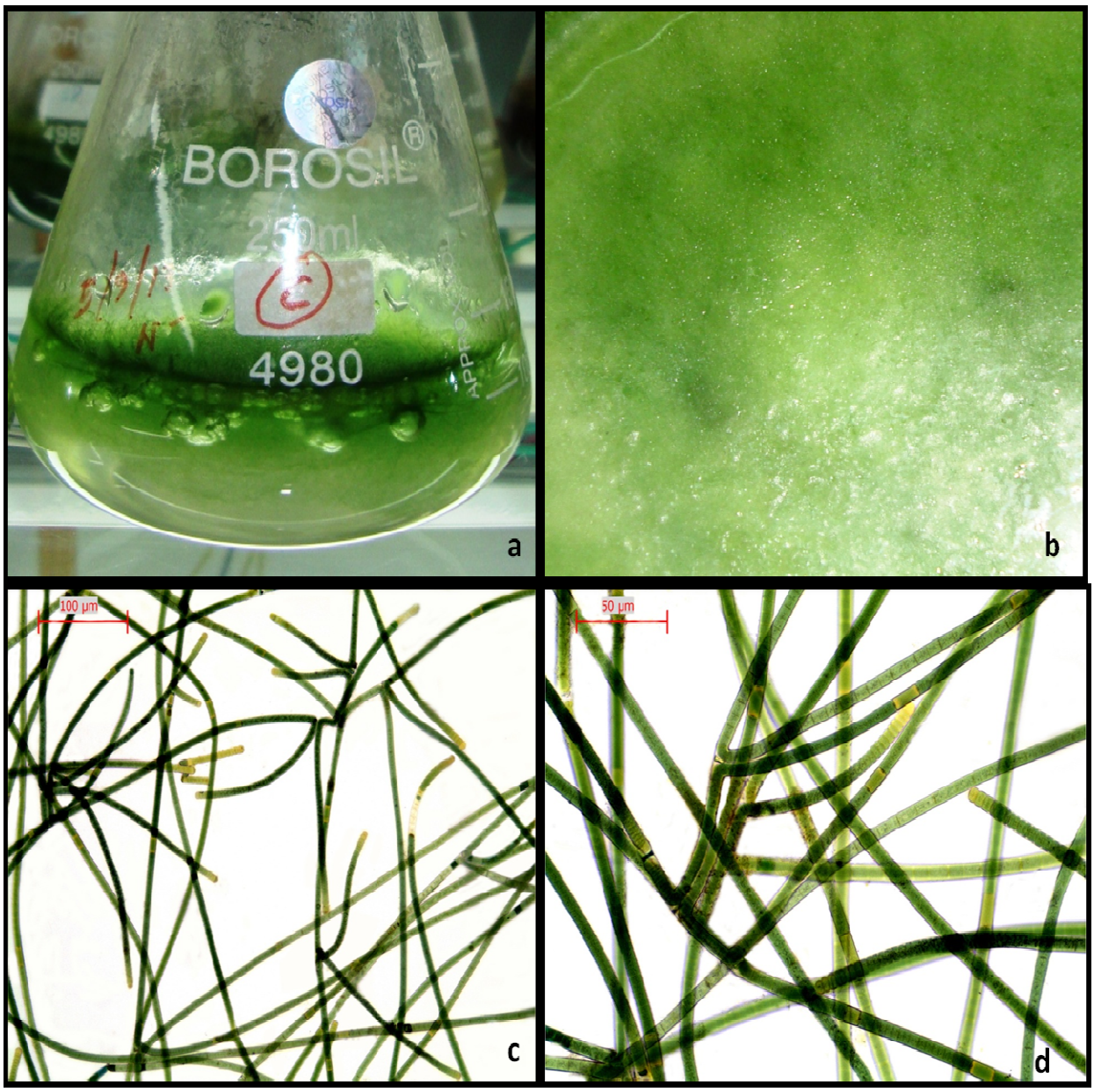


Figure 2

Morphology of S. tolypothrichoides. a, b. Liquid culture and plate view. c, d. Microphotographs showing repeated branching (bar length = 100µm) and false branching with broader apical cells olive-yellowish in colour (bar length = 50µm).

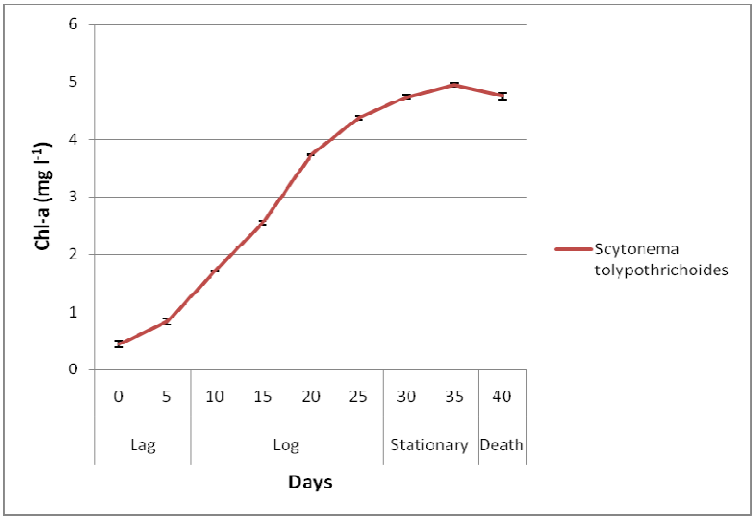


Figure 3
Cyanobacterial growth in terms of chlorophyll a (Chl-a).

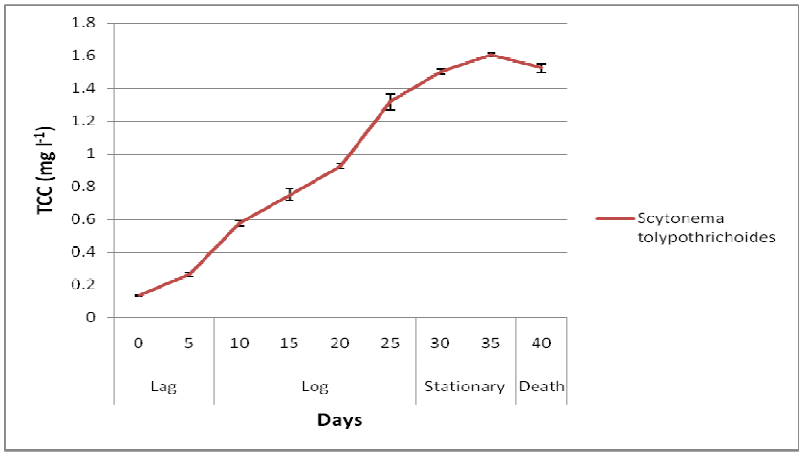


Figure 4
Cyanobacterial growth in terms of total carotenoid content (TCC).

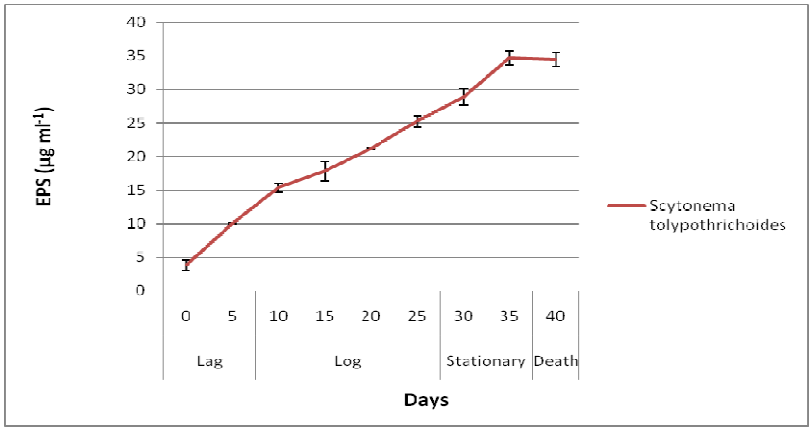


Figure 5
Cyanobacterial growth in terms of exopolysaccharides (EPS) production.

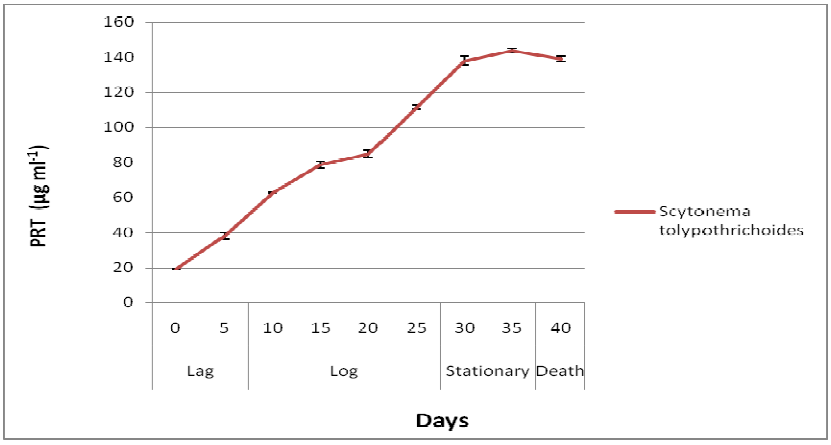


Figure 6
Cyanobacterial growth in terms of total proteins (PRT).

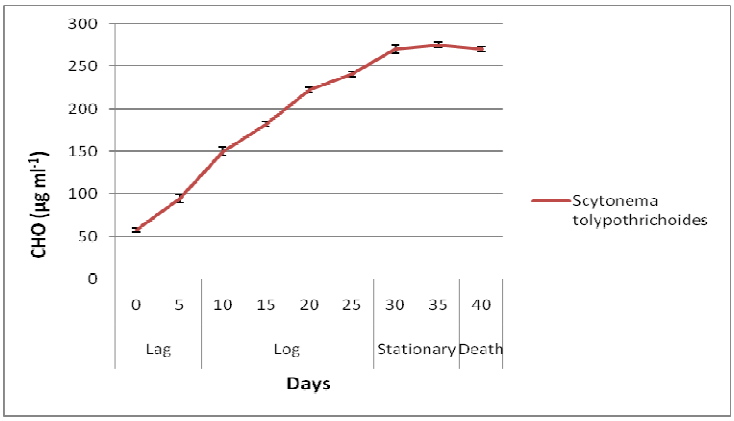
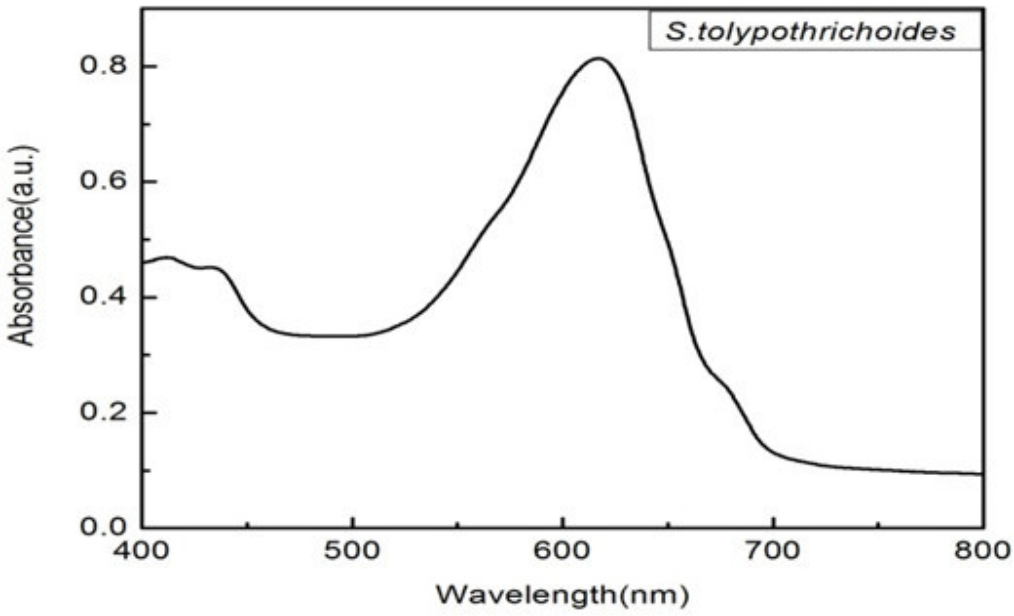


Figure 7
Cyanobacterial growth in terms of total carbohydrate content (CHO).

Table 1
Phycobiliprotein concentration (mean ± SD) of the Scytonema strain

SI.No.	Phycobiliproteins (mg g^{-1})	
1	Phycocyanin (PC)	30.89 ± 2.25
2	Allophycocyanins (APC)	14.98 ± 3.49
3	Phycoerythrin (PE)	5.49 ± 1.33

Figure 8
Absorption spectrum of Phycobiliproteins



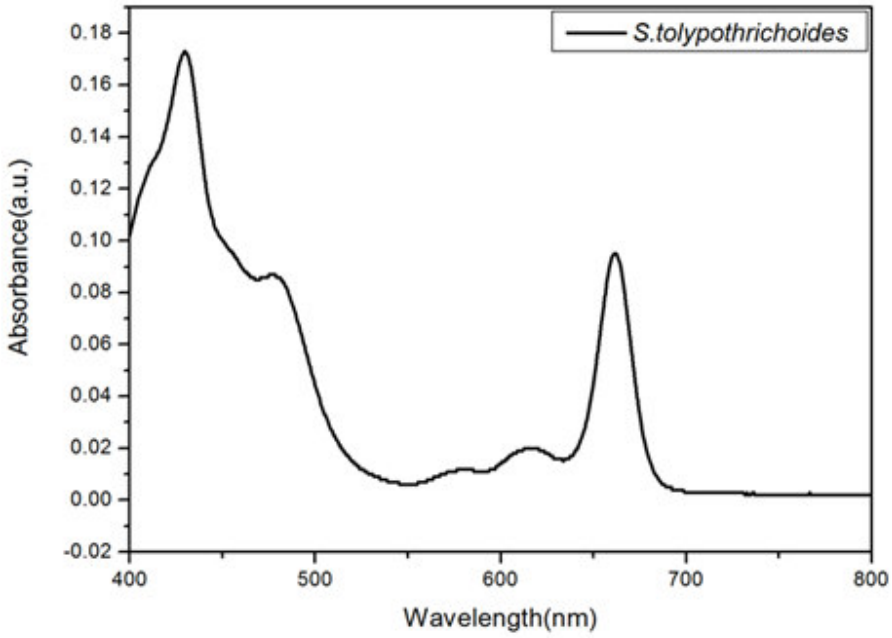


Figure 9
Absorption spectrum of Chlorophyll a

Choudhary (2011) concluded that application of nitrogen-fixing cyanobacteria in the rice fields can be used for sustainable management of nitrogen fertilizer at different stages of paddy cultivation. *S. tolypothrichoides* demonstrated an average nitrogen fixation potential (3.9 ± 0.12 nmoles C_2H_4/mg chl/h). Antimicrobial activity of some *Scytonema* sp. was studied by Bharat (2013) against six different clinical isolates of *Staphylococcus aureus. S. tolypothrichoides* in the present work showed only moderate antimicrobial activity with zone of inhibition of 10.1 ± 0.1mm (Figure 10-a) and a minimum inhibitory concentration of 19.2mg ml^{-1} against *Staphylococcus aureus* (KT68-07036). Correlation analysis of growth and biochemical characteristics such as chlorophyll-*a* (Chl-*a*), total carotenoid contents (TCC) and exopolysaccharides (EPS), total proteins (PRT) and total carbohydrates (CHO) revealed a significant positive correlation among the variables (Table 2).

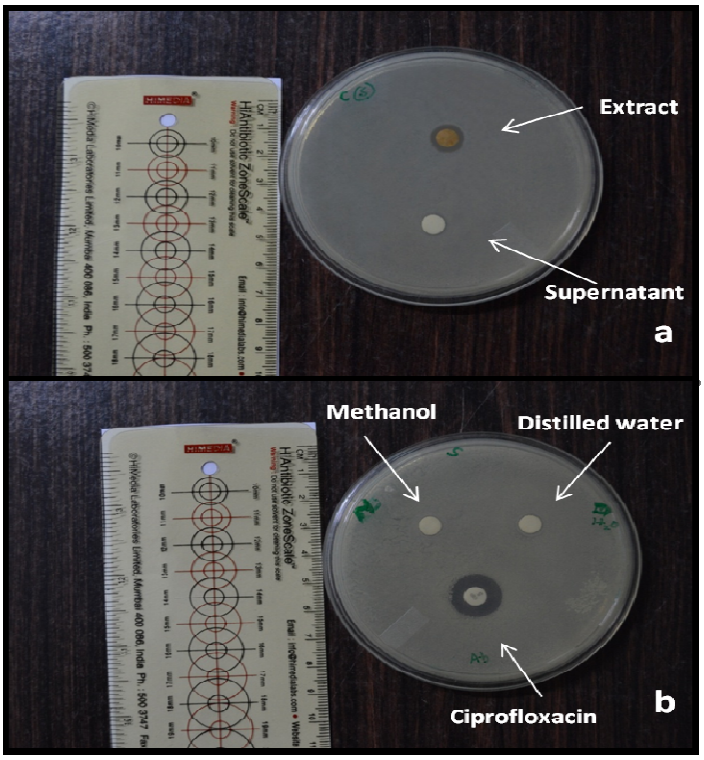


Figure 10

Antimicrobial activity S. tolypothrichoides. a. Zone of inhibition against Staphylococcus aureus (KT68-07036). b. Control : Zone of inhibition for methanol, distilled water and ciprofloxacin against Staphylococcus aureus (KT68-07036)

Table 2

Correlation analysis of growth and biochemical parameters of S. tolypothrichoides

	EPS	PRT	CHO	TCC	Chl-*a*
EPS					
PRT	.988(**)				
CHO	.972(**)	.979(**)			
TCC	.982(**)	.995(**)	.981(**)		
Chl-*a*	.965(**)	.975(**)	.991(**)	.987(**)	

*** Correlation is significant at the 0.01 level (2-tailed).*

CONCLUSION

The paper highlights the importance of combined morphological and biochemical studies in characterization of cyanobacterial species as a part of polyphasic approach. The species isolated in the present work was found to possess a higher phycocyanin content compared to other phycobiliprotein pigments. High carbohydrate and protein content found in this alga contributes to the nutritional value of the species. A moderate nitrogen fixation potential and antimicrobial activity was noted for the species.

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