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RESEARCH ARTICLE

CULTURAL STUDIES, PRODUCTION OF NITROGEN, TOTAL SUGAR, PIGMENTS AND LIPID BY CHLOROGLOEOPSIS FRITSCHII BTA9016 A CYANOBACTERIUM FROM PADDY FIELDS OF ALLAHABAD, INDIA

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ABSTRACT

Cyanobacterium *Chlorogloeopsis fritschii* Mitra 1950 displayed irregular rounded and elongated cell packets during in-situ germination and controlled culture conditions. The strain was investigated for acetylene reduction activity and also assessed for lipid profiling and fatty acid composition by Nile red method followed GC-FID using Supelco standard. Investigation expressed high and considerable amount of acetylene reduction activity i.e., 4.47 and 11.27 nmole C_2H_4/µg of Chl-a/hr during 15th and 30th days growth phase respectively. Investigated organism also rigorously worked out for the production and release of pigments, total soluble proteins, total sugar and ammonia excretion during mid and last phase of growth cycle. The 16S rRNA sequences was compared with the retrieved cultures from NCBI GenBank database. Phylogenetic analysis was largely consistent which was obtained from 16S rRNA gene sequence analysis. Phylogenetic trees was constructed by neighbour joining and the findings indicate that the genus *Chlorogloeopsis fritschii* with accession number KJ562182.1 was distantly related to the genus *Nostochopsis* and *Fischerella*.

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INTRODUCTION

Cyanobacteria are small prokaryotic mostly aquatic organisms. Some cyanobacterial species can be genetically engineered in order to produce compounds of interest by utilizing light and carbon dioxide. These compounds of interest can include biofuels, industrial bio chemicals, pharmaceutical, food supplements and other compounds such as lipids. The *Chlorogloeopsis fritschii* is a heterocyst forming nitrogen fixing cyanobacterium which can among others be isolated from hot springs. *Chlorogloeopsis fritschii* has a diverse morphology and diversity of function. Thallus in form of a compact stratum of indefinite size, composed irregular-rounded cell-packets and aggregates, of uniserial up to multiserial short rows of cells (trichomes with 3-20 cells), usually without distinct mucilaginous envelopes, single cell-packets enclosed in thin, firm sheaths. Cells rounded, with pale blue-green, granula content. Heterocyst terminal and intercalary irregularly disposed (Komerak, 1989). The role of heterocystous cyanobacteria in N fixation in paddy fields has been appreciated (Barbosa, 2002) a long time. Regarding the diversity of cyanobacteria, especially in paddy fields and the evaluation of the ability of each species to fix atmospheric N can lead to identification of the most common species as biofertilizers. (Thiel, 2001; Irisarri, 2001). In addition characterizations based on polyphasic studies improve the resolution of cyanobacterial taxonomy and currently constitute the best defined base-line for diversity and ecological studies (Taton *et al.,* 2006; Heath *et al*., 2010). In recent years, the studies based on combined genetic and phenotypic properties of new isolates have increased the reliability of identification. The aim of this study was a thorough characterization of *Chlorogloeopsis fritschii* isolated from paddy fields of Allahabad, U.P., India through analysis of their genetic,

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morphological and biochemical characteristics and this polyphasic approach will allow us a better understanding of the *Chlorogloeopsis* diversity their identification and their functions in different environments.

MATERIALS AND METHODS

Strain isolation and growth condition

The studied strain was obtained from Department of Botany, University of Allahabad, Allahabad, U.P, India which was originally isolated from the paddy fields Uttar Pradesh, near Allahabad, India. Unialgal biomass was inoculated in Erlenmeyer flask containing BG-11 (-N) broth medium (Stanier *et al*., 1971). The flasks were kept in culture room under light: dark cycles of 14:10h conditions maintained at 28±2ºC under illumination provided by cool white fluorescent tubes of 54-67µmol photons $m^{-2}s^{-1}$.

Morphological study

The trinocular Carl Zeiss microscope with Axio Vision Viewer 4.8 software was used for image analysis. The length and width of vegetative cells, positions, frequency, size and shape of heterocysts and akinetes and thallus morphology and behaviour were the major parameters for morphological characterization.

Determination of acetylene reduction activity

Nitrogenase activity was measured by acetylene reduction technique described by Hardy *et al*., (1973). Activity was performed in calibrated triplicate serum bottles. A known volume of algal biomass was taken into 13 ml capacity serum bottles. Stopper the bottle and remove the gas phase equivalent to 10% of the remaining volume of the tubes and injected equivalent volume of acetylene (C_2H_2). Serum bottles were incubated for 90 min under light conditions 54-67 µmol photons $m^{-2}s^{-1}$at 28±2°C interval shake was done and reaction was terminated by injecting 0.8ml of 15% trichloroacetic acid. Ethylene produced in the bottle was analyzed in gas chromatograph (Ceres 800 Plus Thermo scientific) using Porapak-R column. Standard was prepared using pure ethylene.

Extracellular ammonium excretion

This was determined by measuring absorbance at 640 using UV-Vis spectrophotometer model 1800 Shimadzu with extinction coefficient described by Solorzano, (1969).

Total sugar

Total sugar was measured at 620 nm and calculated from the standard graph followed the method described by Spiro, 1966.

Pigment analyses

Phycobiliproteins (PBS)

Phycobiliproteins was recorded by measuring OD at 615, 652 and 562 as described by Bennett and Bogorad, (1973).
PC-Phycoerythrin; PE-Phycocyanin; APC-Allophycocyanin

Total carotenoids

Estimation of total carotenoids was determined by the method described by Jensen, (1978) and O.D was measured at 450 nm using 85% acetone as blank.

Chlorophyll-a: Chlorophyll-a was determined by measuring O.D. at 665nm as described by Mckinney, (1941).

Total soluble proteins: Estimation of total soluble proteins was determined by measuring OD at 650 nm as described by Herbert *et al*., (1971).

Lipid profiling: The total lipid and fatty acid composition were extracted described by Bligh & Dyer (1959).

Genotypic characterization: Mechanical disruption of cell was done in present experiment by following Xanthogenate method (Tillett & Neilan 2000).

Analysis of sequence data: Nucleotide sequence obtained from DNA sequence was compared with the sequence available in the NCBI database using BLAST (http://www.ncbi.nml.nih.gov /BLAST). Trees based on 16S rRNA were constructed using the available cyanobacterial gene sequences along with the sequence determined in this study using the neighbour-joining method (Saitou *et al*., 1987; Thompson *et al*.,1994**)** by using Kimura 2-parameter model (Kimura, 1980) contained in the MEGA 4.0 software (Tamura *et al*., 2007). Sequences were aligned using CLUSTALW to produce working alignment of 16S rDNA sequences for the target strains. The final alignments were obtained by manual refinement. The analysis of similarity matrix and phylogenetic tree was done using statistical significance level of interior nodes was determined by bootstrap analysis (1,000 data re-samplings) (Felsenstein, 1985) and values above 50% were reported.

RESULTS

Thallus forms an amorphous mat of a deep blue-green colour, composed of irregular rounded, uniseriate filaments upto16 cells. The packets arise after 3-dimensional division of cells. Cells were angular without distinct mucilaginous envelopes. Heterocysts terminal and intercalary and akinetes in form of enlarged cells (Fig 1). *Chlorogloeopsis fritschii* produced high amount of chlorophyll-a 7.29 $\mu g^{-1}ml^{-1}$ and 7.94 $\mu g^{-1}ml^{-1}$; total carbohydrates 36.3 $\mu g^{-1}ml^{-1}$ and 19.33 $\mu g^{-1}ml^{-1}$; ammonia excretion 5.10 $\mu g^{-1}ml^{-1}$ and 10.20 $\mu g^{-1}ml^{-1}$; phycoerythin 2.44 $\mu g^{-1}ml^{-1}$and 0.81 $\mu g^{-1}ml^{-1}$; phycocyanin 1.96 $\mu g^{-1}ml^{-1}$and 0.88 $\mu g^{-1}ml^{-1}$; allophycocyanin 2.88 $\mu g^{-1}ml^{-1}$ and 1.28 $\mu g^{-1}ml^{-1}$; total soluble proteins 109.0 $\mu g^{-1}ml^{-1}$and 104.3 $\mu g^{-1}ml^{-1}$during 15th day and 30th day respectively as shown in (Table 1). The lipid production showed high amount of Linolelaidic Acid Methyl Ester (C18:2n6t) followed by *cis*-11-Eicosenoic Acid Methyl Ester (C20:1) (Table 2). The acetylene reduction activity was recorded higher in 30th day growth i.e. 11.27 nmole C_2H_4/µg of Chl-a hr^{-1} than in 15th day growth (Table 1) (Fig 2). 16S rRNA sequences of cyanobacteria belong to heterocystous filamentous retrieved from NCBI genbank were obtained.

Table 1. Biochemical and physiological characterization of *Chlorogloeopsis fritschii* BTA9016

Name of the strain and NCBI accession no.	ARA activities, pigmentation, total soluble protein, sugar and ammonia excretion in culture conditions			
			15th day	30th day
Chlorogloeopsis fritschii BTA 9016 NCBI Accession No.: KJ562182	Acetylene reduction activity (nmole C_2H_4/µg of Chl-a/hr)		4.47±0.19	11.2±1.52
	Chlorophyll-a (µg/ml)		7.29±0.01	7.94 ±0.44
	Ammonia excretion (µg/ml)		5.10±1.59	10.20±1.37
	Total sugar (µg/ml)		36.33±14.98	19.33±4.62
	Total soluble protein (µg/ml)		109.00±1.73	104.33±5.13
	Phycobiliproteins (µg/ml)	PE	2.44±1.94	0.81±0.10
		PC	1.96±0.55	0.88±0.09
		APC	2.88±1.43	1.28±0.28
	Carotenoids (µg/ml)		6.84±0.04	10.77±0.00

Table 2. Extraction of lipid from *Chlorogloeopsis fritschii* BTA 9016

Fatty acid composition	Fatty acid content (%)
Butyric Acid Methyl Ester (C4:0)	0.31755
Caproic Acid Methyl Ester (C6:0)	0.606748
Caprylic Acid Methyl Ester (C8:0)	5.920045
Capric Acid Methyl Ester (C10:0)	0.416785
Undecanoic Acid Methyl Ester (C11:0)	1.003686
Lauric Acid Methyl Ester (C12:0)	4.218883
Tridecanoic Acid Methyl Ester (C13:0)	0.16161
Myristic Acid Methyl Ester (C14:0)	0.05387
Myristoleic Acid Methyl Ester (C14:1)	0.079388
Pentadecanoic Acid Methyl Ester (C15:0)	0.20981
cis-10-Pentadecenoic Acid Methyl Ester (C15:1)	2.041395
Palmitic Acid Methyl Ester (C16:0)	0.496172
Palmitoleic Acid Methyl Ester (C16:1)	0.099234
Heptadecanoic Acid Methyl Ester (C17:0)	0.297703
cis-10-Heptadecenoic Acid Methyl Ester (C17:1)	2.174653
Stearic Acid Methyl Ester (C18:0)	1.40913
Oleic Acid Methyl Ester (C18:1n9c)	9.861072
Linolelaidic Acid Methyl Ester (C18:2n6t)	22.85228
Linoleic Acid Methyl Ester (C18:2n6c)	0.130422
Arachidic Acid Methyl Ester (C20:0)	3.308761
γ-Linolenic Acid Methyl Ester (C18:3n6)	4.573292
cis-11-Eicosenoic Acid Methyl Ester (C20:1)	31.78906
Linolenic Acid Methyl Ester (C18:3n3)	0.127587
Heneicosanoic Acid Methyl Ester (C21:0)	1.145449
cis-11,14-Eicosadienoic Acid Methyl Ester (C20:2)	0.41395
Behenic Acid Methyl Ester (C22:0)	0.385597
Erucic Acid Methyl Ester (C22:1n9)	0.121917
cis-11,14,17-Eicosatrienoic Acid Methyl Ester (C20:3n3)	0.144599
Arachidonic Acid Methyl Ester (C20:4n6)	0.062376
Tricosanoic Acid Methyl Ester (C23:0)	0.666289
cis-13,16-Docosadienoic Acid Methyl Ester (C22:2)	0.592572
Lignoceric Acid Methyl Ester (C24:0)	0.859087
cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester (C20:5n3)	0.41395
Nervonic Acid Methyl Ester (C24:1)	3.045081

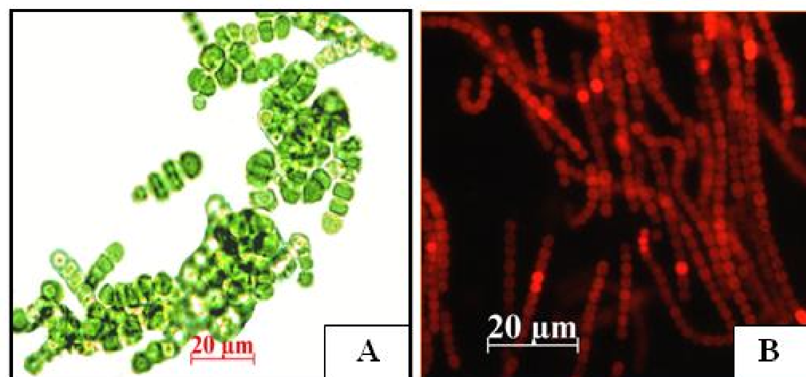
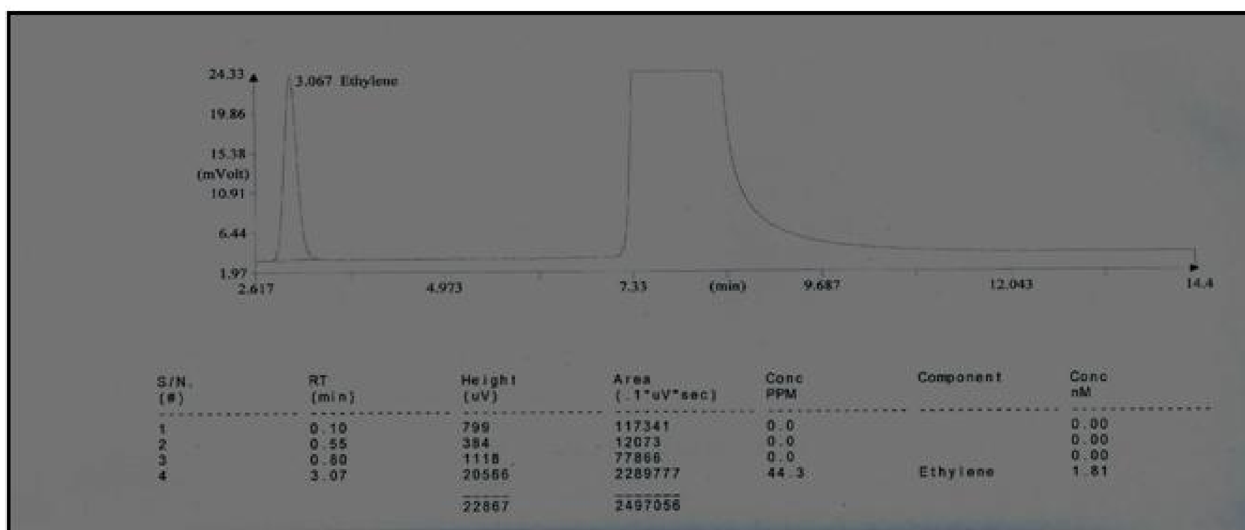


Fig. 1. A. Photomicrograph of *Chlorogloeopsis fritschii* BTA9016; B. Nile red fluorescence of *Chlorogloeopsis fritschii* BTA9016 in (63x)



S/N. (#)	RT (min)	Height (uV)	Area (.1*uV*sec)	Conc PPM	Component	Conc nM
1	0.10	799	117341	0.0		0.00
2	0.55	384	12073	0.0		0.00
3	0.80	1118	77866	0.0		0.00
4	3.07	20566	2289777	44.3	Ethylene	1.81
		22867	2497056			

Fig. 2. Representing the peak of conversion rates of acetylene to ethylene in *Chlorogloeopsis fritschii* BTA9016 data on the peak are retention times [min]

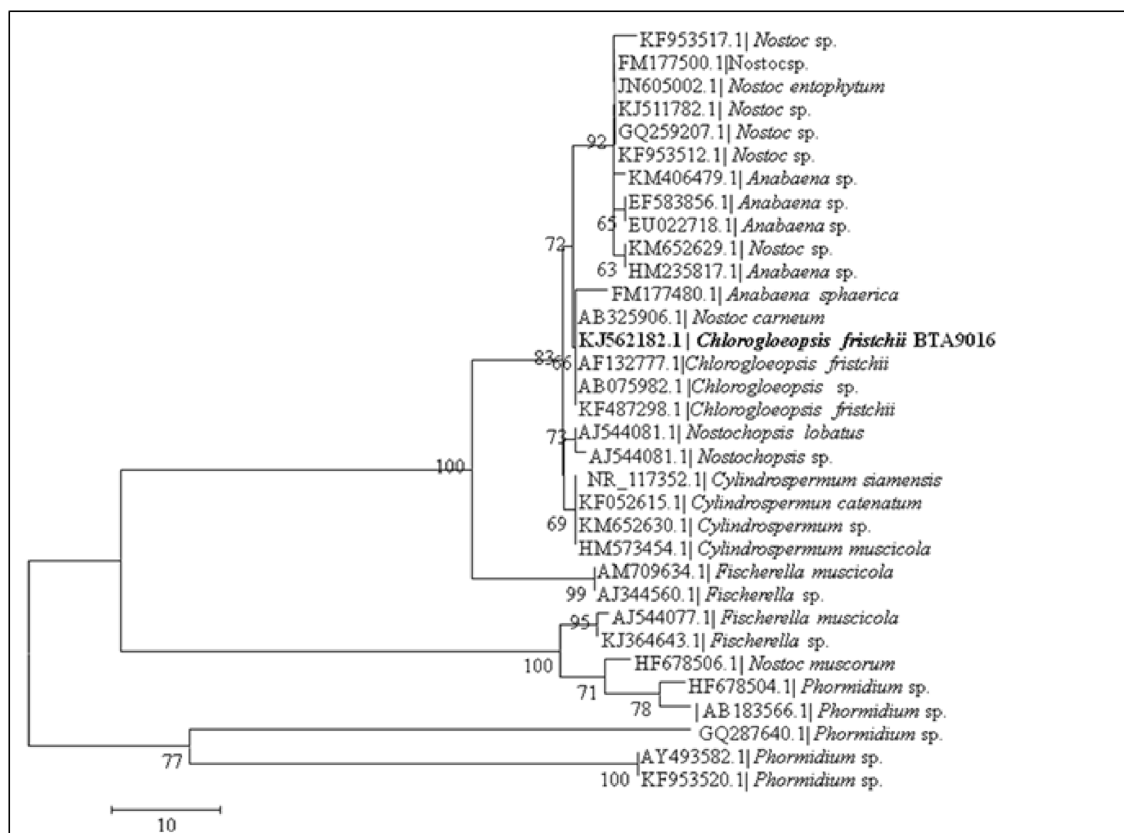


Fig. 3. Neighbour joining tree based on 16S rRNA gene sequences showing the clustering of studied *Chlorogloeopsis fritschii* with *Anabaena*, *Nostoc*, *Cylindrospermun*, *Nodularia*, *Fischerella, Phormidium*. Numbers near nodes indicate bootstrap values over 50% for analysis

The nucleotide sequence of the PCR amplified 16S rRNA gene of the genus *Chlorogloeopsis fritschii* BTA9016 was aligned with known 16S rRNA sequences of 33 other cyanobacteria of filamentous heterocystous group. Phylogenetic analysis showed that heterocyst bearing cyanobacteria under nostocalesform a monophyletic clade. The monophyletic supported by neighbor joining method (Fig 3).

DISCUSSION

The acetylene reduction activity was recorded higher in 30th day growth than in 15th days growth probably due to much frequency of heterocyst in later phase of growth cycle. The rate of acetylene reduction activity by cyanobacteria generally ranges from 1-10 nmole C_2H_4/µg of Chl-a hr^{-1} (Fogg *et al.*,

1973).These specific activities are comparable to the level of ARA with *Chlorogloeopsis fritschii* BTA9016. The cyanobacterium *Chlorogloeopsis fritschii* BTA9016 may be exploited because of its merits on yield and utility of cellular constituents. The rate of acetylene reduction increases with maturity of heterocysts and maximum activity was observed when the frequency of heterocysts was higher. This strain expressed high amount of ARA and may be considered as a good candidate for their utilization as cyanobacterial biofertilizer. The lipid including linolelaidic Acid Methyl Ester and *cis*-11-Eicosenoic Acid Methyl Ester for this strain may be important for nutraceutical and pharmaceutical industry.

Nucleotide sequences of 16S rRNA gene obtained from DNA sequences were compared with other cyanobacterial sequences from the NCBI database designated as *Chlorogloeopsis fritschii* by using BLAST (http:// www.ncbi.nlm.nih.gov/ BLAST). For the purpose of phylogenetic analysis, we have selected mainly NCBI sequences of identified and described members of these genera at the species level trying to avoid sequences of those members determined only at the generic level. Multiple sequence alignment was performed using the CLUSTAL W (Thompson *et al*., 1994) tool within alignment function of MEGA 4.0 (Tamura *et al*., 2007) phylogenetic package. Phylogenetic trees were computed by MEGA 4.0 using neighbor-joining (NJ) algorithms. Algorithm was performed with 1,000 bootstrap replicates. Nucleotide positions contained gaps and missing data were eliminated from the data set. Based on phylogenetic relationships of 16S rRNA nucleotide sequences, it could be interpreted that *Chlorogloeopsis fritschii* was evolved among the nitrogen fixing strains. The clustering of morphological distinct groups in the present study was supported by the previous work described the phylogeny of cyanobacteria obtained from 16S rRNA gene sequences (Giovannoni *et al*., 1988).

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