Physiological and Biochemical Characterization of a Thermophilic Diazotrophic Cyanobacterium *Mastigocladus* species

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ABSTRACT

A heterocystous branched filamentous cyanobacterium was isolated from hot spring at Jakrem, Meghalaya (India), and identified as *Mastigocladus* sp. (cell division occurring in more than one plane). Physiological and biochemical characterization of this strain showed that this cyanobacterium is a thermophile capable of growth, N_2-fixing and nitrogen assimilation at elevated temperatures. When grown at different temperatures, the *Mastigocladus* sp. grew better and showed higher heterocyst frequency and nitrogenase activity at 45°C than at 25°C. There was a significant increase in activities of nitrate reductase activity and glutamine synthetase, and rates of photosynthesis and respiration in cultures grown at 45°C than those grown at 25°C.

Key words: Hot springs, Thermophilic cyanobacterium, Mastigocladus *species, Nitrogen fixation, Biofertilizers*

Introduction

Temperature is one of the most important environmental factors controlling the activities and evolution of organisms, and is one of the easiest variables to measure. High temperature and low combined nitrogen source in the hot springs, favour the growth of N_2-fixing organisms including cyanobacteria (Ward and Castenholz, 2000). However, very few studies have been conducted on thermophilic cyanobacteria. *Mastigocladus* species are known to be a component of algal-bacterial mats in neutral to alkaline thermal springs (Castenholz, 1976, 1977; Fagerberg and Arnott, 1979), being capable of cell division and growth at temperature ranging from 5°C to 64°C (Holton, 1962; Castenholz, 1969; Stevens *et al.*, 1985) and a pH range of 4.8 to 9.8 (Brock and Brock, 1970; Binder *et al.*, 1972). Such an organism may be of great use as biofertilizer in tropical rice fields. We examined a hotspring at Jakrem, Meghalaya (India) for presence of thermophilic N_2-fixing cyanobacteria and isolated a heterocystous cyanobacterium that has been identified as *Mastigocladus* species. Morphological features, growth, heterocyst frequency, enzymes of nitrogen metabolism (activities of nitrogenase, nitrate reductase, glutamine transferase synthetase), photosynthesis, respiration, and phycobiliproteins of the isolated *Mastigocladus* species were studied in cultures grown at 25°C and 45°C, and the results are reported here.

Materials and Methodology

Culture conditions

Mastigocladus sp. was grown under continuous light (photon flux rate of 50 μmol. m^{-2} s^{-1} on the surface of the vessels) in axenic aerated batch cultures in D-medium (Castenholz, 1981) at 45°C inside a B.O.D. incubator or at 25°C in a sterile culture room. The cultures were maintained on agar slants as well as in liquid media (D-N_2 medium, D-nitrate medium or D-NH_4 medium).

Morphology

The cultures were studied by light microscope and whenever necessary, light micrographs were taken using the Jenaval (Carl Zeiss Jena) Research Microscope.

Growth, heterocyst frequency and nitrogenase activity

Growth was measured as increase in concentration of Chl *a* as described by Mackinney (1941). Heterocyst frequency was calculated as percentage of total cells by light microscopic observations after 96 h of incubation in different nitrogen media. Acetylene reduction assay was used to measure nitrogenase activity (Stewart *et al.*, 1967).

Glutamine synthetase (transferase) and nitrate reductase activities

Glutamine synthetase (transferase) activity was measured as described by Sampaio *et al.* (1979). Nitrate reductase (NR) activity was measured as described by Manzano *et al.* (1976) using the ultrasonicated culture suspension.

Oxygen exchange

Oxygen evolution and consumption was measured by using a Clark-type oxygen electrode installed in a 3 ml Plexiglass container with magnetic stirring (Rank Brothers, England).

Phycobiliprotein and protein content

The phycobiliprotein content [phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE)] was determined according to Bennett and Bogorad (1973). Protein content was measured according to Lowry *et al.* (1951).

Results

Jakrem hotsprings contain extensive cyanobacterial mats (Fig. 1a). An examination of these mats under light microscope revealed that *Mastigocladus* was the only cyanobacterium present. This organism is a heterocystous branched-filamentous cyanobacterium belonging to Section V as per Rippka's classification of cyanobacteria (Rippka *et al.*, 1979). It exhibits true branching, undergoes cell division in more than one plane, and consists of three cell types: vegetative cells, heterocysts, and akinetes (Fig. 1b).

Studies on growth of *Mastigocladus* sp. at 25°C and 45°C in media containing N_2, NO_3^- or NH_4^+ as inorganic nitrogen-sources are presented in Fig 2. Nitrate served as the best source of nitrogen for growth (measured as increase in Chl *a*), followed by ammonium and then N_2. However, unlike most heterocystous cyanobacteria, *Mastigocladus* sp. was found to grow better at 45°C. While the trend of relative growth performance in different nitrogen-media remained similar to that at 25°C, the growth was significantly higher at 45°C than that at 25°C in all cases. A

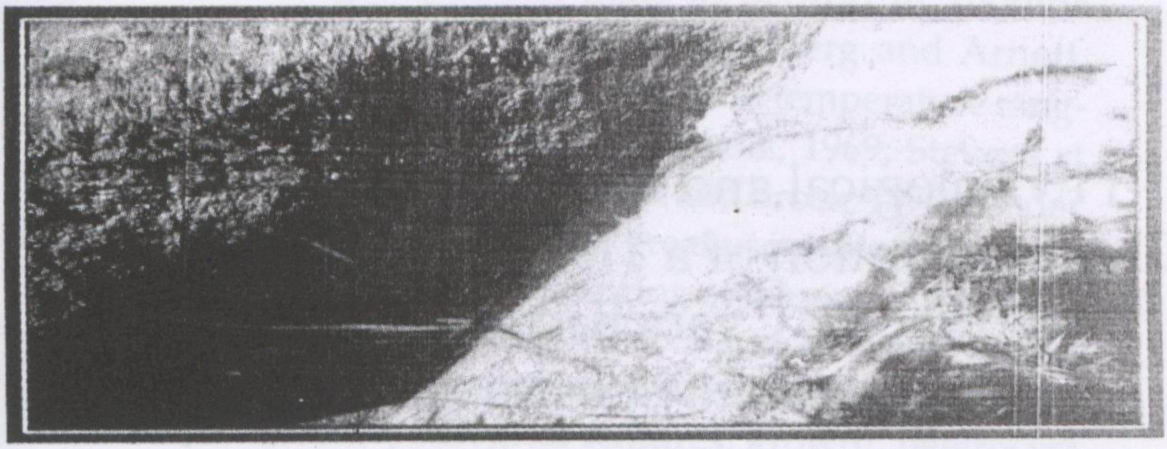

Fig. 1a. *Mastigocladus* sp. mats in the hot spring at Jakrem (Meghalaya, India).

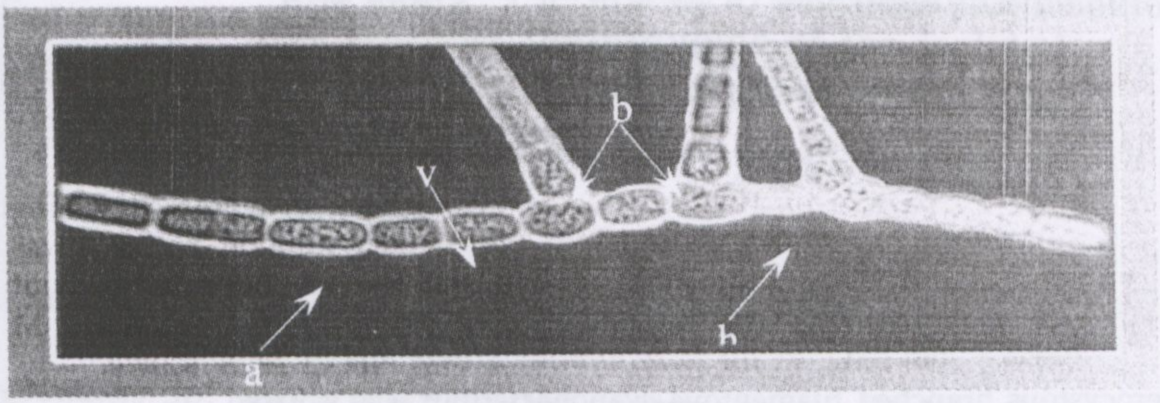

Fig. 1b. A filament of *Mastigocladus* sp. with heterocyst (h), akinete (a), vegetative cells (v) and branches (b). Magnification 40X.

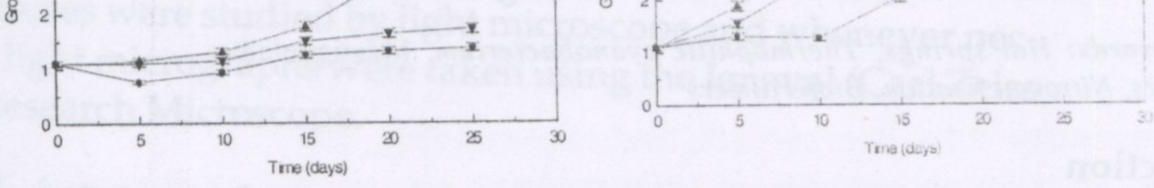

Fig. 2. Growth of *Mastigocladus* sp. in media containing different nitrogen sources at 25ºC (A) and 45ºC (B). Growth in terms of µg Chl *a*. ml^{-1} was determined after intervals of five days. The initial inoculum concentration was 1.1 µg Chl *a*. ml^{-1}. N_2 refers to medium D without any N (•), NO_3^- to medium D with 10 mM $NaNO_3$ (▲), and NH_4^+ to medium D with 2 mM NH_4Cl as N source (▼%).

similar trend was observed with regard to protein content (Fig. 3). *Mastigocladus* sp. differentiated heterocysts and showed nitrogenase activity in N_2-medium (Table 1). Both heterocyst frequency and nitrogenase activity were higher at 45°C than that at 25°C. No

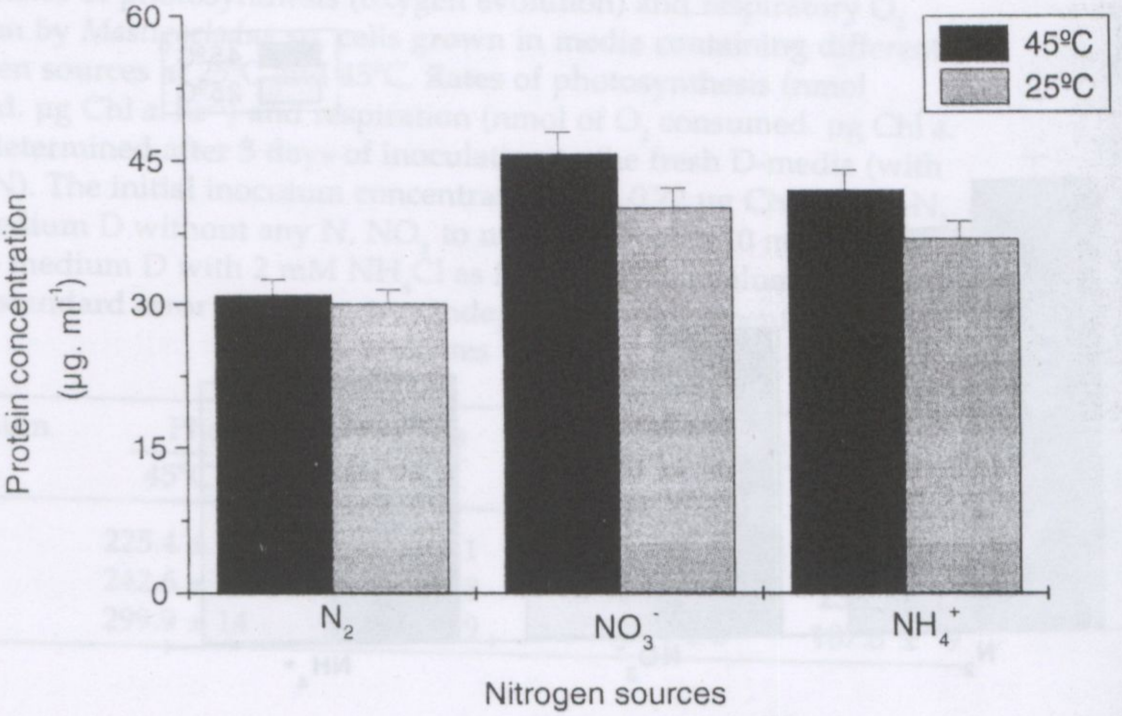

Fig. 3. Protein content of *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 25°C and 45°C. Protein concentration (μg. ml^{-1}) was determined after 4 days of inoculation into the fresh D-media (with and without N). N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl as N source.

Table 1. Heterocyst frequency and nitrogenase activity of *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 25°C and 45°C.

Heterocyst frequency (%) and nitrogenase activity (nmol of C_2H_4 formed. μg^{-1} Chl *a*. hr^{-1}) were determined after 4 days of inoculation into the fresh medium-D with and without N (Castenholz, 1981). The initial inoculum concentration was 1.5 μg Chl *a*. ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl as N source

Medium	Heterocyst frequency (%)		Nitrogenase activity	
	45°C	25°C	45°C	25°C
N_2	10 ± 0.5	3 ± 0.2	4.2 ± 0.2	2 ± 0.1
NO_3^-	0.0	0.0	0.0	0.0
NH_4^+	0.0	0.0	0.0	0.0

heterocyst formation or nitrogenase activity was detected in NO_3^- or NH_4^+-medium at either of the temperatures.

The nitrate reductase activity in cyanobacteria is reported to vary in response to the nitrogen sources in the growth medium (Bagchi and Singh, 1984; Bagchi *et al.*, 1985*a*, *b*). Nitrate reductase activity was studied in *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 25°C and 45°C (Fig. 4). Cells grown at 25°C in N_2-medium showed a NR activity of 8.5 nmol NO_2^- formed. min^{-1}. mg^{-1} protein. The activity was similar in cells grown in NO_3^--medium (9 nmol NO_2^- formed. min^{-1}. mg^{-1} protein) but it was repressed by 54 % in cells grown in NH_4^+-medium (3.9 nmol NO_2^- formed. min^{-1}. mg^{-1} protein). A similar pattern of NR activity was found in cells grown at 45°C in media containing different nitrogen sources. Furthermore, in all the media, NR activity in cells grown at 45°C was higher than that in corresponding cells grown at 25°C. These results indicate that in *Mastigocladus* sp., NR is ammonium-repressible that is derepressed in absence of ammonium. Similarly, the activities of the primary ammonia-assimilating enzyme glutamine synthetase were higher in cells grown at 45°C than those at 25°C. GS activity was significantly higher in N_2-grown cells than in nitrate- or ammonium-grown cells both at 25°C and 45°C.

Photosynthesis is very intimately linked to nitrogen status of the cells since nitrogen is a vital constituent of several photosynthetic components. Nitrogen deficiency is known to cause impairments of photosynthesis (Apte, 1996). N_2-fixing cultures grown at 25°C showed O_2 evolution rates of 240 nmol O_2 evolved. μg^{-1} Chl *a*. h^{-1}. The rates were higher in NO_3^-- and NH_4^+-grown cultures (Table 2). While the trend of photosynthetic O_2 evolution by cells grown in N_2-, NO_3^--, and NH_4^+-media at 45°C was similar to those obtained at 25°C, the rates were higher at 45°C than the corresponding rates at 25°C.

Respiration rates (respiratory O_2 consumption) of cells grown in different nitrogen media showed a trend that was reverse of photosynthesis. At 25°C, higher rate of O_2 consumption occurred in N_2-grown cells (196 nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1}), followed by NO_3^--grown cells (142 nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1}) and NH_4^+-grown cells (122 nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1}). Similar trend was observed at 45°C but cells grown in all the three media (N_2-, NO_3^-- and NH_4^+-media) showed rates of O_2 consumption that were more than 50 % higher than the corresponding rates at 25°C (Table 2).

The phycobiliproteins [phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE)] are accessory photosynthetic pigments in cyanobacteria. Phycobiliprotein contents of cells grown in NO_3^-- and NH_4^+-media were higher than those in N_2-grown cells

Table 2. Rates of photosynthesis (oxygen evolution) and respiratory O_2 consumption by *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 25°C and 45°C. Rates of photosynthesis (nmol of O_2 evolved. µg Chl *a*. hr^{-1}) and respiration (nmol of O_2 consumed. µg Chl *a*. hr^{-1}) were determined after 5 days of inoculation to the fresh D-media (with or without N). The initial inoculum concentration was 0.72 µg Chl *a*. ml^{-1}. N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl as N source. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates

Growth-medium	Photosynthesis rates		Respiration rates	
	45°C	25°C	45°C	25°C
N_2	225.4 ± 11	236.2 ± 11	196.5 ± 9	293.5 ± 11
NO_3^-	242.6 ± 12	363.8 ± 18	142.9 ± 7	235.2 ± 11
NH_4^+	299.9 ± 14	390.3 ± 19	122.4 ± 6	187.8 ± 9

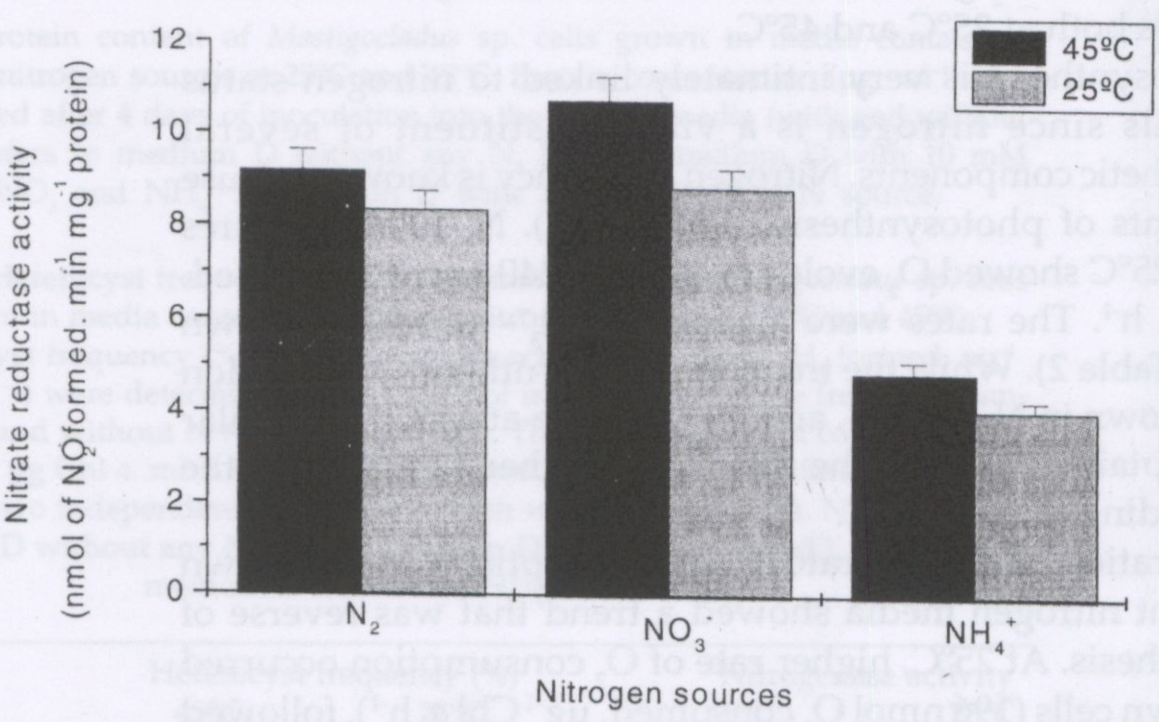

Fig. 4. Nitrate reductase (NR) activity of *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 25°C and 45°C. Nitrate reductase (nmol NO_2^- formed. $min^{-1}.mg^{-1}$ protein) was determined after 4 days of inoculation into the fresh D-media (with and without N). N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl as N source.

(Table 3). Furthermore, cells grown in NO_3^-- and NH_4^+-media at 45°C had significantly higher phycobiliprotein content than cells grown at 25°C.

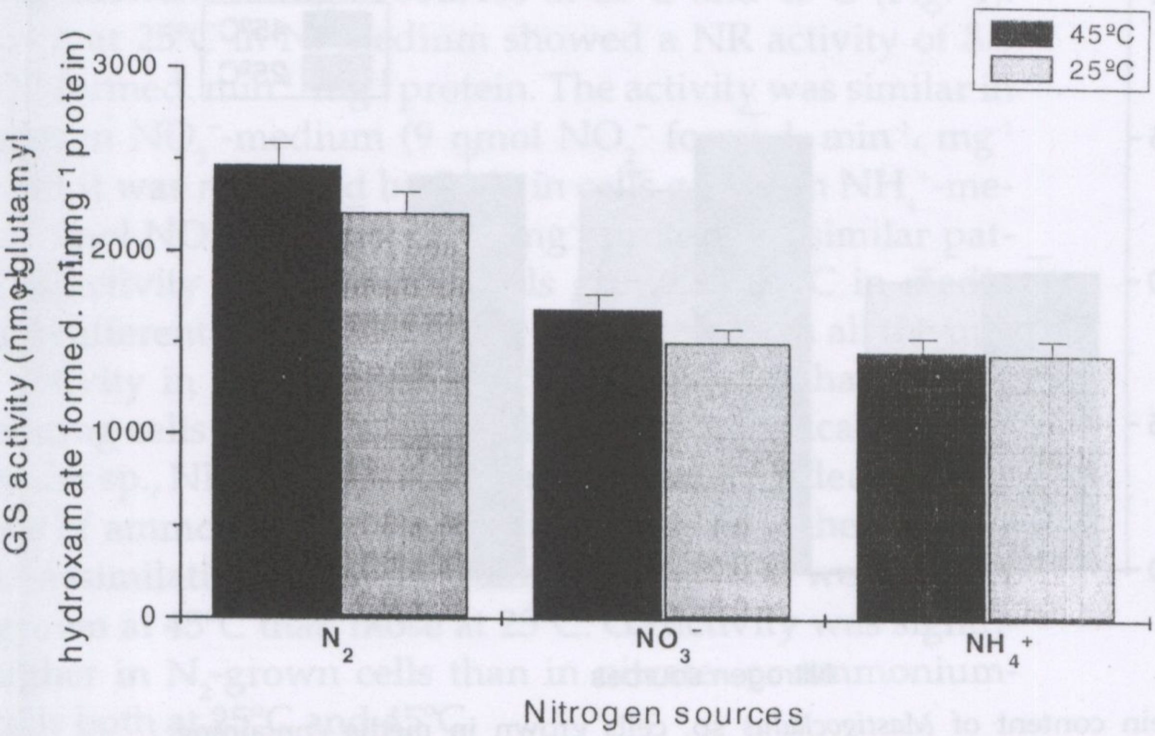

Fig. 5. Glutamine synthetase (transferase) activity of *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 25ºC and 45ºC. Glutamine synthetase (transferase) activity (nmol γ-glutamyl hydroxamate formed. min^{-1}. mg^{-1} protein) was determined after 4 days of inoculation in to the fresh D-media (with and without N). N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl as N source.

Discussion

The results presented here on *Mastigocladus* sp. being able to utilize all the three inorganic nitrogen sources for growth at 25ºC are consistent with earlier reports in other cyanobacteria (Herrero and Flores, 1990; Flores and Herrero, 1994; Herrero *et al.*, 2001; Bhattacharya *et al.*, 2002). The observations of higher heterocyst frequency and nitrogenase activity at 45ºC than that at 25ºC are consistent with reported growth of *Mastigocladus* sp. in hotsprings with temperatures upto 60ºC (Binder *et al.*, 1972). The repressive effect of NO_3^- and NH_4^+ on heterocyst formation and nitrogenase are also consistent with other heterocystous cyanobacteria (Stewart, 1980; Bhattacharya *et al.*, 2002).

The ammonium-repressible nature of NR in *Mastigocladus* sp. is consistent with findings in other cyanobacteria (Bagchi and Singh, 1984; Herrero *et al.*, 1981, 1985; Bagchi *et al*, 1985*a*, *b*; Martin-Nieto *et al.*, 1989; Rai *et al.*, 1992; Bhattacharya *et al.*, 2002). The derepressible nature of NR in *Mastigocladus* sp. is consistent with observations of Bagchi *et al.* (1985*a*) on *Nostoc muscorum* NR but in

Table 3. Phycobiliprotein (phycocyanin, allophycocyanin, phycoerythrin) contents of *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 25°C and 45°C. Phycobiliprotein [phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE)] contents ($\mu g. ml^{-1}$) were determined after 4 days of inoculation to the fresh D-media (with and without N). The initial inoculum concentration was 1.5 μg Chl a. ml^{-1}. N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl as N source. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates

Growth-medium	PC		APC		PE	
	25°C	45°C	25°C	45°C	25°C	45°C
N_2	2.12 ± 0.1	2.13 ± 0.1	2.01 ± 0.1	2.36 ± 0.1	1.48 ± 0.1	1.44 ± 0.1
NO_3^-	2.89 ± 0.1	3.65 ± 0.2	2.55 ± 0.1	2.74 ± 0.1	1.85 ± 0.1	2.17 ± 0.1
NH_4^+	2.79 ± 0.1	6.08 ± 0.3	2.40 ± 0.1	5.57 ± 0.3	1.79 ± 0.1	1.94 ± 0.1

contrast to the NO_3^--inducible nature of NR in *Anabaena cycadeae* (Bagchi *et al.*, 1985*b*). The lower GS activity in nitrate- and ammonium-grown cells is consistent with earlier observations that sources of combined-nitrogen repress GS activity in cyanobacteria (Merida *et al.*, 1991; Frias *et al.*, 1994; Bhattacharya *et al.*, 2002). The observations that NR and GS remain active, and infact show higher activity at 45°C, indicate that *Mastigocladus* sp. is indeed a thermophile and possesses mechanisms for NR and GS to function at higher temperatures.

Higher photosynthetic rates in NO_3^-- and NH_4^+-grown cultures are probably due to the increased nitrogen status leading to higher levels of photosynthetic accessory pigments (phycobiliproteins) of the cells grown in media containing combined-N sources such as nitrate and ammonium. Higher photosynthetic activity coupled with lower respiration rates may explain the better rates of growth in media containing nitrate and ammonia. Higher phycobiliprotein content in cells grown with combined-N (NO_3^-- or NH_4^+-media) is consistent with earlier observations (Stewart, 1980). However, higher phycobiliprotein content at 45°C is unique to *Mastigocladus*, consistent with its thermophilic nature and observed better growth, activities of nitrogen metabolizing enzymes, photosynthesis and respiration at elevated temperatures.

In conclusion, these results show that the *Mastigocladus* sp. utilized the inorganic nitrogen sources for growth. Nitrate served as the best N-source for growth followed by ammonium and N_2. The growth was significantly higher at 45°C than at 25°C while the trend of relative growth performance in different nitrogen media remained similar. Heterocyst frequency, nitrogen fixation, phycobiliprotein content, photosynthesis, respiration, and activities of NR and GS were all significantly higher in *Mastigocladus* cultures grown at 45ºC than those grown at 25ºC Thus, this *Mastigocladus* species appears to be a thermophile possessing mechanisms for functioning of its metabolism at elevated temperatures.

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