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ORIGINAL ARTICLE

Isolation and characterization of a *Mastigocladus* species capable of growth, N_2-fixation and N-assimilation at elevated temperature

Nonibala Khumanthem · Mayashree B. Syiem · Arvind K. Singh · Amar Nath Rai

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Abstract A *Mastigocladus* species was isolated from the hot spring of Jakrem (Meghalaya) India. Uptake and utilization of nitrate, nitrite, ammonium and amino acids (glutamine, asparagine, arginine, alanine) were studied in this cyanobacterium grown at different temperatures (25ºC, 45ºC). There was 2–3 fold increase in the heterocyst formation and nitrogenase activity in N-free medium at higher temperature (45ºC). Growth and uptake and assimilation of various nitrogen sources were also 2–3 fold higher at 45ºC indicating that it is a thermophile. The extent of induction and repression of nitrate uptake by NO_3^- and NH_4^+, respectively, differed from that of nitrite. It appeared that *Mastigocladus* had two independent nitrate/nitrite transport systems. Nitrate reductase and nitrite reductase activitiy was not NO_3^--inducible and ammonium or amino acids caused only partial repression. Presence of various amino acids in the media partially repressed glutamine synthetase activity. Ammonium (methylammonium) and amino acid uptake showed a biphasic pattern, was energy-dependent and the induction of uptake required *de novo* protein synthesis. Ammonium transport was substrate (NH_4^+) - repressible, while the amino acid uptake was substrate inducible. When grown at 25ºC, the cyanobacterium formed maximum akinetes that remained viable upto 5 years under dry conditions.

Keywords Thermophiles · Cyanobacterium · *Mastigocladus* · Nitrogen fixation · Sporulation · Nitrogen metabolism.

N. Khumanthem · M. B. Syiem · A. K. Singh · A. N. Rai (✉)
Department of Biochemistry,
North Eastern Hill University,

Vice Chancellor,
Mizoram University,
Aizawl-796009, Mizoram,
India
Tel: +91 / 389 / 2330650
Fax: +91 / 389 / 2330644
E-mail: raiamarnath@gmail.com

Introdcution

Most N_2-fixing cyanobacteria are mesophilic and show optimum N_2-fixing ability in the moderate temperature range of 20ºC–25ºC. Their various metabolic processes including N_2-fixing ability are adversely affected at high temperature. Information about N_2-fixation and assimilation is scanty in case of thermophilic cyanobacteria. One such thermophilic cyanobacterium is *Mastigocladus*. *Mastigocladus* is a heterocystous, branched filamentous cyanobacterium generally found in thermal waters on every continent[1]. They are the most thermophilic among N_2-fixing cyanobacteria with an upper temperature limit of 60ºC for N_2-fixation[2]. This feature of *Mastigocladus* sp. makes it ecologically important as a component of algal-bacterial mats in neutral to alkaline thermal springs[3,4]. We isolated a *Mastigocladus* sp. locally and characterized its potential for growth, N_2-fixation and nitrogen metabolism at 45ºC with a view to identify a thermophilic diazotrophic cyanobacterium that can be used as biofertilizer in tropical rice fields particularly during summer months.

Akinetes (spores) of cyanobacteria can serve as better inocula than vegetative filaments, since they can withstand adverse environmental conditions on storage, and can be transported in dry form requiring no special storage

conditions or packaging. Therefore, it is of great interest to study akinete differentiation in diazotrophic cyanobacteria and factors that may lead to quick and profuse formation of akinetes with high viability and germination frequency.

Materials and Methods

Isolation and purification: Samples were collected from the hot spring of Jakrem in the state of Meghalaya, India. These collections were examined under microscope and were found to be unialgal in nature. They were then serially diluted and plated on sterile D-nitrate medium[5] with 1.5% agar and incubated at 25ºC under light (photon fluence rate 50 µmol photons m^{-2} s^{-1}). When colonies appeared, individual colonies were picked up and viewed under microscope before subsequent re-plating in D-nitrate medium. The process was repeated several times until well separated colonies were obtained. These colonies were then purified by plating on solidified D-nitrate medium containing Polymixin-B sulphate (10 µg mL^{-1}) and Cyclohexamide (100 µg mL^{-1}). The individual *Mastigocladus* colonies were picked up under aseptic conditions and transferred to sterile liquid D-nitrate medium in test tubes. This procedure was repeated till axenic cultures of *Mastigocladus* were obtained.

Culture conditions: Mastigocladus sp. was grown in batch cultures using D-nitrate medium (10 mM $NaNO_3$)[5] at 25ºC (culture room) or at 45ºC (inside a B.O.D. incubator) with a photon fluence rate of 50 µmol m^{-2} s^{-1}. Whenever needed, cultures were also grown in N_2-medium (D-medium without any source of combined nitrogen), NH_4^+-medium (N_2-medium supplemented with 2 mM NH_4^+) or in N_2-medium supplemented with 1 mM L-glutamine, asparagine, arginine or alanine. The medium was buffered with equimolar concentration of HEPES and its pH was adjusted to 7.5 before autoclaving**.**

Growth, heterocyst frequency and nitrogenase activity: Growth was measured as increase in concentration of Chl *a* content[6]. Heterocyst frequency was calculated as percentage of total cells by light microscopic observations after 96 h of incubation in different media. Acetylene reduction assay was used to measure nitrogenase activity[7].

Nitrate and nitrite uptake assay: The N_2-, NO_3^--, and NH_4^+-grown cultures of *Mastigocladus* sp. were harvested by centrifugation during the exponential growth phase (after 4 days of growth), washed and resuspended in Tricine-$NaHCO_3$ buffer (25 mM, pH 8.1). Uptake experiment was started by addition of $NaNO_3$ (100 µM) or KNO_2 (100 µM) to the cell suspension. Uptake of nitrate and nitrite was measured by determining the rate of their depletion from the medium. The choice of 100 µM external concentration was based on earlier studies in *Anabaena* sp. PCC 7120 and *Synechococcus* sp. strain PCC 7942[8,9]. Samples were withdrawn after 3 h of incubation, subjected to rapid centrifugation at 5000g and the cell-free supernatants analyzed for residual nitrate or nitrite. Nitrate and nitrite concentrations were measured by the method of Cawse[10] and Snell and Snell[11], respectively.

Nitrate reductase, nitrite reductase and glutamine synthetase (transferase) activity: Glutamine synthetase (transferase) activity was measured as described by Sampio *et al*[12]. Ferredoxin-dependent nitrate reductase and nitrite reductase activities were measured using dithionite-reduced methyl viologen as reductant[13,14]. However, the incubation for the enzyme assays was performed at higher temperature (45ºC) for the cultures grown at 45°C, while for cultures grown at 25°C, the incubation was performed at 30°C. Protein concentration was measured according to Lowry *et al*[15].

Ammonium and amino acid transport assay: Ammonium transport assay was done using the radioactive analogue of ammonium, [^{14}C]-methyammonium (sp. activity 370 KBq. µmol^{-1}). Glutamine, arginine, alanine and asparagine uptakes were measured using ^{14}C-labeled glutamine (sp. activity 256 KBq. µmol^{-1}), arginine (sp. activity 65 KBq. µmol^{-1}), alanine (sp. activity 71 KBq. µmol^{-1}) and asparagine (sp. activity 63 KBq. µmol^{-1}). The NO_3-grown cultures of *Mastigocladus* sp. were harvested during the exponential growth phase, washed in N_2-medium and then incubated in N_2-medium, NO_3^--medium and N_2-medium supplemented with 1mM glutamine, arginine, alanine or asparagine for 48 h at 25ºC or 45ºC. After incubation in different nitrogen-media, the cells were harvested by centrifugation, washed and resuspended in 10 mM HEPES-NaOH buffer (pH 7.0). After equilibration for 1 h at 25ºC or 45ºC, radiolabeled methylammonium or amino acids were added to a final concentration of 50 µM. The uptake experiments were carried out at 25ºC or 45ºC at a photofluence rate of 50 µmol. m^{-2}. s^{-1}. Whenever needed, dichlorophenyldimethylurea (DCMU, 10 µM), or carbonyl cyanide chlorophenyl hydrazone (CCCP, 25 µM) were added to the cell suspension 30 minute prior to the addition of labeled amino acid and were present during the experiments. At different time intervals, 400 µl samples were taken out rapidly and the cells were separated from their suspension medium by centrifugation through silicon oil DC 550/dinonyl phthalate (40/60, v/v) into perchloric acid/water (15/85, v/v)[16]. The [^{14}C] in perchloric acid fraction was measured using a liquid

Scintillation counter (Model 1801, Beckman Instruments). Non-specific binding of [^{14}C]-methylammonium was determined by measuring its incorporation in toluene-treated cells[16].

Akinete frequency: Akinete frequency was calculated as percentage of total cell population by light microscopic observations using an Olympus BX-51 light microscope fitted with a JVC digital video camera in different media compositions and at different temperatures.

Results and Discussion

The morphology of the isolated cyanobacterium was studied under light microscope. The organism showed fluorescence for phycobiliproteins (data not shown), and heterocystous branched filamentous habit having cell division in more than one plane. The primary trichome becomes partly multiseriate with lateral uniseriate branches. Heterocysts in the primary trichome were predominantly terminal or lateral. The cyanobacterium was identified as *Mastigocladus* species in keeping with earlier description by Castenholz[1,3–5].

Mastigocladus sp. was initially grown in N_2-medium at a temperature range of 20ºC to 60ºC. Since maximum growth was observed at 45ºC, further studies were conducted only at 45ºC. However, parallel experiments were run at 25ºC for comparative purposes. *Mastigocladus* sp. was able to grow at the expense of N_2, NO_3^- or NH_4^+ as nitrogen source (Table 1). In addition, amino acids such as glutamine, asparagine, arginine and alanine also served as nitrogen source for growth. Among the inorganic N-sources NO_3^- supported best growth whereas among the organic nitrogen sources asparagine and glutamine served as best N-sources followed by arginine and alanine. In all cases significantly higher growth occurred at 45ºC than at 25ºC (Table 1). When grown in medium lacking combined nitrogen (N_2-medium), *Mastigocladus* sp. developed heterocysts and nitrogenase. Both the frequency of heterocysts and the nitrogenase activity were > 2-fold higher in *Mastigocladus* grown at 45ºC than that grown at 25ºC. However, no heterocyst were formed and nitrogenase activity was absent in media containing NO_3^-, NH_4^+ or amino acids (Table 1). These data indicate that *Mastigocladus* sp. was able to use N_2 as N-source for growth in absence of other utilizable sources of nitrogen and that when NO_3^-, NH_4^+, gln, asn, arg or ala were available there was complete repression of heterocyst formation and N_2-fixation. Thus, apart from being thermophilic in nature, this strain's growth and N_2-fixation characteristics were similar to those reported for most mesophilic cyanobacteria[17–20]. However, the complete repression of heterocyst formation and nitrogenase activity in arginine-supplemented media was in contrast to only a partial repression reported in *Anabaena* 7120[18] and *Nostoc* ANTH[20].

Cells grown at 25ºC in N_2-medium showed a nitrate uptake rate of 9.5 nmol nitrate taken up min^{-1} mg^{-1} Chl *a* and nitrite uptake rate was 8.7 nmol nitrite taken up min^{-1} mg^{-1} Chl *a* (Table 2). When the cells were grown in NO_3^--medium, the nitrate and nitrite uptake rates increased by 20% and 40%, respectively. This indicated that nitrate and nitrite uptakes were NO_3^--inducible. In contrast, the nitrate and nitrite uptake rate declined by 68% and 21% in cells grown

Table 2 Nitrate and nitrite uptake by *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 45ºC and 25ºC.

Nitrogen source in growth medium	Nitrate uptake (µmol min^{-1} mg^{-1} Chl *a*)		Nitrite uptake (µmol min^{-1} mg^{-1} Chl *a*)	
	45ºC	25ºC	45ºC	25ºC
N_2	27.04 ± 0.4	9.52 ± 0.4	21.74 ± 1.0	8.72 ± 0.4
NO_3^-	32.92 ± 1.6	12.29 ± 0.6	30.41 ± 1.5	11.17 ± 0.5
NH_4^+	8.81 ± 0.4	3.96 ± 0.2	17.29 ± 0.8	7.93 ± 0.3

N_2 = No combined nitrogen; NO_3^- = +10 mM $NaNO_3$ and NH_4^+ = +2 mM NH_4Cl.

Table 1 Growth (Gr), heterocyst frequency (HF) and nitrogenase (N_2ase) activity of *Mastigocladus* sp. grown in presence of different nitrogen sources.

Nitrogen source in growth medium	Growth (µg chl *a* mL^{-1})		HF (%)		N_2ase (nmol C_2H_4 (µg chl *a* h^{-1})	
	45ºC	25ºC	45ºC	25ºC	45ºC	25ºC
N_2	2.3 ± 0.1	1.6 ± 0.1	10.0 ± 0.5	3.3 ± 0.2	4.6 ± 0.2	2.6 ± 0.1
NO_3^-	3.1 ± 0.2	1.8 ± 0.1	0.0	0.0	0.0	0.0
NH_4^+	2.6 ± 0.1	1.7 ± 0.1	0.0	0.0	0.0	0.0
Gln	4.2 ± 0.2	2.2 ± 0.1	0.0	0.0	0.0	0.0
Asn	4.3 ± 0.2	2.3 ± 0.1	0.0	0.0	0.0	0.0
Arg	4.1 ± 0.2	1.8 ± 0.1	0.0	0.0	0.0	0.0
Ala	3.0 ± 0.2	1.3 ± 0.1	0.0	0.0	0.0	0.0

Chl *a* at 0 h = 1.5 µg mL^{-1}

Table 3 Nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (transferase) activities of *Mastigocladus* sp. cells grown in media containing different nitrogen sources at 45ºC and 25ºC.

Nitrogen source in growth medium	NR (nmol NO_2^- formed min^{-1} mg^{-1} protein)		NiR (nmol NO_2^- consumed min^{-1} mg^{-1} protein)		GS (nmol -glutamyl hydroxamate formed min^{-1} mg^{-1} protein)	
	45ºC	25ºC	45ºC	25ºC	45ºC	25ºC
NH_4^+	9.16 ±0.4	8.33 ± 0.4	162.53 ± 8	127.80 ± 6	2464 ± 123	2217± 110
Gln	10.72 ±0.5	8.81± 0.4	175.03 ± 8	133.03 ± 6	1689 ± 84	1507 ± 75
NH_4^+	4.87 ± 0.2	4.07 ± 0.2	52.65 ± 7	112.00 ± 5	1466 ± 73	1456 ± 72
Gln	7.65 ± 0.4	6.35 ± 0.3	65.12 ± 3	48.23 ± 2	1222 ± 61	693 ± 34
Asn	7.20 ± 0.4	6.25 ± 0.3	67.22 ± 3	52.00 ± 2	1355 ± 67	701 ± 35
Arg	5.88 ± 0.3	5.16 ± 0.3	55.63 ± 2	43.86 ± 2	979 ± 48	684 ± 34
Ala	6.98 ± 0.3	6.12 ± 0.3	69.46 ± 3	54.81 ± 3	788 ± 39	546 ± 27

in NH_4^+-medium, indicating a repression by ammonium that was more severe for nitrate uptake than nitrite uptake. When grown at 45ºC, a similar trend was found except that actual respective values were 2-3 fold higher in cultures grown at 45ºC than at 25ºC. While the induction of the uptake rates by NO_3^- is in keeping with reports on other cyanobacteria[21], it is interesting to note that NH_4^+ repressed nitrate uptake only partially and had an even lesser impact on nitrite uptake. This is in contrast to a complete repression of nitrate and nitrite uptake by ammonium in other cyanobacteria[21,22]. It is interesting to note here that the extent of induction by NO_3^- and repression by NH_4^+, varied for NO_3^- and NO_2^- uptake. This argues against a common NO_3^-/ NO_2^- transport system suggested for cyanobacteria[21]. Indeed based on genomic sequences and mutational studies, two independent nitrate/nitrite transport systems have been suggested in *Nostoc punctiforme*[23] and *Nostoc* ANTH[22], respectively.

Cells grown in N_2-medium at 45ºC showed nitrate reductase activity of 9.16 nmol nitrite formed min^{-1} mg^{-1} protein. The nitrate reductase activity was repressed by 16% in cells grown in presence of glutamine, 21% in presence of asparagine, 36% in presence of arginine and 24% in presence of alanine. In NH_4^+-grown cells, the NR activity was repressed by 47% and in NO_3^--grown cells the activity increased by approximately 10% (Table 3). Thus, the maximum repression of NR occurred in cells grown in NH_4^+-medium, substantial NR activity remained in cells grown in various nitrogen-containing media, and NO_3^- had little effect on NR induction. It appears therefore that NR in *Mastigocladus* sp. is an NH_4^+ repressible-derepressible system. The corresponding activities of NR in cells grown at 25ºC in various nitrogen-containing media followed a pattern similar to that at 45ºC, but the actual activities were lower (Table 3). These results are in agreement with earlier reports of partial repression of nitrate reductase activity by arginine in *Anabaena* 7120[18] and *Nostoc* ANTH[20], but in contrast to the reported induction of NR activity by arginine in *Oscillatoria chalybea*[24]. The fact that NR activity was repressed by NH_4^+ and derepressed in its absence is consistent with earlier reports that in some cyanobacteria NR is NH_4^+ repressible-derepressible[25-28].

Nitrite reductase (NiR) activity in N_2-grown cultures of *Mastigocladus* sp. was 162.53 nmol NO_2^-consumed min^{-1} mg^{-1} protein. NiR activity was repressed by 60% in cells grown in presence of glutamine, 59% in presence of asparagine, 66% in presence of arginine and 57% in presence of alanine. The NiR activity increased by 7.7% in NO_3^--grown cells and decreased by 6.2% in NH_4^+-grown cells (Table 3). The corresponding activities of NiR in cells grown at 25ºC in various nitrogen-containing media followed a pattern similar to that at 45ºC except that the actual activities were significantly lower (Table 3). While addition of amino acids in the growth media caused substantial repression in NiR in *Mastigocladus* sp. NO_3^- and NH_4^+ had little effect (<10%) on NiR activity. It appears therefore that unlike other cyanobacteria[21] nitrite assimilation in *Mastigocladus* sp. is not subjected to induction and repression by NO_3^- and NH_4^+, respectively.

Cells grown in N_2-medium at 45ºC showed glutamine synthetase (GS) activity of 2464 nmol -glutamyl hydroxamate formed min^{-1} mg^{-1} protein. This activity was repressed by 50% in cells grown in presence of glutamine, 45% in presence of asparagine, 60% in presence of arginine and 68% in presence of alanine. The corresponding activities of GS in cells grown at 25ºC in various nitrogen-containing media followed a pattern similar to that at 45ºC, but the actual activities were significantly lower, particularly in cells grown in amino acid containing media (Table 3). These results are in contrast to the earlier reports that there was no significant repression of GS activity in presence of amino acids in *Nostoc muscorum*[19] and *Nostoc* ANTH[20]. However, it is noteworthy that the cells grown in amino acid-containing media retained 32–50% GS activity. This is

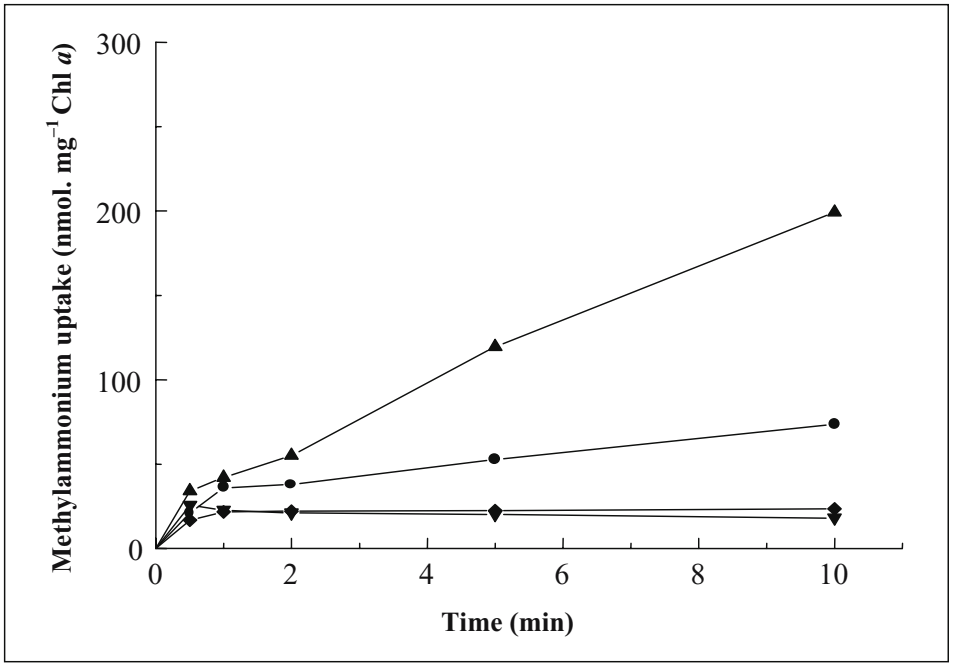


Fig. 1 [^{14}C]-methylammonium uptake in *Mastigocladus* sp. grown in N_2-medium (25ºC, ; 45ºC,), NO_3^--medium (45ºC,), and NH_4^+-medium (45ºC,). Nitrate–grown cultures (exponential phase) were washed and transferred to dif ferent nitrogen media and incubated for 48 h. The cultures were then harvested, washed and resuspended in HEPES buffer, and used for [^{14}C]-methylammonium uptake as described in Materials and Methods. The values presented are means from two independent experiments, each with two replicates. The values shown are corrected for non-specific binding of ^{14}C-methylammonium using toluene-treated cells.

not surprising since *Mastigocladus* sp. is able to use these amino acids as sole N-source for their growth and GS activity is essential for them to do so[19].

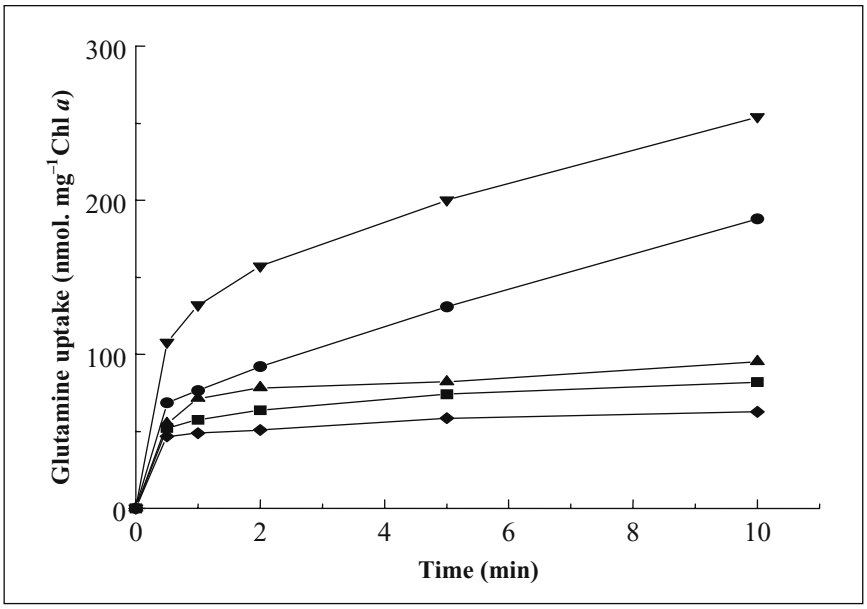
Using ^{14}C-methylammonium as probe, an energy-dependent and NO_3^-- and NH_4^+- repressible ammonium transport system, that is essential for cyclic retention of ammonia in the cells, has been characterized in several cyanobacteria[16,17,29,30]. Typically, in all cases, ^{14}C-methylammonium uptake shows a biphasic curve, a rapid first phase of accumulation lasting 60 s followed by a slower second phase linked to ^{14}C-methylammonium assimilation. ^{14}C-methylammonium uptake in N_2-grown *Mastigocladus* sp. also showed the characteristic biphasic pattern (Fig. 1) representing a rapid initial phase of accumulation lasting 60 s followed by a second slower phase. In N_2-medium, the methylammonium uptake rates in cultures grown at 45ºC were 42.00 and 15.77 nmol. mg^{-1} Chl *a* min^{-1} and at 25ºC , 36 and 3.7 nmol mg^{-1} Chl *a* min^{-1}, during the first and second phase, respectively. Higher uptake rates at 45ºC than at 25ºC are in keeping with that *Mastigocladus* sp. being a thermophile. As in other cyanobacteria[19,20], the ^{14}C-methylammonium uptake was repressed in cells grown in NO_3^-- and NH_4^+-media (Fig. 1). DCMU (an inhibitor of non-cyclic photosynthetic electron-transport) and CCCP (an uncoupler) inhibited intracellular accumulation of ^{14}C-methylammonium by 64% and 62%, respectively. This implies that the cellular uptake of ammonium is energy-dependent (Table 4) as reported for other cyanobacteria[16,20,29].

Table 4 Effect of inhibitors (DCMU and CCCP) on [^{14}C]-methylammonium and ^{14}C-glutamine uptake by *Mastigocladus* sp. at 45ºC.

Treatment	[^{14}C]-methylammonuptake rate (nmol mg^{-1} Chl *a* min^{-1})
Control	42.00 ± 2.1
Control + DCMU (10 μM)	15.15 ± 0.8
Control + CCCP (25 μM)	16.12 ± 0.8

[^{14}C]-methylammonium uptake measured in N_2-grown cells and [^{14}C]-glutamine uptake measured in cells grown in glutamine medium. Control refers to N_2-medium for [^{14}C]-methylammonium uptake and glutamine-medium for [^{14}C]-glutamine uptake.

Uptake of amino acids (Glutamine, asparagine, arginine and alanine) was studied in *Mastigocladus* sp. grown in media containing different nitrogen sources at 25ºC and 45ºC. The glutamine uptake by the cells was biphasic in nature consisting of an initial phase representing intracellular accumulation followed by a slower second phase representing assimilation. This pattern was independent of nitrogen-source used and temperature at which the cells



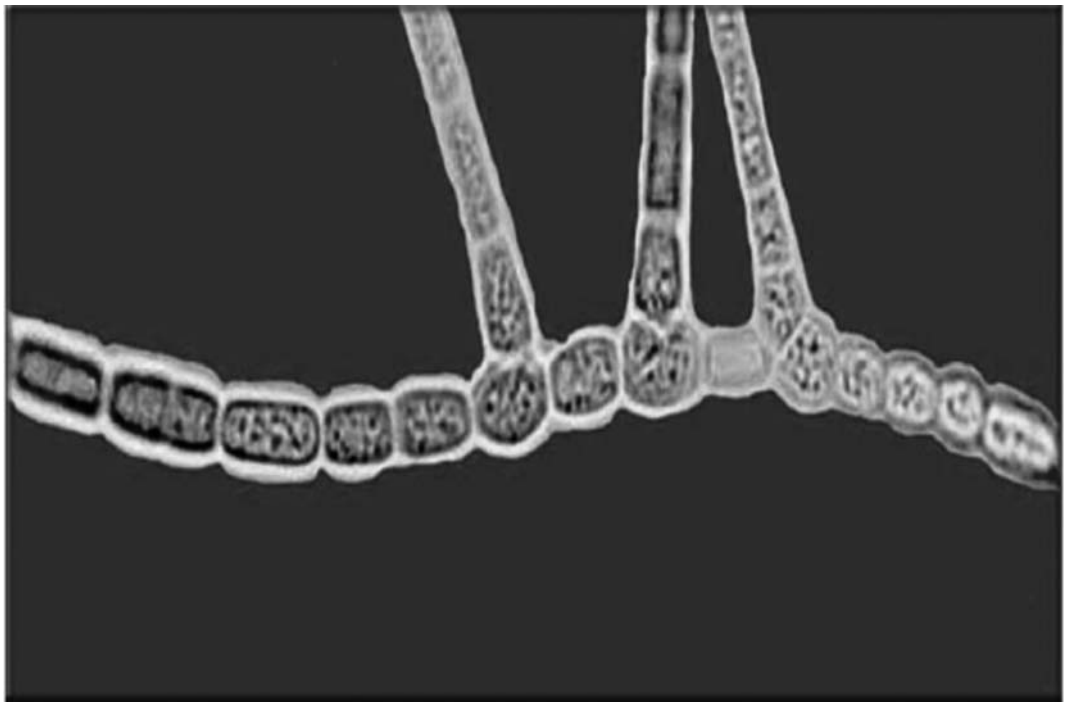


Fig. 2 [^{14}C]-glutamine uptake in *Mastigocladus* sp. grown in N_2-medium (25ºC, ; 45ºC,), glutamine-medium (25ºC, ; 45ºC,) and chloramphenicol treated glutamine-medium (45ºC,). Nitrate–grown exponential cultures were washed and transferred to N_2- or glutamine-medium (N_2-medium + 1mM glutamine) and incubated for 48 h at 45ºC or 25ºC. Chloramphenicol (1 µg. ml^{-1}) was added at the beginning of incubation to one set of glutamine-medium cultures (at 45ºC). After incubation, the N_2- and glutamine-grown cells were washed, resuspended in HEPES buffer and used for [^{14}C]-glutamine uptake as described in Material and Methods. Values are means from two independent experiments, each with two replicates.

were grown (Fig. 2). Cells grown in N_2-medium at 25ºC, showed a glutamine uptake rate of 57.61 and 5.25 nmol min^{-1} mg^{-1} Chl *a*, during the first and second phase, respectively. When grown in glutamine-containing medium, the uptake rate by the cells during the first phase increased by nearly 24% while the second phase was largely unaffected. Thus, growth in glutamine-medium resulted in increased rate of glutamine accumulation. Glutamine uptake rates by the cells grown at 45ºC responded similarly; however, the rates were much higher than those in cells grown at

Table 5 Time course of akinete differentiation and germination in *Mastigocladus* sp. on transfer to different growth media at 25ºC and 45ºC.

Time (days)	Akinete frequency (%)					
	N_2-medium		NO_3^--medium		NH_4^+-medium	
	45ºC	25ºC	45ºC	25ºC	45ºC	25ºC
0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.0	0.0
10	12.0	0.0	0.0	0.0	0.0	0.0
20	30.0±1.5	0.0	0.0	0.0	30.0±1.5	0.0
30	52.0±2.6	0.0	0.0	0.0	50.0±2.5	0.0
40	80.0±4.0	0.0±1.0	0.0	0.0	75.0±3.0	15.0±0.8

Table 6 Germination frequency of *Mastigocladus* sp. akinetes in media containing different inorganic nitrogen sources at 25ºC and 45ºC. Akinetes of *Mastigocladus* sp. were suspended in media containing different inorganic nitrogen sources at 25ºC and 45ºC to a concentration of 1.23 x 10^6 akinetes.ml^{-1} at time zero. Germination frequency (%) was determined in samples withdrawn at different time intervals. Other details as in legends to Table 1.

Time (days)	Germinationfrequency (%)					
	N_2-medium		NO_3^--medium		NH_4^+-medium	
	45ºC	25ºC	45ºC	25ºC	45ºC	25ºC
0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	1.0 ± 0.1	0.0	7.7 ± 0.4	0.0	6.0 ± 0.3
48	0.0	25.0 ± 1.2	0.0	30.0 ± 1.5	0.0	32.0 ± 1.6
72	0.0	55.0 ± 2.7	2.5 ± 0.1	60.0 ± 3.0	3.0 ± 0.1	75.0 ± 3.7
96	0.0	90.0 ± 4.5	8.5 ± 0.4	92.0 ± 4.6	6.2 ± 0.3	92.0 ± 4.6

25ºC (33% and 85% higher during first phase in N_2- and glutamine-grown cultures, respectively, and nearly 100% higher during second phase in both N_2- and glutamine-grown cultures). The induction of glutamine uptake (accumulation during first phase) in cells grown in glutamine-medium required *de novo* protein synthesis since chloramphenicol prevented such induction (Fig. 2). DCMU inhibited glutamine accumulation by 61% and CCCP by 83% (Table 4), indicating that the cellular accumulation of glutamine was energy-dependent. These results are consistent with energy-dependent amino acid uptake in *Anabaena* PCC 7120, *A. variabilis* and *Nostoc* ANTH[18,20,31,32]. Uptake of asparagine, arginine and alanine showed a pattern and characteristics similar to that observed for glutamine uptake described above (data not shown).

Vegetative cells of *Mastigocladus* differentiated into akinetes when grown in N_2- or NH_4^+-media, however, no akinetes were observed in cultures grown in NO_3^--media even after 40 days (Table 5). In cultures grown in N_2-medium at 25ºC, akinetes become visible by day 10 and 80% of the vegetative cells become akinetes after 40 days. However, when grown at 45ºC akinete differentiation was much delayed and even after 40 days only 20% of vegetative cells became akinetes. In cultures grown in NH_4^+-medium at 25ºC akinetes became visible by day 20 and reached a frequency of 75% after 40 days. As in the case of N_2-medium, akinete differentiation in NH_4^+-grown cultures at 45ºC was much delayed with 15% vegetative cells becoming akinetes after 40 days. There are no published reports on sporulation in *Mastigocladus* sp. but our data suggest that less favourable growth conditions result in quick and profuse sporulation in this thermophilic strain.

Germination of akinetes varied according to the nitrogen source in the growth media and temperature at which the akinetes were incubated. At 45ºC, akinete germination started within 24 h in N_2-medium, NO_3^-- and NH_4^+-medium (Table 6). In contrast, it took 3 days in NO_3^-- and NH_4^+-medium, and 20 days in N_2-medium for akinete germination to start at 25ºC. Thus, more favourable growth conditions favours quicker akinete germination.

The *Mastigocladus* sp. akinetes showed prolonged viability. Even after 5 years of storage in dry state at room temperature, over 95% of the akinetes germinated on transfer to NO_3^--medium at 45ºC. The ability of *Mastigocladus* sp. to grow and fix N_2 at high temperature, makes it suitable for application as biofertilizer for tropical/summer rice crop. It's ability to spontaneously sporulate profusely at lower temperature is an added advantage that will be useful for inoculum preparation and distribution.

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