Journal of Experimental Sciences 2011, 2(9): 36-40
ISSN: 2218-1768
www.scholarjournals.org

journal of experimental sciences

www.jexpsciences.com

JES-Life Sciences

Nostoc cyanobiont in the Cyanolichen, *Sticta weigelii* of Eastern Himalayan Region: Isolation, Physiological and Biochemical Characterization

Mayashree B. Syiem*, Luxemburgh Hynniewta, Athokpam Pinokiyo*

Department of Biochemistry, North Eastern Hill University, Shillong 793022, Meghalaya, India
**Presently at P.G. Department of Botany, D.M. College of Science, Imphal, Manipur, India*

Article Info

Article History

Received : 05-05-2011
Revised : 11-07-2011
Accepted : 12-07-2011

*Corresponding Author

Tel : +91-9863063294
Fax : +91-3642550108

Email: mayashreesyiem@yahoo.co.in

Abstract

A cyanobacterium identified as *Nostoc* sp. was isolated from the cyanolichen, *Sticta weigelii* (Ach.) Vainio collected from tree barks in the state of Meghalaya and its various physiological and biochemical characters were evaluated. When the lichen thalli were incubated in liquid medium, the *Nostoc* colonies from these thalli appeared initially in distinct sacs that released individual long heterocystous filaments into the liquid medium upon sub-culturing. The well spaced heterocystsin the filaments had a frequency of 7-8%. Chlorophyll *a* concentration of the cyanobacterium was higher in comparison to many other *Nostoc* species isolated from soil and water from the same region. Its photosynthetic activity was 519.24±0.7 nmol O_2 evolved μg^{-1} chl *a* h^{-1} whereas respiration was 286.35±0.5 nmol O_2 consumed μg^{-1} chl *a* h^{-1}. Enzyme assays reflected nitrogenase activity of 6.98 nmol C_2H_4 produced μg^{-1} chl *a* h^{-1} and GS transferase activity of 712±3.0 nmol γ-glutamyl hydroxymate formed min^{-1} mg^{-1} protein. Nitrate reductase activity was substrate inducible. Ammonium uptake was biphasic in nature and glutamine uptake was energy dependent.

Key Words: Cyanolichen, cyanobacteria, nitrogenase, biochemical activities.

Introduction

Lichens are amongst the most widely distributed eukaryotic organisms in the world. They are symbiotic associations between two (bipartite lichen) unrelated organisms- a fungus (mycobiont) and an alga (phycobiont) or a cyanobacterium (cyanobiont) - or among three (tripartite lichen) unrelated organisms- a fungus, an alga and a cyanobacterium. Lichens have an organized and stable morphology distinct from either of the symbiotic partners. A wide range of cyanobacteria are found in lichen symbiosis. Most common are the heterocystous forms such as *Nostoc, Calothrix, Scytonema* and *Fischerella* and unicellular forms such as *Gloeocapsa*, *Gloeothece* and *Synechocystis*. The cyanobiont may be localized in distinct layer or may be dispersed throughout the thallus. The cyanobiont-mycobiont specificity is low as same cyanobacteria can be found in different lichens or *vice versa*. Within the lichen thalli cyanobiont shows structural and functional changes that facilitates close interaction and nutrient exchange between the partners. These changes include loss of filamentous appearance, larger cell size, altered cell shape, slower growth and cell division and reduced polyphosphate reserves and carboxysomes. Therefore, it is almost impossible to identify and characterize the cyanobacterial partners inside the lichen thalli. In all bipartite lichens studied till date, the cyanobiont is photosynthetic and transfers fixed carbon to the mycobiont in most cases in the form of glucose (Richardson *et al*.,1968). In lichens, heterocyst frequency is altered. In bipartite lichens, the cyanobiont has heterocyst frequency close to its free-living counterpart (~5%) while in tripartite lichens, the heterocyst frequency is much higher. This may be caused by stimulation of heterocyst formation due to photosynthate movement from phycobiont to the cyanobiont (Hitch and Millbenk, 1975). ^{15}N tracer studies indicated that 90% of N fixed in *Peltigera aphthosa* and about 50% of that fixed in *P. canina* was released by the cyanobiont (*Nostoc*) because of repression of GS in the heterocysts (Rai and Bergman, 2002). Glutamine synthetase activity in the thallus of *P. aphthosa* was 25.7 nmol product formed min^{-1} mg^{-1} protein while in free living *Nostoc* isolate was 70 nmol product formed min^{-1} mg^{-1} protein and for *P. canina* was 0.8 nmol product formed min^{-1} mg^{-1} protein and for free living *Nostoc* isolate of *P. canina* was 72.4 nmol product formed $min^{-1}mg^{-1}$ protein. When the cyanobiont was isolated and cultured in its free-living state, it showed different characteristic values for various enzyme activities than those it exhibited while in symbiosis in the lichen thallus.

The present study describes the procedure of isolation of the cyanobiont from *Sticta weigelii*, its identification based on morphological studies and characterization of the free-living cyanobacterium for various growth parameters and enzyme activities.

Materials and Methods

Collection and identification of the cyanolichen

The lichen thalli were collected from the bark of a dicot tree from the permanent campus of North Eastern Hill University, Umshing, Shillong (Fig. 1). The specimen was carefully removed from its substratum. Some of the thallus was kept fresh while some were dried and transferred to a suitable herbarium packet. It was identified with the help of well established lichenological methods. Morphological characters were studied under binocular microscope Olympus D SZ 61. Thin vertical sections of thallus were prepared with the help of a sharp blade, stained in cotton blue–lacto-glycerol solution for anatomical studies under Olympus BX 60. For determining lichen chemicals, spot colour test were performed with the chemicals such as 10% K (Potassium hydroxide solution), C (Calcium hypochloride solution), KC and P (*p*-phenylene diamine solution), by putting directly on the thallus and medulla of the specimen and TLC (thin-layer chromatography) was done following the method described by White & James (1985). The specimen was identified with the help of authentic literature (Awasthi, 2000; Sinha and Singh, 2005) and consulting ASSAM herbarium at Botanical Survey of India, Eastern circle, Shillong. The specimen was deposited in the Herbarium at NEHU, Shillong.

Isolation of the cyanobacterium

10 mg of lichen thalli were cleaned and treated with sodium hypochloride (1%), for 5 minutes, followed by rinsing thoroughly with double distilled water and BG-11_0 media (Rippka et al., 1979) and then inoculated in to the same medium for isolation of the associated cyanobacterium. Isolated cyanobacterium was purified by serial dilution and pour plating on 1.2% nutrient agar. Pure cultures of the cyanobacterium were maintained in culture room at 25 ± 2°C and under light at a photon fluence rate of 50 µmol m^{-2} s^{-1}.

Identification of cyanobacterium

The cyanobacterium was identified with the help Olympus BX 60 microscope with reference to Desikachary (1959), Komárek et al. (2003) and Kondrateva (1968).

Growth Measurement

Growth was measured as increase in chlorophyll *a* content of the sample using spectrophotometric method described by McKinney (1941).

Heterocyst Frequency

Heterocyst frequency was calculated as percentage of total cells by using an Olympus BX 60 light microscope (Wolk, 1965).

Nitrogenase activity

5 ml exponentially growing liquid culture was used to measure nitrogenase enzyme (EC1.18.6.1) activity *in vivo* by gas chromatography using acetylene reduction assay (Stewart *et al.,* 1967). The culture was placed in 15 ml serum vial and 1 ml air in the experimental vial was replaced by 1 ml of pure acetylene. The experiment was done in duplicate. These vials were incubated for 1 hour at 25 ± 2°C with continuous shaking. Ethylene produced was measured using a Varian 3900 gas chromatograph fitted with a porapak 'T' column and a flame ionization detector.

Assay of electron transport activities

O_2 exchange in terms of O_2 evolution and consumption were measured using a Clark-type polarographic O_2 electrode installed in a 3 ml Plexi glass container with magnetic stirring (Robinson *et al.*, 1982). This measurement was done at 25 ± 2°C and at a photon fluence rate of 20 µmol m^{-2} s^{-1}.

Glutamine synthetase (Transferase) and nitrate reductase activity

Glutamine synthetase activity was measured as described by Sampaio *et al.* (1979) after the cells were treated with cetyltrimethylamoniumbromide (CTAB) which permeabilized the cells. Ferredoxin-dependent nitrate reductase activity was measured using dithionite-reduce methyl viologen as reductant (Manzano *et al.,* 1976). Nitrate was estimated spectrophotometrically as described by Snell & Snell (1949).

Ammonium transport activity

This was measured using ammonium analogue [^{14}C] methyl ammonium as described by Rai *et al*., (1984). Exponentially growing *Nostoc* cells were harvested after centrifugation, washed twice in 10 mM HEPES-NaOH buffer (pH 7) and resuspended in the same buffer to a chlorophyll concentration of 5µg ml^{-1}. Cells were equilibrated for 30 minutes. Methyl ammonium uptake activity experiments were carried out by adding [^{14}C] methyl ammonium (sp. activity 172 KBq.μmol^{-1}) to a final concentration of 50 µM. 400 µL samples were withdrawn at specific time intervals and cells were separated from the medium by centrifugation through silicon oil DC 550/dinonylphthalate (40/60, v/v) into perchloric acid/water (15/85, v/v). The [^{14}C] quantity in perchloric acid fraction was determined by using liquid scintillation counter .

Results

Cyanolichen

The cyanolichen was identified as *Stictaweigelii* (Fig 2a).A short taxonomic account is described below:

Sticta weigelii (Ach.) Vainio, Acta Soc. Fauna Fl. Fenn.7: 189, 1890; Joshi & Awasthi, Biol. Mem. 7: 186, 1982.

Thallus not gelatinous, foliose, mostly ascending or loosely appressed, greenish grey to green, irregularly lobed, heteromerous, with algal free medulla; lobes variable in shape, with over 5mm wide, margin wavy or incised, lower surface provided with small white dots representing cyphellae, upper surface isidiate or smooth; cyphellae white, restricted to lower surface, with prominent white margin; cortex uncoloured to slightly greenish, medulla compact, outer portion red-pigmented; central axis solid, colourless.

This specimen was sterile. Chemistry: Cortex- K-, P-, KC-; Medulla, K-, P-, KC-, C-; No lichen substances are present.

Morphology of the isolated cyanobacterium

Within 30-45 days, minute green colonies were seen growing out of the lichen thallus into the medium (Fig. 2c). The cyanobacterium emerging out of the lichen thallus showed special morphogenesis which is different from its morphology within the lichen thallus (Fig 2b). They were present in distinct packets (Fig 2d-e). On further transfers into liquid media enclosed colonies escaped from the sacs in form of long filaments with distinct, well spaced heterocysts (Fig 2f, g). The cyanobacterium was identified as *Nostoc* sp. with close similarity to *Nostoce daphicum* Kondrateva and *Nostoc*

calcicola Bréb. which are characterized by terminal conical heterocysts and narrow, straight hormogonia.

Growth

Once purified and cultured in liquid medium, the cyanobacteria showed profuse growth with increase in chlorophyll *a* and protein content. The heterocyst frequency reached 7-8% in second generation cells. It exhibited sigmoidal growth curve that extended over 40 day period (Fig. 3).

Physiological and biochemical studies

The chlorophyll *a* concentration in the cyanobacterium was found to be higher in comparison to many other *Nostoc* species isolated from soil and water samples (6.18 ± 0.4 as against 4-6 µg chlorophyll *a* ml^{-1} of culture on 15th day). Under optimum conditions, the organism showed extended growth period (Fig. 3). The heterocyst frequency was found to be 7-8% in the filaments. The nitrogenase activity was 6.98 ± 0.9 nmol C_2H_4 produced μg^{-1} chl *a* h^{-1}. Photosynthetic oxygen evolution in the cyanobacterium was 519.24 ± 0.7 nmol O_2 evolved μg^{-1} chl *a* h^{-1} while respiratory oxygen consumption was 286.35 ± 0.5 nmol O_2 consumed μg^{-1}chl*a* h^{-1}. GS transferase activity was 712 ± 3.0 nmol γ-glutamylhydroxymate formed min^{-1} mg^{-1} protein, nitrate reductase activity was 4.2 ± 0.1 nmol nitrite formed min^{-1} mg^{-1} protein and was substrate inducible, ammonium uptake was biphasic in nature. Methylammonium uptake marked by an initial rapid phase lasting for 60 s, followed by a slower second phase, which remained linear during the next 10 min of the experimental period. A value of 129 ± 0.3 nmol$^{14}CH_3NH_3^+$ taken up mg^{-1}chl*a* min^{-1} was observed at 10 minutes after addition of [^{14}C] methyl ammonium to the experimental medium (Table 1). Glutamine uptake was energy dependent in the free-living cyanobacterium as addition of CCCP (protonophore) and DCCD (inhibitor of F_0 part of ATPase) led to drastic reduction in glutamine uptake in the cyanobacterium (data not shown).

Table 1. Characteristic features and enzyme activities in the cultured *Nostoc* sp. isolate of the cyanolichen*Sticta weigelii*

Growth (µg chlorophyll *a* ml^{-1} of culture) on 15th day	6.18 ± 0.4
Heterocyst frequency (percentage of total cells)	7 - 8%
Nitrogenase activity (nmol C_2H_4 produced μg^{-1} chl *a* h^{-1})	6.98 ± 0.9
Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})	519.24 ± 0.7
Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	286.35 ± 0.5
Glutamine synthetase (Transferase) activity (nmol γ-glutamylhydroxymate formed min^{-1} mg^{-1} protein)	712 ± 3.0
Nitrate reductase activity (nmol nitrite formed min^{-1} mg^{-1} protein) in nitrate medium	4.2 ± 0.1
Ammonium uptake (nmol$^{14}CH_3NH_3^+$ taken up mg^{-1}Chl *a*) at 10 minutes after addition of [^{14}C] methyl ammonium to the experimental medium	129 ± 0.3

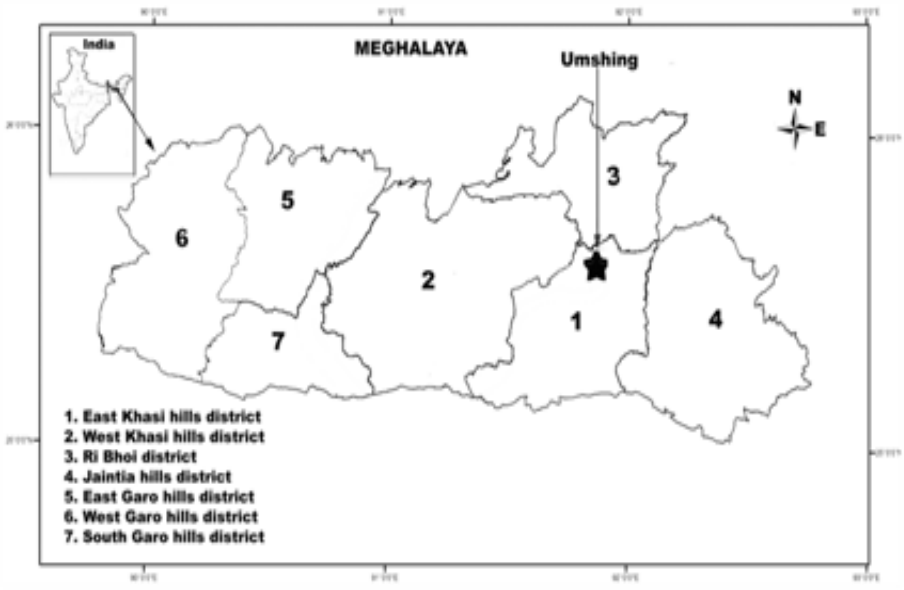

Fig 1. Map showing location of collection site of *Sticta weigelii*

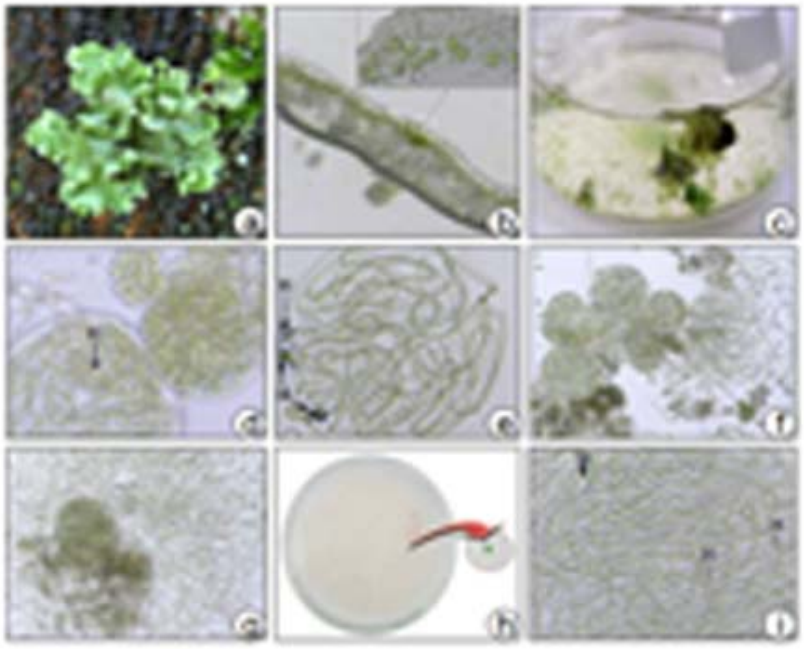

Fig 2. a. *Sticta weigelii* in nature b. Cyanobacteria in distinct layer within lichen thallus c. Cyanobacteria growing out of lichen thallus in liquid medium d-e. Cyanobacterial development within distinct packets in the early stages of isolation, (H-Heterocyst), f-g. Bursting of sacs releasing the cyanobacterium h. Purification of cyanobacterium i. Pure culture of cyanobacterium a *Nostoc* species.

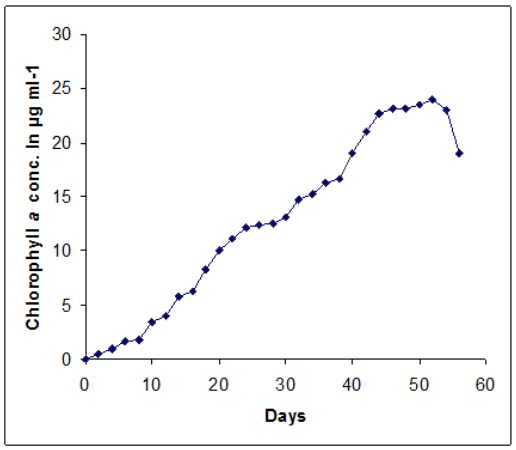

Fig 3. Growth Curve of isolated *Nostoc* sp.

Discussion

The cyanolichen*Sticta weigelii* is comparatively rare in distribution in the pine populated forests of Meghalaya where *Parmotrema tinctorum* is a very common and dominant species. No algal partner was seen/isolated from the lichen. The cyanobacterial partner that was isolated belonged to the genus *Nostoc* characterized by terminal conical heterocysts and showed close resemblance to *Nostoc edaphicum* Kondrateva and *Nostoc calcicola* Bréb. Definite identification is certainly not possible using classical method such as morphological study under microscope. Hence, we are working towards establishing the identity of the cyanobacterium using partial sequence of 16S rRNA gene.The cyanobacterium *Nostoc* seems to be the lichen's primary photobiont, thus confirming *Sticta weigelii* in the group of bipartite lichens. Isolation of the cyanobiont was achieved in nitrogen free medium where it first appeared as cyanobacterial packets with distinct heterocysts (Fig. 2). On further subculture, the cyanobacteria appeared as long filaments in the medium without any outer covering. It is possible that when not in symbiosis, the control on its growth is removed and hence the cyanobacterium shows higher multiplication and growth parameters in its free-living environment. The various physiological and biochemical studies also pointed to the fact that at early stages of free-living life cycle, the cyanobacterium is completely engaged in its growth and development activities. The photochemical activity measured as O_2-evolution and

respiratory activities measured as O_2-consumption were comparable to other free-living *Nostoc* species suggesting that the organism is fully concentrating on optimizing its growth. Efficient photosynthetic and respiratory activities may be contributing towards generating ATP and reductants for various enzyme activities. Definite differences were observed in the morphology of the cyanobacterium when in symbiosis and when it is in its free-living state. Within lichen thallus, the cyanobacterium was seen restricted to a distinct layer just below the upper cortex (Fig 2 b). In the early stages of isolation distinct packets of *Nostoc* were visible in the medium. However, outside the symbiotic conditions, in its free-living state, the cyanobacterium grew unrestricted into long filaments devoid of any outer covering. Once isolated, various enzyme activities of the cyanobacterium were comparable to other free-living *Nostoc* sp.

Acknowledgements

The authors would like to thank University with Potential for Excellence (UPE) scheme of the University Grants Commission, New Delhi to life sciences, North Eastern Hill University, for financial support.

References

Awasthi, D.D. (2000). *Lichenology in Indian subcontinent: A supplement to A hand book of lichens*. Bishen Singh Mahendra Pal Singh Dehra Dun, India.

Desikechary, T.V. (1959). *Cyanophyta*, Indian Council of Agricultural Research, New Delhi.

Hitch, C.J.B. and Millbank, J.W. (1975). Nitrogen metabolism in lichens. VII. Nitrogenase activity and heterocyst frequency in lichens with blue-green phycobiont. *New Phytol*. 75: 239-244.

Joshi, M. And Awasthi, D.D. (1982). The lichen family stictaceae in India and Nepal. *Biol. Mem*. 7: 165-196.

Komárek, J., Kling, H., Komárková, J. (2003,). Filamentous cyanobacteria, In *fresh water algae of North America: ecology and classification* (ed. J.D. Wehr & R.G. Sheath) Academic Press: 117-191.

Kondrateva, N.V. (1968). Voprosymorfologii; sistematiki*Microcystisaeruginosa*Kuetz. Emend. Elenk i blizkichnemuvidov in Cvetenievody, Kiev. 13-42.

Manzano, C., Candau, P., Gomez-Moreao, C., Relimpio, A.M. and Losada, M. (1976). Ferrodoxin dependent photosynthetic reduction of nitrate and nitrite in particles of Anacystisnidulans. *Mol. Cell Biochem*. 10: 161-169.

Mckinney, G. (1941). Absorption of light by chlorophyll solutions. *J. Biol. Chem.*140: 315 -322.

Rai, A. and Bergman, B. (2002). Cyanolichens. *Biol. Environ. Proc. R I A,* 102B (1): 19-22.

Rai, A.N., Rowell, P. and Stewart, W.D.P. (1984). Evidence for an ammonium transport system in free–living and symbiotic cyanobacteria. *Arch. Microbiol*. 137: 241-246.

Richardson, D.H.S., Hill, D.J. and Smith, D.C. (1968). Lichen physiology. IX The role of the alga in determining the pattern of carbohydrate movement between lichen symbionts. *New Phytol.* 67: 469.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology*, 111: 1-61.

Robinson, S.J., Deroo, C.S. and Yocum, C.F. (1982). Photosynthetic electron transfer in preparation of the cyanobacterium *Spirulina platensis*. *Plant Physiology*. 70: 154-161.

Sampio, M.J.A.M., Rowell, P. and Stewart, W.D.P. (1979). Purification and some properties of glutamine synthetase from nitrogen fixing cyanobacterium *Anabaena cylindrical* and *Nostoc* species. *J. Gen. Microbiol.* 111: 181-191.

Sinha, G.P. and Singh, K.P. (2005) *Macrolichens of Sikkim*. Kolkata: Botanical Survey of India.

Snell, F.D. and Snell, C.T. (1949). *Colorimetric Methods of Analysis*, Vol. 2D, New York: Van Nostrand Co. Inc., pp. 804-805.

Stewart, W.D.P., Fitzgerald, G.P. and Burris R.H. (1967). *In situ* studies of nitrogen fixation using acetylene reduction technique. *Proc. Natl. Acad. Sci.*, USA 58: 2071-2078.

White, F.J. and James, P.W. (1985). A new guide to microchemical techniques for the identification of lichen substances. *British Lichen Soc. Bull*., No. 57 (Suppl.): 1-41.

Wolk, C.P. (1965). Control of sporulation in a Blue Green Alga*. Dev. Biol.* 12: 15-35.