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Role of Cyanolichens in biological Nitrogen fixation

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Abstract

Lichens are symbiotically associated of algae and fungi, actively involved in conversion of atmospheric nitrogen into organic form. Lichens are also involved in phosphate solubilization and biological nitrogen fixation. Lichens possess cyanobacteria as its primary phycobiont commonly referred as cyanolichens, but in some lichens cyanobacteria are located in special structure called cephalodia. The heterocyst of cyanobacteria convert nitrogen to amonia form in an anaerobic environment and translocate to the mycobiont. Nitrogen fixation has been estimated in lichen samples by $^{15}N_2$ method or acetylene reduction assay. Acetylene reduction or Nitrogen fixation have been directly affected by environmental factors like desiccation, light, temperature, carbon fixation rate and photosynthesis.

Key words: Lichens, Cephalodia, Cyanobacteria,Bi-partate lichen, Tri-partate lichen, Acetylene reduction assay, Nitrogen fixation and Environmental factors.

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Introduction

Lichens are symbiotic association between a mycobiont (fungus) and a photobiont (green alga and /or cyanobacteria). Lichens which possess cyanobacteria as their primary or secondary photobiont is called cyanolichens. Lichen systematics has been based on the mycobiont, and much less information has been available to identify the lichen photobionts [1]. Although lichens are commonly described as a mutualistic symbiosis between fungi and algae, a wide number of internal bacterial communities have also been documented [2]. Lichens comprise 6% of the Earth's terrestrial vegetation, and are dominant in certain polar ecosystems, being primary colonists of rocks where they play a major role in the biogeochemical cycling of elements and contribute to soil formation. Many lichens are colorful, mostly due to the presence of secondary metabolites which are of fungal origin. In some cases, color may reflect chemical coordination reactions involving lichen biomass components and dissolved cations which would lead to metal complex and mineral formation [3].

Among the fungal species 20% represents lichenized fungi [4], 85% of lichenized fungi comprised withgreen micro-algae, 10%with cyanobacteria and 4% with both cyanobacteria and green algae [1]. In the lichen symbiotic system, mycobiont provides a suitable environment for the photobionts, enabling gas exchange with the atmosphere, water and organic nutrient supply, and protection against drying. Meanwhile, green algae provide a sourceof organic matter which are synthesized during the photosynthesis and cyanobacteria provide a combined nitrogenated source byN_2 fixation process [5].

Earlier, Henriksson [6] has shown that Nostoc isolated from *Collema* sp. actively fixed Nitrogen. Bond and Scott [7] and Scott [8] demonstrated that nitrogen fixation occured in *Collema granosum, Leptogium lichenoides* and *Peltigera praetextata*. All these lichens contain *Nostoc* as the phycobiont. Cyanobacterial species of *Collema granosum, Leptogium lichenoides* and *Peltigera praetextata* were able to fix the nitrogen equally compare to the free-living form of nitrogen fixers. Scott [8] provided the evidence by using *Peltigera praetexatata*, where lichen mycobiont was the recipient of nitrogen fixed by cyanobacteria. Fogg and Stewart [9] revealed that Cephalodia of a lichen *Stereocaulon* actively fixed nitrogen however there was no data available about rate of nitrogen fixation. Further lichens species like *Collemma pulposum* and *Stereocaulon species* are actively involved in nitrogen fixation, both of these lichen contained Nostoc as phycobiont,Nostoc cells are also found in the cephalodia of *Stereocaulon*.

Nitrogen fixation was demonstrated *In-situ* on Signey Island, South Orkney,by using $^{15}N_2$ tracer techniques. Nostoc commune and two lichen species *Collema pulposum* and *Stereocaulon* Sp. were chosen for their study. Both of these lichens contained Nostoc as a phycobiont. Nitrogen fixation by Nostoc and lichens were influenced by the type of rock, amphibolite and marble in these areas. The appreciable nitrogen fixation occurred at 0^oC, and the rate of nitrogen fixation increased rapidly with rise in temperature, When the microenvironment of lichen reached at 10^o C or more, the bulk amount of N_2 fixation accomplished in short duration [9].Cephalodial lichen, *Peltigera aphthosa* from arctic and sub-arctic region of Scotland which was susceptible to air pollution and their mechanism of nitrogen fixation was reported.Lichen sample was kept in growth chamber with controlled conditions/environment like temperature, light, high humidity and pollutant free air. Approximately $8cm^2$thallus was cleaned with distilled water then sterile water and inserted into sterilized 20 ml tube and connected with gas generating apparatus, which contained O_2, CO_2, argon and radiolabelled nitrogen($^{15}N_2$) and the unit was illuminated by white fluorescent light and maintained 25^oC. Later,from the lichen sampleN_2fixation was recognized and the

cephalodia contained a phycobiont(Cyanobacteria) and mycobiont, further they were separated. $^{15}N_2$ tracer technique was used for confirmation of Cephalodia of lichen *Peltigera aphthosa* as a site of nitrogen fixation. Cephalodial Nostoc cells fixed the nitrogen and translocated to thallus, fixed nitrogen was secreted to the lichen thallus at a rate equal to the rate of fixation [10].

Lichens are grouped into bi-partite and tri-partite, based on the number of photobionts present. In bipartite lichen fungi have been associated with algae or cyanobacteria, and tri-partite lichen thallus has been made up of fungi with algae as primary photobiont and cyanobacteria present in special structure cephalodia. Nostocalean cyanobacteria, can participate in several types of lichen symbiosis. They provide both photosynthate and N_2 compounds to their symbiotic partners, and the relative importance of these two functions might vary between different types of lichen symbiosis. In bipartite cyanolichens, cyanobacteria are the only photosynthetic component and play a major role in photosynthesis. In tripartite lichens, green algae occupy most of the thallus and also produce most of the photosynthate. In this situation, Nostoc perform only N_2 fixation, and are restricted to delimited portions of the thallus called cephalodia. Cephalodia can occur either in the medulla (internal cephalodia) or on the surfaces (external cephalodia). The Nostoc cyanobionts of tripartite, cephalodiate lichens generally show higher heterocyst frequencies and higher rates of N_2 fixation than free-living cyanobacteria. The heterocyst frequencies of lichenized, cephalodial Nostoc commonly range from 15 to 35% of trichome cells, as opposed to the 5–10% of free-living Nostoc trichomes and lichenized cyanobionts of bipartite cyanolichens [11-13].

Lichen-associated cyanobacteria have shown enhanced N_2 fixation rates compared with non-associated cells [14]. $^{15}N_2$ experiments on *Peltigera aphthosa* proved that almost all N_2 fixed by cyanobacteria was transferred to the fungi [12].

Experimentally, a mutualistic interaction between a heterotrophic N_2 fixer and lichen fungi in the presence of a carbon source could contribute to enhanced release of organic acids, leading to improved solubilization of the mineral substrate. Three lichen fungi were isolated from *Xanthoparmelia mexicana*, foliose lichen, and they were cultured separately or with a heterotrophic N_2 fixer in nutrient broth media in the presence of a mineral substrate. Cells of the N_2-fixing bacteria attached to the mycelial mats of all fungi, forming biofilms. All biofilms showed higher solubilizations of the substrate than cultures of their fungi alone. This study indicated the significance of origin and existence of N_2-fixing activity in the evolution of lichen symbiosis. Nitrogen fixing photobionts and non- fixing photobionts (green algae) both were present in some remarkable lichens such as *Placopsis gelida* [15].

Nitrogen metabolism

Almost 10% of lichens contain cyanobacteria, as its primary symbiont, which comprised 50 genera and 1000 species. Meanwhile,cyanobacteria as a secondary symbiont in lichens comprised 20 genera and 500 species. Common cyanobacterial species found in lichensare Calothrix, Fischerella (Stigonema), Gloeocapsa, Nostoc, or Scytonema, fixing Nitrogen. Cyanobacteria are primitive form of autotrophic bacteria which are prokaryotic in cellular structure. It is also called Blue green algae because they contain the photosynthetic pigments like phycocyanin, phycoerythrine and chlorophyll [16].These cyanobacteria have some specialized cells called Heterocysts which are enlarged cell present terminally or intercalary in filamentous structure and made up of three different cell wall layers. Outer fibrous and middle homogenous layers made up of non cellulose polysaccharide and inner layer made up of glycolipids. Special cell wall layers permits the nitrogen gas diffuse inside, and inhibits

the entry of atmospheric oxygen. Nitrogenase enzyme which is sensitive to air present inside the heterocyst plays important role in nitrogen fixation. In the N_2 fixation process hydrogen is evolved as byproduct, in this, 40% of hydrogen gas recycled by hup (hydrogen uptake) genes. Photosystem I (PS I) is present in the heterocyst, while Photosystem II (PS II) absent because Photosystem II (PS II) does photolysis of water and generates the free oxygen gas. Whenever the oxygen enters to the heterocyst from polar plugs, Oxidase enzyme converts to water in the presence of hydrogen molecules which are present inside the heterocyst. This process helps to maintain the internal reducing environment of heterocyst [17]. Nitrogen fixation mechanism mainly takes place in heterocyst of cyanobacterial member of lichen. Nitrogen fixation utilizes 8 electrons and 16 Mg ATPs to convert one N_2 to two NH_3 by the enzyme complex nitrogenase. Nitrogenase contains both a Fe-protein and a Fe-Mo protein. Ferredoxin provides the electrons to the Fe-protein; it becomes a strong reducing agent when bound with ATP. The electrons are transferred to the Fe-Mo proteinand responsible for the transfer of electrons and protons to N_2 to form the two NH_3.Among two NH_3 one molecule converted into Glutamine, it moves to the adjacent vegetative cell for other biochemical process. Mean time in vegetative cell carbon fixation process takes place by Photosystem I (PS I) and Photosystem II (PS II). Atmospheric CO_2 is taken up by the RUBISCO (Ribulose Biphosphate Carboxylase) enzyme and changed into 3-PGA (3-Phosphoglyceraldehyde), which enters into the Calvin Cycle, and produce Maltose and Oxalo gluterate. Two molecules of Oxalo gluterate react with one molecule of Glutamine and enter into the GOGAT Pathway (Glutamine Oxalo Gluterate Amino Transferase) and two molecules of Glutamate are formed, one molecule of Glutamate will be cycled back to the Heterocyst. On other hand maltose enters to heterocyst in different intermediate carbon compounds as a carbon skeleton. Different intermediate compounds are Glucose-6-Phosphate, which will be changed into 6-Phospho-Gluconate, and ultimately changed into Ribose-5-Phosphate. Each and every step of transformation of these different Carbon Compounds, give rise to the sufficient amount of energy molecules, ATP to help in the fixation of atmospheric N_2 into solid Ammonia molecule. The Hydrogen ions (H^+) formed in the energy transfer process are taken up by the enzyme N_2 Mediated Hydrogenase/Bi directional/Reversible Hydrogenase encoded by hox gene, and are changed into molecules of Hydrogen (g) (H_2). However, 40% of gaseous Hydrogen gas will be recycled through Plastoquinone and Plastocyanin, by uptake Hydrogenase enzyme encoded by hup (hydrogen uptake) gene [17]. In free-living cyanobacteria heterocysts constitute 5–10% of total cells and similar percentages of heterocysts are found in the cyanobiont when it is the primary photobiont. In higher percentage of heterocysts (15–36%) may be found in the cyanobiont of tripartite lichens higher heterocyst occurrence in lichen was proportionally higher N_2-fixation activity was reported [10].

Estimation of Nitrogen fixation in Lichen by *Invitro* experiments

Nitrogen fixation process involves the reduction of atmospheric N_2 into ammonia, catalyzed by nitrogenase enzyme.Nitrogen fixation mechanism in lichens has been mediated through the prokaryotes or cyanobacterium. Millbank and Kershaw [10] used $^{15}N_2$ isotopic method, and estimated the nitrogenase activity by using Acetylene Reduction assay. Hitch and Stewart [18] collected 36 species of metabolically active lichens mainly from Scottish coastal habitats, among them, eight showed reduction of acetylene to ethylene. This included with *Lichina confinis, Peltigera rufescens, P. aphthosa, P. polydactyla, P. canina, Collema crispum* and *Placopsis gelida.* These contained a

blue-green alga as the primary or secondary phycobiont. Acetylene reduction rates were sometimes high in the field and showed marked seasonal variations. Nitrogenase activity was higher in vegetative cell than in fruiting thalli of *Peltigera rufescens*, and in *P. canina* was higher nearer the apices of the thalli than in older parts. Environmental factors affected the rate of N_2 fixation, among those desiccation was the most important, temperature was less important and the lichens, while requiring light, could continue to fix N_2 in the dark for up to 18 hours (*Lichina confriis*) and 26 hours (*Peltigera rufescens*). The average percentage N_2 content of nitrogen fixing lichens (2.20%) was significantly higher than the non-nitrogen-fixing lichens like *Anaptychia fusca, Buellia canescens, Caloplaca ferriiginea, C. thallincola, Cetraria glauca, Cladonia arbuscula, Cladonia cormita, Cladonia foliacea, Cladonia furcata* etc (0.83%). The maximum and mean rates of N_2 fixation by *P. rufescens* were 38.1 and 4.1 µg N (mg $N)^{-1}$ day^{-1} respectively. The corresponding values for *Lichina cottfinis* were 29.4 and 0.4.

In ananother study, Antoine [18a] used the tripartite lichen *Lobaria oregana* to check the nitrogenase activity using the acetylene reduction assay (ARA). Acetylene was generated by reacting calcium carbide with water, and then injected into 300 ml air-tight Plexiglass incubation chambers to a concentration of 10% by volume. The chambers were shaken vigorously, vented to atmospheric pressure. Lichen thalli were incubated for 60 minutes, and empty control chambers were used to quantify background levels of C_2H_4 present along with the C_2H_2. After the incubation period, syringes were used to withdraw three 1- ml gas samples from each experimental and control chamber. Ethylene content of each sample was determined using gas chromatography method and quantified the nitrogen fixation at sampled sites and was formed to be 1.5 kg ha^{-1} and 2.6–16.5 kg N_2 ha^{-1} yr^{-1}.

Environmental factors affecting acetylene reduction in lichens

Estimation of N_2 fixation by acetylene reduction assay depends on number of environmental factors such as desiccation, temperature and light.

Moisture

N_2 fixation activities in cyanolichens were controlled by moisture [19]. Lichens, which are inactive in the field, reduce acetylene when moistened either in the field or in the laboratory. A total 100 lichen samples were found to be inactive in reducing acetylene, over 90% of these developed Nitrogenase activity within 1 hour of moistening. The recovery of Nitrogenase activity was observed in *Lichina confinis, Peltigera canina* and *Collema crispum*, when wetted with distilled water. In an another study, with *Lichina confinis* and *Peltigera canina,* thalli with water contents as low as 6.7% and 3.8% of their oven dry weights respectively, could recover their capacity to reduce acetylene within 1-2 hours where being moistened [18]. Lichenized *Nostoc* desiccated for few days takes minutes to one hour time to activate or initiate the nitrogenase activity [11].

Light

The effect of light on nitrogenase activity or acetylene reduction assay is not clear because some other factors like desiccation play important role in N_2 fixation process. In some laboratory experiments intensity of light directly affects the acetylene reduction test when all other factors are normal were recorded. Lichen species like *Lichina confinis* and *Peltigera rufescens* were subjected to acetylene reduction test in that Nitrogenase activity of *Lichina confinis* increased with increase in light intensity within the range 2000-16000 lm m^{-2} while *Peltigera rufescens* was light saturated at 3600 lm $^{m-2}$. In dark conditions the N_2 fixation varies, period of fixation in dark was shorter however in some cases it continued to several hours [18].

Temperature

Temperature also controls the nitrogenase activity, whenever sufficient moisture and

light are present.The effects of temperature on acetylene reduction by *Lichina confinis* and *Peltigera rufescens* species used for nitrogenase activity, in which *Peltigera* showed maximum activity near 32.5^{o} C and *Lichina*, showed maximum nitrogenase activity at temperature 20-25^{o} C. *Peltigera* appeared more resistant to temperature extremes than *Lichina* and showed fixation rates over at -2.5^{o} C which were approximately 50% of those at 42.5° C where activity was still appreciable. Further nitrogenase activity by *Lichina confinis*on the other hand, was completely inhibited at -2.5^{o} C and at 42.5^{o} C. The Q_{10} for Nitrogenase activity is high and approached 3-4 times in the temperature range 10-20° C (Hitch and Stewart, 1973).

Conclusion

Lichens are symbiotic organisms which play an important role in biological Nitrogen fixation. Lichens contain fungi, algae/cyanobacteria, or both. Cyanobacteria associated with the fungi forms a cyanolichens, cyanobacteria present in lichen photosynthetically active and fixes the atmospheric Nitrogen, then fixed Nitrogen passes to the mycobiont. Nitrogenase enzyme in the cyanobacteria actively converts the atmospheric non-usable Nitrogen into usable form. Nitrogenase enzyme found active at anaerobic condition only. Acetylene reduction assay, N_2 tracer methods are widely used to find out the rate of N_2 fixation by lichens.Environmental factors like light, temperature, and moisture are important factors which directly affect the Nitrogen fixation or nitrogenase enzyme activity.

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