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Growth and Nitrate Metabolism in the Thermohalophillic Cyanobacterium *Leptolyngbya*.

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Abstract

Extremophilic microorganisms are by definition those that live at the outer limits of the physical parameters that define the boundaries for life on earth. Such common niches that encompass a number of extreme physical stresses are found in the endolithic rock environment of hot and cold deserts with regard to temperature as well as marine environments and hot springs which are highly saline in nature. In most extreme habitats, microbial communities which are dominating, are composed primarily of photosynthetic microorganism mainly the cyanobacteria. Among them a group of extremophiles which are receiving a lot of attention lately are the halophilic microorganisms. In the present study cyanobacterial species *Leptolyngbya* isolated from a hot hyper saline lake that exhibits 2 extreme characters i.e. thermal and saline. Such species are important as they come from an environment with two extremes. This strain was found to grow on a wide range of salinities and temperatures in the laboratory. High rates of growth and nitrate reductase activity were reported that points to flexibility and stability of the enzyme at high temperature and salinity. It also gives an in site to understanding the nitrate metabolism of this interesting organism. The study has unraveled a lot of significant data that opens up many new avenues of research in industrial use of extremophiles, and seeks to answer fundamental questions about survival methods of this poly-extremophylic organism.

Key words*: Leptolyngbya*, Nitrate reductase, Halophiles, Extremophiles.

Short Title: Growth and nitrate metabolism in *Leptolyngbya.*

Introduction

Extremophillic prokaryotes are characterized by inhabiting ecosystems that are from a human perspective, extreme. Extremophilic microorganisms include members of all three domains of life, the Archaea, Bacteria and Eukarya. Often, these microbes are not only challenged by one extreme, but multiple, and thus they are "polyextremophile" (Mesbah and Wiegel, 2008, Banerjee et al 2009). Examples would be life at hot alkaline springs or hypersaline and alkaline lakes or hot and acidic springs. Ever since extremophiles were discovered, their physiology and their adaptation to the unhostile environment have attracted much interest. This was not only because of the interest in their lifestyle, but also for exploring their biotechnological potential (Banerjee et al 2002 a, b, Banerjee and Sharma 2004 a,b,2005, Norris and Castenholz 2006, Fleming and Castenholz 2007, Roeselers *et al*., 2007). In most extreme habitats the microbial communities which dominate are composed primarily of photosynthetic microorganism mainly the cyanobacteria. Cyanobacteria are able to adapt to a wide range of environmental conditions (Tandeau de Marsac and Houmard 1993, Banerjee and Sharma 2004, 2005), among them; salinity is one of the most important factors that limits the growth and productivity of plants, eukaryotic microorganisms, and bacteria (Inabha *et al*., 2001, Oren 2007). Inhabitants of these saline environments are called halophiles. This is the group of extremophiles which is receiving a lot of attention lately (Fleming and Castenholz 2007).

Soil salinity is an important agricultural problem and high NaCl content is a principal deterrent of plant growth in saline habitats. Salinity has a considerable effect on agriculture, affecting almost half of the world's irrigated areas of land to a moderate or high degree. Usar soils (Alfisol, solonetz, alkaline, sodic, saline) extensively distributed in the northern part of India (2.5 million hectares) are characterized as alkaline or saline depending on the salt content. These soils are unproductive, impermeable, and hard due to the presence of undesirable salts on the surface. Saline soils are characterized by exchangeable Na^+ (more than 15%), low quantities of Ca^{2+} and pH values that usually range between 8.5 and 10.5 (Bhaduriya *et al*., 2009). Physical and chemical methods do not completely remove the soluble salts and exchangeable sodium in soil. The photoautotrophic nitrogen-fixing cyanobacteria, in general, exhibit considerable tolerance to salt or osmotic stress (Thomas and Apte 1984) and reclamation of saline soils using cyanobacteria has been attempted with some success by Singh (1950). He suggested that cyanobacteria could be used to reclaim saline soils because they form a thick stratum on the surface of the soil in the rainy season. (Pandey *et al*., 2005, Pandhal *et al*., 2008). NaCl in trace amounts appears essential for some of the metabolic functions in cyanobacteria (Apte and Thomas 1984 a, Thomas and Apte 1984 b), but elevated levels might inhibit growth (Pandey *et al*., 2005).

The cyanobacteria from extreme environments have attracted great attention in recent years because of their ability to endure extreme environmental conditions and which may be a result of inheritance of ancient character which were prominent in the early period of earth history. In the present study the extremophile studied is important not only because it is biologically and scientifically very interesting but because there is a great lacuna of knowledge regarding the physiology of nitrate metabolism in this organisms and which are essential for its growth and survival in those niches. Almost nothing is known about the mechanism adopted by this organism to perform photosynthesis, nitrate reduction under extreme condition. Knowledge in this aspect can be used to reclaim usar soils or other ecological niches that are inaccessible due to the adverse physicochemical properties. This has a big application for Indian soils and soil reclaimation in general. Inspired by such considerations the present work is an attempt to throw light on the physiological aspects related to nitrogen metabolism thermohalophilic cyanobacterium *Leptolyngbya*.

Materials and Methods

Leptolyngbya is an isolate from Iceland which grows in a hot lake in which the salt concentration thrice that of sea water. *Leptolyngbya* was cultured, purified, identified and made axenic by standard microbiological procedure by Professor (Dr.) Meenakshi Banerjee Department of Biosciences, Barkatullah University Bhopal during her visit to the Centre for Ecology and Evolutionary Biology, University of Oregon, USA and brought to the laboratory of Algal Biotechnology, Department of Biosciences, Bhopal, India, for further work. Identification of the strains was made according to the standard literature like Desikacharya, 1959.

Out of the several media tested, *Leptolyngbya* showed best growth in BG-11 (N^+). Cultures were incubated in air-conditioned culture room and illuminated by three 40 W fluorescent tubes from a distance of 50 cm for 12 hours daily. The cultures received 2500 to 3000 flux light intensity at 25 $\pm$2^{o}C unless otherwise stated.

Glassware used was of Corning make. Cultures were maintained in culture tubes (15 * 1.5 cm) each containing 10 ml of medium or in 100 ml Erlenmeyer's flask containing 30 ml medium respectively. Tubes and flasks were plugged with non-absorbent cotton and covered with aluminum foil. Culture media and culture vessels were sterilized by heat at 15 Lb. per square inch pressure for 15 minutes. Solutions of inorganic nitrogen sources were autoclaved separately and added to cold sterile medium. Most chemicals used were sterilized through Millipore filters (pore size 0.45µm).

The growth of the organisms was measured by extracting Chlorophyll *a* (Chl a) in 100% methanol and the optical density was read with a spectrophotometer model 106 at 663 nm wavelength. The amount of Chl *a* was calculated according to the equation of Mackinney (1941) and the generation time (doubling time) was calculated by the growth equation of Kratz and Myers (1955). The estimation of *in vivo* nitrate reductase activity was done by the method of Camm and Stein (1974) was slightly modified by Kumar and Kumar (1980). All chemicals were available at highest purity level from British Drug House (Glaxo) Mumbai.

Results

Growth

Table 1 shows the results of growth of *Leptolyngbya* expressed as fold increase and decrease in growth over control in different temperature, pH and light intensity at 96 h under ideal laboratory conditions. *Leptolyngbya* is a halophile and therefore after obtaining the suitable media for its growth the effect of different concentration of NaCl (30 g.l^{-1} to 140 g.l^{-1}) was first studied. Maximum growth was observed with 90g.l^{-1} so all other environmental parameter were studied on growth with this concentration. During this study temperature (25±2ºC) and NaCl concentration (90 g.l^{-1}) were kept constant.

The effect of different temperatures was studied on the growth of *Leptolyngbya*. It was found that there was an increase in growth with increase in temperature. The maximum growth was observed at 45±2ºC (1.34 µg Chl *a* ml^{-1}) at 96 h and the fold increase was found to be 1.10 over control (25±2ºC) at 96 h. With the increase in temperature i.e. 55±2ºC and 65±2ºC decrease in growth rate was observed. It was 0.56 and 0.52 fold decrease at 96 h over control at 55±2ºC and 65±2ºC respectively Table 1. The effects of different pH ranging from 5 to 10 was studied on *Leptolyngbya.* Maximum growth was observed at pH-9 (1.38 µg Chl *a* ml^{-1}) at 96 h and the next best result was found at pH-10 followed by control pH-8 at 96 h. In this cyanobacterium at pH-9 there was a fold increase of 1.14 over control pH-8 at 96 h.

Studies showed that the growth of cyanobacteria generally sensitive to the light. Experiments with different light intensities on *Leptolyngbya* showed optimum growth at 2500±200 Lux light (1.21 µg Chl *a* ml^{-1}) under laboratory conditions at 96 h. With other increasing light intensities there was a decrease in growth. In *Leptolyngbya* with 5000±200 Lux light intensity there was a fold decrease of 0.48 over control (2500±200 Lux light) at 96 h (Table 1). The effect of darkness on *Leptolyngbya* under laboratory conditions showed an increase in growth in dark condition (0.65 µg Chl *a* ml^{-1}) at 96 h compare to low light intensities i.e. 500±200 Lux light and 1000 Lux light but It was 0.537 fold decrease over control (2500±200 Lux light) at 96 h, (Table 1).

Table-1: Fold increase and fold decrease in growth over control with different temperature, pH and light intensity at 96 h under ideal laboratory conditions for *Leptolyngbya*

Environmental Parameters		*Leptolyngbya*	
		Fold Increase	Fold Decrease
Temperature	45° C	1.10	
	55° C		0.56
	65° C		0.52
pH	5		0.239
	6		0.26
	7		0.52
	8		
	9	1.14	
	10		0.97
Darkness			0.537
Light Intensity	500 Lux		0.446
	1000 Lux		0.462
	2500* Lux		
	5000 Lux		0.48
	10,000 Lux		0.396

Nitrate Reductase Activity

While studying nitrate reductase enzyme activity, different concentration of NaCl (30 $g.l^{-1}$ to 140 $g.l^{-1}$) was first studied on the basal nitrate reductase activity of *Leptolyngbya*. Maximum enzyme activity was observed with 90 $g.l^{-1}$ (1.45 µg NO_3/µg chl *a*) therefore the effect of other environmental parameter on the enzyme activity was studied with this concentration of NaCl (Fig.1). When the effect of different temperatures was studied on the nitrate reductase activity of *Leptolyngbya*, a marked increase in the enzyme activity was obtained with increase in temperature. Maximum nitrate reductase activity was recorded at 45±2ºC at 120 h (1.55 µg NO_3/µg chl *a*). This activity was a1.4 fold increase over control. Higher temperatures of 55 and 65 ^{o} C resulted in decrease in the enzyme activity. (Fig. 2).

Fig. 1: Effect of different salinity (30$g.l^{-1}$ to 140$g.l^{-1}$) on nitrate reductase activity of *Leptolyngbya*. Temperature 25ºC± 2ºC, Light Intensity 2500±200 Lux, pH- 8.2 (Results mean ± SD n=3)

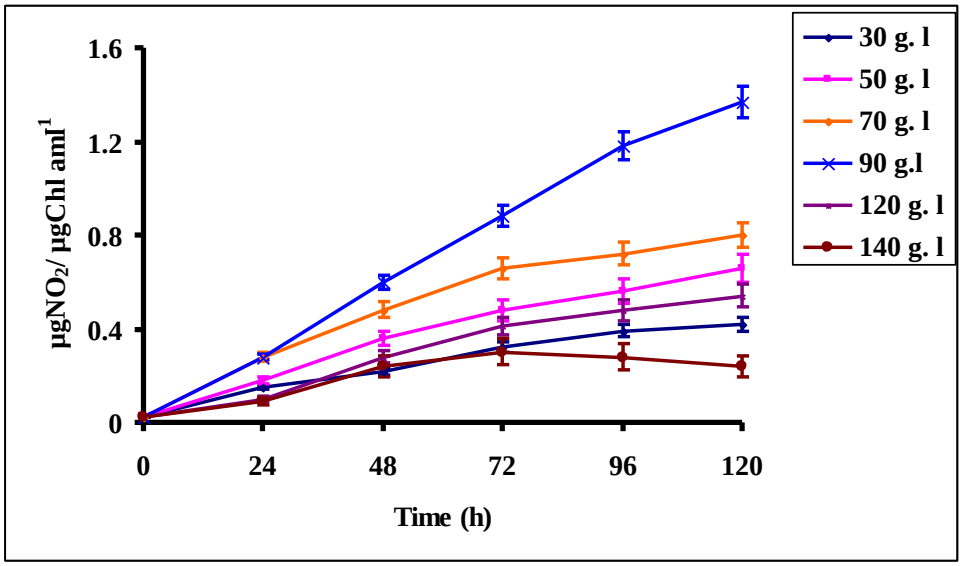
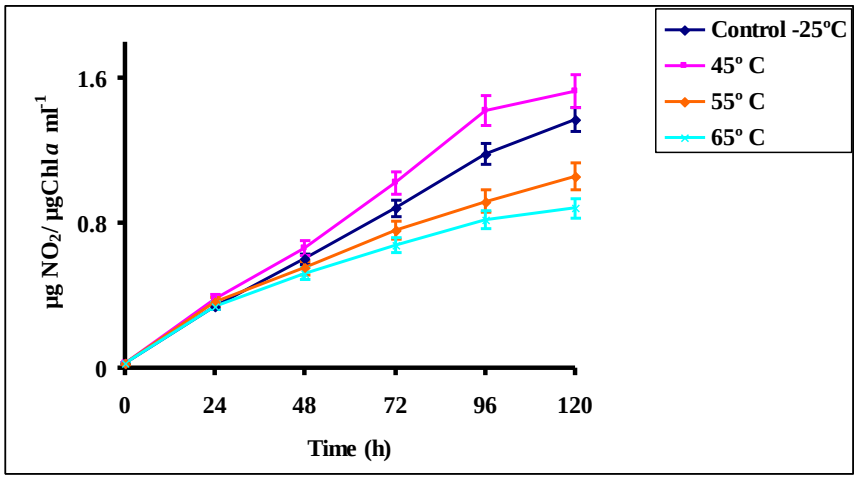


Fig. 2: Effect of different temperatures on nitrate reductase activity of *Leptolyngbya*. Light Intensity 2500±200 Lux, pH- 8.2 (Results mean ± SD n=3)



Effects of pH on nitrate reductase activity were also studied over a wide range (4-12) of pH values. The pH optimum for nitrate reductase of *Leptolyngbya* was observed to be 9 (1.6 µg NO_3/µg chl *a*). This activity was 1.2 fold higher than in control (pH 8.2).(Fig. 3). An effect of different light intensities was also studied on the nitrate reductase activity of the organism. Maximum nitrate reductase activity for the cyanobacterium was obtained at 5000±200 Lux light at 120 h (1.4 µg NO_3/µg chl *a*). This was 1.2 fold higher than control (2500 Lux)(Fig.4). The effect of darkness was also studied on nitrate reductase activity of the strains and dark condition produced a decrease in the enzyme activity (Fig.5).The fold decrease in darkness compared to control was 0.86.

Fig.3: Effect of different pH on nitrate reductase activity of *Leptolyngbya* Temperature 25ºC± 2ºC, Light Intensity 2500±200 Lux (Results mean ± SD n=3)

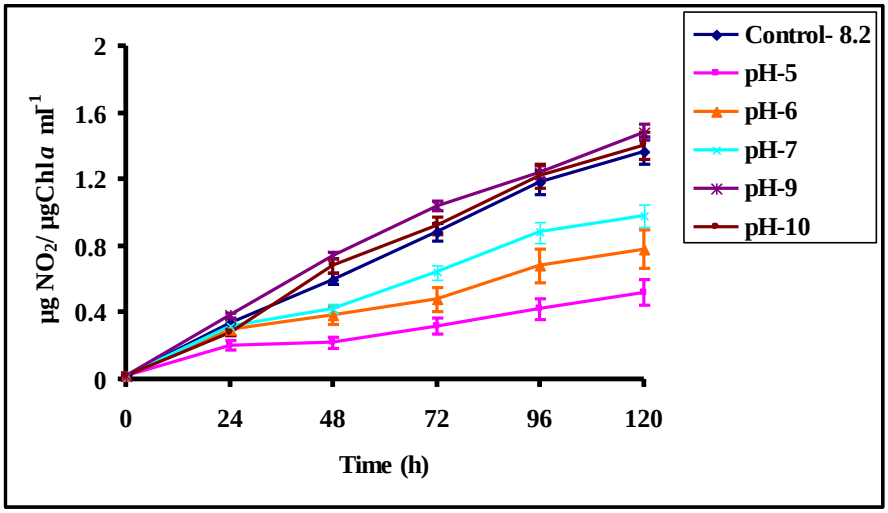


Fig.4: Effect of different light intensity on nitrate reductase activity of *Leptolyngbya.* Temperature 25ºC± 2ºC, pH- 8.2 (Results mean ± SD n=3).

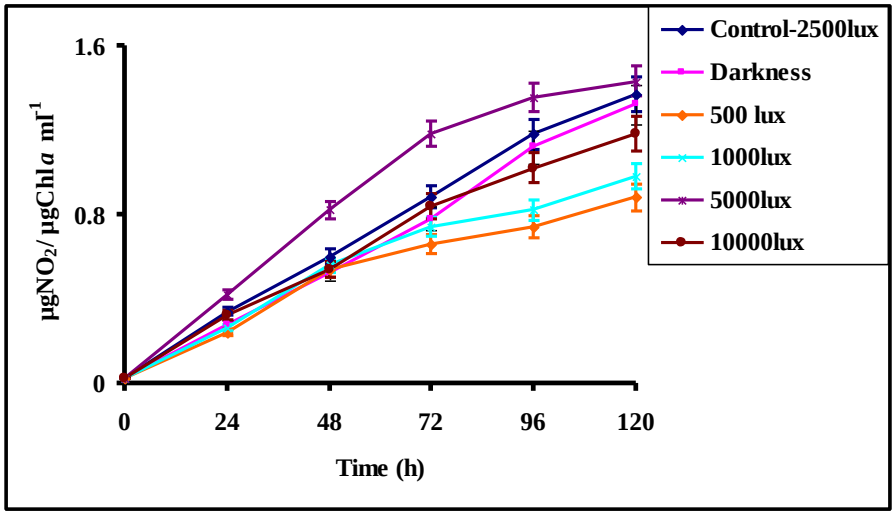
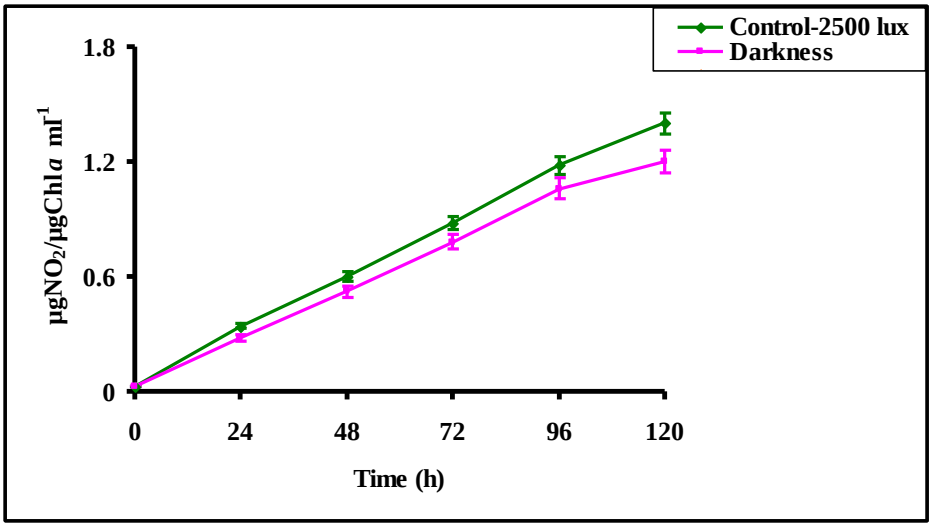


Fig. 5: Effect of darkness on nitrate reductase activity. Temperature 25ºC± 2ºC, pH- 8.2, (Results mean ± SD n=3).



Discussion

The last two decades have shown a surge in the interest in the world of microorganisms that live in extreme conditions. Extreme environment that could not be accessed before because of technical limitations are now being explored, and this has led to the isolation of the wealth of new organisms. Their study of growth with different physiological parameters shows us how life functions at extreme environmental conditions. Recent development in the field of algal biology enhances our understanding about the microorganisms those that lived at extreme environmental condition especially cyanobacteria. Cyanobacteria are gram negative prokaryotes with simple morphological structures.

The observation of present study showed that *Leptolyngbya* is able to grow under wide variety of environmental conditions. It can survive at higher NaCl concentration (90g.l^{-1}) is probably because in its natural environment it was found in the salinity thrice that of sea water (it is already documented that the salinity of seawater is 35 g.l^{-1}) and this coincides with laboratory grown cultures. Apart from this a considerable amount of Na^+ is required for the growth of halophilic cyanobacteria, because it stimulates photosynthesis so rapidly, and therefore it is tempting to speculate HCO_3^- transport into the cell which raises the

intracellular CO_2 concentrations. Survival at high temperature is a characteristic of halotolerant cyanobacteria because life in such hypersaline waters, which, due to high specific heat of brines, easily reach elevated temperature with sunlight. In halophiles, temperature causes increased cell density but above optimum temperature, cell density was reduces because it adversely affects the chlorophyll and carotenoid pigment.

The pH is the most important factor, which determines the cyanobacterial occurrence, which is reported from a wide range of acidic to alkaline pH. The pH optimum for the growth of the strain was 9. At alkaline pH large number of cations such as Na^+, Mg^{++}, Ca^{++} etc are probably taken up by the organism and thus increases the photosynthetic efficiency of cells. The negligible growth at low pH proves that acidic medium is detrimental for growth of the strain. Acidic pH might affect the photosynthetic apparatus, moreover chlorophyll *a* is very acid labile and decomposes to pheophytin under acidic conditions.Light availability influences cyanobacterial growth the observed effects in this study shows that growth of cyanobacteria was generally sensitive to high light intensities. In halophiles high light intensity may reduce the functioning of reaction center of PS I and PS II therefore the observed decrease in growth of *Leptolyngbya* at high intensity although the presence of sheath and high carotenoid content might be an adaptation to survive under high irradiation in halophilic cyanobacterium.

It was observed in present study that nitrate reductase activity was high in thermohalophile *Leptolyngbya*. The high rate of nitrate reductase activity of *Leptolyngbya* in the saline medium points to the halo -tolerance of enzyme in the halophilic cyanobacterium. Every living cell is challenged by changing water activities in its ecosystem, thus constant monitoring and adapting to changing water activities is a prerequisite for life. This is of particular importance to halophiles especially the moderate ones. In its natural environment *Leptolyngbya* found in the salinity more than sea water, so surviving at high salt concentrations and participating in the nitrogen metabolism of that particular niche from where this cyanobacterium was isolated may be an osmo-adaptation of this organism for that extreme condition. The biggest challenge is to adjust the tugor pressure and living cells have developed two principal strategies to re-establish turgor pressure and to circumvent the detrimental consequences of water loss when exposed to increasing osmolarity. On the other hand, the vast majority of prokaryotes cope with increasing osmolarity by uptake or synthesis of compatible solutes, which are defined as small, highly soluble, organic molecules which do not interfere with the central metabolism, even if they accumulate at high concentrations (Brown, 1976).This strategy is widespread and evolutionarily well conserved in all three domains of life (Bohnert et al 1995; Kempf and Bremer, 1998; Roeßler and Mu¨ ller, 2001 a,b; Saum and Mu¨ller, 2008). The uptake and biosynthesis of compatible solutes is induced by high salinity or high osmolarity on both the DNA and protein level. The pathways for the biosynthesis of various solutes have been identified but how the environmental signal "salinity" is sensed and how this signal is transmitted to various output modules at the level of gene, enzyme or transporter activation is completely obscure (Wood 1999). This is even more important if one considers that the overall cellular response of cells to hypersalinity is not only the accumulation of solutes but a reprogramming of cellular metabolism and structure in general.

In high salt concentration it may be possible to keep its cytoplasm isoosmotic with the extracellular environment. The results suggested that nitrate reductase can be an inducible enzyme in this organism and when nitrogen fixation is not operative as presumably in *Leptolyngbya*, being a non heterocystous strain nitrate reductase can replace it. Studies on nitrate reductase activity showed that in the presence of external sources of nitrogen, this extremophile has the ability to metabolize it for its own growth and metabolism and also drive the nitrogen dynamics of the environment where it is present. One important observation was that under extreme conditions the morphology of the cyanobacterium changed remarkably and this could cause enormous effect on the whole metabolism of the organism especially at biochemical level. The cells of the filament were found to be enlarged when grown under high temperature and salinity which obviously provides more surface area for CO_2 fixation and hence more reductant and ATP generation. Nitrate assimilation via nitrate reductase is dependent upon the CO_2 fixation that provides the reductants and ATP for the process. Under high temperature conditions as studied and similar to that found in nature for *Leptolyngbya*, the nitrate reductase activity increased in the cyanobacterium which can be explained with the increased photosynthetic activity. The high nitrate reductase activity in the extremophile at normal temperature (25ºC±2ºC) as well as at high temperature showed the flexibility of the enzyme, in these organisms.

Higher values of nitrate reductase activity in alkaline pH is probably because under alkaline conditions large number of cations such as Na^+, Mg^{++}, Ca^+ etc, are taken up by the organisms and these cations enhances photosynthetic efficiency of the organisms by activating necessary enzymes as cofactor that ultimately results in high rates of nitrate reductase activity in alkaline pH. It is also known that cyanobacteria prefer an alkaline range of pH for its metabolic activities. *Leptolyngbya* showed maximum nitrate reductase activity at reasonably high light intensities. Nitrate uptake under different light intensities in extremophiles is very reduced at low light intensities but with high light intensity the activity increases, this indicates that stimulation of non-cycling electron flow may be induced by nitrate at saturating photon flux density suffices to support the reductant demand for nitrate assimilation, while at low light it does not. Nitrate is frequently found in the environment at relatively low concentrations,

specific nitrate uptake systems are required to concentrate this nutrient inside the cells before nitrate reduction can take place. In organisms performing oxygenic photosynthesis, i.e., the cyanobacteria, algae and plants, nitrate reduction can be functionally linked to the photosynthetic process, and this is especially true for the case of nitrate assimilation in cyanobacteria. The results achieved in this study with different intensities of light showed that the nitrate uptake system probably dependent on light as the dark incubated cells showed a decrease in the enzyme activity as compared to its light grown counterparts. Although the nitrate reductase activity in *Leptolyngbya* decreases in the dark compared to light but there is detectable activity. This suggests that in addition to photosynthetically derived reductant and ATP, dark metabolic cycles like the pentose phosphate pathway may be operative and oxidative phosphorylation was involved in low levels of dark driven nitrate reductase activity in *Leptolyngbya*.

Conclusion

Study of *Leptolyngbya* with regard to its growth and nitrate reductase activity provides an insight to understanding the fundamental mechanism involved in their survival where nitrogen is required. This is important in the present context as we have now entered into a new era of biotechnology where extremophilic cyanobacteria are being considered as potential organism for various biotechnological processes and search for thermotolerant enzymes for industrial processes and soil reclamation.

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