International Journal of Innovative Research in Technology and Science
ISSN: 2321-1156 www.ijirts.org Volume 12 Issue 2, March 2024

Study of Phosphate Metabolism of Cyanobacterium Leptolyngbya

Vidhi Verma, Meenakshi Banerjee
SAM Global University, Bhopal, India
Centre for Applied Algal Research, Rice University, USA
vidhiverma2021@gmail.com, meenakshi.b.bhattacharjee@rice.edu

Selection and peer review of this article are under the responsibility of the scientific committee of the International Conference on Current Trends in Engineering, Science, and Management (ICCSTEM-2024) at SAM Global University, Bhopal.

Abstract- Leptolyngbyais is a polyextremophile found in more than one extreme condition. It thrives in lukewarm water and grows readily in high salinity. This cyanobacterium gains considerable importance, considering recent studies about halophilic enzymes and proteins. In the present study, some important conclusions will take us a long way in formulating principles for the beneficial use of this extreme organism. The high phosphatase activity rate indicates the enzyme's flexibility and stability at high temperatures and salinity. Phylogenetic studies with 16S rDNA show some very interesting results in Leptolyngbya. The study suggests that it evolved from a common ancestor and diverged into discrete species in dissimilar habitats separated by great distances geographically. The study has unravelled a lot of significant data and opened many new avenues of research in the industrial use of extremophiles, which answer the fundamental questions of the existence of extremophilic organisms on Earth and their use in various biotechnological processes.

Keywords- Leptolyngbya, Phosphatase Activity, Halophilic Enzymes, Phylogenetic

INTRODUCTION

Organic phosphorus plays a pivotal role in the global phosphorus cycle. Its availability for biological uptake is facilitated by phosphatase enzymes, which are primarily derived from soil microorganisms associated with plant roots (Clark et al., 1998; Wagner et al., 1995; Eberl et al., 1996; Healy, 1982; Banerjee, 2000a, b, 2001; Whitton et al., 2005; Banerjee et al., 2007). When phosphate supply becomes limited, organisms may experience phosphorus deficiency. In such conditions, one strategy to mitigate this limitation involves the more efficient utilization of inorganic phosphate (Mann, 1994). Cyanobacteria have evolved diverse mechanisms to cope with phosphate scarcity. While most cyanobacteria cannot directly uptake large organic phosphate molecules, they can utilize extracellular alkaline phosphatase to cleave inorganic phosphate from various substrates (Whitton et al., 2005; Banerjee, 2007). In response to phosphorus deficiency, cyanobacteria, like other microorganisms, synthesize alkaline phosphatase enzymes (Bhaya et al., 2000; Hernández et al., 2002; Banerjee et al., 2007). The kinetics of phosphate uptake by cyanobacteria have been investigated across various strains, revealing significant variability influenced by medium composition and cellular nutritional status (Marco & Ouré, 1988; Thiel,

1988; Garbisch et al., 1993; Banerjee, 2007; Banerjee et al., 2007). Cultivating cyanobacteria under phosphorus-limited conditions enhances phosphate uptake rates (Thiel, 1988). In response to phosphorus limitation, many cyanobacteria accumulate polyphosphate reserves and induce extracellular phosphatase synthesis to acquire phosphate from organic substrates in their environment (Wagner et al., 1995; Wagner and Falkner, 2001; Banerjee and John, 2003). While the measurement of phosphatase activity has become a practical biochemical tool in biotechnological studies, knowledge in this area regarding extremophiles remains scarce. The first report on phosphatase activity in extremophiles originated from the Antarctic deserts by Banerjee et al. (2000a, b). Despite this, much remains to be elucidated regarding phosphatase activity in thermophiles or halophiles.

With the rapid advancement of extremophile research, it is conceivable that this field may catalyze the emergence of novel scientific domains. Fossil studies suggest the possibility that microorganisms, potentially cyanobacterial analogues akin to those thriving in the limits of existence in the Antarctic deserts, might have flourished on Mars (Friedman et al., 1994; Wynn-Williams, 2000; Banerjee et al., 2000; Banerjee and Sharma, 2004, 2005). Pioneering investigations on extremophilic organisms were conducted in the Negev deserts (Friedman et al., 1967). Cyanobacteria serve as fundamental colonizers of hot springs and hydrothermal vents. Their significance in these extreme habitats stems from their remarkable resilience to extreme temperatures and high salinity and their ability to undergo photosynthesis under adverse conditions. Production of compatible solutes enables them to withstand osmotic stresses resulting from desiccation, high temperature, and extreme salinity. Numerous cyanobacteria exhibit thermophilic traits (Ward and Castenholz, 2000), with many identified as halophiles, including extreme halophiles (Oren, 2000). However, limited information exists on cyanobacteria exhibiting both traits. Although initial molecular research has been conducted on the thermo-halophilic cyanobacterium Leptolyngbya from dominant ecological niches, there is a shortage of knowledge concerning its behaviour when isolated from its natural habitat and subjected to laboratory conditions. The investigation of this organism is crucial not only due to its biological and scientific significance but also because of the substantial gap in understanding its phosphate metabolism, which is essential for its growth and survival in its niche. Little is known about the mechanisms this organism employs to undertake phosphate metabolism under extreme conditions and how it supplements its phosphate requirements. Therefore, studying the extremophile Leptolyngbya holds significance in assessing the potential contribution of this organism and other extremophiles inhabiting similar ecological niches to the phosphorus dynamics of those environments. Additionally, it is inherently intriguing to explore organisms surviving on the brink of life, sustaining metabolic activities where their natural counterparts would perish. Such investigations are imperative before considering any biotechnological applications involving these organisms.

MATERIALS AND METHODS

Collection and identification of the organism

The Leptolyngbya Iceland clone two utilized in this study, a thermo-halophilic extremophile, was isolated from siliceous crusts at temperatures ranging from 40 to 45 degrees Celsius in a

geothermal seawater lagoon in southwest Iceland. Among Leptolyngbya morphotypes, Iceland Clone 2 was specifically chosen for subsequent investigation. This culture exhibited growth only within the 45 to 50 degrees Celsius temperature range, thriving in a medium with total dissolved solids (TDS) concentrations ranging from 28 to 94 g L(-1). Following an in-depth phylogenetic analysis of this strain in comparison with similar strains, conducted by Professor (Dr.) Meenakshi Banerjee from the Department of Bioscience at Barkatullah University, Bhopal, India, during her visit to the Center of Ecology and Evolutionary Biology at Oregon University, USA, the Leptolyngbya Iceland clone two was transported back to her laboratory of Algal Biotechnology in India for further research. Identification procedures were previously conducted in the USA through 16S RNA analysis (Banerjee et al., 2009).

Cultural media and cultural conditions

The filaments (trichomes) could migrate and grow outward from the central inoculum source on solid plates prepared with BG-11 medium and 15% Bacto Difco Agar. Small agar blocks containing individual self-isolated trichomes (clones) were carefully excised using sterile watchmaker's forceps under 40–60× magnification with a dissecting microscope and transferred into 125 mL flasks containing liquid IO BG-11 medium. After successful growth in the flasks, cultures were replated to ensure greater clonality. Cultures of Iceland Clone 2 were maintained at a constant temperature of 45 ± 1°C and continuously illuminated with cool white fluorescent lamps. The ambient temperature was maintained at 25°C ± 2°C with a light flux of 2500-3000 Lux and a light-dark cycle of 16:8 hours. Before commencing the experiment, the cultures were allowed to grow for six months under the above conditions to acclimate the organisms to their new environment, distinct from their original habitat.

Growth measurement by Chlorophyll analysis

The growth experiment was conducted in culture tubes containing 10 mL of basal medium. Growth was assessed by chlorophyll extraction using the method outlined by Banerjee et al. (2000) and Marker (1995). Chlorophyll was extracted using either 100% methanol or 80% acetone. Tubes were agitated for 5 minutes and left overnight in darkness in a refrigerator to ensure complete extraction. The optical density of the extract was determined using a Systronic 169 spectrophotometer at 663 nm. The quantity of Chlorophyll extracted was calculated according to the equation proposed by Mackinney (1941). A represents the absorbance of light by the sample, k is a constant dependent on the solvent used, and c is the chlorophyll concentration in the sample. The chlorophyll content of the algae was then calculated as:

$$\text{Chlorophyll } (\mu g/mL) = \text{optical density} \times 12.63 \times \text{dilution factor.}$$

The generation time (K) was calculated using the growth equation developed by Kratz and Myers (1955):

$$K = 2.303 (\log N2 - \log N1) / (T2 - T1)$$

Where N1 represents the initial optical density divided by the protein concentration at time T1, and N2 represents the final optical density divided by the protein concentration at time T2.

Measurement of phosphatase enzyme activity

The study of alkaline phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activity in the Iceland clone was conducted following the methods outlined by Manasseh et al. (1990), Whitton et al. (1990), and Banerjee et al. (2007). PMEase activity was routinely assessed using a colourimetric method employing para-nitro-phenyl-phosphate (p-NPP), while PDEase activity was evaluated using bis-para-nitro-phenyl-phosphate (bis-p-NPP). Cyanobacterial cells were subjected to a P-minus medium for two weeks before the phosphatase assay to deplete cellular phosphorus. The assays were performed in a phosphorus-free version of the medium, buffered with 100 µM glycine (final concentration) to achieve a pH of 8.0. Further practical details can be found in Whitton et al. (1992). The results are presented as µmol (pNP) µg $chla^{-1}h^{-1}$ for PMEase activity and µmol (bis-pNP) µg $chla^{-1}h^{-1}$ for PDEase activity. All results are expressed as mean ± standard deviation of three independent replicates.

Study of environmental factors

Effect of salt concentrations

The effect of different salt (NaCl) concentrations from 30g.l^{-1} to 140g.l^{-1} on phosphatase activity (PMEase and PDEase) of Leptolyngbya was studied (Banerjee et al. 2009).

Effect of pH

The impact of pH ranging from 5 to 10 on the growth and the organism's PMEase and PDEase activity was investigated. Various pH conditions were regulated using a universal electrode pH meter (Systronics Model No. 361), and adjustments were made by adding 2 N NaOH and 1 N HCl to achieve the desired pH levels. To maintain pH stability, the medium was buffered with Tris (Hydroxymethyl) aminomethane/HCl buffer, following the protocols outlined by Banerjee et al. (2000) and Whitton et al. (1992).

Effect of light intensity

The cultures were maintained in the culture room for light intensity, illuminated by three 150-watt fluorescent tubes. The light source was obtained from a cool fluorescent tube of about 150W. To avoid overheating, cultures were incubated in water-jacket boxes. Variation in light intensity was obtained by increasing and decreasing the distance of cultures from the source of light using lux meter LX-101 Lutron, and dark conditions were made by wrapping the culture tube or flasks twice with aluminium foil and carbon paper (Banerjee et al. 2000 a,b).

Statistical Analysis

Statistical analysis consisted of mean values and standard errors, which are given in the figure and tables. Means were compared using student's t-test at $\alpha = 0.01$ and 0.05.

Result

Effect of environmental factors on phosphatase activity.

After obtaining results on growth in this Iceland strain Leptolyngbya, with different environmental factors, the same conditions were chosen to study the effect on phosphatase activities. Like growth, maximum phosphomonoesterase and phosphodiesterase activity were observed with 90g. The L^{-1} of Nacl, therefore this concentration of NaCl, was kept constant in further studies on phosphomonoesterase and phosphodiesterase activity, as shown in Figures 1A and 1B. The effect of different pH on the phosphomonoesterase and phosphodiesterase activity was studied, and the strain was rated from 5 to 10. In Leptolyngbya, maximum phosphomonoesterase activity was observed at

pH-10 (0.062 µmol p-NP µg Chla^{-1}h^{-1}) at 72 h and followed by the pH-9 (0.058 µmol p-NP µg Chla^{-1}h^{-1}) at 72 h (Figure.-8A). In this cyanobacterium at pH-10, there was a fold increase of 1.19 over the control pH-8 at 72 h (Figure 3B). Maximum phosphodiesterase activity was observed at pH 9 (0.056 µmol bis-p-NP µg Chla^{-1} h^{-1}) at 96 hours, representing a 1.27-fold increase over the control at pH 8 at the same time point (Fig. 5.5b). However, acidic pH levels 5 and 6, as well as neutral pH 7 (as depicted in Figures 3A and 3B), failed to elicit any significant impact on the phosphomonoesterase and phosphodiesterase activity of Leptolyngbya when compared to the control conditions (as depicted in Figures 3A and 3B).

The impact of various light intensities on the strain's phosphomonoesterase and phosphodiesterase activity was investigated. Experiments conducted with different light intensities on Leptolyngbya revealed maximum phosphomonoesterase activity at 2500±200 lux light (0.052 µmol p-NP µg Chla^{-1} h^{-1}) at 72 hours (Figure 1A). The next most favourable result was observed at 5000±200 lux light (0.046 µmol p-NP µg Chla^-1 h^-1). In Leptolyngbya exposed to 5000±200 lux light intensity, there was a 0.882-fold decrease compared to the control (2500±200 lux light) at 72 hours (Figure 1A). Conversely, increasing light intensities decreased phosphomonoesterase activity at 72 hours (Figure 1A).

Phosphodiesterase activity was also highest at 2500±200 lux light (0.044 µmol bis-p-NP µg Chla^{-1}h^{-1}) at 96 hours (Figure 1B). However, increasing light intensities led to decreased phosphodiesterase activity at 96 hours. The effect of darkness on the phosphomonoesterase and phosphodiesterase activity of the organism is illustrated in Figure 2 A, and B. Leptolyngbya exhibited a nominal decrease in phosphomonoesterase activity under dark conditions (0.050 µmol p-NP µg Chla^{-1}h^{-1}) at 72 hours, representing a 0.96-fold decrease compared to the control under light conditions (2500±200 lux light) at 72 hours.

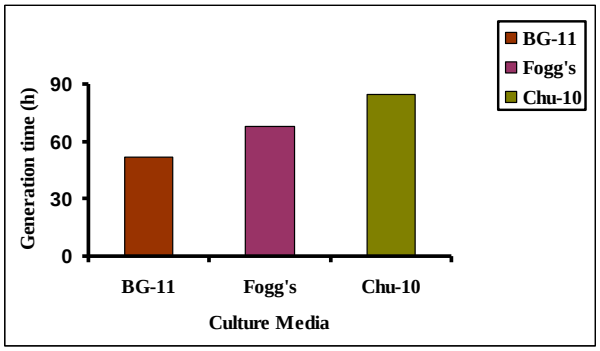

Figure 1. The generation time of leptolyngbya is in three different cultural media. Cultures grown under25^{o}c± 2^{o}c, light intensity 2500±200 lux, pH- 8

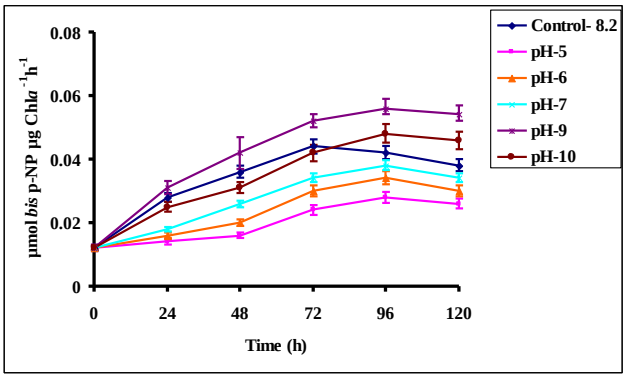

Figure 2A. Effect of different pH on phosphomonoesterase activity of leptolyngbya. Temperature 25^{o}c± 2^{o}c, light intensity 2500±200 lux. (Results mean ± SD, N=3).

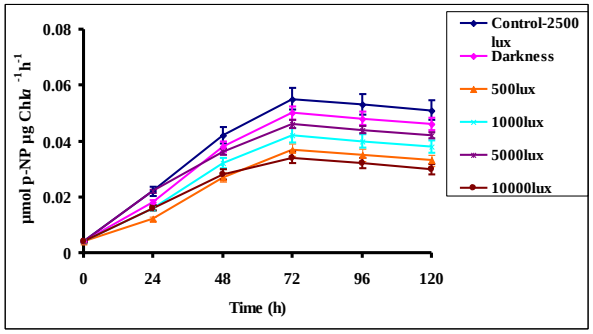

Figure 2B. Effect of different pH on phosphodiesterase of leptolyngbya. Temperature 25°c± 2°c, light intensity 2500±200 lux. (Results mean ± SD N=3).

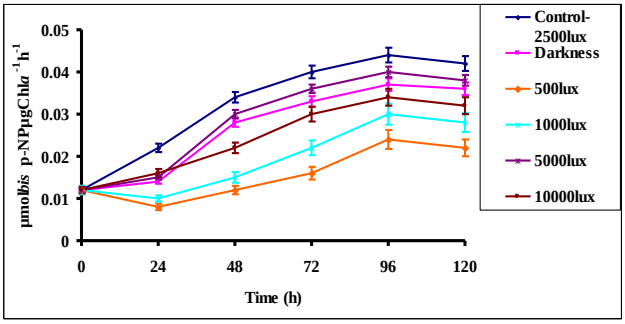

Figure 3A. Effect of different light intensities on phosphomonoesterase activity of leptolyngbya. Temperature 25°c± 2°c pH- 8 (results mean ± SD N=3).

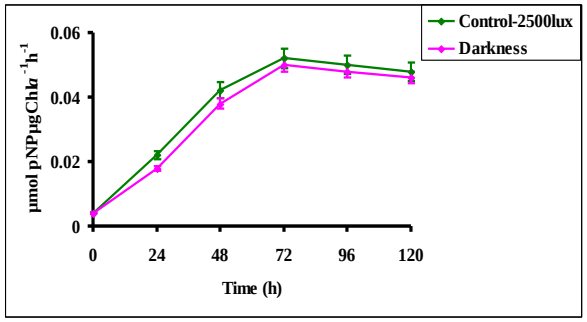

Figure 3B. Effect of different light intensities on phosphodiesterase activity of leptolyngbya. Temperature 25°c± 2°C, pH- 8 (results mean ± SD N=3).

Discussion

In the present study, some significant observations point to the variation in response of extremophilicLeptolyngbya when exposed to different environmental conditions. It is even more significant since there is little information on cyanobacteria that combines these two extreme traits, such as the Iceland clone 2 Leptolyngbya. The members of the common and ecologically important cyanobacterial form–the genus Leptolyngbya, are widely distributed in numerous ecosystems all over the globe (Komarék 1989). The present study shows that Leptolyngbya can also grow under various extreme conditions. The ability ofLeptolyngbyato to survive at a super higher NaCl concentration ($90g.l^{-1}$) is probably because of the adaptation to the conditions in the hyper saline-thermal lake of Iceland, where it was isolated. The significant growth in thrice the salt concentration of seawater can be explained by the fact that a considerable amount of Na^+ is required for the growth of halophilic cyanobacteria because it stimulates photosynthesis so rapidly that HCO_3^- transport into the cell raises the intracellular CO_2 concentrations. In halophiles, adaptation to higher concentrations of NaCl is also dependent upon the osmotic adaptation of the organism, and organic solutes play an important role in osmotic adaptation.

The pH of an ecological niche is the most important factor determining the cyanobacterial occurrence, which is reported from a wide range of acidic to alkaline environments. The pH optimum for the growth ofLeptolyngbyawas 9. Many cations, such as Na+, Mg++, Ca++, etc, exist at alkaline pH, which are taken up by the organism and thus increase the photosynthetic efficiency of cells. The negligible growth at low pH proves that an acidic medium is detrimental to the growth of the strain. Acidic pH might affect the photosynthetic apparatus.

Moreover, Chlorophyll a is very acid-labile and decomposes into pheophytin under acidic conditions. Light availability influences cyanobacterial growth. The observed effects in this study show that the growth of cyanobacteria is generally sensitive to high light intensities. In halophiles, the high light intensity may reduce the functioning of the reaction centre of PS I and PS II; therefore, the observed decrease in the growth of Leptolyngbya at high intensity. Laboratory experiments using white light have generally indicated that cyanobacteria have low light requirements for growth at very low irradiance intensities. Natural hot spring mats suggest that many cells making up the algal layer might not become lightly saturated even at high light intensities because of self-shading, except for some thermophilic microorganisms that avoid high light irradiance by active gliding motility response, causing a move downward into the soft microbial mats 'or' sediments with increasing irradiance, thereby avoiding the stress entirely. The Chlorophyll a to phycobiliprotein ratio remained constant during light-limited growth despite reducing pigments as irradiance increased. This indicates that the number of photosynthetic units per cell declines in response to increasing irradiance. Light is an important factor in growth and photosynthesis, as demonstrated in this study. The slight decrease in growth rates in the dark is related to the non-availability of reductant and energy in the form of ATP, affecting the photosynthetic process and leading to a decrease in the organism's growth rate compared to light conditions.

Cyanobacteria can synthesize phosphates in response to phosphorus deficiency in their external environment. Phosphate-solubilizing microorganisms produce extracellular enzymes like phosphatases and organic acids for phosphate solubilization. Cyanobacteria secrete alkaline phosphatase into the surrounding medium under phosphate-starved conditions. During a long P-limitation period, Cyanobacteria excrete extracellular phosphatases, which frequently release inorganic phosphate (Pi) from a few complex organic phosphate substrates (Stihl et al. 2001) that cannot be transported through the cell membrane. Alkaline phosphatase is the first enzyme active in a dissolved state in natural water. The high alkaline phosphatase activity obtained in the saline medium in the present study indicates halotolerance of phosphatase enzyme in Leptolyngbya. In the natural environment, Leptolyngbya is found in salinity thrice that of seawater, which coincides with the laboratory-grown cells (90 $g.l^{-1}$). NaCl stress at lower concentrations might be caused by increased cellular PMEase activity, while a higher concentration of NaCl might favour the release of the extracellular PMEase. An increase in activity in higher concentrations of NaCl ($90g.l^{-1}$) suggests that the higher concentrations of salt stimulate the release of the enzyme. The release of cell-bound enzymes into the medium proves that alkaline phosphatases are loosely bound to structural elements of the cell wall and can be easily released from cells by osmotic shock. NaCl has a concentration-dependent dual effect on phosphatase activity in this strain.

The results suggested that phosphomonoesterase and phosphodiesterase might be inducible enzymes in this organism. This study also suggests that high salinity seems to trigger phosphate uptake, thereby providing the necessary energy in the form of ATP, resulting in a marked increase in alkaline phosphatase activity. In this study, significantly less PDEase activity was observed compared to PMEase activity. This is probably because the PDEase

releases about 25% of the inorganic phosphate from bis-pNPP compared to that released by pNPP. The monoesters can provide the necessary phosphorus levels to the cells as substrates and are easily available compared to diesterases and their activity. The pH values that gave maximum growth and phosphatase activity were quite close to those reported for most cyanobacteria, and this also coincides with the pH conditions present in the hot springs. Thermophilic cyanobacteria occur in environments with pH values of 7.0-9.5.

Na^+ is also known to regulate the internal pH of the cell in cyanobacteria. The negligible rates of alkaline phosphatase activity at low pH, i.e., 5-7, proved that an acidic medium is detrimental for the alkaline phosphatase activities as this enzyme gets induced well above pH-7 and which goes per the name of the enzyme alkaline phosphatase. The energy dependence of the phosphate uptake process was observed in different light-intensity conditions. It might be expected that the activity of an enzyme occurring at the cell surface would not be closely linked to photon irradiance, at least in the short term. In the present study, it has been observed that light is likely to enhance phosphate uptake from the vicinity of the enzyme, thus reducing possible substrate inhibition. This suggests that light had a positive effect on phosphate activity. Approximately similar enzyme activity in light conditions suggests that although a distinct light regime exists in hot springs, fluctuations and decreases in light intensity may not affect phosphatase activity in extremophilic cyanobacteria. Apart from that, these cyanobacteria are found in layers in their natural environment. This ability allows the naturally occurring organisms and laboratory-grown cultures to be grown under dark conditions. The almost similar effect of light and darkness on PMEase and PDEase activities was unexpected. Much of the effect occurred during the early stage of assays, so the decreased percentage dropped during long-term assays.

Nevertheless, this effect was consistent and repeatable, and there was no obvious source of an artefact. Most studies on surface phosphatases show no effect of light during short-term studies. However, a few studies on seaweeds have shown enhancement in the light (Hernandez et al. 2000), where the effect is thought to reflect the demand for energy to transport the phosphate released by hydrolysis into the cell. The red alga Corallina elongata is the only organism reported to show slightly higher activity in the dark than under low light flux (Hernandez et al. 1996). It was suggested that phosphatase activity might help fulfil P requirements in this organism when direct uptake of inorganic P is reduced under conditions of limited reducing power. This might apply to the Iceland clone Leptolyngbyaand, and possibly the surface phosphatase(s) in this organism undergoes some form of change on transfer from dark to light. Any factor that affects the metabolism of phosphates, which is crucial for biomolecules associated with energy exchange and genetic processes at the limits of survival, is of great importance.

CONCLUSION

Despite its exposure to high salinity and temperature, significant phosphatase activity in the Icelandclone 2 Leptolyngbya strain suggests that this cyanobacterium does not lose the capacity to form phosphatase enzymes even under those adverse conditions. The presence of cyanobacterial phosphatases may have very important ecological implications in diverse ecological niches, including the extremophilic environments where there is a constant

fluctuation in nutrients, especially of phosphate, and organic P may be a frequent alternative to inorganic P as these cyanobacteria are the dominant organisms in the algal/cyanobacterial flora in these environments. Significant enzyme activity in both light and dark for Leptolyngbya implies that changes in light regimes in thermophilic environments may not affect phosphatase activity. Cyanobacterial cells within the immobilized mats of cells on the boundary of the thermal lake from where the cyanobacterium was isolated could become light-limited by the shading effect in the lower layers. Still, even under these conditions, results predict that limiting light conditions does not affect phosphatase activity. Observations of the present study suggest that in similar extreme ecological niches, alkaline phosphatase activity can contribute significantly to maintaining the growth of cyanobacteria for prolonged periods, even under unfavourable conditions of phosphorus depletion. This island strain has two extreme traits: a thermos halotolerant phosphatase enzyme, making it extremely important for industrial applications.

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