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Growth and Nitrogen (N) Metabolizing Enzymes of Mesophilic and Psychrophilic Heterocystous Cyanobacteria—In Response to Temperature Regimes

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Abstract

The increased NR & NiR activity, GS and nitrogenase activities at 25°C and 35°C in psychrophilic and mesophilic organisms indicates that the Arctic isolate is a psychrotolerant as it shows increased enzyme levels than its original temperature in which we isolated the organism though biomass was greater at 3°C. The biomass and enzyme data indicates cyanobacteria are the initiators of primary colonization in polar and alpine environments and are responsible for the nitrogen and carbon incorporation into biosphere. The nitrogenase activity as measured by ARA was high at 37°C mesophilic and 25°C was found to be optimum for psychrophilic. Likewise, NR & NiR was also optimal at 25°C.

Keywords: *Arctic, cyanobacteria, glutamine synthetase, nitrate reductase, nitrite reductase, nostoc calcicole*

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INTRODUCTION

The prokaryotic cyanobacteria are characterized by their oxygenic photosynthetic ability. Globally wide range of environments are made up of complex, micro-scale ecosystems constitutes microbial mats of photosynthetic cyanobacteria [1]. The cyanobacterial existence in diverse ecological niches from polar to desert climate, due to their structural diversification with diverse metabolism and environmental plasticity [2]. The habitats, includes freshwater lakes, temperate soils, vast oceanic areas, and even in hot springs arid deserts and icy lakes [3]. Temperature is the critical regulatory factor affect the photosynthesis, growth and nutrient uptake by influencing enzyme activity, electron transfer chain, membrane fluidity [4]. Based on temperature influenced habitation in the planet, cyanobacteria can be classified into psychrophilic, psychrotolerant, mesophilic, and thermophilic forms [5]. The algal microflora of freshwater and terrestrial habitats are important in extreme ecosystems of polar hemisphere [6]. Temperature below 5°C is common throughout the cold environments of polar region [7]. Persistent cold due to extreme fluctuations in irradiance, freeze-thaw cycles leads large variations in nutrient supply and salinity. The biomass and species richness of prokaryotes in these ecosystems denotes restricted biodiversity as a result of these constraints. Fossil record states, cyanobacteria were the only organisms achieved complete morphological diversity 2 billion years ago [8]. In Antarctic ponds, streams, rivers, and lakes covered with perennial ice cover most of the year. The microbial mats primary photosynthetic production these habitats were controlled by PAR (photosynthetically active radiation) [9]. The factors such as salinity and nutrients also influence the cyanobacterial diversity [10]. In addition to other biota of Arctic ice shelves, glaciers, provide habitats for cyanobacteria [11,12**[AQ: Please verify if the citation given here for Säwström et al., 2002; Mueller et al., 2005 is correct.]** However, the total area of ice shelves is lower than in Antarctica, where ice shelves fringe 40% of coastline. Nostocales, Oscillatoriales, and Chroococcales constitute the most common groups. Incorporation of Nitrogen into the

biosphere by cyanobacterial assimilation—a process known as N_2 fixation, an inherent ability, thereby making an important contribution to the earth's nitrogen as well as other nutrient cycle. [13]. The assimilation of nitrate, nitrite and urea even amino acids such as arginine and glutamine provides intracellular ammonium, an ideal nitrogen source. The repression of permease genes and nitrogen assimilatory enzymes in the presence of ammonium, a process called "nitrogen control" [14]. The cohesive layering of microbial mats in a benthic region of lakes, established through an extra polysaccharide matrix with characteristic pigment stratification [15]. Under low-nutrient conditions, organisms, especially, cyanobacteria grow efficiently [16]. In an Arctic ecosystem, among the different nutrients, nitrogen is found to be limiting [17]. Under this N limiting conditions, amazing ability of diazotrophic cyanobacteria (mostly *Nostoc* and other genera) contribute a larger extent toward carbon and nitrogen economy[18,19]. The main factors which affect diazotrophic nitrogen fixation in polar environments are temperature and soil moisture [20]. Hence, in this study, the influence of temperature on growth and their nitrogen fixation/assimilation ability of selected *Nostoc* strains belongs to psychrophilic and mesophilic groups were planned.

MATERIALS AND METHODS

Source of Strains and Maintenance

Both the psychrophilic *Nostoc sp* BDU ARC 10641 and mesophilic *Nostoc calcicola BDU 40302* from germplasm of NFMC are maintained at 15 ± 2°C and 25 ± 2°C under cool white fluorescent light at 1500 lux intensity with 24/0 L/D cycle.

Light Microscopy

The strains were observed under Leica inverted microscope DMI 3000B (Leica, Germany) equipped with digital camera DFC 425C and the documented images were processed with the Leica application suite software. Morphological confirmation of strains was done by standard identification literatures [21,22].

DNA Extraction and Amplification

Xanthogenate nucleic acid isolation protocol [23] was used to extract entire genomic DNA from the selected *Nostoc* strains. 16S rDNA gene was split into 3 regions and amplified with different set of primers to generate the complete sequence of the DNA. The 16S-8 forward primer–AGAGTTTGATCCTGGCTCAG and 16S-740 reverse primer–TCTACGCA TTTCACCGCTAC [24], cyanobacterial specific primer CYA-359 forward–GGG GAATTTTCCGCAATGGG and equimolar ratio of CYA-781(A) reverse primer–GACTACTGGGGTATCTAATCCCATT, and CYA781(B) reverse primer–GACTACAGG GGTATCT AATCCCTTTI [25] and CYA-359 forward primer–GGGGAATTTTCCGCA ATGGG [25] and 16S-1494 reverse primer–GTACGGCTACCTTGTT ACGAC[26] were used to amplify I region, II region, and the III region of 16s rRNA gene sequence, respectively. Eppendorf master cycler Pro S PCR machine was used for amplification with GoTaq colorless master mix (Promega, USA), 0.3 lM of each primer, and template DNA quantity of 100 ng in a 100 µl reaction. The reaction steps were, initial denaturation (94°C for 5 min) followed by 30 cycles of denaturation (94°C for 1 min), annealing (I region: 55.5°C, II region: 60°C, and III region: 56.4°C for 1 min) and extension (72°C for 1 min) and a final extension (72°C for 7 min). The amplified products electrophoresed and visualized under UV transilluminator.

16S rRNA Gene Sequence Analysis

After visual confirmation of amplified bands, with QIAquick PCR purification kit (Qiagen, Germany) purified products were sequenced. The forward/reverse sequences of 3 regions were retrieved from electropherograms. The sequences were manually checked and assembled into a single contiguous sequence of whole 16S rRNA gene using the software DNA Baser version 3.5.1 (http://www.dnabaser.com). To identify the closely related taxa, nucleotide blast of NCBI (http://blast.ncbi.nlm.nih.gov) was performed online for sequence comparison. Clustal X was used to multiple sequence alignment [27] and the phylogenetic tree was generated with MEGA software version 6 [28].

Nucleotide Sequence Accession Numbers

The accession numbers for psychrophilic *Nostoc sp* BDU ARC 10641 and mesophilic

Nostoc calcicola BDU 40302 are KY662381, KC883980, respectively.

Growth Conditions

Selected strains were scaled up using ASN III, BG 11 medium. The cultures were grown under white fluorescent light with an intensity of 20 μmol m^{-2} s^{-1}. The mesophile *Nostoc calcicola* BDU 40302 was maintained at 25 ± 2°C in ASN III medium and psychrophilic *Nostoc sp* BDU ARC 10641 was maintained at 15 ± 2°C in BG 11 medium with a photoperiod of 24:0 h light/dark.

To study the effect of temperature on nitrogen assimilation, the mid log phase cultures both were, respectively, inoculated in ASN III N, BG11 N media and incubated at different temperatures varying from 3 ± 2°C, 15 ± 2°C, 25 ± 2°C, 35 ± 2°C. At 12th and 24th hr, cultures were analyzed for nitrogen-fixing enzymes.

Dry Weight Estimation

On the seventh day of growth, both the cyanobacterial cultures were centrifuged and with distilled water the pellet washed, dried by hot air oven at 60°C for at least two replicate values.

Chlorophyll Estimation

After centrifugation the pellet mixed with 80% methanol in the dark and Chl-a was extracted. Cary 100 bio UV–VIS spectrophotometer was set at 663 nm and the optical density of the supernatant was measured and estimated following [29].

Carotenoid Estimation

After centrifugation, the pellet mixed with 85% acetone in the dark and carotenoid was extracted. Cary 100 bio UV–VIS spectrophotometer was set at 450 nm and the optical density of the supernatant was measured and the amount of carotenoids were estimated taking acetone as blank [30].

Protein Estimation

The protein content culture extract was measured using the method suggested by [31] with bovine serum albumin as standard.

Nitrogen Metabolizing Enzymes

The spectrophotometric assay for Nitrate reductase (NR, E.C.1.6.6.1) and Nitrite reductase (NIR, E.C.1.7.7.2) enzymes were carried out by nitrate reduction with sodium dithionite and reduced methyl viologen as the electron donor [32]. The enzyme activity was expressed as μg nitrite formed/removed mg^{-1} protein. Glutamine synthetase (GS, E.C.6.3.2.3) assay for cyanobacterial pellet permeabilized with toluene was performed at 4°C. Temperature incubated cells were mixed with 1 ml of reaction mixture for 30 min for estimating GS activity in vitro. After stopping the reaction, Cary 100 bio UV–VIS spectrophotometer was set at 540 nm and the optical density of the supernatant was measured and the enzyme activity was expressed as μg γ-glutamyl hydroxamate formed mg^{-1} protein [33]. Acetylene reduction assay was used to determine Nitrogenase (ARA, E.C. 1.18.6.1) activity [34] in the selected two *Nostoc* strains grown at varied temperatures of 3, 15, 25, and 35°C in sealed airtight vials. 10% gas phase was withdrawn from the above vials, to replace acetylene. At respective temperatures, the assay vials were incubated under cool white fluorescent light of 20 μmol photons m^{-2} S^{-1} with an illuminated shaker (100 rpm). To stop enzyme activity, 0.2 ml of 20% trichloroacetic acid (TCA, Sigma Aldrich, USA) was injected to each vial. The gaseous phase of 100 μl was injected into Porapak-T column with oven temperature of 75°C; detector temperature of 120°C; nitrogen as carrier gas with flow of 30 ml min^{-1}) in a gas chromatograph model Clarus 500 (Perkin Elmer, USA) equipped with a flame ionization detector to determine concentration of reduced ethylene. Ethylene puriss ≥99.95% (Sigma Aldrich, USA) was used as standard. And the nitrogenase activity was expressed as nanomoles of ethylene formed h^{-1} G^{-1}dryweight.

Statistics

The error in the graph represents N ± SE, where $N = 3$.

RESULTS

Though the harsh environment forcibly put an end to all life forms there, the cyanobacteria, the only microbial forms not only grow in lower temperatures but also play significant role in nitrogen and carbon cycle of this extreme habitat. Both the isolated psychrophilic and mesophilic *Nostoc sp* were compared for N_2 fixation and assimilatory reduction in their suitable and different temperatures for

understanding the nitrogen metabolism. Chosen strains under microscopic observation shows trichomes unbranched, rounded, or subspherical heterocysts with barrel shaped cells [21] (Figure 1).

Morphological observation of polar isolate *Nostoc sp* ARC 10641 showed compact trichomes, cells linked, colorless sheath with cell length of 4.5 μm, breadth of 4.3 μm with rounded heterocyst (Figure 1A). The other strain, mesophilic *Nostoc calcicola* BDU 40302 shows loosely arranged trichomes with colorless sheath with cell length, breadth of 4.1 to 4.4 μm and 3.1 μm and subspherical heterocyst (Figure 1B). Using 16S RNA gene marker sequence, molecular identification of organisms was carried out to infer their phylogenetic position. The neighbor joining method was used to construct phylogenetic tree with nearly complete 16S rRNA gene sequence (*1380) using closely related 20 cyanobacterial sequences. The Arctic isolate *Nostoc sp* BDU ARC 10641 and *Nostoc calcicola* BDU 40302, clustered with *Nostoc sp*. 152 AJ133161 and *Nodularia sp* PCC 7507, respectively (Figure 2**[AQ: Please verify Figure 3 has been renumbered to Figure 2 to maintain sequential citation.]**).

Fig. 1: *Microphotographs of Selected Strains: (A) Nostoc sp BDU ARC 10641 and (B) Nostoc calcicola BDU 40302.*

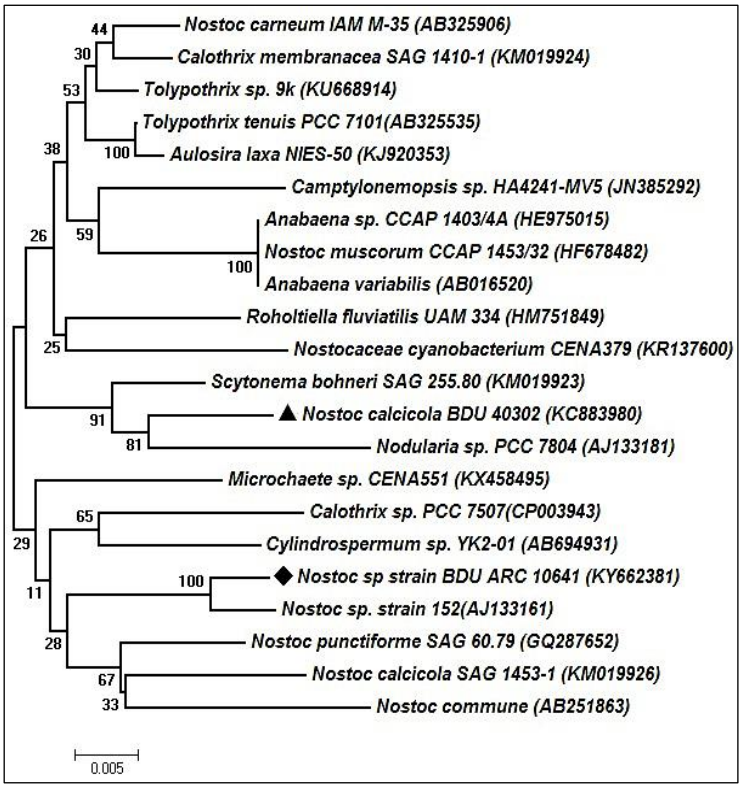

Fig. 2: *Phylogenetic Tree of Selected Psychrophilic Nostoc sp BDU ARC 10641 and Mesophilic Nostoc Calcicola BDU 40302.*

The microscopic observation of temperature incubated strains showed clumps of aggregated cells of mesophilic *Nostoc* at 3 and 15°C (Figures 3E and 3F**[AQ: Please verify Figures 2E and 2F has been renumbered to Figures 3E and 3F to maintain sequential citation.]**). Psychrophilic *Nostoc* formed extensive sheath and aggregated cells at 3 and 35°C (Figures 3A and 3D**[AQ: Please verify Figures 2A and 2D has been renumbered to Figures 3A and 3D to maintain sequential citation.]**). Whereas in other temperatures both organisms showed dispersed filaments throughout the medium. Chlorophyll, carotenoids, and biomass (dry weight), was estimated for selected strains at different temperatures (3°C, 15°C, 25°C, 35°C). Though the biomass component and chlorophyll of Arctic *Nostoc* initially elevated from 3°C to 15°C, and got drastically reduced about 50% at 37°C (Figures 4A and 4C). The above components were started to decline at 25°C for an Arctic isolate implying, as a psychrophile, it could not grow well at higher temperature. On the other hand, mesophile showed a decrease in biomass and chlorophyll with respect to lower temperature as expected which also indicate its mesophilic nature (Figure 4A and 4C).

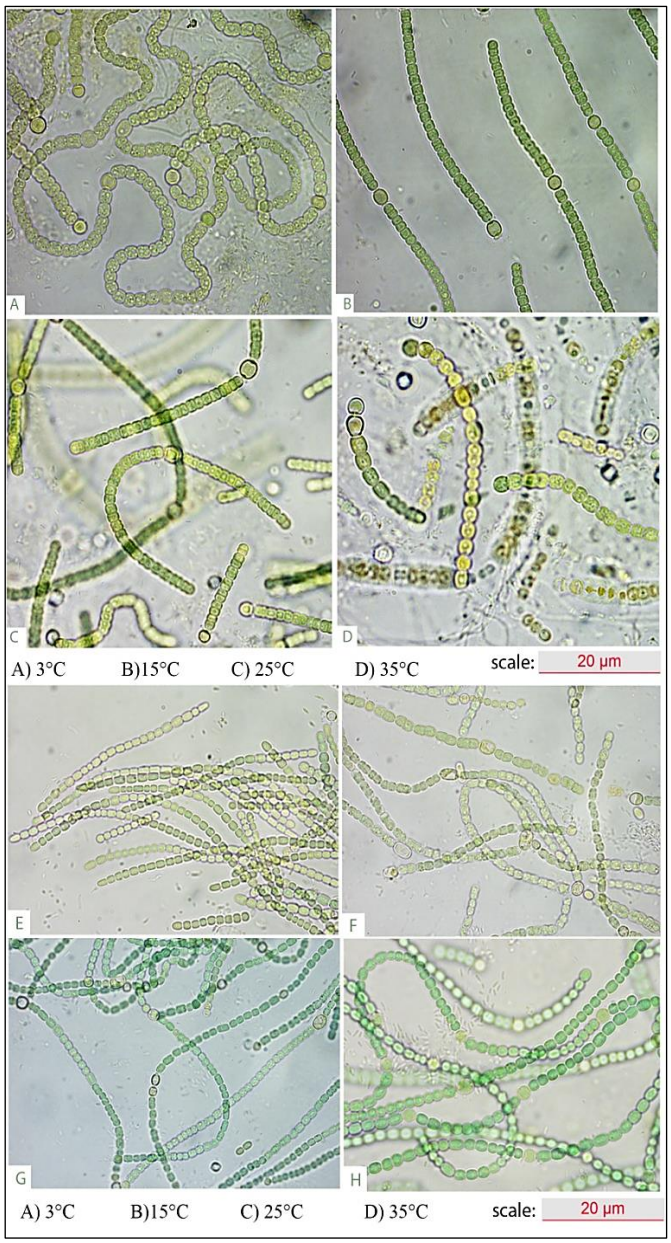

Fig. 3: *Bright field microphotographs of psychrophilic Nostoc sp BDU ARC 10641 (A-D) and mesophilic Nostoc calcicola BDU 40302 (E-G) grown at different temperatures.*

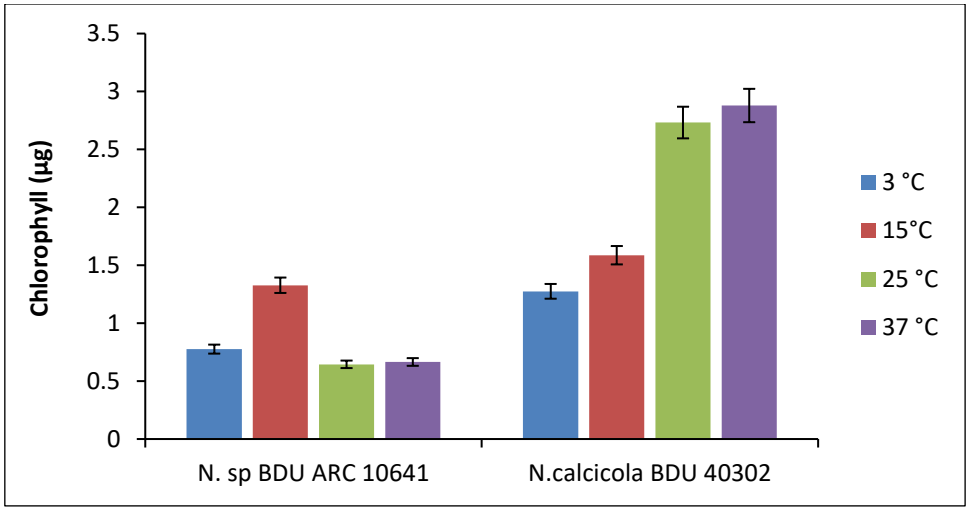

Fig. 4A: *Growth Analysis of Selected Psychrophilic and Mesophilic Strains in Terms of Chlorophyll*.

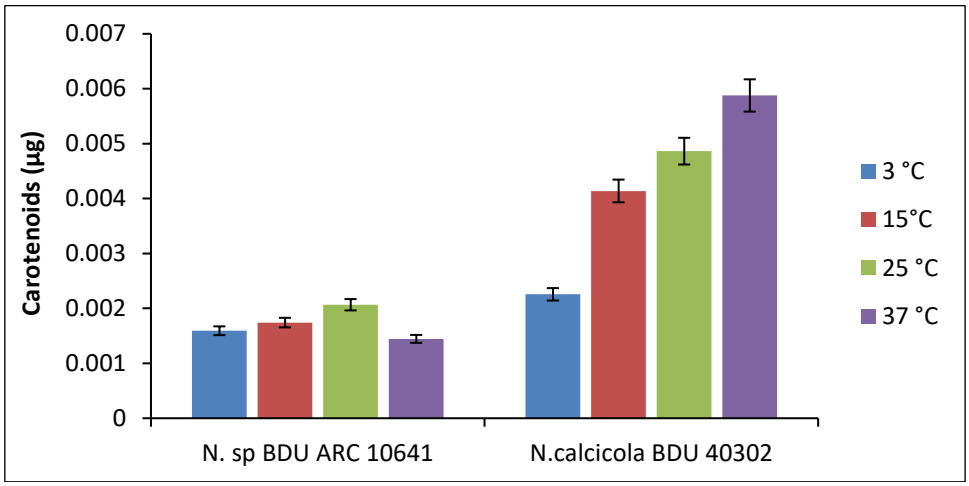

Fig. 4B: *Growth Analysis of Selected Psychrophilic and Mesophilic Strains in Terms of Carotenoids*.

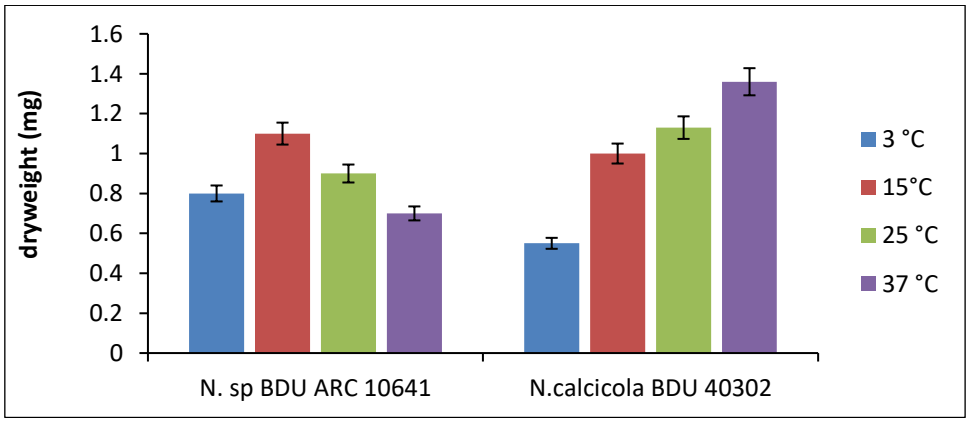

Fig. 4C: *Growth Analysis of Selected Psychrophilic and Mesophilic Strains in Terms of Dry Weight*.

The carotenoid content of mesophilic *Nostoc* was maximum at 37°C, while in psychrophilic *Nostoc*, carotenoids increased reciprocally up to 25°C. This correlates with the previous study [5] which showed reciprocal decrease of carotenoids to temperature enhancement in the Arctic strain. In the current study, Arctic *Nostoc* (psychrophilic form) synthesized high carotenoids at low temperature. In contrast, cartenoids of mesophilic *Nostoc* got reduced at low temperature and increased at high temperature (Figure 4B), again adding evidence

that mesophilic/psychrophilic genetic makeup is different. To understand the nitrogen assimilation in different temperature regimes, the tropical *Nostoc calcicola* BDU 40302 (mesophilic form) and psychrophilic *Nostoc sp* BDU ARC 11641 were grown in their native and altered temperatures. The N assimilatory enzymes such as Nitrate, Nitrite Reductases (NR), (NiR), and Glutamine Synthetase (GS) and N fixing Nitrogenase were measured during 12 and 24 hours. Mesophilic *Nostoc* shows higher NR activity at 35°C, whereas the psychrophilic *Nostoc* grew at 25°C showed high NR activity (Figures 5 and 6).

Similarly, a higher nitrite reductase (NiR) activity was found at 25°C in psychrophilic *Nostoc* and at 35°C, mesophilic *Nostoc* showed maximum activity (Figures 7 and 8). Increase NiR levels up to 25°C in psychrophilic *Nostoc*, 35°C in the case of mesophilic *Nostoc.*

In psychrophilic *Nostoc*, glutamine synthetase (GS) levels were high at 3 and 25°C 12th hr (Figure 10). In mesophilic *Nostoc*, GS levels actively got increased at 25°C and 35°C (Figure 9). Apart from their native temperatures, the psychrophilic *Nostoc*, showed nitrogenase activity also got increased up to 25°C, 24th hr indicating it is a psychrotolerant (Figure 12). Similarly, in mesophilic *Nostoc*, the nitrogenase activity was increased up to 35°C (Figure 11), whereas it was declined in the other organism at this temperature.

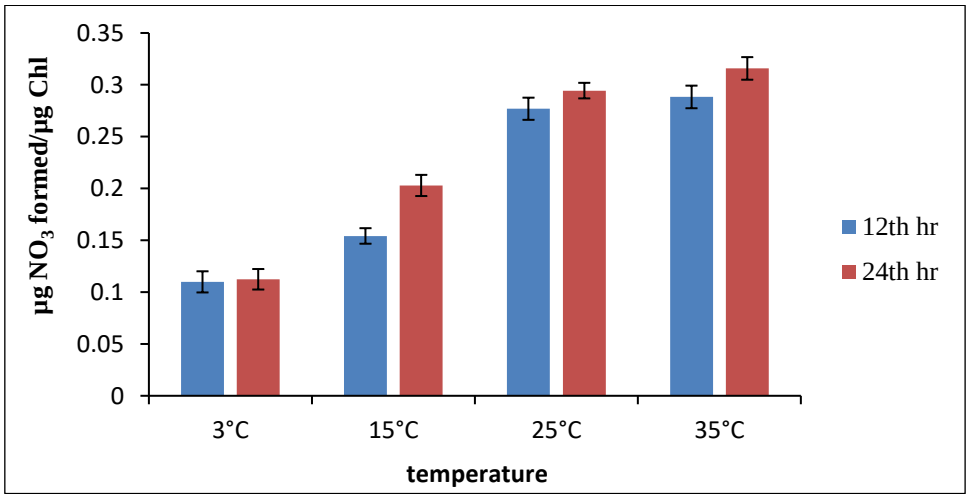

Fig. 5: *Nitrate Reductase Activity of Nostoc Cacicola BDU 40302 Grown at Four Different Temperatures.*

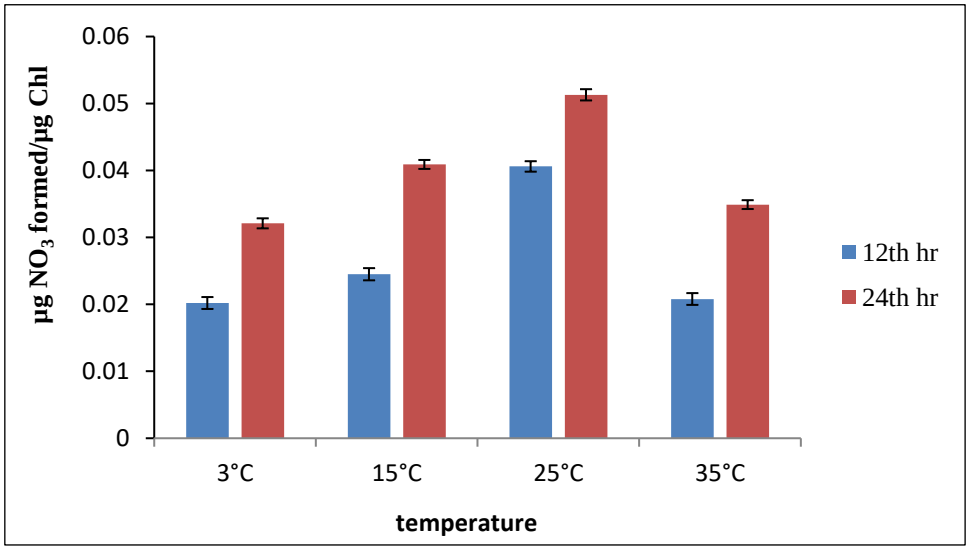

Fig. 6: *Nitrate Reductase Activity of Nostoc sp BDU ARC 10641 Grown at Four Different Temperatures.*

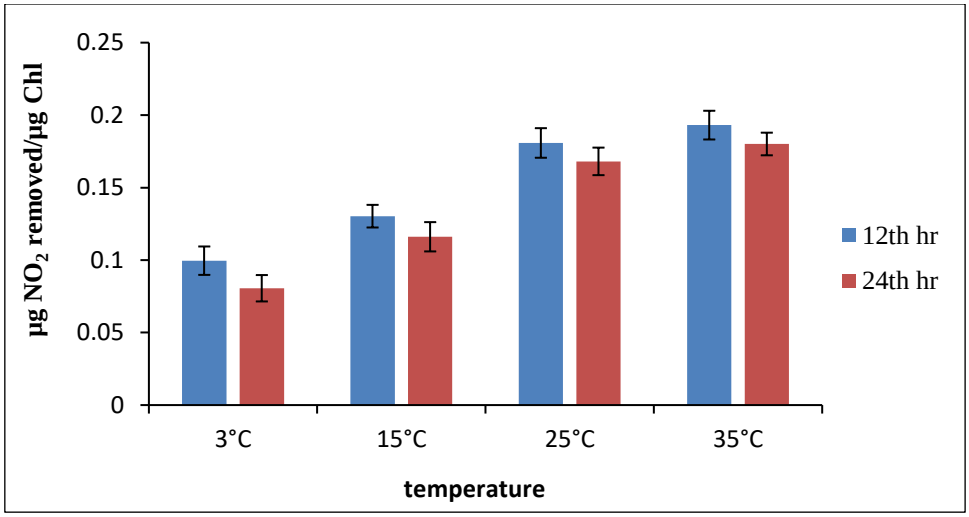

Fig. 7: *Nitrite Reductase Activity of Nostoc Calcicola BDU 40302 Grown at Four Different Temperatures.*

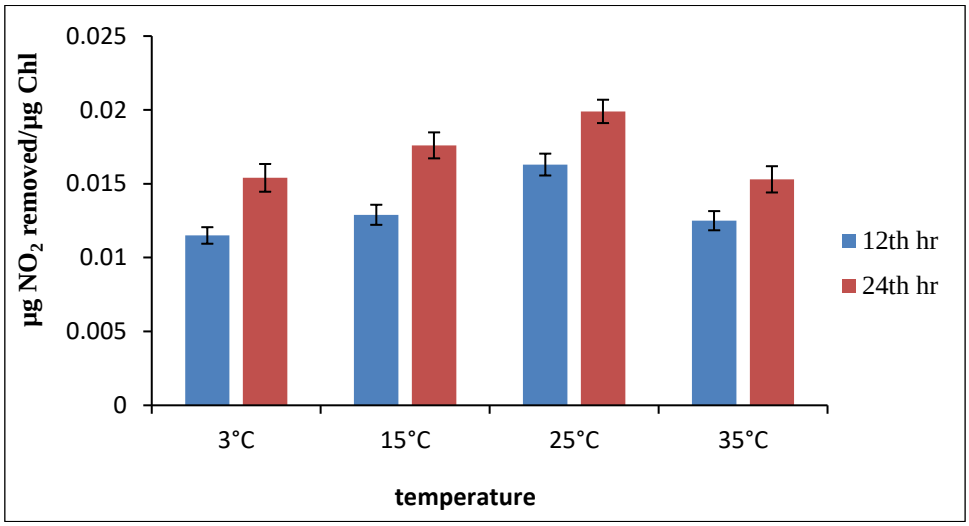

Fig. 8: *Nitrite Reductase Activity of Nostoc sp BDU ARC 10641 Grown at Four Different temperatures.*

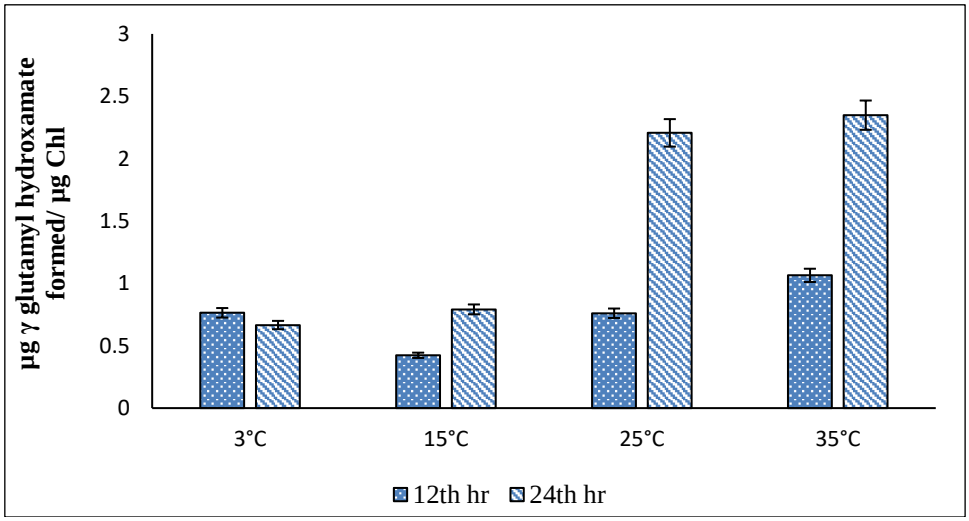

Fig. 9: *Glutamine Synthetase Activity of Nostoc Cacicola BDU 40302 Grown at Four Different Temperatures.*

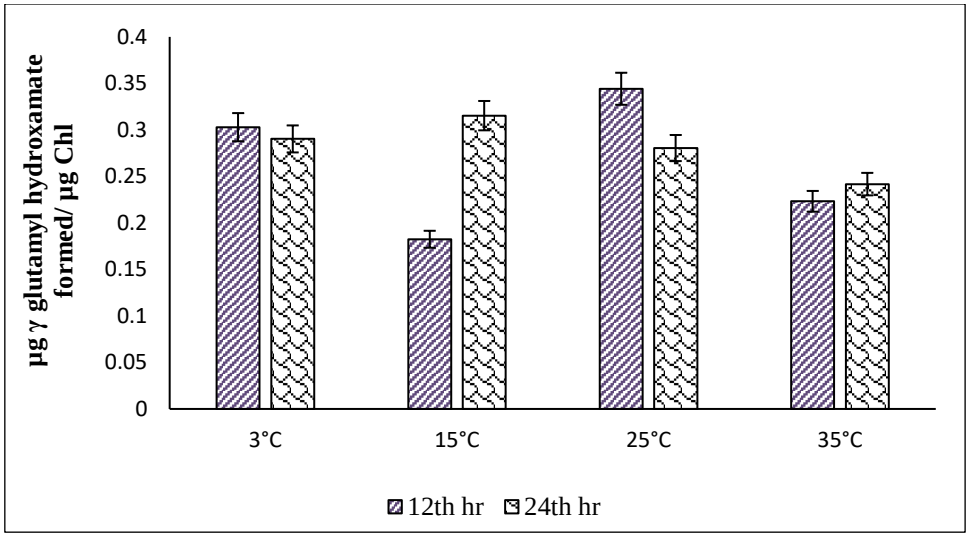

Fig. 10: *Glutamine Synthetase Activity of Nostoc sp BDU ARC 10641 Grown at Four Different Temperatures.*

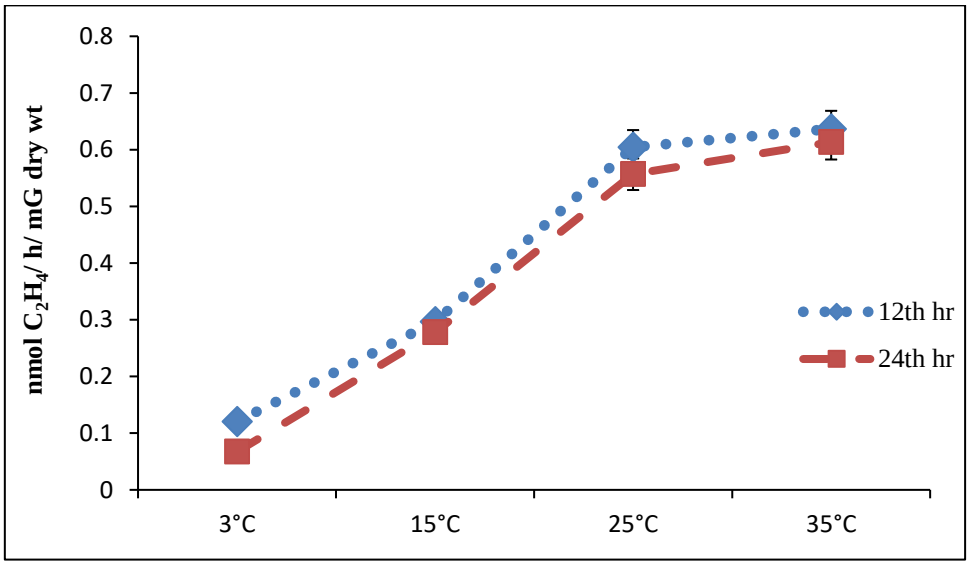

Fig. 11: *Nitrogenase Activity of Nostoc Calcicola BDU 40302 Grown at Four Different Temperatures.*

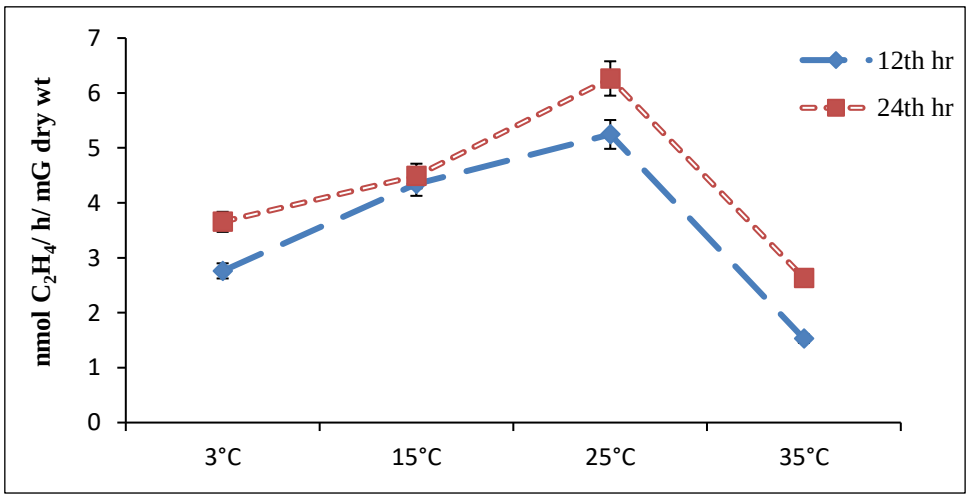

Fig. 12: *Nitrogenase Activity of Nostoc sp BDU ARC 10641 Grown at Four Different Temperatures.*

DISCUSSION

The icy environments such as sea ice, glaciers, and snow are dominated by complex microbial consortia, dominated by the cyanobacteria which are microbial oxygenic prokaryotic phototrophs, with the *Phormidium*, *Nostoc*, *Leptolyngbya* as prominent genera and *Gloeocapsa* [12]. The identification of genes (*nifH*) responsible for nitrogen cycling and targeting these genes through metagenomics in both Arctic and Antarctic soils studies facilitated the understanding of functional capability of diazotrophy [35]. The major problems which hamper growth of cyanobacteria in polar regions, oligotrophic environments are freezing temperatures, extreme fluctuations in UV irradiance and scarcity of nitrogen [36]. The stress-related damage is alleviated by the accessory photosynthetic pigment, carotenoid protects against singlet oxygen damage in turn plays vital role in buffering membrane fluidity [37]. According to Hille classification, all prokaryotic nitrate reductases subgrouped as respiratory, periplasmic, and assimilatory nitrate reductases and belongs to dimethyl sulfoxide (DMSO) reductase family [38]. For assimilatory purposes the 2-electron reduction of nitrate to nitrite was catalyzed by cyanobacterial nitrate reductases with a molecular weight of about 80 kDa contain a [4Fe–4S] cluster and a Mo-cofactor and they are monomers of the *nar*B gene product [39]. They are partially associated with the thylakoid membrane and can accept electrons from physiological electron donors such as photosynthetically reduced ferredoxin or flavodoxin, and from artificial electron donors like methyl viologen. The interaction of lysine and arginine of nitrate reductase with ferredoxin facilitates electrostatically stable complex between nitrate reductase and negatively charged ferredoxin [40]. In cytoplasm, the process of N assimilation involves reduction of nitrate to nitrite (NO_{2-}), using NAD(P)H as the electron donor by the enzyme nitrate reductase (NR), [41]. Nitrite reductase (NiR), further convert reduced nitrite to ammonium which is incorporated into amino acids by action of glutamine synthetase [42]. In cyanobacteria, ammonium assimilation takes place mainly by the sequential action of GS and glutamate synthase (GOGAT; GS-GOGAT pathway) [43]. The availability of nitrogen source strictly regulate synthesis of glutamine synthetase in the cell and plays a vital role in nitrogen metabolism of prokaryotes. Microbial nitrate reduction can be used for the following reasons. (1) nitrate utilization as a nitrogen source for growth (nitrate assimilation), (2) as the electron donor for metabolic energy generation (nitrate respiration), (3) excess reducing power dissipation for redox balancing (nitrate dissimilation) [44]. The critical step of the nitrogen cycle in the biosphere is the assimilatory nitrate reduction which is carried out by many bacteria, fungi, cyanobacteria, and plants involving sequential electron reductions catalyzed by nitrate reductases, nitrite reductases. The resulting ammonia incorporation into C skeletons via ammonium assimilation pathway is done by phototrophic organisms predominantly [45]. Temperature is increasing at higher rates in the polar regions than anywhere else on the planet. It was found that cyanobacterial mat communities and associated protozoans and metazoans are affected by the consequences of climate change. They are an ideal model system for the exploration of climate-induced changes due to their simple trophic structure and sensitivity to climate change. Freshwaters, soils, and glacier environments in polar and alpine regions are commonly dominated by nitrogen-fixing cyanobacteria and serve as indicators to climate change, recent study results that they are psychrotolerant rather than psychrophilic. The optimal growth temperature lie above the temperature range of the ambient environment [46]. To decipher the temperature associated changes in the cyanobacterial nitrate assimilation enzymes, the selected polar and tropical cyanobacterial isolates were incubated in psychrophilic, mesophilic temperature conditions, and vice versa. The results reveal that, in psychrophilic *Nostoc*, both NR, NiR showed maximum activity at 25°C than its normal temperature. At low temperature, the cyanobacterial cells have reduced their metabolic activity resulting in lesser enzyme activity. The increase in temperature from 15 to 25°C and from 25 to 35°C, might have caused high cellular permeability and uniform dissipation in medium which enables light penetration thereby increased the activity of NR, NiR, and GS [47]. At low temperature, low

permeability due to reduced membrane fluidity leads lessened activity of these enzymes. At high temperature (35°C), enhanced membrane fluidity and uniform dissipation of cells in medium enabled the mesophile metabolically to fetch better with increased NR, NiR, and GS activity; on the contrary at 3°C, sudden plasmolytic shock lead release of cellular constituents. The study mainly edges on NR, NiR, GS, and Nitrogenase enzyme systems. The increase of NR, NIR, GS at 25°C in psychrophile and at 35°C in mesophile revealed the activity of these enzyme systems based on altered temperatures, which might be the critical survival behavior of these organisms in the harsh environments with temperature fluctuations. It could be concluded that temperature plays a vital role in cyanobacterial N_2 fixation and assimilation.

CONCLUSION

Though both psychrophilic and mesophilic strains belong to the genera *Nostoc*, the study shows that based on the habitats at their habitat temperatures the enzyme system, pigment synthesis either get down or upregulated to combat the harsh environment. Both the strains, though isolated from different environments show maximum enzyme activities at their respective environments, they are responsible for "C" and "N" fixation.

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