Original Research Article
ISSN: 2583-7419
biologyforum.actabotanica.org

Exalted Level of Nitrogen Metabolism In the Presence of Sodium Sulphide in *Nostoc ellipososporum* under Thermal Stress

Manpreet[1], Lovepreet Kaur[2], Shveta[3], Jasvirinder Singh Khattar[4]

[1]*Department of Botany, Sri Guru Teg Bahadur Khalsa College, Sri Anandpur Sahib, Punjab, India*
[2]*Government College for Girls, Chandigarh, India*
[3]*Department of Botany and Environmental Science, Sri Guru Granth Sahib World University, Fatehgarh Sahib, Punjab, India*
[4]*Department of Botany, Punjabi University, Patiala, India*

Citation: Manpreet, Lovepreet Kaur, Shveta, Jasvirinder Singh Khattar (2024). Exalted Level of Nitrogen Metabolism In the Presence of Sodium Sulphide in *Nostoc ellipososporum* under Thermal Stress. *Acta Biology Forum*. **01 to 08.**
DOI: https://doi.org/10.51470/ABF.2024.3.3.01

Corresponding Author: **Manpreet** | E-Mail: **(manpreet.botany@gmail.com)**

Received 7 September 2024 | Revised 9 September 2024| Accepted 5 October 2024 | Available Online 2 November 2024

ABSTRACT

It is well-recognized that thermophilic cyanobacteria flourish in sulfur-rich thermal springs. Mesophilic cyanobacteria are available at temperatures ranging from 28 to 37°C. This study sought to determine if mesophilic cyanobacteria can also grow at elevated temperatures in the presence of sulphide, and if yes ,then what role sulfide would have in reducing high temperature stress in mesophilic cyanobacterial organisms. The prolifecation of Nostoc ellipososporum, a mesophilic cyanobacterium, was examined at its optimal temperature, both in the presence and absence of sulphide under thermal stress. During the current investigation, nitrate uptake was seen to slow down under temperature stress, but when sulphide was added to the cultures, nitrate uptake increased. This demonstrates that increased levels of nitrogen metabolism in the presence of sulphide contributed to the survival of the test organism under temperature stress.

Keywords: *mesophilic cyanobacteria; nitrogen metabolism; sulphide; high-temperature stress.*

1. INTRODUCTION

Cyanobacteria are believed to be ubiquitous, inhabiting a broader range of environments than any other organism on Earth. They can thrive in varied environmental conditions, such as hot water springs, hypersaline areas, chilling climates, and deserts. Additionally, they are commonly found in freshwater, marine, and terrestrial ecosystems [70,40]. One of the most remarkable features of cyanobacteria is their ability to thrive in a wide range of temperatures [40,17] They are classified into psychrophilic, mesophilic, or thermophilic based on their optimal growth temperature ranges. Psychrophilic cyanobacteria are found in Arctic and Antarctic lakes with average temperatures of 0 to 10°C [37,63]. Mesophilic species thrive at 25 to 37°C, while thermophilic cyanobacteria flourish at 45 to 60°C. Some cyanobacteria have even adapted to survive in extreme heat, thriving at temperatures between 73 and 90°C [14].

Microorganisms play a crucial role in sulfur springs. Sulfur is frequently found in many ecosystems, either consistently due to its natural presence in water or occasionally through biological sulfate reduction. Hot springs that contain sulfide in which temperatures ranges from 42 to 85°C [15,16].

Cyanobacteria can thrive in anoxic conditions, as evidenced by their growth in the presence of hydrogen sulfide. Additionally, a diverse range of sulfur-dependent, hyperthermophilic bacteria exists at temperatures of 90°C and higher. Some cyanobacteria in sulfur containing environments can perform oxygenic photosynthesis using only PS- I, provided that electron donors like hydrogen sulfide are available.

Oscillatoria amphigranulata, an isolate from New Zealand's hot springs with sulfur dioxide at 56°C, has been shown to perform both anoxygenic and oxygenic photosynthesis [16]. Likewise, various isolates of *Microcoleus chthonoplastes* from around the world have been shown to perform both anoxygenic and oxygenic photosynthesis in environments with high sulfide concentrations [16]. However, tolerance to sulfide varies among cyanobacteria, as this substance can be toxic to strains that are typically sensitive to it, When exposed to an even lower concentration of this chemical. [14,30, 50]. This study aimed to determine whether mesophilic cyanobacteria can bear temperatures exceeding their optimal range in the presence of sulfide, and to explore the role of sulfuric acid in enhancing heat resistance. This approach could be useful for enriching fields with mesophilic cyanobacteria that typically cannot survive at temperatures between 42 and 50°C. For heterocystous cyanobacteria, photosynthesis is the main source of reductant and energy for nitrogen fixation. In contrast, unicellular *Gloeothece* relies on aerobic respiration or sunlight (both in dark and light conditions) for the same purpose. In sulfide-dependent anoxygenic metabolism, PS-I generates the ATP needed for nitrogen synthesis, while sulfide acts as an electron donor for carbon dioxide fixation. Cyanobacteria are capable of fixing nitrogen while simultaneously producing oxygen through their photosynthetic processes.Many researchers have suggested that photosynthesis and nitrogen fixation should occur in different locations and times. [11]. This type of adaptation enables cyanobacteria to thrive in low oxygen environments while facilitating both oxygenic photosynthesis and nitrogen fixation simultaneously.

Reports indicate that the filamentous, non-heterocystous cyanobacterium *Plectonema boryanum* can fix nitrogen at oxygen levels as low as 1.5 percent in the dark and 0.5 percent in the light [65,47]. The cyanobacterium *Phormidium* has been shown to fix nitrogen in anaerobic environments. Sulfidic conditions are particularly conducive to anaerobic nitrogen fixation, as anaerobic photosynthesis is driven by the activity of PS-I [64].

Some researchers propose that the presence of sulfide enhances the rate of nitrogen fixation. *Pseudanabaena* FS39 has demonstrated nitrogen assimilation during anoxygenic photosynthesi [39].

Certain cyanobacteria have adapted to aerobic environments by performing photosynthesis during the day and nitrogen fixation at night, as they require an anoxygenic environment for the nitrogen fixation process. Research has been conducted on unicellular *Cyanothece, Gloeothece*, and *Simploca* to examine the temporal separation of these two processes [41]. These non-heterocystous organisms seem to have evolved a mechanism that allows nitrogen fixation and oxygenic photosynthesis to take place at different times, with ATP and reducing power generated through dark metabolism [45, 38].

Giddings et al. (1981) studied the metabolism of sulfur compounds in *Anabaena variabilis* and proposed that while vegetative cells initially activated sulfate reduction, metabolically active heterocysts were incapable of doing so. Singh (1978) investigated nitrogen fixation in Nostoc calcicola by changing pH levels in the presence of sulfur dioxide. Gupta and Talpasayi (1984) studied the effects of three sulfur compounds: cysteine, sodium sulfide, and sodium dithionite) on the growth of *Synechococcus cedrorum.*

2. MATERIALS AND METHODS

Selection of test organism

Some mesophilic cyanobacteria from our collection of cyanobacterial cultures were studied for six days at four distinct temperatures: 37, 42, 45 and 50°C. The current study used *Nostoc ellipososporum*, which grows at 42°C.

Microorganism

The lab at Punjabi University Patiala has isolated the cyanobacterium *Nostoc ellipososporum* from paddy fields in Patiala, Punjab (India).

2.1 Methodology

Conditions for culture growth

The organisms were grown in a Chu-10 medium [24]. The pH of the medium was adjusted to 7.8. Except when stated differently noted, the stock and experimental cultures were kept at 28°C. All tests used cultures of the test organism that were six days old and exponentially growing.

Sulphide endurance of the organism

To assess its endurance threshold, the test organisms were cultivated in different concentrations of sodium sulphide (0.5, 1.0 , 2.0, 2.5 and 5.0 mM) in 100 mL of sterile Chu- 10 media. Every two days for up to 12 days, 15 mL samples were collected and an absorbance reading was taken with a Spectronic 20D+ spectrophotometer.

The higher temperature of 42°C was selected to induce thermal stress in the cultures since the ideal temperature range for mesophilic cyanobacteria growth is 28 to 37°C.

Thus, the organism's growth was measured by observing an increase in absorbance at 720 nm at 28 and 42°C in the presence and absence of sodium sulphide. To assess the organism's growth at 42°C, cultures were cultured in a BOD incubator equipped with fluorescent tube lighting.

3. Nitrogen Source Uptake and Nitrogen Assimilation Enzymes

Nitrogenase activity:

The acetylene reduction assay was employed to investigate nitrogenase activity following Stewart et al. (1970). The effect of sulfide on nitrogenase activity was tested by adding the medium with known concentrations of sulphide. Enzyme activity is measured in units of 1 nmol of ethylene hr^{-1} mg^{-1}protein.

Nitrate uptake:

Nitrate uptake by the selected organisms was followed by measuring the rate of their
depletion from the liquid medium. Suitable aliquots were taken out at predetermined intervals, and the amount of nitrate still present in the medium was calculated after centrifuging the cells to separate them. Nitrate remaining in the medium was estimated.

Nitrate estimation

Modified method of Robinson et al. (1959) was used to estimate nitrate where chloroform was replaced by glacial acetic acid.

The nitrate uptake was expressed as µmol nitrate uptake μg^{-1} protein.

Nitrate reductase (NR) and Nitrite reductase (NiR) activities:

In accordance with Herrero et al., the total cell NR and NiR activities were assessed (1981) the amount of nitrite formed was calculated using the Nicholas and Nason method (1957). The NiR activity was measured using the same process, but $NaNO_2$ rather than KNO_3 was utilized. NiR activity was measured in terms of nmol of nitrate decreasing per minute.

Glutamine synthetase (GS) activity:

The Shapiro and Stadtman (1970) method was used to study the GS activity.

The quantity of enzyme needed to make 1 mol of -glutamylhydroxamate min^{-1} mg of protein is referred to as one unit of enzyme.

Statistical Analysis

The entire dataset is the average of three different experiments, with a standard deviation of 5%. (SD). The data was statistically examined with one-way ANOVA and Tukey's honest significance difference test. All statistical analyses were compared to probability values at the 95 percent confidence level using GraphPad Prism 6.0 version 6.0. (www.graphpad.com).

4. RESULT AND DISCUSSION

Tolerance Level of *Nostoc ellipososporum* to Sulphide

At 37, 42, 45 and 50°C over the course of six days, the growth of nine mesophilic cyanobacteria was measured in early experiments using an increase in absorbance at 720 nm.

None of the studied mesophilic cyanobacteria survived at 45°C, according to the results as shown in Table 1.

However, *Nostoc ellipososporum* grew at 42°C (Fig.1). Therefore *Nostoc ellipososporum* was selected for the further research

experiments. Its growth was compared at 37, 42 and 45°C, and it was observed that after 12 days, the growth of the organism at 37 and 42°C declined.

Additionally, the development of test organisms was examined at various temperatures in both the presence and absence of sulphide (Fig. 2).

We examined sulphide dosages of 0.5, 1.0, 2.0 and 2.5 mM. In comparison to he absorbance of control cultures at 42°C, the absorbance of the cultures at 42°C in the presence of 0.5, 1.0, 2.0 and 2.5 m M sulphide was 21, 36.8, 52.5 and 73.6 % higher. The organism continued to exist in 5.0 mM concentration of sulphide but did not grow. This indicated that 5.0 mM was lethal to organism at 42°C.

Table 1. *Temperature-dependent growth of mesophilic cyanobacteria*

Organism Temperature(°C)	37 42 45 50
Anabaena naviculoides	+ - - -
Nostoc muscorum	+ - - -
Nostoc calcicola	+ - - -
Plectonema boryanum	+ - - -
Phormidium molle	+ - - -
Nostoc ellipososporum	- - - -
Nostoc punctiforme	+ + - -
Lyngbya faveolarum	+ - - -

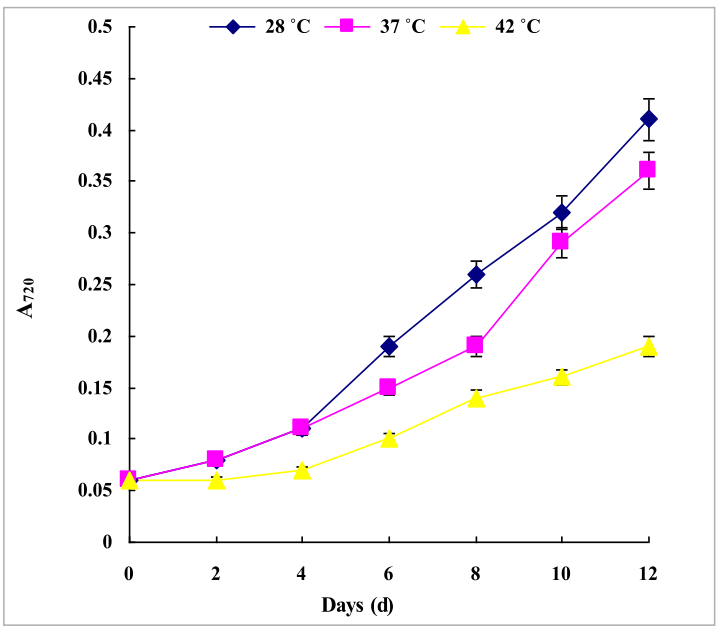

Figure 1. *Growth of Nostoc ellipososporum in basal culture medium*

This occurred as a result of sulphide's poisonous properties, which targets and permanently disables PSII. The photosynthetic electron transfer chain's electron flow is inhibited by sulphide toxicity due to its interaction with metalloproteins. Sulphide is toxic because of its interaction withthe electron chain [14,13,32].

At 42°C, presence of 2.5 mM sulphide in the culture medium had a favourable impact on the growth of *Nostoc ellipososporum*, hence this concentration of sulphide was chosen for further studies.

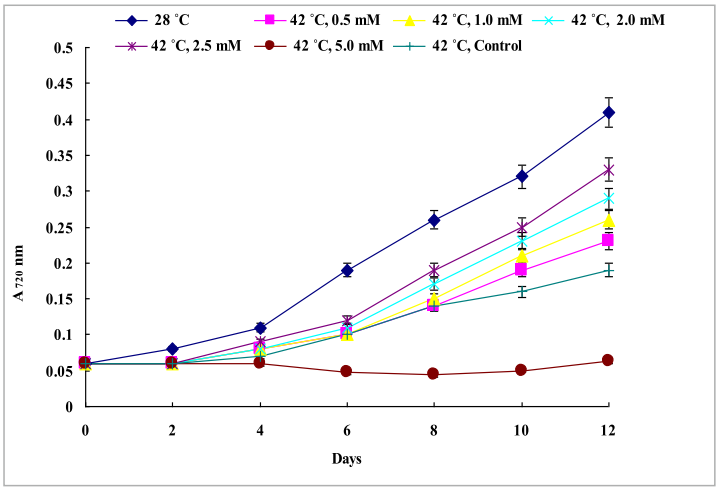

Figure 2. *Growth of Nostoc ellipososporum in absence of sulphide at 28 , 42 oC and with addition of sulphide at 42 oC.*

The impact of thermal stress on enzymes involved in nitrogen assimilation and nitrogen metabolism during nitrogen absorption.

During the present work, potassium nitrate (10 mM) was added as a nitrogen source. When compared to cultures at 28°C, growth of *Nostoc ellipososporum* in control cultures at 42°C was significantly reduced by 54%. Growth of the cyanobacterium increased by 23% when nitrate was supplied in cultures at 42°C, but it grew by over 100% when sulphide and nitrate were also added, compared to control cultures at 42°C (Fig. 3).

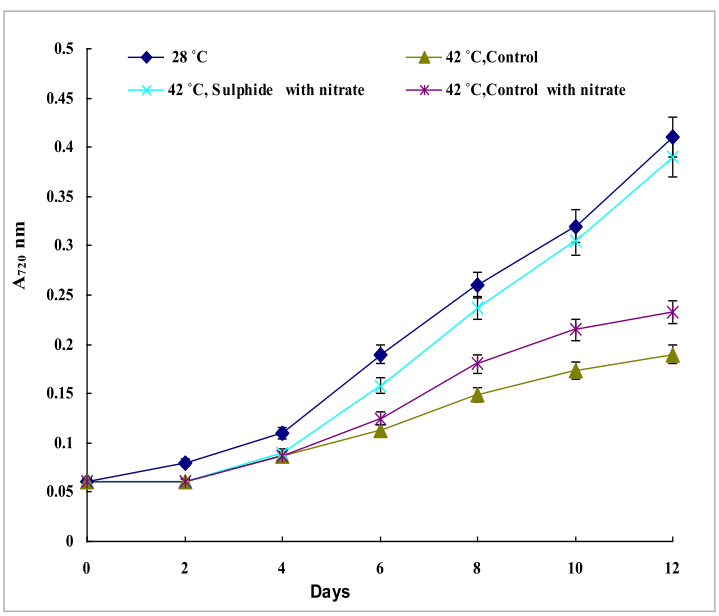

Figure 3: *Development of Nostoc ellipososporum in absence of sulphide at 28 , 42oC and with addition of sulphide and nitrate to culture media at 42 oC .*

Nitrate uptake

The study examined nitrate uptake by the test organism and its depletion from the solution over time. The cultures were cultivated in the appropriate conditions for two, four, and six days. Cells were removed from the medium, and nitrate uptake from the solution was monitored.

Similarly, *Nostoc ellipososporum* cultures grown at 42°C had a 28%, 31%, and 33.33% lower rate of nitrate uptake than cultures grown at 28°C after 2 days, 4 days, and 6 days, respectively. Nitrate uptake in sulphide cultures increased by 12-13% during 4 to 6 days compared to control cultures at 42°C (Fig. 4).

At 42°C, growth of *Nostoc ellipososporum* was reduced . When basal media was added with 2.5 mM sulphide, *Nostoc ellipososporum* developed more quickly than control cultures at 42°C. Increased nitrate uptake compared to control cultures may contribute to improved development of test organisms during temperature stress.

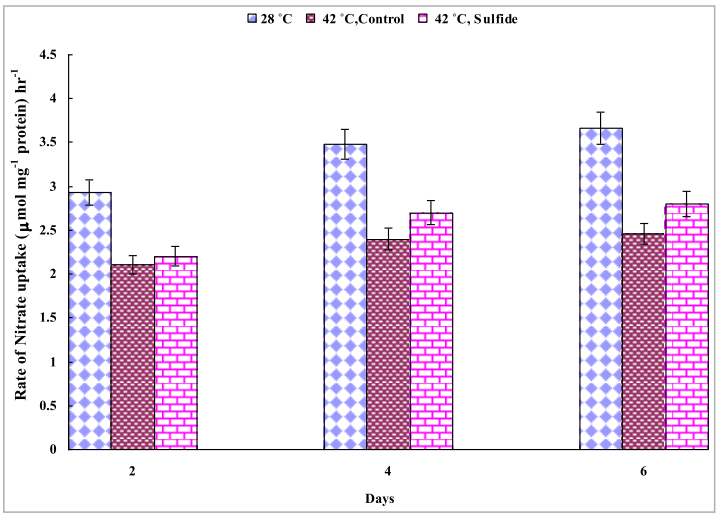

Figure 4: *Nitrate uptake in Nostoc ellipososporum when grown at 28 and 42oC in the absence of sulphide and with addition of sulphide to culture media at 42 oC.*

Effect of thermal stress on nitrogen assimilating enzymes

Nitrogenase activity of the test microorganism *Nostoc ellipososporum* cultivated at 28°C was 12.6 U and 14.4 U after 24 and 48 hours, respectively (Fig. 5). At 24 and 48 hours, there was no significant difference in nitrogenase activity between control and sulphide cultures at 42°C

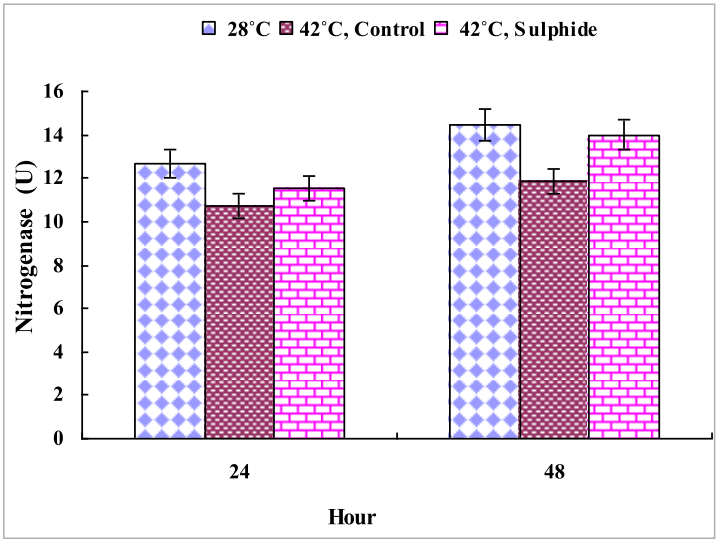

Figure 5: *Nitrogenase inNostoc ellipososporum when grown at 28 and 42 °C in absence of sulphide and with addition of sulphide to culture media at 42°C.*
U: nmol ethylene produced mg^{-1} protein hr^{-1}

Nitrate reductase (NR) activity

Cultures of the test organism *Nostoc ellipososporum* cultivated at 42°C showed a 19.2% and 23.5% decrease in NR activity on 4th and 6th day , respectively, compared to control cultures at 28°C. There were no significant variations in NR activity between control and sulphide cultures at 42°C for the first four days, but on the sixth day, there was a 23% increase in NR activity, 3.2 U in sulphide cultures compared to control cultures at 42°C.

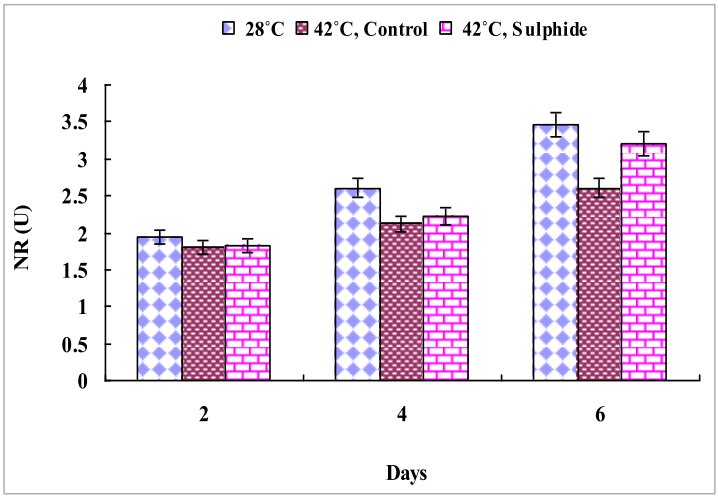

Figure 6: *Activity of Nitrate reductase inNostoc ellipososporum when grown at 28 and 42°C in absence of sulphide and with addition of sulphide to culture media 42°C. Nitrate reductase activity on zero day: 1.7 U*
U: nmol nitrite formed mg^{-1} protein min^{-1}

Nitrite reductase (NiR) activity

NiR activity in control and sulphide-treated cultures was nearly identical, but when compared to sulphide-containing *Nostoc ellipososporum* cultures, it was essentially identical for up to 2 days. NiR activity decreased by 7.1% and 18.7% on the fourth and sixth days, respectively, in cultures cultivated at 42°C. Cultures' NiR activity did not change in the presence of sulphide, although it increased by 23% after 6 days (Fig. 7).

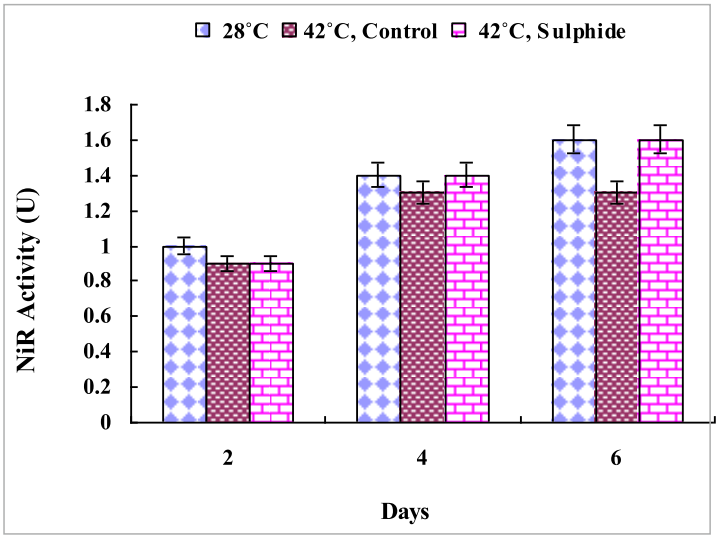

Figure 7: *Activity of Nitrite reductase inNostoc ellipososporum when grown at 28 and 42°C in absence of sulphide and with addition of sulphide to culture media at 42°C.*
Nitrate reductase activity on zero day: 3.0 U
U: nmol nitrite decreased mg^{-1} protein min^{-1}

Glutamine synthetase (GS) activity

GS activity in *Nostoc ellipososporum* cultures grown at 28 and 42°C in the presence and absence of sulphide was almost the same. On 4 d and 6 d GS activity in cultures grown at 42°C decreased by 41.1 and 44.7%. Though GS activity got increased by 4 d and 6 d, respectively, even that was supported less than GS activity of cultures grown at 28°C (Fig. 8)

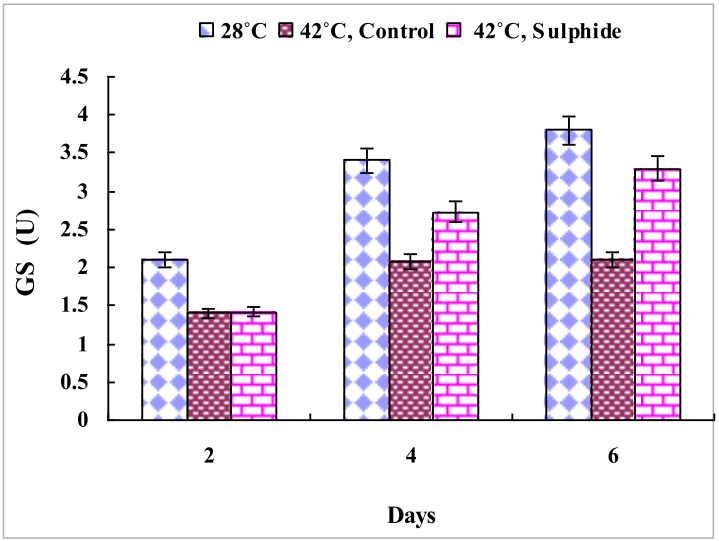

Figure 8 : *Activity of Glutamine synthetase inNostoc ellipososporum when grown at 28and 42°C in absence of sulphide and with addition of sulphide to cuture media at 42°C.*
Glutamine synthetase activity on zero day: 1.3 U
U: nmol γ-glutamate hydroxamate formed mg^{-1} protein min^{-1}

Nitrogen metabolism in cyanobacteria is photosynthesis-dependent.

Nostoc ellipososporum nitrogenase activity in control and sulphide cultures did not differ significantly after 48 hours of thermal stress.

Nitrogen sources for cyanobacteria include nitrate, nitrite, and ammonium [25].

Cyanobacteria absorb nitrate and then reduce it to ammonium [7,35,25]. Cyanobacteria reduce nitrate in two phases using enzymes nitrate reductase (NR) and nitrite reductase (NiR), resulting in the creation of ammonium. The study examined how temperature stress affected the NR and NiR activities of test cyanobacteria. Thermal stress reduced NR activity by 23.5% after 6 days in *Nostoc ellipososporum.*

According to our findings, sulphide had a key role in the survival of test organisms under temperature stress, as NR activity increased by 23% in the above test organisms. Similar results were found when NiR activity was assessed. Sulphate has been shown to play a positive impact in the regulation of nitrate reductase and ATP-sulphurylase in wheat and mustard. Naturally occurring thiol chemicals, cysteine, and glutathione, have been demonstrated to impact nitrate reductase activity in mustard [24]. NR and NiR are membrane-bound enzymes whose activity in cyanobacteria rely on reduced ferredoxin generated during oxygenic photosynthesis [26].

Decreased nitrate absorption at elevated temperatures may result in decreased enzyme activity in control cultures at high temperatures.In cyanobacteria, ammonium generated by nitrate and nitrite reductase activities or direct absorption is integrated into the carbon skeleton by glutamine synthetase (GS) activity via the GS-GOGAT system [25]. Thus, the impact of high temperatures on the GS activity of test microorganisms in the absence and presence of sulphide was investigated.GS activity of *Nostoc ellipososporum* decreased by 44.7% after 6 days of heat stress. However, sulphide addition in the cultures dramatically boosted GS activity in all of the tested species. It was discovered that nitrate uptake/nitrogen fixation by test organisms was influenced by high temperature for 2-4 days, whereas enzymes of nitrogen metabolism were affected for 4-6 days. Thus, temperature stress affected nitrogen. nitrogen metabolism at the uptake level not at the reduction level.

The test organisms' capacity to develop at 42°C in the presence of sulphide and perform improved N metabolism demonstrates their adaptability to higher temperatures. Elevated temperatures significantly impacted photosynthesis and nitrogen metabolism in test organisms. The presence of sulfide improved these activities. Sulphide mitigated the deleterious impact of temperature stress on photosynthesis and nitrogen metabolism in test microorganisms.

The effect of thermal stress on GS activity appears to be correlated with low rates ofnitrate uptake rather than the direct toxic effect of high temperature on GS activity. Under thermal stress when nitrate uptake by the test organism was low, low GS activity was observed but in the presence of sulphide when nitrate uptake relatively increased GS activity also increased.

High activities of nitrogen assimilation enzymes in cells grown in sulphide at elevated

temperature as compared to control cultures indicated that test microorganisms assimilated more amount of nitrogen in the presence of sulphide.

The effect of heat stress on GS activity appears to be connected to decreased rates of nitrate uptake rather than the direct toxic effect of high temperatures on GS activity. Under temperature stress, when nitrate uptake by the test organism was low, GS activity was low; but,when sulphide was present, GS activity rose.

Nitrogen absorption enzyme activity was higher in cells grown in sulphide at greater temperatures compared to control cultures, indicating that test microorganisms absorbed more efficiently.

Elevated temperature indirectly affects nitrogen metabolism via thermal stress on photosynthesis, as nitrogen fixation and metabolism in cyanobacteria are photosynthesis-dependent [25]. Elevated temperatures affected photosynthesis, resulting in reduced N metabolism.

5. CONCLUSIONS

Nitrate uptake by the test organisms was shown to slow down under temperature stress, but when sulphide was added to the cultures, nitrate uptake increased. Supplementation of sulphide to elevated temperature cultures positively affected nitrate uptake, as the rate of uptake increased in *Nostoc ellipososporum*. This increased level of nitrogen metabolism in the presence of sulphide contributed to the survival of mesophilic cyanobacteria under thermal stress.

COMPETING INTERESTS

The authors have declared that there are no competing interests.

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