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Research Paper

Effects of temperature and sulfide on freshwater cyanobacteria isolated from a sulfur spring in the western ghat forests of Karnataka

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Abstract

The study deals with the effect of different temperature regimes on the growth, photosynthetic and phycobilin pigment contents in some cyanobacterial species isolated from sulfur spring in the Western Ghats of Karnataka. In the present study four isolates from sulfur spring namely, *Leptolyngbya purpurascens, Planktolyngbya limnetica*, *Scytonema bohnerii* and *Calothrix fusca* were selected for the study. The study noticed that all the four isolated species of sulfur spring showed proper growth up to temperature 45°C ($p<0.01$). Interestingly, it was also noticed that all the four species showed moderate growth up to 50°C, while *Scytonema bohnerii* has tolerated up to 65°C, where it showed proper growth up to 50°C. *Planktolyngbya limnetica* showed slight growth up to 60°C, *Leptolyngbya purpurecens* exhibited the growth up to 55°C. Similarly, *Calothrix fusca* showed growth up to 50°C. The species namely, *Leptolyngbya purpurecens, Planktolyngbya limnetica* and *Calothrix fusca* did not show any growth below 25°C temperature. *Scytonema bohnerii* does not exhibited sustainable growth below 30°C. In the study, sulfur spring isolates produced higher carotenoids at the temperature between 40 and 45°C. In this study the effect of sulfide (Na_2S) on their growth, photosynthetic ability and heterocyst frequency was also determined. It was observed that sulfide (Na_2S) content at a particular concentration (50 to 55 ppm) significantly increased their growth, pigment production and also heterocyst numbers. But it was also observed that after 55 ppm. of sulfide concentration, there was a slight decline in the growth indicating that sulfide at higher concentration becomes toxic to that species, hence growth was inhibited.

Keywords: Cyanobacteria, Sulfur spring, Temperature, Sulfide, Biomass, Pigments, Western Ghats

Introduction

Cyanobacteria are generally found in fresh, brackish and marine waters and also in Antarctic lakes and hot springs[1,2]. They are of interest due to their ability to grow in high temperatures and in other extreme environments[3-5]. Temperature is one of the important abiotic factors for cyanobacterial cultivation. Many cellular processes of cyanobacteria are temperature dependent, their rates changing exponentially with temperature increases and maximum values occurring between 25°C and 40°C[6]. Most of the thermophilic cyanobacteria grow poorly below 30 to 35°C. Certain species of *Synnechococcus* did not grow below 50°C[7]. Effects of high-temperature stress on photosynthetic organelles of cyanobacteria have been studied by many workers [8,9]. The other variable that can vary greatly across cyanobacterial habitats is sulfide[10], but is absent from stably oxygenated environments, sulfide is either permanently or periodically present in many ecosystems where cyanobacteria are found[4]. Although few researchers have studied the effect of temperature and sulfide on cyanobacteria, the study in the tropical waters is limited except the reports of Debnath et al. (2009), Rajeshwari and Rajashekhar (2012), Mongra (2014) and Roy et al. (2014) [11-14].

In the present study, effect of different temperature regime and sulfide concentration on the growth characteristics of different species of cyanobacteria isolated from a sulfur spring near Uppinangady, Dakshina Kannada District in the Western Ghats of Karnataka is reported.

Materials and Methods

Effect of temperature

For the study, four sulphur spring isolates namely, *Leptolyngbya purpurascens, Planktolyngbya limnetica, Scytonema bohnerii* and *Calothrix fusca* were selected. They were cultured and maintained in BG- 11 culture medium. About 5 ml of homogenized culture suspension of each of the 15- day old culture was inoculated into each of the 250 ml conical flasks containing 100 ml BG – 11 culture medium[15]. The culture flasks were incubated at different temperature regimes (ranging from 0^oC, 5, 10, 15, 20, 25… up to 80^oC) using shaker incubator fitted with thermostat and white fluorescent light (Rotek, India Ltd.). (In order to maintain the temperature of < 20 ^oC, an cold room was used). For each culture, three replicates were maintained. The cultures were grown at continuous illumination (2000 lux), with 14:10 hour light: dark regime. The nitrogen free BG-11 medium was used for nitrogen fixing cyanobacteria. The growth in terms of chlorophyll-a was assessed after 20 days of incubation.

Effect of sulfide

Four species of cyanobacteria isolated from sulfur spring namely, *Leptolyngbya purpurascens, Planktolyngbya limnetica*, *Scytonema bohnerii* and *Calothrix fusca* were selected for this study. About 5 ml of homogenized culture suspension of each of the 15- day old culture was inoculated into each of the 250 ml conical flasks containing 100 ml BG - 11 culture medium (pH 7.2). In order to study the effect of sulfide on the growth rate, sulfide in the form of sodium sulfide (Na_2S) was added to each of the sample. Sodium sulfide stock solution was prepared in the sterilized media to obtain the desired concentration (1, 5, 10, 15, 20….. up to 200 ppm) of sodium sulphide and BG-11 medium without containing sodium sulfide solution served as control. For each concentration, three replicates were maintained. The cultures were grown at illumination (2000 lux) at a temperature of 28 ± 2^0C with 14:10 hour light: dark regime. The nitrogen free BG-11 medium was used for nitrogen fixing cyanobacteria. The growth in terms of chlorophyll-a was assessed after 20 days of incubation.

Growth Measurements

Determination of biomass

The biomass of the cultures was evaluated by dry weight method in different pH regime for 25 days[16]. 10 ml culture was concentrated by centrifugation at 3,000 rpm for 10 min. Pellet was washed by deionized water and centrifuged followed by drying (at 60^oC). Dry weight was expressed as g/L. All experiments were conducted three times. Data were reported as the average value of three sets.

Estimation of chlorophyll-a

Chlorophyll-a was estimated by Jeffrey and Humphrey method[17]. Chlorophyll-a content was extracted with 90% acetone (v/v). About 10 ml of homogenized cyanobacterial suspension was pelleted by centrifugation and 10 ml of 90% acetone was added. The centrifuge tube was vigorously shaken so as to dissolve completely in the solvent. Then all the tubes were placed in a refrigerator for 24 hours for complete extraction of the pigments. After the extraction period, the samples were centrifuged and the supernatant was collected. The supernatant was made up to 10 ml with 90% acetone and absorbance measured at 665 nm in a UV visible spectrophotometer (Systronics India, Ltd.) against 90% acetone as blank. The amount was calculated using the extinction coefficient given by Jeffrey and Humphrey[17]. Mean values of triplicates ± SD were recorded.
The amount of chlorophyll-a was determined using the equation:

$$\text{chlorophyll- a } (\mu g/ml) = 11.85\ A_{664} - 1.54\ A_{647} - 0.08\ A_{630}$$

Estimation of total carotenoids

Carotenoids were estimated according to the protocol prescribed by Parsons and Strickland[18]. Carotenoids present in the samples were extracted using 80% acetone. After complete extraction, samples were centrifuged for about 10 minutes at 5000 rpm, and the absorbance of the clear solution was measured at 480 and 510 nm wavelengths using UV visible spectrophotometer, taking 80% acetone solution as the blank. The absorbance of the sample was also obtained at 750 nm, which was subtracted from the values at 480 and 510 nm, thus minimizing the error.
The amount of carotenoids was determined using the equation:

Total carotenoids (µg/ml) = 7.6 (E480 - E750) - 1.49 (E510 - E750)

Estimation of phycobilin pigments

Estimation of phycobilin pigments like phycocyanin, allophycocyanin and phycoerythrin were done by spectrophotometric method[19,20]. The known volumes of cyanobacterial suspensions were centrifuged and the pellets were suspended in 5 ml of 50 mM phosphate buffer (pH 7.0). The contents were repeatedly frozen and thawed and centrifuged in order to facilitate complete extraction. The supernatants were pooled and the absorbance was measured at 565, 620 and 650 nm against phosphate buffer blank[21]. Calculations were done using the following equations given by Tandeau De Marsac and Houmard[22].

$$\text{Phycocyanin (PC)}\,\mu g/ml = \frac{A620 - (0.7 \times A650)}{17.38}$$

$$\text{Allophycocyanin (APC)}\mu g/ml = \frac{A650 - (0.208 \times A620)}{15.09}$$

$$\text{Phycoerythrin (PE)}\,\mu g/ml = \frac{A565 - 2.41\,(PC) - 0.849\,(APC)}{19.62}$$

Determination of heterocyst frequency

Heterocyst frequency was determined by counting the number of heterocysts (late proheterocysts/early heterocysts were recognized by their thickened cell wall and pale appearance, and mature heterocysts were recognized by their poles) and vegetative cells that were present along the filaments of cyanobacteria. The heterocyst frequency was determined by counting the number of heterocysts per hundred vegetative cells in at least 20-25 healthy and equal length filaments by the following formula explained by Vintila and El-Shehawy (2007) and Shukla et al. (2009)[23, 24].

$$\text{Heterocyst frequency (\%)} = \frac{\text{Total number of heterocysts} \times 100}{\text{Total number of vegetative cells}}$$

Statistics

The data of dry weight, chlorophyll-a, carotenoids, phycobilin pigment content and heterocyst frequencies were expressed as mean of the triplicate values ± standard deviation (SD). The obtained data was subjected to analysis of variance (ANOVA) and then followed by Bonferroni post hoc analysis was carried out (using SPSS software, version 21.0) in order to verify the significant difference between cyanobacterial species cultured at different temperature and sulfide regimes.

Results and Discussion

Effect of temperature

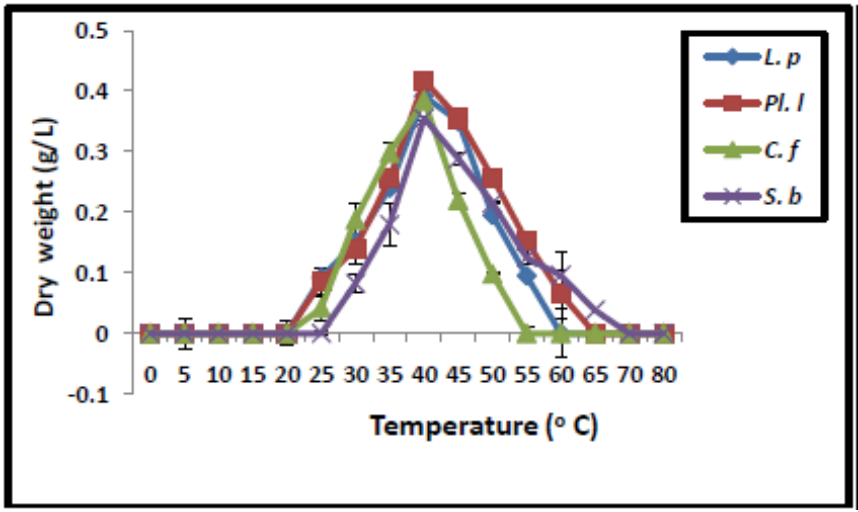
Effect of different temperature regimes on the growth pattern of four species of cyanobacteria isolated from sulfur spring is given in Table 1. The study noticed that all the four isolates of sulfur spring showed proper growth up to temperature 45^{o}C. Interestingly, it was also noticed that all the four species showed moderate growth up to 50^{o}C, while *Scytonema bohnerii* has tolerated up to 65^{o}C, where it showed proper growth up to 50^{o}C. *Planktolyngbya limnetica* showed slight growth up to 60^{o}C, *Leptolyngbya purpurecens* exhibited the growth up to 55^{o}C. Similarly, *Calothrix fusca* showed growth up to 50^{o}C. The species namely, *Leptolyngbya purpurecens, Planktolyngbya limnetica* and *Calothrix fusca* did not show any growth below 25^{o}C temperature. *Scytonema bohnerii* does not exhibited sustainable growth below 30^{o}C.

Growth in terms of dry weight measurements in the four species at different temperature level is shown in Figure 1(a). Growth in terms of chlorophyll-a content in the four species at different temperature level is shown in Figure 1(b). In these cases, the species isolated from sulphur spring showed higher biomass and chlorophyll-a contents at 35 to 40^{o}C, ($p<0.01$) where *Leptolyngbya purpurecens* showed higher values of chlorophyll-a (4.250 µg/ml) at 40^{o}C ($p<0.001$).

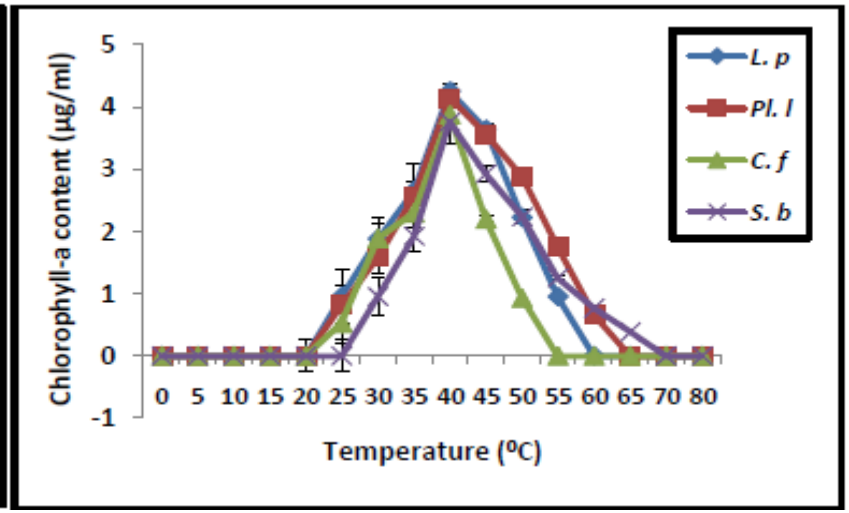
Table 1: Growth of eight species of cyanobacteria isolated from different aquatic habitats at different temperature regimes

Species	Temperature range (in °C)															
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
Leptolyngbya purpurecens	-	-	-	-	-	+	++	+++	++	+	+	+	-	-	-	-
Planktolyngbya limnetica	-	-	-	-	-	+	++	+++	+++	++	+	+	+	-	-	-
Calothrix fusca	-	-	-	-	-	+	++	+++	++	+	+	-	-	-	-	-
Scytonema bohnerii	-	-	-	-	-	-	++	+++	+++	+++	++	+	+	+	-	-

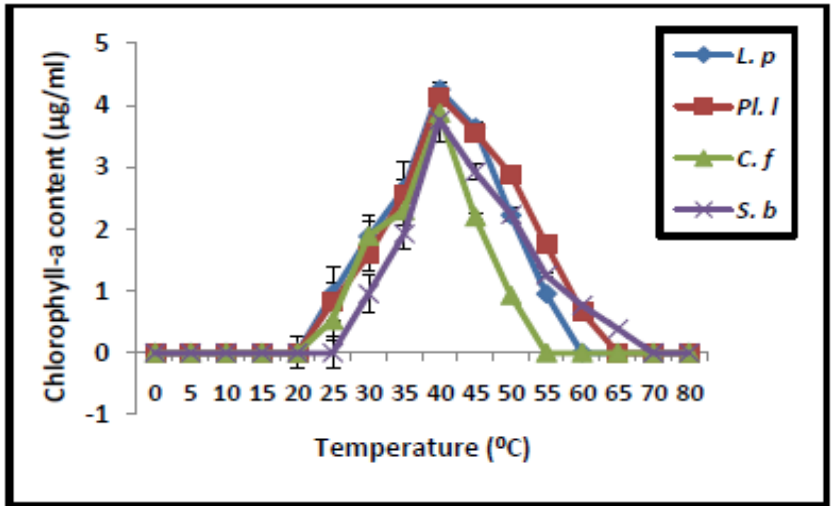
+ : slight growth; + + : moderate growth; + + + : good growth; - : no growth



(a)

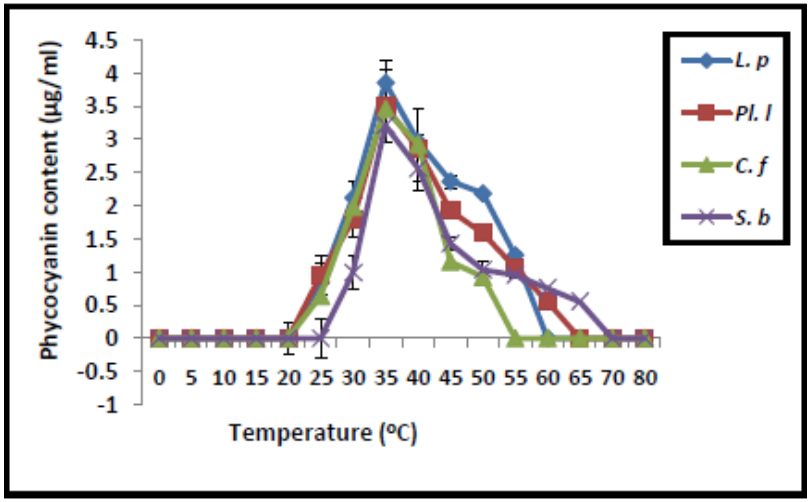


(b)

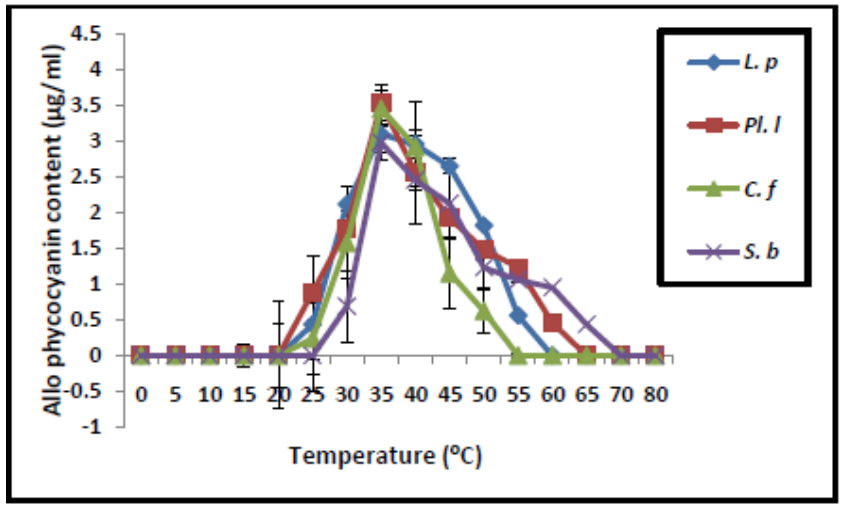


(c)

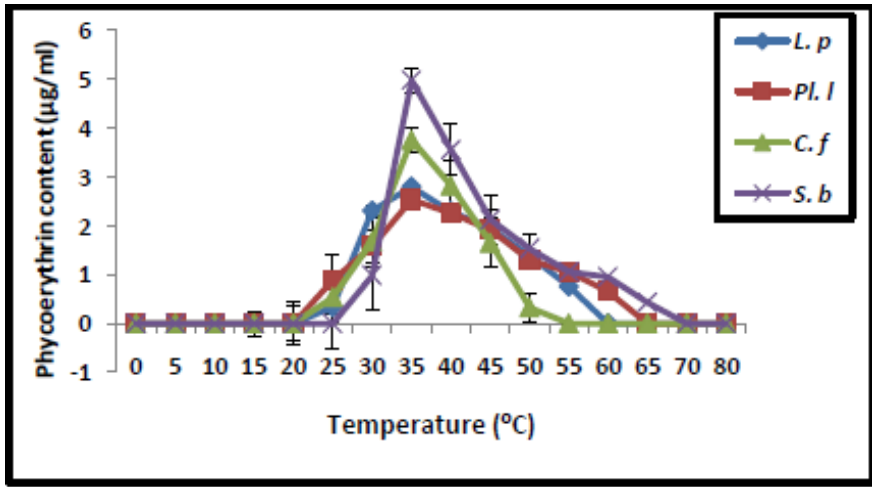
Figure 1 (a-c): Effect of different temperature regimes on the growth in terms of dry weight, chlorophyll-a and carotenoid content in the eight species of cyanobacteria* isolated from a sulfur spring



(a)

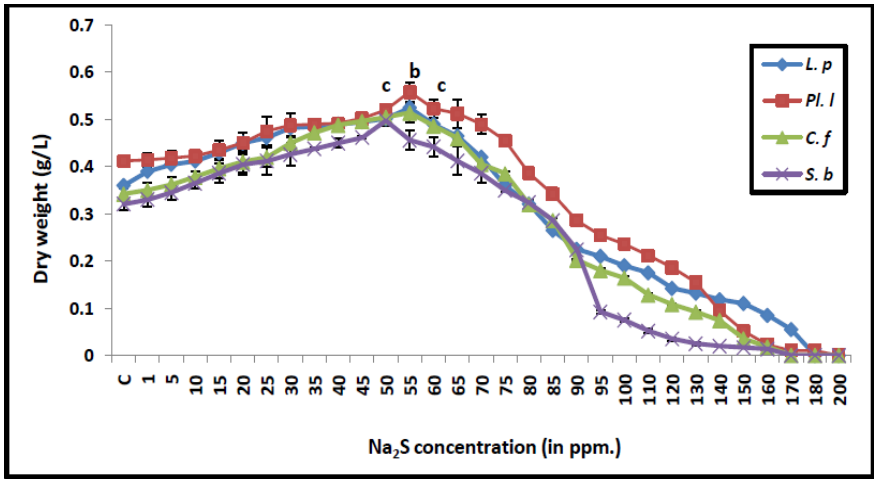


(b)

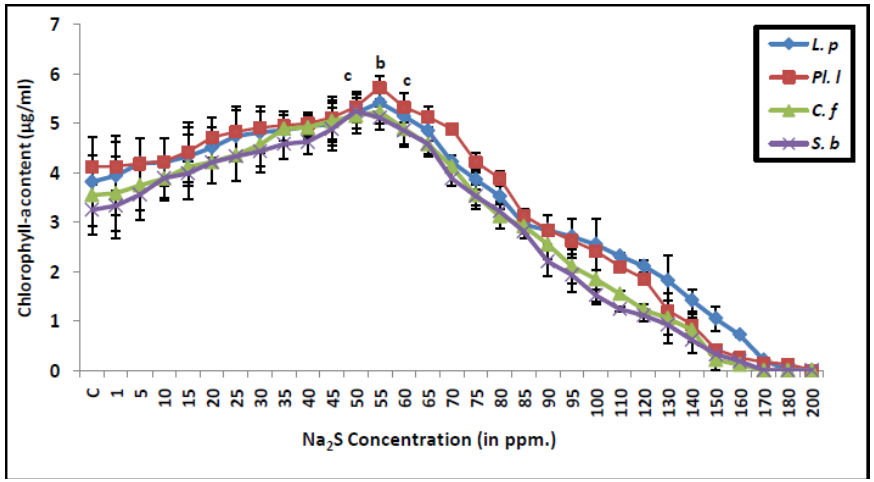


(c)

Figure 2 (a-c): Effect of different temperature on the phycobilin pigments in (Phycocyanin, allophycocyanin and phycoerythrin) eight species of cyanobacteria* of different habitats



(a)



(b)

Figure 3 (a & b): Effect of Na_2S on the growth of cyanobacteria* in terms of dry weight and chlorophyll-a isolated from a sulphur spring
C: control $^{b}p<0.01$, $^{c}p<0.05$

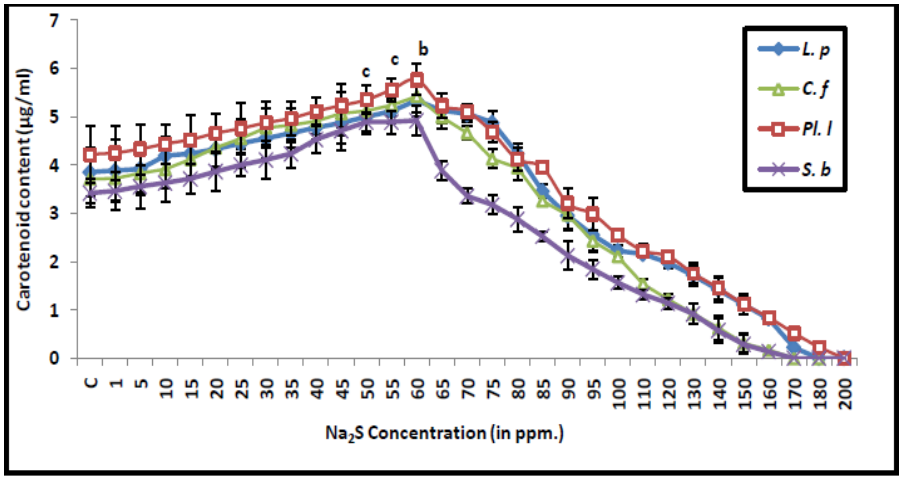


Figure 4: Effect of Na_2S on carotenoid pigments in cyanobacteria* isolated from sulphur spring

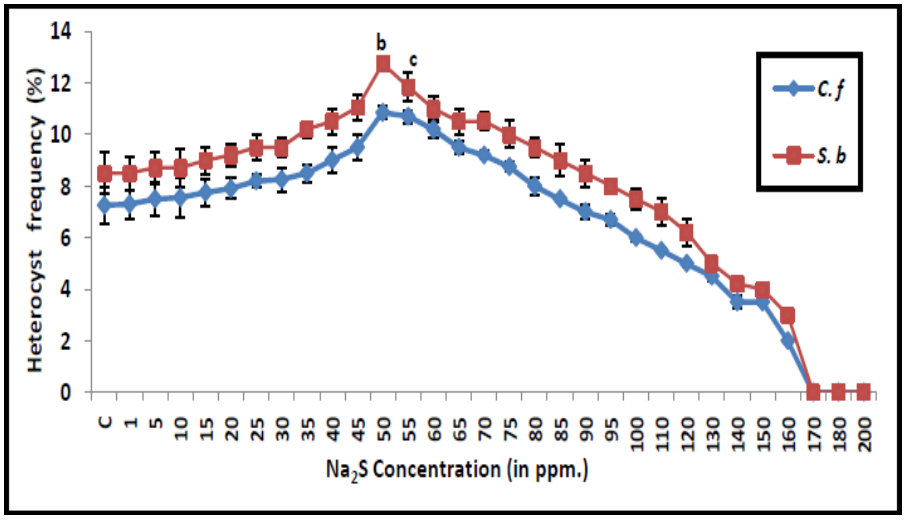


Figure 5: Effect of Na_2S on heterocyst frequency in two species of sulphur spring cyanobacteria* C: control [b] p<0.01, [c] p<0.05
**** L. p*: *Leptolyngbya purpurecens*; *Pl. l*: *Planktolyngbya limnetica*; *C. f* : *Calothrix fusca S. b*: *Scytonema bohnerii***

Similar type of observations was made in the carotenoid contents of these species at different temperatures (Figure 1c). Here, at higher temperature levels cyanobacteria synthesize more carotenoids. In the study, it was noticed that sulfur spring isolates produced higher carotenoids at the temperature of 45^{o}C ($p<0.001$), where, *Leptolyngbya purpurecens* showed significant carotenoid content of 5.65µg/ml followed by *Planktolyngbya limnetica* (4.925 µg/ml) at 45^{o}C ($p<0.001$).

The effect of temperature on the phycobilin pigments in cyanobacteria is shown in Figure 2 (a-c). The study revealed that, cyanobacteria synthesize high content of phycocyanin, allophycocyanin and phycoerythrin at moderate temperature levels. In this study, species isolated from sulphur spring exhibited higher phycocyanin values at 35^{o}C, while *Leptolyngbya purpurecens* showed maximum phycocyanin composition of 3.855 µg/ml ($p<0.05$). Similar trend was observed in allophycocyanin contents in these species at different temperature regimes (Figure 2b). In case of phycoerythrin composition (Figure 2c.) at various temperatures the species showed similar type of results like phycocyanin and allophycocyanin contents, where four spring isolates showed higher content of phycoerythrin at 35^{o}C regime ($p<0.01$). Among the four species of sulphur spring isolates, *Calothrix fusca* (3.756 µg/ml) ($p<0.01$) and *Scytonema bohnerii* (4.981 µg/ml) showed high phycoerythrin content at 35^{o}C ($p<0.001$).

Effect of sulfide on cyanobacteria

Effect of sulfide on the growth of four species in terms of dry weight and chlorophyll-a composition is shown in Figure 3 (a & b). The study revealed that, sulfide (Na_2S) content at lower concentration significantly increased the growth of sulphur spring isolates. In the present study, sodium sulfide solution incorporated with BG-11 cultured medium showed that from the initial concentration (1.0 ppm) up to 55.0 ppm, there was an increase in the biomass and chlorophyll-a content when compared to control. At 55 ppm significant growth was noticed in all the four species of sulphur spring cyanobacteria ($p<0.01$). But it was also observed that after 55 ppm of sulfide concentration, there was a slight decline in the growth and above 160 to 170 ppm., the growth was almost nil. Among the four species studied, *Leptolyngbya purpurecens* and *Planktolyngbya limnetica* respond well against various sulfide concentration. Similar trend was noticed in carotenoid composition in these species (Figure 4), where there was gradual increase in the carotenoid content from 5.0 ppm to 60.0 ppm. It was also found that, maximum carotenoid composition was observed at 60.0 ppm ($p<0.01$), and decreased gradually after 80.0 ppm ($p>0.05$) and thereafter sudden decline in the carotenoid composition.

Effect of sulfide on the heterocyst frequency in the two species of cyanobacteria is shown in Figure 5. In this study also sulfide solution at moderate level induced more number of heterocysts when compared to control. The present study has revealed that sodium sulfide at 50.0 ppm induced more number of heterocysts ($p<0.01$), hence the heterocyst frequency slightly increased from the initial concentration to slightly higher concentration level (up to 50.0 ppm). Similarly, it was also noticed that sulfide at higher doses becomes toxic to the species, hence the heterocyst frequency gradually decreased. In the present study, *Scytonema bohnerii* (12.75 %) and *Calothrix fusca* (10.8 %) exhibited higher heterocyst frequency at 50.0 ppm.

Discussion

Several thermophilic cyanobacteria have been reported from hot springs. These include *Synechococcus lividus*, *Oscillatoria terebriformis*, *Mastigocladus laminosus* and *Phormidium laminosum*. They were widely used as bio-tools for realizing some physiological and ecological mechanisms (i.e. photosynthesis, heat tolerance, heat tolerant-related protein and succession change) under high temperature environments[25-27]. Based on earlier studies, *S. lividus* probably represent the photosynthetic organism that can survive at the highest temperature up to 73°C[28].

Synechocystis cells can grow within the temperature range of 15 and 45^{o}C, but below 25 and above 43^{o}C it suffers from severe stress symptoms[15,29] which supports our study, where similar type of results were obtained for sulfur spring isolates. Temperatures above 45^{o}C are considered to be lethal for *Synechocystis* although cells can acquire thermo tolerance through pre-exposure to sub-lethal temperatures[30]. During the first phase of the heat response, cells produce rapidly large amounts of heat shock proteins which act as molecular chaperones that enable the proper folding of proteins to their active state or solubilise aggregates of misfolded proteins[31] .

Temperature stress in cyanobacteria has mainly focused on heat stress while less attention has been paid to lower temperature. But some mechanisms of cold responses and acclimation have been identified[32,33]. At low temperatures chaperones act to protect proteins that are susceptible to damage. Lower temperatures make cell membranes more rigid, and as a consequence the *desA* and *desB* genes encoding desaturase enzymes in *Synechocystis* sp. are activated[34]. Desaturases increase the number of double bonds in fatty acid hydrocarbon chains thus increasing membrane fluidity[35,36].

Cyanobacteria are temperature dependent, their growth rates accelerating exponentially with increasing temperature, with maximal values occurring between 25 and 40°C which is in agreement with our findings. The effect of temperature on photosynthetic growth rate of cyanobacteria has been studied[37,38]. Cyanobacterial dominance generally occurs at higher (> 20°C) water temperatures because of temperature optima above this value, whereas temperature optima for other algal groups tend to be lower. Light-limited growth and photosynthetic rates of *Oscillatoria* sp. were however, temperature independent [39,40].

In the present study, none of the species showed growth below 15^{o}C which agreed with the findings of Konopka[41]. Earlier reports on the experiments with natural samples indicate that temperatures below 15^{o}C do not limit cyanobacterial growth. At 4^{o}C, the rate of photosynthesis was usually 50% of that at the optimal temperature[42]. The species like *Microcystis* have been found to have an optimal temperature for growth and photosynthesis at or above 25^{o}C[43,44] which supports our study. Furthermore, the cellular toxin content of multiple genera of cyanobacteria increases with increasing temperature to a maximum above 25^{o}C [45,46].

The chlorophyll-a content in sulfur spring cyanobacterial biomass was influenced mainly by temperature. It began to affect more strongly on the chlorophyll-a level, only after increase in temperature. The slight decrease in the chlorophyll-a content at higher temperature (>45^{o}C), because of an inceptive photoinhibition and partial destruction of photosynthetic pigments[47,48]. It was known that as the temperature increases, there was reduction in the phycobilin pigment synthesis. Cyanobacterial phycobiliproteins are known to be degraded by heat treatment which agrees with our findings where higher temperature has resulted in the decrease of the phycobilin pigment composition[49].

Brock[42] has suggested that the photosynthetic apparatus itself is the most temperature sensitive component of cyanobacteria with the higher temperature tolerance up to 74^{o}C, which agrees with our results where four species of sulphur spring isolates showed moderate growth up to 55^{o}C and among them, *Sytonema bohnerii* tolerated up to 65^{o}C and *Planktolyngbya limnetica* up to 60^{o}C. It is known that certain macromolecules such as enzymes and membranes are more heat stable in thermophyllic cyanobacteria[50,51]. A large number of enzymes from thermophiles have been found to be very thermo-stable and interestingly, these enzymes also have unusual stability to organic solvents and pH extremes[52,53].

Sulfide is absent from stably oxygenated environments. It may be either permanently or periodically present in many ecosystems where cyanobacteria are found, as a result of its presence in water. These include benthic microbial mat communities that are commonly found in hot springs and both hypersaline and marine sediments[54]. Cyanobacteria likewise vary in sulfide tolerance and strains that typically are not exposed to sulfide in nature, including most planktonic and heterocystous cyanobacteria are extremely sensitive to and are irreversibly poisoned by this toxin[55-57]. In contrast, those from sulfidic habitats exhibit one or more adaptations for maintaining their photoautotrophic metabolism under such conditions. These include the maintenance of oxygenic photosynthesis by the resistance of photosystem (PS) II to sulfide and the ability to perform Photosystem II- independent, anoxygenic photosynthesis with sulfide as an electron donor to photosystem (PS)I [57,58]. This supports our study where sulfide at lower concentration increased the growth and induced more number of heterocysts.

Sulfide is known to bind metallo proteins and could conceivably interfere with one or more components involved in photosynthetic electron transport[59]. These include the manganese-containing, water-splitting complex on the donor side of PS II as well as cytochromes on the acceptor side, whereas some reports suggested that, sulfide inhibits oxygenic photosynthesis by blocking

electron flow from the donor side of PS II [57, 60], other results have implicated the acceptor side as the target [57].

Cyanobacteria are often found in sulfide-rich environments in coexistence with anoxygenic photosynthetic bacteria[54,61]. Sulfide is toxic to eukaryotic photosynthetic organisms and to non-adapted cyanobacteria [56,62,63]. It inhibits the electron transport chain by reacting with cytochromes and hemeproteins and by binding to metal proteins. Mere exposure to low redox potential may drastically inhibit oxygen evolution in some cyanobacteria[64]. The occurrence of oxygenic cyanobacteria in sulfide waters which requires some adaptation to cope with toxicity. Some of the earlier studies showed that, cyanobacteria possess a versatile metabolism and to tolerate conditions of high sulfide[57,65] and thereby cyanobacteria probably may be switched from oxygenic photosynthesis to anoxygenic photosynthesis at higher temperatures, by using alternatively sulfide as an electron donor, which supports our study where sulphur spring isolates showed steady luxuriant growth up to 50.0 ppm of Na_2S solution.

Growth of cyanobacteria in the presence of sulfide has been studied by several investigators[66]. Cohen et al.[58] showed that sulphide can act as an alternative electron donor in the process of anoxygenic photosynthesis of the species *Oscillatoria fimnetica.* Garlick et al. [55] examined 21 strains of cyanobacteria for anoxygenic photosynthesis and showed that this is a common phenomenon in a number of cyanobacteria. *Dactylococcopsis salina* was not found in Solar Lake under high sulphide concentrations, yet it was found to occur at sulphide concentrations of 10 µm during the night. Sulfide may thus be a limiting factor for the occurrence of cyanobacteria [67,68].

Conclusion

Sulfur spring cyanobacteria are qualitatively different from other freshwater and mesophilic forms. Certain species of the latter group possess the ability to adapt to high temperature and sulfide content only by developing special adaptive morphological features which vary in response to environmental conditions. To the contrary for thermophilic cyanobacteria, sulphur springs are their natural environments with relative constancy in their physic-chemical properties where the organisms have evolved to meet the environmental challenges of high temperature. They do not develop any such adaptive morphological features in their cells to survive at high temperature. There is a need for detailed investigation on the effects of environmental factor such as temperature on cyanobacterial physiology. It is of interest not only to characterise the temperature range of growth but also specify the stress inducing values of these factors.

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