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POTENTIAL EFFECT OF pH AND UV-B ON GROWTH BEHAVIOR AND LIPID PROFILING OF *NOSTOC MUSCURUM*

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ABSTRACT

Cyanobacteria are diversified group of photosynthetic prokaryotic microorganisms widely distributed in nature (Potts, 2002). Earlier cyanobacteria was known for their key role in conversion of early Earth's reducing atmosphere into an oxidizing one by carrying out oxygenic photosynthesis.

Present study demonstrates the potential effect of two major abiotic stress factors pH and UV-B radiation on growth, lipid yield and lipid profiling of cyanobacteria *Nostoc muscurum.*

This was a preliminary study on evaluating the potential effects pH and UV-B on growth behavior and lipid profiling of *Nostoc muscurum.* However, knowledge of physiological responses at cellular level in cyanobacterial populations affected by pH and UV radiation is important to understand the connection of environmental changes and persistence of species in aquatic ecosystems.

Keywords: Cyanobacteria, *Nostoc muscurum,* Effect Of pH, UV-B, Growth Behavior and Lipid Profiling

INTRODUCTION

Cyanobacteria are diversified group of photosynthetic prokaryotic microorganisms widely distributed in nature (Potts, 2002). Earlier cyanobacteria was known for their key role in conversion of early Earth's reducing atmosphere into an oxidizing one (Rasmussen et al., 2008) by carrying out oxygenic photosynthesis. Further, they are ubiquitous in nature growing not only in freshwater, marine and terrestrial ecosystems but are also found in extreme habitats such as hot springs, hypersaline localities, freezing environments and arid deserts (Fogg et al., 1973). Their widespread ecological importance encompasses sorority with other organisms forming microbial mats, biofilms and benthic communities and such sororities are of predominant importance and sometimes the only life forms found in certain extreme habitats and impacts on nutrient cycling (Capone et al., 1999). Some heterocystous strains of cyanobacteria fix nitrogen thereby reducing the demand for nitrogen fertilizer and the associated production of nitrous oxide, a major greenhouse gas (Flores and Herrero, 2010 and Kumar et al., 2010). It is also evident from reports that several species of cyanobacteria can tolerate high pH, high salinity and conditions that help to control contaminants and predators in outdoor ponds by producing a mucilaginous envelope for protection against predators and desiccation. Cyanobacteria is not only diversified in terms of their physiological and ecological variety but are also diverse in terms of morphology, including multicellular filamentous species that may bioflocculate or float to the surface of a pond for easier harvesting (Arnaud et al., 2012).

In recent past, several strains of cyanobacteria heralded as a valuable source of lipid with wide spectrum applications (Liu et al., 2005) Among the various potential application of cyanobacterial derived lipid, production of biodiesel has gained much attraction since lipid content in microalgae can account from 20 to 50% by weight of dry biomass and sometimes it was even found to be more than 80% as postulated by Spolaore et al. (2006). Furthermore, neoteric research has flourished employing genetically engineered cyanobacteria for the production of biofuels including ethanol (Deng and Coleman, 1999), isobutyraldehyde and isobutanol (Atsumi et al., 2009), and free fatty acids (Roessler et al., 2009). Not only this, lipids and fatty acids play a vital role in the tolerance of cyanobacterial cells to various environmental stresses such as desiccation, salt induced damage, low temperature, high light induced photoinhibition (Singh et al., 2002). *Nostoc* (cyanobacteria) is one of the five genera in the family *Nostocaceae* of subgroup 4, section A, of the oxygenic phototrophic bacteria. *Nostoc muscurum* belongs to the filamentous, heterocystous cyanobacteria commonly occurred in the free-living state (Potts, 2000). *Nostoc* play a vital role in nitrogen fixation and found in symbiotic association with diverse group of green plants, and about 10% of lichens have cyanobacteria (e.g., *Nostoc*) as the main or only photosynthetic partner (Rai et al., 2002). Also, they are rich source of protein, vitamin and unsaturated fatty acids that are advantageous for humans and animals.

Moreover, microalgal-derived lipids depend on biomass concentration and also on the lipid content of individual cells (Li et al., 2008; Griffiths et al., 2009). These two factors affected by numerous roadblocks such as availability of nutrients (e.g. nitrogen, phosphorus, carbon, silicon and iron), temperature, light, pH, salinity, aeration rate, physiological state of cells, and cultivation time besides genetic background. Therefore, ideal conditions are prerequisite to produce lipid at the highest productivity with the highest lipid cell content. Unfortunately, this is not always the case since, high lipid yields are usually produced by cells under stress, often during nutrient limitation associated with low biomass productivity and low overall lipid productivity thereof. In this study in order to maintain a culture successfully and to optimize the culture growth conditions, we demonstrate the potential effect of two major abiotic stress factors pH and UV-B radiation on growth, lipid yield and lipid profiling of cyanobacteria *Nostoc muscurum.*

MATERIAL AND METHODS

Test organisms

The test cultures of heterocystous cyanobacterium *Nostoc muscurum* was obtained from the University of Allahabad, Uttar Pradesh, India.

Maintenance of stock cultures

For maintenance of laboratory culture, 3 mL of a 3 week older cyanobacterial stock culture was used as inoculum in 50 mL of autoclaved BG-11 media in 150 mL Erlenmeyer flasks. These samples were maintained at 26 ± 2 ^{0}C, illuminated with white fluorescent tubes providing an intensity of 75 mol $m^{-2}s^{-1}$ and a photoperiod of 14:10 hrs. The stock cultures were maintained for 20-30 days.

Optimization of pH of culture media for enhanced production of total lipid

For optimization of pH, culture media with different levels of pH (5-9) were used, keeping other components of BG-11 media and environmental conditions constant. The media were made in 100ml BG-11 Erlenmeyer flasks and the pH of the culture media was adjusted to 5, 6, 7, 8 and 9 with the help of 1 N NaOH and 0.5 N HCl. The cultures were maintained for 30 days.

Exposure of UV-B radiation for enhanced production of total lipid content

The 20 days old cultures of cyanobacterium *Nostoc muscurum* i.e. cyanobacteria in their early stationary phase were exposed to UV-B radiation for 6, 12, 18, 24, 30 hours by using UV-illuminator (Philip TEK 40W, ACTENIC BL240, Germany) .

Growth analysis of experimental organisms in terms of Chlorophyll-'a' content

Chlorophyll 'a' was estimated by the method of McKinney (1941) from day 5 to day 30 at the gap of 5 days. For extraction, cells were harvested and pellet down by centrifugation at 5000 rpm for 10 min at 4ºC. Supernatant was discarded. Chlorophyll 'a' was extracted in 3ml of 100% chilled methanol and left for 24 hours at 4°C. After 24 hours samples were centrifuged for 10 min at 4°C and supernatant was collected. The absorbance of the supernatant was recorded at 680nm against methanol serving as blank. *Chlorophyll 'a' was calculated using the equation: A=Kcl* [Where: A-absorbance at 680nm (A680), K-Molar extinction coefficient, which is constant (13.42), C- Concentration (mg/ml) and L - Path length which is usually 1].

Biomass harvest

The biomass were harvested by filtration between day 20 to 25 i.e. during exponential, early stationary phase and late stationary phase. After filtration and washing of biomass with deionized water, biomass was concentrated by centrifugation at 15,000 rpm for 15 min. The cell pellets were then again re-suspended in deionized water and re-centrifuged two times to remove residual medium and further used for extraction of total lipid content.

Extraction of lipid

Total lipid from *Nostoc muscurum* was extracted according to the Bligh and Dyer method (1959). Briefly, methanol–chloroform 2:1 (v/v) was added to extract the lipids from the cells of *Nostoc muscurum* in their early stationary phase. The mixture was sonicated for 15 min, and then centrifuged. Supernatant was transferred by means of a pipette to another tube. Precipitated residual material was then re-suspended in methanol–chloroform–water 2:1:0.8 (v/v/v) and the mixture was again vortexed for 15 min. Phase separation was obtained by centrifugation and lower chloroform phase was collected. The chloroform phase was dried in a rotatory evaporator and the lipids obtained were dissolved in chloroform and stored at -20 °C.

Quantification of total lipid

Total lipid content was quantified by gravimetric method. The weight of the crude lipid obtained from each sample was estimated by following equation:

Weight of lipid = (weight of container + extracted lipid) – (weight of container)

Lipid content (%) = [amount of lipid extracted (g)] ×100/ [weight of original sample (g)]

Characterization of extracted lipid by thin layer chromatography

Thin layer chromatography was carried out on plates of size 20cm×20cm using prepared silica plates (MERCK). Plates were activated at 120°C for 2h in hot air oven. After the application of extracts, the plates were developed in the chromatographic tank saturated with the solvent system. The solvent system used was chloroform: methanol: 25% ammonia solution (65:25:4v/v/v) (Ramadan et al., 2008). Plates were exposed to iodine vapor in an air tight tank in fume hood. All unsaturated and saturated lipid appeared as brown spots (Randerath, 1964).

Determination of R_f values

The R_f value was calculated in relation to the solvent front and the distance of each band migrated was measured by the following formula:

R_f value = (Distance travelled by sample)/ (Distance travelled by solvent)

Statistical Analysis

Every experiment was repeated thrice and all the results were expressed as mean value ± SD for three replications.

RESULTS AND DISCUSSION

Growth behavior of Nostoc muscurum during pH optimization

Cyanobacteria like other bacteria have four different phases of growth viz., lag phase, exponential (or log) phase, stationary phase and the death phase earlier growth rate was measured in terms of cell counting and/or the changes of total biomass (dry or wet weight). However, the growth rate of cyanobacteria can also be measured indirectly by determination of the changes of cellular components such as total organic carbon, lipids, protein, or chlorophyll (Moheimani et al., 2013). Among all the known methods the total chlorophyll concentration has been the frequently studied method to measure the growth rate/behavior of phototrophic organisms, particularly in the case of filamentous cyanobacteria, where the number of cells cannot be counted directly. In this study also growth behavior of *Nostoc muscurum* was determined in terms of chlorophyll 'a'content. The results revealed that growth rate had direct correlation with increasing pH (5 to 9) (Fig. I) and the maximal growth of *Nostoc muscurum* was observed in media with pH-8. Moreover, a continuous increase in chlorophyll 'a' content was observed from day 5 to day 20 followed by a sharp decline in later stages of growth. The drop in chlorophyll 'a' content in later stages of growth might be due to the reason that cells have reached in stationary phase which resulted in reduced photosynthesis and thereby resulted in reduced growth. Moreover, pH can affect algal growth in a number of ways. It can change the distribution of carbon dioxide species and carbon availability, alter the availability of trace metals and essential nutrients, and at extreme pH levels potentially cause direct physiological effects (Chen Celia and Durbin Edward, 1994).

Total percentage content of lipid in media with altered pH values

The gravimetric analysis of lipid content during variable pH treatment showed that the total lipid content of the cultured organism tends to increase with increasing pH found maximum at pH-8 (14%) followed by a decreased lipid content at pH-9 (12%) in terms of fresh weight of *Nostoc muscurum* during early stationary phase of growth (Table 1). Further, there was a gradual increase in percentage content of lipid in all cases except at pH-5 during stationary phase which might be due to accumulation of higher content of neutral lipid triglyceride which tends to accumulate during stressed or stationary phase of growth (Miao et al., 2004).

The present study is also in agreement with reports demonstrating that pH between 7.4 and 8.0 were favorable for the optimum growth of cyanobacterial species (Rippka et al., 1979, Bano and Siddiqui, 2004 and Rai and Rajashekhar, 2014). Several other studies also speculated acidic conditions for growth of cyanobacteria which connotes that cyanobacterium can adapt to variable pH conditions for their growth (Buck and Smith, 1995 and Burja et al., 2002). In the study performed by Subramanian et al., (2014), it was observed that marine cyanobacterial genera showed considerable variation in total lipid content from 11.54 to 14.11 % of dry weight. Several other studies were also performed to unraveling the effect of light, temperature and salinity on the lipid and fatty acid composition in some species isolated from different habitats. (Rezanka et al., 2003;

Maslova et al., 2004; Liu et al., 2005; Rosales et al., 2005). Moreover, this study was also in agreement with study conducted by Rippka *et al.,* 1979; Bano and Siddiqui, 2004; Sushanth Vishwanath Rai and Madaiah Rajashekhar, 2014 demonstrating pH between 7.4 and 8.0 favorable for the optimum growth of cyanobacterial species. There were also many reports demonstrating acidic conditions for growth of cyanobacteria which indicates that cyanobacteria can adapt to variable pH conditions for their growth as suggested by Buck and Smith (1995) and Burja *et al.* (2002). The preference of cyanobacterial strains towards alkaline pH further solve the problem of preventing cultures from invading microorganisms in cultures as it has been reported that highly alkaline environment could be the possible strategy for controlling contamination by other organisms. Eleftherios Touloupakis et al (2016) conducted a study on *Synechocystis* PCC 6803 demonstrating effect of high pH on growth of *Synechocystis sp.* PCC 6803 cultures and their contamination by golden algae (Poterioochromonas sp.).

Preliminary identification of lipid classes by thin layer chromatography

The preliminary identification of lipid classes of *Nostoc muscurum* cultured in variable pH (5-9) by thin layer chromatography tabulated in Table 2. The TLC chromatogram of *Nostoc muscurum* showed the presence of bands of lipids corresponding to different classes. The cyanobacterial strain was cultivated with varying pH ranging from pH-5 to pH-9. The TLC results of extracted lipid had shown the presence of glycolipids, neutral lipids and phospholipids. The culture with pH-5 had shown the presence of two bands of lipid, in which one might be of neutral lipid (Sterol esters) and the other one of glycolipids (Monogalactosyldiacyl glycerol). The culture with pH-6 had shown the presence of four bands which might be of glycolipids (monogalactosyldiacylglycerol) and neutral lipids (free fatty acids, triacylglycerols and sterol esters). In the culture with pH-7, three bands were observed, which might be of neutral lipid corresponding to the lipid subclasses triacylglycerols and sterol esters, glycolipids The culture with pH-8 had shown the presence of maximum six bands of lipid out of which three might be of neutral lipids belonging to the sub-classes of lipid free fatty acids, triacylglycerols sand sterol esters), two bands of glycolipids corresponding to sub-class monogalactosyldiacylglycerol and esterified steryl glucoside and one of phospholipid of lipid sub-class phosphatidylcholine. While the cultures with pH-9 had shown the presence of only four bands in which two bands were of neutral lipid (triacylglycerols and sterol esters) and one band of glycolipids (esterified steryl glucoside and monogalactosyldiacylglycerol) and one band of phospholipid (phosphatidylcholine). The bands corresponding to respective R_f value depicted in Table 2.

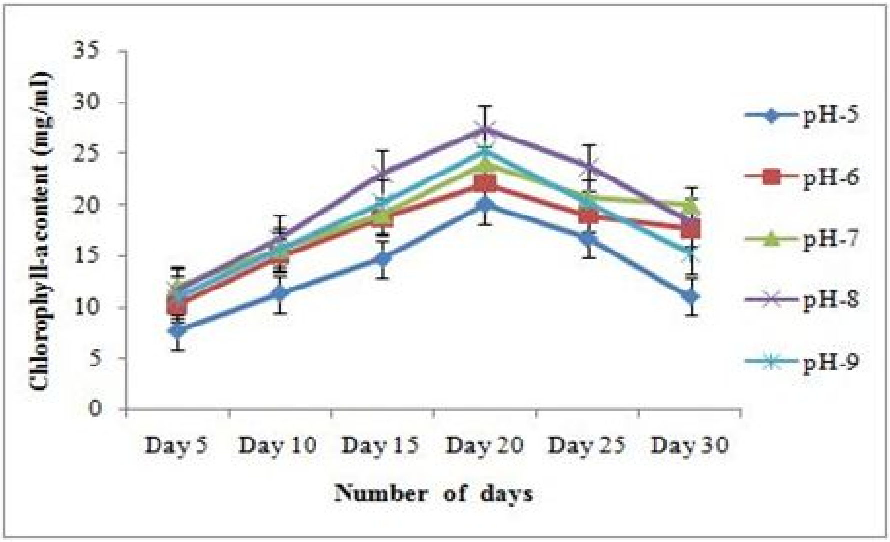

Fig. I: Growth behavior of *Nostoc muscurum* during different pH treatment

Table 1: Percentage of lipid content of *Nostoc muscurum* at different pH values

Different pH treatment	Percentage of lipid content in terms of fresh wt of cyanobacteria *Nostoc muscurum*		
	Exponential phase	Early exponential phase	Stationary phase
pH 5	9±0.085	11±0.65	14±0.60
pH 6	10±0.06	12±0.05	13±0.064
pH 7	10±0.065	13±0.50	13±0.058
pH 8	13±0.01	14±0.85	14±0.08
pH 9	11±0.50	12±0.05	12±0.10

Table 2 TLC chromatogram of *Nostoc muscurum* during pH optimization

Lipid class	TLC chromatogram of *Nostoc muscurum* under pH stress					
	R_f	5	6	7	8	9
Sulphoquinovosyldiacylglycerol	0.06	-	-	-	-	-
Phosphatidylinositol (PL)	0.11	-	-	-	-	-
Digalactosyldiacylglycerol	0.17		-	-	-	-
Phosphatidylcholine (PL)	0.20	-	-	-	+	+
Phosphatidylethanolamine (PL)	0.30	-	-	-	-	-
Steryl glucoside	0.41	-	-	-	-	-
Phosphatidylserine (PL)	0.47	-	-	-	-	-
Free fatty acids(NL)	0.56	-	+	-	+	-
Monogalactosyldiacylglycerol	0.64	+	+	+	+	+
Esterified steryl glucoside	0.76	-	-	-	+	++
Triacylglycerols (NL)	0.79	+	+	+	+	+
Sterol esters	0.95	+	++	++	++	++

++ - moderate intensity; + - low intensity ± not clearly visible

Effect of UV-B exposure on growth behavior of Nostoc muscurum

From the result obtained during pH optimization of media, pH8 was found to be most suitable for cultivation of cyanobacterial strain *Nostoc muscurum* in terms of optimum biomass and lipid yield. Therefore, to study the effect of UV-B exposure on growth and lipid content for other study, the cultures were grown in pH-8 media. Cyanobacteria harvest light energy during the process of photosynthesis and assimilate it into carbon compounds. Any adverse effect of UV-B may have adverse affect on the photosynthesis and related metabolic processes thereby hindering overall growth performance of cyanobacteria. (Germán et al.,2012). The cyanobacterial cells in their early stationary phase were exposed to UV-B radiation for 6, 12, 18 and 24 hours. The growth behavior in terms of chlorophyll-a content of cyanobacteria was determined after different period of exposure and it was observed that with increased duration of UV-B exposure, a sharp decline in chlorophyll-‘a’content was observed (Fig. II). It was seen that there was approximately 3 fold decline in chlorophyll-‘a’content after 30 hour exposure of UV-B as compared to the control avowing detrimental effect of exposure on cyanobacteria. The results are further in compliance with earlier studies (Bhandari and Sharma, 2007; Xue et al., 2005; Ganapathy Kavitha et al., 2015) reporting a sharp decline in growth and chlorophyll-a content with increased exposure of UV-B. Similarly, in report by Han et al. (2003), it was demonstrated that exposure to UVA + UVB radiation led to the inhibition of growth of a rice field cyanobacterium *Anabaena sp*., while inhibition was estimated up to 40% in *Anabaena sp*. PCC 7120 after solar UV radiation (Gao et al., 2007). Other work with *Nostoc muscorum and Phormidium foveolarum* revealed that UV-B dose of 1.0 μmol m^{-2} s^{-1} induced a 14–21% growth decrease, while in other treatment employing a lower UV-B dose (0.1 μmol m^{-2} s^{-1}) did not influence growth (Singh et al., 2012). Study with various strains of cyanobacteria demonstrated decline in growth of 48% after 30 min of exposure to UV-B alone (14.4 ± 1 Wm^{-2}), (Kumar et al., 2003); However, all cell strains died after 90 min exposure (Kumar et al., 2003). Ganapathy et al., 2015 observed 28 and 40% growth decrease after 30 and 60 min exposure of lower intensity of UV-B (3 Wm^{-2}) in case of cyanobacterium strain (*Artrospira platensis)*. It was also evident that lowering photosynthetic pigment indirectly led to decreased rate of photosynthesis and biomass production. Furthermore, cyanobacteria have several strategies for protection and mitigation of UV-B exposure which include mat or crust formation (Omoregie et al., 2004), vertical migration of individuals within the mat, or self shading due to changes in morphology as observed in *Arthrospira platensis* (Wu et al., 2005). Also, some strain of cyanobacteria such as *Spirullina* produce UV-absorbing substances including MAAs and/or scytonemins, to avoid excessive solar UV radiation (Dillon et al., 2002; Liu et al., 2004; Sinha and Haider et al., 2002). Not only have this cyanobacteria also possesses both non-enzymatic and enzymatic antioxidants to withstand oxidative stress for instance ascorbate, tocopherol, carotenoids and reduced glutathione and superoxide dismutase, catalase, glutathione peroxidase and the enzymes involved in the ascorbate-glutathione cycle to detoxify the ROS such as ascorbate peroxidase, monodehydroascorbate reductase, deghdroascorbate reductase and glutathione reductase (He and Hader, 2002).

Effect of UV-B exposure on total lipid content

Only very low proportions of solar UV radiation received by Earth's surface (UVC: 0%, 100–290nm; UVB: <1%, 280–315nm; and UVA: <7%, 315–400 nm), this portion of the solar spectrum is highly energetic (Kirk, 1994) and affect several processes, among which some are advantageous while other have adverse effects. For instance, UVB induces a reduction in the metabolism of heterotrophic bacteria (Amado et al., 2015), as well as aquatic primary producers, such as cyanobacteria, mainly due to DNA damage (Buma et al., 2001; Helbling et al., 2001; Rastogi et al., 2010). Furthermore, UVA was found to have negative effect on primary production, pigment degradation and changes in nitrogen metabolism of phytoplankton (Kim and Watanabe, 1994; Döhler and Buchmann, 1995; Palffy and Voros, 2006). In our study we have observed that UV-B exposure had detrimental effect on the growth as well as lipid content of both *Synechococcus* PCC7942 and *Nostoc muscurum.* The results of UV-B exposure revealed sharp decrease in total lipid content of cyanobacterium *Nostoc muscurum* (Figure 4). The maximum percentage of lipid content (11%) was obtained after 6 hrs of UV-illumination followed by gradual decline with advancement of UV-B exposure which might be due to the result of primary UV-B mediated events such as direct photosynthetic damage, loss of permeability/membrane changes, pigment destruction, protein/enzyme inactivation, reduced DNA and protein synthesis, reduced uptake of nutrients, hormone inactivation and signal transduction through phytochrome or signal transduction via a specific UV-B photoreceptor (Sinha and Hader., 2002; Kumar et al.,2003). Bhandari and Sharma (2006) studied the effect of UV-B exposure on photosynthesis, membrane lipids and MAAs in marine cyanobacterium, *Phormidium corium* attesting that UV-B radiation resulted in decline in sugar content, peroxidation of membrane lipids as well as quantitative and qualitative changes in phosphoglycolipids and neutral lipids.

TLC analysis of lipids of Nostoc muscurum exposed to UV-B radiation

Thin layer chromatogram of UV-B exposed culture had shown presence of glycolipids and neutral lipids while the bands of phospholipids were not observed (Table 3) which might be due to the negative impact of UV-B exposure on structural lipids. In 6 and 12 hours of UV-B treated culture two bands of Rf value 0.76 and 0.79 were observed, which might be of esterified steryl glucoside and triacylglycerols. In 18 hour UV-B treated cultures, two bands of Rf value 0.76 and 0.95 were revealed which might be of esterified steryl glucoside and sterol esters. In 24 hours and 30 hour UV-B treated culture maximum number of bands i.e. four were observed in which two might be of monogalactosyldiacylglycerol and esterified steryl glucoside and two of triacylglycerols and sterol esters respectively. However, in 30 hours UV-B exposed cultures two bands i.e., monogalactosyldiacylglycerol and triacylglycerols were not clearly visible.

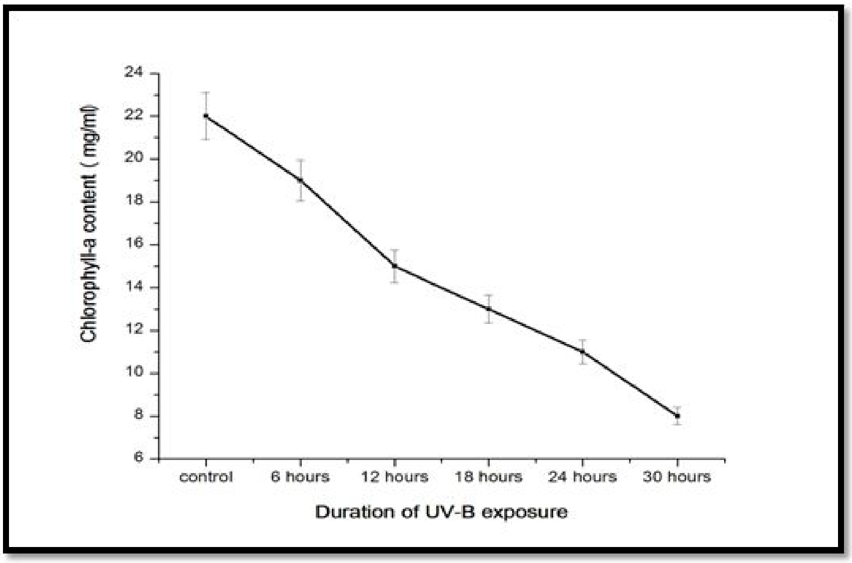

Fig. II: Growth behavior of *Nostoc muscurum* after UV-B exposure

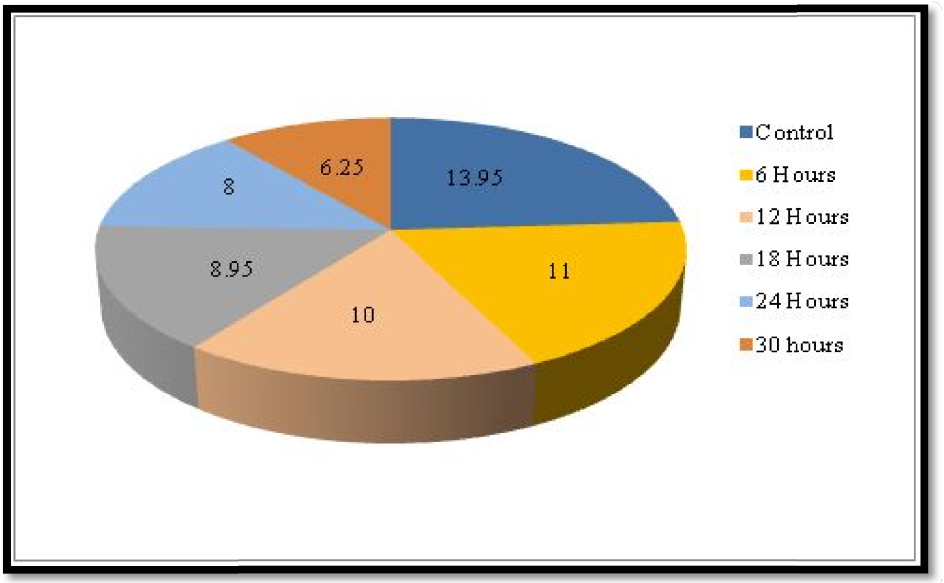

Fig. III: Percentage lipid yield of *Nostoc muscurum* after UV-B exposure

Table 3: TLC chromatogram of *Nostoc muscurum* after UV-B exposure

Lipid class	Duration of UV-B exposure (hours)					
	Rf	6	12	18	24	30
Sulphoquinovosyldiacylglycerol	0.06	-	-	-	-	-
Phosphatidylinositol	0.11	-	-	-	-	-
Digalactosyldiacylglycerol	0.17	-	-	-	-	-
Phosphatidylcholine	0.20	-	-	-	-	-
Phosphatidylethanolamine	0.30	-	-	-	-	-
Steryl glucoside	0.41	-	-	-	-	-
Phosphatidylserine	0.47	-	-	-	-	-
Free fatty acids	0.56	-	-	-	-	-
Monogalactosyldiacylglycerol	0.64	-	-	-	+	±
Esterified steryl glucoside	0.76	++	+	±	+	+
Triacylglycerols	0.79	++	+	-	+	±
Sterol esters	0.95	-	-	+	+	+

++ = moderate intensity; + = low intensity ± = not clearly visible

CONCLUSION

From the above mentioned data, it could be concluded that cyanobacteria have good capacity to tolerate variable pH moreover; pH-8 was best suited for cultivation of *Nostoc muscurum* for high lipid yield with maximal biomass production. In contrast, the UV-B exposure had shown detrimental effect on biomass production rate as well as total lipid content of cyanobacteria expanding the importance of UV radiation as an environmental stressor. This was a preliminary study on evaluating the potential effects pH and UV-B on growth behavior and lipid profiling of *Nostoc muscurum.* However, knowledge of physiological responses at cellular level in cyanobacterial populations affected by pH and UV radiation is important to understand the connection of environmental changes and persistence of species in aquatic ecosystems.

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