International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com

Research Article

# Random sampling of cyanobacterial diversity from five locations within Eastern Himalayan Biodiversity hot spot

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## Abstract

Five randomly selected locations within Eastern Himalayan biodiversity hot spot were used to assess cyanobacterial diversity. Jagiroad and Ghilamara of Assam and Mawlai, Umiam and Sohra of Meghalaya were taken for the diversity study. A total of 103 cyanobacteria were isolated from these sites which belonged to ten different genera. The genus *Nostoc* found to be most abundant. *Nostoc*, *Anabaena* and *Fischerella* strains were widely distributed in all the sites emphasizing their adaptability and resilience under diverse environmental conditions. Shannon-Wiener diversity index showed Mawlai with the highest cyanobacterial diversity (1.74) and the lowest was found in Jagiroad (1.0395). Similarly, Simpson's diversity index was also highest in Mawlai (0.773) whereas Jagiroad recorded lowest (0.625). However, evenness index was highest in Jagiroad (0.953) and lowest was found in Umiam (0.77). Simpson's dominance index was maximum in Jagiroad (0.375) and was minimum in Mawlai (0.227). Apart from diversity studies, thirty different cyanobacterial strains were evaluated for their growth rate in terms of chlorophyll *a* and protein content. Heterocyst frequency and nitrogenase activities were analyzed in order to determine biofertilizer potentiality. *Nostoc* sp.6 had highest chlorophyll *a* (3.38 µg/mL) and protein content (64.4 µg/mL). The lowest chlorophyll *a* was found in *Oscillatoria* sp.1 (0.84µg/mL) whereas protein content was lowest in *Scytonema* sp.3 (21.1µg/mL). Nitrogenase activity was highest in *Nostoc* sp.6 (2.18 nmol $C_2H_4$ produced/µg Chl *a*/hr). Along with high chlorophyll *a*, protein content and nitrogenase activity, *Nostoc* sp.6 can be an ideal candidate for biofertilizer application in crop cultivation in the region. Nitrogenase activity was lowest in *Oscillatoria* sp.2 (0.135 nmol $C_2H_4$ produced/µg Chl *a*/hr) indicating a wide difference in various parameters within the members of cyanobacteria isolated from the region.

**Keywords:** Cyanobacteria, Shannon-Wiener diversity index, Simpson's dominance index, chlorophyll *a*, Nitrogenase activity.

## Introduction

Cyanobacteria are ubiquitous gram negative microbes found in diverse habitats ranging from fresh to saline water, desert to Antarctica, soil surface to bark of trees and clean to contaminated surroundings (Torsvik *et al.*, 1996; Garcia-Pichel *et al.*, 2001; Thajuddin and Subramanian, 2005). They are resilient and highly adaptive to moderate to extreme environments. They are known to have evolved and diversified at some stage in the Precambrian era (2.8-3.5 $\times 10^9$ years ago). They are the first photosynthetic (Schopf, 2000) as well as many members of the group are capable of diazotrophic growth. They are morphologically diverse and can be single celled, filamentous non-heterocystous, filamentous heterocystous and filamentous branched heterocystous forms (Humm *et al.*, 1980; Clayton *et al.*, 1990). They have immense agricultural importance as they enrich soil fertility and soil quality by virtue of their ability to fix atmospheric nitrogen and secreting various polysaccharides that help soil particle binding. Cyanobacteria and algae are primary producers of many eco-systems and therefore the entire food webs are dependent on the members of these organisms. In recent times various other biotechnological potentials such as their use as food and feed, production of pigments, restriction enzymes as well as in the field of bioremediation of toxic compounds (Patterson, 1995; Moore, *et al.*, 1988; Lee, *et al.*, 1989). To realize various such potential of cyanobacteria, diverse members of the group need to be evaluated in detail. Thus a study into the diversity

of cyanobacteria of a region is important as the various climatic, geographical and ecological factors shape their diversity and allow expression of different traits relevant for survival in the unique environment. This would provide an understanding of region based cyanobacterial population that could be exploited for different biotechnological applications. Many researchers have worked out cyanobacterial diversity of different regions of all over the world including India (Nillson, *et al.*, 2000; Song, *et al.*, 2005; Valerio, *et al.*, 2008; Deshmukh, *et al.*, 2010; Chaurasia, 2015). However, North Eastern region of India is less explored although this region falls under Eastern Himalaya biodiversity hot spot (Tiwari, *et al.*, 2005; Devi, *et al.*, 2010; Syiem, *et al.*, 2010).

As pointed out earlier, diversity and distribution of cyanobacteria in any region is greatly affected by many factors such as pH, temperature, salinity, moisture, pollutants, etc. Among these factors, pH and temperature play an important role for cyanobacterial optimal growth (Singh, 1961; Kaushik, 1994). In India, North-Eastern region presents varied ecological niches which provide excellent growth conditions for various cyanobacteria. Five sites within Assam and Meghalaya i.e. Jagiroad, Ghilamara, Mawlai, Umiam and Sohra were randomly chosen for assessing cyanobacterial diversity within the region. They fall in 92°4 E, 94°41 E, 91°88 E, 91°88 E and 91°7 E latitude and 26°2 N, 27°31 N, 25°63 N, 25°65 N and 25°3 N longitudes, respectively. Within the selected sites, soil and water pH varied from acidic to neutral to alkaline (5.8-8.2) which presented adverse to favourable conditions for cyanobacterial growth and diversity (Nongbri, *et at.*, 2012). Other physico-chemical parameters like elevation, longitude, latitude, total dissolved solids (TDS), dissolved oxygen (DO) also influence in growth and diversity.

This is a pilot study to establish the pattern of cyanobacterial diversity within the region and to add to the extensive list of cyanobacteria that has already been compiled earlier by Nongbri *et al.*, in 2012. Various diversity indices were worked out in order to compare and contrast cyanobacterial diversity among the sites. This would provide certain understanding about the role of pH, temperature, elevation, altitude, longitude, etc. on the growth and diversity of the cyanobacteria that populated the selected ecological niches in the region. Apart from the diversity analysis, some representative cyanobacterial isolates were also partially characterized in terms of chlorophyll *a* and protein content to evaluate their growth rate. Heterocyst frequency and nitrogenase activities were also measured since these parameters are indicative of their potential as biofertilizer in crop cultivation.

## Materials and Methods

### Collection sites

Soil and water samples were randomly collected in sterile sample collection bottles from Assam (Jagiroad and Ghilamara) and Meghalaya (Mawlai, Umiam and Sohra), India.

### Physico-chemical parameters

Temperature, latitude, longitude and elevation of the all sample collection sites were recorded. The physico-chemical properties namely pH, Dissolved oxygen (DO), Total dissolve solids (TDS) and Total dissolve solids (TDS) of the samples were analysed according to the method described by APHA, (1985).

### Isolation and purification

Samples were transferred into flasks containing BG-$11_0$ media with/without nitrogen supplement under optimum temperature of 25 ± 2°C, pH 7.5 under continuous light at a photon fluence rate of 50µmol photons $m^{-2}$ $s^{-1}$ (Rippka *et al.*, 1979). When the cyanobacterial filaments were visible after 8-10 days, purification process was followed by using repeated pour plate method on 1.2% nutrient agar made in BG-$11_0$ media until single colonies of cultures were obtained. These were transferred to sterilized conical flask containing fresh BG-$11_0$ media and allowed to grow. The cyanobacterial isolates were identified by looking under Olympus BX 53 light microscope by using reference from Rippka *et al.*, 1979 and Desikachary, 1959.

### Diversity Analysis

Total cyanobacterial strains under each genus were identified and counted for estimating diversity and richness of each study area. Shannon-Wiener and Simpson's diversity indices are most commonly used diversity indices for measuring species diversity of a region. Therefore, the diversity was calculated using Shannon-Weiner Index (H) (Shannon and Weaver, 1949), Simpson's diversity (1-D) (Simpson, 1949) and Hill's Index (Hill, 1973) using following formulae:

***Shannon-Weiner Index,*** $H = -\sum pi \ln pi$

***Simpson's Dominance,*** $D = \sum (pi)^2$

***Simpson's Diversity Index,*** $1 - D = 1 - \sum(pi)^2$

Where, pi = total no. of strains of genus i/total number of all strains.

The Percent abundance was calculated as follows:

***Percent Abundance*** $= \frac{Y}{X} \times 100$

Where, X = Total number of isolates

Y = Number of isolates belonging to a particular genus

***McIntosh Evenness Index:***

$McE = [N - \sqrt{(\sum ni)}] / [N - (N/\sqrt{S}]$, (McIntosh, 1967)

Where, ni= No. of strains of genus i and S= Total no. of genera and N= Total no. of strains

## Estimation of chlorophyll *a* content

Growth rate of cyanobacteria were measured in terms of increase in chlorophyll *a* concentration. 3mL of each cyanobacterial sample was centrifuged at 2500 rpm for 3 minutes and the supernatant was discarded. To the pellet containing centrifuge tube equal volume of methanol was added and made the volume level to 3mL. Samples were vortexes and placed at 4°C overnight for the extraction of chlorophyll *a*. At the end of this incubation period, volume of the solutions were checked, which was followed by centrifuged and absorbance of the supernatant was read spectrophotometrically (SmartSpec Plus, BioRad, USA) at 663 nm (MacKinney, 1941). Chlorophyll *a* concentration was calculated by using the formula: chlorophyll *a* concentration (µg/mL) = Absorbance at 663 nm × 12.63.

## Protein estimation

3mL of cyanobacteria culture was centrifuged at 2500 rpm for 3 minutes and the pellet resuspended in 3mL distilled water. The cells were then disrupted by ultrasonication using Sonic Vibra cell sonicator (USA) fitted with a microprobe. 0.5mL of sonicated cyanobacterial cell was taken and made the volume to 1mL with distilled water. 5mL of solution C solution was added to each test tubes of 1mL culture and incubated for 20 minutes in room temperature. Immediately after the incubation, 0.5mL of 1N Folin-Ciocalteu's phenol reagent was added and incubated for 10 minutes. The coloured solution was read at 750 nm against blank and concentration was calculated using standard curve (Lowry *et al.*, 1951).

## Heterocyst frequency

Number of heterocyst present on the glass slide was counted under Olympus BX 53 light microscope and heterocyst frequency was calculated as percentage of total cell population. At least 500 cells were counted for each study (Wolk 1965).

## Nitrogenase activity

Nitrogenase activity was measured by the method described by Stewart *et al.*, (1967) where acetylene reduction to ethylene was quantified. Tubes (15mL) containing 5mL of cyanobacterial suspension were sealed and 1mL of air was replaced by 1mL of commercial grade acetylene gas. These tubes were incubated in light under constant shaking for one hour. After one hour of incubation the ethylene gas produced from the reduction of acetylene was quantified with Varian 3900 Gas chromatography. The gas chromatography is equipped with a flame ionization detector and a Porapak T column, the carrier gas was nitrogen (30 mL/min), the oven temperature was maintained 120℃. The nitrogenase activity was expressed as nmol of $C_2H_4$ produced/µg of Chl *a*/h.

# Results

## Physico-chemical parameters

A few physico-chemical parameters such as elevation, temperature, latitude, longitude, pH, total dissolved solids (TDS) and dissolved oxygen (DO) of the collected samples were analyzed. All these parameters are summerized in Table 1.

**Table 1.** Physico-chemical parameters of the five different sites of Assam and Meghalaya.

| | Locations | Elevation (metre) | Lattitude | longitude | Temp (°C) | pH | TDS (mg/L) | DO (mg/L) |
|---|---|---|---|---|---|---|---|---|
| **Assam** | Jagiroad | 62 | 92.40 E | 26.20 N | 30 | 8.2 | 1350 | 1.3 |
| | Ghilamara | 94 | 94.41 E | 27.31 N | 32 | 6.7 | 327 | 7.3 |
| **Meghalaya** | Mawlai | 1496 | 91.88 E | 25.63 N | 21 | 6.2 | 17 | 7.4 |
| | Umiam | 1005 | 91.88 E | 25.65 N | 23 | 5.9 | 31 | 7 |
| | Sohra | 1484 | 91.70 E | 25.30 N | 16 | 5.8 | 14 | 5 |

## Collection, isolation and purification

A total of 103 cyanobacteria were isolated from five different randomly chosen sites of Assam and Meghalaya. All purified samples were maintained in the culture room under optimum temperature, pH and light condition in BG-$11_0$ medium. These isolates belonged to both heterocystous and non-heterocystous forms. Morphologically they were either single celled, filamentous or branched filamentous forms. Microscopic observation showed presence of 10 different cyanobacterial genera that are listed in Table 2. (*Anabaena*, *Nostoc*, *Calothrix*, *Scytonema*, *Fischerella*, *Gloeocapsa*, *Plectonema*, *Oscillatoria*, *Synechococcus* and *Cylindrospermum*).

**Table 2.** Genera wise abundance in the various locations.

| | | Assam | | Meghalaya | | |
|---|---|---|---|---|---|---|
| **Genus** | Total no. of isolates | Jagiroad | Ghilamara | Mawlai | Umiam | Sohra |
| ***Anabaena*** | 24 | 5 | 6 | 7 | 4 | 2 |
| ***Nostoc*** | 45 | 10 | 4 | 15 | 10 | 6 |
| ***Calothrix*** | 7 | - | 1 | 4 | 1 | 1 |
| ***Scytonema*** | 4 | - | - | 2 | 1 | 1 |
| ***Fischerella*** | 12 | 5 | 1 | 3 | 2 | 1 |
| ***Gloeocapsa*** | 1 | - | - | - | 1 | - |
| ***Plectonema*** | 1 | - | - | 1 | - | - |
| ***Oscillatoria*** | 3 | - | - | 2 | 1 | - |
| ***Synechococcus*** | 2 | - | 2 | - | - | - |
| ***Cylindrospermum*** | 4 | - | - | 3 | 1 | - |
| **Total** | 103 | 20 | 14 | 37 | 21 | 11 |

## Cyanobacterial abundance

Percent abundance of cyanobacterial genera is presented in percent in the Fig. 1. Mawlai recorded highest abundance of cyanobacterial genera followed by Umiam, Jagiroad, Ghilamara and lowest was seen in Sohra which are summarized in table 2. The most abundant genera among all isolates were found to be *Nostoc* (44%) followed by *Anabaena* (23%), *Fischerella* (11%) and *Calothrix* (7%). The occurrence of rest of the genera were sporadic i.e. *Cylindrospermum* and *Scytonema* (4%), *Oscillatoria* (3%), *Synechococcus* (2%) and *Plectonema* and *Gloeocapsa* (1%) in these study sites.

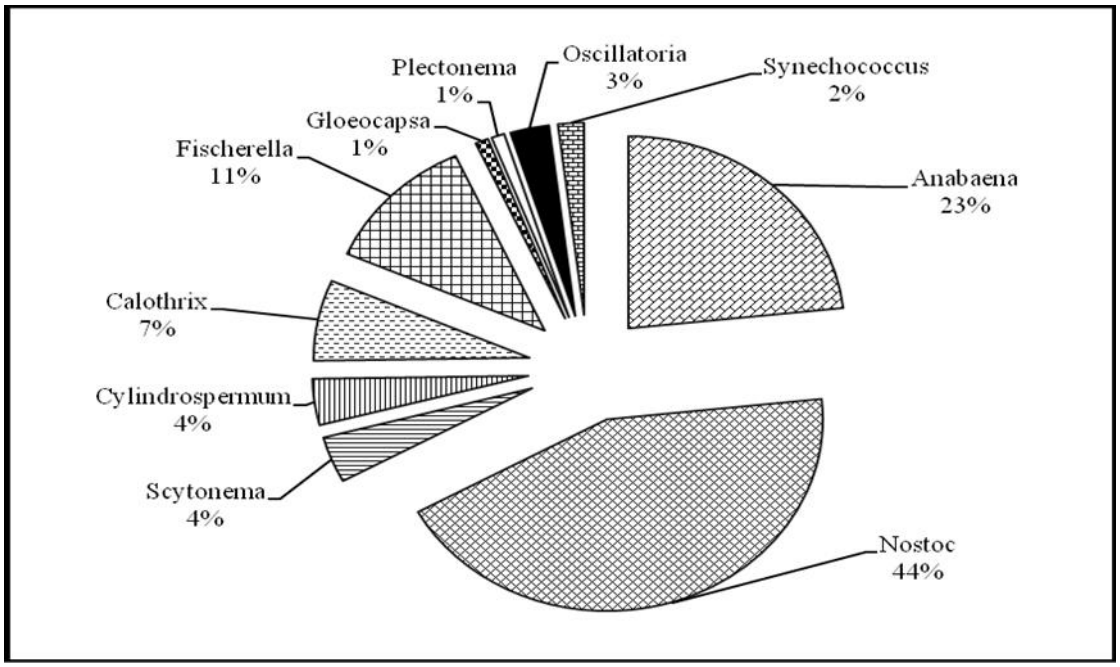


**Fig. 1:** Percent abundance of cyanobacterial genera in all sites.

## Richness and Diversity Analysis

Richness and diversity in a site is measured by the number of species. More the number higher is the richness. Diversity indices take into accounts both relative abundance and richness to predict how well species are distributed within a community. Four different statistical analyses were used to encompass various diversity parameters in predicting the diversity indices of the individual sites as well as the overall diversity of all the study sites undertaken for analyses. These analyses revealed that of the five different sites Malawi had the highest richness of cyanobacteria whereas Jagiroad showed lowest (Table 2.). *Nostoc*, *Anabaena* and *Fischerella* strains were widely distributed in all the sites emphasizing their adaptability and resilience under diverse environmental conditions. Calculations based on Shannon-Wiener diversity index showed Mawlai has the highest diversity (1.74) followed by Umiam (1.609), Ghilamara (1.371), Sohra (1.29) and the lowest was found in Jagiroad (1.0395).

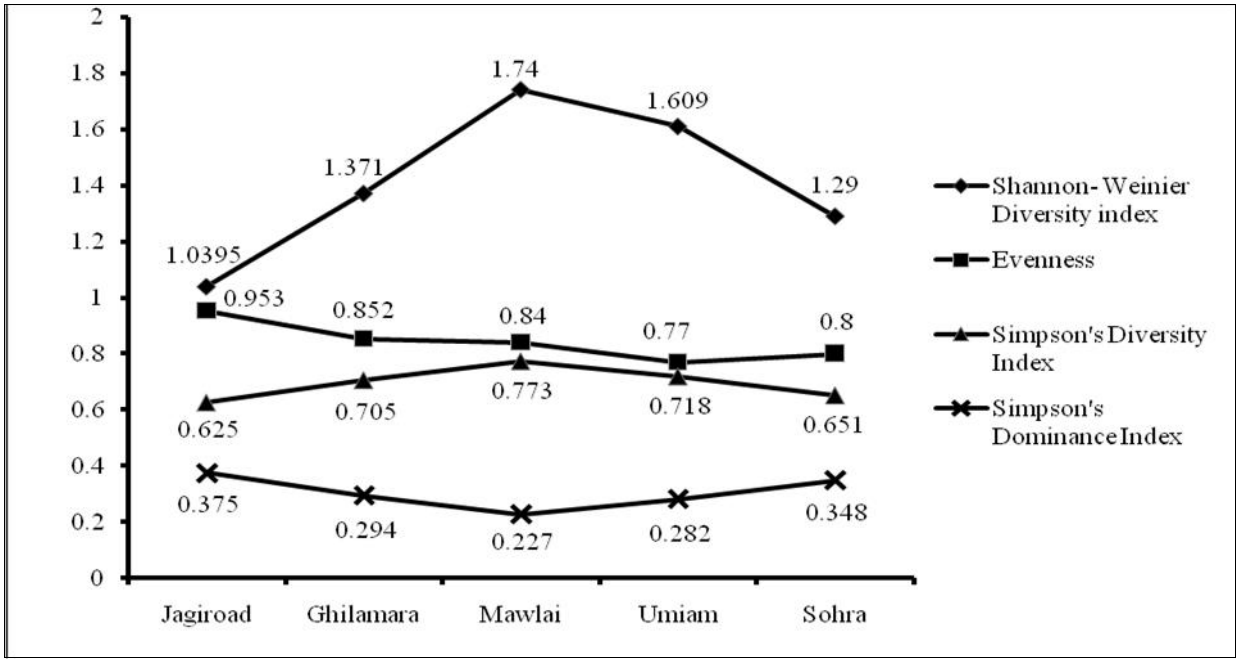


**Fig. 2:** Generic diversity (Shannon's-Weinier index, evenness, Simpson's diversity and Simpson's dominance) of cyanobacterial populations.

However, evenness index was highest for Jagiroad (0.953) followed by Ghilamara (0.852), Mawlai (0.84), and Sohra (0.8) and lowest in Umiam (0.77). Simpson's diversity index revealed the sequence of diversity: Mawlai (0.773) > Umiam (0.718) > Ghilamara (0.705) > Sohra (0.651) > Jagiroad (0.625). Simpson's dominance index was maximum in Jagiroad (0.375) and minimum in Mawlai (0.227). All these diversity indices for the various locations are graphically represented in the Fig. 2.

## Physiological and biochemical characterization of some isolates

Thirty different isolates were further characterized in terms of chlorophyll *a* and protein content as their measure of growth. Heterocyst frequency and nitrogenase activity were measured as parameters for their ability to add soil fertility.

## Estimation of growth in terms of chlorophyll *a* content

Growth rate vary from cyanobacterial strains to strains. Some cyanobacterial were fast growing while some showed slower growth. Therefore, chlorophyll *a* estimation was done when the culture age attained 7 day. Thirty different cyanobacteria were randomly chosen from the total cyanobacteria isolates for chlorophyll *a* estimation. Strains were selected in the manner that the thirty different cyanobacteria represented all the ten genera. Chlorophyll *a* concentration among the thirty selected isolates ranged between 0.84-3.38 µg/mL (Fig. 3). Most of the *Nostoc* and *Anabaena* strains showed high chlorophyll *a* content compared to the rest of the genera. *Nostoc* sp.1, *Nostoc* sp.3 and *Nostoc* sp.6 showed high chlorophyll *a* content (>3 µg/mL). On the other hand *Synechococcus* sp.1, *Oscillatoria* sp.1, *Gloeocapsa* sp.1 and *Scytonema* sp.3 showed low chlorophyll *a* content (<1 µg/mL).

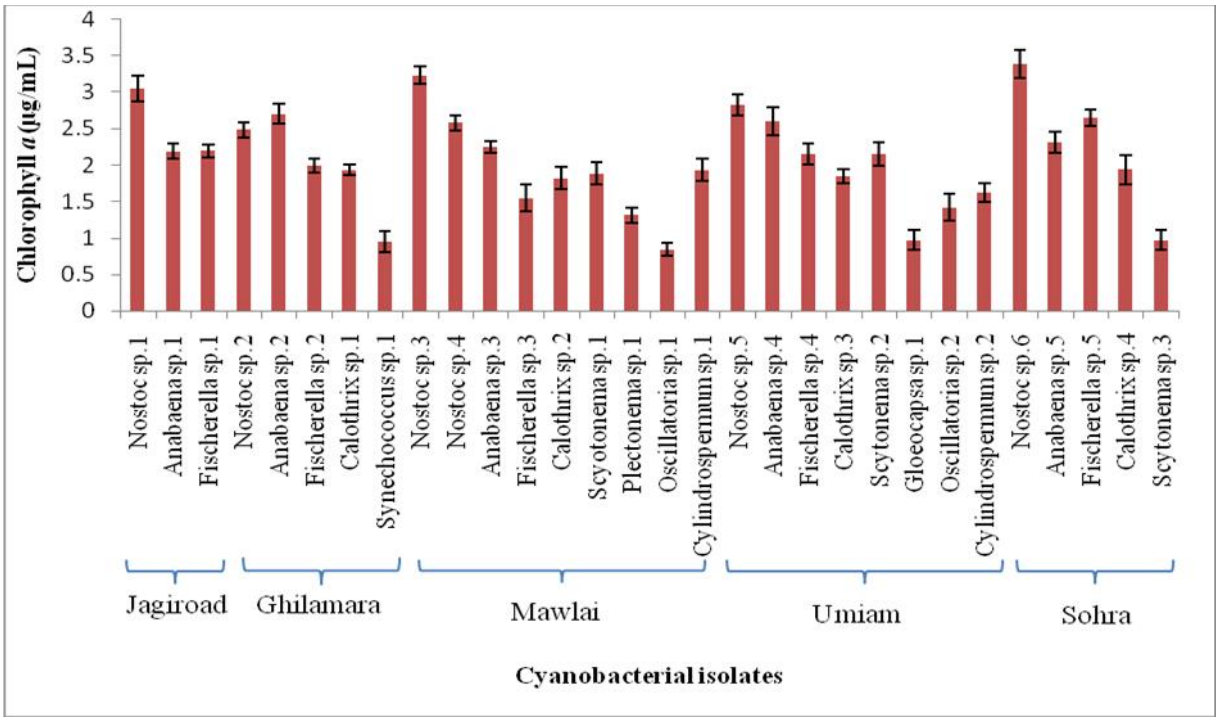


**Fig. 3**: Chlorophyll *a* content of the thirty randomly selected cyanobacteria which belonged to five different sites.

## Estimation of protein content

The protein content of the selected cyanobacterial strains were estimated in 7 day exponentially growing cultures. The result showed that the protein concentrations ranged between 21.1-64.4 µg/mL (Fig. 4). *Nostoc* sp.6 showed highest protein content (64.4 µg/mL) and lowest was found in *Scytonema* sp.3 (21.1 µg/mL). *Nostoc* sp.1, *Anabaena* sp.1, *Nostoc* sp.2, *Nostoc* sp.3, *Nostoc* sp.4, *Anabaena* sp.3, *Nostoc* sp.5, *Nostoc* sp.6 showed high protein contents and may have possible implications of their use in animal feed and fodder.

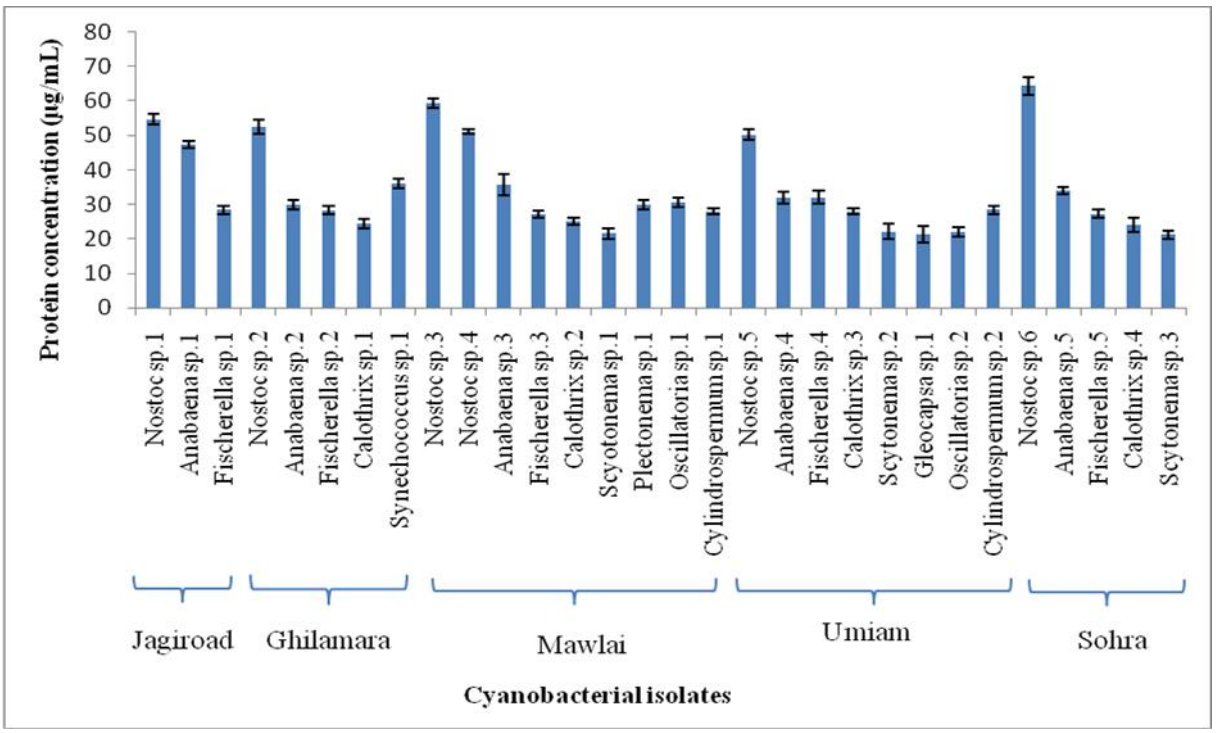


**Fig. 4:** Protein concentration of cyanobacterial isolates.

## Heterocyst frequency

Heterocyst frequency was counted in the selected 7 day old cyanobacterial strains to estimate the percentage of heterocyst present. The frequency in the heterocystous cyanobacteria varied from species to species within the range 0.91-8.02% (Fig. 5). High heterocyst frequency was seen in *Nostoc* sp.1 (7.48%), *Anabaena* sp.1 (7.4%), *Nostoc* sp.2 (7.45%), *Nostoc* sp.3 (7.23%), *Nostoc* sp.5 (7.5%), *Nostoc* sp.6 (8.02%) and *Anabaena* sp.5 (7.14%). *Calothrix* sp.2 showed very low heterocyst frequency of 0.91%.

All isolates of the genera *Anabaena* and *Nostoc* showed heterocyst frequency above 5% and *Fischerella* isolates showed more than 4%. Non-heterocystous isolates were *Synecococcus* sp.1, *Plectonema* sp.1, *Gloeocapsa* sp.1 and *Oscillatoria* sp.1 and 2.

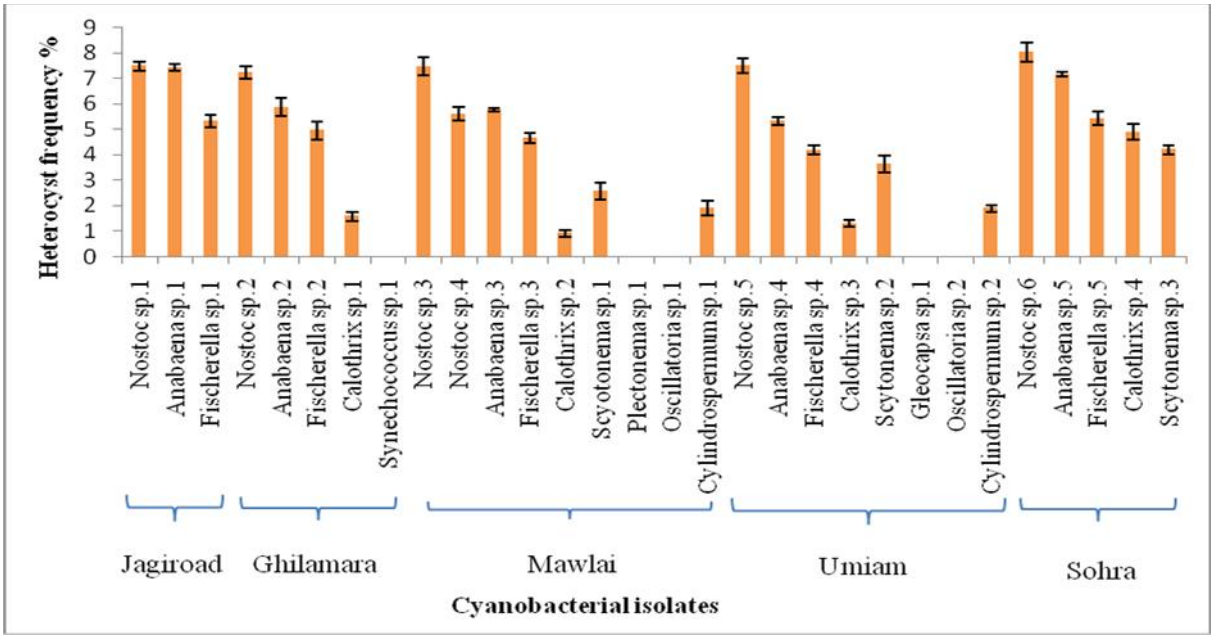


**Fig. 5:** Heterocyst frequency percentage of thirty different cyanobacterial isolates.

## Nitrogenase activity

The nitrogenase activities of the thirty selected cyanobacteria measured on day 7$^{th}$ are represented in the Fig.6. The activity ranged between 0.135 -2.18 nmol $C_2H_4$ produced/µg Chl *a*/hr where the highest activity was observed in strain *Nostoc* sp.6 followed by *Nostoc* sp.1, *Nostoc* sp.3, *Anabaena* sp.1, *Nostoc* sp.5, etc. and lowest was recorded in *Oscillatoria* sp.2.

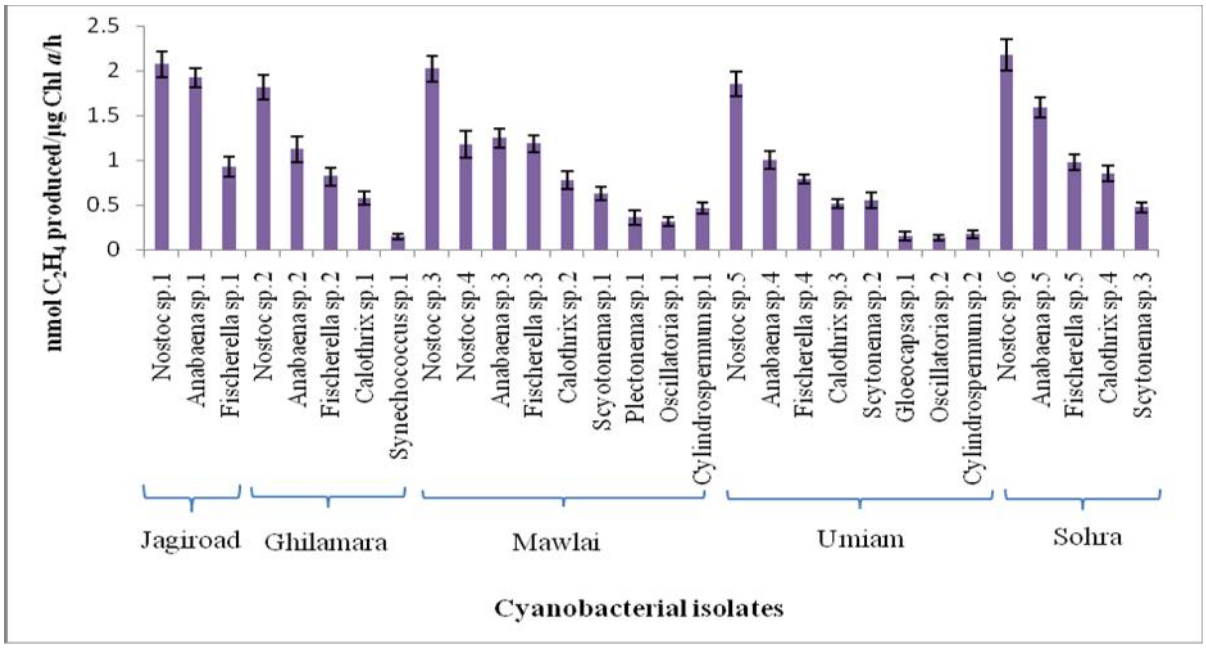


**Fig. 6:** Acetylene reduction assay of few selected cyanobacteria.

## Discussion

A total of 103 cyanobacterial isolates were purified from the five randomly selected sites. The pH of these sites ranged from 5.8-8.2. Ten different genera that included both heterocystous and non-heterocystous cyanobacterial isolates were found in these sites although the number of heterocystous isolates were far more in number than the non-heterocystous types. Small number of genera represented in the collection highlights the fact that growth and distribution of cyanobacteria in any ecosystem are affected by the interplay among different physico-chemical parameters that constitute their immediate environment. The higher number of heterocystous forms further emphasizes the fact that heterocystous forms are more adjustable than the non-heterocystous forms in any environment. Many researchers have already shown importance of physico-chemical parameters on the distribution and diversity of the cyanobacteria (Prasanna and Nayak, 2007; Dey *et al.*, 2010; Selvi and Sivakumar, 2011). Among these factors, pH is a significant determining factor in cyanobacterial diversity and cyanobacteria are known to thrive in slightly alkaline pH. However, in our study, Sohra with lowest pH of 5.8 had higher cyanobacterial diversity (Shannon-Weiner's index: 1.29) than Jagiroad that recorded pH of 8.2 (Shannon-Weiner's index: 1.0395). This finding revealed that pH alone cannot be the determining reason in cyanobacterial diversity. Other factors such as elevation of a place, the prevailing average temperature, dissolved oxygen and total dissolved solids etc. as well as pollutants in the surrounding environment collectively play a decisive role in shaping the cyanobacterial diversity and distribution. This inference was deduced from our study where we have looked into these factors individually for each site under study. Mawlai that recorded maximum diversity among the sites had highest elevation of 1496 m, low TDS (17 mg/L), highest DO (7.4 mg/L) and a ambient temperature of 21°C, although, pH of Mawlai was slightly acidic (6.2) in nature. Jagiroad that recorded minimum diversity among all sites studied recorded an elevation of 62 m, very high TDS (1350 mg/L) and lowest DO (1.3 mg/L). Although the temperature was 30°C and pH of this site was alkaline (8.2) that favours cyanobacterial growth. These favourable conditions were overridden by the effect of other environmental factors mentioned above in determining the cyanobacterial diversity of the site. In addition, effluents from the Hindustan paper mill in Jagiroad have critically affected the soil and water bodies of the place. There are records of high levels of pollutants such as heavy metals (Cu, Zn, As, Ni, etc.) as well as high levels phosphates, nitrates and sulphates in these effluents. Thus, these findings reiterate the fact that a combination of various factors is crucial in shaping the diversity in a location. Again, highest value for Simpson's dominance was recorded in Jagiroad (0.375) with ten isolates out of twenty belonging to the genus *Nostoc*. A look into the different genera isolated from all sites showed that the genus *Nostoc* was most abundant (44%) in almost all sites. This reflects the resilience and versatility of this genus that allowed its members to populate diverse ecosystems. The abundance of this genus was followed by *Anabaena* (23%) and *Fischerella* (11%). The rest of the genera were found sporadically indicating their limitations in populating areas that are varied in terms of various ecological factors including pH, TDS, DO, temperature etc.

Biochemical characterization proved that the isolates varied from each other in their growth and other parameters. As seen from protein and chlorophyll *a* content on day seventh, the growth of individual isolates varies significantly and same was true for nitrogenase activity. Highest value for protein and chlorophyll *a* content was seen in *Nostoc* sp.6 (isolated from Sohra). Most of the *Nostoc* and *Anabaena* isolates recorded high value of chlorophyll *a* and protein content. In many cyanobacteria, heterocyst frequency increases if the fertility of the soil is low. Both *Anabaena* and *Nostoc* isolates from all sites recorded >5% heterocyst frequency. Most members of the genus *Fischerella* showed heterocyst frequency ~4%. Similarly, most of the *Nostoc* and *Anabaena* showed higher nitrogenase activities in line with their high heterocyst frequency. Their ubiquitous presence, high biomass and high nitrogenase activity could be exploited in applying these cyanobacterial strains as biofertilizer in crop cultivation in these areas. Also many isolates from Jagiroad could be further researched for their application as bioremediators of heavy metal ions as well as other contaminants as they are able to grow in presence of such pollutants in their vicinity.

## Conclusion

Random sampling of cyanobacterial diversity from five locations within Eastern Himalayan Biodiversity hot spot showed the members of the genus *Nostoc* to be most abundant in the region. This was followed by *Anabaena*. Even in Jagiroad where there was high amount of pollutants in the soil and water samples, the cyanobacterial isolates predominantly belonged to *Nostoc*. Although, this study revealed that the

cyanobacterial diversity of a location is determined by interaction among various prevailing environmental factors such as pH, temperature, TDS, DO and pollutants, the genus *Nostoc* showed highest resilience and members of this genus were most abundant in all five study sites. Biochemical analyses revealed that generally isolates from the genera *Nostoc* and *Anabaena* were fast growing. They also recorded higher heterocyst frequency and nitrogenase activity.

## Acknowledgments

The authors would like to thank University Grants commission for financial assistance and Department of Science and Technology, New Delhi for providing INSPIRE Fellowship.

## Conflict of interest

Authors declare there is no conflict of interest.

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