International Journal of Biotechnology and Microbiology
www.biotechnologyjournals.com
Online ISSN: 2664-7680, Print ISSN: 2664-7672
Received: 13-12-2024, Accepted: 12-01-2025, Published: 27-01-2025
Volume 7, Issue 1, 2025, Page No. 17-22

Chlorophyll-a, crude proteins, lipids contents and nitrogen scavenging potential of filamentous nitrogen-fixing cyanobacteria

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Abstract
Total nitrogen, chlorophyll-a, crude proteins, lipids contents of *filamentous*, heterocystous soil cyanobacterial species was estimated by standard methods. Cyanobacteria when grown under laboratory conditions total nitrogen, chlorophyll-a, crude proteins, lipids contents were obtained in the range of 1.52- 5.28%, 0.52- 5.67 µg gm^{-1}, 9.50- 33% and 1.4- 19.2% respectively on fresh weight basis. *Anabaena circinalis*, *Nostoc punctiforme*, *Nostoc muscorum*, *Nostoc carneum*, *Anabaena orientalis* were found to be the dominant nitrogen fixers encountered from the soils of the study area. However as far as chlorophyll-a content was concerned not much variation was found in the tested cyanobacterial species. *Nostoc* and *Anabaena* were found to be the alternative sources of algal proteins and could be effectively used as protein rich food. Almost all cyanobacterial species exhibited better lipids content and therefore could be considered as good sources of biodisel. In most species of *Nostoc* and *Anabaena* growth appeared to be correlated to crude proteins and lipids contents. A general trend of more crude proteins content than the lipids was observed in all the studied cyanobacterial forms.

Keywords: Cyanobacteria, total nitrogen, chlorophyll-a, crude proteins, lipids, *Nostoc*, *Anabaena*

Introduction

Cyanobacteria comprises a heterogenous assemblage of photosynthetic prokaryotes having an extraordinary biosynthetic potential and a repertoire of diverse metabolic activities. These are often referred to as "Miniature Factories" of the biological world and represent an alternate source of a variety of bioactive compounds, lipids, proteins, enzymes, pigments and compounds of pharmaceutical value. Cyanobacteria are unique in their ability to conduct the two incompatible processes of oxygen evolving photosynthesis and oxygen sensitive nitrogen fixation within specialized cell heterocyst [1]. These photosynthetic cyanobacteria make a valuable contribution to the nitrogenous soil fertility by fixing atmospheric nitrogen [2]. The use of biofertilizers based on local strains of cyanobacteria shows promise to increase nitrogen use efficiency in rice [3].

By considering amplified world population it can be expected that the food requirement in the prospect could not be covered by conventional agriculture [4]. Therefore, in recent years there have been innumerable attempts to search non-conventional, renewable and alternate sources of food in order to feed ever-increasing population of the world. Microalgae are not only used for the production of health foods and animal feed but also for wastewater treatment, aquaculture and pharmaceutical manufacture [5, 6].

Development in algal biotechnology industry requires an extensive screening of microalgae, which is expected to result in discovery of better strains of interest. This is because of microalgae are abundant in proteins, with species such as *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Anabaena platensis*, *Anabaena maxima* containing 50-70% proteins by dry weight, which comprises all nine essential amino acids [7, 8]. This makes algae valuable protein sources equivalent to those derived from animals. Algal biotechnology at the commercial level is at present centered around a few species of *Chlorella*, *Spirulina*, *Scenedesmus* and *Dunaliella salina*, reporting a proteins content of 38 to 70% dry weight [9, 10, 11, 12, 13]. A number of other species are reported to have biochemical molecules important for agricultural and biomedical research, cosmetics, veterinary, printing and pharmaceutical applications. Hence isolation of strains of filamentous cyanobacteria and optimization of growth parameters is essential to further explore their utilization value.

Cyanobacteria are unique in that, besides chlorophyll-a, which is directly involved in photosynthesis, a special class of pigments- phycobiliproteins such as phycocyanin and phycoerythrin which exhibit different colors are the major light harvesting complexes of the cell. Cyanobacterial pigments are not only used as nutritional ingredients and natural dyes for food and cosmetics, but also fluorescent markers in biomedical studies and as phycoflours in fluorescence activated cell sorting, flow cytometry and histochemistry [14]. Increasing consciousness about the harmful effects of synthetic or chemical dyes encouraged people to give more preference towards the usage of natural products, such as plant or microbial-derived colors in food and cosmetics [15].

By considering all these issues along with social responsibilities as well as to fulfill such lacunae, the present investigation was carried out to estimate the nitrogen fixation potential, chlorophyll-a, crude proteins and lipids content of cyanobacteria isolated from agro-practices areas of Ahmednagar district, Maharashtra state.

Materials and Methods

A total 136 soil samples were collected from various sampling sites belonged to rainfed and riverside areas as per the procedure given by [16] and were brought to the laboratory for growth study of cyanobacteria. The isolation and purification of cyanobacterial strains was carried using BG-11 Culture medium [17]. The composition of BG-11 liquid medium per litre of glass-distilled water was $NaNO_3$ (1.5 gm), K_2HPO_4 (40 mg), $MgSO_4$ $7H_20$ (75 mg), $CaCl_2.H_20$ (36 mg), Na_2CO_3 (20 mg), Citric Acid (6 mg),

Ferric Ammonium Citrate (6 mg), Disodium Magnesium EDTA (1 mg), H_3BO_3 (2.86 gm), $MgCl_2$ (1.81 gm), $ZnSO_4$ (79 mg), Na_2MoO_4 (39 mg), $CuSO_4$ (79 mg), $Co(NO_3)_2$ (49 mg).

All the ingredients of above BG-11 medium were completely dissolved in distilled water one by one and the final volume 1000 ml was made by using glass distilled water. After adjusting pH to 7, it was then sterilized in autoclave for 20 minutes at 121^0 C and 15 lbs pressure.

Isolation of cyanobacteria

For isolation and identification of cyanobacterial forms, 5gm of soil samples were inoculated in sterilized flasks having 100 ml of BG-11 medium. Inoculation was done in laminar airflow unit. These inoculated flasks were incubated at 28 ± 2^0C under 2.5 K lux fluorescent light for 2-3 weeks under 16/ 8 hours light/ dark cycles. After 20-25 days of incubation period, the visible growth of algae appeared in the enrichment cultures.

Solid agar medium was also prepared by adding 1.2% agar-agar powder in the above-mentioned BG-11 liquid medium. The medium was sterilized and used to prepare plates and slants to obtain unialgal cultures.

Purification of cyanobacteria

Cyanobacterial growth appeared in the plates and tubes were isolated and purified by repeated sub-culturing in BG-11 liquid medium followed by serial dilution and streak plate method on solid BG- 11 medium.

Direct isolation: After examined under a microscope, aliquots of materials with uniform colonies or filament were immediately seeded in test containers on BG-11 nutrient medium solidified with 1.2% agar. Some samples were immediately inoculated in culture flasks with 30 ml sterilized BG-11 medium.

Serial dilution: For isolation of single species from the dispersed sample mixtures, subsequent dilutions was carried out by adding 1 gm of material in 9 ml of sterile distilled water. After that, 1 ml of this combination was mixed with 9 ml of sterile distilled water. This procedure was done until the material was diluted 1/10 (10-fold) [18, 19]. Following that, 1ml of this combination was used as an inoculum and was distributed on newly made solidified nutrient media on agar plates.

Streak plate method: The streak plate technique was used to separate specific cyanobacteria species and isolate cultures that were healthy and developing quickly. This aids in the separation of individual cyanobacterium clusters on agar. For this, 20 ml BG11 medium was poured into each petri plate and left to settle and solidify using 1.2% Agar. The sample culture was streaked with an inoculating (nichrome) loop onto solid BG-11 medium. The Petri plates were covered with parafilm and incubated in a culture chamber. The complete procedure was carried out aseptically in front of laminar air flow.

Taxonomic identification of the cyanobacteria:

Slides were made and examined under the trinocular microscope to identify the cyanobacterial forms. Morphometric studies were carried out by using ocular and stage micrometers. Microphotography and measurements of the species were made by ocular and 10 X, 40 X, 100 X objectives with 10X and 15X eyepieces. The taxonomic identification was done by following the monographs and keys given by [20, 21, 22]. The identified cyanobacteria species were arranged as per the system of classification followed by Desikachary.

Estimation of total nitrogen

Total nitrogen content was estimated by conventional Micro-kjeldahl distillation method [23]. 10 ml aliquot of cyanobacterial species extract was pipette out into distillation flask and 10ml distilled water + 10 ml 50 % NaOH was added to it. Distillation was carried out, collecting the distillate in 20 ml of 4% boric acid solution in a 150 ml beaker with mixed indicator (Methyl red 5 drops + Bromocresol green 5 drops). The distillate was then titrated with standard H_2SO_4 (0.02 N) until pink colour turns green. A blank was run without plant extract and readings were noted.

Isolation and quantification of chlorophyll- a

Chlorophyll-a isolation and its quantitative estimation from cyanobacterial species was carried using the method of [24]. 1 gm of cyanobacterial species sample was thoroughly grinded in 80% acetone with mortar and pestle. The extract was centrifuged at 5000 rpm for 05 minutes and the supernatant was transferred to a volumetric flask. Complete extraction was done by adding 20 ml of the solvent to pellet and the above process was repeated. The final volume of the supernatant was made up to 100 ml with 80% acetone. The absorbance of the solution was read at 645 nm and 663 nm in a spectrophotometer against the solvent (80% acetone) blank.

Crude proteins estimation

The crude proteins content was quantified by estimating total nitrogen (Micro-kjeldahl method) and multiplied it by a factor 6.25 as per the method given in the book of [25]. In general, the nitrogen content is multiplied by the factor 6.25 to arrive at the percentage of crude proteins which is based on the assumption that nitrogen constitutes to 16% of a proteins.

Estimation of lipids

The lipids were extracted from cyanobacterial strains and were estimated by the method of [26]. The powdered material weighing one gram was homogenized in mortar with 10 ml of chloroform: methanol (2:1 v/v) solvent. The homogenate was filtered through Whatman filter paper No. 1 so as to collect filtrate. Same procedure was repeated again using 5 ml solvent mixture so as to extract remaining lipids from residue. One-third volume of water was added to extract, to remove water soluble impurities. The upper layer of water was separated. The filtrate was then transferred to pre-weighed glass beaker (A) and then kept in water bath for drying. After complete drying, weight of beaker was reweighed (B) and lipids content of alga was calculated by using the formula: Weight of lipid (Oil) = B - A mg gm^{-1}

Results and Discussion

A total of 16 cyanobacterial species isolated from collected soil samples belonging to *Nostoc* and *Anabaena* genera were studied for their total nitrogen, chlorophyll-a, crude proteins, lipids contents (Table 1). Both *Nostoc* and *Anabaena* belongs to the order Nostocales [27], a group of dinitrogen (N_2) fixing cyanobacteria that develops heterocysts when fixed nitrogen is limiting [28] and utilizes

the enzyme nitrogenase to convert nitrogen to ammonia [29]. The raised cyanobacterial cultures in nitrogen free BG-11 medium gave the evidence of its nitrogen fixing potential.

Total Nitrogen:

In the present investigation, wide variation was observed in the total nitrogen content of the studied species of cyanobacteria (Table- 1). The highest total nitrogen content was observed in *Anabaena circinalis* (5.28%) followed by *Nostoc punctiforme* (4.94%), *Nostoc muscorum* (4.89%), *Nostoc carneum* (4.84%), *Anabaena orientalis* (4.80%), *Anabaena spiroides* (3.78%), *Anabaena fertilissima* (3.65%). The least amount of total nitrogen content was recorded in *Nostoc calcicola* (1.52%).

The calculated range of total nitrogen content within the studied genus was discussed here with great details (Table-1). The four species of the genus *Nostoc viz*. *N. punctiforme, N. paludosum, N. carneum* and *N. muscorum* exhibited higher range of total nitrogen content. Whereas, six species in the genus *Nostoc* showed medium range and only *Nostoc calcicola* exhibited lower range. On the other hand, higher range of total nitrogen contents was obtained in all the studied species of the genus *Anabaena*. The mean amount of total nitrogen content obtained in the studied genera was *Nostoc* (3.21%) and *Anabaena* (4.08%). The practical results revealed that, 56.25 percent studied cyanobacterial species were feel right in the higher range of total nitrogen, while 37.50 percent species showed medium range and only 6.25 percent species were situated in the lower range of total nitrogen content.

Chlorophyll-a

Altogether 16 cyanobacterial species were analyzed for their chlorophyll-a content using Arnon method (1949) and values are expressed as μg gm^{-1} of fresh weight basis (Table 1). Amongst the studied cyanobacterial isolates tested for chlorophyll-a, an array of 0.52 μg gm^{-1} to 5.67 μg gm^{-1} chlorophyll-a content was obtained. The maximum chlorophyll-a was observed in *Nostoc punctiforme* (5.67 μg gm^{-1}) followed by *Anabaena fertilissima* (5.40 μg gm^{-1}), *Nostoc muscorum* (4.98 μg gm^{-1}), *Anabaena circinalis* (4.90 μg gm^{-1}), *Anabaena spiroides* (4.06 μg gm^{-1}) while the least value was obtained in *Nostoc linckia* (0.52 μg gm^{-1}).

The calculated range of chlorophyll-a content within the genus *Nostoc* revealed that only *Nostoc punctiforme* exhibited higher range of chlorophyll-a (Table 1). While the two species *viz*. *Nostoc carneum* and *Nostoc muscorum* showed medium range and the remaining eight *Nostoc* species recorded lower range of chlorophyll-a. Furthermore, within the genus *Anabaena*, all the tested species for chlorophyll-a content illustrated higher range of chlorophyll-a content. The mean amount of chlorophyll-a content obtained in the genus *Nostoc* and *Anabaena* was 2.42 μg gm^{-1} and 4.09 μg gm^{-1} respectively.

Table 1: Total nitrogen, chlorophyll-a, crude proteins, lipids contents of filamentous cyanobacteria

Sr. No.	Cyanobacterial species	Total nitrogen (%)	Range within genus	Chlorophyll- a (μg gm^{-1})	Range within genus	Crude proteins (%)	Range within genus	Lipids (%)	Range within genus
1.	*Nostoc punctiforme*	4.94	H	5.67	H	30.87	H	9.7	H
2.	*Nostoc entophytum*	2.67	M	1.26	L	16.68	M	10.4	H
3.	*Nostoc paludosum*	4.09	H	2.66	L	25.56	H	6.1	M
4.	*Nostoc linckia*	2.68	M	0.52	L	16.75	M	7.7	M
5.	*Nostoc sporangiaeforme*	2.75	M	1.93	L	17.18	M	7.4	M
6.	*Nostoc ellipsosporum*	2.29	M	0.64	L	14.31	M	8.2	M
7.	*Nostoc carneum*	4.84	H	3.49	M	30.25	H	1.4	L
8.	*Nostoc calcicola*	1.52	L	3.34	L	9.500	L	3.6	L
9.	*Nostoc muscorum*	4.89	H	4.98	M	30.56	H	5.7	L
10.	*Nostoc microscopicum*	2.31	M	0.55	L	14.43	M	6.9	M
11.	*Nostoc verrucosum*	2.41	M	1.65	L	15.06	M	4.8	L
12.	*Anabaena spiroides*	3.78	H	4.06	H	23.62	H	14.7	H
13.	*Anabaena oryzae*	2.89	H	2.78	H	18.06	H	19.2	H
14.	*Anabaena fertilissima*	3.65	H	5.40	H	22.81	H	12.8	H
15.	*Anabaena orientalis*	4.80	H	3.32	H	30.00	H	7.3	L
16.	*Anabaena circinalis*	5.28	H	4.90	H	33.00	H	18.1	H

The values represented are mean of three observations and expressed as percentage (%) of fresh weight basis.
Where, L- Lower, M- Medium, H- Higher

Note: In the Table- 1, the lower, medium and higher range within the genus was formed by subtracting the lowest amount from the highest and then divided by the number of ranges i.e. 3. The value thus obtained is of range 'L' and is multiplied by 2 and 3 to get range 'M' and 'H' respectively.

Crude proteins:

The analyzed cyanobacterial species showed an array of 9.5% to 33% crude proteins. The highest crude proteins was recorded in *Anabaena circinalis* (33%). It was followed by *Nostoc punctiforme* (30.87%), *Nostoc muscorum* (30.56%), *Nostoc carneum* (30.25%), *Anabaena orientalis* (30%) and *Nostoc paludosum* (25.56%). The least amount was obtained in *Nostoc calcicola* (9.5%).

The calculated range of crude proteins amongst the eleven species of the genus *Nostoc*, four species *viz. N. punctiforme, N. paludosum, N. carneum* and *N. muscorum* revealed higher range of crude proteins content (Table 1). The rest analyzed species in the genus *Nostoc* showed signs of medium range except *Nostoc calcicola,* where lower range was found. Furthermore, within the genus *Anabaena,* higher range of crude proteins was recorded in all the

studied species. The mean amount of crude proteins content obtained in the *Nostoc* and *Anabaena* was 20.10% and 25.49% respectively. It was found that about 56.25 percent studied cyanobacterial species were fit right in the higher range of crude proteins, while 37.5 percent species preoccupied medium range and only 6.25 percent species were positioned in the lower range of crude proteins content.

Lipids:

The paramount lipids content was recorded in the cyanobacteria, *Anabaena oryzae* (19.2%), then it was followed by *Anabaena circinalis* (18.1%), *Nostoc verrucosum* (14.7%), *Anabaena fertilissima* (12.8%) and *Nostoc entophytum* (10.4%). The least amount of lipids was obtained in *Nostoc carneum* (1.4%).

The calculated range of lipids within the genus *Nostoc* revealed that, *N. punctiforme* and *N. entophytum* exhibited higher range of lipids (Table 1). Whereas, *N. paludosum, N. linckia, N. ellipsosporum* and *N. microscopicum* showed medium range and the remaining four species *viz. N. carneum, N. cacicola, N. muscorum* and *N. verrucosum* revealed lower range of lipids content. In the genus *Anabaena,* all the studied species exhibited higher range except *A. orientalis* where lower range of lipids content was recorded. The mean amount of lipids content obtained in the studied genera was *Nostoc* (6.53%) and *Anabaena* (14.42%). It was found that about 37.50 percent studied cyanobacterial species were belonged to the higher range of lipids, while 31.25 percent species possessed medium range and 31.25 percent species were positioned in the lower range of lipids content.

Discussion

The practical results for total nitrogen, chlorophyll-a, crude proteins, lipids contents of 16 cyanobacterial species are discussed and impended here in great details. The total nitrogen recorded in *Nostoc muscorum* (4.89%) appears close to the value of 5.4% and 5.09% as reported by [30, 31] respectively. Similarly, the estimated value of total nitrogen in *Nostoc sporangiaeforme* (2.75%) is in agreement with that of the reported value 3.38% by [32]. In the present study, the amount of total nitrogen fixed by *Nostoc linckia* was 2.68% is found similar to reports of [33] and is lower than the value reported by [34] as (4.86%).

The obtained value of chlorophyll-a in *Nostoc sporangiaeforme* (1.93 μg gm^{-1}) is found to be identical to that of the recorded value (1.91 μg gm^{-1}) for the same species by [34]. The estimated chlorophyll-a in *Anabaena oryzae* (2.78 μg gm^{-1}) is found to be very close to the reported value of [35]. At the same time *Anabaena orientalis* (3.32 μg gm^{-1}) showed almost equal chlorophyll-a content with reports of [36]. However, the estimated chlorophyll-a in *Nostoc muscorum* (4.98 μg gm^{-1}) appears to be more than the reported value for *Nostoc muscorum* (2.75 μg gm^{-1}) by [37] and (1.79 μg gm^{-1}) by [38].

The chlorophyll-a in cyanobacteria reported by [39] revealed that, he observed chlorophyll-a of *Nostoc linckia* (0.52 μg gm^{-1}) is in agreement with the reported value (0.7 μg gm^{-1}). Likewise in *Nostoc carneum,* the amount of chlorophyll-a recorded was 3.49 μg gm^{-1}, which is nearly identical with the reported value (3.70 μg gm^{-1}). While in *Nostoc calcicola,* the estimated value (3.34 μg gm^{-1}) of chlorophyll-a is close to the reported value 2.0 μg gm^{-1}.

The estimated crude proteins in *Nostoc punctiforme* (30.87%), *Nostoc muscorum* (30.56%) and *Nostoc carneum* (30.25%) are in close proximity with the reported amount in *Nostoc commune* (29.4%) by [40]. The obtained crude proteins in *Anabaena oryzae* (18.06%) appears close to the reported value by [41]. Whereas *Anabaena circinalis* studied for its crude proteins, fit right with the previously reported value by [42] and [43].

At the same time estimated crude proteins in *Nostoc calcicola* (9.5%) appears identical with that of the reported value by [44, 45]. Similarly, in the genus *Anabaena*, the estimated crude proteins 18.06% is found to be in line with that of the reported value by [36]. However, the crude proteins obtained in *Anabaena spiroides* and *Anabaena oryzae* appears very less than the recorded value by [35].

The proteins in cyanobacteria reported by [39] revealed that, the obtained crude proteins in *Anabaena fertilissima* (22.81%) and *Anabaena spiroides* (23.62%) is in agreement with the reported value. Similarly, the estimated crude proteins in *Nostoc calcicola* (9.5%) matches with the reported value 11.2%. To the contrary in the genus *Nostoc viz. N. carneum* (30.25%), *N. muscorum* (30.56%) and *N. ellipsosporum* (14.31%), the estimated crude proteins appears to be more than that of the reported values.

The pragmatic value of lipids recorded in *Nostoc sporangiaeforme* (7.4%) appears close to that of the reported value by Bhandari and Sharma (2007). The estimated minimum lipids content within the genus *Anabaena* (*A. orientalis*, 7.3%) is much closer to the reported value by [42]. Similarly, amount of lipids obtained in *Nostoc carneum* (1.4%) is in agreement with the reported value of [46, 47]. The tested lipids in *Nostoc* species from a minimum of 1.4% to maximum of 10.4%, which is not in agreement with the reported value by [48].

Summary and Conclusions

The pragmatic results revealed that *Anabaena circinalis, Nostoc punctiforme, Nostoc muscorum, Nostoc carneum, Anabaena orientalis* were found to be the dominant nitrogen fixers encountered from the soils of the study area. These forms capable of growing on moist and shaded soil surface hold promise for crops like sugarcane, tomato, mungbeans and maize. However as far as chlorophyll-a content was concerned not much variation was experiential in the studied cyanobacterial species. It was also revealed that, 37.50 percent studied cyanobacterial species were feel right in the higher range of chlorophyll-a, while 12.50 percent species showed medium range and 50 percent species were situated in the lower range of chlorophyll-a content.

Nostoc and *Anabaena* were found to be the alternative sources of algal proteins and could be effectively used as protein rich food. Almost all cyanobacterial species exhibited better lipids content and therefore could be considered as good sources of biodiesel. In general, higher range of chlorophyll-a content accompanied with lower range of lipids composition, but in *Nostoc* and *Anabaena*, although it varied at species level but did not depend on growth. In most species of *Nostoc* and *Anabaena* growth appeared to be correlated to crude proteins and lipids contents. A general trend of more crude proteins content than the lipids was observed in all the studied cyanobacterial forms.

Acknowledgement
The author express deep sense of gratitude to The Principal, K.J. Somaiya College, Kopargaon (M.S.) for providing laboratory facilities during the entire research investigation.

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