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Regular Article

Enhanced Carotenoid Synthesis of *Phormidium* sp. in Stressed Conditions

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ABSTRACT: Cyanobacteria, also known as blue-green algae or blue-green bacteria obtain their energy through photosynthesis. Phormidium, Filamentous unbranched, non nitrogen fixing, microscopic and later macroscopic up to several cm in diameter, usually covering substrates of different types. Phormidium sp. was selected for the experiment and grown in BG11 medium containing different concentration (0, 1, 2, 3, 4 µg/ml) after the growth period the cells were collected and used to estimate the amount of chlorophyll 'a', carotenoids, phycocyanin, free amino acids and proteins in different concentration. An increase in growth period days (0, 2, 4, 6, 8, 10) increase the cellular content namely chlorophyll 'a', carotenoids, phycocynin, free amino acids and proteins. Carotenoids are responsible for many of the red, orange and yellow hues of plant leaves, fruits, and flowers as well as the colors of some birds, insects, fish, and crustaceans. The carotenoid content showed positive increase by lowering the nitrogen concentration in which the modified BG11-A medium showed the highest value (85.2 µg/ml) in half strength. It is concluded that enhanced carotenoid synthesis in stressed conditions and it increases two ecological functions, providing photoprotection and increasing photosynthetic performance of surface cyanobacterial populations.

Key words: *Phormidium*, chlorophyll 'a', carotenoids, phycocynin, carotenoids

Introduction

Cyanobacteria, also known as blue-green algae or blue-green bacteria obtain their energy through photosynthesis. The name "cyanobacteria" comes from the colour of the bacteria (Greek: κυανός (kyanós) = blue). Cyanobacteria are found in almost every conceivable environment, from oceans to fresh water to bare rock to soil. Most are found in fresh water, while others are marine, occur in damp soil, or even temporarily moistened rocks in deserts. Cyanobacteria are the only organisms that actively evolve oxygen as a by-product of oxygenic photosynthesis within the same cell or colony of cells where nitrogen fixation occurs. The presence of oxygen triggered biochemical and morphological adaptations in diazotrophic phototrophs aimed at limiting the inhibitory effects of oxygen on nitrogenase (Gallon, 1992; Bergman *et al.,* 1997; Berman-Frank *et al.* 2003). Cyanobacteria get their name from the bluish pigment phycocyanin, which they use to capture light for photosynthesis. They also contain chlorophyll a, the same photosynthetic pigment present in higher plants.

Nitrogen fixation cannot occur in the presence of oxygen therefore nitrogen is fixed in specialized cells called heterocysts. These cells have thickened wall that provides an anaerobic environment. Such larger cells are common among the filaments of *Nostoc, Anabaena, Cylindrospermum* etc., (Min, H and Sherman, 2010)

Cyanobacteria are used as biofertilizers and have a great advantage in the cultivation of rice. The floating fern Azolla which is actively distributed among the paddy fields, houses colonies of the cyanobacterium *Anabaena* in its leaves, which fixes nitrogen. The ferns then provide an inexpensive natural fertilizer and nitrogen source for the rice plants when they die at the end of the season. Cyanobacteria also form symbiotic relationships with many fungi, forming complex symbiotic "organisms" known as lichens. (Dobson, 2000)

Cyanobacteria show a remarkable variation in physiological properties a collection of Cyanobacteria from various habitats provides an excellent opportunity for the experimental selection of suitable strains. Cyanobacteria secrete enzymes that can be exploited commercially. Marine cyanobacteria have been used in large-scale production of enzymes such as beta lactamase, protease and lipase (Prabhakaran *et.al,* 1994). Unique sequence-specific endonucleases have been obtained from *Anabaena cylindrica* (Acy I), *Anabaena flos-aquae* (Afl I & Afl II), *A.variabilis* UW (AvrII), *Microcoleus sp*. UFEX 2220 (MstII), *Nostoc sp*. PCC 7524 (Nsp C I), which can be marketed at low cost since relative biomass production of cyanobacteria is much less expensive than bacteria (Elhai, and Wolk, 1988). Cyanobacterial isolates with capacity to mineralize organic phosphorus have been reported with alkaline phosphotase activity. Enzymes such as chitinase, L-asparaginase, L-glutaminase, amylase, protease, lipase, cellulase, urease and superoxide dismutase have been reported from cyanobacteria (Wikstrom. *et al.,* 1997). Analysis of extracellular growth-promoting substances liberated by *Nostoc muscorum* and *Hapalosiphon fontinalis* was found to contain amino acids like serine, arginine, glycine, aspartic acid, threonine, glutamic acid, cystine, proline, valine, ornithine, lysine, histidine and iso-leucine (Misra, and Kaushik, 1989). In addition, cyanobacteria are a rich source of several polyols, polysaccharides, lipids, fatty acids, halogenated compounds, etc (Becker, 1994).

Cyanobacteria in general and marine species in particular are one of the richest sources of known and novel bioactive compounds including toxins with wide pharmaceutical applications. Gustafson *et al.,* (1989) reported anti-HIV activity of marine cyanobacterial compounds from *Lyngbya lagerheimii* and *Phormidium tenue*. Screening of extracts from the large culture collection of marine cyanobacteria for antiviral, anti-bacterial, anti-fungal and immuno-modulatory activities have been carried out recently.

Cyanobacterial cells have developed natural methods of responding to metals such as copper, lead, and cadmium through passive accumulation in cells and through surface binding to various functional groups. They have also been found to remove harmful metals from the environment. For example, *Spirulina platensis*, was shown to contain detectable levels of mercury and lead when grown under contaminated conditions (Slotton *et al.*1989), implying that this cyanobacterium was taking up the toxic metal ions from its environment.

Bioactive compounds of cyanobactera

Dolastatin

The pharmacological properties of the major mollusc commonly known as the "sea hare". The unique linear pentapeptide is composed of four novel amino acid residues (dolavaline, dolaisoleucine, dolaproline and valine). The residue sequence was assigned on the basis of several low resolution mass spectral fragmentation techniques. It was proposed that dolastatin10 binds with the β sub unit of tubulin. It is an anti-tumour agent in patients of platinum-sensitive ovarian carcinoma and degree of toxicity (Gerwick et al., 2001).

Symplostatin

It was discovered by direct isolation from the cyanobacterium symploca hydnoides. This compound exhibited cytotoxicity against a human carcinoma cell line. These effects were observed at concentrations of interphase microtubules. Cell cycle analysis indicated that symplostatin 1 caused with the effects on mitotic spindles. It inhibit both endothelial cell proliferation and invasion with in vivo against murine colon 38 and murine mammary 16/c cell lines (Gerwick et al., 2001).

Curacin A:

It is isolated from the collection of cyanobacterium majuscula. It was originally active against a vero cell line and subsequent

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assesmrnt in the NCI 60 cell line assay with some selectivity for colon, renal, and breast cancer cell lines (Gerwick et al., 2001).

Materials and Methods

Organism used for the study

Phormidium sp is a filamentous cyanobacterium. The taxonomic position is Cyanophyceae, Oscillatoriales, Phormidiaceae, Phormidioideae. The cultures were obtained from the Culture Collection Centre, Centre of Advanced study in Botany, University of Madras and made axenic by antibiotic treatment following the method of Droop (1967). The cells were axenized by the following method, shown in Table-1.

Table 1. Concentration of antibiotics in µg/mL for axenization

Antibiotic mixture	Dilution tube 1	2	3	4	5	6
Benzyl penicillin	500	250	125	2.5	31.25	15.63
Streptomycin	250	125	62.5	31.25	15.63	7.82
Chloramphenicol	20	10	5	2.5	1.25	0.63

Growth conditions

Cultures were grown at 24 ± 1ºC in a thermostatically controlled room illuminated with cool white fluorescent tubes (Philips 40 W) providing an irradiance of 50 µE/m^2/s in a 12h: 12h light/dark regime (Fig 1).

Growth measurements

Phormidium sp., was cultured and shaken with glass beads to get a uniform cell suspension and growth was measured by in terms of fresh weight.

Estimation of pigments

Phormidium sp*.,* was grown in a suitable medium which is modified by changing the nitrogen concentration while the normal medium serves as the control. At the end of growth period, cells were pelleted by centrifugation at 10000 rpm for 10 minutes. The pellet was suspended in sterile medium, centrifuged and was cold extracted with 90% acetone and left overnight in dark. After complete extraction, it was again centrifuged at 10000 rpm for 10 minutes and the clear supernatant was used for spectrophotometric estimation of pigments. Absorbance was measured using 10 mm width cuvette at 663 nm and 480 nm (Mackinney , 1941).

The amount of chlorophyll *a* was calculated using the formula

$$\text{Chl. a} \quad \text{mg/L} = 12.63 \times E_{663}$$

$$\text{Carotenoids} = E_{480} \times 100$$

Similarly, after extraction in 100% methanol the cyanobacterial pellet was washed and centrifuged twice with distilled water and resuspended in 0.05 M phosphate buffer (pH 6.7). Cells were repeatedly freezed and thawed in phosphate buffer below 5°C, cells were disrupted for 10-15 minutes and then centrifuged at 5000 rpm for 5 minutes. The supernatant was carefully collected. The absorbance of the supernatant was measured at 562, 615 and 652 nm in a spectrophotometer using 0.05M phosphate buffer as blank to determine C.Phycocyanin (PC), and Allophycocyanin (APC) (Bennet and Bogoras, 1973).

$$\text{C.Phycocyanin (PC)} = \frac{E_{615} - 0.475\,(E_{652})}{5.34}$$

$$\text{Allophycocyanin(APC)} = \frac{E_{652} - 0.208\,(E_{615})}{5.09}$$

Estimation of free amino acids & Protein

Free amino acids and Proteins were estimated by *Modified methods of Sadasivam and Manikam and* Lowry *et al.*, 1951 respectively.

Column Chromatography

Mass culture

The mass culture of cyanobacterial culture can be done with 5litre Hopkins flask .The BG11 medium is used for the culture.10 ml of culture was inoculated in two litres of medium. The culture is aerated using an aerator and illuminated using a table lamp. The *Phormidium* culture was inoculated in the flask inside the laminar air flow and maintained in the culture room.

Preparation of extract from *Phormidium* sp.

The cells were separated by centrifugation at 10000 rpm for 10 min, the pellet was collected. The separted cells were incubated overnight with 100% methanol and centrifuged at 10000 rpm for 10 min, the supernatant was collected. Then the solvent was evaporated and the extract was saved for further analysis.

Packing of the column:

A clean, dry column was aligned in a vertical position. A beaker was placed under the column outlet. The column was slowly and evenly filled about two-thirds with silica gel slurry. The stop cock was opened to allow liquid to drain into the beaker. The side of the chromatographic tube was gently tapped with a cork ring during the packing process, to make the silica gel compact without air bubbles. Meanwhile the stop cock was opened to allow the excess of eluting solvent to run out. The bottom outlet of the separation column was closed.

Procedure:

Using a volumetric pipet the methanol extract of cyanobacteria was added directly. Then the mobile phase (7:3 Petroleum ether and Acetone) was drained continuously. The bottom outlet of the column was opened. The eluent flows down through the column. The column, with the adsorbent and the sample, was developed. As the eluent passes down the column, the components of the mixture begin to move down the column. The separated zones flow out of the column, where the eluates are collected in test tube. The golden yellow colour is collected at the bottom which is carotenoid.

Results

Growth of *Phormidium* sp.

The dry weight was found to increase constantly with increase in the number of days. The dry weight was found to be lowest in day zero (50.1 µg/ml) and highest (521.2 µg/ml) in the tenth day. The growth curve was in the lag phase during the first two days after that it enters into the exponential phase which was up to sixth day later it was in the stationary phase.

Chlorophyll a content of *Phormidium* sp.

The chlorophyll a content was found to increase drastically from the day zero after eighth day the growth was found to be stationary. There was a constant increase from the day zero to the eighth day after that there was decrease in the concentration. The minimum value was (0.09 µg/ml) at day zero and the maximum value was (12.40 µg/ml) at day ten.

Carotenoid content of *Phormidium* sp.

The highest value was (76.2 µg/ml) at day ten and the lowest was (4.3 µg/ml) at day zero. Until day four the carotenoid content was increasing slowly (20.1 µg/ml) after fourth day it was found to increase drastically which was the expotential growth (50.1 µg/ml) and later that the carotenoid content was found to maintained stationarily.

Phycobiln content of *Phormidium* sp.

The Phycobilin content was at the lowest (3.2 µg/ml) in day zero and highest (68.1 µg/ml) in day ten. The phycobilin content was started increasing from the day zero and it was very high after day four which was in the expotnential phase of growth after that the growth rate started decreasing from the day eight which leeds to the stationary phase of growth.

Figure 1: Culture of *Phormidium* sp., maintained in the culture room

A) Culture in a conical flask

B) Biomass in solution

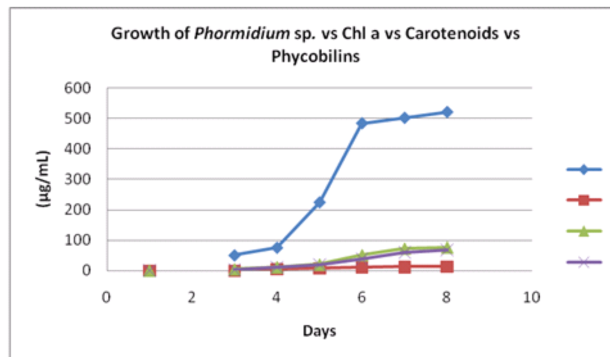
Figure 2. Mass cultivation of *Phormidium* sp. for extraction of carotenoids and metabolites

Figure 3. Separation of carotenoid from methanol extract

A) Separated carotenoid B) Column chromatography

Table 2: Growth of *Phormidium* sp. vs Chl a vs Carotenoids vs Phycobilins

Days	Dry weight µg/mL	Chl a (µg/mL)	Carotenoids (µg/mL)	Phycobilins (µg/mL)
0	50.1	0.09	4.3	3.2
2	75.3	4.21	11.2	10.5
4	224.5	8.14	20.1	19.2
6	484.4	10.22	50.6	38.4
8	502.1	12.25	72.3	59.4
10	521.2	12.40	76.2	68.1

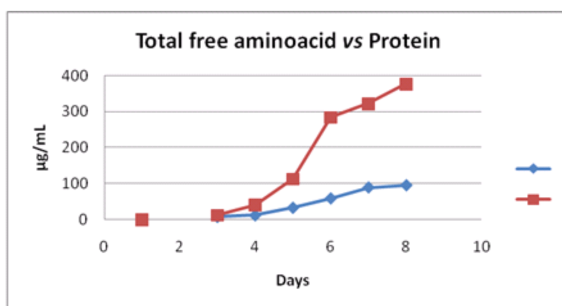


Total free aminoacid content of *Phormidium* sp.
Total free aminoacid was found to be (8.1 µg/ml) at day zero which was the lowest and (95.6 µg/ml) at day ten which was the highest value. The curve shows that the total free Aminoacid content was found to increase slowly until the day two (12.1 µg/ml) after that it was found to increase drasyically (33.5 µg/ml) from the next day and was incresing expotentially until day eight (89.5 µg/ml) then the growth rate was found to be decreased and was maintained in stationary phase.

Total protein content of *Phormidium* sp.
The lowest value was (13.5 µg/ml) at day zero and the highest value was (377.2 µg/ml) at day ten. The drastic growth was achieved from the day four (113.8 µg/ml) which was under the exponential increase in the total protein content then there was a little lag, after that the growth was found to be little decreased and decrease in the total protein-content.

Table 3: Total free aminoacid & Protein content of *Phormidium* sp. during the growth period

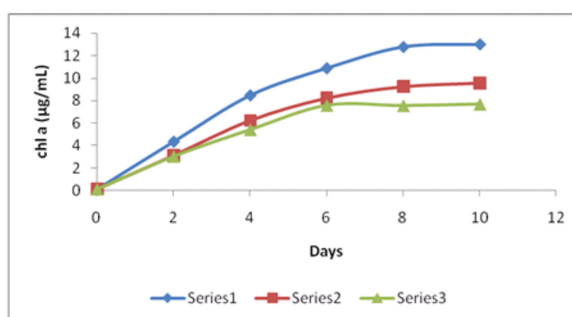
Days	Amino acids (µg/mL)	protein (µg/mL)
0	8.1	13.5
2	12.6	41.9
4	33.5	113.8
6	59.4	284.5
8	89.5	322.1
10	95.6	377.2



Influence of N content of the medium on chlorophyll a
In this two modified medium BG11-A and BG11-B were used which was half strength and quarter strength respectively and the regular medium was used as a control. The lowest value was (0.12 µg/ml) for BG11-A and (0.11 µg/ml) for BG11-B and the highest was (9.58 µg/ml) BG11-A and (7.69 µg/ml) BG11-B while the control was (12.98) which shows clearly that the less Nitrogen content in the medium has negative impact on the chlorophyll a content. The chlorophyll a content was found to be decreased in the half strength medium and still decreased in the quarter strength medium.

Table 4: Influence of N content (in terms of $NaNO_3$ mg/L) of the medium on chl a in *Phormidium sp.*

Days	chl a (µg/mL)		
	Control BG11	Modified BG11-A	Modified BG11-B
0	0.11	0.12	0.11
2	4.34	3.14	3.04
4	8.45	6.25	5.42
6	10.86	8.24	7.58
8	12.75	9.26	7.56
10	12.98	9.58	7.69

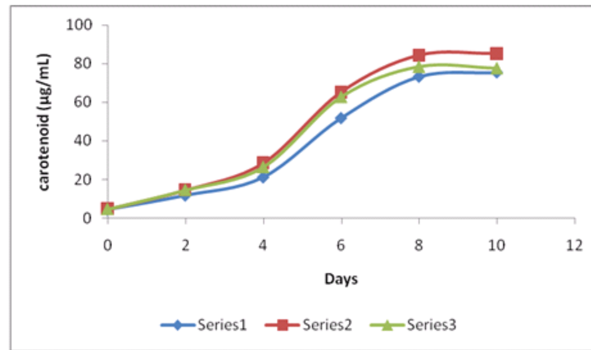


Influence of N content of the medium on carotenoid

The carotenoid content in the modified mediums has the lowest value of (4.6 µg/ml) in both BG11-A and BG11-B and the highest value was (85.2 µg/ml) and (77.6 µg/ml) in BG11-A and BG11-B respectively while the control was (75.4 µg/ml). These values shows that the decreased nitrogen concentration in the modified mediums increased the carotenoid content which is highest in the half strength and second highest in the quarter strength. This shows clearly that decreased nitrogen concentration showed positive impact on the carotenoid content of the modified mediums.

Table 5: Influence of N content of the medium on carotenoid in *Phormidium sp.*

Days	**carotenoid (µg/mL)**		
	Control BG11	**Modified BG11-A**	**Modified BG11-B**
0	4.5	4.6	4.6
2	11.8	14.5	14.5
4	21.2	28.5	26.4
6	51.8	65.2	62.7
8	73.2	84.3	78.4
10	75.4	85.2	77.6

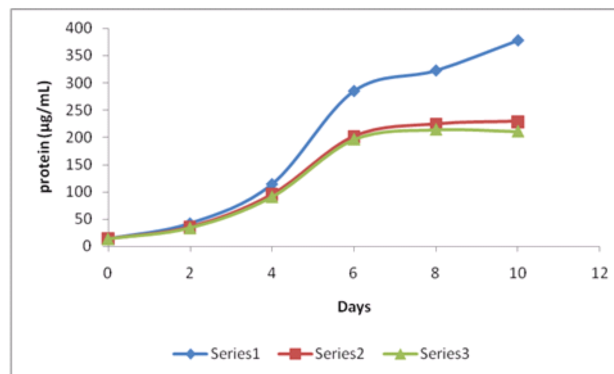


Influence of N content of the medium on protein

The lowest value was found to be (13.5 µg/ml) in BG11-A and (13.6 µg/ml) in BG11-B at day zero and the highest value was (228.9 µg/ml) in BG11-A and (211.0 µg/ml) in BG11-B while the control was (377.2 µg/ml). It shows that the modified medium both half and quarter strength has less protein content due to the decrease in the nitrogen content of the medium which can be compared with the control. The decreased nitrogen concentration of the two modified mediums shows negative impact on the protein content.

Table 6: Influence of N content of the medium on protein in *Phormidium sp.*

Days	protein (µg/mL)		
	Control BG11	Modified BG11-A	Modified BG11-B
0	13.5	13.5	13.6
2	41.9	35.4	33.7
4	113.8	95.2	90.8
6	284.5	201.4	196.2
8	322.1	224.1	214.3
10	377.2	228.9	211.0



Discussion

Phormidium, Filamentous unbranched, non nitrogen fixing, microscopic and later macroscopic up to several cm in diameter, usually covering substrates of different types (Komárek, 1992). *Phormidium* was cultured in BG11 medium and its growth was analyzed by determining its dry weight for ten days at 2 days intervals. The dry weight was lowest in day zero and highest on the tenth day.

It was found that the *Phormidium tenue* exhibits optimal growth in the presence of B, Mn, I and Fe in a medium having a pH 8-2.It responds to treatment with trace elements and exhibits normal growth until the salinity of the medium reaches 8-5 per cent, beyond which range there is a definite fall in the growth rate. It absorbs nitrogen from the media; and no evidence is obtained to show that it fixes atmospheric nitrogen when sufficient quantity of nitrogen is available in the media. The maximum limit of salinity of the outside water that *Phormidium* can tolerate appears to be about 8 - 5 per cent. Within this range growth is normal and but beyond this salinity there is very little growth. (Pillai, 1954)

Phormidium sp. strain C86 changes the phycobilisome type depending on light quality. It is the first organism examined that exhibits a new type of complementary chromatic adaptation by altering the composition of the phycobilisome core and the number and composition of peripheral rods and by changing the ratio of photosystem II to phycobilisomes. (Martin and Werner, 1995). The highest value of phycobilins was found to (68.1 µg/ml) at day ten.

Carotenoids are responsible for many of the red, orange and yellow hues of plant leaves, fruits, and flowers as well as the colors of some birds, insects, fish, and crustaceans. Modification of normal composition of BG-11 culture medium, by altering nitrogen and carbon sources resulted in 25-38% increase in carotenoid content in *Nostoc* sp, *Anabaena* sp and *Phormidium* sp. The carotenoid content showed positive increase by lowering the nitrogen concentration in which the modified BG11-A medium showed the highest value (85.2 µg/ml) in half strength.

In Synechocystis sp., 15 and 30 days old cultures, the amount of Chlorophyll 'a' decreased at all irradiation doses. The concentration of chlorophyll 'a' and Carotenoid increased as the growth period increased (Trimurtula *et al*, 1994). Carotenoid is found to increase in less nitrogen content. It is concluded that enhanced carotenoid synthesis in stressed conditions and it increases two ecological functions, providing photoprotection and increasing photosynthetic performance of surface cyanobacterial populations (Pearl, 1984).

Protein content was lower on the first day (13.5µ/ml) and maximum at the end of tenth day (377.2µg/ml). Several benthic cyanobacteria were found to produce significant amounts of extracellular flocculants. The *Phormidium* flocculant is a sulfated heteropolysaccharide to which fatty acids and protein are bound. The polysaccharide backbone is composed of uronic acids, rhamnose, mannose, and galactose. (Bar and Shilo, 1987)

Sastry and Chaudhary (1990) reported some selected biological variables, such as pigment absorbance, spectral characteristics, protein and carbohydrate levels and ratio of nucleic acid which affect the overall metabolic activities and influence the growth of the algae. Rai and Dubey (1989) study the effect of metals on proteins constituents in *Anabaena doliolum*.

Influence of Nitrogen content on the growth of *Phormidium* sp was determined by using two modified mediums BG11 A and BG11 B each with different concentrations of Nitrogen. All the parameters were determined and compared with that of control. Chlorophyll a content of the control was the highest (12.98µg/ml) than the modified mediums BG11-A and BG11-B which are found to be 9.58µg/ml and 7.69µg/ml respectively. Carotenoid content of *Phormidium* cultured in BG11-A was the highest (85.2µg/ml) than BG11-B (77.6µg/ml) at the tenth day. Carotenoid content was low (75.4 µg/ml) in control culture. Hence carotenoid content was found increased in nitrogen modified BG11-A and BG11-B mediums. Influence of nitrogen content on total protein of *Phormidium* was found to decrease comparatively with control; protein content was high (377.2µg/ml) in control at tenth day. Protein content of *Phormidium* on BG11-A was high (228.9µg/ml) compared to (211.0µg/ml) in BG11-B. Thus protein content was found to decrease with decrease in nitrogen content.

Cyanobacteria have been known to be an enormous resource for compounds with varying bioactivities including antimicrobial, antiviral and enzyme inhibitory effects. Several compounds such as cyanopeptolins, micropeptins, oscillapeptins, nostopeptins, agardhipeptins, anabaenopeptins, nodulapeptins, microviridins and aeruginosins are protease inhibitors. They are widely distributed in such species of cyanobacteria as *Microcystis*, *Oscillatoria*, *Nostoc*, *Nodularia* and *Anabaena* and most compounds are cyclic depsipeptides containing the 3-amino-6-hydroxy-2-piperidone (Ahp) unit. (Zainnudin. *et.al*, 2007)

Summary

Phormidium sp. was selected for the experiment and grown in BG11 medium containing different concentration (0, 1, 2, 3, 4 µg/ml) after the growth period the cells were collected and used to estimate the amount of chlorophyll 'a', carotenoids, phycocyanin, free amino acids and proteins in different concentration. An increase in growth period days (0, 2, 4, 6, 8, 10) increase the cellular content namely chlorophyll 'a', carotenoids, phycocynin, free amino acids and proteins.

Nitrates in the form of NaNO3 were essential component of the high growth yielding medium that is BG11 and this double source of Nitrate may be attributed to the high yield of algae (Singh and Srivastava, 1968).

The experimental conditions are very simple and operational cost is low. The handling of biomaterial is very easy and harmless. *Phormidium* sp. was cultured and shaken with glass beads to get a uniform cell suspension and growth was measured in terms of fresh weight.

Blue green algae are renewable bio-fertilizers and they represent a self supporting biological agent because they can photosynthetically provide energy for nitrogen fixation. Producing mass culture of *Phormidium* sp. is based upon the use of local resources and simple techniques.

The carotenoids present in the extract can be separated using the column chromatography method. Carotenoid synthesis in stressed conditions and it increases two ecological functions, providing photoprotection and increasing photosynthetic performance of surface cyanobacterial populations. (Pearl, 1984)

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