

Isolation, optimization and characterization of selected Cyanophycean members

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Ten spp. of Cyanobacteria (non-heterocytous) were isolated from various marine habitats. Three bioactive potential forms, were observed for their optimization of growth, subjecting them to various concentrations of NaCl, pH, light and nutrients. Proteins, lipids, and carbohydrates were also evaluated from some of the forms. Exponential growth rates of (0.062 and 0.01 g day⁻¹) were exhibited by *Phormidium tenue* and *S. cedrorum*, respectively, in the first two weeks. Wide range (0-100%) of salinity tolerance was noticed in *Phormidium tenue*, *S. cedrorum* and *S. pevalekii*. Optimum growth and maximum biomass were obtained in media with NaCl concentrations of 5-25%. All three species exhibited maximum growth in media with pH 7.5, and responded well with increasing concentrations of NO₃-N. The quantum of nitrate required for maximum growth varied with species. *Synechococcus cedrorum* produced higher biomass in the artificial light, compared to the same in direct sunlight, and diffused light. *Spirulina major* showed maximum (66.72%) proteins, while *Oscillatoria* sp. was rich (28.82%) in carbohydrate contents. *Phormidium* showed relatively higher (11.3%) lipid contents.

[**Keywords:** Cyanobacteria, Isolation, Optimization, Characterization, Marine habitats]

Introduction

Constantly increasing interest in biotech and bioenergy potentials, necessities exploration, identification, isolation and culture of marine blue green algae (BGA). Although they exist with diverse morphology, in a wide variety of habitats, studies have been limited to a relatively few of their representatives, mainly due to the difficulties encountered in their isolation, and the subsequent purification. The techniques normally used to isolate the algal species, which could readily be cultured¹. Various approaches have been adopted to develop efficient methods to isolate culture and purify fresh water BGA²⁻⁷. Efforts also have been reported on isolation and culture of marine BGA⁸⁻⁹. Formulation of a suitable culture medium is prerequisite in achieving high production, as standard media have been observed to yield low biomass of algae. Several attempts have been made to establish the nutritional requirements of BGA and to optimize media formulation^{7,9,10}.

Culture experiments have been successful where ecophysiological requirements of particular species are known. A comprehensive study for optimization of the culture conditions was carried out in order to maximize growth of marine BGA, with the objective to determine the effects of different ecological factors

on the growth of selected species. Data generated during present investigations could be useful in the understanding of a commercial/biotech potential BGA.

Materials and Methods

Isolation

Samples collected from intertidal rocky and swampy regions, along the open shore, estuarine regions, and salt pans were examined under the light microscope to evaluate relative abundance of BGA. Samples with very low counts and mixed forms were concentrated by centrifugation, while those with high counts were diluted with suitable medium. Enrichment and isolations were carried out using culture media (ASN-III N⁺, ASN-III N⁻) till unialgal forms of various species were obtained^{1,11}.

Isolation techniques included simple enrichment and direct manual isolation. In simple enrichment method, the inoculum was prepared by mixing the samples with the selected medium and then serial dilutions were made in test tubes containing similar media. Direct isolations were done by picking up single filament or single cell using sterile Pasteur pipettes, which were pulled out to a very thin capillary, using a dissecting microscope. In some cases series of dilutions were made in sterile medium

using homogenized cell suspension of natural sample of BGA. Irrespective of the procedure, during isolation and enrichment, all the media contained $50 \mu\text{g ml}^{-1}$ cycloheximide (Hi-Media) for inhibiting growth of eukaryotes¹²⁻¹³ like green algae, diatoms or even fungi. As soon as unialgal cultures were obtained, cycloheximide was removed from the media. Unialgal stocks were maintained in 50 ml of appropriate liquid medium in 100 ml Erlenmeyer flasks, as well as on agar slants at room temperature ($29^\circ\text{C} \pm 1^\circ\text{C}$), and at low light intensity ($10\text{-}15 \mu\text{E m}^{-2} \text{s}^{-1}$).

Axenic cultures of stock algae were obtained by treating a cell suspension with UV irradiation or standard antibiotics (Streptomycin, Ampicillin and Chloramphenicol). The antibiotics treated cell suspension was diluted in sterile medium and streaked on agar plates. The purity of cultures were checked by streaking individual species (from the culture) on medium supplemented with 0.2% (w/v) glucose, as well as on nutrient agar¹¹. The absence of heterotrophic forms, after two weeks of incubation in the dark at room temperature, was considered as evidence for a pure culture. Purity was then routinely checked by phase contrast microscope.

Isolated strains of BGA were grown in ASN III media for checking growth. Their major biochemical contents (protein, carbohydrates and lipids) were carried out using standard methods^{14,15 & 16}. *Synechocystis pevalekii*, *Synechococcus cedrorum* and *Phormidium tenue* were selected for optimization of biomass studies.

Optimization of culture media for salinity, pH and nutrients

Salinity concentrations (0 to 200 ‰) of ASN III media (100 ml) were made. For studying pH optimization, culture media with different levels of pH (2.5 to 11.5) were used. All the other components of media and environmental conditions were kept constant. Nitrate-N requirement of selected BGA were tested at concentrations in the range of 0.1225 to 3.6 gm l^{-1} of NaNO_3 . Nitrate:Phosphate ratio of 20:1 was maintained by increasing K_2HPO_4 proportionally to NaNO_3 . For optimizing Phosphate-P, media were prepared with varying concentrations (0.005 to 0.16 gm l^{-1}) of K_2HPO_4 .

Exposure to light

Synechococcus cedrorum exposed to direct sunlight ($800\text{-}1020 \mu\text{E m}^{-2} \text{s}^{-1}$), diffused light ($50\text{-}120 \mu\text{E m}^{-2} \text{s}^{-1}$), artificial light ($25\text{-}35 \mu\text{E m}^{-2} \text{s}^{-1}$),

and to dark conditions. All the above experimental culture media concentrations were made in 100 ml ASN III. Initial inoculum of 2% from the stock cultures were introduced in all the experiments, and cultures were incubated at room temperature ($29 \pm 1^\circ\text{C}$), under white fluorescent lights ($25\text{-}35 \mu\text{E m}^{-2} \text{s}^{-1}$) except in cultures subjected to light effect studies. Media without cultures of BGA were also kept as a common control. Growth was measured by estimating dry weight (dw) biomass and chlorophyll *a* contents¹⁷.

Results

Isolation of BGA

Total eight forms of BGA namely *S. pevalekii*, *S. cedrorum*, *Phormidium tenue*, *Lyngbya* sp., *Aphanothece pallida*, *Oscillatoria* sp., *Spirulina major*, and Unidentified species were isolated. Different media supported growth of different strains of BGA in varying quantum; however, better growth and more number of forms were exhibited in ASN III N⁺. Typical sigmoid growth curve was observed in *Phormidium*. *Phormidium tenue* and *Synechococcus* showed exponential growth rates of 0.062 and $0.01 \text{ g (dw) day}^{-1}$, respectively, in the first two weeks (Fig. 1). The growth declined thereafter; however, the biomass steadily increased reaching maximum $2.48 \text{ gm (dw) l}^{-1}$ of *P. tenue* and $1.903 \text{ gm (dw) l}^{-1}$ of *S. cedrorum* at the end of 28th day. *Synechocystis* showed gradual increase with average growth rate $0.04 \text{ mg Chl } a \text{ day}^{-1}$, and produced maximum biomass (chl *a*) 2.62 mg l^{-1} , on 21st day. The growth rate in the different strains varied from 0.01 to $0.1179 \text{ g (dw) day}^{-1} \text{ l}^{-1}$, with minimum growth rate of $0.0507 \text{ g (dw) day}^{-1} \text{ l}^{-1}$ in *A. pallida*. In general, the optimum growths of various strains occurred during the period of 15-30 days (Fig. 1).

Biochemical constituents

The maximum proteins were found in *S. major* (66.72%) followed by *Oscillatoria* sp. (50.96%), *P. tenue* (46.56%) and *A. pallida* (31.16%) (Table 1). Carbohydrate contents were observed to be maximum (23.82%) in *Oscillatoria* sp., followed by *P. tenue* (19.42%), and *S. major* (15.56%). *Oscillatoria* sp. (11.3%) and *P. tenue* (10.95%) showed relatively higher lipid contents.

Salinity and pH optimization

Phormidium tenue, *S. cedrorum* and *S. pevalekii* exhibited wide range (0-100 ‰) of salinity tolerance,

and maximum biomass in *Synechococcus* (1.52 gm l^{-1}) and *Phormidium* (1.6 gm l^{-1}) occurred in salinity concentration of 25‰, while that of *Synechocystis* (0.0049 gm l^{-1}) in 15 ‰ (Fig. 2), and observed to be significantly ($r = 0.83$) correlated with salinity concentrations. Growth increased up to 15‰ and then decreased with increasing concentration of salinity.

The growth also exhibited correlation with increasing pH between 6.5 to 7.5, however, they could tolerate pH up to 9.5. The optimum growth was observed in media with pH 7.5 (Fig. 3). Very low pH 2.5 and pH >10.5 were found to inhibit the growth of algae (Fig. 3).

Nutrient optimization

Species reported to grew well in the media enriched with nitrate and phosphate (Figs. 4 & 5). Maximum growth of *Synechocystis* and *Synechococcus* occurred in media with 0.9 gm l^{-1} , while *Phormidium* showed

Table 1—Biochemical characteristics of BGA

Species	Protein Contents (%)	Carbohydrate Contents (%)	Lipid Contents (%)
<i>Aphanothece pallida</i>	31.16	2.28	6
<i>Lyngbya sp.</i>	21.16	7.10	7.5
Unidentified sp	16.55	4.62	3.5
<i>Synechocystis pevalekii</i>	17.6	6.85	3
<i>Spirulina major</i>	66.72	15.65	9.8
<i>Phormidium tenue</i>	46.56	19.42	10.95
<i>Synechococcus cedrorum</i>	45.9	12.8	1.2
<i>Oscillatoria sp</i>	50.96	23.82	11.3

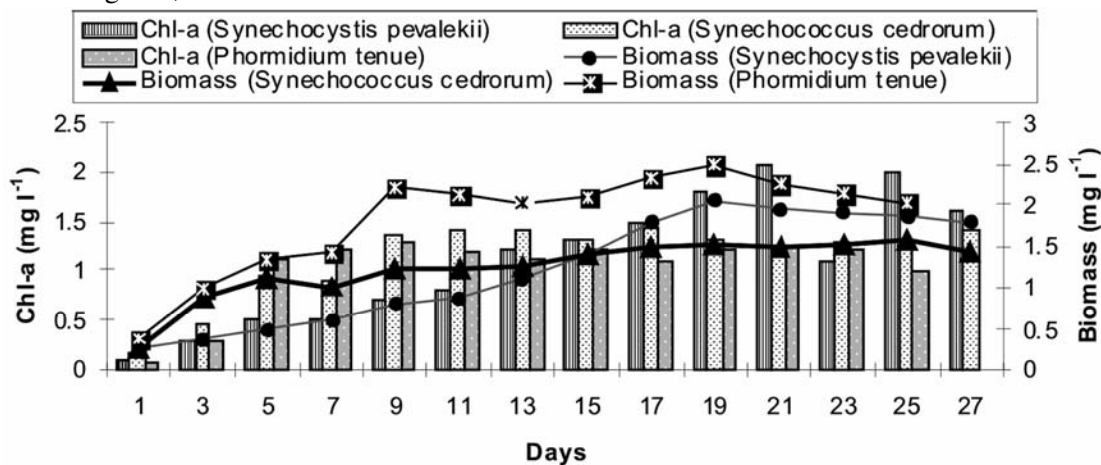


Fig. 1—Growth of BGA in enriched media.

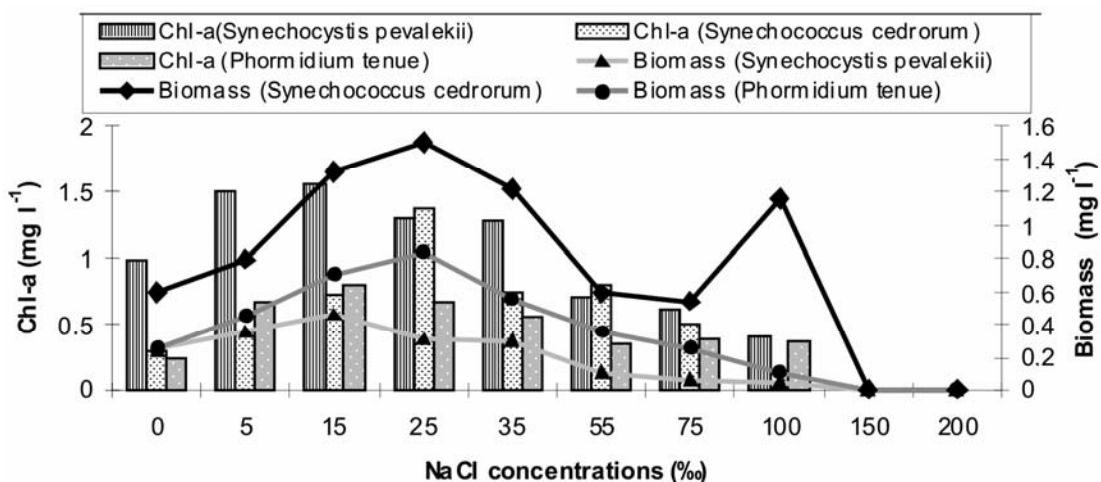


Fig. 2—Effect of different concentrations of Salinity on the growth of BGA.

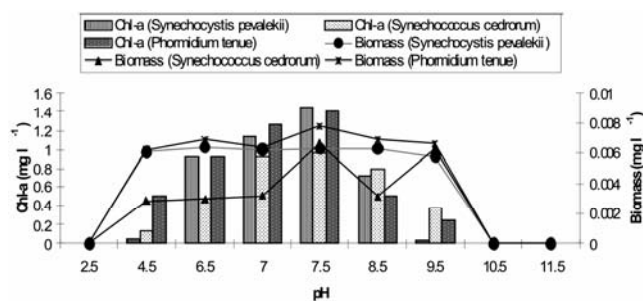


Fig. 3— Effect of different pH on the growth of BGA.

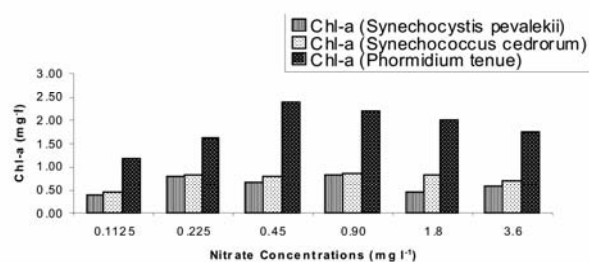


Fig. 4—Effect of different concentrations of nitrate on growth of BGA.

maximum growth in media with 0.45 gm l^{-1} of Nitrate. Maximum biomass in *Synechocystis* and *Synechococcus* was expressed with 0.02 gm l^{-1} concentration of phosphate in the media while *Phormidium* required relatively low (0.01 gm l^{-1}) concentration of $\text{PO}_4\text{-P}$ for it's maximum yield (Fig. 5).

Effect of light

Synechococcus cedrorum preferred low light intensity ($25\text{-}35 \mu\text{E m}^{-2} \text{ s}^{-1}$) for its growth (Fig. 6). In artificial light it produced maximum Chl *a* ($254.32 \mu\text{g l}^{-1}$) compared to the same in the diffused light ($247.9 \mu\text{g l}^{-1}$), and direct sunlight ($67.94 \mu\text{g l}^{-1}$). However, in total dark conditions it continued to survive with low ($23.7 \mu\text{g l}^{-1}$) concentration of Chl *a*.

Discussion

Primary importance for the successful cultivation of algae, and particularly BGA is the careful elimination of living contaminants from desired algae to be inoculated. Biomass production and optimum period of growth greatly varies from species to species, which could mainly be attributed to the quantum and type of nutrient availability in the ambience^{18,19}, and preference of individual species

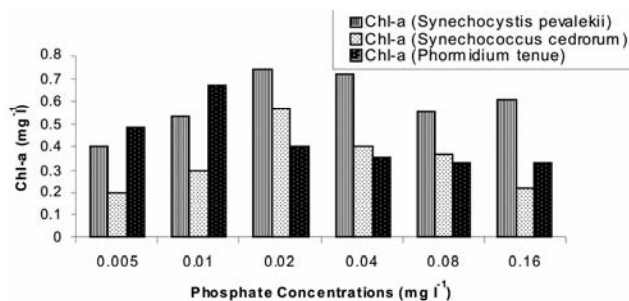
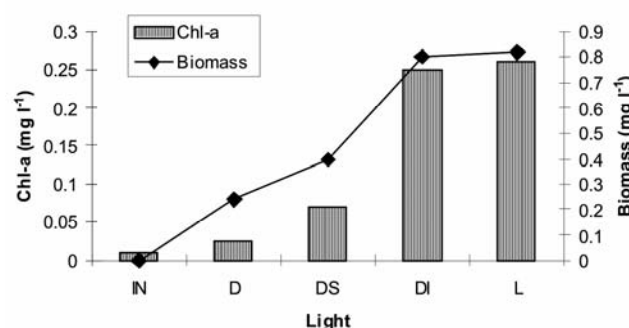


Fig. 5—Effect of different concentrations of phosphate on growth of BGA.

Fig. 6—Effect of different types of light on the growth *Synechococcus cedrorum*. IN-Initial; D-Dark; DSL-Direct sunlight; DL-Diffused light; L-Artificial Light

for the particular nutrient²⁰. In general, growth rate in the present BGA were much lower than reported for other algal species^{21,22}. Blue-green algae exhibited relatively better growth in ASN III N^+ compared to the same in other media.

Spirulina contains ~ 50-70% protein, and has been reported of having outstanding nutritional profile^{23,24}. Protein contents in *S. major* was found to be higher (~ 60%). Carbohydrates, known as an energy-yielding nutrient of organisms. In *Anabaena*, dry matter contained 14.6 to 34.15% carbohydrates¹²⁵. However, the carbohydrate contents in BGA were observed to be relatively lower (0.8% to 7.1% dw). Similarly BGA were reported to be poor in lipids. *Spirulina* contained only 6-13% lipids, half of which were fatty acids^{24,26 & 27}. Present results were also revealed low concentrations of lipids in BGA.

Salinity affects living organisms by decreasing the water potential, resulting in loss of cellular water and simultaneous influx of ions into the cells²⁸. Organisms posses ability for salinity tolerance but the range varies with different form. A number of BGA, grow in a wide range of salinity^{29,30,31}, and hence their halotolerance and mechanism of adaptation are of

much interest. Present data also indicated potentials of these algae in acclimatizing to the varying salinity concentrations. During adaptation to salt stress, balanced water potential is achieved by the accumulation of osmoprotective compounds while inorganic ions are extruded from the cell^{32,33,34}. Heteroside glucosylglycerol [2-O-(α -D-glucopyranosyl) glycerol] (GG) was reported as the first osmoprotective substance in the marine strain *Synechocystis* sp.³³. Photosynthetic apparatus has been considered to be the most efficient system that adapts to salinity³⁵ and once the lost pigments are synthesized the algae slowly and gradually regains its other metabolic activities. Salinity tolerance up to 100‰ in present BGA, indicative of their moderately halotolerant nature which accumulate glucosyl-glycerol (GG) as osmoprotective substance³³. Number of BGA studied were found to be versatile in nature by having wide range of salinity (15-35‰) tolerance. *Phormidium* species found to tolerate wide range (0-100‰) of salinity^{36,37}. Wide range of salinity tolerance indicates that salinity variations in the ambience are of minor importance for the ecology of studied BGA⁸.

Cyanobacteria during present investigations grew well in pH range 6.5 to 8.5 with variation in their degree of tolerance. They could also survive in pH < 4.5. Though, BGA showed tolerance³⁸ to wide pH range, they generally reported to grow well in pH range of 7.5 to 9^{39,40}. Different algae have different pH optima. The pH determines the solubility of carbon dioxide and minerals in the ambience influencing the metabolism of algae⁴¹. Species such as *Phormidium valderianum* from Indian waters does not grow⁴² in pH < 5. However, the present, algal forms expressed their tolerance to pH of 4.5, though the growth was suppressed compared to same in media with pH between 6.5 to 8.5 (Fig. 3). Moderate growth of *Phormidium* and *Synechococcus* in culture media with pH value of 4.5, are suggestive of their greater adaptability to the acidic ambience. Many extremophiles have evolved to grow best at extremes of pH. Cyanobacteria have been found in acid lakes (pH 4.1-5) and dominate in ambience with low pH⁴³.

Growth of algae, in general, depends upon the availability of nitrogen and/or phosphate^{44,45,46}. The increased nutrients resulted in better growth and higher biomass of BGA⁴⁷. Phosphorus was reported to be essential element for pigment development^{48,49}. Algae are known to assimilate phosphorus in excess of their requirements⁵⁰.

The poor growth of *S. cedrorum* in direct sunlight compared to the same in diffused and artificial lights could be attributed to its acclimatization to the low light intensity. Though there was no increase in biomass, BGA continued to survive in the dark. The ability to survive during a long period in darkness depends on endogenous respiration of stored carbohydrates or is linked to the assimilation of exogenous carbon by heterotrophic mechanism. The BGA strains studied showed increased productivity at higher concentrations of nutrients, and preferred low intensity of light. Present studies may be of a great value in culture and purification of marine BGA of bioenergy and biotech potentials.

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