EVALUATION OF THREE SPECIES OF CYANOBACTERIA FOR PRODUCTION OF PHYCOBILIPROTEINS

Thesis submitted in partial fulfillment

of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

ENVIRONMENTAL BIOTECHNOLOGY

Under

The Faculty of Environmental Studies

Cochin University of Science and Technology

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February 2017

Certificate

This is to certify that this thesis entitled ***"Evaluation of three species of cyanobacteria for production of phycobiliproteins"*** *is a bonafide record of research carried out by Ms.Divya Sivaji under my guidance and supervision in partial fulfillment of the requirements for the degree of Doctor of Philosophy under the Faculty of Environmental Studies, Cochin University of Science and Technology and that no part thereof has been included for the award of any other degree. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate has been incorporated in the thesis.*

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Declaration

I hereby declare that the thesis entitled ***"Evaluation of three species of cyanobacteria for production of phycobiliproteins"*** *is a authentic record of the research work carried out by me under the guidance of Dr. Ammini Joseph, Professor, School of Environmental Studies, Cochin University of Science and Technology in partial fulfillment of the requirements for the award of Doctor of Philosophy under the Faculty of Environmental Studies, Cochin University of Science and Technology and no part of this thesis has been submitted for the award of any other degree, diploma, associateship, or other title or recognition from any University/ Institution.*

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February 2017

Acknowledgement

This thesis work would not have been possible without the help and support from the various people I got the opportunity to be associated with. On completing this thesis successfully, I would like to express my heartfelt thanks to all those people.

Firstly I would like to thank and express my deep gratitude to my supervising guide Dr. Ammini Joseph, Professor, School of Environmental Studies for guiding me throughout my thesis work, for encouraging me at each phase of my research work and offering her continuous support throughout. Her expertise and sincerity inspired me to complete my research work successfully.

I would like to thank Dr. S. Rajathy, Director, School of Environmental Studies, Cochin University of Science and Technology, for providing me the necessary facilities for completing my research work. My sincere and heartfelt thanks to all the Faculty members, Office staff and Technical staff of SES for all their help and encouragement throughout the period of my research work.

I express my sincere thanks to my labmates Dhanya, Lakshmi, Rakesh and Smitha for their constant support, encouragement, valuable suggestions and discussions. I am very fortunate to have them as my colleagues as well as friends.

I would like to thank my parents (Mrs. Asha Sivaji and Mr. V. Sivaji), sister (Ms. Drishya Sivaji), grandfather (Late Mr. M.G. Dayanandan), grandmother (Mrs. Rajamma Dayanandan) and uncle (Mr .Anoop. D) for encouraging and supporting me throughout my student life as well as research life. I don't have enough words to express my deepest gratitude to them. I

would also like to especially thank my husband Mr. Dharmendra Kumar Singh and my son Mr. Dhruv Dharmendra Singh for offering me their unfailing moral support, patience, co-operation and encouragement for completing my research work successfully. So I take this opportunity to dedicate this research work to my family members.

Divya Sivaji

Contents

1. Introduction -- 1
1.1 Applications of Cyanobacteria -- 2
1.1.1 Cyanobacteria as Food -- 2
1.1.2 Cyanobacteria as Biofertiliser --- 3
1.1.3 Source of Pigments --- 5
1.1.4 Bioactive Compounds from Cyanobacteria ---------------------------------- 6
1.1.5 Exopolysaccharides from Cyanobacteria ---------------------------------- 10
1.1.6 Biopolyesters from Cyanobacteria --------------------------------------- 11
1.1.7 Cyanobacteria for Environmental Bioremediation ------------------------ 12
1.1.8 Biofuels --- 13
1.2 Cultivation Systems --- 14
1.2.1 Open Ponds --- 15
1.2.2 Photobiorectors -- 16
1.3 Commercial Products from Microalgae/Cyanobacteria ------------------------ 19
1.4 Scope of the Present Study -- 23
1.5 Objectives of the Present Study --- 24
2. Development of Cyanobacterial Cultures ------------------------------------ 27
2.1 Introduction -- 27
2.2 Methods of Isolation -- 31
2.3 Purification of Cultures -- 33
2.4 Outcome of Isolation -- 34
2.5 Maintenance of Cultures --- 37
3. Production of Phycobilin Pigments by Phormidium tenue, Oscillatoria acuminata and Synechococcus elongatus ------------------ 39
3.1 Introduction -- 39
3.2 Materials and Methods --- 44
3.2.1 Evaluation of Growth --- 44
3.2.2 Evaluation of Phycobiliproteins --------------------------------------- 45

3.2.3 Evaluation of Media and Incubation Conditions -------------------- 46

3.3 Experimental Assessments --47

3.3.1 Evaluation of Culture Media--- 47

3.3.2 Effect of Salinity on Pigment Production ------------------------------ 54

3.3.3 Effect of pH on Pigment Production ------------------------------------ 59

3.3.4 Effect of Light Intensity on Pigment Production --------------------- 63

3.3.5 Effect of Light Quality on Pigment Production ----------------------- 69

3.3.6 Effect of UV Exposure on Pigment Production ---------------------- 78

3.3.7 Effect of Temperature on Pigment Production ------------------------ 83

3.3.8 Combined Effect of Light and Temperature on Pigment Production ----- 90

3.3.9 Purity and Yield of Phycocyanin, Allophycocyanin and Phycoerythrin ---- 102

3.4 Discussion---105

4. Aquatic Pollutants and Pigment Production by Synechococcus elongatus --- 109

4.1 Introduction --- 109

4.2 Materials and Methods -- 112

4.2.1 Effect of Heavy Metals on Pigment Production ---------------------112

4.2.2 Effect of Pesticides on Pigment Production --------------------------113

4.3 Results --- 114

4.3.1 Effect of Heavy Metal Contamination -------------------------------114

4.3.2 Effect of Pesticide Contamination -------------------------------------119

4.4 Discussion --- 126

5. Summary and Conclusion--- 129

References --- 137

Appendix --- 165

Publication -- 231

Chapter 1

INTRODUCTION

Contents

1.1 Applications of Cyanobacteria
1.2 Cultivation Systems
1.2 Commercial Products from Microalgae/Cyanobacteria
1.3 Scope of the Present Study
1.4 Objectives of the Present Study

Cyanobacteria (otherwise known as blue-green algae) are gram-negative bacteria which are one of the most widely distributed prokaryotes. They can be found growing in diverse ecological regions exposed to varying conditions of environment. They exhibit a variety of morphological forms: (1) Unicellular cyanobacteria - either found to be free-living or are enclosed inside a mucilaginous membrane (2) Cyanobacteria in the form of a row of cells, known as a trichome and when a trichome is surrounded by a sheath, it is called a filament (3) More than one trichome within a filament and the filament can be unbranched or branched. A branched filament can be composed of a single row of cells or multiple rows of cells. Over the past many years, the variations in the morphological characteristics have been used to classify cyanobacteria. Some of these morphological features are unicellular forms, filamentous forms, colonial forms, branched or unbranched filaments, false or true branching of filaments, presence or absence of heterocysts, presence of exospores or endospores etc. According to Lee (2008) cyanobacteria are generally divided into three orders : (1) Order 1 : Chroococcales - single cells or cells which are loosely bound to form gelatinous irregular colonies, (2) Order 2 : Oscillatoriales – filamentous cyanobacteria, and (3) Order 3 : Nostocales – filaments cyanobacteria having

heterocysts. www.algaebase.org classified cyanobacteria into seven orders : (1) Order : Chroococcales (2) Order : Cyanophyceae ordo incertaesedis (3) Order: Gloeobacterales (4) Order : Nostocales (5) Order : Oscillatoriales (6) Order : Stigonematales (5) Order: Oscillatoriales (6) Order: Stigonematales (7) Order : Synechococcales. According to the classification followed by Desikachary (1959) there are five orders : (1) Order : Chroococcales (2) Order : Chamaesiphonales (3) Order : Pleurocapsales (4) Order : Nostocales (5) Order : Stigonematales.

In recent years cyanobacteria have grabbed a lot of attention for their potential application in biotechnology.

1.1 Applications of Cyanobacteria

1.1.1 Cyanobacteria as Food

Historically there has been many reports of cyanobacteria being used as food for human consumption. It has been reported that for many decades, *Spirulina (Arthrospira)* biomass has been used for preparing food by the people living in the Republic of Chad, Mexico and Spain (Yi *et al.*, 2017). The *Spirulina (Arthrospira)* biomass is harvested from lakes and sun dried. This sun dried biomass is used in the preparation of various traditional dishes like meat and vegetable soups. *Nostoc commune*, which grows in the form of large gelatinous sheets, is widely used in Asian cuisine. This cyanobacterium is consumed raw or stir fried, is used for preparing soups, and can also be used as a thickening agent in various other foods. *Nostoc flagelliforme* is commonly sold in Chinese markets as dry filaments which appears as a black hair-like vegetable. This is locally known as "*fa cai*" and usually served during the festive season. *Nostoc flagelliforme* is a terrestrial cyanobacterium that grows very slowly as a mat attached to the substrate in desert steppe regions of northern and northwestern China. *Nostoc punctiforme* has been traditionally

used as human food in China, Mongolia and South America. This terrestrial cyanobacterium grows as ball-shaped colonies and is locally known as "Lakeplum". A unicellular cyanobacterium, *Aphanotheca sacrum*, is consumed in Japan as a special delicacy known as "suizenji-nori". Cyanobacterial biomass has been marketed for human consumption from many years. Out of these the most commonly marketed cyanobacterium in around 70 countries is *Spirulin*a. Many studies have been conducted to assess the nutritional value of cyanobacteria. They have been found to be a rich source of vitamins (like provitamin A, vitamin E, thiamine, cobalamine, biotin and inositol), proteins and lipid (Chew *et al.*, 2017). Cyanobacterial biomass are found to be easily digestible. One of the limiting factors known is that cyanobacteria have a high content of nucleic acids, which are metabolized to uric acid. Uric acid in excessive quantities is known to cause many adverse health effects like kidney stones (Gantar and Svircev, 2008).

Yang *et al.* (2011) evaluated the *in vitro* and *in vivo* toxicity of the edible blue-green algae, *Nostoc commune var. sphaeroides kutzing* and *Spirulina platensis*. They concluded from their results that both these blue-green algae are free from microcystin (MC) and that there will be no side effects by using 5 % BGA as a dietary supplement.

1.1.2 Cyanobacteria as Biofertiliser

Cyanobacteria have a great potential to be used as a ecofriendly and cost effective alternative to chemical fertilizers. The use of a defined mixture of cyanobacterial species to inoculate soil is known as algalization. The mass production of cyanobacterial biofertilser can be by either open-air or indoor production systems. An increase in grain yield of rice by 15-20% have been reported in field studies (Mishra and Pabbi, 2004).

Cyanobacterial inoculation of soil have been shown to improve the physico-chemical properties of soil, aids in gradually increasing the amount of soil nitrogen and carbon, improving electrical conductivity and soil pH and the quality of the grain in terms of the protein content also improved. A very good yield can be obtained by integrating minimal use of chemical fertilizers along with a excellent biofertlizer like cyanobacteria (Kaushik, 2014).

Lakshmi *et al*. (2012) evaluated the potential of biofertilizers such as cyanobacteria, phosphobacteria and azolla in minimizing the impact of climate change on rice farming, by nutrient supplementation, by aerating the water through photosynthetic activity, hence reducing the emission of methane and sequestration of carbon by carbon concentrating mechanism during which carbon dioxide is concentrated at the site of photosynthetic carboxylation. They reported that in their study, the combined application of cyanobacteria and organic manure recorded both reduction in the emission of methane during cultivation and also a higher rice yield. Saadatnia and Riahi (2009) showed that the germination of rice seeds treated with four species of *Anabaena* was faster compared to the control.

Palaniappan *et al*. (2010) reported that treatment of cow pea (*Vigna unguiculata* L.) seeds with a 5% aqueous extract of the cyanobacterium *Phormidium* immobilized in coir pith as a combination of basal and foliar application, significantly increased the seed germination, plant height, plant weight, number of flowers, root nodules and biomass in comparison with the control. Begum *et al*. (2011) reported that the treatment of two high yielding varieties of rice with a mixed cyanobacterial inoculam along with urea-N, produced a significant increase in number of tillers/hill, length of panicle, weight of grains and yields of grain and straw in comparison with the control. Bhuvaneshwari *et al*. (2011) reported a significant improvement in both

morphometric and yield parameters in sunflower (*Helianthus annuus* L.) treated with coir pith based cyanobacterial biofertiliser. Pazhanivel *et al.* (2011) reported a significant improvement in quality of fatty acids in groundnut (*Arachis hypogaea* L.) with a coir pith based cyanobacterial biofertiliser. Moorthy and Malliga (2012) evaluated the effect of different concentrations of cyanospray (supernatant of *Oscillatoria annae* culture inoculated in coirpith) on *Aloe barbadensis* Miller (*Aloe vera*). They concluded that application of 0.4% cyanospray on *A. barbadensis* significantly improved its growth characteristics and yield and hence it is suitable to be used commercially as a biofertilizer.

1.1.3 Source of Pigments

The three important groups of cyanobacterial pigments namely chlorophyll, carotenoids and phycobilins have great potential for quick commercial success as functional food in aquaculture, cosmetics, food technology and pharmaceuticals. The commercial uses of chlorophyll products are as food additive for colouration to provide green colouring to a variety of food stuffs and beverages. The carotenoid pigments are commercialized as β-carotene as vitamin supplement, in poultry farming to provide orange colour to egg yolk, astaxanthin is used in sunscreen creams, in aquaculture to provide typical reddish colour to salmonids and other aquatic organisms and for cancer prevention and anti-tumor therapy. The food additive bixin derived from carotene is used to provide peach colour to various dairy products, xanthophylls – lutein and zeaxanthin are used to provide colour to chicken skin and to prevent macular degeneration and cataract in humans. Violaxanthin is used as a food additive; canthaxanthin is used as a food additive, used in cosmetic industry and in tanning pills (Koller *etal.*, 2014).

The phycobilin pigments are commercially used as chemical tags, as food colourants and in cosmetics. Investigations have proven that along with their primary application as natural dyes, they also have many health promoting aspects and a wide range of pharmaceutical applications (Simeunovic *et al.*, 2012). Phycocyanin have been used as food colourants in place of the synthetic food colours used traditionally. They are being used to impart colour in foods like chewing gum,ice sherbets, popscicles, candies, soft drinks, dairy products, and in cosmetics like lipstick and eyeliners. Phycobiliproteins are also widely used in clinical and immunological laboratories as labels for antibodies, as receptors, and other biological molecules in fluorescence studies and they are also used in immunolabelling experiments, fluorescence microscopy and diagnostics (Richa *et al.*, 2011). Phycocyanin has also been shown to have a major influence on serum cholesterol concentrations, which imparted a strong hypocholesterolemic activity (Sekar and Chandramohan, 2008). It has been reported that phycocyanin have antioxidant properties and this phycobiliprotein scavenge oxygen free radicals and react with other oxidants having pathological relevance. These properties of phycocyanin have been reported to cause the cataract preventive action in wistar rats having naphthalene and galactose induced cataract (Kothadia *et al.*, 2011).

1.1.4 Bioactive Compounds from Cyanobacteria

There are several reports of the potential applications of cyanobacteria in medicine, pharmaceuticals, fine chemicals, enzymes, and as herbicides (Kumar *et al.*, 2010; Rajneesh *et al.*, 2017). Apart from the primary metabolites like proteins, fatty acids, vitamins, pigments etc, they produce several useful secondary metabolites having antifungal, antibacterial, antiviral, antineoplastic, and antialgal activities (Volk and Furkert, 2006; Silva-Stenico *et al.*, 2011). Cyanobacteria are also known to produce many photoprotective compounds like scytonemin and

mycosporine-like amino acids, for protection against the harmful ultraviolet radiation. These compounds have the potential for many commercial applications (Fleming and Castenholz, 2007).Other compounds produced by microalgae having commercial importance are – glycerol, vitamins, polysaccharides and polyhydroxyalkanoate (bioplastics) (Markou and Nerantzis, 2013).

- **Antialgalactivity**

Alleopathy is a complex chemical signaling in which secondary metabolites produced by an organism has a harmful or beneficial effect on another organism. Allelopathy is considered as an adaptation for achieving a competitive edge over other organisms belonging to the same community.

The studies on allelopathic activity of cyanobacteria has received attention primarily because of the following reasons: (1) strains of some cyanobacteria are known to produce cyanotoxins like microcystins, nodularins etc. Such cyanobacteria are of particular interest because they can be used to combat harmful algal blooms, (2) they can be used for developing eco-friendly and tributylin-free antifouling paints for ships, (3) the knowledge about such allelochemicals can be useful for a better understanding of interactions between organisms competing for various resources within the same habitat, and (4) most of the cyanobacterial algicides are known to affect photosynthesis, so they can be used as natural herbicides (Smith and Doan, 1999; Volk, 2005; Gantar *et al.*, 2008).

It has been experimentally proven that eventhough allelopathy does occur under natural conditions, the competition for nutrients between the two species can also be a major influential factor (Zuo *et al.*, 2016).

- **Antifungal activity**

Cyanobacterial secondary metabolites having antifungal activity are considered to have the potential to be used as effective drugs since they are

more safer and they are also reported to be patented for agricultural use (Frankmolle *et al.*, 1991; Biondi *et al.*, 2004). Bertin *et al.*, 2016 reported the isolation and characterization of a compound Kalkipyrone B from the cyanobacterium *Leptolyngbya* sp. possessing both cytotoxic and antifungal activities.

- **Antibacterial activity**

Cyanobacterial secondary metabolites having antibacterial and antimicrobial effects are being presently considered for use in food preservation. The antibacterial effects have been partly attributed to the presence of unsaturated fatty acids having chain lengths above 10 carbon atoms. So compounds showing the above mentioned properties, derived from cyanobacteria are considered to have great potential to be used as preservatives in food or feed formulations, in place of synthetic antimicrobial compounds or antibiotics presently in use (Guedes *et al* ., 2011).

- **Antineoplastic and antitumor activity**

There has been many reports of cyanobacteria producing compounds having antineoplastic properties. This activity has been demonstrated in some cyanobacterial genera such as *Nostoc*, *Scytonema*, *Hapalosiphon*, *Lyngbya* and *Symploca*. A compound called Cryptophycin isolated from cyanobacteria has been identified to cause depolymerization of microtubule, and is also known to induce apoptosis in human prostrate cancer cells. Such reports indicate that cyanobactertia produce various compounds with anticancer activity (Svircev *et al.*, 2008). Since cancer is still one of the leading causes of mortality worldwide identification of new naturally derived compounds is essential for overcoming the increasing tumour resistance and undesirable side effects. Currently around 17

anticancer compounds derived from marine cyanobacteria are in various phases of clinical trials (Freitas *et al.*, 2016).

- **Sunscreen activity**

Cyanobacteria have evolved numerous defense mechanisms against UV radiation earlier in their evolutionary history. These adaptations include avoiding exposure, presence of various active repair mechanisms such as the SOS repair response, reactive oxygen species removal by carotenoids, and biosynthesis of secondary metabolites such as mycosporine-like amino acids (MAA) and scytonemin, which act as UV protective pigments (Fleming and Castenholz, 2007). Scytonemin is a extracellelar pigment which is localized in the extracellular polysaccharide sheaths (EPS). This pigment is observed as a yellow-green or yellow-brown pigmentation in the sheaths of cyanobacteria. Scytonemin has been reported to have anti-inflammatory and antiproliferative activity. It has passive UV absorption properties in the UV-A region; 85 to 90% of the incident UV-A is absorbed by this pigment. Therefore, they are considered to have the potential to be used as sunscreen agents (Sorrels *et al*., 2009).

Mycosporine-like amino acids (MAA) have strong UV absorption maxima between 310 and 362 nm. They are found to have great potential as sunscreen compounds, which can replace various commercially available sunscreens used in many cosmetic skin care products (Ferroni *et al*., 2010). They are small hydrophilic compounds found in different taxonomic groups. Strong UV absorption (307–362 nm), high molar extinction coefficients (e = 28,000 - 50,000 M^{-1} cm^{-1}), , resistance to various abiotic factors and photostability in the presence of photosensitizers signify the potentials of MAAs as effective natural photoprotectants. It has been shown that due to their strong antioxidant and free radical scavenging ability they can prevent the adverse consequences of oxidative stress. Certain abiotic factors like desiccation, UV radiation and nutrient

concentrations are shown to greatly influence the synthesis of MAAs. Due to their potential antioxidant and sunscreen activity they can be used in various pharmaceutical and cosmetics industries (Rastogi *et al.*, 2016).

1.1.5 Exopolysaccharides from Cyanobacteria

Many cyanobacteria are known to possess sheaths, capsules and slimes in their outer walls. Polysaccharide producing strains are known to release their capsule or slime as water -soluble polymer into the medium. These strains have been studied extensively as they are considered to have many industrial applications. Some of these are: (1) Most of the cyanobacterial polysaccharides are known to have abundant uronic acid subunits having carboxyl groups, making them very efficient metal ion binders. Therefore, water-soluble polymer producing cyanobacterial strains are considered to have great potential for removing metals from polluted waters, (2) The cyanobacterial polysaccharides were found to be complex heteropolymers. They are mostly composed of 6 to 10 monosaccharides like : glucose, galactose and mannose (hexoses), ribose, arabinose and xylose (pentoses), fucose and rhamnose (deoxyhexoses), and glucuronic and galacturonic acid (acidic hexoses). These polysaccharides have been found to have greater number of monomers than those currently used in industries. So they have the potential for various industrial applications, (3) The presence of significant amounts of sulphate groups in cyanobacterial RPSs, has been reported to confer them antiviral properties, (4) cyanobacterial polysaccharides are found to have capability to alter the rheological behavior of water and they can stabilize the flow properties of aqueous solutions under drastic changes of temperature, ionic strength and pH; because of these properties cyanobacterial polysaccharides have the potential to be used as thickening agents, and (5) These polysaccharides can be used for stabilization of emulsions or as bioflocculants because of the presence of both hydrophilic and

hydrophobic groups in them (Philippis *et al*., 2001 and Yunyi *et al*., 2007). Exopolysaccharides in cyanobacteria can either remain associated to the cell surface (cell-bound polymers) (classified into three -sheath, capsule and slime) or liberated into the surrounding environment as released polysaccharides (released polysaccharides – RPS). The synthesis of exopolysaccharides in cyanobacteria occurs in the cytoplasm (Delattre *et al*., 2016).

1.1.6 Biopolyesters from Cyanobacteria

The accumulation of polyhydroxyalkanoates (PHAs) occurs as a response towards stress experienced by microorganisms while residing at various ecological niches. Many cyanobacteria are known to produce a type of polyester polymer known as polyhydroxyalkanoates (PHAs). The most commonly encountered PHAs is polyhydroxybutyrate (PHB), which can be used as raw ematerial for the production of biodegradable plastics. Cyanobacteria are known to accumulate PHBs as a storage reserve for carbon and energy in the presence of excess source of carbon, and usually under conditions of nutrient limitations such as deficiency of nitrogen, phosphorus, oxygen or trace metals in the growth medium. Usually they are known to occur inside the cyanobacterial cell as inclusion bodies, and as granules in the cytoplasm. PHAs have many applications like : (1) due to their biological characteristics and biodegradability, they have the potential to be a ecofriendly alternative to plastics produced from petrochemical sources, (2) their biodegradability, producing CO_2 and H_2O as products, are indicative of their biocompability. So they have many potential application like in manufacture of packaging films, usage in medical applications such as in heart valves, surgical suture, wound dressing, drug delivery systems, bone implants, tissue engineering, disposable diapers etc (Miyake *et al*., 2000; Lopez-Cortes *et al*., 2008; Rebah *et al*., 2009; Wong *et al*., 2000; Quillaguaman *et al*., 2010). Some

other applications of PHAs are - their potential to act as a precursor for production of renewable biofuels, as a sizing agent and in surface coating of paper in the paper industry, potential use of PHAs to control bacterial pathogens in some aquaculture applications like the administrations of these biopolymers in the aquaculture feeds which has been reported to confer protection against virulent bacterial pathogens, and PHA can be used as controlled release agents for application of herbicides in agriculture (Gumel *et al.*, 2012). As the load of industrial effluents on soil is increasing, currently researchers are studying the production of PHA producing strains using various effluents like distillery effluent, textile effluent, oil mill wastes, dairy wastes, paper mill wastes, starchy wastes, petroleum refinery wastes, animal residues and agricultural wastes (Saharan *et al.*, 2014). Due to their inherent biodegradability, sustainability and environment friendly properties, biodegradable plastics have a promising future (Anjum *et al.*, 2016). It has been demonstrated that the sustainable production of PHA is multi-facetted and various hotspots in the processing chain needs to be addressed in order to make the industrial scale production of PHA feasible both economically and environmentally (Koller *et al.*, 2016).

1.1.7 Cyanobacteria for Environmental Bioremediation

Cyanobacteria are known to have the potential to be utilized in bioremediation treatments like –wastewater treatment, heavy metal removal, pesticides, crude oil and dye degradation (Fatma, 1999; Idi *et al.*, 2015). Cyanobacteria have been shown to have the ability to degrade textile dyes that are recalcitrant compounds that resist conventional biological treatments of effluents. This in turn helps in solving the environmental problems caused by hazardous dyes (Dellamatrice *et al.*, 2017).

Animal and household wastewaters have been treated in ponds for many years. In algal ponds, the wastewaters are treated wholly by algae or in

combination with other microorganisms. *Spirulina* has been successfully cultivated in a high rate algal pond situated in Thailand using digested tapioca factory effluent as a substrate (Kojima and Lee, 2001).

Dubey *et al*. (2011) investigated the biodegradation and biosorption capacity of some cyanobacterial species isolated from Pharmaceutical and Textile industries in Bhopal. They found the most dominant species among the cyanobacterial isolates were *Oscillatoria* sp., *Synechococcus* sp., *Nodularia* sp., *Nostoc* sp. and *Cyanothece* sp. They reported that these cyanobacteria were highly efficient in the removal of toxic contaminants from the industrial effluents, which indicated that the cyanobacteria used in their study are highly beneficial for bioremediation applications.

Shashirekha *et al*. (2011) reported effective removal of Cr^{3+} from exhaust chrome liquor (ECL), which is a tannery effluent, and from synthetic basic chromium sulfate (BCS) solution along with significant reductions in sulfates, BOD, COD, TDS, TSS etc. when treated with three cyanobacterial species – *Spirulina*, *Oscillatoria* and *Synechocystis*, individually or as a consortium.

Large volumes of oily wastewaters are generated in petroleum refineries storage and transport facilities. Many cyanobacteraia are known to have a strong affinity to hydrocarbons. Some cyanobacterial isolates like *Dermocarpella*, *Mysosarcina*, *Chroococcidiopsis*, *Nodularia*, *Scytonema*, *Calothrix*, *Fischerella* and *Chlorogloeopsis* are reported to be capable of photoheterotrophic growth utilizing oil fractions as substrates (Kojima and Lee, 2001).

1.1.8 Biofuels

There is a good potential for production of many biofuels like biodiesel, biohydrogen, biomethane and bioethanol from microalgae. Biohydrogen was produced out of *Scenedesmus obliquus* in 1942 and presently it is produced out of

Chlamydomonas reinhardtii. There is a potential for production of bioethanol out of *Chlorococcum littorale* and *Spirulina*.

Parmar *et al.* (2011) reported that cyanobacteria and microalgae can be potentially used for the production of biofuels in an economically feasible and ecofriendly manner and at rates high enough to replace a majority of the society's use of fossil fuels. Some of the aspects of the biofuel production by cyanobacteria and microalgae that have contributed to capture the interest of researchers and entrepreneurs around the world are : ability to perform oxygenic photosynthesis using water as electron donor, they have high growth densities and consequently high per-acre productivity, they are a non-food based feedstock resource, they use non-productive and non-arable land, they can grow in a wide variety of water sources (fresh, brackish, seawater and wastewater) and they produce valuable co-products along with biofuels. Before energy products from these systems can enter the commercial market, they have to overcome different biotechnical, economic and environmental constraints.

Many filamentous strains of cyanobacteria that are capable of fixing atmospheric nitrogen have emerged as promising candidates for production of fuels and chemicals. Researchers have engineered many filamentous cyanobacterial strains for producing high value chemicals and potential next generation biofuels like famesene, limonene and linalool (Johnson *et al.*, 2016). The ability of cyanobacteria to produce hydrogen has given rise to its potential use in the field of bioenergy and bioelectricity (Sarma *et al.*, 2016).

1.2 Cultivation Systems

The success of the various culture methods used for mass cultivation of microalgae including cyanobacteria lies in obtaining the maximum productivity of the cultured organism, and the quality of the final product. The

efficiency of the mass cultivation system is indicated by the optimal utilization of the growth conditions by the cultured strain. Some of the commonly used culture systems are discussed below (Richmond, 2004; Andersen, 2005).

1.2.1 Open Ponds

Large scale cultivation of microalgae is most commonly done in open ponds. They are considered to be more durable than closed reactors. Open ponds vary in size, shape, construction material used, type of agitation, and inclination. Some of the commonly used open ponds are:

- **Inclined systems**

In inclined systems, the culture suspension flows down a sloping surface and as a result of this, turbulence is created by gravity. Inclined systems have many advantages like very high turbulent flow, the capability to maintain very thin culture layers (less than 1 cm), this inturn facilitates higher cell concentration (upto 10 g L^{-1}) and a higher surface-to-volume ratio. This culture system also has several disadvantages like sedimentation of cells at points of lower turbulence, evaporative losses of culture suspension, very high rate of CO_2 desorption, and requirement for great amount of energy for pumping the culture to the top of the inclined surface.

- **Circular ponds**

Circular ponds have a depth of about 30 cm and the pond size is limited to about 10,000 m^2 as even mixing of the culture is not possible in larger ponds. In Japan and Taiwan, circular ponds with a centrally pivoted rotating agitator are used for the cultivation of *Chlorella*.

- **Raceway ponds**

Raceway ponds are most widely used for the production of *Spirulina*, *Dunaliella salina* and *Haematococcus*. Raceway ponds are considered to have a very efficient design for large scale cultivation of microalgae. Raceway ponds are constructed in the form of endless loops and the circulation of the culture is done by paddle wheels. Individual ponds have an area of upto 1 ha and have an average depth of around 20 to 30 cm.

- **Cascade pond**

These sloped ponds are very shallow with a circulating pumpand is reported to be used for the cultivation of *Chlorella* in Trebon, Czech Republic. Cascade ponds are considered to be highly suitable for cultivating *Chlorella* and *Scenedesmus*. As the slopes of the pond has extremely shallow water depth (~10-20 mm), very high productivities and cell densities can be achieved.

1.2.2 Photobiorectors

Photobioreactors are culture systems for phototrophs in which more than 90% of light does not impinge directly on the culture surface, but passes through the reactors transparent walls to reach the cultivated cells. Consequently photobioreactors strongly limit or prevent the exchange of gases and contaminants between the culture suspension and the atmosphere. Photobiorectors are classified as:

- **Tubular photobioreactors**

(a) Serpentine photobiorectors

In this photobioreactor, several straight transparent tubes are joined in a series by U-bends to form a flat loop (photostage) that can be arranged either vertically or horizontally. A separate vessel is used for gas exchange and

nutrient addition. Circulation between this vessel and the photostage is achieved by using a pump or an airlift.

(b) Manifold photobiorectors

In manifold photobioreactors, a series of parallel tubes are connected at the ends by two manifolds, one of which is used for distribution and the other is used for the collection of the culture suspension. Near-horizontal manifold photobioreactors have been developed and reported to be used for the cultivation of *Arthrospira platensis*, *Anabaena siamensis*, *Nannochloropsis* sp. and *Phaeodactylum tricornutum*.

(c) Helical photobioreactors (bio-coilphotobioreactor)

Helical photobioreactors consist of usually flexible tubes of small diameter, which are wound around an upright structure. A biocoil consist of a photostage of polyethylene or PVC tubing (between 2.5 and 5 cm diameter), which are wound helically around a cylindrical support which is typically 8 m in height with a core diameter of 2 m.

(d) Fence arrangement with manifolds

A photobiorector known as bio-fence was developed by Applied Photosynthetics Limited (APL) (Manchester, UK), specifically designed for the cultivation of marine algae used in aquaculture or used in wastewater treatment. The bio-fence is comprised of an array of rigid transparent tubes racked together in banks and connected by manifolds in a fence-like arrangement. A centrifugal pump or an airlift is used used to circulate the culture suspension between the photostage and a holding tank. pH of the culture is controlled by injecting CO_2 in the photostage.

- **Flat photobioreactors**

Flat photobioreactors have the advantage that irradiance at the culture surface can be measured.

(a) Flat alveolar panels

This photobioreactor is usually constructed from commercially available, transparent PVC, polycarbonate or polymethyl methacrylate sheets, which are internally partitioned to form narrow channels called alveoli. This culture system is reported to be used for cultivating various microalgae and cyanobacteria (eg: *Chlorella* and *Arthrospira platensis*).

(b) Glass plates

This is an inclined modular photobioreactor which consists of a series of flat glass chambers, 0.7 m high and 0.9 m long, connected in cascade and tilted at the appropriate angle to maximize capturing of solar radiation. This culture system has been reported to be used for cultivating *Arthrospira platensis* and *Nannochloropsis*.

Vertical cylinders and sleeves

This is a simple system otherwise known as column reactors, in which mixing is achieved by injecting compressed air. The first units of this photobioreactor constructed, consisted of glass columns 1.8 m in height and 10 cm in diameter, constricted at the bottom to prevent algal settling. This culture system is reported to be used for cultivating *Chlorella*, *Microcystis aeruginosa*, *Nostoc*, *Anacystis*, *Isochrysis galbana*, *Porphyridium*, *Dunaliella*, and *Phaeodactylum tricornutum*.

- **Axenic photobioreactors**

The first axenic photobioreactor developed for the axenic cultivation of microalgae was a glass tubular reactor. This was a vertical flat coiled

photobiorector having a capacity of 110 L, which could be sterilized by steam. The microalgal suspension was circulated with the help of a glass centrifugal pump. The temperature was controlled using a water-jacketed section, a carbon dioxide-air mixture was injected into an external exchanger for the exchange of gas and the lighting was provided by using artificial illumination. Some of problems encountered while using this system were reported to be cell damage to fragile cyanobacteria due to the centrifugal pump, formation of foam and adhesion of cells to the glass walls (Richmond, 2004; Andersen, 2005).

1.3 Commercial Products from Microalgae/Cyanobacteria

The first commercial large – scale culture of microalgae was started by Nihon Chlorella (Taipei, Taiwan) in the early 1960's by culturing *Chlorella*. The second commercial facility was started in the early 1970's by Sosa Texcoco S. A. (Mexico City, Mexico) by establishing a facility for the culturing and harvesting of *Arthrospira*. By the start of the 1980's, it was reported that there were around 46 large – scale commercial establishments in Asia, together producing more than 1000 kg of microalgae (majority producing *Chlorella*). In 1986, with the establishment of production facilities by Western Biotechnology (Hutt Lagoon, Australia) and Betatene (Whyalla, Australia), the third major microalgal industry came into existence by commercially producing *Dunaliella salina* as a source of β-carotene. This was soon followed by the establishment of other commercial plants in Israel and the USA. At around the same time large – scale commercial production of cyanobacteria began in India (Spolaore *et al.*, 2006).

Presently four species of microalgae -*Arthrospira*, *Dunaliella salina*, *Chlorella* and *Aphanizomenon flos-aquae* have found applications in food industry. Microalgae are used in the food industry as tablets, capsules and liquids. *Arthrospira* is a valuable foodstuff because it is rich in protein and other nutrients and it has been reported to aid in the alleviation of

hyperlipidemia, protection against renal failure, suppression of hypertension, suppression of elevated serum glucose levels and promotion of intestinal *Lactobacillus*. *Chlorella* contains β-1,3-glucan, which is an active immune stimulator, a reducer of blood lipids and a free-radical scavenger. *D.salina* is exploited for its β-carotene content, that makes it an important ingredient of many dietary supplements and functional foods. *A.flos-aquae* can be used alone or along with other nutraceuticals and natural food products to promote human health (Wang *et al.*, 2015).

Some microalgal nutritional and health food are marketed in the form of : Micro algal tablet or capsule – Qizheng *Spirulina* tablet are produced by Shenzhen Lanzao Biotechnology Corporation and Guangzhou Guanghua Pharmaceutical Company Ltd and Mingxing Hukangbao tablets are produced by Guangzhou Mingxing Pharmaceutical Factory. Yunan Green-A Biotechnology Co. Ltd. Manufactures *Spirulina* capsules; Microalgal nutritional liquid– Presently *Spirulina* liquid is manufactured by Wuhan Plant Research Institute in cooperation with Wuhan Pharmaceutical Factory and by Guanzhou Maoyuan Imp. & Exp. Corporation. There has been reports of potential commercial use of *Chlorella* growth factor; Microalgal noodles– *Spirulina* noodle cakes are presently produced by Guangzhou Nanfang Flour factory and marketed in China and some other potential products that can be developed using microalgae are microalgal bread, microalgal biscuits, microalgal drink, microalgal green tea, microalgal beer and microalgal candy (Liang *et al.*, 2004). Some of the best worldwide known *Spirulina* producing companies are: Earthrise Farms (USA), Cyanotech (USA), Hainan DIC Microalgae Co., Ltd (China), Marugappa *Chettiar* Research Center (India), Genix (Cuba) and Solarium Biotechnology (Chile).

There is a huge potential for marketing of microalgal products as functional foods and currently biscuits rich in omega-3 fatty acids are produced by adding *Isochrysis galbana* biomass (Borowitzka, 2013). It was reported that the market for carotenoids in the year 2010 was around US $ 1.2 billion, which included both chemically synthesized and naturally produced carotenoids (Borowitzka, 2013). The commercial production of β-Carotene from the microalga *Dunaliella salina* or *Dunaliella bardawil* was started in the 1980s by four companies –Koor Foods (Nature Beta Technology) in Israel, Western Biotechnology Ltd and Betatene Ltd in Australia, and Nutralite in the USA. There has been reports of small-scale commercial production from *Dunaliella* in India and China. It has been reported that currently around 30% of microalgal biomass produced is used as fodder additives. Some of the microalga used as fodder additives are *Arthrospira*, *Chlorella*, and *Scenedesmus*. Microalgae are also reported to be used for improving the properties of various aquaculture products by adding it in the feed -(a) addition of *Haematococcus* increases the colour of salmonid tissues and shells, (b) increase of red and yellow inclusions in the muscles of carp by adding carotenoid rich *Arthrospira*, (c) use of *Hasleaostearia* in France to create blue and green shade on labial feelers and gills of oysters and (d) change of colouring in fish is achieved by addition of phycocyanin rich biomass of *Spirulina* or by adding carotenoid rich biomass of *Dunaliella* (Varfolomeev and Wasserman, 2011).

Astaxanthin is commercially produced from the green alga *Haematococcus pluvialis*. The culture is usually a two-stage process and since this is a fresh water alga, the first stage of production is always in a closed photobioreactor and the second stage is either in open outdoor ponds or in closed photobioreactors. Presently the astaxanthin produced from *Haematococcus* is marketed as a neutraceutical and antioxidant product. It is

also used for anti-tumor therapies and prevention as well as treatment of neural damages related with age related macular degeneration like Alzheimer and Parkinson disease. Other than these uses it is considered to be a natural superfood which enhances athletic performance by increasing stamina and reducing the time taken for muscle recovery (Panis and Carreon, 2016).

Phycocyanin is marketed in Japan as 'LinaBlue' by DIC CORP., Japan and it has applications as food and cosmetic colours. It has been reported that depending upon the purity it sells for between US $ 500 to 100,000 kg^{-1}. It has been estimated that the current total market value for phycobiliprotein products is greater than US $ 60 million (Borowitzka, 2013). *Spirulina (Arthrospira)* has been commercially produced in open raceway ponds from a long time and the current annual world production is in excess of 5000t (Borowitzka, 2013). This cyanobacterium is considered to be a very good source of C-phycocyanin.

Commercial production of various fatty acids has been reported from various microalgae – docosahexaenoic acid (DHA) is extracted from *Crypthecodinium cohnii* and marketed as a DHA product – DHASCO™ (main commercial use is in infant formula) and an oil rich in both eicosapentaenoic acid (EPA) and DHA, extracted from a strain of *Schizochytrium* is marketed (Borowitzka, 2013). Aurora Algae, situated in Hayward, USA has announced a EPA product from the marine eustigmatophyte *Nannochloropsis*. The best algal source of γ–linolenic acid is *Spirulina platensis*. There is a potential use of microalgal oils as a component of animal feeds (Borowitzka, 2013).

A mixture of polysaccharides extracted from heterotrophic green algae, is currently marketed with the trade name 'Alguronic acid'. Several other ingredients for cosmetics currently available in the market include polysaccharides from *Porphyridium*, extracts of *Chlorella*, *Spirulina* and *Aphanizomenon* etc. (Borowitzka, 2013).

Lu *et al.* (2011) investigated the status of *Spirulina (Arthrospira)* industry in Inner Mongolia region of China, which has a temperate continental climate. They found that several native species of *Spirulina (Arthrospira)* along with the thermophilic *S. platensis* and *S. maxima*, are commonly used for commercial production in Inner Mongolia.

1.4 Scope of the Present Study

In India, the commercial ventures in microalgal cultivation has been limited to the cyanobacterium *Spirulina*. Aurospirul situated in Auroville, Tamil Nadu has been growing *Spirulina* for sales in India and abroad. *Spirulina* is cultivated organically in this farm and is marketed as : Aurospirul A V Super Greens, Aurospirul Spirulina Tablets and Aurospirul Spirulina Crunchy with CHILI (www.auroville.com). Py Farms situated in a remote village in Pondicherry has been cultivating *Spirulina* as well as manufacturing and marketing *Spirulina* products since 2008. Their marketed *Spirulina* products are : Pondicherry Spirulina Capsules and Spirulina Powder (www.pyfarms.com). Algene Biotech located at Surat, Gujarat has been commercially producing *Spirulina* and marketing it as Spirugene – Powder and Spirugene – Capsules (www.algenebiotech.com). Parry Neutraceuticals situated at Chennai, India has been manufacturing *Spirulina* organically and marketing it as: Parry's Spirulina (www.parrynutraceuticals.com). Hydrolina Biotech, Chennai has been cultivating *Spirulina* and marketing it as : VITALINAA–Spirulina capsules, tablets and spray dried powder (www.hydrolinabiotech.com). Batra Herbals located in Madhya Pradesh, India has been marketing *Spirulina* as Spirulina powder (www.steviaplants.in). Presently the majority of commercial production of cyanobacteria in India is for marketing the products as nutritional supplements. So there is immense scope for commercially producing high value products and exploring the suitability of cyanobacteria other than *Spirulina*. The future of

large scale production of microalgae will depend on not only the market potential of the product but also on the production cost. Production of *Spirulina* in the wet tropics is limited by its requirement of alkaline water with high pH (8.5 -11.0)and salinity > 30 g/L. Therefore alternative species adapted to local conditions have to be identified for low cost cultivation.

There is a growing awareness on the use of natural colourants in food and cosmetic industry. Cyanobacteria is identified as the natural source of the blue colouring pigment C-phycocyanin. This pigment also has diagnostic and pharmaceutical applications. In the absence of commercial production from India and the growing market demand for this pigment it is useful to screen new isolates of cyanobacteria for their potential to yield these valuable pigments.

1.5 Objectives of the Present Study

The present study was devised with the following objectives:

i. To isolate and develop cultures of cyanobacteria from the environs of Kochi and quantify the production of phycobilin pigments. Presently the commercial production of phycobiliproteins is limited to *Spirulina, Galdieria sulphuraria* and *Porphyridium*. Since phycobiliproteins have been shown to have a wide range of applications, it is essential to explore the potential of more cyanobacteria for large scale production of phycobiliproteins.

ii. To optimize the production of phycobilin pigments by the species isolated. Optimization of phycobiliprotein production by the three species in different growth media with various pH and salinity conditions were undertaken. Different incubation conditions of light and temperature were applied to assess the production of the pigments.

iii. To study the effect of certain pollutants on production of phycobilins. This was done to know the tolerance level of cyanobacteria to trace pollutants in the water used for cultivation and to what extent it will affect the production of the pigments.

Chapter 2

DEVELOPMENT OF CYANOBACTERIAL CULTURES

Contents

2.1 Introduction
2.2 Methods of isolation
2.3 Purification of cultures
2.4 Outcome of Isolation
2.5 Maintenance of cultures

2.1 Introduction

The isolation, identification and culture of cyanobacteria is important because of the vast potential of its use in biotechnology. In spite of the presence of wide variety of cyanobacteria in diverse habitats, only a few of them have been isolated and purified because of the difficulties encountered.

Developing single species cultures of cyanobacteria, further purification, and maintenance of these cultures are the preliminary steps in algal biotechnology. The process though tedious, has certain standard protocols which needs modification depending on the species being cultured. The important steps towards successful isolation of cyanobacteria are: (1) Understanding and replicating the conditions present in the natural environment from which the samples are collected. (2) Successfully eliminating contaminants like other algal species, bacteria etc.

The method of sample collection is often crucial for survival of cyanobacterial cells. The water samples must be collected in clean containers and transported to the laboratory under stable temperatures as soon as possible without much delay. This increases the chances of viable cells in the samples for isolation (Anderson, 2005).

The difficulties encountered during isolation and cultivation of axenic cyanobacterial strains limits their use in various biotechnological processes and production of various bioactive compounds especially those having pharmaceutical and neutraceutical value. The establishement of a axenic culture requires two steps : (1) development of pure culture from the progeny of a single cell, (2) cells has to be free from contaminants like eukaryotes, prokaryotes and viruses (Sulcius et al., 2016).

For use in research, the culture must at least be unialgal and in some cases it should be axenic. The methods of isolation generally used are – selection of sources of microalgae, enrichment of a culture, direct isolation and producing axenic cultures. Microalgal samples can be isolated from different sources like water or soil and by scraping from the surface of various substrates. The enrichment of a culture medium is done to provide a suitable environment for the growth and reproduction of the target species of microalgae while eliminating the non-target organisms. Direct isolation of single cells or filaments can be done by using micropipettes to pick them up under a dissecting microscope and transferring them onto a sterile agar medium or a fresh sterile liquid medium for isolation. Axenic cultures can be produced using various techniques – washing individual cells under a dissecting microscope using sterile medium and transferring them through a series of sterile medium; microalgal cells can be separated using density gradient centrifugation; UV irradiation can be used to obtain bacteria free microalgal cultures; membrane filters can be used to separate filamentous algae from bacteria and various antibiotics have been used for eliminating bacteria from microalgal cultures (Richmond, 2004). It has been reported that the isolation and purification of freshwater strains is much faster in comparison with marine strains which has slower growth rate (He *et al.*, 2012).

As cyanobacteria have vast potential in various biotechnological and industrial applications it is necessary to maintain cultures indefinitely in culture collections. For small scale long-term maintenance of microalgae, the most commonly used method is serial transfer of an aliquot of the culture to fresh medium on a regular time schedule which varies from 2-4 weeks depending upon the strain being cultured (Acreman, 1994). Another method to preserve cyanobacterial cultures for long-term is cryopreservation of the strains (Urmeneta *et al.*, 2003).

For the successful cultivation of a cyanobacterial species, the ecophysiological requirements of the species must be known. So in order to know the effect of various ecological factors on the growth of a selected species, and consequently to maximise its growth, optimization of the culture conditions are done (Nagle *et al.*, 2010). Cyanobacterial growth in culture is strongly influenced by the culture medium. Algal growth can be affected in different ways by variations in pH, changes in the distribution of carbon dioxide and carbon availability, alterations in the trace metals and essential nutrients.

The optimum growth of microalgal culture requires adequate amounts of nutrients. The various growth media in use contain macronutrients such as phosphorus, nitrogen, carbon, potassium, sulphur, iron, magnesium, calcium, silicon in case of diatoms etc. and trace elements like cobalt, molybdenum, manganese, boron, copper, zinc, vanadium, selenium,and vitamins (eg. B12, thiamine) (Richmond, 2004). Accordingly a variety of nutrient media for culturing microalgae have been devised and for mass production of microalgae special formulations are made up using commercial fertilisers and natural waters. Many compounds are used for formulating the recipes of media and the various media differ according to their respective formulations. Examples of some commonly used freshwater growth media are - AF6 media used for culturing volvocalean algae, xanthophytes, cryptophytes, dinoflagellates,

synurophytes, euglenoids and green ciliates; Allen's Blue-Green Algal Medium used for culturing of freshwater and marine cyanobacteria; BG-11 Medium for culturing freshwater, soil, thermal and marine cyanobacteria; Bold's Basal Medium used for culturing many algae including chlorococcalean algae, volvocalean algae, filamentous green algae, xanthophycean algae, euglenoids and cyanobacteria; Chu # 10 Medium used for culturing a variety of algae including green algae, diatoms, cyanobacteria and glaucophycean alga; Spirulina Medium, Modified for culturing *Spirulina* sp.; Zarrouck medium used for culturing *Spirulina*. Examples of some commonly used marine media are – ASN-III medium for culturing marine algae; f/2 Medium for culturing coastal marine algae; K Medium used for culturing oligotrophic (oceanic) marine phytoplankters; Walne's Medium for culturing marine phytoplankton; Antia's Medium for culturing of a crytophyte; Plymouth Erd-schreiber Medium for culturing of many marine algae (Andersen, 2005). The threshold concentrations in nutrients in culture medium of algae should not be exceeded and so in order to quantify possible nutrient limitation, the Redfield ratio of 106C: 16N: 1P is commonly used. BG-11 medium and Modified Allen's Nutrient Solution have N:P ratios of 45:1, Bold's Basal medium have N:P ratio of 4:1 and Zorrouck solution have N:P ratio of 6:1 (Richmond, 2004). The presence of nutrients in right proportions is important (eg. N:P, N:Si).

Most photoautotrophically growing algae prefer free CO_2, however yields may be increased upto two or three times by adding organic sources of carbon like glucose or acetate. The bioavailability of nutrients in the culture medium is mainly controlled by the pH. Due to the photosynthetic activity of the microalgae, in too high pH the free carbon dioxide becomes unavailable, phosphorus is precipitated, and ammonia is released into the air. So addition of free carbon dioxide is beneficial to growth of algae. For small scale microalgal culture, complex and expensive media are generally used; but for large scale

culturing, as cost of the media becomes a limiting factor, organic and inorganic fertilisers are usually used (Noue and Pauw, 1988). For commercial aquaculture based production of algae, regulating the concentration and elemental ratios of a number of nutrients that includes carbon, ammonium, orthophosphate, silicate and nitrate is important. These nutrients are the major elements required by plants. To properly understand the abundance and production of microalgae in natural environments, a in-depth understanding of these nutrients is necessary (Ringuet *et al.*, 2011).

The nutrient requirements of each species differ based on its niche such that optimization of growth medium is a necessary prerequisite for successful biomass production in culture systems. The culture media and optimum conditions for various algal species have been investigated by many (Pandey *et al.*, 2010; Ilavarasi *et al.*, 2011; Jitendra *et al.*, 2012).

2.2 Methods of Isolation

The first process in this investigation was to isolate cyanobacteria from the local environment, and develop them in cultures. For this water and sediment samples were collected from the fresh water, marine water and brackish water environs of Kochi (Latitude : 9°55'52.44"N, Longitude : 76°16'2.29"E).

2.2.1 Sampling procedure

Samples for isolation of cyanobacteria were collected from fresh water ponds, traditional pokkali-prawn farms and Cochin backwaters. The sites were visited every fortnight and water and sediment samples were collected. A total of sixty water and sediment samples each were collected during the sampling season.

Water samples were collected using a water sampler (where there was sufficient depth) or using a polyethylene bottle and transferred to lab in

polyethylene bottles from the site. The sediment samples were collected using a sediment grab and the surface layer was removed into polyethylene bags. The samples were immediately transported to the laboratory. Several samples were collected every fortnight for isolation. Serial dilution technique and agar plating technique were adopted for the isolation of cyanobacteria in this study.

❖ ***Serial dilution technique***

The water samples collected were thoroughly mixed and 1 mL each inoculated into defined liquid media in test tubes followed by serial dilution. Sediment suspensions were also inoculated similarly. The media was selected such that it included freshwater as well as marine, inorganic as well as organic and with or without added nitrogen. The media used were f/2 medium, f/2 medium with addition of vitamins, ASN-III medium, sediment extract medium (full strength) and sediment extract medium (half strength). The sediment extract for sediment extract medium was prepared by suspending 1 kg sediment in 1 L water, followed by digestion in autoclave. After cooling, the supernatant was decanted, filtered and sterilised. Sediment extract medium (full strength) was prepared by adding 50 ml sediment extract, 0.1 g KNO_3 and 0.02 g NaHPO4 to 1 L GF/C filtered estuarine water. The half strength medium was prepared by adding 25 mL of extract along with nitrate and phosphate as before.

The inoculated test tubes were incubated in a light panel of day light fluorescent lamps at an intensity of 2200 lux and temperature of 28± 2^0 C and a 12 : 12 light – dark cycle. These tubes were observed for growth for two weeks to one month. Tubes that developed blue-green colour were selected, observed under microscope, and those with blue-green algal filaments were recultured to another set of serial dilution.

❖ ***Agar plating technique***

The liquid culture media used above were solidified using 1.5 % agar. The agar based media were autoclaved and cooled to 45 – 50^0 C. The freshly prepared and cooled medium was poured into sterile glass petridishes to a uniform depth of about 4 – 5 mm. The agar plates were allowed to cool further to room temperature. Unless the plates were used on the same day, they were stored in a refrigerator in sealable plastic bags to prevent drying of agar.

The water samples and sediment suspension was streaked on the agar surface in the petridish by using quadrant streaking technique. The inoculated petriplates were incubated as above. The petriplates were incubated until colonies appeared on the agar surface. Blue-green coloured single colonies were picked up using a sterile inoculation loop and introduced carefully into respective sterile liquid medium in test tubes.

2.3 Purification of Cultures

The isolation was especially targeted to development of culture of filamentous cyanobacteria. The test tubes positive for filamentous growth were selected, the cyanobacteria were picked by fine needles and introduced into fresh media. The process was repeated several times. Once monocultures were obtained, they were checked for bacterial growth by inoculating 1mL of the liquid culture onto the surface of a nutrient agar plate. The cultures with bacterial contamination was known by the colonies appearing on the nutrient agar plate and these cultures were exposed to UV for 30 minutes and then were subcultured repeatedly to get axenic cultures.

The cyanobacterial cultures were scaled up slowly by introducing into 50 mL conical flasks containing liquid medium, then to media in 250 mL conical flasks, and subsequently to media in 1000 mL conical flasks plugged with sterile non – absorbent cotton (Fig. 2.1).

serial dilution agar plating

Pure cultures

Fig 2.1 Isolation of cyanobacterial culture

2.4 Outcome of Isolation

Although a variety of filamentous forms developed in the initial stages of culture, purification to single species was tedious. As purification proceeded many species were excluded from the mainstream purification process as it was difficult to remove the unicellular microalgae from among the tangled filaments. The visual observation on the development of filamentous cyanobacteria is represented in table 2.1 and 2.2. The test tubes positive for filamentous cyanobacterial growth and free of unicellular forms were selected and further purified.

Table 2.1 Results of primary inoculation of samples - observation of blue green growth

(+ present/ - absent)

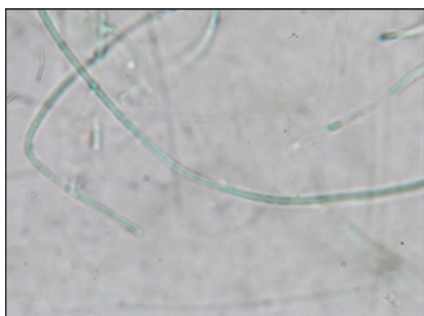
Growth media	Sample	Presence/absence of growth		
		1st stage	2nd stage	3rd stage
f/2	Water	+	+ +	-
	Sediment	+ +	+	+
f/2 with vitamin solution	Water	-	-	-
	Sediment	+ + +	+ +	+
ASN-III	Water	-	-	-
	Sediment	+	-	+ +
Sediment extract medium – full strength	Water	+	+	+
	Sediment	+	-	+
Sediment extract medium – half strength	Water	-	+	-
	Sediment	+	-	+

Table 2.2 Results of microscopic examination of reinoculated samples

Growth media	Filamentous cyanobacteria	Unicellular cyanobacteria
f/2	+ + + + + + + + +	+
f/2 with vitamin solution	+ + + + + +	-
ASN-III	+ + + + + + +	-
Sediment extract medium – full strength	+ + + + + + + +	-

Finally a single species was obtained growing in ASN-III medium. This was identified as *Phormidium tenue* based on morphotaxonomic description of Desikachary (1959). The species identity was not confirmed by a expert. This isolate was assigned the strain number *Phormidium tenue* (strain D2008). Two cultures of cyanobacteria, *Oscillatoria acuminata* and *Synechococcus elongatus* were acquired from the culture collection of School of Environmental Studies, CUSAT for this investigation. The stock cultures obtained were sub-cultured into modified BG-11 medium as they were being maintained in this medium. The cultures that developed within two weeks of inoculation were confirmed for purity and scaled up for this study. These isolates were assigned as *O. acuminata* (strain D2009) and *S. elongatus* (strain D2010). The description of the three species is given below.

1. Phormidium tenue (Menegh.) Gomont



Division : Cyanophyta
Class : Cyanophyceae
Order : Nostocales
Family : Oscillatoriaceae
(Desikachary, 1959, p.259, Pl. 43, Figs. 13-15 & Pl. 44, Figs.7-9)

Thallus pale blue-green, thin, membranous; trichome straight, densely entangled, slightly constricted at the cross- walls, attenuated at the ends, 1-2 µm broad; sheath thin, diffluent; cells up to three times longer than broad, 2.5-5 µm long, septa not granulated, cross-walls visible; end-cell acute-conical, calyptra absent. The present strain is isolated from a brackish water prawn farm in Kochi.

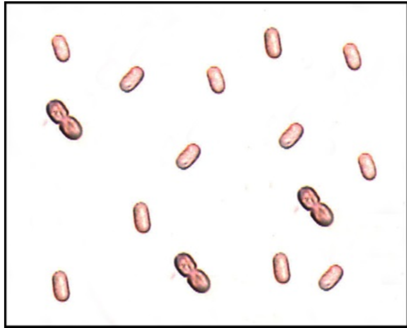
2. *Oscillatoria acuminata* Gomont

Division : Cyanophyta
Class : Cyanophyceae
Order : Nostocales
Family : Oscillatoriaceae
(Desikachary, 1959, p. 240, Pl. 38, Fig. 7 & Pl. 40, Fig. 13)

Thallus blue-green; trichome more or less straight, slightly constricted at the cross-walls, 3 – 5 µm broad, at the ends briefly tapering, sharply pointed, bent; cells were sub quadrate, granulated at the cross walls; end cell mucronate, without calyptra.

This strain of *O. acuminata* was formerly isolated from soil and water samples collected from sewage drains in Kochi.

3. ***Synechococcus elongatus*** **Nag.**



Division	:	Cyanophyta
Class	:	Cyanophyceae
Order	:	Chroococcales
Family	:	Chroococcaceae
		(Desikachary, 1959, p. 143, Pl. 25, Figs. 7,8)

Cells cylindrical, 1.4 – 2 µm broad, 1 ½ - 3 times as long as broad, single or 2 cells together; contents homogenous and light blue-green. This strain of *S. elongatus* was formerly isolated from salinity prone paddy fields (Pokkali fields) of Kochi.

2.5 Maintenance of Cultures

S. elongatus and *O. acuminata* were sub-cultured monthly in BG-11 modified medium (Rippka *et al*., 1979) as this was the medium used for maintaining these cultures for the past years. *Phormidium tenue* was sub-cultured monthly in ASN- III medium as this was the medium of isolation. The three were maintained as batch cultures in 1 L borosilicate culture flask at 28± 2^0C below light panel of day light fluorescent lamps and 12:12 light/dark cycle.

Chapter 3

PRODUCTION OF PHYCOBILIN PIGMENTS BY *PHORMIDIUM TENUE, OSCILLATORIA ACUMINATA* AND *SYNECHOCOCCUS ELONGATUS*

Contents

3.1 Introduction
3.2. Materials and Methods
3.3 Experimental Assessments
3.4 Discussion

3.1 Introduction

The pigments of cyanobacteria comprise chlorophyll *a* in the thylakoid membrane, carotenoids – carotenes (α-carotene and β-carotene) and xanthophylls (violaxanthin, lutein, zeaxanthin, peridinin, fucoxanthin) and the water soluble chromoproteins known as phycobiliproteins, which are assembled into macromolecular aggregates (phycobilisomes) attached to the outer surface of the thylakoid membranes. Some cyanobacteria contain chlorophyll *b* and the cyanobacterium *Acaryochloris marina* contains chlorophyll *d* (Richmond, 2004; Lee, 2008).

Phycobilisomes are the light-harvesting antenna pigment complexes of cyanobacteria, which compose around 40% of the soluble protein of the cell and are responsible for the colour of the cyanobacterial cell (Grossman and Kehoe, 1997). In cyanobacteria, phycobilisomes are found to be regularly arranged in parallel rows on the thylakoid membrane and there are four

different morphological types of phycobilisomes, seen by electron microscopy : (1) Hemidiscoidal , (2) Hemiellipsoidal, (3) Bundle-shaped, and (4) Block – shaped. Phycobiliproteins are usually found to be consisted of hetero-monomers, which have two subunits – α and β. The α and β subunits differ in their molecular mass, amino acid sequence and chromophore content.

Phycobiliproteins are classified into three groups (Gault and Marler, 2009) based on the different chromophores present or their respective absorption spectra: (1) Phycoerythrin (PE; λ_{max}= 490 - 570 nm), (2) Phycocyanin (PC; λ_{max}= 590 – 625 nm) and phycoerythrocyanin (PEC; λ_{max}= 560 – 600 nm), and (3) Allophycocyanin (APC; λ_{max}= 650 – 665 nm).

Photosynthesis is carried out in cyanobacteria by the absorption of light in green, orange and red region of the spectrum collectively by the phycobiliproteins. The molecular structure of phycobilisomes allows phycobiliproteins to transfer the excitation energy absorbed by them to the PS II reaction centre with approximately 80-90% efficiency. The light energy harvested by the phycobilisome is transferred to the reaction centre of PS II via the antenna chlorophylls and the efficiency of this transfer is known to be affected by a wide range of environmental factors by affecting the pigment protein interaction. Some of these environmental factors are heat treatment, low temperature, nitrogen stress, heavy metal stress etc. (Middepogu *et al.*, 2012). Environmental conditions can cause alterations in the ratio of phycobiliprotein pigments and are influenced by the availability of nutrients as well as by environmental factors like temperature, light, water and pH (Simeunovic *et al.*, 2012; Pandhey *et al.*, 2013).

Many cyanobacteria are known to be able to optimize light harvesting and acclimatize to light quality, especially green-to-red light ratios, by a

process known as complementary chromatic adaptation. During this process, cyanobacteria sense the abundance of red light and green light in their environment and produce more of the light harvesting phycobiliprotein, phycocyanin in red light and produce more phycoerythrin in green light, the transition occurring at a wavelength of around 590 nm. Cyanobacteria capable of complementary chromatic adaptation have been classified into four groups based on how their phycobilisomes respond to their light environment :

(1) Group I species contain both phycocyanin and phycoerythrin but do not alter their composition in response to abundance of specific light wavelengths. (2) Group II species start or increase the production of phycoerythrin when exposed to green light but do not alter the production of phycocyanin in response to abundance of certain light wavelengths.

(3) Group III species inhibit the production of phycoerythrin and increase the production of phycocyanin when exposed to red light and decrease the production of phycocyanin and increase the production of phycoerythrin when exposed to green light, and

(4) Group IV found to occur recently in the marine environment and is responsive to changes in blue and green light. During group IV chromatic adaptation there are no significant changes in PBS protein composition unlike group II and Group III chromatic adaptation. Instead the change occurs in the two bilin isomers attached to PE II. A very high ratio of the blue–light absorbing chromophore phycourobilin (PUB ; λ_{max} = 495 nm) to the green–light absorbing chromophore phycoerythrobilin (PEB ; λ_{max} = 545 nm) occurs while growing in blue light and when growing in green light this ratio decreases significantly. The ability to chromatically adapt to the light environment allows the cyanobacterial species to inhabit a niche where

competition from other organisms may limit the availability of light wavelengths (Marsac, 1977; Palenik, 2001; Stowe *et al.*, 2011; Gutu and Kehoe, 2012, Parmar *et al.*, 2013).

Phycobiliproteins have a wide variety of applications and there is great economic potential for its commercial production. Commercially phycobiliproteins are high-value natural products having actual and potential biotechnological applications in pharmaceuticals, neutraceuticals, cosmetic industry, food industry, biomedical research and clinical diagnostics (Manirafasha *et al.*, 2016). A common limitation of marketing of purified phycobiliproteins is their high cost at around $ 10 to 50 per mg of the purified pigment (Ramos *et al.*, 2010). Phycocyanin and phycoerythrin are used as food colourants in chewing gums, jellies, beverages, sweets etc. and they can also be used as colourants while manufacturing cosmetics. These phycobiliproteins are also used in fluorescent labelling of antibodies in diagnostic kits utilised in immunology, cell biology and biomedical research. Phycobiliproteins have also been reported to have anti-inflammatory, hepato-protective, antioxidant and anticancer properties (Mishra *et al.*, 2012; Ores *et al.*, 2016). Phycoerythrin has been reported to be extensively used commercially as a fluorescent dye and in photodynamic therapy (Karseno *et al.*, 2009).

Phycoerythrin has been reported to be widely used in fluorescent probes and many companies are marketing antibodies conjugated with phycoerythrin (Chakdar and Pabbi, 2012). Phycoerytrhin is considered useful for development of fluorescent probes because of its high extinction coeffiecients, mora than 0.8 fluorescence quantum efficiency and stability as hexamers even at lower concentrations without showing decrease in fluorescence (Tang et al., 2016). Presently phycoerythrin is commercially produced from the red algae *Porphyridium* and marketed by various

companies like Ana Spec Inc., Sigma-Aldrich Corporation and Invitrogen Corporation (Pumas *et al.*, 2012, Khattar *et al.*, 2015).

C-phycocyanin (C-PC) has been reported to have antioxidative, anti-inflammatory and anticarcinogenic activities. C-PC has also been reported to be used for the treatment of Alzheimer's disease and Parkinson's disease (Ramos *et al.*, 2010; Lee *et al.*, 2017). Phycocyanin is commercially produced from the cyanobacterium *Spirulina* (*Arthrospira*) *platensis* (Sloth *et al.*, 2006; Martinez *et al.*, 2016).

Allophycocyanin (APC) has been reported to be extensively used as a fluorescent probe especially for flow cytometry. APC has also been reported to have many other applications because of its antioxidant and antienterovirus properties (Su *et al.*, 2010; Chen *et al.*, 2016).

The extraction and purification of phycocyanin and phycoerythrin has been successfully done by many (Rossano *et al.*, 2003; Ranjitha and Kaushik, 2005; Niu *et al.*, 2006; Minkova *et al.*, 2007; Zhu *et al.*, 2007; Tripathi *et al.*, 2007; Ramos *et al.*, 2010; Gupta and Sainis, 2010; Su *et al.*, 2010; Kawsar *et al.*, 2011; Chakdar and Pabbi, 2012; Pumas *et al.*, 2012)and the purity standards for industry has been defined(Kuddus *et al.*, 2013). However the commercial potential of phycobilin pigments has some major obstacles such as widespread utilization, lower product yield which needs to be increased through low-cost production and harvesting technologies, along with evaluation of novel species and environmental conditions for algal production. This investigation is an attempt to find the potential of the three local strains of cyanobacteria for the production of phycobilin pigments, and study the environmental conditions that favour the production of these pigments, in culture conditions.

3.2 Materials and Methods

3.2.1 Evaluation of Growth

The growth of the cultures of the filamentous algae *P.tenue* and *O.acuminata* was measured in terms of biomass as dry weight. The multiplication of the unicellular cyanobacterium *S.elongatus* was determined as cell count. As an additional measure of growth, chlorophyll *a* was also determined.

P. tenue and *O. acuminata* were harvested by filtering through nylon cloth; the filtered sample was rinsed thoroughly with distilled water to wash of any remnants of the culture medium. The samples were blotted dry in a filter paper, and dried at 50^0C overnight to determine the biomass as dry weight. The result was expressed as mg L^{-1}.

The cell count of *S. elongatus* was estimated by counting the cells using a haemocytometer after fixing the cells with lugol's iodine. The result was expressed as cell count x 10^6 mL^{-1}.

To determine the chlorophyll *a* content of *P. tenue* and *O. acuminata*, the samples were blotted dry and 40 mg of sub-samples were suspended in 8 mL of 90% acetone, and kept overnight in dark at 4^0C. These samples were thawed to room temperature and centrifuged at 5000 rpm for 15 minutes. The extract was made upto 10 ml with 90% acetone. The absorbance of the supernatant were read at 664 nm, 665 nm and 750 nm in a spectrophotometer, before and after acidification and chlorophyll a computed as per the equation by Lorenzen (1967) given below.

$$\text{Chlorophyll } a,\ \mu g/L = \frac{26.7\ (664_b - 665_a)\ x\ V_1}{V_2 x\ L}$$

Where:

V_1 = volume of extract, mL

V_2 = volume of sample, L

L = light path length or width of cuvette, cm and

664_b, 665_a = optical densities of 90% acetone extract before and after acidification, respectively.

The value 26.7 is the absorbance correction

The yield of chlorophyll *a* was expressed as mg g^{-1} dry weight of the sample.

To determine the chlorophyll *a* content of *S. elongatus*, 15 mL of the cultures were filtered through a cellulose membrane filter paper and suspended in 90% acetone overnight in dark at 4^0C. These samples were thawed to room temperature, and centrifuged at 5000 rpm for 15 minutes. The extract was made upto 10 mL using 90% acetone. Chlorophyll *a* was estimated according to the method of Lorenzen (1967) and expressed as fg $cell^{-1}$.

3.2.2 Evaluation of Phycobiliproteins

To estimate the phycobiliprotein content of *P. tenue* and *O. acuminata*, the biomass was dried at 50^0C overnight and 0.002 g of the dried biomass was mixed thoroughly with 5 mL of 0.1 M phosphate buffer (pH : 7.0) in a screw capped culture tube. These samples were kept in dark overnight at - 20^0C, and thawed to room temperature. The freezing and thawing was repeated thrice. The extract was centrifuged at 5000 rpm for 15 minutes. The absorbencies of the supernant was read at 562 nm, 615 nm, 652 nm and 750 nm (Siegelman and Kycia, 1978).

Phycocyanin (PC) = $\{A_{615} - (0.474 \text{ x } A_{652})\} / 5.34$ [mg mL^{-1}]

Allophycocyanin (APC) = $\{A_{652} – (0.208 \times A_{615})\} / 5.09$ [mg mL^{-1}]

Phycoerythrin (PE) = $\{A_{562} – (2.41 \times PC) – (0.849 \times APC)\} / 9.62$ [mg mL^{-1}]

The phycobiliprotein content was expressed in mg g^{-1} dry weight.

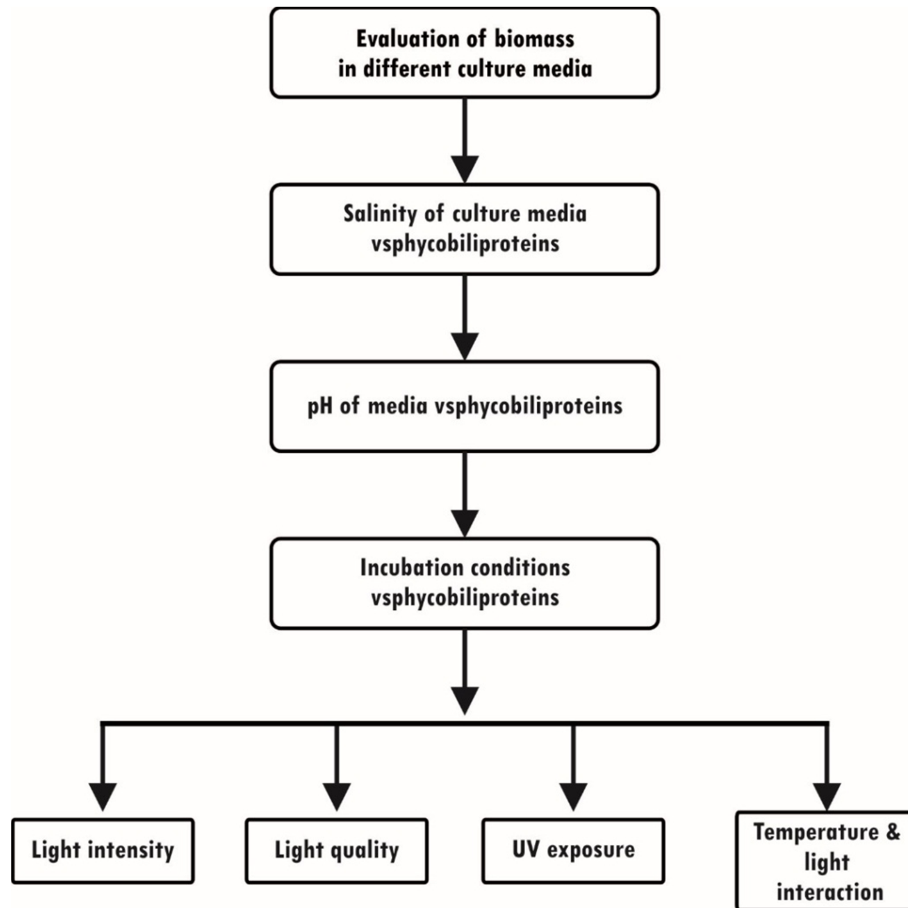
The procedure of extraction of phycobiliproteins followed for *P. tenue* and *O. acuminata* was adopted for *S. elongatus*, but the efficiency of extraction was very low. It was found from literature that *S. elongatus* cells were mechanically more resistant and these cells can be broken by using either a french pressure cell (Wyman, 1992), ultrasonication (Six *et al.*, 2004) or extraction using lysozyme (Vernet *et al.*, 1990). So lysozyme extraction was tried and standardized using 14 day old culture.

- **Extraction of phycobiliproteins using lysozyme**

S. elongatus culture was centrifuged at 5000 rpm for 15 minutes. Supernatant was discarded and pellet was rinsed thrice with distilled water. The pellet was resuspended in 2 mL of 5, 10 and 15 mg L^{-1} lysozyme in eppendorf tubes. These eppendorf tubes were kept at -20^{0}C overnight, and thawed at room temperature. The freezing and thawing was repeated thrice. The three extracts were centrifuged at 5000 rpm for 15 minutes. The supernatants were compared visually, and the absorbance of the extracts were measured. The phycobiliproteins were estimated as before and expressed as fg $cell^{-1}$.

3.2.3 Evaluation of Media and Incubation Conditions

The growth media and incubation conditions for the production of phycocyanin, phycoerythrin and allophycocyanin production were optimized through independent experiments carried out in a sequence, deriving conclusions in every step. The schematic diagram of the evaluation protocol is given below.



Protocol for evaluation of phycobiliproteins

3.3 Experimental Assessments

3.3.1 Evaluation of Culture Media

Cyanobacteria normally follows photoautotrophic production and is employed in open pond cultivation in the tropics and sub-tropics, though it can also be raised under mixotrophic and heterotrophic conditions indoor. In this study the photoautotrophic method is used wherein the species are cultivated in inorganic media. The biomass and yield of phycobiliproteins was tested in different culture media.

Phormidium tenue was grown in f/2 medium, sediment extract medium and ASN-III medium as these three media had shown better growth of the species during the initial isolation phase. f/2 medium is a general enriched medium for growing marine algae from the coastal ecosystems. It has a N:P ratio of 15 : 1 and contains the vitamins - thiamine. HCl (vitamin B1), biotin (vitamin H) and cyanocobalamin (vitamin B12). ASN-III medium is a artificial seawater medium for growing marine algae. It has a N:P ratio of 1 : 1 and contains the vitamin - cyanocobalamin (vitamin B12). Sediment extract medium is a derivative of biphasic soil-water medium for isolation and growing of algae (Pringsheim, 1946).

The media tested were modified BG-11, SN medium and E31 medium for *Oscillatoria acuminata* and *Synechococcus elongatus*. Modified BG-11 medium is a freshwater medium for culturing freshwater, soil, thermal and marine cyanobacteria. It has a N:P ratio of 49.1 : 1 and has no added vitamins. SN medium was prepared as a freshwater medium in place of the original SN medium, which was a marine medium for culturing cyanobacteria. It has a N:P ratio of 4.9 : 1 and contains the vitamins - thiamine. HCl (vitamin B1), biotin (vitamin H) and cyanocobalamin (vitamin B12). E31 medium was prepared as a freshwater medium while the original medium was a marine medium formulated to culture marine algae. It has a N:P ratio of 10 : 1 and contains the vitamins - thiamine. HCl (vitamin B1), biotin (vitamin H) and cyanocobalamin (vitamin B12). The composition of the media is given in Appendix 1.

The three species were inoculated into the respective test media in 250 mL conical flask and plugged with non-absorbent cotton. The inoculum was 30 mg wet weight (1.40 mg dry weight) of *P. tenue* and 30 mg wet weight

(1.32 mg dry weight) of *O. acuminata*. The initial cell density of *S. elongatus* was 1 x 10^6 mL^{-1}. The cultures were incubated at a temperature of 28 ± 2^0C and a photoperiod of 12 L : 12 D at a light intensity of 2200 lux from day light fluorescent lamps. Six replicates were maintained for each. The cultures were incubated for twenty four days. *P.tenue* and *O. acuminata* were evaluated for biomass on the 14th day and *S. elongatus* on the 7th day of incubation.

After incubation of *P. tenue* and *O. acuminata* for 14 days, the entire culture of three replicates were harvested by filtering through nylon cloth and biomass determined as dry weight. After incubation of *S. elongatus* cultures for 7 days, the cell counts were estimated from sub-samples.

The remaining three replication of the cultures of *P. tenue* and *O. acuminata* were sampled for the estimation of pigments phycocyanin, allophycocyanin and phycoerythrin . Sub samples were withdrawn from these replicates on 14th day and 24th day for pigment determination. Sub samples were withdrawn from the three replicates of *S. elongatus* on the 7^{th}day and 24th day for pigment determination. The phycobilin pigments were determined as described in section 3.2.2.

- **Results**

Phormidium tenue produced the highest yield of 94.42 mg L^{-1}dry weight in ASN-III, closely followed by 81.94 mg L^{-1}dry weight in f/2, and 72.56 mg L^{-1} dry weight in sediment extract medium. The analysis of variance of the yield revealed that the difference was significant with p = 7.21E-08 (Table 3.1). Among the three, ASN-III was selected for further studies.

Table 3.1 Biomass production by *Phormidium tenue* in different growth media (growth period – 14 days)

Replications	Biomass (mg L^{-1}dry weight)		
	f/2	Sediment extract medium	ASN-III
1	81.76	72.56	95.06
2	82.84	73.18	93.73
3	81.23	71.93	94.48
Mean	81.94	72.56	94.42

Analysis of Variance

Source of Variation	Sum of squares	df	Mean Square	F	P-value	F crit
Between Groups	722.01	2	361.01	717.94	7.21E-08*	5.14
Within Groups	3.02	6	0.50			
Total	725.028	8				

*Significant at 0.01 level

Oscillatoria acuminata produced a highest yield of 58.76 mg L^{-1}dry weight in modified BG-11, followed by 40.38 mg L^{-1}dry weight in E31 medium and 36.53 mg L^{-1}dry weight in SN medium. The analysis of variance of the yield revealed a significant difference with p = 1.36E-09 (Table 3.2). As the yield was highest in modified BG-11 medium, it was inferred as the most suitable medium for growth of *O. acuminata.*

Table 3.2 Biomass production by *Oscillatoria acuminata* in different growth media (growth period – 14 days)

Replications	Biomass (mg L^{-1}dry weight)		
	Modified BG-11	SN	E31
1	59.04	36.12	40.71
2	58.41	37.05	40.45
3	58.83	36.41	39.97
Mean	58.76	36.53	40.38

Analysis of Variance

Source of Variation	Sum of squares	df	Mean Square	F	P-value	F crit
Between Groups	847.09	2	423.55	2701.95	1.36E-09*	5.14
Within Groups	0.94	6	0.16			
Total	848.03	8				

*Significant at 0.01 level

The growth of *Synechococcus elongatus* in the three selected growth media showed that the highest biomass of 5.65 x 10^6 mL^{-1} was produced in modified BG-11, followed by 4.97 x 10^6 mL^{-1} in E31 medium and 3.22 x 10^6 mL^{-1} in SN medium. The analysis of variance of the yield revealed a significant difference with p = 0.02 with respect to cell yield (Table 3.3). The cell count was highest in modified BG-11; therefore modified BG-11 medium was inferred as the most suitable medium for growth of *S. elongatus*.

Table 3.3 Biomass production by *Synechococcus elongatus* in different growth media (growth period – 7 days)

Replications	Cell count (x 10^6 mL^{-1})		
	Modified BG-11	SN	E31
1	6.80	3.36	4.30
2	4.50	2.79	5.30
3	5.65	3.52	5.30
Mean	5.65	3.22	4.97

Analysis of Variance						
Source of Variation	Sum of squares	df	Mean Square	F	P-value	F crit
Between Groups	9.39	2	4.70	7.82	0.02*	5.14
Within Groups	3.61	6	0.60			
Total	13.00	8				

*Significant at 0.05 level

- **Culture media and phycobiliproteins**

Phycocyanin content of *P. tenue* was 32.57 mg g^{-1} dry weight on the 14th day and 34.51 mg g^{-1} dry weight on the 24^{th}day. Allophycocyanin was 10.78 mg g^{-1} dry weight on the 14th day and 12.29 mg g^{-1} dry weight on the 24th day. Phycoerythrin yield was 3.88 mg g^{-1} dry weight on the 14th day and 5.05 mg g^{-1} dry weight on the 24th day. The analysis of the data by student's t test revealed that there was no significant difference in yield between 14 an 24 days of incubation (Table 3.4).

Table 3.4 Production of phycobilins by *Phormidium tenue*

Days of growth	mg g^{-1} dry weight		
	Phycocyanin	Allophycocyanin	Phycoerythrin
14	32.57	10.78	3.88
24	34.51	12.29	5.05
P value	0.08	0.06	0.05

n = 3; level of significance $P < 0.05$

Phycocyanin content of *O. acuminata* was 98.80 mg g^{-1} dry weight on the 14th day and 100.82 mg g^{-1} dry weight on the 24th day. Allophycocyanin content was 48.07 mg g^{-1} dry weight on the 14th day and 50.08 mg g^{-1} dry weight on the 24th day. Phycoerythrin content of *O. acuminata* was 21.02 mg g^{-1} dry weight on the 14th day and 23.04 mg g^{-1} dry weight on the 24th day. The analysis of the data by student's t test revealed that there is significant difference in yield between 14 an 24 days of incubation; the yield on 24th day is higher (Table 3.5).

Table 3.5 Production of phycobilins by *Oscillatoria acuminata*

Days of growth	mg g^{-1} dry weight		
	Phycocyanin	Allophycocyanin	Phycoerythrin
14	98.80	48.07	21.02
24	100.82	50.08	23.04
P value	0.02	0.002	0.003

n = 3; significant at $P < 0.05$

- **Standardisation of lysozyme extraction of *S. elongatus***

Adoption of the lysozyme extraction method for *S. elongatus* served to increase the yield of phycobiliproteins. Fig. 3.1 provides a visual comparison of the extracts of phycobiliproteins from *S. elongatus* at 5, 10 and 15 mg L^{-1} lysozyme and the regular freeze and thaw extraction control without lysozyme. The lysozyme concentration of 15 mg L^{-1} gave the best extraction. The quantitative determination of the pigments based on their absorbance also

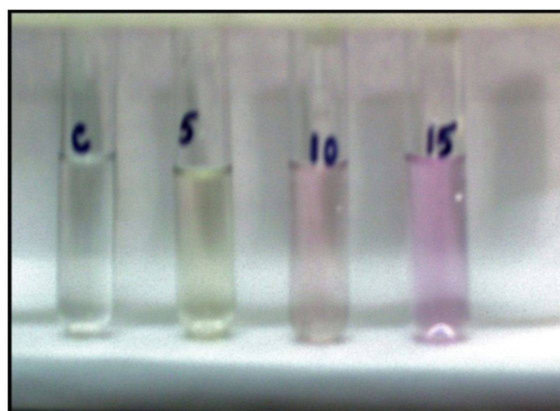


Fig. 3.1 Extracts of phycobiliproteins from *S. elongatus* using 5, 10 and 15 mg L^{-1} of lysozyme and control without lysozyme

yielded similar results (Table 3.6). The highest phycocyanin of 2.36 fg $cell^{-1}$ was obtained upon extraction of *S. elongatus* with 15 mg L^{-1} lysozyme. Similarly the allophycocyanin and phycoerythrin yield were higher at 15 mg L^{-1} of lysozyme. Therefore, 15 mg L^{-1} was selected for further extraction of phycobiliproteins from *S. elongatus* in all the evaluation steps.

Table 3.6 Yield of phycobiliproteins of *S. elongatus* at different concentrations of lysozyme (mean of duplicate values)

Pigment (fg $cell^{-1}$)	Control	5 mg L^{-1}	10 mg L^{-1}	15 mg L^{-1}
Phycocyanin	0.02	1.52	2.03	2.36
Allophycocyanin	0.03	1.75	3.17	3.55
Phycoerythrin	0.02	1.08	2.10	2.94
Total Phycobiliproteins	0.07	4.35	7.30	8.84

The *S. elongatus* cultures raised in BG-11 medium yielded 1.99 fg $cell^{-1}$ on the 7th day and 2.46 fg $cell^{-1}$ on the 24th day. Allophycocyanin content of *S. elongatus* was 2.71 fg $cell^{-1}$ on the 7th day and 3.47 fg $cell^{-1}$ on the 24th day. Phycoerythrin content of *S. elongatus* was 1.83 fg $cell^{-1}$ on the 7th day and 1.95 fg $cell^{-1}$ on the 24th day. The analysis of the data by student's t test revealed that there was no significant difference in yield between 7 and 24 days of incubation (Table 3.7).

Table 3.7 Production of phycobilins by *Synechococcus elongatus*

Days of growth	fg $cell^{-1}$		
	Phycocyanin	Allophycocyanin	Phycoerythrin
7	1.99	2.71	1.83
24	2.46	3.47	1.95
P value	0.12	0.13	0.46

n = 3; significant at $P < 0.05$

Based on the above observations ASNIII medium and an incubation period of 14 days is selected for *Phormidium tenue.* Modified BG-11 medium and an incubation period 24 days yield higher quantity of pigments in *Oscillatoria acuminata*; but the increase of the pigments after the 14th day is only 2-8%; therefore an incubation period of 14 days is selected for further experiments. BG-11 medium and an incubation period of 7 days is selected for *Synechococcus elongatus* based on the experimental results.

3.3.2 Effect of Salinity on Pigment Production

The most significant effects of salinity on algal growth are the osmotic consequences of movements of water molecules along water-potential gradients, and the flow of ions along electrochemical gradients. When any change in salinities occur, algae respond with an osmo-acclimation process or turgor pressure regulation. It has been found that some organisms are known to be osmotically adaptive and take very little effort in adjusting to the external environment, while others are more sensitive to osmotic changes. It has been suggested that variations in salinity could be a factor in the growth rate of algae (Fu and Bell, 2003). During the process of adaptation to varying amount of salinity, the balance in water potential occurs by extruding inorganic ions from the cell and the accumulation of osmoprotective compounds in the cell. One of the systems in the cell which adapts very quickly to the salt stress caused by salinity is the photosynthetic system, and after the pigments which were

bleached are synthesized again, the other metabolic activities are regained by the algae. It is important to evaluate the salinity tolerance of microalgae as salt concentration of the culture medium in open pond cultures increases because of the daily evaporation in arid and semiarid conditions (Pal *et al.*, 2011). Cyanobacteria are known to accumulate glucosyl-glycerol as the osmoprotective compound (Nagle *et al.*, 2010). Different cyanobacteria are reported to have varying degrees of tolerance to salinity (Srivastava *et al.*, 2009; Swapnil *et al.*, 2015; Yadav *et al.*, 2016). Cyanobacteria have been successfully applied for reclamation of saline soils, and because of the potential economic implications there has been a focus on research regarding the salinity tolerance of cyanobacteria (Rai and Abraham, 1993; Kheirfam *et al.*, 2017).

The influence of salinity of the selected medium on production of biomass and pigments by the three cyanobacterial species was evaluated by growing them in medium of different salinities prepared by adjusting the salinity by adding NaCl.

Phormidium tenue was being maintained in ASN III medium at salinity 25 x 10^{-3}. This strain did not grow in any freshwater media during the process of isolation. Therefore the tolerance of this culture to higher levels of salinity than that of the maintenance medium was studied, and how the salinity influenced the pigment production was analysed.

30 mg samples of *P. tenue* were inoculated into ASN III media of salinities 25 (control), 30 and 35 x 10^{-3} and incubated for 14 days as before. Each level of salinity had six replicates. The biomass of three replicates were determined separately as dry weight. Sub samples from the rest of the three were withdrawn to determine chlorophyll *a*, phycocyanin, allophycocyanin and phycoerythrin.

Oscillatoria acuminata was similarly grown in modified BG-11 medium (freshwater control) and media of salinity levels 5,15,25 and 35 x 10^{-3} . After incubation of *P. tenue* and *O. acuminata* for 14 days, the entire culture of three replicates were harvested and dry weight determined. Subsamples were withdrawn from the other three replicate test cultures to estimate the pigments. Chlorophyll *a* and phycobiliproteins were determined as before.

Synechococcus elongatus was inoculated into modified BG-11 medium (freshwater control), 5,15,25 and 35 x 10^{-3} at a cell concentration of $1x10^6$ mL^{-1} in triplicates. The cell count was measured after 7 days of incubation and the pigments were estimated. Data were subjected to one way ANOVA followed by Tukey's test at the 5% significant level (P-value < 0.05) using the program Ky plot (Appendix 2). PC: PE ratio was computed.

- **Results**

Microscope observations revealed morphological changes in *P.tenue* as the salinity of the medium increased. The filaments were bent and cells were seen to separate within the membrane at higher salinities. The biomass of *P. tenue* was significantly affected by salinity of the media. The highest biomass of 86.97 mg L^{-1} dry weight was obtained in 25 x 10^{-3} and lowest biomass of 40.87 mg L^{-1} dry weight in 35 x 10^{-3}. The amount of chlorophyll *a* did not show any significant difference with salinity of the medium (Table 3.8).

Table 3.8 Biomass and production of chlorophyll a by *Phormidium tenue* following growth in different salinities

Salinity (x 10^{-3})	*P. tenue*	
	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry Weight)
25	86.97 a*	11.56 a
30	57.00 b	11.37 a
35	40.87 c	10.78 a

* Figures sharing the same letters in the same column do not differ significantly at P < 0.05

Oscillatoria acuminata exhibited changes in morphology and pigmentation of cells when grown in saline media. Shorter filaments were observed in cells grown at 15 x 10^{-3} and it was noticed that there was slight fading of the blue green colour. The fading of the blue green pigment was more prominent among cells grown at 25 x 10^{-3}. It was observed that cells grown at 35 x 10^{-3} had the blue green colour only in some portions of the filaments and the remaining filament was completely devoid of colour. The biomass of *O. acuminata* was significantly affected by salinity of the media. The highest biomass of 93.24 mg L^{-1} dry weight was obtained in the control in fresh water. Biomass was significantly reduced even at salinity 5 x 10^{-3}. The lowest biomass was 19.71 mg L^{-1} dry weight in 35 x 10^{-3} . Chlorophyll *a* was significantly reduced at salinity 25 x 10^{-3} and above (Table 3.9).

Changes in morphology and leaching of pigments was observed when *S. elongatus* was grown in saline media . The cell density of *S. elongatus* culture was similar in control, 5 and 15 x 10^{-3} and it decreased significantly at >15 x 10^{-3}. Chlorophyll *a* was significantly lower at > 5 x 10^{-3} (Table 3.9).

Table 3.9 Biomass and production of chlorophyll a by *Oscillatoria acuminata* and *Synechococcus elongatus* following growth in different salinities

Salinity (x 10^{-3})	*O. acuminata*		*S. elongatus*	
	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry weight)	Cell count (x 10^6 ml^{-1})	Chlorophyll *a* (fg $cell^{-1}$)
Control	93.24 a*	7.17 a	9.42 a	4.93 a
5	81.96 b	7.84 a	10.05 a	4.60 a
15	42.60 c	6.77 a	9.19 a	2.55 b
25	29.33 d	4.90 b	6.14 b	1.97 b
35	19.71 e	0.92 c	1.78 c	1.66 b

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

➢ Salinity and phycobiliproteins

Phycocyanin content of *P. tenue* was 32.56 mg g^{-1} dry weight at 25 x $10^{-3}$and it increased to 38.76mg g^{-1} dry weight at 30 x 10^{-3}; phycocyanin content was statistically similar at 30 and 35 x 10^{-3}. The yield of allophycocyanin and phycoerythrin was not affected by salinity of the medium. The ratio of PC/PE was nearly constant (Table 3.10).

Table 3.10 Phycobiliprotein content of *Phormidium tenue* in media of different salinity

Salinity (x 10^{-3})	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)	PC/E
25	32.56 a*	10.27 a	3.61 a	9.02
30	38.76 b	12.67 a	4.33 a	8.95
35	36.67 b	12.13 a	4.11 a	8.92

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

The phycobiliprotein production of *O. acuminata* was affected by salinity of the media. The medium of salinity 5 x 10^{-3} produced the highest yield of phycocyanin and allophycocyanin; while the production of phycoerythrin was similar in both control and 5 x 10^{-3} . The ratio of PC/PE was nearly constant (Table 3.11).

Table 3.11 Phycobiliprotein content of *Oscillatoria acuminata* in media of dfferent salinity

Salinity (x 10^{-3})	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)	PC/PE
Control	96.54 a*	46.64 a	20.53 a	4.70
5	99.87 b	48.59 b	21.20 a	4.71
15	85.75 c	41.76 c	18.25 b	4.70
25	72.51 d	35.30 d	15.40 c	4.71
35	64.16 e	31.46 e	13.64 d	4.70

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

The pigments – phycocyanin and allophycocyanin of *S.elongatus* increased with salinity of the media and was highest in 25 x 10^{-3}. The phycoerythrin content was not significantly different. The ratio of PC/PE fluctuated with salinity (Table 3.12).

Table 3.12 Phycobiliprotein content of *Synechococcus elongatus* in media of different salinity

Salinity (x 10^{-3})	PC (fg $cell^{-1}$)	APC (fg $cell^{-1}$)	PE (fg $cell^{-1}$)	PC/PE
Control	1.13 a b*	1.63 a b	1.03 a	2.83
5	1.15 a b	1.65 a b	1.06 a	1.98
15	1.28 a b c	1.87 a b	1.17 b	1.51
25	1.67 c	2.60 c	1.54 c	2.14
35	1.06 b	1.53 b	0.97 d	3.03

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

It is inferred that *P.tenue* can be raised at 25 x 10^{-3} ASN III medium as it produces the largest biomass and pigments in this medium. *O.acuminata* could be best grown in freshwater medium for biomass; but chlorophyll *a*, phycocyanin and allophycocyanin are higher at 5 x 10^{-3}. Considering the higher biomass and chlorophyll *a* which will support sustained growth of the culture, the freshwater medium is selected for further evaluation. Although salinity seems to favour the production of phycobilins by *S.elongatus* there are no distinct trends observed. Therefore the freshwater medium is selected for further evaluation.

3.3.3 Effect of pH on Pigment Production

The pH of freshwater may fluctuate dramatically in environments where the carbonate buffering system is weak. It has been shown by a number of studies that in spite of the strong buffering capacity of the carbonate system in seawater, significant pH changes also occur in marine

ecosystems (Xu *et al.*, 2017). So in many marine environments pH may significantly regulate algal abundance and distribution. Generally changes in pH level appear to correlate with changes in temperature, dissolved oxygen and phytoplankton production (Chen and Durbin, 1994; Gonçalves-Araujo, 2016). The pH value of the culture medium along with dry cell weight may be a indirect method for determination of the extent of cell growth, because the pH gradually rises as bicarbonate added to the medium dissolves to produce carbon dioxide which in turn releases OH^- during the culture period. It has been suggested that the pH of the culture medium has to be controlled, as pH increases, it causes autoinhibition of cell growth (Pandey *et al.*, 2010; Ai *et al.*, 2017).

The effect of pH on the production of chlorophyll *a*, phycocyanin , allophycocyanin and phycoerythrin by the three species was investigated by growing them in the selected culture media modified to different pH.

The pH of the ASN-III medium used for maintenance is 7.3 and that of modified BG-11 is 7.4. These media were modified by adding Na_2CO_3/ HCl to obtain five levels of pH for testing the effect of pH on pigment production. These test pH were 6.5, 7.5, 8.5, 9.5 and 10.5. The test media were inoculated with *P. tenue* and *O. acuminata* at the rate of 30 mg (wet weight) and *S. elongatus* at the rate of 1×10^6 mL^{-1}. The test setup was same as given in section 3.3.1. In case of both *P. tenue* and *O. acuminata*, six replicates were maintained; three for evaluating biomass, and three for estimating pigments. Three replicates were maintained for *S. elongatus*.

After incubation for 14 days, for *P. tenue* and *O. acuminata*, and 7 days for *S. elongatus*, the biomass and pigments of all test cultures were determined as before.

Data were subjected to one way ANOVA at the 5% significant level (p-value < 0.05) using excel data analysis package and Tukey test using Ky plot (Appendix 3). PC : PE ratio was computed.

- **Results**

Microscope observations did not reveal any morphological changes in *P.tenue* due to variation in pH. The biomass of *P. tenue* was significantly affected by pH of the media. At pH : 7.5 the highest biomass of 86.96 mg L^{-1} dry weight was obtained. Chlorophyll *a* content did not differ significantly from pH 6.5 to pH 8.5 (Table 3.13).

Table 3.13 Biomass and production of chlorophyll *a* by *Phormidium tenue*, *Oscillatoria acuminata*, and *Synechococcus elongatus* following growth in different pH

pH	*P. tenue*		*O. acuminata*		*S. elongatus*	
	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry Weight)	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry Weight)	Cell count (x 10^6 ml^{-1})	Chlorophyll *a* (fg $cell^{-1}$)
6.5	64.09 a*	8.98 a b	72.63 a	5.72 a	5.50 a	3.45 a c
7.5	86.96 b	11.52 a	89.92 b	8.00 b	6.46 a	4.93 b d
8.5	73.20 c	10.61 a b	85.90 c	6.11 a	6.75 a	3.89 a d
9.5	66.61 d	8.46 b	61.69 d	5.64 a	7.35 a	2.37 c e
10.5	39.82 e	5.64 c	39.13 e	0.97 c	5.83 a	1.73 e

* Figures sharing the same letters in the same column do not differ significantly at P < 0.05

Microscope observations did not reveal any morphological changes in *O. acuminata* at different levels of pH. The biomass production of *O. acuminata* was significantly affected by pH of the media. At pH : 7.5 the highest biomass of 89.92 mg L^{-1}dry weight was obtained. Chlorophyll *a* was also similarly high at pH 7.5 (Table 3.13).

Microscope observations did not reveal any morphological changes in *S. elongatus* with variation in pH. The highest cell count of 7.35x 10^6 mL^{-1} was obtained at pH : 9.5. Analysis of variance revealed that there was no

significant difference in cell count at different pH levels. Chlorophyll *a* was significantly high at pH 7.5 (Table 3.13).

➢ pH and phycobiliproteins

The growth media of pH 6.5 and 7.5 favoured the production of phycocyanin. There was no significant reduction in allophycocyanin and phycoerythrin even at pH 9.5. The ratio of PC/PE was nearly constant(Table 3.14)

Table 3.14 Phycobiliprotein content of *Phormidium tenue* in media of different pH

pH	PC ($mg\ g^{-1}$ Dry weight)	APC ($mg\ g^{-1}$ Dry weight)	PE ($mg\ g^{-1}$ Dry weight)	PC/PE
6.5	31.01 a b*	10.33 a b	3.67 a b	8.45
7.5	32.49 a	10.76 a b	3.87 a b	8.40
8.5	28.67 b c	9.54 a c	3.42 a b c	8.38
9.5	26.75 c	9.18 b c	3.08 b c	8.69
10.5	21.45 d	7.16 d	2.51 c	8.55

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

The yield of phycocyanin, allophycocyanin and phycoerythrin of *O.acuminata* was similar from pH 6.5 to 8.5 . Beyond pH 8.5 the yield decreased. The PC/PE ratio was constant throughout(Table 3.15).

Table 3.15 Phycobiliprotein content of *Oscillatoria acuminata*` in media of different pH

pH	PC ($mg\ g^{-1}$ Dry weight)	APC ($mg\ g^{-1}$ Dry weight)	PE ($mg\ g^{-1}$ Dry weight)	PC/PE
6.5	93.87 a*	45.64 a	19.97 a	4.70
7.5	97.58 a	47.48 a	20.76 a	4.70
8.5	95.50 a	46.45 a	20.32 a	4.70
9.5	81.33 b	39.58 b	17.40 b	4.67
10.5	72.75 c	35.39 b	15.48 b	4.70

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

The amount of phycocyanin and allophycocyanin of *S.elongatus* was distinctively higher in medium at pH 7.5. The content of phycoerythrin did not differ significantly at pH 6.5 and 7.5. Towards higher pH it decreased significantly. The ratio of PC to PE varied considerably. At pH 6.5 the ratio was 0.74 indicating that lower pH would favour the production of phycoerythrin (Table 3.16).

Table 3.16 Phycobiliprotein content of *Synechococcus elongatus* in media of different pH

pH	PC (fg cell^{-1})	APC (fg cell^{-1})	PE (fg cell^{-1})	PC/PE
6.5	1.13 a*	1.63 a	1.53 a	0.74
7.5	2.42 b	2.69 b	1.78 a	1.36
8.5	1.38 a	1.79 a	0.57 b	2.42
9.5	1.23 a	1.40 a	0.23 b	5.35
10.5	1.02 a	1.23 a	0.14 b	7.29

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

It is inferred that *P.tenue* can be cultivated at any pH from 6.5 to 8.5 for the production of phycobilins; but the production of biomass is favoured at pH 7.5 so that this pH may be selected for the pigment production. *O.acuminata* also shows the same response; therefore the pH 7.5 is selected. *S.elongatus* favours pH 7.5 for production of phycocyanin and allophycocyanin; but when the pH is lowered to 6.5 there occurs a higher production of phycoerythrin over phycocyanin resulting in lowering of PC/PE to 0.74. The cell density acquired by this species is not significantly different at pH 6.5 and 7.5. So it can be concluded that for a production system aiming at phycoerythrin , the pH of the medium may be set at 6.5.

3.3.4 Effect of Light Intensity on Pigment Production

As a result of changes in the environmental conditions like light and temperature, cyanobacteria are known to exhibit a wide variety of cellular

responses. Apart from the main source of energy, light can be a limiting substrate in low light conditions and a inhibiting substrate in high light conditions. In comparison with all the other factors, light is considered to be a important factor for determining the growth rate and CO_2 biofixation of a photosynthetic organism. It has been experimentally proven that light intensity is a significant factor for biomass production and also for the synthesis of proteins (Jitendra *et al.*, 2012; Szwaja *et al.*, 2016). In pure water there is a selective absorption of light and photons with wavelengths of ~ 700 nm (red) having a higher chance of being absorbed than those with wavelengths ~ 400 nm (blue) which are poorly captured by the water molecules. During vertical migration of cyanobacteria in water, there occurs changes in light intensity as well as light quality. Algae in aquatic ecosystems encounter seasonal variations in day length and temperature. Genetic and physiological differences have been cited as reasons for the high species specificity of the optimal daylength and growth per unit light dose (Tang and Vincent, 2000; Martinez *et al.*, 2016).

The effect of exposure to two different light intensities on the growth and pigment production of the three species of cyanobacteria was evaluated for this investigation.

30 mg wet weight of samples of *P. tenue* and *O.acuminata* were inoculated into ASN III media and modified BG-11 medium respectively in 250mL culture flasks in six replicates as in previous experiments. *S. elongatu*s was inoculated into modified BG-11 medium at a cell concentration of $1x10^6$ mL^{-1} in triplicate.The test cultures were incubated at a temperature of 28 ± 2^0C and a photoperiod of 12 L : 12 D but under high and low light intensities. These were from 2200 lux white day light fluorescent lamps, and 500 lux, the ambient light of the laboratory, when cultures were kept on a southern window sill.

After incubation for 14 days, the entire culture of *P. tenue* and *O. acuminata* in triplicates were harvested and biomass determined as dry weight. After incubation of *S. elongatus* cultures for 7 days, samples were withdrawn from the triplicates and the cell counts were estimated. Subsamples were withdrawn from the respective replicate cultures and pigments were estimated as before. Data were subjected to the student's t-test (P-value ≤ 0.05) using excel data analysis package.

- **Results**

There was no visible difference between the cultures of *P.tenue* grown at 2200 lux and 500 lux . Upon incubation for 14 days the biomass increased to 54.73 mg L^{-1} dry weight at a light intensity of 2200 lux, and 60.09 mg L^{-1}dry weight at a light intensity of 500 lux . From t-test it was inferred that there was significant increase in biomass at 500 lux . Chlorophyll *a* production was also higher at the lower light intensity (Table 3.17; Appendix 4).

Table 3.17 Biomass and production of chlorophyll *a* by *Phormidium tenue, Oscillatoria acuminata,* and *Synechococcus elongatus* following growth in 2200 lux and 500 lux of light intensity

Light intensity (lux)	*P. tenue*		*O. acuminata*		*S. elongatus*	
	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry weight)	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry weight)	Cell count (x 10^6 ml^{-1})	Chlorophyll *a* (fg $cell^{-1}$)
2200	54.73	11.43	48.05	9.08	9.42	5.47
500	60.09*	13.91*	54.42*	6.74*	4.80*	5.34

n=3; significant level * P ≤ 0.05

There was no visible difference between the cultures of *O. acuminata* grown at 2200 lux and 500 lux . Upon incubation the biomass increased to 48.05 mg dry weight at a light intensity of 2200 lux and 54.42 mg dry weight at a light intensity of 500 lux. The production of chlorophyll *a* was significantly lower at low light (Table 3.17).

There was visible difference in the appearance of *S. elongatus* cultures grown at 2200 lux and 500 lux. The former appeared reddish brown and the latter blue-green in colour (Fig. 3.2).

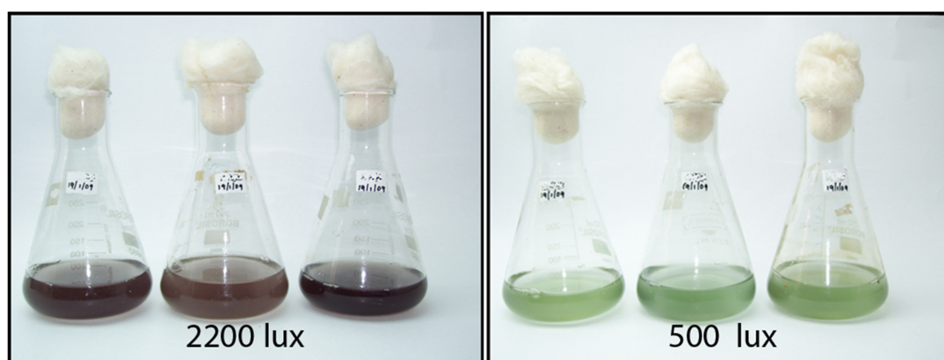


Fig. 3.2 *S. elongatus* cultures grown at two different light intensities

The cell density of *S. elongatus* increased from the initial inoculam of 1 x 10^6 ml^{-1} to 9.42 x 10^6 mL^{-1} when grown at 2200 lux and to 4.80 x 10^6 ml^{-1} at 500 lux. It was inferred from the t-test that there was significant decrease in cell density at low light. The chlorophyll *a* content was similar in both (Table 3.17).

- ***Light intensity and phycobiliproteins***

The amount of phycobilin pigments produced by *P.tenue* under the two light intensities is presented in table 3.18. The phycocyanin component increased by 33.63%, allophycocyanin increased by 13.31% and phycoerythrin decreased by 41.69% under 500 lux compared to that at 2200 lux (Fig. 3.3).

Table 3.18 Production of phycobilin pigments by *Phormidium tenue, Oscillatoria acuminata,* and *Synechococcus elongatus* following growth at 2200 lux and 500 lux

Species	Light intensity (lux)	PC	APC	PE	PC : PE
P. tenue	2200	32.57	10.78	3.88	8.39
(mg g^{-1} dry weight)	500	43.52*	12.21*	2.26*	19.26
O. acuminata	2200	98.80	48.07	21.02	4.70
(mg g^{-1} dry weight)	500	116.04*	50.48*	24.28*	4.78
S. elongatus	2200	2.11	2.57	2.08	1.01
(fg cell^{-1})	500	2.24	3.43	1.48	1.51

n=3; significant level * $p < 0.05$; PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

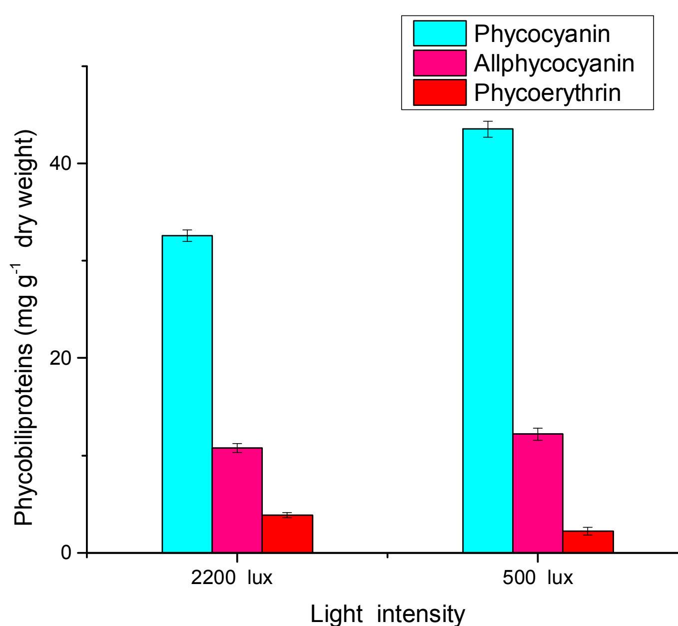


Fig. 3.3 Effect of light intensity on relative production of phycobiliprotein pigments of *Phormidium tenue*

The ratio of phycocyanin to phycoerythrin was 8.39 at 2200 lux and 19.26 at 500 lux. The change in the relative distribution of the phycobilin pigments was significant.

The amount of phycobilin pigments produced by *O.acuminata* under the two light intensities is presented in table 3.18. The phycocyanin component increased by 17.45%, allophycocyanin increased by 5.01% and phycoerythrin increased by 15.51% under 500 lux compared to that at 2200 lux (Fig. 3.4). The ratio of phycocyanin to phycoerythrin was 4.70 at 2200 lux and 4.78 at 500 lux.

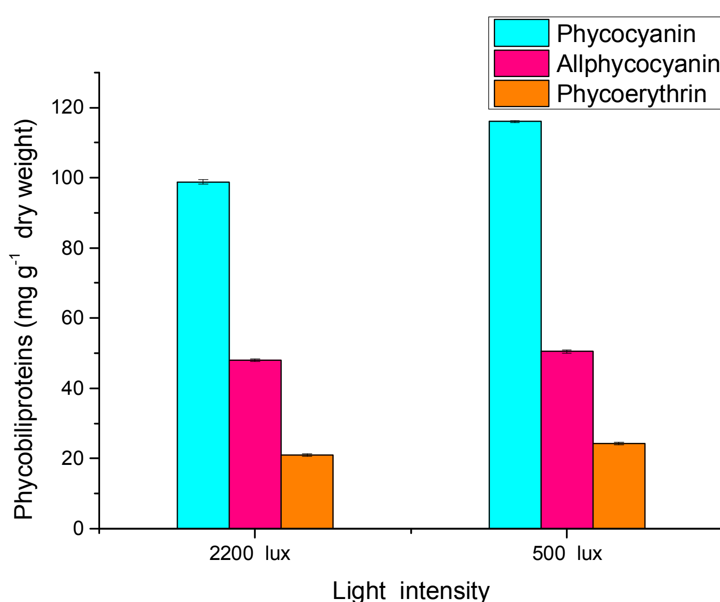


Fig. 3.4 Effect of light intensity on relative production of phycobiliprotein pigments of *Oscillatoria acuminata*

The amount of phycobilin pigments produced by *S. elngatus* under the two light intensities is presented in table 3.18. The phycocyanin component increased by 6.26%, allophycocyanin increased by 33.53% and phycoerythrin decreased by 28.86% under 500 lux compared to that at 2200 lux(Fig. 3.5). The ratio of phycocyanin to phycoerythrin was 1.01 at 2200 lux and 1.52 at 500 lux.

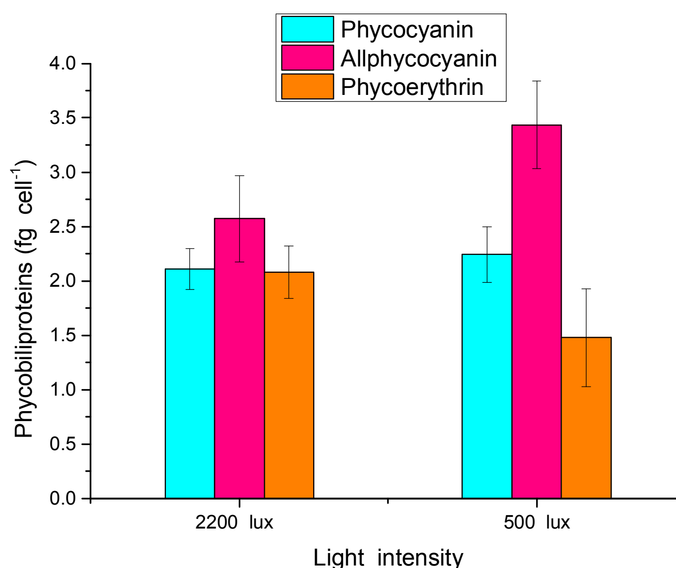


Fig. 3.5 Effect of light intensity on relative production of phycobiliprotein pigments of *Synechococcus elongatus*

In general the lower light intensity seems to be favourable for production of phycocyanin and allophycocyanin. *P. tenue* and *S. elongatus* have higher phycoerythrin in high light intensity while *O. acuminata* increase the phycoerythrin production at lower light. However in *P.tenue* and *O.acuminata* the production of phycocyanin and allophycocyanin is considerably high compared to phycoerythrin thereby imparting the blue green colour to the cells. The relative proportion of phycocyanin and phycoerythrin is equal in *S.elongatus* when grown under 2200 lux. At 500 lux both phycocyanin and allophycocyanin is increasing and phycoerythrin is decreasing with a PC/PE ratio of 1.52. Therefore the colour of the cells appears blue green. Therefore in a production system for phycoerythrin using *S.elongatus* higher light intensity is preferred.

3.3.5 Effect of Light Quality on Pigment Production

Many cyanobacteria are known to be able to optimize light harvesting and acclimatize to light quality, especially green-to-red light ratios, by a process

known as complementary chromatic adaptation. During this process, cyanobacteria sense the abundance of red light and green light in their environment and produce more of the light harvesting phycobiliprotein, phycocyanin in red light and produce more phycoerythrin in green light, the transition occurring at a wavelength of around 590 nm. Cyanobacteria capable of complementary chromatic adaptation have been classified into three groups based on how their phybilisomes respond to their light environment : (1) Group I species contain both phycocyanin and phycoerythrin but do not alter their composition in response to abundance of specific light wavelengths. (2) Group II species start or increase the production of phycoerythrin when exposed to green light but do not alter the production of phycocyanin in response to abundance of certain light wavelengths. (3) Group III species inhibit the production of phycoerythrin and increase the production of phycocyanin when exposed to red light and decrease the production of phycocyanin and increase the production of phycoerythrin when exposed to green light and Group IV has been found to occur until recently in the marine environment and is responsive to changes in blue and green light. During group IV chromatic adaptation there are no significant changes in PBS protein composition unlike group II and Group III chromatic adaptation. Instead the change occurs in the two bilin isomers attached to PE II. A very high ratio of the blue – light absorbing chromophore phycourobilin (PUB ; λ_{max} = 495 nm) to the green – light absorbing chromophore phycoerythrobilin (PEB ; λ_{max} = 545 nm) occurs while growing in blue light and when growing in green light this ratio decreases significantly. The ability to chromatically adapt to the light environment allows the cyanobacterial species to inhabit a niche were competition from other organisms may limit the availability of light wavelengths (Marsac, 1977; Palenik, 2001; Stowe *et al.*, 2011; Gutu and Kehoe, 2012; Herbstova *et al.*, 2015).

The effect of different light wavelengths on the phycobilin composition of three species of cyanobacteria was evaluated.

30 mg wet weight of samples of *P. tenue* and *O.acuminata* were inoculated into ASN III media and modified BG-11 medium respectively in 250mL culture flasks in six replicates as in previous experiments. *S. elongatus* was inoculated into modified BG-11 medium at a cell concentration of $1x10^6$ mL^{-1} in triplicate.

The test cultures were incubated at a temperature of 28 ± 2^0C and a photoperiod of 12 L : 12 D at 2200 lux as in previous experiments. The three species were exposed to different light wavelengths – white (400-700 nm), red (620-700 nm), green (490-570 nm) and blue light (450-495 nm). The red, green and blue wavelengths were simulated by covering the culture flasks with cellophane papers of the respective colours (Mishra *et al.*, 2012; Parmar *et al.*, 2013). White light refers to the day light fluorescent lamps.

The inoculated cultures of *P. tenue* and *O. acuminata* were incubated for 14 days. The respective biomass and pigments were determined as before. *S. elongatus* was incubated for 7 days, and biomass and pigments estimated as before.

As *S. elongatus* showed chromatic adaptation, fresh cultures from maintenance stock were inoculated as before, and exposed to green and red light for 14 days. These fourteen day old cultures were reversed by exposing red light grown cultures to green light and vice versa, and incubated for another 14 days. The cultures were observed for their visual appearance.

- **Results**

There was no visual change in the colour of *P.tenue* cultures grown at different light wavelengths. Microscope observations did not reveal any morphological changes. *Phormidium tenue* produced highest yield of 125.62 mg L^{-1}dry weight under green light, and lowest yield of 93.58 mg L^{-1}dry weight under red light . The chlorophyll *a* content was significantly high in white light (Table 3.19; Appendix 5).

Table 3.19 Biomass and production of chlorophyll a by *Phormidium tenue, Oscillatoria acuminata*, and *Synechococcus elongatus* following growth in different light wavelengths

Light wavelength	*P. tenue*		*O. acuminata*		*S. elongatus*	
	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry weight)	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry weight)	Cell count (x 10^6 ml^{-1})	Chlorophyll *a* (fg $cell^{-1}$)
White	104.22 a*	8.83 a	104.29 a	5.11 a	8.30 a	5.63 a
Red	93.58 b	2.69 b	76.93 b	3.55 b	3.53 b	6.77 b
Green	125.62 c	3.21 b	67.80 c	6.08 a	3.26 b	3.57 c
Blue	105.38 a	0.35 c	48.04 d	1.95 c	1.42 b	2.54 c

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

Microscope observations did not reveal any morphological changes and there was no visual change in the colour of *O. acuminata* cultures grown at different wavelengths of light. *Oscillatoria acuminata* produced a highest yield of 104.29 mg L^{-1}dry weight under white light and lowest yield of 48.04mg L^{-1}dry weight under blue light. Production of chlorophyll *a* was statistically same in white and green light (Table 3.19).

There was visual change in the colour of *S. elongatus* cultures grown at different light wavelengths. Cultures grown under red light appeared blue-green in colour while the other three were reddish brown in appearance (Fig. 3.6). *Synechococcus elongatus* produced a highest biomass of 8.30x 10^6 mL^{-1}under white light and lowest biomass of 1.42x 10^6 mL^{-1}under blue light .The chlorophyll *a* content was significantly high in red light (Table 3.19).

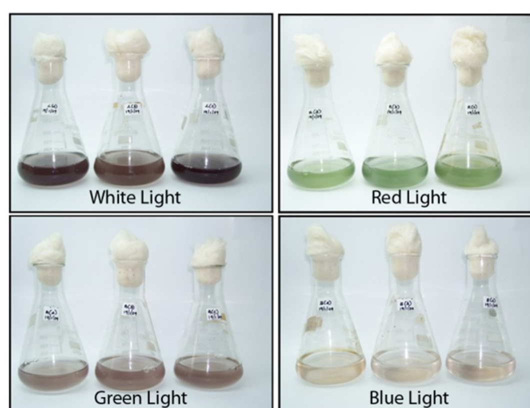


Fig. 3.6 Effect of light quality on the growth of *S. elongatus*

➢ *Light quality and phycobiliproteins*

In *Phormidium tenue* the production of phycobiliproteins was considerably high under white light and it is reflected in the quantity of component pigments phycocyanin, allophycocyanin and phycoerythrin (Fig. 3.7) as well. A one third reduction in the quantity of total phycobiliproteins occurred in blue light. The respective quantities in white, red, green and blue light were 46.25, 34.92, 32.66 and 14.20 mg g^{-1} dry weight.

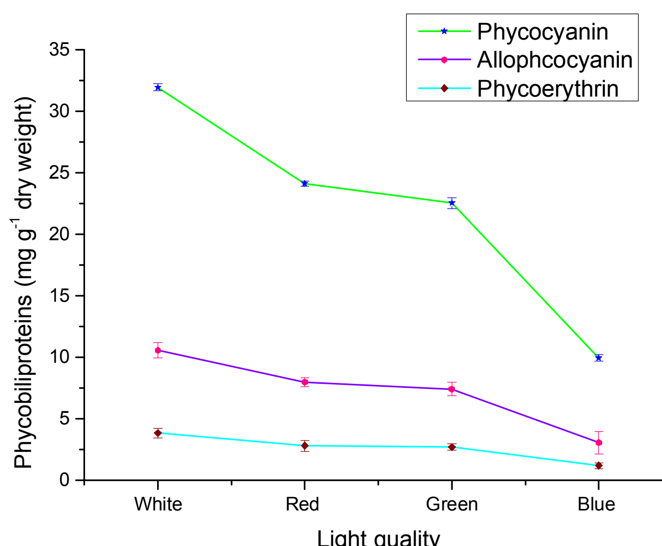


Fig. 3.7 Effect of light quality on phycobiliproteins of *Phormidium tenue* (production in 14 day growth period)

The relative amounts of phycocyanin, allophycocyanin and phycoerythrin also varied similarly and PC/PE ratio was constant (Table 3.20).

Table 3.20 Phycobiliprotein content of *Phormidium tenue* upon growth in different light wavelengths

Light wavelength	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)	PC/PE
White	31.94 a	10.56 a	3.84 a	8.32
Red	24.12 b	7.99 b	2.81 b	8.58
Green	22.55 c	7.42 b	2.69 b	8.38
Blue	9.95 d	3.06 c	1.19 c	8.36

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

In *O. acuminata* the highest total phycobiliprotein value of 83.166 mg g^{-1} dry weight was obtained under white light and lowest total phycobiliprotein value of 38.734 mg g^{-1} dry weight blue light (Fig. 3.8). The same trend was exhibited by phycocyanin, allophycocyanin and phycoerythrin. Phycocyanin was the major component followed by allophycocyanin and phycoerythrin in all light environs. The PC/PE ratio was constant (Table 3.21).

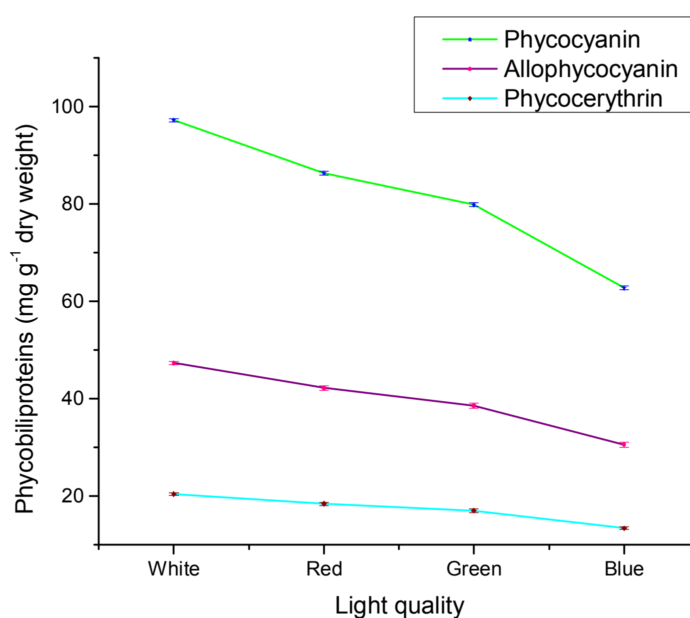


Fig. 3.8 Effect of light quality on phycobiliproteins of *Oscillatoria acuminate*

Table 3.21 Phycobiliprotein content of *Oscillatoria acuminata* upon growth in different light wave lengths

Light wavelength	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)	PC/PE
White	97.25 a	47.32 a	20.35 a	4.78
Red	86.32 b	42.17 b	18.35 b	4.70
Green	79.83 c	38.54 c	16.97 c	4.70
Blue	62.74 d	30.53 d	13.35 d	4.70

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

In *S. elongatus* the highest total phycobiliprotein value of 6.111 fg cell^{-1} was obtained under red light and lowest total phycobiliprotein value of 4.254 fg cell^{-1} in blue light (Fig. 3.9). The production of phycocyanin was comparatively higher in red compared to white and green light. However a statistically significant decrease in pigments was observed only in blue light. Production of allophycocyanin is not significantly affected by the wavelength differences. The PC/PE ratio is varying from 0.98 to 2.16. As given in Table 3.22 the PC/PE ratio of the red light grown culture is the highest showing that the relative amount of phycoerythrin is low and is overtaken by phycocyanin and this imparts the blue green colour to the culture .

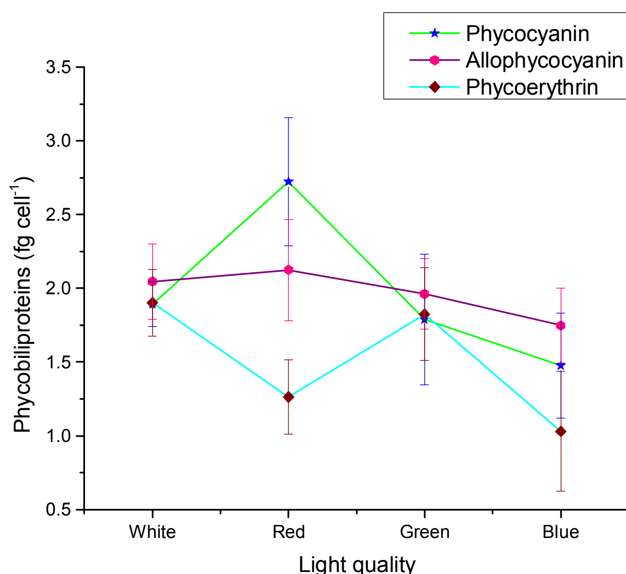


Fig. 3.9 Effect of light quality on phycobiliproteins of *Synechococcus elongatus*

Table 3.22 Phycobiliprotein content of *Synechococcus elongatus* upon growth in different light wave lengths

Light wavelength	PC (fg cell^{-1})	APC (fg cell^{-1})	PE (fg cell^{-1})	PC/PE
White	1.89 a b	2.05 a	1.90 a	0.99
Red	2.72 a	2.13 a	1.26 a b	2.16
Green	1.79 a b	1.96 a	1.83 a b	0.98
Blue	1.48 b	1.75 a	1.03 b	1.44

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE – Phycoerythrin

The chromatic adaptation exhibited by *S. elongatus* was further confirmed by exchanging the cultures grown in red light to green light and vice versa. The cultures grown for 14 days in red light which appeared blue-green in colour, gradually changed to reddish brown when transferred to green light (Fig. 3.10). These reddish brown cultures turned blue-green upon reexposure to red light (Fig. 3.11). The ratio of PC red light/ PC green light was 1.52 and PE green light/ PE red light was 1.45. Therefore it is clear that red light promotes production of phycocyanin and green light favour more production of phycoerythrin. Hence this strain of *S. elongatus* comes under Group III chromatic adaptation.

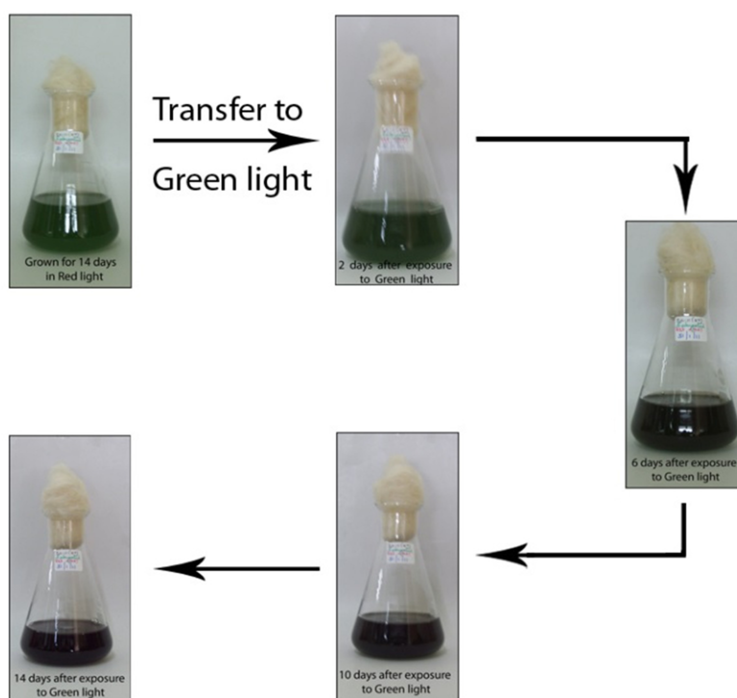


Fig. 3.10 Visual observation of *Synechococcus elongatus* after transfer from red light to green light for a period of 14 days

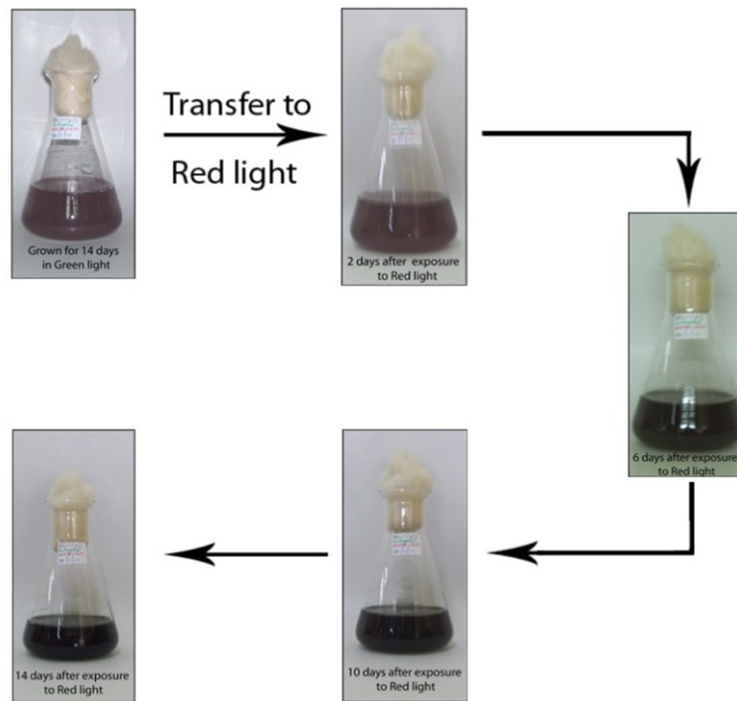


Fig. 3.11 Visual observation of *Synechococcus elongatus* after transfer from green light to red light for a period of 14 days

It is inferred that the production of pigments chlorophyll a, phycocyanin, allophycocyanin and phycoerythrin by *P. tenue* is best supported by the white light though high biomass accumulation occurs in green light. Probably sustained growth may be achieved only under the full spectrum as the pigment production is favoured by this . *O.acuminata* has the maximum yield of biomass as well as pigments in white light. *S.elongatus* exhibits chromatic adaptation. The culture density is significantly high in white light; but chlorophyll a increases in red light. Although the absolute quantities of the phycobilin pigments does not vary significantly with a few exceptions, the relative quantity of phycocyanin and phycoerythrin varies as in Group III chromatic adaptation. This property could be exploited for production of phycoerythrin by this strain of *S.elongatus*.

3.3.6 Effect of UV Exposure on Pigment Production

Depletion of stratospheric ozone levels and subsequent increase in the levels of the incoming solar UV radiation is much discussed. Even though cyanobacteria have been shown to exhibit many adverse reactions to UV exposure that includes impairment of physiological and biochemical processes, they have developed numerous mechanisms like avoidance of UV exposure by migration, the synthesis of UV absorbing substances or quenching agents and DNA repair mechanisms to deal with exposure to UV radiation (Priyadarshani *et al.*, 2012; Grumezesco, 2016). The biosynthesis and accumulation of UV screening photoprotective compounds like mycosporine-like amino acids (MAAs) and scytonemin has been reported to be a crucial physiochemical adaptational mechanism of cyanobacteria against harmful UV radiation. Occurrence of MAAs have been reported widely from cyanobacterial strains from habitats exposed to strong soalr radiation and this compound protects the cell by absorbing the UV radiation. MAAs have absorption maxima between 280 and 360 nm and are small, water soluble molecules of imino carbonyl derivatives of cyclohexanone. Scytonemin is also a photoprotective compound and it is a water insoluble molecule that occurs in extracellular mucilaginous sheath of cyanobacterial cells (Bhandari and Sharma, 2011; Rastogi *et al.*, 2014; Hartmann *et al.*, 2015). In this context it was decided to examine how the growth and pigment production of the species under investigation is influenced by exposure to UV.

30 mg wet weight of samples of *P. tenue* and *O.acuminata* were inoculated into ASN III medium and modified BG-11 medium respectively in 250mL culture flasks in six replicates as in previous experiments. *S. elongatus* was inoculated into modified BG-11 medium at a cell concentration of $1x10^6$ mL^{-1} in triplicate.

The test cultures were incubated at a temperature of 28 ± 2^0C and a photoperiod of 12 L: 12 D. Light was provided from a panel of day light fluorescent lamps to provide 2200 lux. Three sets of cultures were inoculated:

Set I : Control cultures grown in white light for 14 days.

Set II : Cultures grown in white light for 7 days and then transferred under 36W UV lamps for another 7 days

Set III : Cultures grown under 36W UV lamps for 14 days.

All the cultures were harvested on the 14th day of incubation and the biomass and pigments were estimated. Data were subjected to one way ANOVA followed by Tukey's test at the 5% significant level (P-value < 0.05) using the program Ky plot (Appendix 6).

- **Results**

Eventhough there was no visible difference in colour between the three sets of cultures of *P.tenue*, the culture density of those grown exclusively in UV was visibly thinner. Cultures that initiated growth under white light for seven days were slightly stimulated by the 7 day UV exposure that followed. *P. tenue* produced highest yield of 113.57 mg L^{-1}dry weight under white light + UV (set II), and lowest yield of 47.16mg L^{-1}dry weight under UV. Chlorophyll *a* was also similarly affected (Table 3.23).

Table 3.23 Biomass and production of chlorophyll a by *Phormidium tenue, Oscillatoria acuminata*, and *Synechococcus elongatus* following exposure to UV

Light condition	*P. tenue*		*O. acuminata*		*S. elongatus*	
	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry Weight)	Biomass (mg L^{-1})	Chlorophyll a (mg g^{-1} dry Weight)	Cell count (x 10^6 ml^{-1})	Chlorophyll *a* (fg $cell^{-1}$)
White	104.22 a*	4.14 a	87.33 a	5.11 a	8.29 a	5.62 a
White + UV	113.57 b	8.83 b	104.29 b	6.72 b	11.83 b	6.50 a
UV	47.16 c	0.96 c	34.64 c	0.98 c	1.16 c	0.92 b

* Figures sharing the same letters in the same column do not differ significantly at P < 0.05

Eventhough there was no visible difference in colour between the three sets of cultures of *O.acuminata*, the culture density of those grown in UV was visibly thinner. *O. acuminata* produced a highest yield of 104.29 mg L^{-1}dry weight under white light + UV and lowest yield of 34.64mg L^{-1}dry weight under UV. Chlorophyll *a* was also similarly affected (Table 3.23).

The cultures of *S. elongatus* grown in white light + UV was reddish brown in colour and those grown in UV were extremely pale reddish brown in colour. *S. elongatus* produced a highest biomass of 11.83x 10^6 mL^{-1}under white light + UV and lowest biomass of 1.16x 10^6 mL^{-1} under UV. Chlorophyll a did not differ significantly between set I and II (Table 3.23).

- ***UV exposure and phycobiliproteins***

In *Phormidium tenue* the production of phycobiliproteins was considerably high under white light + UV and it is reflected in the quantity of component pigments phycocyanin, allophycocyanin and phycoerythrin (Fig. 3.12) as well.

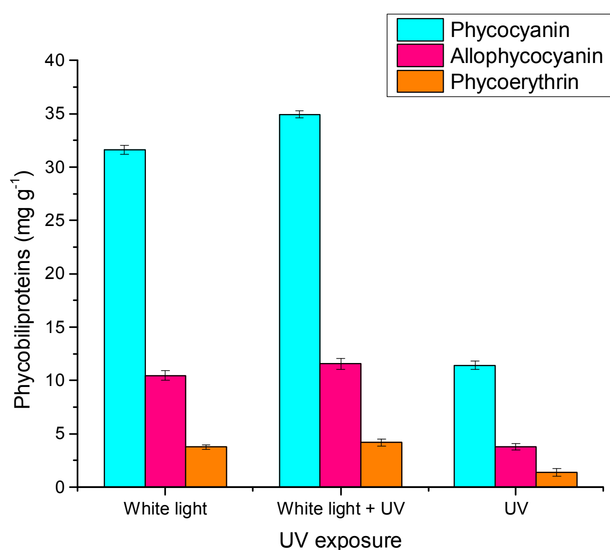


Fig. 3.12 Effect of UV exposure on phycobiliproteins of *Phormidium tenue*

The production of all phycobilin pigments was high in cultures exposed to UV following 7 day growth under white light. The relative proportion of phycocyanin and phycoerythrin was constant in all three.(Table 3.24).

Table 3.24 Phycobiliprotein content of *Phormidium tenue*

Light condition	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)	PC/PE
White	31.59 a*	10.45 a	3.77 a	8.38
White + UV	34.93 b	11.56 b	4.16 a	8.40
UV	11.41 c	3.77 c	1.36 b	8.39

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

In *O. acuminata* the highest level of pigments was obtained under white light + UV and lowest was obtained under UV alone(Fig. 3.13). However the ratio of PC/PE did not alter (Table 3.25).

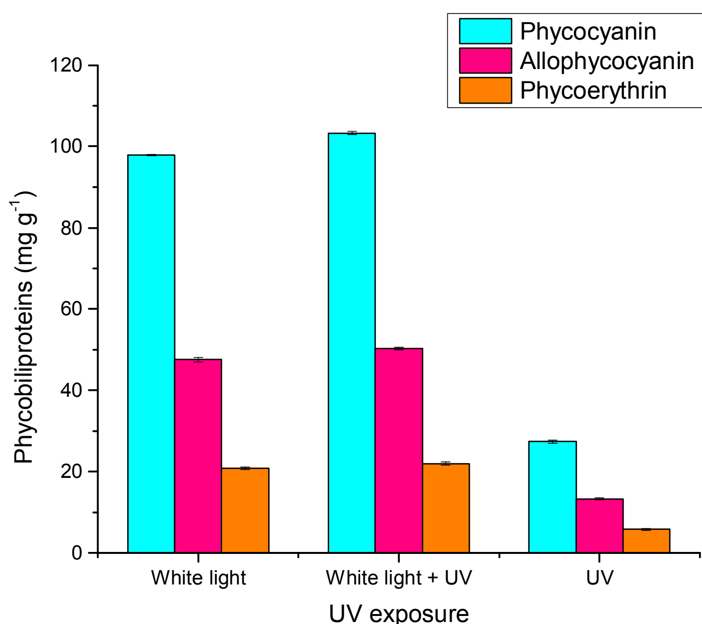


Fig. 3.13 Effect of UV exposure on phycobiliproteins of *Oscillatoria acuminata*

Table 3.25 Phycobiliprotein content of *Oscillatoria acuminata*

Light condition	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)	PC/PE
White	97.84 a*	47.59 a	20.82 a	4.70
White + UV	103.32 b	50.27 b	21.98 b	4.70
UV	27.36 c	13.31 c	5.82 c	4.70

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

In *S. elongatus* the highest level of pigments was obtained under white light and UV and lowest was obtained under UV alone(Fig. 3.14). The ratio of PC/PE fluctuated from 0.8 to 1.0 (Table 3.26).

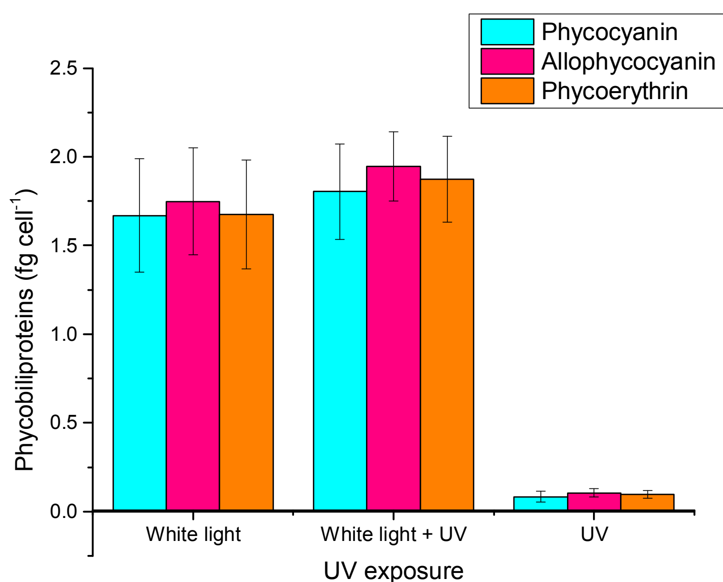


Fig. 3.14 Effect of UV exposure on phycobiliproteins of *Synechococcuselongatus*

Table 3.26 Phycobiliprotein content of *Synechococcus elongatus*

Light condition	PC (fg cell^{-1})	APC (fg cell^{-1})	PE (fg cell^{-1})	PC/PE
White	1.67 a*	1.75 a	1.67 a	1
White + UV	1.80 a	1.95 a	1.87 a	0.96
UV	0.08 b	0.11 b	0.10 b	0.80

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$
PC – Phycocyanin; APC – Allophycocyanin; PE - Phycoerythrin

It is inferred that in general these species may tolerate short term exposures to UV once they have picked up active growth in a normal environment; but long term exposure will considerably affect the pigments and consequently growth and accumulation of biomass.

3.3.7 Effect of Temperature on Pigment Production

Temperature along with various other physico-chemical factors can influence the tetrapyrrole structure and content of phycobiliproteins in the cyanobacterial cell. They have been shown to influence the synthesis of phycobiliproteins. Therefore the phycobiliprotein content of cyanobacteria can be increased by optimizing the various culture conditions along with temperature (Johnson *et al.*, 2014; Pruvost *et al.*, 2015).

The effect of temperature on the three species of cyanobacteria were evaluated at five levels of temperature above the ambient in the controlled environment of a plant growth chamber (Fig. 3.15). The plant growth chamber was illuminated through three panels of day light fluorescent lamps which could be set at different light intensities. The temperature and humidity could be controlled.

Fig. 3.15 Plant growth chamber

Phormidium tenue and *Oscillatoria acuminata* were filtered out from two week old maintenance cultures; pressed well in a blotting paper and 0.03 g of the samples were ground in a clean glass mortar to dissociate the floc. Each sample of *P. tenue* was inoculated to 150 mL 0f ASN-III medium in 250 mL conical flask*;* *O. acuminata* was transferred to 150 mL of modified BG-11 medium in 250 mL conical flask. Six replicates were maintained for both species; three for evaluating biomass, and three for estimating chlorophyll *a* and phycobiliprotein content. *Synechococcus elongatus* was inoculated in triplicates into 150 mL modified BG-11 medium in 250 mL conical flasks, at a cell concentration of 1 x 10^6 mL^{-1}. The cultures were incubated in growth chamber at a humidity of 60% at temperatures of 30^0C, 35^0C, 40^0C, 45^0C and 50^0C with a photoperiod of 12 L: 12 D at light intensity 2000 lux in different sets of experiment.

After incubation for 7 days, the entire culture of three replicates of *P.tenue* and *O. acuminata* were harvested by filtering through nylon cloth; and the filtered sample was rinsed thoroughly with distilled water to wash off the culture medium. The samples were blotted dry in a filter paper and dried at 50^0C overnight to determine the biomass as dry weight. To determine the chlorophyll *a* content of the species, sub-samples were withdrawn on the 7th day of growth and the method of Lorenzen (1967) was followed as given before. To estimate the phycobiliprotein content of *P.tenue* and *O. acuminata*, subsamples were withdrawn from the respective replicate cultures and the method of Siegelman and Kycia (1978) was followed as given before.

After incubation of *S. elongatus* cultures for 7 days, samples were withdrawn from the triplicates and the cell counts were estimated by counting the cells using a haemocytometer after fixing the cells with lugol's iodine. The chlorophyll *a* content of *S. elongatus* was determined according to the method of Lorenzen (1967) and phycobiliprotein content was estimated according to the method of Siegelman and Kycia (1978) as given before.

The data were plotted graphically to understand the effect of temperature using the excel software. Data were subjected to one way ANOVA followed by Tukey's test at the 5% significant level (P-value < 0.05) using the program Ky plot (Appendix 7).

- **Results**

There were no visible change in colour of *P.tenue* cultures grown at different temperatures in the plant growth chamber (Fig. 3.16). *P. tenue* produced highest yield of 38.17 mg L^{-1}dry weight at 35^0C and lowest yield of 33.54 mg L^{-1}dry weight at 50^0C. The difference in biomass was not

statistically significant. Chlorophyll a was observed to double at > 3 5^0C (Table 3.27).

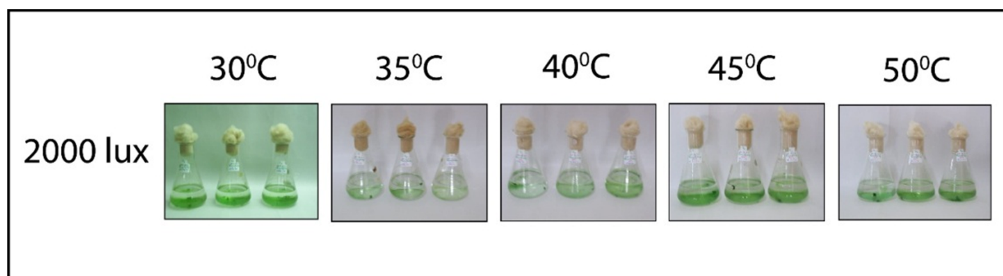


Fig. 3.16 Effect of temperature on *Phormidium tenue*

Table 3.27 Biomass and production of chlorophyll a by *Phormidium tenue*, *Oscillatoria acuminata*, and *Synechococcuselongatus* following growth in different temperatures at 2000 lux.

Temperature (°C)	*P. tenue*		*O. acuminata*		***S. elongatus***	
	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry Weight)	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} dry Weight)	Cell count (x 10^6 ml^{-1})	Chlorophyll *a* (fg $cell^{-1}$)
30	37.90 a	3.53 a	71.51 a	6.17 a	11.99 a	5.25 a b
35	38.17 a	2.24 a	45.16 b	4.01 b	11.28 a	4.30 a
40	37.17 a	5.09 a	43.13 b	4.38 a b	12.03 a	5.73 a c
45	34.43 a	11.01 b	38.40 b	5.06 a b	10.07 a	7.41 b c d
50	33.54 a	12.78 b	36.64 b	4.55 a b	8.96 a	9.16 d

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

There were no visible changes in colour of *O. acuminata* cultures grown at different temperatures in the plant growth chamber (Fig. 3.17). *Oscillatoria acuminata* produced highest yield of 71.51 mg L^{-1}dry weight at 30^0C and lowest yield of 36.64mg L^{-1}dry weight at 50^0C (Table 3.27).Production of chlorophyll *a* was also high at 30^0C .

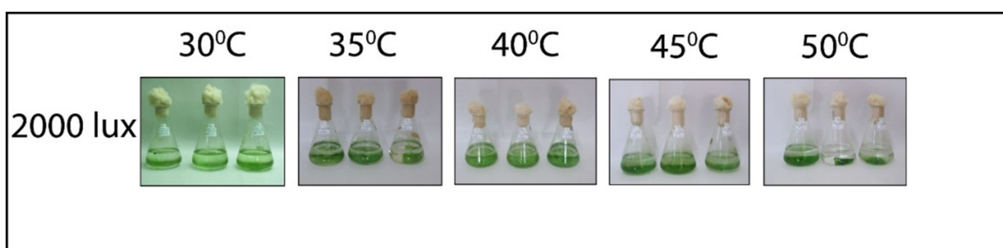


Fig. 3.17 Effect of temperature on *Oscillatoria acuminata*

All the cultures of *S. elongatus* grown inside the growth chamber at different temperatures appeared grass green in colour, which was different from its original blue green or reddish tint (Fig. 3.18).

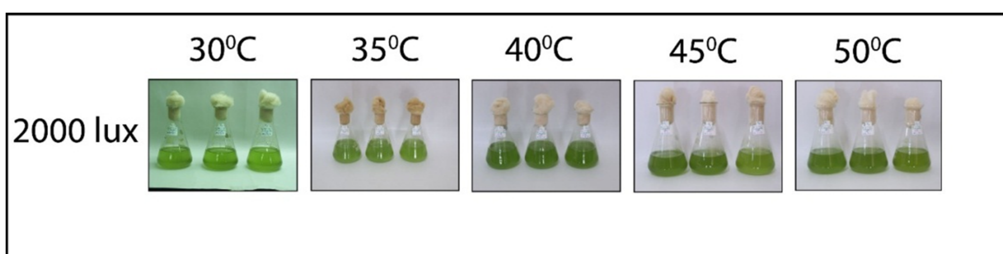


Fig. 3.18 Effect of temperature on *Synechococcus elongatus at different temperature*

S. elongatus produced a cell count of 12.03x 10^6 mL^{-1}at 40^0C and lowest of 8.96x 10^6 mL^{-1} at 50^0C; but there was no significant statistical variation. However the chlorophyll a seems to have been stimulated by temperatures 45^0C and 50^0C (Table 3.27).

- ***Temperature and phycobiliproteins***

In *Phormidium tenue* the production of phycocyanin was considerably high at 35^0C to 40^0C. Allophycocyanin also followed the same trend; but the amount of phycoerythrin reduced from 30^0C upwards (Fig. 3.19, Table 3.28).

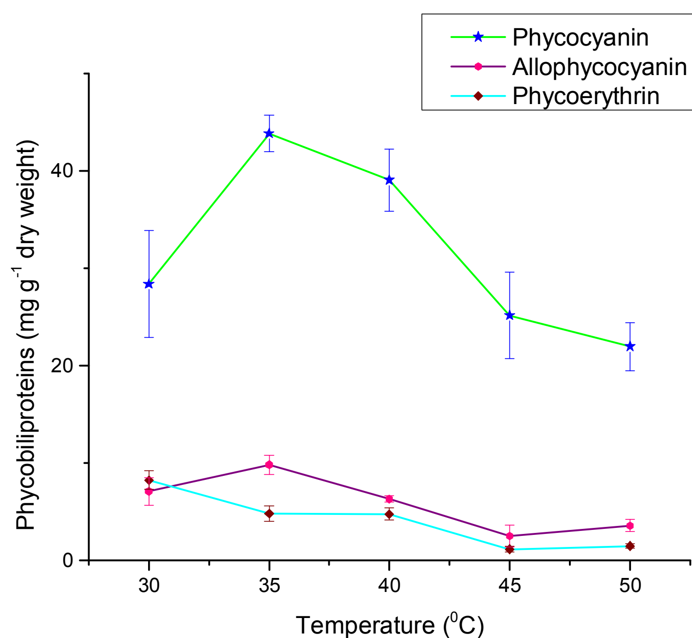


Fig. 3.19 Effect of temperature on phycobiliproteins of *Phormidium tenue*

Table 3.28 Phycobiliprotein content of *Phormidium tenue grown at different temperature*

Temperature (^{0}C)	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)
30	28.38 a	7.11 a	8.26 a
35	43.82 b	9.83b	4.84 b
40	39.04 b	6.31 a	4.77 b
45	25.17 a	2.52 c	1.17 c
50	21.97 a	3.59 c	1.50 c

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

In *O.acuminata* the highest production of phycocyanin and allophycocyanin occurred at 40^0C. Phycoerythrin production was low at >30^0C (Fig. 3.20). The statistical analysis of the data is presented in table 3.29.

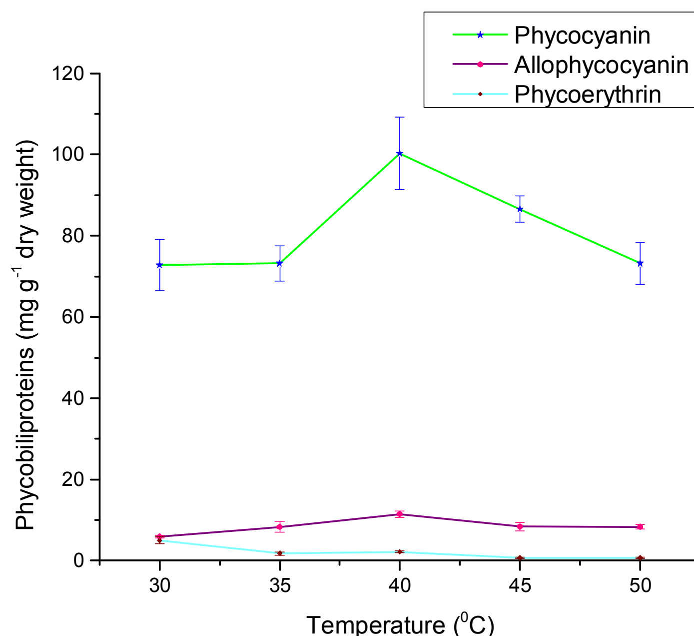


Fig. 3.20 Effect of temperature on phycobiliproteins of *Oscillatoria acuminata*

Table 3.29 Phycobiliprotein content of *Oscillatoria acuminata*

Temperature (^{0}C)	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)
30	72.75 a	5.92 a	4.99 a
35	73.17 a	8.33 b	1.78 b
40	100.28 b	11.45 c	2.18 b
45	86.56 a b	8.39 b	0.72 b
50	73.170 e	8.333 d	0.72 a

* Figures not sharing the same letters in the same column differ significantly at $P < 0.05$

The general observation is that the growth of the three species will be supported even at 50^{0}C. The production of phycobilin pigments is sensitive to temperature variation. In *Phormidium tenue* the production of phycocyanin and allophycocyanin is at its maximum at 35^{0}C-40^{0}C while that of *Oscillatoria*

acuminata it is 40^0C-45^0C. Production of phycoerythrin is at its highest at 30^0C in both. It decreases as the temperature is increased.

The unique observation in this set of experiments was that there was visible change of colour of all cultures from reddish brown to grass green exhibited by *Synechococcus elongatus* when grown inside the growth chamber. The behaviour of the phycobilin pigments could not be studied so that this phenomenon cannot be explained at this stage; but definitely further studies has to be undertaken as this species shows chromatic adaptation which is an important property of many cyanobacteria to adapt to changes in their environment.

3.3.8 Combined Effect of Light and Temperature on Pigment Production

The growth and photosynthesis of photoautotrophs including microlage are affected by the light conditions (intensity and quality). In aquatic systems these conditions have been known to change at different depths of water and under changing weather conditions (Kim *et al.*, 2012; Bland and Angement, 2016). Temperature is associated with absorption of light and they do interact in deciding the ecophysiology of organisms. It has been reported that growth rate is independent of temperature and proportional to light exposure (photoperiod x irradiance) with a negligible maintenance requirement when cyanobacteria encounter subsaturating irradiances in their environment, and growth is temperature dependent and not proportional to photoperiod length at saturating irradiances (Foy and Gibson, 1993; Pelechata *et al.*, 2016).

The combined effect of light and temperature on three species of cyanobacteria were evaluated in this study at five levels of temperature and light intensity in the controlled environment of the plant growth chamber.

The experiment was repeated as in the section 3.3.7. The cultures were incubated in the growth chamber at humidity of 60% and temperatures of 30^0C, 35^0C, 40^0C, 45^0C and 50^0C with a photoperiod of 12 L: 12 D at light intensities of 1000, 2000, 2500, 3000 and 3500 lux from day light fluorescent lamps of the chamber.

After incubation for 7 days, the entire culture of three replicates of *P.tenue* and *O. acuminata* were harvested and biomass determined. Chlorophyll a and phycobiliproteins were determined from the sub-samples of the other three replicates.

After incubation of *S. elongatus* cultures for 7 days, samples were withdrawn from the triplicates and the cell counts and chlorophyll a were estimated.

Data were subjected to two way ANOVA at the 5% significant level (P-value < 0.05) using origin 8.1 data analysis package (Appendix 8). The least significant differences (LSD) between means was calculated as :

$$\text{LSD} = t \text{ x SED}$$

where

t = critical value from the t-distribution table

$$\text{SED} = \frac{\sqrt{2}\text{X EMS}}{\text{r}}$$

EMS = error mean square

r = number of replications

The LSD was compared with the differences between the pairs of means and a decision was made to find which pairs are significantly different.

➢ Results

There were no visible change in colour of *P.tenue* cultures grown at different light and temperature combinations. A visual comparison of the cultures is given in figure 3.20.

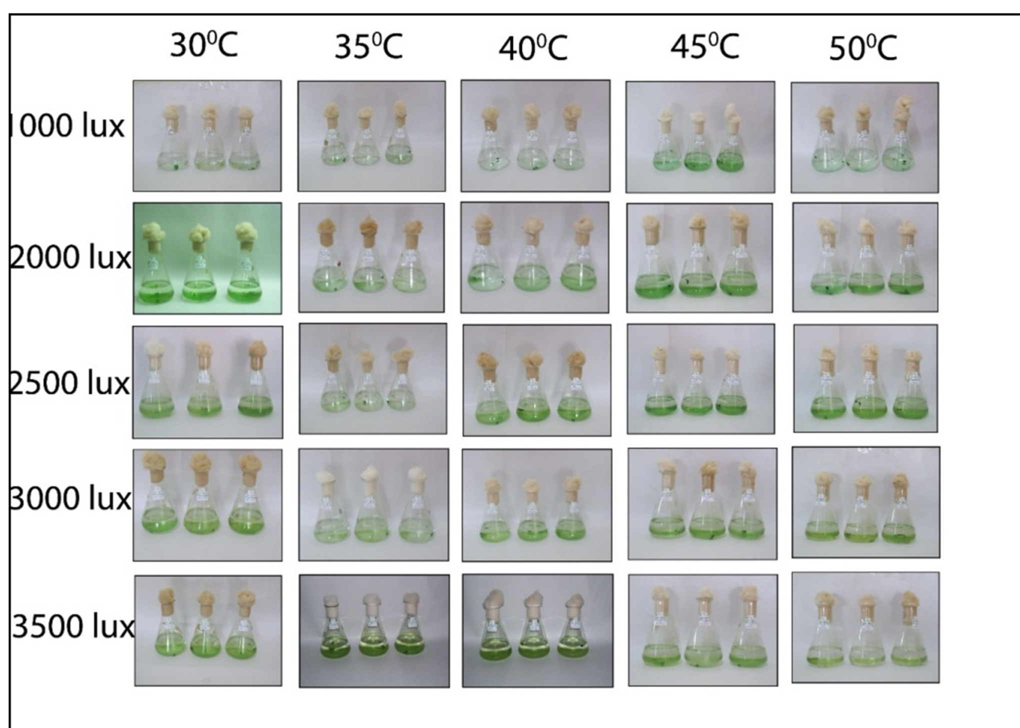


Fig. 3.20 Combined effect of light and temperature on growth of *P. tenue*

The analysis of variance of the biomass yield revealed that the population means of different light levels were significantly different, population means of temperature were significantly different and the interaction between light and temperature were significant (Table 3.30). The LSD between the treatment means was 4.69. According to this the yield of biomass of *P.tenue* did not differ at 2500 lux between 30 and 35^{0}C and at 2000 lux between 30-45^{0}C. This shows that at lower light intensity higher temperatures are tolerated. The data of individual results are given in Appendix 8.

Table 3.30 Biomass (mg L^{-1}dry weight) production by *Phormidium tenue* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	4450.508	4	1112.63	136.18	3.13E-26	2.56
Columns	290.15	4	72.54	8.88	1.7E-05	2.56
Interaction	989.04	16	61.82	7.57	1.49E-08	1.85
Within	408.52	50	8.17			
Total	6138.22	74				

Treatment Means					
	30^{0}C	35^{0}C	40^{0}C	45^{0}C	50^{0}C
1000 lux	9.47	14.36	14.45	21.26	15.64
2000 lux	37.84	38.64	37.55	36.99	30.47
2500 lux	41.22	38.75	34.67	30.53	30.08
3000 lux	34.40	24.56	32.09	33.60	31.94
3500 lux	32.80	31.33	27.45	22.82	17.80

LSD = 4.69

P.tenue exhibited strong interaction between light and temperature in the production of chlorophyll *a*. The least significant difference was 1.64 and it showed that the highest chlorophyll *a* value of 12.78 mg g^{-1} dry weight was obtained at 50^{0}C under 2000 lux and 12.15 mg g^{-1} dry weight at 50^{0}C under 2500 lux (Table 3.31).

Table 3.31 Chlorophyll *a* (mg g^{-1} dry weight) of *Phormidium tenue* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	261.04	4	65.26	65.39199	3E-19	2.56
Columns	216.65	4	54.16	54.27	1.37E-17	2.56
Interaction	229.90	16	14.37	14.39	1.73E-13	1.85
Within	49.89	50	0.99			
Total	757.48	74				

Treatment Means					
	30^{0}C	35^{0}C	40^{0}C	45^{0}C	50^{0}C
1000 lux	3.66	1.31	1.17	1.33	2.06
2000 lux	3.53	2.24	5.09	11.01	12.78
2500 lux	4.72	4.06	4.98	8.49	12.15
3000 lux	5.46	5.11	5.25	8.14	7.08
3500 lux	4.66	5.86	5.27	7.04	5.55

LSD = 1.64

There were no visible changes in colour of *O. acuminata* cultures grown at different light and temperatures. A visual comparison of the cultures is given in figure 3.21.

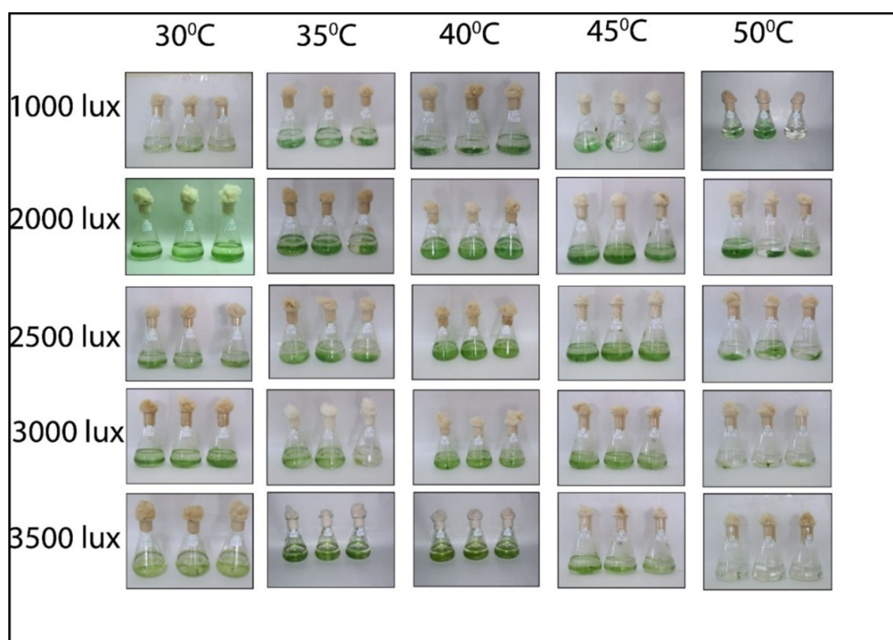


Fig. 3.21 Combined effect of light and temperature on *O. acuminata*

The analysis of variance of the yield revealed that the population means of light were significantly different, population means of temperature were significantly different and the interaction between light and temperature were significant (Table 3.32). The overall ANOVA showed significant interaction between light and temperature with P= 1.56E-16. The least significant difference was 6.00 and it showed that *O. acuminata* produced a highest yield of 71.51mg L^{-1} dry weight at 30^0C under 2000 lux .

Table 3.32 Biomass (mg L^{-1}dry weight) production by *Oscillatoria acuminata* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	7477.27	4	1869.32	139.53	1.79E-26	2.56
Columns	7681.53	4	1920.38	143.34	9.68E-27	2.56
Interaction	4374.52	16	273.41	20.41	1.56E-16	1.85
Within	669.88	50	13.39			
Total	20203.2	74				

Treatment Means					
	30⁰C	35⁰C	40⁰C	45⁰C	50⁰C
1000 lux	16.64	22.84	29.07	21.26	16.47
2000 lux	71.51	45.16	43.13	38.39	36.64
2500 lux	59.95	26.56	33.45	30.53	25.49
3000 lux	59.62	22.73	15.36	13.78	12.71
3500 lux	34.18	29.80	24.91	16.22	0

LSD = 6.00

In *O. acuminata*, the production of chlorophyll a was significantly reduced at >2500 lux. The least significant difference was 1.18. At lower light intensity higher temperatures even at 45^{0}C, the pigment production was high (Table 3.33).

Table 3.33 Chlorophyll *a* (mg g^{-1} dry weight) of *Oscillatoria acuminata* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	73.75	4	18.44	35.56	4.65E-14	2.56
Columns	33.97	4	8.49	16.38	1.23E-08	2.56
Interaction	34.42	16	2.15	4.15	5.5E-05	1.85
Within	25.92	50	0.52			
Total	168.06	74				

Treatment Means					
	30⁰C	35⁰C	40⁰C	45⁰C	50⁰C
1000 lux	5.56	5.38	5.53	5.90	5.10
2000 lux	6.17	4.01	4.38	4.73	4.55
2500 lux	5.27	5.33	6.43	4.46	2.95
3000 lux	4.33	5.45	4.43	3.99	2.96
3500 lux	2.67	3.73	3.95	2.55	0

LSD = 1.18

S. elongatus cultures grown inside the growth chamber at different light and temperatures were grass green in colour irrespective of temperature and light conditions (Fig. 3.22).

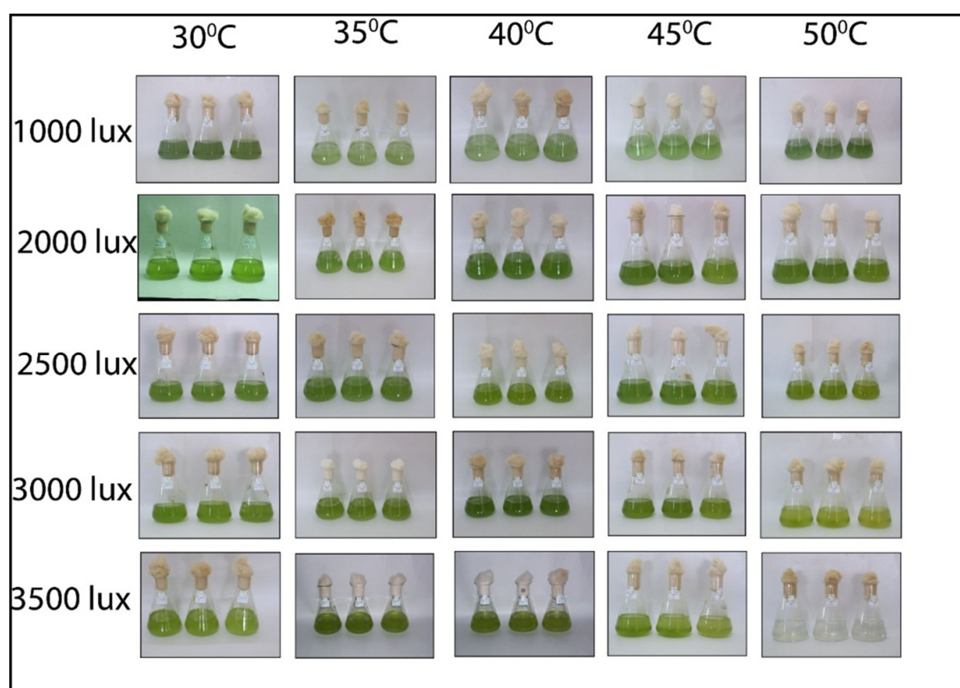


Fig. 3.22 Combined effect of light and temperature on *S. elongatus*

The analysis of variance of the yield revealed that the population means of light were significantly different, population means of temperature were significantly different and the interaction between light and temperature were significant. The overall ANOVA showed significant interaction between light and temperature with P= 9.04E-09. The least significant difference was 2.13. The cell density was maximum at 2500 - 3000 lux at 30-40^{0}C (Table 3.34).

Table 3.34 Cell count (x 10^6 mL^{-1}) of *Synechococcus elongatus* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	207.42	4	51.85	30.85	5.91E-13	2.56
Columns	316.45	4	79.11	47.07	2.32E-16	2.56
Interaction	209.95	16	13.12	7.81	9.04E-09	1.85
Within	84.04	50	1.68			
Total	817.86	74				

Treatment Means					
	30⁰C	35⁰C	40⁰C	45⁰C	50⁰C
1000 lux	7.21	8.28	8.42	8.84	8.61
2000 lux	11.99	11.28	12.03	10.07	8.96
2500 lux	14.27	12.99	12.84	10.11	8.43
3000 lux	14.73	13.35	13.06	9.15	6.15
3500 lux	11.47	11.90	8.63	6.34	0

LSD = 2.13

In *S. elongatus*, the least significant difference was 1.27 and it showed that the highest chlorophyll *a* value of 9.16 fg $cell^{-1}$ was obtained at 50^0C under 2000 lux (Table 3.35).

Table 3.35 Chlorophyll *a* (fg $cell^{-1}$) of *Synechococcus elongatus* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	189.91	4	47.48	79.07	5.4E-21	2.56
Columns	62.44	4	15.61	25.99	1.09E-11	2.56
Interaction	65.28	16	4.08	6.79	7.79E-08	1.85
Within	30.02	50	0.60			
Total	347.65	74				

Treatment Means					
	30⁰C	35⁰C	40⁰C	45⁰C	50⁰C
1000 lux	5.09	4.42	5.44	6.76	7.19
2000 lux	5.25	4.30	5.73	7.41	9.16
2500 lux	3.64	3.65	4.01	6.91	5.69
3000 lux	3.18	3.68	3.51	6.68	5.24
3500 lux	3.17	1.91	1.65	2.05	0

LSD = 1.27

➢ *Combined effect of light and temperature on phycobiliproteins*

In *P. tenue*, the highest phycocyanin content was obtained at 1000 - 2500 lux. The least significant difference was 5.02. The temperature range was 45-50^{0}C at 1000 lux, 35^{0}C at 2000 lux and 35-40^{0}C under 2500 lux (Table 3.36).

Table 3.36 Phycocyanin (mg g^{-1} dry weight) of *Phormidium tenue* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	6214.02	4	1553.50	166.06	3.26E-28	2.56
Columns	1300.98	4	325.24	34.77	7.02E-14	2.56
Interaction	4286.98	16	267.94	28.64	1.12E-19	1.85
Within	467.75	50	9.35			
Total	12269.72	74				

Treatment Means					
	30⁰C	35⁰C	40⁰C	45⁰C	50⁰C
1000 lux	21.05	31.12	39.72	48.75	43.79
2000 lux	28.38	47.15	39.04	25.17	21.97
2500 lux	33.10	43.82	44.66	25.91	25.39
3000 lux	34.69	24.94	20.07	7.96	9.61
3500 lux	19.99	11.96	17.12	13.15	6.74

LSD = 5.02

The least significant difference was 1.87 and it showed that the highest allophycocyanin value of 9.83 mg g^{-1} dry weight was obtained at 35^{0}C under 2000 lux, 9.93 mg g^{-1} dry weight at 30^{0}C under 2500 lux, 9.33 mg g^{-1} dry weight at 35^{0}C under 2500 lux and 9.92mg g^{-1} dry weight at 40^{0}C under 2500 lux (Table 3.37). The light requirement is from 2000 – 2500 lux and temperature 30- 40^{0}C.

Table 3.37 Allophycocyanin (mg g^{-1} dry weight) of *Phormidium tenue* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	280.49	4	70.12	54.21	1.4E-17	2.56
Columns	103.14	4	25.78	19.93	7.28E-10	2.56
Interaction	247.51	16	15.47	11.96	5.77E-12	1.85
Within	64.68	50	1.29			
Total	695.82	74	695.82			
Treatment Means						
	30⁰C	35⁰C	40⁰C	45⁰C	50⁰C	
1000 lux	4.09	6.79	7.47	8.42	8.07	
2000 lux	7.11	9.83	6.31	2.52	3.59	
2500 lux	9.93	9.33	9.92	3.53	2.90	
3000 lux	6.66	3.05	2.50	1.73	2.06	
3500 lux	4.22	1.73	2.00	2.29	2.10	

LSD = 1.87

The least significant difference was 0.82 and it showed that the highest phycoerythrin value of 10.72 mg g^{-1} dry weight was obtained at 30^0C under 2500 lux (Table 3.38). The individual results are presented in Appendix 8.

Table 3.38 Phycoerythrin (mg g^{-1} dry weight) of *Phormidium tenue* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	63.22	4	15.81	63.49	5.55E-19	2.56
Columns	204.17	4	51.04	205.02	2.37E-30	2.56
Interaction	125.82	16	7.86	31.59	1.29E-20	1.85
Within	12.4	50	0.25			
Total	405.66	74				
Treatment Means						
	30⁰C	35⁰C	40⁰C	45⁰C	50⁰C	
1000 lux	3.53	2.82	3.11	2.36	2.19	
2000 lux	8.26	4.84	4.77	1.17	1.49	
2500 lux	10.72	4.18	4.34	1.89	1.64	
3000 lux	6.39	1.97	1.97	1.29	1.09	
3500 lux	2.71	1.74	1.53	3.25	1.84	

LSD = 0.82

In *O. acuminata*, the least significant difference was 7.23 showed that the highest phycocyanin value of 100.28 mg g^{-1} dry weight was obtained at $40^{0}C$ under 2000 lux (Table 3.39).

Table 3.39 Phycocyanin (mg g^{-1} dry weight) of *Oscillatoria acuminata* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	41824.79	4	10456.20	538.66	1.98E-40	2.56
Columns	2740.56	4	685.14	35.29	5.34E-14	2.56
Interaction	3352.39	16	209.52	10.79	3.72E-11	1.85
Within	970.57	50	19.41			
Total	48888.32	74				

Treatment Means					
	$30^{0}C$	$35^{0}C$	$40^{0}C$	$45^{0}C$	$50^{0}C$
1000 lux	32.20	28.29	21.58	19.91	17.28
2000 lux	72.75	73.17	100.28	86.56	80.53
2500 lux	47.33	55.92	78.52	59.85	53.19
3000 lux	30.85	37.47	46.83	37.49	29.89
3500 lux	21.65	33.19	25.24	16.60	0

LSD = 7.23

The least significant difference was 1.44 and it showed that the highest allophycocyanin value of 14.64 mg g^{-1} dry weight was obtained at $45^{0}C$ under 1000 lux (Table 3.40).

Table 3.40 Allophycocyanin (mg g^{-1} dry weight) of *Oscillatoria acuminata* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	297.51	4	74.38	95.99	7.98E-23	2.56
Columns	74.88	4	18.72	24.16	3.63E-11	2.56
Interaction	427.46	16	26.72	34.48	1.84E-21	1.85
Within	38.74	50	0.77			
Total	838.61	74				

Treatment Means					
	$30^{0}C$	$35^{0}C$	$40^{0}C$	$45^{0}C$	$50^{0}C$
1000 lux	4.86	6.21	8.67	14.64	1.88
2000 lux	5.92	8.33	11.45	8.39	9.18
2500 lux	6.50	4.54	6.29	4.75	4.29
3000 lux	7.96	5.18	1.82	2.04	3.44
3500 lux	6.96	4.69	3.09	1.61	0

LSD = 1.44

The least significant difference was 0.95 and it showed that the highest phycoerythrin value of 4.99 mg g^{-1} dry weight was obtained at 30^0C under 2000 lux, 4.62 mg g^{-1} dry weight at 30^0C under 2500 lux and 5.01mg g^{-1} dry weight at 30^0C under 3000 lux (Table 3.41).

Table 3.41 Phycoerythrin (mg g^{-1} dry weight) of *Oscillatoria acuminata* following growth in different light and temperature conditions

Analysis of Variance						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	6.04	4	1.51	4.49	0.003512	2.56
Columns	106.34	4	26.59	79.21	5.21E-21	2.56
Interaction	30.26	16	1.89	5.63	1.16E-06	1.85
Within	16.78	50	0.34			
Total	159.42	74				

Treatment Means					
	30⁰C	35⁰C	40⁰C	45⁰C	50⁰C
1000 lux	1.69	2.63	0.98	1.93	0.50
2000 lux	4.99	1.78	2.18	0.72	0.51
2500 lux	4.62	3.17	1.62	1.03	0.65
3000 lux	5.01	1.73	1.56	1.99	0.37
3500 lux	3.69	1.49	1.29	1.40	0

LSD = 0.95

It is inferred that *P. tenue* can accumulate maximum biomass upto 45^0C at 2000 lux . Phycocyanin production is at its best at 1000 to 2000 lux and can proceed to 50^0C at 1000 lux. The suitable condition for production of allophycocyanin is 2000 to 2500 lux and temperature $30\text{-}40^0C$. The production of phycoerythrin is favoured at 2500 lux at 30^0C.

The yield of *O. acuminata* is highest at 2000 lux and 30^0C. Phycocyanin production is at its best at 2000 lux and 40^0C, allophycocyanin at 1000 lux and 45^0C while the suitable condition for phycoerythrin is 2000 to 3000 lux and 30^0C.

The yield of *S. elongatus* is highest at 2500 to 3000 lux and upto 40^0C. These results clearly show that in culture systems the light and temperature must be regulated for each product.

The result of optimization of growth conditions for *Phormidium tenue*, *Oscillatoria acuminata* and *Synechococcus elongatus* are summarised in Table 3.42.

Table 3.42 Result of optimization of growth conditions

Growth condition	*P. tenue*	*O. acuminata*	*S. elongatus*
Medium	ASN -III	Modified BG - 11	Modified BG - 11
Salinity	25×10^{-3}	Freshwater	Freshwater
pH	7.5	7.5	7.5
Light intensity	500 lux	500 lux	500 lux
Light quality	Fluorescent lamps / outdoor	Fluorescent lamps / outdoor	Fluorescent lamps / outdoor
Temperature	30^0C	30^0C	30^0C

3.3.9 Purity and Yield of Phycocyanin, Allophycocyanin and Phycoerythrin

Various methods are employed for extraction of phycobiliproteins, but no standard technique to quantitatively extract these pigments from microalgae exists. A method that works well in one organism may not be the method of choice for another organism (Ranjitha and Kaushik, 2005) . C-PC is often extracted by subjecting the biomass to cycles of freezing and thawing, mechanical cell disruption, lysozyme treatment, sonication , and high pressure exposure (Furuki *et al.*, 2003; Patil *et al.*, 2006; Patil and Raghavarao, 2007). These crude extracts are purified by different methods such as ultrafiltration, charcoal adsorption, ammonium sulphate precipitation , ion-exchange

chromatography, aqueous two phase system extraction etc. (Herrera *et al.*, 1989; Benedetti *et al.*, 2006; Soni *et al.*, 2006; Minkova *et al.*, 2007; Wang *et al.*, 2017).

The purity of C-PC preparations is evaluated based on the ratio between absorbencies from phycocyanobilin at 620 nm and aromatic amino acids in all proteins in the preparation at 280 nm . C-phycocyanin with A_{620}/A_{280} greater than 0.7 were considered food grade , while of 3.9 were reactive grade and > 4.0 analytical grade (Rito-Palomares *et al.*, 2001). Ramos *et al.* (2010) developed a rapid and scalable method for extraction and purification of C-phycocyanin from *Anabaena marina*. They estimated the purity of phycocyanin with absorbance ratio values A_{615}/A_{280}. Su *et al.* (2010) devised a simple and highly efficient method for extracting allophycocyanin (A-PC) from *Spirulina (Arthrospira) platensis*. They noted that the purity ratio (A_{650}/A_{280}) of the crude extract was 0.3 and the purity ratio of the extract after ammonium sulfate precipitation was 0.5. Tripathi *et al.* (2007) evaluated the extraction and purification of phycoerythrin from the terrestrial cyanobacterium *Lyngbya arboricola* by subjecting the cyanobacterial biomass to acetone precipitation, gel filtration by applying the extract to a Sephacryl column and ion exchange chromatography by introducing the extract to DEAE-cellulose column. They observed that the purity ratio of phycoerythrin after acetone precipitation was 2.57 (A_{560}/A_{280}). They found that the purity ratio of phycoerythin increased to 3.16 (A_{560}/A_{280}) after being subjected to Sephacryl gel filteration. After further purification of the extract by DEAE-cellulose ion exchange chromatography, the purity ratio further increased to 5.25 (A_{560}/A_{280}).

Pumas *et al.* (2012) evaluated the purification of phycoerythrin from the thermostable cyanobacterium *Leptolyngbya* sp. and also studied the

thermostability of the purified phycoerythrin. They purified the phycoerythin fromn the harvested cyanobacterial cells by the following methods - sonication, ammonium sulfate precipitation, hydroxyapatite column, Q-Sepharose coloumn and Sephacryl^{TM} S-200 HR. They were able to purify phycoerythrin with a purity index of 17.38 (A_{565}/A_{280}). They also comapared the purity of PE in the extract with PC, by using a purity index of A_{615}/A_{565} and observed a PC/PE index of 0.006, which showed a very low PC contamination.

Ranjitha and Kaushik (2005) evaluated the purification of phycoerythrin and phycocyanin from the cyanobacterium *Nostoc muscorum*. They observed that after ion exchange chromatography on DEAE cellulose-52 column, the purity ratio of PE (A_{562}/A_{280}) was 8.12. They also noted that rechromatography of the blue fraction obtained after this, showed purity ratio of PC (A_{615}/A_{280}) as 3.89.

In the present investigation the phycobiliprotein yield from the three cyanobacterial species were evaluated based on the crude extracts and the purity was determined by measuring the absorbance in spectrophotometer at 280 nm, 562 and 615 nm. The purity was calculated using the following relation:

A_{615}/A_{280} = Phycocyanin

A_{562}/A_{280} = Phycoerythrin

- **Results**

The phycobilin extracts of *Phormidium tenue* and *Oscillatoria acuminata* were coloured blue typical of phycocyanin pigment, and that of *Synechococcus elongatus* was pink indicative of the higher proportion of phycoerythrin (Fig. 3.23). The highest yield of phycocyanin obtained in this investigation was 43.52 mg g^{-1} dry weight for *P. tenue* and 116.04 mg g^{-1} dry weight for *O.*

acuminata. The highest yield of phycoerythrin obtained from *S. elongatus* was 2.08 fg $cell^{-1}$. Therefore these extracts were checked for purity.

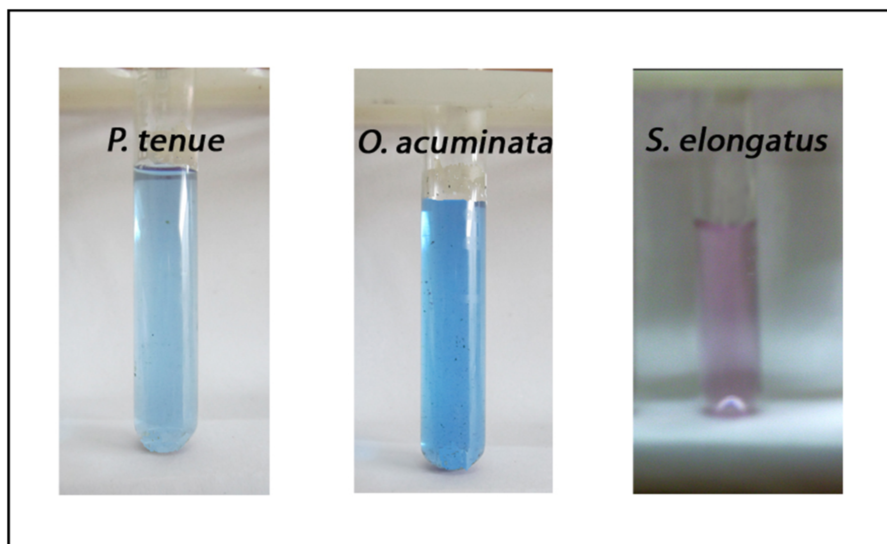


Fig. 3.23 Crude extracts from three cyanobacterial species

The purity of phycocyanin of the crude extract from *P. tenue* was obtained as 0.96 and that of *O. acuminata* was 1.21. The purity of phycoerythrin of *S. elongatus* was 0.05. It is possible to enhance the purity by adopting different purification methods, though it was not attempted in this study.

3.4 Discussion

Phycobiliproteins are the major photosynthetic accessory pigments in cyanobacteria. Many studies have shown that the production of phycobiliproteins varies with factors like pH, temperature, light intensity, light quality etc. and is related to the ecology of competitive utilisation of resources by the species(Takano *et al.*, 1995; Chaneva *et al.*, 2007; Hemlata and Fatma, 2009, Maurya *et al.*, 2014; Baer *et al.*, 2016). Based on the relative production of these pigments in the laboratory cultures many potential species are

identified for commercial production. In this investigation, the conditions of culture leading to the production of these pigments have been explored. It is found that the filamentous cyanobacteria *Phormidium tenue* produces the largest amount of phycocyanin *i.e* 43.52 µg mg^{-1} dry weight when grown at low light intensity of 500 lux in the ambient day light in ASN-III medium of salinity 25 x 10^{-3} at pH 7.5 and temperature 28 ± 2^{0}C for fourteen days. The corresponding production of allophycocyanin is only 28% of phycocyanin and that of phycoerythrin is 5% of phycocyanin. Therefore this strain of *P. tenue* can be considered as a phycocyanin producer.

The second species of cyanobacteria investigated in this study is *Oscillatoria acuminata*. According to the results obtained this strain produces the highest amount of phycocyanin *i.e* 116.04 µg mg^{-1}dry weight when grown at low light intensity of 500 lux in the ambient day light in modified BG-11 medium prepared in freshwater at a pH of 7.5 and temperature 28 ± 2^{0}C for fourteen days. The corresponding allophycocyanin content was 44% of phycocyanin and phycoerythrin was 21% of phycocyanin. Therefore this strain of *O. acuminata* may be considered as a phycocyanin producer.

Comparing both these species *O. acuminata* has higher production of phycocyanin per unit weight. The purity of the extract is also higher than that of *P. tenue*. The relative distribution of the component pigments phycocyanin, allophycocyanin and phycoerythrin are different for the two. *Phormidium tenue* has higher proportion of phycocyanin compared to *O. acuminata*. Therefore the phycobilin extract of *P. tenue* seems to be better for easy purification as it has more of phycocyanin. It has been reported that *Phormidium* sp. BTA-1048 can produce 168.15 µg mg^{-1} of phycocyanin at pH 6.0 (Keithellakpam *et al.*, 2015).

According to Roman *et al.* (2002) the synthesis of phycobiliproteins increases under light limiting conditions. Many authors including Hong and Lee (2008) and Hemalata and Fatma (2009) have concluded that light intensity near to 25 $\mu molm^{-2}s^{-1}$ is most suitable for phycocyanin production. Johnson *et al.* (2014) studied the effect of different factors on phycobiliproteins. They have obtained the maximum yield of phycobiliproteins of 0.13 g g^{-1} dry cell weight of *Nostoc* sp. when grown in green light. Sharma *et al.* (2013) demonstrated in Anabaena strains that carotenoids and phycobilins decrease with increase in temperature and osmotic potential. Khattar *et al.* (2015) has projected *Anabaena fertilissima* strain grown in blue light as a promising candidate for phycobiliprotein production commercially. Hosseini *et al.* (2015) have reported that *Spirulina platensis* produces 44.27 mg g^{-1} dry weight of phycocyanin in laboratory cultures at pH 9.6.The present strain of *Phormidium tenue* (D 2008) has the advantage that it produces a similar amount of phycocyanin in the ambient day light of the indoor environment at pH 7.5 which is an important factor in the cost reduction of production system.

The third species of cyanobacteria in this investigation is *Synechococcus elongatu*s, which is unicellular. The strain was proved to exhibit chromatic adaptation. The extraction and estimation of the water soluble pigments showed that in general the relative proportion of phycocyanin, allophycocyanin and phycoerythrin is constant *i.e* ≈1. Exceptionally, under exposure to pH 6.5 and light intensity of 2200 lux at 28±2^0C the PC/PE ratio reduces to 0.74. The species maybe further investigated for production of phycoerythrin at low pH.

The commercial production of phycocyanin is based on a very few species. They are *Spirulina platensis* (*Arthrospira platensis*) produced

through photoautotrophic or mixotrophic process. The rhodophyte *Galdieria sulphuraria* is grown heterotrophically and C-phycocyanin is produced. Allophycocyanin is commercially produced from *Spirulina platensis*. Phycoerythrin occurs in cyanobacteria, cryptomonads and Rhodophyta. The commercial production is from the Mediterranean red algae *Corallina elongata* and from the red algae *Porphyridium cruentum*. All these species require unique conditions of growth for the production of the specific pigments. Therefore search for new candidate species is required and this study has identified two potential species i.e. *Phormidium tenue* and *Oscillatoria acuminata* for production of phycocyanin. *Oscillatoria acuminata* would be the right candidate for upscaling the production of phycobiliproteins Among the three species investigated, *Synechococcus elongatus* has higher proportion of phycoerythrin; but the yield and purity and purity is low for a viable utility..

Chapter 4

AQUATIC POLLUTANTS AND PIGMENT PRODUCTION BY *SYNECHOCOCCUS ELONGATUS*

Contents

4.1 Introduction
4.2. Materials and Methods
4.3 Experimental Assessments
4.4 Discussion

4.1 Introduction

Water pollution is a major environmental concern and is defined as the introduction of contaminating pollutants into the natural waters leading to adverse changes. Numerous reports suggest that the earth's water resources are being depleted, polluted and being rendered un-potable at a very fast rate (Malik *et al.*, 2014). Pollution of the aquatic environment by both organic and inorganic chemicals poses a major threat to the survival of aquatic organisms including fish (Saeed and Shaker, 2008).

In India 70 percent of its surface water resources and a growing percentage of its groundwater reserves are contaminated by toxic, biological, organic and inorganic pollutants. So water pollution is a serious environmental problem in India. In many cases, these water sources have been rendered unsafe for human consumption as well as for other activities like industrial needs and irrigation. The degradation in water quality can contribute to water scarcity because this limits its availability for both human use and for the ecosystem. The Central Pollution Control Board (CPCB) identified severely polluted

stretches on 18 major rivers in India in 1995. A majority of these polluted stretches were situated in and around large urban areas, implying that the contribution of industrial and domestic sector's to water pollution is very high.

Agricultural activities are also a major contributor to water quality. Agricultural run-offs containing pesticides and fertilizer residues affect groundwater and surface water sources. Fertilizers have an indirect adverse impact on water resources by increasing the nutritional content of water sources and allowing microorganisms (maybe disease vectors or algae) to proliferate.

Diverse classes of chemical pollutants are released into the aquatic environment from various sources like industry, agriculture, domestic effluents and medical wastes. Heavy metals are any metallic element that has a relatively high density and is poisonous or toxic even at low concentrations. The term "heavy metals" applies to the group of metals and metalloids with atomic density greater than 4 g cm^{-3} or 5 times or more, greater than water. But being a heavy metal is more in concern with their chemical properties rather than its density. Heavy metals include lead (Pb), cadmium (Cd), zinc (Zn), mercury (Hg), arsenic (As), silver (Ag), chromium (Cr), copper (Cu), iron (Fe) and the platinum group elements. More prominence of heavy metal pollution occurs in areas of mining and old mine sites and as the distance from the mining site increases, pollution decreases. Lead, cadmium, mercury and arsenic are the heavy metals causing major threat to human health. The release of heavy metals to the environment occurs by means of various processes and pathways – emission to air (eg. during combustion, extraction and processing), to surface waters (through surface runoff and releases from storage and transport) and to the soil (therefore into groundwaters and crops). Because of the quantities of heavy metals involved and the widespread dispersion and

potential for exposure that often follows, atmospheric emissions causes the greatest concern to human health.

The environmental pollution caused by accumulation of oraganic pollutants (pesticides, PCBs, DDT etc.) and heavy metals (Cd, Pb, Se, As etc.) can be solved to some extent by the use of phytoremediation technology that uses algae or aquatic plants to remove these pollutants from the environment.

The environmental fate of metals are influenced by microbes employing diverse physicochemical and biological mechanisms that bring about changes in the mobility and speciation of metals. Cyanobacteria respond and adapt to most stress conditions and are often found to be abundant in metal-contaminated environments. They can affect the mobility and bioavailability of metals as they can tolerate, accumulate and detoxify metal contaminants in aquatic environments. The attributes of cyanobacteria in this regard are the ability to modify the metal speciation thus leading to decreased or increased mobility of metals. These mechanisms include – extracellular sequestration, organic or inorganic precipitation, intracellular compartmentalization, active transport and the synthesis of metal-binding proteins like metallothioneins (Acharya and Apte, 2013).

The U.S Environmental Protection Agency (EPA) states that " By their very nature, most pesticides create some risk of harm to humans, animals, or the environment because they are designed to kill or otherwise adversely affect living organisms". Many studies involving the major rivers and streams reveal that around 96% of all fish, 33% of major aquifers and approximately 100% of all surface water samples contain one or more pesticides at detectable levels. When pesticides enter the water system, they have been reported to reach non-target organisms (including plants, animals and humans). Many pesticides are

toxic to humans eventhough it might take a larger dose to harm humans in comparison with harming pests like insects. These pesticides harm humans in many ways like endocrine disruption, disruption in the functioning of sex hormones and negatively impacting the reproductive performance (Agrawal et al., 2010). Many studies in India have reported contamination in various environmental realms because of the accumulation of pesticide residues in biota and humans (Carvalho *et al.*, 2014; Singare, 2015).

In this investigation the effect of two heavy metals and three pesticides on the production of phycobiliproteins of *Synechococcus elongatus* was studied. It has been shown that the pollutants present in the water of aquaculture systems, whether indoor or outdoor can be accumulated by the organisms and this in turn can affect human health adversely after consumption (Martins *et al.*, 2011; Squadrone *et al.*, 2016; Islam *et al.*, 2017). This study aims to identify how the pollutants interfere in the growth and production of pigments by cyanobacteria thereby affecting the efficiency and viability of the culture systems.

4.2 Materials and Methods

4.2.1 Effect of Heavy Metals on Pigment Production

Synechococcus elongatus was inoculated into modified BG-11 medium in one liter conical flasks, at a cell concentration of 1 x 10^6 mL^{-1}. The cultures were incubated for seven days under day light fluorescent lamps as described in section 3.3.1. On the seventh day the cultures were divided into two sets and added different test doses of cadmium and copper. The test doses were 0.5, 1.0, 3.0, 6.0 and 9.0 mg L^{-1} of the respective element. Cadmium was added to the culture medium as cadmium chloride and copper as copper sulphate in

solution. Three replicates were maintained for each with untreated control. All the cultures were again incubated for 96 hours.

After 96 hrs of growth, the cell count was determined using a haemocytometer; phycobiliproteins and total protein content were estimated.

- ***Estimation of proteins***

The cyanobacterial culture was centrifuged at 5000 rpm for 10 minutes and the pellet was washed twice with distilled water. 5ml of 10% trichloro acetic acid was added to the pellet and left for 30minutes in a boiling water bath. The contents were cooled and centrifuged at 5000 rpm for 5 minutes. The resulting pellet was dissolved in 1 mL of 1N NaOH and from this 0.1 mL was taken and made upto 1 mL with distilled water. 5 mL of alkaline reagent was added to this and incubated for 3 minutes. Then 0.5 mL of folin-ciocalteu's reagent was added to it and mixed thoroughly and allowed to stand for 30 minutes. The absorbance was measured at 750 nm in a spectrophotometer. The standard protein solution was prepared in a graded series to determine the concentration in sample (Lowry *et al.*, 1951). The amount of protein in the sample was expressed as fg $cell^{-1}$.

4.2.2 Effect of Pesticides on Pigment Production

S. elongatus culture was inoculated at a cell concentration of 1 x 10^{-6} mL^{-1} into modified BG-11 medium in one liter conical flasks and grown under a light panel of 2200 lux with a light : dark cycle of 12 :12hrs at 28 ± 2^0 C. After 7 days of growth, the following pesticides were introduced into the culture . The pesticides assayed are given in table 4.1.

Table 4.1 Pesticides assayed against in vitro cultures of *Synechococcus elongatus*

Trade name	Chemical name	Class	Chemical category
Classic 20	Chlorpyriphos	Insecticide	Organophosphorus
Indofil M-45	Mancozeb	Fungicide	Dithiocarbamate
Roundup	Glyphosate	Herbicide	N-(phosphonomethyl) glycine

Two test doses of each pesticide was assayed against a control at a sublethal and lethal concentration based on literature (Table 4.2).

Table 4.2 Test doses of pesticides assayed

Pesticide	Sublethal	Lethal
Chlorpyriphos	0.01%	0.2%
Mancozeb	10 mg L^{-1}	32 mg L^{-1}
Glyphosate	0.5%	2%

After 96 hrs of growth cell count was determined using a haemocytometer; phycobiliproteins and protein content were estimated. The effects were statistically evaluated by ANOVA (Appendix 9).

4.3 Results

4.3.1 Effect of Heavy Metal Contamination

➢ ***Effect of cadmium contamination***

There was visual change in the colour of *S. elongatus* cultures grown in media incorporated with different concentrations of cadmium. The culture grown as control was reddish brown in colour . The test cultures with cadmium were pale yellow to green depending on increasing cadmium level (Fig. 4.1).

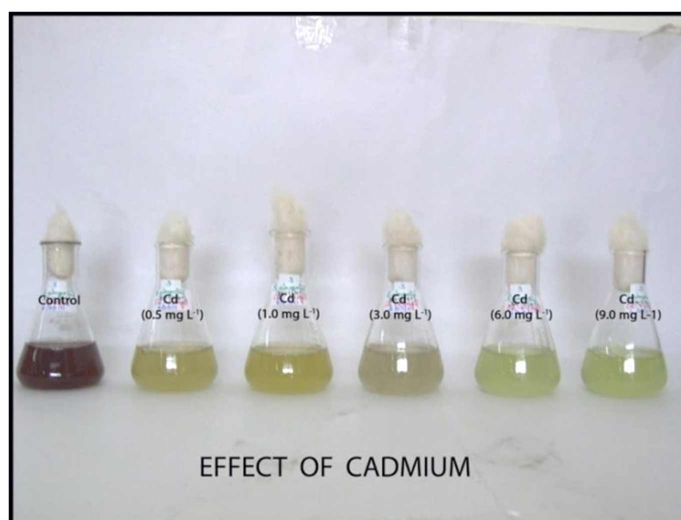


Fig. 4.1 Effect of cadmium on growth of *Synechococcus elongatus*

S. elongatus produced a highest biomass of 29.56 x 10^6 mL^{-1} in the control culture and lowest biomass of 4.66 x 10^6 mL^{-1} in media incorporated with 9.0 mg L^{-1} cadmium (Fig. 4.2, Table 4.3). The culture density, protein content and phycobiliproteins decreased gradually with increasing cadmium level.

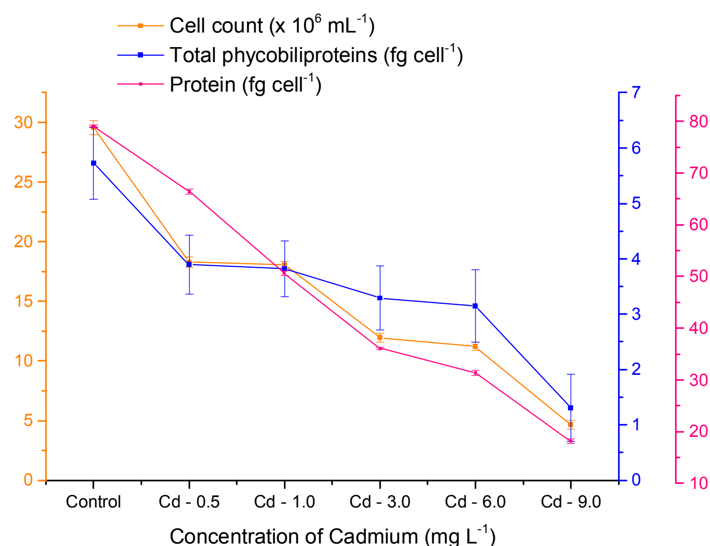


Fig. 4.2 Effect of cadmium on growth of *Synechococcus elongatus*

Table 4.3 Effect of different concentrations of cadmium on growth, total phycobiliproteins and protein of *Synechococcus elongatus*

Concentration of Heavy metal (mg L^{-1})	Cell count (x 10^6 mL^{-1})	Total phycobiliproteins (fg $cell^{-1}$)	Protein (fg $cell^{-1}$)
Control	29.56 a*	5.726 a	79.084 a
0.5	18.28 b	3.895 b	66.446 b
1.0	18.07 b	3.816 b c	50.642 c
3.0	11.96 c	3.291 b c d	36.105 d
6.0	11.21 c	3.145 b c d	31.374 e
9.0	4.66 d	1.307 e	18.131 f

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

The quantity of component pigments phycocyanin, allophycocyanin and phycoerythrin also decreased when exposed to cadmium. The least amount was produced in media incorporated with cadmium 9.0 mg L^{-1}. The PC/ PE ratio was distributed equally across the different concentrations of cadmium (Table 4.4).

Table 4.4 Phycobiliprotein content of *Synechococcus elongatus* grown in media incorporated with Cadmium

Concentration of Cd (mg L^{-1})	PC (fg $cell^{-1}$)	APC (fg $cell^{-1}$)	PE (fg $cell^{-1}$)	PC/PE
Control	1.846 a*	2.029 a	1.851 a	1.00
0.5	1.286 b	1.317 b	1.291 b	1.00
1.0	1.259 b c	1.291 b c	1.266 b c	0.99
3.0	1.074 b c	1.134 b c	1.082 b c	0.99
6.0	1.029 b c	1.073 b c d	1.043 b c	0.99
9.0	0.405 d	0.489 d	0.413 d	0.99

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

➢ *Effect of copper contamination*

There was visual change in the colour of *S. elongatus* cultures grown in media incorporated with different concentrations of copper. The control culture and those incorporated with 0.5 and 1.0 mg L^{-1} of copper, were reddish brown in colour while cultures incorporated with copper (3.0 – 9.0 mg L^{-1}) were light green in appearance (Fig. 4.3). *S. elongatus* produced a highest biomass of 29.56 x 10^6 mL^{-1} in the control culture and lowest biomass of 16.18 x 10^6 mL^{-1} in media incorporated with 9.0 mg L^{-1} copper (Fig. 4.4, Table 4.5).

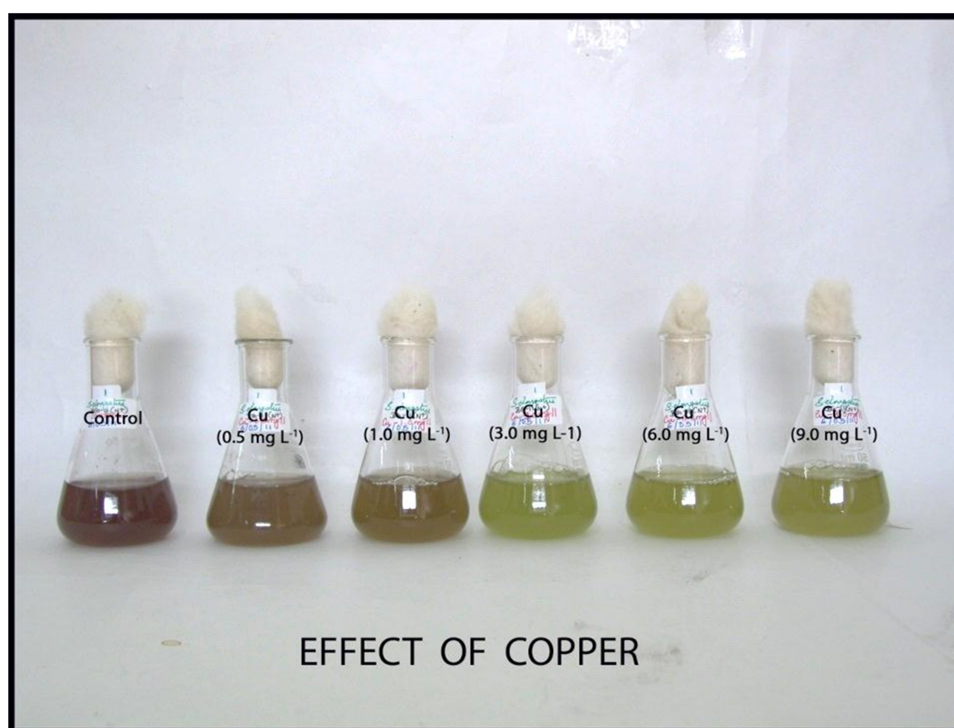


Fig. 4.3 Effect of copper on growth of *Synechococcus elongatus*

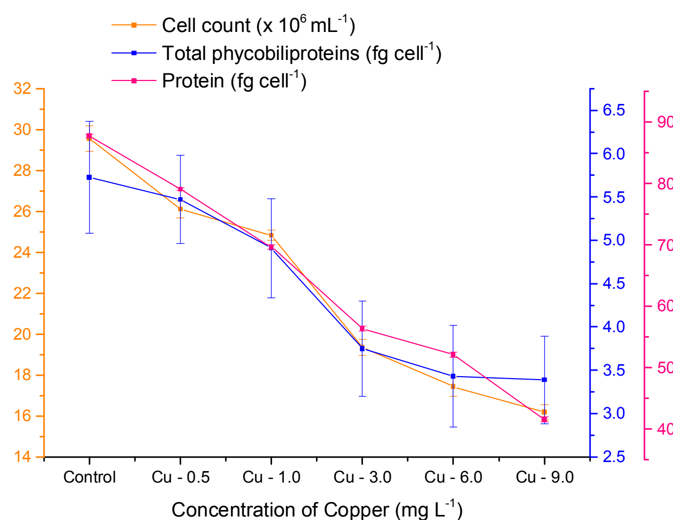


Fig. 4.4 Effect of copper on growth of *Synechococcus elongatus*

Table 4.5 Effect of different concentrations of Cu on growth, total Phycobiliproteins and protein

Concentration of Heavy metal (mg L^{-1})	Cell count (x 10^6 mL^{-1})	Total phycobiliproteins (fg $cell^{-1}$)	Protein (fg $cell^{-1}$)
Control	29.56 a*	5.726 a	87.74 a
0.5	26.11 b	5.470 a b	79.08 b
1.0	24.83 c	4.910 a b c	69.62 c
3.0	19.35 d	3.749 c d	56.39 d
6.0	17.44 e	3.432 c d e	52.11 e
9.0	16.18 f	3.390 c d e	41.59 f

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

In *Synechococcus elongatus* grown in media incorporated with different concentrations of copper, the production of phycobiliproteins was statistically similar in the control cultures and those treated with 0.5 and 1.0 mg L^{-1} of copper. The production of phycobiliproteins decreased as the concentration of copper increased from 3.0 to 9.0 mg L^{-1}. These trends were reflected in the quantity of component pigments phycocyanin, allophycocyanin and phycoerythrin as well. The highest PC/PE ratio did not vary considerably (Table 4.6).

Table 4.6 Phycobiliprotein content of *Synechococcus elongatus* grown in media incorporated with Copper

Concentration of Cu (mg L^{-1})	PC (fg $cell^{-1}$)	APC (fg $cell^{-1}$)	PE (fg $cell^{-1}$)	PC/PE
Control	1.846 a*	2.029 a	1.851 a	1.00
0.5	1.726 a	1.952 a b	1.792 a	0.96
1.0	1.551 a b	1.773 a b c	1.586 a b	0.98
3.0	1.171 b c	1.365 b c d	1.212 b c	0.97
6.0	1.073 b c	1.271 c d	1.094 b c d	0.98
9.0	0.883 c	1.077 d	0.895 c d	0.99

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

4.3.2 Effect of Pesticide Contamination

- ***Effect of Chlorpyriphos on growth*** **and phycobiliproteins**

There was visual change in the colour of *S. elongatus* cultures grown in media incorporated with different percentages of the insecticide chlorpyriphos. The cultures grown as control and those grown in media incorporated with 0.01% of chlorpyriphos were reddish brown in colour while cultures incorporated with 0.2% of chlorpyriphos were pale green in appearance (Fig. 4.5). *S. elongatus* produced a highest biomass of 22.53 x 10^6 mL^{-1} in the control culture and lowest biomass of 13.24 x 10^6 mL^{-1} in media incorporated with 0.2% of chlorpyriphos (Fig. 4.6, Table 4.7).

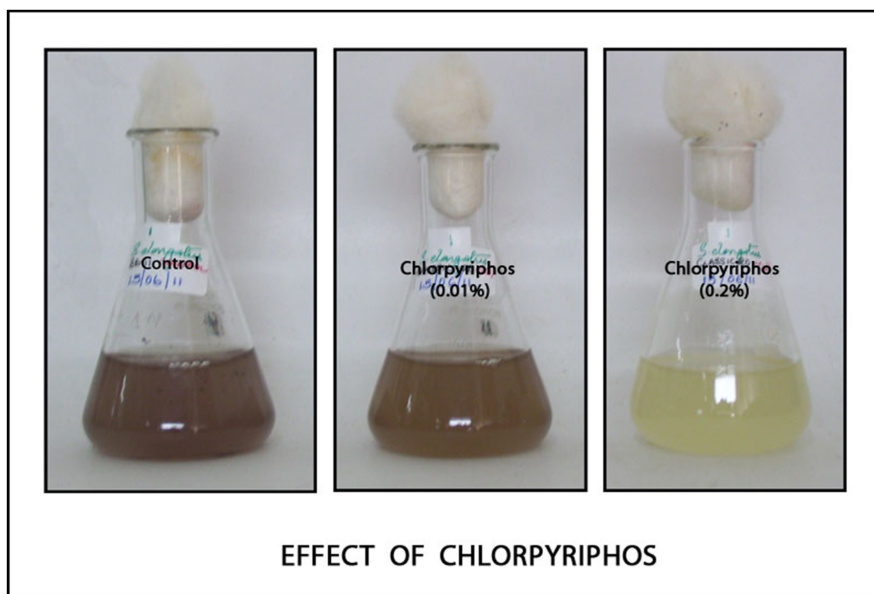


Fig. 4.5 Effect of chlorpyriphos on growth of *Synechococcus elongates*

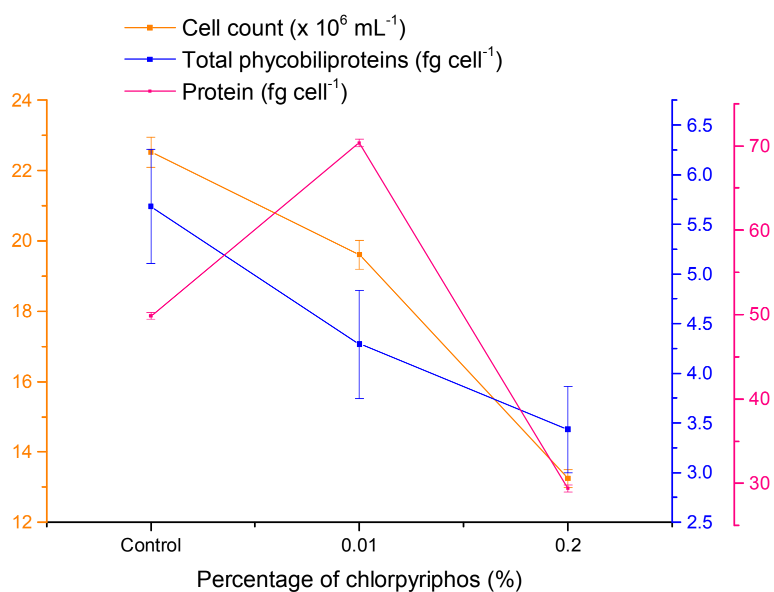


Fig. 4.6 Effect of chlorpyriphos on growth of *Synechococcus elongatus*

Table 4.7 Effect of different percentage of chlorpyriphos on growth, total phycobiliproteins and protein

Percentage of chlorpyriphos (%)	Cell count (x 10^6 mL^{-1})	Total phycobiliproteins (fg $cell^{-1}$)	Protein (fg $cell^{-1}$)
Control	22.53 a*	5.681 a	49.850 a
0.01	19.61 b	4.293 b	70.334 b
0.2	13.24 c	3.434 b	29.407 c

* Figures sharing the same letters in the same column do not differ significantly at P < 0.05

The production of phycobiliproteins decreased as the percentage of chlorpyriphos increased from 0.01% to 0.2%. These trends were reflected in the quantity of component pigments phycocyanin, allophycocyanin and phycoerythrin as well. The least amount was produced in media incorporated with chlorpyriphos at 0.2%. The PC/ PE ratio was distributed equally across the different percentages of chlorpyriphos (Table 4.8).

Table 4.8 Phycobiliprotein content of *Synechococcus elongatus* grown in media incorporated with chlorpyriphos

Percentage of chlorpyriphos (%)	PC (fg $cell^{-1}$)	APC (fg $cell^{-1}$)	PE (fg $cell^{-1}$)	PC/PE
Control	1.824 a*	2.022 a	1.835 a	0.99
0.01	1.367 b	1.543 b	1.383 b	0.99
0.2	1.079 b	1.243 b	1.112 b	0.97

* Figures sharing the same letters in the same column do not differ significantly at P < 0.05

➢ ***Effect of mancozeb on growth and phycobiliproteins***

The fungicide mancozeb depressed the cell multiplication, reduced the production of pigments, but increased the protein production. The cultures grown as control were reddish brown in colour while those grown in media incorporated with 10 mg L^{-1} and 32 mg L^{-1} of mancozeb were green in appearance (Fig. 4.7). *S. elongatus* produced a highest biomass of 22.53 x 10^6 mL^{-1} in the control culture and lowest biomass of 13.99 x 10^6 mL^{-1} in media incorporated with 32 mg L^{-1} of mancozeb (Fig. 4.8, Table 4.9).

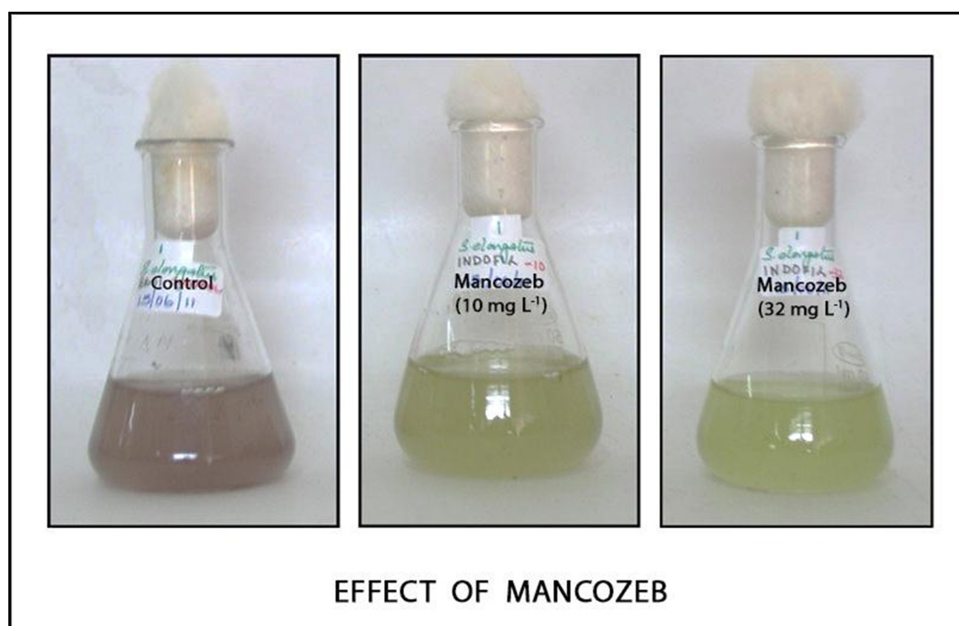


Fig. 4.7 Effect of Mancozeb on growth of *Synechococcus elongatus*

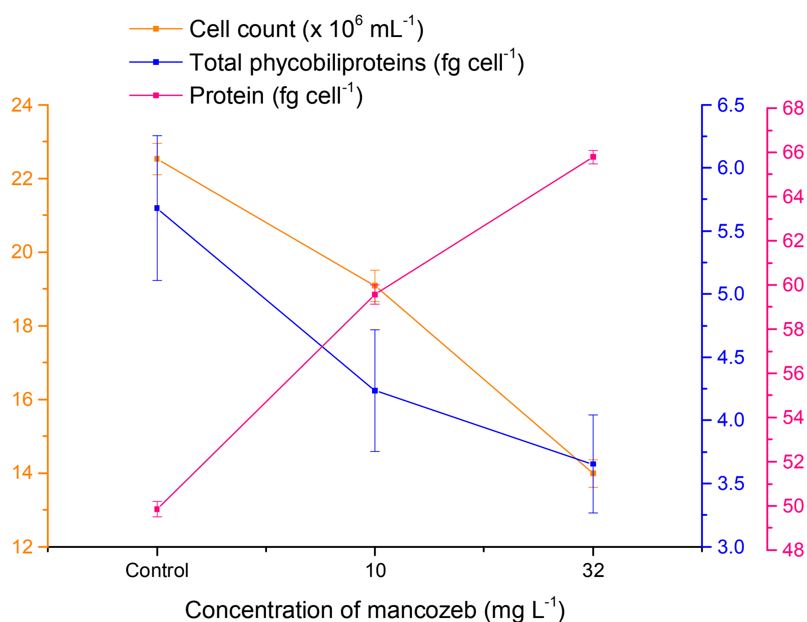


Fig. 4.8 Effect of mancozeb on growth of *Synechococcus elongatus*

Table 4.9 Effect of different concentrations of mancozeb on growth, total phycobiliproteins and protein

Concentration of mancozeb 45 (mg L^{-1})	Cell count (x 10^6 mL^{-1})	Total phycobiliproteins (fg $cell^{-1}$)	Protein (fg $cell^{-1}$)
Control	22.53 a*	5.681 a	49.850 a
10	19.08 b	4.237 b	59.563 b
32	13.99 c	3.655 b	65.788 c

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

These trends were reflected in the quantity of component pigments phycocyanin, allophycocyanin and phycoerythrin as well. The least amount was produced in media incorporated with Mancozeb 32 mg L^{-1}. The respective quantities in control, Mancozeb 10 mg L^{-1} and 32 mg L^{-1} were 5.681, 4.237 and 3.655 fg $cell^{-1}$. The PC/PE ratio was nearly same (Table 4.10).

Table 4.10 Phycobiliprotein content of *Synechococcus elongatus* grown in media incorporated with Mancozeb

Concentration of Mancozeb (mg L^{-1})	PC (fg $cell^{-1}$)	APC (fg $cell^{-1}$)	PE (fg $cell^{-1}$)	PC/PE
Control	1.824 a*	2.022 a	1.835 a	0.99
10	1.349 b	1.526 b	1.362 b	0.99
32	1.128 b	1.336 b	1.190 b	0.95

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

- ***Effect of Glyphosate on growth and phycobiliproteins***

The herbicide glyphosate drastically affected the growth as well as pigment production of *S. elongatus* . The cultures grown as control were reddish brown in colour while those grown in media incorporated with 0.5% and 2% of glyphosate were pale green in appearance (Fig. 4.9). *S. elongatus* produced a biomass of 22.53 x 10^6 mL^{-1} in the control culture and 9.75 x 10^6 mL^{-1} in media incorporated with 2% of glyphosate (Fig. 4.10, Table 4.11).

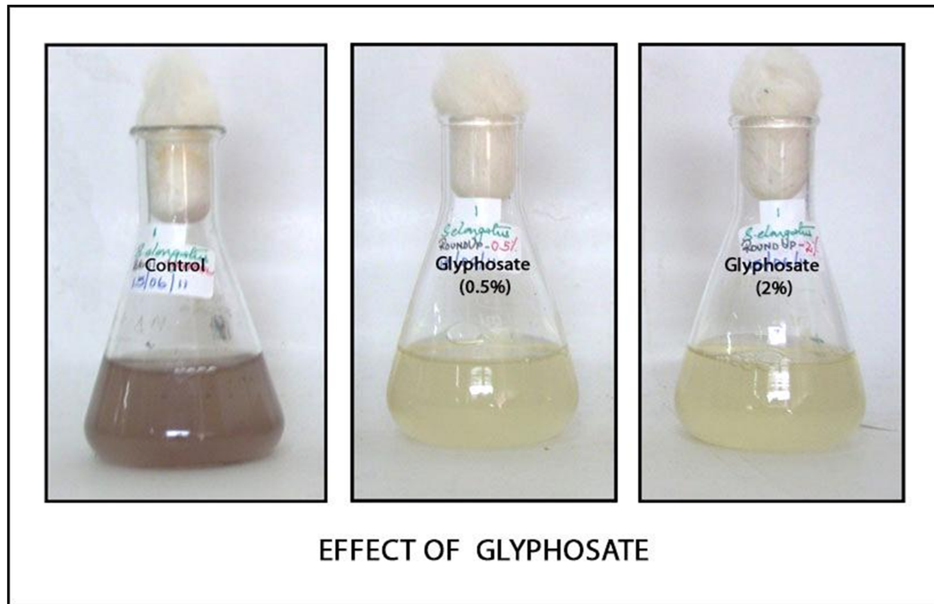


Fig. 4.9 Effect of glyphosate on growth of *Synechococcus elongatus*

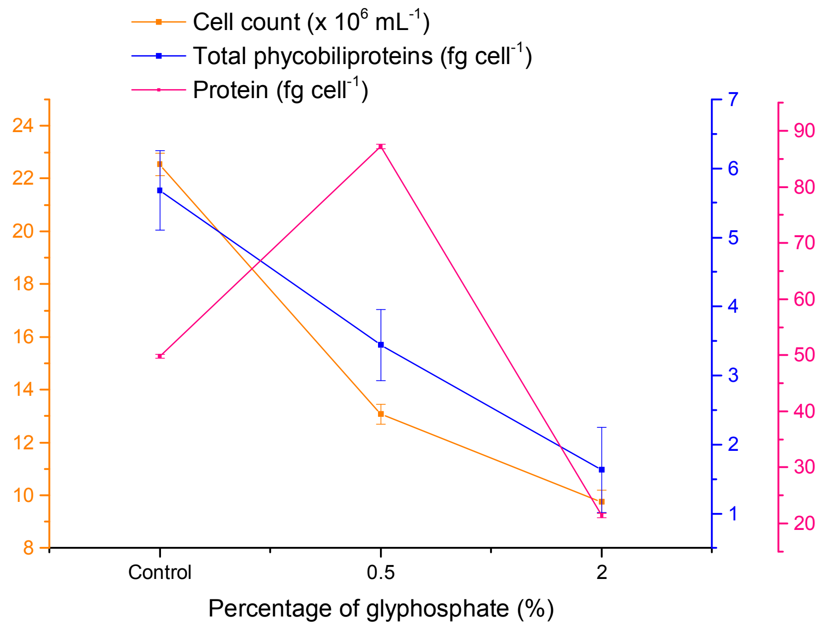


Fig. 4.10 Effect of glyphosate on growth of *Synechococcus elongatus*

Table 4.11 Effect of different percentage of glyphosate on growth, total phycobiliproteins and protein

Percentage of glyphosate (%)	Cell count ($x 10^6 mL^{-1}$)	Total phycobiliproteins (fg $cell^{-1}$)	Protein (fg $cell^{-1}$)
Control	22.53 a*	5.681 a	49.850 a
0.5	13.07 b	3.440 b	87.215 b
2.0	9.75 c	1.634 c	21.415 c

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

The production of phycobiliproteins decreased as the percentage of glyphosate increased from 0.5% to 2%. These trends were reflected in the quantity of component pigments phycocyanin, allophycocyanin and phycoerythrin as well. The least amount was produced in media incorporated with 2% glyphosate. The respective quantities in control, 0.5% and 2% were 5.681, 3.440 and 1.634 fg $cell^{-1}$. The highest PC/PE ratio was 0.99 in the control, closely followed by 0.96 at 0.5% and 0.95 at 2.0% (Table 4.12).

Table 4.14 Phycobiliprotein content of *Synechococcus elongatus* grown in media incorporated with glyphosate

Percentage of glyphosate (%)	PC (fg $cell^{-1}$)	APC (fg $cell^{-1}$)	PE (fg $cell^{-1}$)	PC/PE
Control	1.824 a*	2.022 a	1.835 a	0.99
0.5	1.064 b	1.270 b	1.106 b	0.96
2.0	0.470 c	0.670 c	0.493 c	0.95

* Figures sharing the same letters in the same column do not differ significantly at $P < 0.05$

It is observed that contamination of culture medium affects the production of the pigments considerably. The extent of damage varies with the contaminant. Cadmium is more injurious than copper. The toxicity of the pesticides vary with their composition. As the proportion of phycocyanin to phycoerythrin is not altered drastically it may be assumed that all the phycobiliproteins are affected equally. As the masking effect of these pigments are reduced the green colour of chlorophyll predominates.

4.4 Discussion

Heavy metals are a group of pollutants widely used for industrial purposes and reported to contaminate aquatic ecosystems. Copper is a common toxicant, though required by autotrophs in low amount, can interfere with many physiological processes to be potentially cytotoxic at high concentrations. Cadmium is another heavy metal implicated in aquatic toxicity. Cadmium is not an essential nutrient for cyanobacteria but it can attach to sulphated groups, metalloproteins and metalloenzymes thereby neutralising their function (Pinto *et al.*, 2003). Priyadarshini and Rath (2012) studied the effect of heavy metals nickel and copper on two species of *Phormidium* and two species of *Oscillatoria*. The pigment concentration as well as protein was found to decrease with increasing test concentration for *O. boryana*, *Oscillatoria* sp. and *P. tenue*. The other species of *Phormidium* had increased pigment content at 0.1 mg L^{-1} Ni/ Cu. This led to the conclusion that there is differential response among inter and intragenic species, as well as between the different metals.

In the present investigation, cadmium concentration of 0.5 mg L^{-1} reduced the growth, total phycobiliproteins and protein content of *S. elongatus* significantly. The production of phycobiliproteins was not affected significantly with copper at 1.0 mg L^{-1}; but the total protein content reduced and cell contents decreased significantly at 1.0 mg L^{-1}. These results only confirm the previous observations that copper and cadmium can inhibit the growth and photosynthetic activity of cyanobacteria.

The contamination of water bodies by fungicides, insecticides and herbicides are of concern as often they are harmful to the aquatic organisms.

Many herbicides inhibit the light reactions in photosynthesis. 2,4-D inhibits algal growth, photosynthesis and chlorophyll *a* synthesis (Wong, 2000).

Several reports are available on the comparative toxicity of different types of pesticides on cyanobacteria (Babu *et al.*, 2001; Nirmal Kumar and Rita, 1996; Kumar *et al.*, 2012; Chalifour and Tam, 2016). Kumar *et al.* (2012) have demonstrated tremendous decrease in photosynthetic pigments, metabolic as well as enzymatic activities in cyanobacteria as a response to pesticide treatment. In the present investigation all the three pesticides tested reduce the growth of the culture and the phycobiliproteins.

Heavy metals and pesticides are commonly found as trace compounds in agricultural and industrial areas and can infiltrate into water sources. Contaminated water when used in culture systems can adulterate algal products as well. Even though they have been found to be less toxic to cyanobacteria at lower concentrations, they have the ability to terminate algal growth at levels toxic to humans (Al-Dhabi, 2013). The permissible limits of cadmium in commercial Spirulina as per the “Cyanotech Gold Standard” is <0.2 ppm and it has to be free of pesticides and herbicides. In the present study it is observed that there is lowering of growth upon addition of heavy metals and pesticides indicating the species is very sensitive to contamination of the growth medium.

SUMMARY AND CONCLUSION

Cyanobacteria have wide applications in medicine, pharmaceuticals and fine chemicals. Apart from the primary metabolites like proteins, fatty acids, vitamins and pigments, they produce several useful secondary metabolites having antifungal, antibacterial, antiviral, antineoplastic, and antialgal activities.

Cyanobacteria have received attention as a promising source of phycobiliproteins, which are natural colourants that are both industrially and pharmacologically important. The content and composition of phycobiliproteins in cyanobacteria have been shown to be influenced by nutrient availability as well as environmental factors like light intensity, light quality, temperature, salinity and pH etc.

The commercial production of phycobiliproteins is based on a very few species. They are *Spirulina platensis* (*Arthrospira platensis*) produced through photoautotrophic or mixotrophic process from which phycocyanin and allophycocyanin is produced. The rhodophyte *Galdieria sulphuraria* is grown heterotrophically for production of C-phycocyanin. Phycoerythrin occurs in cyanobacteria, cryptomonads and Rhodophyta. The commercial production is from the Mediterranean red algae *Corallina elongata* and from the red algae *Porphyridium cruentum*. All these species require unique conditions of growth for the production of the specific pigments. Therefore search for new candidate species is required.

The objectives of this investigation were:

i. To isolate and develop cultures of cyanobacteria from the environs of Kochi and quantify the production of phycobilin pigments.
ii. To optimize the production of phycobilin pigments by the species isolated.
iii. To study the effect of certain pollutants on production of phycobilins.

Three species of cyanobacteria were evaluated for the production of phycobiliproteins under different growth conditions during this investigation. These three species are – *Phormidium tenue* isolated for this investigation from the brackish water prawn farms of Kochi, *Oscillatoria acuminata* isolated originally from sewage drains in Kochi, and *Synechococcus elongatus* formerly isolated from pokkali paddy fields of Kochi. The species were maintained as batch cultures under illumination from day light fluorescent lamps at 28 ± 2^0C. The growth and production of phycobiliproteins of the three species were evaluated through independent experiments carried out in a sequence, deriving conclusions in every step.

P. tenue was grown in f/2 medium, sediment extract medium and ASN-III medium for evaluation of the culture media. For *O. acuminata* and *S. elongatus* the media tested were modified BG-11 , SN medium and E31 medium . Biomass and phycobiliproteins content were evaluated for all three cyanobacterial cultures.Based on the observations ASN III medium and an incubation period of 14 days was selected for *Phormidium tenue ,* modified BG-11 medium and an incubation period 14 days was selected for *Oscillatoria acuminata*; and modified BG-11 medium and an incubation period of 7 days was selected for *Synechococcus elongatus*.

The influence of salinity of the selected medium on production of pigmentsby the three cyanobacterial species was evaluated by growing them in medium of different salinities. Biomass, chlorophyll *a* and phycobiliproteins content were estimated. It was inferred from the results that *P.tenue* can be raised at 25 x 10^{-3} ASN III medium. For *O.acuminata* and *S.elongatus* freshwater medium was selected for further evaluation.

The effect of pH on the production of chlorophyll *a* and phycobiliproteins production by the three species was investigated by growing them in the selected culture media modified to different pH. Biomass, chlorophyll *a* and phycobiliproteins content were estimated.It was inferred from the results that *P.tenue* and *O. acuminata* can be cultivated at pH 7.5. *S.elongatus* favours pH 7.5 for production of phycocyanin and allophycocyanin; but when the pH was lowered to 6.5 there occured a higher production of phycoerythrin over phycocyanin resulting in lowering of PC/PE to 0.74. The cell density acquired by this species is not significantly different at pH 6.5 and 7.5. So the pH of the medium can be set at pH 6.5 when the aim is production of phycoerythrin.

The effect of exposure to two different light intensities on the growth and pigment production of the three species of cyanobacteria was evaluated. Biomass, chlorophyll *a* and phycobiliproteins content were estimated. From the results it was inferred that lower light intensity was the most suitable for production of phycobiliproteins in *P. tenue* and *O. acuminta*. But for the production of phycoerythrin from *S.elongatus,* higher light intensity is preferred.

The effect of different light wavelengths on the phycobilin composition of three species of cyanobacteria was evaluated. The biomass, chlorophyll *a* and phycobiliproteins content were estimated. It was

inferred that the production of pigments by *P.tenue* and *O. acuminata* was the maximum when grown under white light. *S.elongatus* exhibits chromatic adaptation. The culture density was the highest under white light. Although the absolute quantities of the phycobilin pigments does not vary significantly, the relative quantity of phycocyanin and phycoerythrin in this species varies as in Group III chromatic adaptation.

The growth and pigment production of the three species after exposure to UV was investigated. It was inferred that in general these species may tolerate short term exposures to UV once they have picked up active growth in a normal environment; but long term exposure will considerably affect the pigments and consequently decrease growth and accumulation of biomass.

The effect of temperature on the three species of cyanobacteria were evaluated at five levels of temperature above the ambient in the controlled environment of a plant growth chamber. The biomass, chlorophyll *a* and phycobiliproteins content were estimated. The unique observation in this set of experiments was that there was visibly change of colour exhibited by *Synechococcus elongatus* when grown inside the growth chamber. The cultures appeared grass-green instead of its normal blue green or reddish brown tint. The behaviour of the phycobilin pigments could not be studied so that this phenomenon cannot be explained at this stage; but definitely further studies has to be undertaken as this species shows chromatic adaptation which is an important property of many cyanobacteria to adapt to changes in their environment.

The combined effect of light and temperature on the three species of cyanobacteria were evaluated in this study at five levels of temperature and light intensity in the controlled environment of the plant growth chamber. The biomass, chlorophyll *a* and phycobiliproteins content were estimated. It is inferred that *P. tenue* can accumulate maximum biomass

upto 45^{0}C at 2000 lux . Phycocyanin production is at its best at 1000 to 2000 lux and can proceed to 50^{0}C at 1000 lux.The suitable condition for production of allophycocyanin is 2000 to 2500 lux and temperature 30-40^{0}C. The production of phycoerythrin is favoured at 2500 lux at 30^{0}C.

The yield of *O.acuminata* is highest at 2000 lux and 30^{0}C. Phycocyanin production is at its best at 2000 lux and 40^{0}C, allophycocyanin at 1000 lux and 45^{0}C while the suitable condition for phycoerythrin is 2000 to 3000 lux and 30^{0}C.

The yield of *S. elongatu*s is highest at 2500 to 3000 lux and upto 40^{0}C. These results clearly show that in culture systems the light and temperature must be regulated for each product and species concerned.

In the present investigation the purity of the crude extracts of phycobiliproteins from the three cyanobacterial species were evaluated. Purity of phycocyanin in the extracts of *P. tenue* was 0.96 and that of *O. acuminata* was 1.21. The purity of phycocerythrin in the extract of *S. elongatus* was 0.05. The purity has to be improved by further purification to obtain pure products.

This study has identified two potential species i.e. *Phormidium tenue* and *Oscillatoria acuminata* for production of phycocyanin. Among the three species investigated, *Synechococcus elongatus* has higher proportion of phycoerythrin; but the yield and purity and purity is low for a viable utility.

In this investigation the effect of two heavy metals and three pesticides on the production of phycobiliproteins of *Synechococcus elongatus* was studied. It was observed that contamination of culture medium affects the production of the pigments considerably. The extent of damage varied with the contaminant. Cadmium was more injurious than copper. The toxicity of

the pesticides varied according to their concentration. As the proportion of phycocyanin to phycoerythrin was not altered drastically it may be assumed that all the phycobiliproteins are affected equally.

This leads to the conclusion that in culture systems for production of phycobilin pigments, use of unpolluted water as growth media is a must for achieving the growth rates and the pigment production.

It is concluded from this investigation:

- *Phormidium tenue* is a promising strain for production of phycocyanin when grown at low light intensity of 500 lux in the ambient day light indoor in ASN-III medium of salinity 25 x 10^{-3} at pH 7.5 and temperature 28 ± 2^0C for fourteen days. The purification of the extract and steps to increase biomass is to be taken up.
- *Oscillatoria acuminata* has higher phycocyanin content when grown at low light intensity of 500 lux in the ambient day light indoor in modified BG-11 medium prepared in freshwater at a pH of 7.5 and temperature 28 ± 2^0C for fourteen days. The purity of phycocyanin in the extract is higher than *P. tenue*; but the amount of allophycocyanin and phycoerythrin are higher compared to *P.tenue*. This may make purification process tedious. Still it may be worth to develop the process of purification.
- *Synechococcus elongatus* has a tendency to produce more of phycoerythrin at higher light intensity of day light fluorescent lamps at pH 6.5. The species also exhibits chromatic adaptation. Further investigation is required in both these directions.
- The results conform to the earlier reports that the production of phycobiliproteins is considerably influenced by the growth medium and

the environmental conditions. So production systems targeting a specific pigment such as phycocyanin, allophycocyanin or phycoerythrin must establish the optimum conditions of growth of the species for the accumulation of that particular compound.

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3. www.auroville.com
4. www.cyanosite.bio.purdue.edu
5. www.hydrolinabiotech.com
6. www.parrynutraceuticals.com.
7. www.pyfarms.com.
8. www.steviaplants.in.

Appendix 1

Table (1.1) f/2 medium (Andersen, 2005)

Component	Stock Solution (g L^{-1} dH_2O)	Quantity used per liter of media
$NaNO_3$	75	1 mL
$NaH_2PO_4 \cdot H_2O$	5	1 mL
$Na_2SiO_3 \cdot 9H_2O$	30	1 mL
Trace metals solution	(recipe given below)	1 mL
Vitamins solution	(recipe given below)	0.5 mL
Composition of trace metals solution (g L^{-1} dH_2O)		
$FeCl_3 \cdot 6H_2O$	-	3.15 g
$Na_2EDTA \cdot 2H_2O$	-	4.36 g
$MnCl_2 \cdot 4H_2O$	180.0	1 mL
$ZnSO_4 \cdot 7H_2O$	22.0	1 mL
$CoCl_2 \cdot 6H_2O$	10.0	1 mL
$CuSO_4 \cdot 5H_2O$	9.8	1 mL
$Na_2MoO_4 \cdot 2H_2O$	6.3	1 mL
Composition of vitamin solution (g L^{-1} dH_2O)		
Thiamine · HCl (vitamin B1)	-	200 mg
Biotin (vitamin H)	1.0	1 mL
Cyanocobalamin (vitamin B12)	1.0	1 mL

Table (1.2) ASN –III medium (Rippka, 1988)

Component	Stock Solution ($g\ L^{-1}\ dH_2O$)	Quantity used per liter of media
NaCl	-	25.0 g
$MgSO_4 \cdot 7H_2O$	-	3.5 g
$MgCl_2 \cdot 6H_2O$	-	2.0 g
$NaNO_3$	-	0.75 g
$K_2HPO_4 \cdot 3H_2O$	-	0.75 g
$CaCl_2 \cdot 2H_2O$	-	0.5 g
KCl	-	0.5 g
$NaCO_3$	-	0.02 g
Citric acid	-	3.0 mg
Ferric ammonium citrate	-	3.0 mg
Mg EDTA	-	0.5 mg
Cyanocobalamin (vitamin B12)	-	10.0 mg
A-5 + Co trace metals sol.	(recipe given below)	1.0 mL
Composition of A-5 + Co trace metalssolution($g\ L^{-1}$ dH2O)		
H3BO3	-	2.860 g
MnCl2 · 4H2O	-	1.810 g
ZnSO4 · 7H2O	-	0.222 g
NaMoO4 · 2H2O	-	0.390 g
CuSO4 · 5H2O	-	0.079 g
Co(NO3)2 · 6H2O	-	49.40 mg

Table (1.3) Sediment Extract medium

Component	**Quantity used per liter of media**
Sediment extract	50 mL
KNO_3	0.1 g
NaHPO4	0.02 g

Table (1.4) Modified BG-11 medium (Andersen, 2005)

Component	**Stock Solution ($g\ L^{-1}\ dH_2O$)**	**Quantity used per liter of media**
Fe Citrate solution		1 mL
Citric acid	6	1 mL
Ferric ammonium citrate	6	1 mL
$NaNO_3$	-	1.5 g
K_2HPO_4	30.53	1 mL
$MgSO_4.7H_2O$	75	1 mL
$CaCl_2.2H_2O$	36	1 mL
Na_2CO_3	20	1 mL
Na_2EDTA	1.04	1 mL
Trace metals solution	(recipe given below)	1 mL
Composition of trace metals solution ($g\ L^{-1}\ dH_2O$)		
H_3BO_3	-	2.860 g
$MnCl_2.4H_2O$	-	1.810 g
$ZnSO_4.7H_2O$	-	0.220 g
$CuSO_4.5H_2O$	79.0	1 mL
$Na2MoO_4.2H_2O$	-	0.391 g
$Co(NO_3)_2.6H_2O$	49.4	1 mL

Table (1.5) SN medium (www.cyanosite.bio.purdue.edu)

Compound	Stock Solution ($g\ L^{-1}\ dH_2O$)	Quantity used per liter of medium
$NaNO3$	76.50	10 mL
$K2HPO4$	15.68	1 mL
$Na2EDTA \cdot 2H2O$	5.58	1 mL
$Na2CO3$	10.70	1 mL
Cyanocobalamin (vitamin B12)	0.001	1 mL
Vitamin solution	(recipe given below)	1 mL
Trace metals solution	(recipe given below)	1 mL
Composition of vitamin solution ($g\ L^{-1}\ dH_2O$)		
Vitamin B_{12}(cyanocobalamin)	1.0	1 mL
Biotin	0.1	10 ml
Thiamine – HCl	-	200 mg
Composition of trace metals solution ($g\ L^{-1}\ dH_2O$)		
Citric Acid · H_2O	-	6.250 g
Ferric ammonium citrate	-	6.000 g
$MnCl_2 \cdot 4H_2O$	-	1.400 g
$Na_2MoO_4 \cdot 2H_2O$	-	0.390 g
$ZnSO_4 \cdot 7H_2O$	-	0.222 g
$Co(NO_3)_2 \cdot 6H_2O$	-	0.025 g

Table (1.6) E31 Medium (www.cyanosite.bio.purdue.edu)

Ingredients	Medium composition
Soil extract*	50.0 ml
KNO_3	0.10 g
K_2HPO_4	0.01 g
$MgSO_4.7H_2O$	0.01 g
Cyanocobalamin	100.00 mg
Thiamine HCl	50.00 mg
Biotin	100.00 mg
Distilled water	1000.0 ml

***Preparation of soil extract**

Air dried soil and twice its volume of distilled water were autoclaved for 2 hr and left to cool. The supernatant was decanted carefully and filtered through Whattman No 1 filter paper. The soil extract thus obtained was autoclaved at 15 lbs pressure for 15 minutes and stored in refrigerator.

Appendix 2

Table (2.1) Effect of salinity of the growth medium on production of biomass of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	3283.17	2	1641.59	23009.31	2.22E-12
Within Groups	0.43	6	0.07		
Total	3283.60	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Salinity (x 10^{-3})	Mean biomass (mg L^{-1})	1 2 3 4 5
1	25	86.97	\
2	30	57	* \
3	35	40.87	* * \
*= significant at $P < 0.05$			

Table (2.2) Effect of salinity of the growth medium on production of chlorophyll *a* of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	0.99	2	0.49	0.58	0.59
Within Groups	5.07	6	0.85		
Total	6.06	8			
Since $P > 0.05$ ACCEPT Ho : All groups equal					

Table (2.3) Effect of salinity of the growth medium on biomass of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	12702.81	4	3175.70	22439.95	1.03E-19
Within Groups	1.42	10	0.14		
Total	12704.22	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean biomass (mg L^{-1})**	**1 2 3 4 5**
1	Control	93.24	\
2	5	81.96	* \
3	15	42.60	* * \
4	25	29.33	* * * \
5	35	19.71	* * * * \
*= significant difference (p = 0.05)			

Table (2.4) Effect of salinity of the growth medium on production of chlorophyll *a* of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	93.650	4	23.413	52.229	1.15E-06
Within Groups	4.483	10	0.448		
Total	98.133	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean chlorophyll *a* (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	Control	7.174	\
2	5	7.841	. \
3	15	6.774	. . \
4	25	4.898	* * * \
5	35	0.923	* * * * \
*= significant at P < 0.05			

Table (2.5) Effect of salinity of the growth medium on cell density of *Synechocococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	142.511	4	35.628	182.028	2.71E-09
Within Groups	1.957	10	0.196		
Total	144.468	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean cell count (x 10^6 mL^{-1})**	**1 2 3 4 5**
1	Control	9.42	\
2	5	10.05	. \
3	15	9.19	. . \
4	25	6.14	* * * \
5	35	1.78	* * * * \
*= significant at P < 0.05			

Table (2.6) Effect of salinity of the growth medium on chlorophyll *a* of *Synechocococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	27.728	4	6.932	22.983	5.01E-05
Within Groups	3.016	10	0.302		
Total	30.744	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean chlorophyll *a* (fg $cell^{-1}$)**	**1 2 3 4 5**
1	Control	4.934	\
2	5	4.597	. \
3	15	2.549	* * \
4	25	1.974	* * . \
5	35	1.659	* * . . \
*= significant at P < 0.05			

Table (2.7) Effect of salinity of the growth medium on production of phycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	59.73	2	29.87	34.87	0.0005
Within Groups	5.14	6	0.86		
Total	64.87	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean phycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4 5**
1	25	32.56	\
2	30	38.76	* \
3	35	36.67	* . \
*= significant at $P < 0.05$			

Table (2.8) Effect of salinity of the growth medium on production of allophycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	9.46	2	4.73	3.25	0.11
Within Groups	8.72	6	1.45		
Total	18.18	8			
Since $P > 0.05$ ACCEPT Ho : All groups equal					

Table (2.9) Effect of salinity of the growth medium on production of phycoerythrin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	0.81	2	0.41	0.89	0.46
Within Groups	2.75	6	0.46		
Total	3.57	8			
Since $P > 0.05$ ACCEPT Ho : All groups equal					

Table (2.10) Effect of salinity of the growth medium on production of phycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2812.15	4	703.04	1619.88	5.21E-14
Within Groups	4.34	10	0.43		
Total	2816.49	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean phycocyanin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	Control	96.54	\
2	5	99.87	* \
3	15	85.75	* * \
4	25	72.51	* * * \
5	35	64.16	* * * * \
*= significant difference (p = 0.05)			

Table (2.11) Effect of salinity of the growth medium on production of allophycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	640.103	4	160.03	447.02	3.18E-11
Within Groups	3.579837	10	0.36		
Total	643.6828	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean allophycocyanin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	Control	46.64	\
2	5	48.59	* \
3	15	41.76	* * \
4	25	35.30	* * * \
5	35	31.46	* * * * \
*= significant at P < 0.05			

Table (2.12) Effect of salinity of the growth medium on production of phycoerythrin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	126.8114	4	31.70	169.76	3.82E-09
Within Groups	1.867515	10	0.19		
Total	128.6789	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean phycoerythrin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	Control	20.53	\
2	5	21.20	. \
3	15	18.25	* * \
4	25	15.40	* * * \
5	35	13.64	* * * * \
*= significant at P < 0.05			

Table (2.13) Effect of salinity of the growth medium on phycocyanin of *Synechocococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	0.994	4	0.248	8.713	0.003
Within Groups	0.285	10	0.029		
Total	1.279	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean phycocyanin (fg $cell^{-1}$)**	**1 2 3 4 5**
1	Control	1.125	\
2	5	1.149	. \
3	15	1.275	. . \
4	25	1.671	* * . \
5	35	1.059	. . . * \
*= significant at P < 0.05			

Table (2.14) Effect of salinity of the growth medium on allophycocyanin of *Synechocococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	2.756	4	0.689	19.274	0.0001
Within Groups	0.357	10	0.036		
Total	3.113	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean allophycocyanin (fg $cell^{-1}$)**	**1 2 3 4 5**
1	Control	1.632	\
2	5	1.648	. \
3	15	1.873	. . \
4	25	2.605	* * * \
5	35	1.529	. . . * \
*= significant at P < 0.05			

Table (2.15) Effect of salinity of the growth medium on phycoerythrin of *Synechocococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	0.61	4	0.15	302.81	2.19E-10
Within Groups	0.01	10	0.001		
Total	0.62	14			
Since P > 0.05 ACCEPT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Salinity (x 10^{-3})**	**Mean phycoerythrin (fg $cell^{-1}$)**	**1 2 3 4 5**
1	Control	1.03	\
2	5	1.06	. \
3	15	1.17	* * \
4	25	1.54	* * * \
5	35	0.97	* * * * \
*= significant at P < 0.05			

Appendix 3

Table (3.1) Effect of pH of the growth medium on biomass of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	3541.55	4	885.39	9407.66	7.94E-18
Within Groups	0.94	10	0.09		
Total	3542.49	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean biomass (mg L^{-1})**	**1 2 3 4 5**
1	6.5	64.09	\
2	7.5	86.96	* \
3	8.5	73.20	* * \
4	9.5	66.61	* * * \
5	10.5	39.82	* * * * \
*= significant difference (P < 0.05)			

Table (3.2) Effect of pH of the growth medium on chlorophyll *a* of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	61.491	4	15.373	15.797	0.0003
Within Groups	9.731	10	0.973		
Total	71.223	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean chlorophyll *a* (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	6.5	8.980	\
2	7.5	11.518	. \
3	8.5	10.609	. . \
4	9.5	8.458	. * . \
5	10.5	5.641	* * * * \
*= significant difference (P < 0.05)			

Table (3.3) Effect of pH of the growth medium on biomass of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	5034.30	4	1258.58	12682.99	1.78E-18
Within Groups	0.99	10	0.10		
Total	5035.30	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean biomass (mg L^{-1})**	**1 2 3 4 5**
1	6.5	72.63	\
2	7.5	89.92	* \
3	8.5	85.90	* * \
4	9.5	61.69	* * * \
5	10.5	39.13	* * * * \
*= significant difference (P < 0.05)			

Table (3.4) Effect of pH of the growth medium on chlorophyll *a* of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	80.951	4	20.238	74.528	2.1E-07
Within Groups	2.715	10	0.272		
Total	83.667	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean chlorophyll *a* (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	6.5	5.720	\
2	7.5	7.998	* \
3	8.5	6.111	. * \
4	9.5	5.645	. * . \
5	10.5	0.970	* * * * \
*= significant difference (P < 0.05)		.= no significant difference	

Table (3.5) Effect of pH of the growth medium on cell counts of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	6.461	4	1.615	2.176	0.145
Within Groups	7.422	10	0.742		
Total	13.883	14			
Since P > 0.05 ACCEPT Ho : All groups equal					

Table (3.6) Effect of pH of the growth medium on chlorophyll *a* of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	19.114	4	4.779	27.217	2.35E-05
Within Groups	1.756	10	0.176		
Total	20.870	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons							
Group	**pH**	**Mean chlorophyll *a* (fg cell^{-1})**	**1**	**2**	**3**	**4**	**5**
1	6.5	3.448	\				
2	7.5	4.934	*	\			
3	8.5	3.894	.	.	\		
4	9.5	2.369	.	*	*	\	
5	10.5	1.730	*	*	*	.	\
*= significant difference (P < 0.05)		.= no significant difference					

Table (3.7) Effect of pH of the growth medium on phycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	222.30	4	55.58	47.15	1.86E-06
Within Groups	11.79	10	1.18		
Total	234.09	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean phycocyanin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	6.5	31.01	\
2	7.5	32.49	. \
3	8.5	28.67	. * \
4	9.5	26.75	* * . \
5	10.5	21.45	* * * * \
*= significant difference (P < 0.05)			

Table (3.8) Effect of pH of the growth medium on allophycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	23.36	4	5.84021	10.66	0.001
Within Groups	5.48	10	0.547649		
Total	28.84	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean allophycocyanin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	6.5	10.33	\
2	7.5	10.76	. \
3	8.5	9.54	. . \
4	9.5	9.18	. . . \
5	10.5	7.16	* * * * \
*= significant difference (P < 0.05)			

Table (3.9) Effect of pH of the growth medium on phycoerythrin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	3.45	4	0.86	5.86	0.01
Within Groups	1.47	10	0.15		
Total	4.92	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean phycoerythrin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	6.5	3.67	\
2	7.5	3.87	. \
3	8.5	3.42	. . \
4	9.5	3.08	. . . \
5	10.5	2.51	* * . . \
*= significant difference (P < 0.05)			

Table (3.10) Effect of pH of the growth medium on phycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	1377.674	4	344.42	154.39	6.08E-09
Within Groups	22.3083	10	2.23		
Total	1399.983	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean phycocyanin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	6.5	93.87	\
2	7.5	97.58	. \
3	8.5	95.50	. . \
4	9.5	81.33	* * * \
5	10.5	72.75	* * * * \
*= significant difference (P < 0.05)		.= no significant difference	

Table (3.11) Effect of pH of the growth medium on allophycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	325.26	4	81.31	24.49	3.78E-05
Within Groups	33.21	10	3.32		
Total	358.46	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean allophycocyanin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	6.5	45.64	\
2	7.5	47.48	. \
3	8.5	46.45	. . \
4	9.5	39.58	* * * \
5	10.5	35.39	* * * . \
*= significant difference (P < 0.05)			

Table (3.12) Effect of pH of the growth medium on phycoerythrin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	SS	df	MS	F	P-value
Between Groups	61.43	4	15.36	21.33	6.96E-05
Within Groups	7.199	10	0.72		
Total	68.63	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean phycoerythrin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	6.5	19.97	\
2	7.5	20.76	. \
3	8.5	20.32	. . \
4	9.5	17.40	* * * \
5	10.5	15.48	* * * . \
*= significant difference (P < 0.05)			

Table (3.13) Effect of pH of the growth medium on phycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	3.821	4	0.955	9.273	0.002
Within Groups	1.030	10	0.103		
Total	4.851	14			
Since P > 0.05 ACCEPT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean phycocyanin (fg cell^{-1})**	**1 2 3 4 5**
1	6.5	1.125	\
2	7.5	2.417	* \
3	8.5	1.379	. * \
4	9.5	1.231	. * . \
5	10.5	1.025	. * . . \
*= significant difference (P < 0.05)			

Table (3.14) Effect of pH of the growth medium on allophycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	3.847	4	0.962	14.114	0.0004
Within Groups	0.681	10	0.068		
Total	4.529	14			
Since P > 0.05 ACCEPT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean Allophycocyanin (fg cell^{-1})**	**1 2 3 4 5**
1	6.5	1.632	\
2	7.5	2.417	* \
3	8.5	1.786	. * \
4	9.5	1.398	. * . \
5	10.5	1.234	. * . . \
*= significant difference (P < 0.05)			

Table (3.15) Effect of pH of the growth medium on phycoerythrin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	6.889	4	1.722	53.867	9.92E-07
Within Groups	0.320	10	0.032		
Total	7.208	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**pH**	**Mean phycoerythrin (fg cell^{-1})**	**1 2 3 4 5**
1	6.5	1.529	\
2	7.5	1.757	. \
3	8.5	0.558	* * \
4	9.5	0.231	* * . \
5	10.5	0.136	* * . . \
*= significant difference (P < 0.05)			

Appendix 4

Table (4.1) Results of student's t-test of growth of *Phormidium tenue* at 2200 lux and 500 lux

Replications	Biomass (mg L^{-1} dry weight)		Chlorophyll a (mg g^{-1} dry weight)	
	2200 lux	500 lux	2200 lux	500 lux
1	54.71	60.52	10.231	14.519
2	54.43	59.81	12.494	13.236
3	55.06	59.93	11.567	13.976
Mean	54.73	60.09	11.431	13.910
Variance	0.10	0.14	1.294	0.415
P value	4.75E-05*		0.05	

*Significant P ≤ 0.05

Table (4.2) Results of student's t-test of growth of *Oscillatoria acuminata* at 2200 lux and 500 lux

Replications	Biomass (mg L^{-1} dry weight)		Chlorophyll a (mg g^{-1} dry weight)	
	2200 lux	500 lux	2200 lux	500 lux
1	47.75	54.78	9.778	7.120
2	47.92	54.56	8.523	6.109
3	48.49	53.93	8.926	6.975
Mean	48.05	54.42	9.076	6.735
Variance	0.15	0.19	0.411	0.299
P value	4.73E-05*		0.01*	

*Significant P ≤ 0.05

Table (4.3) Results of student's t-test of growth of *Synechococcus elongatus* at 2200 lux and 500 lux

Replications	Cell count (x 10^6 mL^{-1})		Chlorophyll a (fg $cell^{-1}$)	
	2200 lux	500 lux	2200 lux	500 lux
1	9.93	4.82	4.789	5.467
2	8.79	5.14	6.235	4.578
3	9.54	4.45	5.386	5.983
Mean	9.42	4.80	5.470	5.343
Variance	0.34	0.12	0.528	0.505
P value	0.001*		0.84	

*Significant P ≤ 0.05

Table (4.4) Results of student's t-test of phycobiliproteins of *Phormidium tenue* at 2200 lux and 500 lux

Replications	Phycocyanin		Allophycocyanin		Phycoerythrin	
	2200 lux	500 lux	2200 lux	500 lux	2200 lux	500 lux
1	33.137	44.492	11.171	11.642	4.092	2.163
2	32.590	43.084	10.267	12.117	3.588	1.953
3	31.975	42.982	10.887	12.867	3.964	2.673
Mean	32.567	43.519	10.775	12.209	3.881	2.263
Variance	0.338	0.712	0.214	0.381	0.069	0.137
P value	5.01E-05*		0.032*		0.003*	

*Significant P ≤ 0.05

Table (4.5) Results of student's t-test of phycobiliproteins of *Oscillatoria acuminata* at 2200 lux and 500 lux

Replications	Phycocyanin		Allophycocyanin		Phycoerythrin	
	2200 lux	500 lux	2200 lux	500 lux	2200 lux	500 lux
1	98.24	115.71	47.76	49.98	20.66	23.87
2	99.47	116.18	47.97	50.61	20.99	24.38
3	98.69	116.23	48.47	50.85	21.41	24.59
Mean	98.80	116.04	48.07	50.48	21.02	24.28
Variance	0.39	0.08	0.13	0.20	0.14	0.14
P value	2.66E-05*		0.002*		0.0004*	

*Significant P ≤ 0.05

Table (4.6) Results of student's t-test of phycobiliproteins of *Synechococcus elongatus* at 2200 lux and 500 lux

Replications	Phycocyanin		Allophycocyanin		Phycoerythrin	
	2200 lux	500 lux	2200 lux	500 lux	2200 lux	500 lux
1	2.315	2.287	3.012	3.565	2.297	1.566
2	1.941	2.474	2.232	3.754	1.823	1.879
3	2.073	1.965	2.468	2.981	2.118	0.991
Mean	2.110	2.242	2.571	3.433	2.079	1.479
Variance	0.036	0.066	0.160	0.162	0.057	0.203
P value	0.513		0.058		0.134	

Appendix 5

Table (5.1) Effect of light wavelengths on biomass of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	1611.56	3	537.19	79.93	2.64E-06
Within Groups	53.77	8	6.72		
Total	1665.33	11			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean biomass (mg L^{-1})**	**1 2 3 4**
1	White	104.22	\
2	Red	93.58	* \
3	Green	125.62	* * \
4	Blue	105.38	. * * \
*= significant difference (P < 0.05)			

Table (5.2) Effect of light wavelengths on chlorophyll *a* of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	116.35	3	38.78	697.82	5.16E-10
Within Groups	0.45	8	0.06		
Total	116.80	11			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean chlorophyll *a* (mg g^{-1} dry weight)**	**1 2 3 4**
1	White	8.83	\
2	Red	2.69	* \
3	Green	3.21	* . \
4	Blue	0.35	* * * \
*= significant difference (P < 0.05)			

Table (5.3) Effect of light wavelengths on biomass of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	4913.98	3	1637.99	143.50	2.71E-07
Within Groups	91.32	8	11.41		
Total	5005.30	11			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean biomass (mg L^{-1})**	**1 2 3 4 5**
1	White	104.29	\
2	Red	76.93	* \
3	Green	67.80	* * \
4	Blue	48.04	* * * \
*= significant difference ($P < 0.05$)			

Table (5.4) Effect of light wavelengths on chlorophyll *a* of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	29.65	3	9.88	46.77	2.04E-05
Within Groups	1.69	8	0.21		
Total	31.34	11			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean chlorophyll *a* (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	White	5.11	\
2	Red	3.55	* \
3	Green	6.08	. * \
4	Blue	1.95	* * * \
*= significant difference ($P < 0.05$)			

Table (5.5) Effect of light wavelengths on cell count of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	77.454	3	25.818	21.305	0.0004
Within Groups	9.695	8	1.212		
Total	87.149	11			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean cell count ($x\ 10^6\ mL^{-1}$)**	**1 2 3 4**
1	White	8.29	\
2	Red	3.53	* \
3	Green	3.26	* . \
4	Blue	1.42	* . . \
*= significant difference ($P < 0.05$)			

Table (5.6) Effect of light wavelengths on chlorophyll *a* of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	27.728	3	11.104	66.943	5.22E-06
Within Groups	3.016	8	0.166		
Total	34.639	11			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean chlorophyll *a* ($fg\ cell^{-1}$)**	**1 2 3 4**
1	White	5.626	\
2	Red	6.774	* \
3	Green	3.567	* * \
4	Blue	2.536	* * . \
*= significant difference ($P < 0.05$)			

Table (5.7) Effect of light wavelengths on phycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	746.25	3	248.75	2511.30	3.11E-12
Within Groups	0.79	8	0.10		
Total	747.05	11			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean phycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4**
1	White	31.94	\
2	Red	24.12	* \
3	Green	22.55	* * \
4	Blue	9.95	* * * \
*= significant difference (P < 0.05)			

Table (5.8) Effect of light wavelengths on allophycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	87.39	3	29.13	71.60	4.03E-06
Within Groups	3.25	8	0.41		
Total	90.65	11			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean allophycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4**
1	White	10.56	\
2	Red	7.99	* \
3	Green	7.42	* . \
4	Blue	3.06	* * * \
*= significant difference (P < 0.05)			

Table (5.9) Effect of light wavelengths on phycoerythrin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	10.72	3	3.57	29.86	0.0001
Within Groups	0.96	8	0.12		
Total	11.68	11			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean phycoerythrin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4**
1	White	3.84	\
2	Red	2.81	* \
3	Green	2.69	* . \
4	Blue	1.19	* * * \
*= significant difference (P < 0.05)			

Table (5.10) Effect of light wavelengths on phycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	1877.88	3	625.96	4225.72	3.89E-13
Within Groups	1.19	8	0.15		
Total	1879.06	11			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean phycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4**
1	White	97.25	\
2	Red	86.32	* \
3	Green	79.83	* * \
4	Blue	62.74	* * * \
*= significant difference (p = 0.05)			

Table (5.11) Effect of light wavelengths on allophycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	448.85	3	149.62	674.60	5.91E-10
Within Groups	1.77	8	0.22		
Total	450.62	11			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean allophycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4**
1	White	47.32	\
2	Red	42.17	* \
3	Green	38.54	* * \
4	Blue	30.53	* * * \
*= significant difference ($p = 0.05$)			

Table (5.12) Effect of light wavelengths on phycoerythrin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	78.54279	3	26.18	225.65	4.56E-08
Within Groups	0.928208	8	0.12		
Total	79.47099	11			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean phycoerythrin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4**
1	White	20.35	\
2	Red	18.35	* \
3	Green	16.97	* * \
4	Blue	13.35	* * * \
*= significant difference ($P < 0.05$)			

Table (5.13) Effect of light wavelengths on phycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	2.553	3	0.851	6.359	0.016
Within Groups	1.071	8	0.134		
Total	3.624	11			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean phycocyanin (fg cell^{-1})**	**1 2 3 4**
1	White	1.895	\
2	Red	2.724	. \
3	Green	1.789	. . \
4	Blue	1.475	. * . \
*= significant difference (P < 0.05)			

Table (5.14) Effect of light wavelengths on allophycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	0.237	3	0.079	1.040	0.426
Within Groups	0.608	8	0.076		
Total	0.845	11			
Since P < 0.05 ACCEPT Ho : All groups equal					

Table (5.15) Effect of light wavelengths on phycoerythrin of*Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	1.634	3	0.545	5.763	0.021
Within Groups	0.756	8	0.095		
Total	2.390	11			
Since P > 0.05 ACCEPT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Light quality**	**Mean phycoerythrin (fg cell^{-1})**	**1 2 3 4**
1	White	1.902	\
2	Red	1.262	. \
3	Green	1.825	. . \
4	Blue	1.030	* . . \
*= significant difference (P < 0.05)			

Appendix 6

Table (6.1) Effect of UV exposure on biomass of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	7754.19	2	3877.10	799.54	5.22E-08
Within Groups	29.10	6	4.85		
Total	7783.29	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean biomass (mg L^{-1})**	**1 2 3**
1	White light	104.22	\
2	White light + UV	113.57	* \
3	UV	47.16	* * \
*= significant difference (P < 0.05)			

Table (6.2) Effect of UV exposure on chlorophyll *a* of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	93.901	2	46.951	1386.587	1.01E-08
Within Groups	0.203	6	0.034		
Total	94.104	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean chlorophyll *a* (mg g^{-1} dry weight)**	**1 2 3**
1	White light	8.826	\
2	White light + UV	4.135	* \
3	UV	0.963	* * \
*= significant difference (P < 0.05)			

Table (6.3) Effect of UV exposure on biomass of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	7914.42	2	3957.21	803.65	5.14E-08
Within Groups	29.54	6	4.92		
Total	7943.97	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean biomass (mg L^{-1})**	**1 2 3**
1	White light	87.33	\
2	White light + UV	104.29	* \
3	UV	34.64	* * \
*= significant difference (P < 0.05)			

Table (6.4) Effect of UV exposure on chlorophyll *a Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	52.629	2	26.3143	463.66	2.66E-07
Within Groups	0.341	6	0.057		
Total	52.969	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean chlorophyll *a* (mg g^{-1} dry weight)**	**1 2 3**
1	White light	5.112	\
2	White light + UV	6.716	* \
3	UV	0.976	* * \
*= significant difference (P < 0.05)			

Table (6.5) Effect of UV exposure on cell count of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	177.028	2	88.514	1259.09	1.34E-08
Within Groups	0.422	6	0.070		
Total	177.450	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean cell count ($x\ 10^6\ mL^{-1}$)**	**1 2 3**
1	White light	8.29	\
2	White light + UV	11.83	* \
3	UV	1.16	* * \
*= significant difference ($P < 0.05$)			

Table (6.6) Effect of UV exposure on chlorophyll *a* of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	53.892	2	26.946	180.909	4.34E-06
Within Groups	0.894	6	0.149		
Total	54.786	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean chlorophyll *a* (fg $cell^{-1}$)**	**1 2 3**
1	White light	5.616	\
2	White light + UV	6.495	. \
3	UV	0.921	* * \
*= significant difference ($P < 0.05$)			

Table (6.7) Effect of UV exposure on phycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	972.29	2	486.15	3328.82	7.3E-10
Within Groups	0.88	6	0.15		
Total	973.18	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean phycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3**
1	White light	31.59	\
2	White light + UV	34.93	* \
3	UV	11.41	* * \
*= significant difference (P < 0.05)			

Table (6.8) Effect of UV exposure on allophycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	106.40	2	53.20	291.11	1.06E-06
Within Groups	1.10	6	0.18		
Total	107.49	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean allophycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3**
1	White light	10.45	\
2	White light + UV	11.56	* \
3	UV	3.77	* * \
*= significant difference (P < 0.05)			

Table (6.9) Effect of UV exposure on phycoerythrin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	13.81	2	6.91	69.05	7.22E-05
Within Groups	0.60	6	0.10		
Total	14.41	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean phycoerythrin ($mg\ g^{-1}$ dry weight)**	**1 2 3**
1	White light	3.77	\
2	White light + UV	4.16	. \
3	UV	1.36	* * \
*= significant difference (P < 0.05)			

Table (6.10) Effect of UV exposure on phycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	10766.80	2	5383.40	56942.33	1.46E-13
Within Groups	0.58	6	0.09		
Total	10767.36	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean phycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3**
1	White light	97.84	\
2	White light + UV	103.32	* \
3	UV	27.36	* * \
*= significant difference (P < 0.05)			

Table (6.11) Effect of UV exposure on allophycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2547.49	2	1273.74	8294.03	4.73E-11
Within Groups	0.92	6	0.15		
Total	2548.41	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean allophycocyanin (mg g^{-1} dry weight)**	**1 2 3**
1	White light	47.59	\
2	White light + UV	50.27	* \
3	UV	13.31	* * \
*= significant difference (p = 0.05)			

Table (6.12) Effect of UV exposure on phycoerythrin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	487.34	2	243.67	2696.57	1.37E-09
Within Groups	0.54	6	0.09		
Total	487.88	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean phycoerythrin (mg g^{-1} dry weight)**	**1 2 3**
1	White light	20.82	\
2	White light + UV	21.98	* \
3	UV	5.82	* * \
*= significant difference (P < 0.05)			

Table (6.13) Effect of UV exposure on phycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	5.484	2	2.742	46.781	0.0002
Within Groups	0.352	6	0.059		
Total	3.624	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean phycocyanin (fg cell^{-1})**	**1 2 3**
1	White light	1.669	\
2	White light + UV	1.804	. \
3	UV	0.084	* * \
*= significant difference (P < 0.05)			

Table (6.14) Effect of UV exposure on allophycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	6.120	2	3.060	71.392	6.56E-05
Within Groups	0.257155	6	0.043		
Total	198.153	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean allophycocyanin (fg cell^{-1})**	**1 2 3**
1	White light	1.748	\
2	White light + UV	1.946	. \
3	UV	0.106	* * \
*= significant difference (P < 0.05)			

Table (6.15) Effect of UV exposure on phycoerythrin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	5.68334	2	2.842	55.83081	0.0001
Within Groups	0.305387	6	0.0509		
Total	5.989	8			
Since P > 0.05 ACCEPT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**UV exposure**	**Mean phycoerythrin ($fg\ cell^{-1}$)**	**1 2 3**
1	White light	1.674	\
2	White light + UV	1.874	. \
3	UV	0.097	* * \
*= significant difference (P < 0.05)			

Appendix 7

Table (7.1) Effect of temperature on biomass of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	35.82	4	8.95	1.20	0.414371
Within Groups	37.39	10	7.48		
Total	73.21	14			
Since P > 0.05 ACCEPT Ho : All groups equal					

Table (7.2) Effect of temperature on chlorophyll *a* of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	263.26	4	65.81	20.62	8.07E-05
Within Groups	31.92	10	3.19		
Total	295.18	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Temperature (^{0}C)	Mean chlorophyll *a* (mg g^{-1} dry weight)	1 2 3 4 5
1	30	3.53	\
2	35	2.24	. \
3	40	5.09	. . \
4	45	11.01	* * * \
5	50	12.78	* * * . \
*= significant difference (P < 0.05)			

Table (7.3) Effect of temperature on biomass of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2401.09	4	600.27	29.93	1.53E-05
Within Groups	200.59	10	20.06		
Total	2601.67	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Temperature (^0C)**	**Mean biomass (mg L^{-1})**	**1 2 3 4 5**
1	30	71.51	\
2	35	45.16	* \
3	40	43.13	* . \
4	45	38.39	* . . \
5	50	36.64	* . . . \
*= significant difference (P < 0.05)			

Table (7.4) Effect of temperature on chlorophyll *a* of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	8.39	4	2.09	4.66	0.02
Within Groups	4.50	10	0.45		
Total	12.89	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Temperature (^0C)**	**Mean chlorophyll a (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	30	6.17	\
2	35	4.01	* \
3	40	4.38	. . \
4	45	5.06	. . . \
5	50	4.55	. . . . \
*= significant difference (P = 0.05)			.= no significant difference

Table (7.5) Effect of temperature on cell count of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	21.18	4	5.29	2.54	0.11
Within Groups	20.87	10	2.09		
Total	42.05	14			
Since P > 0.05 ACCEPT Ho : All groups equal					

Table (7.6) Effect of temperature on chloropjhyll *a* of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**df**	**SS**	**MS**	**F**	**P-value**
Between Groups	44.39	4	11.09	12.26	0.0007
Within Groups	9.05	10	0.91		
Total	53.44	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons							
Group	**Temperature (^0C)**	**Mean chlorophyll *a* (fg cell^{-1})**	**1**	**2**	**3**	**4**	**5**
1	30	5.25	\				
2	35	4.30	.	\			
3	40	5.73	.	.	\		
4	45	7.41	.	*	.	\	
5	50	9.16	*	*	*	.	\
*= significant difference (P < 0.05)							

Table (7.7) Effect of temperature on phycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	1047.10	4	261.78	18.91	0.000118
Within Groups	138.42	10	13.84		
Total	1185.52	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Temperature (^0C)**	**Mean phycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4 5**
1	30	28.38	\
2	35	43.82	* \
3	40	39.04	* . \
4	45	25.17	. * * \
5	50	21.97	. * * . \
*= significant difference (P < 0.05)			

Table (7.8) Effect of temperature on allophycocyanin of *Phormidium tenue* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	101.53	4	25.38	26.62306	2.6E-05
Within Groups	9.53	10	0.95		
Total	111.06	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Temperature (^0C)**	**Mean allophycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4 5**
1	30	7.11	\
2	35	9.83	* \
3	40	6.31	. * \
4	45	2.52	* * * \
5	50	3.59	* * * . \
*= significant difference (P < 0.05)			

Table (7.9) Effect of temperature on phycoerythrin of Phormidium tenue (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	101.03	4	25.26	61.68	5.21E-07
Within Groups	4.10	10	0.41		
Total	105.12	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Temperature (⁰C)**	**Mean phycoerythrin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4 5**
1	30	8.26	\
2	35	4.84	* \
3	40	4.77	* . \
4	45	1.17	* * * \
5	50	1.49	* * * . \
*= significant difference (P < 0.05)			

Table (7.10) Effect of temperature on phycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	1555.48	4	388.87	11.05	0.001
Within Groups	351.98	10	35.19		
Total	1907.454	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Temperature (⁰C)**	**Mean phycocyanin ($mg\ g^{-1}$ dry weight)**	**1 2 3 4 5**
1	30	72.75	\
2	35	73.17	. \
3	40	100.28	* * \
4	45	86.56	. . . \
5	50	80.53	. . * * \
*= significant difference (P < 0.05)			

Table (7.11) Effect of temperature on allophycocyanin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	47.23	4	11.81	14.98	0.0003
Within Groups	7.88	10	0.79		
Total	55.11	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Temperature (⁰C)**	**Mean allophycocyanin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	30	5.92	\
2	35	8.33	* \
3	40	11.45	* * \
4	45	8.39	* . * \
5	50	9.18	* . . . \
*= significant difference (P < 0.05)			

Table (7.12) Effect of temperature on phycoerythrin of *Oscillatoria acuminata* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	38.70	4	9.68	21.90	6.19E-05
Within Groups	4.42	10	0.44		
Total	43.12	14			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	**Temperature (⁰C)**	**Mean phycoerythrin (mg g^{-1} dry weight)**	**1 2 3 4 5**
1	30	4.99	\
2	35	1.78	* \
3	40	2.18	* . \
4	45	0.72	* . . \
5	50	0.51	* . . . \
*= significant difference (P < 0.05)			

Appendix 8

Table (8.1) Biomass, chlorophyll *a* and phycobiliproteins of *Phormidium tenue* after 7 days growth in different light and temperature conditions

Light and temperature	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} Dry weight)	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)
L_1T_1	9.47	3.664	21.053	3.759	2.865
L_1T_2	14.36	1.306	31.124	6.799	3.149
L_1T_3	14.45	1.167	39.715	7.469	3.106
L_1T_4	21.26	8.329	48.748	8.424	2.363
L_1T_5	15.64	2.061	43.795	8.070	2.192
L_2T_1	37.84	2.199	28.383	7.108	8.261
L_2T_2	38.64	3.239	47.152	7.495	4.838
L_2T_3	37.55	6.092	39.036	6.312	4.773
L_2T_4	36.99	10.339	21.834	2.183	1.170
L_2T_5	30.47	12.779	31.970	4.923	1.829
L_3T_1	41.22	4.724	13.103	9.926	10.722
L_3T_2	38.75	4.062	43.822	5.993	2.850
L_3T_3	34.67	4.981	51.323	9.919	4.339
L_3T_4	30.53	8.489	25.911	3.533	1.885
L_3T_5	30.08	12.146	25.399	2.905	1.640
L_4T_1	32.79	5.464	38.031	6.664	6.391
L_4T_2	31.33	5.112	24.945	3.054	1.969
L_4T_3	27.45	5.253	20.065	2.503	1.969
L_4T_4	22.82	7.138	7.955	1.732	1.287
L_4T_5	17.80	6.409	9.611	2.055	0.422
L_5T_1	31.15	5.997	19.986	4.215	2.705
L_5T_2	29.53	6.523	11.962	1.732	1.738
L_5T_3	29.24	5.940	17.119	1.002	1.535
L_5T_4	29.04	7.036	13.147	2.297	3.246
L_5T_5	25.19	6.548	6.744	1.770	1.839

L_1 =1000 lux, L_2 =2000 lux), L_3 =2500 lux, L_4 =3000 lux, L_5 =3500 lux, T_1 =30^{0}C, T_2 =35^{0}C, T_3 =40^{0}C, T_4 =45^{0}C and T_5 =50^{0}C

Table (8.2) Biomass, chlorophyll *a* and phycobiliproteins of *Oscillatoria acuminata* after 7 days growth in different light and temperature conditions

Light and temperature	Biomass (mg L^{-1})	Chlorophyll *a* (mg g^{-1} Dry weight)	PC (mg g^{-1} Dry weight)	APC (mg g^{-1} Dry weight)	PE (mg g^{-1} Dry weight)
L_1T_1	16.64	5.561	72.203	3.524	1.356
L_1T_2	22.84	4.714	28.298	6.206	2.626
L_1T_3	29.07	5.529	21.576	6.004	0.985
L_1T_4	21.26	5.905	23.244	13.971	1.929
L_1T_5	16.47	4.100	20.610	1.545	0.834
L_2T_1	71.51	6.166	72.751	5.922	4.993
L_2T_2	45.16	4.005	73.170	8.333	1.777
L_2T_3	43.13	4.383	100.280	11.451	2.185
L_2T_4	38.39	5.727	86.562	8.394	0.719
L_2T_5	36.64	4.547	80.528	10.185	0.508
L_3T_1	59.95	2.602	47.334	2.836	2.952
L_3T_2	26.56	5.333	55.919	4.203	1.615
L_3T_3	33.45	2.952	78.520	6.293	3.171
L_3T_4	30.53	4.464	59.854	4.747	1.031
L_3T_5	25.49	6.432	53.191	6.285	0.980
L_4T_1	59.62	1.995	30.846	10.289	5.013
L_4T_2	22.73	5.450	37.468	4.514	1.731
L_4T_3	15.36	4.431	29.892	1.816	1.562
L_4T_4	13.78	3.992	46.829	2.036	0.371
L_4T_5	12.71	3.289	37.495	3.436	1.992
L_5T_1	48.38	2.001	21.654	6.963	3.690
L_5T_2	29.42	3.726	73.198	8.025	2.832
L_5T_3	29.18	3.947	51.903	7.091	1.964
L_5T_4	24.04	4.221	49.933	5.277	1.736
L_5T_5	18.26	0	0	0	0

L_1 =1000 lux, L_2 =2000 lux), L_3 =2500 lux, L_4 =3000 lux, L_5 =3500 lux, T_1 =30^{0}C, T_2 =35^{0}C, T_3 =40^{0}C, T_4 =45^{0}C and T_5 =50^{0}C

Table (8.3) Biomass and chlorophyll *a* of *Synechococcus elongatus* after 7 days growth in different light and temperature conditions

Light and temperature	Cell count (x 106 mL-1)	Chlorophyll *a* (fg cell-1)
L_1T_1	8.21	6.925
L_1T_2	8.28	4.419
L_1T_3	8.42	5.437
L_1T_4	8.84	6.760
L_1T_5	8.95	7.185
L_2T_1	11.32	5.584
L_2T_2	11.28	4.300
L_2T_3	12.03	5.728
L_2T_4	10.07	7.412
L_2T_5	8.96	9.156
L_3T_1	14.27	3.641
L_3T_2	12.49	3.652
L_3T_3	12.84	4.010
L_3T_4	10.45	6.913
L_3T_5	9.10	5.685
L_4T_1	13.39	2.850
L_4T_2	13.35	1.910
L_4T_3	13.06	1.845
L_4T_4	9.15	7.011
L_4T_5	6.15	5.244
L_5T_1	11.47	4.169
L_5T_2	11.90	3.677
L_5T_3	8.63	5.317
L_5T_4	6.34	5.714
L_5T_5	0	0

L_1 =1000 lux, L_2 =2000 lux), L_3 =2500 lux, L_4 =3000 lux, L_5 =3500 lux, T_1 =30^{0}C, T_2 =35^{0}C, T_3 =40^{0}C, T_4 =45^{0}C and T_5 =50^{0}C

Table (9.1) Effect of different concentrations of cadmium on biomass of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	1081.30	5	216.26	1201.74	9.17E-16
Within Groups	2.16	12	0.18		
Total	1083.46	17			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cd (mg L^{-1})	Mean cell count (x 10^6 mL^{-1})	1 2 3 4 5 6
1	Control	29.56	\
2	0.5	18.28	* \
3	1.0	18.07	* . \
4	3.0	11.96	* * * \
5	6.0	11.21	* * * . \
6	9.0	4.66	* * * * * \
*= significant at P < 0.05			

Table (9.2) Effect of different concentrations of cadmium on protein of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	7851.15	5	1570.23	10641.08	1.93E-21
Within Groups	1.77	12	0.15		
Total	7852.92	17			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cd (mg L^{-1})	Mean protein (fg $cell^{-1}$)	1 2 3 4 5 6
1	Control	79.08	\
2	0.5	66.45	* \
3	1.0	50.64	* * \
4	3.0	36.11	* * * \
5	6.0	31.37	* * * * \
6	9.0	18.13	* * * * * \
*= significant at $P < 0.05$			

Table (9.3) Effect of different concentrations of copper on biomass of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	430.33	5	86.07	459.78	2.86E-13
Within Groups	2.25	12	0.19		
Total	432.57	17			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cu (mg L^{-1})	Mean cell count (x 10^6 mL^{-1})	1 2 3 4 5 6
1	Control	29.56	\
2	0.5	26.11	* \
3	1.0	24.83	* * \
4	3.0	19.35	* * * \
5	6.0	17.44	* * * * \
6	9.0	16.18	* * * * * \

*= significant at P < 0.05

Table (9.4) Effect of different concentrations of copper on protein of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	4569.67	5	913.93	7383.86	1.72E-20
Within Groups	1.49	12	0.12		
Total	4571.15	17			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cu (mg L^{-1})	Mean protein (fg $cell^{-1}$)	1 2 3 4 5 6
1	Control	87.74	\
2	0.5	79.08	* \
3	1.0	69.62	* * \
4	3.0	56.39	* * * \
5	6.0	52.11	* * * * \
6	9.0	41.59	* * * * * \
*= significant at $P < 0.05$			

Table (9.5) Effect of different percentage of chlorpyriphos on biomass of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	135.18	2	67.59	481.89	2.37E-07
Within Groups	0.84	6	0.14		
Total	136.02	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Chlorpyriphos (%)	Mean cell count (x 10^6 mL^{-1})	1 2 3
1	Control	22.53	\
2	0.01	19.61	* \
3	0.2	13.24	* * \
*= significant at $P < 0.05$			

Table (9.6) Effect of different percentage of chlorpyriphos on protein of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2512.49	2	1256.24	7576.79	6.2E-11
Within Groups	0.99	6	0.17		
Total	2513.48	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Chlorpyriphos (%)	Mean protein (fg $cell^{-1}$)	1 2 3
1	Control	70.33	\
2	0.01	49.85	* \
3	0.2	29.41	* * \
*= significant at $P < 0.05$			

Table (9.7) Effect of different percentage of mancozeb on biomass of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	110.83	2	55.42	328.02	7.44E-07
Within Groups	1.01	6	0.17		
Total	111.85	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Mancozeb (mg L^{-1})	Mean cell count (x 10^6 mL^{-1})	1 2 3
1	Control	22.53	\
2	10	19.08	* \
3	32	13.99	* * \
*= significant at P < 0.05			

Table (9.8) Effect of different percentage of mancozeb on protein of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	387.13	2	193.56	1406.71	9.64E-09
Within Groups	0.83	6	0.14		
Total	387.95	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Mancozeb (mg L^{-1})	Mean protein (fg $cell^{-1}$)	1 2 3
1	Control	65.79	\
2	10	59.56	* \
3	32	49.85	* * \
*= significant at P < 0.05			

Table (9.9) Effect of different percentage of glyphosate on biomass of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	263.84	2	131.92	775.39	5.72E-08
Within Groups	1.02	6	0.17		
Total	264.86	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Glyphosate (%)	Mean cell count ($x\ 10^6\ mL^{-1}$)	1 2 3
1	Control	22.53	\
2	0.5	13.07	* \
3	2	9.75	* * \
*= significant at $P < 0.05$			

Table (9.10) Effect of different percentage of glyphosate on protein of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	6534.27	2	3267.14	22285.2	2.44E-12
Within Groups	0.88	6	0.15		
Total	6535.15	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Glyphosate (%)	Mean protein ($fg\ cell^{-1}$)	1 2 3
1	Control	87.21	\
2	0.5	49.85	* \
3	2	21.42	* * \
*= significant at $P < 0.05$			

Table (9.11) Effect of different concentrations of cadmium on phycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	3.27	5	0.65	19.57	2.15E-05
Within Groups	0.40	12	0.03		
Total	3.67	17			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cd (mg L^{-1})	Mean phycocyanin (fg $cell^{-1}$)	1 2 3 4 5 6
1	Control	1.85	\
2	0.5	1.29	* \
3	1.0	1.26	* . \
4	3.0	1.07	* . . \
5	6.0	1.03	* . . . \
6	9.0	0.40	* * * * * \
*= significant at $P < 0.05$			

Table (9.12) Effect of different concentrations of cadmium on allophycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	3.69	5	0.74	14.31	0.0001
Within Groups	0.62	12	0.05		
Total	4.31	17			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cd (mg L^{-1})	Mean allophycocyanin (fg $cell^{-1}$)	1 2 3 4 5 6
1	Control	2.03	\
2	0.5	1.32	* \
3	1.0	1.29	* . \
4	3.0	1.13	* . . \
5	6.0	1.07	* . . . \
6	9.0	0.49	* * * * . \
*= significant at P < 0.05			

Table (9.13) Effect of different concentrations of cadmium on phycoerythrin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	3.26	5	0.65	18.89	2.58E-05
Within Groups	0.41	12	0.03		
Total	3.67	17			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cd (mg L^{-1})	Mean phycoerythrin (fg $cell^{-1}$)	1 2 3 4 5 6
1	Control	1.85	\
2	0.5	1.29	* \
3	1.0	1.27	* . \
4	3.0	1.08	* . . \
5	6.0	1.04	* . . . \
6	9.0	0.41	* * * * * \
*= significant at $P < 0.05$			

Table (9.14) Effect of different concentrations of copper on phycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2.25	5	0.45	13.69	0.0001
Within Groups	0.39	12	0.03		
Total	2.65	17			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cu (mg L^{-1})	Mean phycocyanin (fg $cell^{-1}$)	1 2 3 4 5 6
1	Control	1.85	\
2	0.5	1.73	. \
3	1.0	1.55	. . \
4	3.0	1.17	* * . \
5	6.0	1.07	* * . . \
6	9.0	0.88	* * * . . \
*= significant at $P < 0.05$			

Table (9.14) Effect of different concentrations of copper on allophycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2.32	5	0.46	9.81	0.001
Within Groups	0.57	12	0.05		
Total	2.88	17			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cu (mg L^{-1})	Mean allophycocyanin (fg $cell^{-1}$)	1 2 3 4 5 6
1	Control	2.03	\
2	0.5	1.95	. \
3	1.0	1.77	. . \
4	3.0	1.37	* . . \
5	6.0	1.27	* * . . \
6	9.0	1.08	* * * . . \
*= significant at P < 0.05			

Table (9.15) Effect of different concentrations of copper on phycoerythrin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2.33	5	0.47	12.78	0.0002
Within Groups	0.44	12	0.04		
Total	2.77	17			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Cu (mg L^{-1})	Mean phycoerythrin (fg $cell^{-1}$)	1 2 3 4 5 6
1	Control	1.85	\
2	0.5	1.79	. \
3	1.0	1.59	. . \
4	3.0	1.21	* * . \
5	6.0	1.09	* * . . \
6	9.0	0.89	* * * . . \
*= significant at P < 0.05			

Table (9.16) Effect of different percentage of chlorpyriphos on phycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	0.85	2	0.42	15.49	0.004
Within Groups	0.16	6	0.03		
Total	1.01	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Chlorpyriphos (%)	Mean phycocyanin (fg cell^{-1})	1 2 3
1	Control	1.82	\
2	0.01	1.37	* \
3	0.2	1.08	* . \
*= significant at P < 0.05			

Table (9.17) Effect of different percentage of chlorpyriphos on allophycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	0.93	2	0.46	13.27	0.006
Within Groups	0.21	6	0.03		
Total	1.14	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Chlorpyriphos (%)	Mean allophycocyanin (fg cell^{-1})	1 2 3
1	Control	2.02	\
2	0.01	1.54	* \
3	0.2	1.24	* . \
*= significant at P < 0.05			

Table (9.18) Effect of different percentage of chlorpyriphos on phycoerythrin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	0.79	2	0.39	13.52	0.01
Within Groups	0.18	6	0.03		
Total	0.98	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Chlorpyriphos (%)	Mean phycoerythrin (fg cell^{-1})	1 2 3
1	Control	1.83	\
2	0.01	1.38	* \
3	0.2	1.11	* . \
*= significant at P < 0.05			

Table (9.19) Effect of different percentage of mancozeb on phycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	0.76	2	0.38	15.14	0.005
Within Groups	0.15	6	0.03		
Total	0.91	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Mancozeb (mg L^{-1})	Mean phycocyanin (fg cell^{-1})	1 2 3
1	Control	1.82	\
2	10	1.35	* \
3	32	1.13	* . \
*= significant at P < 0.05			

Table (9.20) Effect of different percentage of mancozeb on allophycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	0.75	2	0.38	10.05	0.01
Within Groups	0.22	6	0.04		
Total	0.98	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Mancozeb (mg L^{-1})	Mean allophycocyanin (fg $cell^{-1}$)	1 2 3
1	Control	2.02	\
2	10	1.53	* \
3	32	1.34	* . \
*= significant at P < 0.05			

Table (9.21) Effect of different percentage of mancozeb on phycoerythrin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	0.67	2	0.33	12.25	0.008
Within Groups	0.16	6	0.03		
Total	0.83	8			
Since P < 0.05 REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Concentration of Mancozeb (mg L^{-1})	Mean phycoerythrin (fg $cell^{-1}$)	1 2 3
1	Control	1.83	\
2	10	1.36	* \
3	32	1.19	* . \
*= significant at P < 0.05			

Table (9.22) Effect of different percentage of glyphosate on phycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2.765051	2	1.382525	45.68084	0.000234
Within Groups	0.181589	6	0.030265		
Total	2.94664	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Glyphosate (%)	Mean phycocyanin (fg cell^{-1})	1 2 3
1	Control	1.82	\
2	0.5	1.06	* \
3	2	0.47	* * \
*= significant at $P < 0.05$			

Table (9.23) Effect of different percentage of glyphosate on allophycocyanin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2.75	2	1.38	33.99	0.0005
Within Groups	0.24	6	0.04		
Total	2.99	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Glyphosate (%)	Mean allophycocyanin (fg cell^{-1})	1 2 3
1	Control	2.02	\
2	0.5	1.27	* \
3	2	0.67	* * \
*= significant at $P < 0.05$			

Table (9.24) Effect of different percentage of glyphosate on phycoerythrin of *Synechococcus elongatus* (Analysis of Variance and Tukey multiple comparison)

ANOVA TABLE					
SOURCE	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	2.71	2	1.35	34.12	0.0005
Within Groups	0.24	6	0.04		
Total	2.94	8			
Since $P < 0.05$ REJECT Ho : All groups equal					

TUKEY multiple comparisons			
Group	Percentage of Glyphosate (%)	Mean phycoerythrin (fg $cell^{-1}$)	1 2 3
1	Control	1.83	\
2	0.5	1.11	* \
3	2	0.49	* * \
*= significant at $P < 0.05$			