EVALUATION OF CYANOBACTERIA FOR BIOMASS PRODUCTION AND EFFLUENT TREATMENT

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IN
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UNDER

THE FACULTY OF ENVIRONMENTAL STUDIES

BY

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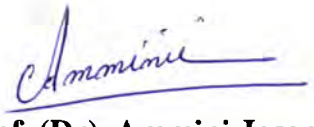
OCTOBER 2005

Dedicated to my Parents, Husband and Son.

CERTIFICATE

This is to certify that this thesis entitled "Evaluation of Cyanobacteria for Biomass production and Effluent treatment" is an authentic record of research work carried out by Mrs. Jitha.G under my guidance and supervision in the School of Environmental Studies, Cochin University of Science and Technology in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in Environmental Biotechnology and no part of this work has previously formed the basis for the award of any other degree in any University.



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DECLARATION

I hereby declare that the thesis entitled "Evaluation of Cyanobacteria for Biomass production and Effluent Treatment" is an authentic record of research work carried out by me under the guidance and supervision of Prof. (Dr). Ammini Joseph, Director, School of Environmental Studies, Cochin University of Science and Technology in partial fulfilment of the requirements for the award of the Degree of ***Doctor of Philosophy*** *in Environmental Biotechnology and no part of this work has previously formed the basis for the award of any other degree in any University.*

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CHAPTER

INTRODUCTION

1.1 Applications of microalgae

Algae have been part of life of coastal population throughout the world for thousands of years. Approximately 200 species of seaweeds are used world-wide (Zemke-White and Ohno, 1999) of which about 10 species or genera such as the brown algae *Laminaria*, red algae *Porphyra*, *Gracilaria,* and green algae *Enteromorpha* are intensively cultivated (Wikfors and Ohno, 2001). An increasing number of people are becoming aware of the benefits and potential of algae. The microalgae gained importance as food and feed as well as source of fine chemicals in the 1960s. Cyanobacteria especially *Spirulina* have been used as a food item in countries of Asia and Africa due to their high protein content. The recorded history of the consumption of Cyanobacteria extends back to the year 1521, when Bernal Diaz del Castillo, described the consumption of a preparation of *Spirulina* by people in the vicinity of Mexico-city (Lee, 1989). Intensive cultivation of microalgae for of commercial products has been accomplished in the past thirty years. The general objective of microalgae biotechnology is to produce specific products that meet the requirement of the consumer. In 1964 'Chlorella tablets' were made from dry powder and were sold in the markets in Japan. In 1975 'Spirulina tablets' were marketed (Yamaguchi, 1997). Algae and their extract are also included in noodles, wine, beverages, breakfast cereals and cosmetics (Lee, 1997). Microalgae extract is available in the market in China, which is easy to drink, easy to digest and suitable for all ages (Liang *et al*, 2004). Edible microalgae *Chlorella* and *Spirulina* are rich in protein, lipid, polysaccharide, edible fiber, microelements and bioactive substances (Li and Qi, 1997). Characteristics of *Spirulina* that makes it particularly suitable for food include

high protein content, essential amino acids, essential fatty acids, and high vitamin A content (Mathew *et al.*, 1995). It is a rich source of vitamin B12 as well as significant source of vitamin B1 and B2 (Lem and Glick, 1985). *Spirulina* is an excellent source of polyunsaturated fatty acids like γ-linolenic acid which has been demonstrated to have therapeutic potential in alleviating or preventing various diseases and disorders (Harris, 1989; Mundt *et al.*, 2003). Trials with poultry, pigs, and ruminants conclude that concentrations of *Spirulina* up to 10% are satisfactory replacements for conventional protein sources, while higher concentrations reduce growth (Patterson, 1996).

The commercially cultivated microalgae include *Chlorella, Spirulina, Dunaliella, Nannochloris, Nitzschia, Tetraselmis, Skeletonema, Isochrysis* and *Chaetoceros. Dunaliella* is mainly cultivated for the production of β-carotene. *Isochrysis* is used widely in commercial mariculture as feed in bivalve hatcheries. *Nannochloris* and *Chaetoceros* are mass cultured for the production of feed for marine species and also in commercial shrimp hatcheries.

A rapid expansion of the microalgal industry in the Asia-Pacific region has occurred in the recent years. There are around 100 commercial producers of microalgae in the Asia-Pacific region, with annual production capacity ranging from 3 to 500 tons (Lee, 1997). Its rapid growth could be attributed to increasing demand in the health food market in Asia, growing affluence in Asian countries, and consumer acceptance of natural herbal products. Japan has long history of large-scale commercial production and consumption of microalgae as health food supplement. Taiwan is still the main producer of

Chlorella and produces over 50 % of world production. In India, Parry Agro Industries Ltd. and Central Food Technological Research Institute is providing food grade *Spirulina* having a national standard. There are two major producers of *Spirulina* in Thailand, namely Siam Algae Co. Ltd. and Neotech Food Co. Ltd. Cyanotech and Earthrise Farms in Hawaii and California, respectively, are the major producers of *Spirulina* in North America. There are two *Dunaliella* producers in Australia. The main markets for these β-carotene products are Japan, USA, Korea and Europe (Lee, 1997). Brazil has a great potential for cultivation of microalgae, with excellent temperature and solar irradiation, but few efforts have been made in this area. China has an extensive cultivation of *Spirulina* in the world.

1.2 Technology of production

There are two main choices in microalgal mass culture systems, open cultivation and bioreactor technology. In both, the main concern in design is efficiency in the harvesting of solar energy. Open systems have the advantage of being cheaper and easier to construct and operate (Tredici and Materassi, 1992). Three different designs of open algal ponds commonly in use are circular ponds, with agitation provided by a rotating arm; raceways with culture mixed by a paddle wheel, and sloped reactors where mixing of algal suspension is achieved by pumping and gradient flow (Becker, 1994). Enclosed systems arose with the need to reduce contamination, and to have control over the environment of the culture, especially in the production of secondary metabolites. Much debate has taken place in recent years concerning the design of photobioreactors (Pulz, 2001). Productivity of *Chlorella* was found to be increased by 1.7 times in a column type

photobioreactor (Degen *et al.*, 2001). Zittelli *et al.* (1999) described outdoor cultivation of *Nannochloropsis* in tubular reactors for the production of eicosapentaenoic acid. Research in the field of reactors, has evolved different models of photobioreactors such as tubular loop reactors, and air lift tubular loop reactor where polyethylene tubes are used, flat plate reactors developed in Israel, α- bioreactor in Singapore, and semi spherical plate reactor developed in Japan. The α- bioreactor consists of two sets of inclined glass tubes crossing over each other whereby the cultures are exposed to high photon flux density in the morning resulting in higher specific growth rate (Lee and Richmond, 1998). Grobbelaar and Kurano (2003) have described a continuous flow photobioreactor, where algal cells are acclimated to different light conditions, and this multi compartment reactor yielded a 37 % higher productivity rate. Gonzalez *et al.* (2005) reported that β carotene could be effectively produced from *Dunaliella salina* using a closed tubular bioreactor. Eventhough it has high productivity, the cost of maintenance will be very high. However enclosed systems are necessary to achieve monoculture systems for the production of biopharmaceuticals (Kojima and Lee, 2001).

In open outdoor systems productivity of microalgae is controlled by a combination of abiotic and biotic factors, of which solar irradiance and temperature are most important. Other abiotic factors include pH, nutrients, salinity, toxicants, oxygen, CO_2 etc. Biotic factors include other algae, bacteria, fungi, virus, zooplankton etc. In addition, operational factors such as mixing, dilution rate, depth etc. are also important. One of the important requisites for successful algal cultivation is the construction of suitable basins, which should be efficient, easy to operate, durable and cheap. Tanticharoen *et al.*

(1994) reported pigments and fatty acid production from *Spirulina* in a high rate algal pond. Borowitzka (1999) reported commercial production of *Spirulina*, *Chlorella*, *Dunaliella,* and *Haematococcus* in circular ponds for production of microalgal products. Contamination is the main problem in the outdoor cultivation. Contamination with bacteria cannot be omitted from open mass cultures; however, contamination with *Salmonella*, *Shigella* and *Clostridium* makes lots of problems (Becker, 1994).

Harvesting of biomass is mainly done by centrifugation or using gravity filtration technique, which is the cheapest method to harvest microalgae. Shimamatsu (2004) has reported mass production of *Spirulina* in outdoor open ponds for the production of food, feed and pigments. Many producers have become successful in the outdoor open pond production of microalgae such that the world production now exceed 3000 tons on a dry weight basis.

1.3 Potential uses of Cyanobacteria

Cyanobacterial protein either as a supplement or as an alternative source has received worldwide attention. *Spirulina* is an important food or feed supplement because of its excellent nutrient composition and digestibility (Palmegiano *et al.*, 2005). Strains of *Anabaena* and *Nostoc* are also consumed as human food (Thajuddin and Subramanian, 2005). Consortia of Cyanobacteria are used as biofertilizer in paddy fields. The beneficial effect of algal inoculation on the grain yield of many rice varieties has been demonstrated in different parts of the world. The studies conducted by Kannaiyan and Kumar (2004) concluded that 10-15 % increase in grain yield could be obtained through algal inoculation, on an average, there is

contribution of 20-30 Kg N/ha/season which means that chemical nitrogen fertilizer could be saved to that extent. It is also observed that continuous application of algae for 4-5 consecutive seasons results in an appreciable population build up. The algal effect could be seen in subsequent years without any further inoculation. The application of Cyanobacteria with the coir waste can replace 50% of the chemical fertilizers (Krishnaveni, 1999). Sharma and Mehta (2002) showed that use of cyanobacteria as biofertilizer yielded better than urea and phosphate on the growth of *Capsicum anum* seedlings. They enhance growth, save the environment from extra load of chemical fertilizers and do not leave any toxic residues.

In addition to the supply of nitrogen, Cyanobacteria could also release a variety of substances such as hormones, vitamins, pigments, amino acids, polysaccharides (Kaushik, 1993) and growth promoting substances (Ravisankar, 2000; Rajula and Padmadevi, 2000 and Kanakalatha, 2001). Cyanobacteria produce a number of enzymes that have commercial importance. Phosphoglycerate kinase produced by *Spirulina platensis* is specific for ATP and may well be used for ATP determination. *Anabaena* sp produces restriction endonucleases that has wide range application in recombinant DNA technology (Mayer, 1981). *Anacystis nidulans* contains aminoacid oxidase. Another enzymes namely superoxide dismutase, and phospho-glycerol kinase have been isolated and purified from *Spirulina* (Houmard and Tandeau, 1988). Although soil actinomycetes and fungi are the source for majority of antibiotics currently known from microorganisms, researchers are seeking new antibiotics or other bioactive metabolites from microalgae. Cyanobacteria produce numerous secondary metabolites which

show characteristic biological activities including cyto-toxic, immunosuppressive, antifungal, antiviral, cardio-active and enzyme inhibitory effects (Namikoshi and Rinehart, 1996; Rao *et al.*, 2002). The carotenoides and phycobiliproteins, characteristic of Cyanobacteria have high commercial value (Thajuddin and Subramanian, 2005). Antioxidant and antiinflammatory activities of phycocyanin have been reported (Romay *et al.*, 1998; Gonzalez *et al.*, 1999). The antioxidative activity against methyl linoleate of the pigment was reported (Hirata *et al.*, 2000). Liu (2000) reported that C-Phycocyanin could be used as a potential antitumor substance. Cyanobacteria are the richest source of known and novel bioactive compounds including toxins with wide pharmaceutical applications (Raghavan *et al.*, 2002). Bioactive molecules from *Spirulina* could neutralize the poisonous effect of heavy metals, and possess antitumour activity (Liu *et al.*, 1991). Gustafson *et al.* (1989) reported anti- HIV activity of marine Cyanobacterial compounds from *Lyngbya lagerheimii* and *Phormidium tenue*. Natural products of *Nostoc* that are effective against secondary fungal infections in AIDS patients have been patented (Potts, 2000). Microalgal toxins are considered to be a threat to public health but once their structure and function are understood well, they can act as a drug like 'atopin' of *Gomphosphaeria* sp which is used to lyse cells of a dinoflagellate *Gymnodium breve.*

Microalgae have been an integral component of oxidation ponds usually incorporated with wastewater treatment. Algae play a major role in supplying oxygen to the bacterial population for the breakdown of complex organic matter (Oswald, 1988). Unicellular green algae such as *Chlorella* and *Scenedesmus* have been widely used in wastewater treatment as they often

colonize the ponds naturally, and have fast growth rates and high nutrient removal capabilities. Deviller *et al.* (2004) reported the use of high rate pond in water treatment in a recirculating fish rearing system. Over the last few decades, efforts have been made to apply intensive microalgal cultures to perform the biological tertiary treatment of secondary effluents (de la Noüe *et al.*, 1992). Identifying oxygen evolving photosynthetic organisms with high growth rate, high biomass yield and high utilization potential which could play a duel role of cleansing the waste water and also serving as a source of feed, fertilizer, and fuel is the primary requirement (Subramanian and Uma, 1996). However, one of the major drawbacks of using microalgae in wastewater purification is the harvesting of biomass (Laliberte *et al.*, 1997). According to Lima *et al.* (2003) the microalgal consortium was able to remove 30 and 50 mg L^{-1} *p*-Nitrophenol from the culture medium, which extends the knowledge on the ability of such organisms to participate in the removal of such compounds from the environment.

Species with a natural tendency to aggregate offer an attractive option and in this respect, epilithic or benthic filamentous Cyanobacteria are excellent candidates for intensive mass cultures intended for wastewater treatment (de la Noüe and Proulx, 1988). *Phormidium bohneri* Schmidle, a tropical Cyanobacterium, shows self-aggregation, capacity to sediment (Talbot and de la Noüe, 1993), and growth features that lend themselves to a bio-treatment system for wastewater in temperate and warm climates (Blier *et al.*, 1996; Sylvestre *et al.*, 1996). Proulx *et al.* (1994) performed preliminary testing under field conditions to assess its capability to remove nutrient, and promising results have been obtained with fish farm effluent (Dumas *et al.*,

1998). Mulbryl *et al.* (2001) reported that benthic fresh water algae could reduce nitrogen and phosphorus present in the dairy manure. The production of benthic algae for the treatment of manure offers considerable potential for on-farm recycling of manure nutrients. Moreover, use of the algal biomass as an animal diet supplement may lead to other benefits beyond nutrient recycling. Philippis (2003) reported that the biomass of *Cyanospira capsulata* and *Nostoc* possesses high affinity and high specific uptake for copper, comparable with the best performances shown by other microbial biomass, and suggest the possibility to use the capsulated trichomes of the two Cyanobacteria for the bioremoval of heavy metals from polluted water bodies.

In general, Cyanobacteria have a variety of characteristics that make them well suited to wastewater treatment. First, they have a high nutrient removal capacity as they can accumulate inorganic phosphorus and nitrogen and store them as polyphosphates and cyanophycin (Fay, 1983); but this feature has been little explored in the context of wastewater treatment. Second, they are likely to tolerate the highly variable conditions that characterise polluted effluents. Finally, many of the mat-forming strains self-aggregate in culture and therefore readily sediment in the absence of stirring, which allows the resultant biomass to be harvested easily (Mespoulède, 1997). The increasing concern about the contamination of water bodies by heavy metals has stimulated, in recent years a large number of researches on the possible ways to remove these toxic substances from the environment. Among the techniques that have been proposed, the use of microorganisms as an alternative to the conventional physicochemical methods encounters an increasing interest owing to a number of potential advantages of this method: (i) the rapid

kinetics of metal removal, (ii) the capability of the microbial biomass to remove metallic ions at concentration levels of the order of micromoles per liter, (iii) the utilization of naturally abundant renewable biomaterials, (iv) the possibility to treat waters containing multiple metals (Wilde and Benemann, 1993). Microorganisms can remove metals from the surrounding environment through various mechanisms (Garnham, 1997): (i) by biosorption, either as a metabolically mediated process or as a passive adsorption of metals on the charged macromolecules of the cell envelope, or (ii) by the chelation of heavy metals due to the release of charged extracellular polysaccharides. In this connection, it has been stressed that the main role in the metal chelation is carried out by the negatively charged groups of the acidic polysaccharides present in the cell envelope, such as carboxyl (Hamdy, 2000 ; Inthorn, 2001) or sulphate groups. Thus, the capability of the charged macromolecules present on the external layers of cell envelopes (i.e., lipopolysaccharide, capsule, slime) to bind heavy metals seems to be quite promising, giving the possibility to use either microbial cultures or dried biomass for the removal of these toxic elements.

Interest in alternatives to non-renewable fossil fuels has increased in the recent past due to both the finite nature of these resources as well as interest in achieving non-pollutive alternative sources of energy. Cyanobacteria, like all photosynthetic microorganisms can be used for generation of biomass, from which methane can be produced either by thermal gasification, catalytic hydrogenation or biological gasification (Patterson, 1996). Among microbes, Cyanobacteria seem to be more promising for biological photoproduction of hydrogen. Nitrogenase and reversible hydrogenase are the major enzymes

responsible for hydrogen production in Cyanobacteria (Prabaharan and Subramanian, 1996).

1.4 Challenges for future

Only a few Cyanobacterial species producing interesting compounds have been well characterized or exploited commercially. The potential of Cyanobacterial biomass for production of biochemicals, viz., pigments, antibiotics, essential fatty acids, vitamins etc. is yet to be exploited. C-phycocyanin from *Spirulina* has been commercialized. This is an odorless, non-toxic blue powder with a slight sweetness and is brilliant blue with faint reddish fluorescence in water. Recently there has been a growing interest and increasing evidence on potentially important physiological functions of phycocyanin, such as antioxidant activities (Romay *et al.*, 1998; Hirata *et al.*, 2000), anti-inflammatory activities (Romay *et al.*, 1998; Gonzalez *et al.*, 1999) and others (Belay *et al.*, 1993; Liu *et al.*, 2000). Phycocyanin can reduce cholesterol in blood (Iwata *et al.*, 1990). It is a recent trend to search microorganisms for the production of essential fatty acids. Poly unsaturated fatty acids like linolenic acid and eicosapentaenoic acid have lot of economic importance. Effluent treatment by physico-chemical methods is energy and cost intensive, often resulting in secondary pollutants. The new challenge is to control pollution in a cost effective way without further polluting the environment (Subramanian and Uma, 2001). Cyanobacteria are the best species in this regard because they show high flexibility and absorb nutrients from wastewaters. Basic research is needed to expand the range of organisms. Screening programmes must identify useful products, and Cyanobacterial strains that produce significant amounts of the product. Genetic engineering of

Cyanobacterial strains holds the promise of new products and improved yields. Recent developments in phycological research focuses on algal cultivation, algal floristic and molecular systematics, algal molecular biology, algal product development and the role of algae in environmental pollution control (Tseng, 2004). Thajuddin and Subramanian (2005) strongly recommend these organisms for exploiting various applications including pollution abatement.

1.5 Significance of the present study

Only a few Cyanobacterial strains have been well characterized or exploited commercially. Basic research is needed to identify new Cyanobacterial strains of high value products (Thajuddin and Subramanian, 2005). The Cyanobacterium *Spirulina* has already been commercially exploited in several ways including applications in health food and wastewater treatment. *Oscillatoria*, *Phormidium*, *Anabaena* and *Nostoc* are the common filamentous Cyanobacteria observed in the local habitat. It is necessary to select and characterize the local strains, which could be mass cultured in the outdoor environments. Biochemical composition of the species has to be defined to assess the nutritional quality of the species. Presence of proteins and unsaturated fatty acids in the Cyanobacteria is an important aspect as far as the nutritional quality is concerned. Phycocyanin, owing to its fluorescent nature, has gained commercial importance as a fluorescent marker, and also as a food colorant. Research continues around the world on the anti-cancerogenic property of phycocyanin. So it is very important to screen out the species with high phycocyanin content. Presence of eicosapentaenoic acid and linolenic acid in Cyanobacteria offers good scope for the study of these organisms.

Oscillatoria and *Phormidium* have been observed to thrive in petroleum refinery effluent and sewage (Joseph and Joseph, 2001a). Verifying the capacity of these algae in intensive cultivation would give the lead for a cost-effective viable treatment system without secondary effect. Therefore the objective of the present research is:

1. To develop pure cultures of local isolates of Cyanobacteria for extraction of biochemicals of commercial value.
2. To couple biomass production with effluent treatment.

2

CHAPTER

ISOLATION AND BIOMASS PRODUCTION

2.1 Introduction

Cyanobacteria occupy a unique position since they posses an autotrophic mode of growth like eukaryotic plant cells and a metabolic system as that of bacteria. The Cyanobacteria formerly known as blue green algae are of special importance in the balance of nature. They are photosynthetic, typically unicellular organisms although cells may some times be connected to form thread like filaments. In filamentous species growth may include differentiation to produce cells for particular function. Any cell of such a filament is potentially capable of binary fission and hence growth in length of the chain is mainly the sum of the individual cells. Often initial isolations of Cyanobacteria from environment may give rise to mixed cultures. Therefore it is essential to purify the individual types of Cyanobacteria from the mixture.

The Culturing of microalgae in the laboratory presents two problems. First, pure culture of a single species is needed to study an organism's characteristics. Second, a medium must be found that will support the growth of the desired organism. Once an organism has been isolated, it can be maintained indefinitely in pure culture called a Stock culture. Stock cultures are maintained by making subcultures in fresh medium at frequent intervals to keep the organisms growing.

The laboratory isolation of microalgae generally follows the following procedures. (1) Simple enrichment (2) Continuous culture (3) Filtration, (4) Direct Isolation (Daft, 1995). Material collected from natural habitat should be examined under the light microscope in order to ascertain the relative abundance of the types of Cyanobacteria present in the sample. If the

population is too, low the sample can be concentrated by centrifugation or if too high, diluted with suitable medium. The samples can be streaked on to agar plate or cultured in liquid medium as serial dilution to obtain monocultures. Filtration technique entails filtration through various sized filters by means of the application of gentle suction. The pore size of the filters is selected to allow contaminated bacteria to pass through while retaining Cyanobacteria. Some species of Cyanobacteria like *Oscillatoria* are capable of gliding motion and shows a tendency to move towards light. This property can be exploited for isolation.

The cultivation of algae involves optimization of various factors like pH, alkalinity, salinity, aeration, temperature, light etc. The pH of the culture medium is one of the important factors in algal cultivation. It determines the solubility of carbon dioxide and minerals in the medium and directly and indirectly influences the metabolism of algae (Becker, 1993). Algae exhibit a clear dependency on the pH of the growth medium and different species vary greatly in their response to the pH. Among soil properties pH is a very important factor in growth, establishment and diversity of Cyanobacteria, which can generally be reported to prefer neutral to slightly alkaline pH for optimum growth. Ciferri and Tiboni (1985) mentioned that *Spirulina* sp could thrive pH range 7 to 11.3 whereas *Dunaliella* prefers optimum pH between 7 and 9. Numerous reports of Cyanobacteria in fresh water and soil indicated that their diversity and abundance were greatest at higher pH (Kanniayan and Kumar, 2004).

Cyanobacteria prefer an optimum temperature between 30 and 35^{0}C. Ray and Bagchi (2001) optimized the culture conditions for growth of *Oscillatoria laetevirens*, at a temperature of 28 ± 2^{0}C and illumination by cool white fluorescent lamps of intensity 25 Wm^{-2} Karna *et al.* (1999) raised *Phormidium valderianum* under continuous white fluorescent light at an intensity of 1500 lux at 25 ± 2^{0}C in a controlled culture room. Kaushik (1999) reported that out of the five different species of Cyanobacteria tested, *Nostoc* was the predominant form followed by *Calothrix* at temperature 10 to 35^{0}C. The optimum temperature of *Spirulina* lies in the range of 30 to 35^{0}C (Tomaselli *et al.*, 1993). Rafiqul *et al.* (2005) optimized environmental factors such as temperature, light and pH of *Spirulina fusiformis* at 32^{0}C, 2500 lux and 7 respectively to obtain specific growth rate 0.138 d^{-1} and biomass 2.9 g L^{-1}

Aeration and agitation are important parameters in algal mass culture. Aeration mode has a direct effect on algal productivity (de la Noue *et al.,* 1984). Agitation is considered with regard to its capacity to aerate the culture and to maintain the filamentous algae in suspension without mechanical stress (Persoone *et al.,* 1980). Pouliot *et al.* (1989) conducted experiments at laboratory scale on the effect of different aeration–agitation modes (stirring and air bubbling) on growth rate and confirmed that maximum growth rate of 0.34 d^{-1} was obtained in the cultures with bubbling on a 14/24 h basis. Costa *et al.* (2001) reported that aeration rate of 20 l h^{-1} is enough for *Spirulina platensis*.

The yield of various species varies under laboratory conditions. *Phormidium bohneri* produced 0.7 g l^{-1} at 30^{0}C from an initial concentration of 0.1 g L^{-1} (Talbot and dela Noue, 1993). Oliveira *et al.* (1999) revealed that *Spirulina* sp

could produce maximum 2.4 g L^{-1} at 30-35^{0}C with 0.1 g L^{-1} inoculum size. Costa *et al.* (2001) reported that maximum biomass yield for *Spirulina platensis* was 1.99 g L^{-1} in medium containing 0.03 M NaNO3 for an initial concentration of 0.1 g L^{-1} in 28 days. Gordillo *et al.* (1999) reported biomass yield of 1.1 g L^{-1} for an initial concentration of 0.15 g L^{-1} in five days in *Spirulina platensis* achieving specific growth rate between 0.45 and 0.5 d^{-1} According to Oliveira *et al.* (1999) *Spirulina platensis* has specific growth rate between 0.46 and 0.58 d^{-1} whereas that of *Spirulina maxima* is between 0.26 and 0.45 d^{-1} at 30-35^{0}C. *Anabaena* sp. showed maximum growth rate of 0.35 d^{-1} in fresh water medium (Rai and Tiwari, 1999).

The present study was carried out to develop Cyanobacteria isolates for mass culturing in the laboratory as well as in outdoor conditions. Cyanobacteria have been isolated and their growth requirements optimized.

2.2 Isolation and purification of Cyanobacteria

Materials and Methods

Soil and water samples were collected from paddy fields and sewage drains for isolating Cyanobacteria. Samples were brought to the laboratory, and inoculated into various algal growth media in test tubes stoppered with non-absorbent cotton. The growth medium was prepared in filtered, boiled tap water by adding required quantity of nutrient stock solutions. The samples were incubated under 12 h light: 12h dark cycles with illumination from cool white fluorescent tubes at an intensity of 2000 lux and a temperature 30 ± 3^{0}C. The samples were incubated for two weeks. Those showing algal growth upon microscope observation were selected, and re-inoculated into new media. The

algal cultures thus obtained in the liquid culture media were an assemblage of different algal groups and ciliates. The following methods were adopted for the purification of Cyanobacteria from the assemblage.

- ***Agar plating method***

Agar medium was prepared by adding 1.5 g of agar into 100 ml of BG-11 medium. The liquid cultures showing blue-green growth were transferred to agar plates and incubated for two weeks. The blue-green spots developed were repeatedly streaked on to agar slants for purification.

- ***Serial dilution technique***

The liquid cultures were serially diluted in BG-11 medium and incubated under light assembly for two weeks. The positive tubes were recultured till monocultures were obtained.

- ***Direct Manual picking***

The liquid cultures were observed under the microscope for the presence of blue-green filaments. The latter were picked manually under the microscope using micro-needles, and transferred to fresh BG-11 medium. Filtration technique and treatment with 0.01 % formalin were also employed to get rid of unicellular algae and ciliates respectively.

2.3 Identification of the isolates

The isolated algae were identified based on morphological and structural features (Desikachary, 1959). Three species of filamentous Cyanobacteria

were isolated and developed into pure cultures. The isolated algae were identified as *Oscillatoria acuminata* Gomont, *Oscillatoria subbrevis* schmidle and *Phormidium tenue* Menegh (Plate I).

Oscillatoria acuminata Gomont

Thallus blue-green; unbranched filamentous trichomes; Cells 4 μm broad and 5μm long. Motile filaments with tapering end and oscillatory movement.

Oscillatoria subbrevis Schmidle

Thallus blue-green; filamentous unbranched trichomes; 7.2 μm broad, nearly straight, not attenuated at the apices. Cells 1.8-μm long, calyptra absent. Hormogones formed by development of separation discs.

Phormidium tenue Menegh

Thallus blue-green, thin, trichome straight and densely entangled. Mucilage sheath present around each trichome. Cells longer than broad (2.7x1.8 μm), calyptra absent.

2.4 Optimization of growth of *Oscillatoria acuminata, Oscillatoria subbrevis,* and *Phormidium tenue*

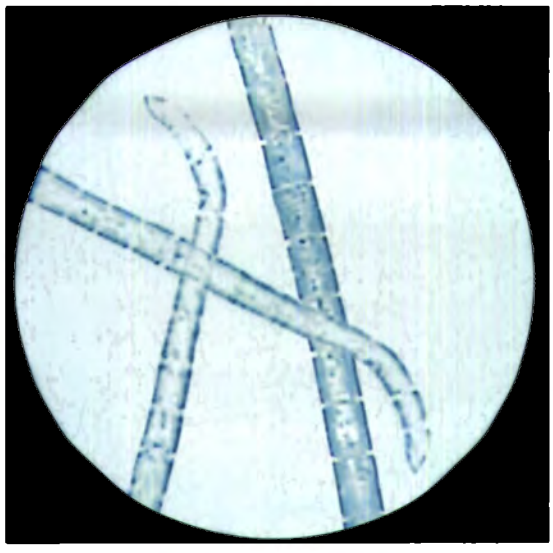
2.4.1 Selection of growth medium

Procedure

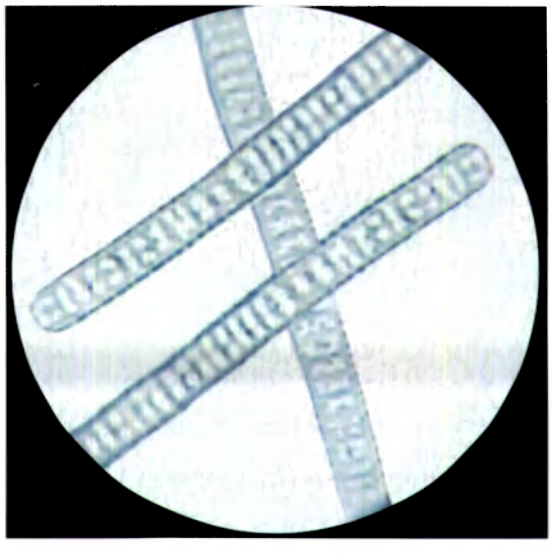
The three species of isolated Cyanobacteria were cultured in ten different growth media by inoculating equal amounts of biomass into triplicate culture flasks. They were grown for two weeks as batch cultures in the laboratory. The

Plate I

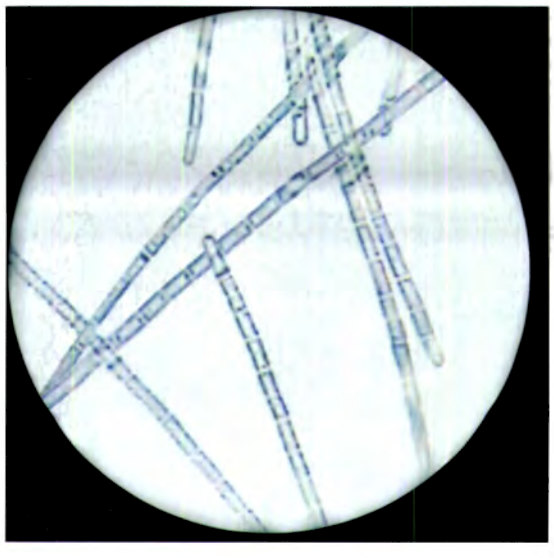
Oscillatoria acuminata



Oscillatoria subbrevis



Phormidium tenue



conditions of incubation were the same as described above in section 2.2 and has been used throughout this investigation for indoor cultivation. The culture media tested were ASM, MN, BG-11 (Daft, 1995), Modified BG-11 (Vonshak, 1997), Allen &Arnon, Modified Allen & Arnon medium (Becker, 1994) Fogg's, Tolypothrix (ICAR, 2000), Zarrouk's (Zarrouk, 1966), and CFTRI (Venkataraman and Becker, 1985) medium. The appearance of bluish green color was observed as an indication of growth on the 12^{th} day. Based on these results five media were selected for further study. The culture media were taken in sterilized 250 ml borosilicate culture flask to 100 ml volume in triplicate, and inoculated with 5 mg each dry weight of the algae. The cultures were incubated under the light assembly as before. The biomass was harvested on 12^{th} day and its dry weight was estimated.

Estimation of dry weight

The biomass was harvested by filtration. The filtered biomass was washed with distilled water, blotted and transferred to pre-weighed watch glass. This was placed in the oven at 60^0C for 12 h. The sample was weighed after cooling in a desiccator to get the dry weight.

Results

The Modified Allen & Arnon nutrient medium did not support the growth of any of the three species. The rest of the nine media were favorable to the growth of *P. tenue*. The growth of *O. subbrevis* was restricted to eight culture media, and that of *O. acuminata* to five growth media (Table 2.1). The confirmatory test of the growth of three species in five selected growth media

showed that *O. acuminata* produced a biomass of 48 mg in BG-11 on the 12th day, *O. subbrevis* produced 51 mg, and *P. tenue* had a yield of 45 mg, which is quite higher than the growth in other media (Table 2.2). Therefore BG-11 was selected as the growth medium for the three species isolated. All the three species formed mats on the sides of the container, which got detached within 8-12 days to form floating scum.

Table 2.1 Growth of *Oscillatoria acuminata, Oscillatoria subbrevis* and *Phormidium tenue* in different culture media (based on visual observation + indicates growth, - indicates no growth

Sl.No	Culture medium	*O. acuminata*	*O. subbrevis*	*P. tenue*
1	ASM		+	+
2	MN		+	+
3	Fogg's	+	+	+
4	Tolypothryx			+
5	BG-11	+	+	+
6	Modified BG- 11	+	+	+
7	Modified Zarrouk's		+	+
8	CFTRI	+	+	+
9	Allen & Arnon	+	+	+
10	Modified Allen & Arnon			

Table 2.2 Yield of *Oscillatoria acuminata, Oscillatoria subbrevis*, and *Phormidium tenue* after 12 days of inoculation (Inoculum size = 5 mg dry weight)

Culture medium	(mg dry weight /100 ml) ± SD		
	O. acuminata	*O. subbrevis*	*P. tenue*
Fogg's	23 ± 3.2	29 ± 1.5	30 ± 3.3
CFTRI	25±2.6	24 ± 4.2	5 ± 1.6
BG-11	48 ± 3.0	51 ± 3.5	45 ± 2.1
Modified BG-11	9± 1.2	31± 5.0	40 ± 3.3
Allen &Arnon	17 ± 2.1	29 ± 2.1	8 ± 3.2

2.4.2 Effect of alkalinity

Procedure

Alkalinity of the BG-11 medium was altered by the addition of bicarbonate and phosphate. The media thus prepared had alkalinities 90 and 150 mg $CaCO_3\ L^{-1}$ Equal amount of biomass was added to these different test media of 100 ml in triplicate culture flasks, and incubated as in the previous experiment. The BG-11 medium of alkalinity 45 mg $CaCO_3\ L^{-1}$ was provided as control. The cultures were incubated as before. Harvesting was done on 12^{th} day by filtering out the mats formed, and dry weight estimated. The ideal alkalinity for the growth of the three species was statistically evaluated.

Results

The alkalinity of the growth medium affected the growth of *O. acuminata* considerably (Figure 2.1).

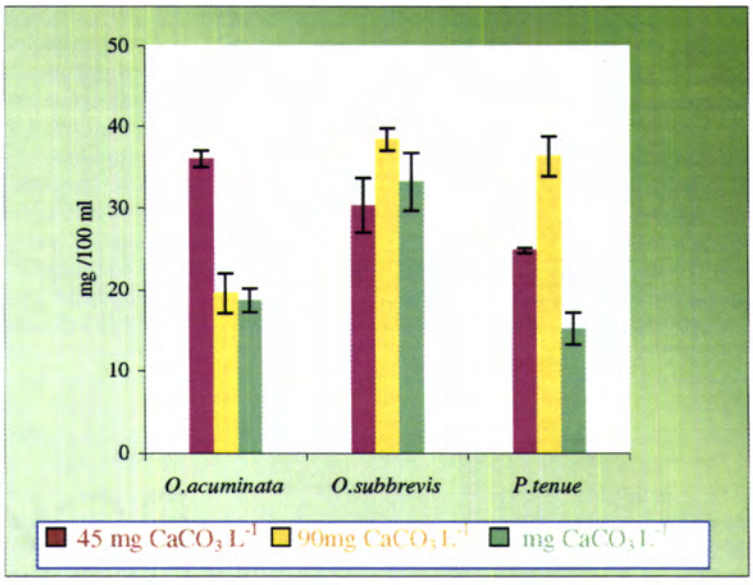


Figure 2.1 Twelve day yield of *Oscillatoria acuminata*, *Oscillatoria subbrevis*, and *Phormidium tenue* in growth media of different alkalinity

The yield at 45 mg $CaCO_3$ L^{-1} was 37 mg compared to 19.6 mg at 90 mg $CaCO_3$ L^{-1} Further increase in alkalinity did not significantly affect growth (Table 2.3). *O. subbrevis* had highest production at an alkalinity of 90 mg $CaCO_3$ L^{-1}. Further hike in alkalinity did not increase yield significantly (Table 2.4)

Table2.3 Analysis of variance and Tukey multiple comparison of effect of alkalinity on growth of *Oscillatoria acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	2	639.742	319.871	287.137
Within (Error)	6	6.687	1.114	
Total	8	646.429		
Critical F value = 5.14 (0.05,2,6) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Alkalinity (Mg $CaCO_3$ L^{-1})	Mean	3 2 1	
3	150	18.667	\	
2	90	19.600	\	
1	45	37.000	* * \	
* = significant difference (p=0.05) . = no significant difference				

P. tenue exhibited highest growth at 90 mg $CaCO_3$ L^{-1}. At 150 mg $CaCO_3$ L^{-1} the growth declined significantly (Table 2.5).

Table 2.4 Analysis of variance and Tukey multiple comparison of effect of alkalinity on growth of *Oscillatoria subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	2	97.847	48.923	5.911
Within (Error)	6	49.653	8.276	
Total	8	147.500		
Critical F value = 5.14 (0.05,2,6) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Alkalinity (Mg $CaCO_3$/L)	Mean	1 3 2	
1	45	30.400	\	
3	150	33.233	.\	
2	90	38.367	* \	
* = significant difference (p=0.05). = no significant difference				

Table 2.5 Analysis of variance and Tukey multiple comparison of effect of alkalinity on growth of *Phormidium tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	2	670.177	335.089	100.779
Within (Error)	6	19.948	3.325	
Total	8	690.126		
Critical F value = 5.14 (0.05,2,6) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Alkalinity (Mg $CaCO_3$/L)		Mean	3 1 2
3	150		15.183	\
1	45		24.933	* \
2	90		36.300	* * \
* = significant difference (p=0.05)			. = no significant difference	

2.4.3 Effect of pH

Range finding test

The BG-11 medium was modified to alkalinity levels optimum for the growth of each of the three species from the above results. Further the pH was altered by adding dil. Hcl / NaOH to obtain the test range 3-13. The test media were taken in test tubes in triplicates. The media were inoculated separately with equal amounts of inoculum of the three species. Growth of the strains in these different pH were compared on 12^{th} day by visual observation, based on the development of blue-green surface scum.

Confirmatory test

P^H range of 7.5- 11.5 were selected based on the above observation. 100 ml each of the test media were taken in conical flasks in triplicate and inoculated with 4 mg dry weight of the algae. The cultures were incubated under fluorescent light assembly as in previous experiments. The dry biomass produced was estimated on the 12^{th} day. The results are represented

graphically. The optimum pH for growth of the three species was estimated statistically.

Results

The three species tested did not grow below pH 4 and above pH 12. There was varying degree of growth between the pH range 4 and 12 (Table 2.6).

Table.2.6 Growth of *Oscillatoria acuminata, Oscillatoria subbrevis* and *Phormidium tenue* in media of different pH (based on visual observation. - no growth, + detectable growth, + + good growth)

pH	*O. acuminata*	*O. subbrevis*	*P. tenue*
3			
4	+	+ +	+ +
5	+	+ +	+ +
6	+ +	+ +	+ +
7.5	+ +	+ +	+ +
8.5	+ +	+ +	+ +
9.5	+ +	+ +	+ +
10.5	+	+ +	+ +
11.5	+	+ +	+ +
12	+	+ +	+
13			

Confirmatory test revealed that in the pH range 7.5 to 11.5, the growth of *O. acuminata* decreased with increasing pH (Figure 2.2). The analysis of variance combined with Tukey analysis showed that pH had significant effect on the growth of the species. The yield at pH 7.5 was significantly higher than all other test pH levels (Table 2.7).

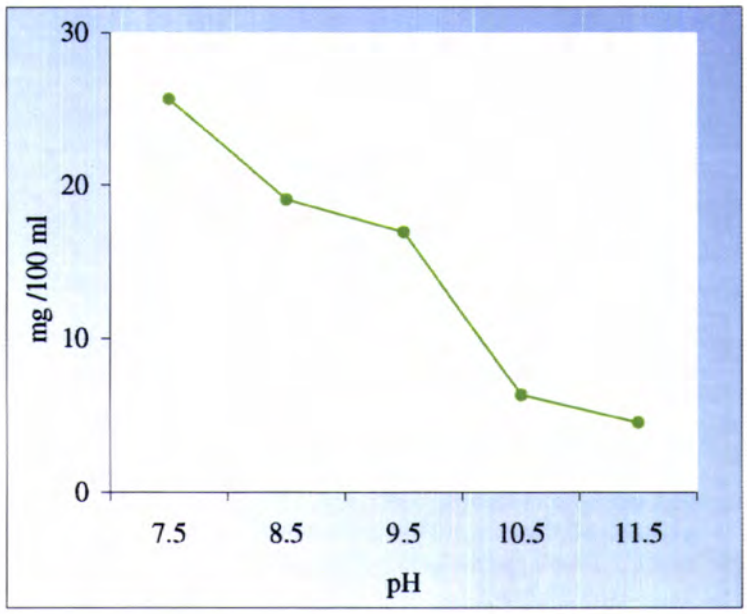


Figure 2.2 Effect of pH on growth of *Oscillatoria acuminata*

Table 2.7 Analysis of variance and Tukey multiple comparison of the effect of pH on the growth of *Oscillatoria acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	952.631	238.158	423.016
Within (Error)	10	5.627	0.563	
Total	14	958.257		
TUKEY method of multiple comparisons				
Group	pH	Mean	5 4 3 2 1	
5	11.5	4.567	\	
4	10.5	6.300	\	
3	9.5	16.967	* * \	
2	8.5	19.067	* * * \	
1	7.5	25.667	* * * * \	
* = significant difference (p=0.05)		= no significant difference		

The growth of *O. subbrevis* was promoted by increasing pH upto 9.5; further up there was gradual reduction in growth (Figure 2.3). Analysis of variance showed that the pH effect is significant at probability level 0.05 (Table 2.8). However there is no significant difference in growth at pH 9.5 and 10.5.

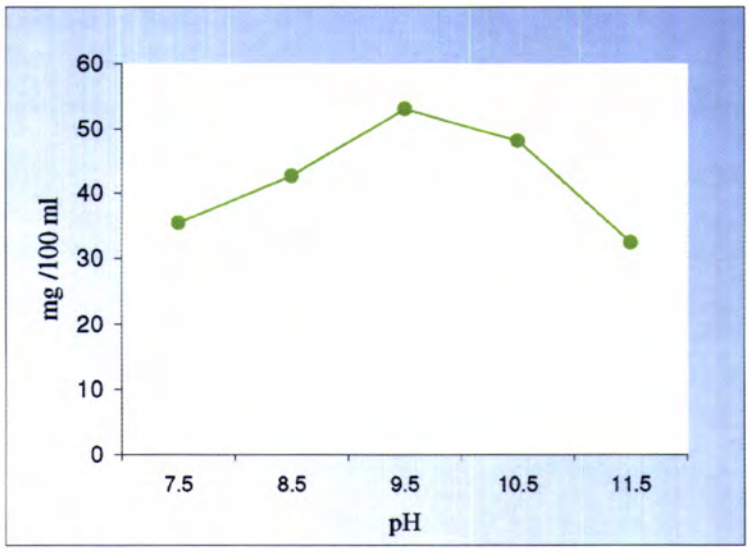


Figure 2.3 Effect of pH on growth of *Oscillatoria subbrevis*

Table 2.8 Analysis of variance and Tukey multiple comparison of the effect of pH on the growth of *Oscillatoria subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	887.317	221.829	34.296
Within (Error)	10	64.680	6.468	
Total	14	951.997		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	pH	Mean	5 1 2 4 3	
5	11.5	32.567	\	
1	7.5 (control)	35.500	.\	
2	8.5	42.700	* * \	
4	10.5	48.233	* * \	
3	9.5	53.233	* * * \	
* = significant difference (p=0.05) . = no significant difference				

The growth of *P. tenue* increased with increasing pH. The highest yield was at pH 10.5 (Figure 2.4). However analysis of variance revealed that the biomass yield was similar in the pH range 7.5 to 10.5. There was significant reduction in yield at pH 11.5 (Table 2.9)

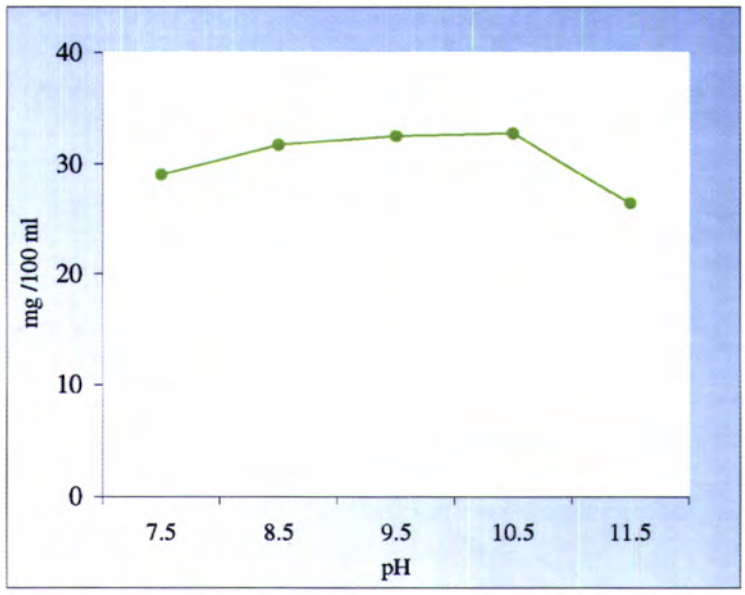


Figure 2.4 Effect of pH on growth of *Phormidium tenue*

Table 2.9 Analysis of variance and Tukey multiple comparison of the effect of pH on the growth of *Phormidium tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	75.829	18.957	3.865
Within (Error)	10	49.047	4.905	
Total	14	24.876		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho: All groups equal				
TUKEY method of multiple comparisons				
Group	pH	Mean	5 3 1 2 4	
5	11.5	26.367	\	
3	9.5	28.967	\	
1	7.5 (control)	29.033	\	
2	8.5	31.700	\	
4	10.5	32.733	* \	
* = significant difference (p=0.05)		. = no significant difference		

2.4.4 Effect of aeration and agitation

Procedure

Batch cultures of the three species were grown in 1 litre borosilicate flasks containing specific growth medium selected, and placed under cool white

fluorescent lamps at 2000 lux. They were incubated at 30± 3^{0}C in 12/12 L/D cycle. Agitation of the cultures was provided in four different ways

1. By continuously bubbling filtered air through the culture during the light period (12/24 basis)
2. By intermittently bubbling filtered air through the culture at one hour intervals during the light period (6/24 basis).
3. By intermittent gentle spinning during the light period
4. Static batch culture as control

The growth of the three species was estimated as the twelve-day yield of biomass, and the effect of aeration was elucidated by statistical analysis.

Results

The type of agitation given to the culture significantly affected the growth of all three species (Figure 2.5). Continuous aeration (12/24 h basis) resulted in poor

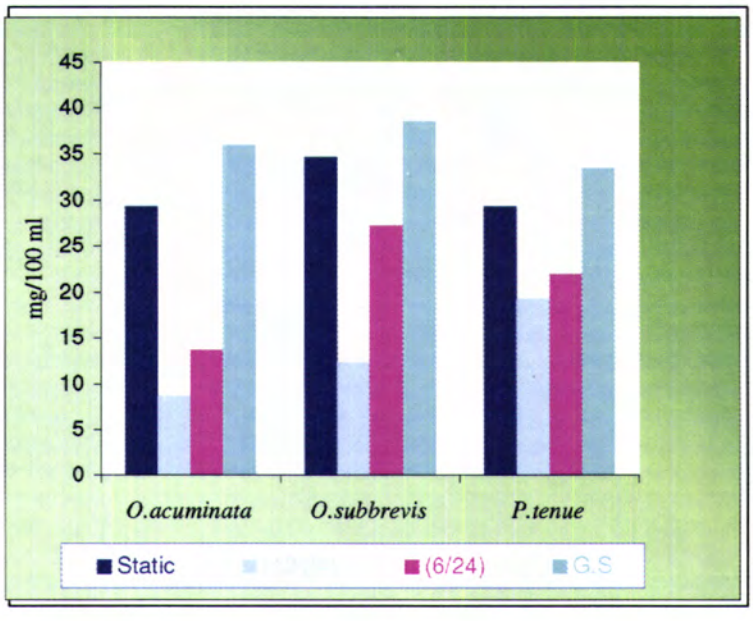


Figure 2.5 Effect of aeration and agitation on growth of *Oscillatoria acuminata* (Aeration 12/24, 6/24, G.S – gentle spinning)

growth. Reduction in the period of aeration to six hours improved the growth of *O. acuminata, O. subbrevis* and *P. tenue.* In static culture the twelve-day yield was 29.3 mg/100 ml. But gentle spinning of the cultures produced a significantly higher biomass of 36 mg (Table 2.10).

Table 2.10 Analysis of variance and Tukey multiple comparison of the effect of aeration on growth of *Oscillatoria acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	3	1490.917	496.972	165.657
Within (Error)	8	24.000	3.000	
Total	11	1514.917		
Critical F value = 4.07 (0.05,3,8) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Aeration	MEAN	2 3 1 4	
2	12/24	8.667	\	
3	6/24	13.667	* \	
1	Static	29.333	* * \	
4	Gentle spinning	36.000	* * * \	
* = significant difference (p=0.05) . = no significant difference				

The growth of *O. subbrevis* was drastically affected by bubbling air. The yield was similar in static batch culture as well as with gentle spinning (Table 2.11)

Table 2.11 Analysis of variance and Tukey multiple comparison of the effect of aeration on growth of *Oscillatoria subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	3	1203.290	401.097	48.671
Within (Error)	8	65.927	8.241	
Total	11	1269.217		
Critical F value = 4.07 (0.05,3,8) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Aeration	Mean	2 3 1 4	
2	12/24	12.333	\	
3	6/24	27.333	* \	
1	Static	34.667	* \	
4	Gentle Spinning	38.533	* * \	
* = significant difference (p=0.05) . = no significant difference				

Similar results were obtained with *P. tenue* (Table 2.12) which yielded a maximum biomass of 33.47 mg upon gentle spinning of the culture which was statistically similar to the static cultures.

Table 2.12 Analysis of variance and Tukey multiple comparison of the effect of aeration on growth of *Phormidium tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	3	381.907	127.302	23.180
Within (Error)	8	43.940	5.492	
Total	11	425.847		
Critical F value = 4.07 (0.05,3,8) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Aeration	Mean	2 3 1 4	
2	12/24	19.333	\	
3	6/24	22.000	\	
1	Static	29.333	* * \	
4	Gentle Spinning	33.467	* * \	
* = significant difference (p=0.05) . = no significant difference				

2.4.5 Effect of salinity

Procedure

The growth media were modified by adding crude sea salt to a salinity range of 5 to 35 x 10^{-3} The respective test media were taken in conical flasks and were inoculated with equal quantities of the three test species, and incubated for 12 days. The growth was measured on the 12^{th} day as dry weight and the effect of salinity on growth of the three species were elucidated through statistical analysis.

Results

Salinity of the growth medium significantly reduced the growth of *O. acuminata* (Figure 2.6, Table 2.13).

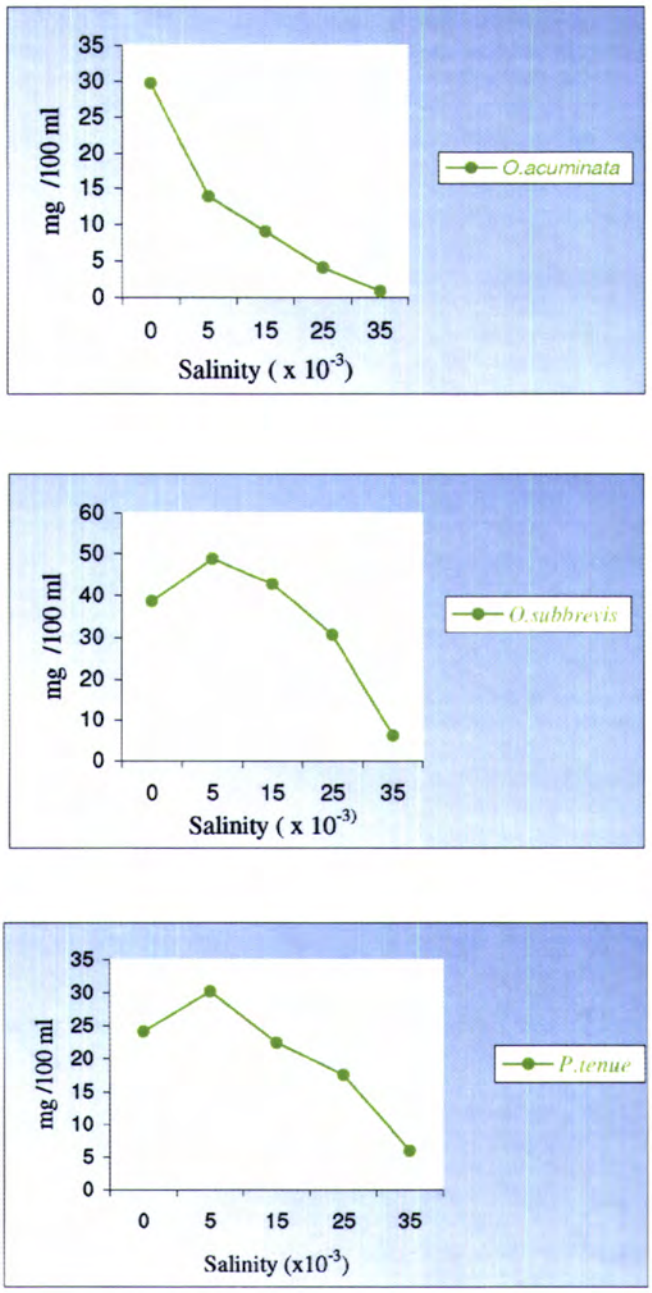


Figure 2.6 Effect of salinity on growth of *Oscillatoria acuminata*, *Oscillatoria subbrevis* and *Phormidium tenue*

Table 2.13 Analysis of variance and Tukey multiple comparison of the effect of salinity on the growth of *Oscillatoria acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	1534.389	383.597	449.177
Within (Error)	10	8.540	0.854	
Total	14	1542.929		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity (x 10^{-3})	Mean	5 4 3 2 1	
5	35	0.800	\	
4	25	4.067	* \	
3	15	9.000	* * \	
2	5	13.833	* * * \	
1	Control	29.667	* * * * \	
* = significant difference (p=0.05) . = no significant difference				

The growth of *O. subbrevis* increased significantly at salinity $5x10^{-3}$ The growth decreased slightly at 15 x 10^{-3}; but it was higher than control (Figure 2.6; Table 2.14). Further increase in salinity decreased growth. The growth of *P. tenue* was significantly higher than control at salinity 5 x 10^{-3}. The growth decreased at higher salinities (Figure 2.6; Table 2.15).

Table 2.14 Analysis of variance and Tukey multiple comparison of the effect of salinity on the growth of *Oscillatoria subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	3309.129	827.282	419.302
Within (Error)	10	19.727	1.973	
Total	14	3328.856		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity(x 10^{-3})	Mean	5 4 1 3 2	
5	35	6.067	\	
4	25	30.633	* \	
1	Control	38.567	* * \	
3	15	42.700	* * * \	
2	5	48.733	* * * * \	
* = significant difference (p=0.05) . = no significant difference				

Table 2.15 Analysis of variance and Tukey multiple comparison of the effect of salinity on the growth of *Phormidium tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	998.627	249.657	51.657
Within (Error)	10	48.327	4.833	
Total	14	1046.953		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity ($x10^{-3}$)	Mean	5 4 3 1 2	
5	35	5.867	\	
4	25	17.533	* \	
3	15	22.433	* \	
1	Control	24.133	* * \	
2	5	30.200	* * * * \	
* = significant difference (p=0.05) . = no significant difference				

2.4.6 Definition of optimum growth medium

The growth medium for best yield of *O. acuminata* was identified as the standard BG-11 medium with alkalinity 45 mg $CaCO_3$/L and pH 7.5. *O. subbrevis* and *P. tenue* were grown in the above medium modified to raise the alkalinity to 90 mg $CaCO_3$ L^{-1} and pH 9.5. The composition of the medium is given below.

Composition	BG-11 for *O. acuminata* (g/L)	Modified BG-11 for *O. subbrevis* and *P. tenue* (g/L)
$NaNO_3$	1.5	1.5
Na_2CO_3	0.02	0.05
K_2HPO_4 3 H_2O	0.04	1.0
$MgSO_4$ 7 H_2O	0.095	0.095
$CaCl_2$ $2H_2O$	0.036	0.036
Citric acid	0.006	0.006
FerricAmmonium Citrate	0.006	0.006
EDTA (Na Mg Salt)	0.001	0.001
H_3BO_3	0.003	0.003
$MnCl_2$ $4H_2O$	0.002	0.002
$ZnSO_4$. 7 H_2O	0.0002	0.0002
Na_2MoO_4. $2H_2O$	0.0004	0.0004
$CuSO_4.5H_2O$	0.00008	0.00008
$Co(NO_3)_2.6H_2O$	0.00005	0.00005

2.5 Growth kinetics of *Oscillatoria acuminata,Oscillatoria subbrevis* and *Phormidium tenue*

Procedure

The culture media selected were autoclaved in 250 ml borosilicate flasks plugged with non-absorbent cotton. The three species were inoculated into the respective media. The inoculum was equivalent to 5 mg dry weight in 100 ml growth medium. Each species was inoculated with sufficient replications to provide samples throughout the period of sampling. The cultures were incubated under the light assembly as in the previous experiments. The biomass produced was filtered, and the dry weight was estimated at intervals of four days. The process was repeated up to 20th day of inoculation. The growth curve was plotted using the data. Dry weights were used to calculate growth rate as

$$r = (\ln DW_t - \ln DW_0) / t$$

where DW_0 = initial dry weight,

DW_t = dry weight for day t

t = time between both measurements (in days)

Results

O. acuminata showed an increase in biomass up to 20th day. The species show didn't show any lag phase. The maximum growth rate observed was 0.36 d^{-1} *O. subbrevis* showed a linear growth up to 12th day. The growth rate declined after 12 days. The maximum growth rate observed was 0.51 d^{-1} The cultures of *P. tenue* reached stationary phase by 16th day (Figure 2.7). The maximum growth rate observed was 0.33 d^{-1}

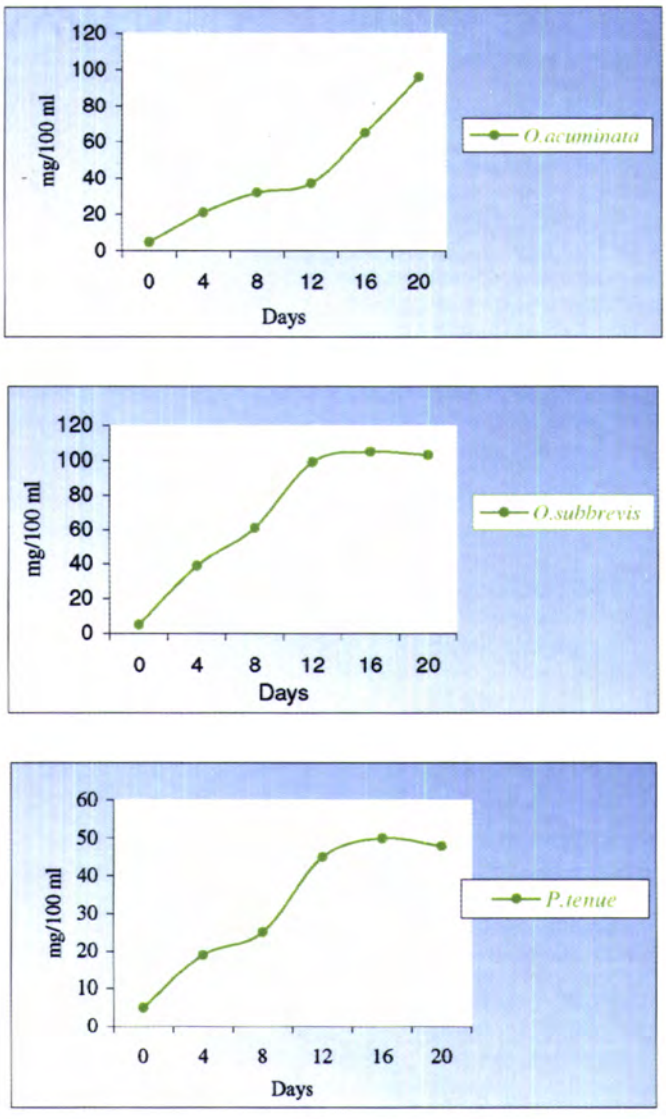


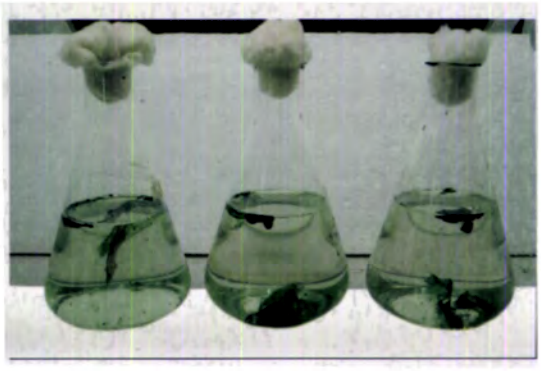
Figure 2.7 Growth curve of *Oscillatoria acuminata*, *Oscillatoria subbrevis* and *Phormidium tenue*

2.6 Biomass production

The biomass produced by the three Cyanobacteria under indoor and outdoor conditions were evaluated. Indoor cultures were grown in 1 and 2 litre borosil culture flasks and incubated under the light assembly as before (Plate II). The outdoor cultures were grown in fibreglass tanks of 10 – 15 L capacity (Plate III). The culture media used for both indoor and outdoor cultivation were BG-

Plate II

Indoor cultivation of *Oscillatoria acuminata*, *Oscillatoria subbrevis* and *Phormidium tenue*



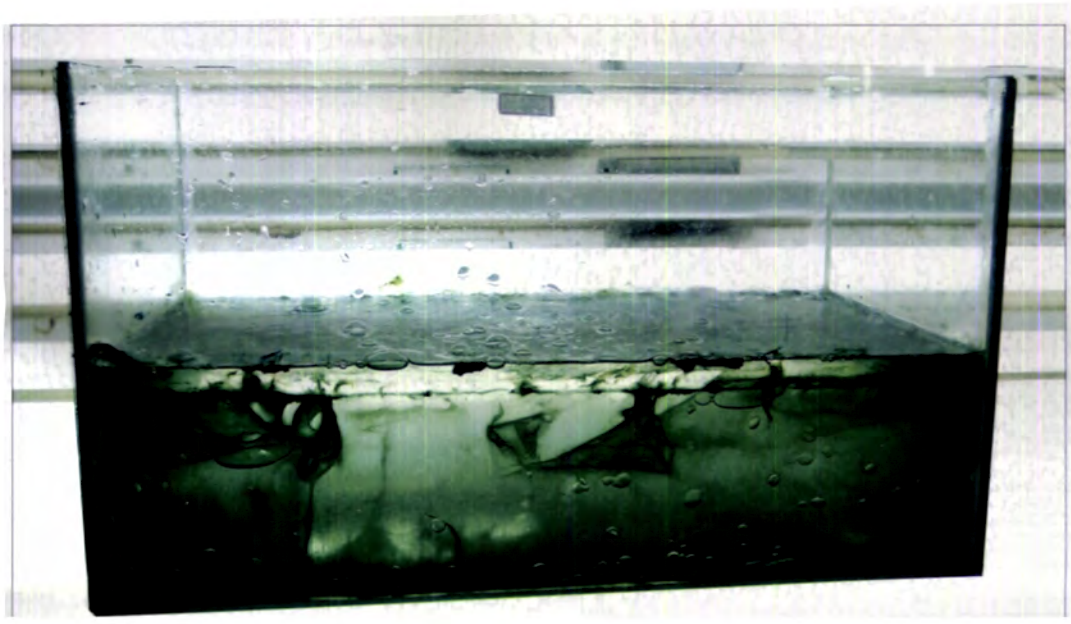
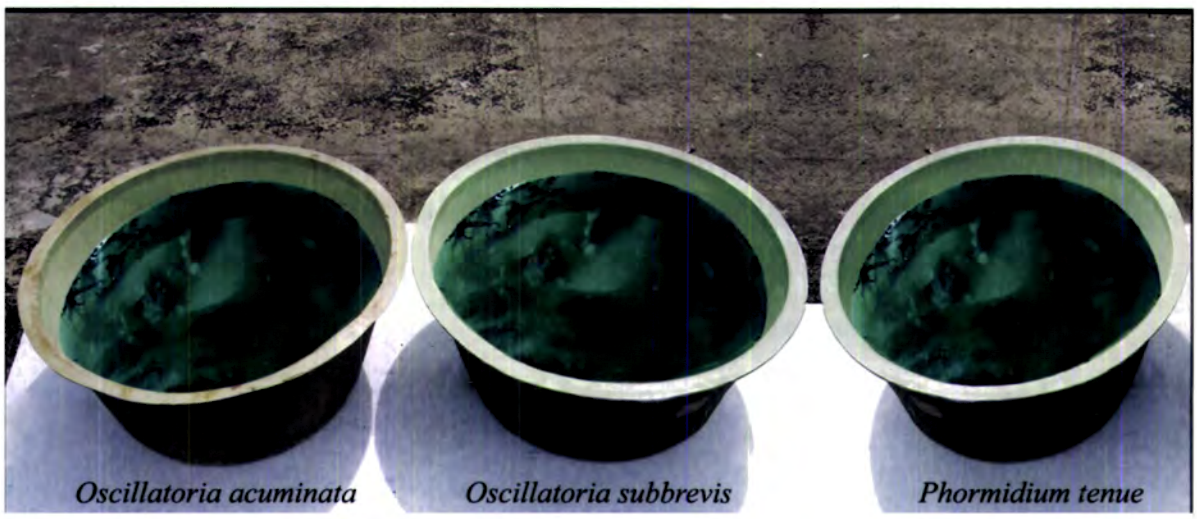


Plate III

Outdoor Cultivation



11 for *O. acuminata,* and modified BG-11 for *O. subbrevis and P. tenue.* The inoculum was equivalent to 10 mg dry weight L^{-1} in indoor cultures. Outdoor cultures were inoculated at the rate of 10 and 15 mg dry weight L^{-1} The cultures were kept exposed to sunlight with intermittent agitation. The cultures were harvested on 12^{th} day and the biomass produced was estimated as dry weight L^{-1}. Chlorophyll *a* and Phycobiliproteins were estimated as additional measure of biomass.

Estimation of chlorophyll a

The algae were harvested and washed twice with distilled water. The chlorophyll was extracted in 90 % acetone and the absorbance of the clear extract was measured at 750, 663, and 645 nm. The amount of chlorophyll *a* per unit biomass was calculated following the relation given by Becker (1994)
Chlorophyll $a = (12.7 \times A_{663}) - (2.69 \times A_{645})$ mg L^{-1}

Estimation of phycobiliproteins

50 mg of fresh biomass was washed with distilled water. It was ground well with 3 –5 ml phosphate buffer (0.05M) using a mortar and pestle. The whole extract was transferred into a clean dry test tube and frozen for 12 h. The content was thawed at 4^0C. The process was repeated, until the extraction was complete. The supernatants were pooled and the absorbance was measured at 652 and 615 nm against phosphate buffer blank (Siegelman and Kycia, 1978).

Results

O. acuminata yielded 93 mg L^{-1} in the indoor conditions and in the outdoor conditions this species yielded only 69 mg L^{-1} for inoculum size 10 mg L^{-1}

The species produced 79 mg L^{-1} for inoculum size 15 mg L^{-1} in the outdoor conditions. The mean chlorophyll content of the biomass was 1.9 mg %, phycocyanin 17.99 mg % and 7.3 mg % allophycocyanin upon indoor cultivation (Table 2.16).

Table 2.16 Biomass, chlorophyll *a* and phycobiliproteins of *Oscillatoria acuminata* in the Indoor and Outdoor conditions upon twelve day growth.

Parameter	Indoor cultivation	Outdoor cultivation	
	Initial Inoculum ($10mgL^{-1}$)	Initial Inoculum ($10mgL^{-1}$)	Initial Inoculum ($15mgL^{-1}$)
Biomass (mg /L)	93	69	79
Chlorophyll *a* (mg %)	1.90 ± 2.57		1.77 ± 2.1
Phycocyanin (mg %)	17.99 ± 1.15		15.97 ± 0.46
Allophycocyanin (mg %)	7.3 ± 1.8		5.8 ± 0.58

In outdoor cultivation the chlorophyll content ranged from 1. 5 to 2 mg % and phycocyanin from 15.4 to 16.3 mg % and allophycocyanin from 5.8 to 5.9 mg %. *O. subbrevis* could produce biomass of 240 mg L^{-1} in the indoor conditions and 115 mg L^{-1} in the outdoor conditions for inoculum size 10 mg L^{-1}; whereas it yielded 152 mg L^{-1} for inoculum size 15 mg L^{-1} in the outdoor conditions (Table 2.17).

Table 2.17 Biomass, chlorophyll and phycobiliproteins of *Oscillatoria subbrevis* in Indoor and Outdoor conditions upon twelve day growth.

	Indoor cultivation	Outdoor cultivation	
	Inotial Inoculum ($10mgL^{-1}$)	Initial Inoculum ($10mgL^{-1}$)	Initial Inoculum ($15mgL^{-1}$)
Biomass (mg/L)	240	115	152
Chlorophyll *a* (mg %)	1.59 ±2.46		1.53 ± 1.08
Phycocyanin (mg %)	13.74 ± 0.37		13.12 ± 0.54
Allophycocyanin (mg %)	6.016 ±0.5		6.36 ± 0.061

Chlorophyll content of this species ranged between 1.3and 1.9 mg % in indoor experiments, and 1.4 to 1.7 mg % in outdoor conditions. The species yielded 13.3 to 14 mg % phycocyanin, and 5.96 to 6.05 mg %

allophycocyanin in indoor experiments and yielded 12.7 to 13.7 mg % phycocyanin and 6.3 mg % allophycocyanin in outdoor conditions. *P. tenue* produced biomass of 80 mg L^{-1} in the indoor experiments and 73.24 mg L^{-1} in the outdoor experiment for inoculum size 10 mg L^{-1} It could yield 73 mg L^{-1} in outdoor conditions for 15mg L^{-1} inoculum size (Table 2.18).

Table 2.18 Biomass, Chlorophyll and phycobiliproteins of *Phormidium tenue* in the Indoor and Outdoor conditions upon twelve-day growth.

Parameter	Indoor cultivation	Outdoor cultivation	
	Initial Inoculum ($10mgL^{-1}$)	Initial Inoculum ($10mgL^{-1}$)	Initial Inoculum ($10mgL^{-1}$)
Biomass (mg L^{-1})	80	73.24	73
Chlorophyll *a* (mg %)	0.83 ± 0.8		0.73 ± 0.37
Phycocyanin (mg %)	8.23 ± 0.13		5.6 ± 2.0
Allophycocyanin (mg %)	5.10 ± 0.15		3.3 ± 0.4

Chlorophyll content of this species ranged from 0.78 to 0.95 mg % in the indoor experiments and 0.68 to 0.77 mg % in outdoor conditions. Phycocyanin content was in between 8.1 to 8.4 mg %, and allophycocyanin between 5.03 and 6 mg % in the indoor conditions. It yielded 3.6 to 7.7 mg % phycocyanin and 3 to 3.8 mg % allophycocyanin in the outdoor conditions.

2.7 Discussion

Cyanobacteria are considered to be important components of biofilms in tropical rockey shores (Nagarkar *et al.*, 2004) and shallow water ecosystems (Vincent, 2000). *Oscillatoria* and *Phormidium* are genera that are frequent in such bio-films. The samples collected for this study had abundant population of Cyanobacteria so that the three species could be isolated following standard methods. The present results have shown that *O. acuminata* grows well in BG-11 medium while *O. subbrevis* and *P. tenue* prefers higher alkalinity and pH.

High alkalinity is mandatory for the growth of *Spirulina* and bicarbonate is used to maintain pH (Venkataraman and Becker, 1985). Rafiqul *et al.* (2005) showed that pH requirement of *Spirulina platensis* was 9 whereas that of *Spirulina fusiformis* was pH 10. Eventhough the three species showed growth at different pH conditions, the species has its own preference for better yield. *O. acuminata* preferred only pH 7.5 for its better biomass production whereas *O. subbrevis preferred* pH 9.5 to 10.5 for its better growth. Even though *P. tenue* yielded more at pH 10.5, there was no significant difference from the yield at pH 9.5, 8.5 and 7.5. This indicates that the species tolerates a wide pH range.

Studies on the salinity tolerance revealed that *O. subbrevis* can grow well at 5- 15 x 10^{-3} and *P. tenue* at 5x 10^{-3} salinity; whereas *O. acuminata* preferred fresh water medium for its better growth. Sekar and Subramanian (1999) have shown the tolerance capacity of *P. tenue* even at high salinity conditions. Decrease in growth rate and enhancement of respiration are major physiological responses reported for halotolerant algae (Sallal *et al.*, 1990). Cyanobacteria are known to show marked morphological change under variable growth conditions including salinity (Mahasneh *et al.*, 1990). Decrease in pigmentation is another response to salt stress noted for Cyanobacteria (Vonshak *et al.*, 1996). Morphological variations observed in the Cyanobacteria strains were apparently an expression of the physiological stress to which the cells were subjected in cultures with higher salinity. This was also recognized by Kebede (1997) in *Spirulina platensis.* Aeration did not appear to be a suitable system to aerate filamentous Cyanobacterial cultures and agitation is considered with regard to its capacity to aerate the

culture and to maintain the filamentous algae in suspension without mechanical stress (Persoone *et al.*, 1980). Studies on effect of aeration showed that all the three species required only intermittent gentle spinning for its better yield. Pouliot (1989) reported that mass cultivation of *Phormidium sp.* requires gentle agitation using paddle wheels, where aeration is too expensive on a large-scale cultivation.

The specific growth rate of the three species studied were 0.36, 0.51 and 0.33 d^{-1} for *O. acuminata*, *O. subbrevis* and *P. tenue* respectively. According to Oliveira *et al.* (1999) *Spirulina platensis* has specific growth rate between 0.46 and 0.58 d^{-1} whereas that of *Spirulina maxima* is between 0.26 and 0.45 d^{-1} at 30-35^{0}C. *Anabaena* sp showed maximum growth rate of 0.35 d^{-1} in fresh water medium (Rai and Tiwari, 1999). The present results are in conformity with the earlier reports.

The mean chlorophyll *a* content of *O. acuminata*, *O. subbrevis* and *P. tenue* was 1.9, 1.59 and 0.83 mg % respectively. The phycocyanin was in the range 8-18 mg % for the three species. Sarada *et al.* (1999) showed that *Spirulina* sp possess 19.4 ± 0.4 mg % phycocyanin. Fatma *et al.* (1999) has reported 18.8 mg % phycocyanin in *Spirulina platensis*. Recently Shimamatsu (2004) has reported 1.2 % Chlorophyll *a* and 16.2 % phycocyanin in dry spirulina powder. The phycocyanin content was reported to vary with nitrogen concentration in the medium (Bousiba and Richmond, 1980) and 22 % of phycocyanin was reported by Proteus corporation (Fox, 1983). These results suggest that out of the three species investigated *O. acuminata* is a potential source for the production of pigments for commercial purpose.

The yield of biomass was highest for *O. subbrevis* both under indoor and outdoor cultivation. This species could produce 240 mg L^{-1} in indoor conditions with 10 mg L^{-1} initial inoculum size. Oliveira *et al.* (1999) revealed that *Spirulina* sp. could produce maximum 2.4 g L^{-1} with 0.1 g L^{-1} inoculum size. Costa *et al.* (2001) reported that maximum biomass yield for *Spirulina platensis* was 1.99 g l^{-1} for an initial concentration of 0.1 g L^{-1} in 28 days. *P. tenue* produced 80 mg L^{-1} in indoor conditions. *Phormidium bohneri* produced 0.7 g L^{-1} from an initial concentration of 0.1 g L^{-1} (Talbot and dela Noue, 1993). Kim *et al.* (2005) has obtained 1.9 g L^{-1} *Spirulina platensis* in a 5 L photobioreactor. Rafiqul *et al.* (2005) reported the biomass yield of 2.7 and 2.9 g L^{-1} for *Spirulina platensis* and *Spirulina fusiformis* respectively. Mass culturing of the species in the outdoor conditions resulted a little reduction in biomass yield and pigment production. This may be because of the environmental fluctuations like temperature, humidity etc. *O. subbrevis* yielded more allophycocyanin content in the outdoor conditions. Venkataraman and Becker (1985) has reported that reduction in *Spirulina* biomass in outdoor experiments is due to loss during harvesting operations, infection, temperature fluctuation, evaporation, mixing etc. Comparing with earlier results *O. subbrevis* is on par with *Spirulina* regarding the yield. *O. acuminata* is a better candidate for pigment production. *O. subbrevis* being more tolerant to salinity, pH and alkalinity may be considered to be the sturdy species, and hence suitable for cultivation under varied environmental conditions. The yield and pigment content of *P. tenue* is significantly low compared to the two species of *Oscillatoria.*

3

CHAPTER

BIOCHEMICAL COMPOSITION

3.1 Introduction

Cyanobacteria are commercially exploited in several countries for their valuable constituents particularly for proteins, pigments and polyunsaturated fatty acids. *Spirulina* is known for its high protein content and quality, and is considered to be a rich source of single cell protein (Reed *et al.,* 1985). Studies conducted by Fatma *et al.* (1999) showed that *Spirulina* possess protein within a range of 45-70 %. Rafiqul *et al.* (2005) reported 61.8 % proteins in *Spirulina fusiformis.* Becker (1994) reported that *Anabaena* sp possess 43-56 % of protein. Nagarkar *et al.* (2004) reported 18.86 % proteins in *Lyngbya martensiana* and 70 % in *Spirulina subsala.* Algae accumulate a variety of carbohydrates as reserve or storage materials; these are often found in large amounts in the cell: the carbohydrates are polyglucans (starch), β-1,3-glucans, fructosans, inulin, sucrose, and poly hydroxy alcohols (Craigie, 1974). Oliveira *et al.*(1999) reported that carbohydrate may range from 10.58 to 19.93 mg % in *Spirulina.* Rafiqul *et al.* (2005) reported 18.2 % carbohydrate in *Spirulina fusiformis. Chlorella pyrenoidosa* has 57 % protein and 26 % carbohydrates (Aarson *et al.,* 1980). Nagarkar *et al.* (2004) reported 16.6 % carbohydrate in *Spirulina subsala* and 15.4 % in *Phormidium tenue.*

The average lipid content of algae varies between 1 and 40 % and under certain conditions; it may be as high as 85 % of the dry weight (Becker, 1994). Algal lipids are typically composed of glycerol, sugars or bases esterified to fatty acids having carbon numbers in the range of C_{12}-C_{22}. They may be saturated or unsaturated. Cyanobacteria especially filamentous species tend to have larger amounts of polyunsaturated fatty acids (Becker, 1994). The major

lipids of Cyanobacteria consist of glyco and sulfolipids as well as sterols, hydrocarbons, and fatty acids (Murata, 1987). Oliveira *et al.* (1999) revealed *Spirulina maxima* possesses 6.2 % lipids and Rafiqul *et al.* (2005) reported that *Spirulina platensis* possesses 7.4 % at 30^0C. Babadzhanov (2004) reported 14.3 % lipids in *Spirulina platensis*. *Scenedesmus* sp possesses 12 to 14 mg % lipids (Soeder, 1981). *Synechococcus* sp possesses 15% lipids (Trubachev *et al.*, 1976). Chu *et al.* (1995) reported that *Ankistrodesmus convolutes* possesses 19.3 to 25 % lipids, 14.72 to 20 % carbohydrates and 17.2 to 21 % proteins. The experiments conducted by Zhu *et al.* (1997) suggested that carbohydrate, protein and lipid content varied with the incubation temperature. *Isochrysis* sp has been widely used as a mariculture feed due to its high content of long chain polyunsaturated fatty acids (Jeffery *et al.*, 1994).

Some polyunsaturated fatty acids (PUFA) are considered to be essential fatty acids (EFA), since; they cannot be synthesized in the human body. Absence of EFA cause schizophrenia (Hollman, 1966), thrombosis (Gorman, 1979), arthritis and cerebro and cardiovascular diseases. In view of its therapeutic uses EFA has gained commercial interest. Certain species of Cyanobacteria are rich in polyunsaturated fatty acids. The fatty acid profiles and lipid content vary between strains of *Isochrysis* (Lopez *et al.*, 1992) and their biochemical composition change in response to environmental factors such as irradiance (Brown *et al.*, 1993), nutrient concentration (Fabregas *et al.*, 1996), preservation procedures, growth rate and temperature (Grima *et al.*,1994). *Spirulina* contains very low level of lipids but is still important in view of the polyunsaturated fattyacids. *Spirulina maxima* contain only 6.8 % lipids (Fatma

et al., 1999). Studies conducted by Oren *et al.* (1985) revealed that *Oscillatoria limnetica* possesses 43.3 % palmitic acid, 16.1 % palmito oleic acid and 35.8 % oleic acid. *Phormidium* sp possesses 42.8 % palmitic acid and 38.5 % oleic acid. Fatma *et al.* (1999) reported that *Spirulina platensis* possesses 28.35 % palmitic acid and 20.5 % oleic acid, and *Spirulina maxima* has 24.08 % oleic acid and 20.4 % linolenic acid of the total fatty acid present. Eicosapentaenoic acid is a polyunsaturated fatty acid that plays an important role in the regulation of biological functions and prevention and treatment of a number of human diseases such as heart and inflammatory diseases. Microalgae contain large quantities of high-quality EPA and they are considered a potential source of this important fatty acid (Wen and Chen, 2003). Eicosapentaenoic acid content of *Isochrysis galbana* possesses 27.66 % of total fatty acid present (Fidalgo, 1998). Suda *et al.* (1998) reported 10.1 % linolenic acid in *Lyngbya hieronymusii* var. and 22 % myristic acid. The ash content in algae is an important criteria for their use as food. Studies conducted by Fatma *et al.* (1999) have obtained 19.2 mg for *Spirulina laxissima*, and 15.4 mg for *Spirulina maxima*; Shimamatsu (2004) reported 7.7 mg % in food grade *Spirulina*. Bureau of Indian Standards has set up a maximum level of 9% for *Spirulina* to be marketed in food grade (IS 12895: 1990).

The photosynthetic pigments of Cyanobacteria are located in thylakoids that lie free in the cytoplasm near the cell periphery. Cell colors vary from blue-green to violet-red. The green of chlorophyll *a* is usually masked by carotenoids (e.g.beta-carotene) and accessory pigments such as phycocyanin, allophycocyanin and phycoerythrin. The pigments are embodied in

phycobilisomes, which are found in rows on the outer surface of the thylakoids All Cyanobacteria contain chlorophyll *a* and phycobiliproteins. Phycobiliproteins are water soluble and stable in solution or as solids. They are classified on the basis of their colour into two large groups, the phycoerythrins (red) and the phycocyanins (blue). The subdivisions of phycocyanin are C-phycocyanin, R-phycocyanin and Allo-phycocyanin. R-phycocyanin is common in red algae whereas other two are present in cyanophyceae. Fatma *et al.* (1999) showed that *Spirulina platensis* has 18.88 % phycocyanin.

Phycocyanin is a blue colored, fluorescent pigment having absorption maxima at 615- 620 nm. Phycocyanin is stable over a pH range of 5-7.5 at 9 ± 1^0C, whereas temperature beyond 40^0C led to instability (Sarada *et al.*, 1999) Phycocyanin obtained from *Spirulina* (*Arthrospira*) has been used for a long time as a natural colorant for food and cosmetics (Chen *et al.*, 1996; Li and Qi, 1997; Liang, 2004). Recently there has been a growing interest and increasing evidence on potentially important physiological functions of phycocyanin, such as antioxidant activity (Romay *et al.*, 1998; Hirata *et al.*, 2000), anti-inflammatory activity (Romay *et al.*, 1998; Gonzalez *et al.*, 1999) and others (Belay *et al.*, 1993; Liu *et al.*, 2000). The commercial use of phycocyanin is expected to increase markedly in the future for pharmaceuticals and coloring additives (Subhashini *et al.*, 2004).

In view of potential applications of fine chemicals of biological origin, the newly developed cultures of Cyanobacteria were analyzed for biochemical composition. The first part of this chapter deals with the estimation of

proteins, carbohydrates, lipids and chlorophyll *a* and then proceeds to the quantification of biochemical composition in wet and dry biomass. Effect of pH on chlorophyll content of the three species and effect of salinity on chlorophyll were also analyzed. The second part deals with the effect of pH and salinity on phycobiliproteins, selection of species for phycocyanin production, and screening of buffers for maximum extraction of phycobiliproteins.

3.2 Materials and Methods

The three species of Cyanobacteria were grown in the laboratory in the respective media under 2000 lux light intensity from day light fluorescent lamps on a 12:12 light-dark cycle in batch culture in replicates. The algae were harvested on the 12^{th} day of inoculation. Further harvesting was done at four-day intervals up to 36 days for determining the biochemical composition.

3.2.1 Estimation of moisture and ash content

The harvested biomass were washed with distilled water, placed over a filter paper to blot the water, and transferred to pre- weighed watch glass. The wet weight was determined. It was dried overnight at 60^0C, cooled in desiccator and weighed to obtain the dry weight. The percent moisture content was determined on wet weight basis. The dried samples were placed in pre-weighed silica crucibles and ashed at 550 ^{0}C for 1 h in a muffle furnace. The weight of the ash was determined and expressed as percentage of dry weight.

3.2.2 Estimation of proteins

The harvested biomass were washed with distilled water, and 50 mg wet biomass was homogenized with 10% TCA. The contents were centrifuged,

and the resulting pellet dissolved in 1N NaOH. The protein content was estimated according to Lowry *et al.* (1951) and expressed as mg %.

The dry biomass of the 12th day harvest was digested, and the content of total N_2 was determined according to the kjeldahl method (AOAC, 1995). Protein content was calculated using a factor of 6.25.

3.2.3 Estimation of Carbohydrates

The carbohydrate content was determined by the anthrone method (Hedge and Hotreiter, 1962). 50mg of the wet biomass was washed with distilled water and ground with 2.5 N HCl using a mortar & pestle. Contents were transferred into a boiling tube, kept in boiling water bath for three hours, and cooled to room temperature. It was neutralized with solid sodium carbonate until the effervescence ceased, and made up to 10 ml. The sample was centrifuged; 4 ml of anthrone reagent was added to 1 ml of the extract and heated for 8 minutes in a boiling water bath. It was cooled rapidly and the absorbance was measured at 630 nm. The analysis was carried out on the dry material also.

3.2.4 Estimation of lipids

50mg of wet biomass was taken and washed well with distilled water. The wet material was ground with glass powder and extracted with chloroform-methanol (2:1). 2 ml of 0.9% aqueous sodium chloride solution was added and allowed it to stand overnight at 4°C to obtain a clear biphasic layer. The lower phase containing the lipids was drawn out and adjusted to 10 ml by the addition of chloroform. 1 ml of this extract was taken in a clean dry test tube and dried in a vacuum desiccator over silica gel. This was dissolved in 1 ml of concentrated

sulfuric acid. The lipid content of this was estimated by the sulpho-phosphovanillin method (Barnes and Blackstock, 1973). The procedure was repeated using the dried algal powder on the 12^{th} day of inoculation.

3.2.5 Fatty acid analysis

The dried algae were extracted in chloroform: methanol (2:1). A known volume of extract was saponified and fatty acid methyl ester were produced followed by extraction of fatty acids in1:1 mixture of hexane and anhydrous diethyl ether (Miller & Berger, 1985). The organic extract was transferred to the GC vials. The gas chromatograph was run in the following conditions.

Column	DEGS (Diethylene glycolsuccinate)
Oven temp	180^0C (Isothermal)
Injection port temperature	200^0C
Detector	FID (Flame Ionization Detector)
Detector temp	210^0C
Carrier gas	Nitrogen, 30 ml min^{-1}

The individual fatty acids were identified and quantified by using the FAME standards under similar conditions. The results were expressed in mg %of lipid.

3.2.6 Chlorophyll production and age of culture

The algae were harvested and washed twice with distilled water. The chlorophyll was extracted in 90 % acetone and the absorbance of the clear extract was measured at 750, 663, and 645 nm. The amount of chlorophyll *a* per unit biomass was calculated following the relation given by Becker (1994)

Chlorophyll *a* = (12.7 x A_{663}) – (2.69 x A_{645}) mg L^{-1}

The amount of chlorophyll *a* was estimated through different stages of progression of the culture from the 10^{th} to 36^{th} day.

3.2.7 Effect of pH on chlorophyll and phycobiliproteins

The growth media of the three species of Cyanobacteria were modified to pH range of 7.5- 11.5. 100 ml each of the test media were taken in conical flasks in triplicate and inoculated with 5 mg dry weight of the algae. The cultures were incubated under fluorescent light assembly as in previous experiments. The chlorophyll content and phycobiliproteins of the three species were determined on 12^{th} day. The effect of pH on chlorophyll and phycobiliproteins were elucidated through statistical analysis.

3.2.8 Effect of Salinity on chlorophyll and phycobiliproteins

The culture medium was modified by adding crude sea salt to a salinity range of 5 to 35 x 10^{-3} The test media taken in conical flasks were inoculated with equal quantities of the three species, and incubated for 12 days. The chlorophyll and phycobiliproteins content of the three species were examined on 12^{th} day and the effect of salinity on chlorophyll and phycobiliproteins were elucidated through statistical analysis.

3.2.9 Augmentation of phycobiliprotein extraction

Effect of Extraction medium

Phycobiliproteins were extracted from wet biomass of *O. acuminata* in distilled water and various buffers. The buffers tested were sodium phosphate, Potassium phosphate, Ammonium acetate, Potassium chloride, Calcium

chloride, Sodium chloride and distilled water. The color of the extract was noticed, and the extract was scanned using spectrophotometer to detect the λ_{max}. Quantitative estimation of the blue colored extracts was done and based on this the buffer for the best extraction of the phycocyanin and allophycocyanin was selected.

Extraction of wet and dry biomass

Replicate samples of *O. acuminata* were weighed and dried in an oven at 60^0C overnight. The wet and dried biomass was ground well with distilled water and it was placed inside the freezer. The contents were thawed at 4^0C and the amount of phycobiliproteins was examined. The wet samples were sun dried for 1-5 h and phycobiliproteins were determined at hourly intervals.

3.3 Results

3.3.1 Moisture and Ash content

The mean moisture content of the three species of Cyanobacteria ranged from 89.71 to 91.96 % (Table 3.1)

Table 3.1 Percentage moisture and percentage ash weight of *Oscillatoria acuminata,Oscillatoria subbrevis* and *Phormidium tenue*

Isolate	% moisture	% Ash weight
O. acuminate	91.96 ± 0.71	8.3±0.45
O. subbrevis	90.23 ±1.59	11.6 ± 1.14
P. tenue	89.71 ± 1.49	12.6 ±1.04

The mean ash weight of *O. acuminata* was 8.3 %. *O. subbrevis* 11.6 % and *P. tenue* 12.6 %.

3.3.2 Protein, Carbohydrate and lipid content

Oscillatoria acuminata

Protein content of this species showed an increasing tendency up to 16th day and then it was decreasing up to 36 day. Maximum protein was obtained on 16th day i.e. 56.28 mg % (Figure 3.1). Maximum carbohydrate was obtained on 16th day ie.14.45 mg %, and with the age of the culture, the carbohydrate level decreased gradually.

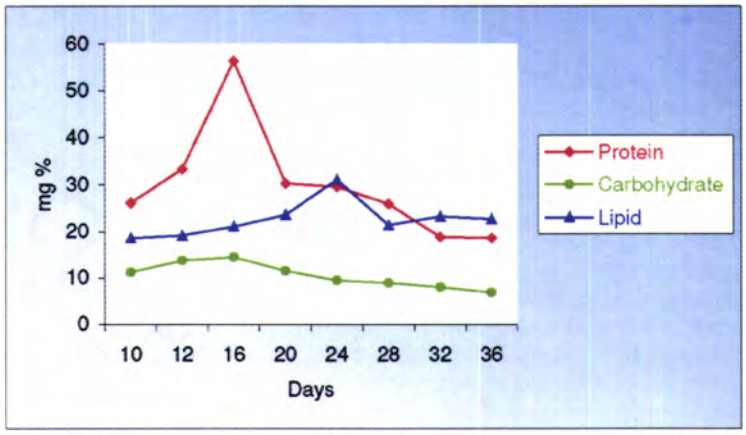


Figure 3.1 Protein, carbohydrate and lipid content of *Oscillatoria acuminata* at different time intervals

Lipid content showed an increasing tendency up to 24th day. The maximum yield of lipid was 31 mg %.

Oscillatoria subbrevis

The highest protein recorded was on 16th day ie. 34 mg %. The carbohydrate level was nearly constant during the first twenty days of culture growth with a maximum content of 22.7 mg %. Beyond twenty days, the protein and carbohydate content decreased and lipid content increased (Figure3.2). The maximum lipid estimated was 40.9 mg % on 28th day.

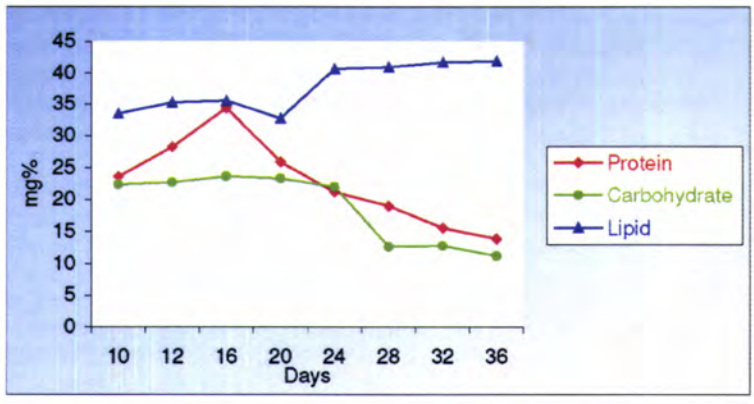


Figure 3.2 Protein, carbohydrate and lipid content of *Oscillatoria subbrevis* at different time intervals

Phormidium tenue

Protein showed an increasing tendency up to 16th day and later had decline.

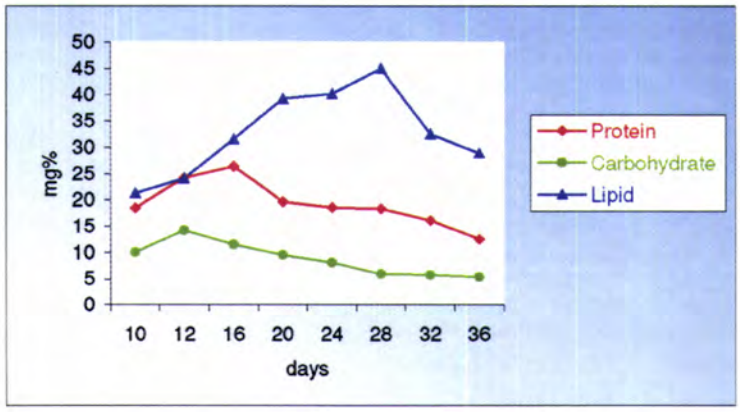


Figure 3.3 Protein, carbohydrate and lipid content of *Phormidium tenue* at different time intervals

26.3 mg % protein content was recorded on 16th day. Carbohydrate increased upto 12th day. Lipid content steadily increased up to 44 mg % on 28th day (Figure 3.3).

Biochemical composition of the three Cyanobacterial species from12th day wet and dry biomass is given in the Table 3.2. The dry biomass yielded higher

amount of protein than the wet biomass of all the three species; while wet biomass had better extraction of lipids. Elution of carbohydrates was higher in dry biomass for *O. subbrevis* and *P. tenue* (Table 3.2).

Table 3.2 Biochemical composition of three species of Cyanobacteria from 12th day wet and dry biomass

Parameters	*O. acuminata*		*O. subbrevis*		*P. tenue*	
	Wet biomass	Dry biomass	Wet biomass	Dry biomass	Wet biomass	Dry biomass
Protein (mg %)	33.29 ±5.8	58.5 ±6.02	28.6 ± 3.5	35.5 ± 3.71	24.16 ± 1.6	26.5 ± 6.02
Carbohydrate (mg %)	13.68 ± 4.3	10.87 ±1.27	22.68 ± 3.8	29.25 ±2.12	14.19 ± 2.9	16.68 ± 4.08
Lipid (mg %)	19.24 ± 6.2	18.82 ± 1.18	35.22 ± 4.5	31.52 ±1.9	24.02 ± 3.1	19.27 ± 1.49

Mean percentages of carbohydrate, protein, lipid and ash content of three species from the 12th day dry biomass are given in the figure (3.4).

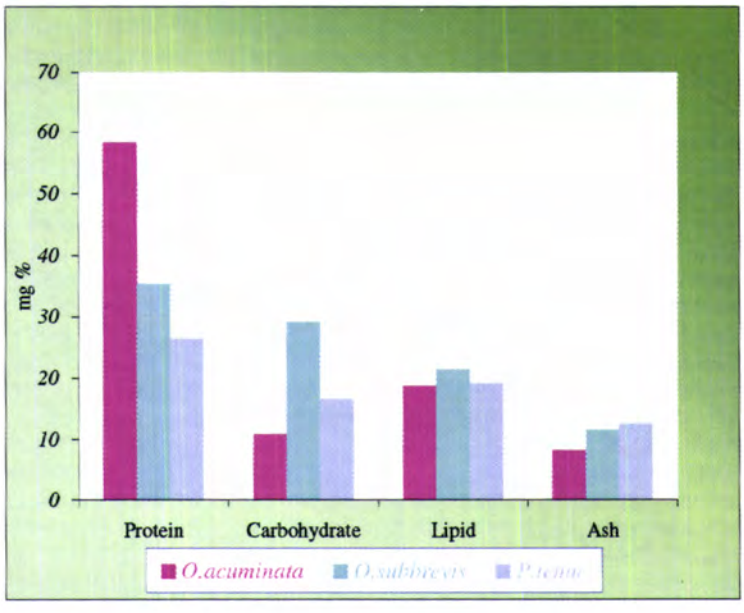


Figure 3.4 Mean mg % of protein, carbohydrate and lipid of *Oscillatoria acuminata*, *Oscillatoria subbrevis* and *Phormidium tenue* from 12th day dry biomass.

3.3.3 Fatty acid profile

Fatty acid composition of the three-Cyanobacterial species is shown in Table 3.3. *O. acuminata* has twelve different kinds of fatty acids. Proportion of unsaturated to saturated fatty acids is 0.81. Palmitic acid (16:0) is the major component of the fatty acids. The species possesses 7.72 % eicosapentaenoic acid 5.33 % linoleic acid.

Table 3.3 Fatty acid composition of *Oscillatoria acuminata, Oscillatoria subbrevis and Phormidium tenue*

Sl.No.	Name of the fatty acids	mg % of lipid		
		O. acuminata	*O. subbrevis*	*P. tenue*
1.	12:0	2.05	10.81	3.94
2.	14:0	7.47	8.59	64.87
3.	15:0			0.290
4.	16:0	17.00	31.92	38.51
5.	21:0	8.35		0.27
6.	26:0	0.04		
7.	16:1	6.52	26.25	16.69
8.	18:1(trans)	0.24		1.57
9.	18:1(cis)	4.80	19.79	12.39
10.	18:2	5.33	18.35	42.16
11.	20:1	0.29		48.27
12.	20:5	7.72		
13.	24:1	3.31		
UFA/ SFA		0.81	1.5	1.12

O. subbrevis has six different kinds of fatty acids. The proportion of unsaturated to saturated fatty acids is 1.5. The major fatty acid present is palmitic acid (31.92 % of total lipid). The species possess 3 different kinds of unsaturated fatty acids i.e. 26.25 % palmitooleic acid, 19.79 % oleic acid and 18.35 % linoleic acid. *P. tenue* has ten different kinds of fatty acids of which five are unsaturated. The proportion of unsaturated to saturated fatty acids is

1.12. Myristic acid is found to be the major fatty acid in this species (64.87 % of lipid). It also contains 38.51 % palmitic acid. Among the unsaturated fatty acids eicosenioc acid (48.27 %) and linoleic acid (42.16 %) dominates.

3.3.4 Pigment Production

Chlorophyll production and age of culture

The amount of chlorophyll *a* of *O. acuminata* per unit weight increased till sixteen days of culture and further declined gradually. Similar trend was shown by the other two species as well (Figure 3.5).

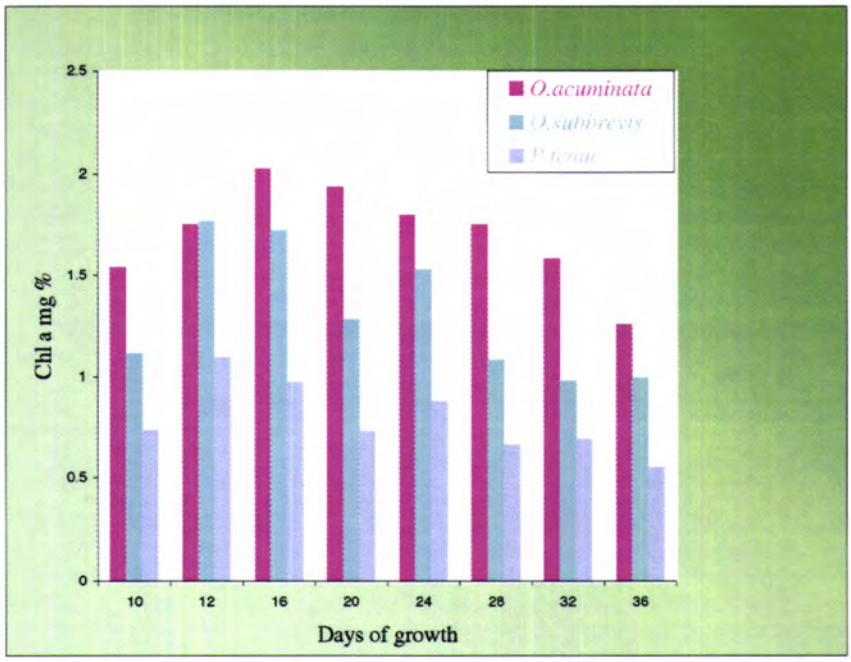


Figure 3.5 Chlorophyll content of *Oscillatoria acuminata*, *Oscillatoria subbrevis*, and *Phormidium tenue* at different time intervals.

The maximum yields of chlorophyll *a* was 2.03 mg % for *O. acuminata*, 1.77 mg % for *O. subbrevis* and 1.09 mg % for *P. tenue*.

Effect of pH on chlorophyll

The pH of the growth medium affected the production of chlorophyll significantly. The chlorophyll content of *O. acuminata* decreased successively with the rise in pH (Figure 3.6)

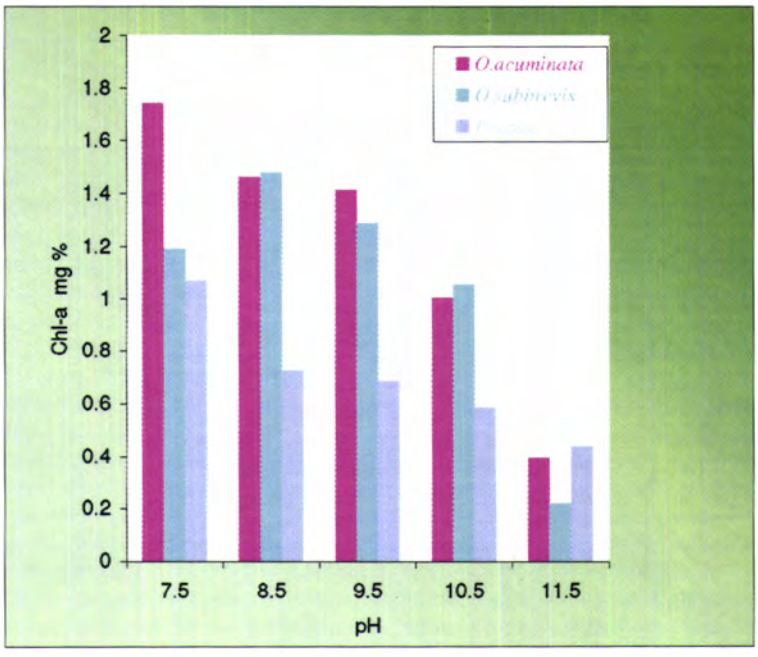


Figure 3.6 Effect of pH on chlorophyll content of *Oscillatoria acuminata*, *Oscillatoria subbrevis* and *Phormidium tenue*

The amount of chlorophyll *a* was estimated to range from 1.75 mg % to 0.40 mg % in the pH range 7.5 to 11.5. Analysis of variance combined with Tukey method of multiple comparisons showed marked significant difference between each pH levels (Table 3.4).

Table 3.4 Analysis of variance and Tukey method of effect of pH on chlorophyll content of *Oscillatoria acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	3.2930	0.8233	8233.000
Within (Error)	10	0.0011	0.0001	
Total	14	3.2942		

Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal			
TUKEY method of multiple comparisons			
Group	pH	Mean	5 4 3 2 1
5	11.5	0.397	\
4	10.5	1.007	* \
3	9.5	1.417	* * \
2	8.5	1.463	* * * \
1	7.5	1.747	* * * * \
* = significant difference (p=0.05) . = no significant difference			

O. subbrevis cultures produced 1.19 mg % Chlorophyll *a* at pH 7.5 and 1.29 mg % at pH 9.5. Further increase in pH seemed to reduce Chlorophyll *a* production (Figure 3.6). Every pair of test pH levels had significant effect as revealed by Tukey analysis (Table 3.5).

Table 3.5 Analysis of variance and Tukey method of effect of pH on chlorophyll content of *Oscillatoria subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	2.8608	0.7152	3576.000
Within (Error)	10	0.0018	0.0002	
Total	14	2.8626		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	pH	Mean	5 4 1 3 2	
5	11.5	0.220	\	
4	10.5	1.057	* \	
1	7.5	1.193	* * \	
3	9.5	1.293	* * * \	
2	8.5	1.480	* * * * \	
* = significant difference (p=0.05) . = no significant difference				

P. tenue yielded 1.07 mg % Chlorophyll *a* at pH 7.5. At pH 8.5, the Chlorophyll *a* decreased significantly to 0.73 mg %. The yield at pH 9.5 was 0.69 mg %, which is statistically insignificant from pH 8.5 (Table 3.6).

Table 3.6 Analysis of variance and Tukey method of effect of pH on chlorophyll content of *Phormidium tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	0.6496	0.1624	541.333
Within (Error)	10	0.0029	0.0003	
Total	14	0.6526		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	pH	Mean	5 4 3 2 1	
5	11.5	0.443	\	
4	10.5	0.587	* \	
3	9.5	0.690	* * \	
2	8.5	0.730	* * \	
1	7.5	1.070	* * * * \	
* = significant difference (p=0.05) . = no significant difference				

At pH > 9.5 there was significant reduction of Chloriphyll *a* content (Figure 3.6).

➢ ***Effect of pH on phycocyanin***

The pH of the growth medium affected the production of phycocyanin significantly. The phycocyanin content of *O. acuminata* decreased successively with the rise in pH (Figure 3.7)

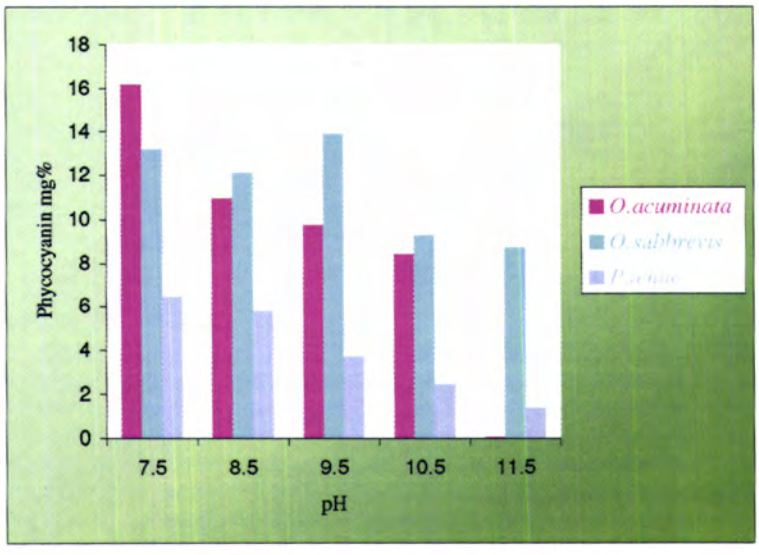


Figure 3.7 Effect of pH on phycocyanin content of *Oscillatoria acuminata*, *Oscillatoria subbrevis*, and *Phormidium tenue*

The amount of phycocyanin was estimated to range from 16.18 mg % to 0.063mg % in the pH range 7.5 to 11.5. Analysis of variance combined with Tukey method of multiple comparisons showed significant difference between each pH levels (Table 3.7)

Table 3.7 Analysis of variance and Tukey method of effect of pH on phycocyanin content of *Oscillatoria acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	408.720	102.180	6812.00
Within (Error)	10	0.147	0.015	
Total	14	408.867		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
GROUP	P^H	Mean	5 4 3 2 1	
5	11.5	0.063	\	
4	10.5	8.420	* \	
3	9.5	9.743	* * \	
2	8.5	10.967	* * * \	
1	7.5	16.187	* * * * \	
* = significant difference (p=0.05) . = no significant difference				

O. subbrevis produced 13.86 mg % phycocyanin at pH 9.5. The yield at 7.5 was 13.18 mg % and at 8.5 it was 12.08 mg %, which is statistically insignificant (Table 3.8).

Table 3.8 Analysis of variance and Tukey method of effect of pH on phycocyanin content of *Oscillatoria subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	64.777	16.194	43.416
Within (Error)	10	3.728	0.373	
Total	14	68.505		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				

TUKEY method of multiple comparisons			
Group	P^H	Mean	5 4 2 1 3
5	11.5	8.700	\
4	10.5	9.263	\
2	8.5	12.080	* * \
1	7.5	13.187	* * \
3	9.5	13.867	* * * \
* = significant difference (p=0.05)		. = no significant difference	

Further increase in pH seemed to reduce the phycocyanin production. (Figure 3.7). The species yielded only 8.7 mg % at pH 11.5 and 9.26 mg % at 10.5. *P. tenue* yielded 6.45 mg % phycocyanin at pH 7.5. Further increase in pH seemed to reduce phycocyanin production. Every pair of test pH levels had significant effect as revealed by Tukey analysis (Table 3.9).

Table 3.9 Analysis of variance and Tukey method of effect of pH on phycocyanin content of *P. tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	56.556	14.139	883.688
Within (Error)	10	0.157	0.016	
Total	14	56.712		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	P^H	Mean	5 4 3 2 1	
5	11.5	1.320	\	
4	10.5	2.433	* \	
3	9.5	3.687	* * \	
2	8.5	5.767	* * * \	
1	7.5	6.453	* * * * \	

➢ ***Effect of pH on allophycocyanin***

The pH of the growth medium affected the production of allophycocyanin content significantly. The allophycocyanin content of *O. acuminata* decreased with the rise in pH (Figure 3.8).

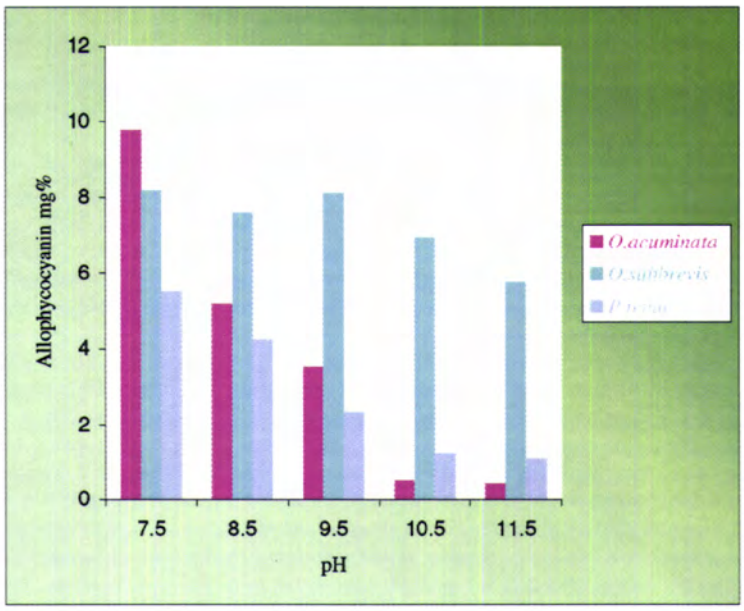


Figure 3.8 Effect of pH on allophycocyanin content of *Oscillatoria acuminata*, *Oscillatoria subbrevis*, and *Phormidium tenue*

The amount of allophycocyanin content ranged from 9.77 mg % to 0.41 mg % in the pH range 7.5 to 11.5 (Table 3.10).

Table 3.10 Analysis of variance and Tukey method of effect of pH on allophycocyanin content of *Oscillatoria acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	179.796	44.949	
Within (Error)	10	1.081	0.108	416.194
Total	14	180.877		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	pH	Mean	5 4 3 2 1	
5	11.5	0.413	\	
4	10.5	0.510	\	
3	9.5	3.523	* * \	
2	8.5	5.183	* * * \	
1	7.5	9.773	* * * * \	
* = significant difference (p=0.05) . = no significant difference				

O. subbrevis yielded similar quantities of allophycocyanin in the pH range 7.5 to 9.5 with a mean value of 7.98 mg %. Above this pH, there was drastic reduction in the production of this pigment (Table 3.11).

Table 3.11 Analysis of variance and Tukey method of effect of pH on allophycocyanin content of *Oscillatoria subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	12.173	3.043	15.849
Within (Error)	10	1.921	0.192	
Total	14	14.095		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho: All groups equal				

TUKEY method of multiple comparisons			
Group	pH	Mean	5 4 2 3 1
5	11.5	5.767	\
4	10.5	6.960	* \
2	8.5	7.610	* \
3	9.5	8.120	* \
1	7.5	8.210	* * \
* = significant difference (p=0.05)		. = no significant difference	

The allophycocyanin content of *P. tenue* decreased successively with the rise in pH (Figure 3.8). The amount of allophycocyanin ranged from 5.51 mg % to 1.08 mg %in the pH range 7.5 to 11.5 (Table 3.12).

Table 3.12 Analysis of variance and Tukey method of effect of pH on allophycocyanin content of *P. tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	45.000	11.250	450.000
Within (Error)	10	0.252	0.025	
Total	14	45.252		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				

TUKEY method of multiple comparisons			
Group	pH	Mean	5 4 3 2 1
5	11.5	1.080	\
4	10.5	1.223	\
3	9.5	2.347	* * \
2	8.5	4.233	* * * \
1	7.5	5.507	* * * * \
* = significant difference (p=0.05)		. = no significant difference	

Analysis of variance combined with Tukey method of multiple comparisons showed significant difference between each pH levels.

➢ ***Effect of salinity on chlorophyll***

Salinity of the growth medium affected the production of Chlorophyll *a* significantly. The chlorophyll content of *O. acuminata* decreased successively with the rise of pH (Figure 3.9)

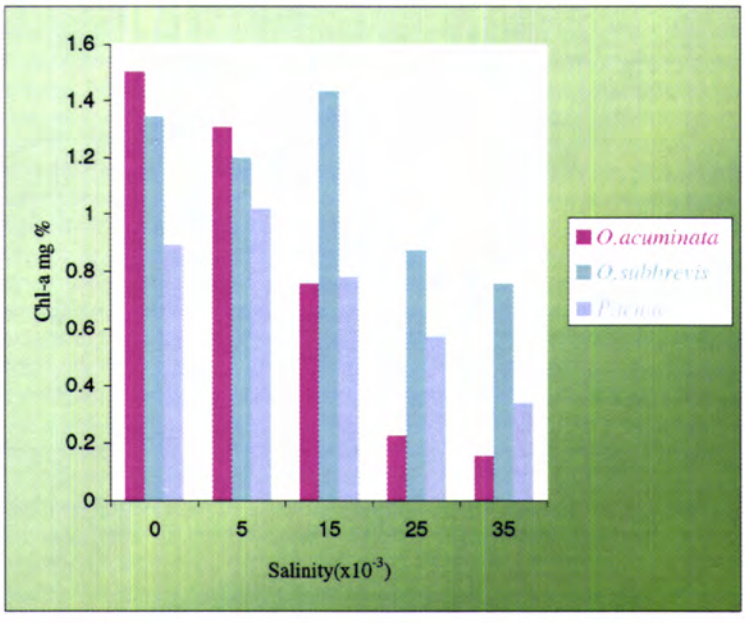


Figure 3.9 Effect of salinity on chlorophyll content of *Oscillatoria acuminata*, *Oscillatoria subbrevis*, and *Phormidium tenue*

The chlorophyll content of the species grown in non-saline medium was 1.5 mg % and at $5x10^{-3}$, it was 1.3 mg %, which is statistically insignificant (Table 3.13).

Table 3.13 Analysis of variance and Tukey method of effect of salinity on chlorophyll content of *Oscillatoria acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	4.496	1.124	102.182
Within (Error)	10	0.106	0.011	
Total	14	4.602		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity (x 10^{-3})	Mean	5 4 3 2 1	
5	35	0.157	\	
4	25	0.227	\	
3	15	0.760	* * \	
2	5	1.310	* * * \	
1	Control	1.503	* * * \	
* = significant difference (p=0.05) . = no significant difference				

Further up increasing salinity reduced the production of Chlorophyll *a*. *O. subbrevis* yielded highest chlorophyll at salinity $15x10^{-3}$ (Figure 3.8). At salinity levels 25 and $35x\ 10^{-3}$, the chlorophyll production was reduced. All salinity levels tested yielded significantly different quantities of Chlorophyll *a* (Table 3.14)

Table 3.14 Analysis of variance and Tukey method of effect of salinity on chlorophyll content of *Oscillatoria subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	1.0464	0.2616	436.000
Within (Error)	10	0.0061	0.0006	
Total	14	1.0524		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				

TUKEY method of multiple comparisons			
Group	Salinity (x 10^{-3})	Mean	5 4 2 1 3
5	35	0.757	\
4	25	0.873	* \
2	5	1.200	* * \
1	Control	1.347	* * * \
3	15	1.433	* * * * \
* = Significant difference (p=0.05)		. = no significant difference	

P. tenue yielded more chlorophyll at $5x10^{-3}$ than the fresh water medium (Figure 3.8). Each pair of test pH levels had significant effect as revealed by Tukey analysis (Table 3.15).

Table 3.15 Analysis of variance and Tukey method of effect of salinity on chlorophyll content of *Phormidium tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	0.8763	0.2191	243.444
Within (Error)	10	0.0086	0.0009	
Total	14	0.8849		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity (x 10^{-3})	Mean	5 4 3 1 2	
5	35	0.337	\	
4	25	0.573	* \	
3	15	0.780	* * \	
1	Control	0.893	* * * \	
2	5	1.020	* * * * \	
* = significant difference (p=0.05)		. = no significant difference		

➢ *Effect of salinity on phycocyanin*

The production of phycocyanin was considerably decreased by salinity of the growth medium. At $5x10^{-3}$, the phycocyanin production was ≈ 50 % of the control and at still higher salinities there was again drastic reduction (Figure 3.10)

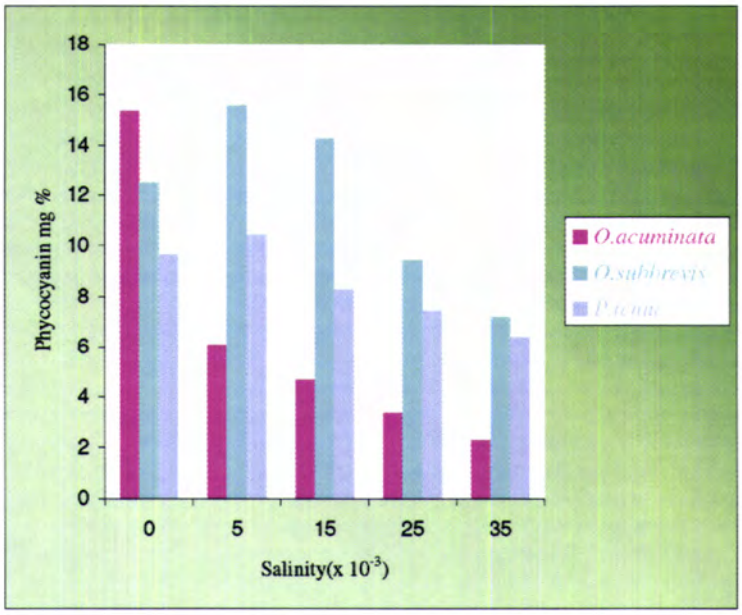


Figure 3.10 Effect of salinity on phycocyanin content of *Oscillatoria acuminata*,*Oscillatoria subbrevis*,and *Phormidium tenue*

At 25 x 10^{-3} it yielded 3.41 mg %, which is statistically insignificant from the yield at 35 x 10^{-3}. All other pair of test salinity levels had significant effect as revealed by Tukey analysis (Table 3.16).

Table 3.16 Analysis of variance and Tukey method of effect of salinity on phycocyanin content of *O. acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	328.298	82.075	383.528
Within (Error)	10	2.145	0.214	
Total	14	330.443		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity (x 10^{-3})	Mean	5 4 3 2 1	
5	35	2.303	\	
4	25	3.410	\	
3	15	4.690	* * \	
2	5	6.103	* * * \	
1	Control	15.383	* * * * \	
* = significant difference (p=0.05)		. = no significant difference		

The phycocyanin content of *O. subbrevis* increased at low salinity levels from 12.50 mg % in fresh water medium to 15.60 mg % in 5x 10^{-3} and 14.3 mg % at salinity 15x 10^{-3} There was no statistically significant difference between these (Table 3.17).

Table 3.17 Analysis of variance and Tukey method of effect of salinity on phycocyanin content of *O. subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	142.925	35.731	63.018
Within (Error)	10	5.668	0.567	
Total	14	148.593		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity (x 10^{-3})	Mean	5 4 1 3 2	
5	35	7.220	\	
4	25	9.453	* \	
1	Control	12.503	* * \	
3	15	14.300	* * \	
2	5	15.597	* * * \	
* = significant difference (p=0.05) . = no significant difference				

P. tenue showed maximum phycocyanin content at 5x10^{-3} (Figure 3.10). Further increase in salinity reduced phycocyanin production significantly (Table 3.18).

Table 3.18 Analysis of variance and Tukey method of effect of salinity on phycocyanin content of *P. tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	32.099	8.025	157.353
Within (Error)	10	0.507	0.051	
Total	14	32.606		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				

TUKEY method of multiple comparisons			
Group	Salinity (x 10^{-3})	Mean	5 4 3 1 2
5	35	6.400	\
4	25	7.467	* \
3	15	8.297	* * \
1	Control	9.667	* * * \
2	5	10.457	* * * * \
* = significant difference (p=0.05) . = no significant difference			

The species yielded 10.45 mg % at $5x10^{-3}$ levels but it could produce only 9.66mg % in fresh water medium, which is found to be statistically significant. The yield at $35x10^{-3}$ was 6.4 mg %. Every pair of test salinity level had significant effect as revealed by Tukey analysis.

➢ ***Effect of salinity on allophycocyanin***

Salinity of the growth medium affected the production of allophycocyanin content. *O. acuminata* showed a decrease in allophycocyanin content with the increase of salinity (Figure 3.11)

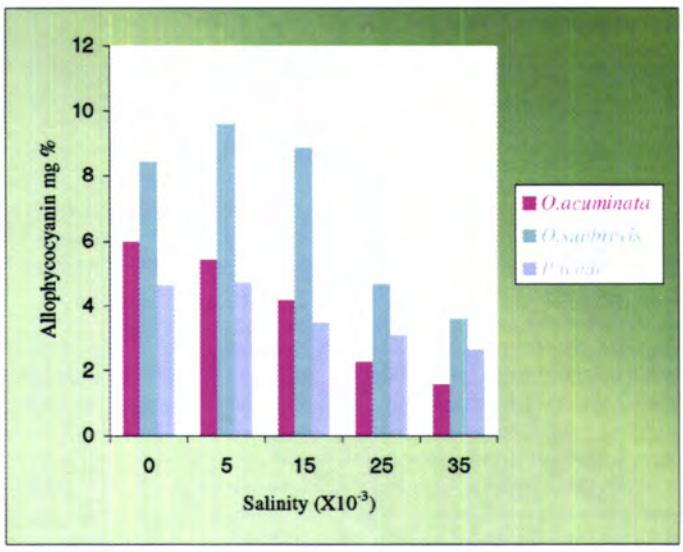


Figure 3.11 Effect of salinity on allophycocyanin content of *Oscillatoria acuminata*, *Oscillatoria subbrevis*, and *Phormidium tenue*

The amount of allophycocyanin was estimated to range from 5.4 to 1.57 mg % in the salinity range $5x10^{-3}$ to 35 x 10^{-3} Analysis of variance combined with Tukey method of multiple comparisons showed significant difference between each salinity levels (Table 3.19).

Table 3.19 Analysis of variance and Tukey method of effect of salinity on allophycocyanin content of *O. acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	45.013	11.253	3751.000
Within (Error)	10	0.033	0.003	
Total	14	45.046		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity (x 10^{-3})	Mean	5 4 3 2 1	
5	35	1.573	\	
4	25	2.240	* \	
3	15	4.163	* * \	
2	5	5.440	* * * \	
1	Control	5.997	* * * * \	
* = significant difference (p=0.05) . = no significant difference				

The allophycocyanin yield of *O. subbrevis* increased with salinity upto $15x10^{-3}$ followed by decrease (Figure 3.11). The species produced 9.59 mg % at $5x10^{-3}$ and 8.87 mg % at 15 x 10^{-3}, which is found to be statistically insignificant (Table 3.20).

Table 3.20 Analysis of variance and Tukey method of effect of salinity on allophycocyanin content of *O. subbrevis*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	87.129	21.782	279.256
Within (Error)	10	0.780	0.078	
Total	14	87.910		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				

TUKEY method of multiple comparisons			
Group	Salinity (x 10^{-3})	Mean	5 4 1 3 2
5	35	3.613	\
4	25	4.700	* \
1	Control	8.443	* * \
3	15	8.867	* * \
2	5	9.593	* * * \
* = significant difference (p=0.05) . = no significant difference			

P. tenue had similar allophycocyanin content in fresh water medium, and at $5x10^{-3}$ Further increase in salinity decreased allophycocyanin content significantly (Figure 3.11). At higher salinity levels there was significant reduction in the production of allophycocyanin (Table 3.21).

Table 3.21 Analysis of variance and Tukey method of effect of salinity on allophycocyanin content of *P. tenue*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	4	10.568	2.642	220.167
Within (Error)	10	0.123	0.012	
Total	14	10.691		
Critical F value = 3.48 (0.05,4,10) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Salinity (x 10^{-3})	Mean	5 4 3 1 2	
5	35	2.647	\	
4	25	3.097	* \	
3	15	3.477	* * \	
1	Control	4.667	* * * \	
2	5	4.733	* * * \	
* = significant difference (p=0.05) . = no significant difference				

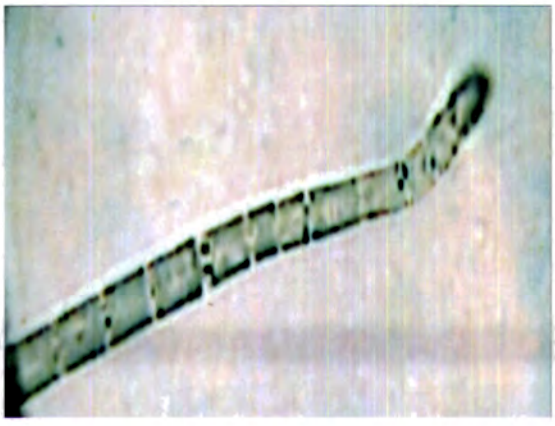
Microscopic observations of the three species grown in the different salinity conditions is given in the Plate IV, V and VI. The filaments were observed to be disintegrated and fragmented with dissolution of cell walls and leaching out of pigments at higher salinity conditions.

Plate IV

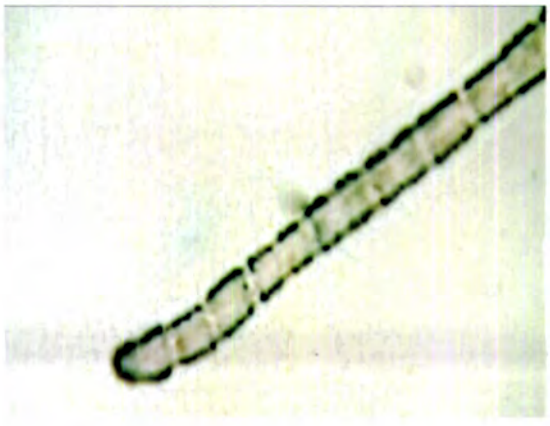
Effect of Salinity on Pigments of *Oscillatoria acuminata*

Control

5×10^{-3}



15×10^{-3}



25×10^{-3}

35×10^{-3}

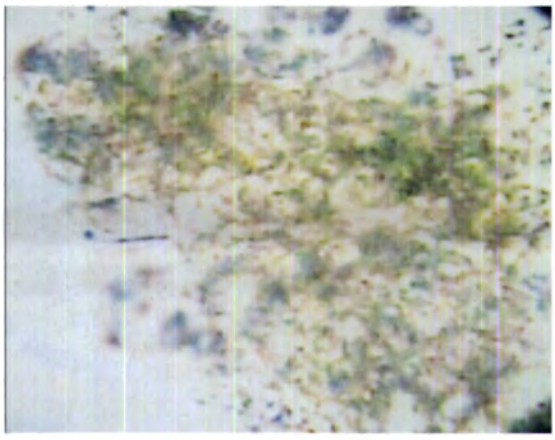
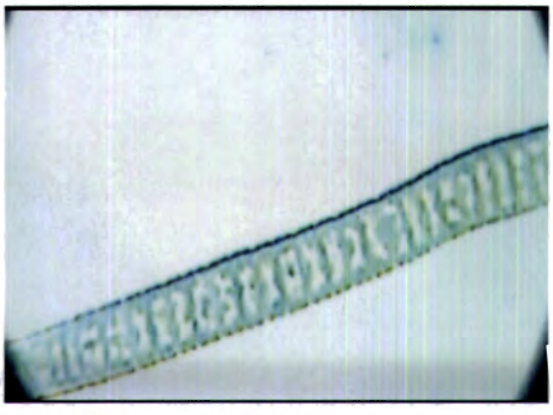


Plate V

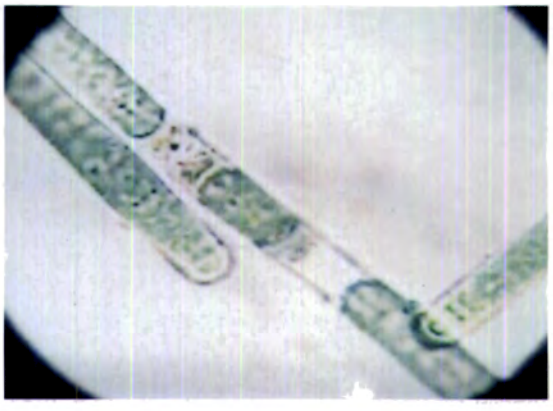
Effect of Salinity on Pigments of *Oscillatoria subbrevis*

Control

5×10^{-3}



15×10^{-3}



25×10^{-3}

35×10^{-3}

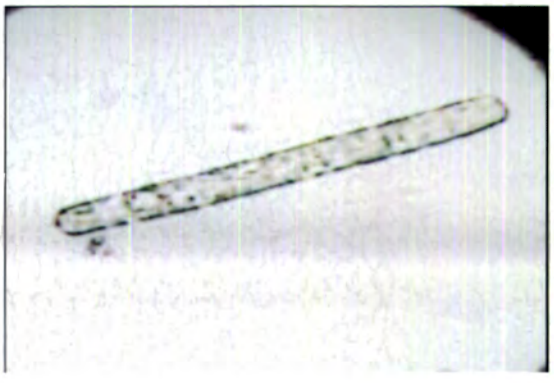
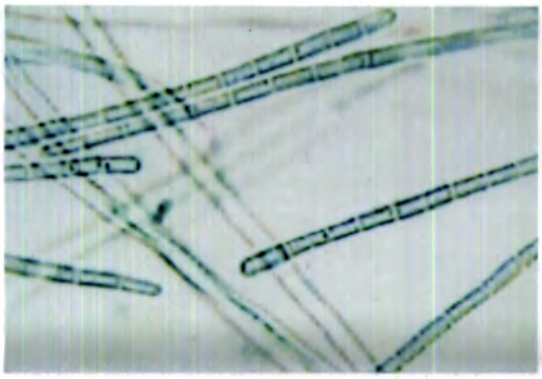


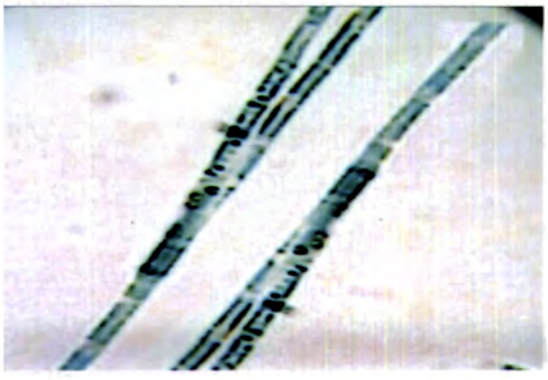
Plate VI

Effect of Salinity on Pigments of *Phormidium tenue*

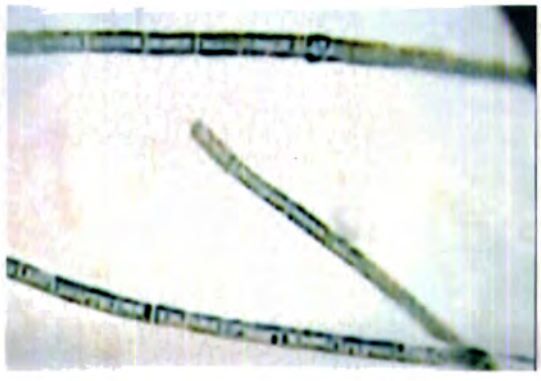
Control



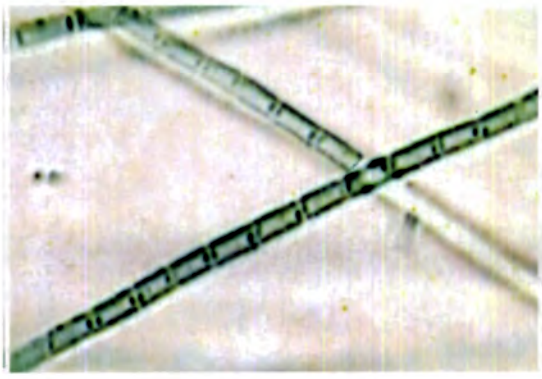
5 x 10^{-3}



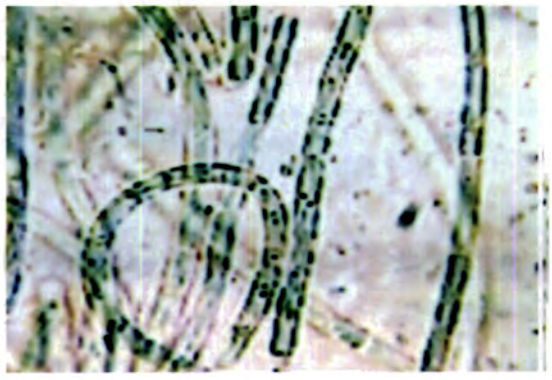
15 x 10^{-3}



25 x 10^{-3}



35 x 10^{-3}



3.3.5 Augmentation of phycobiliproteins extraction

Effect of extraction medium

The blue pigment phycocyanin was extracted in eight different media ranging from distilled water to various buffers (Table 3.22). The distilled water yielded a clear blue extract characteristic of phycocyanin with λmax 618 nm (Figure 3.12). Out of the six buffers tested, sodium phosphate, potassium phosphate, and ammonium acetate yielded blue coloured extract with λ_{max} at 618nm. The rest of the three buffers did not yield blue color extract. The measure of the absorbance of the extracts in distilled water, sodium phosphate, potassium phosphate, and ammonium acetate led to the yield estimate of phycocyanin as 23.81, 19.40, 19.15 and 17.63 mg % respectively. Anova proved distilled water to be the medium for maximum extraction of phycocyanin and allophycocyanin from *O. acuminata* (Table 3.23 and 3.24)

Table 3.22 Effect of different buffers on phycocyanin release in *Oscillatoria acuminata*

Extraction medium	Colour of Extract	Absorbance Peak (λ)
Sodium phosphate	Blue	618
Potassium phosphate	Blue	618
Ammonium acetate	Blue	618
Potassium chloride	Pale green	589
Calcium Chloride	Colorless	651
Sodium Chloride	Blue green	588
Distilled Water	Blue	618
Distilled Water (pH-5)	Blue green	609

Table 3.23 Analysis of variance and Tukey method of the effect of different buffers on phycocyanin extraction in *O. acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	3	63.675	21.225	17.585
Within (Error)	8	9.658	1.207	
Total	11	73.333		
Critical F value = 4.07 (0.05,3,8) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Buffer	Mean	3 1 2 4	
3	Ammonium acetate	17.630	\	
1	Potassium phosphate	19.147	\	
2	Sodium phosphate	19.397	\	
4	Distilled water	23.810	* * * \	
* = significant difference (p=0.05) . = no significant difference				

Table 3.24 Analysis of variance and Tukey method of the effect of different buffers on Allophycocyanin extraction in *O. acuminata*

ANOVA TABLE				
SOURCE	DF	SS	MS	F
Between	3	26.857	8.952	23.313
Within (Error)	8	3.073	0.384	
Total	11	29.929		
Critical F value = 4.07 (0.05,3,8) Since F > Critical F REJECT Ho:All groups equal				
TUKEY method of multiple comparisons				
Group	Buffer	Mean	1 2 3 4	
1	Potassium phosphate	6.060	\	
2	Sodium phosphate	7.800	* \	
3	Ammonium acetate	8.820	* \	
4	Distilled water	10.157	* * \	
* = significant difference (p=0.05) . = no significant difference				

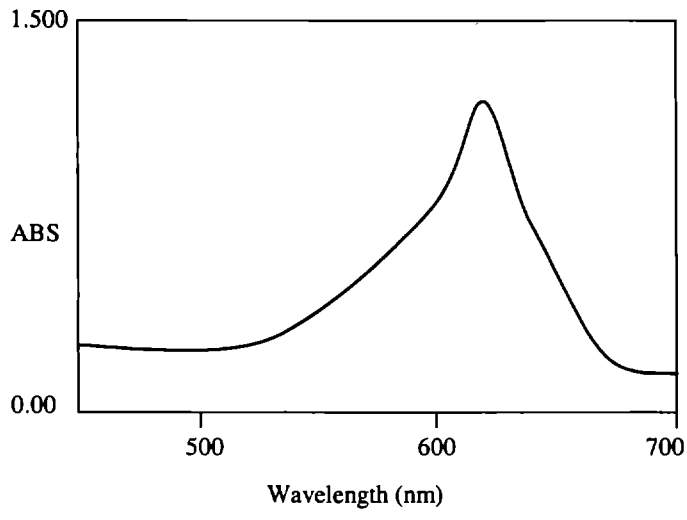


Figure. 3.12 Absorption spectrum of C-Phycocyanin from *Oscillatoria acuminata*

Effect of drying

Dryness of the biomass affected the yield of phycocyanin and allophycocyanin (Table 3.25). The wet biomass of *O. acuminata* yielded 26 mg % phycocyanin and 6.48 mg % allophycocyanin. Solar drying for one hour yielded 16.5 mg % phycocyanin and 5.6 mg % allophycocyanin.

Table 3.25 Effect of sundrying and oven drying on phycobiliproteins release in *Oscillatoria acuminata*

Parameter	Wet material	Solar drying					Oven drying 60^0C (Over night)
		1 h	2h	3h	4h	5h	
PC	26.3	16.5	12.03	9.1	8.3	8.1	3.6
APC	6.48	5.6	4.4	1.3	1.9	1.7	4.3

After 2 h of drying the phycocyanin was reduced to 12.03 mg % and allophycocyanin to 4.4 mg %. The yield of pigments decreased as drying period was prolonged. The oven-dried biomass had the lowest yield of phycocyanin, while the allophycocyanin was comparable to that of 2 h solar drying.

3.4 Discussion

The biochemical composition of algae varies with species, light, temperature and growth stage, which in turn is related to the culture age and nutrient depletion (Venkataraman and Becker, 1985). Biochemical composition of the three species currently isolated were analysed at different phases of growth. The three species have higher protein and carbohydrate in the early stages of culture and lipids accumulated as the culture aged. *O. acuminata* showed 31 % lipid on 24th day. *O. subbrevis* and *P. tenue* showed maximum 40 and 45 % respectively in the stationary phase. The present study clearly supports the observations by Zhu *et al.* (1997), they have obtained high protein content in the exponential phase and high lipid accumulation in stationary phase. Carbohydrate content was low compared to proteins and lipids in all the three species and it was decreasing after attaining maximum in the exponential phase. In photosynthetic microalgae, the biochemical composition of rapidly growing cells is generally characterized by a higher protein and low carbohydrate content and under growth limiting conditions, when cells have reached stationary growth phase more photo assimilated carbon is incorporated into carbohydrate or lipid (Aarson *et al.*, 1980).

Analysis of protein revealed that *O. acuminata* has high protein content ie. 58.5 % compared to other two species. Nagarkar *et al.* (2004) have reported 50.85 % in *Oscillatoria Formosa*. *Spirulina* is the commercially cultivated species having high protein content. Rafiqul *et al.*(2005) have reported 58.6 %, and Fatma *et al.* (1999) reported 55 % proteins in *Spirulina platensis*. Liang *et al.* (2004) reported that *Spirulina* possess 55-70 % proteins. This strongly

suggests that *O. acuminata* has a similar range of proteins when compared to *Spirulina*. *O. subbrevis* and *P. tenue* can be considered as better lipid accumulators as growth of the culture proceeds. The lipid content of *O. subbrevis* was 40.9 mg % and that of *P. tenue* 44 mg % upon growth for 28 days. Oliveira *et al.* (1999) reported that *Spirulina maxima* possesses 6.2 % lipids and Rafiqul *et al.* (2005) reported that *Spirulina platensis* possesses 7.4%. The present results showed that all the three species possess higher lipid content than recorded values. Babadzhanov (2004) reported 14.3 % lipids in *Spirulina platensis*. Chu *et al.* (1995) reported that *Ankistrodesmus convolutes* possesses 19.3 to 25 % lipids. Comparison of wet and dry powder for the analysis of proteins, carbohydrates and lipids showed that more protein was yielded from dry powder, whereas more lipid was obtained from wet biomass. Dry material gives better extraction of carbohydrate from *O. subbrevis*. The fatty acid profile of *O. subbrevis* promises higher nutritional quality with a proportion of unsaturated to saturated fatty acids of 1.5 to be clearly followed by *P. tenue* with a proportion of 1.12.

A survey of fatty acid composition of filamentous Cyanobacteria by Oren *et al.* (1985) revealed that many of them contain saturated and monounsaturated fatty acids only. The present study revealed the presence of polyunsaturated fatty acids like eicosapentaenoic acid (20:5) and linoleic acid (18:2). Eicosapentaenoic acid content of *Isochrysis galbana* ranged between 2.4 to 5.3 % (Otero, 1997). Polyunsaturated fatty acids like 20:4, 20:5 and 22:6 are of increasing interest since they have been demonstrated to have several biotechnological and pharmaceutical applications. The ash content in algae is an important criteria for their use as food. In the present study *O. acuminata*

has the lowest ash content of 8.3 %. *O. Subbrevis* has got 11.6 % whereas *P. tenue* has 12.6 %. The low ash content of *O. acuminata* which is within the stipulated standards of food grade *Spirulina* and the high protein content are commendable properties for its potential use as a source of protein in diet.

The yield of chlorophyll *a* is 2.03 % from *O. acuminata*, 1.77 % from *O. subbrevis* and 1.09 % from *P. tenue*. The corresponding phycobiliproteins yield (PC+APC) is 25.95, 19.37 and 11.95 % respectively. When the three species are compared, *O. acuminata* is again the better isolate for the production of pigments. The yield of pigments is comparable or rather better than the reported results of *Spirulina*. Pigment composition of algae also varies with nutrients, culture age, pH and temperature. Studies on effect of pH on phycocyanin showed that *O. acuminata* and *P. tenue* requires pH 7.5 for maximum production of chlorophyll and phycobiliproteins, whereas *O. subbrevis* prefers pH 8.5 to 9.5 for the better production of these pigments. Salinity has great effect on chlorophyll and phycocyanin. *O. acuminata* showed reduction in pigment content with the increase of salinity and phycocyanin leaching was observed at 5 $x10^{-3}$ and 15 $x10^{-3}$ Failure of the organisms to tolerate higher salinities was also evidenced by a rapid drop in the pigments.

O. subbrevis had high pigment production at salinity 15 $x10^{-3}$. At higher levels of salinity morphological deformities and pigment leaching occurred. *P. tenue* could tolerate upto 25 x 10^{-3} The present observation agrees well with the former reports. Decrease in growth rate and enhancement of

respiration are major physiological responses reported for halotolerant algae (Sallal *et al.*, 1990). Cyanobacteria are also known to show marked morphological changes under variable growth conditios including salinity (Mahasneh *et al.*, 1990). Decrease in pigmentation is another response to salt stress noted for Cyanobacteria (Vonshak *et al.*, 1996). Kebede (1997) reported that specific growth rate of *Spirulina platensis* was decreased with increase of salinity. Anand *et al.*, (1986) reported that cell disruption, release of phycobilin pigments and declining photosynthesis were the responses of *Oscillatoria sancta* and *Nostoc piscinale* under salinity (240 mM). Marin *et al.* (1998) have reported responses of *Dunaliella* sp under different salinity conditions and showed that cells were larger and more spherical in cultures grown at 9% salinity.

As the results showed that *O. acuminata* possesses high phycoyanin content, the extraction was further standardized using different buffers. Ammonium acetate, potassium phosphate, sodium phosphate and distilled water gave similar results. The results also confirmed that distilled water provide better extraction quantitatively. In *Spirulina* phycocyanin is produced by enzymatic disintegration of cell walls, followed by purification of the crude extract through a series of procedure including centrifugation, fractional precipitation, dialysis, and column chromatography (Boussiba and Richmand, 1979). Different treatments are used for cell wall disruption, such as homogenizer (Oranda *et al.*, 1975; Boussiba and Richmand, 1979), sonication (Murakami *et al.*, 1981), cell mill with glass beads (Scheer and Kufer, 1977) and hand grinding (Devi *et al.*, 1981). In industrial process, phycocyanin is extracted

using aqueous extraction, after which the crude extract is centrifuged to remove other pigments, then concentrated by ultrafiltration, and finally freeze dried as a phycocyanin product (Kato, 1985). The phycocyanin content in *S. platensis* depends on where it is grown and/or the growth conditions (Chen *et al.*, 1996; Tomaselli *et al.* 1997). For consistency and assurance of high product quality of the phycocyanin itself and/or phycocyanin-including products, there is a need to establish a method for extracting phycocyanin rapidly and selectively from *S. platensis*. For the extraction of phycobiliproteins commercially, species having high pigment content and easily breakable cell wall has to be discovered and normally cynobacterial cell wall are thick and extremely resistant (Stewart and Farmer, 1984). The experiments with three species showed that *O. acuminata* possess easily breakable cell wall compared to the other two species. In this study hand grinding with mortar and pestle followed by repeated freezing and thawing was adopted for the release of phycobiliproteins. Phycocyanin can be released from *O. acuminata* in fresh distilled water by hand grinding. This property makes more pure phycocyanin and further reduces effort for purification. Zhang and Chen (1999) separated and purified C-Phycocyanin and allophycocyanin by precipitation with ammoniulm sulphate, ion exchange, chromatography and pure phycocyanin and allophycocyanin were finally obtained with an A_{620}/A_{280} value of 5.06 and an A_{655}/A_{280} value of 5.34 repectively. Minkova *et al.* (2003) purified C-phycocyanin and its homogeneity was demonstrated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Recently Fusuki *et al.* (2003) reported that phycocyanin

can be efficiently extracted from *Spirulina*, by cell disruption by ultrasonic irradiation. Eventhough phycocyanin is obtained by the above processes, further purification is needed for commercializing the product. Patel *et al.* (2005) described single step chromatographic method for purification of C-phycocyanin from *Spirulina*, *Phormidium* sp and *Lyngbya* sp.

4

CHAPTER

CYANOBACTERIA FOR SEWAGE AND INDUSTRIAL EFFLUENT TREATMENT

4.1 Introduction

Biological treatment of wastewater offers the advantage of greater flexibility and lower operational costs over conventional physical treatment systems to reduce the pollution load of effluents (Subramanian and Uma, 1997; Surendran *et al.*, 2001). The development of newer technologies emphasize on detoxification, metabolisation, accumulation, and disinfection of the effluent systems by introducing selected microbes replacing the conventional biological systems relying on natural populations (Subramanian and Uma, 2001). Algae and Cyanobacteria are widely reported to be of potential use in wastewater treatment of variety of industrial effluents and pollutants (Uma and Subramanian, 1990; Roymohapatra and Padhi, 2000; Thajuddin and Subramanian, 2005). The biological treatment of wastewater with algae to remove nutrients was first proposed by Oswald *et al.* (1988). Joseph and Joseph (2002) reported that an oil refinery effluent holding pond is a eutrophic system with a resistant microalgal community with distinct seasonal variation in species composition. Sharma *et al.* (2004) have described treatment of 'Dairy effluent' by Cyanobacteria. Bender *et al.* (2004) have developed an effluent treatment system using microbial mats and sand filters dominated by Cyanobacteria.

The usefulness of algal systems more particularly, the Cyanobacteria in not only treating the wastes but also producing a variety of useful by-products is being understood and is commercially practiced (Kojima and Lee, 2001); but their precise role in treating effluents, as well as the effect of effluents on the organisms are not clearly understood. The role of algae in increasing pH is

also made use of in wastewater treatment. A marine Cyanobacterium *Phormidium valderianum* was found to degrade phenol completely at 100 mg L^{-1} by its intracellular oxidases and laccase enzymes, and such strains could be effectively used for treament of phenol containing wastes (Shasirekha *et al.*, 1997). Recently Abed and Koster (2005) reported that Cyanobacteria along with aerobic heterotrophic bacteria can degrade petroleum compounds. Hirooka *et al.*(2003) reported removal of hazardous phenols by micoalgae including *Anabaena variabilis* under photoautotrophic conditions. Studies conducted by Joseph and Joseph (2001b) in petrochemical effluent revealed reduction of total dissolved solids content in the effluent samples inoculated with *Oscillatoria quadripunctulata*. Lima *et al.* (2003) reported that micro algal consortium could effectively degrade p-nitrophenol (50 mg L^{-1}) within 5 days. Cohen (2002) described that Cyanobacterial mat dominated by *Oscillatoria* sp and *Phormidium* sp could be effectively used for bioremediation of oil spills. Chevalier *et al.* (2000) reported excellent performance of *Phormidium bohneri* in the removal of nitrogen and phosphorus from the wastewater and described its potential use in tertiary wastewater treatment.

Raw waste waters as well as treated effluents most often contain significant amounts of bio-available inorganic substances such as NH_4^+, NO_3^-, and PO_4^{-3}, which contribute to eutrophication of receiving bodies of water. Sharma *et al.* (2004) revealed that cultivation of Cyanobacteria in the dairy effluent leads to utilization of free CO_2 and also it absorbed significant amounts of both Ca^{++} as well as Mg^{++} Addition of Cyanobacteria also brings about solubilisation of insoluble phosphates, improves soil aggregation, soil water holding capacity

and soil density. Dumas *et al.* (1998) described bio-treatment of fish farm effluents using the Cyanobacterium *Phormidium bohneri.* Bender *et al.* (2004) reported that microbial mats dominated by Cyanobacteria can transform nitrogenous wastes in to cellular protein and rapidly metabolize other fish wastes. Removal of trace elements is the problem still unresolved due to very low concentration of contaminants. Chojnacka *et al.* (2004) presented the applicability of *Spirulina* sp for trace elements removal from refinery and smelting industries. Studies conducted by Tien (2002) on biosorption of metal ions in *Oscillatoria limnetica* revealed that the species has highest sorption capacity for lead. Gong *et al.* (2005) reported 92 % removal of lead by pre treated biomass of *Spirulina maxima.* Rangsayatorn *et al.* (2004) studied biosorption of cadmium by immobilized *Spirulina platensis* on alginate gel and silica gel. Mohapatra and Gupta (2005) described concurrent sorption of Zn (II), Cu (II) and Co (II) by *Oscillatoria angustissima.*

The mat forming species of filamentous Cyanobacteria self aggregate in culture (Talbot and de la Noue, 1993). Therefore after the Cyanobacteria have taken up the nutrients in the effluents, the purified water can be decanted and the Cyanobacteria can then be harvested with ease (Proulx *et al.*, 1994). Potential uses of harvested biomass include the extraction of commercially valuable pigments (Glazer, 1994); the organic matter can be used to harvest other valuable by-products as well (Gill *et al.*, 1999).

Aim of the present work was to evaluate the growth response of the isolated strains in both effluents. First part of this chapter deals with the biomass production of the three isolated species of Cyanobacteria in the ETP effluent

of a petroleum refinery and in the STP effluent. Second part deals with the treatment of these two effluents by *O. subbrevis*. Pigment composition, heavy metal content and protein profile of the harvested biomass were elucidated to evaluate its further applicability.

4.2 Materials and methods

4.2.1 Collection of industrial effluent

Effluent was collected from the final polishing pond of the effluent treatment plant of the Kochi Refineries Ltd (KRL). The effluent emanating from the process unit of the refinery contains sulphides, hydrocarbons, phenols and other organic matter. The ETP comprise the API separator to skim off oil, trickling filter and two polishing ponds. The effluent is discharged out from the final polishing pond from which the samples for this study were collected.

Effluent was collected from different locations of the pond at different depths using a water sampler, and pooled to obtain a composite sample. The samples were collected in polyethylene bottles and transported to the laboratory.

4.2.2 Collection of STP effluent

Effluent was collected from a Sewage Treatment Plant (STP) in Kochi. The STP has a collection tank from which sewage is fed into an aeration tank. After aeration, the sludge settles out in the settling tank and the overflow is pumped out into the civil drains. The sewage samples for this study were collected from the aeration tank and transported to the laboratory in polyethylene bottles.

4.2.3 Effluent analysis

The samples brought to the laboratory were immediately analyzed for the physico-chemical parameters. Parameters like conductivity, turbidity, and pH were determined directly using instruments. BOD was estimated as the difference in the dissolved oxygen content of the initial and final samples following incubation at 20^0C in a BOD incubator for 5 days. The samples for estimation of phosphate, nitrate and sulphate were filtered through whatman GF/C filter paper and the filtrate was used for analysis. The phosphate was estimated by ascorbic acid method. The cadmium reduction method was used for the estimation of nitrate. Sulphate content was determined by turbidimetric method. Alkalinity was determined by titrimetric method. Chlorides were determined by argentrometric method, and hardness was determined by EDTA titrimetric method. The analyses were done as per the Standard Methods (APHA, 1998). Conductivity was measured as mmho/cm, using conductivity meter. Turbidity was measured in NTU units in a Nephelo turbidity meter.

4.2.4 Algal Assay of Effluents

- ***Indoor cultivation***

The effluent collected from the refinery and the sewage treatment plant (STP) were filtered through absorbent cotton followed by successive filtration through whatman No.2 filter paper and GF/C to remove the turbidity. 100 ml each of these filtered effluents were taken in clean borosilicate culture flasks plugged with non-absorbent cotton. The three species of Cyanobacteria, *O. acuminata, O. subbrevis* and *P. tenue* were inoculated separately in triplicates to both these effluents. Control cultures in standard growth medium

were raised alongside. The cultures were incubated under light assembly as in previous experiments. The dry biomass was estimated on 8^{th}, 12^{th} and 16^{th} day.

➢ ***Outdoor cultivation***

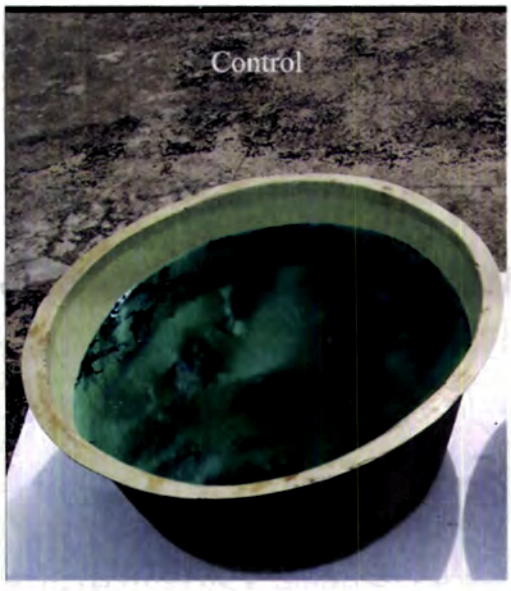
The refinery effluent was transferred into fiberglass tanks of 15 L capacity and separately inoculated with the three species of Cyanobacteria. The STP effluent was similarly kept in fibreglass tanks and inoculated with *O. subbrevis*. Control cultures in standard growth medium were raised alongside (Plate VII). The inocula were equivalent to 10 mg dry weight L^{-1} The inoculated cultures were kept exposed to sunlight with intermittent aeration. The cultures were harvested on 12^{th} day and the biomass produced was estimated as dry weight L^{-1}

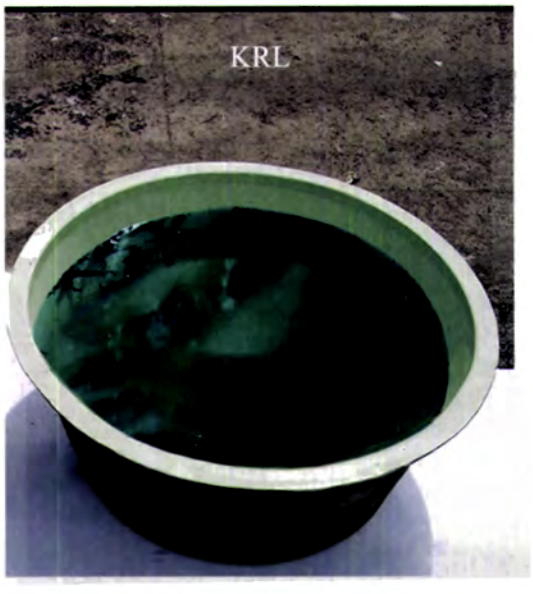
4.2.5 Treatment of sewage and refinery effluent by *O. subbrevis*

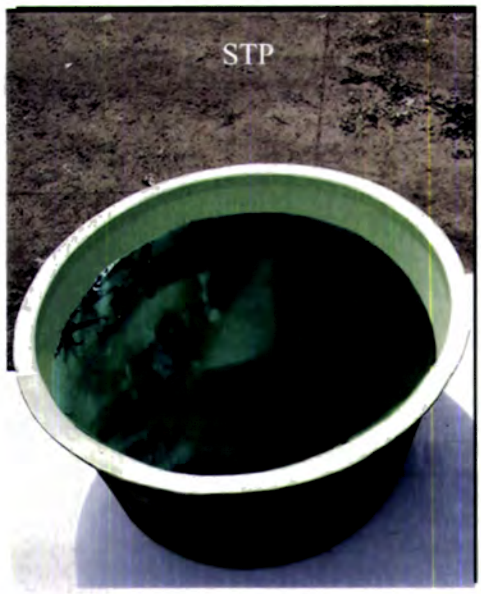
Based on the algal assay results, both under indoor and outdoor conditions, *O. subbrevis* was identified for the treatment of effluents. Fibreglass tanks of 15L capacity were filled with filtered STP and refinery effluents and kept outdoor at day temperature 30± 3^0C and inoculated with *O. subbrevis* (10 mg dry weight L^{-1}). Intermittent agitation was provided. The biomass developed was filtered out on the 12^{th} day. The effluent was analysed for its physico-chemical properties. The initial and final results of this analysis were used to evaluate treatment capability of the species. Pigment status of the treated as well as control biomass was carried out. The protein profile of the control and treated biomass was elucidated by electrophoresis. The treated and control biomass were dried and heavy metal analyses were done.

Plate VII

Outdoor Cultivation in KRL and STP effluents







4.2.6 Determination of Protein Profile

Extraction of protein

500 mg of Cyanobacterial pellet was taken and sonicated in Tris-HCl buffer (0.0625M, pH 6.8) for 10 min and was centrifuged at 5000 x g for 10 min and the supernatant was obtained. 5 ml of 10 % cold TCA was added to the supernatant and incubated overnight; centrifuged at 12,000 x g for 10 minutes and the pellet was obtained. The pellet was washed with the ethanol- ether (1:1 V/V) repeatedly to remove TCA. The TCA precipitate was dissolved in 0.0625 M Tris HCl, pH 6.8. Analysis of proteins was carried out by SDS-PAGE according to the method of (Laemmli, 1970).

Procedure

The protein samples were diluted (1:1 v/v) with sample buffer and heated 5 min at 100^0C in a sealed screw cap micro centrifuge tube. Separating gel solution and stacking gel solution were prepared. The glass plates were assembled and freshly prepared separating gel solution was immediately poured to this sandwich until the height of the solution is 11 cm. A layer of Iso-butyl alcohol was poured over it and allowed the gel to polymerize for 30-60 min at room temperature. The layer of Iso-butyl alcohol was poured off and rinsed with 1xTris Cl /SDS. The freshly prepared stacking gel solution was poured into the sandwich until the height of the solution is 1 cm from the top of the plates. Teflon comb was inserted into the stacking gel solution and this was allowed to polymerize for 30 - 40 min at room temperature. The wells were filled with 1 x SDS/ electrophoresis buffer after removing the Teflon comb without damaging the wells. Protein samples were loaded into the wells as thin layer at the bottom

of the wells. Equal volumes of sample buffer were added to the empty wells to prevent spreading of adjoining lanes. The cell was connected to the power supply at 10 mA of constant current for a slab gel 1.5 mm thick, until the bromophenol blue tracking dye enters the separating gel; then the power supply was increased to 15 mA. When the dye reached the bottom of the separating gel the power supply was stopped and disconnected. Carefully removed the sandwich from the chamber and placed on a sheet of absorbent paper. The molecular weights of various bands were calculated with the R*f* of the standard protein bands using the quantity one software of Bio-Rad.

4.2.7 Determination of heavy metal content in the biomass

Procedure

The effluent treated biomass were harvested and dried at 60^0C overnight. 500 mg of powdered biomass were digested with concentrated H_2SO_4. After digestion the samples were diluted to appropriate volume with de-ionized water and the heavy metal content was determined by atomic absorption spectro photometry (Perkin Elmer AAS 3110). In AAS, the sample was first converted in to an atomic vapor and then the absorption of atomic vapor was measured at a selected wavelength, which was characteristic of each individual element. Quantitative measurements in AAS were based on Beers law and therefore, the measured absorbance is proportional to the concentration. For the estimation of lead the instrument was calibrated using a standard solution of 20 ppm lead and the concentrations of lead in the samples were then directly measured from AAS. The lamp used was hollow cathode lamp of lead and the flame used was air-acetylene. The monochrometer wavelength

selected was 283.3 nm. For the estimation of cadmium, hollow cathode lamp of cadmium was used as the primary light source. The instrument was calibrated using the standard solution of cadmium (1.5ppm). The monochrometer wavelength selected was 228.8 nm.

4.3 Results

4.3.1 Effluent properties

The results of analysis of KRL and STP effluent is given in the (Table 4.1).

Table 4.1 Physico-chemical characteristics of effluents from refinery (KRL) and sewage (STP)

Parameter	KRL	STP
Turbidity (NTU)	8.4	3.8
pH	7.5	7.1
Nitrate (mg L^{-1})	6.1	34
Phosphate (mg L^{-1})	0.199	1.8
Sulphate (mg L^{-1})	103	17
Conductivity (mmho/cm)	0.35	0.42
BOD (mg L^{-1})	59	53
Alkalinity (mg $CaCO_3$ mg L^{-1})	65	78.2
Total hardness (mg $CaCO_3L^{-1}$)	55.5	66.7
Ca Hardness (mg $CaCO_3$ L^{-1})	51	56
Chlorides (mg L^{-1})	147.2	49.3

The samples collected were turbid with pH > 7. The nitrate content of the STP effluent was 34 mg L^{-1} and that of refinery effluent was 6.1 mg L^{-1}. Both effluents were of organic in nature with BOD 53 mg L^{-1} and 59 mg L^{-1} respectively for the STP and refinery effluent. Sulphate content was relatively high in refinery effluent. STP effluent had alkalinity of 78.2 mg $CaCO_3$ L^{-1} whereas refinery effluent showed 65 mg $CaCO_3$ L^{-1}.

4.3.2 Algal assay

➢ ***Indoor experiment***

The three species of Cyanobacteria could grow in both refinery and STP effluent. *O. acuminata* produced a biomass of 19.3 mg /100 ml in KRL effluent and 14.3 mg/100 ml in STP effluent within 8 days (Figure 4.1).

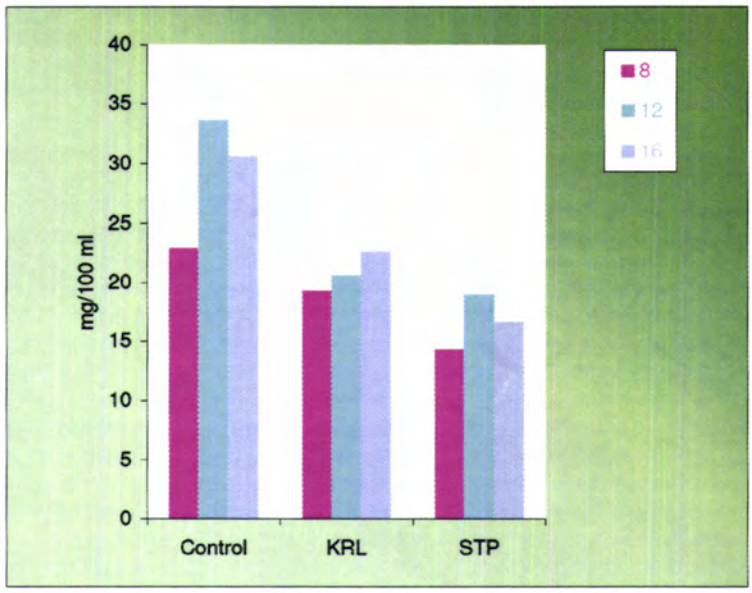


Figure 4.1 Growth of *O. acuminata* in KRL and STP effluent in 8, 12, and 16 days

The species produced 20.7 mg on 12^{th} day and 22.6 mg on 16^{th} day in refinery effluent. It produced 19 mg in STP effluent on 12^{th} day. The 16^{th} day yield was 16.67 mg in 100 ml STP. The 16^{th} day biomass of *O. acuminata* in KRL effluent was significantly lower than control (Calculated t value = 3.05, $p<0.05$). The growth in STP significantly lowered (Calculated t value = 5.61, $p<0.05$) upon 16 day growth. *O. subbrevis* could grow well in both KRL and STP effluent. The growth was also comparable with the control. (Figure 4.2)

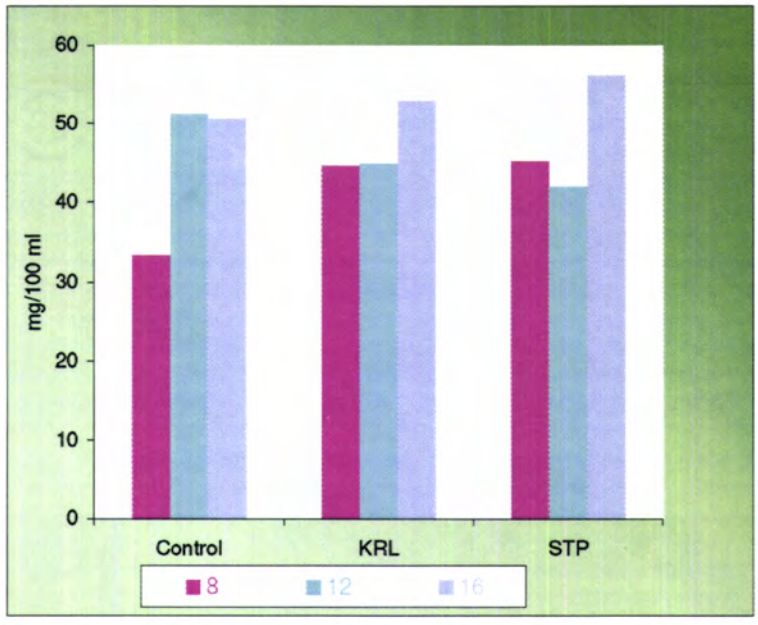


Figure 4.2 Growth of *O. subbrevis* in KRL and STP effluent in 8, 12, and 16 days

The species yielded 44.6 mg/ 100 ml within 8 days and 53 mg/100 ml within 16 days in KRL effluent. The 16th day yield in KRL effluent didn't show any significant difference from control (Calculated t value = 1.94, $p>0.05$). It could yield 56.3 mg in STP effluent within 16 days and it was significantly higher than the control (Calculated t value = 3.80, $p< 0.05$).

P. tenue yielded 19.6mg/100 ml within 12 days and 22 mg/100 ml within 16 days in KRL effluent, the biomass was significantly lower than the control (Calculated t value = 5.13, $p< 0.05$) and it produced only 18.7 mg/100 ml in STP effluent on 12th day (Figure 4.3).

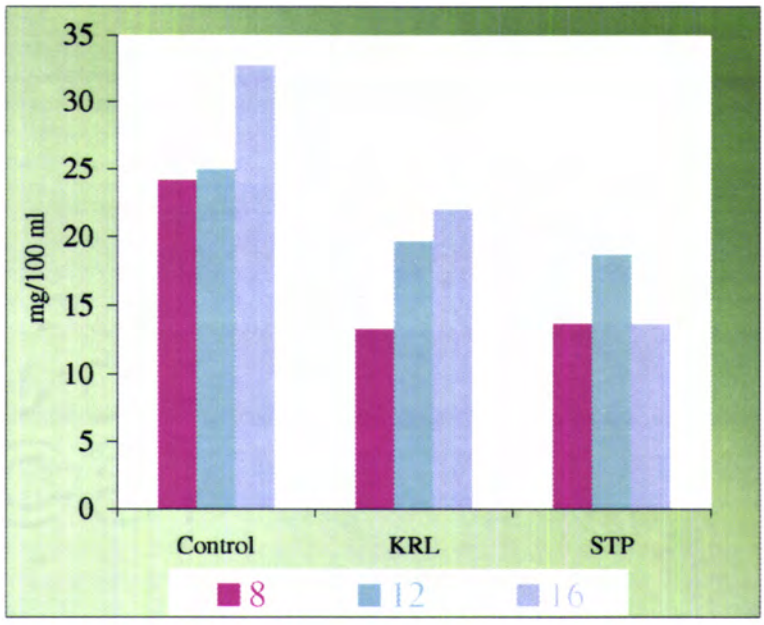


Figure 4.3 Growth of P. *tenue* in KRL and STP effluent in 8, 12, and 16 days

The biomass was reduced to 13.5 mg in STP effluent with in 16 days and it was significantly lower than the control (Calculated t value = 12.12, $p< 0.05$).

➢ ***Outdoor experiment***

Outdoor cultivation studies showed a reduction in biomass production by *O. acuminata* and *P. tenue* in KRL effluent. *O. subbrevis* could grow well in KRL and STP effluent in outdoor conditions. *O. acuminata* produced only 21.3 mg dry wt L^{-1} in KRL effluent whereas *O. subbrevis* yielded 106 mg dry wt L^{-1} in KRL effluent and 116 mg dry wt L^{-1} in STP effluent (Figure 4.4). *P. tenue* produced 52.2 mg dry wt L^{-1}. Based on the above results *O. subbrevis* was finally confirmed for the treatment of both KRL and STP effluents.

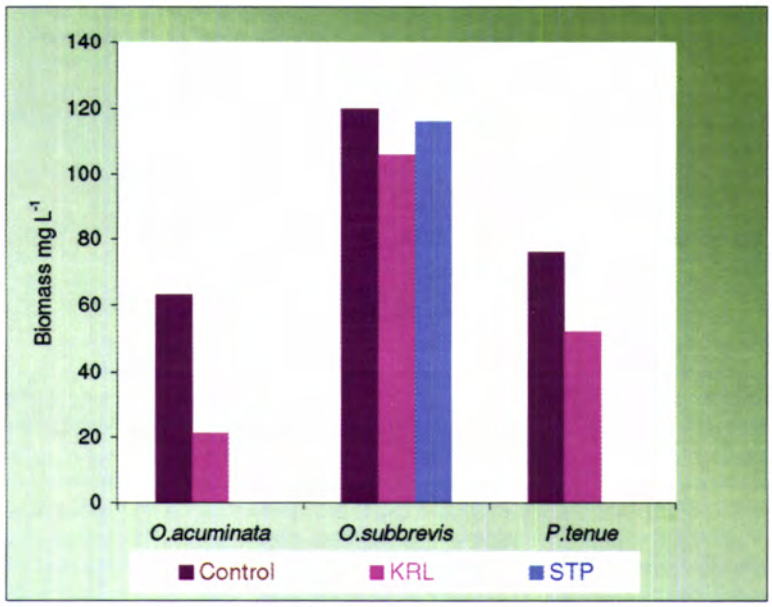


Figure 4.4 Twelve day yield of *O. acuminata* & *P. tenue* in KRL effluent and *O. subbrevis* in both KRL and STP effluent

4.3.3 Treatment of KRL and STP effluent by *O. subbrevis*

Initial pH of the STP effluent and KRL effluent were 7.4 and 7.9, respectively. The growth of *O. subbrevis* increased the pH to about 9.49 in KRL effluent and to 8.28 in STP effluent (Table 4.2). Nitrate level of KRL effluent was reduced to 3.9 mg L^{-1} from 5.6 mg L^{-1} Nitrate level of STP was reduced to 8 mg L^{-1} from 30 mg L^{-1} Phosphate content of KRL was reduced to 0.087 from 0.192 mg L^{-1} 25 % reduction of phosphate was observed in STP effluent. 26 % and 34 % reduction in sulphate content was recorded by the growth of *O. subbrevis* in KRL and STP effluent respectively. Initial conductivity of KRL and STP effluent were 0.25 and 0.35 mmho/cm, it was reduced to 0.18 and 0.20 mmho/cm respectively. Alkalinity of KRL effluent was reduced to 8.5 mg $CaCO_3$ L^{-1} from 50 mg $CaCO_3$ L^{-1}, STP effluent showed only 17 mg $CaCO_3$ L^{-1} after treatment with *O. subbrevis*. 50 %

reduction of total hardness was observed in both effluents. Ca hardness was reduced to 15 mg $CaCO_3$ L^{-1} and 20 mg $CaCO_3$ L^{-1} from 40 and 50 mg $CaCO_3$ L^{-1} of KRL and STP effluent respectively.

Table 4.2 Effluent properties of the refinery (KRL) and sewage (STP) before and after treatment with *Oscillatoria subbrevis*

Parameter	KRL		STP	
	Untreated effluent	Treated effluent	Untreated effluent	Treated effluent
pH	7.9	9.49	7.4	8.28
Nitrate (mg L^{-1})	5.6	3.9	30	8
Phosphate (mg L^{-1})	0.192	0.087	1.6	1.2
Sulphate (mg L^{-1})	100.2	75.5	14.3	9.4
BOD (mg L^{-1})	35	10	45	12
Conductivity (mmho/cm)	0.25	0.18	0.35	0.20
Alkalinity (mg $CaCO_3$ L^{-1})	50	8.5	76.5	17
Total hardness (mg $CaCO_3$ L^{-1})	50	25	60	30
Ca Hardness (mg $CaCO_3$ L^{-1})	40	15	50	20
Chlorides (mg L^{-1}))	146.8	144.6	48.92	45.3

4.3.4 Effect of effluents on pigments

O. subbrevis didn't show much reduction in pigment composition after treatment in both KRL and STP effluents. The species yielded 1.44 mg % chlorophyll *a* in control and 1.2 mg % after treatment after treatment in KRL and STP effluent. It yielded 12.19 mg % phycocyanin in KRL effluent and 12.07 % in STP effluent (Figure 4.5). The species could yield 5.06 % allophycocyanin in KRL effluent,while in STP effluent it could yield 6.5 %.

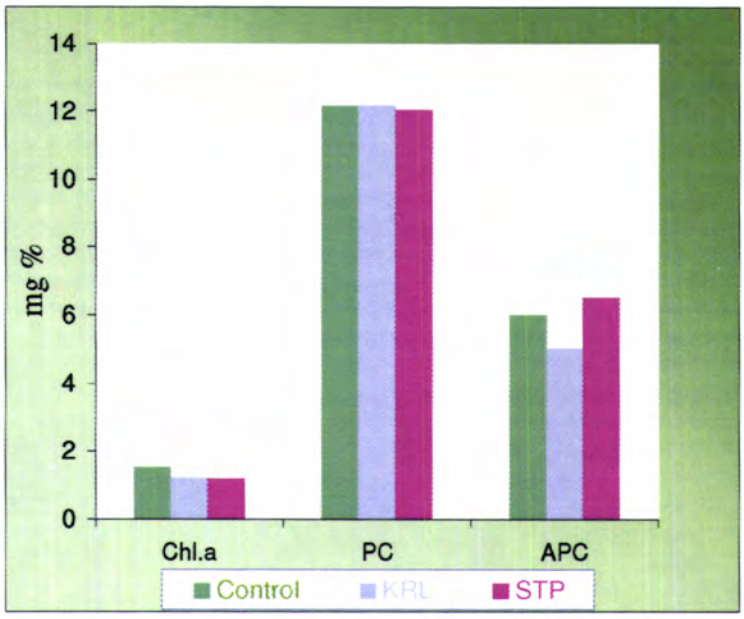


Figure 4.5 Chlorophyll *a*, Phycocyanin, Allophycocyanin content of *O. subbrevis* in KRL and STP effluent

4.3.5 Protein profile of untreated and treated biomass

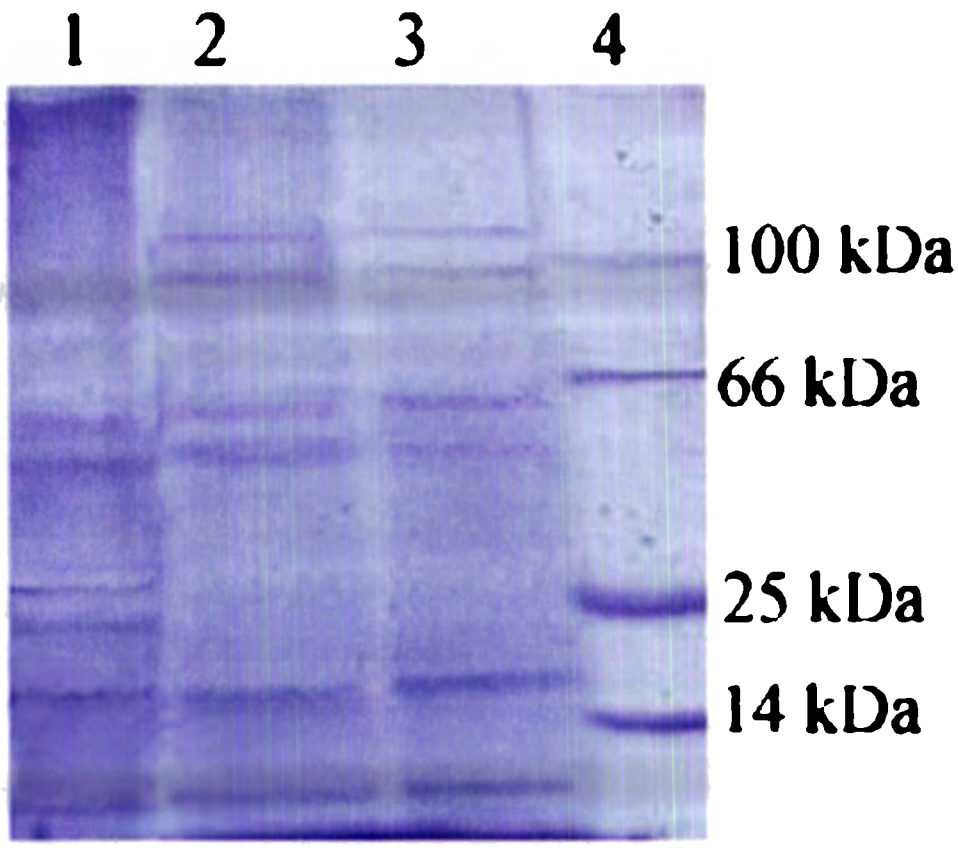
Protein profile of *O. subbrevis* upon growth in KRL and STP effluent is shown in the Plate VIII. The protein extract of the species showed visible protein bands from 10 to 62 kDa and above that bands were not clear. Eventhough the banding pattern was almost retained in the species grown in both effluents, new protein bands at 93 and 114 kDa were clearly visible in the case of biomass grown in KRL effluent. Biomass grown in STP effluent also showed clear protein bands at 97 and 116 kDa region. The protein bands at 21 kDa and 26 kDa exhibited by the species was absent in treated biomass. This indicates the difference in the protein profile of the species when grown in the effluent.

4.3.6 Heavy metal content in the harvested biomass of *O. subbrevis*

Heavy metal analysis of *O. subbrevis* grown in both KRL and STP effluent showed that the species possesses heavy metal accumulation capacity. Lead

plate VIII

Protein profile of *O. subbrevis* grown in KRL and STP effluents



Lane 1 Control
Lane 2 - ***O. subbrevis*** **grown in KRL effluent**
Lane 3 - ***O. subbrevis*** **grown in STP effluent**
Lane 4 - **Marker**

content in the biomass grown in sewage was 33.8 µg/g whereas harvested biomass from KRL contained 27.8 µg/g (Table 4.3).

Table 4.3 Heavy metal content of the biomass treated in refinery (KRL) and sewage (STP)

Parameter (µg/g)	Control	KRL	STP
Lead	2.28	27.8	33.8
Cadmium	3.42	22.61	8.85

Biomass grown in KRL effluent showed 22.61 µg/g cadmium whereas the biomass grown in STP showed 8.85 µg/g. The accumulation of lead was 12-15 times that of control. Cadmium was accumulated to about 3 -7 times.

4. 4 Discussion

The three species of Cyanobacteria isolated and characterized in this investigation are capable of growing in the effluent media and without any nutrient enrichment. Cyanobacteria are known for its high adaptability to various environments. The present results confirm the earlier observations that Cyanobacteria can effectively utilize the dissolved substances in the effluents. Fresh water Cyanobacterial strains *Phormidium* (de la Noue and Prolux, 1988) and *Oscillatoria* sp (Manoharan and Subramanian, 1992 a) have shown increased growth and resulted in the removal of phosphate and nitrogen in sewage to an appreciable extend. In the present investigation the yield of *O. acuminata* and *P. tenue* was lower than the control., but that of *O. subbrevis* was comparable or greater than the control. The pigment content of the species was also similar to the control. The growth of *O. subbrevis* increased the pH to about 9.49 and 8.28 within 12 days in KRL and STP

effluent respectively. Similar results were obtained by several workers (Manoharan and Subramanian, 1992 a; Phang, 2000; Sharma *et al.*, 2004) Cultivation of Cyanobacteria in the effluent leads to utilization of free CO_2 for its photosynthetic activity and results in increase in pH. The increase in pH is due to photosynthetic activity of the algae, and can also lead to other desirable effects for waste water treatment such as precipitation of phosphate with the formation of hydroxy-apatite (Oswald, 1988) and stripping of ammonia by volatilization. Rapid alteration in the pH of the surrounding medium was known to be brought by the marine cyanobacterium *Phormidium valderianum* BDU 30501 in chloralkali effluent of pH 2.8 to 8.5 in 48 h; such a phenomenon probably eliminates the coliforms in sewage (Uma and Subramanian, 1994).

The organism could reduce BOD considerably. Studies conducted by Manoharan and Subramanian (1992 a) reported considerable reduction in BOD of domestic sewage inoculated with *Oscillatoria pseudogeminata.* Kirkwood (2003) reported that Cyanobacteria remove small organic molecules during mixotrophic and heterotrophic growth. Nitrate level of STP effluent was fairly high compared to KRL effluent; *O. subbrevis* could reduce it to 8 mgL^{-1} from 30 mgL^{-1}. 30 % of nitrate was reduced in the case of KRL effluent. The algae could reduce 54.68 % phosphate from KRL effluent. Two fresh water forms of Cyanobacteria *Phormidium* sp and *Lyngbya* sp immobilized to deacylated form of chitin, was shown to remove 95% of phosphate and 98% of nitrogen in 24 h (de la Noue and Prolux, 1988). Olguin *et al.* (2003) have described 72- 87 % removal of phosphate from effluent containing digested pig waste. Manoharan and Subramanian (1992 a) showed 100 % removal of nitrate from sewage by

Oscillatoria. Phang (2000) have obtained 99.4 % removal of phosphate from wastewater by *Spirulina.* Microalgae have a high capacity for inorganic nutrient uptake and they can be grown in mariculture in outdoor solar bioreactors. *Oscillatoria willei* with a holding time of 96 hours was able to show 96 % bacterial removal (Kalaichelvi, 1997). *O. subbrevis* could reduce 24.6 % sulphate content of KRL effluent within 12 days. Sharma *et al.* (2004) reported 60 % reduction in sulphate within 7 days from dairy effluent by Cyanobacteria.

Initial conductivity of KRL and STP effluent were 0.25 and 0.35 mmho/ cm, it was reduced to 0.18 and 0.20 mmho/cm respectively. Joseph and Joseph, (2001 b) reported 32.6 % reduction in TDS within 96 h. Fresh water Cyanobacteria *Oscillatoria pseudogeminata* along with natural population removed various anions like nitrate, phosphorus, ammonia, chloride and sulphate in paper mill and ossein effluent (Manoharan and Subramanian, 1992 b). 50 % reduction of hardness was observed after treatment with *O. subbrevis.* Sharma *et al.* (2004) reported that Cyanobacteria can absorb significant amounts of both Ca^{++} as well as MG^{++} from dairy effluent. Alkalinity of KRL effluent was reduced to 8.5 mg $CaCO_3$ L^{-1} from 50 mg $CaCO_3$ L^{-1}

Effect of effluent on the organism was studied by analyzing chlorophyll and phycobiliproteins. *O. subbrevis* didn't show much reduction in pigment composition after treatment in both KRL and STP effluents. The species could yield 1.2 % chlorophyll *a* after treatment and it yielded 12.19 % phycocyanin in KRL effluent and 12.07 % in STP effluent. Dash and Mishra (1996) studied the effect of paper mill waste on the pigment and protein content of a blue green alga, *Westiellopsis prolifica*, showed increased chlorophyll *a*, carotenoid

and protein content for upto 14 days of incubation followed by a decline. Protein profile analysis of treated and untreated biomass showed that certain proteins are disintegrated but certain other proteins are newly formed. The species showed clear bands at 21 kDa and 26 kDa but it was totally absent in treated biomass. The species grown both in KRL and STP showed clear protein bands in between 93 kDa and 116 kDa which were not observed in untreated biomass. This indicates the formation of new protein after treatment. This may be attributed to the synthesis of degrading enzymes or stress- related proteins. Shashirekha *et al.* (1997) reported increase in protein content of *Phormidium valderianum* is due to the de *novo* synthesis of phenol degrading enzymes and stress- related proteins. Bhagwat and Apte (1989) demonstrated that salinity inhibited the synthesis of a large number of polypeptides in *Anabaena torulosa*. In this study also, when strains grown in effluent inhibition of proteins in the range 21-26 kDa was observed. Recently Saha *et al.* (2003) reported nitrogen stress induced changes in *Oscillatoria willei*, including modifications of protein synthesis leading to the repression of three polypeptides and synthesis of two new polypeptides. A desiccation- tolerant Cyanobacterium *Nostoc commune* accumulates a novel group of acidic proteins when colonies are subjected to repeated cycles of drying and rehydration.

O. subbrevis has been shown to accumulate heavy metals lead and cadmium in this investigation. The species could accumulate 33.8 µg/g of lead from sewage and 22.6 µg/g of cadmium from KRL effluent. Raveender *et al.* (2002) reported mutant strains of Cyanobacteria could adsorb 250 µg Cd^{2+} / mg dry weight contributing about 30 % of the total cadmium removal from the

solution after 200 h exposure. Rangsayatorn *et al.* (2004) reported that the maximum biosorption capacities for alginate immobilized cells and silica immobilized cells were 70.92 and 36.63 mg Cd/g biomass, respectively by *Spirulina platensis*. Gong *et al.* (2005) reported 92 % of lead could be removed by pre treated biomass of *Spirulina maxima* from waste water and also showed that lead adsorbed could be desorbed effectively by 0.1 M nitric acid, EDTA and hydrochloric acid.

Manoharan and Subramanian (1992 a) reported enhancement in the carbohydrate and reduction in protein content of *Oscillatoria* after treatment in sewage. Environmental stress induced modification of protein synthesis have already been reported (Bhagwat and Apte, 1989). Manoharan and Subramanian (1993) studied the influence of effluents on fatty acid content of Cyanobacterium. Manoharan and Subramanian (1995) reported 12-52 % reduction in lipid content with Ossein and tannery effluents, and 34 to 41 % with domestic sewage and paper effluent. Analysis of protein and carbohydrate content of harvested biomass from domestic wastewaters after treatment showed that it could be used for the preparation of animal feed (Talbot and de la Noue, 1993; Gill et al. 1999) commercially valuable pigments (Glazer, 1994; Tang *et al.* 1997). Recently Brown *et al.* (2005) showed that Cyanobacteria can exhibit an elevated tolerance for iron in culture media and suggested that the isolate may be an important component of an iron-depositing microbial community. The results in this study strongly suggest *O. subbrevis* can be effectively used for the removal of dissolved substances at tertiary treatment level of petroleum refineries and also of STPs; but the resulting pH of the effluent has to be taken care of. As the species has

the capacity to accumulate metals from the effluent, the resulting biomass needs thorough screening for its further use as feed or as a source of pigments or proteins.

5

CHAPTER

SUMMARY AND CONCLUSION

Summary and Conclusion

The microalgae gained importance as food and feed as well as source of fine chemicals since the 1960's. *Spirulina* became the trend setter due to its easily culturable properties as well as nutritional composition. A rapid expansion of microalgal industry occurred in the Asia-Pacific region as microalgae came to stay as a health food supplement.

Microalgae have been an integral component of oxidation ponds usually incorporated with wastewater treatment. Over the last few decades, efforts have been made to apply intensive microalgal cultures to perform biological tertiary treatment of secondary effluents. Given the limited number of species still available for commercial exploitation, it is imperative to isolate and cultivate those photosynthetic organisms with high growth rate and biomass accumulation, which could play the dual role of cleaning the wastewater and also providing useful biomass. This has been the objective of this study *ie.*

- To develop pure cultures of local isolates of Cyanobacteria for extraction of biochemicals of commercial value
- To couple biomass production with effluent treatment

Cyanobacteria for this investigation were isolated from soil and water samples collected from paddy fields and sewage drains of the region. Three species of filamentous Cyanobacteria were developed into pure cultures in BG-11 medium. They were *Oscillatoria acuminata*, *Oscillatoria subbrevis* and *Phormidium tenue*.

The culture requirements of the three species were optimized with respect to culture medium, pH, alkalinity, salinity, and aeration for the maximum production of biomass. The results are given in the table below.

Sl.No	Growth condition for maximum biomass production	*O.acuminata*	*O.subbrevis*	*P.tenue*
1	Culture medium	BG-11	Modified BG-11	Modified BG-11
2	Alkalinity(mg $CaCO_3L^{-1}$)	45	90 - 150	90
3	pH	7.5	9.5 - 10.5	7.5 - 10.5
4	Aeration/Agitation	Gentle shaking	Gentle shaking or static culture	Gentle shaking or static culture
5	Salinity	Nil	$5-15 \times 10^{-3}$	5×10^{-3}

The growth kinetics of the three species was defined in the above culture media at ambient temperature (30 ± 3^0C) and light intensity 2000 lux from day light fluroscent lamps at 12:12 light/ dark cycle. The maximum growth rates were recorded as 0.36 d^{-1}, 0.51 d^{-1} and 0.33 d^{-1} for *O. acuminata*, *O. subbrevis* and *P. tenue* respectively. The biomass obtained in indoor and outdoor cultivation is given below.

Sl.No.	yield	*O. acuminata*		*O. subbrevis*		*P. tenue*	
		Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
1	*Biomass (mg L^{-1})	93	69	240	115	80	73
2	Chlorophyll *a* (mg %)	1.90	1.77	1.59	1.53	0.83	0.73
3	Phycocyanin (mg %)	17.99	15.97	13.74	13.12	8.23	5.60
4	Allophycocyanin(mg %)	7.30	5.80	6.02	6.36	5.10	3.30

* Initial inoculum : 10 mg L^{-1}
No.of days of growth : 12

The biochemical compostion of the biomass of the three species was estimated in terms of carbohydrates, proteins, lipids and fatty acid composition. The ash

weight of the three species was in the range 8.3 to 12.6 %. The highest yield of carbohydrate, protein and lipids were recorded as below.

Species	Carbohydrate (mg %)	Protein (mg %)	Lipid (mg %)
O.acuminata	14.45	56.28	31.00
O.subbrevis	22.70	34.00	40.90
P.tenue	14.19	26.30	44.00

The relative proportion of carbohytdrate, protein and lipid changed with the age of culture. The three species have higher protein and carbohydrate in the early stages of growth and lipids accumulated as the culture aged. The protein content of *O. acuminata* is comparable to that of *Spirulina*. *O. subbrevis* and *P. tenue* have proved to be better lipid accumulators than *Spirulina platensis*. The fatty acid profile showed the presence of both unsaturated and saturated fatty acids in the ratio 0.81, 1.5 and 1.12 for *O. acuminata, O. subbrevis* and *P. tenue* respectively. The presence of polyunsaturated fatty acids is of interest as they have demonstrated to be of food value, and in biotechnological applications. The ash content of *O. acuminata* (8.3 %) is within the stipulated standards of food grade *Spirulina*. Along with its high protein content, the species can be identified for food and feed. *O. acuminata* is again the better isolate for the production of pigments - chlorophyll *a* and phycocyanin, the yield being better than that of *Spirulina*. The production of pigments by the three species of Cyanobacteria was studied with respect to age of culture, pH and salinity of the growth medium. The optimum conditions for the best accumulation of pigments are as given below;

SI.No.	Parameter	*O. acuminata*	*O.subbrevis*	*P.tenue*
1	Age of culture (days)	16	12-16	12
2	pH(for *Chl *a)*	7.5	8.5	7.5
3	pH (for *PC)	7.5	7.5-9.5	7.5
4	pH (for *APC)	7.5	7.5-9.5	7.5
5	Salinity (for Chl. *a)*	$0-5 \times 10^{-3}$	15×10^{-3}	5×10^{-3}
6	Salinity (for PC)	0	$5-15 \times 10^{-3}$	5×10^{-3}
7	Salinity (for APC)	0	$5-15 \times 10^{-3}$	$0-5 \times 10^{-3}$

* Chl *a* - Chlorophyll *a*, PC - Phycocyanin, APC- Allophycocyanin

As *O. acuminata* was identified to be the better accumulator of pigments among the three species studied, further experiments were concducted to improve the extraction of phycocyanin, a product of high commercial value. Phycocyanin was extracted from *O. acuminata* using different extraction media. Ammonium aceatate, potassium phosphate, sodium phosphate and distilled water gave similar results qualitatively. Upon quantitative estimation, distilled water proved to be superior extraction medium for phycocyanin. The dryness of biomass affected the yield of phycocyanin pigments upon extraction. It was concluded from the above results that in term of environmental tolerance *O. subbrevis* and *P. tenue* would be more promising. *O. subbrevis* is a high biomass accumulator as well. Therefore this species would be of potential application in wastewater treatment. An industrial effluent and sewage effluent was subjected to evaluation of treatment by the Cyanobacteria under investigation. Industrial effluent was collected from the effluent treatment plant of Kochi Refineries Ltd. Sewage effluent was

collected from the aeration tank of a sewage treatment plant. The effluent properties were determined and assayed against three species to determine the survival of the species in the effluents. The growth was estimated as biomass on 8 th, 12th and 16 th day of inoculation. The biomass of *O. acuminata* was significantly low in both the effluents. The growth of *O. subbrevis* was similar to control in the industrial effluent, and significantly higher than the control in sewage effluent. *P. tenue* yielded significantly lower biomass in both effluents. Therefore *O. subbrevis* was identified to be the potential isolate for wastewater treatment. Anlysis of the effluent prior to and after growth of *O. subbrevis* for a period of twelve days showed that the conductivity of the industrial effluent decreased from 0.25 mmho/cm to 0.18 mmho/cm and that of sewage effluent from 0.35 to 0.20 mmho/cm indicating that the total dissolved substances in the effluent has been utilized for algal growth. However the pH of the effluent had risen from 7.4 to 9.5 (in refinery effluent) and from 7.9 to 8.3 (in sewage effluent).

The utilization of biomass resulting from biological treatment of wastewaters needs thorough investigation. The quantitative yield of chlorophyll *a* and phycocyanin by *O. subbrevis* when raised in the effluents was similar to that of the control cultures indicating that the effluent has no toxic action on the pigments. However the protein profile of the bomass had been affected. The protein extract of *O. subbrevis* raised in the regular growth medium had visible bands in the electrophoretic gel column from 10 to 62 kDa. In the effluent grown biomass the bands at 21 kDa and 26 kDa were absent. New protein bands were observed at 93 and 114 kDa in biomass raised on refinery effluent. The sewage grown biomass had new protein bands at 97 and 116 kDa region.

These results indicate that the effluent can affect the protein profile of the species. Further the heavy metal content of the biomass was analysed with respect to lead and cadmium. The species could accumulate 33.8 µg/g of lead from sewage and 22.6 µg/g of cadmium from the petroleum refinery effluent. The results strongly suggests that *O. subbrevis* can be effectively used for the removal of dissolved substances including metals at tertiary treatment level of petroleum refinery effluent and sewage treatment plants; but the resulting pH of the effluent has to be taken for. Therefore Cyanobacteria may be an effective tool for treatment of acidic effluents, as it may be possible to raise the pH of the effluent to neutral following growth of the species. This leaves ample scope for further research on the isolation and development of Cyanobacteria from extreme environments exhibiting wide environmental tolerance.

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