"Isolation, characterization and nutritional evaluation of cyanobacteria from Cochin estuary and the efficacy of *Synechococcus* sp. and *Synechocystis* sp. as feed supplement for *Oreochromis mossambicus* (Peters, 1852)"

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Under the faculty of

MARINE SCIENCES

By

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Certificate

This is to certify that the thesis entitled "**Isolation, characterization and nutritional evaluation of cyanobacteria from Cochin estuary and the efficacy of *Synechococcus* sp. and *Synechocystis* sp. as feed supplement for *Oreochromis mossambicus* (Peters, 1852)"** is a bonafide record of research work carried out by **Ms. Lekshmi S** under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements of the degree of **Doctor of Philosophy** under the faculty of Marine Sciences of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any University or Institution. I also certify that all the relevant corrections and modifications as suggested by the audience during the pre synopsis seminar and recommended by the Doctoral committee have been incorporated in this thesis.

Kochi-682016
September-2018

Dr. A.V. Saramma
(Supervising Guide)

Declaration

*I hereby declare that the thesis entitled "**Isolation, characterization and nutritional evaluation of cyanobacteria from Cochin estuary and the efficacy of Synechococcus sp. and Synechocystis sp. as feed supplement for Oreochromis mossambicus (Peters, 1852)**" is a genuine record of research work done by me under the supervision and guidance of Dr. A.V. Saramma, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology and that no part of this work has been presented for the award of any degree, diploma, associateship in any University or Institution earlier.*

Cochin
September, 2018

Lekshmi S

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Lekshmi S

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ABBREVIATIONS

A/G	Albumin to globulin ratio
AE	Ascorbic acid equivalents
AFA	*Aphanizomenon flos-aquae*
ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
APC	Allophycocyanins
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BLAST	Basic Local Alignment Search Tool
BMAA	β-N-methylamino-L-alanine
CFU/ml	Colony-forming unit/milliliter
Chl *a*	Chlorophyll *a*
CRD	Completely randomized design
cu.mm	cubic millimeter
CV-N	Cyanovirin-N
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2- diphenyl -1-picrylhydrazyl
ESR	Electron spin resonsnce
et al.	Co-authors
etc.	et cetera
FCR	Feed conversion ratio

g	Gram
g/dl	Gram per deciliter
g/Kg	Gram per kilogram
GAE	Gallic acid equivalence
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HLIPs	High light inducible polypeptides
IBM	International Business Machines
Isi A	Iron stress induced antenna protein
m	Meter
MAA	Mycosporine-like AminoAcids
MDA	Malondialdehyde
mg	Milligram
mg/L	Milligram per liter
mm	Millimeter
MVN	Microvirin
NCBI	National Center for Biotechnology Information
nm	Nanometer
O.D	Optical density
OAA	*Oscillatoria agardhii* agglutinin
OCP	Orange Carotenoid Proteins
OH	Hydroxyl radical
P3HB	Poly-3-hydroxybutyrate

PB	Phycobilins
PE	Phycoerythrin
PG	Propyl gallate
ppm	Parts per million
psu	Practical salinity unit
PUFA	Poly unsaturated fatty acids
QE	Quercetin equivalents
RBC	Red blood cells
ROS	Reactive oxygen species
RP-HPLC	Reverse phase High performance liquid chromatography
rpm	Revolutions per minute
Scp	Small CAB-like proteins
SGR	Specific growth rate
SODs	Superoxide dismutases
Sp.	Species
SPSS	Statistical Package for Social Sciences
SWEM	Sea water enrichment medium
TBHQ	Tert-butylhydroxyquinone
TCA	Trichloroacetic acid
TW	Terawatt
WBC	White blood cells
YEPD	Yeast extract peptone dextrose
$\cdot O_2^-$	Superoxide

˚C	Degree Celsius
µg/mg	Microgram per milligram
µg/ml	Microgram per milliliter
µl/L	Microliter per liter
µmol/L	Micromoles per liter
$^{1}O_{2}$	Singlet oxygen

Chapter 1

General Introduction

Contents

Cyanobacteria, the Gram negative photoautotrophs, constitute one of the oldest living organisms on the earth. Their evolutionary history dates back to 2.85 million years (Miller and Wheeler, 2012). They are the sole prokaryotic algal group belonging to the Kingdom Eubacteria. It is fascinating that these organisms share the characters of both bacteria as well as algae. Their distribution is ubiquitous and occur in diverse habitats such as freshwater, seawater and on damp soils and even in extreme and inhospitable places such as glaciers, desserts, hot springs and salt lakes (Hoek *et al*., 1995). They have an amazing ability to adapt to a wide range of environmental conditions such as high light intensities, temperature changes, salinity and pH variations, nutrient stress, eutrophication etc. (Whitton, 2012). They also exist in symbiotic relationships with a wide variety of organisms from plant and animal kingdom such as *Nostoc* sp. in Cycas roots, *Anabaena* and *Azolla*, *Symploca hydnoides* and *Dollabella auricularia*, *Richelia intercellularis* with *Hemiaulus hauckii*, *Rhizosolenia formosa* and *Chaetoceros* species (Miller and Wheeler, 2012).

Structurally, cyanobacteria are typical prokaryotes with a murein cell wall covered in a mucilaginous sheath. Membrane bound organelles are absent. The primary photosynthetic pigment chlorophyll *a* is present in the thylakoids which lie free in the cytoplasm. The accessory photosynthetic pigments, phycobiliproteins are responsible for the characteristic pigmentation. The genetic material DNA lies bundled up in the centre of the protoplasm. They reproduce asexually and sexual reproduction is completely absent. The reserve polysaccharide is cyanophycean starch (Hoek *et al*., 1995).

Morphologically the cyanobacteria can be unicellular, colonial or filamentous forms. The filamentous forms may be branched or unbranched with or without heterocysts which are specialized cells for performing nitrogen fixation. A total of 2000 species belonging to 150 genera have been described worldwide (Hoek *et al*., 1995). In all, 85 genera and 750 species were reported from India and its neighborhood (Desikachary, 1959). *Calothrix indica* was the first described species from India.

1.1 Importance of cyanobacteria

'Proterozoic era' also known as the age of cyanobacteria paved the way for the evolution of obligate aerobic organisms. Cyanobacteria have played a significant role in increasing the oxygen content of the atmosphere and making the earth a more habitable planet. Cyanobacteria contribute 20-30% of the Earth's total photosynthetic productivity (Hseih and Pedersen, 2015). They convert solar energy into chemical energy at the rate of 450 TW (Waterbury *et al*., 1979). They are a major component of the phytoplankton community in the aquatic environments. Recent studies revealed that two major picoplanktonic cyanobacterial species *Synechococcus* and *Prochlorococcus* account for about 20-90% of the total primary productivity in the tropical

ecosystems (Hoffmann, 1999). In addition to primary production, they also form food for numerous zooplankton including flagellates, ciliates, macro invertebrates, fish larvae etc. (Yahel *et al*., 1998).

Atmospheric nitrogen fixation is another important function carried out by cyanobacteria. They are the major source of nitrogen in oligotrophic oceanic waters. They also form an indispensible part in paddy cultivation, providing large amounts of nitrogen and phosphorus to the field. In addition they also secrete organic acids which increase and improve soil fertility, nutrient availability and water holding capacity. Besides nitrogen fixation they also help in phosphorus fixation and metabolism of carbon dioxide, hydrogen and oxygen (Singh *et al*., 2014).

In addition to the beneficial effects they can also have negative impacts. They are considered as nuisance algae in freshwater reservoirs (Watanabe *et al*., 1995). Cyanobacteria can form extensive blooms in both freshwater and seawater leading to many harmful effects such as oxygen depletion, vertebrate and invertebrate mortality and acute intoxication in mammals including humans (Paerl and Otten, 2013). They also play a major role in the deformation and deterioration of historical monuments (Macedo *et al*., 2009).

1.2 Human nutrition

The consumption of cyanobacteria as food dates back to early centuries. The people of the African country Chad have been harvesting and consuming *Spirulina* from time immemorial. The *Spirulina* delicacy is known as ‘dihe’ in local language. The biomass is used to prepare broths and soups and is sold locally and internationally. In Asia, *Nostoc commune* was harvested as large gelatinous sheets and consumed raw and dried. Similarly, *Nostoc punctiforme* was consumed in China, Mongolia and South America.

Aphanotheca sacrum is considered as a special delicacy in Japan known as "Suizenji-nori" (Gantar and Svircev, 2008). Though they are consumed since ancient times they are still considered in the unconventional food category (Marques *et al*., 2012). Currently only two species are widely marketed *Spirulina* and *Aphanizomenon flos-aquae*. The DIC Corporation Japan, Hainan Simai Pharmacy Co. China, Earthrise Nutritionals USA, Cyanotech Corp. USA, Blue Green Foods USA etc. are some of the industrial giants involved in the production. Approximately well over 3000 tons are produced worldwide and marketed in the form of powders, capsules, tablets, extracts, crystals etc. New products such as alga-based pastas, biscuits, bread, candy bars, yogurts and soft drinks are also finding entry into the market (Marques *et al*., 2012).

Studies report that the consumption of the nutrient dense cyanobacterial biomass is associated with numerous health benefits such as immune boosting, anticancerous, hypocholesterolemic and weight-loss effects (Henrikson, 1997). High protein, the presence of different vitamins, antioxidant rich pigments and easy digestibility are the main features that make them suitable for human consumption. Presence of different toxins such as β-N-methylamino-L-alanine (BMAA) and high nucleic acid content are some of the issues concerned with the consumption of cyanobacteria. On account of all these, cyanobacteria based food products have to pass through a series of rigorous food safety regulations. Currently, they are categorized as health foods or nutraceuticals.

1.3 Animal nutrition

Microalgae are the basic component of the food chain. They form food for a variety of organisms ranging from unicellular to multicellular forms. The survival, growth, development, productivity and fertility of the animals are a reflection of their health. Feed quality is the most important factor affecting

the animal health (Pulz and Gross, 2004). Studies have indicated that the microalgae can positively influence the health and overall performance of the animal. Even small quantities can enhance growth, improve non-specific immune response and disease resistance, antiviral and antibacterial action, improved gut function, better feed conversion, reproductive performance and weight control (Marques *et al*., 2012 and Pulz and Gross, 2004). They also improve the external appearance resulting in healthy skin and lustrous coat in both pets and farming animals (Certik and Shimizu, 1999). A study on the effect of *Spirulina* supplementation in genetically divergent Australian sheep showed that it increased body weight, growth and body conformation significantly (Holman *et al*., 2012). *Spirulina* can be used to improve the yolk colour of eggs (Saxena *et al*., 1983).

Over 30% of the world algal production is used in animal feed sector and over 50% of the *Spirulina* produced is used as feed supplements (Milledge, 2011). Feed quality and feed efficiency form an important concern in the aquaculture field. *Spirulina* has been used as a formulated feed ingredient in aquaculture feeds (Shields and Lupatsch, 2012). It has been proven that fish fed on *Spirulina* have better flavor, firmer flesh, brighter skin colour and reduced mortality rates (Liao *et al*., 1990). It is used for improving colouration of the gold fish in Japan and China (Wikfors and Ohno, 2001). *Spirulina* is also used in diets of various prawn species to enhance carotenoid content and survivability (Sivakumar *et al*., 2011 and Gadelha *et al*., 2013).

1.4 Bioactive compounds from cyanobacteria

Cyanobacteria are considered to be a rich source of novel bioactive metabolites. The first record of medicinal properties of cyanobacteria was made as early as 1500 BC when *Nostoc* species were used to treat gout, fistula and

several forms of cancer (Pietra, 1990). A total of 424 bioactive compounds have been reported from this group. Nostocales and Oscillatoriales are the prominent producers. These compounds are reported to have antibacterial, antifungal, antiviral, anticancerous, enzyme-inhibiting, immunomodulating, anti-inflammatory and antioxidant activities (Burja *et al*., 2001). Cyanovirin-N is an antiviral compound isolated from *Nostoc ellipsosporum*, which effectively inhibited the HIV virus. Borophycin, Cryptophycin and Curacin are also cyanobacterial metabolites which have excellent anticancerous activity (Marques *et al*., 2012). Cyanobacterin produced by *Scytonema hofmanni* and Fisherellin produced by *Fischerella muscicola* are potent herbicides and can also be used to control algal blooms (Borowitzka, 1995). There are numerous reports on the antimicrobial activity of cyanobacteria (Kulik, 1995 and Kreitlow *et al*., 1999). They can inhibit a wide range of Gram positive and Gram negative bacteria and also fungal pathogens. The alkaloid neurotoxins and the cyclic peptide hepatotoxins from different strains of cyanobacteria are used in tumour inhibition and anticancerous studies (Burja *et al*., 2001). The studies on the bioactivity of cyanobacteria indicate that they are potential sources of pharmacologically active extracts that could be further developed into potent drugs and medicines.

1.5 Other uses of cyanobacteria

The pigments from cyanobacteria serve as sources of natural colourants and dyes. They are applied in food, pharmaceutical and cosmetic industries (Pulz and Gross, 2004). Besides being safe, they also act as antioxidants and provide health benefits too (Kovac *et al*., 2013). The water soluble phycobilin pigments are commercially produced by DIC Corp, in the name of 'Lina Blue'. In China and Japan, it is already being used in food products such as chewing gums, candies, dairy products, jellies, soft drinks etc. (Kovac *et al*., 2013). They are also employed as fluorescent tags in flow cytometry and

immunology and as photosensitisers in photodynamic therapy for the treatment of cancers (Borowitzka, 2013).

Cyanobacteria are storehouses of high-value molecules. They can produce polyhydroxyalkonates such as poly-3-hydroxybutyrate (P3HB). *Spirulina*, *Synechocystis* and *Nostoc* can accumulate significant amounts of P3HB (Borowitzka, 2013). They may serve as potential sources of biodegradable plastics. Scytonemins and Mycosporine-like Amino Acids (MAA) are specific compounds produced by cyanobacteria to combat the effect of reactive oxygen species and free radicals. These compounds are incorporated into sunscreens for protection against ultraviolet radiation (Priyadarshini and Rath, 2012). *Spirulina* is reported to contain substantial quantities of γ-Linolenic acid (Vonshak, 2004). *Synechocystis* sp. serves as an important model organism for genetic studies (Ikeuchi and Tabata, 2001).

1.6 Significance of the study

Cyanobacteria represent a group of organisms with high untapped potential for both industrial and biotechnological applications. The study area, Cochin estuary, has rich cyanobacterial diversity but very few studies have been conducted in this respect. In the light of the recent researches, the cyanobacterial biomass could be utilized as sources of wholesome food materials, natural colourants, bioplastics, fine chemicals and bioactive substances. Natural resources are safe and eco-friendly. Present study is an attempt to identify potential cyanobacterial strains with good nutritional value, high antioxidant potential and antimicrobial activity. Since antibiotic resistance is a serious issue affecting both humans as well as the aquaculture sector there is a need for newer alternatives. Studies indicate that cyanobacteria supplementation is associated with numerous positive effects on

growth, immunity, survival and overall appearance of the cultured organism. In this context, an attempt was made to determine the efficacy of cyanobacteria supplementation on the growth and survival of a major cultured fish species *Oreochromis mossambicus* commonly known as the Java Tilapia.

1.7 Objectives of the present study:

i. Isolation, identification and purification of cyanobacteria from Cochin estuary.

ii. Biochemical characterization of selected strains.

iii. Evaluation of *in vitro* antioxidant activity of the strains.

iv. Screening of the strains for antimicrobial activity.

v. Determination of efficiency of selected strains as feed additives in aquaculture.

The thesis is divided into seven chapters. The first chapter gives a general introduction to the topic including the significance and objectives of the work. The second chapter deals with the isolation of cyanobacteria from Cochin estuary, their purification and morphological identification. The optimization of culture conditions and biochemical characterization of the strains are presented in the third chapter. In the fourth chapter, an analysis of the antioxidant potential of the strains using various *in vitro* assays is presented. The fifth chapter describes the antimicrobial activity of the strains. Sixth chapter deals with the effect of supplementing *Synechococcus* sp. and *Synechocystis* sp. on the growth and survival of *Oreochromis mossambicus*. The final chapter includes the summary and conclusions of the study.

Chapter 2

Isolation and culturing of cyanobacteria from Cochin estuary

Contents

2.1 Introduction

Cyanobacteria, the simplest autotrophic organisms with the longest evolutionary history, have inhabited almost all known environments on the earth. They are the principal contributors to open ocean and benthic primary production. The picoplanktonic cyanobacteria *Synechococcus* and *Prochlorococcus* contribute 20-90% of the photosynthetic productivity in different tropical and equatorial oceans (Hoffmann, 1999). Besides primary production, they form food for the planktonic heterotrophic flagellates, ciliates and numerous macroinvertebrates (Yahel *et al*., 1998). Another important role played by these organisms is their ability to fix atmospheric nitrogen. The filamentous non-heterocystous *Trichodesmium* species is the most important source of nitrogen in oligotrophic tropical oceans (Hoffmann, 1999).

The algal culturing started in the early nineteenth century. Beijerinck successfully cultured *Anabaena* in a nitrogen free medium for the first time (Andersen, 2005). Later on many algal culturing media and techniques were developed by algologists. The successful isolation depends on the

understanding and mimicking of the natural environmental conditions of the microalgae (Andersen, 2005). Temperature, salinity, pH, water quality and metal toxicity are the major factors affecting the establishment of microalgal culture. The elimination of potential contaminants is also a major concern during isolation and culturing (Andersen, 2005). Purification of cyanobacteria is equally challenging as isolation and identification. The simple morphology of cyanobacteria makes it difficult for precise identification. Though molecular techniques are available traditional taxonomy is still highly relied upon and helps in the basic identification process. There is a need for the development of advanced and sophisticated techniques for identification and culturing of these organisms.

Cochin estuary is the northward extension of the Vembanad lake. It is the second largest wetland ecosystem in India with an area of~ 25600 ha. It extends from Azhikode in the north to Thannermukkam bund in the south (9˚ 30'-10˚ 12' N to 76˚ 10'- 76˚ 29' E). Periyar and Muvattupuzha are the major perennial rivers draining into the estuary. It is connected to the Arabian Sea through the Cochin bar mouth which is responsible for the salt water intrusion and tidal fluxes in the estuary. The unique ecological conditions of the backwaters support diverse species of flora and fauna. It serves as a nursery ground for many species of finfishes, molluscs and crustaceans. The fishery resources provide raw material for the different industrial units located along the banks of the estuary. Intense fish and shell fish farming activities in the backwaters provide employment to a large section of the local population. The rich biodiversity and dynamic environmental conditions prevailing in the estuary make it suitable for different ecological and biotechnological studies.

The objective of the present chapter was to isolate different species of cyanobacteria from the Cochin estuary and to culture them under laboratory conditions.

2.2 Review of literature

The basic algal culturing started in late 1800s. Beijerinck (1890) successfully cultured pure algal cultures and later on phycologists developed many culture media and introduced several techniques for algal isolation and culturing. Newton and Herman (1979) developed a procedure for the successful isolation of cyanobacteria associated with the aquatic fern *Azolla*. An extensive account of different isolation and purification techniques and also the compositions of various culture media were given by Rippka, Waterbury and Stanier (1981). Stal and Krumbein (1985) isolated and characterized twenty two strains of unicellular, heterocystous and non-heterocystous cyanobacteria from a marine microbial mat. They also analyzed the salt tolerance and atmospheric nitrogen fixing ability of the strains. Waterbury and Wiley (1988) reported the importance of using new culture media such as natural sea water medium and artificial sea water medium for isolation and culturing of marine planktonic cyanobacteria. A solid culture medium containing agarose was developed by Shirai *et al*. (1989) for the isolation of *Microcystis* species.

Ferris and Hirsch (1991) employed nutrient saturated glass fibre filters to isolate axenic strains of cyanobacteria. Chazal, Smaglinski and Smith (1992) successfully used methods involving light variations for the isolation of cyanobacteria from remote arid regions of central Australia. They also analyzed the nitrogen fixation modes in the isolated strains. Hoffmann (1999) studied the diversity of cyanobacteria of the tropical marine ecosystem and concluded that temperature played a major role in their geographic distribution. The importance of major picoplanktonic cyanobacterial species and nitrogen fixing *Trichodesmium* was also highlighted in the study. A comparative study on the phylogenetic and morphological diversity of

cyanobacteria from four different soil desert crusts of the Colorado plateau were done by Garcia, Lopez and Nubel (2001).

Urmeneta *et al*. (2003) isolated twelve filamentous cyanobacteria from the Erbo Delta of Spain. They also determined the effects of various cryoprotectants such as methanol, glycerol and dimethyl sulphoxide for the preservation of the samples. The genetic diversity of thermophilic cyanobacteria from Japan, New Zealand, Italy and North American hot springs was studied by Papke *et al*. (2003). Sompong *et al*. (2005) studied the distribution of thermophilic cyanobacteria from different hot springs of northern Thailand. *Synechococcus lividus*, *Synechococcus* sp. and *Phormidium boryanum* were the predominant species in all sites.

Andersen (2005) made a broad account of the basic algal culturing techniques, which is highly useful in algal isolation, culturing and maintenance. An interesting study on the species composition and spatial distribution of cyanobacteria inhabiting tombstones from Bratislava, Slovakia was done by Uher (2008). Similarly, the biodiversity of cyanobacteria on historical monuments of the Mediterranean Basin was carried out by Macedo *et al*. (2009). Lopes *et al*. (2012) isolated forty four strains from three estuaries in Portugal and the diversity analyses showed that Chroococcales, Oscillatoriales and Nostocales were the most abundant groups in the study area. Pentecost (2014) observed and recorded the distribution and ecology of cyanobacteria in the rocky littoral zone of an English lake.

Wanigatunge *et al*. (2014) studied the genetic diversity of cyanobacteria from different habitats of Sri Lanka. They could identify a total of twenty four different genera belonging to orders Chroococcales, Oscillatoriales, Pleurocapsales and Nostocales. Liu *et al*. (2016) reported the distribution and diversity of blue green algae from Qinghai-Tibetan lakes and concluded that the relative abundance of the organisms was affected by

salinity. The cyanobacterial diversity of alkaline saline lakes in the Brazilian Pantanal wetlands was done by Costa *et al*. (2016) using morphological and molecular approaches. The study revealed that *Anabaenopsis denikinii* and *Arthrospira platensis* were the major bloom forming cyanobacteria. Celepli *et al*. (2017) followed a metagenomic approach for the identification of cyanobacterial community of the Baltic Sea. Unicellular picocyanobacteria such as *Synechococcus* and *Cyanobium* dominated the community. Kong *et al*. (2017) reported the diversity and community structure of cyanobacteria from the recycling irrigation reservoirs of Virginia. *Synechococcus* sp. was the most abundant cyanobacteria in the study sites. Dvorak *et al*. (2017) studied the cryptic diversity of cyanobacteria from less explored habitats using molecular techniques based on the 16S rDNA gene sequences.

The taxonomical identification of the cyanobacteria is highly challenging. One of the greatest works on the identification of cyanobacteria was done by Desikachary (1959) which is widely used for reference all over the world. An attempt to record the coastal and marine biodiversity of the Indian seas and their various ecosystems were carried out by Venkataraman and Wafar (2005). The biodiversity study on the epilithic cyanobacteria from the fresh water streams of Kakoijana reserve forest of Assam revealed the presence of twenty nine species of which majority were new records (Saha *et al*., 2007). Nagle *et al*. (2010) isolated ten cyanobacteria from different marine habitats such as open shore, estuarine and saltpans with the objective to characterize them for various biotechnological applications. The diversity of cyanobacteria from various fresh water bodies of Jodhpur showed the presence of thirteen major genera with high morphotypic diversity (Makandar and Bhatnagar, 2010).

A broad review on the state of knowledge of the coastal and marine biodiversity of the Indian Ocean countries was made by Wafar *et al*. (2011).

Pramanik *et al*. (2011) isolated and characterized eight halophilic cyanobacteria from the mangrove forests of Sunderbans. A study on the distribution pattern of cyanobacteria from hot water springs of Tattapani indicated that *Synechococcus elongatus, Phormidum* and *Mastigocladus laminosus* were present in all the sampling sites irrespective of the seasons (Mongra, 2012).

Suresh *et al*. (2012) could identify forty one species of cyanobacteria from both the Western and Eastern Ghats of India. In their study the microalgal community showed positive relationship with dissolved oxygen, salinity, nutrients and negative relationship with temperature and turbidity. Silambarasan *et al*. (2012) isolated marine cyanobacteria from the mangrove habitats of south east coast of India and this comprised of thirty nine different species. *Synechocystis salina, Oscillatoria salina, Phormidium ambiguum*, *Phormidium tenue* and *Spirulina major* were distributed in all mangrove sampling sites. The biodiversity of cyanobacteria from the estuarine sample from the south east coast indicated the dominance of unicellular forms such as *Aphanocapsa, Aphanothece, Chroococcus, Microcystis, Synechocystis* and *Synechococcus*. However *Chroococcus minutus, Oscillatoria subbrevis* and *Lyngbya aestuarii* were found to be the versatile species (Ramanathan *et al*., 2013).

A study on the cyanobacteria from polluted wetland waters of industrialized Sambalpur district of Odisha indicated that *Anabaena, Oscillatoria*, *Chroococcus* and *Phormidum* were the dominant species and their diversity was positively correlated to dissolved oxygen levels (Deep *et al*., 2013). Dadheech *et al*. (2013) analyzed the cyanobacterial diversity from three different habitats such as hot springs, pelagic and benthic sites. A metagenomic approach was also employed for the study. The prevalent phylotype mainly belonged to the Oscillatoriaceae family.

Singh *et al*. (2014) identified a total of twenty cyanobacterial species from high altitude lakes of Lahaul-Spiti. They could distinguish three distinct

groups based on the nutrient and physical environment demands. Keshari and Adhikary (2014) reported that the species of cyanobacteria belonging to genera *Hassallia*, *Tolypothrix*, *Scytonema*, *Lyngbya* and *Calothrix* were the major genera involved in the disfigurement of stone sculptures and monuments in India. They also conducted molecular phylogenetic studies to compare them with the temperate counterparts.

Cyanobacteria play a major role in agriculture by acting as nitrogen fixers. The diversity and distribution pattern analysis of cyanobacteria from the paddy fields of Chhattisgarh was analyzed by Singh *et al*. (2014). Vijayan and Ray (2015) studied the ecology and diversity of cyanobacteria from the Kuttanad paddy wetlands. *Chroococcus turgidus* and *Gloeotheca rupestris* were found to be the abundant species. Epipelic, epiphytic and planktonic forms of cyanobacteria were studied from the Barasat area of Kolkata by Mukhopadhyay and Naskar (2015). *Microcystis* and *Phormidium* were recorded for the first time from this site. Thingujam *et al*. (2016) studied the factors affecting the cyanobacterial productivity in Loktak Lake, Manipur and concluded that *Nostoc* sp. and *Anabaena* sp. were the most commonly occurring genera in the study area. Sincy and Saramma (2016) made an elaborate record of cyanobacteria of the Cochin estuary. A total of seventy five species were observed and the water temperature, salinity and euphotic depth positively affected the cyanobacterial density. Ram and Shamina (2017) studied the cyanobacterial diversity of different mangrove habitats of Kerala. The genus *Oscillatoria* exhibited the maximum distribution.

2.3 Materials and methods

2.3.1 Sampling sites

Ten stations from Cochin estuary were selected for the sampling. The details of the sampling sites are presented in Table 2.1 and Figure 2.1

Fig. 2.1 Map showing the sampling sites

Table 2.1 Details of the sampling sites

Sl.No	Stations	Position		Description
		Latitude	Longitude	
1	Eloor	10.0697˚N	76.3029˚E	Industrial belt
2	Varapuzha	10.0743˚N	76.2714˚E	Industrial belt
3	Vaduthala	10.0153˚N	76.2754˚E	Disposal of domestic waste
4	Bolgatty	9.987˚N	76.266˚E	Inland navigation and tourism operations
5	Marine Science jetty	9.9636˚N	76.2832˚E	Sewage outfall
6	Thevara	9.9426˚N	76.2986˚E	Sewage outfall
7	Kumbalam	9.9038˚N	76.3106˚E	Disposal of domestic waste
8	Aroor	9.8733˚N	76.3029˚E	Industrial belt
9	Panavally	9.8163˚N	76.3352˚E	Waste dumping from house boats
10	Murinjapuzha	9.8167˚N	76.3940˚E	Disposal of domestic waste

The sampling was carried out during March 2012 (pre-monsoon season). Out of the ten stations five stations recorded salinity near zero and hence were considered as freshwater stations and the rest five stations had salinity ranging between 10-30 psu.

2.3.2 Sampling procedures and processing

2.3.2.1 Hydrography of the study area

Standard procedures were followed for determining the physico-chemical parameters of the sampling sites.

Physico-chemical parameters	Methodology followed
Temperature	Standard mercury thermometer
pH	pH meter (Eutech eco Tester pH2)
Salinity	Hand held refractometer (Erma-Japan)
Euphotic depth	Secchi disc
Dissolved oxygen	Winkler method (Strickland and Parsons,1972)
Nitrite	Strickland and Parsons, 1972
Nitrate	Resorcinol method (Zhang and Fischer, 2006)
Phosphate	Strickland and Parsons, 1972
Silicate	Strickland and Parsons, 1972

2.3.2.2 Isolation, identification and purification of cyanobacteria

Enrichment technique was followed for the isolation of cyanobacteria (Andersen 2005). Ten liters of surface water was collected and concentrated to one liter. 150 ml of the water sample was then transferred to 250 ml conical flasks and enriched with Allen and Nelson medium (1910). Germanium dioxide (10 mg/l) was added to inhibit the growth of diatoms and cyclohexamide (100 mg/l) was used to eliminate the eukaryotes. The samples were incubated under 2000 lux light intensity at 24±2˚C with a photoperiodicity of 12:12 light and dark periods. The culture flasks were regularly shaken for proper mixing and aeration. This also prevented the cyanobacteria from attaching to the inner sides of the conical flask and ensured even distribution of cells.

To isolate nitrogen fixing cyanobacteria the sample was enriched with Allen and Nelson medium devoid of nitrate component. The culture conditions were same as above.

Identification was carried out based on the morphology as per the methods of Desikachary (1959) and Skulberg *et al*. (1993) using a Leica DM phase contrast microscope.

The initial cyanobacterial isolates from the natural environment may often give rise to mixed cultures. The purification of the mixed cultures was carried out by agar plating and serial dilution methods (Andersen, 2005). In the serial dilution technique 1ml of the cyanobacterial culture was inoculated into 9 ml of Allen and Nelson medium and mixed well. The dilutions were made up to 10^{-7} and the cultures were incubated. In agar plating technique samples from the different serial dilutions and directly from the initial culture flask were streaked onto Allen and Nelson agar medium containing 1.5% agar. The culture conditions for incubation were same as described for isolation.

2.3.2.3 Culture setup

The purified strains were maintained as semi continuous batch cultures in the algal culture laboratory of the Department. The culture conditions followed were same as that of isolation. The uni algal strains were regularly observed for cross contamination using light microscopy.

2.4 Results

2.4.1 Hydrography of the sampling sites

The hydrographical parameters of the sampling sites are represented in Table 2.2.

Table 2.2 Hydrography of the sampling sites

Stations	1	2	3	4	5	6	7	8	9	10
Temperature (˚C)	23	23	24	24	27	23	23	24	27	27
pH	7.5	7.6	7.8	7.8	8	7.8	7.8	8.1	7.8	7.6
Salinity (psu)	0	0	5	15	30	15	15	15	10	0
Depth (m)	4	4.8	4.3	3.8	3.1	3.3	3.2	3.4	4.9	5.4
Dissolved Oxygen (mg/L)	2.36	3.31	3.86	3.52	2.48	2.42	2.57	3.15	4.23	4.28
Nitrite (µmol)	0.02	0.19	0.09	0.13	0.19	0.20	0.22	0.16	0.09	0.05
Nitrate (µmol)	4.10	2.51	5.83	3.22	2.99	5.52	2.37	5.87	2.92	2.87
Phosphate (µmol)	1.128	0.157	1.110	0.054	0.010	2.033	1.572	2.045	1.874	1.262
Silicate (µmol)	4.216	3.189	6.081	3.865	2.723	7.15	3.28	6.21	2.35	2.27

2.4.2 Isolation, identification and purification

A total of twelve species of cyanobacteria consisting of both unicellular and filamentous forms were isolated from the sampling stations and purified. The isolated members belonged to four different orders such as Chroococcales, Synechococcales, Oscillatoriales and Nostocales of the class Cyanophyceae. The isolated strains were *Aphanocapsa litoralis, Chroococcus minutus, Lyngbya baculum, Oscillatoria limosa, Oscillatoria chlorina, Synechocystis salina, Synechocystis aquatilis, Synechococcus elongatus, Anacystis nidulans, Synechococcus cedrorum, Anabaena* sp. and *Gloeocapsa gelatinosa* (Plate 1 and 2). *Anabaena* sp. was the only heterocystous form among the isolated species.

Although twelve cyanobacterial strains were isolated and purified only seven strains could establish well in culture. The following were the stable cultures:

1. *Aphanocapsa litoralis*

Division : Cyanophyta

Class : Cyanophyceae

Order : Synechococcales

Family : Merismopediaceae

Genus : *Aphanocapsa*

Species : *litoralis*

Thallus was amorphous without a definite shape and mucilaginous. Cells were blue-green in colour and spherical to subspherical (4-6 µ diameters). They were densely or sparsely aggregated. The species occurs in marine environments.

2. *Chroococcus minutus*

Division : Cyanophyta

Class : Cyanophyceae

Order : Chroococcales

Family : Chroococcaceae

Genus : *Chroococcus*

Species : *minutus*

Cells were spherical or oblong, single and light bluish-green in colour with sheath 6-15 µ diameter and without sheath 4-10 µ diameter. The sheath was non-lamellated and colourless.

3. *Gloeocapsa gelatinosa*

Division : Cyanophyta

Class : Cyanophyceae

Order : Chroococcales

Family : Microcystaceae

Genus : *Gloeocapsa*

Species : *gelatinosa*

Cells were about 2.5 µ without sheath and with sheath 6.2-10 µ in diameter. Bluish-green colouration and the sheath are colourless. It becomes thick and lamellated in older cells.

4. *Oscillatoria limosa*

Division : Cyanophyta

Class : Cyanophyceae

Order : Oscillatoriales

Family : Oscillatoriaceae

Genus : *Oscillatoria*

Species : *limosa*

Thallus dark blue-green to brown, trichome was more or less straight and was not constricted at the cross-walls. 11-22 µ long, commonly 13-16 µ broad. Cells were 1/3-1/6 as long as broad and cross-walls were frequently granulated. The end-cell was flatly rounded with slightly thickened membrane.

5. *Synechocystis aquatilis*

Division : Cyanophyta

Class : Cyanophyceae

Order : Synechococcales

Family : Merismopediaceae

Genus : *Synechocystis*

Species : *aquatilis*

Cells were spherical. Occurs mostly as single or in twos. 5-6 µ broad and are pale blue-green in colour.

6. *Synechocystis salina*

Division : Cyanophyta

Class : Cyanophyceae

Order : Synechococcales

Family : Merismopediaceae

Genus : *Synechocystis*

Species : *salina*

Single, spherical cells join to form chains without mucilage envelopes. They were 2-3 µ broad and bluish-green in colour.

7. *Synechococcus cedrorum*

Division : Cyanophyta

Class : Cyanophyceae

Order : Synechococcales

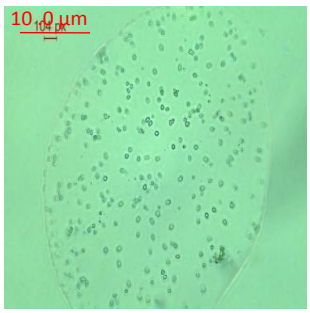
Family : Synechococcaceae

Genus : *Synechococcus*

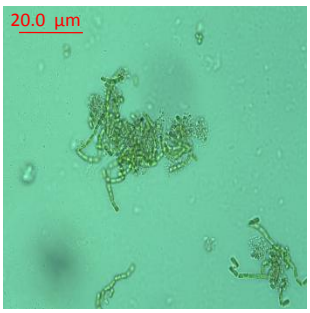
Species : *cedrorum*

Cells were elongate, ellipsoidal and finely rounded. Occurs as single or two together. 3-4 µ broad, 1¼-2 times as long as broad. Cells exhibited bluish-green colouration.

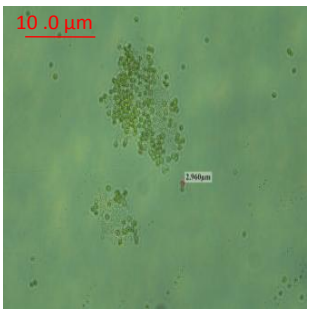
PLATE 1



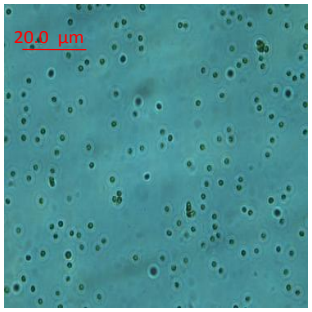
Aphanocapsa litoralis



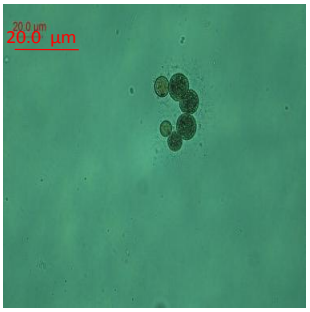
Anacystis nidulans



Chroococcus minutes



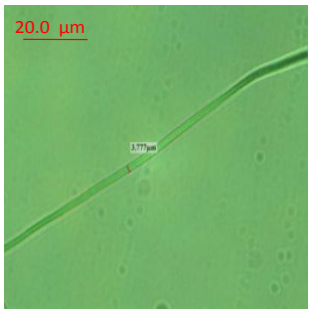
Gleocapsa gelatinosa



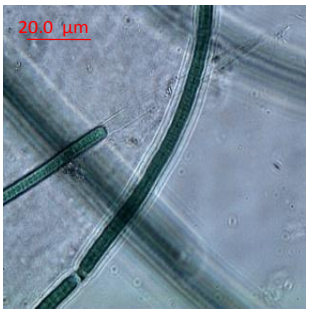
***Anabaena* sp.**

Oscillatoria limosa

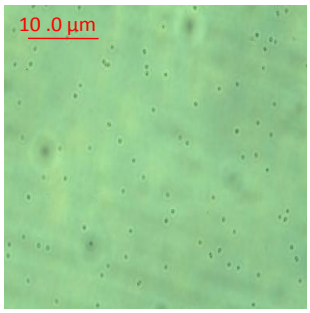
PLATE 2



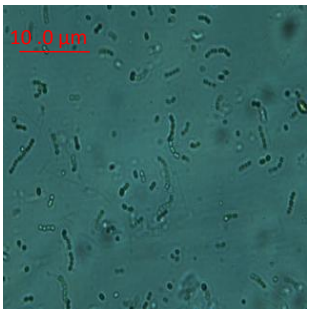
Oscillatoria chlorina



Lyngbya baculum

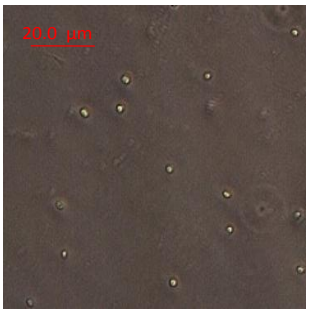


Synechococcus cedrorum



Synechocystis salina

Synechococcus elongatus



Synechocystis aquatilis

2.5 Discussion

Cyanobacteria are ubiquitous organisms widely distributed in both terrestrial and aquatic habitats. The long evolutionary history of the blue green algae has enabled them to survive in almost all hostile and unique environments on the earth. In all, 2000 cyanobacterial species belonging to 150 genera and five orders have been described so far (Hoek *et al*., 1995). Limited studies are available on the diversity and overall distribution of these organisms globally though ample information is available from numerous local habitats. The isolation and identification of cyanobacteria is a tedious process which might be a probable reason. Morphologically cyanobacteria can be unicellular or filamentous heterocystous and non-heterocystous forms and the distribution mainly depends on the environmental conditions.

Cochin estuary is a highly dynamic tropical positive estuary with an interconnected system of bays, lagoons, and swamps penetrating the mainland and enclosing many islands in between. The backwaters are bestowed with rich cyanobacterial diversity (Sincy, 2005). The phytoplankton analysis of Cochin estuary revealed that cyanobacteria formed the third most abundant group after diatom and dinoflagellates (Dayala *et al*., 2014). The isolation of cyanobacteria is a strenuous process requiring high skill and patience. In the present study twelve cyanobacterial species, both unicellular and filamentous forms, were isolated and identified. The isolated species were *Aphanocapsa litoralis, Chroococcus minutus, Lyngbya baculum, Oscillatoria limosa, Oscillatoria chlorina, Synechocystis salina, Synechocystis aquatilis, Synechococcus elongatus, Anacystis nidulans, Synechococcus cedrorum, Anabaena* sp. and *Gloeocapsa gelatinosa*. These isolated species represented the major cyanophycean flora of Cochin estuary. In elaborate seasonal studies, one hundred and sixteen species belonging to thirty one genera were reported

by Newby (2002) and seventy five species from twenty four genera were recorded by Sincy and Saramma (2016). *Synechocystis*, *Gloeocapsa*, *Chroococcus*, *Synechococcus*, *Gloeothece*, *Microcystis*, *Aphanocapsa*, *Aphanothece*, *Merismopedia*, *Oscillatoria*, *Phormidium* and *Anabaena* were the major cyanobacteria reported in their studies. In the current study only twelve species could be isolated as the sampling was done only once. In a similar study on cyanobacteria from Cochin estuary, Rajishamol (2013) could isolate seven species consisting of *Synechococcus elongatus*, *Synechocystis aquatilis*, *Spirulina gigantea*, *Lyngbya baculum* and three species of *Oscillatoria*.

Synechococcus species are widely distributed in freshwater as well as in marine systems (Hoffmann, 1999 and Kong *et al*., 2017). The community structure analysis of cyanobacteria from Cochin estuary revealed that *Synechococcus* was the dominant genus (Anas *et al*., 2015). In the present study also two species, *Synechococcus elongatus* and *Synechococcus cedrorum* were isolated. Unicellular and non-heterocystous forms were more abundant than heterocystous forms which indicate that the estuary is not nitrogen limited. Earlier studies also indicate similar observation (Newby, 2002 and Sincy 2005). The eutrophication of the coastal waters might be one of the reasons for the growth and abundance of unicellular and non-heterocystous cyanobacteria (Paerl, 1999). The isolated strains comprised both freshwater and marine species. The occurrence of freshwater strains in saline water indicates the halotolerant nature of the organisms. Newby (2002) and Sincy (2005) also reported the halotolerant nature of the cyanobacteria from the Cochin estuary.

The most outstanding study on the cyanobacteria of the Indian waters was made by Desikachary (1959). A detailed description and identifying

features of about seven hundred and fifty species have been recorded. In the current study also, the identification of the strains was mainly based on this work. In a recent study, ten different cyanobacteria were isolated from intertidal, rocky and swampy regions of the Western Ghats and the dominant groups were found to be *Synechocystis pevalekii*, *Synechococcus cedrorum*, *Oscillatoria* and *Spirulina* species (Nagle *et al*., 2010). However, the mangrove ecosystems were dominated by *Oscillatoria* and *Nostoc* species (Ram and Shamina, 2017). Studies from estuarine regions of south eastern India indicated that *Chroococcus*, *Microcystis*, *Synechococcus*, *Spirulina*, *Oscillatoria*, *Phormidium*, *Calothrix* and *Lyngbya* were the predominant genera irrespective of the seasons (Ramanathan *et al*., 2013 and Ramaswamy and Chandran, 2015). All these studies indicate the wide occurrence of the common cyanobacterial genera like *Synechocystis*, *Synechococcus*, *Nostoc*, *Oscillatoria*, *Spirulina* etc. The distribution of the group was universal irrespective of the habitats and environmental conditions. In comparison to the diversity of cyanobacteria of the Indian waters, the temperate waters were dominated by three major groups, Chroococcales, Oscillatoriales and Nostocales. The predominant species were found to be *Cyanobium*, *Synechocystis*, *Synechococcus*, *Leptolyngbya*, *Microcoleus*, *Phormidium*, *Romeria*, *Nostoc* and *Nodularia* (Lopes *et al*., 2012).

The physico-chemical parameters such as dissolved oxygen, salinity and nutrients positively influenced the distribution of cyanobacteria whereas, temperature and turbidity had a negative relationship (Suresh *et al*., 2012). However, Hoffmann (1999) had reported that temperature was the major factor limiting the geographic distribution of specific cyanobacterial microflora in the tropical marine ecosystems. Sincy and Saramma (2016) also observed that temperature, salinity and euphotic depth positively affected the

cyanobacterial density while nutrients had a negative impact when they were in excess.

The diversity and distribution of cyanobacteria is highly dependent on the nature of the habitat and the physico-chemical parameters prevailing in the area. However the versatile estuarine environments provide a congenial habitat for potential cyanobacterial strains which could be exploited for various biotechnological and industrial purposes. They can be employed in various physiological, biochemical and toxicological studies. They also provide an insight into the ecological conditions prevailing in the study area.

Chapter 3

Biochemical characterization of the isolated cyanobacteria

Contents

3.1 Introduction

Cyanobacteria are cultivated and consumed worldwide as an unconventional source of protein. *Spirulina*, *Nostoc*, *Aphanizomenon*, *Anabaena* and *Rivularia* are considered as high protein sources (Loaiza *et al*., 2016). The local populations in Mexico and Africa have been using *Spirulina* as food for centuries (Kovac *et al*., 2013). *Nostoc flagelliforme* is served as a delicacy in China. Similarly *Nostoc punctiforme* is used in human diets in Asia and South America (Gantar and Svircev, 2008). The unique nutritional composition of cyanobacteria comprising of proteins, polyunsaturated fatty acids, vitamins, amino acids, minerals, fibre content etc. makes them attractive candidates for the food and nutraceutical industries. The dried cyanobacterial biomass typically contains 46-63% protein, 8-17% carbohydrates and 4-22% lipids (Gantar and Svircev, 2008). They can synthesize all amino acids and contain starch, glucose, sugars and complex polysaccharides which make them suitable food for both animals and humans. The lipids found in cyanobacteria are mainly in the form of glycerol and fatty acids of ω-3 and ω-6 families

(Loaiza *et al*., 2016). Also compared to other microalgal species the cyanobacteria are easily digestible as their cell wall is made of peptidoglycan.

Microalgae are claimed to be "Super foods for Super Health" (Gantar and Svircev, 2008), as they possess antimicrobial, anticancerous, antidiabetic, immune boosting, hypocholesterolemic and weight-loss effects (Henrikson, 1997). Industrial giants such as Dainippon Ink & Chemicals and Cyanotech are manufacturing tons of *Spirulina* and *Apanizomenon flos-aquae* which are utilized as health foods and as formulated feed ingredients (Kovac *et al*., 2013 and Shields and Lupatsch, 2012). The cyanobacterial biomass can be used for the production of third-generation biofuels such as bioethanol, biodiesel and biogas (Mollers *et al*., 2014 and Silva *et al*., 2016). The pigments chlorophyll and phycobiliproteins can serve as natural colourants and dyes in the food industry and are available in the market as 'Lina-Blue'. The natural pigments have excellent antioxidant properties, minimum side effects and could also be employed as photosensitisers in photodynamic therapy for treatment of cancers (Borowitzka, 2013).

Cyanobacteria are a major component in the phytoplankton community and hence they serve as food for various aquatic organisms including fish larvae. Feed production is one of the most costly affairs in the aquaculture sector. So there have been several attempts to develop microalgae-based diets. An algae-based diet can positively affect the physiology and external appearance such as healthy skin and lustrous coat of the cultured organism (Spolaore *et al*., 2006). The efficacy of *Spirulina* supplementation has been studied in livestock, poultry, shrimp and various aquaculture feeds (Yaakob *et al*., 2014). Though the positive effects are proven the commercialization is limited to very few strains. The presence of toxins and the high cost of production are major issues affecting the microalgal industry. Current focus

should be on identifying potent strains from local habitats. Gene manipulation and new advances in algal biotechnology hold hope for the development of new and improved strains for human and animal feeds.

The present study analyses the biochemical and pigment content of seven cyanobacterial strains. Culture conditions such as light, temperature, culture medium, pH and salinity were optimized before the biochemical analysis.

3.2 Review of literature

3.2.1 Optimization of culture conditions

The growth of microalgae in both natural and controlled environments is strongly influenced by abiotic factors such as light intensity, temperature, pH, salinity and the availability of nutrients (Elias *et al*., 2012). Light is the most important factor affecting the growth of photoautotrophs. Zarrouk (1966) determined the response of *Spirulina maxima* to different levels of light intensity. Maloney (1966) observed that the microalgal physiology was affected by the nutrient concentrations in the culture medium. pH is also an important factor as it determines the solubility of carbon dioxide in the culture medium (Markl, 1977). Temperature affects the metabolic activities, influences the nutrient availability and its uptake by cells in the aqueous environment. Konopka and Brock (1978) studied the effect of temperature on the growth and photosynthesis of blue green algae from Lake Mendota and found that the optimum temperature for photosynthesis was between 20 and 30˚C.

Salinity plays a major role in the distribution and abundance of phytoplankton community. It also determines the density, viscosity and solubility of gases in the water column. Borowitzka (1986) found that organic compounds produced by cyanobacteria could act as osmoregulants. Kirst (1989) observed that salinity variations cause various physiological responses in

microalgae leading to osmotic adjustments. Zhu *et al*. (1997) studied the effects of two different temperatures on the growth and biochemical composition of the haptophyte *Isochrysis galbana* TK 1. A detailed account of the effect of light intensity, temperature and salinity on the growth of different *Spirulina* species was given by Vonshak (2004). He also described how the abiotic factors affected photosynthesis and respiration. Kitaya *et al*. (2005) observed that the light requirement for optimum growth varies depending on microalgae. Most of the microalgae including cyanobacteria prefer alkaline pH usually between 7 and 8 (Andersen, 2005). The optimum temperature, light and pH for improving the biomass production in *S. platensis* and *Spirulina fusiformis* was carried by Rafiqul *et al*. (2005). They concluded that favorable environmental conditions led to higher biomass production and improved the protein content.

Nagle *et al*. (2010) optimized the salinity, pH and the nutrient concentrations of *Synechococcus cedorum*, *Synechocystis pevalekii* and *Phormidium tenue*. The cyanobacteria showed wide salinity tolerance and they also observed that acidic pH was inhibitory to the growth of cyanobacteria. Ifeanyi *et al*. (2011) evaluated the optimum light intensity and salt concentrations affecting the growth and proliferation of *Aphanocapsa* cultures. The effect of different nutrient concentrations, temperatures, light intensities and pH on the growth of three microalga *Chlorella*, *Spirogyra* and *Oedogonium* was studied by Munir *et al*. (2015). They also observed that aerating the algal cultures gave better growth rates than static cultures. Abirami *et al*. (2017) optimized the nutrient conditions and temperature for enhancing the omega 3 fatty acid content in *Nannochloropsis gaditana*. Kushwaha *et al*. (2018) optimized the culture conditions of *Oscillatoria obscura* and *Lyngbya limnetica* for enhanced carbohydrate production.

3.2.2 Biochemical characterization

The mass production of microalgae and cyanobacteria gained momentum back in early 1940's and they were grown as food and feed by many countries (Burlew, 1953 and Borowitzka, 1997). A systematic evaluation of the chemical and environmental factors that influence the production of bioactive compounds in blue green algae were studied by Patterson *et al*. (1994). They also investigated the importance of optimizing the culture conditions to increase the yield of the bioactive compounds. Pushparaj *et al*. (1995) investigated the biomass output and biochemical composition of the marine cyanobacteria *Nodularia* sp. in open ponds and tubular photo bioreactors and compared the results. Borowitzka (1995) made an elaborate review on the ability of microalgae to produce pharmacologically important molecules.

The biochemical composition and fatty acid content of filamentous heterocystous nitrogen fixing cyanobacteria was studied by Vargas *et al*. (1998). In 2001, Mundt *et al.* screened and selected cyanobacteria for their biochemical composition and pharmacological activities. An extensive review on various commercial applications of microalgae in human nutrition, animal feed, cosmetics etc. was done by Spolaore *et al*. (2006). The fatty acid composition of twelve different microalgae including cyanobacteria *Chroococcus* sp. and *Synechococcus* sp. were reported by Patil *et al*. (2007). Gantar and Svircev (2008) highlighted the importance of the cyanobacteria as a food source and the issues and regulations associated with the marketing and consumption of cyanobacterial products. Lopez *et al*. (2010) compared the efficiency of Lowry's and Kjeldahl's methods for protein analysis in microalgal biomass. Rajeshwari and Rajashekhar (2011) analyzed the biochemical components of seven cyanobacteria isolated from different aquatic habitats of Western Ghats, Southern India.

Milledge (2011) reviewed the application of microalgae in various fields such as health foods, stable isotopic biochemicals, animal feed and human food. Hemaiswarya *et al*. (2011) made a review on the aspect of sustainability of microalgae as a feed source in aquaculture industry. Fernandez and Ballesteros (2012) analyzed the factors affecting the carbohydrate accumulation in cyanobacteria. Priyadarshani and Rath (2012) reviewed the commercial and industrial application of microalgae such as *Spirulina*, *Isochrysis*, *Chaetoceros*, *Chlorella* and *Dunaliella*. They projected the importance of exploring the microalgal resources for future applications. Shields and Lupatsch (2012) reported that though the good nutritional properties of algal biomass enable them to be utilized as animal feed the high cost of production acts as a hindrance to their future exploitation in the industrial arena. Hassan *et al*. (2012) determined the effect of different growth conditions on the biochemical composition of *Anabaena laxa*, *Anabaena fertilissima* and *Nostoc muscorum*. Markou *et al*. (2013) analyzed the potential of bioethanol production using carbohydrate-enriched biomass obtained from *A. platensis*. Borowitzka (2013) reviewed the various high-value products obtained from microalgae and the factors affecting their development and commercialization. Similarly Yaakob *et al*. (2014) made an over view on the different molecules produced by microalgae and cyanobacteria that could be utilized in the animal feed and aquaculture industries. Mollers *et al*. (2014) determined the effectiveness of cyanobacterial biomass as nutrient feedstock for bioethanol production by yeast fermentation. They observed that nitrogen limitation in the medium caused carbohydrate accumulation by 60% in *Synechococcus* sp. PCC7002.

Encarnacao *et al*. (2015) made a broad review of bioactive compounds and the fine chemicals obtained from cyanobacteria and microalgae. Flor and

Pilar (2015) analyzed the biochemical composition and the nutrient utilization pattern of the cyanobacteria *Geitlerinema lemmermanii*. Rai and Rajashekhar (2015) determined the pigment content of nine cyanobacteria including filamentous species isolated from Arabian Sea coast. Munir *et al*. (2015) reported the lipid content of chlorophycean members *Spirogyra*, *Chlorella* and *Oedogonium*. They found that artificial fluorescent light increased the growth of algae as compared to the natural light. Akgul *et al*. (2015) analyzed the protein content and amino acid composition of two cyanobacteria *Rivularia bullata* and *Nostoc pongiaeforme* isolated from Ayazma stream of Canakkale of Turkey. Hossain *et al*. (2016) measured the phycobiliproteins of four cyanobacteria species and concluded that the strain *Lyngbya* could be a potent source of the phycobiliproteins.

Abeer and Mohamad (2016) investigated the fatty acid profile, pigment and biochemical composition of cyanobacterial mats isolated from lakes of north western desert of Egypt. The effect of different concentrations of sodium bicarbonate on biomass production and carbohydrate accumulation in the cyanobacterial strain *Synechococcus* sp. PCC7002 was analyzed by Silva *et al*. (2016). The study by Finkel *et al*. (2016) threw light on the elemental stoichiometry of macromolecules present in microalgae. They also analyzed the phylogenetic differences in macromolecular composition leading to differences in cellular architecture and biochemistry. Loaiza *et al*. (2016) determined the importance of nitrogen concentration on the biochemical composition of *Nostoc* and *Anabaena* species. A review on the potential of employing microalgae as a natural functional food was given by Lopez *et al*. (2017). Arias *et al*. (2018) analyzed the effect of different photoperiods and nutrient conditions such as nitrogen and phosphorus limitation on the carbohydrate and polyhydroxybutyrates production in mixed cyanobacterial cultures.

3.3 Materials and methods

3.3.1 Optimization of culture conditions

3.3.1.1 Light

The optimum light intensity for the growth of the seven isolated strains was determined by growing them at three different light intensities 700, 1400 and 2100 lux in a versatile environmental chamber (Sanyo, Japan). The experiment was conducted for a period of thirty days and growth was measured in terms of chlorophyll *a*, every third day to determine the optimum light intensity for maximum growth.

3.3.1.2 Temperature

To determine the optimum temperature for growth, the strains were grown at three different temperatures 20, 25 and 30˚C in the environmental chamber. Growth was analyzed as described above.

3.3.1.3 pH

The pH supporting the maximum growth of the cyanobacteria was analyzed by determining the growth at different pH ranging from acidic to alkaline (pH 6-10). The experiment was conducted for thirty days and chlorophyll *a* was measured for determining the growth.

3.3.1.4 Salinity

The optimum salinity for growth was determined by growing the strains at different salinity ranging from 5 to 30 psu for a period of thirty days and Chlorophyll *a* was measured on every third day.

3.3.1.5 Medium

The isolated cyanobacterial strains were cultured in natural seawater containing four different algal culture media such as Allen and Nelson (1910), BG-11 (modified, Andersen, 2005), SN (Waterbury *et al*., 1986) and Sea water enrichment medium (Subramanian *et al*., 1999) for a period of thirty days. The medium supporting the highest biomass production was selected based on the chlorophyll *a* content. The chlorophyll content was determined every third day of the experiment up to thirtieth day.

3.3.1.5a Determination of chlorophyll *a*

Chlorophyll *a*, the primary photosynthetic pigment in cyanobacteria was analyzed by the method of Mackinney (1941). 5 ml of the cultures in the logarithmic phase was centrifuged at 5000 rpm for 5 minutes. The pellets were then suspended in 4 ml methanol (80%) and vortexed thoroughly. The sample was incubated in a water bath set at 60˚C for 1 hour in dark, with occasional shaking to prevent photooxidation. The sample was cooled and centrifuged for 10 minutes at 5000 rpm. The supernatants were pooled till the complete extraction and were made up to a known volume with methanol. The absorbance was read at 663 nm against methanol blank. The values were expressed as µg/mg.

Amount of Chl *a* = A663 x 12.63

'A' is the absorbance of the sample at 663 nm

12.63 is the extinction co-efficient of Chl *a* at 663 nm

3.3.2 Determination of biochemical content

3.3.2.1 Estimation of total protein

The procedure described by Lowry *et al*. (1951) was followed for protein estimation. The algal sample was treated with trichloroacetic acid

(TCA) to precipitate the proteins (Berges *et al*., 1993). The crude extract was mixed with 25% cold TCA and kept in an ice bath for 30 minutes followed by centrifugation at 15000 rpm for 20 minutes at 4˚C. The pellet was rinsed with TCA (10%), centrifuged for 2 minutes at 40˚C. The pellet was again solubilized in 5% TCA and centrifuged (15000 rpm) for 2 minutes at 20˚C. The protein pellet was suspended in 2 ml of 0.1 N NaOH for protein assay.

1 ml of protein sample was mixed with 1 ml of reagent E and kept for incubation at room temperature for 15 minutes. 3 ml of reagent D was added and mixed immediately. The sample was incubated for 45 minutes at room temperature. The intense blue colour developed was spectrophotometrically measured at 540 nm against a reagent blank. A standard curve was plotted with bovine serum albumin (20-100 µg/ml).

Reagent A: 100g Na_2CO_3 dissolved in 1000 ml 0.5 N NaOH

Reagent B: 1g $CuSO_4.5H_2O$ dissolved in 100ml of double distilled water

Reagent C: 2g Potassium tartarate dissolved in 100ml of double distilled water

Reagent D: 5 ml of Folin Ciocalteau reagent diluted with 50 ml of double distilled water

Reagent E: 15 ml reagent A, 0.75 ml reagent B and 0.75 ml reagent C mixed together.

3.3.2.2 Estimation of total carbohydrates

The total carbohydrate present in the algal sample was determined by the phenol sulphuric acid method of Dubois *et al*. (1956). To 1 ml of the cyanobacterial sample 1 ml of 5% phenol was added followed by 5 ml of sulphuric acid (96%). This was mixed well and the sample was allowed to stand

for 30 minutes at room temperature. The absorbance was measured at 490 nm. The standard calibration curve was plotted with glucose (20-100µg/ml).

3.3.2.3 Estimation of total lipids

Phosphovanillin method was followed for the estimation of lipids. The method was a combination of procedures described by Folch *et al*. (1957) and Barnes and Blackstock (1973). 5 ml of algal sample was filtered through Whatman No.1 filter paper and 5 ml of chloroform: methanol solvent (2:1) was added. The sample was macerated and incubated in a water bath at 60˚C for 30 minutes. The volume was made up to 5 ml with the solvent and from this 1 ml was taken and kept in the dessicator for overnight incubation. 1 ml of concentrated sulphuric acid was added and incubated for 10 minutes at 60˚C. Finally 5ml of phosphovanillin reagent was added and incubated for 30 minutes at room temperature. The colour complex developed was read at 520 nm.

3.3.3 Estimation of pigments

3.3.3.1 Determination of chlorophyll *a*

The chlorophyll *a* content of the strains was determined as per the procedure described in section 3.3.1.5a.

3.3.3.2 Determination of carotenoids

Carotenoids were estimated according to the method of Jensen (1978). 5 ml of cyanobacterial culture was centrifuged at 5000 rpm for 5 minutes and the pellet was suspended in 3 ml 85% acetone and incubated in dark for 45 minutes. The sample was then centrifuged for 5 minutes at 5000 rpm. The process was repeated till the supernatants became clear. The supernatants were pooled similar to the procedure for chlorophyll extraction and the volume was

made up to 10 ml with acetone. The absorbance was read at 450 nm with acetone as blank. The values were expressed as µg/mg.

$$C= (D \times V \times f) \times 10 /2500$$

Where, D= O.D. at 450 nm; V= Volume of the extract; f = Dilution factor; 2500= average extinction co-efficient of pigment

3.3.3.3 Determination of phycobiliproteins

Phycobiliproteins were estimated according to Siegelman and Kycia (1978). 5 ml cyanobacterial culture was centrifuged at 5000 rpm for 5 minutes to obtain the pellet which was then suspended in 3 ml of phosphate buffer followed by repeated freezing and thawing and the contents were centrifuged at 5000 rpm for 5 minutes. The supernatant was refrigerated and the absorbance was taken at 565, 615 and 652 nm against phosphate buffer blank. The values were expressed as µg/mg. The amounts of phycobiliproteins were calculated as shown below:

Amount of c- phycobilins (PB) = $A615 - 0.474(A652) \div 5.34$

Amount of c- allophycocyanin (APC) = $A652 - 0.208(A615) \div 5.09$

Amount of c- phycoerythrin (PE) = $A562 - 2.41(PC) - 0.849(\text{APC}) \div 9.6$

3.3.4 Statistical analysis

All experiments were done in triplicates and the results were expressed as mean value ± standard deviation. One-way ANOVA was carried out to determine significant difference if any, between the various culture conditions chosen. To determine whether there was any significant difference in the biochemical composition of the strains, univariate analysis of variance was done followed by post hoc tukey tests in IBM SPSS statistics 22 software package. $p < 0.05$ was considered as significant.

3.4 Results

3.4.1 Optimization of culture conditions

The results of the optimization of culture conditions of the test cyanobacteria indicate that all strains follow the typical sigmoid growth pattern. The initial lag phase is followed by an exponential phase which extends up to the 24th day followed by the stationary phase and decline phase. The maximum chlorophyll *a* content was obtained on the 24th day for all the cyanobacterial cultures and the content decreased towards the stationary phase. The test cyanobacteria exhibited growth at all the culture conditions tested.

3.4.1.1 Light

The strains showed growth at the three light intensities tested. The maximum biomass production for all strains was obtained at 1400 lux followed by 2100 and 700 lux. There was no significant difference in growth between the light intensities tested ($p>0.05$). The growth of the strains at different light intensities is shown in the figures 3.1 to 3.7.

A. litoralis exhibited the maximum chlorophyll *a* content of 3.86±0.06 µg/ml on 24th day at 1400 lux. The least chlorophyll *a* content was recorded at 700 lux (3.12±0.05 µg/ml).

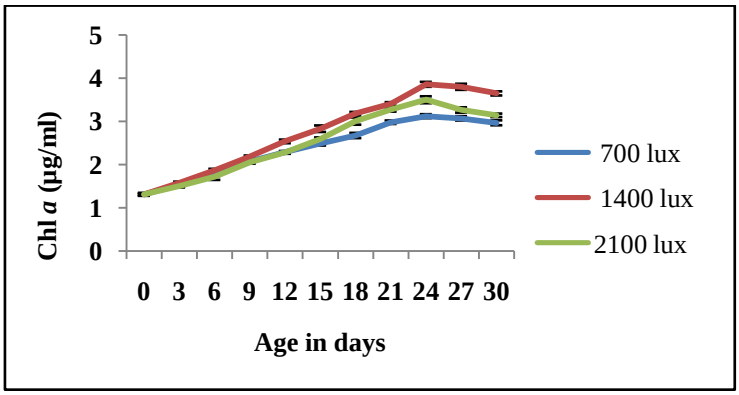


Fig. 3.1 Growth of *A. litoralis* at different light intensities

C. minutus showed the maximum chlorophyll *a* of 5.97±0.07µg/ml at 1400 lux on the 24th day. The second highest was obtained at 2100 lux and least chlorophyll *a* content was observed at 700 lux.

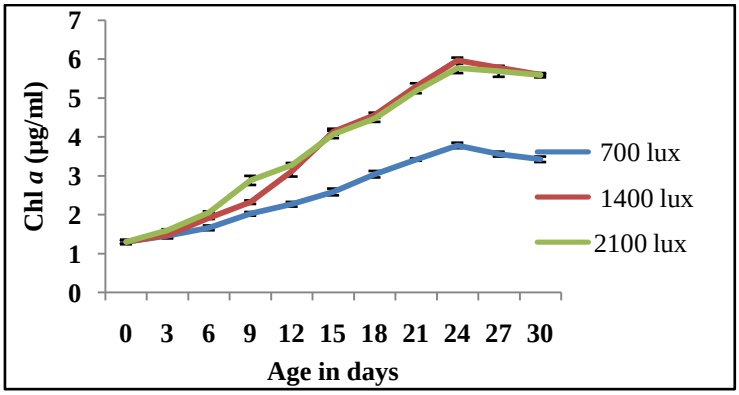


Fig. 3.2 Growth of *C. minutus* at different light intensities

Maximum chlorophyll *a* of 5.97±0.09 µg/ml was obtained for *G. gelatinosa* at 1400 lux on 24th day. At 2100 lux the strain recorded 5.77±0.12 µg/ml chlorophyll *a*. Least value was observed at 700 lux (4.72±0.04µg/ml).

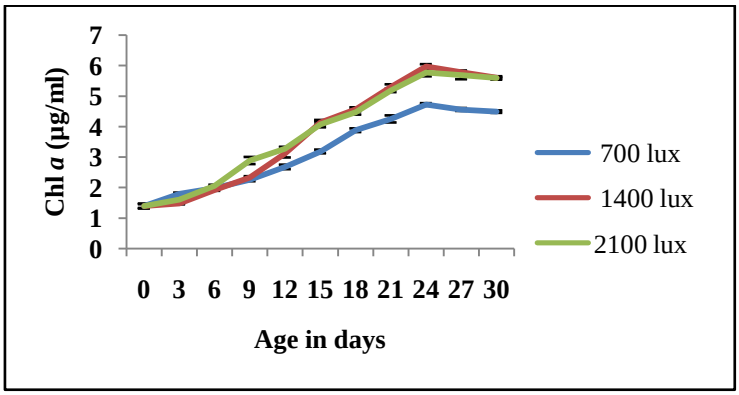


Fig. 3.3 Growth of *G. gelatinosa* at different light intensities

O. limosa showed maximum chlorophyll *a* content of 4.42±0.05 µg/ml at 1400 lux and 4.22±0.07 µg/ml at 2100 lux.

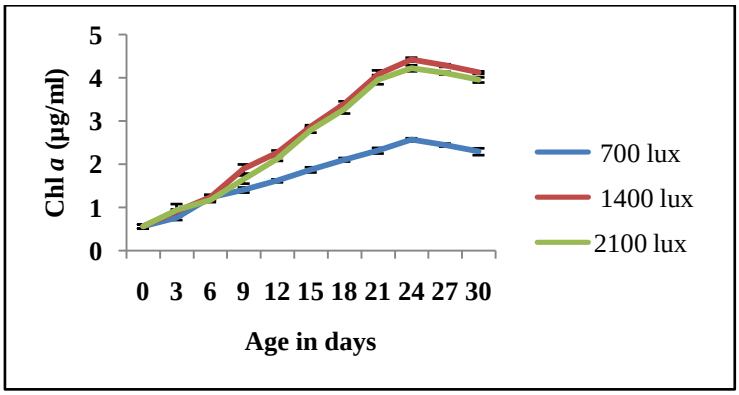


Fig. 3.4 Growth of *O. limosa* at different light intensities

The chlorophyll *a* content recorded by *S. aquatilis* at 1400 and 2100 lux was almost comparable. 3.67±0.04 µg/ml was recorded at 1400 lux and 3.55±0.05 µg/ml was obtained at 2100 lux on the 24th day of the experiment. Least value of 3.26±0.04 µg/ml was observed at 700 lux illumination.

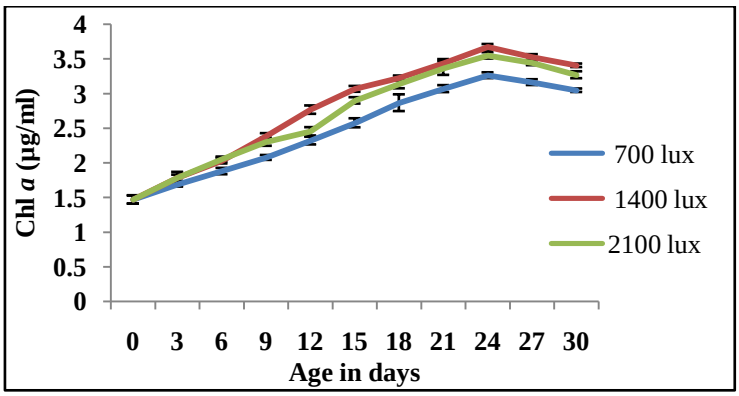


Fig. 3.5 Growth of *S. aquatilis* at different light intensities

S. salina expressed highest chlorophyll *a* content of 4.70±0.04 µg/ml at 1400 lux and least chlorophyll *a* content of 4.18±0.07 µg/ml at 700 lux.

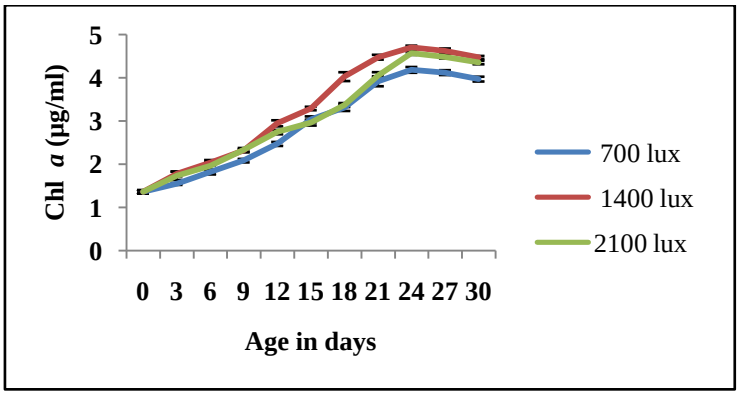


Fig. 3.6 Growth of *S. salina* at different light intensities

S. cedrorum exhibited maximum chlorophyll *a* content at 1400 lux illumination and minimum at 700 lux. Highest value obtained was 4.52±0.06 µg/ml and least value recorded was 3.79±0.10 µg/ml on the 24th day.

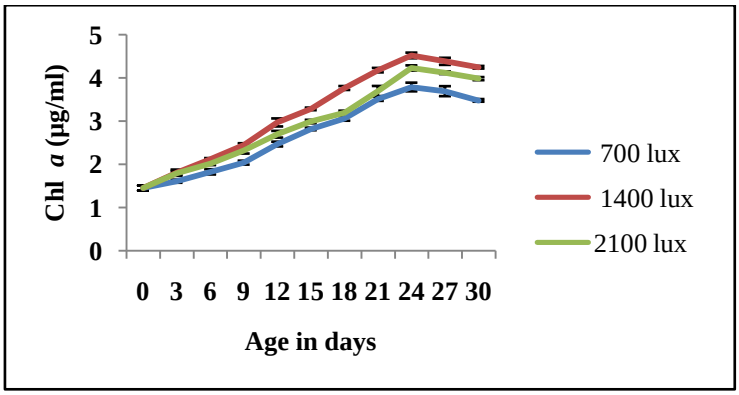


Fig. 3.7 Growth of *S. cedrorum* at different light intensities

3.4.1.2 Temperature

All the tested temperatures (20, 25 and 30˚C) were found to be suitable for the growth of cyanobacteria. 25˚C favored highest growth for all the strains. The second best growth was obtained at 20˚C and comparatively minimum growth was observed at 30˚C. The statistical analysis indicated that there was no significant difference in growth at different temperatures tested ($p>0.05$). The figures 3.8 to 3.14 represent the growth of the test strains at different temperatures.

The maximum chlorophyll *a* content of 4.20±0.04 µg/ml was obtained in *A. litoralis* cultures at 25˚C on 24th day. The second highest value was observed at 20˚C (3.75±0.12µg/ml) and minimum at 30˚C (3.59±0.08 µg/ml).

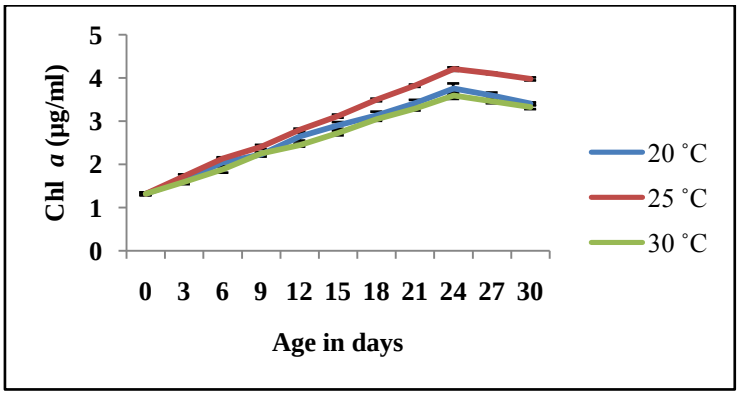


Fig. 3.8 Growth of *A. litoralis* at different temperatures

The highest chlorophyll *a* content for *C. minutus* was observed at 25˚C (6.16±0.05 µg/ml) and least at 30˚C (5.94±0.07 µg/ml).

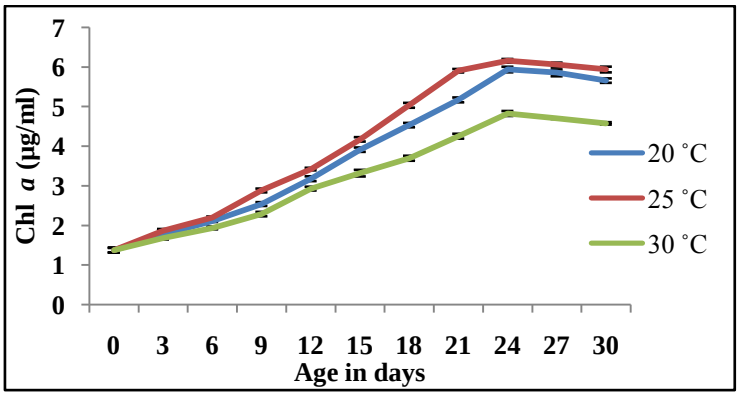


Fig. 3.9 Growth of *C. minutus* at different temperatures

G. gelatinosa showed a maximum of 6.44±0.05 µg/ml chlorophyll *a* at 24˚C and 6.24±0.05 µg/ml at 20˚C and a minimum of 5.35±0.04 µg/ml at 30˚C.

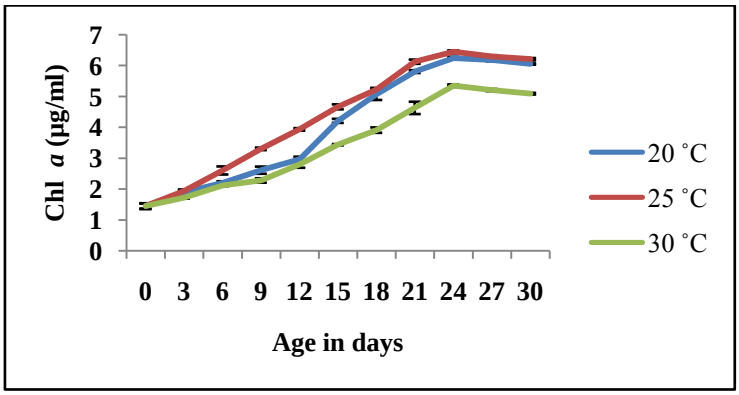


Fig. 3.10 Growth of *G. gelatinosa* at different temperatures

The maximum chlorophyll *a* content recorded in *O. limosa* at 25˚C was 2.81±0.03 µg/ml on the 24th day and least value observed was 2.47±0.04 µg/ml at 30˚C.

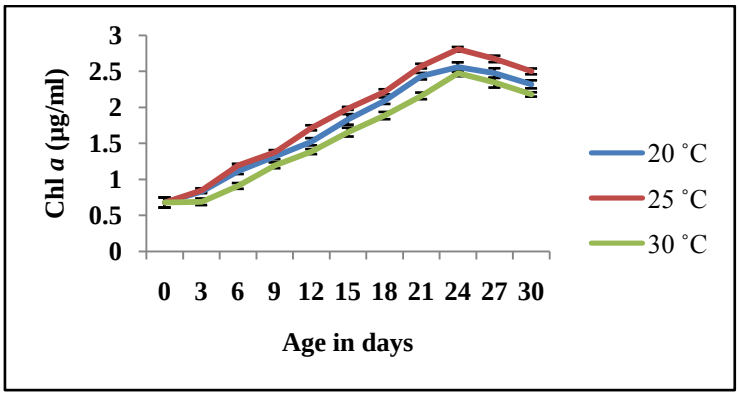


Fig. 3.11 Growth of *O. limosa* at different temperatures

S. aquatilis registered a maximum of 4.54±0.06 µg/ml chlorophyll *a* on the 24th day at 25˚C and the second highest value of 4.32±0.06 µg/ml was obtained at 20˚C.

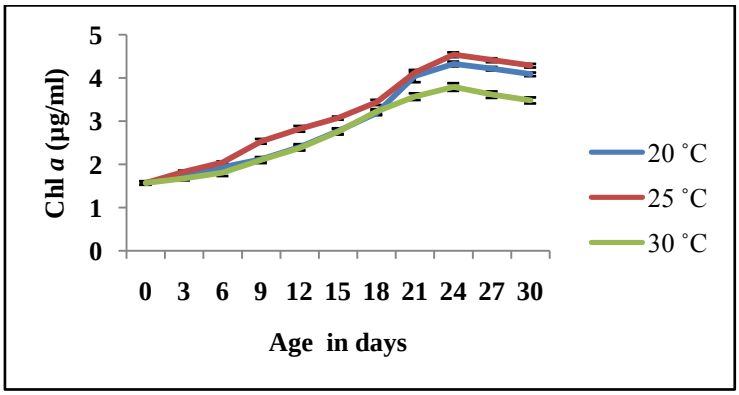


Fig. 3.12 Growth of *S. aquatilis* at different temperatures

S. salina recorded a maximum chlorophyll *a* of 4.23±0.05 µg/ml at 25˚C and the second highest was obtained at 20˚C (4.11±0.04 µg/ml) and the lowest value was observed at 30˚C (3.59±0.07 µg/ml).

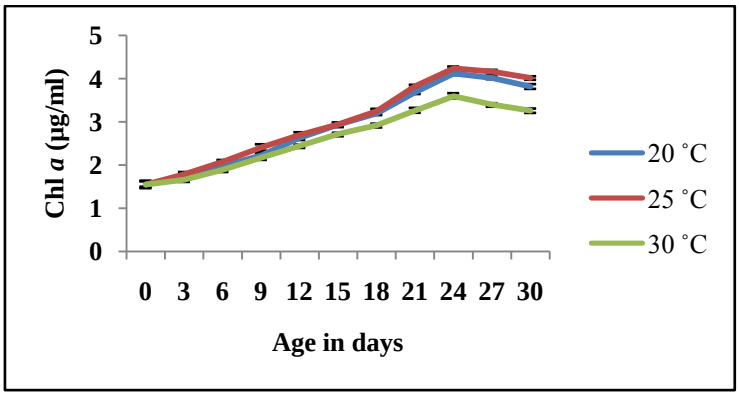


Fig. 3.13 Growth of *S. salina* at different temperatures

S. cedrorum showed a maximum of 4.59±0.04 µg/ml chlorophyll *a* at 25˚C and a minimum value of 3.54±0.07 µg/ml at 30˚C on the 24th day of the culture.

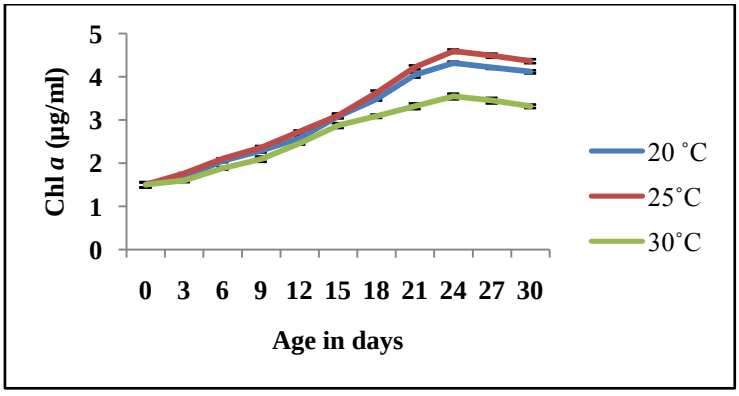


Fig. 3.14 Growth of *S. cedrorum* at different temperatures

3.4.1.3 pH

The strains could grow at all the pH tested (6-10). pH 8 was found to be the optimum for all the seven strains. The second highest growth was

supported by pH 9 followed by pH 10. The growth was minimum in pH 6 indicating that the cyanobacteria preferred alkaline pH. There was significant difference in the growth of the strains at different pH ($p<0.05$) except for *A. litoralis* ($p>0.05$). Figures 3.15 to 3.21 represent the results.

Maximum chlorophyll *a* obtained in *A. litoralis* culture was 6.09±0.02 µg/ml at pH 8 followed by 4.56±0.01 µg/ml at pH 9 and least at pH 6 (3.41±0.04 µg/ml).

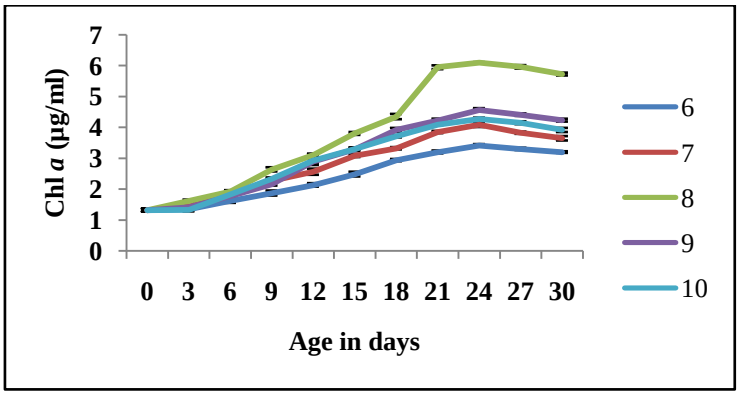


Fig. 3.15 Growth of *A. litoralis* at different pH

C. minutus recorded the highest chlorophyll *a* value of 4.50±0.07 µg/ml at pH 8. 4.19±0.05 µg/ml and 3.28±0.07 µg/ml were registered at pH 9 and pH 10 respectively. Least value observed was 3.11±0.07 µg/ml at pH 6.

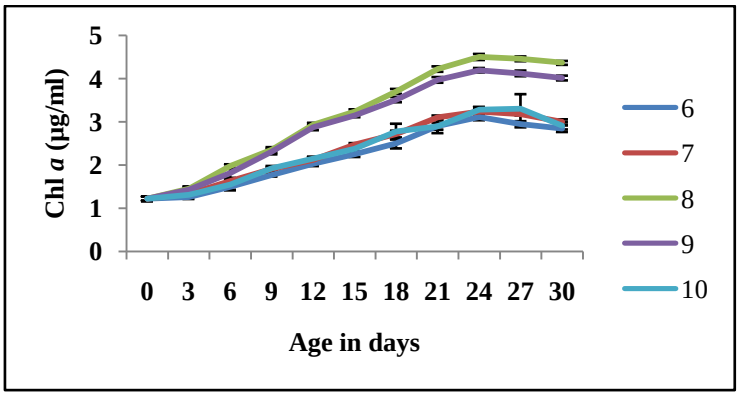


Fig. 3.16 Growth of of *C. minutus* at different pH

The maximum chlorophyll *a* observed in *G. gelatinosa* was 5.65±0.09 µg/ml at pH 8. The second highest value of 4.91±0.08 µg/ml was observed at pH 9. The minimum value recorded was 3.02±0.05 µg/ml at pH 6.

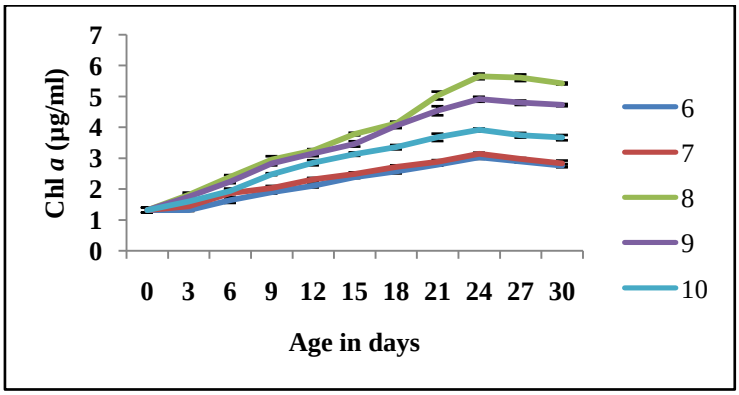


Fig. 3.17 Growth of *G. gelatinosa* at different pH

O. limosa showed maximum growth at pH 8 (3.36±0.08 µg/ml). The second best value obtained was 2.69±0.05 µg/ml at pH 9. 2.30±0.06 µg/ml was recorded at pH 10 and least value of 1.42±0.06 µg/ml was found at pH 6.

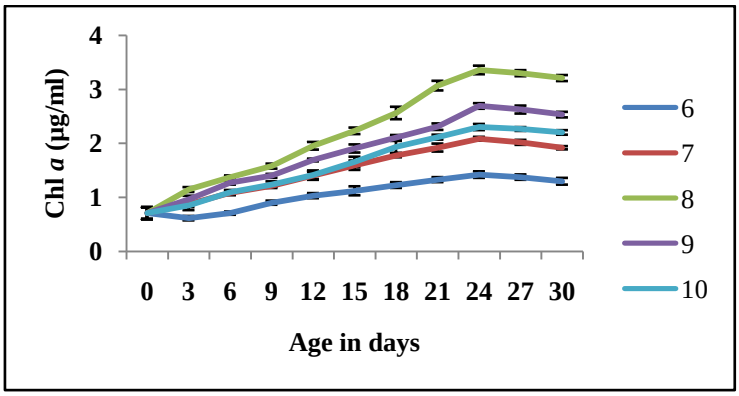


Fig. 3.18 Growth of *O. limosa* at different pH

S. aquatilis showed maximum chlorophyll *a* content of 3.88±0.04 µg/ml at pH 8. 3.34±0.09µg/ml and 3.30±0.08 µg/ml were recorded at pH 9 and 10 respectively. A maximum of 2.94±0.03 µg/ml chlorophyll *a* was observed at pH 7 and 2.67±0.07 µg/ml was observed at pH 6.

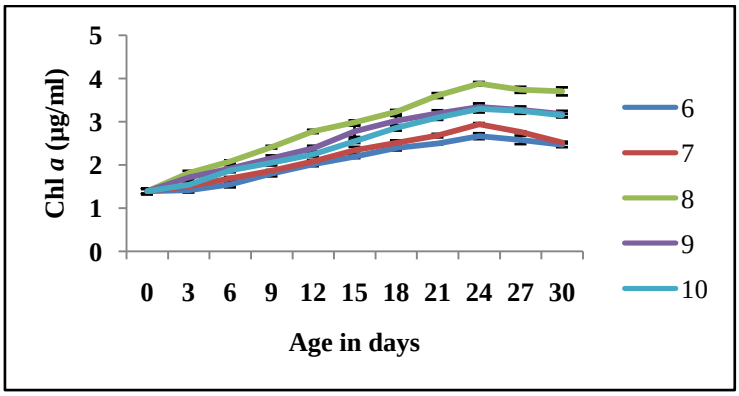


Fig. 3.19 Growth of *S. aquatilis* at different pH

4.05±0.10 µg/ml was the maximum chlorophyll *a* content recorded in *S. salina* at pH 8. At pH 9 the chlorophyll *a* was 3.37±0.06 µg/ml and the least value obtained was 2.27±0.04 µg/ml at pH 6.

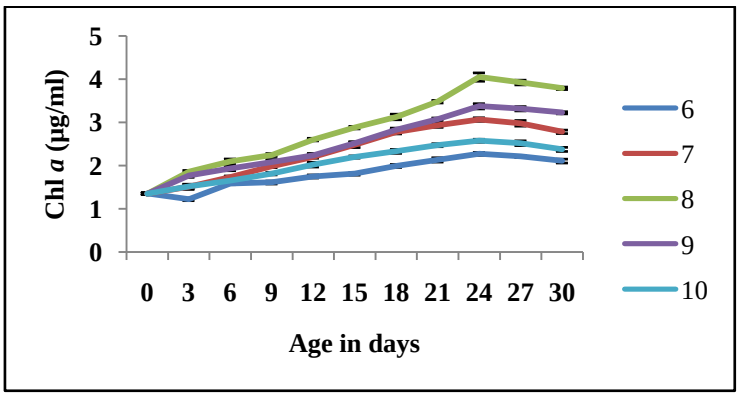


Fig. 3.20 Growth of *S. salina* at different pH

S. cedrorum showed the maximum chlorophyll *a* content of 4.35±0.07 µg/ml at pH 8. The second highest was observed at pH 9 (3.53±0.08 µg/ml) followed by pH 10 (2.62±0.05 µg/ml). The minimum value was recorded at pH 6 (2.29±0.02 µg/ml).

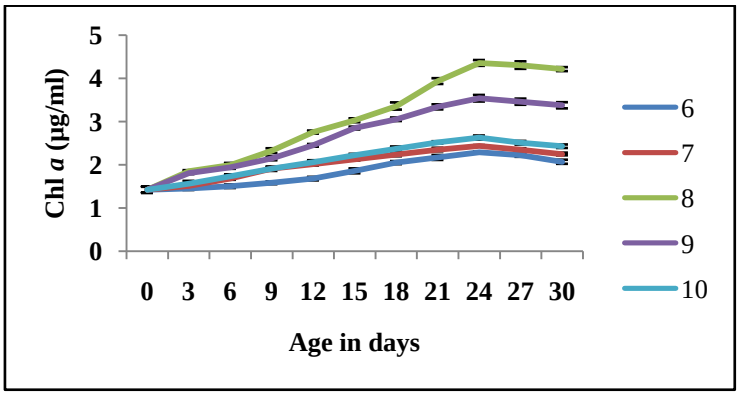


Fig. 3.21 Growth of *S. cedrorum* at different pH

3.4.1.4 Salinity

The growth of cyanobacteria was studied at different salinity (5-30 psu).The tested cyanobacteria could tolerate wide ranges in salinity (5-30 psu). The optimum salinity varied between species. All the species tend to be of marine nature. There was significant difference in growth of the strains at different salinities ($p<0.05$). The figures 3.22 to 3.28 represent the growth of the strains at different salinity tested.

The maximum growth for *A. litoralis* was recorded at 15 psu salinity in the late logarithmic phase. The chlorophyll *a* value obtained was 6.07±0.04 µg/ml, comparatively better growth was observed at 20 psu (5.44±0.08 µg/ml) and 10 psu salinity (5.12±0.06 µg/ml). Least growth was observed at 30 psu (3.54±0.06 µg/ml).

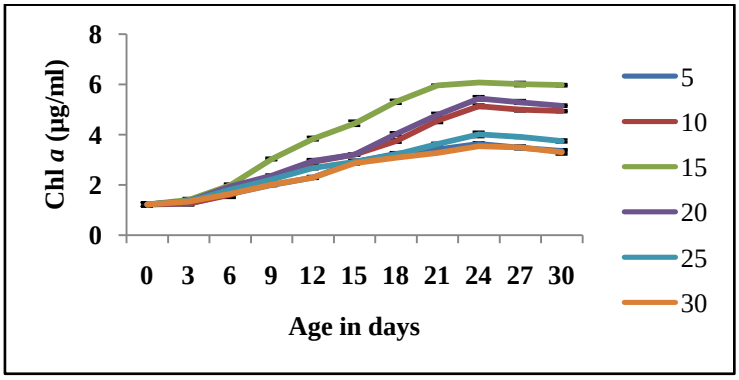


Fig. 3.22 Growth of *A. litoralis* at different salinity

The maximum growth for *C. minutus* was recorded at 20 psu with chlorophyll *a* content of 5.16±0.06 µg/ml. 4.56±0.10 µg/ml was the chlorophyll *a* content obtained at 25 psu. At 30 psu and 15 psu almost similar values were obtained (3.78±0.09 µg/ml and 3.47±0.06 µg/ml respectively). Least growth was registered at 5 psu (3.00±0.06 µg/ml).

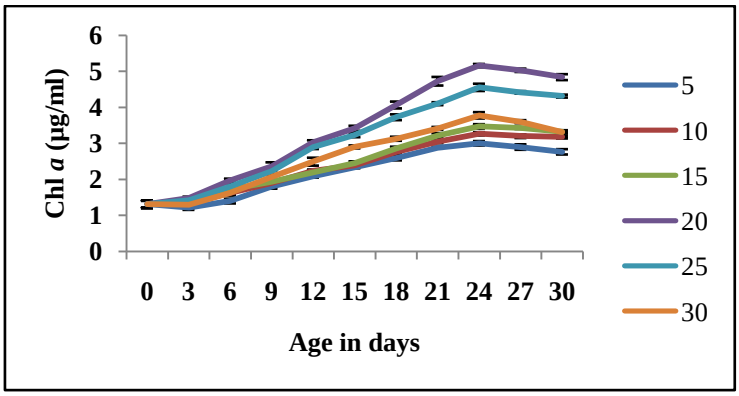


Fig. 3.23 Growth of *C. minutus* at different salinity

G. gelatinosa exhibited maximum growth at 20 psu (6.43±0.04 µg/ml). Second highest growth was observed at 25 psu (5.04±0.06 µg/ml). Almost similar growth was recorded at 30 and 15 psu salinity (4.78±0.04 µg/ml and 4.63±0.04 µg/ml). Minimum growth was recorded at 5 psu (3.28±0.04 µg/ml).

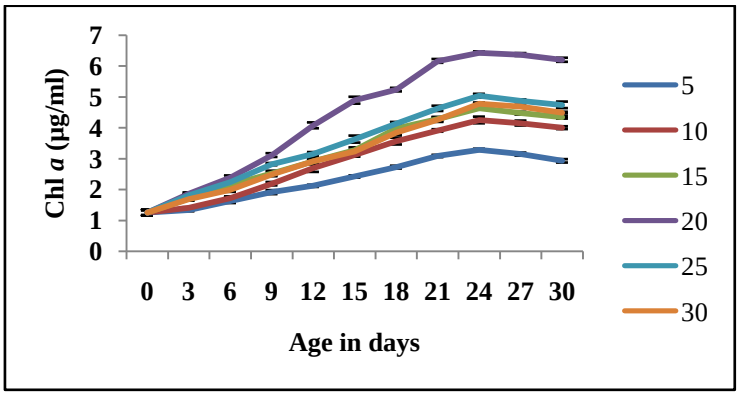


Fig. 3.24 Growth of *G. gelatinosa* at different salinity

The maximum growth for *O. limosa* was registered at 25 psu (4.05±0.07 µg/ml). 20 psu and 30 psu also supported fairly good growth. Least chlorophyll *a* value was observed at 5 psu salinity (1.61±0.06 µg/ml).

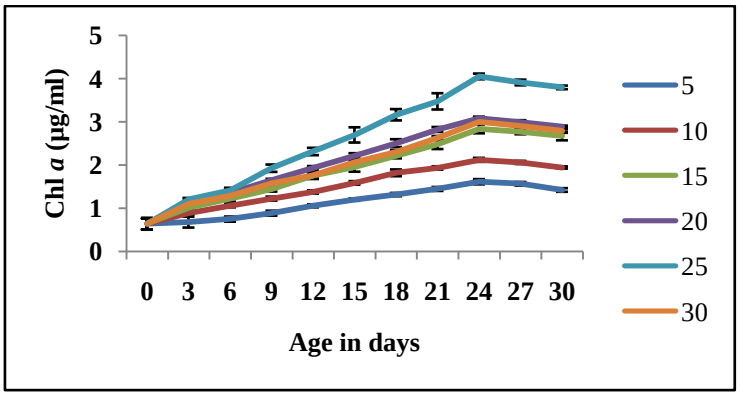


Fig. 3.25 Growth of *O. limosa* at different salinity

S. aquatilis exhibited highest growth at 10 psu (4.06±0.07 µg/ml) followed by 5 psu (3.36±0.05 µg/ml) and 15 psu (3.08±0.05 µg/ml). The growth of the species decreased with increasing salinity and minimum growth was observed at 30 psu (2.09±0.04 µg/ml).

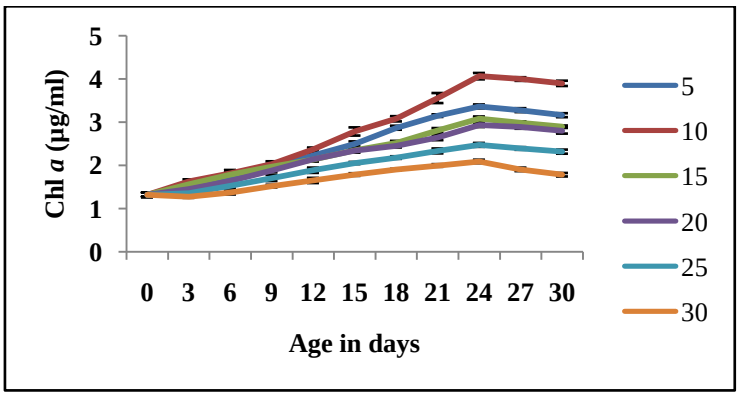


Fig. 3.26 Growth of *S. aquatilis* at different salinity

Growth of *S. salina* increased with increasing salinity. Highest growth was obtained at 30 psu salinity (4.32±0.03 µg/ml). 3.78±0.10 µg/ml

chlorophyll *a* was observed at 25 psu. Least chlorophyll *a* content was recorded at 5 psu salinity (2.05±0.04 µg/ml).

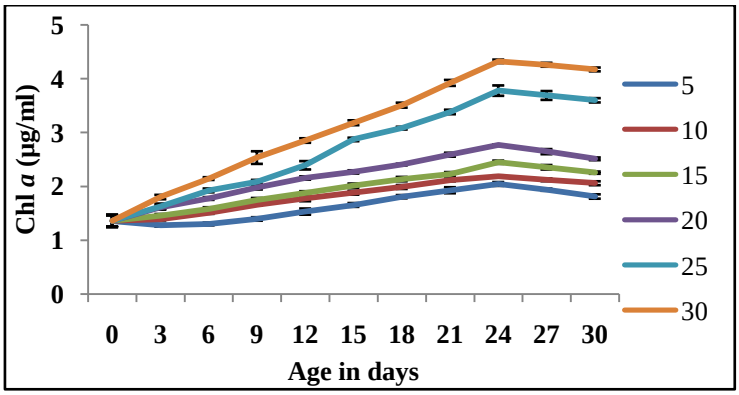


Fig. 3.27 Growth of *S. salina* at different salinity

S. cedrorum showed maximum growth at 10 psu (4.18±0.04 µg/ml) and second best growth at 15 psu (3.02±0.04 µg/ml). Minimum growth occurred at 30 psu salinity (2.21±0.05 µg/ml).

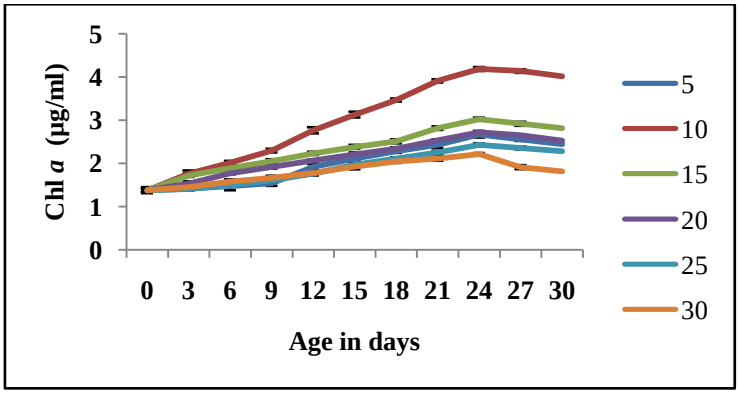


Fig. 3.28 Growth of *S. cedrorum* at different salinity

3.4.1.5 Medium

Effect of culture medium on growth of cyanobacteria was studied by determining growth in different media such as BG11, SN, Allen and Nelson and Seawater enrichment medium. The cyanobacterial strains were able to grow in all the culture media tested. The maximum biomass production was supported in SN medium and BG 11 medium, comparatively lower growth was found in Allen and Nelson and Sea water enrichment medium. SN medium was found to be the optimum medium for all strains except *A. litoralis* which showed highest chlorophyll *a* content in BG11 medium. There was no significant difference in growth of the strains in different media ($p > 0.05$) except for *A. litoralis* and *S. salina.* The results are presented in figures 3.29 to 3.35.

BG11 medium supported the maximum growth in *A. litoralis* (6.25±0.04 µg/ml), followed by SN medium (5.11±0.05 µg/ml). Almost similar growth was observed in Allen & Nelsen (4.37±0.09 µg/ml) and SWEM media (4.27±0.04 µg/ml).

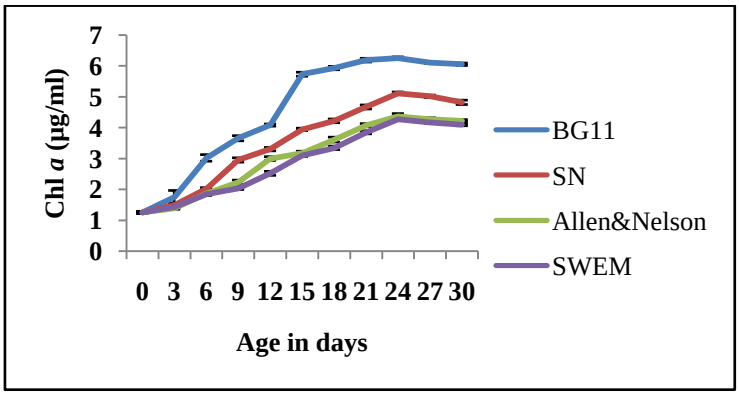


Fig. 3.29 Growth of *A. litoralis* in different media

C. minutus exhibited maximum growth in SN medium with chlorophyll *a* content of 5.98±0.06 µg/ml. The second best growth was observed in BG11 medium (4.91±0.04 µg/ml). Least growth was observed in Allen & Nelsen medium (4.50±0.07 µg/ml).

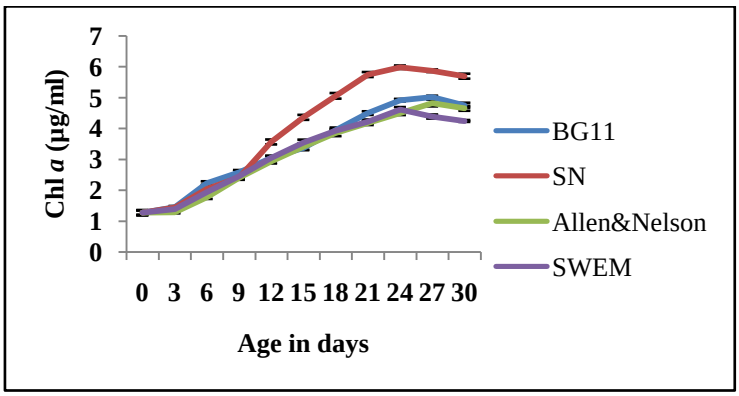


Fig. 3.30 Growth of *C. minutus* in different media

SN medium supported the maximum growth of *G. gelatinosa* (6.60±0.12 µg/ml). The strain showed fairly good growth in BG11 (6.18±0.04 µg/ml) and Allen & Nelson media (5.56±0.07 µg/ml) also. Minimum growth was observed in SWEM medium (5.07±0.09 µg/ml).

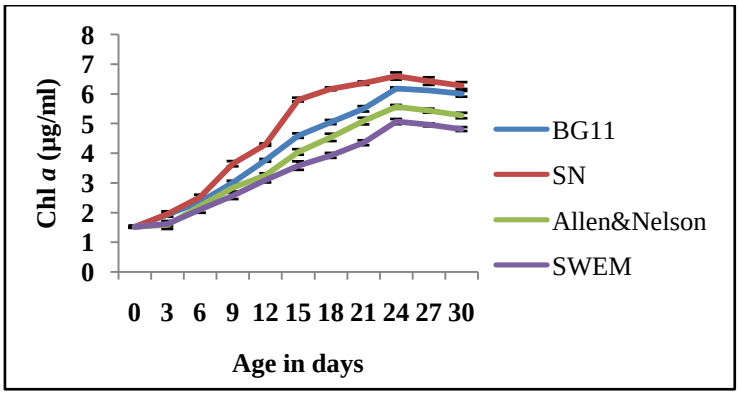


Fig. 3.31 Growth of *G. gelatinosa* in different media

O. limosa exhibited highest growth in SN medium (4.26±0.06 µg/ml). 3.59±0.09 µg/ml chlorophyll *a* was obtained in BG11 medium. Lowest chlorophyll *a* content was observed in SWEM medium (2.61±0.06 µg/ml).

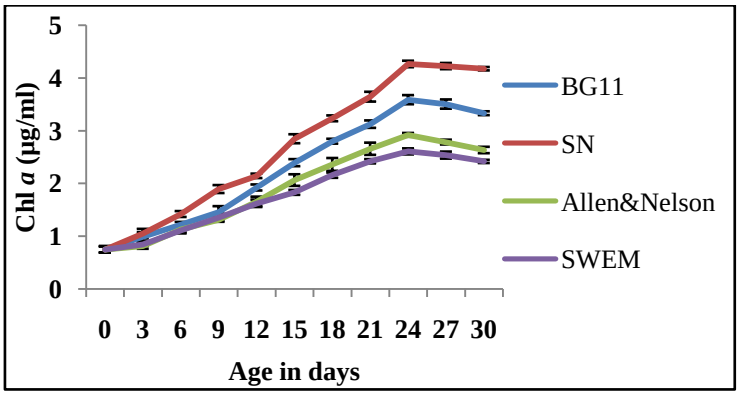


Fig. 3.32 Growth of *O. limosa* in different media

Highest growth of *S. aquatilis* was obtained in SN medium (4.58±0.12 µg/ml) followed by BG11 medium (4.17±0.06 µg/ml). Least growth was observed in SWEM medium (3.31±0.07 µg/ml).

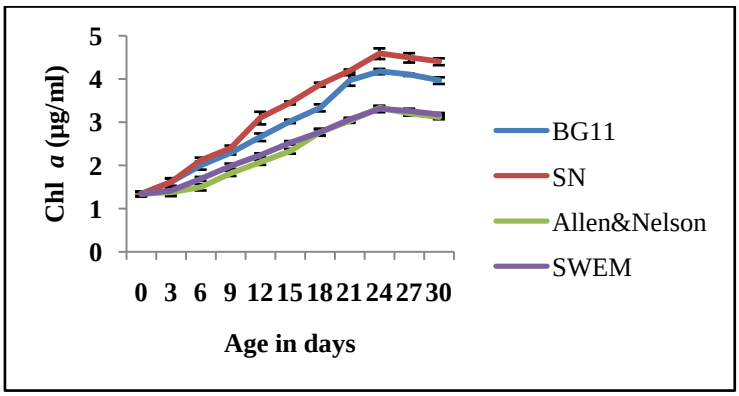


Fig. 3.33 Growth of *S. aquatilis* in different media

SN medium supported the maximum growth in *S. salina* (4.86±0.07 µg/ml). 4.21±0.06 µg/ml chlorophyll *a* content was registered in BG11 medium. Lowest chlorophyll *a* content was found in SWEM medium (3.05±0.06 µg/ml).

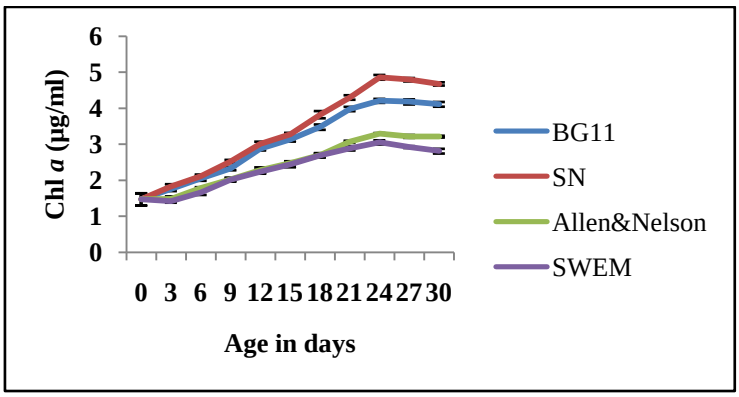


Fig. 3.34 Growth of *S. salina in* different media

S. cedrorum exhibited maximum growth in SN medium (4.27±0.04 µg/ml) and BG 11 medium (4.15±0.04 µg/ml). Lowest growth was observed in Allen & Nelson (3.02±0.04 µg/ml) and SWEM media (3.12±0.05 µg/ml).

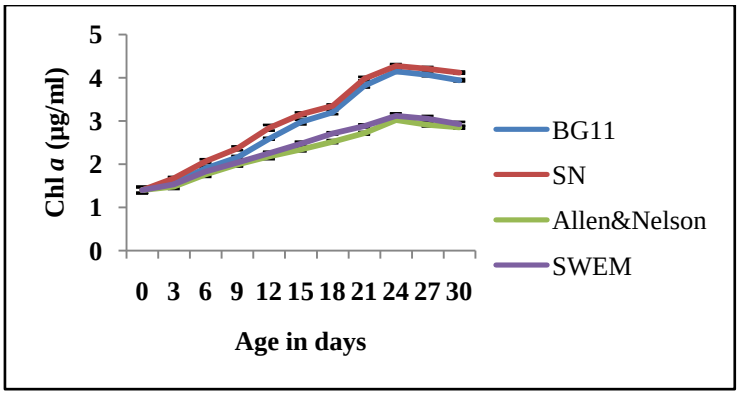


Fig. 3.35 Growth of *S. cedrorum* in different media

3.4.2 Biochemical characterization

Biochemical characterization of the cyanobacterial strains were done in the logarithmic, stationary and decline phases of growth.

3.4.2.1 Protein content

The test strains showed maximum protein content in the logarithmic phase and the content was found to be in the range of 23-48% of dry weight of cells. *S. salina* showed the highest protein content of 47.6%. The filamentous cyanobacterium *O. limosa* showed 43.9% protein content. *C. minutus* had the least protein content (23.1%) among the test strains. ANOVA results indicated that there was significant difference in the protein content of the test strains ($p<0.05$).

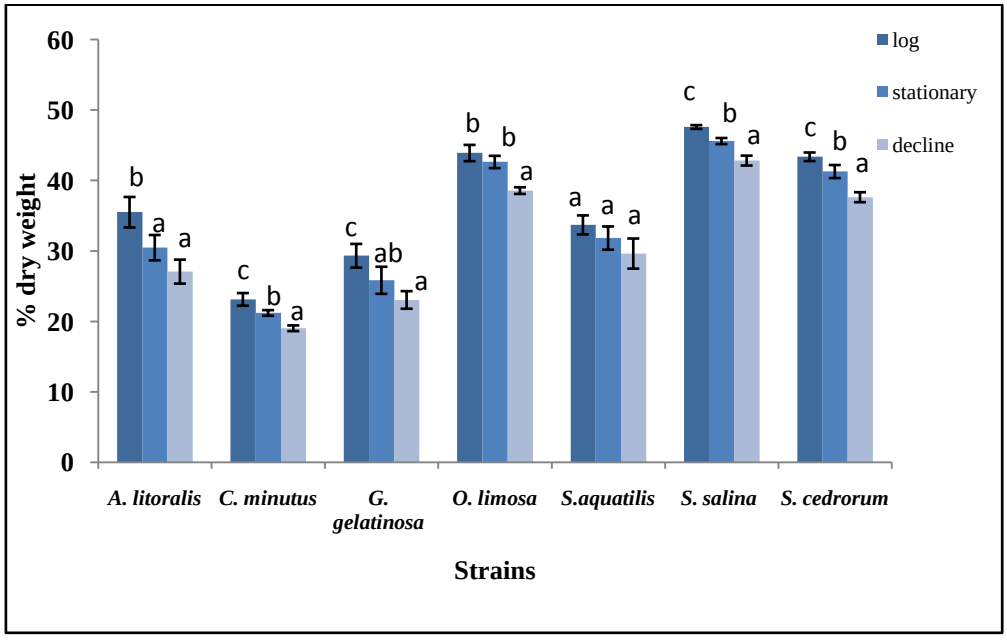


Fig. 3.36 Protein content of the cyanobacterial strains

3.4.2.2 Carbohydrate content

The carbohydrate content of the strains ranged between 27-38% and was highest in the stationary phase of the culture. The highest carbohydrate content was recorded in *S. salina* (38.2%) followed by *S. cedrorum* (36.5%). *C. minutus* exhibited the lowest carbohydrate content (26.6%). There was significant difference in the carbohydrate content of the test strains ($p<0.05$).

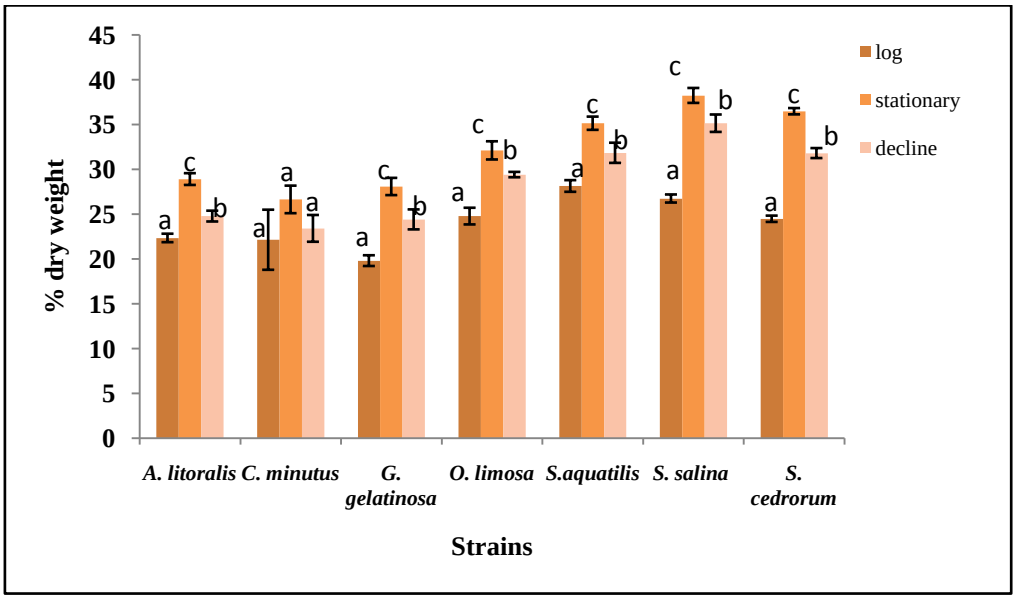


Fig. 3.37 Carbohydrate content of the cyanobacterial strains

3.4.2.3 Lipid content

The lipid content was found to be generally low for all the strains (5 to 8% of the total dry weight). Highest lipid content was observed in *G. gelatinosa* (8.4%) and *C. minutus* (8 %) and least in *A. litoralis* (5.5%). The lipid content was maximum at the decline phase. There was significant difference in the lipid content of the test cyanobacteria ($p<0.05$).

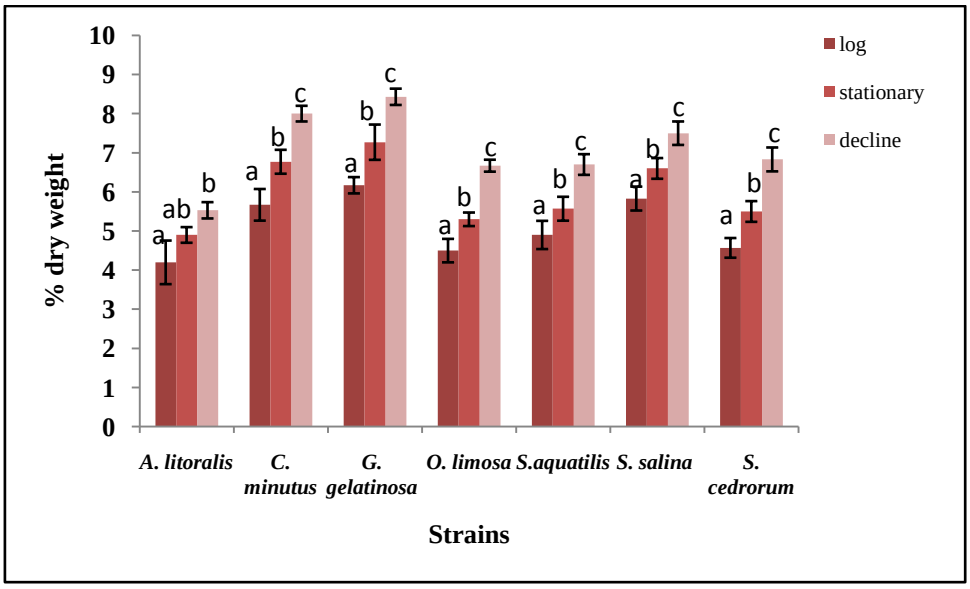


Fig. 3.38 Lipid content of the cyanobacterial strains

3.4.3 Pigments

3.4.3.1 Chlorophyll *a*

Chlorophyll *a* is the primary photosynthetic pigment present in cyanobacteria. The highest chlorophyll *a* content was recorded in *G. gelatinosa* (8.14±0.09µg/mg) and *C. minutus* (7.44±0.33 µg/mg). The lowest value was obtained for *O. limosa* (5.01±0.18 µg/mg). The ANOVA results showed that there was significant difference in the chlorophyll *a* content of the test strains ($p<0.05$).

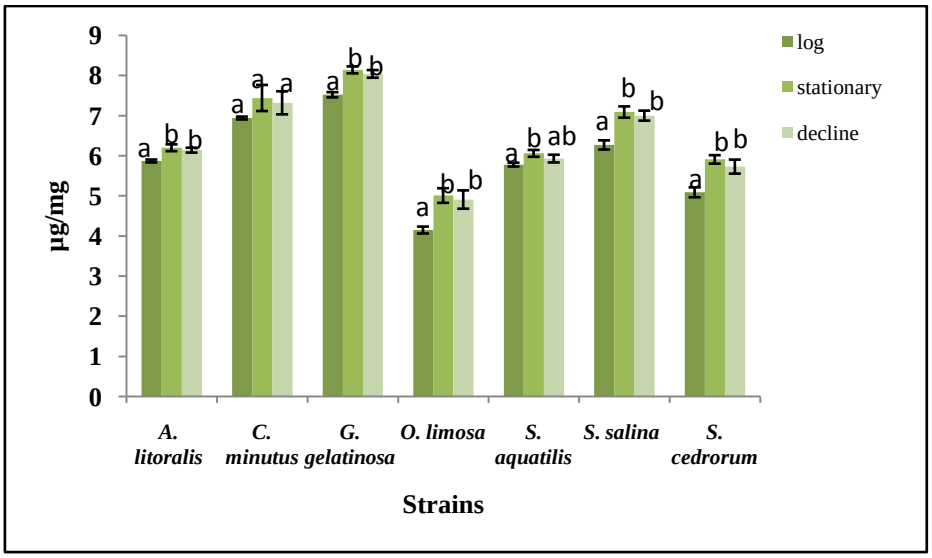


Fig. 3.39 Chlorophyll *a* content of the cyanobacterial strains

3.4.3.2 Carotenoids

Carotenoid content was found to be the highest in *G. gelatinosa* (4± 0.14µg/mg) followed by *C. minutus* with 3.93±0.09µg/mg. *O. limosa* had the lowest carotenoid content of 2.21 ±0.072µg/mg. There was significant difference in the carotenoid content of the test strains ($p<0.05$).

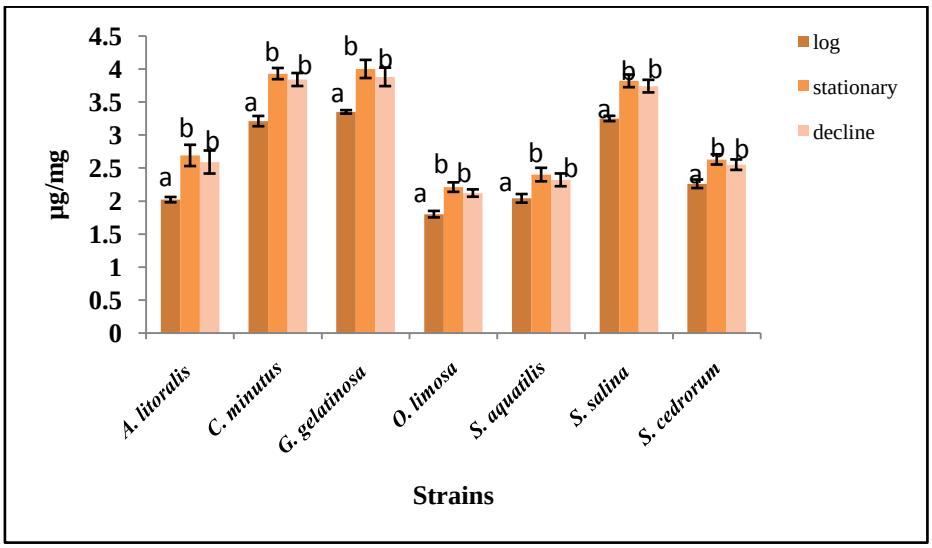


Fig. 3.40 Carotenoid content of the cyanobacterial strains

3.4.3.3 Phycobiliproteins

Among the phycobiliproteins, phycocyanin content was found to be the highest in all strains followed by phycoerythrin and allophycocyanin. *C. minutus* showed the highest phycocyanin content of 2.02± 0.12 µg/mg, second highest was found in *S. salina* (1.98± 0.21µg/mg) and lowest value was exhibited by *A. litoralis* (1.43±0.07µg/mg). *S. salina* showed the maximum phycoerythrin content of 1.66±0.05 µg/mg and *C. minutus* had 1.59 ±0.04 µg/mg. Lowest value was observed for *O. limosa* (1.17±0.05µg/mg). Allophycocyanin was maximum in *S. aquatilis* (1.13±0.09 µg/mg) and *G. gelatinosa* (1.09±0.12µg/mg). *A. litoralis* recorded the lowest allophycocyanin content (0.81±0.075 µg/mg). There was significant difference in the phycobiliproteins content of the test strains ($p<0.05$).

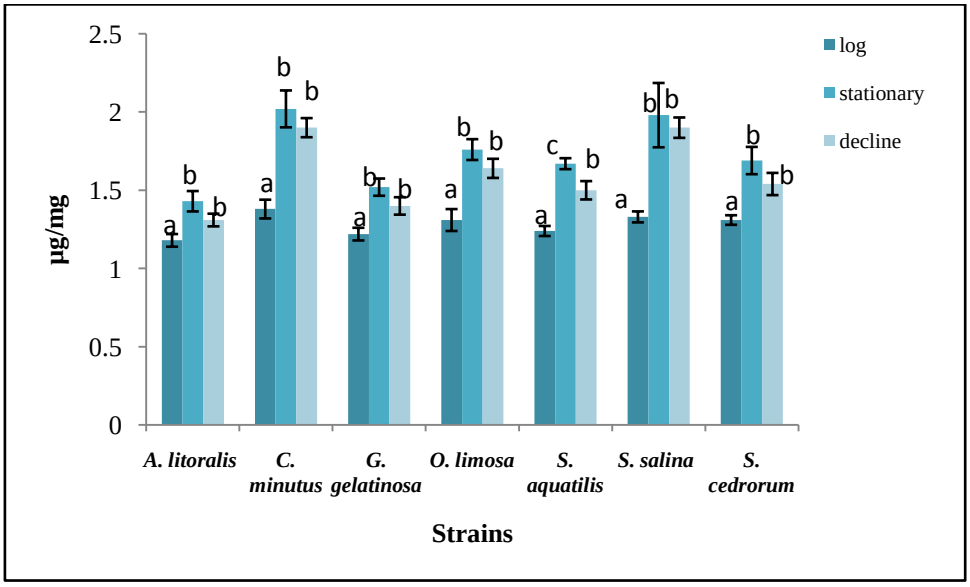


Fig. 3.41 Phycocyanin content of the cyanobacterial strains

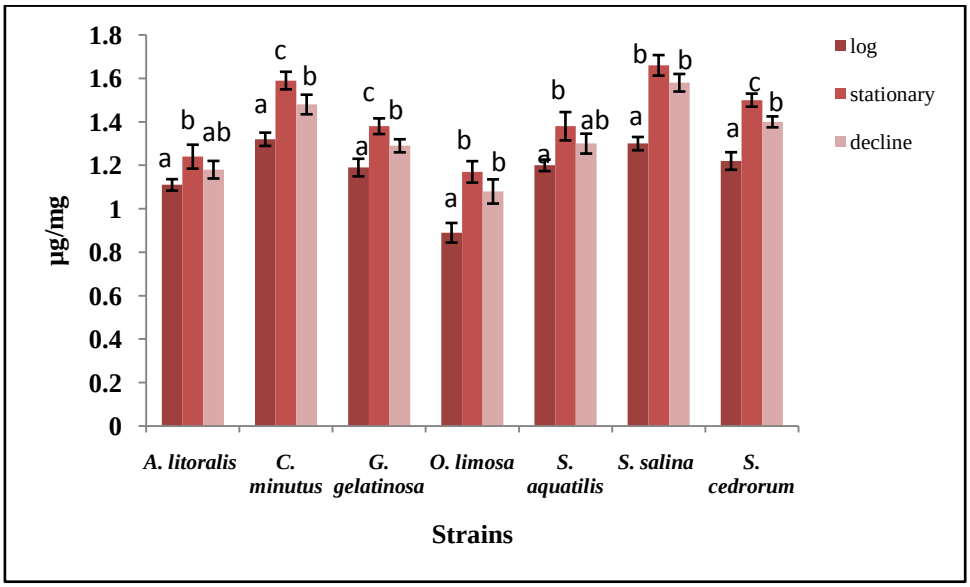


Fig. 3.42 Phycoerythrin content of the cyanobacterial strains

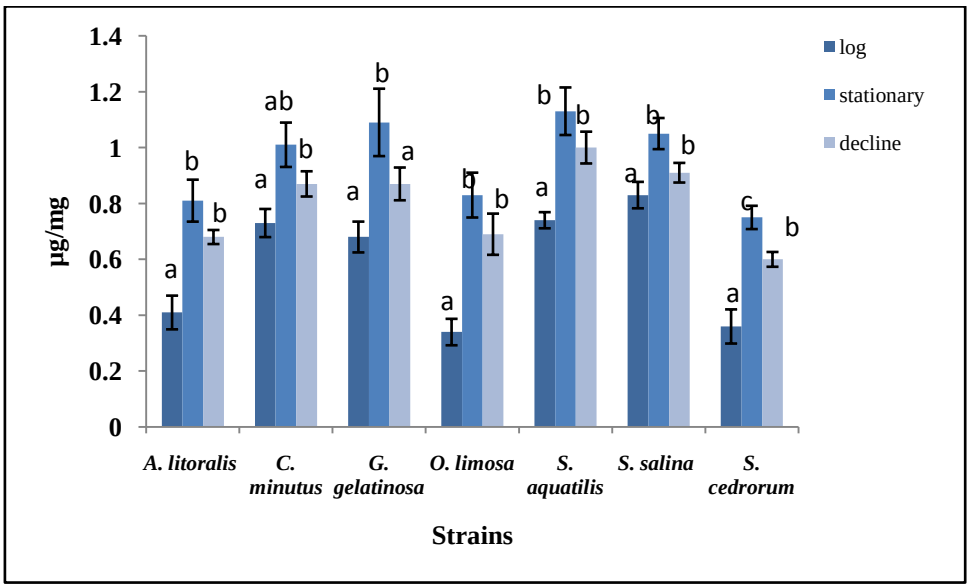


Fig. 3.43 Allophycocyanin content of the cyanobacterial strains

3.5 Discussion

The biochemical composition is one of the most important criteria for an organism to be considered as food. Cyanobacteria with remarkably high protein content have been utilized as food since prehistoric times (Gantar and Svircev 2008). Feed production is a major cost involving process in the aquaculture industry; therefore efforts are being made to develop cost-effective and nutritious feed. Microalgae and cyanobacteria being the basic component of the food chain could be exploited as aquaculture feed or as a feed supplement. From an industrial point of view the biomass of the microalgae is also an important factor. Therefore before considering for commercial applications it is necessary to optimize the conditions favouring the growth and biomass of the species.

The major factors affecting the growth of microalgae are light intensity, temperature, pH, nutrient concentrations, carbon dioxide concentrations, salinity etc. (Becker, 1994). In the present study, the culture conditions of the test strains such as light, temperature, pH, salinity and culture medium supporting the maximum growth was found out. The cyanobacteria could grow at all the culture conditions tested, clearly indicating their wide ecological tolerance (Carr and Whitton, 1982). The optimum growth and biomass production is highly dependent on the species (Nagle *et al*., 2010) and the favorable culture conditions have a positive effect on the biomass production (Rafiqual *et al*., 2005). However, in the present study, the optimum light, temperature and pH conditions for all the test strains were found to be the same. The optimization of culture conditions significantly increased the biomass of the cultured cyanobacteria.

In the present study, 1400 lux favoured the highest growth in *A. litoralis* and all the other test strains and interestingly the growth decreased with increase in light intensity, whereas Ifeanyi *et al*. (2011) showed that high light intensity of 5000 lux enhanced growth of *Aphanocapsa* sp. cultures. Nevertheless, Vonshak (2004) indicated that higher light intensities inhibited growth in *Spirulina* cultures.

Studies indicate that the optimum temperature for microalgae lies between 16 and 24˚C and they can grow well up to 35˚C (Cassidy, 2011 and Aishvarya *et al*., 2015). The temperature changes not only affect the growth but also influence the lipid and protein content of the microalgae (Richmond, 2004 and Munir *et al*., 2015). In the present study, the optimum temperature for all the strains was found to be 25˚C and they exhibited good growth even at 30˚C. This may be due to the fact that these strains have been isolated from a tropical estuary. Complementing the findings of the present study the different cyanobacterial species isolated from the mangrove ecosystems of Sundarbans, also exhibited highest growth at 25˚C (Pramanik *et al*., 2011). However, for the growth of *Oscillatoria obscura* and *Lyngbya limnetica*, the optimum temperature was found to be 20˚C (Kushwaha *et al*., 2018). Hence, it is evident that the optimum temperature for growth varied depending on the species.

The pH of the medium determines the solubility of carbon dioxide and different minerals which, in turn, influence the metabolism of the algae (Markl, 1977 and Richmond 2000). The cyanobacteria generally prefers alkaline pH ranging from 7.5-10 (Prasad *et al*., 1978 and Aishvarya *et al*., 2015). Sincy (2005) observed that the different unialgal cyanobacterial species isolated from Cochin estuary could tolerate wide ranges in pH (6-9). The findings of the present study also indicate the same. The test strains exhibited

growth at acidic, neutral and alkaline pH. However, maximum growth was obtained at pH 8 and pH 9 and minimum growth at pH 6 further confirming the group's affinity towards alkaline pH. In agreement to the present study, the optimum pH for several species of cyanobacteria was found to be between 8 and 9 (Abalde *et al*., 1995, Nagle *et al*., 2010 and Kushwaha *et al*., 2018). However, there are cyanobacteria which could grow even at a lower pH of 4.5 (Pramanik *et al*., 2011).

All the test cyanobacteria, in the present study, preferred saline conditions (10-30 psu) for growth though they could grow even at fresh water conditions thus implying their euryhaline nature. Studies by Subramanian and Thajuddin (1995) also support the above finding. Newby (2002) found that the growth of the cyanobacterial strains *S. salina*, *Synechococcus elongatus* and *Gloeocapsa crepidinum* isolated from the Cochin backwaters enhanced at salinities above 30 psu. In the current study, the salinity variations influenced the growth of the strains. While strains like *A. litoralis*, *C. minutus*, *S. aquatilis*, *O. limosa* and *S. salina* preferred higher salinities (15-30 psu), *S. aquatilis* and *S. cedrorum* (5-10 psu) showed affinity to lower salinities. Sincy (2005) also observed that the cyanobacteria from Cochin estuary could tolerate salinities ranging from 0-40 psu. The study also revealed that the optimum salinity for *Gloeocapsa livida* was 20 psu and *Oscillatoria salina* was 10 psu. The strains *G. gelatinosa* and *O. limosa* of the present study showed highest growth at 20 psu and 25 psu respectively. Therefore, it is clear that the optimum salinity varied with species. Recent reports on the cyanobacteria of mangrove habitats also state that they could tolerate wide salinities ranging from 32 to 82‰ (Pramanik *et al*., 2011).

BG11 and SN media are widely used for the culturing and maintenance of cyanobacterial species (Andersen, 2005). In the present study also, these

two media favoured the highest biomass production and chlorophyll *a* content in the test strains. The concentrations of nitrate and phosphate salts are very high in both SN and BG 11 media compared to the other simpler media such as Allen & Nelson and Seawater enrichment medium used in the study which can be one of the reasons for the high growth. The presence of trace metals and vitamins in the media has also significantly influenced the growth of the test strains. Studies indicate that nitrate and phosphate are the key nutrients involved in the growth of microalgae (Lin and Lin, 2011). SN medium was initially designed for the culturing of the species *Synechococcus sensu lato* (Andersen, 2005); in the present study also the strain *S. cedrorum* exhibited highest growth in the SN medium. Complementary to the findings of the present study, Rajishamol (2013) also found that SN medium supported the highest biomass and chlorophyll *a* production in *Synechococcus elongatus*, *Synechocystis aquatilis* and *Oscillatoria sancta*.

Microalgae including cyanobacteria, in general, are primarily composed of macromolecules such as proteins, carbohydrates, lipids and nucleic acids in varying proportions which differ from species to species and with the growth conditions (Encarnacao *et al*., 2015). The high protein content and the presence of various vitamins, minerals and fatty acids make cyanobacteria an attractive candidate for human and animal nutrition industries. The overall concentration of nutrients in the algal biomass depends largely on the availability of potassium, sodium and nitrogen content in the medium (Gantar and Svircev, 2008). The growth phases also affect the content of different nutrients. Zhu *et al*. (1997) reported that the protein content tends to concentrate in the exponential phase whereas; the carbohydrate content was high in the stationary phase and lipid accumulation increased towards the

decline phase. This was clearly evident in the present study also. Therefore, the harvesting of the algae should be done based on the requirement.

The protein, carbohydrate and lipid content of the strains in the present study were found to be comparable with those reported in the previous studies. Protein content varied between 46 to 63%, carbohydrates 8 to 17% and lipids 4 to 22% of the total biomass (Becker, 1994 and Gantar and Svircev, 2008). The protein content was high followed by carbohydrate and the lipid content was low, which was observed in the current study also.

Studies indicate that protein constitutes the major part of the biochemical content of the cyanobacteria (Abeer and Mohamad, 2016) and it generally varies from 40 to 60% of the dry weight (Becker, 1994). The protein content of the cyanobacteria in the present study varied between 23 to 48%. Among the test strains *S. salina* (47.6%), *O. limosa* (43.9%) and *S. cedrorum* (43.4%) exhibited the highest protein content. Cyanobacteria such as *Spirulina* and *Synechococcus* sp. can concentrate up to 70% and 63% protein (Gantar and Svircev, 2008 and Spolaore *et al*., 2006). But in the present study, the maximum protein content observed was 43% in the strain *S. cedrorum*. Complementary to the present study, Nagle *et al*., (2010) also could record only 46% protein in *Synechococcus cedrorum*. However, Sincy (2005) reported that the protein content of the cyanobacteria isolated from Cochin estuary varied between 4-39% which is low compared to the current study. She could report only 9% protein in *G. gelatinosa*, whereas the protein content in *G. gelatinosa* strain of the present study was 29%. This difference could be due to the difference in the strain as well as the culture conditions. *S. aquatilis* recorded 34% protein content but a similar study on the species could report only 28% protein (Lopez *et al*., 2010). The protein content of filamentous cyanobacteria varied between 37 to 52% (Vargas *et al*., 1998). The

filamentous *O. limosa* strain in the present study, registered 43.9% protein in accordance with the above study.

The carbohydrate content of microalgae varied between 4.1 to 37% with an average value of 23% (Finkel *et al*., 2016). In the present study the total carbohydrate content was found to be between 27 to 38%. All the strains registered fairly good carbohydrate content of above 25% of dry weight especially the Synechococcales group. *S. salina* exhibited a maximum of 38% followed by *S. cedrorum* (36.5%) and *S. aquatilis* (35.1%). According to previous reports the maximum carbohydrate reported from cyanobacteria was 28.4% in unicellular forms (Rajeshwari and Rajashekhar, 2011) and 38% was recorded in filamentous forms (Vargas *et al*., 1998). On the contrary, Sincy (2005) observed low carbohydrate content in various strains of unicellular and filamentous cyanobacteria (<15% of dry weight) from Cochin estuary. Similarly Campa-Avila (2002) could record only 16.68% carbohydrate in *Synechococcus* sp. But in the present study, the strain *S. cedrorum* recorded 36.5% carbohydrate content. Studies report that the carbohydrate content of cyanobacteria varied with the nitrogen concentration in the culture medium (Mollers *et al*., 2014). This might be the reason for the variation of carbohydrate content in the species. Sheekh *et al*. (2015) reported that the carbohydrate content varied between different species of filamentous cyanobacteria and they recorded 29.7% in *Nostoc calcicola* and 17.83% in *Anabaena variabilis*. *O. limosa* in the present study contained 32.1% carbohydrate which is much higher compared to previous reports on filamentous cyanobacteria (Rajeshwari and Rajashekhar, 2011, Sheekh *et al*., 2015).

The algal lipids are mainly composed of glycerol, bases of esterified saturated or unsaturated fatty acids (Abeer and Mohamad, 2016). Most studies indicate that the lipid content of cyanobacteria is generally less than 20% of

dry weight (Spolaore *et al*., 2006, Kovac *et al*., 2013 and Nagle *et al*., 2010). The results of the present study also follow the same trend. Studies report up to 11% lipid in *Synechococcus* sp. (Griffiths and Harrison, 2008) but only 6% lipid was obtained in the present study. A study on lipid content of *S. platensis*, *Synechococcus* sp. and *Aphanothece microscopica* showed that the lipid content varied from 7.9 to 11%, indicating that the lipid content is highly variable with species (Encarnacao *et al*., 2015). The lipid content of *O. limosa* (6.7%) was comparable with those reported on filamentous cyanobacteria (8-13%) by Vargas *et al*. (1998). Contrary to the results of the present study high lipid content (20%) was reported in cyanobacteria of Cochin estuary (Sincy, 2005) and Western Ghats (Rajeshwari and Rajashekhar, 2011). The difference in lipid content of the strains may be due to the difference in carbohydrate content of the medium. Increasing the glucose concentration of the medium by 1% increases the lipid content of cyanobacteria (Hassan *et al*., 2012).

Pigments such as chlorophyll and carotenoids are indispensible components of the photosynthetic process. The microalgal pigments, as well as the whole biomass can be used as food colourants (Kovac *et al*., 2013). The pigments can also act as potential antioxidants. Chlorophyll *a*, the major photosynthetic pigment accounts for 1.1% of dry weight of the algae (Finkel *et al*., 2016). The chlorophyll *a* content varied from 5 to 8 µg/mg and carotenoid content varied from 2 to 4 µg/mg. Higher pigment content was observed in unicellular strains than filamentous forms. *G. gelatinosa* exhibited the maximum and *O. limosa* recorded the lowest pigment content. Sincy (2005) reported high chlorophyll *a* (3%) and carotenoid content (0.6%) in *G. gelatinosa* and *Oscillatoria* species. *S. elongatus* PCC 7942 exhibited maximum chlorophyll *a* content of 6.5µg/mg (Suzuki *et al*., 2010) and in the

present study the strain *S. cedrorum* had 5.91 µg/mg. Therefore it can be concluded that the pigment content varied with species.

The accessory pigments phycobiliproteins generally account for 24% dry weight of the soluble proteins of the cyanobacterial cells (Marsac, 2003). In the present study, the phycocyanin content (1.4 to 2.02µg/mg) was more in all the test cyanobacteria than phycoerythrin (1.2 to 1.59µg/mg) and allophycocyanin (0.81 to 1.13µg/mg). *C. minutus* (2.02µg/mg, 1.59µg/mg) and *S. salina* (1.98µg/mg, 1.66µg/mg) exhibited the highest phycocyanin and phycoerythrin content among the test strains. The values obtained for the test strains were low compared to the earlier studies, which have reported that phycobiliproteins form up to 10% in cyanobacteria (Sincy, 2005). Similarly, the phycobiliproteins in different cyanobacteria from Arabian Sea varied from 4 to 208 mg/g (Rai and Rajashekhar, 2015). *O. limosa* had high phycocyanin and low phycoerythrin content and this constituted less than 5% of the total pigment composition. This is in contrast to earlier reports on *Oscillatoria* species, where up to 80 mg/g was recorded (Hossain *et al*., 2016). The test cyanobacteria showed high chlorophyll *a* content followed by carotenoid content and comparatively low phycobiliproteins.

The biochemical composition is highly dependent on the strain, the growth phase, the type of nutrients present in the medium and the environmental conditions (Vargas *et al*., 1998 and Rajeshwari and Rajashekhar, 2011). Altering the culture conditions by inducing nutrient starvation or the addition of specific nutrients can have a positive influence on the biochemical composition of the desired algae (Fernadez and Ballesteros, 2012 and Patterson *et al*., 1994). The cyanobacteria tend to accumulate carbohydrates under nitrogen and phosphorus limiting conditions (Arias *et al*.,

2018 and Kushwaha *et al*., 2018). *Synechococcus* sp. PCC 7002 accumulated carbohydrate up to 60% under nitrogen starvation (Mollers *et al*., 2014).

The present study was an attempt to identify potential cyanobacterial strains with desirable biochemical composition from local habitats. *S. salina* and *S. cedrorum* emerged to be potential candidates with good protein and carbohydrate content for aquaculture application. *G. gelatinosa C. minutus* and *S. salina* could serve as pigment sources. The strains are to be further analyzed for the presence of toxic genes, if any, and studies need to be carried out to modify the biochemical profile of the prospective strains.

Chapter 4

Antioxidant activity of the isolated cyanobacteria

Contents

4.1 Introduction

Cyanobacteria, the primordial photoautotrophs, played a major role in the evolution of myriad number of aerobic organisms. They are ubiquitous in distribution, and are found thriving in the earth's most hostile environments. Photosynthesis and respiration which are indispensible part of an autotrophic organism generate some undesirable and destructive molecules called the reactive oxygen species (ROS). They are continuously produced during the photosensitization of chlorophyll *a* and phycobiliproteins through type I and type II photochemical reactions, when the electrons and light energy are transferred between the excited chlorophyll, photosystem II, photosystem I and molecular oxygen. Environmental stress conditions such as extreme temperatures, ultraviolet radiations, high salinity, iron starvation, carbon dioxide limitation, etc. can also result in the production of ROS. The ROS generation in the cell leads to photobleaching of the photosynthetic pigments ultimately leading to the inhibition of photosynthesis, breakage of nucleic acids, biological membrane disintegration, apoptosis and programmed cell

death (Hseih and Pedersen, 2015). In addition to these, they damage the photosystems, react with amino acids such as histidine, tryptophan, methionine and cysteine (Halliwell and Gutteridge, 1990) leading to the breakage of peptide bonds. They also degrade the thylakoids, producing higher amounts of malondialdehyde (MDA) which acts as a DNA mutagen by reacting with DNA forming adducts and ultimately leading to genetic mutation (Hseih and Pedersen, 2015).

The ROS mainly consist of two types of species, the radical and non-radical. The radical species are singlet oxygen ($^1O_2$), hydroxyl radical (OH), superoxide ($^{\cdot}O_2^-$) and non-radical species include hydrogen peroxide (H_2O_2). Each ROS species have characteristic physical and chemical properties that make them specific in their reactivity, toxicity and target specificity (Hseih and Pedersen, 2015). ROS is produced in all living organisms and they are the major culprits involved in food spoilage by lipid peroxidation. In humans, they are associated with the cause of many disease conditions such as artherosclerosis, cancer, coronary heart disease and ageing (Li *et al*., 2007).

Cyanobacteria employ a combination of different structural and functional strategies to combat the effect of free radicals. First and foremost, they avoid excess illumination by moving away from it which is termed as vertical migration. Secondly, they have protective pigments in the extracellular sheath known as scytonemins and mycosporins and mycosporine-like aminoacids (MAA). The excess light energy is effectively dissipated as heat energy by protein complexes known as Orange Carotenoid Proteins (OCP) in combination with phycobilisome complex and Iron stress induced antenna protein (IsiA) which is formed during iron deficiency and oxidative stress. Stabilization of the photosystems is carried out by High light inducible polypeptides or proteins (HLIPs) and small CAB-like proteins (Scp). The

system also produces powerful antioxidant enzymes like superoxide dismutases (SODs), catalases and peroxidases (glutathione peroxidases, ascorbate peroxidases and peroxiredoxins) which catalysis the conversion of free radicals into simpler non-toxic forms. The non-enzymatic antioxidants such as ascorbate (vitamin C), α-tocopherol and the phycobiliproteins also strengthen the antioxidant defense system (Hseih and Pedersen, 2015).

Antioxidants are substances that prevent the oxidation of other substances by different mechanisms such as prevention of chain initiation, chelation of transition metal ion catalysts, degradation of peroxides, prevention of hydrogen abstraction and scavenging free radicals (Valko *et al*., 2006). They help in preserving food quality and also have health benefits. They are widely used in various industries such as food, pharmaceuticals, nutraceuticals, cosmetics, aquaculture etc. The antioxidants currently in use are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroxyquinone (TBHQ) which are synthetic in nature. The synthetic antioxidants are suspected carcinogens (Namiki, 1990 and Pokorny, 1991). Due to the undesirable effects arising from the use of synthetic antioxidants there is a requisite for finding safe and natural dietary antioxidants. Current natural antioxidants are obtained from rosemary, green tea and grape seeds (Pokorny, 1991). Natural antioxidants not only improve the shelf life of the food but also provide various health benefits such as prevention of cardiovascular disorders, Alzheimer's disease and also certain types of cancer (Goiris *et al*., 2012).

Microalgae including cyanobacteria are a promising source of natural antioxidants due to their biochemical composition and the possibility of culturing them in non-arable lands. Their amenability to metabolic modulation and lower generation time makes them attractive candidates for industrial

application (Maadane *et al*., 2015). Cyanobacteria contain significant amount of phenolics, phycobiliproteins, carotenoids, vitamins, sulphated polysaccharides, mycosporine-like aminoacids, and scytonemins which could be employed as antioxidant agents in the food and nutraceutical industries. *Spirulina* liquid CO_2 extracts, a powerful antioxidant is a recent addition in the microalgal market and is gaining increasing demand (Pulz and Gross, 2004). In Japan and China phycocyanin derived from *Spirulina* is marketed under the trade name Lina-blue which is used as an antioxidant and natural colourant in the food industry (Kovac *et al*., 2013).

In the present study, the antioxidant properties of the seven cyanobacterial strains were evaluated by phytochemical analysis, quantification of pigments and *in vitro* antioxidant assays. The phytochemical analysis involved the determination of total phenolic content and total flavonoid content. The free radical scavenging ability was analyzed by different assays such as DPPH radical scavenging assay, deoxyribose radical scavenging assay, ferric reducing antioxidant power assay, total antioxidant capacity and hydrogen peroxide radical scavenging assay.

4.2 Review of literature

The plant kingdom has been extensively screened for antioxidant activity including the algal resources. Among the myriad number of microalgae identified only a few have been screened for the antioxidant capability. *Spirulina* has been the most exploited microalgae in the algal world due to its unique biochemical composition. Miranda *et al*. (1998) evaluated the *in vitro* and *in vivo* antioxidant property of *Spirulina maxima* and found that they exhibited antioxidant protection for both the systems. Benedetti *et al*. (2004) analyzed the protective ability of *Aphanizomenon flos-aquae* (AFA)

extract on the human erythrocytes and plasma samples against oxidative damage induced by 2,2 L Azobis (2-amidinopropane) dihydrochloride and cuprous chloride *in vitro*. The results indicated that the phycocyanin from AFA had potential for clinical application.

The antioxidant properties of macroalgae are more extensively looked into than microalgae due to the easy availability of sample. Kuda *et al*. (2005) recorded the antioxidant properties of edible macroalgae *Scytosiphon lomentaria, Papenfussiella kuromo, Nemacystus decipiens* and *Porphyra* sp. by various *in vitro* assays such as DPPH radical scavenging activity, reducing power assay, ferrous ion chelating assay etc. Heo *et al*. (2005) used ten different enzyme extracts of seven species of brown algae along the Jeju coast of South Korea to determine their antioxidant properties and from the results concluded that the enzymatic extracts were better antioxidants than the algal extracts.

Sincy (2005) analyzed the *in vivo* antioxidant activity of selected cyanobacteria from Cochin estuary on *Oreochromis mossambicus*. Patel *et al*. (2006) studied the antioxidant activity of C-phycocyanin isolated from *Lyngbya*, *Phormidium* and *Spirulina* sp. They analyzed the covalently linked tetrapyrrole chromophore phycocyanobilin by electron spin resonance (ESR) which was the pioneering study in this area. The antioxidant activities of an aerial microalga *Coelastrella striolata* var. *multistriata* which is capable of accumulating high amounts of carotenoids were checked by Abe *et al*. (2007). Their study concluded that the alga can be a promising candidate as an antioxidant in the food industry. In another study by Li *et al*. (2007) twenty three microalgae from different classes such as Cyanophyceae, Chlorophyceae and Dinophyceae were extracted into hexane and ethyl acetate and also the aqueous extract were taken to study the total phenolic content and antioxidant capacities. The strains *Synechococcus* sp. FACHB 283, *Chlamydomonas*

nivalis and *Nostoc ellipsosporum* CCAP 1453/17 were found to have high antioxidant capacities.

In a study on the antioxidant activity of ethanolic extracts of microalgae *Porphyridium cruentum*, *Phaeodactylum tricornutum* and *Chlorella vulgaris* by Ignacio and Jose (2008) beta carotene linoleate model system was used and the antioxidant activity of *C. vulgaris* was found to be higher than that of butylated hydroxytoluene and butylated hydroxyanisole. The high content of fatty acids was stated as the reason for high antioxidant activity of *C. vulgaris*. Hajimahmoodi *et al*. (2010) evaluated the antioxidant properties and total phenolic content of twenty four different microalgae from Cyanophyceae and Chlorophyceae, their study highlighted the role of extracellular substances and phenolic compounds towards the antioxidant property of the cell.

Lopez *et al.* (2011) employed reverse phase high performance liquid chromatography (RP-HPLC) to identify and quantify the phenolic compounds present in the alga *Stypocaulon scoparium*. Karunamoorthy *et al*. (2012) analyzed the antioxidant properties of *Chlorella marina* using various *in vitro* assays and the study concluded that the phenolic compounds were a major contributor to the antioxidant activity. The relationship between phenolic content and carotenoid content and their contribution to the antioxidant defense mechanism was analyzed by Goiris *et al*. (2012).

Shanab *et al*. (2012) reported that the aqueous extracts of cyanobacteria exhibited antioxidant as well as anticancerous activities. Sharathchandra and Rajashekhar (2013) found that the cyanobacteria isolated from sulphur spring exhibited very high antioxidant activity indicating that environmental conditions had great effect on the antioxidant potential of the organism. Guedes *et al*. (2013) made an extensive study on the antioxidant

property of eighteen species of cyanobacteria and twenty three species of eukaryotic microalgae. The antioxidant properties and phenylalanine ammonia lyase enzyme activity were studied for the first time from the cyanobacteria *Synechocystis*, *Leptolyngbya* and *Oscillatoria* by Selcen *et al*. (2013). They could obtain high amounts of phenolic contents and good free radical-scavenging activity. Azza *et al*. (2014) studied the antioxidant properties of *Oscillatoria agardhii* and *Anabaena sphaerica*.

Goiris *et al*. (2015) determined whether nutrient stress affected the antioxidant properties in microalgae and concluded that it had a positive influence on the amount of tocopherols and ascorbic acid. Rai and Rajashekhar (2015) analyzed the phenolic, pigment and vitamin C content in nine cyanobacteria, with the objective of applying them in the pharmaceutical industries. The antioxidant activity and total phenolic contents of marine diatoms from southeast coast of India was done by Hemalatha *et al*. (2015). The phenolic content of Moroccan microalgae was analyzed by Maadane *et al*. (2015).

Rajishamol *et al*. (2016) determined the antioxidant activity of cyanobacteria isolated from Cochin estuary using *in vitro* assays. Anas *et al*. (2016) screened *Limnothrix* sp. and *Leptolyngbya* sp. from Arabian Sea for potent antioxidant and cytotoxic activity. The antioxidant properties of cyanobacteria isolated from the freshwater bodies of Sri Lanka were studied by Hossain *et al*. (2016). The phenolic compounds are a major contributor to the antioxidant activity therefore a phenolic profile was generated for cyanobacteria and microalgae clones using RP-HPLC by Idaira *et al*. (2017).

4.3 Materials and methods

4.3.1 Preparation of cyanobacterial extracts

The test cyanobacteria were harvested in the late logarithmic phase by centrifugation at 10000 rpm for fifteen minutes. The biomass obtained was lyophilized and the extracts for the antioxidant assays were prepared as per the procedure of Maadane *et al*. (2015). 100 mg of lyophilized cyanobacteria were extracted with 10 ml of five different solvents such as acetone, chloroform: methanol (2:1), dimethyl sulphoxide, ethanol and methanol for three hours in dark at room temperature. After incubation the extracts were centrifuged at 3000 rpm for 10 minutes. The supernatants were collected and evaporated to dryness under vacuum in a rotary evaporator. The extracts were stored at -20˚C for various antioxidant assays. The extracts were dissolved in double distilled water for the antioxidant assays.

4.3.2 Antioxidant assays

4.3.2.1 Total phenolic content

The total phenolic content of the test cyanobacteria was estimated by Folin-ciocalteau method described by Singleton and Rossi (1965). 200 µl of the extracts were treated with 1ml of Folin ciocalteau reagent (1:10). After 4 minutes of incubation 0.8 ml of saturated sodium carbonate (7.5% w/v) was added and mixed well. The reaction mixture was kept undisturbed for 2 hrs. The absorbance was measured at 765 nm in a Hitachi U3900-spectrophotometer. The total phenolic content was expressed in terms of gallic acid equivalence (GAE).

4.3.2.2 Total flavonoid content

Total flavonoid content was determined according to the method of Zhishen *et al*. (1999). To 1ml of different solvent extracts of the strains, 1.25 ml of double distilled water and 75 µl of 5% sodium nitrite solution were added. After 5 minutes, 150 µl of aluminium chloride trihydrate (10%) solution was added and the total volume was made up to 2.5 ml with double distilled water, this was left to stand undisturbed for 6 minutes. 1 ml of NaOH (1M) was added and thoroughly mixed and the absorbance was read against blank at 510 nm after 15 minutes incubation. The values were expressed as quercetin equivalents (QE).

4.3.2.3 Total antioxidant capacity

The antioxidant capacity was determined by the method of Prieto *et al*. (1999). 300 µl of the samples were mixed with 3.0 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample was mixed well and was incubated for 90 minutes at 95˚C in a water bath. The absorbance was measured at 695 nm. Results were expressed in terms of ascorbic acid equivalence (AE).

4.3.2.4 Deoxyribose radical scavenging activity

Deoxyribose non-site specific hydroxyl radical scavenging assay is based on the generation of hydroxyl radicals in the Fenton reaction (Fe^{2+} + $H_2O_2 \rightarrow Fe^{3+}$ + OH^- + $^{\bullet}OH$). The assay was carried out according to the method of Chung *et al*. (1997). The Fenton reaction mixture for the assay was prepared by mixing 1.0 ml sample with 1.0 ml of $FeSO_4.7H_2O$ (10 mM), 0.1 ml EDTA (10 mM) and 0.1 ml deoxyribose (10 mM). 0.9 ml of phosphate buffer (0.1 M, 7.4 pH) and 0.1 ml H_2O_2 (10 mM) were added to this mixture

and incubated at 37˚C for 4 hrs in dark. 0.5 ml of trichloroacetic acid (2.8%) and 0.5 ml of tertiary butyl alcohol (1%) were added and incubated in a boiling water bath for 10 minutes. The absorbance was measured at 532 nm. The scavenging ability was calculated as per Heo *et al*. (2005).

4.3.2.5 Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) is a reducing assay which is based on the ability of the sample to reduce ferric (III) to ferrous (II) in a redox-linked colorimetric reaction. The reducing power assay was done according to Oyaizu (1986). 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and potassium ferrricyanide (1%) were added to the extracts (100-300 µl) and incubated at 50˚C for 20 minutes followed by addition of 2.5 ml of 10% trichloroacetic acid and centrifuged at 1500 rpm for 10 minutes. 2.5 ml of solution was removed from the upper layer and was mixed with an equal volume of distilled water. 0.5 ml of ferric chloride solution (0.1%) was added and the absorbance of the reaction mixture was measured at 700 nm.

4.3.2.6 DPPH radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) molecule is a commonly used stable free radical to determine antioxidant activity of natural compounds. The delocalization of spare electron present in the molecule gives rise to a deep violet colour which gives an absorption maximum at 517nm in ethanol solution. When DPPH is mixed with a substrate that can donate a hydrogen atom, it leads to the reduced form of DPPH with the loss of colour. The difference in the absorbance value before and after reduction gives the scavenging ability of the sample. The protocol of Yen and Chen (1995) was followed for the assay. 3.0 ml of 0.16 mM DPPH prepared in ethanol was added to the extracts and incubated for 30 minutes at room temperature in

dark. The absorbance of the sample was measured at 517 nm. The scavenging effect was calculated by the following formulae:

$$\% \text{ inhibition of DPPH radical} = \frac{\mathbf{A}\ \text{br} - \text{A ar}}{\text{A ar}} \quad \text{X } 100$$

Where A br is the absorbance before reaction and A ar is the absorbance after reaction.

4.3.2.7 Hydrogen peroxide (H_2O_2) radical scavenging assay

The method of Ruch *et al*. (1989) was followed for the assay. 100-300 µl of algal extracts were mixed with 2.5 ml of 10mM hydrogen peroxide solution prepared in phosphate buffer (pH 7.4). The final volume of the reaction mixture was made up to 1ml with phosphate buffer. The absorbance of the sample was measured after 10 and 60 minutes of the reaction at 232 nm. Phosphate buffer without hydrogen peroxide served as the control. The values were compared with ascorbic acid.

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \quad \text{X } 100$$

4.3.3 Statistical analysis

All the experiments were conducted in triplicates. The results were given as mean value with standard deviations. To determine whether there was any significant difference in the antioxidant activities of the strains, univariate analysis of variance was done followed by post hoc tukey tests in IBM SPSS statistics 22 software packages. $p < 0.05$ was considered as significant.

4.4 Results

4.4.1 Total phenolic content

Total phenolic content was analyzed in seven cyanobacterial strains using different solvent extracts that is acetone, chloroform:methanol, DMSO, ethanol and methanol. *S. salina* showed the highest phenolic content of 44.33±0.433 µg GAE/mg in the methanol extract, followed by 37.73±0.24 µg GAE/mg in ethanol extract. The remaining six test strains exhibited values below 15 µg GAE/mg. *C. minutus* showed 12.90 ±0.105 µg GAE/mg in methanol extract and 12.73±0.057 µg GAE/mg in DMSO extract. *S. aquatilis* had 12.53±0.207 µg GAE/mg phenolic content in DMSO extract. The DMSO extract of *A. litoralis* showed 10.35±0.172 µg GAE/mg. The *G. gelatinosa* and *O. limosa* exhibited similar values in the acetone extract (9.83±0.030 µg GAE/mg and 9.1 ± 0.115 µg GAE/mg). The phenolic content was least in *S. cedrorum* 6.02±0.230 µg GAE/mg (Fig. 4.1). The chloroform:methanol extracts of all the strains recorded the minimum values. The univariate analysis of variance showed that there was significant difference ($p<0.05$) in the phenolic content of the test strains.

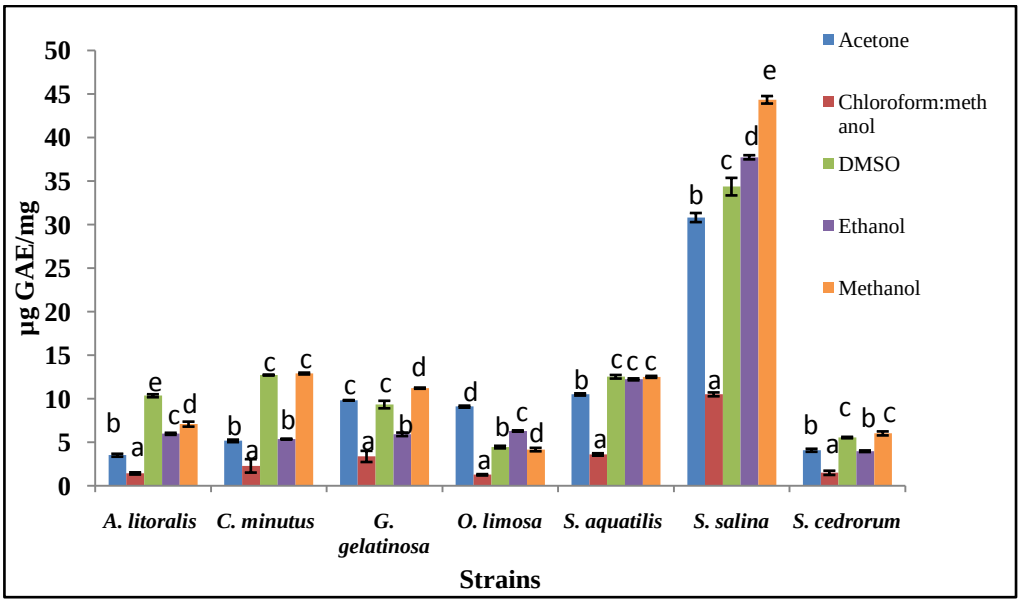


Fig. 4.1 Total phenolic content of the cyanobacterial strains

4.4.2 Total flavonoid content

In the present study, the methanolic extracts of the test strains expressed better flavonoid content than other extracts. The highest content was recorded in the methanol extract of *S. salina* 18.30±1.37 µg QE/mg, while the ethanol extract showed 13.41 ± 0.31 µg QE/mg. The methanol extracts of *G. gelatinosa* and *S. cedrorum* had 7.40±0.176 µg QE/mg and 7.04±0.10 µg QE/mg of flavonoids respectively. The DMSO and methanol extracts of *S. aquatilis* had similar amount of flavonoid (3.7 µg QE/mg). The DMSO extract of *A. litoralis* had 3.77± 0.76 µg QE/mg flavonoid and the methanol extract of the same had 2.89 ± 0.28 µg QE/mg. The ethanol extract of *O. limosa* showed 1.91 ± 0.04 µg QE/mg (Fig. 4.2). The results indicated that the flavonoid compounds were more soluble in methanol and DMSO. The univariate analysis of variance showed that there was significant difference ($p<0.05$) in the flavonoid content of the test cyanobacteria.

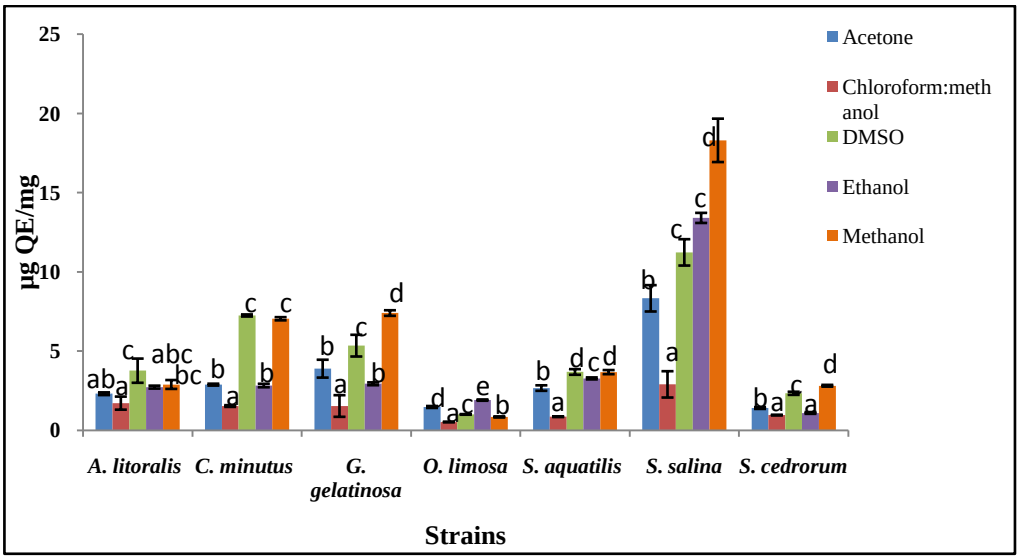


Fig. 4.2 Total flavonoid content of the cyanobacterial strains

4.4.3 Total antioxidant capacity

The total antioxidant capacity was maximum in DMSO extract of *S. salina* (83.63 ± 0.503 µg AE/mg), the methanol extract of the strain recorded 69.8 ± 1.562 µg AE/mg. *S. salina* was followed by *S. cedrorum* and *S. aquatilis* with 61.67 ± 5.32 and 60.9 ± 1.493 µg AE/mg respectively in the DMSO extract. The acetone and methanol extracts of *S. cedrorum* had almost similar activity (43.73 ± 0.83 µg AE/mg and 42.41 ± 1.30 µg AE/mg). *A. litoralis* showed a maximum of 49.63 ± 1.209 µg AE/mg in the methanol extract. The DMSO extract of *C. minutus* had the highest value of 46.8 ± 1.6 µg AE/mg. The DMSO extract of *G. gelatinosa* exhibited 26.93 ± 0.058 µg AE/mg antioxidant capacity. The lowest value for total antioxidant capacity was shown by *O. limosa* in the acetone extract, 21.53 ± 0.702 µg AE/mg (Fig. 4.3). The solvent chloroform:methanol was found to be the least effective solvent for determination of total antioxidant capacity. The univariate analysis of variance showed that there was significant difference ($p<0.05$) in the antioxidant capacity of the test cyanobacteria. It was significantly correlated with the phenolic content (R^2 0.504).

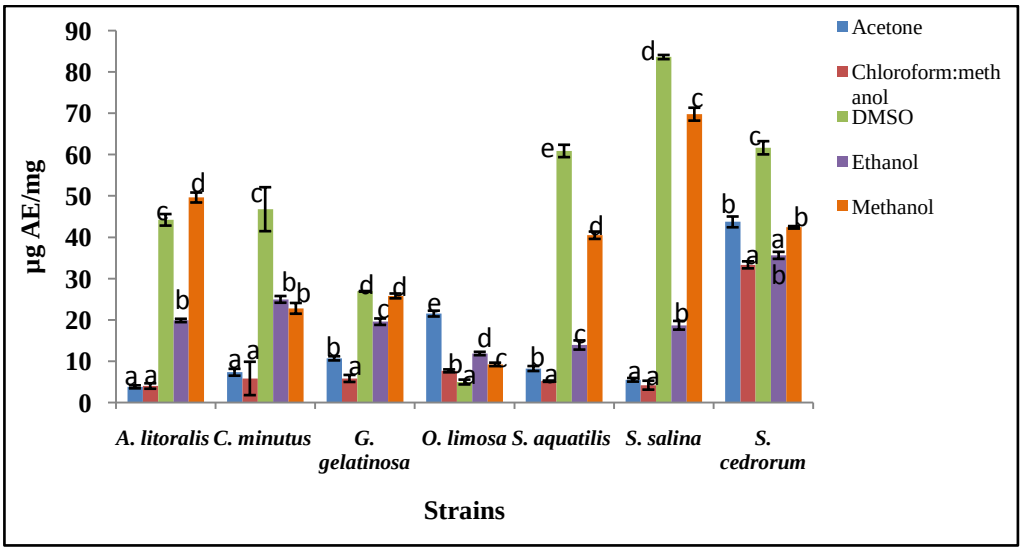


Fig. 4.3 Total antioxidant capacity of the cyanobacterial strains

4.4.4 Deoxyribose radical scavenging activity

The deoxyribose free radical was maximally scavenged by the DMSO extract of *S. salina* at 22.9% (Fig. 4.4). DMSO extract of *S. cedrorum* could scavenge at 20.2% and the methanol extract scavenged 17.9% of hydroxyl radical. The ethanol and methanol extracts of *S. salina* exhibited similar activity of 21.5% and 21%. DMSO extracts of *C. minutus* showed 15.25% scavenging ability while the acetone extract marked 13% activity, the ethanol and methanol extracts had similar ability. DMSO extract of *S. aquatilis* showed maximum value of 13.2% followed by ethanol (12.5%) and acetone extracts (12.3%). *G. gelatinosa* showed 13.3% scavenging ability in DMSO extract. Acetone extract of *A. litoralis* could scavenge 10% of the free radical generated. Among the seven strains least free radical scavenging ability was shown by *O. limosa* (7.9%). The lowest values for all the strains were obtained in the chloroform:methanol extract. DMSO, ethanol and methanol were found to be better solvents for determining the deoxyribose free radical scavenging activity. The univariate analysis of variance showed that there was significant

difference ($p<0.05$) in the deoxyribose radical scavenging activity of the test strains. It was significantly correlated with the phenolic content (R^2 0.738).

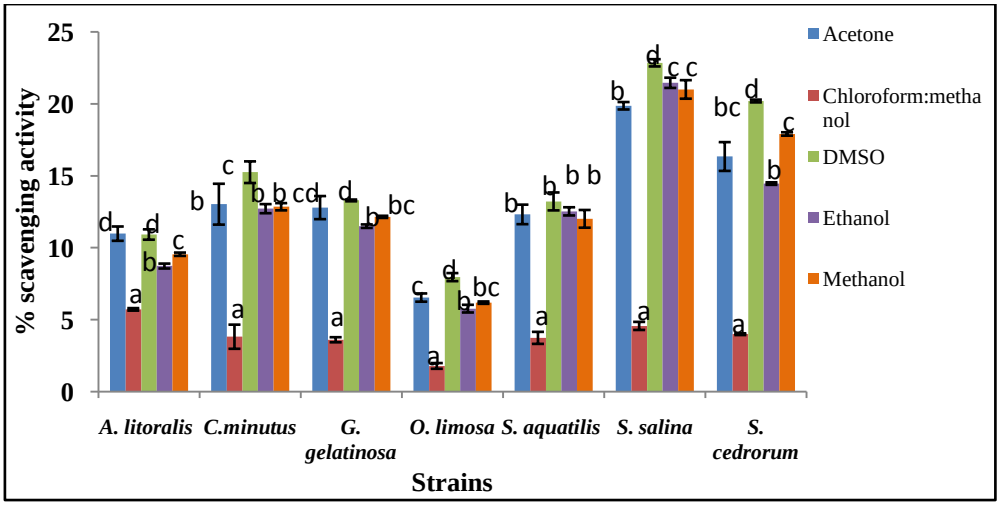


Fig. 4.4 Deoxyribose radical scavenging activity of the cyanobacterial strains

4.4.5 Ferric reducing antioxidant power

The highest ferric reducing power was obtained in the DMSO extracts of *S. salina* (55.18±0.517 µg AE /mg) followed by methanol (53.92±0.075 µg AE /mg) and ethanol extract (49.84±0.050) (Fig. 4.5). In case of *S. cedrorum* the methanol extract had better reducing power than the DMSO and ethanol extracts. The methanol extract exhibited 46.02±0.35 µg AE /mg activity DMSO and ethanol extracts had 40.87±0.50 and 38.7±0.36 µg AE /mg reducing activity. The methanol extracts of *S. aquatilis* had highest reducing activity of 43.18±0.81 µg AE/mg and the ethanol extracts recorded 40.28 ±0.32 µg AE/mg. In *G. gelatinosa* the DMSO, methanol and ethanol extracts exhibited comparable reducing power. The methanol extract had maximum reducing activity in case of *A. litoralis* but acetone extract was more effective in the case of *O. limosa*. The lowest reducing

power among the test strains were recorded for *C. minutus* with a reducing power of 23.10 ± 0.38 µg AE /mg. The values clearly indicate that the assay is dose dependent since the reducing power of the algal extracts increased with increasing concentration. The univariate analysis of variance showed that there was significant difference ($p<0.05$) in the ferric reducing antioxidant power of the test cyanobacteria. It was significantly correlated with total phenolic content (R^2 0.727).

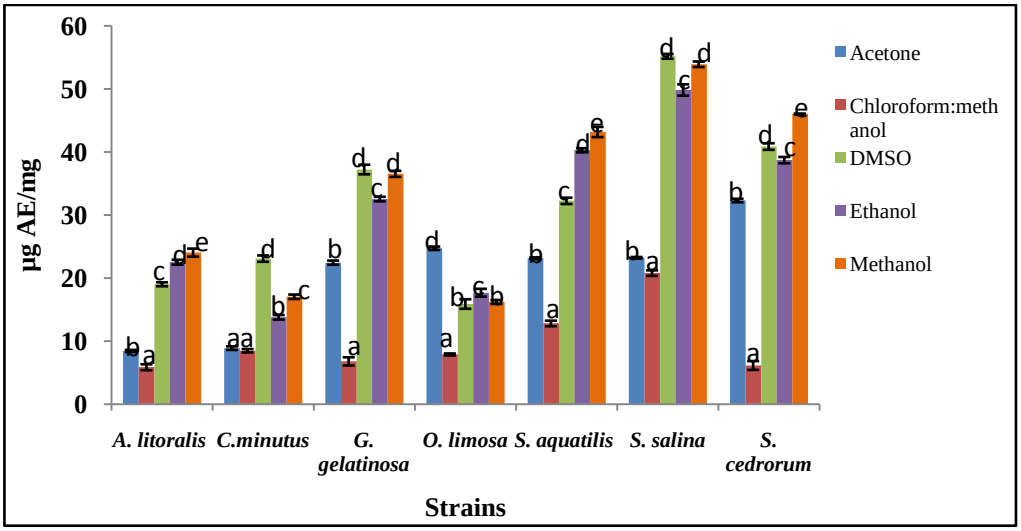


Fig. 4.5 Ferric reducing antioxidant power of the cyanobacterial strains

4.4.6 DPPH radical scavenging activity

S. cedrorum was able to scavenge the maximum amount of DPPH free radical. The methanol extract inhibited 65% and the acetone extract showed 63.6% inhibition. The DMSO and ethanol extracts inhibited the free radical at 54.8%. *S. salina* showed the second best scavenging ability, the methanol extracts of the species could inhibit 55.8% of DPPH. The DMSO extract of *A. litoralis* recorded 52.4% while the acetone and methanol extracts had similar

inhibitory effect. In case of *C. minutus* the methanol extract was able to scavenge the maximum at 49.6%; the DMSO extract, the ethanol extract and acetone extract showed scavenging ability at 47.4 %, 42.2% and 40.2% respectively. *S. aquatilis* showed 46.8% scavenging activity in DMSO extracts. *G. gelatinosa* recorded 37.2% and 35.8% inhibitory action in acetone and methanol extracts respectively. The least scavenging ability was observed in the methanol extracts of *O. limosa* (25.9%). The chloroform:methanol extract of all the strains exhibited least values of scavenging activity. The univariate analysis of variance showed that there was significant difference ($p<0.05$) in the DPPH radical scavenging activity of the test strains. It was significantly correlated with phenolic content (R^2 0.452). The DPPH radical scavenging activity of the seven strains is presented in Fig. 4.6.

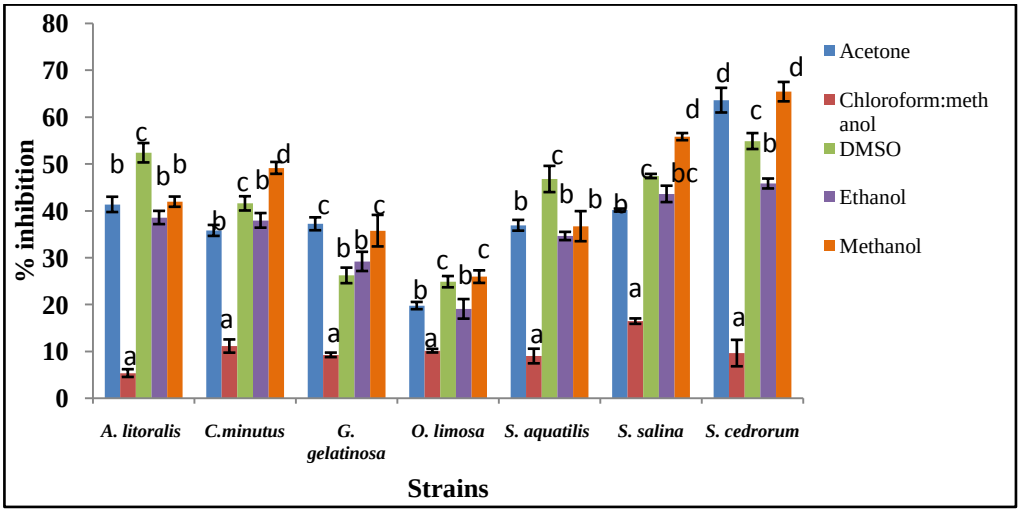


Fig. 4.6 DPPH radical scavenging activity of the cyanobacterial strains

4.4.7 Hydrogen peroxide (H_2O_2) radical scavenging assay

The hydrogen peroxide scavenging activity of the test strains is depicted in Fig. 4.7. All the test strains generally exhibited low hydrogen peroxide (H_2O_2) radical scavenging activity (<10%). The DMSO extract of *S.*

cedrorum and *S. salina* recorded the highest value of 8.1%. The acetone and methanol extracts of *S. salina* showed 6.9% and 6.6% scavenging ability. The methanol and DMSO extracts of *A. litoralis* showed 6.5% and 6.1% activity. The DMSO extracts of *S. aquatilis* and *C. minutus* exhibited 5.2% and 5.1% inhibition of the hydrogen peroxide free radical respectively. The DMSO extracts of *G. gelatinosa* had 4.9% scavenging activity. The least activity of 4.1% was shown by the DMSO extract of *O. limosa*. The chloroform:methanol extracts of the test strains registered below 3% scavenging ability. The univariate analysis of variance showed that there was significant difference ($p<0.05$) in the hydrogen peroxide radical scavenging activity of the test cyanobacteria. The activity was significantly correlated with phenolic content present in the strains (R^2 0.342).

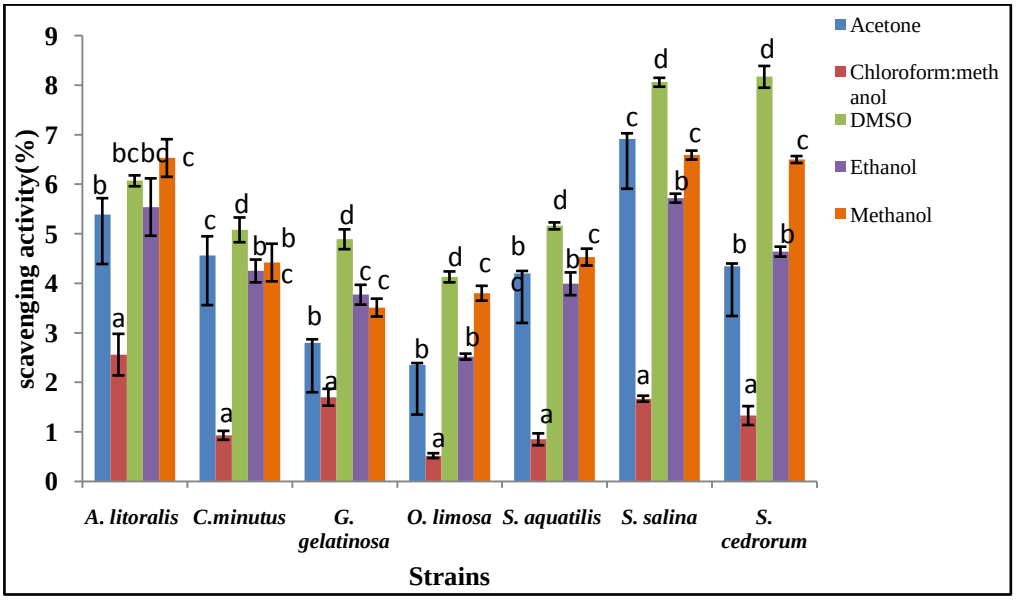


Fig. 4.7 Hydrogen peroxide radical scavenging activity of the cyanobacterial strains

4.5 Discussion

Cyanobacteria, the phylogenetically oldest plants, have effective mechanisms to protect cells from oxidative damage caused by free radicals. Polyphenolic compounds constitute one of the major antioxidants; they neutralize free radicals through single electron or hydrogen atom transfer. They play a major role in biological defense and serves as a primary structural component of cell walls. Besides these they are reported to have antimicrobial, antioxidant and enzyme inhibiting activities (Li *et al*., 2007 and Balboa *et al*., 2013).

Phenolic compounds contribute significantly to the antioxidant activities of cyanobacteria (Shanab *et al*., 2012). The total phenolic content of the test cyanobacteria varied from 6 to 44 µg GAE/mg. *S. salina* exhibited the maximum value (44.3 µg GAE/mg) and all other test strains had lower phenolic content. This is in agreement with earlier studies on cyanobacteria (Selcen *et al*., 2013 and Sharathchandra and Rajashekhar, 2013). Though the phenolic content of *S. salina* was comparable, the strains *S. aquatilis* and *O. limosa* exhibited much lower values. Phenolic content of *Oscillatoria* species from Sri Lankan waters was found to be lower than the present study (Hossain *et al*., 2016). The strain *Synechococcus* sp. FACHB 283 contained 10.56 mg GAE/g (Li *et al*., 2007), but the present study could record only 6.02 µg GAE/mg in *S. cedrorum*. The phenolic content varied depending on the species and the solvent used for extraction.

The phenolic compounds of the strains were highly extractable in methanol. This is in accordance with previous studies (Hemalatha *et al*., 2015, Azza *et al*., 2014 and Karunamoorthy *et al*., 2012). DMSO and ethanol were also found to be suitable for extraction, whereas, chloroform:methanol was

found to be the least efficient solvent for phenolic extraction. Microalgae isolated from Moroccan waters also exhibited high phenolic activity in the ethanol extracts (Maadane *et al.,* 2015). Contrary to the above findings, the aqueous extracts were reported to have more phenolic content than that of their solvent counterparts (Hajimahmoodi *et al*., 2010). The phenolic content of microalgae may be influenced by the environmental conditions. The exposure of the microalgae to the UV- light increased the production of phenolic substances (Maadane *et al.,* 2015). Hence, exposure of the test strains to stress conditions such as intense light radiations might increase the phenolic content.

Flavonoids are phenolic compounds widely distributed among the plants and algae with free radical scavenging abilities. The flavonoid content of the test strains can be one of the reasons for their remarkable antioxidant potential. It followed the same trend as the phenolic content and varied from 1.9 to 18.3 µg QE/mg. *S. salina* showed the highest content and this clearly indicates that it is a major contributor to the total phenolic content of the strain. Similarly, the strain *O. limosa* exhibited the lowest value. The flavonoid content of the test strains were comparable with those obtained for *Oscillatoria agardhii* and *Anabaena sphaerica* (Azza *et al*., 2014). Methanol was found to be the best solvent for extraction of flavonoids also, followed by ethanol and DMSO. This is in accordance with the previous reports of Azza *et al*. (2014), Rai and Rajashekhar (2015) and Anas *et al*. (2016). However, the values obtained for *S. salina* was comparatively higher than the other studies. Hossain *et al*. (2016) reported that the flavonoid content of freshwater forms was much higher than the marine counterparts, but the results of the present study suggest otherwise. Since flavonoids are reported to have therapeutic effects, the cyanobacteria may be exploited as a potential source of flavonoids.

The antioxidant capacity assay is based on the reduction of Molybdenum (VI) to Molybdenum (V) by the antioxidant principle present in the algal extract at an acidic pH resulting in the formation of a green phosphomolybdenum complex. The test strains expressed good antioxidant capacities. *S. salina*, *S. cedrorum* and *S. aquatilis* exhibited the highest antioxidant capacities and *O. limosa* showed the minimum value. Previous studies reported lower antioxidant capacities in cyanobacteria especially *Synechocystis* sp. and *S. salina* (Catarina *et al*., 2013) which was contrary to the findings of the present study. The values reported were 7.98 ± 1.01 mg AE/g and 2.97 ±0.46 mg AE/g whereas 83.63±0.50 µg AE and 60.9 ±1.50 µg AE could be recorded in the test strains. The total antioxidant activity reported in different *Oscillatoria* species by Rai and Rajashekhar (2015), were comparable with the values obtained for *O. limosa* in the present study. Earlier researches suggest that the solvents used for the extraction have dramatic effect on the antioxidant properties (Ganesan *et al*., 2008). The DMSO, methanol and ethanol were found to be effective for determining the antioxidant capacity of the test cyanobacteria. Studies by Hemalatha *et al.* (2015) suggested that methanol and acetone extracts expressed better antioxidant capacities than hexane extracts. Therefore, it is evident that the antioxidant capacity is highly dependent on the chemical nature of the test species.

Hydroxyl radical produced by the Fenton reaction is the most toxic and highly reactive ROS in cyanobacteria. It has got a half-life less than 1µs due to its ability to participate in addition, hydrogen abstraction and electron transfer reactions. It attacks the different biological molecules non selectively and has a very short diffusion path from the site of production (Hseih and Pedersen, 2015). The hydroxyl radical scavenging activity of the test strains were found

to be at a maximum of 23%. This was low compared to earlier studies on other algae and commercial antioxidants, which recorded above 30% scavenging activity (Athukorala *et al*., 2006 and Guedes *et al*., 2013). However, some studies have reported low activity in *Chlorella marina* (Karunamoorthy *et al.*, 2012) and also different cyanobacterial species (Guedes *et al*., 2013). All the solvent extracts except chloroform:methanol exhibited similar scavenging abilities.

The ferric reducing antioxidant activity was analyzed in increasing concentrations of the algal extracts. *S. salina* and *S. cedrorum* exhibited the highest ferric reducing antioxidant activities. Complementing the results of the present study, similar FRAP activity was reported in the cyanobacterial species *Fischerella ambigua* and *Chroococcus disperses* (Hajimahmoodi *et al*., 2010). Besides, the activities obtained are comparable with those recorded by Hossain *et al*. (2016) and Rai and Rajashekhar (2015). The dose dependency was clearly visible; this was reported in earlier studies on both microalgae and macroalgae. The FRAP activity of the different solvent extracts of marine diatoms *O. aurita, C. curvisetus* and *T. subtilis* were found to increase with increasing concentrations of the algal extracts (Hemalatha *et al*., 2015). Similarly, the green algae *C. marina* also exhibited dose dependency (Karunamoorthy *et al*., 2012). Athukorala *et al*. (2006) reported that the macroalgal enzymatic hydrolysate from *Ecklonia cava* showed dose dependency in the FRAP assay. Though the antioxidant power varied between different microalgal species, it was positively correlated with phenolic content, indicating that the phenolic compounds played a role in the ferric reducing activity of the strains.

DPPH radical scavenging activity is one of the most commonly used colorimetric assay to determine the antioxidant activity of natural compounds.

The cyanobacterial extracts in the current study could effectively scavenge the DPPH free radical. The methanol extracts of *S. cedrorum* and *S. salina* could scavenge more than 50% of the free radical. The scavenging activity of the test strains was comparable with that of previous studies on cyanobacteria (Shanab *et al*., 2012). The assay was also dose dependent as the free radical scavenging ability exponentially increased with the increase in algal concentration. The dose dependency of the assay was reported earlier in the cyanobacteria *Limnothrix* sp. and *Leptolyngbya* sp. (Anas *et al*., 2016) and in the macroalgal extracts of brown algae *Scytosiphon lomentaria*, *Papenfussiellla kuromo* and *Nemacystus decipiens* (Kuda *et al*., 2005). In the present study, the maximum radical scavenging activity was observed in the methanol extracts followed by DMSO and ethanol extracts whereas, Maadane *et al*. (2015) observed that the ethanol extracts of the tested microalgae possessed higher radical scavenging activity than the water extract and water/ethanol extract.

Selcen *et al*. (2013) observed that there was significant difference between filamentous and non-filamentous cyanobacteria in their ability to scavenge the DPPH free radical and the study reported very high scavenging activity in *Oscillatoria* sp. BASO703. However, in the present study, unicellular strains exhibited the highest scavenging ability and surprisingly *O. limosa* recorded the lowest scavenging activity. Studies indicate that the solvent polarity significantly affected the free radical inhibition and also the extracts with higher phenolic compounds and flavonoids tend to be better antioxidants (Lopez *et al*., 2011 and Hossain *et al*., 2016).

Hydrogen peroxide is a major non-radical reactive oxygen species generated in cyanobacteria in the photosystem II. It is relatively stable but can form hydroxyl radical through Fenton reaction (Hseih and Pedersen, 2015). The present study recorded low hydrogen peroxide radical scavenging activity

though the extracts showed dose dependency. The maximum scavenging activity obtained was 8.1% in *S. salina*. This is contrary to the studies in both microalgae and macroalgae where fairly good values were obtained up to 55% (Hemalatha *et al*., 2015). Some researches indicate that the C-phycocyanin present in cyanobacteria was an excellent scavenger of the peroxyl radicals and hydroxyl radicals (Patel *et al*., 2006). The lower activity of the test strains in the current study may be due to lower concentration of the algal extracts.

In conclusion, the test strains exhibited good antioxidant activity and free radical scavenging ability. More studies are to be conducted to increase and modify the concentration of the antioxidant substances present in the strains. Sophisticated techniques involving GC-MS and HPLC are to be applied to determine the chemical nature of the antioxidant substances present in the test strains. *S. salina* and *S. cedrorum* strains have the potential for application as antioxidant agents in nutraceutical, cosmetic and aquaculture industries.

Chapter 5

Antimicrobial activity of the isolated cyanobacteria

Contents

5.1 Introduction

Cyanobacteria constitute one of the most promising groups of organisms for novel bioactive compounds after Actinomycetes and Hyphomycetes among the microbial world. The use of *Nostoc* species for treating gout and fistula as early as 1500 BC highlights the importance of these prokaryotic organisms. They are reported to have antibacterial, antifungal, antialgal and antiviral activities (Burja *et al*., 2001). The antimicrobial activities are mainly due to secondary metabolites produced by cyanobacteria. These secondary metabolites can be chemically fatty acids, phenolic compounds, lipopeptides, pure amino acids, macrolides, lactones, esters, aromatic indoles, alkaloids, amides etc. (Burja *et al*., 2001 and Encarnacao *et al*., 2015). The production of bioactive molecules is affected by many factors such as temperature of incubation, incubation period, medium constituents, light intensity and pH of the culture medium (Madhumathi *et al*., 2011). The cyanobacterial secondary metabolites are mainly biosynthesized through non-ribosomal peptide synthetase and polyketide synthetase systems (Singh *et al*.,

2011). Both freshwater and marine forms are equally capable of producing bioactive compounds, but the latter have been least exploited.

Anabaena, *Microcystis*, *Nostoc*, *Lyngbya* and *Oscillatoria* are some of the potent bioactive metabolite producers (Burja *et al*., 2001 and Marques *et al*., 2012). Microvirin (MVN), a cyanobacterial lectin isolated from *Microcystis aeruginosa*, is highly inhibitory to a wide variety of HIV-1 strains, similarly *Oscillatoria agardhii* agglutinin (OAA) and Cyanovirin-N (CV-N) are also potent antiviral compounds (Encarnacao *et al*., 2015). The lipopeptides from cyanobacteria are highly effective against tumor cells. Borophycin is a boron-containing compound produced by *Nostoc linckia* and *Nostoc spongiaeforme* which is reported to have potential cytotoxicity and antitumor activity, similarly Cryptophycin is another anticancerous agent discovered from *Nostoc* sp. ATCC 53789. The cyanobacteria also produce numerous protease inhibitors known as cyanopeptolins which can be used in the treatment of diseases like lung emphysema (Marques *et al*., 2012).

The emergence of antibiotic resistance has led to the search for potent organisms which are commonly available and would cause minimum side effects. Wide screening has been carried on microalgae isolated from different habitats but it would take years for the development of an effective drug. Studies on preliminary screening of cyanobacteria prove that they have wide activity against human, food and aquaculture pathogens. Most researches were conducted on crude extracts of the algae and sometimes the purification leads to loss of activity. The compounds might lose their activity when applied *in vivo* and some exert toxic effects on the living systems (Borowitzka, 1995). These are some of the problems associated with the antimicrobial compounds obtained from microalgae. Despite these facts there are encouraging sides to the use of cyanobacteria for producing antimicrobial compounds such as their

wide ecological tolerance, amenability to genetic modifications, faster growth rates compared to higher organisms, least affected by climate change and the possibility of cultivation even in non-arable lands (Amaro *et al*., 2011 and Maadane *et al*., 2015).

One of the objectives of the present study was to determine the antibacterial and antifungal activities of seven strains of cyanobacteria against major human and aquaculture pathogens.

5.2 Review of literature

Cyanobacteria have been used as a medicine since ancient times (Pietra, 1990), but they were screened for their antimicrobial action only during the past few decades. Kulik reviewed the ability of cyanobacteria to control various plant pathogenic bacteria and fungi in 1995. Kreitlow *et al*. (1999) analyzed the effect of hydrophilic and lipophilic extracts of twelve commonly occurring cyanobacteria on the growth of Gram negative bacteria and yeast *Candida maltosa*. The antibacterial activity of the diatom *Skeletonema costatum* against different species of Vibrios and *Aeromonas hydrophila* was determined by Naviner *et al*. (1999). Antifungal compounds have been developed from cyanobacteria, Tanikolide, a lactone was isolated from marine cyanobacterium *Lyngbya majuscula* by Singh *et al*. (1999) and two antifungal cyclic peptides were obtained from *Tolypothrix byssoidea* (EAWAG 195) by Jaki *et al*. (2001). Burja *et al*. (2001) made an elaborate review on the numerous bioactive compounds identified from marine cyanobacteria and reported that a total of 424 compounds had been isolated from them. They concluded that this prokaryotic group may serve as a potential candidate for drug discovery and development of novel antimicrobial compounds. Ghasemi *et al*. (2003) isolated one hundred and fifty

cyanobacterial isolates from the paddy fields of northern Iran and subjected the culture supernatants and methanolic extracts to antimicrobial activity and identified two potential strains *Fischerella* sp. and *Stigonema* sp.

Noaman *et al*. (2004) reported that the antimicrobial activity of *Synechococcus leopoliensis* was affected by temperature, culture and nutrient conditions. An antifungal glycosylated lipopeptide from *Hassallia* sp. effectively inhibited *Aspergillus fumigatus* and *Candida albicans* (Neuhof *et al*., 2005). Safonova and Reisser (2005) suggested that two types of antibacterial effects are caused by cyanobacteria one is constitutive and the other is induced type. Rechter *et al*. (2006) determined the antiviral activity of *Arthrospira platensis* derived spirulan-like substances and concluded that they could inhibit HIV and herpes viruses. The antibacterial, anti algal and antifungal activity of norharmane, which is an exometabolite produced by cyanobacteria during growth was tested by Volk and Furkert (2006). Volk (2008) screened seven cyanobacteria for the production of norharmane and found that the production varied with species.

Prasanna *et al*. (2008) evaluated the ability of seventy cyanobacterial isolates against phytopathogenic fungi and analyzed the role of hydrolytic enzymes in the fungicidal activity. The antimicrobial and cytotoxic activities of marine *Synechocystis* and *Synechococcus* species were analyzed by Martins *et al*. (2008). Ruangsomboon *et al*. (2010) determined the antibacterial activity of different cyanobacteria and concluded that *Nostoc commune* was the most potent strain inhibiting several pathogenic bacteria. Sethubathi and Prabu (2010) found that *Oscillatoria* sp. isolated from Palk Bay exhibited powerful antibacterial activity against various human pathogenic bacteria. Deshmukh and Puranik (2010) applied Plackett-Burman design to evaluate the media components responsible for the antibacterial activity of alkaliphilic cyanobacteria.

Michele *et al*. (2011) reported that the phenolic extract from *S. platensis* significantly inhibited *Aspergillus flavus*. The antibacterial activity of *Oscillatoria latevirens*, *Phormidium*, *Lyngbya*, *Chroococcus* and *Microcystis aeruginosa* isolated from Thanjavoor area of Tamil Nadu was studied by Madhumathi *et al*. (2011). A novel systematic approach for identifying optimal culture conditions that significantly affected the antimicrobial activity of marine cyanobacteria was introduced by Caicedo *et al*. (2011). Dobrestov *et al*. (2011) determined the effect of polar and non-polar extracts of cyanobacterial mats isolated from different hot springs of Oman on the growth of *Bacillus* sp., *Micrococcus luteus*, *Shigella sonnei*, *Salmonella enterica* and *Klebsiella pneumoniae*. The antidiatom and quorem sensing inhibitory activities of the cyanobacterial extracts were also analyzed. A review on the potential of cyanobacteria as an emerging source of bioactive compounds was given by Singh *et al*. (2011); similarly Amaro *et al*. (2011) gave a broad insight into the antimicrobial activities of microalgae in general. Gantar *et al*. (2011) determined the antibacterial activity of different cyanobacterial species such as *Leptolyngbya*, *Phormidium*, *Pseudoanabaena*, *Synechococcus* along with cyanobacteria isolated from coral affected with black band disease against bacterial coral pathogens. The antimicrobial activity of methanolic extracts of cyanobacteria such as *Oscillatoria sancta* and *Lyngbya birgei* against human bacterial pathogens was tested by Prakash *et al*. (2011). Pramanik *et al*. (2011) isolated eight cyanobacterial species from the Sundarbans mangrove forest and checked its antibacterial activity against major human pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* and concluded that temperature, salinity, pH and nutrient concentrations affected the antimicrobial activity of the strains.

Pradhan *et al*. (2012) evaluated the antibacterial activity of *S. platensis* against different aquatic pathogens while Kokou *et al*. (2012) reported the inhibitory action of the same species against different Vibrio strains. Yadav *et al*. (2012) could find that methanolic extracts of *Anabaena*, *Nostoc* and *Scytonema* effectively inhibited *Pseudomonas* sp. The exopolysaccharides from *Gloeocapsa* sp. and *Synechocystis* sp. could act as effective antimicrobial agents against food pathogens (Najdenski *et al*., 2013). Cyanobacteria can be used for the production of silver nanoparticles which inhibits various pathogens especially *Pseudomonas vulgaris, Salmonella typhi, Vibrio cholerae*, *Streptococcus* sp., *Bacillus subtilis*, *S. aureus* and *E. coli* as reported by Sudha *et al*. (2013). Shaeib *et al*. (2014) determined the antimicrobial activity of aqueous and ethanolic extracts of *N. commune* and *S. platensis*. The GC/Mass spectrometry data was analyzed for the acetone and methanolic extracts of *Nostoc* sp. which was revealed to be a cocktail of different chemical compounds such as phenols, plasticizers, phytols, alkenes, esters and flavonoids (Salem *et al*. 2014). The antimicrobial activity of methanolic extracts of microalgae isolated from Baharia oasis of Egypt was reported by Ahmed (2016). Maadane *et al*. (2017) analyzed the antibacterial and antifungal activity of *Dunaliella salina*, *Nannochloropsis gaditana*, *Dunaliella* sp., *Phaeodactylum tricornutum* and *Isochrysis* sp. against *E. coli*, *P. aeruginosa*, *S. aureus*, *Candida albicans* and *Aspergillus niger*.

5.3 Materials and methods

5.3.1 Preparation of cyanobacterial extract

The cyanobacterial extracts were prepared according to the procedure of Naviner *et al*. (1999). The cyanobacteria in the late logarithmic phase of growth were harvested by centrifugation at 10000 rpm for fifteen minutes followed by lyophilization of the sample. 100 mg of lyophilized sample was extracted with 10 ml of five different solvents such as acetone, ethanol, methanol, dimethyl sulphoxide and diethyl ether. The extracts were centrifuged at 3000 rpm for 10 minutes. The extraction was repeated thrice and the supernatants were pooled and evaporated to dryness under vacuum at low temperature (40˚C). The samples were then stored at 4˚C until use.

The aqueous extract of cyanobacteria was prepared according to Somasekharan *et al*. (2016). The cyanobacterial culture was sonicated at 60% amplitude with 20 seconds of short bursts and 30 seconds of intermittent cooling. The extract obtained was filtered through Whatman filter paper of pore size 0.2µm diameter and was stored at 4˚C for the antimicrobial assay.

5.3.2 Test organisms

A total of ten bacterial strains including Gram negative and Gram positive human and aquaculture pathogens were used for the antibacterial assay. The Gram negative strains included *Aeromonas hydrophila* (KC549803), *Pseudomonas aeruginosa* (MF099861), *Escherichia coli* (KT804408) and Vibrios which included *Vibrio alginolyticus* (KT005561), *Vibrio anguillarum* (KC549801), *Vibrio harveyi* (MTCC 7954), *Vibrio mimicus* (KT187246) and *V. parahaemolyticus* (KM406325). *Staphylococcus aureus* (MTCC 3160) and *Bacillus* sp. (KT833383) were the Gram positive bacteria tested. All the strains were obtained from the culture collection

maintained in the fish pathology laboratory of the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Cochin-16.

Candida albicans and *Saccharomyces cerevisiae* were the fungal strains used for the antifungal assay. *C. albicans* was obtained from the culture collections maintained in the Microbiology laboratory of the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Cochin-16. *S. cerevisiae* granules were purchased from a local supermarket and subjected to purification procedures. The granules were initially inoculated into yeast extract peptone dextrose (YEPD) broth and incubated at 37˚C for 24 hours. Quadrant streaking was carried out on YEPD agar for obtaining pure colonies which was then separated and kept for carrying out the antifungal assay.

5.3.3 Determination of antimicrobial activity

The antibacterial activities of the extracts were determined by agar well diffusion method (Spooner and Sykes, 1972). Overnight cultures of bacterial strains grown in nutrient broth (10^7 CFU/ml) were plated onto nutrient agar plates using a sterile swab. For the Vibrios and *A. hydrophila,* plates were prepared with nutrient agar containing 1% salt. Five wells of diameter 9 mm were made, each extract was added as 50, 100, 150, 200 µl and the centre well was added with 200 µl of solvent alone which served as the negative control. The antibacterial activity was determined after 24 hrs of incubation at 37ºC by measuring the diameter of the inhibition zone in the agar layer with calipers (in mm). Extracts giving zones less than 13 mm indicated lack of activity.

Antifungal activity was also tested according to the procedure described above. Only difference was in the incubation temperature and the

culture media used. *C. albicans* was cultured on potato dextrose medium and *S. cerevisiae* on yeast extract peptone dextrose medium. The fungal strains were incubated at 27˚C for 24 hrs.

5.4 Results

5.4.1 Antibacterial activity

The cyanobacterial strains exhibited excellent antibacterial activity against the pathogens tested and the activity varied with the solvents used. Only the highest concentration (200 µl) of the extracts showed the inhibitory effect. The diethyl ether extract could inhibit almost all the pathogens. The ethanol extracts of the strains also exhibited significant antimicrobial activity. No antibacterial activity was observed for the acetone, DMSO, methanol and aqueous extracts of the strains. The antibacterial activity of the test cyanobacteria is depicted in Table 5.1.

A. litoralis could inhibit three test pathogens. The ether extract was inhibitory to *V. anguillarum* (16 mm) and *S. aureus* (15 mm). *V. alginolyticus* (23 mm) was found to be sensitive to the ethanol extract.

C. minutus inhibited only two pathogens. The diethyl ether extract inhibited *A. hydrophila* (14 mm) and *Bacillus* sp. (13 mm) was sensitive to ethanol extract.

G. gelatinosa ether extract inhibited four of the tested pathogens. *E. coli* (28 mm) and *Bacillus* sp. (27 mm) were effectively inhibited while *V. harveyi* (16 mm) and *V. mimicus* (15 mm) were moderately inhibited. The ethanol extract was found to be effective against *V. parahaemolyticus* (22 mm) and *P. aeruginosa* (13 mm).

The strain *O. limosa* did not exhibit much antibacterial action compared to other test strains. Only the ethanol extract of the strain was found to inhibit *P. aeruginosa* (16 mm).

The antibacterial activity of *S. aquatilis* showed that the ethanol extract effectively inhibited *S. aureus* (22 mm). All other extracts of the strain were ineffective against the pathogens tested.

The diethyl ether extracts of *S. salina* inhibited the maximum number of test pathogens. It could effectively inhibit *S. aureus* (31 mm), *A. hydrophila* (28 mm), *Bacillus* sp. (22 mm) and *V. mimicus* (21 mm). Moderate inhibitory effect was observed for *V. harveyi* (16 mm) and *E. coli* (14 mm). No antibacterial action was observed against *V. anguillarum*, *V. alginolyticus*, *V. parahaemolyticus* and *P. aeruginosa*. The ethanol extracts of the strain lacked antibacterial activity.

The ether extract of *S. cedrorum* could inhibit four of the tested pathogens. They were *V. parahaemolyticus* (33 mm), *V. mimicus* (19 mm), *E. coli* (17 mm) and *V. anguillarum* (16 mm). *V. parahaemolyticus* (23 mm) was found to be highly sensitive to the ethanol extract of the strain.

5.4.2 Antifungal activity

All the test cyanobacterial strains showed good antifungal activity. Even lower concentrations of different cyanobacterial extracts could exhibit inhibitory action compared to the antibacterial activity. The ether and ethanol extracts were highly effective and moderate inhibitory effect was observed for acetone, DMSO and methanol extracts. *C. albicans* was found to be more sensitive to the cyanobacterial extracts than *S. cerevisiae*. The aqueous extracts had no antifungal activity similar to the results of antibacterial activity. The antifungal activities of the strains are presented in Table 5.2.

The ether extract of *A. litoralis* showed antifungal activity against *C. albicans* (20 mm) whereas the DMSO extract inhibited both *C. albicans* and *S. cerevisiae* (16 mm and 14 mm).

The ether extract of *C. minutus* showed inhibition towards *C. albicans* at 150 and 200 µl concentrations of the extracts (13 mm and 26 mm) and towards *S. cerevisiae* (15 mm) at 200 µl concentrations. All other extracts of the cyanobacteria lacked antifungal action.

C. albicans was found to be sensitive to the acetone and DMSO extracts of *G. gelatinosa* (13 mm and 13 mm). The ether extract of the strain inhibited *S. cerevisiae* (15 mm).

The ether and ethanol extracts of *O. limosa* was highly effective against *C. albicans* (25 mm and 22 mm) but no antifungal activity was observed against *S. cerevisiae*.

S. aquatilis ether and ethanol extracts showed antifungal action against *C. albicans* (23 mm and 22 mm) but *S. cerevisiae* was not inhibited by any of the extracts.

S. salina exhibited the lowest antifungal activity among the test strains and none of the extracts of the strain could inhibit *C. albicans*. However, the ether extract effectively inhibited *S. cerevisiae* (22 mm).

S. cedrorum extracts showed high antifungal activity against *C. albicans*. The acetone and ethanol extracts of the cyanobacteria inhibited the pathogen even at 150 µl concentrations. The zone of inhibition obtained for acetone extracts were 13 mm and 26 mm and for ethanol extracts were 20 mm and 31 mm. *C. albicans* was also sensitive to ether extract (15 mm) of *S. cedrorum*. No antifungal activity was observed against *S. cerevisiae*.

Table 5.1 Antibacterial activity of the cyanobacteria

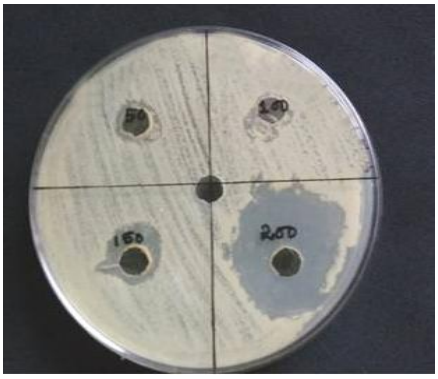
Pathogens	Diethyl ether extract (Zone of inhibition in mm)							Ethanol extract (Zone of inhibition in mm)						
	A. litoralis	*C. minutus*	*G. gelatinosa*	*O. limosa*	*S. aquatilis*	*S. salina*	*S. cedrorum*	*A. litoralis*	*C. minutus*	*G. gelatinosa*	*O. limosa*	*S. aquatilis*	*S. salina*	*S. cedrorum*
S. aureus	15±0.76	-	-	-	-	31±0.58	-	-	-	-	-	22±1.53	-	-
Bacillus sp.	-	-	27±1.53	-	-	22±1.53	-	-	13±0.58	-	-	-	-	-
A. hydrophila	-	14±0.58	-	-	-	28±1.53	-	-	-	-	-	-	-	-
V. anguillarum	16±0.58	-	-	-	-		16±1.01	-	-	-	-	-	-	-
V. alginolyticus	-	-	-	-	-		-	23±0.58	-	-	-	-	-	-
V.harveyi	-	-	16±1.15	-	-	16±0.58	-	-	-	-	-	-	-	-
V. mimicus	-	-	15±0.64	-	-	21±0.58	19±0.58	-	-	-	-	-	-	-
V.parahaemolyticus	-	-	-	-	-		33±1.53	-	-	22±1.15	-	-	-	23±0.58
E. coli	-	-	28±0.58	-	-	14±0.58	17±0.76	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-		-	-	-	13±1	16±0.58	-	-	-

Table 5.2 Antifungal activity of cyanobacteria

Pathogens	*C. albicans*								*S. cerevisiae*							
	Zone of inhibition in mm															
	Acetone extract		Diethyl ether extract		DMSO extract		Ethanol extract		Acetone extract		Diethyl ether extract		DMSO extract		Ethanol extract	
	150µl	200µl	150µl	200µl	150µl	200µl	150µl	200µl	150µl	200µl	150µl	200µl	150µl	200µl	150µl	200µl
A. litoralis	-	-	20±1	-	16±1.53	-	-	-	-	-	-	-	14±0.58	-	-	-
C. minutus	-	-	13±0.58	26±0.58	-	-	-	-	-	-	-	15±0.58	-	-	-	-
G. gelatinosa	-	13±1.53	-	-	-	13±0.58	-	-	-	-	-	15±1.01	-	-	-	-
O. limosa	-	-	-	25±1.53	-	-	-	22±0.58	-	-	-	-	-	-	-	-
S. aquatilis	-	-	-	23±1.53	-	-	-	22±0.58	-	-	-	-	-	-	-	-
S. salina	-	-	-	-	-	-	-	-	-	-	-	22±1.53	-	-	-	-
S. cedrorum	13±0.2	26±1.53	-	15±1.04	-	-	20±0.58	31±1.53	-	-	-	-	-	-	-	-

ANTIBACTERIAL ACTIVITY

PLATE 3



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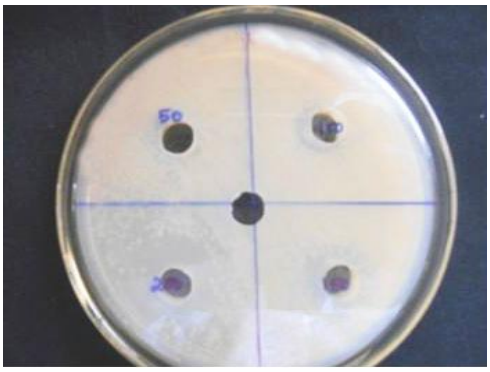

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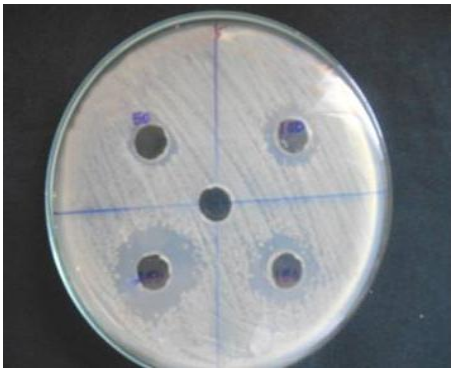

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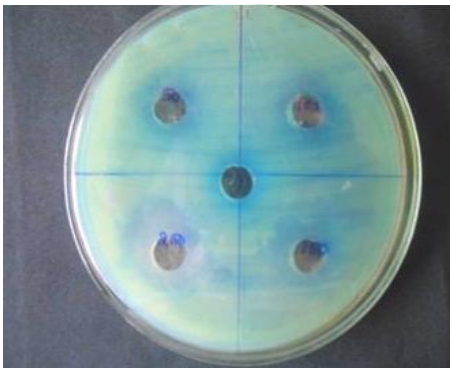
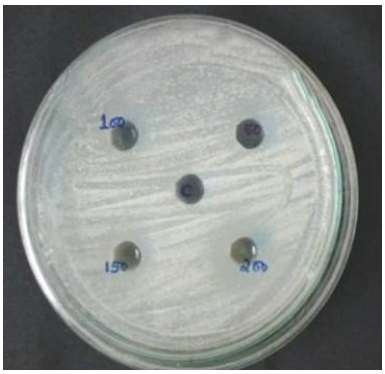


D

A: Antibacterial activity of ether extract of *S. cedrorum* against *V. parahaemolyticus*
B: Antibacterial activity of ether extract of *A. litoralis* against *S. aureus*
C: Antibacterial activity of ether extract of *A. litoralis* against *V. anguillarum*
D: Antibacterial activity of ethanol extract of *O. limosa* against *P. aeruginosa*

ANTIFUNGAL ACTIVITY

PLATE 4



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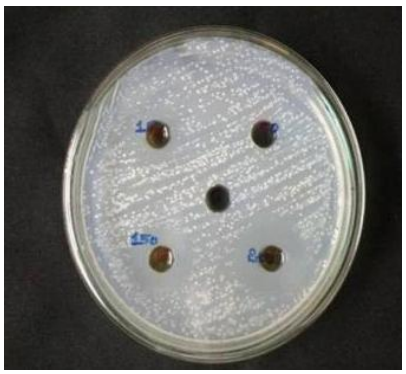

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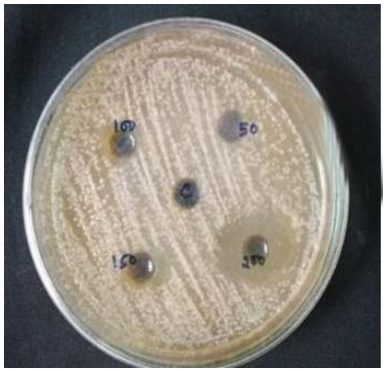
C



D

A: Antifungal activity of acetone extract of *G. gelatinosa* against *C. albicans*

B: Antifungal activity of acetone extract of *S. cedrorum* against *C. albicans*

C: Antifungal activity of ether extract of *G. gelatinosa against S. cerevisiae*

D: Antifungal activity of ethanol extract of *S. salina* against *S. cerevisiae*

5.5 Discussion

Marine cyanobacteria are a prolific source of novel bioactive compounds (Kreitlow *et al*., 1999 Burja *et al*., 2001 and Volk and Fulkert, 2006). Earlier studies have indicated the broad antibacterial, antifungal and antiviral activities of cyanobacteria (Kulik *et al*., 1995, Singh *et al*., 1999 and Rechter *et al*., 2006). In the present study, seven cyanobacterial cultures were tested for their antibacterial and antifungal activities. The strains were able to inhibit the tested pathogens including the fungal strains. The antimicrobial activity was highly dependent on the test strain, the solvent used for extraction and the pathogen. Each cyanobacteria was able to inhibit one or more of the test pathogens. *S. salina*, *S. cedrorum* and *G. gelatinosa* exhibited the maximum antibacterial activity among the test strains.

The highest inhibitory effect was observed in the diethyl ether extracts of the strains which could effectively inhibit almost all pathogens followed by ethanol extracts. This indicates that both non-polar and polar constituents exhibited antibacterial activity. Low antibacterial action (<11 mm) was observed in acetone and methanol extracts of the test cyanobacteria. Prior studies report highest activity in acetone, methanol and ethanol extracts and medium activity in ether extracts (Madhumathi *et al*., 2011, Ghasemi *et al*., 2003 and Shaieb *et al*., 2014). However, Rao *et al*. (2010) has reported that organic solvents with low polarity are most effective in extracting antimicrobial compounds from microalgae which is highly applicable in the present study. Moreover, both ether and ethanol are considered as safe solvents. Various studies have reported the effectiveness of aqueous extract of cyanobacteria in inhibiting various food-borne and human pathogens (Najdenski *et al*., 2013 and Ghasemi *et al*., 2003) but several reports suggest otherwise (Azza *et al*., 2014, Pradhan *et al*., 2012 and Ghosh *et al*., 2008) and

the results of the present study also indicate the ineffectiveness of the aqueous extract. No positive results were obtained in DMSO extracts in accordance with the observations of Najdenski *et al*. (2013).

Cyanobacterial extracts were effective in inhibiting both Gram positive and Gram negative bacteria. They could equally affect *S. aureus*, *Bacillus* sp. and different Vibrios. Some of the studies indicate that the Gram positive bacteria were more inhibited rather than the Gram negatives since the cyanobacteria was Gram negative in nature (Kreitlow *et al*., 1999). Study by Pradhan *et al*. (2012) pointed out that the ethanolic extract from the cyanobacteria *S. platensis* significantly inhibited *E. coli*, *A. hydrophila* and *Vibrio* species especially *V. anguillarum* and *V. fischeri* which is in agreement with our findings. All the *Vibrio* species were inhibited by the cyanobacterial extracts in the present study. Similarly, Rania and Hala (2008) reported that the ethanolic, acetone, ether and methanolic extracts of *S. platensis* inhibited *E. coli*, *S. aureus* and *P. aeruginosa*. In accordance with the observations of the present study, high antibacterial activity was recorded against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* by *Lyngbya*, *Oscillatoria* and *Synechocystis* genera of Sundarbans mangroves (Pramanik *et al*., 2011). Co-culturing of axenic *S. platensis* with different Vibrio species significantly reduced their growth indicating the antagonistic nature of the cyanobacteria and this could be applied in the rearing of fish larvae leading to higher survivability (Kokou *et al*., 2012). Based on the antibacterial activity of the cyanobacterial strains, *S. cedrorum* and *S. salina* could be utilized in the aquaculture field as an antibacterial agent against Vibrios and *A. hydrophila*.

G. gelatinosa and *S. salina* ether extracts were found to be highly inhibitory to *E. coli* and *Bacillus* sp. Some related studies indicate that the ethanolic extracts of *Gloeocapsa* sp. and *Synechocystis* sp. inhibited *S. aureus*

and *E. coli* (Yadav *et al*., 2012 and Najdenski *et al*., 2013) but no activity was observed in ethanol extracts of the strains. Results of the present study indicate that *S. cedrorum* was the most potent strain with high antibacterial and antifungal activity. The high antibiotic activity of the strain may be caused by the production of norharmane which is a biologically active co-mutagenic indole alkaloid reported to be produced by this group (Volk, 2008). Martins *et al*., (2008) could observe high antibacterial and cytotoxic activities in marine *Synechocystis* sp. and *Synechococcus* sp. similar to our findings. The high antibacterial activity of *Synechococcus* sp. and *Gloeocapsa* sp. has been reported in earlier studies also (Ruangsomboon *et al*., 2010 and Najdenski *et al*., 2013). Researches on *Oscillatoria* sp. showed that it is a highly potent antibacterial strain but our study indicated otherwise though high antifungal activity was recorded for the same (Sethubathi and Prabu, 2010, Madhumathi *et al*., 2011 and Prakash *et al*., 2011). Acetone extracts of *S. aquatilis* was highly inhibitory to *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* (Deshmukh and Puranik, 2010) but in the present study only the ethanol extracts of the strain exhibited antibacterial activity against *S. aureus* and was inactive against *E. coli*, *P. aeruginosa* and *Bacillus* sp.

The fungal pathogen *C. albicans* was highly sensitive to the cyanobacterial extracts. Antifungal activity could be observed in acetone, ether, DMSO and methanol extracts of the strains. The effectiveness of cyanobacterial extracts in inhibiting different fungal organisms have been reported earlier (Jaki *et al*., 2001, Ghasemi *et al*., 2003, Neurhof *et al*., 2005, Michele *et al*., 2011 and Najdenski *et al*., 2013). *S. cedrorum* exhibited the highest antifungal action against *C. albicans*, this is contrary to the results of Martins *et al*. (2008), who reported that strains belonging to the genera of *Synechocystis* and *Synechococcus* did not have antifungal activity against *C.*

albicans. However, *S. salina* was not inhibitory to *C. albicans*. In agreement with the findings of the present study, *Fisherella* and *Stigonema* culture supernatants and methanolic extracts were found to be highly inhibitory to *Candida krusei*, *Candida kefyr* and *Candida neoformans* (Ghasemi *et al*., 2003). Though *O. limosa* did not exhibit antibacterial effect, its antifungal action against *C. albicans* was commendable. The allelo-chemical compounds produced by *Oscillatoria* species were known to exert antimicrobial effect (Shanab, 2007). Studies report that the aqueous extracts of cyanobacteria like *Anabaena* and *Nostoc muscorum* had antifungal action against *Aspergillus flavus* (Shaieb *et al*., 2014), but no antifungal effect was observed for aqueous extracts in the present study.

Microalgae produce bioactive compounds as a defensive mechanism and for better survival. The antimicrobial compounds produced by cyanobacteria are chemically peptides, depsipeptides, macrolides, indole alkaloids, saponins, phenolics etc. (Burja *et al*., 2001). The production of these compounds depends on the growth and changes in the culture conditions (Caicedo *et al*., 2011). Elevated temperatures and presence of leucine, citrate and acetate in the medium induced maximum antibacterial activity in *Synechococcus leopoliensis* (Noaman *et al*., 2004). Similarly, the antibacterial activity of *S. aquatilis* could be modified by changing the concentrations of magnesium sulphate and ferric ammonium citrate in the culture medium (Deshmukh and Puranik, 2010). Change in temperature, pH, salinity and nutrient concentrations in the culture medium significantly affected the antibacterial activities of cyanobacteria isolated from mangrove habitats (Pramanik *et al*., 2011). The antimicrobial activity of the test strains in the current study may be enhanced by changing the nutrient concentrations and culture conditions.

The present study highlights that the cyanobacterial strains possess excellent antimicrobial action especially *S. cedrorum* and *S. salina* which could be exploited in the pharmaceutical and food industry. They may also find application in the larval hatcheries, improving the larval performance and survival without any adverse effects on the environment. However, further studies are required to determine the chemistry of the crude extracts of the potent strains.

Chapter 6

Efficacy of supplementing *Synechococcus* sp. and *Synechocystis* sp. on the growth and survival of *O. mossambicus*

Contents

6.1 Introduction

Aquaculture, the fastest growing farming sector meets the protein requirements of the ever expanding world population. The global aquaculture sector will surpass the yield of wild fisheries by 2020-2025 (Tacon, 2003). The success of aquaculture depends on numerous factors such as improved feed and feeding strategies, better water management, environment friendly practices, genetically fit stocks, improved health management and integration with agriculture (Hemaiswarya *et al*., 2011). One of the serious issues affecting this sector is the frequent outbreak of various pathogen-driven and stress-related diseases which, at times, may wipe out the entire farmed organisms leading to heavy economic and financial setbacks. Though the use of antibiotics for the treatment was viewed as a boon, it has turned out to be a curse leading to the emergence of antibiotic resistance.

Studies are being conducted worldwide to determine the effect of administration of a wide variety of probiotics, immunostimulants, plant

products and oral vaccines for controlling different bacterial fish diseases (Zhou and Wang, 2012 and Newaj-Fyzul and Austin, 2015). Probiotics have been associated with beneficial effects such as growth enhancement, improved feed utilization and carcass composition, disease resistance and elevated health status, reduction in intestinal microbes and need for less chemotherapy (Merrifield *et al*., 2010 and Newaj-Fyzul and Austin, 2015). Their possible mode of action is mainly by competitive exclusion, immunomodulation, production of inhibitory molecules, inhibition of virulence gene expression, disruption of quorum sensing, improvement of water quality and enzymatic contribution to digestion (Aditya *et al*., 2008 and Merrifield *et al*., 2010). A wide range of microorganisms such as bacteriophages, Gram positive bacteria, Gram negative bacteria, microalgae and yeasts are being screened for probiotic and immunostimulatory effects (Irianto and Austin, 2002 and Newaj-Fyzul and Austin, 2015).

Microalgae being the natural diet of many aquatic organisms will be a promising candidate and minimum side effects can be expected from their application. *Dunaliella salina*, *Dunaliella tertiolecta*, *Isochrysis galbana*, *Navicula*, *Chlorella*, *Tetraselmis suecica*, *Spirulina*, *Synechococcus* etc. are already being investigated for their various probiotic effects in the fish and shrimp feeds (Spolaore *et al*., 2006 and Newaj-Fyzul and Austin, 2015). Their positive effects have been proven in poultry, mice, cattle and even humans (Milledge, 2011). Worldwide studies are carried out to standardize and to find the most effective strain to be used as a potential supplement for various aquaculture species.

One of the objectives of the present investigation was to determine the dietary effect of 0.5% and 1% of *Synechococcus* sp. and *Synechocystis* sp. and a combination diet containing both species on growth parameters,

haematology and serology of *Oreochromis mossambicus*. The survival of the fish following *A. hydrophila* challenge was also studied.

6.2 Review of literature

Cultured microalgae form an integral part of the aquaculture hatcheries. They serve as food for the larvae of fish species, molluscs and shrimps. Many studies have been carried out to determine the effect of supplementing microalgae as protein source in the aquaculture feed. Presently the emphasis is on the development of environment friendly feed supplements that improves the growth, enhances the stress and disease resistance and overall performance of the cultured organism. Microalgae can positively affect the physiology by providing a large profile of vitamins, minerals, essential fatty acids which improves immune response and also influence their appearance like healthy skin and a lustrous coat (Spolaore *et al*., 2006).

Spirulina has been investigated for its dietary and immunostimulant effects in various fish and shrimp species. Nandeesha *et al*. (1998) analyzed the effect of feeding *S. platensis* on growth, proximate composition and organoleptic quality of common carp, *Cyrinus carpio*. Kim *et al*. (2002) reported that a 2% supplementation of *Chlorella* powder would improve growth, feed utilization, serum cholesterol level and whole body fat contents in juvenile Japanese flounder *Paralichthys olivaceus*. Studies were conducted on spawning and egg quality of the tilapia *Oreochromis niloticus* solely fed on *Spirulina* throughout three generations by Lu and Takeucchi in 2004. Misra *et al*. (2006) reported that 250 mg of β-glucan/kg supplementation in the diet of *Labeo rohita* enhanced growth and survival and also had a significant influence on the haematological and immunological parameters. Kumari *et al*. (2007) analyzed the effect of a proprietary herbal mixture containing *Ocimum sanctum*,

Withania somnifera, *Tinospora cordifolia* and *Emblica officinalis* in rohu fingerlings fed at 1g/Kg for thirty days followed by *A. hydrophila* challenge.

Liu *et al*. (2008) reported that the GH (growth hormone) transgenic *Synechocystis* (2%) enhanced growth of flounder *P. olivaceus*. *Shizochytrium* is a marine thraustochytrid alga rich in DHA; addition of 2% of this alga in the diet of Channel cat fish *Ictalurus puctatus* markedly improved the weight gain, feed efficiency ratio and level of n3 LC-PUFA in the edible tissues of fish (Li *et al*., 2009). Effect of supplementing *Thalassiosira weissflogii* and *Nannochloropsis* cultures to *Litopenaeus vannamei* was studied by Ju *et al*. (2009). Low level dietary application of *Chlorogloeopsis* to *O. niloticus* increased the haematocrit levels but no significant differences were observed for growth parameters or body composition (Merrifield *et al*., 2010). Kirubakaran *et al*. (2010) studied the effect of supplementing 0.1-1% night jasmine (*Nyctanthes arbortristis*) extract in tilapia challenged with *A. hydrophila*. Ngamkala *et al*. (2010) analyzed the effects of glucan and *Lactobacillus rhamnosus* GG (LGG) supplementation on the intestinal morphology after *Aeromonas hydrophila* challenge in *O. niloticus*.

Vasudhevan and James (2011) determined the effect of *Spirulina* supplementation along with vitamin C-incorporated diets on growth, reproduction and coloration in gold fish *Carassius auratus*. The effect of supplementing different levels of *Spirulina* on growth, haematology and survival following *Aeromonas hydrophila* challenge was done in *Labeo rohita* by Andrews *et al*. (2011). Sivakumar *et al*. (2011) proved the efficiency of feeding *Phormidium* and *Chlorella* sp. to *Penaeus monodon*. 20% supplementation of *Spirulina* improved growth in *Puntius gelius* (Hajahmadian and Vajargah, 2012). Up regulating effects were exhibited by *Sparus aurata* fed on dietary *Bacillus subtilis*, *Tetraselmis chuii* and

Phaeodactylum tricornutum singly or in combination (Cerezuela *et al.*, 2012a). The study also reported the histological alterations and microbial ecology caused by the microalgae supplementation (Cerezuela *et al.*, 2012b).

Zhou and Wang (2012) gave a detailed account of different probiotics used in aquaculture, their health benefits, technological applications and safety concerns associated with their administration. *Spirulina* was reported to act as a nutritionally efficient feeding attractant for *Litopenaeus vannamei* (Jose *et al*., 2012). Immunostimulatory effects of *S. platensis* in *Oreochromis niloticus* were determined by studying the non specific-defense mechanisms including serum bactericidal activity, phagocytosis and lysozyme activity by challenging the test fish with *A. hydrophila* (Ragap *et al*., 2012). Fadl *et al*. (2013) could observe that 15% of *Anabaena* supplementation in the ration of *O. niloticus* had a significant effect on growth performance, survival and haematological parameters. Similarly, Ibrahem *et al*. (2013) reported that 10% *Spirulina* supplementation could improve growth, immunity and disease resistance in *O. niloticus*. *L. vannamei* fed on 40% *Spirulina* in the diet exhibited improved amino acid profile and also showed better productive performance (Gadelha *et al*., 2013).

Studies indicate that the incorporation of different cyanobacteria significantly improved the survival rate of the cultured fish (Ramamurthy *et al*., 2013 and Das *et al*., 2013). Teimouri *et al*. (2013) found that inclusion of *S. platensis* (7.5%) ensured better pigmentation and enhanced growth performance in rainbow trout. The effect of replacing *Spirulina* as a food supplement for common carp fingerlings was studied by Abdulrahman (2014). Kuhlwein *et al*. (2014) determined the effect of supplementing dietary glucans in *C. carpio* at 1.0 to 2.0 % for eight weeks. They also determined its effect on the immune system of the test fish. The effect of dietary administration of lycogen, a commercialized carotenoid extract obtained from *Rhodobacter*

sphaeroides WL-APD911 on growth and immune system of seawater red tilapia was determined by Chiu and Liu (2014).

Liang *et al*. (2015) reported that the lower doses of cyanobacteria promoted growth whereas, high doses above 30% inhibited growth. Yeganeh *et al*. (2015) found that 10% supplementation of *S. platensis* in the diet of rainbow trout influenced the haematological and serological parameters positively and recommended it as an immunostimulant in diets of rainbow trouts. Ramamurthy *et al*. (2015) studied the effect of *Nostoc muscorum* on the survival of *Aeromonas salmonicida* infected *Mugil cephalus*. Newaj-Fyzal and Austin (2015) reviewed the different probiotics, immunostimulants, plant products and oral vaccines used in the control of the bacterial fish diseases. The article also gave an insight into different aspects such as the dosage of probiotics, mode of action, their effect on the immune system and future prospects in the aquaculture field. Radhakrishnan *et al*. (2016) reported that 50% *Arthrospira platensis* inclusion in the feed would improve the growth performance in the freshwater prawn *Macrobrachium rosenbergii*.

6.3 Materials and methods

6.3.1 Experimental fish and husbandry

Healthy *Oreochromis mossambicus* fishes with an average weight of 8±2g were purchased from a fish farmer in the locality. The fishes were maintained in well aerated chlorine free tap water tanks of 60 liter capacity. They were acclimatized to the laboratory conditions for fifteen days and during this period they were fed on commercial diet at 3% body weight. The daily ration was subdivided into two and was fed at 08:00 and 16:00 hours. 50 % water exchange and the removal of uneaten feed and faecal matter by siphoning were conducted on a daily basis. Weekly monitoring of the physico-

chemical parameters were done to maintain the quality of the test water at optimum level. The dissolved oxygen ranged from 6.9 to 7.1 mg/L, pH varied between 7-7.2 and water temperature ranged from 24-26˚C. The physico-chemical parameters were measured using Eutech water proof cyberscan PCD 650 meter. The nitrite, nitrate and ammonia levels were maintained below 0.1 ppm. The alkalinity of the test water was also in the permissible range (60-150 mg/L). APHA (1998) was followed for analyzing the hardness of water. All these conditions were followed till the end of the experimental period.

6.3.2 Test cyanobacteria

The cyanobacteria *Synechocystis salina* and *Synechococcus cedrorum* were selected based on their biochemical composition, antimicrobial activity and antioxidant potential for the preparation of the fish feed. The strains were subjected to molecular identification at OmicsGen LifeSciences Pvt.Ltd., Cochin-03, Kerala. The NCBI BLAST analysis results revealed the strains to be *Synechocystis* sp. (MF444861) and *Synechococcus* sp. (MG694463).

S.No	Strain code	Strain name	GenBank Accession number	Maximum similar species	NCBI Acc. No. of matching strains	Coverage /identity% (no of base pairs
1.	MBCC1	*Synechocystis salina*	MF444861	*Synechocystis* sp.PCC6714, *Synechocystis* sp. PCC6702	AB041937 AB041936	100/99 (737)
2.	MBCYB01	*Synechococcus cedrorum*	MG694463	*Synechococcus* sp. PCC8807, *Synechococcus* sp. PCC8807	CP016483 CP016477	100/100 (385)

6.3.3 Preparation of the experimental cyanobacterial diets

The test cyanobacteria were harvested by centrifugation in the late logarithmic phase. A total of six diets were prepared. The cyanobacteria were incorporated at 0.5% and 1% level and a combination diet containing both cyanobacteria (0.5%+0.5%) was prepared. The control feed was prepared without cyanobacteria.

Table 6.1 The different experimental treatments and the denotations used

Treatment	Denotation
Control	C
Synechococcus sp. at 0.5%	Sa 0.5%
Synechococcus sp. 1%	Sa 1%
Synechocystis sp. 0.5%	Sb 0.5%
Synechocystis sp.1%	Sb 1%
Synechococcus sp.+ *Synechocystis* sp.	Sa+Sb (0.5%+0.5%)

All the ingredients were obtained from the local market, weighed as per requirement and were mixed together with adequate amount of water to form a dough. The dough was pressure cooked for 30 minutes and was cooled. After cooling vitamin mixtures, oils and the test cyanobacteria were added and mixed thoroughly and the dough was pressed through a hand pelletizer (diameter 1 mm). The pellets obtained were uniformly spread on butter paper for drying. The dried pellets were stored in air tight containers at 4˚C until use.

Table 6.2 Composition of the fish feed (per 100 g)

Ingredient (g)	Control	Sa 1%	Sa 0.5%	Sb 1%	Sb 0.5%	Sa+Sb
Soya bean meal	43	42	42.5	42	42.5	42
Anchovy meal	38	38	38	38	38	38
Copra meal	12	12	12	12	12	12
Corn starch	3	3	3	3	3	3
Rice bran	1.8	1.8	1.8	1.8	1.8	1.8
Soya bean oil (ml)	1	1	1	1	1	1
Cod liver oil (ml)	1	1	1	1	1	1
Vitamin B	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin C	0.1	0.1	0.1	0.1	0.1	0.1

6.3.4 Proximate composition of the diets

The standard methods recommended by AOAC (2000) were followed for proximate analysis of the experimental feeds.

6.3.4.1 Moisture

A known weight of homogenized sample (5 g) was taken in a pre-weighed aluminium cup and this was heated at 100˚C for two hours in an electric oven. The sample was allowed to cool in a dessicator and repeatedly weighed until a constant weight was obtained. The moisture content was calculated and expressed using the following formulae:

$$\text{Moisture (\%)} = \frac{\text{loss in weight (g)}}{\text{weight of sample taken (g)}} \times 100$$

Subtracting moisture content from cent percent gives the total dry matter contained in the feed.

6.3.4.2 Ash content

3 g of the sample was taken in a pre-weighed silica crucible and was kept in a hot air oven at 55˚C overnight followed by incineration in a muffle

furnace at 600˚C for four hours until a grey/white ash was obtained. It was then cooled in a dessicator and weighed. The ash content was calculated using the following formulae:

$$\text{Ash (\%)} = \frac{\text{Weight of ash (g)}}{\text{Weight of sample ignited (g)}} \times 100$$

6.3.4.3 Crude fat

3 g of the sample was taken in a Whatman filter paper made into a thimble which was plugged with absorbent cotton. The weight was noted and the thimble was placed in a soxhlet extraction unit with an attached receiving flask (previously weighed). Petroleum ether (40-60^{0}C boiling point) was poured washing into the thimble through a glass funnel. Connected the extraction unit and receiving flask to the soxhlet condenser. The flask was heated on a boiling water bath. Extraction was continued at a condensation rate of 5-6 drops per second, till the solvent in the extraction unit becomes clear (10 hours). After completing the extraction the flask was removed, dried in a hot air-oven maintained at 100^{0}C and weighed, the crude fat was calculated and expressed in percentage.

$$\text{Fat (\%)} = \frac{\text{Weight of Fat (g)}}{\text{Weight of Sample}} \times 100$$

6.3.4.4 Crude protein

Crude protein content analysis involved digestion and distillation of the sample. 0.5 g of the moisture free sample was transferred into a Kjeldahl flask of 100 ml capacity. To the sample 1g digestion mixture [K_2SO_4: $CuSO_4$ (8:1)] along with a few glass beads were added followed by the addition of 10 ml concentrated sulphuric acid. It was digested over a heating coil until the solution turned colourless.

To the digested and cooled solution distilled water was added in small quantities with intermittent shaking and cooling until the addition of water did not generate heat. It was transferred qualitatively into a 100 ml standard flask and made up to the volume. 10 ml of the made-up solution was transferred to the reaction chamber of the micro kjeldahl distillation apparatus. 10 ml of sodium hydroxide (40%) and two drops of phenolphthalein indicator were added and distilled for six minutes. The ammonia liberated was collected in a 100 ml conical flask containing 10 ml of boric acid (4%) with a few drops of Tashiro's indicator. Distillation was continued for 4 minutes once the solution turned from pink to the green colour. The amount of ammonium liberated was determined by titrating with 0.1N standard hydrochloric acid. Crude protein content was calculated by multiplying total nitrogen content with conversion factor of 6.25 and expressed as percentage.

$$\text{Total nitrogen (\%)} = \frac{\text{Volume of HCl} \times 0.1 \times 0.014}{\text{Weight of sample}} \times 100$$

6.3.4.5 Carbohydrates

The percentage carbohydrate content of the feed was determined by subtracting the protein and fat contents from 100%.

Table 6.3 Proximate analysis of the feed

Content (%)	Control	Sa 1%	Sa 0.5%	Sb 1%	Sb 0.5%	Sa+Sb
Crude protein	36.73	36.95	36.82	37.11	36.85	36.73
Crude fat	7.90	7.84	7.92	7.76	7.91	7.94
Total carbohydrate	53.98	53.86	53.87	53.76	53.94	54.00
Ash	1.39	1.35	1.39	1.37	1.30	1.33
Dry matter	90.78	90.67	90.81	90.71	90.84	90.75

6.3.5 Experimental design

A total of two hundred and sixteen fishes were randomly divided into six experimental groups. Each experimental group consisted of three replicates. This is a completely randomized design (CRD). The duration of the experiment was for seventy days and was divided into two phases. The first phase was the dietary phase which lasted for sixty days. The second phase started with the challenging of the fishes from different experimental groups with *Aeromonas hydrophila* and lasted for ten days during which the mortality of the fishes was monitored. In the dietary phase, the growth was recorded at an interval of fifteen days and blood samples were collected from all the groups at the end of the dietary phase which was later analyzed for hematological and serological parameters.

6.3.5.1 Growth

Growth was recorded by weighing the fishes (triplicate) from each tank separately with a domestic weighing balance at an interval of fifteen days. Growth performance was analyzed by parameters such as weight gain (%), specific growth rate (SGR) and feed conversion ratio (FCR) which was determined using the following formulae:

Weight gain (%) = [Final weight – Initial weight/Initial weight] x 100

SGR (%) = [ln Final weight – ln Initial weight /Number of days] x100

FCR = Total dry feed intake (g) / Wet weight gain (g)

6.3.5.2 Sampling

At the end of the experiment, three fish from each tank were taken randomly for the blood sampling. Feed was discontinued twenty four hours prior to blood sampling. The fish were anesthetized with clove oil (50 µl/L).

Blood was drawn from the caudal peduncle region using a sterile 1 ml syringe. The collected blood was immediately transferred into heparinized eppendorf tube for hematological studies. For serum analysis the collected blood was left for clotting for two hours. The serum was then analyzed for total protein, albumin and globulin content.

6.3.5.3 Haematology

6.3.5.3.1 Haemoglobin content

Haemoglobin content was determined by the cyanmethaemoglobin method using Drabkins reagent (Andrews *et al*., 2011). 20 µl of blood was mixed with 5 ml of Drabkin's diluent and allowed to stand for five to ten minutes for the formation of cyanmethaemoglobin. The absorbance was measured at 540 nm with Drabkins diluent as blank. A standard curve was plotted using cyanmethhaemoglobin standard and the haemoglobin values obtained were expressed as g/dl.

6.3.5.3.2 Total erythrocyte and leucocyte count

For analyzing the erythrocyte and leucocyte counts 20 µl of blood was taken separately and was diluted two hundred times with corresponding diluting fluid (RBC diluting fluid and WBC diluting fluid) (Andrews *et al*., 2011). The sample was then loaded into the counting chamber of the haemocytometer (Neubauer's counting chamber) and the number of cells was calculated using the following formulae:

Total number of cells ($cu.mm^{-1}$) = (Number of cells counted x Dilution)/Area counted x Depth of fluid).

6.3.5.4 Serum parameters

6.3.5.4.1 Estimation of total proteins

The total serum protein was analyzed according to Lowry *et al*. (1951). 200 µl of serum was mixed with 1 ml of sodium hydroxide and 5 ml of alkaline copper reagent, mixed well and incubated for ten minutes. 0.5 ml of Folins phenol reagent was added and the reaction mixture was kept undisturbed for 30 minutes. The absorbance was measured at 500 nm post incubation. The standard curve was plotted with bovine serum albumin. Total protein was expressed as gram per deciliter of blood.

6.3.5.4.2 Estimation of albumin and globulin

The albumin content was determined according to the modified method of Kingsley (1939). To 300 µl serum 5.7 ml of sodium sulphite (28%) was added, the mixture was rotated gently between palm. 3 ml ether was added and the reaction tube was gently shaken upside down for 20 times. After ten minutes a 'globulin button' was formed at the ether saline interphase. Then it was centrifuged for fifteen minutes for the hardening of the 'globulin button'. After centrifugation the tube was tilted and a pipette was inserted carefully to obtain the clear solution below the globulin layer. To 2 ml of the clear solution 5 ml of biuret reagent was added and incubated at 37˚C in a water bath for ten minutes. The absorbance was read at 555 nm. Globulin was determined by the difference between total protein and albumin. The values were expressed as g/dl. The albumin/globulin (A/G) ratio was also analyzed.

6.3.5.5 Challenge study with *Aeromonas hydrophila*

After sixty days of feeding trial, the fishes were challenged with *A. hydrophila* (KC549803) obtained from the Fish Pathology laboratory of the

Department. Cells were inoculated into nutrient broth for twenty four hours at 37˚C. The culture was then centrifuged at 3000 rpm for 10 minutes to obtain the pellet. The pellet was resuspended in phosphate buffer saline (pH 7.4). The final bacterial concentration was adjusted to 10^6 CFU/ml by serial dilution. 100 µl of the bacterial culture was injected intraperitonially into the experimental fishes. Mortality was recorded daily. During the challenge study also the fishes were fed on the experimental diet and the water quality parameters were maintained as described in section 6.3.1. The mortality was observed for ten days. To confirm the cause of death the bacterium was re-isolated from the dead fish.

The survival (%) was calculated based on the following formula:

$$\text{Survival (\%)} = \text{Number of fish surviving after challenge} \div \text{Number of fish injected with bacteria} \times 100$$

6.3.6 Statistical analysis

The whole experiment was performed in triplicates and the results were expressed as mean value ± standard deviation. One-way ANOVA was carried out to determine significant difference if any, between the different treatments. $p<0.05$ were considered as statistically significant. IBM SPSS statistics version 22 was the software package employed for statistical analysis.

6.4 Results

6.4.1 Growth

Growth was measured in terms of increase in body weight. All the treatment groups exhibited marked increase in body mass and length. The fishes fed on experimental diet containing *Synechococcus* sp. at 1% recorded the maximum growth. Feeds containing *Synechocystis* sp. at 0.5%, combination

feed containing both *Synechococcus* sp. and *Synechocystis* sp. at 0.5% and *Synechococcus* sp. at 0.5% exhibited similar values. Fishes fed on *Synechocystis* sp. at 1% achieved values slightly higher than the control group which recorded the least values in the whole experiment. The highest weight gain was 82.53% and lowest was 79.35%. The increase in weight gain between different experimental groups was statistically significant ($p < 0.05$). The mean body weight of *O. mossambicus* at 15- day interval is presented in Fig. 6.1.

The feed conversion ratios were found to be improved for the group fed on Sa 1% (4.14±0.22) while the least efficient value was obtained for the control group (4.34±0.17). The group supplemented with Sb 1% showed a FCR ratio of 4.27±0.15. The differences in FCR values between different treatment groups were statistically not significant ($p > 0.05$).

The specific growth rate was maximum in Sa 1% (2.91±0.09) experimental group and least in the control group (2.62±0.07). The group fed on 0.5 % *Synechocystis* sp. recorded a value of 2.76±0.05. There was significant difference in the SGR of different experimental groups ($p<0.05$). Table 6.4 represents the values of the growth parameters tested.

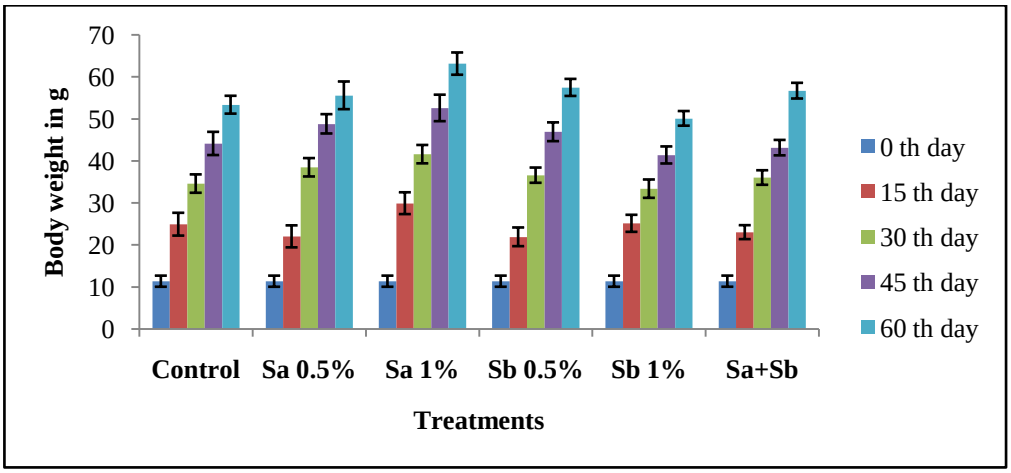


Fig. 6.1 Mean body weight of *O. mossambicus* fed on different experimental feeds

Table 6.4 Growth parameters of *O. mossambicus*

Treatment	Weight gain (%)	FCR	SGR
Control	79.35 ± 0.85[a]	4.34 ±0.17[a]	2.62 ±0.07[a]
Sa 0.5%	80.49 ± 1.42[ab]	4.32 ±0.20[a]	2.69 ±0.083[ab]
Sa 1%	82.53 ±0.92[c]	4.14 ± 0.22[a]	2.91 ±0.09[c]
Sb 0.5%	80.84 ±0.57[b]	4.29 ±0.12[a]	2.76 ±0.05[b]
Sb 1%	79.94 ±0.74[ab]	4.27±0.15[a]	2.68 ±0.06[ab]
Sa+Sb	80.57 ± 0.65[ab]	4.31 ±0.14[a]	2.73 ±0.06[b]

6.4.2 Haematology

The highest haemoglobin content of 9.03g/dl was observed in Sa 1%. The second highest value was observed in control group. Sa 0.5%, Sb 0.5% and Sa+Sb recorded similar values. Lowest value of 7.84g/dl was estimated in group supplemented with Sb 1%.

The erythrocyte count was maximum in Sa 1% (32522 cells/mm^3). Sa 0.5% and control feed showed 30659 cells/mm^3 and 30442 cells/mm^3 respectively. The experimental feeds Sb 0.5% and Sa+Sb showed almost similar values. The lowest erythrocyte count was measured in fishes fed with Sb 1%.

O. mossambicus fed on Sa 1% exhibited the highest WBC count of 10806 cells /mm^3. Control group and Sa 0.5% had 10177 cells/mm^3 and 10220 cells/mm^3. Least value was recorded in Sb 1%. The results of the haematological parameters tested are presented in Table 6.5.

Statistical analysis showed that there was significant difference in the haematological parameters between different treatment groups ($p<0.05$).

Table 6.5 Haematological parameters of *O. mossambicus*

Treatment	Haemoglobin (g /dl)	WBC (cells/mm^3)	RBC (cells/mm^3)
Control	8.78 ±0.08^{d}	10176 ±54.49^{d}	30442 ±119.2^{d}
Sa 0.5%	8.64 ±0.09^{c}	10220 ±32.6^{d}	30659 ±103.64^{d}
Sa 1%	9.03 ±0.12^{e}	10806 ±121.21^{e}	32522 ±386.71^{e}
Sb 0.5%	8.50 ±0.05^{b}	9748 ±73.6^{b}	29311 ±242.28^{b}
Sb 1%	7.84 ±0.1^{a}	9341 ±122.94^{a}	28099 ±375.6^{a}
Sa+Sb	8.47±0.05^{b}	9931 ±103.05^{c}	29864 ±268.04^{c}

6.4.3 Serum biochemical parameters

The serum total protein varied from 2.7 to 4.3 g/dl. The group fed on Sa 1% showed the highest total serum protein and globulin levels and the same group exhibited lowest albumin levels and A/G ratio. Sb 1% showed the maximum albumin value of 1.30 g/dl and highest A/G ratio of 0.91. Groups having higher globulin content showed lower albumin and hence lower A/G ratio. Statistical analysis showed that there was significant difference in the serological parameters between different experimental groups ($p<0.05$). Table 6.6 presents the values of the serological parameters.

Table 6.6 Serological parameters of *O. mossambicus*

Treatment	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio
Control	4.10 ±0.04^{d}	1.21 ±0.07^{ab}	2.89 ±0.03^{d}	0.42 ±0.03^{a}
Sa 0.5%	3.63 ±0.07^{c}	1.29 ±0.06^{bc}	2.33±0.12^{c}	0.56 ±0.05^{b}
Sa 1%	4.35 ±0.08^{e}	1.26 ±0.08^{abc}	3.10 ±0.08^{e}	0.41 ±0.03^{a}
Sb 0.5%	3.25 ±0.07^{b}	1.16 ±0.06^{a}	2.09±0.06^{b}	0.55 ±0.56^{b}
Sb 1%	2.75 ±0.09^{a}	1.30 ±0.06^{c}	1.44±0.06^{a}	0.91 ±0.05^{c}
Sa+Sb	3.26 ±3.27^{b}	1.19 ±1.20^{a}	2.06±2.06^{b}	0.58 ±0.59^{b}

6.4.4 Survival

No mortality was recorded during the sixty days of feeding experiment. But mortality of the experimental fish was observed from the third day of post *A. hydrophila* challenge. At the end of the challenge period highest mortality was observed in control group. Above 50% survival was recorded in all the groups fed on experimental diets. The highest survival percentage was for the group fed on Sa 1% (88.89%). The group fed on the combination diet and Sa 0.5% also showed remarkable surviving ability. Among the experimental groups fed on cyanobacteria least survival percentage was recorded for Sb 1% (52.78%) (Fig. 6.2).

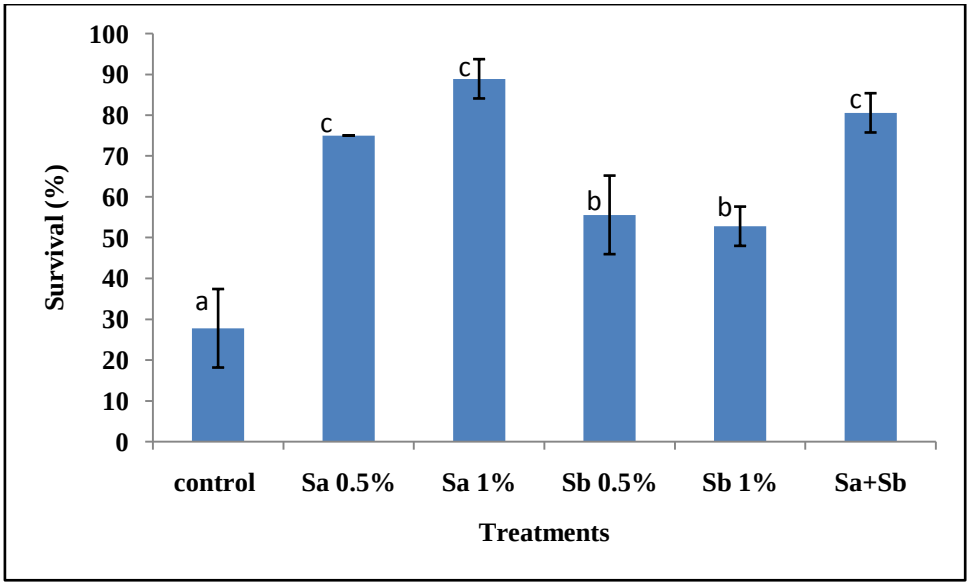


Fig. 6.2 Survival (%) of different experimental groups after *A. hydrophila* challenge

6.5 Discussion

Aquaculture sector is a rapidly emerging industry providing food and employment to a large section of the society. A number of factors affect the successful running of the industry such as good water management practices, quality of the stocks, feeding strategies, health and economic management (Hemaiswarya *et al*., 2011). The disease outbreaks and emergence of antibiotic resistance have been affecting the aquaculture industry badly. Development of safe, cost- effective and eco-friendly alternatives have been of prime concern to the scientific community in this field. Administration of probiotics and immunostimulants hold hope for solving the problem of antibiotic resistance. A wide variety of microorganisms including microalgae have been considered for the application as probiotics and immunostimulants (Irianto and Austin, 2002 and Zhou *et al*., 2009). Cyanobacteria being a principal component in the natural diet of many fishes including tilapia (Dempster *et al*., 1993) may have the potential to enhance the growth and survivability of the cultured organism.

Studies indicate that the cyanobacteria supplementation in different fish species significantly improved the growth, carcass quality, pigmentation, organoleptic quality, resistance to bacterial infections and survivability of the fish (Nandeesha *et al*., 1998, Overa-Novoa *et al*., 1998, Andrews *et al*., 2011, Vasudhevan and James, 2011, Hajiahmadian and Vajargah, 2012, Teimouri *et al*., 2013 and Ibrahem *et al*., 2013). The positive effects exerted by cyanobacteria on aquaculture organisms led to the formulation of the present study.

The dietary intake of cyanobacteria in fish may cause hormesis effect (Liang *et al*., 2015). It is a biological phenomenon whereby a beneficial effect such as improved health, stress tolerance, growth or longevity results from the exposure to low doses of an agent that is otherwise toxic or lethal when given at higher doses. Hence the current experiment was designed by incorporating a

maximum of 1% cyanobacteria into the fish feed. The effect of combination diet containing two cyanobacteria on the growth and survival of *O. mossambicus* was done for the first time to our knowledge.

A significant increase in growth parameters such as weight gain and final body weight, improved FCR and SGR were observed in the experimental group fed on 1% *Synechococcus* sp. A general increase in growth was observed for all the groups and the least was observed in the group fed on control diet. The promotional effect on growth by cyanobacteria was probably due to the proteins, polysaccharides and pigment nutrients (Liang *et al*., 2015). In accordance with the present study similar results were obtained in *L. rohita* fingerlings fed on 1%, 2% and 4% of *S. platensis* incorporated diets (Andrews *et al*., 2011). In another study, 0.5-2% GH- transgenic *Synechocystis* in the feed of *Paralichthys olivaceus* had expressed improved growth though the feed intake was not affected significantly (Liu *et al*., 2008).

Most studies point out that only higher supplementation of the cyanobacteria causes positive impacts (Hajiahmadian and Vajargah, 2012, Ibrahem *et al*., 2013 and Fadl *et al*., 2013). This is in contrast to the observations of the present study where enhancement in growth parameters was observed even at 0.5% supplementation. Recent researches highlight that the supplementation of whole organisms as well as active components derived from the organisms result in more or less similar impacts on the test species. 1% lycogen supplementation in red tilapia a hybrid of *O. mossambicus* and *O. niloticus*, significantly increased the weight gain, SGR and improved FCR indicating a probiotic effect (Chiu and Liu, 2014). In the fish *Dicentrachus labrax* the dietary supplementation of glucan, α-tocopherol and ascorbic acid at 1-2% increased the weight gain over 600% and significantly improved the FCR and enhanced the SGR (Bagni *et al*., 2000).

Contrary to the above positive effects, some studies have reported adverse and neutral effects of higher supplementation of dietary cyanobacteria (Badwy *et al*., 2008 and Liang *et al*., 2015). In the present study, the supplementation of *Synechocystis* sp. resulted in reduced growth and the haematological and serological parameters exhibited lower values compared to other experimental feeds, hence all cyanobacteria are not beneficial to the cultured fish. The antinutritional effect was more clearly evident at higher concentrations (1%) of the cyanobacteria.

The haemoglobin content, erythrocyte and leucocyte count were all found to be enhanced in the *Synechococcus* sp. supplemented experimental group (Sa 1%). Similarly, Merrifield *et al*. (2010) observed that 0.5% and 1% *Chlorogloeopsis* supplemented diet increased the haematocrit levels in red tilapia. Andrews *et al*. (2011) also observed increased values of haematological parameters in *L. rohita* supplemented with *S. platensis* (1, 2 and 4%). The probiotic or immunostimulant supplementation sometimes leads to neutral effect or affect only specific parameters (Bagni *et al*., 2000). Glucan supplementation in Sea bass increased the leucocyte count but there was not much variation in the haemoglobin and erythrocyte count (Bagni *et al*., 2000). Similarly, in the present study the erythrocyte and leucocyte count was found to be lowered in fish fed on Sb 1%.

The leucocytes play a major role in the non-specific or innate immunity of the fish. Hence the increase in count can be considered as an indicator of the health status of the fish (Roberts, 1978). The phycocyanin present in the cyanobacteria promotes the production of white blood cells which was earlier proved in mice (Zhang, 1994). The increase in serum protein and globulin content in cyanobacteria fed groups were reported in previous studies also (Andrews *et al*., 2011 and Ramamurthy *et al*., 2013). Yeganeh *et al*. (2015)

reported significant increase in the RBC, WBC, haemoglobin, total protein and albumin levels in *S. platensis*-fed rainbow trouts. Similar results were reported by Das *et al*. (2013) in *L. rohita* fed on *Microcystis aeruginosa* incorporated diets. Hence it can be concluded that the *Synechococcus* sp. significantly improved the haematological parameters of the test fish.

The present study recorded an increase in serum protein and globulin in *O. mossambicus* fed on Sa 1%. Increase in serum protein and globulin indicates a stronger innate response in fish (Jha *et al*., 2007) and the gamma globulin is the immunologically active protein part of the blood. Lower albumin globulin ratio means the globulin content was higher and hence higher resistance in the test fish. The albumin and globulin are important for maintaining a healthy immune system (Nya and Austin, 2009).

The probiotic administration in finfish causes stimulation of innate and cellular immunity. They cause expression of cytokines, stimulation of phagocytic and lysozyme activities, respiratory burst activity, complement activity, cytotoxicity, increase in total protein content, enhanced leucocytes, lymphocytes, erythrocytes, neutrophils production, plasma bactericidal activity, increased enzyme activity etc. (Newaj-Fyzul and Austin, 2015). In the present study, *O. mossambicus* exhibited incredible survivability against *A. hydrophila* infection. Above 50% survival was recorded even in the least efficient experimental group (Sb 1%) and high mortality was observed in the control group. Previous studies indicate that cyanobacterial supplementation improves the survival of the challenged fish and exerts immunostimulatory effects (Watanuki *et al*., 2006, Abdel-Tawwab and Ahmed, 2009, Andrews *et al*., 2011, Ramamurthy *et al*., 2013 and Das *et al*., 2013). Ibrahem *et al*. (2013) reported that 10% supplementation of *S. platensis* increased the resistance of *O. niloticus* towards *Pseudomonas fluorescenes* infection. *Microcystis aeruginosa* is a toxic

cyanobacteria but the supplementation of the same alga at 1% in the diet of *L. rohita* increased the survival percentage of *A. hydrophila* infected fish by 72% (Das *et al*., 2013). 89% survival was observed for the *A. hydrophila* infected *O. mossambicus* in the present study. Ramamurthy *et al*. (2015) reported 85% survivability in *Nostoc muscorum* fed *Mugil cephalus* challenged with *A. salmonicida*. Though the supplementation of *Synechocystis* sp. at 1% had an antinutritional effect it significantly improved the survival of the test fish. This indicates that the cyanobacteia evoked an immunostimulatory effect and increased the resistance against the bacterial infection.

Studies have proved that the supplementation of cyanobacteria improves the non-specific immune parameters such as serum bactericidal activity, phagocytic activity and lysozyme activity which lead to lower mortality rate in test species (Ragap *et al*., 2012). In the present study, the enhanced leucocytes and globulin content might have played a role in the increased survivability of the fish against *A. hydrophila* infection. The intake of hot water extract of *S. platensis* enhanced the innate immunity by increasing the lysozyme levels in *Litopenaeus vannamei* and thus protected it against *Vibrio alginolyticus* infection (Tayag *et al*., 2010).

The supplementation of 1% *Synechococcus* sp. was found to be highly effective in enhancing the growth, haematology, serology and survival of *O. mossambicus*. It improved the general health status of the fish. Interestingly, the combination feed containing both *Synechococcus* sp. and *Synechocystis* sp. also showed high survival rate but had little effect on the growth performance. Generally, the survival rate was high for all cyanobacteria-treated groups. In conclusion, the strain *Synechococcus* sp. at 1% incorporation into the fish feed has the potential to serve as a dietary supplement and probiotic for *O. mossambicus*.

Chapter 7

Summary and Conclusion

Cyanobacteria, the Gram negative oxygenic phototrophs are cosmopolitan in distribution and are characterized by wide ecological tolerance. They have been an excellent area of research due to their immense biotechnological potential. They are reported to have antimicrobial, antiviral, antioxidant, anti-cancerous, antialgal and anti plasmodial effects. They have gained significance as source of food materials, natural colouring agents, biofuels, fine chemicals, bioactive substances etc. Cyanobacteria are also gaining importance in the aquaculture field as feed and feed additives due to their unique biochemical composition. The study area, Cochin estuary has a rich cyanophycean microflora which is largely under-exploited in terms of bioprospecting. In the present study, an attempt was made to isolate, characterize and to determine the antimicrobial, antioxidant and dietary effect of cyanobacteria from Cochin estuary.

- A total of twelve species of cyanobacteria belonging to four different orders such as Chroococcales, Synechococcales, Oscillatoriales and Nostocales of the class Cyanophyceae were isolated from ten stations of Cochin estuary.
- The isolated strains were *Aphanocapsa litoralis, Chroococcus minutus, Lyngbya baculum, Oscillatoria limosa, Oscillatoria chlorina, Synechocystis salina, Synechocystis aquatilis, Synechococcus elongatus, Anacystis nidulans, Synechococcus cedrorum, Anabaena* sp. and *Gloeocapsa gelatinosa*.

- Although twelve cyanobacterial strains were isolated and purified only seven strains could establish well in culture. The stable cultures were *A. litoralis, C. minutus, G. gelatinosa, O. limosa, S. aquatilis, S. salina* and *S. cedrorum*.
- They were maintained as semi-continuous batch cultures in the Algal culture laboratory of the Department.
- All the seven cultures were optimized with regard to their culture conditions such as light, temperature, pH, salinity and medium.
- The optimum light intensity and temperature were found to be 1400 lux and 25˚C for all strains.
- All the test strains could tolerate wide ranges in pH and salinity. pH 8 supported the highest biomass followed by pH 9.
- Optimum salinity varied between 10 psu to 30 psu depending on the species and all strains exhibited growth at freshwater conditions also.
- Nutrient rich media such as SN and BG11 favoured maximum growth.
- The biochemical characterization of the strains revealed that protein content (23- 48 % of dry weight) was the highest followed by carbohydrate (19-38%) and lipid (5-8%).
- The protein content was the highest in the logarithmic phase, carbohydrate was the highest in the stationary phase and lipid content of the cyanobacteria was maximum at the decline phase.
- *Synechocystis salina* showed the highest protein (47.6%) and carbohydrate content (38.2%).

- *Synechococcus cedrorum* had 43.4% protein and 36.5% carbohydrate. Lipid content was the highest in *G. gelatinosa* (8.4%).
- The chlorophyll *a* and carotenoid content were maximum in *G. gelatinosa*
- Phycobiliprotein content analysis showed that *C. minutus* exhibited the highest phycocyanin content. *Synechocystis salina* had the highest phycoerythrin content and *Synechocystis aquatilis* showed the highest allophycocyanin content.

  - Cyanobacteria employ several structural and functional strategies to neutralize the effects of free radicals. Total phenolic content, total flavonoid content, total antioxidant activity, ferric reducing antioxidant power, DPPH radical scavenging assay, deoxyribose radical scavenging activity and hydrogen peroxide radical scavenging activity of the cyanobacterial strains were analyzed.

- The phenolic content of the strains ranged from 6 to 44 µgGAE/mg and the strain *Synechocystis salina* showed the highest phenolic content. *Synechococcus cedrorum* had the lowest phenolic content among the test strains
- The flavonoid content was found to be varying from 2-18 µgQE/mg. The highest flavonoid content obtained was in *S. salina*, and lowest value was recorded in *O. limosa*.

- All the test strains exhibited fairly good antioxidant capacity except *O. limosa*. The *S. salina* recorded the highest total antioxidant activity followed by *S. cedrorum.*
- The highest deoxyribose radical scavenging was found to be 23%. *S. salina* and *S. cedrorum* exhibited the highest scavenging activity and *O. limosa* showed the lowest value.
- Maximum ferric reducing antioxidant power was recorded in *S. salina* followed by *S. cedrorum.*
- The maximum amount of DPPH free radical was scavenged by the strains *S. cedrorum* and *S. salina*.
- The hydrogen peroxide radical scavenging activity was low and the maximum value recorded was 8% in *S. cedrorum*.
- Ferric reducing antioxidant power, DPPH radical scavenging assay and hydrogen peroxide radical scavenging activity exhibited dose dependency.
- *Synechocystis salina* showed the highest phenolic content, flavonoid content, total antioxidant activity, ferric reducing activity and deoxyribose radical scavenging activity.
- *Synechococcus cedrorum* exhibited the maximum DPPH and H_2O_2 scavenging activity.

➢ The antimicrobial activity of different crude extracts of the strains was highly commendable. Vibrios were found to be highly sensitive to the cyanobacterial extracts.

- *Synechocystis salina* showed wide range of antibacterial activity against various human and aquaculture pathogens. The ether extract of the strain effectively inhibited *S. aureus*, *A. hydrophila*, *Bacillus* sp., *V. mimicus*, *V. harveyi*, and *E. coli*.
- *Synechococcus cedrorum* was highly inhibitory to the aquaculture pathogen *V. parahaemolyticus*.
- *G. gelatinosa* could effectively inhibit the pathogens *E. coli* and *Bacillus* sp. *A. litoralis* extracts inhibited *S. aureus* and *V. anguillarum*.
- The strains, *C. minutus*, *O. limosa* and *S. aquatilis* could inhibit only a single test pathogen each. *A. hydrophila*, *P. aeruginosa* and *S. aureus* were pathogens inhibited by the strains respectively.
- Acetone, methanol, dimethyl sulphoxide and aqueous extracts of the strains exhibited no antibacterial activity.

  - The cyanobacteria also exhibited good antifungal activity. All the test cyanobacteria except *S. salina* could effectively inhibit *C. albicans*.

- The ethanol extract of *S. cedrorum* was highly inhibitory to *C. albicans*. Similarly, the ether extract of *C. minutus*, *O. limosa* and *S. aquatilis* also exhibited high antifungal activity against the pathogen.

- *S. cerevisiae* was comparatively more resistant than *C. albicans*. However, it was found to be sensitive to the ethanol extract of *S. salina.*

  - The cyanobacteria supplementation in fish feed have positive effects on growth, survival, reproductive performance and overall appearance and health status of the fish.

- 1% *Synechococcus cedrorum* (Sa 1%) supplementation significantly improved the growth parameters in *O. mossambicus.*
- The food conversion ratios improved and the specific growth rate increased for the Sa 1% group. The haemoglobin content, the total erythrocyte and leucocyte count were also found to be enhanced in the Sa 1% group.
- The serological parameters such as total serum protein and globulin content were found to be enhanced in Sa 1% group.
- 1% *Synechocystis salina* (Sb 1%) had an antinutritional effect; it reduced the haematological and serological parameters of the test fish. All other cyanobacterial diets did not show much variation from the control diet.

  - The challenge study with *Aeromonas hydrophila* showed that the group fed on 1% *Synechoccoccus cedrorum* exhibited the highest survival (89%). The combination diet also supported high survivability. The cyanobacterial diets might have exerted an immunostimulatory effect on the test fish resulting in high

survivability. Though Sb 1% did not have much dietary effect it improved the survival rate of the challenged fish.

The present study shows that cyanobacteria have immense potential for producing economically important compounds. They serve as very good sources of proteins, carbohydrates, lipids, pigments, antioxidants, antimicrobial compounds etc. The cyanobacteria such as *G. gelatinosa* and *C. minutus* could be subjected to detailed analysis for extracting industrially important natural colourants. *S. salina* and *S. cedrorum* have the potential for application as antioxidant and antimicrobial agents in pharmaceutical, nutraceutical, cosmetic and aquaculture industries. By manipulating the culture conditions, they may be induced to produce desired products. The antimicrobial agents produced by the cyanobacteria need to be characterized further. Cyanobacteria such as *S. salina* and *S. cedrorum* can serve as dietary supplement to improve the resistance of fishes to pathogens thus reducing the mortality rate and for the overall well being of fishes. The strain *Synechococcus cedrorum* at 1% incorporation into the fish feed has the potential to serve as a dietary supplement and probiotic for *O. mossambicus*. However, detailed investigations on the presence of toxic genes, if any, easy method of cell harvesting etc. need to be carried out before recommending them for aquaculture or commercial application.

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APPENDIX I

MEDIUM COMPOSITION

Allen and Nelson medium (1910)

Solution A

	Ingredients	**Quantity**
	KNO_3	2.2 g
	Distilled water	100 ml
Solution B	Na_2HPO_4	4 g
	$CaCl_2$	4 g
	$FeCl_3$	2 g
	Conc. HCl	2 ml
	Distilled water	80 ml

Solution A and Solution B were prepared separately and 2 ml of Solution A and 1 ml of Solution B were added to 1000 ml sea water. The pH was adjusted to 7.

BG11 medium (modified, Andersen, 2005)

Ingredients	**Stock solution (g/l dH2O)**	**Quantity used**
Citric acid	6	1 ml
Ferric ammonium citrate	6	1 ml
$NaNO_3$	-	1.5 g
$K_2HPO_4.3H_2O$	40	1 ml
$MgSO_4.7H_2O$	75	1 ml
$CaCl_2.2H_2O$	36	1 ml
Na_2CO_3	20	1 ml
MgNa2EDTA.H_2O	1.0	1 ml
Trace metal solution		1 ml

Ingredients	Stock solution (g/l dH2O)	Quantity
H_3BO_3		2.860 g
$MnCl_2.4H_2O$		1.810 g
$ZnSO_4.7H_2O$		0.220 g
$CuSO_4.5H_2O$	79.0	1 ml
$Na_2MoO_4.2H_2O$		0.391 g
$Co(NO_3)_2.6H_2O$	49.4	1 ml

Prepare the ferric ammonium citrate stock solution and other stock solutions separately. Into 900 ml of distilled water, add 1 ml of the ferric citrate solution, and then add the remaining components. Autoclave and after coolong the final pH was adjusted to 7.4.

SN medium (Andersen, 2005)

Ingredients	Stock solution (g/l dH2O)	Quantity
$NaNO_3$	76.50	10 ml
K_2HPO_4	15.68	1 ml
$Na_2EDTA.2H_2O$	5.58	1 ml
Na_2CO_3	10.70	1 ml
Cyanocobalamine	0.001	1 ml
Cyano trace metal solution		
Citric acid.H_2O		6.250 g
Ferric ammonium citrate		6.000 g
$MnCl_2$. $4H_2O$		1.400 g
$Na_2MoO_4.2H_2O$		0.390 g
$ZnSO_4.7H_2O$		0.222 g
$Co(NO_3)_2.6H_2O$		0.025 g

To prepare, autoclave 750 ml of filtered natural sea water and separately autoclave 236 ml of double distilled water. Cool and aseptically combine the two solutions. Aseptically, add 10 ml of NaNO3 solution and 1 ml of the other five stock solutions.

Sea water enrichment medium (Subramanian *et al*., 1999)

Solution A

Ingredients	Quantity
$NaNO_3$	0.5 g
Sea water	100 ml

Solution B

KNO_3	2.02 g
K_2HPO_4	0.35 g
$FeCl_3$	9.70 g
$MnCl_2$	0.75
EDTA	100 mg
Thiamine HCl	0.1 mg
Sea water	75 ml
Distilled water	25 ml

Solution A and solution B were prepared separately and autoclaved. 2 ml solution A and 1 ml solution B were added to 1000 ml sterilized sea water.

APPENDIX II

Table 1. Growth of *A. litoralis* at different light intensities

	Chl *a* (µg/ml)										
Light intensity	Days 0	3	6	9	12	15	18	21	24	27	30
700	1.31±0.03	1.54±0.04	1.78±0.05	2.07±0.04	2.29±0.03	2.49±0.05	2.67±0.06	2.98±0.04	3.12±0.05	3.07±0.05	2.97±0.06
1400	1.31±0.03	1.57±0.03	1.87±0.04	2.18±0.03	2.54±0.04	2.83±0.08	3.18±0.04	3.40±0.04	3.86±0.06	3.8±0.07	3.65±0.05
2100	1.31±0.03	1.50±0.03	1.73±0.07	2.05±0.03	2.28±0.03	2.59±0.04	3.00±0.08	3.27±0.04	3.50±0.08	3.26±0.06	3.14±0.04

Table 2. Growth of *C. minutus* at different light intensities

	Chl *a* (µg/ml)										
Light intensity	Days 0	3	6	9	12	15	18	21	24	27	30
700	1.3±0.05	1.45±0.06	1.66±0.06	2.02±0.04	2.27±0.06	2.59±0.09	3.04±0.08	3.42±0.03	3.78±0.07	3.56±0.06	3.43±0.07
1400	1.3±0.05	1.48±0.03	1.92±0.03	2.32±0.05	3.12±0.13	4.14±0.08	4.56±0.07	5.3±0.08	5.97±0.07	5.78±0.04	5.60±0.02
2100	1.3±0.05	1.6±0.026	2.06±0.03	2.88±0.12	3.27±0.06	4.07±0.09	4.47±0.08	5.18±0.06	5.77±0.12	5.69±0.14	5.59±0.06

Table 3. Growth of *G. gelatinosa* at different light intensities

	Chl *a* (µg/ml)										
Light intensity	Days 0	3	6	9	12	15	18	21	24	27	30
700	1.39±0.07	1.79±0.04	1.98±0.05	2.25±0.05	2.67±0.074	3.18±0.06	3.88±0.06	4.25±0.12	4.72±0.04	4.56±0.05	4.48±0.04
1400	1.39±0.07	1.48±0.03	1.92±0.03	2.32±0.05	3.11±0.13	4.14±0.08	4.56±0.07	5.30±0.08	5.97±0.07	5.78±0.04	5.60±0.03
2100	1.39±0.07	1.60±0.03	2.06±0.03	2.88±0.12	3.27±0.06	4.07±0.09	4.47±0.08	5.18±0.06	5.77±0.12	5.69±0.14	5.59±0.06

Table 4. Growth of *O. limosa* at different light intensities

	Chl *a* (µg/ml)										
Light intensity	Days 0	3	6	9	12	15	18	21	24	27	30
700	0.56±0.05	0.76±0.05	1.23±0.07	1.40±0.06	1.62±0.04	1.87±0.06	2.10±0.04	2.31±0.07	2.57±0.04	2.44±0.04	2.29±0.08
1400	0.56±0.05	0.91±0.04	1.24±0.05	1.89±0.11	2.26±0.06	2.87±0.03	3.40±0.06	4.08±0.09	4.42±0.05	4.28±0.03	4.12±0.03
2100	0.56±0.05	0.94±0.14	1.17±0.05	1.65±0.09	2.12±0.05	2.79±0.06	3.27±0.10	3.96±0.11	4.22±0.07	4.11±0.03	3.95±0.06

Table 5. Growth of *S. aquatilis* at different light intensities

	Chl *a* (µg/ml)										
Light intensity	Days 0	3	6	9	12	15	18	21	24	27	30
700	1.47±0.06	1.69±0.03	1.88±0.05	2.08±0.04	2.32±0.06	2.57±0.07	2.87±0.12	3.07±0.05	3.26±0.04	3.16±0.04	3.05±0.03
1400	1.47±0.06	1.78±0.05	2.03±0.04	2.39±0.04	2.77±0.06	3.07±0.04	3.22±0.04	3.44±0.06	3.67±0.04	3.53±0.04	3.41±0.03
2100	1.47±0.06	1.78±0.09	2.05±0.04	2.30±0.06	2.45±0.06	2.9±0.05	3.14±0.07	3.37±0.10	3.55±0.05	3.44±0.04	3.27±0.05

Table 6. Growth of *S. salina* at different light intensities

	Chl *a* (µg/ml)										
Light intensity	Days 0	3	6	9	12	15	18	21	24	27	30
700	1.36±0.04	1.54±0.02	1.82±0.06	2.08±0.04	2.47±0.05	3.04±0.07	3.31±0.07	3.92±0.12	4.18±0.07	4.12±0.06	3.97±0.06
1400	1.36±0.04	1.78±0.06	2.04±0.06	2.31±0.04	2.95±0.07	3.29±0.04	4.03±0.10	4.48±0.06	4.70±0.04	4.62±0.06	4.47±0.04
2100	1.36±0.04	1.73±0.10	1.96±0.08	2.33±0.05	2.74±0.05	2.96±0.07	3.37±0.05	4.05±0.08	4.57±0.04	4.48±0.04	4.35±0.04

Table 7 Growth of *S. cedrorum* at different light intensities

	Chl *a* (µg/ml)										
Light intensity	Days 0	3	6	9	12	15	18	21	24	27	30
700	1.45±0.06	1.61±0.04	1.82±0.06	2.04±0.05	2.47±0.06	2.82±0.03	3.06±0.05	3.51±0.05	3.79±0.10	3.69±0.11	3.48±0.03
1400	1.45±0.06	1.80±0.07	2.12±0.03	2.44±0.05	2.97±0.09	3.28±0.03	3.76±0.05	4.18±0.05	4.52±0.06	4.38±0.08	4.24±0.03
2100	1.45±0.06	1.79±0.05	2.01±0.03	2.32±0.07	2.69±0.08	2.99±0.04	3.19±0.05	3.69±0.12	4.23±0.06	4.12±0.03	3.98±0.04

Table 8. Growth of *A. litoralis* at different temperatures

	Chl *a* (µg/ml)										
Temp	Days 0	3	6	9	12	15	18	21	24	27	30
20°C	1.32±0.03	1.63±0.06	2.05±0.08	2.23±0.05	2.64±0.09	2.9±0.08	3.13±0.09	3.41±0.08	3.75±0.12	3.59±0.07	3.40±0.03
25°C	1.32±0.03	1.72±0.05	2.13±0.03	2.40±0.05	2.8±0.03	3.12±0.04	3.49±0.03	3.82±0.03	4.20±0.04	4.10±0.02	3.98±0.04
30°C	1.32±0.03	1.59±0.05	1.88±0.07	2.25±0.03	2.45±0.04	2.73±0.06	3.04±0.04	3.29±0.04	3.59±0.08	3.46±0.05	3.32±0.05

Table 9. Growth of *C. minutus* at different temperatures

	Chl *a* (µg/ml)										
Temp	Days 0	3	6	9	12	15	18	21	24	27	30
20°C	1.38±0.06	1.78±0.03	2.12±0.03	2.54±0.05	3.18±0.06	3.92±0.06	4.54±0.06	5.17±0.06	5.94±0.07	5.86±0.09	5.66±0.06
25°C	1.38±0.06	1.87±0.04	2.20±0.03	2.89±0.05	3.43±0.03	4.18±0.06	5.04±0.06	5.91±0.06	6.16±0.05	6.06±0.05	5.94±0.07
30°C	1.38±0.06	1.69±0.04	1.94±0.03	2.29±0.06	2.93±0.05	3.32±0.08	3.7±0.06	4.26±0.06	4.83±0.06	4.71±0.02	4.58±0.03

Table 10. Growth of *G. gelatinosa* at different temperatures

	Chl *a* (µg/ml)										
Temp	Days 0	3	6	9	12	15	18	21	24	27	30
20°C	1.45±0.09	1.9±0.08	2.20±0.06	2.61±0.12	2.96±0.09	4.21±0.07	5.07±0.18	5.81±0.05	6.24±0.05	6.18±0.04	6.05±0.02
25°C	1.45±0.09	1.94±0.04	2.60±0.13	3.30±0.04	3.93±0.04	4.66±0.08	5.22±0.06	6.13±0.07	6.44±0.05	6.29±0.05	6.21±0.04
30°C	1.45±0.09	1.72±0.03	2.12±0.03	2.28±0.07	2.79±0.10	3.44±0.02	3.91±0.09	4.63±0.20	5.35±0.04	5.21±0.04	5.09±0.03

Table 11. Growth characteristics of *O. limosa* at different temperatures

	Chl *a* (µg/ml)										
Temp	Days 0	3	6	9	12	15	18	21	24	27	30
20°C	0.68±0.07	0.83±0.02	1.12±0.04	1.32±0.04	1.52±0.05	1.83±0.07	2.09±0.05	2.43±0.05	2.56±0.07	2.48±0.07	2.32±0.06
25°C	0.68±0.07	0.85±0.03	1.20±0.02	1.38±0.03	1.72±0.04	1.99±0.02	2.22±0.04	2.57±0.03	2.81±0.03	2.67±0.05	2.50±0.04
30°C	0.68±0.07	0.69±0.05	0.91±0.04	1.20±004	1.39±0.04	1.66±0.06	1.89±0.05	2.16±0.05	2.47±0.04	2.34±0.07	2.18±0.03

Table 12. Growth characteristics *S. aquatilis* at different temperatures

	Chl *a* (µg/ml)										
Temp	Days 0	3	6	9	12	15	18	21	24	27	30
20°C	1.57±0.04	1.72±0.08	1.93±0.03	2.12±0.04	2.40±0.07	2.77±0.07	3.18±0.04	4.04±0.14	4.32±0.06	4.21±0.04	4.08±0.04
25°C	1.57±0.04	1.83±0.03	2.04±0.03	2.53±0.06	2.82±0.07	3.07±0.04	3.43±0.06	4.12±0.03	4.54±0.06	4.41±0.05	4.28±0.04
30°C	1.57±0.04	1.67±0.04	1.81±0.08	2.10±0.07	2.38±0.05	2.76±0.07	3.22±0.06	3.57±0.08	3.79±0.09	3.61±0.08	3.48±0.07

Table 13. Growth of *S. salina* at different temperatures

	Chl *a* (µg/ml)										
Temp	Days 0	3	6	9	12	15	18	21	24	27	30
20°C	1.55±0.08	1.74±0.04	1.98±0.06	2.23±0.05	2.63±0.06	2.93±0.05	3.19±0.04	3.68±0.04	4.11±0.04	4.02±0.03	3.82±0.06
25°C	1.55±0.08	1.78±0.06	2.08±0.04	2.41±0.07	2.69±0.07	2.94±0.04	3.24±0.06	3.82±0.04	4.23±0.05	4.16±0.05	4.01±0.04
30°C	1.55±0.08	1.66±0.03	1.89±0.03	2.17±0.05	2.45±0.03	2.72±0.03	2.92±0.04	3.26±0.06	3.59±0.07	3.4±0.03	3.26±0.05

Table 14. Growth of *S. cedrorum* at different temperatures

	Chl *a* (µg/ml)										
Temp	Days 0	3	6	9	12	15	18	21	24	27	30
20°C	1.5±0.06	1.68±0.05	2.05±0.03	2.28±0.04	2.58±0.06	3.08±0.04	3.48±0.03	4.04±0.06	4.31±0.04	4.21±0.03	4.11±0.04
25°C	1.5±0.06	1.76±0.01	2.10±0.02	2.36±0.04	2.73±0.03	3.10±0.05	3.63±0.05	4.22±0.05	4.59±0.04	4.49±0.05	4.36±0.05
30°C	1.5±0.06	1.59±0.03	1.88±0.03	2.09±0.06	2.45±0.02	2.87±0.06	3.09±0.04	3.32±0.07	3.54±0.07	3.45±0.06	3.32±0.04

Table 15. Growth of *A. litoralis* at different pH

	Chl *a* (µg/ml)										
pH	Days 0	3	6	9	12	15	18	21	24	27	30
6	1.32±0.05	1.35±0.03	1.62±0.07	1.87±0.08	2.13±0.06	2.48±0.08	2.94±0.04	3.2±0.05	3.41±0.04	3.30±0.04	3.20±0.03
7	1.32±0.05	1.43±0.04	1.79±0.05	2.27±0.04	2.56±0.10	3.08±0.04	3.32±0.04	3.85±0.04	4.08±0.07	3.82±0.04	3.64±0.07
8	1.32±0.05	1.62±0.04	1.92±0.04	2.64±0.07	3.1±0.05	3.8±0.05	4.35±0.09	5.94±0.06	6.09±0.02	5.96±0.06	5.72±0.05
9	1.32±0.05	1.41±0.06	1.77±0.04	2.16±0.05	2.89±0.11	3.29±0.06	3.92±0.06	4.22±0.06	4.56±0.01	4.41±0.04	4.23±0.05
10	1.32±0.05	1.33±0.06	1.82±0.04	2.33±0.04	2.92±0.06	3.30±0.03	3.71±0.04	4.09±0.02	4.27±0.04	4.15±0.05	3.91±0.07

Table 16. Growth of *C. minutus* at different pH

	Chl *a* (µg/ml)										
pH	Days 0	3	6	9	12	15	18	21	24	27	30
6	1.22±0.05	1.26±0.05	1.49±0.08	1.77±0.04	2.02±0.05	2.25±0.06	2.50±0.11	2.90±0.08	3.11±0.07	2.94±0.07	2.84±0.08
7	1.22±0.05	1.33±0.06	1.63±0.07	1.91±0.04	2.12±0.07	2.48±0.03	2.72±0.08	3.10±0.05	3.24±0.06	3.18±0.03	3.00±0.06
8	1.22±0.05	1.45±0.04	1.98±0.04	2.35±0.06	2.93±0.05	3.23±0.06	3.70±0.06	4.22±0.06	4.50±0.07	4.46±0.06	4.36±0.05
9	1.22±0.05	1.43±0.08	1.81±0.11	2.31±0.06	2.88±0.07	3.15±0.04	3.51±0.06	3.97±0.07	4.19±0.05	4.12±0.07	4.01±0.06
10	1.22±0.05	1.31±0.04	1.55±0.12	1.92±0.06	2.15±0.04	2.38±0.07	2.77±0.18	2.89±0.15	3.28±0.07	3.30±0.34	2.91±0.09

Table 17. Growth of *G. gelatinosa* at different pH

	Chl *a* (µg/ml)										
pH	Days 0	3	6	9	12	15	18	21	24	27	30
6	1.32±0.08	1.32±0.03	1.64±0.09	1.89±0.04	2.10±0.05	2.39±0.03	2.57±0.07	2.79±0.03	3.02±0.05	2.89±0.02	2.76±0.05
7	1.32±0.08	1.45±0.03	1.87±0.12	2.03±0.07	2.31±0.05	2.48±0.06	2.71±0.06	2.88±0.06	3.14±0.04	2.97±0.04	2.83±0.10
8	1.32±0.08	1.81±0.07	2.40±0.05	2.95±0.11	3.24±0.05	3.78±0.04	4.12±0.06	5.03±0.13	5.65±0.09	5.61±0.11	5.42±0.03
9	1.32±0.08	1.76±0.06	2.24±0.05	2.83±0.06	3.15±0.08	3.46±0.09	4.05±0.07	4.54±0.15	4.91±0.08	4.80±0.07	4.72±0.04
10	1.32±0.08	1.60±0.11	1.94±0.08	2.48±0.03	2.85±0.08	3.13±0.06	3.36±0.07	3.68±0.12	3.92±0.04	3.74±0.08	3.67±0.09

Table 18. Growth of *O. limosa* at different pH

	Chl *a* (µg/ml)										
pH	Days 0	3	6	9	12	15	18	21	24	27	30
6	0.71±0.11	0.62±0.04	0.71±0.03	0.90±0.04	1.03±0.05	1.12±0.08	1.23±0.05	1.33±0.05	1.42±0.06	1.38±0.05	1.30±0.06
7	0.71±0.11	0.88±0.06	1.09±0.05	1.22±0.03	1.41±0.07	1.60±0.09	1.78±0.04	1.92±0.07	2.09±0.04	2.02±0.05	1.92±0.03
8	0.71±0.11	1.15±0.05	1.38±0.03	1.58±0.05	1.96±0.07	2.23±0.06	2.56±0.11	3.07±0.09	3.36±0.08	3.3±0.06	3.21±0.06
9	0.71±0.11	0.96±0.07	1.27±0.04	1.4±0.04	1.69±0.03	1.91±0.08	2.11±0.05	2.31±0.06	2.69±0.05	2.63±0.07	2.53±0.05
10	0.71±0.11	0.85±0.09	1.09±0.04	1.24±0.06	1.41±0.09	1.66±0.10	1.94±0.11	2.12±0.05	2.30±0.06	2.27±0.04	2.20±0.04

Table 19. Growth of *S. aquatilis* at different pH

	Chl *a* (µg/ml)										
pH	Days 0	3	6	9	12	15	18	21	24	27	30
6	1.39±0.06	1.41±0.05	1.54±0.06	1.80±0.06	2.01±0.04	2.19±0.03	2.39±0.06	2.49±0.02	2.67±0.07	2.58±0.10	2.46±0.05
7	1.39±0.06	1.50±0.03	1.69±0.04	1.86±0.02	2.08±0.04	2.34±0.08	2.51±0.06	2.68±0.04	2.94±0.03	2.76±0.02	2.52±0.02
8	1.39±0.06	1.81±0.06	2.08±0.03	2.41±0.03	2.78±0.04	2.99±0.05	3.23±0.05	3.61±0.06	3.88±0.04	3.74±0.07	3.70±0.09
9	1.39±0.06	1.71±0.06	1.92±0.08	2.16±0.07	2.38±0.07	2.78±0.13	3.02±0.11	3.19±0.08	3.34±0.09	3.27±0.09	3.18±0.07
10	1.39±0.06	1.54±0.08	1.87±0.04	2.05±0.06	2.24±0.05	2.55±0.04	2.86±0.06	3.10±0.06	3.30±0.08	3.25±0.05	3.15±0.04

Table 20. Growth of *S. salina* at different pH

	Chl *a* (µg/ml)										
pH	Days 0	3	6	9	12	15	18	21	24	27	30
6	1.35±0.02	1.22±0.03	1.58±0.02	1.61±0.04	1.75±0.04	1.81±0.03	1.99±0.04	2.13±0.05	2.27±0.04	2.21±0.03	2.11±0.05
7	1.35±0.02	1.51±0.06	1.74±0.03	1.99±0.04	2.20±0.03	2.48±0.07	2.78±0.05	2.93±0.05	3.07±0.04	2.98±0.07	2.78±0.04
8	1.35±0.02	1.86±0.03	2.10±0.06	2.25±0.04	2.60±0.03	2.88±0.03	3.13±0.07	3.48±0.03	4.05±0.10	3.92±0.06	3.79±0.03
9	1.35±0.02	1.77±0.04	1.93±0.05	2.08±0.06	2.23±0.05	2.52±0.04	2.83±0.04	3.07±0.03	3.37±0.06	3.32±0.05	3.22±0.03
10	1.35±0.02	1.52±0.05	1.66±0.03	1.82±0.03	2.02±0.06	2.20±0.03	2.33±0.05	2.47±0.04	2.58±0.04	2.52±0.06	2.38±0.05

Table 21. Growth of *S. cedrorum* at different pH

	Chl *a* (µg/ml)										
pH	Days 0	3	6	9	12	15	18	21	24	27	30
6	1.42±0.07	1.45±0.04	1.50±0.04	1.58±0.03	1.68±0.04	1.86±0.06	2.05±0.04	2.17±0.06	2.29±0.02	2.22±0.04	2.07±0.05
7	1.42±0.07	1.51±0.04	1.68±0.03	1.91±0.03	2.01±0.03	2.12±0.03	2.24±0.04	2.35±0.04	2.44±0.02	2.35±0.03	2.25±0.03
8	1.42±0.07	1.85±0.03	1.99±0.05	2.32±0.06	2.75±0.04	3.03±0.05	3.36±0.08	3.93±0.07	4.35±0.07	4.30±0.09	4.21±0.05
9	1.42±0.07	1.81±0.03	1.94±0.04	2.15±0.05	2.45±0.03	2.85±0.03	3.05±0.04	3.34±0.06	3.53±0.08	3.46±0.07	3.37±0.07
10	1.42±0.07	1.56±0.06	1.73±0.05	1.91±0.05	2.06±0.05	2.22±0.03	2.37±0.05	2.52±0.03	2.62±0.05	2.51±0.04	2.43±0.05

Table 22. Growth of *A. litoralis* at different salinity

	Chl *a* (µg/ml)										
Salinity (PSU)	Days 0	3	6	9	12	15	18	21	24	27	30
5	1.23±0.07	1.26±0.07	1.65±0.07	2.00±0.05	2.3±0.05	2.92±0.08	3.24±0.06	3.41±0.06	3.63±0.06	3.48±0.03	3.36±0.06
10	1.23±0.07	1.27±0.04	1.61±0.13	2.26±0.07	2.91±0.08	3.21±0.04	3.75±0.07	4.56±0.09	5.12±0.06	4.99±0.06	4.93±0.04
15	1.23±0.07	1.41±0.06	1.99±0.06	3.03±0.05	3.84±0.07	4.45±0.10	5.30±0.08	5.95±0.03	6.07±0.04	6.01±0.08	5.96±0.04
20	1.23±0.07	1.36±0.07	1.94±0.04	2.37±0.04	2.96±0.06	3.21±0.04	4.03±0.06	4.79±0.07	5.44±0.08	5.30±0.07	5.15±0.04
25	1.23±0.07	1.35±0.04	1.82±0.08	2.22±0.06	2.68±0.06	2.91±0.06	3.27±0.07	3.63±0.04	4.01±0.10	3.90±0.03	3.75±0.06
30	1.23±0.07	1.35±0.04	1.65±0.06	2.02±0.05	2.29±0.05	2.87±0.06	3.08±0.04	3.28±0.03	3.54±0.06	3.49±0.07	3.30±0.09

Table 23. Growth of *C. minutus* at different salinity

	Chl *a* (µg/ml)										
Salinity (PSU)	Days 0	3	6	9	12	15	18	21	24	27	30
5	1.31±0.1	1.22±0.06	1.40±0.07	1.81±0.06	2.09±0.04	2.34±0.03	2.60±0.07	2.88±0.03	3.00±0.06	2.90±0.08	2.77±0.07
10	1.31±0.1	1.33±0.04	1.62±0.07	1.90±0.03	2.23±0.04	2.39±0.05	2.76±0.06	3.05±0.04	3.27±0.05	3.2±0.05	3.17±0.03
15	1.31±0.1	1.37±0.05	1.72±0.03	1.93±0.06	2.19±0.04	2.45±0.05	2.86±0.05	3.22±0.07	3.47±0.06	3.43±0.03	3.32±0.05
20	1.31±0.1	1.48±0.05	1.97±0.06	2.36±0.11	3.03±0.06	3.42±0.07	4.07±0.09	4.73±0.12	5.16±0.06	5.03±0.05	4.84±0.08
25	1.31±0.1	1.41±0.03	1.80±0.09	2.22±0.07	2.89±0.05	3.23±0.06	3.73±0.08	4.10±0.04	4.56±0.10	4.42±0.04	4.31±0.04
30	1.31±0.1	1.29±0.04	1.62±0.05	2.07±0.06	2.49±0.11	2.90±0.04	3.13±0.06	3.42±0.04	3.78±0.09	3.60±0.04	3.31±0.03

Table 24. Growth of *G. gelatinosa* at different salinity

	Chl *a* (µg/ml)										
Salinity (PSU)	Days 0	3	6	9	12	15	18	21	24	27	30
5	1.25±0.08	1.33±0.04	1.62±0.06	1.91±0.07	2.12±0.04	2.42±0.04	2.72±0.05	3.08±0.04	3.28±0.04	3.14±0.05	2.93±0.06
10	1.25±0.08	1.40±0.02	1.72±0.06	2.19±0.06	2.70±0.13	3.12±0.06	3.57±0.11	3.91±0.04	4.25±0.11	4.15±0.08	4.00±0.05
15	1.25±0.08	1.71±0.07	2.16±0.07	2.52±0.09	2.91±0.07	3.27±0.09	3.97±0.07	4.28±0.07	4.63±0.04	4.47±0.05	4.34±0.04
20	1.25±0.08	1.86±0.04	2.39±0.07	3.11±0.06	4.08±0.10	4.89±0.11	5.23±0.06	6.16±0.06	6.43±0.04	6.37±0.05	6.20±0.07
25	1.25±0.08	1.81±0.04	2.23±0.04	2.82±0.04	3.15±0.06	3.63±0.11	4.13±0.06	4.63±0.09	5.04±0.06	4.87±0.05	4.74±0.11
30	1.25±0.08	1.68±0.04	1.98±0.06	2.48±0.05	2.91±0.10	3.23±0.04	3.85±0.09	4.26±0.07	4.78±0.04	4.68±0.03	4.49±0.03

Table 25. Growth of *O. limosa* at different salinity

	Chl *a* (µg/ml)										
Salinity (PSU)	Days 0	3	6	9	12	15	18	21	24	27	30
5	0.64±0.13	0.68±0.12	0.75±0.06	0.89±0.06	1.06±0.04	1.20±0.03	1.32±0.04	1.45±0.05	1.61±0.06	1.57±0.04	1.42±0.04
10	0.64±0.13	0.88±0.04	1.06±0.04	1.22±0.05	1.38±0.04	1.59±0.04	1.82±0.08	1.93±0.04	2.12±0.05	2.06±0.04	1.94±0.03
15	0.64±0.13	1.01±0.03	1.22±0.06	1.44±0.06	1.75±0.08	1.96±0.11	2.22±0.06	2.48±0.11	2.84±0.10	2.77±0.06	2.67±0.10
20	0.64±0.13	1.14±0.04	1.35±0.08	1.65±0.04	1.93±0.05	2.21±0.06	2.51±0.09	2.823±0.06	3.08±0.04	3.00±0.04	2.89±0.03
25	0.64±0.13	1.20±0.04	1.41±0.06	1.93±0.09	2.31±0.09	2.70±0.17	3.16±0.13	3.47±0.19	4.05±0.07	3.91±0.07	3.80±0.04
30	0.64±0.13	1.10±0.03	1.28±0.04	1.57±0.04	1.76±0.05	2.07±0.05	2.30±0.05	2.63±0.05	3.00±0.05	2.91±0.04	2.79±0.05

Table 26. Growth of *S. aquatilis* at different salinity

	Chl *a* (µg/ml)										
Salinity (PSU)	Days 0	3	6	9	12	15	18	21	24	27	30
5	1.32±0.05	1.51±0.03	1.68±0.05	1.98±0.03	2.23±0.05	2.50±0.06	2.87±0.05	3.15±0.03	3.36±0.05	3.28±0.05	3.16±0.05
10	1.32±0.05	1.63±0.05	1.82±0.08	2.03±0.06	2.37±0.05	2.78±0.09	3.08±0.06	3.56±0.12	4.06±0.07	4.00±0.04	3.90±0.06
15	1.32±0.05	1.56±0.05	1.79±0.07	1.97±0.06	2.13±0.05	2.35±0.04	2.52±0.05	2.81±0.01	3.08±0.05	2.99±0.03	2.90±0.03
20	1.32±0.05	1.46±0.03	1.64±0.06	1.88±0.04	2.14±0.04	2.34±0.05	2.45±0.04	2.64±0.06	2.93±0.05	2.89±0.04	2.80±0.07
25	1.32±0.05	1.36±0.04	1.52±0.06	1.71±0.07	1.89±0.06	2.05±0.03	2.18±0.03	2.33±0.06	2.47±0.05	2.39±0.03	2.32±0.05
30	1.32±0.05	1.27±0.03	1.37±0.04	1.52±0.03	1.65±0.06	1.79±0.03	1.90±0.02	2.00±0.03	2.09±0.04	1.91±0.04	1.78±0.04

Table 27. Growth of *S. salina* at different salinity

	Chl *a* (µg/ml)										
Salinity (PSU)	Days 0	3	6	9	12	15	18	21	24	27	30
5	1.36±0.11	1.28±0.03	1.30±0.03	1.40±0.03	1.53±0.06	1.66±0.04	1.81±0.03	1.93±0.06	2.05±0.04	1.94±0.02	1.81±0.04
10	1.36±0.11	1.39±0.02	1.51±0.02	1.66±0.01	1.77±0.05	1.88±0.04	1.99±0.04	2.12±0.02	2.19±0.02	2.12±0.03	2.06±0.04
15	1.36±0.11	1.46±0.03	1.58±0.03	1.75±0.04	1.88±0.04	2.01±0.04	2.14±0.04	2.23±0.04	2.45±0.03	2.36±0.04	2.26±0.02
20	1.36±0.11	1.61±0.03	1.78±0.03	1.98±0.04	2.16±0.03	2.27±0.03	2.41±0.02	2.59±0.04	2.77±0.02	2.65±0.05	2.51±0.03
25	1.36±0.11	1.63±0.06	1.93±0.04	2.09±0.04	2.39±0.08	2.87±0.03	3.08±0.03	3.38±0.04	3.78±0.10	3.69±0.08	3.6±0.04
30	1.36±0.11	1.80±0.04	2.15±0.03	2.54±0.12	2.85±0.04	3.18±0.05	3.51±0.05	3.92±0.06	4.32±0.03	4.26±0.04	4.17±0.04

Table 28. Growth of *S. cedrorum* at different salinity

	Chl *a* (µg/ml)										
Salinity (PSU)	Days 0	3	6	9	12	15	18	21	24	27	30
5	1.38±0.08	1.42±0.07	1.47±0.05	1.54±0.10	1.92±0.06	2.11±0.07	2.28±0.04	2.43±0.04	2.66±0.07	2.55±0.07	2.44±0.04
10	1.38±0.08	1.77±0.07	2.01±0.07	2.29±0.04	2.76±0.04	3.13±0.07	3.46±0.07	3.91±0.04	4.18±0.04	4.13±0.05	4.01±0.04
15	1.38±0.08	1.72±0.06	1.89±0.04	2.05±0.05	2.23±0.04	2.38±0.04	2.51±0.05	2.81±0.05	3.02±0.04	2.91±0.04	2.81±0.05
20	1.38±0.08	1.53±0.03	1.77±0.05	1.92±0.03	2.06±0.04	2.20±0.02	2.34±0.05	2.53±0.03	2.71±0.03	2.65±0.04	2.52±0.02
25	1.38±0.08	1.41±0.04	1.50±0.03	1.61±0.04	1.77±0.06	1.97±0.05	2.11±0.03	2.25±0.03	2.43±0.03	2.35±0.03	2.28±0.03
30	1.38±0.08	1.45±0.03	1.58±0.03	1.67±0.05	1.78±0.04	1.92±0.04	2.04±0.06	2.11±0.03	2.21±0.05	1.91±0.03	1.81±0.05

Table 29. Growth of *A. litoralis* in different media

	Chl *a* (µg/ml)										
Medium	Days 0	3	6	9	12	15	18	21	24	27	30
BG 11	1.25±0.02	1.75±0.22	3.02±0.11	3.65±0.08	4.08±0.04	5.73±0.07	5.93±0.06	6.18±0.06	6.25±0.04	6.11±0.04	6.05±0.04
SN	1.25±0.02	1.49±0.07	2.02±0.04	2.95±0.07	3.30±0.06	3.94±0.04	4.22±0.06	4.67±0.07	5.11±0.05	5.01±0.04	4.82±0.07
Allen & Nelson	1.25±0.02	1.38±0.03	1.86±0.05	2.23±0.07	3.00±0.06	3.17±0.07	3.61±0.08	4.08±0.05	4.37±0.09	4.28±0.04	4.22±0.04
SWEM	1.25±0.02	1.43±004	1.85±0.04	2.03±0.04	2.52±0.07	3.10±0.04	3.34±0.06	3.85±0.05	4.27±0.04	4.16±0.03	4.09±0.04

Table 30. Growth of *C. minutus* in different media

	Chl *a* (µg/ml)										
Medium	Days 0	3	6	9	12	15	18	21	24	27	30
BG 11	1.27±0.08	1.45±0.03	2.23±0.06	2.58±0.07	3.04±0.07	3.37±0.06	3.95±0.08	4.49±0.06	4.91±0.04	5.02±0.04	4.75±0.08
SN	1.27±0.08	1.44±0.05	2.04±0.10	2.41±0.03	3.56±0.08	4.36±0.08	5.05±0.09	5.74±0.08	5.98±0.06	5.87±0.04	5.69±0.08
Allen & Nelson	1.27±0.08	1.28±0.04	1.77±0.06	2.4±0.07	2.93±0.07	3.41±0.11	3.85±0.10	4.17±0.07	4.50±0.07	4.82±0.11	4.65±0.08
SWEM	1.27±0.08	1.38±0.02	1.93±0.05	2.46±0.07	3.05±0.06	3.54±0.09	3.93±0.05	4.22±0.07	4.62±0.07	4.38±0.07	4.24±0.03

Table 31. Growth of *G. gelatinosa* in different media

	Chl *a* (µg/ml)										
Medium	Days 0	3	6	9	12	15	18	21	24	27	30
BG 11	1.52±0.03	1.93±0.06	2.36±0.11	3.01±0.06	3.76±0.05	4.59±0.08	5.05±0.07	5.50±0.09	6.18±0.04	6.12±0.04	6.00±0.10
SN	1.52±0.03	1.95±0.09	2.52±0.08	3.65±0.09	4.29±0.04	5.81±0.07	6.17±0.05	6.36±0.06	6.60±0.12	6.43±0.13	6.28±0.11
Allen & Nelson	1.52±0.03	1.58±0.14	2.22±0.06	2.84±0.13	3.25±0.07	4.04±0.09	4.53±0.12	5.08±0.11	5.56±0.07	5.43±0.07	5.27±0.09
SWEM	1.52±0.03	1.61±0.07	2.10±0.11	2.57±0.11	3.09±0.08	3.58±0.14	3.93±0.08	4.35±0.08	5.07±0.09	4.96±0.05	4.81±0.07

Table 32. Growth of *O. limosa* in different media

	Chl *a* (µg/ml)										
Medium	Days 0	3	6	9	12	15	18	21	24	27	30
BG 11	0.75±0.06	0.99±0.08	1.22±0.05	1.46±0.11	1.92±0.06	2.39±0.07	2.80±0.05	3.12±0.07	3.59±0.09	3.50±0.09	3.33±0.03
SN	0.75±0.06	1.05±0.08	1.42±0.06	1.89±0.08	2.14±0.04	2.85±0.09	3.23±0.06	3.64±0.09	4.26±0.06	4.22±0.06	4.17±0.03
Allen & Nelson	0.75±0.06	0.82±0.06	1.13±0.06	1.31±0.04	1.66±0.09	2.06±0.11	2.36±0.12	2.66±0.12	2.92±0.04	2.78±0.05	2.63±0.06
SWEM	0.75±0.06	0.85±0.03	1.11±0.06	1.36±0.07	1.62±0.07	1.83±0.05	2.16±0.05	2.42±0.04	2.61±0.06	2.54±0.07	2.42±0.03

Table 33. Growth of *S. aquatilis* in different media

	Chl *a* (µg/ml)										
Medium	Days 0	3	6	9	12	15	18	21	24	27	30
BG 11	1.34±0.05	1.61±0.08	2.00±0.09	2.29±0.03	2.65±0.09	3.02±0.04	3.33±0.08	3.97±0.12	4.17±0.06	4.10±0.03	3.96±0.08
SN	1.34±0.05	1.61±0.09	2.12±0.07	2.40±0.06	3.10±0.15	3.45±0.04	3.87±0.05	4.19±0.04	4.58±0.12	4.49±0.11	4.40±0.08
Allen & Nelson	1.34±0.05	1.38±0.09	1.49±0.07	1.82±0.06	2.07±0.06	2.33±0.06	2.77±0.08	3.03±0.04	3.33±0.04	3.20±0.05	3.12±0.05
SWEM	1.34±0.05	1.42±0.07	1.69±0.05	1.98±0.06	2.24±0.05	2.52±0.04	2.77±0.07	3.06±0.04	3.31±0.07	3.27±0.06	3.17±0.04

Table 34. Growth of *S. salina* in different media

	Chl *a* (µg/ml)										
Medium	Days 0	3	6	9	12	15	18	21	24	27	30
BG 11	1.47±0.17	1.76±0.06	2.05±0.06	2.33±0.05	2.87±0.04	3.13±0.05	3.48±0.07	3.98±0.06	4.21±0.06	4.18±0.07	4.11±0.07
SN	1.47±0.17	1.83±0.06	2.11±0.05	2.52±0.05	3.01±0.07	3.27±0.05	3.82±0.10	4.30±0.06	4.86±0.07	4.79±0.05	4.68±0.05
Allen & Nelson	1.47±0.17	1.48±0.07	1.78±0.03	2.02±0.06	2.28±0.08	2.48±0.05	2.70±0.06	3.07±0.03	3.30±0.02	3.22±0.04	3.21±0.03
SWEM	1.47±0.17	1.42±0.04	1.66±0.07	2.01±0.03	2.27±0.05	2.44±0.08	2.69±0.06	2.87±0.06	3.05±0.06	2.92±0.03	2.81±0.07

Table 35. Growth of *S. cedrorum* in different media

	Chl *a* (µg/ml)										
Medium	Days 0	3	6	9	12	15	18	21	24	27	30
BG 11	1.40±0.07	1.58±0.03	1.91±0.04	2.15±0.04	2.59±0.02	2.97±0.06	3.20±0.04	3.81±0.04	4.15±0.04	4.06±0.03	3.94±0.03
SN	1.40±0.07	1.68±0.03	2.06±0.05	2.36±0.05	2.84±0.07	3.15±0.04	3.35±0.03	3.98±0.04	4.27±0.04	4.21±0.04	4.12±0.03
Allen & Nelson	1.40±0.07	1.48±0.06	1.76±0.06	1.99±0.05	2.18±0.07	2.34±0.04	2.52±0.04	2.71±0.03	3.02±0.04	2.91±0.04	2.85±0.03
SWEM	1.40±0.07	1.53±0.05	1.83±0.06	2.04±0.03	2.25±0.04	2.47±0.05	2.71±0.03	2.88±0.04	3.12±0.05	3.06±0.06	2.93±0.05

Table 36. Protein content of the cyanobacterial strains

Strains	Growth phase		
	log	stationary	decline
A. litoralis	35.5±2.12	30.47±1.80	27.07±1.70
C. minutus	23.13±0.90	21.2±0.40	19.03±0.40
G. gelatinosa	29.32±1.69	25.84±1.92	23.05±1.25
O. limosa	43.9±1.15	42.63±0.87	38.57±0.47
S. aquatilis	33.7±1.35	31.83±1.65	29.63±2.14
S. salina	47.6±0.26	45.6±0.44	42.83±0.70
S. cedrorum	43.37±0.61	41.27±0.93	37.63±0.71

Table 37. Carbohydrate content of the cyanobacterial strains

Strains	Growth phase		
	log	stationary	decline
A. litoralis	22.33±0.47	28.9±0.66	24.77±0.60
C. minutus	22.13±3.35	26.63±1.54	23.40±1.49
G. gelatinosa	19.8±0.60	28.07±0.96	24.4±1.11
O. limosa	24.77±0.93	32.10±1.01	29.4±0.30
S. aquatilis	28.13±0.65	35.13±0.74	31.83±1.12
S. salina	26.73±0.45	38.23±0.83	35.13±0.97
S. cedrorum	24.47±0.35	36.47±0.35	31.80±0.56

Table 38. Lipid content of the cyanobacterial strains

Strains	Growth phase		
	log	stationary	decline
A. litoralis	4.20±0.56	4.90±0.20	5.53±0.21
C. minutus	5.67±0.40	6.77±0.31	8.00±0.20
G. gelatinosa	6.17±0.21	7.27±0.45	8.43±0.21
O. limosa	4.50±0.30	5.30±0.17	6.67±0.15
S. aquatilis	4.90±0.36	5.57±0.31	6.70±0.26
S. salina	5.83±0.31	6.60±0.26	7.50±0.30
S. cedrorum	4.57±0.25	5.50±0.26	6.83±0.31

Table 39. Chlorophyll *a* content of the cyanobacterial strains

Strains	Growth phase		
	log	stationary	decline
A. litoralis	5.87±0.04	6.20±0.09	6.14±0.060
C. minutus	6.94±0.04	7.44±0.33	7.32±0.29
G. gelatinosa	7.52±0.07	8.14±0.09	8.04±0.10
O. limosa	4.15±0.09	5.01±0.18	4.91±0.23
S. aquatilis	5.78±0.05	6.06±0.09	5.93±0.10
S. salina	6.27±0.12	7.09±0.14	7.00±0.12
S. cedrorum	5.09±0.12	5.91±0.11	5.73±0.18

Table 40. Carotenoid content of the cyanobacterial strains

Strains	Growth phase		
	log	stationary	decline
A. litoralis	2.02±0.04	2.69±0.16	2.59±0.17
C. minutus	3.21±0.08	3.93±0.09	3.84±0.10
G. gelatinosa	3.35±0.03	4.00±0.14	3.88±0.14
O. limosa	1.80±0.05	2.21±0.07	2.12±0.06
S. aquatilis	2.04±0.07	2.40±0.10	2.32±0.10
S. salina	3.25±0.04	3.82±0.10	3.74±0.10
S. cedrorum	2.26±0.06	2.63±0.08	2.55±0.08

Table 41. Phycocyanin content of the cyanobacterial strains

Strains	Growth phase		
	log	stationary	decline
A. litoralis	1.18±0.04	1.43±0.07	1.31±0.04
C. minutus	1.38±0.06	2.02±0.12	1.90±0.06
G. gelatinosa	1.22±0.04	1.52±0.06	1.40±0.06
O. limosa	1.31±0.07	1.76±0.07	1.64±0.06
S. aquatilis	1.24±0.03	1.67±0.04	1.50±0.06
S. salina	1.33±0.04	1.98±0.21	1.90±0.06
S. cedrorum	1.31±0.03	1.69±0.09	1.54±0.07

Table 42. Phycoerythrin content of the cyanobacterial strains

Strains	Growth phase		
	log	stationary	decline
A. litoralis	1.11±0.03	1.24±0.06	1.18±0.04
C. minutus	1.32±0.03	1.59±0.04	1.48±0.05
G. gelatinosa	1.19±0.04	1.38±0.04	1.29±0.03
O. limosa	0.89±0.05	1.17±0.05	1.08±0.06
S. aquatilis	1.20±0.03	1.38±0.07	1.30±0.05
S. salina	1.30±0.03	1.66±0.05	1.58±0.04
S. cedrorum	1.22±0.04	1.50±0.03	1.40±0.03

Table 43. Allophycocyanin content of the cyanobacterial strains

Strains	Growth phase		
	log	stationary	decline
A. litoralis	0.41±0.06	0.81±0.08	0.68±0.03
C. minutus	0.73±0.05	1.01±0.08	0.87±0.05
G. gelatinosa	0.68±0.06	1.09±0.12	0.87±0.06
O. limosa	0.34±0.05	0.83±0.08	0.69±0.07
S. aquatilis	0.74±0.03	1.13±0.09	1.00±0.06
S. salina	0.83±0.04	1.05±0.06	0.91±0.04
S. cedrorum	0.36±0.06	0.75±0.04	0.60±0.03

Table 44. Total phenolic content

Strains	Extracts				
	Acetone	Chlo:Meth	DMSO	Ethanol	Methanol
A. litoralis	3.52±0.17	1.43±0.13	10.35±0.17	5.98±0.12	7.09±0.29
C. minutus	5.17±0.14	2.30±0.77	12.73±0.06	5.37±0.06	12.9±0.11
G. gelatinosa	9.83±0.03	3.38±0.64	9.34±0.43	5.92±0.20	11.22±0.07
O. limosa	9.10±0.12	1.27±0.08	4.46±0.14	6.30±0.22	4.17±0.24
S. aquatilis	10.50±0.13	3.61±0.14	12.53±0.21	12.23±0.11	12.52±0.11
S. salina	30.81±0.52	10.51±0.22	34.36±1.00	37.73±0.24	44.33±0.43
S. cedrorum	4.09±0.17	1.50±0.24	5.55±0.08	3.98±0.09	6.03±0.23

Table 45. Total flavonoid content

Strains	Extracts				
	Acetone	Chlo:Meth	DMSO	Ethanol	Methanol
A. litoralis	2.31±0.09	1.72±0.41	3.77±0.76	2.73±0.09	2.90±0.28
C. minutus	2.89±0.06	1.54±0.06	7.25±0.07	2.83±0.11	7.04±0.10
G. gelatinosa	3.89±0.56	1.54±0.68	5.35±0.68	2.94±0.09	7.40±0.18
O. limosa	1.48±0.06	0.53±0.00	1.01±0.019	1.91±0.04	0.85±0.03
S. aquatilis	2.67±0.17	0.86±0.02	3.69±0.18	3.28±0.060	3.68±0.13
S. salina	8.33±0.83	2.90±0.83	11.23±0.83	13.41±0.32	18.30±1.37
S. cedrorum	1.41±0.06	0.97±0.02	2.34±0.10	1.10±0.06	2.82±0.06

Table 46. Total antioxidant capacity

Strains	Extracts				
	Acetone	Chlo:Meth	DMSO	Ethanol	Methanol
A. litoralis	3.80±0.36	4.00±0.66	44.23±1.40	19.87±0.40	49.63±1.21
C. minutus	7.33±0.83	5.83±4.04	46.80±5.32	24.97±0.81	22.80±1.30
G. gelatinosa	10.70±0.50	5.83±0.85	26.93±0.06	19.57±0.78	25.81±0.59
O. limosa	21.53±0.70	7.67±0.35	4.97±0.59	11.87±0.40	9.23±0.42
S. aquatilis	8.23±0.60	5.17±0.15	60.90±1.50	13.93±1.11	40.50±0.89
S. salina	5.50±0.44	4.20±1.08	83.63±0.50	18.70±1.05	69.80±0.85
S. cedrorum	43.73±1.31	33.33±0.85	61.67±1.60	35.63±0.85	42.41±0.30

Table 47. Deoxyribose radical scavenging activity

Strains	Extracts				
	Acetone	Chlo:Meth	DMSO	Ethanol	Methanol
A. litoralis	10.98±0.50	5.72±0.09	10.92±0.36	8.73±0.17	9.55±0.12
C. minutus	13.03±1.42	3.82±0.84	15.25±0.75	12.72±0.32	12.85±0.25
G. gelatinosa	12.79±0.80	3.61±0.18	13.29±0.06	11.50±0.12	12.15±0.08
O. limosa	6.54±0.29	1.79±0.20	7.96±0.28	5.77±0.27	6.19±0.08
S. aquatilis	12.32±0.68	3.74±0.42	13.22±0.62	12.53±0.28	12.01±0.62
S. salina	19.87±0.26	4.57±0.28	22.85±0.25	21.46±0.36	21.00±0.64
S. cedrorum	16.34±1.00	4.00±0.06	20.20±0.10	14.46±0.09	17.91±0.12

Table 48. Ferric reducing antioxidant power

Strains	Conc	Acetone	Chlo:Meth	DMSO	Ethanol	Methanol
A. litoralis	100 µl	3.18±0.12	1.46±0.31	6.92±0.16	1.98±0.12	5.90±0.54
	200 µl	5.28±0.27	3.61±0.48	15.44±0.66	12.41±0.54	14.85±1.16
	300 µl	8.46±0.16	5.87±0.47	19.03±0.31	22.53±0.39	24.05±0.64
C. minutus	100 µl	2.46±1.12	2.36±0.19	7.95±0.99	3.46±0.27	6.69±1.04
	200 µl	6.13±0.57	5.99±0.80	16.33±0.43	8.56±0.39	14.80±0.72
	300 µl	8.90± 0.28	8.49±0.27	23.10± 0.50	13.79±0.36	17.05±0.35
G. gelatinosa	100 µl	6.92±0.40	2.15±0.28	8.82±0.45	9.46±0.28	8.85±0.61
	200 µl	12.23±0.58	3.44±0.38	30.28±0.44	24.08±0.71	30.54±0.42
	300 µl	22.46± 0.33	6.80±0.65	37.23± 0.76	32.54± 0.36	36.54±0.48
O. limosa	100 µl	8.90±0.27	2.57±0.44	7.03±0.38	6.62±0.21	5.03±0.16
	200 µl	19.92±0.47	6.18±0.23	9.95±0.35	11.70±0.60	11.85±0.95
	300 µl	24.74±0.27	7.9±0.16	15.90±0.75	17.69±0.62	16.23± 0.31
S. aquatilis	100 µl	7.87±0.98	5.87±0.31	9.51±0.05	9.26±0.36	11.46±0.48
	200 µl	16.44±0.66	11.33±0.62	25.10±0.38	26.34±0.10	25.64±0.45
	300 µl	23.15±0.16	12.82±0.44	32.26± 0.50	40.28±0.32	43.18±0.81
Synechocystis salina	100 µl	8.49±0.43	5.10±0.35	10.95±0.35	13.20±0.31	13.90±0.20
	200 µl	16.79±0.43	8.26±0.14	47.41±0.38	38.38±0.43	47.03±0.96
	300 µl	23.23±0.13	20.80±0.44	55.18±0.38	49.84±0.90	53.92± 0.44
Synechococcus cedrorum	100 µl	7.51±0.59	0.95±0.27	8.61±0.64	8.20±0.54	7.44±0.51
	200 µl	27.64±0.32	4.59±0.27	31.08±1.35	31.33±0.93	38.87±1.06
	300 µl	32.31±0.28	6.15±0.70	40.87±0.52	38.72±0.50	46.02±0.08

Table 49. DPPH radical scavenging activity

Strains	Conc	Acetone	Chlo:Meth	DMSO	Ethanol	Methanol
A. litoralis	100 µl	9.56±2.01	0.95±0.51	11.60±1.63	5.61±1.15	5.16±0.69
	200 µl	28.05±1.08	3.70±0.58	30.72±3.47	28.55±1.44	33.08±0.69
	300 µl	41.36± 1.63	5.35±0.83	52.39± 2.07	38.56± 1.41	41.94 ±1.09
C. minutus	100 µl	0.7±0.83	0.23±0.77	3.05±1.77	1.86±0.80	3.09±0.40
	200 µl	15.73±1.14	3.90±2.44	25.78±0.77	16.98±2.13	31.04±1.34
	300 µl	35.8 ±1.17	11.12± 1.41	41.58 ±1.52	37.95±1.56	49.15±1.27
G. gelatinosa	100 µl	0.38±0.19	0.63±0.11	1.08±0.55	0.77±0.51	1.71±0.51
	200 µl	23.46±1.73	4.91±0.77	7.27±0.51	11.22±3.76	21.03±2.13
	300 µl	37.22 ±1.38	9.24± 0.48	26.20± 1.66	29.19± 2.05	35.75± 3.36
O. limosa	100 µl	1.15±0.58	0.44±0.29	2.74±0.67	0.64±0.30	1.47±0.47
	200 µl	8.03±1.44	3.82±0.77	10.14±2.13	8.79±2.07	13.83±2.89
	300 µl	19.76± 0.77	10.13± 0.39	24.86 ±1.20	19.06±2.09	25.94 ±1.34
S. aquatilis	100 µl	1.47±0.44	0.61±0.13	2.17±0.40	0.76±0.57	2.23±0.62
	200 µl	21.16±1.78	4.97±0.51	25.49±2.20	11.73±1.17	21.35±1.49
	300 µl	36.90 ±1.15	8.99 ±1.56	46.78± 2.80	34.61±0.88	36.71 ±3.21
Synechocystis salina	100 µl	0.95±0.08	0.57±0.03	3.50±0.09	1.97±0.19	3.95±0.06
	200 µl	20.33±0.26	8.16±0.27	32.56±1.54	23.96±1.18	31.74±2.08
	300 µl	40.15± 0.31	16.44±0.58	47.42 ±0.45	43.60 ±1.75	55.83 ±0.76
Synechococcus cedrorum	100 µl	5.16±0.39	1.15±0.19	3.31±0.48	1.21±0.40	2.74±0.40
	200 µl	46.53±1.17	4.97±2.46	40.54±1.27	36.26±2.11	48.76±1.82
	300 µl	63.61±2.63	9.62±2.82	54.88± 1.70	45.82±1.06	65.43± 2.07

Table 50. Hydrogen peroxide radical scavenging assay

Strains	Conc	Acetone	Chlo:Meth	DMSO	Ethanol	Methanol
A. litoralis	100 µl	2.08±0.07	0.29±0.17	2.58±0.28	1.96±0.12	2.72±0.19
	200 µl	3.57±0.30	1.19±0.13	4.12±0.15	3.92±0.11	4.28±0.19
	300 µl	5.39 ±0.33	2.56± 0.42	6.07± 0.11	5.54 ±0.58	6.53± 0.38
C. minutus	100 µl	0.54±0.28	0.32±0.04	0.64±0.12	0.46±0.12	0.51±0.14
	200 µl	2.44±0.14	0.61±0.07	2.9±0.22	2.47±0.45	2.71±0.27
	300 µl	4.56± 0.39	0.93±0.09	5.08±0.25	4.25± 0.23	4.42± 0.38
G. gelatinosa	100 µl	0.27±0.03	0.21±0.07	0.35±0.06	0.32±0.04	0.41±0.03
	200 µl	1.09±0.11	1.13±0.09	2.65±0.20	2.09±0.21	1.2±0.07
	300 µl	2.80± 0.07	1.70 ±0.17	4.89± 0.20	3.77± 0.20	3.51± 0.18
O. limosa	100 µl	1.10±0.07	0.11±0.01	1.40±0.03	1.22±0.05	1.28±0.05
	200 µl	1.80±0.16	0.29±0.05	2.24±0.05	1.88±0.08	2.38±0.07
	300 µl	2.35± 0.04	0.52± 0.05	4.13± 0.11	2.52± 0.06	3.80± 0.15
S. aquatilis	100 µl	1.25±0.09	0.19±0.08	1.39±0.08	1.40±0.08	1.31±0.04
	200 µl	2.72±0.08	0.52±0.04	2.89±0.17	2.86±0.16	2.49±0.07
	300 µl	4.20 ±0.05	0.85±0.12	5.16 ±0.07	3.99±0.23	4.53 ±0.17
Synechocystis salina	100 µl	0.76±0.08	0.25±0.04	0.87±0.08	0.64±0.03	0.81±0.08
	200 µl	3.25±0.07	0.68±0.10	3.37±0.07	3.00±0.07	3.25±0.03
	300 µl	6.91± 0.12	1.67± 0.06	8.06± 0.09	5.72± 0.09	6.59± 0.09
Synechococcus cedrorum	100 µl	1.47±0.12	0.48±0.04	1.86±0.10	1.16±0.10	1.57±0.02
	200 µl	3.03±0.12	0.93±0.01	3.80±0.21	2.92±0.06	3.48±0.03
	300 µl	4.34 ±0.06	1.33±0.19	8.17± 0.22	4.64± 0.10	6.5± 0.07

Table 50. Mean body weight of *O. mossambicus* fed on different experimental feeds

Time interval	Treatments					
	Control	Sa 0.5%	Sa 1%	Sb 0.5%	Sb 1%	Sa+Sb
0th day	11.33±1.32	11.33±1.32	11.33±1.32	11.33±1.32	11.33±1.32	11.33±1.32
15th day	24.89±2.71	22±2.62	29.89±2.60	21.89±2.22	25.10±2.03	23±1.66
30th day	34.56±2.19	38.44±2.19	41.56±2.19	36.56±1.81	33.35±2.19	36±1.73
45th day	44.11±2.76	48.78±2.30	52.56±3.15	46.89±2.24	41.37±2.03	43.11±1.74
60th day	53.33±2.12	55.56±3.30	63.11±2.65	57.44±2.03	50.08±1.74	56.67±1.87

Table 51. Survival (%) of different experimental groups after *A. hydrophila* challenge

Parameter	Treatments					
	Control	Sa 0.5%	Sa 1%	Sb 0.5%	Sb 1%	Sa+Sb
Survival (%)	27.78±9.61	75±0	88.89±4.82	55.56±9.62	52.78±4.81	80.55±4.81

Appendix-III

STATISTICAL ANALYSIS

Biochemical characterization

ANOVA results

1. Growth of *A. litoralis* at different light intensities

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.786	2	.393	.786	.466
Within Groups	13.489	27	.500		
Total	14.275	29			

Homogeneous Subsets

	Light intensity	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	700 lux	10	2.4973
	2100 lux	10	2.6327
	1500 lux	10	2.8877
	Sig.		.444
Means for groups in homogeneous subsets are displayed.			
a. Uses Harmonic Mean Sample Size = 10.000.			

2. Growth of *C. minutus* at different light intensities

Chl *a* **ANOVA**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.539	2	5.769	2.902	.072
Within Groups	53.686	27	1.988		
Total	65.225	29			

Homogeneous Subsets

	Light intensity	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	700 lux	10	2.7230
	1400 lux	10	4.0183
	2100 lux	10	4.0580
	Sig.		.105
Means for groups in homogeneous subsets are displayed.			
a. Uses Harmonic Mean Sample Size = 10.000.			

3. Growth of *G. gelatinosa* at different light intensities

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.928	2	1.464	.670	.520
Within Groups	59.027	27	2.186		
Total	61.955	29			

Homogeneous Subsets

Chl *a*

	Light intensity	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	700 lux	10	3.3763
	1400 lux	10	4.0183
	2100 lux	10	4.0580
	Sig.		.564
Means for groups in homogeneous subsets are displayed.			
a. Uses Harmonic Mean Sample Size = 10.000.			

4. Growth of *O. limosa* at different light intensities

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.067	2	3.533	2.876	.074
Within Groups	33.168	27	1.228		
Total	40.234	29			

Homogeneous Subsets

Chl *a*

	Light intensity	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	700 lux	10	1.8590
	2100 lux	10	2.8170
	1400 lux	10	2.9477
	Sig.		.090

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

5. Growth of *S. aquatilis* at different light intensities

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.589	2	.295	.758	.478
Within Groups	10.496	27	.389		
Total	11.086	29			

Homogeneous Subsets

Chl *a*

	Light intensity	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	700 lux	10	2.5950
	2100 lux	10	2.8263
	1400 lux	10	2.9303
	Sig.		.462

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

6. Growth of *S. salina* at different light intensities

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.886	2	.443	.384	.685
Within Groups	31.169	27	1.154		
Total	32.055	29			

Homogeneous Subsets

Chl *a*

	Light intensity	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	700 lux	10	3.0453
	2100 lux	10	3.2560
	1400 lux	10	3.4663
	Sig.		.660

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

7. Growth of *S. cedrorum* at different light intensities

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.463	2	.732	.900	.418
Within Groups	21.954	27	.813		
Total	23.417	29			

Homogeneous Subsets

Chl *a*

	Light intensity	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	700 lux	10	2.8277
	2100 lux	10	3.1010
	1400 lux	10	3.3687
	Sig.		.385

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

8. Growth of *A. litoralis* at different temperatures

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.929	2	.464	.785	.466
Within Groups	15.971	27	.592		
Total	16.899	29			

Homogeneous Subsets

Chl *a*

	temp	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	30°C	10	2.7600
	20°C	10	2.8743
	25°C	10	3.1770
	Sig.		.456

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

9. Growth of *C. minutus* at different temperatures

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.642	2	2.321	1.031	.370
Within Groups	60.766	27	2.251		
Total	65.408	29			

Homogeneous Subsets

Chl *a*

	temp	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	30°C	10	3.4250
	20°C	10	4.0703
	25°C	10	4.3673
	Sig.		.353

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

10. Growth of *G. gelatinosa* at different temperatures

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.373	2	2.686	1.038	.368
Within Groups	69.884	27	2.588		
Total	75.256	29			

Homogeneous Subsets

Chl *a*

	temp	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	30°C	10	3.6530
	20°C	10	4.3230
	25°C	10	4.6730
	Sig.		.346

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

11. Growth of *O. limosa* at different temperatures

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.455	2	.227	.552	.582
Within Groups	11.134	27	.412		
Total	11.589	29			

Homogeneous Subsets

Chl *a*

	temp	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	30°C	10	1.6883
	20°C	10	1.8507
	25°C	10	1.9897
	Sig.		.553

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

12. Growth of *S. aquatilis* at different temperatures

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.105	2	.552	.615	.548
Within Groups	24.247	27	.898		
Total	25.352	29			

Homogeneous Subsets

Chl *a*

	temp	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	30°C	10	2.8380
	20°C	10	3.0783
	25°C	10	3.3080
	Sig.		.517

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

13. Growth of *S. salina* at different temperatures

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.888	2	.444	.664	.523
Within Groups	18.055	27	.669		
Total	18.943	29			

Homogeneous Subsets

Chl *a*

	temp	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	30°C	10	2.7313
	20°C	10	3.0330
	25°C	10	3.1370
	Sig.		.517

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

14. Growth of *S. cedrorum* at different temperatures

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.763	2	.881	1.015	.376
Within Groups	23.454	27	.869		
Total	25.217	29			

Homogeneous Subsets

Chl *a*

	temp	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	30°C	10	2.7600
	20°C	10	3.1813
	25°C	10	3.3330
	Sig.		.368

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

15. Growth of *A. litoralis* at different pH

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.093	4	3.273	2.350	.068
Within Groups	62.670	45	1.393		
Total	75.763	49			

Homogeneous Subsets

Chl *a*

	pH	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	6	10	2.5503	
	7	10	2.9847	2.9847
	10	10	3.1837	3.1837
	9	10	3.2863	3.2863
	8	10		4.1143
	Sig.		.634	.221

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

16. Growth of *C. minutus* at different pH

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.404	4	2.101	2.886	.033
Within Groups	32.756	45	.728		
Total	41.160	49			

Homogeneous Subsets

Chl *a*

	pH	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	6	10	2.3080
	10	10	2.4453
	7	10	2.4693
	9	10	3.1387
	8	10	3.3187
	Sig.		.078

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

17. Growth of *G. gelatinosa* at different pH

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	20.981	4	5.245	5.835	.001
Within Groups	40.454	45	.899		
Total	61.435	49			

Homogeneous Subsets

Chl *a*

	pH	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	6	10	2.3377		
	7	10	2.4660	2.4660	
	10	10	3.0363	3.0363	3.0363
	9	10		3.6453	3.6453
	8	10			4.0020
	Sig.		.476	.058	.171

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

18. Growth of *O. limosa* at different pH

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.802	4	2.201	6.796	.000
Within Groups	14.571	45	.324		
Total	23.373	49			

Homogeneous Subsets

Chl *a*					
	pH	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	6	10	1.1047		
	7	10	1.5913	1.5913	
	10	10	1.7083	1.7083	1.7083
	9	10		1.9510	1.9510
	8	10			2.3797
	Sig.		.142	.622	.080

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

19. Growth of *S. aquatilis* at different pH

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.563	4	1.391	4.374	.005
Within Groups	14.309	45	.318		
Total	19.871	49			

Homogeneous Subsets

Chl *a*

	pH	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	6	10	2.1550	
	10	10	2.1627	
	9	10	2.6413	2.6413
	7	10	2.6923	2.6923
	8	10		3.0220
	Sig.		.225	.562

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

20. Growth of *S. salina* at different pH

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.296	4	2.074	6.425	.000
Within Groups	14.527	45	.323		
Total	22.823	49			

Homogeneous Subsets

Chl *a*

	pH	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	6	10	1.8143		
	10	10	2.1493	2.1493	
	7	10	2.4397	2.4397	2.4397
	9	10		2.6353	2.6353
	8	10			3.0060
	Sig.		.118	.326	.188

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

21. Growth of *S. cedrorum* at different pH

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.535	4	3.134	9.043	.000
Within Groups	15.594	45	.347		
Total	28.129	49			

Homogeneous Subsets

Chl *a*

	pH	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	6	10	1.8487		
	7	10	2.0853	2.0853	
	10	10	2.1923	2.1923	
	9	10		2.7940	2.7940
	8	10			3.2097
	Sig.		.689	.071	.518

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

22. Growth of *A. litoralis* at different salinity

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21.967	5	4.393	2.741	.028
Within Groups	86.564	54	1.603		
Total	108.530	59			

Homogeneous Subsets

Chl *a*

	salinity	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	30	10	2.6870	
	5	10	2.7250	
	25	10	2.9470	2.9470
	10	10	3.4620	3.4620
	20	10	3.6550	3.6550
	15	10		4.4020
	Sig.		.532	.123

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

23. Growth of *C. minutus* at different salinity

ANOVA

Chl a

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.508	5	2.502	2.778	.026
Within Groups	48.624	54	.900		
Total	61.132	59			

Homogeneous Subsets

Chl *a*

	salinity	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	5	10	2.2997	
	10	10	2.4917	2.4917
	15	10	2.5960	2.5960
	30	10	2.7623	2.7623
	25	10	3.2673	3.2673
	20	10		3.6077
	Sig.		.220	.108

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

24. Growth of *G. gelatinosa* at different salinity

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.689	5	5.338	3.855	.005
Within Groups	74.768	54	1.385		
Total	101.456	59			

Homogeneous Subsets

Chl *a*

	salinity	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	5	10	2.4563	
	10	10	3.1003	
	15	10	3.4253	3.4253
	30	10	3.4357	3.4357
	25	10	3.7050	3.7050
	30	10		4.6727
	Sig.		.184	.185

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

25. Growth of *O. limosa* at different salinity

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15.644	5	3.129	6.570	.000
Within Groups	25.716	54	.476		
Total	41.360	59			

Homogeneous Subsets

Chl *a*

	salinity	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	5	10	1.1720		
	10	10	1.6003	1.6003	
	15	10	2.0363	2.0363	2.0363
	30	10		2.1410	2.1410
	20	10		2.2577	2.2577
	25	10			2.7947
	Sig.		.073	.288	.155

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

26. Growth of *S. aquatilis* at different salinity

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.730	5	1.746	4.911	.001
Within Groups	19.198	54	.356		
Total	27.928	59			

Homogeneous Subsets

Chl *a*

	salinity	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	30	10	1.7277		
	25	10	2.0220	2.0220	
	20	10	2.3177	2.3177	2.3177
	15	10	2.4097	2.4097	2.4097
	5	10		2.5717	2.5717
	10	10			2.9220
	Sig.		.126	.323	.226

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

27. Growth of *S. salina* at different salinity

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.005	5	.001	3.573	.007
Within Groups	.015	54	.000		
Total	.020	59			

Homogeneous Subsets

Chl *a*

	salinity	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	10	10	.0268	
	20	10	.0291	
	15	10	.0348	.0348
	5	10	.0356	.0356
	30	10	.0474	.0474
	25	10		.0520
	Sig.		.085	.213

28. Growth of *S. cedrorum* at different salinity

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.043	5	2.609	9.574	.000
Within Groups	14.714	54	.272		
Total	27.757	59			

Homogeneous Subsets

Chl *a*

			Subset for alpha = 0.05		
	salinity	N	1	2	3
Tukey HSD[a]	30	10	1.6837		
	25	10	1.9620	1.9620	
	5	10	2.0843	2.0843	
	20	10	2.2243	2.2243	
	15	10		2.4343	
	10	10			3.1660
	Sig.		.206	.343	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

29. Growth of *A. litoralis* in different media

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	20.113	3	6.704	4.103	.013
Within Groups	58.827	36	1.634		
Total	78.940	39			

Homogeneous Subsets

Chl *a*

	medium	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	SWEM	10	3.0653	
	A&N	10	3.2190	
	SN	10	3.7513	3.7513
	BG11	10		4.8737
	Sig.		.631	.221

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

30. Growth of *C. minutus* in different media

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.717	3	1.572	.852	.475
Within Groups	66.409	36	1.845		
Total	71.126	39			

Homogeneous Subsets

Chl *a*

	medium	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	SWEM	10	3.3747
	A&N	10	3.3783
	BG11	10	3.5787
	SN	10	4.2137
	Sig.		.519

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

31. Growth of *G. gelatinosa* in different media

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.953	3	3.651	1.585	.210
Within Groups	82.916	36	2.303		
Total	93.869	39			

Homogeneous Subsets

Chl *a*

	medium	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	SWEM	10	3.6073
	A&N	10	3.9817
	BG11	10	4.4493
	SN	10	5.0057
	Sig.		.186

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

32. Growth of *O. limosa* in different media

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.025	3	2.008	2.370	.087
Within Groups	30.503	36	.847		
Total	36.528	39			

Homogeneous Subsets

Chl *a*

	medium	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	SWEM	10	1.8907
	A&N	10	2.0340
	BG11	10	2.4333
	SN	10	2.8890
	Sig.		.090

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

33. Growth of *S. aquatilis* in different media

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.479	3	2.160	2.814	.053
Within Groups	27.634	36	.768		
Total	34.113	39			

Homogeneous Subsets

Chl *a*

	medium	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	A&N	10	2.4457
	SWEM	10	2.5420
	BG11	10	3.1103
	SN	10	3.4203
	Sig.		.079

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 10.000.

34. Growth of *S. salina* in different media

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.333	3	2.778	3.835	.018
Within Groups	26.075	36	.724		
Total	34.408	39			

Homogeneous Subsets

Chl *a*

	medium	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	SWEM	10	2.4140	
	A&N	10	2.5537	2.5537
	BG11	10	3.2083	3.2083
	SN	10		3.5200
	Sig.		.177	.071

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

35. Growth of *S. cedrorum* in different media

ANOVA

Chl *a*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.959	3	1.653	2.788	.054
Within Groups	21.342	36	.593		
Total	26.300	39			

Homogeneous Subsets

Chl *a*

	med	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	A&N	10	2.3757
	SWEM	10	2.4823
	BG11	10	3.0360
	SN	10	3.2023
	Sig.		.095

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

36. Protein content of the cyanobacterial strains

Tests of Between-Subjects Effects

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4557.403[a]	20	227.870	140.743	.000
Intercept	72977.677	1	72977.677	45074.447	.000
strain	4216.490	6	702.748	434.050	.000
phase	315.297	2	157.649	97.371	.000
strain * phase	25.616	12	2.135	1.318	.245
Error	68.000	42	1.619		
Total	77603.080	63			
Corrected Total	4625.403	62			

a. R Squared = .985 (Adjusted R Squared = .978)

Homogeneous Subsets

protein

strain=*A. litoralis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	decline	3	27.0667	
	stationary	3	30.4667	
	logarithmic	3		35.5000
	Sig.		.151	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 3.612.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	decline	3	19.0333		
	stationary	3		21.2000	
	logarithmic	3			23.1333
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .376.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	decline	3	23.4667	
	stationary	3	27.0333	27.0333
	logarithmic	3		29.2667
	Sig.		.083	.292

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 2.700.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	decline	3	38.5667	
	stationary	3		42.6333
	logarithmic	3		43.9000
	Sig.		1.000	.259

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .772.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	phase	N	Subset
			1
Tukey HSD[a,b]	decline	3	29.6333
	stationary	3	31.8333
	logarithmic	3	33.7000
	Sig.		.065

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 3.042.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	decline	3	42.8333		
	stationary	3		45.6000	
	logarithmic	3			47.6000
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .251.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	decline	3	37.6333		
	stationary	3		41.2667	
	logarithmic	3			43.3667
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .580.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

37. Carbohydrate content of the cyanobacterial strains

Tests of Between-Subjects Effects

Dependent Variable: carbohydrate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1588.711[a]	20	79.436	64.192	.000
Intercept	50512.686	1	50512.686	40819.641	.000
strain	799.242	6	133.207	107.645	.000
phase	704.381	2	352.191	284.608	.000
strain * phase	85.088	12	7.091	5.730	.000
Error	51.973	42	1.237		
Total	52153.370	63			
Corrected Total	1640.684	62			

a. R Squared = .968 (Adjusted R Squared = .953)

Homogeneous Subsets

carbohydrate

strain=*A. litoralis*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	22.3333		
	decline	3		24.7667	
	stationary	3			28.9000
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .339.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	phase	N	Subset
			1
Tukey HSD[a,b]	logarithmic	3	22.1333
	decline	3	23.4000
	stationary	3	26.6333
	Sig.		.116

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 5.279.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	19.8000		
	decline	3		24.4000	
	stationary	3			28.0667
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .841.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	24.7667		
	decline	3		29.4000	
	stationary	3			32.1000
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .661.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	28.1333		
	decline	3		31.8333	
	stationary	3			35.1333
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .743.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	26.7333		
	decline	3		35.1333	
	stationary	3			38.2333
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .613.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	24.4667		
	decline	3		31.8000	
	stationary	3			36.4667
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .186.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

38. Lipid content of the cyanobacterial strains

Tests of Between-Subjects Effects

Dependent Variable: lipid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	82.607[a]	20	4.130	45.731	.000
Intercept	2318.680	1	2318.680	25672.555	.000
strain	39.920	6	6.653	73.666	.000
phase	41.212	2	20.606	228.153	.000
strain * phase	1.474	12	.123	1.360	.223
Error	3.793	42	.090		
Total	2405.080	63			
Corrected Total	86.400	62			

a. R Squared = .956 (Adjusted R Squared = .935)

Homogeneous Subsets

lipid

strain=*A. litoralis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	4.2000	
	stationary	3	4.9000	4.9000
	decline	3		5.5333
	Sig.		.121	.161

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .131.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	5.6667		
	stationary	3		6.7667	
	decline	3			8.0000
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .099.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	6.1667		
	stationary	3		7.2667	
	decline	3			8.4333
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .097.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	4.5000		
	stationary	3		5.3000	
	decline	3			6.6667
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .048.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	4.9000	
	stationary	3	5.5667	
	decline	3		6.7000
	Sig.		.089	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .098.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	5.8333		
	stationary	3		6.6000	
	decline	3			7.5000
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .084.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	4.5667		
	stationary	3		5.5000	
	decline	3			6.8333
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .076.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

39. Chlorophyll *a* content of the cyanobacterial strains

Tests of Between-Subjects Effects

Dependent Variable: chla

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	67.890[a]	20	3.394	162.182	.000
Intercept	2508.667	1	2508.667	119858.943	.000
strain	62.851	6	10.475	500.484	.000
phase	4.351	2	2.175	103.934	.000
strain * phase	.688	12	.057	2.738	.008
Error	.879	42	.021		
Total	2577.436	63			
Corrected Total	68.769	62			

a. R Squared = .987 (Adjusted R Squared = .981)

Homogeneous Subsets

Chl *a*

strain=*A. litoralis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	5.8700	
	decline	3		6.1367
	stationary	3		6.2000
	Sig.		1.000	.488

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .004.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	phase	N	Subset
			1
Tukey HSD[a,b]	logarithmic	3	6.9400
	decline	3	7.3233
	stationary	3	7.4400
	Sig.		.111

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .063.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	7.5167	
	decline	3		8.0400
	stationary	3		8.1433
	Sig.		1.000	.347

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .007.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	4.1533	
	decline	3		4.9100
	stationary	3		5.0067
	Sig.		1.000	.787

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .031.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	5.7767	
	decline	3	5.9267	5.9267
	stationary	3		6.0567
	Sig.		.127	.189

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .006.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	6.2667	
	decline	3		7.0000
	stationary	3		7.0867
	Sig.		1.000	.697

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .016.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	5.0867	
	decline	3		5.7267
	stationary	3		5.9100
	Sig.		1.000	.305

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .019.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

40. Carotenoid content of the cyanobacterial strains

Tests of Between-Subjects Effects

Dependent Variable: caro

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	33.669[a]	20	1.683	184.896	.000
Intercept	525.316	1	525.316	57696.803	.000
strain	29.942	6	4.990	548.099	.000
phase	3.460	2	1.730	190.005	.000
strain * phase	.267	12	.022	2.444	.016
Error	.382	42	.009		
Total	559.367	63			
Corrected Total	34.051	62			

a. R Squared = .989 (Adjusted R Squared = .983)

Homogeneous Subsets

caro

strain= *A. litoralis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	2.0200	
	decline	3		2.5900
	stationary	3		2.6900
	Sig.		1.000	.670

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .019.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain= *C. minutus*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	3.2067	
	decline	3		3.8400
	stationary	3		3.9267
	Sig.		1.000	.489

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .008.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain= *G. gelatinosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	3.3500	
	decline	3		3.8767
	stationary	3		4.0000
	Sig.		1.000	.435

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .013.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.7967	
	decline	3		2.1200
	stationary	3		2.2100
	Sig.		1.000	.235

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .004.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	2.0367	
	decline	3		2.3200
	stationary	3		2.4033
	Sig.		1.000	.526

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .008.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	3.2500	
	decline	3		3.7400
	stationary	3		3.8233
	Sig.		1.000	.469

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .007.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	2.2633	
	decline	3		2.5500
	stationary	3		2.6267
	Sig.		1.000	.461

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .005.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

41. Phycocyanin content of the cyanobacterial strains

Tests of Between-Subjects Effects

Dependent Variable: phycocyanin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.268[a]	20	.213	38.454	.000
Intercept	148.980	1	148.980	26847.066	.000
strain	1.668	6	.278	50.083	.000
phase	2.230	2	1.115	200.957	.000
strain * phase	.370	12	.031	5.557	.000
Error	.233	42	.006		
Total	153.481	63			
Corrected Total	4.501	62			

a. R Squared = .948 (Adjusted R Squared = .924)

Homogeneous Subsets

phycocyanin

strain=*A. litoralis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.1767	
	decline	3		1.3133
	stationary	3		1.4267
	Sig.		1.000	.072

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .002.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.3800	
	decline	3		1.8967
	stationary	3		2.0200
	Sig.		1.000	.249

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .007.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.2233	
	decline	3		1.4000
	stationary	3		1.5233
	Sig.		1.000	.057

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .003.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.3100	
	decline	3		1.6433
	stationary	3		1.7567
	Sig.		1.000	.169

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .004.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	1.2367		
	decline	3		1.4967	
	stationary	3			1.6733
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .002.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.3267	
	decline	3		1.9733
	stationary	3		1.9767
	Sig.		1.000	.999

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .016.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.3067	
	decline	3		1.5433
	stationary	3		1.6900
	Sig.		1.000	.082

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .005.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

42. Phycoerythrin content of the cyanobacterial strains

Tests of Between-Subjects Effects

Dependent Variable: phycoeryhtrin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.114[a]	20	.106	61.044	.000
Intercept	107.565	1	107.565	62113.496	.000
strain	1.418	6	.236	136.497	.000
phase	.626	2	.313	180.883	.000
strain * phase	.070	12	.006	3.345	.002
Error	.073	42	.002		
Total	109.752	63			
Corrected Total	2.187	62			

a. R Squared = .967 (Adjusted R Squared = .951)

Homogeneous Subsets

phycoeryhtrin

strain=*A. litoralis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.1100	
	decline	3	1.1767	1.1767
	stationary	3		1.2367
	Sig.		.211	.268

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .002.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	1.3167		
	decline	3		1.4767	
	stationary	3			1.5867
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .002.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	1.1933		
	decline	3		1.2900	
	stationary	3			1.3800
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .001.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	.8867	
	decline	3		1.0800
	stationary	3		1.1667
	Sig.		1.000	.167

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .003.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.2000	
	decline	3	1.3000	1.3000
	stationary	3		1.3833
	Sig.		.098	.168

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .002.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	1.3033	
	decline	3		1.5767
	stationary	3		1.6633
	Sig.		1.000	.084

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .002.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	1.2167		
	decline	3		1.3967	
	stationary	3			1.5000
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .001.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

43. Allophycocyanin content of the cyanobacterial strains

Tests of Between-Subjects Effects

Dependent Variable: allophycocyanin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.006[a]	20	.150	39.498	.000
Intercept	38.360	1	38.360	10082.209	.000
strain	1.465	6	.244	64.162	.000
phase	1.436	2	.718	188.763	.000
strain * phase	.104	12	.009	2.288	.024
Error	.160	42	.004		
Total	41.526	63			
Corrected Total	3.165	62			

a. R Squared = .950 (Adjusted R Squared = .925)

Homogeneous Subsets

allophycocyanin

strain=*A. litoralis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	.4133	
	decline	3		.6833
	stationary	3		.8133
	Sig.		1.000	.072

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .003.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	.7267	
	decline	3	.8667	.8667
	stationary	3		1.0100
	Sig.		.066	.060

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .004.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	.6833	
	decline	3	.8733	
	stationary	3		1.0867
	Sig.		.071	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .007.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	.3367	
	decline	3		.6933
	stationary	3		.8333
	Sig.		1.000	.102

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .005.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	.7433	
	decline	3		.9967
	stationary	3		1.1267
	Sig.		1.000	.091

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .004.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	phase	N	Subset	
			1	2
Tukey HSD[a,b]	logarithmic	3	.8333	
	decline	3	.9133	
	stationary	3		1.0500
	Sig.		.171	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .002.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	phase	N	Subset		
			1	2	3
Tukey HSD[a,b]	logarithmic	3	.3567		
	decline	3		.6000	
	stationary	3			.7467
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .002.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

Antioxidant activity

44. Total phenolic content

Tests of Between-Subjects Effects

Dependent Variable: gae

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11058.733[a]	34	325.257	4093.488	.000
Intercept	11239.584	1	11239.584	141454.671	.000
strain	8244.139	6	1374.023	17292.632	.000
solvent	1424.448	4	356.112	4481.813	.000
strain * solvent	1390.146	24	57.923	728.981	.000
Error	5.562	70	.079		
Total	22303.879	105			
Corrected Total	11064.295	104			

a. R Squared = .999 (Adjusted R Squared = .999)

Homogeneous Subsets

GAE

strain=*A. litoralis*

	solvent	N	Subset				
			1	2	3	4	5
Tukey HSD[a,b]	Chlo:Meth	3	1.4333				
	Acetone	3		3.5200			
	Ethanol	3			5.9800		
	Methanol	3				7.0933	
	DMSO	3					10.3467
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .034.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:Meth	3	2.2967		
	Acetone	3		5.1733	
	Ethanol	3		5.3700	
	DMSO	3			12.7267
	Methanol	3			12.9000
	Sig.		1.000	.153	.236

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .009.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:Meth	3	3.3833			
	Ethanol	3		5.9167		
	DMSO	3			9.3400	
	Acetone	3			9.8267	
	Methanol	3				11.2167
	Sig.		1.000	1.000	.495	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .129.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:Meth	3	1.2733			
	Methanol	3		4.1733		
	DMSO	3		4.4567		
	Ethanol	3			6.3000	
	Acetone	3				9.1000
	Sig.		1.000	.058	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .012.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:Meth	3	3.6100		
	Acetone	3		10.4967	
	Ethanol	3			12.2333
	Methanol	3			12.5167
	DMSO	3			12.5300
	Sig.		1.000	1.000	.234

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .026.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	solvent	N	Subset				
			1	2	3	4	5
Tukey HSD[a,b]	Chlo:Meth	3	10.5067				
	Acetone	3		30.8133			
	DMSO	3			34.3633		
	Ethanol	3				37.7333	
	Methanol	3					44.3333
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .316.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:Meth	3	1.5000		
	Ethanol	3		3.9833	
	Acetone	3		4.0900	
	DMSO	3			5.5533
	Methanol	3			6.0267
	Sig.		1.000	.942	.051

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .031.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

45. Total flavonoid content

Tests of Between-Subjects Effects

Dependent Variable: qe

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1523.164[a]	34	44.799	247.915	.000
Intercept	1652.505	1	1652.505	9144.870	.000
strain	948.110	6	158.018	874.464	.000
solvent	263.781	4	65.945	364.937	.000
strain * solvent	311.273	24	12.970	71.774	.000
Error	12.649	70	.181		
Total	3188.319	105			
Corrected Total	1535.814	104			

a. R Squared = .992 (Adjusted R Squared = .988)

Homogeneous Subsets

qe

strain=*A. litoralis*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:Meth	3	1.7200		
	Acetone	3	2.3133	2.3133	
	Ethanol	3	2.7300	2.7300	2.7300
	Methanol	3		2.8967	2.8967
	DMSO	3			3.7667
	Sig.		.078	.456	.069

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .169.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:Meth	3	1.5367		
	Ethanol	3		2.8300	
	Acetone	3		2.8867	
	Methanol	3			7.0433
	DMSO	3			7.2500
	Sig.		1.000	.907	.066

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .007.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:Meth	3	1.5383			
	Ethanol	3		2.9400		
	Acetone	3		3.8933		
	DMSO	3			5.3467	
	Methanol	3				7.4033
	Sig.		1.000	.222	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .258.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	solvent	N	Subset				
			1	2	3	4	5
Tukey HSD[a,b]	Chlo:Meth	3	.5287				
	Methanol	3		.8487			
	DMSO	3			1.0080		
	Acetone	3				1.4767	
	Ethanol	3					1.9100
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .001.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:Meth	3	.8617			
	Acetone	3		2.6700		
	Ethanol	3			3.2800	
	Methanol	3				3.6767
	DMSO	3				3.6867
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .016.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:Meth	3	2.9033			
	Acetone	3		8.3333		
	DMSO	3			11.2317	
	Ethanol	3			13.4067	
	Methanol	3				18.3000
	Sig.		1.000	1.000	.083	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .809.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:Meth	3	.9660			
	Ethanol	3	1.1000			
	Acetone	3		1.4100		
	DMSO	3			2.3400	
	Methanol	3				2.8167
	Sig.		.136	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .004.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

46 Total antioxidant capacity

Tests of Between-Subjects Effects

Dependent Variable: ae

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	31921.577[a]	10	3192.158	20.053	.000
Intercept	69557.202	1	69557.202	436.954	.000
strain	10834.856	6	1805.809	11.344	.000
solvent	21086.721	4	5271.680	33.116	.000
Error	14963.531	94	159.187		
Total	116442.310	105			
Corrected Total	46885.108	104			

a. R Squared = .681 (Adjusted R Squared = .647)

Homogeneous Subsets

ae

strain=*A. litoralis*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Acetone	3	3.8000			
	Chlo:Meth	3	4.0000			
	Ethanol	3		19.8667		
	DMSO	3			44.2333	
	Methanol	3				49.6333
	Sig.		.999	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .830.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:Meth	3	5.8333		
	Acetone	3	7.3333		
	Methanol	3		22.8000	
	Ethanol	3		24.9667	
	DMSO	3			46.8000
	Sig.		.472	.175	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 1.160.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:Meth	3	5.8333			
	Acetone	3		10.7000		
	Ethanol	3			19.5667	
	Methanol	3				25.8667
	DMSO	3				26.9333
	Sig.		1.000	1.000	1.000	.289

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .385.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	solvent	N	Subset				
			1	2	3	4	5
Tukey HSD[a,b]	DMSO	3	4.9667				
	Chlo:Meth	3		7.6667			
	Methanol	3			9.2333		
	Ethanol	3				11.8667	
	Acetone	3					21.5333
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .259.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	solvent	N	Subset				
			1	2	3	4	5
Tukey HSD[a,b]	Chlo:Meth	3	5.1667				
	Acetone	3		8.2333			
	Ethanol	3			13.9333		
	Methanol	3				40.5000	
	DMSO	3					60.9000
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .930.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:Meth	3	4.2000			
	Acetone	3	5.5000			
	Ethanol	3		18.7000		
	Methanol	3			69.8000	
	DMSO	3				83.6333
	Sig.		.547	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 1.033.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:Meth	3	33.3333		
	Ethanol	3	35.6333	35.6333	
	Methanol	3		42.4667	
	Acetone	3		43.7333	
	DMSO	3			61.6667
	Sig.		.886	.057	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 9.551.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

47. Deoxyribose radical scavenging activity

Tests of Between-Subjects Effects

Dependent Variable: scavengingactivity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3272.467[a]	34	96.249	415.573	.000
Intercept	13826.514	1	13826.514	59698.503	.000
strain	1379.006	6	229.834	992.352	.000
solvent	1573.203	4	393.301	1698.148	.000
strain * solvent	320.258	24	13.344	57.616	.000
Error	16.212	70	.232		
Total	17115.194	105			
Corrected Total	3288.679	104			

a. R Squared = .995 (Adjusted R Squared = .993)

Homogeneous Subsets

scavengingactivity

strain=*A. litoralis*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	5.7233			
	Ethanol	3		8.7333		
	Methanol	3			9.5533	
	DMSO	3				10.9167
	Acetone	3				10.9800
	Sig.		1.000	1.000	1.000	.999

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .085.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:meth	3	3.8200		
	Ethanol	3		12.7167	
	Methanol	3		12.8500	
	Acetone	3		13.0267	
	DMSO	3			15.2500
	Sig.		1.000	.914	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .207.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	3.6133			
	Ethanol	3		11.5000		
	Methanol	3		12.1467	12.1467	
	Acetone	3			12.7900	12.7900
	DMSO	3				13.2900
	Sig.		1.000	.285	.289	.509

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .140.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	1.7900			
	Ethanol	3		5.7700		
	Methanol	3		6.1867	6.1867	
	Acetone	3			6.5400	
	DMSO	3				7.9600
	Sig.		1.000	.268	.408	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .056.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	solvent	N	Subset	
			1	2
Tukey HSD[a,b]	Chlo:meth	3	3.7400	
	Methanol	3		12.0133
	Acetone	3		12.3167
	Ethanol	3		12.5300
	DMSO	3		13.2233
	Sig.		1.000	.120

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .296.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	4.5700			
	Acetone	3		19.8667		
	Methanol	3			21.0033	
	Ethanol	3			21.4633	
	DMSO	3				22.8500
	Sig.		1.000	1.000	.610	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .150.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	3.9967			
	Ethanol	3		14.4600		
	Acetone	3		16.3400	16.3400	
	Methanol	3			17.9067	
	DMSO	3				20.1967
	Sig.		1.000	.110	.217	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .687.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

48. Ferric reducing antioxidant power

Tests of Between-Subjects Effects

Dependent Variable: ae

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	21077.189[a]	34	619.917	2763.071	.000
Intercept	67611.814	1	67611.814	301356.697	.000
strain	9068.701	6	1511.450	6736.776	.000
solvent	8603.529	4	2150.882	9586.827	.000
strain * solvent	3404.959	24	141.873	632.352	.000
Error	15.705	70	.224		
Total	88704.708	105			
Corrected Total	21092.894	104			

a. R Squared = .999 (Adjusted R Squared = .999)

Homogeneous Subsets

ae

strain=*A. litoralis*

	solvent	N	Subset				
			1	2	3	4	5
Tukey HSD[a,b]	chlo:meth	3	5.8700				
	acetone	3		8.4633			
	dmso	3			19.0267		
	ethanol	3				22.5367	
	methanol	3					24.0533
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .180.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	chlo:meth	3	8.4867			
	acetone	3	8.9967			
	ethanol	3		13.7933		
	methanol	3			17.0500	
	dmso	3				23.1033
	Sig.		.752	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .271.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	chlo:meth	3	6.7967			
	acetone	3		22.4633		
	ethanol	3			32.5400	
	methanol	3				36.5367
	dmso	3				37.2300
	Sig.		1.000	1.000	1.000	.551

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .296.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	chlo:meth	3	7.9000			
	dmso	3		15.8967		
	methanol	3		16.2300		
	ethanol	3			17.6933	
	acetone	3				24.7433
	Sig.		1.000	.907	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .228.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S.aquatilis*

	solvent	N	Subset				
			1	2	3	4	5
Tukey HSD[a,b]	chlo:meth	3	12.8200				
	acetone	3		23.1533			
	dmso	3			32.2567		
	ethanol	3				40.2800	
	methanol	3					43.1800
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .246.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	chlo:meth	3	20.7967			
	acetone	3		23.2300		
	ethanol	3			49.8433	
	methanol	3				53.9233
	dmso	3				55.1800
	Sig.		1.000	1.000	1.000	.050

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .219.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	solvent	N	Subset				
			1	2	3	4	5
Tukey HSD[a,b]	chlo:meth	3	6.1533				
	acetone	3		32.3067			
	ethanol	3			38.7200		
	dmso	3				40.8700	
	methanol	3					46.0233
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .131.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

49. DPPH radical scavenging activity

Tests of Between-Subjects Effects

Dependent Variable: percentageinhibiton

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26739.957[a]	34	786.469	277.181	.000
Intercept	123294.893	1	123294.893	43453.710	.000
strain	7236.725	6	1206.121	425.082	.000
solvent	16208.428	4	4052.107	1428.113	.000
strain * solvent	3294.805	24	137.284	48.384	.000
Error	198.617	70	2.837		
Total	150233.468	105			
Corrected Total	26938.574	104			

a. R Squared = .993 (Adjusted R Squared = .989)

Homogeneous Subsets

percentageinhibiton

strain=*A. litoralis*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:meth	3	5.3500		
	Ethanol	3		38.5600	
	Acetone	3		41.3633	
	Methanol	3		41.9433	
	DMSO	3			52.3933
	Sig.		1.000	.103	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 2.155.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	11.1167			
	Acetone	3		35.8000		
	Ethanol	3		37.9533		
	DMSO	3			41.5767	
	Methanol	3				49.1500
	Sig.		1.000	.099	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .857.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:meth	3	9.2433		
	DMSO	3		26.1967	
	Ethanol	3		29.1933	
	Methanol	3			35.7533
	Acetone	3			37.2200
	Sig.		1.000	.415	.895

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 4.080.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:meth	3	10.1333		
	Ethanol	3		19.0567	
	Acetone	3		19.7600	
	DMSO	3			24.8567
	Methanol	3			25.9400
	Sig.		1.000	.959	.838

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 1.672.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:meth	3	8.9867		
	Ethanol	3		34.6067	
	Methanol	3		36.7100	
	Acetone	3		36.9033	
	DMSO	3			46.7833
	Sig.		1.000	.685	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 4.532.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	16.4400			
	Acetone	3		40.1500		
	Ethanol	3		43.5967	43.5967	
	DMSO	3			47.4200	
	Methanol	3				55.8333
	Sig.		1.000	.348	.262	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 4.625.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	9.6233			
	Ethanol	3		45.8233		
	DMSO	3			54.8767	
	Acetone	3				63.6067
	Methanol	3				65.4300
	Sig.		1.000	1.000	1.000	.527

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 1.940.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

50. Hydrogen peroxide radical scavenging assay

Tests of Between-Subjects Effects

Dependent Variable: scavengingactivity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	402.916[a]	34	11.850	255.356	.000
Intercept	1877.147	1	1877.147	40449.122	.000
strain	114.538	6	19.090	411.347	.000
solvent	251.222	4	62.806	1353.345	.000
strain * solvent	37.156	24	1.548	33.360	.000
Error	3.249	70	.046		
Total	2283.312	105			
Corrected Total	406.165	104			

a. R Squared = .992 (Adjusted R Squared = .988)

Homogeneous Subsets

scavengingactivity

strain=*A. litoralis*

	solvent	N	Subset		
			1	2	3
Tukey HSD[a,b]	Chlo:meth	3	2.5567		
	Acetone	3		5.3900	
	Ethanol	3		5.5367	5.5367
	DMSO	3		6.0733	6.0733
	Methanol	3			6.5300
	Sig.		1.000	.280	.068

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .154.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*C. minutus*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	.9300			
	Ethanol	3		4.2500		
	Methanol	3		4.4167	4.4167	
	Acetone	3			4.5567	
	DMSO	3				5.0767
	Sig.		1.000	.258	.404	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .009.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*G. gelatinosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	1.6967			
	Acetone	3		2.8033		
	Methanol	3			3.5133	
	Ethanol	3			3.7667	
	DMSO	3				4.8867
	Sig.		1.000	1.000	.419	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .029.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*O. limosa*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	.5200			
	Acetone	3		2.3467		
	Ethanol	3		2.5233		
	Methanol	3			3.7967	
	DMSO	3				4.1333
	Sig.		1.000	.196	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .008.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. aquatilis*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	.8500			
	Ethanol	3		3.9933		
	Acetone	3		4.2033	4.2033	
	Methanol	3			4.5333	
	DMSO	3				5.1633
	Sig.		1.000	.427	.103	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .021.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. salina*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	1.6667			
	Ethanol	3		5.7233		
	Methanol	3			6.5900	
	Acetone	3			6.9133	
	DMSO	3				8.0633
	Sig.		1.000	1.000	.117	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .021.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

strain=*S. cedrorum*

	solvent	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	Chlo:meth	3	1.3333			
	Acetone	3		4.3367		
	Ethanol	3		4.6400		
	Methanol	3			6.5000	
	DMSO	3				8.1733
	Sig.		1.000	.702	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .083.
a. Uses Harmonic Mean Sample Size = 3.000.
b. Alpha = 0.05.

Correlations

51. Correlation between total phenolic content and total antioxidant activity

Correlations

		tpc	frap
tpc	Pearson Correlation	1	.727**
	Sig. (2-tailed)		.000
	N	35	35
frap	Pearson Correlation	.727**	1
	Sig. (2-tailed)	.000	
	N	35	35

**. Correlation is significant at the 0.01 level (2-tailed).

53. Correlation between total phenolic content and deoxyribose radical scavenging activity

Correlations

		tpc	drsa
tpc	Pearson Correlation	1	.738**
	Sig. (2-tailed)		.000
	N	35	35
drsa	Pearson Correlation	.738**	1
	Sig. (2-tailed)	.000	
	N	35	35

**. Correlation is significant at the 0.01 level (2-tailed).

54. Correlation between total phenolic content and DPPH radical scavenging activity

Correlations

		tpc	dpph
tpc	Pearson Correlation	1	.452**
	Sig. (2-tailed)		.006
	N	35	35
dpph	Pearson Correlation	.452**	1
	Sig. (2-tailed)	.006	
	N	35	35

**. Correlation is significant at the 0.01 level (2-tailed).

55. Correlation between total phenolic content and H_2O_2 radical scavenging activity

Correlations

		tpc	h2o2
tpc	Pearson Correlation	1	.342*
	Sig. (2-tailed)		.044
	N	35	35
h2o2	Pearson Correlation	.342*	1
	Sig. (2-tailed)	.044	
	N	35	35

*. Correlation is significant at the 0.05 level (2-tailed).

56. Correlation between total phenolic content and carotenoids

Correlations

		tpc	carotenoids
tpc	Pearson Correlation	1	.465
	Sig. (2-tailed)		.293
	N	7	7
carotenoids	Pearson Correlation	.465	1
	Sig. (2-tailed)	.293	
	N	7	7

DIETARY EFFECT

57. Weight gain

ANOVA

Weight gain

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	52.206	5	10.441	12.835	.000
Within Groups	39.047	48	.813		
Total	91.253	53			

Homogeneous Subsets

Weight gain

	treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	control	9	79.3456		
	Sb1	9	79.9367	79.9367	
	Sa0.5	9	80.4911	80.4911	
	Sa+Sb	9	80.5689	80.5689	
	Sb0.5	9		80.8356	
	Sa1	9			82.5289
	Sig.		.062	.298	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

58. FCR

ANOVA

FCR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.214	5	.043	1.468	.218
Within Groups	1.402	48	.029		
Total	1.616	53			

Homogeneous Subsets

FCR

	treatment	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Sa1	9	4.1478
	Sb1	9	4.2689
	Sb0.5	9	4.2900
	Sa+Sb	9	4.3078
	Sa0.5	9	4.3233
	Control	9	4.3389
	Sig.		.187

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

59. SGR

ANOVA

SGR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.439	5	.088	18.106	.000
Within Groups	.233	48	.005		
Total	.672	53			

Homogeneous Subsets

SGR

	treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Control	9	2.6211		
	Sb1	9	2.6756	2.6756	
	Sa0.5	9	2.6944	2.6944	
	Sa+Sb	9		2.7300	
	Sb0.5	9		2.7567	
	Sa1	9			2.9089
	Sig.		.242	.153	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

60. HB

ANOVA

HB

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.347	5	1.469	195.441	.000
Within Groups	.361	48	.008		
Total	7.708	53			

HB

	treatment	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	Sb 1	9	7.8356				
	Sa+Sb	9		8.4689			
	Sb0.5	9		8.5033			
	Sa0.5	9			8.6433		
	Control	9				8.7867	
	Sa1	9					9.0322
	Sig.		1.000	.958	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

61. RBC

ANOVA

RBC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	98671286.093	5	19734257.219	265.427	.000
Within Groups	3568752.889	48	74349.019		
Total	102240038.981	53			

Homogeneous Subsets

RBC

	treatment	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	Sb1	9	28099.5556				
	Sb0.5	9		29311.5556			
	Sa+Sb	9			29864.0000		
	Control	9				30442.5556	
	Sa0.5	9				30659.6667	
	Sa1	9					32522.5556
	Sig.		1.000	1.000	1.000	.545	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

62. WBC

ANOVA

WBC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11010857.870	5	2202171.574	264.929	.000
Within Groups	398991.333	48	8312.319		
Total	11409849.204	53			

Homogeneous Subsets

WBC

	treatment	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	Sb 1	9	9341.3333				
	Sb0.5	9		9748.2222			
	Sa+Sb	9			9931.4444		
	Control	9				10176.6667	
	Sa0.5	9				10220.6667	
	Sa1	9					10806.2222
	Sig.		1.000	1.000	1.000	.908	1.000

Means for groups in homogeneous subsets are displayed.

63. Total protein

ANOVA

Total protein

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.015	5	3.203	604.779	.000
Within Groups	.254	48	.005		
Total	16.270	53			

Homogeneous Subsets

Total protein

	treatment	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	Sb 1	9	2.7467				
	Sb0.5	9		3.2522			
	Sa+Sb	9		3.2556			
	Sa0.5	9			3.6256		
	Control	9				4.1033	
	Sa1	9					4.3544
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

64. Albumin

ANOVA

Albumin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.140	5	.028	6.496	.000
Within Groups	.207	48	.004		
Total	.348	53			

Homogeneous Subsets

Albumin

	treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Sb0.5	9	1.1644		
	Sa+Sb	9	1.1978		
	Control	9	1.2100	1.2100	
	Sa1	9	1.2556	1.2556	1.2556
	Sa0.5	9		1.2933	1.2933
	Sb1	9			1.3044
	Sig.		.054	.096	.617

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 9.000.

65. Globulin

ANOVA

Globulin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.458	5	3.292	477.180	.000
Within Groups	.331	48	.007		
Total	16.789	53			

Homogeneous Subsets

Globulin

	treatment	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	Sb1	9	1.4422				
	Sa+Sb	9		2.0578			
	Sb0.5	9		2.0878			
	Sa0.5	9			2.3322		
	Control	9				2.8933	
	Sa1	9					3.0989
	Sig.		1.000	.972	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 9.000.

66. AG ratio

ANOVA

AG Ratio

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.468	5	.294	136.637	.000
Within Groups	.103	48	.002		
Total	1.571	53			

Homogeneous Subsets

AG Ratio

	treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Sa1	9	.4056		
	Control	9	.4189		
	Sb0.5	9		.5533	
	Sa0.5	9		.5567	
	Sa+Sb	9		.5844	
	Sb1	9			.9056
	Sig.		.990	.713	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

67. Survival

ANOVA

Survival

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7565.784	5	1513.157	35.661	.000
Within Groups	509.185	12	42.432		
Total	8074.969	17			

Homogeneous Subsets

Survival

	treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey B[a]	Control	3	27.7767		
	Sb1	3		52.7767	
	Sb0.5	3		55.5567	
	Sa0.5	3			75.0000
	Sa+Sb	3			80.5533
	Sa1	3			88.8900

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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Articles Published

- Lekshmi S & Saramma AV. (2018) *In vitro* antibacterial and antioxidant activities of cyanobacteria isolated from Cochin estuary, India. Ecology, Environment and Conservation, 24(1) 559-567.
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- Lekshmi S & Saramma AV. Isolation and Characterization of cyanobacteria from Cochin estuary. National seminar on 'Marine Ecosystem Health' (MEH 2015), Department of Marine Biology, Microbiology and Biochemistry, CUSAT, Kochi. 12-13 March 2015.

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