Putative Probiotic *Cyanobacterium* spp. from the Coastal Waters of Cochin: Polyphasic Taxonomy, Fate of Axenic Cultures and Heterotrophic Bacterial Associates

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Ph.D. Thesis under the Faculty of Environmental Studies

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Guru Brahma, Guru Vishnu,

Guru Devo Maheshwara,

Guru Sakshal Param Brahma,

Tasmai Sri Gurave Namah

I dedicate this thesis to my Guiding Lights

Professor A. Mohandas

&

Professor I. S. Bright Singh

Certificate

*This is to certify that research work presented in the thesis entitled "**Putative Probiotic Cyanobacterium spp. from the Coastal Waters of Cochin: Polyphasic Taxonomy, Fate of Axenic Cultures and Heterotrophic Bacterial Associates**" is based on the original work done by Mrs. Deepa G.D. under our guidance at National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associate-ship, fellow-ship or any other similar title or recognition. We further certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in the thesis.*

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Declaration

*I hereby do declare that the work presented in the thesis entitled "**Putative Probiotic Cyanobacterium spp. from the Coastal Waters of Cochin: Polyphasic Taxonomy, Fate of Axenic Cultures and Heterotrophic Bacterial Associates**" is based on the original work done by me under the guidance of Dr. A. Mohandas (Supervising Guide), Emeritus Professor, National Centre for Aquatic animal Health, School of Environmental Studies, Cochin University of Science and Technology, Cochin-682016 and Dr. I. S. Bright Singh (Co-guide),UGC BSR Faculty, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.*

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Chapter 1

General Introduction

Contents

In an ecosystem, every group of organisms interacts with non-living components and carries out their specific role in nutrient cycling and energy flow. Cyanobacteria are organisms with prokaryotic cellular organizations showing similarity as well as distinction from Gram-negative group of bacteria and fulfil their ecosystem functions in a well discernible manner (Hoiczyk & Hanzel, 2000). They are unique among the prokaryotic group by having the property of oxygenic photosynthesis. It is believed that cyanobacteria were the first major group of phototrophs to develop a two-stage photosynthetic pathway capable of oxidizing water to produce molecular oxygen (Giovannoni *et al*., 1998). Cyanobacteria play an important role on earth as primary producers (Robertson *et al*., 2001). There are cyanobacteria having the capacity for atmospheric nitrogen fixation (Howarth *et al*., 1998). By possessing all these properties, the cyanobacteria are one among the most successful and widespread group of prokaryotic organisms found in diverse

aquatic and terrestrial environments in most parts of the earth (Robertson *et al.*, 2001; Palinska *et al.*, 2006).

1.1Origin of Cyanobacteria

There is a widespread consensus that life occurred on earth 3500 Ma (million years ago). Geologists and geochemists agree that cyanobacteria have a long evolutionary history, starting at least 3500Ma. Blank (2004) stated that cyanobacteria originated at least 1500Ma and possibly 2700 Ma. Cavalier-Smith (2006) approximated this figure to 2900 Ma. Though arguments still go on regarding the period of origin, by 2450-2320 Ma, cyanobacteria became the dominant primary producers on earth's oceans and remained so till 700-600 Ma. Geochemical and fossil evidence indicate that cyanobacteria caused the transition of earth's atmosphere from its primordial, anaerobic state to its aerobic present state (Giovannoni *et al.*, 1998). Considering age of the earth as 4500 Million years (Dalrymple, 1994), cyanobacteria were present there through most of the earth's history with utmost influence on its environment and life. Sholpf and Walter (1982) have termed the Proterozoic era (2500-570 Ma) as the Age of cyanobacteria as they are the most abundant in fossil record during this period. Some of the reasons for the success of cyanobacteria in modern habitats can be related to their long evolutionary history. Theirs were not mere existence, but, altered life history of the earth by changing the reducing environment that existed on earth to an oxidizing one.

1.2 Cell Morphology and Ultrastructure of Cyanobacteria

Cyanobacteria range from unicellular to filamentous and colonial forms. Yet all the cells share basic cellular features of bacteria (Castenholz *et al.*, 2001). Certain unique and diagnostic characters that cyanobacteria possess are briefly described below.

1.2.1 Cell Envelope

The cell wall of cyanobacteria is of Gram-negative type, but the structural peptidoglycan layer is often considerably thicker than in Gram-negative *Proteobacteria* (Hoiczyk & Hansel, 2000). Numerous unicellular, colonial, and filamentous cyanobacteria possess an envelope outside of the outer membrane. This is variously called the sheath, glycocalyx, or capsule, or depending on the consistency, referred as gel, mucilage, or slime. The sheaths of cyanobacteria are predominantly composed of polysaccharides, but in some strains, >20% of the weight may consist of polypeptides. In the firm sheaths of many colonial and filamentous cyanobacteria, yellow, red or blue pigments may accumulate and mask the colour of cells. A typical yellow-brown pigment was characterized as scytonemin which has UV-absorbance and protective properties.

Cyanobacteria also synthesize and secrete extracellular polymeric substances (EPS) (Neu & Marshall, 1990), mainly of polysaccharidic in nature, which can remain covalently linked or loosely attached to the cell surface, or be released into the surrounding environment (de Philippis &Vincenzini, 2003).

1.2.2 Cell Exterior and Motility

Fimbriae (or pili) occur abundantly with diverse patterns in many cyanobacteria. Though prokaryotic flagella have never been reported in cyanobacteria, swimming motility by small unicellular forms has been described. Gliding type of motility has been known to exist in large filamentous forms.

1.2.3 Cell Interior

Thylakoids of cyanobacteria are attached to cytoplasmic membrane and arranged either concentrically or radially. On both surfaces of thylakoids are upright hemidiscoidal or hemispherical phycobilisomes, complex protein-pigment aggregates which harvest light (Bryant *et al.*, 1979). The pigments contained in phycobilisome are allophycocyanin (APC) in the core, which touches the surface of thylakoid membrane, usually directly in contact with light-harvesting Chlorophyll a of PSII, and phycocyanin (PC) in the rods. Phycoerthrocyanin (PEC) or phycoerythrin (PE) if present occurs as outer discs of the rods.

1.2.4 Cell Inclusions

In the cytoplasm of cyanobacteria, there are other inclusions, readily visible under TEM (Allen, 1984) and these include:

a. glycogen (polyglucose) granules either, ovoid or elongate and rod-shaped located between thylakoids

b. cyanophycin granules, structured granules having radiating substructure pattern, are polymers of arginine and aspartic acid, which serve as reserves of nitrogen

c. carboxysomes (polyhedral bodies), which are large angular structures, serve as reserves of ribulose bisphosphate carboxylase/ oxygenase (RUBISCO)

d. polyphosphate (volutin) granules reaching 100-300nm in diameter, and

e. gas vacuoles composed of many elongate cylindrical gas vesicles bearing a pointed cap at each end.

1.2.5 Centroplasm

The nucleoid region or centroplasm containing DNA fibrils is found in a complex folded arrangement, circular when unfolded. The total molecular weight of cyanobacterial genomes ranges between 1.6 and $8.6x10^9$daltons.

1.2.6 Protoplasm

The protoplasm of cyanobacteria is more viscous compared to other bacteria. Ribosomes of prokaryotic nature are found throughout the cytoplasm.

1.2.7 Heterocysts

In filamentous cyanobacteria, specialized cells called heterocysts with the function of fixing atmospheric nitrogen are found in between vegetative cells. They are usually yellowish with no granular reserve materials or gas vacuoles. It has a thick wall which projects into cell interior as a knob at cell ends.

1.2.8 Akinetes (Spores)

Cyanobacteria are traditionally best adapted to drought and desiccation, probably through the presence of modified vegetative cells (spores or akinetes) that are resistant to desiccation.

1.3 Metabolic Evolution through Cyanobacteria

Cyanobacteria, arguably, are the most important group of organisms to exist on our planet. The most important metabolic innovation in earth's history was oxygenic photosynthesis. Their ability to use water as electron source freed primary production from the earlier limits set by alternative electron sources such as H_2S, H_2 and Fe^{2+}(Knoll, 2008). The new scenario, deriving energy and reducing power from sunlight and water, increased primary

productivity and re-structured ecosystems (Fischer *et al.*, 2016). The release of oxygen as end product dramatically altered the redox state of earth's atmosphere and oceans, and permanently changed all biogeochemical cycles.

Photosynthesis in many cyanobacteria is stimulated by low oxygen concentration, the oxygen competing with carbon dioxide for the enzyme ribulose-1,5-bisphosphate carboxylase/ oxygenase (RUBISCO). When cyanobacteria first appeared, the earth's atmosphere was rich in carbon dioxide (10-100 times that of present levels) and oxygen was sparse (about 10^{-8} times that of present levels). Thus, cyanobacteria dominated earth's biosphere for at least one billion years or probably more. As oxygen-evolving cyanobacteria became abundant, the oxygen in the atmosphere gradually built up, creating a protective ozone (O_3) layer in the atmosphere at the same time. This layer removed harmful ultraviolet radiation from atmosphere paving way for the evolution of radiation-sensitive organisms (Whitton & Potts, 2012).

Many cyanobacteria have the ability to photosynthesize under aerobic and anaerobic conditions. Under aerobic conditions, electrons for photosystem I are derived from photosystem II. Under anaerobic conditions, in the presence of sulfur, electrons are derived from the reduction of sulfur. These cyanobacteria are facultative phototrophic anaerobes and fill an important ecological niche in aquatic systems. While other photosynthesizers like eukaryotic algae were restricted to aerobic habitats, and photosynthetic bacteria restricted to photoanaerobic habitats, cyanobacteria possessed a selective advantage in habitats that fluctuated between the above conditions in different seasons. Two of the sulfide rich ecosystems i.e., hot sulfur springs and marine littoral sediments, reminiscent of old ecosystems that pre-dated oxidized atmosphere, have high numbers of cyanobacteria.

Biological carrying capacity of oceans was maximized when they coupled oxygenic photosynthesis with nitrogen fixation. Diazotrophic cyanobacteria are crucial players in the nitrogen budget. Among nitrogen-fixing microorganisms, nitrogen-fixing cyanobacteria are unique in their ability to carry out oxygen-evolving photosynthesis and oxygen-labile nitrogen fixation within the same organisms. These seemingly incompatible reactions take place in heterocystous cyanobacteria by the spatial separation of the site of nitrogen fixation (heterocysts) from the site of photosynthesis (vegetative cells) (Stanier & Cohen-Bazire, 1957; Haselkorn, 1978). Mitsui *et al.*, (1986) demonstrated nitrogen fixation in unicellular cyanobacteria by temporal separation of the photosynthesis and nitrogen fixation phases.

Chloroplasts, organelle for photosynthesis, evolved from cyanobacteria living symbiotically within plant cells. Sometime in the late Proterozoic, or in the early Cambrian, cyanobacteria began to take up residence within certain eukaryote cells, making food for the eukaryote host in return for a home. This event is known as endosymbiosis. Concept of a cyanobacterium-chloroplast transition during evolution was based on the discovery of cyanelles, chloroplast-like cyanobacterial endosymbionts, found in a number of taxonomically unrelated eukaryotes. The cyanobacterial nature of these cyanelles was evident from their peptidoglycan-containing envelopes and their usage of phycobilisomes as light-harvesting complexes. More direct evidence for the cyanobacterial nature of chloroplasts came later with the discovery of the prokaryote-type chloroplast genetic system and protein translation machinery, especially their 70S ribosomes, and their double-layered envelopes. Molecular and phylogenetic studies have revealed that the chloroplasts of plants and algae evolved from a cyanobacterial ancestor (Margulis, 1992; Delwiche & Palmer, 1997; Giovannani, 1998).

1.4 Adaptation of Cyanobacteria to extreme environments

The long evolutionary history gave cyanobacteria the selective advantage to survive in diverse aquatic and terrestrial habitats exhibiting wide ranges in temperature, salinity, water potential, pH, and irradiance (Miller & Castenholz, 2000). They often form microbial mats with other bacteria, from Antarctic ice to continental hot springs. Cyanobacteria can develop in hypersaline and alkaline lakes, support high metal concentrations and tolerate xerophilic conditions (*i.e.*, low availability of water), forming endolithic communities in desert regions. However, cyanobacteria are rarely found in acidic environments at pH values lower than 5–6 (Rampelotto, 2013). In a cold environment they can adapt to constant low temperatures and freezing, high UV radiation and desiccation. By keeping their nitogenase enzyme active at -7^{o}C, they are able to metabolize while freezing (Pandey *et al.*, 2004). For living in a hot spring at temperatures between 45 and 73°C, the thermal maximum for photosynthetic life, they shift their temperature optimum to a higher value and trade off their low temperature adaptation genes (Miller & Castenholz, 2000). It was found that single celled *Synechococcus* in hotsprings had a peculiar adaptation: when sun goes down, they switch off photosynthesis and start nitrogen fixation leading to production of nitrogenous compounds (Steunou *et al.*, 2006). In hypersaline environments, intact cells and cell extracts were examined using ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy and it was found that quaternary ammonium compound glycine betaine was responsible for osmotic adjustment (Reed *et al.*, 1984). In highly alkaline extreme environments that form in closed drainage basins exposed to high evaporation rates, there is scarcity of Mg^{2+} and Ca^{2+} in the water chemistry, the lakes become enriched in CO_3^{2-} and Cl^{-}, with pHs in the range 8 to >12. Here, photosynthetic primary production by chroococcalean and

oscilattorian cyanobacteria appears to be the basis of all nutrient recycling (Jones *et al.*, 1998).

1.5 Cyanobacterial Association

Periphyton is the assemblage of microorganisms attached to submerged surfaces. Cyanobacteria are dominant in such associations. These microbial mats span kilometers in spread with meters in thickness. Epilithon are crusts and films of living organisms over rocks commonly in desertified areas and epilithic cyanobacteria are a common presence in this assemblage all over the planet. Epiphytes are biofilms found growing on plants and epiphytic cyanobacteria are found in terrestrial and aquatic environments. In a study by Yamamuro (1999), cyanobacteria living on the surface of seagrass *Syringodium isoetifolium*, was found responsible for the supply of carbon and nitrogen compounds to heterotrophs living in the ecosystem, sparing seagrass from grazing. Epipsammon are assemblages found on sand by non-motile or partially mobile organisms. In aquatic environments, filamentous and colonial cyanobacteria attach to sand surfaces by way of a pad or a small stalk of exopolysaccharides, and harbor a lot of biodiversity (Underwood, 2010). Epipelon are defined as organisms that live in sediments. Bottom of lakes and rivers are colonized by unicellular, filamentous or colonial heterocystous cyanobacteria contributing to nitrogen budget of lakes (Scott & Marcarelli, 2012).

Bloom-formers are found in warm, stable, nutrient-rich lakes and are largely absent from the polar and alpine regions. The genera that are particularly common include *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Microcystis*, *Nodularia*, *Nostoc* and *Planktothrix* (Carmichael, 2001). These genera can produce a wide variety of different toxic compounds

(Sivonen & Jones, 1999). The toxins can roughly be classified as hepatotoxins, neurotoxins and dermatotoxins. The factors causing dominance of bloom-forming cyanobacteria are of great interest to water quality managers because of the production of toxins and other secondary metabolites by these organisms.

Certain cyanobacterial taxa are often found in loose association with emergent plants in ponds and wetlands. This community is referred to as the metaphyton, and includes genera such as *Chroococcus* along with other organisms like protists, bacteria and fungi, planktonic and attached cyanobacteria viz. *Leptolyngbya, Merismopedia*, and *Phormidium*. In peat bogs, genera such as *Aphanothece, Chroococcus, Hapalosiphon, Merismopedia,* and *Tolypothrix* are found in association with the Sphagnum moss, and occur at acidic pHs as low as four.

Some cyanobacteria occupy both benthic as well as planktonic habitats. For example, the saline lake species *Aphanothece halophytica* commonly forms mucilaginous, benthic films, but it is also often found in the phytoplankton of such lakes, sometimes at high concentrations (Zohary *et al*., 1998).

The symbiotic interactions of cyanobacteria include close associations with fungi to form lichens, sometimes found in semiaquatic habitats but usually in the surrounding terrestrial environment; on or inside mosses; as an endosymbiont within the semiaquatic angiosperm *Gunnera*; and within the floating fern *Azolla,* which is often cultivated in rice fields for its nitrogen content (Moore, 1969; Meeks, 1998).

1.6Applications of Cyanobacteria

Cyanobacteria are food factories that can survive on minimal nutrients. A cyanobacterial culture can be grown in a bioreactor if it is prone to contamination by other organisms under natural growth. They can be grown in open ponds if they can over-power contaminating organisms. Whichever way we look at it, biotechnology industry is sure to gain a lot from them. Much work has been going on worldwide to elucidate the secondary metabolites of cyanobacteria. The drug industry has been getting several antiviral, antifungal, antibacterial, antiplasmodial, antialgal, immunosuppresent and anticancer compounds from them (Abed *et al*., 2009; Singh *et al*., 2011; Dixit & Susheela, 2013; Vijayakumar *et al*., 2015. Polyhydroxyalkanoates (PHAs) are another array of compounds isolated from cyanobacteria. These biodegradable compounds have properties similar to polythene and polypropylene and hence offer tremendous potential in all applications currently done using plastics (Steinbuchel *et al.*, 2003, Verlinden *et al*., 2007; Balaji *et al*., 2012). Bioremediation is an area which is getting new additions with every successful research project. Vast array of organisms were found to decompose various components in oil spills, surfactants and herbicides (Abed *et al*., 2009). Mostly they work along with other bacteria as consortia and eat away the pollutants. Waste water treatment processes also make use of cyanobacteria (Uma &Subramanian, 1990; Lincoln *et al*., 1996; Chevalier *et al*., 2000). Cyanobacteria have been used to produce hydrogen gas which can be used as an alternative energy source replacing fossil fuel (Aoyama *et al*., 1997; Dutta *et al.*, 2005; Rupprecht *et al*., 2006)

Cyanobacteria are also part of food industry due to the nutritional benefits they offer. It has more than 60% proteins, beta carotene and vitamins. Genera like *Arthrospira*, *Nostoc* and *Anabaena* are grown in bioreactors or

open ponds and made into powders, tablets and capsules for human consumption (Abed *et al*., 2009). They also benefit the aquaculture industry as fish and crustacean feed (Preetha *et al*., 2007). Exoploysaccharides produced by hypersaline cyanobacteria have application in textile industry as emulsifiers.

Agriculture sector has made tremendous gain with these organisms (Singh, 1980; Wagner, 1997; Vaishampayan *et al*., 2001). Ferns like *Azolla* that harbor nitrogen fixing cyanobacteria called *Anabaena* are able to fix 20-25Kg N/ hectare of soil in a year. Other than the symbiont *Azolla*, many free living cyanobacteria in the genera *Anabaena, Aulosira* and *Nostoc* have potential as biofertilizers, and are employed in fields in China and Vietnam (Sahu *et al*., 2012). In addition to nitrogen fixation, they have mineralization potential that degrades pesticides, organic wastes and residues, detoxify heavy metals and improve bioavailability of phosphorus. They suppress certain pathogenic microorganism and secrete plant growth hormones (Singh *et al.*, 2016).

Use of successful indigenous strains of cyanobacteria as the potential biofertilizer not only improves the physico-chemical and biological properties of the soil but also helps in promoting yield of various agricultural crops such as rice, wheat and pearl millet under saline, drought and contaminated agroecosystems (Singh *et al*., 2011). Regular application of cyanobacterial strains adapted to various extreme environments seems promising for wasteland management and improvement of soil stability, nutrient status, soil microbial activities, nutrient mineralization and crop growth in ecologically sustainable manner (Singh *et al*., 2011).

1.7 Cyanobacteria as Model Organisms

Cyanobacteria are used as model organisms in studies relating to photosynthesis, endosymbiotic origin of chloroplasts and gene regulation (Bryant, 1994; Campbell *et al.*, 1998; Seo & Yokota, 2003). Genetic engineering of the cyanobacterium *Synechocystis*, conferring the ability to generate volatile isoprene hydrocarbons from CO_2 and H_2O to be used as photosynthetic biofuels, is a promising scenario (Lindberg *et al.*, 2010). Photo physiology (Six *et al.*, 2004), circadian oscillation mechanisms (Nakajima *et al.*, 2005), transcriptional regulation of photosynthesis (Hernández-Prieto *et al.*, 2016) etc. are some of the studies which were done using cyanobacteria as model organisms.

1.8Classification of Cyanobacteria

Plants and animals exhibit consistent features by which they can be distinguished and can be segregated into distinct groups. Such a classification system has to be present for every organism including viruses or bacteria. Traditionally, classification of cyanobacteria was based on morphological features such as cell size, shape and arrangement of cells, planes of division, pigmentation, trichome width, presence of characters like gas vacuoles and sheath. (Valerio *et al*, 2009) Such a mode of identification calls for considerable expertise and familiarity with a whole lot of strains. Subjective judgment from the part of operators also leads to errors. As a result of this, more than 50% of strains in culture collections across the world are thought to be misidentified (Komarek & Anagnostidis, 1989; Rudi *et al*., 1997). Some diagnostic features like gas vacuoles and akinetes can show variations in differing environment or growth conditions. Some morphological features might be lost when cultures were maintained in laboratory conditions (Lyra *et*

al., 2001). Identical genotypes of cyanobacterial strains show varying phenotypes in natural conditions and may become identical in laboratory conditions (Palinska *et al*., 1996). Complex behavior of filamentous cyanobacteria in laboratory cultures sometimes makes identification difficult. Despite the large size of some of the colonial forms, morphological identification is often challenging due to coloration and variable growth forms (Sumina, 2006; Engene *et al*., 2010).

Earlier, cyanobacterial diversity studies were included in the studies of eukaryotic algae due to their shared property of photosynthesis, green pigmentation and presence in similar habitats. Because of the structural complexity of the group, traditional classification was based on morphological characters that can be elucidated by light microscopy (Palinska *et al.*, 2006).Their relationship with bacteria was recognized by Cohn in 1875. It took a long time to be reckoned in prokaryotic group. Bergey's Manual of Systematic Bacteriology (2001) became the first official treatise to take on this stand.

Despite all sorts of pressure for a unified code, systematic treatment of cyanobacteria or cyanophyceae is still guided by two codes of nomenclature, the Botanical and Bacteriological codes, which were derived from historically separate traditions founded on different principles, and which accordingly proscribe different treatments. Taxonomic treatment of cyanobacteria, including formal description of new taxa, can presently be carried out under the aegis of either of these codes. However, the rules of the codes are quite different and, when applied to the same group of organisms, confusions are unavoidable. The classification system of these prokaryotic organisms has been in a flux since they are clubbed along with algae by botanists and along with bacteria by bacteriologists through their respective codes of

classification. The morphological variability of this group varies from single cells to filaments which in turn are un-branched, false branched or true branched with cell specialization for nitrogen fixation and reproduction. In the botanical system of classification, scientists take into consideration factors such as morphology, cell division, pigmentation etc. By Bacteriological code this is replaced by mean G+C composition, growth requirements and sequencing data based on small subunit ribosome gene and other protein coding genes.

Presently there are 475 Strains of cyanobacteria in pure culture in Pasteur Culture Collection of Cyanobacteria, the reputed collection in the world (research.pasteur.fr, 2017)

Polyphasic taxonomy, the current concept in cyanobacterial classification,aims to bring together both systems of classification and make it a coherent one, combining morphology and gene sequencing data.

1.9 Objectives of the Present Work

Preetha *et al.*, (2007) carried out an investigation of strains of cyanobacteria with anti-vibrio activity and zeroed in on six strains, and proposed two strains as promising probiotics in aquaculture. Among these six strains three were unicellular organisms belonging to the order Chrococcales. These Chroococcalean strains were selected for further investigation by a polyphasic approach to ascertain their phylogeny.

Cyanobacterium sp. MCCB 114, MCCB 115 and MCCB 238 existed as non-axenic cultures in the culture collection of National Centre of Aquatic Animal Health, Cochin University of Science and Technology, Kerala India.

This research is aimed to classify the three isolates of *Cyanobacterium* from the coastal waters of Cochin by polyphasic approach.

A serendipity that happened during the course of this investigation was that, axenic cultures of *Cyanobacterium* strains deteriorated faster compared to non-axenic cultures. Hence, this study focused on understanding the differences between axenic and non-axenic cultures with regard to cell ageing or senescence.

Cyanobacteria are always associated with a rich flora of heterotrophs by virtue of their thick glycocalyx which provides nutrients. Whether the bacteria living along with cyanobacteria are unique or just occur by chance is a topic of interest. This research investigated the heterotrophic bacteria associated with three isolates of *Cyanobacterium,* and tried to find out whether the associated microorganisms were any specialized group scavenging on cyanobacterial exudates or just a chance occurrence.

Thus the objectives of this study were:

- **Polyphasic taxonomy of *Cyanobacterium* MCCB 114, MCCB 115 and MCCB 238**
- **Fate of axenic cultures of *Cyanobacterium* MCCB 114, MCCB 115 and MCCB with regard to senescence**
- **Identification of associated heterotrophs of *Cyanobacterium* MCCB 114, MCCB 115 and MCCB 238**

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Chapter 2

Polyphasic Taxonomy of Putative Probiotic *Cyanobacterium* spp. MCCB 114, MCCB 115 and MCCB 238

Contents

2.1 Introduction

Cyanobacteria are a group of organisms that are known by many other names: cyanophyta, blue green bacteria and blue green algae. The terms blue green algae or cyanophyta were found reasonable until around 1960, as they were classified as a major taxon among the algae governed by the Botanical Code of classification. When the fundamental differences between prokaryotes and eukaryotes were clearly defined based on cell structure, two facts concerning cyanobacterial taxonomy became evident: prokaryotic cell structure is the only character common to and distinctive of all bacteria; and cyanobacteria are typical prokaryotes in terms of cellular organization (Stanier *et al.*, 1978). In this context, the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974), the standard treatise in bacterial classification, divided *Procaryotae* into two divisions, The Cyanobacteria and The Bacteria. In the subsequent edition of Bergey's Manual, the Cyanobacteria were clustered along with photosynthetic prokaryotes as Photobacteria (Holt, 1994). This was followed in the

comprehensive second edition of *Bergey's Manual of Systematic Bacteriology,* where cyanobacteria were figured in the Volume 1 dealing with archaea and the deeply branching and phototrophic bacteria (Boone & Castenholz, 2001).

Though these changes were happening on one side, the Botanical Code was still the followed nomenclatural system. Stanier *et al.* (1978) published a proposal to place the nomenclature of the cyanobacteria under the rules governing the International Code of Nomenclature of Bacteria (ICNB), now the International Code of Nomenclature of Prokaryotes (ICNP). This created a potential conflict in Botanical system. A judicial commission which met at Munich, Germany decided that scientists were at a liberty to follow whichever system they want. Since then the issue was raised in many committees, but till now no consensus has been arrived at (Oren, 2004; Oren, 2005; Oren and Garritty, 2014).

Modern taxonomy is a method to recognize and register diversity of organisms which may either be found in natural populations or maintained in cultures (Komarek & Mares, 2012). As research field advances, more information is gathered on the genetic, phenotypic and ecological characters of organisms. As a result, earlier assignments of genera or other higher groupings may face a sea change and new logical organizations may be created.

Presently the systematic treatment of Cyanobacteria or Cyanophyceae is guided by both Codes of classification derived from historically separate traditions, based on fundamentally different principles and hence requiring different treatments (Palinska *et al.,* 2006). Komarek (2006); and Komarek and Jiri (2012) had stressed on a polyphasic approach that integrated morphological variations in nature as well as the recently generated morphological, ultrastructural, ecophysiological and biochemical characters.

There is tremendous diversity among strains of cyanobacteria, but the extent of diversity is not discernible on morphological examination. Strains that look similar are genetically much different. A comprehensive biodiversity assessment is incomplete as far as these organisms are concerned. This chapter describes some steps to be taken for ascertaining identity of strains based on polyphasic approach. It is very surprising to note that even the biggest cyanobacterial culture collection in Pasteur Institute, France, has only 475 strains in pure culture. Strains collected from India were seven which is far low by any standards (CRBIP, 2017). Considering the potential application of cyanobacteria in bringing down carbon dioxide levels in the environment as well as a raw material for many industries, biodiversity assessment and screening for bioactive metabolites should be taken up in all parts of the world, so that we do not lose any organism by unintentional anthropogenic activities.

2.2 Materials and Methods

2.2.1 Strains used in the study

Preetha *et al.* (2007) isolated 54 strains of cyanobacteria from the water samples collected from Vypeen, off-shore Cochin by enrichment method and carried out an investigation of strains with anti-vibrio activity. Six strains of cyanobacteria with antibacterial activity against 14 isolates of pathogenic vibrios were identified. Of these, two strains with higher activity were proposed as probiotics in aquaculture. They were identified using morphological characters and ascertained strain numbers as *Synechocystis* MCCB 114 and *Synechocystis* MCCB 115. Another cyanobacteria strain belonging to the order *Chroococcales* was present in the group, which showed similar property though slightly lesser, was also included in the present study,

since possession of antivibrio activity was common to all three strains, and all were unicellular, and round, chroococcalean cyanobacteria. The third strain was assigned the culture collection number MCCB 238.

Synechocystis sp. MCCB 114, MCCB 115 and MCCB 238 existed as non-axenic, unicyanobacterial cultures in the culture collection of National Centre of Aquatic Animal Health, Cochin University of Science and Technology, Kerala India. The objective of the present chapter was to work out the polyphasic taxonomy of these three potential cultures. The first step undertaken was to bring them into pure culture.

2.2.2 Liquid Medium for Isolation and Maintenance

Cyanobacteria being photoautotrophs, simple mineral media can support their growth. The media used should mimic the environment of isolation and they must be supplemented by essential nutrients such as sources of nitrogen, phosphorus and trace elements. Since the cyanobacterial strains used for the study were isolated from coastal environment, MN medium (Waterbury & Stanier, 1981) was used as the medium for isolation and maintenance. The medium had the following composition (per litre): $NaNO_3$ 0.75g; K_2HPO_4. $3H_2O$ 0.02g; $MgSO_4$ 0.038g; $CaCl_2$ 0.018; Na_2CO_3 0.02g; Disodium EDTA 0.0005g; Citric acid 0.003g; and Ferric ammonium citrate 0.0.003g. The medium was prepared in 75% aged sea water filtered through Whatman No. 1 filter paper to remove impurities. Trace minerals were added as a solution A5+Co (Rippka *et al.*, 1979) in the following concentrations B 46µM; Co 0.17µM; Mn 9.2µM; Zn 0.77µM. In the original composition of A5+Co, Cu at 0.32µM was to be added. But this ingredient was eliminated from the medium as it was reported that copper may be toxic for certain cyanobacteria (Waterbury, 2006). The medium was prepared as 50mL aliquots

in 100mL Erlenmeyer flasks. Inoculation with the required organism was carried out using platinum loop (Cole-Palmer, India)

2.2.3 Solid Media for Purification

Obtaining axenic cultures of cyanobacteria is a difficult process and this has led to a great variety of purification techniques. Extensive reviews on the topic were made by pioneers in the field including Stanier *et al.* (1971), Waterbury (1986) and Rippka (1988), among others. Several hundred cyanobacterial strains, including representatives from most of the major groups have been isolated and purified using standard microbiological techniques. Despite this success, there are still many uncultured cyanobacteria, particularly those found in oligotrophic waters of oceans and fresh water lakes.

Streak plate techniques, techniques making use of gliding motility and phototaxis, treatment with toxic chemicals, antibiotics, heat, ultraviolet, gamma radiation, mechanical techniques like micromanipulation, filtration, equilibrium centrifugation are commonly used for the purpose depending on the characteristics of cyanobacteria to be purified (Vaara *et al.*, 1979). Wide varieties of bacteria are often associated with cyanobacteria and hence application of a single antibiotic or chemical may be insufficient to eliminate them all. So, combinations of chemicals, antibiotics or physical methods are in use (Jones *et al.*, 1973). Agar plating is sometimes impossible due to the impurities present, and alternatives to agar are proposed by some workers. Ferris and Hirsch (1991) suggested the use of glass fiber filters in petri plates as a substitute for agar. However, in our study, we resorted to the use of agar in a purified form.

Purification of most bacterial strains can be accomplished by streaking onto media in petri plates solidified using agar. But cyanobacterial strains

under study failed to grow on agar plates of MN media on which they were growing in broth culture. It is reported that sterilizing mineral salts along with agar may produce compounds toxic to cyanobacteria (Rippka, 1988). Hence, two modifications were made in the preparation of agar. Agar was washed and dried according to Waterbury (1986). A sample of 100g of Difco Bacto-agar (BD, USA) was washed by stirring with 3 L of double distilled water in a beaker for almost 30 minutes. When the agar settled in the bottom, water was removed by filtering. This procedure was repeated till the filtrate became clear. Then the agar was washed with 3L of 95% ethanol and finally with 3L of analytical grade acetone. The agar was spread on a tray and dried at 50^{o}C in an oven for 2-3 days and stored in air tight containers. This agar was used as solidification media for all experiments using cyanobacteria. Concentration of agar was also kept low. Solid media prepared with 0.7% purified agar was firm enough to support streaking.

Cyanobacterial colonies did not grow on MN agar plates when agar and media components were autoclaved together. Hence, double strengths of media and washed agar were autoclaved separately; cooled to 45^{o}C, mixed and poured into plates. Since the incubation time for cyanobacteria was longer, approximately 40mL media were added onto each plate and dried sufficiently. After streaking with the required culture with a platinum inoculation loop, the sides of the petri plates were sealed with strips of Parafilm (3M, USA) to minimize evaporation loss.

2.2.4 Incubation

The liquid medium prepared in 50mL Erlenmeyer flasks was incubated under light intensity of 40 $\mu Em^{-2}s$ provided by clear while fluorescent lamps

for a 14:10 photoperiod. The agar plates were kept inverted on a glass top and illumination was provided from below.

2.2.5 Subculturing

Cyanobacterial strains always have nearly invisible heterotrophic bacteria as associates and hence purification is a step that takes time and diligence. To aid in the process of picking up colonies for sub-culturing, a microscope with 10X objective is invaluable. For picking up new colonies for sub-culturing, incubated colonies were examined under microscope. Most heterotrophic bacteria appear within 2-3 days as discrete colonies. Cyanobacterial colonies may appear as minute dots between 5 and 10 days. Cyanobacterial colonies free from any contaminating bacterial colonies were carefully selected by microscopic examination, marked and incubated further so that they become big enough to be picked by inoculation needle. The process of sub-culturing was repeated 8-14 times for obtaining axenic cultures.

2.2.6 Checking for axenicity

Three methods were employed for checking the axenicity of cultures.

2.2.6.1 Employment of bacterial test media

Carbon compounds were added onto MN media in the following concentrations and dispensed into test tubes (Vaara *et al*, 1979; Anderson, 2005).

BT medium 1: MN with 1g proteose peptone/L

BT medium 2: MN with 5g yeast extract/L

BT medium 3: MN with 5g peptone+ 3g yeast extract/L

BT medium 4: MN with 1g glucose + 1g peptone/L

BT medium 5: MN with 0.5g sodium acetate +0.5g glucose+0.5g tryptone+0.3g yeast extract/L

BT medium 6: MN with 1g yeast extract + 2g tryptone/L

TYG medium : MN with 5g tryptone + 2.5g yeast extract + 1g glucose/L

A loopful of culture was inoculated into the medium and incubated in dark for 14 days. Absence of growth and turbidity in the media was taken as positive result. This was further confirmed with Gram's staining and observation under microscope.

2.2.6.2 Epifluorescence using DAPI staining

Cyanobacteria have pigment auto fluorescence which when examined under fluorescent microscope gives bright red colour. But heterotrophs cannot be distinguished using the technique. Using a nucleic acid stain such as DAPI (4', 6-diamidino-2-phenylindole), (Sigma, USA) which stains all the cells in the culture, was also employed for testing axenicity.

2.2.6.3 16S rRNA gene amplification of the isolate using the universal primers and sequencing

If the single colony obtained after the purification step is subjected to 16S r RNA gene amplification using universal primers of bacteria followed by sequencing, we can ascertain the purity of the culture, if the sequence corresponds to a cyanobacterial strain. (The protocol is described in section 2.2.7.4.2.1).

2.2.7 Guidelines for characterization of cyanobacteria

Traditional methods laid out by Geilter (1932); Desikachary (1959); and Bourelly (1970) as cited by Rippka *et al.* (1979) were used to infer the taxonomical identity of the strains. Morphological and biochemical

characterization of strains as per bacteriological systems were carried out as described by Castenholz (2001). Modern phycological characterization as per Komarek *et al.* (2005) was also followed.

2.2.7.1 Cell morphology

Cell shape, polarity, cell dimensions, colour and plane of division were studied using light, phase contrast and fluorescence optics of Olympus CX 41 microscope (USA) equipped with Q Imaging Miropublisher 3.3 RTV for image capture and Q Capture Pro 7 for image analysis. For fluorescence microscopy, additional to Olympus CX 41, Olympus CX 51 with multiple wavelength emission was also used as per the requirement.

Colony morphology was studied by observation under 10X objective of bright field microscope.

Though majority of unicellular cyanobacteria are immotile, some may exhibit movement in liquid media or on surfaces. This may be due to gliding motility or by the presence of Type IV pili. Motility was assessed by growing the strains on petri plates and exposed to unilateral illumination. Positive response was indicated by phototactic movement clearly visible under 10X objective of bright field microscope.

For sheath or glycocalyx description, negative staining with India ink (Nigrosin) was carried out. Depending on the features of the polysaccharidic outer layer varying in thickness, consistency and appearance after staining may be referred to as sheath, capsule and slime. The sheath is a thin electron dense layer which loosely surround cells and even visible without staining by light microscopy. The capsule is closely associated with cell surface, has sharp outlines and a coherent structure and visible by negative staining. The slime is

a mucilaginous layer dispersed around the cells but does not reflect the shape of cells.

2.2.7.2 Ultrastructure

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were carried out for studying the ultrastructure. Thylakoid arrangement, cell wall structural appearance, internal inclusions, storage granules and sheath structure were the characters observed.

Samples of 2mL of exponential phase cultures were pelleted by centrifugation in a refrigerated centrifuge (Sigma, USA) at 6000g and washed twice with 0.1M sodium cacodylate buffer and fixed with 2.5% glutaraldehyde and incubated at 4^{o}C overnight. The pellets were washed with buffer and post fixed using 2% osmium tetroxide at 4^{o}C for 2 hours. Subsequently the suspension was pelleted by centrifugation at 8000g and washed twice with sterile Milli-Q water (Millipore, USA) and dehydrated using an acetone series of 70-100%. For SEM, the dry cells were sputter coated with gold using SC 7620 Sputter Coater and observed using Vega3 Scanning Electron Microscope (Tescan, Brno, Czech Republic) at Maharajas College, Ernakulam.

For TEM, the dehydrated cells were embedded in resin, sectioned using ultramicrotome, stained using uranyl acetate and observed under TECNAI 200 Kv Transmission Electron Microscope (Fei, Electron Optics, USA) at AIIMS, New Delhi.

2.2.7.3 Physiology/Biochemistry

All the studies in this section dealing with growth in culture media were done in triplicates. Cell growth was estimated by cell counting using Neubeur haemocytometer, and correlated with absorbance at 750nm by preparing a standard curve.

2.2.7.3.1 Absorption spectra

Chlorophyll a, phycobiliproteins (C-phycocyanin, allo-phycocyanin and phycoerythrin) and an array of carotenoids were the photopigments present in cyanobacteria. In vivo absorption spectra revealed an absorption maxima of chlorophyll a at 670-683nm. The phycocyanin peak showed a variation extending from 620 to 638nm. Phycoerythrin if present peaks at approximately 570nm.

In vivo absorption spectra were measured using Shimadzu UV-1601 UV-Visible spectrophotometer, Japan. The scanning wavelength was in the range 190 to 1100.

2.2.7.3.2 Temperature optimum and maximum

The temperature range for growth was determined in MN media at 4, 10, 15, 18, 20, 25, 28, 30, 35, 40 and 45°C and incubated in an illuminated rotary shaker (Orbitek, Scigenics Biotech Pvt. Ltd., India). Cell count was estimated by counting using Neubeur haemocytometer.

2.2.7.3.3 Salinity tolerance

MN media was prepared using fresh water and NaCl was added to the media in the range 0, 10, 20, 30, 40, 50g NaCl/L. 1mL of corresponding cultures were inoculated and incubated under light at 26^{o}C.

2.2.7.3.4 pH range and tolerance

The pH for growth in MN media was in the range of 4.0 to 12 with an interval of 1 unit using 40 mM of the following buffers to maintain pH: MES (for pH 4.5–6.0), PIPES (for pH 6.5–7.5), Tricine (for pH 8.0–8.5) and CAPSO (for pH 9.0–10.0) of corresponding cultures were inoculated and incubated under light at 26^{o}C.

2.2.7.3.5 Capacity for dark chemoheterotrophy

Most cyanobacteria are obligate phototrophs, though a few strains grow in dark at the expense of organic compounds. When grown in light, even obligate phototrophs may assimilate those using photochemical reactions for the generation of ATP and reducing power. The photosynthetic growth rate may be increased by the provision of organic substrates, if they serve as a general source of cellular carbon and if the rate of photosynthetic growth is limited by the rate of CO_2 assimilation. However, for growth in dark, a photosynthetic organism must be able to use carbon through respiration or fermentation and not merely as a source of assimilable carbon (Stanier, 1971). The organic compounds tested were glucose (0.25%, w/v); acetate, succinate, pyruvate and glutamate (furnished as sodium salts at a concentration of 0.1%, w/v). For photoheterotrophic growth experiments 10µM DCMU (3-(3,4-dichlorophenyl)-1,1dimethyl urea) which inhibits photosystem II was added. The experiment was conducted using plates of MN agar where $NaNO_3$ as nitrogen source was replaced by NH_4NO_3 to preclude the possibility that nitrate is not a utilizable nitrogen source in the dark, as might be the case if its assimilatory reduction was a light dependent process. The plates were inoculated by placing small drops of liquid cultures on agar surface. One set of plates was incubated at 25^{o}C in dark and one set at the same temperature in light. The plates were checked for signs of growth over a period of one month.

2.2.7.3.6 $CaCO_3$ deposition

$CaCO_3$ crystals were formed at the bottom of the conical flask when growth was in stationary phase. It was photographed using bright field microscope.

2.2.7.3.7 Nitrogen Fixation

It was thought that nitrogen fixation was a property invariably associated with the formation of heterocysts. But some unicellular cyanobacteria seem to fix atmospheric nitrogen in nutritional deficient waters.

The test was done by inoculating the cells in MN medium prepared in freshwater with added NaCl (2.5%w/v) and devoid of $NaNO_3$. Ability to grow well in the medium through repeated transfer was considered as positive result.

2.2.7.3.8 Anaerobic growth with Sulphide as electron donor

To check whether sulphide can be used as electron donor for photoassimilation of CO_2, protocol of Cohen *et al*. (1986) was followed. The growth media had mM of K_2HPO_4, 0.33; NH_4Cl, 0.33; $MgCl_2.6H_2O$, 0.33; KCl, 0.33; Vit B12, 10^{-5}; Na_2CO_3 1.5 as well as trace elements solution of MN. 0.5mM Na_2S and 5mM DCMU were added and pH adjusted to 6.8 with HCl. Pre-inoculated cells were completely filled into 10mL screw cap tubes sealed with paraffin wax and incubated under light. Growth indicated positive result.

2.2.7.3.9 Exopolysachharide Production by Alcian Blue Staining

There is a growing industrial interest towards polysaccharides from microbial sources. Samples for microscopic evaluation of the EPS were filtered onto a 0.4-µm filter and stained for 2s with a 0.02% Alcian Blue solution in 0.06% acetic acid, followed by rinsing with Milli-Q water (Passow and Alldredge, 1994).

2.2.7.4 DNA Sequence analysis

Scientists interested in taxonomic studies should not assume that the strains they get from culture collections are correctly identified as there are many misidentified strains in them (Komarek, 1994). When we carry out sequence analysis of cyanobacteria and deposit in Genbank database of National Centre for Biological Information (NCBI), USA, many sequences may be loners as there may not be sufficient sequences to compare them with. Such a situation will improve with time as more and more investigators work with pure strains and sequence deposits will increase.

2.2.7.4.1 DNA extraction

Ten day old cyanobacterial cells grown in MN media were harvested by centrifugation and DNA extracted by Xanthogenate method (Tillet and Neilan, 2000). A sample of 2mL of log phase culture was taken and cells were harvested by centrifugation at 8000g for 5min and washed twice with TE buffer (10mM Tris, 1mM EDTA, pH 8.0). Cell pellet after centrifugation was resuspended in 1mL of freshly prepared xanthogenate buffer (1% potassium ethyl xanthogenate (Fluka, Buchs, Switzerland); 100 mM Tris-HCl, pH 7.4; 20 mM EDTA, pH 8; 1% sodium dodecylsulfate; 800 mM ammonium acetate) and 50µL of RNase (100ug/mL) was added to the suspension. It was incubated at 65^{o}C for 2 hours. The tube was mixed well and incubated in ice for 30 min and centrifuged at 14000g for 10 min. The supernatant was transferred to a new tube and 1mL of ice-cold ethanol (100%) was added and kept overnight for DNA to precipitate into the solution. It was pelleted by centrifugation at 14000g for 10 min. The pellet was washed twice with 75% ethanol, and air dried and dissolved in 100uL Milli Q water and used for PCR.

The DNA was quantified and purity assessed using Hitachi U-2800 UV-VIS spectrophotometer with multiple wave length reads.

2.2.7.4.2 Ribosomal RNA (rRNA) Gene Sequence Determination

Bacterial rRNA genes are having an operon structure with genes arranged in the order 16S rRNA-23S rRNA-5S rRNA, with an internal transcribed spacer (ITS) region between each gene. After transcription, the primary transcript that is produced, shows pairing at complementary regions and looks like two stems which carry unprocessed 16S and 23S rRNA sequences and other necessary sites for process and release of mature rRNA molecules. Antiterminator sites or box A sequences are present either as paired or upaired regions immediately adjacent to processing stems and are well conserved within all bacterial groups. Box B stem that immediately follows Box A is highly variable.

2.2.7.4.2.1 Small Subunit Ribosomal RNA gene (16S rRNA gene)

16S ribosomal RNA gene is present as multiple copies in prokaryotic DNA. The most important aspect of this gene is the presence of conserved and variable regions within them. The conserved regions are regions which are attached to other components of the ribosome whereas the sequences between the conserved portions mutate at a faster rate. The conserved regions thus give an idea of the distant relationships whereas the variable regions indicate close relationship with similar organisms. The universality of the conserved regions has enabled us to design primers targeting the entire span of eubacteria.

In this study, universal primers for 16 S rRNA were derived from *E.coli*. The forward primer ranging from 9-27 base pairs, and reverse primer ranging from 1477-1498 base pairs of *E.coli* 16S rRNA gene (Reddy *et al.*,

2000) was used as primers for polymerase chain reaction done in Eppendorf Mastercycler Gradient, USA.

Forward primer: GAGTTTGATCCTGGCTCA

Reverse primer: ACGGCTACCTTGTTACGACT

The reaction mixture comprised of 12.5µL of Emerald Master Mix (Takara, Japan), 1µL each of forward and reverse primers, 50ng of DNA template and Milli Q to make up a total reaction volume of 25µL. The thermal cycling steps were as follows: initial denaturation at 95^{o}C for 7 min was followed by 25 cycles of 95^{o}C for 20s, 58^{o}C for 40s and 72^{o}C for 90s followed by a final extension of 72^{o}C for 7 min. Agarose gel electrophoresis of an aliquot of the PCR product was run along with 1KB marker (New England Biolabs, USA) and documented using Gel documentation System (BioRad, USA).The PCR product of approximately 1500bp cloned into pGEMT easy vector by TA cloning and used to transform competent cells of *E.coli* cells JM 101. Upon colony PCR using vector specific primers T7 and SP6, and checking the orientation of insert in the vector, the positive cultures were grown overnight in Luria bertani Broth (Sigma, USA) harvested and plasmid extracted using HP plasmid mini prep kit (Sigma, USA). The Plasmid was sequenced using vector specific sequences in an automated sequencer (Sanger ABI 3730xl Big Dye Terminator Sequencer, GATC Biotech, UK).

The sequences obtained were aligned using Genetool Lite Version 1.1 (BioTools Inc., Double twist, USA) and homology assessed using Blast algorithm with sequences in NCBI and RDP database and identified.

For comparing sequences with those present in databases, Geneious R11.0.2 software (www.geneious.com) was used. By using this, pairwise and

multiple alignments could be done and neighbor joining phylogenetic trees could be constructed.

2.2.7.4.2.2 Large Subunit Ribosomal RNA gene (23S rRNA gene)

23S rRNA is a 2904 nucleotides long component of the large subunit (50S) of the bacterial ribosome. The universality of the conserved genes of 16S rRNA due to its functional rigidity is applicable to 23S rRNA also. So far, the main focus of phylogenetic study was 16 S rRNA because of its smaller length and a very robust database of whole lot of species for comparison. Since the cost of sequencing has come down significantly now, 23S rRNA is gaining importance for sequence comparisons. Because of its length, it possesses greater sequence variation and characteristic sequence stretches, unique insertions/deletions, thus aiding a better phylogenetic characterization.

Sequences of 23S rRNA of cyanobacteria are meagre, the only sequences at present being those derived from whole genome shotgun sequences restricted to certain groups of cyanobacteria (del Campo *et al*., 2010). This sequence was mainly selected for this study to assess the robustness of this sequence database and to ascertain whether it corroborates well with the phylogenetic tree made from 16S rRNA database. DNA was extracted and PCR was done using 23S rRNA specific primers.

Forward primer: AGGGGTAAAGCACTGTTTCG

Reverse primer: CCTTCTCCCGAAGTTACG

The PCR cycling conditions were as follows: an initial denaturation at 94^{o}C for 2 min was followed by 40 cycles of 94^{o}C for 30s, 55^{o}C for 60s and 72^{o}C for 120 s followed by a final extension of 72^{o}C for 7 min.

PCR product of 800bp was directly sequenced and identified as described in 2.2.7.4.2.1.

2.2.7.4.2.3. 16S-23S Internally Transcribed Spacer (ITS)

The operons containing the genes coding for the three ribosomal RNAs (16S, 23S, 5S) including their spacers are present in multiple copies in prokaryotes. The heterogeneity shown by ribosomal RNA gene is reflected in the spacers also. The internally transcribed spacer present between 16S and 23S rRNA is a region that is suited for evolutionary and phylogenetic studies. In the case of *E.coli* some ITS regions contain a gene for tRNA for glutamine while others code for isoleucine and alanine. Such heterogeneities can make out interspecies variation.

In this study, the sequence of ITS was also amplified by specific primers described by Iteman et al (2000); Laloui et al, (2002)

Forward primer 322: TGTACACACCGCCCGTC

Reverse primer 340: CTCTGTGTGCCTAGGTATCC

The PCR cycling conditions were as follows:

Initial denaturation at 95^{o}C for 5 min was followed by 30 cycles of 94^{o}C for 60s, 55^{o}C for 60s and 72^{o}C for 60 s followed by a final extension of 72^{o}C for 10 min.

PCR product of 500bp was cloned into pGEMT easy vector, plasmid extracted, sequenced and identified as described in 2.2.7.4.2.1.

2.2.7.4.2.4 DNA-Dependent RNA Polymerase

The DNA-dependent RNA polymerase of cyanobacteria contains a unique core component, gamma, which is absent from the RNA polymerases

of other eubacteria. RNA polymerase in prokaryotes generally consists of a catalytic core of four subunits (ββ'α2) and a dissociable sigma factor, which confers promoter specificity. The basic structure of this enzyme was believed to be the same in all eubacteria until the RNA polymerase of the filamentous cyanobacterium *Anabaena* strain was purified. The cyanobacterial core polymerase was found to contain, in addition to a β, a β' and two α's, a novel core subunit of 70kDa designated γ, which is absent from the RNA polymerases of other eubacteria. A region of DNA homologous to part of the *E.coli* β' (*rpoC*) is split between two linked genes *rpoC1* and *rpoC2*. Palenik and Haselkorn (1992) suggested that rpoC1 can serve as a discriminatory marker for cyanobacterial identification. Thus, the partial sequence of rpoC1 was amplified, sequenced and its similarity assessed with other sequences in databases and described in 2.2.7.4.2.1

Primers described by Wilson & Schembri (2000) were used.

Forward primer: GAGCTCYAWNACCATCCAYTCNGG

Reverse primer: GGTACCNAAYGGNSARRTNGTTGG

The PCR cycling conditions were as follows:

An initial denaturation at 95^{o}C for 10 min was followed by 35 cycles of 92^{o}C for 90s, 58^{o}C for 60s and 72^{o}C for 120 s followed by a final extension of 72^{o}C for 10 min.

PCR product of approximately 500 bp was cloned into pGEMT easy vector, plasmid extracted, sequenced and identified as described in 2.2.7.4.2.1

2.2.7.4.2.6 Intergenic spacer between Phycocyanin B and A subunits of Phycocyanin Operon

The genes encoding the major light-harvesting accessory pigment proteins, particularly the phycocyanin operon (*cpc*), including the IGS between *cpc*B and *cpc*A and the corresponding flanking regions (*cpc*BA-IGS), have also been targeted for phylogenetic studies of cyanobacteria. The nucleotide substitution rate in this region is potentially higher leading to higher polymorphism than that of the widely used 16S rRNA gene sequence (Lyra *et al.* 2001). Sequence analyses of these genes are thus helpful in differentiating genotypes below the generic level. Besides, they are relatively large-sized in comparison with other genes encoding for the photosynthetic pigments (~700-800bp) and are ubiquitous in cyanobacteria. In this study a part of the operon coding for *cpc*B, *cpc*A and the intergenic spacer between them (IGS) could be amplified and its heterogeneity studied.

Primers

Forward primer:
TAGTGTAAAACGACGGCCAGTTGYYTKCGCGACATGGA

Reverse primer:
TAGCAGGAAACAGCTATGACTATGACGTGGTGTARGGGAAYTT

The PCR cycling conditions were as follows:

An initial denaturation at 94^{o}C for 7 min was followed by 40 cycles of 94^{o}C for 20s, 50^{o}C for 30s and 72^{o}C for 60 s followed by a final extension of 72^{o}C for 7 min.

PCR product of approximately 500 bp was cloned into pGEMT easy vector, plasmid extracted, sequenced and identified as described in 2.2.7.4.2.1

2.2.7.4.2.1 Ribulose bisphosphate carboxylate large subunit (rbcL)

Ribulose bisphosphate carboxylase is a key enzyme for carbon dioxide fixation in the Calvin cycle, thus making it universal in cyanobacteria and plastids. As an important enzyme, its sequence is conserved throughout CO_2 fixing groups. Hence, the sequence analysis of this enzyme in a partial state was attempted in the current work. It was PCR amplified using the following primers.

Forward primer: GGCGGCAGGTAAGAAAGGGTTTCGTA

Reverse primer: CGTAGCTTCCGGTGGTATCCAGT

The PCR cycling conditions were as follows:

An initial denaturation at 94^{o}C for 10 min was followed by 35 cycles of 94^{o}C for 30s, 56^{o}C for 30s and 72^{o}C for 120 s followed by a final extension of 72^{o}C for 10 min.

PCR product of approximately 800 bp was cloned into pGEMT easy vector, plasmid extracted, sequenced and identified as described in 2.2.7.4.2.1

2.3 Results

Cyanobacterial strains MCCB 114, MCCB 115 and MCCB 238 were maintained in MN media. When they were subcultured into MN media solidified with 2% agar, cyanobacteria did not grow. But when it was grown in MN media solidified using 0.7% purified Difco bacto agar described by Waterbury (1986), distinct colonies developed after 10 days of incubation. These colonies had bacteria growing on periphery, when observed under 10X

objective of bright field microscope. They were then streaked again of fresh media and upon 9-12 transfers, bacterial free cultures were obtained (Fig 1).

Axenicity of cyanobacterial cultures was proven under the following conditions:

No growth was observed in the dark incubated bacterial test media

No bacterial cells were found when cultures were observed under fluorescent microscope after staining with DAPI

The DNA sequence for 16S rRNA corresponded with genus *Cyanobacterium* in the NCBI database.

The characteristics of the three strains are described below:

2.3.1 *Cyanobacterium* MCCB 114

Cyanobacterium MCCB 114 had round, entire and convex colonies on MN agar (Fig 2). Upon examination under microscope, the cells were spherical, approximately 2µ in diameter and mode of cell division was symmetrical transverse binary fission in a single plane (Fig 3). In an exponential phase culture, most of the cells were in the state of binary fission. The cells were non-motile and they did not exhibit phototaxis. They clumped together and settled down when kept undisturbed. The glycocalyx appeared thick on negative staining (Fig 4). They were producing copious amounts of exopolysaccharides visible by staining with alcian blue (Fig 5) and the cells were embedded in them as culture aged. Examination under scanning electron microscope showed that the cell wall was having a thick, loosely attached layer of glycocalyx corresponding to mucilaginous slime (Fig 6). Sections observed under transmission microscope (Fig 7) revealed that the cells were

having thylakoids in parallel rows and located throughout the cell. There were subcellular granules of polyphosphate and starch.

The morphological and ultrastructural features placed them in the Subsection 1 of Cyanobacteria (Order Chroococcales). Symmetrical binary fission in single plane, spherical cells of approximately 2μ and arrangement of thylakoids placed them in Form Genus *Cyanobacterium*. The cells possessed the following characters:

The cellular absorption spectra (Fig 8) revealed pigment peaks at approximately 670nm and approximately 620nm revealing the presence of chlorophyll a and phycocyanin. Allophycocyanin and phycoerythrin were absent. They were having a wide pH range tolerance from 5 to 11 and a tilt towards alkaline pH with the optimum being 8 (Fig 9). They preferred fresh water media corresponding to a value 0% w/v of NaCl. They however tolerated upto 40% w/v of NaCl. For a marine isolate, this was an indicator that they were fresh water organisms having tolerance to high salinities (Fig 10). The cells grew in the temperature range 18-40^{o}C (Fig 11). Optimum temperature was 28^{o}C. The peculiarity of this cyanobacterium is that it can tolerate high temperatures. Results of chemoheterotrophy (incubation in darkness) revealed that all the plates were negative. In photoheterotrophy experiments (incubation under light) they showed a weak response to succinate and failed to grow in pyruvate, acetate, glutamate and dextrose. They did not fix atmospheric nitrogen. They did not use sulphide as electron donor under anaerobic conditions. They did not deposit $CaCO_3$ crystals on the sides of conical flask.

DNA sequence determination studies revealed that all the genes studied were showing similarity to *Cyanobacterium aponinum* isolated from the

thermal waters in Padua, Italy whose whole genome sequence has been published. This particular isolate was thermotolerant and living in fresh water. So, isolate MCCB 114 is undoubtedly belonging to the genus *Cyanobacterium* by sequence analysis also.

The gene sequences deposited in Genbank for this strain were as follows:

Cyanobacterium MCCB 114 16S rDNA complete sequence : KM275588
Cyanobacterium MCCB 114 23S rDNA partial sequence : KM359967

Cyanobacterium MCCB 114 phycocyanin beta subunit (cpcB) gene, partial cds; cpcB-cpcA intergenic spacer, complete sequence; and phycocyanin alpha subunit (cpcA) gene, partial cds : KM359971

Cyanobacterium MCCB 114 DNA-directed RNA polymerase subunit gamma (rpoC1) gene, partial cds : KM376969

Cyanobacterium MCCB 114 ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit (rbcL) and chaperonin family protein RbcX (rbcX) genes, partial cds : KM376970

The phylogenetic trees comparing the sequences of all the studied isolates as well as sequence deposits in the genbank database are shown in figures 12 to 16.

2.3.2 *Cyanobacterium* MCCB 115

Cyanobacterium MCCB 115 had irregular, undulate and flat colonies (Fig 17) on MN agar. Upon examination in microscope, the cells were spherical, approximately 2µ in diameter, and mode of cell division was symmetrical transverse binary fission in a single plane (Fig 18). In an exponential phase culture, most of the cells were in the state of binary fission. The cells were

motile as revealed by positive phototaxis when subjected to unilateral illumination. This may be gliding motility or attributed to Type 1V Pili. Thick glycocalyx was visible on negative staining (Fig 19). Exopolysaccharides were visible by staining with alcian blue (Fig 20) but they were less in comparison to MCCB 114. Examination under scanning electron microscope revealed that cell wall was having protrusions all over (Fig 21) and whether it was glycocalyx or any motility organelle like pili was to be identified. On TEM, thylakoids were arranged parallel to each other and found in the entire area of cell other than the area of nucleoplasm. There were subcellular granules of polyphosphate and starch. The outer layer of visible wall layer was very peculiar and whether it was pili was to be identified (Fig 22).

The morphological and ultrastructural features placed them in the Subsection 1 of Cyanobacteria (Order Chroococcales). Symmetrical binary fission in single plane, spherical cells of approximately 2m, and arrangement of thylakoids placed them in Form Genus *Cyanobacterium*. The cells possessed the following characters:

The cellular absorption spectra revealed pigment peaks at approximately 670nam and approximately 620nm revealing the presence of chlorophyll a and phycocyanin (Fig 23). Allophycocyanin and phycoerythrin were absent. They were having a wide pH range tolerance from 5 to 11 and a tilt towards alkaline pH with the optimum being 8 (Fig 24). The cells grew in the temperature range of 15-35^{o}C. Optimum temperature was 28^{o}C. They preferred fresh water media corresponding to a value 0%w/v of NaCl. They, however, grew in 20% w/v NaCl, but failed to grow in 30% w/v of NaCl (Fig 25).Since this organism came from a marine water source, it was due to its salt tolerance that it survived repeated subculturing in MN media with 25%w/v of NaCl. Results of chemoheterotrophy (incubation in darkness) revealed that

all the plates were negative. In photoheterotrophy experiments (incubation under light) they showed a very good response to pyruvate and weak response to acetate and succinate. They failed to grow in glutmate and dextrose. They did not fix atmospheric nitrogen. They did not use sulphide as electron donor under anaerobic conditions. They deposited $CaCO_3$ crystals on the sides of conical flask (Fig 26).

DNA sequence determination studies revealed that all the genes studied were showing similarity to *Cyanobacterium aponinum*. The isolate MCCB 115- undoubtedly could be placed under genus *Cyanobacterium* by sequence analysis also.

The gene sequence accession numbers in NCBI Genbank were as follows:

Cyanobacterium MCCB 115 16S rDNA complete sequence :KM275589

Cyanobacterium MCCB 115 23S rDNA partial sequence :KM359968

Cyanobacterium MCCB 115 phycocyanin beta subunit (cpcB) gene, partial cds; cpcB-cpcA intergenic spacer, complete sequence; and phycocyanin alpha subunit (cpcA) gene, partial cds :KM359972

Cyanobacterium MCCB 115 cpc*A* and IGS partial sequence:KM359973

Cyanobacterium MCCB 115 DNA-directed RNA polymerase subunit gamma (rpoC1) gene, partial cds :KM376968

Cyanobacterium MCCB 115 ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit (rbcL) and chaperonin family protein RbcX (rbcX) genes, partial cds :KM376971

The phylogenetic trees comparing the sequences of all the studied isolates as well as sequence deposits in the genbank database are shown in figures 12 to 16.

2.3.3 *Cyanobacterium MCCB 238*

Cyanobacterium MCCB 238 had round, entire and convex colonies on MN agar just like MCCB 114 (Fig 27). Upon examination under a microscope, the cells were spherical, approximately 2µ in diameter and mode of cell division was symmetrical transverse binary fission in a single plane (Fig 28). In an exponential phase culture, most of the cells were in the state of binary fission. The cells were non-motile but it lacked cell aggregation unlike MCCB 114. The glycocalyx was visible on negative staining and it was having a capsule like appearance. They were producing copious amounts of exopolysaccharides visible by staining with alcian blue. The examination under scanning electron microscope showed that the cell wall was having a thin layer of glycocalyx with small hair like projections. TEM analysis revealed that the cells were having thylakoids that were in parallel rows and located throughout the cell. There were polyphosphate granules and starch in cytoplasm.

The morphological and ultrastructural features placed them in the Subsection 1 of Cyanobacteria (Order Chroococcales). Symmetrical binary fission in single plane, spherical cells of approximately 2.5m and arrangement of thylakoids placed them in Form Genus *Cyanobacterium*.

The cellular absorption spectra revealed pigment peaks at approximately 670nam and approximately 620nm indicating the presence of chlorophyll a and phycocyanin. Allophycocyanin and phycoerythrin were absent. They were having a wide pH range tolerance from 5 to 11 and a tilt towards alkaline pH with the optimum being 8. They preferred fresh water media corresponding to a value 0%w/v of NaCl. They, however, tolerated up

to 30% w/v of NaCl. This can very well adapt to marine environment with ease. The cells grew in the temperature range of 18-35^{o}C. Optimum temperature was 28^{o}C. Results of chemoheterotrophy (incubation in darkness) revealed that all the plates were negative. In photoheterotrophy experiments (incubation under light) they showed a high response to acetate and weak response to succinate and dextrose. They failed to grow in pyruvate and glutamate. They did not fix atmospheric nitrogen. They did not use sulphide as electron donor under anaerobic conditions. They deposited $CaCO_3$ on the sides of glass surface.

DNA sequence determination studies revealed that all the genes studied were showing similarity to *Cyanobacterium aponinum* described earlier. This particular isolate was thermotolerant and living in fresh water. So, isolate MCCB 238 undoubtedly belonged to genus *Cyanobacterium* by sequence analysis also.

The gene sequence accession numbers in NCBI Genbank were as follows:

Cyanobacterium MCCB 238 16S rDNA complete sequence :KM275590

Cyanobacterium MCCB 238 23S rDNA partial sequence :KM359969

Cyanobacterium MCCB 238 phycocyanin beta subunit (cpcB) gene, partial cds; cpcB-cpcA intergenic spacer, complete sequence; and phycocyanin alpha subunit (cpcA) gene, partial cds :KM363254

Cyanobacterium MCCB 238 ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit (rbcL) and chaperonin family protein RbcX (rbcX) genes, partial cds :KM376972

The phylogenetic trees comparing the sequences of all the studied isolates as well as sequence deposits in the genbank database are shown in figures 12 to 16.

2.3.4 Comparing the three strains

The cyanobacterial strains under study existed in the culture collection as *Synechocystis* when the identity was ascertained by morphological characters. Polyphasic study placed them in a different genus *Cyanobacterium*.

They might have originated in fresh waters and adapted themselves to marine waters. The phylogenetic analysis see them clustering closely to each other be it 16S, 23S, rbCl, rpoC1or cpcBA IGS. Though they differ in their glycocalyx structure, motility and utilization of certain growth compounds; they have a common ancestor and all other differences were dependent on the external environment. Hence, they may be termed ecotypes. It is to be noted that there are not much sequence deposits for comparison and construction of robust phylogenetic trees right now, but as sequence deposits increase in database, the results will be useful. So no attempt is made to assign specific (species) name to these organisms at this juncture.

2.4 Discussion

It is true that confusions and ambiguities exist in the taxonomy of Phylum Cyanobacteria. The second edition of Bergey's manual of systematic bacteriology attempted a comprehensive classification of this phylum which changed orders into Subsections and Genera to Form-Genera. In this edition, Phylum Cyanobacteria comprises of a single Class Cyanobacteria which is further divided into five subsections.

Subsection I (Formerly Chroococcales)

Subsection II (Formerly Pleurocapsales)

Subsection III (Formerly Oscillatoriales)

Subsection IV (Formerly Nostocales)

Subsection V (Formerly Stigonematales)

Each of these subsections is divided into families and then into Form-Genera.

Form-Genus III Cyanobacterium comes under Subsection I (Formerly Chrococcales). In this genus phycobiliproteins and phycobilisomes are present in thylakoids. Reproduction is symmetrical transverse binary fission in single plane and absence of a structured sheath. Cells are spherical to rod-shaped, 1.7-2.3µm in diameter, thylakoids are parallel and dispersed throughout the cell and mean DNA base composition is 39-41 mol% G+C. The type species as per Botanical code is *Cyanobacterium stanieri.*

Morphological and ultrastructural features placed the three isolates *Cyanobacterium* MCCB 114, MCCB 115 and MCCB 238 into Subsection I and Form Genus III *Cyanobacterium*. 16S rRNA gene sequence was the next robust dataset available to establish the identity of the organisms. When DNA sequence comparisons were made using NCBI blast, *Cyanobacterium* MCCB 114, 115 and 238 had 99% sequence similarity with each other. When neighbor-joining phylogenetic tree was constructed, they all showed 99% similarity to the following deposits in Genbank; *Cyanobacterium aponinum* PCC 10206 isolated from thermal pond in Italy, Cyanobacterium MBIC 10216 from Japan, *Cyanobacterium aponinum* ThrSCCsp4 and *Cyanobacterium aponinum* IklSCC30 isolated from hot spring mats of Greece and *Cyanobacterium aponinum* KSU-WH-5 from freshwater bodies of Saudi Arabia, They showed 97% similarity to two uncultured bacterium clones LVB1, VDB19 isolated from hot springs in Velingrad region, Bulgaria. They

showed only 96% similarity to *Cyanobacterium stanieri*, the type strain as per botanical classification.

Polyphasic taxonomical studies of *Cyanobacterium aponinum* PCC 10206, isolated from Euganean Thermal Springs, Padua, Italy (Moro *et al*., 2007) showed that it was similar morphologically and ultrastructurally to the isolates under study. *Cyanobacterium aponinum* ThrSCCsp4 and *Cyanobacterium aponinum* IklSCC30 isolated from two separate hot spring mats of Greece (Bravakos *et al*., 2016) also showed morphological similarity to the isolates under study. So, all the isolates can be considered as sister species of *Cyanobacterium aponinum* PCC 10206.

Two inferences that can be made from the results were that, *Cyanobacterium* sp. MCCB 114, MCCB 115 and MCCB 238 were closely related to *Cyanobacterium aponinum* discovered from freshwater bodies from Italy, Japan, Greece and Saudi Arabia and two uncultured organisms from Bulgaria. All the related isolates were thermotolerant. Moreover, *Cyanobacterium* MCCB 114, 115 and 238 were isolated from the marine environment but showed optimum growth at 0%w/v NaCl, which showed it was also a freshwater species adapted to higher salinities.

The cyanobacteria are an excellent group of organisms that demonstrate the evolution of tolerance. During its evolutionary history, this ancient lineage of oxygen-evolving, photoautotrophic bacteria has established itself in diverse aquatic and terrestrial habitats exhibiting wide ranges in temperature, salinity, water potential, pH, and irradiance (Whitton & Potts, 2012). Castenholz (1996) had identified the invasion of the genus *Synechococcus* in alkaline hot spring habitats across western North America, Asia, Africa, and possibly

Europe. It has to be noted that similar to genus *Synechococcus*, genus *Cyanobacterium* is also showing a widespread distribution in water bodies at elevated temperatures.

Cyanobacteria are hardy organisms that are well represented in planet's harshest environments; boiling deep-sea rift vents, volcanic craters, and polar glaciers. Among extremophiles, thermophiles are widely studied as hot spring mat communities are teeming with cyanobacteria. The heat tolerance of thermophilic microorganisms is conferred by configurations of protein and nucleic acid components within the cell, which is less reliant on hydrogen bonding to preserve their spatial structure (Stetter, 1999).

Papke et al. (2003) studied island-like hot spring cyanobacterial communities in which geographical isolation should be prominent and detectable if it influences the evolution of bacteria. The genetic diversity of cyanobacteria indigenous to North American, Japanese, New Zealand and Italian springs was surveyed and they concluded that geographical isolation was acting at global and local spatial scales leading to phylogenetic and distribution patterns now present in these communities and proposed that geographical isolation (i.e. genetic drift) must in part be responsible for driving the observed evolutionary divergences.

Though there have not been many studies to support or disprove their findings, the presence of thermotolerant *Cyanobacterium* with 99% 16S rRNA gene similarity from different parts of the world shows that there are indeed some organisms unchanged in these environments. The widespread intercontinental dispersion of *Cyanobacterium aponinum* brings to fore so many unanswered questions. Did they originate from a common ancestor that

was widespread millions of years ago, even before the oceans and continents were separated? Otherwise, was there a natural dispersion mechanism for these species which brought them to their current habitats? For an aquatic organism, seasonal inundation by rains or floods would have taken them to oceans. But these organisms reaching lakes and thermal springs at faraway places are such a rare occurrence. Such observations call for in-depth studies on the diversity and distribution of such an evolutionary significant group of prokaryotes.

Cyanobacterial diversity is a largely neglected component of biodiversity and diversity evaluations and conservations efforts so far have not focused on this group of organisms (Rejmankova et al., 2004). It is not easy to work out the taxonomy of this large group and different approaches had to be taken to study the diversity. Hence, studies can be taken up to divide cyanobacteria into functional groups and then understand their role in global productivity. If organisms showing specific characters need to be studied, for example, toxin producers or nitrogen fixers, the presence of functional genes associated with that physiology can be detected by the metagenomic approach and then, actual isolation of the organism from the environment can be attempted. If there is a need to understand the detailed dynamics and evolution of cyanobacterial communities, a sophisticated taxonomic system is needed, one that identifies organisms to a level that defines species boundaries (Hayes et al., 2007).

The principal aim of systematics is to discover, describe and classify the diversity of living organisms. Systematists have concluded that the basic unit of biological diversity is the species. However, there is no widely

accepted concept of species for prokaryotes, and assignment of isolates to species is based on measures of phenotypic or genome similarity. The current methods for defining prokaryotic species are inadequate and incapable of keeping pace with the levels of diversity that is being uncovered in nature (Stackebrandt et al., 2002).

Attempts to classify cyanobacteria by polyphasic approach is widely undertaken (Abed et al., 2002; Comte et al., 2007; Moro et al., 2007; Zapomělová et al., 2009; Dadheech et al., 2012; Sciuto et al., 2012; Lee et al., 2014; Bravakos et al., 2016; Jancusova, 2016; Kurmayer et al., 2017). It is necessary that classification of cyanobacteria need to be continually updated in accordance with revisions based on molecular sequence comparisons and combined with morphological features, ecophysiological characters and biochemical and molecular markers (Komarek and Mares, 2012).

Almost all traditional (Geitlerian) cyanobacterial genera were confirmed by molecular level. There were also divisions of many existing genera into more generic units, characterized both genetically and morphologically. Even though generic names were changed for many organisms, unfortunately, incorrect names are still used in the database of sequences like Genbank (For eg. Anacystis nidulans = Synechococcus nidulans, Anabaena variabilis = Trichormus variablils). Hence taxonomists must continually update to use the most modern taxonomic methods and necessary nomenclatural corrections should be applied to all strain collections and experimental studies. Though there does not exist any authority which can implement such changes, experimental workers should accept those revisions and move forward for the sake of science (Komarek, 2010).

Cyanobacterium MCCB 114

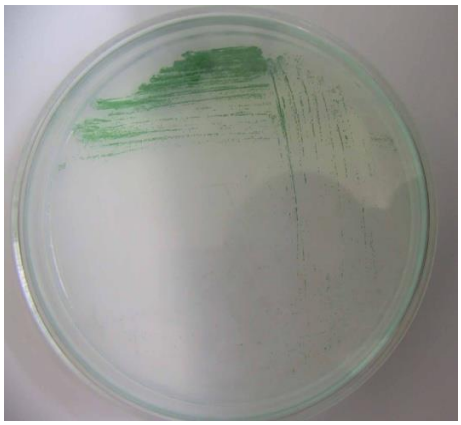


Fig 1. Axenic culture

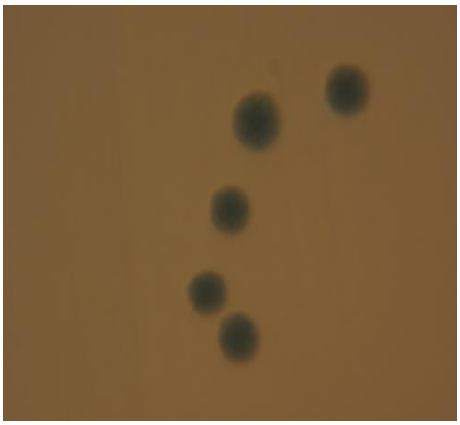


Fig 2. Colony morphology

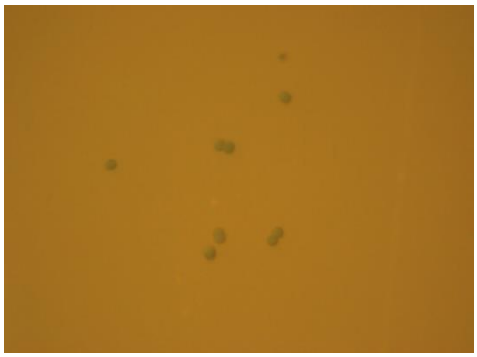


Fig 3. Cells (40X) bright field

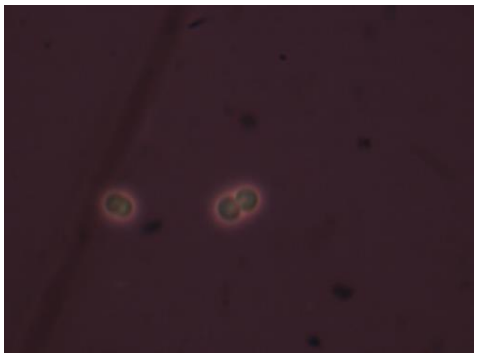


Fig 4. Capsule structure by nigrosin staining

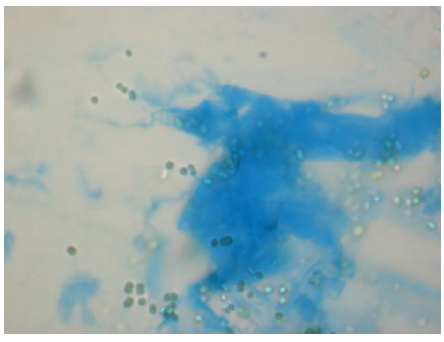


Fig 5. Alcian blue staining for EPS

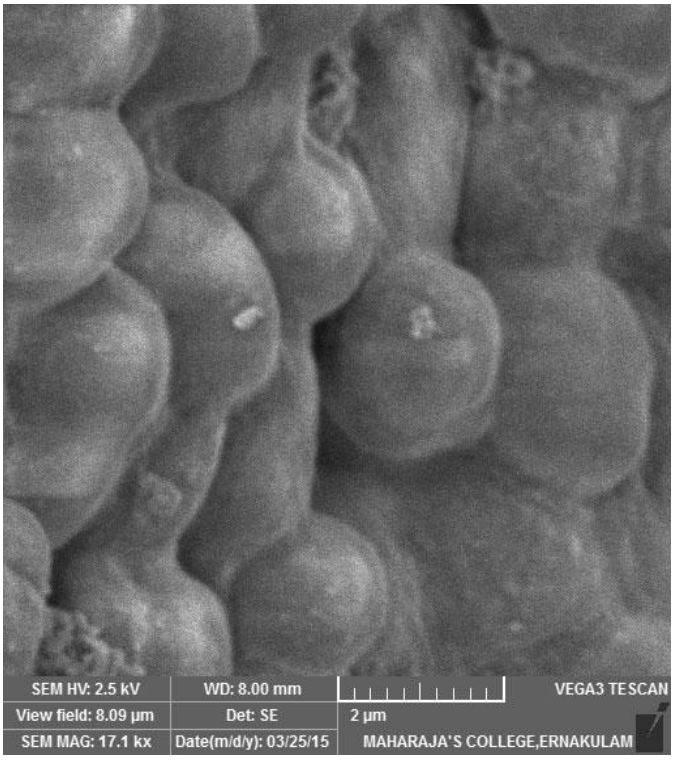


Fig 6. Scanning electron micrograph showing cell aggregation and glycocalyx

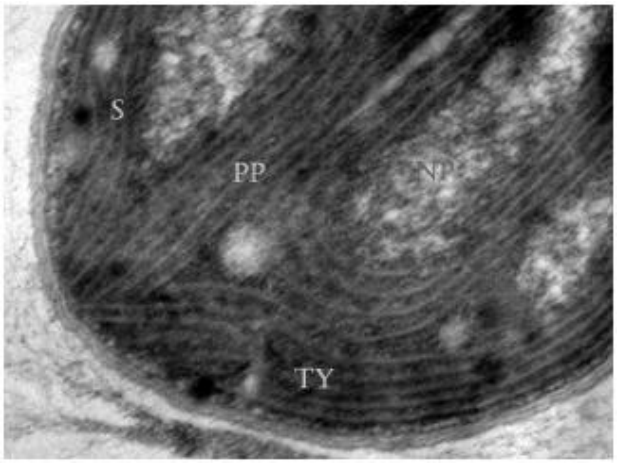


Fig 7. Transmission electron micrograph showing thylakoid (TY)arrangement, nucleoplasm (NP), polyphosphate granules(PP) and starch(S)

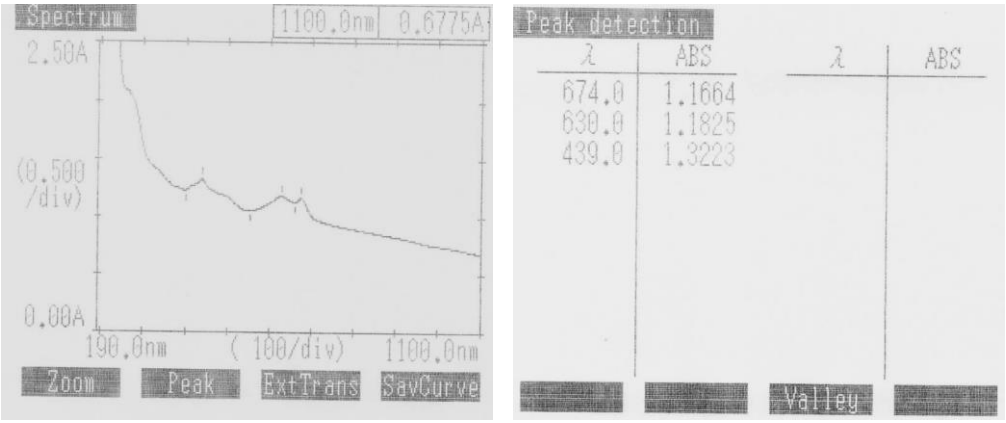
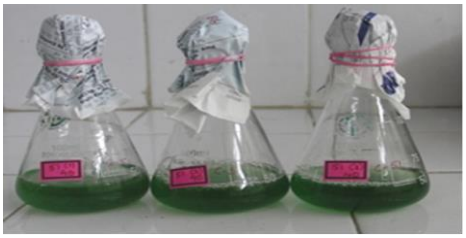


Fig 8. Cellular absorption spectra showing peaks: 674 corresponding to Chlorophyll a and 638 corresponding to Phycocyanin

Fig 9. pH range and tolerance

Fig 10. Salinity range and tolerance. From left, 0, 10, 20, 30. 40, 50g w/v NaCl/


a


b

Fig 11. Growth at a temperature of a. 40°C and b.45°C

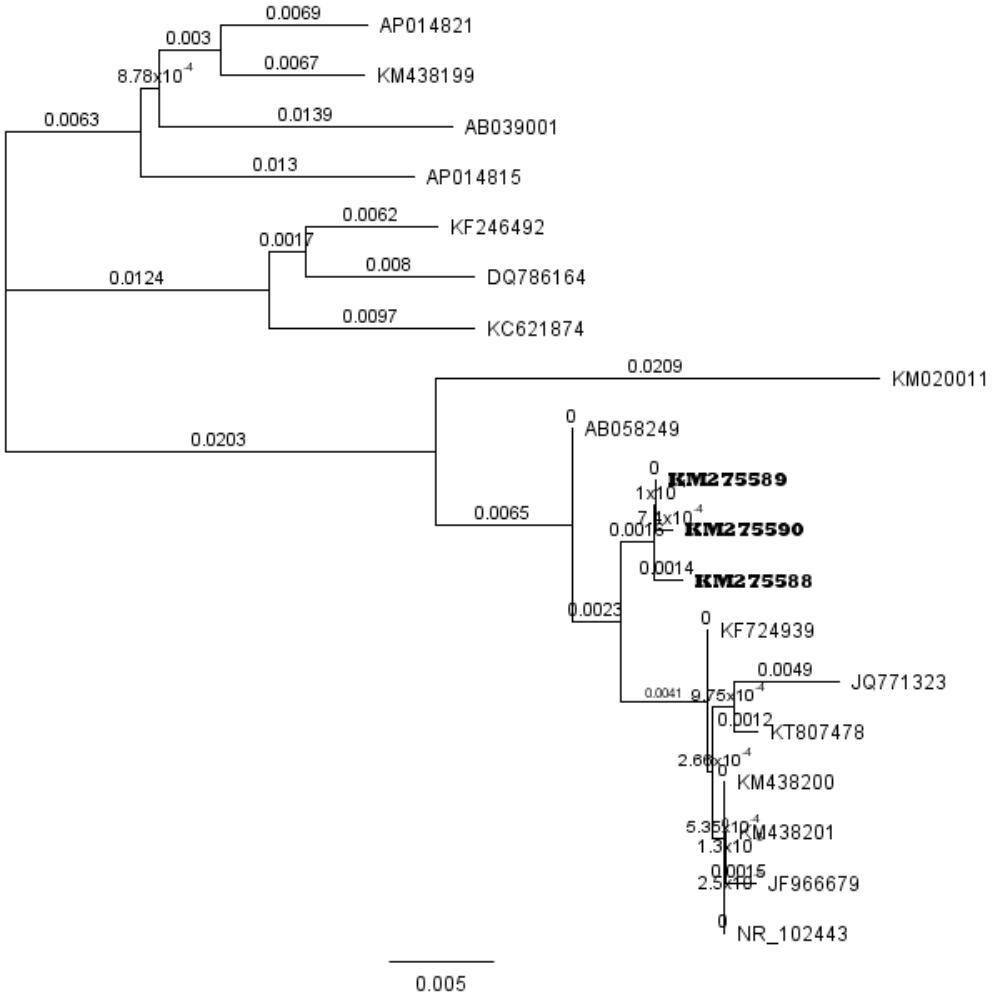


Fig. 12. Neighbour joining phylogenetic tree of 16S DNA of three strains (shown in bold). Please refer Appendix 1 for correlating organisms with accession numbers.

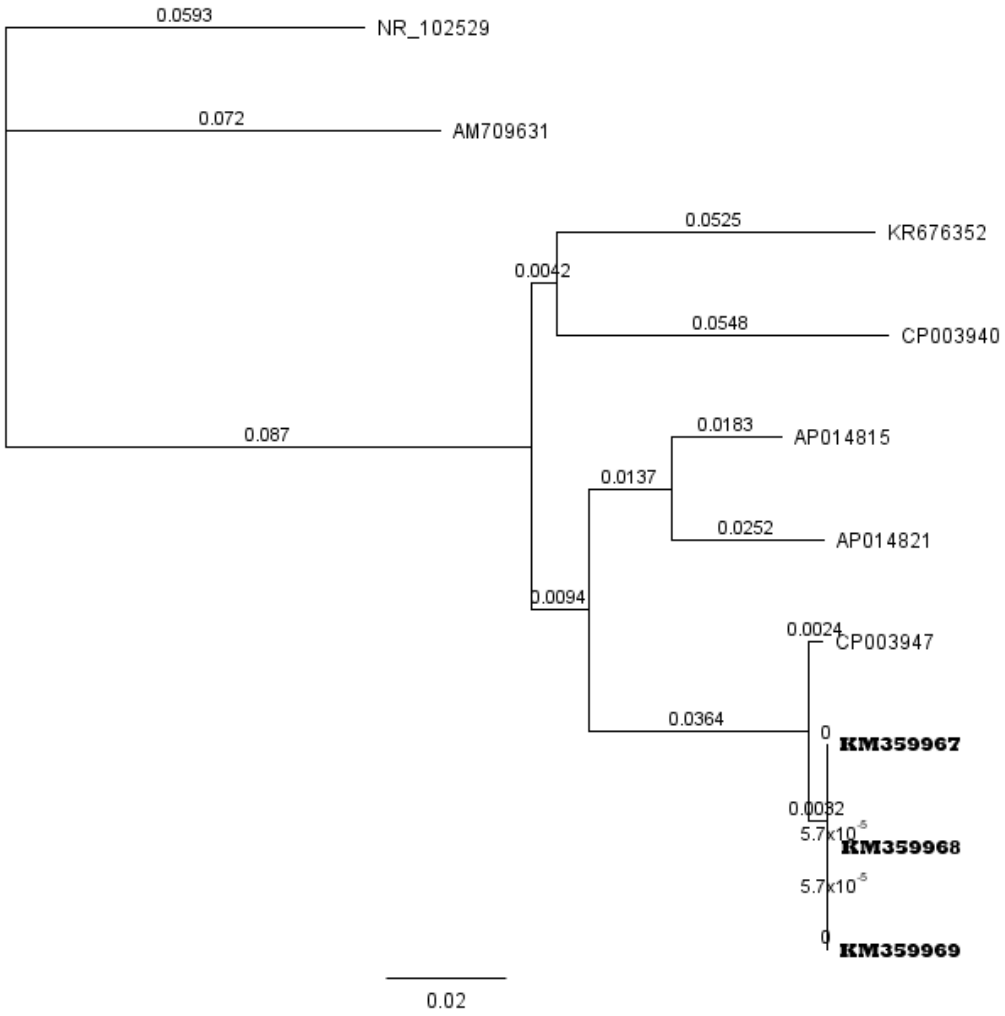


Fig. 13. Neighbour joining phylogenetic tree of 23 S DNA of three strains (shown in bold). Please refer Appendix 2 for correlating organisms with accession numbers.

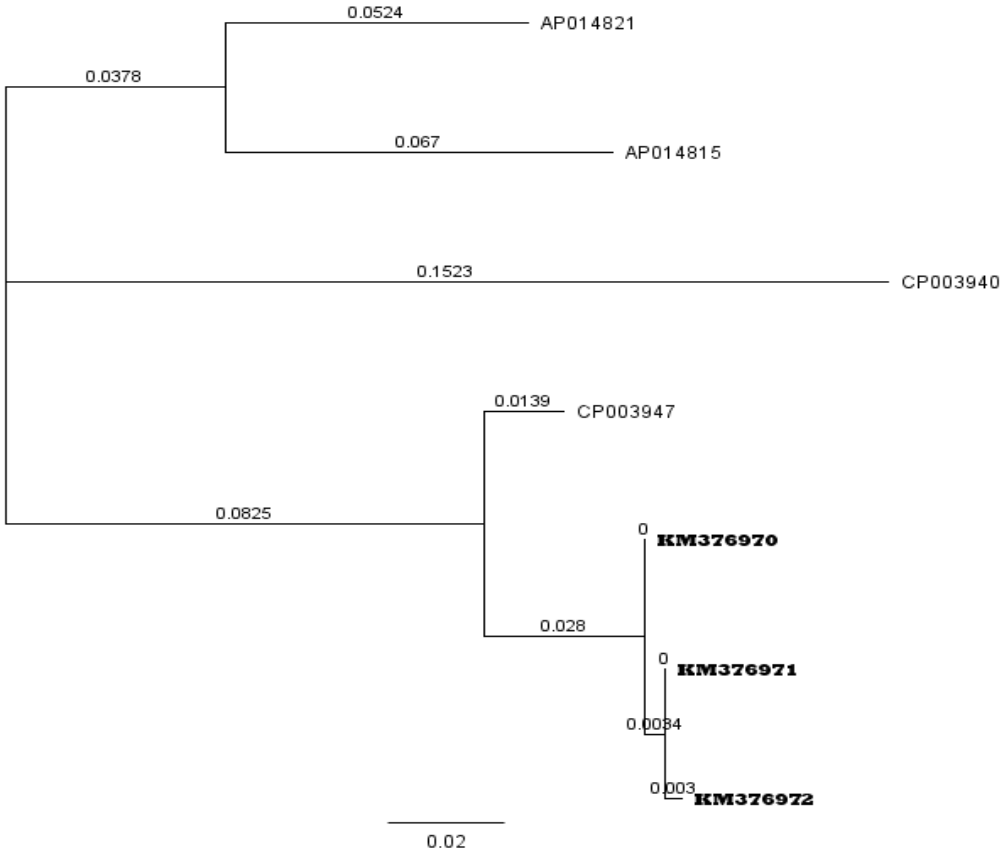


Fig. 14. Neighbour joining phylogenetic tree of rbcL of three strains (shown in bold). Please refer Appendix 3 for correlating organisms with accession numbers. Only a single sequence shows 96% similarity to the cyanobacterial strains used in the study.

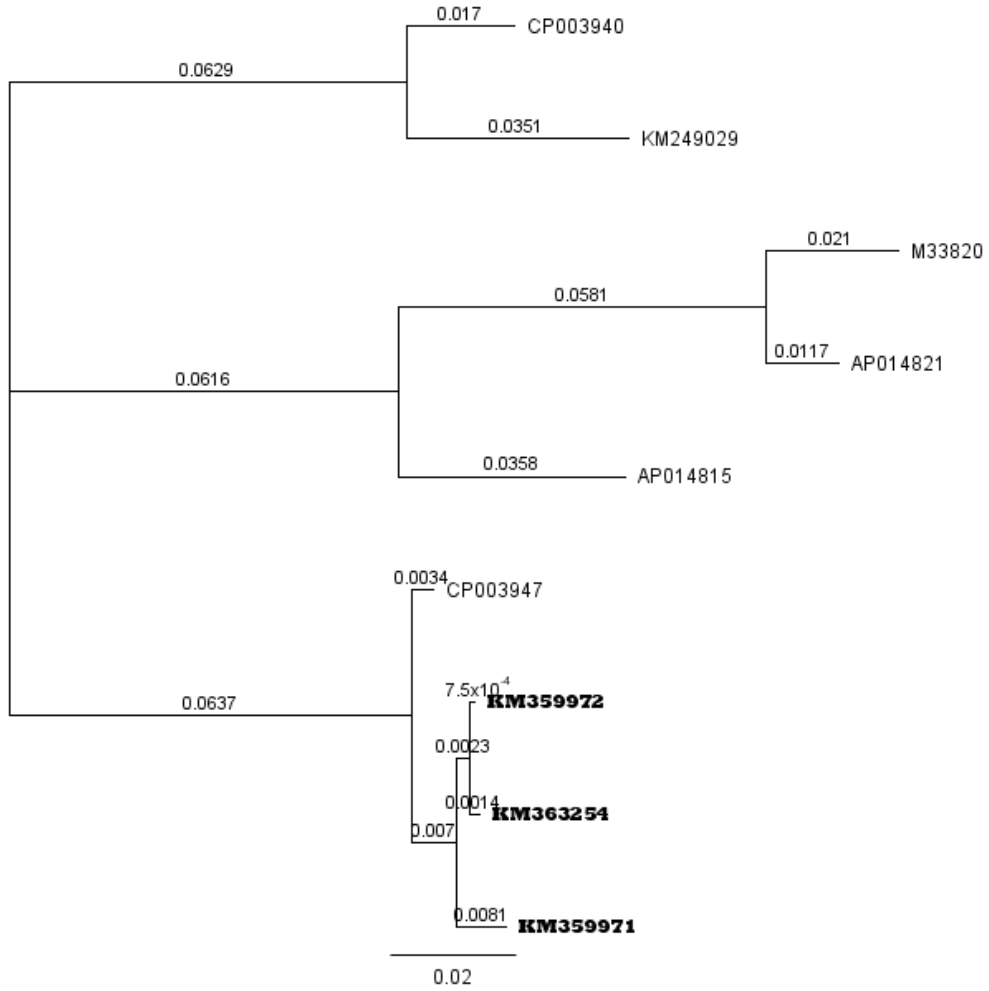


Fig. 15. Neighbour joining phylogenetic tree of phycocyanin subunit of three strains (shown in bold). Please refer Appendix 4 for correlating organisms with accession numbers. Only a single sequence shows 99% similarity to the cyanobacterial strains used in the study.

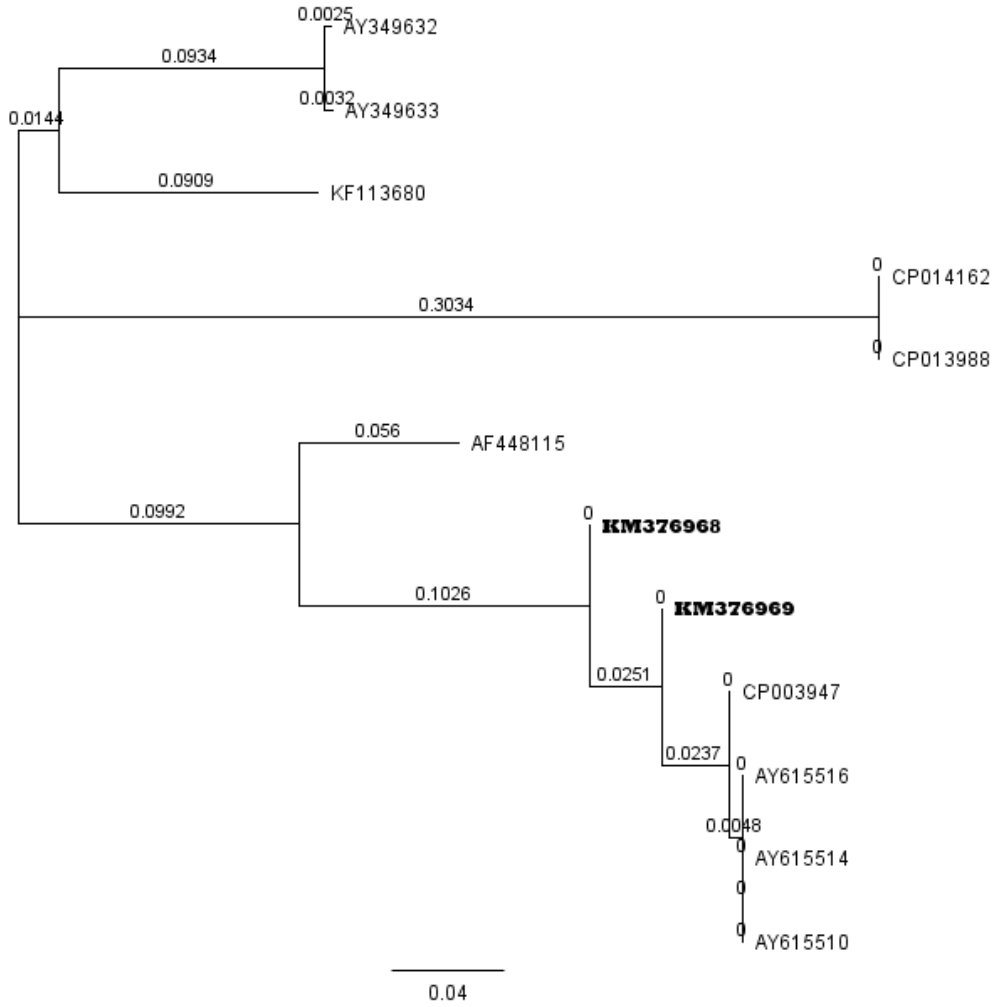


Fig. 16. Neighbour joining phylogenetic tree of rpoC1 subunit of two strains (shown in bold). Please refer Appendix 4 for correlating organisms with accession numbers. Only a single sequence shows 99% similarity to the cyanobacterial strains used in the study.

Cyanobacterium MCCB 115

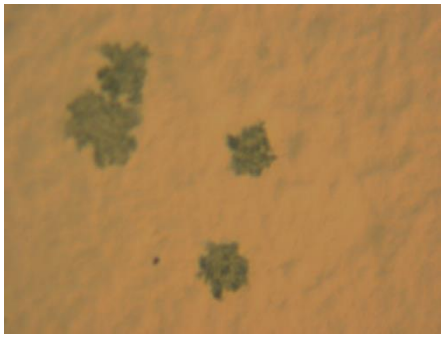


Fig. 17. Colony morphology

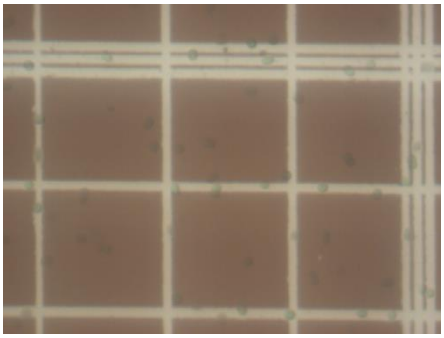


Fig. 18. Single cells in haemocytometer grid

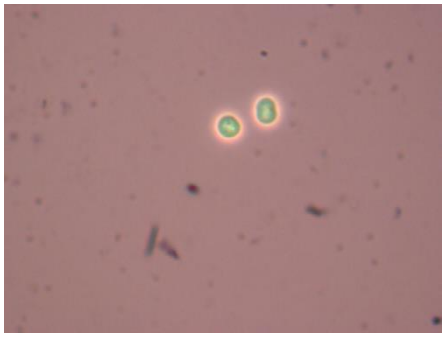


Fig 19. Capsule staining by Nigrosin

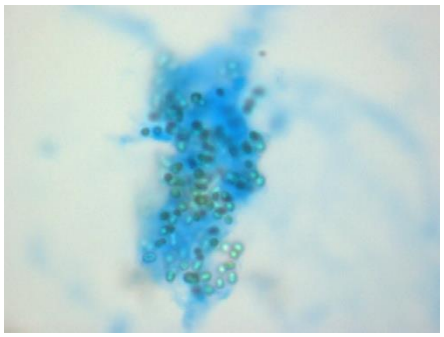


Fig 20. Alcian blue staining for-EPS

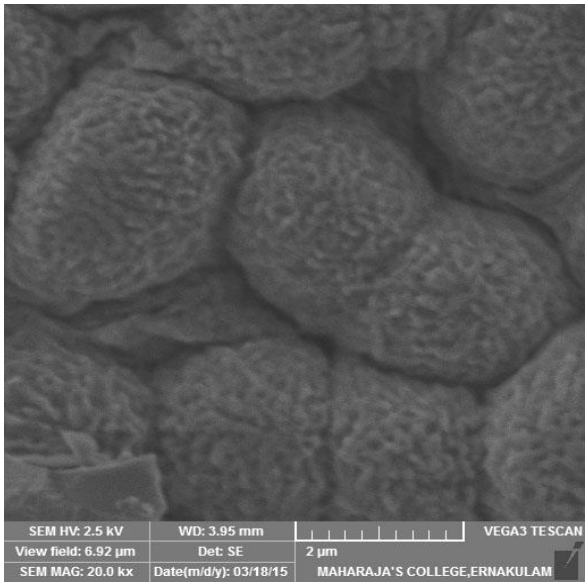


Fig 21. Scanning electron micrograph showing glycocalyx with irregular surface

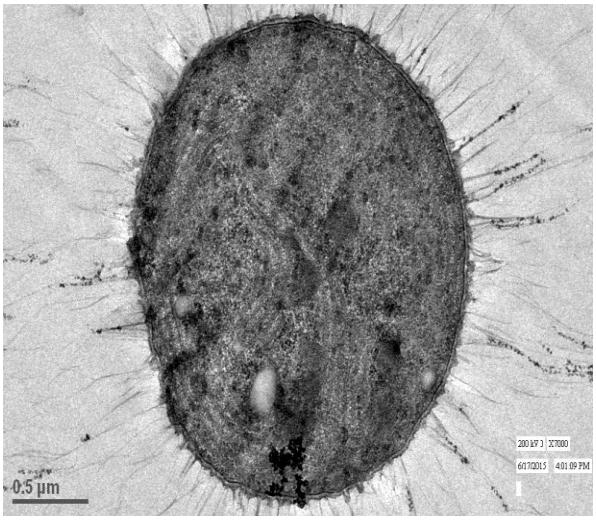
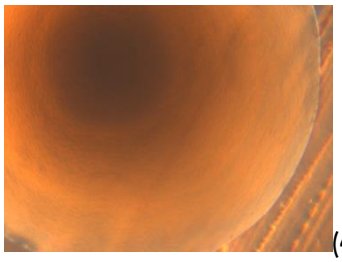


Fig 22. Transmission electron micrograph with pili like protrusions

Fig 23. Cellular absorption spectra showing peaks: 673 corresponding to Chlorophyll a and 622 corresponding to Phycocyanin

Fig 24. pH range and tolerance

Fig 25. Salinity range and tolerance. From left, 0, 10, 20, 30. 40, 50g w/v NaCl/L


(400x)

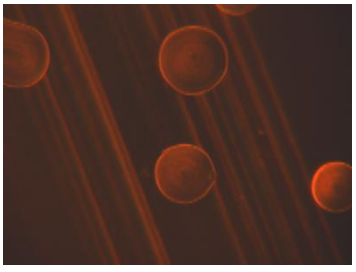

(100x)

Fig 26. $CaCO_3$ crystals on sides of conical flask

Cyanobacterium MCCB 238

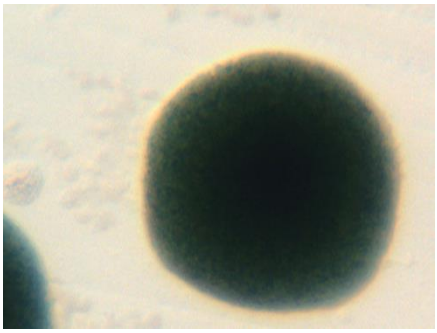


Fig 27. Colony morphology

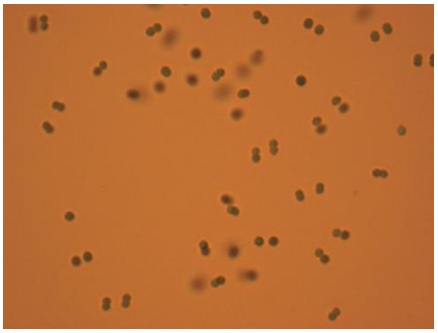


Fig 28. Single cells (40X)

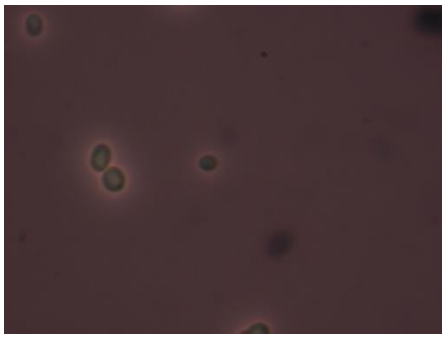


Fig 29. Capsular staining by Nigrosin

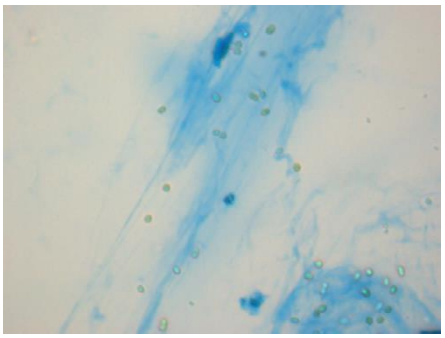


Fig 30. Alcian blue staining for EPS

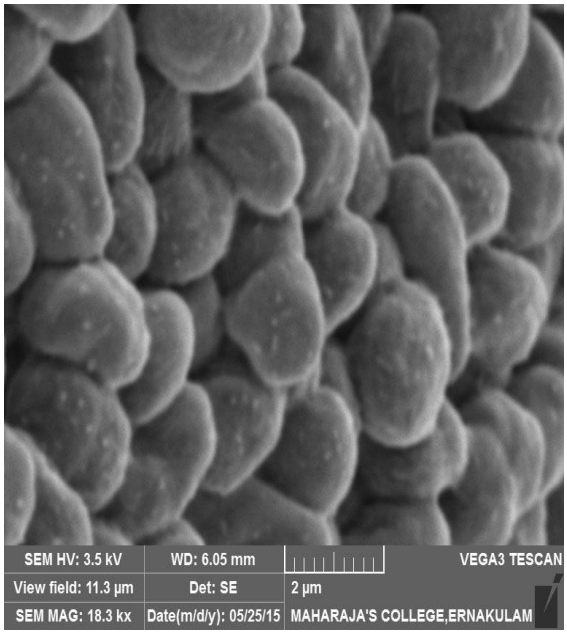


Fig 31. Scanning electron micrograph showing cells with thin sheath and widely dispersed projections on surface

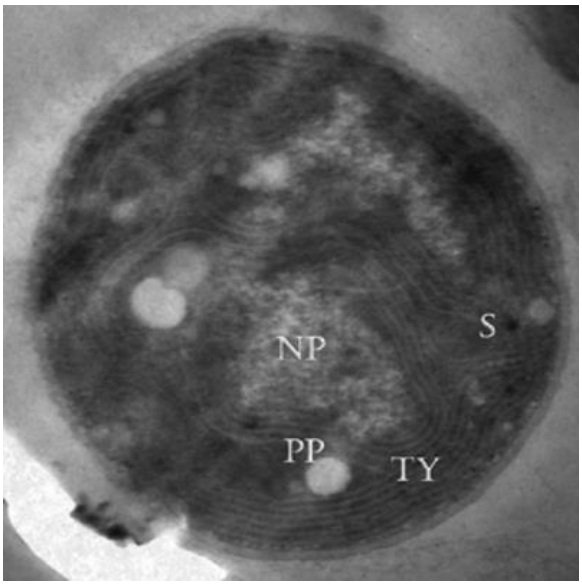


Fig 32. Transmission electron micrograph showing thylakoid (TY)arrangement, nucleoplasm (NP), polyphosphate granules(PP) and starch(S)

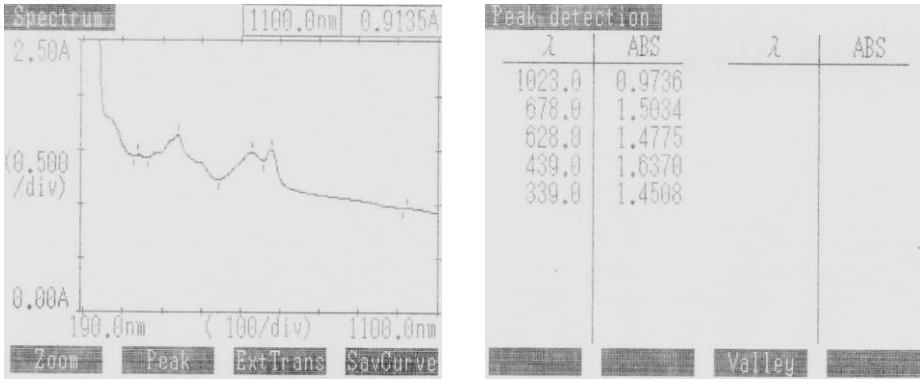


Fig. 33. Cellular absorption spectra showing peaks: 673 corresponding to Chlorophyll a and 622 corresponding to Phycocyanin

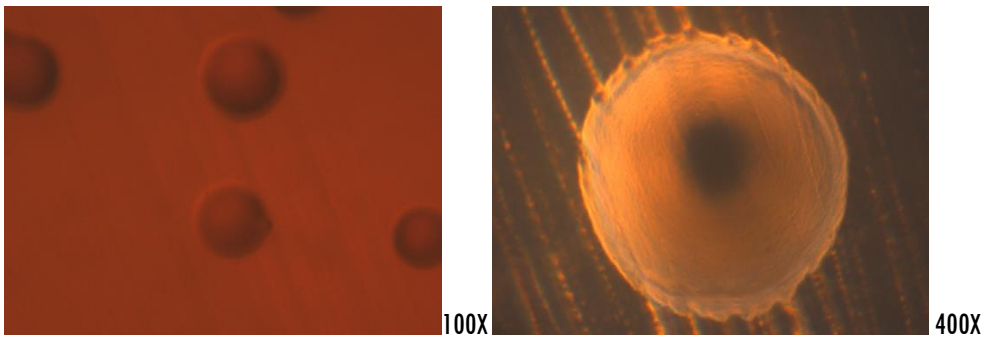


Fig. 34 $CaCO_3$ deposition on the sides of conical flask

Appendix 1. 16SrRNA Gene Alignments: NCBI Accession Number and Identity

- KM275590.1 Cyanobacterium sp. MCCB 238 16S ribosomal RNA gene, partial sequence
- KM275589.1 Cyanobacterium sp. MCCB 115 16S ribosomal RNA gene, partial sequence
- KM275588.1 Cyanobacterium sp. MCCB 114 16S ribosomal RNA gene, partial sequence
- NR_102443.1 Cyanobacterium aponinum strain PCC 10605 16S ribosomal RNA, partial sequence
- CP003947.1 Cyanobacterium aponinum PCC 10605, complete genome
- KM438201.1 Cyanobacterium aponinum IklSCC30 16S ribosomal RNA gene
- JF966679.1 Cyanobacterium IHB-410 16S ribosomal RNA gene, partial sequence
- AM238427.1 Cyanobacterium aponinum ETS-03 16S rRNA gene (partial)
- KM438200.1 Cyanobacterium aponinum ThrSCCsp4 16S ribosomal RNA gene
- KT807478.1 Cyanobacterium aponinum KSU-WH-5 16S ribosomal RNA gene, partial sequence
- KJ465919.1 Uncultured bacterium clone LVB1 16S ribosomal RNA gene, partial sequence
- KJ465958.1 Uncultured bacterium clone VDB19 16S ribosomal RNA gene, partial sequence
- JQ771323.1 Synechococcus elongatus str. Ramsar 16S ribosomal RNA gene, partial sequence

- KX062019.1 Uncultured bacterium clone PHS5 16S ribosomal RNA gene, partial sequence
- AB058249.1 Cyanobacterium sp. MBIC10216 gene for 16S rRNA, partial sequence
- KM020011.1 Synechocystis aquatilis SAG 90.79 16S ribosomal RNA gene, partial sequence
- KF724939.1 Synechococcus elongatus ISC 106 16S ribosomal RNA gene, partial sequence
- AP014821.1 Geminocystis sp. NIES-3709 DNA, complete genome
- JX023443.1 Uncultured Cyanobacterium sp. clone Fardillapur 16S ribosomal RNA gene, partial sequence
- FJ866623.1 Uncultured cyanobacterium clone BK-45-25 16S ribosomal RNA gene, partial sequence
- AP014815.1 Geminocystis sp. NIES-3708 DNA, complete genome
- DQ786164.1 Cyanobacterium sp. LLi5 16S ribosomal RNA gene, partial sequence
- KF246492.1 Geminocystis sp. CENA526 16S ribosomal RNA gene, partial sequence
- AB039001.1 Synechocystis PCC6308 gene for 16S rRNA, partial sequence
- KC621874.1 Cyanobacterium sp. THH 16S ribosomal RNA gene, partial sequence

Appendix 2. 23SrRNA Gene Alignments: NCBI Accession Number and Identity

- KM359969.1 Cyanobacterium sp. MCCB 238 23S ribosomal RNA gene, partial sequence

- KM359967.1 Cyanobacterium sp. MCCB 114 23S ribosomal RNA gene, partial sequence
- KM359968.1 Cyanobacterium sp. MCCB 115 23S ribosomal RNA gene, partial sequence
- CP003947.1 Cyanobacterium aponinum PCC 10605, complete genome
- NR_102522.1 Cyanobacterium aponinum PCC 10605 strain 23S ribosomal RNA, complete sequence
- AP014815.1 Geminocystis sp. NIES-3708 DNA, complete genome
- AP014821.1 Geminocystis sp. NIES-3709 DNA, complete genome
- KR676352.1 Geminobacterium atlanticum LEGE 0745923S ribosomal RNA gene, partial sequence
- CP024912.1 Cyanobacterium stanieri HL-69 chromosome, complete genome
- CP003940.1 Cyanobacterium stanieri PCC 7202, complete genome
- NR_102523.1 Cyanobacterium stanieri PCC 7202 strain PCC 7202 23S ribosomal RNA, complete sequence
- AP018281.1 Chondrocystis sp. NIES-4102 DNA, complete genome
- NR_102529.1 Stanieria cyanosphaera PCC 7437 strain PCC 7437 23S ribosomal RNA, complete sequence

Appendix 3. rbcL Gene Alignments: NCBI Accession Number and Identity

- KM376970.1 Cyanobacterium sp. MCCB 114 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) and chaperonin family protein RbcX (rbcX) genes, partial cds
- KM376971.1 Cyanobacterium sp. MCCB 115 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) and chaperonin family protein RbcX (rbcX) genes, partial cds

- KM376972.1 Cyanobacterium sp. MCCB 238 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) and chaperonin family protein RbcX (rbcX) genes, partial cds
- CP003947.1 Cyanobacterium aponinum PCC 10605, complete genome
- AP014821.1 Geminocystis sp. NIES-3709 DNA, complete genome
- AP014815.1 Geminocystis sp. NIES-3708 DNA, complete genome

Appendix 4. *cpc* BA-IGS Alignments: NCBI Accession Number and Identity

- KM359972.1 Cyanobacterium sp. MCCB 115 phycocyanin beta subunit (cpcB) gene, partial cds; cpcB-cpcA intergenic spacer, complete sequence; and phycocyanin alpha subunit (cpcA) gene, partial cds
- KM359971.1 Cyanobacterium sp. MCCB 114 phycocyanin beta subunit (cpcB) gene, partial cds; cpcB-cpcA intergenic spacer, complete sequence; and phycocyanin alpha subunit (cpcA) gene, partial cds
- CP003947.1 Cyanobacterium aponinum PCC 10605, complete genome
- KM363254.1 Cyanobacterium sp. MCCB 238 phycocyanin beta subunit (cpcB) gene, partial cds; cpcB-cpcA intergenic spacer, complete sequence; and phycocyanin alpha subunit (cpcA) gene, partial cds
- AP014815.1 Geminocystis sp. NIES-3708 DNA, complete genome
- CP003940.1 Cyanobacterium stanieri PCC 7202, complete genome
- AP014821.1 Geminocystis sp. NIES-3709 DNA, complete genome
- M33820.1 Synechocystis sp. phycocyanin beta and alpha subunit (cpcBA) genes, complete cds
- KM249029.1 Cyanobacterium stanieri KD02 phycocyanin beta subunit (cpcB) and phycocyanin alpha subunit (cpcA) genes, partial cds

Appendix 5. *rpo*C1 Gene Alignments: NCBI Accession Number and Identity

- KM376969.1 Cyanobacterium sp. MCCB 114 DNA-directed RNA polymerase subunit gamma (rpoC1) gene, partial cds
- KM376968.1 Cyanobacterium sp. MCCB 115 DNA-directed RNA polymerase subunit gamma (rpoC1) gene, partial cds
- CP003947.1 Cyanobacterium aponinum PCC 10605, complete genome
- AF448115.1 Synechococcus sp. PCC 8806 RNA polymerase subunit (rpoC1) gene, partial cds
- KF113680.1 Uncultured Cyanobacterium sp. clone St2-45m-8 DNA-dependent RNA polymerase subunit gamma (rpoC1) gene, partial cds
- AY349632.1 Uncultured Prochlorococcus sp. clone SCS-s30 RNA polymerase subunit (rpoC1) gene, partial cds
- AY349633.1 Uncultured Prochlorococcus sp. clone SCS-80-34 RNA polymerase subunit (rpoC1) gene, partial cds

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Chapter 3

Fate of axenic cultures of *Cyanobacterium* MCCB 114, MCCB 115 and MCCB 238 with regard to senescence

Contents

3.1 Introduction

Life always has been based on the phenomenon of give and take. All life forms derive a lot from their surroundings, by way of chemical exchange. Concept of populations, food chains, ecosystems, all centers on this principle. When such a beneficial exchange happens between organisms, we term it symbiosis.

3.1.1 Symbiosis

Symbiosismeans the interaction between two different organisms living in close physical association, typically to the advantage of both; or a mutually beneficial relationship between different people or groups.

The most important symbioses in biology might have been the formation of mitochondria and chloroplasts, playing critical roles in energy metabolism. Mitochondria, which are found in almost all eukaryotic cells, are the sites of oxidative metabolism and are thus responsible for generating most of the ATP derived from the breakdown of organic molecules. Chloroplasts are the sites of photosynthesis and are found only in the cells of plants and

green algae. Mitochondria and chloroplasts originated from the endosymbiotic association of aerobic bacteria and cyanobacteria, respectively, with the ancestors of eukaryotes.

3.1.2 Cyanobacteria and Symbiosis

The significance of chloroplast formation and the effect this organelle had on the evolution of plant kingdom is huge. Thus it was some ancient cyanobacterial cell that influenced life on earth to such a great extent. It can be said that cyanobacteria have been symbionts from the very beginning.

3.1.2 Cyanobacteria and Eukaryotes

Cyanobacteria form associations with lichenized and non-lichenized fungi, lower and upper plants in terrestrial environment. Cyanobacteria that get into symbiotic associations are commonly referred to as cyanobionts.

Terrestrial cyanobionts so far studied are limited and commonly belong to heterocystous genera *Nostoc*. *Nostoc-Gunnera* symbiosis is well explained (Johanssen and Bergman, 1992). *Gunnera* is a gymnosperm with a long evolutionary history and they form symbiotic association with different species of *Nostoc*. In the presence of *Nostoc* cell, Gunnera secretes mucilage and once inside this, *Nostoc* produces hormogonia (motile reproductive filaments) and migrates to intercellular spaces. Once *Nostoc*hormogoniaare near cells, the cell wall dissolves and *Nostoc* goes inside the cell. Such chemoattraction happens in most plant-cyanobacteria symbioses. Similarly *Calothrix* and *Scyotonema* also form such associations with lichens and cycads. Gehringer et al, (2010) investigated the endosymbiotic cyanobacteria in cycads all along Australia and found out that species commonly found in aquatic and marine environments were present in the terrestrial hosts.

Lichens are symbiotic associations between a mycobiont (fungus) and a photobiont (green alga or cyanobacterium). Estimates of the number of lichen species range from 13,000 to 17,000, of which about 10% contain a cyanobacterium. The most frequent cyanobacterial genus in lichens is *Nostoc (*Paulsrud and Lindblad, 1998).

Cyanobacteria can infect the thallus of bryophytes (Adams and Duggen, 2008). The cyanobionts undergo morphological and physiological changes, including reduced growth rate and CO_2 fixation, and enhanced N_2 fixation, and release to the plant much of the dinitrogen fixed.

The most studied symbiosis in fresh water is that of *Azolla-Anabaena* which has now become the backbone of organic farming. Azolla, an aquatic fern, harbours *Anabaena azollae* in its leaf cavities and provides them with carbon sources and in turn gets their nitrogen requirements fulfilled (Pabby et al, 2003). When Azolla is applied to a hectare of paddy field, the plants will be able to derive at least 18Kg of nitrogen from them.

Cyanobacteria are also present as epiphytes on the surface of an alga called *Chara*. *Chara* has $CaCO_3$ crystals over them. They live on the surface and fixes N_2 for the host (Ariosa *et al.*, 2004)

The most common marine organisms harboring cyanobacteria are corals, diatoms, dinoflagellates, seagrass and sponges.

In photosynthetic hosts like diatoms, cyanobacteria supply the host with nitrogen. *Richelia intracellularis and Calothrix rhizosoleniae* are filamentous heterocystous cyanobacteria that live in symbioses with many diatoms like *Hemiaulus, Rhizosolenia and Chaetoceros* (Foster *et al.,* 2011). Episodic blooms of the diatom *Hemiaulus hauckii* and its diazotrophic

cyanobacterial symbiont *Richelia intracellularis* can reach very high densities (Yeung *et al.,* 2012).

Cyanobionts are usually found associated with the leaves of the sea grass *Cymodocea rotundata* as small adhering patches of pigmented microbial aggregates of thin biofilms (Hamisi *et al*., 2013).

Dinoflagellates exhibit a wide range of nutritional modes, being free living autotrophs, symbiotic autotrophs, mixotrophs or heterotrophs. Some of them lack photosynthetic pigments. Some of the unpigmented organisms have coloured bodies inside them which are symbiotic cyanobacteria. Representatives of the genera *Ornithocercus, Histioneis and Citharistes* had at least two and possibly three distinct forms of symbiotic cyanobacteria (Lucas 1991).

Cyanobacterial endosymbionts are almost ubiquitous in marine sponges and their presence can cause significant modifications in sponge morphology. They are usually intercellular, but sometimes occur in specialized vacuoles termed 'cyanocytes'. *Synechococcus* spp. are the most common cyanobacterial symbionts found in sponges, while *Oscillatoria spongeliae* has been reported over a wide geographic range. Sponges mostly get their colour from cyanobacterial symbionts based on their phycobiliprotein content. (Usher *et al*., 2004).In certain marine invertebrates, cyanobionts provide the host with carbon.Colonies of the Carribean coral *Montastraea cavernosa* harbour endosymbiotic cyanobacteria that fix nitrogen for the host (Lesser *et al.,* 2004).

Ascidians live either permanently attached to a solid object or buried in the sand. Some ascidians showed association with *Synechocystis*, a cyanobacterium which seems to provide host with dissolved organic carbon (Lambert *et al.,* 1996).

Cyanobacteria occur in cells of the sub-epidernal connective tissue of two worms *Ikedosoma gogoshiminse* and *Bomellia fulginosa*. The former lives in muddy sand and the latter on coral reefs. Nothing is known about the cyanobacteria or their role in the host (Rai, 1990).

Symbiotic associations between cyanobacteria and marine organisms are important in the oligotrophic waters of the global ocean, where cyanobionts make it possible for a wide range of marine organisms to survive in nutrient depleted waters. The nature of associations was studied to a certain extent in sponges and ascidians. Much interesting results will come about if studies are taken up in the case of other symbiotic associations involving cyanobacteria.

3.1.3 Cyanobacteria-Bacteria Symbiosis

Cyanobacteria often live in association with heterotrophic bacteria. Cyanobacteria provide a perfect habitat for bacteria with their polysaccharide and peptide containing envelopes. Filamentous cyanobacteria exhibit close and specific associations with bacteria during active growth. Associations range from the general presence of a variety of heterotrophic bacteria in mucilaginous sheaths to highly specific associations between physiologically distinct cyanobacterial cells and bacterial epiphytes (Paerl & Gallucci, 1985).

Anabaena and *Aphanizomenon* are cyanobacterial genera having heterocysts, thick-walled cells which are sites of nitrogen fixation. Heterocysts are often covered by bacteria, while neighboring non-nitrogen-fixing vegetative cells remain relatively free of them (Paerl, 1976). Independent studies have shown that cellular nitrogen fixation and growth of host cyanobacteria are enhanced in these types of associations (Paerl & Gallucci, 1985). Molecular oxygen (O_2) is a potent inhibitor of key microbial processes,

including photosynthesis, nitrogen fixation, denitrification, sulfate reduction, methanogenesis, iron, and metal reduction reactions. Prokaryote survival and proliferation in aquatic environments is often controlled by the ability to tolerate exposure to oxic conditions. Localized removal of O_2 will happen in regions having bacterial epiphytes, thus maintaining optimal N_2 fixation rates in such symbiotic associations (Paerl & Pickney, 1996).

Another well studied phenomenon is cyanobacterial mat formation. Cyanobacteria that build microbial mats include a variety of filamentous and unicellular species. Filamentous cyanobacteria like *Microcoleus chthonoplastes* dominates marine intertidal microbial mats all over the world. A notable characteristic of such mats are twisted trichomes embedded in common polysaccharide sheath. D'Amelio *et al.* (1986) reported a filamentous unculturable purple bacterium found in close association with M. *chthonoplastes* in a hypersaline pond in Mexico, and in Solar Lake, Egypt. These bacteria were found inside the cyanobacterial bundle, enclosed by the cyanobacterial sheath.

Hotspring microbial mats in Yellowstone National Park in the USA and similar microbial mats are dominated by rod-shaped unicellular cyanobacterium *Synechococcus lividus* (Allewalt *et al*., 2006). Cyanobacteria often dominate in aquaculture ponds with high nutrient loading of phosphorus relative to nitrogen with low relative mixing and warm waters. In such cases, their ability to fix nitrogen, ability to make floating mats by buoyancy regulation by the gas vacuoles plays a role.

Production of siderophores to scavenge iron during iron starvation in environments by marine cyanobacteria and heterotrophic bacteria was revealed by Barbeau *et al.,* (2003).

The degradation of Microcystin, a toxic peptide produced by cyanobacteria during harmful algal blooms (HABs) could be done by a bacterium *Sphingomonas* (Bourne *et al.,* 1996). This bacterium isolated from irrigation drainage water, had no direct association with cyanobacteria, but had a high potential in microcystindegradation, supposedly due to the structure of the oligopeptide in microcystin being similar to the ones found in peptone.

Hube*et al*. (2009) attempted the classification of heterotrophic bacteria associated with filamentous strains *Oscillatoria brevis* and *Nodularia harveyana* and found three groups belonging to *Rhodobacter, Porphyrobacter, Roseobacter* and *Muricauda*.

Shen *et al.*, 2011 studied the morphological and physiological changes caused by heterotrophic bacteria in *Microcystis aeruginosa*, a colony forming cyanobacteria and found out that non axenic organisms produced exopolysaccharides whereas axenic ones lived as single cells.

Since we have discussed about the symbiotic associations that happen in cyanobacteria, we now have to think of a different aspect, what if they are alone. In nature no organism gets space to live on their own. But in a laboratory, many microorganisms exist in pure culture (axenic). What happens to an organism in a pure culture is an important question that will reveal interesting answers.

3.1.4 Senescence

Senescence is defined as the condition or process of deterioration with age. When applied to individual cells it is the loss of a cell's power of division and growth.

Current research into senescence is mainly performed in medical disciplines like Gerantology (Beck & Scheibe, 2001). Programmed cell death

(PCD) is defined as internally programmed degeneration of cells, tissues, organs and whole organisms leading to death (Nooden *et al.,* 1997). Apoptosis refers to programmed death of small numbers of animal cells, and it shows some special features at the cell level. Some senescing plant cells show some symptoms typical of apoptosis, while others do not. Traditionally, the ideas on senescence mechanisms fall into two major groupings, nutrient deficiencies (e.g., starvation) and genetic programming (i.e., senescence-promoting and senescence-inhibiting genes). Considerable evidence indicates that nutrient deficiencies are not central senescence programme components, while increasing evidence supports genetic programming.

Prokaryotes like bacteria and cyanobacteria that divide by binary fissionwere considered as organisms that do not age. This was because binary fission of bacteria has been assumed to proceed with a non-conservative dispersion of both undamaged and damaged constituents, such that there are no adult forms of bacterial cells and the bacterial population is not age structured (Nystrom, 2007).

Bacteria enjoy an infinite capacity for reproduction as long as they reside in an environment supporting growth. However, their rapid growth and efficient metabolism ultimately results in depletion of growth-supporting substrates and the population of cells enters a phase defined as the stationary phase of growth. In this phase, their reproductive ability is gradually lost. These apparently sterile/non-culturable cells initially remain intact and metabolically active (Nystrom, 2003).

Starved and growth-arrested bacterial cells show the same signs of senescence as ageing cells of higher organisms. Free radicals may be involved in the gradual loss of bacterial culturability observed in a stationary phase culture.

Senescence of growth-arrested *Escherichia coli* cells is like mandatory aging in higher eukaryotes, accompanied by increased oxidative modifications of macromolecules. Similar to aged flies, this senescence-related oxidation targets enzymes of the Krebs cycle. Additional targets include the universal stress protein A, the Hsp70 chaperone DnaK, translation elongation factors, and histone-like proteins (Nystrom, 2002).

There is another view that the apparent loss of viability of starved cells is a programmed and adaptive response in which the cells enter a reversible non-culturable state; the theory of the formation of viable but non-culturable cells (Nystrom, 2001)

Literature on cyanobacterial senescence has been few. Hence it was assumed that unicellular cyanobacteria that divide by binary fission may follow similar ageing process like *Escherichia coli*.

Comparative accounts on the viability of cyanobacterial cultures in pure (axenic) state and as consortia also could not be found in literature.

3.1.5 Background of the study

The array of symbiotic associations found in nature, always present a view of stable ecosystems. We cannot imagine such stability in a man-made culture medium where a single type of organism is allowed to multiply. A similar situation came to the fore while maintaining cyanobacterial cultures in laboratory as part of this work. From the culture collection of cyanobacteria maintained as consortia (xenic state), selected cultures were made axenic. Comparing cyanobacterial cultures in their xenic state (with other unknown organisms living along) and their axenic state(pure culture free of all contaminants), it was found that pure cultures deteriorated significantly faster when compared to xenic state cultures.

3.2 Materials and Methods

3.2.1 Cultures

The three cultures of *Cyanobacterium* MCCB 114, MCCB 115 and MCCB 238 described in Chapter 2 and their non-purified counterparts were the cultures exhibiting this feature.When both the cultures were maintained under uniform conditions, the pure cultures reached their stationery phase faster, lost their pigmentation and reached a stage of senescence in a short period compared to non-axenic cultures which remained stable for lot more days. This prompted us to go indepth into the aging of cultures and the associated bacteria present in them.

The cultures of *Cyanobacterium* MCCB 114, MCCB 115 and MCCB 238 existed in xenic and axenic states in the culture collection of National Centre for Aquatic Animal Health, Kerala, India. Thus, the 6 groups of cyanobacteria used in this study were designated names given below.

Group	Strain	Designation
1	*Cyanobacterium* MCCB 114 axenic/pure	114 P
2	*Cyanobacterium* MCCB 114 xenic/consortia	114 C
3	*Cyanobacterium* MCCB 115 axenic/pure	115 P
4	*Cyanobacterium* MCCB 115 xenic/consortia	115 C
5	*Cyanobacterium* MCCB 238 axenic/pure	238 P
6	*Cyanobacterium* MCCB 238 xenic/consortia	238 C

3.2.2 Culture medium

MN medium was selected for the study as it was nutrient deficient, and used as acommon maintenance media in laboratories. The preparation of this medium is described in section 2.2.2 in detail.

For culturing each group of cyanobacteria, 36 nos of 100mL Erlenmeyer flasks were taken and 50mL aliquot of MN medium was added.The respective cyanobacterial cultures in their log phase were added to the flasks. The cell numbers of each group were estimated by measuring their absorbance at 750nm. Absorbance of 0.5 approximately corresponded to 10^7 cells/mL of cyanobacteria. So prior to inoculation, the absorbance was adjusted to 0.5 by dilution with sterile MN media. 50µL of cultures were added to each flask. The flasks were incubated under light intensity of 40 µEm-2sprovided by clear while fluorescent lamps for a 14:10 photoperiod and the temperature in the incubation room was approximately 26^{o}C. Every 10thday, triplicates were taken from each set and the following observations were made.

3.2.3 Cell count of live cyanobacteria by Flourescin diacetate

Live cell counts of cyanobacteria were done using Neubeur haemocytometer and Olympus CX 41 microscope having a FluoLEDEasyBlue (480 nm) filter by Fluorescin diacetate (FDA) assay. This assay is based on the cleavage of fluorescin diacetate in actively respiring cells. Non polar hydrophobic FDA is hydrolyzed by non-specific esterases present in intact cells yielding fluorescin. The polar fluorescin is retained within intact cell membrane. (Garvey *et al*, 2007) The retained fluorescin fluoresces green under blue light excitation. Fluorescence excitationand emission of FDA were at 485 nm and at 540 nm respectively.

The stock solution of FDA (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO), (Merck, India) to a concentration of 5 mg/mL and stored at 4^{o}C. 1 µL of FDA stock was added to 1 mL cyanobacterial culture and incubated for 20 min. It was loaded onto haemocytometer and placed under 40X objective of the microscope in fluorescence mode. After focusing on the

central square (RBC Counter) of haemocytometer grid, cells fluorescing green were counted.

3.2.4 Total cell count of cyanobacteria

The same field of the slide fixed for FDA staining was observed under phase contrast objective and counted.

3.2.5 Estimation of chlorophyll

The first sign of cyanobacterial ageing was loss of chlorophyll from cells. So this was included in the parameters for comparison. 20mL of algal suspension was centrifuged at 1000 rpm and the supernatant discarded. The pellet was suspended in 6mL of 80% Acetone (Merck, India) which was kept in ice. The whole material was transferred into a cold tissue grinding tube and macerated well with 2-3 washings. Then the volume was made upto 10mL and once again centrifuged at 1000 rpm. Absorbance of the samples were taken at A_{663}, A_{645} and values deducted from the formula given. (Becker, 2008)

Chlorophyll a = $(12.7 \times A_{663})-(2.69 \times A_{645})$

Chlorophyll b was calculated as $(22.9 \times A_{645})-4.64 \times A_{663})$

Chlorophyll a+b=$(8.02 \times A_{663})+(20.2 \times A_{645})$

3.2.6 Cellular absorptionspectra using UV-Vis spectrophotometer

Cellular absorption spectra also revealed peaks of pigments present in cell suspension. As cultures aged they showed loss of pigmentation which could be deciphered by the flattening of peaks in the spectra.

In vivo absorption spectra were analyzed using Shimadzu UV-Vis spectrophotometer. The scanning wavelength was in the range 190 to 1100.

Significant observations revealed by absorption spectra are given in Figs 40 to 45.

3.2.7 Microscopic Observation

Samples were prepared for scanning electron microscopy and observations made using the protocol described in section 2.2.7.2.

3.3 Results

All parameters selected for comparing the longevity of consortia over pure culture answered in the affirmative. Fig. 35 shows the photograph of cultures on 110th day. It was evident that consortia were superior to axenic cultures as far as viability of cells was concerned.

3.3.1 Cell Counts of Cyanobacteria

3.3.1.1 Comparing *Cyanobacterium* MCCB 114: Axenic cultures and Consortia

Axenic MCCB 114 reached stationary phase by 30th day. The total cell count did not show much variation after it attained stationary phase. Live cyanobacterial cells showed a declining trend beyond 40th day. From a cell count of 4.48±0.13x10^{7}, it reached a level of 1±0.04 x10^{6} cells on 90th day. But thereafter a rapid decline followed and by 100th day, all the cells were dead.

In the consortia of MCCB 114, stationary phase was reached at 40th day. Though the cells showed a declining trend, 1.7±0.14x10^{7}cells were alive on 110th day. The results of are shown in Tables 1 and 2. Diagrammatic representation of the results is shown in Fig36.

3.3.1.2 Comparing *Cyanobacterium* MCCB 115: Axenic cultures and Consortia

Axenic MCCB 115 reached stationary phase by 40th day (4.69±0.13x10^{7}). Live cells showed a declining trend beyond 40th day. On 90th day the cell numbers reached 3±0.025x10^{5}. 100th day observations did not show the presence of live cyanobacteria in the cultures.

In the consortia of MCCB 115, though there was a slight decline of cells beyond 40th day, 1.24±0.16 x107cells were viable beyond 110th day. The results are shown in Tables 3 and 4 and Fig 37

3.3.1.3 Comparing*Cyanobacterium* MCCB 238: Axenic cultures and Consortia

Axenic MCCB 238 reached stationary phase by 30th day. From a cell count of 4.81±0.27x10^{7}, it reached 5.6±0.035 x10^{5}on 90th day. Thereafter there was a rapid decline and no cells were found live on 100th day.

In the case of consortia, the cultures reached stationary phase on 40th day (4.81±0.27x10^{7}). Though there was slight decline, 1.31±0.04x10^{7} were alive beyond 110 days. The results are shown in Tables5, 6 and Fig 38.

3.3.2 Chlorophyll content

Chlorophyll a (Chla)is the main photosynthetic pigment present in cyanobacteria and quantification of this pigment will give an estimate of actively photosynthesizing cells in the cultures.

In Axenic culture of *Cyanobacterium* MCCB 114, Chla content was highest on 40th day and after that showed a declining trend. Chl a got exhausted on 100th day. In *Cyanobacterium* MCCB 114 consortia, Chl a

peaked on 30th day. Though the values declined, Chla was present in detectable amounts even on 110th day.

In Axenic culture of *Cyanobacterium* MCCB 115, Chl a peaked on 20th day and showed a decline thereafter and it went undetected by 90th day. In the consortia of *Cyanobacterium* MCCB 115, chlorophyll peaked on 30th day and it was present in modest amounts even after 110th day.

In *Cyanobacterium* MCCB 238 axenic culture, Chlorophyll was almost equal on 40th and 50th day. But it went undetected on 100th day. In *Cyanobacterium* MCCB 238 consortia, Chl a peaked on 50th day and was detectable beyond 110th day.

The values of Chl a in the cultures under study are given in Table 7. Graphical representation of results are given in Fig 39.

3.3.3 Cellular Absorption Spectra

This investigation gives an indication of major pigments in an easily decipherable way. Pigments show peaks when detectable and peaks flattens when values are undetectable.

In axenic *Cyanobacterium* MCCB 114 peaks flattened on 100th day whereas detectable peaks were present in consortia beyond 110th day.

In axenic *Cyanobacterium* MCCB 115 peaks flattened on 80th day whereas detectable peaks were found in consortia beyond 110th day.

In axenic *Cyanobacterium* MCCB 115 peaks flattened on 100th day whereas detectable peaks were found in consortia beyond 110th day.

The spectral diagrams are given in Figs. 40 to 45.

3.3.4 Microscopic Observations

Fig. 46 to 48 reveal the associated bacteria found in the consortia of *Cyanobacterium* MCCB 114, *Cyanobacterium* MCCB 115 and *Cyanobacterium* MCCB 238 respectively. In all the pictures, it was found that bacteria were found stringently attached to glycocalyx of cyanobacteria.

3.4 Discussion

The symbiotic association of cyanobacteria with various organisms were extensively studied and the dominant role of cyanobacteria in the association was always projected. In this comparative account of axenic and non-axenic cultures of cyanobacteria in three different strains belonging to same genus, it was found that the bacteria played in key role in keeping the cyanobacteria robust after it reached the stationary phase. Nutrient depletion is the reason that leads to the ageing or death of cells after the stationary phase. When this situation is overcome by nutrient recycling, cell growth and division can continue for a significant period. In the case of cyanobacteria, the associated bacteria were playing a significant role in nutrient cycling.

Berg et al. (2009) isolated 460 bacterial strains from a cyanobacterial bloom and reported that some of the isolates had an enhancing effect on cyanobacterial growth.

The most important inference from this study was that the three cultures of Cyanobacterium showed identical results. All the axenic cultures more or less deteriorated beyond 80-90 days whereas when they were associated with consortia all the cultures lived beyond 110 days.

This result holds significance in many ways. Biotechnologists mostly have to work with pure cultures for isolation of compounds or biomass. In such a

system, axenic cultures take time and diligence for purity maintenance. If cyanobacteria survives better in an environment if they have their symbiotic association of bacteria, such cultures will be easy to maintain. So, more focus has to be given on studying the bacterial association of these useful microorganisms.

Senescence was an aspect that comes to fore when we find the changes happened in cyanobacteria. The axenic cells underwent a process of senescence and reached a death phase. But more detailed investigations pertaining to senescence could not be undertaken, but it was there. Consortia, on the other hand, were robust till the end of the study, with viable cell counts. Immortality of consortia is a field of study that has to be taken up working out the expression of senescence related genes and biochemical investigations.

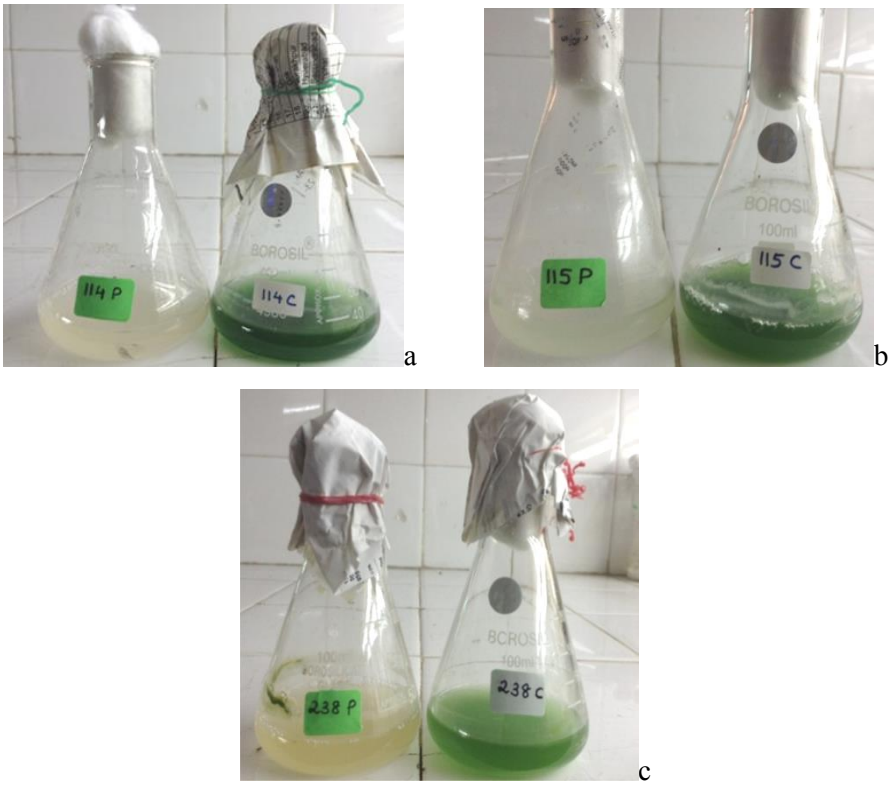


Fig. 35 Cyanobacterial cultures on 110th day. (a) shows 114P and 114C; (b) shows 115 P and 115 C; (c) shows 238 P and 238 C

Table 1 Cell Count of *Cyanobacterium* MCCB 114 Axenic culture

	Total ($x10^7$ cells)	Live($x10^7$ cells)
10th day	0.48±0.08	0.43±0.03
20th day	3.34±0.26	3.41±0.23
30th day	4.45±0.12	4.45±0.2
40th day	4.46±0.14	4.47±0.13
50th day	4.54±0.24	1.75±0.08
60th day	4.79±0.2	0.67±0.05
70th day	4.58±0.28	0.48±0.04
80th day	4.61±0.26	0.32±0.06
90th day	4.64±0.29	0.11±0.04
100th day	4.68±0.26	0
110th day	4.67±0.22	0

Table 2 Cell Count of *Cyanobacterium* MCCB 114 Consortia

	Total ($x10^7$ cells)	Live($x10^7$ cells)
10th day	0.48±0.08	0.45±0.02
20th day	3.88±0.23	3.41±0.23
30th day	4.6±0.24	4.49±0.25
40th day	4.46±0.09	4.64±0.26
50th day	4.81±0.18	3.91±0.19
60th day	4.53±0.23	2.81±0.32
70th day	4.59±0.32	2.5±0.24
80th day	4.64±0.16	2.3±0.19
90th day	4.66±0.19	2.18±0.2
100th day	4.73±0.17	1.82±0.17
110th day	4.63±0.25	1.79±0.15

Table 3 Cell Count of *Cyanobacterium* MCCB 115 Axenic culture

	Total ($x10^7$ cells)	Live($x10^7$ cells)
10th day	0.47±0.04	0.45±0.02
20th day	1.27±0.23	1.23±0.09
30th day	3.57±0.26	3.73±0.06
40th day	4.69±0.14	4.69±0.24
50th day	4.62±0.28	3.59±0.27
60th day	4.54±0.32	2.59±0.34
70th day	4.62±0.26	1.9±0.24
80th day	4.56±0.17	0.81±0.16
90th day	4.57±0.17	0.03±0.03
100th day	4.54±0.23	0
110th day	4.63±0.26	0

Table 4 Cell Count of *Cyanobacterium* MCCB 115 Consortia

	Total ($x10^7$ cells)	Live($x10^7$ cells)
10th day	0.59±0.07	0.57±0.07
20th day	1.93±0.35	1.88±0.26
30th day	4.37±0.29	4.44±0.25
40th day	4.68±0.39	4.8±0.17
50th day	4.72±0.31	3.7±0.21
60th day	4.36±0.34	3.3±0.2
70th day	4.62±0.27	2.93±0.2
80th day	4.55±0.23	2.56±0.24
90th day	4.82±0.2	1.99±0.17
100th day	4.69±0.16	1.48±0.16
110th day	4.68±0.16	1.24±0.16

Table 5 Cell Count of *Cyanobacterium* MCCB 238 Axenic Culture

	Total (x10^7 cells)	Live(x10^7 cells)
10th day	0.45±0.03	0.46±0.03
20th day	1.89±0.27	1.79±0.17
30th day	4.67±0.22	4.82±0.13
40th day	4.66±0.38	4.52±0.28
50th day	4.88±0.25	3.98±0.2
60th day	4.78±0.13	3.61±0.26
70th day	4.48±0.24	2.21±0.32
80th day	4.72±0.21	1.54±0.27
90th day	4.7±0.22	0.06±0.04
100th day	4.69±0.22	0
110th day	4.75±0.08	0

Table 6. Cell Count of *Cyanobacterium* MCCB 238 Consortia

	Total (x10^7 cells)	Live(x10^7 cells)
	Total	Live
10th day	0.42±0.04	0.42±0.05
20th day	1.89±0.27	1.69±0.14
30th day	4.76±0.2	4.71±0.17
40th day	4.76±0.13	4.8±0.21
50th day	4.52±0.21	3.44±0.2
60th day	4.65±0.25	2.53±0.32
70th day	4.56±0.28	1.95±0.55
80th day	4.66±0.32	1.42±0.21
90th day	4.75±0.23	1.27±0.07
100th day	4.53±0.22	1.2±0.18
110th day	4.78±0.17	1.31±0.04

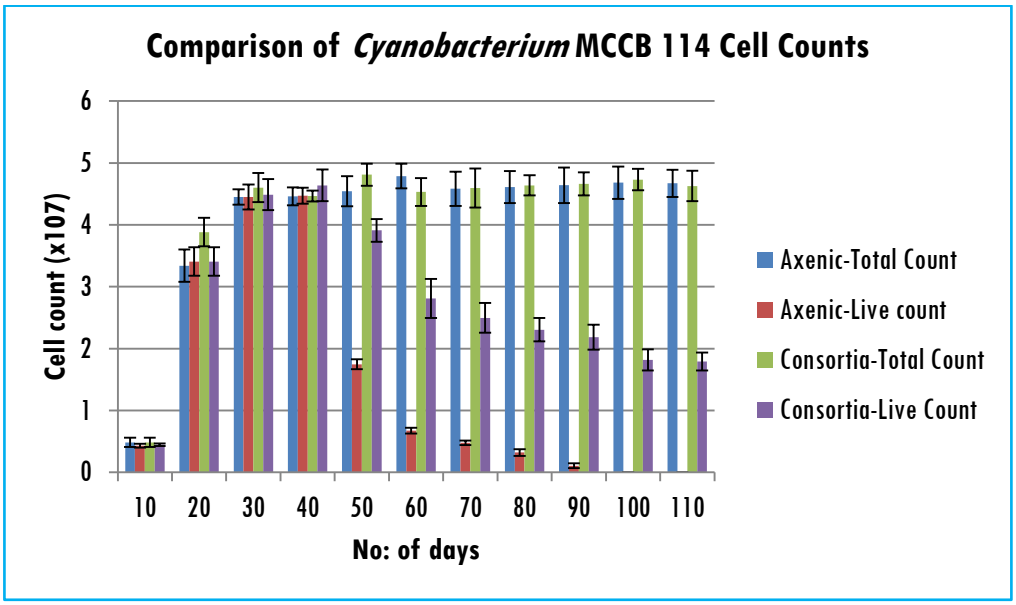


Fig. 36 Cell count of axenic and non-axenic cultures of *Cyanobacterium*MCCB 114

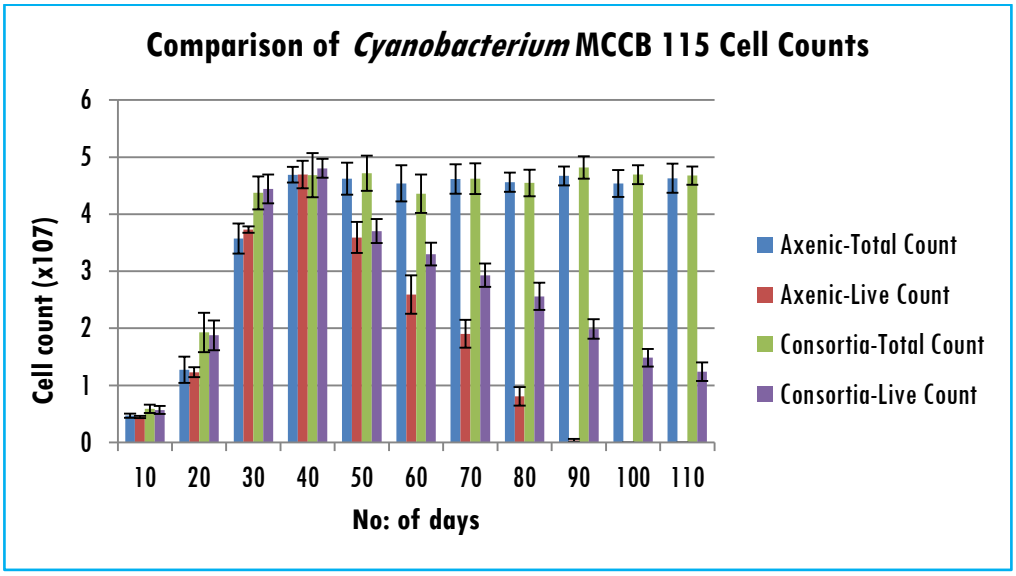


Fig. 37 Cell count of axenic and non-axenic cultures of *Cyanobacterium*MCCB 115

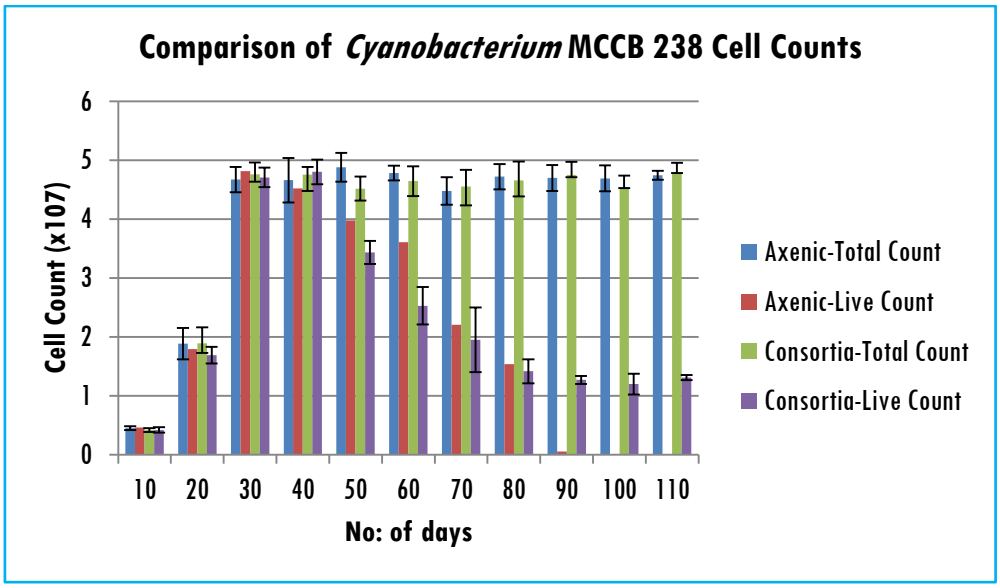


Fig. 38 Cell count of axenic and non-axenic cultures of *Cyanobacterium*MCCB 238

Table 7 Chlorophyll a content in cyanobacterial cultures as mg /L

	MCCB 114 Axenic	MCCB 114 Consortia	MCCB 115 Axenic	MCCB 115 Consortia	MCCB 238 Axenic	MCCB 238 Consortia
10th day	0.52	0.55	1.08	1.75	2.44	2.02
20th day	1.41	0.96	5.63	2.75	4.01	4.40
30th day	5.37	5.78	5.09	5.45	4.35	4.01
40th day	5.81	4.97	4.74	4.37	4.40	4.11
50th day	3.22	3.80	3.71	4.10	4.40	4.29
60th day	1.83	3.11	3.28	3.68	2.66	3.87
70th day	0.99	3.14	1.29	3.14	2.58	3.43
80th day	0.88	3.95	0.94	3.02	1.30	3.23
90th day	0.37	3.57	0.00	2.96	0.14	2.53
100th day	0.00	2.97	0.00	2.78	0.00	2.24
110th day	0.00	2.63	0.00	2.78	0.00	2.26

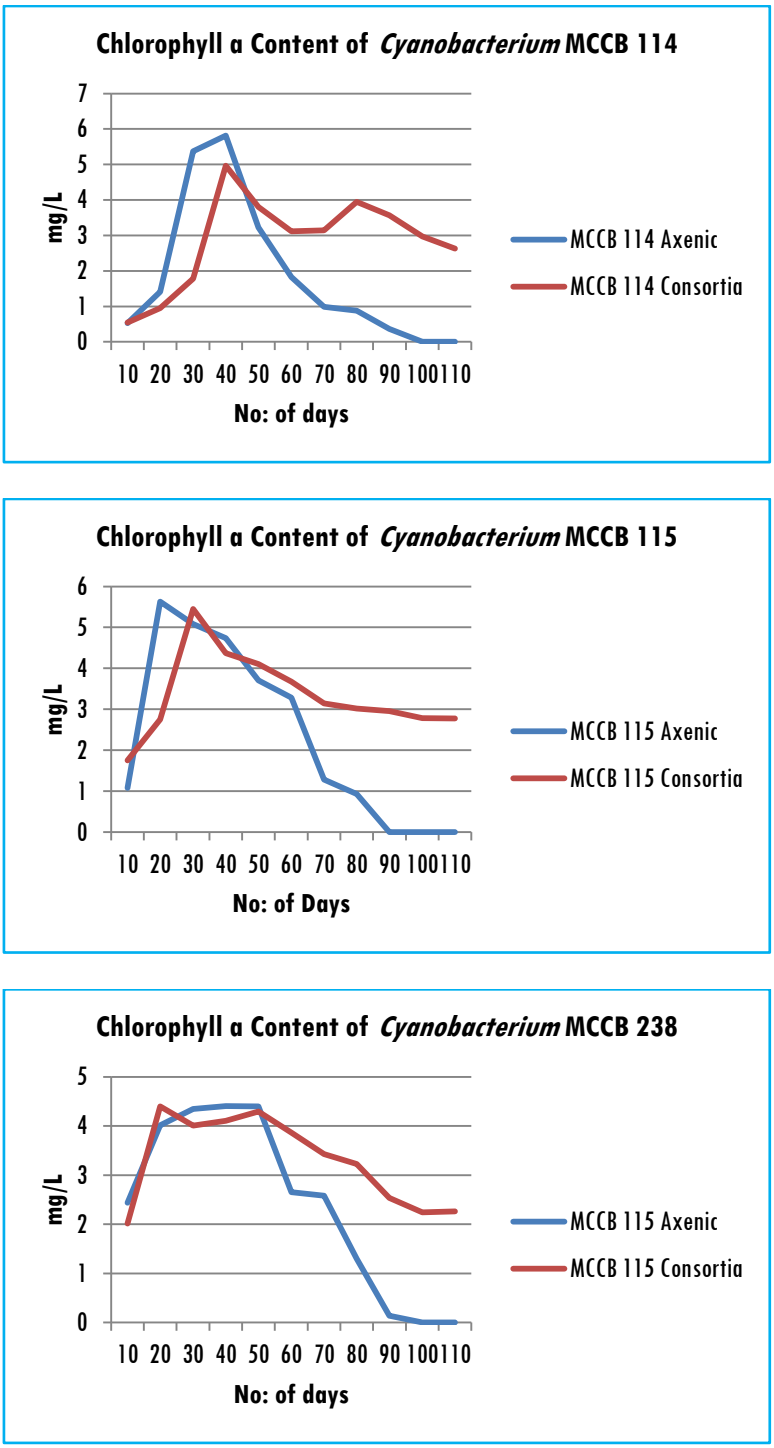


Fig. 39 Chlorophyll content comparisons

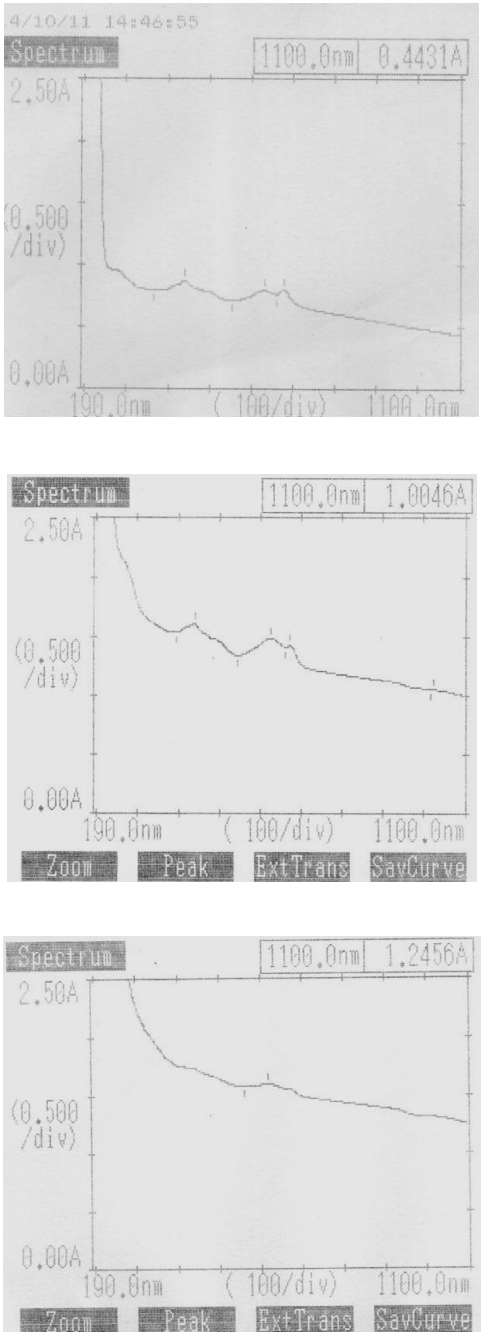


Fig. 40 Cyanobacteria MCCB 114 Axenic culture: Cellular absorption spectra of 10th day, 30th day and 100th day

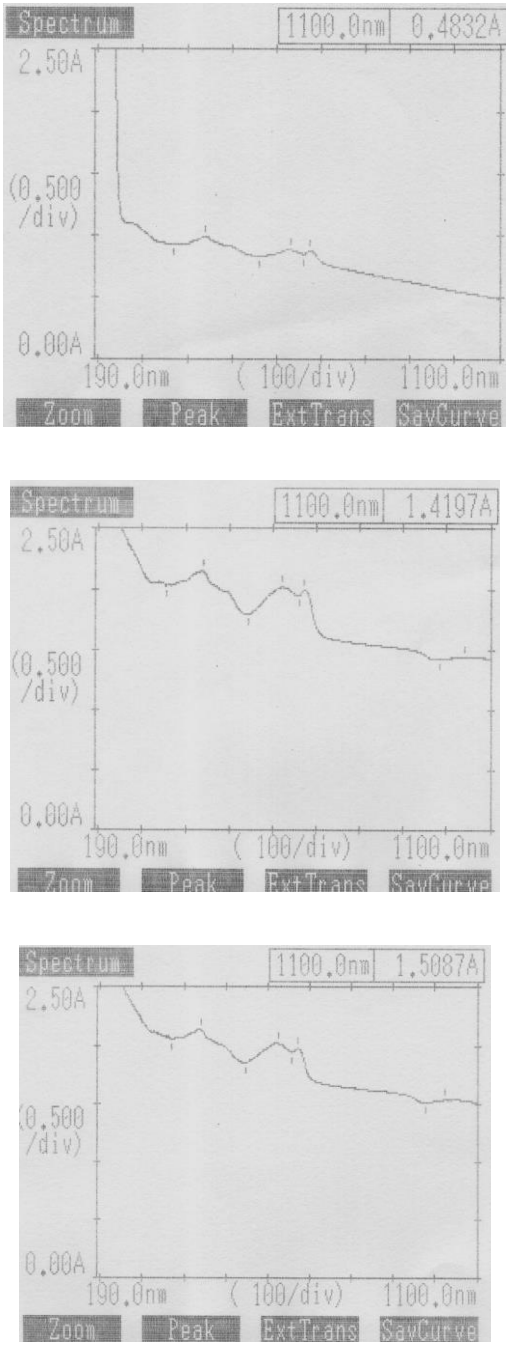


Fig. 41 *Cyanobacterium* MCCB 114 Consortia: Cellular absorption spectra of 10th day, 30th day and 110th day

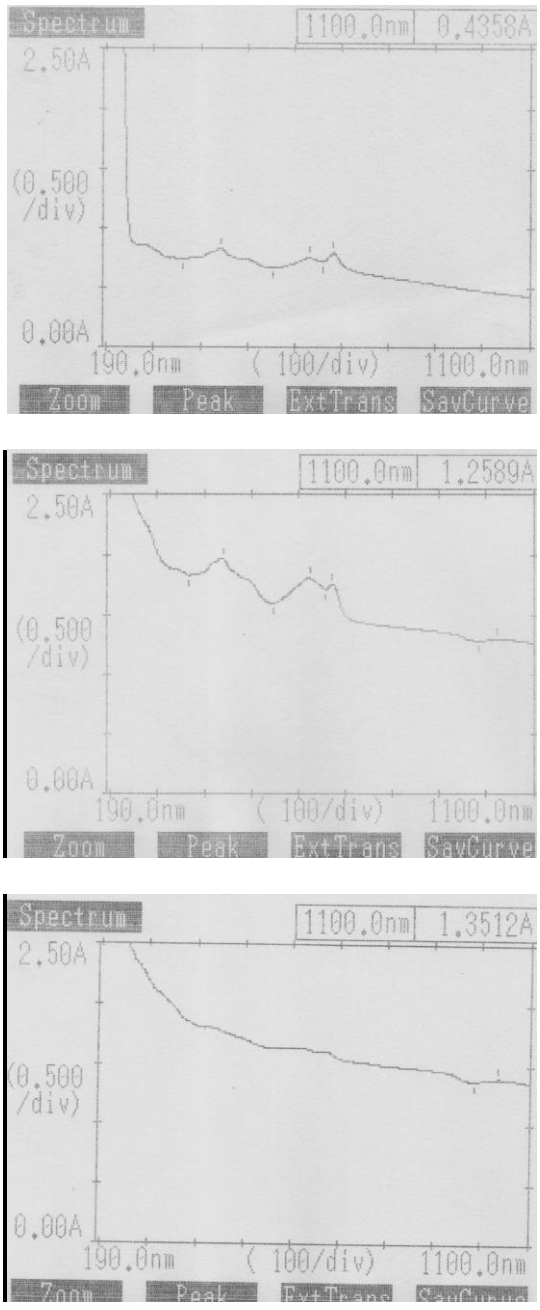


Fig. 42 *Cyanobacterium* MCCB 115 Axenic culture: Cellular absorption spectra of 10th day, 30th day and 90th day

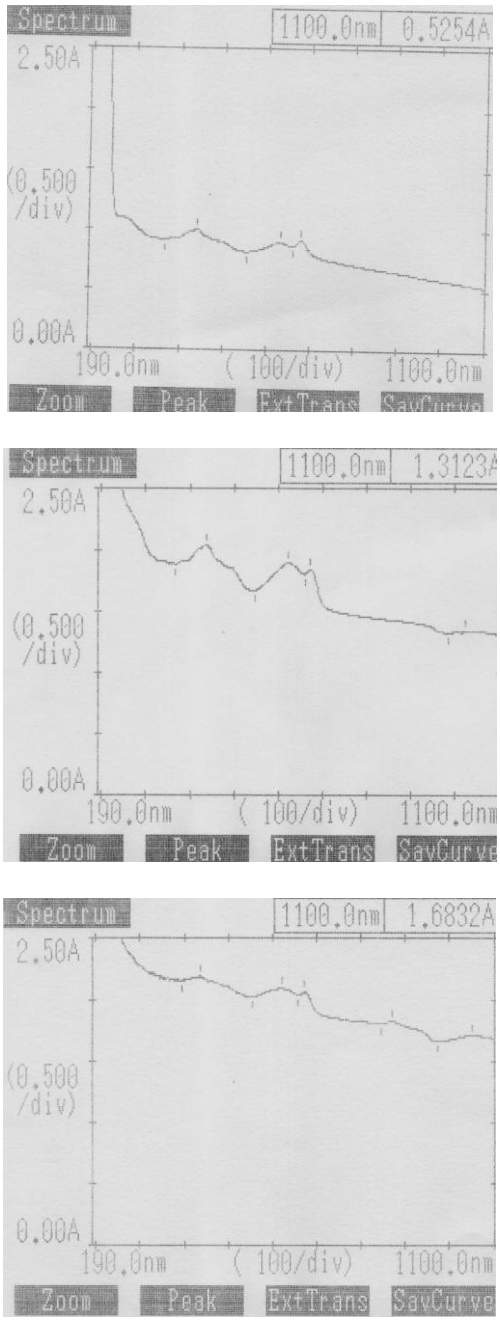


Fig. 43 *Cyanobacterium* MCCB 115 Consortia: Cellular absorption spectra of 10th day, 30th day and 110th day

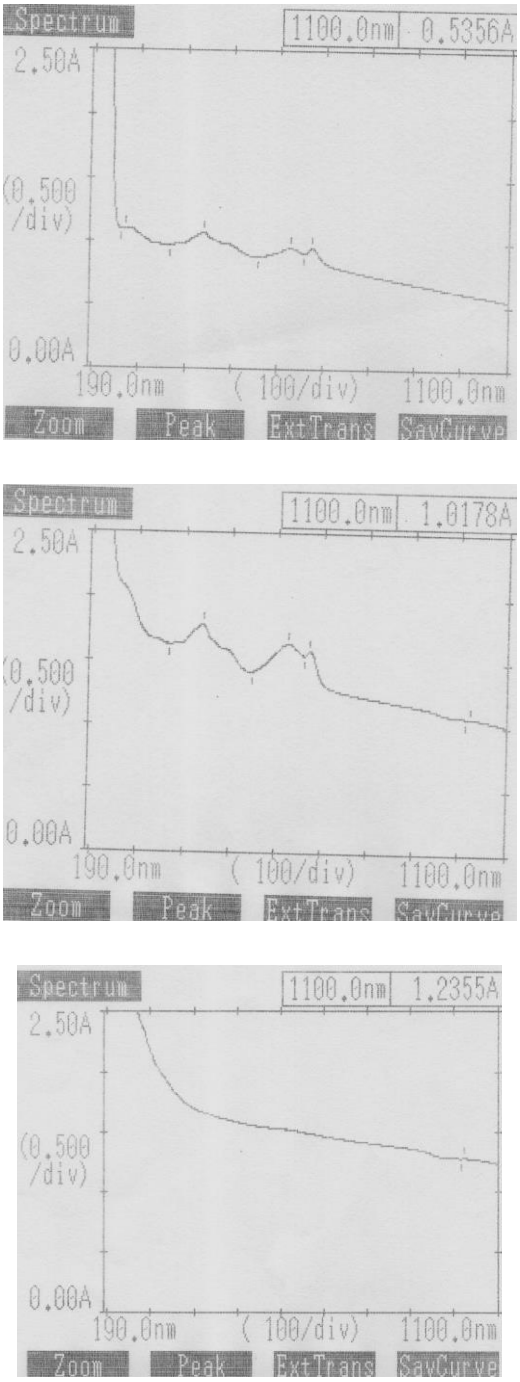


Fig. 44 *Cyanobacterium* MCCB 115 Axenic culture: Cellular absorption spectra of 10th day, 30th day and 100th day

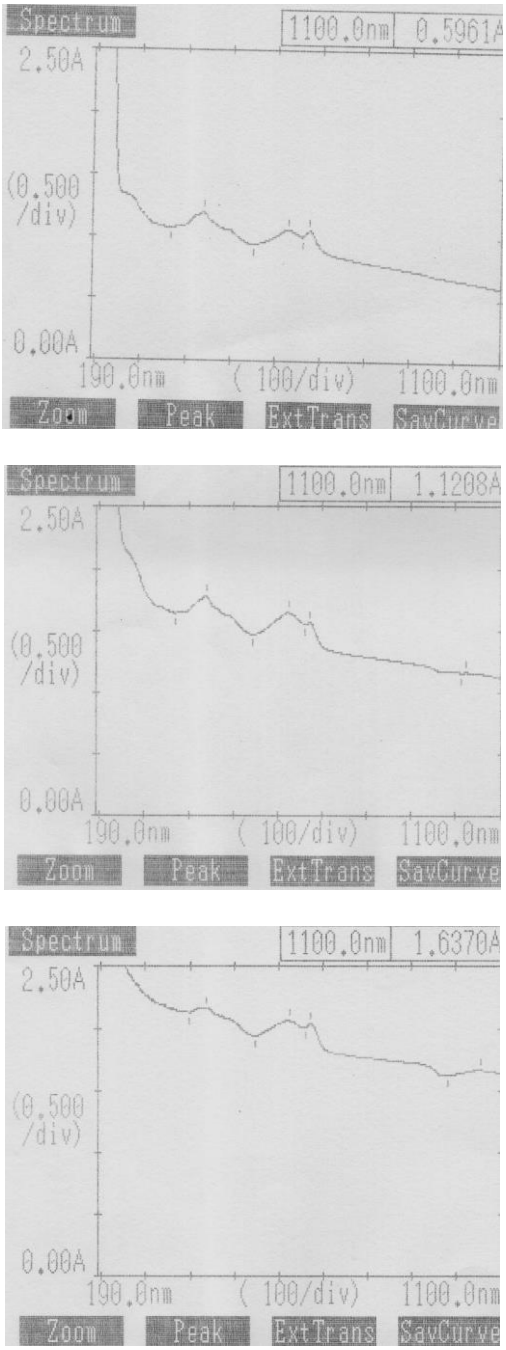
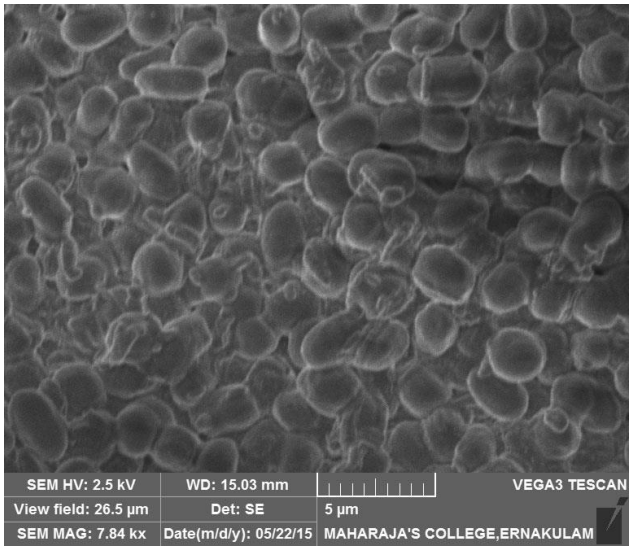


Fig. 45 *Cyanobacterium* MCCB 115 Consortia: Cellular absorption spectra of 10th day, 30th day and 110th day



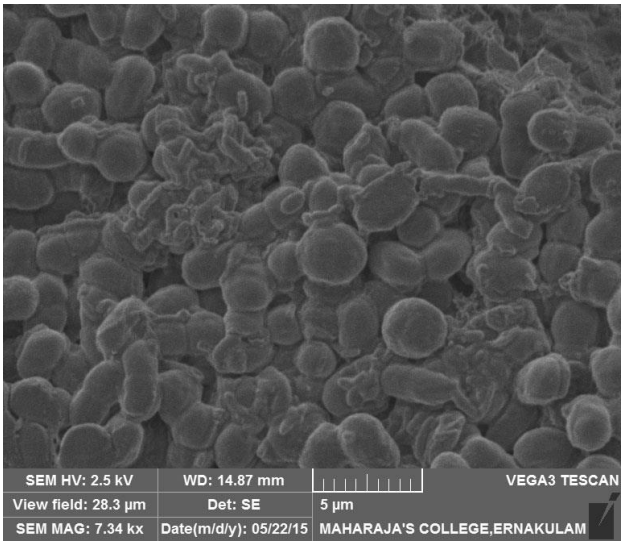


Fig. 46 Scanning microscopic images of *Cyanobacterium* MCCB 114 Consotia

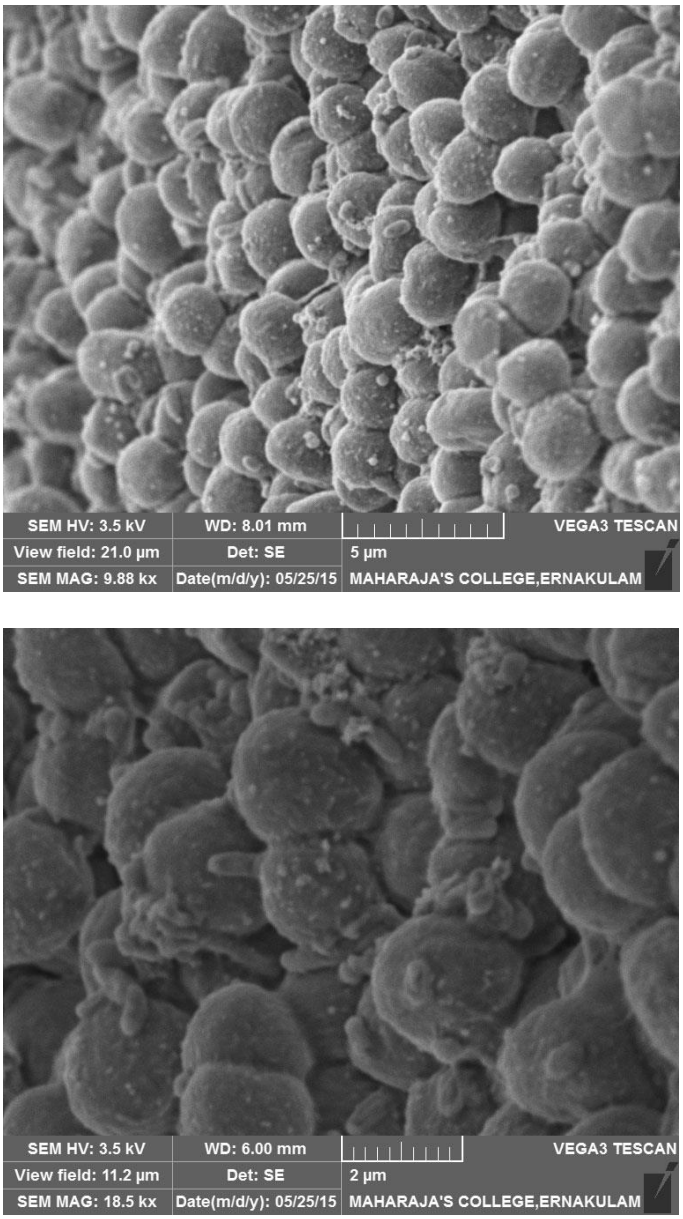
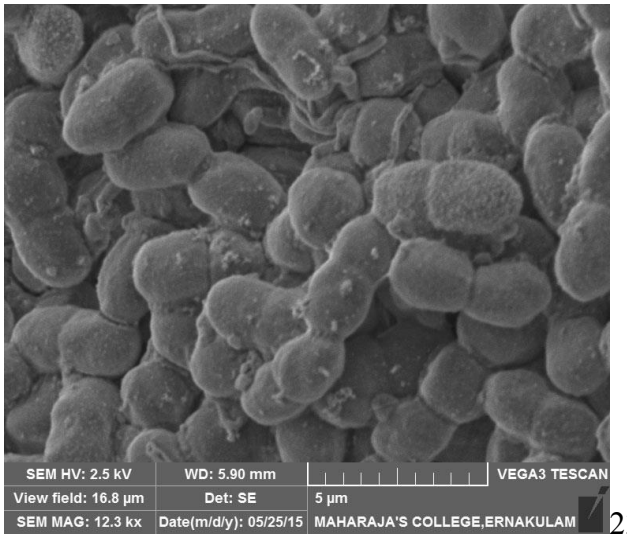
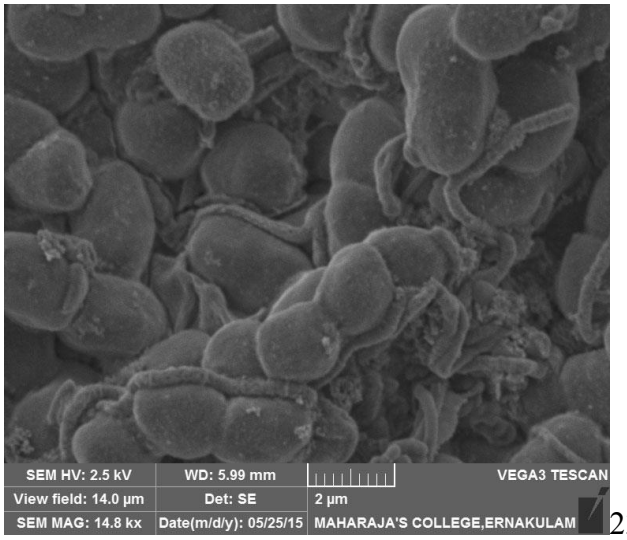


Fig. 47 Scanning microscopic images of *Cyanobacterium* MCCB 115 Consotia



238



238

Fig. 48 Scanning microscopic images of *Cyanobacterium* MCCB 238 Consortia

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Chapter 4

Identification of associated heterotrophs of *Cyanobacterium* MCCB 114, MCCB 115 and MCCB 238

Contents

4.1 Introduction

Cyanobacteria are one of the abundant and evolutionarily older organisms in nature. This would have given them the advantage of gene selection pertaining to adaptation and survival. When ecological conditions are not suitable for survival of individual organisms they have the know-how to form associations with other organisms that will ensure the longevity of cells by give and take mechanism. This concept is vivid when we look at the long range of associations formed by cyanobacteria.

Cyanobacteria are phototrophic bacteria obtaining their carbon and energy by photosynthesis, while heterotrophic bacteria derive carbon and energy from organic compounds. Bacteria associated with cyanobacteria always instilled interest in the researchers. In 1888, Pfeffer reported that *Sprirogyra* filaments attract bacterial cells towards them chemotactically at the time of photosynthesis.

It has been known for many years that filtrates from axenic cyanobacterial cultures may be enriched with organic compounds including

simple amino acids and peptides, sugars, polyalcohols and occasionally, vitamins, enzymes and toxins, commonly known by the term extracellular products (Fogg, 1966). In marine ecosystem, these may play an important role in food chains, especially as potential nutrients for bacteria (Bell & Mitchell, 1972). Upto 50% of the bacterial heterotrophic activity in natural waters can be supported by extracellular products from algae and cyanobacteria (Bell, 1983).

In natural environment, there can be antagonistic, neutral and symbiotic interaction between cyanobacteria and bacteria. In an environment where organic matter is sufficient for bacterial growth, bacteria may carry out aerobic respiration and use up the dissolved oxygen and inhibits cyanobacteria during night. During daytime, oxygen will be sufficient due to photosynthesis and cyanobacteria will be unaffected in photic zones (Cole, 1982). There are also reports of lytic bacteria viz. *Cytophaga, Cellvibrio, Bacillus brevis* and *Bdellovibrio* attacking various genera of cyanobacteria and photosynthetic algae (Cole, 1982). Some bacteria produce pigments that are inhibitory to cyanobacteria to avoid competition (Darveau & Lynch, 1977).

Competition for limiting nutrients has given interesting adaptations to cyanobacteria. Cyanobacterial growth is usually limited by nutrients like nitrogen and phosphorus. Bacteria grow rapidly and assimilate phosphorus. Hence cyanobacteria which grow slowly have the capacity to store phosphorus as intracellular polyphosphate (Rhee, 1972). For nitrogen deficiencies, many cyanobacterial species fix atmospheric nitrogen with specialized cells called heterocysts (Paerl, 1978). Heterotrophic bacterial growth in nature is probably limited by organic carbon (Rhee, 1972).

In a closed system, autotrophic growth would be impossible without heterotrophic activity. *In situ* remineralization of organic material by heterotrophs, especially limiting nutrients like nitrogen and phosphorus are crucial for autotrophs. (Cole, 1972). Some cyanobacteria require B vitamins which may also be produced by specialized heterotrophic bacterial groups (Hains & Guillard, 1974)

Sometimes cyanobacterial microenvironment may be altered by bacteria in ways that stimulate cyanobacterial growth. *Nostoc* strain grew well in the presence of *Caulobacter* than its absence and the two membered cultures had enhanced abilities to fix atmospheric nitrogen (Bunt, 1961).

Decomposition of dead cyanobacteria is carried out by heterotrophs. At the onset of cell death, there is usually a large initial release of soluble materials by autolysis which are metabolized by bacteria. (Cole, 1982).

Albertano and Urzi (1999) working on ancient architectural and natural hypogeal in Rome found evidence of physical attachment between phototrophic microbes and various rod shaped and filamentous heterotrophic bacteria, but a strong evidence of the specificity of the association was lacking.

The degradation of Microcystin, a toxic peptide produced by cyanobacteria during harmful algal blooms (HABs) could be done by a bacteria *Sphingomonas* (Bourne *et al.,* 1996). This bacterium isolated from irrigation drainage water, had no direct association with cyanobacteria, but had a high potential in microcystin degradation, supposedly due to the structure of the oligopeptide in microcystin being similar to the ones found in peptone.

HABs always show tight interaction between algae and associated bacteria. In such an event, there is spatial and temporal association wherein the

metabolites produced by both groups tend to be inhibitory or useful to each other. Working on a toxic metabolite domoic acid that is produced during bloom of *Pseudo-nitzschia multiseries*, Bates *et al. (*2004) recorded that the neither the algae nor the associated heterotrophic bacteria alone in culture could not produce domoic acid, but when algae and bacteria were present as a group domoic production occurred.

Production of siderophores to scavenge iron during iron starvation in environments by marine cyanobacteria and heterotrophic bacteria was discussed by Barbeau *et al.*(2003).

Recent studies focuses on cyanobacterial communities, their associates and their composition by an array of new techniques presently available.

Maruyama *et al.* (2003) used Fluorescence in situ hybridization (FISH) to enumerate bacterial community associated with cyanobacteria and also to target a specific toxin degrading bacteria in the mucilage of cyanobacterium and found that presence of bacteria enhanced toxin production.

The diversity of bacterial communities associated with cyanobacterial blooms in Swedish lakes was studied by Eiler & Bertilsson (2004) using Terminal Fragment Length Polymorphism (T-RFLP) and identified Proteobacteria*, Bacteriodetes, Actinobacteria, Verrucomicrobia* and *Plactomycetes*divisions.

Bruno *et al.* (2006) inspecting the rocks of underground cemeteries and catacombs exposed to artificial light, found strains of cyanobacteria *Leptolyngbya* and associated with them were heterotrophs *Pseudomonas, Stenotrophomonas, Agrobacterium* and *Bacillus*.

Toumainen *et al.* (2006) elucidated the microbial community associated with cyanobacterium*Nodulariaspumigina* (Mertens) from Baltic sea using

Temperature Gradient Gel Electrophoresis of a fraction of 16SrRNA gene and reported that the bacterial community varied a lot when compared to community structure elucidated by culture dependent methods.

Study by Eiler *et al.*(2006) on the phycosphere of cyanobacterium *Gloeotrichia echinulata* revealed a diverse bacterial community that included populations affiliated with *Proteobacteria, Bacteriodetes, Acidobacteria, Fusobacteria, Firmicutes, Verrucomicrobia,* and other cyanobacteria.

Hube *et al*. (2009) characterized heterotrophic bacterial strains associated with two filamentous cyanobacteria *Nodularia harveyana* and *Oscillatoria brevis* using Fluorescence in situ hybridization as well as fingerprinting by Amplified ribosomal DNA restriction analysis followed by sequencing.

Berg et al. (2009) isolated 460 bacterial strains from the site of an algal bloom and classified them into *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes* and *Deinococcus-Thermus*.

Pyrosequencing study on bacterial assemblages in Baltic Sea suggested that there were seasonal changes in community structure (Andersson *et al*, 2010)

Though most of the studies focused on community structure of cyanobacteria, there were attempts to learn about the interaction of cyanobacteria and bacteria. Shen *et al., 2011* cocultured unicellular *Microcystis aeroginosa* with natural bacterial community associated with the organism and noted that presence of bacterial lead to aggregation of cells in colonies and increase in exopolysaccharides secretion.

Svercel *et al.* (2011) studied the interaction between two newly isolated filamentous heterotrophic bacteria *Fibrella aesturina* and *Fibrisoma limi* with

axenic strain of cyanobacterium *Nostoc muscorum* from a culture collection and reported antagonistic behaviour.

Most of the studies on cyanobacterial associates so far deciphered population dynamics and classified organisms into higher taxonomic units. Workers generally undertook metagenomic approaches or culture independent methods for their study. Population studies of uni-cyanobacterial cultures and their associates had been few.

Most studies reported were from bloom forming, filamentous nitrogen fixing bacteria wherein cyanobacteria were the providers of nitrogen to the associates.

In the present study, the three cyanobacteria selected were unicellular and did not fix atmospheric nitrogen. But they were having associated bacterial population that helped in the viability of cells. Hence it was decided to assess the diversity and identity of the organisms by DNA sequence based analysis and biochemical tests.

4.2 Materials and Methods

4.2.1 Media for isolation of heterotrophic bacteria

Anderson (2005) described bacterial test media (BT-1 to BT-6) of the following composition. Since MN medium was supporting the growth of cyanobacteria, modification of MN medium with various organic compounds were tried. Vaara *et al* (1979) described TYG medium which is designated as BT-7 medium.

MN medium was prepared as described in section 2.2.2. To it was added organic compounds to prepare BT-1 to BT-7 of the following composition.

BT-1: MN medium with 1g protease peptone/L

BT-2: MN medium with 5g yeast extract/L

BT-3: MN medium with 5g peptone and 3g beef extract/L

BT-4: MN medium with 1g dextrose and 1g peptone/L

BT-5: MN medium with 0.5g sodium acetate, 0.5g glucose+0.5g tryptone+0.3g yeast extract/L

BT-6: MN medium with 1g yeast extract and 2g tryptone/L

BT-7: MN medium with 5g tryptone+2.5g yeast extract+1g dextrose/L

The media were prepared and pH was adjusted to 7.2±0.5. For making agar plates they were solidified with 2% agar and poured into petri plates.

Cyanobacterial cultures were serially diluted upto 10^{-8} dilution and each dilution were poured into plates in triplicates. They were incubated under dark for 21 days. The bacterial numbers were enumerated.

4.2.2 Heterotrophic bacterial isolation

The medium which supported maximum bacterial growth was selected as the medium for bacterial isolation. From triplicate plates at 10^{-6} dilution, 30 colonies were picked at random from the three cyanobacterial consortia MCCB 114, MCCB 115 and MCCB 238. Each colony was purified by streak plate method and designated strain numbers. Quadrant streaking was done on BT-6 agar plates and incubated in dark for 21 days. Some of the organisms were slow growers. The colony morphology of the cultures was recorded and biochemical tests performed.

4.2.3 Clustering of bacteria

The bacterial cultures obtained were showing similar morphologies. Identification of all cultures by sequence analysis would be a costly affair. Hence it was decided to cluster the bacteria into operational taxonomic units based on biochemical tests and DNA profiling technique called Amplified Polymorphic DNA restriction Analysis (ARDRA)

After performing the biochemical tests, the results were tabulated as a matrix which scored the test result as 1 it is positive and 0, if it was negative. This matrix was analysed by NTSYS PC software which generated a dendrogram based on numerical taxonomy (employing UPGMA method) by analyzing 48 unit characters or operational taxonomic units (OTUs). From each branch of the dendrogram, representative cultures were selected for sequencing.

Phenotypic characters are invaluable in clustering organisms for taxonomy. But they tend to vary a lot among strains based on the environment in which they live. Hence molecular sequencing data and analysis are also valued in taxonomy. Restriction analysis of DNA is a reliable technique for clustering of environmental isolates. 16S rDNA is universally present in prokaryotes and it codes for ribosome which is an assortment of conserved and variable regions. The restriction analysis of this gene with tetra cutter restriction enzymes will give representative patterns which can be distinguished from one another by analysis.

For performing Amplified Ribosomal DNA Restriction Analysis, the cultures were grown in BT-6 medium and incubated at 25^{o}C with constant shaking of 150 rpm. After attaining sufficient growth, DNA was extracted using phenol-chloroform method. After quantifying the DNA using dual

wavelength spectrophotometer (260/280nm), polymerase chain reaction was performed. The forward primer ranging from 9-27 base pairs and reverse primer ranging from 1477-1498 base pairs of *E.coli* 16S rRNA gene (Reddy et al., 2000) were used as primers for polymerase chain reaction done in EppendorfMastercycler Gradient, USA. The reaction mix comprised of 12.5µL of Emerald Master Mix (Takara, Japan), 1µL each of forward and reverse primers, 50ng of DNA template and Milli Q to make up a total reaction volume of 25µL. The thermal cycling steps were as follows: an initial denaturation at 95^{o}C for 7 min was followed by 25 cycles of 95^{o}C for 20s, 58^{o}C for 40s and 72^{o}C for 90s followed by a final extension of 72^{o}C for 7 min. Agarose gel electrophoresis of an aliquot of the PCR product was run along with 1KB marker (New England Biolabs, USA) and documented using Gel documentation System (BioRad, USA). This PCR product was used for restriction digestion by ARDRA.

Selection of restriction enzymes

Three tetra cutter restriction enzymes were selected for performing ARDRA.

AluI : Derived from *Arthrobacter luteus*, Alu I restriction enzyme cuts DNA having a sequence AG/CT.

Hae III : Derived from *Haemophilus aegypteus*, Hae III enzyme cuts a DNA strand having sequence GG/CC

Hpa II : Derived from *Haemophilus parainfluenza*, Hpa II cuts a DNA sequence having C/CGG sequence

Each PCR product was digested with Alu 1, Hae III and Hpa II (New England Biolabs, USA) in separate reactions. The digestion was performed in Eppendorf tubes containing 15 µl PCR product, 2 µl buffer (50mM NaCl, 10mM, Tris – HCl, 10mM $MgCl_2$, 1nM dithiothreitol),2.8 µl milliQ water, and 0.2 µl

(10U/ µl) each restriction enzyme thus having a 20µl reaction volume. The reaction tubes were incubated for 3 hours in a water bath set at 60°C which was the optimal temperature for the action of the three enzymes used. When the time for restriction had elapsed, the enzyme was deactivated by incubation at 80°C in a water bath. Restricted products were run on 3% agarose gel in 1X TAE buffer for 3hours at 100V.

After digesting each PCR product with the restriction enzyme, the gel images were carefully studied and the band patterns obtained were also scored according to the presence or absence of a gene fragment of appropriate length. If a fragment was present it was given a score 1 and if absent, it was 0. The matrix so generated were clustered using UPGMA method.

4.2.4 Denaturing Gradient Gel Analysis (DGGE)

A finger printing technique introduced by Muyzer *et al.* (1993), DGGE can separate DNA fragments of same length but different sequences. This technique makes use of denaturing or partial melting of polyacrylamide gels using a linear gradient of denaturants (urea and formamide) which hampers DNA migration. A GC rich clamp added to 5' end of one of the reaction primers prevents the complete dissociation of DNA strands. As a DNA fragment enters the concentration of denaturant where its lowest-temperature melting domain begins to denature, the domain 'opens' forming a DNA molecule with a branched structure. This branched DNA has a greatly retarded mobility in the gel matrix. If a DNA sample contains a point mutation in this domain which differs from the normal sequence, the Tm of the domain will be different; the DNA molecule will melt out at a slightly different Tm and thus will have an altered mobility in the gel compared to control DNA.

PCR amplification was performed in 1 50uL solution containing 1uL of PCR product as template, 10mM of each of deoxynucleotide triphosphates, 10 pico moles of each of the primers, Taq precision buffer(10X) and 5U of Taq polymerase. The forward primer 341 f (CCTACGGGAGGAGCAG) was with GC clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGC). 907r (CCGTCAATTCCTTTGAGTTT) was used as the reverse primer. PCR was performed on Thermal Cycler Gradient (Eppendorf ,USA) using a touch down program. Initial denaturation was at 94^{o}C for 5 min followed by 30 cycles at 94^{o}C for 30 s and a final extension at 72^{o}C for 5 min. Presence of PCR product was confirmed by agarose gel electrophoresis.

Denaturing gels were prepared by combining two solutions containing acrylamide and differing amounts of denaturants (Muyzer et al. 1993). Electrophoresis was performed in 1 × Tris-acetate EDTA buffer at 60 °C and at a constant voltage of 70 V for 16 h using a DCode system (BioRad). The DGGE gels were stained in a 1 : 2000-diluted SybrGold (Molecular Probes) in water for 30 min and gel image captured using Bio Rad XR+ Gel documentation System (BioRad, USA). Each DNA band in a lane was indicative of DNA sequences with same melting temperature.

4.3 Results

The seven media selected for heterotrophic bacterial were compared on the basis on bacterial counts and the results are given in Table 9.

BT-6 medium having 2% tryptone and 1% yeast extract supported maximum bacterial growth. Hence this medium was selected as the medium for enumeration, isolation and purification of cyanobacteria associated heterotrophic bacteria.

4.3.1 Colony Morphology of Bacteria

From the three cyanobacterial consortia 30 colonies each were isolated and purified. Isolates from *Cyanobacterium* MCCB 114 were given sequential numbers A1 to A 30. Isolates from *Cyanobacterium* MCCB 115 were given sequential numbers B1 to B30. Isolates from *Cyanobacterium* MCCB 238 were given sequential numbers C1 to C30. The colony morphology of the isolates is given in Tables 10 to 12.

4.3.2 Biochemical tests

Once purity of each heterotrophic isolate was established, biochemical tests were performed. Table 13 lists the biochemical tests performed on each bacterial isolate.

The processing of data of biochemical test results was done using NTSys Software. For processing by software, positive result was denoted as 1 and negative result denoted as 0. This scoring pattern as given in Tables14, 15, 16, 17, 18, 19 were analysed by software and dendrogram generated as given in Figures 49, 50 and 51.

4.3.3 Amplified Polymorphic DNA restriction analysis

Restriction of PCR products using three tetra cutter enzymes resulted in distinct band patterns as shown in Fig. 52. Band patterns obtained in ARDRA were scored based on the approximate size of bands. Approximate fragment sizes obtained after digestion by enzymes are given in Table 20. A matrix was prepared based on the presence or absence of a restriction fragment. These wereanalysed by NTSys software and they gave dendrograms given in Figures 53, 54 and 55.

Isolates from Cyanobacterium MCCB 114 resulted in 5 clusters. Isolates from Cyanobacterium MCCB 238 formed 4 clusters. Isolates from Cyanobacterium MCCB 238 formed 4 clusters.

4.3.4 Sequence Analysis

From each cluster obtained from the dendrogram, one representative sequence was selected for sequencing.

The sequences obtained were aligned using Genetool Lite Version 1.1 (BioTools Inc., Double twist, USA) and homology assessed using Blast algorithm with sequences in NCBI and RDP database and identified.

Representatives of *Cyanobacterium* MCCB 114 belonged to the following genera. *Labrenzia, Alkanivorax, Thalassobaculum, Maricaulis and Hoeflea.*

Representatives of *Cyanobacterium* MCCB 114 belonged to the following genera. *Hoeflea, Maricaulis, Labrenzia* and *Alkanivorax.*

Representatives of *Cyanobacterium* MCCB 114 belonged to the following genera. *Alkanivorax, Mesorhizobium, Maricaulis* and *Labrenzia.*

Sequences were deposited in NCBI Genbank with the following accession numbers.

Table 8 NCBI Accession Numbers of Heterotrophic Bacteria

Isolates of Cyanobacterium MCCB 114

Seq1	*Alcanivorax*sp.	MG025890
Seq2	*Alcanivorax* sp.	MG025891
Seq3	*Thalossobaculum* sp.	MG025892
Seq4	*Maricaulis* sp.	MG025893
Seq5	*Hoeflea* sp.	MG025895
Seq6	*Labrenzia* sp.	MG025896

Isolates of Cyanobacterium MCCB 115

Seq7	*Alcanivorax*sp.	MG025897
Seq8	*Hoeflea* sp.	MG025898
Seq9	*Labrenzia* sp.	MG025899
Seq10	*Maricaulis* sp.	MG025900

Isolates of Cyanobacterium MCCB 238

Seq11	*Alcanivorax* sp.	MG025901
Seq12	*Mesorhizobium* sp.	MG025902
Seq13	*Labrenzia*sp.	MG025903
Seq14	*Maricaulis* sp.	MG025904

4.3.5 Denaturing Gradient Gel Electrophoresis

Denaturing gel band numbers are indicative of the number of different species. Image of DGGE gel showing bands in a lane is indicative of species diversity and is shown as Fig.56.

It was found that diversity of organisms in the cyanobacterial cultures were low as evident by the number of bands in each line of the gel. In lane

showing DGGE of *Cyanobacterium* 114, six distinct bands could be identified. The thick band may be the dominant species in the culture i.e., *Cyanobacterium* sp.

In the lane showing DGGE of *Cyanobacterium* 115, five distinct bands are visible. In the lane showing DGGE of *Cyanobacterium* 238, five distinct bands are visible.

4.4 Discussion

This study on the identification of heterotrophic bacteria associated with cyanobacteria put on light some interesting observations. The three consortia used for the study were from off coast, Cochin. They were selected based on their antivibrio activity on 14 isolates of *Vibrio* and was suggested as a probiotic for aquaculture (Preetha *et al.,* 2007). The three cyanobacteria were identified as *Cyanobacterium* (Genus) with 99% similarity with *Cyanobacterium aponinum* reported from Italy. Further studies revealed that pure (axenic) cultures deteriorated faster when compared to consortia. So the associated bacteria were significant in the viability of cyanobacteria. This led to the isolation and identification of these bacteria.

The significant observation from the study was that the diversity of these consortia was low. DGGE gel patterns only showed limited number of bands which was representative of species diversity. Also in all the three groups, there was the presence of *Labrenzia, Alkanivorax* and *Maricaulis*. *Hoeflea,* a nitrogen fixing photobacteria was found in MCCB 114 and 115. A close relative of *Hoeflea, Mesorhizobium* was present in MCCB 238. *Thalassobaculum* was present in MCCB 114.

Labrenzia was reported to be an aerobic anoxygenicphototroph (Biebl et al., 2007). They belong to the group of alpha proteobacteria and have *puf*L

and *puf*M genes coding for photosynthetic reaction centers. This explained why this was growing in a photic environment. The light requirements of this bacteria and cyanobacteria were same and they were co-existing. Cyanobacteria produced oxygen during photosynthesis, whereas, as an aerobe, these bacteria utilized them.

Hoeflea and *Mesorhizobium* are two very similar groups of bacteria. *Hoeflea phototrophica* (Biebl *et al*. 2006) is a member of the family *Phyllobacteriaceae* in the order *Rhizobiales*. This marine bacterium contains the photosynthesis reaction-center genes *pufL* and *pufM* and is of interest because it lives in close association with toxic dinoflagellates such as *Prorocentrumlima*. They represent an array of physiological diversity, including carbon fixers, photoautotrophs, photoheterotrophs, nitrifiers, and methanotrophs. So *Hoeflea* might be capable of utilizing carbon compounds, if they are abundant in the environment. Also genus *Cyanobacterium* was not reported to be a nitrogen fixer and having a nitrogen fixing bacteria as an associate would have been beneficial for them. *Hoeflea* was also reported to be associated with toxic dinoflagellates and hence it would capable of metabolizing toxins. So their association would have certainly benefitted cyanobacteria.

Mesorhizobium, commonly found in soil, as a nitrogen fixer associated with root nodules of leguminous plants. *M. sediminum* was also reported from deep sea sediments in Indian Ocean (Yuan et al., 2016). Fu et al. (2017) also isolated *M. oceanicum* from deep sea water of South China Sea. In our study, it was found that *Mesorhizobium* was very slow growing and colonies took a long time to develop (7-14 days). So there is a chance that these bacteria prefer a symbiotic existence with cyanobacteria. As a nitrogen fixer, the cyanobacteria also would have benefitted from them.

Alkanivorax was reported to be a hydrocarbon degrading bacteria and capable of tolerating toxicity of organic compounds and decomposition products of hydrocarbon degradation (Naether *et al*., 2013). Such an organism could have utilized exocellular secretions that might have been harmful to or secreted by cyanobacteria. Hence its place as a cyanobacterial isolate is justified.

Maricaulis, a member of Caulobacteria, has two different morphologies. Reproduction results in the separation of two cells that are morphologically and behaviourally different from each other. One sibling is non-motile, sessile by virtue of adhesive material and prosthecate, possessing at least one elongated, cylindrical appendage (a prostheca) that is an outgrowth of the cell envelope, including the outer membrane, the peptidoglycan layer and the cell membrane (Staley, 1968). Caulobacteria are ubiquitous in water. As typical aquatic bacteria, they may be second only to pseudomonads in the breadth of their distribution and numbers (Lapteva, 1987). These two bacterial groups together are presumed to be responsible for considerable mineralization of dissolved organic material in aquatic environments, whereby the oligotrophic caulobacters are especially important when nutrient concentrations and ambient temperatures are low (Staley *et al.,* 1987). So, *Maricaulis* was present in Cyanobacterial culture due to two reasons: it can survive in low nutrient environment and that they play a significant role in mineralization of dissolved organic material. So it was indeed a symbiotic association.

Another associated heterotroph of *Cyanobacterium* MCCB 114 was *Thalassobaculum*. It was Gram negative and pleomorphic. *Thalassobaculum* with slightly curved and straight rod-shaped cells was isolated from coastal seawater (Zhang *et al.,* 2008). A novel strain of bacteria, Gram negative, rod-

shaped, non-motile without flagella, isolated from deep seawater also was reported as *Thalassobaculum* (Su *et al.,* 2016). Significance of the association of *Thalassobaculum* and cyanobacteria could not be understood with available literature as species reports were few.

The three Cyanobacterial consortia were isolated in 2004 and they have been repeatedly sub-cultured and maintained in the laboratory. During the passage of time, some of the bacteria present in the initial phase would have died off due to minimal availability of nutrients. Bacteria that showed adaptation to assimilate cyanobacterial exudates, detoxify toxic chemicals, utilize organic compounds or synthesize utilizable compounds for cyanobacteria would have survived in the long run.

Ecosystem concept, which is all about co-existence and food chains can happen in vitro and bring about stability in the system. This is a reminder that we also do not have existence without our environment and the organisms present in it, be it a microbe, bird or bee. Documentation and conservation of biodiversity is the need of the hour, before they go into oblivion by anthropogenic activities.

Table 9 Comparing of bacterial test media for growing heterotrophic associated bacteria of *Cyanobacterium* MCCB 114, 115 and 238. Counts are in cfu./mL

	BT-1	BT-2	BT-3	BT-4	BT-5	BT-6	BT-7
MCCB 114	$1.5x10^6$	$2x10^6$	$1.8x10^6$	$1.9x10^6$	$2x10^6$	$5.6x10^6$	$2.5x10^6$
MCCB 115	$8.8x10^6$	$6x10^6$	$9x10^6$	$8.9x10^6$	$9.7x10^6$	$14..2x10^6$	$7.5x10^6$
MCCB 238	$8.5x10^6$	$6.1x10^6$	$9.6x10^6$	$9.3x10^6$	$9.2x10^6$	$11.5x10^6$	$9.2x10^6$

Table 10 Colony characteristics of heterotrophic bacterial aggregates of Cyanobacterium MCCB 114

	Colony Characteristics
A1	Cream, round, mucoid when young, irregular and brown on ageing
A2	Cream, round, mucoid when young, irregular and brown on ageing
A3	Cream, round, mucoid when young, irregular and brown on ageing
A4	Pale white, irregular, flat, translucent, swarming colonies
A5	Cream, round, mucoid when young, irregular and brown on ageing
A6	Small, cream, round, entire, convex, slow growing,
A7	Pale white, irregular, flat, translucent, swarming colonies
A8	Cream, round, mucoid when young, irregular and brown on ageing
A9	Small, cream, slow growing,
A10	Pale white, irregular, flat, translucent, swarming colonies
A11	Small, white, round, entire
A12	Pale white, irregular, flat, translucent, swarming colonies
A13	Pale white, irregular, flat, translucent, swarming colonies
A14	Pale white, irregular, flat, translucent, swarming colonies
A15	Small, white, round, entire
A16	Small, cream, round, translucent
A17	Small, cream, slow growing,
A18	Pale white, irregular, flat, translucent, swarming colonies
A19	Small, cream, slow growing,
A20	Pale white, irregular, flat, translucent, swarming colonies
A21	Pale white, irregular, flat, translucent, swarming colonies
A22	Small, white, round, entire
A23	Small, cream, slow growing,
A24	Pale white, irregular, flat, translucent, swarming colonies
A25	Pale white, irregular, flat, translucent, swarming colonies
A26	Pale white, irregular, flat, translucent, swarming colonies
A27	Small, white, round, entire
A28	Pale white, irregular, flat, translucent, swarming colonies
A29	Small, white, round, entire
A30	Small, white, round, entire

Table 11 Colony characteristics of heterotrophic bacterial aggregates of Cyanobacterium MCCB 115

	Colony Characteristics
B1	Small, cream, slow growing,
B2	Cream, round, mucoid when young, irregular and brown on ageing
B3	Cream, round, mucoid when young, irregular and brown on ageing
B4	Pale white, irregular, flat, translucent, swarming colonies
B5	Small, white, round, entire
B6	Cream, round, mucoid when young, irregular and brown on ageing
B7	Pale white, irregular, flat, translucent, swarming colonies
B8	Small, white, round, entire
B9	Small, white, round, entire
B10	Small, white, round, entire
B11	Small, white, round, entire
B12	Small, white, round, entire
B13	Pale white, irregular, flat, translucent, swarming colonies
B14	Cream, round, mucoid when young, irregular and brown on ageing
B15	Cream, round, mucoid when young, irregular and brown on ageing
B16	Cream, round, mucoid when young, irregular and brown on ageing
B17	Cream, round, mucoid when young, irregular and brown on ageing
B18	Pale white, irregular, flat, translucent, swarming colonies
B19	Small, white, round, entire
B20	Pale white, irregular, flat, translucent, swarming colonies
B21	Pale white, irregular, flat, translucent, swarming colonies
B22	Small, white, round, entire
B23	Small, white, round, entire
B24	Small, white, round, entire
B25	Cream, round, mucoid when young, irregular and brown on ageing
B26	Cream, round, mucoid when young, irregular and brown on ageing
B27	Cream, round, mucoid when young, irregular and brown on ageing
B28	Cream, round, mucoid when young, irregular and brown on ageing
B29	Cream, round, mucoid when young, irregular and brown on ageing
B30	Cream, round, mucoid when young, irregular and brown on ageing

Table 12 Colony characteristics of heterotrophic bacterial aggregates of Cyanobacterium MCCB 115

	Colony Characteristics
C1	Small, white, round, entire
C2	Small, white, round, entire
C3	Small, white, round, entire
C4	Pale white, irregular, flat, translucent, swarming colonies
C5	Cream, round, mucoid when young, irregular and brown on ageing
C6	Small, white, round, entire
C7	Small, white, round, entire
C8	Pale white, irregular, flat, translucent, swarming colonies
C9	Pale white, irregular, flat, translucent, swarming colonies
C10	Cream, round, mucoid when young, irregular and brown on ageing
C11	Cream, round, mucoid when young, irregular and brown on ageing
C12	Small, white, round, entire
C13	Cream, round, mucoid when young, irregular and brown on ageing
C14	Cream, round, mucoid when young, irregular and brown on ageing
C15	Pale white, irregular, flat, translucent, swarming colonies
C16	Pale white, irregular, flat, translucent, swarming colonies
C17	Small, white, round, entire
C18	Pale white, irregular, flat, translucent, swarming colonies
C19	Small, cream, round, entire, convex, slow growing,
C20	Cream, round, mucoid when young, irregular and brown on ageing
C21	Small, white, round, entire
C22	Small, white, round, entire
C23	Small, white, round, entire
C24	Small, white, round, entire
C25	Pale white, irregular, flat, translucent, swarming colonies
C26	Small, white, round, entire
C27	Small, cream, round, entire, convex, slow growing,
C28	Small, white, round, entire
C29	Small, white, round, entire
C30	Small, white, round, entire

Table 13 Biochemical tests performed for clustering bacteria

Biochemical Test	Notation used	Biochemical Test	Notation used
MOF Dextrose Slant	T1	Sucrose Oxidation	T26
MOF Dextrose Butt	T2	Sucrose Fermentation	T27
Citrate	T3	Sorbitol Oxidation	T28
Galactose	T4	Sorbitol Fermentation	T29
Maltose	T5	Adonitol Oxidation	T30
Indole	T6	Adonitol Fermentation	T31
TSI test glucose fermentation only	T7	Lactose Oxidation	T32
TSI test glucose & lactose/or sucrose fermentation	T8	Lactose Fermentation	T33
Casein	T9	MannitolOxidation	T34
Fructose	T10	Mannitol Fermentation	T35
Xylose	T11	Glycerol Oxidation	T36
Ribose	T12	Glycerol Fermentation	T37
Raffinose	T13	Inositol Oxidation	T38
Melibiose	T14	Inositol Fermentation	T39
Rhamnose	T15	Dulcitol Oxidation	T40
Trehalose Oxidation	T16	Dulcitol Fermentation	T41
Trehalose Fermentation	T17		
Mannose Oxidation	T18		
Mannose Fermentation	T19		
Cellobiose Oxidation	T20		
Cellobiose fermentation	T21		
Arabinose Oxidation	T22		
Arabinose Fermentation	T23		
Inulin Oxidation	T24		
Inulin Fermentation	T25		

Table 14 Result of Biochemical Tests for Cyanobacterium MCCB 114 isolates A1-A15

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15
T1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
T2	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
T3	1	1	1	0	1	0	0	1	0	1	0	1	1	1	0
T4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	1	1	1	0	1	0	0	1	0	1	0	1	1	1	0
T8	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1
T9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T16	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T18	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T22	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0
T23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T26	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T28	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T29	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T30	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
T31	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
T32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T34	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T35	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T37	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1
T38	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T39	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
T40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 15 Result of Biochemical Tests for *Cyanobacterium* MCCB 114 isolates A16-A30

	A16	A17	A18	A19	A20	A21	A22	A23	A24	A25	A26	A27	A28	A29	A30
T1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0
T2	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0
T3	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0
T8	1	0	1	0	0	0	1	0	1	0	1	1	0	1	1
T9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T16	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T18	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T22	0	1	1	1	1	1	0	1	1	1	1	0	1	0	0
T23	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T26	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T28	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T29	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T30	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0
T31	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0
T32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T34	1	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T35	1	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T36	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T37	0	0	1	0	1	1	1	0	1	1	1	1	1	1	1
T38	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T39	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0
T40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 16 Result of Biochemical Tests for *Cyanobacterium* MCCB 115 isolates B1-B15

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15
T1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T2	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T3	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1
T4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1
T8	0	0	0	1	1	0	1	1	1	1	1	1	1	0	0
T9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T16	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T18	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T22	1	1	1	1	0	1	1	0	0	0	0	0	1	1	1
T23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T26	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T28	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T29	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T30	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T31	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T34	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T35	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T37	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T38	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T39	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 17 Result of Biochemical Tests for *Cyanobacterium* MCCB 115 isolates B16-B30

	B16	B17	B18	B19	B20	B21	B22	B23	B24	B25	B26	B27	B28	B29	B30
T1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T2	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T3	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1
T4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1
T8	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0
T9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T16	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T18	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T22	1	1	1	0	1	1	0	0	0	1	1	1	1	1	1
T23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T26	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T28	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T29	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T30	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T31	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T34	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T35	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T37	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T38	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T39	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 18 Result of Biochemical Tests for *Cyanobacterium* MCCB 238 isolates C1-C15

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
T1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
T2	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
T3	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0
T4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0
T8	1	1	1	1	0	1	1	0	1	0	0	1	0	0	1
T9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T16	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T18	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T22	0	0	0	1	1	0	0	1	1	1	1	0	1	1	1
T23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T26	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T28	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T29	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T30	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
T31	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
T32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T34	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T35	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T37	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
T38	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T39	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1
T40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 19 Result of Biochemical Tests for *Cyanobacterium* MCCB 238 isolates C16-C30

	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30
T1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T2	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
T8	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1
T9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T16	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T18	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T22	1	0	1	1	1	0	0	0	0	1	0	1	0	0	0
T23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T26	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T28	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T29	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T30	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T31	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T34	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T35	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T37	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1
T38	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T39	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0
T40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

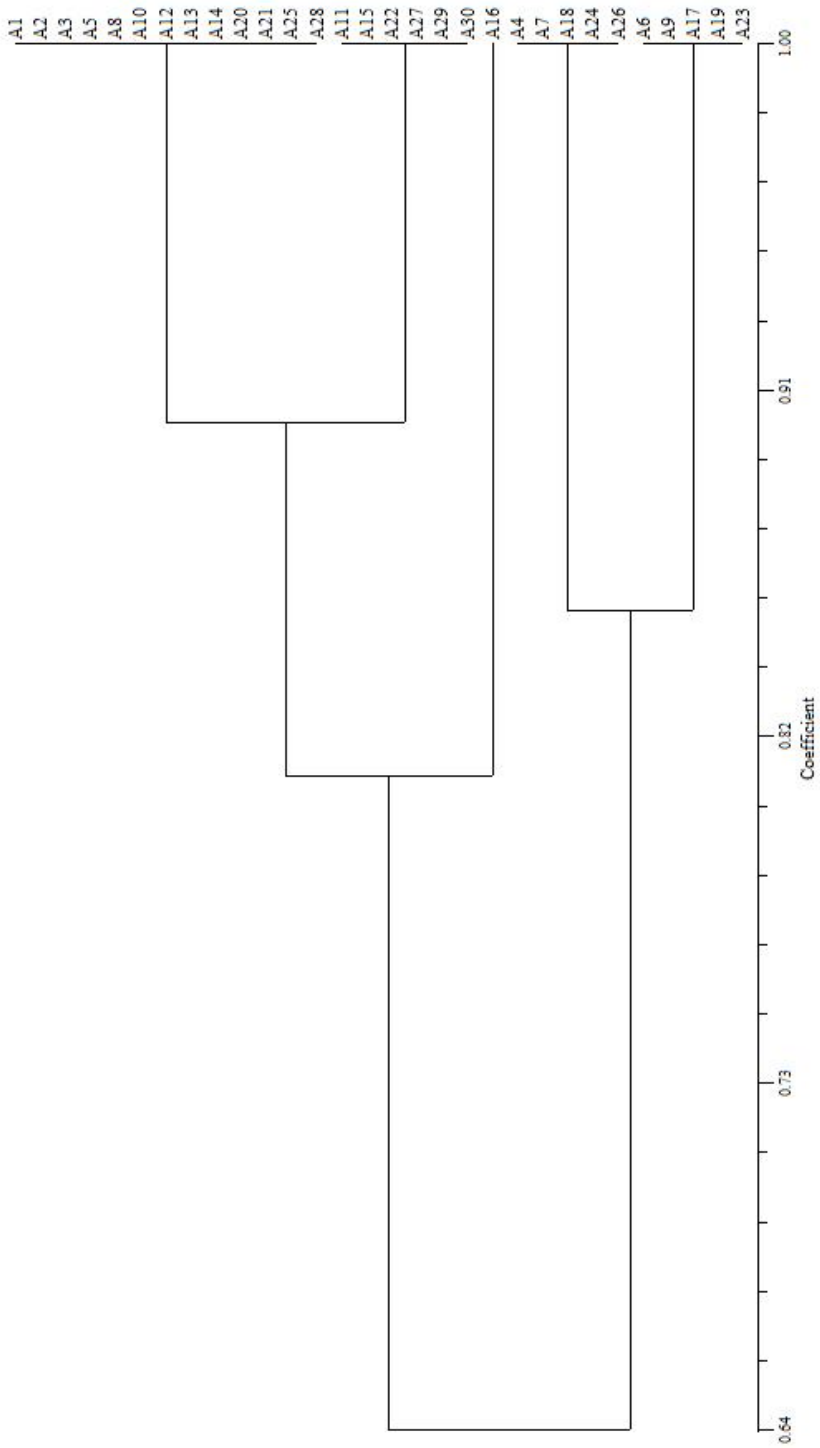

Fig. 49 Dendrogram showing the clustering of bacterial isolates of *Cyanobacterium* MCCB 114 by biochemical tests

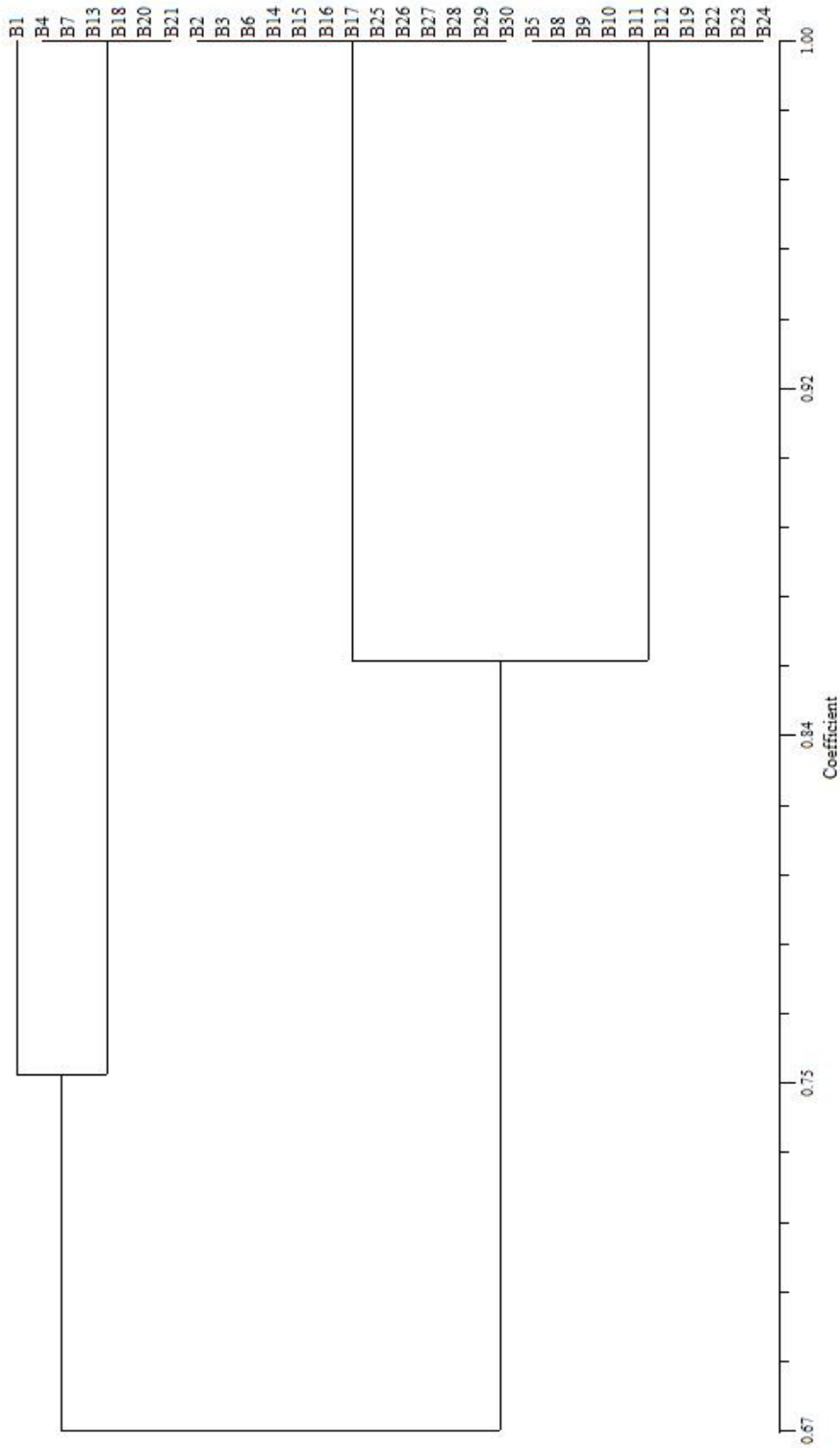

Fig. 50. Dendrogram showing the clustering of bacterial isolates of *Cyanobacterium* MCCB 115 by biochemical tests

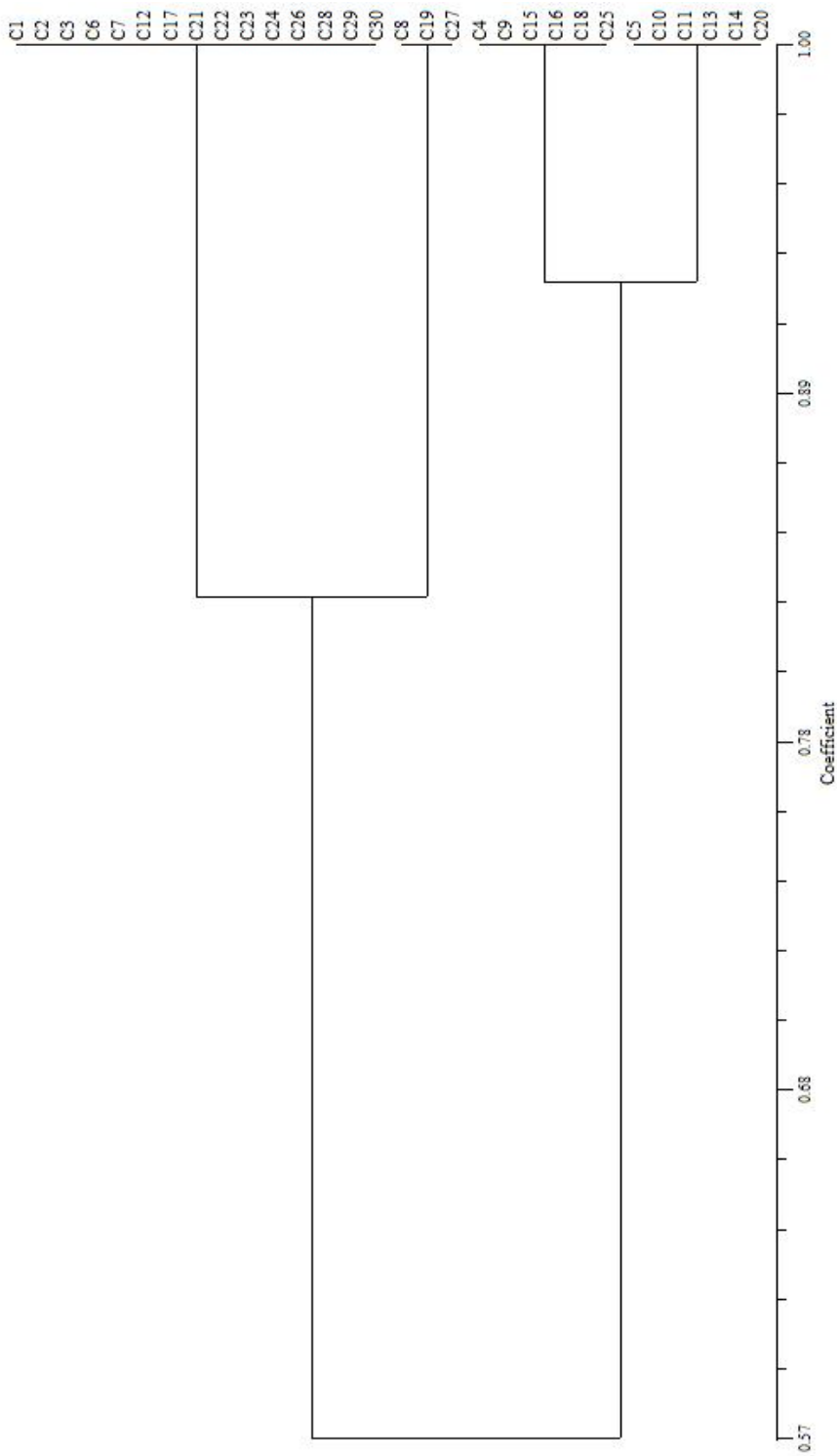

Fig. 51 Dendrogram showing the clustering of bacterial isolates of *Cyanobacterium* MCCB 238 by biochemical tests

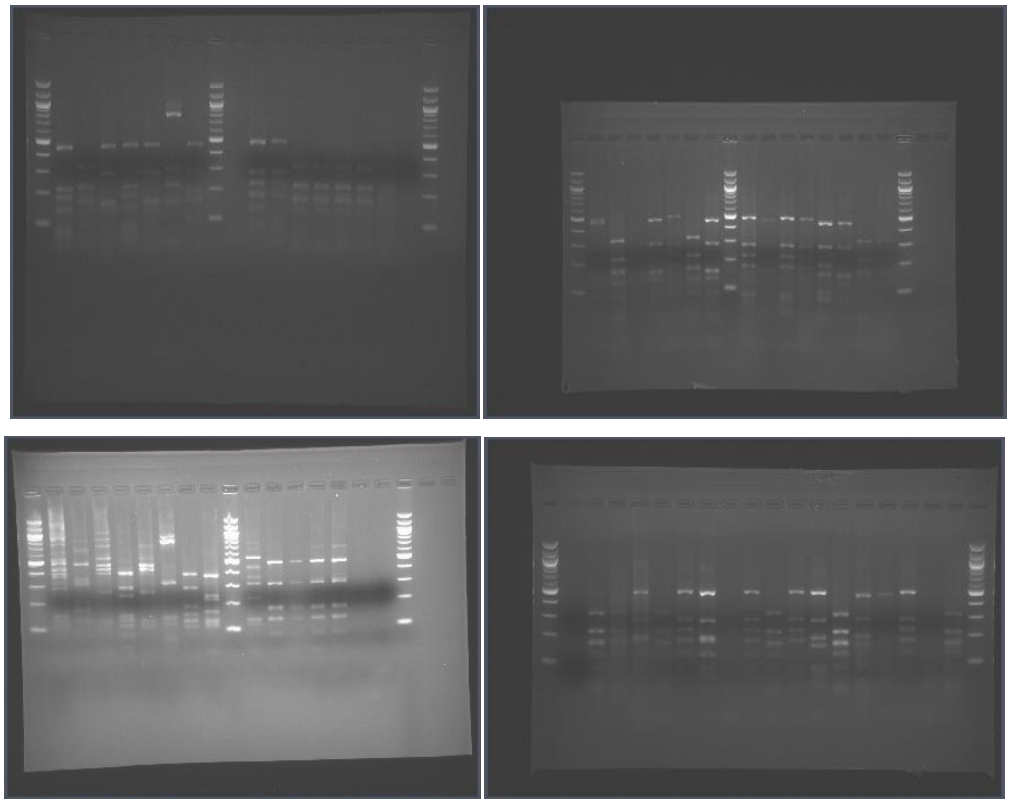

Fig 52. Some restriction patterns generated by ARDRA

Table 20 Approximate fragment length which served as operational taxonomic unit for scoring ARDRA Gels

Enzyme	Segment size after restriction digestion	Designation used for scoring
Alu I	100	T1
Alu I	150	T2
Alu I	200	T3
Alu I	300	T4
Alu I	350	T5
Alu I	500	T6
Alu I	600	T7
Alu I	750	T8
Hae III	100	T9
Hae III	150	T10
Hae III	200	T11
Hae III	300	T12
Hae III	350	T13
Hae III	450	T14
Hae III	500	T15
Hpa II	100	T16
Hpa II	150	T17
Hpa II	200	T18
Hpa II	250	T19
Hpa II	300	T20
Hpa II	400	T21
Hpa II	500	T22

Table 21 Scoring of ARDRA Gels of *Cyanobacterium* MCCB 114 isolates A1-A15

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15
T1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
T2	1	1	1	0	1	1	0	1	1	1	0	1	1	1	0
T3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T4	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
T5	1	1	1	0	1	1	0	1	1	1	0	1	1	1	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
T8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T9	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1
T10	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
T11	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1
T12	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1
T13	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
T14	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
T15	1	1	1	0	1	0	0	1	0	1	0	1	1	1	0
T16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T17	0	0	0	1	0	1	1	0	1	0	1	0	0	0	1
T18	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
T21	1	1	1	1	1	0	1	1	0	1	0	1	1	1	0
T22	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1

Table 22 Scoring of ARDRA Gels of *Cyanobacterium* MCCB 114 isolates A16-A30

	A16	A17	A18	A19	A20	A21	A22	A23	A24	A25	A26	A27	A28	A29	A30
T1	1	0	0	0	0	0	1	0	0	0	0	1	0	1	1
T2	0	1	0	1	1	1	0	1	0	1	0	0	1	0	0
T3	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T4	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0
T5	0	1	0	1	1	1	0	1	0	1	0	0	1	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1
T8	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T9	0	0	1	0	0	0	1	0	1	0	1	1	0	1	1
T10	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0
T11	1	1	0	1	1	1	1	1	0	1	0	1	1	1	1
T12	0	0	1	0	1	1	1	0	1	1	1	1	1	1	1
T13	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0
T14	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0
T15	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0
T16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T17	0	1	1	1	0	0	1	1	1	0	1	1	0	1	1
T18	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1
T19	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0
T21	1	0	1	0	1	1	0	0	1	1	1	0	1	0	0
T22	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1

Table 23 Scoring of ARDRA Gels of *Cyanobacterium* MCCB 115 isolates B1-B15

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15
T1	0	0	0	0	1	0	0	1	1	1	1	1	0	0	0
T2	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1
T3	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T4	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T5	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1
T6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	0	0	1	0	0	1	1	1	1	1	0	0	0
T8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T9	0	0	0	1	1	0	1	1	1	1	1	1	1	0	0
T10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T11	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1
T12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T14	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T15	1	1	1	0	0	1	0	0	0	0	0	0	0	1	1
T16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T17	0	0	0	1	1	0	1	1	1	1	1	1	1	0	0
T18	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
T21	0	1	1	1	0	1	1	0	0	0	0	0	1	1	1
T22	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1

Table 24 Scoring of ARDRA Gels of *Cyanobacterium* MCCB 115 isolates B16-B30

	B16	B17	B18	B19	B20	B21	B22	B23	B24	B25	B26	B27	B28	B29	B30
T1	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0
T2	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1
T3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T4	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T5	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0
T8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T9	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0
T10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T11	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1
T12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T14	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T15	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1
T16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T17	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0
T18	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
T21	1	1	1	0	1	1	0	0	0	1	1	1	1	1	1
T22	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1

Table 25 Scoring of ARDRA Gels of *Cyanobacterium* MCCB 238 isolates C1-C15

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
T1	1	1	1	0	0	1	1	0	0	0	0	1	0	0	0
T2	0	0	0	0	1	0	0	1	0	1	1	0	1	1	0
T3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T4	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
T5	0	0	0	0	1	0	0	1	0	1	1	0	1	1	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	1	1	1	0	0	1	1	0	0	0	0	1	0	0	0
T8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T9	1	1	1	1	0	1	1	0	1	0	0	1	0	0	1
T10	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
T11	1	1	1	0	1	1	1	1	0	1	1	1	1	1	0
T12	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
T13	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
T14	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
T15	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0
T16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T17	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1
T18	1	1	1	0	1	1	1	1	0	1	1	1	1	1	0
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
T21	0	0	0	1	1	0	0	0	1	1	1	0	1	1	1
T22	1	1	1	0	1	1	1	1	0	1	1	1	1	1	0

Table 26 Scoring of ARDRA Gels of *Cyanobacterium* MCCB 238 isolates C16-C30

	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30
T1	0	1	0	0	0	1	1	1	1	0	1	0	1	1	1
T2	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
T3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T4	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T5	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	1	0	0	0	1	1	1	1	0	1	0	1	1	1
T8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T9	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1
T10	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
T11	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1
T12	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1
T13	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
T14	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T15	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
T16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T17	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
T18	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1
T19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T20	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T21	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0
T22	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1

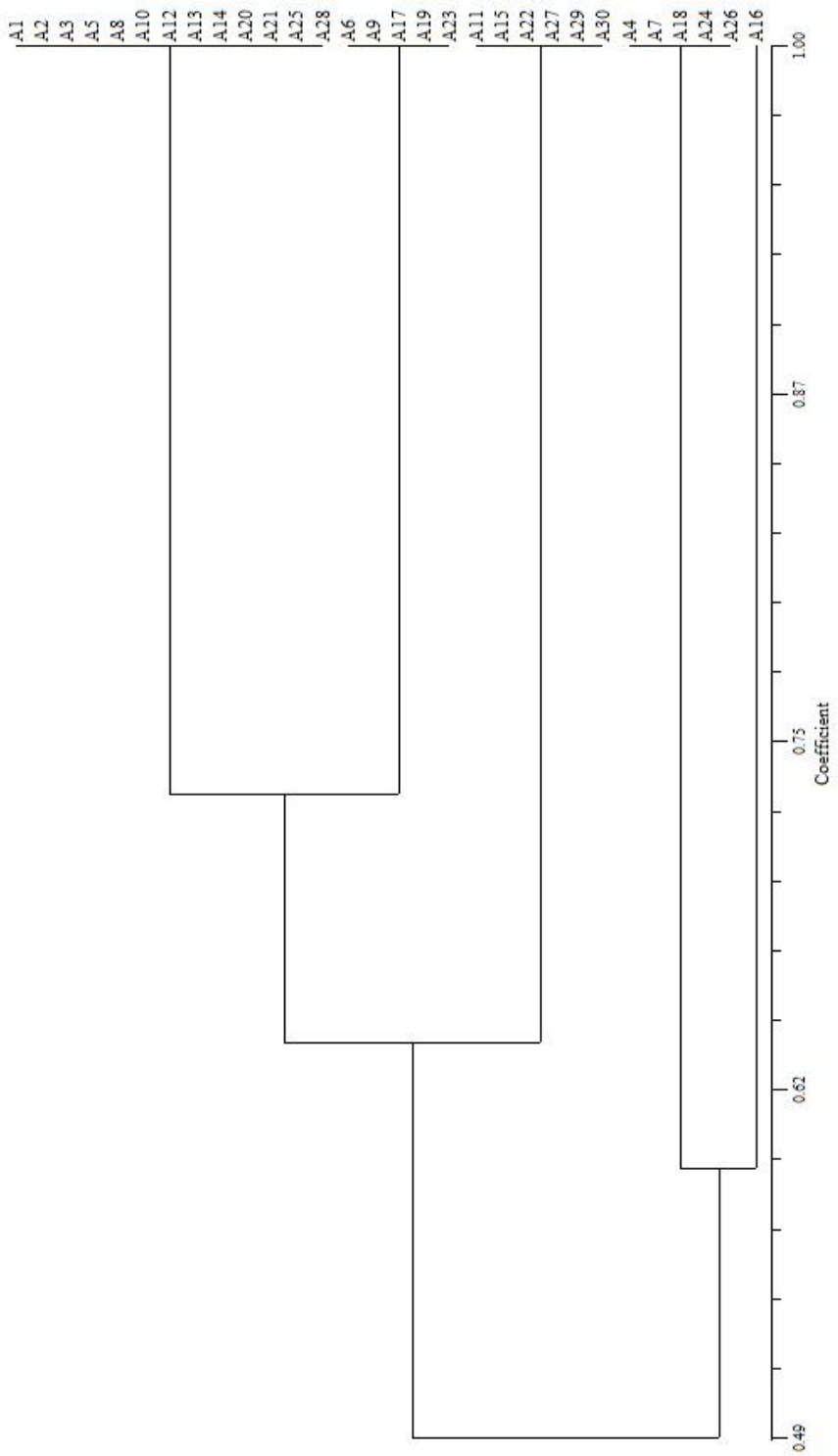

Fig. 53. Dendrogram showing the clustering of bacterial isolates of *Cyanobacterium* MCCB 114 by ARDRA

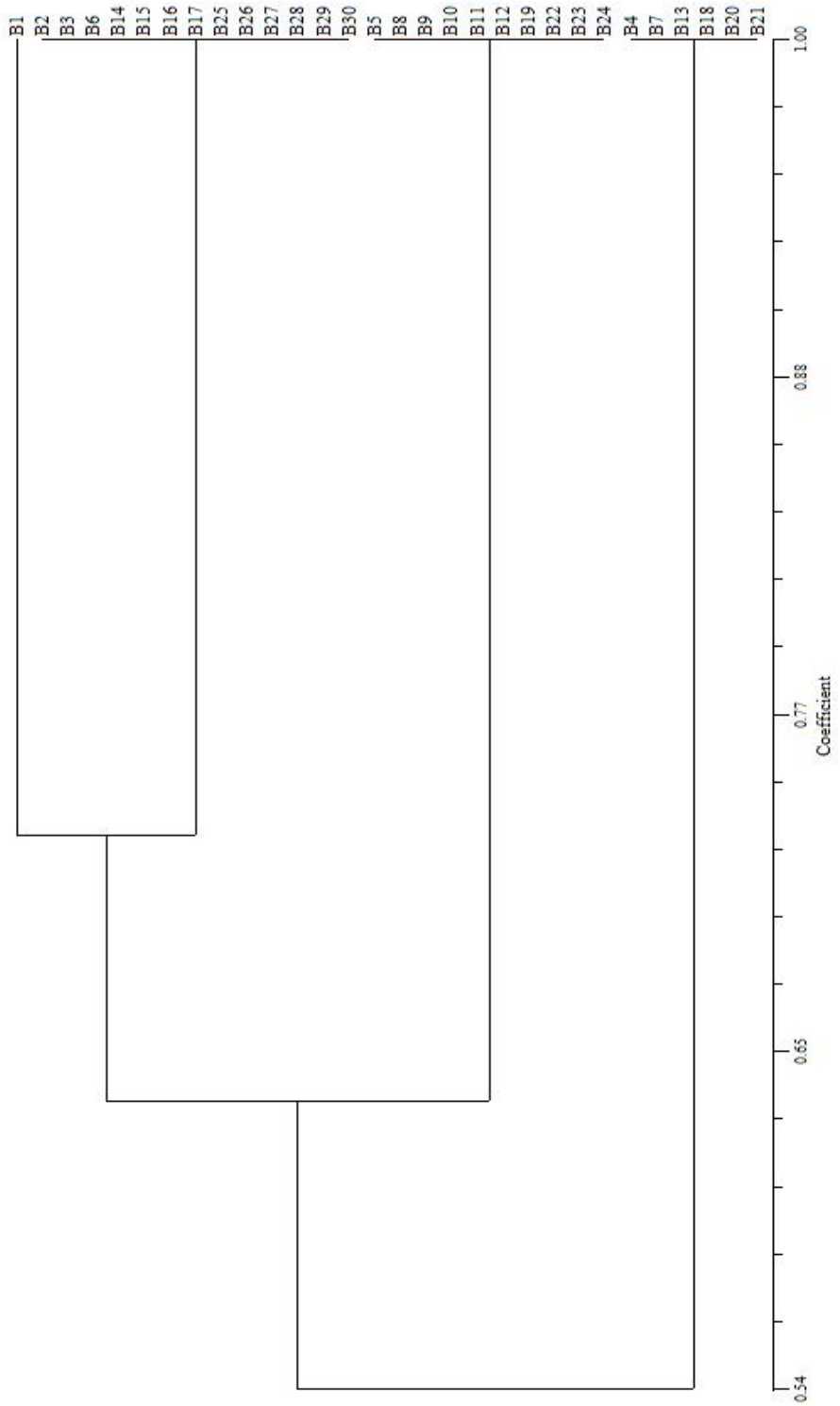

Fig. 54. Dendrogram showing the clustering of bacterial isolates of *Cyanobacterium* MCCB 115 by ARDRA

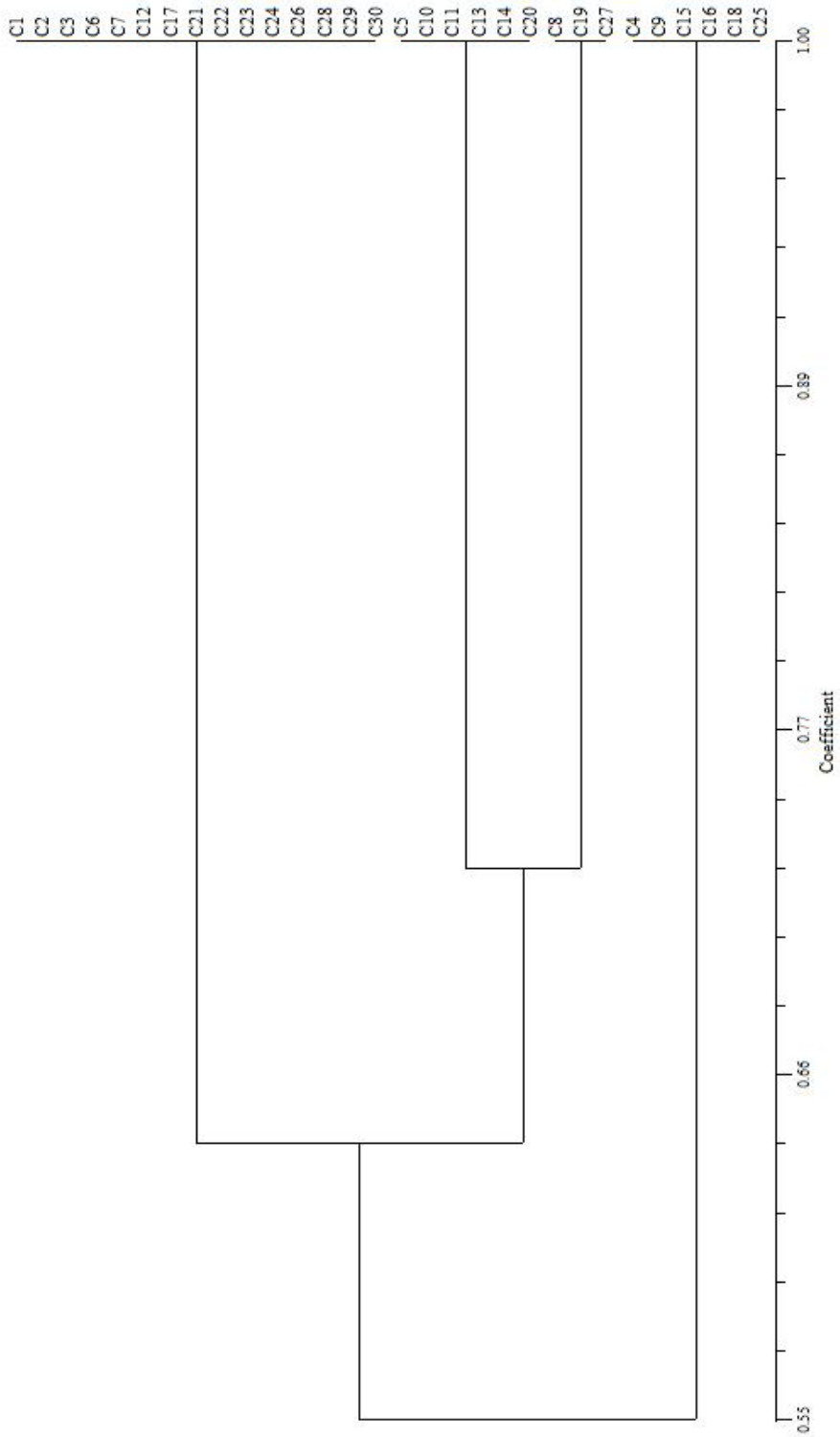

Fig. 55. Dendrogram showing the clustering of bacterial isolates of *Cyanobacterium* MCCB 238 by ARDRA

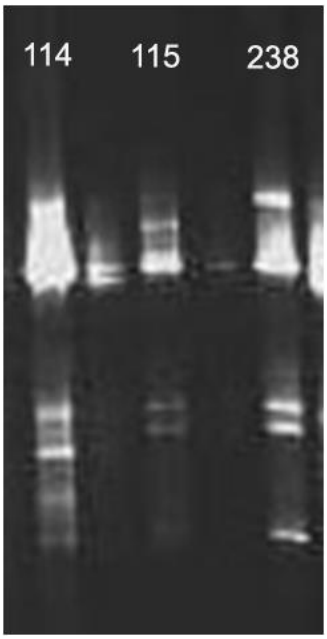

Fig 56. Gel image of Denaturing Gradient Gel Electrophoresis

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Chapter 5

Conclusion and Scope for Further Research

Cyanobacteria, called as blue green algae by phycologists and Cyanophyta by botanists are prokaryotes in terms of cellular organization. There are no hard and fast rules pertaining to the classification of cyanobacteria. Earlier Geitlerian system was followed for classification which followed morphological characterization of collected specimens. Cyanobacteria were divided into 5 major divisions based on morphology.

But when DNA sequence analysis became widespread, small subunit ribosomal RNA gene (16S RNA gene), large subunit ribosomal RNA gene (23S RNA gene), Internally transcribed spacer (ITS), Intergenic spacer between Phycocyanin B and A subunits of Phycocyanin Operon (*cpc* BA IGS), Ribulosebisphosphate carboxylate large subunit (*rbc*L), DNA dependent RNA polymerase (*rpo*C1), all found applications in the field of cyanobacterial taxonomy.

Though there was no authority to define how ideal classification systems should be, there was a movement across the world to follow a polyphasic approach which incorporates morphological data, biochemical data, environmental data and genomic data.

Polyphasic taxonomy of three cyanobacterial isolates from the coastal waters of Cochin classified them under Genus *Cyanobacterium*. In the case of prokaryotes, we cannot apply species concept as they are constantly evolving and adapting to new environments, resulting in phenotypic changes. But genotypic changes occur at a slower pace. So sequence analysis is quintessential in the field of taxonomy.

Sequence analysis is easy to perform with axenic cultures. Hence purification was undertaken by repeated streaking until axenicity was established. Cyanobacteria took approximately 10 days to form discrete colonies that could be sub cultured. As the number of cultures to be purified was less, streak plate technique could be resorted to. Mechanical methods and addition of antibiotics were alternatives to purification by streaking if the large numbers of cyanobacteria need to be purified.

Morphological differences among cyanobacterial strains were less. External appearance of glycocalyx was the only difference noted. They showed some difference in their affinity towards carbon compounds, but those were also not of significance for species designations. Hence these Cyanobacteria were designated as ecotypes, which showed differences based on the external environment.

The isolation source of these organisms was sea water. In the experiment to understand salinity optimum and tolerance, it was found that they thrived in fresh water i.e., 0%w/v of NaCl. But tolerance was in the range 20-40%w/v of NaCl. This shows that they were fresh water isolates that reached marine environment and survived there.

All the cyanobacterial cultures reached a pH above 8 when they reached stationary phase. With growth optima between pH 7.5 and approximately 10, cyanobacteria can therefore be considered alkaliphiles (Brock, 1973). Their preference for alkaline conditions suggests that there is an acid barrier that these micro-organisms have not been able to overcome, as a consequence of which cyanobacteria have been excluded from acidic environments.

These organisms were mesophiles, though their nearest relative as per Genbank database was a thermophile.

Concluding the results of polyphasic taxonomy, it can be said that more questions than answers were raised during the course of study. Isolation of this genus from fresh water and brackish water environments, their mode of survival in differing salinities and temperatures, the structural changes of glycocalyx, distribution patterns, isolation of economically important compounds are some good areas to work on.

Axenic cultures of *Cyanobacterium* deteriorated faster when compared to consortia. This phenomenon was observed in all the three isolates. Interrelationships between cyanobacteria and bacteria were keeping the consortia robust for longer periods. Re-mineralization, detoxification, nitrogen fixation: anything or everything can be the answer. Population dynamics, succession and toxins present in cyanobacterial mats and blooms were investigated by metagenomic approaches in recent times. But studies on comparison of axenic and non-axenic cultures have been few. Shen et al. (2011) reported that in a unicellular colony forming cyanobacteria *Microcystis aeruginosa* grown along with a group of natural isolates, non-axenic organisms aggregates into colonies by producing copious amount of exopolysaccharides whereas axenic ones continued living as single cells. In our study, in the case of MCCB 114, axenic cultures aggregated, settled down and deteriorated. Hence this observation may be different in different groups. Such a phenomenon was not found in other two cultures of MCCB 115 and MCCB 238.

From cell counts of live cells, chlorophyll content and peaks of cellular absorption spectra, it was found that consortia was viable for a longer period compared to axenic cultures. More investigations are needed to elucidate the mechanisms that lead to ageing of cyanobacterial cells. There are many assumptions on bacterial ageing. The free radical hypothesis states that aging results from random deleterious events, and that self-inflicted oxidative damage is the primary contributor in the degeneration of organisms.Another

hypothesis that explains ageing is sigma factor competition. In bacteria, proliferation activities are carried out by housekeeping factor σ^{70} and maintenance activities are directed by σ^{S}. Conflict in the expression of these factors also plays a role in senescence. Asymmetry in cell division in *E.coli* was also reported as a cause for ageing (Nystrom, 2002). Though these are some preliminary observations, senescence of microbes undergoing binary fission also calls for further investigation with modern techniques.

Study on the associated bacterial heterotrophs in the three closely related strains, isolated from same locality and repeatedly sub-cultured for 14 years showed similar community structure. This observation shows natural selection and ecosystem concept operating in vitro. Bacteria that were stringent associates of cyanobacteria and performing a role in maintaining the ecosystem healthy, survived the passage of time. All common marine bacteria that would have been present at the time of isolation, not associated with cyanobacteria were wiped out. The strains that remained: *Hoeflea*, *Mesorhizobium* and *Labrenzia* were phototrophic organisms. They were also mineralizers, cleaning up the exudates produced by cyanobacteria. *Maricaulis* was a prosthecatebacterium with two types of cells, stalk cell which is sessile and swarmer cell, motile by means of polar flagellum. It is ubiquitous in all waters due to active swarming. *Alkanivorax* was a hydrocarbon degrading bacteria, capable of tolerating toxicities associated with intermediate compounds of hydrocarbon degradation. So it would also have had an active role in ecosystem maintenance in the case of cyanobacterial consortia. *Thalassobaculum,* a member of Rhodospirillaceae was present in Cyanobacteria MCCB 114. Not much could be assumed regarding its function in the company of cyanobacteria.

Each and every organism has a role in ecosystem. If there is a passion to observe things, there will be so many things lying out there for discovery.

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