REVIEW OF LITERATURE

2.1. Cyanobacteria

Cyanobacteria constitute a highly diverse prokaryotic group and inhabit a wide variety of environments offering different climatic conditions, including the most extreme ones, such as hot springs and deserts as well as cold areas in the Antarctic (Whitton and Potts, 2000). Cyanobacteria evolved approximately 3.5 billion years ago (Schopf, 2000) and their ancestors introduced oxygenic photosynthesis using water as electron donor. Through the evolutionary trend cyanobacteria have remained a critical component of terrestrial and aquatic ecosystems as primary producers and are often important agents for nitrogen fixation (Knoll, 2008).

Cyanobacteria are microscopic in size ranging from 1-80 μm in cell diameter and show substantial morphological diversity. Following botanical nomenclature, they were earlier classified as blue-green algae, based primarily on their algal and plant-like photosynthesis, as well as their distinct pigmentation caused by phycobiliproteins (phycocyanin and phycoerythrin) and chlorophyll-a. This classification depends on observations of field material, represented by herbarium specimen (Rippka et al., 1979). Hence, the identification is based on phenotypic rather than genotypic characters, such as morphology of cells and filaments, shape of the terminal cells, presence or absence of sheaths, gas vacuoles, motile hormogonia, heterocysts and akinetes/spores (Thuret, 1875; Gomont, 1892; Komarek and Anagnostidis, 1998, 2005).

Following the discovery using electron microscopy in the 70’s that the cell wall is typically Gram negative and the sub-cellular arrangement typically prokaryotic (lack of membrane bound organelles), the group was reclassified as cyanobacteria following the bacteriological approach of nomenclature (Stanier et al., 1978). Identification based on
physiological and genotypic characters of live specimen in culture, cyanobacteria have been
categorised into five principle morphological groups or sections, representing different
ascending complexities (Rippka et al., 1979) as shown below:

I. Unicellular cyanobacteria that reproduce by binary fission or budding

II. Unicellular cyanobacteria that reproduce by multiple fission

III. Filamentous non-heterocystous cyanobacteria that divide in one plane

IV. Filamentous heterocystous cyanobacteria that divide in one plane

V. Filamentous heterocystous cyanobacteria that divide in more than one plane

However, both the botanical and the bacterial approaches have drawbacks. For example, the unavailability of appropriate media/conditions that can support growth of all
cyanobacteria still makes only a limited number of cyanobacteria isolated from nature being
culturable (Kumari et al., 2009). Also changes of phenotypic characters due to environmental
variations are common and were considered as problematic using the culture independent
approach (Komarek and Anagnostidis, 1989). Recently, the use of molecular techniques in the
culture independent approach was introduced to identify cyanobacteria in various habitats, but
it may still be problematic to identify characters signifying individual taxa (Komarek and

To avoid these constrains, the identification of cyanobacteria is currently often based
on a combination of the more traditional botanical and the more modern bacteriological
approach. It was opined that cyanobacteria be classified using this combined set of markers,
using molecular data (as a genetic basis) combined with structural/ ultrastructural, phenotypic
and ecological data, whenever possible. Recently genomic methods, such as whole genomic
sequencing and metagenomic analyses, have been used to establish the diversity of
cyanobacteria both from cultured species as well as from natural communities. The molecular
methods expedite the exploration of many habitats and have demonstrated that some
cyanobacteria are habitat specifics and many habitats contain a still largely un-described genetic diversity. The diversity may have been masked using simple morphological features as a base for identifications (Garcia-Pichel, 2008).

Molecular methods currently being used include for example: DNA-DNA hybridization, nucleotide (nucleic acid) cloning and sequencing, several polymerase chain reaction based fingerprinting methods, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), single strand conformation polymorphism (SSCP), DNA microarrays, as well as PCR independent methods like G+C content. Various marker genes, encoding house-keeping or functional genes, have been useful to identify as well as to study the function of cyanobacteria. The 16S rRNA gene (partial and whole sequence) has so far been the most frequently and successfully used cyanobacterial identifier and has expanded our knowledge substantially when it comes to identification of cyanobacteria from various natural habitats including tropical marine habitat (Nubel et al., 1997; Lundgren et al., 2003; Uku et al., 2007; Diez et al., 2007; Bauer et al., 2008; Foster et al., 2009b).

The 16S rRNA gene is highly conserved between different species of bacteria and archaea and has become an important molecular marker for microbial phylogenetic analysis (Woese, 1987; Woese et al., 1990; Case et al., 2007). The most commonly used 16S rRNA cyanobacteria specific PCR primers set was designed by Nubel et al. (1997). Comparison of 16S rRNA gene sequence analysis of natural communities and strains from culture collections provides insights into differences (Garcia-Pichel et al., 2001) and/or similarities between cyanobacteria taxa. However, the 16S rRNA gene is considered to be more conserved in function and structure than protein encoding genes and thus the genetic diversity
found using 16S rRNA as marker may not sufficiently deeply reflect the physiological diversity of the organisms present (Fox et al., 1992).

2.2. Historical perspective

Farmintzin (1871) was apparently the first to culture algae, although the work of Beijerinck (1890, 1891, 1893) and Miquel (1890/92a-e) represented the first serious studies on algae culture. Moore (1903) and Kuster (1907) were the first to publish reviews of algal culturing techniques. Algal culturing techniques are also described in detail in several books and articles started from 1930s and continuing (Guillard, 1995; Richmond, 2003). The importance of sterile technique and the concept of perpetual maintenance were adapted from microbiological research (Beijerinck, 1890, 1891, 1893; Miquel, 1890/92a-e). Many of the methods and basic media concepts that are used today were developed in the late 1800’s and early 1900’s. Advances in instrumentation are too numerous to summarize, but significantly, Warburg (1919) and Hartmann (1921) were the first to describe the use of electric lights as an illumination source. To avoid heating, they used a screen of cold water, between the lights and the cultures, to absorb the infra-red radiation.

2.2.1. Isolation

Single cell isolation techniques using a capillary pipette were developed (Allen and Nelson, 1910) and the techniques were improved by Pringsheim (1921b) and Lwoff (1923, 1929). Beijerinck (1893) was the first to isolate algae using gelatin plates (a method with limited application because bacteria dissolve the gelatin). Klebs (1896) was apparently the first to use agar for the culture of algae. Tischutkin (1897) and Ward (1899) used petriplates containing agar in their efforts for isolating algae. Ward (1899) recommended through washing of agar with dilute acetic acid to remove contaminating salts, a step modified much later by Waterbury et al. (1986) for isolating oceanic picoplankters. Chodat (1904, 1913) cultured algae on agar in Erlenmeyer flasks and he cut out blocks of agar for transfer the alga
to new flasks. Skinner (1932) isolated soil algae by embedding them in agar filled glass test tubes and when colonies formed, he broke the test tubes to recover the isolated colonies.

Serial dilutions were developed very early as an isolation technique (Allen and Nelson, 1910). Mainx (1927) was the first to publish a method for isolating algae by centrifugation and Bold (1942) was the first to isolate algae using phototaxis and various refinements have been added (Paasche, 1971). Klebs (1896) isolated filamentous algae by first isolating their zoospores onto agar. Vischer (1937) first reported a method of dragging filaments through agar to remove epiphytes. The following are a few of the first reports to isolate specific algae into culture: *Zoochlorellae* (Beijerinck, 1890), freshwater and marine diatoms (Miquel, 1893), *Nostoc* (Bouilhac, 1897) and dinoflagellates (Kuster, 1908).

### 2.2.2. Axenic cultures and antibiotics

Remarkably, axenic (“pure” in older literature) algal cultures were established almost immediately after the advent of algal culturing. There is some controversy surrounding the honors for the first axenic culture. Beijerinck (1890, 1893) claimed to have success, but as Pringsheim (1946) reminds us, Klebs (1896), who was a student of Koch, questioned this claim. Miquel (1893) addressed the problems associated with contaminating fungi, protozoa and bacteria, he illustrated a rinsing apparatus for removing bacteria and he reported that he obtained axenic (“pure”) cultures of diatoms. Richter (1903), unaware of Miquel’s success, claimed to be the first to establish an axenic culture. One year later, Chodat (1904) described his success in obtaining axenic cultures.

After the discovery of antibiotics in the mid 1900’s, these were quickly applied to the process of purifying algal strains. Godzwiz-Shelubsky (1951), Provasoli *et al.* (1951) and Reich and Kahn (1951) reported the results of several antibiotics used to purify algal cultures. Spenser (1952), Droop (1967) and others (Stein, 1973) published other early papers describing antibiotic methods. Lewin (1959) pointed out that despite this success, many of
the strains rendered axenic by antibiotics could have been purified using traditional methods; this remains true today.

2.2.3. **Culture medium and its preparation**

In the beginning, culture media were usually prepared by adding chemical compounds to lake water or seawater (Miquel, 1893; Molisch, 1895, 1896). Distilled water was tried; however, the tin/platinum stills of those days added considerable amounts of metals to the water, making the culture medium toxic to most algae. Pringsheim (1912) recommended the use of a glass still for preparing distilled water. Similarly, culture media were often sterilized by pasteurization rather than autoclaving because of the metal contamination from the copper autoclaves. Naegeli (1893) was the first to report copper toxicity for algae.

Early workers discovered that the exact concentrations and ratios of chemicals were not always critical and subsequent scientists modified early media recipes - a process that continues till today. Beijerinck (1901) discovered that certain cyanobacteria would grow without a nitrogen source and hypothesized that somehow they could produce their own nitrogen source. Furthermore, early workers recognized that chemicals contained impurities, implying that unknown contaminating trace nutrients were being added inadvertently to the culture medium, contributing to better algal growth (Allen and Nelson, 1910; Allen, 1914). The situation was summarized succinctly by Provasoli and Pintner (1960) as they explained why early media required only Fe and later media required Co, Cu, Mn, Mo, V and Zn. The “impurities” of distilled water, seawater and glassware were recognized; metal toxicity was described and the importance of pH, vitamins and iron (as a trace metal) were established (Allen and Nelson, 1910; Allen, 1914).

2.2.4. **Organic additions**

While Farmintzin (1871) was apparently the first to grow algae by adding the inorganic salts used for photosynthetic plants (Knop’s solution), organic additions to algal
culture media began almost immediately. Miquel (1893) added some material from an organic maceration to his mineral culture medium. Bouilhac (1897) cultured *Nostoc* in an organic medium and similarly, Matruch and Molliard (1902) cultured *Stichococcus* using an organic medium. *Polytoma* was apparently the first colourless alga brought into culture (Jacobsen, 1910) and a few years later, Pringsheim (1921a) reported the use of acetate as an organic source (*Chlamydomonas, Carteria* and *Polytoma*).

2.3. **Immobilization**

In the past 20 years, the use of immobilized enzymes or cell components for the production of a series of metabolites has become a branch of biotechnology of rapidly growing importance. Although in the initial stage most of the research work on immobilization dealt with systems designed for the release of products, synthesized by enzymes or multi-enzyme complexes, a more recent development focuses on the immobilization of complete cells or cell agglomerates (Becker, 1995). An immobilized cell is defined as a cell that by natural or artificial means is prevented from moving independently of its neighbours to all parts of the aqueous phase of the system under study (Tampion and Tampion, 1987). To a certain extent these systems resemble natural environmental conditions as many microorganisms grow in a biotype, where they are also immobilized by encapsulation in slimes or as a partner of symbiotic systems. Although the pioneering work with immobilized cells mostly employed heterotrophic organisms, a number of scientific reports today deal with studies on plant cells, algae, cyanobacteria and photosynthetic bacteria. These phototrophic organisms offer several prospects for use in immobilization techniques, because they can use sunlight as their sole, or major, energy source to make products from the substrates of photosynthesis.
2.3.1. Immobilization techniques

In principal, six different types of immobilization methods can be distinguished. They are covalent coupling, affinity immobilization, adsorption, confinement in liquid-liquid emulsion, capture behind semi-permeable membrane and entrapment (Mallick, 2002). Entrapment is the most frequently used method in laboratory experiments. Entrapment methods are based on the confinement of the cells in a three dimensional gel lattice. The cells are free within their compartments and the pores in the material allow substrates and products to diffuse to and form the cells. Several synthetic (acrylamide, polyurethane, polyvinyl, etc.) and natural polymers (collagen, agar, agarose, cellulose, alginate, carrageenan, etc.) are used for this purpose. However, for algal immobilization the most frequently used natural gels are alginate and carrageenan.

2.3.2. Current uses of immobilized algae

Using the biomass for production of modern energy carriers such as electricity has a wide range of other environmental, social and economic benefits. Direct generation of electricity has been demonstrated by immobilizing the cyanobacterial species Mastigocladus laminosus (Ochiai et al., 1980) and Phormidium (Ochiai et al., 1983) onto SnO$_2$ optically transparent electrode with calcium alginate, functioned as an anodic photoelectrode on continuous illumination for periods of time adequate for use in a conventional electrochemical cell. This “living electrode” shows promise of use as a long-lived photo converter of solar radiant energy to electric energy and as a suitable replacement for unstable chloroplast systems. Such “living electrodes” have been used to generate photocurrents and have been operated for 20 days or more (Ochiai et al., 1980).
2.4. Preservation

A technique which can prevent loss of algal cultures or their characteristics is cryopreservation. Currently, it is used only by some of the large collections e.g. CCAP where one third of their 2000+ algal strains are stored under liquid nitrogen. The cryoprotectant is usually 5% DMSO but methanol and sucrose can be effective. The rate of recovery is often a problem and where loss of viability is severe, freeze-resistant mutants may actually be selected. Unicellular green algae and cyanobacteria are most successfully cryopreserved. For cyanobacteria, an alternative technique of lyophilization is very convenient for culture collections. Vaulot et al. (1989) recommended immediate fixation with 1% glutaraldehyde followed by storage in liquid nitrogen. This method has been proven to be suitable for cyanobacteria and small cells but presents many drawbacks such as cell loss and chlorophyll fluorescence variation for larger cells. The main physical problem linked to preservation methods concerns the cellular damage caused by intracellular freezing.

The amount of damage depends upon the freezing rate i.e. at ultra-rapid freezing rate, little or no freezable water leaves the cell and intra-cellular ice forms; at moderate freezing rate, only part of the freezable water leaves cells which are not sufficiently dehydrated to cause injury; at slow freezing rate, all freezable water leaves cells which may suffer severe dehydration injuries. One possibility for avoiding internal freezing would be the addition of cryoprotectant just above the freezing temperature prior to chemical fixation, before super cooling (Withers, 1985). Indeed, cryoprotectants have colligative properties and maintain water in the liquid state by forming hydrogen bonds, preventing the diffusion of water molecules to the ice front and participating in the crystallization process (Finkle et al., 1985).

2.5. Ultra structure

A number of investigators have shown that the ultrastructure and organization of the cyanobacterial cell is fundamentally different from that found in higher plants and animals
Stanier and Van Neil (1962) suggested that it is a prokaryotic cell. Two regions within the protoplast are recognized. The outer or peripheral region is defined by the presence of paired membranes, which are presumed, on the basis of cell-fraction studies, to be associated with the photosynthetic pigments (Shatkin, 1960). The central region, which merges into the peripheral region, includes structures which closely resemble the nuclear equivalent of the bacterial cell, both under the electron microscope (Hopwood and Glauert, 1960) and in their reaction to nuclear stains (Cassel and Hutchinson, 1954).

Comparatively little is known about the functional roles of the two regions or of the distribution of enzymatic processes within the structure of the protoplast. The cell wall structure is similar to that of a Gram-negative bacterium (Gerba et al., 2000). Some cyanobacteria form a complex and multilayered photosynthetic membrane system composed of mucilaginous envelopes or sheaths that bind filaments or groups of cells together. Some filamentous cyanobacteria form heterocysts which are rounded, seemingly empty cells that are generally distributed along a filament or at one end of the filament.

2.6. Heterocyst

When plants emerged from aquatic habitats to terrestrial ones in the devonian period, they encountered the problem of obtaining CO$_2$ for photosynthesis while conserving water, which they solved by the evolution of adjustable pores, called stomata (Chaloner, 1970). Over a billion years earlier, cyanobacteria encountered the parallel problem of obtaining N$_2$ for their oxygen-sensitive nitrogenase while excluding O$_2$. The problem was of their own making: they developed oxygenic photosynthesis and the O$_2$ they generated accumulated in the atmosphere (Gallon, 1992). In one group of cyanobacteria the problem was solved by separating the two incompatible processes, restricting oxygenic photosynthesis to the more
abundant vegetative cells and confining N$_2$ fixation to a morphologically different cell, the heterocyst (Fay et al., 1968; Elhai and Wolk, 1990).

The heterocyst is distinguished by its hyaline envelope and a distinctive pore at each end. During differentiation, heterocysts synthesize nitrogenase and other proteins involved in the assimilation and transport of fixed nitrogen to vegetative cells (Elhai and Wolk, 1990; Murry et al., 1984). In N$_2$ fixation, one molecule of N$_2$ is reduced to produce two molecules of ammonia; concomitantly, at least two protons are reduced to hydrogen (N$_2$ + 8H$^+$ + 8e$^- = 2$NH$_3$ + H$_2$). The process requires 16 ATPs and eight or more electrons (Gallon, 1992). Heterocysts retain photosystem-I. In the light, they synthesize ATP by cyclic phosphorylation but, without photosystem-II, they depend on the dissimilation of organic substrates provided by the vegetative cells for supplies of reductant.

In the dark, ATP is generated by catabolism of imported sugar phosphates or organic acids; increasing pO$_2$ stimulates N$_2$ fixation, indicating that the ATP supply is normally limiting (Wolk et al., 1994; Staal et al., 2003). Organic acids are also needed for assimilation.
of ammonia into amino acids; glutamic acid is amidated to make glutamine, which is transported to neighbouring vegetative cells (Thomas et al., 1977). All of this traffic in organic substances passes through the pore. Excess fixed nitrogen is stored in cyanophycin, (a polymer of aspartic acid and arginine), which often accumulates at each end of the heterocyst (Thomas et al., 1977).

### 2.7. Nitrogen fixation

Nitrogen is one of the major constituents of biological systems. The shortage of available nitrogen is the reason why fertilizer is used in agriculture. The only relevant inorganic source of nitrogen is our atmosphere. Accessing atmospheric dinitrogen, however, requires breaking one of the strongest chemical bonds. For this purpose, nature developed an enzyme of exciting complexity, namely nitrogenase.

### 2.8. Nitrogenase

While nitrogen is abundant in huge amounts in the atmosphere and is required by plants as fertilizer (nutrient), they cannot directly access it. Gaseous nitrogen is bound by one of the strongest covalent bonds in nature. Industry uses the Haber-Bosch process to break this bond and convert dinitrogen to ammonia, requiring about 500°C and 450 bar pressure. Around 80-10^9 kg of ammonia is manufactured annually in that way. Nature, in contrast, converts dinitrogen to ammonia at ambient conditions by employing the enzyme nitrogenase. Biological nitrogen fixation produces two or three times more ammonia per year than the industrial process. The structure of nitrogenase came to be known in 1992; however, the mechanism is still elusive today. Howard and Rees (1996) have unravelled the structure of nitrogenase.
2.8.1. Proteins of nitrogenase

Nitrogenase consists of two component proteins named the Fe-protein (or dinitrogenase reductase) and the MoFe-protein (or dinitrogenase). They may occur independently of each other in different concentrations within the cell. The Fe-protein hydrolyzes Mg ATP and uses the required energy to provide electrons to the MoFe-protein. The later contains the active site of nitrogen fixation, the FeMo-cofactor.

2.8.2. The Fe-protein

The Fe-protein is the smaller of the two nitrogenase proteins, a homo-dimer with a molecular mass of approximately 60-64 kDa\(^2\) (Burges and Lowe, 1996; Mayer et al., 2002) contained one [Fe\(_4\)S\(_4\)] cluster. Its structure was determined by Georgiadis et al. (1992). The functional role is the hydrolysis of two molecules of Mg ATP per molecule Fe-protein and the subsequent transfer of one electron to the MoFe-protein. A high concentration of Fe-protein compared to MoFe-protein is called a “highflux” condition. In this case, electrons (and subsequently also protons) are transferred rather fast to the substrate conversion site.

2.8.3. The MoFe-protein

The MoFe protein is a \(\alpha_2\beta_2\) tetramer which contained two \(\alpha\) and two \(\beta\) subunits, with a total mass of approximately 240-250 kDa (Burges and Lowe, 1996; Mayer et al., 2002). Two metal sulfur cluster, called P-clusters, are located at the interface between \(\alpha\) and \(\beta\) subunits and two FeMo cofactors (M-cluster, FeMoco) are contained within \(\alpha\) subunits. The FeMo cofactors are assumed to be the location of substrate conversion. The structure was first unravelled in 1992. Since then, increasingly more refined crystallographic structure determinations have been published. The structure determinations have provided the only reliable information about the stoichiometry of the cofactor. An additional central ligand, which could either be C, N or O was found in the FeMoco using an accurate structure
determination by Mayer et al. (2002). Theoretical investigations (Dance, 2003; Lovell et al., 2003; Vrajmasu et al., 2003; Schimpl, 2003) ruled out oxygen and showed that the most plausible is nitrogen. Thus, the composition of FeMoco is MoFe₇S₉N. FeMoco is linked to the protein via a sulfur atom from a cysteine residue (Cysα275) and a nitrogen atom of the imidazole ring of a histidine.

2.8.4. Nitrogenase and Acetylene

Nitrogenase is not only able to catalyze the conversion of N₂ to NH₃, but can also reduce a number of other substrates. One of the most detailed studied among these alternative substrates is acetylene, C₂H₂. While N₂ is fully reduced to NH₃ by the enzyme, C₂H₂ is only reduced to C₂H₄, ethylene (Dilworth, 1966). No further reduction to ethane, C₂H₆, takes place at the wild-type enzyme.

\[ \text{C}_2\text{H}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{C}_2\text{H}_4 \]

C₂D₂ as substrate allows studying the stereo-selectivity of the reduction. It is nearly quantitatively converted to cis- C₂H₂H₂, only about 4% of trans product is found (Fisher et al., 2000; Benton et al., 2001). The main reason why C₂H₂ is better studied than N₂ is the fact that acetylene binds to less reduced levels of the cofactor than N₂ does. This makes it easier to access the C₂H₂ binding mode experimentally. While dinitrogen is not able to bind to states less reduced than E₂H₂. EPR/ENDOR experiments (McLean, 2002) show that C₂H₂ even interacts with the resting state E₀H₀ of the cofactor. Kinetic studies (Lowe et al., 1990) however, conclude that C₂H₂ is not reduced before bound to the E₁H₁ state. In the N₂ conversion process, H₂ is a necessary by-product (Hadfield and Bulen, 1969). H₂ production takes reduction equivalents from N₂ reduction. In general, H₂ is also produced during the conversion of acetylene. However, in this case the enzyme is theoretically able to completely suppress hydrogen production at infinite partial pressure of C₂H₂ (Rivera-Ortiz and Burris, 1975).
2.8.5. Acetylene Reduction Assay

The measurement of in situ $\text{N}_2$-fixation rates on the basis of total nitrogen changes or $^{15}\text{N}_2$ uptake is not entirely satisfactory, the former method is insufficiently sensitive and accurate, and the $^{15}\text{N}$ method is time consuming, expensive and requires a mass spectrometer. The discovery by Schollhorn and Burris (1966) and by Dilworth (1966) that the nitrogen-fixing complex (nitrogenase) reduces acetylene to ethylene (Dilworth, 1966) suggested that the rate of acetylene reduction may be used as an index of the rate of $\text{N}_2$-fixation. Subsequently, the measurement of ethylene production from acetylene (Koch and Evans, 1966; Koch et al., 1967; Schollhorn and Burris, 1967; Sloger and Silver, 1967; Kelly et al., 1967) and the measurement of cyanide (Hardy and Knight, 1967), isocyanide (Kelly et al., 1967) and azide reduction (Hardy and Knight, 1967; Schollhorn and Burris, 1967) have been used to aid in laboratory studies of $\text{N}_2$-fixation. However, the potential of the method for field investigations of $\text{N}_2$-fixation generally has not been appreciated by limnologists, marine biologists and soil scientists.

2.9. Fatty acid/ lipid profiles

The analysis of the overall fatty acid profiles as well as the occurrence of fatty acids (FAs) in different lipid classes in microalgae is an emerging field which is expected to reveal the identification of novel FAs with a variety of new functional groups (Berge and Barnathan, 2005). Despite a number of reports has been carried out and published, describing the contents as well as the composition of polyunsaturated fatty acids (PUFAs) in mostly marine microalgae (Visco and Marty, 1993; Dunstan et al., 1994; Tonon et al., 2002), systematic approaches that include different or even many genera of microalgae and particularly those from freshwaters or terrestrial habitats are still missing (Hardwood and Guschina, 2009).

Based on current knowledge, FA composition divides microalgae roughly into two groups, i.e. on one hand the cyanobacteria and green algae (Chlorophyta and Streptophyta)
which contain low amounts of FAs, predominantly saturated and mono unsaturated FAs as well as trace amounts of PUFAs, mostly linoleic acid [LA, 18:2 (9Z, 12Z); where x:y (z) is a fatty acid containing x carbons and y double bonds in position z counting from the carboxyl end]. On the other hand, chromalveolate algae contain significant amounts of PUFAs (Watson, 2003). Among the various biochemical markers, FA or lipid profiles represent a chemically relatively inert class of compounds that is easy to isolate from biological material and FA profiles are considered as chemotaxonomic markers to define groups of various taxonomic ranks in flowering plants, trees and other embryophytes (Spitzer, 1999; Mongrand et al., 2005).

Beside the identification of novel FAs, some recent studies report on the use of FAs and lipid profiles of algae as biomarkers (Volkman et al., 1998; Berge and Barnathan, 2005; Schweder et al., 2005; Rossi et al., 2006). Viso and his team determined profiles of FAs of nine different marine algal groups and they were able to define even species-specific lipid compositions (Visco and Marty, 1993). Moreover, they found a roughly taxon specific profile when the cells were cultured under identical growth conditions. Various strains and species of the cyanobacterium Nostoc were screened for their FA content and the application of a FA-based cluster analysis has been described for their identification (Temina et al., 2007). FA and lipid composition have also been used as biomarkers to distinguish closely related microalgae at the species and the generic levels (Volkman et al., 1998; Leblond et al., 2005).

Hitherto no systematic analysis has been carried out on a large scale basis on either the profiles of lipids or FAs in microalgae. The FA profiles of all available microalgal strains of the SAG culture collection of microalgae (http://www.epsag.uni-goettingen.de) is one of the most diverse and comprehensive resources of microalgae. Till March 2011, 2291 strains of mainly microscopic algae including a considerable variety of cyanobacteria were available. They comprise almost all phyla and classes of eukaryotic algae, but an emphasis is put on
algae from freshwaters and terrestrial habitats. Distribution patterns of FAs may be valuable also as a proxy to identify certain groups, species and strains of microalgae of particular interest for applied research, i.e. due to the presence of certain FAs and/or high percentages of total FA content.

They also tested whether the detected FA distribution patterns were meaningful in a phylogenetic context at various taxonomic levels, i.e. to define taxonomic groups of microalgae by their FA patterns. It would assist predicting FA content and/or presence of other valuable compounds if the phylogenetic relationships of algae were reflected in their FA distribution patterns. On investigating the lipids in five cyanobacterial species, Holton et al. (1968) found that the level of the fatty acid unsaturation correlated with the morphological complexity of the organisms. Kenyon and Stanier (1970) observed that unsaturated fatty acid content was correlated with the morphology and physiology-based classification of the cyanobacteria.

Unicellular and filamentous cyanobacteria have been grouped in five clusters depending on the number and position of double bonds counted from the carboxyl terminus (Δ) or from the methyl terminus (ω) of 16 carbon (C\textsubscript{16}) and 18 carbon (C\textsubscript{18}) fatty acids (Kenyon, 1972; Kenyon et al., 1972; Murata et al., 1992; Cohen et al., 1995). Four groups were first found by Kenyon et al. (1972) and confirmed by Murata et al. (1992). The fifth group described by Cohen et al. (1995) was positioned according to the Kenyon-Murata classification system between groups 1 and 2. The strains of group 1 were devoid of polyunsaturated fatty acid (PUFA) and contain only saturated and monounsaturated fatty acids.

The strains of the group defined by Cohen et al. (1995) contain 18:2\textsubscript{Δ9,12} (18:2ω6, linoleic acid) as the only C\textsubscript{18} PUFA. Group 2 consists of cyanobacterial strains containing 18:3\textsubscript{Δ9,12,15} (18:3ω3, α-linolenic acid) as the only C\textsubscript{18} PUFA. The strains of group 3 have
18: 3Δ6, 9, 12 (18:3ω6, γ-linolenic acid) as the major C18 PUFA, but no or only traces of 18:3Δ9, 12, 15. Strains of group 4 contain either 18:3Δ9, 12, 15 or 18:3Δ6, 9, 12 or both, but also produce 18:4Δ6, 9, 12, 15 (18:4ω3, octadecatetraenoic acid). The double bonds in the hydrocarbon chains of PUFA are introduced by fatty acid desaturases, which play an important role in the acclimation of the various organisms to changes in environmental temperatures (Murata and Wada, 1995; Nishida and Murata, 1996). The DesA (Δ12), DesB (ω3 or Δ15) and DesC (Δ9) acyl-lipid desaturases and their corresponding genes, desA, desB and desC, have been identified in Synechococcus PCC 7002 (Sakamoto et al., 1994a,b,c, 1998; Sakamoto and Bryant, 1997) and DesD (Δ6), encoded by desD was found in Synechocystis PCC 6803 (Reddy et al., 1993).

Cyanobacteria have also been classified by analysing the composition of whole cell fatty acids. Caudales and Wells (1992) showed that the filamentous heterocystous Nostoc and Anabaena genera could be distinguished on the basis of their cellular fatty acids. Vargas et al. (1998) found that the nitrogen fixing genera were also differentiated by their 3-hydroxy and non-polar fatty acid content. Recently, Caudales et al. (2000) showed that the fatty acid composition of unicellular cyanobacterial strains of subsections I and II generally agreed with their morphological distinctions. The study also highlighted the differences between the fatty acid compositions of Chroococcidiopsis and the other baecocytes-forming strains of the order Pleurocapsales.

2.10. Polyphasic approaches

The following criteria are important for cyanobacteria: (i) ecological, ecophysiological and biogeographical studies, (ii) morphological variation limits in nature and in cultures, (iii) data about ultrastructure including explanation of cytological structures in cyanobacterial cells, (iv) biochemical characteristics and their stability and (v) molecular analyses, mainly those concerning diversity, diversification processes and speciation and
phylogenetic relations (Anagnostidis and Komarek, 1985; Komarek and Caslavska, 1991; Castenholz, 2001; Suda et al., 2002; Flechtner et al., 2002). The molecular (phylogenetic) data should be accepted as a basic criterion. However, the correct classification is impossible without the careful combination of genetic data with morphological diversity and variation, ecological and ecophysiological characteristics, ultra structural studies and without the correct application of convenient formal prescriptions for designation of taxa and strains (nomenclatoric rules).

Recently, this combined methodology is usually presented as “polyphasic approach.” The application of all the mentioned modern criteria is quite necessary, but it creates also numerous problems. The discrepancy between the fact, that up to date known cyanobacterial diversity from nature is based only on morphological characters and on the other side, the modern molecular analyses are pursued almost only in populations and clones, transferred in cultures, belong to most serious complications. Also only small portion of natural eco and morphotypes are cultivable. The further complication is that the transfer of ecologically variable and easily adaptable cyanobacterial types into more or less unified culture conditions is always some kind of stress for them and majority of cyanobacterial species grow in culture in atypical stages, or in monstrosities. Another problem is a very arbitrary (taxonomic) identification of strains and their designation by nomenclatoric incorrect names, which complicates usually the final taxonomic evaluation.

The arbitrary use of the both, the Bacteriological and Botanical Nomenclatoric Codes were principally accepted for cyanobacteria. However, no one from these codes was found quite satisfactory for cyanobacteria (neither after some additions). Both codes are often used without coincidences with different prescriptions. The result is that only few cyanobacterial taxa can be considered as “validly described” (Oren, 2004; Oren and Tindall, 2005). In spite of all complications mentioned, the modern cyanobacterial review should be already
modified substantially according to results following from the modern evaluations; the revised classification system can only be a basis for further complex studies of cyanobacterial diversity.

2.11. Combination of molecular and traditional methods

Molecular evaluations of cyanobacterial diversity indicated prospective results, leading to the compilation of cyanobacterial system in agreement with genetic basis as well as with phenotype variation (Rippka et al., 1979; Wilmotte and Golubic, 1991). The tendencies to omit molecular or traditional taxonomic methods and criteria are evidently misleading.

2.11.1. RAPD

Random Amplified Polymorphic DNA (RAPD) allows the detection of multi-locus genetic variation using short primers of arbitrary sequence (Welsh and McClelland, 1990; Williams et al., 1990). This molecular technique is very easy to perform and requires no prior knowledge of the genomes under investigation (Weising et al., 1995). This technique has been used for the analysis of diversity within germplasm populations (Virk et al., 1995), phylogenetic relationships (Lunge et al., 1994) and identification to the strain level (Welsh and McClelland, 1990). It has helped to develop tools for rapid characterization of *Lactobacillus* strains both at species and sub-species level (Berthier and Ehrlich, 1999). In some cases, single oligonucleotides (oligo) of 10-34 base pairs have been used for RAPD-PCR reaction (Mullis and Faloona, 1987).

Nishihara et al. (1997) have used RAPD analysis to discriminate genotypes of *Microcystis*. However, multiplex RAPD-PCR with two 10-mer random oligonucleotides in a single PCR was particularly useful for strain differentiation of cyanobacterial isolates because it increased the number of informative genetic markers (Neilan, 1995). Multiplex RAPD with
two 10-mer random oligonucleotides in a single PCR has also been used to differentiate strains of *Lactobacillus* from the gastrointestinal tract (Daud Khaled *et al.*, 1997).

### 2.11.2. 16S rRNA

The detection of the slightly different 16S rRNA gene sequences is not sufficient to prove the presence of *Schizothrix, Phormidium, Scytonema, Nostoc* and *Chlorogloeopsis* in an environmental sample. The *rpoC1* gene encoding the subunit of RNA polymerase has been described as an alternative target for the analysis of cyanobacterial phylogeny (Bergsland and Heselkorn, 1991) and community structure. However, the sequence data available for these genes are rather limited, whereas the determination of 16S rRNA gene sequences is a routine procedure in prokaryotic taxonomy today. Gugger *et al.* (2002a) reported that the planktonic *Anabaena* strains were not distinguishable from *Aphanizomenon* strains by morphological analysis. But sequencing of the 16S rRNA gene, the spacer region of the ribosomal operon (ITS1) and the *rbcLX* (RuBisCo) region performed on 26 *Anabaena* strains and 14 *Aphanizomenon* strains isolated from several lakes in Denmark, Finland and France revealed differences between them.

DNA fingerprinting has been found to yield significant diversity among nitrogen fixing cyanobacteria in soil samples collected from different ecosystems (Lowe, 1980). PCR methods using DNA dependent RNA polymerase (*rpoC1*) have been found quite useful in understanding molecular phylogeny of *Anabaena circinalis* (Fergusson and Saint, 2000). Likewise 16S rRNA gene sequences have been used to study *Azolla-Anabaena* endosymbiont (Eskew *et al*., 1993). DNA-DNA hybridization has been found to be very precise (Sigler *et al*., 2003) in delineating the 2 *Chroococcidiopsis* species isolated from hot and cold deserts. 16S rRNA gene analysis revealed that the cyanobacterial phylotypes observed in dolomite were related to known diazotrophs including *Anabaena, Calothrix, Scytonema* and *Nostoc*. Svenning *et al.* (2005) studied the phylogeny of symbiotic cyanobacteria within the genus
Nostoc based on 16S rDNA sequence analysis. Since the genera, Nostoc and Anabaena exhibit very similar properties, the absence of hormogonia, the motile stage of the filaments in Anabaena has been used to distinguish them (Wilmotte and Herdman, 2001), although hormogone formation is dependent on growth conditions (Ward et al., 1998).

Analysis of the genetic diversity of symbiotic Nostoc strains using molecular methods revealed heterogeneity reflecting high genetic diversity (West and Adams, 1997; Costa et al., 1999; Nilsson et al., 2000). Sequence heterogeneity between strains at a higher taxonomic level has also been demonstrated using 16S-RFLP and ITS-RFLP combined with DGGE analyses of the functional hetR gene (Svenning et al., 2005). Phylogenetic analysis revealed that Nostoc strains were intermixed within the genus Anabaena and vice-versa. Notwithstanding, Dyble et al. (2002) characterized Cylindrospermopsis raciborski isolates from diverse geographic origins based on nifH gene. Ten 16S rDNA probes to identify the cyanobacteria genera, Microcystis, Plankothrix, Anabaena and Aphanizomenon; in addition a probe which corresponds to the Nostoc (which includes Nostoc, Anabaena and Aphanizomenon sp.) has been reported by Rudi et al. (2000).

Within bacteria, sequence information from the gene coding for the small subunit of ribosomal RNA, 16S rDNA, is widely regarded as one of the most valid criteria for determining relationships between closely related groups, such as species or genera (Weisburg et al., 1991). The conservative nature of the gene, its universal distribution and the availability of information in public databases (GenBank, EMBL, DDBJ and RDP) make the 16S rRNA gene very useful for phylogenetic studies and taxonomy. Furthermore, its validity for phylogeny of cyanobacteria was recently documented by Oksanen et al. (2004).