CHAPTER 1

Introduction

The simple and common green algae of the genus *Chlorella* (Beijerinck, 1890) are placed below the order Chlorellales and class Trebouxiophyceae (Krienitz *et al*., 2004). It is one of the archetypical forms of coccoid green algae and best-studied phototropic eukaryotes (Lothar *et al.*, 2004). The reproduction mode of *Chlorella* is asexual and achieved by producing non-motile autospores.

Warburg (1919) discovered that pure cultures of the fast-growing *Chlorella* can be used as the ideal experimental materials for research on photosynthesis, physiology, nitrate reduction and biochemistry (Wu *et al*., 2001). Recently, *Chlorella* has been extensively studied and employed in various applications in biotechnology and agriculture. *Chlorella* has also been used as a bio-indicator of wastewater and the protein rich biomass produced can be used as animal feed (Kessler, 1982). According to Gouveia *et al*. (2007), the biomass of *C. vulgaris* is useful as a colouring agent in traditional butter cookies, as feed and as food products. *Chlorella* is also rich in lipids, amino acids and vitamins (Kralovec *et al*., 2004). In Japan, *Chlorella* is used to cure peptic ulcers, gastric ulcers and as an agent for slow healing wound (Jensen, 1987). Commercial utilization of *Chlorella* was started for the first time in 1961 by Nihon *Chlorella* INC (Richmond, 2003).

According to Andersen (1998), the correct identification of algae is essential for their biotechnological applications, understanding the ecology of aquatic ecosystems and global biogeochemistry. Many microalgae possess only a few morphological characteristics that are useful for species characterization (Fawley, 2004). *Chlorella* is one of such algae which do not exhibit distinct characteristics that differentiate them

from other algae in terms of the morphological properties. In addition, certain biochemical and physiological characteristics are not species specific for *Chlorella* (Kalina and Puncocharova, 1987). For instance, there have been many reports on fatty acid composition of *Chlorella* (Shinichi *et al*., 1983; Zhukova and Aizdaicher, 1995; Vanderploeg *et al*., 1996 and Rosa *et al*., 2005). However, the data on fatty acid composition of *Chlorella* are frequently discrepant even within the same species because of the environmental effects such as light and temperature (Zhukova and Aizdaicher, 1995). Therefore, classifying unknown isolates of *Chlorella* can be difficult.

In view of the limited morphological and biochemical features for *Chlorella*, molecular tools such as phylogenetic analysis are useful to unravel the taxonomy of *Chlorella*. Therefore, many scientists have focused on molecular studies which have made significant contributions to our understanding of the evolutionary history of algae. Phylogenetic analyses are very useful in identifying structural features that are well correlated with genetic or physiological differences among species (Graham and Wilcox, 2000). Gene sequencing has been the most active field of phycological systematics in the last decade and has provided important new information on the relationships between *Chlorella* species (Lee, 1999). For instance, Huss *et al*. (1999) reported four true *Chlorella* species, namely *C. vulgaris*, *C. kessleri*, *C. lobophora* and *C. sorokiniana* based on analyses of small subunit ribosomal RNA (SSU rRNA) genes. These four species are spherical, solitary living and do not possess any mucilage or cell wall ornamentation like spines or bristles (Andreyeva, 1975). Later, *C. kessleri* was found to be a member of a sister clade of *Chlorella* and therefore transferred to the genus *Parachlorella* (Krienitz *et al*., 2004).

In spite of the many studies on the taxonomy of *Chlorella*, there have been no reports on the relationship of *Chlorella* isolates from different latitude regions. The primary objective of this project was to find out how *Chlorella* isolates from different latitudes, ranging from tropical, temperate, sub-Antarctic to Antarctic regions are related in terms of their morphological, biochemical (fatty acid profiles and pigmentation) and 18S rDNA phylogeny.

To achieve the objectives, the following were conducted:

i) Characterizing the *Chlorella* isolates based on morphological and biochemical features such as fatty acid composition and pigmentation

ii) Molecular analysis of *Chlorella* isolates based on 18S rDNA gene

An outline of the approaches used to achieve the objectives of this research is shown in Figure 1.1

The following hypotheses were tested:

Ho: Morphological features, fatty acid profiles and pigmentation cannot be used to differentiate the *Chlorella* isolates from different regions.

H_1: Morphological features, fatty acid profiles and pigmentation can be used to differentiate the *Chlorella* isolates from different regions.

H_O: *Chlorella* isolates from the same latitudinal regions are not closely related in terms of phylogeny based on 18S rDNA

H_1: *Chlorella* isolates from the same latitudinal regions are closely related in terms of phylogeny based on 18S rDNA.

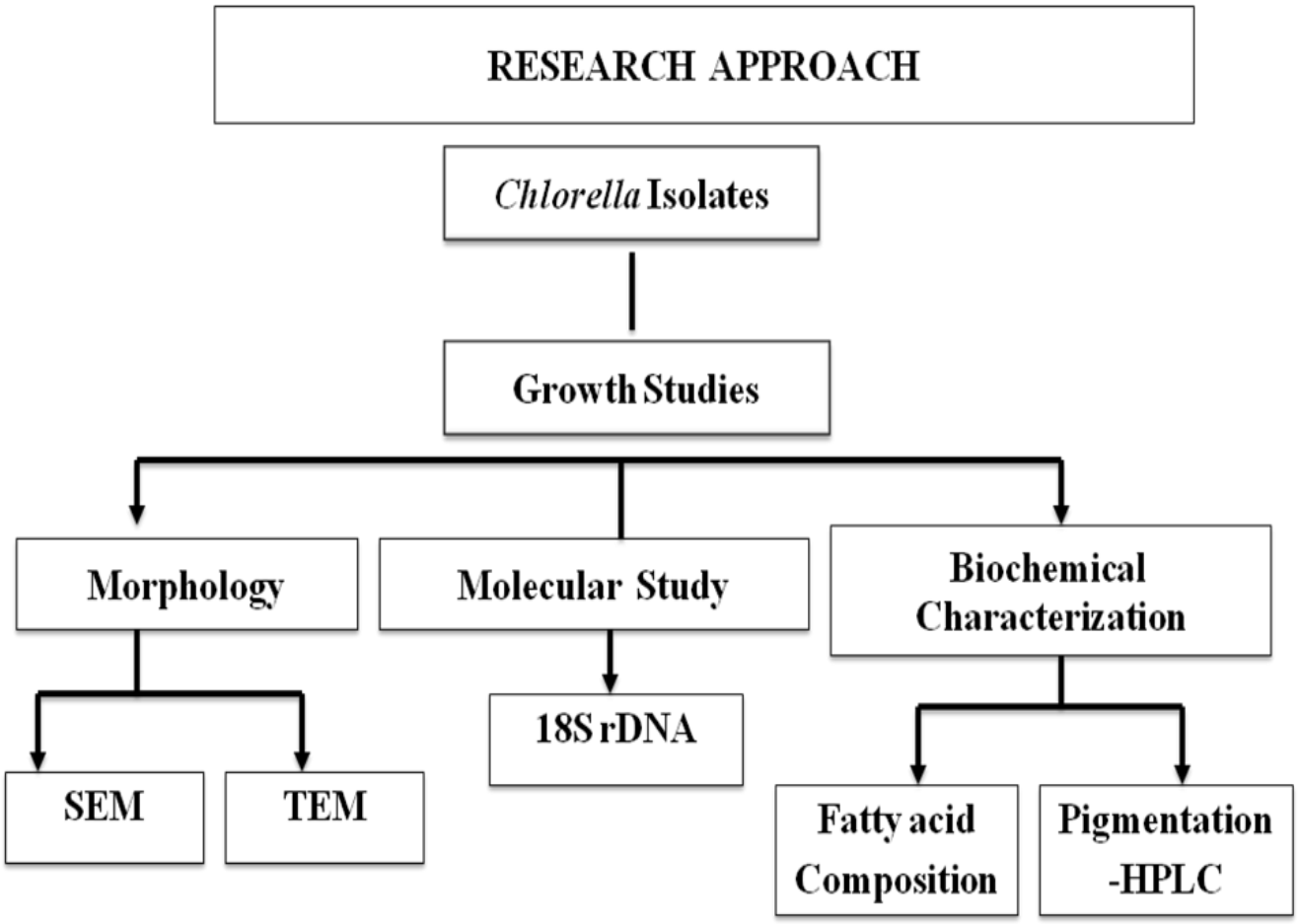


Figure 1.1 Outline of research approaches used to achieve the objective of this study

CHAPTER 2

Literature Review

2.1 *Chlorella*

Chlorella is a cosmopolitan alga which is placed under the phylum Chlorophyta and class Trebouxiophyceae (Krienitz *et al*., 2004). They are one type of eukaryotic green algae (Lothar *et al*., 2004). According to Graham and Wilcox (2000), eukaryotic cells are defined by the presence of a double-bound nucleus, mitochondria and chloroplasts.

Chlorella species are commonly found in water, freshwater lakes, ponds, marine or brackish waters. They also can be found almost in every other environment such as in the snow and damp areas. Some have been identified in hot springs where the temperature is 180^{o}C (Jensen, 1987). Alongi (1990) reported that the mud on temperate shores contained greater standing stocks of *Chlorella* than other microalgae. *Chlorella* also can be found in the Arctic and Antarctica. Antarctica comprises the area of the Earth south of 60^{o}C which includes ice-covered continent, isolated islands and a large part of the Southern Ocean (King and Turner, 1997). It presents an extremely harsh environment for terrestrial and freshwater algae with very low temperatures, frequent and rapid fluctuations from freezing to thawing, severe winds, low humidity and long periods of light and darkness (Becker, 1982). Studies have been carried out on algal distribution and habitat in terrestrial Antarctic environments (Ohtani, 1991). It is really an interesting phenomenon to discover how microalgae especially Chlorella can grow and survive in such extreme and low temperatures.

In terms of carbon nutrition, *Chlorella* can grow under autotrophic, heterotrophic and mixotrophic conditions (Phang and Chu, 2004). Heterotrophic organisms obtain their materials and energy from organic compounds produced by other organisms. Under autotrophic conditions, organisms obtain their energy through the absorption of light energy for the reduction of CO_2 by the oxidation of substrates (H_2O) with the release of O_2 (Richmond, 2003). Mixotrophic growth is equivalent to the combination of autotrophy and heterotrophy where both organic compounds such as vitamins and amino acids as well as CO_2 are necessary for growth (Richmond, 2003).

Mixotrophic mass cultivation of *Chlorella* was replaced photoautotropically in 1964 as the initial stage of commercial production and to facilitate enough supply of an inexpensive protein source for food and feed in protein-deficient areas of the world (Richmond, 2004). According to Richmond (2000) *Chlorella* is able to utilize glucose more efficiently in the presence of light. However, Kowallik and Kamiya (1987) reported that glucose uptake in mixotrophic culture of some strains of *C. vulgaris* is inhibited by light unlike other strains of *Chlorella* and microalgal species which are able to undergo mixotrophic growth.

Chlorella that can be cultured mixotrophically include *C. regularis* which utilizes acetate, *C. sorokiniana* and *C. vulgaris* which grows on glucose (Lee *et al*., 1996). The growth yield and productivity of mixotrophic *Chlorella* grown in glucose was found to be the greatest when compared to heterotrophic and photoautotrophic cells (Lee *et al*., 1996). Martinez and Orus (1991) reported that the specific growth rate of mixotrophic *Chlorella* increased with light intensity. According to Richmond (2003) *Chlorella* is able to utilize glucose more efficiently in the presence of light.

Many *Chlorella* species can also be cultured heterotrophically. According to Tan and Johns (1991), *C. saccharophila* and *C. sorokiniana* use glucose as their organic carbon substrate whereas *C. vulgaris* uses acetate, glucose, lactate and glutamate (Gladue and Maxey, 1994). Lee *et al*. (1996) reported that *C. sorokiniana* grew heterotrophically on glucose at night and during the day they were mixotrophic by utilizing both glucose and CO_2. Recently, high quality biodiesel production from *C. protothecoides* through the technology of transesterification was carried out by Xu *et al*. (2006). The algal cells were grown heterotrophically in fermenters. Corn powder was used as organic carbon source instead of glucose to increase the biomass of the production. This particular method has great potential in the industrial production of liquid fuel from microalgae. According to Ikegami *et al*. (2004), *Chlorella* cultured under heterotrophic conditions in the dark are able to grow with Zn^{2+} at 10–40 mM which is ten times the lethal concentration compared to autotrophically grown cells, making this *Chlorella* useful for bioremediation.

Chlorella also grows in symbiosis with other organisms. For instance, the first observation of a symbiotic relationship among fungus, bacteria and *Chlorella* was reported by Watanabe *et al*. (2005). The results showed that the interaction between *Chlorella* and its symbionts under photoautotrophic conditions involves both mutualism and commensalism. Two kinds of symbiotic conformation were observed in *C. sorokiniana*. In one consortium, the symbionts were harbored on the sheath excreted from *C. sorokiniana* and in another; bacterial and fungal strains were directly adhered to the cell surface of *C. sorokiniana*.

2.2 Characteristics of *Chlorella*

2.2.1 Morphological Features of *Chlorella*

The cells of *Chlorella* have a thin cell wall which is spherical or ellipsoidal (Richmond, 2003). Previously, they were placed in four general groups, namely a) spherical group, (ratio of the two axes equals one), b) ellipsoidal group (ratio of the longest axis to the shortest axis being 1.45 to 1.60), c) spherical or ellipsoidal cells, d) globular to sub-spherical cells (Richmond, 1990). The common characteristics of Chlorella are small cells, (<10µm), roughly spherical in shape, containing single parietal chloroplast and pyrenoid. The most common species is *C. pyrenoidosa* which has small cells (3-5µm) and C. vulgaris which has larger cells, ranging from 5 to 10µm (Bellinger, 1992). However the size (2-12 µm) of Chlorella species changes with culture conditions (Huss and Sogin, 1990).

Chlorella contains parietal shaped chloroplast which is bound by a double-membrane envelope (Lee, 1989). It is the most conspicuous organelle in the algal cell and its form is an important criterion in the classification of the green algae. The chloroplasts have membrane sacs known as thylakoid that carry out the light reactions of photosynthesis whereas carbon dioxide fixation occurs in the stroma (Lee, 1989). The thylakoid that penetrates into the pyrenoid matrix is uniformly double layered in *C. vulgaris, C. lobophora, C. kessleri and C. sorokiniana* (Ikeda and Takeda, 1995). The lipoproteins inside the chloroplast are known as plastoglobules. They play an important role in thylakoid membrane as a reservoir for certain lipids in *Chlorella* (Kessler and Vidi, 2007). According to Brehelin *et al*. (2007), they are defined as plastid-localized lipoprotein particles that contain tocopherols, other lipid isopyrenoid derived metabolites and structural proteins called plastoglobulins.

Several types of cell wall structure are exhibited in *Chlorella*, including electron-dense, electron-transparent and layered structures (Nemkova and Kalina, 2000). Yamamoto e*t al*. (2004) examined the structural changes of premature thin electron-dense daughter cell walls during the cell-growth and cell-division phase in *C. vulgaris*. They found that the cell begins to synthesize a new daughter cell wall shortly after the release of the spores from the sporangium and during the protoplast division-phase; the daughter cell wall expands on the surface of the invaginating plasma membrane of the cleavage furrow. In another study, Yamamoto *et al.* (2005) reported on cell wall structure and synthesis of the daughter cell wall in four species by electron microscopy and found that the cell wall of *C. vulgaris, C. sorokiniana and C. lobophora* consisted of an electron-dense thin layer with an average thickness of 17-22 nm and the cell wall of Parachlorella kessleri was electron-transparent and 54-59 nm in thickness. There are few distinguishing characters that can be used to differentiate Chlorella from Parachlorella, which include the thickness of cell wall, electron density of cell wall and daughter cell wall synthesis.

According to Burczyk *et al*. (1995), two types of cell wall ultrastructure have been used to distinguish the algal strains belonging to Chlorococcales where the first type is the homogenous structure of the outer cell wall layer and the second type is membrane-like trilaminar structure of this layer. The type of cell wall is often a problem in the studies of *Chlorella*. In some strains of Chlorella, the trilaminar outer layer contains sporollenin (Bellinger, 1992). Sporollenin, an extraordinarily resistant polymer of carotenoids acts as the protector against enzymatic attack (Burczyk *et al*., 1995). Chlorella can also be divided into two groups according to the sugar constituents of the rigid cell wall which can be either the glucose-mannose type or the glucosamine-type (Takeda, 1993). *Chlorella vulgaris* Beijerinck, *C. sorokiniana* Shihira et Krauss, *C.*

lobophora Andreyeva and *Parachlorella kessleri* Krientiz have the glucosamine type cell wall (Takeda, 1993).

2.2.2 Fatty acids of *Chlorella*

Fatty acids are primary metabolites of acetyl CoA pathway which is genetically determined, evolutionary very old and therefore they are conservative (Petkov and Garcia, 2007). There have been several studies on fatty acid composition of different *Chlorella* strains. Most studies reported that Chlorella strains have similar composition. The common fatty acids of *Chlorella* include 14:0, 16;0, 16:1,16:2, 16:3, 18:0, 18:1, 18:2 and 18:3 (Yu *et al*., 2007). The absence of fatty acids such as 16:4, 18:3 and 18:4 can be useful as taxonomic marker for *Chlorella* (Murakami *et al*., 1997).

Marine *Chlorella* also contains the same fatty acids as other species of the genus but then there are differences in the proportions of specific fatty acids. For instance, the percentage of 14:0 can reach about 10 % of the total fatty acids in marine Chlorella (Petkov and Garcia, 2007). Some *Chlorella* species, especially *C. minutissima* produce up to 45 % of eicosapentaenoic acid (EPA) (20:5) of the total fatty acids (Seto *et al*., 1984) and this particular species has been shown to be a promising source of EPA for commercial exploitation. EPA obtained from these algae is claimed to be superior to fish oil (Vidyavati and Mahato, 1999). *Chlorella* also contains significant quantities of fats and oils (lipids) which are similar to vegetable oil and contain up to 85% of the dry weight as lipids (Borowitzka, 1968).

There have been several reports on fatty acids of Antarctic *Chlorella*. For instance, Nagashima *et al.* (1995) studied the temperature acclimation and fatty acid

composition of two Antarctic *Chlorella*. *Chlorella vulgaris* strain SO-26 cells cultured at 20^{o} C photosynthesize between 0 - 35^{o} C, with a maximum rate at 20^{o} C, while cells cultured at 10^{o} C show a shift of the optimum to about 2 to 3^{o}C lower and show higher activity than cells cultured at 20^{o} C. In both strains tested, the major fatty acids are palmitic, linoleic and linolenic acids. When culture temperature was lowered from 20 to 10^{o} C, the increase of the ratio of unsaturated fatty acid to total fatty acid in strain SO-26 was considerably greater than that in strain C-133. The results indicate that in photosynthesis, the properties of the Antarctic strain Chlorella SO-26 is more psychrophilic than those of the mesophilic *Chlorella* C-133. Both strains can acclimate to the culture temperature, at least partly because of fatty acid unsaturation.

2.2.3 Pigmentation of *Chlorella*

In general, Chlorophyceae are generally believed to have the same major pigments as higher plants. The pigments usually found in *Chlorella* include chlorophyll a, b, ß-carotene, neoxanthin, zeaxanthin, antheraxanthin and violaxanthin and lutein is the dominant carotenoid in *Chlorella* (Jensen, 1987).

The pigmentation of *Chlorella* may vary in response to stressed conditions. For instance, exposure to combined conditions of high light intensity and nitrogen deficiency results in the accumulation of secondary carotenoids in *C. zofingiensis* (Bar *et al*., 1995). These secondary carotenoids function as a light filter to reduce irradiation of the cell components to prevent photooxidative damage and to reduce water losses. *C. zofingiensis* also produces large amounts of the valuable ketocarotenoid, astaxanthin under dark, heterotrophic growth conditions, making it potentially useful for commercial production of the pigment as feed additives, colorants, and health products.

There have been studies to unravel the biosynthetic pathways of *Chlorella* pigments. Huang et al. (2005) reported on the identification and characterization of a β-carotene oxygenase (CRTO) gene which is directly involved in the biosynthesis of ketocarotenoids in C. zofingiensis. This gene not only exhibits normal CRTO activity by converting β-carotene to canthaxanthin via echinenone, but also displays a high enzymatic activity of converting zeaxanthin to astaxanthin via adonixanthin. Based on the bifunctional CRTO, a predicted pathway for astaxanthin biosynthesis in C. zofingiensis is described, and the CRTO is termed as carotenoid 4,4′-β-ionone ring oxygenase. There have also been attempts to produce lutein from Chlorella under heterotrophic conditions. High productivity of biomass and lutein was attained from Chlorella protothecoides grown on glucose under heterotrophic conditions (Shi *et al*., 1997).

2.3 Taxonomic studies on *Chlorella*

Many microalgae possess only few morphological characteristics that are useful for species characterization, leading to the possibility of numerous cryptic species (Fawley, 2004). According to Potter and Anderson (1997), a comparison of the morphology of Chlorellacean organisms grown in suspension cultures with those grown on solid media demonstrated that morphological features could be profoundly influenced by culture conditions (Krienitz *et al*., 2004).

Some of the most commonly reported microalgae are coccoid organisms referred as little green balls or little round green things and difficult to identify because of their small size and simple morphology (Callieri and Stockner, 2002). As a result, many

floristic studies identify these organisms of little green balls as *C. vulgaris* or *Chlorella* species (Fawley, 2004).

Beijerinck (1890) stated that the identification of *Chlorella* species become one of the most difficult tasks in the systematics of coccoid green algae. Taxonomy of the genus *Chlorella* Beijerinck based on light microscopy observations was published by Fott and Novakova (1969). In this study, they established the nomenclature types and documented the phenoplasticity of eight species and four varieties. After numerous chemotaxonomical investigations by Kessler and Huss (1992), it became evident that the genus Chlorella represents a taxonomically heterogeneous assembly of simple unicells.

Kessler and Huss (1992) presented a classification system of *Chlorella* strains that includes 16 taxa. They are differentiated by a combination of 12 physiological and biochemical species-specific properties (Huss and Sogin, 1990). The guanine + cytosine (G+C) content of Chlorella species ranges from 43 to 78mol % G+C, which suggests that *Chlorella* represents an assemblage of morphologically similar species of polyphyletic origin rather than a natural genus (Kessler, 1984). Indeed, several authors have nearly dismantled the taxonomic unit of *Chlorella* and at least six out of the 16 taxa defined by Kessler have been renamed and classified in different genera or families (Kalina and Puncocharova, 1987).

Even though the morphological species concept is frequently used for eukaryotic microorganisms (Coleman, 2001), the taxonomy of *Chlorella* species is very difficult because their morphological and physiological characteristics normally change with the

environment, making species identification difficult (Wu *et al*., 2001). More than 100 Chlorella species have been described from freshwater, marine and soil habitats and most of them need to be revised (Komarek and Fott 1983). Only a few taxonomic studies have been carried out based on morphological characteristics (Fott and Novakova, 1969; Kalina and Puncocharova, 1987). Pyrenoid occurs in every class of eukaryotic algae and its presence within a class is considered to be one of primitive evolutionary characteristic (Richmond, 2003). Pyrenoid is known to occur in most of the *Chlorella* species.

To solve problems in the classification of *Chlorella*, Kessler (1982) did a comparative study on many physiological and biochemical properties as well. Takeda (1991) classified *Chlorella* species by the sugar composition of the cell wall where *Chlorella* species were divided into two groups by the chemical composition of their rigid cell wall which is composed of glucosamine. He found that the rigid cell wall of *C. vulgaris, C. sorokiniana and C. kessleri* consisted of glucosamine, while other *Chlorella* species possessed glucose and mannose as a main constituent of the rigid cell wall. Another character indicating a closer evolutionary relationship of species with glucosamine cell walls is the identical pyrenoid structure. The thylakoid that penetrates into the pyrenoid matrix is uniformly double-layered (Ikeda and Takeda, 1995).

Mourente *et al.* (1990) studied the total fatty acid as a taxonomic index of some marine microalgae used as food in marine aquaculture. They found that *Chlorella* species showed high proportions of short-chained polyunsaturated fatty acids, with 16:2, 16: 3, 18:2 and 18:3 as major fatty acids. In this study, fatty acid composition was used as a taxonomic marker to differentiate Chlorophyceae from other Chlorophytes.

The major light-harvesting complexes from *Mantoniella squamata* (Prasinophyceae) and from *C. fusca* (Chlorophyceae) were analyzed with respect to polypeptide composition and pigmentation by Wilhem (1990). It was found that the polypeptides of Chlorella are higher than those of Mantoniella and bind twice the amount of pigment and the amount of pigment per polypeptide was of ecological as well as of taxonomic importance.

Morphological and physiological analyses could not completely resolve the taxonomy problems in Chlorella, therefore molecular approaches to algal systematics and phylogeny are now applied to revise the systematics of *Chlorella* (Graham and Wilcox, 2000).

2.3.1 Molecular studies on *Chlorella*

The systematics of algae has been driven by observational tools due to the apparent simplicity of the organisms to the naked eye, their small size and cryptic features (Coleman, 2001). Molecular data have also indicated patterns of local adaptation and endemism among some algae where morphological differences are difficult to detect (Coleman, 2001). Sequences of 18S ribosomal DNA (rDNA), Rubisco large subunit gene (rbcL) and Internal Transcribed Spacer (ITS) regions are the most commonly used molecular data for green algae phylogenetic analysis. 18S rDNA and ITS are located in the nucleus, whereas rbcL is located in the chloroplast (Liu *et al*., 2006). Their structural character and fast evolutionary rate make them a reliable tool for taxonomy.

The use of molecular tools such as PCR amplification and direct sequencing of the 18S rRNA gene has resolved taxonomic problems in several microalgae (Huss *et al*. 1999). There are three methods used to isolate nucleic acid for sequencing namely gene cloning using recombinant DNA technology, direct amplification of target DNA from whole genomic DNA and isolation and sequencing of RNA transcription of genes of interest (Hilis *et al*., 1996). Studies of the nuclear-encoded, 18S rDNA gene have proven useful in elucidating cryptic diversity among autosporic and coccoid green algae (Huss and Sogin 1990; Huss *et al*., 1999). Quantitative DNA hybridization procedures have been employed to investigate intraspecific relationships of Chlorella species (Huss et al., 1999). DNA hybridization data provided little conclusive information about the evolution of *Chlorella* species and their relation to other green algae (Huss and Sogin, 1990). Although most studies within eukaryotes have focused upon phylogenetic relationships between divergent organisms (Sogin, 1989), it has been demonstrated that it is also possible to infer reliable relationships between species of a single genus (Sogin *et al.*, 1986).

There are several reasons why molecular data especially DNA and amino acid sequence data are more suitable for evolutionary studies than morphological and physiological data. According to Hilis *et al*. (1996), DNA and protein sequences are strictly heritable entities whereas many morphological traits can be influenced to varying extents by environmental factors and molecular traits generally evolve in a much more regular manner than do morphological and physiological characters.

A new insight into the taxonomy of coccoid green algae was achieved by employment of 18S rDNA sequence data. In 18S rRNA phylogenetic trees, *Chlorella*

taxa were dispersed over two classes: the Chlorophyceae and the Trebouxiophyceae (Friedl, 1995; Huss *et al*., 1999). The class Trebouxiophyceae was established by Friedl (1995). The existence of a distinct group containing *C. vulgaris* Beijerinck, *C. lobophora* andreyena, *C. sorokiniana* Shihira et Krauss and *C. kessleri* Fott Novakova has been also resolved in the 18S rRNA phylogeny (Huss *et al*., 1999). According to Huss *et al*. (1999), only four species, namely *C. vulgaris, C. sorokiniana, C. lobophora* and *C. kessleri* should be kept in the genus *Chlorella* where *C. vulgaris* is closely related to *C. sorokiniana, C. lobophora and Parachlorella kessleri*. The separation of Parachlorella from the true *Chlorella* species sensu Huss *et al.,* (1999) is based on molecular data as well. Ustinova *et al*. (2001) also confirmed that the P. kessleri represents its own phylogenetic lineage within the Trebouxiophyceae separated from the other lineage. Consequently, Krienitz *et al*. (2004) also suggested that *C. kessleri* be transferred into a new genus, *Parachlorella* and this was supported by the difference in the cell wall structure of *Chlorella* and *Parachlorella*.

In 18S rDNA sequence analyses, a growing number of morphologically different algae are found to cluster within the clade of true *Chlorella* sense stricto species. For instance, Hegewald and Hanagata (2000) found that the coenobial ellipsoid, *Dicloster acuatus* formerly classified within the family *Scenedesmaceae,* closely related to *C. kessleri*. Ustinova et al. (2001) revealed a close relationship of the needle-shaped *Closteriopsis acicularis* with *C. kessleri*. Wolf *et al*. (2002) found *Actinastrum hantzschii* which is characterized by spindle-shaped elongated cells that are arranged in coenobiae, to be closely related to *C. sorokiniana*.

According to *Huss et al*. (1999) several members of the genus *Chlorella* have been transferred to other lineages of Chlorophyta using molecular analysis. Within the Trebouxiophyceae, *C. reniformis* S. Watanabe represents the new genus Watanabea Hanagata (Hanagata et al., 1998) and *C. protothecoides* Kruger was established as the type species of the genus *Auxenochlorella* Shihara and Krauss (Kalina and Puncocharova, 1987). Different varieties of *C. fusca* transferred to the genus *Scenedesmus Meyen* (Hegewald and Silva, 1988; Kessler and Huss, 1992), a member of Chlorophyceae.

The analyses of SSU rDNA sequences revealed that a growing number of morphologically different algae clusters within the clade of *Chlorella* species such as the coenobial spindle-shaped *Actinastrum hantzschii* (Wolf *et al*., 2002), the colonial mucilagineous *Dictyosphaerium pulchellum*, the single-celled, spiny Diacanthos *belenophorus*, the coenobial bean-shaped *Didymogenes anomala* and *D. palatine* and the colonial form with cell wall bristles, *Micractinium pusillum* (Krienitz *et al*., 2004). Because of their morphology differs markedly from the spherical shape of *Chlorella*, these taxa were formerly classified in other families of coccoid green algae, namely *Coelastraceae, Botryococcaceae, Oocyctaceae, Scenedesmaceae and Micractitiaceae* (Komarek and Fott, 1983). Therefore, integrated approaches combining morphological, molecular phylogenetic and ecophysiological methods are important.

According to Lothar *et al*. (2004) complete sequences of both 18S rRNA gene and the ITS 2 regions were used to elucidate the phylogenetic positions of several genera and species of the *Chlorellaceae*; which is a monophyletic lineage within the Trebouxiophyceae. The phylogenetic trees revealed that the organisms investigated

belong to two sister groups within the Chlorellaceae, which were designated as the *Chlorella* and *Parachlorella* clades. The distinction of the new genus *Parachlorella* from the true *Chlorella* is based on molecular data (Huss et al., 1999).

Recently '*Nannochloropsis*' from a prawn farm in Hainan, China was investigated and identified as *Chlorella*, a member of Chlorophyceae based on ultrastructure, fatty acid composition and 18S rDNA by Yu *et al.* (2007). 18S rDNA sequence analyses indicated that this alga was a species of *Chlorella*. Wu *et al*. (2001) carried out study the on the identification of *Chlorella* spp. using nuclear-encoded and chloroplast encoded small-subunit rDNA sequences. Phylogenetic analyses were compared to morphological and biochemical data. The sequences closely resemble each other and the morphological and biochemical data indicated that the phylogenetic analysis was in line with the results. Besides using the common gene, 18S rDNA for green algae, Burja *et al*. (2001), identified C. vulgaris using cyanobacteria derived 16S rDNA primers. They proved that these primers not only targeted cyanobacterium sequences but also can be used in the identification of eukaryotic algae such as *Chlorella*.

Recently, Summerer *et al*. (2008) carried out phylogenetic studies based on 18S rDNA, the internal transcribed spacer (ITS)-1 region and the chloroplast 16S rDNA of several *Chlorella* from lakes, both symbionts of the ciliate *Paramecium bursaria* and free living *Chlorella* to elucidate phylogenetic relationships of *Chlorella*-like algae and to assess their host specificity. Sequence analyses resulted in well-resolved phylogenetic trees providing strong statistical support for a homogenous '*zoochlorellae*' group of different ciliate species from one lake, but different *Chlorella* in one of those ciliate species occurring in another lake (Summerer *et al*., 2008). The results strongly suggest a

high degree of species specificity and importance of physiological adaptation in symbiotic *Chlorella*.

Kiss *et al*. (2008) described a new Chlorophyte isolate from a low temperature pond on the Ross Ice Shelf near Bratina Island, Antarctica. The phylogenetic analysis of 18S rDNA and 16S rDNA and morphological analyses place this new strain in the *Chlorella* clade together with the 'true' *Chlorella* clade named as *Chlorella* B1. It's a psychrophilic species exhibiting optimum temperature for growth around 10^{o} C.

2.4 Nutritional value of *Chlorella*

Chlorella contains high amounts of lipids, minerals, B complex, beta-carotene, vitamins C and E, iron and calcium. The proteins of *Chlorella* contain at least 19 of the 22 known amino acids (Kralovec *et al*., 2004). In 1917, a German microbiologist named Lindner came up with the idea of making food from Chlorella which is over 80% protein (Jensen, 1987). Commercial utilization of *Chlorella* was started in 1961 by Nihon *Chlorella* Inc., which was established using the mass cultivation installations at the Microalgae Research Institute of Japan.

Due to its high nutritional value, *Chlorella* has been marketed as a health food in the form of tablets, granules and drinks (Yamaguchi, 1997). The first addition of *Chlorella* to food was in the production of fermented milk where *Chlorella* extracts were used to promote the growth of Lactobacillus (Yamaguchi, 1997). Dried biomass or extracts of *Chlorella* are used as additives to fermented soybeans, vinegar, green tea, candies, bread and noodles, added to western and Japanese cakes as a coloring agent (Maruyama and Ando, 1992). The dried biomass of *Chlorella* is added to aquaculture

feed to improve the quality of cultured fish and flesh quality of the fish (Nakagawa *et a*l., 1984). The protein content of *Chlorella* is higher (50% of dry weight) than that of the best vegetable sources used in animal feed (Vass and Bhanou, 1973). Therefore a higher proportion of proteins (33%) can be replaced by Chlorella without any negative symptoms in animal feed (Nakagawa *et al*., 1984).

2.5 Biotechnological Applications of *Chlorella*

There are numerous functions of *Chlorella*. It is the most interesting species to scientists because of its nutritional value, its composition can be controlled through the chemicals used in the growth medium, reproduction is very fast and the cell wall is thick enough to protect its nutrients (Richmond, 2003).

Fatty acid composition of *Chlorella* plays an important role in biotechnological application. Polyunsaturated fatty acids (PUFA) such as linoleic acid (18:2), ß-linolenic acid (18:3), dihomo-linoleic acid (20:3), arachidonic acid (20:4) and eicosapentaenoic acid (20:5) are commercially important (Richmond, 2004). They are essential components of the diet of humans and animals (Borowitzka, 1968) and are becoming important feed additives in aquaculture (Watanabea *et al*., 1983).

The pigments of *Chlorella* used as natural colourants for farmed products and as antioxidants (Gouveia et al., 2003). The 0.2 % carotenoids extracted from *C.vulgaris* was used as an ingredient in rainbow trout, Oncorhyncus mykiss, diets (Gouveia *et al*., 1997). According to Sano (1993), certain strains of *Chlorella* become red or orange in a nitrogen-limited and hypersaline medium, accumulating a large quantity of astaxanthin. Such a biomass is considered to be an effective feed supplement for pigmentation of

cultured fish and shellfish. *Chlorella* is also applied in the production of zooplankton such as the rotifer and copepod which are important larval and juvenile feeds of fish (Fukusho, 1981 and Kitajima, 1983).

Chlorella was one of the first algae grown as a food additive in Japan and Taiwan (Vidyavati and Mahato, 1999). Technologies for mass cultivation and production of dried biomass with good digestibility as well as offering it to the public in easy-to-use tablet form, helped to establish the *Chlorella* industry as a novel health food in Japan, Taiwan, Malaysia and Indonesia for the last 35 years (Richmond, 2004). It also has been studied as food and part of bioregulatory systems for space travel. This is because *Chlorella* can photosynthesize and grow continuously and the O_2 released can be used by the space travelers while the CO_2 exhaled by them used in the photosynthesis of such algae (Vidyavati and Mahato, 1999).

C. vulgaris has a great biotechnological potential for producing valuable substances for food, cosmetics and pharmaceutical products (Muller *et al*., 2005). In Japan, antitumor activity of *C. vulgaris* was examined (Tanaka *et al.*, 1998): the growth of methyloanthrene-induced fibro-sarcomas in a syngeneic or semisyngeneic host was inhibited by injection of a hot water extract of *C. vulgaris* into the tumor near the regional lymph nodes. Both T-cells and macrophages appear to participate in the antitumor effect of the algal extract. *C. pyrenoidosa* has been shown to enhance the antibody response in subjects receiving influenza vaccination (Kralovec *et al*., 2004). Studies also have shown that in Japan, *Chlorella* used to cure peptic ulcers and gastric ulcers and as an agent of slow healing wound (Jensen, 1987). The most important substance for human health discovered in *Chlorella* cell is ß-1, 3-glucan, which is an

active immunostimulator, control the ulcerative colitis and hypertension free radical scavenger and a reducer of blood lipids (Richmond, 2004).

Some essential unsaturated fatty acids are used in the pharmaceutical industry as precursors of prostaglandins, prostacyclins and leucotrienes (Martin and Elert, 2004). Patents have been applied for their use as antihypertensive, for treatment of hyperlipidemia, cholesterol reduction and as health food and certain fatty acids which are isolated from *Chlorella* called chlorellin and eicosapentaenoic as antibiotic properties (Richmond, 2004). *C. minutissima* contains high amounts of PUFA and is an important source of PUFA-rich nutraceutical supplements (Seto *et al*., 1984). The effects of these fatty acids on free radical generation in tumor cells, tocopherols during marginal protein nutrition and living metabolism during cardiovascular also have been reported (Martin and Elert, 2004).

Hawkins and Nakamura (1999) studied the effect of *Chlorella* on the expression of the human growth hormone. *Chlorella* is an attractive plant system for both genetic and molecular studies because it is unicellular, has a small genome size is easy to culture and is able to serve as a food supplement. Recently, successful transformation of *Chlorella* by using microprojectile bombardment has been reported (Dawson *et al*., 1997). Chow and Tung (1999) established conditions for the transformation of C. *vulgaris* using hygromycin B resistance as a marker for selection.

Of all the *Chlorella* species, *C. sorokiniana* Shihira et Krauss was found to be most resistant to heat and high light intensity. Therefore it has been studied mainly for biomass production and photosynthesis research (Morita *et al*., 2002). They are

considered as potential substitutes for petroleum products (Princen, 1982). Even though *Chlorella* strains have a great potential to be a resource for biodiesel production due to its faster growth and easier cultivation but then the lipid content in *Chlorella* under general growth conditions is 14-30% by weight of dry biomass (Spolaore *et al.*, 2006) which cannot meet the commercial requirement for biodiesel production. Cock *et al.* (1990) reported that *Chlorella* strains grown in low nitrogen medium including four freshwater strains *C. protothecoides, C. vulgaris, C. emersonii* and *C. sorokiniana* and one marine strain *C. minutissima* are suitable for diesel replacement. According to Scrag *et al*. (2003) a kind of emulsion consisting of transesterified rapeseed oil, a surfactant and slurry of *C. vulgaris* could be used as a fuel in an unmodified single cylinder diesel engine. Recently, high quality of biodiesel production from the heterotrophic microalgae, *C. protothecoides* was obtained by Xu *et al*. (2006).

Another application of *Chlorella* is in wastewater treatment where *C. vulgaris* UMACC 001 was successfully used to treat rubber and palm oil mill effluents in high rate algal ponds (Phang, 1990). It also has been employed successfully for the tertiary wastewater treatment mainly for removal of nitrogen and phosphorus and heavy metals (Tam and Wong, 2000). It has been found to be particularly suitable for large-scale microalgal biomass production, tests for metal ion toxicity, the production of a new polyvinyl chloride *Chlorella* composite material (Zhang *et al*., 2000) and as an additive in sustainable fuel (Scragg *et al*., 2003). Recently, Khan and Yoshida (2007) studied the effect of L-glutamic acid on the growth and ammonium removal from ammonium solution and natural wastewater by *C. vulgaris* NTM 06. The results show that treating C.vulgaris NTM 06 with 1.5% of L-glutamic acid can increase levels of enzymes in treated Chlorella cells capable of assimilating increase amount of ammonia.

CHAPTER 3

Materials and Methods

3.1 *Chlorella* Cultures

A total of 11 isolates of *Chlorella* from the University of Malaya Algae Culture Collection (UMACC) were included in this study. This comprised seven tropical, two Antarctic, one temperate and one sub-Antarctic isolate (Table 3.1). The *Chlorella* isolates were cultured in Bold's Basal Medium (Nichols and Bold, 1965) (Appendix B.1) except for Chlorella UMACC 245 (marine isolate) which was cultured in Provasoli Medium (CCMP, 1996) (Appendix B.2). The cultures were grown in triplicate in 250 mL conical flask containing 100 mL. Inoculum (10 %) used was from the exponential phase cultures, standardized at an optical density at 620 nm of 0.2 (OD_{620}= 0.2).

The tropical and temperate cultures were maintained in controlled-environment incubators set at 26 ^{o}C and 18 ^{o}C respectively with irradiance 42 $\mu mol^{-}m^{-2}s^{-1}$ on a 12:12 h light-dark cycle. The Antarctic and sub-Antarctic isolates were maintained at 4 ^{o}C and illuminated with cool white fluorescent lamps (42 $\mu mol^{-}m^{-2}s^{-1}$) on a 12:12 h light-dark cycle.

Table 3.1 Origin of the *Chlorella* isolates used in this study

Isolate	Origin
Tropical isolate	
Chlorella UMACC 001	Fish pond, Institute of Postgraduate Studies, UM
Chlorella UMACC 103	Klang Estuary, Selangor
Chlorella UMACC 104	Sementa Mangrove, Selangor
Chlorella UMACC 050	Tenamaran Palm Oil Mill, Selangor
Chlorella UMACC 245	Coastal waters, Terengganu
Chlorella UMACC 187	Chinese graveyard, Pahang
Chlorella UMACC 193	Tyre shop, Pahang
Temperate isolate	
Chlorella UMACC 251	Richmond River, Hobart, Australia
Sub-Antarctic isolate	
Chlorella UMACC 250	Marion Island
Antarctic isolates	
Chlorella UMACC 234	Snow sample, Casey Station
Chlorella UMACC 237	Soil sample, Casey Station

3.1.1 Growth Studies

Growth of the cultures was monitored every 2 days for 14 days by measuring OD_{620} using a spectrophotometer (UV-160A UV-Visible Recording Spectrophotometer, Shimadzu) (Appendix A). The specific growth rate (μ, day^{-1}) was determined using the following formula:

$$\mu\ (day^{-1}) = (\ln N_2 - \ln N_1) / (t_2 - t_1)$$

Where, $N_{2=}$ OD_{620} at t_2

$N_{1=}$ OD_{620} at t_1

$t_2 - t_{1=}$ time within the exponential phase

3.3 Morphological Studies

3.2.1 Light Microscopy

The *Chlorella* isolates were examined using the light microscope (Leica Microsystem Ac). The diameter of the cells was measured using an optical micrometer at 400 x magnification. Features such as cell shape, cell wall, cell size and present of chloroplast and pyrenoid were recorded.

3.2.2 Preparation of Samples for Scanning Electron Microscopy

The cells were grown on agar plates. The agar with the algae was cut into cubes before being post-fixed with 2 % OsO_4 overnight at 4 ^{o}C. Then, the samples were dehydrated through a series of increasing concentrations of ethanol (10 % - 95 %). The samples were fixed in a series of ethanol: acetone (3:1, 1:1 and 1:3) for 15 min each. After critical-point drying (CPD), the samples were mounted on stubs, dried overnight in a vacuum dessicator. The samples were coated with gold and kept in the dessicator before viewing under a JSM 6400 Scanning Electron Microscope, (SEM) (JEOL, Tokyo, Japan).

3.2.3 Preparation of Samples for Transmission Electron Microscopy

The *Chlorella* isolates were fixed in 4 % glutaraldehyde, 0.1 % M cacodylate buffer (pH 7.4) overnight at 4 ^{o}C. The specimens were washed with 0.1 % M cacodylate buffer three times before being post-fixed in 1 % OsO_4 overnight at 4 ^{o}C. Then, the samples were fixed in 8 % uranyl acetate for 10 min and dehydrated through a series of increasing concentrations of ethanol (10 % - 95 %). The samples were fixed in propylene oxide followed by propylene oxide: epon (1:1) and propylene oxide: epon (1:3) for 15 min each. Finally, the samples were embedded in epon (Spurr's resin) at 60 ^{o}C overnight. Ultrathin sections were cut with a diamond knife, sequentially stained every 10 min with 2 % uranyl acetate and 1.33 % lead citrate. The sections were then examined under the Transmission Electron Microscope (Carl Zeiss SMT- Nanotech. System Division).

3.3 Biochemical Characterization

3.3.1 Lipid Extraction

Lipids from the samples were extracted using methanol-chloroform-water (MeOH-$CHCl_3$, 2: 1: 0.8; v/v/v) and the content determined by gravimetry (Bligh and Dyer, 1959). The algae samples (100 mL) from exponential phase were filtered using Whatman filter paper 47 mm diameter x 0.45 µm. The filtered cells were extracted with 5 mL of MeOH-$CHCl_3$ and mashed with a hand homogenizer. After centrifugation at 3000 rpm for 10 min, 2 mL of solvent mixture and 2 mL of distilled water were added to the supernatant to give a final methanol-chloroform-water ratio of 1: 1: 0.9 (v/v/v) to initiate phase separation. The upper aqueous phase containing salts and water-soluble material was discarded and the lipids were recovered in the lower chloroform phase. The lower fraction was transferred to a pre-weighed vial. The vials were previously dried in the oven for overnight at 100 ^{o}C. After drying, the vials were cooled in a dessicator and weighed. The solvent was evaporated by a gentle stream of nitrogen gas. The vial containing the dried extract was kept in a dessicator containing silica gel for 24h.

3.3.2 Fatty Acid Transesterification

The dry lipid extracts were transesterified to form fatty acid methyl esters (FAME) before being analyzed by gas chromatography. The lipid extracts were redissolved in 1mL of 1 % sulfuric acid-methanol (H_2SO_4-MeOH) and heated at 90 ^{o}C for an hour to convert free fatty acids to FAME. The esters were extracted with 1mL of hexane (GC grade). The mixture was vortexed vigorously for few minutes and left for a while to initiate phase separation. Then, the upper phase which contained FAME was transferred to a glass vial before being blown dry with a gentle stream of nitrogen gas

and redissolved in 0.5 mL of hexane. The FAME samples were stored in a freezer before being analyzed by gas chromatography.

3.3.3 Gas Chromatographic Analysis of Fatty Acids

The FAME was analyzed using gas chromatography (Shimadzu GC 13A). The peaks were identified based on comparison with the retention times of authentic FAME standards and quantified based on the integrated areas of each peak using the external standards (Sigma Chemicals) (Appendix C). The quantification of the FAME was based on the integrated areas of each peak. The GC system consisted of a flame ionization detector (FID) and an integrator. The system was equipped with a polar capillary column with dimensions of 30 m x 0.25 mm and 0.25 µm thickness. The split ratio of the injector was set at 1:60 and the carrier gas (N) was at a flow rate of 0.6 mL min^{-1}. The injector and detector of the machine were set at 300 ^{o}C and the oven temperature was set at 130 ^{o}C (2 min) and gradually increased to 200 ^{o}C (1 min) at the rate of 3 ^{o}C min^{-1}. The temperature was further increased to 230 ^{o}C at 2 ^{o}C min^{-1} and held for 5 min. Each cycle was run for 45 minutes. Then, 1 µl of samples was injected into the machine.

3.3.4 High Performance Liquid Chromatography (HPLC) Analysis of Pigments

Pigment analysis was carried out using reverse-phase HPLC equipped with C18 reverse-phase column. The cultures of *Chlorella* isolates were examined under light microscopy before HPLC pigment analysis to ensure the cells were in good condition. A total of 100 ml of culture were harvested by centrifugation at 3,000 rpm, for 10 min at 4 ^{o}C. The cells were then transferred to a microcentrifuge tube (1.5 mL). The pigments were extracted under low light with 1 mL of 99 % acetone (HPLC grade). The tubes were chilled in a beaker of ice and sonicated for 5 min. The extracts were centrifuged at

10,000 rpm for 5 min, and 50 uL of the supernatant was immediately injected into the HPLC, a reverse-phase column. The pigments were eluted with methanol: water (9:1) for 0.5 min, with a linear gradient to 100 % methanol in 10 min. The eluted pigments were detected with a UV-visible detector at 440 nm and a Prostar 330 Photodiode Array Detector (Varian Analytical Instruments, USA). During a single analysis, the full spectral range of 350-700 nm with steps of 1nm and a 0.4 s time resolution was recorded. The pigments were identified based on the retention time and absorption spectra.

3.4 Molecular Analysis

3.4.1 Genomic DNA Extraction

The cells from exponential phase cultures (100 ml) were harvested by centrifugation (3,000 rpm, for 10 min) at 4 ^{o}C. Genomic DNA extraction was carried out using modified phenol chloroform extraction method (Sambrook, 2003). The sample was lysed in Nuclei Lysis Solution and homogenized using a mortar and pestle. The lysate was then incubated at 65 ^{o}C for 30 min before adding RNase Solution and 20 % of sodium dodecyl sulfate (SDS). Then, potassium acetate (5M) was added and incubated on ice for 20 min. The protein was precipitated with chloroform: isoamyl (24:1), protein precipitation solution. The tube was gently mixed for 10min before centrifugation at 13,000 rpm for 10 min at 4 ^{o}C to pellet the protein and cell debris. The supernatant containing the DNA was added with isopropanol and centrifuged at 13,000 rpm for 10 min at 4 ^{o}C. The supernatant was decanted and the pellet was washed with 70 % ethanol. The DNA was recovered by centrifugation at 13,000 rpm for 3 min at 4 ^{o}C. The pellet was air-dried and dissolved in 30 µL of TE buffer (DNA Rehydration Solution) and incubated at 65 ^{o}C. The DNA was stored at -20 ^{o}C until use.

3.4.2 Quantification of Genomic DNA

The quantity of genomic DNA was determined by measuring the absorbance at 260 nm to 280 nm. The ratio of OD_{260} to OD_{280} was used to assess the purity of DNA. The integrity of the DNA sample was verified with 0.8 % agarose electrophoresis in 1x TAE buffer and stained with ethidium bromide.

3.4.3 Primer Design for PCR Amplification of 18S rDNA Gene

Two sets of primers were used to amplify the 18S rDNA from all the *Chlorella* isolates (Table 3.2). The primers SHF1 and SHR1 were selected based on the study by Liu et al. (2006). The partial and complete rDNA sequence for 18S gene from *Chlorella* was derived from the database of Gene Bank of the National center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). The second forward primer (SHF2) was designed using the Primer 3 Programme.

Table 3:2 Primers for Amplification of 18S rDNA

Primers	Sequences (5 → 3')	Length (nt)	Tm (^oC)	GC Content (%)
Forward (SHF1)	AACCTGGTTGATCCTGCCAGT	21	49.2	52.4
Reverse (SHR1)	TGATCCTTCTGCAGGTTC ACCTAC	24	52.3	50.0
Forward (SHF2)	CAATCC TGACACAGGGAGGT	20	48.7	55.0

3.4.4 Amplification of 18S rDNA with Polymerase Chain reaction (PCR) Technique

Standard double stranded PCR amplifications were performed. The 25 μL of PCR reaction mixture consisted of 1x PCR buffer, 0.5 μL $MgCl_2$ (2 mM), 0.4 μL dNTP mix (0.16 mM), 1 μL forward and reverse primer (0.4 μM), 0.4 μL Taq DNA polymerase, 1 μL of genomic DNA (100 μg/μL). The PCR conditions were as follows: 5 min at 95 ^{o}C for pre-denaturation followed by 30 cycles, each including 1 min at 95 ^{o}C to denature the DNA strand, 1min at 56 ^{o}C to anneal the primers and 2 min at 72 ^{o}C to extend the PCR amplified product. This was followed by a final extension at 72 ^{o}C for 10 min. The quality of preselective amplifications was analyzed with 1.2 % agarose gel electrophoresis in 1x TAE buffer. Then, the putative PCR amplified bands were excised and extracted with the QIAEX II Agarose Gel Extraction kit.

3.4.5 Purification of DNA Fragments

The putative DNA fragments were purified from the agarose gel using QIAEX II Agarose Gel Extraction Kit according to the supplier's protocols. The excised gel slice was dissolved in Membrane Binding Solution at a ratio 300 uL solution to 100 mg agarose gel slice. The mixture was vortexed every two minutes during the 10 min incubation at 50 ^{o}C to solubilize the agarose and to bind the DNA. The dissolved gel was transferred into a mini-column and incubated for 1 min at room temperature followed by centrifugation at 13,000 rpm for 1 min and the flow through was discarded. The column was washed with 700 uL membrane wash solution and centrifuged at 13,000 rpm for 1 min at room temperature. The washing step was repeated with 500 uL of Membrane Wash Solution followed by centrifugation at 13,000 rpm for 5 min at room temperature. The DNA was eluted in 30 uL of Nuclease-Free Water and incubated at room temperature for 1 min before centrifugation at 13,000 rpm for 1 min. Finally,

the purified products were analyzed with 1.2 % agarose gel electrophoresis and sent for direct sequencing (ABI 3730 XL, Applied Biosystems). All the *Chlorella* sequences were subjected to NCBI BLAST searches for identification of the gene and species.

3.4.6 Phylogenetic Analysis

The 18S rDNA sequences were compared with published sequences in NCBI using BLAST searches to identify the closest matching sequences for inclusion in the phylogenetic analysis. Then, the sequences were aligned together with other published *Chlorella* sequences (Table 3.3) using Bio Edit Sequence Alignment Editor Programme (Hall, 1999). Multiple sequence alignment was obtained using the CLUSTAL W computer programme (Thompson *et al*., 1994).

Phylogenetic relationships were inferred using Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods with PAUP*4.0b10 (Swofford, 2002). Sites showing the insertions or deletions as well as missing data were excluded from phylogenetic analysis. A total of 1040bp were included in PAUP analysis. The distance matrix and MP methods were used to construct the trees. For the distance matrix method, Hasegawa-Kishino-Yano (HKY85) (Hasegawa *et al*., 1985) method was used where the pairwise distances were determined.

For NJ analysis, the HKY 85 model was used to convert pairwise sequence similarities into evolutionary distances and 1000 replicates tree were constructed with random sequence addition replicates, Tree Bisection Reconnection (TBR)-branch-

swapping algorithm. Bootstrap analysis (Felsenstein, 1985), was used to evaluate the statistical reliability.

MP analyses were done using the heuristic search option under the following conditions: TBR branch swapping, 10 random addition sequences, and gaps (insertions/deletions) treated as missing. Bootstrap analysis was performed in 1000 replicates with random sequence addition (10 replicates).

The ML tree was constructed by likelihood analysis using HKY 85 model with starting tree obtained via NJ. Bootstrap analysis was done for 100 replicates. The best confirmed transition / transversion (Ti / Tv) parameter, base frequencies and among-site rate heterogeneity were estimated. In the ML bootstrap analysis, empirical base frequencies were used. Number of substitution types set as 2, the Ti / Tv ratio was set to 2.0, a gamma distribution of 0.5 was assumed for variable sites with the estimation of the shape parameter.

Isolate	Accession No	Species	Habitat	Locality	Reference
CCAP 211/90	FM205861	*Chlorella* sp.	freshwater	Easter Island, Chile	Huss *et al*.(1999)
SAG 211-8K	X62441	*C. sorokiniana*	freshwater	USA	Huss *et al*. (1994)
SAG 211-11b	X13688	*C. vulgaris*	freshwater	Netherlands	Huss and Sogin (1990)
SAG 1.80	AB006046	*C. minutissima*	freshwater	Bermuda, mangrove swamp	Phycologia 38: 1
SAG 211-18	X73992	*Chlorella* sp.	freshwater	cliff pool on Island, Sweden	Huss *et al.* (1994)
SAG 211/1a	X63520	*C. ellipsoidea*	freshwater	-	Huss *et al*.(1999)
SAG 211-11g	X56105	*Parachlorella kessleri*	freshwater	New York, Temperate	Krietniz *et al*.(2004)
SAG 11.86	Y17470	*Closteriopsis acicularis*	freshwater	Germany, plankton of Grömitzer See	Ustinova *et al*. (2001)
SAG 2046	AY323841	*Parachlorella beyerinckii*	freshwater	Germany, Tollensesee lake	Krienitz, *et al*.(2004)
CBS 15	AY948419	*C. vulgaris*	Freshwater	temperate	Huss and Sogin (1990)
CCAP 211/82	FM205855	*C. vulgaris*	Freshwater	Micheln,Germany	
CCAP 211/ 11F	AY591515	*C. vulgaris*	Freshwater	temperate	Muller *et al*. (2005)
Taka P-3-A2	AB191207	*C. vulgaris*	Freshwater	Oita, Japan	-
Antarctic 2 E(9)	AB001374	*Chlamydomonas sp.*	-	Antarctic	Uchida *et al*. (1997)
MES-A1-2	AB006048	*C. trebouxioides*	Freshwater	Bermuda mangrove swamp	Hanagata (1997)
IAM C-531	AB080309	*Parachlorella kessleri*	Freshwater	temperate	Yamamoto *et al*. (2002)
-	AJ439399	*C. protothecoides*	-	Germany	Huss *et al*.(2002)
Singapore-67781	AB017121	*Pyramimonas disomata*	Freshwater	Singapore	Nakayama *et al*. (1998)
UTEX 76	X73994	*Desmodesmus communis*	-	Bristol, England	Kessler *et al*.(1997)

Table 3.3 Details of reference *Chlorella* strains from Gene Bank

CHAPTER 4

Results

4.1 Growth Studies

The *Chlorella* isolates from different geographical regions were grown at different temperatures according to their ambient environment. These isolates have different specific growth rates (μ). The tropical isolates have the highest μ followed by sub-Antarctic, temperate and Antarctic isolates. The Antarctic isolates grew much slower than other isolates. The exponential phase for *Chlorella* isolates from the tropical, sub-Antarctic and Antarctic regions began on the fourth day (Figure 4.1a, c & d). Figure (4.1b) shows that the early stage of exponential phase for temperate *Chlorella* isolate was on the fifth day. The specific growth rates (μ*)*, of *Chlorella* isolates from the tropics were within the range of 0.20 - 0.27 day $^{-1}$ (Table 4.1). The specific growth rates of temperate, sub-Antarctic and Antarctic isolates were 0.18, 0.20 and 0.12 - 0.13 day $^{-1}$ respectively.

.

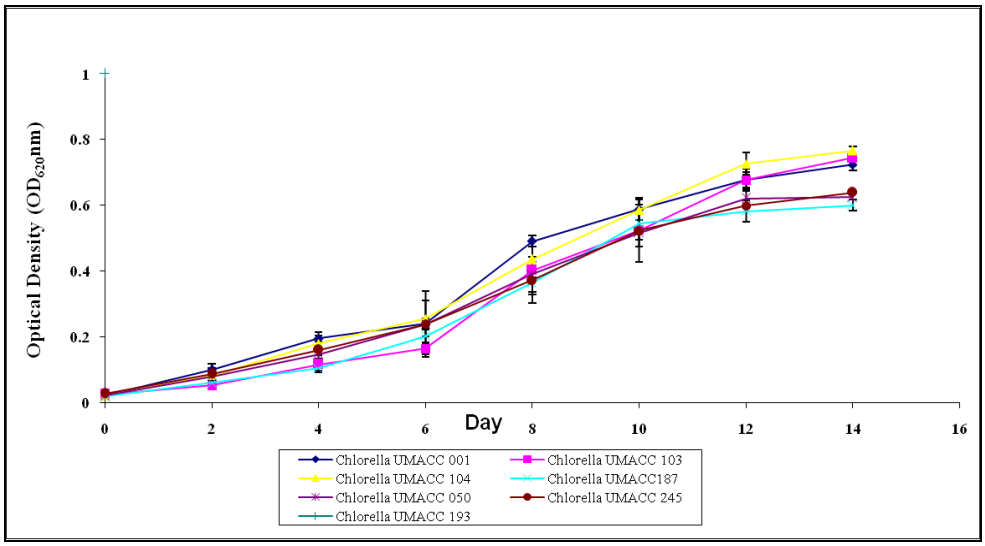


Figure 4.1(a) Semi-logarithmic growth curves of the tropical isolates based on OD_{620}nm

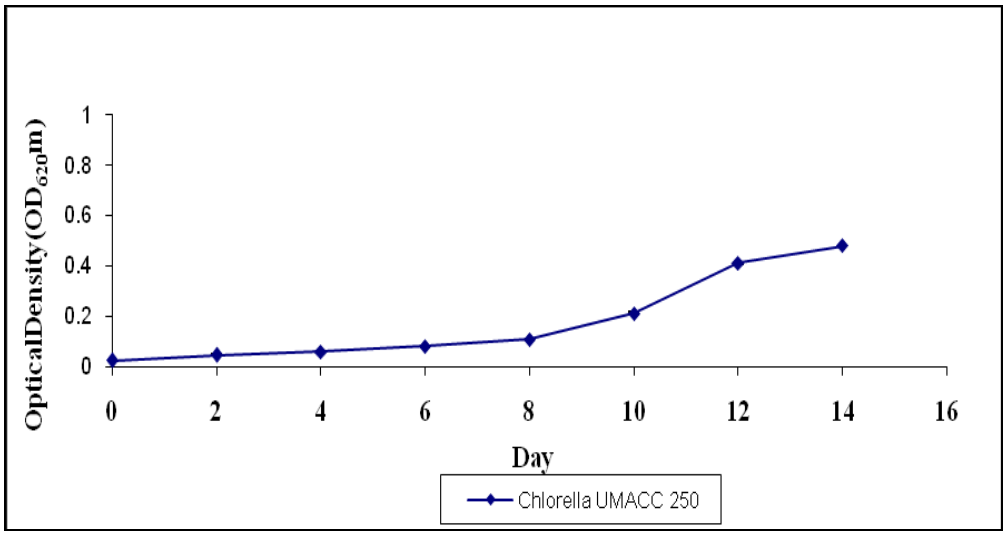


Figure 4.1(b) Semi-logarithmic growth curve of the temperate isolate based on OD_{620}nm

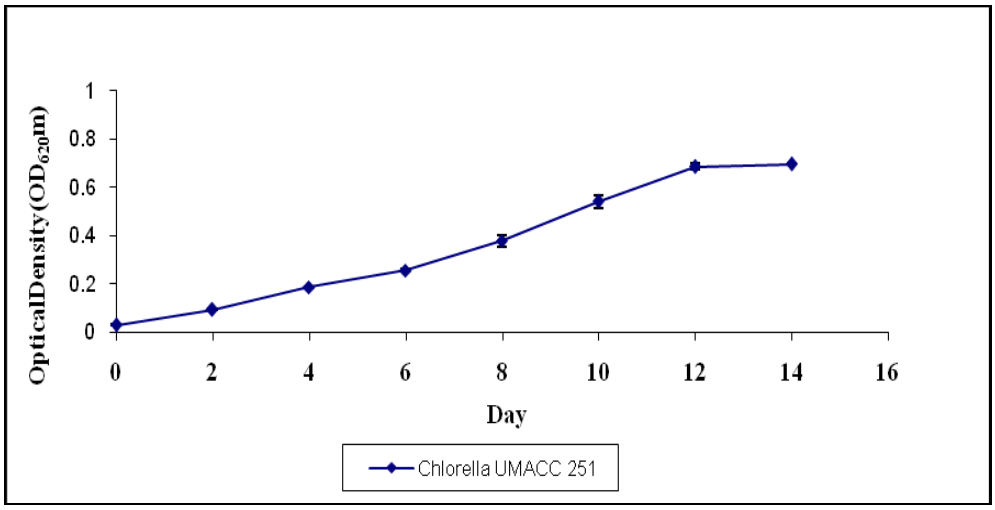


Figure 4.1(c) Semi-logarithmic growth curve of the Sub Antarctic isolate based on OD_{620}nm

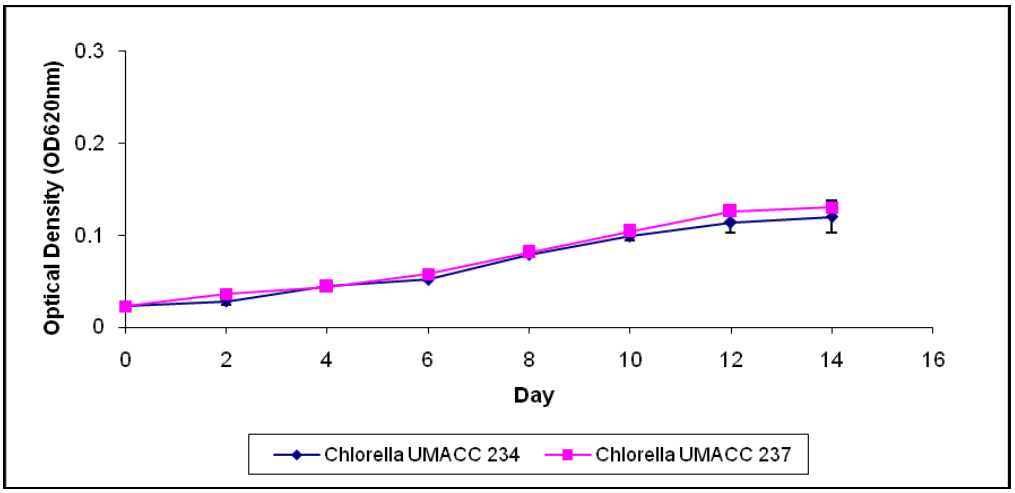


Figure 4.1(d) Semi-logarithmic growth curve of the Antarctic isolates based on OD_{620}nm

Table 4.1 Specific growth rates *(*μ, day^{-1}*)* of *Chlorella* isolates from various regions

Isolate	Specific Growth Rate (μ, day^{-1})
Tropical Isolates	
Chlorella UMACC 001	0.214 ± 0.002
Chlorella UMACC 103	0.220 ± 0.001
Chlorella UMACC 104	0.253 ± 0.003
Chlorella UMACC 050	0.274 ± 0.002
Chlorella UMACC 245	0.210 ± 0.002
Chlorella UMACC 187	0.197 ± 0.002
Chlorella UMACC 193	0.223 ± 0.005
Temperate Isolate	
Chlorella UMACC 251	0.178 ± 0.002
Sub-Antarctic Isolate	
Chlorella UMACC 250	0.201 ± 0.001
Antarctic Isolates	
Chlorella UMACC 234	0.116 ± 0.004
Chlorella UMACC 237	0.132 ± 0.001

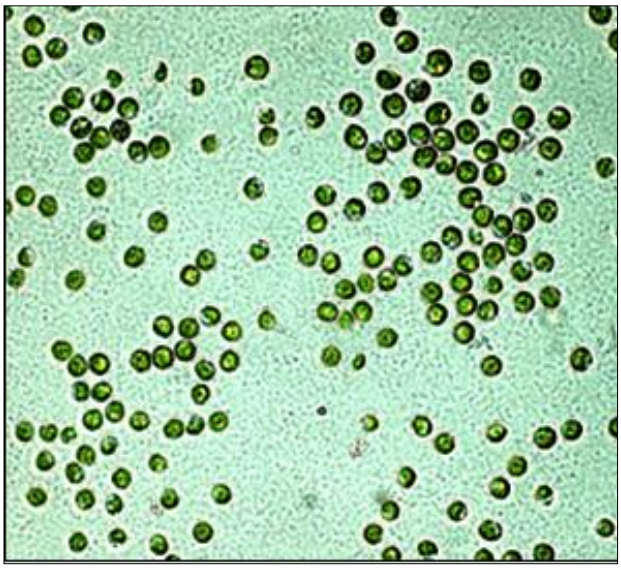
4.2 Morphological Features

4.2.1 Morphological Features by Light Microscopy

The light micrographs of the 11 *Chlorella* isolates are shown in Figure 4.2. All the *Chlorella* isolates consisted of single cells either spherical or ellipsoidal (Figure 4.3). Four tropical *Chlorella* isolates and both Antarctic isolates were spherical in shape while two tropical isolates, *Chlorella* UMACC 103 and *Chlorella* UMACC 104 and the sub-Antarctic isolate *Chlorella* UMACC 250 were ellipsoidal (Table 4.2). The diameters of the tropical, Antarctic and temperate isolates ranged from 6.2-7.6µm, 7.0-7.3µm and 6.9-7.4µm respectively. The cell contained a nucleus, vacuole, nucleolus and a single parietal cup-shaped chloroplast (Figure 4.4). Pyrenoid was found to be present in almost all the isolates. *Chlorella* UMACC 050 and *Chlorella* UMACC 245 contained big and round shape pyrenoid (Table 4.2). Cells with thickened walls were frequently observed in *Chlorella* UMACC001 during the late exponential phase (Figure 4.5).

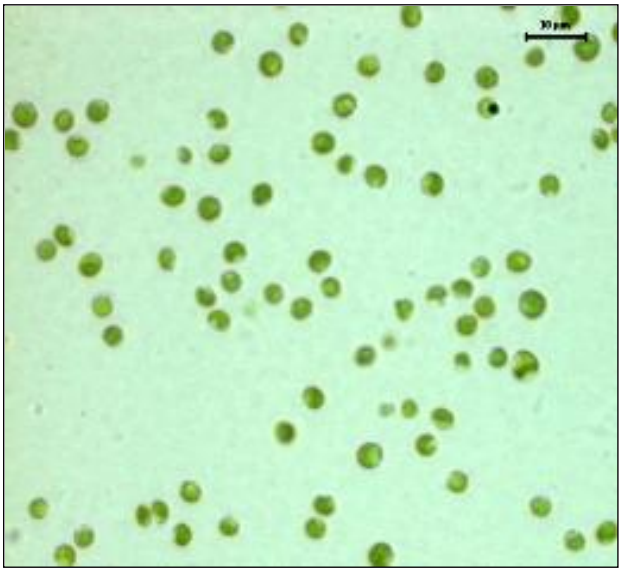
The propagation mode of the *Chlorella* isolates was by autosporulation where autospores were discharged from an aperture that developed by a localized dissolution of the parent cell wall (Figure 4.6). Both ellipsoidal and spherical cells were capable of producing two forms of autospores, spherical and ellipsoidal.

(a)



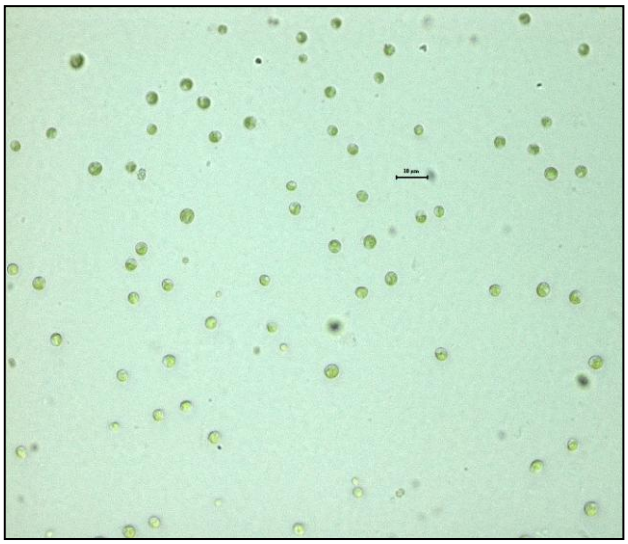
Chlorella UMACC 001 (Tropical isolate)

(b)



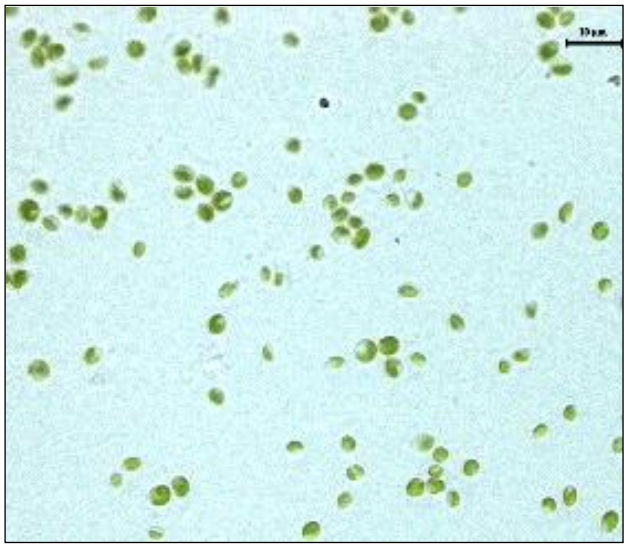
Chlorella UMACC 050 (Tropical isolate)

(c)



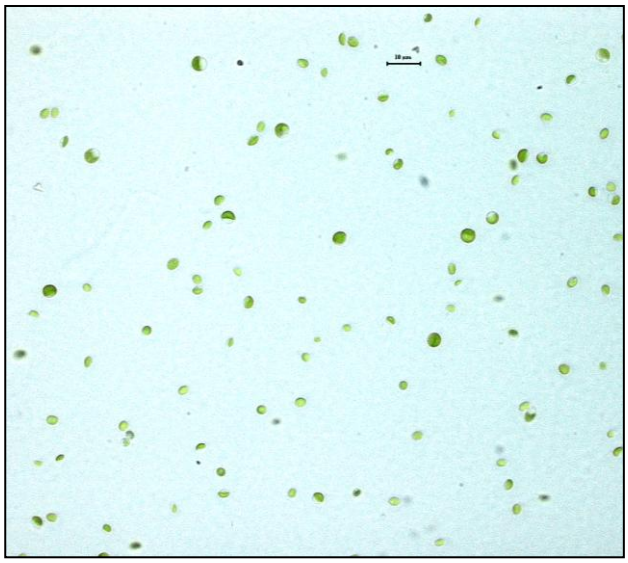
Chlorella UMACC 187 (Tropical isolate)

(d)



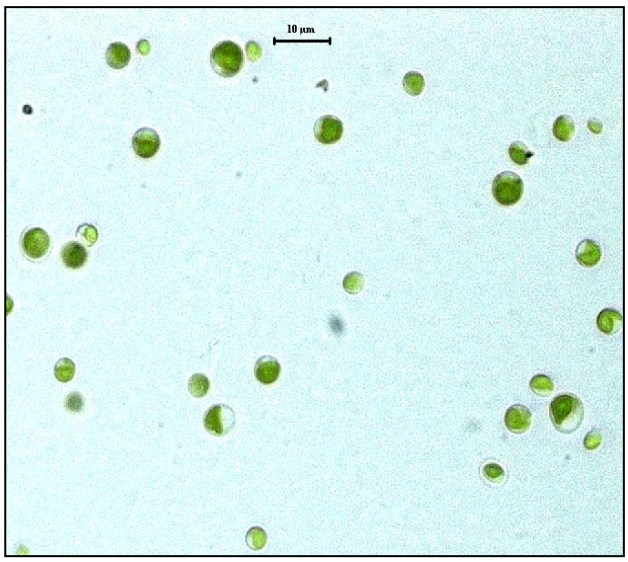
Chlorella UMACC 103 (Tropical isolate)

(e)



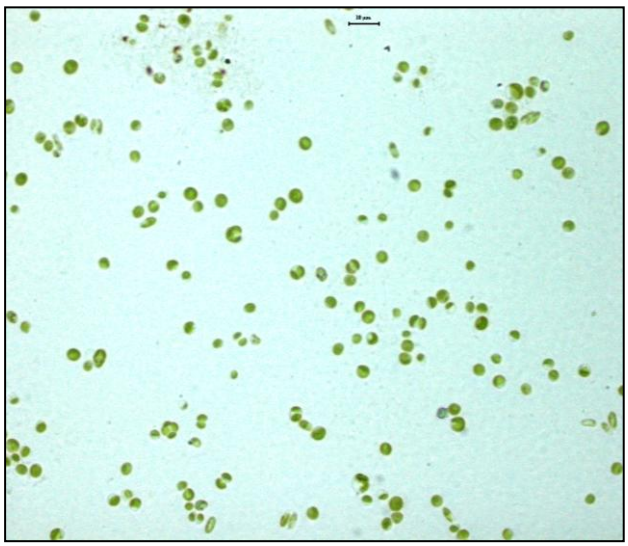
Chlorella UMACC 104 (Tropical isolate)

(f)



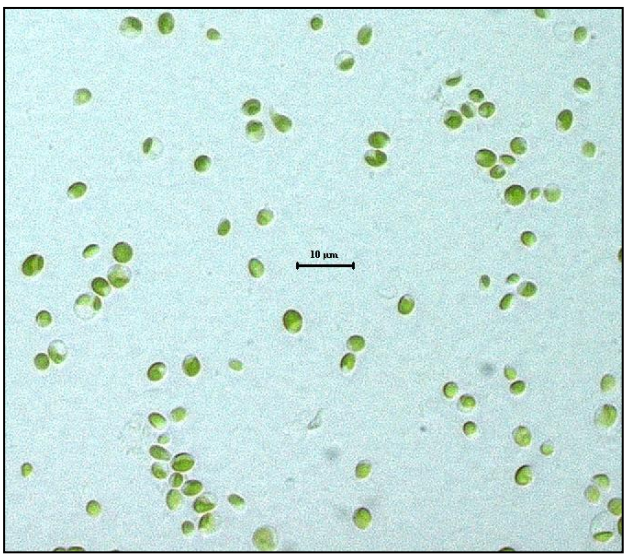
Chlorella UMACC 245 (Tropical isolate)

(g)



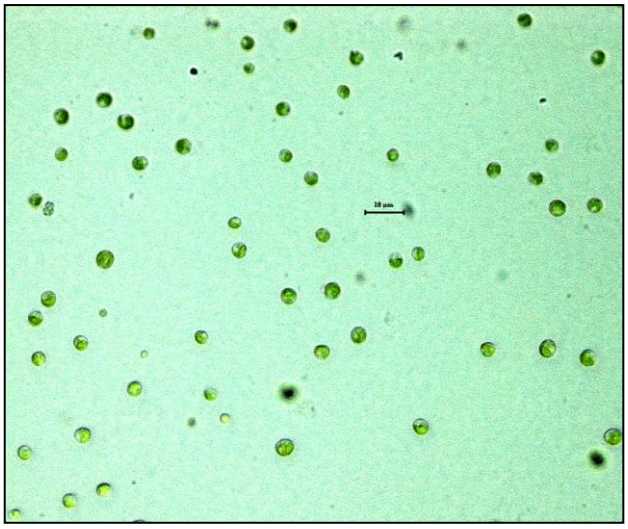
Chlorella UMACC 193 (Tropical isolate)

(h)



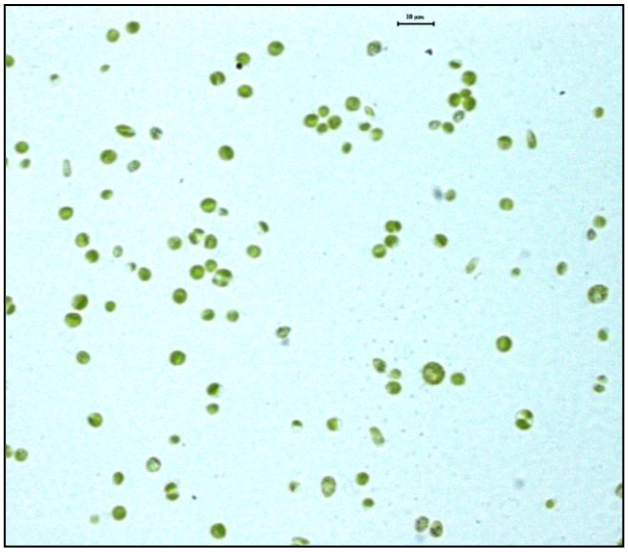
Chlorella UMACC 251 (Temperate isolate)

(i)



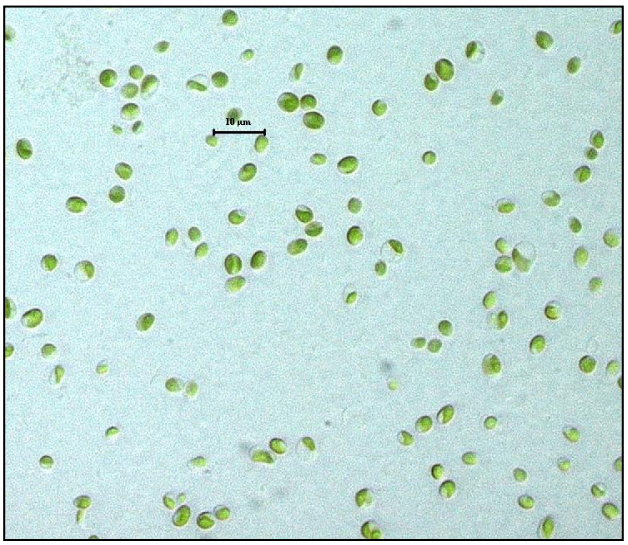
Chlorella UMACC 237 (Antarctic isolate)

(j)



Chlorella UMACC 234 (Antarctic isolate)

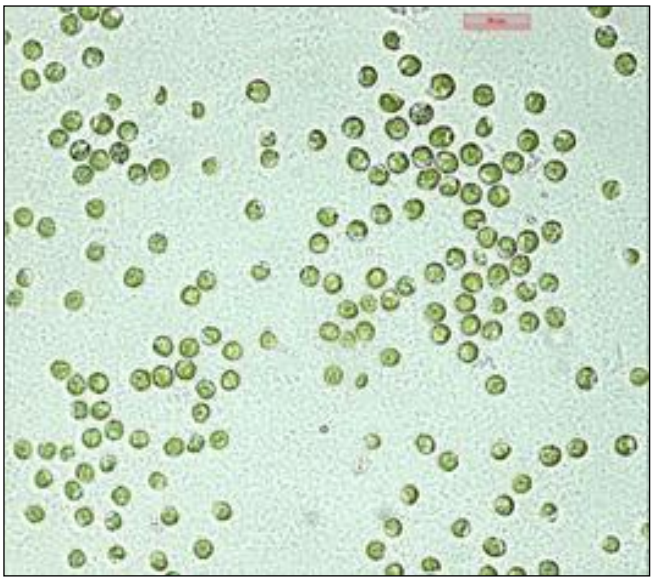
(k)



Chlorella UMACC 250 (Sub Antarctic isolate)

Figure 4.2 *Chlorella* isolates from (a-g) tropical, (h) temperate, (k) Sub-Antarctic and (i-j) Antarctic regions

(a)



(b)

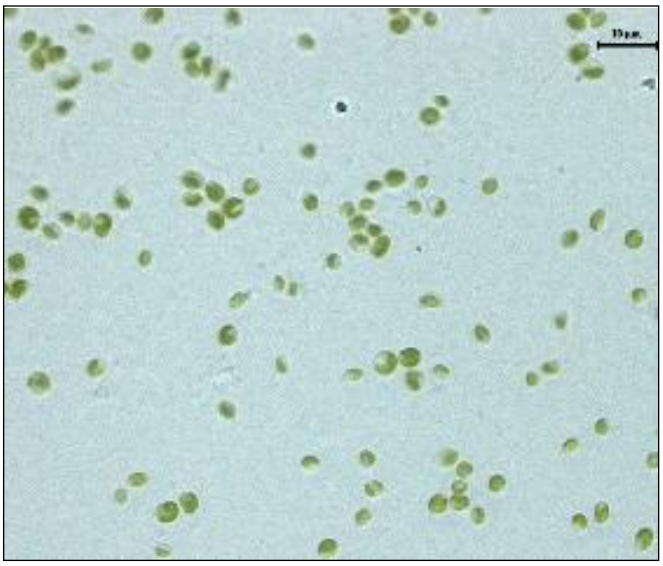


Figure 4.3 Light micrograph showing the typical spherical and ellipsoidal cells of *Chlorella* a) *Chlorella* UMACC 001, spherical b) *Chlorella* UMACC 103, ellipsoidal

Table 4.2 Morphological features of the *Chlorella* isolates based on light microscopic examination

	Characters				
Isolate	Cell shape	Cell size (µm)	Cell wall	Chloroplast	Pyrenoid
Tropical					
Chlorella UMACC 001	spherical	6.8-7.0	thick outer wall	single and large cup-shaped	present
Chlorella UMACC 050	spherical	6.6-6.8	thin outer wall	Single cup-shaped	round and big
Chlorella UMACC 103	ellipsoidal	6.7-7.4	thin outer wall	single cup-shaped	present
Chlorella UMACC 104	ellipsoidal	6.5-7.3	thin outer wall	single cup-shaped	present
Chlorella UMACC 187	spherical	7.4-7.6	thin outer wall	single cup-shaped	present
Chlorella UMACC 193	spherical	6.8-7.4	thin outer wall	single cup-shaped	present
Chlorella UMACC 245	spherical to oval	6.2-6.5	thick outer wall	single and large cup-shaped	round and big pyrenoid present
Antarctic					
Chlorella UMACC 234	spherical	7.0-7.2	thin outer wall	single cup-shaped	present
Chlorella UMACC 237	spherical /ellipsoidal	7.1-7.3	thin outer wall	single cup-shaped	present
Sub-Antarctic					
Chlorella UMACC 250	ellipsoidal	6.9-7.4	thin outer wall	single cup-shaped	present
Temperate					
Chlorella UMACC 251	spherical to oval	6.7-7.0	thin outer wall	single cup-shaped	round and big pyrenoid present

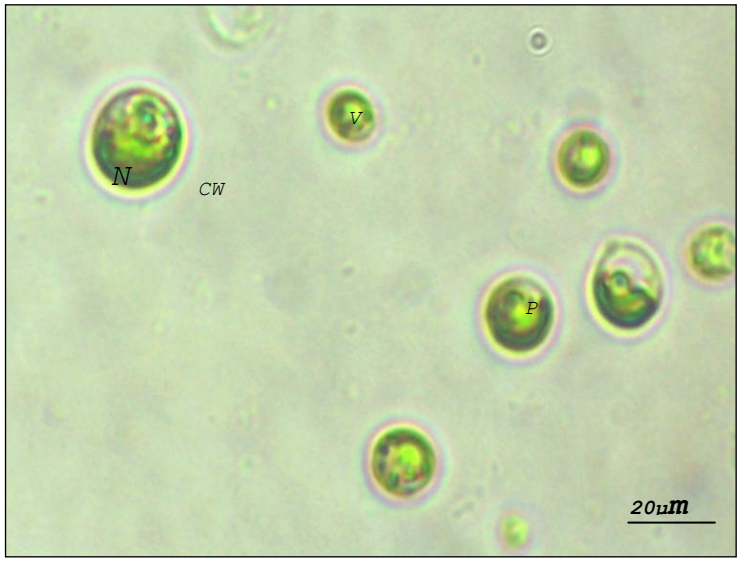


Figure 4.4 Light micrograph showing the typical structure of *Chlorella* vacuole (V), nucleus (N), pyrenoid (P), and cell wall (CW). This picture was taken from *Chlorella* UMACC 245, a tropical isolate

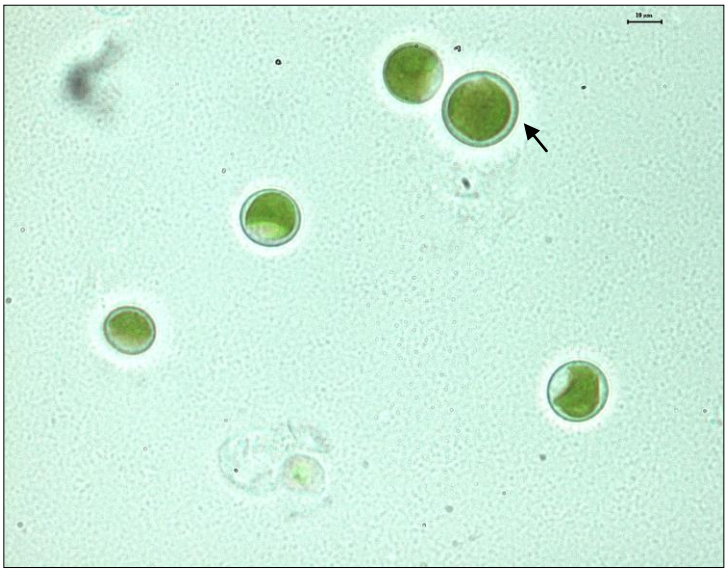


Figure 4.5 Cells of *Chlorella* UMACC 001with thickened wall (arrow) during the late exponential phase

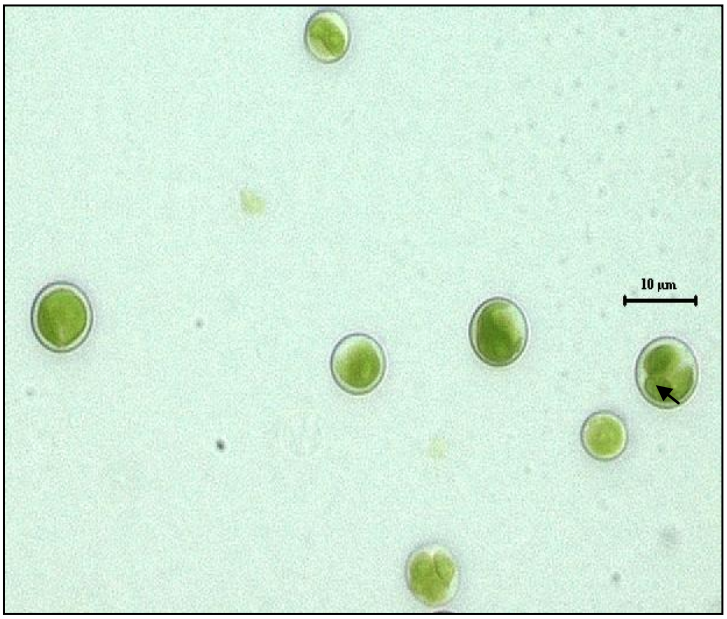


Figure 4.6 Sporangium with four autospores, enclosed by their cell wall (arrow)

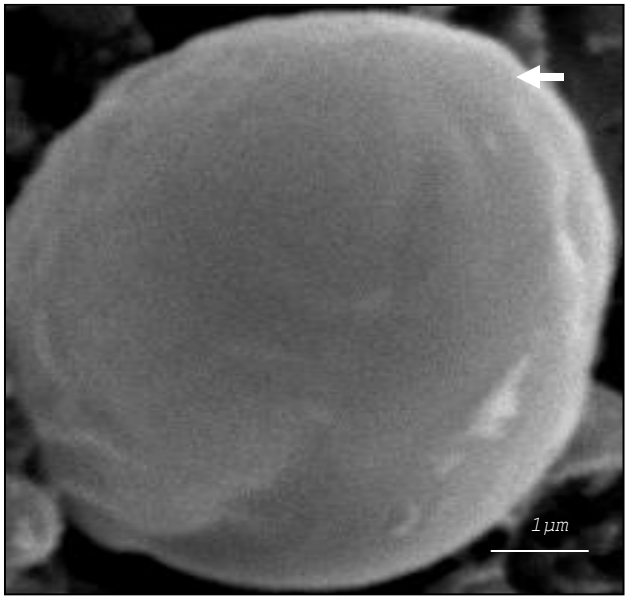
4.2.2 Morphological Features Revealed by Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy showed that all the *Chlorella* isolates contained smooth cell wall except for *Chlorella* UMACC 193 which was covered by an irregular network of subtle ribs (Figure 4.7). Two types of cell wall shape, ellipsoidal and spherical were identified though SEM (Figure 4.8). No ornamentation was found in all the *Chlorella* isolates.

4.2.3 Morphological features revealed by Transmission Electron Microscopy (TEM)

The ultrastructure of all the *Chlorella* isolates contained the typical and common characteristic of *Chlorella* such as nucleus which contained the entire DNA, nucleolus, mitochondrion, starch, vacuole, lipid droplets and pyrenoid (Figure 4.9). The cell wall consisted of either double or trilaminar layers (Figure 4.10). The trilaminar cell wall layer was only found in the temperate isolate *Chlorella* UMACC 251.

(a)



(b)

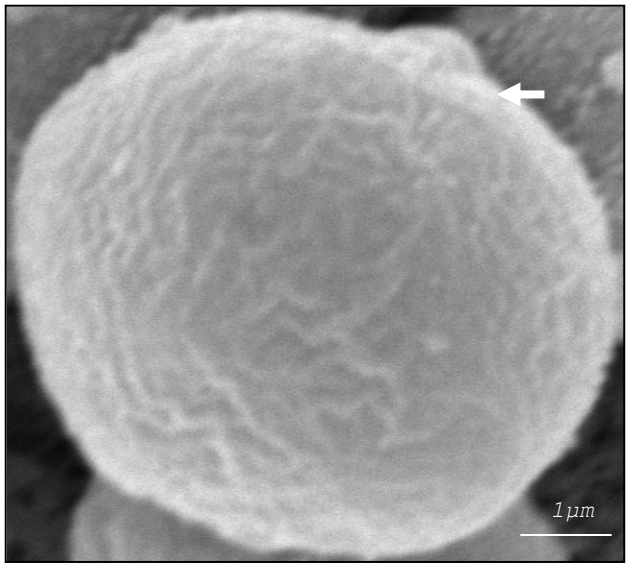
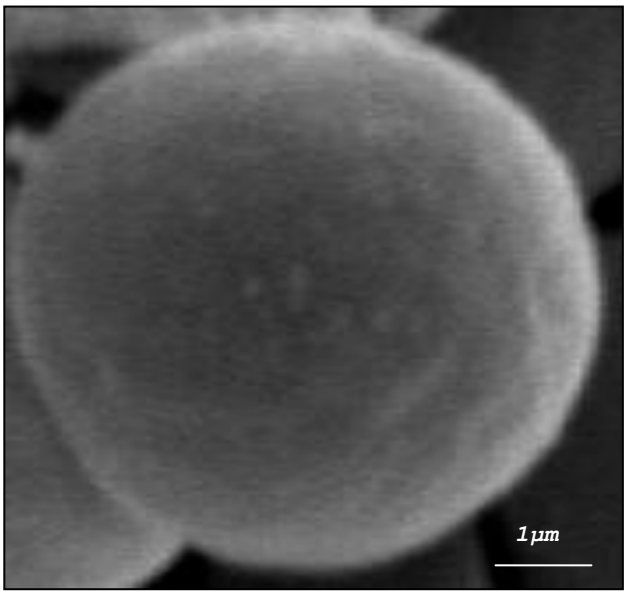


Figure 4.7 Scanning electron micrographs of *Chlorella* showing the a) smooth cell wall of Chlorella UMACC 001 (tropical isolate) and b) irregular network of subtle ribs of *Chlorella* UMACC 193 (tropical isolate), as indicated by the arrows.

(a)



(b)

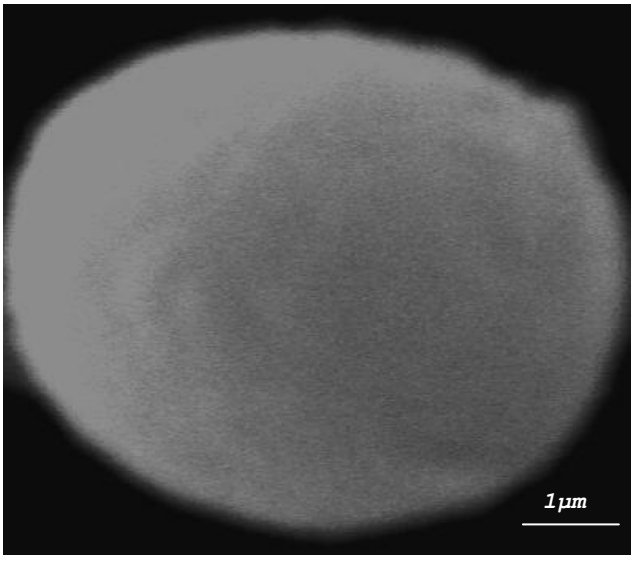


Figure 4.8 Scanning electron micrographs of *Chlorella* showing the a) spherical cell shape of *Chlorella* UMACC 001 (tropical isolate) b) ellipsoidal cell shape of *Chlorella* UMACC 103 (tropical isolate)

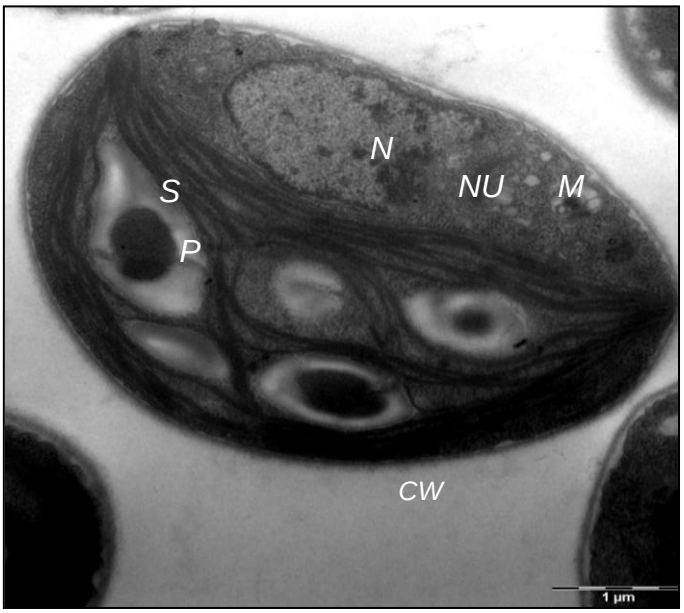
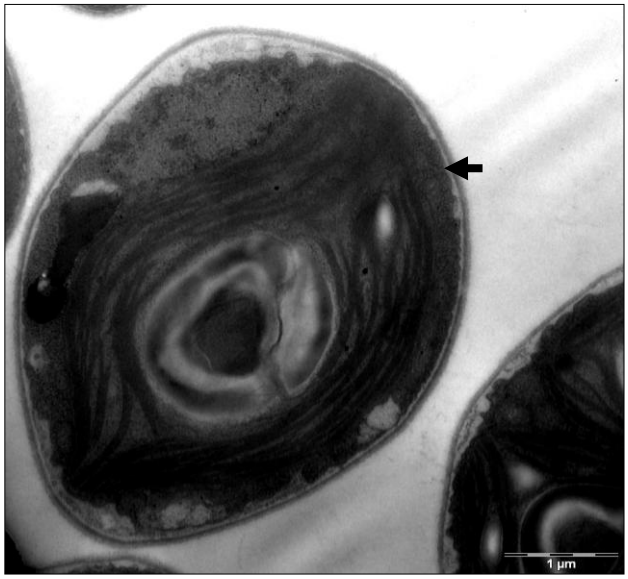


Figure 4.9 Typical uultrastructural features of the *Chlorella* isolates as shown by the TEM micrograph of C*hlorella* UMACC 245 (marine tropical isolate). P- Pyrenoid, N-Nucleus, NU-nucleolus, M-mitochondrion, CW-cell wall, S-starch granule

(a)



(b)

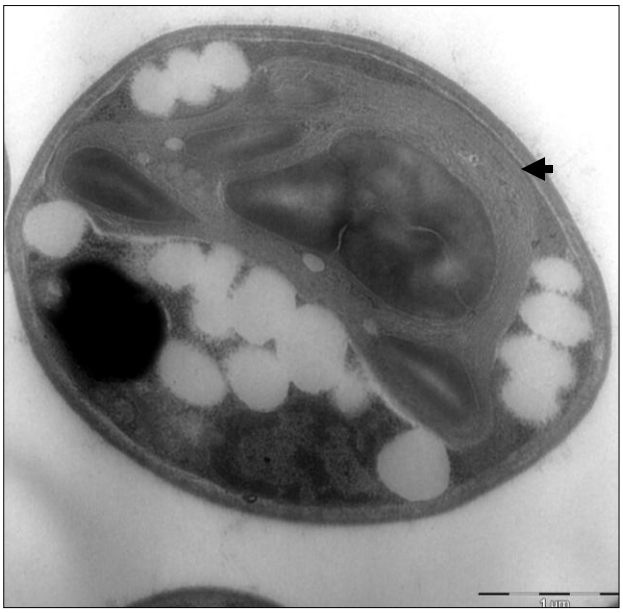


Figure 4.10 Two types of cell wall layer (a) Double layer (arrow), *Chlorella* UMACC 237 (Antarctic isolate) (b) Trilaminar layer (arrow), *Chlorella* UMACC 251(temperate isolate)

The chloroplast of the *Chlorella* isolates was either parietal or cup-shaped with a clearly visible pyrenoid covered by starch grains. The chloroplast contained double membrane enclosed by an envelope consisting of an inner and an outer phospholipid membrane (Figure 4.11). Abundant thylakoids were found and thylakoid lamellae were arranged in several parallel rows and loose stacks (Figure 4.12). The thylakoids were found to have a flattened disk shape which was contained in an empty area called the thylakoid space or lumen (Figure 4.13). Abundant lipid droplets were present under the envelope.

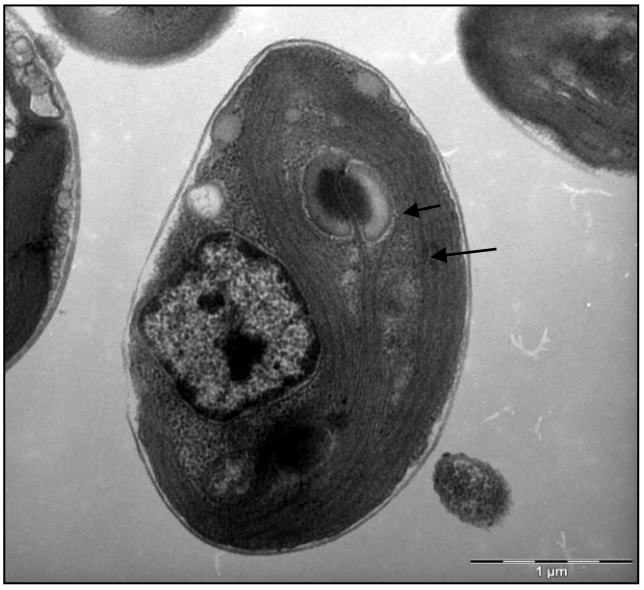


Figure 4.11 Chloroplast membranes (arrows indicate the inner and outer layer) of *Chlorella* UMACC 104 (tropical isolate)

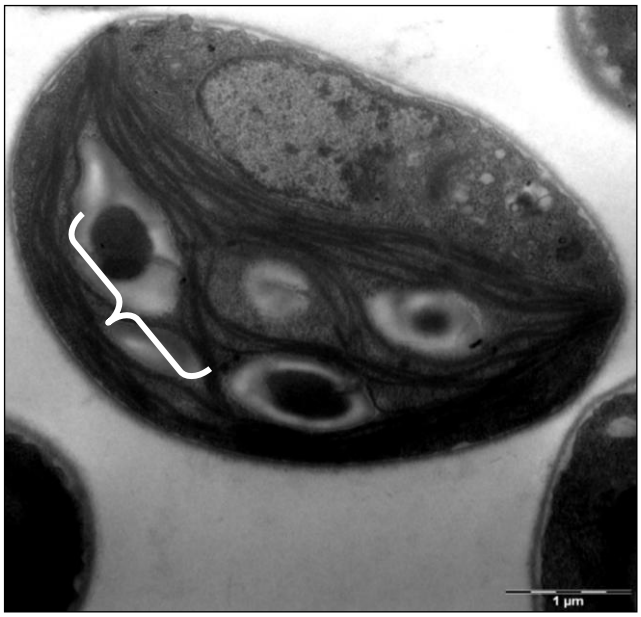


Figure 4.12 Spindle-shaped chloroplast with abundant pyrenoids of *Chlorella* UMACC 104 (tropical isolate)

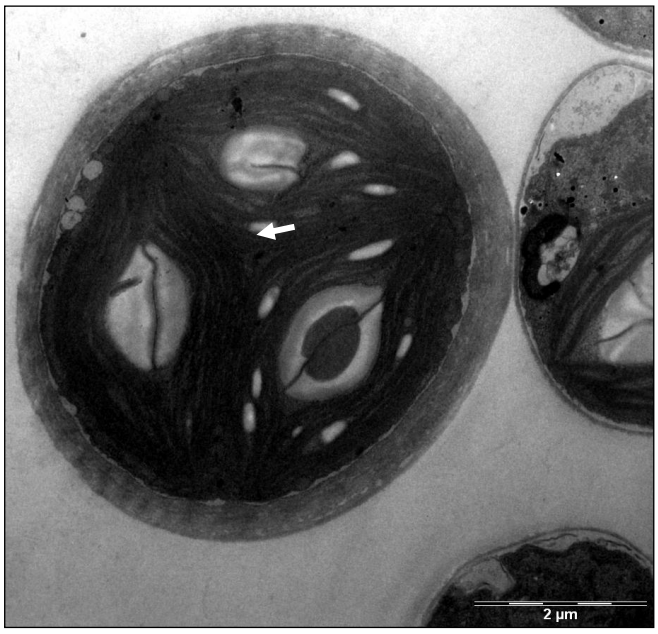


Figure 4.13 Thylakoid structure (arrow). The thylakoids are arranged in several parallel rows and loose stacks in *Chlorella* UMACC 001(tropical isolate).

4.2.3 (a) Reproduction Mode of *Chlorella* Isolates

The reproduction mode of the *Chlorella* isolates was by autosporulation. The autospores formed were either ellipsoidal or spherical shape or the number ranged from 2,4,6,8 to 16 (Figure 4.14). The autospores had a central nucleus and a single parietal chloroplast with a pyrenoid and some interthylacoidal starch grains (Figure 4.15). Most sporangia contained unequally divided cells. Some sporangia contained a large number of autospores. Mitochondria were also found near the concave side of the chloroplast.

4.1.3 (b) Plastoglobules in *Chlorella* Isolates

Another form of cellular inclusion in the *Chlorella* isolates was plastoglobules. The plastoglobules were found mainly within the chloroplast. The sub-Antarctic and Antarctic *Chlorella* isolates contained more plastoglobules compared to the tropical and temperate isolates (Figure 4.16).

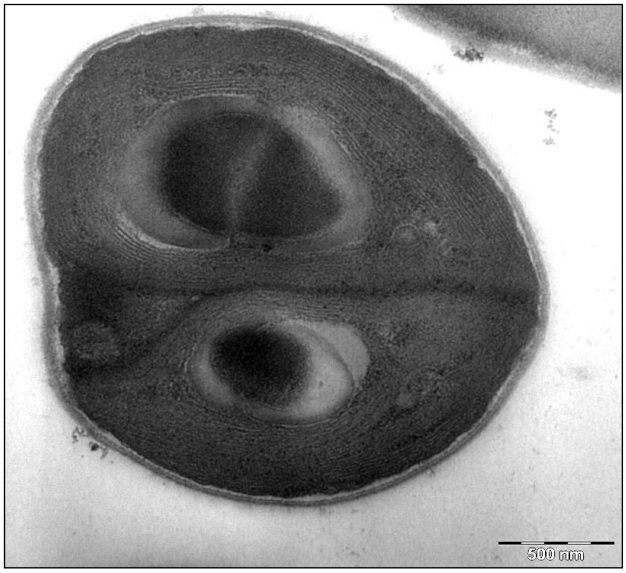


Figure 4.14 Autosporulation mode of *Chlorella* UMACC 001 (a) Two and (b) four autospores were packed in a triangular shape and enclosed by their own wall within the parental wall.

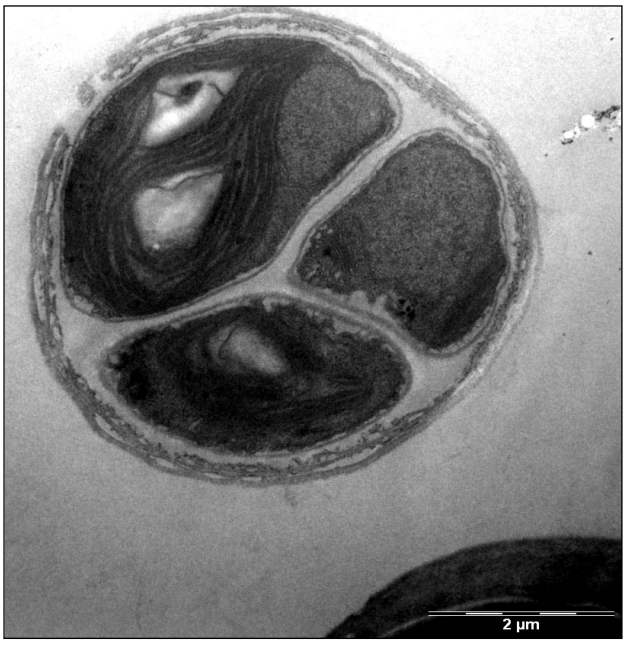
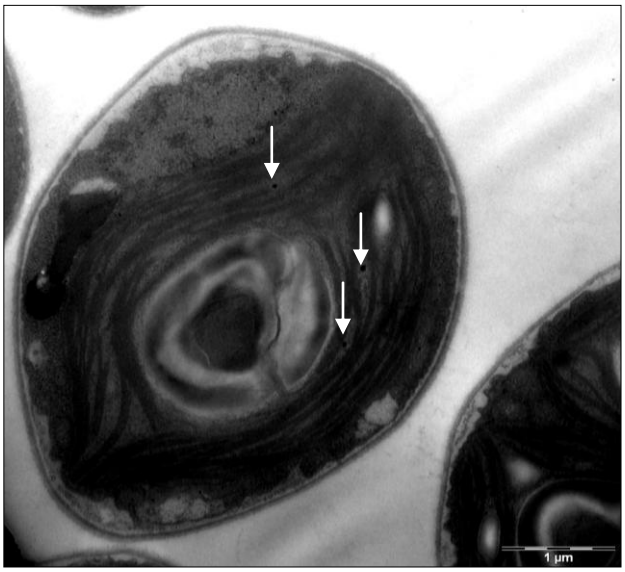


Figure 4.15 Ultrastructure of autospore of *Chlorella* UMACC 001. T-Thylakoid, N-Nucleus, ST-Starch granule, P-Pyrenoid, DC-Daughter cell wall, MC-mother cell wall

(a)



(b)

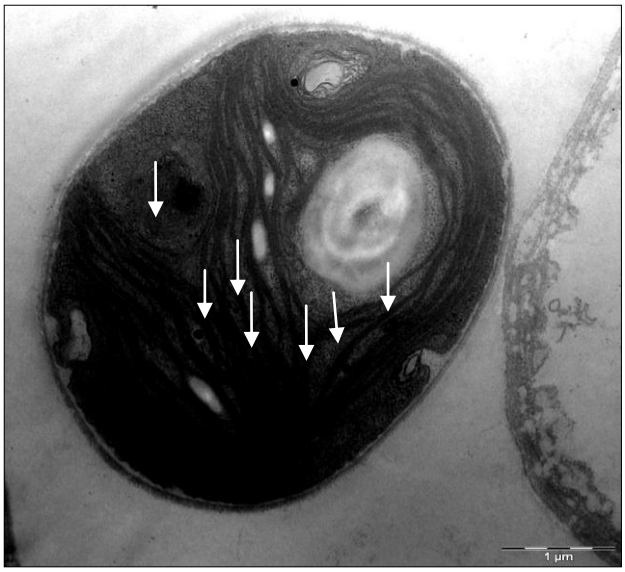


Fig 4.16 Plastoglobules in (a) *Chlorella* UMACC 050 (Tropical isolate) (b) *Chlorella* UMACC 237 (Antarctic isolate)

4.3 Biochemical Characterization

4.3.1 Fatty Acid Composition

There were no marked differences in the fatty acid composition among the 11 *Chlorella* isolates. In general, all the *Chlorella* isolates produced mainly saturated fatty acid (SFA) followed by polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA) except for the marine isolate, *Chlorella* UMACC 245 which contained mainly PUFA (Figure 4.17). The SFA were 14:0; 16:0, and 18:0, while the PUFA include 16:2.16:3, 16:4, 18:2 and18:3. Two types of MUFA were detected, namely 16:1, 18:1 (Table 4.2). The major PUFA in all the tropical isolates was 18:3. The dominant type of SFA was 18:0 followed by 16:0 while the MUFA were dominated by 18:1.

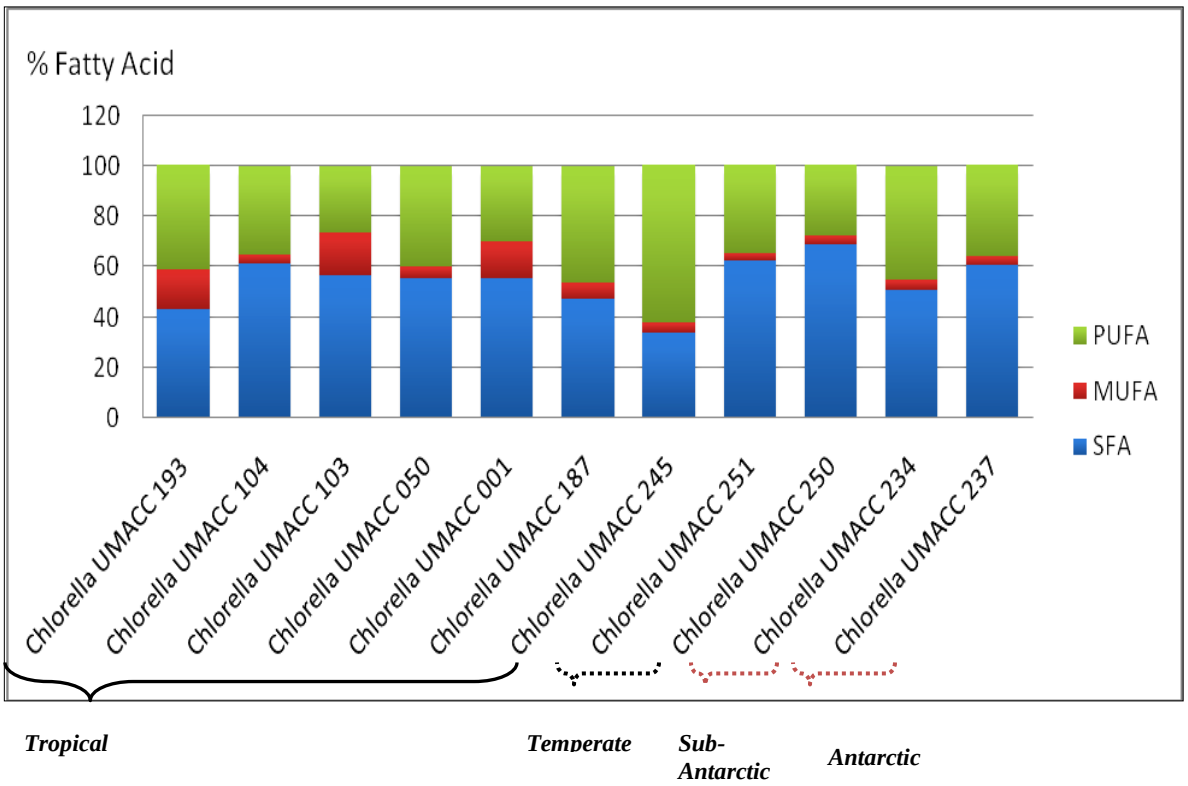


Figure 4:17 Fatty acid composition (% total fatty acids) of the tropical, temperate sub-Antarctic and Antarctic *Chlorella*

PUFA- Polyunsaturated fatty acid
MUFA- Monounsaturated fatty acid
SFA- Saturated fatty acid

Table: 4:3 Fatty acid composition (% total fatty acids) of the tropical, temperate, sub-Antarctic and Antarctic *Chlorella* isolates

	Tropical Isolates						
Fatty acid	*Chlorella* UMACC 193	*Chlorella* UMACC 104	*Chlorella* UMACC103	*Chlorella* UMACC 245	*Chlorella* UMACC 001	*Chlorella* UMACC 187	*Chlorella* UMACC 050
SFA							
14:0	1.5±0.2	Not Detected	1.3±0.1	0.3±0.5	1.6±0.4	Not Detected	1.2±0.1
16:0	19.8±14.1	40.6±8.2	24.5±0.9	23.1±0.7	16.8±3.9	28.7±1.3	21.7±6.2
18:0	21.9±13.6	20.5±2.3	30.8±1.5	10.4±1.9	37.0±1.5	18.3±3.0	32.5±6.0
Total	43.2	61.1	56.6	33.9	55.4	47.0	55.4
MUFA							
16:1	1.7±0.4	Not Detected	1.3±0.1	0.3±0.5	0.9±0.8	Not Detected	0.4±0.7
18:1	14.3±2.3	3.6±0.8	15.8±0.1	3.9±1.0	14±1.5	6.6±0.6	4.5±1.6
Total	16.0	3.6	17.0	4.2	14.9	6.6	4.9
PUFA							
16:2	Not Detected	Not Detected	0.1±0.2	5.1±0.9	Not Detected	Not Detected	0.8±0.2
16:3	0.3±0.6	9.3±8.0	4.6±0.5	15.8±1.6	2.7±0.3	3.3±2.8	4.7±0.4
16:4	10.9±2.9	7.3±0.5	4.6±1.3	Not Detected	3.9±1.6	Not Detected	4.9±1.2
18:2	4.5±0.5	3.8±3.7	5.4±1.4	14.5±0.9	4.0±0.2	11.9±0.7	4.9±1.5
18:3	25.4±4.2	14.8±4.5	7.6±5.9	26.5±3.0	19.1±6.9	31.2±1.3	24.4±1.7
Total	41.1	35.3	26.4	62.0	29.7	46.4	39.7

Table 4.3 Continued data are shown as mean values of three replicates (n=3) with standard deviations (SD).

	Temperate isolate	**Sub-Antarctic isolate**	**Antarctic Isolates**	
% Fatty acid	*Chlorella* UMACC 251	*Chlorella* UMACC 250	*Chlorella* UMACC 234	*Chlorella* UMACC 237
SFA				
14:0	3.3±0.5	3.2±0.7	2.6±0.4	3.3±0.6
16:0	24.4±1.8	24.5±4.0	2.6±6.8	24.8±2.6
18:0	34.9±4.6	45.9±3.1	25.5±0.7	34.5±1.8
Total	62.6	68.9	50.65	62.9
MUFA				
16:1	Not Detected	0.2±0.3	3.19±0.1	Not Detected
18:1	3±0.3	3±0.2	0.73±1.3	3.5±1.1
Total	3	3.2	3.92	3.5
PUFA				
16:2	Not Detected	Not Detected	Not Detected	Not Detected
16:3	2.1±0.8	3.2±0.5	2.7±0.3	0.7±1.1
16:4	5.5±0.6	7.9±1.7	10.7±2.1	6.9±1.0
18:2	6.4±0.7	6±1.7	5.0±0.3	4.6±0.7
18:3	11.9±1.8	10.9±6.2	27.1±3.9	21.4±1.0
Total	35.0	28.0	45.4	33.6

4.4 Pigmentation

A total of six pigments from the *Chlorella* isolates were identified based on the spectral data of the peaks separated by HPLC (Table 4.4) (Appendix D). The identified pigments were chlorophyll *a*, chlorophyll *b*, ß- carotenoid, lutein, violaxanthin and cis-neoxanthin (Figure 4.18). The pigment composition of all the *Chlorella* isolates was similar (Table 4.5).

Table 4.4 Absorption maxima of the pigments of *Chlorella* separated by HPLC

Pigment	**Absorption maxima (Wavelength, nm)**
Lutein	450-460
Chlorophyll *a*	420-430; 640
Chlorophyll *b*	450; 640
ß-carotene	449-475
Cis-neoxanthin	430
Violaxanthin	440

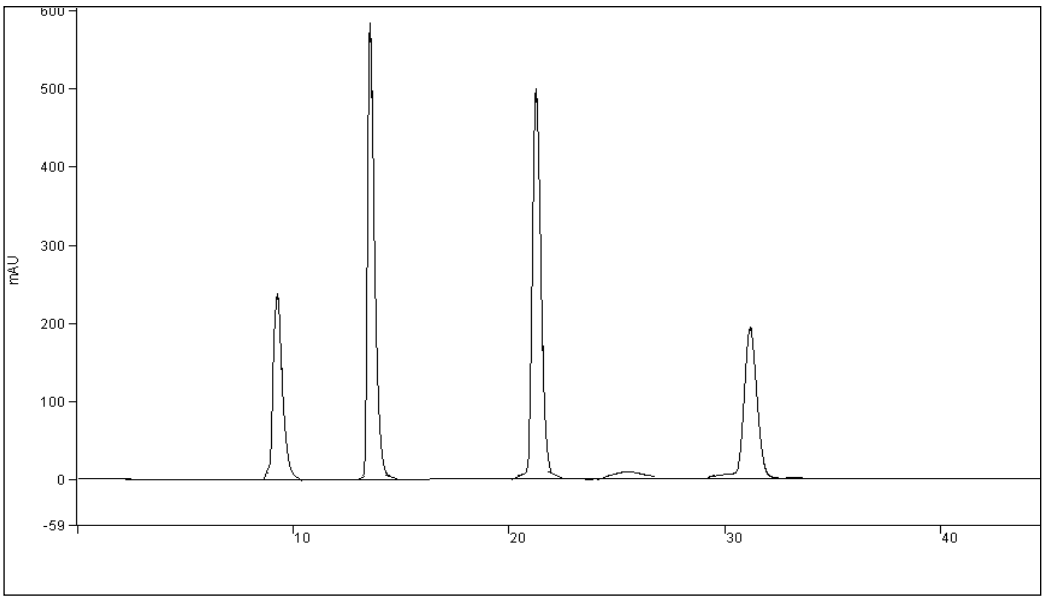


Figure 4.18 A typical chromatogram of the pigments of *Chlorella* by HPLC

Table 4.5 The major pigments of the tropical, temperate, sub-Antarctic and Antarctic *Chlorella* isolates

Isolate	Pigment					
	Chl-*a*	Chl-*b*	Lutein	ß-carotene	Cis-neoxanthin	Violaxanthin
Tropical Isolates						
Chlorella UMACC 001	+	+	+	+	+	+
Chlorella UMACC 050	+	+	+	+	+	+
Chlorella UMACC 103	+	+	+	+	+	+
Chlorella UMACC 104	+	+	+	+	+	+
Chlorella UMACC 187	+	+	+	+	+	+
Chlorella UMACC 193	+	+	+	+	+	+
Chlorella UMACC 245	+	+	+	+	+	+
Antarctic Isolate						
Chlorella UMACC 234	+	+	+	+	+	+
Chlorella UMACC 237	+	+	+	+	+	+
Sub-Antarctic Isolate						
Chlorella UMACC 250	+	+	+	+	+	+
Temperate Isolate						
Chlorella UMACC251	+	+	+	+	+	+

4.5 Molecular analysis

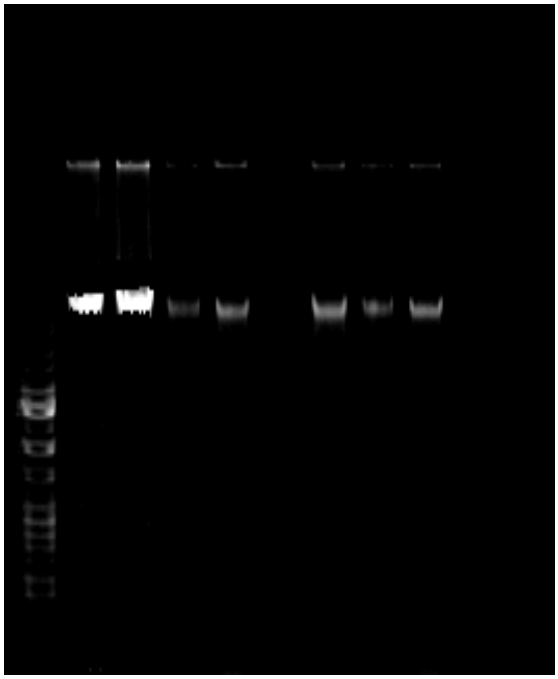
4.5.1 Extraction of Genomic DNA from the *Chlorella* isolates

The quantity and purity of the DNA were determined based on OD_{260}/ OD_{280} (Table 4.6). The purity of the samples ranged from 1.84 -2.00 while the total yield of the genomic DNA ranged from 0.36-0.54µg / mg. The quality and integrity of the DNA sample were verified with 0.8% agarose gel electrophoresis (Figure 4.19).

Table 4.6 The purity and quantity of the extracted genomic DNA from the *Chlorella* isolates

Sample	Purity OD_{260}/OD_{280}	Quantity (µg/mg)
Chlorella UMACC 001	1.84	0.50
Chlorella UMACC 103	1.94	0.52
Chlorella UMACC 104	1.87	0.49
Chlorella UMACC 050	1.98	0.36
Chlorella UMACC 187	1.99	0.53
Chlorella UMACC 193	1.82	0.49
Chlorella UMACC 234	1.96	0.53
Chlorella UMACC 237	1.91	0.50
Chlorella UMACC 245	2.00	0.54
Chlorella UMACC 250	2.00	0.53
Chlorella UMACC 251	1.89	0.51

(a) M 1 2 3 4 5 6 7



(b) M 8 9 10 11

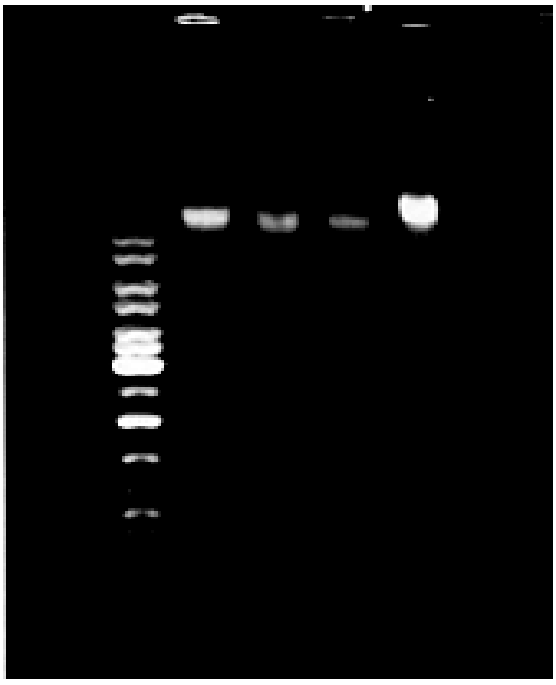


Figure 4.19 Agarose gel electrophoresis of the genomic DNA isolated from (a) Tropical *Chlorella* isolates (Lane 1: *Chlorella* UMACC 001, 2: *Chlorella* UMACC 103, 3: *Chlorella* UMACC 104, 4: *Chlorella* UMACC 193, 5: *Chlorella* UMACC 187, 6: *Chlorella* UMACC 050, 7: *Chlorella* UMACC 245. (b) Temperate, Sub-Antarctic and Antarctic isolates (Lane 8: *Chlorella* UMACC 251, 9: *Chlorella* UMACC 250, 10: *Chlorella* UMACC 234 and 11: *Chlorella* UMACC 237. Lane M contained the Crystalgen Inc.1kb plus marker.

4.5.2 Amplification of 18S rDNA with PCR Technique

The genomic DNA extracted from all the isolates were used for the amplification of 18S rDNA with PCR Technique. A total of three different primers were used in the PCR reaction. The 18S rDNA gene was successfully amplified using the primers SHF1 and SHR1. The PCR products were analyzed with 1.2% agarose gel electrophoresis in 1x TAE buffer. The sizes of the bands were within the expected range of 1300-1350bp (Figure 4.20).

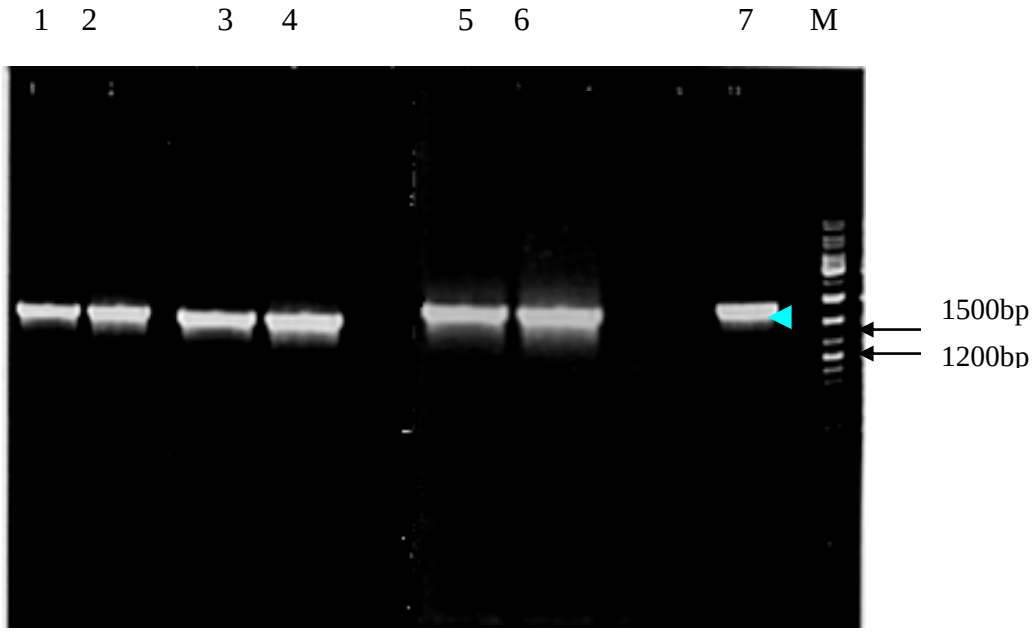


Figure 4.20 Agarose gel electrophoresis of the DNA fragments obtained from PCR. The expected size of the band is between 1200bp to 1500bp. Arrowhead shows the expected size, 1327bp. M is the 1 kb plus marker. (Lane 1: *Chlorella* UMACC 001, 2: *Chlorella* UMACC 103, 3: *Chlorella* UMACC 104, 4: *Chlorella* UMACC 193, 5: *Chlorella* UMACC 187, 6: *Chlorella* UMACC 050, 7: *Chlorella* UMACC 245 and Lane M represent the Crystalgen Inc. 1kb plus marker)

4.5.3 Phylogenetic Analysis

A total of 35 sequences comprising sequences from 11 *Chlorella* isolates of this study and 24 reference sequences from Gene Bank were used for the phylogenetic analysis. In addition, *Monostroma grevillei* (AF 015279) and *Ulva curvata* (AF 189078) were included as the outgroups for the analysis. Phylogenetic trees based on ML, MP and NJ analyses were constructed (Figure 4.21, 4.22 and 4.23).

A distance tree was constructed with the NJ method based on HKY85 model method. The branch length was proportional to evolutionary distances. The phylogenetic tree obtained from NJ analysis shown in Figure 4.22. The 100 and 1000 replicates of bootstrap value were conducted to evaluate the statistical reliability. The distance tree was rooted with weighted least squares with power=2, weighted sum-of-squares =0.16296 and average %SD=5.493. Pair-wise distances were calculated using HKY 85 model.

ML analysis was performed using first the HKY 85 model. The number of substitution types was set at 2, Ti/Tv at 2 and the distribution at variable sites were equal and the assumed proportion of invariable was none. The shape parameter (alpha) was estimated. The HKY 85 model has the highest likelihood score (-ln L: 8261.60737). The base frequencies for A, C, G and T were 0.25978, 0.20838, 0.26607 and 0.26577 respectively. The estimated Ti/Tv ratio was 1.502821 (Kappa=3.009634).

The estimated value of gamma shape parameter was 0.594344. This parameter describes the distribution of substitution probabilities among sites in a sequence. The ML tree is shown in Figure 4.21.

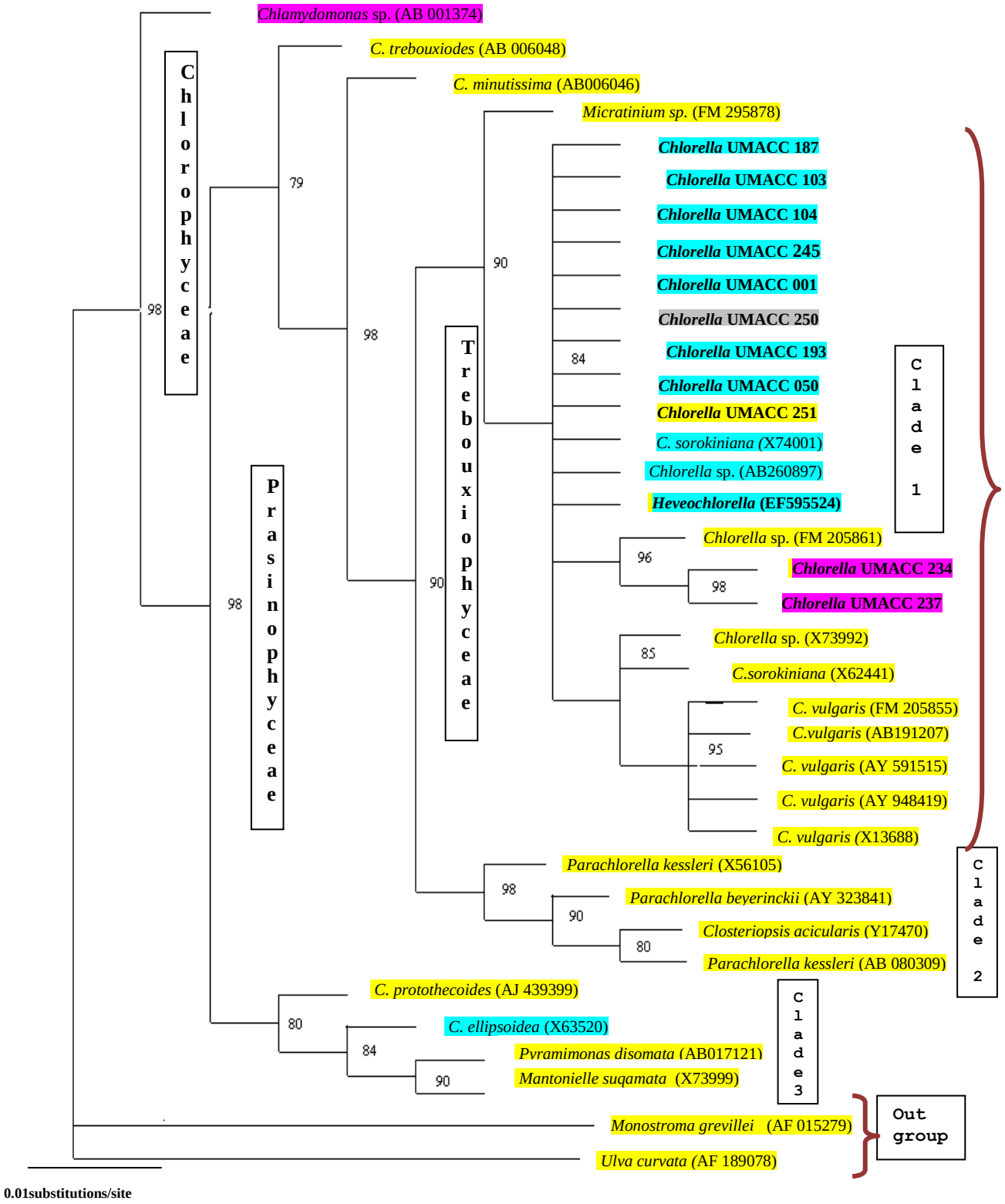
The most parsimonious tree obtained with MP is shown in Figure 4.23. The tree length was 1376 with high indices of consistency (CI=0.7302) and retention index (RI=0.7496) suggesting that a strong phylogenetic signal was present in the dataset. The Homoplasy index (HI) excluding the informative characters was 0.5039, CI excluding uninformative characters was 0.6454 and the rescaled consistency index was 0.3546. The majority of nodes in the tree were supported by bootstrap values >50%. In the analysis, 1314 characters were constant which included 223 (16.97%) parsimony informative variable sites and 134 (10.20%) parsimony uninformative variable sites.

In this study, according to ML analysis of the 18S rDNA sequences revealed that all the 11 isolates are from the genus *Chlorella* (Figure 4.21). This is because they clustered together with other 'true' *Chlorella* in the class of Trebouxiophyceae. The NJ, MP and ML analyses showed that the bootstrap values were >70%. Within the Trebouxiophyceae, there are three major clades (Clade 1, 2 and 3). All the *Chlorella vulgaris* including the type species (X13688) were clustered together. All these isolates are from the temperate region.

The seven tropical isolates studied grouped together and closely related with each other (Figure 4.21). The sub-Antarctic (*Chlorella* UMACC 250) and temperate isolates (*Chlorella* UMACC 251) also clustered together with the tropical isolates. Both the Antarctic isolates (*Chlorella* UMACC 234 and *Chlorella* UMACC 237) were clustered in the same clade with high bootstrap value.

Within the Trebouxiophyceae, *P. beijerinck*, *P. kessleri*, *Closteriopsis acicularis* and *Discloster arcuatus* formed a sister clade. These four species belong to the *Parachlorella*-clade reported by Krienitz *et al*. (2004). The species from the class

Prasinophyceae clustered together with *Chlorella prothecoides* (AJ439399) and *Chlorella ellipsoidea* (X63520). *Chlamydomonas* sp. (AB 001374) from the class of Chlorophyceae formed a separate clade. The topologies of the NJ and MP trees were similar to the MJ tree although the bootstrap values were different (Fig 4.22 and 4.23).


Chlamydomonas sp. (AB 001374)
C. trebouxiodes (AB 006048)
C. minutissima (AB006046)
Micratinium sp. (FM 295878)
Chlorella UMACC 187
Chlorella UMACC 103
Chlorella UMACC 104
Chlorella UMACC 245
Chlorella UMACC 001
Chlorella UMACC 250
Chlorella UMACC 193
Chlorella UMACC 050
Chlorella UMACC 251
C. sorokiniana (X74001)
Chlorella sp. (AB260897)
Heveochlorella (EF595524)
Chlorella sp. (FM 205861)
Chlorella UMACC 234
Chlorella UMACC 237
Chlorella sp. (X73992)
C.sorokiniana (X62441)
C. vulgaris (FM 205855)
C.vulgaris (AB191207)
C. vulgaris (AY 591515)
C. vulgaris (AY 948419)
C. vulgaris (X13688)
Parachlorella kessleri (X56105)
Parachlorella beyerinckii (AY 323841)
Closteriopsis acicularis (Y17470)
Parachlorella kessleri (AB 080309)
C. prototheco ides (AJ 439399)
C. ellipsoidea (X63520)
Pyramimonas disomata (AB017121)
Mantonielle suqamata (X73999)
Monostroma grevillei (AF 015279)
Ulva curvata (AF 189078)
Chlorophyceae
Trebouxiophyceae
Prasinophyceae
Clade 1
Clade 2
Clade 3
Out group
79
98
90
84
96
85
95
80
0.01substitutions/site

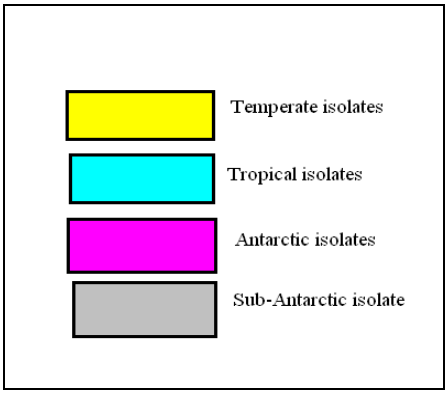
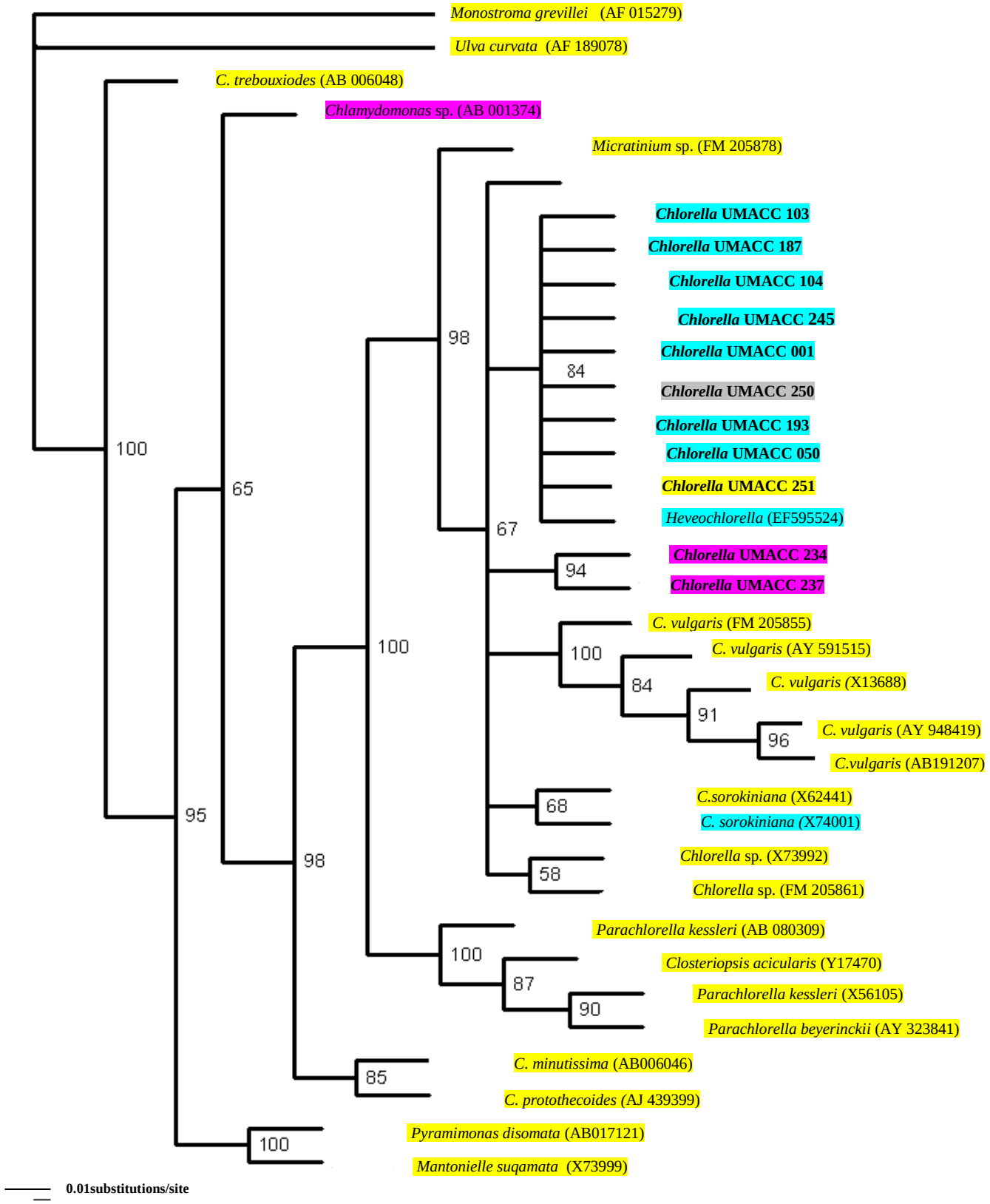


Figure 4.21 Maximum likelihood tree based on 18S rDNA gene sequences. The numbers at the branches indicate bootstrap values obtained by ML (100 replicates) larger than >70%. The taxa shown in bold letters are the species for which 18S rDNA sequences were determined in this study.


Monostroma grevillei (AF 015279)
Ulva curvata (AF 189078)
C. trebouxiodes (AB 006048)
Chlamydomonas sp. (AB 001374)
Micratinium sp. (FM 205878)
Chlorella UMACC 103
Chlorella UMACC 187
Chlorella UMACC 104
Chlorella UMACC 245
Chlorella UMACC 001
Chlorella UMACC 250
Chlorella UMACC 193
Chlorella UMACC 050
Chlorella UMACC 251
Heveochlorella (EF595524)
Chlorella UMACC 234
Chlorella UMACC 237
C. vulgaris (FM 205855)
C. vulgaris (AY 591515)
C. vulgaris (X13688)
C. vulgaris (AY 948419)
C.vulgaris (AB191207)
C.sorokiniana (X62441)
C. sorokiniana (X74001)
Chlorella sp. (X73992)
Chlorella sp. (FM 205861)
Parachlorella kessleri (AB 080309)
Closteriopsis acicularis (Y17470)
Parachlorella kessleri (X56105)
Parachlorella beyerinckii (AY 323841)
C. minutissima (AB006046)
C. protothecoides (AJ 439399)
Pyramimonas disomata (AB017121)
Mantonielle suqamata (X73999)
100
65
98
84
67
94
100
100
84
91
96
68
58
98
100
87
90
85
95
100
0.01substitutions/site

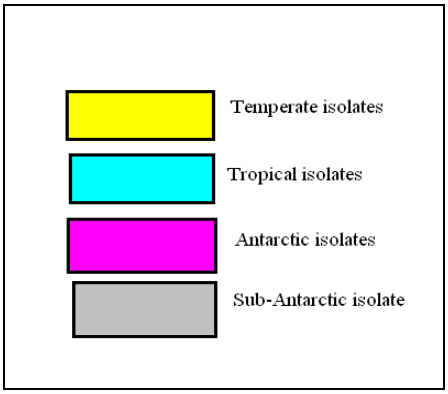
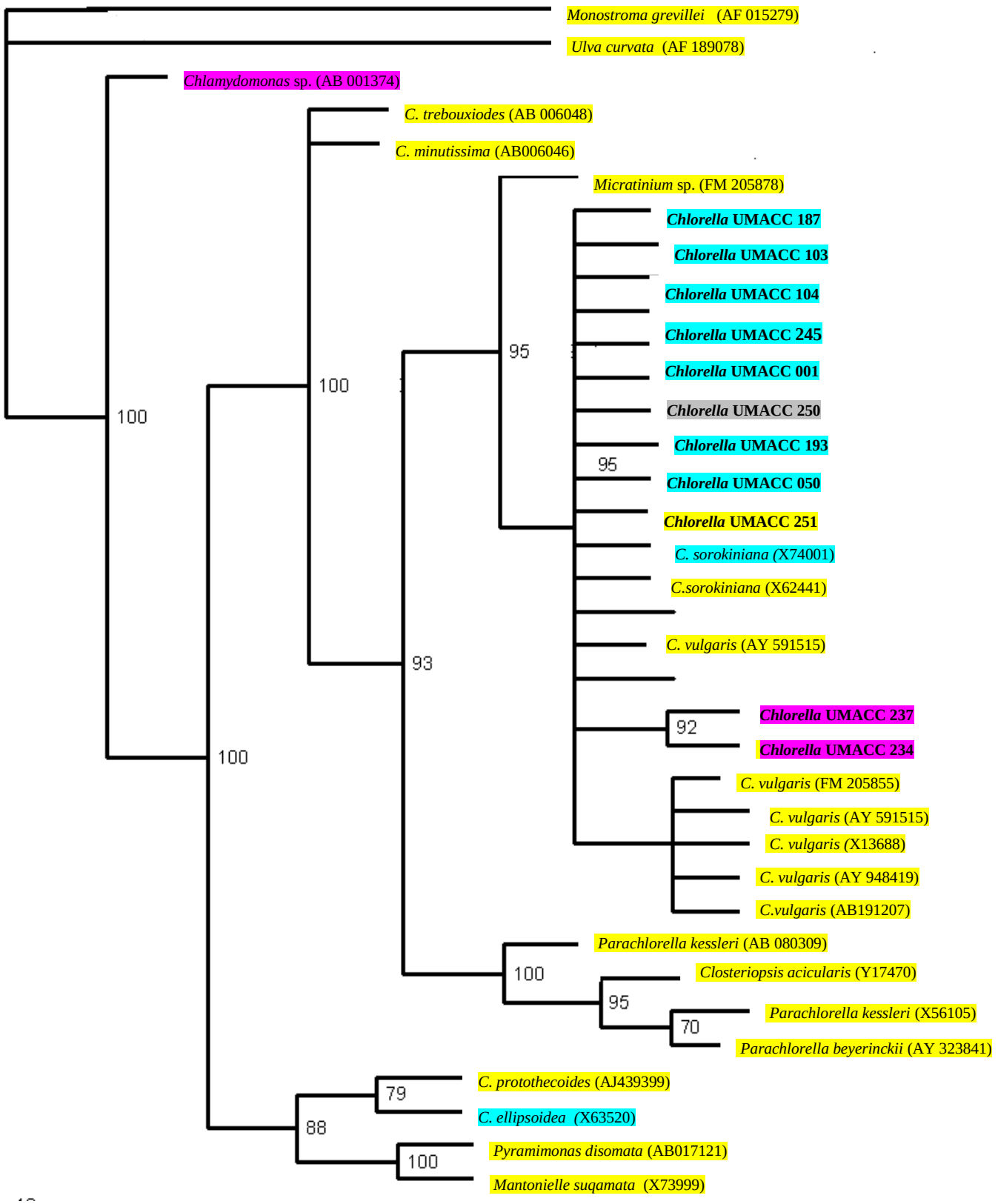


Figure 4.22 Neighbour-Joining tree based on 18s rDNA gene sequences. The numbers at the branches indicate bootstrap values obtained by MP (1000replicates) larger than 60%.


Monostroma grevillei (AF 015279)
Ulva curvata (AF 189078)
Chlamydomonas sp. (AB 001374)
C. trebouxiodes (AB 006048)
C. minutissima (AB006046)
Micratinium sp. (FM 205878)
Chlorella UMACC 187
Chlorella UMACC 103
Chlorella UMACC 104
Chlorella UMACC 245
Chlorella UMACC 001
Chlorella UMACC 250
Chlorella UMACC 193
Chlorella UMACC 050
Chlorella UMACC 251
C. sorokiniana (X74001)
C.sorokiniana (X62441)
C. vulgaris (AY 591515)
Chlorella UMACC 237
Chlorella UMACC 234
C. vulgaris (FM 205855)
C. vulgaris (AY 591515)
C. vulgaris (X13688)
C. vulgaris (AY 948419)
C.vulgaris (AB191207)
Parachlorella kessleri (AB 080309)
Closteriopsis acicularis (Y17470)
Parachlorella kessleri (X56105)
Parachlorella beyerinckii (AY 323841)
C. prototheco ides (AJ439399)
C. ellipsoidea (X63520)
Pyramimonas disomata (AB017121)
Mantonielle suqamata (X73999)
100
100
100
95
95
93
92
100
95
70
79
88
100

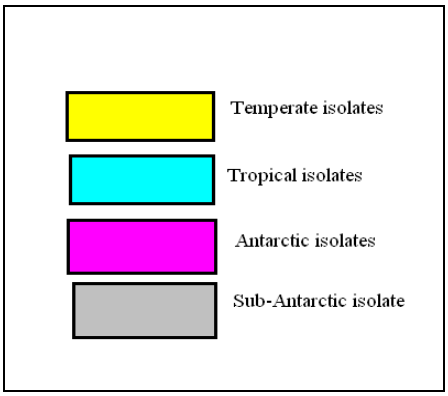


Figure 4.23 Maximum Parsimony tree based on 18S rDNA gene sequences. The numbers at the branches indicate bootstrap values obtained by MP (1000 replicates) larger than >70%.

CHAPTER 5

DISCUSSION

5.1 Morphological Characterization

In this study, morphological characterization of the *Chlorella* isolates was carried out using LM, SEM and TEM. Since the late 1950s, the advent of the electron microscope and the effective preparation techniques has led to new concepts in the study of phycology (Lee, 1980). Therefore, the ultrastructure of the isolates was identified using both SEM and TEM. The isolates from all the regions contained typical characteristics of the genus *Chlorella* although they were from different environments. Light micrographs revealed that the isolates contained single cells, spherical or ellipsoidal in shape with a thin cell wall. The cells also contained nucleus, nucleolus, mitochondria and single parietal cup-shaped chloroplast. *Chlorella* UMACC 001 and *Chlorella* UMACC 050 were identified as *C. vulgaris* because of their spherical shape, 5-10um in size and dark-green in colour. Such observations agree with the data reported by Agarwal and Manisha (2007).

Two types of cell wall, smooth and irregular network of subtle ribs were observed based on SEM identification. All the *Chlorella* isolates contained smooth cell wall except *Chlorella* UMACC 193 (tropical isolate) which has irregular network of subtle ribs. This is similar to the type of cell wall of *Chlorella* reported by Huss *et al*. (2002).

The sample processing for TEM was carried out according to the method by Huss *et al*. (2002) with some minor modifications. TEM revealed that all the isolates contained the typical structure of the genus *Chlorella*, characterized by nucleus,

nucleolus, mitochondrion, single parietal cup-shaped chloroplast with many pyrenoid, lipid droplets and thylakoids with granum and starch. A similar characteristic for the genus *Chlorella* was reported by Huss and Sogin (1990). It is further supported by Yu *et al*. (2007) who showed that *Chlorella* cells are spherical in shape, enclosed by thin cell wall with fine floss and contain many pyrenoids with 2-3 thylakoids. In this study, the reproduction mode of the isolates was by autosporulation. The autospores were spherical or ellipsoidal in shape, and contained parietal chloroplasts with pyrenoid in most species. Yamamoto *et al*. (2005) also reported that the autosporulation is a common mode of propagation for unicellular green algae and the daughter cells are formed in the wall of the mother cell. The autospores are important in the taxonomy of *Chlorella*. For instance, the coccoid green algae Heveochlorella hainangensis was described recently based on their both triangular and spherical autospores and is different from other Chlorella (Zhang *et al*., 2008)

Plastoglobules may be important for the adaptation of the Antarctic *Chlorella* to the extreme conditions. The TEM micrographs showed that both the Antarctic *Chlorella* isolates consisted of more plastoglobules then the other isolates. During the interannual changes, summer melt and long day length the Antarctic algae are exposed to various stress factors ranging from the extremely low temperature, high salinity to hyperoxic conditions (Rex *et al*., 2000). During this time, upregulation of several plastoglobules has been observed as a consequence of photooxidative stress such as cold and high light (Thomas and David, 2005).

5.2 Biochemical Characterization

5.2.1 Fatty Acid Composition

In general, SFA (14:0, 16:0, and 18:0), PUFA (16:2.16:3, 16:4, 18:2, 18:3) and MUFA (16:1, 18:1) were produced by all the *Chlorella* isolates. The present study showed that the fatty acid composition was similar to that of other reports. Yu *et al*. (2007) reported that the specific features of Chlorophyceae are high concentrations of C16 PUFA such as 16:2. 16:3 and 16:4 and C18 PUFA such as 18:2 and 18:3. Thus, it was confirmed that these *Chlorella* isolates were from the family Chlorophyceae. SFA were the dominant type of fatty acid in all the Chlorella isolates followed by PUFA and MUFA except for the marine isolate, *Chlorella* UMACC 245. It contained more PUFA followed by SFA and MUFA. Marine *Chlorella* is known to contain high amount of PUFA such as arachidonic and eicosapentaenoic acids (Shinichi et al. 1983; Hirata *et al*. 1985; Seto *et al*., 1984; Yongmanichai and Ward, 1991; Zhukova and Aizdaicher, 1995; Liu and Lin, 2001; Wacker *et al*., 2002; Rosa *et al*., 2005 and Petkov and Garcia, 2007).

The major fatty acids in the *Chlorella* isolates were 16:0, 18:0 and 18:3. This similar to that reported in a previous study by Teoh *et al*. (2004) who found that the Antarctic *Chlorella* strains also contained high amount of SFA with the dominance of 16:0 and 18:0. Even though several studies have been conducted using different *Chlorella* strains under different conditions, similar fatty acid composition was found (Miura *et al*., 1993). According to Yongmanichai and Ward (1991), *C. minutissima* also contained the similar type of fatty acids including 16:0, 18:0, 18:1, 18:3 and 20:5. The dominant fatty acid in this species was 18:3. Recently, similar fatty acid profile of another Chlorella was reported by Petkov and Garcia (2007).

According to Certik and Shimizu (1999), fatty acid composition offers valuable taxonomic and physiologic information as to its relationship with other organisms, which may not be available from morphologic characteristics alone. For instance, Yu *et al*. (2007) reported that the fatty acid profile is an taxonomic character for *Chlorella* at the genus level where Nannochloropsis (eustigmatophyte) was transferred to the genus Chlorella because it does not produce 20:5ẃ3. Fatty acid composition however may not be a reliable taxonomic marker to differentiate Chlorella at the species level (Shinichi *et al*., 1983; Zhukova and Aizdaicher, 1995; Vanderploeg *et al*., 1996 and Rosa *et al*., 2005).

5.2.2 Pigmentation

Six major pigments, namely lutein, chlorophyll a, chlorophyll b, ß -carotene, cis-neoxanthin and violaxanthin were identified in all the Chlorella isolates despite they come from different geographical regions. These six pigments were the characteristics of the genus *Chlorella.* This was supported by Bellinger (1992) who reported similar pigmentation in *Chlorella* species. Lutein is a common pigment found in Chlorophytes (Anderson *et al*., 1998).

In some studies, pigmentation is found to be useful as a taxonomic marker at the species level. For instance, Van Lenning *et al*., (2003) found that the pigmentation plays an important role as taxonomic marker to differentiate some of the species of Pavlovaceae despite their similarity in terms of phylogenetic relationships based on 18S rDNA and morphological characteristics.

5.3 Molecular Analysis of 18S rDNA

In this study, 18S rDNA from a total of 11 *Chlorella* isolates were sequenced for phylogenetic analysis. These data are useful especially with regards to tropical *Chlorella*. To date, there are only few references of 18S rDNA tropical *Chlorella* sequences in Gene Bank. In this study, DNA sequences were used to infer phylogenetic relationships of *Chlorella* isolates using NJ, MP and ML methods with PAUP*4.0b10 (Swofford, 2002). *Monostroma grevillei* (AF 015279) and *Ulva curvata* (AF 189078) was chosen as outgroups. In gene families, an appropriate outgroup share at least one conserved domain with the group of interest (Harrison and Langdale, 2006). According to Hilis *et al*. (1996) no method of phylogenetic reconstruction can be claimed to be better than others under all conditions because each has advantages and disadvantages, depending on the evolutionary process. Thus, all the three analysis, NJ, MP and ML were conducted.

Phylogenetic evidence on the basis of nuclear 18S sequence analysis confirmed that all the *Chlorella* isolates from different geographical regions formed a clade within the Trebouxiophyceae family in the MP tree as well as in the NJ tree. Molecular phylogenetic analyses have shown that *Chlorella* formed clades within the Trebouxiophyceae. Krienitz *et al*. (2004) also positioned *Chlorella* in the lineage of Trebouxiophyceae. The majority of nodes in the tree were supported by strong bootstrap values. The bootstrap values of the NJ and MP analysis were much stronger compared with ML. Statistical tests are important in testing the reliability of the obtained tree. Felsenstein's bootstrap test is one of the most commonly used tests (Felsenstein, 1985). Thus, we used Felsenstein's bootstrap test in this study.

All the seven tropical isolates were grouped together and closely related with each other. *Heveochlorella* (EF595524) isolated from rubber tree in the tropics (Zhang *et al*., 2008) was grouped together with these tropical isolates. The temperate and sub-Antarctic isolates also clustered together with the tropical isolates. Both the cold-inhabiting isolates from the Antarctic region were grouped together formed another clade. The marine isolate, *Chlorella* UMACC 245 clustered together with the freshwater and the Antarctic isolates even though they were from different habitats. Hence, this shows that the phylogenetic relationship does not correlate with the original habitats of the *Chlorella* isolates. According to Emden (2008), no clear geographical distribution was observed in *Hemiselmis amylosa* where the freshwater and marine strains from different geographical regions occurred in mixed clades throughout the phylogenetic tree.

In this study, all the *C. vulgaris* strains from Gen Bank clustered together in the same clade. The phylogenetic analysis seems to indicate that the species factor is more important than the geographical factor in determining the phylogenetic grouping. Recently, Liu *et al*. (2006) reported that 18S rDNA of the two *C. sorokiniana* strains from Gen Bank were clustered together in the same clade. This was further supported by Wu *et al*. (2001), where 18S rDNA revealed that all the *C. sorokiniana* isolates were clustered together.

The evidence from the molecular data suggested that the Antarctic isolates were closely related with the tropical isolates. There is a possibility that the Antarctic *Chlorella* might have been introduced into the Antarctic ecosystems by natural dispersal or by human activities. Broady *et al*. (1997) reported that some of the Antarctic '*Xanthonema*' strains are closely related to the European strains, where they might have

been introduced to the Antarctic ecosystem by human activity or natural dispersal. Evolutionary relationship of psychrophilic and psychrotolerant polar *Oscillatoria* were examined based on small subunit 16S rDNA sequences by Nadeau *et al*., (2001). Their findings support the hypothesis that Oscillatorians in both Polar Regions originated from temperate species. Furthermore, morphological designations of these filamentous cyanobacteria often do not have phylogenetic significance.

According to Vincent (2000), Antarctica has been isolated from the rest of the world since the separation from Gondwanaland and the formation of the Polar Front more than 10 million years ago and therefore there is a need to understand Antarctic microbial ecosystems before they are damaged by human activities. It is well known that the Antarctic microbial communities have been subjected to threats of major environmental disturbance and loss through human activities (Vincent, 1999) as well as by anthropogenic changes in the global environment. According to Marshall (1996a) the invasion process for Antarctic microbes were more varied than for larger biota. These transport processes can operate over long distances to allow gene flow from outside the Antarctic region and also provide pathways for the redistribution of microorganisms within Antarctica. However, there has been no detailed information on the microalgae.

The combined data based on morphological, biochemical, pigmentation and molecular analysis confirmed that the 11 isolates are true *Chlorella*. There were separated from the Parachlorella-clade. In terms of morphology, the *Parachlorella* consists of transparent electron density of cell wall, 54-59nm thickness of cell wall and late formation of daughter cell wall synthesis. In this study, all the *Chlorella* isolates do not have such features.

Krienitz *et al*. (1996) compared the morphology and nuclear encoded SSU rDNA of green algae and found them closely related. Currently it is accepted that *Chlorella* evolved independently in different evolutionary lineages of Chlorophyta, within the Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Prasinophyceae (Friedl, 1997; Chapman *et al*., 1998). For instance, in this study, *P.beijerinck, P.kessleri, Closteriopsis acicularis* and *Discloster acuatus* which were identified and named as *Parachlorella* by previous studies formed another clade called *Parachlorella*-clade. According to Huss *et al*. (1999) four true *Chlorella* species are *C. vulgaris, C. lobophora, C. sorokiniana* belong to the *Chlorell*a-clade and *C. kessleri* is closely related with *C. vulgaris* (Huss and Sogin, 1990). Later it was transferred from *C. kessleri* to the *Parachlorella*-clade (Krienitz *et al*., 2004). High bootstrap value support was found for the monophyletic origin of the newly described *P. beijerinckii* and *C. kessleri*. Therefore, new combination of *P. kessleri* was proposed. In *Parachlorella*-clade, *Dicloster* and *Closteriopsis* cluster together. It's further supported by Hegewald and Hanagata (2000) and Ustinova e*t al*. (2001) who found a close relationship of both species with *P.kessleri*. *D. acuatus* was formerly a member of the *Scenedesmaceae* and *C. acicularis* was a member of the *Selenastraceae* (Komarek and Fott, 1983). Both were transferred to the genus *Parachlorella* because of a relatively high number of sequence differences in the 18S rRNA gene and the ITS region.

A novel unicellular *Chlorella*-like green alga was isolated from the rubber tree was analyzed using electron microscopy and phylogenetic analysis inferred using both chloroplast-encoded 16S and nuclear-encoded 18S rDNA (Zhang *et al*., 2008). The combination of the morphological and phylogenetic analysis identified the species *C.*

luteoviridis and *C. saccharophila*. Therefore, they named a new genus as *Heveochlorella*. Recently, Hoshina and Imamura (2008), conducted phylogenetic analysis of most widely studied symbiotic Paramecian algal strain, *Chlorella sp*. and *C. vulgaris* using large subunit ribosomal RNA genes (LSU rDNA). Even though LSU rDNA is very advanced in phylogenetic studies, no sequence data from Trebouxiophyceae have been published except *C. ellipsoidea* Gerneck. Most of the substitutions (65%) were encountered in the first 800bp of the alignment and it is 8 times faster then the complete SSU rDNA, making it good candidate for a phylogenetic marker and giving a resolution level intermediate between SSU rDNA and ITS.

From the analysis, the classical and molecular biological approaches do not readily corroborate each other and the systematics of widely distributed Chlorella remains enigmatic. According to Huss *et al*. (2002), the availability of increasing information on phylogenetic relationship among coccoid green algae has given rise to the difficulties faced when a traditional system of classification is compared with molecular data. According to Kaczmarska *et al*. (2005), molecularly derived trees provide no special insights into morphological evolution unless morphology is examined in a theoretically rigorous framework. Other than that, strain variation and distribution should be examined using a molecular metric, other than small subunit ribosomal gene sequence data which would provide greater phylogenetic resolution among closely related species (Nadeau *et al*. 2001).

5.4 Areas for Further Studies

In this study, more tropical isolates were included in the phylogenetic analysis. Only one temperate isolate (*Chlorella* UMACC 251) was sequenced. However, many

sequences of temperate strains from Gen Bank were included in tree analysis. Only one sub-Antarctic and two Antarctic *Chlorella* isolates were included in this study. Data from more isolates are required in order to unravel the relationship amongst *Chlorella* from the different geographical regions.

This study should also be expanded by including more *Chlorella* isolates from the Arctic region to examine the relationships of *Chlorella* from North Pole to South Pole. This will give further insights into the evolutionary relationships of *Chlorella* isolates from different latitude regions.

The question of the ancestry of *Chlorella* cannot be regarded as being completely solved by 18S rDNA gene. Even though SSU DNA has been extensively studied in large number of organisms, covering most eukaryotic organism and large number of sequences has been published, but then, incongruent phylogenetic relationships between SSU rDNA and some other genes have been reported suggesting that depending on SSU rDNA sequences alone are not sufficient. This is because 18S rDNA is a highly conserved gene.

In this study, only one gene marker, 18S rDNA was used for the phylogenetic analysis. A combined analysis using more gene markers such as Rubisco and ITS has to be carried out to resolve the phylogenetic relationship of *Chlorella* isolates from different regions. Nevertheless, studies on other aspects such as morphology, biochemical and physiology (nutritional mode) characterization need to be done for species identification.

Chlorella is one of the cosmopolitan algae that can be found in every part of the world. It is really worth-while to further pursue this study in this direction to contribute to the understanding of biodiversity and systematics of *Chlorella*.

Therefore, more isolates from all the regions especially from the temperate and sub-Antarctic regions should be included in this study. Further detail analysis of morphological characterization using TEM need to be done for the species identification. Instead of using only one marker for molecular analysis, few highly variable molecular markers should be included in future to anticipate a clear understanding of geographical distribution of *Chlorella* isolates from various regions.

CHAPTER 6

CONCLUSION

This study has contributed 11 new 18S rDNA sequence data from *Chlorella* isolates to Gen Bank. The data from the Antarctic, sub-Antarctic and tropical *Chlorella* is particularly valuable as such information is still lacking in the database. The molecular analysis has given some information on the relationship of *Chlorella* isolates from different latitudes. In comparison, morphological and biochemical (fatty acid profiles and pigmentation) features were not able to distinguish the *Chlorella* isolates. Based on the findings from the study, the following conclusions can be made:

1) The morphological characters strongly indicate that the 11 isolates are from the genus *Chlorella*. The isolates from all the regions contained typical characteristics of the genus *Chlorella* although they were from different environment.

2) The similar fatty acid composition also indicates that the 11 isolates belong to the genus *Chlorella.*

3) The pigmentation of the 11 isolates of *Chlorella* was characterized by chlorophyll *a*, chlorophyll *b*, lutein, ß-carotene, cis-neoxanthin and violaxanthin.

4) There were no marked differences in terms of fatty acid profiles indicating that the 11 isolates belong to the genus *Chlorella.*

5) Molecular analysis of 18S rDNA based on MP, ML and NJ methods revealed that all the isolates clustered in the *Chlorella*-clade. It also showed that the tropical isolates are closely related with the Antarctic isolates.

In conclusion, the following hypotheses were accepted:

Ho: Morphological features, fatty acid profiles and pigmentation cannot be used to differentiate the *Chlorella* isolates from different regions.

H_1: *Chlorella* isolates from the same latitudinal regions are closely related in terms of phylogeny based on 18S rDNA.