

ISOLATION AND CHARACTERIZATION OF CAROTENOID PRODUCING
MICROALGAE FROM KWAZULU-NATAL (SOUTH AFRICA)

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DECLARATION

I, Ayodeji Emmanuel Adedoyin, declare that

1. The research reported in this thesis, except where otherwise indicated, is my own and has been generated by me as the result of my own original research.
2. This thesis has not been submitted for any degree or examination at any other university.
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ABSTRACT

Natural carotenoid pigments from green microalgae are known to exhibit beneficial effects in the treatment of cardiovascular diseases, high blood pressure, gastrointestinal discomfort, and even specific types of cancer. This is mostly due to their antioxidant capacity, which is able to neutralize reactive radical species involved in such diseases. Green algae such as *Parachlorella* spp., *Haematococcus* spp., and *Scenedesmus* spp. are known producers of such carotenoids, among which astaxanthin is one of the best known. Therefore, the isolation and characterization of carotenoid producing microalgae was conducted using environmental samples collected in Pietermaritzburg, KwaZulu-Natal, as inocula. Algal isolates were obtained using three different media under strictly photoautotrophic conditions. Three selected purified axenic isolates were analyzed via light and electron microscopy, and the 18S rRNA gene sequence for two of these isolates was analyzed as they show lesser morphological features enabling taxonomic assignment. In addition, growth rates of isolates were determined under photoautotrophic and photoheterotrophic conditions. Moreover, the production of pigments was analyzed using thin layer chromatography (TLC), UV-VIS spectrometry and antioxidant capacity measurements using the diphenylpicrylhydrazyl (DPPH) organic radical based assay. Based on the cell morphology and cytological structures, the three isolates were provisionally identified as *Parachlorella* sp., *Scenedesmus* sp. and *Haematococcus* sp. The analysis of the 18S rRNA sequence confirmed that isolate AA2 belongs to the genus *Scenedesmus* while for isolate AA1 the best match was *Parachlorella*. Light and electron microscopy indicated pigment accumulation in the three strains. Using acetone extracts and TLC analysis, in addition to chlorophyll a, four major carotenoids were detected from the *Haematococcus* sp. cells namely; astaxanthin, lutein, echinenone and β -carotene. Two major carotenoids were identified for *Scenedesmus* sp. (hydroxyechinenone and β -carotene), while only β -carotene was identified in *Parachlorella* sp. The R_f values and spectral properties matched those reported in the literature for the identified carotenoids. The DPPH assay showed that methanol extracts of all three isolates as well as individual carotenoids isolated via preparative TLC, were able to reduce the organic radical DPPH, thereby confirming the antioxidant activity of carotenoids present in these isolates. To establish whether sufficient cell numbers can be generated under photoautotrophic and photoheterotrophic conditions, growth kinetics were established for all three isolates in batch experiments. *Parachlorella* sp strain AA1 showed the highest growth

rate of 0.020 h^{-1} with a generation time of about 35 h when grown photoheterotrophically with acetate (25 mM), followed by *Scenedesmus* sp. at a growth rate of 0.019 h^{-1} and a generation time of 36 h, while the same applied to *Haematococcus* sp. (growth rate = 0.016 h^{-1} and generation time = 43 h) with glucose (50mM). However, for the production of the carotenoid astaxanthin by *Haematococcus* sp. photoautotrophic conditions were better.

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CHAPTER 1

Literature Review

1.1. Introduction

Algae are a major group of eukaryotes present in almost every habitat on our planet (Schlichting, 1974; Weiss, 1983). Most algae are capable of performing oxygenic photosynthesis as a means to generate biomass. However, many of these can utilize simple organic acids or sugars in addition to carbon dioxide as their source of carbon and energy in the presence or absence of light. The dimensions of algae range from 1µm in the case of the microscopic picoeukaryote *Micromonas pusilla* to 50m in the case of *Macrocystis pyrifera* (Joint et al., 1986). Microscopic algae are single-celled microorganisms that occur as discrete individual cells alone, some in pairs, in small groups or in sheets of individuals that have the same resemblance. The most abundant green microalgae present in the phytoplankton freshwater are single-celled drifters such as *Chlorella* spp., *Euglena* spp., and *Scenedesmus* spp. (Fitzgerald, 1964; Andersen, 2005). Microalgae are highly diverse as they may be irregular, non-motile or motile by flagella, they might undergo complex lifecycles, may be naked, have a cell wall, or a cell covered with silica or proteinaceous plates.

Pulz and Gross (2004) stated that microalgae represent a distinct group of microorganisms of great ecological benefits, the spread of which is enormous since they live in all major natural habitats, from cold, arctic regions, through highly alkaline or saline habitats, to hot springs and arid soils. Microalgae play a significant role in the global primary biomass production and form the source of the food chain in the aquatic environment (Sunrek, 2008). Microalgae are naturally divided into five classes based on the different photosynthetic assimilatory pigments (*Chlorophyceae*, *Euglenophyceae*, *Xanthophyceae*, *Bacillariophyceae*, *Rhodophyceae*) and were also additionally categorized by their developmental life cycle, and cell structure. However, many species are highly variable in their physiology and morphology, which can make this differentiation difficult and undependable (Krienitz, 2003; Sunrek, 2008). Currently, algae consist of nine eukaryotic

phyla, the *Rhodophyta*, *Cryptophyta*, *Heterokontophyta*, *Haptophyta*, *Dinophyta*, *Apicomplexa*, *Chlorophyta*, *Euglenophyta*, and *Chlorarachniophyta* (Croft et al., 2006; Leliaert et al., 2012). The most ubiquitous algae found in natural environments are the diatoms, golden-algae (*Heterokontophyta*), and green-algae (*Chlorophyta*) (Long, 1980; Luo et al., 2011; Krienitz and Bock, 2012). Microalgae are an incredibly distinct group of organisms (Croft et al, 2006). Although between 200 000–800 000 species are believed to exist, so far only limited numbers are kept in collections and a handful are cultivated on a large commercial scale (Parmar et al, 2011).

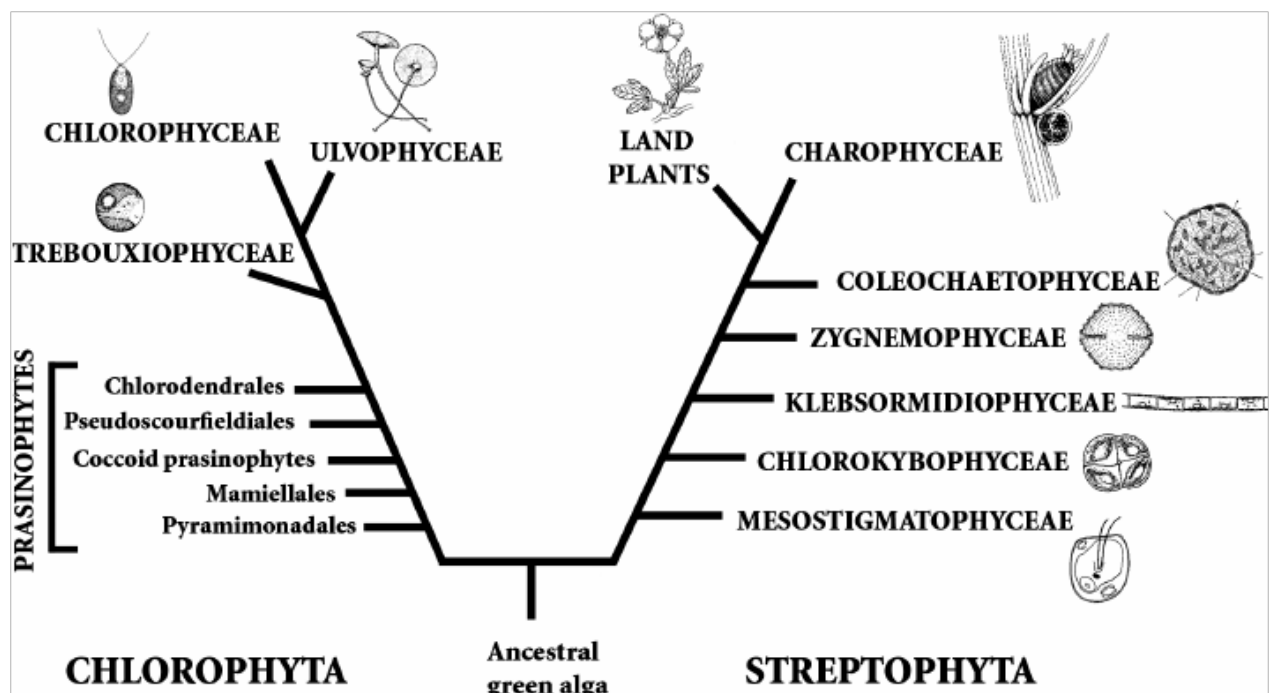


Figure 1.1 Phylogenetic overview of the green-algae (left) and other phylogenetically related eukaryotes (right). Source: Rindi et al. (2011).

Microalgae play an important ecological role in global chemical cycles. They contribute to the global CO₂ fixation due to their high photosynthetic conversion efficiency and ability to thrive in diverse ecosystems (Zeng et al., 2011). Their bioactive compounds and extracellular polymers for example can affect soil particle adherence, plant growth and water storage (Pulz and Gross, 2004). They have carbon concentrating mechanisms that suppress photorespiration and increase biomass production rates. It was long believed that microalgae only required CO₂, light, and inorganic nutrients to enable high growth rates. However, many algae species have lineages found to be dependent on vitamins for growth. Interestingly, those microalgae dependent on external sources of vitamins frequently form mutual

relationships with bacteria capable of synthesizing B-type vitamins (Croft et al, 2006). The green algae consist of more than 7000 different species growing in a variety of habitats and are especially abundant in fresh water. Most green algae are unicellular and microscopic, forming the slimy green scum found in freshwater environments especially in the case of *Chlorella* spp (*Parachlorella*). Others are bigger and more complex, forming spherical colonies composed of many cells as in the case of *Volvox* spp. (Elliot, 1934; Kim et al. 2015). Green algae cells contain high-value molecules that are of interest in biotechnology, such as lipids, proteins, carbohydrates and numerous pigments (Mendes et al., 2003; Batista et al., 2013). These pigments can help to improve gastrointestinal health and protect against free radical damage to preserve immune-system defences. For instance, carotenoid pigments such as astaxanthin serve as a therapeutic agent against atherosclerotic cardiovascular diseases and inhibit the growth of fibrosarcoma cancer cells. This is mostly due to their super antioxidant capacity to scavenge reactive radical species involved in such diseases (Cuellar-Bermudez et al. 2015; Shah et al. 2016). The high-protein content of some green-algal species is one of the major reasons why they are considered as a non-conventional source of proteins. Members of the genus *Chlorella* and *Scenedesmus* are known to contain between 50-60 percent crude protein while *Haematococcus* spp. contains up to 60 percent crude protein (Kofrany and Jekat, 1967; Soeder and Binsack, 1978). For example, *Chlorella* spp. was early on commercialised as a health food in Japan, Taiwan and Mexico (Garcia Sanchez et al., 2003; Borowitzka, 2013). Carotenoid pigments such as astaxanthin, echinenone, lutein, canthaxanthin, β -carotene and lycopene are synthesized by green algae (Ambati et al., 2014). Of all the pigments, astaxanthin has the widest range of applications in nutraceuticals, pharmaceuticals, cosmetics, chemicals and aquaculture due to its strong antioxidant activity and pigmentation (Borowitzka, 2013; Perez-Lopez et al., 2014).

The freshwater green algae *Haematococcus pluvialis* and *Chlorella zofingiensis* are generally beneficial as a natural source of astaxanthin, *Chlorella vulgaris* as a supplementary food byproduct and the algal genera *Dunaliella* and *Scenedesmus* as a source of β -carotenoids. The nutritional value of proteins from these green-algae compares favourably to egg, soy, and wheat protein as well as to WHO/FAO protein requirements (Becker, 2007; Chacon-Lee et al., 2010).

1.2. *Haematococcus* spp.

The freshwater unicellular biflagellate green-algae *Haematococcus pluvialis* belonging to the class *Chlorophyceae*, order *Volvocales* and the family *Haematococcaceae* (Elliot, 1934; Eom et al., 2006). *Haematococcus pluvialis* is ubiquitous in fresh natural or artificial water environment and naturally present in many habitats globally (Genitsaris et al., 2016). Knowledge of the unique developmental life cycle and morphology of *Haematococcus pluvialis* has become increasingly important due to the interest in this organism as a biotechnological natural source of cellular astaxanthin. The developmental life cycle of *Haematococcus pluvialis* is divided into four types of unique and distinguishable stages, these are motile zoospores (macrozooids), an immature cyst (microzooids), the resting (palmella) stage, and an immotile spore (aplanosporic) stage (Elliot, 1934; Klochkova et al. 2013). The macrozooids of *Haematococcus pluvialis* are spherical, oval shaped, or pear-shaped cells, biflagellate with a cup-shaped chloroplast. Due to the swift developmental stages and cell division leading to 2-8 daughter cells, zoospores are mostly common in the early vegetative biflagellate developmental stage of a batch experimental culture under favourable cultivation conditions. When the zoospores are subjected to unfavourable or adverse cultivation conditions, they change into an immotile 'palmella' form by losing their two swimming flagella while the cell size increases (Triki et al., 1997). This stage is referred to as encystment. As the adverse condition persist, the so called palmella develops into the immotile asexual aplanospore with rigid and thick cell walls. At the maturation of the aplanospore, a high quantity of the carotenoid astaxanthin accumulate, resulting into intense reddish colour of the cells while the green pigmented vegetative stage contains mostly lutein (75-80%) and 10-20% of β -carotene, violaxanthin, neoxanthin and zeaxanthin (Harker et al., 1996). It has been reported that the flagellate stage of some *Haematococcus pluvialis* strains are competent of accumulating natural astaxanthin and related pigments without the formation of aplanospores (Hagen et al., 2002; Brinda et al., 2004). When the adverse cultivation conditions become favourable, the aplanosporic cells will develop into active swimming zoospores with two flagella and begin a new vegetative developmental growth stage, however aplanospores can alternatively divide into aplanospores as well.

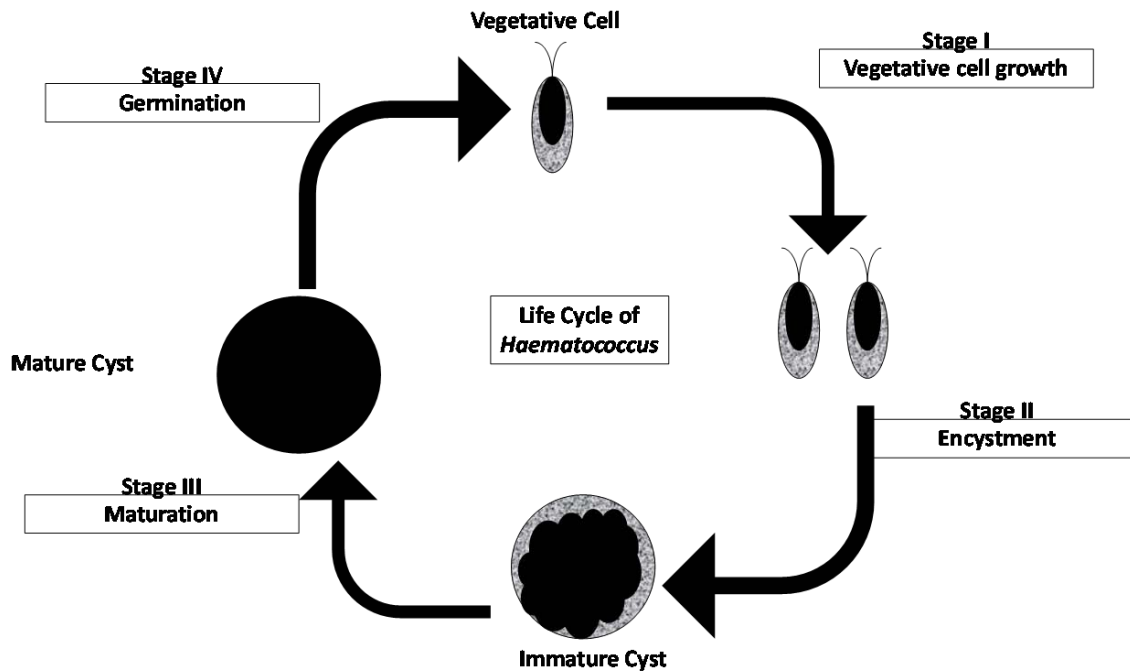


Figure 1.2 Schematic diagram of the morphological changes in the life cycle of *Haematococcus pluvialis*.

1.3. *Chlorella* spp.

Higher plants cannot supply all animal nutritional requirements; some of them contain anti-nutritive or toxic components that make them unsuitable for the aquaculture industry. The use of microalgae could therefore be a solution. One of the early ideas for the use of microalgae was their introduction into human or animal food as a protein source. The protein content of microalgae is very high compared to higher plants (Guerin et al., 2003). It is known that *Chlorella* spp. due to the presence of carotenoids possesses immunomodulating activity and can act as a preventive agent against several tumours, can decrease systematic high blood pressure or high serum cholesterol levels (Baker, 2002).

Chlorella beijerinck, a member of the genus *Chlorella*, is one of the best known microalgae species worldwide. Notwithstanding, members of the genus generally lack morphological characters, more than 100 *Chlorella* species have been named since the description of the type species *Chlorella vulgaris* by Beijerinck in 1890. Their habitats vary from fresh water, marine to edaphic habitats (Luo, et al., 2010; Khaybullina et al., 2010). Pyrenoids, starch grains, and a nucleus can readily be seen in some species, with the absence of flagella. *Chlorella*, a broad oval green microscopical alga belongs to the large group of

algae in the order *Chlorellaceae*. However, recent approaches (Bock et al., 2011), have reclassified *Chlorella* based on ultrastructure of the cell wall, (Atkinson et al., 1972; Yamada and Sakaguchi, 1982), pyrenoid ultrastructure (Ikeda and Takeda, 1995; Nemkova and Kalina, 2000), chemical composition of cell walls, biochemical and physiological characters, (Kessler and Huss, 1992; Hegewald and Kneifel, 1982), sequence analyses of the 18S rRNA gene (Huss et al., 1989; Huss and Sogin, 1990), and numerical classification strategies (DaSilva and Gyllenberg, 1972). True *Chlorella* species according to Huss et al. (1999), have to be divided as follows: *Chlorella vulgaris*, *Chlorella lobophora* and *Chlorella sorokiniana* belong to the *Chlorella*-clade, whereas *Chlorella kessleri* (now *Parachlorella kessleri*) belongs to the *Parachlorella*-clade. The distinction of the new genus *Parachlorella* from the true *Chlorella* species is based on sequence analysis of the 18S rRNA gene. However, more recent work has reclassified *Chlorella* species on the basis of biochemical and molecular data, the genus currently consists of five “true” *Chlorella* species: *Chlorella vulgaris*, *C. lobophora andrejeva*, *C. sorokiniana shihira* et Krauss, *C. heliozoae* Pröschold et Darienko and *C. variabilis shihira* et Krauss (Krienitz et al. 2004; Pröschold et al. 2011).

Chlorella zofingiensis, current standing as *C. variabilis shihira* et Krauss, was grouped within the *Chlorophyceae* (Hegewald and Kneifel, 1982), it is an aerobic heterotroph that utilizes mannose and glucose and is also able to accumulate copious quantities of carotenoids. It has a rigid cell wall, whereas other species such as *Chlorella vulgaris* have a glucosamine containing cell wall without the ability to accumulate secondary carotenoids, similarly they possess a double thylakoid bisecting the pyrenoid matrix (Takeda, 1991; Ikeda, 1995). Under adverse conditions such as high light irradiance, nitrogen deprivation, canthaxanthin and astaxanthin are produced in their free form or as monoesters and diesters with concomitant degradation of chloroplast membranes (Rise et al., 1994; Bar et al., 1995; Del Campo et al., 2004). Strains of *Chlorella zofingiensis* have been cultivated photoautotrophically, mixotrophically and photoheterotrophically, with highest carotenoid concentrations per biomass of $0.72\text{g} \times \text{L}^{-1}$ and $15\text{mg} \times \text{L}^{-1}$ under photoautotrophic conditions, which makes it a promising potent producer of natural astaxanthin (Del campo et al., 2007). However, *Haematococcus pluvialis* has the highest intracellular astaxanthin content (Zhang et al., 1997; Oncel et al. 2011). *Chlorella zofingiensis* accumulates over 80% of astaxanthin during the late exponential phase and up to 20% of lutein, β -carotene, canthaxanthin and violaxanthin (Del Campo et al., 2004). *Chlorella vulgaris* and *Chlorella protothecoides* synthesize primary carotenoids such as lutein. However, they are mostly selected by

researchers for lipid production due to their high lipid content and rates of growth (Singh and Gu, 2010).

1.4. *Scenedesmus* spp.

The genus *Scenedesmus* (*Chlorophyceae*) is ubiquitous in fresh water environments in the temperate world (Vincent 1980; Trainor 1963; Keller *et al.*, 2008). About 1300 *Scenedesmus* taxonomies have been described using morphological characters (Hegewald *et al.*, 1994; Hegewald and Silva, 1988), and only between 13-30 species are recognised (Egan and Trainor, 1990).

Trainor (1998) is of the opinion that a single-celled isolate of *Scenedesmus* spp. could show a variety of morphology that spans the characteristic morphology of two genera. It was therefore the understanding of phenotypic plasticity in *Scenedesmus* spp. that led to the assumption that only about 30 species occur in the genus, in comparison to the over 300 stated. It was the revision of this class- the *Scenedesmaceae*- that brings about the suggestion that morphologically differentiated subgenera should be upgraded to genetic status. As a result, the genus *Desmodesmus* was separated from *Scenedesmus* by An *et al.* (1999). According to Hegewald *et al.* (2010), 13 species of the genus *Scenedesmus* are presently phylogenetically and morphologically well defined. *Scenedesmus* spp. is a common part of the phytoplankton in nutrient rich waters (Krienitz and Bock, 2012). Its ability to grow in eutrophic media makes it desirable for use in high rate oxidation ponds for wastewater treatment to remove nitrogen and phosphorus for the production of algal biomass (Nair *et al.*, 1981; Fingerhut *et al.*, 1984).

Scenedesmus spp. also serves as food source for herbivorous zooplankton like copepods (Boersma and Vijverberg, 1995). *Scenedesmus vacuolatus* accumulates secondary carotenoids such as β -carotene, canthaxanthin, lutein, echinenone, and adonirubin (Qin *et al.* 2008). Orosa *et al.* (2000) and Qin *et al.* (2008) reported a low concentration of astaxanthin at $0.041\text{mg} \times \text{L}^{-1}$ using a two stage culture, with astaxanthin accumulation recorded under induction conditions (incubation at 30°C with illumination at $180\mu\text{mol m}^{-2} \times \text{s}^{-1}$ for 48h). Lewis and Flechtner (2004) also reported that *Scenedesmus deserticola* changes color during early growth stages from olive to bright orange, then turns reddish during pigment accumulation signifying astaxanthin accumulation. The additionally produced lutein is part of the pigments recommended for the prevention of some specific types of cancer and retinal

degeneration (Del Campo, 2004). Maximal biomass and lutein productivities of $0.87\text{g} \times \text{L}^{-1}$ and $4.77\text{ mg} \times \text{L}^{-1} \text{ day}^{-1}$ were measured in *Scenedesmus almeriensis* under high irradiances of $1625\text{ }\mu\text{E m}^{-2} \times \text{s}^{-1}$ and an optimal growth temperature of 35°C . *Scenedesmus* biomass contains between 50-60% crude proteins (Kofranyi and Jekat, 1967; Muller-Wecker and Kofranyi, 1973). The systematic German studies on the nutritional value of *Scenedesmus* spp. revealed that the biological value, digestibility and wholesomeness of green microalgae depend on processing technology (Kraut et al., 1966; Pabst, 1975). Soeder et al (1970) stated that the protein content in *Scenedesmus acutus* is higher than that of fruits and vegetables. Lipids are also part of algal nutritional value and so are many pigments (Deventer and Heckman, 1996; Borowitzka, 2013).

The size of *Scenedesmus* spp. ranges from 2-20 μm , with a smooth, non-ornamented cell wall (Hegewald, 2000). This wall is layered with cellulose, sporopollenin, and mucilage (Hegewald, 1997). This size makes them consumable for protozoa, fish, bivalves, rotifers, and copepods (Boing et al, 1998). Since the experiments of Egan and Trainor (1989) and Hegewald (1997), *Scenedesmus* spp. is known to reproduce by asexual reproduction. This involves the production of autospores by means of two successive, non-vegetative cell divisions, each *Scenedesmus* spp. cell is capable of producing either a single colony or a new 4-celled colonies.

1.5 Algal cultivation systems

Laboratory scale batch cultivation systems have been implemented for the cultivation of microalgae since the 1940s. Naturally, they are designed to suit a typical strain, product or purpose. Essentially, two methods of large scale cultivation of microalgae for the benefit of higher biomass productivity exist: firstly, by culturing in a large uncontrolled open reservoir (ponds and raceways) while the second stage involves the use of controlled closed vessels i.e. fermentors or photobioreactors (Pulz, 2001). Microalgae are cultured in properly illuminated environments naturally or artificially, and they use CO_2 as a carbon source. Additional chemicals are required for the large scale cultivation of microalgae, such chemicals help either in regulating biosynthetic pathways, inducing oxidative stress responses, microalgal metabolism or as metabolic precursors. Yu et al. (2015) recommended the use of propyl gallate and butylated hydroxyanisole as enhancers on the large scale cultivation of microalgae due to low cost of purchase. However, to increase the productivity of the carotenoid astaxanthin, the report of Gao et al (2015) indicated that jasmonic acid (JA), salicylic acid

(SA), gibberellic acid (GA₃) and 2, 4 epibrassinolide (EBR) stimulated astaxanthin productivity to $1.458\text{mg} \times \text{L}^{-1}$, $2.74\text{mg} \times \text{L}^{-1}$, $2.39\text{mg} \times \text{L}^{-1}$, $2.26\text{mg} \times \text{L}^{-1}$, respectively, depending on the concentrations used. Biofouling and biofilms of microalgae that are often seen as obstacles for suspended culture have recently been examined as cultivation methods for commercial-scale microalgal biomass production (Bendy and Sureshkumar, 2017). Interestingly, diverse microalgae also produced valuable fine chemicals such as propylene glycol, poly-3-hydroxy butyrate, glycerol-tert-butyl ether, and the omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) (Leu and Boussiba, 2014). Autotrophic cultivation takes more time, with low biomass production because it uses sunlight as the energy source and carbondioxide as the only source of carbon to form chemical energy via photosynthesis. Under autotrophic cultivation, *Chlorella* spp. gave the highest lipid yield of $54\text{mg} \times \text{L}^{-1}\text{d}^{-1}$ compared to $23\text{mg} \times \text{L}^{-1} \times \text{d}^{-1}$ in *Scenedesmus* spp. (Chiu et al., 2009).

1.5.1 Heterotrophic and photoheterotrophic cultivation

Some selected microalgae can use organic carbon compounds as both energy and carbon source in the nonexistence of light illumination, this condition is referred to as heterotrophic cultivation (Chojnacka and Marquez-Rocha, 2004). The heterotrophic growth of microalgae was reported to record high cell biomass on a commercial scale (Chen and John, 1994) and can increase the saturation of storage lipids and associated natural pigments in comparison to autotrophic conditions (Perez-Garcia et al., 2011). Photoheterotrophic conditions are described by the presence of two carbon and energy sources (CO₂ and another reduced carbon compound), and light. Simultaneous assimilation of organic carbon and CO₂ via photosynthesis was reported to result in higher growth yield and biomass production (Orus et al., 1991). Strains such as *Euglena* spp., *Haematococcus pluvialis*, and *Chlorella regularis* have been reported to grow photoheterotrophically (Martinez and Orus, 1991; Takeyama et al., 1997). The most generally used carbon source for the photoheterotrophic and heterotrophic culture of microalgae is glucose. Respiration and increased rates of growth are established with glucose compared to other substrate such as fructose, acetate, organic acids and other monohydric alcohols. This is mainly because glucose has higher energy content per mol than other carbon sources (Boyle and Morgan, 2009). Glucose enhances changes in *Chlorella vulgaris* physiology, which firmly affect the metabolic pathways of carbon assimilation, the cell volume, size of the cells, as well as size of storage materials such as carotenoids, chlorophyll, lipids, RNA, proteins, starch and vitamins (Martinez et al., 1991). In

some open pond systems, organic carbon sources such as glucose, acetate and fructose are supplied continuously in low dose. This is done to enhance higher microbial biomass, however, it might lead to excessive bacterial growth. Lee (2001) is of the opinion that organic carbon should be added during the day time hours, as the light would control fast growing bacteria. Below are some of the limitations to the use of heterotrophic and photoheterotrophic algal cultivation systems.

- Only a minimal number of microalgae species can develop under heterotrophic conditions.
- High energy demand and costs by supplying organic substrates.
- Competition and contamination with other microorganisms such as bacteria.
- Reduction of cell growth by surplus organic substrates.
- Incapability to produce light-induced metabolites in case of heterotrophic growth (Chen, 1996).

Nevertheless, many current studies indicate that heterotrophic and photoheterotrophic cultures are gaining increasing interest for producing various kinds of microalgae metabolites including pigments at various levels, from bench scale experiments to large commercial scale (Brennan and Owende, 2010). The use of new, better suited strains is required due to their ability of producing increased biomass and to accumulate pigments as well as lowering risk of contamination.

1.5.2 Closed systems

There are different types of closed control systems used for the cultivation of microalgae. The controlled closed system hinders contact between the immediate environment and the enclosed algae. For collection, pre and post harvesting, precautions measures must be put in place to reduce the interaction with the immediate environment. Fermentor-type reactors, flat-plate reactors, tubular systems, and even bags are used in the closed system cultivation of greenalgae (Borowitzka, 1999). Recently, biodomes and floating bags have been used (Carvalho et al., 2006). Closed control systems are generally known as photobioreactors (PBRs). They can be located outdoors, however in special situations, they are located inside greenhouses to accommodate more controlled environments at the value of increased running cost. Closed photobioreactors remain the best cultivation system for

Haematococcus spp. with haematochrome content at 1.5-3% of dry biomass at the aplanosporic stage (Olaizola, 2000; Guerin et al., 2003). However, of all the bioreactor types, biofilm photobioreactors (PBRs) have the highest light distribution rate and direct gas exchange between gas phase and biofilm (Podola et al., 2016). Generally, photobioreactors have the following benefits over open ponds:

- Hindered or reduced contamination thereby allowing the culturing of algal species that cannot be cultivated in open ponds.
- Provide better control over cultivation conditions (pH, temperature, nutrient supply).
- Stop evaporation and minimize the amount of used water.
- Reduced CO₂ losses due to outgassing.
- Achieve higher cell concentrations and, therefore, higher pigment per volume productivity.

Disadvantages of photobioreactors:

- Production is capital intensive. This is parts of the challenges hindering the use of photobioreactors for pigment production.
- Regardless of higher biomass concentration recorded and better control of cultivation conditions, the production cost and productivity in some enclosed photobioreactor systems are only slightly higher than those attainable in uncontrolled open-pond cultures.
- The technical challenge in sterilizing these photobioreactors has limited their uses for green-algae cultures for targeted high valued products such as chemical and pharmaceutical products.

1.5.2.1 Horizontal tubular bioreactors

Tubular bioreactors appear in 3 forms: (i) The simple airlift and bubble columns consist of vertical tubing that is transparent to allow light circulation: (ii) helical tubular bioreactors, consist of a flexible plastic tube coiled in a circular framework (Lee et al., 1995). (iii) In horizontal tubular reactors, exchange of gas takes place in the tube connection or through a dedicated gas exchange unit, and the angle towards sunlight is specifically adequate for efficient light harvesting. Such cultivation systems can control high volumes of culture

because they are not liable to easy contamination (Richmond et al., 2001). Gudín and Chaumont (1984) and Venkataraman et al. (1985), have been designing tubular bioreactor configurations as early as 1984. They reported a bioreactor system bearing a capacity of 7000L in total, in which a productivity of $36\text{g} \times \text{m}^{-2} \times \text{d}^{-1}$ of lipids was recorded in the cultivation of *Phorphyridium cruentum* (Chaumont et al., 1988). The major challenge to this bioreactor was its capital intensity, along with the essential fragility of its glass components. Richmond (1993) reported similar layouts of horizontal tubular reactors, which resulted in productivities as high as $1.5\text{g} \times \text{L}^{-1} \times \text{d}^{-1}$ of protein with *Spirulina platensis* and $0.32\text{g} \times \text{L}^{-1} \times \text{d}^{-1}$ with *Isochrysis galbana*. These controlled systems were based on an external unit designed for light circulation, connected to a gas exchange tower through an airlift pump. García et al (2009) reported that the highest yield of $0.7\text{g} \times \text{L}^{-1} \times \text{d}^{-1}$ of biomass was recorded when *Haematococcus pluvialis* was cultivated in a tubular photobioreactor with astaxanthin productivity of $8\text{mg} \times \text{L}^{-1} \times \text{d}^{-1}$. However, recent work by Park et al. (2014) recorded a much higher astaxanthin productivity of $602\text{mg} \times \text{L}^{-1} \times \text{d}^{-1}$ using a photobioreactor in a two stage perfusion cultivation method. The airlift tubular photobioreactor was reported to have the ability of stimulating astaxanthin pigments better than the bubble column, due to better liquid circulation ($460\text{mg} \times \text{L}^{-1}$ astaxanthin in the airlift and $390\text{mg} \times \text{L}^{-1}$ in the bubble column) (Poonkum et al., 2015).

1.5.2.2 Flat plate bioreactors

Flat plate bioreactors (FPR) are naturally built to make proper use of natural light such as sun, hence, narrow panels are mostly designed to achieve a high surface area to volume ratio. In the early 1980s, FPR reactors were costly and showed limitation in culture circulation (Pirt, 1983). A 500l FPR bioreactor was designed by Pulz (1992), in which the culture flow from an open gas exchange unit through several parallel panels placed in horizontal position. The cell flow rate increased at a high linear speed ($1.2\text{m} \times \text{s}^{-1}$). The greatest benefits of this system are its provision of a closed gas transfer unit, which has proven its efficiency in overcoming the problems of oxygen build up. Nevertheless, such a closed zone limits the effect of contamination (Richmond, 2001). Fermenter type (FPR) bioreactors are scarcely in use due to low area-to-volume ratio and poor sunlight harvesting capacity, however Pohl, et al.(1988) provided a solution to this problem by building a 250l FTR with stainless steel and internally illuminated by fluorescent lamps. With these parameters, axenic cultures can be maintained for long periods and used for pigment

production. Poonkum et al. (2015) recently reported the use of flat panel airlift photobioreactors (FP-APBRs) which enhanced astaxanthin production to 26.63mg/m³ (5.34% wet weight).

1.5.3 Open air system

Open ponds systems were the oldest configuration proposed for microalgae cultivation and are currently in use in large scale-commercial processes. Borowitzka (1992) stated several factors to be considered in using the open air system, some of these factors are strain types, land procurement, manpower, water, nutrient types, climatic conditions, energy source and the type of final product. There are four different kinds of open air systems available presently, the raceway ponds, the shallow big ponds, circular ponds, and tanks. There are two major benefits of culturing microalgae using the open pond cultivation system. Firstly, the open pond uncontrolled system is not difficult to design and requires a limited technical knowhow (Richmond, 1986). Secondly, an open pond is affordable and cheaper than closed bioreactors because closed bioreactors require a cooling system, proper monitoring and expensive materials. Although cheaper and easy to build, they are not commonly used because of factors such as light intensity, temperature conditions, evaporation, and protection from contamination are very difficult to maintain if intended to be used for supplement production, and therefore it has a limited use for pure cultures.

Kawaguchi (1980) and Belays (1997) suggested the use of a raceway system for the cultivation of *Chlorella* spp. to attain higher growth rates and to reduce the tendency of overgrowth by related microalgae. Open ponds also lack room for development as well as improvement compared to closed photobioreactor systems (Pulz, 2001). The only uncontrolled open air cultivation system which attained significantly sustainable cell densities is the cascade system designed by Masojidek and Prasil (2010), which is currently in use in Trebon, the Czech Republic for the culture of *Chlorella* spp. However, the largest algal cultivation systems currently in use are open ponds, due to their minimal capital and managing cost (Morweiser et al., 2010). In addition, open pond remains the conventional method used for pigment production for such as β -carotene, astaxanthin, lutein, violaxanthin from *Dunaliella* spp, *Chlorococcum* spp, *Chlorella* spp, *Haematococcus* spp, and *Scenedesmus* spp, with an estimated yield value of 1200t per year (Ben-Amotz, 2004).

1.5.3.1 Raceway pond

The raceway pond system is a unique type of an open system designed as a shallow closed loop channel that permits the free flow of water. Raceway ponds are not difficult to construct and, for this purpose, they are mostly used for the large scale cultivation of microalgae. They are generally constructed in compacted or cemented earth. Their sizes and shapes may differ, but for a bigger size area with several raceways, long stretched ponds with 180° curves on either ends are the most efficient and compactable. For circulation and mixing, a paddle wheel is usually in use. It operates uninterruptedly to prevent sedimentation (Venkataraman, 1985). The temperature condition in an open raceway varies within a diurnal cycle and the season thereby limiting its use to temperate regions. Temperature is very hard to manage. Cooling takes place by evaporation. Evaporative water loss can be important, causing an increase in salt concentration and other compounds (Burlew, 1953). However, raceway ponds allowed the induction of pigment production such as astaxanthin and its accumulation in short periods of time (Milledge, 2011). *Haematococcus pluvialis* was cultivated photoautotrophically in a raceway pond with optimum pigment production (Olaziola, 2000).

Table 1.1. Comparison of the properties of different large-scale algal culture systems (Borowitzka, 1999)

Reactor type	Mixing	Light utilisation efficiency	Temperature control	Gas transfer	Hydrodynamic stress on algae	Species control	Sterility	Scale-up	Reference
Unstirred shallow ponds	Very poor	Poor	None	Poor	Very low	Difficult	None	Very difficult	Borowitzka and Borowitzka, 1989
Tanks	Poor	Very poor	None	Poor	Very low	Difficult	None	Very difficult	Fox, 1983
Circular stirred ponds	Fair	Fair-good	None	Poor	Low	Difficult	None	Very difficult	Tamiya, 1957; Stengel, 1970; Soeder, 1981
Paddle-wheel Raceway Ponds	Fair-good	Fair-good	None	Poor	Low	Difficult	None	Very difficult	Weissman and Goebel, 1987; Oswald, 1988
Stirred Tank reactor (internal or external lighting)	Largely uniform	Fair-good	Excellent	Low-high	High	Easy	Easily achievable	Difficult	Pohl et al., 1988
Air-Lift reactor	Generally uniform	Good	Excellent	High	Low	Easy	Easily achievable	Achievable	Jüttner, 1977
Bag Culture	Variable	Fair-good	Good (indoors)	Low-high	Low	Easy	Easily achievable	Achievable	Baynes et al., 1979
Flat-Plate reactor	Uniform	Excellent	Excellent	High	Low-high	Easy	Achievable	Achievable	Hu et al., 1996; Tredici and Zitelli, 1997
Tubular reactor (Serpentine type)	Uniform	Excellent	Excellent	Low-high	Low-high	Easy	Achievable	Reasonable	Richmond et al., 1993; Torzillo, 1997
Tubular Reactor (Biocoil type)	Uniform	Excellent	Excellent	Low-high	Low-high	Easy	Achievable		

1.5.4. Harvesting and product formation

Proper harvesting remains part of the challenge in microalgae biomass production. For the commercial scale harvest of pigments, centrifugation is the most commonly used method. In a recent study, Shah et al. (2016) describe the use of floatation and disk-stack centrifugation achieving 95% biomass recovery. Bead milling and expeller pressing remains the most appropriate method for cell disruption of algae cells (Mendes-Pinto et al, 2001; Olaizola, 2000). In commercial pigment production, spray drying is considered as the most suitable method to dry algal biomass containing high value carotenoids such as astaxanthin, compared to solar and freeze drying. The recovery efficiency of dry biomass (powder) using this method exceeds 95% (Leach et al., 1998). The extraction of desired pigments from dried biomass can be done by dissolving the powder in solvents and oils. Microwave-assisted, enzyme-assisted approaches and increasingly supercritical carbon dioxide (SC-CO₂) extraction can also be used. Supercritical carbon dioxide (SC-CO₂) extraction provides shorter extraction time and reduces the use of toxic organic solvents, it is the most compatible and efficient method for pigment extraction (Guedes et al. 2011).

1.6. Beneficial carotenoids from microalgae

Many natural carotenoids are synthesized by microorganisms such as yeasts, bacteria, algae, fungi and even higher plants (Van den Berg, et al, 2000). Up to date, more than 600 carotenoids have been isolated and detected from natural habitats (Straub, 1987). Carotenoids are known for their potential to prevent serious human health disorders such as some specific types of cancer, heart disease, and macular degeneration, which are usually linked to their potential antioxidant scavenging capacity (Krinsky, 1989; Maoka et al., 2001). Xanthophylls for example have been shown to be the most effective free radical neutralizer and scavengers (Krinsky, 1994; Mortensen et al., 2001). Minhas et al. (2016) reported that lutein enhanced pigmentation in poultry and aquaculture, thereby qualifying lutein as one of the fastest growing natural carotenoids on the commercial market with a value worth of \$150 million per year in the USA (Borowitzka, 2013). In 2010, the poultry industry reported the use of \$233 million of lutein, however lutein productivity is estimated to grow up to \$309 million by 2018 but cannot still compete with \$1 billion market worth of astaxanthin which is projected to double before 2018. In addition, the carotenoid canthaxanthin also enhanced egg yolk pigmentation in poultry farming and is used as a feed additive. It is also widely used as a colorant in food industries and as a tanning agent in the cosmetic industry (Higuera-Ciapara,

2006). Canthaxanthin was reported by Vachali et al. (2012) to prevent UV-induced immune suppression in mice, being protective against skin cancer in experimental animals and to be of importance in the treatment of skin infections such as photodermatitis. The super antioxidant activities of natural carotenoids have been reported to reflect not only the rates of free radical neutralization and scavenging but also the reaction of the resultant natural carotenoid derived radicals. The natural carotenoid radical-cations have shown to be stabilised and presumed to be unreactive, they may further undergo bimolecular decay to produce non-radical products or, in the case of carotene radical-adducts, may destroy radical reactions by binding to the attacking free radicals thereby neutralizing the radicals (Krinsky, 1989; Everett et al., 1996). Astaxanthin for example has higher radical scavenging capacity than canthaxanthin, β -carotene, zeaxanthin, and lutein and 100 times more than α -tocopherol (Shah et al., 2016). Due to the super antioxidant capacity of astaxanthin, its use as supplement has been ascribed with potential properties against a considerable number of diseases. Li et al. (2015) demonstrated the use of astaxanthin as a safe treatment for autoimmune hepatitis by down regulation of c-Jun N-terminal Kinase (JNK/phosphorylated-JNK) mediated apoptosis and autophagy. Zeaxanthin and astaxanthin can inhibit the growth of breast, fibrosarcoma, prostate cancer cells and embryonic fibroblasts by suppressing the tumor growth and stimulating the immune response against the antigen (Palozza et al., 2009). These two carotenoids prevent skin thickening, collagen reduction and prevent ultraviolet induced skin damage (Rao et al., 2013). Astaxanthin supplementation inhibits oxidation of low density lipoprotein (LDL), it is carried by (very low density lipoprotein (VLDL), LDL and high density lipoprotein (HDL) in human blood and protects LDL-cholesterol against oxidation (Iwamoto et al., 2000). Carotenoids are known to be reactive towards singlet oxygen, with lycopene being the most effective quencher in vitro (Di Mascio et al., 1989; Di Mascio et al., 1991). β -Carotene is an important natural antioxidant compound and a precursor of vitamin A in food and feed products. Minhas et al. (2016) estimated the market worth of β -carotene to be \$261 million in 2010 and predicted it to grow up to \$334 million in 2018. Since hydrogen production also depends on photosynthesis like pigment production, as an alternative to improve the production of the bioproduct, stress induced by sulfur deprivation has been proposed and applied in a two stage process for hydrogen production under anaerobic conditions (Kari Skjanes et al., 2013; Chen et al., 2015). Batch experimental cultures are grown aerobically in the first growth phase, followed immediately by a second phase where the algae are denied of sulfur and produce hydrogen anaerobically (Melis et al., 2000). The algae can be collected from the growth phase via centrifugation and then

transferred to a different bioreactor for hydrogen production. *Chlorella* spp., *Scenedesmus* spp. and *Haematococcus* spp. are among the 30 genera of green-algae considered as most competent of producing hydrogen and related valuable products in the same process (Kari Skjanes et al., 2013). However, two newly isolated strains of *Chlorella vulgaris* have demonstrated the ability to produce hydrogen under atmospheric oxygen concentrations (Hwang et al., 2014). The use of anaerobiosis induction method, avoiding the competition for electrons from other pathways and increasing the sources of electrons, and increasing hydrogenase activity (or decreasing its oxygen sensitivity), are major steps that could be improved through genetic engineering for hydrogen production (Dubini and Ghirardi, 2014; Gimpel et al., 2015; Bajhaiya et al., 2016).

Table 1.2. Summary of potential high-value products from microalgae, alternative sources and their applications (Borowitzka, 2013).

Product	Potential or existing algae source	Some alternate source(s)	Applications	Selected references
Carotenoids				
β -carotene	<i>Dunaliella salina</i>	<i>Blakesleya trisporea</i> , synthetic	Pigmenter (food), pro-vitamin A, antioxidant	Borowitzka and Borowitzka 1989; Choudhari et al. 2008; Borowitzka 2010
Astaxanthin	<i>Haematococcus pluvialis</i> , <i>Chlorella zoofingensis</i>	<i>Xanthophyllomyces dendrorhous</i> , synthetic	Pigmenter (aquaculture), antioxidant	Cysewski and Lorenz 2004; Lemoine and Schoefs 2010; Rodriguez-Saiz et al. 2010; Schmidt et al. 2011
Canthaxanthin	<i>Chlorella</i> spp., other green algae	<i>Dietzia natronolimnaea</i> , synthetic	Pigmenter (aquaculture, poultry and food)	Arad et al. 1993; Hanagata 1999; Nasrabadi and Razavi 2010
Zeaxanthin	<i>Chlorella ellipsoidea</i> , <i>Dunaliella salina</i> (mutant)	<i>Paprika (Capsicum annuum)</i> ; <i>Tagetes erecta</i> , synthetic	Antioxidant, food pigmenter	Jim et al. 2003; Koo et al. 2012
Lutein	<i>Scenedesmus</i> spp., <i>Muriellopsis</i> sp., other green algae	<i>Tagetes</i> sp., <i>Blakesleya trisporea</i>	Antioxidant, poultry	Piccaglia et al. 1998; Blanco et al. 2007; Choudhari et al. 2008; Sánchez et al. 2008; Fernández-Sevilla et al. 2010
Phytoene, phytofluene	<i>Dunaliella</i> spp.	Tomato (<i>Solanum lycopersicum</i>)	Antioxidant, cosmetics	von Oppen-Bezalel and Shaish 2009
Echinenone	<i>Botryococcus braunii</i> , cyanobacteria	—	Antioxidant	Jäger et al. 2002; Matsuura et al. 2012
Fucoxanthin	<i>Phaeodactylum tricornutum</i>	Brown algae	Antioxidant	Kim et al. 2012

Product	Potential or existing algae source	Some alternate source(s)	Applications	Selected references
Phycobilins (phycocyanin, phycoerythrin, allophycocyanin)	Cyanobacteria, Rhodophyta, Cryptophyta, – Glaucophyta		Natural pigment (e.g. cosmetics and food products), fluorescent conjugates, antioxidant, etc.	Oi et al. 1982; Glazer and Streyer 1984; Arad et al. 1996; Eriksen 2008
Fatty acids				
Arachidonic acid	<i>Parietochloris incise</i>	<i>Mortierella</i> spp.	Nutritional supplement	Bigogno et al. 2002; Solovchenko et al. 2008; Streekstra 2010
Eicosapentaenoic acid	<i>Nannochloropsis</i> spp., <i>Phaeodactylum tricornutum</i> , <i>Monodus subterraneus</i> .	Fish oil	Nutritional supplement	Hu et al. 1997; Molina Grima et al. 1999; Sukenik 1999; Lu et al. 2001
Docosahexaenoic acid	<i>Cryptocodinium cohnii</i> , <i>Schizochytrium</i> spp., <i>Ulkenia</i> spp	Fish oil	Nutritional supplement	Barclay 1994; Mendes et al. 2009; Barclay et al. 2010; Wynn et al. 2010
Sterols	Many species	Various plants	Nutraceutical	Fabregas et al. 1997; Volkman 2003; Francavilla et al. 2010
Squalene	<i>Aurantiochytrium</i> spp.	Shark liver	Cosmetics	Kaya et al. 2011
Polyhydroxyalkonates	<i>Nostoc</i> spp, <i>Synechocystis</i> and other cyanobacteria	<i>Ralstonia</i> sp, GM <i>Escherichia coli</i>	Biodegradable plastics	Vincenzini and De Philippis 1999; Philip et al. 2007; Haase et al. 2012
Polysaccharides	<i>Porphyridium</i> spp., <i>Rhodella</i> spp., various cyanobacteria	Guar gum, xanthan	Thickeners, gelling agents etc., cosmeceuticals	De Philippis et al. 2001; Pereira et al. 2009; Arad and Levy-Ontman 2010
Mycosporine-like amino acids	Cyanobacteria, Dinophyta and other algal phyla		Sunscreens	Garcia-Pichel and Castenholz 1993; Llewellyn and Ains 2010

Astaxanthin (3,3'-dihydroxy- β,β' -carotene-4,4-dione) is one of the high-value carotenoids from microalgae that is achieving commercial success (Bajhaiya et al., 2016; Klochkova et al., 2013). Astaxanthin is ubiquitous in nature, particularly in the freshwater and marine habitat, and is possibly best known for eliciting the pinkish-red color to the flesh of salmonids, as well as shrimps, crayfish, croaker fish and lobsters (Bowen et al., 2002). Because these animals cannot synthesize astaxanthin *de novo*, natural pigments must be supplied to their food menu. In the marine and freshwater environment, astaxanthin is biosynthesized in the food chain, with microalgae as the major production source (Guerin et al., 2003).

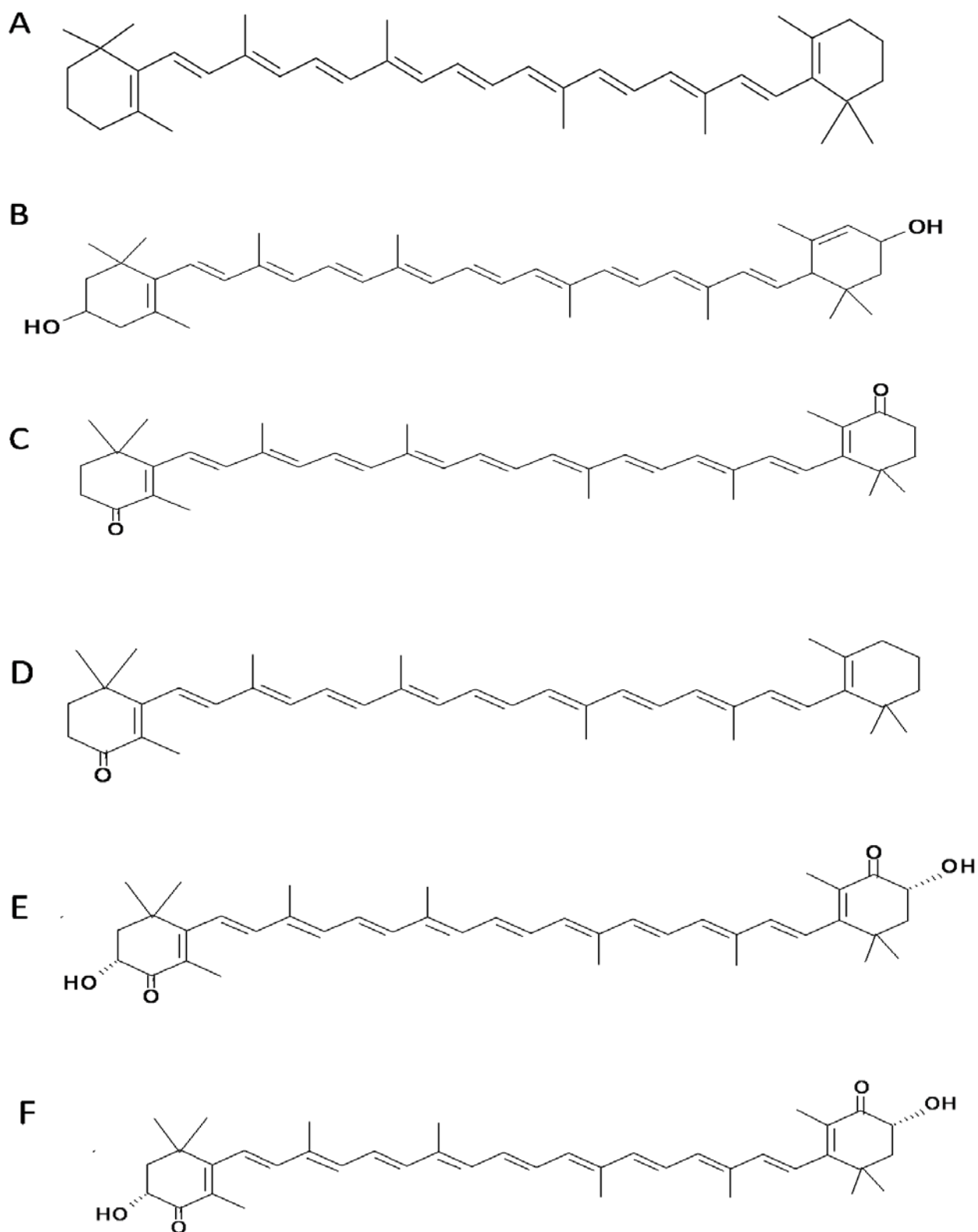


Figure 1.3. Selected carotenoids produced by green algae that are of beneficial importance. (A) β-Carotene (B) Lutein (C) Canthaxanthin (D) Echinenone (E) Adonirubin, and (F) Astaxanthin.

According to Ma and Chen (2001), astaxanthin can be produced synthetically from petrochemical sources or obtained from natural sources such as *Haematococcus pluvialis*, and *Chlorella zofingiensis* (Guerin et al., 2003). It has special chemical properties due to its molecular structure. The presence of the hydroxyl (-OH) and keto [C=O] moieties on each ionone ring explains some of its special features, such as the ability to be esterified, ability to possess higher antioxidant capacity due to stable radical scavenging and more polar nature than other natural carotenoids such as β -carotene, lutein, canthaxanthin and lycopene. Hussein et al (2006) stated that astaxanthin in its free form is considerably unstable and easily affected by oxidation. Thus, 4% were oxidized under high irradiance, salinity and high temperature condition. However, free astaxanthin is commercially more beneficial than astaxanthin esters (Ma and Chen, 2001). Synthetic astaxanthin is identical to that produced in living organisms but it consists of a 1:2:1 mixture of three isomers (3S, 3'S), (3R, 3'S) and (3R, 3'R) respectively (Danxiang Han et al., 2013). Currently, only a minimal number of natural astaxanthin producers from the microbial origin can compete profitably with synthetic astaxanthin, this include the green microalgae *Haematococcus pluvialis*, *Chlorella zofingiensis* and the red yeast *Phaffia rhodozyma* (Johnson and An, 1991). The 3S, 3'S astaxanthin isomer is the most valuable and predominantly synthesized by the green microalgae (Barbosa et al., 1999).

The process of accumulation of astaxanthin in *Haematococcus* spp. has been analysed by optical and electron microscopy (Lang, 1968; Fatima Santos and Mesquita, 1984). In the motile biflagellate cells, astaxanthin pigment first appears in small round inclusions in the perinuclear cytoplasm, the pigment granules are not within any organelle or vesicle. In maturing cysts (encystment stage), the pigment accumulation increases in number and takes on different kinds of shapes. Coalescence of the globular granules results from increasing quantities of astaxanthin formed as the cell matures. In mature cysts (aplanosporic stage), the cytoplasm is almost completely reddish with no natural pigment in the nucleus or chloroplast. Astaxanthin disperses towards the periphery of *Haematococcus* spp. cells under continuous light illumination and moves back towards the centre after illumination is stopped (Yong and Lee, 1991). No measurable changes occur during this migration. The red aplanosporic cysts are more resistant to photoinhibition than the green biflagellate cells, strongly signifying a photoprotective role for astaxanthin (Yong and Lee, 1991). The specific rate of astaxanthin accumulation is a function of the photon flux density. Continuous illumination and nutrient deprivation are most favourable for astaxanthin accumulation, and the carotenoid content is

correlated proportionally to light intensity (Lee and Ding, 1994). Salinity is another intricate environmental factor affecting pigment accumulation in green algae. Salama et al. (2013) reported that NaCl inhibits photosynthesis in the freshwater alga *Scenedesmus* sp. to stimulate higher pigment accumulation. *Dunaliella salina* and *Haematococcus pluvialis* are among the green-algae that can tolerate high salinity stress in the cultivation medium to produce large quantities of pigments (Minhas et al., 2016). To increase astaxanthin productivity, Gao et al. (2015) employed salicylic acid and jasmonic acid to induce higher expression levels of genes involved in carotenogenesis (*ipi-1*, *ipi-2*, *psy*, *pds*, *lyc*, *bkt2*, *crtR-B* and *crtO*), which resulted higher astaxanthin productivity of $2.74\text{mg} \times \text{L}^{-1}$ and $1.458\text{mg} \times \text{L}^{-1}$ as compared to $1.25\text{mg} \times \text{L}^{-1}$ without inducer in *Haematococcus pluvialis*.

Astaxanthin can be produced quantitatively as percentage of dry weight from the following microalgae species; *Botryococcus braunii* 0.01% (Grung, 1994), *Chlamydocaps* spp. 0.04% (Leya et al., 2009), *Chlorella zofingiensis* 0.7% (Orosa et al., 2000), *Chlorococcum* sp. 0.7% (Liu and Lee, 2000), *Neochloris wimmeri* 1.9% (Orosa et al., 2000), *Scenedesmus* sp. 0.3% (Qin et al., 2008), and *Haematococcus pluvialis* 4% (Lee and Ding, 1994). Out of all the above mentioned microalgae, *Haematococcus pluvialis* is recognised as one of the promising producers of natural astaxanthin pigments due to its outstanding ability to accumulate significant quantities of astaxanthin pigment under adverse conditions (Boussiba, 2000). The production of astaxanthin by *Haematococcus pluvialis* has been examined intensively (Harker et al., 1996; Cordero et al., 1996). However, efficient astaxanthin production by *Haematococcus pluvialis* is difficult because of the slow growth rate, changes in cell cytology under various environmental conditions and the sensitivity to hydrodynamic stress. Under ideal cultivation conditions, most cells are actively swimming and biflagellate and accumulate only low concentrations of astaxanthin. Thus, the pigment accumulation stage differs from those of vegetative cell growth under which astaxanthin pigment is synthesized. The separation of the astaxanthin production phase from the cell growth phase is therefore necessary to obtain high cell biomass with high pigment content. Kobayashi et al. (1997) reported that the potential of obtaining high cell concentrations in heterotrophic culture is high because the cells can be maintained in the vegetative form for a relatively long time. However, astaxanthin accumulation is relatively low under heterotrophic conditions due to the absence of light (Kobayashi et al., 1992). To address the issue of astaxanthin production, Park et al. (2014) established a two-stage perfusion cultivation system for *Haematococcus pluvialis* with proper increased light illumination ($150 - 450 \mu\text{E} \times$

$\text{m}^{-2} \times \text{s}$). The first step promotes algal growth under favourable culture conditions, in the second stage reddish cells were subjected to high stress conditions (high light intensity, excess acetate, high salinity, cell division inhibitors) with an astaxanthin productivity of $602\text{mg} \times \text{L}^{-1}$ (Park et al., 2014).

Column and thin layer chromatography were originally used and are well established for the analysis of pigments. Preparative and analytical TLC enables separation, identification and easy recovery of pigments, the separation is based on the interaction of the compound with the mobile and the stationary phase, thus the lipophilicity of the carotenoid influences the retention factor (R_f). However, recovery of pigments from thin layer chromatography plates can be a challenge (Steinberg, 1989; Mercadante and Rodriguez-Amaya, 1989). Sturzenegger et al. (1980) classified carotenoids into essential conservative, intermediate, or essentially non- conservative using circular dichroism (CD) based on their temperature dependence. Carotenoids with the absorbance maxima between 200-500nm were categorized as essentially conservative due to low temperature requirement and their tendency to invert upon all-trans to mono-cis isomerisation. As an alternative method, column chromatography such as high-performance liquid chromatography (HPLC) can be used to separate carotenoids. In HPLC analysis of carotenoids, detection takes place via a UV-VIS detector using an appropriate wavelength or a photodiode array detector (DAD), which allows a continuous collection of spectrophotometric data during the analysis (Huck et al., 2000).

Liquid chromatography-mass spectrometry is another analytical tool for the identification of carotenoids. Electrospray ionization (ESI) and atmospheric pressure chemical ionization mass spectrometry (APCI/MS) provide information about the carotenoid structure via mass spectral analysis of fragments (Rivera and Canela-Garayoa, 2012). LC-MS in the APCI mode was developed for the separation and identification of carotenoids in mixtures containing zeaxanthin, β -carotene, canthaxanthin, echinenone, and astaxanthin, as well as cis-trans isomers of β -carotene (Lacker et al, 1999). Mass spectra are typically obtained by scanning the mass range from m/z 200-700. The limit of detection for β -carotene determined in positive ion APCI-MS was apparently 1pmol (Lacker et al., 1999). Even though most mass spectra of carotenoid pigments have been acquired using positive ion mode, negative ion mode has also been reported (Van Breeman et al., 2012).

1.7. Conclusion

The use of microalgae *in vivo* and *in vitro* is receiving special attention due to the high valued pigments produced by them. These pigments are of importance in nutraceuticals, pharmaceuticals, chemical formulations, poultry and aquaculture industries. Newly improved technologies are implemented continuously to improve natural pigment production over the synthetic route. The antioxidant and radical scavenging properties exhibited by pigments such as astaxanthin, lutein, or β -carotene qualifies them to be used in treatment of diseases such as cancer, gastrointestinal ulcer as well as eye and skin diseases. The green microalgae have been cultivated photoautotrophically, heterotrophically, mixotrophically and photoheterotrophically, even under two stage perfusion. However, factors such as light, medium composition, temperature, pH, cost of microalgae production, cultivation practice, contamination control, market demand, pigment isolation and characterization still limit the production of natural pigments from microalgae. This in particular has affected the aspiration relating to the use of green algae pigments in pharmaceuticals, nutraceuticals, biotechnology and as supplementary food for animal and human consumption. Technology improvements and screening for new producer strains is still required to enable laboratory to large scale commercial production in order to overcome the use of synthetic products.

The aim of this research was to isolate and characterize green-algae from environmental samples in KwaZulu-Natal (South Africa), capable of producing carotenoid pigments (such as astaxanthin).

The main objectives of this study were:

- To isolate, purify and characterize microalgae from environmental samples able to produce carotenoid pigments.
- To determine growth kinetics and to analyze pigments in extracts from algal biomass produced under photoautotrophic and photoheterotrophic conditions.
- To detect carotenoids by Thin Layer Chromatography (TLC) and UV-VIS spectrometry.
- To determine the antioxidant capacity of algal cell extracts and pigments using an organic radical based assay.

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CHAPTER 2

Isolation and characterization of microalgae from KwaZulu-Natal (South Africa)

2.1. Introduction

Microalgae have been extensively studied for various purposes, such as for the production of biomass, as source of valuable chemicals such as pigments or as health food supplement (Guerin et al., 2003). They are used in environmental biotechnology for biofuel production, waste water treatment and lipid production (Glombitza and Koch, 1989; Chen et al., 2011). These microorganisms are commonly found in freshwater, soil and marine habitats and are classified as unicellular, eukaryotic organisms (Henley et al., 2004; Krienitz et al., 2004). *Chlorella* spp., *Scenedesmus* spp., and *Haematococcus* spp. are examples for commercially important microalgae that can produce carotenoids, which are fat soluble pigments that are associated with lipid fractions. Examples of such pigments are astaxanthin, β -carotene, and lutein, which have all attracted commercial interest for medicinal and nutraceutical uses (Miki, 1991; Ruiz et al., 2016). Among the microalgal producers for astaxanthin, *Chlorella zofingiensis* and *Scenedesmus vacuolatus* have been reported to achieve high yields of astaxanthin under mixotrophic conditions, although *Haematococcus pluvialis* is considered the most potent producer (Johnson and An, 2008). The intracellular changes during the accumulation of astaxanthin in this microalga differ from those of *Chlorella* spp. and *Scenedesmus* spp. (Hanagata and Dubinsky, 1999; Klochkova et al., 2013). Under favourable growth conditions, *Haematococcus pluvialis* appears as green motile biflagellate cells, but as conditions become unfavourable, the cells lose their motility, increase cell size, and form non-motile red cysts with a thick cell wall. These aplanospores of *Haematococcus* spp. accumulate copious quantity of the secondary natural carotenoid astaxanthin, mostly in the form of fatty acid mono and diesters (Kobayashi et al., 1991). The subjection of *Scenedesmus* spp. to varying growth conditions such as light intensity, nitrogen starvation, and redox stress brings about a color change in the cells, from green to brick red (Fatima and Mesquita, 1984; Hanagata and Dubinsky, 1999). *Scenedesmus komarekii* synthesizes secondary carotenoids such as astaxanthin when growing under high light irradiance and nitrogen limitation. The synthesized carotenoids protect the photosynthetic apparatus against photo-oxidative damage under high irradiance (Lee and Ding, 1992; Lee and Ding, 1994; Hagen et al., 1994).

Scenedesmus spp., and *Chlorella* spp., additionally served as food supplements in countries like Japan, Mexico, and Taiwan, due to lipids, proteins, vitamins, and carotenoids present in the algal biomass. *Chlorella* spp. contains up to 0.7% dry weight, *Scenedesmus* spp. contains up to 0.3% dry weight while over 4% dry weight of astaxanthin was recorded in *Haematococcus pluvialis* (Orosa et al., 2000; Qin et al., 2008; Wayama et al., 2013). Photosynthetic microalgae can be cultivated autotrophically using CO₂ as a carbon source and light as energy source in open or closed photobioreactors (Chen et al., 2009). However, the photoautotrophic culture mode has both disadvantages, such as slow growth, and advantages, such as lower risk of contamination, while carbon sources present in heterotrophic conditions enhance the growth of bacteria and fungi. In contrast to photoautotrophic cultivation, heterotrophic cultivation can be experimented in conventional microbial bioreactors, which makes it convenient to alter conditions to increase the quantities of biomass and minimize the production cost of microalgal biomass (Borowitzka, 1999). The mixotrophic culture differs slightly from the heterotrophic culture, where CO₂ and organic carbon are simultaneously absorbed and both photosynthetic metabolism and respiration operate concurrently (Lee, 2004). Many microalgae can grow mixotrophically, heterotrophically or photoheterotrophically and photoautotrophically. Photoheterotrophic cultures are characterized by the presence of two carbon sources (CO₂ and another carbon compound), in addition to light. Simultaneous assimilation of organic carbon and CO₂ via photosynthesis was reported to result in higher growth yield and biomass production (Orus et al., 1991). Strains such as *Euglena* spp., *Haematococcus pluvialis*, and *Chlorella regularis* have been reported to grow photoheterotrophically (Martinez and Orus, 1991; Takeyama et al., 1997).

Most research on microalgae centered on the selection of photoautotrophic strains and cultivation conditions that lead to the highest yield in the shortest possible time. Cultivation conditions such as light intensity, temperature and carbon sources are major factors that determine the growth rate of microalgae, which are cultivated under photoheterotrophic conditions. Thus far, knowledge about the isolation of green microalgae for the production of carotenoids such as astaxanthin is limited in South Africa. Therefore, this study aimed to isolate and characterize microalgae from environmental samples in KwaZulu-Natal, South Africa, that are able to produce carotenoids such as β -carotene and astaxanthin.

2.2. Materials and Methods

2.2.1. Media

Bourrelly medium

The medium according to Bourrelly (1968) was used for the isolation and purification of microalgae. It was generated by dissolving KNO_3 0.2 g, K_2HPO_4 0.04 g, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 0.03g, $\text{Ca}(\text{NO}_3)_2$ 0.03g, and FeCl_3 0.001g, in 1L of demineralized millipore water, adjusting the pH to 7.0 using HCl. 10 ml of soil extract was added to 990ml medium. Preparation of soil extract was done by adding 50ml of Millipore water to 40g of soil sample collected from botanical garden, with boiling for 1 hour on a hot plate with stirring. Slurry was allowed to settle at room temperature, followed by filtration (Whatman, number 1001240). It was then autoclaved at 121°C for 20 minutes. For solid media, 15 g/L of bacteriological agar was added to the medium prior to autoclaving.

Chu medium

The medium according to Chu (1942) was prepared by dissolving $\text{Ca}(\text{NO}_3)_2$ 0.04g, K_2HPO_4 0.01g, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 0.025g, Na_2CO_3 0.02g, and FeCl_3 0.0008g, in 1L of demineralized millipore water adjusting the pH to 7.4 using HCl. It was autoclaved at 121°C for 20 minutes. For solid media, 15 g/L of bacteriological agar was added to the medium prior to autoclaving.

Bristol medium

The medium according Bristol (1919) was generated by preparing stock solutions each of NaNO_3 10g, CaCl_2 1g, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 1g, K_2HPO_4 3g, KH_2PO_4 7g, NaCl 1g, and FeCl_3 1% dissolved in 400ml of millipore water. To obtain 1L medium, 10ml of each of the above first six stock solutions and 50 μl of the FeCl_3 stock solution was added to 940ml of demineralized millipore water. The pH of the medium was adjusted to 7.2 with HCl. It was autoclaved at 121°C for 20 minutes. For solid media, 15 g/L of bacteriological agar was added to the medium prior to autoclaving.

2.2.2. Isolation and purification of microalgae

Sample collection, enrichment and isolation

Water samples were collected in sterile Schott glass bottles from the Msunduzi River (S 29°37'27.40", E 30°22'22.49") in the city of Pietermaritzburg (KwaZulu-Natal, South Africa). Samples were transported to the laboratory on ice to inoculate Bristol, Chu and Bourrelly medium. Additional samples were collected from a bird bath showing red coloration indicating the presence of *Haematococcus* spp. in Hayfields, Pietermaritzburg (KwaZulu-Natal).

Enrichment and isolation

Serially diluted samples (in a range of 10^{-1} – 10^{-6}) in sterile Chu, Bourrelly, and Bristol liquid medium were spread plated on the matching solidified medium and incubated at $21 \pm 1^\circ\text{C}$ with 24 hour light cycles at $40 \mu\text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1}$ for 10 days. After regular subculturing under the above conditions, axenic cultures of three green algal strains were established. To ascertain purity, samples culture were routinely streaked on to nutrient agar (Merck) and regularly examined by phase contrast microscopy.

To preserve the microalgae cultures, the isolated strains were grown in their preferred liquid medium for 10 days and then stored in 20% (v/v) glycerol (200 μL glycerol and 800 μL culture sample) at -80°C . In addition, working cultures were maintained on Bristol, Chu and Bourrelly medium.

2.2.3. Microbiological characterization

The characterization of the isolates was carried out by examining the cell morphology of isolates and by analysis of the 18S rRNA gene sequences of two out of the three isolates selected for further analysis showing insufficient morphological characteristics for reliable taxonomic assignment.

Light Microscopy

Wet mounts of all the three isolates were examined by bright field and phase contrast microscopy (Motic BA 310 and Axioscope, Carl Zeiss, Germany). Images were obtained using an AxioCam ICC 3 (Carl Zeiss GmbH, Germany). To determine the presence and number of pyrenoids in microalgal isolates, cells were stained with 0.2 % (w/v) aqueous potassium iodide in an Eppendorf tube for 30 mins according to Lang (1963). After 30 mins, stained cells were examined by bright field microscopy and images were captured as stated above.

Electron Microscopy

TEM

The cytology of isolates was analyzed using Transmission electron microscopy (TEM). The cells collected from individual colonies from agar plates were suspended in sterile water and fixed in 3% glutaraldehyde and washed twice in 50mM Sodium cacodylate (pH= 7.4), then post-fixed in 2% osmium tetroxide, dehydrated in an ethanol series (10%, 30%, 50%, 70%, 90%, and 100%), followed by 100% propylene oxide, and finally embedded in 50:50 Spurr resin (Spurr, 1969). The samples were sectioned using an ultra-microtome (EM UC7: Leica Microsystems) and stained with 2% uranyl acetate and examined using a JEOL 1400 Transmission Electron Microscope (Jeol, Japan).

SEM

For the scanning electron microscopy (SEM), microalgal cells were fixed in 3% glutaraldehyde, then dehydrated in an ethanol series (10%, 30%, 50%, 70%, 90%, and 100%), dried using critical point drying (Quorum K850) and then sputter coated with gold and examined with a Zeiss EVOLS15 Scanning Electron Microscope (Carl Zeiss, Germany).

2.2.4. Phylogenetic analysis of isolates

Genomic DNA of the microalgal isolates strain AA1 and AA2 was extracted from individual colonies collected from axenic cultures grown for 10 days on Chu and Bourrelly agar at $21\pm1^{\circ}\text{C}$ with 24 hours light cycles at $40\ \mu\text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1}$, using a ZR fungal/Bacterial DNA Microprep kit (Zymo Research, USA) according to the manufacturer's instructions. The DNA extracts were immediately frozen and stored at -20°C .

Amplification of the 18S rRNA gene

Previously reported primers were used for the amplification of the partial eukaryotic 18S rRNA gene (Katana et al., 2001). These primers were synthesized by Inqaba, South Africa.

Forward primer S3F24 5' -AACCTGGTTGATCCTGCCAGT- 3'

Reverse primer S3F25 5' - TGATCCTTCTGCAGGTTACCTACG- 3'

Table 2.1. Composition of the PCR reaction mix used for the amplification of the 18S rRNA gene for strain AA1 and AA2.

Reagents	per reaction(μL)
Forward primer S3F24 (10 μM)	0.5
Reverse primer S3F25 (10 μM)	0.5
Master Mix	12.5
Nuclease free water	10
DNA Template	1.5
Total Volume	25

The following PCR parameters were employed: Initial denaturation at 94°C for 2 mins followed immediately by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1min and extension at 72°C for 1 min. The final extension was for 7 mins at 72°C and the holding temperature at 4°C indefinitely.

The size of the amplification products (expected size= 1800bp) was verified on 1.5% (w/v) agarose gels using 1X Tris, Borate, EDTA buffer (pH=8.3) at 120V for 45 mins. Gels were stained using Novel juice DNA gel stain (GenedireX, USA). Prior to loading of the gel, 5µL PCR product was mixed with 8µL/well orange green loading dye (GenedireX). For comparison, a size marker was used (100bp DNA ladder, GenedireX).

Sequencing

The amplification products were sequenced by the Centre for Analytical Facilities, Stellenbosch University (South Africa). Sequences obtained were trimmed to the same threshold quality level and sequence not shorter than to match 1300 bp. The resulting sequences obtained were then compared with those published in GenBank using the BLAST search algorithm from the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov).

Phylogenetic analysis

The partial 18S rRNA gene sequences of the isolated strains were aligned with 18S rRNA gene sequences of type strains and environmental isolates obtained from the National Centre Biotechnology Information (www.ncbi.nlm.nih.gov) for 18S rRNA gene. The sequence alignment was done using ClustalW; phylogenetic trees were then generated using the neighbour joining method with resampling for 1000 bootstrap replicates. This was conducted using MEGA (Molecular Evolutionary Genetic Analysis) version 7.0 (Kumar et al., 2016). The 18S rRNA gene sequences of *Ochromonas danica* SAG 933.7 GU 935657.1 and *Chlamydomonas reinhardtii* CC- 849 KR 904894.1 were used as out-group for strain AA1 and strain AA2, respectively. The tree topology was verified by applying the Maximum Likelihood phylogeny method.

2.3. Results

2.3.1. Characterization of the isolated strains

Phenotypic Characteristics

A provisional characterization of the three selected microalgal isolates was performed, based on light and electron microscopy of cells (morphology, size and shape) and appearance of colonies after growth on solid medium. Three isolates that could be clearly distinguished via colony and cell morphology were selected for further analysis.

Table 2.2. Basic macroscopic and light microscopic features of three selected isolates.

Feature	Strain AA1	Strain AA2	Strain AA3
Colony morphology and size	Bright green colonies on Chu and Bristol medium, 4.5mm in diameter on both media after 10days of incubation.	Bright green colonies on Chu and Bristol medium (8mm in diameter), faint green colour on Bourrelly medium, 5mm in diameter after 10days of incubation.	Dark green colonies on Bourrelly medium (12mm in diameter) and faint green on Bristol and Chu medium with 6mm in diameter after 14days of incubation.
Cell shape and size	Ovoidal shape, 2.5-5.6 μm	Spindle shape, 8 -15 μm	Spherical shape, 12.6-15.4 μm , encystment, 20-50 μm , mature cyst $\geq 50 \mu\text{m}$
Cell wall	Smooth cell wall	Smooth cell wall	Smooth cell wall
Motility	Non-motile	Non-motile	Biflagellate at vegetative stage, encystment at exponential stage, immature cyst at late exponential stage and mature cyst at late stage
Organelles	Pot-shaped parietal chloroplast, single pyrenoid and nucleus	Cup-shaped parietal chloroplast with single pyrenoids and nucleus	Cup-shaped chloroplast, single pyrenoid and nucleus

The light microscopy analysis of the three selected purified isolates revealed that Chu and Bristol medium (liquid and solidified), supported the growth of strain AA1, (ovoidal shaped non-motile cells, presumptive *Parachlorella* sp.) (Figure 2.1a) and strain AA2 (spindle shaped, non-motile cells, presumptive *Scenedesmus* sp.) (Figure 2.1b) but only enabled poor growth of strain AA3 (spherical shaped motile biflagellate cell, presumptive *Haematococcus* sp.). However, *Haematococcus* sp. strain AA3 grew well using Bourrelly liquid and solidified medium, undergoing the developmental life cycle showing the different cellular stages expected for this genus (Figure 2.1c, Figure 2.4 and 2.5). Between 3-5days, motile biflagellated cells were observed, followed by encystment with increase in cell size and colonies showing greenish-reddish color, indicating pigment accumulation (Figure 2.4-2.5). As stress conditions such as continuous illumination persisted, the palmelloid stages lost their flagella, with thicker cell walls being formed.

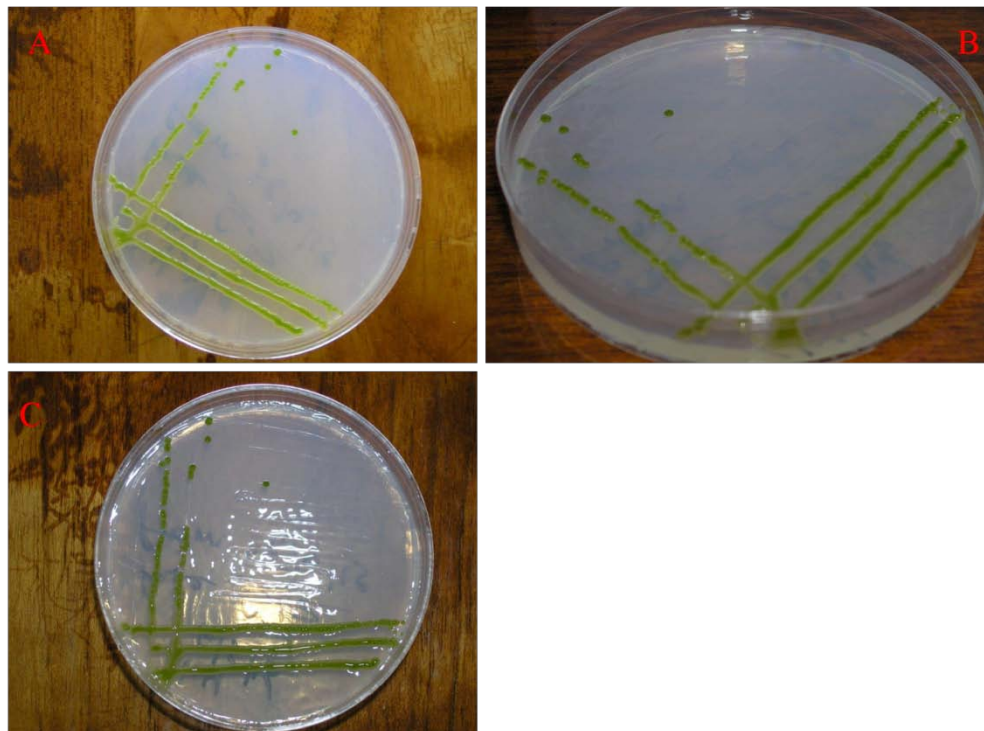


Figure 2.1. Growth of isolated strains on solid media.

(A) Growth of *Parachlorella* sp. strain AA1 on Chu medium.

(B) Growth of *Scenedesmus* sp. strain AA2 on Chu medium.

(C) Growth of *Haematococcus* sp. strain AA3 on Bourrelly medium.

Culture samples of *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2, and *Haematococcus* sp. strain AA3 grown in their preferred medium were further examined by bright field and phase contrast microscopy for morphological characterization.

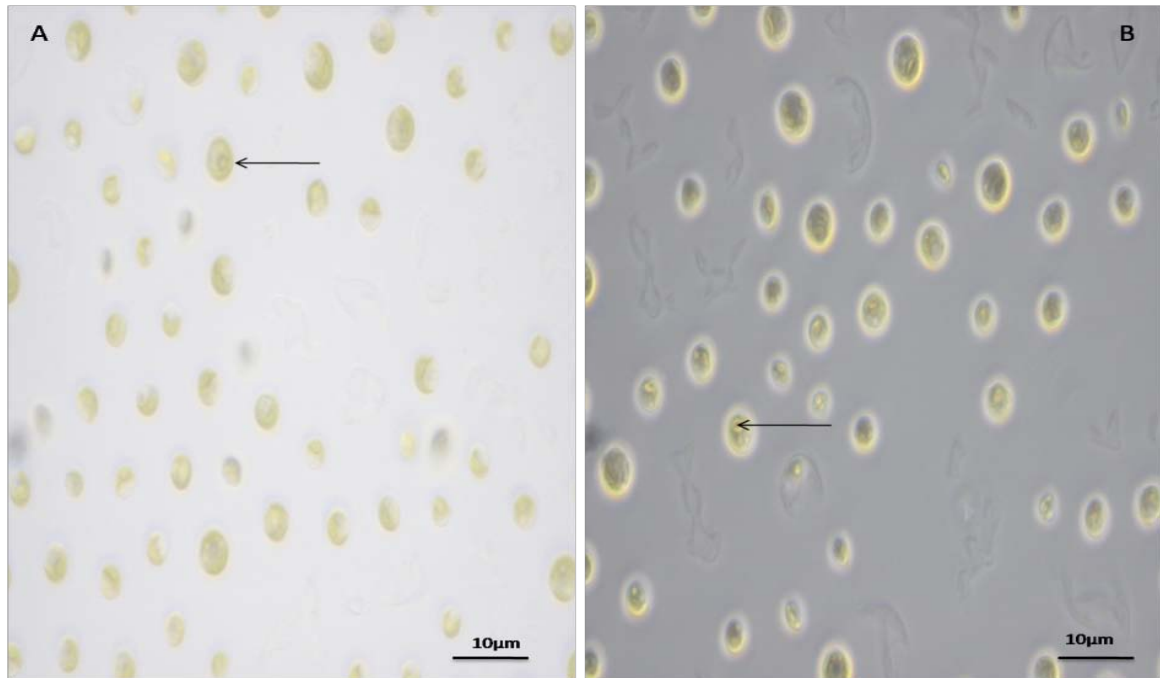


Figure 2.2. The cells of *Parachlorella* sp. strain AA1 during the exponential stage.

(A) Bright field microscopy of *Parachlorella* sp. strain AA1 during the exponential stage. The arrow highlights the presence of a pyrenoid.

(B) Phase contrast microscopy of *Parachlorella* sp. strain AA1 during the exponential stage. The arrow highlights the presence of a pyrenoid.

Vegetative cells with a pot shaped chloroplast in a size range of 3–10µm (Figure 2.2) were observed in strain AA1. The cells are planktonic, non-motile, and ovoidal shaped with a single pyrenoid.

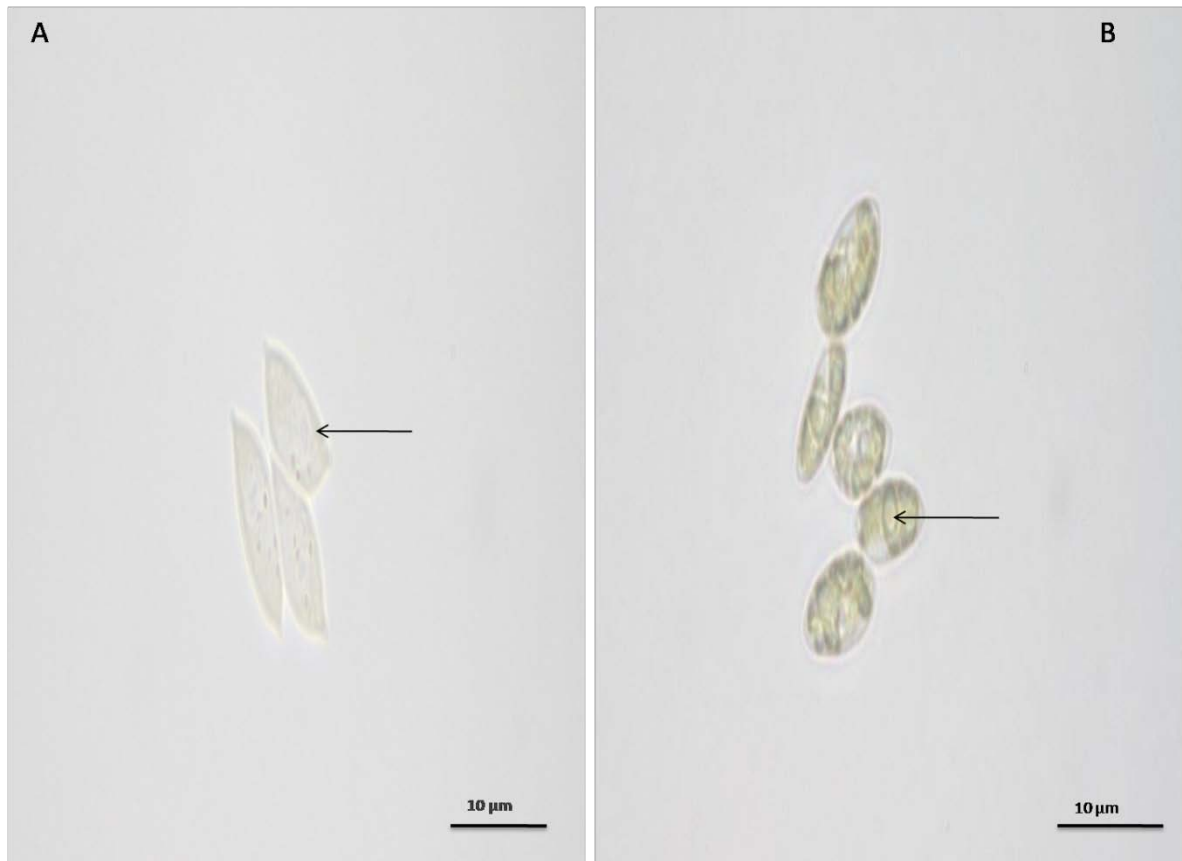


Figure 2.3. The cells of *Scenedesmus* sp. strain AA2 during the exponential stage.

(A) Bright field microscopy of *Scenedesmus* sp. strain AA2 during the early exponential stage. The arrow highlights the presence of some general cytology.

(B) Phase contrast microscopy of *Scenedesmus* sp. strain AA2 during the late exponential stage. The arrow highlights the presence of a pyrenoid.

As demonstrated by bright field and phase contrast microscopy (Figure 2.3), the cells of *Scenedesmus* sp. strain AA2 were non-motile, spindle-shaped (8–15µm) and planktonic with a cup shaped chloroplast and a single pyrenoid. Cells were typically attached side by side with nipple-shaped projections on the obtuse apices of the cells.

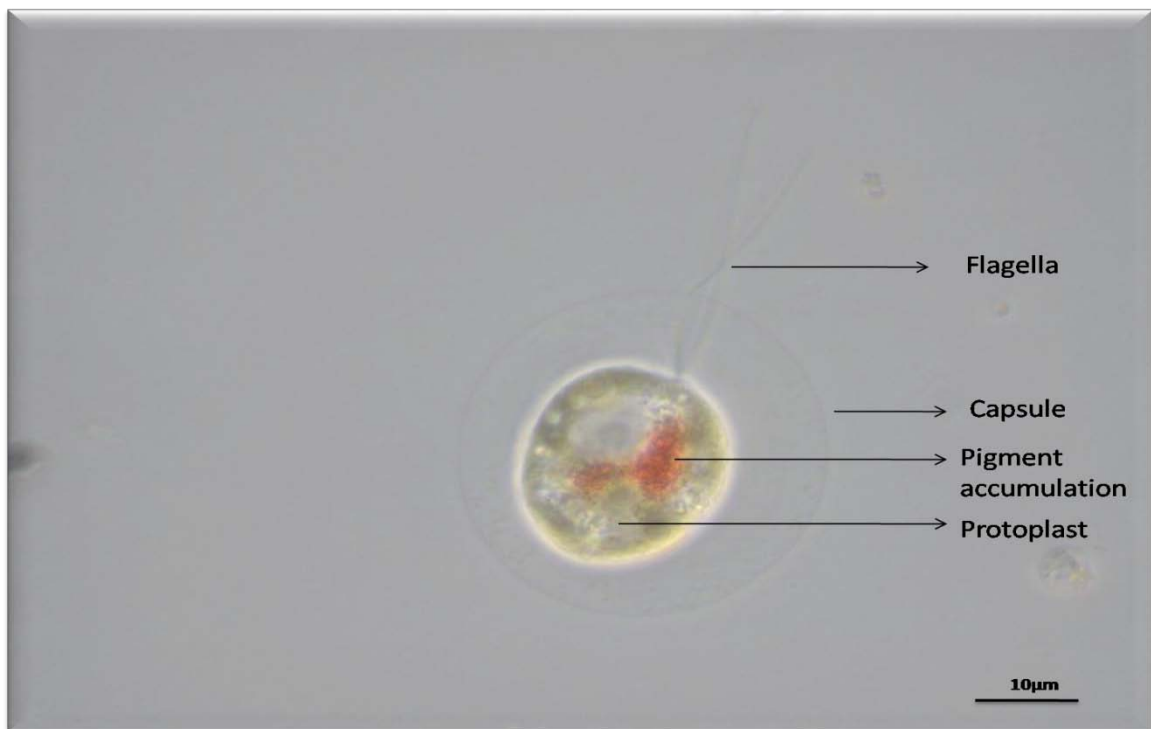


Figure 2.4. A zoospores of *Haematococcus* sp. strain AA3 from a liquid culture in the exponential growth phase.

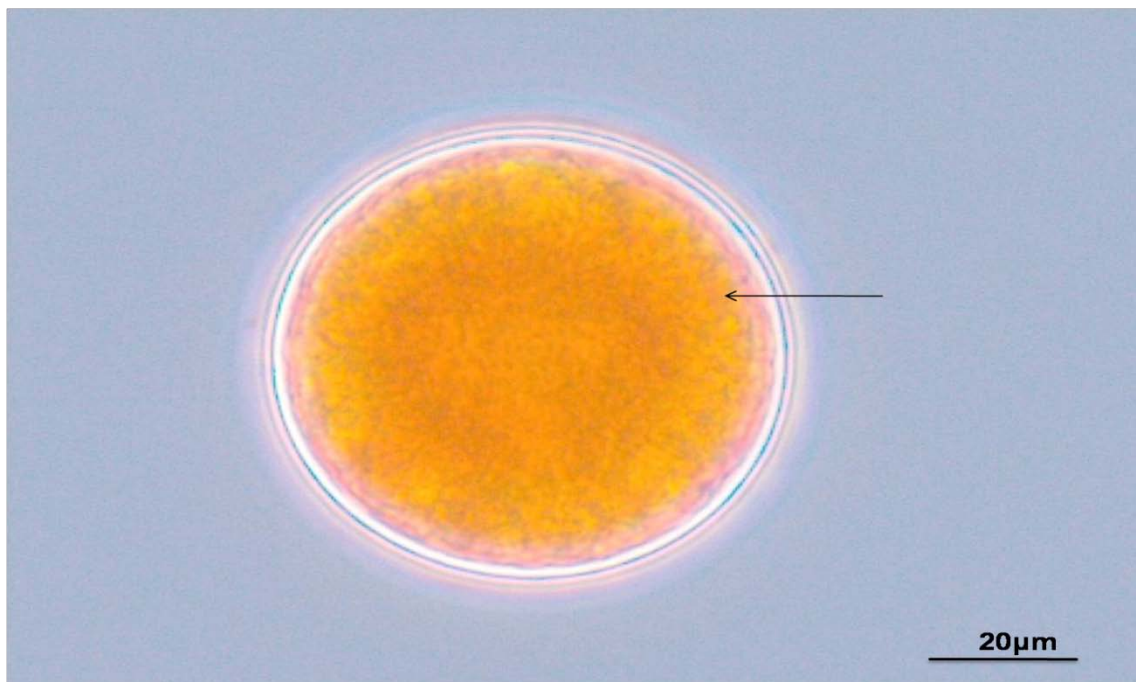


Figure 2.5. An aplanospore of *Haematococcus* sp. strain AA3 showing pigment accumulation in the late life cycle stage. The arrow highlights accumulated pigment.

Motility was observed in *Haematococcus* sp. strain AA3, with flagellate stages showing two flagella (Figure 2.4). The flagellate vegetative cells were spherical in shape and typically between 10 –15 μm in diameter and surrounded with a capsule (plasma strands). Pigment accumulation was observed closed to the protoplast, with the protoplast not reaching the capsule. In addition, in the late stage of the life cycle haematochrome in thick-walled red cysts $\geq 50\mu\text{m}$ was observed with an apparent gelatinous extracellular matrix (Figure 2.5).

Pyrenoid staining

The number and shape of pyrenoids is a useful taxonomic marker. Therefore, pyrenoid staining using iodine was performed. The presence of oval cells, with one pyrenoid present in mucilage was observed in *Parachlorella* sp. strain AA1 (Figure 2.6). Flat, spindle- shaped cells with a single oval, angular shaped pyrenoid were detected in *Scenedesmus* sp. strain AA2 (Figure 2.7). A single spherical, centrally located pyrenoid was observed in *Haematococcus* sp. strain AA3 (Figure 2.8).



Figure 2.6. Phase contrast microscopy of cells of *Parachlorella* sp. strain AA1 stained with 0.2% (w/v) aqueous potassium iodide. The arrow highlights the presence of a stained pyrenoid.

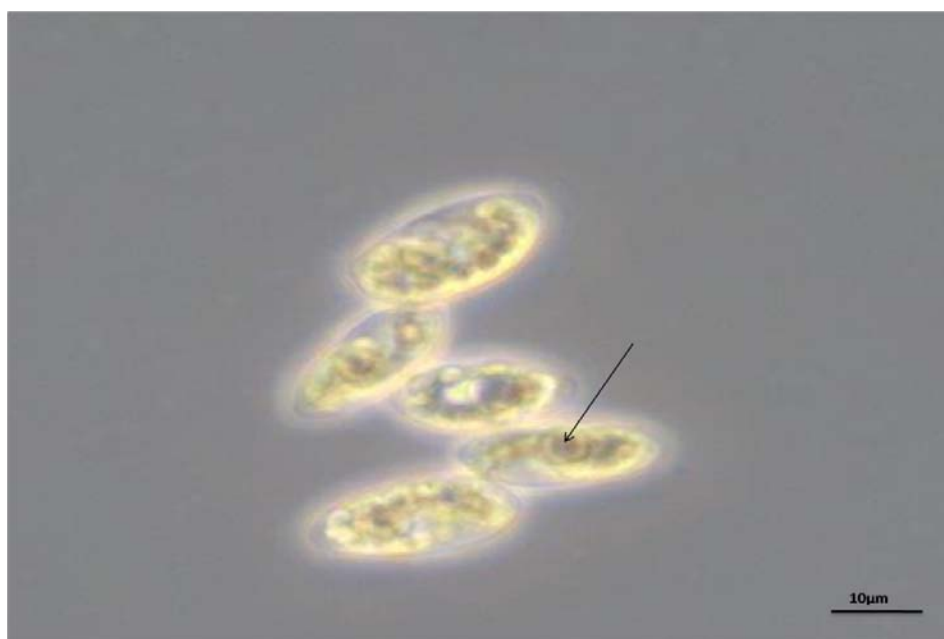


Figure 2.7. Phase contrast microscopy of cells of *Scenedesmus* sp strain AA2 stained with 0.2 % (w/v) aqueous potassium iodide. The arrow highlights the presence of a stained pyrenoid.



Figure 2.8. Phase contrast microscopy of a *Haematococcus* sp. strain AA3 cell stained with 0.2 % (w/v) aqueous potassium iodide. The arrow highlights the presence of a stained pyrenoid.

Transmission electron microscopy confirmed the presence of a double layered single pyrenoid in the isolated *Parachlorella* sp. strain AA1 (Figure 2.9b). Figure 2.9a revealed that isolate AA1 undergoes asexual reproduction by cell division. Pigment accumulation was observed (figure 2.9c) with a double layered cell wall shown in figure 2.9d. Clusters of cells are shown in the SEM micrograph in Figure 2.10b. The SEM analysis further confirmed that *Parachlorella* sp. strain AA1 reproduced by binary fission (Figure 2.10a).

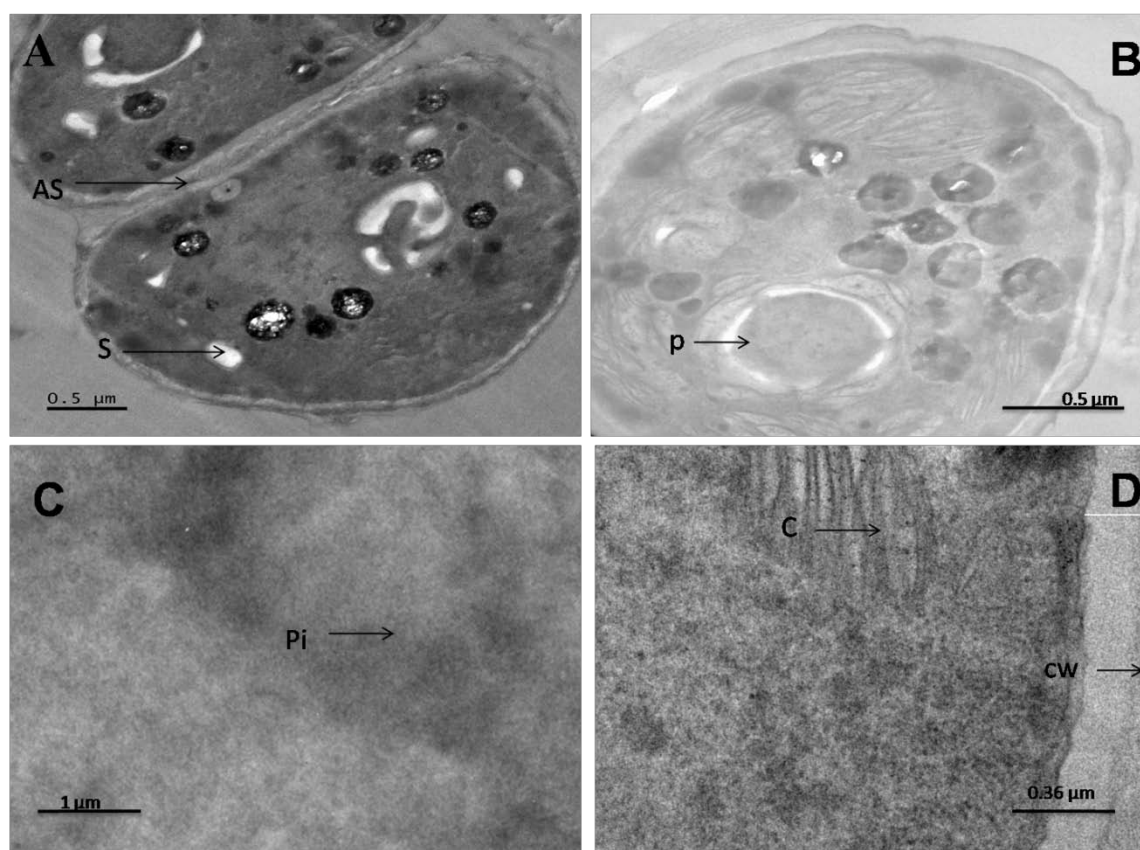


Figure 2.9. TEM analysis of cells of *Parachlorella* sp. strain AA1.

- (A) Thin section of a dividing cell of strain AA1 during asexual reproduction at the exponential stage.
- (B) General cytology of a single cell with a single pyrenoid during the exponential stage.
- (C) Section of cytoplasm with pigment accumulation.
- (D) Cell section with double layered cell wall and chloroplast (AS= Asexual reproduction, P= pyrenoids, Pi= pigment accumulation, CW= cell wall, S= starch, C= chloroplast).

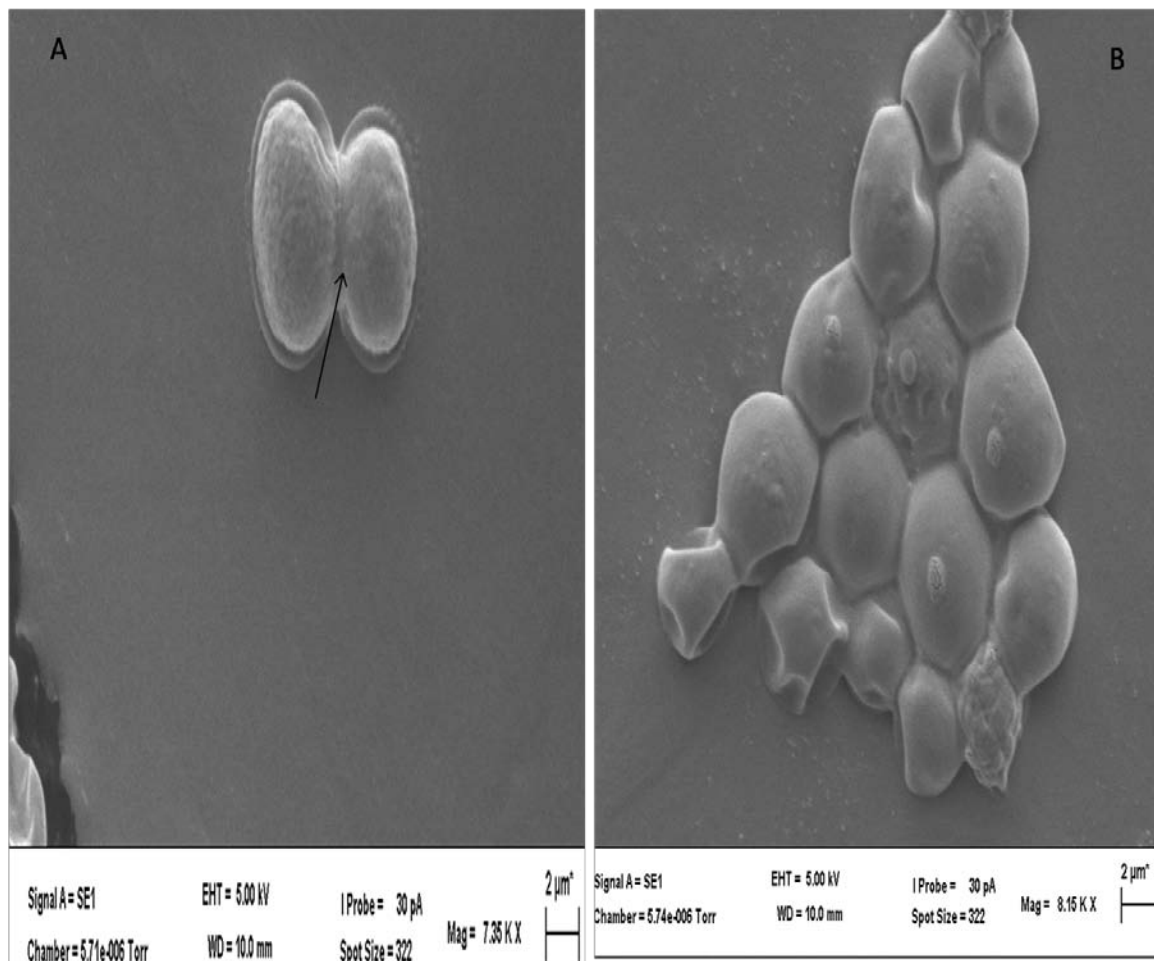


Figure 2.10. SEM analysis of cells of *Parachlorella* sp. strain AA1.

(A) Cell reproduction by binary fission during the exponential phase. The arrow highlights binary dividing cell.

(B) Clusters of cells during the exponential phase.

Analysis by transmission electron microscopy of thin sections of *Scenedesmus* sp. strain AA2 showed that the cell wall had an outer trilaminar layer and an inner polysaccharide layer (Figure 2.11a). The pyrenoid was surrounded by starch grains and the chloroplast was observed at the periphery of the cell close to the pyrenoid (Figure 2.11b and c). Lipid bodies containing pigment were seen almost in the center. The scanning electron micrographs (Figure. 2.12) show a single cell from the exponential phase and asexual reproduction by multiple fission as a coenobium of *Scenedesmus* sp. strain AA2 formed (Figure 2.12A).

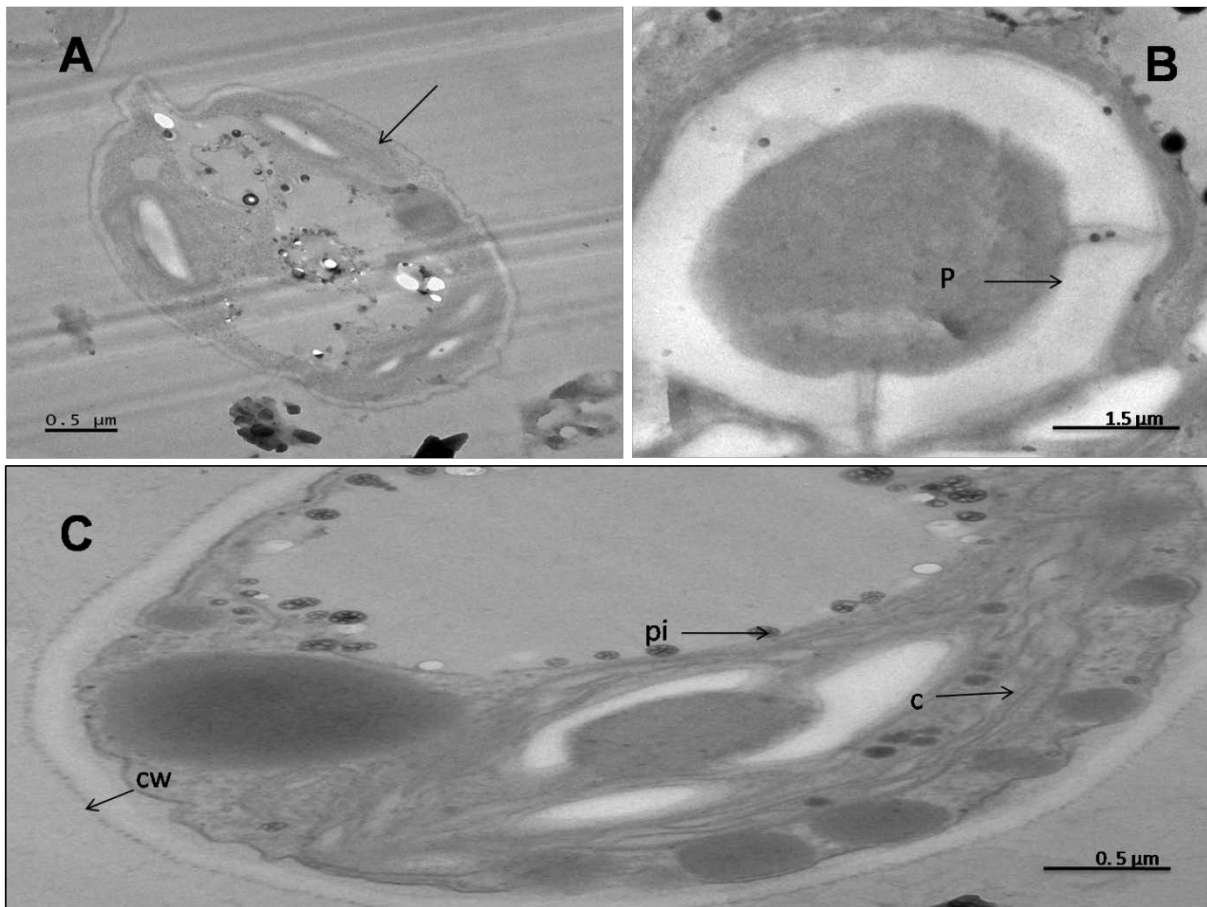


Figure 2.11. TEM analysis of cells of *Scenedesmus* sp. strain AA2.

- (A) Thin section showing a single cell during the exponential phase. The arrow highlights thin section of a single cell.**
- (B) Pyrenoid surrounded by starch granules during the exponential phase. The arrow highlights the presence of a pyrenoid.**
- (C) Cell section with double layered cell wall, pigment accumulation and chloroplast. (CW= cell wall, P=Pyrenoids, Pi =pigments accumulation, C= chloroplast).**

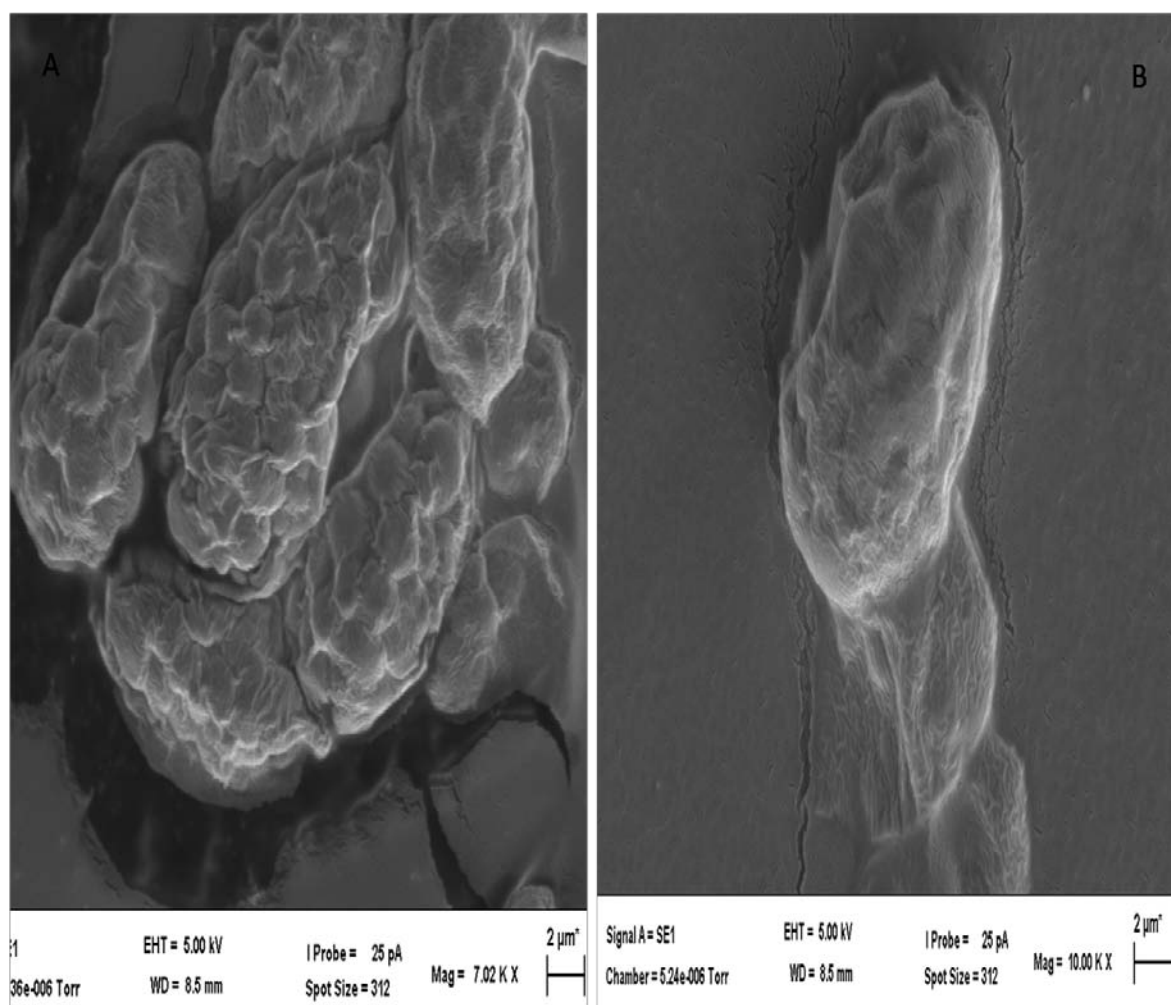


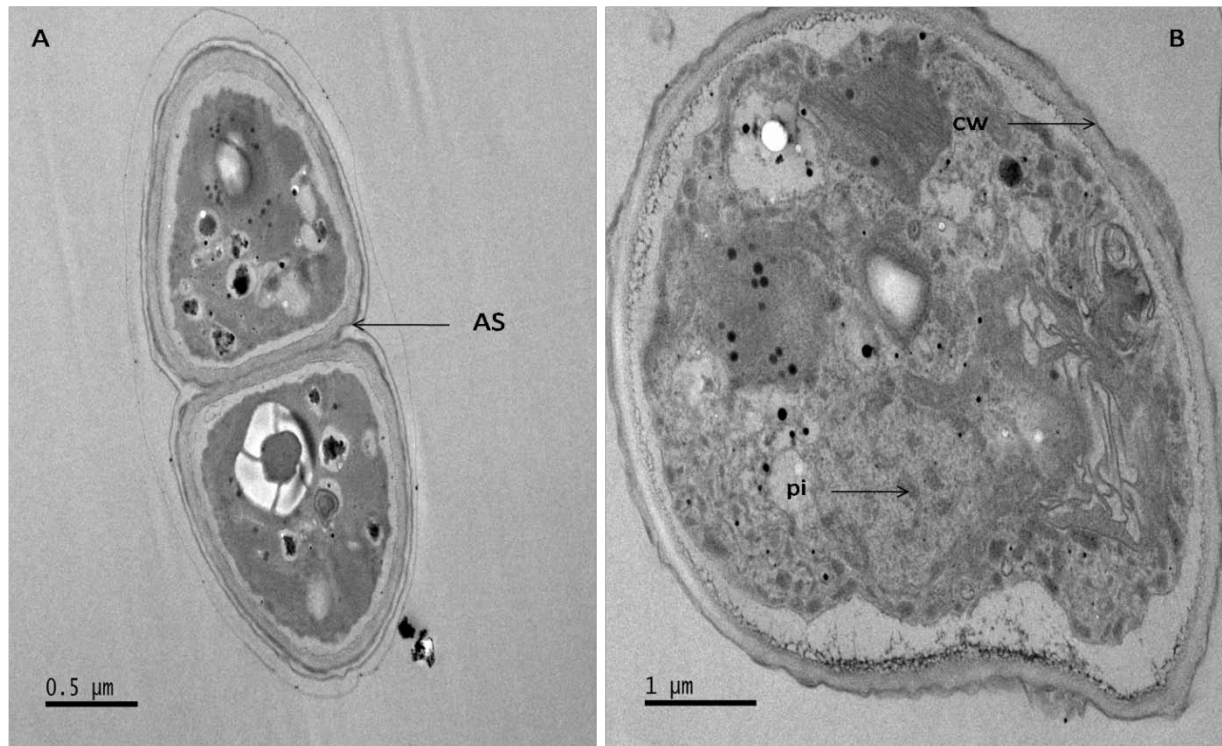
Figure 2.12. SEM analysis of cells of *Scenedesmus* sp. strain AA2.

(A) Asexual production by multiple fission forming a coenobium during the exponential phase.

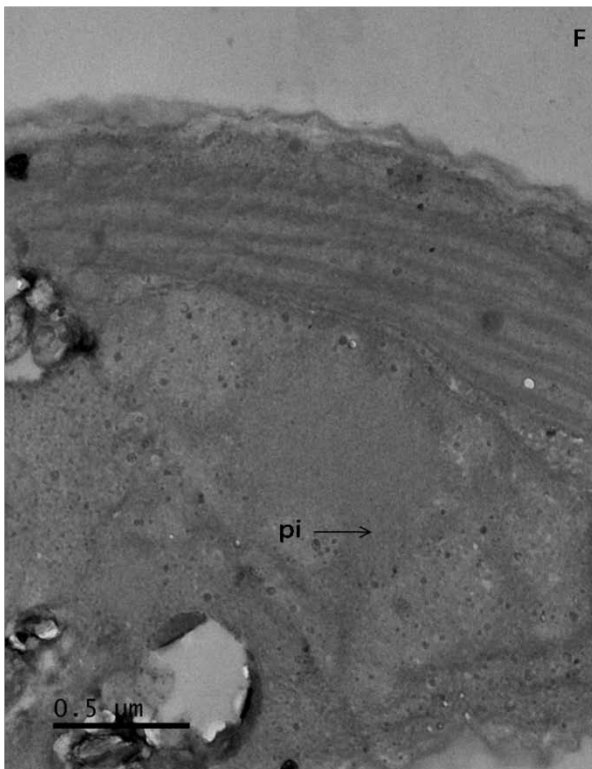
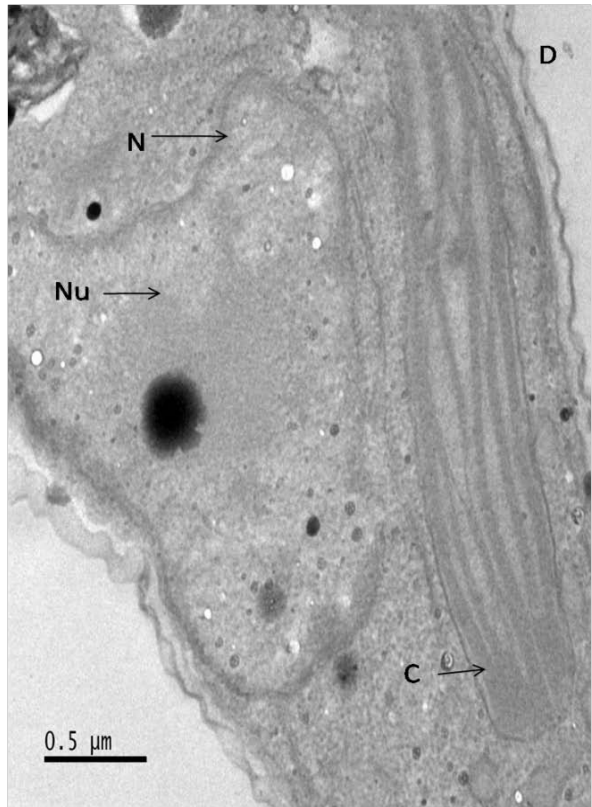
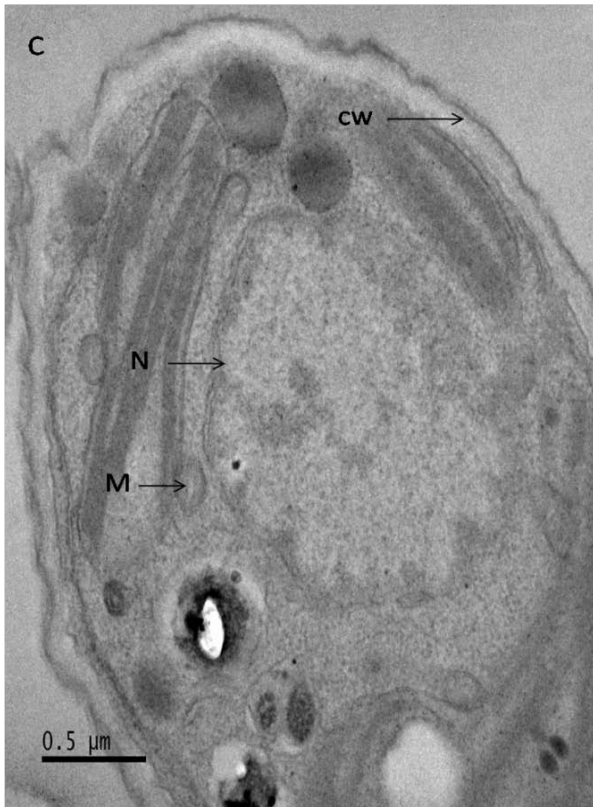
(B) Single parental cell during the exponential phase.

Ultrastructural analysis of thin sections of *Haematococcus* sp. strain AA3 by transmission electron microscopy revealed zoospores of *Haematococcus* sp. strain AA3 undergoing asexual reproduction by binary fission (Figure 2.13a). In thin sections, a thick electron dense cell wall surrounding the cells, and two layers of extracellular matrix were observed near the cell wall in addition to pigment accumulation (Figure 2.13b). Mitochondria are located close to the chloroplast and the nucleus during the early stage (Figure 2.13c). The nucleus was located in the centre of the cells during the early growth stage (Figure 2.13c) but more developed with a nucleolus during the late growth stage (Figure 2.13d). A highly developed chloroplast was located

at the periphery of the cells in figure 2.13d, and lipid droplets containing astaxanthin were observed, which build up due to stress conditions during the late growth stage (Figure 2.13e and 2.13f). Starch grains were located around the pyrenoids in figure 2.13g. The SEM micrographs show individual and cell groups of *Haematococcus* sp. strain AA3 which may lead to asexual reproduction by gametogenesis (Figure 2.14). The surface of the outer cell sheath was irregularly wavy and protuberates



(Matching legend on page 69).



(Matching legend on page 69).

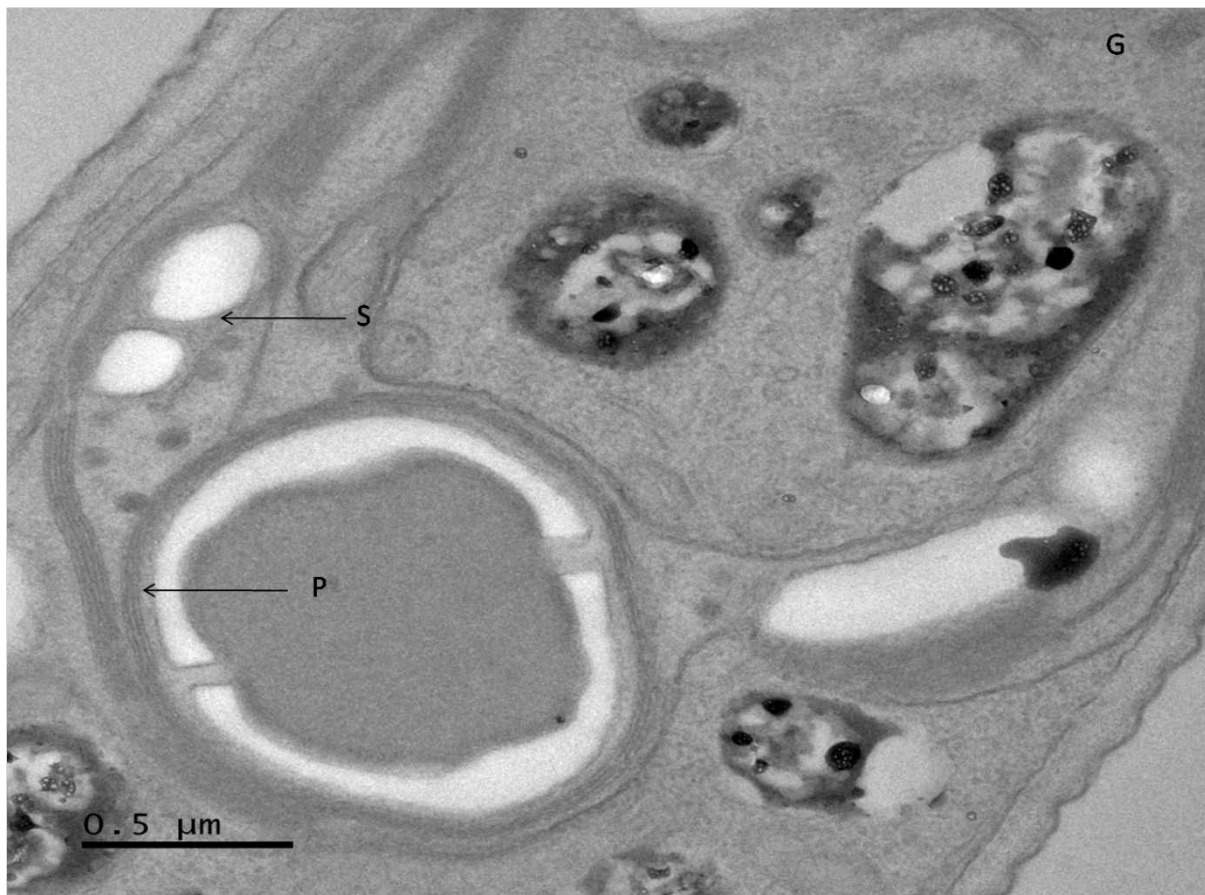


Figure 2. 13. TEM analysis of thin sections of cells of *Haematococcus* sp. strain AA3.

- (A) Asexual reproduction during the early vegetative stage.**
- (B) Thin section of a single cell with pigment accumulation during the early stage.**
- (C) Cell wall with plasmalemma and some intermediate tripartite crystalline layers at the early stage.**
- (D) Nucleus containing nucleolus at the late stage.**
- (E) Astaxanthin containing lipid droplet during the late stage.**
- (F) Section of chloroplast at the late stage.**
- (G) Thin section of the pyrenoids with starch. (N= Nucleus, Nu= Nucleolus, P = Pyrenoid, S = Starch, M = Mitochondrion, CW= Cell wall, C= Chloroplast, PI= Pigment accumulation).**

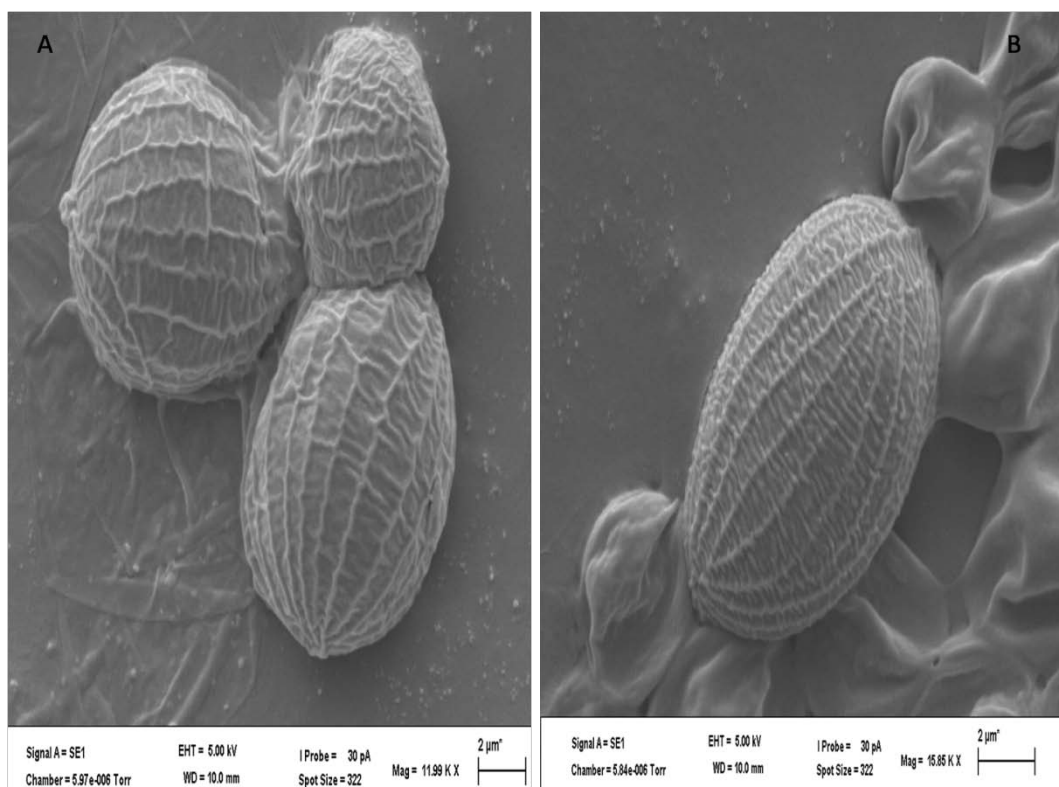


Figure 2.14. SEM analysis of the cell morphology of *Haematococcus* sp. strain AA3 from the exponential stage.

(A) Cluster of cells.

(B) Single cell revealing the wavy surface of the cell.

2.3.2. Phylogenetic analysis of two isolates

The 18S rRNA gene sequences are most widely used for the phylogenetic analysis of green algae as they are suitable for identifying even distantly related species (Buchheim et al., 2013). To determine the phylogenetic relationship of the two isolates, the nucleotide sequences of the 18S rRNA gene were compared with those published in GenBank using the Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov). The best matches based on the sequences and the E-value from the database are listed in Table 2.2-2.3.

Table 2.3. Best three matching sequences obtained from NCBI using blast for strain AA1.

Accession No	Description	Max score	E Value	Identity
KX021356.1	<i>Parachlorella kessleri</i> strain TY02 18S ribosomal RNA gene, partial sequence	1304	0.0	100%
KM020114.1	<i>Parachlorella kessleri</i> strain SAG 211-11c 18S ribosomal RNA gene, partial sequence	1304	0.0	100%
KM020113.1	<i>Parachlorella kessleri</i> strain SAG 27.87 18S ribosomal RNA gene, partial sequence	1304	0.0	100%

Accession date= 11/10/2016.

Table 2.4. Best three matching sequences obtained from NCBI using blast for strain AA2.

Accession No	Description	Max score	E value	Identity
X81966.1	<i>Scenedesmus ovalternus</i> 18S ribosomal RNA gene	1825	0.0	99%
KP726264.1	<i>Desmodesmus denticulatus</i> strain KLL-G003 18S ribosomal RNA gene, partial sequence	1820	0.0	99%
FR865716.1	<i>Scenedesmus bacillaris</i> genomic CCAP 276/29 18S ribosomal RNA	1812	0.0	99%

Accession date= 11/10/2016.

Comparison of the 18S rRNA gene sequence of the isolated strains AA1 and AA2 to the sequences contained in GenBank gave an apparent similarity level of more than 98% for strain AA1 and strain AA2; this is a similarity level regarded as sufficient to assign isolates to genus level (Bock et al. 2011). Accordingly, strain AA1 can be assigned provisionally to the genus *Parachlorella* with 100% similarity to *Parachlorella kessleri*, while strain AA2 can be assigned provisionally to genus *Scenedesmus* with 99% similarity to *Scenedesmus ovalternus*. The high score between strain AA2 and *Desmodesmus* spp. indicated the level of similarity between the two genera, which can only be distinguished by their ultrastructural cell wall (An et al., 1999). Based on the observed cytological and distinctive features and the lifecycle, isolate AA3 was assigned to the genus *Haematococcus*. *Haematococcus pluvialis* zoospores are characterized by long flagella

tubes at right angles to each other, a separation of cell wall and protoplast that are connected only by thin cytoplasmic threads thereby matching the characteristics observed for isolate AA3.

Phylogenetic trees using the 18S rRNA sequences of strain AA1 and strain AA2 and the 18S rRNA sequences for type strains obtained from the GenBank were constructed using MEGA version 7.0. The phylogenetic relatedness of the isolated strains AA1 and AA2 with type strain sequences are demonstrated in figure 2.15 to 2.16.

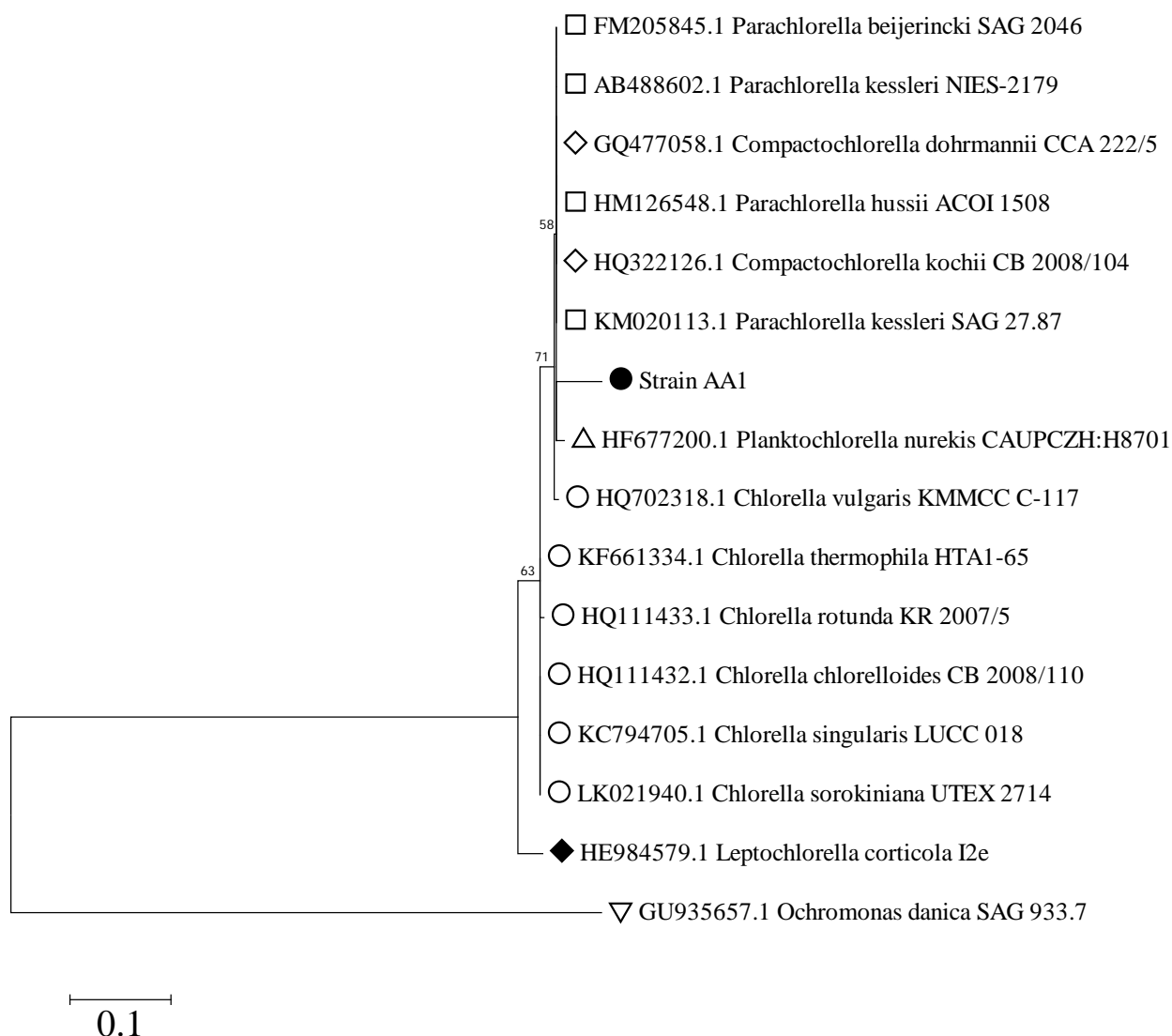


Figure 2.15. Phylogenetic assignment of strain AA1 (●) using the neighbour joining method based on its 18S rRNA gene sequence in comparison with 18S rRNA gene sequences of selected type strains of *Chlorella*, *Compactochlorella*, *Leptochlorella*, *Planktochlorella* and *Parachlorella* (□). The 18S rRNA gene sequence of *Ochromonas danica* (▽) was used as out-group. The scale bar represents 10 estimated change per 100 nucleotides. Only bootstrap values ≥ 50 are shown.

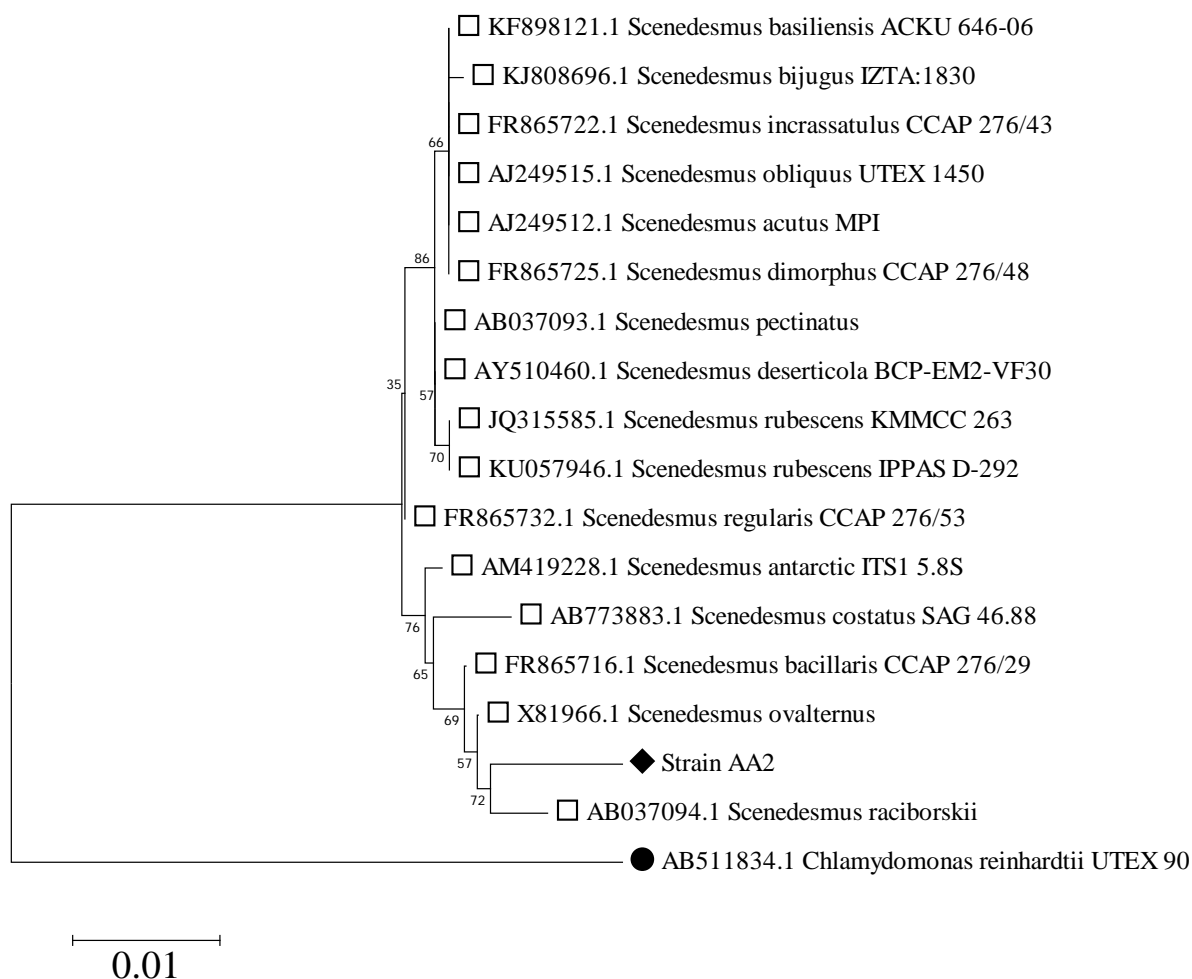


Figure 2.16. Phylogenetic assignment of strain AA2 (◆) using the neighbour joining method based on its 18S rRNA gene sequence in comparison with 18S rRNA gene sequences of selected type strains of *Scenedesmus* (□). The 18S rRNA gene sequence of *Chlamydomonas reinhardtii* (●) was used as out-group. The scale bar represents 1 estimated change per 100 nucleotides. Only bootstrap values ≥ 50 are shown.

The phylogenetic trees illustrate the relationship of the isolated strains AA1 and strain AA2 with selected type strains (Figure 2.15 to 2.16). The phylogenetic analysis revealed that strain AA1 was closely related to the species within the family *Chlorellaceae*. Strain AA2 clustered with the species of genus *Scenedesmus*, and was most closely related to *Scenedesmus raciborskii*.

2.4. Discussion

Isolation and characterization

Algae are the major group of eukaryotes present in almost every habitat on our planet (Schlichting, 1974; Weiss, 1983). However, not all of these algae can produce beneficial pigments at sufficient quantities for use by humans and animals. Some of these are truly *Chlorophyceae* most commonly belonging to families such as the *Chlorellaceae*, *Scenedesmaceae* and the *Haematococaceae* (Elliot, 1934; Atkinson et al., 1972; Hegewald et al., 1994).

The following well established media were used for the isolation and purification of pigment producing microalgae, Bourrelly medium, Chu medium and Bristol medium (Bristol, 1919; Chu, 1942; Bourrelly, 1968), with the pH of the media ranging between 7.0–7.4. Only an appropriate culture medium enables pigment production. Isolation of axenic cultures from mixed populations is a necessity in the study of microalgae as only this allows for the proper physiological analysis of the organisms (Krienitz et al, 1996). While Chu and Bristol medium (liquid and solidified) supported the growth of *Parachlorella* sp. strain AA1 (with high similarity to *Chlorella*) and *Scenedesmus* sp. strain AA2, they enabled only poor growth of *Haematococcus* sp. strain AA3. However, *Haematococcus* sp. strain AA3 grew well on Bourrelly medium. It has been previously reported that the composition of Chu and Bristol medium provides required mineral salts for the growth of freshwater microalgae and enhances the typical cell morphology in *Chlorella* spp. and *Scenedesmus* spp. (Bristol, 1919; Chu, 1942). This corresponded with Fabregas et al. (2000), reporting that to maximize the growth of *Haematococcus pluvialis*, only Bourrelly medium enabled both high growth rates and increased pigment yields.

The microscopy analysis of strain AA1 allowed the preliminary identification of the isolate as a presumptive member of the genus *Parachlorella*. The morphological features corresponded to those of a typical *Parachlorella* spp. (Bock et al., 2011; Skaloud et al., 2014). The cells of strain AA1 were light green in color, non-motile, of ovoidal shape and ranged in size between 3-10 µm. The cell walls were smooth and double layered and cells possessed a parietal pot shaped chloroplast (Table 2.1).

The transmission electron microscopy of thin sections of cells of *Parachlorella* sp. strain AA1 confirmed the presence of very thick gelatinous cell walls comprising of at least two layers. This is a typical feature of the genus *Chlorella* and *Parachlorella*. The cytological observations corresponded to previous observations of Krienitz et al. (2004) and Bock et al. (2011) for *Chlorella* spp. Asexual reproduction occurred in *Parachlorella* spp. strain AA1 at the exponential phase and a single pyrenoid structure was observed under photoautotrophic conditions, matching the result for *Parachlorella* spp. (Figure 2.9a and 2.10a). However, Hase et al. (1957) reported that *Chlorella* spp. can produce spores under heterotrophic and mixotrophic conditions, which were not detected for strain AA1.

Strain AA2 showed light microscopic features typical of *Scenedesmus* spp., when compared to previous studies of Trainor (1998) and Hegewald et al. (1994). The cells of strain AA2 are parallel, spindle shaped with light green color, and ranged in size between 8 - 15 μm . They possessed a smooth cell wall with a nipped-shaped projection on the obtuse apices of cells, a cup-shaped parietal chloroplast and a single pyrenoid (Table 2.1), which was reported for *Scenedesmus* spp. by Lewis and Flechtner (2004).

Scenedesmus is a genus known to reproduce through multiple fission. This was evident for isolate AA2 (Figure 2.12a) according to Rading et al. (2011). This helps in maximizing light exploitation for photosynthesis and permitting higher growth rates compared with species that undergo only the binary fission. The TEM analysis of strain AA2 showed a cell wall with an outer trilaminar and an inner polysaccharide layer (Figure 2.11a), which is typical for *Scenedesmus* spp. (Hegewald et al. 1994). The curlyness of the cell wall is typical of the genus *Scenedesmus* and is due to sporopollenin and corresponded to previous observations of Krienitz et al. (1996). The detection of coenobia (Figure 2.12a) was in agreement with the report of Egan and Trainor (1989) and Hegewald et al. (1994) whereby a single mother cell can give rise to daughter cells ranging from 2–16 coenobia.

The morphological analysis of strain AA3 via light microscopy matched reports for *Haematococcus pluvialis* (Pocock, 1960; Lee and Ding, 1994; Triki et al., 1997; Klochkova et al., 2013). The zoospores are biflagellate, dark green in color with a spherical shape and ranged between 10.6–15.4 μm (Figure 2.4 and table 2.2). The lifecycle of *Haematococcus* sp. strain AA3 was characterized by four different cellular stages, starting with the macrozooid, which was spherical, ellipsoidal with two flagella and a cup-shaped chloroplast (Figure 2.4), and present in the early vegetative stage under favourable conditions. When the conditions become

unfavourable, these zoospores develop into immotile “Palmella stages” by losing their two flagella along with increasing the cell size (encystment). When stress persists, the palmella develops into the non-motile asexual aplanospore with a rigid and thick cell wall (Figure 2.5). Large quantities of astaxanthin pigment accumulate giving rise to a bright red color during the maturation stage (Gong and Chen, 1997; Brinda et al., 2004). Cytological analysis of strain AA3 agreed with previous reports of Fatima and Santo (1984) and Kim et al. (2015) on carotenogenesis and pigment accumulation in *Haematococcus pluvialis* and that when the conditions become favourable, gametocysts can give rise to zoospores depending on the culture condition. Figure 2.13a revealed that the vegetative stage of *Haematococcus* sp. strain AA3, with cells undergoing asexual reproduction by cell division with the protoplast separated from the capsule in the zoospores (Figure 2.4). Motile stage diversity in the green algae, type and number of flagella, insertion and general morphology has been accorded substantial weight in making taxonomic and phylogenetic pronouncements (Bold and Wynne, 1985; Thompson and Wujek, 1989; Pegg et al., 2015). The thick cell wall, pigment accumulation and protoplast observed in strain AA3 are unique and are typical only of *Haematococcus pluvialis* at the akinetes stage (Pegg et al., 2015). According to Buchheim et al. (2013), only *Haematococcus pluvialis* accumulates the natural carotenoid astaxanthin at the vegetative stage thereby matching isolate strain AA3.

Pyrenoids as taxonomic markers

Chloroplasts of most algae are known to contain large bodies termed pyrenoids (Fritsch, 1935). According to Drawert and Mix (1962), algal pyrenoids consist of finely granular, densely packed, homogeneous material. Staining with iodine can help distinguish some algal groups based on the number and types of pyrenoids present. The stain showed for strain AA1 the presence of one pyrenoid covered in mucilage (Figure 2.6). This corresponded to the previous report of Nemcova and Kalina (2000) of a typical *Chlorella* pyrenoid and that a pyrenoid bisected by four thylakoids has never been observed in *C. vulgaris*, *C. sorokiniana* and *C. kessleri* now reassigned to *Parachlorella kessleri*. According to Atkinson et al. (1972), Corre et al. (1996), and Huss et al. (1999), *Chlorella* species with a glucan-type cell wall exhibit quite diverse pyrenoid structures, which may indicate that the different *Chlorella* species are not closely related. Nevertheless, some ultrastructural features of *Chlorella luteoviridis* such as thin electron dense layer, microfibrillar structure and cell wall remnants persisting in the medium resembles that of glucosamine-type *Chlorella* spp. Species with a glucosamine cell wall such as *Chlorella kessleri*, *Chlorella sorokiniana*, and *Chlorella vulgaris* are virtually identical in pyrenoid morphology, indicating a

closer evolutionary relationship. In the species with glucosamine cell walls, the thylakoid that penetrates into the pyrenoid matrix is uniformly double layered (Kapaun et al., 1992; Ikeda and Takeda, 1995), which was similar to the pyrenoid present in the isolated strain AA1.

Flat, spindle - shaped cells with a single oval, angular shaped pyrenoid were observed in *Scenedesmus* sp. strain AA2 (Figure 2.7), which is similar to the pyrenoids observed by Bisalputra and Weier (1964) in *Scenedesmus quadricauda*. Gibbs (1962) described that the pyrenoid of *Scenedesmus quadricauda* in old cultures has large starch plates around the fibrillar matrix and no lamellar structures within the pyrenoid. Smith (1914) and Bisalputra and Weier (1964) reported, that pyrenoids of *Scenedesmus* spp. which are very large before the beginning of cell division, disappear during cell cleavage and a new pyrenoid is formed in each of the daughter cells, indicating that new starch platelets can be formed in *Scenedesmus* spp. without any interaction with the chloroplast thylakoids.

A single spherical centrally located pyrenoid was observed in *Haematococcus* sp. strain AA3 (Figure 2.8 and 2.11b). This observation was in accordance with the previous findings of Thompson and Wujek (1989) and Wayama et al. (2013) that the structure and shape of pyrenoids in *Haematococcus pluvialis* does not change at the exponential stage and that a single pyrenoid is present at the late stage. When the algal cells are under suitable nutritional conditions, amino acids form a substantial proportion of the early product of photosynthesis and as a result, pyrenoids of green algae could be referred to as a temporary storage region of the early products of photosynthesis, which under overproduction conditions could be easily changed into more permanent storage material in the form of the starch sheath (Holm-Hansen et al., 1959; Syrett, 1962).

Taxonomic identification of microalgal isolates involves a combination of morphological examination and increasingly genetic characterization of taxonomic marker genes (Huss et al. 1999; Bock et al. 2011; Buchheim et al., 2013), as many closely related species lack distinct morphological features, making them difficult to identify (Pulz and Gross, 2004). As microalgal cells can also structurally change (size and cell shape) during their developmental lifecycle and under certain cultivation conditions, morphology can be misleading (Surek, 2008).

18S rRNA gene sequencing has become the reference method for algae classification as it can provide and confirm identification at genus and even species level (Buchheim et al., 2013). The homology search using the Basic Logical Alignment Search Tool (BLAST) showed that isolates

strain AA1 and strain AA2 had sequence similarity scores $\geq 99\%$ when compared to sequences deposited in GenBank for type strains of *Parachlorella* and *Scenedesmus* which enabled assignment at genus level for the two isolates (Table 2.3 – 2.4). The 18S rRNA gene of strain AA1 had a highest similarity score of 100% to the 18S rRNA gene sequence from a species of the genus *Parachlorella* (*Parachlorella kessleri* formerly *Chlorella kessleri*), strain AA2 had a similarity value of 99% to the 18S rRNA gene sequence from a species of the genus *Scenedesmus* (*Scenedesmus ovalternus*).

The phylogenetic trees based on the 18S rRNA gene sequences reveal the phylogenetic relationship between the strains AA1 and AA2 with selected representatives of closely related genera. It is evident from the phylogenetic analysis that strain AA1 belongs to the genus *Parachlorella*-clade in the family *Chlorellaceae* (Skaloud et al., 2014). Strain AA2 forms a cluster with *Scenedesmus raciborski* in the family *Scenedesmaceae* (An et al., 1999; Lewis and Flechtner, 2004).

2.5. Conclusion

This research aimed at isolating and characterizing pigment producing microalgae that are of potential benefit to humans and animals. The media and culture conditions used enabled the isolation of three different isolates that produced pigments, which was confirmed by light microscopy and TEM analysis revealing pigment accumulation in the three isolates. The three isolates were provisionally assigned based on the analysis to the family level *Parachlorellaceae*, *Scenedesmaceae* and *Haematococeae*.

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CHAPTER 3

Growth and carotenoid production by isolated microalgae

3.1. Introduction

The need for alternate sources of valuable bioproducts such as pigments has drawn the attention of many researchers to microalgae. *Scenedesmus* spp. (Class *Chlorophyceae*, order *Chlorococcales*) for example is considered as a potent producer of carotenoids and other high value products. Pigments from *Scenedesmus* spp. are known to possess antioxidant properties, and hence are used in disease protection and prevention, for example (eye diseases) and other health related ailments, as they inhibit the development of cataracts and macular degeneration (Krinsky, 1989; Guerin et al., 2003). In additional studies, lutein was recommended for the prevention of specific types of cancer (Del Campo et al., 2000; Eisenhauer et al., 2017). Marigold (*Tagetes erecta* L.) is the commercially known source of lutein. However, microalgae such as *Chlorella protothecoides* and *Chlorella zofingensis* (Class *Trebouxiophyceae*, order *Chlorellales*) are also considered as potent producers of lutein (Del Campo et al. 2004, 2007) and *Scenedesmus almeriensis* has been discovered to contain high lutein quantities (Sanchez et al., 2008). Del Campo et al. (2001) reported up to 0.03% dry weight of lutein in *Scenedesmus almeriensis* cells compared to only 0.01% dry weight in marigold flowers. *Scenedesmus almeriensis* was reported to attain a lutein productivity of $8.5 \text{ mg} \times \text{L}^{-1} \times \text{day}^{-1}$, which makes it attractive for commercial purposes (Molina et al., 2005; Lin et al., 2015).

Haematococcus pluvialis (Class *Chlorophyceae*, order *Volvocales*) is one of the numerous well known microalgal species that can synthesize high value compounds naturally (Elliot, 1934; Klochkova et al., 2013). It is known to accumulate and synthesize high levels of the natural antioxidant astaxanthin (3,3'-dihydroxy- β,β' -carotene-4,4'-dione) under adverse conditions such as nutrient deprivation, temperature variation, increased salinity, or chemical induction (such as by salicylic acid) and combinations of other types of stress (Kobayashi et al., 1992; Kakizono et al., 1992; Fan et al., 1994; Li et al., 2008; Lemoine and Shoefs, 2010). As the unfavourable conditions persist, the *Haematococcus* cell loses its motility (biflagellate stage), the cell size increases, and orange-red colored cysts with thick cell walls are formed. The antioxidative effect makes astaxanthin useful in pharmaceutical, cosmetic, food, and aquaculture industries and generates a vast demand in the world market. The global market of astaxanthin is estimated to be over US\$1 billion and projected to double before 2018 (De Holanda and Netto, 2006; Minhas et al., 2016). Carotenoids such as astaxanthin help in protecting skin and eye cells from UV-light

mediated photooxidation, inhibit the growth of fibrosarcoma and selected cancer cells and hepatitis, prevent high cholesterol, age-related macular degeneration and high blood pressure due to their effectiveness in scavenging free radicals, which in case of astaxanthin is exceeding that of other carotenoids and vitamin E (Guerin et al., 2003; Rao et al., 2013; Li et al., 2015). Of all the species producing astaxanthin, *Haematococcus pluvialis* is known to be able to accumulate up to 65% of carotenoids in the form of astaxanthin and over 4% by dry weight as astaxanthin, which makes it the richest source of natural astaxanthin among astaxanthin producing microorganisms like *Chlorella zofingiensis* or *Phaffia rhodozyma* (Lee and Ding, 1994; Del Campo, 2004; Kim et al., 2015). This is due to its potential to synthesize and accumulate high quantities of astaxanthin pigment under adverse conditions (Boussiba 2000; Lemoine and Schoefs, 2010). As animals cannot synthesize carotenoids de novo, they have to get natural carotenoids via their feeds or food (Bowen et al., 2002). Nowadays, the industrial astaxanthin pigment for aquaculture is mostly chemically synthesized from petrochemical sources, whereas natural astaxanthin contributes only to a small fraction of this market (Guerin et al., 2003). Astaxanthin is also preferred over other carotenoids such as canthaxanthin due to better assimilation by the digestive tract of salmonids and its higher colour intensity (Storebakken and No, 1992). Its red pigmentation makes it beneficial for use in marine industry for pigmenting ornamental fish (Ako and Tamaru, 1999; Bowen et al., 2002). Being globally used in aquaculture farming as a feed additive and colorant, the astaxanthin pigment market in the United State was calculated to be over US\$200 million per year at a price of about US\$2,500 per kg (Minhas et al., 2016). The increasing demand of customers for natural foods has therefore increased the production of astaxanthin pigment from natural sources such as crustacean by-products, microalgae, and transgenic plants (Misawa, 2009; Li et al., 2011).

The beneficial importance of carotenoids as the main dietary source of vitamin A has been known for many years. Carotenoids in foods are usually less well assimilated from the intestinal system than preformed Vitamin A. As the quantity of carotenoids in the diet increases, the absorption efficiency decreases (Olson et al., 1996). A low plasma level of β -carotene has also been associated with cardiovascular diseases (Gey, 1993; Kontush et al., 1999). Dugas et al. (1998) reported that β -carotene functions as antioxidant in protecting low-density lipoprotein from cell-mediated oxidation. Over 90% of commercially produced β -carotene is chemically synthesized. Only a few selected countries like Israel, China, Australia, and Mexico produced natural β -carotene at large commercial scale from microalgae of the genus *Dunaliella* (Johnson and Schroeder, 1995; Ye et al., 2008).

Growth conditions are one of the factors that determine the yield of carotenoid pigments such as astaxanthin from microalgae. In this study, the growth of three microalgal isolates, *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2, and *Haematococcus* sp. strain AA3 under photoautotrophic and photoheterotrophic conditions using glucose and acetate as organic carbon sources was examined. Carotenoid production was confirmed via thin-layer chromatography (TLC) and UV-VIS spectrometry. The antioxidant potential of algal cell extracts and isolated pigments was determined using a stable organic radical based assay.

3.2. Materials and Methods

3.2.1. Chlorophyll extraction

Different methods have been recommended for the routine assay of chlorophyll (a, b, c and total chlorophyll) content in algal cells using acetone, methanol, and ethanol as solvents. However, of all the solvents, ethanol is the most preferable due to safety reasons, cost considerations and because it can give sharp chlorophyll absorption maxima like acetone. Chlorophyll a was extracted from algal cells harvested in the exponential growth phase (1×10^7 cells/ml) of all the three strains thrice by using 100% ethanol, glass beads and centrifugation ($10,000 \times g$ for 5 mins) at varying extraction time intervals (0, 5, 10, 20, 30, 60, and 120mins) to establish the optimum conditions. The chlorophyll a content in ethanol extracts was estimated spectrophotometrically using a UV-VIS spectrophotometer (Shimadzu, 1240, Japan) at 665nm as reported by Winthermans and Demots (1965).

3.2.2. Growth kinetics of isolates

The growth of microalgae depends on the salt composition of the mineral media, organic substrates if applicable, and cultivation conditions such as light intensity. For biomass production, growth rates under photoautotrophic and photoheterotrophic conditions using glucose and acetate as supplements were compared.

Photoautotrophic growth

100ml Erlenmeyer flasks containing 10ml of sterile mineral salts medium without carbon source were inoculated to an initial cell density of about 8×10^5 cells/ml. For *Parachlorella* sp. strain AA1 and *Scenedesmus* sp. strain AA2, the medium according to Chu (1942) was used while *Haematococcus* sp. strain AA3 was cultivated in the medium according to Bourrelly (1968). The cultures were incubated at 20 ± 1 °C under $40 \mu\text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1}$ continuous illumination for up to 14 days while shaking at 100 rpm. The cell counts were estimated in 20 μ L culture sample at 12 hour intervals with a Neubauer counting chamber (Wertheim, Germany) using a light microscope (Zeiss Primo Star). The chlorophyll a (100 μ L culture sample) content in ethanol extracts (30mins, procedure 3.2.1) was estimated spectrophotometrically using a UV-VIS spectrophotometer (Shimadzu, 1240, Japan) at 665nm as reported by Winthermans and Demots (1965).

Photoheterotrophic growth

Photoheterotrophic growth of the microalgae was performed on an orbital shaker (100 rpm) under $40\mu\text{mol photon} \times \text{m}^{-2} \times \text{s}^{-1}$ light intensity at $20 \pm 1^\circ\text{C}$. 100ml Erlenmeyer flasks containing 10ml of sterile medium as for the photoautotrophic growth experiments supplemented with 0.025M of acetate as carbon source were inoculated to an initial cell density of about 7×10^5 cells/ml. For *Parachlorella* sp. strain AA1 and *Scenedesmus* sp. strain AA2, the medium according to Chu (1942) was used while *Haematococcus* sp. strain AA3 was cultivated in the medium according to Bourrelly (1968). In an alternate approach, acetate was substituted by using 0.05M of glucose. The cell counts and the chlorophyll a content in ethanol extracts were estimated as stated above (Section 3.2.1).

3.2.3. Large scale growth of *Haematococcus* sp. strain AA3 for pigment production

A 30ml portion of 7 days old *Haematococcus* sp. strain AA3 culture (4×10^5 cells/ml) was inoculated into 200ml of fresh sterile Bourrelly medium into each of three 1L flasks, each of the flasks were incubated photoautotrophically at $20 \pm 1^\circ\text{C}$, with 24 hour illumination at $40\mu\text{mol photon} \times \text{m}^{-2} \times \text{s}^{-1}$ until the very late stationary phase. The flasks were shaken manually at once per day. Cell counts, chlorophyll a content and pigment accumulation were estimated at 48 hours intervals. Biomass comprising of encysted cells from all three flasks at the aplanosporic stage with deep reddish coloration has confirmed by phase contrast light microscopy was harvested by centrifugation at $10,000 \times g$ for 15 mins, pigment was extracted in the dark with acetone with the aid of 0.3mm sterile glass beads over 30 mins. Extraction was repeated three times until debris became colorless. The cell debris was removed by centrifugation prior to analysis. Astaxanthin content was determined spectrophotometrically using a UV-VIS spectrophotometer (Shimadzu, 1800) at 474nm according to Dragos et al. (2010)

Pigment extraction and analysis using *Parachlorella* sp. strain AA1 and *Scenedesmus* sp strain AA2

Stationary cells (5.58×10^6 cells/ml) of *Parachlorella* sp. strain AA1 grown photoautotrophically were harvested from 200ml culture by centrifugation ($10,000 \times g$ for 15 mins). Pigments were isolated from cell pellets by 30 mins of extraction in acetone in the dark using sterile glass beads (0.3mm diameter) to crush cells. The extraction procedure was repeated three times until cell debris became colorless. The presence of pigments in the combined acetone extract was determined spectrophotometrically (UV-VIS spectrophotometer, Shimadzu, 1800, Japan) between 400-700nm according to Cho (1970).

Exponential phase cells of *Scenedesmus* sp. strain AA2 (1.83×10^6 cells/ml) were inoculated into three 1000ml conical flasks each containing 200ml of fresh sterile Chu medium (Chu, 1942). The culture was incubated photoautotrophically under $40 \mu\text{mol photon} \times \text{m}^{-2} \times \text{s}^{-1}$ continuous illumination until the late stationary phase. The three flasks were manually shaken once a day. Cells were harvested by centrifugation at $10,000 \times g$ for 15 mins after microscopy and visible inspection revealed color change indicating carotenoid accumulation in cultures. The cell pigments were extracted repeatedly with acetone in the dark, after disrupting cells with 0.3mm glass beads. Pigments were analyzed spectrophotometrically as above (Section 3.2.3).

3.2.4. Chromatographic detection of pigments

Analytical-TLC was carried out on silica gel 60 TLC plates (5 cm \times 10 cm, Merck, Germany). Acetone extracts (10 μl) of *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2 and *Haematococcus* sp. strain AA3 were applied at 1 cm of the base of the TLC plate and allowed to dry for a few minutes in the dark. Afterwards, the plates were developed in a closed glass chamber using acetone/n-hexane (30% /70%) (v/v) or acetone /petroleum ether (25%/75%) (v/v) as the mobile phase according to Khanafari et al. (2007) and Jaime et al. (2010). Authentic β -carotene (Sigma Aldrich, U.S.A) was used as a standard.

For preparative TLC, pre-coated silica gel H60 thin layer chromatography plates (20 cm x 20 cm Merck, Germany) were used for the isolation of pigments at larger quantities. Acetone and n-hexane in the ratio 30%/70% (v/v) was used as mobile phase. 50 μl of highly concentrated pigment extracts (*Parachlorella* sp. AA1, *Scenedesmus* sp. AA2, *Haematococcus* sp. AA3) were spotted on the TLC plate. All separations were carried out in a closed glass chamber in the dark

according to Kobayashi et al. (1991). For the isolation of different carotenoids, distinct individual bands were scrapped from the TLC plate, eluted into 1.5ml acetone followed by centrifugation ($10,000 \times g$ for 15 mins). Absorption spectra of pigments extracted from individual bands were determined spectrophotometrically between 400nm-700nm using a UV-VIS spectrophotometer (Shimadzu, 1800, Japan) according to Dragos et al. (2010).

3.2.5. Antioxidant capacity of methanolic extracts of algae cells and of isolated pigments

Antioxidant capacity of Vitamin C

Radical scavenging ability of the known antioxidant vitamin C using 2,2-Diphenyl-1-picrylhydrazyl was determined according to the method of Blois et al. (1958). A 5mM stock solution of 2,2-diphenyl-1-picrylhydrazyl was prepared in methanol. Ascorbic acid was used as a reference antioxidant and dissolved to 10mM in methanol. In a total volume of 1ml, the assay cuvette contained 100 μ M DPPH and varying concentrations of ascorbic acid (0-2.5mM). A mixture of methanol (980 μ L) and 20 μ L of DPPH (5mM) was used as control and methanol served as blank. After 30 mins incubation in the dark, a violet to yellow color change was observed visually and the absorbance was measured at 517nm using a UV-VIS spectrophotometer (Shimadzu, 1800, Japan).

Antioxidant capacity of β -carotene

β -carotene was dissolved to 10mM in methanol. Precipitate was removed by centrifugation ($10,000 \times g$ for 5 mins). In a total volume of 1ml, the assay cuvette contained 100 μ M DPPH and varying concentrations of β -carotene (0-900 μ M). A mixture of 980 μ L of methanol and 20 μ L of DPPH (5mM) was used as control. The absorbance of the reaction mixture was measured at 517nm against a methanol blank using a UV-VIS spectrophotometer (Shimadzu, 1800, Japan) after 30 mins of incubation in the dark according to Blois et al (1958). The molar extinction coefficient of β -carotene at 450nm in methanol was taken as $136\,400\text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ (Craft and Soares, 1992).

Antioxidant capacity of cell extracts

Cells of *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2, and *Haematococcus* sp. strain AA3 after being grown photoautotrophically under continuous illumination were harvested from 10 day old plates and extracted using methanol. After 30 mins of extraction in methanol in the dark using sterile glass beads (0.3mm diameter) to crush cells, supernatants were recovered by centrifugation ($10,000 \times g$ for 5 mins). Extraction was repeated thrice until cell debris became colorless. In a total volume of 1ml, the assay cuvette contained 100 μ M DPPH and varying concentrations of cell extracts of strains AA1, AA2, AA3 in a range of 0-900 μ M based on β -carotene using the reported molar extinction at $136\,400\text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ (Craft and Soares, 1992). The reaction mixtures were incubated for 30 mins in the dark. A mixture of 980 μ L of methanol and 20 μ L of DPPH (5mM) was used as control. The absorbance of the reaction mixtures was measured at 517nm against a methanol blank using a UV-VIS spectrophotometer (Shimadzu, 1800, Japan) according to Blois et al. (1958).

Antioxidant capacity of TLC purified astaxanthin

Varying concentrations of TLC purified astaxanthin in methanol (0-900 μ M) from *Haematococcus* sp. strain AA3 was added to a 1ml assay cuvette containing 100 μ M DPPH. A mixture of 980 μ L of methanol and 20 μ L of DPPH (5mM) was used as control. The mixtures were incubated in the dark for 30 mins. The absorbance of the reaction mixture was measured at 517nm against a methanol blank using a UV-VIS spectrophotometer (Shimadzu, 1800, Japan) according to Blois et al (1958). The molar extinction coefficient of astaxanthin at 474nm in methanol ($12300\text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$) was used to quantify astaxanthin (Buchwald and Jencks, 1968).

3.3. Results

3.3.1. Optimum time for chlorophyll a extraction

For the quantification of chlorophyll a in algal biomass, 30 mins was established as optimum extraction time using an identical number of algal cells (1×10^7 cells) of all the three strains (Figure 3.1).

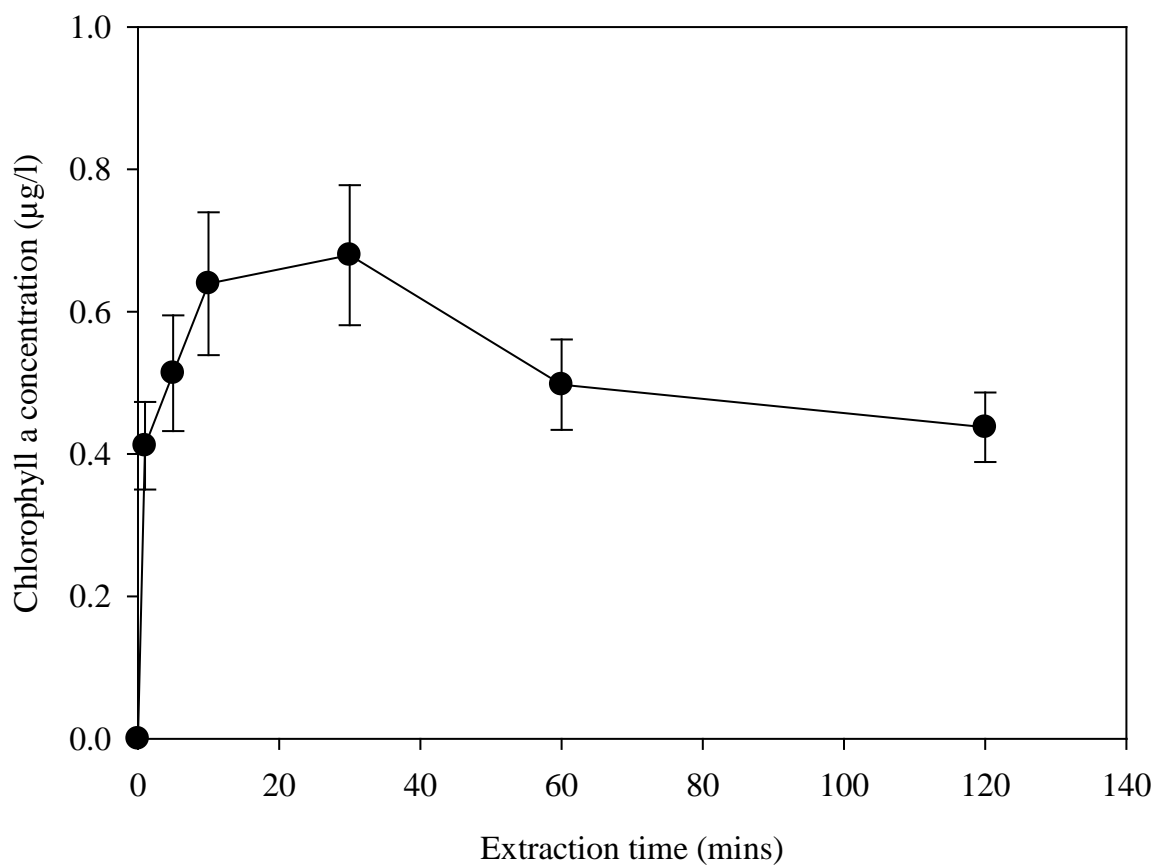


Figure 3.1. Optimum time for chlorophyll a extraction from photoautotrophically produced algal biomass of all three strains using ethanol. The bar indicates the standard error of the mean.

3.3.2. Photoautotrophic and photoheterotrophic growth of microalgal isolates

To determine the best cultivation condition for the isolated strains AA1, AA2, and AA3, the growth under photoautotrophic and photoheterotrophic conditions using glucose and acetate as substrates was analyzed over time by determining total microscopic cell counts along with chlorophyll a content. Results are shown in figures 3.2-3.5.

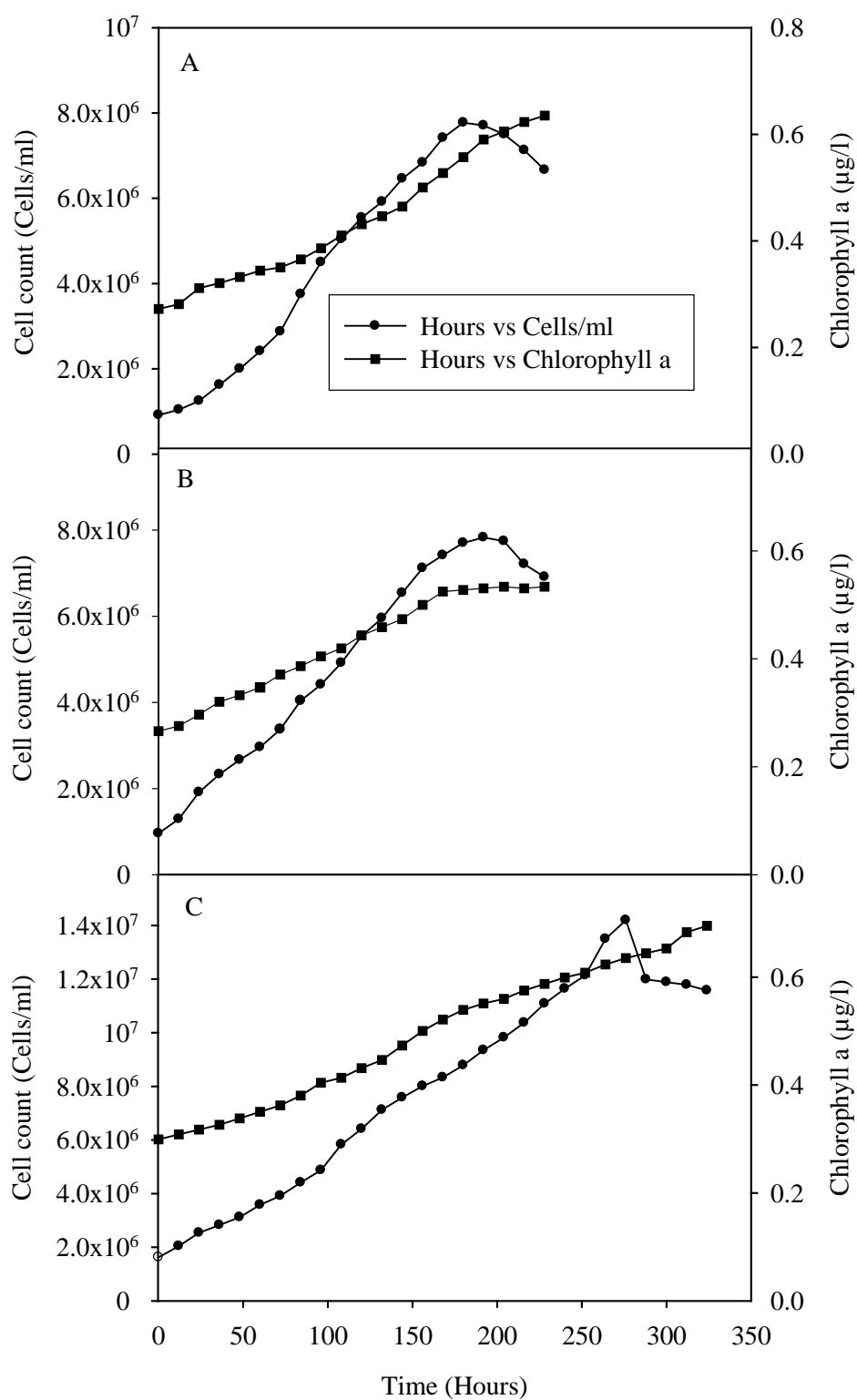


Figure 3.2. The growth of *Parachlorella* sp. strain AA1 (A), *Scenedesmus* sp. strain AA2 (B), and *Haematococcus* sp. strain AA3 (C) under photoautotrophic conditions in Chu (A, B) and Bourrelly medium (C).

Based on the cell counts, three to four growth phases were observed (Figure 3.2a -3.2c). A lag phase for about 12-24 hours in strains AA1, and AA2, an exponential phase lasting for about 132 hours in strain AA1, 144 hours in strain AA2 and 252 hours in strain AA3, followed by a stationary phase of about 50 hours in strain AA1 and 40 hours in strain AA2. A visible death phase started at 204 hours in strain AA1, at 192 hours in AA2 and at 288 hours for strain AA3.

Strain AA1 had a growth rate (μ) of 0.015 h^{-1} , a generation time of 46 hours, and a doubling time of 210 hours (based on chlorophyll a content), strain AA2 had a growth rate (μ) of 0.011 h^{-1} with a generation time of 63 hours, and a doubling time of 187 hours (based on chlorophyll a content). For strain AA3, the growth rate (μ) was 0.009 h^{-1} with a generation time of 72 hours, and a doubling time of 203 hours (based on chlorophyll a content). Low growth rates were recorded under photoautotrophic conditions for all the three isolates, indicating the need for carbon sources to improve growth rates.

Effect of acetate on the growth of the three isolated strains

The effect of 0.025M of acetate as additional carbon source on growth rates and on chlorophyll a content was determined in the three isolated strains.

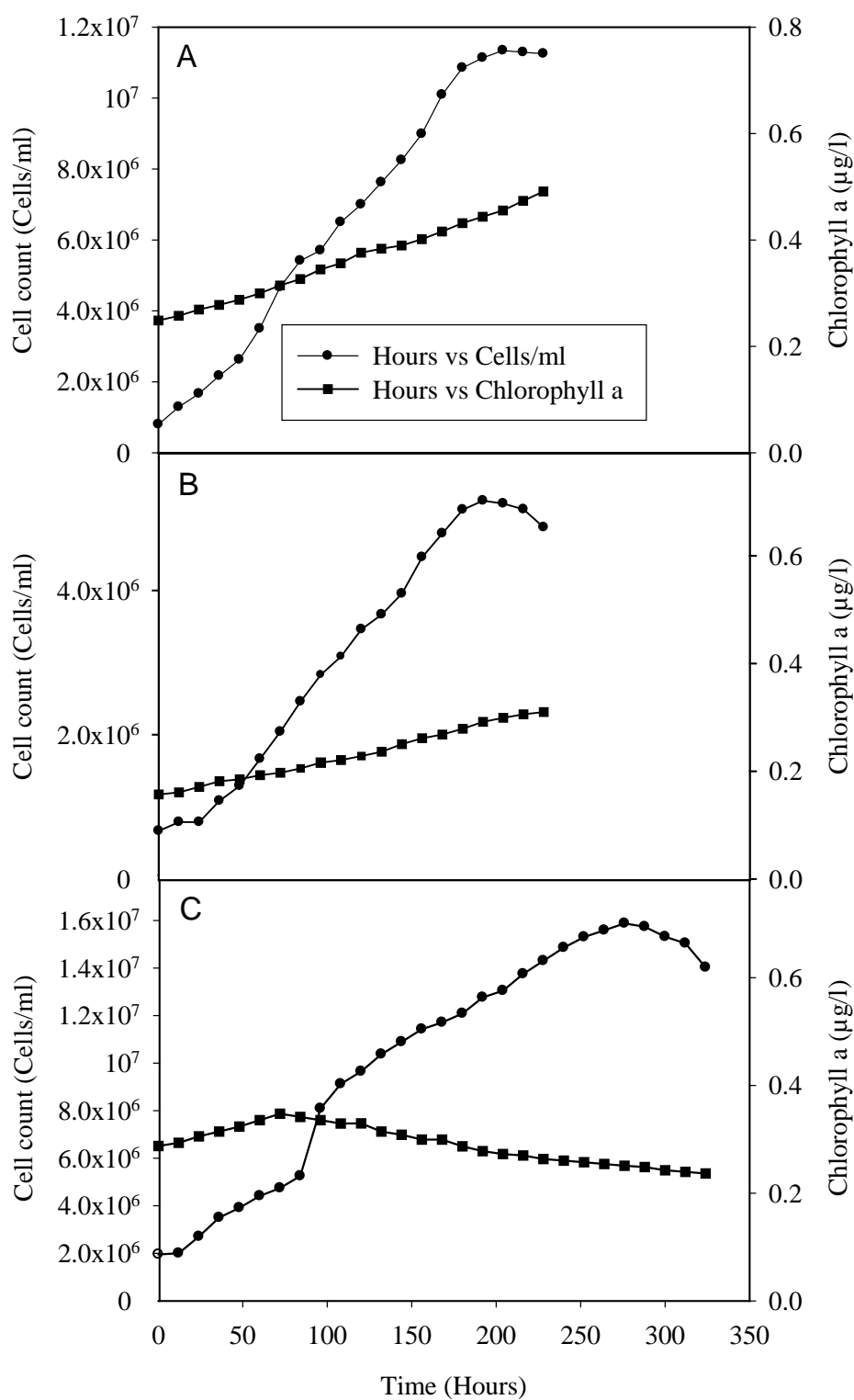


Figure 3.3. Photoheterotrophic growth of *Parachlorella* sp. strain AA1 (A), *Scenedesmus* sp. strain AA2 (B), and *Haematococcus* sp. strain AA3 (C), with 0.025M acetate as the carbon source in Chu (A, B) and Bourrelly medium (C).

As expected, the cell numbers increased for all three algal strains (Figure. 3.3A-C). However, in case of chlorophyll a content, a decreasing chlorophyll a concentration was observed in *Haematococcus* sp. strain AA3 from about 80 hours. The exponential phase in strain AA1 lasted until about 168 hours followed by a stationary phase. Strain AA1 had a specific growth rate (μ) of 0.020 h^{-1} , a generation time of about 35 hours with a doubling time of 210 hours based on chlorophyll a content.

For *Parachlorella* sp. strain AA1 and *Scenedesmus* sp. strain AA2, cell counts increased concomitant with the increase in chlorophyll a content over time in the presence of acetate (Figure 3.3 A and B). *Scenedesmus* sp. strain AA2 grew with 0.025M acetate as carbon source at a growth rate (μ) of 0.019 h^{-1} , generation time of about 36 hours with a doubling time of 216 hours based on chlorophyll a content.



Figure 3.4. Cells of *Haematococcus* sp. strain AA3 from the late growth stage (276 hours) when grown with 0.025M acetate as the carbon source photoheterotrophically in Bourrelly medium.

As for strain AA1 and AA2, growth of isolate AA3 with acetate represented a typical growth curve, the lag phase lasted for about 24 hours, followed by an exponential phase of about 264 hours, a stationary phase that started at about 276 hours and was directly followed by death phase starting after about 288 hours (Figure 3.3C). While cell numbers increased, the chlorophyll a content already decreased after about 80 hours, indicating inhibition of chlorophyll a synthesis

which was not observed in the other two strains analyzed. Greenish-orange color indicating carotenoid accumulation was observed in the aplanosporic cells from the stationary phase (Figure 3.4). A specific growth rate (μ) of 0.015 h^{-1} with a generation time of 46 hours, and a doubling time of 256 hour (based on chlorophyll a content) was recorded.

Effect of glucose on the growth rate of the three isolated strains

To determine the effects of glucose when present as additional carbon source, the isolated strains were grown photoheterotrophically in the presence of 0.05M of glucose.

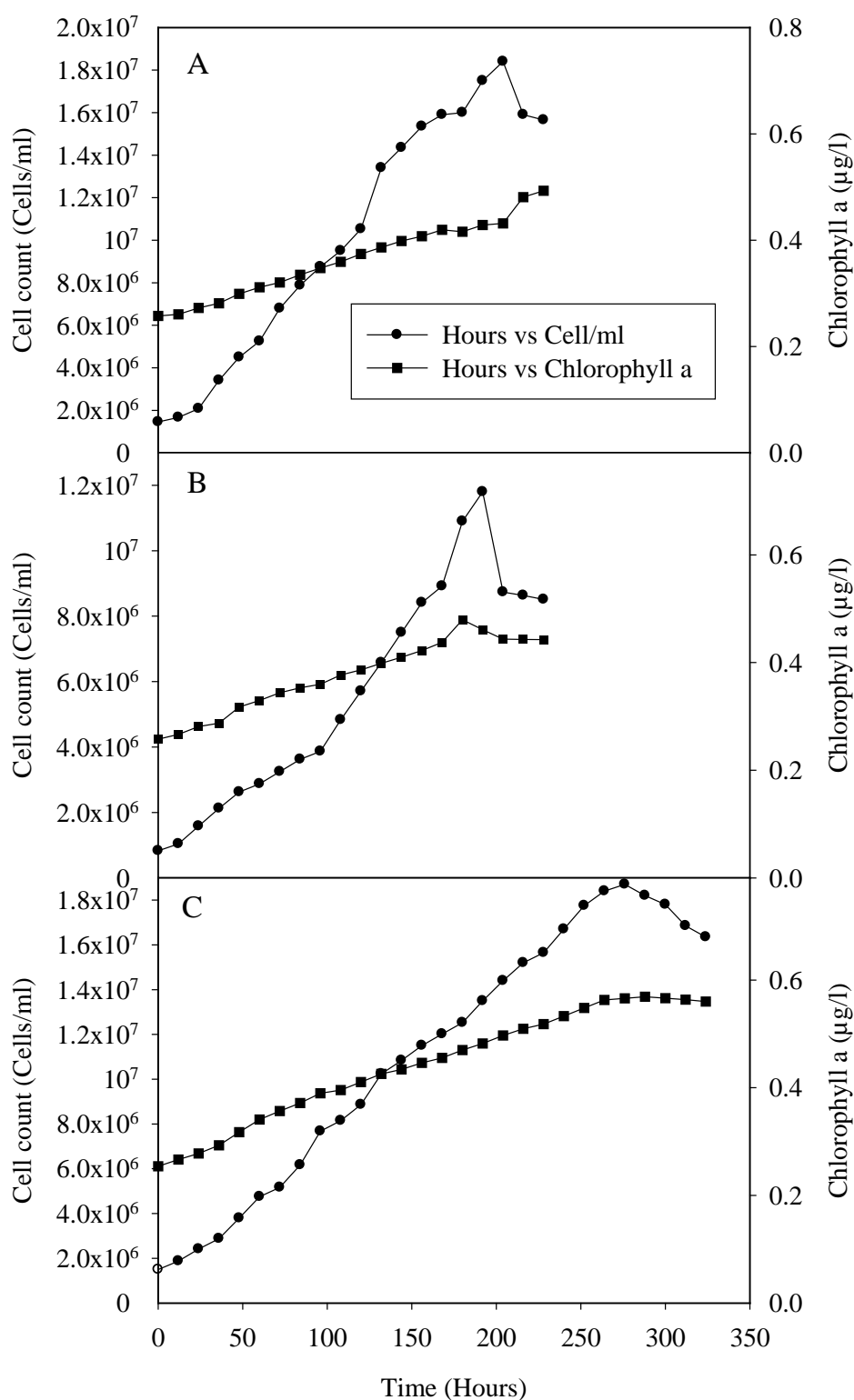


Figure 3.5. Photoheterotrophic growth of *Parachlorella* sp. strain AA1 (A), *Scenedesmus* sp. strain AA2 (B), and *Haematococcus* sp. strain AA3 (C) with 0.05M glucose as the carbon source in Chu (A, B) and Bourrelly medium (C).

In the presence of glucose, cell counts and chlorophyll a content increased overtime for all three microalgal strains. However, for strain AA1, chlorophyll a content apparently increased even though cell counts decreased after about 216 hours (Figure 3.5A).

With 0.05M of glucose, under photoheterotrophic conditions strain AA1 had a growth rate (μ) of 0.017 h^{-1} and a generation time of about 41 hours. The doubling time was established as 223 hours based on the chlorophyll a content. The photoheterotrophic growth rate of *Parachlorella* sp. strain AA1 was lower with glucose (0.017 h^{-1}) than with acetate (0.020 h^{-1}) but higher than under photoautotrophic conditions with no carbon source (0.015 h^{-1}). This implies that glucose enhanced growth for strain AA1 to a lesser degree than acetate.

Strain AA2 had a growth rate (μ) of 0.012 h^{-1} , a generation time of about 58 hours with a doubling time of 266 hours based on chlorophyll a content. This showed that glucose enabled only a slightly faster increase in cell number over time under photoheterotrophic conditions when compared to photoautotrophic growth conditions.

When strain AA3 was grown in Bourrelly medium with 0.05M glucose, cell numbers increased along with chlorophyll a content overtime (Figure 3.5C), which is different from photoheterotrophic growth with acetate, where chlorophyll a biosynthesis was clearly inhibited after about 80 hours. Strain AA3 had a growth rate (μ) of 0.016 h^{-1} , a generation time of about 43 hours and a doubling time of 266 hours based on chlorophyll a content.

Table 3.1. Growth kinetic data for all three isolates under photoautotrophic and photoheterotrophic conditions using acetate and glucose supplemented medium based on cell counts.

Isolate	No supplement μ (h ⁻¹)	Generation time (Hrs)	Acetate supplemented media μ (h ⁻¹)	Generation time (Hrs)	Glucose supplemented media μ (h ⁻¹)	Generation time (Hrs)
<i>Parachlorella</i> sp. AA1	0.015	46.20	0.020	34.65	0.017	40.70
<i>Scenedesmus</i> sp. AA2	0.011	63.01	0.019	36.48	0.012	57.76
<i>Haematococcus</i> sp. AA3	0.009	72.20	0.015	46.20	0.016	43.32

In summary, slower growth was recorded for *Parachlorella* sp. strain AA1 under photoautotrophic conditions in comparison to photoheterotrophic with acetate and growth with glucose. Acetate enhanced growth in *Scenedesmus* sp. strain AA2 compared to glucose, and glucose in turn enabled better growth than no carbon supplement. Glucose, however, enhanced growth of *Haematococcus* sp. strain AA3 more than acetate, which in turn enabled higher growth than photoautotrophic conditions.

3.3.3. Pigment production

Carotenoid detection in cells of *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2 and *Haematococcus* sp. strain AA3

To verify the presence of carotenoids in *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2, and *Haematococcus* sp. strain AA3, pigments were extracted in the dark with acetone from about 32mg and 20mg wet weight of cell biomass, respectively collected after photoautotrophic growth (late growth phase), as photoautotrophically grown cells enabled higher carotenoid accumulation. The extracts were analyzed with UV-VIS spectroscopy over a range of 400 to 700nm. Maxima were detected at a wavelength of 450nm (shoulder at 437nm, 475nm) and 665nm, indicating the presence of carotenoids and chlorophyll a.

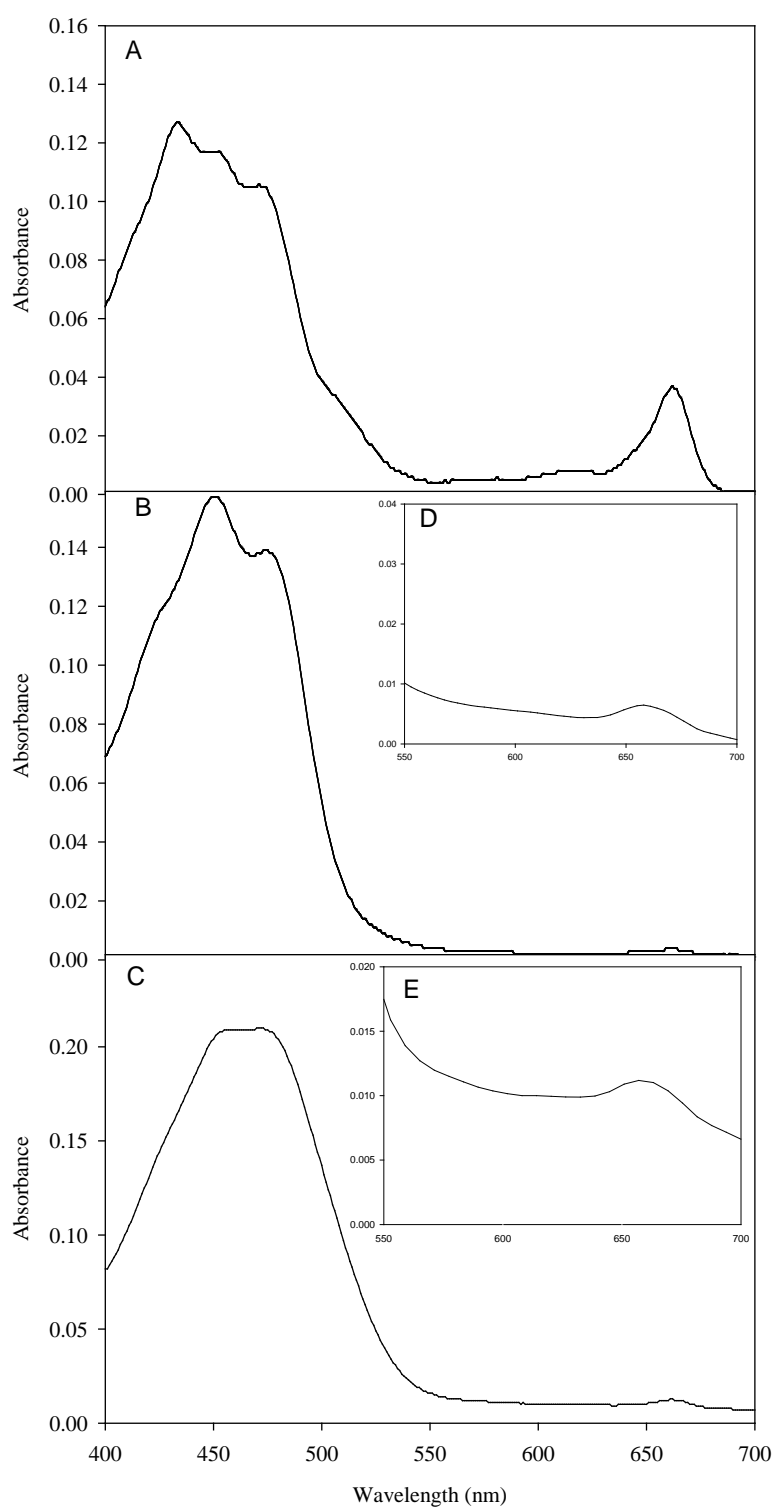


Figure 3.6. The visible absorption spectrum of diluted *Parachlorella* sp. strain AA1 (A), *Scenedesmus* sp. strain AA2 (B), and *Haematococcus* sp. strain AA3 (C) acetone extracts of cell biomass grown photoautotrophically and harvested in the late stationary phase. The inserts D and E shows the region of chlorophyll *a* absorbance.

Figure 3.6A shows an absorbance maximum at 450nm plus shoulders at about 437nm and 475nm, indicating the presence of β -carotene in addition to the presence of chlorophyll a indicated by the maximum at 665nm. These spectral properties match the literature spectroscopic data for β -carotene and chlorophyll a in acetone. The visible spectrum of acetone extract from *Scenedesmus* sp. strain AA2 (Figure.3.6B) indicated a higher ratio of β -carotene (A450nm) to chlorophyll a (665nm) content than in strain AA1. Based on the absorbance at 450nm (β -carotene) and at 665nm (chlorophyll a), the apparent β -carotene to chlorophylla ratio was about 30 in strain AA2 and only about 3 in strain AA1. The spectrum for *Haematococcus* sp. strain AA3 (Figure 3.6C) indicated low chlorophyll a content at 665nm and a maximum at 474nm, indicating astaxanthin accumulation. To verify the presence of pigments in acetone extracts, analytical TLC was performed.



Figure 3.7. Analytical thin layer chromatography of *Parachlorella* sp. strain AA1 cell pigment extract in acetone using two different solvent systems.

(A) Mobile phase = Acetone and n-hexane (30/70%) (v/v).

(Band 1= Pheophytin like compound, band 2= Chlorophyll a, band 3= Possible canthaxanthin isomer, band 4= β -carotene, band 5= Authentic β - carotene)

(B) Mobile phase = Acetone and petroleum ether (25/75%) (v/v).

(Band 1= Pheophytin like compound, band 2= Chlorophyll a, band 3= Possible canthaxanthin isomer, band 4= β -carotene, band 5= Authentic β - carotene)

Figure 3.7 shows major pigments detected by TLC in *Parachlorella* sp. strain AA1 acetone extracts subsequently characterized by their R_f values and spectral properties after isolation from preparative TLC using an acetone/n-hexane solvent systems. Authentic β -carotene served as standard. Two major carotenoids were detected, band 4 with the yellow coloration was identified as β -carotene with a R_f value of 0.95 in the two solvent systems while band 3 matched literature data for canthaxanthin isomers with a R_f value of 0.60 and 0.65 in acetone/ n-hexane and acetone/ petroleum ether respectively. Band 2 with greenish colouration was identified as chlorophyll a (R_f = 0.53) in acetone /n-hexane mobile phase and (R_f = 0.56) in petroleum ether/ acetone solvent, as identical R_f values were established for authentic chlorophyll a from spinach. The first band (R_f =

0.42) in acetone/n- hexane and ($R_f = 0.45$) in petroleum ether/ acetone as mobile phase corresponded to pheophytin-like compounds that result from the partial degradation of chlorophyll a (Cho, 1970).

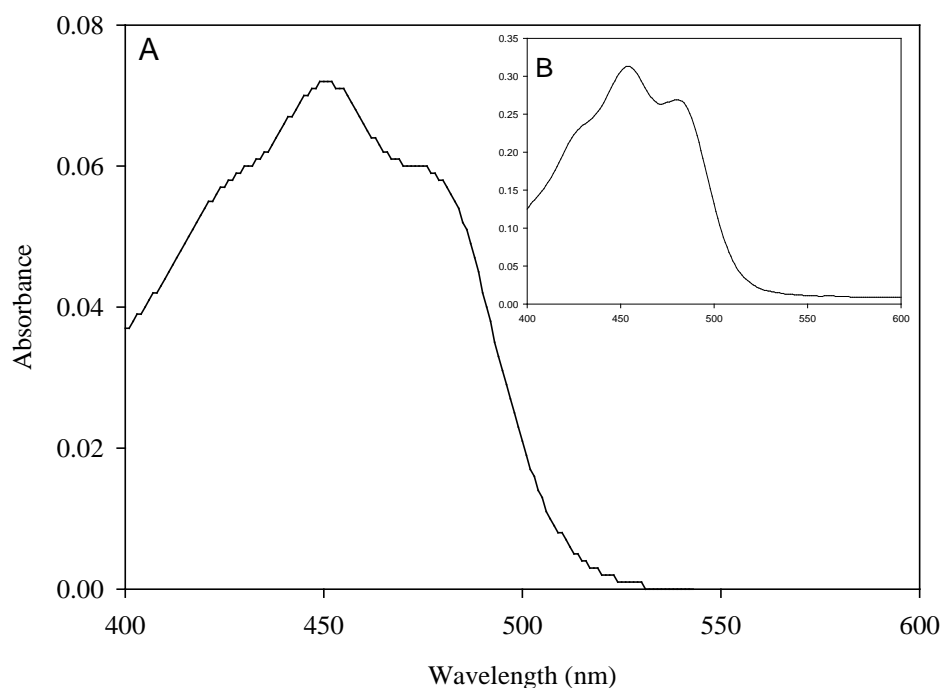


Figure 3.8. The visible absorption spectrum of the major pigment isolated by preparative TLC from *Parachlorella* sp. strain AA1 acetone extract with an R_f value of 0.95 in acetone/n-hexane (30/70%)(v/v). Spectra were measured in acetone.

(A) β -carotene (maximum at 450nm, shoulder at 437nm and 475nm)

(B) Authentic β -carotene (maximum at 450nm, shoulder at 435nm and 475nm).

Scenedesmus sp. strain AA2 cells from the late stationary phase showed by light microscopy that carotenoids had accumulated in the cells. A change of cultures from green to light orange at the end of the stationary phase in cultures of *Scenedesmus* sp. strain AA2 was therefore regarded as an indicator for the accumulation of carotenoids. This was also shown by the increased A450nm/A665nm ratio in the late stationary phase (Figure 3.6B).



Figure 3.9. Analytical thin layer chromatography of *Scenedesmus* sp. strain AA2 cell pigment extract in acetone using two different solvent systems.

(A) Mobile phase = Acetone and n-hexane (30/70%) (v/v).

(Band 1= Pheophytin like compound, band 2= unknown carotenoid, band 3= β -carotene, band 4= Authentic β -carotene).

(B) Mobile phase = Acetone and petroleum ether (25/75%) (v/v).

(Band 1= Pheophytin like compound, band 2= unknown carotenoid, band 3= β -carotene, band 4= Authentic β -carotene).

Three distinct bands were detected chromatographically when using acetone/n-hexane (30/70%) as mobile phase (figure 3.9A). Band 1 ($R_f = 0.42$) was probably a pheophytin-like compound resulting from the partial degradation of chlorophyll a, while the second band showed a R_f of 0.58. The third yellowish band was identified as β -carotene ($R_f = 0.95$). However, when using acetone and petroleum ether as mobile phase (25/75%). The first band matches with $R_f = 0.40$ while the second band showed a R_f of 0.53. The third band was identified as β -carotene ($R_f = 0.95$). To obtain further information about the pigments produced, the major bands eluted from acetone were analyzed with UV- VIS spectroscopy after isolating these using preparative TLC and acetone and n-hexane as mobile phase.

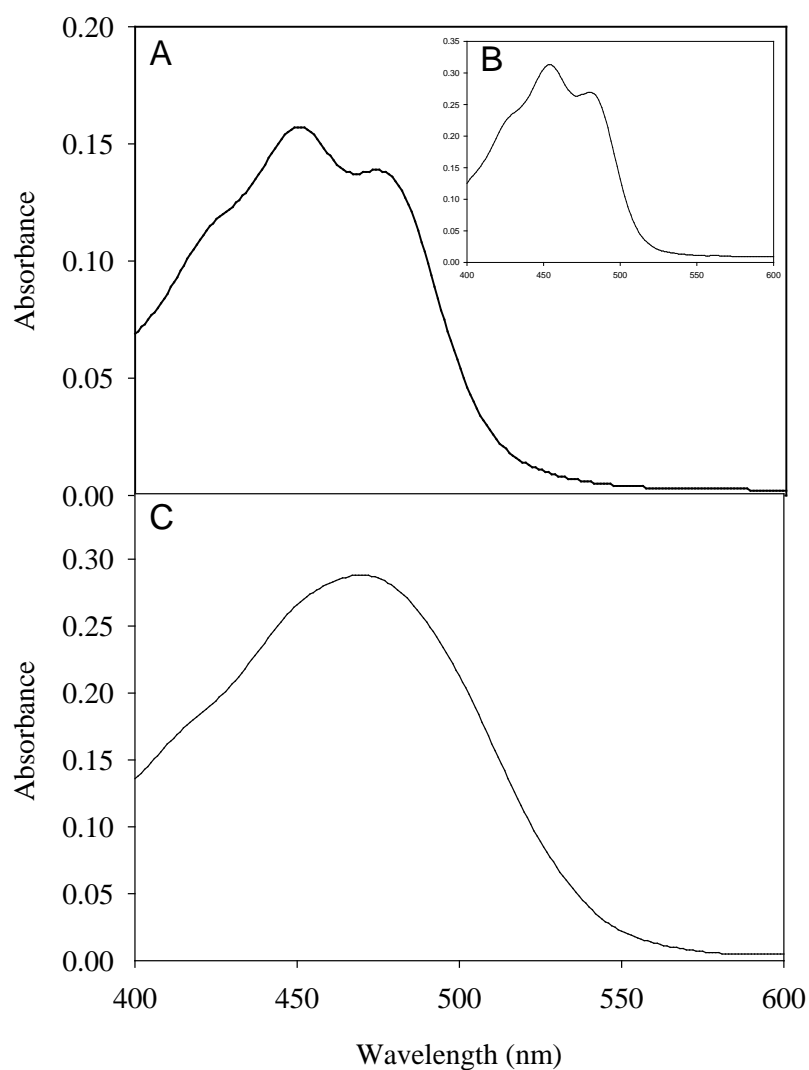


Figure 3.10. The visible absorption spectrum of two major pigments isolated by preparative TLC from *Scenedesmus* sp. strain AA2 acetone extract with R_f values of 0.95 and 0.58 using acetone and n-hexane (30/70%) as mobile phase. Spectra were measured in acetone.

(A) β -carotene (maximum at 450nm, shoulder at 437 and 475nm).

(B) Authentic β -carotene (maximum at 450nm, shoulder at 435nm and 475nm).

(C) Unknown carotenoid (maximum at about 469nm).

The visible absorption spectrum and retention values of pigments obtained from acetone extracts confirmed the presence of β -carotene and indicated the presence of one additional carotenoid, which might represent hydroxyechinenone based on the R_f value (figure 3.9) in *Scenedesmus* sp. strain AA2.



Figure 3.11. Bright field light microscopy of pigmented aplanosporic cells from the late stationary growth phase of *Haematococcus* sp. strain AA3 grown photoautotrophically in Bourelly medium.

Cells from the late stationary phase of the large scale cultivation experiment with *Haematococcus* sp. strain AA3 showed by light microscopy that carotenoids had accumulated (Figure 3.11). The aplanosporic cells of strain AA3 appeared orange/reddish and had an average diameter of about 25 μm in the late stationary growth phase. This indicates the ability of strain AA3 to produce and to accumulate carotenoids of beneficial importance for human and animals use. Figure 3.12 reveals pigment ratio accumulation for 3 weeks.

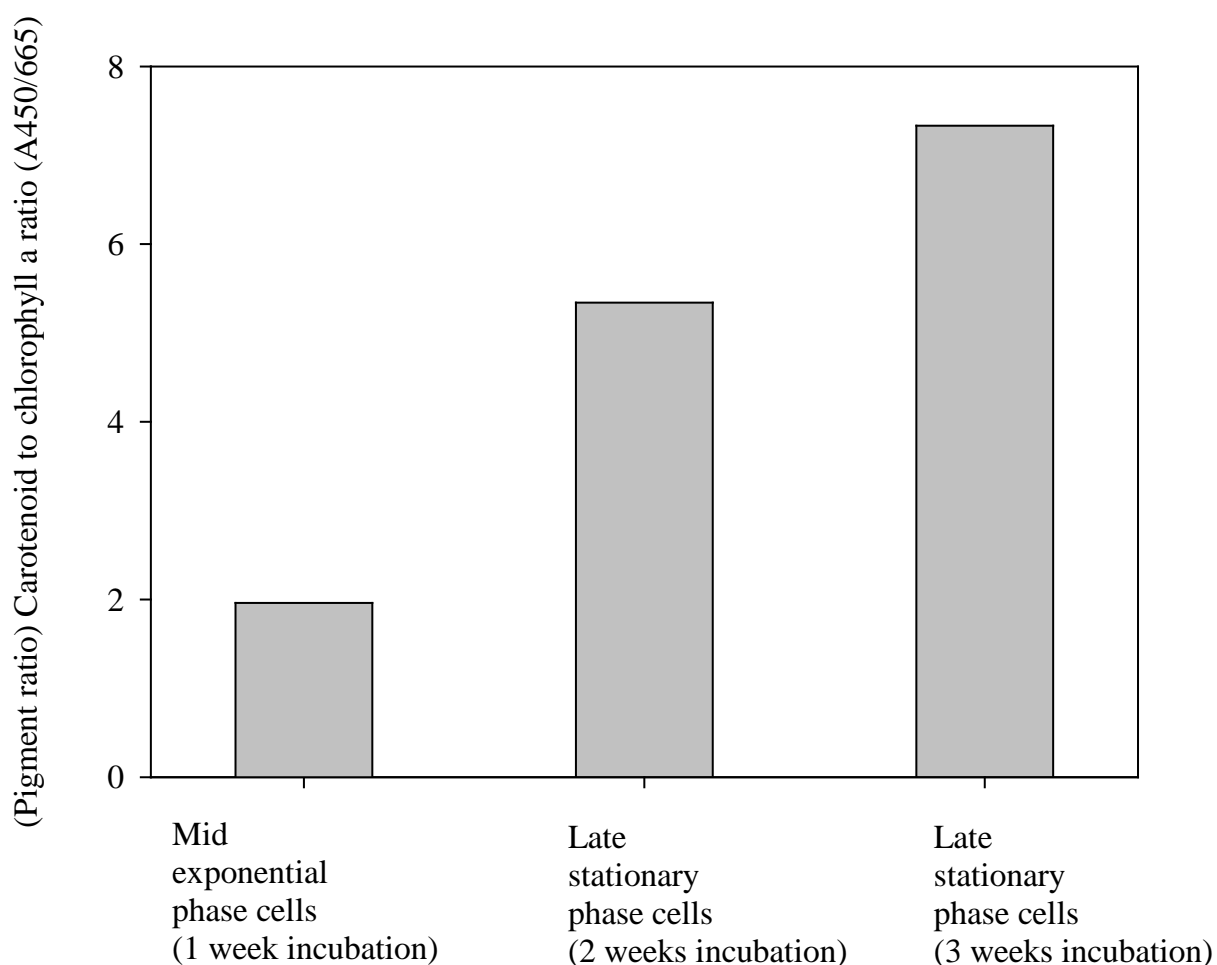


Figure 3.12. Pigment ratio comparison between *Haematococcus* sp. strain AA3 cell extracts from the mid exponential growth phase to the late stationary growth phase.

The analysis of *Haematococcus* sp. strain AA3 acetone extracts indicated a shift in the ratio of chlorophyll a content based on the maximum at 665nm to carotenoids based on the maximum at 450nm representing β -carotene. An increased ratio of β -carotene to chlorophyll a content was recorded at the late stationary phase under $40\mu\text{mol photon} \times \text{m}^{-2} \times \text{s}^{-1}$ continuous illumination. In addition, figure 3.12 was mainly for large scale pigment production that requires high volume of biomass reflecting the hydrophobicity of strain AA3 cells in comparison to figure 3.6 that was majorly for growth rate determination and higher pigment ratio was recorded due to low volume of biomass used.

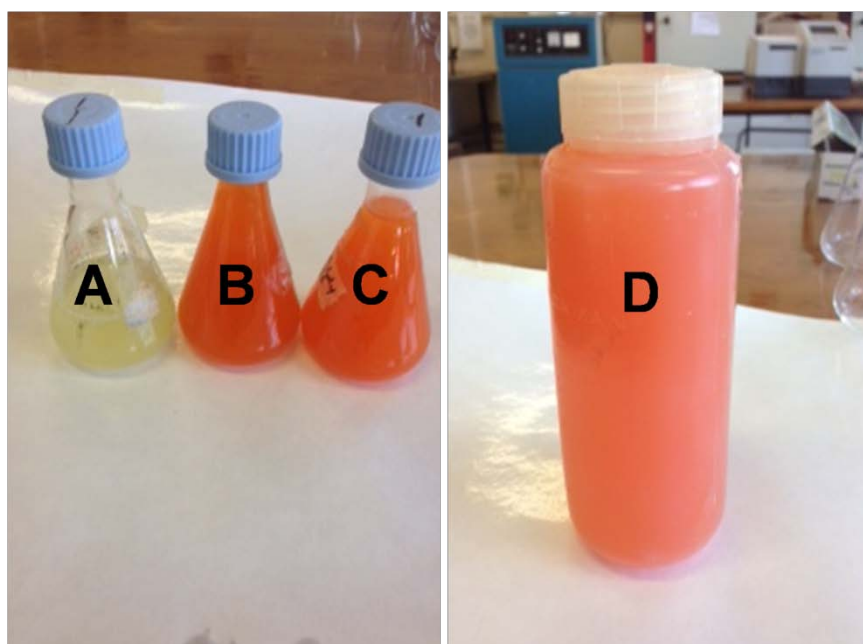


Figure 3.13. Visible carotenoid accumulation in cell suspensions of *Haematococcus* sp. strain AA3 grown photoautotrophically under continuous illumination in Bourrelly medium.

- (A) Early growth phase culture sample of *Haematococcus* sp. strain AA3.**
- (B) Stationary phase culture sample of *Haematococcus* sp. strain AA3.**
- (C) Late stationary phase culture sample of *Haematococcus* sp. strain AA3.**
- (D) Late stationary culture sample of *Haematococcus* sp. strain AA3 at time of harvesting.**

The shift in the pigment ratio shown in figure 3.12 was evident in the coloration of cultures as well (Figure 3.13). In the early growth stages with mostly biflagellated cells, cultures appeared green due to higher chlorophyll a content while older cultures showed a strong orange-red coloration, highlighting the accumulation of carotenoids in the aplanospores.

Preparative thin layer chromatography revealed the presence of five distinct pigment bands in *Haematococcus* sp. strain AA3 acetone extract obtained from cells at the late stationary growth phase (Figure 3.14).

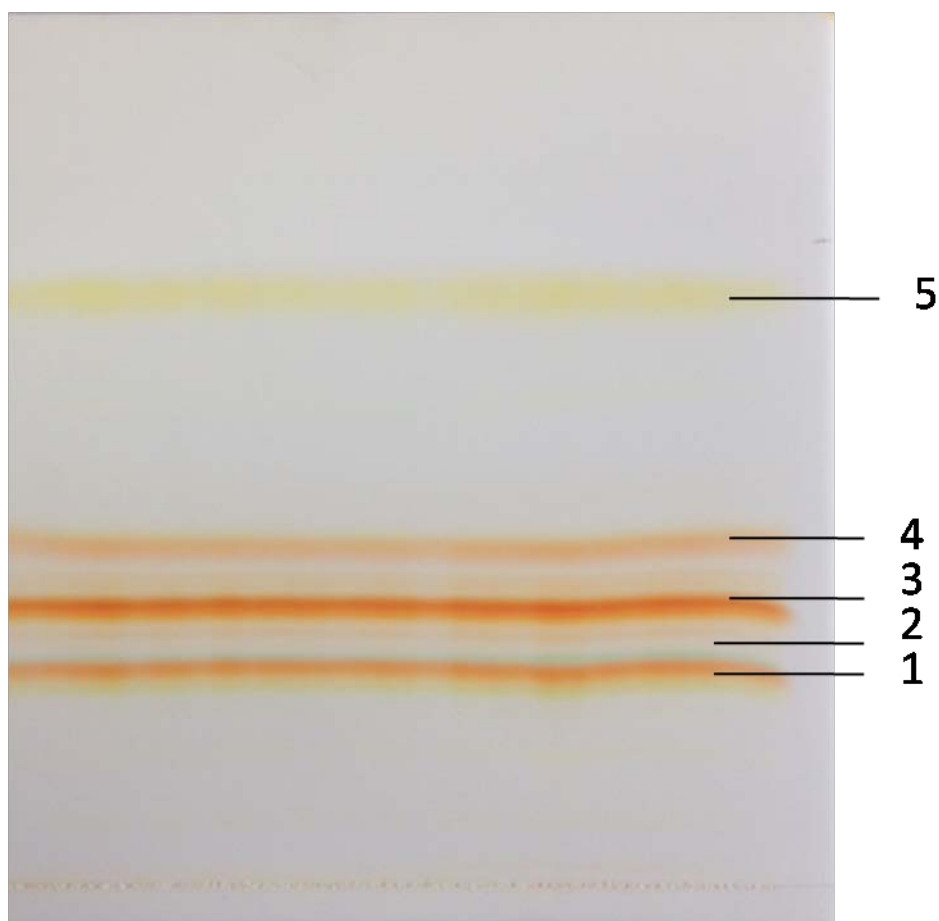


Figure 3.14. Preparative thin layer chromatography showing five major carotenoids present in *Haematococcus* sp. strain AA3 acetone extract. Acetone and n-hexane (30/70) (v/v) were used as the mobile phase.

(band 1=lutein, band 2 = unknown carotenoid, band 3= astaxanthin, band 4= unknown carotenoid, band 5= β -carotene).

Pigments from *Haematococcus* sp. strain AA3 were further analyzed via analytical thin layer chromatography for efficient separation and detection of carotenoids via their retention values (R_f) in two different solvent systems (Figure 3.15).

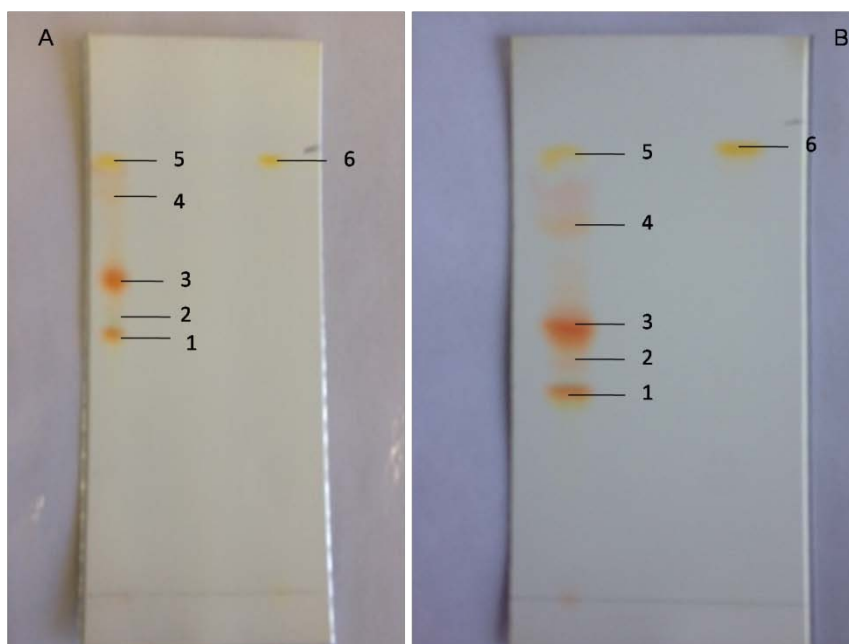


Figure 3.15. Analytical thin layer chromatography of *Haematococcus* sp strain AA3 extracts using two different solvent systems. Authentic β -carotene served as standard.

(A) Mobile phase = Acetone and n-hexane (30/70%) (v/v).

(band 1=lutein, band 2= unknown carotenoid, band 3= astaxanthin, band 4 = unknown carotenoid, band 5= β -carotene, band 6= authentic β -carotene).

(B) Mobile phase = Acetone and petroleum ether (25/75%) (v/v).

(band 1=lutein, band 2= unknown carotenoid, band 3= astaxanthin, band 4= unknown carotenoid, band 5= β -carotene, band 6= authentic β -carotene).

Using acetone/n-hexane as mobile phase (30/70%), lutein was identified using analytical TLC with a R_f value of 0.54, the second band had a R_f value of 0.56. The strong third reddish band was identified as astaxanthin (R_f value of 0.71) while the fourth band matched R_f value of 0.91. The fifth band was assigned to β -carotene ($R_f = 0.95$). Using the acetone and petroleum ether mobile phase, the first yellow band was identified as lutein with a R_f value of 0.42 while the second band had a R_f value of 0.46. Based on the R_f values and spectroscopic properties the third band was identified as astaxanthin ($R_f = 0.52$). The fourth band had a $R_f = 0.76$ while the fifth band was identified as β -carotene ($R_f = 0.95$).

Table 3.2. Retention value and absorption maximum (λ_{max} , nm) of the major carotenoid in *Parachlorella* sp. strain AA1 acetone extracts using two different solvent systems. Standard R_f values were obtained by using authentic reference material (β -carotene) and literature values. Solvent a; acetone/n-hexane (30:70%, v/v), solvent b; acetone/petroleum ether (25:75%, v/v).

Carotenoid	Retention value (Rf) solvent a	Literature (Rf) value *solvent a	Retention value (Rf) solvent b	Literature (Rf) value **solvent b	λ_{max} (nm) in acetone	Literature λ_{max} (nm) in acetone ***
Authentic β -carotene	0.98	0.98	0.95	0.95	450	450
β -carotene	0.95	0.98	0.95	0.95	450	450

*. Qin et al. (2008). Process Biochemistry, 43(8), 795-802.

**. Jaime et al. (2010). Food Science and Technology, 43(1), 105-112.

*** Cho, (1970). Biochimica et Biophysica Acta (BBA)-Bioenergetics, 216(1), 139-150.

Table 3.3. Retention value and absorption maximum (λ_{\max} , nm) of two major carotenoids in *Scenedesmus* sp. strain AA2 acetone extracts using two different solvent systems. Standard R_f values were obtained by using authentic reference material (β -carotene) and literature values. Solvent a; acetone/n-hexane (30:70%, v/v), solvent b; acetone/petroleum ether (25:75%, v/v).

Carotenoid	Retention value (Rf) solvent a	Literature (Rf) value *solvent a	Retention value (Rf) solvent b	Literature (Rf) value **solvent b	λ_{\max} (nm) in acetone	Literature λ_{\max} (nm) in acetone ***
Authentic β -carotene	0.98	0.98	0.95	0.95	450	450
β -carotene	0.95	0.98	0.95	0.95	450	450
Unknown carotenoid	0.58	0.58 ⁺	0.53	0.54 ⁺⁺	469	463 ⁺⁺⁺

* Qin et al. (2008). Process Biochemistry, 43(8), 795-802.

** Jaime et al. (2010). Food Science and Technology, 43(1), 105-112.

*** Dragos et al. (2010). Romanian Society for Cell Biology, 15(2), 353-361.

+ Qin et al. (2008). Process Biochemistry, 43(8), 795-802, reported this R_f value for hydroxyechinenone.

++ Krinsky et al. (1964). Plant Physiology, 49(3), 441- 447, reported this R_f value for hydroxyechinenone.

+++ Dragos et al. (2010). Romanian Society for Cell Biology, 15(2), 353-361, reported this absorbance maximum for hydroxyechinenone.

Table 3.4. Retention value and absorption maximum (λ_{max} , nm) of four major carotenoids identified in *Haematococcus* sp. strain AA3 acetone extracts using two different solvent systems. Standard R_f values were obtained by using authentic reference material (β -carotene) and literature values. Solvent a; acetone/n-hexane (30:70%, v/v), solvent b; acetone/petroleum ether (25:75%, v/v).

Carotenoid	Retention value (R_f) solvent a	Literature value (R_f) * solvent a	Retention value (R_f) solvent b	Literature value (R_f) ** solvent b	λ_{max} (nm) in acetone	Literature λ_{max} (nm) in acetone ***
Authentic β -carotene	0.98	0.98	0.95	0.95	450	450
β -carotene	0.95	0.98	0.95	0.95	450	450
Astaxanthin	0.71	0.75	0.52	0.50	474	474
Lutein	0.54	0.55	0.42	0.47	446	446
Unknown carotenoid	0.91	0.87 ⁺	0.76	0.76 ⁺⁺	475	462 ⁺⁺⁺

* Khanafari et al. (2007). Journal of Environmental Health Science and Engineering, 4(2), 93-98.

** Jaime et al. (2010). Food Science and Technology, 43(1), 105-112.

*** Dragos et al. (2010). Romanian Society for Cell Biology, 15(2), 353-361.

+ Khanafari et al. (2007). Journal of Environmental Health Science and Engineering, 4(2), 93-98 reported this R_f value for echinenone.

++ Jaime et al. (2010). Food Science and Technology, 43(1), 105-112, reported this R_f value for echinenone

+++ Dragos et al. (2010). Romanian Society for Cell Biology, 15(2), 353-361, reported this absorbance maximum for echinenone.

From above tables 3.2- 3.4, β -carotene was identified has the major common carotenoid pigment in the three isolated microalgal strains at a retention value (R_f) value of 0.98 and 0.95 in acetone/petroleum ether (25/75) and acetone/n-hexane (30/70). Lutein and astaxanthin were detected in strain AA3 in the two different solvent systems while the unknown carotenoid identified in strain AA2 was possibly hydroxyechinenone based on the R_f value albeit its spectral data did not match exactly. The R_f value of the unknown carotenoid in strain AA3 corresponded with echinenone although its 2 max differed from the values reported in literature. The absorption

spectrum of β -carotene isolated from the three strains showed a maximum at 450nm, while lutein from strain AA3 showed an absorbance maximum at 446nm and astaxanthin at 474nm.

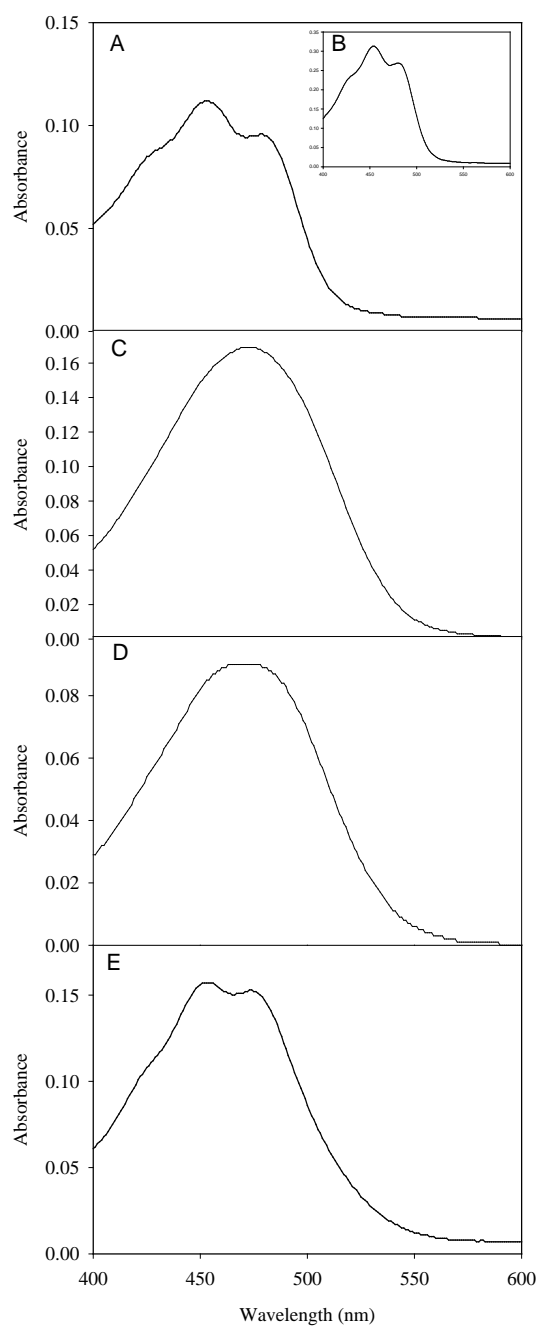


Figure 3.16. The absorption spectra of pigments isolated using preparative TLC and *Haematococcus* sp. strain AA3 acetone extract. Spectra were measured in acetone

- (A) β -carotene** (maximum at 450nm, shoulder at 437nm and 475nm).
- (B) Authentic β -carotene** (maximum at 450nm, shoulder at 435nm and 475nm).
- (C) Unknown carotenoid**, (maximum at about 475nm).
- (D) Astaxanthin** (maximum at 474nm).
- (E) Lutein** (maximum at 446nm, shoulder at 475nm).

The detection of carotenoids in cell extracts of all three strains confirmed their potential to produce value compounds (Table 3.2 - 3.4). The visible absorption spectra of the four major pigments detected in *Haematococcus* sp. strain AA3 are evidence of the strains ability to produce beneficial natural carotenoid pigments.

3.3.4. Evaluation of antioxidant activity

All three microalgal strains are a natural source of bioactive compounds such as carotenoids, which are known antioxidants. Therefore, the antioxidant capacity of isolated pigments and cell extracts was assessed using the DPPH assay.



Figure 3.17. Demonstration of the antioxidant capacity of varying concentrations of vitamin C in the DPPH assay causing decolorization of the stable organic radical after 30 mins of incubation in the dark. Left cuvette (0mM vitamin C) to right cuvette (2mM vitamin C).

The antioxidant capacity was further determined using UV-VIS spectrometry at an absorbance of 517nm (Figure 3.18).

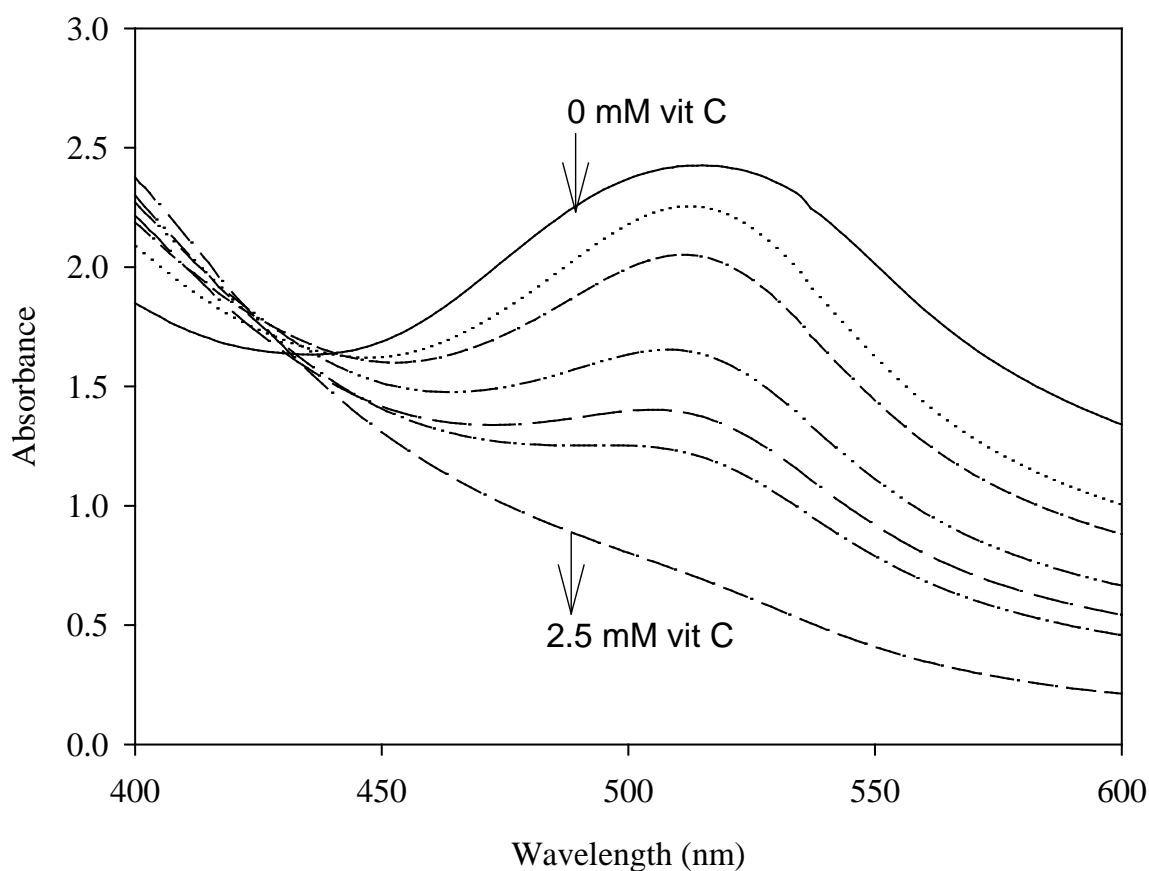


Figure 3.18. Spectrophotometric demonstration of the antioxidant capacity of Vitamin C by using the stable organic radical DPPH.

The reduction of the stable radical DPPH in the presence of varying concentrations of vitamin C compared to the control without vitamin C indicated hydrogen atom donation causing a decrease in absorbance at 517nm. The EC_{50} value for vitamin C was established as $150\mu\text{M}$ based on assay results.

Similarly, the antioxidant effect of authentic β -carotene was determined by its ability to scavenge the stable DPPH radical. After 30 mins of incubation in the dark, decolorization was observed when compared to the control without β -carotene, showing that the antioxidant had scavenged the radical. The EC_{50} was established as the concentration of β -carotene required to scavenge 50% DPPH as determined at A_{517} relative to a control mixture to which only methanol was added, resulting in an EC_{50} value of $225\mu\text{M}$. For *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2, and *Haematococcus* sp. strain AA3, algae cells grown photoautotrophically to the exponential phase were extracted with methanol over 30 mins, particular matter was removed by

centrifugation and the supernatant used in the DPPH assay. Non-diluted methanolic extracts of *Parachlorella* sp. strain AA1 were able to completely decolourize 100µM of DPPH within 30 mins of incubation in the dark. This indicates that *Parachlorella* strain AA1 methanolic cell extracts possess antioxidant capacity.

The EC₅₀ was established as the concentration of *Parachlorella* sp. strain AA1 extract based on β-carotene required to produce 50% reduction at A₅₁₇ relative to the control (only methanol) using the reported molar extinction coefficient for β-carotene at 450nm in methanol taken as 136 400 L × mol⁻¹ × cm⁻¹ (Craft and Soares, 1992). This resulted in an EC₅₀ value of 330µM. For *Scenedesmus* sp. strain AA2 extract an EC₅₀ value of 280µM was recorded while for *Haematococcus* sp. strain AA3 extract an EC₅₀ value of 264µM was determined for DPPH reduction (Table 3.5).

Similarly, the antioxidant capacity of the isolated pigment astaxanthin was determined via UV-VIS spectral analysis using DPPH. Loss of violet color was observed in the presence of astaxanthin compared to the control after 30mins of incubation in the dark, demonstrating that astaxanthin scavenged the stable free radical. An estimated EC₅₀ value of 249µM was established using the reported molar extinction coefficient at 474nm reported for astaxanthin in methanol (Buchwald and Jencks, 1968).

Table 3.5. Effect concentration values of standards (Vitamin C and β-carotene) and microalgal samples causing a 50% reduction in DPPH.

Antioxidants	EC ₅₀ value (µM)	Literature value (µM)
Vitamin C	150	165 ¹
β-carotene	225	250 ²
<i>Chlorella</i> sp. strain AA1 extract*	330	340 ³
<i>Scenedesmus</i> sp. strain AA2 extract*	280	312 ⁴
<i>Haematococcus</i> sp. strain AA3 extract*	264	300 ⁵
Astaxanthin (Purified TLC band)	249	275 ⁶

Literature values were selected based on EC₅₀ values reported for the same antioxidants in similar strains.

¹ Liu et al. 2008. Food Science and Technology, 41(7), 1344-1349.

² Jimenez et al. 2000. Journal of the Science of Food Agriculture, 80(11), 1686-1690.

³ Hajimahmoodi et al. 2010. Journal of Applied Phycology, 22(1), 43-50.

⁴ Natrah et al. 2007. Journal of Applied Phycology, 19(6), 711-718.

⁵ Cerón, et al. 2007. Applied Microbiology and Biotechnology, 74(5), 1112-1119.

⁶ Liu and Osawa, 2007. Biochemical and Biophysical Research Communications, 357(1),

187-193.

*Based on β -carotene concentration.

The above table 3.5 demonstrated the antioxidant capacity of pigments from all the three isolated strains as they all scavenged the organic radical DPPH.

3.4. Discussion

Growth analysis

Different methods have been recommended for the routine assay of chlorophyll (a,b, and c) content in algae. As a low extraction efficiency for chlorophyll has been recorded using acetone for many green algae and cyanobacteria (Riemann, 1982), ethanol can be used instead for both freshwater and marine algae. The extract optimization results for three different microalgae corresponded with Winthermans and Demots (1965), where chlorophyll a content was extracted most efficiently using ethanol, and 30mins of extraction time (figure 3.1).

Cultivation conditions, light intensity, salinity and temperature are some of the factors that affect microalgal growth (Chen et al., 2011). Growth kinetic studies on photoheterotrophic cultures of various microalgae such as *Chlorella* spp., *Chlorococcum* spp., *Scenedesmus* spp. and *Haematococcus* spp. have shown that the growth response depends on both the light intensity and the strain (Follmann et al., 1978; Ogawa and Aiba, 1981; Kobayashi et al., 1992; Marquez et al., 1995; Sansawa and Endo, 2004). The use of a counting chamber is a simple, reliable and fast method of quantifying cell growth. Under photoautotrophic conditions, *Parachlorella* sp. strain AA1 showed the highest growth rate at 0.015 h^{-1} and a generation time of 46 hours, followed by *Scenedesmus* sp. strain AA2 at 0.011 h^{-1} and generation time of 63 hours, while *Haematococcus* sp. strain AA3 grew at 0.009 h^{-1} and a generation time of 72 hours (Table 3.1). For all three microalgae strains, an increase in chlorophyll a content was detected. The highest growth rate (0.015 h^{-1}) recorded under photoautotrophic conditions for strain AA1 agrees well with an earlier report of Liang et al. (2009), where a similar growth rate was recorded for a *Parachlorella* strain under photoautotrophic conditions (Figure. 3.2a). However, Farooq et al. (2013) recently reported a two stage cultivation method for *Parachlorella*, which enhances biomass and lipid production. During the photoautotrophic growth stage, microalgal biomass was produced, while in the second stage (photoheterotrophic conditions), lipid is produced using organic carbon sources such as glucose and acetate at high concentrations. The combination of photoautotrophic-photoheterotrophic conditions enabled a biomass yield of $2.74\text{g} \times \text{L}^{-1}$ and a lipid productivity of $50.23\text{mg} \times \text{L}^{-1} \times \text{day}^{-1}$. The growth rate of *Scenedesmus* sp. strain AA2 (0.011 h^{-1}) is consistent with that reported by Xin et al. (2011), where the optimum temperature range of 20-25°C supported moderate growth of *Scenedesmus* sp. The low growth rate recorded for *Haematococcus* sp. strain AA3 (0.009 h^{-1}) was in agreement with that of Hata et al. (2001) who found that high

illumination and an additional carbon source are required to achieve high cell numbers and pigment production in *Haematococcus* sp.

Under photoheterotrophic conditions with 0.025M of acetate, higher growth rates were recorded for all three strains compared to photoautotrophic growth (Table 3.1). Among the three strains tested, *Parachlorella* sp. strain AA1 recorded the highest growth rate of 0.020 h^{-1} and shortest generation time of about 35 hours, followed by *Scenedesmus* sp. strain AA2 with a growth rate of 0.019 h^{-1} and a generation time of 36 hours. However, similar to photoautotrophic condition, morphological changes were observed in cells of *Haematococcus* sp. strain AA3 with a growth rate 0.015 h^{-1} and a generation time of 46 hours but with a decrease in chlorophyll a content. This implies that acetate enhances cell growth under moderate illumination when compared to photoautotrophic growth. This finding is similar to Killam and Meyers (1956) and Martinez et al. (1997), who reported that organic carbon stimulates cell growth under light saturating conditions although some reports showed that the stimulatory effects of organic carbon was observed only under minimal light conditions (Kobayashi et al., 1991). On the other hand, Ogawa and Aiba (1981) reported that the growth rate of *Scenedesmus acutus* was lower under photoheterotrophic conditions using acetate as carbon source than under photoautotrophic conditions even at a moderate light intensity of $60\mu\text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1}$, this is in contrast to the results for strain AA2. Chlorophyll a content increased under photoautotrophic and photoheterotrophic conditions using acetate as carbon source in strain AA2, indicating that acetate did not inhibit chlorophyll a biosynthesis (Martinez and Orus, 1991).

Light microscopy of *Haematococcus* sp. strain AA3 cells grown under photoheterotrophic conditions with acetate revealed a gradual change from the green motile biflagellate stage to the aplanosporic stage (figure 3.4). This observation corresponded to the reports by Kobayashi et al. (1992, 1993), which showed that acetate as substrate induced quicker morphological changes in *Haematococcus* sp. from the green vegetative stage to the aplanosporic stage than under photoautotrophic conditions. The spectrophotometric analysis revealed a reduction in chlorophyll a content with simultaneous accumulation of carotenoid pigments. The result for AA3 agreed with the report of Lebedeva et al. (2005), Ryu et al. (2004), and Kobayashi et al. (1992), that chlorophyll a increases or decreases depending on the species and the culture conditions in glucose and acetate supplemented medium. Kobayashi et al. (1992a, b) and Kakizono et al. (1992) reported that the concentration of sodium acetate in a culture medium, together with a high light illumination, inhibited growth and photosynthesis, thus stimulating cyst formation, which, in the

presence of a high C/N ratio, induced the synthesis of astaxanthin and similar pigments. In acetate supplemented medium, *Haematococcus* sp. strain AA3 cells entered the encystment stage within a shorter period (5-7 days) compared to about 14 days required under photoautotrophic conditions (figure 3.3c and 3.4).

Various types of organic carbon sources such as acetate, fructose, and glucose can be used to enhance growth of green algae (Zheng et al., 2012). The growth rate in *Parachlorella* sp. strain AA1 in glucose supplemented medium (0.017 h^{-1}) was higher than that in *Scenedesmus* sp. strain AA2 (0.012 h^{-1}) and *Haematococcus* sp. strain AA3 (0.016 h^{-1}) (Table 3.1). This suggested that acetate and glucose stimulated the vegetative growth of all three strains, and possibly stimulated carotenoid accumulation at the expense of chlorophyll a (Liang et al., 2009). This also implies that in the presence of an organic substrate cell growth was not strictly dependent on photosynthesis. The onset of a light yellow coloration of cultures was observed at the late exponential growth phase of strain AA1 in glucose supplemented medium. The color change was related to the production of carotenoids, presenting an advantage of carbon supplementation for pigment production. Ogawa and Aiba (1981) noted that the effect of glucose depends on the concentration. Low glucose concentrations (5g/L) did not have any effect on photosynthetic activity, but at higher glucose concentrations (50g/L) the rate of photosynthesis decreased. The results obtained in this study were opposite to the findings of Lewitus and Kana (1995), where glucose caused a decrease in chlorophyll a content as the *Parachlorella* sp. strain AA1 and *Scenedesmus* sp. strain AA2 algal cells grew. However, the results for strain AA1 are consistent with those of Farooq et al. (2013) who reported that photoheterotrophic cultures of *Chlorella vulgaris* grew better than photoautotrophic cultures.

Scenedesmus sp. strain AA2 grew better with acetate than glucose. However, growth of strain AA2 with higher concentrations of acetate should be monitored to avoid acetate inhibition. A green to light yellowish color change was observed in *Scenedesmus* sp. strain AA2 supplemented with glucose at the late exponential phase. This agrees with Heredia-Arroyo et al. (2010) reporting that the color of photoautotrophic cultures remained green while that of photoheterotrophic cultures turned yellowish during cultivation. The (highest) growth rate recorded for *Haematococcus* sp. strain AA3 (0.016 h^{-1} and with a generation time of 43 hours) with 50mM of glucose matched the study of Oncel et al. (2011), where 50mM of glucose was reported to enhance growth yields of *Chlorella zofingiensis* and *Haematococcus pluvialis*. Glucose similarly,

supported better growth of *Haematococcus* sp. strain AA3 better than for *Scenedesmus* sp. strain AA2.

According to Wang et al. (2012), microalgae can be cultivated with glucose concentrations up to 50g/L, however, it needs to be monitored because a high glucose supplement can inhibit microalgal growth and enable bacterial or fungal growth. Glucose is the most commonly used carbon source due to its ability to produce $\sim 2.8\text{KJ/mol}$ of energy compared to only $\sim 0.8\text{KJ/mol}$ for acetate (Boyle and Morgan, 2009). *Chlorella protothecoides* and *Scenedesmus* sp. were reported to give the highest lipid productivity of $54\text{mg} \times \text{L}^{-1} \times \text{day}^{-1}$ and $37\text{mg} \times \text{L}^{-1} \times \text{day}^{-1}$ when grown with 1% and 1.5% glucose supplemented medium (Liang et al, 2009; Mandal and Mallick, 2009). Carbon sources such as acetate, glucose, or glycerol are known to give optimal yields under photoheterotrophic conditions, hence when these carbon sources are combined with optimal light intensity, higher yields are obtained as seen in the case of strain AA1 to 1.8×10^7 cells/ml, strain AA2 to 1.2×10^7 cells/ml and strain AA3 that increased up to 1.8×10^7 cells/ml under photoheterotrophic conditions (Figure 3.5).

However, photoheterotrophic cultures are known to be more sensitive to photoinhibition than photoautotrophic cultures (Chonjnacka and Marquez- Rocha, 2004). Therefore, moderate light intensity ($40\text{-}60 \mu\text{mol photon} \times \text{m}^{-2} \times \text{s}^{-1}$) is recommended for the growth of green algae. Liang et al. (2009) reported that when a *Chlorella vulgaris* strain was grown on 1% glucose with continuous day and night illumination (photoheterotrophic conditions), the highest biomass production (2g/l) and lipid productivity ($540\text{mg} \times \text{L}^{-1} \times \text{d}^{-1}$) was achieved, superior to yields obtained under heterotrophic conditions with the same glucose concentration. Marquez et al. (1993; 1995) observed that the growth of *Chlorella* sp. under mixotrophic conditions supplemented with glucose was much better than under photoautotrophic conditions. This report corresponds with results for strain AA1 supplemented with glucose, as higher growth was recorded photoheterotrophically compared to photoautotrophic conditions. Thus, to optimize microalgal growth, both light intensity and duration, and the use organic carbon source should be taken into account simultaneously.

Microalgae are known to accumulate large quantities of carotenoids (Godwin and Britton, 1988). To detect carotenoid accumulation over time, UV-VIS spectroscopy was employed. When *Haematococcus* strain AA3 was grown photoautotrophically for up to 3 weeks, a decrease in chlorophyll a content was observed spectrophotometrically from the late stationary growth phase as cells grew and stress persisted causing orange/reddish color change of cultures matching the

cell morphological observation by Hata et al. (2001) who reported that when photoautotrophically grown cells of *Haematococcus* sp. are appropriately illuminated, they accumulate high concentrations of natural carotenoid pigments (3.11-3.12). Thus, the separation of cell growth phases from carotenoid production phases is necessary to obtain cell with high carotenoid content. Carotenoid biosynthesis in *Haematococcus pluvialis* is due to nutrient deprivation, high irradiance, environmental oxidative stress, and other cultivation conditions inducing stress (Kobayashi et al., 1992a, b; 1993, Fan et al., 1994; Hagen et al., 1993; Tan et al., 1995). The higher carotenoid pigment accumulation recorded at the very late stage under the $40\mu\text{mol photon} \times \text{m}^{-2} \times \text{s}^{-1}$ illumination was in agreement with the previous findings of Oncel et al (2011) and Zhang et al. (2016) who found that higher irradiance from about $40\mu\text{mol photon} \times \text{m}^{-2} \times \text{s}^{-1}$ enabled higher carotenoid pigment accumulation in *Haematococcus pluvialis* and *Chlorella zofingiensis* (figure 3.12). As reported by Boussiba and Vonshak (1991) and Hagen et al. (2000), astaxanthin production by *Haematococcus pluvialis* can be induced by modifying the environmental conditions.

Pigment production

To determine carotenoid accumulation in *Parachlorella* sp. strain AA1, UV-VIS spectroscopy of acetone extracts was used and two maxima at 450 and 665nm were detected (Figure 3.6A). β -carotene exhibits an absorption maximum at 450nm in acetone with shoulders at 437nm and 475nm, while 665nm is the absorption maximum for chlorophyll a (Cho, 1970). β -carotene isolated from *Parachlorella* strain AA1, for *Chlorella vulgaris* and *Chlorella zofingiensis*, as previously reported by Del Campo et al. (2007) and Gors et al. (2010) can serve as active immunostimulator, reducer of blood lipids and free-radical scavenger. Other health beneficial effects of *Chlorella* spp. carotenoids are the lowering of cholesterol (Zhao et al. 2015), preventive action against atherosclerosis or antitumor action (Panahi et al., 2016). Increasing light intensity enhances lipid accumulation in *Chlorella* species, according to Anderson (2005), optimal light and dark regimes have been found to vary from 12:12 hour to 16:08 hour for most cultures. Light intensity above the saturation point causes light inhibition, which can be counterbalanced by exposing microalgal cells to very short cyclic periods of light and dark conditions (Imamoglu et al., 2009).

Light microscopy of *Scenedesmus* sp. strain AA2 cells from the late stationary stage revealed a faint orange color change overtime due to light intensity and stress conditions. The cell wall of the orange cells was observed to be thick. This matches the recommendation by Boussiba and

Vonshak (1991) and Zlotnik et al. (1993), that culturing conditions and high irradiance can induce and enhance natural carotenoid accumulation in *Scenedesmus* spp. Hanagata and Dubinsky (1999) reported that secondary carotenoids accumulate in the chloroplastidal plastoglobuli of *Haematococcus pluvialis* while in *Scenedesmus komarekii* they appeared in lipoidal globules. Bar et al. (1995) concluded that *Chlorella zofingiensis* accumulates secondary carotenoids both in lipoidal globules and in the chloroplast. UV-VIS spectroscopy of *Scenedesmus* sp. strain AA2 acetone extracts between 400-700nm revealed a low concentration of chlorophyll a and the presence of β -carotene due to a maximum at 450nm in the apparent ratio of 30 (A450/A665) (Figure 3.6B), resembling absorption spectra of acetone cell extracts described by Qin et al. (2008) for *Scenedesmus* spp. The absorption spectrum for *Haematococcus* sp strain AA3 acetone extracts revealed the presence of an astaxanthin maximum at 474nm in a ratio 10 to chlorophyll a at 665nm. This is similar to that described by Minguez et al. (1992); Buchwald and Jencks (1968); Kim et al. (2015) of a typical astaxanthin and chlorophyll a (Figure 3.6C). As expected, dominance of the aplanosporic stage was observed with the light microscope at the late stationary phase. Haematocysts became increasingly reddish (Figure 3.11) due to accumulation of carotenoids in the synthesized thick cell wall and chloroplasts. This corresponds to the morphological changes observed by Kobayashi (1991); Han et al. (2013); and Klochkova et al. (2013) for species in the genus *Haematococcus*. Continuous illumination with high light intensity resulted in mature cysts leading to maximal pigment productivity. The pigment ratio (A450/A665) in strain AA3 matches that of Kobayashi et al. (2007) where pigments ratio of 7.0 was recorded for *Haematococcus pluvialis* at the late stationary phase.

Thin layer chromatography.

The R_f values for β -carotene from *Parachlorella* sp. strain AA1 were similar to those of the standard β -carotene and those reported by Qin et al. (2008) and Jaime et al. (2010) using the same mobile phase as done in the present study. The identification of β -carotene (Figure. 3.8) was based on the spectral properties that matched authentic β -carotene and correlated to data reported by Cho (1970), where β -carotene had a maximum at 450nm plus shoulders at 435nm and 475nm.

In the case of *Scenedesmus* sp. strain AA2, two major pigments were chromatographically detected based on spectral properties and R_f values. The first pigment was assigned to β -carotene. The second carotene was possibly hydroxyechinenone based on the R_f values that was similar to that of hydroxyechinenone, although the maxima in the UV-Vis spectra characteristics differ slightly (Figure 3.9 and 3.10). This observation is in accordance with the previous findings of Qin

et al. (2008), where seven carotenoids were identified in *Scenedesmus obliquus* using three different solvent systems, with β -carotene showing a maximum at 450nm in acetone and R_f value at 0.95 in acetone/ n-hexane (30/70%) solvent while hydroxyechinenone had maximum at 463nm different from strain AA2 and a R_f value 0.45 in acetone/petroleum ether (20/80%) solvent system.

Four major carotenoids were detected using TLC and UV spectroscopy in acetone extracts of *Haematococcus* sp. strain AA3 (figure 3.15 and 3.16). The R_f values (0.95, 0.91, 0.71, 0.54) of the four major carotenoids using acetone and n-hexane (30/70%) as mobile phase matched with previously reported data by Khanafari et al. (2007), while the R_f values (0.98, 0.87, 0.75, 0.55) of the major carotenoids identified using acetone/petroleum ether (25/75%) as mobile phase was in agreement to Jaime et al. (2010). The UV-VIS characteristics of strain AA3 are in accordance with the previous findings of Jaime et al. (2010) and Dragos et al. (2010) where astaxanthin had an absorption maximum of 474nm, β -carotene at 450nm, lutein at 446nm, echinenone at 462nm, although the absorption maximum of strain AA3 at 475nm was slightly higher by 13nm but showing similar spectrum characteristics. According to Khanafari et al. (2007), R_f values for astaxanthin monoesters are in a range of (0.50-0.58), while those for diesters range from (0.73-0.75) in acetone/n-hexane (30/70%) as mobile phase. However, Jaime et al. (2010) reported a range of (0.37-0.50) for astaxanthin monoesters and (0.79-0.91) for diesters using acetone/petroleum ether (25/75) as mobile phase, indicating that the extract of strain AA3 is probably a mixture of mono and diesters. β -carotene had been previously isolated from *Chlorella zofingiensis* by Del Campo et al. (2007). Both β -carotene and hydroxyechinenone detected in *Scenedesmus* sp. strain AA2 were previously reported by Qin et al. (2008) to be synthesized by *Scenedesmus obliquus*, exhibiting maxima at 450nm and 463nm respectively. Based on the chemical structures of carotenoids identified from *Haematococcus* sp. strain AA3 and previous reports on the biosynthesis of astaxanthin by Han et al. (2013), two pathways of carotenogenesis are proposed for this organism.

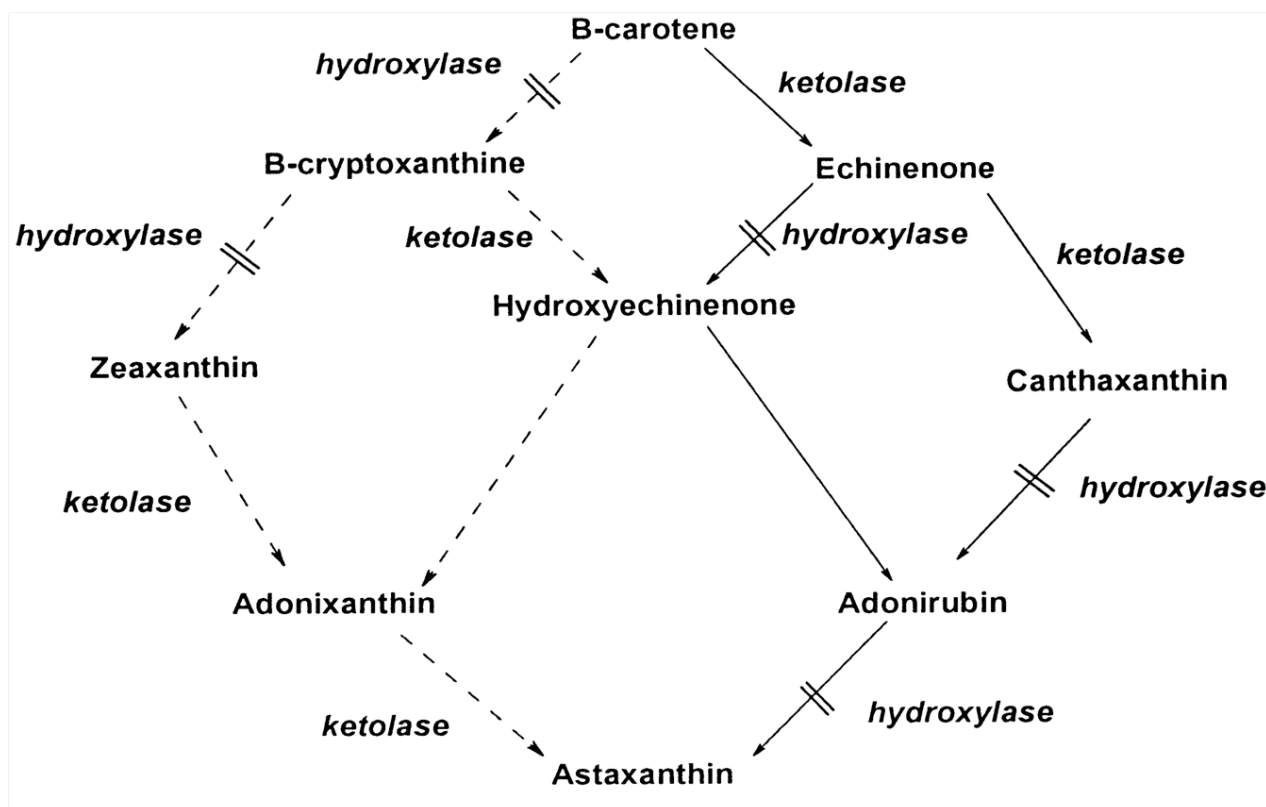


Figure 3.19. Possible pathways for astaxanthin biosynthesis from β -carotene proposed in the literature (Han et al., 2013). The signs ‘||’ and ‘ \rightarrow ’ indicate the enzymatic reactions that ellipticine and cerulenin inhibit, respectively.

As shown in Figure 3.19, echinenone can be synthesized by a direct oxidation of β -carotene by β -carotenoid ketolase (BKT), then acting as a substrate for hydroxylation or oxidation to produce hydroxyechinenone and canthaxanthin. Hydroxylation of canthaxanthin and hydroxyechinenone can be catalyzed by β -carotenoid hydroxylase (CrtR-b) to produce adonirubin and adonixanthin. Adonirubin and adonixanthin are converted to astaxanthin by hydroxylation, followed by oxidation. This biosynthetic pathway has been detected in strains such as *Chlorella zofingiensis*, *Chlorococcum* sp., *Scenedesmus obliquus* and *Haematococcus pluvialis* (Kobayashi, 2003; Han et al., 2013). In acetone extracts of *Haematococcus* sp. strain AA3, β -carotene, and echinenone were detected in addition to astaxanthin, indicating that astaxanthin was possibly synthesized from β -carotene via echinenone and adonirubin. The characterization by TLC and UV-Vis spectral analysis showed that the major natural carotenoid pigment isolated from strain AA2 was β -carotene while in strain AA3 it was β -carotene and astaxanthin. Other carotenoids were only detected as minor pigments. Strain AA3 achieved a carotenoid pigment productivity of $26 \text{ mg} \times \text{L}^{-1} \times \text{d}^{-1}$ which is not sufficient to make this organism a realistic candidate for the production of

natural astaxanthin. Wang et al. (2012) reported yields of $602 \text{ mg} \times \text{L}^{-1} \times \text{d}^{-1}$ for astaxanthin when produced on an industrial scale. Commercial production on a large scale is therefore required, possibly by using a two stage cultivation method to enhance pigment productivity and to obtain higher growth yields.

Antioxidant properties

2, 2 -Diphenyl-1-picrylhydrazyl (DPPH) is a well established stable free organic radical that can be used for antioxidant assays (Blois, 1958). It is a simple, rapid, and convenient method for screening (many) biological samples with regards to their antioxidant and radical scavenging ability (Molyneux, 2004).

The antioxidant capacity of extracts of the isolates *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2 and *Haematococcus* sp. strain AA3 was analyzed based on their radical scavenging potentials. Antioxidants are classified based on their mode of action, as oxygen scavengers, chelators of metal ions capable of catalysing lipid oxidation and as free radical terminators (Shahidi et al., 1992). Carotenoids are substances that protect humans and animals against oxidative damage, this qualifies them to be used as food supplements and in nutraceuticals (Sies and Stahl, 1995). Decolorization of DPPH was observed for the antioxidant reference compound vitamin C, resulting in an EC_{50} value of $150 \mu\text{M}$. A similar result with vitamin C was previously reported by Liu et al. 2008, where the DPPH radical method was used to evaluate antioxidant activity resulting in an EC_{50} value for vitamin C of $165 \mu\text{M}$. An EC_{50} of $225 \mu\text{M}$ was recorded for β -carotene. This observation is in agreement with the reported value of Jimenez et al. (2000) on the reaction of β -carotene with free radicals reporting an EC_{50} of $250 \mu\text{M}$. Hence, β -carotene and vitamin C are potentially beneficial to human health by scavenging harmful free radicals causing degenerative diseases such as cardiovascular diseases or cancer (Gey et al., 1991; Ames et al., 1993). β -carotene quenches singlet oxygen ($^1\text{O}_2$) by electron transfer and free radicals by donating hydrogen. The carotenoid then changes from the ground state to a triplet state. After electron exchange the carotenoid triplet state (^3CAR) can turn to the ground state by dissipating the energy as heat. This quenching reaction is responsible for carotenoid photoprotection against $^1\text{O}_2$ (Krinsky, 1993; Edge and Truscot, 1999). Methanolic extracts of all three strains exhibited similar antioxidant activity when compared with the reference antioxidants (Vitamin C) and β -carotene. Methanolic cell extracts of *Parachlorella* sp. strain AA1 gave an EC_{50} value of $330 \mu\text{M}$ based on its β -carotene content. This result is in agreement with previous studies of Hajimahmoodi et al.

(2010), where *Parachlorella* sp. extracts exhibited DPPH radical scavenging ability with an EC₅₀ value of 340µM. The *Parachlorella* sp. strain AA1 extract exhibited good antioxidant activity, which may be expected mainly due to the presence of large quantities of lipophilic carotenoids.

Similarly, *Scenedesmus* sp. strain AA2 extracts had a good scavenging ability against DPPH with an EC₅₀ value of 280µM based on the β-carotene content. The antioxidant capacities of *Scenedesmus* spp. extracts have been examined by Natrah et al. (2007) and El Baky et al. (2014), where *Scenedesmus* extracts showed high antioxidant capacity with an EC₅₀ value of 312µM.

Haematococcus sp. strain AA3 extracts reacted quickly with DPPH to scavenge it, resulting in an EC₅₀ of 264µM based on β-carotene content. This observation is in accordance with reports of Kobayashi and Sakamoto, (1999) and Ceron et al. (2007), where *Haematococcus pluvialis* extract was reported to possess high antioxidative capacity. The composition and concentration of microalgae pigments may vary depending on the species analyzed, thus illustrating why the antioxidant activities of (certain) microalgae (may) also differ from one another (Metting and Pyne, 1986). However, for the TLC purified astaxanthin from strain AA3, an EC₅₀ value of 249µM was established, indicating the radical scavenger ability of this carotenoid. This value corresponds to the previous findings of Liu and Osawa (2007), where the antioxidative ketocarotenoid, astaxanthin, exhibited a high antioxidative capacity. Numerous findings have demonstrated the antioxidant capacity of carotenoids in reducing free radicals and neutralizing oxidative effects to help the body maintain a healthy state. Of all the natural carotenoids, natural astaxanthin was shown to possess high antioxidative capacity, thus surpassing the antioxidative benefits of β-carotene, lutein, canthaxanthin, and zeaxanthin (Shah et al., 2016). The protection against oxidation by astaxanthin is due to the quenching of singlet oxygen and scavenging of free radicals, thereby terminating chain reactions (Kobayashi et al., 1997; Kobayashi and sakamoto, 1999). Foods ingredients of plant source not only provide certain significant antioxidant vitamins, such as vitamin C (ascorbic acid), provitamin A (β-carotene) and vitamin E (a-tocopherol), but also a complex mixture of other natural materials with antioxidant potential. These antioxidants are beneficial to human health and serve as food preservatives by retarding deterioration, rancidity and discolouration caused by oxidation (Dziezak, 1986).

This study investigated the effects of certain growth conditions on biomass and carotenoid production by three green microalgae, *Parachlorella* sp. strain AA1, *Scenedesmus* sp. strain AA2 and *Haematococcus* sp. strain AA3. Photoheterotrophic growth with acetate led to the highest cell yield in *Parachlorella* sp. strain AA1, while glucose led to the highest cell yield in

Haematococcus sp. strain AA3. In addition, the detection of carotenoids by TLC and UV-VIS spectrometry showed that these isolates are capable of producing natural carotenoid pigments for human beneficial purposes. The DPPH organic radical assay revealed the beneficial antioxidant potential of these isolates, making them promising sources for the production of natural carotenoid pigments particularly astaxanthin and potential food additives.

3.5. References

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CHAPTER 4

CONCLUSION AND OUTLOOK

Green microalgae are ubiquitous in nature as they are commonly found in freshwater, marine and terrestrial habitats (Aslam et al., 2007; Leliaert et al., 2012). Three green microalgae strains were isolated by selective enrichment from environmental samples. Based on morphological and molecular characteristics, strain AA1, forming small unicellular, ovoidal, non-motile cells, was assigned to the genus *Parachlorella*, while strain AA2, a colonial, irregular, non-motile shaped alga was assigned to the genus *Scenedesmus*. Strain AA3 showing spherical cells with a unique developmental life cycle including motile biflagellate stage, immature stage, encystment stage, and mature stage was assigned to the genus *Haematococcus*. Interest in studying *Haematococcus* spp. has increased in recent years due to its ability to accumulate the high value antioxidant ketocarotenoid astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione), which is of great beneficial importance to humans and animals (Higuera-Ciapara et al., 2006; Shah et al., 2016). All three isolates grew well with acetate and glucose as source of carbon and energy under photoheterotrophic growth conditions compared to photoautotrophic growth condition. Glucose enabled higher growth rates (0.016 h^{-1}) in *Haematococcus* sp. when grown photoheterotrophically and so acetate did in *Chlorella* sp. (0.020 h^{-1}). Glucose can promote physiological changes in cells, which affect the metabolic pathways of carbon absorption, volume of storage materials, such as lipids grains, starch, (Martinez and Orus, 1991) protein, chlorophyll, RNA, vitamin content, growth rates, as well as size of the cells (Yeh and Chang, 2012). Oxidative assimilation of glucose begins with a phosphorylation of hexose, yielding glucose-6-phosphate, which is readily available for storage, biosynthesis, and respiration. *Chlorella* spp. possess an inducible hexose transport systems that allows them to utilize glucose for growth even at low concentrations. The metabolism of acetate starts with formation of acetyl-CoA by the enzyme acetyl-CoA synthetase. Acetate is metabolized through two pathways; the glyoxylate cycle in glyoxysomes or the tricarboxylic acid cycle (TCA) (Boyle and Morgan, 2009).

β -carotene was identified in *Parachlorella* sp strain AA1 as the major carotenoid pigment in accordance to the report of Del Campo et al. (2007). Two distinct natural carotenoid pigments were present in *Scenedesmus* sp strain AA2. extracts, as was reported by Qin et al. (2008) and Guedes et al. (2011). Four major different natural carotenoid pigments were identified in *Haematococcus pluvialis* extract using thin layer chromatography and UV-VIS spectroscopy, as was reported by Khanafari et al. (2007) and Jaime et al. (2010). Synthetic astaxanthin is currently

dominating the carotenoid market. The successful isolation of astaxanthin and similar carotenoid producing microorganisms from terrestrial and freshwater environments indicates that natural astaxanthin producing microalgae are present in KwaZulu-Natal. Natural astaxanthin is more stable, has greater antioxidative capacity and shelf-life than synthetic astaxanthin (Perez-Lopez et al., 2014), and provides a stronger pigmentation to shrimps than the synthetic form (Bowen et al., 2002). The biosynthetic pathways of astaxanthin accumulation were analysed in *Chlorococcum* sp., *Coelastrella* sp., *Phaffia rhodozyma*, and *Chlorella zofingiensis* (Liu et al., 2014). Besides echinenone and canthaxanthin that are involved in *Chlorococcum* sp. and *Chlorella zofingiensis*, the alga *Haematococcus* sp. strain AA3 in the present study accumulated pigment indicating that astaxanthin was probably synthesized from β - carotene to adonirubin via echinenone and canthaxanthin (Han et al., 2013). The current increasing demand for natural antioxidants as alternative to synthetic products requires the production of astaxanthin and similar carotenoids from natural sources, although the high cost of production and low productivity of astaxanthin hinders the large scale production of natural astaxanthin. However, the use of astaxanthin as an antioxidant and its high marked value will boost the commercial production once optimized with lowered costs (Leu and Boussiba, 2014; Minhas et al., 2016). The knowledge of astaxanthin producing *Haematococcus* species from South Africa is limited (Pocock, 1960), therefore, research should be carried out on how natural astaxanthin can be produced from South African isolates on a large scale to provide food additives and drive the biotechnology economy (Pringsheim, 1966; Klochkova et al., 2013). However, all the three microalgae isolates demonstrated the ability to scavenge radicals, signifying their antioxidant capacity and potentially beneficial role that they can play in treating health degenerative diseases, gastrointestinal discomforts, and in aquaculture as part of animal meals and also as food additives (Palozza et al., 2009; Rao et al., 2013; Li et al., 2015).

The results obtained from this study indicate that additional work is required in the following areas:

- Growth and pigment production should be determined under varying light intensities and cycles to assess the impacts of light exposure on pigment accumulation. In addition, the carotenoid biosynthesis should be investigated to identify metabolic bottle necks. Carotenoids should be analyzed using additional methods such as High-performance liquid chromatography and liquid chromatography mass spectrometry.
- The effects of varying concentrations of different carbon sources on the pigment production under photoheterotrophic conditions.

Glucose and acetate (0.05M and 0.025M) enhanced the growth of all three isolates. Higher concentrations of substrates should be tested to determine if higher growth yields will be recorded and to assess at which concentrations growth will be inhibited. Fructose, glycerol and urea can be tested as alternative carbon sources to analyse their impact on growth rates and pigment production.

- The semi-pilot scale production of natural carotenoid pigments should be established by using the raceway pond method and photobioreactors.

Two stage cultivation methods should be further investigated as a means to enhance biomass production and carotenoid productivity.

- Determining the antioxidant capacity of isolates and their pigments using other assays.

The antioxidant capacity of the strains and their pigments should be evaluated by using additional assays such as ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid), TRAP (total radical trapping antioxidant parameter), and electron spin resonance spectroscopy (ESR).

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APPENDIX A

Gene sequencing for strain AA1 and AA2

The partial 18S rRNA gene sequence established for strain AA1

5'-GAAAGCATTTGCCAAGGAGGTCTTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAGATATTG
ATACCGTCCTAGTCTCAACCATAACACGATGCCGTATAGGGATCGGCTGATGTTTCTTCGATGAC
TCCGCCGGCACATTATGAGAAATCAAAGTTTTTGGGTTCGGGGGGAGTACGGTCGCAAGGCTGA
AACTTAAAGGAATCTACGGAAGGGCACCACCAGGCGTGGAGCATGCGGCTTAATTCACCTAACAC
GGGAAACTTACCAGGTCCAGACATAGTGAGGATTGTCAGATTGAGAGCTCTTTCTTGATTATAT
GGGTGGTGGTGCATGGCCTTTATTAGTTGCAGGGATGCCTCGTCAGGTTGATTCCGGTAACGAAC
TAGACCTCATCCTGCTAAATAGTCACGGCCTCCTCGGGGGCCGGCAGACTTCTTAGAGGGACTAT
TGGCGACTAGCCAATGGAAGCATGAGGCAATAACAGGTCTGTGATGCCCTTAGATGATCGGGCCG
CACGCGCGCTTCATTGAAGCAATCAACGAGCCTAGCCTTGCCCGAGAGGCCCGGGTAATCTTCAA
AACTGCATCGTGATGGGGATAGATTATAGCAATTACTAATCTTCAACGAGGAATTCCTAGTAAGC
GCAAGTCATCACCTTGCGTTGATTACGTCCCTTCCCTTTGTACACACCGCCCGTCGCTCCTACCG
ATTGGGTGTGCTGGTGAAGTGTTCCGATTGGCAACCCGGGGCGGTTTCCGCCCTGGGCTGCCGAG
AAGTTCATTAAACCCTCCACCTAG-3'

The partial 18S rRNA gene sequence established for strain AA2

5'-GTATAAACTGCTTATACTGTGAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGG
TGGTACCTTACTATTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCGTAAATCCCGACTCC
TGGAAGGGACGTATATATTAGATAAAAGGCCGACCGGACTTTGTCCGACCCGCGGTGAATCATGA
TATCTTCACGAAGCGCATGGCCTCGCGCCGGCGCTGTTCCATTCAAATTTCTGCCCTATCAACTT
TCGATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGTTCGATTCCGG
AGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCC
TGATACGGGGAGGTAGTGACAATAAATAACAATATCGGGCATTTAATGTCTGGTAATTGGAATGA
GTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCCCTGGTGCCAGCAGCCGCGGT
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GGGTGGGTTCCTAGCGGTCCGCCTTATGGTTGAGTACTGCTATGGCCTTCCTTATCTGTGCGGGGAC
GGGCTTCTGGGACTGCATCTGTCCGGGCACTCGGAGTCGACGTGGTTACTTTTAGTAAATTAAGA
AGTCGTTCAAAGCAGGCTTACGCCAGAATACTTTATGCATGGAATAACACGATAGGACTCTGGCC
TATCTTGTTGGTCTGTAGGACCGGAGTAATGATTAAGAGGGACAGTCGGGGGCATTCGTATTTCA
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GTTTTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCGTTAGTCTCAAC
CATAAACGATGCCGACTAGGGATTGGCGAATGTTTTTTTAATGACTTCGCCAGCACCTTATGAG
AAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGG
AAGGGCACCACCAGGCGTGGAGCTTGCGGCTTAATTTGACTCAACACGGGAAAACCTTACCAGGTC
CAGACATAGTGAGGATTAACCTGATTGAGAACTCTTTCTTGTTTCTATGGGTGGTGGTGCATGGCC
GTTTTTAGTTGGTTGGTTTCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAA
ATAGTCTCAGTTGCTTTTTGCAGCTGGCTGACTTCTTAGAGGGACTATTGGCGTTTAGTCAATGG
AAGTATGAGGCAATAACAGGTTTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTTCACTG
ATGCATTCAACAAGCCTATCCTTGACCGAAAGGTCCGGGTAATCTTTTAAACTGCATCGTGATGG
GGATAGATTATTGCAATTTTTAGTCTTCAACGAGGAATACCTAGTAGGCGCAAGTCATCATCTTG
CGTCGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTGCTGGTA
TATTTTTCGGATTGGCAGCTTAGGGTGGCAACTCCTCATGTCTGCCTAGAAGTTCTTTAAACCCT
CCCACCTAGAGGAAGAGAAGTCTAGAATGCAC-3 '

