

Chapter one

1. Introduction

Microalgae cultivation is receiving much attention globally (Borowitzka, 1992; Bosma and Wijffels, 2003; Benemann, 2008). Nowadays, microalgae are cultivated as a source of highly valuable molecules such as pigments, lipid and protein that can be incorporated into important industries (Spolaore *et al.*, 2006). There are numerous applications of microalgae such as simple biomass for human food and animal feed (Aksu *et al.*, 1997; Ben-Amtoz, 2003). Microalgae are mostly used in the field of human and animal nutrition (Stottrup and McEvory, 2003). They can act as a nutritional supplement, or represent a source of natural food colorants, as well as other commercial uses (Borowitzka, 1999; Soletto *et al.*, 2005).

The increasing interest in microalgae cultivation has emerged due to microalgae have many advantages over higher plants, first, microalgae have higher productivity than plant (rapidly growth, doubling in biomass every few hours to a few days) (Ben-Amtoz *et al.*, 2009). Second, the cultivation of microalgae does not require arable land or freshwater (it can be carried out in shallow ponds on hardpan soils, using saline or brackish water) (Ben-Amtoz, 2003). Many species of microalgae can be cultivated using natural seawater, and land that is not competitive for resources with conventional agriculture (Chen *et al.*, 2009). The combination of high biomass productivities and the lack of need for arable land and freshwater support the large-scale production of microalgal biomass without affecting

agricultural commodities prices. Thereby avoiding the ethical conflict that arises when diverting crops that are desperately needed to feed a growing world population for biomass production (Ben-Amtoz *et al.*, 2009).

The production of microalgal biomass is not new as a technology. It has been described in several earlier books and articles in the late 1800s and early 1900s (Andersen, 2005). The utilization of algae by human has a very long history. The first collection of algae as food appeared 2,500 years ago in China, in where people used edible algae as *Nostoc* to survive (Spolaore *et al.*, 2006).

Fewer species of microalgae have economical importance because they are highly productive, easy to maintenance and ideally to culture (Borowitzka, 1992). The main species grown being *Chlorella* and *Spirulina* for health food, *Haematococcus pluvialis* for astaxanthin and some other species for aquaculture (Meng *et al.*, 2009). Among the various microalgae that have been explored for their suitability for commercial potentials is *Dunaliella* sp. *Dunaliella salina* is a unicellular green alga belonging to the class of Chlorophyceae (Avron and Ben-Amotz, 1992). *Dunaliella salina* is a unique species of this class due its stress adaptation and carotenoids production (Ben-Amtoz, 2003). It has a great ability to thrive in high salinity environments such as: brine lakes, salt marshes and hypersaline soil (Ben-Amtoz *et al.*, 2009). It's proven to be successful due to accumulation of significant amount for valuable chemicals, such as glycerol and β -carotene (Hadi *et al.*, 2008). Many studies have been reported that *D. salina* is the best commercial source of natural β -carotene (Borowitzka, 1995), which has many applications in nutrition, cosmetic and pharmaceutical products (Cantrell *et al.*, 2003). In recent years, mass cultivation of *D. salina* for commercial production has

being accomplished in several countries such as Australia, United States and China (Spolaore *et al.*, 2006).

However, microalgae-based technologies are not yet developed and the economics of such processes are relatively expensive (Chen *et al.*, 2009). Nutrient cost is a major challenge of algae cultivation (Carlsson *et al.*, 2007). Algae culture techniques involve consumption of nutrient to achieve maximum algal production. The disposal of sewage water can prove beneficial in this field (Costa *et al.*, 2004). The economic benefits of using partially treated sewage water, attributed primarily to its high content of nutrients (Lubello *et al.*, 2004). It contains valuable nutrients for algae culture such as phosphates, nitrates and sulphates (Allam and Abd-El.Kareem, 2001). Indeed, the application of partially treated sewage water for algae cultivation is a new method of reusing sewage water (Chen *et al.*, 2009). In Saudi Arabia, very limited quantities of the treated effluents are using for benefit purposes. The utilized water quantity doesn't reach more than 6% while more than 60% of municipal wastewater can be used for non-potable uses (NWC, 2010).

In addition, water supply represents one of the most critical variables in *D. salina* cultivation. *Dunaliella salina* requires highly salt content to achieve optimal production of β -carotene (Ben-Amotz, 2003; Lamela *et al.*, 2010). Therefore, the using of brine water is likely favorite sustainable solution for water required to cultivate *Dunaliella*. Many companies have attempted to reuse natural brine water or saline waste stream in order to reduce the cultivation cost (Curtain, 2000; Lamela *et al.*, 2010). Indeed, some of *Dunaliella* production plants have been established close to available sources of salt water. For example, sea salt industries usually in warm and sunny areas where the rate of water evaporation is high and non-agricultural land abundant (Richmond, 2003; Ben-Amotz, 2003).

Hence, it is a worthwhile to develop a new technique to cultivate this highly economically value species with cost effectively method. The reuse of discharged desalination water (DDW) and partially treated sewage water (PTSW) as a culture medium for mass microalgal production could be of great interest. Such utilization with further dilution with natural sweater could reduce the impact on local environment. Furthermore, recycle wasted water to produce high value bioproducts is another advantage. Development of cultivation techniques, microalgal biotechnology can ready to meet the high demands of both the food and pharmaceutical industries.

1.1 Aim of study

The aim of this study is to develop low cost sustainable cultivation technique for *D. salina*. In addition, evaluate *D. salina* quality and quantity (biomass and its biochemical composition) under Dual-phase culturing method; develop by using unused free local water sources. Also, reducing the damage on marine environment especially in Jeddah region partially treated sewage (PTSW) and discharged desalination water (DDW).

1.2 Objectives

- Developing new method using free local water sources for *D. salina* cultivation to maximize *D. salina* growth performance.
- Establishing the best dilution of medium with seawater for mass *D. salina* cultivation.
- Study the effect of discharged desalination water (DDW) and partially treated sewage (PTSW) as a shock to boost the biochemical composition production of the algae biomass.

Chapter two

2. Literature review

2.1 Historical perspective of microalgae cultivation

The development in algal culture techniques allowed the expansion of hatcheries for aquaculture (Stottrup and McEvoy, 2003). Today, microalgae are used extensively in aquaculture for shrimp and fish feeding and biotechnology for producing high value bioproducts. The success of commercial large-scale production of microalgae depends on many factors such as: selection of good strain, high productivity, and cost effectiveness (Borowitzka, 1992).

There are many challenges faced during algal cultivation. These challenges include the cost of nutrients, large lands, light, water, and carbon dioxide source (Benemann, 2008). Therefore, many studies have tested the possibility of using different types of wastewater to cultivate microalgal biomass (Costa *et al.*, 2004; Chen *et al.*, 2009; Lamela *et al.*, 2010). Microalgae have a great ability to absorb nitrogen and phosphors from aquatic source and are able to use large quantities of organic carbon which would otherwise be emitted to the atmosphere (Ben-Amotz, 2003). Using wastewater for growing algae seems to be environmental and cost effective method for algal biomass production (Lubello *et al.*, 2004). Growing algae on wastewater streams have a number of benefits. It will offset additional costs for nutrient removal from wastewater streams, and the costs associated with nutrient and

water supplies for algae growth will be greatly reduced or eliminated. If it is grown on municipal wastewater, algae help remove nutrients particularly phosphorous and nitrogen. While cultivation systems that do not utilize wastewater must add nutrients such as phosphoric acid and urea or ammonium nitrate to enrich algal culture (Chen *et al.*, 2009).

2.2 Cultivating algae using different water sources

Many studies have been conducted to test the possibility of using different types of wastewater to cultivate microalgae such as swine wastewater (Siranee *et al.*, 2002), soy sauce waste treatment solution (Shirai *et al.*, 1998), and different wastewater taken from different treatment stages (Kong *et al.*, 2010). Wang *et al.* (2010) suggest that growing algae in nutrient-rich centrate offers a new option to reduce the nutrient load from municipal wastewater treatment plant.

Different species of marine microalgae can grow well if they are cultivated on wastewater or mixture of wastewater and seawater. Goldman and Stanly (1974) demonstrated the feasibility of utilizing treated wastewater effluent as the nutrient source for the mass cultivation of marine algae in large outdoor ponds. Craggs *et al.* (1995) cultured two species of marine microalgae on wastewater (primary sewage effluent) diluted 1:1 with sterile seawater. The results indicate the potential of using marine algae for wastewater treatment. Ashly (2007) reported that *Dunaliella tertiolecta* is likely an ideal candidate for saline wastewater treatment due its ability to grow in saline environments as well as it can reduce concentrated nutrient loads when very high concentrations of nitrogen-ammonia are present. Allam and Abd-El.Kareem (2001) found that *Dunaliella salina* showed higher cell densities at high concentrations of sewage and industrial wastewater. Costa *et al.* (2004) examined the possibility of using urban secondary sewage as an alternative medium for the culture

of *Tetraselmis chuii* and *Dunaliella viridis*. They found that using urban secondary treated sewage for cultivation of microalgae achieved satisfactory cell densities and growth rates when compared with other culture media such as bovine and chicken manure extracts.

2.3 Cultivating marine microalgae using seawater

In general, marine microalgae may be grown in either synthetic or natural seawater culture media. The latter have; however been found to be more suitable than the former (Stein, 1973). The basic idea of using seawater for algal cultivation is the availability of source especially in coastal regions. The need for mass cultivation of microalgae and the available of water source for cultivation are good investment of marine (Andersen , 2005). Indeed, many algae culture collection centers and producer maintained marine algal strain in using oceanic seawater. For example, CSIRO Marine and Atmospheric Research (CMAR), university of Texas laboratory (UTEX) and Culture Collection of Algae and Protozoa (CCAP) (personal communication). In case of using seawater based media, it may be necessary to collect off-shore because it has low concentration of metal and organic pollutants (Stein, 1973). Near-shore water sources are seldom acceptable because it may seasonally vary due to rainfall and runoff inputs, These sources may have elevated nutrients, sediments and decreased salinity and large phytoplankton blooms may also alter the organic compounds in the seawater (Andersen , 2005).

Using Natural seawater (NW) as a culture medium may be preferred if large quantities are required. However, various additions must be made to the NW to enhance the algal yield. Routinely, seawater enriched with artificial nutrients or commercial salts (Herrero *et al.*, 1991). It was well known that chemicals that were

added to seawater contained impurities such as trace elements, and these often improved growth (Andersen, 2005).

Most of seawater-based media requires addition of vitamins and soil extract. As the constituents of the soil extract vary depending on the origin of the soil, the exact soil composition is undefined (Andersen, 2005). Moreover, the using of soil extract is less acceptable due the presence of fertilizer, pesticide and other contaminants. In most media recipes, synthetic vitamins of (B₁₂, Thiamin and Biotin) are required with different concentrations (Ben-Amtoz *et al.*, 2009). The addition of synthetic vitamins is not practical due to the reason that vitamins are heat sensitive and expensive. The purpose of media is to enhance growth while fulfilling this purpose, a medium should be easy (as simple as possible) in preparation and should also be inexpensive (Stein, 1973).

2. 4 Species selection

The first step in developing large scale algal culture is to choose the algal species. Pulz and Gross (2004) observed that: “successful algal biotechnology mainly depends on choosing the right alga with relevant properties for specific culture conditions and products”. A variety of desirable characteristics reported for large-scale algal culture are summarized in Table 2.1. Selection of fast-growing, productive strains, optimized for the local climatic conditions is of fundamental importance to the success of any algal mass culture. A single algal species is unlikely to excel in all categories, hence prioritization is required. Environmental conditions, availability of water supply and choice of culture system influence species choice. For example, some algae are most productive at high temperatures and bright light, while growth of others is retarded by full sunlight (Sheehan *et al.*, 1998).

Table 2.1 Summary of desirable algae characteristics for mass culture.

Characteristic	Advantages	Disadvantages	Reference
Growth in extreme environment	Reduces problems with competing species and predators	Only limited numbers of species available and some extreme environments difficult to maintain on a large scale (i.e. cold)	Borowitzka (1992)
Rapid growth rate	Provides competitive advantage over competing species and predators; reduces pond area required	Growth rate is usually inversely related to cell size; i.e. fast growing cells are usually very small	Borowitzka (1992)
Large cell size, colonial or filamentous morphology	Reduces harvesting costs	Large cells usually grow slower than smaller cells	Borowitzka (1992)
Wide tolerance of environment conditions	Less control of culture conditions required. Growth over range of seasons and ambient weather conditions		Borowitzka (1992) and Grobbelaar (2000)
Tolerance of shear force	Allows cheaper pumping and mixing methods to be used		Borowitzka (1992)
High cell content of product	Higher value of biomass	Products are usually secondary metabolites; high concentrations mean slower growth	Borowitzka (1992)
CO ₂ tolerance and uptake	Greater potential for CO ₂ sequestration and use of waste CO ₂		Grobbelaar (2000)
Tolerance of contaminants	Potential growth in polluted water and on flue gases containing high CO ₂ , NO _x and SO _x		Borowitzka (1992)
No excretion of autoinhibitors	Reduces autoinhibition of growth at high biomass densities		Grobbelaar (2000)

In this study, *D. salina* was chosen due its fast growth, high productivity as well as its multiple promising applications.

2.5 The genus of *Dunaliella*

Dunaliella is a unicellular, bi-flagellate, green alga belonging to the class Chlorophyceae (Avron and Ben-Amotz, 1992). *Dunaliella* is morphologically similar to *Chlamydomonas*, with the main difference being the absence of a cell wall in *Dunaliella* species. *Dunaliella* cell has two flagella of equal length and a single, cup-shaped chloroplast, which in the marine and halophilic species has a central pyrenoid (Oren, 2005). Cell shape in this genus is very variable, being oval, spherical, cylindrical, ellipsoidal, egg-, pear- or spindle-shaped with radial, bilateral or dorsoventral symmetry or being asymmetrical. Cells in any given species may change shape with changing conditions, often becoming spherical under unfavorable conditions. Cell size also varies with growth conditions and light intensity (Ben-Amotz *et al.*, 2009). A formal description of the genus *Dunaliella*, named in honor of Dunal who had first seen these organisms in salterns in France almost seventy years earlier, and of the first two species within the genus, *D. salina* and *D. viridis* (Oren, 2005).

2.5.1 *Dunaliella* environments

Understanding of *Dunaliella* environment is very important to achieve successful isolation and optimization of the *Dunaliella* growth. That will be through simulation of naturally occurring environmental conditions (Andersen, 2005). *Dunaliella* species often isolated from marine habitats, such as oceans, brine lakes, salt marshes, and salt water ditches near the sea, predominantly in water bodies containing more than 2M salt and high levels of magnesium as well as soils (Brock, 1975). *Dunaliella* usually flourishes on a wide range of chemical compositions and

salt concentrations ranging from 0.5% to saturation (around 35%) (Borowitzka and Borowitzka, 1988). *Dunaliella* species also occurs in wide range of pH tolerance from pH 1 *D. acidophila* ; to pH 11 *D. salina* (Gimmler *et al.*, 1989). Ben-Amotz and Avron isolated *Dunaliella* from a salt pond near Bardawil Lagoon in the North Sinai in 1976 and called it *D. bardawil* (Ben-Amotz *et al.*, 2009). Several investigators used this name although its characteristics suggest that it is in fact *D. salina* (Ben-Amotz *et al.*, 1982). Many species are extremely halotolerant and thrive in many 'bittern' habitats from seawater to salterns and evaporation ponds. *Dunaliella* species have been previously isolated from different natural sources in Saudi Arabia. *Dunaliella minuta* have been reported for the first time in Saudi Arabia from saline soil of Al-Shiggah in Al-Qaseem (Arif, 1992).

Joly (1873) described changing color of cells during aging from green to brick-red and then blood-red in Mediterranean "salt fields". He also noted an apparent relationship of color to salinity, including the reddest water color after a record dry summer in 1839. The phenomenon of orange-red algal bloom in such marine environments is usually related to combined sequential growth of *Dunaliella* first, and then halophilic bacteria, and occasionally protozoa, as commonly observed in concentrated saline lakes such as the Dead Sea, the Pink Lake in Western Australia, the Great Salt Lake in Utah, U.S.A. and in many other places around the globe (Ben-Amotz *et al.*, 2009).

The wide distribution of species of the genus *Dunaliella* may be contributed to their tolerance to a wide range of salinities, light intensities, and temperatures as well as their ability to survive for many years among salt crystals (Ben-Amotz *et al.*, 2009). Several strains of *D. salina* Teodoresco were isolated from different locations,

for example Romania, Spain, Iran, Egypt and Kuwait (Ben-Amotz *et al.*, 2009; Tafreshi and Shariati, 2006 ; Abu-Rezq *et al.*, 2010).

2.5.2 Life cycle and reproduction in *Dunaliella*

Dunaliella salina and some of the other species undergo complex life cycles that encompass, in addition to division of motile vegetative cells, the possibility of sexual reproduction. Fusion of two equally sized gametes (iso-gametes) to form a zygote was documented in many of the early studies. Some *Dunaliella* species can also develop a vegetative palmelloid stage consisting of round non-motile cells. This phenomenon has been documented in *D. salina* cultures at lowered salinities (Oren, 2005). Brock, 1975 observed such palmelloid forms of *Dunaliella* sp. in benthic algal mats of Great Salt Lake, Utah. *Dunaliella* cells resorted to sexual reproduction only in harsh conditions. Sexual reproduction is rarely observed in cultures, but more often in the field. It is by isogamy, with conjugation proceeding in a manner similar to that observed in *Chlamydomonas*. The zygote is green or red and is surrounded by a very thick smooth wall. After a resting stage the zygote nucleus divides meiotically, forming up to 32 cells which are liberated through a rupture in the mother cell wall (Borowitzka *et al.*, 1982). It has been reported that zygote formation in *D. salina* to be induced by a reduction in salt concentration from 10 to 3%. In the process first the flagella touch, and then the gametes form a cytoplasmic bridge and fuse (Oren, 2005). For successful large scale cultivation of *D. salina* , it is important to minimize the sexual reproduction and maximize the vegetative reproduction through fulfill the optimum culture conditions (Ben-Amotz *et al.*,2009).

2.5.3 Previous studies on *Dunaliella salina*

Unicellular green algae of the genus *Dunaliella* were studied since the early 19th century and numerous species were characterized and classified since then.

There are 23 salt-water species of *Dunaliella* which are divided into four sections: *Dunaliella*, *Peirceinae*, *Tertiolectae* and *Virtidis* of which only members of *Dunaliella* accumulate carotenoids. *Dunaliella salina* stands out as an organism that accumulates a high content of carotenoids in response to environmental stress (Ben-Amtoz *et al.*, 2009). Therefore *D. salina* is one of the most studied alga worldwide. However, the local and semi-local researches are almost rare. Al Rashed and Arif (1998) studied the effect of crude oil extract and hydrocarbons on *D. salina*. Later, Allam and Abd-El.Kareem (2001) have been tested the effect of sewage and industrial wastewater on *Dunaliella* cells. Al-Ghamdi (2004) studied the effect of six anions on the growth and production of carotene in *D. salina* isolated from Arabian Gulf. Recently, Al-Adali *et al.* (2012) were determined optimal salinity, temperature, nutrient level and CO₂ supply required for achieving maximal growth of *Dunaliella salina* (Dunal) Teodoresco. Abu-Rezq *et al.* (2010) found out the optimum indoor culture conditions required for the highest growth rate of *D. salina* isolated from Kuwait's puddle stagnant waters off Bubiyan Island.

2.6 *Dunaliella salina* applications

Dunaliella salina has many economical promising applications. It could be used in a various high-value chemicals production such as β -carotene (Borowitzka and Borowitzka, 1990), glycerol (up to 50% of dry weight) which have been used in the cosmetic, pharmaceutical and food industries (Ben-Amotz *et al.*, 1982), polyunsaturated fatty acids (PUFA) which have important benefits and functions in dietetics and therapeutic uses (Abd El-Baky *et al.*, 2004 b), protein (57% of dry weight) (Spolaore *et al.*, 2006), minerals (Supamattaya *et al.*, 2005), enzymes (Ben-Amotz and Avron, 1990) and antioxidants which decrease the risk of cancer, aging, inflammation, stroke and neurodegenerative diseases (Abd El-Baky *et al.*, 2004 a ;

Chidambara Murthy *et al.*, 2005; Milko, 1963). In addition, *D. salina* produces other bioactive compounds like antibiotics. It is demonstrated that *D. salina* showed good antimicrobial activity against several microorganisms of importance for the food industry (Herrero *et al.*, 2006). Moreover, *D. salina* is a promising source of biofuel due to its high content of lipids. Park *et al.* (1998) proved that the hydrocarbon productivity of *D. salina* was similar to that from *Botryococcus braunii*, which was well known to economically produce liquid fuels. Table 2.2 shows the promising applications of *D. salina*.

2.6.1 Biofuels from *Dunaliella salina*

Dunaliella salina is a particularly promising alga for biofuels production for several reasons: First, the high productivity of biomass. Second, it has a high content of commercially valuable chemicals such as: β -carotene, starch, glycerol, and lipids. Third, the lack of need for arable land and freshwater (it can be carried out in shallow ponds on hardpan soils, using saline or brackish water) (Avron and Ben-Amotz 1992; Ben-Amotz and Avron 1983; Oren 2005).

There are several alternatives for using *D.salina* as fuels including: 1) Drying the biomass followed by direct combustion for power generation or other thermochemical conversions (pyrolysis) to generate gas or oils. 2) Chemical or physical separation of lipids for the production of biodiesel, 3) Fermentations to produce ethanol, methane or other fuels by microbial action (Ben-Amotz *et al.*, 2009).

2.6.2 Carbohydrate content and production of ethanol from *Dunaliella salina*

The carbohydrate content is important parameter in production of ethanol and other solvents from microalgae. The basic idea of ethanol generation from *Dunaliella*

Table 2.2 Promising applications of *Dunaliella salina* .

Chemicals	Content	Proposed application	Reference
Source of carotinoids, vitamins and antioxidants		Cosmatic and pharmaceutical industries	Chidambara Murthy <i>et al.</i> (2005); Milko (1963)
Beta-carotene	>5% of dry weight	Cosmatic, pharmaceutical and food industries	Borowitzka and Borowitzka (1988)
Biological reactors			Geng <i>et al.</i> (2003)
Minerals		Pharmaceutical industries	Supamattaya <i>et al.</i> (2005)
Enzymes		Pharmaceutical industries	Ben-Amotz and Avron (1990)
Treating wastewater		Bioremediation	Thakur and Kumar (1999)
Hydrocarbons		Biofuel	Park <i>et al.</i> (1998)
Source of glycerol	up to 50% of dry weight	Cosmetic, pharmaceutical and food industries	Tornabene <i>et al.</i> , (1980) Borowitzka and Borowitzka (1988)
Polyunsaturated fatty acids (PUFA)		Benefits and functions in dietetics and therapeutic uses	Abd El-Baky <i>et al.</i> (2004 b)
Protein	57% of dry weight	Food supplements and animal feeds	Spolaore <i>et al.</i> (2006)
Antibiotics		Pharmaceutical industries	Herrero <i>et al.</i> (2006)

is fermentation of starch or glycerol (that is accumulated at high salinities by *Dunaliella* to ethanol. Shirai *et al.* (1998) cultivated *Dunaliella* on a high-salinity (3% wt NaCl) soy sauce waste, disrupted the cells and saccharified the intracellular starch using glucoamylase produced by *Rhizopus* species. The saccharified starch was then fermented by *Saccharomyces cerevisiae*, yielding 11 mg ethanol per gram *Dunaliella* biomass (dry weight basis). Nakas *et al.* (1983) found that glycerol produced by *Dunaliella* can be fermented to solvents, including ethanol. The ethanol yields depending on fermentation conditions.

2.6.3 *Dunaliella salina* in aquaculture industry

Recently, the microalga was introduced to the aquaculture industry in two forms, liquid paste and dry powder. *D. salina* biomass containing high levels of protein, carotenoids, minerals, vitamins, and fatty acids (Ben-Amtoz *et al.*, 2009). *D. salina* used in aquaculture, as a sole food source for filter feeders, a food additive for many fish and crustacean species, as well as enrichment for rotifers and artemia (Stottrup and McEvoy, 2003).

2.6.4 Beta-carotene production from *Dunaliella salina*

Dunaliella salina accumulates large amount of β -carotene under stress conditions such as high salinity, high temperature, and high light intensity (Abu-Rezq *et al.*, 2010). *Dunaliella salina* has served and still as the major source for the extraction of β -carotene for commercial applications (Ben-Amtoz, 2003). Beta-carotene has been used for many years as a food coloring agent, as pro-vitamin A (retinol) in food and animal feed, as an additive to cosmetics, multivitamin preparations, and in the last decade as a health food product under the as an antioxidant agent. Beta-carotene production by *Dunaliella* sp. is the major success story of halophile biotechnology (Oren, 2010). Large scale commercial β -carotene

production by *D. salina* is only viable because of a combination of factors. Firstly, the ideal production sites are arid or semi-arid areas with a high salinity water supply. Secondly, it is possible to grow *D. salina* in highly selective medium, inducing the production of β -carotene and at the same time minimizing the risk of possible contamination by predators or competitors for the algae (Ben-Amtoz *et al.*, 2009). Thirdly, *D. salina* accumulates β -carotene more than other species (Ansen *et al.*, 1969). *Dunaliella* cell can accumulate thousands of times more β -carotene than a carrot cell (Klausner, 1986). It can yield about 400 mg β -carotene/m² of cultivation area (Finney *et al.*, 1984). Mokady *et al.* (1989) have shown that *Dunaliella* is safe and can be a potential source of food supplement. Therefore, it is an attractive "cell factory" for the commercial production of β -carotene by using salinity shock (Moulton *et al.*, 1987). Finally, the high commercial value of bulk beta-carotene, from US\$300 to US\$3000 per kg, makes it a very attractive investment (Spolaore *et al.*, 2006).

Nowadays, β -carotene via *D. salina* is produced in places such as Australia, Israel, USA, China and some pilot-scale projects in Spain, Kuwait, Chile, Iran and others (Spolaore *et al.*, 2006). The largest algae farms in the world are located in Hutt Lagoon and Whyatta, Australia, and cover over 800 ha, owned by Cognis Australia (Tafreshi and Shariati , 2009). Different strategies have been proposed to maximize β -carotene production per unit time and per culture volume. The scientific basis behind all strategies is the same as mentioned before; stress conditions in *D. salina* will trigger β -carotene production. In fact, optimization of both cultivation parameters and cultivation systems is needed in order to achieve maximization of β -carotene production.

2.6.4.1 Neutral beta-carotene versus synthetic beta-carotene

Beta-carotene source can be natural and also be synthesized chemically. While β -carotene is present in many natural such as fruits and the fungus *Blakeslea trispora*; its concentration in these sources is relatively low (Dufosse *et al.*, 2005). Natural β -carotene is a mixture composed mainly of 9-cis, the balance being all-trans (Prieto *et al.*, 2011). Beta-carotene is present in most plants in small amounts of ~0.2% of the dry weight with approximately 1/3 as 9-cis β -carotene (Ben-Amotz *et al.*, 2009). The β -carotene biosynthesized by *Dunaliella* contains of the high bioavailability 9-cis and all-trans stereoisomers, representing 40% and 50%, respectively. Thus, the content of 9-cis β -carotene is highest in *Dunaliella* among all the natural sources (Hsu *et al.*, 2008). The chemical product differs from that of *Dunaliella*: the synthetic form contains only the all-trans isomer (fig. 2.1), while the alga produces a high percentage of 9- cis β -carotene (Oren, 2010).

Although synthetic forms of β -carotene are much less expensive than the natural ones, extensive research have been compared the effects of synthetic and natural carotenoids and concluded that natural carotenoids are safer and more effective than synthetic carotenoids (Ben-Amotz *et al.*, 2009). It is accepted today that the natural isomer composition of β -carotene is superior to the synthetic all-trans form, for example in terms of antioxidant and anticancer activities (Spolaore *et al.*, 2006). Furthermore, certain studies that tested the role of synthetic β -carotene supplements in cancer prevention highlighted a toxicity risk due to the possible chemical contamination derived from the production and purification processes.

2.6.4.2 Demands of β -carotene in global markets

Beta-carotene pigment has a high economical value. Its demand is increasing in global markets due their antioxidant activity and colorant properties (Berset, 1990;

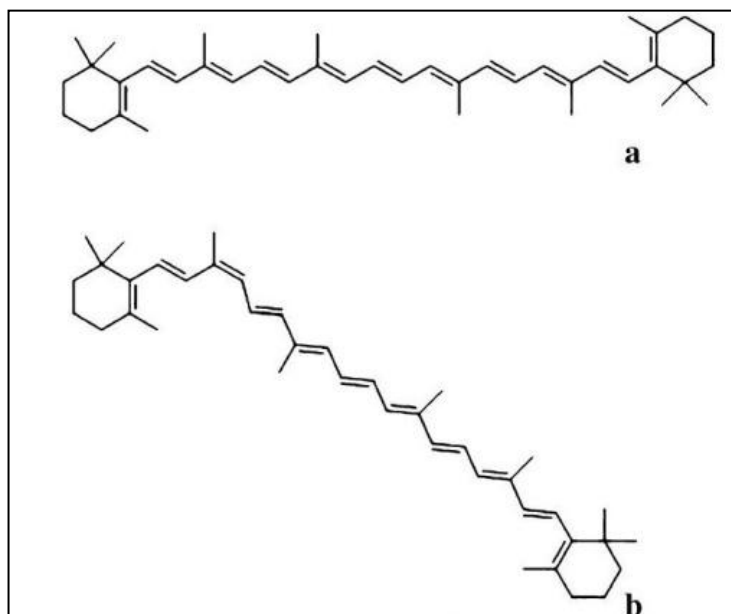


Fig. 2.1 Chemical structures of beta-carotene isomers. a. 9-cis-beta -carotene, b. all-trans-beta-carotene (Wang *et al.*, 1994).

Gomez and Gonzalez, 2004). The cost of natural *D. salina* β -carotene varies from \$ 300 to \$ 3000 / kilogram (Bettenbaugh *et al.*, 2008). Although natural β -carotene price is much higher than synthetic (\$400 to \$ 800 / kilogram for synthetic β -carotene, the price difference reflects that the consumers prefer the natural products (Abu-Rezq *et al.*, 2010). The natural products of *Dunaliella* sp. are widely distributed in many different markets under different categories: dried *Dunaliella* powder which used for fishes and prawns feed in aquaculture, soft capsules for human use, β -carotene extract which utilized for pharmacy, cosmetics and nutrition supplements production (Ben-Amotz and Avron 1990; Ben-Amotz, 2003). Saudi consuming market is promising in natural products. Therefore, investment in such *D. salina* products will fulfill both local and international markets.

2.7 Cultivation systems of *Dunaliella salina*

The production of high-value compounds using light and seawater is a unique property of photoautotrophic microalgae. Several cultivation techniques that are used for production microalgal biomass have been developed by researchers and commercial producers. The photoautotrophic microalgae are most commonly grown in enclosed systems which could be ponds, tank or photobioreactors (Patil *et al.*, 2005). *Dunaliella* cultivated in closed photobioreactors to reduce the influence of external factors on the culture, this method is highly intensive cultivation. Many trials of different models of closed photobioreactors, with attempts to design the best sunlight-harvesting unit for β -carotene optimization (Hejazi, 2003). However, none of these trials have taken production beyond the laboratory or small pilot plant volume, mainly due to economic limitations and non-feasible large-scale development. The few industrial ventures of high intensity-closed photobioreactors became insolvent and no longer exist (Ben-Amotz *et al.*, 2009).

The open pond *D.salina* cultures are economically more favorable, but raise the issues of land use cost, water availability, and appropriate climatic conditions. Neither temperature nor solar radiation can be controlled in open ponds (Ben-Amtoz, 1995). Further, there is the problem of contamination by fungi, bacteria and protozoa and competition by other microalgae. In *D. salina* open ponds, contamination is managed mainly by maintaining high salinities (Andersen, 2005). Several strategies have been used open ponds to grow commercial scale *Dunaliella* biomass. These strategies summarized as follows:

a) Extensive cultivation: is a shallow and unmixed pond. In this system the growth conditions are poorly controlled. Therefore, very high-salinity water employed to decrease fungal contamination and attacks by zooplanktonic predators (Brock, 1975). This system are currently used in (Cognis plant) due to the low operating costs in Australia (Ben-Amtoz *et al.*, 2009)

b) Intensive cultivation uses high biotechnology to control all factors affecting cell growth and chemistry. The ponds are usually oblong, lined, constructed raceways varying in size up to a production surface area of approximately 3000 m². The use of long arm, slow revolution paddle wheels is presently common in the large-scale facilities in Israel, USA, China, and Chile (Ben-Amotz, 2003).

c) Semi-intensive cultivation: between the extensive and intensive modes, where ponds are enlarged ten times, to about 50.000 m² each, with partial control and no mixing (Ben-Amotz, 2003).

Each of culture systems has advantages and disadvantages, and the choice of system used will depend on a number of factors such as the feasibility and the desired product. The intrinsic properties of the algae are one of the most important

factors to be considered for the selection of a practical culture system (Ben-Amtoz *et al.*, 2009).

2.7.1 Two-phase cultivation method

Accumulation of carotenoids is main characteristic of *D. salina* when growing under stress conditions. In 1981, Chen and Chi proposed a two-stage method for *Dunaliella* cultivation (Ben-Amtoz, 2003). Shaish *et al.* (1992) show that the carotenogenesis of *Dunaliella* and cell growth are two separate biological processes of different controls, and that carotenogenesis can be induced physiologically at any stage of the cell cycle. Based on this laboratory study, a large scale, two-phase *Dunaliella* cultivation system for production of β -carotene was developed and is described by (Ben-Amtoz, 1995). The two-stage process is one of the most common strategies used to grow β -carotene rich *Dunaliella* biomass. The concept of this strategy include using lower salinity conditions for optimal growth to ensure maximum production of biomass in first stage then changing to high salinity stress conditions to maximize induction and accumulation of desired products. Practically, in the first stage *Dunaliella* grown in nutrients-rich medium and low NaCl concentration. Then, the culture transferred to the second stage, diluted with high salt and limited nutrient medium (Ben-Amtoz, 2003).

2.7.2 Salt requirement of *D.salina* culture

Some considerations should be taken in *D. salina* cultivation. Salt is the major constituent of the *Dunaliella* growth medium. *Dunaliella* cells grow best at 5-10% NaCl, but concentrations exceeding 12% are required to eliminate predators (Ben-Amotz *et al.*, 2009). This requirement of NaCl could be fulfilled through using NaCl salt to adjust the medium salinity. The source of the brine can have a major effect on the economics of *D.salina* cultivation (FAO, 1990). The relatively high cost

of NaCl forces the large scale producer to use alternatives. Therefore, worth consideration is culture facility should have cheap sources of high salinity water or brines (Curtain, 2000). Therefore, commercial *Dunaliella* ponds employ evaporated concentrated seawater or seawater augmented with dry salt to reach the desired concentration in the medium. Many present *Dunaliella* production plants are located close to available sources of salt water, e.g., sea-salt industries, usually in warm and sunny areas where the rate of water evaporation is high and non-agricultural land is abundant (Ben-Amotz, 2003). There are many companies seeking to develop projects to cultivate marine algae on a large-scale at a low cost as well as in sustainable manner. For example, Australian companies Henkel-Cognis and Western Biotechnology Ltd already utilized natural salt lakes for *D. salina* cultivation (Curtain, 2000). Each of the production ponds used by Western Biotechnology Ltd. at Hutt Lagoon located on the west coast of Australia. They cultivate *Dunaliella* sp. at high salinities in a medium made up of NaCl-saturated brines obtained from Hutt Lagoon (FAO, 1990). The salinity controlled by the addition of seawater obtained from a bore located on the seaward side of the lagoon. Nutrients are added as required. When the pond has reached the appropriate β -carotene content the culture is pumped to a harvesting plant on the shore (Ben-Amotz *et al.*, 2009). After harvesting the remaining medium is returned to the growth pond and the salinity and nutrient content is adjusted as appropriate. Hence, several lines of algal research are examining the utilization of using different water sources such as brine water or sea water to diminish the salt water requirement (Lamela *et al.*, 2010; Craggs *et al.*, 1995). Recently, *D. salina* has been cultivated successfully at a laboratory scale in brine from a desalination plant in Spain (Lamela *et al.*, 2010). However, this brine

has a high salt content but low amount of nutrients. Therefore, brine could be suitable solution for shocking the biomass to induce carotenogenesis.

2.8 Overview of desalination

In the last couple of decades, significant growth has occurred in the human population. Due to population growth, reusing is considered to be an important problems throughout the world. A number of such countries and communities have turned to desalination as a solution for supplying clean potable water. As of 1992, there were 18 desalination plants operating along Saudi Arabia's Red Sea coast with a total combined productivity of 726,343 m³/ day (UNEP, 1997). Currently, Saudi Arabia is the largest producer of potable water (Danoun, 2007). The Saline Water Conversion Corporation (SWCC) of Saudi Arabia is producing a large quantity of potable water of 1014.21 million cubic meters in 2009 (SWCC, 2010).

2.8.1 Brine water

Brine water is generally defined as saturated or nearly saturated water with salt (usually sodium chloride). Brine is water containing more than 100 ppt. Brine refers to naturally occurring salt water or industrial generated water. Brine is commonly produced during some industries such as tanneries, pickling and desalination of seawater.

Desalination is a separation process used to reduce the dissolved salt content of saline water to a usable level. Desalination techniques have been used since the early stages of life by removing the salt from the seawater to use as drinkable water (Einav *et al.*, 2002). Desalination processes involve the separation of saline feedwater into two streams:

1- Low-salinity product water (the fresh water or desalinated water) which is suitable for consumption by humans.

2- Very saline concentrate (brine or discharged desalination water) which containing the remaining dissolved salts (Danoun, 2007).

2.8.2 Discharged desalination water

The amount of flow discharged to waste as a brine discharge varies from 20 to 70 percent of the feed flow, depending on the technology that is used in the plant (UNEP, 1997). In the reverse osmosis (RO) technology (which currently used in Saudi Arabia), fifty percent of the feedwater will be potable water and the other fifty percent will be the discharge brine. A desalination plant is the same as any other industrial activity could have the potential to cause several environmental impacts to the surrounding area. The major adverse environmental impact of desalination plants is the double salt concentration that has been extracted from the seawater and then returns back to the sea. Jeddah desalination plants dispose a large quantity of such brine into the sea. The discharges of Jeddah plants include cooling seawater (at about 39°C), chlorine and anti-sealant chemicals as well as brine (with a concentration of 50,000 ppm) which exceeds by 1.3 times the ambient salinity of the Red Sea, at a temperature of 41°C (approximately 9°C above the average ambient Red Sea temperature) (UNEP,1997). This discharge brine has the ability to change the salinity, alkalinity and the temperature averages of the seawater and can affect marine habitat. It has been estimated that the expected salinity level of the discharge brine is approximately double which equals to 64-70 ppt (UNEP, 1997). This highly concentrated waste product has harm effect for marine environment while enhancing the presence of algae. To clarify the brine impacts, it is helpful to compare some chemical characteristic of feed and brine water (Table 2.5). The brine water usually

contain: magnesium, sodium, potassium, calcium, chloride, sulfate, and bicarbonate (Alameddine and El-Fadel, 2007).

Table 2.5 Comparison between the chemical characteristic of the feed and brine water of different desalination plants (Alameddine & El-Fadel 2007).

Parameter	Plant A		Plant B		Plant C		Plant D	
	Intake water	Brine water	Intake water	Brine water	Intake water	Brine water	Intake water	Brine water
Magnesium(mg/l)	1,612	3,625	1,655	3,500	1,821	3,606	NR	NR
Sodium (mg/l)	11,806	21,750	13,250	26,142	12,103	22,437	NR	NR
Potassium (mg/l)	574	870	610	830	542	845	NR	NR
Calcium (mg/l)	516	1,850	659	1,775	563	1,818	NR	NR
Iron (ppb)	NR	NR	NR	NR	NR	NR	19.0	25.0
Copper (ppb)	NR	NR	NR	NR	NR	NR	4.3	8.0
Chloride (mg/l)	26,921	37,227	28,113	38,821	27,135	37,779	25,134	41,748
Sulfate (mg/l)	3,723	4,560	3,227	4,319	3,115	4,321	NR	NR
Bicarbonate (mg/l)	115	190	131	187	126	185	NR	NR
TDS (mg/l)	45,340	70,278	47,737	71,689	45,490	71,204	46,710	79,22

NR :Not reported

Chapter three

3. Material and Methods

3.1 Stock cultures

A Unialgal culture of *Dunaliella salina* CCAP 19/18 was obtained from CCAP (Culture Collection of Algae and Protozoa, Oban, UK). Stock cultures of the algae were grown in 250 ml Erlenmeyer flasks containing 100 ml of Johnson's medium (Johnson *et al.*, 1968) and maintained in a controlled environment room at $20 \pm 1^\circ\text{C}$, under a continuous light regime using day-light fluorescent tubes (PHILIPS TL-D 36 W/34 -765), with light irradiance of 2000 LUX ($28 \mu\text{mol. m}^{-2}\text{s}^{-1}$), light intensity was measured using (Digital luxmeter, model MS6610). pH was maintained at 8. Stocks were maintained in semi-continuous culture by transferring 10 ml of old culture to a new flask containing fresh medium every 2 weeks.

3.2 Water sampling

Three types of water were used in the experiments: Seawater (Sw), Partially Treated Sewage Water (PTSW) and Discharged Desalination Water (DDW). Seawater was obtained from Offshore Eastern Coast of the Red Sea, (Sharm Obhur, Jeddah) using boat. Partially Treated Sewage Water (PTSW) was obtained from Bani Malek Treatment Plant at Jeddah. It was collected from mobile tankers after primary treatment. Discharged Desalination Water (DDW) was taken from out take desalination plant of Saline Water Conversion Corporation (SWCC), Al-Shoaibh. All water samples were stored in reinforced glass bottles at refrigerator for no more than

one week. Water samples were filtered gradually through glass microfiber filters (934-AH, Whatman) to remove large particles and suspended solids, (GF/F, Whatman) to remove smaller particulate matter and contaminants, sterilized by autoclaving at 121 °C for 27 min. Water parameters (salinity and conductivity) were measured using conductivity meter. Turbidity was measured using turbidity meter. The chemical elements in water samples were analyzed in Center of Excellence in Desalination Technology laboratory (CEDT). Phosphate, nitrate, sulphate, potassium, calcium and magnesium were determined using ion chromatography whereas copper was determined using Inductively Coupled Plasma-Optical Emission Spectrophotometer (ICP-OES).

3.3 Media preparation

Two stocks media were prepared with different water sources. The first one was Johnson's medium prepared with distilled water (Johnson *et al.*, 1968) and the second one based on seawater as a solute and source of NaCl. Seawater was enriched with the major elements required for *D. salina* growth according to (Loeblich, 1982), calcium and magnesium salts were reduced to avoid precipitation (Harrison *et al.*, 1980). For preparation Johnson's medium, each component were dissolved into 980 ml of distilled water using cellulose nitrate membrane filter (Whatman, pore size 0.2 µm) as described in Table 6.1. Then, 10 ml of each stock solution was added; pH was adjusted on 8 before autoclaving (121°C for 20 minutes) after that cooled to room temperature.

3.4 Experimental culture conditions

The culture conditions of this study were as described before according to (Takagi, *et al.*, 2006; AbuSaraa *et al.*, 2011; Kim *et al.*, 2011; Ben-Amotz *et al.*,

2009). Gloves were worn through all steps to prevent any possible contamination. Sterile conditions were throughout the experiments applied during cultivation.

Table 3.1 Components of the media used in the study

Medium	Johnson's medium (J)	Seawater medium (Sw)
Water source salinity	Distilled water 58.44	Seawater -
MgCl ₂ .6H ₂ O	1.5 g	-
MgSO ₄ .7H ₂ O	0.5 g	0.5 g
KCl	0.2 g	-
CaCl ₂ .2H ₂ O	0.2 g	-
KNO ₃	1 g	1 g
NaHCO ₃	0.043 g	0.043 g
KH ₂ PO ₄	0.035 g	0.035 g
Fe-solution	10 ml	10 ml
Trace-element solution	10 ml	10 ml

Table 3.2 Components of stock solutions (Johnson *et al.*, 1968).

Fe solution (per 1 liter)	
Na ₂ EDTA	189 mg
FeCl ₃ ·6H ₂ O	244 mg

Trace-element solution (per 1 liter)	
H ₃ BO ₃	61.0 mg
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	38.0 mg
CuSO ₄ ·5H ₂ O	6.0 mg
CoCl ₂ ·6H ₂ O	5.1 mg
ZnCl ₂	4.1 mg
MnCl ₂ ·4H ₂ O	4.1 mg

3.5 Primary experiment

The experiment was designed to allow the selected algae to grow in different water sources through two stages. First step was to compare the effects of different concentrated media (PTSW and DDW) on *D. salina* growth. Both water types were filtered and autoclaved separately, cooled to room temperature then inoculated with 10 ml of stock culture. All cultivations were done in 250 ml Erlenmeyer flasks at controlled room temperature (20°C) and placed under continuous light regime using day-light fluorescent tubes (PHILIPS TL-D 36 W/34 -765), with light irradiance of 2000 LUX (28 $\mu\text{mole}/\text{m}^2\cdot\text{sec}$). Cell concentration of each sample was measured by counting the cell number every three days. Triplicate cultures of each medium were prepared. Since neither PTSW nor DDW enhances *D. salina* growth in the first stage, the PTSW was diluted with seawater to give different concentrations 25%, 50% and 75%. Additional medium was prepared using seawater. The second stage was aimed to determine the optimal and maximum concentration of PTSW for *D. salina* growth. *Dunaliella salina* cells exhibited clumps in seawater media. Therefore, the experiment was designed using PTSW and DDW as a shocking factors in the second stage of dual phase technique as described by (Ben-Amotz, 1995).

3.6 Experimental design

The experiments were designed into two stages: the first stage was aimed to determine the best dilutions of Johnson's medium for optimum growth of *D. salina*. The second stage aimed to use two type of water (PTSW and DDW) as a shocking factor to impose *D. salina* products. The two stages of experiment were conducted in the same time on the same culture conditions.

3.6.1 First stage

In 500 ml Erlenmeyer flasks, 1:0, 1:1, 2:1 and 3:1 dilutions of Johnson's medium versus enriched seawater were used. *Dunaliella salina* was grown in 150 ml of media. Each treatment was done in six replicates divided into two groups to be used in the next stage. Flasks were gently shaken at least once a day. All cultures were incubated in the same culture conditions used for stock cultures. During the experiment time, one ml sample was taken every 48 h to calculate the growth rate for 24 days. Once the growth rate reached the maximum, 10 ml samples were taken for protein, ten ml for carbohydrates, 2 ml for β -carotene and 20 ml lipid determination. Then, all samples were stored in the refrigerator for less than one day.

3.6.2 Second stage

The same cultures that have been previously divided into two groups in the first stage have been shocked. The first group was shocked with 1/3 sterilized DDW. The second group was shocked with 1/2 of the sterilized PTSW that was done due to (Ben-Amotz, 1995) study. The duration was ten days for both groups. The biochemical analysis were done immediately after shock and stored for less than one day.

3.7 Growth measurement

3.7.1 Growth rate

Cell number was determined using every two days, using a Model 3 Coulter Multimixer, and associated software V 5.53. The specific growth rate (μ) of *D.salina* was calculated as the slop by plotting the natural log of the cell concentration vs. time in days using equation below (Garcia *et al.*, 2007).

$$\mu = \frac{\ln C_1 - \ln C_0}{t_1 - t_0}$$

where C_0 and C_1 (cells per ml) are cell density values at the beginning (t_0) and the end (t_1) of a selected time interval between inoculation and maximum cell density, respectively.

3.7.2 Dry weight

For dry weight, *Dunaliella* cells were harvested by centrifugation at 3500 rpm for 15 min using refrigerated centrifuge and washed three times with sterile 4% (w/v) NaCl solution to eliminate the residual salts. Samples were filtered onto (Whatman GF/C) glass microfiber filters. Filters were dried at 40 °C oven for 48h before weighting. The weights of the dried samples were measured, until two successive weight give constant value. Triplicates samples of each treatment were taken.

3.8 Biochemical analysis of algal biomass

3.8.1 Beta-carotene content

According to Craft and Soares (1992) the solubility of β -carotene in Tetrahydrofuran (THF) is higher than in other solvents. Beta-carotene was extracted in THF as described by (Hejazi , 2003). A sample of 2 ml was taken from the aqueous phase of each culture medium that had been mixed thoroughly. After 15 min centrifugation at 3500 rpm the upper phase was decanted and 2 ml of tetrahydrofuran (THF) was added to the biomass. Each sample was mixed by vortex for 1-2 min to reach complete extraction. Then, samples were centrifuged again for 15 min at 3500 rpm for separation of the biomass and the solvent phase. The extracted pigments in the solvent phase were quantified by the spectrophotometric method at wavelength 455 nm. Synthetic all-trans β -carotene (Sigma, type I) was used as stranded.

3.8.2 Carbohydrate content

Total cell carbohydrate was extracted in acidic medium of sulfuric acid, carbohydrate content was determined by the reaction with phenol in the presence of sulfuric acid (Dubois *et al.*, 1956). Carbohydrate was extracted from samples as following: 10 ml of culture was centrifuged at 3500 rpm for 15 min. In ice bath, one ml of sulfuric acid was added to the pellet overnight. Then, 6 ml of sulfuric acid was added and vortex. To perform carbohydrate assay, about 200 μ l of the digested cell walls were mixed with 10 μ l of 80% phenol in water and 1.5 ml conc. H₂SO₄. The tubes were left for 5 min. in a boiling water bath followed by 30 min. at room temperature. The absorbance was measured at 485 nm against a blank. The quantity of the sugars was determined from a standard curve of D-glucose.

3.8.3 Total protein content

Protein was quantified using Lowry method (Lowry *et al.*, 1951), the total protein contents were extracted from the pellet in 1 N NaOH placed in 90°C water bath. The reagents were prepared freshly and stored indefinitely as following:

- A. 2% Na₂CO₃ in 0.1 N NaOH
- B. 1% NaK Tartrate in H₂O
- C. 0.5% CuSO₄.5 H₂O in H₂O
- D. 48 ml of A, 1 ml of B, 1 ml C
- E. Phenol Reagent - 1 part Folin-Phenol [2 N] : 1 part water

This assay were performed in a test tube contain 1 ml of protein extract mixed with 2 ml of solution D , left for 10 min at room temperature. Then, 0.2 ml of dilute Folin-phenol solution was added to each tube and vortex immediately. Test

tubes were incubated at room temperature for 30 minutes. Protein was determined by spectrophotometry at 600 nm, using Bovine Serum Albumin (BSA) as standard.

3.8.4 Total lipids content

Total lipids were determined by a modified Bligh and Dyer (1959) method. This method extracts the lipids from harvested cells using a mixture of methanol, chloroform, and water. Firstly, the cells were washed three times with 1% NaCl. The supernatant was discarded in order to minimize sample to 2 ml of cell suspension. 7.5 ml of (chloroform/methanol) 1:2 mixture was added with mixing for 10 min. Then, 2.5 ml of chloroform and 2.5 ml of 1% NaCl solution were added with mixing to adjust the ratio of methanol, chloroform and water to 2:2:1 (v/v/v). Tubes were centrifuged for 10 minutes at 3500 rpm. After centrifugation, two layers were formed, a water-methanol upper layer and chloroform lower layer which contains the extracted lipids. The upper layer was removed, the chloroform evaporated and the extracts weighed as the total lipid.

3.9 Statistical analysis

Growth rate and the effect of dilutions and stages on biochemical contents were analyzed by using One-way ANOVA. Tukey HSD tests were performed for multiple comparisons. The effect of dilution on β -caroten content during second stage was analyzed by One-way ANOVA test. Data always passed tested for normality, homogeneity of variance by Levene's test. For all analysis, the statistical significance was at ($P < 0.05$). The statistical analysis was performed using SPSS, version 21. All graphs were performed using Sigmaplot software, version 9.0

Chapter four

4. Results

4.1 Chemical characteristic of water samples

In this study, the available phosphate concentration in partially treated sewage water (PTSW) was 1.76 mg/l and it was 0.088 mg/l for seawater, whereas there was no phosphate content detected for desalination water (DDW) (Table 4.1). Nitrate content was very low (only 9.54 mg/l) in PTSW followed by seawater (1.21 mg/l). No nitrate content was detected in DDW.

The highest calcium content was recorded for DDW (784.41 mg/l), followed by seawater (544.34 mg/l), whereas the lowest content of this element was in PTSW (10.215 mg/l). The maximum magnesium content was recorded for DDW (2493.10 mg/l), followed by seawater (1488.75 mg/l), whereas the minimum content was in PTSW (14.695 mg/l). The highest potassium level was recorded for DDW (680.19 mg/l), followed by seawater (399.66 mg/l), then 1.81 mg/l for PTSW. The highest sulphate was observed in DDW (5469.1 mg/l) secondly seawater (3283.4 mg/l) and the lowest was PTSW (119.1 mg/l). The micronutrient (copper) was conducted for DDW, whereas was not detected for either seawater or PTSW (Table 4.1).

The salinity follow conductivity pattern, the highest conductivity and salinity level was observed in PTSW (584 μ S/cm), (373 mg/l), respectively followed by value (93.3 mS/cm), (59,700 mg/l) for DDW whereas the lowest conductivity

Table 4.1 Chemical characteristic of water types used in the study.

Parameter	Seawater	Discharged desalination water	Partially treated sewage
Phosphate	0.088	ND	1.76 mg/l
Nitrate	1.21 mg/l	ND	9.54 mg/l
Calcium	544.34 mg/l	784.41 mg/l	10.215 mg/l
Magnesium	1488.75 mg/l	2493.10 mg/l	14.695 mg/l
Potassium	399.66 mg/l	680.19 mg/l	1.81 mg/l
Sulphate	3283.4 mg/l	5469.1 mg/l	119.1 mg/l
Copper	ND	0.002 mg/l	ND
pH	8.06	8.27	8.90
Conductivity	60.2 mS/cm	93.3 mS/cm	584 μ S/cm
Salinity	38,500 mg/l	59,700 mg/l	373 mg/l

and salinity was recorded for seawater (60.2 mS/cm), (38,500 mg/l). All pH level was almost is the same range for all types of water about 8.

4.2 Effect of media dilution on growth parameters (first stage)

In this study, *Dunaliella salina* was capable to grow in a different dilution of Johnson's medium with seawater. Data shows that there was significant difference in growth rate between dilutions. The highest growth rate was obtained in 1:0 with mean slope $0.183 \pm 0.00 \text{ d}^{-1}$ and the lowest for 1:3 with mean slope $0.143 \pm 0.00 \text{ d}^{-1}$. There was no significant difference in the growth rate between 1:0 and 1:1; also between 1:2 and 1:3 (Fig 4.1).

4.3 Effect of Dual-phase system on biochemical composition content

4.3.1 Protein

There were variations in the means of protein contents per cell and per liter between dilutions and stages. In general, protein content in the first stage was higher than the second stage per cell and per liter. In the first stage, maximum protein content was for 1:0 ($13.06 \pm 0.28 \text{ pg/cell}$) and the lowest was for 1:3 ($9.53 \pm 0.28 \text{ pg/cell}$).

The second stage whether effects DDW or PTSW showed the same pattern (Fig 4.2). In DDW and PTSW, the dilution 1:0 was highest in the amount of protein content per cell and per liter (6.57 to 8.06 pg/cell). The lowest protein content was for 1:3 (1.9 to 4.53 pg/cell). There was no significant difference between dilutions 1:1, 1:2, 1:2 and 1:3 in protein content per cell. Protein content per liter shows that there was no significant difference between the dilutions 1:1 and 1:2. No significant differences in protein content between shocked with DDW and PTSW per liter, whereas there were significant differences in protein content per cell between all stages (Table 4.2).

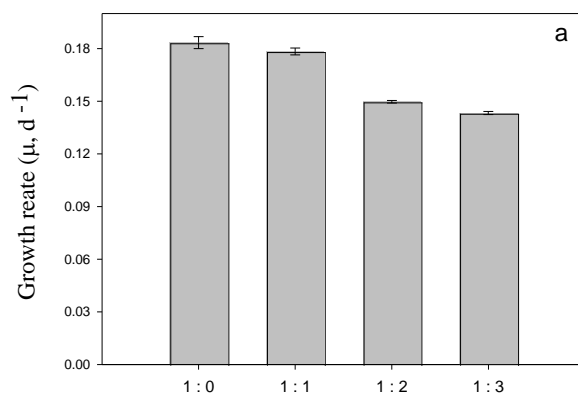


Fig.4.1 Growth rate of *Dunaliella salina* grown in different dilutions of Johnson's medium during the first stage (μ , day^{-1}), Bars are one standard error \pm .

Table 4.2 One-way ANOVA and Tukey HSD test for the effect of Johnson's medium dilutions and stages on biochemical contents. Turkey's test data underscoring indicates where there were no significant differences. Biochemical content of *D.salina* cells expressed as pg per cell.

Parameter	One-way ANOVA (groups and stages)	Turkey's test Between dilutions				Turkey's test Between stages		
Protein (pg cell ⁻¹)	**	1:0	<u>1:1</u>	<u>1:2</u>	1:3	First	PTSW	DDW
Carbohydrate (pg cell ⁻¹)	**	<u>1:0</u>	<u>1:1</u>	1:2	1:3	First	PTSW	DDW
Lipid (pg cell ⁻¹)	**	1:0	1:1	1:2	1:3	First	PTSW	DDW
Beta-carotene (pg cell ⁻¹)	**	1:0	1:1	<u>1:2</u>	<u>1:3</u>	First	PTSW	DDW

** p- value ≤ 0.05

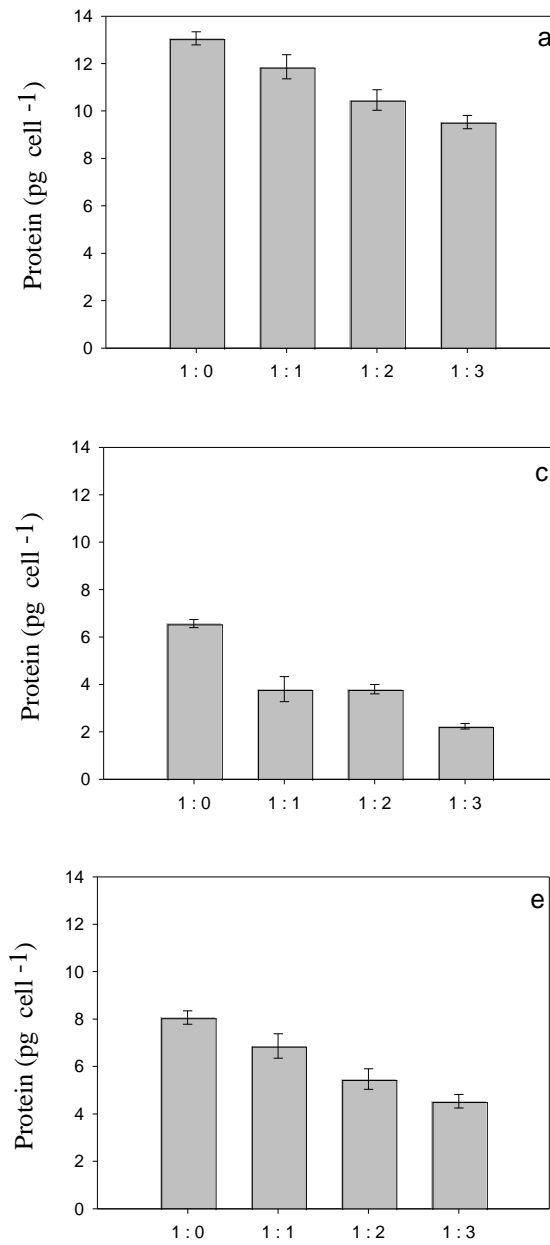


Fig. 4.2 Protein content of *Dunaliella salina* , a. first stage (pg cell⁻¹) ,b. second stage (shocked with DDW) (pg cell⁻¹), c. second stage (shocked with PTSW) (pg cell⁻¹), of different dilutions of Johnson's medium (1:0 , 1:1 , 1:1 , 1:3). Bars are one standard error ±.

4.3.2 Carbohydrate

The highest carbohydrate content was observed in the first stage compared to the second stage shocked by DDW and PTSW (Fig 4.3).

In the first stage, the highest carbohydrate content was observed for 1:0 (14.37 ± 0.16 pg/cell). In the other hand, the lowest carbohydrate content was recorded for 1:3 (10.3 ± 0.15 pg/cell) (Fig 4.3 a). In the second stage, generally carbohydrate content was higher in the DDW shocked cultures than shocked by PTSW. Carbohydrate content ranged from 6.8 to 8.23 pg/cell for DDW shocked cultures. Dilution 1:0 in PTSW shocked cultures was highest in carbohydrate content (7.13 ± 0.11 pg/cell) (Fig 4.3). There was no significant difference in carbohydrate content between dilutions 1:0 and 1:1 pg/cell. There were also significant differences in carbohydrate content per cell between all stages (Table 4.2).

4.3.3 Lipids

In general, lipid content was enhanced by shocking either by DDW or PTSW. In the second stage, the cultures shocked by DDW were achieved better lipid content (5.66 to 15.13 pg/cell) than cultures shocked by PTSW (3.8 to 8.5 pg/cell) (Fig 4.4). There were significant differences in the lipid content between all stages per cell and per liter. Also, there were significant differences between all dilutions in lipid content per cell whereas there was no significant difference in lipid content per liter between 1:0 and 1:1 (Table 4.2).

4.3.4 Beta-carotene

Overall, β -carotene content per cell and per liter were higher in the second stage than first stage. In the first stage, the highest β -carotene content was in dilution of 1:0 (3.65 ± 0.08 pg/cell) and the lowest was in dilution 1:3 (1.13 ± 0.10 pg/cell) (Fig.4.5a).

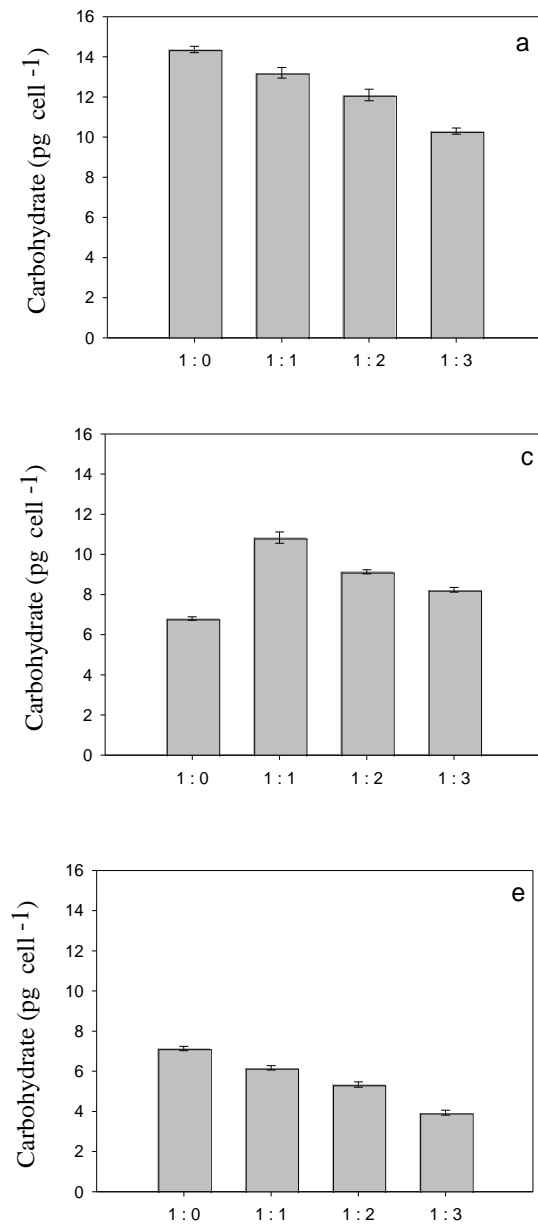


Fig. 4.3 Carbohydrate content of *Dunaliella salina* , a. first stage (pg cell⁻¹) ,b. second stage (shocked with DDW) (pg cell⁻¹), c. second stage (shocked with DDW) (pg cell⁻¹), of different dilutions of Johnson's medium (1:0 , 1:1 , 1:1 , 1:3). Bars are one standard error ±.

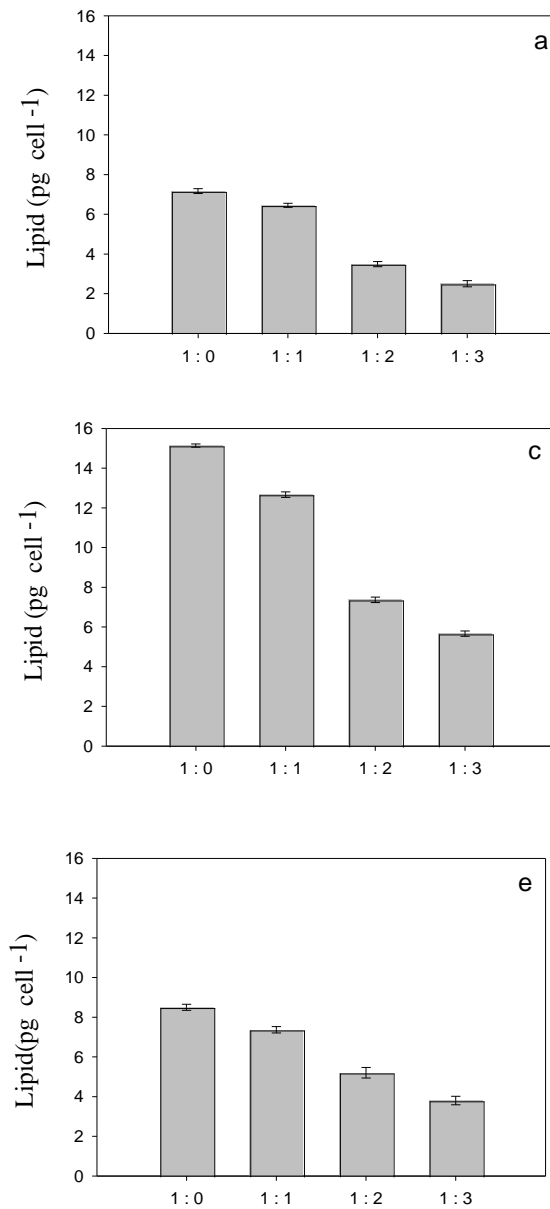


Fig. 4.4 Lipid content of *Dunaliella salina* , a. first stage (pg cell⁻¹) , b. second stage (shocked with DDW) (pg cell⁻¹), c. second stage (shocked with PTSW) (pg cell⁻¹), of different dilutions of Johnson's medium (1:0 , 1:1 , 1:1 , 1:3). Bars are one standard error ±.

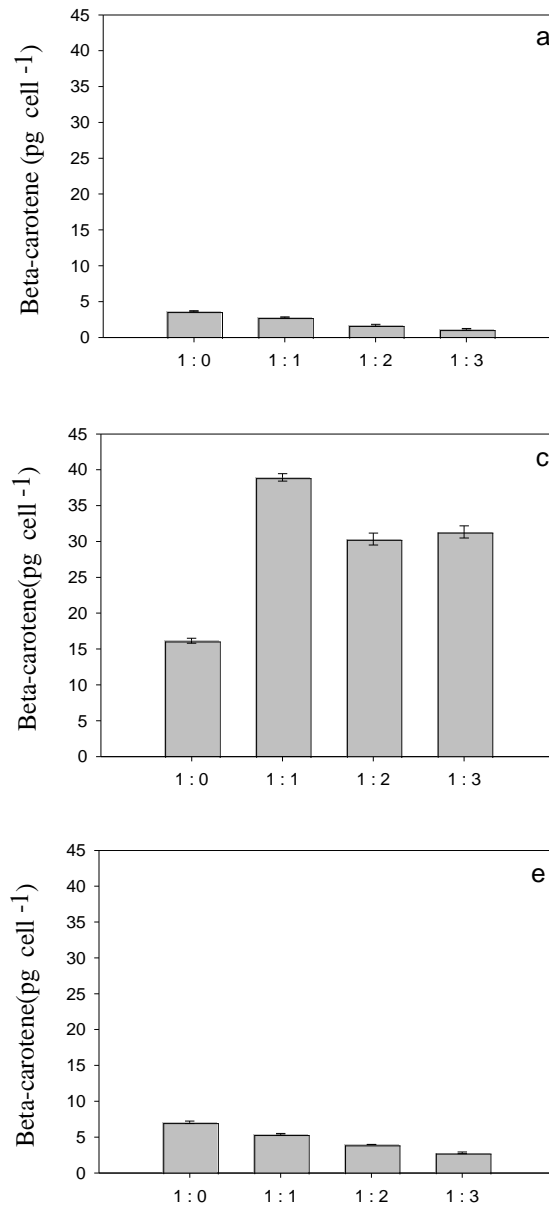


Fig. 4.5 Beta-carotene content of *Dunaliella salina* , a. first stage (pg cell⁻¹) ,b. second stage (shocked with DDW) (pg cell⁻¹), e. second stage (shocked with PTSW) (pg cell⁻¹), of different dilutions of Johnson's medium (1:0 , 1:1 , 1:1 , 1:3). Bars are one standard error ±.

The highest β -carotene content per liter was obtained in second stage shocked by DDW ranged from 16.17 to 38.93 pg /cell compared to PTSW shocked cultures (Fig 4.5). There was no significant difference in β -carotene between dilutions 1:2 and 1:3 pg/cell (Table 4.2).

In addition, β -carotene content per cell and per liter showed no significant differences between first stage and second stage shocked by PTSW (Table 4.5) whereas there were significant differences in β -carotene content per cell between all stages.

In the second stage, (shocked by DDW) the highest content of β -carotene was for dilution 1:1 with means of 38.93 ± 0.51 pg/cell and the lowest was for 1:0 with means of 16.17 ± 0.35 pg/cell (Fig 4.5b). One-way ANOVA showed that there was no significant difference in β -carotene content between dilution 1:2 and 1:3 per cell whereas the rest of dilution has significant differences between them in β -carotene pg/cell (Table 4.3).

Table 4.3 One-way ANOVA and Tukey HSD test for the effect of DDW shock on beta-carotene content. Turkey's test data underscoring indicates where there were no significant differences.

Parameter	p-level	Turkey's test
Beta-carotene (pg cell ⁻¹)	**	1:0 1:1 <u>1:2 1:3</u>

** p- value ≤ 0.05

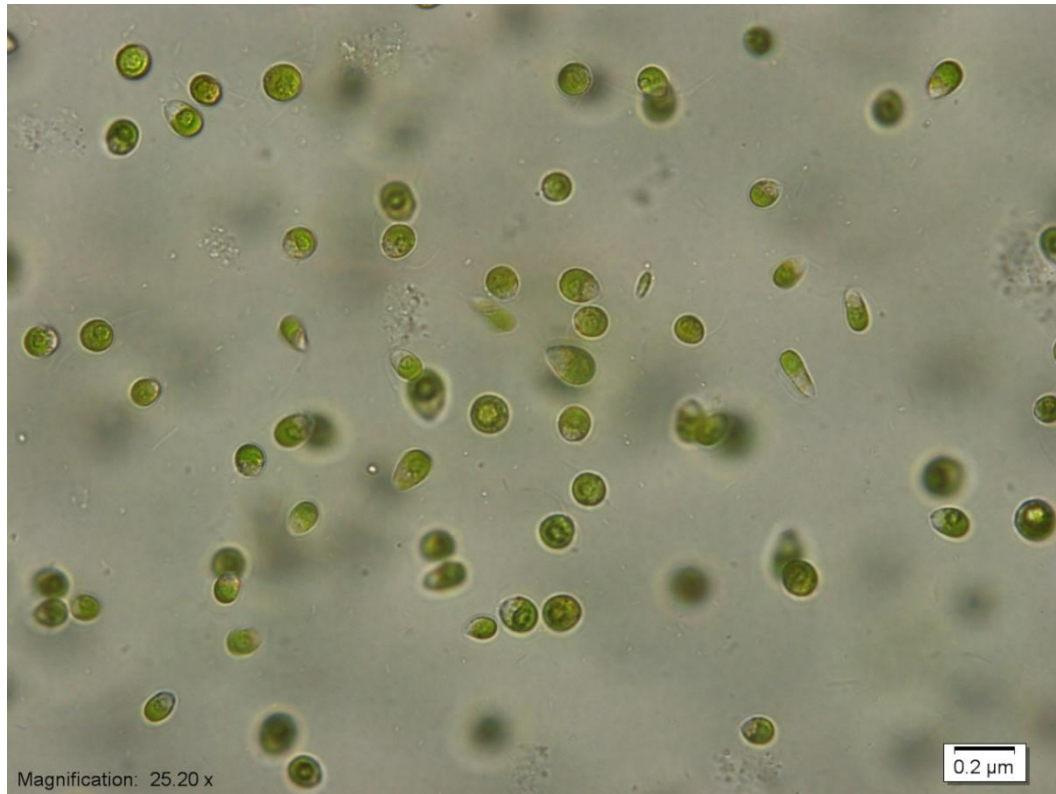


Fig. 4.6 Microscopic photograph of *Dunaliella salina* cells through the first stage (15 days old) at a magnification of 500x.



Fig. 4.7 Photograph of *Dunaliella salina* cultures through the first stage (15 days old).

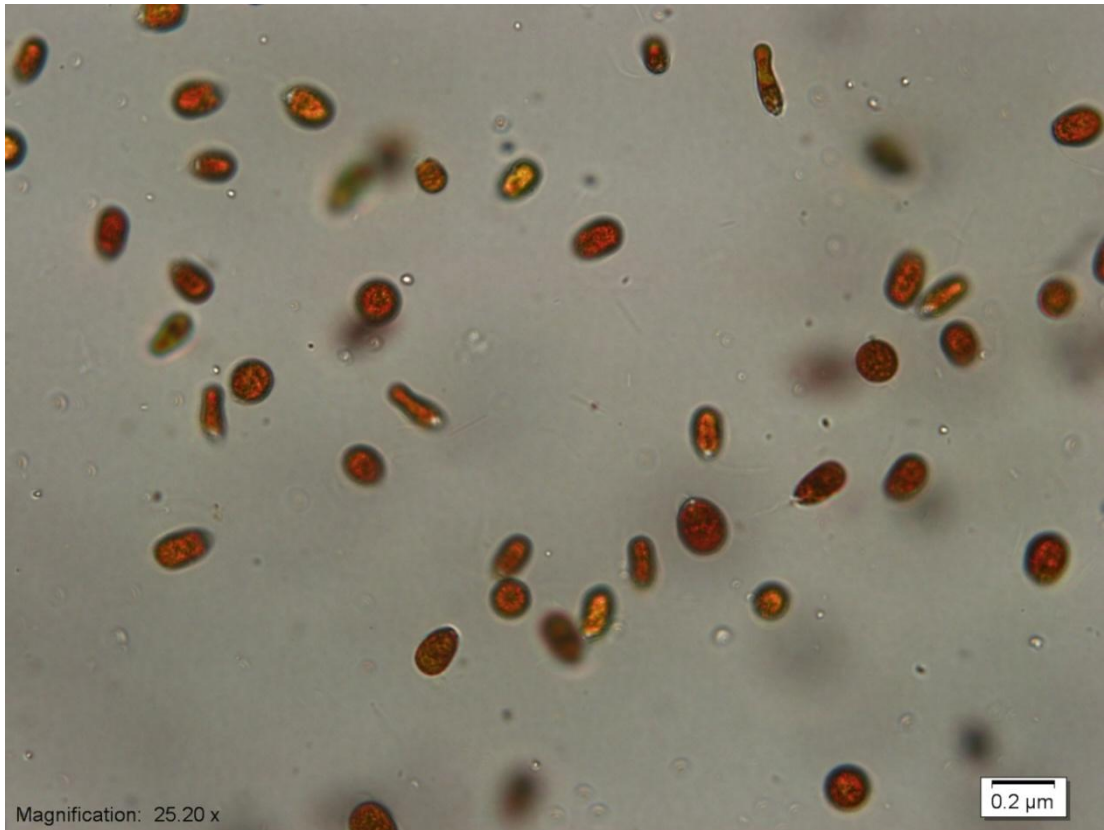


Fig. 4.8 Microscopic photograph of *Dunaliella salina* shocked cells with discharged desalination water (DDW) at a magnification of 500x.

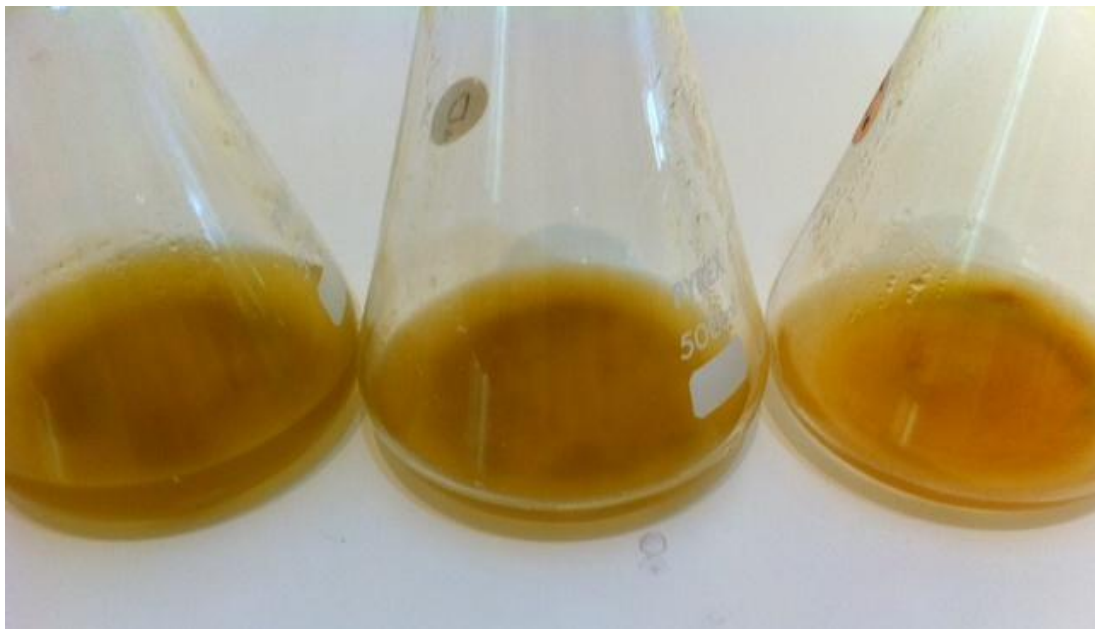


Fig. 4.9 Photograph of *Dunaliella salina* cultures through the second stage (after 15 days) of shocking with discharged desalination water (DDW).

Chapter five

5. Discussion

Growth parameters reflect the response of microalgae cells to favorite growth conditions (Stein, 1973), however biochemical composition content doesn't follow this trend (Gottumukala, 2010). In this study, Dual-phase system tested the first stage to achieve higher growth performance for *D. salina* whereas the second stage was designed to boost some specific biochemical composition content.

The maximum growth rates reflect the optimal culture conditions (Ben-Amotz *et al.*, 2009). Mainly, phosphorus and nitrogen are important macronutrients to build up material for microalgae growth (Ben-Amotz, 2003). Therefore, any limitation in these macronutrients could clearly reflected by growth rate value. Although chemical analysis of natural seawater that used in this study show no phosphate content was detected and low nitrate content (see Table 4.1), nutrients provided by diluted Johnson's medium 1:1 (J/Sw) was sufficient enough to support growth of *D. salina*. Whereas further dilutions in seawater were lead to dilute the minimum phosphate and nitrate content (Andersen, 2005).

On the other hand, the biochemical composition content of any microalgal species is clearly related to their growth phase (exponential or stationary) and to culture conditions such as light frequency (Brown *et al.*, 1993), light intensity (Thompson *et al.*, 1993), temperature (Thompson *et al.*, 1992) or culture media

(Wikfors *et al.*, 1996). Literature results have been obtained mainly with small experimental volumes under various controlled, but highly variable, conditions (batch/continuous, L/D cycle, period of harvesting). Gross composition does not always correlate directly with nutritional value owing to possible deficiency in some essential nutrients. However, when specific essential nutrients are in adequate proportion, the gross composition may be important (Stottrup and McEvoy, 2003). Brown *et al.* (1997) reported that overall biochemical of 40 algal species grown under standard condition showed protein as a major organic component (15-52% of dry weight), followed by lipid (2-20%), then carbohydrate (5-12%).

In this study, protein and carbohydrate content in the first stage was higher comparing to the second stage. The first stage present the favorite culture conditions therefore, physiological process of photosynthesis was maximum to enhance carbohydrate content (Wainman and Smith, 1997). In addition, phosphate and nitrate concentration was sufficient to build up protein content in the first stage compared to the second stage (Gottumukala, 2010). In the second stage, the available phosphorus concentration in PTSW was lower than the optimal level required for *D. salina* (Ben-Amtoz *et al.*, 2009) (see Table 4.1). Nitrogen and phosphorus were limiting macronutrients in DDW (see Table 4.1) that maybe the reason for the lowest protein content that recorded in this study for DDW.

Lipids are known to be a storage product of many algal species (Andersen, 2005). It was found that there are many microalgae species capable of producing high amounts of lipids under nitrogen deficiency (Roessler, 1990). Literature also showed that increasing salinity has significant effect on increasing lipid content (Gautam *et al.*, 1994 ; Takagi *et al.*, 2006).

This study supported these phenomena as highest lipid content was observed in DDW that has no P and N content recorded (Table 4.1). That suggesting that DDW could be good addition to *D.salina* cultivation system in Saudi Arabia and other Arab region, that has proven to be successful in Spain (Lamela *et al.*, 2010).

Studies, carried out with different *D. salina* strains found variations in β -carotene content depend on stress factor such as: high light intensity, high temperature, high salinity, and nitrogen deficiency (Abu-Rezq *et al.*, 2010). The most highly effective factor is salinity (Ben-Amtoz *et al.*, 2009). Ben-Amtoz (2003) stated that most *Dunaliella* cells turning orange or red in color under the wide range of salt concentrations. High salt content in *Dunaliella* culture media known to induced carotenogenesis (Ben-Amtoz, 1995). The data of this work shows that β -carotene content in *D. salina* shocked with DDW was the maximum recorded content due to high level of salinity. Abd-El-Baky *et al.* (2004a) showed similar β -carotene content with similar salinity concenterntation

Furthermore, literature shows that the carotenogenesis and growth *Dunaliella* sp. are two separate biological processes (Shaish *et al.*, 1992) that explains the minimum β -carotene content recorded in the first stage of this work.

This study suggests that the dilution of 1:1 is the best to achieve maximum production and quality composition compared to the other dilution. Nevertheless, DDW could be a new addition to be applied in *D. salina* cultivation system. It is known that the amount of flow DDW varies from 20 to 70 % of feed flow of desalination process (UNEP, 1997). Saudi Arabia has the highest desalination capacity worldwide, the use of brine could be a good idea in *D. salina* cultivation to

minimize the environmental impacts and benefit from the huge capacity of this wasted unused water source for lipid and β -carotene production.

Further studies could improve such application in Saudi Arabia. Temperature is known to affect the biochemical of *D. salina* and any other microalgal species. Therefore, it could be selected as the first factor to improve *D. salina* β -carotene production application (Gomez and Gonzalez, 2005).

It is also recommended to search for local strain for any selected cultivation system (Thinh *et al.*, 1999). Using locally isolated is better than using international because it would be adapted to local environmental conditions especially in countries with high temperature level (Chen *et al.*, 2009). Dual-phase technique is suitable for outdoor cultivation system especially with local strain. Furthermore that could be even improve further by genetic modification to fit outdoor cultivation system need and for the specific product targeted (Ben-Amtoz, 1995; Tafreshi and Shariati, 2006).

6. References

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