Evaluation of *Dunaliella* isolates from Sua Pan (Botswana) solar salt evaporation ponds for production of β-carotene

Prepared by: Malebogo Pinkie Ralefala

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Department of Chemical Engineering
University of Cape Town
Cape Town, South Africa

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Supervisor: Prof S.T.L. Harrison

ABSTRACT

The overall objective of this study was to characterise, functionally, *Dunaliella* species naturally inhabiting Sua Pan solar saltworks evaporation ponds in Botswana and to evaluate the species for potential application in commercial production of β-carotene. The main goal was to establish if the species are of commercial value with regard to massive accumulation of carotenoids, specifically β-carotene. The study assessed the productivity of the environmental isolates by means of growth and carotenoid biosynthesis properties at indoor laboratory scale. Further, the study aimed at establishing conditions for control of non-carotenogenic *Dunaliella* species co-existing with the carotenogenic ones in the Sua Pan solar salt evaporation ponds. The commercially used *D. bardawil* UTEX 2538 was used as basis for comparison.

The *Dunaliella* cultivation system was initially established by studying growth properties of *D. bardawil* UTEX 2538 and *D. salina* UTEX 1644 under varying levels of light intensity, sodium chloride concentration, nitrate concentration and temperature. Univariate or multivariate experiments were used. Optimum starting cell concentration and cultivation media selection were established at the beginning of the thesis. Light intensity levels were from 300 to 2500 lux (4.04 to 33.75 $\mu mol.m^{-2}.s^{-1}$) while temperature levels were at 25°C and 30°C. Growth of *D. bardawil* UTEX 2538 and *D. salina* UTEX 1644 in Modified Johnson's medium and BAAM were compared. Starting concentration of 10% v/v was selected to give the highest maximum cell concentration. This, however, was due to the highest starting concentration rather than the highest specific growth rate. The 10% v/v inoculum resulted in a concentration of 94 x 10^4 $cells.mL^{-1}$ with a specific growth rate of 0.16 day^{-1}. Within the light intensity range used, higher cell growth rates of 0.45 day^{-1} were observed at 2500 lux compared to 0.13 day^{-1} at 300 lux. However, these light intensities remained light limiting. The *Dunaliella* species grew better at 25°C than at 30°C. BAAM medium was selected for successive experiments. The cell concentrations achieved in this section were in the average of 159 x 10^4 $cells.mL^{-1}$ for *D. salina* UTEX 1644 and 115 x10^4 $cells.mL^{-1}$ for *D. bardawil* UTEX 2538. The specific

growth rates for *D. salina* UTEX 1644 and *D. bardawil* UTEX 2538 were 0.48 day^{-1} and 0.44 day^{-1} respectively.

The brines collected from solar evaporating ponds of the Sua Pan saltworks contained two *Dunaliella* species: larger cells that appeared red at high salt concentration and small cells that remained green across all salt concentrations. Based on cell morphology and physiological properties, the large carotenogenic cells were identified as *D. salina* while the small non-carotenogenic type was identified as *D. viridis*. The isolates were studied under varying gradients of light intensity, temperature, sodium chloride concentration and nitrate concentration to identify common points for optimum growth of carotenogenic cells while limiting growth on the non-carotenogenic cells. Increase in temperature from 25°C to 30°C reduced growth of non-carotenogenic and carotenogenic cells. Increase in sodium chloride concentration from 2 M to 3 M reduced growth of non-carotenogenic and carotenogenic cells by 20% and 10% respectively. There was no trend observed with nitrate gradient. The growth rate of the environmental isolates was a function of growth conditions. The highest maximum specific growth rate obtained for carotenogenic cells was 0.52 day^{-1} for cultures cultivated in BAAM media containing 3M NaCl and 5 mM KNO_3 and incubated at 50-80 $\mu mol.m^{-2}.s^{-1}$ light intensity. For non-carotenogenic cells the highest maximum specific growth rate realised was 1.40 day^{-1} obtained in cultures cultivated in BAAM media containing 3 M NaCl, 1.25 mM KNO_3 and incubated under 140 $\mu mol.m^{-2}.s^{-1}$ light intensity and 37°C.

Factorial experiments (3^3) were designed to determine the optimum conditions for β-carotene biosynthesis by environmental isolates. Nitrate concentration (mM) (X_1), sodium chloride concentration (M) (X_2) and light intensity ($\mu mol.m^{-2}.s^{-1}$) (X_3) were chosen as variables for the experiments. Results and analysis was reported for change in cell concentration, cellular carotenoid content and volumetric carotenoid content. The 3^3 factorial experiments gave the following regression model for cellular total carotenoid content ($pg.cell^{-1}$):

From the study, it is observed that carotenogenic *Dunaliella* isolates from Sua Pan salt evaporation ponds compare to other identified carotenogenic *Dunaliella* species that are used commercially for production of β-carotene. Further, the Makgadikgadi salt pan brines can be used for cultivation of *Dunaliella* because of the similarities in composition. *Dunaliella* cultivation and massive accumulation of carotenoids involves a compromise between the growth and carotenoid biosynthesis phases; highest carotenoid biosynthesis occurs at lowest rates of growth. High volumetric cellular content ($mg.L^{-1}$) can be achieved by high light intensity and sodium chloride which increases individual carotenoid biosynthesis but adversely affects growth or by lowering light intensity and sodium chloride levels to obtain cells with lower individual cell carotenoid biosynthesis but higher growth rates. Dual phase allows separate maximum cell concentration first than carotenoids; however, under conditions favouring growth, growth of non-carotenogenic *Dunaliella* competes with carotenogenic *Dunaliella*.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE:

Abbreviations

ABA	Abscisic Acid
ADP	Adenosine diphosphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BASF	Badische Anilin & Soda Fabrik
BCC	Business Communications Company
BSA	Bovine Serum Albumin
Ca	Concentration of chlorophyll a
Ca+b	Concentration of chlorophyll a and b
Cb	Concentration of chlorophyll b
Cbr	Carotene biosynthesis related
Ccs	capsanthin-capsorubin synthase
Crtl:	phytoene desaturase (bacterial type)
CrtL-b:	lycopene β-cyclase
CrtL-e:	lycopene ε-cyclase
CrtO:	*β*-C-4-oxygenase (ketolase)
CrtR-b:	*β*-ring hydroxylase
CrtR-e:	ε-ring hydroxylase
CS ponds	Cooling water sump
Cx+c	Concentration of xanthophylls and carotenoids
DMADP	dimethylallyl diphosphate
E ponds	Evaporating ponds
ED	Effective dose
EDTA	Ethylene tetra-acetic acid (EDTA)
Ggps:	geranylgeranyl diphosphate synthase
GLA	Gamma Linolenic Acid
IDP:	isopentenyl diphosphate
Ipi:	IDP isomerase
MJ(J/1)	Modified Johnson's Medium (J/1)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate with reducing power
NEDI	N-(1-naphthyl)-ethylenediamine dihydrochloride solution
NS ponds	NaCl (N-brine) storage ponds
Pds:	phytoene desaturase
Psy:	phytoene synthase
ST	Trauna storage ponds

UTEX	University of Texas
Vde:	violaxanthin deepoxidase
X ponds	Crystalliser ponds
Zds:	ζ-carotene desaturase
Zep (*aba2*):	zeaxanthin epoxidase

Symbols

μ	Specific growth rate
Abs	Absorbance at a known wavelength
dry $wt.m^{-2}.day^{-1}$	dry weight per meter squared per day
E%	Extinction coefficient for carotenoids
K	Kelvin
Kerg	Kilo erg (1 erg = 1 $kg.m^2.s^{-2}$= 10^{-7} J)
M	Molar
$mg.m^{-2}.day^{-1}$	milligram per meter squared per day
mM	Millimolar
MPa	Mega Pascals
$pg.cell^{-1}$	picogram per cell
$pg.mL^{-1}$	picogram per millilitre
Ppm	Parts per million
t_1	time 1
t_2	time 2
v/v	Volume per volume
X_0	Initial cell concentration
X_1	Cell concentration at time 1
X_2	Cell concentration at time2
X_{max}	maximum cell concentration

CHAPTER 1: INTRODUCTION

1.1 Background and objectives

Beta-carotene (β-carotene) belongs to a group of some 600 compounds called carotenoids. The carotenoids are divided into two subgroups. The hydrocarbon subgroup called carotenes form the main group. Their oxygenated forms are called xanthophylls (Davies, 1976; Sandmann, 2001a, 2001b). The principal characteristic of the carotenoids is their conjugated double bond system that determines their biological properties and colour (Sandmann, 2001a). Carotenoids are synthesised naturally by a range of algae, plants, fungi and bacteria. In these organisms, they function as components of light-capturing systems (Davies, 1976; Sandmann, 2001a, 2001b) or as protectants from photooxidative damage (Salguero *et al.*, 2003). It is the carotenoids that are responsible for imparting colour to fruits, vegetables, leaves and other coloured living species (Goodwin, 1988; Davies, 1976; Sandmann, 2001a, 2001b).

Animals, including man, are incapable of synthesising carotenoids and, therefore, rely on the primary producers for sources of carotenoids. Several properties are attributed to carotenoids in animals. Carotenoids with at least one unsubstituted β-ionone ring including β-carotene, α-carotene and β-cryptoxanthin have provitamin A activity. Of these, β-carotene has the highest activity (Rock, 1997). Further, carotenoids function as antioxidants because of their ability to inactivate the damaging oxidative molecules such as reactive oxygen singlets and to quench the carboxyl radicals (Rock, 1997; Sandmann, 2001a). Carotenoids can also function as colouring agents. For example, β-carotene is used as a colourant in chicken feed to enhance the yellow colour of the egg yolk and astaxanthin is used in aquaculture to enhance the pink colour in salmon and trout (Boussiba and Vonshak, 1991; Kobayashi *et al.*, 2001; Cifuentes *et al.*, 2003).

According to Business Communications Company (BCC, Inc) (Ulrich, 2000), the global market for the commercially available carotenoids was estimated at $US 887 million in 2004. The major commercial carotenoids reported were β-carotene valued at $US 242 million (a $US30 million rise from 1999), astaxanthin at $U S234 million, canthaxanthin at $US 140 million and lutein at $US 130 million. A 2.9% annual increase in the β-carotene demand is projected, resulting in a $US 1 billion market by 2009. The major application of β-carotene is in food and feed, neutraceuticals, pharmaceuticals and cosmetics industries where it is used as a colourant and a precursor to vitamin A (Ulrich, 2000).

The β-carotene demand is met by two major sources: the synthetic form accounts for 92% of the market while the natural β-carotene derived from living organisms, mainly *Dunaliella* algal species, constitutes the remaining fraction (Ulrich, 2000). The carotenoid producing *Dunaliella* species are known to be the highest natural producers of β-carotene synthesising about 12% by weight of the dry algal weight (Ben-Amotz *et al.*, 1983). The fungus, *Blakeslea*, has also been used in Europe for commercial production of natural β-carotene by conventional submerged culture (Ulrich, 2000; Dufossé *et al.*, 2005).

The natural and the synthetic β-carotene have different structures and hence the different physiological properties. Natural β-carotene contains both the all-*trans* and 9-*cis* isomers with the ratio of the isomers proportional to the amount of light reaching the cells during cell division (Ben-Amotz *et al.*, 1988). The synthetic form, however, is comprised only of the all-*trans* isomer (Ausich, 1997; Rock, 1997; Ben-Amotz, 1999; Fuller *et al.*, 2001; Garcia-Gonzalez *et al.*, 2005; Chidambara Murthy *et al.*, 2005). A comparison between *Dunaliella bardawil* derived β-carotene and synthetic all-trans β-carotene by Ben-Amotz and Levy (1996) demonstrated that 9-*cis* β-carotene isomer is a more efficient *in vivo* lipophilic antioxidant than the all-trans β-carotene isomer is. Further, the 9-*cis* β-carotene isomer is more soluble in hydrophobic solvents than the all-trans isomer. The 9-*cis* β-carotene does not crystallise while its synthetic counterpart crystallises easily. According to Ben-Amotz (1999), the bioavailability of the two isomers is improved when

supplied together as in natural β-carotene products. This shows that where biological activity is of importance such as in the health food and pharmaceuticals, natural β-carotene is of higher value (Ausich, 1997; Rock, 1997; Ben-Amotz, 1999; Fuller *et al.*, 2001; Garcia-Gonzalez *et al.*, 2005; Chidambara Murthy *et al.*, 2005). In addition to the presence of the two isomers, naturally produced β-carotene products often contain significant amounts of other carotenoids. This is beneficial in applications such as in health foods (Dufossé *et al.*, 2005).

Commercial cultivation of carotenogenic *Dunaliella* species for natural β-carotene production represents one of the successes of microalgal biotechnology. These species are cultivated in open ponds under high salt concentration conditions to reduce chances of contamination by other organisms (Borowitzka and Borowitzka, 1988; Borowitzka, 1999; Pulz, 2001). Currently the *Dunaliella* alga is cultivated industrially for production of natural β-carotene in Australia, Israel, China, UK, USA, India (Ulrich, 2000; Oren, 2005).

Although microalgal biotechnology gained recognition in the 1940s (Burlew, 1953; Benemann, 1989), there have been many barriers to its successful economic exploitation. These range from reliance on the ambient environmental conditions for algal productivity, natural limitations of the physiology of the species involved, the optimisation of algal mass cultivation systems, harvesting methods, specifically in the case of single cell microalgae, downstream processing of the algae or of its products as well as the high capital involved in the technology required to carry out these activities (Richmond, 2000). Ongoing microalgal research aims to improve the efficiency of the technology to enable production of natural carotenoids at competitive prices. New methods of cultivation (Borowitzka *et al.*, 1990; Ben-Amotz, 1995; Garcia-Gonzalez *et al.*, 2003; Hejazi and Wijffels, 2004) and improved photobioreactor design have focused on improved productivity (Chaumont, 1993; Singh *et al.*, 2000; Pulz, 2001; Hejazi and Wijffels, 2004; Barbosa *et al.*, 2003). Investigations into new strains and improvement of some existing strains (Shaish *et al.*, 1992; Markovits *et al.*, 1993; Hirschberg *et al.*, 1997; Misawa and Shimada, 1998; Jahnke, 1999) remain studies of interest.

This project focuses on the isolation and characterisation of β-carotene accumulating *Dunaliella* species from the Sua Pan solar saltworks evaporation ponds in the north-eastern part of Botswana. Overall, the project aims at evaluating the potential for exploitation of these *Dunaliella* isolates for industrial production of β-carotene. The thesis also explores the possibility of commercial cultivation of *Dunaliella* in Botswana owing to the potentially favourable climatic and environmental conditions, as well as the occurrence of natural saline water suitable for *Dunaliella* mass culture. The presence of β-carotene-producing *Dunaliella* species at the Sua Pan solar saltworks was reported by Masemola (1999). Her study focused on the effects of the *Dunaliella* species on the quality of the saltworks' products i.e. sodium carbonate (soda ash) and sodium chloride (common salt). Formal studies to assess feasibility for commercial application of these *Dunaliella* isolates for industrial production of β-carotene and the possibility of cultivating the algae in Botswana have not been reported.

The objectives of this study are:

1. To isolate and characterise high carotenoid-producing *Dunaliella* isolates of Sua Pan solar saltworks evaporation ponds
2. To establish conditions for optimum growth and carotenoid biosynthesis at indoor laboratory conditions
3. To study the extent and control of the contamination with non-carotenogenic *Dunaliella* species in dual phase mode of cultivation
4. To study the influence of non-carotenogenic *Dunaliella viridis* on process in open system

1.2 Scope of the thesis

The principal objective of the thesis is to evaluate the productivity of β-carotene accumulating *Dunaliella* species isolated from Sua Pan solar saltworks ponding systems focusing on their growth rates and carotenoid biosynthesis properties. This is done through the comparison to type culture strains. The thesis also studies the establishment of conditions for optimum production of carotenoids, specifically β-carotene, at indoor laboratory scale and the establishment of conditions minimising growth of the co-cultured non-carotenogenic species present in the ponding system.

1.3 Structure of the thesis

The literature review presented in Chapter 2 introduces algal biotechnology and further highlights the utilisation of the genus *Dunaliella* in the natural production of β-carotene. The factors influencing *Dunaliella* growth and carotenoid biosynthesis are discussed. The literature review concludes by discussing various industrial cultivation systems for *Dunaliella*. Methods for conducting experiments follow in Chapter 3. To provide a basis for comparison, the establishment of the culture system is conducted using *Dunaliella bardawil* UTEX 2538 and *Dunaliella salina* UTEX 1644 from University of Texas algal culture collection is reported in Chapter 4. *Dunaliella* isolates from Sua Pan solar saltworks are discussed in Chapters 5 and 6. This is followed by enrichment for and characterisation of the natural Sua Pan *Dunaliella* isolates, their growth, and the control of competing non-carotenogenic species in co-culture and finally, carotenoid production studies of the *Dunaliella* species are presented. The implications of the presence of non-carotenogenic *Dunaliella* species in the outdoor open cultivation system using dual mode system are further discussed. In Chapter 7, the conclusions from the study are drawn and recommendations for further work presented. Detailed methods of analysis are provided in the appendices.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

On recognising the commercial potential of algal biotechnology around 1940s (Burlew, 1953; Benemann, 1989), several potential applications were targeted. These included the use of different algal species in the food and feed industry as protein sources, vitamin supplements and colourants, as well as the use of the algae as agents of wastewater treatment (Benemann, 1989; Becker, 1994). Further to these applications, algal biotechnology has evolved to produce high value products with potential use as pharmaceuticals and health foods (Benemann, 1989; Borowitzka *et al.*, 1990; Becker, 1994). Dufossé *et al.* (2005) highlighted the increasing demand for natural colours for use in food, pharmaceuticals, cosmetics, textiles and as printing dyes.

The first algal species to undergo commercialisation were *Chlorella* and *Spirulina* which had application in the health food industry as food supplements (Becker, 1994). *Spirulina* is exploited for its high protein content, carotenoid content, presence of the essential fatty acid gamma linolenic acid (GLA) required for growth stimulation, polyunsaturated fatty acids and some other micronutrients (Becker, 1994; Andrade and Costa, 2007). *Spirulina* also produces the pigment phycocyanin used as a natural colorant in the food and cosmetics industries (Benemann, 1989; Dufossé *et al.*, 2005).

After the establishment of *Chlorella* and *Spirulina* biotechnology, cultivation of the halotolerant microalga, *Dunaliella*, followed (Ben-Amotz *et al.*, 1982; Borowitzka and Borowitzka, 1989; Becker, 1994). The carotenogenic *Dunaliella* changes from green to orange or red on accumulation of β-carotene and other carotenoids when subjected to adverse growth conditions such as high salinity, high light intensity, nutrient deprivation and extreme temperatures (Borowitzka and Borowitzka, 1989; Ben-Amotz and Shaish, 1992; Zamir, 1992; Ben-Amotz, 1999).

β-carotene has formed an important colourant in the food and feed industry. Dufossé *et al.* (2005) identified β-carotene as one of the world's leading colourants. It is applied to a range of food and beverage products to improve their appearance. β-carotene is used in the manufacturing of foods such as margarine, cheese, fruit juice, baked goods, dairy products, canned goods, confectionary and health foods (Dufossé *et al.*, 2005). Further, β-carotene is converted to vitamin A by cleavage of the C-15, 15'double bond by β-carotene 15,15' dioxygenase in body tissues (Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989a; Rock, 1997; Ben-Amotz, 1999). Vitamin A aids in alleviating various human ailments including eye diseases, night blindness, skin conditions and various forms of cancer (Rock, 1997; Handelman, 2001). β-carotene also functions as a potent antioxidant (Rock, 1997; Salguero *et al.*, 2003; Garcia-Gonzalez *et al.*, 2005; Chidambara Murthy *et al.*, 2005) in the pharmaceutical industry. It protects the human body by scavenging oxidants, such as singlet oxygen, carboxyl and the peroxyl radicals implicated in carcinogenesis and therefore protects the human body against tumour formations (Jimenez, 1993; Kai-Xian *et al.*, 1998). In an *in vitro* assay, Miki (1991) investigated the effects of carotenoids as free radical scavengers using thiobarbituric acid (TBA) method with ferrous ion of haemeprotein as a free radical producer with heat and linoleic acid as an acceptor. They established an effective dose 50 (ED_{50}) of 960 nM for β-carotene, the amount required to produce an effect on 50% of the population.

Formulations of the natural β-carotene products include β-carotene extracts, sold as vegetable oil, capsules of *Dunaliella* powder for human use and dried *Dunaliella* for feed use (Oren, 2005). In purified natural β-carotene preparations, β-carotene accounts for 85 to 90% of the total carotenoids. The remaining 10 to 15% comprises of chlorophyll a and b, lycopene, α-carotene, lutein, zeaxanthin and cryptoxanthin (Ben-Amotz and Avron, 1983; Kai-Xian *et al.*, 1998; Ben-Amotz, 1999).

The microalga, *Haematococcus,* is the most recent species to attract attention in microalgal biotechnology. *Haematococcus* is important in the natural production of the carotenoid pigment astaxanthin. Astaxanthin functions as an antioxidant and a colourant,

providing the source of pigmentation for fish in aquaculture and for eggs in the poultry industry (Boussiba and Vonshak, 1991; Kobayashi *et al.*, 2001; Cifuentes *et al.*, 2003). Because of its powerful antioxidant activity, demonstrated by an ED_{50} of 200 nM, the pigment also carries potential for clinical application (Kobayashi *et al.*, 2001).

The genus *Dunaliella* is wide spread, inhabiting environments of varying conditions. The habitats of the β-carotene rich *Dunaliella* species, however, are characterised by salt concentrations reaching as high as 5.5 M (35 % w/v) (Avron, 1992), high light intensity, low nutrient availability and extreme temperatures. The presence of β-carotene rich *Dunaliella* species in nature have been recorded in areas including Dead Sea in Israel, Great Salt Lake in Utah, USA (Richmond, 1986), Pink Lake in Australia (Avron, 1992) and the Sua Pan solar salt evaporation ponds in Botswana (Masemola, 1999).

Whilst widely distributed in nature, the main commercial cultivation of *Dunaliella* species for β-carotene production is currently restricted to limited regions worldwide including USA, Israel, Australia, UK, Japan, India, Taiwan and People's Republic of China (Benemann, 1989; Borowitzka and Borowitzka, 1989; Ben-Amotz, 1999; Dufossé *et al.*, 2005). There are small plants in Chile, Mexico, Cuba, Iran, Taiwan and Japan (Dufossé *et al.*, 2005).

2.2 Overview of carotenoids

Carotenoids are 40-carbon (C_{40}) compounds belonging to the tetraterpenoids (Goodwin, 1981). Structurally, the tetraterpenoids are made up of eight 5-carbon (C_5) branched units formed into two 20-carbon (C_{20}) units which are then joined head to head to form the C_{40} compound (Goodwin, 1980; Bramley, 1997). Carotenes are hydrocarbons that are either linear or cyclised at one or both ends of the molecule while their oxygenated derivatives are called xanthophylls (Hirschberg, 1999). The prominent characteristic of carotenoids is their polyene chain, consisting of conjugated double bonds, which determines biological action and colour (Hirschberg, 1999; Sandmann, 2001b).

2.2.1 Description of β-carotene

The carotene, β-carotene has the formula $C_{40}H_{56}$. Its structure is shown in Figure 2.1. β-carotene is the orange pigment forming an important part of the light harvesting photosynthetic system in plants and algae.

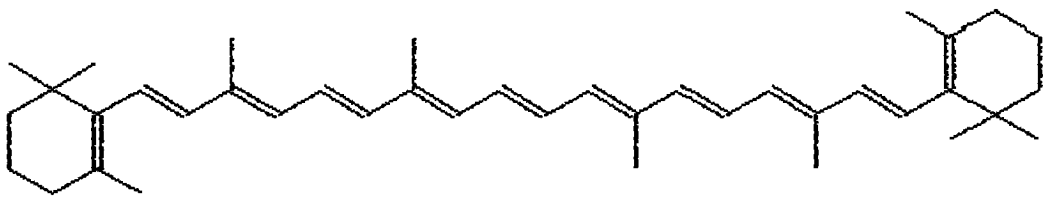


Figure 2.1: Structure of β-carotene

2.2.2 Structural functions of β-carotene in naturally producing organisms

The conjugated double bond system of the carotenoids facilitates light harvesting apparatus. This is critical for photosynthesis. β-carotene also provides a photoprotectant function, protecting the cells and tissues of synthesising organisms from photochemical reaction products (Goodwin, 1980; Rock, 1997; Sandmann, 2001a). This is achieved by quenching high energy oxygen molecules that may result from photosynthesis such as oxygen singlets, oxygen triplets and oxygen radicals (Miki, 1991; Jimenez, 1993; Salguero *et al.*, 2003).

Mechanisms for neutralising free radicals may follow two routes (Miki, 2010). One way is demonstrated in equation 2.1 where the free radical scavenging antioxidants (IH) stabilises active free radicals (X•) by donating a hydrogen atom. The second route is by proton transfer (equation 2.2) for production of a stable compound (XH) and antioxidant-derived radical (I•). Carotenoids scavenge radicals by addition to the double bond (Miki, 2010).

$$X\bullet + IH \longrightarrow XH + I$$ **Equation 2.1**

$$X\bullet + IH \longrightarrow X^{-} + IX\bullet^{+} \longrightarrow XH + I\bullet \qquad \textbf{Equation 2.2}$$

2.2.3 Biochemical pathways for biosynthesis of β-carotene in algae

Carotenoids are synthesised via the central isoprenoid pathway in the plastids (Hirschberg, 1999). Plastids are membrane-bound organelles in plant and algal cells which serve as centres of specialised metabolic activities. The 5-carbon compound, isopentenyl diphosphate (IDP), is synthesised from pyruvate and glyceraldehyde-3-phosphate. This is used as the starting material for the isoprenoids, as shown in Figure 2.2. The definitions and abbreviations of the enzymes are presented in the nomenclature. The IDP isomerase enzyme converts IDP to dimethylallyl diphosphate. Following this, three sequential additions of IDP molecules by the enzyme geranylgeranyl diphosphate synthase (*Ggps*) lead to the formation of a 20 carbon molecule geranylgeranyl diphosphate (GGDP) (Hirschberg, 1999).

The next step in the synthesis of carotenoids is marked by the head to head condensation of the two GGDP molecules forming phytoene. This step is catalyzed by phytoene synthase. Phytoene is then converted to ζ-carotene by phytoene desaturase which, in turn, is converted to lycopene by ζ-carotene desaturase in plants. In bacteria, the step from phytoene to lycopene is catalysed by the bacterial type of phytoene desaturase, *crtI*.

The branching of the pathway at the cyclisation of lycopene molecule leads to two routes. One route leads to the synthesis of β-carotene and its oxygenated derivates. β-cyclase catalyses the conversion of lycopene to β-carotene via γ-carotene. The other route leads δ-carotene and other compounds containing one β-ring and one ε-ring including lutein and α-carotene.

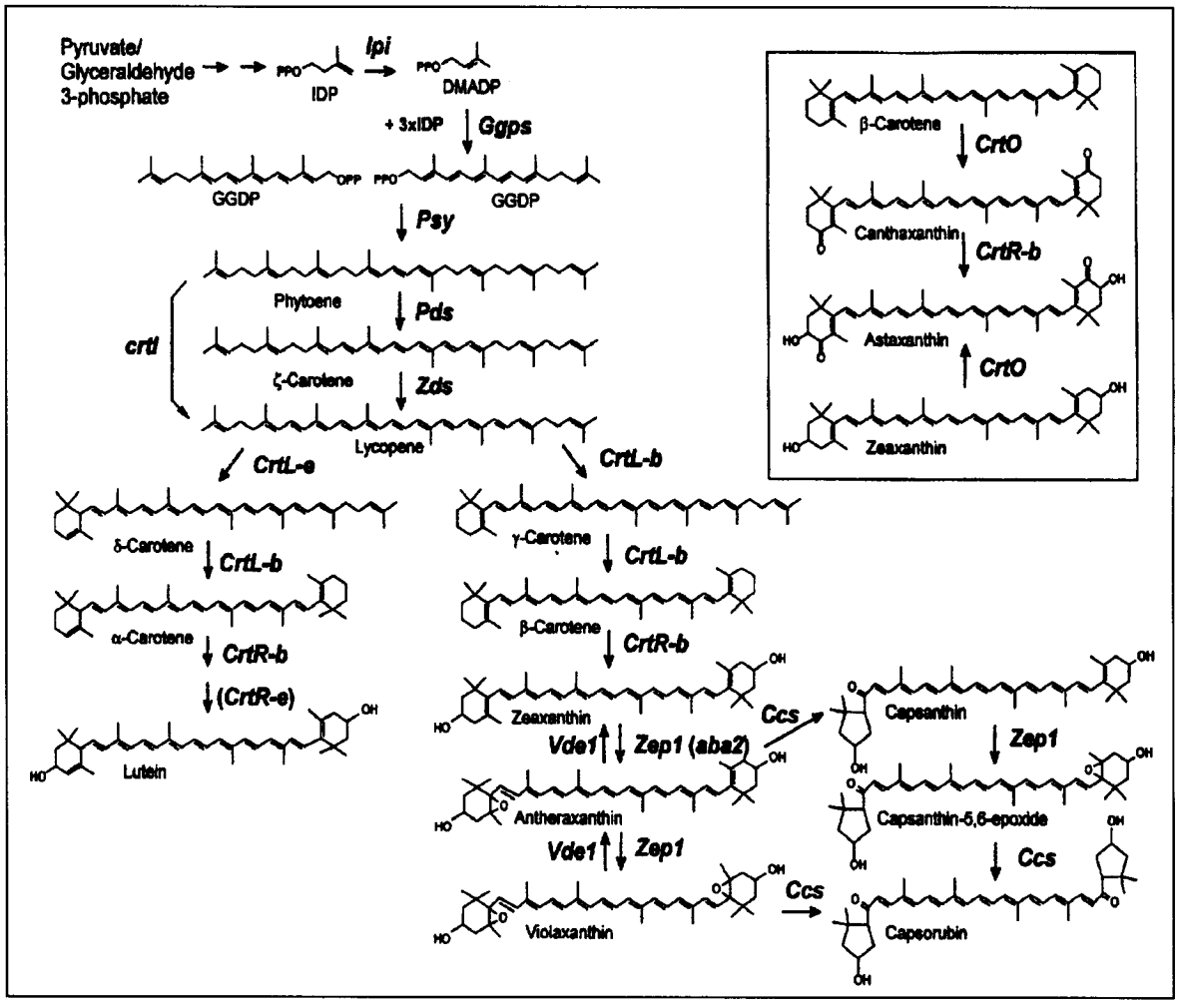


Figure 2.2: The carotenoid biosynthesis pathway depicting the carotenoid structures and their respective enzymes in plants (Adapted from Hirschberg, 1999).

Beyond β-carotene, a reversible xanthophyll cycle involving zeaxanthin, antheraxanthin and violaxanthin exists (Figure 2.2). In light stress, violaxanthin, with limited energy quenching abilities, is converted to zeaxanthin via antheraxanthin. In such conditions, zeaxanthin functions as a photoprotective pigment. It is postulated that this reduces the amount of energy reaching the photosynthetic reaction centres thus protecting the organisms against photoinhibition. Further, the carotenoids of the xanthophyll cycle are precursors of the plant hormone, abscisic acid (ABA). ABA is hypothesised to be involved in signal induction of massive β-carotene accumulation due to salt stress (Cowan and Rose, 1991), discussed in Section 2.4.4.

2.2.4 Chemical synthesis of β-carotene

The methods for production of synthetic β-carotene vary. The two major processes are the Badische Anilin and Soda-Fabrik (BASF) and F. Hoffman-La Roche Co. Ltd (Roche) (University of Bristol, n.d.). The BASF process is based on the Wittig reaction where two C_{20} compounds, β-retinyltriphenylphosphonium chloride and retinal react to form β-carotene. The Roche process involves the reaction between two C_{19} compounds and a C_2 compound. The C_{19} compounds are β-C_{19}-aldehyde while the C_2 compound is acetylenedimagnesium bromide. These two chemical synthesis processes yield β-carotene in an *all-trans* isomer.

2.3 The microalga: *Dunaliella*

Dunaliella is a halotolerant, biflagellate, unicellular motile green alga shown in Figure 2.3a. Originally, the genus *Dunaliella* was placed under class Chlorophyceae and the order Volvocales (Ben-Amotz and Avron, 1989b). However, in their review, Borowitzka and Siva (2007) placed the *Dunaliella* genus under class Chlorophyceae, order Dunaliellales and family Dunaliellaceae.

2.3.1 Morphology and Structure of *Dunaliella*

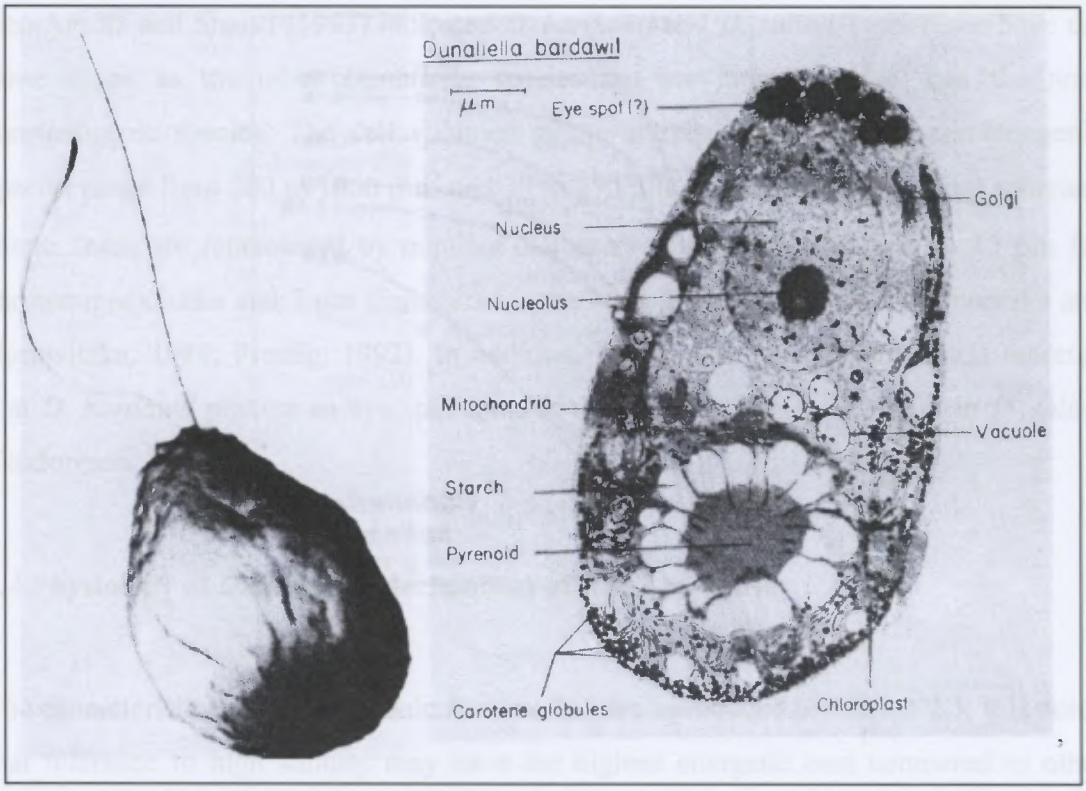
The members of this genus are typically ovoid although the cell morphology and size vary through spherical, cylindrical, egg-shaped, pear-shaped or ellipsoidal with changing environmental conditions (Ben-Amotz and Avron, 1989b; Avron, 1992). Unlike other algal genera, *Dunaliella* lacks a rigid cell wall. The cell envelope is a thin elastic plasma membrane which allows for adjustment in cell shape and size due to glycerol synthesis or breakdown in response to the osmotic stress imposed by prevailing salt concentrations (Avron, 1992). One large, cup-shaped chloroplast is located at the cell posterior, shown

in Figure 2.3b. In species capable of massive accumulation of carotenoids, the carotenoids are located in oily globules in the inter-thylakoid space of the chloroplast illustrated also in Figure 2.3b (Ben-Amotz *et al.*, 1982; Ben-Amotz and Avron, 1989b).

2.3.2 Taxonomy of *Dunaliella* genus

According to Massyuk (1973), as cited by Preisig (1992), 28 species of *Dunaliella* genus exist. The genus can be subdivided into 2 subgenera: *Pascheria* and *Dunaliella*. The *Pascheria* subgenus comprised of 5 freshwater species while the remaining 23 species constituted the marine and halophilic species contained in the *Dunaliella* subgenera. The distinction between species was based on the physiology, morphology, size, shape and ability to accumulate carotenoids Preisig (1992).

Two species are known to be halotolerant: *Dunaliella salina* (*D. salina*) and *Dunaliella viridis* (*D. viridis*), the former capable of accumulating massive amounts of carotenoids while the latter remains green at all salt concentrations (Oren, 2005). However, owing to the misnaming in the genus, there exist strains of *D. salina* that are incapable of massive carotenoids accumulation.



(a) (b)

Figure 2.3: Monogram showing a structure of *Dunaliella* (Ben-Amotz and Avron, 1989a).

(a) *Dunaliella* morphology **(b) *Dunaliella* cell structure showing carotenoid globules location**

Regarding carotenogenesis, *Dunaliella bardawil* (*D. bardawil*) is reported as another *Dunaliella* species capable of massive carotenoids accumulation (Ben-Amotz *et al.*, 1982; Borowitzka and Borowitzka, 1988; Lers *et al.*, 1990; Preisig, 1992; Salguero *et al.*, 2003; Oren, 2005). There is a debate as to whether *D. salina* and *D. bardawil* are indeed different species. Preisig (1992) and Borowitzka and Borowitzka (1988) indicated *D. bardawil* is a *nomen nudum* for *D. salina* Teodoresco, the name used by Ben-Amotz and Shaish (1992) for carotenogenic *D. salina* strain to differentiate from *D. bardawil*. All the strains capable of accumulating massive amounts of carotenoids are characterised as *D. salina* (Preisig, 1992; Borowitzka and Borowitzka, 1988).

Ben-Amotz and Shaish (1992) indicated *D. bardawil* and *D. salina* Teodoresco have the same shape as the other *Dunaliella* species but are larger in size than the non-carotenogenic species. The cell volumes of the carotenogenic and non-carotenogenic species range from 300 to 1000 μm^3 and 30 to 150 μm^3 respectively. Assuming spherical shape, these are represented by nominal diameters of 8 to 25 μm and 5 to 15 μm for carotenogenic cells and 7 μm diameters for the non-carotenogenic cells (Borowitzka and Borowitzka, 1989; Preisig, 1992). In addition, Ben-Amotz and Shaish (1992) reported that *D. bardawil* possess an eye spot towards the flagellate which is absent in *D. salina* Teodoresco.

2.4 Physiology of *Dunaliella*: Mechanisms of stress responses

The characteristics of carotenogenic *Dunaliella* were introduced in Section 2.1. It is noted that tolerance to high salinity may have the highest energetic cost compared to other stress factors. High energy is required for maintenance of proper osmotic conditions and specific ion concentrations. This is plausible in view of association of high salinity with generally reduced maximum growth rates compared to low salinity levels (Henley *et al.*, 2002) as demonstrated in Figure 2.4. Under these conditions, *D. salina* grew optimally at 2 M NaCl, followed by 1 M NaCl. Increase of salinity from 2 M to 5 M lead to reduced growth. The organism does not thrive well at low salinities (0.1 M NaCl). The effect of other factors on halotolerance remains an area of interest. However, Henley *et al.* (2002) established that salinity increases also increase tolerance to high irradiance and temperature in *D. parva*.

The survival of *Dunaliella* species under conditions of stress in extreme environments can be accounted for by several mechanisms: elimination of sodium (Na^+) ions, massive accumulation of glycerol, overproduction of β-carotene (Ben-Amotz *et al.*, 1982; Shaish *et al.*, 1991; Borowitzka, 1988) and expression of various stress related proteins (Lers *et al.*, 1990; Levy *et al.*, 1993; Pick *et al.*, 2002; Henley *et al.*, 2002; Zamir, 2002).

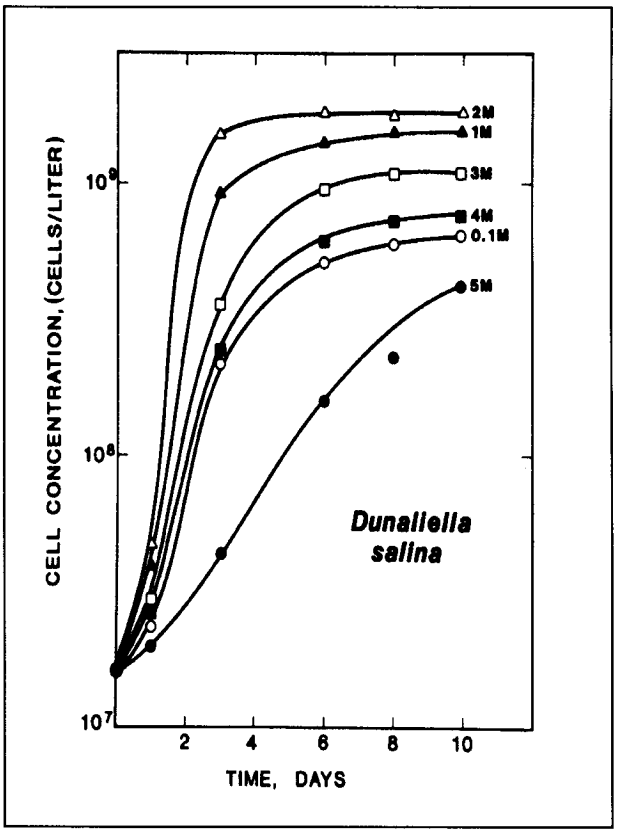


Figure 2.4: Effects of salinity on the growth rate of *Dunaliella salina* (Ben-Amotz and Avron, 1989b).

The ability of *Dunaliella* to tolerate the high salt concentrations lies with its unique physiology. The organism is capable of eliminating the detrimental sodium (Na^+) ions through several mechanisms. Katz *et al.* (1986) demonstrated that when *Dunaliella* was grown in media containing 0.5 M or 4 M Na^+ ions, the intracellular Na^+ ion levels did not exceed 100 mM. Following this discovery, several distinct Na^+ transporters were identified by Pick and co-workers (2002). These are shown in Figure 2.5.

The Na^+-redox pump driven by a plasma membrane electron transport system and the Na^+-ATPase are involved in the elimination of the sodium ions (Na^+ ions). The sodium ions enter the cell via two routes: the Na^+/H^+ antiporter and Na^+/anion symporter (Katz *et al.*, 1986; Katz and Pick, 2001; Weiss *et al.*, 2001). The Na^+/H^+ antiporter is used in pH balancing by inward movement of Na^+ ions and elimination of H^+ ions. The Na^+/anion

symporter is responsible for the uptake of sulphate (Na^+/SO_4^-) and phosphate (Na^+/P_i) across the cell membrane (Weiss *et al.*, 2001).

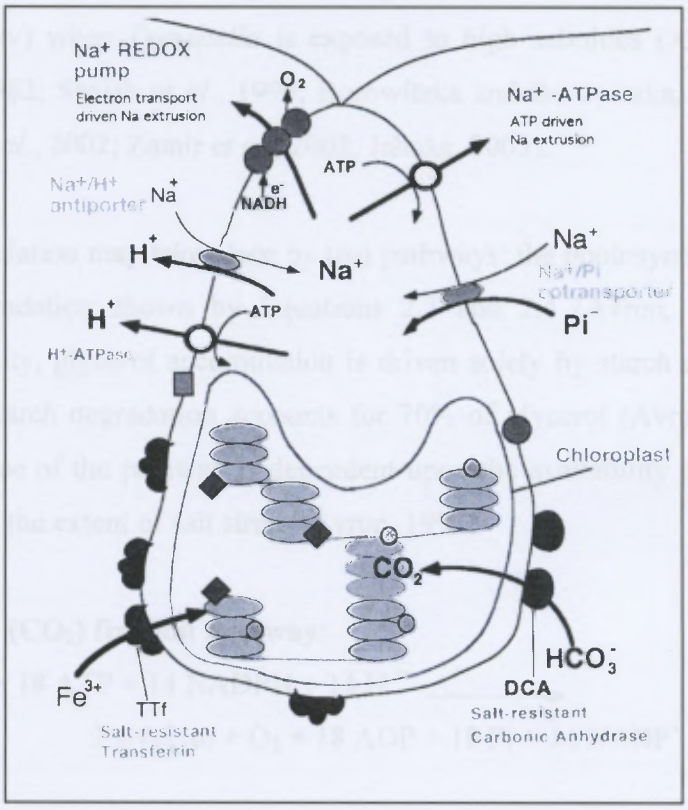


Figure 2.5: Molecular basis of salinity tolerance in *Dunaliella* (Pick *et al.*, 2002).

2.4.1 Massive glycerol accumulation

With lack of cell wall, *Dunaliella* cells respond rapidly to change in environmental osmotic pressure by water movement across the plasma membrane coupled with synthesis or elimination of glycerol. This causes changes in cell shape and size (Ben-Amotz and Avron, 1983; Avron, 1992).

Through a process for accumulation of compatible solutes, *Dunaliella* cells accumulate excess amounts of glycerol as a mechanism for balancing high environmental salt

concentrations. In this manner, glycerol is compatible as it does not interfere with the physiology of the cells especially the stability of the enzymes (Borowitzka and Borowitzka, 1988; Oren, 1999; Oren 2005). The intracellular glycerol concentration is directly proportional to the osmotic pressure in the environment and its concentration can exceed 50% (w/v) when *Dunaliella* is exposed to high salinities (>3 M NaCl) (Ben-Amotz *et al.*, 1982; Shaish *et al.*, 1990; Borowitzka and Borowitzka, 1988; Pick *et al.*, 2002; Henley *et al.*, 2002; Zamir *et al.*, 2002; Jahnke, 2003).

Glycerol accumulation may take place by two pathways: the photosynthetic CO_2 fixation and starch degradation shown by Equations 2.3 and 2.4 (Avron, 1992). Following increase in salinity, glycerol accumulation is driven solely by starch degradation in the dark. In light, starch degradation accounts for 70% of glycerol (Avron, 1992; Phillips, 1993). The choice of the pathway is dependent upon the availability of light, the starch reserve pool and the extent of salt stress (Avron, 1992).

Carbon dioxide (CO_2) fixation pathway:

$$6\ CO_2 + 8\ H_2O + 18\ ATP + 14\ NADPH + 14\ H^+ \longrightarrow 2\ glycerol + O_2 + 18\ ADP + 18\ Pi + 14\ NADP^+$$ **Equation 2.3.**

Starch degradation pathway:

$$[Glucose] + 2\ H_2O + ATP + 2\ NADH + H^+ \longrightarrow 2\ glycerol + O_2 + ADP + Pi + NAD^+$$ **Equation 2.4.**

Massive accumulation of glycerol at high salt concentrations results in production of glycerol as a by-product in industrial production of β-carotene from *Dunaliella* species. *Dunaliella salina* at 1.5 M NaCl produced approximately 2 M intracellular glycerol (Avron and Ben-Amotz, 1980). Further, cultivation in 3 M NaCl, 4 mM nitrate and sunlight illumination produced approximately 4 M glycerol (25% dry algal weight glycerol) (Avron and Ben-Amotz, 1980). At this level the carotenes were at least 3% dry algal weight.

2.4.2 Massive β-carotene accumulation

The ability of some *Dunaliella* species to synthesise massive amounts of β-carotene and the conditions inducing its formation are well established (Ben-Amotz *et al.*, 1982; Borowitzka and Borowitzka, 1988; Lers *et al.*, 1990; Preisig, 1992; Salguero *et al.*, 2003; Oren, 2005). Under normal growth conditions, *Dunaliella* can synthesise about 0.4% of its dry algal weight as β-carotene and thus appears green in colour. Under these conditions, the β-carotene functions primarily as an accessory for light harvesting. Under high light intensity (1 700 to 2 000 μmol photons.m^{-2}.s^{-1}) (Pulz, 2001), high NaCl concentration (>3 M NaCl), temperature extremes and nutrient deprivation, some *Dunaliella* are reported to synthesise β-carotene at about 10% of its algal dry weight (Ben-Amotz *et al.*, 1982; Borowitzka and Borowitzka, 1988; Lers *et al.*, 1990; Preisig, 1992; Salguero *et al.*, 2003; Oren, 2005). The cells appear orange-red due to carotenoid accumulation of which approximately 90% is β-carotene which is believed to function as a photoprotectant (Ben-Amotz and Avron, 1983; Borowitzka, 1988; Shaish *et al.*, 1991; Kai-Xian *et al.*, 1998). Jimenez and Pick (1993) hypothesised that the β-carotene protects the alga by prevention of formation of reactive oxygen species, by quenching the triplet state chlorophylls or by neutralising the already formed singlet oxygen (O^{-}).

2.4.3 Regulation of carotenoid biosynthesis

The signal for induction of carotenoid biosynthesis remains to be elucidated. The plant hormone, abscisic acid (ABA) is associated with induction of carotenoid biosynthesis resulting from salinity stress in *Dunaliella salina* (Cowan and Rose, 1991). Present in all photosynthetic organisms, ABA plays an important role in the plant life cycle, including responses to various environmental stresses such as high light intensity, drought, temperature and salinity (Cutler and Krochko, 1999). According to Cowan and Rose (1991), accumulation of β-carotene occurs in two stages, the first concomitant with increase in ABA biosynthesis and the second as a result of the newly synthesised ABA.

This proposed mechanism is supported by the fact that ABA is derived from C_{40} carotenoid, the xanthophyll violaxanthin, via xanthoxin (Figure 2.6).

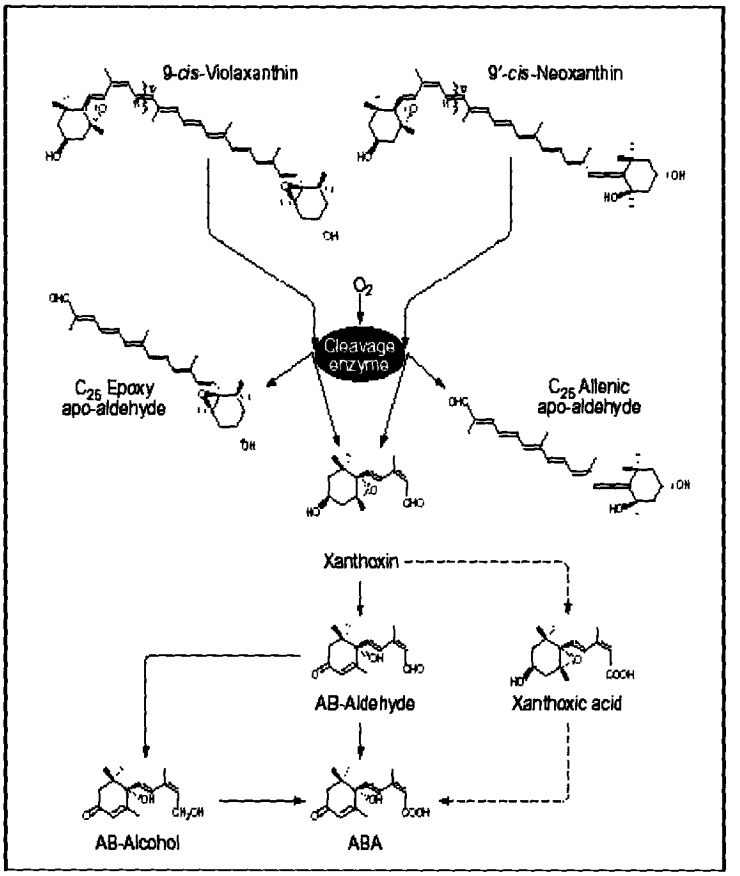


Figure 2.6: Formation of Abscisic acid (ABA) (Cutler and Krochko, 1999).

The search for carotenoid biosynthesis signals driven by other inducing stresses identified different pathways from that of ABA biosynthesis. A Cbr (carotene biosynthesis related) protein was found to accompany increase in carotenoid content due to light stress and sulphate deprivation (Lers *et al.*, 1991). Following the evaluation of Cbr as a participant in carotenoid biosynthesis signalling, Levy *et al.* (1993) proposed that Cbr binds zeaxanthin to form photoprotective complexes in the light harvesting antennae and is therefore not a carotenoid biosynthesis signal.

2.4.4 Stress induced proteins

Two proteins induced by high salt concentration have been identified in *Dunaliella.* One protein is the 60 kD plasma membrane protein which was identified as the salt resistant carbonic anhydrase (Zamir *et al.*, 2002; Pick *et al.*, 2002). The carbonic anhydrase is increased at high salinities for CO_2 sequestering (Avron, 1992; Pick *et al.*, 2002) as CO_2 solubility in water is greatly reduced at high salinities (Pick *et al.*, 2002). The second is a 150 kD transferin-like plasma membrane protein that aids in the uptake of Fe^{3+} at high salt concentration (Zamir *et al.*, 2002; Pick *et al.*, 2002). From this, it was established that CO_2 and Fe^{3+} availability are major rate limiting steps at high salinities (Pick *et al.*, 2002).

Liska and co-workers (2004) studied changes in protein profile in *Dunaliella* following salt stress. Seventy-six (76) different salt induced proteins were identified using molecular techniques. These included proteins of the Calvin cycle, enzymes participating in starch mobilisation and redox energy production, regulatory factors in protein synthesis and degradation as well as homologs of the Na^+-redox transporters. The use of molecular techniques showed *Dunaliella* responds to increase in high salinity by enhancement of photosynthetic CO_2 assimilation as well as redirection of carbon and energy resources to synthesize glycerol. The functions of the stress-related proteins are yet to be elucidated.

2.5 Production of *Dunaliella* biomass

2.5.1 Reproduction of *Dunaliella*

The life cycle of *Dunaliella* is complex involving both asexual and sexual reproduction processes. Lengthwise division of a motile vegetative cell occurs in asexual reproduction (Richmond, 1986). Sexual reproduction, however, involves fusion of two equally sized gametes to form the zygote. A thick outer layer protects the zygote during exposure to

harsh conditions. On germination, the zygote releases 32 cells (Preisig, 1992). Non-motile asexual cysts with thick walls are also formed at extreme conditions (Preisig, 1992). Biomass production in *Dunaliella* cultivation involves asexual reproduction of the motile vegetative cells (Borowitzka, 1990; Oren, 2005). Sexual reproduction of *Dunaliella* is uncommon in forced cultivation. It is more typical in nature where the algae need to survive cyclic harsh conditions (Borowitzka, 1990).

2.5.2 Factors influencing growth of *Dunaliella*

The growth of *Dunaliella* is influenced by the interaction of a range of parameters. These include availability of the photosynthetically active radiation, availability of nutrients, salinity, temperature and pH (Borowitzka and Borowitzka, 1988; Borowitzka and Borowitzka, 1989). In outdoor open systems used in commercial cultivation of carotenogenic *Dunaliella*, the presence of other halotolerant organisms, especially non-carotenogenic *Dunaliella* species, may present competition and result in mixed cultures containing both carotenogenic and non-carotenogenic *Dunaliella* species.

2.5.2.1 Light intensity

Light intensity is viewed as the principal limiting factor in photobiotechnology (Richmond, 1986) where photosynthetic rates are directly dependent on photon flux availability (Benemann, 1989). Outdoor light intensities may reach ranges of 1700 to 2000 μmol photons.m^{-2}.s^{-1} (Pulz, 2001) depending on the geographical zones and climatic conditions. Photosynthesis reaches saturation at about 30% of this light intensity, after which the photosynthetic apparatus may get damaged through photooxidation from unquenched singlet oxygen free-radicals resulting in photoinhibition (Pulz, 2001). When reviewing the *Dunaliella* taxonomy, Borowitzka and Siva (2007) indicated that carotenogenic *Dunaliella* should be cultivated at high light intensities (> 500 μmol photons.m^{-2}.s^{-1}) but when low light intensities are employed (< 200 μmol photons.m^{-2}.s^{-1}), cultivation should be longer (weeks). The latter is often the

case with most indoor laboratory experiments. Table 2.1 includes summaries of the light intensity levels utilised by various authors.

Typically, high light intensity combined with reduced growth rates brought on by adverse conditions favour β-carotene accumulation in carotenogenic *Dunaliella* species. Outdoor biomass production requires the moderation of light intensity reaching the cultures, otherwise extremely high light intensities can cause photo-bleaching of susceptible cells leading to death (Ben-Amotz and Avron, 1983). In such cases attempts are made to reduce the amount of light by shading the cultivation reactors, or by increasing the culture density in order to promote self-shading. The amount of light reaching the cells is controlled in indoor laboratory scale cultivation or bioreactor cultivation.

On the other hand, excessive shading in algal culture may occur in dense cultures, deep cultures or under limited culture mixing, where light availability may become growth limiting resulting in significantly reduced algal productivities. It is thus necessary to ensure that the algal culture receives optimal irradiance. Both the depth of the culture and mixing of the culture are important as these two parameters influence the tendency towards self shading in the culture and consequently the microalgal growth rate (Ogbonna and Tanaka, 2000).

Table 2.1: Summary of cell density and growth rate achieved for *Dunaliella* from literature[2]

Species (Author)	LI	NaCl	Nitrate (mM)	Sulphate (mM)	T (°C)	Growth rate (day^{-1})	Cell concentration ($cells.mL^{-1}$)
1. a. ***D. bardawil*** b. ***D. salina*** (noncarotenogenic) (Ben-Amotz and Avron, 1983)	a. 10 kergs[1] b. 5 to 550 kergs	3.5 M (21%w/v)	Nitrate: 0 to 10	Sulphate: 0 to 10	25	0.3 (calculated) 0.60 (calculated)	$X_o = 4 \times 10^4$ $X_{max}=8 \times 10^5$ $X_o = 4 \times 10^4$ $X_{max} = 5 \times 10^6$
2. ***D. salina*** (Thailand) (Powtongsook *et al.*, 1995)	270 $\mu mol.m^{-2}.s^{-1}$	10% w/v (1.7 M) 20% w/v (3.3 M) 30%w/v (5 M)	10	10	30 to 37	10% NaCl: 0.13 to 0.36 20% NaCl: 0.11 to 0.26 30% NaCl: 0.08 to 0.14	-
3. ***D. salina*** **Teodoresco** (Chile) (Cifuentes *et al.*, 1992)	150 $\mu mol.m^{-2}.s^{-1}$	12.5 %w/v (2.1 M) and 25% w/v (4.2 M)	10	10	25	0.14 to 0.32	overall: 6.7 to 17.4 x 10^5
4. ***D.viridis*** **Teodoresco** (Jimenez and Niell, 1990)	150 $\mu mol.m^{-2}.s^{-1}$	1M (6% w/v) 2 M (12% w/v) 3 M (18%w/v) 4 M (24%w/v)	0.5 1.5 5.0 10	5	20 25 30	Nitrate stress: 0.77 to 1.27 NaCl stress 0.46 to 1.40	Nitrate stress: 3.5 to 13.60 x 10^6 NaCl stress 6.3 to 18.5 x 10^6
5. ***D. salina*** (Iran) (Tafreshi and Shariati, 2006)	Outdoor, 33°58 N, 51° 29 E	1st Stage: 2 M (12%) 2nd Stage: 2.5 M (15%)	1st Stage: 5 2nd Stage: 0.1	1st Stage: 2 2nd Stage: 2	Outdoor	1st Stage: 0.19 to 0.48 2nd Stage: 0.16 to 0.18	1st Stage: 3.20 to 3.50 x 10^6 2nd Stage: 2.70 to 3.00 x 10^6

[1] 1 erg = 1 $kg.m^2.s^{-2}$= 10^{-7} J

[2] Growth phase and carotenoid phase are averaged

While a depth of 5 cm is considered ideal, typically 15 to 30 cm depth is used in outdoor pond mass culture, owing to the requirements of other engineering factors, such as agitation required for keeping the algal culture well mixed and preventing settling.

2.5.2.2 Nutrients concentration and availability

Dunaliella requires supply of an appropriate carbon source and other macro-elements including nitrogen, phosphorus, magnesium, sulphur and calcium. Trace elements required include zinc, iron, cobalt, copper, molybdenum and manganese. Following is a brief discussion of important nutrients in *Dunaliella* biomass production. A summary of optimal levels is presented in Table 2.2 (Adapted from Ben-Amotz and Avron, 1989b).

Table 2.2: Summary of nutrients requirements for *Dunaliella* (Ben-Amotz and Avron, 1989b).

PARAMETERS	Concentrations for optimal growth
NaCl	6-12 % (1-2 M)
Nitrogen. Supplied in the form of Ammonia, Nitrate, or Urea	5 mM
Phosphorus	Less than 0.1 mM
Sulphate	About 2 mM
Magnesium	About 1 mM
Potassium	1 mM
Calcium	0.1 mM
Micronutrients; Manganese Zinc Cobalt Copper	 5 μM 1 μM 0.1 μM 0.01 μM
Carbon dioxide	At laboratory scale, the carbon source used is usually $NaHCO_3$, at concentration 10mM
Temperature	Optimum 32°C, range for good growth 25 - 35 °C
pH control	pH 7 to 9

Dunaliella species are strict photoautotrophs. They grow on inorganic carbon sources such as gaseous carbon dioxide, sodium hydrogen carbonate or sodium bicarbonate (Aizawa and Miyachi, 1992; Ben-Amotz and Avron, 1989b).

Nitrogen is a requirement for *Dunaliella* growth. It can be supplied in the form of nitrate, ammonia or urea. Nitrate is the preferred form. Ammonia is toxic at concentrations above 5 mM when the pH exceeds 8. Urea is a source of organic carbon which may encourage growth of contaminants.

Phosphorus is required at low concentrations of below 0.1 mM (Table 2.2). The presence of phosphorus together with calcium at pH above 8 may cause flocculation. This may reduce the growth rate. Sulphate is required at 2 mM. In the presence of calcium (Ca^{+}) ions, a calcium sulphate precipitate may form. Calcium is required at a very low concentration of 0.1 mM. At pH in excess of 8, reactions with some other medium components may result in the formation of precipitates including $Ca(HCO_3)_2$, $CaCO_3$, $CaSO_4$ and $Ca_3(PO_4)_2$ (Ben-Amotz and Avron, 1989b). Magnesium and potassium are required, each at 1 mM. *Dunaliella* can store magnesium and potassium at 300 mM and 200 mM respectively. A range of trace nutrients including manganese, zinc, cobalt and copper are typically provided although the functions have not been identified (Ben-Amotz and Avron, 1989b).

2.5.2.3 Temperature

The optimal growth temperature for *Dunaliella* is around 32°C. Growth across a broad range of 25 to 35°C is reported (Ben-Amotz and Avron, 1989b). Temperature affects glycerol leakage from the cells. There is leakage of glycerol at 40°C. At 50°C all the glycerol may be lost to the medium. Glycerol leakage in open cultures can be used as an organic carbon source supporting growth of halophilic and halotolerant bacterial contaminants. However, it is noted that the optimum temperature increases with high light intensity and high salinity. Increased high light intensity up to 30% of saturation

light intensity (2000 μmol photons.m^{-2}.s^{-1}) supports higher rates of photosynthesis hence increased growth (Vorst, 1995; Pulz, 2001).

2.5.2.4 Presence of other organisms

2.5.2.4 (a) Presence of non-carotenogenic *Dunaliella* species

The physiology of *Dunaliella* enables for cultivation in highly selective medium not tolerated by the majority of organisms, except for the similarly halophilic and halotolerant species. The occurrence of non-carotenogenic *Dunaliella* species, *D. viridis*, under carotenoid-producing conditions was mentioned in Section 2.3.2. Other possibilities include *D. parva, D. tertiolecta* and *D. minuta* (Preisig, 1992; Masemola, 2000). These species are smaller in size than the carotenoid-producing *Dunaliella*. When growing at low salt concentration and nutrient balanced conditions, the non-carotenogenic species outcompete the carotenoid-producing *Dunaliella* strains (Borowitzka and Borowitzka, 1988). The proliferation of non-carotenogenic *Dunaliella* species and other non-*Dunaliella* species leads to competition for nutrients or reduction of light available for carotenogenic *Dunaliella* (Borowitzka and Borowitzka, 1988).

The growth properties of carotenogenic and non-carotenogenic *Dunaliella* species are summarised in Table 2.1. The table shows examples of growth rates and biomass attained by various authors. It should, however, be noted that comparison of *Dunaliella* productivities between studies from literature can be hampered by the varying conditions under which these experiments are conducted. The parameters evaluated include light intensity, temperature and sodium chloride, nitrate and sulphate concentrations. Further, the effects of these parameters were studied as either univariate or multivariate where the multivariate represents the multiplicative effects of the parameters in natural setting. From Table 2.1, it is noted that the maximum specific growth rates achieved for non-carotenogenic *D. salina* and *D. viridis* were 0.6 and 1.40 day^{-1} respectively. For the carotenogenic species, *D. bardawil* and *D. salina,* the authors reported specific growth rates of 0.3 and 0.48 day^{-1} respectively.

The differences in growth rates between non-carotenogenic and carotenogenic species dictate the need for control of growth conditions for reduction of the non-carotenogenic species in mixed cultures to be achieved. This forms the topic of the current thesis. Borowitzka and Borowitzka (1988) emphasised the importance of experimenting with mixed cultures at laboratory and pilot scale level as industrial cultivation are prone to infection with non-carotenogenic halotolerant *Dunaliella* species.

2.5.2.4 (b) Presence of non-*Dunaliella* species

High salinities (> 35% w/v; 6 M NaCl) and high alkalinity favours the survival of the Cyanobacteria including *Spirulina, Oscillatoria* and *Coccochloris* (Masemola, 1999; Oren and Rodriguez-Valera, 2001). These may compete with *Dunaliella* cultures for growth limiting conditions. Moreno-Garrido and Cavavte (2001) reports the occurrence of *Dunaliella* predators in the open ponds systems such as ciliates and amoeba which were able to survive high salinities of around 5 M NaCl (30% w/v).

2.5.2.4 (c) Halotolerant and halophilic bacteria

Halotolerant bacteria such as *Halobacterium*, *Haloarcula* and *Haloanaerobiceae* are typically found at concentrations of 10^6 to 10^9 cells.mL^{-1} in high salt environments (Oren, 2002). The concentration of these is dependent upon, among other factors, organic carbon availability. Less contamination is expected when *Dunaliella* is grown in medium containing minimum organic matter. *Dunaliella*, however, produces glycerol that may leak into the growth medium and act as a source of organic matter for the bacteria.

2.5.3 Kinetics of biomass production

The growth kinetics influences the economy of production for the carotenogenic *Dunaliella* species. Kinetics studies are necessary for process design so that the duration of the cultivation phase and space-time relationship of the key parameters under study

can be determined. *Dunaliella salina*, like other microalgae, exists at low biomass concentrations in cultures.

2.6 Carotenoid biosynthesis

2.6.1 Factors influencing carotenoid biosynthesis

Table 2.3 shows the effects of environmental parameters, namely high light intensity, high salt concentration, macronutrient (nitrate, sulphate, phosphate) availability and temperature on biomass yield and carotenoid production in *Dunaliella salina*. Increases in salinity and irradiance are the strongest promoters of carotenoid production, followed by nitrogen deficiency and then increases in temperature. Compared to nitrogen deficiency, phosphate deficiency is not a strong inducer of carotenoid biosynthesis. From the table, an inverse relationship between biomass and carotenoid production can be deduced.

Table 2.3: Effect[3] of operating parameters on *D. salina* biomass production and β-carotene accumulation (Adapted from Borowitzka, 1990)

Factor	Biomass	β-carotene
Salinity increase	--	++++
Salinity decrease	+	-
Nitrogen deficiency	--	+++
Phosphorus deficiency	--	+
Increase in inorganic carbon	+++	0
Increase in light	+	++++
Decrease in light	-	---
Temperature increase	+	++
Temperature decrease	-	-

[3] - = inhibitory effect; + = stimulating effect; 0= no effect

Application of these parameters as univariate and multivariate factors in β-carotene production is shown in Table 2.3. In industrial production of β-carotene, the cultures are subjected to the synergistic effects of these factors. At controlled settings, these parameters and their levels vary between studies.

2.6.1.1 Light intensity

The amount of β-carotene accumulated intracellularly by *Dunaliella* is a direct function of the amount of irradiance to which the alga is subjected during a division cycle (Ben-Amotz and Avron, 1983). Further, the 9-*cis* to all-*trans* β-carotene isomers ratio is promoted at low light intensities (20 to 50 μmol photons.m^{-2}.s^{-1}) compared to high light intensities (200 to 1 250 μmol photons.m^{-2}.s^{-1}) (Orsert and Young, 2000). The potential function of β-carotene in relation to high light intensity was discussed in Section 2.4.2.

2.6.1.2 Nutrient concentration and availability

Increased NaCl has been mentioned as one of the key parameters for inducing β-carotene accumulation. It can be used as a sole stressor to achieve the β-carotene accumulation indicated in Table 2.3. Carotenogenic *D. salina* and *D. bardawil* grow optimally at 1-2 M NaCl. Reduced growth rates, accompanied by β-carotene accumulation in *Dunaliella,* result at salinities exceeding 2 M and up to NaCl saturation (approximately 5 M).

Deprivation of nutrients including nitrates, sulphates and phosphates lead to the promotion of carotenoid biosynthesis (Ben-Amotz and Avron 1983). Reductions in the availability of these nutrients have negative effects on cell growth rate. To induce carotenoid biosynthesis, the *Dunaliella* cells are grown in a nutrient replete medium and then transferred to medium which is growth-limiting in nitrate, sulphate or phosphate content.

2.6.1.3 Effects of temperature

Extreme temperatures also promote substantial β-carotene production although to a lesser extent than high sodium chloride concentration level, high light intensity level and nitrate deprivation as indicated in Table 2.3. Garcia *et al.* (2007) reported maximal β-carotene accumulation of 35.14 pg.cell^{-1} at 38°C, their highest experimental temperature value for optimal β-carotene biosynthesis.

2.6.2 Kinetics of carotenoid biosynthesis

In their work to determine the kinetics and characteristics of β-carotene accumulation induced by light stress, Lers *et al.* (1990) grew *Dunaliella* cells at 27 μeinsteins.m^{-2}.s^{-1} (27 μmol photons.m^{-2}.s^{-1}) and transferred them to 1650 μeinsteins.m^{-2}.s^{-1} (1650 μmol photons.m^{-2}.s^{-1}). They observed that β-carotene accumulated at two distinct stages: the first stage lasted 24 hours and occurred immediately after exposure to the higher light intensity. The second stage occurred at the beginning of stationary phase and continued until culture collapse. It was deduced that the first stage of accumulation could be due to photo-induced gene activation of anabolic β-carotene enzymes or controlling factors. The signal for the second stage of β-carotene accumulation, however, remains undetermined.

In addition, for understanding algal growth dynamics, kinetics studies are also required for process design. Table 2.4 collates literature values of β-carotene yields which vary according to algal strains and cultivation conditions.

2.7 Commercial systems for biosynthesis of β-carotene by *Dunaliella*

Systems for the mass cultivation of *Dunaliella* for β-carotene production vary. Systems can be extensive or intensive (Borowitzka and Borowitzka, 1989) and could further be

classified as open or closed (Pulz, 2001). Ben-Amotz (1999) further defines different levels of mass culture: extensive, intensive, semi-intensive and highly intensive.

Table 2.4: Carotenoid accumulation of *Dunaliella* species reported in the literature

Species	LI	Stress factor	Carotenoid content	Author
1. *D. bardawil*	a. 10^4 kergs b. natural illumination	Salinity 3M, Outdoors	β-carotene ($pg.cell^{-1}$) 25-45	Ben-Amotz *et al.*, 1982
2. *D. bardawil*	a. 10 kergs b. 5 to 550 kergs	3.5 M (21%) NaCl Nitrate: 0 to 10 mM Sulphate: 0 to 10 mM	β-carotene ($pg.cell^{-1}$) Light intensity stress: 7.9 to 25.7 Nitrate: 35 to 45 Sulphate: 20 to 30	Ben –Amotz and Avron, 1983
3. *D. salina*, Thailand	270 $\mu mol.m^{-2}.s^{-1}$	1.8 – 5 M NaCl	Carotenoid ($pg.cell^{-1}$) 24- 80.3	Powtongsook *et al.*, 1995
4. *D. salina*, Chile isolates 8 strains	150 $\mu mol.m^{-2}.s^{-1}$	30% NaCl	Carotenoid ($pg.cell^{-1}$) 42.4	Cifuentes *et al.*, 1992
5. *D. viridis* Teodoresco	150 $\mu mol.m^{-2}.s^{-1}$	Nitrate stress (mM): 0.5 to 10 NaCl stress 1 to 4 M (6%)	Carotenoid ($mg.L^{-1}$) Nitrate stress: 0.6 to 5.4 NaCl stress 2.0 to 5.5	Jimenez and Niell, 1990
6. *D. salina* (Iran) 3 strains	Outdoor, 33°58 N, 51° 29 E	Stage 1: 2 M (12%) NaCl; 5 mM Nitrate; 2 mM Sulphate Stage 2: 2.5 M (15%) NaCl; 0.1 mM Nitrate; 2 mM Sulphate	β-carotene: $pg.cell^{-1}$ 7.0 to 23	Tafreshi and Shariati, 2006

2.7.1 Extensive systems

Extensive systems are low technology systems where algal cultivation is dependent upon natural conditions. These systems include natural solar evaporation ponds and artificial ponds such as raceways, tanks and circular ponds (Borowitzka and Borowitzka, 1989; Pulz, 2001). The key difference between natural and artificial systems is the provision for stirring in artificial systems. Artificial open ponds are designed with paddle wheels which

aid in stirring thus contributing to improved productivity. Mixing reduces the settling tendency of the cultures by providing sufficient turbulence and aids in support of denser cultures where light availability may be a limiting factor (Borowitzka and Borowitzka, 1989; Borowitzka, 1999; Pulz, 2001).

The extensive systems suffer the drawbacks of having limited control on the cultivation parameters. Cultivation is subject to climatic conditions including temperature and light intensity fluctuations. Salinity changes due to evaporation losses, rate of CO_2 diffusion from the atmosphere, dilutions resulting from rainfall and the likelihood of contamination from competitor species or algal grazers are potential problems encountered in extensive systems. Cultivation of *Dunaliella* in open ponds exploits the ability of the alga to prosper in extreme conditions and hence limits contamination from competitors and grazers (Borowitzka and Borowitzka, 1989; Borowitzka, 1999; Pulz, 2001). According to Pulz (2001) and Borowitzka (1999), *Dunaliella* is cultivated for production of carotenoids in shallow open ponds of approximately 250 hectares in Australia and Ukraine. Earthen wall lined unstirred ponds are also used in Australia. Raceways are used in both Israel and USA.

Typically, commercial *Dunaliella* plants are situated along seashores, or close to salt lagoons and salt producing plants (Ben-Amotz, 1999). The nutrient composition of the brines or seawater sustains growth of *Dunaliella* with minimum requirement for additional nutrients. Salt is often added to increase the salt concentration to induce carotenoid biosynthesis. Light is also freely available. There is, however, a need for large areas of land with access to seawater or brine.

Key factors of extensive system (raceway ponds) design are presented and discussed in Table 2.5. Raceway ponds are typically oblong with depth or 10 to 20 cm for efficient solar radiation, gaseous exchange and mixing. For even mixing, velocities ranging from 10 to 30 $m.s^{-1}$ are preferred.

Table 2.5: Key parameters for raceway pond design (Benemann, 1989).

Open Ponds Parameters	Description and Requirements
Pond design	- Oblong raceways made of bricks, concrete, fibreglass, PVC - Size depends on the area that can be stirred with one paddle wheel but the common average surface area1000-4000 m^2
Depth	Range from 10 – 20 cm in depth to allow for even solar radiation as well as oxygen and carbon dioxide exchange
Mixing	Should be > 10 $cm.s^{-1}$ to prevent deadspots and allow all nutrients to be distributed around the pond. Velocities of > 30$cm.s^{-1}$ may create turbidity.
Pond liner	- Liners are needed to prevent loss of media as well as to avoid introduction of dirt and soil into the pond. - The plastic, concrete, fibre-glass and other material that can be used for pond walls can be used.

2.7.2 Intensive systems

The intensive systems employ higher control of cultivation parameters and are mainly designed as photobioreactors. Flexible designs including tubular, flat panel and reactor-like designs exist. Light availability and temperature are controlled. Nutrients are added to improve the productivity of the alga. Mixing systems are designed for even and reproducible distribution of medium components including CO_2 and O_2. Medium loss through evaporation, and culture contamination are absent or minimal (Pulz, 2001).

The ability to control the cultivation parameters comes with added expenses and technical challenges, especially the provision of artificial light. Furthermore, designing closed photobioreactors is often technically challenging. Of importance are temperature control, reactor fouling and the discharge of oxygen produced during photosynthesis (Pulz, 2001). Efforts to cultivate *Dunaliella* intensively in closed photobioreactors have not been successful economically (Ben-Amotz, 1999).

The intensive methods offer significantly increased biomass productivities and carotenoid yields. For example, Ben-Amotz and Avron, 1989a) reported biomass productivities in the ranges of 4 to 10 g dry $wt.m^{-2}.day^{-1}$ and carotenoid yields of between 250 to 500 $mg.m^{-2}day^{-1}$ with intensive cultivation. Extensive cultivation, however, yields

productivities ranging from 0.1 to 10 $mg.m^{-2}day^{-1}$ (Phillips, 1993). From an economic perspective, running costs incurred in extensive cultivation are considerably lower than those in intensive cultivation. However, the former can have much lower productivities (Benemann, 1989; Phillips, 1993).

2.7.3 Mode of *Dunaliella* cultivation for β-carotene production

The mode of *Dunaliella* cultivation for β-carotene production follows two strategies: single mode, involving averaged biomass production and carotenoid accumulation processes; and dual mode, where the alga is first cultivated in conditions promoting optimal biomass production followed by transfer of the biomass to conditions promoting carotenoid biosynthesis and accumulation (Ben-Amotz, 1995).

The dual phase mode is preferred in intensive systems, specifically with closed photobioreactors. Biomass production phase and carotenoid biosynthesis and accumulation are controlled and optimised separately (Ben-Amotz, 1995) which promotes higher biomass productivities and carotenoid yields. Ben-Amotz (1995) demonstrated the productivity differences between single and dual phase mode of cultivation using two separate nurseries. Single stage cultivation yielded 200 $mg.m^{-2}.day^{-1}$ *Dunaliella* biomass whilst productivities of up to 450 and 300 $mg.m^{-2}.day^{-1}$ were attained in first and second stage respectively in the dual phase mode of cultivation.

2.8 Downstream processing in commercial cultivation of *Dunaliella*

Dunaliella products in the market can take a variety of forms. Whole cells can be dried and sold as β-carotene rich algal powder. Algal pigments can also be extracted into vegetable oil. Dufossé *et al.* (2005) illustrated production steps from biomass production and harvesting to product preparation as shown in Figure 2.7. Briefly, *Dunaliella* biomass is harvested via centrifugation, with or without flocculation for later carotenoid extraction. The carotenoids are evaluated for quality before they are processed into the

different products in varying industries including pharmaceuticals, food industries and cosmetics.

2.8.1 Harvesting of algal biomass

The challenge in *Dunaliella* harvesting lies with the characteristic low cell density in the cultures and the *Dunaliella* cell morphology. *Dunaliella* cultures typically reach densities of 200 to 500 $mg.L^{-1}$. This presents economic and technical difficulties in separating the biomass from the medium to produce slurries with 5 to 15% solids content (Borowitzka and Borowitzka, 1989; Ben-Amotz and Avron, 1989b). Harvesting biomass can account for 20 to 30% of the total cost of *Dunaliella* production (Grima *et al.*, 2003).

Dunaliella's lack of cell wall renders the cells prone to rupture during harvesting. In addition, the mucilage coating leads to cell aggregation and clogging when filter membranes are used for harvesting (Ben-Amotz, 1999). Members of the *Dunaliella* genus are motile enabling them to disperse in the culture making settling difficult. High salinity is used in *Dunaliella* culture exposing much of the harvesting machinery to corrosion. Harvesting methods are presented in Table 2.6. Filtration, pressure filtration, large-scale filtration, centrifugation, flocculation and hydrophobic binding can be used. Centrifugation is preferred when *Dunaliella* are cultivated for high value products such as carotenoids (Grima, *et al.*, 2003). The harvest method of choice is dictated by ultimate use of the final product and economics.

Table 2.6: Potential harvesting methods for *Dunaliella*

Harvesting method	Comments
Filtration (Borowitzka and Borowitzka, 1988)	- Sand filtration, cellulose fibres not efficient because of clogging. Backwashing has been tried but this leads to breakage of cells and glycerol leakage
Pressure filtration (Grima *et al.*, 2003)	- The system clogs easily
Large scale filtration (Grima *et al.*, 2003)	- Culture medium is passed through diatomaceous earth. Algae extracted with an organic solvent. This is concentrated and purified.
Centrifugation (Grima *et al.*, 2003; Borowitzka and Borowitzka, 1988)	- This method causes high degree of cell destruction; it is also very costly with high initial investment.
Flocculation (Borowitzka and Borowitzka, 1988)	- Flocculants were used to aggregate the microalgal cells, which can then float, or sediment. - Beta-carotene can, however, be extracted directly from flocculated cells - A new flocculation, flotation, and dehydration method patented by Borodyanski and Konstantinov (2003) uses modified starch as a flocculating agent. The cells do not rupture
Hydrophobic binding (Curtain and Snook, 1985)	- This method is based on the fact that at high salt concentrations (about 4M), the *Dunaliella* cells form hydrophobic surface coat. This property is used to adsorb the cells to hydrophobic surfaces. Beta-carotene is then extracted from the cells.

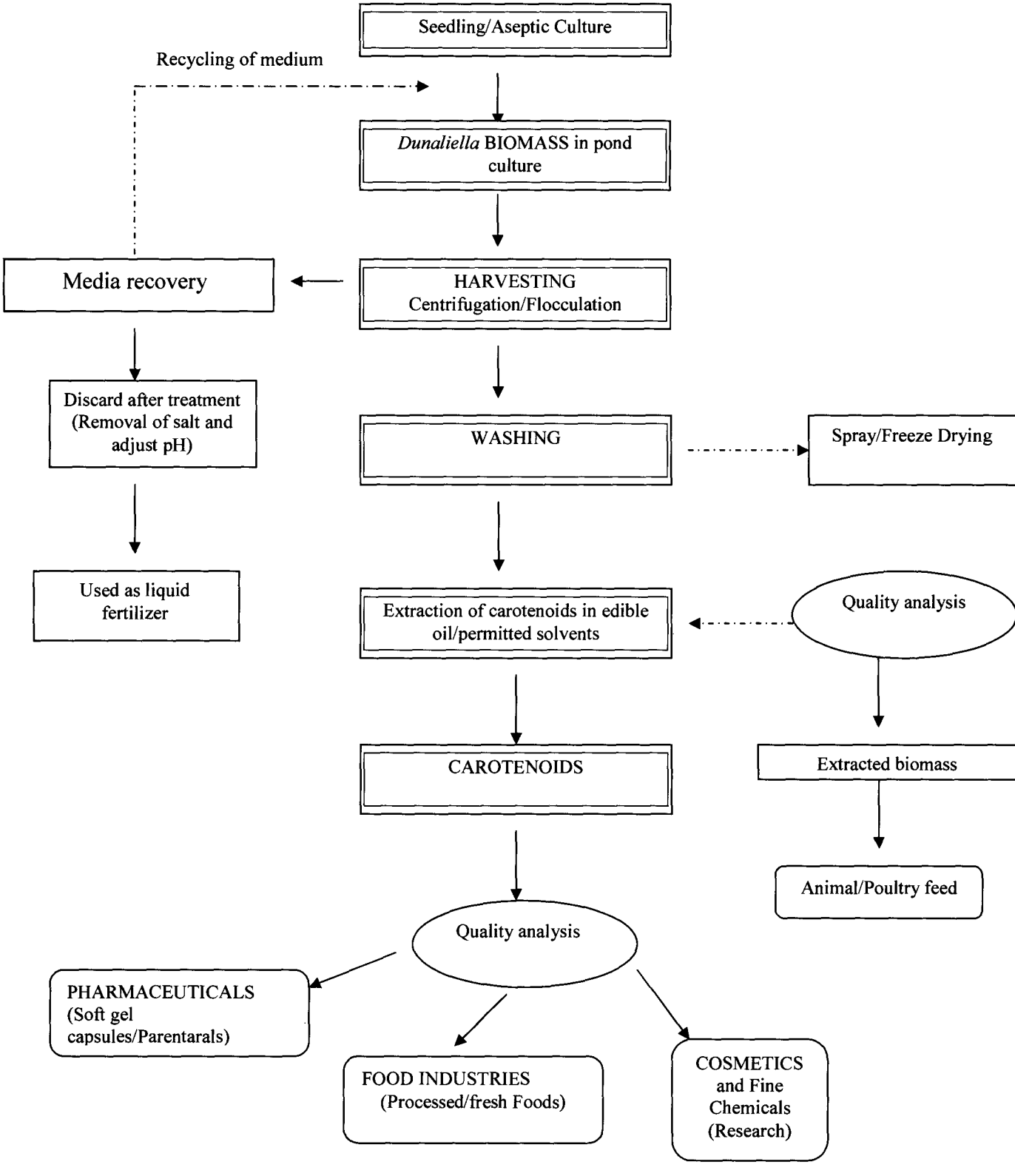


Figure 2.7: Flow chart for production and utilisation of *Dunaliella*. Adapted from Dufossé *et al.* (2005)

2.8.2 Production of the final product

The *Dunaliella* slurry resulting from harvesting can be dried using several methods: spray drying, freeze-drying, drum drying and sun drying (Benemann, 1989). The high water content of the process stream that needs to be evaporated off makes spray drying expensive. Spray drying is, however, more economical with the high value products although it may cause deterioration of the carotenoids (Benemann, 1989; Grima *et al.*, 2003) as heat and air are introduced. β-carotene is susceptible to oxidation in heat, air and light.

Benemann (1989) and Grima *et al.* (2003) highlighted the difficulty of using solvent extraction from wet biomass without previously disrupting the cells. However, direct extraction of carotenoids from *Dunaliella* using oil appears to be a better method (Nonomura, 1987). The carotenoids are extracted directly into oil as the final product.

Mendes *et al.* (2003) presented supercritical carbon dioxide extraction as an alternative method for carotenoids extraction from *Dunaliella* and other microalgae. When subjected to 30.0 MPa and temperature of 313.1 K, the natural β-carotene isomers is more soluble in the liquid CO_2 solvent than the synthetic *trans*-β-carotene, a point exploited in application of natural β-carotene in the pharmaceutical industry.

Leon *et al.* (2003) and Hejazi and Wijffels (2004) have introduced the use of aqueous–organic two phase photobioreactor for simultaneous cultivation of *Dunaliella* and extraction of β-carotene. Cultures are first grown under growth-supporting conditions. Following this, excess light is used to induce carotenogenesis. A biocompatible organic phase is added for β-carotene extraction. This is achieved by continuous re-circulation of the biocompatible organic phase containing the cells (Hejazi and Wijffels, 2004). The use of two-phase aqueous/decane system proved to be stable and showed an increase in β-carotene content in 4 days with 8% of total carotenoid extracted into the decane phase (Leon *et al.*, 2003). It is anticipated that when optimised, this method of cultivation will

aid in reduction of costs associated with downstream processing of *Dunaliella* in the commercial cultivation as well as improved productivity of the alga.

2.9 Conclusion

The literature review provides an insight into mass cultivation of *Dunaliella* for natural production of β-carotene. The natural production of β-carotene from *Dunaliella* is dependent on the understanding of both the physiology of the organism and the parameters that control the process. This is necessary to make both the biomass production and downstream processing of biomass and extracted β-carotene product streams commercially viable.

Dunaliella is widely distributed in nature with species having the best commercial potential being isolated from areas of extreme environmental conditions, such as high salinities, high irradiances and extreme temperatures, and low nutrient availability. This organism is exploited worldwide for its ability to produce massive amounts of β-carotene, which finds use as provitamin A, an antioxidant and colourant in the pharmaceuticals, food and feed industries. Currently, 90% of the β-carotene in the market is of synthetic origin. It has been shown that there is increased demand for natural β-carotene in the marketplace because of its advantage of having a higher biological activity than its synthetic counterpart.

The occurrence of β-carotene rich *Dunaliella* species in the Sua Pan solar evaporation ponds in north-eastern Botswana has been reported before but there are no published studies on the potential of these species for commercial production of β-carotene. Both carotenogenic and non-carotenogenic *Dunaliella* species are found in these solar evaporating ponds. These organisms have been studied with regard to their effect on the quality of soda ash produced by the nearby Botswana Ash mine (Masemola, 1999). This project focuses on evaluating the potential of the Sua Pan *Dunaliella* isolates for commercial natural β-carotene production. Here β-carotene production is assessed under different environmental parameters that are known to induce β-carotene biosynthesis, as

well as to quantify these parameters for optimum β-carotene production at laboratory scale.

The hypotheses addressed in the project are:

1. *Dunaliella* isolates from the Sua Pan solar evaporation ponds have the potential for economic large-scale production of natural β-carotene.
2. An investigation of the environmental parameters that impact on biomass productivity and β-carotene biosynthesis and accumulation in these organisms at laboratory scale, will provide the data required to evaluate the feasibility of commercial cultivation of Sua Pan isolates of carotenogenic *Dunaliella* species at Sua Pan.
3. An understanding of growth conditions on both the carotenogenic and non-carotenogenic *Dunaliella* species will aid process design to maximise carotenogenic growth

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Introduction

This chapter details the methodology used to isolate β-carotene-producing strains of *Dunaliella* from the Sua Pan solar saltworks and to investigate their productivity. Type culture collection species are used for comparison. Evaluation of culture medium used for cultivation of *Dunaliella* is discussed in Section 3.2. Section 3.3 details the algal species used in this study. Section 3.3 also covers the isolation and enrichment of the environmental isolates. Algal cultivation is discussed in Section 3.4. Provision of light follows in Section 3.5. The general analytical methods and the experimental design are described in Sections 3.6 and 3.7 respectively. Where necessary, the detailed protocols are included as appendices. The conclusion of the chapter is in Section 3.8.

3.2 Culture medium

A variety of cultivation media have been used for culturing *Dunaliella* (Ben-Amotz and Avron, 1983; Borowitzka and Borowitzka, 1988; Cifuentes *et al.*, 1992; Masemola, 1999; Powtongsook *et al.*, 1995; Tafreshi and Shariati, 2006). Two media were selected for this work. Modified Johnson's Medium, abbreviated MJ (J/1) in the thesis, was used in *Dunaliella bardawil* cultivation and carotenoid biosynthesis studies (Ben-Amotz and Avron, 1983; Borowitzka and Borowitzka, 1988; Cifuentes *et al.*, 1992; Powtongsook *et al.*, 1995). BAAM medium was used previously for cultivation of *Dunaliella* species from the Sua Pan solar saltworks evaporation ponds (Masimola, 1999). The constituents of the two media are compared in Table 3.1.

With the exception of the presence of potassium chloride (KCl) and magnesium chloride ($MgCl_2.6H_2O$) contained in the MJ (J/1) medium, both BAAM and MJ (J/1) media contained similar macronutrients but at differing concentrations. The potassium nitrate

concentration in MJ (J/1) medium was double that in BAAM medium. The chelation agent, ethylenediamine tetra-acetic acid (EDTA) contained in BAAM was at a micromolar concentration compared with the nanomolar concentration contained in MJ (J/1). The trace elements contained in the two media were similar except for the ferric chloride ($FeCl_3.6H_2O$), ammonium molybdate ($(NH4)_6Mo_7O_{24}.2H_2O$) and copper sulphate ($CuSO_4.5H_2O$) were absent in the BAAM medium. The BAAM medium, however, contained higher quantities of the remaining trace elements such as B, Mn, Zn and Co than MJ (J/1) medium.

Table 3.1: Chemical composition of the BAAM medium (Masimola, 1999) and Modified Johnson's medium (J/1) (Ben-Amotz *et al.*, 1982).

Major constituents	BAAM composition	MJ (J/1) composition
NaCl	2 M	2 M
$NaHCO_3$	50 mM	50 mM
KNO_3	5 mM	10 mM
$MgSO_4.7H_2O$	5 mM	2 mM
KH_2PO_4	0.2 mM	0.3 mM
$CaCl_2.2H_2O$	0.3 mM	1 mM
KCl		3 mM
$MgCl_2.6H_2O$		7 mM
Ferrous solution		
$FeCl_3.6H_2O$	-	9 nM
Na_2EDTA	-	5 nM
Trace elements		
EDTA	75 µM	
H_3BO_3	0.4 µM	10 nM
$MnCl_2.4H_2O$	0.01 µM	0.2 nM
$ZnCl_2$	0.002 µM	0.3 nM
$CoCl_2$	0.04 nM	0.2 nM
$(NH_4)_6Mo_7O_{24}.2H_2O$		0.3 nM
$CuSO_4.5H_2O$		0.2 nM

The components of the growth media were dissolved individually in appropriate volume of distilled water. The pH was adjusted to approximately pH 7.5 using concentrated hydrochloric acid (HCl) and 2 N sodium hydroxide (NaOH). The media were autoclaved at 121°C for 20 min. For solid medium, 0.9% (w/v) agar was added.

3.3 Microorganisms

In order to characterise the *Dunaliella* isolates from the Sua Pan solar saltworks, two *Dunaliella* culture collection species were used as positive controls: *Dunaliella bardawil* UTEX 2538 and *Dunaliella salina* UTEX 1644.

3.3.1 Culture collection *Dunaliella* cultures

3.3.1.1 *Dunaliella bardawil (D. bardawil)* UTEX 2538

Dunaliella bardawil (UTEX 2538) has been studied by a number of algal researchers for massive accumulation of β-carotene. *D. bardawil* was purchased from the University of Texas (UTEX) algal culture collection. It was isolated by Ben-Amotz and Avron from the salt ponds of Bardawil Lagoon, North Sinai, Israel, in 1978. R. Adams deposited the same species as American Type Culture Collection (ATCC) 30861 in 1980. *D. bardawil* UTEX 2538 is patented for production of glycerol, carotenes, and protein rich material by Avron and Ben-Amotz (University of Texas, n.d.).

3.3.1.2 *Dunaliella salina (D. salina)* UTEX 1644

As previous *Dunaliella* isolates from Sua Pan solar saltworks evaporation ponds have been identified as *D. salina* (Masemola, 1999) and the cultivation of *D. salina* for β-carotene production is cited in the literature, *D. salina* UTEX 1644 was also purchased as a positive control from the University of Texas algal culture collection. It was isolated by L. Loeblich from Point Colorado Salinas, La Paz, Baja California, Mexico and deposited by A.R. Loeblich as D-18-f. *D. salina* UTEX 1644 grows on high salinity seawater (5 to 15% w/v NaCl) (University of Texas, n.d.).

3.3.2 Enrichment of *Dunaliella* cultures from Sua Pan solar salt works evaporation ponds

Two (2) batches of brine samples were obtained from Sua Pan solar salt works evaporation ponds during this project. The first batch of samples was taken in August 2004 from five sites: CS, E3, E6, NS and X2. The second samples were taken in November 2004 from X2 crystalliser ponds. The properties of the ponds are discussed in depth in Chapter 5. CS is an inlet pond which also functions as the cooling sump of the plant effluent, E3 and E6 represent the evaporation ponds, NS (derived from NaCl storage) is a holding pond for brine exiting the evaporation ponds while X2 is crystalliser ponds number 2. The ponds vary primarily in NaCl concentration. The general pond layout is shown in Figure 3.1. It should be noted that the partitioning of the ponds and their use may vary from the diagram depending on the season and their need (Masemola, 1999).

For the first and second batch of brine samples, samples were collected in 0.25 L and 2 L plastic bottles respectively. Samples were shipped from Sua Pan, Botswana to Cape Town, South Africa over 3 days. On arrival, the brine samples were observed microscopically to estimate biomass concentration and to record algal cell morphology to assist in identification of the observed species.

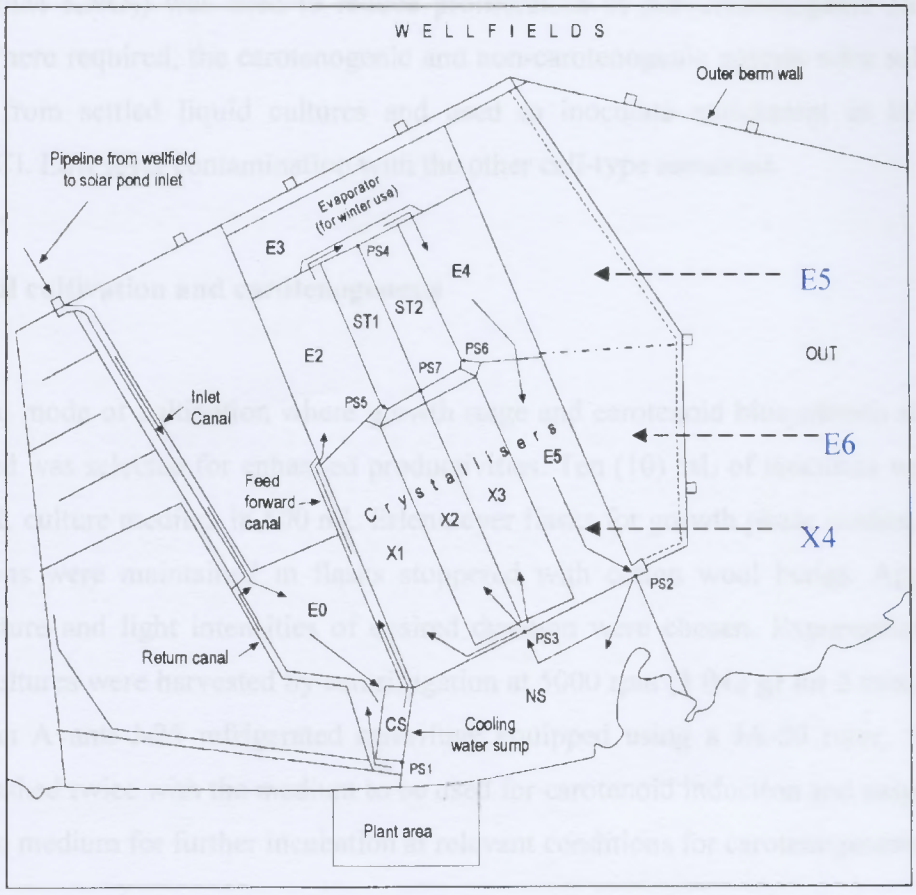


Figure 3.1: The layout of the solar evaporating ponds (Masemola, 1999). Changes to the original layout are marked in blue of E5, E6 and X4 as per Botswana Ash (1998).

3.3.3 The *Dunaliella* stock cultures

Stock cultures of the type culture species were maintained as 50 mL suspension cultures in 250 mL Erlenmeyer flasks, plugged with cotton wool, in BAAM media containing 2 M NaCl at 25°C under continuous irradiance of approximately 50 µmol photons.m^{-2}.s^{-1} using light system A, described in Section 3.5. Cultures were subcultured once a month.

Environmental *Dunaliella* stock cultures were maintained as non-axenic cultures in BAAM medium under the same conditions, except 3 M NaCl and 1.25 mM KNO_3

(25% 5mM KNO_3) was used to reduce proliferation of non-carotenogenic *Dunaliella* cells. Where required, the carotenogenic and non-carotenogenic species were selectively chosen from settled liquid cultures and used to inoculate enrichment in BAAM at 3 M NaCl. Low level contamination with the other cell-type remained.

3.4 Algal cultivation and carotenogenesis

The dual mode of cultivation where growth stage and carotenoid biosynthesis stages are separated was selected for enhanced productivities. Ten (10) mL of inoculum was added to 90 mL culture medium in 500 mL Erlenmeyer flasks for growth phase studies. Aseptic conditions were maintained in flasks stoppered with cotton wool bungs. Appropriate temperature and light intensities of desired duration were chosen. Exponential growth phase cultures were harvested by centrifugation at 5000 rpm (3 042 g) for 5 minutes with Beckman Avanti J-25 refrigerated centrifuge equipped using a JA-20 rotor. The cells were washed twice with the medium to be used for carotenoid induction and suspended in the same medium for further incubation at relevant conditions for carotenogenesis.

The growth phase of *Dunaliella* cultures resulted in cell concentrations of the order of 10^6 cells.mL^{-1}. In order to ensure optimum light intensity at induction of carotenoids, appropriate dilutions of cells were made to achieve cell concentrations of 10^4 cells.mL^{-1}. Culture volumes of 100 mL in 500 mL Erlenmeyer flasks with manual shaking twice a day were employed for carotenogenesis.

3.5 Provision of light intensity

The required light intensity was provided by two systems: system A and system B shown in Figures 3.2 and 3.3 respectively. System A provided low light intensity ranging from 300 to 3500 lux (4 to 50 $\mu mol.m^{-2}.s^{-1}$). Initial studies on the establishment of *Dunaliella* culturing system using type culture species were based on light system A. Continuous light was provided by three double 120 cm 36 W cool white fluorescent light bulbs positioned to provide light from above the cultures through a distance of 40 and 60 cm at

the top and bottom shelf respectively. To control the light reaching the cultures, the number of bulbs could be varied between 2 and 3 double bulbs.

Light system B was designed for higher light intensity ranging from 80 to 200 $\mu mol.m^{-2}.s^{-1}$. Three (3) light boxes were constructed using three double 60 cm 18 W cool white fluorescent bulbs (OSRAM L36 W/20) providing continuous light at the bottom of the Erlenmeyer flasks (Figure 3.3). Provision was made to control the number of bulbs used at a given time, controlling the light intensity reaching the cultures. A polyethylene lining on the perspex on the light boxes was used to control the light intensity further. Ventilation holes were created for air circulation to allow temperature control inside the light boxes. The light intensity achieved varied with position in the three boxes. The regions of similar intensity are illustrated in Figure 3.3c and light intensity ranges measured in $\mu mol.m^{-2}.s^{-1}$ are given in Table 3.2

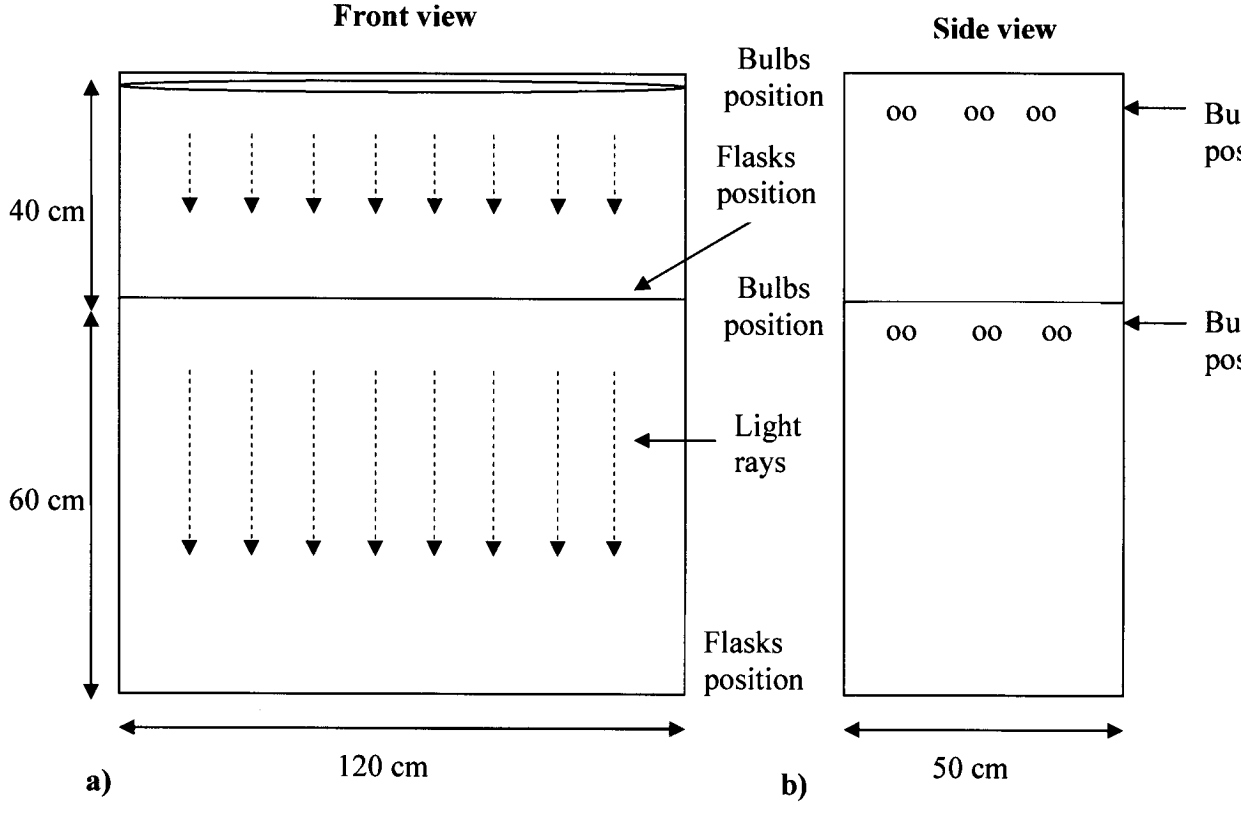
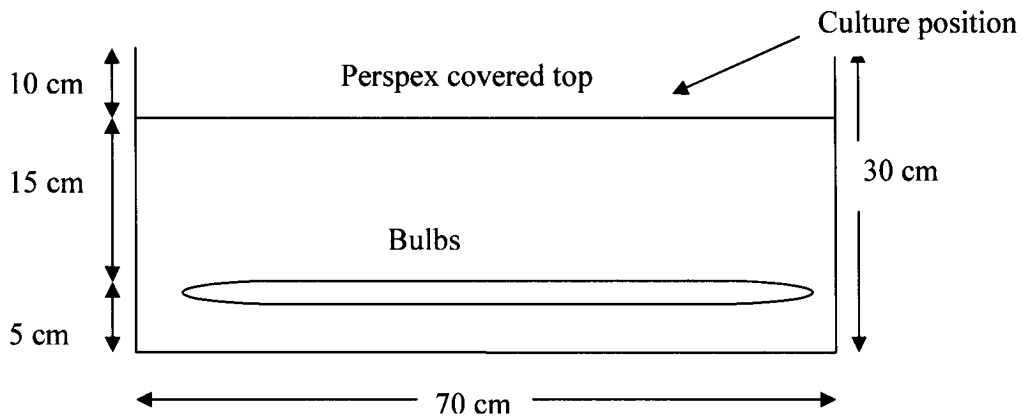
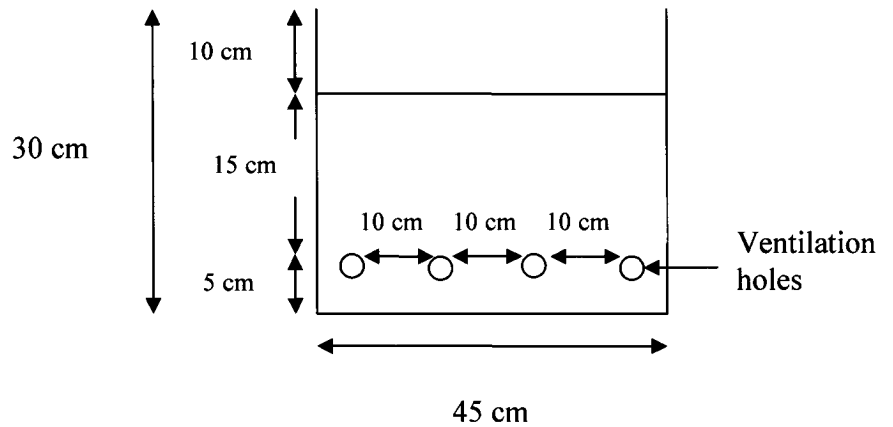


Figure 3.2: Length dimension of light system A used for provision of low light intensity a) Front view , b) Side view

a) Front view



b) Side View



c) Top view of the light boxes

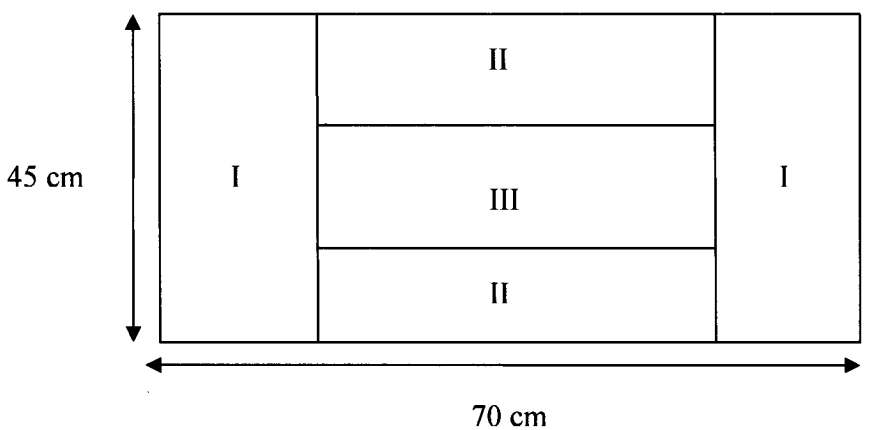


Figure 3.3: Light system B used to provide light intensity of 80 to 200 µmol.m^{-2}.s^{-1}. (a) The length dimension of the light boxes used in the project (b) The width dimension of the light box set ups (c.) An illustration of light boxes showing light intensity relative to position (top view).

Table 3.2 Light intensities (μmol photons.m^{-2}.s^{-1}) of the light boxes used for provision of light

Box 1:	Box 2:	Box 3:
I: 80 ± 5	I: 150 ± 5	I: 150 ± 5
II: 100 ± 5	II: 180 ± 5	II: 180 ± 5
III: 100 ± 5	III: 200 ± 5	III: 200 ± 5

3.6 General analytical methods

3.6.1 Light intensity measurements

Two instruments were used for measuring light intensity. The LX-101 lux meter, manufactured by Electronics Express, was used in the early phase of the work, in results presented in Chapter 4: Establishment of the system. The instrument measures the intensity of light as perceived by human eye. The unit of lux represents illumination per square meter. The radiometer model LICOR-250A fitted with a LI-210SZ quantum sensor, manufactured by LI-COR Biosciences, was used for the remainder of the study. The radiometer measures irradiance as light energy utilised for photosynthesis. The energy is delivered in minute packets called photons, typically presented as photon flux in plant biology. Photon flux depicts the number of photons falling per square meter per second (μmol.m^{-2}.s^{-1}) where 1 mole represents 6 x 10^{23} photons.

3.6.2 Biomass analysis

3.6.2.1 Microscopic observation and cell count

The phase contrast microscope, Olympus BX40 was used for morphological observations and cell concentration estimation of cell cultures. An improved Neubauer haematocytometer counting chamber was used. The *Dunaliella* life cycle included period of high and low mobility. In cases of high cell mobility, an iodine solution was added to

inhibit cell motion. An aliquot of 10 µl of iodine was added per 1 mL culture introducing dilution error of 1% which was considered minimal.

The cell concentration was to determine suitable parameters for comparison of growth: the maximum specific growth rate (μ_{max}), the average cell concentration achieved at stationary phase (X_{max}) and the extent of growth (X_{max}/X_0).

The specific growth rate was calculated during the exponential growth phase according to Malthus equation:

$$\frac{dX}{dt} = \mu X, \text{ integrated to give}$$

$$\mu = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)} \qquad \textbf{Equation 3.1}$$

where X_1 and X_2 are the cell concentration at time t_1 and t_2 respectively. Time was reported in days.

The extent of growth was given by:

$$\frac{X_{max}}{X_0} \qquad \textbf{Equation 3.2}$$

where X_{max} and X_0 are the average stationary phase cell count and initial cell count respectively.

3.6.2.2 Cell size determination

The image analysis software, AnalySIS, designed by Soft Imaging System, GmbH was used for determination of cell size and morphology of *Dunaliella* cells from micrographs. Micrographs of cells suspended in growth medium were taken at 400X and 1000X

magnification to estimate size. Morphology and cell size were evaluated microscopically as detailed by Preisig (1992) and Borowitzka and Siva (2007).

3.6.2.3 Cell size determination using Malvern Mastersizer particle analyser

Cell size determination using Malvern Mastersizer manufactured by Malvern Instruments Ltd was attempted using MS1 sampler at 2500 rpm stirring speed. The Malvern Mastersizer uses laser diffraction technology to measure the particles sizes in dispersion. NaCl salt solution at 12% w/v was used as a dispersant. For this project, there was no correlation between microalgal cell size data obtained via Malvern particle analyser and image analysis software. Microscopical evaluation of cultures leaving the particle analyser revealed ruptured *Dunaliella* cells.

3.6.2.4 Scanning electron microscopy

Detailed cell morphology of *Dunaliella* cells was studied using a Leo Stereoscan 440 model scanning electron microscope at the Electron Microscope Unit, Department of Physics, University of Cape Town. Osmium tetraoxide was used to fix the cells and to provide a cell coating to aid in the viewing of the cell surface using the electron beam. The detailed fixation procedure and sample preparation are provided in Appendix AI. Morphology studies were carried out on cultures from both growth and carotenogenesis phase.

3.6.3 Carotenoid quantification

Various methods exist for extraction, identification and quantification of carotenoids based on sample properties. Owing to the large number of samples handled in the thesis, a rapid spectrophotometric method was employed for carotenoid analysis. The method

was validated by a detailed spectrophotometric method within which a High Performance Liquid Chromatography (HPLC) quantification step was incorporated. Extraction was carried out under a low oxygen, low light and low temperature environment to avoid degradation of carotenoids.

Carotenoid quantity was presented in two formats: the specific carotenoid concentration as pigment per cell ($g.cell^{-1}$) and the volumetric carotenoid concentration ($mg.Litre^{-1}$). The pigment per cell parameter provided information on how much pigment each cell produced on average. The pigment per litre measurement, on the hand, gave an indication on how much pigment was produced in volume of the culture.

3.6.3.1 Detailed spectrophotometric measurements of carotenoids

The detailed spectrophotometric method is an improvement of an American Official Analytical Chemists (AOAC) Official Methods of Analysis (1995): 941.15 developed for quantification of β-carotene and total carotenoids in dried vegetables. This method was validated using HPLC analysis as per Section 3.6.3.3 and Appendix AIII. Validation of the method with HPLC showed accuracy to within 5% (Cyanotech Corporation, 2001).

Extraction was first carried out in methanol to precipitate carbohydrates and to dehydrate tissues to allow for easy penetration of tissues in subsequent extraction with organic solvents. This was followed by a saponification step for degradation of lipids and chlorophyll constituents. For β-carotene, a two phase extraction in heptane and spectrophotometric quantification at 436 nm was employed while a two phase extraction in diethyl ether and spectrophotometric quantification at 450 nm was used for total carotenoids. The detailed method is provided in the Appendix AIII.

The absorbance of the heptane extract was read at 436 nm against a heptane blank using a spectrophotometer. Calculations were made as follows:

Specific β-carotene concentration ($g.cell^{-1}$)

$$= \frac{Abs\ 436}{E\%,cm} x \frac{1}{Cellcount} x\ volume\ x\ dilution\ factor\ x\ \%\ beta-carotene$$

Equation 3.3a

Specific β-carotene concentration ($g.cell^{-1}$)

$$= \frac{Abs\ 436}{196} x \frac{1}{Cell\ count} x\ \ 25\ ml\ x\ 1 \cdot 25\ x\ 0.84$$

Equation 3.3b

Total carotenoids were calculated using the diethyl ether extraction. Absorbance of the diethyl ether extract was read against the ether blank at 450 to 453 nm using ThermoSpectronic Genesys 10UV spectrophotometer. The β-carotene was estimated from the literature, using the method adopted from the Cyanotech Corporation (2001), based on the work of Schuep and Schierle (1995). Results obtained via this method were compared with that of HPLC method.

Total specific carotenoid concentration ($g.cell^{-1}$)

$$= \frac{Abs\ (450-453)}{E\%,cm} x \frac{1}{Cell\ count} x\ 25\ ml\ x \frac{ether\ volume}{2}$$

Equation 3.4a

Total specific carotenoid concentration ($g.cell^{-1}$)

$$= \frac{Abs\ (450-453)}{259.2} x \frac{1}{Cell\ count} x\ 25\ ml\ x \frac{ether\ volume}{2}$$

Equation 3.4b

The protocol for conducting this analysis is outlined in Appendix AIII.

3.6.3.2 Rapid spectrophotometric measurement

The ability of acetone to penetrate the hydrophilic matrix was employed for rapid extraction of carotenoids. For complete extraction, the tissue were homogenised in the presence of the extracting solvent.

Two (2) mL culture sample was centrifuged using a Biofuge pico microcentrifuge model manufactured by Heraeus Instruments, at 12 000 rpm for 5 minutes. The pellet was re-suspended in known amount of 100% ice cold acetone and the cells ruptured using a Virsonic VirTis cell sonicator for 15 seconds at power 3. Cells were then centrifuged and the supernatant was drawn into clean tubes. Washing in solvent and centrifugation was employed to achieve complete pigment removal while noting the solvent amount used. Absorbance of the clear extracts was measured at 470 nm wavelength with a spectrophotometer. Where necessary, the extracts were filtered with 0.45 μm Millipore filters. Spectrophotometric readings were kept approximately in the range 0.2 - 1.25 by dilution with ice cold acetone when required.

Total volumetric carotenoid concentration ($mg.litre^{-1}$)

$$= \frac{Abs\,(450-453)}{E\%,cm} x \frac{1}{volume\ acetone(l)} x\ dilution$$

Equation 3.5a

$$= \frac{Abs\,(450-453)}{262} x \frac{1}{volume\ acetone(l)} x\ dilution$$

Equation 3.5b

3.6.3.3 HPLC analysis of β-carotene

For accurate measurement of carotenoids, especially β-carotene, the Schuep and Schierle (1995) HPLC method for quantification of astaxanthin from *Haematococcus* algae was adapted for β-carotene from *Dunaliella*. The method uses a non-aqueous mobile phases in conjunction with reversed phase columns.

The protocol for extraction and quantification of carotenoids is outlined in Appendix AIII. Total carotenoids were extracted using 100% ice cold acetone and sample was analysed using Beckman System Gold HPLC with a System Gold 168 detector. The Luna 3 μm analytical silica column was used in this analysis. A hexane/acetone isocratic (82:18 v/v) mixture was used as running solvent. A flow rate of 1.2 $mL.mn^{-1}$, 20 μl sample and ambient temperature was used. Twenty minutes running time was used with β-carotene eluting at around 1.4 minutes. β-carotene standards manufactured by Sigma-Aldrich were prepared fresh on the day of HPLC analysis and these were used to construct the standard curve for quantification of β-carotene in samples. The procedure for preparation of standards is also included in Appendix AIII.

3.6.4 Measurements of biochemical cell components (cell composition)

Further algal characterisation was done by measurement of biochemical components of both type culture *Dunaliella* species and environmental isolates. Massive accumulation of carotenoids is typically accompanied by changes in protein, carbohydrate and chlorophyll concentrations and cell size.

Carotenogenic cells were analysed for chlorophylls and carotenoids before and after massive carotenoid accumulation. Following extraction of these pigments, the remaining pellet was analysed for protein and carbohydrate content.

3.6.4.1 Measurements of chlorophylls and carotenoids

To measure the chlorophyll content, aliquots of 1 mL cultures of green and red cells in triplicate were harvested and treated for pigment extraction as described for the rapid spectrophotometric method using 100% acetone. Quantification of the pigments in the extracted sample was conducted according to Lichtenthaler (1987), using Equations 3.6 to 3.9.

$C_a = 11.24A_{661.6} - 2.04A_{644.8}$ **Equation 3.6**

$C_b = 20.13A_{644.8} - 4.19A_{661.6}$ **Equation 3.7**

$C_{a+b} = 7.05A_{661.6} + 18.09A_{644.8}$ **Equation 3.8**

$C_{x+c} = [(1000\ A_{470} - 1.90C_a - 63.14\ C_b)/214]$ **Equation 3.9**

where C_a is the concentration of chlorophyll a, C_b the concentration of chlorophyll b, C_{a+b} the concentration of chlorophyll a and b, and C_{x+c} the concentration of xanthophylls and carotenes (total carotenoids).

To determine the concentration of pigment in algal system, the ratio of sample volume and solvent volume are used. For example, the concentration of chlorophyll a in the algal suspension, chl a, is calculated as follows:

Chl a ($mg.L^{-1}$) = $C_a = 11.24A_{661.6} - 2.04A_{644.8}$ x acetone volume (mL)/sample volume (mL)

Equation 3.10

3.6.4.2 Protein Determination

The modified Lowry method (Chang *et al.*, 1994) using albumin bovine serum (BSA) as a standard was used for protein determination. The biuret reaction (reaction of copper ions with peptides bonds under alkaline conditions) combined with the reduction of the

Folin-Ciocalteau phenol reagent by tyrosine and tryptophan residues in the proteins produce a bluish colour that is quantified spectrophotometrically at 450 nm. The modified Lowry method is detailed in Appendix AIV.

3.6.4.3 Carbohydrate Determination

The phenol-sulphuric method (Mohammed and Rayas-Duarte, 1995) which measures total carbohydrate was used for determination of cellular carbohydrate. The method is based on the reaction of carbohydrate with phenol in the presence a strong acid, sulphuric acid. The acidic conditions lead to carbohydrate dehydration, forming furfural and hydroxyl methyl furfural compounds which condense with phenol resulting in yellow-orange colour. The colour can be read spectrophotometrically at 488 nm. A standard curve was prepared using glucose as a standard. The phenol-sulphuric acid method is detailed in Appendix AIV.

3.6.5 Nitrate and phosphate consumption by *Dunaliella*

In order to establish the effect of nutrient depletion on cell concentration during the growth phase, consumption of nitrate and phosphate was followed. The two nutrients implicated in induction of carotenogenesis..

3.6.5.1 Nitrate measurement

Nitrate in solution was determined according to Koroleff (1983) detailed in Appendix AII. Nitrate was first converted to nitrite using cadmium with copper sulphate in a column as a reducing agent in the presence of ammonium chloride (NH_4Cl). The nitrite formed reacts with sulphanilamide to form a compound which then reacts further with N-(1-naphthyl)-ethylenediamine dihydrochloride solution (NEDI) to form a red azo dye. This was measured at 543 nm.

3.6.5.2 Phosphate measurement

The phosphate in solution was determined according to Koroleff (1983) detailed in Appendix AII. The phosphate in the culture medium was reacted with a composite reagent comprising ammonium molybdate, sulphuric acid, ascorbic acid and potassium antimonyl-tartrate to form a phosphor-molybdate complex. This was reduced to a blue colour in the presence of ascorbic acid. The colour intensity was read at the wavelength of 885 nm.

3.7 Experimental design

3.7.1 Establishment of starting cell concentration

To establish the optimum starting cell concentration, three cell concentrations of *D. bardawil* UTEX 2538 were inoculated into Modified Johnson's Medium. The cultures were then grown at 1000 lux (13.5 μmol. $m^{-2}.s^{-1}$) light intensity and 25°C. Change in cell concentration was monitored for 11 days.

3.7.2 *Dunaliella* growth properties under varying growth conditions

Modified Johnson's (MJ (J/1)) medium and BAAM medium were compared with respect to biomass productivity using the type culture species *D. bardawil* UTEX 2538 and *D. salina* UTEX 1644.

Sodium Chloride (NaCl) at 2 M concentration, identified as optimum in Section 2.4, was used for both Modified Johnson's (MJ (J/1)) and BAAM media. Light system A was used for provision of continuous light intensity at 300, 1000, 1500 and 2500 lux (4.1, 13.5, 20.3 and 33.8 μmol. $m^{-2}.s^{-1}$). Temperature was set at two levels: 25°C and 30°C. Light system A was placed in a temperature-controlled laboratory to achieve 25°C temperature.

The light intensities at 30°C where achieved by placing the flasks in a constant temperature room.

3.7.3 Isolation and characterisation of salt evaporation ponds *Dunaliella* isolates

Following isolation and enrichment of the environmental *Dunaliella* isolates, studies were conducted. For the thesis, salt evaporation ponds samples were studied as mixed cultures of carotenogenic and non-carotenogenic cells representing most probable conditions in outdoor cultivation of *Dunaliella* using dual phase mode. The growth kinetics studies were conducted to establish the growth rate of the carotenoids species at set parameters. The presence of the non-carotenogenic species would affect the growth of the carotenogenic species, hence the inclusion of the studies on control of non-carotenogenic species.

The multivariate effects of light intensity, nitrate and sodium chloride concentration on growth of both carotenogenic and non-carotenogenic cells from environmental samples were evaluated. Light system B, providing 80 – 200 $\mu mol.m^{-2}.s^{-1}$ light intensity, was used for this section. A summary of these conditions is presented in Table 3.3.

Table 3.3: Summary of parameters used in kinetics studies of salt evaporating samples with BAAM medium

NaCl concentration (M)	KNO_3 concentration (mM)	Light intensity ($\mu mol.m^{-2}.s^{-1}$)	Temperature (°C)
2	0.5 (10% full nitrate)	80	32 ± 2
3	1.25 (25% full nitrate)	100	37 ± 2
	2.5 (50% full nitrate)	150	
	5 (100% full nitrate)	180	
		200	

Light intensity was evaluated at 80, 100, 150, 180 and 200 $\mu mol.m^{-2}.s^{-1}$. Potassium nitrate was evaluated at 0.5 mM (10% full nitrate), 1.25 mM (25% full nitrate), 2.5 mM (50% full nitrate) and 5 mM (100% full nitrate). The light intensities and nitrate concentrations were evaluated using BAAM medium containing 2 M and 3 M sodium chloride. Cultures were incubated at 32°C and 37°C. The growth studies were monitored by change in cell concentration.

3.7.4 Optimisation of carotenoid inducing conditions at laboratory scale

3.7.4.1 Factorial design and optimisation

A 3^3 factorial experiments were used to determine optimum conditions for of β-carotene biosynthesis. The following variables: nitrate concentration, sodium chloride (NaCl) concentration and light intensity were used at different levels as in table 3.4. Not all the sodium chloride could be dissolved at 5 M hence 4.5 M NaCl was used.

Table 3.4: Factorial design parameters for carotenoid biosynthesis

	Actual values		
Coded values	**Nitrate concentration (mM)**	**Sodium chloride concentration (M)**	**Light intensity ($\mu mol.m^{-2}.s^{-1}$)**
-1	0 (0% full nitrate)	3	100
0	0.5(10% full nitrate)	4	150
1	1.25 (25% full nitrate)	5	200

X1: nitrate concentration

X2: sodium chloride concentration

X3: light intensity

Twenty seven (27) experiments in triplicates were conducted and the average was calculated. Changes in cell concentration for both carotenogenic and non-carotenogenic species were monitored during the carotenoid biosynthesis phase. Carotenoids (β-carotene) were quantified at the end of carotenogenic phase. Excel was used to establish the regression equation used to determine the significant variables. Mathematical modelling was carried out by using equation 3.11 to obtain a second order polynomial equation (Armstrong and James, 1996).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_1^2X_{11} + b_2^2X_{22} + b_3^2X_{33} + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{123}X_1X_2X_3$$

Equation 3.11

Where Y is the dependent variable: cell concentration and β-carotene content in carotenoid biosynthesis studies. The b_0 is the intercept while the b_i (b_1, b_2 and b_3), b_{ij} (b_{12}, b_{23}, b_{13}) and b_{ijk} (b_{123}) are regression coefficients for the second order polynomial. X_i represents different levels of independent variables. Coefficients are an indication of how much each factor influences the independent variable. A regression equation was established by inputting regression coefficients into equation 3.11. To assess the observed values, the mathematical model derived from the coefficients was used to establish the predicted values. Error obtained was established between the two. The quality of the experiments was evaluated using the student "t" test and *p-value*.

Once the critical variables were identified, surface response methodology was used to determine the optimum β-carotene biosynthesis. β-carotene was presented as both cellular content and in volumetric form. Response surface methodology as a function of two factors at a time while maintaining the third variable constant was used to demonstrate main and interaction effects of the two variables.

3.7.5 Comparison of the carotenogenic property of different *Dunaliella* species

To test the established conditions for optimum β-carotene biosynthesis described in Section 3.7.5, the pooled samples isolates, the X2 isolates and *D. bardawil* UTEX 2538 were tested to evaluate the model. Cultures of 100 mL in 500 mL Erlenmeyer flasks plugged with cotton wool bungs were used as before. Cultures were incubated under continuous illumination of average light intensity 200 $\mu mol.m^{-2}.s^{-1}$ using light system B at 25°C with manual shaking once a day. Flasks were rotated through positions on the light box on daily basis to achieve average light intensity. Samples were taken on a daily basis for cell count estimation and quantification of carotenoids and β-carotene.

3.8 Conclusion

The isolates were evaluated in terms of growth and carotenoid biosynthesis. The effect of several parameters including light intensity, sodium chloride concentration, nitrate concentration and temperature were evaluated for the growth phase. For carotenoid biosynthesis, factorial experiments were used to determine the optimal parameters and ranges for production of natural β-carotene. The parameters included high levels of NaCl, nitrate deprivation and light intensity. During the course of the studies, the productivity of the environmental isolates was compared with that of the commercial species. Further, for environmental isolates, studies were conducted to evaluate measures for control of non-carotenogenic species using the mentioned parameters. Total carotenoids were estimated using spectrophotometric methods while the HPLC method was used for more accurate quantification of β-carotene.

CHAPTER 4: CULTIVATION OF *Dunaliella bardawil* and *Dunaliella salina* –ESTABLISHMENT OF THE SYSTEM

4.1. Introduction

Laboratory scale studies of *Dunaliella* species sourced from the type culture collection are reported in this chapter in order to define operating systems. Through establishing growth kinetics and *Dunaliella* productivities, under varying conditions, the type culture species form a positive control for environmental isolate studies. The chapter focuses on biomass production. The effects of starting cell concentration, media, light intensity and temperature on *Dunaliella* growth were evaluated and growth was monitored using maximum specific growth rate, μ_{max}, maximum cell concentration attained, $X_{max,}$ and extent of growth, X_{max}/X_0.

Establishment of starting cell concentration is covered in Section 4.2.1. The synergistic effects of culture media, light intensity and temperature on *D. bardawil* UTEX 2538 are investigated in Section 4.2.2. Following establishment of favourable growth conditions using the *D. bardawil*, a comparison is made between the performance of *D. bardawil* UTEX 2538 and *D. salina* UTEX 1644 in BAAM and MJ (J/1) media in Section 4.2.3. Discussions are covered in Section 4.3.

4.2 Studies on key parameters affecting growth of *Dunaliella* species

Starting cell concentration effects on *Dunaliella* growth was established under univariate experimental design. Selection of media, effects of light intensity and temperature on *Dunaliella* growth profiles were conducted via multivariate experimental design. Thereafter, species performance was compared.

4.2.1 Establishment of optimum starting cell concentration for *Dunaliella bardawil* UTEX 2538

The effect of inoculum on the rate and extent of growth of *D. bardawil* was evaluated using Modified Johnson's Medium (MJ (J/1)) at 25°C and continuous light intensity of 1000 lux (approximately 13.5 $\mu mol.m^{-2}.s^{-1}$) using light system A. Studies on the optimum starting biomass concentration were conducted at three levels: 1%, 5% and 10% (v/v) inoculum yielding initial cell concentrations of $2.2x10^4$, $11x10^4$ and $22x10^4$ cells.mL^{-1} respectively. Studies were done in duplicate.

Working volumes of 100 mL of MJ (J/1) medium containing 2 M NaCl in 500 mL Erlenmeyer flasks were used. The flasks were kept monoseptic with cotton wool bungs. Cultures were shaken manually twice a day for 11 days. During incubation, cultures were rotated on the light shelf to average the light intensity reaching the cultures. Aliquots of 1 mL were taken from the well mixed culture at given times for cell count estimation. The cell count as a function of batch growth time is shown in Figure 4.1. The experimental points are connected as a guide to the eye and are not fitted to a model. The final cell concentration, X_{max}, the maximum specific growth rate, μ_{max} and the extent of growth, X_{max}/X_0 were determined from the cell count and are compared in Figure 4.2.

Figures 4.1 and 4.2a show that the higher starting cell concentration supports a higher final cell concentration, X_{max} within 11 days. The 10% inoculum gave the highest X_{max} of 91.5 ± 3.5 x10^4 cells.mL^{-1}, followed by the 5% inoculum at 76.0 ± 15.6 x10^4 cells.mL^{-1} and 1% inoculum at 38.0 ± 8.5 x 10^4 cells.mL^{-1}. The latter did not reach stationary phase in 11 days. The ANOVA analysis gave a *p-value* < 0.05 indicating that the starting cell concentration was significant for optimal cell concentration

The specific growth rate, shown in Figure 4.2a, was highest at 5% (v/v) inoculum at 0.23 ± 0.02 day^{-1}, followed by 0.21 ± 0.04 day^{-1} obtained for the 1% inoculum. The

lowest specific growth rate was 0.16 ± 0.01 day^{-1} attained for the 10% inoculum. The slightly lower specific growth rate at 10% could be attributed to increased shading. According to Richmond (2004), in high cell density cultures, cell density becomes the most dominant factor in determining cell culture performance. With a *p*- value > 0.05, the ANOVA analysis indicated no significant difference between the growth rates at the given starting cell concentration.

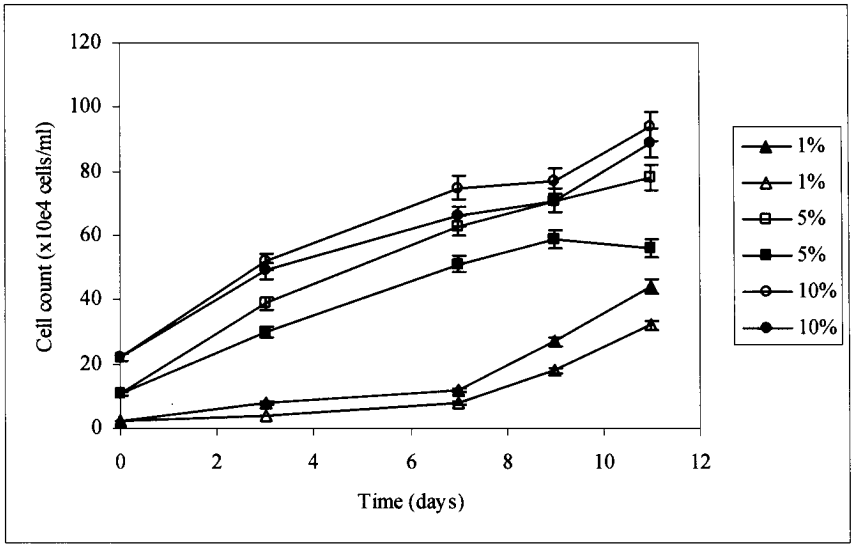
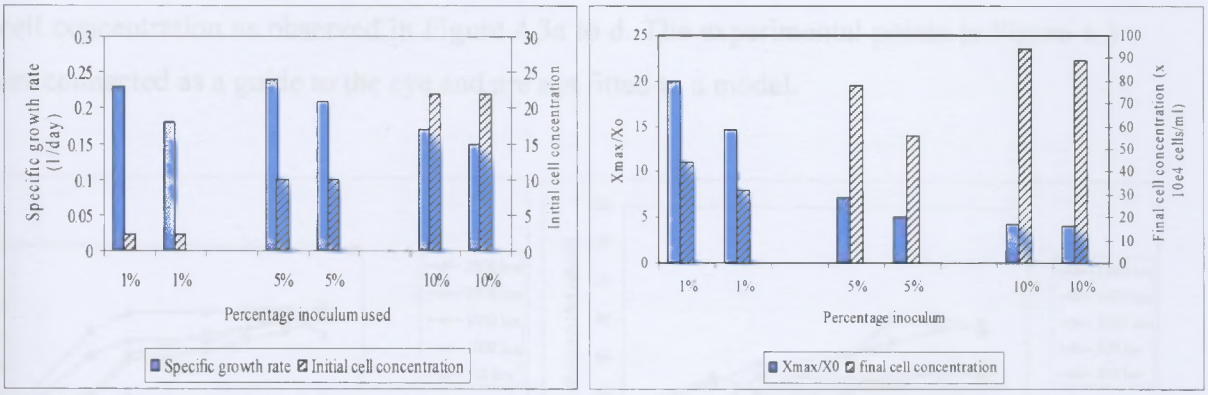


Figure 4.1: Growth profiles as a function of time and inoculum cell concentration for *D. bardawil* in Modified Johnson's Medium at 1000 lux (13.5 µmol.m^{-2}.s^{-1} light intensity) and 25°C.

The extent of growth was the highest at inoculum of 1% at 17 fold where the maximum cell concentration was the lowest, 38.0 ± 8.5 x 10^4 cells.mL^{-1}. The lowest extent of growth was observed at 10% inoculum at 5 fold where the highest maximum cell concentration of 91.5 ± 3.5 x10^4 cells.mL^{-1} was reached. At *p-value* < 0.05, the ANOVA analysis confirmed the significance of the starting concentration on the extent of growth, $X_{max}/X_{0.}$

From the results, it is deduced that the highest maximum cell concentration, 91.5 ± 3.5 x 10^4 cells.mL^{-1} attained at 10% inoculum was due to the high starting cell

concentration and not a high specific growth rate. This is shown by the low extent of growth of 5 fold. The 1% inoculum had the highest extent of growth of 17 fold. This could be accounted for by favourable conditions and abundant light intensity experienced at low algal concentrations and negligible self shading or sufficient CO_2. The high rate of growth did not yield the highest final cell concentration. From this work, a 10% inoculum was selected for the remaining studies for production of maximal cell concentration.



(a) (b)

Figure 4.2: Effect of inoculum concentration on the extent of growth of *D. bardawil* in Modified Johnson's Medium at light intensity of 1000 lux (approximately 13.5 $\mu mol.m^{-2}.s^{-1}$) and 25°C measured as X_0, X_{max}, μ_{max} and X_{max}/X_0.

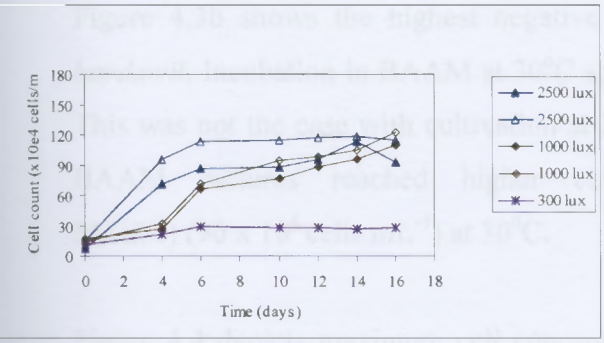
a: X_0 and μ_{max} b: X_{max}/X_0 and X_{max}

4.2.2 Effects of culture media, light intensity and temperature on growth profile of *Dunaliella bardawil* UTEX 2538

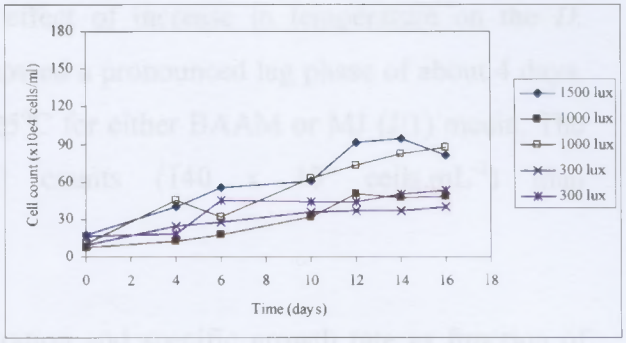
Working volumes of 100 mL using MJ (J/1) and BAAM culture media were inoculated with 10% starting cell culture for maximum productivity. Flask preparation and treatment at incubation conditions were as presented in Section 4.2.1. Light system A was used for provision of continuous light intensity at 300, 1000, 1500 and 2500 lux (4.05, 13.5, 20.25 and 33.75$\mu mol.m^{-2}.s^{-1}$). Temperature at 25°C and 30°C was used. Incubation at 25°C was achieved by placing light system A in a temperature-controlled laboratory. The light

intensities at 30°C were achieved by placing the flasks in a constant temperature room. The cultures were observed over 16 days.

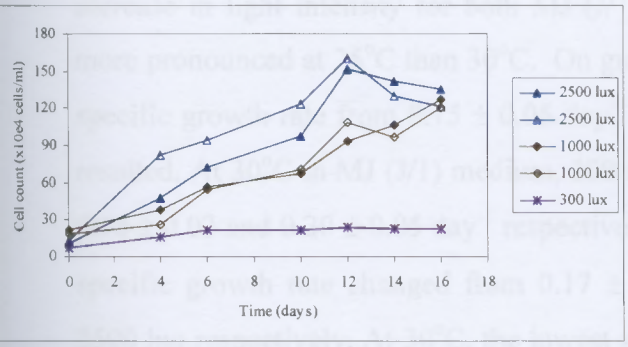
Figure 4.3 shows the growth profiles of *D. bardawil* under synergistic effects of light intensity, media and temperature. The figure also shows the growth kinetics of the alga and the final concentrations attained at indicated incubation conditions. The term 'maximum' cell concentration was introduced instead of the 'final' cell concentration as there were occurrences of cell concentration reduction following attainment of maximum cell concentration as observed in Figure 4.3a to d. The experimental points in Figure 4.3 are connected as a guide to the eye and are not fitted to a model.



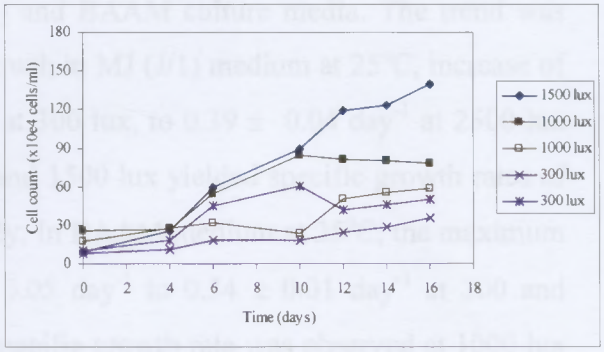
(a)



(b)



(c)



(d)

Figure 4.3: Evaluation of multiplicative effects of varying light intensity (300 to 2500 lux) and temperature on growth kinetics of *D. bardawil* in BAAM and MJ (J/1) media using 10% inoculum.

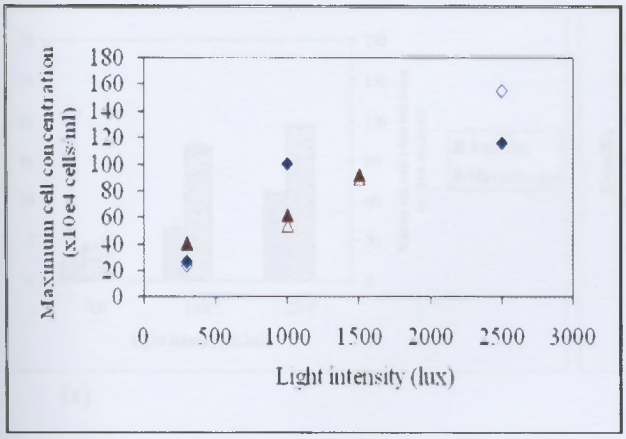
a) Modified Johnsons medium, 25°C **b) Modified Johnsons medium, 30°C**
c) BAAM medium, 25°C **d) BAAM medium, 30°C**

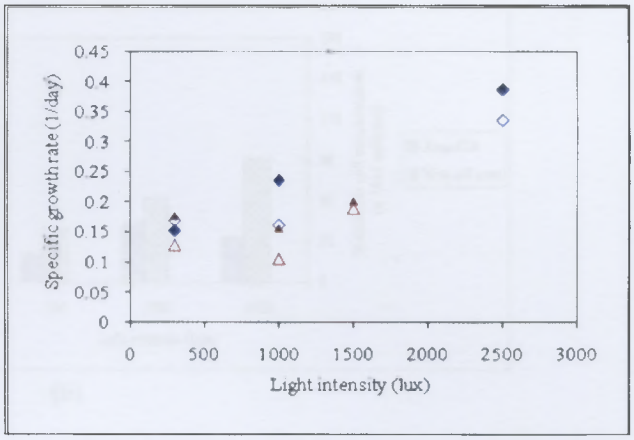
Influence of light intensity can be observed from Figures 4.3a to d. The highest maximum cell concentration was achieved at higher light intensities: 119 x 10^4 cells.mL^{-1} attained under 2500 lux at 25°C in MJ (J/1) medium in Figure 4.3a and 160 x 10^4 cells.mL^{-1} under 2500 lux at 25°C in BAAM in Figure 4.4c.

At 30°C, cell concentrations of 90 x 10^4 cells.mL^{-1} and 140 x 10^4 cells.mL^{-1} were achieved in MJ (J/1) medium (Figure 4.3b) and BAAM medium (Figure 4.3d) respectively, under light intensity of 1500 lux.

The comparison of results at 25°C and 30°C indicates higher maximum cell concentrations at 25°C in both media with cell increasing with increasing light intensity. Figure 4.3b shows the highest negative effect of increase in temperature on the *D. bardawil*. Incubation in BAAM at 30°C showed a pronounced lag phase of about 4 days. This was not the case with cultivation at 25°C for either BAAM or MJ (J/1) media. The BAAM cultures reached higher cell counts (140 x 10^4 cells.mL^{-1}) than MJ (J/1) (90 x 10^4 cells.mL^{-1}) at 30°C.

Figure 4.4 depicts maximum cell concentration and specific growth rate as function of light intensity. Increase in the maximum specific growth rates was observed with increase in light intensity for both MJ (J/1) and BAAM culture media. The trend was more pronounced at 25°C than 30°C. On growth in MJ (J/1) medium at 25°C, increase of specific growth rate from 0.15 ± 0.05 day^{-1} at 300 lux, to 0.39 ± 0.04 day^{-1} at 2500 lux resulted. At 30°C in MJ (J/1) medium, 300 and 1500 lux yielded specific growth rates of 0.18 ± 0.03 and 0.20 ± 0.05 day^{-1} respectively. In BAAM medium at 25°C, the maximum specific growth rate changed from 0.17 ± 0.05 day^{-1} to 0.34 ± 0.01 day^{-1} at 300 and 2500 lux respectively. At 30°C, the lowest specific growth rate was observed at 1000 lux at 0.10 ± 0.01 day^{-1}. A slight increase in the growth rate was noted at 300 lux resulting in the value of 0.12 ± 0.07. Light intensity of 1500 lux resulted in the highest specific growth rate of 0.19 ± 0.05 day^{-1} at 30°C.





(a) **(b)**

Figure 4.4: Evaluation of multiplicative effects of varying light intensity (300 to 2500 lux) and temperature on growth kinetic indicators of *D. bardawil* in BAAM and MJ (J/1) media using 10% inoculum, Maximum cell concentration and maximum specific growth rate as function of light.

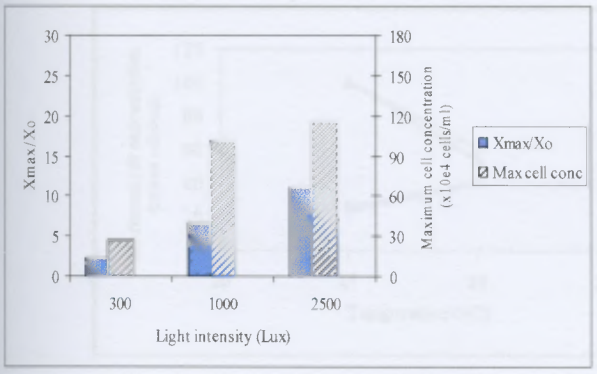
▲ **MJ (J/1) 30°C** ◆ **MJ (J/1) 25°C**

△ **BAAM 30°C** ◇ **BAAM 25°C**

a) Maximum cell concentration **b) Maximum specific growth rate**

Growth properties of *D. bardawil* were compared in MJ (J/1) and BAAM media at same light intensities (300 to 1500 lux) and temperatures (25°C and 30°C). Higher maximum cell concentration of 160 x 10^4 cells.mL^{-1} was obtained with BAAM at 25°C, compared to 120 x 10^4 cells.mL^{-1} at 25°C for MJ (J/1) medium.

In Figure 4.5, the interaction between temperature and light intensity is observed for each media. At high light intensities (1000 and 2500 lux), the maximum cell concentration decreased with increase in temperature. A reduction from 100 to 54 x 10^4 cells.mL^{-1} (BAAM) and 100 to 61 x 10^4 cells.mL^{-1} (MJ (J/1)) were observed. However at 300 lux (4.05µmol.m^{-2}.s^{-1}), a temperature increase led to increase in maximum cell concentration from 24 to 40 x 10^4 cells.mL^{-1} (BAAM) and an increase from 48 to 62 x 10^4 cells.mL^{-1} (MJ (J/1)) was noted (Figure 4.5a).



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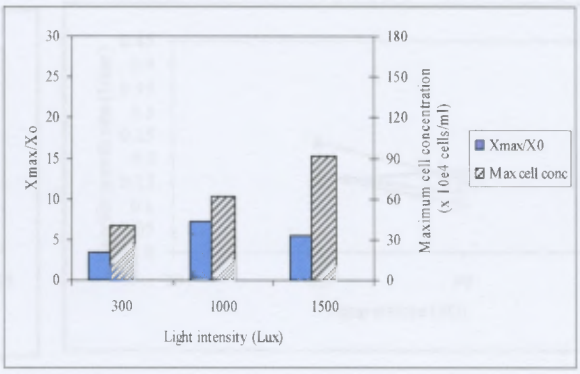

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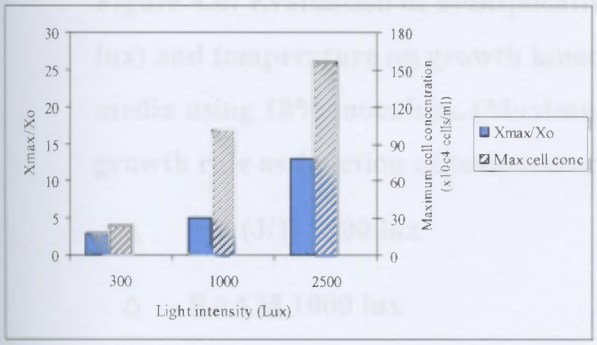

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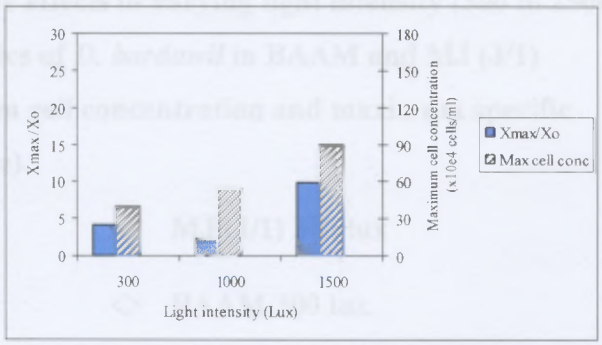


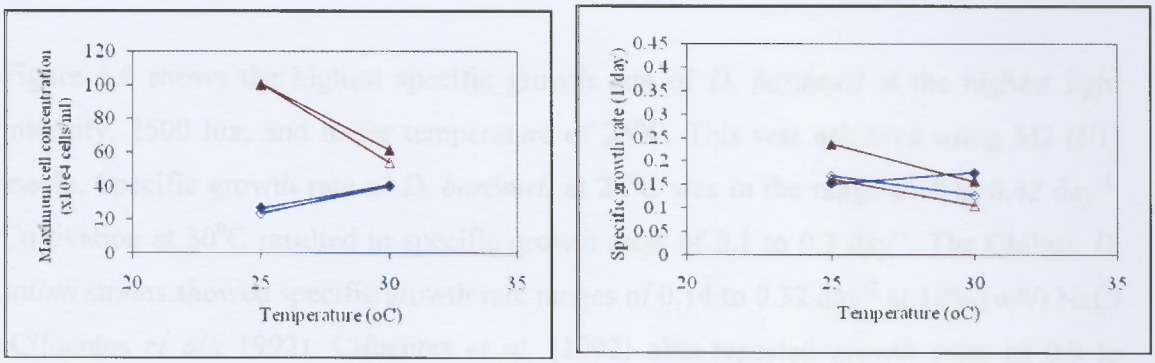
(d)

Figure 4.5: Evaluation of multiplicative effects of varying light intensity and temperature on the extent of growth of *D. bardawil* in BAAM and MJ (J/1) media using 10% inoculum

(a) MJ (J/1) at 25^{0}C **(b) MJ (J/1) at 30^{o}C**

(c) BAAM at 25^{o}C **(d) BAAM at 30^{o}C**

The maximum growth rate decreased with increase in temperature for MJ (J/1) at 1000 lux, and BAAM at both 1000 and 300 lux. The specific growth rate in MJ (J/1) at 300 lux was largely unchanged between 25 and 30^{o}C (Figure 4.5b). This relationship is also viewed in Figure 4.6. In this case, the experimental points are also connected as a guide to the eye and are not fitted to a model.



a) b)

Figure 4.6: Evaluation of multiplicative effects of varying light intensity (300 to 2500 lux) and temperature on growth kinetics of *D. bardawil* in BAAM and MJ (J/1) media using 10% inoculum. (Maximum cell concentration and maximum specific growth rate as function of temperature).

▲ **MJ (J/1) 1000 lux** ◆ **MJ (J/1) 300 lux**

△ **BAAM 1000 lux** ◇ **BAAM 300 lux**

a) Maximum cell concentration **b) Maximum specific growth rate**

The maximum cell concentrations attained in this part of the project ranged from around 20 to 160 x 10^4 cells.mL^{-1} corresponding to lowest light intensity (300 lux) and highest light intensity (2500 lux) respectively. These values, at the higher end, are in the same range as those reported by Cifuentes *et al.* (1992) for the Chilean *Dunaliella salina* Teodoresco of 67 to 174 x 10^4 cells.mL^{-1}. The algae were cultivated in J/1 medium at light intensity of 60 µmol. m^{-2}.s^{-1} (4440 lux) under a 12:12 (Light:Dark) ratio photo-period at 20°C. The Chilean *D. salina* is carotenogenic (Cifuentes *et al.*, 1992).

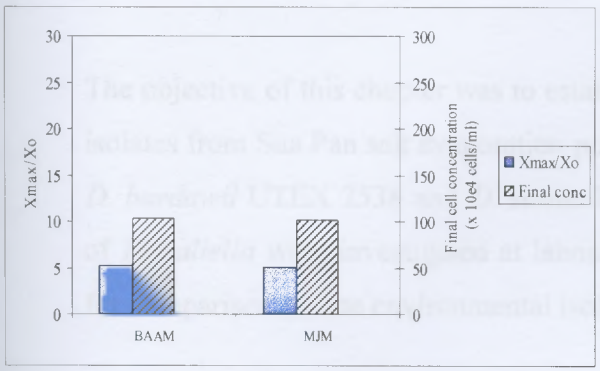
Investigations into the growth cycle of *D. bardawil* under the stipulated conditions showed that the exponential growth phase at 25°C in MJ (J/1) medium occurred from day 0 to 6, reaching a stationary phase at day 6 as indicated in Figure 4.3a. Cultivation in BAAM at 25°C, as shown in Figure 4.3c, showed continued growth to day 12 following the exponential phase from day 0 to 6. The linear nature of this growth suggested limitation of the culture through either the supply of light or of carbon source.

Figure 4.6 shows the highest specific growth rate of *D. bardawil* at the highest light intensity, 2500 lux, and lower temperature of 25°C. This was achieved using MJ (J/1) media. Specific growth rate of *D. bardawil* at 25°C was in the range 0.15 to 0.42 day^{-1}. Cultivation at 30°C resulted in specific growth rates of 0.1 to 0.2 day^{-1}. The Chilean *D. salina* strains showed specific growth rate ranges of 0.14 to 0.32 day^{-1} at 10% (w/v) NaCl (Cifuentes *et al.*, 1992). Cifuentes *et al.* (1992) also reported growth rates of 0.9 to 1.0 day^{-1} under continuous aeration illustrating the need for good CO_2 mass transfer. The cultivation of *D. salina* isolates from Thailand in 10% NaCl resulted in growth rates of 0.13 to 0.36 day^{-1} (Powtongsook *et al.*, 1995).

4.2.3. Performance of *Dunaliella bardawil* and *Dunaliella salina* in MJ (J/1) and BAAM media

Following establishment of growth conditions, comparison of *D. bardawil* UTEX 2538 and *D. salina* UTEX 1644 growth was conducted. A higher light intensity in the range 30 to 50 $\mu mol.m^{-2}.s^{-1}$ (2220 to 3700 lux) was provided by light system A. A working volume of 100 mL in 500 mL Erlenmeyer flasks maintained monoseptic with cotton wool bungs was used for the two media and the cultures were incubated in a room set at 25°C. A 10% inoculum was used.

The final cell concentration and the extent of growth for *D. salina* were higher than those for *D. bardawil* in the both BAAM and MJ (J/1) media under the conditions already described, shown in Figure 4.7a and 4.7b. The average for the final cell concentration of *D. salina* were 192 x 10^4 $cells.mL^{-1}$ and 162 x 10^4 $cells.mL^{-1}$ in BAAM and MJ (J/1) respectively compared to 102 x 10^4 $cells.mL^{-1}$ and 103 x 10^4 $cells.mL^{-1}$ in BAAM and MJ (J/1) respectively for *D. bardawil*. The extent of growth for *D. salina* were 7.0 and 10.2 in BAAM and MJ (J/1) respectively while values of 5.2 and 5.1 were reported for *D. bardawil* in BAAM and MJ (J/1) medium.



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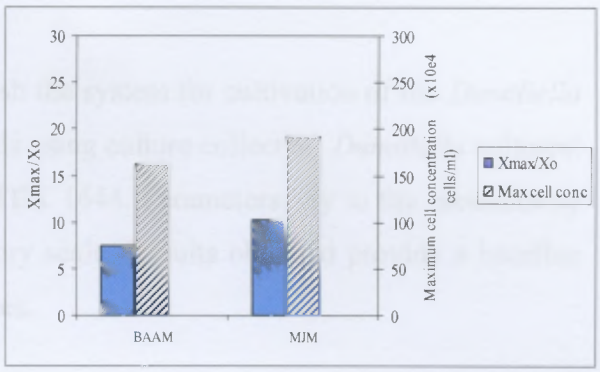

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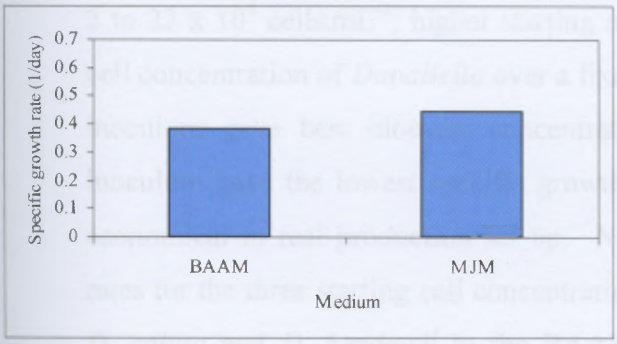

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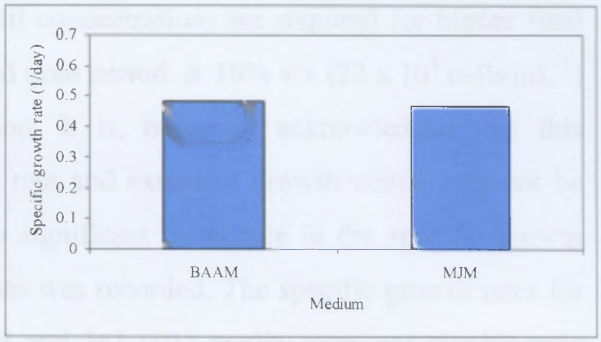


d)

Figure 4.7: Evaluation of multiplicative effects of varying light intensity and temperature on the extent of growth of *D. bardawil* and *D. salina* in BAAM and MJ (J/1) media using 10% inoculum, 30 to 50 µmol.m^{-2}.s^{-1} Light intensity at 25°C.

a) *D. bardawil* in BAAM and MJ (J/1) **b) *D. salina* in BAAM and MJ (J/1)**

c) *D. bardawil* in BAAM and MJ (J/1) **d) *D. salina* in BAAM and MJ (J/1)**

The specific growth rates of *D. bardawil* in BAAM and MJ (J/1) media were 0.39 ± 0.04 day^{-1} and 0.44 ± 0.01 day^{-1} respectively under the set conditions depicted in Figure 4.7c, compared with values of 0.48 ± 0.07 day^{-1} in BAAM media and 0.47 ± 0.19 day^{-1} in MJ (J/1) media for *D. salina*, as shown in Figure 4.7d. Comparison of the specific growth rates of *D. bardawil* and *D. salina* showed, using an ANOVA evaluation, insignificant difference. The corresponding extent of growth for *D. salina* (7.40 ± 1.30 (BAAM) and 6.72 ± 1.29 (MJ (J/1)) was also marginally higher than that of *D. bardawil* (5.92 ± 1.27 (BAAM) and 5.43 ± 0.31 (MJ (J/1)).

4.3 Discussion and conclusion

The objective of this chapter was to establish the system for cultivation of the *Dunaliella* isolates from Sua Pan salt evaporation ponds using culture collection *Dunaliella* cultures: *D. bardawil* UTEX 2538 and *D. salina* UTEX 1644. Parameters key to the productivity of *Dunaliella* were investigated at laboratory scale. Results obtained provide a baseline for comparison for the environmental isolates.

Section 4.3.1 showed that, on using an inoculum concentration in the range 2 to 22 x 10^4 cells.mL^{-1}, higher starting cell concentrations are required for higher final cell concentration of *Dunaliella* over a fixed time period. A 10% v/v (22 x 10^4 cells.mL^{-1}) inoculum gave best biomass concentration. It is, however, acknowledged that this inoculum gave the lowest specific growth rate and extent of growth which may not be economical in real production set up. No significant difference in the specific growth rates for the three starting cell concentrations was recorded. The specific growth rates for *D. salina* and *D. bardawil* in the BAAM and MJ (J/1) media were not significantly different hence either medium could be used for cultivation of *Dunaliella*. BAAM medium was chosen for the extended study. Higher light intensities (2500 to 3700 lux) support *Dunaliella* growth better than lower light intensities (300 lux). The two *Dunaliella* species grew better at 25°C than at 30°C. The conditions in this section gave specific growth rates in the ranges of 0.39 to 0.48 day^{-1} which compare to literature values (Table 2.3).

Studies on multiplicative effects of light intensity, temperature and the medium used showed that the *D. bardawil* grows better at 25°C than at 30°C and at high light intensities (2500 lux) when selected from 300 to 2500 lux. In Section 4.3, it was seen that even higher light intensities of 30 to 50 μmol.m^{-2}.s^{-1} (2220 to 3700 lux) supported higher growth rates of *D. bardawil* and *D. salina*. The specific growth rates attained in the study are in the range of those reported in literature.

The growth cycle was studied to determine the period at which to transfer *Dunaliella* biomass to carotenogenesis-inducing conditions as done in dual mode of *Dunaliella* cultivation. The *Dunaliella* growth was followed for a period of maximum of 16 days. From these studies it was observed that transfer to carotenoid biosynthesis inducing conditions can be carried out around day 6 when higher light intensities are used as observed with 2500 lux and MJ (J/1) cultures in Fig 4.3a where cultures did not have a pronounced lag period. This is the termination of the exponential phase of *Dunaliella* after which stationary growth suggests Carbon or light limitation and a reduced growth rate. While incubation of *Dunaliella* in BAAM medium at 2500 lux light intensity did not result in cultures reaching stationary phase, the cultures at 2500 lux had reached higher cell concentrations on day 6 compared cultures at lower light intensities (300 to 1500 lux). Transfer to carotenoid inducing conditions at day 6 is in agreement with the findings by Phillips (1994). The carotenogenesis phase of *Dunaliella* requires much higher light intensities and lower cell concentrations. These conditions should be accounted for when determining when to transfer the biomass to the stressing conditions for biosynthesis of carotenoids.

CHAPTER 5: CHARACTERISTICS OF SUA PAN SALT EVAPORATION PONDS BRINES AND THE *Dunaliella* ISOLATES

5.1 Introduction

Chapter 5 discusses the composition of Sua Pan brines and conditions in the solar saltworks evaporation ponds. Factors influencing the occurrence of β-carotene rich *Dunaliella* species in such environments are emphasised. The chapter also focuses on the morphological identification and enrichment of these environmental isolates.

Section 5.2 presents the background of Sua Pan brines. The geographic location and occurrence of the brines are presented in Section 5.3. The characterisation of the Sua Pan solar evaporation ponds in Section 5.4 describes the location of the saltworks, the existing climatic conditions at the Sua Pan solar saltworks and the pond system. The *Dunaliella* isolates are characterised in Section 5.5. Section 5.6 reviews separation of the two isolates. Section 5.7 provides the chapter conclusions.

5.2 Background on Sua Pan brines

Exploitation of Sua Pan brines for natural commercial production of sodium carbonate (soda ash) and sodium chloride (common salt) dates back to the 1950s (Gould, 1986). A series of geological and chemical studies at the time revealed abundant supplies of sodium salts including common salts (NaCl), soda ash (Na_2CO_3) and salt cake (Na_2SO_4). Traces of other chemical compounds included muriate of potash (KCl), bromine (Br_2) and lithium chloride (LiCl). The ionic and chemical compositions of the brines, based on Gould (1986), are presented in Table 5.1.

Brine reserves occurred primarily at two points: the aquifer and the overlaying sand/clay sequence. At the time quantities were estimated at 8 013 million m^3 in the aquifer and

5 502 million m^3 in the overlying sand/clay sequence (Gould, 1986). The reserves are expected to run the Botswana Ash soda ash producing plant, operating at full capacity, to 2020 (Botswana Ash 1998).

Table 5.1: Chemical composition [4]of Sua Pan Brines in wells. Adapted from Gould (1986).

Ions	Concentration (M)	Compounds	Concentration (M)
Na^+	2.93	NaCl	2.19
K^+	0.06	KCl	0.06
Cl^-	2.25	Na_2SO_4	0.10
CO_3^-	0.22	Na_2CO_3	0.22
HCO_3^-	0.11	$NaHCO_3$	0.11
SO_4^{2-}	0.10		

Information in Table 5.1 was obtained by averaging the chemical compositions from several wells of the Sua Pan. Gould (1986) noted that the brines from different areas of the pan were similar in composition but not identical.

5.3 Geographic location and occurrence of brines

Sua Pan is located in the Makgadikgadi pan in the north-eastern part of Botswana at 20°40'S and 25°45'S (Figure 5.1). Makgadikgadi salt pan is a result of an ancient lake that resulted in a series of arid and pluvial periods about 100 000 years ago. The ultimate drying up of Lake Makgadikgadi resulted in alkaline brines rich in sodium compounds that exist in the aquifer in the internal basin drainage (Gould, 1986).

[4]Data was obtained by averaging the chemical composition from Sua Pan wells. Gould (1986) noted the brines from different pan areas were similar in composition but not identical.

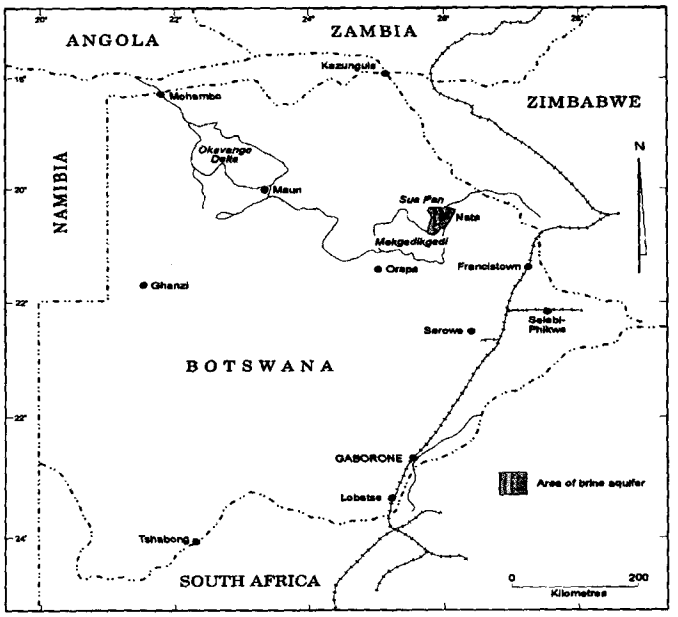


Figure 5.1: Map of Botswana showing the location of Sua Pan at the Makgadikgadi area (Gould, 1986)

5.4 Characteristics of the Sua Pan solar salt evaporation ponds

5.4.1 Location of the Sua Pan solar salt works

Following investigations on potential exploitation of the Sua Pan brines for commercial purposes, Soda Ash, was formed in 1991 for natural production of soda ash (Na_2CO_3) with common salt (NaCl) as a byproduct and was later transformed to Botswana Ash (BotAsh) (PTY) Ltd. The company is located in Sua Pan in the north-eastern part of Botswana (Masemola, 1999).

Like most of the solar salt works around the world, the Sua Pan soda ash and salt producing plant is located in the area with ease access to brine. Conditions which favour optimal evaporation of brines or seawater for salt crystallisation and concentration of compounds of interests are also key determinants for location identification (Gould, 1986; Garcia and Acquah, 1993; Masemola, 1999). High light intensities, high

temperatures and relatively scarce annual rainfall are desired for evaporation of the brines (Garcia and Acquah, 1993).

The brine in the salt evaporation ponds is pumped from the wells situated 20 km from the ponds. These wells are in the wellfield situated in the northern part of Sua Pan which covers an area of approximately of 200 km^2. The brine is concentrated and evaporated in a series of evaporating ponds using the natural environmental conditions discussed in Section 5.4.3 (Masemola, 1999).

5.4.2 Climatic conditions at the Sua Pan solar salt works

The climate of Botswana is arid to semi-arid (Gould, 1986; Jain *et al.*, 2003) with a short dry winter from May to August and a longer summer from September to April (Gould, 1986). The country is one of the sunniest countries in Southern Africa with little cloud cover, low humidity and very low wind speed through the year for most parts of the country (Jain *et al.*, 2003). The annual rainfall is on average 400 mm (Gould, 1986; Meteorological Services, Botswana, 2003). The rains start around October/November and increase with intensity till December from which it declines, stopping around March/April. The average highest rainfall for peak months is around 100 mm (Gould, 1986). It should be noted, however, that rainfall varies over the years (Gould, 1986: Meteorological Services, Botswana, 2003). The winds are light to variable except from August/October when steady easterly winds prevail. The effects of the temperature and the wind lead to fresh water evaporation rates of around 100 to 200 mm per month. The lower rate of evaporation is experienced at lowest temperatures in July and the higher rate in October (Gould, 1986).

The average temperatures vary with the time of the year with average maximum temperatures ranging from 23°C in July to 32°C (extremes of 38°C) in January and average minimum ranging from 5°C in July to 18°C in January. Frost can occur in winter, especially during June and July (Gould, 1986; Masemola, 1999).

5.4.3 Sua Pan solar salt evaporation ponds system

Brine is pumped into a series of shallow ponds and is retained in each pond for a certain period to reduce the water content. The brine evaporation ponds are arranged to crystallise different salts or impurities that occurs with evaporation. Evaporation and brine flow results in gradients of brine constituents with brine flow in the various ponds.

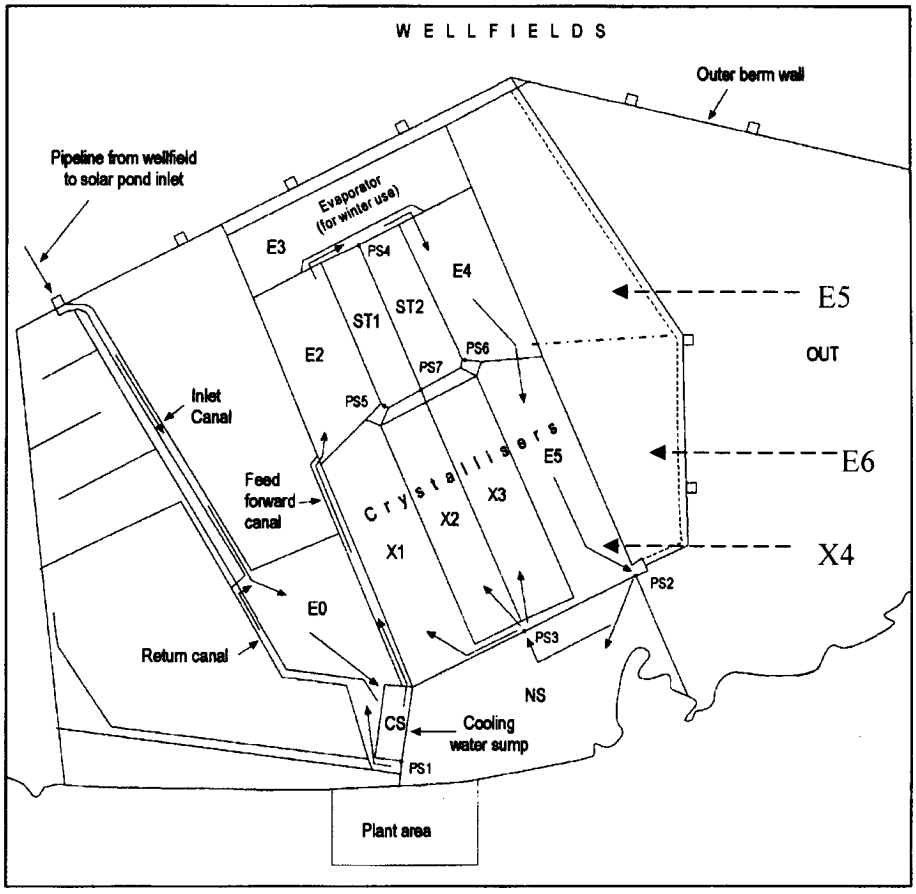


Figure 5.2: The layout of the solar evaporating ponds (Masemola, 1999). Changes to the original layout are marked in blue of E5, E6 and X4 as per Botswana Ash, 1998).

The arrangements of ponds at the Sua Pan Saltworks in the 1990s is shown in Figure 5.2. Some modification has occurred as indicated in the diagram. The brine is first pumped into E0, from the wellfields. E0 also serves as a cooling sump from the processing plant as well as collection point for the influent. The brine from the plant is cooled by mixing

with the influent brine and passed into the deep pond CS. From here, the brine is passed to the evaporation ponds E2, E3, E4, E5 and E6 for concentration. At these ponds, the brine evaporates till the salt reaches saturation and starts to crystallise out of solution. This brine is called N-brine and it is stored in NS ponds to be fed to the crystalliser ponds X1, X2, X3 or X4, depending on the number of crystallisers being used at the time, where NaCl deposition on the pond floor occurs. Also, further evaporation, resulting in further enrichment of the other sodium salt, takes place in the crystalliser ponds. The brine remains in the crystalliser ponds until just prior to deposition of saturated secondary salts which may contaminate the NaCl product. At this stage, the brine is rich in sodium carbonate and is called T-brine (trauna). Natural soda ash (sodium carbonate) is deposited as trauna ($NaCO_3.NaHCO_3.2H_2O$). The T-brine is stored at the two storage ponds, ST1 and ST2 where it is kept for an average of 50 days for further evaporation. The sodium chloride is harvested from the crystalliser for processing and refining. The brine is then pumped to the processing plant (Botswana Ash, 1998). The arrangement of the ponds used may be adjusted by controlled removal or additions of some areas to allow for changes in climatic seasons.

5.4.3.1 Chemical composition across the evaporation ponds system

The changes in chemical composition of the brines caused by evaporation, adapted from Masemola (1999), are illustrated in Table 5.2. The data shows a monthly summary for July 1992. Most significant are the increases in concentrations of the sodium chloride, sodium carbonate and potassium chloride. Sodium chloride increased from 2.14 M at the inlet to 3.58 M at T-brine holding ponds. Sodium sulphate and sodium bicarbonate concentration also increase but at a lower degree than when compared to NaCl.

Table 5.2 shows increases in concentration of the major brine compounds: soda ash (Na_2CO_3), salt cake (Na_2SO_4), salt (NaCl) and potash (KCl) with evaporation as the brine runs from the inlet through the cooling sump (CS), principal evaporating pond (E0), storage of NaCl saturated brine (NS), one of the crystalliser pond (X2) and the trauna brine (T-brine).

Table 5.2: Major compounds in the solar ponds. Adapted from Masemola (1999).

Location	NaCl (M)	KCl (M)	Na_2SO_4 (M)	Na_2CO_3 (M)	$NaHCO_3$ (M)
Inlet	2.14	0.03	0.10	0.21	0.11
CS	2.44	0.04	0.11	0.23	0.16
E0	2.64	0.05	0.10	0.26	0.17
NS	3.67	0.07	0.20	0.35	0.17
X2	3.69	0.09	0.24	0.40	0.16
T-Brine	3.58	0.11	0.26	0.40	0.16

In addition the inlet contains the following: Phosphorus: 1.6 mM; Nitrogen: 0.5 mM; Sulphide: 0.09 mM (Masemola 1999). Consistency is observed in the brine composition entering the ponds in 1986 and 1992, based on the concentrations of the key compounds in Table 5.1 and the inlet concentration in Table 5.2.

5.4.3.2 Biological activity in the solar salt evaporation ponds

The gradient in chemical composition across the evaporation ponds influences the biological population in the ponds. Shortly after the establishment of the Soda Ash plant, challenges were encountered with soda ash and common salt quality resulting from the biological system of the ponds. The halotolerant algal genus *Dunaliella*, halophilic and halotolerant bacteria as well as archaea were the primary suspects. *Dunaliella* species were identified as one of the primary photosynthetic producers in saline and hypersaline environments. It was thought that both alga and bacteria produced organic compounds that affected the quality of soda ash and NaCl produced negatively. However, the presence of these organisms aids in the absorption of solar energy thus aiding in the evaporation processes in the ponds. The presence of these organisms, is thus, desired up to certain levels beyond which they impact on the products negatively (Masemola, 1999; Botswana Ash, 1998).

The biological management of the solar salt evaporation ponds forms a crucial aspect of the operations of the plant to maintain the required quality of the products. The presence

of *Dunaliella* species at Sua Pan salt evaporation ponds is currently monitored as part of biological management of the evaporation ponds. The interest in the organism is centred on quality control of the products of the plant.

Studies on the behaviour of *Dunaliella* species in the natural environment was based on information obtained from Botswana Ash (PTY) Ltd database for the period of December 2002 to October 2003. Growth characteristics of *Dunaliella* in response to salinity and temperature gradient are evaluated. The data were obtained by taking *Dunaliella* species counts in brine flow across the evaporation ponds during the evaporation process over a period of time. Further, as the data was collected over a period of time and across climatic seasons, effects of temperature were also evaluated. The *Dunaliella* cell counts presented include all the *Dunaliella* species in the ponds.

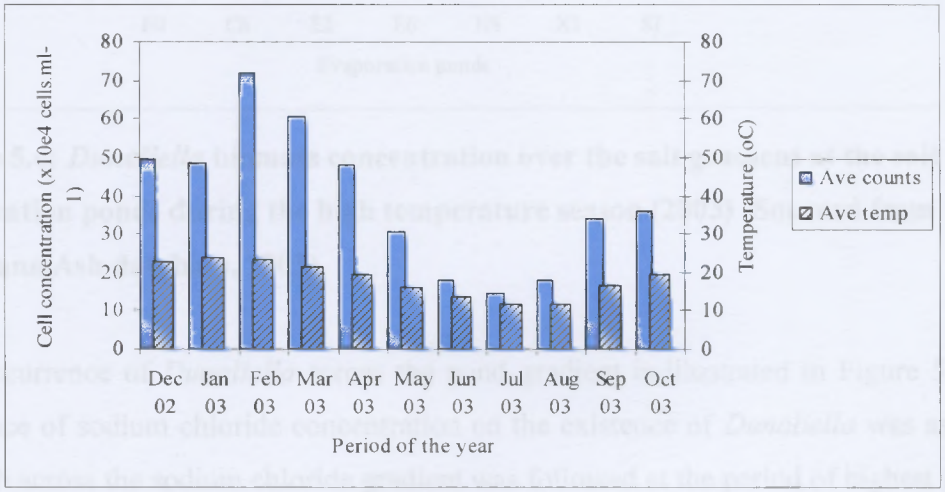


Figure 5.3: *Dunaliella* biomass monitored in the inlet pond (E0) over time (derived from Botswana Ash database, 2003)

Figure 5.3 illustrates the growth profile of the *Dunaliella* species over a year period as deduced from evaporation ponds database obtained from Botswana Ash Company. The information is obtained by averaging readings of the inlet pond, E0, which contained the highest *Dunaliella* concentration. Samples were taken every three days. Maximum algal concentration of approximately 71 x 10^4 cells.mL^{-1} was attained around the February

when brine temperatures are the highest in summer, at around 23°C. The cell concentrations decrease with decrease in temperature to reach the lowest concentration of 14 x 10^4 cells.mL^{-1} in July at an average temperature of 11°C in winter. A summary of brine sample temperatures is included in Appendix B, table B1 and B2.

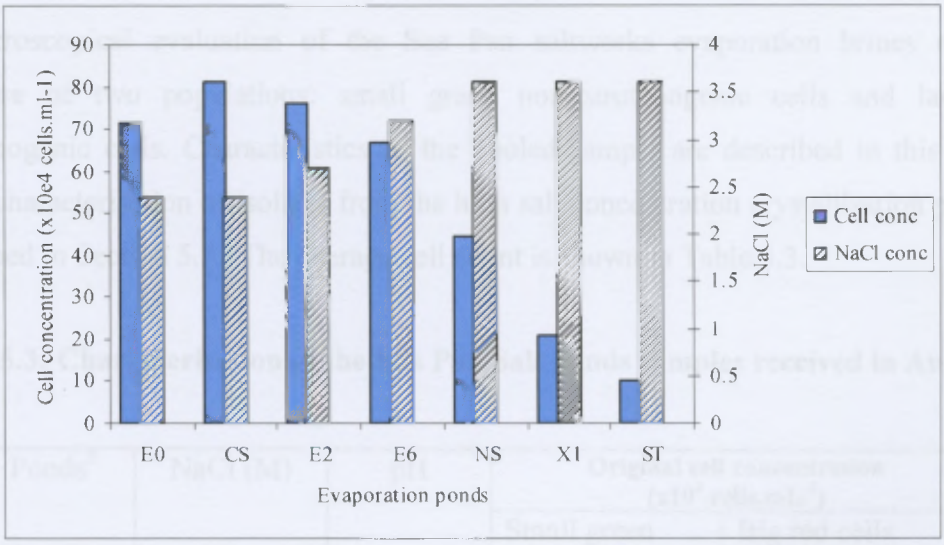


Figure 5.4: *Dunaliella* biomass concentration over the salt gradient at the salt evaporation ponds during the high temperature season (2003) (Sourced from Botswana Ash database, 2003)

The occurrence of *Dunaliella* across the pond gradient is illustrated in Figure 5.4. The influence of sodium chloride concentration on the existence of *Dunaliella* was assessed. Growth across the sodium chloride gradient was followed at the period of highest growth, February. The highest cell concentrations, 72 to 82 x 10^4 cells.mL^{-1}, were observed at the lowest sodium chloride concentration of approximately 2.4 M. These were found in the E0, CS and E2 ponds. Thereafter, the cell concentration decreases with increase in sodium chloride concentration. When the brine reaches the NS pond, sodium chloride reached 3.6 M with a cell concentration of 44 x 10^4 cells.mL^{-1}. Although the sodium chloride remained at 3.6 M in the crystalliser ponds X2 (used alternatively with X1) and ST brine pond, the cell counts decreased significantly to 21 x 10^4 cells.mL^{-1} and 10 x 10^4 cells.mL^{-1} respectively. This may be an indication of additional factors controlling the

Dunaliella growth at these ponds including entrapment by NaCl crystals, destruction of cells by the NaCl crystals and high alkalinities.

5.5 Characterisation of *Dunaliella* isolates from the evaporation ponds

A microscopical evaluation of the Sua Pan saltworks evaporation brines revealed presence of two populations: small green non-carotenogenic cells and large red carotenogenic cells. Characteristics of the pooled sample are described in this section while characterisation of isolates from the high salt concentration crystallisation ponds in described in Section 5.7. The average cell count is shown in Table 5.3.

Table 5.3: Characterisation of the Sua Pan Salt ponds samples received in August 2004

Ponds[5]	NaCl (M)	pH	Original cell concentration ($x10^4$ cells.mL^{-1})	
			Small green cells	Big red cells
CS	2.4	9.48	12	Not detected
E3	2.7	9.51	60	Not detected
E6	3.2	9.37	6	4
NS	3.6	9.38	8	2
X2	3.6	9.47	5	4

5.5.1 Cell type composition

The two algal cell types observed using microscopic evaluation were characterised as small green cells with an average diameter of 7 μm and the large green or red cells depending on their state of carotenogenesis, with an average diameter of 15-20 μm. The small cells remained green throughout the entire salt gradient. Based on the literature review, the small green cells were implicated as *Dunaliella viridis* while the large red

[5] The values are obtained from BOTASH database for August 2003

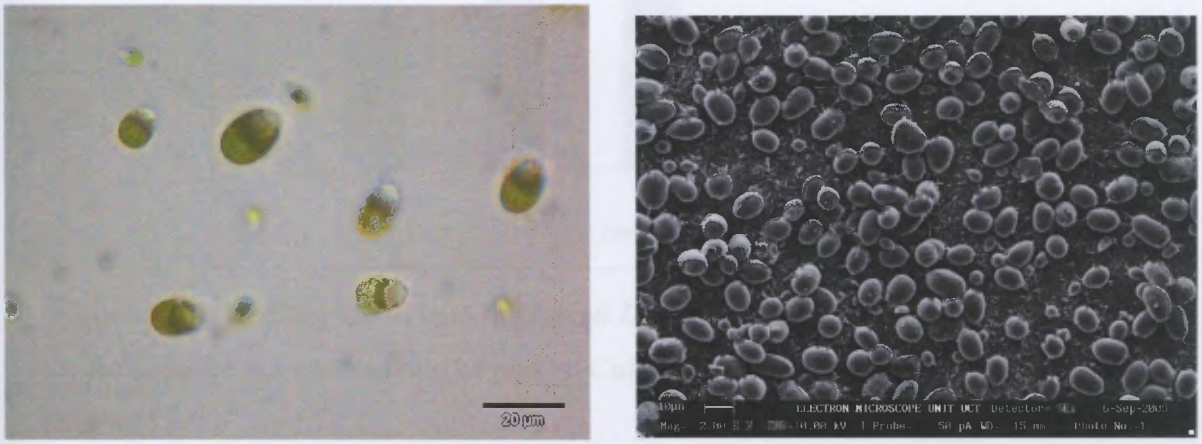
cells were proposed as *Dunaliella salina* (Preisig, 1992: Masemola, 1999; Borowitzka and Siva, 2007). It was recognised that the morphology and size of the *Dunaliella* species change according to their environment. According to Preisig (1992) the length of *D. salina* was 18-23 μm with a width of 10-15 μm. Only *D. salina* and *D. viridis* reported to exist at salt saturation with *D. salina* being carotenogenic and *D. viridis* being non-carotenogenic. The subsequent studies were carried out using pooled cell cultures from the above samples to give a uniform representation of the environment to represent the population of the evaporation ponds.

The light micrograph in Figure 5.5a shows the two *Dunaliella* isolates taken from the brine stock cultures derived following isolation of the cultures. The cultures were kept in 3M NaCl and BAAM media with 25% of typical nitrate concentration at a light intensity of approximately 50 $\mu mol.m^{-2}.s^{-1}$. The scanning electron micrograph of the two *Dunaliella* cell types found in the brine samples in Figure 5.5b shows presence of both big cells and small cells in the growth phase. The *Dunaliella* cells are known to have two flagella which do not show in Figure 5.5. There is a possibility that the cells may have lost their flagella during sample preparation for the electron microscope. Further, the low low magnification used with the microscopy may have prevented their visualisation.

5.5.2. Studies on growth properties

To characterise the growth properties, 100 mL BAAM with 2 M NaCl medium in 500 mL Erlenmeyer flasks was inoculated with a 10% inoculum of the isolate. Cultivation for one week at 50 $\mu mol.m^{-2}.s^{-1}$ and 25°C was used to reverse carotenogenesis of the already red cells. The process was intended to move the red cells from massive carotenoid accumulation phase to growth phase. Studies were done in duplicate and average cell counts presented in Figures 5.6 and 5.7. It should be noted that while total cell number is used to generate data presented in Figure 5.6 and 5.7, the cultures comprised mostly of the small non-carotenogenic cells. The relative sizes of populations

of large (*D. salina*) and small (*D. viridis*) cells and their change in number is presented in Table 5.4. At the end of the growth phase, the large cells appeared green.



a) **b)**

Figure 5.5: Morphology of Sua Pan *Dunaliella* in the mixed culture under growth phase conditions

a) Light micrograph of *Dunaliella* isolates **b) Scanning electron micrograph of *Dunaliella* isolates**

The specific growth rate of *Dunaliella* cells at the given incubation conditions ranged from 0.44 to 0.85 day^{-1} with the highest growth rate reported for the isolates from pond E3 (Figure 5.6). At the *p-value* < 0.05, ANOVA confirmed the significant differences in the specific growth rates of the cells. *D. viridis* dominated these cultures. A maximum specific growth rate for *D. viridis* of 1.4 day^{-1} has been reported (Jimenez and Niell, 1990). The lower growth rates reported in these experiments may be due to the low light intensity used in this experiment.

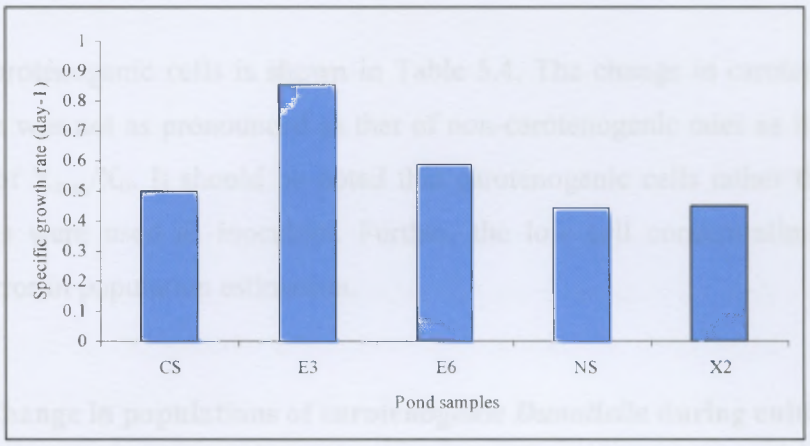


Figure 5.6: Specific growth rate for mixed *Dunaliella* cultures obtained from the brine samples across the train of ponds. Cultivation in BAAM with 2 M NaCl, 50 µmol.m^{-2}.s^{-1} and 25°C

Regarding the biomass production, the maximum algal concentrations attained were 4.6 to 5.3 x 10^6 cells.mL^{-1}. The maximum cell concentration showed little dependence on the pond source (Figure 5.7). This could have been due to the introduction of limiting factors such as light intensity availability to individual cells which could have been reduced with increase in cell density or depletion of crucial media components such as Carbon. The extent of growth, however, increased with increase in sodium chloride concentration in the pond from which inoculums was sourced.

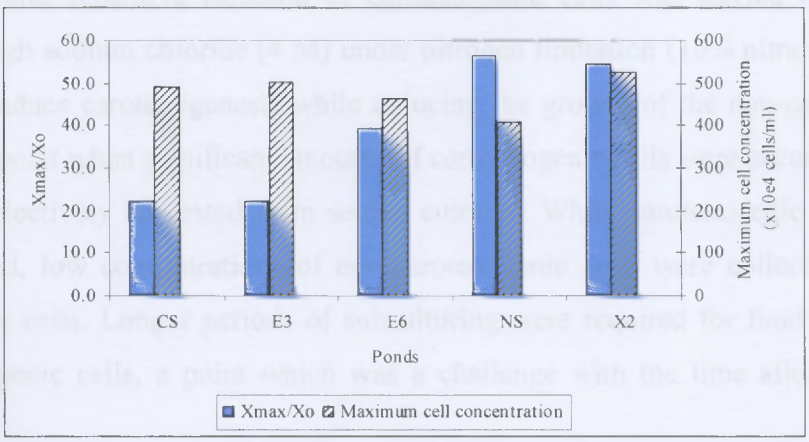


Figure 5.7: Evaluation of X_{max}/X_0 for *Dunaliella* mixed cultures obtained from the brine samples. Cultivation in BAAM with 2 M NaCl, 50 µmol.m^{-2}.s^{-1} and 25°C

Growth of carotenogenic cells is shown in Table 5.4. The change in carotenogenic cell concentration was not as pronounced as that of non-carotenogenic ones as illustrated by comparison of X_{max}/X_0. It should be noted that carotenogenic cells rather than actively growing cells were used as inoculum. Further, the low cell concentration may have introduced error in population estimation.

Table 5.4: Change in populations of carotenogenic *Dunaliella* during cultivation in BAAM with 2 M NaCl, 50 µmol.m^{-2}.s^{-1} and 25°C

	Dunaliella conc (x 10^4 cells.mL^{-1})		
Pond samples	Day 0	Day 5	Day 8
CS	<1	<1	<1
E3	<1	<1	<1
E6	<1	4	4
NS	1.5	1.5	3
X2	1.5	2.5	5.5

5.6 Separation of carotenogenic and non-carotenogenic *Dunaliella* cells

From this point, repetitive isolation of carotenogenic cells was carried out, through cultures in high sodium chloride (4 M) under nitrogen limitation (10% nitrate content of BAAM) to induce carotenogenesis while reducing the growth of the non-carotenogenic cells. At the point when significant amounts of carotenogenic cells were accumulated, red cells were selectively harvested from settled cultures. While carotenogenic populations were enriched, low concentrations of non-carotenogenic cells were collected with the carotenogenic cells. Longer periods of subculturing were required for final isolation of the carotenogenic cells, a point which was a challenge with the time allocated to the thesis.

Attempts to isolate the cultures using solid media were made. A solid media comprising 0.9% agar of BAAM with 3.5 M NaCl and a nitrate content reduced to 10% of that of the standard media was inoculated with 1mL *Dunaliella* cultures from the pooled pond samples. The inoculated solid media was incubated in petri dishes under light system A at approximately 80 $\mu mol.m^{-2}.s^{-1}$. The petri dishes were wrapped with parafilm to reduce the evaporation of media and were incubated for approximately 21 days. The resulting colonies of red carotenogenic cells were transferred to fresh solid media while minimising contamination with the non-carotenogenic cells. The cultures obtained from 'picked' carotenogenic cells still contained traces of non-carotenogenic cells. At 2 M NaCl, the non-carotenogenic cells tended to outgrow the carotenogenic cells, hence stock cultures of the mixed species were maintained at 3 M NaCl.

5.7 Crystalliser ponds samples

The second batch of brine samples were collected from the X2 crystalliser pond. Owing to the highest sodium chloride concentration, the *Dunaliella* strains occurring here were expected to be enriched in carotenogenic species. These are compared with the pooled *Dunaliella* samples in Section 6.5.1.

5.8 Conclusion

The chemical composition of the brines and the changes in the evaporation pond system were evaluated in this chapter. Isolation of *Dunaliella* species from the Sua Pan brines and identification of the species based on morphology and literature was presented.

Brines at the Makgadikgadi Pan contain sodium salts including common salts (NaCl), soda ash (Na_2CO_3) and salt cake (Na_2SO_4) as key compounds. *Dunaliella* species were shown to exist in the solar salt evaporation ponds and their growth was shown to be influenced by changing chemical composition of the ponds. Sodium chloride concentrations increased through the train of ponds because of evaporation. Low sodium

chloride concentrations of 2.4 M supported higher algal concentrations in the ponds. Growth was reduced at higher sodium chloride concentrations of 3.6 M. Analysis of data from Botswana Ash database also showed that the existence of the alga in the ponds was also influenced by changes in temperature with highest counts observed during summer when average temperatures reached 23°C. Lowest counts were observed in winter at temperatures of 11°C.

The Sua Pan salt evaporation ponds favour evaporation through high light intensities, high temperatures, relatively low rainfall and low humidity. These conditions also favour outdoor cultivation of *Dunaliella* for β-carotene production; hence potential for commercial exploitation of the alga exists.

Two *Dunaliella* species were isolated from the brines: carotenogenic *D. salina* and non-carotenogenic *D. viridis*. Growth studies showed *D. viridis* exhibited higher growth rates than *D. salina*, particularly at lower NaCl concentrations of 2 M and higher nitrogen concentrations. *D. viridis* dominated the ponds at the inlet to the salt works.

CHAPTER 6: PHYSIOLOGICAL CHARACTERISATION OF SOLAR SALT EVAPORATION PONDS *Dunaliella* ISOLATES

6.1 INTRODUCTION

This chapter investigates *Dunaliella* species isolated from the Sua Pan solar salt evaporation ponds. It presents the study of both growth and carotenoid biosynthesis properties with emphasis on the major pigment, β-carotene. The primary objective of this section is to evaluate the suitability of the isolates for application in commercial production of carotenoids under defined indoor culture conditions. The results of this study provide the basis for further evaluation of the species in terms of scale up and the outdoor cultivation of the isolates.

Industrial carotenoid biosynthesis from *Dunaliella* requires good carotenoid productivity. This is dependent upon the biomass productivity (influenced by the biomass concentration and specific growth rate) as well as the β-carotene content of the cells. The objective is thus to optimise biomass production as well as synthesis of carotenoids in each cell. It is well recognised that carotenoid synthesis is maximised under stress conditions under which growth is compromised. Hence, dual phase mode of cultivation is used in this study. In the dual phase mode application, the goal of optimisation at the growth phase level is thus to produce optimum cell concentrations that are further diluted to maximise light intensity before the cell culture is induced for carotenoid biosynthesis.

Two *Dunaliella* species were isolated from the Sua Pan salt evaporation ponds brines: the carotenogenic *Dunaliella salina* (*D. salina*) and the non-carotenogenic *Dunaliella viridis* (*D. viridis*) as described in Section 5.5.1. Studies on the co-existence of the two species are of importance to the commercial cultivation of the carotenogenic *D. salina* as the non-carotenogenic species may interfere with the growth of the carotenogenic species, especially under dual phase operation in open culture systems. The efficiency of the process is influenced by non-carotenogenic species competing for limiting C source and

light, amongst other resources. The quality of the final carotenoid product may also be compromised.

Section 6.2 details the growth phase studies of the environmental samples comprising *D. salina* and *D. viridis*. The objective of the experiments conducted was to establish favourable conditions for *D. salina* while restricting the growth of the non-carotenogenic cells (*D. viridis*). Nutrient consumption studies are reported in Section 6.3 while the carotenogenesis studies, including the optimisation of carotenoid biosynthesis, are carried out in Sections 6.4 and 6.5. Section 6.6 reports characterisation studies based on biochemical composition of *D. salina* with focus on macromolecules present. Conclusions drawn from key findings are collated in Section 6.7.

6.2 Growth studies on solar salt evaporation ponds isolates

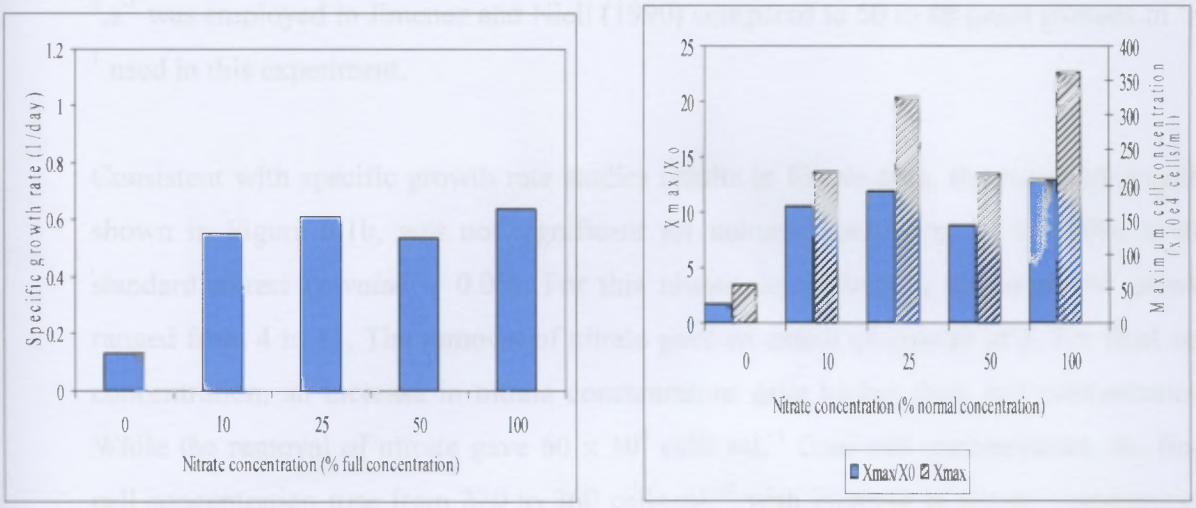
The studies of growth of *D. bardawil* UTEX 2538 presented in Section 4.2 were used, in conjunction with the literature review, to inform selection of conditions to characterise the environmental isolates. The factors chosen as operating variables include sodium chloride concentration, light intensity, and temperature. In addition, nitrate deprivation was included to control growth of non-carotenogenic species.

6.2.1 Effects of nitrate concentration on growth of mixed culture

The use of nitrate deprivation to promote carotenogenesis and limit growth was discussed in Section 2.5 and 2.6. Nitrate deprivation was identified as a potential means for reducing the growth of the non-carotenogenic cells in the *Dunaliella* species cultures. The objective of this section was to establish the nitrate concentration at which growth of the non-carotenogenic cells was compromised while minimising the effect on the growth of the carotenogenic cells.

Studies were conducted using the mixed culture isolates prepared by pooling the brine samples, described in Section 5.5. A 10% (v/v) inoculum volume was used to inoculate 100 mL BAAM medium in 500 mL Erlenmeyer flasks with cotton wool bungs. The nitrate concentrations are provided as percentages, representing the specific fractions of 5 mM KNO_3 concentration in balanced BAAM medium. This is the optimum concentration reported by Borowitzka (1992) and quantified as 100% in this study. Section 2.5.2.3 indicates optimum potassium concentration for *Dunaliella* growth is 1 mM (Ben-Amotz and Avron, 1989b), hence no replacement of the potassium in KNO_3 was considered. Light intensity, sodium chloride and temperature were kept constant at 50 - 80 μmol photons.m^{-2}.s^{-1}, 2 M NaCl and 25°C respectively. Continuous illumination was provided with light system A. Flasks were rotated randomly daily to minimise the variations in light intensities. The cultures were monitored over a period of 8 days with manual shaking once daily. The growth indicators reported in Section 4.1 are used here: maximum specific growth rate μ_{max}, maximum cell concentrations attained X_{max} and the degree of growth X_{max}/X_0. An ANOVA was used to evaluate the data.

Results of this experiment are presented in Figure 6.1. Microscopic evaluation of the cultures revealed the cultures were comprised mainly of non-carotenogenic cells hence the data were presented as total cells (carotenogenic and non-carotenogenic). Maximum specific growth rates are demonstrated in Figure 6.1a while the maximum cell concentration and the extent of growth are depicted in Figure 6.1b. Large cells comprised 5 to 10% of the total cells for this section.



a) **b)**

Figure 6.1: Growth indicators for total *Dunaliella* cells in pooled environmental samples as a function of KNO_3 gradient (100% = 5 mM KNO_3). Cultivation in BAAM with 2 M NaCl at 50- 80 µmol.m^{-2}.s^{-1} light intensity and 25°C. a. Specific growth rate, µ; b. X_{max}/X_o and maximum cell concentration

The average maximum growth rate observed ranged from 0.13 to 0.64 day^{-1} as the NO_3 concentration increased from 0 to 5 mM KNO_3, as shown in Figure 6.1a. The specific growth rates of cultures containing 10 to 100% of the standard nitrate (0.5 to 5 mM KNO_3) did not show significant difference (*p-value* > 0.05) in specific growth rate (0.53 to 0.63 day^{-1}) with average of 0.58 day^{-1}. However, in the absence of nitrate, a lower maximum specific growth rate of 0.12 day^{-1} was recorded. With a *p-value* < 0.05, the one-way ANOVA showed the nitrate concentration was a significant factor in the cultivation of *Dunaliella* cultures at the given conditions. It is observed that 0 mM NO_3 restricts cell concentration, the extent of growth and the specific growth rate. The specific growth rates observed by Cifuentes *et al.* (1992) for *D. salina* (non-carotenogenic) ranged from 0.46 to 1.40 day^{-1} (Table 2.1). The experimental cell concentrations reached were approximately 50 to 350 x 10^4 cells.mL^{-1} while those for literature *D. viridis* were 1360 to 1850 x 10^4 (1.35 to 1.85 x10^7) cells.mL^{-1} (Jimenez and Niell, 1990). The values reported from the literature pertain to unicultures. Further, light intensity of 150 µmol photons.m^{-}

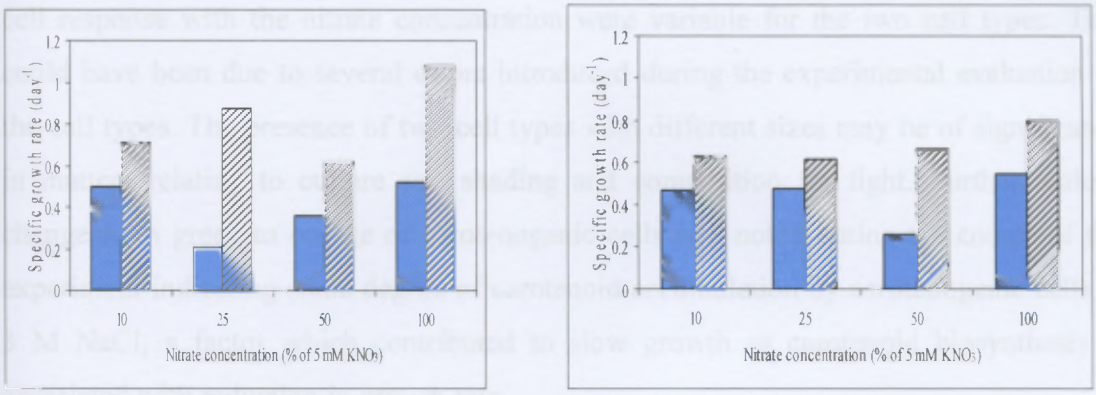
$^{2}.s^{-1}$ was employed in Jimenez and Niell (1990) compared to 50 to 80 μmol photons.$m^{-2}.s^{-1}$ used in this experiment.

Consistent with specific growth rate studies results in Figure 6.1a, the extent of growth, shown in Figure 6.1b, was not significant for cultures containing 10 to 100% of the standard nitrate (*p-value* > 0.05). For this nitrate concentration, the extent of growth ranged from 4 to 11. The removal of nitrate gave an extent of growth of 2. For final cell concentration, an increase in nitrate concentration gave higher final cell concentration. While the removal of nitrate gave 60 x 10^{4} cells.mL^{-1} final cell concentration, the final cell concentration rose from 220 to 360 cells.mL^{-1} with increase in nitrate concentration from 0.5 to 5 mM. There was, however, a reduction in final cell concentration at 2.5 mM (50% KNO_3), which could have resulted in an error in the experiment, possibly culture self shading of cells. This is plausible as the extent of growth is relatively lower than of the other nitrate concentrations.

6.2.2 Effect of nitrate and sodium chloride concentrations on growth of the mixed culture

As an extension to Section 6.2.1, the control of non-carotenogenic *Dunaliella* using nitrate availability was explored through the possible interactive effect of nitrate and sodium chloride concentration. The same nitrate concentrations were used as in Section 6.2.1 with omission of 0% nitrate, found to have adverse effect on the cultures. The sodium chloride concentrations were set at 2 M and 3 M. Growth characteristics of both carotenogenic cells and non-carotenogenic cells were followed at these conditions.

The inoculation procedures and experimental set up are described in Section 3.4. The growth indicators specified in Section 6.2.1 were used. The maximum specific growth rates of carotenogenic and non-carotenogenic *Dunaliella* cells are shown in Figure 6.2.



a) **b)**

Figure 6.2: A comparison of specific growth rate for the carotenogenic (car) and non-carotenogenic (non-car) cells at (a) 2 M NaCl and (b) 3 M NaCl on exposure to increasing NO_3^- availability (0.5 mM -5 mM KNO_3).

Figure 6.2 showed differences in behaviour of the two cell types at 2 M and 3 M NaCl. The non-carotenogenic cells had higher specific growth rates than the carotenogenic cells at both NaCl concentrations. Further, the increase in sodium chloride concentration from 2 to 3 M reduced the growth rate of the non-carotenogenic cells by approximately 20% while that of the carotenogenic cells type increased by approximately 10%. A summary of two-factor ANOVA showed that for the carotenogenic cell type, the change with sodium chloride concentration was not significant (*p-value* > 0.05). Also at the *p-value* > 0.05, the interaction of sodium chloride and nitrate factors at the given concentrations was also not significant for carotenogenic cells. The two factors were, however, significant for non-carotenogenic cells at *p-value* < 0.05 each. The interaction of the two parameters was also significant although with *p-value* = 0.05.

A comparison between carotenogenic and non-carotenogenic cells showed higher specific growth rates for non-carotenogenic than those of carotenogenic cells at all conditions shown in Figure 6.2. The specific growth rate for carotenogenic cells ranged from 0.21 to 0.52 day^{-1} while that of non-carotenogenic cells ranged from 0.62 to 1.08 day^{-1}. Overall, based on the specific growth rates, increase in the sodium chloride concentration facilitated a reduction in specific growth rate of non-carotenogenic cells. Results for the

cell response with the nitrate concentration were variable for the two cell types. This could have been due to several errors introduced during the experimental evaluation of the cell types. The presence of two cell types with different sizes may be of significance in matters relating to culture self shading and competition for light. Further, colour change from green to orange of carotenogenic cells was noted during the course of the experiment indicating some degree of carotenoid accumulation by carotenogenic cells at 3 M NaCl, a factor which contributed to slow growth as carotenoid biosynthesis is associated with reduction in growth rate.

Specific growth rates for carotenogenic and non-carotenogenic *Dunaliella* species from literature are shown in Table 2.1. Specific growth rates of 0.08 to 0.48 day^{-1} for carotenogenic cells were noted while that of the non-carotenogenic species were in the ranges of 0.46 to 1.40 day^{-1}. Further, in Section 4.2, the specific growth rates attained by type culture carotenogenic *Dunaliella* species were given. *D. bardawil* gave specific growth rates of 0.02 to 0.39 day^{-1} while the non-carotenogenic *D. salina* gave 0.48 day^{-1}. It is observed that the non-carotenogenic cells outcompete the carotenogenic as explained by Borowitzka and Borowitzka (1988) in Section 2.5.2.4a. While the carbon flux in carotenogenic cells is diverted to carotenoid biosynthesis during stress, this does not occur in non-carotenogenic cells. Further, the non-carotenogenic cells do not expend energy in carotenoid biosynthesis hence higher growth rates. Although different conditions were employed for different settings, the carotenogenic environmental isolates had slightly higher specific growth rates compared to literature (Table 2.1) and the type culture *Dunaliella*.

6.2.3 Effects of light intensity gradient on mixed culture

Light system B, delivering light intensities of 80 to 200 μmol photons.m^{-2}.s^{-1}, was used for this section of this study. The cultures were incubated at 25°C. The sodium chloride was maintained at 3 M to limit growth of non-carotenogenic cells. The nitrate was 25%

of the full concentration (1.25 mM KNO_3) to reduce the growth of the non-carotenogenic cell type. Section 2.6 highlighted nitrate deprivation as one cause of growth reduction.

The inoculation protocol, growth conditions and growth indicators described in Section 3.4 were used. The effects of the light intensity on total cell growth (carotenogenic and non-carotenogenic cells) and carotenogenic cell type are reported in this section. Evaluation of the non-carotenogenic cell type with light intensity and temperature is presented in Section 6.2.4.

The results shown in Figures 6.3 and 6.4 illustrate that the light intensity across the range of 80 to 200 μmol photons.$m^{-2}.s^{-1}$ did not affect the growth rate of the combined carotenogenic and non-carotenogenic cells nor the growth rate of the carotenogenic cells (in the mixed culture) when evaluated separately.

Figure 6.3 shows the average specific growth rate of the total *Dunaliella* population ranged from 0.56 to 0.71 day^{-1}. The specific growth rate of the carotenogenic cell type, classified as *D. salina*, was in the range of 0.48 to 0.61 day^{-1}. Contradictory to the literature, at *p-value* > 0.05, the one-way ANOVA showed that the variation in light intensities was not significant for the growth rate of the carotenogenic cells under the experimental conditions. The extent of growth of the carotenogenic cells was between 5.90 and 7.64. The *p-value* > 0.05 showed that the given light intensities did not affect the extent of growth.

The fact that light intensity gradient did not change the growth rate of the alga may mean that either the levels of the light intensities were not significantly different from each other or the growth was controlled by another parameter under the conditions of this experimental set up. For instance, carbon availability may have limited algal growth; carbon was supplied as $NaHCO_3$. The light intensity employed in the literature ranged from 150 μmol.$m^{-2}.s^{-1}$ to outdoor conditions, as shown in Table 2.1, compared with the range of 80 to 200 μmol.$m^{-2}.s^{-1}$ used in this study.

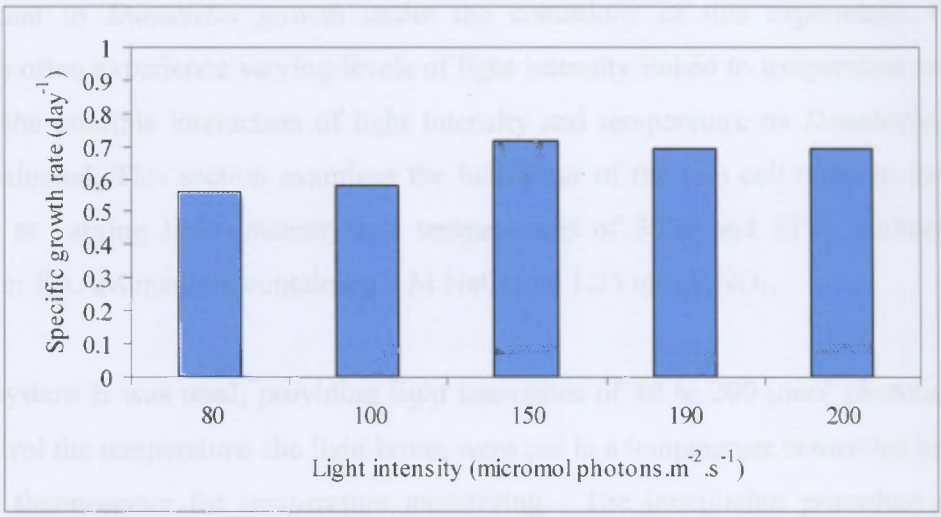
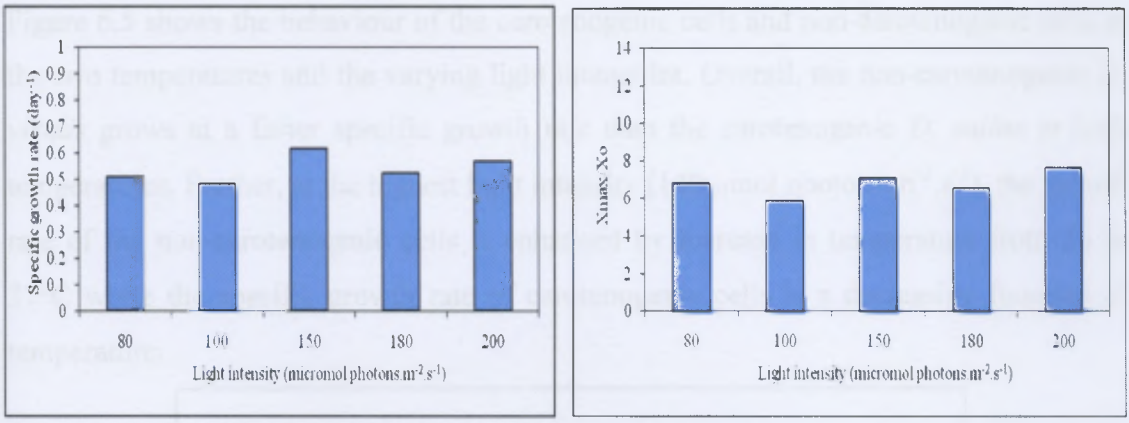


Figure 6.3: Effect of light intensity on growth of total (carotenogenic + non-carotenogenic) cells in BAAM media containing 3 M NaCl and 25°C.



a. **b.**

Figure 6.4: Effect of light intensity on growth of carotenogenic cells in BAAM media containing 3 M NaCl and 25°C

6.2.4 Effects of light intensity and temperature on the mixed culture

Temperature is also noted as an important factor for growth of *Dunaliella* species as illustrated in Section 5.4.3.2. Section 6.2.3 studied the evaluation of light intensity effects on the environmental *Dunaliella* isolates. The result showed that light intensity was not

significant to *Dunaliella* growth under the conditions of this experiment. Outdoor cultures often experience varying levels of light intensity linked to temperature variation. Hence the possible interaction of light intensity and temperature on *Dunaliella* growth was evaluated. This section examines the behaviour of the two cell types in the mixed culture at varying light intensity and temperatures of 30°C and 37°C. Cultures were grown in BAAM medium containing 3 M NaCl and 1.25 mM KNO_3.

Light system B was used, providing light intensities of 80 to 200 µmol photons.m^{-2}.s^{-1}. To control the temperature, the light boxes were put in a temperature controlled box fitted with a thermometer for temperature monitoring. The inoculation procedure and the experimental set up in Erlenmeyer flasks, described in Section 3.4, were used. Growth monitoring and indicators reported in Section 3.6.2.1 were used.

Figure 6.5 shows the behaviour of the carotenogenic cells and non-carotenogenic cells at the two temperatures and the varying light intensities. Overall, the non-carotenogenic *D. viridis* grows at a faster specific growth rate than the carotenogenic *D. salina* at both temperatures. Further, at the highest light intensity (140 µmol photons.m^{-2}.s^{-1}), the growth rate of the non-carotenogenic cells is enhanced by increase in temperature from 30 to 37°C while the specific growth rate of carotenogenic cells is a decreasing function of temperature.

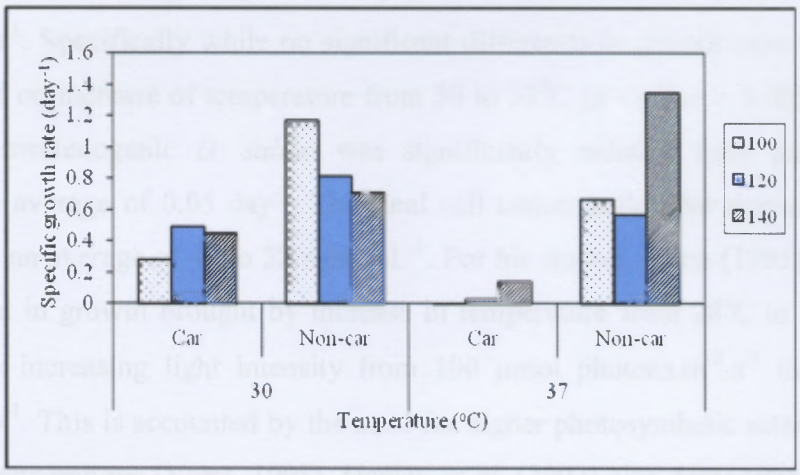


Figure 6.5: Effect of light and temperature on specific growth rate of carotenogenic (car) and (non-car) cells in BAAM containing 1.25 mM nitrate and 3 M NaCl.

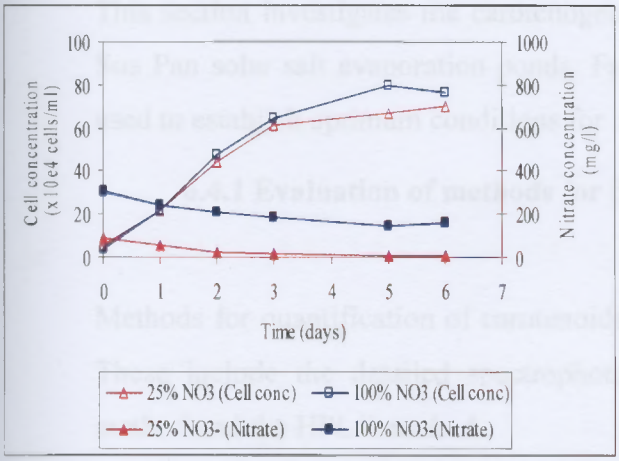
The ANOVA for the carotenogenic cells indicated both light intensity and temperature were significant at the given levels. At 95% confidence level, *p-value* < 0.05 for light intensity and temperature, there was interaction between light intensity and temperature for carotenogenic cells. With *p-value* > 0.05 both light intensity and temperature were not significant to the growth of the non-carotenogenic cells.

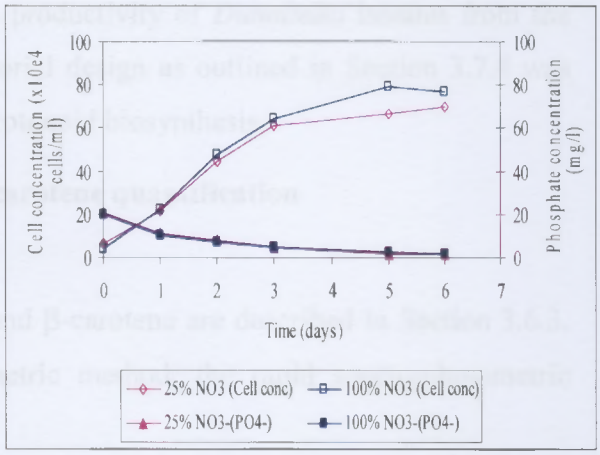
At lower temperature (30°C), the carotenogenic cells had the lowest average specific rate (0.29 day^{-1}) at the lowest light intensity (100 μmol photons.m^{-2}.s^{-1}). The specific growth rate differences at 120 and 140 μmol photons.m^{-2}.s^{-1} were not significant at 0.49 and 0.46 day^{-1} respectively as shown in Figure 6.5. The highest growth rate for the non-carotenogenic cells at 30°C was 1.17 day^{-1}at 100 μmol photons.m^{-2}.s^{-1}. At 120 and 140 μmol photons.m^{-2}.s^{-1} the specific growth rates were 0.82 and 0.71 day^{-1} respectively. At 37°C, growth rates for carotenogenic cells were 0.01 day^{-1} at 100 and 0.49 day^{-1} at 120 μmol photons.m^{-2}.s^{-1} and there were no significant differences of rates at the levels of light intensities. At 140 μmol photons.m^{-2}.s^{-1}, the growth rate for the carotenogenic cells was slightly higher at an average of 0.13 day^{-1}. A reduction in cell concentration, from an average of 36 to 12 cells.ml^{-1} for all light intensities, was observed for carotenogenic cells at incubation at 37°C. The specific growth rates averages for non-carotenogenic cells at 37°C were not significantly different at 100 and 120 μmol photons.m^{-2}.s^{-1} (0.68 and 0.57 day^{-1} respectively). A higher growth rate of 1.35 day^{-1} was observed at 140 μmol photons.m^{-2}.s^{-1}. Specifically while no significant difference in growth rates of *D. viridis* was observed on increase of temperature from 30 to 37°C (*p - value* > 0.05), the growth rate of the carotenogenic *D. salina* was significantly reduced from an average of 0.41 day^{-1} to average of 0.05 day^{-1}. The final cell concentration for non-carotenogenic cell fell from an average of 42 to 32 cells.mL^{-1}. For his studies, Vorst (1995), established that reduction in growth brought by increase in temperature from 28°C to 34°C can be overcome by increasing light intensity from 100 μmol photons.m^{-2}.s^{-1} to 2500 μmol photons.m^{-2}.s^{-1}. This is accounted by the need for higher photosynthetic rates for survival at the high temperatures (Vorst, 1995). Henley *et al.* (2002) also demonstrated that high salinity increases tolerance to high growth radiance and temperature.

6.3 Nutrient consumption studies of *Dunaliella salina* isolates

Nutrient consumption studies were carried out to establish the depletion of the key nutrients: nitrate and phosphate during growth phase of the alga. The objective was to further investigate the results obtained on effect of nitrate concentration on the growth of *Dunaliella* in Section 6.2 and to determine if the nutrients could be limiting cell growth. Two levels of nitrate were chosen: 25% (1.25 mM KNO_3) and 100% (5 mM KNO_3). The BAAM standard concentration of phosphate of 0.02 mM was used with 2 M NaCl. The mixed culture described in Section 5.5 was used for this experiment. Light system B provided an average light intensity of 180 μmol photons.m^{-2}.s^{-1} at 25°C. Inoculation and incubation were performed as described in Section 3.4 using 500 mL Erlenmeyer flasks with manual shaking once a day. Standard procedures for growth monitoring and growth indicators were used. The cell types were not differentiated.

The cell concentrations attained at both 25% and 100% KNO_3 were similar as shown in Figure 6.6. The specific growth for the total cell population at 25% and 100% KNO_3 were 1.0 day^{-1} (R^2: 0.976) and 1.2 day^{-1} (R^2: 0.9602) respectively. The extent of growth (X_{max}/X_o) was 11.7 and 17.7 for 25% (1.25 mM KNO_3) and 100% KNO_3 (5 mM KNO_3) respectively. Figure 6.6a further indicates that, on addition of 308 mg.L^{-1} nitrate (5 mM KNO_3), 162.73 mg.L^{-1} nitrate remained at day 6 when the cells have reached stationary phase. However, on addition of 84 mg.L^{-1} (1.25 mM KNO_3) only 11.42 mg.L^{-1}nitrate remained day 6 of the experiment. These represented the utilisation of 162 and 69 mg nitrate.L^{-1} respectively, equivalent to 9 and 6 mg nitrate.L^{-1} per doubling of the inoculated population.





a) **b)**

Figure 6.6: Evaluation of nutrient consumption in BAAM medium with 2 M NaCl and light intensity of 180 µmol photons.$m^{-2}.s^{-1}$ at 25°C.

a) Evaluation of nitrate consumption **b) Evaluation of phosphate consumption**

Phosphate consumption was studied simultaneously with nitrate consumption, using a starting concentration of phosphate (KH_2PO_4) of 0.02 mM (20 mg.L^{-1}). Data are shown in Figure 6.6b. The phosphate concentration remaining at day 6 was 1.25 mg.L^{-1} and 1.41 mg.L^{-1} for the 1.25 mM KNO_3 and 5 mM KNO_3 samples respectively. Phosphate utilisation was similar on a volumetric basis (19.52 and 18.42 mg.L^{-1} respectively), representing 1.76 and 1.04 mg.L^{-1} phosphate for 1.25 mM KNO_3 and 5 mM KNO_3 per doubling of the inoculated population respectively. The results show that at a starting concentration of 84 mg.L^{-1} nitrate (1.25 mM KNO_3) and 20 mg.L^{-1} phosphate, the cultures become nutrient-depleted following incubation for greater than 6 days based on nutrient depletion.

6.4 Carotenogenesis studies

This section investigates the carotenogenic productivity of *Dunaliella* isolates from the Sua Pan solar salt evaporation ponds. Factorial design as outlined in Section 3.7.4 was used to establish optimum conditions for carotenoid biosynthesis.

6.4.1 Evaluation of methods for β-carotene quantification

Methods for quantification of carotenoids and β-carotene are described in Section 3.6.3. These include the detailed spectrophotometric method, the rapid spectrophotometric method and the HPLC method.

6.4.1.1 Detailed spectrophotometric method

In the detailed spectrophotometric method, described in Section 3.6.3.1, extraction of carotenoids from wet biomass harvested from 2 mL cultures was carried out in methanol. β-carotene was then extracted into heptane following saponification by saturated potassium hydroxide in methanol. Following method in Appendix IIIA, β-carotene was measured extracted into heptane at absorbance 436 nm. To establish the total carotenoid concentration, diethyl ether was used as in Appendix IIIA, part C. Total carotenoids were determined by measuring absorbance of diethyl ether extract at 450 – 453 nm. Equations 3.3 and 3.4 were used for quantification. Samples were analysed in duplicate and the results are presented in Table 6.1.

6.4.1.2 Rapid spectrophotometric method

The rapid spectrophotometric method is outlined in Section 3.6.3.2. The method employed extraction of carotenoids using ice cold acetone. Where necessary, samples were filtered through a 0.45 μm filter paper. The absorbance was measured at 470 nm and the concentration calculated according to Equation 3.5 using the extinction coefficient of 2620 for β-carotene in 100% acetone. Samples were analysed in triplicate. The results are shown in Table 6.1. For confirmation of maximum absorbance, the

spectrum for β-carotene in acetone is shown in Figure 6.7. The rapid spectrophotometric extraction procedure was also used for quantification of chlorophyll pigments as described in Section 6.6.

6.4.1.3 HPLC quantification method

For HPLC quantification, the rapid extraction method described in Section 6.4.1.2 was used. Following filtering of the acetone extract, a known volume of extract was evaporated under nitrogen and re-suspended in HPLC running solvent for analysis as detailed in Appendix AIII.

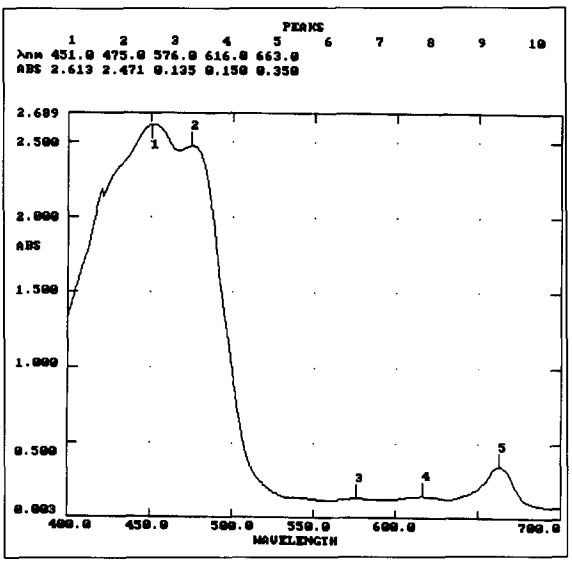


Figure 6.7: Algal β-carotene spectrum in acetone solvent

6.4.1.4 Results obtained from comparison of carotenoid quantification methods

The results for the different methods are presented in Table 6.1. With a *p-value* < 0.05, the methods are significantly different based on the method accuracy. The HPLC method, specific for β-carotene, gave the highest amount of carotenoid (β-carotene) followed by

the detailed spectrophotometric and the rapid spectrophotometric methods. The spectrophotometric methods give carotenoids as total carotenoids.

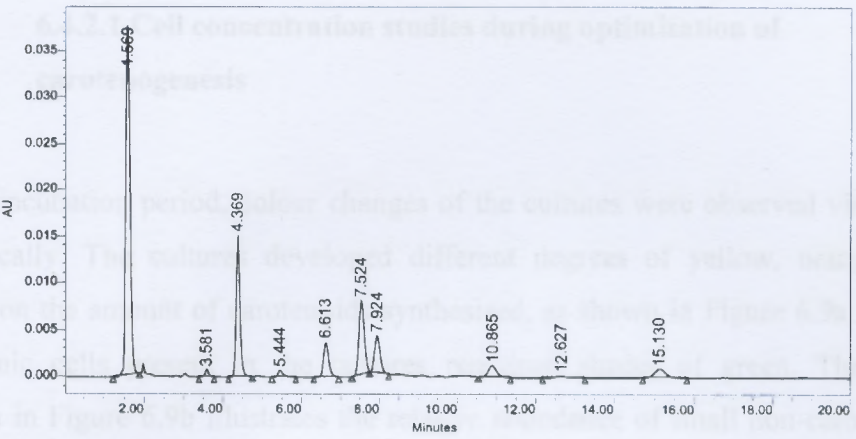
Table 6.1: Comparison of β-carotene quantification methods for environmental samples.

Quantification method	*D. salina* (Carotenogenic phase)	*D. salina* (Growth phase)
Detailed spectrophotometric ($pg.cell^{-1}$)	32.1 ± 0.8	7.6 ± 1.2
Rapid spectrophotometric ($pg.cell^{-1}$)	30.9 ± 1.6	11.3 ± 1.9
HPLC ($pg.cell^{-1}$)	39.64 ± 1.4	5.9 ± 1.5

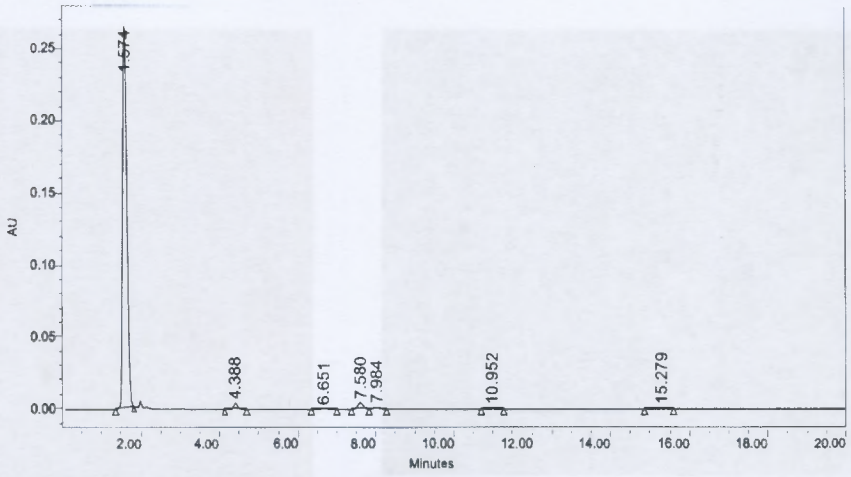
The HPLC chromatograms in Figure 6.8a and 6.8b show β-carotene quantification from carotenogenic cells in growth phase and β-carotene accumulation phase respectively. β-carotene eluted at around 2.0 minutes. Massive β-carotene accumulation is seen in Figure 6.8b. β-carotene concentration at growth phase is lower and is accompanied by relatively significant quantities of other pigments including lutein, zeaxanthin and cryptoxanthin.

6.4.2 Optimisation for carotenoid biosynthesis

The optimisation experiments were conducted with culture volumes of 6 mL in 15 mL test tubes in triplicate. Growth phase cells at exponential growth were harvested at 3500 rpm using a Beckman centrifuge, rotor JA-10. Cultures were washed twice with 3 M NaCl and 0% nitrate BAAM medium and suspended in medium under study to a cell concentration of approximately 35 to 45 x 10^4 $cells.mL^{-1}$ carotenogenic cells. Pooled environmental sample cultures were incubated at the appropriate light intensity at 25°C for 6 days using light system B at the end of which cultures were evaluated for carotenoid content.



(a)



(b)

Figure 6.8: Beta-carotene HPLC chromatogram

(a) HPLC chromatogram for carotenogenic cells at growth phase

(b)HPLC chromatogram for carotenogenic cells after massive carotenoid accumulation

Differential cell counts of the carotenogenic cells and non-carotenogenic cells were established in triplicate using the Neubaeur cell counting chamber (Appendix B, Table B4). Data obtained were analysed with ANOVA Statistica software to determine the main effect and interaction of the indicated parameters