GLYCEROL FROM NOVEL STRAINS OF HALOPHYTIC MICROALGAE

By

Aminu Lailaba Abubakar

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School of Science

University of Greenwich, Medway Campus

Chatham Maritime, Kent ME4 4TB, UK

DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any purpose, other than that of the Doctor of Philosophy (PhD) being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarized the work of others.

Aminu Lailaba Abubakar.....Date.....
(Student)

Professor Patricia J Harvey.....Date.....
(First Supervisor)

Dr. John Orchard.....Date.....
(Second Supervisor)

DEDICATION

This thesis is dedicated to my family and entire good people of Lailaba District.

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ABSTRACT

Glycerol is a new biofuel which underpins a commercial CHP technology as a result of the novel McNeil combustion cycle patented by Aquafuel, UK. This research explored glycerol production in new strains of halotolerant microalgae *Dunaliella* (T35, T36 and T37) and *Asteromonas* (T33a, T33b and T33c) obtained from Namibia. The aim was to: (a) Determine the optimum conditions of pH value, temperature and salinity on the specific growth rate and doubling time and parameters of glycerol production, (b) Investigate metabolites associated with glycerol accumulation and dissimilation in *Dunaliella* cells exposed to salinity stress under continuous light or dark regimes. (c) Maximize glycerol recovery from microalgal cultures using novel supercritical carbon dioxide (SC-CO_2) extraction technology.

Dunaliella and *Asteromonas* strains were shown to withstand large variations in the salinity of the growth medium 0.5 - 4.0 M NaCl. The optimum conditions for their growth were 1.0 M NaCl, 30 ^{o}C, pH 7.5 to 9.0 and 45 µmol m^{-2} s^{-1}. Cultivation at 1.0 M NaCl produced 46.1 - 65.2 pg/cell of glycerol in *Dunaliella* and 53.8 to 67.1 pg/cell of glycerol in *Asteromonas*, but transfer of cells from 1.0 to 4.0 M NaCl for 240 min maximized glycerol production to 179.5 - 237.6 pg/cell in *Dunaliella* and 128.7 - 184.2 pg/cell in *Asteromonas*. The amount of glycerol produced by *Dunaliella* isolated from Namibia was between 3.3 and 5.2 fold greater than other species investigated (*D. salina, D. quartolecta*, *D. parva* and *D.* p*olymorpha*). Transfer of cells to high salinities did not disrupt cell integrity, although cells shrank by 35 to 62 % of their original volume due to water efflux from the cell.

The starch and glycerol pools of *Dunaliella* T35 were investigated after 24 h exposure to 1.0 and 4.0 M NaCl in dark and light regimes in order to investigate how starch degradation contributed to glycerol production. In 1.0 M NaCl, the amount of starch was 45 and 222 µg/mg of dry biomass under dark and light regimes respectively, and glycerol, 163 and 174 µg/mg of dry biomass. Under parallel conditions at 4.0 M NaCl, the amount of starch decreased to 27 and 146 µg/mg of dry biomass and glycerol increased to 592 and 706 µg/mg of dry biomass in the dark and light, respectively. This suggested that the contribution of starch breakdown to glycerol synthesis increased with increasing salinity stress in the Namibian strain of *Dunaliella*. Hyperosmotic shock decreased the pool size of proline and increased the pools size of pyruvate and glycerol.

The degree of cell rupture after SC-CO_2 extraction of glycerol from *Dunaliella* was dependent on the applied pressure. Cells were ruptured and glycerol was released at 100 bar and the degree of cell rupture increased with pressure to 200 bar in the presence of chloroform. At 350 bar in the absence of chloroform SC-CO_2 extraction of glycerol produced ~6 % higher glycerol levels than sonication.

Namibian strains of *Dunaliella* and *Asteromonas* T33c investigated are best suited for glycerol production compared to *Asteromonas* T33a, T33b and other strains of *Dunaliella* (*D. salina, D. quartolecta*, *D. parva* and *D.* p*olymorpha*). Cells cultivated in 1.0 M NaCl to generate biomass followed by transfer to hyperosmotic stress for glycerol accumulation was shown to be optimum for glycerol production. SC-CO_2 is shown to be an effective, 'green' technology for glycerol extraction from *Dunaliella*.

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LIST OF ABBREVIATION AND SYMBOL

Abbreviation/symbol	Meaning
–	Minus sign
%	Percent
$(NH4)_6Mo_7O_{24}·4H_2O$	Ammonium molybdate tetrahydrate
+	Plus sign
+/–	Plus-minus sign
<	Less-than sign
>	Greater-than sign
µ	Specific growth rate
µg	Microgram
µl	Microliters
µm	Micrometer
μm^3	Cubic micrometer
ρ	Rho factor
1H-NMR	Proton nuclear magnetic resonance
A	*Absorbance*
Å	Angstrom
A.	*Asteromonas*
AAAs	Aromatic amino acids
ADP	Adenosine diphosphate
AFDW	Ash free dry weight
Al	Aluminium
AMDIS	Automated mass spectral deconvolution and identification system
ANOVA	Analysis of variance
aq	Aqueous
atm	Atmosphere
ATP	Adenosine triphosphate
bar	Atmospheric pressure

BCAAs	Branched-chain amino acids
C	Carbon
CA	Carbonic anhydrase
Ca	Calcium
$CaCl_2$	Calcium chloride
CCAP	Culture collection of algae and protozoa
$CHCl_3$	Chloroform/ trichloromethane
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
CHP	Combined heating and power
Cl	Chlorine
cm	Centimeter
cm^2	Centimeter squared
CO_2	Carbon dioxide
CO_2^{-3}	Carbonate
CO_3^{2-}	Carbonate ions
CoA	Coenzyme A
$CoCl_2.6H_2O$	Cobalt(II) chloride hexahydrate
CPD	Critical point dryer
$CuC1_2.2H_2O$	Copper(II) chloride dihydride
$CuSO_4.5H_2O$	Copper(II) sulfate pentahydrate
CV	Coefficient of variance
D.	*Dunaliella*
d^{-1}	Per day
D_{AB}	Binary diffusion coefficient
ddH_2O	Double distilled water
DF	Dilution factor
dH_2O	Distilled water
DHA	Dihydroxyacetone
DHAK	Dihydroxyacetone kinase
DHAP	Dihydroxyacetone-phosphate
DHAPR	Dihydroxyacetone phosphate reductase

DHAR	Dihydroxyacetone reductase
DIC	Dissolved inorganic carbon
div.	Division
DNA	Deoxyribonucleic acid
DW	Dry weight
E.C.	Enzyme commission
EDX	Energy-dispersive X-ray
EI	Electron ionization
eV	Electron volt
FBPA	Fructose-1,6-diphosphate aldolase
$FeC1_3.6H_2O$	Iron(III) chloride hexahydrate
G	Generation time
g	Grams
g	Centrifugal force
g/cm^3	Gram per cubic centimeter
g/l	Gram per liter
GC	Gas chromatography
GDH	Glycerol dehydrogenase
GHGs	Greenhouse gasses
GLYK	Glycerol kinase
GMD	Golm metabolome database
$gmol^{-1}$	Gram per mole
GPDH	Glycerol-3-phosphate dehydrogenase
GPI	Glucose-6-phosphate isomerase
GPP	Glycerol-3-phosphate phosphatase
H^+	Hydrogen ion
H_2O	Water
H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
HCl	Hydrochloric acid
HCO_3	Bicarbonate
HCO^{-3}	Bicarbonate

hr	Hour
IAA	Indole-3-acetic acid
J	Joules
K	Potassium
KCI	Potassium chloride
KH_2PO_4	Potassium hydrogen phosphate
KNO_3	Potassium nitrate
KV	Kilovolts
L	Length
LC	Liquid chromatography
L-DOPA	3,4-Dihydroxy-L-phenylalanine
LSD	Least significant difference
M	Molar
m/z	Mass-to-charge ratio
MBA	Marine biological association
MeOH	methanol
Mg	Magnesium
$MgCl_2$-$6H_2O$	Magnesium chloride hexahydrate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
min	Minute
min^{-1}	Per minute
ml	Milliliter
mmol	Millimoles per liter
$MnCl_2.4H_2O$	Manganese(II) chloride tetrahydrate
ms	Millisecond
MS	Mass spectrometry
MSTFA	N-Methyl-N-trimethylsilyl-trifluoroacetamide
N	Number of samples
N	Nitrogen
Na	Sodium
Na $(CH_3)_2$ $AsO_2.3H_2O$	Solution of sodium cacodylate
Na^+	Sodium ion

Na_2EDTA	Disodium ethylenediaminetatraacetate
$Na_2MoO_4.2H_2O$	Sodium molybdate
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaH_2PO_4.2H_2O$	Sodium dihydrogen phosphate dihydrate
$NaHCO_3$	Sodium bicarbonate/sodium hydrogen carbonate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
ND	Not detected
NH	Amino group
NH^+_4	Ammonium ion
NIST	National Institutes of Standards and Technology
nm	Nanometer
NMR	Nuclear magnetic resonance
N_o	Number of cells at a starting point
N_t	Number of cells after the period of time
O	Oxygen
^oC	Degree Celsius
OH	Hydroxyl group
P	Pressure
P	Phosphorus
PA	Phosphatidic acid
PAR	Photosynthetically active irradiation
P_c	Critical pressure
PCA	Principal component analysis
PFK	Phosphofructokinase
pg	Pico gram
PGM	Phosphoglucose mutase
pH	Potential of hydrogen
Pi	Inorganic phosphate

PIP	phosphatidylinositol 4-monophosphate
PIP_2	phosphatidylinositol 4,5-biphosphate
PPi	Pyrophosphate
q	“Studentized range” or Student's *q*
R^2	Coefficient of determination
RNA	Ribonucleic acid
RT	Retention time
RV1	High-pressure reaction vessel
s	Second
S	Sulphur
SC	Supercritical
SC-CO_2	Supercritical carbon dioxide
SD	Standard deviation
SE	Standard error
SEM	Scanning electron microscope
Si	Silicon
T	Temperature
t	Time
$t_2 - t_1$	Time interval in days
T_c	Critical temperature
TCA	Tricarboxylic acid
TD	Tyrosine decarboxylase
TMAO	Trimethylamine N-oxide
TMS	Trimethylsilyl
TPI	Triose phosphate isomerase
U	Unfolded
UV	Ultraviolet
v/v	Volume per volume
v/w	Volume per weight
VOCs	Volatile organic compounds
W	Width
w/v	Weight per volume

w/w	Weight per weight
Wm^2	Watts per meters squared
$ZnCl_2$	Zinc chloride
$ZnSO_4.7H_2O$	Zinc Sulfate Heptahydrate
δ-ALA	δ-Aminolevulinic acid

CHAPTER 1.0: GENERAL INTRODUCTION

1.1 Background

Biofuels, broadly defined as solid, liquid, or gaseous fuels produced from biomass, have been the focus of considerable research due to their potential as substitutes for fossil fuels (Fangrui and Milford, 1999; Ooi *et al*., 2004; Nigam and Singh, 2011). Biofuels are similar in physical characteristics to mineral-based fuels, but differ in that they are produced from renewable natural resources. Biomass-derived fuels can play an important role in filling the gap between limited fuel supplies and increasing worldwide demand. Biomass is the only low carbon alternative source for the production of liquid fuels (Dimitriou and Bridgwater, 2010). Liquid biofuels in general offer environmental benefits because they have 'low carbon' emissions. According to Lendle and Schaus (2010) not all biofuels are carbon neutral because their use will never lead to a 100 % reduction in atmospheric greenhouse gasses (GHGs) emissions. Biofuels need to be sustainable and also not compete with land for food. In addition to reducing the adverse environmental effects of the fossil fuels, biofuels have the potential to reduce vulnerability to fossil fuel depletion (Ooi *et al*., 2004; Nigam and Singh, 2011).

The technology for the production of biofuels is not new: humans have been making ethanol and fatty acid derivatives for combustion in engines for decades (Demirbas, 2003; Balat, 2006). One of the inventors to convince people of the use of ethanol was a German scientist named Nikolaus August Otto in 1861. Starch-based crops such as corn are widely used for bioethanol production (Ziska *et al*., 2009). Rudolf Diesel invented the diesel engine in 1900 to run on peanut oil (Demirbas, 2003; Balat, 2006). Biodiesel that serves as a replacement for hydrocarbon fuels in diesel engines is generally comprised of fatty acid methyl-esters from oleaginous crops such as palm oil, soybean oil, sunflower oil, coconut oil, rapeseed oil and tung oil (Ma and Hanna, 1999; Balat, 2006; Huber *et al.*, 2006; Gouveia and Oliveira, 2009). Recently research has focused on the development of concepts such as renewable resources, sustainable development, green energy and eco-friendly processes (Demirbas, 2006). Developed countries are employing modern technologies and efficient bio-energy conversion

to produce a range of biofuels, which are becoming cost-wise competitive with fossil fuels (Demirbas, 2006).

The major types of biofuels include ethanol, biodiesel, methanol, and butanol; however, the two principal biofuels are ethanol and biodiesel. Biofuels that are presently in commercial production are the so-called "first generation biofuels" that are produced from food sources: these include bioethanol produced from sugar or starch crops such as sugar cane, corn, sugar beet or wheat, and biodiesel made from oily plants such as rape seed and soya beans, and from animal fats. Biodiesel is produced from triglycerides of plant and animal origin by transesterification (also called alcoholysis) by reaction with alcohol, usually methanol (Antoni *et al*., 2007; El-Mashad *et al*., 2007). The by-products of the transesterification process include glycerol, un-reacted catalyst and alcohol. Glycerol has some value as a feedstock for a wide range of industrial processes to provide higher value chemicals. However, recent research has shown that glycerol can also be considered as a biofuel. Glycerol fuel can be used to generate power in combined heating and power (CHP) engines and thermal batteries (Day *et al*., 2011). It could also be used to power ships. The potential of glycerol in power generation, transport and fuel storage could result in a high demand, which will increase the requirement for oil crops for making biodiesel. There are some problems in using oil crops as renewable feedstock for glycerol production. Apart from competing with oil crops for human consumption the oil has to be esterified to obtain glycerol as a by-product. However, there are alternative approaches. This thesis presents results on research to optimize the production of glycerol from microalgae, and become a "second generation biofuel" produced from non-food sources.

1.1.1 Occurrence of glycerol

Glycerol can be sourced from (i) transesterification of plant oil and animal fat, (ii) saponification and (iii) microorganisms; as a by-product of fermentative metabolism and *de novo* synthesis (Taherzadeh *et al*., 2002).

Glycerol is a prominent metabolite produced by several microorganisms through the process of fermentation. Studies concerning glycerol formation have been carried out using baker's

yeast, *Sacchromyces cerevisiae,* since 1858 (Wang *et al*., 2001; Taherzadeh *et al*., 2002). *S. cerevisiae* was employed for glycerol production because:

(1) Glycerol is a predominant by-product of the fermentative metabolism of sugar to ethanol in a redox-neutral process in the organism (Taherzadeh *et al*., 2002); substantial production of glycerol from monosaccharides was achieved in yeast by forming a complex of acetaldehyde with the bisulphite ion that limits ethanol production and promotes reoxidation of glycolytically-formed NADH by glycerol synthesis.

(2) Glycerol serves as a functional osmolyte (balancing external osmotic pressure) in osmotolerant yeasts (Rehm, 1988; Agarwal, 1990; Taherzadeh *et al*., 2002). In addition to yeasts there are a number of microorganisms that serve as potential candidates for glycerol production. The disadvantages of fermentative processes using these microbes have been the low yield of glycerol and difficulties in the recovery of glycerol from the fermentation broth (Taherzadeh *et al*., 2002); yeasts are also heterotrophic. Table 1.1 shows a number of microorganisms that have been studied as potential candidates for glycerol production. The advantage of microalgae as compared with other sources is that they are photosynthetic and use CO_2 and water to produce glycerol. They are able to grow in liquid medium such as saline water and waste water streams, thereby reducing the use of freshwater.

Table 1-1 Example of microorganisms in which glycerol production has been studied (adapted from Taherzadeh *et al*., 2002)

Genera	Strain
Yeast	*Saccharomyces cerevisiae*
	Saccharomyces ellipsoideus
	Zygosaccharomyces rouxii
Mold	*Debaryomyces mogii*
	Rhizopus nigricans
	Rhizopus javanicus
Bacteria	*Bacillus subtilis*
	Bacillus coli
	Bacterium orleanense
Protozoa	*Trypanosoma cruzi*
Microalgae	*Dunaliella tertiolecta*
	Dunaliella salina
	Dunaliella viridis

1.1.2 Properties of Glycerol

Glycerol (glycerine and glycerin), propan-1, 2, 3-triol (figure 1.1), is a simple alcohol with two primary and one secondary alcohol. Glycerol has a molar mass of 92.09 $gmol^{-1}$, density of 1.261 g/cm^3, melting point of 17.8 ^oC, and boiling point of 290 ^oC. It is miscible with water and alcohol; insoluble in ether, benzene, and chloroform. Glycerol is a clear, colourless, odourless, sweet-tasting syrupy liquid. This trivalent alcohol is widely used for a variety of purposes in a number of industries such as food, tobacco, pharmaceutical, cosmetic, paint, pulp and paper, leather, textile and chemical industries (Zhuge *et al*., 2001; Kacka and Donmez 2008). In addition to these commonly known applications, glycerol also possesses desirable fuel characteristics. Glycerol is (i) a liquid, (ii) highly combustible but not explosive, (iii) has a high energy to mass ratio, (iv) stable on long-term storage, and (v) is transportable by pipeline (Wackett, 2008).

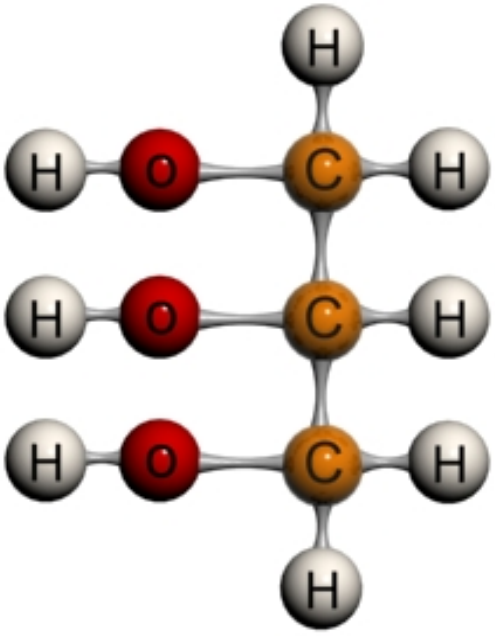


Fig. 1-1 Structure of glycerol (propan - 1, 2, 3-triol)

1.1.3 Commercial production of glycerol

In the 1970s and 80s, many research articles were published on the commercial production of carotenoids from halotolerant microalgae (Ben-Amotz *et al*., 1987). In addition to β-carotene production, *Dunaliella* was reported to also produce abundant quantities of glycerol, although during that time less importance was placed on glycerol production compared with β-carotene. The intracellular glycerol concentration of *D. parva* was up to 2.1 M when grown in a medium containing 1.5 M NaCl (Ben-Amotz and Avron, 1973). Chen and Chi (1981) speculated that *Dunaliella* may be the best agent to be used for commercial production of glycerol, but to-date glycerol has not been considered as a fuel. This situation changed in 2010 when Aquafuel Research Limited Kent, United Kingdom developed a technology that showed that glycerol has the potential as the cleanest and most efficient fuel for combined heating and power (CCHP) application.

Dunaliella species grow globally and have the ability to produce glycerol at high salinity. This research explored new strains of halotolerant microalgae *Dunaliella* (T35, T36 and T37) and *Asteromonas* (T33a, T33b and T33c) isolated from saline marine water in Namibia.

These three strains of *Dunaliella* were shown by the Marine Biological Association (UK) to be genetically distinct from all other known *Dunaliella* strains as evidenced by sequencing and phylogenetic analyses of the 28S rRNA gene (see Figure 1.2). This research will investigate and compare the level of glycerol produced by these three strains of *Dunaliella* and *Asteromonas* as well as other strains.

This research will also provide detailed information that could be used to maximise the growth of *Dunaliella* and *Asteromonas* strains for glycerol accumulation by revealing effects of the following variables: temperature, light regime, initial pH and level of salinity.

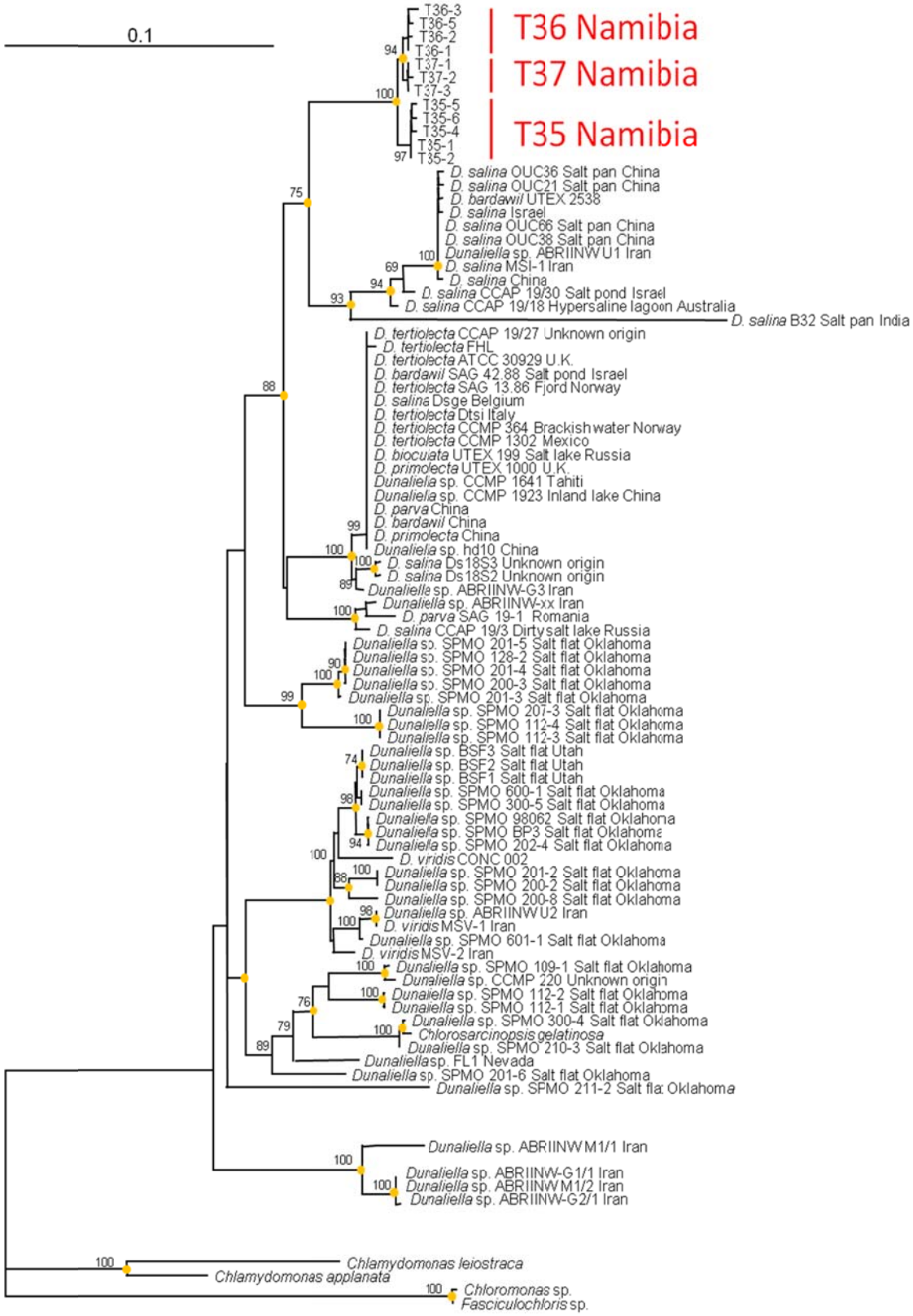


Fig. 1-2 Microalgal phylogenetic tree constructed for *Dunaliella* showing the distance in relatedness of strains T35, T36 and T37 isolated from Namibia (Harvey *et al*., 2013)

1.1.4 Research objectives

In order to develop a mass culture of halotolerant microalgae to supply a large amount of renewable glycerol, an in-depth investigation of the cell growth kinetics, substrate utilization, salinity tolerance and metabolic profile of these microalgal strains is needed. This information is crucial to the future optimization of a mass microalgal glycerol production process. The aim of this research is to identify the conditions under which two new strains of halotolerant microalgae, *Dunaliella* (T35, T36 and T37 and *Asteromonas* (T33a, T33b and T33c) obtained from saline river of Namibia produce glycerol. This research has four major objectives:

1. Determination of the optimum conditions of pH value, temperature, light and salinity for biomass and glycerol production in strains of *Dunaliella* and *Asteromonas* isolated from saline waters in Namibia;
2. Comparison of the morphological and biochemical characteristics of algae cultivated in media of different salinities;
3. Identification of metabolites associated with glycerol accumulation and dissimilation in osmotically stressed cells of *Dunaliella*; and
4. Development of an extraction procedure to maximize glycerol recovery from algal cultures using supercritical carbon dioxide (SC-CO_2) technology.

1.1.5 Organization of Thesis

The introductory chapter 1.0 sets out briefly the background and general objectives of this study, followed by literature review in chapter 2.0 relating to halotolerant microalgae, osmolytes, metabolomic study and SC-CO_2 extraction. Chapter 3.0 presents the general materials and methods used during this study with the quality assurance and control measures adopted in order to provide confidence in the results obtained. Chapter 4.0 describes the growth parameters of two species of halotolerant microalgae. Method Development for Quantifying Glycerol in *Dunaliella* Cells is presented in chapter 5.0. Chapter 6.0 describes glycerol accumulation in *Dunaliella* and *Asteromonas* at different salinity and determination of glycerol and starch production under hyperosmotic and hypo-osmotic stress. It also

describes cell's size and volume characterization of the organisms stressed at the various salinities. Chapter 7.0 describes the effect of salinity on cell elemental composition of *Dunaliella* and *Asteromonas* species. Metabolomics approach for assessing hyperosmotic shock of microalgae, *Dunaliella* T35, is reported in chapter 8.0. Chapter 9.0 aims to develop a technique using supercritical carbon dioxide (SC-CO_2) technology to extract glycerol from halotolerant microalgae. The thesis summary and future work are presented in chapter 10.0.

CHAPTER 2.0: LITERATURE REVIEW

2.1 Microalgae flexibility for biofuels production

Microalgal cells are known to have the potential to survive competitive/severe environments by developing defence strategies that result in significant production of diverse protective chemicals from different metabolic pathways (Cardozo *et al*., 2007). Similar to plants, microalgae use the processes of photosynthesis to convert solar energy into chemical energy. This natural process of harvesting solar energy has been used in the development of algal cultivation systems for waste water treatment, animal feeds, fertilizers, biologically-active substances, essential compounds for human nutrition as well as a number of secondary metabolites of pharmaceutical and nutraceutical potential (Chaumont, 1993; Cardozo *et al*., 2007; Singh and Gu, 2010). In addition to all these, microalgae are gaining special interest in the present day energy scenario due to their fast growth, which is coupled with the accumulation of relatively high concentration of lipid, proteins and carbohydrate. These properties render microalgae as an excellent potential source for biofuels such as biodiesel, bioethanol and biomethane, biohydrogen and glycerol (Schenk *et al*., 2008; Singh and Gu, 2010; Patil *et al*., 2011).

Microalgae comprise a vast group of several thousand diverse species that enables the selection of preferred species for the production of the desired biofuel. The yield of biofuels (such as biodiesel, bioethanol and bio-methane) from microalgae is species- and strain-dependent because they respond differently to environmental conditions and nutrients. For example, the oil content of *Botryococcus braunii, Chlorella emersonii, Chlorella protothecoides, Chlorella vulgaris, Dunaliella tertiolecta, Nannochloropsis oculata, Neochloris oleoabundans, Phaeodactylum tricornutum, Porphyridium cruentum and Scenedesmus obliquus* can reach up to 55-75 % by dry weight biomass (Mata *et al*., 2010).

Algae that can accumulate considerable amounts of biomass in the form of starch, which can be converted to fermentable sugars, are considered to be suitable agents for bioethanol production. Species such as Chlorella, Spirulina and Chlamydomonas are known to contain a large amount (> 50 % of the dry weight) of starch (John *et al*., 2011). Algae with high

accumulation of polysaccharides in the form of alginate, laminaran and mannitol with limited amount of cellulose and zero lignin are considered as an excellent material/resource for the production of biomethane by anaerobic digestion processes. Vergara-Fernandez *et al*. (2008) described two species of marine algae, *Macrocystis pyrifera* and *Durvillea antarctica,* which are considered suitable for biomethane production.

Green algae without cell wall such as *Dunaliella* and *Asteromonas* species are types of halophilic algae found in sea water. These microalgae accumulate polysaccharides (starch), as well as large concentrations of β-carotene to protect themselves against the intense light, and high concentrations of glycerol as an osmolyte (protection against osmotic pressure) (Ben-Amotz and Avron, 1973; Ben-Amotz and Grunwald, 1981; Ben-Amotz and Avron, 1990). The mass production of β-carotene using *Dunaliella* species is well developed under large-scale conditions for commercialization (Ben-Amotz and Avron, 1990).

2.2 Algae classification

Algae are a large and diverse group of eukaryotic photosynthetic and non-photosynthetic organisms growing in almost every habitat; they can be aquatic or subaerial. Algae tolerate a broad range of environmental factors such pH, temperature, turbidity, salinity and CO_2 concentration (Tartar and Boucias, 2004; Barsanti and Gualtieri, 2006). Cyanobacteria were historically called blue-green algae but are now excluded from the algal group as they are prokaryotic organisms. The four major groups of eukaryotic organisms are: (1) Unikonts which include Opisthokonts and Amoebozoa (2) Archaeplastida, (3) Rhizaria, Alveolates and Stramenopiles (RAS) and (4) Excavates, mitochondriate and core amitochondriate excavates (Burki *et al*., 2007; Hackett *et al*., 2007; Baldauf, 2008). Figure 2.1 shows how algae and other already characterized eukaryotes were assigned to one of eight groups based on molecular phylogenetic and ultrastructural data (Baldauf, 2003; Baldauf, 2008).

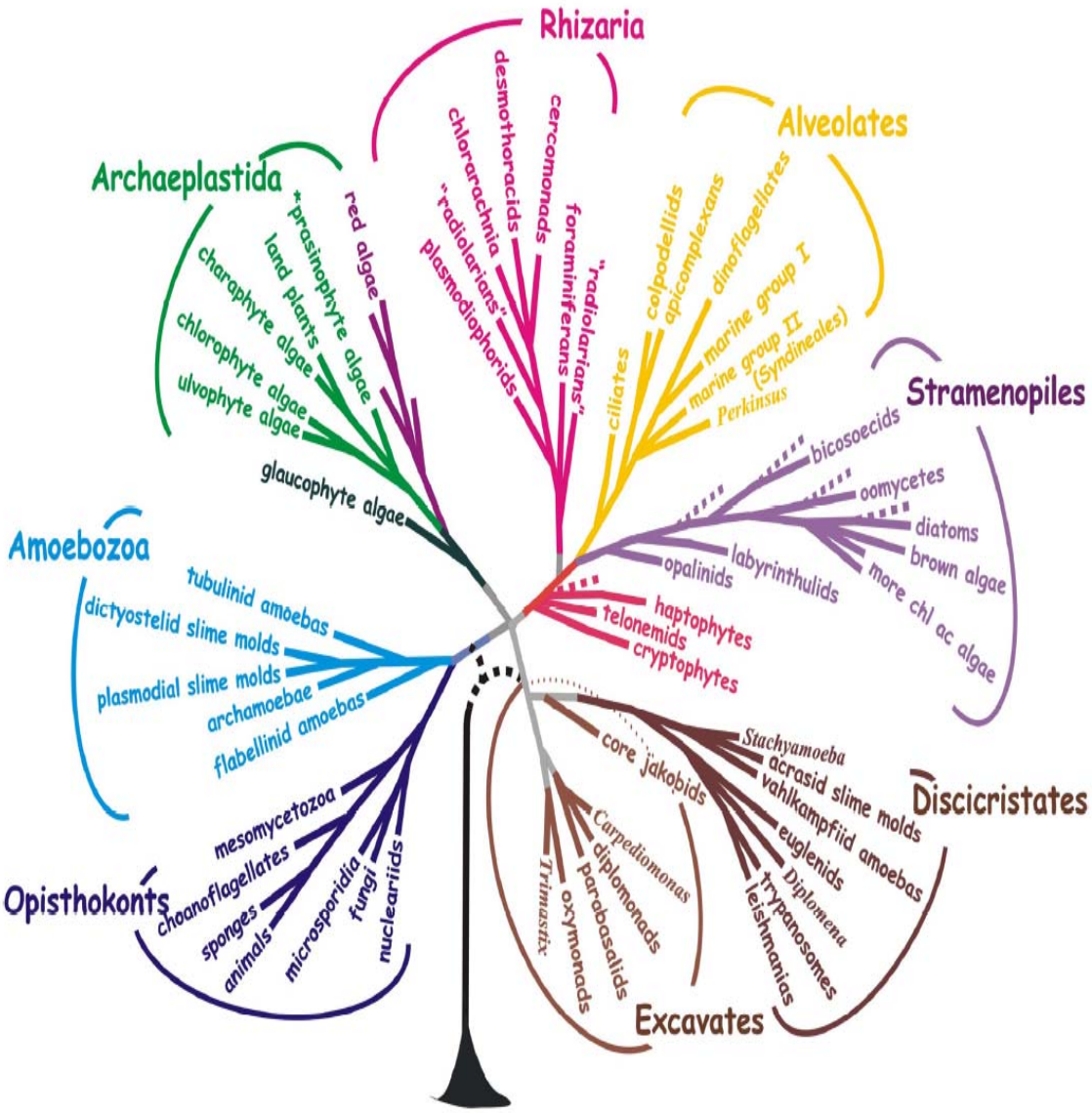


Fig. 2-1 The major groups of eukaryotes based on published molecular phylogenetic and ultrastructural data showing the groups of algae and other eukaryotes (Baldauf, 2003; Baldauf, 2008).

This review will focus on *Chlorophyta* (green algae) of the group Archaeplastida. *Chlorophyta* is a large group of organisms with a large variability; they are divided into two phyla: prasinophytes and core chlorophytes (Leliaert *et al*., 2012; Barsanti *et al*., 2013). Green algae possess synapomorphic characters that include a chloroplast with a double membrane (containing chlorophyll *a* and *b*), an eyespot and a chloroplast-embedded pyrenoid (Richmond, 2004; Barsanti and Gualtieri, 2006; Barsanti *et al*., 2013). The classification of

the *Chlorophyta* presented here relies on a combination of morphological, ultrastructural and molecular phylogenetic features. Within the core *chlorophytes* clade there are three well-supported groups: *Ulvophyceae*, *Trebouxiophyceae* and *Chlorophyceae* (UTC) (Lewis and McCourt, 2004; Leliaert *et al*., 2012; Barsanti *et al*., 2013).

Members of the group *Chlorophyta* are commonly found in marine, freshwater and terrestrial environments. All members of core *chlorophytes* have swimming cells with two or four anterior flagella. The flagella basal bodies are associated with four microtubular rootlets that are cruciately arranged, which alternate between two and higher numbers of microtubules, according to the formula (X-2-X-2) (Lewis and McCourt, 2004; Barsanti and Gualtieri 2006; Leliaert *et al*. 2012; Barsanti *et al*., 2013). The orientations of the basal bodies were used to discriminative between these algae. According to Lewis and McCourt (2004) the *Chlorophyceae* have a direct opposite or clockwise orientation of the basal bodies, whereas *Trebouxiophyceae* and *Ulvophyceae* have a counter-clockwise orientation of the basal bodies. UTC characterization is mediated by a phycoplast mode of cell division (a new system of microtubules that develops parallel to the plane of nuclear division), which was later lost in the *Ulvophyceae* (Leliaert *et al*., 2012). UTC shared certain number of ultrastructural characteristic between them (Table 2.1).

Molecular phylogenetic analyses have been considered as a new method for reconstructing the evolutionary relationships between UTC algae. Molecular phylogenetic studies of nuclear ribosomal DNA (18S nrDNA) sequences (Lewis and Lewis, 2005; Watanabe and Nakayama, 2007), chloroplast genome (Pombert *et al.*, 2005; Turmel *et al.*, 2008), mitochondrial genome (Turmel *et al*. 2003; Pombert *et al.*, 2004) and plastid genome (Matsumoto *et al*., 2011) have supported the monophyly of UTC groups.

The initial molecular phylogenetic studies based on 18S nrDNA sequences shows that the *Ulvophyceae* branch first, leaving *Chlorophyceae* and *Trebouxiophyceae* as sisters (Krienitz *et al*. 2001; Lopez-Bautista and Chapman, 2003; Lewis and McCourt, 2004). Other phylogenetic studies using 18S revealed a sister relation between *Chlorophyceae* and *Ulvophyceae* (Lewis and Lewis 2005; Watanabe and Nakayama, 2007). Phylogenetic analysis of chloroplast genes was also supported a sister relation between *Trebouxiophyceae* and *Ulvophyceae* (Pombert *et al*., 2005). Many plastid genes have been recognized as

homologs in UTC algae (Matsumoto *et al*., 2011). The relationship based on molecular data between the UTC has been reviewed by Leliaert *et al*. (2012) (Figure 2.2). This molecular evidence has contributed in confirming the ultrastructural-based hypothesis which shows that the green algae diverged into two discrete clades: the core *chlorophytes* and *prasinophytes*. According to Leliaert *et al*. (2012) phylogenetic relationships among and within the main clades of the core chlorophytes (UTC) have not been fully resolved.

Table 2-1 The differences and similarities in cellular organization, chloroplast morphology, cell wall composition, and life histories of UTC algae (Lewis and McCourt, 2004; Baldauf, 2008; Leliaert *et al*., 2012)

	Chlorophyceae	***Ulvophyceae***	***Trebouxiophyceae***
Habitats	Freshwater and terrestrial algae	Freshwater, marine and terrestrial algae	Mainly include marine green algae
Morphological diversity	Unicells (flagellates and non-flagellates)	Non-flagellate unicells or colonies, unbranched or branched filaments, or small blades	Ranges from flagellate and non-flagellate unicells and colonies to branched and unbranched filaments, foliose blades and multinucleate life forms
Mode of reproduction	Asexual and sexual reproduction	Commonly produce asexual, non-motile autospores	Sexual reproduction
Ultrastructural characteristics	Clockwise basal body orientation	Counter clockwise orientation of the basal bodies	Counter clockwise orientation of the basal bodies

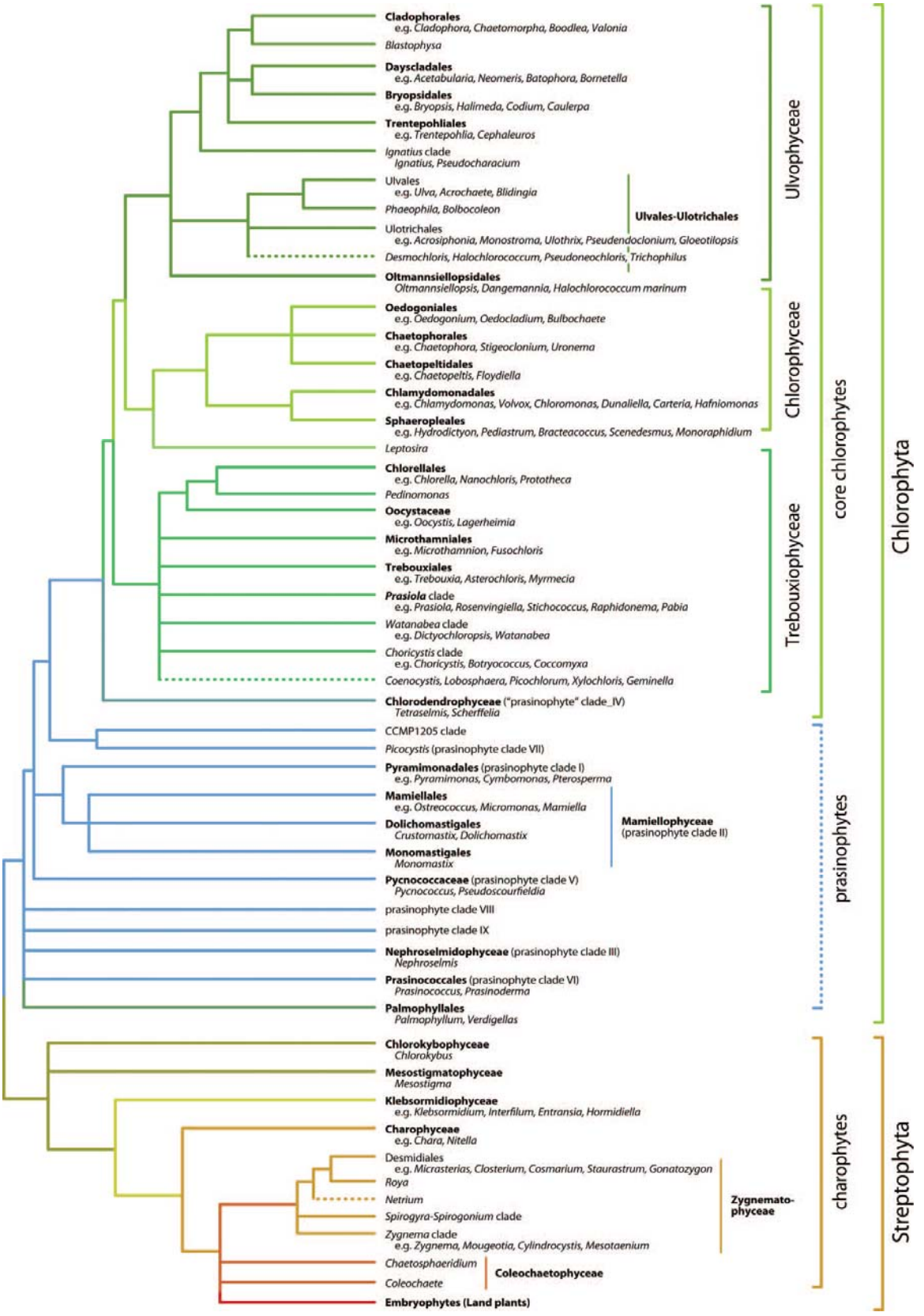


Fig. 2-2 Molecular phylogenetic relationships between green algae. For the details explanations of the various clades see Leliaert *et al*. (2012).

2.3 The Description of the genus *Dunaliella*

The microalgal genus, *Dunaliella*, comprises halophilic species adapted to hypersaline environments. It was first identified by a French Scientist Michel Felix Dunal in (1838) and was later established by Teodoresco in 1905 (Chen and Jiang 2009; Jayappriyan *et al*., 2010; Ramos *et al*., 2011). Algae of the *Dunaliella* genus have been the subject of many physiological studies due to the ability of some members/species to accumulate glycerol and carotenoids (Borowitzka, 1988).

2.3.1 Taxonomy

The genus *Dunaliella* was placed in the class *Chlorophyceae*, the order *Volvocales* and the family *Polyblepharidaceae*. Borowitzka and Siva (2007) reported a different classification that placed *Dunaliella* in the new order *Dunaliellales* with the family *Dunaliellaceae*. The present taxonomic classification of the genus is based both on the morphological and physiological or biochemical attributes (Gonzalez *et al*., 1998; Borowitzka and Siva 2007). The common morphological descriptions reported were cell shape, cell size and symmetry, chloroplast shape and presence or absence of pyrenoid (a proteinaceous structure found within the chloroplast of certain algae; function as a center for starch production) and refractile granules in the cytoplasm. In addition to all those features tolerance to osmotic shock (salinity tolerance) and accumulation of large amount of β-carotene in the chloroplast have been used to differentiate within the genus (Gonzalez *et al*., 1998; Borowitzka and Siva, 2007). *Dunaliella* varies in size from 3-13 μm width and 5-25 μm in length (Avron and Ben-Amotz, 1992; Oren, 2005; Jayappriyan *et al*., 2010; Ramos *et al*., 2011). The total number of species in the genus *Dunaliella* remains at twenty eight. Twenty three occur in saline environment and five in fresh water (Jayappriyan *et al*., 2010). The best known examples of freshwater species are *D. acidophila, D. lateralis, D. flagellata, D. obliqua* and *D. paupera* and the marine species are *D. maritima, D. polymorpha, D. primolecta, D. quartolecta* and *D. tertiolecta*, with most favourable salinities for growth of about 2 to 4 % NaCl, and the hypersaline species are (*D. viridis, D. parva and D. salina*) with optimum salinity for growth >6 % NaCl (Jimenez and Niell, 1991; Borowitzka and Siva, 2007; Tafreshi and Shariati, 2009).

2.3.2 Morphology

Dunaliella species are photosynthetic (Ben-Amotz and Avron, 1978), unicellular, rod shaped or ovoid forms, ellipsoid, pyriform or fusiform. The cells become spherical under extreme conditions such as osmotic shock before returning to their normal shape. Giant and irregular structures are formed under low growth temperature and when the cultures become old (Borowitzka and Siva, 2007).

Dunaliella species lack a rigid cell wall and hence are regarded as naked green microalgae. They are enclosed by a thin plasma membrane or periplast that is made up of a mucilaginous glycoprotein coat called a glycocalyx, which permits rapid changes in cell shape and volume in response to osmotic changes (Avron and Ben-Amotz, 1992; Oren, 2005; Jayappriyan *et al*., 2010; Ramos *et al*., 2011). According to Borowitzka and Siva (2007) a glycocalyx cell covering can be seen under the electron microscope which is also possible to visualise under a light microscope using Indian ink or stain such as alcian blue or ruthenium red. The cells are biflagellate and motile. The flagella length varies with species (Borowitzka and Siva, 2007). The eyespot is present and has an anterior peripheral location in the chloroplast. In some species (such as *D. salina),* the eyespot may be hardly visible in the light microscope (Borowitzka and Borowitzka, 1988; Avron and Ben Amotz, 1992). The presence or absence of eyespots is a distinguishing feature between *D. salina* and *D. viridis*. Parra *et al.* (1990) observed that the *D. salina* strain UTEX-209 did not demonstrate the presence of the eyespot.

2.3.3 Vegetative reproduction

Dunaliella can reproduce by either vegetative (asexual) or sexual means (Oren, 2005; Borowitzka and Siva, 2007). Asexual reproduction is by longitudinal division into two or four morphologically equal daughter cells (binary fission) (figure 2.3). In their description of vegetative reproduction, Borowitzka and Siva (2007) found that *D. salina* cell division begins with nuclear division followed immediately by an infurrowing (a groove formed in the membrane of a dividing algae) of the cell. As the cell volume increases a longitudinal division plane is formed. The cell infurrowing occurs in vegetative reproduction, but different opinions exist as to whether this infurrowing begins at the posterior or anterior end of the

cell. In their findings Borowitzka and Siva (2007) concluded that cell infurrowing started at the flagella (anterior) end of the cell, which was followed immediately at the opposite (posterior) end of the cell. Thus, flagella duplication can be completed prior to the completion of cell division, such that large cells with four flagella may be visible.

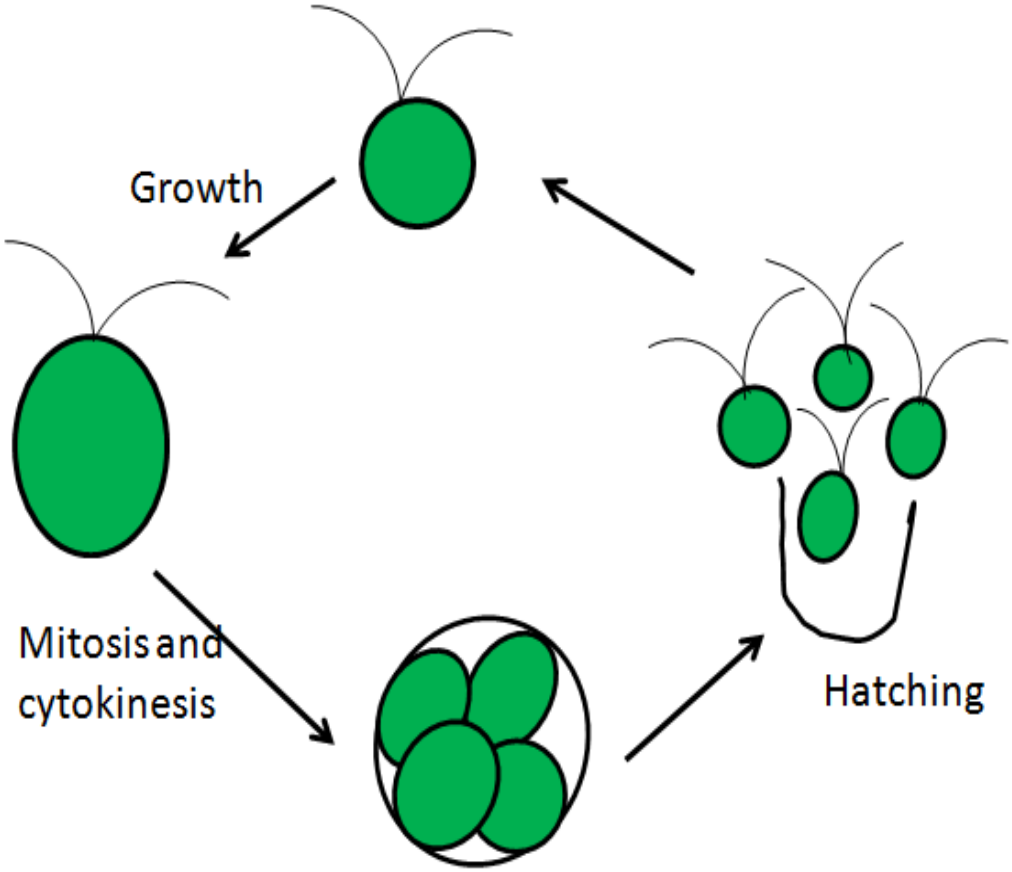


Fig. 2-3 Diagrammatic representation of *Dunaliella* asexual reproduction. During asexual reproduction, cells grow and undergo two or more rounds of mitosis and cytokinesis before the daughter cells hatch from the old cell membrane

As part of the vegetative mode of reproduction algae in the *Dunaliella* genus form a spherical non-motile cell (palmella stage) when exposed to extreme conditions such as hyperosmotic and hypo-osmotic stress. *Dunaliella* was reported to produce vegetative cysts (non-motile spores called aplanospores) at about 10 % NaCl or less (Borowitzka and Huisman, 1993; Borowitzka and Siva, 2007).

2.3.4 Sexual reproduction

Although *Dunaliella* reproduces asexually, adverse conditions such hypoosmotic shock (about 10 % NaCl or less) and nitrogen depletion of the culture medium can stimulate the initiation of sexual reproduction. This process of gamete formation (gametogenesis) can either be heterogamous or isogamous. The latter is the form of sexual reproduction in *Dunaliella* in which the gametes produced are identical in size, shape and motility. According to Borowitzka and Siva (2007) mating occurs between different (+/–) mating strains. The two cells first join their flagella, beginning at the tips of the flagella. Once the flagella have come together for most of their length, the (+) gamete then produces a thin mating tube between its flagella which connects with the (–) gamete between its flagella (figure 2.4). Two gametes fuse and a zygote is formed. The zygote has a thick outer layer that can withstand exposure to fresh water and also survive prolonged period of dryness (Oren, 2005). Germination of these zygotes could give rise to 4, 6, 8, 16 and up to 32 haploid daughter cells (Oren, 2005; Borowitzka and Siva, 2007).

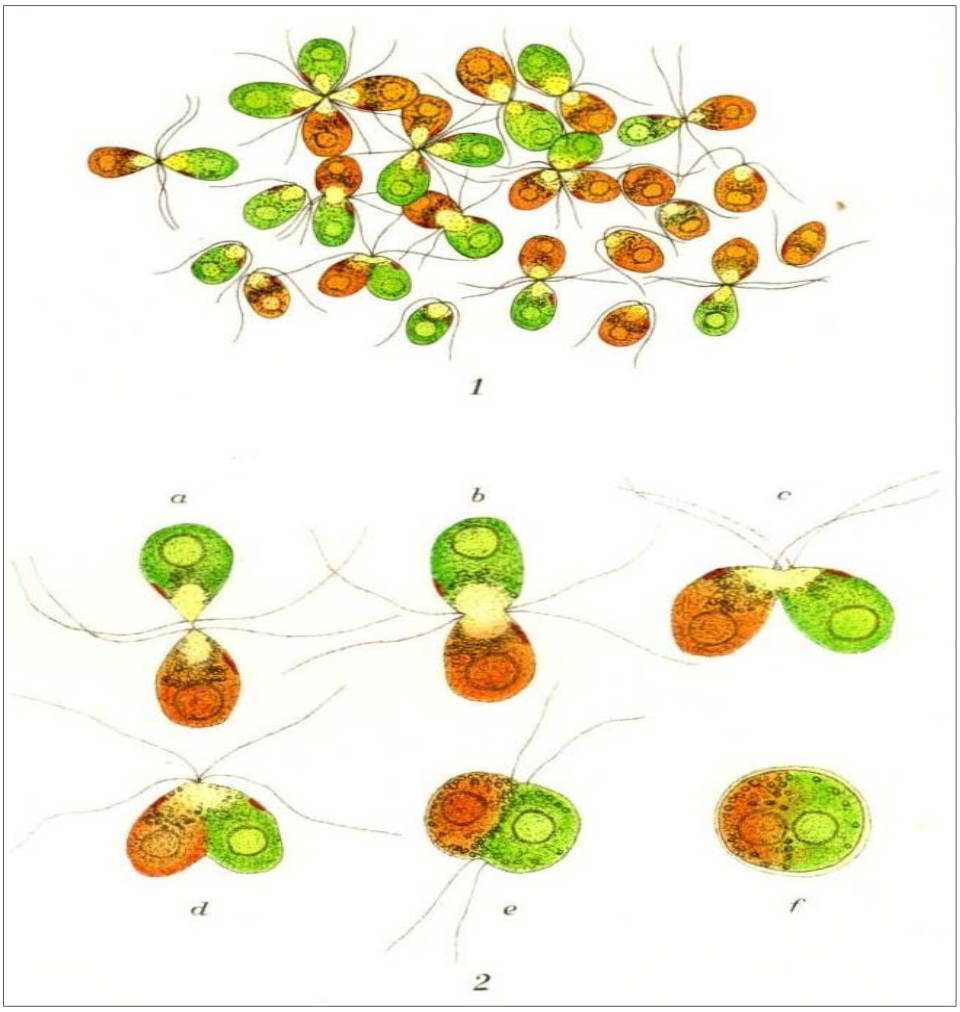


Fig. 2-4 Diagrammatic representation showing aggregation and zygote formation of *D. salina* (green and red form). Fusion of gametes, (b, c and d) (plasmogamy and karyogamy) to form a zygote, (e) meiosis occurs to form four haploid progeny cells and (f) shows how each of which grows into an asexual cell of the respective mating type. Adapted from Oren, (2005)

2.4 The Description of the genus *Asteromonas*

Species of the genus *Asteromonas* algae are halophytic and green wall-less unicellular flagellate algae of the class *Prasinophyceae* present in salt marshes and small brine ponds (Peterfi and Manton, 1968; Ben-Amotz and Grunwald, 1981; Hotos, 2003). *Asteromonas gracilis* Artari was first described by Artari (1913 and 1916) and has subsequently been encountered in many other parts of the globe in related habitats. *Asteromonas* cells are biflagellate and pointed (sometimes bluntly so) at both ends. Division occurs during

locomotion. The chloroplast is cup shaped, with an anterior stigma and a large pyrenoid. Similar to *Dunaliella* the absence of a cell wall and the flexibility of the periplast permit rapid changes of shape to take place when cells are brought to hypoosmotic shock/dilution (Peterfi and Manton, 1968; Hotos, 2003). Although *Asteromonas* and *Dunaliella* differ profoundly in the general organisation of the cell and in the structural details of the pyrenoid, both of them show a similar external appearance; they are naked, biflagellated, green cells with anteriorly attached flagella and the starch staining brownish with iodine. Other similarities exist on the basis of fine structure which are too numerous to discuss individually and can also be considered to be beyond the scope of this chapter (for review see Peterfi and Manton, 1968).

Halophytic microalgae are often subjected to widely fluctuating salt concentrations and can tolerate a broad range of salt, from low to high saline medium. Contrary to the amount of literature available that elucidates the mechanism of osmotic adaptation and glycerol accumulation in *Dunaliella* under osmotic stress, the information on osmoregulation and glycerol accumulation in *Asteromonas* is rather limited (Ben-Amotz and Grunwald, 1981). *Asteromonas* and *Dunaliella* can maintain a high concentration gradient of glycerol and thus are able to survive in a saline medium (Wegmann *et al*., 1980; Ben-Amotz and Grunwald, 1981).

2.5 Growing at various geographical locations

When Dunal first identified *Dunaliella*, he described it as a microalga living in concentrated brines (Oren, 2005). Microalgae similar to the one reported by Dunal were observed by other scientists in hypersaline lakes and in oceans all over the world (Africa, America, Asia, Australia and Europe) (Ginzburg, 1987; Borowitzka and Borowitzka, 1988; Tran *et al*., 2013).

The current research was set to investigate *Dunaliella* obtained from Swakopmund in the Erongo region of Namibia, Africa. In the west, Erongo has a shoreline on the Atlantic Ocean. On land, it borders the following regions: Kunene to the north, Otjozondjupa to the east, Khomas to the southeast and Hardap to the south (See Figure 2.5) (Central Bureau of Statistics, 2010).

The western Erongo region falls entirely within the Namib Desert and the cold Atlantic Ocean to the west. The annual mean temperature ranges between 16 to 25 °C, mean maximum temperature in summer ranges between 20 to 36 ^{o}C and mean minimum temperature in winter 8 to 12 ^{o}C. The mean annual rainfall is < 50 to 300 mm and most rain falls between October to April (88 – 95 %) (Erongo Regional Council, 2005). Figure 2.6 shows Swakopmund's brown salt lakes; the brown colour of the lake is due to the high concentration of salt tolerant algae such as *Dunaliella* and *Asteromonas*.

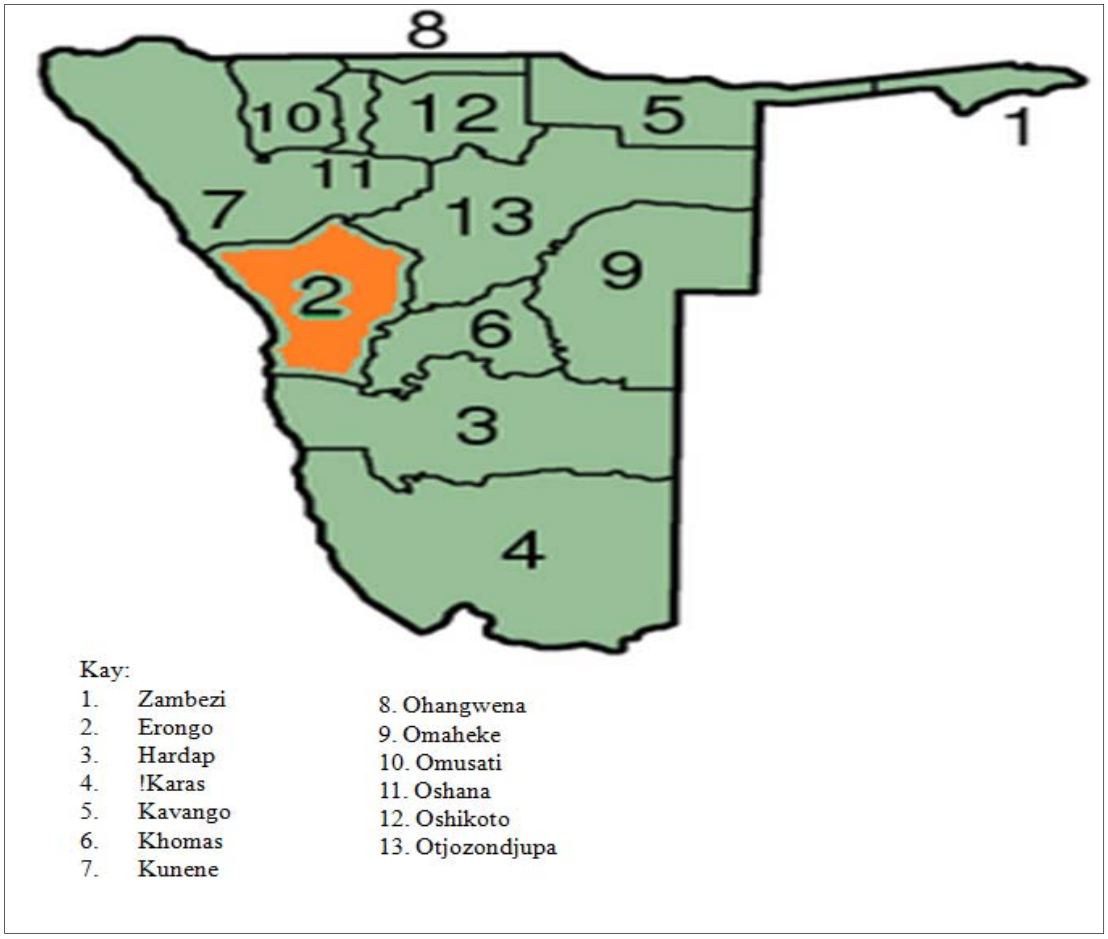


Fig. 2-5 Map of Namibia showing different regions.

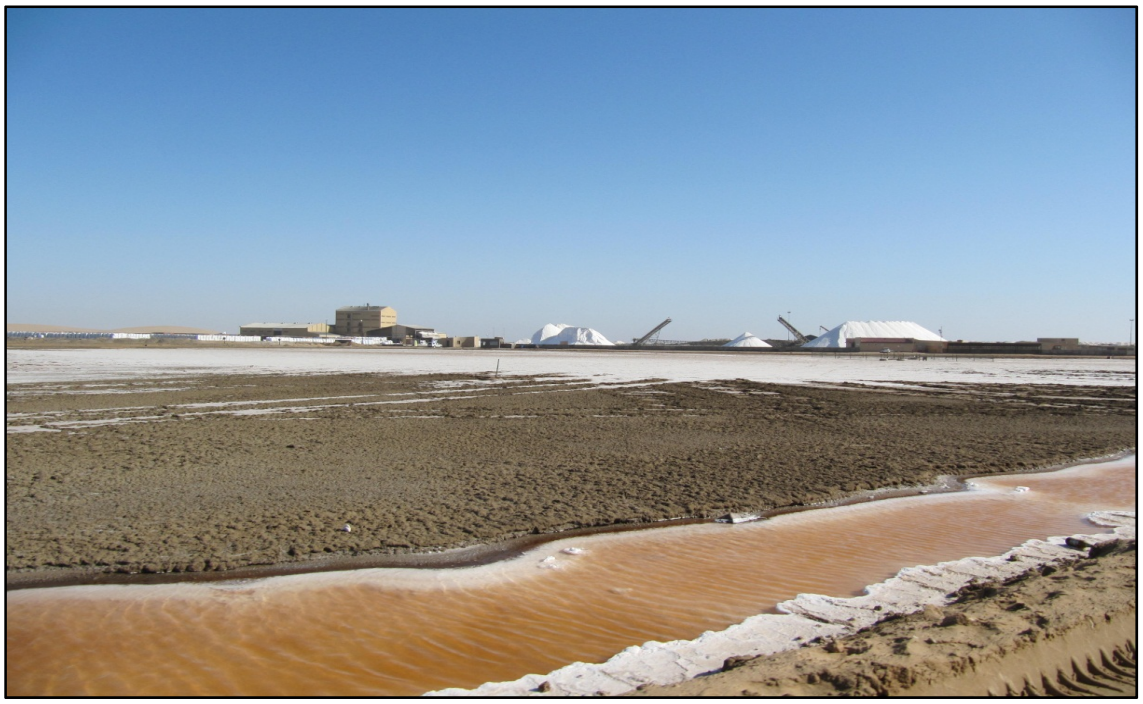


Fig. 2-6 Salt Lake at Swakopmund of Erongo region. The brown colour is due to a bloom of halotolerant microalgae

2.6 Cultivation of halotolerant microalgae

Research to date has focused on the ability of halotolerant microalgae to accumulate concentrations of β-carotene and glycerol. Optimizing microalgae growth rates by manipulating environmental parameters such as salinity, temperature and pH is an effective approach to increase microalgae biomass production (Avron and Ben-Amotz, 1978; Ben-Amotz, 1980; Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989; Avron, 1992; Mata *et al*., 2010)

2.6.1 Salinity

The ability of *Dunaliella* cells to grow in a saline environment under the influence of osmotic stress has received considerable attention. These microalgae demonstrate a remarkable degree of adaptation to salinity in their natural habitats, growing in media ranging from 0.05 M to saturation 5.0 M NaCl depending on the species (Richmond, 1986; Katz and Avron 1985;

Borowitzka and Borowitzka, 1988; Avron, 1992; Garcia, *et al*., 2007; Mishra *et al*., 2008). *Dunaliella* species are regarded as the most salinity tolerant microorganism known, having a growth range broader than the most salinity tolerant prokaryotes with which they share environments (Borowitzka and Borowitzka, 1988).

The optimal salinity for growth (increase in cell number) is different in various species. For example, the optimal growth of *D. salina* and *D. viridis* isolated from Gave khooni salt marsh of Iran was obtained at 2.0 and 1.0 M NaCl, respectively (Hadi *et al*., 2008). The optimum growth rate for *D. salina*, *D. parva* and *D. psuedosalina* isolated from salt marshes of Iran ranged between 0.5 to 2.0 M NaCl (Shariati 2003; Ben-Amotz *et al*., 2009). The growth of *D. tertiolecta* was optimum at 0.2 to 0.5 M (Jahnke and White, 2003). The ability of *Dunaliella* to tolerate ranges of NaCl concentrations and undergo rapid changes in those concentrations is brought about by the unique ability of these microalgae to produce intracellular glycerol (Ben-Amotz and Avron, 1980; Borowitzka and Borowitzka, 1988). As the salinities increased (0.5 to 3.5 M NaCl) glycerol production increased probably for osmoregulation in extreme environments; protein synthesis also increased (Ginzburg and Ginzburg, 1993). In *D. viridis* cell carbon and nitrogen increased with increase in salinity and the cell accumulated glycerol, nitrate, structural proteins and free amino acids to adjust to high salinities (Jimenez and Niell, 1991; Ben-Amotz *et al*., 2009).

2.6.2 Temperature

Temperature is an important factor for both *Dunaliella* and *Asteromonas* growth. The growth rate increases with the increase in temperature up to its optimum level and when the optimum level is reached, the growth rate will decrease drastically with further increase in temperature.

The habitats of *Dunaliella* spp. are diverse, ranging from the Antarctic to the tropical lagoons indicating its wide tolerance to temperatures from -35 to about 40 ^{o}C (Wegmann *et al*., 1980 in Ben-Amotz *et al*., 2009). The optimum temperature range varies with the species i.e. for *D. salina* 20-40 ^{o}C (Borowitzka, 1981), for *D. viridis* 14-30 ^{o}C, for *D. bioculata* 25 ^{o}C, *D. primolecta* 29 ^{o}C and for *D. tertiolecta* 20 ^{o}C (Borowitzka and Borowitzka, 1988).

According to Jimenez and Neill (1991) the cell division rate depends on environmental factors such as temperature. A sudden increase of temperature by 5-15 ^{o}C above the preconditioning temperature accelerated cell division for the short term. Photosynthesis steadily increased between 15 and 31^{o}C, reached its peak followed by decrease up to 42 ^{o}C in *D. parva*; its halotolerance and thermo-resistance are evidenced by its net photosynthesis and positive oxygen evolution at 42 ^{o}C (Jimenez *et al*., 1991). This thermo-resistance ability is attributed to the protective effects of intracellular glycerol (Borowitzka and Borowitzka 1988).

2.6.3 pH value

The pH of the culture growth medium is an important factor affecting the optimal growth of microalgae cells. pH value of the medium plays a vital role in the rate of metabolic activity of microalgae and hence for the biosynthesis of secondary metabolites (Liu and Lee, 2000; Khalil *et al*., 2010). It can change the distribution of carbon dioxide species and carbon availability and also alter the availability of trace metals and essential nutrients (Chen and Durbin, 1994). Change in pH value of the medium can affect growth rates in *Asteromonas* and *Dunaliella* species (Celekli and Donmez, 2006; Ben-Amotz *et al*., 2009).

Dunaliella and *Asteromonas* species, except *D. acidophila*, grow in media with a pH value > pH 6.0. These species could grow at a pH range from 6 to 10. There is considerable evidence to demonstrate that changes in pH value of culture media influence the capacity of the cells to utilize dissolved inorganic carbon (DIC) and HCO_3 as carbon sources during photosynthesis (Latorella and Vadas, 1973). For example the pH value of a freshly inoculated batch culture of *D. tertiolecta* was 7.89 and increased as the culture grew and utilized CO_2 during photosynthesis. This caused a shift in bicarbonate buffer system (see below) and the pH value increased to 8.81 by day 3, and to pH 11 by day 7 (Ben-Amotz *et al*., 2009).

2.6.4 Carbon dioxide (CO_2) solubility and its effect on microalgae cells growth

The carbonate system in the sea comprises three species of dissolved inorganic carbon (DIC), namely molecular dissolved CO_2 [or aqueous carbon dioxide, CO_2 (aq)], HCO_3^-

(bicarbonate) and CO_2^- (carbonate) (Dieter *et al*., 1999). The relative amounts of the three DIC species are a function of pH value. Typical seawater with a value of pH 8.2 has 90 % of its DIC in the form of HCO_3^-, 9 % as CO_2^- and less than 1 % as CO_2. The rise in atmospheric CO_2 and the resulting net flux of CO_2 into the surface of the ocean causes a continuous change in seawater carbonate chemistry. The dissolved CO_2 reacts with water according to the following reactions (Equation 1.1 and 1.2) (Dieter *et al*., 1999; Riebesell, 2004).

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \qquad \textbf{(Eq. 2-1)}$$

$$HCO_3^- \rightleftharpoons H^+ + CO_3^{2-} \qquad \textbf{(Eq. 2-2)}$$

The reaction of CO_2 with water generates one proton for each HCO_3^- and two protons for each CO_3^{2-} formed. These acidifications cause a shift of the pH-dependent equilibrium between CO_2, HCO_3^- and CO_3^- towards higher CO_2 levels and lower carbonate ions concentrations $[CO_3^{2-}]$. The net result of CO_2 addition therefore is an increase in the concentration of dissolved CO_2, $[CO_2]$, a decrease in $[CO_3^{2-}]$ and seawater pH, and a slight increase in the concentrations of HCO_3^- and DIC (Huertas *et al*., 2000; Riebesell, 2004).

The responses of microalgae to CO_2 enrichment can be expected to occur in phase with the increase in atmospheric CO_2. Some algae possess an external carbonic anhydrase (CA), an enzyme that catalyzes the interconversion of HCO_3^- and CO_2 at the plasmalemma and maintains a constant equilibrium concentration of CO_2 at the site of its transporter (Burns and Beardall, 1987; Colman and Rotatore, 1995; Williams and Turpin, 1987; Huertas *et al*., 2000; Riebesell, 2004). Huertas *et al*. (2000) reported that the direct uptake of bicarbonate by an active transport system has been found in several species of algae. The catalyzed conversion of HCO_3^- to CO_2 via extracellular carbonic anhydrase would tend to either diminish or abolish potential effects of CO_2 limitation (Dieter *et al*., 1999). The uptake of all forms of DIC has been described in detail in marine diatoms (see Colman and Rotatore, 1995; Korb *et al*., 1997).

However, reactions within the carbonate system, particularly the interconversion between HCO_3^- and CO_2 are slow compared to rates of carbon uptake by microalgae. Conditions in the diffusive boundary layer around a photosynthesizing cell therefore deviate significantly

from those in the bulk medium. Still, even for a cell restricted to CO_2 uptake, the photosynthetic carbon demand in most cases will be met by the diffusive supply of CO_2 from its environment (Wolf-Gladrow and Riebesell, 1997; Dieter *et al*., 1999).

2.6.5 Photosynthesis efficiency

Photosynthetic efficiency on a global basis may be defined as the fraction of photosynthetically active radiation (PAR) that falls on the earth's surface, which is converted to stored energy by photosynthesis in the biosphere (Hall and Rao, 1999). The annual value of total solar energy received at the Earth's surface is equivalent to about 2,500,000 Joules (J) of heat. Of this, roughly half is reflected back by the clouds and by the gases in the upper atmosphere. For the remaining radiation that reaches the earth's surface, only 50 % is the spectral region of the light that could bring about photosynthesis, the other half being weak infra-red radiation (Hall and Rao, 1999; Janssen *et al*., 2001; Larkum, 2010). According to Larkum (2010) on a cloudless day the temperate zones offer good levels of solar energy, but the season is shorter than other zones. Table 2.2 relates the zones of PAR and its impact on yield.

Table 2-2 Budget for solar energy at various sites on the Earth's surface and theoretical primary productivity at various latitudes. The three values for daily carbon fixation shown are for the summer solstice, the equinox and the winter solstice respectively. The Equator has only one solstice. (Adapted from Ritchie, 2010; Larkum, 2010).

Latitude	**Growing seasons**	**Daily carbon fixation [g(C) m^{-2} d^{-1}]**	**Total irradiance (Em^{-2})**	**Total carbon fixation [g(C) m^{-2}]**
Equator	All year (365 d)	19.8 & 17.6	20238	6823
Darwin (12°28'S)	All year (365 d)	20.3, 19.2 & 13.9	19710	6602
Tropic of Cancer 23°30'N	All year (365 d)	21.8, 17.6 & 9.90	18615	6136
37°N	7 months (214 d)	22.3, 14.2 & 4.98	12208	4031
55°N	5 months (153 d)	20.8, 8.09 & 0.606	8 130	2533

The energy efficiency of photosynthesis is the ratio of the energy stored to the energy of light absorbed. The amount of energy stored can only be estimated because many products are formed, and these vary with the microalgae species and environmental conditions. Janssen *et al*. (2001) studied the efficiency of photosynthesis in *D. tertiolecta* under short light/dark cycle (3/3 s, 94/94 ms and 31/156 ms) and the efficiency was determined and expressed as the biomass yield on light energy in gram protein produced per mol of photons absorbed. The yield under the 94/94 ms cycle was higher than the yield under continuous light of 440-455 mmol m^{-2} s^{-1}. This shows that under the 94/94 ms cycle light energy is integrated with dark processes and the photosynthetic efficiency is higher than under continuous light in *D. tertiolecta* (Janssen *et al*., 2001). Ben-Amotz (2011) elucidated the productivity of two different species of microalgae, *Dunaliella* and *Nannochloropsis*, cultivated in 10 and 0.1 hectares respectively. Table 2.3 summarized the theoretical values of the biomass production.

Table 2-3 Theoretical biomass productivity of *Dunaliella* and *Nannochloropsis* (Adapted from Ben-Amotz, 2011)

Productivity	***Dunaliella***
Growth area (hectares)	10.0
Pond biomass (g/L)	0.3
Biomass (g/m^2/year)	70.0
Biomass (g/m^2/day)	2.0
Photosynthetic efficiency (%)	0.24
	β-Carotene
Production (tons/year)	3.5
Production (g/m^2/day)	0.1

2.7 Osmolytes

Osmolytes are naturally occurring small organic molecules often referred to as ‘‘chemical chaperones”, and are normally accumulated in the intracellular location at relatively high concentrations and can increase thermodynamic stability of folded proteins without

perturbing/disturbing other cellular processes (Kumar, 2009). They are usually accumulated at relatively high concentrations when cells/tissues are subjected to stress conditions such as temperature, pressure, and the presence of salts and other solutes (Kumar, 2009). Naturally occurring organic osmolytes can be grouped into three classes: polyols, amino acids, and methylamines. The polyols include glycerol, sorbitol, trehalose, glucose, and sucrose. Amino acids and amino acid derivatives that serve as osmolytes are, for example, glycine, proline, betaine, taurine, and glutamic acid. Commonly known methylamines are trimethylamine N-oxide (TMAO), sarcosine, and choline-*O*-sulphate (Harries and Rosgen, 2008; Kumar, 2009) (Figure 2.7).

Naturally occurring organic osmolytes are normally found in a range of organisms, including animals, plants and microorganisms (Kumar, 2009). Organic osmolytes exert a dramatic influence on the protein folding reaction, without making or breaking covalent bonds. With the exception of urea, they are often called 'compatible solutes', a term indicating lack of perturbing (distressing) effects on cellular macromolecules and implying interchangeability (Yancey, 2005; Street *et al*., 2006).

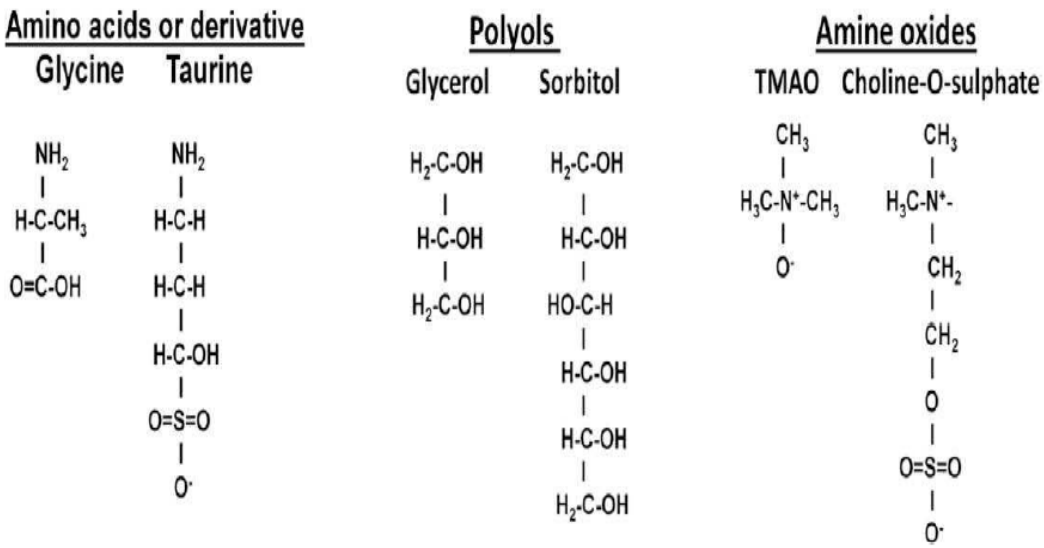


Fig. 2-7 Diagram showing chemical structures of two examples each in the three major categories of osmolytes. Each class interacts with both the peptide backbone and to some extent, also with amino acid side chains (Kumar, 2009)

2.8 Osmoregulatory mechanism

The osmotic response of halotolerant microalgae *Dunaliella* and *A. gracilis* involves both structural and metabolic changes. According to Ben-Amotz *et al*. (1982) *Dunaliella* cells behave like perfect osmometers rapidly shrinking or swelling under hypertonic or hypotonic conditions, respectively (Figure 2.8), which includes three stages when the salinity is changed. The first stage (within a few minutes) is termed "**instant response**": cell shape, size structure and the concentration of ions in cells change in this stage. The second stage, **short-term response**, is a 2 to 3 hours process of balancing the osmotic pressure inside and outside of *Dunaliella* cells by regulating the intracellular concentration of glycerol. The last stage is **long-term response** which involves osmotic stress-induced gene expression and the accumulation of some salt-induced proteins (Chen and Jiang, 2009). In order to respond to a high external osmotic environment, the cells accumulate glycerol, seemingly to compensate for differences between the extracellular and intracellular water potential. The mechanisms by which glycerol can confer such tolerance are not clear. According to Shen *et al*., (1999) a physiological mechanism is that glycerol is involved in osmotic adjustment by facilitating water flux across the plasma membrane.

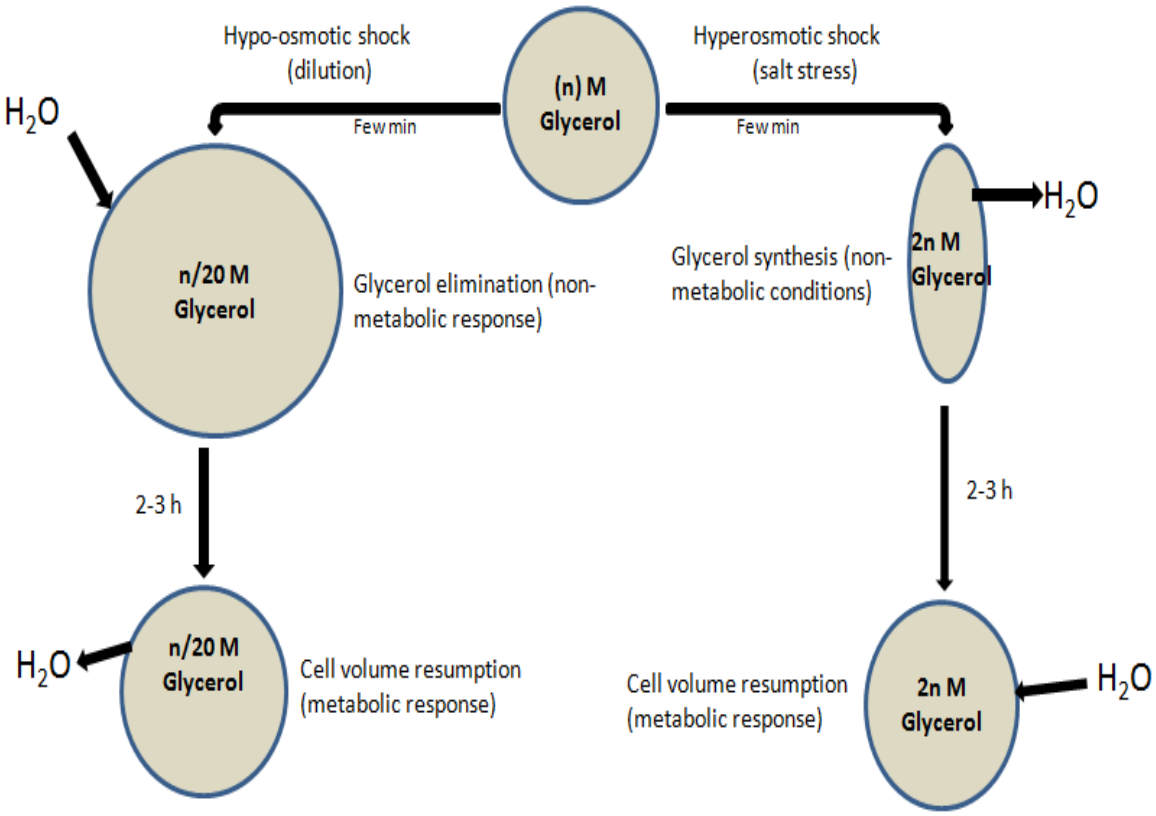


Fig. 2-8 Schematic representations of the volume changes of halotolerant microalgae in response to hypertonic and hypotonic conditions (modified from: Ben-Amotz, *et al*., 1982; Cheng and Jiang, 2009). Where n= amount of glycerol in mole, 2n= twice amount of glycerol in mole and n/20= amount of glycerol in mole divide by 20.

The lack of a rigid cell wall permits a rapid adjustment of the intracellular osmotic pressure by fluxes of water through the cytoplasmic membrane. Subsequently the cells gradually return to their original shape through a phase of metabolic adjustment. During this metabolic adjustment period under hyperosmotic condition the halotolerant microalgae produce and accumulate glycerol above the original level, while under hypotonic conditions the algae decrease the glycerol content lower than the original level (Ben-Amotz *et al*., 1982). In both hypertonic and hypotonic conditions, water flows through the cytoplasmic membrane in response to the new level of intracellular glycerol so that at the steady state the original cell volume is regained (Ben-Amotz and Avron, 1973; Ben-Amotz, 1975; Ben-Amotz *et al*., 1982).

2.9 Cell membrane and effect of temperature

As previously noted *Dunaliella* species do not contain a rigid cell wall but have a thin elastic plasma membrane that responds rapidly to changes in osmotic pressure by changes in cell volume. *Dunaliella* cells can withstand 3 to 4 fold increases or decreases in osmotic pressure, shrinking or swelling in response, respectively (Avron and Ben-Amotz, 1992; Shariati and Lilley, 1994).

Both *Dunaliella* and *Asteromonas* can maintain extremely high concentration gradients ($>10^4$) of glycerol between the intracellular space and the medium. This unique ability is highly temperature-dependent (Wegmann *et al*., 1980). Treating these microalgae for 10 minutes at temperatures above 60 ^{o}C caused complete release of all the internally held glycerol, 50 % of glycerol was released at around 50 ^{o}C, but essentially none was released below 40 ^{o}C (Wegmann *et al*., 1980). This is independent of the salt concentration of the medium (1.0 to 4.0 M NaCl). The underlying mechanism may involve a temperature-dependent conformational transition of a component of the cellular membrane which is essential for glycerol impermeability (Wegmann *et al*., 1980), indicating that halotolerant microalgae possess a special membrane which enables a rapid increase or decrease, respectively, in cell volume that prevents apoptosis (cell death) (Chen and Jiang, 2009). According to Fujii and Hellebust (1992) sudden hypo-osmotic shocks cause transient formation of small non-specific pores in the plasma membrane of *D. tertiolecta* through which intracellular glycerol is released to the medium.

2.10 Mechanism of glycerol-starch interconversion in *Dunaliella*

There are two metabolic pathways identified to be responsible for glycerol production in halophytic microalgae (Figure 2.9): one using the product of photosynthesis and the other via the metabolic breakdown of starch, the storage product in *Dunaliella* (Ben-Amotz and Avron, 1973; Cui *et al*., 2010; Zhao *et al*., 2013). Under hyperosmotic stress glycerol is synthesized in the light by using products of photosynthesis and starch breakdown, whereas in the dark glycerol is synthesized only from the products of starch breakdown (Goyal, 2007). The contribution of starch breakdown for glycerol formation increased steadily with the

augmentation of salinity, and glycerol was synthesized only from the products of starch breakdown in continuous dark regime (Chen and Jiang, 2009).

The mechanism of osmoregulation involves unique enzymes that include glycerol-3-phosphate dehydrogenase (GPDH) which catalyses the conversion of dihydroxyacetone to glycerol-3-phosphate. The second unique enzyme is glycerol-3-phosphate phosphatase (GPP) which specifically dephosphorylates glycerol-3-phosphate to glycerol. The third enzyme is dihydroxyacetone kinase (DHAK) which catalyses the conversion of dihydroxyacetone to dihydroxyacetone phosphate leading to starch production (Ben-Amotz *et al*., 1982; Zhao *et al*., 2013). GPDH is the first enzyme involved in glycerol synthesis, and may therefore play a central role in glycerol accumulation. There are three isoforms of GPDH in *D. salina*: GPDH-1 and GPDH-2 are nicotinamideadenine dinucleotide (NAD+)-dependent and are located in the chloroplast or cytoplasm, whereas GPDH-3 is a flavin adenine dinucleotide-(FAD) dependent isoform located in the mitochondria (He *et al*. 2007; Yang *et al*. 2007; Zhao *et al*., 2013).

Other enzymes such as glucose-6-phosphate isomerase (GPI), fructose-1,6-biphosphate aldolase (FBPA) and phosphofructokinase (PFK) are considered as important enzymes that are also involved in the glycerol metabolic pathway (Pick, 1991; Chen and Jiang, 2009; Zhao *et al*., 2013). GPI is related to starch breakdown for glycerol formation, it was reported to be induced by high salinity (3.5 M) and inhibited by light (Cui *et al*., 2010). According to Zhao *et al*. (2013) neither the transcription nor enzyme activity of GPI changed over 24 h exposure of *Dunaliella* to salinity stress under light/dark regime. The level of FBPA was also found to be increased by high salinity stress (Zhang *et al*., 2003; Zhao *et al*., 2013). Pick (1991) demonstrated that hyperosmotic shock induces a decrease in fructose-6-phosphate and an increase in fructose-1, 6-bisphosphate indicating the activation of phosphofructokinase.

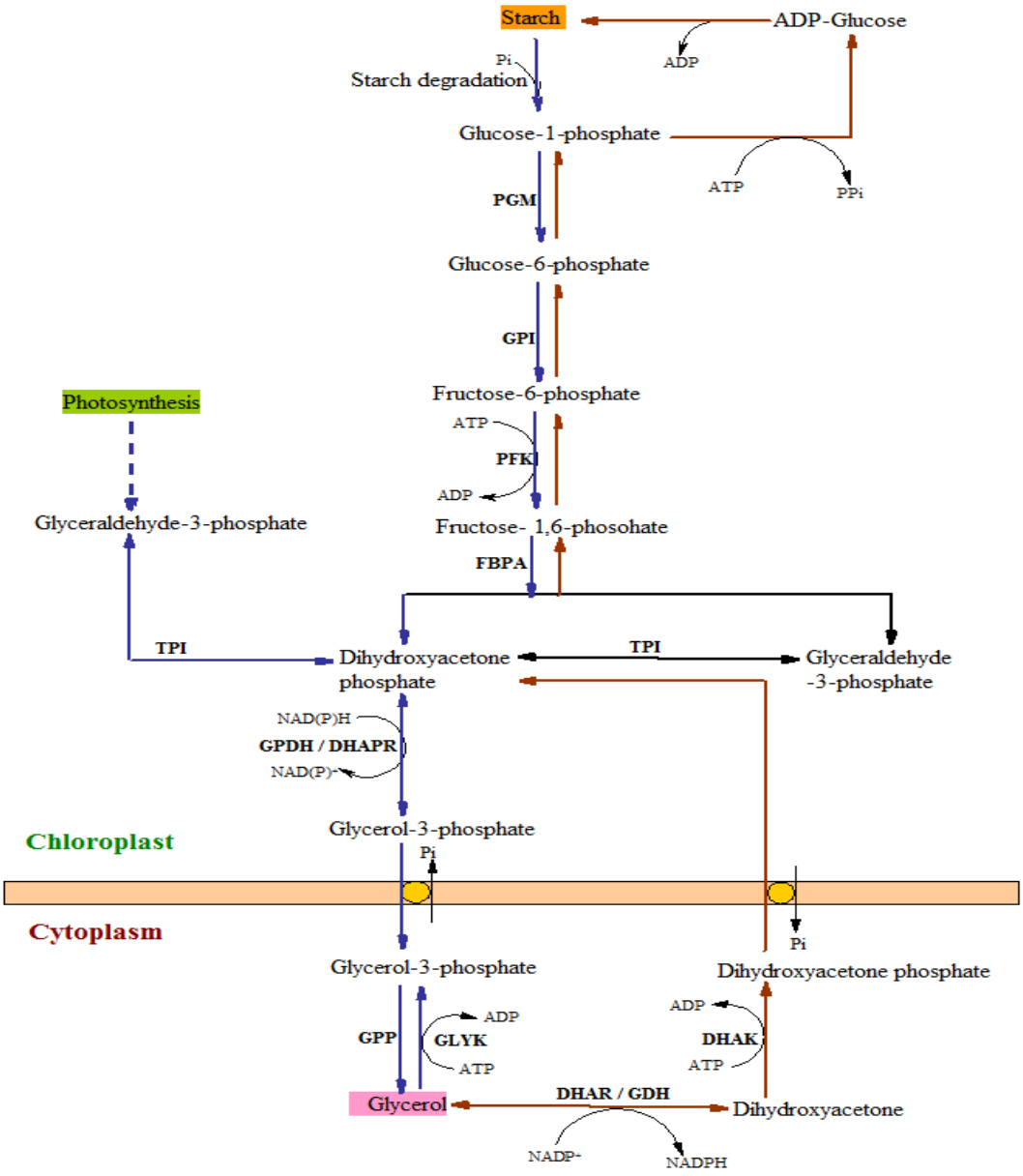


Fig. 2-9 Hypothetical pathways of glycerol synthesis and dissimilation following osmotic shock (Modified from Bental *et al*., 1990; and Zhao *et al*., 2013). ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHAK, dihydroxyacetone kinase; DHAPR, dihydroxyacetone phosphate reductase; DHAR, dihydroxyacetone reductase; FBPA, fructose-1,6-diphosphate aldolase; GDH, glycerol dehydrogenase; GLYK, glycerol kinase; GPDH, glycerol-3-phosphate dehydrogenase; GPI, glucose-6-phosphate isomerase; GPP, glycerol-3-phosphate phosphatase; NAD, nicotinamide adenine dinucleotide trihydrate; NADH, nicotinamide adenine dinucleotide; PFK, phosphofructokinase; PGM, phosphoglucose mutase; Pi, orthophosphate; PPi, pyrophosphate; TPI, triose phosphate isomerase.The blue arrows represent pathways activated by hyperosmotic shock, the brown arrows, pathways activated following hypo-osmotic shock. The circles in the chloroplast membrane represent the Pi/triose-phosphate translocator.

On exposure to hypertonic stress the glucose-6-phosphate obtained via starch breakdown is converted to fructose-1,6-diphosphate and then to dihydroxyacetone phosphate (DHAP), which can be converted to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase (GPDH). Glycerol-3-phosphate is converted to glycerol by glycerol-3-phosphate phosphatase (Goyal *et al*., 1987; Chen and Jiang, 2009). In the reverse direction, when subjected to hypotonic stress, excess glycerol is dissimilated by oxidation via glycerol dehydrogenase to dihydroxyacetone (DHA) and then phosphorylation to form DHAP via dihydroxyacetone kinase (Ben-Amotz, 1975; Taherzadeh *et al*., 2002; Zhao *et al*., 2013). It should be noted that most of these reactions are reversible and under redox regulation via ATP or NAD(P)H, or regulated by H^+.

2.11 Ions in osmotic adjustment

Halophytic microalgae have an outstanding ability to eliminate Na^+ ions and to maintain low internal Na^+ concentrations below those of the outside medium even at external NaCl of 3.0-4.0 M concentrations. It was shown that *D. salina* cells contain only 20-100 mM Na^+ when grown over the range of 1.0 - 4.0 M NaCl, showing that Na^+ is effectively excluded from the cells (Pick *et al*., 1986; Chen and Jiang, 2009). *D. salina* retains intracellular Na^+ concentrations using four distinct Na^+ transporters in its plasma membranes; **(1)** a Na^+/H^+ antiporter, **(2)** a redox-driven Na^+ pump, which functions in pH homeostasis and in maintaining the intracellular Na^+ concentrations low, respectively (Katz *et al*., 1986). *Dunaliella* utilizes the electrochemical Na^+ gradient for energization of phosphate uptake through **(3)** Na^+/Pi symporters and **(4)** Na^+-ATPase (Weiss *et al*., 2001). These suggest that uptake of phosphate and of sulphate in *D. salina* are mediated by Na^+/anion symporters and are driven by sodium concentration gradient across the plasma membrane (Weiss *et al*., 2001) (Figure 2.10).

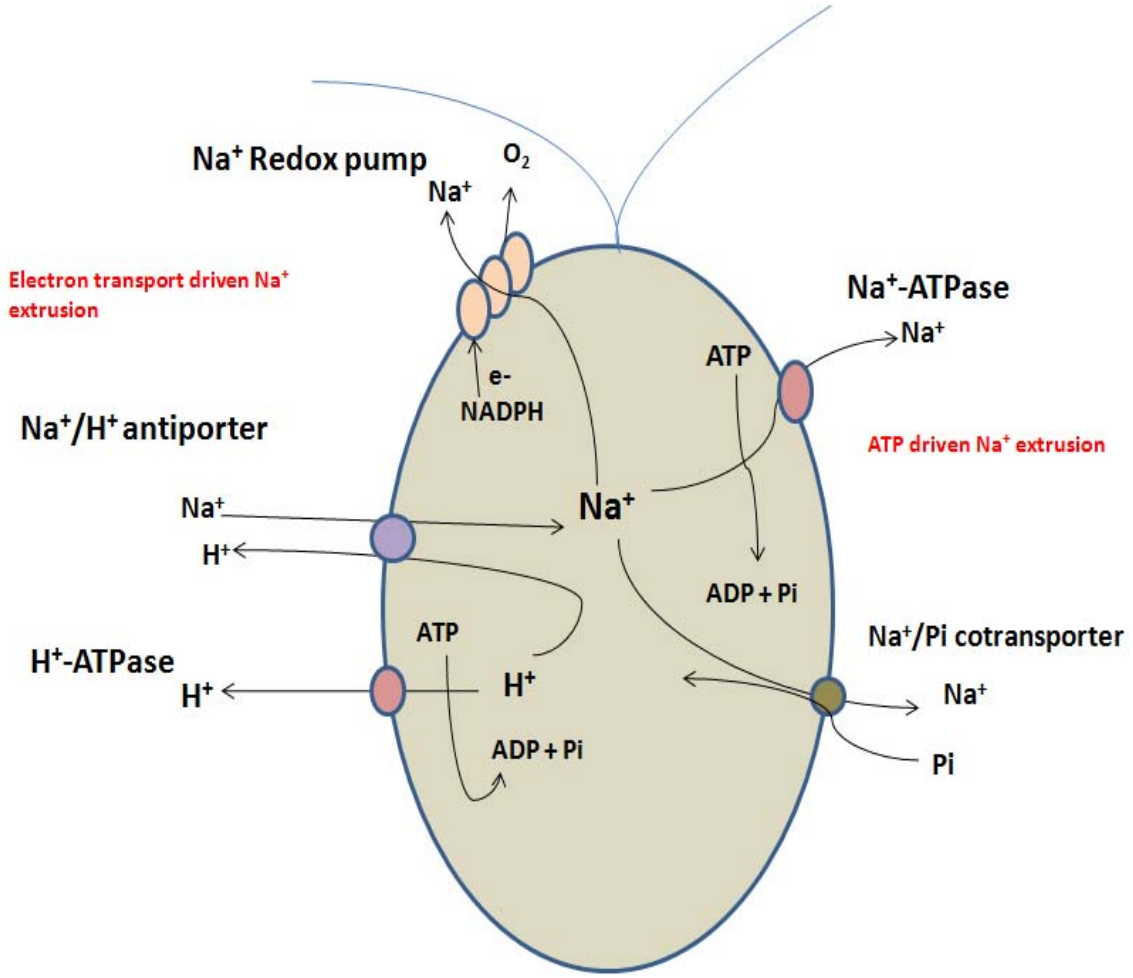


Fig. 2-10 Diagrammatic representation of ion transporters involved in salt resistance in *Dunaliella* (Modified from Cheng and Jiang, 2009)

The mechanism of osmotic adjustment in figure 2.10 shows that Na^+ can be excluded from the cells by Na^+/ H^+ antiporter in the plasma membrane of *Dunaliella* coupled to H^+ transport into cells, then the H^+ in cells is excluded from the cells by H^+-ATPase in the plasma membrane (Katz *et al.*, 1986). It was suggested that the antiporter Na^+/H^+ catalyzes influx rather than extrusion, and that it was activated by internal acidification or by hyperosmotic shocks (Katz *et al*., 1991) and a putative Na^+-ATPase might carry out extrusion in *D. salina* (Pick, 1992; Chen and Jiang, 2009). This leads to the operation of an electrogenic NADPH-driven redox system coupled to Na^+ extrusion in *Dunaliella* plasma membrane (figure 2.10). The redox-driven Na^+ extrusion and recycling in *Dunaliella* evolved as a means of adjustment to hypersaline medium (Katz and Pick, 2001; Chen and Jiang, 2009).

2.12 Phospholipid metabolism and Pi/triose-P translocator in halotolerance microalgae

The nature of signal transduction induced by hyperosmotic shock is fundamentally different from that induced by hypoosmotic shock. It appears that the response to hypoosmotic shock involves rapid alteration in membrane morphology (Maeda and Thompson, 1986; Oren, 2005) accompanied by phospholipid metabolism (Einspahr *et al*., 1988; Anderca *et al*., 2002). The increase in cell volume following rapid transfer to hypotonic medium is accommodated by increased plasmalemmal surface area through rapid vesicle fusion. The response to dilution also involves the rapid hydrolysis of the polyphosphoinositides, phosphatidylinositol 4,5-biphosphate (PIP_2), and phosphatidylinosito 4-monophosphate (PIP) through the apparent activation of phospholipase C (Einspahr *et al*., 1988; Muradyan *et al*., 2004). The rapid hydrolysis of the polyphosphoinositides after hypoosmotic shock is accompanied by a sharp increase in the levels of phosphatidic acid (PA), leading to the activation of an inositol phospholipid signal transduction pathway (Einspahr *et al*., 1988), which in turn leads to the activation of a specific phospholipase C in the plasma membrane of the algae. Chitlaru and Pick (1991) analyzed changes in different phosphometabolites following osmotic shocks and found that hypo-osmotic shock induces an increase in inositolbisphosphate but not phosphorylcholine, supporting the previous indications for differential activation of phospholipases by both hypo-osmotic and hyperosmotic shocks in *Dunaliella*. These metabolites (phospholipids) play a fundamental role in many extracellular signal transductions that lead to alterations of intracellular physiology (Einspahr *et al*., 1988; Muradyan *et al*., 2004)

When *Dunaliella* cells are subjected to hyperosmotic shock they respond with a decrease in cell volume accompanied by plasmalemma infolding without overall loss of the surface area. This contrasts with the dramatic increase in plasmalemma surface area after hypo-osmotic shock (Einspahr *et al*., 1988). Hyperosmotically shocked *Dunaliella* also react by inducing rapid changes in the metabolism of phospholipid. This is achieved by reducing their content of phosphotidic acid accompanied by increased in PIP_2 and PIP, suggesting an inhibition of the key enzyme, phospholipase C, or an increase in polyphosphoinositol synthesis, or both. Phospholipases are known to regulate a variety of physiological responses not only in *Dunaliella* but also in both animals and plants (Einspahr *et al*., 1988; Muradyan *et al*., 2004; Oren, 2005).

When halotolerant microalgae were subjected to hypo-osmotic or hyperosmotic shocks the Pi/triose-phosphate shuttle operates (Bental *et al*., 1990). The osmotic response is initiated by differential volume changes of the cytoplasm and chloroplast that alter cytoplasmic orthophosphate (Pi) concentration. This in turn activates chloroplast enzymes in the direction of either glycerol or starch biosynthesis. The direction of transport via the translocator is controlled by the relative concentrations of its substrates in the chloroplast and cytoplasm (Pi, phosphoglycerate, triose-phosphates as DHAP or glycerol-P) (see section 2.10; figure 2.9). The role of Pi in glycerol cycle is supported by the hypotheses presented by Gimmler and Möller (1981) and Bental *et al*. (1990) who reported the role of Pi as a trigger in controlling the carbon distribution between starch and glycerol.

2.13 Metabolomic Study

Metabolomics deals with the non-targeted identification and quantification of all metabolites within an organism or system, under a given set of conditions (Dettmer *et al*., 2007; Allwood *et al*., 2008; Kluender, 2009). Just like the study of gene and protein expressions within samples is called genomics and proteomics, respectively, metabolomics is concerned with biochemical profiling of all the metabolites (e.g. amino acids, glucose, ATP and glycerol) in a cell, tissue, or organism. Metabolomics science plays a vital role in discovering which cellular biochemical processes have been altered under specific environmental, stress or toxic conditions. The biochemical processes that involve the interaction of chemicals with an organism, may significantly contribute to understanding their stress or toxic actions. Metabolomics has effectively been applied in environmental studies (Lin *et al*., 2006; Hines *et al*., 2007), freezing tolerance of plants (Cook *et al*., 2004), temperature stress (Guy *et al*., 2008), nutrient deprivation in *Chlamydomonas reinhardtii* (Bolling and Fiehn, 2005), and *D. salina* under light stress and nitrogen deprivation (Lamers, 2011).

2.13.1 The use of metabolomics in studying microalgae stress responses

Metabolomics is being increasingly used for understanding the cellular phenotypes in response to various types of stresses - biotic or abiotic (Bhalla *et al*., 2005). Subjecting

microbial cells to stress could lead to manipulation of their metabolic functions and can cause changes in the activity, not only of the target pathway, but also of multiple pathways interacting directly or indirectly with the target pathway. Such interactions may involve, for example, competition for common precursors. When the activity of a target pathway is enhanced, this could result in the decrease in substrate supply to a competing pathway. On the other hand, suppression of a target pathway may result in increased substrate supply for a competing pathway (Ishihara *et al*., 2007).

A microalga, *Chlamydomonas reinhardtii,* has been used as the model organism of two studies aimed at optimizing metabolite profiling methods (Lee and Fiehn, 2008; Kempa *et al*. 2009). A study on the effects of various nutrient limitations on the polar compounds of *C. reinhardtii* showed that phosphorus depletion leads to a different phenotype compared to nitrogen, iron or sulphur depletion (Bolling and Fiehn, 2005). Another metabolomic study using *Scenedesmus vacuolatus* showed that the inhibitor prometryn impaired energy metabolism and affected various other parts of the algal metabolism (Kluender *et al*., 2009). Lamers (2011) compared the polar metabolite profiles in *D. salina* cells in response to stress induced by high light and nitrogen starvation. In his research, Lamers (2011) used gas chromatography/mass spectrometry (GC/MS) and detected 44 polar metabolites for both conditions. In order to assess the influence of nitrogen starvation on the intracellular concentrations of nitrogenous metabolites, Lamers (2011) determined the stress-induced changes of 19 nitrogen-containing compounds. The most notable decreases were for the amino acids asparagine and glutamine. In high-light stress he detected 12 nitrogen-containing metabolites, of which 2 increased (guanine and L-DOPA), and 4 other metabolites decreased significantly in their intracellular concentrations. The polar metabolite profiles obtained were correlated to the stress-induced changes in lipid-soluble isoprenoids, including β-carotene, and composition of fatty acids.

2.13.2 Instrumentation applied to metabolomic analysis

Analyses of metabolites consist of a sequence of steps that includes sample preparation, metabolite extraction, derivatization, metabolite separation, detection, and data treatment. However, not every step is always needed. Only detection and data analysis have been

essential steps in all reported metabolomics studies. The choice of the analytical steps depends on the type of sample (e.g. solids vs. liquids), instrumentation to be used for separation (e.g. GC vs. LC) and detection method (e.g. MS vs. NMR).

For example, proton nuclear magnetic resonance (1H-NMR) spectroscopy is suitable for the analysis of bulk metabolites, liquid chromatography (LC)-MS is greatly applicable to the analysis of a wide range of semi-polar compounds including many secondary metabolites, and GC-MS for the analysis of volatile organic compounds (VOCs) and derivatised primary metabolites.

2.13.3 The application of GC/MS for metabolomic analysis

GC/MS is the popular choice for quantitative and qualitative metabolite profiling. Most of the plants' metabolites are non-volatile and cannot be analyzed directly by this method. Such compounds (e.g. amino acids, sugars and organic acids) have to be converted to less polar, more volatile derivatives before they are applied to the GC column (Bhalla *et al*., 2005; Allwood *et al*., 2008). GC-MS analysis of plant and algae extracts commonly employs a two-stage derivatisation process: (1) O-alkylhydroxylamine which converts sample carbonyl groups to oximes for thermal stabilisation and (2) A secondary treatment with a silylating compound [e.g. N-methyl-N-(trimethylsilyl)trifluoroacetamide] causes the formation of volatile trimethylsilyl esters (Roessner *et al*., 2000; Du *et al*., 2011).

The metabolites from the samples are automatically identified by comparing and matching the retention time or retention index with those from mass spectra of chromatograms generated from pure chemical standards. Mass spectral comparisons can be made with commercially available databases such as the National Institute of Standards and Technology library (www.nist.gov) or more commonly via in-house libraries (Lisec *et al*., 2006; Allwood *et al*., 2008; Lamers, 2011).

2.14 Extraction of Glycerol

2.14.1 Supercritical and liquid carbon dioxide (CO_2)

There are well established procedures for extraction of glycerol from *Dunaliella* and *Asteromonas spp*., these include enzymatic extractions and ultrasonic-assisted solvent extraction. Chloroform is the most common organic solvent used. Microalgal cells are collected from the cultures by centrifugation. The harvested algal pellet is resuspended in water/chloroform to lyse the cells. The resultant chloroform-glycerol-water solution is then separated from the bio-mass by centrifuge (Ben-Amotz and Avron, 1973; Chen *et al*., 2011). Supercritical carbon dioxide (SC-CO_2) is regarded to safe process, offering mild operating conditions, no environmental impact and a high-quality final product without any trace of solvent (Perrut, 2001). Some literature is available on the SC-CO_2 extraction of lipids from microalgae strains, including *Spirulina (Arthrospira) platensis*, *Spirulina (Arthrospira) maxima*, *Chlorella vulgaris*, *Ochronomas danica*, *Botryococcus braunii*, *sochrysis galbana* and *Skeletonema costatum* (Polak *et al*., 1989; Mendes *et al*., 1994; Mendes *et al*., 1999; Perretti *et al*., 2003; Mercer and Armenta, 2011). There is no information on the extraction of glycerol from microalgae using SC-CO_2 method.

At pressures and temperatures above ambient, carbon dioxide can exist in forms usable as a solvent (*i.e.* as a liquid or supercritical fluid) (McKenzie *et al*., 2004; Herrero *et al*., 2006). Supercritical fluids are fluids at a higher pressure and temperature than the critical point, where distinct liquid and gas phases do not exist, as shown in the phase diagram in figure 2.11 (Herrero *et al*., 2006). CO_2 is a liquid under relatively mild pressures and temperatures, in the ranges of 5.2 to 73.8 bar and -56.6 to 31.0 °C. Supercritical carbon dioxide (SC-CO_2) is produced at temperatures higher than the critical temperature (31.0 °C) and between the critical pressure (73.8 bar) and extremely high pressures (approximately 104 bar). Supercritical fluids have no distinct liquid or vapour phase but retain the properties of both (McKenzie *et al*., 2004). Table 2.4 shows the relative properties of liquid, gas, and supercritical phases of CO_2. It also reveals that a supercritical fluid has the capability to have a higher mass transfer rate over a liquid solvent. CO_2 is chosen as a solvent to replace organic solvent, because it is nontoxic, and non-flammable with low critical point (Gomez and de la

Ossa, 2000). In addition, SC-CO_2 can be removed easily from the extract (no solvent residue) and there are no costs associated with solvent waste disposal (Stahl *et al*., 1980).

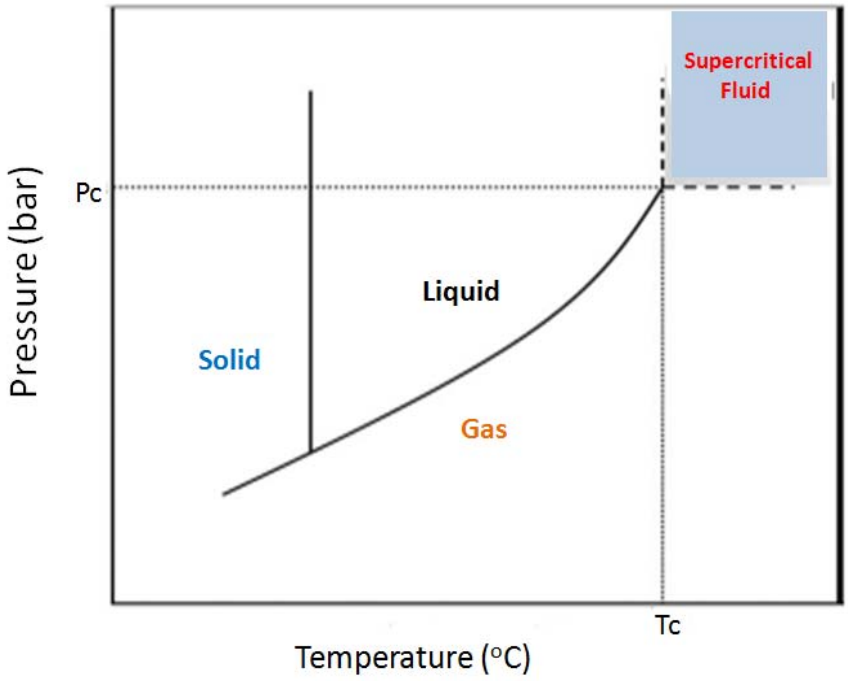


Fig. 2-11 Typical phase diagram for a pure compound (adapted from Herrero *et al*., 2006).

Table 2-4 Range values of several physicochemical properties of gases, liquids and supercritical fluids (adopted from Herrero *et al*., 2006)

State of Fluid	**Density (ρ, g/cm^3)**	**Diffusivity (D_{AB}, cm^2/s)**	**Viscosity (μ, g s/cm)**
Gas *P*= 1 atm; *T*=21 °C	10^{-3}	10^{-1}	10^{-4}
Liquid *P*=1 atm; T=15-30 °C	1	$<10^{-5}$	10^{-2}
Supercritical $P=P_c$; $T=T_c$	0.3 - 0.8	10^{-3} - 10^{-4}	10^{-4} - 10^{-3}

2.14.2 Applications of CO_2 as an alternative solvent

Over the past decade, SC-CO_2 extraction has been considered as an alternative to liquid-liquid extraction or distillation for the isolation of valuable compounds from natural products (Randolph, 1990; Hartono *et al*., 2001). Commercial applications of SC-CO_2 extraction are used particularly for the extraction of biological compounds in situations where there is a requirement for low-temperature processing, high mass-transfer rates and negligible carryover of solvent into the final product (Randolph, 1990). SC-CO_2 technology is well established for the extraction of various food products, including essential oils and hops, and for the decaffeination of coffee and tea, flavonoid compounds from Spearmint (*Mentha spicata L.*) leaves (Bimakr *et al*., 2009), chlorophyll a from *Spirulina platensis* (Mendiola *et al*., 2007; Tong *et al*., 2010), oil from microalgae for biodiesel production (Halim *et al*., 2012), lipids from the microalgae *Nannochloropsis* sp (Andrich *et al*., 2005), Astaxanthin from *Haematococcus pulvialis* (Thana *et al*., 2008), and palm kernel oil (Zaidul *et al*., 2007).

The application of SC-CO_2 in cell disruption and the extraction of lipophilic compounds from microalgae has been reported; the details of parameters used and targeted compounds are summarized in Table 2.5. To-date there has been no study that reported the use of SC-CO_2 in extraction glycerol from *Dunaliella* cells. It could be used as an alternative and attractive means of extracting pure glycerol from microalgae biomass. The main focus of chapter 9.0 presented in this research is the use of SC-CO_2 for glycerol extraction in *Dunaliella* with the aim of producing biofuels.

Table 2-5 Application of SC-CO_2 for disruption of different cells

Microorganism	SC-CO_2 Disruption Condition			Criteria for verifying cell rupture	Reference
	P (bar)	T (°C)	Time (h)		
Nannochloropsis gaditana, Synechococcus sp. and D. salina	200-500	40-60	180	Release of carotenoids and chlorophyll	Macias-Sanchez *et al*., 2009
D. salina	183-437	15-45	nr	Release of carotenoid	(Jaime *et al*., 2007)
Spirulina platensis	220-320	55-75	nr	Release of carotenoid	(Mendiola *et al*., 2007)
Dunaliella salina	100-500	40-60	nr	Release of carotenoid	(Macias-Sanchez *et al*., 2009)
Nannochloropsis gaditana	100-500	40-60	nr	Release of carotenoid	(Macias-Sanchez *et al*., 2005)
Synechococcus sp	100-500	40-60	nr	Release of carotenoid	Macias-Sanchez *et al*., 2007)
Saccharomyces cerevisiae	71-218	25-35	0-10	Release of protein	(Lin *et al*., 1992)
Saccharomyces cerevisiea	34-345	10-85	1-15	Release of protein	(Lin and Chen, 1994)
Saccharomyces cerevisiea	41	40	3	Agar plate count/ direct observation by electron microscopy	(Nakamura *et al*., 1994)
Saccharomyces cerevisiea, E. coli, Bacillus subtilis	Near critical	Near critical	nr	Released of protein and nucleic acid	(Castor and Hong, 1995)
Ralstonia eutropha	SC	SC	0.33	Released of protein and Poly (hydroxybutyrate)	(Khosravi *et al*., 2004)

nr= not reported

CHAPTER 3.0: GENERAL MATERIALS AND METHODS

This chapter presents the general methodologies that were used in maintaining the stock cultures of the experimental algae, the methods used to estimate the growth of the cells under experimental treatments, and the extraction and detection methods used for glycerol and other metabolites. The subsequent chapters contain more detailed information relating to the set up and conditions of the experiments conducted and the results. The chemicals, equipments/software used in the experimental studies are listed in Table 3.1 and 3.2, respectively.

Table 3-1 Materials

Chemicals	**Chemical Formula**	**Percentage purity**	**Product number, Company, Town and Country**
3,4-Dihydroxy-L-phenylalanine (L-DOPA)	$C_9H_{11}NO_4$	≥98%	D9628, Sigma-Aldrich, Dorset, UK
6-Phosphogluconic acid trisodium salt	$C_6H_{10}Na_3O_{10}P$	≥95%	P6888, Sigma-Aldrich, Dorset, UK
Acetic acid	$C_2H_4O_2$	≥99.7%	320099, Sigma-Aldrich, Dorset, UK
Acetone	C_3H_6O	≥99.9%	270725, Sigma-Aldrich, Dorset, UK
Acetyl acetone	$C_5H_8O_2$	≥99.5%	00900, Sigma-Aldrich, Dorset, UK
Adenine	$C_5H_5N_5$	≥99%	A8626, Sigma-Aldrich, Dorset, UK
Adonitol (rabitol)	$C_5H_{12}O_5$	≥99%	A5502, Sigma-Aldrich, Dorset, UK
Alanine	$C_3H_7NO_2$	≥98.5%	A9920, Sigma-Aldrich, Dorset, UK
Ammonium bicarbonate	NH_4HCO_3	≥99.5%	09830, Fluka-Sigma-Aldrich, Dorset, UK
Ammonium molybdate tetrahydrate	$(NH4)_6Mo_7O_{24}\cdot 4H_2O$	81.0-83.0%	A7302, Sigma-Aldrich
Glycerol Dehydrogenase Assay kit	-	-	G3512, Sigma-Aldrich, Dorset, UK
Biotin	$C_{10}H_{16}N_2O_3S$	≥99%	B4639, Sigma-Aldrich, Dorset, UK
Boric acid	H_3BO_3	99.5+%	10112140, Fisher Scientific, Leicestershire, UK

Calcium chloride	$CaCI_2$	≥99%	C3306, Sigma, Dorset, UK
Chloroform/ trichloromethane	$CHCl_3$	99.8+%	10020090, Fisher Scientific, Leicestershire, UK
Citric acid	$C_6H_8O_7$	≥99.5%	251275, Sigma-Aldrich, Dorset, UK
Cobalt(II) Chloride Hexahydrate	$CoCl_2.6H_2O$	-	192092500, Acros Organics, New Jersey, USA
Copper(II) Chloride Dihydride	$CuC1_2.2H_2O$	99.0%	C3279, Sigma-Aldrich, Dorset, UK
Copper(II) sulfate pentahydrate	$CuSO_4.5H_2O$	≥98%	C8027, Sigma-Aldrich, Dorset, UK
Cyanocobalamin (Vit. B_{12})	$C_{63}H_{88}CoN_{14}O_{14}P$	99%	S24/25, Acros Organics, New Jersey, USA
D-(−)-3-Phosphoglyceric acid disodium salt	$C_3H_5Na_2O_7P$	≥93%	P8877, Sigma-Aldrich, Dorset, UK
D-(+) Glucose	$C_6H_{12}O_6$	≥99.5%	G8270-1KG, Sigma-Aldrich, Dorset, UK
D-Fructose 1,6-bisphosphate trisodium salt hydrate	$C_6H_{11}Na_3O_{12}P_2$	≥98%	F6803, Sigma-Aldrich, Dorset, UK
D-Glucose 6-phosphate disodium salt hydrate	$C_6H_{11}Na_2O_9P$	≥98%	G7250, Sigma-Aldrich, Dorset, UK
D-Glucose 6-phosphate sodium salt	$C_6H_{13}O_9PNa.xH_2O$	> 98 %	G7879, Sigma-Aldrich, Dorset, UK
Dihydroxyacetone phosphate dilithium salt	$C_3H_5Li_2O_6P$	≥93%	D7137, Sigma-Aldrich, Dorset, UK
Disodium ethylenediaminetatraacetate (Na_2EDTA)	$C_{10}H_{16}N_2O_8$	99%	BPE120, Fisher Scientific, Leicestershire, UK
D-Malic acid	$C_4H_6O_5$	-	46940-U, Sigma-Aldrich, Dorset, UK
D-Ribose 5-phosphate disodium salt hydrate	$C_5H_{11}O_8P \cdot xH_2O$	≥98%	R7750, Sigma-Aldrich, Dorset, UK
D-Threitol	$HOCH_2[CH(OH)]_2CH_2OH$	99%	377619, Sigma-Aldrich, Dorset, UK
Ethanol	C_2H_6O	99+%	12478730, Fisher Scientific, Leicestershire, UK
Formaldehyde	CH_2O	36.5-38% in H_2O	F8775, Sigma-Aldrich, Dorset, UK
Glutaraldehyde	$CH_2(CH_2CHO)_2$	25% in H_2O	G5882, Sigma-Aldrich, Dorset, UK
Glycerol	$C_3H_8O_3$	≥99%	G5516, Sigma-Aldrich, Dorset, UK

Glycerol phosphate disodium salt hydrate	$C_3H_7Na_2O_6P \cdot 5H2O$	≥99%	G6501, Sigma-Aldrich, Dorset, UK
Glycine	$C_2H_5NO_2$	≥99%	G7126, Sigma-Aldrich, Dorset, UK
Guanine	$C_5H_5N_5O$	98%	G11950, Sigma-Aldrich, Dorset, UK
Hydrochloric acid	HCl	99+%	10254100, Fisher Scientific, Leicestershire, UK
Iron(III) Chloride Hexahydrate	$FeC1_3 \cdot 6H_2O$	≥98.5 %	196690010, Acros Organics, New Jersey, USA
Isopropanol	C_3H_8O	99.5+%	10477070, Fisher Scientific, Leicestershire, UK
L-Asparagine	$C_4H_8N_2O_3$	≥98%	A0884, Sigma-Aldrich, Dorset, UK
L-Glutamic acid	$C_5H_9NO_4$	98.5-100%	G8415, Sigma-Aldrich, Dorset, UK
L-Glutamine	$C_5H_{10}N_2O_3$	≥99%	G3126, Sigma-Aldrich, Dorset, UK
L-Lysine	$C_6H_{14}N_2O_2$	≥98%	L5501, Sigma-Aldrich, Dorset, UK
L-Phenylalanine	$C_9H_{11}NO_2$	≥98%	P2126, Sigma-Aldrich, Dorset, UK
L-Proline	$C_5H_9NO_2$	≥99%	P0380, Sigma-Aldrich, Dorset, UK
L-Pyroglutamic acid	$C_5H_7NO_3$	≥99.0%	83160, Sigma-Aldrich, Dorset, UK
L-Serine	$C_3H_7NO_3$	≥99%	S4500, Sigma-Aldrich, Dorset, UK
L-Threonine	$C_4H_9NO_3$	≥98%	T8625, Sigma-Aldrich, Dorset, UK
L-Tyrosine	$C_9H_{11}NO_3$	≥98%	T3754, Sigma-Aldrich, Dorset, UK
Magnesium Chloride Hexahydrate	$MgCl_2$-$6H_2O$	≥99.0%	M2670, Sigma-Aldrich, Dorset, UK
Magnesium Sulphate Heptahydrate	$MgSO_4 \cdot 7H_2O$	-	101514Y, BDH Chemical Ltd, Poole, England
Manganese (II) Chloride Tetrahydrate	$MnCl_2 \cdot 4H_2O$	99.5-103.0%	S-6646, Sigma, Dorset, UK
Methanol	CH_4O	99.8%	322415, Sigma-Aldrich, Dorset, UK
Methoxyamine hydrochloride	$CH_3ONH_2 \cdot HCl$	98%	226904, Sigma-Aldrich, Dorset, UK
myo-Inositol	$C_6H_{12}O_6$	≥99%	I5125, Sigma-Aldrich,

			Dorset, UK
Nicotinamide adenine dinucleotide (NAD)	$C_{21}H_{27}N_7O_{14}P_2$		N8285, Sigma-Aldrich, Dorset, UK
N-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA). for GC derivatization	$CF_3CON(CH_3)Si(CH_3)_3$	≥98.5%	69479, Fluka-Sigma-Aldrich, Dorset, UK
Potassium chloride	KCI	≥99.0%	P9333, Sigma-Aldrich, Dorset, UK
Potassium hydrogen phosphate	KH_2PO_4	98.0 - 100.5 %	P2222, Sigma, Dorset, UK
Potassium Nitrate	KNO_3	> 99.0 %	P8291, Sigma-Aldrich, Dorset, UK
Potassium phosphate buffer solution	-	-	Sigma-Aldrich, Dorset, UK
Putrescine dihydrochloride	$C_4H_{12}N_2$	≥98%	P7505, Sigma-Aldrich, Dorset, UK
Pyridine	C_5H_5N	99.8%	270970, Sigma-Aldrich, Dorset, UK
Pyruvic acid	$C_3H_4O_3$	98%	107360, Sigma-Aldrich, Dorset, UK
Sodium bicarbonate/sodium hydrogen carbonate	$NaHCO_3$	99.5-100.5%	S5761, Sigma-Aldrich, Dorset, UK
Sodium cacodylate	$(CH_3)_2AsO_2Na \cdot 3H_2O$	≥98%	C0250, Sigma-Aldrich, Dorset, UK
Sodium Chloride	NaCl	≥99.5%	S7653, Sigma-Aldrich, Dorset, UK
Sodium dihydrogen phosphate dihydrate	$NaH_2PO_4 \cdot 2H_2O$	≥99.0%	71505, Sigma-Aldrich, Dorset, UK
Sodium hydroxide	NaOH	-	10346330, Fisher Scientific, Leicestershire, UK
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	≥98%	243655Sigma-Aldrich, Dorset, UK
Sodium nitrate	$NaNO_3$	≥99.0%	S5022, Sigma, Dorset, UK
Sodium periodate	NaIO4	≥99.8%	311448, Sigma-Aldrich, Dorset, UK
Spermidine	$C_7H_{19}N_3$	≥99.0%	S2626, Sigma-Aldrich, Dorset, UK
Starch (GO/P) Assay Kit	-	-	STA20-1KT, Sigma-Aldrich, Dorset, UK
Succinic acid	$C_4H_6O_4$	≥99.5%	14078, Sigma-Aldrich, Dorset, UK
Sulphuric acid	H_2SO_4	-	10090250, Fisher

			Scientific, Leicestershire, UK
Thiamine HCl (Vit. B_1)	$C_{12}H_{17}N_4OS \cdot HCl$	> 99 %	T1270, Sigma, Dorset, UK
Trichloroacetic acid	$C_2HCl_3O_2$	≥99.0%	T6399, Sigma-Aldrich, Dorset, UK
Zinc chloride	$ZnCl_2$	98+%	10000540, Fisher Scientific, Leicestershire, UK
Zinc Sulfate Heptahydrate	$ZnSO_4 \cdot 7H_2O$	> 99.0 %	Z0251, Sigma-Aldrich, Dorset, UK
β-Nicotinamide adenine dinucleotide (reduced) (NADH)	$C_{21}H_{27}N_7Na_2O_{14}P_2 \cdot x H_2O$	> 95 %	N4505, Sigma-Aldrich, Dorset, UK

Table 3-2 Equipments and software

Equipments	**Uses for this research**	**Manufacturers**
Autoclave machine	Sterilization of culture media for microalgal cultivation	BOXER Laboratory Equipment Limited, UK
Neubauer haemocytometer	Counting microalgal cells	Sigma-Aldrich, UK
67 Series UV/Visible spectrophotometer	Absorbance measurement	Jenway (Bibby Scientific Limited, Beacon Road, Stone, Staffordshire), UK
Cuvette macro disposable polystyrene 4.0 ml	Absorbance measurement	FB55143, Fisher Scientific
Eppendorf centrifuge	Harvesting microalgal cells	Eppendorf- Fisher Scientific, UK
Oven	Determination of cell dry weight	Memmert, Germany
Furnace	Ashed samples after oven drying for determination of ash and ash free dry weight	Vecstar Ltd, Chesterfield, UK
Water Bath	Extraction of glycerol and other metabolites from microalgae	Grant Instruments (Cambridge) Ltd, UK
pH meter (HI 2210)	pH measurement	HANNA Instruments, UK
Light microscope with software	The software is a monochrome digital camera for Ultra-fast live cell imaging and superb fluorescence documentation Leica DFC365 FX)	Leica Microsystems, UK

EMITECH K-850 critical point dryer (CPD)	Drying microalgal cells for Scanning electron microscopic experiment	Quorum Technologies Ltd 1 & 3 Eden Business Centre South Stour Avenue Ashford, Kent, UK
Scanning electron microscope (SEM)	Microalgal cells imaging	S360 Cambridge model scanning electron microscope, UK
SEM/Energy-Dispersive X-Ray (EDX) Analysis	Qualitative EDX analysis (elemental analysis of microalgae samples).	Oxford Instruments Co., Abingdon, Oxford-Shire, UK
Gas chromatography - mass spectrometry (GC-MS)	Identification and detection of species based on their retention time and mass spectrum (a compound's specific fragmentation pattern)	Agilent, UK
Centrifugal concentrator (Speedvac-Savant SPD20110)	Sample drying for GC-MS analysis	Thermo Fisher Scientific, US
Supercritical fluid extraction system	Microalgae cell rupture and glycerol extraction	Thar Technology, Inc., USA
Software		
Automated Mass Spectral Deconvolution and Identification System (AMDIS)	Spectral deconvolution software	National Institute of Standards and Technology (NIST), Gaithersburg, USA
MassLynx 4.0	Metabolites were identified using this software coupled with a commercially available compound library: (NIST) GOLM metabolome database.	PerkinElmer Life and Analytical Sciences, USA
SIMCA-Q 13.0.3	Software for multivariate data analysis such as principal component analysis (PCA)	UMETRICS an MKS company, Crewe, UK
Sigmaplot version 11	Statistical analysis of data	Systat Software Inc., London, UK.

3.1 Biological material and culture maintenance condition

The biological material studied in this thesis consists of six strains of halotolerant microalgae, three each of *Dunaliella* and *Asteromonas* species, respectively (Table 3.3). All the strains were isolated by Richard Pipe of the Marine Biological Association (MBA), United Kingdom. These strains were maintained in modified Johnson's media (Table 3.4) (Johnson

et al., 1968; Borowitzka, 1988). Five ml of the *Dunaliella* inoculum were first transferred to 100 ml of Johnson's modified medium. For *Asteromonas* inoculum five ml was first inoculated to 100 ml of f/2 medium (Table 3.5) then transferred to Johnson's modified medium in a 150 ml flask plugged with cotton wool. Stock cultures of algae were allowed to grow and the media became dense after 2-3 weeks; samples were taken at this stage for experiments. All the inoculation procedures were conducted under sterile conditions. No air was bubbled through the cultures and they were agitated by hand once every day.

Table 3-3 Strains of *Dunaliella* and *Asteromonas* investigated for glycerol production

Microalgal species	**Strain code**	**Habitat**	**Growth medium for culture maintenance**
Dunaliella	T35	Saline water	Johnson's modified medium
	T36	Saline water	Johnson's modified medium
	T37	Saline water	Johnson's modified medium
Asteromonas	T33a	Saline water	f/2 medium and Johnson's modified medium
	T33b	Saline water	f/2 medium and Johnson's modified medium
	T33c	Saline water	f/2 medium and Johnson's modified medium

Table 3-4 Modified Johnson's medium composition for maintenance of *Dunaliella* and *Asteromonas*

To 980 ml of distilled water add	
NaCl*	X g
$MgCl_2$-$6H_2O$	1.5 g
$MgSO_4.7H_2O$	0.5 g
KCI	0.2 g
$CaCI_2$	0.2 g
KNO_3	1.0 g
$NaHCO_3$	0.043 g
KH_2PO_4	0.035 g
Fe-solution (1)	10 ml
Trace-element solution (2)	10 ml
(1) Fe-solution (for 1 litre)	
Na_2EDTA	1.89 g
$FeC1_3.6H_2O$	2.44 g
(2) Trace-element solution (for 1 litre)	
$ZnCl_2$	4.1 mg
H_3BO_3	61.0 mg
$CoCl_2.6H_20$	5.1 mg
$CuC1_2.2H_2O$	4.1 mg
$MnCI_2.4H_2O$	4.1 mg
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	38.0 mg
Adjust pH to 7.5 with HCl	

*The amounts of NaCl varied in the culture medium experiments. The level of sodium chloride in the medium is indicated for each experiment (as needed to obtain the desired salinity) in the appropriate experimental chapter. 1.0 M NaCl was used in culture maintenance.

The medium was sterilized by autoclaving at 120 °C, 15 psi for 15 min. The phosphate components were autoclaved separately and the medium was adjusted to pH 7.5 with hydrochloric acid (HCl).

Table 3-5 f/2 medium composition used for maintenance of *Asteromonas*

To 998 ml of distilled water add	
$NaNO_3$	0.075 g
$NaH_2PO_4.2H_2O$	0.0057 g
Trace metals stock solution (1)	1.00 ml
Vitamin mix stock solution (2)	1.00 ml
(1) Trace elements	
Na_2EDTA	4.16 g
$FeCl_3.6H_2O$	3.15 g
$CuSO_4.5H_2O$	0.01 g
$ZnSO_4.7H_2O$	0.022 g
$CoCl_2.6H_2O$	0.01 g
$MnCl_2.4H_2O$	0.18 g
$Na_2MoO_4.2H_2O$	0.006 g
(2) Vitamin mix	
Cyanocobalamin (Vit. B_{12})	0.0005 g
Thiamine HCl (Vit. B_1)	0.1 g
Biotin	0.0005 g

Adjust pH to 8.0 with HCl or NaOH. The medium was sterilized by autoclaving at 120°C for 15 min.

3.2 Cultivation condition and inoculation

Inoculation: Either 1.0 ml or 1.5 ml of stock cultures, *Dunaliella* and *Asteromonas* ($x10^6$ cells/ml) were inoculated in 100 ml or 150 ml of growth medium respectively, to bring the final concentration of cells at the start of growth experiments to 10^4 cells /ml.

Cultivation: After inoculation cultures were placed in a temperature controlled growth chamber (23 ± 2 °C) with light intensity of 45 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) provided by cool white fluorescent lamps, under a 12/12 h light/dark cycle. These cultures were manually agitated once per day with the exception of the first three days of

inoculation (cultures were kept static) under basal maintenance conditions at 1.0 M NaCl condition. For experiments aimed at measuring cell growth and glycerol production, the starting inoculum was always taken from the exponential growth phase of the stock culture. During this phase the rate of growth is constant and the cells have uniform chemical and physical properties.

3.2.1 Microalgal cell growth

Count method: The culture flasks were agitated by hand and the cell numbers were determined by sampling each unit every 48 h by taking an aliquot of 400 – 500 µl from each culture flask. The aliquot samples were fixed with 2.0 % formaldehyde and counted using a 0.2 mm deep Neubauer haemocytometer, with a light microscope. Counting considered the cells presented in the four red, large squares of the haemocytometer (A, B, C and D) (Figure 3.1), and mean cell number were calculated and expressed as the number of cells per ml using equation 3.1.

$$\text{Number of cells/ml} = \frac{\text{Total cells}}{\text{Squares counted}} \times \text{DF} \times 10^4 \quad \text{---------- ➔ } \textbf{\textit{Eq}}.\ 3\text{-}1$$

Where DF = dilution factor and 10^4 = is the volume conversion factor for the haemocytometer

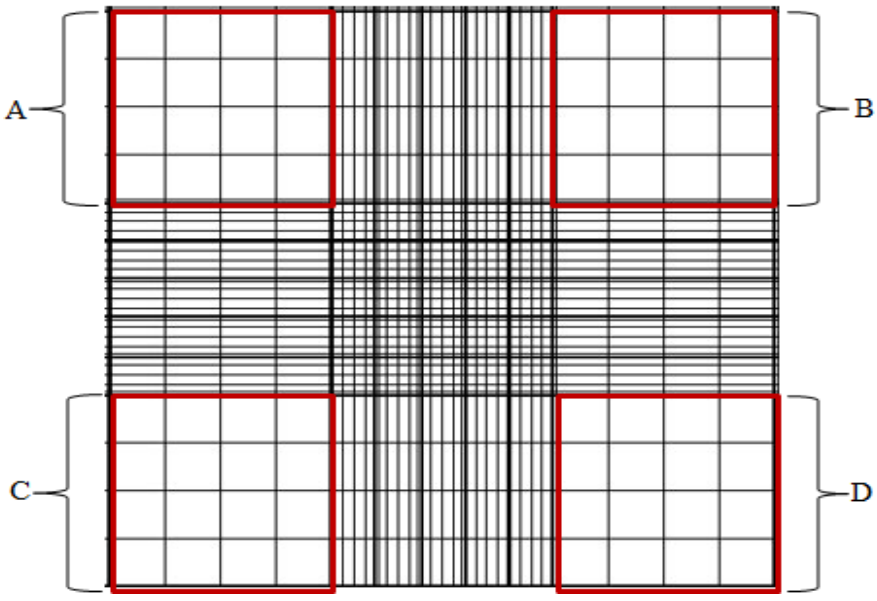


Fig. 3-1 A haemocytometer chamber showing counting grids (A, B, C and D)

Absorbance (A_{630}) method: growth monitoring was also performed using absorbance measurements at 630 nm on a Jenway 67 Series UV/Visible Spectrophotometer. Depending on the dilution required, 100-200 µl of sample was added to a 3 ml 10 mm optical path length cuvette. 1900-1800 µl (depending on dilution) of modified Johnson's medium was pipetted into the cuvette followed by mixing. Samples were diluted such that the measured absorbance was in a range of 0.1 to 0.5, in order to ensure sensitive measurement. Absorbance measurements were plotted against the number of cells counted

3.2.2 Determination of specific growth rate (µ) and generation time (G)

Experiments were conducted using three replicate cultures. The cell density measurements started at day 4, to allow adjustment to ionic strength of the medium. The measurements were continued every 2 days for 28 days. Determination of the doubling time (i.e. the time taken for the number of cells in a population to double) and the specific growth rate (increase in cell mass per unit time) of the microalgae under the influence of varying temperature and salinity were calculated using the following equations (Eq. 3.2, 3.3 and 3.4).

$$\text{Specific growth rate } (\mu) = \frac{\ln N_{t2} - \ln N_{t1}}{(t_2 - t_1)} \quad \text{---------->} \ \textbf{\textit{Eq}}.\ 3\text{-}2$$

$$\text{Divisions per day} = \frac{\mu}{\ln 2} \quad \text{---------->} \ \textbf{\textit{Eq}}.\ 3\text{-}3$$

$$\text{Doubling time (G)} = \frac{1}{\text{Divisions per day}} \quad \text{---------->} \ \textbf{\textit{Eq}}.\ 3\text{-}4$$

$$\text{Doubling time (G)} = \frac{(t_2 - t_1)\log 2}{\log N_t - \log N_o}$$

Where $(t_2 - t_1)$ = time interval in days, N_o = number of cells at a starting point, N_t = the number of cells after the period of time.

3.2.3 Harvesting microalgae

Unless otherwise stated, cells were routinely harvested by centrifugation at 3,000g for 8 min at 25 ^{o}C.

3.2.4 Determination of chlorophyll content

The chlorophyll content of *Dunaliella* cells was determined according to the procedure described by Ben-Amotz and Avron (1983). 10 ml of microalgae culture was centrifuged at 3000 g for 10 min; the supernatant was discarded and the pellet was resuspended in 100 % acetone. The acetone served to extract the chlorophyll from the pellet during a 1 hr incubation period (in the dark). Thereafter, the samples were subjected to vortexing for a few minutes followed by centrifugation at 3000 g for 10 minutes. The supernatant was diluted to 80 % by the addition of distilled water to a final volume of 10 ml. Chlorophyll *a* and *b* was assayed on a Jenway 67 Series UV/Visible Spectrophotometer at 663 and 645 nm against acetone (blank). Concentration of chlorophyll *a* and *b* and total chlorophyll were calculated by equation 3.5, 3.6 and 3.7 respectively (Porra *et al*., 1989). When chlorophyll was extracted using chloroform; the amount of chlorophyll *a* and *b* and total chlorophyll were calculated by equation 3.8, 3.9 and 3.10 respectively (Lichtenthaler 1987; and Wellburn 1994) and expressed as mg/l.

$$\text{Chl } a = 12.25 \times A_{663.6} - 2.55 \times A_{646.6} \quad \text{---------- } \textbf{\textit{Eq}}\text{. 3-5}$$

$$\text{Chl } b = 20.31 \times A_{646.6} - 4.91 \times A_{663.6} \quad \text{---------- } \textbf{\textit{Eq}}\text{. 3-6}$$

$$\text{Total Chl} = [\text{Chl } a + \text{Chl } b] = 17.76 \times A_{646.6} + 7.34 \times A_{663.6} \quad \text{---------- } \textbf{\textit{Eq}}\text{. 3-7}$$

$$\text{Chl } a = 11.47 \times A_{665.6} - 2.0 \times A_{647.6} \quad \text{---------- } \textbf{\textit{Eq}}\text{. 3-8}$$

$$\text{Chl } b = 21.85 \times A_{647.6} - 4.53 \times A_{665.6} \quad \text{---------- } \textbf{\textit{Eq}}\text{. 3-9}$$

$$\text{Total Chl} = [\text{Chl } a + \text{Chl } b] = 19.85 \times A_{647.6} + 6.94 \times A_{665.6} \quad \text{---------- } \textbf{\textit{Eq}}\text{. 3-10}$$

3.2.5 Determination of biomass dry weight

The microalgae cells in the culture at log phase were harvested by centrifugation at 3,000 g for 10 min at room temperature. The pellet was placed in a crucible and dried at 105 °C for 24 hr in oven to determine the dry weight. These oven-dried samples were then ashed in a furnace at 500 ^{o}C for 6 hr once cooled in a dessicator. Ash free dry weight was calculated using equation 3.11.

Ash Free Dry Weight (AFDW) = Cells Dry Weight - Ashed weight - - - - - - - - - ➤ ***Eq***. 3-11

3.3 Light Microscopy

Microalgae samples were examined using a Leica microscope (the software used was monochrome digital camera for Ultra-fast live cell imaging and superb fluorescence documentation Leica DFC365 FX). This software can be calibrated to allow the operator to programme the desired measurements which could be automatically repeated accurately and reproducibly on subsequent occasions.

3.4 Scanning Electron Microscopy:

3.4.1 Preparation of fixatives

Cacodylate buffer (pH 7.2) **Solution A:** 0.2 M solution of sodium cacodylate [42.8g of Na $(CH_3)_2$ $AsO_2.3H_2O$ in 100 ml of dH_2O]. **Solution B:** 0.2 M hydrochloric acid solution (HCl). 4.5 ml of solution B were added to 50 ml of solution A, and made up to 200 ml with dH_2O (Hayat, 1986).

Glutaraldehyde-Cacodylate fixative

8 ml of 25 % (w/v) glutaraldehyde was added to 50 ml of 0.2 M cacodylate buffer, and made up to 100 ml with distilled H_2O. The concentration of prepared glutaraldehyde is 2 % (w/v), and the molarity of the buffer is 0.1M (Hayat, 1986)

Fixation Steps

Microalgal pellets were harvested by centrifugation at 2,500 g for 4 minutes at 4 ^oC. The pellets were immersed in a 2 % (v/v) glutaraldehyde solution buffered with 0.1 M sodium cacodylate (final concentration) for 1 hr at 4.5 ^oC. Microalgae cells were filtered using Velin filter paper. After filtration the filters were immediately enclosed in small squares of aluminium foil. This step was completed in less than 30 sec to avoid air drying of the filters. The filters were placed on the square of foil and loosely folded in half. Samples were identified by indented writing on the folded foil.

3.4.2 Dehydration Steps

After 1-hr fixation, samples were dehydrated by transfer though a series of vials of increasing concentrations of ethanol as Table 3.6.

Table 3-6 Series of ethanol solutions used in microalgae cell dehydration steps

Solution	% Ethanol	% dH_2O	No. of washes	Duration of each wash (min)
Solution 1	10	90	1	30
Solution 2	25	75	1	30
Solution 3	50	50	1	30
Solution 4	75	25	2	30
Solution 5	90	10	1	30
Solution 6	100	0	1	30

Note: samples could be left in the 75 % ethanol solution for up to 2 weeks, if critical point drying could not be done shortly after the last step.

3.4.3 Critical point drying

Microalgal cells were dried using an EMITECH K-850 critical point dryer (CPD) to avoid surface tension and prevent sample distortion. Samples in 100 % ethanol were placed in the critical point dryer (CPD) sample chamber and cooled for 30 min at near 0 ^{o}C. The chamber was then flushed several times with CO_2, to replace 100 % ethanol intermediate solvent in drying process, until no ethanol was present in the vented gas. The sample temperature was then raised to 35 ^{o}C and 1350 Psi (above the CO_2 critical point constants 31.1^{o}C and 1072 Psi) before allowing the CO_2 to purge very slowly (1000 ml min^{-1}) from the sample chamber.

3.4.4 Mounting and viewing with the SEM

Small squares were cut from dried filters with a razor blade and mounted directly on aluminium stubs (Sigma Aldrich) using carbon tabs (Sigma Aldrich), coated with gold for 2 minutes with a sputter coater (model: Edwards S150B; pressure: 1mbar), and viewed with an S360 Cambridge model scanning electron microscope with a 20 KV electron beam at an 18 mm working distance. All samples were coated in a vacuum with two layers of gold, each at a thickness of 10 nm.

3.4.5 Determination of cell volume

The cell volume was calculated using the equation of a prolate ellipsoid, which has been recognised as the shape of algae (Hellebust, 1976; Ehrenfeld and Cousins, 1982; Berube *et al*., 1994). The volume formula for a prolate ellipsoid is presented in equation 3.12.

$$V = \frac{4}{3}\pi ab^2 \qquad \textbf{\textit{Eq}}.\ 3\text{-}12$$

Where *a* is the half maximum length (A) and *b* is the half maximum width (B). Detail of where the measurement was conducted is shown by figure 3.2.

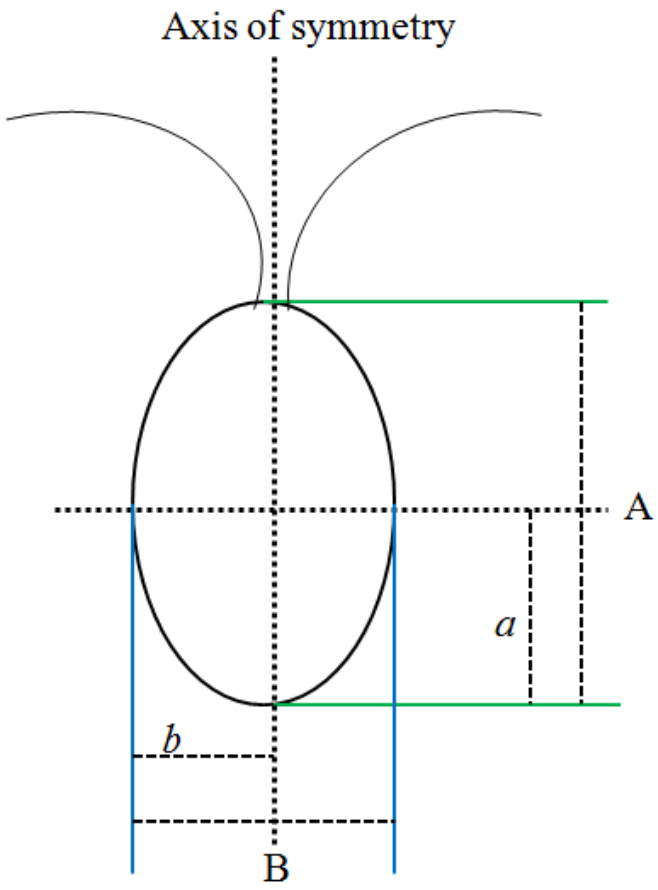


Fig. 3-2 Schematic diagram of microalgal cell showing A, *a*, B, *b* and axis of symmetry. Adapted from Berube *et al*. (1994)

3.5 Determination of glycerol

Three methods were used to measure glycerol: chemical, enzymatic and GC-MS method.

3.5.1 Chemical Method

The glycerol content in each microalgal sample was measured according to the method of Chen *et al*., (2011) with some modifications. The algal pellet harvested by centrifugation at 3000 g for 5 min was resuspended in 1.0 ml of distilled water and 0.2 ml of chloroform. The suspension was sonicated for 8 minutes in an ultrasonic cell disruptor at room temperature, and the supernatant was collected by centrifugation at 10,000 g for 10 min at room

temperature. 100 µl of supernatant of each sample was put in a test tube and made up to 0.2 ml with distilled water; then 1 ml of sodium periodate reagent (3 mmol/l sodium periodate and 100 mmol/l ammonium acetate in 100 ml 6 % acetic acid) was added with mixing to each sample. Five minutes after the addition of sodium periodate reagent, 2.5 ml of acetyl acetone reagent (acetyl acetone: isopropanol =1:99) was added to each sample with mixing. All tubes were placed in a water bath at 60 °C for 30 min. After the tubes had been cooled to room temperature, the absorbance of each sample was read at 410 nm in a spectrophotometer.

The glycerol carbon chain is cleaved twice, and two molecules of formaldehyde and one molecule of formic acid are liberated; in the process, two molecules of periodate are consumed. Glycerol concentration was determined by measuring the formaldehyde from reactions 1 and 2 (Figure 3.3).

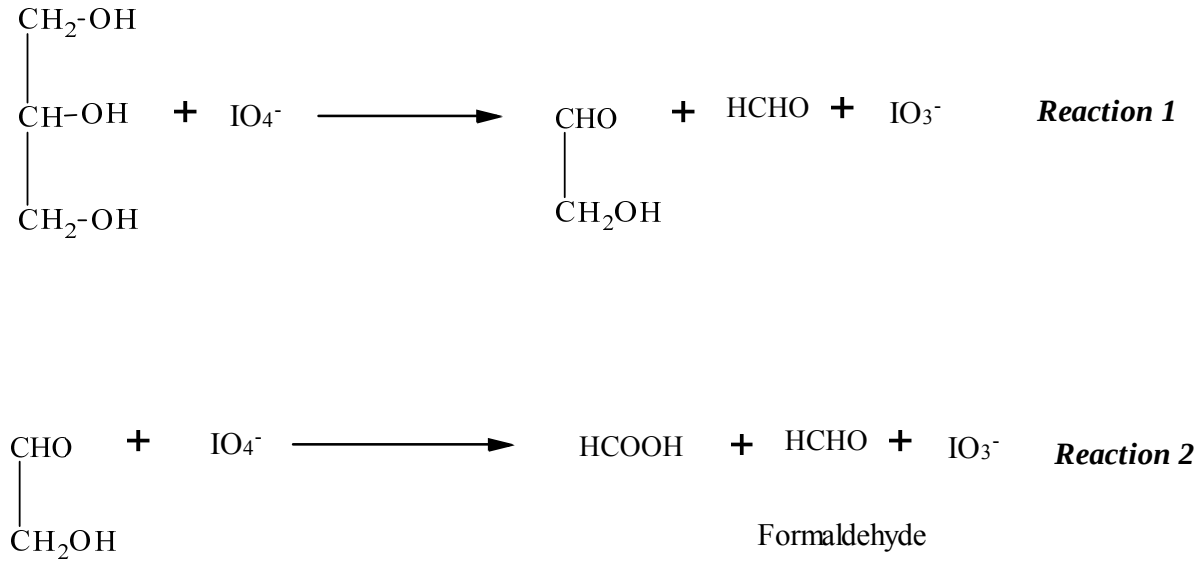


Fig. 3-3 Oxidation of glycerol by periodate reagent method

3.5.2 Enzymatic method

Microalgal extract preparation

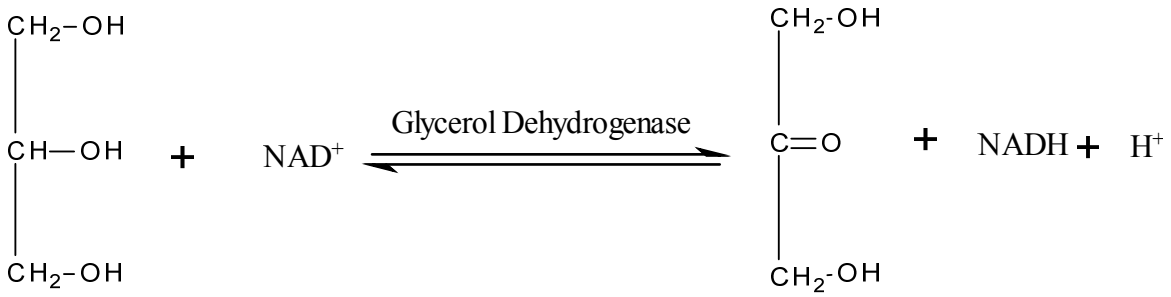
This method was adopted from Ben-Amotz and Avron (1973) with some modifications. The algal pellet harvested by centrifugation was resuspended in 1.5 ml of distilled water. The suspension was sonicated for 5 minutes in an ultrasonic cell disruptor at room temperature,

and the broken cells were removed by centrifugation at 12,000 g for 10 min. The supernatants obtained from these algae were deproteinized with 2 % trichloroacetic acid, neutralized with NaHCO, and analyzed for glycerol by the enzymic method described below.

Enzymic Determination of glycerol

The glycerol content in the algal cells was determined by a specific enzymic oxidation of glycerol by NAD to dihydroxyacetone and NADH (Figure 3.4). A sample of the algal extract was added to the reaction mixture containing: 0.1 M NAD, 0.125 M carbonate/bicarbonate buffer, pH 10.0. The reaction was started by the addition of 10 µl of glycerol dehydrogenase enzyme at concentration of 100 unit/ml in 0.05 M potassium phosphate buffer solution (Worthington Biochemical Enzyme catalogue adopted from Lin and Magasanik, 1960). The final volume was 1.0 ml. The reduction of NAD was followed using a Jenway 67 Series UV/Visible Spectrophotometer, at 340 nm.

The extinction coefficient for NADH (ε_{340} = 6.220 x 10^3 $M^{-1}cm^{-1}$) was used to calculate the amount of glycerol in the samples. The amount of NADH formed in this reaction is stoichiometric with the amount of glycerol. NADH is determined by means of its light absorption at 340 nm.



Glycerol Dihydroxyacetone

Fig. 3-4 Oxidation of glycerol by NAD catalysed by glycerol dehydrogenase. NAD refers to Nicotinamide Adenine Dinucleotide

3.6 Metabolite Extraction

Metabolites were extracted using a method reported by Lamers (2011) with some modification. A 0.5 g pellet of microalgae cells was suspended in 1.0 ml of 4 ^oC ice-cold

aqueous methanol (MeOH: ddH_2O), containing 2.5 µg/ml adonitol (rabitol) as internal standard. The mixtures were briefly vortexed and then sonicated for 20 minutes in an ultrasonic disruptor. The metabolites were then extracted by transferring the tubes into a waterbath at 70 °C with agitation for 40 minutes. The tubes were cooled on ice, vortexed, sonicated for 20 minutes and then centrifuged at 5000 g for 5 minutes at 4 ^oC.

A volume of 200 µl was transferred to a clean tube and 200 µl of ddH_2O and 143 µl of $CHCl_3$ were added, after which the samples were vortexed. The samples were centrifuged (13000 g, 10 min) and 200 µl of the upper (polar) phase was transferred into a GC-MS vial. The solvents were evaporated in a centrifugal concentrator (Speedvac-Savant SPD20110) for 9 hours at 40 °C.

3.6.1 Derivatization of samples

The dried sample's "polar phase" was derivatized by oximation with 150 µl of methoxyamination reagent (2 % methoxyamine hydrochloride dissolved in pyridine) at 40 ^oC for 30 minutes. The main targets of methoxymation reaction are carbonyl groups (Figure 3.5). Subsequent silylation was carried out with 150 µl of N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) at 50 ^oC for 60 min. Here hydroxyl groups are the primary targets for silylation (Figure 3.6). After derivatization was completed, the vials containing samples were briefly centrifuged for 5 seconds and the vials were left to cool in the fume hood for a few minutes prior to the GC-MS analysis. All standards analyzed on the GC–MS/MS were prepared to give a final concentration of 1.43 mM.

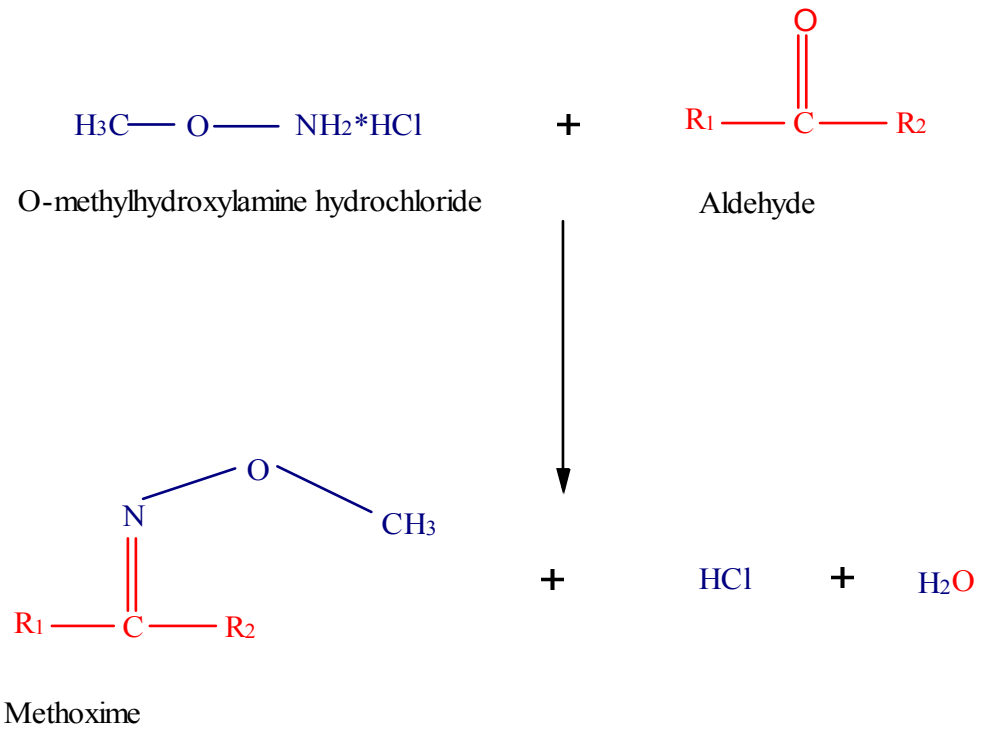


Fig. 3-5 An example of oximation (Accessed from Dettmer *et al.*, 2007). Chemical derivatization reactions commonly used for GC-MS-based plant metabolite analysis

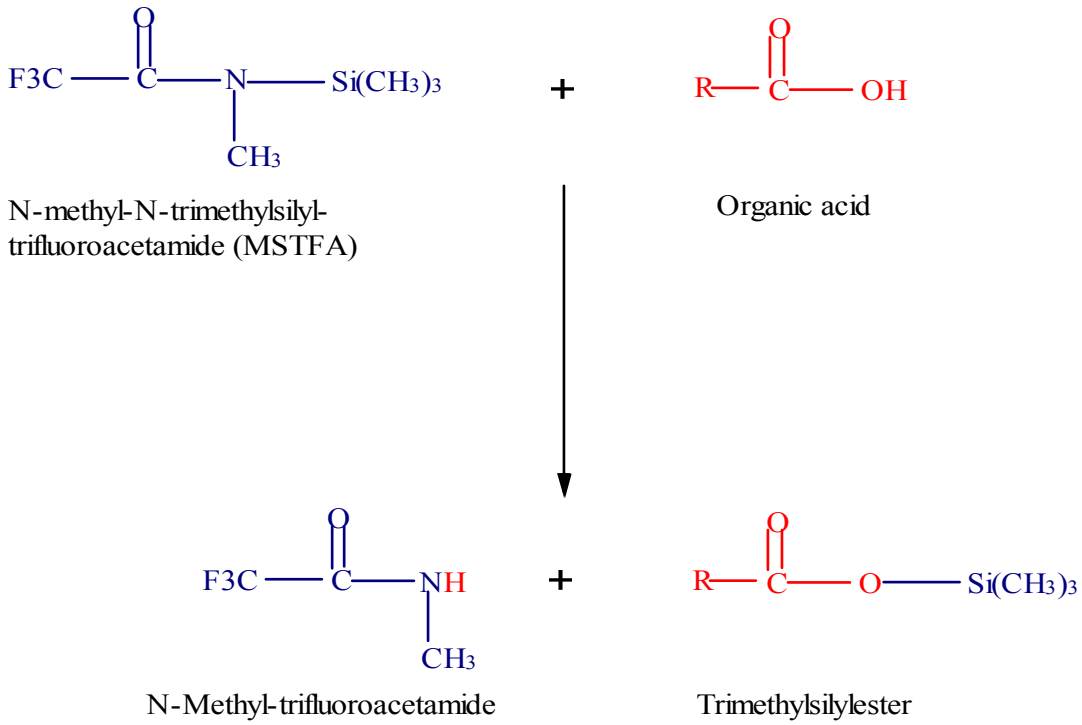


Fig. 3-6 An example of silylation (Accessed from Dettmer *et al.*, 2007).

3.6.2 Preparation of standard

A test solution containing eleven amino acids (β-alanine, L-glutamate, L-lysine, L-glycine, L-glutamine, L-phenylalanine, L-proline, L-asparagine, L-threonine, L-serine, and L-tyrosine), citric cycle intermediates (pyruvate, succinate, citrate and malate), glycolytic pathway intermediates (D-Glucose, D-glucose 6-phosphate and D-fructose 1, 6-bisphosphate), Kelvin cycle intermediates (D-ribose 5-phosphate and D-3-phosphoglyceric acid), glycerol cycle intermediates (glycerol, glycerol-3-phosphate and dihydroxyacetone phosphate) and other metabolites (Adenine, D-threitol, guanine, L-pyroglutamic acid, spermidine, *myo*-inositol, 6-phosphogluconic acid, putrescine dihydrochloride and 3,4-dihydroxy-L-phenylalanine "L-DOPA") were used. A 0.71 mM solution of these in double distilled water (ddH_2O) was prepared from standards. Prior to GC–MS analysis, samples were derivatized (see section 3.6.1).

3.6.3 GC-MS analysis

The GC-MS analysis was conducted according to the method reported by Lamers, (2011) with some modifications. A 0.2 µl aliquot of the derivatised extract was injected into an Agilent DB5 capillary column (30 m × 0.25 mm × 0.25 µm). The inlet temperature was set at 220 ^{o}C. After a 5-minute solvent delay, initial GC oven temperature was set at 60 ^{o}C. Two minutes after injection, the GC oven temperature was raised to 240 ^{o}C (6 ^{o}C min^{-1}), and finally held at 250 ^{o}C for 6 minutes. Helium was used as the carrier gas with a constant flow rate set at 1ml min^{-1}. The measurements were made with positive electron impact ionisation (70 eV) in the full scan mode (m/z 30-550). The microalgal metabolites were identified using MassLynx 4.0 software (PerkinElmer Inc.) coupled with a commercially available compound library: National Institutes of Standards and Technology (NIST) Mass Spectral Database 5.0 (PerkinElmer Inc., Waltham, MS), Golm Metabolome Database (Kopka *et al*., 2005), and the fragmentation patterns of the analytes. The GC-MS conditions for is summarised as follows:

Column: Agilent DB5 (30 m × 0.25 mm × 0.25 µm)

Carrier gas: Helium at 1ml min^{-1}

Oven temperature: 60 ^{o}C for 2 minutes; then the temperature was raised to 240 ^{o}C (6 ^{o}C min^{-1}),

MSD transfer Temperature: Held at 250 ^{o}C for 5 minutes

Data system: Agilent Chemstation

Injector: Split/splitless at 220 ^{o}C

Injection volume: 0.2 µl

Detector: Transfer line temperature at 250 ^{o}C; Source temperature at 230 ^{o}C; Quad temperature at 150 ^{o}C

3.6.4 Data processing

The individual spectra and chromatograms from GC-MS data files were deconvoluted with the aid of Automated Mass Spectral Deconvolution and Identification System (AMDIS) spectral deconvolution software package v2.69 (NIST, Gaithersburg), freely available at http://chemdata.nist.gov/mass-spc/amdis/, to carry out baseline corrections and purify spectrum from background noise. Target compounds were identified by fragmentation patterns and matching spectra in a reference library (NIST Mass Spectral Database v. 5.0) and (Golm Mass Spectral Database).

3.6.5 Selection criteria for the identification of compounds

The total-ion chromatograms (TIC) of the polar compounds extracted from *Dunaliella* T35 cells were identified by matching samples with spectra of the standards and using the GC-MS Metabolites Spectral Database (GOLM and NIST 2005) mass spectral library. Based on the identification results, the quantitation ion, reference ion(s), retention time, mass spectrum and retention index for each compound were registered in the compound table. That table was used to automatically detect each compound. Appendix A1.1 shows the standards and their TMS-derivatives that were used in identify metabolites from microalgae samples.

3.6.6 Multivariate data analysis

To determine the changes or differences between the total ion currents or chromatograms (TIC) of various algal samples, principal component analysis (PCA) was performed using SIMCA-Q 13.0.3 (MKS Umetrics AB, UK). PCA was performed to represent low dimensionality of the multivariate data set that captures most of the variance.

3.7 Starch Determination

The amount of starch was determined using a starch assay kit (Amylase/Amyloglucosidase Method) reported by Sigma-Aldrich® technical bulletin, with some modifications.

3.7.1 Sample preparation

50 mg of microalgae wet pellet (equivalent to 20 mg dry pellet). For wheat and corn starch controls, and samples with high starch content, the sample size was reduced to 10 mg. To the harvested microalgae pellets and control samples (corn and wheat starch) 5.0 ml of 80 % ethanol in dH_2O solution was added. Following 5 minutes incubation at 80–85 °C, the contents of the tubes were mixed and another 5.0 ml of the 80 % ethanol solution added. The tubes were then centrifuged for 10 minutes at 1,000 *g*. The supernatant was discarded. The pellet was resuspended in 10 ml of 80 % ethanol solution and mixed and centrifuged for 10 minutes at 1,000 *g*, and the supernatant was carefully poured off and discarded. Starch digestion was undertaken as presented in the next section.

3.7.2 Starch digestion

0.2 ml of 80 % ethanol solution was added to each pellet and to an empty tube labelled "Starch Digestion Blank" and mixed. 3.0 ml of water and 0.02 ml of α-Amylase (heat stable) was added into each sample and blank tube, mixed and then incubated for 5 minutes in a 100 ^{o}C water bath. The tubes were removed from the water bath and cooled to room temperature. The volume in each tube was made up to 10 ml with dH_2O and mixed.

To 1.0 ml of each test and blank solution above, 1.0 ml of the starch assay reagent was added. The solutions were mixed and incubated for 15 minutes at 60 °C in a water bath. The tubes were removed from the water bath and cooled to room temperature. 1.0 ml of each sample and blank was diluted to 10 ml with water. Glucose was determined as described in the next section (Table 3.7).

3.7.3 Glucose assay

Table 3-7 Glucose assay reaction mixture

Reagent	Standard Blank	Standard	Reagent Blank	Test
Distilled water (ml)	1.0	0.950	-	-
Glucose standard reagent (ml)	-	0.05	-	-
Blank from starch digestion (ml)	-	-	1.0	-
Samples from starch digestion (ml)	-	-	-	1.0

At zero time, the reaction was started by adding 2.0 ml of the glucose assay reagent to the first tube and mixed. A 30–60 second interval was allowed between additions of glucose assay reagent to each subsequent tube (see Figure 3.7 for the reactions). The tubes were incubated for 30 minutes at 37 °C. Each reaction was stopped at 30–60 second intervals by adding 2.0 ml of 12 N H_2SO_4 into each tube. The tubes were mixed thoroughly. The absorbance of each tube was measured at 540 nm corresponding to the formation of oxidized *o*- Dianisidine in an enzyme coupled reaction with glucose oxidase and peroxidase (method is called Trinder technique).

Reactions:

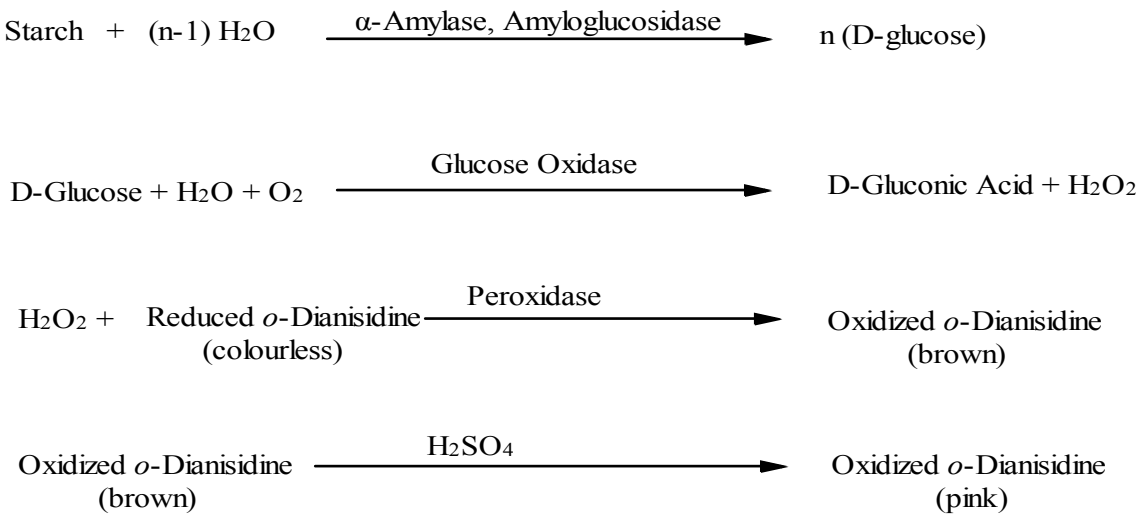


Fig. 3-7 Hydrolysis of starch to glucose (catalyzed by α-amylase and amyloglucosidase) and glucose oxidizedation to gluconic acid and hydrogen peroxide by glucose oxidase. Adopted from Sigma-Aldrich® technical bulletin. (http://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/Bulletin/sta20bul.pdf).

Calculations:

$$\Delta A_{STANDARD} = A_{STANDARD} - A_{STANDARD\ BLANK}$$

$$\Delta A_{TEST} = A_{TEST} - A_{REAGENT\ BLANK}$$

$$\%Starch = \frac{(\Delta ATEST)\,(F)\,(V)\,(SF)\,(SDF)\,(VGA)\,(MWF)\,(100)}{(\text{Conversion factor for } \mu g \text{ to mg})\,(\text{Sample weight in mg})}$$

$$= \frac{(\Delta A_{TEST})\,(50/\Delta A_{STD})\,(10)\,(2)\,(10)\,(1.0)\,(0.9)\,(100)}{(1000)\,(\text{Sample weight in mg})}$$

$$= \frac{(\Delta A_{TEST})\,(900)}{(\Delta A_{STD})\,(\text{Sample weight in mg})} \dashrightarrow \textbf{\textit{Eq}}.\ 3\text{-}13$$

F = µg glucose in standard ÷ $\Delta A_{STANDARD}$ at 540 nm = $50/\Delta A_{540}$

V = Initial sample volume (from sample preparation)

SF = Total assay volume from starch assay/sample volume from starch assay

SDF = Dilution factor from end of starch assay

VGA = Initial sample volume from glucose assay

MWF = Molecular weight of starch monomer/molecular weight of glucose = 162/180 = 0.9

3.8 Super critical CO_2 (SC-CO_2) extraction

A glass beaker containing algae (1.5 - 2.0 g wet pellet) was placed in the high-pressure reaction vessel (RV1) (figure 3.8). The pressurized CO_2 was pumped into the reaction vessel and heated to desired temperature. A series of experiments were conducted in temperatures ranging from 40-50 °C and pressures from 100-200 bar to determine the optimum condition for glycerol extraction. Only one variable was changed at a time, for example temperature was kept constant and pressure was gradually increased by 10 bar at a time until the desired value was achieved. The experiments were used to establish best condition for maximum cell rapture. Extraction of glycerol was performed in a system presented in figure 3.8.

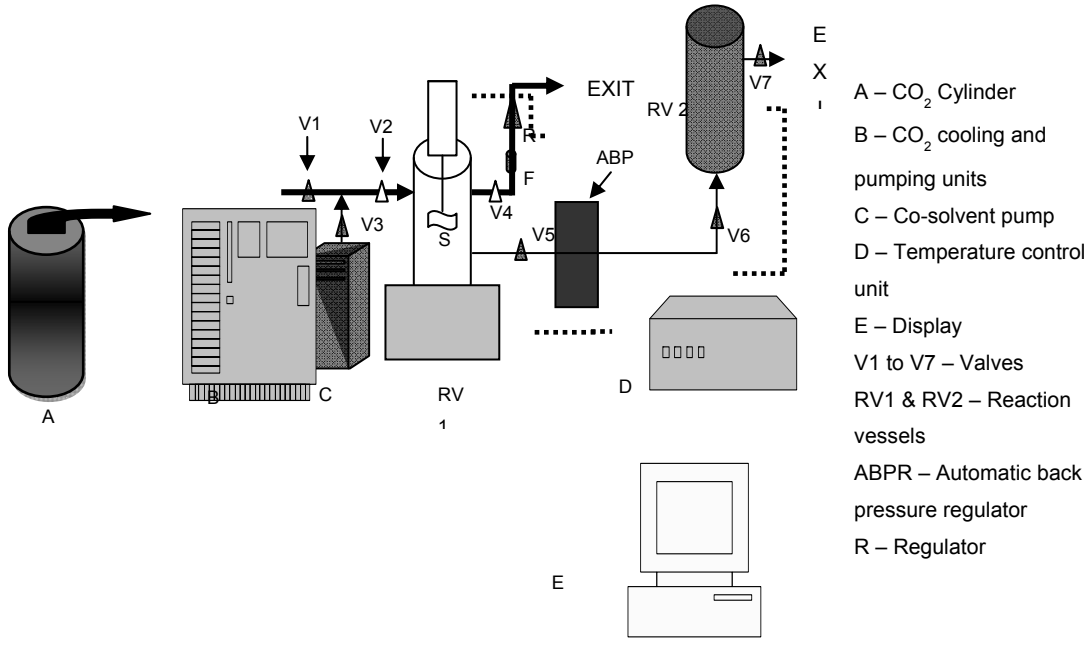


Fig. 3-8 Schematic diagram of supercritical fluid equipment

CHAPTER 4.0: EFFECT OF SALINITY, TEMPERATURE AND INITIAL pH ON THE GROWTH PARAMETERS OF HALOTOLERANT MICROALGAE (*DUNALIELLA* AND *ASTEROMONAS*)

4.1 Introduction

Halophytic microalgae (*Dunaliella* and *Asteromonas*) are ubiquitous microorganisms in hypersaline environments; they are able to survive in saline media ranging from about 0.5 M NaCl to saturated salt solutions of around 5.0 M NaCl (Ben-Amotz and Avron, 1983; Ben-Amotz and Avron, 1990). The microalgal growth responses involve complex interactions to a number of variables such as temperature, pH, salinity and nitrogen concentration. The production of glycerol is crucial to the survival of *Dunaliella* in various saline media. Unlike the case with β-carotenoids there is no commercial production of glycerol from microalgae. Carotene production by mass cultivation of *Dunaliella* is one of the foremost successes in applied algal biotechnology as reported by Ben-Amotz and Avron, (1989). Glycerol production from halophytic microalgae is dependent on the amount of salinity stress applied (Ben-Amotz and Avron, 1990).

In order to promote mass production and reduce glycerol production costs, it is essential to identify the set of conditions required for the cultivation of halotolerant microalgae. Studies on production of glycerol by *Dunaliella* strains have been conducted (Ben-Amotz *et al.,* 1982; Kacka and Donmez, 2008; Chen *et al*., 2011), but there is a need to understand whether Namibian strains of *Dunaliella* and *Asteromonas* have the same capacity to produce glycerol. The wide distribution of species of the genera *Dunaliella* and *Asteromonas* may be attributed to their tolerance to a wide range of salinities, temperature and pH. These factors influence the rate of growth of numerous halotolerant microalgae species (Ginzburg and Ginzburg, 1981; Borowitzka and Borowitzka, 1988). In this research the aim is to establish a laboratory cultivation system in which both species of *Dunaliella* and *Asteromonas* obtained from Namibia could be grown at a high specific growth rate. Previous research has shown that *D.*

viridis grows optimally in 1.0 – 1.5 M NaCl and tolerates up to 4.0 M NaCl whereas *D. salina* grows best in 2.0 M NaCl and tolerates up to 6.0 M NaCl (Borowitzka *et al*., 1977). However *D. viridis* has been reported to grow optimally at 5.8 M NaCl and temperature of 30 ^{o}C (Jimenez and Niell, 1991).

This chapter presents results of an investigation on the effect of temperature, pH and salinity on growth and chlorophyll of *Dunaliella* (T35, T36 and T37) and *Asteromonas* (T33a, T33b and T33c). These microalgae were grown alongside temperate species (*D. salina, D. quartolecta, D. parva and D. polymorph*a) obtained from Culture Collection of Algae and Protozoa (CCAP) and their growth parameters were compared.

4.2 Methods

4.2.1 Monitoring of microalgae cell culture growth

See section 3.2.1

4.2.2 Effect of different factors (pH, temperature and salinity) on growth parameters

Halotolerant microalgae *Dunaliella* strains (T35, T36 and T37) and *Asteromonas* strains (T33a, T33b and T33c) were maintained in the cultivation media containing 1.0 M NaCl as described in section 3.1. A 1.0 ml (x10^6 cells) microalgal samples were taken at the exponential growth phase from the maintenance culture and added to Johnson's medium with different amounts of NaCl as shown in Table 4.1. The effect of initial culture media pH and temperature was also assessed on microalgae grown in Johnson's medium with 1.0 M NaCl. *D. salina, D. quartolecta, D. parva and D. polymorph*a were cultivated under the same conditions described for *Dunaliella* strains in Table 4.1 and their growth parameters were compared with those of *Dunaliella* (T35, T36 and T37) and *Asteromonas* (T33a, T33b and T33c) strains.

Table 4-1 Different experimental conditions during batch culture experiments for the determination of growth parameters

Experiment	Factor investigated
Salinity	0.5, 1.0, 2.0, 3.0 and 4.0 M NaCl
Temperature	15, 20, 25, 30 and 35 ^{o}C
Initial pH*	6.0, 7.0, 7.5, 8.0 and 9.0

* The initial pH of the growth medium was adjusted to 6, 7, 7.5, 8 and 9 with 0.01 M HCl and 0.01M NaOH similar to experiments conducted by Celeki and Donmez, (2006) with some modification

4.2.3 Data analysis

Analysis of variance (ANOVA) was performed to test the differences between the means of growth rates at each environmental factor (salinity, temperature and pH). When the ANOVA results showed that the treatments were significant ($p < 0.05$), Tukey's multiple comparison tests was used to compare the value of the mean of each treatment.

4.3 Results

4.3.1 Monitoring of microalgae cell growth

Figure 4.1 shows a strong linear correlation ($R^2 = 0.9995$) between number of cells counted using a haemocytometer and optical density measurement at 630 nm. This suggests that either absorbance measurement or cell number count could be used in monitoring microalgal culture growth. In this research, optical density measurement was used as a more rapid alternative to haemocytometer count in monitoring microalgal cell culture growth, and where necessary the absorbance measurement were converted to cell density (number of cells/ml) using the calibration curve below (Figure 4.1).

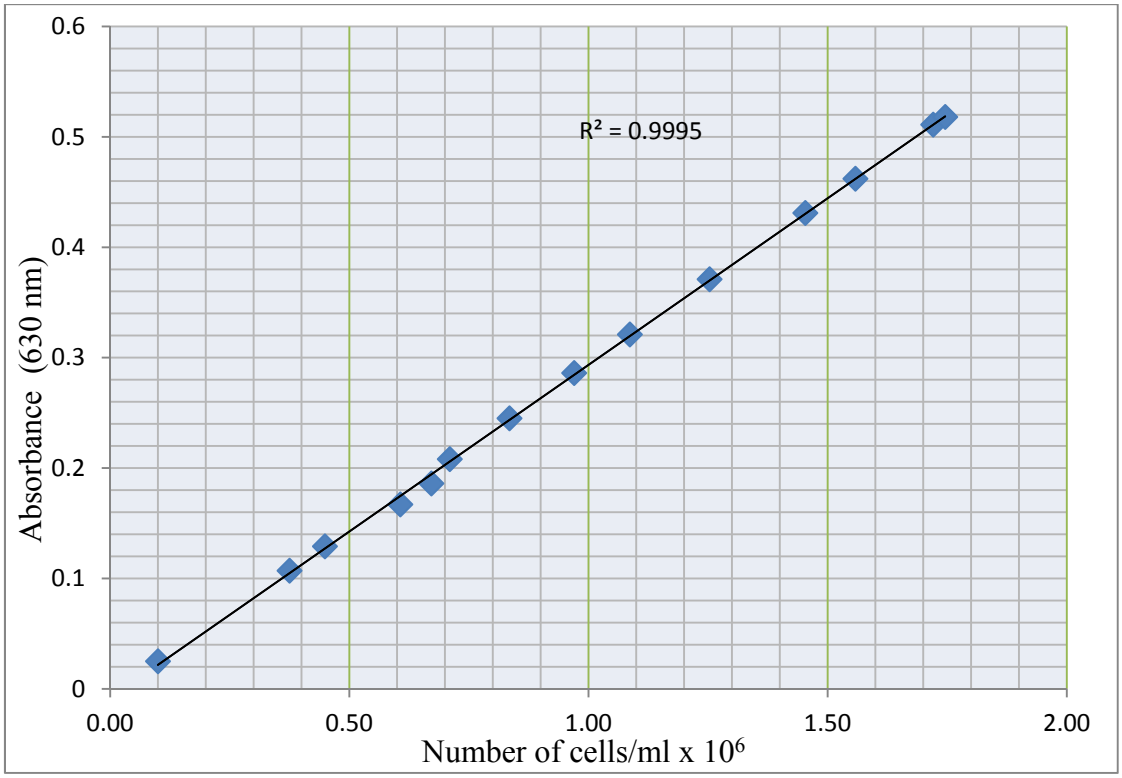


Fig. 4-1 Absorbance measurement versus haemocytometer count of *Dunaliella* T35 grown at 1.0 M NaCl concentration at 25 °C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium.

4.3.2 Chlorophyll composition and dry weight of *Dunaliella* and *Asteromonas*

It is worth pointing out that both the *Dunaliella* and *Asteromonas* strains have chlorophyll *a* and *b* (Masuda *et al*., 2002), therefore, the extraction yields presented in this work represent the sum of both of these.

Two experiments were undertaken: a preliminary study of chlorophyll levels of T35 in 1.0 and 4.0 M NaCl over the first 10 days of cultivation, and secondly, a study of all algal strains in 1.0 and 4.0 M NaCl over the first 14 days.

The amount of chlorophyll (mg/L per cultures) measured was plotted against the number of cells counted for *Dunaliella* T35 grown in 1.0 and 4.0 M NaCl medium. A positive correlation (R^2 = 0.9537 and 0.9567) was found between number of cells counted using haemocytometer and amount of chlorophyll measurement at 1.0 and 4.0 M NaCl, respectively (Figure 4.2).

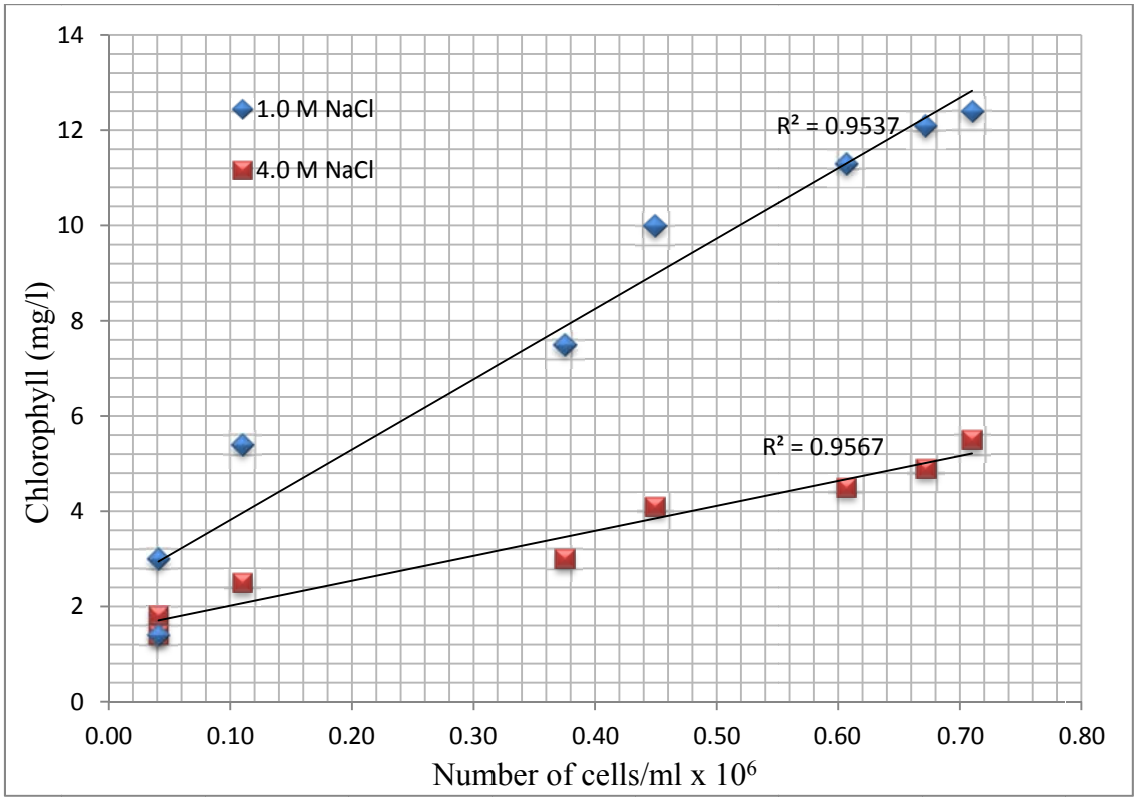


Fig. 4-2 Correlation between amount of chlorophyll and cell number of *Dunaliella* T35 grown at 1.0 and 4.0 M NaCl concentration at 25 ^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium over 10 days.

In the second experiment, the chlorophyll content per ml of culture for 1.0 M NaCl reached a plateau after 10 days of inoculation for *Dunaliella* strains but continued to increase in 4.0 M NaCl (Figure 4.3). Similar results were observed for the three strains of *Asteromonas* (Figure 4.4). However, since the content of chlorophyll per cell at the end of 14 days was the same in both 1.0 M NaCl and 4.0 M NaCl for each strain investigated, the results show that lower chlorophyll levels per volume of culture in 4.0 M NaCl negatively affected by low cell number growth compared to 1.0 M NaCl (Table 4.2).

These data also show that *Dunaliella* strain T36 had a significantly lower content of chlorophyll (6.4 ± 0.02 pg/cell) than either T35 or T37; similarly *Asteromonas* strain T33b differs from either T33a or T33c based on chlorophyll contents. The result shows that chlorophyll determination can be used to estimate photosynthetic production in cells.

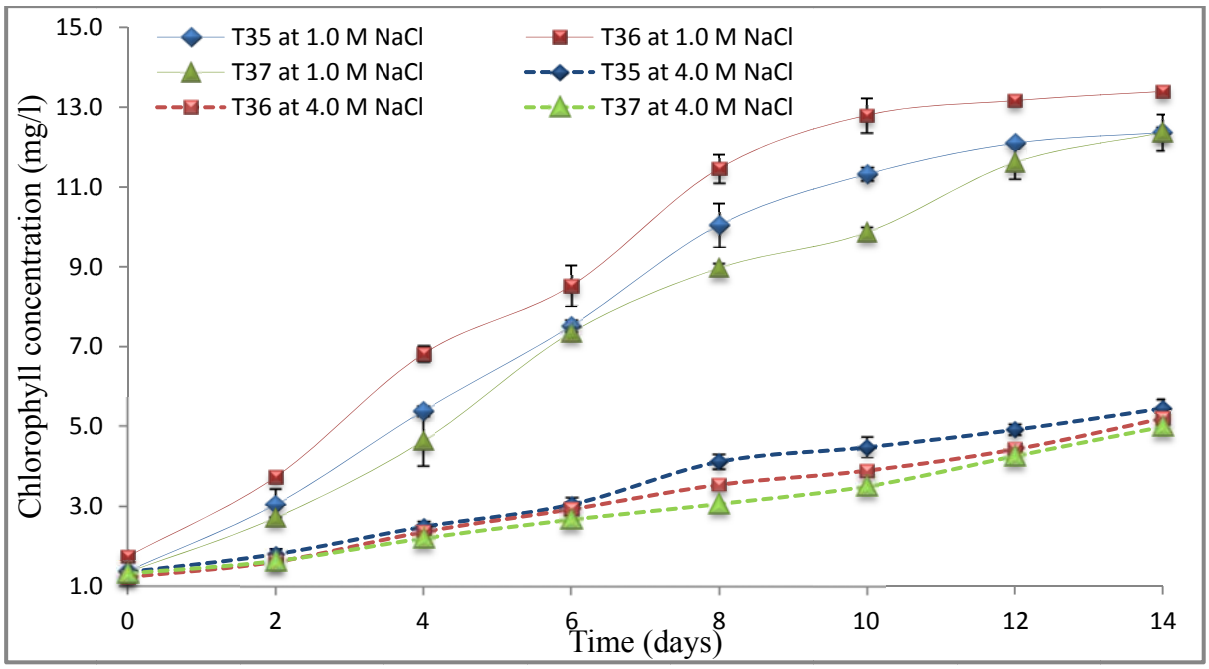


Fig. 4-3 Chlorophyll content of *Dunaliella* T35, T36 and T37 cultivated at 25 °C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in 1.0 and 4.0 M NaCl medium. Error bars represent the standard deviations (n=3) of the measured chlorophyll.

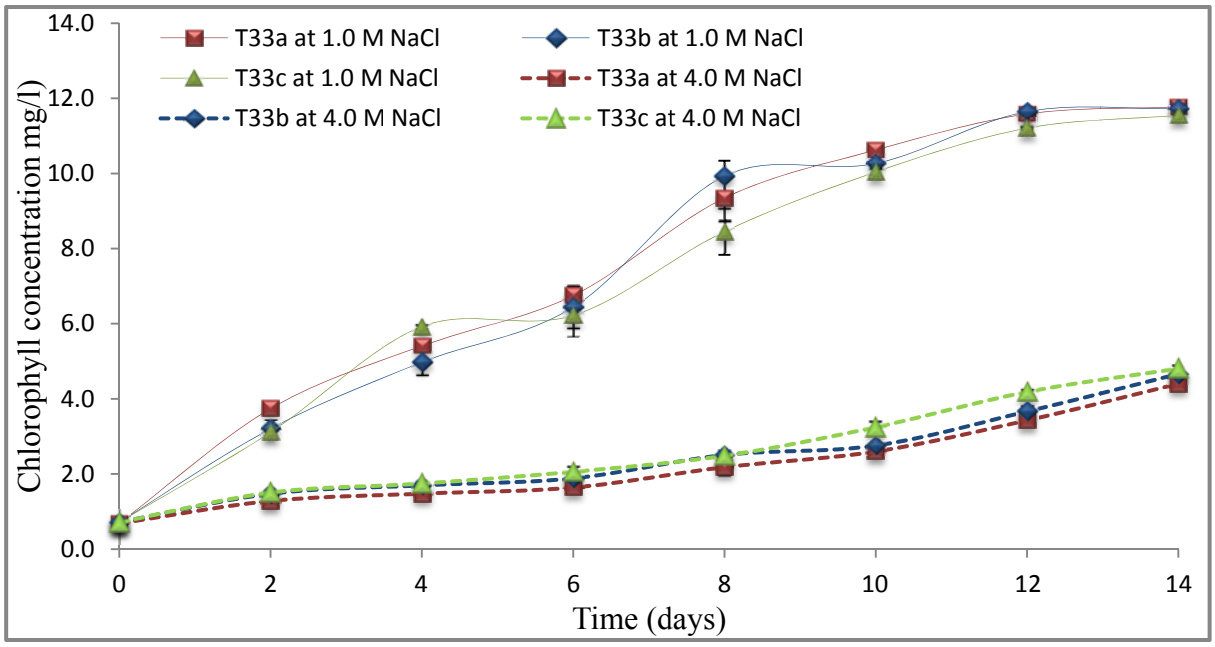


Fig. 4-4 Chlorophyll content of *Asteromonas* T33a, T33b and T33c at 25 °C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in 1.0 and 4.0 M NaCl medium. Error bars represent the standard deviations (n=3) of the measured chlorophyll.

Table 4-2 Effect of NaCl concentrations on the total chlorophyll of *Dunaliella* and *Asteromonas* strains after 14 days growth in Johnson's medium at 25 ^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod

Strain	Salinity (NaCl M)	Chlorophyll (mg/l)	Chlorophyll (Pg/cell)
Dunaliella			
T35	1.0	12.4 ± 0.12	7.1 ± 0.07
	4.0	5.5 ± 0.22	7.0 ± 0.29
T36	1.0	13.4 ± 0.05	6.4 ± 0.02
	4.0	5.2 ± 0.13	6.3 ± 0.17
T37	1.0	12.4 ± 0.45	7.2 ± 0.02
	4.0	5.0 ± 0.14	7.0 ± 0.19
Asteromonas			
T33a	1.0	11.8 ± 0.04	6.9 ± 0.02
	4.0	4.7 ± 0.11	6.7 ± 0.16
T33b	1.0	11.7 ± 0.16	5.9 ± 0.08
	4.0	4.7 ± 0.07	5.9 ± 0.09
T33c	1.0	11.5 ± 0.17	6.6 ± 0.10
	4.0	4.8 ± 0.08	6.6 ± 0.12

The amount of dry weight (DW) produced by *Asteromonas* strains appeared slightly higher compared to three strains of *Dunaliella*, although the difference in DW between *Dunaliella* and *Asteromonas* is not statistically significantly (Table 4.3). The Ash-Free Dry Weight (AFDW; a measurement of the weight of organic material) of microalgal sample was about 85-80 % of DW. The amount of water in the wet pellet was between 60 to 63.5 % of the ww.

Table 4-3 Dry weight and ash free dry weight of 14 days old cultures of *Dunaliella* and *Asteromonas* strains grown at 1.0 M NaCl, 25 ^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. Dry weight was corrected for NaCl, samples were washed with ammonium bicarbonate to reduce the effect of salt in weight measurement.

Strain	Cell density (x10^6 cells/ml)	WW (g/l)	DW (g/l)	AFDW (g/l)	Moisture content (%)	Organic matter (%)	AFDW/ DW
T35	1.75	1.37 ± 0.03	0.50 ± 0.01	0.44 ± 0.02	63.5	36.5	0.88
T36	2.10	1.39 ± 0.08	0.54 ± 0.02	0.47 ± 0.02	61.2	38.8	0.87
T37	1.73	1.33 ± 0.02	0.52 ± 0.02	0.45 ± 0.01	60.9	39.1	0.87
T33a	1.70	1.45 ± 0.05	0.58 ± 0.02	0.49 ± 0.02	60.0	40	0.85
T33b	2.00	1.48 ± 0.03	0.57 ± 0.01	0.49 ± 0.01	61.5	38.5	0.86
T33c	1.75	1.54 ± 0.02	0.58 ± 0.02	0.51 ± 0.02	62.3	37.7	0.88

WW= Wet weight, DW= Dry weight; AFDW= Ash free dry weight

4.3.3 Effect of salinity on cell number growth, specific growth rate and doubling time of *Dunaliella* strains

After inoculation of microalgae cells in modified Johnson's medium, the cultures undergo a lag phase as described in section 3.2. This is the time represented for adaptation of microalgae to the new environment; the cultures were clear and transparent (Figure 4.5A). For *Dunaliella* the lag phase lasted approximately 5 days after inoculation (Figure 4.6). On approximately day 6-7, there was a visibly detectable increase in cell density (Figure 4.5B). Development of green colour in the cultures indicated that the microalgal cells were in the exponential growth phase (increase in cell number, at a constantly growing rate). Cell cultures were allowed to grow continuously throughout the exponential growth phase under defined culturing conditions. By day 25 the cultures were opaque and dark green in colour (Figure 4.5C).

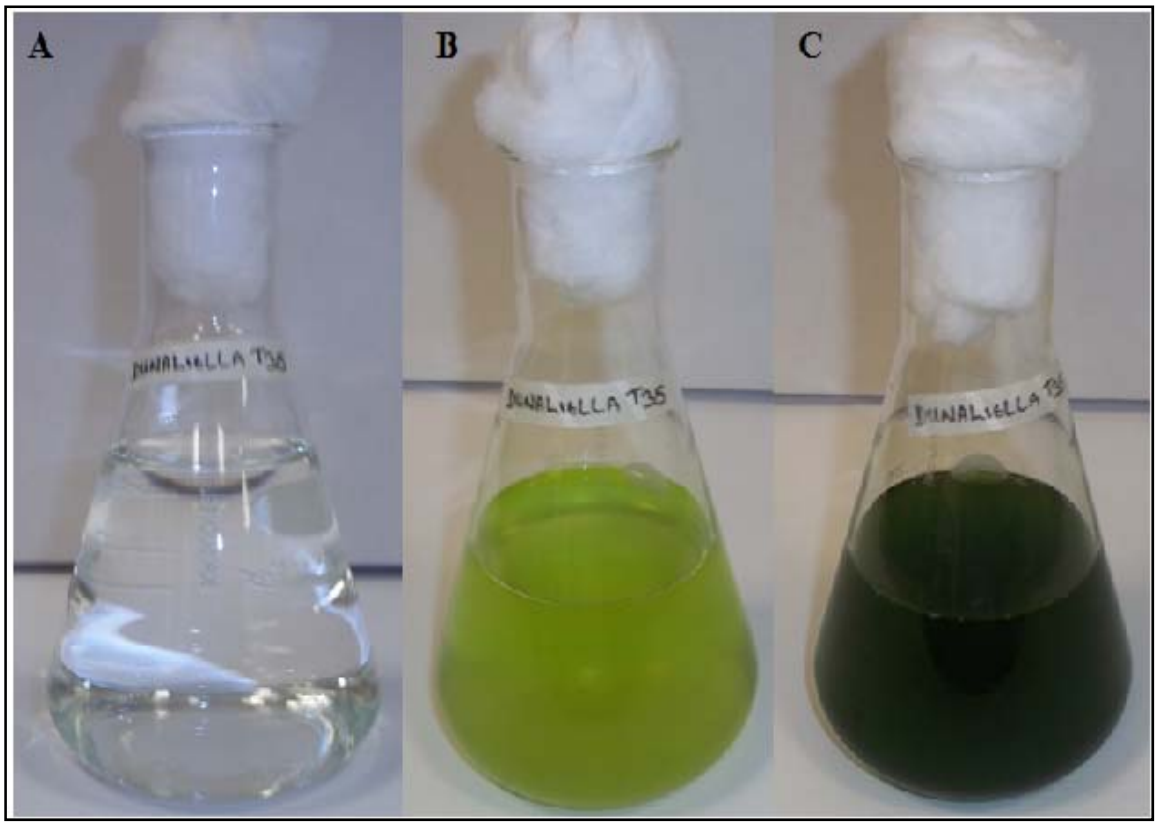


Fig. 4-5 *Dunaliella* T35 cultures grown at 1.0 M NaCl concentration at 25^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium; (A) Cultures after 24 hours of inoculation, (B) cultures on day 7 of inoculation and (C) cultures on day 25 of inoculation.

Figure 4.6 describes the growth kinetics for *Dunaliella* strains. The specific growth rate and doubling time for each strain (Table 4.5) was obtained by plotting the natural log of cell number against cultivation time for the period from day 5 to day 23 (Figure 4.7).

In culture medium with 1.0 M NaCl cell division for all strains of *Dunaliella* was more rapid (0.119-0.123 Div.d^{-1}) than at any other salt concentrations investigated (Figure 4.6). Higher cell density was found at the end of 25 days cultivation period (Table 4.4). Maximum cell density (1.75x10^{6} cells/ml) for T35 was obtained at 1.0 M NaCl concentration and the lowest cell density (1.06 x 10^{6} cell/ml) was obtained at 4.0 M NaCl. A comparison of the cell densities of the strains is shown in Table 4.4. There was a decrease of the cell number with increasing salinity above 1.0 M NaCl and in salinity below 1.0 M NaCl. Notably, *Dunaliella*

T36 had a faster doubling time than either T35 or T37. It is considered the best strain in terms of rapid increase in cell number.

Under all conditions of NaCl there was an apparent higher rate of growth from day 3 to 5 compared to the later, exponential phase (Figure 4.6). The possible reason for that is from 1 to 3 days after inoculation the divided cells are present at too low in number to be seen in the haemocytometer. The maximum cell density was observed at day 27 of the experimental period, which may indicate that under the specified conditions the amount of nutrients in the growth medium was able to support the cultures for 27 days. After 27 days of experiment the cells density declined at 4.0 M NaCl medium. The cell may not withstand constant exposure to high salinity, which may relate to drain of ATP in the starch to glycerol conversion (Kaplan *et al*., 1980).

Appendix A2.1 describes a double reciprocal plot (Lineweaver-Burk plot) constructed from the data. Despite the limited number of data points below 1.0 M NaCl, it shows that in *Dunaliella* increasing NaCl concentration from 1.0 to 4.0 M NaCl is inhibitory to growth.

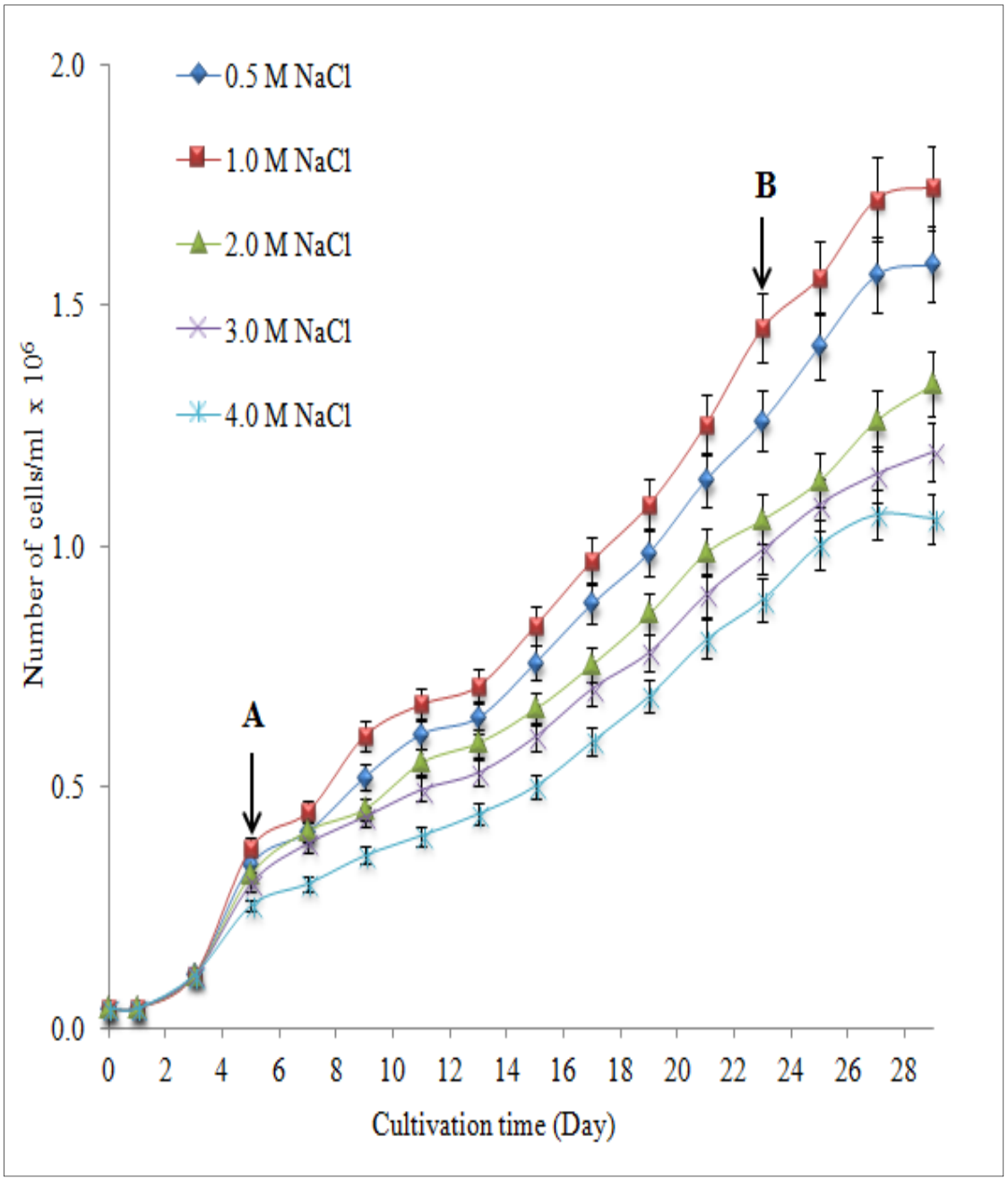


Fig. 4-6 Effect of increasing NaCl (0.5 to 4.0 M) on the growth of *Dunaliella* T35 at 23°C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. A to B are the data points used in estimating specific growth rate. Error bars represent the standard deviations (n≥3) of measured values of cell density

Table 4-4 Comparison of the effect of salinity on cell density of *Dunaliella* strains at 23 ^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium for 23 days.

***Dunaliella* strain**	**Cell number/ml x 10^6 (1.0 M NaCl)**	**Cell number/ml x 10^6 (4.0 M NaCl)**	**% Reduction**
T35	1.75	1.06	39
T36	4.38	1.60	63
T37	2.66	1.04	61

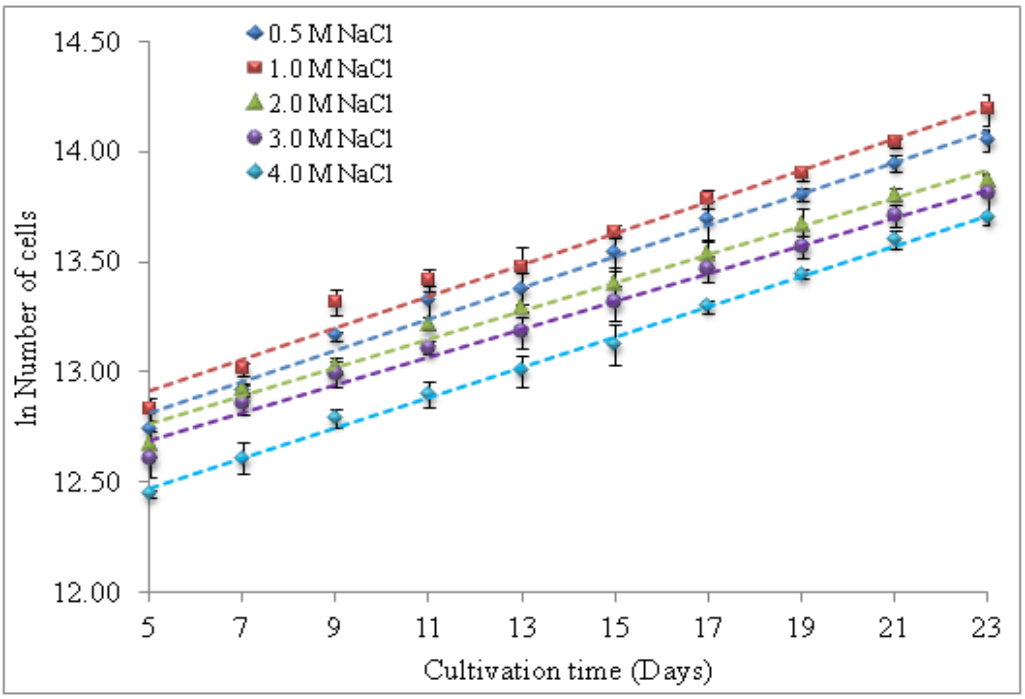


Fig. 4-7 Natural log plot for the effect of increasing NaCl (0.5 to 4.0 M) on the growth of *Dunaliella* T35 at 23^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. Error bars represent the standard deviations (n≥3) of measured values of cell density (natural log).

Table 4-5 Specific growth rate and doubling time of *Dunaliella* T35, T36 and T37 grown in a range of NaCl concentrations at 23 ^{o}C, pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium.

Salinity	***Dunaliella***					
	T35		T36		T37	
	SGR ($Div.d^{-1}$)	Doubling (days)	SGR ($Div.d^{-1}$)	Doubling (days)	SGR ($Div.d^{-1}$)	Doubling (days)
0.5	0.114	6.1	0.120	5.8	0.116	6.0
1.0	0.119	5.8	0.123	5.6	0.119	5.8
2.0	0.112	6.2	0.117	5.9	0.116	6.0
3.0	0.103	6.7	0.109	6.4	0.109	6.4
4.0	0.102	6.8	0.108	6.4	0.104	6.7

SGR= Specific growth rate

4.3.4 Effect of salinity on cell number growth, specific growth rate and doubling time of *Asteromonas* strains

Similar to *Dunaliella*, the optimum NaCl concentration for growth is 1.0 M (Figure 4.8) and growth was retarded with increasing salt concentration. A plot of natural log of absorbance against cultivation time (8 to 22 day) was used to determine the specific growth rate and doubling time (Figure 4.9 and Table 4.6). The lag period however was longer for these microalgae (4 to 6 days) compared to 3 to 4 for *Dunaliella.* After 26 days of inoculation, cultures in 1.0 M NaCl entered the stationary phase and the growth rate decreased. Cultures in 0.5, 2.0, 3.0 and 4.0 M NaCl were still in the exponential growth phase at 26 days after inoculation. Both specific growth rate and doubling time did not show a significant difference ($p = <0.001$ and $F = 34.364$) between three *Asteromonas* strains at the same salinities. There was statistically significant differences in specific growth rate between cultures cultivated in 1.0 and 4.0 M NaCl for all the three strains of *Asteromonas*; this was confirmed by Tukey's multiple comparison test ($p < 0.05$).

Similar to *Dunaliella*, NaCl was inhibitory to the growth of *Asteromonas* cells above 1.0 M NaCl as evidenced by double reciprocal plot (see Appendix A2.1).

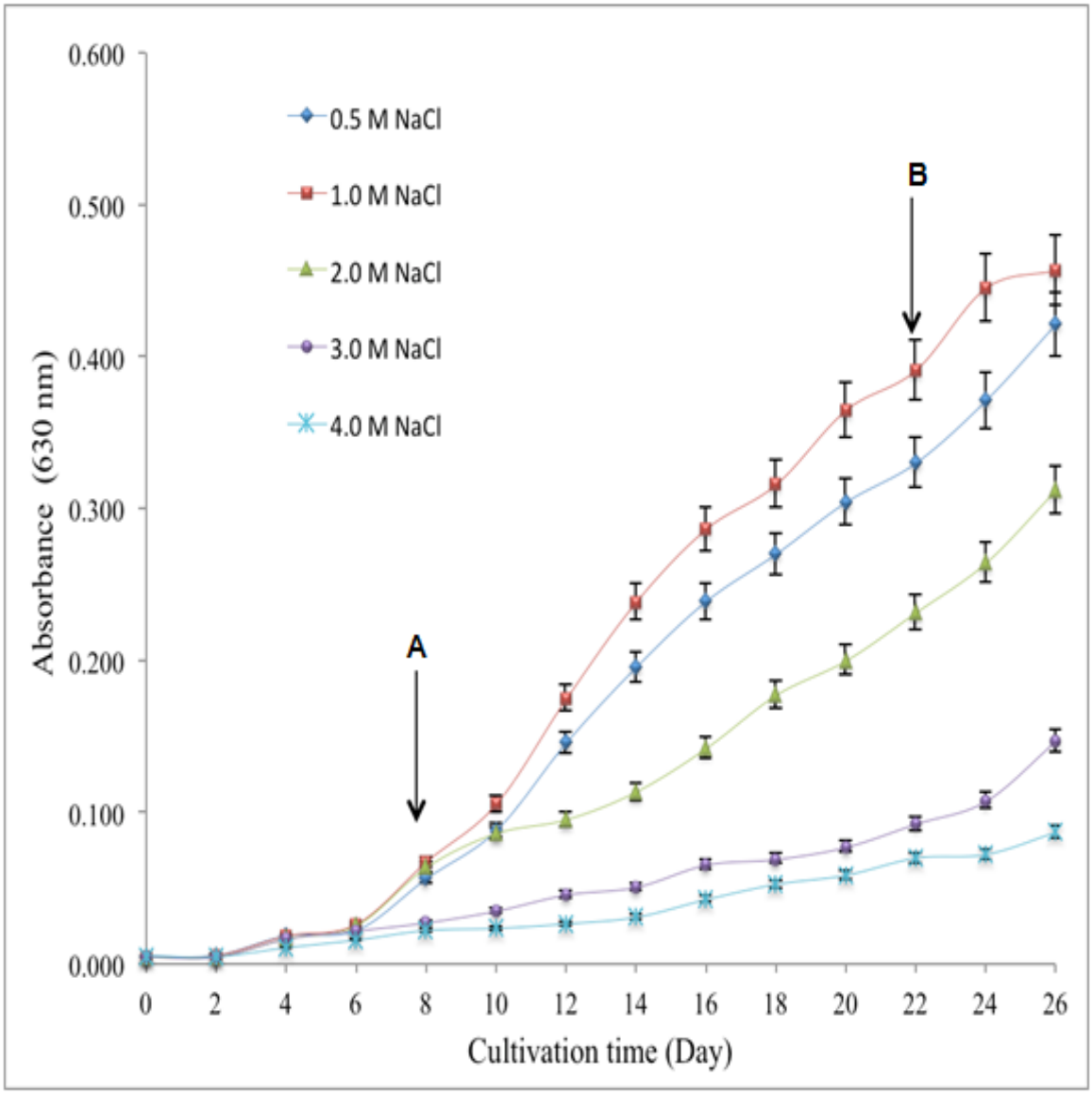


Fig. 4-8 Effect of increasing NaCl (0.5 to 4.0 M) on the growth of *Asteromonas* T33a at 25 ^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. A to B are the data points used in estimating specific growth rate. The data represent the mean (± SE) of the three experiments. Error bars represent the standard deviations (n≥3) of measured values of cell density

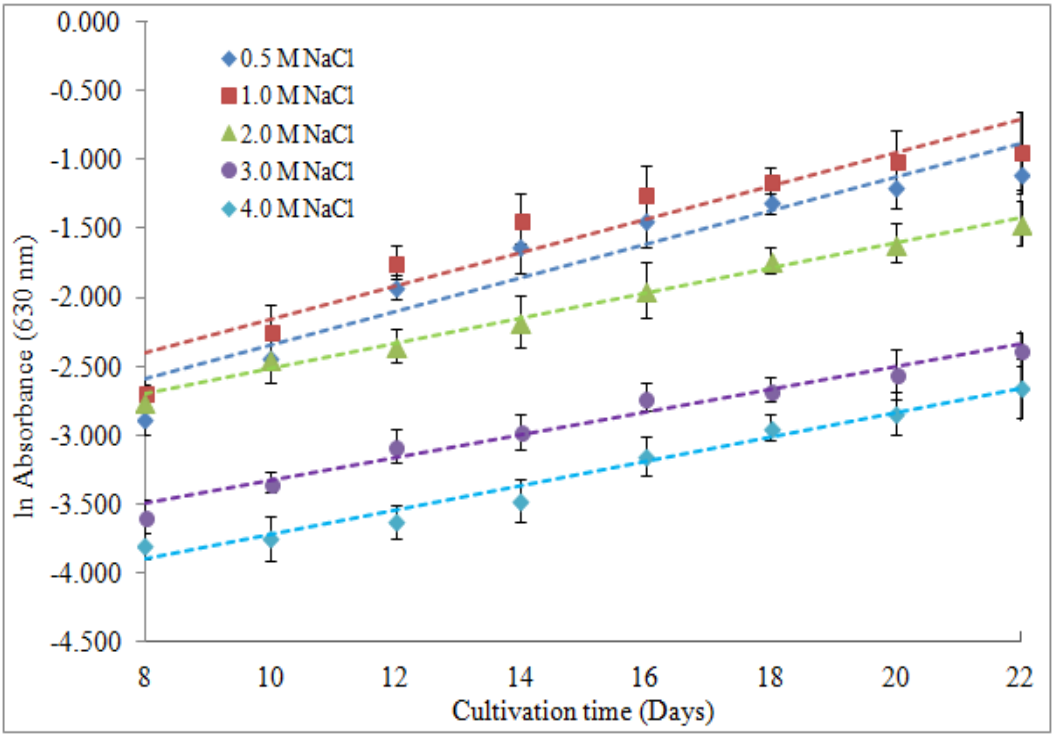


Fig. 4-9 Natural log plot for the effect of increasing NaCl (0.5 to 4.0 M) on the growth of *Asteromonas* T33a at 25 °C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. Error bars represent the standard deviations (n≥3) of measured values of cell density (natural log).

Table 4-6 Specific growth rate and doubling time of *Asteromonas* (T33a, T33b and T33c) grown in a range of NaCl concentration (0.5 to 4.0 M) at 25 °C, pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium.

Salinity (M)	***Asteromonas***					
	T33a		**T33b**		**T33c**	
	SGR ($Div.d^{-1}$)	Doubling time (days)	SGR ($Div.d^{-1}$)	Doubling time (days)	SGR ($Div.d^{-1}$)	Doubling time (days)
0.5	0.120	5.8	0.129	5.4	0.133	5.2
1.0	0.122	5.7	0.134	5.2	0.136	5.1
2.0	0.097	7.1	0.108	6.4	0.114	6.1
3.0	0.089	7.8	0.093	7.5	0.098	7.1
4.0	0.083	8.4	0.086	8.1	0.087	8.0

SGR = Specific growth rate

4.3.5 Effect of temperature on cell number, growth, specific growth rate and doubling time of *Dunaliella*

All the three strains of *Dunaliella* used in this research were able to grow at the five different temperatures (15 to 35 ^{o}C) tested but growth was optimal at 30 °C (Figure 4.10). At 30 ^{o}C the exponential growth phase was shorter and finished at 18 days.

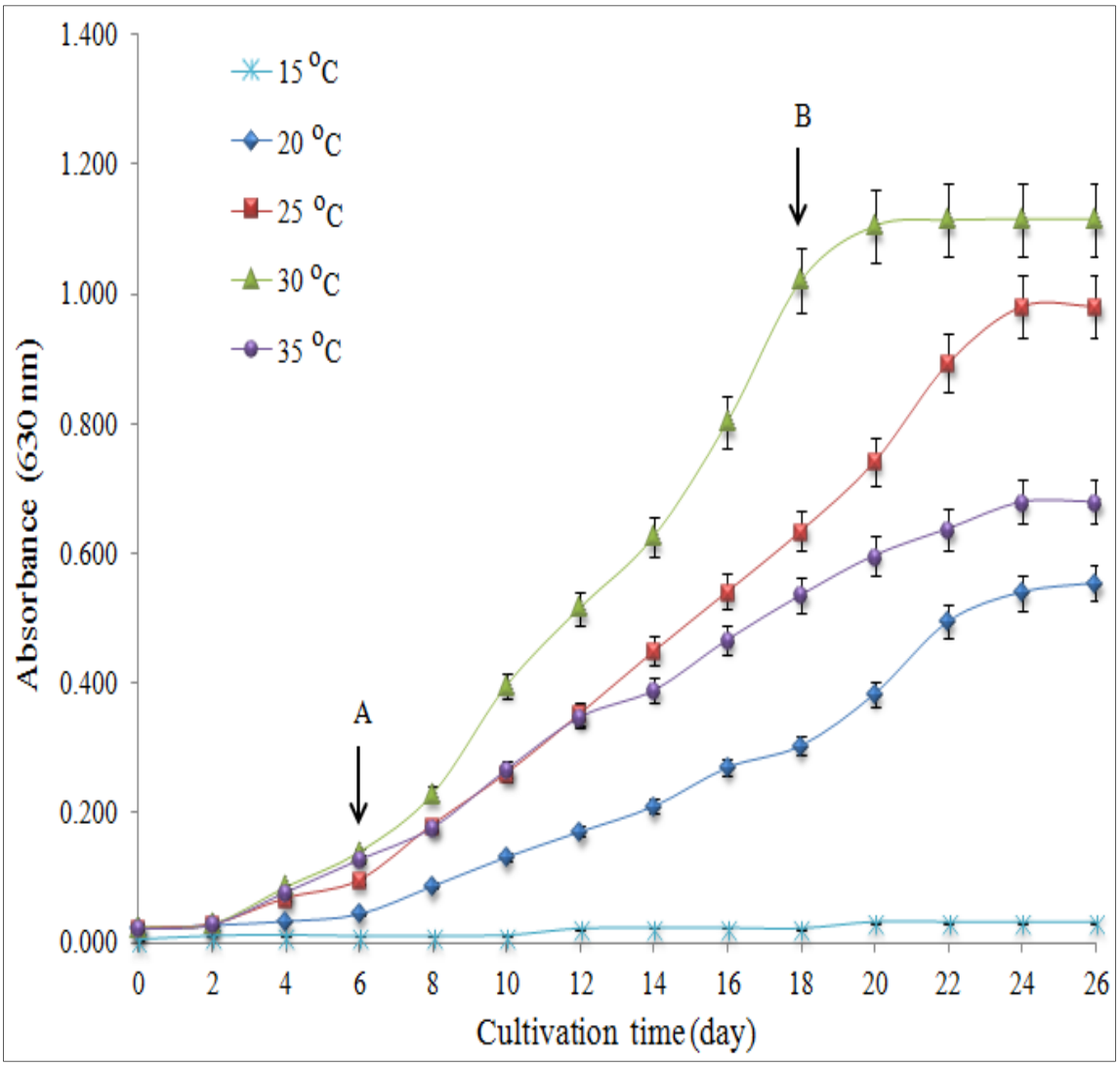


Fig. 4-10 Effect of temperature (15 to 35 ^{o}C) on the growth *of Dunaliella* T35 at 1.0 M NaCl, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. A to B are the data points used in estimating specific growth rate. Error bars represent the standard deviations (n≥3) of measured values of cell density

Dunaliella strains reacted in similar way at various temperatures (Table 4.7). There is no statistically significant difference in the cell density among the three strains T35, T36 and T37, at each of the incubation temperatures (p=0.542 and F = 0.679).

Table 4-7 Effect of temperature on growth of *Dunaliella* strains after 20 days at 1.0 M NaCl, pH 7.5, 45 μmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. The values represent the mean of three replicate experiments

Temperature (^{o}C)	***Dunaliella* (cell number/ml x 10^{6})**		
	T35	**T36**	**T37**
15	0.13	0.04	0.04
20	1.67	1.76	1.90
25	3.00	3.13	2.80
30	3.73	4.43	3.58
35	2.15	2.30	2.07

The specific growth rate and doubling of growth at different temperatures was determined from a plot of natural log against cultivation time (Figure 4.11). The highest specific growth rate and fastest doubling time was obtained at 30 ^{o}C (Table 4.8). At temperatures lower and higher than 30 ^{o}C the cells were normal and active, but had a greatly lower specific growth rate. There is no statistical significant difference (p>0.005) in doubling time between strains (T35, T36 and T37) at the same temperatures. The doubling time for T36 at 15 ^{o}C could not be calculated as there was no growth observed during the experimental period.

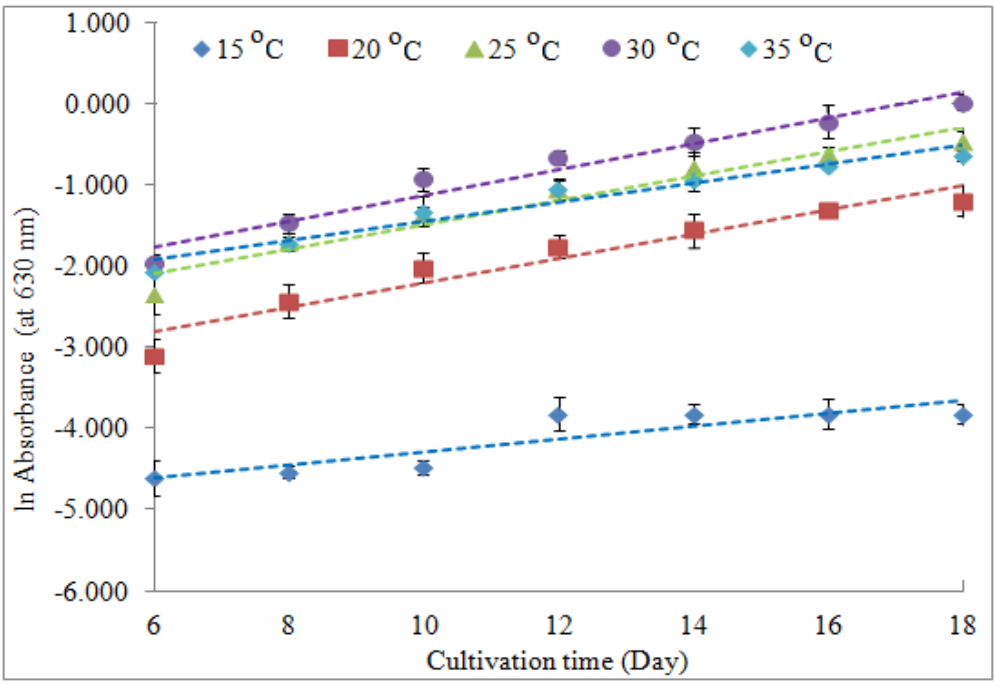


Fig. 4-11 Natural log plot for the effect of temperature (15 to 35 °C) on the growth of *Dunaliella* T35 at 1.0 M NaCl, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. Error bars represent the standard deviations (n≥3) of measured values of cell density (natural log).

Table 4-8 Specific growth rate and doubling time of *Dunaliella* T35, T36 and T37 grown in a range of temperatures at 1.0 M NaCl, pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. The data represent the mean (± SE) of the three experiments.

Temp. (°C)	***Dunaliella***					
	T35		T36		T37	
	SGR ($Div.d^{-1}$)	Doubling time(days)	SGR ($Div.d^{-1}$)	Doubling time(days)	SGR ($Div.d^{-1}$)	Doubling time(days)
15	0.078	8.9	0	0	0.053	13.1
20	0.139	5.0	0.136	5.1	0.135	5.1
25	0.159	4.4	0.154	4.5	0.156	4.4
30	0.168	4.1	0.165	4.2	0.166	4.2
35	0.118	5.9	0.124	5.6	0.116	6.0

SGR = Specific growth rate

4.3.6 Effect of temperature on cell number growth, specific growth rate and doubling time of *Asteromonas*

The effect of temperature on the growth kinetics of *Asteromonas* T33a is shown in Figure 4.12, from which data were abstracted to derive specific growth rate and doubling time (see Figure 4.13).

After an initial lag phase, cultures grew exponentially for 18 days before ceasing incremental growth at 30 and 25 ^{o}C or in the case of 35 ^{o}C subsiding. On day 22 it appears that the cultures at 35 ^{o}C were not able to tolerate the additional heat. The cultures in 20 ^{o}C were still in the exponential after 26 days. Very little growth was observed at 15 ^{o}C for these microalgae. The maximum specific growth rates and fastest doubling time for all three strains of *Asteromonas* was obtained at 30 ^{o}C and very low specific growth rates were obtained at 15 ^{o}C (Table 4.9). However, there is not a statistically significant difference among strains at each of the incubation temperatures. Doubling time increases from 30 ^{o}C to 15 ^{o}C and also increases when the cells were exposed to 35 ^{o}C.

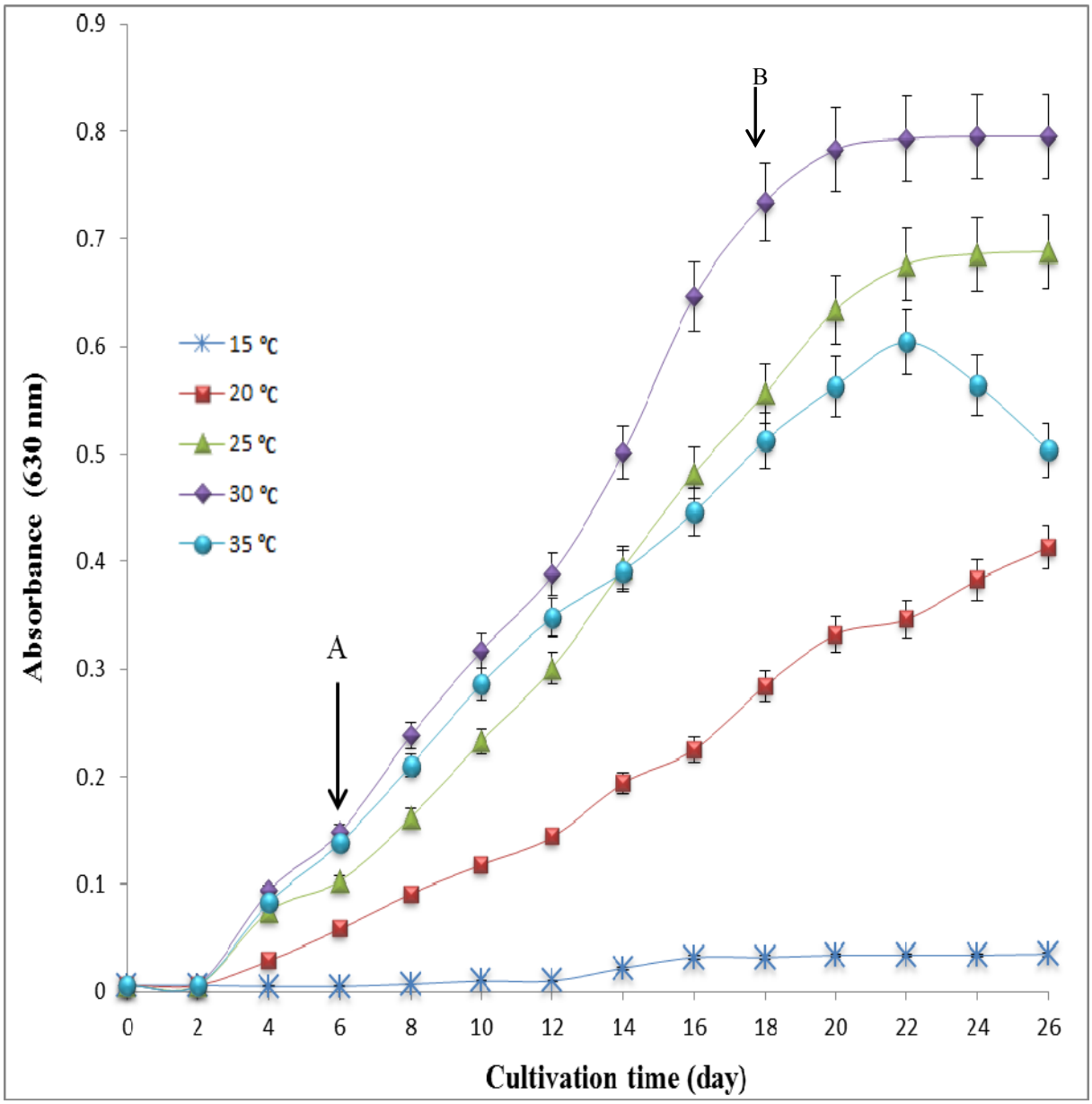


Fig. 4-12 Effect of increasing temperature (15 to 35 ^{o}C) on the growth of *Asteromonas* T33a at 1.0 M NaCl, pH 7.5, 45 μmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. A to B are the data points used in estimating specific growth rate. Error bars represent the standard deviations (n≥3) of measured values of cell density.

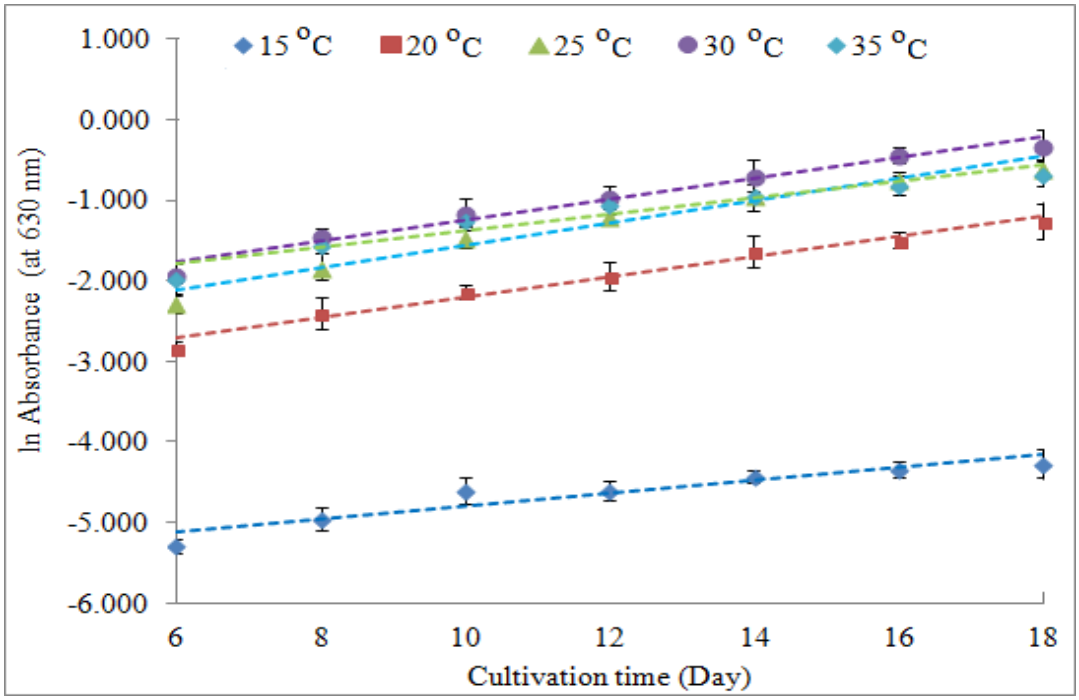


Fig. 4-13 Natural log plot for the effect of temperature (15 to 35 °C) on the growth of *Asteromonas* T33a at 1.0 M NaCl, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. Error bars represent the standard deviations (n≥3) of measured values of cell density (natural log).

Table 4-9 Specific growth rate and doubling time of *Asteromonas* (T33a, T33b and T33c) grown at different temperature (15 to 35°C) at 1.0 M NaCl concentration, pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. The data represent the mean (± SE) of the three experiments.

Temp. (°C)	***Asteromonas***					
	T33a		T33b		T33c	
	SGR (Div. d^{-1})	Doubling time(days)	SGR (Div.d^{-1})	Doubling time(days)	SGR (Div.d^{-1})	Doubling time(days)
15	0.061	11.4	0.053	13.1	0.048	14.4
20	0.120	5.8	0.112	6.2	0.110	6.3
25	0.130	5.3	0.127	5.5	0.125	5.5
30	0.139	5.0	0.134	5.2	0.136	5.1
35	0.103	6.7	0.110	6.3	0.110	6.3

SGR = Specific growth rate

4.3.7 Investigation of temperature effect using Arrhenius plot for *Dunaliella* and *Asteromonas* strains

A modified version of the Arrhenius equation (equation. 4.1) adopted from Goldman and Carpenter (1974) was used to investigate the effect of different temperatures on specific growth rate of *Dunaliella* and *Asteromonas*.

$$K = Ae^{-\mu/T} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \textbf{\textit{Eq}}.\ 4.1$$

Where μ is a temperature characteristic that describes the specific growth rate, T is the temperature, K is called constant and A is frequency factor (Y-intercept).

Arrhenius plot (Figure 4.14) shows that 30 ^{o}C (33.0 x 10^4 ^{o}K) is the optimum temperature for achieving maximum specific growth rate in both strains of *Dunaliella* and *Asteromonas*. The growth reaction of these microalgae at all temperatures investigated shows similar kinetic process (Figure 4.14). The temperature coefficient (Q_{10}) is the factor by which the reaction rate increases (specific growth rate) when the temperature is raised by ten degrees. The mean Q_{10} calculated between 20 and 30 ^{o}C for *Dunaliella* and *Asteromonas* strains was 1.20 and 1.22, respectively (Figure 4.14).

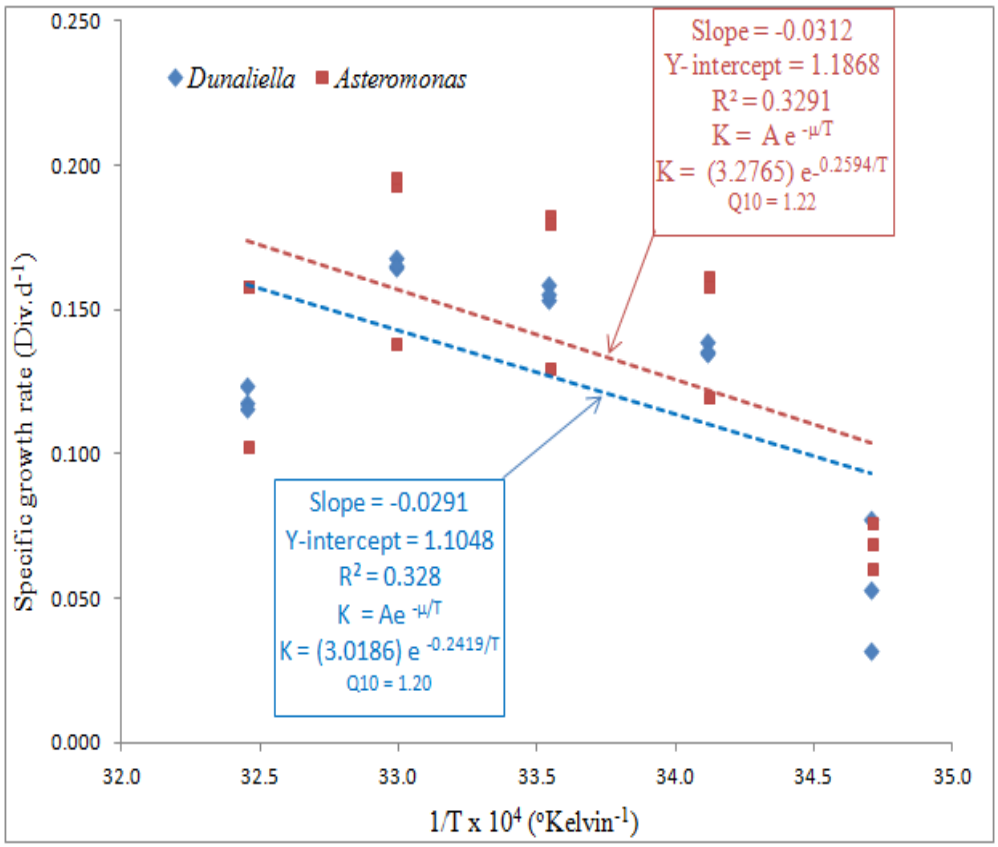


Fig. 4-14 Relationship between the specific growth rate of *Dunaliella* and *Asteromonas* strains and temperatures at 1.0 M NaCl, pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium.

4.3.8 *Dunaliella* and *Asteromonas* growth at different initial pH

Figure 4.15 and 4.16 shows the effect of initial pH on cell density of *Dunaliella* T36 *Asteromonas* T33a respectively. Both *Dunaliella* and *Asteromonas* showed consistent increase in cell number and growth rates at higher initial pH level of 7.5, 8.0 and 9.0 compared to 6.0 and 7.0. The final pH on day 20 in all cases was found to be 9.0.

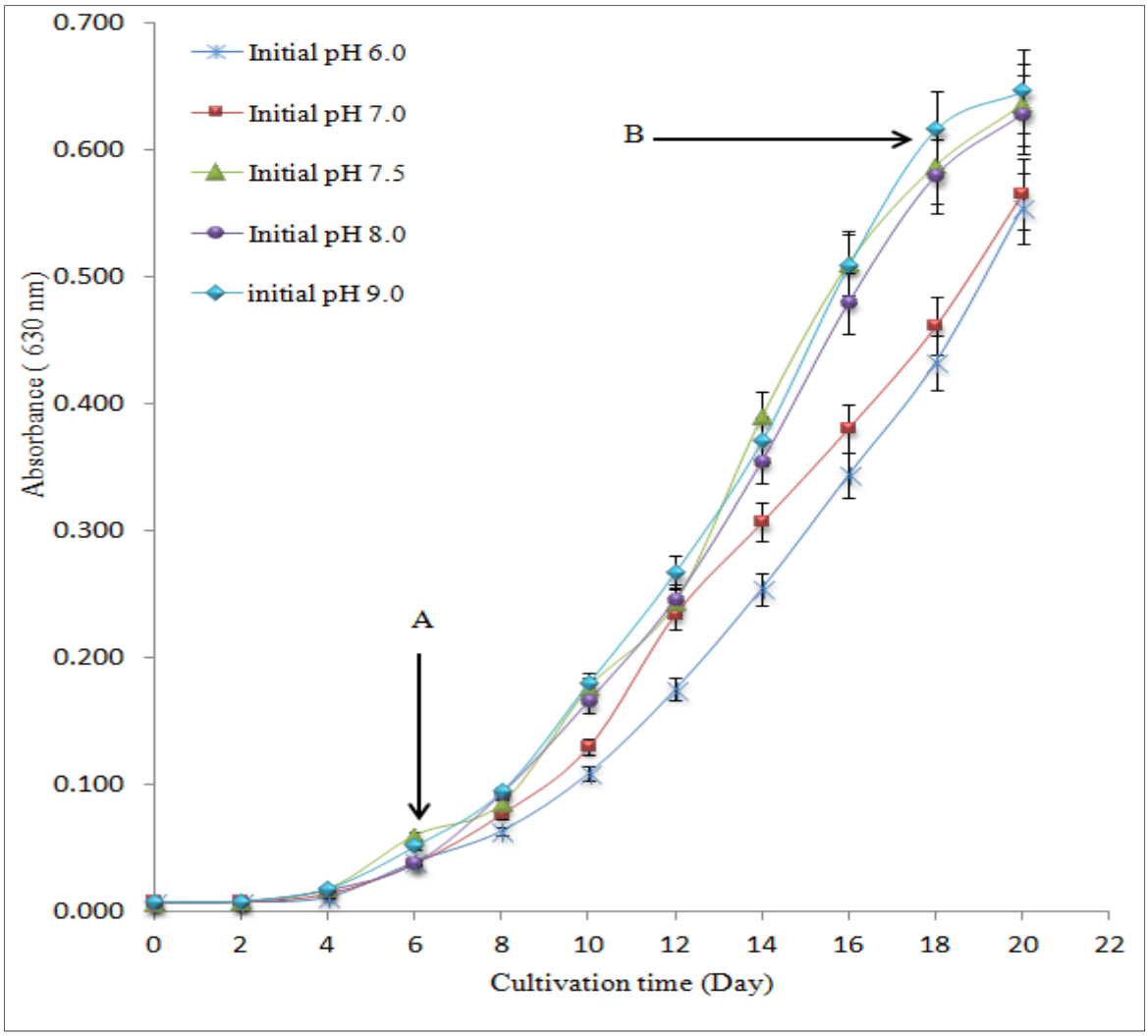


Fig. 4-15 *Dunaliella* T36 cell densities in relation to initial pH growing in modified Johnson's media with 1.0 M NaCl concentration at 25 ^{o}C, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. A to B are the data points used in estimating specific growth rate. Error bars represent the standard deviations (n≥3) of measured values of cell density

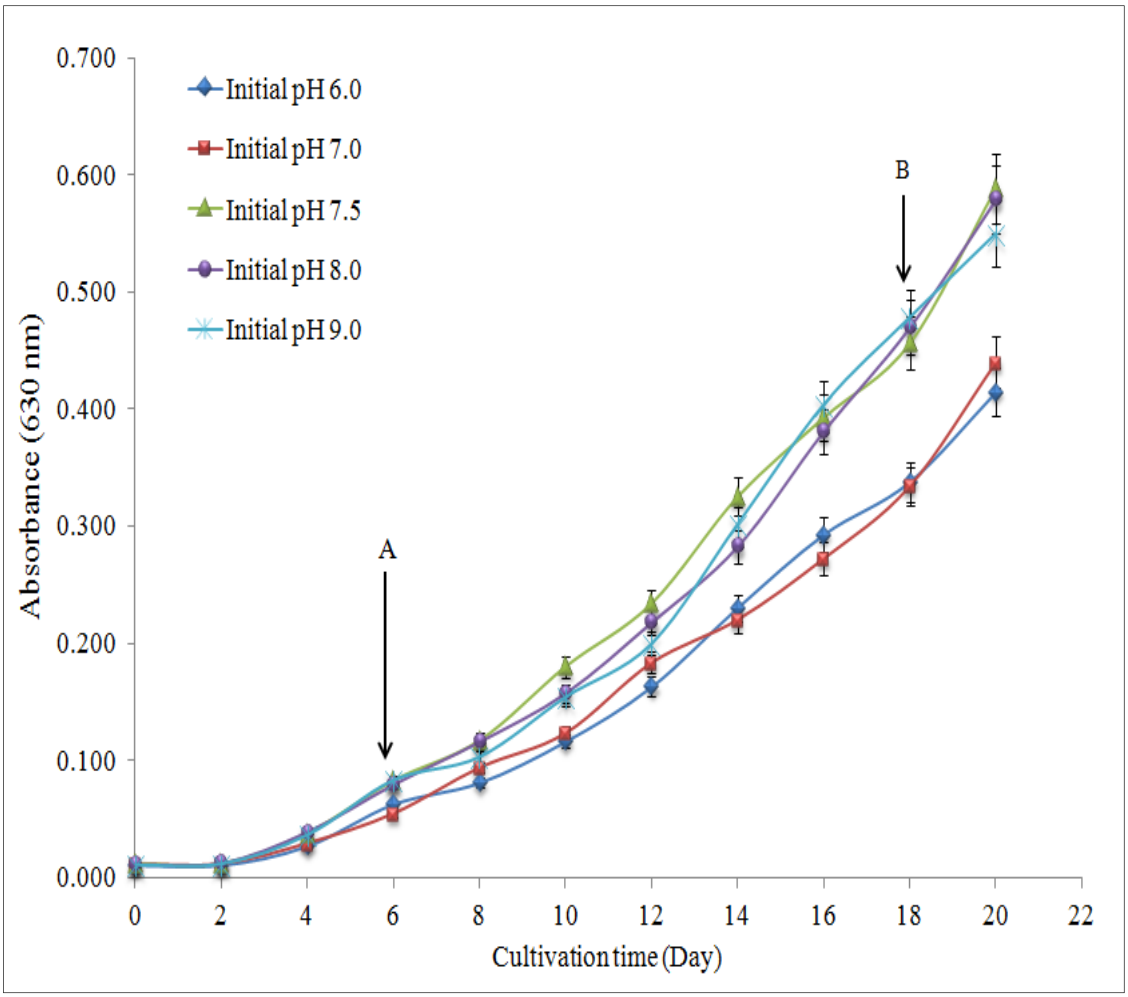


Fig. 4-16 *Asteromonas* T33a cell density in relation to initial pH growing in modified Johnson's media with 1.0 M NaCl concentration at 25°C, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. A to B are the data points used in estimating specific growth rate. Error bars represent the standard deviations (n≥3) of measured values of cell density

Specific growth rate and doubling time was determined at exponential growth phase (6 to 18 days) by plotting natural log of absorbance values against time (Figure 4.17 and 4.18). There was no significant differences between the initial pH values investigated (P>0.050) for all the three strains of *Dunaliella* (Table 4.10). Similar response was observed in *Asteromonas* strains (Table 4.11). A fastest doubling time was obtained in *Dunaliella* as compared to *Asteromonas*.

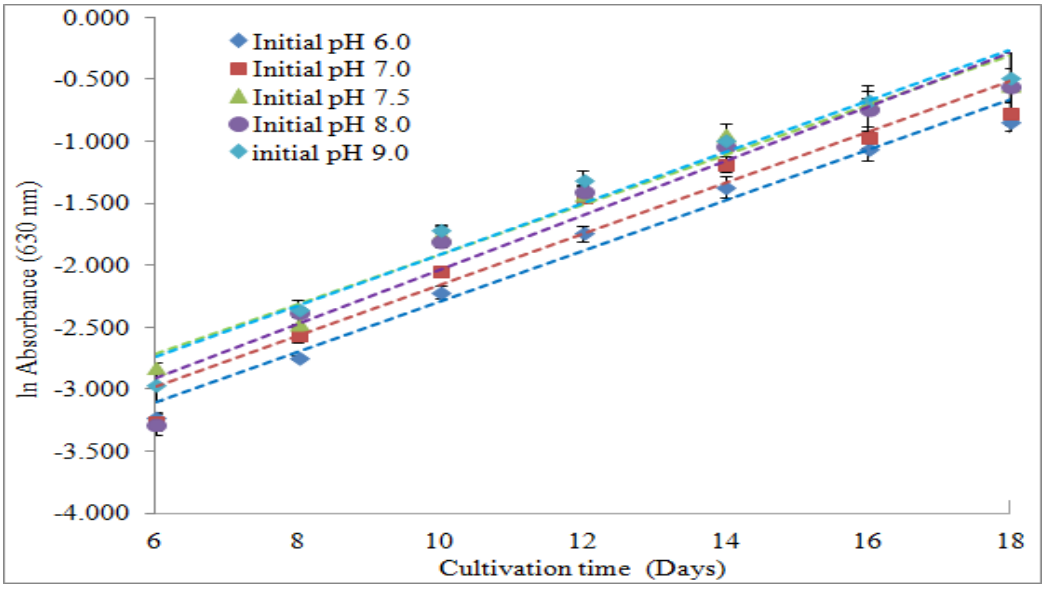


Fig. 4-17 Natural log plot of *Dunaliella* T36 cell densities in relation to initial pH growing in modified Johnson's media with 1.0 M NaCl concentration at 25 ^{o}C, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. Error bars represent the standard deviations (n≥3) of measured values of cell density (natural log).

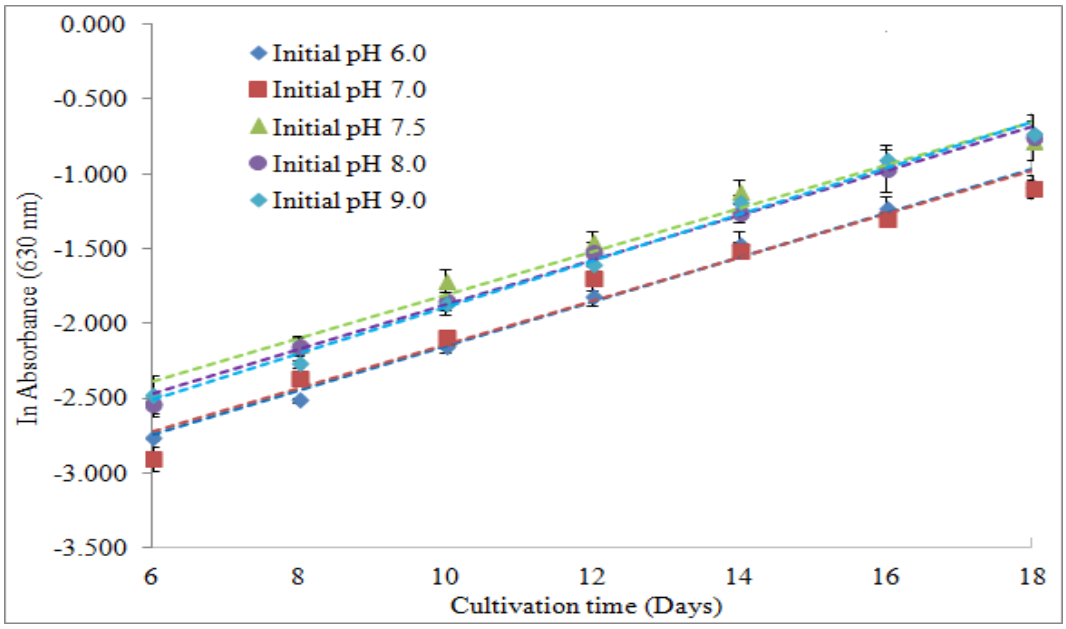


Fig. 4-18 Natural log plot of *Asteromonas* T33a cell densities in relation to initial pH growing in modified Johnson's media with 1.0 M NaCl concentration at 25 ^{o}C, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. Error bars represent the standard deviations (n≥3) of measured values of cell density (natural log).

Table 4-10 Specific growth rate and doubling time of *Dunaliella* T35, T36 and T37 grown at different initial pH at 25 ^{o}C, 1.0 M NaCl concentration and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. The data represent the mean (± SE) of the three experiments

pH	***Dunaliella***					
	T35		T36		T37	
	SGR ($Div.d^{-1}$)	Doubling time(days)	SGR ($Div.d^{-1}$)	Doubling time(days)	SGR ($Div.d^{-1}$)	Doubling time(days)
6.0	0.156	4.4	0.157	4.4	0.155	4.5
7.0	0.157	4.4	0.157	4.4	0.156	4.4
7.5	0.158	4.4	0.158	4.4	0.157	4.4
8.0	0.163	4.3	0.161	4.3	0.164	4.2
9.0	0.163	4.3	0.16	4.3	0.166	4.2

SGR = Specific growth rate

Table 4-11 Specific growth rate and doubling time of *Asteromonas* T33a, T33b and T33c grown at different initial pH at 25 ^{o}C, 1.0 M NaCl concentration and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. The data represent the mean (± SE) of the three experiments

pH	***Asteromonas***					
	T33a		T33b		T33c	
	SGR ($Div.\ d^{-1}$)	Doubling time(days)	SGR ($Div.d^{-1}$)	Doubling time(days)	SGR ($Div.d^{-1}$)	Doubling time(days)
6.0	0.127	5.5	0.122	5.7	0.121	5.7
7.0	0.129	5.4	0.122	5.7	0.122	5.7
7.5	0.131	5.3	0.125	5.5	0.126	5.5
8.0	0.130	5.3	0.124	5.6	0.127	5.5
9.0	0.132	5.3	0.125	5.5	0.126	5.5

SGR = Specific growth rate

4.3.9 Comparisons of growth parameters of *Dunaliella* (T35, T36 and T37) with species of *Dunaliella* obtained from the Culture Collection of Algae and Protozoa (CCAP).

In order to determine and compare growth rates and doubling time, three strains of *Dunaliella* (Namibian isolates; T35, T36 and T37) and four other *Dunaliella* strains (*D. salina, D. quartolecta, D. parva* and *D. polymorph*a) were cultivated under the same laboratory condition at 23 ^{o}C in four different NaCl concentration (Figure 4.19 and 4.20). The highest specific growth rate and fastest corresponding doubling time was obtained for *D. quartolecta* (0.230 and 3.01 days respectively) cultivated in 1.0 M NaCl. Doubling time increased with increase salinity for *D. parva* (9.23 days) *and D. polymorph*a (9.108), *D. quartolecta* (8.12 days) and *D. salina* (7.76 days).

The specific growth rate for *Dunaliella* (T35, T36 and T37) at 4.0 M NaCl was higher when compared with *D. salina, D. quartolecta, D. parva and D. polymorph*a. There was no difference in the specific growth rate at 2.0 and 3.0 M NaCl for all the strains investigated.

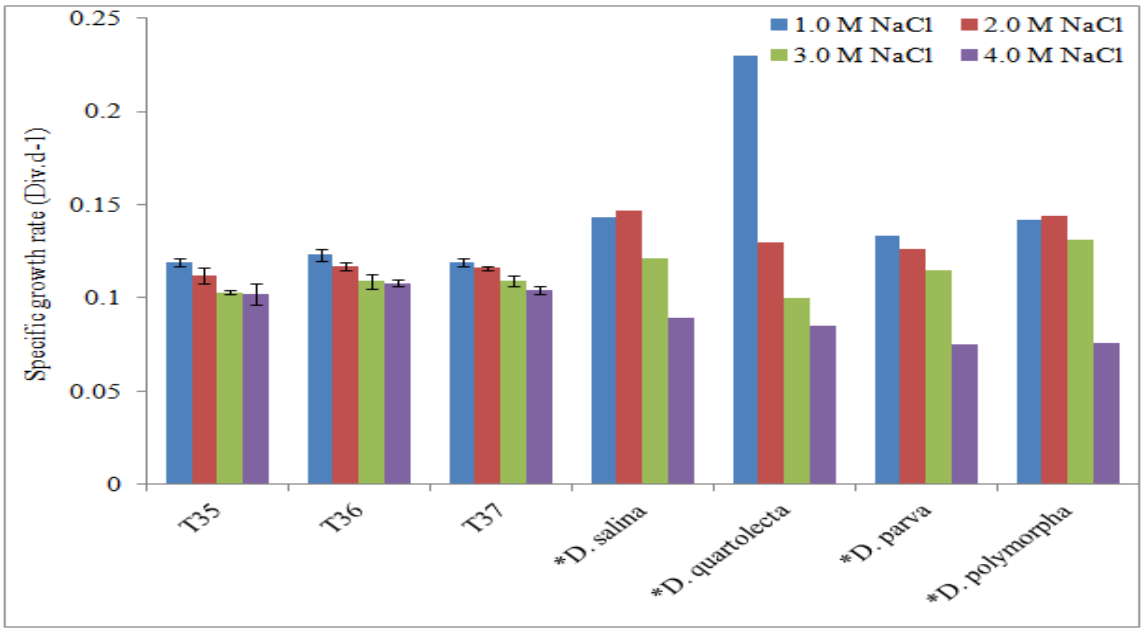


Fig. 4-19 Specific growth rate of *Dunaliella* (T35, T36 and T37) and other species (*D. salina*, *D. quartolecta*, *D. parva* and *D. polymorpha*) determined at 23 ^{o}C in 1.0 M NaCl concentration at pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. *Data courtesy of Rekha Swamy

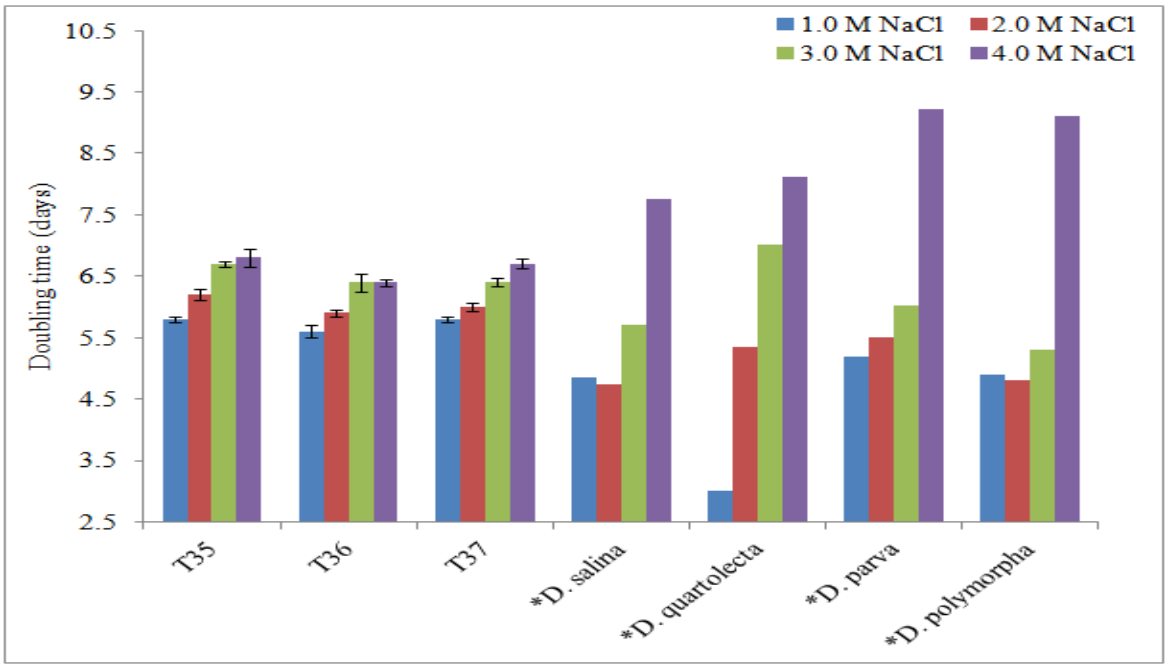


Fig. 4-20 Doubling time of *Dunaliella* (T35, T36 and T37) and other species (*D. salina*, *D. quartolecta*, *D. parva* and *D. polymorpha*) determined at 23 ^{o}C in 1.0 M NaCl concentration at pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. *Data courtesy of Rekha Swamy

4.4 Discussion

This research highlights the remarkable diversity of halotolerant microalgal strains that exists is even within the same physical location. The collection of strains, *Dunaliella* and *Asteromonas*, isolated from the saline river of Namibia, differed not only in their genetic construction, but also expressed differences in response to different environmental conditions of NaCl concentration, temperature and pH. Table 4.12 shows the temperature, pH and salinity-dependent growth of *Dunaliella* and *Asteromonas* obtained by this study and values reported by other researchers for *D. salina* and *D. viridis*. Although there have been only few studies reported, these data show that there is a relatively broad range of doubling time exhibited by *Dunaliella*, ranging from 0.75 (Ginzburg and Ginzburg, 1981) to 4.4 (this study) and 5.35 (Munoz *et al*., 2004). The longer doubling times (slow specific growth rates) reported here reasons may reflect the use of sodium bicarbonate as the chief source of carbon for the algae. Whereas Ginzburg and Ginzburg (1981) gassed their algal cultures with CO_2, a source of carbon in the growth media.

Dunaliella and Asteromonas cultures are able to continue growing at a steady rate (under the same growth conditions) for many generations provided that the NaCl concentration remains constant. Also Giordano (1997) observed higher growth rates in *D. salina* grown at 30 ^{o}C, but at different nitrogen enrichment (NH^{+}_{4}). And the two Chilean strains of *D. salina* CONC-007 and CONC-006 have division rates of 0.76 and 0.65 d^{-1} at 0.86 M NaCl concentration (Cifuentes *et al*., 1996). These are similar to values reported by Markovits *et al*. (1993) of *D. salina* minimum growth rate ranging from 0.093 to 0.23 d^{-1} at 25 ^{o}C.

Table 4-12 Doubling time and specific growth rate reported by others; salinity-dependent, pH and temperature-dependent growth

Species	NaCl (M)	Temp (^{o}C)	pH	Specific growth rate (d^{-1})	Doubling time (d^{-1})	References
D. salina	2.0	29	7.7 & 9.0	-	0.75– 1.25	(Ginzburg and Ginzburg, 1981)
D. salina	-	25	8.0 ± 0.5	0.093 – 0.23	-	(Markovits *et al*., 1993)
D. salina	0.5	20	7.5	-	1.98	(Munoz *et al*., 2004)
D. salina	2.0	20	7.5	-	1.17	(Munoz *et al*., 2004)
D. salina	3.0	20	7.5	-	5.35	(Munoz *et al*., 2004)
D. salina CONC-006 &007	0.86	30 ± 2	-	0.76 & 0.65	-	Cifuentes *et al*., 1996
D. salina	1.5	30	8.0	0.3 to 0.5	-	Giordano, 1997
D. bardawil	3.0	25	8 ± 0.5	0.169	-	(Markovits *et al*., 1993)
D. bardawil	-	25	-	-	1.5 - 2.0	Ben-Amotz *et al*. (1991).
Dunalialla T35	1.0	30	7.5 - 9.0	0.168	4.1	
Dunalialla T36	1.0	30	7.5 - 9.0	0.165	4.2	
Dunalialla T37	1.0	30	7.5 - 9.0	0.166	4.4	
Asteromonas T33a	1.0	30	7.5 -9.0	0.139	5.0	
Asteromonas T33b	1.0	30	7.5 -9.0	0.134	5.2	
Asteromonas T33c	1.0	30	7.5 - 9.0	0.136	5.1	
D. viridis	2.0	29	7.7 & 9.0	-	0.58 – 1.0	(Ginzburg and Ginzburg, 1981),
D. viridis	1.0	30	7.5	-	0.5	(Jimenez and Niell, 1991)
D. viridis	1.0	25	-	0.77	0.90	(Ilknur *et al*., 2008)
D. viridis	2.0	25	-	0.72	0.94	(Ilknur *et al*., 2008)
D. viridis	3.0	25	-	0.58	1.18	(Ilknur *et al*., 2008)
D. viridis	1.0	28	-	0.31	2.25	(Ilknur *et al*., 2008)
D. viridis	2.0	28	-	1.08	0.64	(Ilknur *et al*., 2008)
D. viridis	3.0	28	-	0.56	1.23	(Ilknur *et al*., 2008)

Effect of temperature on growth: Temperature is one of the most important factor to be considered in microalgal cultivation because at higher temperatures (e.g. 45 ^{o}C) some enzymes and proteins will be denatured and lose functionality. Microalgal cell divisions cannot be prolonged under these conditions. Eppley (1972) used the Arrhenius equation to investigate the effect of temperature on specific growth rate of microalgae grown in batch culture. He found an interesting relationship which shows that the specific growth rate of microalgae increased almost exponentially with increasing temperature from 10 to 40 ^{o}C.

Goldman and Carpenter (1974) used the Arrhenius plot/equation to analyse the result of continuous culture experiment and came to the same conclusion as Eppley (1972).

In the present study both *Dunaliella* and *Asteromonas* strains obtained from Namibia had 30 ^{o}C as the optimum temperature for their growth, with minimal growth below 15 ^{o}C. Since the prevailing temperatures of Namibia are 20 to 36 ^{o}C in summer, 8 to 12 ^{o}C in winter, these strains are only ever likely to grow in summer. Garcia *et al*. (2007) reported 22 ^{o}C as the optimum temperature for growth for Mexican species of *Dunaliella,* and Ben-Amotz and Avron (1989), 25-35 ^{o}C for D. *bardawil*. Borowitzka (1981) reported 20-30 ^{o}C for *D. salina,* but there appears to be a broad range of temperature tolerances depending on the strain for example: *D. bioculata* 25 ^{o}C, *D. primolecta* 29 ^{o}C, *D. tertiolecta* 20 ^{o}C and *D. viridis* 14-30 ^{o}C (Borowitzka and Borowitzka, 1988).

Effect of salinity on growth: *Dunaliella* has been shown ability to grow in saturated brine (similar to natural conditions) (Johnson *et al*., 1968), but optimum growth always occurs at lower salinity (0.5 to 2.0 M NaCl). In this regard, optimal growth rates at 1.0 to 2.0 M NaCl by Namibian isolates (*Dunaliella* and *Asteromonas*) are therefore typical of the species. Increasing NaCl concentration of the growth medium from 1.0 to 3.0 and 4.0 M inhibited growth (see section 4.3.3 and 4.3.4), but growth increased from 0.5 to 1.0 M which points to their obligate halotolerant nature.

Jimenez and Niell (1991) recorded similar findings on *D. viridis* from an athalassic (inland) lake in Spain that were grown over 4.0 M NaCl with an optimal growth at 1.0 M NaCl. These NaCl concentrations in the growth media were lower than the NaCl growth ranges found for *D. viridis* isolated in Yucatan (2.6 and 3.4 M NaCl) (Garcia *et al*., 2007). However, Davis (1990) reported similar results for *D. viridis* as being one of the main components of planktonic community in a solar saltworks at intermediate salinities (from 1.7 to 3.4 M NaCl). The optimum salinity for growth of *D. salina, D. parva* and *D. psuedosalina* isolated from salt marshes of Iran was at 0.5 to 2.0 M NaCl (Sharati, 2003).

Effect of initial pH on growth: Dunaliella and *Asteromonas* cells divide faster at initial pH 7.5 to 9.0 when compared to initial pH 6.0 and 7.0 at 1.0 M NaCl modified Johnson's medium. This is similar to *Dunaliella* species isolated from Lake Tuz of Turkey, which grew steadily in Johnson's medium and attained maximum cell density at pH 9.0 (Celekli and

Donmez, 2006). *D. tertiolecta* grew at pH 6.0 to 9.3 with maximum cell number at pH 6.4 - 8.3 (Humphrey, 1975). The pH for maximum growth for *D. viridis* was between pH 7.2 and 7.6 and pH 8.0 to 9.1 for *D. salina* (Ben-Amotz *et al*., 2009). The pH-dependent changes in algal growth rate may be due to dissolved inorganic carbon (DIC) speciation and concentration (Gensemer *et al*., 1993).

Chlorophyll estimation: Chlorophyll content was used for estimating biomass of microalgae in culture and can be used to measure growth as well as productivity. According to Ramaraj *et al*. (2013) the conventional method of chlorophyll measurement might not be a good index for biomass estimation: chlorophyll has been reported to be the primary target of salt toxicity, limiting net assimilation rate, and results in reduced photosynthesis and reduced growth (Rai 1990; Rai and Abraham 1993). The lower chlorophyll content of T36 and T33b may reflect a greater susceptibility to salt toxicity. According to Moradi and Ismail (2007) the lower amount of chlorophyll per ml at higher salinities is due to decrease in photosynthetic rate because of salt osmotic and toxic ionic stress.

4.5 Conclusions

This research determined the optimum temperature, salinity and initial pH for the growth of both the *Dunaliella* and *Asteromonas* strains isolated from Namibia. These strains can withstand large variations in the external salinity of the growth medium from 0.5 to 4.0 M NaCl concentration. By investigating the cell growth kinetics of microalgae using cells density determination, it was demonstrated that the rate of cell growth (increase in cell number) was maximum at 1.0 M NaCl concentration. The growth of *Dunaliella* and *Asteromonas* strains measured at different temperatures showed that as temperature increased from 15 ^{o}C microalgal growth increased, reached an optimum at 30 ^{o}C, and then decreased. The highest mean specific growth rate of 0.168 div/day for *Dunaliella* and 0.139 div/day for *Asteromonas* was obtained at 30 ^{o}C. The optimum temperature recorded in this study was 30 °C for both *Dunaliella* and *Asteromonas* strains. However, the temperate strains (*D. salina, D. quartolecta, D. parva and D. polymorph*a) showed optimal growth at 20 ^{o}C. Furthermore, the results indicated that the growth performance for the two microalgal strains was better at initial pH levels (7.5 to 9.0).

CHAPTER 5.0: METHOD DEVELOPMENT FOR QUANTIFYING GLYCEROL IN *DUNALIELLA* CELLS

5.1 Introduction

Since *Dunaliella* species lack a rigid cell wall, they are able to respond rapidly to sudden salinity changes. This offers advantages in osmoregulation: *Dunaliella salina* cells for instance are able to respond to osmotic shock by promptly adjusting in shape and volume (Avron and Ben-Amotz, 1992; Borowitzka and Siva, 2007). Somewhat surprisingly not all *Dunaliella* species may respond in the same way to sudden osmotic shock. *Dunaliella tertiolecta* cells have been reported to remain relatively unchanged in volume and instead, may either take up exogenous glycerol (Lin *et al*., 2013) or excrete it (Chow *et al*., 2013). The possibility exists that this phenomenon may be more widely spread than previously thought: in *Dunaliella salina* there have been reports of glycerol leakage (Gilmour *et al*, 1984; Wegmann *et al,* 1980), possibly caused by membrane re-organisation (Ben-Amotz and Avron, 1989) or by the formation of transient, non-specific pores in the cell membrane (Fujii and Hellebust, 1992), but this phenomenon has generally been attributed to a temperature-dependent effect because no glycerol leakage was observed below 40 ^{o}C, while exposure to temperatures above 60 ^{o}C resulted in the release of all intracellular glycerol.

This chapter sought to evaluate the size of glycerol pools in the external environment and internal cells of the Namibian strains to assess whether they utilised the external environment in the course of managing internal cellular glycerol concentration. However since it is possible that exposure to high centrifugal force during cell harvesting could lead to cell membrane rupture causing glycerol to disperse to the external environment, this chapter also sought to examine the effect of centrifugal force during *Dunaliella* cell harvesting. A wide variety of centrifugation forces ranging from 1,000 to 12,000 g have been used by other researchers without mentioning a reason for a particular choice (Ben-Amotz and Avron, 1973; Chen et al., 2011; Chow *et al*., 2013; Fu *et al*., 2014). For this study a reliable and reproducible method for glycerol quantification is required. Two different methods are in routine use in the literature: the first, an indirect chemical method based on the use of sodium

periodate. This forms a coloured derivative with any formaldehyde formed after the cleavage of glycerol with periodate. The second is a more specific enzyme –based assay based on the measurement of NADH after enzymic oxidation with glycerol dehydrogenase (Ben-Amotz and Avron, 1973; Chitlaru and Pick 1989; Chen *et al*., 2011; Chow *et al*., 2013; Zhao *et al*., 2013).

The aims of this chapter were therefore:

1. To develop a reliable, sensitive and reproducible method for measuring trace quantities of glycerol within cells of *Dunaliella* and in media of varying salinities
2. To examine the effect of centrifugal force on the distribution of glycerol both within cells of *Dunaliella* and *Asteromonas* strains from Namibia and in the external environment. This would enable a reliable framework to be established in order to assess the mechanism by which these strains responded to sudden changes in osmotic shock.

5.2 Method

5.2.1 Comparison of the chemical and enzymatic methods of glycerol estimation

In order to evaluate the sensitivity of these methods samples of 200 μg/ml of glycerol were dissolved in a modified Johnson's medium that contained 4.0 M NaCl (see section 3.1; Table 3.4) for 2 hours before glycerol estimation was carried out. The experiment was run on three separate days and two measurements were conducted on each day for each method. For the details of sodium periodate reagent method and enzymatic method see section 3.5.1 and 3.5.2.

5.2.2 Effect of centrifugal force on harvesting microalgae

This experiment was conducted to investigate the optimum centrifugation force (g) for harvesting *Dunaliella* and *Asteromonas* strains and also to investigate whether glycerol

leaked from cells during centrifugation. In this experiment cells from 14 day-old culture of *Dunaliella* grown in 1.0 M NaCl at 25 ^{o}C were counted in a haemocytometer prior to the centrifugation experiment and after 1.5 ml of culture was centrifuged at 3000, 5000, 7000, 9000 and 11000 g for 10 min at 25 °C. The harvested cell pellet was carefully suspended into the medium and the cell number was re-counted (i.e. to check cell recovery). The centrifugation speed (g) at which maximum cell recovery is achieved was used in harvesting microalgal cells in subsequent experiments.

The level of glycerol from the harvested cell pellet and cell medium exposed to different centrifugal force was measured using the sodium periodate oxidation method (see section 3.5.1). The sample size for this experiment was 1.5 ml.

GC-MS analysis was undertaken to compare the level of glycerol in the supernatant and pellet centrifuged at 3000 and 9000 g. This experiment was conducted using 50 ml centrifuge tubes. The samples sizes were increased from the 1.5 to 50 ml because this method comprises a number of washing steps (phase separations) between the organic solvents and aqueous phase. For the glycerol extraction, derivatization and GC-MS analysis (see section 3.6).

5.3 Results

5.3.1 Comparison of the use of the sodium periodate method and glycerol dehydrogenase enzymatic method to estimate glycerol in culture media

Table 5.1 compares the results obtained using either sodium periodate or glycerol dehydrogenase to quantify a known amount (200 μg/ml) of glycerol added to Johnson's medium containing 4.0 M NaCl. Two determinations were made each day over a period to 3 days to test reliability and reproducibility. The mean percent recovery of glycerol using the sodium periodate (chemical) method was 99.9 % whereas the % recovery using glycerol dehydrogenase (enzymatic method) was 33.2 %. The chemical method resulted in a standard deviation of 0.69 μg/ml and coefficient of variance of 0.3 %. The enzymatic method resulted in a standard deviation of 9.0 μg/ml and coefficient of variance of 13.6 %. The result shows

that chemical method is more reproducible (on a day to day basis) than enzymatic method as proved by the standard deviation and coefficient of variance.

Table 5-1 Comparison of the recovery and reproducibility of the chemical and enzymatic methods using 200 µg/ml of glycerol in 4.0 M modified Johnson's medium.

Day	**Chemical Method Absorbance at 410 nm**		**Enzymatic method Absorbance at 340 nm**	
	Determination 1 (glycerol µg/ml)	Determination 2 (glycerol µg/ml)	Determination 1 (glycerol µg/ml)	Determination 2 (glycerol µg/ml)
1	199.3	200.9	73.9	69.1
2	200.3	199.3	57.1	78.0
3	199.3	199.7	65.1	55.5
Mean	199.8		66.4	
SD	0.69		9.0	
CV, %	0.3		13.6	
% Recovery	99.9		33.2	

In addition to the lower level of reproducibility, glycerol dehydrogenase used for the enzymatic method was inhibited strongly by NaCl, (33.2 % recovery) the principal component of modified Johnson's medium, in contrast to the situation with sodium periodate (99.9 % recovery) (see table 5.1).

The standard curve for glycerol measured using sodium periodate resulted in a linear relationship between absorbance and glycerol concentration with a correlation coefficient of 0.996 (Figure 5.1).

Based on these data, the enzymatic method with glycerol dehydrogenase was ruled out in favour of the use of sodium periodate for routine assay of glycerol. The sodium periodate method offered the necessary reproducibility, reliability and sensitivity required of this work and was free from interference by NaCl. The use of sodium periodate is also easy to use and

relatively inexpensive. However chemical method lacks specificity compared to glycerol dehydrogenase.

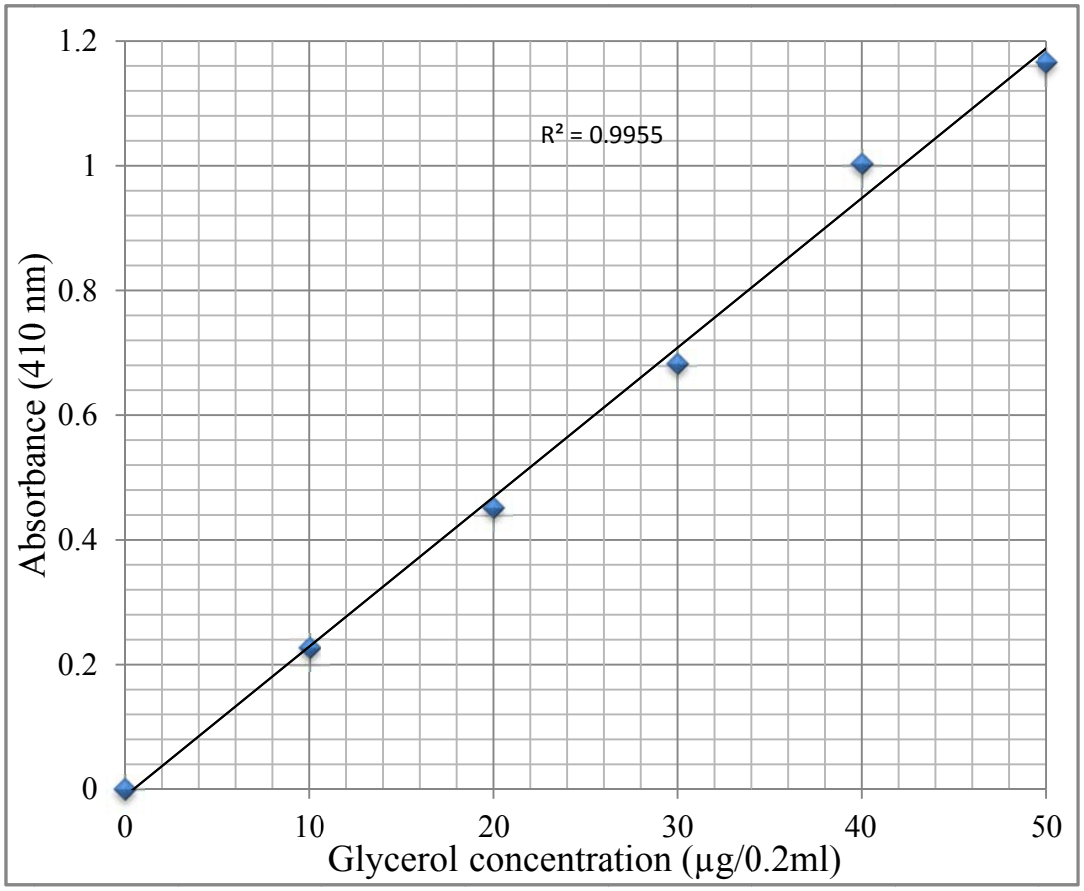


Fig. 5-1 Glycerol standard curve for sodium periodate reagent method (at 410 nm)

5.3.2 Identification and quantification of glycerol using GC-MS

The use of GC-MS offers a third method of quantifying glycerol in culture media. Since it is totally different from the colourimetric (chemical and enzymic) methods, which rely on a series of reactions to produce a pigment that is further measured by a spectrophotometer, it offers the possibility to back up any results obtained using either colourimetric method. Glycerol and internal standard (ribitol) were silylated by N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) reagent in derivatization reaction and converted to tris(trimethylsilyl) glycerol ether and ribitol, 1, 2, 3, 4, 5-pentakis-O- (trimethylsilyl),

respectively (see section 3.6.1). The two derivatives were separated by the optimized GC-MS method and eluted at retention time 14.69 min for the glycerol ether and 24.17 min for the internal standard ether (figure 5.2).

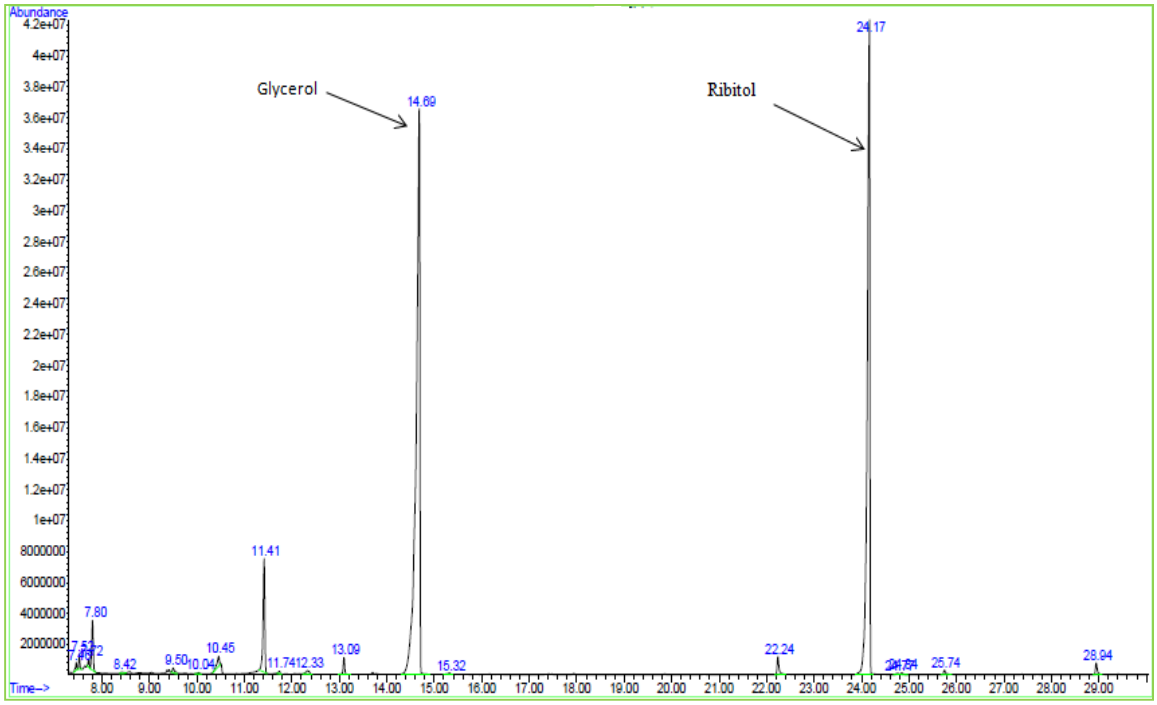


Fig. 5-2 Chromatogram of glycerol and standard (ribitol) after the derivatization

Based on the mass spectrums of tris(trimethylsilyl) glycerol ether, two of the prominent fragment ions are 73 and 147 m/z, which were formed by the loss of a trimethylsilyl group (figure 5.3). In additional to the fragment ions of 73 and 147 m/z, one important fragment ion of tris(trimethylsilyl) glycerol ether was 205 m/z and formed by the loss of another trimethylsilyl group (figure 5.3). The response peak areas of the selected ions were obtained by the selected ion monitor mode of GC-MS to eliminate the background noise interference in the peak integrations. Figure 5.3 and 5.4 shows how glycerol was identified by matching the retention time and mass spectrum characteristics against those of standard NIST library data base (www.nist.org). In general, the sensitivity of the GC-MS methods was much higher than any photometric method that uses a UV detector (Li *et al*., 2006).

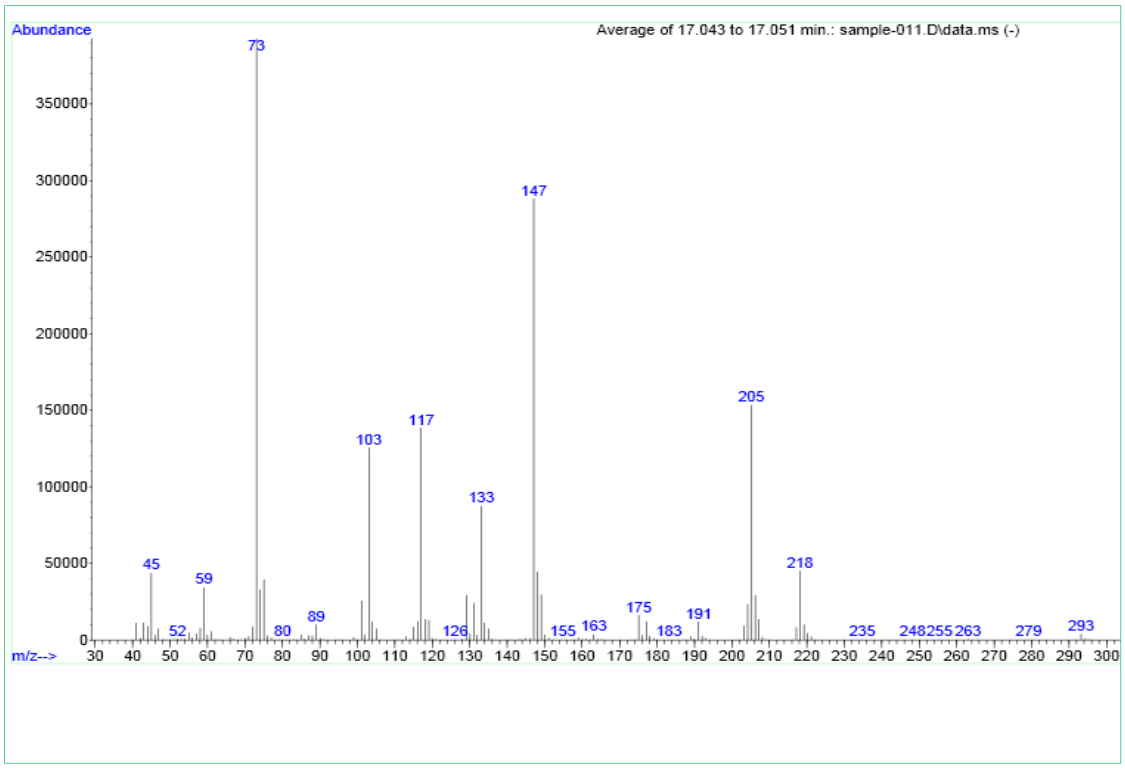


Fig. 5-3 Spectra of derivatized glycerol (standard) from mass spectrometry

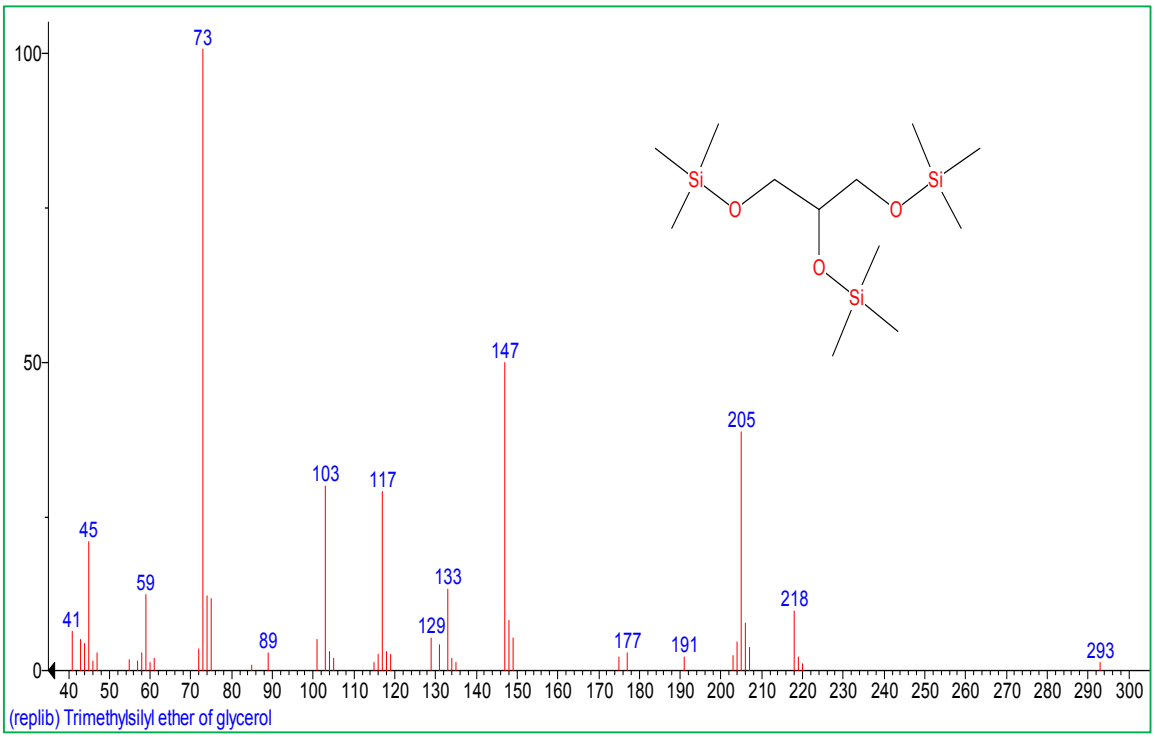


Fig. 5-4 Spectra of derivatized glycerol from NIST library

5.3.3 Effect of centrifugation on *Dunaliella* cell rupture and glycerol levels

Dunaliella cells were exposed to gravitational force ranging from 3000, 5000, 7000, 9000 and 11000 g at 25 ^{o}C, using an Eppendorf centrifuge with 1.5 cm head-height. After resuspending the pelleted cells, they were visually inspected for cell damage. At 3000 g no loss in cell number occurred after 10 min exposure of *Dunaliella* cells in the centrifugal force (Figure 5.5). However, there was about 20 to 65 % decrease in intact cells after centrifugation between 5000 to 11000 g in the pellet and at 11,000 g up to 70 % of cells were lost.

Figure 5.6 shows the amount of glycerol retained in cells at different centrifugation forces using the same centrifugation conditions. Glycerol was determined using the sodium periodate method. The largest amount of glycerol was retained in cells at lower g force (glycerol in cells not the culture medium). The result shows leakage of glycerol into the medium above 5000 g.

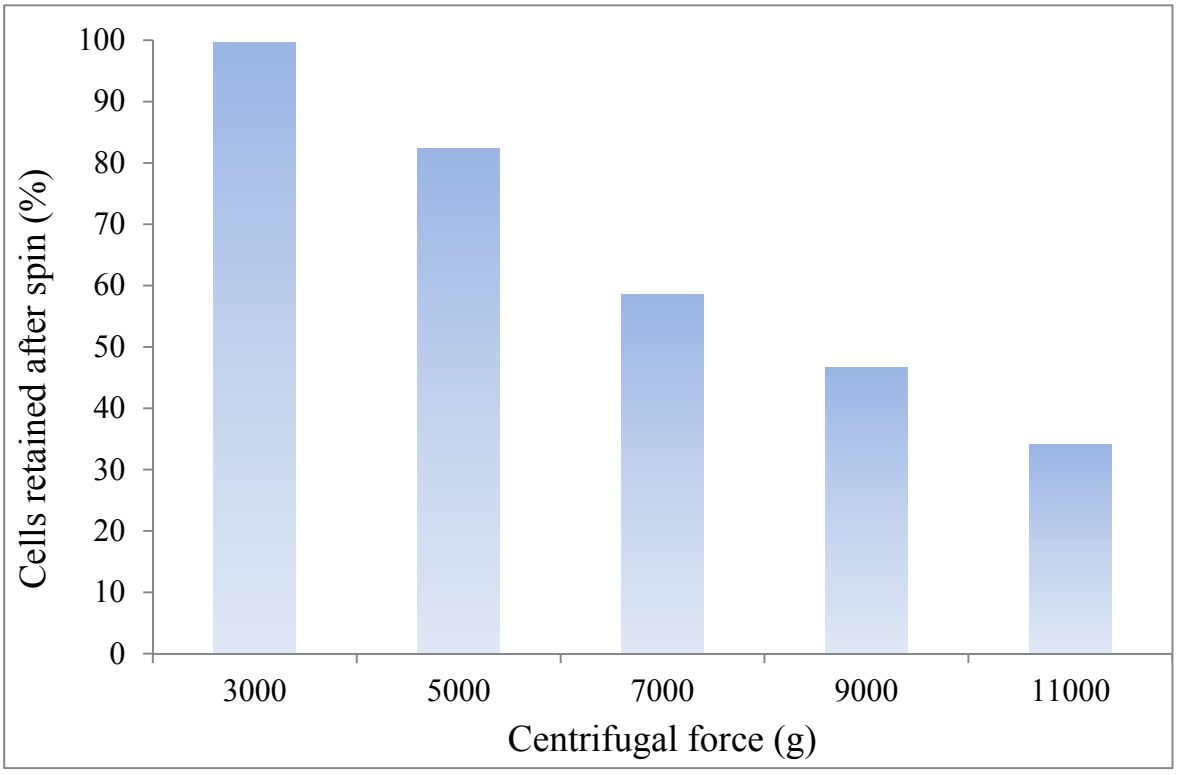


Fig. 5-5 Effect of centrifugal force (g) on cell recovery from the pellet of *Dunaliella* T35 cells at different centrifugation force for 10 minutes 25 ^{o}C.

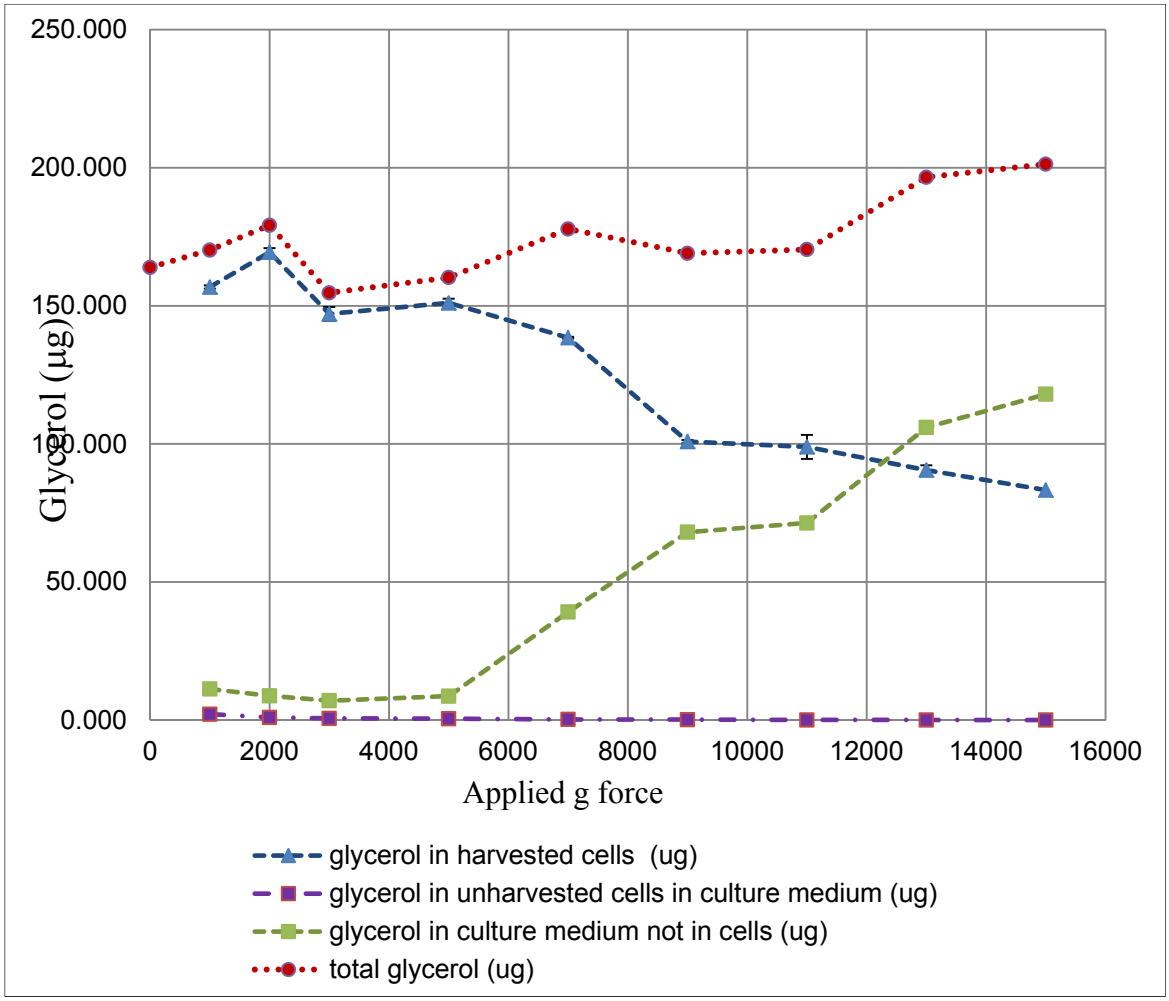


Fig. 5-6 Effect of centrifugation on *D*unaliella cells spun at 1000 – 15000 g, 25 ^{o}C for 10 min. The figure shows cells recovery and amount of glycerol detected. Error bars represent the standard deviations (n≥3) of measured values of glycerol content

5.3.4 Determination of glycerol after centrifugation using GC-MS analysis

GC-MS analysis was conducted to investigate the amount of glycerol in both pellet and supernatant (medium) of *Dunaliella* cultures after centrifugation at 3000 or 9000 g. This experiment was conducted using 50 ml centrifuge tubes in a Sorvall centrifuge i.e. a different head-height compared to the previous experiment. The chromatograms shown in Figure 5.7 and Figure 5.9 represent the results obtained after analysis of *Dunaliella* cells collected after centrifugation at either 3,000 g or 9,000g respectively; those in Figure 5.8, and Figure 5.10, for the corresponding media in which the cells were growing, also collected after centrifugation at either 3,000 g or 9,000g respectively. The peak at ~14.7 min was identified

as glycerol whilst that at ~24 min corresponded to the internal standard (ribitol). After centrifugation at 3,000 g the ratio of the abundance of glycerol: ribitol in the supernatant was large relative to that corresponding in the pelleted cells. By contrast, after centrifugation at 9,000 g the corresponding ratio of glycerol: ribitol in the supernatant was smaller (Figure 5.10) and that in the cells correspondingly larger (Figure 5.9).

Figure 5.11 shows the mean glycerol concentration determined by reference to the peak area obtained for a glycerol standard measured after adjustment to the concentration of ribitol recovered, for all the samples investigated. The amount of glycerol presented in Figure 5.11 using GC-MS analysis was used to confirm glycerol identity after periodate method. The amount of glycerol determined from the cell pellet obtained at 3000 g was over 13 % greater than that obtained at 9000 g, whereas the amount of glycerol detected in the supernatants of samples centrifuged at 9000 g was 94 % greater than that at centrifuged 3000 g. This result indicated cell rupture at 9000 g.

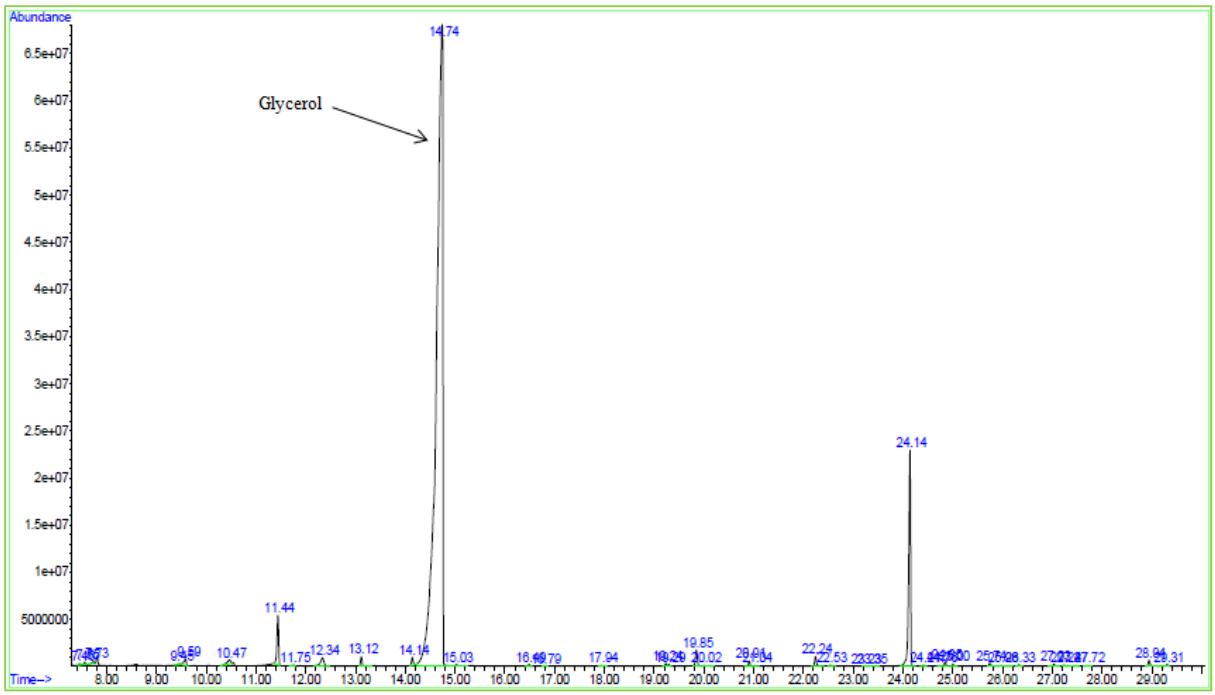


Fig. 5-7 Chromatogram of *Dunaliella* cell pellet harvested by centrifugation for 10 min. at 3000 g. It showed that derivatized glycerol was successfully separated from other components occurring in the sample

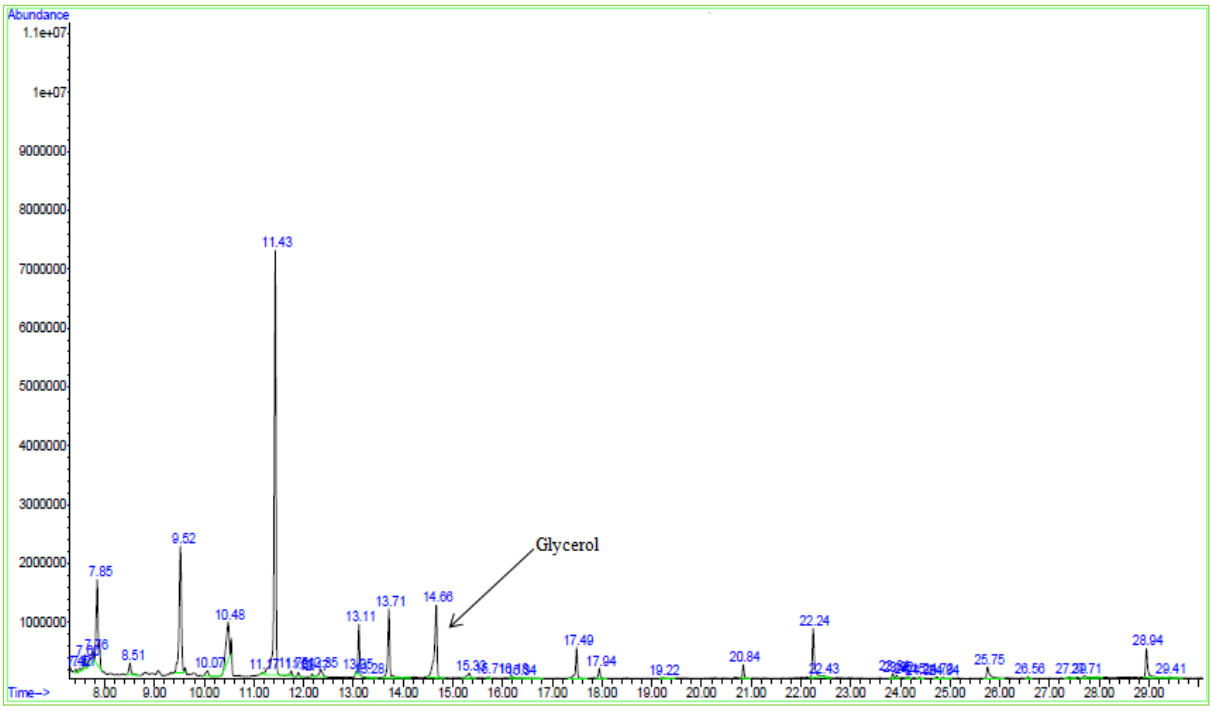


Fig. 5-8 Chromatogram of supernatant (culture medium) separated from cells after they had been centrifuged for 10 min at 3000 g.

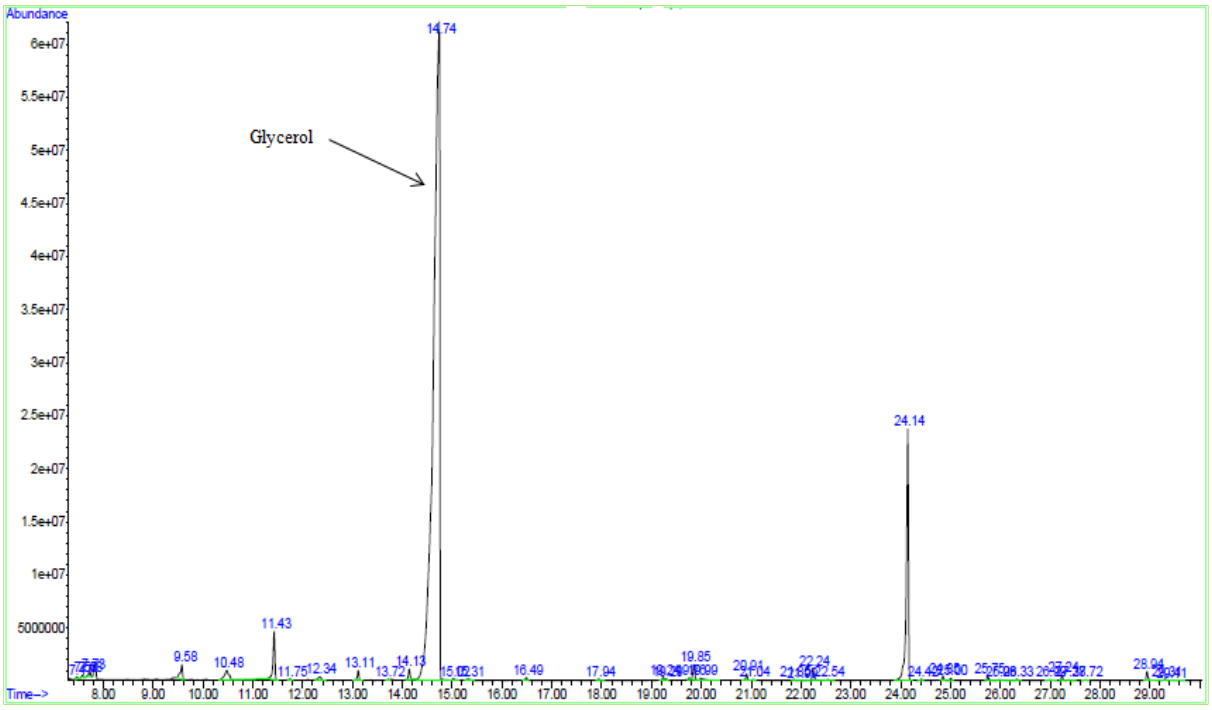


Fig. 5-9 Chromatogram of *Dunaliella* cell pellet collected after centrifugation for 10 min at 9000 g.

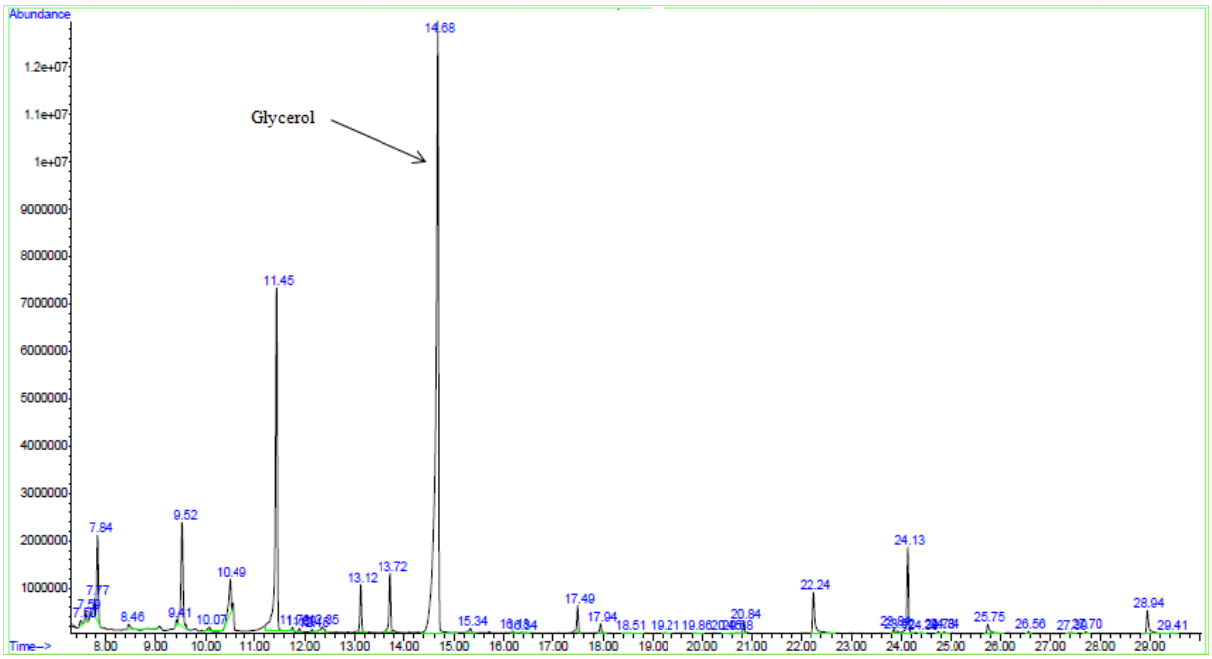


Fig. 5-10 Chromatogram of supernatant (culture medium) after centrifugation of cells for 10 min at 9000 g.

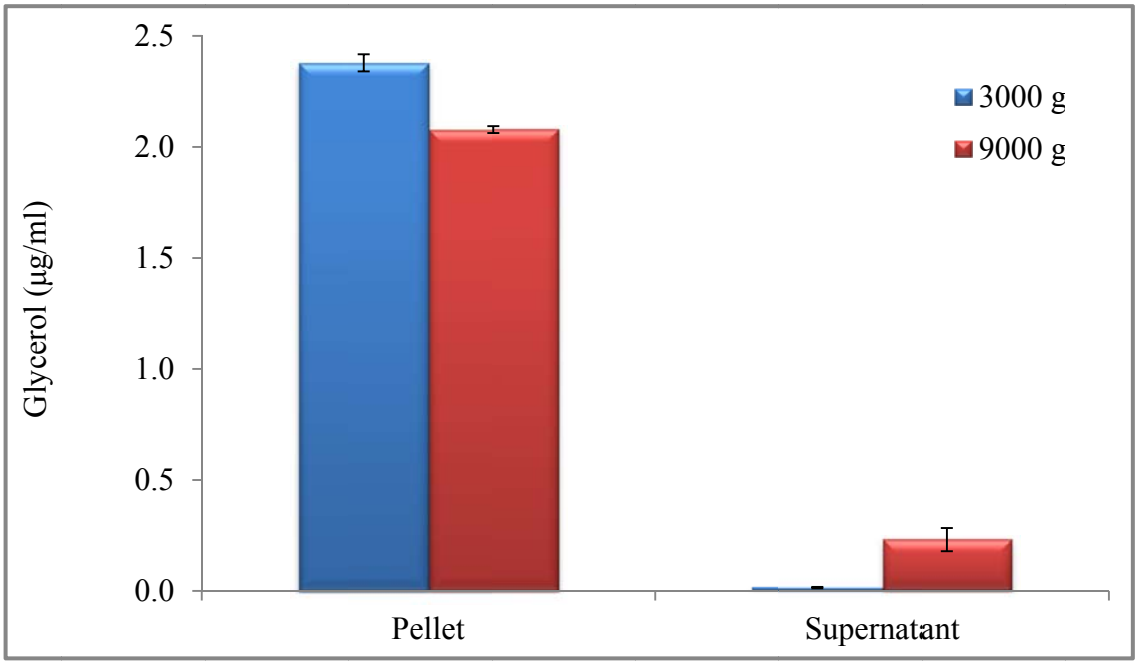


Fig. 5-11 Amount of glycerol obtained from GC-MS analysis of samples centrifuged at 3000 g and 9000 g. Calculations were based on the correlation between the area of the peak obtained for a glycerol standard and that corresponding for samples after adjustment for the concentration of the internal standard recovered. Error bars represent the standard deviations (n≥3) of measured values of cell density and measured values of glycerol content.

5.4 Discussion

This study compared chemical and enzymatic methods of glycerol determination. The validation of chemical method was conducted using GC-MS analysis. The effect of centrifugal force on the distribution of glycerol both within cells and in the external medium was also investigated.

The necessity for accurate measurement of small amounts of glycerol in the presence of high NaCl concentrations in the microalgal growth medium also limits the choice of technique.

Enzymatic method has been used to quantify glycerol in *Dunaliella* species (Ben-Amotz and Avron, 1973; Borowitzka *et al*., 1977; Fujii and Hellebust, 1992). This assay is costly because of the use of commercially produced enzyme kits. This study shows that only 33.2 % of glycerol was recovered using enzymatic method. It is also not as sensitive and reproducible as other methods such as the chemical method and GC-MS analysis.

Chemical method showed that the absorbance of glycerol was linear with its concentration. High salt concentrations did not interfere with this method. The sensitivity of this method at high salt concentrations, make it an attractive alternative for glycerol quantification. This method has a much greater sensitivity than enzymatic assays for estimation of glycerol in *Dunaliella* grown in saline medium.

GC analysis has been reported in the quantification of glycerol in microalgal samples (Gehron and White, 1983; Borowitzka *et al*., 1977). Lamers (2011) described a derivatisation method which involved the silyation of glycerol by N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and methoxyamine hydrochloride, to form tri-methyl-silyl glycerol. This silyation procedure is similar to the one adopted in this study. This study confirmed that GC-MS analysis is also a reliable method for glycerol estimation in *Dunaliella*. However GC-MS analysis comprises a number of washing steps (phase separations) between the organic chloroform and aqueous phase. It also required solvent evaporation in a centrifugal concentrator (Speedvac-Savant SPD20110), which takes 9-24 hours.

Enzymatic analysis was not an appropriate method for the quantification of glycerol in *Dunaliella*, whereas both chemical method and GC-MS analysis were suitable for glycerol quantification. This research adopted chemical method for the routine determination of glycerol.

For all the centrifugal forces tested very few number of cells remained in the supernatant after centrifugation. However the number of intact cells decreases with increasing centrifugation force from 2000 g to 11,000 g. Centrifugal forces beyond 5000 g was not suitable for harvesting intact cells of *Dunaliella* as a significant number of cells would be damaged. The larger amount of glycerol detected in the supernatant of the samples treated at higher centrifugal force (9000 g) served as another evidence of cell membrane damage during centrifugation. This could be a reason why a study conducted by Chow *et al.* (2013) on continuous extraction of glycerol from *D. tertiolecta* exposed to 10,000 g released glycerol in the medium.

CHAPTER 6.0: RESPONSES OF NAMIBIAN STRAINS OF *DUNALIELLA* AND *ASTEROMONAS* TO EXTREME LEVELS OF SALINITY

6.1 Introduction

Cellular homeostasis by strains of *Dunaliella* exposed to extremely varied salinities is thought to be maintained both by balancing synthesis and catabolism of glycerol (Borowitzka and Borowitzka, 1988), as well as through the ability to change cell volume and shape. Not all algae appear to exhibit these traits to the same extent: *D. tertiolecta* shows only limited ability to change shape and has been reported to possess a membrane-bound O-acetyltransferase protein which acts as a glycerol-uptake protein to transport glycerol from the external medium into the intracellular space (Lin *et al.,* 2013; Chow *et al,.* 2013).

Cell shape in halotolerant microalgae is highly variable: ellipsoid, oval, spherical, cylindrical, ellipsoidal and pyriform (pear-shaped) (Borowitzka and Siva, 2007). Cell size of halotolerant microalgae is correlated with changes in growth conditions. Changing salinity of the culture medium has been found to induce variations in cell length, width and volume. An increase in cell size and volume has been directly correlated with the accumulation of glycerol which serves as the major compatible solute in halotolerant microalgae species (Borowitzka and Siva, 2007; Coesel *et al*., 2008).

In *D. salina* glycerol concentration can reach 50 to 85 % of the cell dry weight when grown in a medium containing >4.0 M NaCl (Avron and Ben-Arnotz, 1978; Ben-Amotz, 1980; Ben-Amotz *et al.,* 1982; Calvin and Taylor, 1989; Kacka and Donmez, 2008; Chen *et al*., 2011). Intracellular glycerol is usually synthesised from the intermediates of the photosynthetic pathway and from starch breakdown (Gimmler and Moller, 1981; Fujii and Hellebust, 1992). The contribution of these metabolic pathways to glycerol synthesis depends on salinity stress applied, the size of starch reserve pool, and availability of light and CO_2 (Avron, 1992). Starch breakdown accounts for 70 % of the glycerol synthesis in the light regime and for all the glycerol synthesis in the dark regime (Borowitzka and Borowitzka, 1988). These two

carbon pools, glycerol and starch, are inter-converted in the *Dunaliella* cell (Beckett *et al,* 1985; Gimmler and Moller, 1981; Degani *et al,* 1985).

Having established a reliable method for harvesting cells of *Dunaliella* and *Asteromonas* intact, as well as a sensitive method free from interference for analyzing glycerol, the mechanism by which the Namibian strains *Dunaliella (*T35, T36 and T37) and *Asteromonas* (T33a, T33b and T33c) responded to sudden changes in osmotic shock was investigated. This study involved assessments of the:

1. Change of shape and volume of the *Dunaliella* and *Asteromonas* cells;
2. Accumulation of intracellular glycerol
3. Change in starch levels, under light and dark conditions.

6.2 Method

6.2.1 Microscopy and morphological study

This experiment was carried out to investigate the changes in *Dunaliella* and *Asteromonas* cell volume following exposure to hyper and hypo-osmotic stress using Leica microscope (see section 3.3)

Major features observed include size, shape, volume and colour of the cell. Scalar measurements such as cell length and width were taken from a minimum of 25 cells from each strain randomly after exposure to different NaCl concentrations. The experiment was conducted using two separate groups (treatments) as follows:

Group 1: *Dunaliella* and *Asteromonas* strains were cultured in 1.0, 2.0, 3.0 and 4.0 M NaCl for 14 days. Cell characteristics such as size, shape, colour and volume were examined using software. For this both the length and width of the cells were measured to determine the size as shown in figure 6.1.

Group 2: After 14 days cells cultured in 1.0 M NaCl were also subjected to osmotic shock , as follows: 0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 M NaCl medium for 20 minutes.

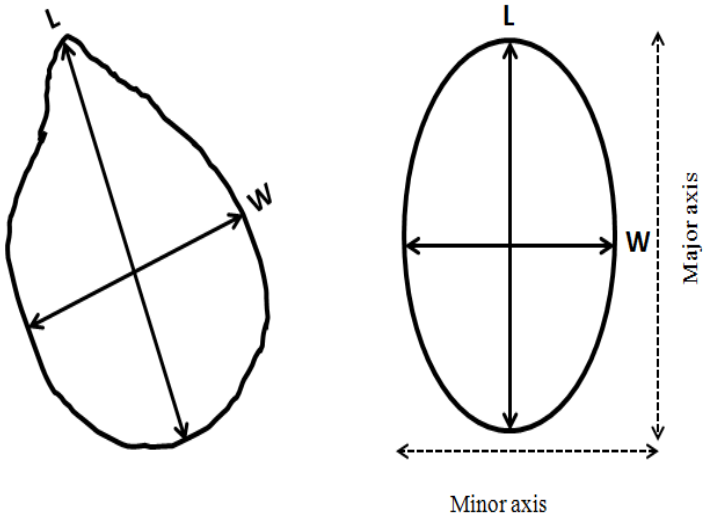


Fig. 6-1 Schematic diagram of ellipsoid microalgal cells showing major and minor axis. The major axis is the maximum length from the one end to the other whereas the minimum axis is the minimum length from the one end to the other. L = length and W= width

6.2.2 Determination of cell volume

See section 3.4.5

6.2.3 Investigation of the intracellular accumulation of glycerol

In experiments designed to measure the intracellular accumulation of glycerol during culture in media of different salinities, 2.5 ml of microalgal cells were transferred to 250 ml fresh media containing either 1.0, 2.0, 3.0 or 4.0 M NaCl concentrations. After culturing for 28 days in light/dark regimes at 25 ^{o}C the cultures were harvested and the pellets were used for glycerol determination.

For experiments to test the effect of hyperosmotic shock, 14 days old microalgal culture grown in 1.0 M NaCl (at exponential phase) was dispensed into centrifuge tubes (10^6 cell/ml), then microalgal cells in these tubes were harvested and resuspended in fresh medium

(10^6 cell/ml), which contained either 0.5, 1.0, 2.0, 3.0 or 4.0, M NaCl, and the cells were observed microscopically to check viability. The cultures were then placed in either constant light of 45 μmol m^{-2} s^{-1} or in the dark for varying lengths of time and analysed for either starch or glycerol content.

6.2.4 Effect of darkness and continuous illumination on starch contents following hyperosmotic shock

Starch content: 20 ml of microalgal culture grown in 1.0 M NaCl (at exponential phase) were harvested by centrifugation at 3000 *g* for 5 min at 25 °C. The resulted microalgae pellets were resuspended in 20 ml of fresh medium, which contained 1.0, 2.0, 3.0 and 4.0, M NaCl, respectively. These microalgae cultures were divided into two groups. Group 1 was kept in continuous illumination for 24 hr. Group 2 were kept under the dark regime for 24 hours. The amount of starch and glycerol was tested after 24 hr of hyperosmotic shock in both the light and the dark regime. Amount of starch was determined using the method described in (section 3.7.)

6.3 Results

6.3.1 Effect of increasing salinity on *Dunaliella* cell shape, size and volume

Figure 6.2 shows the light microscopic images of *Dunaliella* and *Asteromonas* species cultured at 1.0 M NaCl modified Johnson's medium. *Dunaliella* strains are unicellular, ovoid in shape, size (5.0-8.5 μm), with sharp apex, rounded posterior, pointed anterior and two equal flagella (Figure 6.3). *Asteromonas* species possess different unit sizes (6.0-10.6 μm). The presence of ridging in *Asteromonas* made it a distinguishing feature from *Dunaliella* (see Figure 6.4). The absence of filament in some of the micrographs occurs as the result of culture age or handling procedure. Appendix A3.1 shows the SEM micrographs of other strains of *Asteromonas* used in this research.

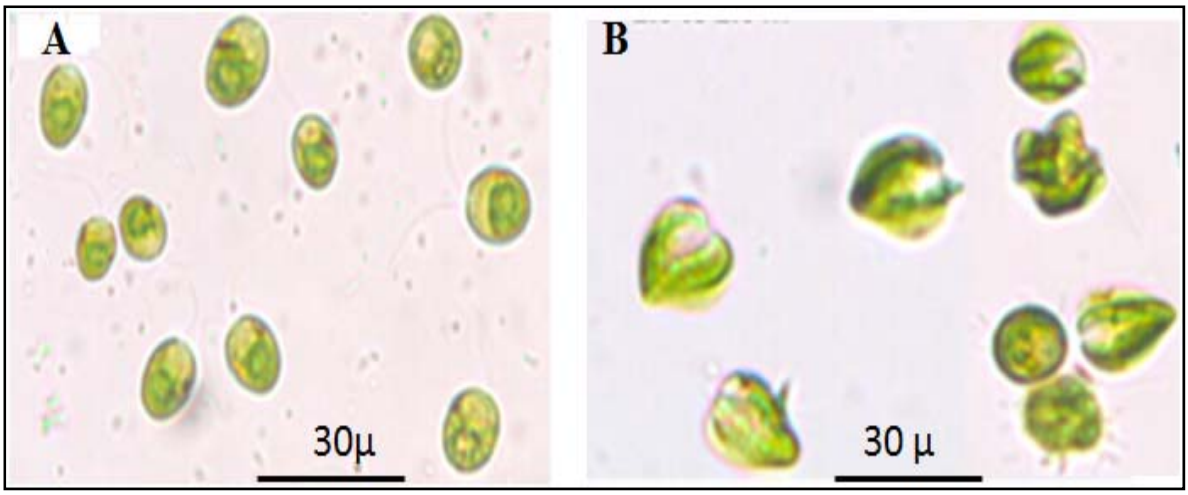


Fig. 6-2 Light microscopic images of halotolerant microalgae A. *Dunaliella* T35 (5.0 -8.5 µm) and B. *Asteromonas* T33a (6.0-12.6 µm), magnification 100x

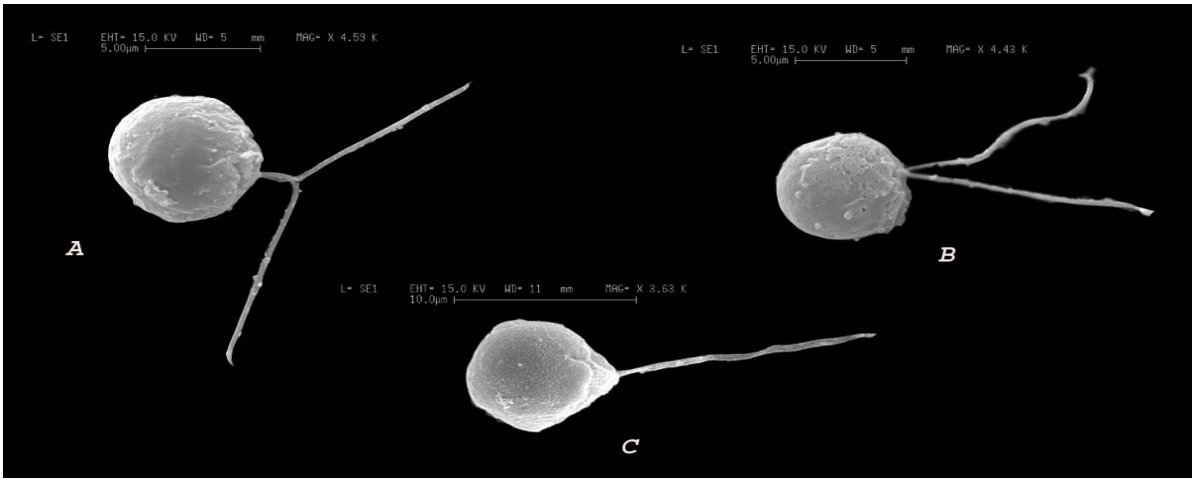


Fig. 6-3 Scanning electron microscopic images of *Dunaliella* strains: (A) *Dunaliella* T35 magnification 2120x; (B) *Dunaliella* T36 magnification 2120x; (C) *Dunaliella* T37 magnification 2130x.

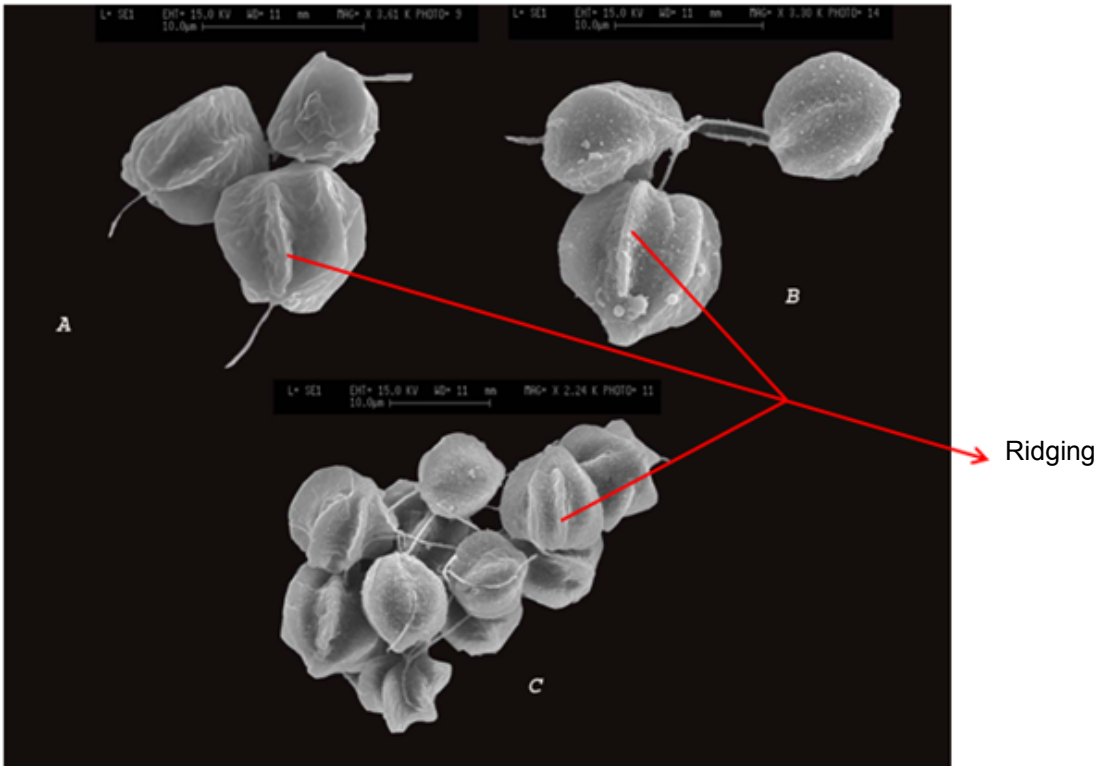


Fig. 6-4 Scanning electron microscopic images of *Asteromonas* strain T33c: (A) magnification 3610x; (B) magnification 3300x; (C) magnification 2240x

Figure 6.5 and 6.6 show the light microscopic images of *Dunaliella* T35 and *Asteromonas* T33a grown in modified Johnson's medium with either 1.0, 2.0, 3.0 or 4.0 M NaCl for 14 days. Images for T36 and T37 as a function of varying salinity were similar with that of T35. The results presented in Figure 6.7 and 6.8 show that the volume of the *Dunaliella* and *Asteromonas* cells decreased in response to an increase in the salinity of the medium. Images for T33b and T33c as a function of varying salinity were similar with that of T33a.

A difference in ionic strength of the culture medium leads to differences in both *Dunaliella* and *Asteromonas* cells volume. Cells grown under optimum salinity for growth (1.0 M NaCl) had the largest volume: 120 μm^3 for T35; 117 μm^3 for T36 and 111 μm^3 for T37, respectively, and 123, 163 and 119 μm^3 for T33a, T33b and T33c, respectively.

As salinity of the growth medium increased the cell volume decreases. There was 6, 20 and 24 % reduction in cell volume between *Dunaliella* T35 cells grown in 1.0 M NaCl than

corresponding cells grown in 2.0, 3.0 and 4.0 M NaCl concentration. Similar result pattern was recorded for *Dunaliella* T36 and T37 (Figure 6.7) and for *Asteromonas* (Figure 6.8).

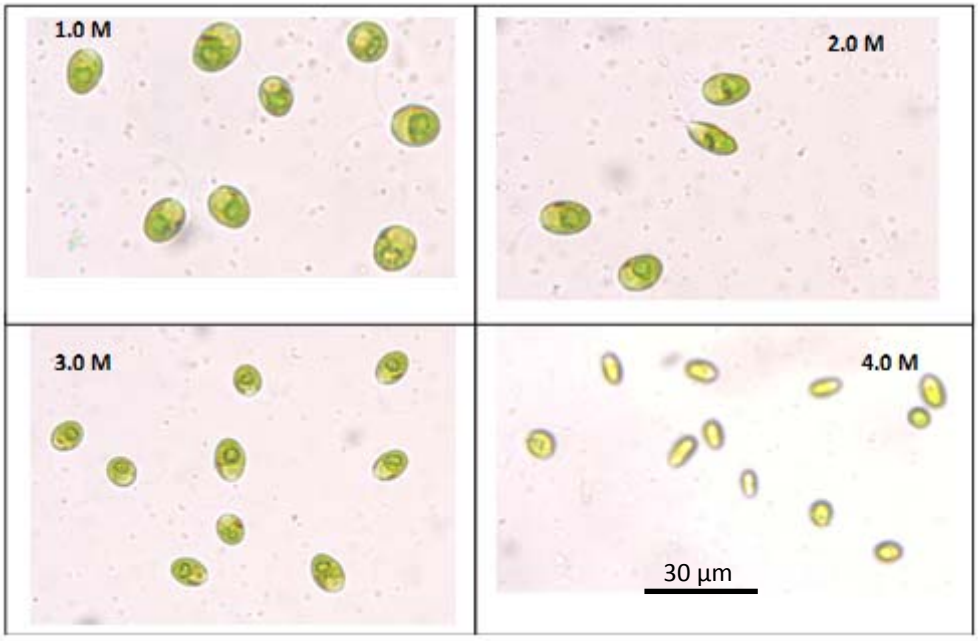


Fig. 6-5 Variation of cell shape of *Dunaliella*; T35 grown at various salinities (1.0 M NaCl, 2.0 M NaCl, 3.0 M NaCl and 4.0 M NaCl) for 14 days at pH 7.5, 25 ^{o}C and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod. Magnification X400

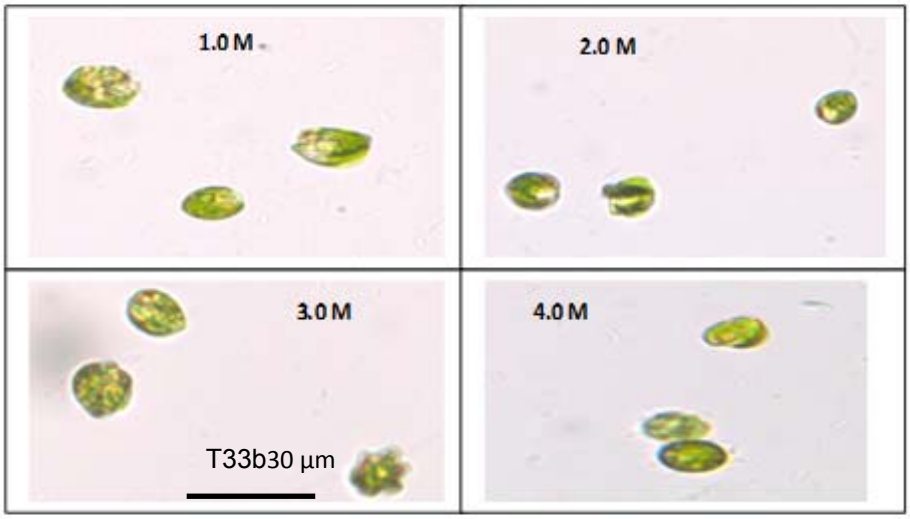


Fig. 6-6 Variation of cell shape of *Asteromonas*; T33a grown at various salinities (1.0 M NaCl, 2.0 M NaCl, 3.0 M NaCl and 4.0 M NaCl) for 14 days at pH 7.5, 25 ^{o}C and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod. Magnification X400

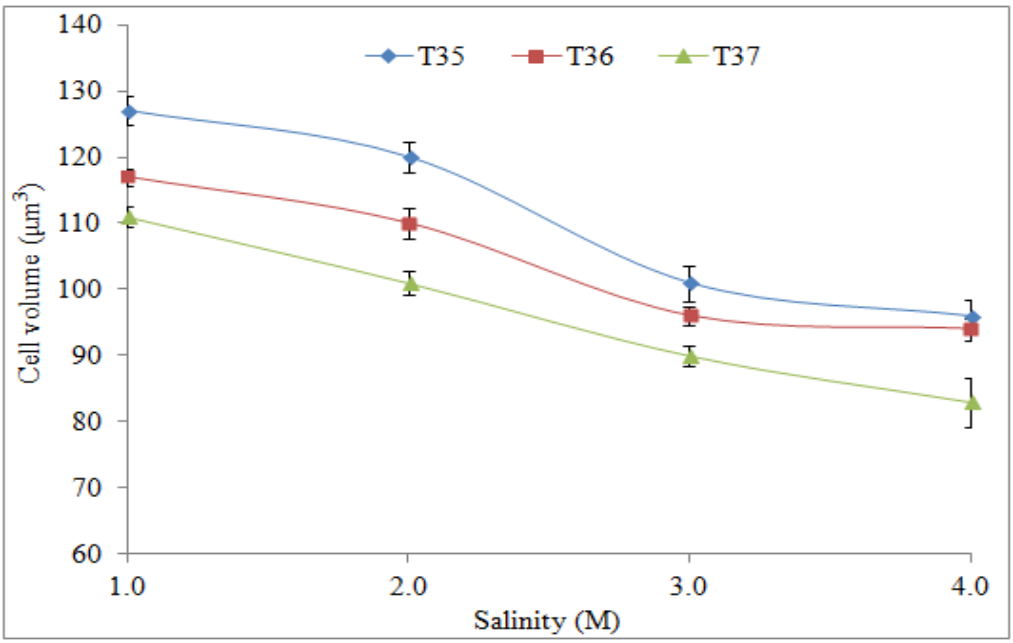


Fig. 6-7 Mean cell volume (μm^3) of *Dunaliella* strains exposed to a range of NaCl concentration at 25 ^oC, pH 7.5 and 45 µmol m^{-2} s^{-1} 12: 12 light/dark after 14 days of inoculation. The data represent the mean of three different experiments. Error bars represent the standard deviations (n=3) of measured cell volume.

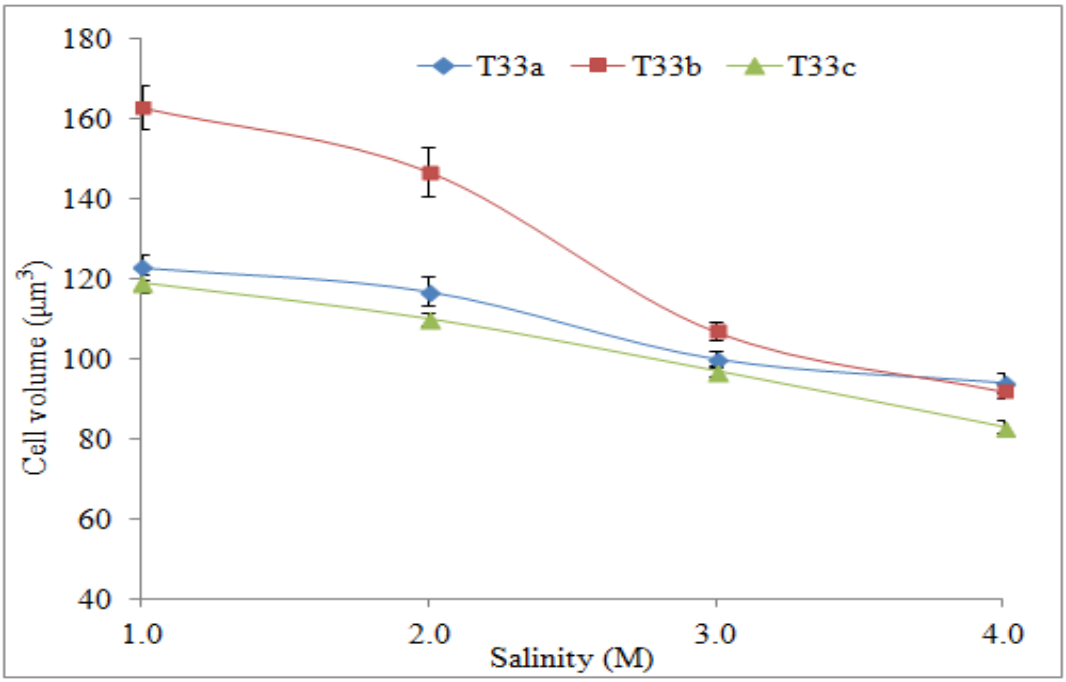


Fig. 6-8 Mean cell volume (μm^3) of *Asteromonas* strains exposed to a range of NaCl concentration at 25 ^oC, pH 7.5 and 45 µmol m^{-2} s^{-1} 12: 12 light/dark after 14 days of inoculation. Error bars represent the standard deviations (n=3) of measured cell volume.

6.3.2 Effect of increasing salinity on the production of glycerol by *Dunaliella* and *Asteromonas*

Increasing salinity in the culture medium caused a decline in cell density for both *Asteromonas* and *Dunaliella* strains, reflecting the negative effect of salinity on cell doubling time as previously recorded (chapter 4) for each strain tested. For *Dunaliella* strains it also caused an increase in glycerol content per cell up to 3.0 M NaCl, beyond which glycerol levels declined (Figures 6.9 - 6.11). In 3.0 M NaCl the glycerol content was at least 3-fold greater than that in 1.0 M NaCl (for T37 nearly 4-old greater). The increase cannot be attributed to the decrease in cell volume alone, and must therefore reflect synthesis of glycerol as well. Interestingly, these results contrast to the situation found with *Asteromonas* strains. In these latter, glycerol levels declined in all strains cultured with increasing salinity above 1.0 M, apart from T33a, whose pool size of glycerol only declined in media with salinity >2.0 M NaCl (see Figures 6.12 - 6.14).

Figure 6.15 shows that there is inter-relationship between salinity stress, cell volume and level of glycerol per cell. For all the three *Dunaliella* strains the highest cell volume was obtained from cell exposed to 1.0 M NaCl medium. The smallest cell volume was obtained from cell exposed to 3.0 and 4.0 after 14 days of inoculation (Figure 6.15). Cell volume and glycerol per cell and salinity stress are related; the higher the salinity stress the higher the glycerol and lower the cell volume. Similar to *Dunaliella*, *Asteromonas* shows a good correlation between cell volume and glycerol accumulation in the cells cultivated for 14 days (Figure 6.16).

The opposite effects in *Dunaliella* strains on cell doubling time and on glycerol production influenced the amount of glycerol that could be achieved per g dry mass, i.e. glycerol productivity (mg glycerol/g dry weight). Glycerol productivity was maximal in cultures maintained in 3.0 M NaCl for all Namibian *Dunaliella* strains tested (Figures 6.9 - 6.11 and Table 6.1), even though glycerol per cell for T37 was greatest in 4.0 M NaCl. After 28 days culture T36 had the lowest concentration of glycerol per cell at all concentrations of salinity tested compared to either T35 or T37, whose glycerol contents were similar (Pairwise comparison of the strains grown at different salinities for glycerol production on pg per cell basis indicated that the two strains (T35 and T37) were not significantly different ($P < 0.050$).

However since the doubling time of T36 was significantly greater than for either T35 or T37, irrespective of the salt concentration (see also Chapter 4), the yields of glycerol produced by each strain after 28 days cultivation were not significantly different. Appendix A3.2 shows ANOVA data comparing the treatments (strains; T35, T36 and T37) means and the multiple comparison tests (Turkey test) between salinity ranges. $p = 0.155$ and $F = 2.588$ between the overall means of the three *Dunaliella* strains based on glycerol per ml of culture medium (see ANOVA table in Appendix A3.3).

In the case of the Namibian *Asteromonas,* strains produced less glycerol than *Dunaliella*. Only strain T33c cultured in 4.0 M NaCl produced a level of glycerol per cell approaching that of the *Dunaliella* strains (~210 pg / cell (*Asteromonas* T33c*)* compared to 170 -235 pg / cell for *Dunaliella*), but the largest amount of glycerol per ml for *Asteromonas* T33c was achieved at 1.0 and 2.0 M NaCl, reflecting the faster doubling time at this salt concentration compared to that at 4.0 M NaCl (Table 6.2). Appendix A3.4 and A2.5 shows ANOVA data comparing the treatments (strains; T33a, T33b and T33c) means and the multiple comparison tests (Turkey test) between salinity ranges.

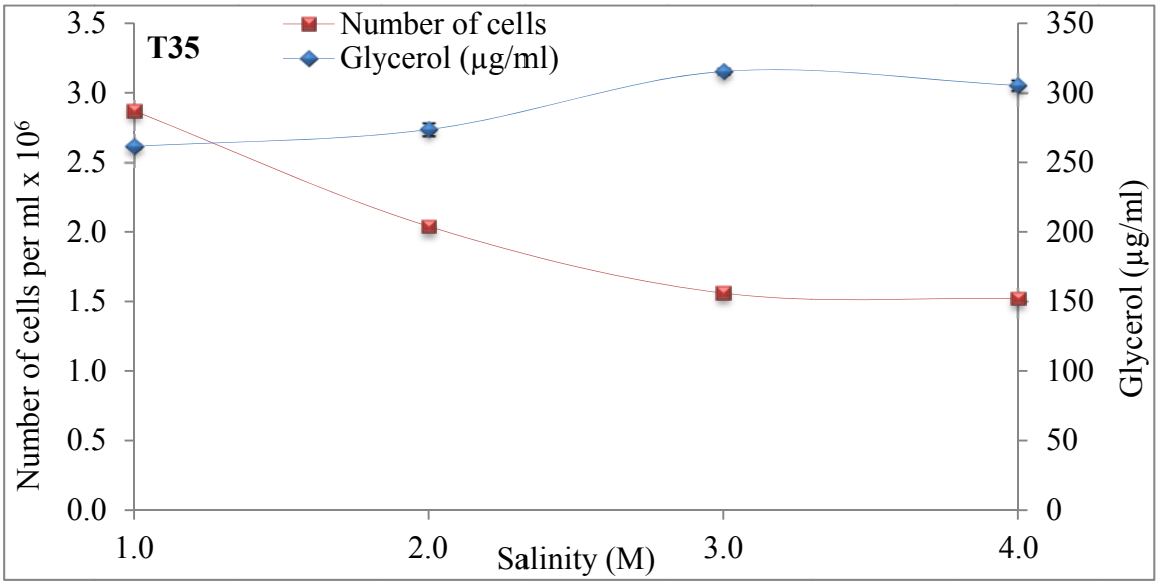


Fig. 6-9 Effect of salinity on the amount of glycerol accumulated by cultures of *Dunaliella* T35 and on cell density after 28 days culture in modified Johnson's medium in 12:12 light/dark regimes at 25 ^{o}C. 2.5 ml of microalgal cells (10^6 cell/ml) were transferred to 250 ml fresh media containing 1.0, 2.0, 3.0 and 4.0 M NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

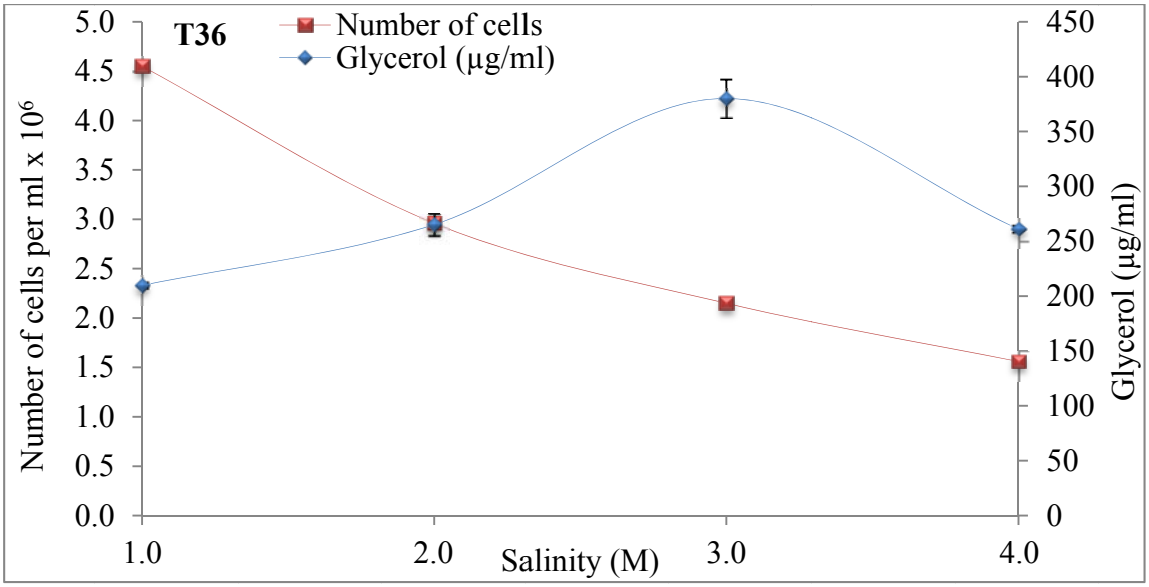


Fig. 6-10 Effect of salinity on the amount of glycerol accumulated by cultures of *Dunaliella* T36 and on cell density after 28 days culture in modified Johnson's medium in 12:12 light/dark regimes at 25 °C. 2.5 ml of microalgal cells (10^6 cell/ml) were transferred to 250 ml fresh media containing 1.0, 2.0, 3.0 and 4.0 M NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

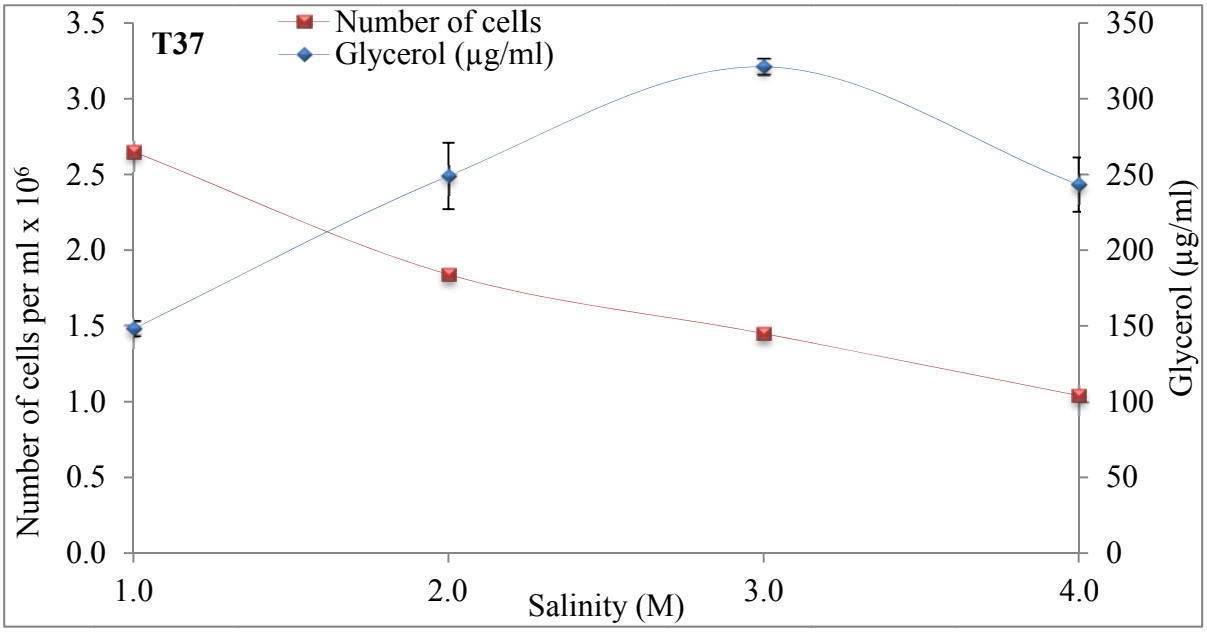


Fig. 6-11 Effect of salinity on the amount of glycerol accumulated by cultures of *Dunaliella* T37 and on cell density after 28 days culture in modified Johnson's medium in 12:12 light/dark regimes at 25 °C. 2.5 ml of microalgal cells (10^6 cell/ml) were transferred to 250 ml fresh media containing 1.0, 2.0, 3.0 and 4.0 M NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

Table 6-1 The amount of glycerol accumulated milligrams per gram dry weight and pg per cell of *Dunaliella*; T35, T36 and T37 grown at various salinities after 28 days cultivation at 23 ^{o}C, initial pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod.

Strain	Salinity (M)	Glycerol (mg/g DW)	Glycerol (pg/cell)	Number of cell x 10^6 /ml
Dunaliella T35	1.0	523	65.2 ± 0.6	2.87
	2.0	547	133.8 ± 2.4	2.04
	3.0	631	203.2 ± 3.1	1.56
	4.0	611	200.5 ± 11.3	1.52
Dunaliella T36	1.0	388	46.1 ± 0.7	4.55
	2.0	491	89.6 ± 8.1	2.96
	3.0	704	177.0 ± 2.6	2.15
	4.0	484	167.4 ± 7.6	1.56
Dunaliella T37	1.0	285	55.9 ± 0.9	2.65
	2.0	479	135.3 ± 4.9	1.84
	3.0	618	221.4 ± 8.7	1.45
	4.0	468	234.9 ± 10	1.04

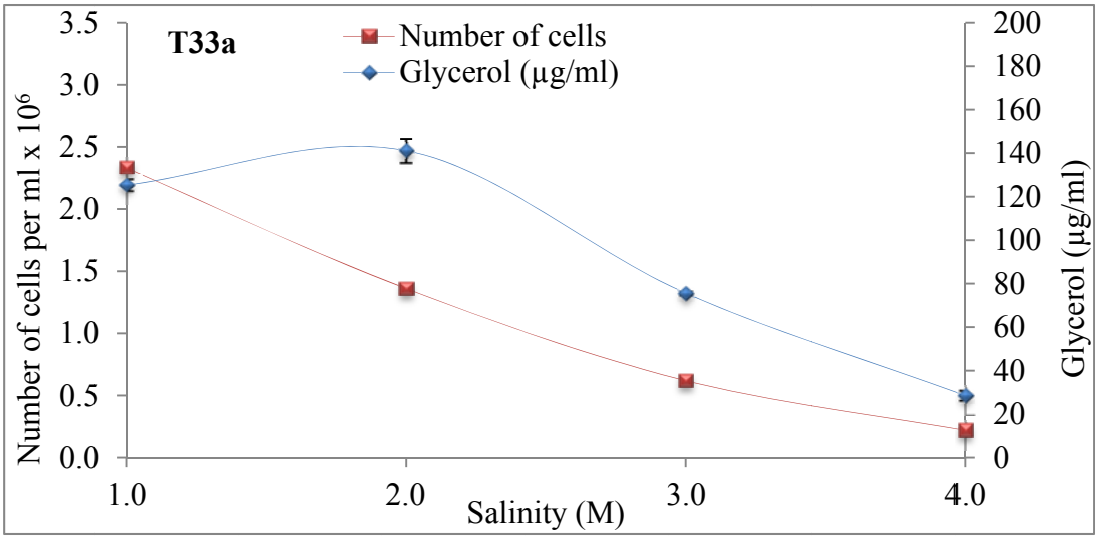


Fig. 6-12 Effect of salinity on the amount of glycerol accumulated by cultures of *Asteromonas* T33a and on cell density after 28 days culture in modified Johnson's medium in 12:12 light/dark regimes at 25 ^{o}C. 2.5 ml of microalgal cells (10^6 cell/ml) were transferred to 250 ml fresh media containing 1.0, 2.0, 3.0 and 4.0 M NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

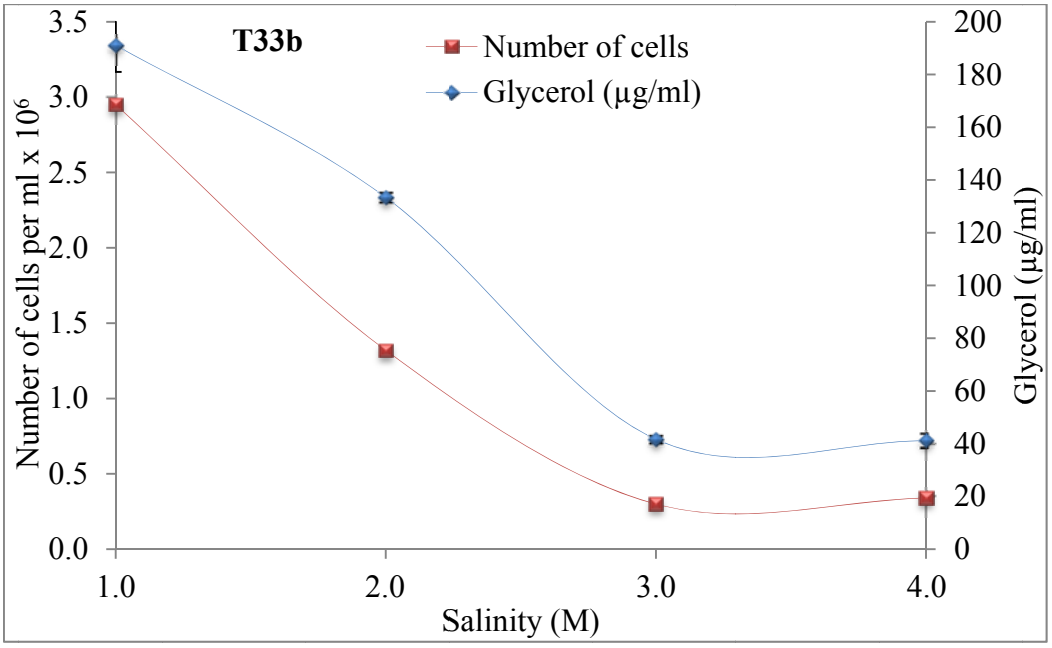


Fig. 6-13 Effect of salinity on the amount of glycerol accumulated by cultures of *Asteromonas* T33b and on cell density after 28 days culture in modified Johnson's medium in 12:12 light/dark regimes at 25 °C. 2.5 ml of microalgal cells (10^6 cell/ml) were transferred to 250 ml fresh media containing 1.0, 2.0, 3.0 and 4.0 M NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

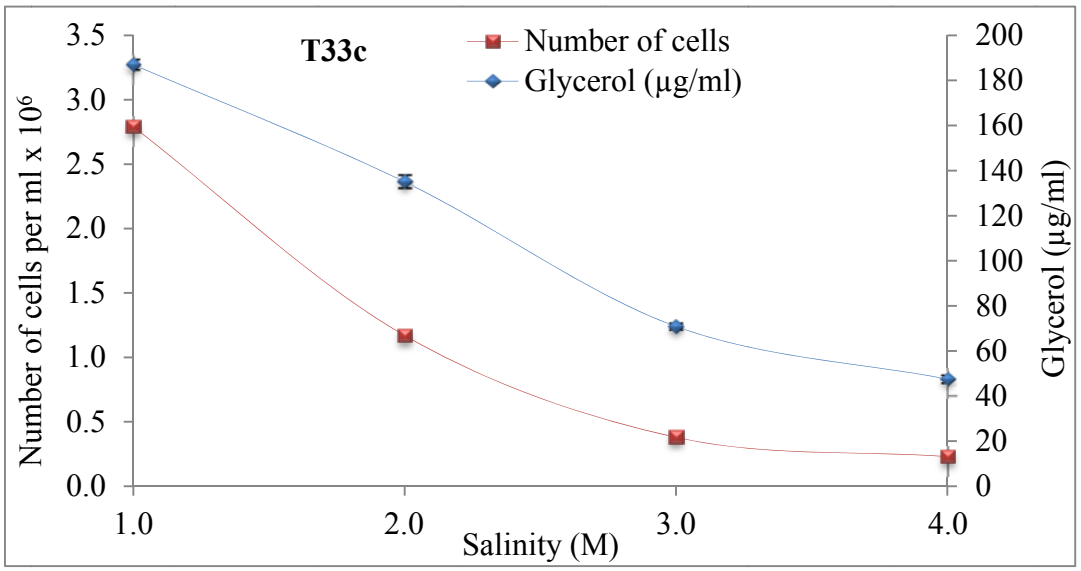


Fig. 6-14 Effect of salinity on the amount of glycerol accumulated by cultures of *Asteromonas* T33c and on cell density after 28 days culture in modified Johnson's medium in 12:12 light/dark regimes at 25 °C. 2.5 ml of microalgal cells (10^6 cell/ml) were transferred to 250 ml fresh media containing 1.0, 2.0, 3.0 and 4.0 M NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

Table 6-2 The amount of glycerol accumulated per mg dry weight and pg per cell of *Asteromonas*; T33a, T33b and T33c at various salinities (after 28 days cultivation)

Strain	NaCl (M)	Glycerol (mg/g DW)	Glycerol (Pg/cell)	Number of cells x10^6
Asteromonas T33a	1.0	216.0	53.8 ± 0.8	2.33
	2.0	243.3	103.5 ± 3.9	1.36
	3.0	130.3	122.6 ± 2.9	0.62
	4.0	49.4	126.3 ± 0.9	0.22
Asteromonas T33b	1.0	329.5	64.8 ± 0.3	2.95
	2.0	229.9	101.3 ± 2.3	1.32
	3.0	71.9	138.0 ± 2.8	0.30
	4.0	70.9	121.0 ± 3.5	0.34
Asteromonas T33c	1.0	322.4	67.1 ± 2.8	2.79
	2.0	232.8	115.2 ± 12.2	1.17
	3.0	122.2	184.9 ± 6.2	0.38
	4.0	81.9	209.6 ± 10.1	0.23

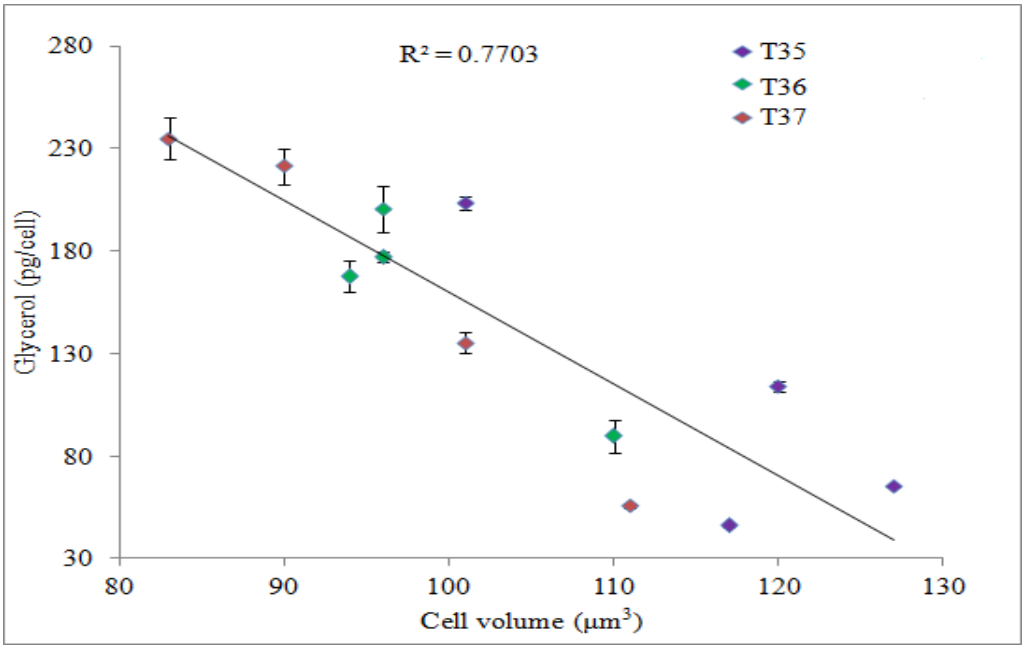


Fig. 6-15 Correlation between mean cell volume and glycerol level per cell of *Dunaliella* strains cultivated in a range of NaCl concentration at 25 ^{o}C, pH 7.5 and 45 µmol m^{-2} s^{-1} 12:12 light: dark for 14 days. Error bars represent the standard deviations (n=3) of measured values of glycerol content

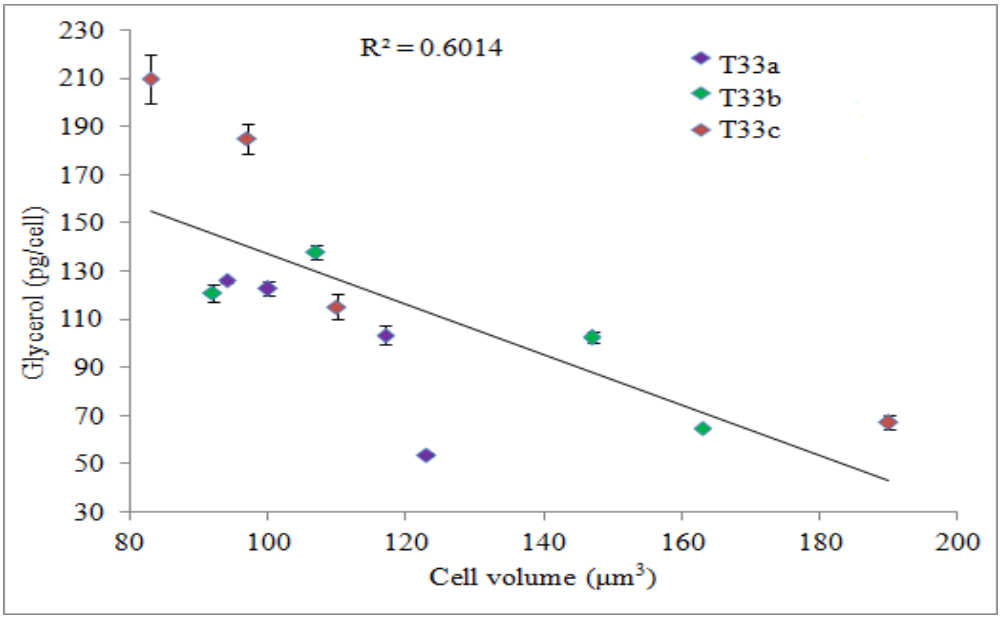


Fig. 6-16 Correlation between mean cell volume and glycerol level per cell of *Asteromonas* strains cultivated in a range of NaCl concentration at 25 ^{o}C, pH 7.5 and 45 µmol m^{-2} s^{-1} 12:12 light: dark for 14 days. Error bars represent the standard deviations (n=3) of measured values of glycerol content

6.3.3 Effect of hyperosmotic shock on cell size, volume and glycerol production by *Dunaliella* and *Asteromonas*

Dunaliella and *Asteromonas* cells were grown in 1.0 M NaCl for 14 days and then exposed to hyperosmotic osmotic shock for either 160 min (*Dunaliella*) or 240 min (*Asteromonas*) under constant light.

After 20 min, the shapes of microalgal cell of three *Dunaliella* strains were ellipsoid under low salinity (0.5 and 1.0 M NaCl), and changed to become long and thin with increased salinity (2.0, 3.0 and 4.0 M NaCl). Furthermore, the chlorophyll pigment of the cells appeared lighter in the cells exposed to 4.0 M NaCl when compared with those in 1.0 M NaCl concentration (Figure 6.17). In *Asteromonas* the length of the cell decreased only slightly in response to an increase in the osmolarity of the culture medium, and simultaneously the cell width decreased drastically (Figure 6.18). In highly saline (4.0 M NaCl) medium, the *Dunaliella* cells were shrunken. The shrinking part of the cells was

almost light in colour. For the transfer from 1.0 to 4.0 M NaCl concentration the volume decreased by 62 %. Transfer of *Dunaliella* cells T35 from 1.0 to 2.0 and 3.0M NaCl shows decreased in cell volume to 6 % and 35 % respectively. Similar results were recorded for *Dunaliella* T36 and T37. For *Asteromonas* T33a, the transfer from 1.0 to 4.0 M NaCl concentration caused the volume to decrease to about 35 %. Similar results (percentage change) were recorded for *Asteromonas* T33b and *Asteromonas* T33c respectively (see Appendix A3.6 and A3.7 for the calculated percent changes).

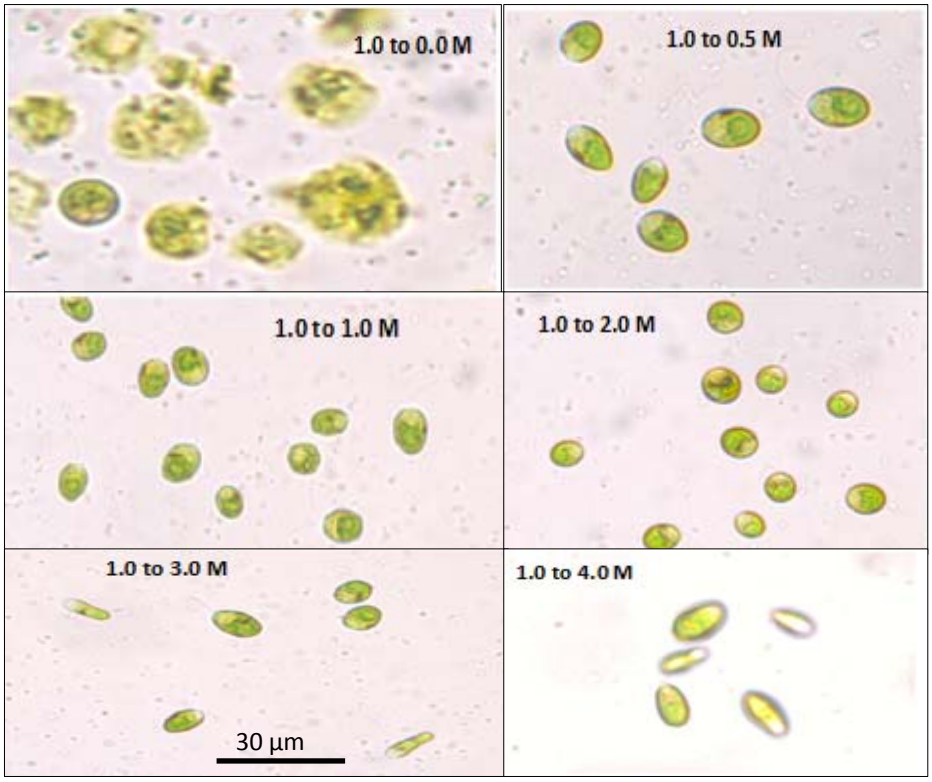


Fig. 6-17 Variation of cell shape of *Dunaliella*; T35 cultured in 1.0 M NaCl and then treated by hyperosmotic or hypo-osmotic shock (from 1.0 to 0.0 M NaCl, from 1.0 to 0.5 M NaCl, from 1.0 to 1.0 M NaCl, from 1.0 to 2.0 M NaCl, from 1.0 to 3.0 M NaCl and from 1.0 to 4.0 M NaCl) for 20 min at pH 7.5, 25 ^{o}C and 45 µmol m^{-2} s^{-1} under light regime. The Figure is representative of three replicated studies with comparable findings. Magnification X400

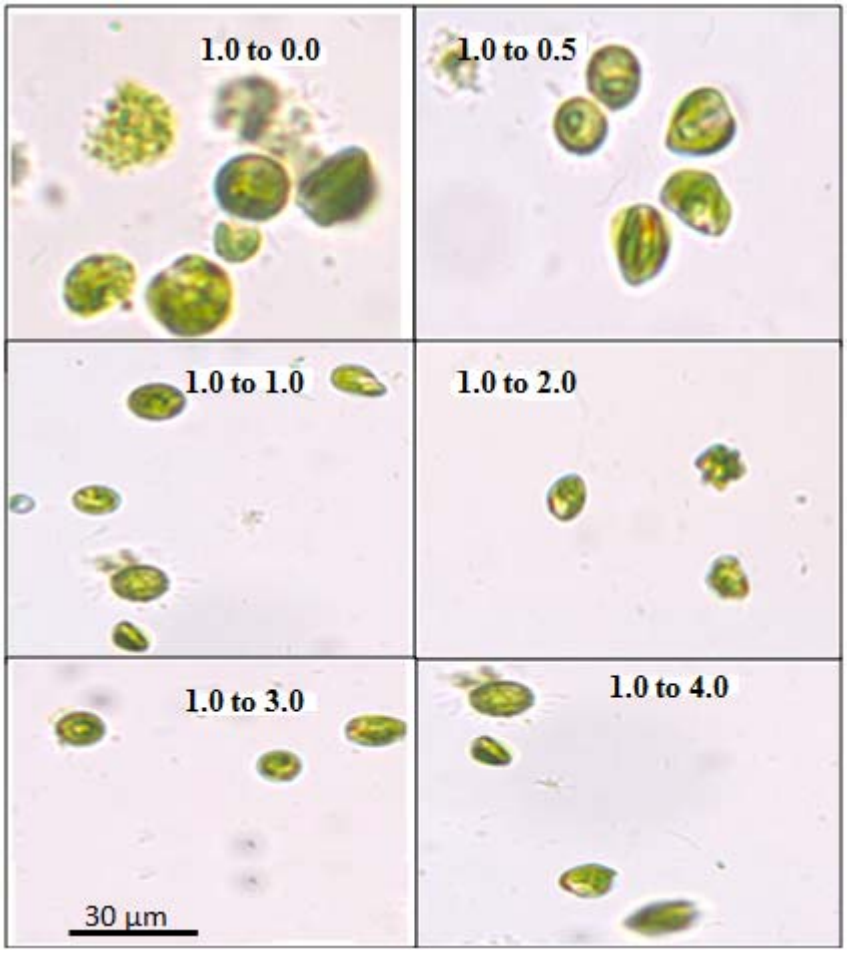


Fig. 6-18 Variation of cell shape of *Asteromonas*; T33a cultured in 1.0 M NaCl for 14 days and then treated by hyperosmotic or hypo-osmotic shock (from 1.0 to 0.0 M NaCl, from 1.0 to 0.5 M NaCl, from 1.0 to 1.0 M NaCl, from 1.0 to 2.0 M NaCl, from 1.0 to 3.0 M NaCl and from 1.0 to 4.0 M NaCl) for 20 min at pH 7.5, 25 ^{o}C and 45 µmol m^{-2} s^{-1} under light regime. The Figure is representative of three replicated studies with comparable findings. Magnification X400, size 5-8 µm

Dunaliella and *Asteromonas* cells cultivated in 1.0 M NaCl for 14 days were transferred to various salinities for 20 min and volume changes were recorded (Figure 6.19 and 6.20). *Dunaliella* and *Asteromonas* cells cultivated in 3.0 and 4.0 M NaCl concentration for 14 days (section 6.3.1) have greater volume compared to the corresponding cells exposed to 20 min hyperosmotic shock. This shows that these cells would require more than 20 minutes to attained new cell volume (as they cannot resume to original volume). Similar to cells

cultivated in 3.0 and 4.0 M NaCl for 14 days, there is a good correlation between cell volume and glycerol accumulation when cell were exposed to hyperosmotic shock for 20 min (Figure 6.21 and 6.22).

Neither *Dunaliella* nor *Asteromonas* cells were disrupted on transfer from 1.0 M NaCl to 0.5 M NaCl media. Figure 6.19 and 6.20 show that in 0.5 M NaCl the cell volume of *Dunaliella* T35, T36 and T37 and *Asteromonas* T33a, T33b and T33c increased to a similar final volume (184, 171 and 184 μm^3) for *Dunaliella* and (189, 198 and 205 μm^3) for *Asteromonas*, respectively. When *Dunaliella* T35 was transferred from 1.0 to 0.5 M NaCl media, the cell volume increased by 42 %. When *Asteromonas* T33a was transfer from 1.0 to 0.5 M NaCl concentration the cell volume increased to 54 %.

When resuspended in culture medium without NaCl all *Dunaliella* cells broke within 20 min due to the large water uptake from the medium (see Figure 6.17). Cell rupture was more prominent in T37 compared to T35 and T36 cultures respectively. In the case of the *Asteromonas* strains, only few (5-10 %) cells were disrupted after 20 min exposure to 0.0 M NaCl. This indicated that *Asteromonas* shows more tolerance to hypo-osmotic shock when compared to *Dunaliella* as previous shown by Figure 6.18.

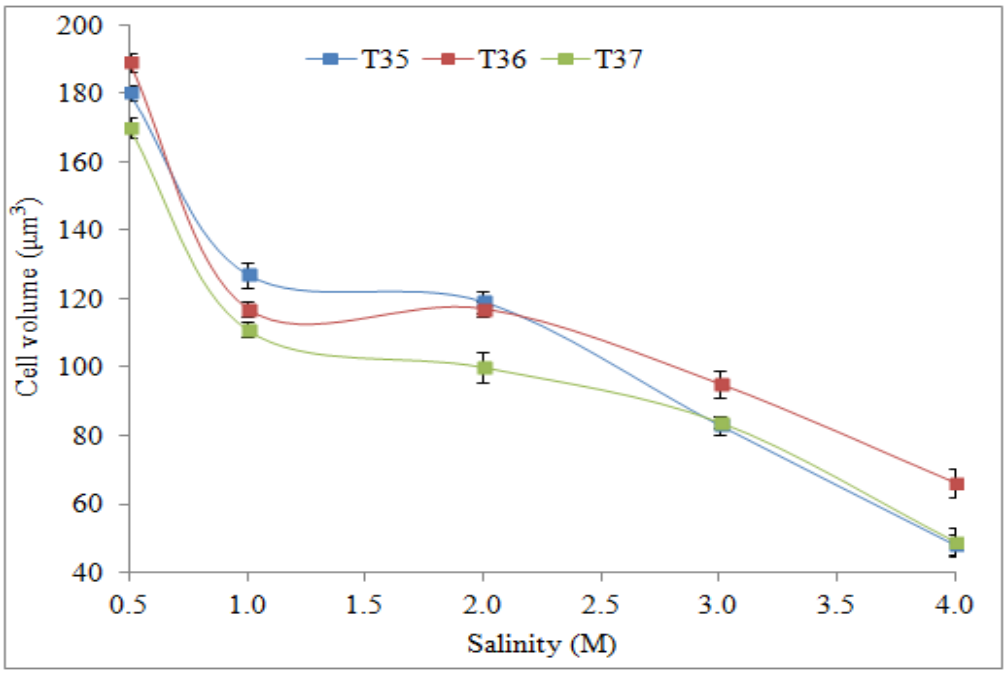


Fig. 6-19 Mean cell volume (μm^3) of *Dunaliella* strains transferred from 1.0 to hyper or hypo-osmotic stress at 25 °C, pH 7.5 and 45 µmol m^{-2} s^{-1} light for 20 min. Error bars represent the standard deviations (n=3) of measured cell volume.

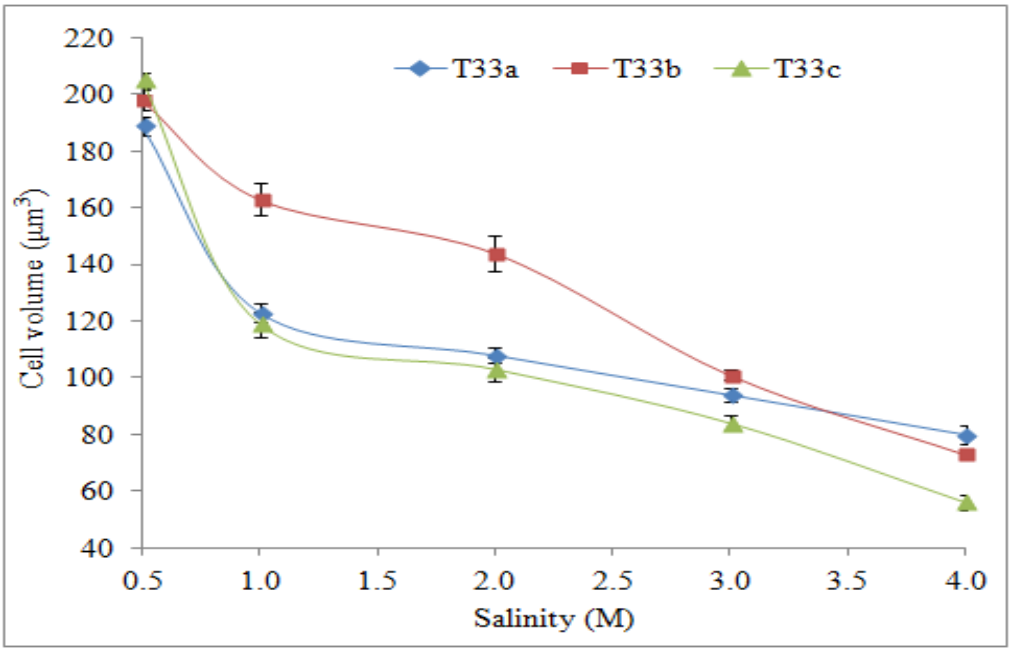


Fig. 6-20 Mean cell volume (μm^3) of *Asteromonas* strains transferred from 1.0 to hyper and hypo-osmotic stress at 25 °C, pH 7.5 and 45 µmol m^{-2} s^{-1} light for 20 min. Error bars represent the standard deviations (n=3) of measured cell volume.

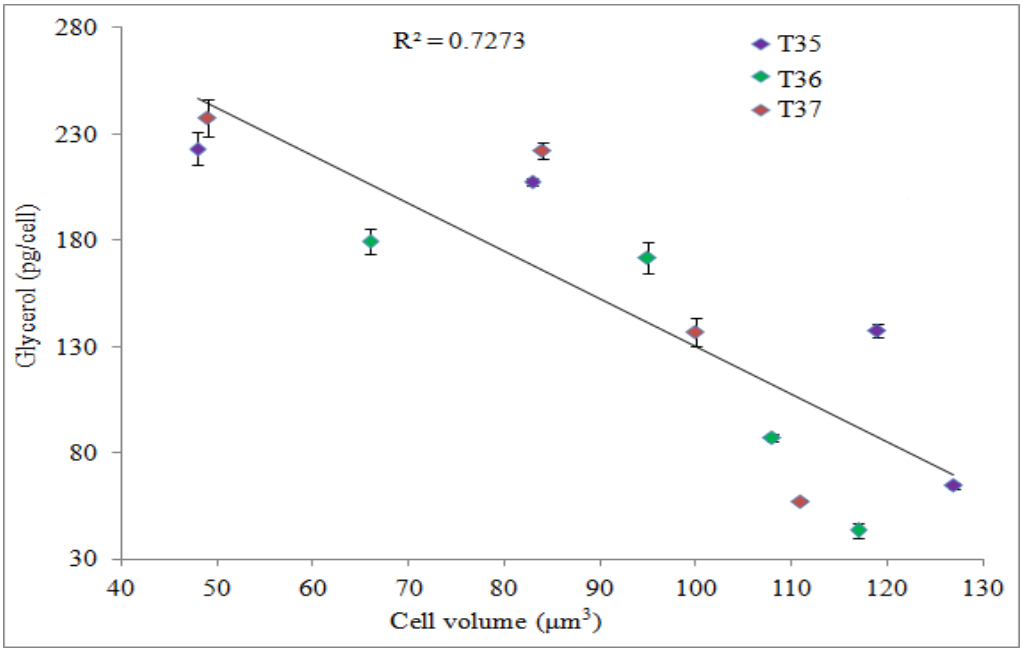


Fig. 6-21 Correlation between mean cell volume and glycerol level per cell of *Dunaliella* strains exposed to a range of NaCl concentration exposure at 25 °C, pH 7.5 and 45 µmol m^{-2} s^{-1} light for 20 min. Error bars represent the standard deviations (n=3) of measured values of glycerol content

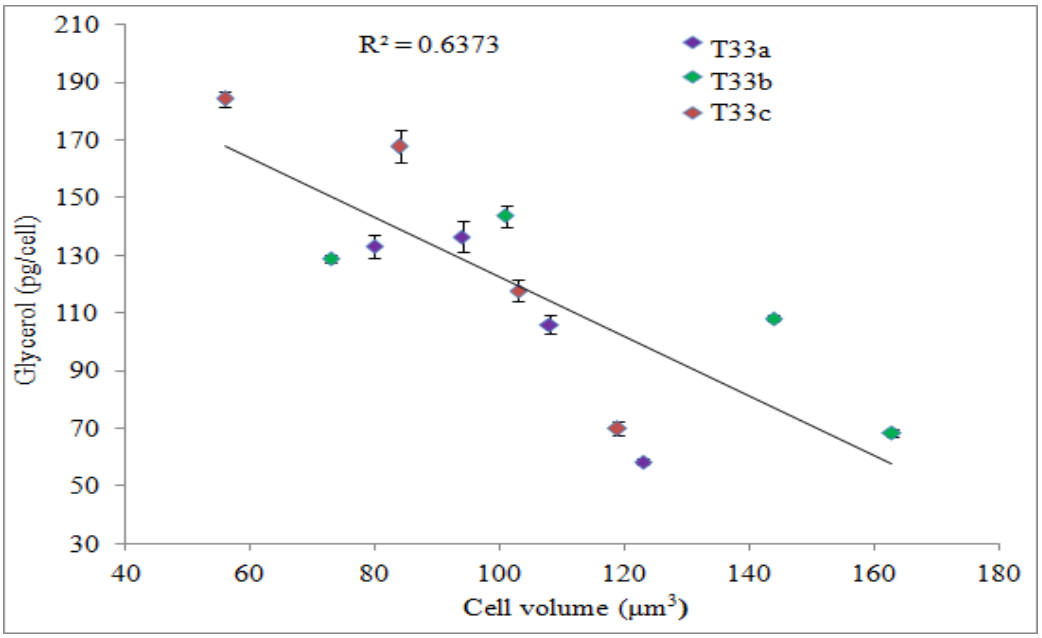


Fig. 6-22 Correlation between mean cell volume and glycerol level per cell of *Asteromonas* strains exposed to a range of NaCl concentration exposure at 25 °C, pH 7.5 and 45 µmol m^{-2} s^{-1} light for 20 min. Error bars represent the standard deviations (n=3) of measured values of glycerol content

There was no change in cell number during the period of hyperosmotic shock (240 min), but all strains each increased the level of intracellular glycerol. The largest amount of glycerol per ml of culture medium was measured in 4.0 M NaCl medium for each of the *Dunaliella* strains and for two of the *Asteromonas* strains (T33a and T33c) (Figure 6.23 - 6.28). *Asteromonas* T33b produced largest amount at 3.0 M NaCl. *Dunaliella* T36 cells produced largest amount of glycerol per ml at 3.0 and 4.0 M NaCl when compared to T35 and T37. This is related to its highest cell density. The amounts of glycerol detected in pg cell were similar to those recorded in Section 6.3.1 for all the strains at different salinity (Table 6.3).

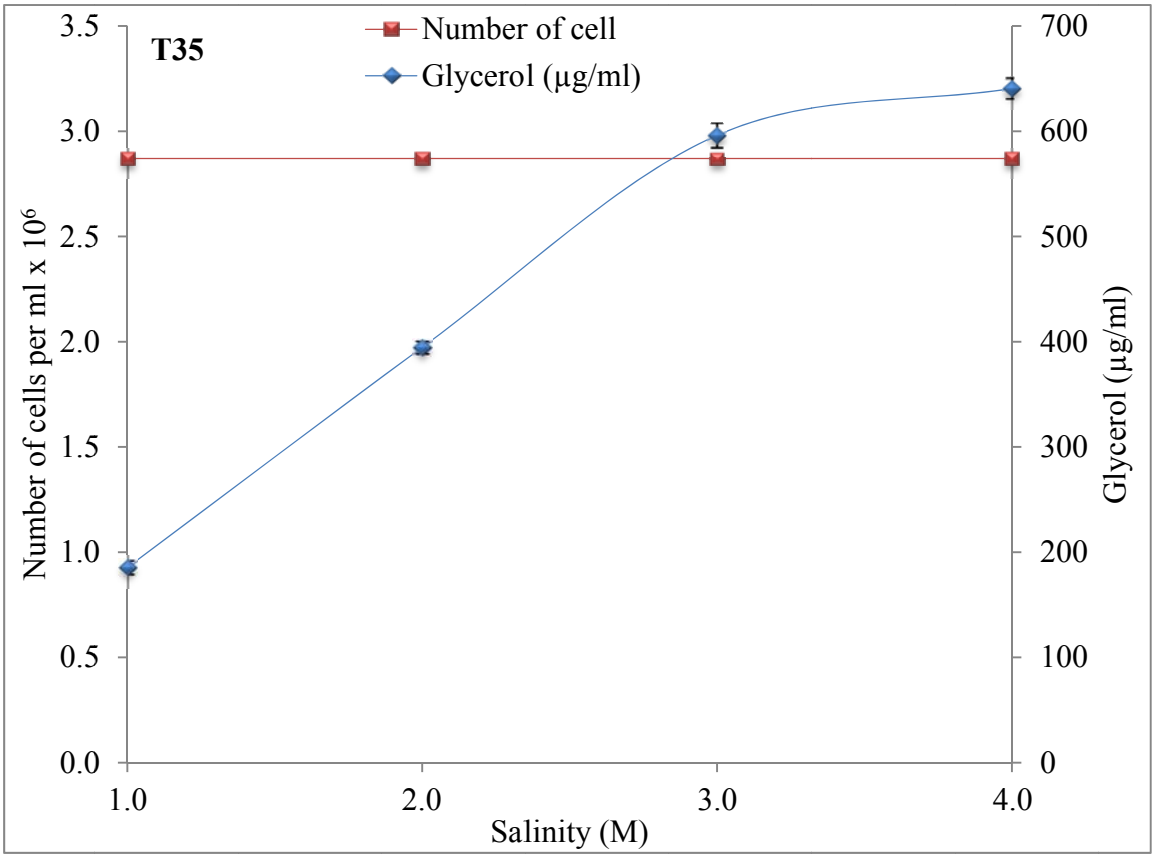


Fig. 6-23 Graphical representation to show the effect on cell number and on glycerol yield per ml of culture medium of *Dunaliella* T35 after 160 min exposure to fresh medium containing varying amounts of NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

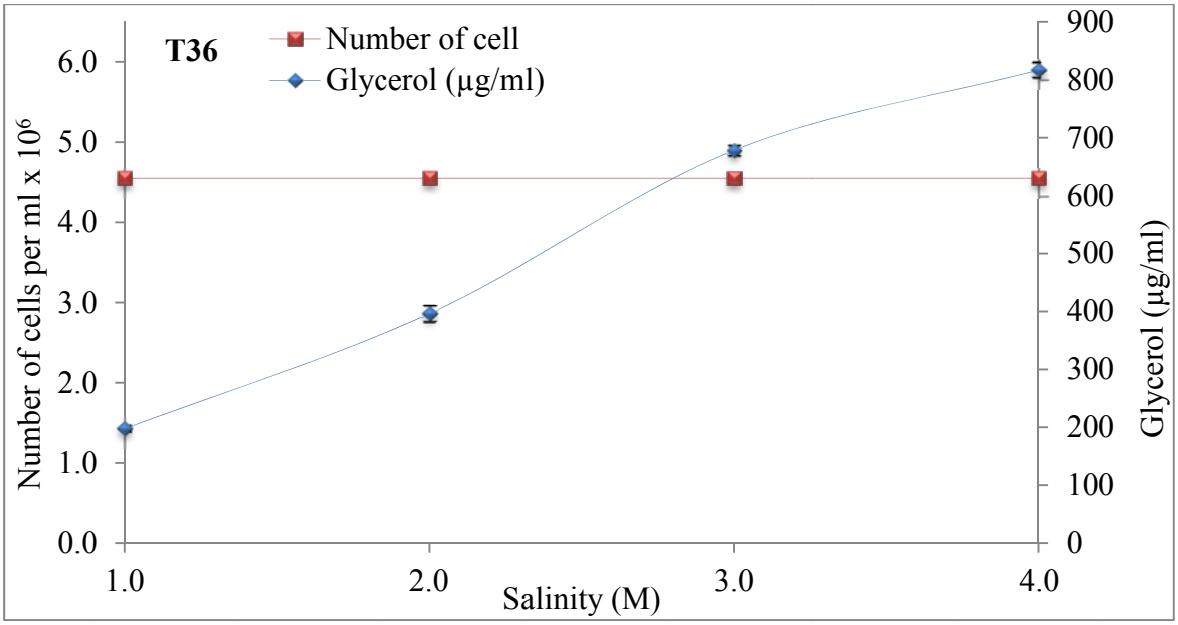


Fig. 6-24 Graphical representation to show the effect on cell number and on glycerol yield per ml of culture medium of *Dunaliella* T36 after 160 min exposure to fresh medium containing varying amounts of NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

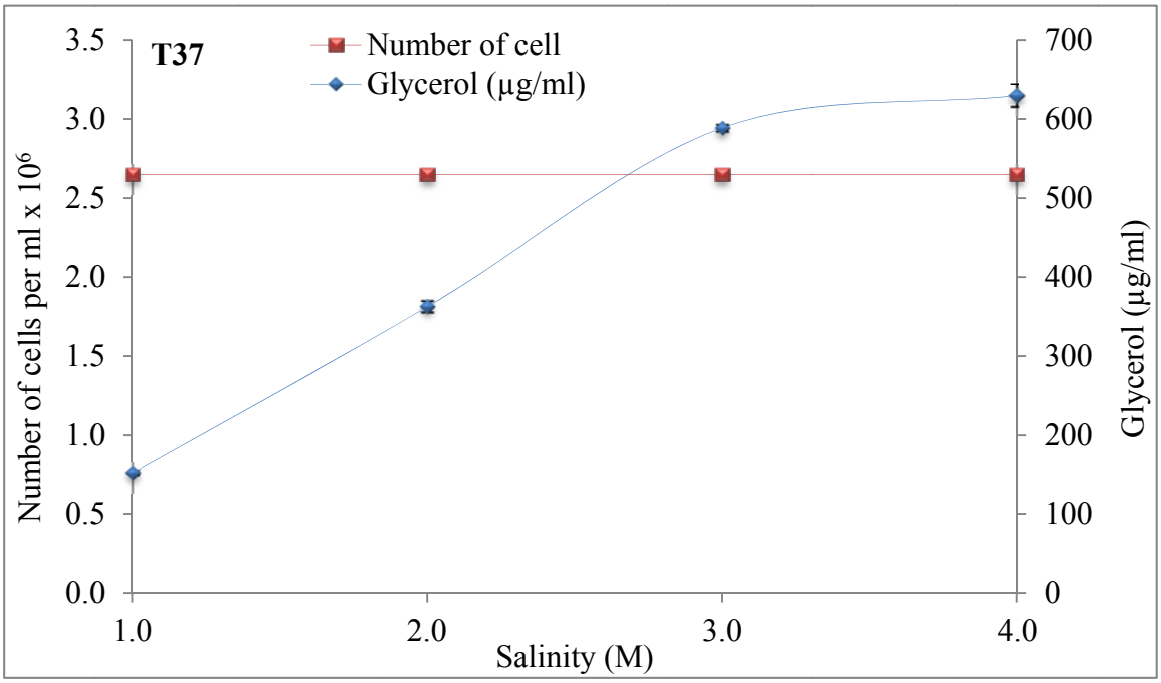


Fig. 6-25 Graphical representation to show the effect on cell number and on glycerol yield per ml of culture medium of *Dunaliella* T37 after 160 min exposure to fresh medium containing varying amounts of NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

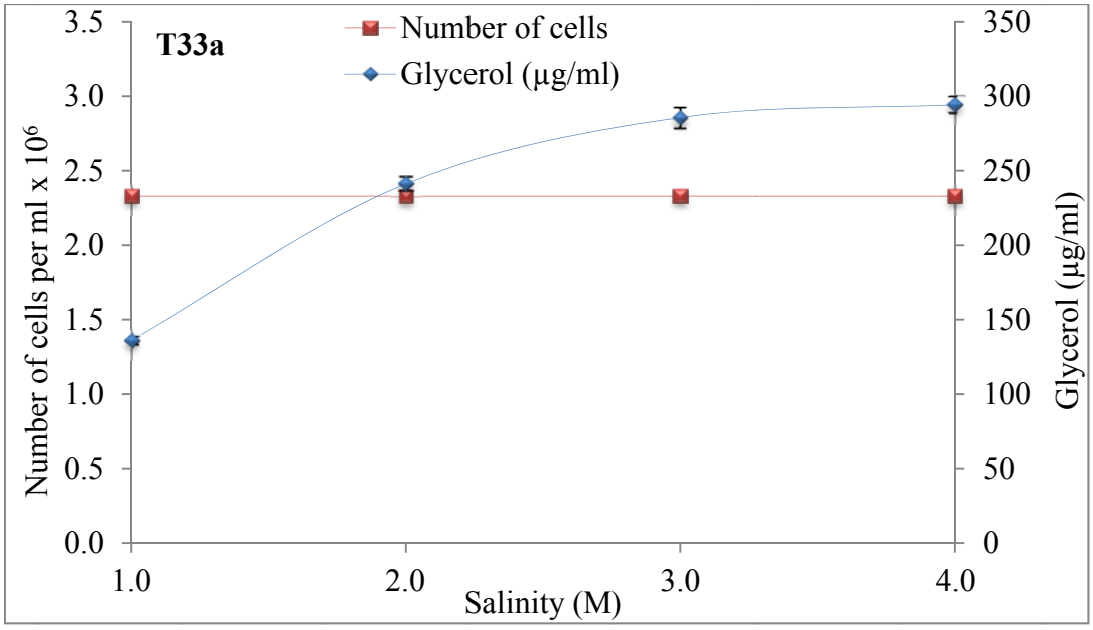


Fig. 6-26 Graphical representation to show the effect on cell number and on glycerol yield per ml of culture medium of *Asteromonas* T33a after 160 min exposure to fresh medium containing varying amounts of NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

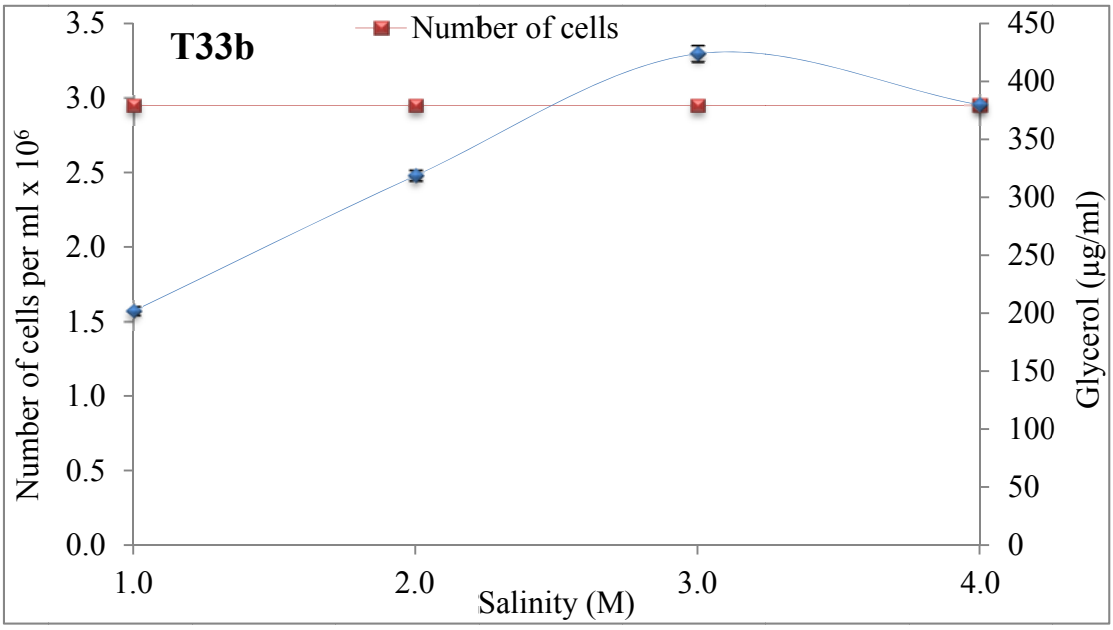


Fig. 6-27 Graphical representation to show the effect on cell number and on glycerol yield per ml of culture medium of *Asteromonas* T33b after 160 min exposure to fresh medium containing varying amounts of NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

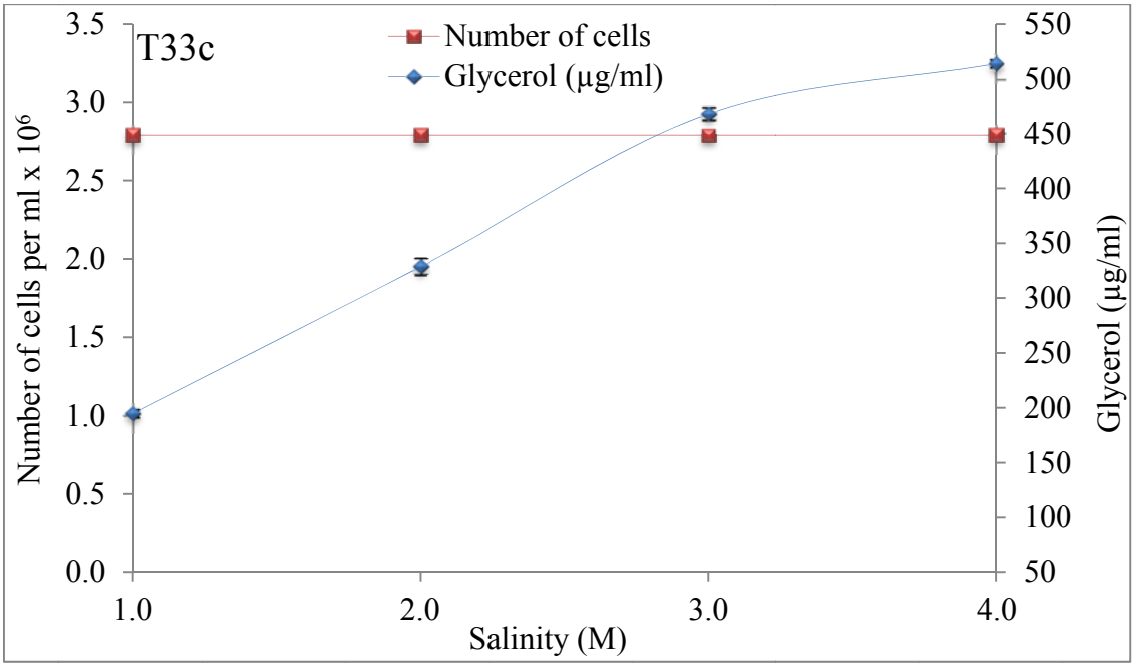


Fig. 6-28 Graphical representation to show the effect on cell number and on glycerol yield per ml of culture medium of *Asteromonas* T33c after 160 min exposure to fresh medium containing varying amounts of NaCl. Error bars represent the standard deviations (n=3) of measured values of glycerol content

Table 6-3 Amount of glycerol in pg per cell of *Dunaliella* and *Asteromonas* after 28 days of cultivation and following 160 min transfer to hyperosmotic shock

Microalgae	**28 days continuous cultivation in 3M NaCl (glycerol pg/cell)**	**14 days cultivation in 1M NaCl followed by 160 min exposure to 3 M NaCl (glycerol pg/cell)**	**28 days continuous cultivation in 4M NaCl (glycerol pg/cell)**	**14 days cultivation in 1M NaCl followed by 160 min exposure to 4 M NaCl (glycerol pg/cell)**
T35	203.2	207.7	200.5	223.3
T36	177.0	171.7	167.4	179.5
T37	221.4	222.1	234.9	237.6
T33a	122.6	136.5	126.3	133.1
T33b	138.0	143.7	121.0	128.7
T33c	184.9	167.7	209.6	184.2

Figure 6.29 describes the kinetics of glycerol production for *Dunaliella*. Glycerol levels increased rapidly within 60 min of exposure to either 3.0 or 4.0 M NaCl and the maximum cell levels were reached by 80 to 160 min depending on strain. The apparent surge in glycerol productivity between 60 min and 80 min observed for *Dunaliella* T35 in particular and to lesser extents for T36 and T37 may reflect recruitment of different pathways known to synthesise glycerol i.e. degradation of stored starch by glycolysis to form dihydroxyacetone phosphate, and thence glycerol, and synthesis *de novo* of glycerol from photosynthesis.

To test this hypothesis, a further set of experiments were undertaken with *Dunaliella* T35 and with *Asteromonas* wherein exposure to hyperosmotic conditions was conducted in either light or dark regimes. No statistically significant difference was found between the amount of glycerol accumulated under light and dark regimes for *Asteromonas* (see Appendix A3.8 Table A1.4). However a different set of results was obtained for *Dunaliella* T35 see Figure 6.30 (no light during osmotic shock) and Figure 6.31 (continuous light). Within 20 min of exposure to extreme salinity, the concentration of glycerol per cell increased and continued to increase at the same rate, irrespective of the lighting regime, for the next 40 min. The surge in glycerol production was only observed after 60 min and in continuous light. The observed increase in glycerol /cell in the first 60 min was therefore likely to be due to activation of the glycolytic pathway associated with starch degradation, which can be carried out in both light and dark. There may be a component of cell volume change as well based on visual observations (up to 24 % cell shrinkage in transferring from media with 1.0 M NaCl to 4.0M NaCl, see above), however these data do not allow deconvolution of the contribution of cell volume change from that of starch degradation.

Between 60 and 80 min. the surge in glycerol production observed previously was only evident in the light, amounting to an increase of ~72% over that produced in the dark after exposure to 4.0 M NaCl. This would be consistent with recruitment of the photosynthetic pathway of glycerol synthesis in the light, over and above other mechanisms and which only becomes evident after 60 min. In the dark the starch degradation pathway seems likely to be the only pathway responsible for glycerol synthesis throughout the time course.

Somewhat surprisingly, by 160 min the level of glycerol in all conditions of increased salinity reached a plateau, irrespective of the lighting regime, and was maintained for the duration of

the experiment. Thus, in 3.0 M and 4.0 M NaCl the levels of glycerol achieved in the dark were ~155 pg/cell and ~180 pg/cell respectively but around 15 % greater (~ 183 pg/cell and ~ 210 pg/cell respectively) in the light, even though light and dark conditions had the same corresponding level of salinity.

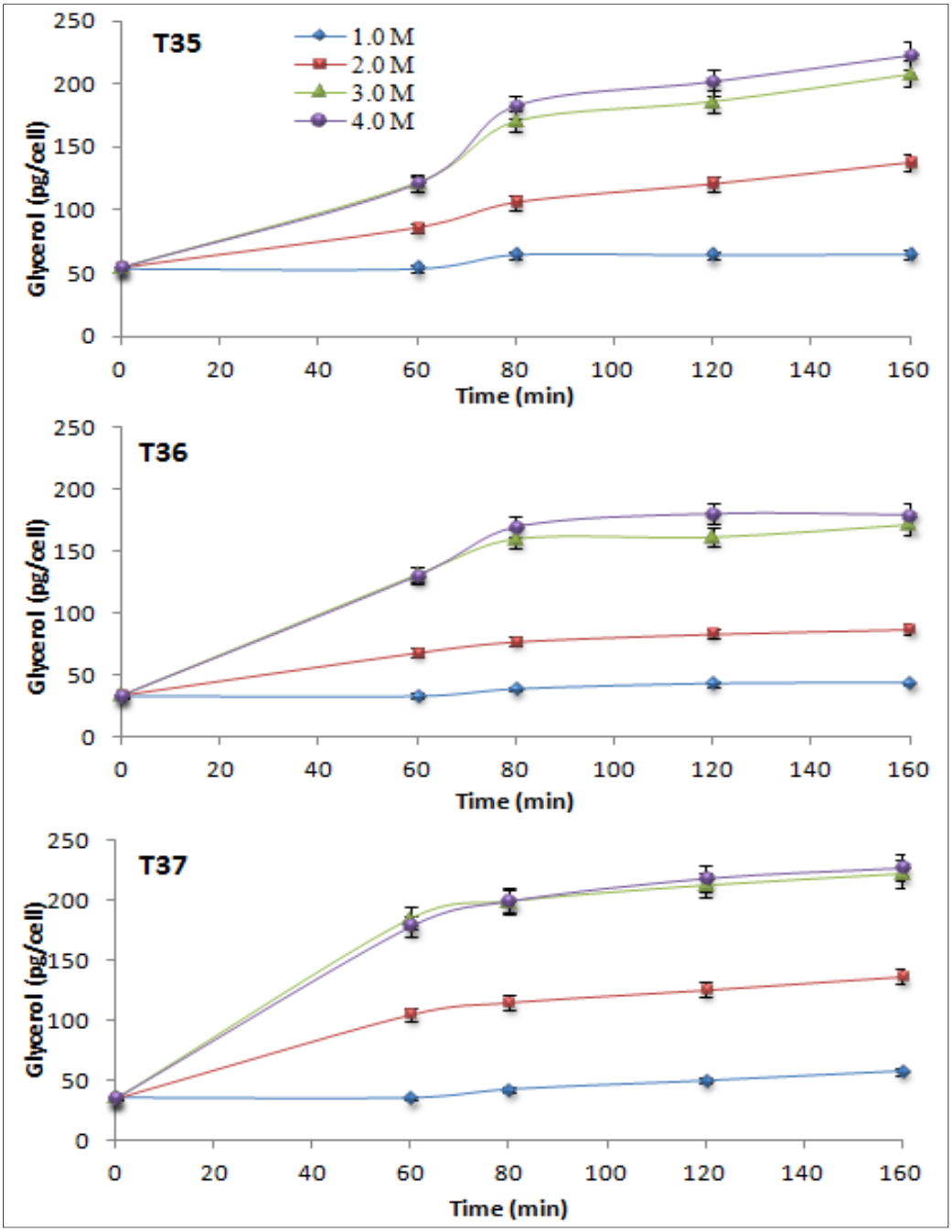


Fig. 6-29 The effect of increasing the NaCl concentration on the glycerol content of Dunaliella strains (T35, T36 and T37) as a function of time at constant light of 45 µmol m^{-2} s^{-1}. Error bars represent the standard deviations (n=3) of measured values of glycerol content

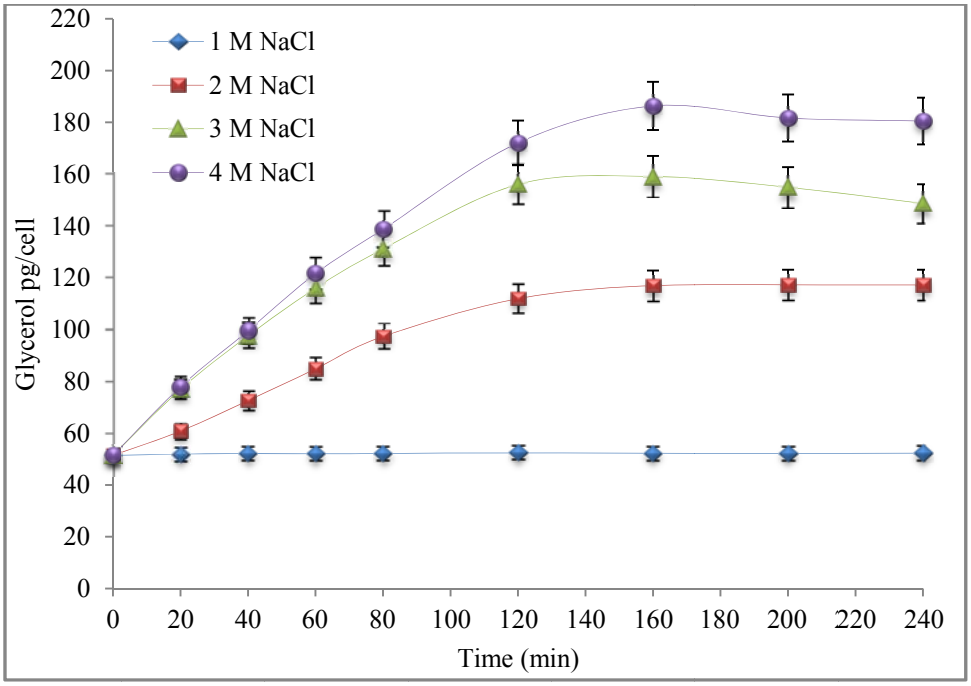


Fig. 6-30 The effect of increasing the NaCl concentration on the glycerol content of *Dunaliella* T35 as function of time (20 to 240 min) under the dark regime. Error bars represent the standard deviations (n=3) of measured values of glycerol content

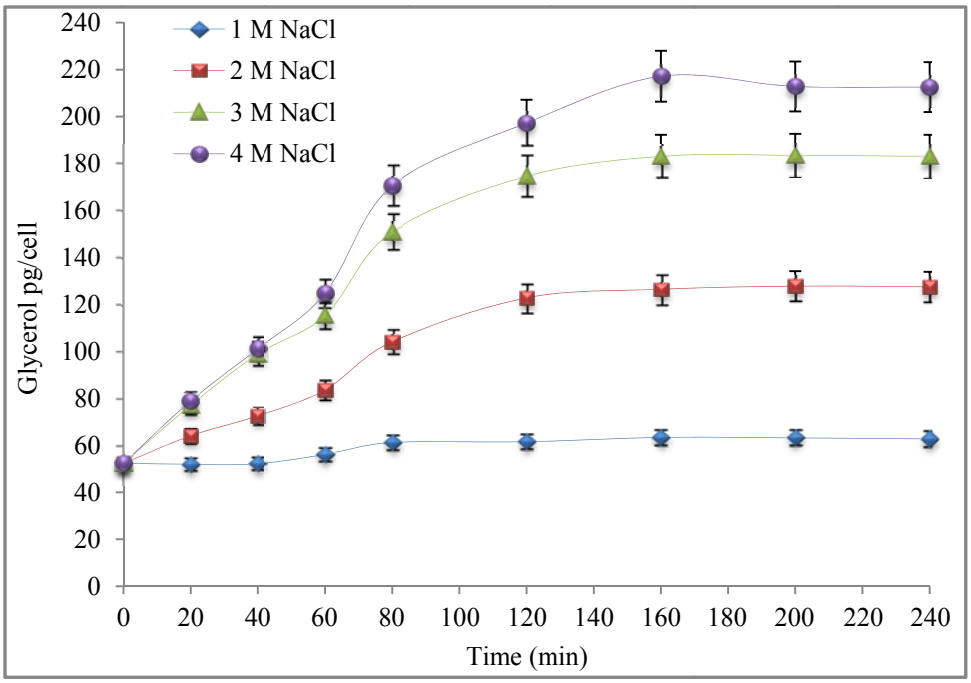


Fig. 6-31 The effect of increasing the NaCl concentration on the glycerol content of *Dunaliella* T35 as function of time (20 to 240 minutes) under the light regime at 45 µmol m^{-2} s^{-1}. Error bars represent the standard deviations (n=3) of measured values of glycerol content

6.3.4 Effect of hyperosmotic stress, darkness and continuous illumination on starch and glycerol contents of *Dunaliella* T35

To understand the extent by which starch degradation contributed to glycerol production, cells of *Dunaliella* T35 cultured in 1.0 M NaCl at 12:12 light/dark were transferred to fresh media containing either 1.0 M NaCl or 4.0 M NaCl and maintained in either continuous light or dark regimes for 24 hours before measurement of the size of both starch and glycerol pools in harvested cells (Figure 6.32 and Figure 6.33). The results are also shown in Table 6.4, by assuming a value of 2 moles glycerol formed for each mole of starch, these figures can be expressed in moles of glycerol or glycerol equivalents / mg biomass.

Table 6-4 Effect of hyperosmotic shock on starch conversion to glycerol in *Dunaliella* T35 under continuous light and dark regime

Treatment	µg starch /mg biomass*	Moles starch /mg biomass	Moles glycerol equival.	µg glycerol /mg biomass	Moles glycerol	Total moles glycerol + glycerol equivalent
1.0 M NaCl no treatment	217	1.205	2.41	174	1.89	4.30
1.0 M NaCl, 24h light	222	1.23	2.47	174	1.89	4.36
1.0 M NaCl 24h dark	45	0.25	0.50	163	1.77	2.27
4.0 M NaCl 24h light	146	0.81	1.60	706	7.67	9.27
4.0 M NaCl 24h dark	27	0.15	0.30	592	6.43	6.73

*Measurements made on the same number of cells for each treatment and corrected to mg biomass.

From inspection of table 6.4, it is apparent that in the dark after 24 h incubation in 1.0 M NaCl, ~1.92 moles glycerol equivalents of starch were consumed. This is attributed to respiratory activity to maintain cellular turnover and energy balance in the dark. Some negligible amount of glycerol (0.12 moles) also disappeared, which may reflect draining of energy reserves including glycerol, without the capacity to replenish them by heterotrophic metabolism under extended applied conditions of darkness, which would be consistent with

an obligate autotrophic mode of metabolism typical of *Dunaliella* species. Equally this falls within the limits of error (~6 %). In 4.0 M NaCl in the dark, however, the amount of glycerol increased to reach 6.43 moles. Since the starch pool decreased in size only by 2.11 moles glycerol equivalents and allowing for 1.92 moles respiratory activity the data suggest recruitment of glycerol from other sources, possibly the breakdown of lipids. In the light in 4.0 M NaCl the amount of glycerol increased from that at 1.0 M NaCl to reach 7.67 moles; however the pool size of starch also decreased by 0.8 mole equivalents of glycerol, suggesting that mechanisms of glycerol synthesis involving both photosynthesis and respiration were recruited to counter the extreme level of salinity.

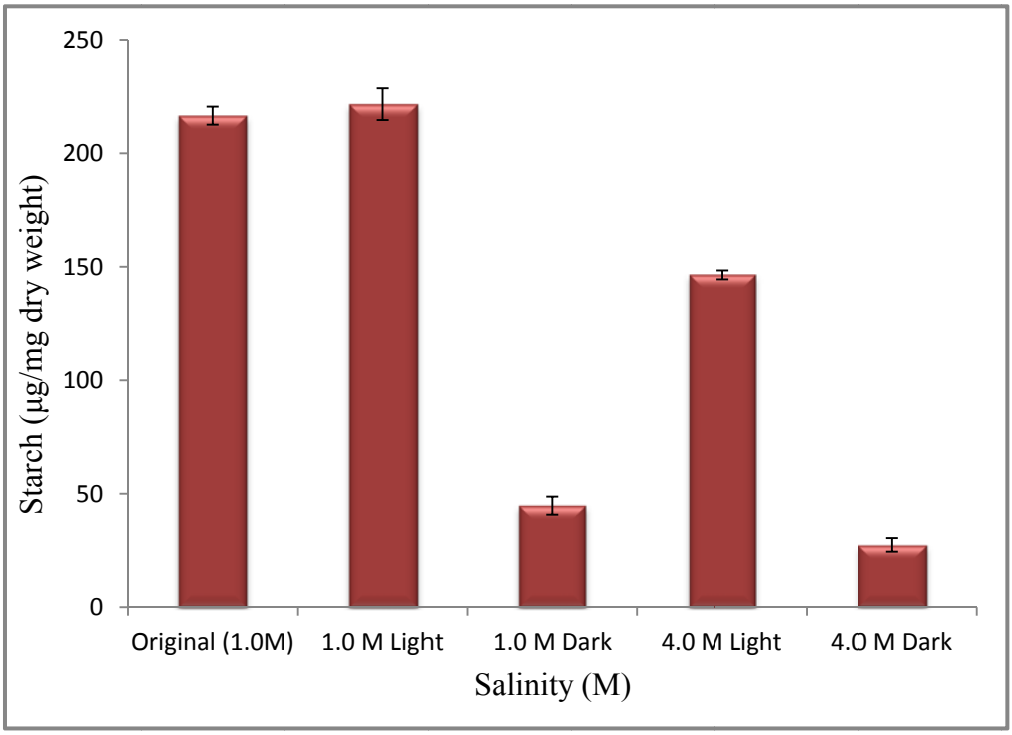


Fig. 6-32 The effect of increasing the NaCl concentration on starch content of *Dunaliella* T35 under a dark and light regime. Error bars represent the standard deviations (n=3) of measured values of starch content.

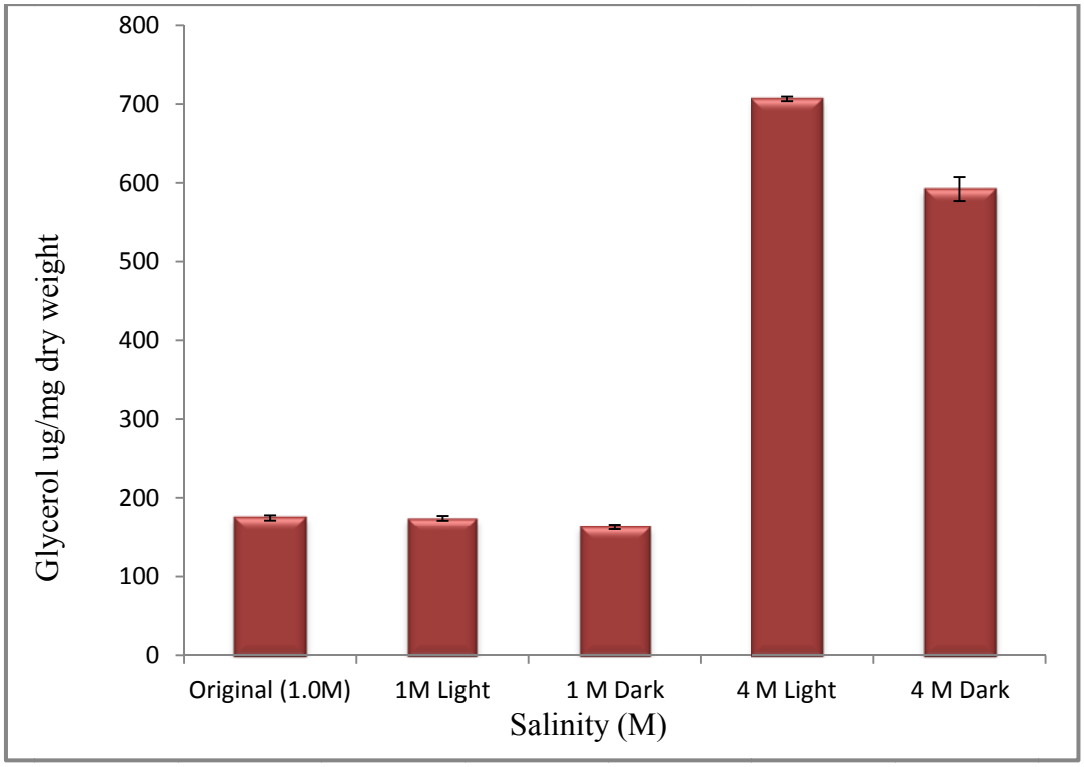


Fig. 6-33 Effect of osmotic shock on glycerol accumulation under both light and dark regime. Each point represents the mean of three replicate experiments. Error bars represent the standard deviations (n=3) of measured values of glycerol content.

6.3.5 Comparison for glycerol production between *Dunaliella* (T35, T36 and T37) with other microalgal species

Dunaliella T35, T36 and T37 show statistically significant greater amounts of glycerol produced per cell at all NaCl concentrations tested in comparison with the *D. salina, D. quartolecta*, *D. parva* and *D. polymorpha* grown under the same laboratory conditions (Table 6.5).

The largest glycerol content (234.9 pg/cell) recorded in *Dunaliella* T37 grown in 4.0 M NaCl was 3.0 to 4.6 fold more than cultures (*D. salina, D. quartolecta*, *D. parva* and *D. polymorpha*) grown under the same salinity concentration (Table 6.5). The amount of glycerol produced by *Dunaliella* T35 in 4.0 M NaCl concentration (212.6 pg/cell) was 2.7 to 4.2 fold high when compared to *D. salina, D. quartolecta*, *D. parva* and *D. polymorpha* species grown under the same salinity concentration (see Table 6.5).

Table 6-5 Mean glycerol accumulation of *Dunaliella* (T35, T36 and T37) and CCAP cultures (*D. salina, D. quartolecta*, *D. parva* and *D. polymorpha*) grown at various salinities after 28 days cultivation at 23 ^{o}C, initial pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod

Strain	**Glycerol mean pg/cell**			
	1.0 M NaCl	2.0 M NaCl	3.0 M NaCl	4.0 M NaCl
Dunaliella T35	92.2	133.8	203.2	200.5
Dunaliella T36	46.1	89.1	177.0	167.2
Dunaliella T37	55.9	135.1	221.4	234.9
*D. salina**	10.2	31.8	57.0	79.5
*D. quartolecta**	10.7	17.5	52.0	52.7
*D. parva**	9.0	20.0	37.0	55.5
*D. polymorpha**	11.8	23.9	45.2	50.6

*Data courtesy of Rekha Swamy

Table 6.6 shows the effect on glycerol production by the three Namibian strains of *Dunaliella* for 240 min hyperosmotic shock compared with *D. salina* and *D. polymorpha*. Increase in the NaCl concentration of the medium from 1.0 to 2.0, 3.0 and 4.0 M causes an increase in the glycerol during 240 min (T35) and 360 min (*D. salina* and *D. polymorpha*) after the salinity was increased.

Dunaliella T35 produced the greater amount (pg/cell) at 4.0 M NaCl whereas *D. salina* and *D. polymorpha* produced greater amount at 3.0 M NaCl medium. The amount of glycerol (183.1 pg/cell) produced by *Dunaliella* T35 in 3.0 M NaCl after 240 min was 2.3 and 3.4 fold greater than the amount of glycerol (79.1 and 54.0 pg/cell) produced by *D. salina* and *D. polymorpha*, respectively, at 360 min in 3.0 M NaCl concentration. The amount of glycerol (212.6 pg/cell) produced by *Dunaliella* T35 in 4.0 M NaCl after 240 min was 3.3 and 5.2 fold greater than the amount of glycerol by *D. salina* and *D. polymorpha*, respectively.

Table 6-6 Mean glycerol content of cell *Dunaliella* T35, *D. salina* and *D. polymorpha* cultured in 1.0 M NaCl and treated by hyperosmotic shock under continuous illumination. Cells were transferred to fresh media of increased salt and glycerol content recorded after 240 min for *Dunaliella* T36 and 360 min for *D. salina* and *D. polymorpha*, respectively.

***Dunaliella* Strains**	**Time of salinity exposure (min)**	**Glycerol mean pg/cell**			
		1.0 M NaCl	2.0 M NaCl	3.0 M NaCl	4.0 M NaCl
Dunaliella T35	240	62.8	127.5	183.1	212.6
*D. salina**	360	22.7	ENC	79.1	64.3
*D. polymorpha**	360	19.4	ENC	54.0	40.6

ENC = Experiment Not Conducted, *Data courtesy of Rekha Swamy

6.3.6 Summary of glycerol production for strains *Dunaliella* and *Asteromonas* at different conditions

Table 6.7 presents summary of glycerol and cell density. There is no statistically significant difference in the amount of glycerol accumulated per cell between the cells exposed to 28 days and 240 min hyperosmotic shocks for all the strains of *Dunaliella* and *Asteromonas*. But the amount of glycerol per ml at 3.0 and 4.0 M NaCl in 240 min hyperosmotic shock was about 2 to 3 fold compared to 28 days cultures; this is because of high cell density of the hyperosmotic treatment. Table 6.8 shows the percentage of the dry matter of glycerol under the best conditions of glycerol production (3.0 and 4.0 M NaCl) for *Dunaliella* and *Asteromonas* strains.

Table 6-7 Mean glycerol content of *Dunaliella* and *Asteromonas* cultured in 1.0, 2.0, 3.0 and 4.0 M NaCl medium for 28 days and cultured in 1.0 M NaCl and treated by hyperosmotic shock; transferred to fresh media of 1.0, 2.0, 3.0 and 4.0 M NaCl and glycerol content recorded after 160 min.

Strain	**Salinity (M)**	**Cultivation under continuous osmotic stress (28 days cultivation)**				**Hyperosmotic shock (160 min)**			
		Glycerol (µg/ml)	% increase from 1.0 M	Glycerol (pg/cell)	Number of cells x 10^6/ml	Glycerol (µg/ml)	% increase from 1.0 M	Glycerol (pg/cell)	Number of cells x 10^6/ml
T35	1.0	261.5	-	65.2	2.87	185.4	-	64.6	2.87
	2.0	273.6	4	133.8	2.04	394.6	53	137.5	2.87
	3.0	315.3	17	203.2	1.56	596.1	69	207.7	2.87
	4.0	305.3	14	200.5	1.52	640.6	71	223.2	2.87
T36	1.0	209.7	-	46.1	4.55	197.5	-	43.4	4.55
	2.0	265.2	21	89.6	2.96	396.3	50	87.1	4.55
	3.0	380.1	45	177.0	2.15	678.2	71	171.7	4.55
	4.0	261.1	20	167.4	1.56	816.7	76	179.5	4.55
T37	1.0	148.1	-	55.9	2.65	151.8	-	57.3	2.65
	2.0	249.0	41	135.3	1.84	362.3	58	136.7	2.65
	3.0	321.1	54	221.4	1.45	588.6	74	222.1	2.65
	4.0	243.3	39	234.9	1.04	629.6	76	237.6	2.65
T33a	1.0	125.3	-	53.8	2.33	136.1	-	58.4	2.33
	2.0	141.1	11	103.5	1.36	241.2	44	105.9	2.33
	3.0	75.6	-40	122.6	0.62	285.7	52	136.5	2.33
	4.0	28.6	-77	126.3	0.22	294.3	54	133.1	2.33
T33b	1.0	191.1		64.8	2.95	201.8	-	68.4	2.95
	2.0	133.4	-30	101.3	1.32	318.6	37	108.0	2.95
	3.0	41.7	-78	138.0	0.30	423.9	52	143.7	2.95
	4.0	41.1	-78	121.0	0.34	379.7	47	128.7	2.95
T33c	1.0	187.0		67.1	2.79	194.7	-	69.8	2.79
	2.0	135.0	-28	115.2	1.17	328.6	41	117.8	2.79
	3.0	70.9	-62	184.9	0.38	467.8	58	167.7	2.79
	4.0	47.5	-75	209.6	0.23	513.9	62	184.2	2.79

Table 6-8 Percentage amount of glycerol in dry weight basis of *Dunaliella* and *Asteromonas* strains after 14 days of cultivation and following 160 min transfer to hyperosmotic shock.

Microalgae	14 days cultivation in 1.0 M NaCl followed by 160 min exposure to 3.0 M NaCl (% glycerol of the dry matter)	14 days cultivation in 1.0 M NaCl followed by 160 min exposure to 4.0 M NaCl (% glycerol of the dry matter)
Dunaliella T35	75	80
Dunaliella T36	58	70
Dunaliella T37	74	79
Asteromonas T33a	36	37
Asteromonas T33b	53	47
Asteromonas T33c	58	68

6.4 Discussion

When halophytic microalgal cells are subjected to hyper or hypo-osmotic shock their volume undergoes short-term changes (Borowitzka and Siva, 2007). The general cell organization has been studied in most detail in *D. salina* (Borowitzka and Siva, 2007) and A. *gracilis* (Peterfi and Manton, 1968) both with the light microscope and the electron microscope.

Dunaliella T36 produced largest amount of glycerol per ml of the culture medium, i.e. 380.1 µg/ml, after 28 days of cultivation at 3.0 M NaCl. The amount of glycerol increased to 816.7 µg/ml when the cell cultivated in 1.0 M NaCl was transferred to medium containing 4.0 M NaCl for 240 min. For all the strains investigated transfer of cultures from 1.0 M NaCl medium to higher salinity for 240 min causes an increase in glycerol per ml of cultures. But there was no different in the amount of glycerol per cell.

The linear relationship from 1.0 to 3.0 M NaCl (section 6.3.2) of intracellular glycerol to the external salinity suggests glycerol is produced by *Dunaliella*, Namibian strains, to maintain the osmotic balance within the cell. Our findings show similarity in glycerol accumulation between all the three *Dunaliella* strains and *Asteromonas* T33c, which are similar with the findings of Ben-Amotz and Avron (1980) who reported that the amount of glycerol per cell in

A. gracilis, as in various species of *Dunaliella*. Ben-Amotz and Grunwald (1981) concluded that the species of *Dunaliella* and *A. gracilis* of different volumes reached similar values of glycerol to chlorophyll ratios, which are linearly related to external salt concentration.

The results described in section 6.3.2 for *Dunaliella* T35 agreed with the published work by Ben-Amotz and Avron (1973) using *D. parva* and Brown and Borowitzka (1979) using *D. tertiolecta* and *D. viridis* both reported that the highest intracellular glycerol level is reached within 90 min of the increase in salinity. However, the results shown in section 6.3.3 for *Dunaliella* T35 indicates that glycerol synthesis reach it maximum for about 160 to 240 min after hyperosmotic shock. The commencement of glycerol biosynthesis in response to an increase in osmotic stress is rapid, since at the 20 minutes of osmotic shock there is an evidence of glycerol production. The result presented in section 6.3.3 shown that at t = 0 *Dunaliella* T35 have 52.36 pg/cell glycerol in 4.0 M NaCl medium and after 20 min exposure to this saline medium the level of glycerol rises to 78.93 pg/cell. This suggests that glycerol accumulation begins in less than 20 min when osmotic stresses have being applied to *Dunaliella* cells. This immediate stimulation of glycerol production by osmotic stress was also found by Borowitzka *et al*. (1977) and Brown and Borowitzka (1979).

Dunaliella species accumulated different amount of glycerol at different saline medium. Phadwal and Singh (2003) showed that *D. salina* and other *Dunaliella* species (isolate investigated) accumulated glycerol at levels of 15.56 pg/cell and 23.24 pg/cell, respectively, in artificial sea water (ASW) media at 2.0 M NaCl concentration. Chen *et al*., (2011) reported the mean cell glycerol content of 87.84 % by *D. salina* at 5.0 M NaCl concentration whereas 94.26 pg/cell of glycerol were discovered from *Dunaliella* species obtained from hypersaline river of India (Phadwal and Singh, 2003). In this study, glycerol content of both *Dunaliella* and *Asteromonas* species were greater than the ones reported above, but is within the ranges of 50 and 400 pg/cell reported by Ben-Amotz and Grunwald (1981) for algae grown at 0.5 and 4.5 M NaCl, respectively.

The presented results in section 6.3.2 have revealed that during the 40 to 160 min series of osmotic shock, glycerol production is increased. In this case (at 160 minutes osmotic shock) a maximum value for the glycerol content has been approached Figure 6.8 and 6.9. This shows

that *Dunaliella* grown under optimum salinity could be subjected to higher saline medium (near saturation) for glycerol accumulation.

The formation of glycerol in response to osmotic shock by *Dunaliella* is independent of light period; as evident by figure 6.9 which shows that glycerol could be produced by *Dunaliella* cell in both light and dark regime following osmotic shock. The result obtained section 6.3.3 proved that glycerol accumulation by *Dunaliella* in response to hyperosmotic stress took place in the presence of the light or in the dark. Glycerol production by *Dunaliella* in the light and the dark regimes was also observed (Ben-Amotz, 1975 and Borowitzka *et al*., 1977).

Hyperosmotic shock in *Dunaliella* T35 induced an increase in glycerol content and a parallel increase in starch content in the light regime and decrease in starch content in the dark regime, indicating metabolic conversion of glycerol to starch. This proposed that *Dunaliella* cells utilize an active inter-conversion between starch and glycerol, and photosynthesis, to meet the osmotic balances which are set by the external salinity stress (Avron and Ben-Amotz, 1992).

According to Goyal (2007) at the highest salt concentration, contribution of photosynthesis in glycerol synthesis was almost completely inhibited, showing that glycerol was synthesized almost exclusively from the products of starch breakdown. The present study shows that on exposure to 4.0 M NaCl concentration *Dunaliella* T35 responded by increasing glycerol synthesis but slower rate of the starch formation in the light regime, the cells exposed to 1.0 M NaCl produced more starch and less glycerol in comparison to those in 4.0 M NaCl medium under a light regime. Under parallel conditions in darkness, starch degradation is increased. The greater the osmotic stress of the growth medium the higher is the rate of glycerol synthesis, whereas starch content and salinity of the medium are inversely related (section 6.3.4). Thus, the contribution of starch breakdown to glycerol synthesis increased progressively with increasing salt stress (Goyal, 2007).

Dunaliella and *Asteromonas* undergo rapid volume change and they slowly re-adjust for high salinity. *Dunaliella* strains undergoes 20 to 25 % decrease in cell volume when transferred from 1.0 M to 4.0 M NaCl after 14 days salinity stress whereas in 20 min salinity stress their cell volume decreased by 44 to 62 %. *Asteromonas* cell volume also decreases under both long and short-term salinity stress. There were 24 to 44 % decrease in cell volume for the

cells exposed to osmotic shock for 14 days and 35 to 55 % decrease in cell volume after 20 min exposure when transferred from 1.0 to 4.0 M NaCl. Similar findings were reported by Chen and Jiang (2009) who also showed that the volume of *Dunaliella* cells was reduced in cultures of higher salinities (4.0-5.0 M NaCl) compared to lower salinity (0.5 M NaCl). The results show that higher salinities have adverse effect on the growth of *D. salina*.

According to Maeda and Thompson (1986) *Dunaliella* when exposed to either hyper or hypo-osmotic stress undergo a change in cell volume within 2-4 min (Maeda and Thompson, 1986). Another study shows that when *Dunaliella* cells are exposed to a hyperosmotic shock they rapidly shrink due to water efflux from the cell and then slowly return to their original volume by accumulating intracellular glycerol after 2 to 3 h (Katz and Avron, 1985; Chen and Jiang, 2009). When *Dunaliella* and *Asteromonas* were exposed to a hyperosmotic shock the cellular volume decreased and vice versa. Due to the lack of a cell-wall in the cell, it is expected to respond osmotically as a perfect osmometer, i.e. the cell osmotic volume should be directly proportional to the inverse of the osmotic pressure under nonmetabolic conditions (Katz and Avron, 1985). This change of *D. salina* cell volume is associated with osmoregulation and glycerol synthesis (Avron and Ben-Amotz, (1992). According to Ben-Amotz and Grunwald (1981) under hyperosmotic conditions, the *Asteromonas* cells produce and accumulate intracellular glycerol until the new equilibrium is achieved.

Dunaliella strains (T35, T36 and T37) exposed to higher salinity from 1.0 M to 4.0 M NaCl concentration showed a decrease in cell volume and changing from green to pale green in colour. Microscopic observations showed that *Asteromonas* are similar to *Dunaliella* cells; they behave like osmometers and rapidly swell or shrink under hypoosmotic or hyperosmotic conditions, respectively. But *Asteromonas* possess a ridging as a distinguishing feature from *Dunaliella*. *Dunaliella* cells are exposed to a hyperosmotic shock they rapidly shrink due to water efflux from the cell and then slowly return to their original volume by accumulating intracellular glycerol (Ben-Amotz and Avron, 1983). This is true for *Dunaliella* and *Asteromonas*, Namibian strains, only at 1.0 and 2.0 M NaCl concentration but cells that were exposed to 3.0 and 4.0 M NaCl did not resume the original volume after both 20 min and 14 days. Chen and Jiang (2009) reported that *Dunaliella* might survive and grow at high salinity which could lead to moderate dehydration of cells and make their shape slender.

This study shows that *Dunaliella* and *Asteromonas* cells undergo changes in cell volume at different NaCl concentration. Under the conditions employed in this study that cell volume is 184 μm^3 for *Dunaliella* T35, 186 μm^3 for *Dunaliella* T36 and 202 μm^3 for *Dunaliella* T37 at 1.0 M NaCl (optimum salinity for growth). A mean cellular volume of *Dunaliella* and *Asteromonas* recorded coincides with what was reported by other studies (Jimenez and Niell, 1991). The changes in cell volume following osmotic changes were also observed in *D*. salina by Avron and Ben-Amotz (1992) who report that *D*. *viridis* cell volume to be below 200 μm^3, which is within the volume range for the strain isolated from Namibia. *Dunaliella* and *Asteromonas*, Namibian strains, responded differently to *D*. *salina* reported by Garcia *et al*. (2007) which shows that the volume in *D*. *salina* increased linearly from 524 to more than 2000 μm^3 in response to increasing NaCl concentrations. But the cell volumes in *D*. *viridis* of 114 to 220 μm^3 are similar to the volume determined from both *Dunaliella* and *Asteromonas* strains used in this study.

6.5 Conclusion

The results of the experiments conducted showed that the amount of glycerol produced by *Dunaliella* and *Asteromonas* as a function of cell density/biomass greatly increased with an increase in NaCl concentration. *Dunaliella* strains produced more glycerol in comparison with *Asteromonas* strains under the same growth condition. This study also confirms that the amount of glycerol produced by *Dunaliella* isolated from Namibia supersedes the amount detected from other species (*D*. *salina, D*. *quartolecta*, *D*. *parva* and *D*. p*olymorpha*). The results suggest that halotolerant microalgae accumulate glycerol in response to an increase in the NaCl concentration of growth medium; strains respond in different way. Following hyperosmotic shock the amount of glycerol produced by *Dunaliella* in light during photosynthesis alone cannot support the rate of glycerol synthesis. During the first 60 min of exposure to high saline medium, glycerol production was linear, which suggests that the production of glycerol by *Dunaliella* depends initially on the degradation of starch. The rapid increase in glycerol production from 60 to 80 min appears to be due to a contribution of both the starch breakdown and the photosynthesis. This data suggested that both in the light and in the dark, the precursor for glycerol synthesis during hyperosmotic stress is derived from starch breakdown. With regards to the precursor of carbon for the *de novo* biosynthesis of

glycerol under hyperosmotic shock, it is logical to conclude that *Dunaliella* T35 can be adapted to higher osmotic stress in the dark, only if the cells contain sufficient amount of starch to provide carbon source for glycerol biosynthesis.

The result of this study also show that microalgal cell transferred from the medium of optimum salinity for growth (1.0 M NaCl) to higher salinity (3.0 to 4.0) rapidly reduced their volume and when transferred to lower growth medium (0.5 M NaCl) they swelled within 20 min. This change in cell volume upon exposure to different salinity concentration is correlated with accumulation or dissimilation of glycerol, the most important osmotically active intracellular solute in halotolerant microalgae.

The largest amount of glycerol was obtained at 4.0 M NaCl in most of the strains. In order to achieves both high cell density and high amount of glycerol. It is therefore, recommended to first grow these halophytic microalgae in 1.0 M NaCl for maximum cell density and then transfer them for 160 min to higher salinity (4.0 M) to maximize glycerol accumulation.

CHAPTER 7.0: EFFECT OF SALINITY ON ELEMENTAL COMPOSITION OF *DUNALIELLA* AND *ASTEROMONAS* SPECIES

7.1 Introduction

SEM-EDX analysis is a powerful technique for elemental analysis of microalgae samples. This technique is rapid and requires little or no sample preparation, which allows the simultaneous determination of all elements (Tazaki *et al*., 1994; Mendoza *et al*., 1999; Kleinubing *et al*., 2010). With the morphological characters obtained from SEM, supported by EDX micro analysis, it is possible to identify elements such as Na, Mg, Al, P, S, Si, Cl, K, Ca, Mn, Fe, Cr, Co, Ni, Cu, and Zn in algae samples. In the present research, SEM-EDX is used to determine the elemental composition *Dunaliella* and *Asteromonas* species.

This study will focus on nitrogen and carbon. Nitrogen is primarily found in proteins, a change in the cells relative amount of protein could reflect in the nitrogen amount. Elements such as carbon content are complex to explain, because it occurs in proteins, carbohydrates and lipids, but it can be used for biomass estimation. It was shown that a number of proteins are characteristic for low NaCl concentration; their synthesis is stopped with enhancing osmotic pressure. Other proteins are only induced in cells adapted to high salinity (Golldack *et al*., 1995). The aim of this study is to investigate the effects of a sudden hyperosmotic stress on cell protein (by using nitrogen estimation) and biomass (carbon estimation) using EDX-analysis. Changes in specific growth and doubling time of *Dunaliella* and *Asteromonas* at different salinity have been described in chapter 4.0. In addition to growth rate and doubling time, changes in the elemental composition of these microalgae may be used as an indicator of metabolic adjustments which could be integrated in understanding the response of *Dunaliella* and *Asteromonas* after short term hyperosmotic shock.

7.2 Method

7.2.1 EDX analysis

Microalgae cells grown in 1.0 M NaCl were transferred to fresh medium that contained 1.0 and 4.0 M NaCl for 240 min. The cells were harvested by centrifugation at 3000 g, the resulting pellet was placed at 40 ^{o}C for 10 hr to remove moisture. After drying, the samples were observed using the Hitachi FEG-SEM scanning electron microscope (10 kV) under vacuum. Samples were analysed without any chemical pre-treatment. A total of 26 samples were analysed, corresponding to a minimum of three replicates per sample, to reduce the risk of analytical error.

7.3 Results

7.3.1 Elemental composition of *Dunaliella*

The SEM photographs and corresponding EDX spectra were taken for all three *Dunaliella* strains and one typical micrograph and spectrum of *Dunaliella* T36 are shown for 1.0 and 4.0 M NaCl, respectively, in Figure 7.1 and 7.2. The atom percentage of elements present in all sample locations with percentage atoms for *Dunaliella* strains obtained from EDX are given in Table 7.1. The major elements detected in these microalgae are C, N, O, and other elements detected were Na, Mg, Al, Si, P, S, Cl, K and Ca.

Elements such as (N and O) show very similar patterns; their atom percent decreased when the cells were transferred from 1.0 to 4.0 M NaCl concentration in all the three *Dunaliella* strains analysed (Table 7.1), suggesting loss of protein from the external surface (EDX penetration is 1.0 μm into the cell). Carbon is the major element for the algae biomass, its presence in stressed and non-stressed microalgae is determined by physiological aspects, percentage C composition is constantly higher in the cells exposed to 4.0 M NaCl than those in 1.0 M NaCl, and this difference was observed in all the three strains investigated, consistent with loss of protein from the cells. Similar pattern was observed with Na, Cl and

Ca ions, consistent with binding of NaCl from the external medium. The Si was only detectable in T35 exposed to both 1.0 and 4.0 M NaCl and T36 exposed to 4.0 M NaCl only. In the case of Mg, Al, P, and S there appears to be no change in relative %, and accumulation is similar in all the three strains at 1.0 M and 4.0 M NaCl concentrations.

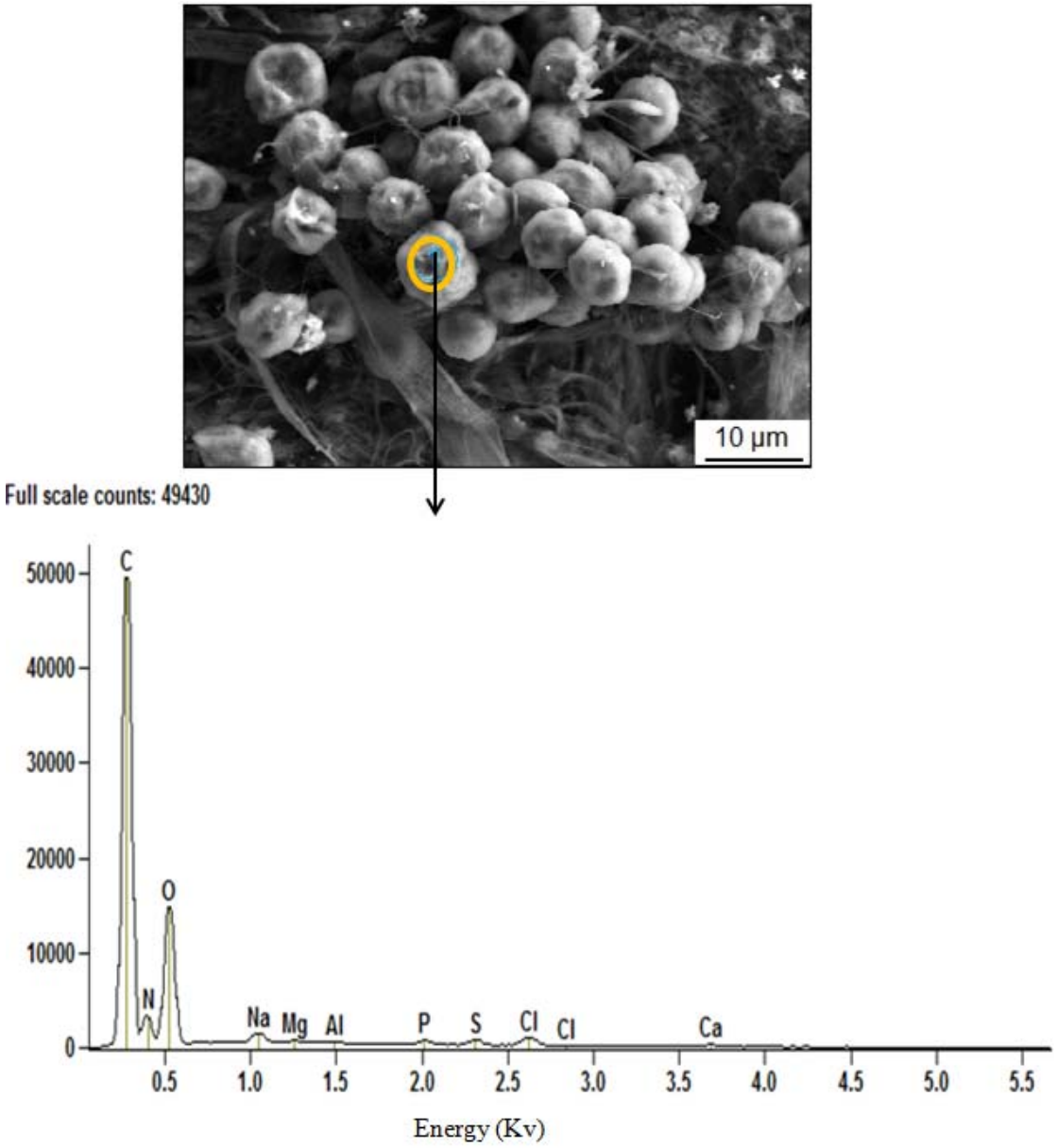


Fig. 7-1 Scanning electron microscopy (SEM) micrographs of *Dunaliella* T36 grown in 1.0 M NaCl concentration and energy dispersive X-rays spectroscopy (EDX) in the region of spectra of the SEM

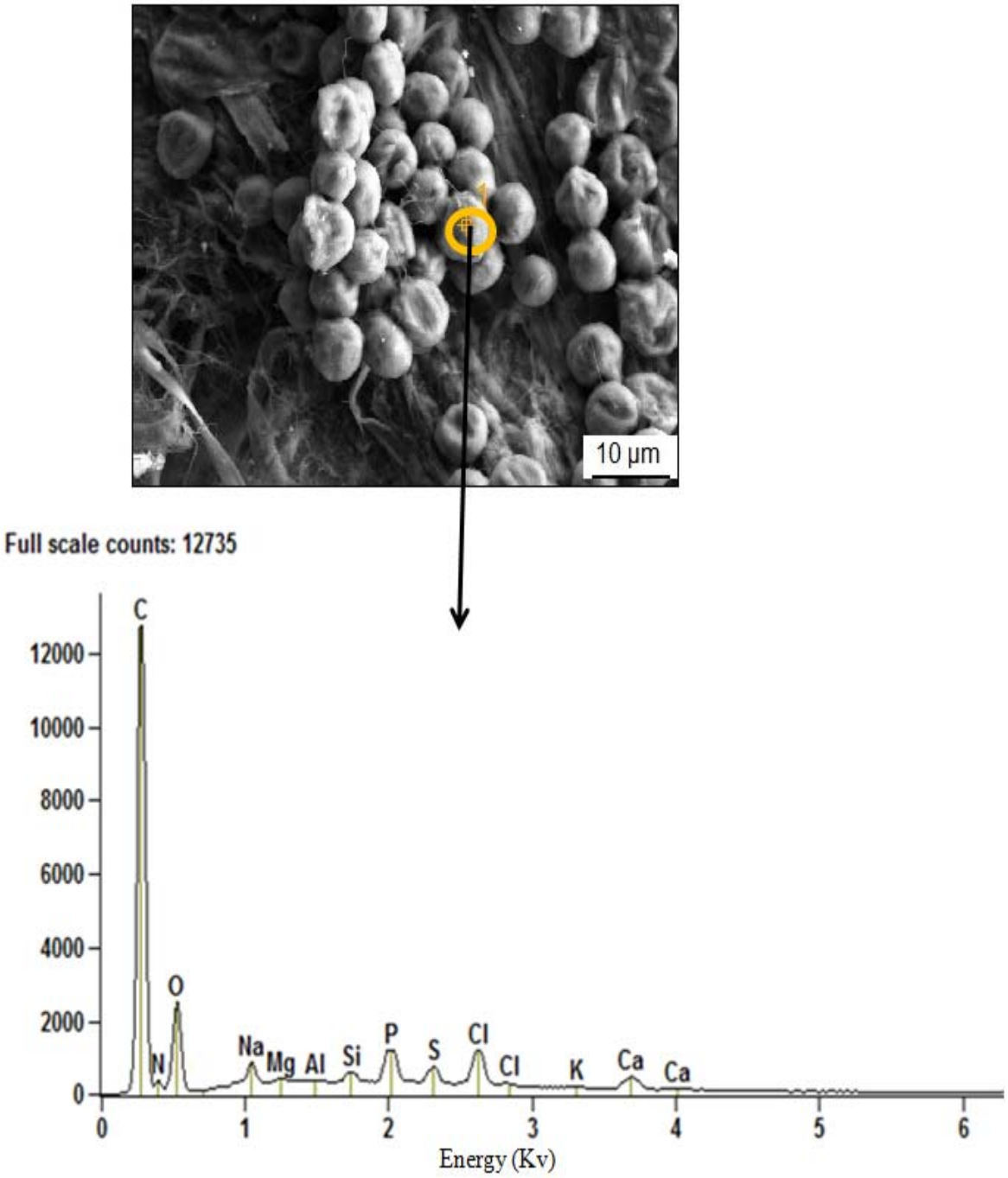


Fig. 7-2 Scanning electron microscopy (SEM) micrographs of *Dunaliella* T36 exposed to 4.0 M NaCl concentration and energy dispersive X-rays spectroscopy (EDX) in the region of spectra of the SEM

Table 7-1 Elemental composition of *Dunaliella* T35, T36 and T37 exposed to 1.0 and 4.0 M NaCl concentrations for 240 min at pH 7.5, 25 ^{o}C and 45 µmol m^{-2} s^{-1} light.

Element	**Composition (Atom %)**								
	***Dunaliella* T35**			***Dunaliella* T36**			***Dunaliella* T37**		
	1.0 M	**4.0 M**	**% Ch.**	**1.0 M**	**4.0 M**	**% Ch.**	**1.0 M**	**4.0 M**	**% Ch.**
C	70.15	77.86	9.9	59.31	73.08	18.8	63.77	72.75	12.3
N	19.03	13.0	-31.7	17.42	9.05	-48.8	13.55	9.14	-32.5
O	9.71	7.51	-22.7	21.45	11.98	-44.1	20.04	14.82	-26.0
Na	0.42	0.58	27.6	0.53	0.86	38.4	1.0	1.22	18.0
Mg	0.07	0.1	30	0.07	0.15	53.3	0.04	0.09	55.6
Al	0.05	0.09	44	0.04	0.13	69.2	0.06	0.07	14.3
Si	0.07	0.03	-57.1	ND	0.34	-	ND	ND	-
P	0.2	0.25	20	0.18	1.38	86.9	0.1	0.22	54.5
S	0.1	0.17	41.2	0.29	0.78	62.8	0.2	0.31	35.5
Cl	0.1	0.26	61.5	0.53	1.9	72	1.06	1.24	14.5
K	ND	ND	-	ND	0.1	-	0.08	ND	-
Ca	0.1	0.15	33.3	0.18	0.25	28	0.1	0.14	28.6
	------	-------		--------	--------		---------	--------	
Total	100	100		100	100		100	100	

ND = Not Detected; % Ch. = percent change from 1.0 to 4.0 M NaCl medium

7.3.2 Elemental composition of *Asteromonas*

Figure 7.3 and 7.4 show the EDX spectrum of *Asteromonas* T33b grown at 1.0 and 4.0 M NaCl concentration. The results in Table 7.2 show the mean values for 12 elements of *Asteromonas* spp. grown at different salinities. Table 7.2 shows that higher percentage values for C, N and O found in the three strains of *Asteromonas* T33b, at 1.0 and 4.0 M NaCl concentration. The percentage C found in three different strains of *Asteromonas* is not statistically significant.

There was a decrease in N and O composition from the cell exposed to 4.0 M NaCl when compared with cells exposed to 1.0 M NaCl. There was also a decrease in S level in T33a and T33b when transferred from 1.0 to 4.0 M NaCl medium. S was not detected in T33c grown in

1.0 M NaCl. The percentage level of Mg, P, S and Ca increased with increasing NaCl concentration (from 1.0 to 4.0 M). Relatively high Na and Cl contents were determined in *Asteromonas strains* grown in 4.0 M NaCl in comparison with those cultures grown in 1.0 M NaCl concentration. Table 7.2 shows that K is detected only in *Asteromonas* T33c grown in 4.0 M NaCl. The distribution of Si in a number of samples was observed and the maximum value was obtained in T33c treated with 1.0 M NaCl concentration.

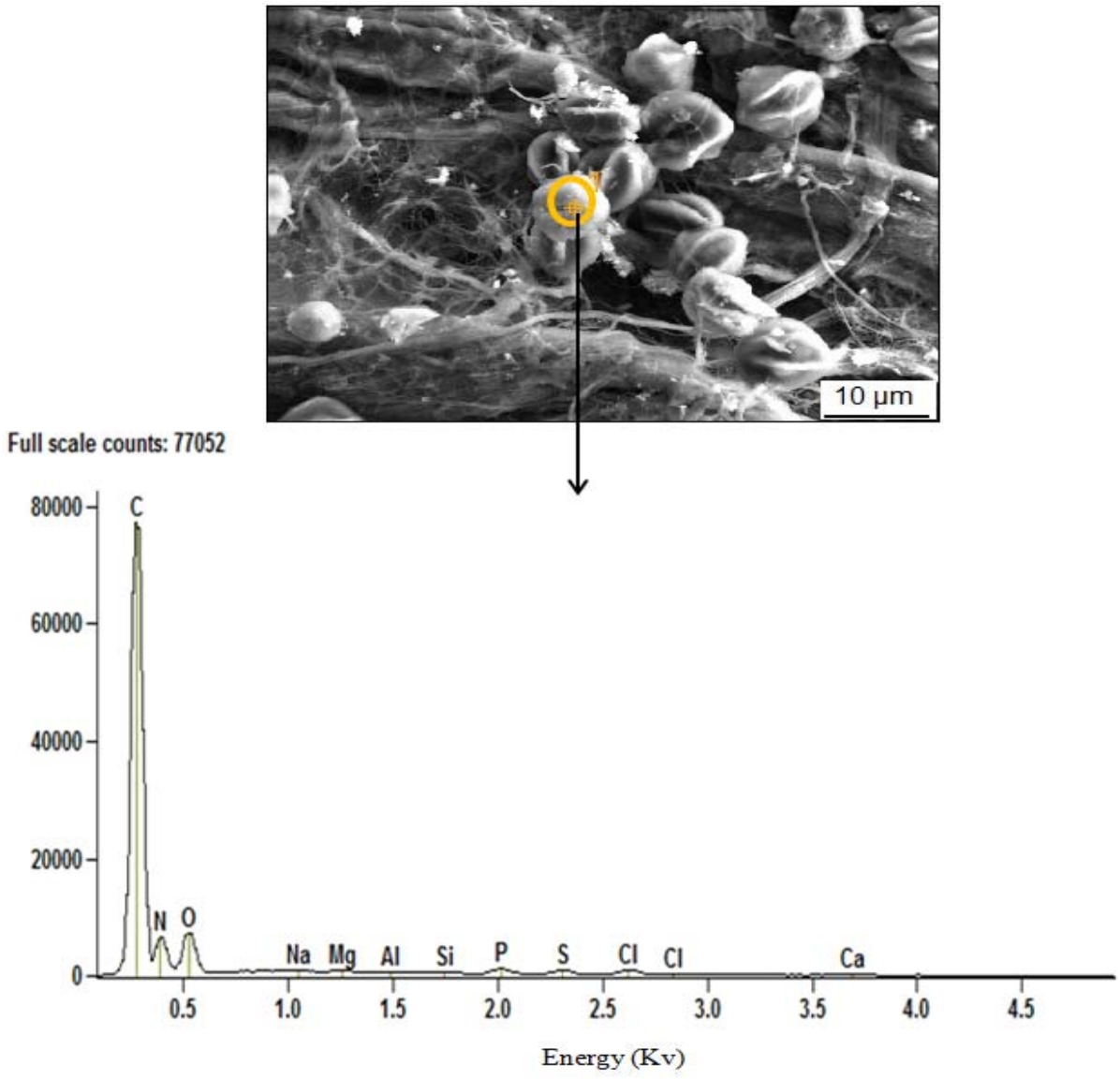


Fig. 7-3 Scanning electron microscopy (SEM) micrographs of *Asteromonas* T33b exposed to 4.0 M NaCl concentration and energy dispersive X-rays spectroscopy (EDX) in the region of spectra of the SEM

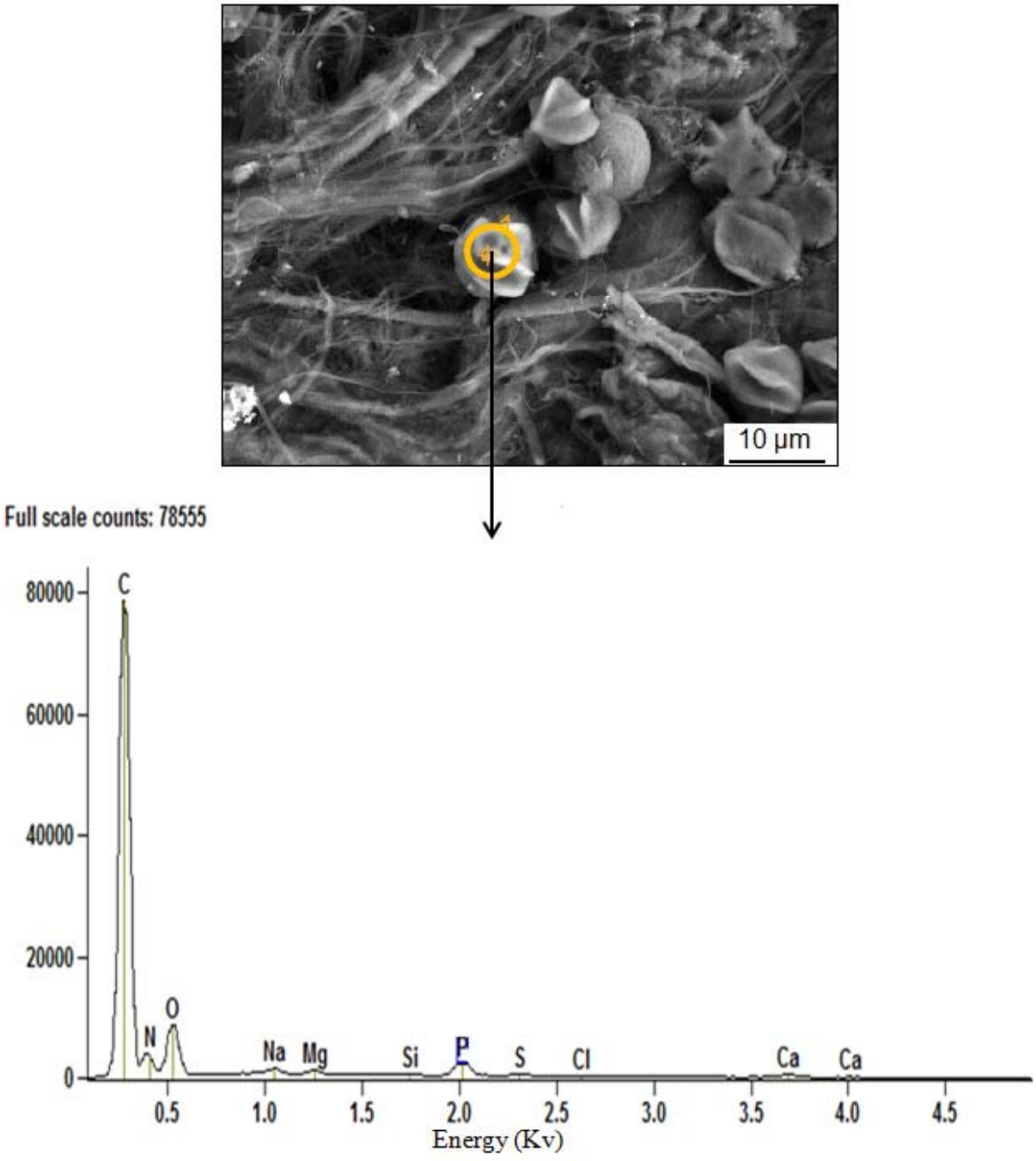


Fig. 7-4 Scanning electron microscopy (SEM) micrographs of *Asteromonas* T33b exposed to 4.0 M NaCl concentration and energy dispersive X-rays spectroscopy (EDX) in the region of spectra of the SEM

Table 7-2 Elemental composition of *Asteromonas* T33a, T33b and T33c exposed to 1.0 and 4.0 M NaCl concentrations for 240 min at pH 7.5, 25 ^{o}C and 45 µmol m^{-2} s^{-1} light.

Element	**Composition (Atom %)**								
	***Asteromonas* T33a**			***Asteromonas* T33b**			***Asteromonas* T33c**		
	1.0 M	**4.0 M**	**%Ch.**	**1.0 M**	**4.0 M**	**% Ch.**	**1.0 M**	**4.0 M**	**% Ch.**
C	76.77	80.90	5.1	73.81	80.10	7.9	69.90	78.08	10.5
N	14.63	11.9	-18.7	15.87	10.74	-32.2	15.39	7.85	-49
O	5.6	3.31	-40.9	8.79	6.71	-23.7	12.53	10.17	-18.8
Na	0.39	0.59	33.9	0.1	0.25	60.0	0.47	1.15	59.1
Mg	0.04	0.05	20	0.08	0.12	33.3	0.11	0.15	26.7
Al	ND	0.05	-	0.04	0.08	50	0.04	0.07	42.8
Si	ND	0.08	-	0.08	ND	-	0.48	0.1	-79.1
P	0.47	0.61	23	0.32	0.55	41.6	0.37	0.63	41.3
S	0.86	0.51	-40.7	0.3	0.17	-43.3	ND	0.44	-
Cl	0.8	1.47	45.5	0.37	0.92	59.8	0.39	0.9	57.8
K	ND	ND	-	ND	ND	-	ND	0.04	-
Ca	0.44	0.53	16.98	0.24	0.36	33.3	0.32	0.42	23.8
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Total	100	100		100	100		100	100	

ND = Not Detected; %Ch. = percent change from 1.0 to 4.0 M NaCl medium

7.3.3 Relationship between cell volume and carbon at different salinity

The values for microalgal cell carbon content as a function of cell volume in media of different salinities are shown in Table 7.3. There was variation in cell volume- carbon ratios in both *Dunaliella* and *Asteromonas* at different NaCl concentrations. The result shows that cells exposed to 4.0 M NaCl contained more carbon per unit volume than cells exposed to 1.0 M NaCl, respectively.

Table 7-3 The relationship between cell carbon and volume for halotolerant microalgae (*Dunaliella* and *Asteromonas*) strains. Cell volume presented here were obtained from chapter 6.0

Microalgae	Salinity (M)	Carbon content (weight %)	Cell volume (μm^3)	Carbon/Cell volume
Dunaliella T35	1.0	70.15	127	0.6
	4.0	77.86	96	0.8
Dunaliella T36	1.0	59.31	117	0.5
	4.0	73.08	94	0.8
Dunaliella T37	1.0	63.77	111	0.6
	4.0	72.75	83	0.9
Asteromonas T33a	1.0	76.77	123	0.6
	4.0	80.90	94	0.9
Asteromonas T33b	1.0	73.81	163	0.5
	4.0	80.10	92	0.9
Asteromonas T33c	1.0	69.90	119	0.6
	4.0	78.08	83	0.9

7.4 Discussion

This study was conducted to determine the elemental composition of *Dunaliella* (T35, T36, and T37) and *Asteromonas* (T33a, T33b and T33c) after short-term exposure to hyperosmotic stress.

The relationship between cell carbon and volume of halotolerant microalgae could be used to predict their biomass. For this reason, it is desirable to establish whether a predictable relationship between cell volume and cell carbon can be derived. No study has been conducted that has directly determined *Dunaliella* and *Asteromonas* cell carbon using EDX and related that to cell volume. This described morphological and physiological relationship of *Dunaliella* and *Asteromonas* strains after exposure to different salinity stress and also investigated the relationship between cells volume and relative abundance of carbon. It

appears that cell size is not a variable affecting the carbon content per unit volume in halotolerant microalgae. *Dunaliella* and *Asteromonas* cells exposed to high salinity (4.0 M NaCl) have smaller volume but accumulated more carbon than those exposed to low salinity 0.5 and 1.0 M NaCl concentrations.

The variation in the elements content of *Dunaliella* and *Asteromonas* species was observed, with a small difference in the microalgae of the same strains. The SEM-EDX, analyses reveal that carbon and nitrogen are the major elements occurring in the greatest concentrations in both *Dunaliella* and *Asteromonas* strains, Namibian isolates. This study shows variation in C: N ratio when microalgal cells were exposed to different salinity. High C:N ratio was determined in the cell inoculated at 4.0 M NaCl concentration when compared to those in 1.0 M NaCl concentration. *Dunaliella* and *Asteromonas* strains accumulates glycerol as an osmolyte 'compatible solute' responsible for the maintenance of the osmotic balance between the cell and the medium when salinity increases; this could cause the cell volume to decrease and the total cell carbon to increase. A study conducted by Ahmad and Hellebust, (1986) shows that when *Chlamydomonas pulsatilla (*euryhaline flagellate) were exposed to high salinity it accumulated glycerol as compatible solute which causes the total cell carbon to increase. It has been shown that *D. viridis* increases total cell carbon and nitrogen following an increase in the salinity of the medium (Jimenez and Niell, 1991).

7.5 Conclusion

This study also illustrates the use of the SEM-EDX technique in rapid assessment of elemental composition of the salinity stressed microalgae. The distribution of elements in *Dunaliella* and *Asteromonas* has been studied by this technique. The SEM-EDX technique is suitable for multi-element determinations in microalgae samples. The samples do not need any chemical pre-treatment. The samples are analysed non-destructively, being retained for re-use and re-evaluation. *Dunaliella* and *Asteromonas* cell have mechanism to regulate NaCl concentration but still accumulated at high salinity.

CHAPTER 8.0: METABOLOMIC PROFILE OF DUNALIELLA T35 AFTER EXPOSURE TO HYPEROSMOTIC SHOCK USING GC-MS APPROACH

8.1 Introduction

From data presented in chapter 6, starch degradation was identified as a major pathway by which glycerol was sythesised in the Namibian strains of *Dunaliella*. In the light, photosynthesis was also recruited to produce glycerol after 60 min exposure to hyperosmotic shock. However, in the dark, elevated levels of glycerol were detected over and above the level that could be accounted for by starch degradation alone, suggesting that other degradative pathways may also be involved in glycerol synthesis. To better understand the response of halotolerant microalgae to increase in NaCl concentration and how such responses underpin glycerol production, a detailed metabolomic study of *Dunaliella* T35 was required.

GC-MS is a very suitable technique for comprehensive analysis of cellular metabolites, as it combines high separation efficiency with versatile, selective and biochemical mass detection (Koek *et al*., 2011). In this chapter an untargeted GC/MS based profiling approach was employed to obtain as much information as possible about key metabolites of *Dunaliella* T35 following exposure for 24 h to 4.0M NaCl under dark and light regimes. The aim was to provide an insight into the metabolites employed by halotolerant microalgae, *Dunaliella* T35, to survive osmotic stress and to support glycerol production. Figure 8.1 shows the summary of the experiment.

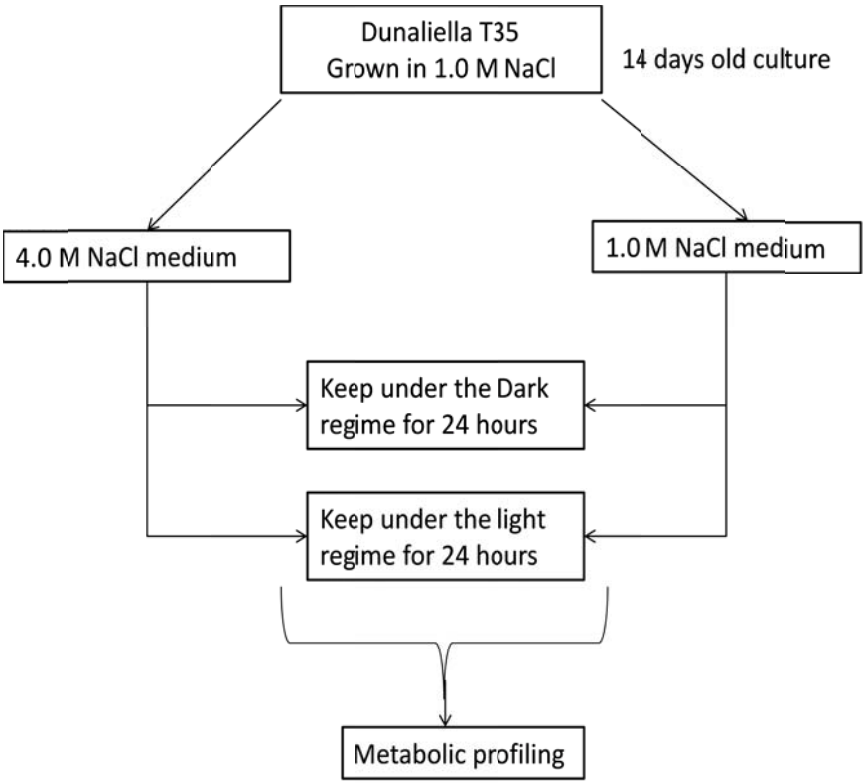


Fig. 8-1 Experimental approach flow chat for metabolomic profiling of *Dunaliella* T35 under osmotic shock

8.2 Methods

For sample and standard preparation, metabolite extraction, derivatization of samples, GC-MS analysis, selection criteria for the identification of compounds, data analysis and multivariate data analysis (see section 3.6)

8.3 Results

8.3.1 Analytical result

Polar metabolites of *Dunaliella* T35 which had been extracted with methanol, then methoxymated and silylated were separated by GC. The baseline corrected total ion chromatograms representing the elution profiles of the metabolites are presented in figure 8.2

(for the cells exposed to 1.0 M NaCl under continuous illumination), figure 8.3 (for the cells exposed to 4.0 M NaCl under continuous illumination), figure 8.4 (for the cells exposed to 1.0 M NaCl under continuous dark regime) and figure 8.5 (for the cells exposed to 4.0 M NaCl under continuous dark regime). They show about 100 peaks in each case. Some of these peaks corresponded to amino acids, sugars alcohol, organic acids and polyamines.

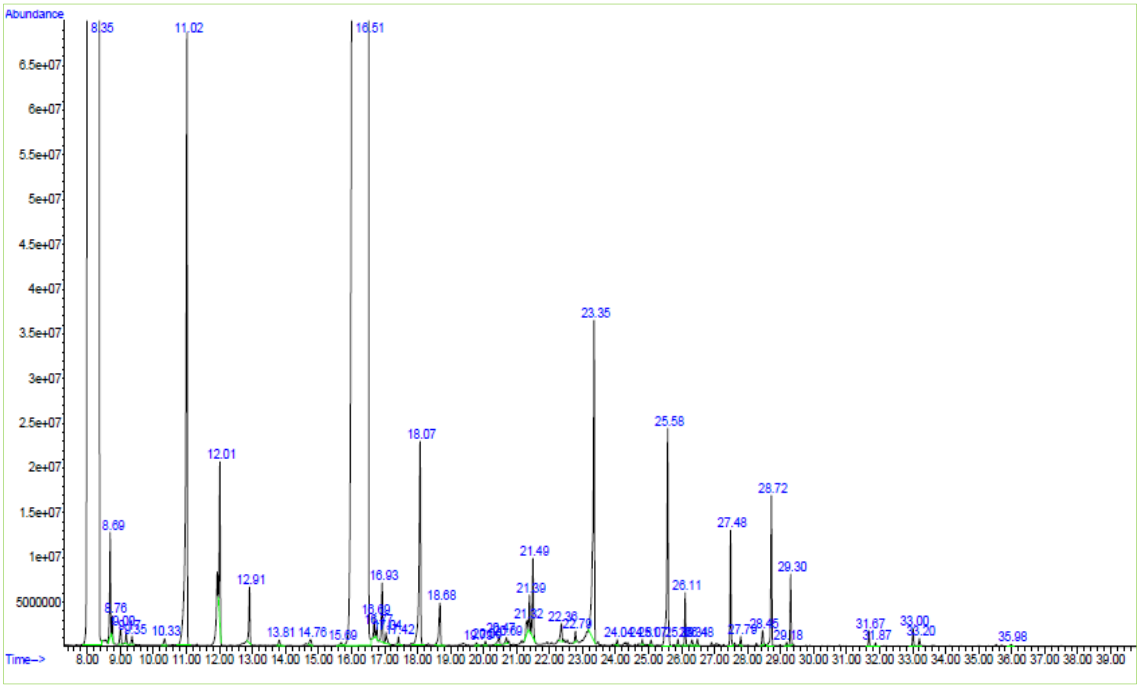


Fig. 8-2 Total ion chromatogram of *Dunaliella* T35 exposed to 1.0 M NaCl concentration under continuous illumination 45 µmol m^{-2} s^{-1} at 25 ^{o}C and pH 7.5 for 24 hours.

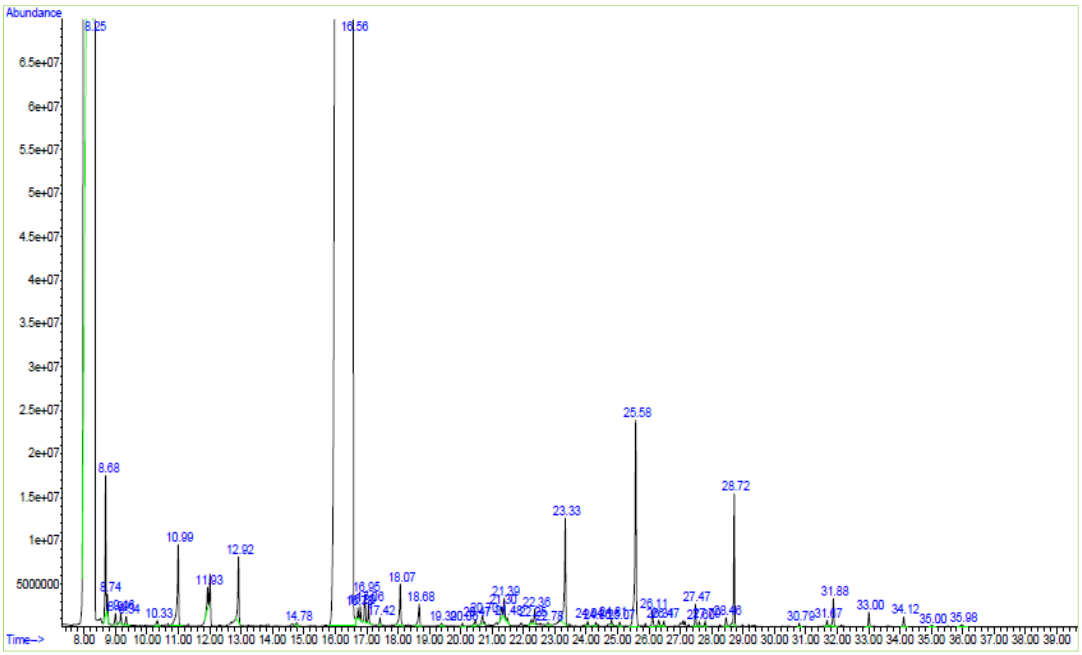


Fig. 8-3 Total ion chromatogram of *Dunaliella* T35 exposed to 4.0 M NaCl concentration under continuous illumination 45 µmol m^{-2} s^{-1} at 25 ^{o}C and pH 7.5 for 24 hours.

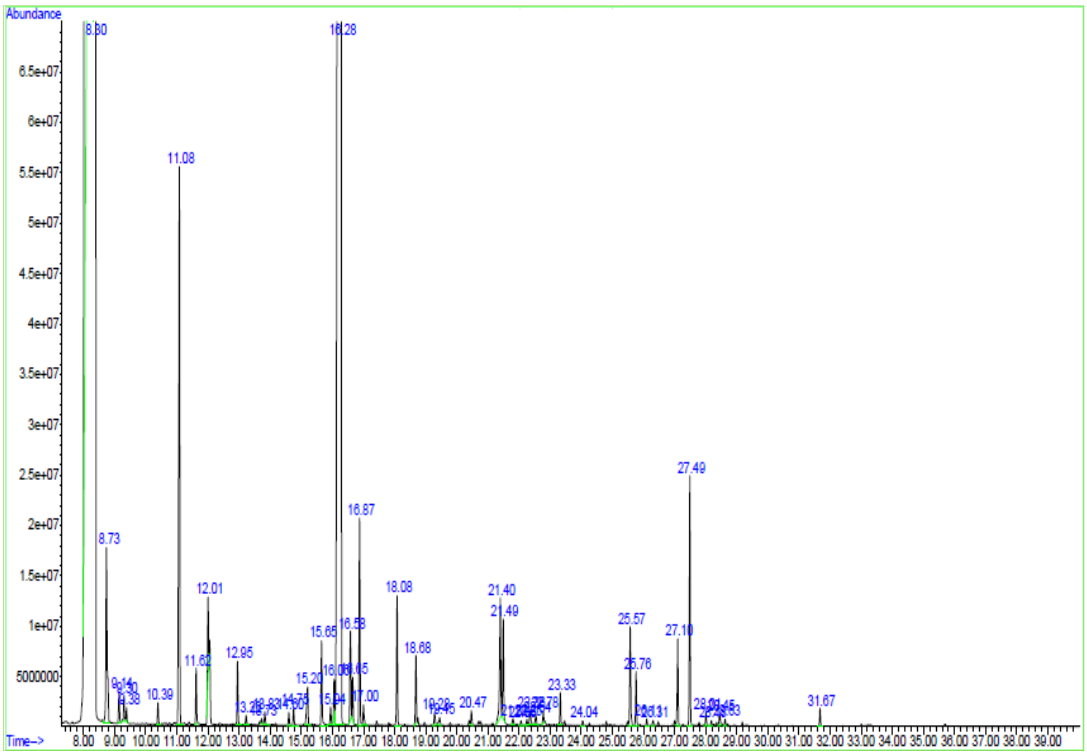


Fig. 8-4 Total ion chromatogram of *Dunaliella* T35 exposed to 1.0 M NaCl concentration under continuous dark regime at 25 ^{o}C and pH 7.5 for 24 hours.

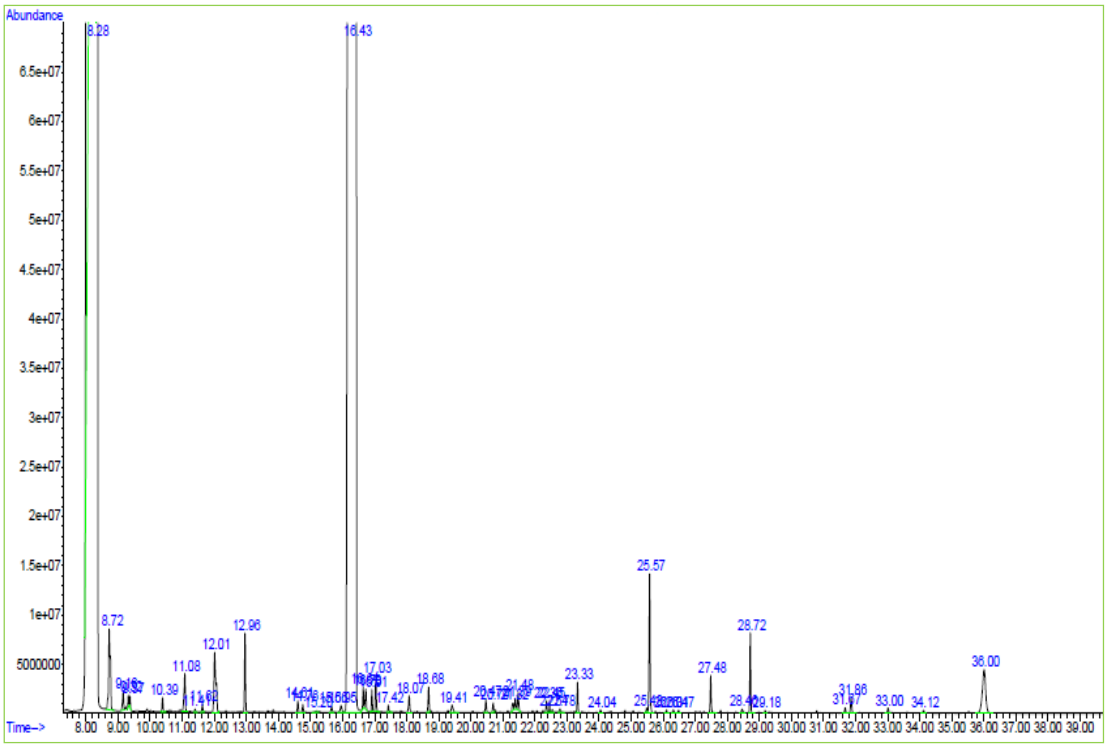


Fig. 8-5 Total ion chromatogram of *Dunaliella* T35 exposed to 4.0 M NaCl concentration under continuous dark regime at 25 ^{o}C and pH 7.5 for 24 hours.

Following peak picking and alignment with MetAlign (data processing software), a total mass signal of about 3272 was obtained for the samples. The data obtained were filtered for noise, normalized using the internal standard (rabitol, which after derivatisation appeared as rabonic acid) and filtered for poor reproducibility. This procedure reduced the number of mass signals by approximately 65 %.

Out of 100 peaks in each total ion chromatogram only 35 compounds were notably more intense than the rest and were selected for further analysis. From these 35 compounds 16 were identified by matching to libraries based on mass fragmentation patterns and 19 by direct comparison with standards (see Table 8.1). This number of metabolites is less in comparison with other untargeted metabolite profiling studies in microalgae (Bolling and Fiehn 2005; Lee and Fiehn 2008; Kluender *et al*. 2009) but in similar amount to the study conducted by Lamers (2011). The reason for the low number of metabolites detected may be due to the low biomass concentrations in the samples and the small sample volumes that were collected during the experiments.

GC-MS analysis of *Dunaliella* cell extracts allowed the detection of low levels of amino acids (asparagine, glutamic acid, isoleucine, L-glycine, L-leucine, L-lysine, L-tyrosine, phenylalanine, proline, threonine, valine and α-alanine), polyamines (spermidine and putrescine), and other metabolites: pyrazine, pyroglutamic acid, pyruvic acid, succinic acid, tryptamine, δ-Aminolevulinic acid, guanine, D-rabonic acid (the internal standard), glycerol, glycerol-3-phosphate, L-ascobic acid, L-DOPA, *myo*-inositol, threitol, tryptamine, citric acid, D-(+)- Glucose, phosphoric acid and two unknown metabolites.

Table 8-1 GC-MS analysis of compounds (metabolites) extracted from *Dunaliella* T35 after 24 h constant light or dark exposure in media containing different NaCl concentrations. Compounds were quantitatively identified based on their retention times using MassLynx 4.0 software and either the Golm Mass Spectral Database (GOL) or National Institute of Standards and Technology library (NIST) mass spectral databases and their relative concentrations expressed as % peak area were determined by reference to standards.

Compound	**Retention time (RT) (min)**	**Method ID**	**1.0 M Light**	**4.0 M Light**	**1.0 M Dark**	**4.0 M Dark**
Asparagine	24.33	GOL	ND	ND	0.02	0.02
Citric acid	27.12	STD	ND	ND	0.61	ND
D-(+)- Glucose	28.95	STD	0.20	ND	ND	ND
D-rabonic acid	24.04	NIST	0.04	0.03	0.04	0.02
Glutamic acid	23.33	STD	1.87	0.73	0.17	0.12
Glycerol	16.51	STD	35.45	56.03	28.93	47.45
Glycerol-3-phosphate	26.12	STD	0.22	0.10	0.04	0.02
Guanine	31.87	GOL	0.03	0.15	ND	0.07
isoleucine	16.69	NIST	0.09	0.06	ND	ND
L-Ascorbic acid	29.3	GOL	0.31	ND	ND	ND
L-DOPA	31.57	STD	0.35	0.23	1.01	0.09
L-Glycine	16.86	STD	ND	ND	1.45	ND
L-Leucine	16.06	NIST	ND	ND	0.29	ND
L-Lysine	28.72	STD	0.65	0.60	ND	0.24
L-Tyrosine	29.02	STD	ND	ND	0.10	ND
myo-Inositol	31.67	STD	0.07	0.03	0.1	0.02
Phenylalanine	21.8	NIST	ND	ND	0.07	ND
Phosphoric acid	13.81	GOL	0.06	0.03	0.07	0.01
Proline	16.69	STD	0.70	0.06	0.95	0.22
Putrescine	25.58	STD	1.31	1.52	0.57	0.64
Pyrazine	12.01	GOL	0.49	0.26	1.40	0.54
Pyroglutamic acid	21.39	STD	0.18	0.11	0.77	0.05
Pyruvic acid	11.02	STD	10.14	0.69	4.28	0.23
Serine	18.07	STD	1.16	0.29	0.66	0.10
Spermidine	33.00	STD	0.09	0.08	ND	0.02
Succinic acid	16.91	STD	0.32	ND	ND	0.12
Threitol	24.81	NIST	0.04	0.03	0.03	0.01
Threonine	18.68	STD	0.26	0.17	0.34	0.12
Tryptamine	15.97	GOL	0.27	0.06	0.12	0.06
UN001	8.69	GOL	0.61	0.83	1.20	0.66
UN002	12.91	GOL	0.38	0.48	0.38	0.44
UN003	21.49	GOL	0.83	ND	0.56	0.08
Valine	14.77	GOL	0.06	0.05	0.14	0.04
α-Alanine	19.49	STD	ND	ND	0.08	0.08
δ-Aminolevulinic	27.48	GOL	1.51	0.13	1.16	0.17

acid						

ND= Not detected, STD= Standard, GOL= Golm Mass Spectral Database and NIST= National Institute of Standards and Technology library

Figure 8.6 shows the results of examination of the differences among *Dunaliella* T35 exposed to salinity stress with different ranks using a principal component analysis (PCA) score plot. The dots are the metabolites and are named accordingly. The principal axes of plot represent the first and second PC scores and metabolites are plotted on that basis. The numerals in figure 8.6 indicate the rankings that had been assigned in the competition. With respect to PC 1, the higher ranked metabolites and the lower ranked ones are positioned separately to the right and left sides of the graph respectively, clearly indicating the differences in ranking. The metabolome in the lower left hand quadrant scored low on both ranking. PC2 seems to flag glycerol abundance. Figure 8.7 shows the loading plot of principal components.

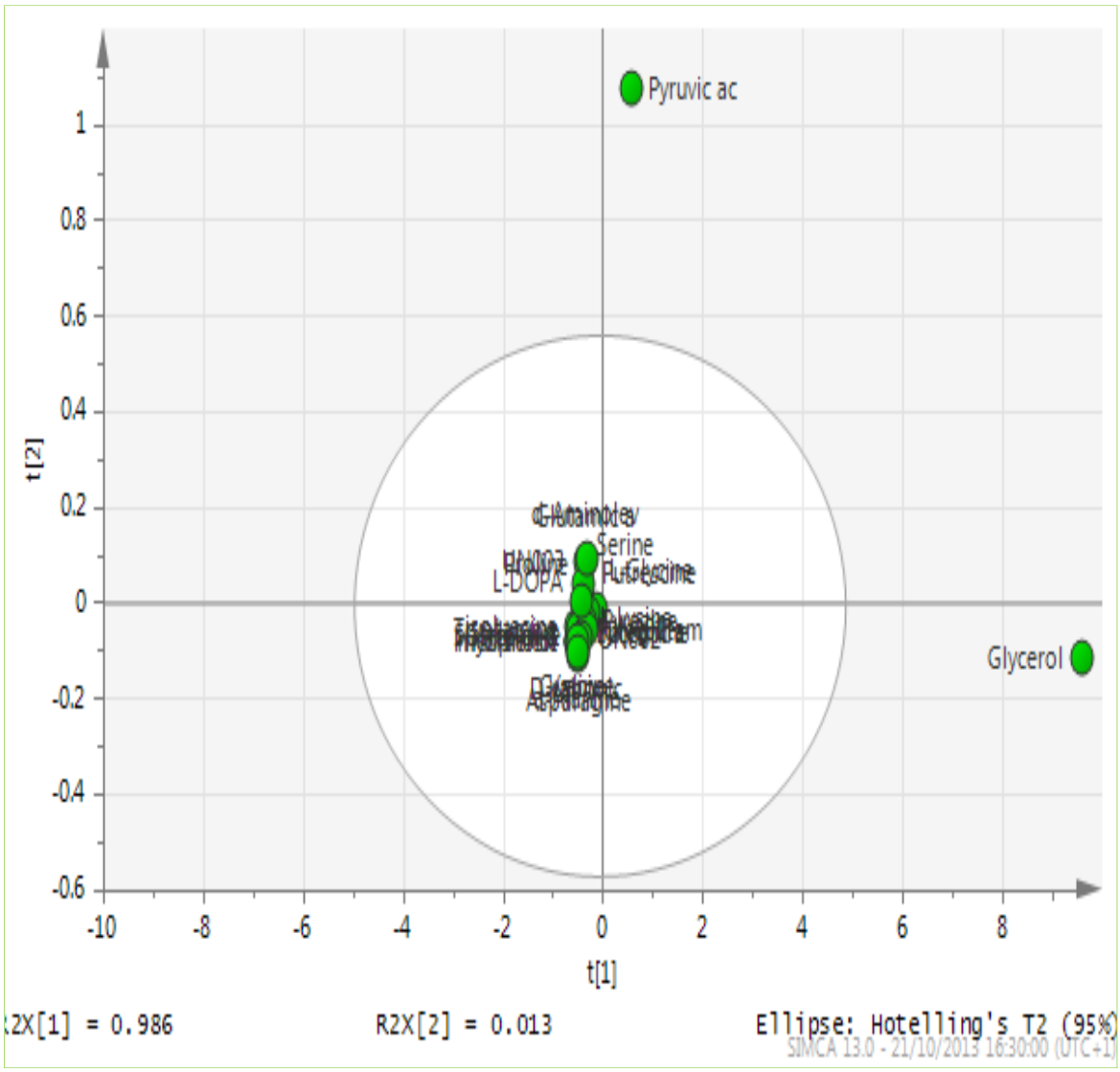


Fig. 8-6 Score plot result of the principal component analysis (SIMCA-Q 13.0.3; UMETRICS, Crewe, UK) of *Dunaliella* T35 cells exposed to 1.0 and 4.0 M NaCl in constant light/dark regimes 45 µmol m^{-2} s^{-1} at 25 ^{o}C and pH 7.5 for 24 hours.

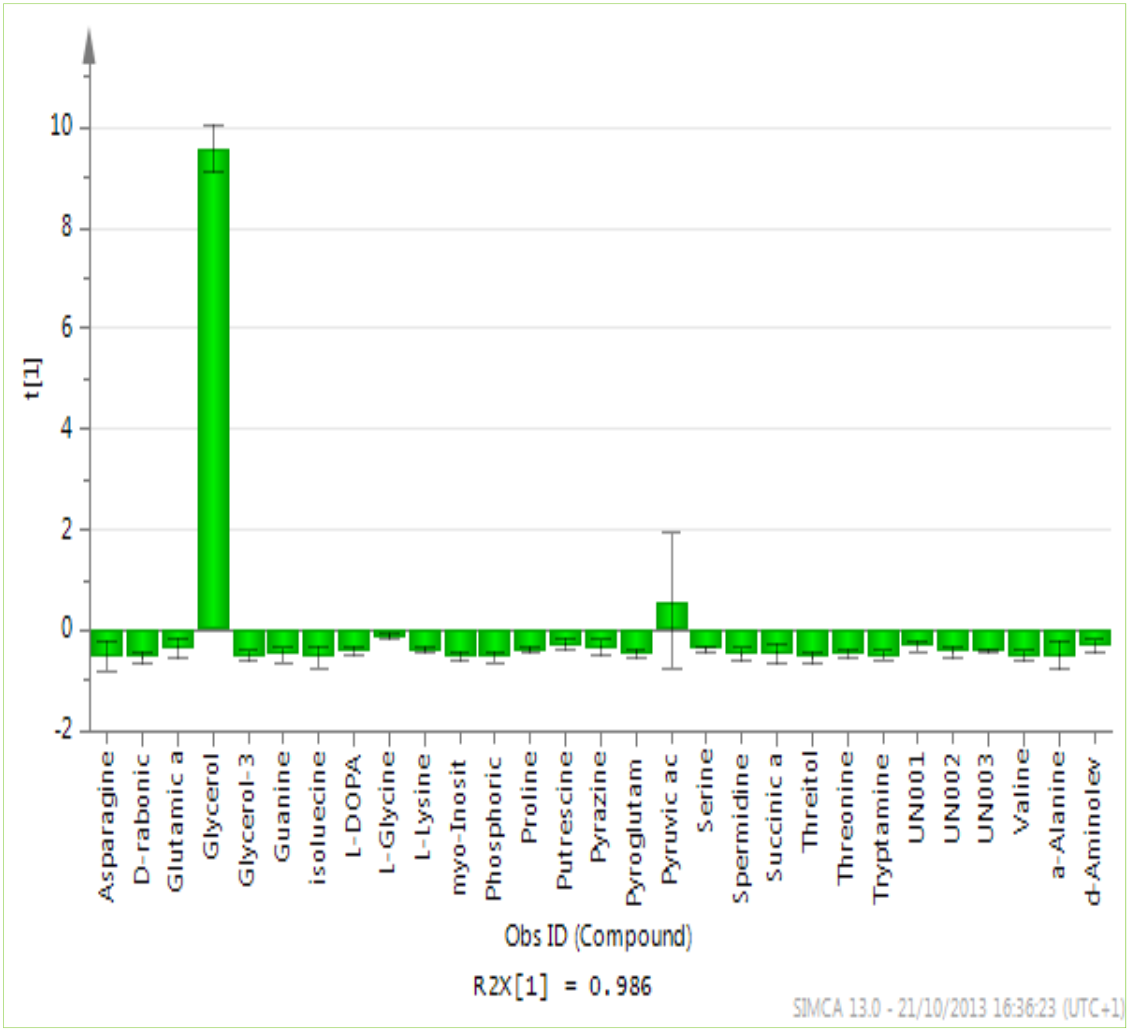


Fig. 8-7 Loading plot of principal component analysis of *Dunaliella* T35 cells exposed to either 1.0 M NaCl or 4.0 M NaCl in constant light/dark regimes45 µmol m^{-2} s^{-1} at 25 ^{o}C and pH 7.5 for 24 hours. The figure is generated using SIMCA-Q 13.0.3

A loading plot was created based on the results of the score plot in order to investigate the relationships between compounds from rank to rank (Figure 8.8) as a function of cell treatments. The results obtained suggest that the metabolome of *Dunaliella* cells exposed to 1.0 M NaCl in continuous illumination had been ranked high by virtue of the large number of metabolites followed by cells exposed to 1.0 NaCl medium in constant dark, 4.0 M NaCl in continuous illumination and 4.0 M NaCl medium in continuous dark, respectively (Figure 8.8).

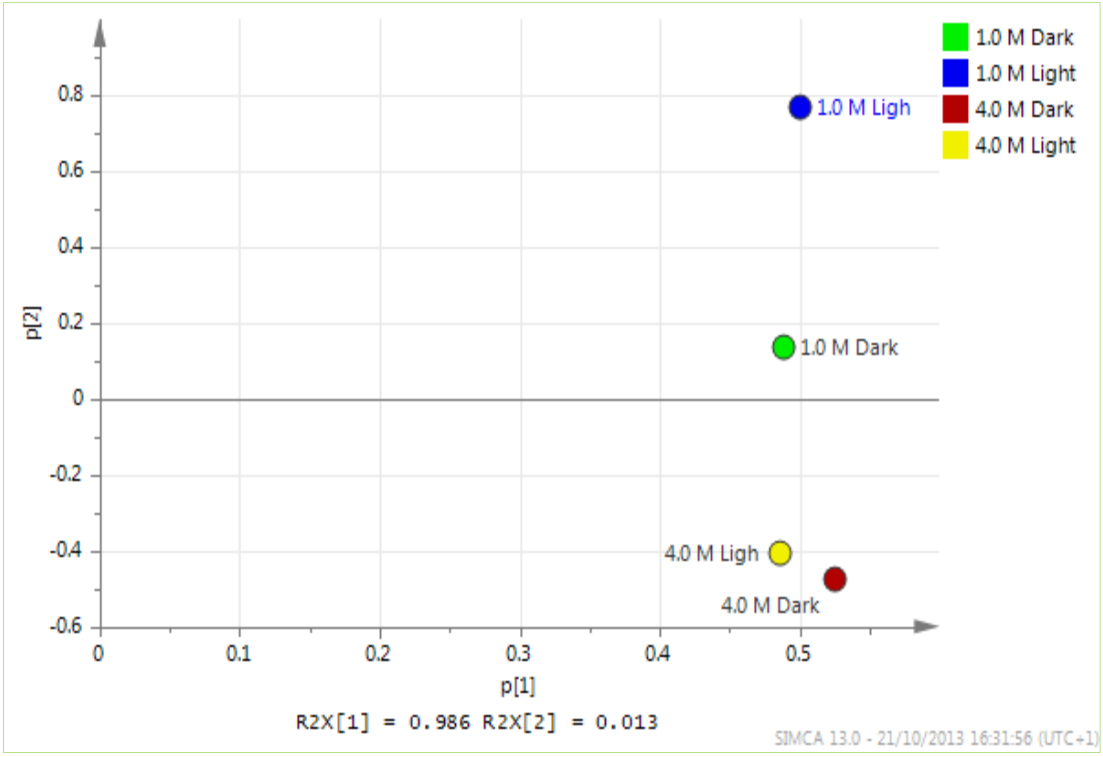


Fig. 8-8 Loading scatter plot result of principal component analysis of *Dunaliella T35* cells exposed to 1.0 and 4.0 M NaCl in constant light/dark regimes 45 µmol m^{-2} s^{-1} at 25 ^{o}C and pH 7.5 for 24 hours. The figure is generated using SIMCA-Q 13.0.3

8.3.2 Effect of NaCl concentration on the overall metabolite profiles

The number of detected metabolites following exposure of *Dunaliella* T35 to 1.0 M NaCl concentration was greater in comparison to those in cells exposed to 4.0 M NaCl, irrespective of the lighting regime. Out of thirty five metabolites identified only six metabolites (asparagine, citric acid, glycine, leucine, tyrosine, phenylalanine and α-alanine) were not recorded in the cell exposed to 1.0 NaCl under constant light. Based on the number of metabolites detected, cells exposed to 1.0 M NaCl in constant light or dark had the largest number of metabolites (28 metabolites) followed by cells exposed to 4.0 M NaCl in constant dark (27 metabolites) and cell exposed to 4.0 M NaCl in constant light (24 metabolites), respectively.

With the exception of pyruvate and glycerol none of these metabolites exhibited significant changes in response to salt stress. Their percentage peak area and retention time are presented in Table 8.1.

The relative concentration of glycerol was significantly larger compared to all the metabolites determined in all experimental conditions and increased significantly after hyperosmotic shock in either the light (58 % increase) or dark (64 %). Interestingly the relative % of glycerol formed on exposure to 4M NaCl in the light was ~15 % greater in the light (56.03 %) than that corresponding in the dark (47.45 %), consistent with previous findings (see 6.3.2). The significant variation in glycerol concentration correlates with the previous result presented in chapter 6.0 which shows under that under hyperosmotic shock *Dunaliella* cell accumulate up to 80 % glycerol dry weight basis (see chapter 6.0; Table 6-8).

After glycerol, pyruvate was the second most abundant metabolite detected in this study. Pyruvate typically represents a control point in carbohydrate metabolism, either entering the mitochondrial matrix for oxidation in the citric acid cycle to generate NADH, and ATP and metabolites, or staying in the cytosol where it can be reduced under anaerobic conditions to recycle NADH to NAD^+ by forming a reduced end product such as lactate, ethanol or glycerol. Pyruvate was detected in all the experimental conditions. However the amount of pyruvate detected decreased 14-fold when cells were transferred from 1.0 M NaCl to 4.0 M NaCl in the light and 18-fold in constant dark.

Gycerol-3-phosphate is a key glycerol cycle intermediate formed with reducing equivalents, either NADH or $FADH_2$, from dihydroxyacetone phosphate in a reversible reaction catalyzed by any one of 3 isomers of glycerol-3-phosphate dehydrogenase that have been found in the cytosol, mitochondria or chloroplast. The differences between treatments were not considered significant relative to the differences shown with glycerol and pyruvate. Nevertheless it is interesting to note that larger relative amounts were detected in the cells exposed to constant illumination (0.22 % and 0.10 % for 1.0 M and 4.0 M NaCl respectively), compared to the dark (0.04 % light; 0.02 % dark), with larger relative amount in 1M NaCl (Table 8.1). This would be consistent with a larger relative pool size of reducing equivalents formed in the light from photosynthesis, shifting the equilibrium in the direction of glycerol-3-phosphate formation.

Table 8.1 shows that proline was detected in all the experimental conditions: the largest relative amount was obtained in the cells exposed to 1.0 M NaCl in either constant light (0.7

%) or dark (0.95 %). However it decreased by 11-fold and 4-fold in the cell exposed to 4.0 M NaCl in constant light and dark regimes, respectively.

Glycine was only detectable in this study in the cells exposed to 1.0 M NaCl in constant dark and it was below the detection limit in cells treated in both 1.0 and 4.0 NaCl in light and in 4.0 M NaCl in constant dark. Unlike glycine, serine and threonine were detected in all experimental conditions (figure 8.9). The amount of serine and threonine was found to be low in cells exposed to 4.0 M NaCl when compared to those exposed to 1.0 M NaCl concentrations.

L-phenylalanine, and L-tyrosine are aromatic amino acids (AAAs) whereas valine, leucine and isoleucine form the small group of branched-chain amino acids (BCAAs) classified by their small branched hydrocarbon residues. Table 8.1 shows that L-phenylalanine and L-tyrosine were detected only on the cells exposed to 1.0 M NaCl in constant dark regime. Of all the BCAA, valine was the only amino acid detected in cells exposed to all experimental conditions. Leucine was detected in cells exposed to both 1.0 and 4.0 M NaCl under constant light with no significant difference between them. L-Leucine was only detected in the cells exposed to 1.0 M NaCl in constant dark and it was below the detection level in other experimental conditions investigated (Table 8.1).

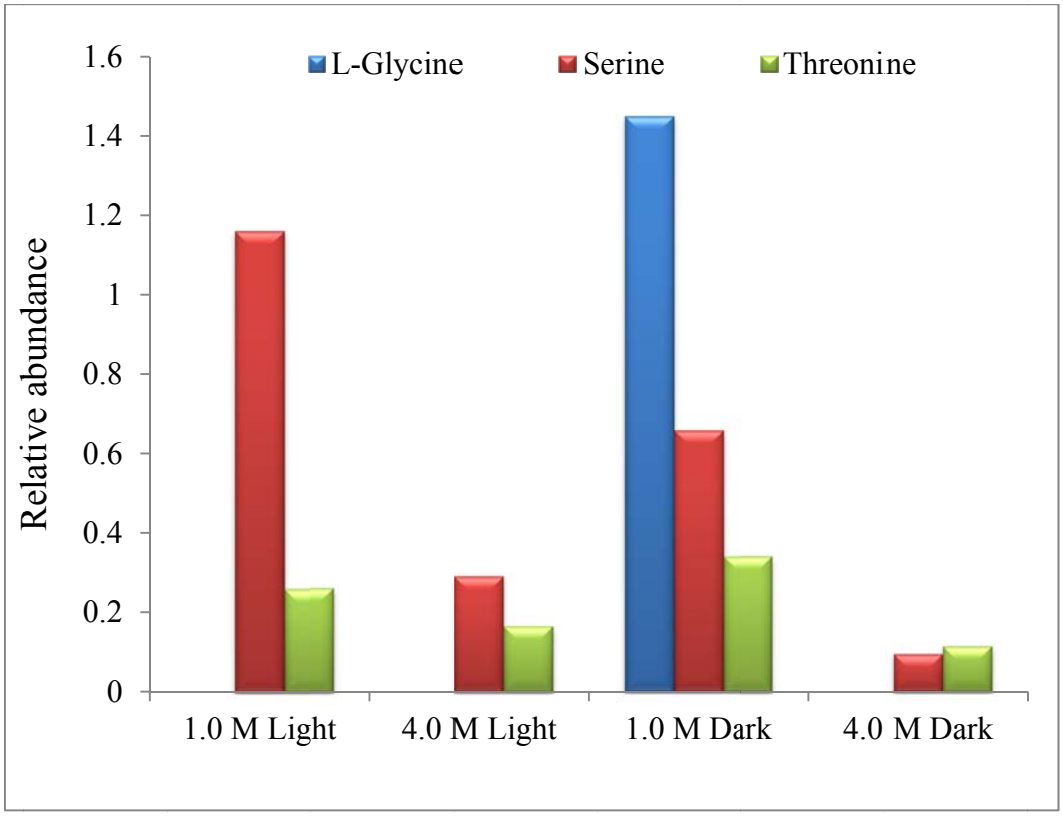


Fig. 8-9 Effect of salinity stress and light/dark regime on the relative abundance of glycine, serine and threonine

The metabolites *myo*-inositol and guanine both responded oppositely to the two different salinity media: figure 8.10 shows that guanine increased when *Dunaliella* T35 cells were exposed to 4.0 M NaCl with high amounts in cells kept under constant light regime and also its level was below detection in 1.0 M NaCl in constant dark regime. The amount of *myo*-inositol was inversely proportional to the amount of guanine under the same experimental conditions. Ascorbic was detected by this study only in the cells exposed to 1.0 M NaCl in constant light (figure 8.10).

Glucose was only detected in the cultures exposed to 1.0 M NaCl in light. L-DOPA was detected in all the samples investigated, but the cells exposed to 1.0 M NaCl in constant dark/light had the largest relative amount (see Table 8.1).

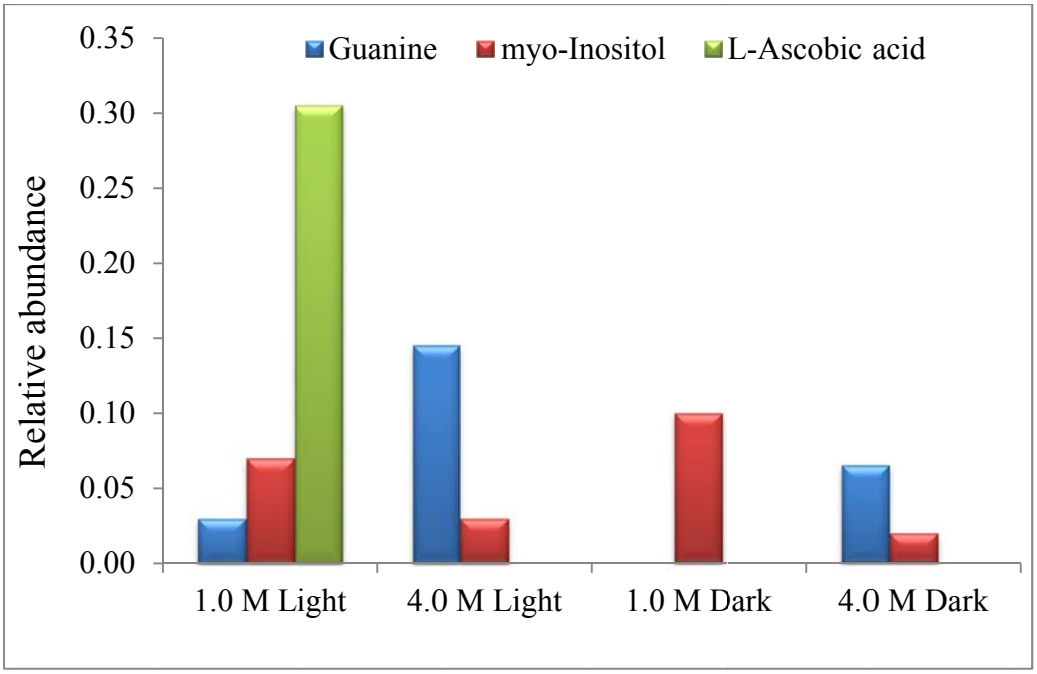


Fig. 8-10 Effect of salinity stress and light/dark regime on the relative abundance of guanine, *myo*-inositol and ascobic acids

The three unknown metabolites (UN001, UN002 and UN003) were detected in relatively high amount and correlated positively or negatively with salinity or light/dark regime exposure. UN001 was detected in all the cells exposed to 1.0 and 4.0 M NaCl in both constant light and dark regime; the cells exposed to 1.0 M NaCl concentrations in constant dark regime had the larger amount (see Table 8.1). UN002 was detected in all the experimental conditions and there was no difference in its amount in all samples. UN003 was not detected in the cells exposed to 4.0 M NaCl in constant light and the amount detected in the cells exposed to 4.0 M NaCl in constant dark regime was 85 % less when compared to cells exposed to 1.0 M NaCl concentrations in dark regime. UN002 was detected in all the experimental conditions and there is no difference in its amount in all samples.

8.4 Discussion

GC coupled with MS has been regarded as the gold standard for analysing many compounds (such as lipid, drugs metabolites and environmental contaminants). GC-MS offers great advantages in identification and detection of species based on their retention time and mass spectrum (a compound's specific fragmentation pattern) (Garcia and Barbas, 2011). Compounds produce reproducible fragmentation patterns when ionized by a fixed electron voltage (Usually -70 eV). Thus, the fragmentation spectra obtained by GC-MS are not instrument dependent and allow for the creation of databases and the sharing of data between users, making the technique particularly valuable. GC-MS also allows for quantitative detection of analytes (Garcia and Barbas, 2011). For examples, volatile and low-molecular-weight metabolites can be sampled and analyzed directly without derivatization (Pauling *et al*., 1971; Koek *et al*., 2011). However, several metabolites contain polar functional groups and are heat stable at the temperatures required for their separation or are not volatile at all. Therefore, derivatization prior to GC analysis is needed to extend the application range of GC based methods (Koek *et al*., 2011).

Dunaliella T35 cells were subjected to both 1.0 and 4.0 M NaCl medium under constant light and dark regimes. Following hyperosmotic stress the untargeted metabolomic study using GC-MS profiling resulted in the identification of total of 35 metabolites. The number of metabolites reported in this study are less in comparison with other untargeted metabolite profiling studies in microalgae as reported by (Bolling and Fiehn 2005; Lee and Fiehn 2008; Kluender *et al*. 2009) but in similar amount to the study conducted by Lamers (2011). The reasons for the low number of metabolites detected may be due to experimental difficulties of working with low biomass concentrations in the samples and the small sample volumes collected during the experiments. Nevertheless the fact that the number of detected metabolites following exposure to 1.0 M NaCl concentration was greater in comparison with those in cells exposed to 4.0 M NaCl, irrespective of the lighting regime, would be consistent with the view that under salinity stress the majority of metabolic pathways were redirected to synthesise glycerol. This may include lipid degradation in the dark, which could account for the increased levels of glycerol over and above the amount that could be expected from starch degradation alone.

Higher levels of glycerol and low levels of other sugar and amino acid, tricarboxylic acid (TCA) cycle intermediates and other metabolites were evident. Some of these compounds exhibited changes in response to salinity stress. In order to get an insight into metabolites that may be related to glycerol accumulation in hyperosmotic shock, the presence/absence and percentage relative abundance of metabolites from different treatments were compared.

Glycerol accumulation: *Dunaliella* is known to produce glycerol by starch breakdown or by photosynthetic CO_2 fixation. In chapter 6 *Dunaliella* and *Asteromonas* accumulated up to 704 mg/g dry weight glycerol when exposed to high salinity. Glycerol accumulated at the expense of starch and possibly other storage compounds. Thus although stored lipid pools were not analysed in the present study, their degradation may also have contributed to the size of the glycerol pool. *D. viridis* for example, accumulates and stores neutral triglycerides and sterols as light intensity increases (Gordillo *et al*., 1998). The present study confirmed that the amount of glycerol increased upon cells' exposure to 4.0 M NaCl (Table 8.1). It also confirmed that in the light the pool size of glycerol was ~15 % greater than in the dark. Possibly under extreme salinity glycerol synthesis may serve as a redox transfer mechanism from the chloroplast, oxidizing excess NAD(P)H in a process akin to photorespiration (Igamberdiev *et al*., 2001).

Glycerol-3-phosphate was the only glycerol cycle intermediate detected in this study. This compound was detected in all the cells but the highest amount was detected in cells exposed to 1.0 M NaCl concentrations in constant illumination. Under these conditions glucose was also detected. Together these data point to a larger relative pool size of reducing equivalents in the light from photosynthesis, shifting the equilibrium between dihydroxyacetone phosphate and glycerol-3-phosphate in the direction of glycerol-3-phosphate formation on the one hand and starch formation on the other. This is in turn consistent with the notion that glycerol-3-phosphate may be involved in regulating redox balance in the light by consuming NAD(P)H from photosynthesis in a cycle that might be coupled with glycerol synthesis from pyruvate in *Dunaliella.* On exposure to high salinity the equilibrium position of the corresponding reactions would be shifted in the direction of glycerol synthesis, thus reducing the relative concentrations of both pyruvate and glycerol-3-phosphate and in the light, maintaining redox balance. Such a mechanism would explain the elevated levels of glycerol in the light compared to those in the dark in 4 M NaCl.

Glycerol-3-phosphate is converted to glycerol by glycerol-3-phosphate phosphatase (Goyal *et al*., 1987; Chen *et al*., 2011).

Glucose was only detected in the cultures exposed to 1.0 M NaCl in light. The accumulation of glucose may be considered as a sign for the storage of carbohydrate reserves (i.e. starch).

Proline as osmoprotectants or osmolytes: Other changes that occurred in hyperosmotic medium were related to potential osmolytes. The major osmolyte responsible for the short-term adaptation of *Dunaliella* cells to high salinities is glycerol. The metabolomic profiles, however, indicated the presence of additional osmolytes, such as proline. However, the amount of proline decreased (11-fold) in the cells exposed to hypersaline (4.0 M NaCl) condition in constant illumination when compared to the amount detected in cells exposed to 1.0 M NaCl under constant illumination. The amount also decreased by 4-fold in the cell exposed to 4.0 M NaCl in constant dark when compared to the amount detected in cells exposed to 1.0 M NaCl under similar condition. The possible reason for proline disappearance at higher salinity could be its contribution to the adjustment of the overall intracellular osmolarity is negligible compared to glycerol (Dittami *et al*., 2011). In the short-term salt stress response, both mannitol and proline might function as local osmolytes in specific cell compartments (Dittami *et al*., 2011).

Accumulation of amino acids: Microalgal cells possess numerous regulated metabolic networks. Among these metabolic networks are those leading to the synthesis of amino acids which are the building blocks for the proteins biosynthesis and may also serve as precursors for a large array of metabolites with multiple functions in plant/microalgae growth and response to various stresses (Less and Galili, 2008; Du *et al*., 2011).

Different amino acids are produced under different abiotic stresses, such as hyper and hypo-osmotic shock, high and low temperature, drought or nutrient deficiency (Du *et al*., 2011). Under salinity stress, synthesis of amino acid such as proline was stimulated to prevent cell hyperosmotic shock. Asparagine production may be stimulated to prevent ammonium toxicity and to reassimilate the nitrogen released (Diaz *et al*., 2005). Du *et al*. (2011) found an increase in the response ratio of asparagine after 6 and 18 days of heat stress in *Cynodon transvaalensis*. The study reported here shows that there was some variations, although not

statistically significant, in the amount of free amino acids in salinity range of 1.0 M to 4.0 M NaCl.

Glycine was only detectable in the cells exposed to 1.0 M NaCl in constant dark whereas alanine was detected in cells exposed to both 1.0 and 4.0 M NaCl under constant dark regime and its level was below the detection limit in cells exposed to similar medium under constant illumination. Glycine is considered as major metabolite in the photorespiratory process. Photorespiration is a defence mechanism which protects plants from photoinhibition/ photooxidation (Akiko and Go, 1996; Niyogi, 1999). In the mitochondria glycine can be oxidized to form serine, their ratio is considered a marker of photorespiration in terrestrial plants and *Ectocarpus* (Foyer *et al*. 2003; Gravot *et al*. 2010).

Glutathione is an antioxidant found to be accumulated in several plants during the acclimation to stress (Tausz *et al*., 2004). In tomato plants, an enzyme called glutamylcysteine synthetase was demonstrated to be an important regulator of glutathione content under salt stress (Mittova *et al*. 2003). In *Ectocarpus*, there are two isomers of glutamylcysteine synthetase, one of these isomers was activated under hypersaline stress while the second one was down-regulated, this is neither supporting nor weakening the hypothesis that glutathione synthesis may be related to the observed changes in glycine and serine ratio. However, this study was not able to substantiate the suggested role of glutathione in *Dunaliella* T35, as it did not detect glutathione in any of the samples. Therefore, additional experimental evidence, in particular measurements of glutathione content, will be required to further investigate this hypothesis.

Glutamate and δ-Aminolevulinic acid: δ-Aminolevulinic acid (δ-ALA) is the initial precursor in the porphyrin synthesis pathway, the pathway that leads to heme in animals (Shemin and Russell, 1953) and chlorophyll in bacteria (Brunham and Lascelles, 1963) and plants (Beale and Castelfranco, 1974); and of the phycobilins in red algae (Troxler and Brown, 1975). In many organisms ALA is formed by the condensation of glycine and succinyl-CoA, catalyzed by ALA synthetase, a pyridoxal- requiring enzyme. The enzymatic activity was first demonstrated in photosynthetic bacteria (Kikuchi *et al*., 1958). Kipe-Nolt and Stevens (1980) reported that blue-green algae synthesize ALA from glutamate in a manner similar to that observed in green plants and green algae. In this study the cells exposed to 1.0 and 4.0 M

NaCl in constant light were found to accumulate high amount of glutamic acid when compared to the similar group of cells kept under constant dark regime. Similar to glutamate, more δ-ALA was accumulated in the cells exposed to 1.0 and 4.0 M NaCl in constant light, the reasons could be *Dunaliella* T35 use glutamate as a precursor for the synthesis of δ-ALA (an initial substrate for chlorophyll biosynthesis).

Pyruvate and other organic acids: Organic acids are mainly produced in mitochondria through the Kreb's cycle. Organic acids like pyruvate and Krebs cycle intermediates such as citrate, succinate and malate are of fundamental importance at the cellular level due to their participation in several biochemical pathways, involving energy production (through the electron transport chain), serving as precursors for amino acid biosynthesis and modulating adaptation of the plant to the environment (Saha *et al*., 2012).

In this study pyruvate was detected in all the cells exposed to 1.0 and 4.0 M NaCl in both constant light and the dark regime with highest amount in the cells exposed to 1.0 M NaCl concentrations in constant illumination. The amount of pyruvate was found to decrease (14-fold) in the cells exposed to 4.0 M NaCl concentration in constant illumination when compared to the amount detected in cells exposed to 1.0 M NaCl under constant illumination. Similarly, the amount of pyruvate also decreased (18-fold) in the cells exposed to 4.0 M NaCl in constant dark when compared to the amount detected in cells exposed to 1.0 M NaCl under similar condition. According to Saha *et al*. (2012) the activity of pyruvate dehydrogenase (E.C. 1.2.4.1) in mung bean seedlings (*Vigna radiata* L. Wilczek) decreased in 50 mM NaCl but increased in 100 mM and 150 mM concentrations. This may be a reason why higher amount of pyruvate was detected in the cell exposed to 1.0 M NaCl concentration. The presence of citric acid was only detected in the cells exposed to 1.0 M NaCl in the constant dark regime whereas succinic acid was detected in cells exposed to 1.0 and 4.0 M NaCl in constant light. According to Hardie (2003) the TCA-cycle intermediates citrate, malate and succinate accumulation are indicative of a cellular energy surplus.

Myo-inositol, guanine and ascorbic acid: The metabolites *myo*-inositol and guanine both responded oppositely to the two different types of stress: guanine increased upon cell exposure to 4.0 M NaCl with a high amount in cells kept under constant illumination and also it was below detection in 1.0 M NaCl in constant dark. The amount of *myo*-inositol was

inversely proportional to the amount of guanine under the same experimental conditions; there was decrease in the *myo*-inositol content in cells exposed to 4.0 M NaCl concentration. The result also showed a decrease in amount of *myo*-inositol in cells exposed to 1.0 M NaCl in comparison to those in 1.0 M NaCl in the dark, this may be due to the rapid utilization of this compound, possibly for the mediation of the high-light stress as suggested by Lamers (2011). However, this study could support the view that *myo*-inositol could be considered as a precursor for the biosynthesis of ascorbic acid under high light stress, as ascorbic acid was detected by this study only in the cells exposed to 1.0 M NaCl in constant light (figure 8.10).

Studies conducted by Lorence *et al*. (2004) have shown that *myo*-inositol acts as the initial substrate for the biosynthesis of the antioxidant ascorbic acid. Ascorbic acid is known to accumulate in *D. salina* under high-light stress and nitrogen limitation (Abd El-Baky *et al*., 2004).

In addition to the metabolites discussed in this section a number of additional metabolites (Table 8.1) was detected but their relationship to *Dunaliella* growth at different salinity, light and dark regime is not clear. A further explanation of these metabolites and their possible roles in *Dunaliella* cells are presented in Appendix A4.1.

8.5 Conclusions

Under salinity stress the majority of metabolic pathways in *Dunaliella* appear to be redirected to synthesise glycerol. This may include lipid degradation in the dark. Glycerol plays a key role in osmoregulatory mechanism at high salinities; other osmoregulatory solutes such as proline, inorganic ions and sugars make a minor contribution to cell osmolarity. Solutes other than glycerol and proline (amino acids, carbohydrates, inorganic ions) are reported to be employed by halotolerant algae in osmoregulatory processes, but the extent to which these solutes contribute to the intracellular osmolarity is still uncertain given their relative abundance. GC-MS analysis is suitable for metabolomics study, as it combines high separation power with sensitive and selective mass detection. It involved unbiased peak picking and clustering of signals into metabolite mass spectra. This study detected 35 metabolites from *Dunaliella* cells exposed salinity stress in light/dark regimes. A pre-

requisite of GC-MS is sample vaporization, and to achieve this without thermal decomposition, sample derivatisation is often required.

CHAPTER 9.0: GLYCEROL EXTRACTION FROM *DUNALIELLA* USING SUPERCRITICAL CARBON DIOXIDE (SC-CO_2) CELL RUPTURE

9.1 Introduction

The aim of the research presented in this chapter was to develop a method for extracting glycerol and other products using SC-CO_2 at moderate pressures, temperatures and time. The application of SC-CO_2 in cell disruption and the extraction of lipophilic compounds from microalgae has been reported, but not for hydrophilic compounds like glycerol. An initial study was conducted to determine efficiency of CO_2 in cell disruption. Cell disruption was verified by SEM and by glycerol determinations (by sodium periodate reagent and GC-MS methods described in sections 3.5.1 and 3.6.3, respectively). This experiment provided information on the optimum pressure, temperature and time for cell rupture and glycerol extraction. The effect of varying the chloroform to water ratio on glycerol extraction yield using SC-CO_2 was also investigated. The study was extended to understand the effect of pressure in the extraction efficiency of glycerol (whether increasing pressure could be used as alternative to the use of chloroform). Experimental design is shown in figure 9.1.

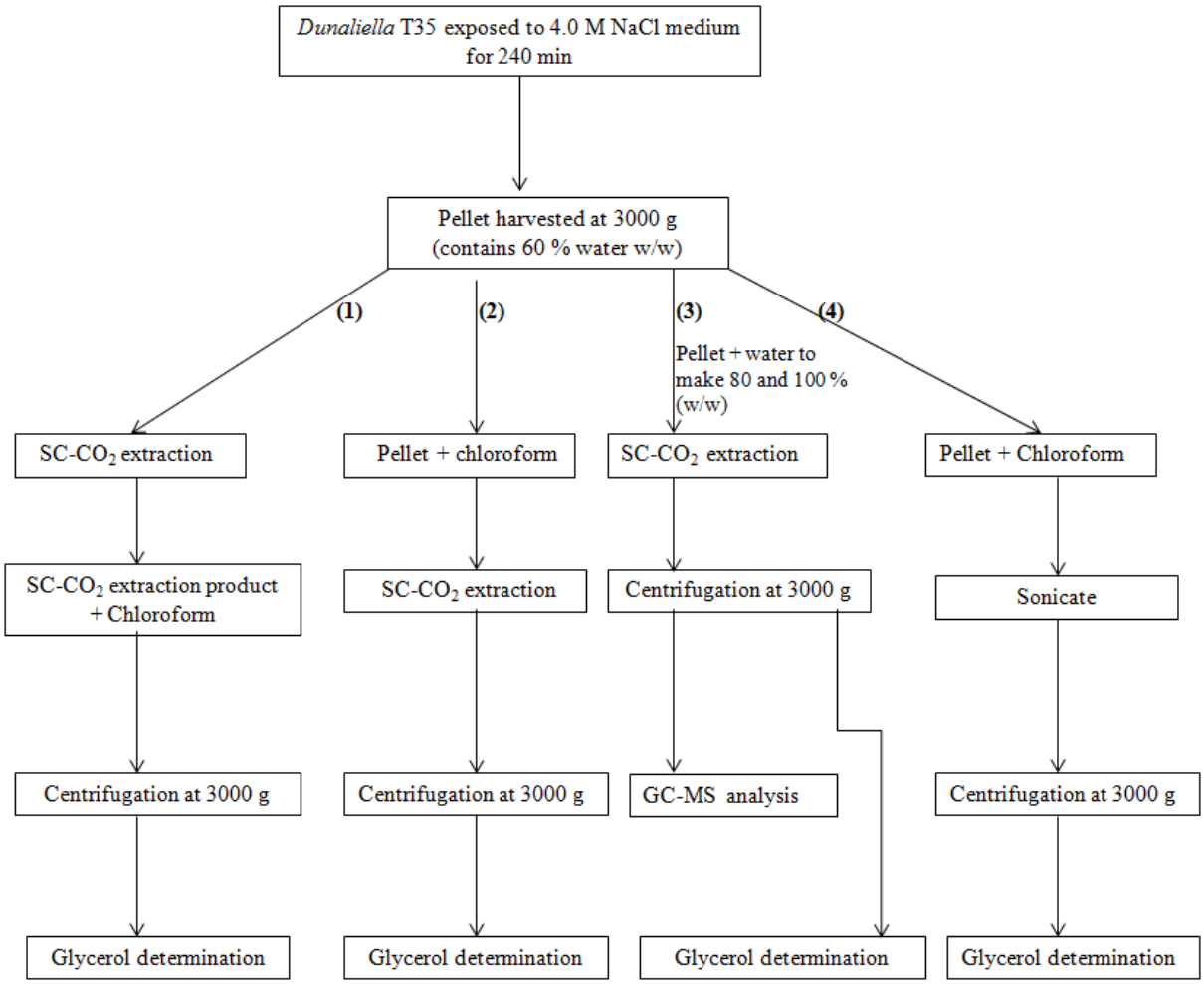


Fig. 9-1 Experimental approach flow chart for the SC-CO_2 extraction of glycerol from *Dunaliella* T35 exposed to 4.0 M NaCl concentration for 160 min. (1) Chloroform was added to the pellet after SC-CO_2 extraction (2) Chloroform was added to the pellet before SC-CO_2 extraction (3) No chloroform added (4) Pellet sonicated in chloroform

Pathway 1 was designed to optimize the process variables for glycerol extraction with SC-CO_2 and examined variables of pressure (100 to 200 bar); temperature (40-50 °C) and dynamic extraction time (15-90 min). Pathway 2 was designed because conventional extraction of glycerol (sonication method) described in section 3.5.1 uses ~16 % chloroform. Consequently this experiment was conducted to verify the influence of SC-CO_2 extraction pressure when using a combination of SC-CO_2 and chloroform with concentrations of 5, 9 and 16 % chloroform in SC-CO_2. Pathway 3 was designed to investigate if glycerol could be

extracted using SC-CO_2 without addition of chloroform. Pathway 4 describes the standard method used to assess glycerol content in cells, namely 8 min sonication in ~16 % chloroform (section 3.5.1) and thus serves as the benchmark for the experimental design. The set-up for SC-CO_2 extraction is described in Chapter 3.0.

9.2 Methods

Pathway 1 (figure 9.1): this experiment was conducted with the original harvested pellet (contains 60 % aqueous culture medium w/w). Experimental methodology was applied to optimize the process variables for glycerol extraction including pressure (100 to 200 bar) temperature (40 - 50 °C) and dynamic extraction time (15 - 90 min) (Table 9.1). The optimization of the method was facilitated using a statistical technique called factorial design (Kazazi *et al*, 2007). The factors and levels investigated were pressure, temperature and time (3x3x4x2= 72 runs).

Table 9-1 Experimental conditions for studying the effect of various factors influencing *Dunaliella* cell rupture using SC-CO_2.

Parameter	**Level**			
	1	**2**	**3**	**4**
Pressure (bar)	100	150	200	-
Temperature (^oC)	40	45	50	-
Extraction time (min)	15	30	60	90

9.2.1 Glycerol determination

Chemical method (see section 3.5.1) and GC-MS analysis (see section 3.6.3)

9.2.2 Scanning electron microscopy

See section 3.4

9.2.3 Statistical analysis

Data generated were subjected to analysis of variance (ANOVA) and multiple comparison tests were performed using a least significant difference (LSD). All analyses were carried out using the statistical software, Sigmaplot version 11.

9.3 Results

9.3.1 Assessment of cell membrane rupture after SC-CO_2 treatment

Cell rupture efficiency was determined on wet pellets of *Dunaliella* T35 cells (60 % moisture content) harvested from the log phase of growth (10^6 cells/ml). Pellets were exposed to SC-CO_2 at various values of temperature pressure and time, and the efficiency of cell rupture assessed using (a) SEM and (b) determination of the amount of glycerol released from pellets of cells using SC-CO_2 treatments.

9.3.1.1 Assessment of cell membrane rupture using SEM

SEM micrographs of *Dunaliella* cells ruptured with SC-CO_2 (Figure 9.2) clearly illustrate that the cell membrane was disrupted after exposure to SC-CO_2 and that the extent of disruption was similar to the results obtained using the standard glycerol extraction protocol (3.5.1) which is based on the use of sonication in ~16 % chloroform. These SEM micrographs are representative of all tested conditions. The degree of rupture was dependent on the applied pressure. Micrographs show that the increase in pressure resulted in higher cell rupture where the maximum cell rupture was observed after SC-CO_2 treatment at 200 bar and 50 ^{o}C. Data for 40 and 45 ^{o}C is not shown.

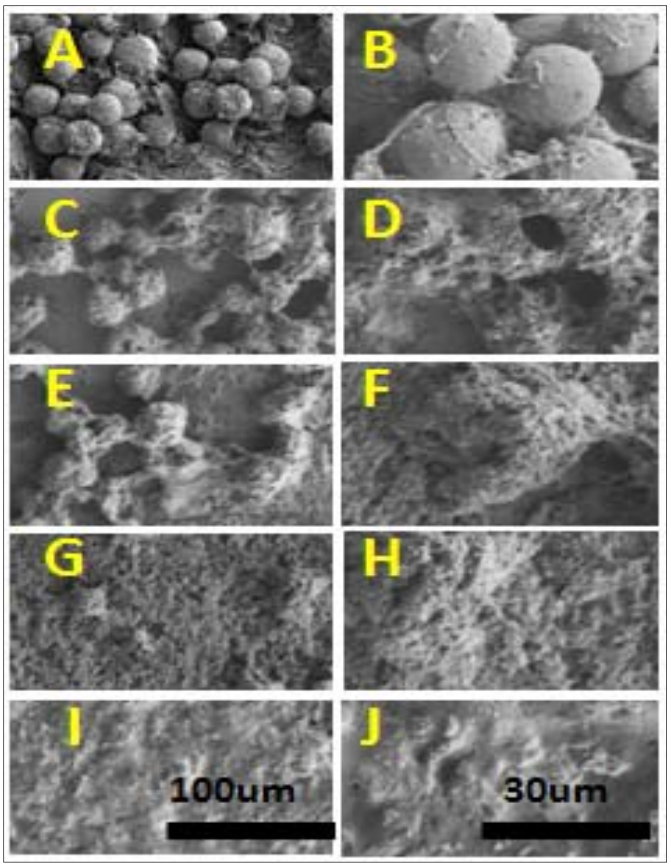


Fig. 9-2 SEM micrographs of *Dunaliella* cells, untreated (A and B); after SC-CO_2 exposure at 100 bar (C and D), at 150 bar (E and F), at 200 bar (G and H) at 50 ^{o}C; and after sonication in ~ 14 % chloroform, 85 % water v/v (I and J). (Magnification X500 for A, C, E, G and I) and (Magnification X1500 for B, D, F, H and J)

9.3.1.2 Glycerol extracted

The benchmark value of the quantity of glycerol that could be extracted from the cells using 8 min. sonication in ~16 % chloroform, without involvement of SC-SCO_2, was 726.5 ± 4.0 mg/g DW. This value was used to assess the efficacy of SC-CO_2 treatments to extract glycerol, instead of using sonication. Thus after SC-CO_2, treated pellets were brought to ~16 % v/v with respect to chloroform and assayed for glycerol using sodium periodate as before (3.5.1). Figure 9.3 shows the experimental yields for the extraction of glycerol from *Dunaliella* T35 using SC-CO_2 under different conditions of pressure, temperature and time; Figure 9.4 shows the kinetics of extraction as a function of pressure and Figure 9.5, the effect of temperature as a function of pressure.

At 50 ^{o}C an increase in pressure from 100 to 150 bar led to a progressive increase in the glycerol extraction yields, which increased again when the pressure was 200 bar. The amount of glycerol extracted increased significantly ($p<0.001$) with pressure from 100 to 200 bar, (See Appendix A5.1 for details of statistical analysis). This trend can be attributed to the variations in diffusivity and density of the solvent system with pressure and temperature (Macias-Sanchez *et al*., 2009) and to increase of *Dunaliella* cell rupture at higher pressures.

The difference in mean amount of glycerol extracted at 200 bar among three different temperatures (40, 45 and 50 ^{o}C) indicated that there is no statistically significant difference ($p>0.05$). This indicates that the variation in the amount of glycerol extracted largely depended on the pressure rather than the temperatures investigated except for 40 ^{o}C at 15 and 30 min.

Cells exposed to 200 bar pressure resulted in the largest amount (735.9 mg/g DW) of glycerol extracted. This value is the same as that estimated in the cells using sonicatation and chloroform (3.5.1) (726.5 ± 4.0 mg/g DW). There was no significant difference in glycerol extraction yield between 60 and 90 min at 200 bar.

Taken together with the results of SEM analysis, these results show that pressure applied during SC-CO_2 extraction is more important than temperature in disrupting *Dunaliella* cells. Moreover confluence in the level of glycerol that could be extracted by this method strengthens the level of confidence in the methodology used in routine laboratory practice to analyse glycerol from *Dunaliella.*

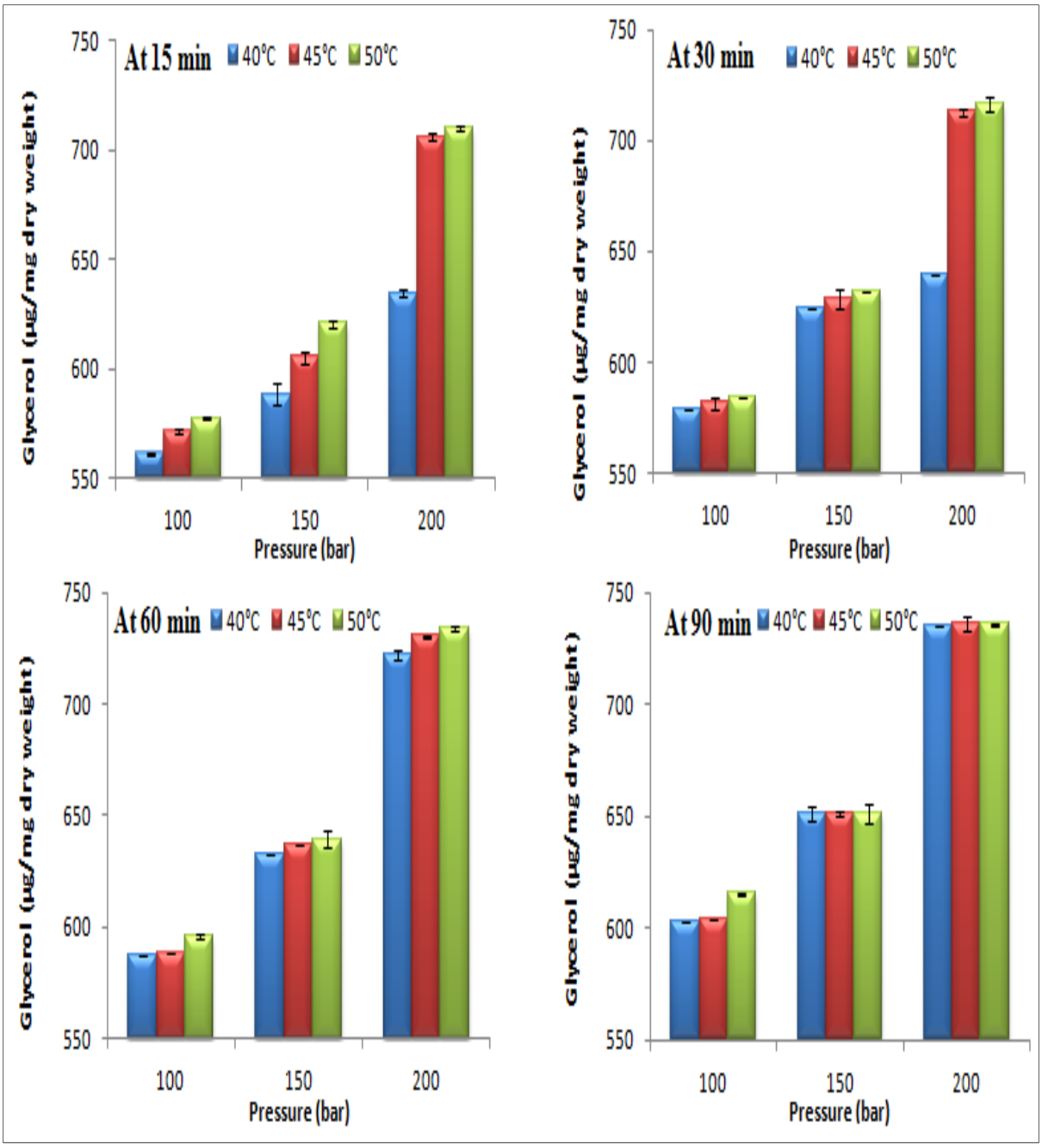


Fig. 9-3 The SC-CO_2 extraction of glycerol from *Dunaliella* T35 at different pressure. The cells were cultured for 14 days at 1.0 M NaCl at 25 ^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. After 14 days of cultivation, microalgal pellets were harvested by centrifugation and exposed to 4.0 M NaCl for 160 min prior to the SC-CO2 experiment. Error bars represent the standard deviations (n=3) of measured values of glycerol content

9.3.1.3 Effect of extraction time and pressure

The mean of values for extraction time from 15 - 90 min and over the temperature range 40 - 50 ^{o}C at different pressure is shown in figure 9.4. Most of the glycerol (depending on the pressure, ~78-94 % of the benchmark value) was extracted within the first 15 min of applied pressure with SC-CO_2. The maximum yield of glycerol (100 – 100 % of the benchmark value) was obtained using 60 min extraction with SC-CO_2 and 200 bar pressure and any further increase in exposure time made no significant difference. At 200 bar an increase in time from 30 to 60 min led to a further increase (~5 %) in the glycerol extraction yield. Hence, it can be concluded that the optimum exposure time for cell rupture is 60 min at 200 bar.

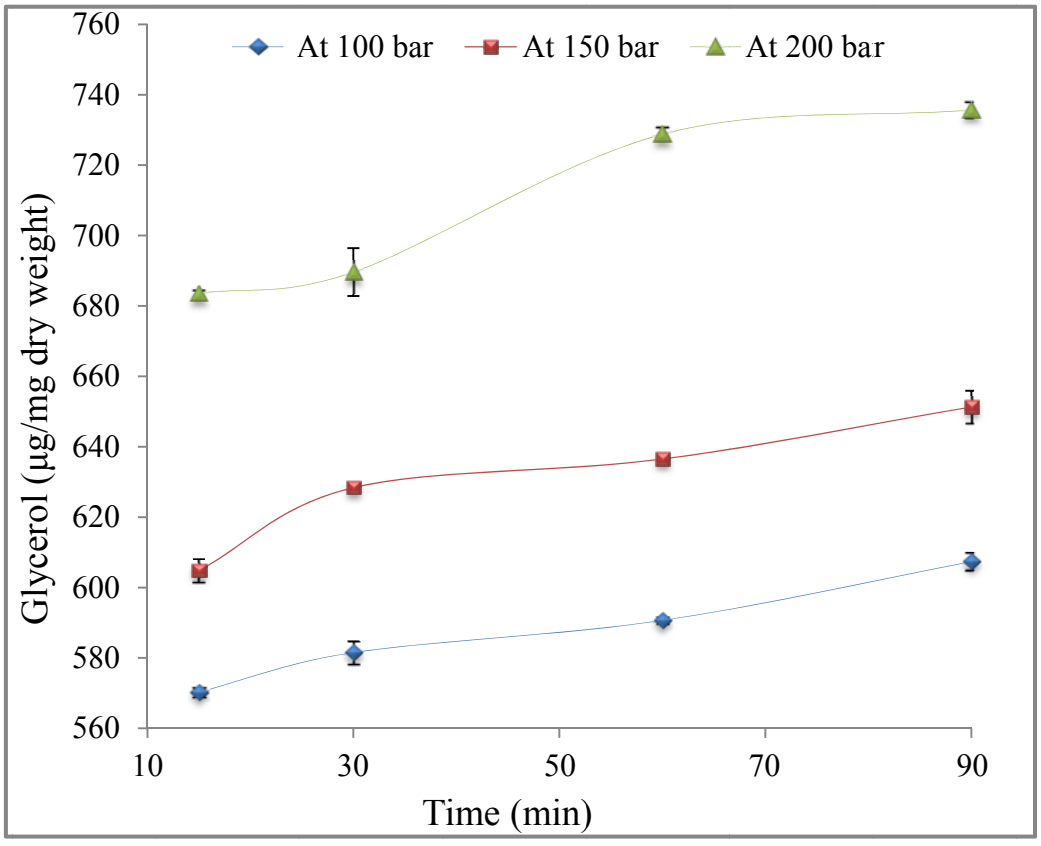


Fig. 9-4 Effect of mean dynamic time at mean temperature on the extraction of glycerol at different pressures. Error bars represent the standard deviations (n=3) of measured values of glycerol content

9.3.1.4 Effect of temperature and pressure

The mean of values for extraction temperature 40 - 50 ^{o}C min and over the time range 15 - 90 min at different pressure is shown in figure 9.5. At the pressure of 100 and 150 bar, a change in temperature from 40 to 50 ^{o}C shows that there is no significant difference in the amount of glycerol extracted ($p>0.05$). However, there was an increase from 682.9 to 724.2 (mg/g DW) with increase in temperature from 40 to 45 ^{o}C at 200 bar (figure 9.5). At 200 bar there is no significant difference between 45 and 50 ^{o}C.

As far as the effect of temperature is concerned, lesser yields were obtained at 100 bar for all operating temperatures studied. At 40 ^{o}C the trend in the experimental results is similar to the trend observed at 45 ^{o}C: there is no increase in glycerol extraction at 100 and 150 bar at both temperatures. At 200 bar, the glycerol extraction yield reached its maximum at the temperature of 50 ^{o}C. Figure 9.5 shows that at 50 ^{o}C the extraction yields increase with pressure.

Both pressure and temperature affected the cell rupture positively. However, it was noticed that as pressure increased from 100 to 200 bar, effect of temperature also increased. Therefore, a small increase in temperature could lead to an increase in cell rupture at higher pressure. At the same temperature, the glycerol yield increases with the pressure. The effect of the temperature is less at 100 and 150 bar of pressures, but at 200 bar an increase in temperature leads to an increase in the yield of extracted glycerol. It can be concluded that the optimum temperature for cell rupture is 45 to 50 ^{o}C.

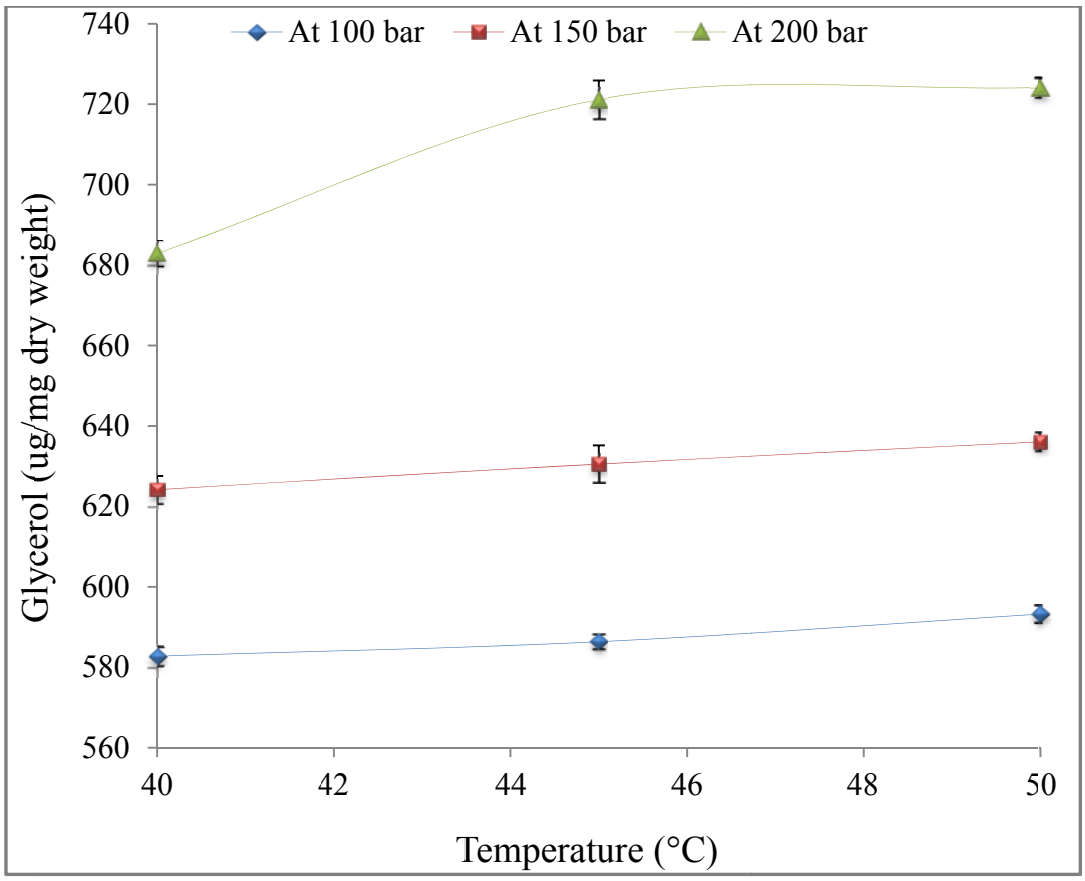


Fig. 9-5 Effect of mean temperature at mean time on the extraction of glycerol at the different Pressure. Error bars represent the standard deviations (n=3) of measured values of glycerol content

9.3.2 Using chloroform as a co-solvent of SC-CO_2 for the extraction of glycerol

The standard method for glycerol extraction described in section 3.5.1 uses sonication of wet pellets containing 60 % w/w culture medium in ~16 v/v chloroform. As one of this chapter's goals was to develop an environmental friendly extraction method, SC-CO_2 experiments were carried out with samples containing smaller volumes of chloroform in the batch extraction system at 50 ^{o}C and 200 bar for 60 min. These experimental conditions were chosen because they are the optimum conditions for the *Dunaliella* cell rupture using SC-CO_2 (see section 9.3.2.1).

Table 9.2 shows that the mean amount of glycerol extracted with SC-CO_2 on wet samples to which chloroform was added to a final concentration of 16 % w/w chloroform (50 % w/w aqueous medium) was 763.4 ± 2.5 (mg/g DW); this was only 5 % greater than the amount

(723.1 ± 3.7 mg/g DW) of glycerol detected using 5 % chloroform (57 % w/w aqueous medium) and is not significant (Table 9.2).

Dunaliella cells disintegrated with the sonication technique using 16 w/w chloroform. The glycerol extracted following sonication with 5 % w/w chloroform is 638.4 ± 2.5, which was 12 % lower when compared to SC-CO_2 rupture under similar condition (5 % chloroform).

Increasing chloroform increased the amount of glycerol extracted for both SC-CO_2 and sonication method. Sonication with 16 % chloroform yielded the same amount of glycerol as SC-CO_2 extraction with 5 % chloroform.

Table 9-2 Effect of chloroform concentration on glycerol extraction from *Dunaliella* T35 using SC-CO_2 under optimum conditions (200 bar and 50 ^{o}C at 60 min) and sonication method

Method of cell rupture	Chloroform % (w/w)	Glycerol (mg/g DW)
SC-CO_2	5	723.1 ± 3.7
SC-CO_2	16	763.4 ± 2.5
sonication	5	638.4 ± 2.5
sonication	16	726.5 ± 4.0

9.3.3 Effect of water content on the extraction of glycerol

Microalgae cells are harvested in aqueous medium. *Dunaliella* cell pellet used for the experiment presented in section 9.3.2 contained 60 % aqueous medium with 4.0 M NaCl (w/w). It is important to determine if the water alone (aqueous Johnson's medium) without chloroform will affect glycerol extraction efficiency. This experiment (pathway 3, see Figure 9.1) was conducted to investigate whether an increase in aqueous medium added to the harvested cell pellet will lead to an increase in cell rupture. A set of experiments was conducted to evaluate the impact of aqueous medium content on extraction efficiency of glycerol using SC-CO_2. Figure 9.6 shows that yields of 367.3 ± 2.2 and 367.4 ± 0.8 glycerol

mg/g DW were obtained from samples treated with SC-CO_2 (using optimum conditions for cell rupture; 50 ^{o}C, 60 min and 200 bar) containing 80 and 100 % medium in the cell pellet, respectively. In these conditions 9 % more glycerol was obtained in comparison to the samples containing 60 % medium w/w (334.4 ± 2.1 glycerol mg/g DW). The efficiency of the extraction is not statistically significant when the amount of medium was increased from 80 to 100 % w/w. This study shows that increasing amount of medium to the microalgae pellet from 60 to 80 % leads to an improvement in the extraction yield.

However, these experiments also confirm that the optimum extraction conditions (50 ^{o}C and 200 bar, for 60 min) to achieve complete cell rupture do not result in complete glycerol extraction compared to extraction procedures that incorporate chloroform. However repetition of the extraction cycle at optimum conditions for cell rupture may result in higher glycerol yield. To assess this possibility, a sample containing 80 % medium w/w was extracted twice at 200 bar and 50 ^{o}C for 60 min. After the first SC-CO_2 treatment the mean amount of glycerol extracted was 367.3 ± 2.2 (mg/g DW). The pellet was re-extracted in SC-CO_2 after addition of 80 % fresh medium (w/w), which resulted in an extra 104.8 ± 3.5 mg/g DW. The sum of glycerol obtained from both first and second extraction (472.1 mg/g DW) is 38 and 34 % lower than 763.4 ± 2.5 mg/g DW and 723.1 ± 3.7 mg/g DW of the amount of glycerol determined using 16 and 5 % chloroform, respectively (see section 9.3.3).

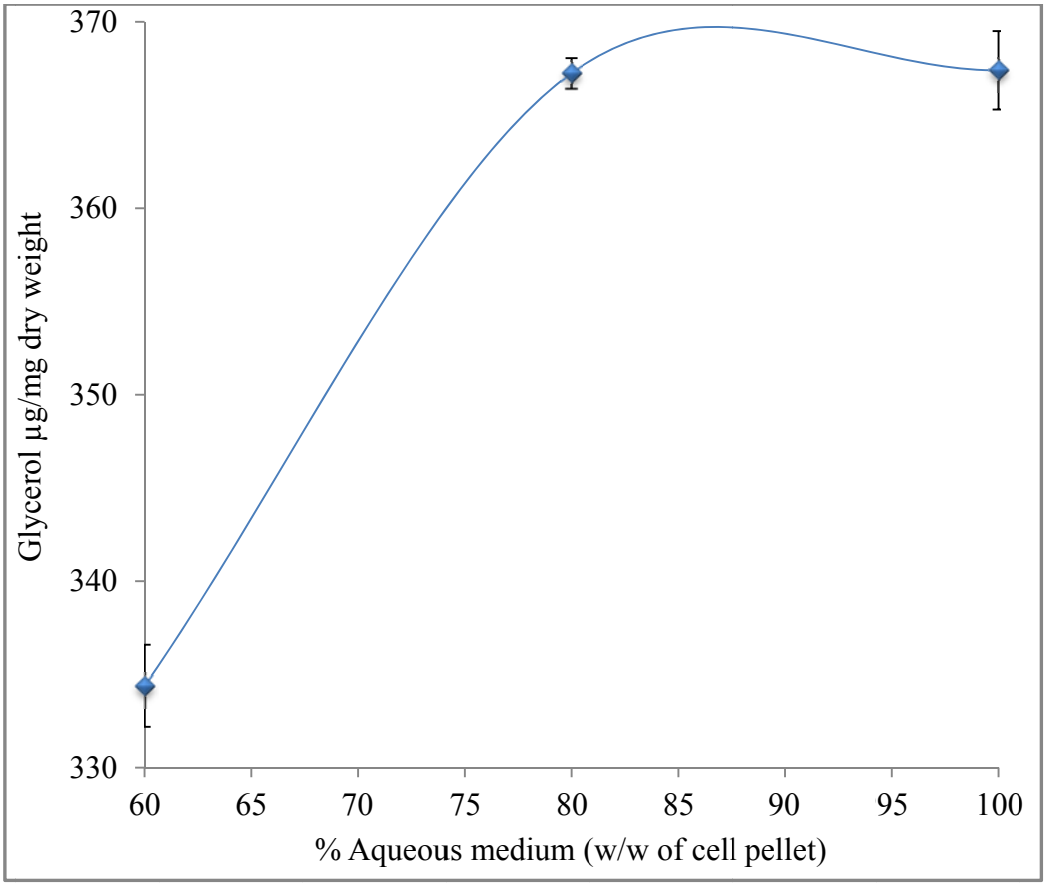


Fig. 9-6 Effect of aqueous medium content on glycerol extraction from *Dunaliella* using SC-CO_2 treatment. The extraction conditions were 50 ^{o}C, 60 min and 200 bar. Error bars represent the standard deviations (n=3) of measured values of glycerol content

Effect of increasing SC-CO_2 pressure: The purpose of the experiments was to investigate if further increase in pressure above 200 bar would improve the glycerol extraction efficiency with aqueous medium. A set of experiments was conducted at 250 and 350 bar using samples containing 80 % aqueous medium (w/w), without chloroform addition. Figure 9.7 shows that at 350 bars the amount of glycerol extracted increased by 14 % in comparison with glycerol extracted at 200 bar under optimized conditions (50 ^{o}C and 60 min). The amount of glycerol extracted from SC-CO_2 at 200, 250 and 350 bar were 367.3 ± 5.8, 397.8 ± 3.1 and 428.1 ± 8.5 (mg/g DW) respectively. The amount of glycerol extracted was greater than the amount extracted by 8 min sonication without chloroform. Figure 9.7 clearly suggests that increase in extraction pressure has a significant effect on the extraction efficiency. It can be seen that

increase of pressure from 200 to 350 bar shows an increased in glycerol extraction by 14 %. Further increase in pressure was not considered.

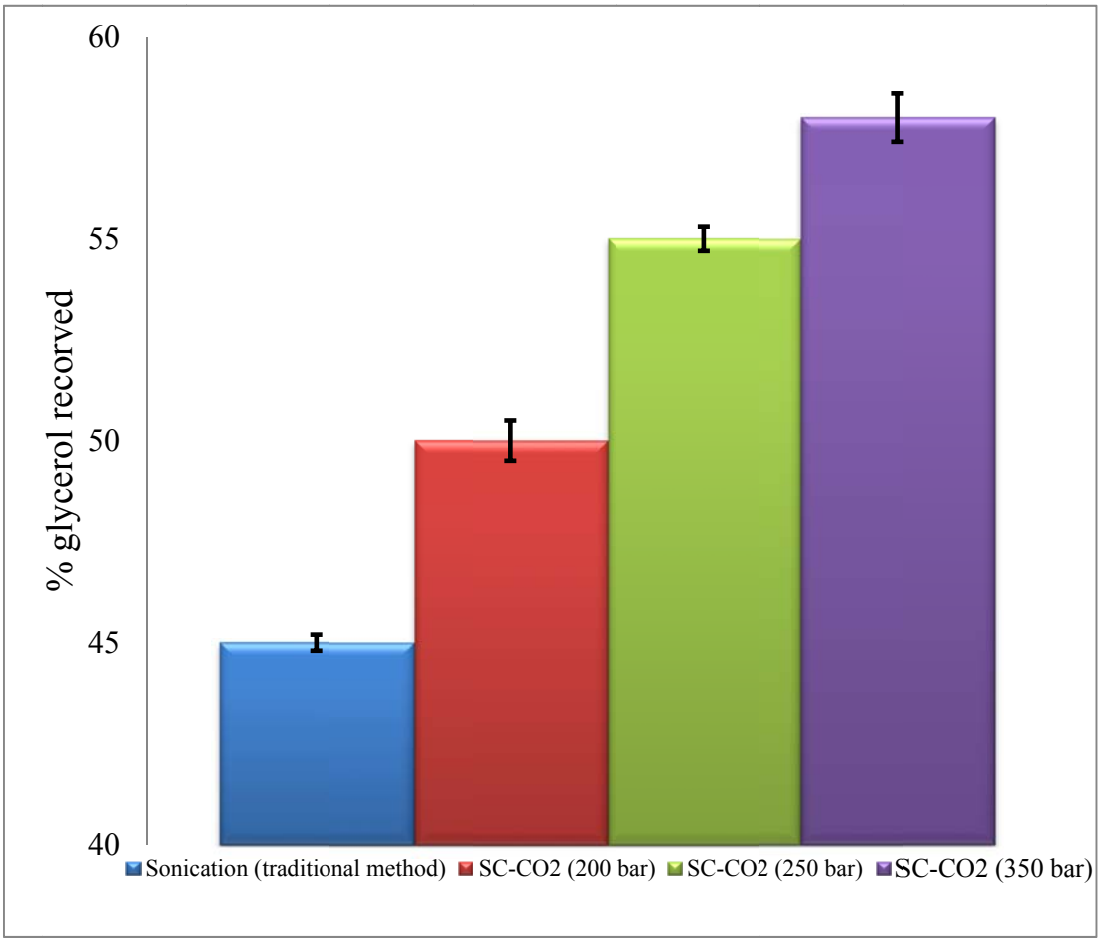


Fig. 9-7 Effect of increasing SC-CO_2 pressure on glycerol extraction from *Dunaliella* in comparison with sonication in the absence of added chloroform. The data represent the mean (± SE) of three different experiments.

9.3.4 Analysis of chloroform phase after SC-CO_2 exposure

Determination of the amount of pigment (chlorophylls and carotenoids) in the chloroform layer after SC-CO_2 extraction represents an alternative method to assess the degree of cell rupture. This experiment was conducted in order to explore whether both the chlorophylls and carotenoids content would be extracted simultaneously with glycerol in the chloroform layer after SC-CO_2 extraction. The experimental conditions for SC-CO_2 extraction used in

this experiment were the same as those reported in section 9.3.2. The absorbance was measured using wavelengths of 350 – 900 nm (figure 9.8).

Both chlorophylls and carotenoids were identified using their characteristic absorption maxima in chloroform (Wellburn, 1994). Similar to Wellburn's observation, chlorophyll exhibited four peaks, at 420, 460, 612 and 668 nm (figure 9.8). The peaks in the absorbance profile at 420 and 612 nm, 460 nm and 668 nm and 540 nm are characteristic for the presence of chlorophyll *a* and *b* of *D. salina* and β-carotene globules from *D. bardawil*, respectively, as reported by Ben-Amotz *et al*. (1982) and Kleinegris *et al*., (2010). Chlorophyll *a* and *b* concentrations were determined using the equations reported by Lichtenthaler (1987) and Wellburn (1994) (see section 3.2.6). The content of carotenoids was measured at 450 nm using the standard extinction coefficient value (2500 M^{-1} cm^{-1}). Table 9.3 shows the amount of carotenoids and chlorophyll determined from samples exposed to both the sonication and SC-CO_2 extractions. This result show that there is more cell rupture in SC-CO_2 treated samples than those treated with sonication in the presence of chloroform.

Table 9-3 The amount of chlorophylls and carotenoids determined from chloroform phase after SC-CO2 extraction at 200 bar, 50 ^{o}C for 60 min.

Method of cell rupture	**Chl a (µg/ml)**	**Chl *b* (µg/ml)**	**Carotenoids (µg/ml)**
Sonication	4.0 ± 0.12	1.0 ± 0.10	2.2 ± 0.15
SC-CO2	4.5 ± 0.15	1.2 ± 0.15	2.5 ± .013
% Recovery for SC-CO_2	113	120	114

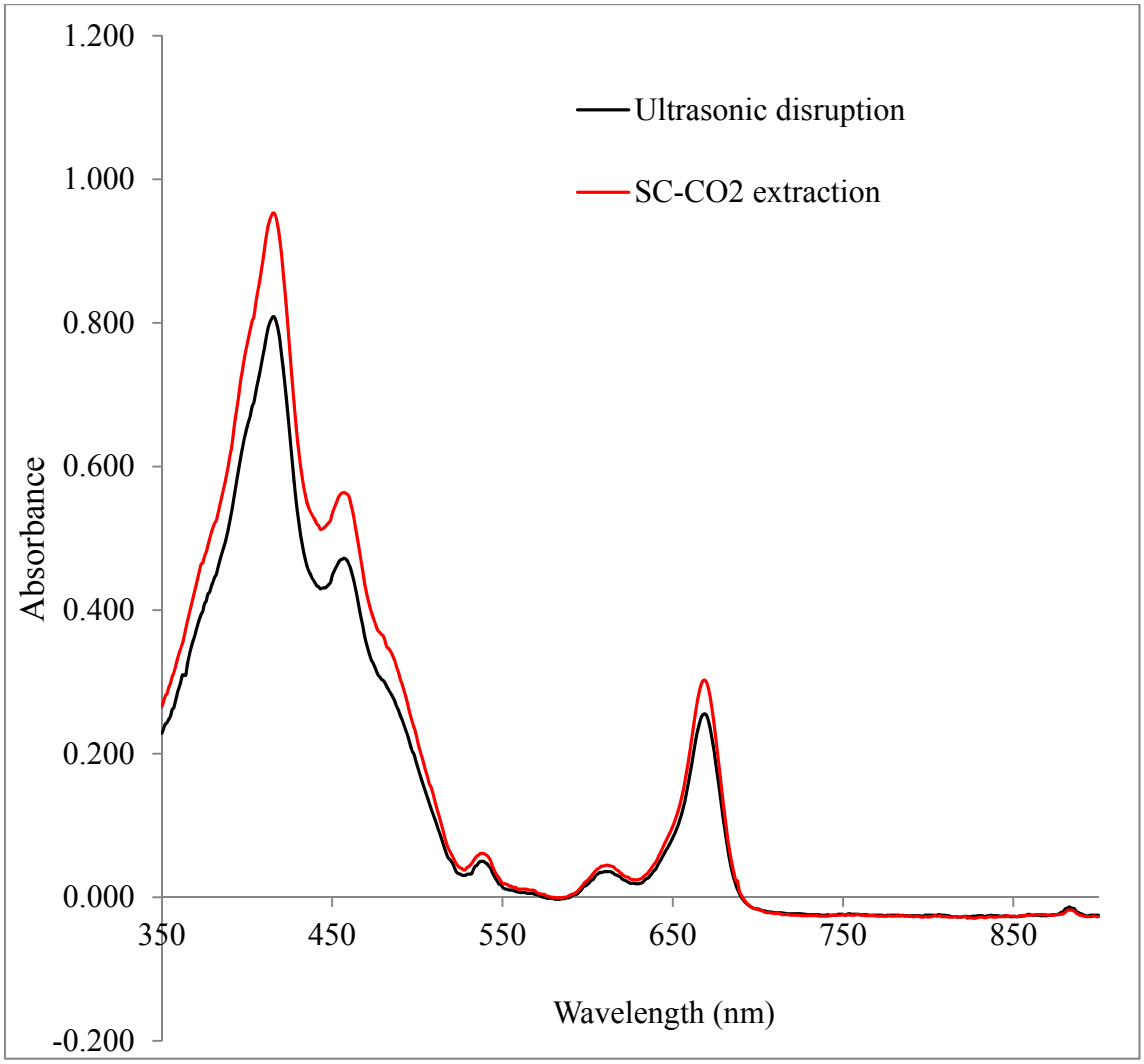


Fig. 9-8 Absorbance spectrum of chloroform phase of *Dunaliella* treated with SC-CO_2 and after ultrasonic disruption. Peaks at 420, 460, 612 and 668 nm are consistent with the presence of chlorophyll. The peaks at 540 nm are consistent with the presence of carotenoids.

9.3.5 Quantification of glycerol using GC-MS

The chromatogram shown in Figure 9.9 represents samples treated using SC-CO_2 at 350 bar at 50 ^{o}C for 60 min. Similar chromatograms were observed for GC-MS on 3 different replicates, which indicates reproducibility of the results (see Appendix A5.2 for other chromatographs). Table 9.4 shows the mean glycerol concentration from all the samples investigated. This result shows a variation in the concentration of glycerol determined. The 350 bar pressure proved to be more efficient in disrupting the cells. The amount of glycerol determined by GC-MS analysis (Table 9.4) is similar to the amount determined using the

chemical method (see section 9.3.2.1. This indicates that both methods can be reliably used for glycerol estimation in *Dunaliella*. In addition to glycerol other compounds were observed, as evidenced by the chromatogram presented in Figure 9.9. Detailed identification and quantification of those compounds were presented in chapter 8.0.

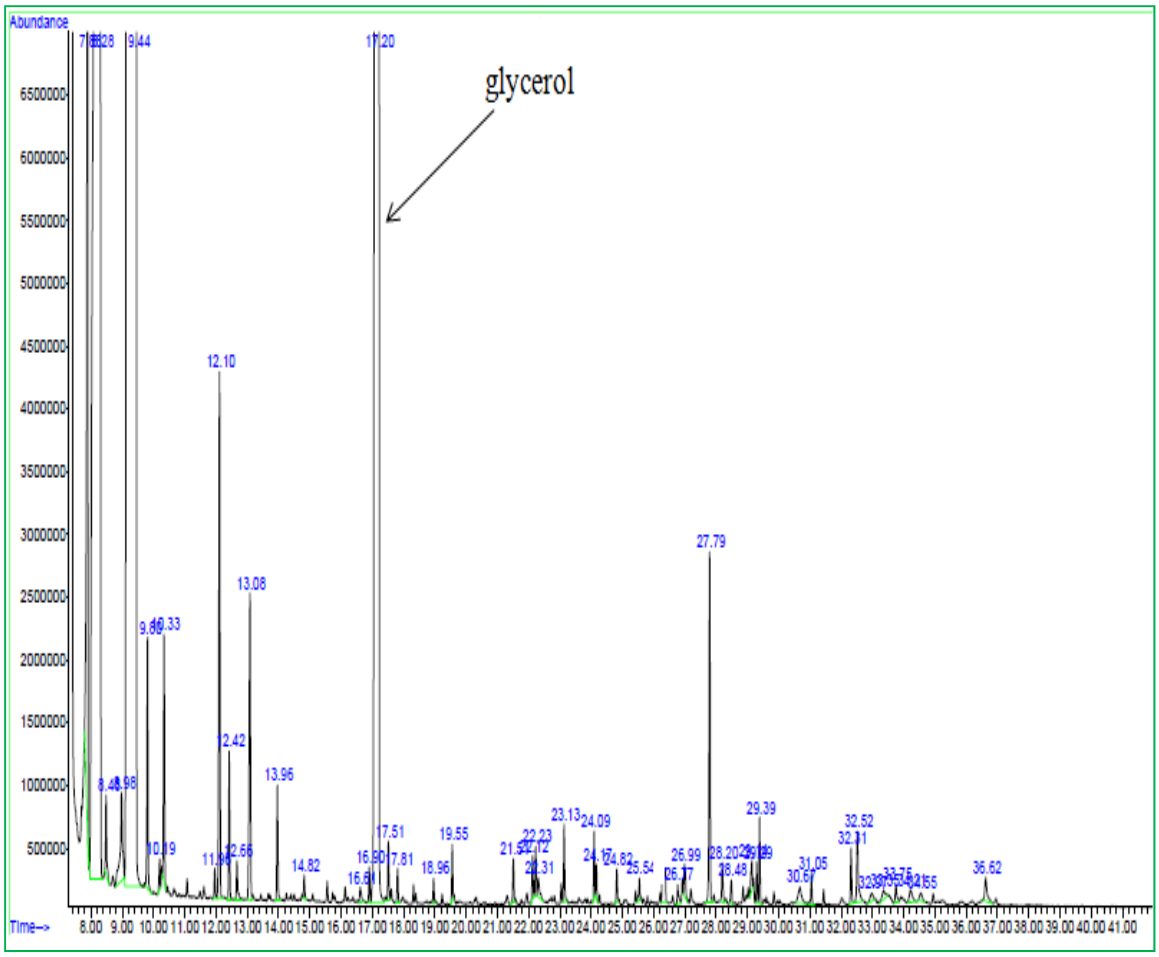


Fig. 9-9 Chromatogram of samples treated with SC-CO_2 at (350 bar, 50 ^{o}C and 60 min), which showed that derivatized glycerol was successfully separated from other components occurring in the sample.

Table 9-4 The amount of glycerol determined from GC chromatogram and compared with chemical method

SC-CO_2 extraction pressure (bar)	Glycerol (µg/ml)	Mean glycerol (mg/g DW). GC-MS method	Glycerol (mg/g DW). Chemical method
350	213.3 ± 3.2	761.7	-
250	196.9 ± 3.5	703.1	-
200	177.5 ± 4.5	633.7	735.9

9.4 Discussion

The experiments discussed in this chapter provide an important insight into methods for glycerol extraction using SC-CO_2. Routine analysis of the glycerol content in cells of *Dunaliella* with sodium periodate requires that the cells are thoroughly disrupted and glycerol extracted, usually achieved using 8 min sonication of the cells in 16% v/v chloroform. In this work, SC-CO_2 was applied under different conditions of temperature, pressure and time to cells in either the presence or absence of chloroform, and the yield of glycerol extracted assessed either with or without further sonication and chloroform addition. The two main approaches are either cell disruption using SC-CO_2 followed by chloroform extraction and sonication to assess the yield of glycerol recovered or addition of chloroform to the samples as part of the SC-CO_2 experiment. Using a mixture of SC-CO_2 and 16 % chloroform recovered more glycerol than treatments based solely on the use of chloroform (and sonication). The use of SC-CO_2 can be considered as environmental friendly because the amount of chloroform used can be recollected and reused in the extraction, and lesser amounts are required for complete extraction.

The effectiveness of microalgal glycerol extraction is known to increase with the degree of cell disruption. When intact cells are disintegrated during SC-CO_2 extraction, cellular glycerol is liberated from the cell and released into the surrounding medium. SC-CO_2 extraction of glycerol from *Dunaliella* requires aqueous medium in the microalgal pellet for

the successful cell rupture. For this reason, SC-CO_2 extraction was conducted with microalgae wet pellet. Figure 9.2 shows that SC-CO_2 was able to rupture must of the cells with 60 % w/w aqueous medium contents of the cell pellet.

The results of this research on the extraction of glycerol by means of SC-CO_2 allowed the evaluation of the factors that have to be considered in trying to apply the method on a large scale. The amount of CO_2 needed depends to a large extent on the pressure and temperature used during extraction. Section 9.3.2.1 shown that the SC-CO_2 at pressure of 200 bar and a temperature of 50 ^{o}C and 60 min, are suitable for rupturing *Dunaliella* cells for glycerol extraction. The yields of glycerol that can be extracted with SC-CO_2 are larger compared to those obtained by ultrasonic disruption method.

Accumulation of carotenoid in *D. salina* and *D. bardawil* has been extensively studied, and it is was reported that it is triggered by high salinity (Borowitzka *et al*., 1990) and under high temperature and high irradiance Ben-Amotz *et al*., 1988; Araneda *et al*., 1992). The accumulation of total carotenoids in other species of *Dunaliella* per volume of cultures is reported with (10.6 to 13.3 µg/ml) by *D. salina* grown in 3.4 M NaCl (Aguilar, *et al*., 2004) and (5.18 to 9.52 µg/ml) by *Dunaliella sp* grown in 1.0 to 3.0 M NaCl (Rad *et al*., 2011). The values of carotenoid reported in the present work were much lower, but may reflect the fact that in the present experiments, cells were not treated to encourage the synthesis of carotenoids but rather, glycerol.

Macias-Sancheza *et al*. (2009) compared SC-CO_2 and ultrasound-assisted extraction of carotenoids and chlorophyll a from *Dunaliella salina* and showed that the best extraction yields were obtained at a temperature of 60 ^{o}C when the operation was carried out at 300, 400 and 500 bar. According to them the behaviour was attributed to the fact that at these pressures (300, 400 and 500 bar) the density of the carbon dioxide is greater and, at the same time, the increase in temperature causes increases in the solvent diffusivity and the vapour pressures of the compounds being extracted, thus favouring their dissolution and giving better extraction yields.

GC-MS analysis confirmed the identity and amount of glycerol measured using the chemical method making both methods reliable in glycerol quantification. This study demonstrates that SC-CO_2 extraction can successfully be applied to *Dunaliella* cells for cellular membrane

rupture and glycerol extraction. Optimizing SC-CO_2 extraction conditions can contribute to obtaining glycerol from microalgae and developing an environmentally friendly extraction method that is very important from a green technology perspective. The best extraction yields of glycerol using *Dunaliella* T35 cells as the raw material were obtained at the maximum operating temperature (50 ^{o}C) and at a pressure of 200 bar when chloroform was used as co-solvent of extraction. It was also demonstrated that glycerol could be extracted at a pressure of 350 bar without the addition of chloroform. Although environmental friendly, the level of glycerol extracted was lower than with the addition of chloroform.

CHAPTER 10.0: SUMMARY AND FUTURE WORK

10.1 Summary

The aim of this research was to characterise the growth and production of glycerol in two novel halophilic microalgae species *Dunaliella* (T35, T36 and T37) and *Asteromonas* (T33a, T33b and T33c) that had been isolated from saline river of Namibia.

This research highlights the remarkable diversity of halotolerant microalgal strains that exists even within the same physical location. The collection of strains, *Dunaliella* and *Asteromonas*, isolated from the saline river of Namibia, differed not only in their genetic construction, but also expressed differences in response to different environmental conditions. Temperature had a profound effect on growth compared to salinity and pH. The temperatures tested were 15, 20, 23, 25, 30, and 35 °C, where 30 °C was considered as an optimum for growth (increase in cell number) for both strains of *Dunaliella* and *Asteromonas* isolated from Namibia. This optimum temperature reflects the environmental condition of Namibia. Different strains of *Dunaliella* and *Asteromonas* have different optimum temperature for their growth which are best suited to the local environment. The UK strains (*D. salina, D. quartolecta, D. parva and D. polymorph*a) grown alongside with the Namibian strains produced different results at 30 ^{o}C; but have the optimum growth at 20 ^{o}C. The optimum temperature for growth of *Dunaliella* across the globe range from <0 to 40 °C (Gimmler *et al*., 1978; Siegel *et al*., 1984; Borowitzka, 1981) depending on the their origin.

Investigation of the cell growth kinetics by cell density determination confirmed that the rates of cell growth of Namibian strains, *Dunaliella* and *Asteromonas*, reached a maximum level in media of 1.0 M NaCl than either lower or high salt concentrations. By contrast all the four UK strains investigated had maximum growth rates at 2.0 M NaCl. This study confirmed that Namibian strains, *Dunaliella* and *Asteromonas*, prefer an intermediate but still saline medium. It also indicated that there is no apparent requirement for high salt concentration for growth but, rather, being both halotolerant and halo-dependent. The optimal salinity for growth is different in various species. *Dunaliella* cells demonstrate a remarkable degree of adaptation to salinity in their natural habitats, growing in media ranging from 0.05 M to

saturation 5.0 M NaCI depending on the species. It is generally believed that *Dunaliella* and *Asteromonas* species respond to salt stress by synthesis or elimination of intracellular glycerol. But the challenges remain the choice of optimum centrifugal force for harvesting intact cells and sensitive method to quantify glycerol.

Efforts were made to find the suitable centrifugal force for harvesting of *Dunaliella* cells. Results have shown that 3000 to 5000 g were suitable for harvesting intact cells of *Dunaliella* T35 and *D. salina* (temperate strain). Harvesting efficiency of 99 % intact cells was achieved at 3000 g. This differs profoundly from the centrifugal force used by other research in harvesting other strains of *Dunaliella* across the globe. As pointed out in chapter 5.0 other studies used high centrifugal force (up to 10,000 g) to harvest *Dunaliella* cells. This study considered 3000 g to be the best centrifugal force for harvesting intact cells of both Namibian and temperate strains of *Dunaliella*.

Growth studies on Namibian strains of *Dunaliella* and *Asteromonas* showed that NaCl concentrations favouring maximal glycerol accumulation are not the same as those required for maximal microalgal productivity. Maximum glycerol yield occurred at 3.0 and 4.0M NaCl concentration whereas maximum microalgal productively occurred at 1.0 M NaCl concentration. This study developed a two stage approach that involved growing the cells at their preferred salinity of 1.0 M NaCl to stimulate biomass productivity, and then to induce high glycerol production the cells were harvested and transferred to medium that contained 4.0 M NaCl, this caused hyperosmotic stress and the high production of glycerol.

Dunaliella and *Asteromonas* cells were grown in 1.0 M NaCl to maximize cell density which followed by hyperosmotic osmotic shock to maximize glycerol production. Glycerol synthesis in *Dunaliella* T35 reaches it maximum at 160 to 240 min after the salinity is increased. *Dunaliella* T36 produced the highest amount of glycerol over other strains and was therefore considered as the most favourable strain for the large production of glycerol. A 3.0 and 4.0 M NaCl were best media for higher glycerol accumulation and *Dunaliella* T36 is considered as best strain in terms of maximum glycerol accumulation and cell density.

Using glycerol amount per ml as benchmarks, this research identifies *Dunaliella* T35, T36 and T37 as well as *Asteromonas* T33c as the strains of choice from other *Asteromonas* (T33a and T33b) and *D. salina, D. quartolecta, D. parva and D. polymorph*a cultures investigated.

The amount of glycerol produced by Namibian strains of *Dunaliella* was 2.7 to 4.2 fold high when compared to *D. salina, D. quartolecta*, *D. parva* and *D. polymorpha* species grown under the same salinity concentration. Generally glycerol production in *Dunaliella* after hyper or hypo-osmotic shock is correlated to change in cell volume. The shrinkage of cells upon exposure to different salinity concentrations is correlated with accumulation or glycerol, the most important osmotically active intracellular solute in *Dunaliella*. Previous research shows that when *Dunaliella* cells were transferred from low to high salinity medium they rapidly shrink due to water efflux from the cell and then slowly return to their original volume by accumulating intracellular glycerol.

Changes in cell volume and glycerol content following hypertonic shock shows that when *Dunaliella* and *Asteromonas* Namibian strains were transferred to high salinity medium they rapidly shrink due to water efflux from the cell and then slowly return to their original volume for the cells that were exposed to 1.0 and 2.0 M NaCl only, whereas cells exposed to 3.0 and 4.0 did not return to the original volume for both 14 days old cultures and those exposed to 20 min hyperosmotic stress. It was also determined that the cells cultivated at 3.0 and 4.0 M NaCl for 14 days have larger volume than corresponding cells exposed for 20 min under the same salinities. This suggested that *Dunaliella* and *Asteromonas*, Namibian strains, require more than 20 min to attain certain volume at high salinity. This study also shows that smaller cells (cells exposed to 4.0 M NaCl concentration) accumulate more glycerol per unit volume than larger cells (cells exposed to 1.0 M NaCl concentration). In addition to glycerol *Dunaliella* cell may produce other metabolites that may help in surviving osmotic stress and to support glycerol production.

Studies were conducted to assess the level of other metabolites and potential osmolytes under salinity stress in constant light and the dark regime. It confirmed some metabolic changes in response to hyper and hypo-osmotic stress. This study confirmed glycerol as the principal compatible osmolyte in halotolerant *Dunaliella* isolated from saline river of Namibia, and showed that, in the short-term response to salt stress, proline and other osmolytes detected are too low to serve the role as additional primary osmolytes in the entire *Dunaliella* cell.

Chloroform is a solvent commonly used for glycerol extraction in the laboratory from *Dunaliella*. This chemical is toxic, hazardous to the environment and its disposal is very

expensive. This research explored more environmentally friendly extraction technique, SC-CO_2 extraction, of glycerol from *Dunaliella* T35 as an alternative to chloroform. Study at optimizing extraction of glycerol examines the use of SC-CO_2 under various conditions of pressure, time and temperature, in the presence and absence of chloroform. Comparison of the results obtained between SC-CO_2 and sonication method (traditional method) shows that the high amount of glycerol was extracted using SC-CO_2 extraction procedure when chloroform was used as co-solvent. Although there is decrease in glycerol yield; SC-CO_2 technique was able to extract 58 % glycerol at pressure of 350 bar without the addition of chloroform. The SC-CO_2 technology haven been successful used for the large scale extraction of β-carotene from *Dunaliella* biomass. However, it is not clear whether SC-CO_2 will be used for glycerol extraction from large scale of *Dunaliella* biomass.

10.2 Future work

This research confirmed that *Dunaliella and Asteromonas* strains from Namibia grow optimally at 1.0 M NaCl concentration, 30 ^{o}C and initial pH of 7.5 to 9.0. Laboratory evaluation of these microalgae shows high production of glycerol by *Dunaliella* strains. However using these strains in large scale production is also a subject matter of future studies. The future work will also consider optimizing growth (increase cell density) of *Dunaliella* and *Asteromonas* using multivariate approach, which will be compared with studying individual factors conducted by this research.

Future work will also investigate molecular mechanism of glycerol biosynthesis and regulation in *Dunaliella* and *Asteromonas* strains obtained from Namibia. This will involve the following: (a) Isolation and characterization of glycerol biosynthetic pathways genes; (b) Gene expression analysis of cells exposed to hyperosmotic shock.

Investigations into stress related proteins and alterations of the gene expressions in salinity stressed microalgae are very important area of research. The future work should focus on exploitation of genetic transformation and metabolic engineering of *Dunaliella* and *Asteromonas* for mass-production of glycerol and high-value proteins such as vaccines, antibiotics and enzymes; this will open an interesting new aspect of microalgae biotechnology in future.

Dunaliella produced glycerol by starch breakdown or by photosynthetic CO_2 fixation. Although stored lipid pools were not analysed in the present study, their degradation may also contribute to the size of the glycerol pool in both *Dunaliella* and *Asteromonas*. Future work will employ nuclear magnetic resonance (NMR) spectroscopy to investigate the amount of lipid accumulated in the cells exposed to hyperosmotic shock.

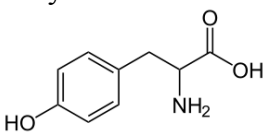
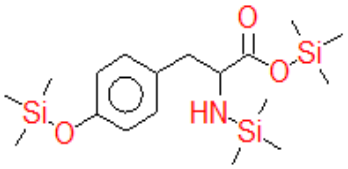
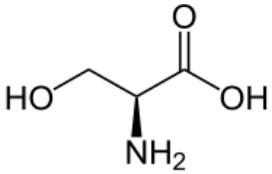
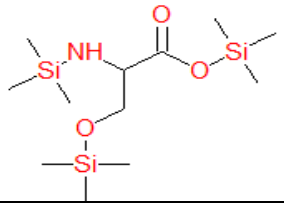
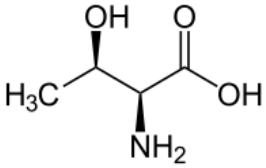
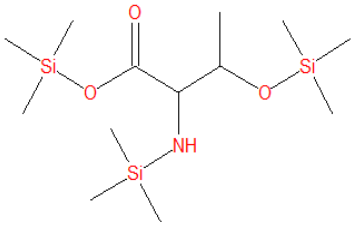
The $SCCO_2$ extraction was used to extract glycerol from *Dunaliella* T35. Although glycerol is the largest compound produced by these microalgae after exposure to hyperosmotic stress (see chapter 6.0 and 8.0), there are many other compounds that were not measured in this study. Other compound such as carbohydrate, proteins and lipid could be included in future analysis.

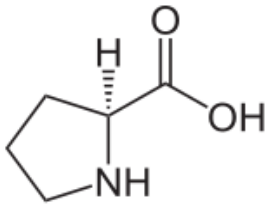
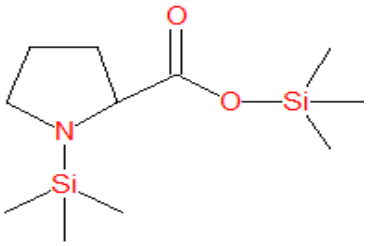
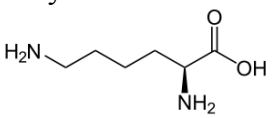
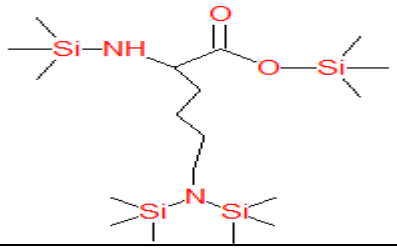
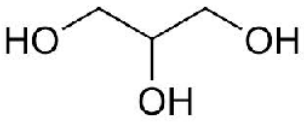
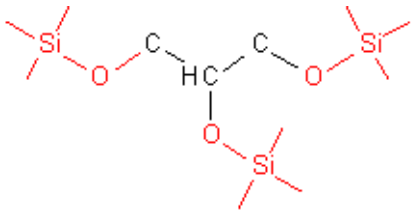
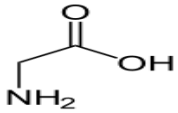
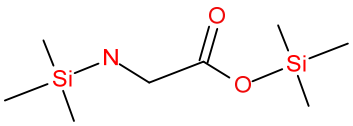
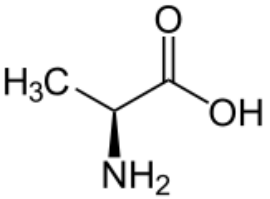
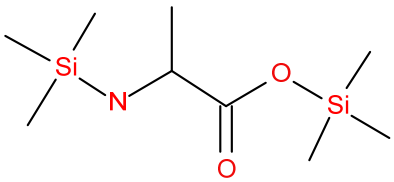
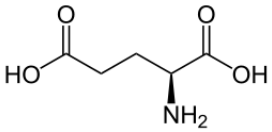
Appendix 1.0

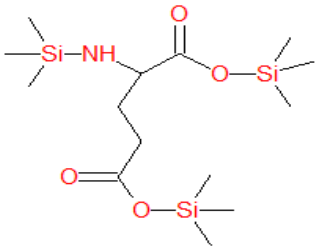
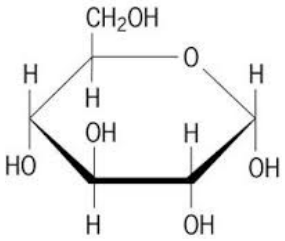
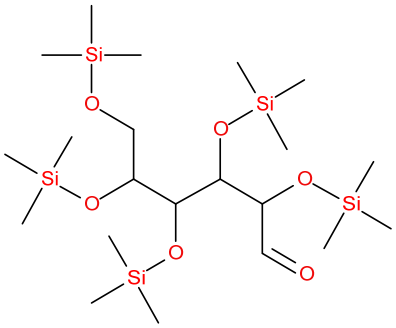
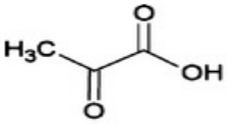
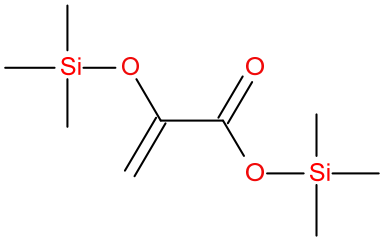
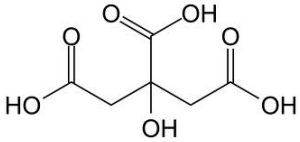
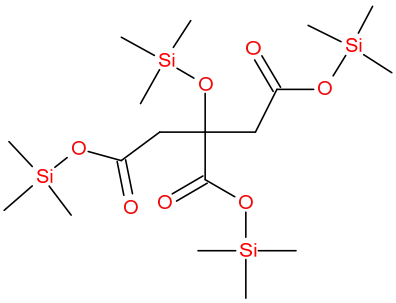
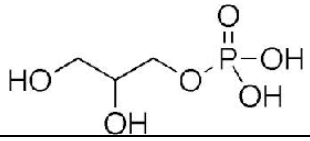
A1.1 Standards and their TMS-derivatives used in identify metabolites from microalgae samples

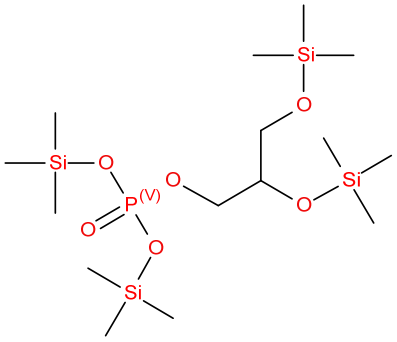
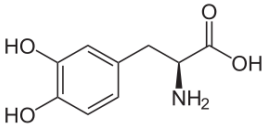
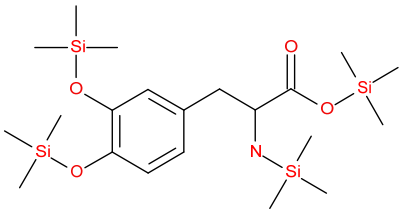
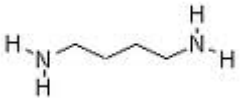
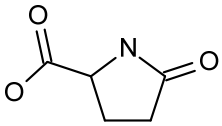
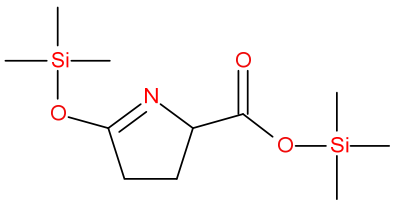
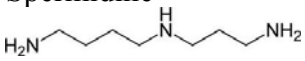
Table A1.1 shows the standards and their TMS-derivatives that were used in identify metabolites from microalgae samples using mass spectra analysis whereas other metabolites were identified by database inspection (GMD and NIST).

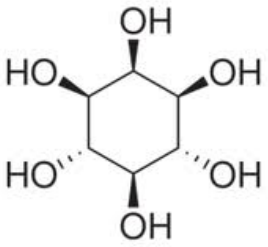
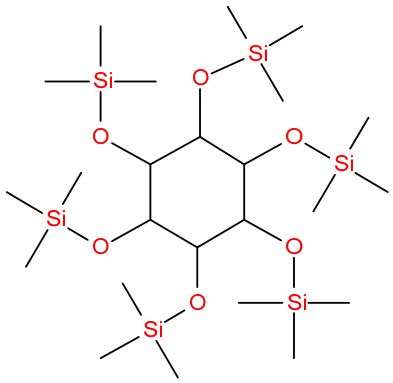
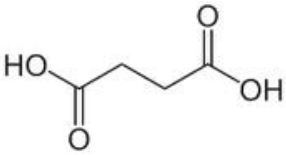
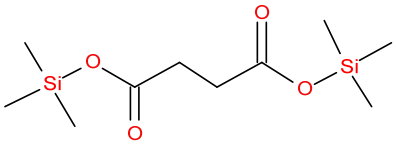
Table A1-1 List of 19 standards and their TMS-derivatives that were used tentatively to identify metabolites from microalgae samples exposed to different experimental conditions using mass spectra analysis

S/N	Retention time (RT) (min)	Compound	Derivative
1	29.02	L-Tyrosine 	3 MTS 
2	18.09	L-Serine 	3TMS 
3	18.68	L-Threonine 	3TMS 

4	16.69	L-Proline 	3TMS 
5	28.75	L-Lysine 	4TMS 
6	16.51	Glycerol 	3TMS 
7	16.86	L-Glycine 	2TMS 
8	19.49	α-Alanine 	2TMS 
9	23.33	L-Glutamic acid 	3TMS

			
10	28.95	D-(+)- Glucose 	**5TMS** 
11	11.02	Pyruvic acid 	2TMS 
12	27.12	Citric acid 	4TMS 
13	26.12	Glycerol phosphate 	4TMS

			
14	31.57	3,4-Dihydroxy-L-phenylalanine (L-DOPA) 	4TMS 
15	25.58	Putrescine 	2TMS
16	21.39	L-Pyroglutamic acid 	2TMS 
17	33.00	Spermidine 	

18	31.67	*Myo*-Inositol 	6TMS 
19	16.91	Succinic acid 	2TMS 

Appendix 2.0

A2.1 Inhibition of *Dunaliella* and *Asteromonas* cell growth by NaCl

Figure A1.1 and A1.2 describes the double reciprocal plot (Lineweaver-Burk plot) constructed from the data. It shows that in *Dunaliella* increasing NaCl concentration from 1.0 to 4.0 M NaCl is inhibitory to growth. Similar to *Dunaliella*, NaCl was inhibitory to the growth of *Asteromonas* cells above 1.0M NaCl as evidenced by double reciprocal plot.

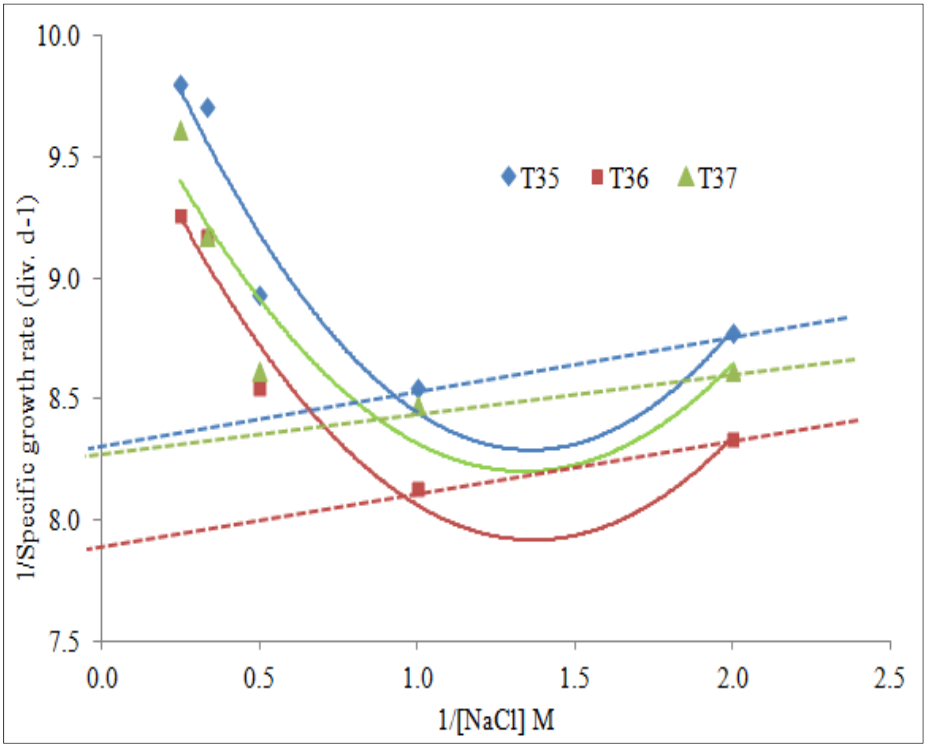


Fig. A1-1 Double reciprocal plot for the effect hyperosmotic shock on the growth of *Dunaliella* strains at 23 ^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium.

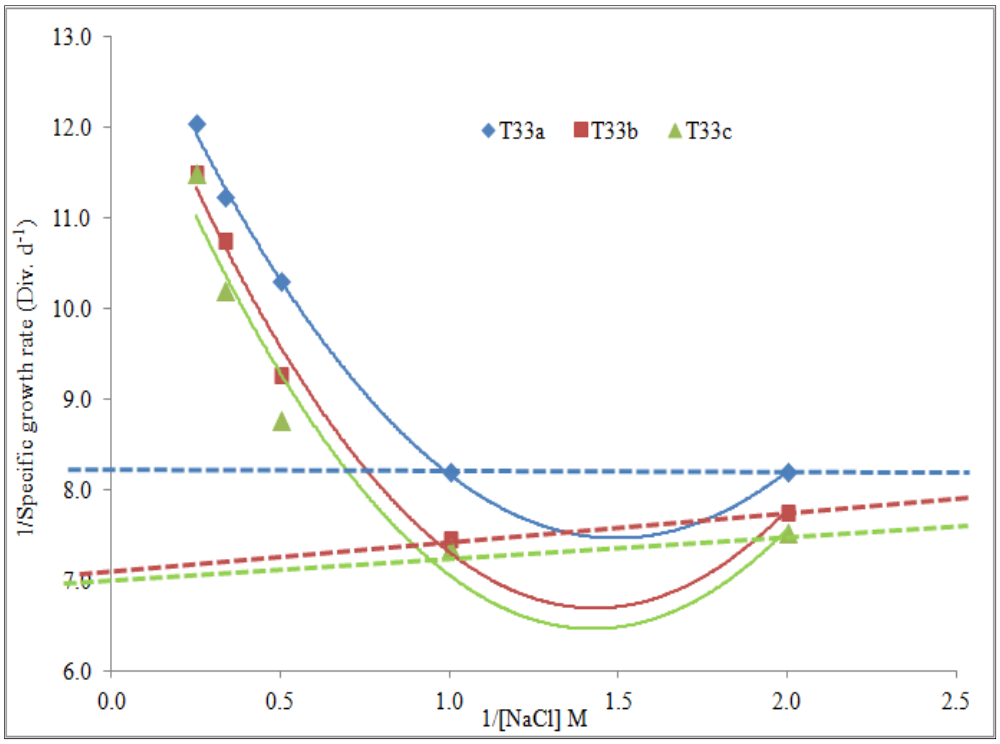


Fig. A1-2 Double reciprocal plot for the effect hyperosmotic shock on the growth of *Asteromonas* strains at 25 ^{o}C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium

Appendix 3.0

A3.1 *Dunaliella* and *Asteromonas* scanning electron microscopy

Figure (A1.3 to A1.7) revealed the morphological characteristics of these microalgae when exposed to 1.0 M NaCl concentration. This study revealed a substantial structure and morphology of the Namibian isolates as seen in the micrographs in. Both *Dunaliella* and *Asteromonas* species possess different textures and unit sizes. The absence of filament in some of the micrographs occurs as the result of cultures age or handling procedure.

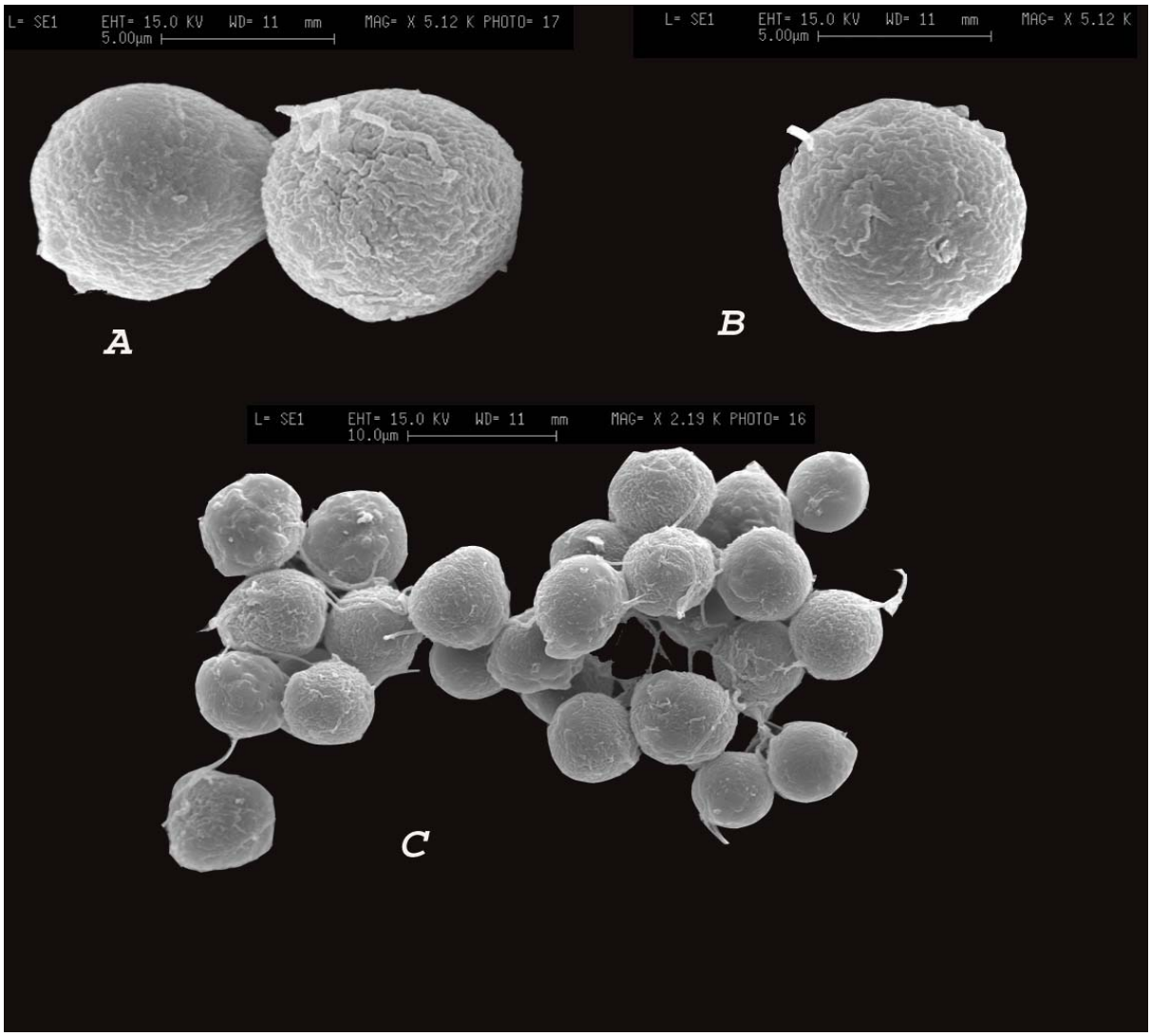


Fig. A1-3 Scanning electron microscopic images of *Dunaliella* T35: (A) (magnification 5120x); (B) magnification 5120x; (C) magnification 2190x

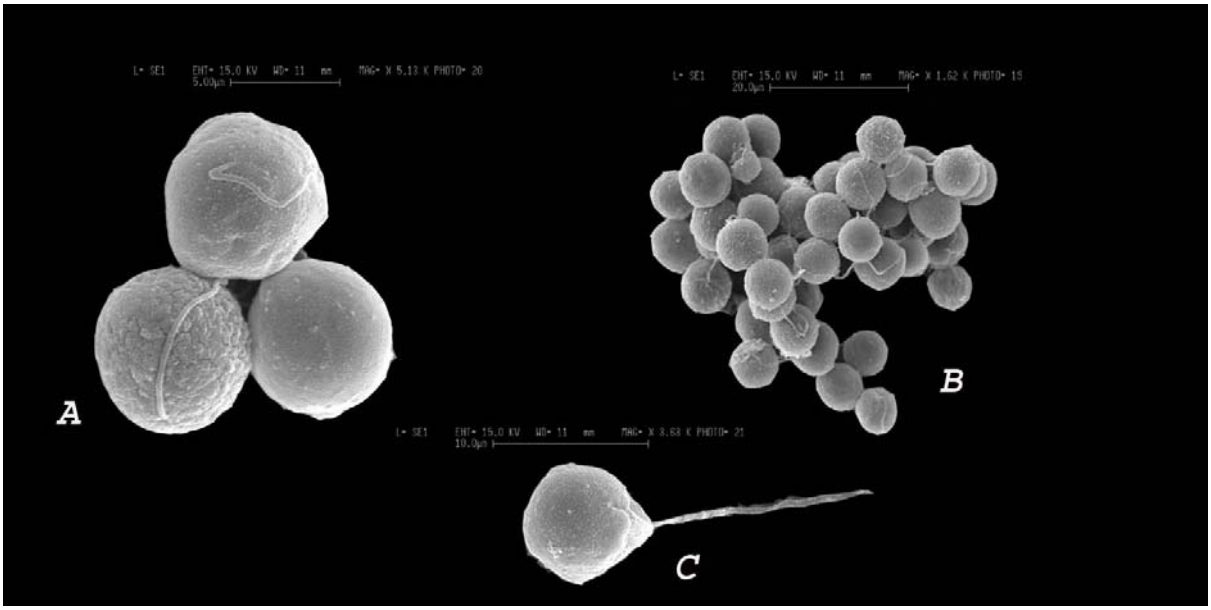


Fig. A1- 4 Scanning electron microscopic images of *Dunaliella* T36: (A) magnifications 5120x; (B) magnification 1420x; and (C) magnification 3630x

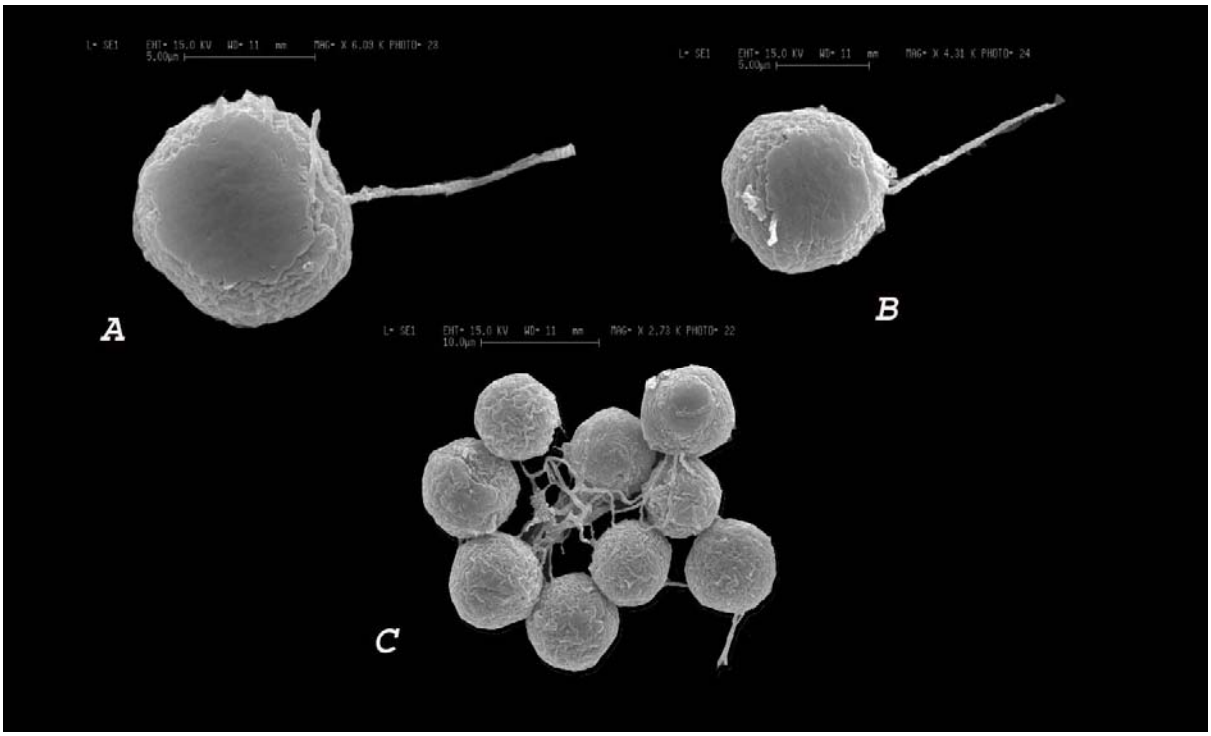


Fig. A1-5 Scanning electron microscopic images of *Dunaliella* T37: (A) magnification 6030x; (B) magnification 4330x; (C) magnification 2730x

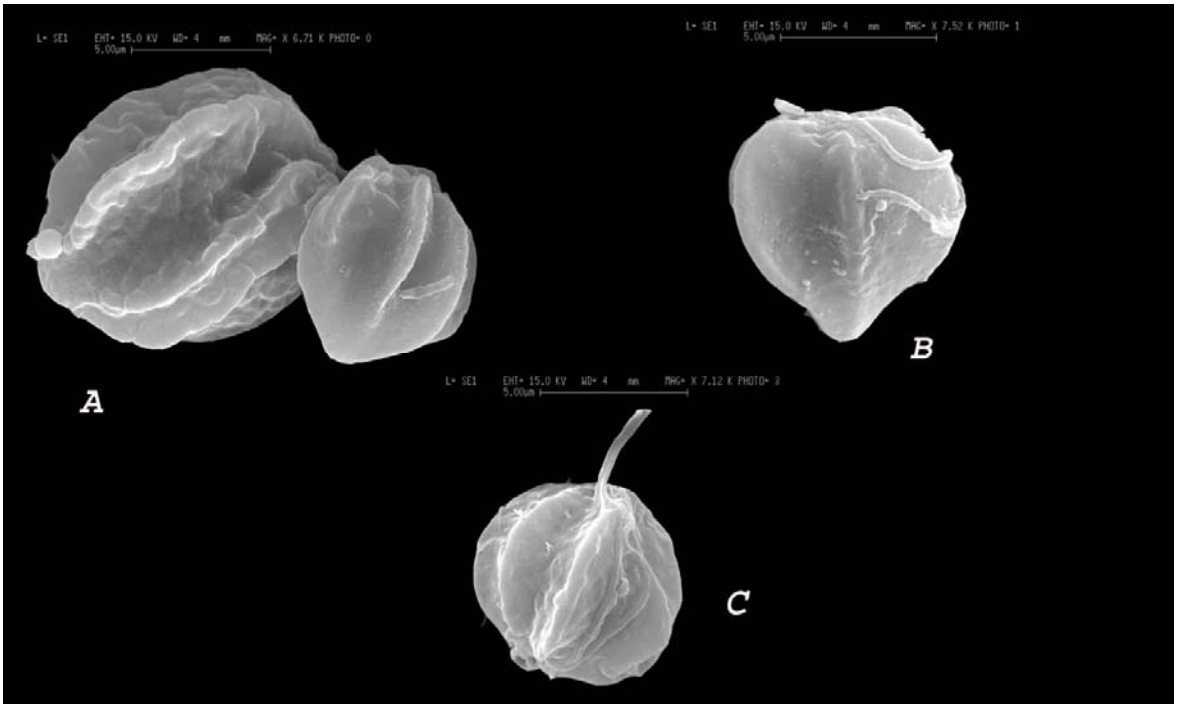


Fig. A1-6 Scanning electron microscopic images of *Asteromonas* strain T33a: (A) magnification 6170x; (B) magnifications 7520; (C) magnification 7120x

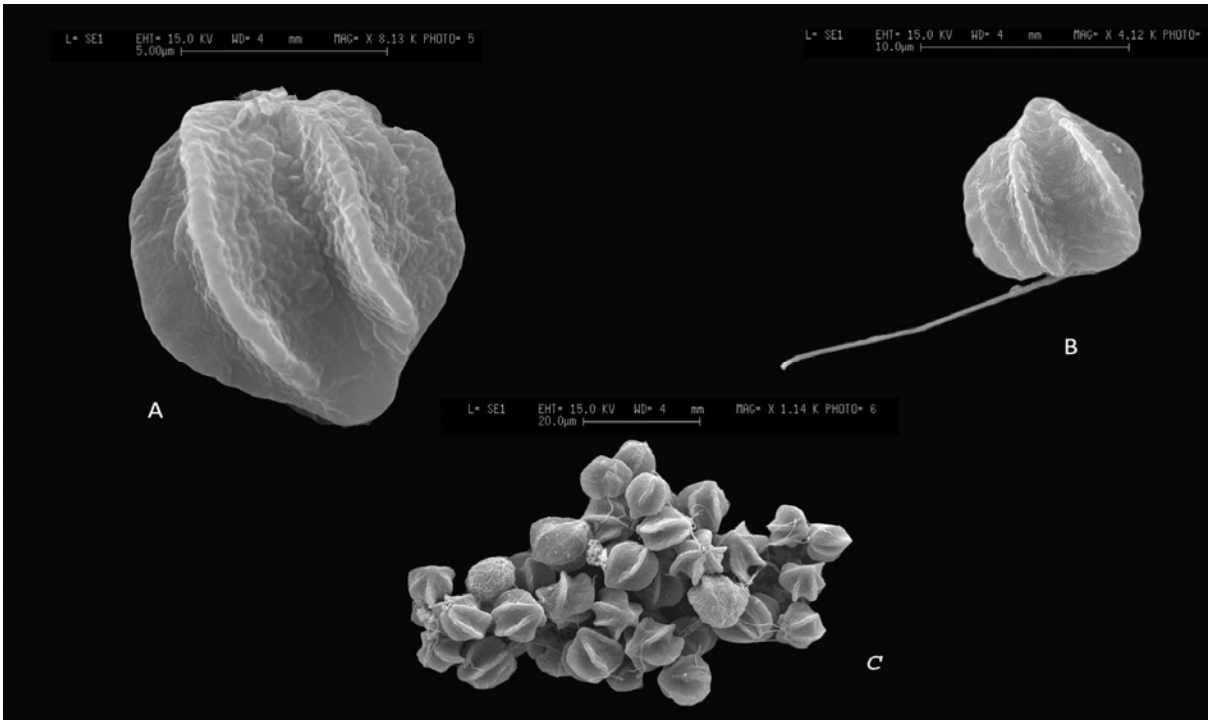


Fig. A1-7 Scanning electron microscopic images of *Asteromonas* strain T33b: (A) magnifications 8130x; (B) magnification 4120x; and (C) magnification 1140x

A3.2 Two way analysis of variance for the effect of salinity on *Dunaliella* for glycerol production (pg/cell) cultivated under continuous osmotic stress (28 days cultivation)

Dependent Variable: Col 3 (Glycerol pg/cell)

Normality Test (Shapiro-Wilk): Passed (P = 0.966)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Col 1 (*Dunaliella* strains)	2	4204.847	2102.423	8.173	0.019
Col 2 (Different salinity)	3	40044.969	13348.323	51.891	<0.001
Residual	6	1543.413	257.236		
Total	11	45793.229	4163.021		

The difference in the mean values among the different levels of Col 1 is greater than would be expected by chance after allowing for effects of differences in Col 2. There is a statistically significant difference (P = 0.019). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Col 2 is greater than would be expected by chance after allowing for effects of differences in Col 1. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.0500: for Col 1: 0.742

Power of performed test with alpha = 0.0500: for Col 2: 1.000

Least square means for Col 1 (*Dunaliella* strains):

Group	**Mean**	**SEM**
T35	157.175	8.019
T36	120.025	8.019
T37	161.875	8.019

Least square means for Col 2 (Different salinity):

Group	Mean	SEM
1.0	64.400	9.260
2.0	119.567	9.260
3.0	200.533	9.260
4.0	200.933	9.260

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 1 (*Dunaliella* strains)

Comparison	Diff of Means	p	q	P	P<0.050
T37 vs. T36	41.850	3	5.219	0.024	Yes
T37 vs. T35	4.700	3	0.586	0.911	No
T35 vs. T36	37.150	3	4.633	0.039	Yes

Comparisons for factor: Col 2 (Different salinity)

Comparison	Diff of Means	p	q	P	P<0.050
4.0 vs. 1.0	136.533	4	14.745	<0.001	Yes
4.0 vs. 2.0	81.367	4	8.787	0.003	Yes
4.0 vs. 3.0	0.400	4	0.0432	1.000	No
3.0 vs. 1.0	136.133	4	14.701	<0.001	Yes
3.0 vs. 2.0	80.967	4	8.744	0.003	Yes
2.0 vs. 1.0	55.167	4	5.958	0.022	Yes

A3.3 Two way analysis of variance for the effect of salinity on *Dunaliella* for glycerol production (per ml of cultures) cultivated under continuous osmotic stress (28 days cultivation)

Dependent Variable: Col 3 (Glycerol per ml of cultures)

Normality Test (Shapiro-Wilk): Passed (P = 0.950)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Col 1 (*Dunaliella* strains)	2	5265.247	2632.623	2.588	0.155
Col 2 (Different salinity)	3	26496.816	8832.272	8.684	0.013
Residual	6	6102.647	1017.108		
Total	11	37864.709	3442.246		

The difference in the mean values among the different levels of Col 1 is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Col 2. There is not a statistically significant difference (P = 0.155).

The difference in the mean values among the different levels of Col 2 is greater than would be expected by chance after allowing for effects of differences in Col 1. There is a statistically significant difference (P = 0.013). To isolate which group(s) differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.0500: for Col 1: 0.217

Power of performed test with alpha = 0.0500: for Col 2: 0.834

Least square means for Col 1(*Dunaliella* strains):

Group	**Mean**	**SEM**
T35	288.925	15.946
T36	279.025	15.946
T37	240.375	15.946

Least square means for Col 2 (Different salinity):

Group	Mean
1.0	206.433
2.0	262.600
3.0	338.833
4.0	269.900

Std Err of LS Mean = 18.413

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 1 (*Dunaliella* strains):

Comparison	Diff of Means	p	q	P	P<0.050
T35 vs. T37	48.550	3	3.045	0.159	No
T35 vs. T36	9.900	3	0.621	0.901	Do Not Test
T36 vs. T37	38.650	3	2.424	0.276	Do Not Test

Comparisons for factor: Col 2 (Different salinity):

Comparison	Diff of Means	p	q	P	P<0.050
3.0 vs. 1.0	132.400	4	7.191	0.009	Yes
3.0 vs. 2.0	76.233	4	4.140	0.094	No
3.0 vs. 4.0	68.933	4	3.744	0.132	Do Not Test
4.0 vs. 1.0	63.467	4	3.447	0.170	No
4.0 vs. 2.0	7.300	4	0.396	0.992	Do Not Test
2.0 vs. 1.0	56.167	4	3.050	0.237	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

A3.4 Two way analysis of variance for the effect of salinity on *Asteromonas* for glycerol production (pg/cell) cultivated under continuous osmotic stress (28 days cultivation)

Dependent Variable: Col 3 (Glycerol pg/cell)

Normality Test (Shapiro-Wilk): Passed (P = 0.507)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Col 1 (*Dunaliella* strains)	2	4372.872	2186.436	4.547	0.063
Col 2 (Different salinity)	3	16141.982	5380.661	11.191	0.007
Residual	6	2884.835	480.806		
Total	11	23399.689	2127.244		

The difference in the mean values among the different levels of Col 1 is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Col 2. There is not a statistically significant difference (P = 0.063).

The difference in the mean values among the different levels of Col 2 is greater than would be expected by chance after allowing for effects of differences in Col 1. There is a statistically significant difference (P = 0.007). To isolate which group(s) differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.0500: for Col 1: 0.436

Power of performed test with alpha = 0.0500: for Col 2: 0.925

Least square means for Col 1 (*Dunaliella* strains):

Group	**Mean**
T33a	101.550
T33b	106.275
T33c	144.200

Std Err of LS Mean = 10.964

Least square means for Col 2 (Different salinity):

Group	Mean
1.000	61.900
2.000	106.667
3.000	148.500
4.000	152.300

Std Err of LS Mean = 12.660

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 1 (*Dunaliella* strains):

Comparison	Diff of Means	p	q	P	P<0.050
T33c vs. T33a	42.650	3	3.890	0.074	No
T33c vs. T33b	37.925	3	3.459	0.109	Do Not Test
T33b vs. T33a	4.725	3	0.431	0.951	Do Not Test

Comparisons for factor: Col 2 (Different salinity):

Comparison	Diff of Means	p	q	P	P<0.050
4.0 vs. 1.0	90.400	4	7.141	0.009	Yes
4.0 vs. 2.0	45.633	4	3.605	0.148	No
4.0 vs. 3.0	3.800	4	0.300	0.996	Do Not Test
3.0 vs. 1.0	86.600	4	6.841	0.012	Yes
3.0 vs. 2.0	41.833	4	3.304	0.191	Do Not Test
2.0 vs. 1.0	44.767	4	3.536	0.157	No

A3.5 Two way analysis of variance for the effect of salinity on *Asteromonas* for glycerol production (per ml of cultures) cultivated under continuous osmotic stress (28 days cultivation)

Dependent Variable: Col 3 (Glycerol per ml of cultures)

Normality Test (Shapiro-Wilk): Passed (P = 0.637)
Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
Col 1 (*Dunaliella* strains)	2	609.545	304.772	0.609	0.574
Col 2 (Different salinity)	3	33064.389	11021.463	22.038	0.001
Residual	6	3000.708	500.118		
Total	11	36674.643	3334.058		

The difference in the mean values among the different levels of Col 1 is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Col 2. There is not a statistically significant difference (P = 0.574).

The difference in the mean values among the different levels of Col 2 is greater than would be expected by chance after allowing for effects of differences in Col 1. There is a statistically significant difference (P = 0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.0500: for Col 1: 0.0502
Power of performed test with alpha = 0.0500: for Col 2: 0.998
Least square means for Col 1 (*Dunaliella* strains):

Group	Mean	SEM
T33a	92.650	11.182
T33b	101.825	11.182
T33c	110.100	11.182

Least square means for Col 2 (Different salinity):

Group	Mean	SEM
1.000	167.800	12.911
2.000	136.500	12.911
3.000	62.733	12.911
4.000	39.067	12.911

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 1 (*Dunaliella* strains):

Comparison	Diff of Means	p	q	P	P<0.050
T33c vs. T33a	17.450	3	1.561	0.547	No
T33c vs. T33b	8.275	3	0.740	0.863	Do Not Test
T33b vs. T33a	9.175	3	0.821	0.835	Do Not Test

Comparisons for factor: Col 2 (Different salinity):

Comparison	Diff of Means	p	q	P	P<0.050
1.0 vs. 4.0	128.733	4	9.970	0.002	Yes
1.0 vs. 3.0	105.067	4	8.137	0.005	Yes
1.0 vs. 2.0	31.300	4	2.424	0.393	No
2.0 vs. 4.0	97.433	4	7.546	0.007	Yes
2.0 vs. 3.0	73.767	4	5.713	0.026	Yes
3.0 vs. 4.0	23.667	4	1.833	0.597	No

A3.6 Mean length, width and volume of *Dunaliella* strains after hyperosmotic shock

Table A1.2 shows the summary of the values and percent changes from 1.0 M NaCl to other concentration of length, width and volume of *Dunaliella* strains after hyperosmotic shock

Table A1-2 Mean cell volume of *Dunaliella* strains exposed to different salinity stress for 20 min at pH 7.5, 25 ^{o}C and 45 µmol m^{-2} s^{-1} under continuous illumination. Estimates are based on 5 independent measurements for each strain (calculated from Length and width in section 6.3.1)

Strain	Suspending Medium NaCl (M)	Length (µm)	% length change from 1.0M	Width (µm)	% width change from 1.0M	Volume (μm^3)	% Volume change from 1.0 M	Glycerol (pg/cell)
T35	0.5	7.7 ± 0.1	4.1	6.7 ± 0.1	17.5	180	42	-
	1.0	7.4 ± 0.1	-	5.7 ± 0.2	-	127	-	64.6
	2.0	7.2 ± 0.2	-2.7	5.6 ± 0.1	-1.8	119	-6	137.5
	3.0	6.9 ± 0.1	-6.8	4.8 ± 0.2	-15.8	83	-35	207.7
	4.0	5.7 ± 0.2	-23.0	4.0 ± 0.1	-40.3	48	-62	223.2
T36	0.5	8.0 ± 0.1	8.1	6.7 ± 0.1	21.8	189	62	-
	1.0	7.4 ± 0.1	-	5.5 ± 0.1	-	117	-	43.4
	2.0	7.2 ± 0.1	-2.7	5.3 ± 0.1	-3.6	108	-8	87.1
	3.0	7.0 ± 0.3	-5.4	5.1 ± 0.1	-7.3	95	-19	171.7
	4.0	6.5 ± 0.2	-12.2	4.4 ± 0.2	-20.0	66	-44	179.5
T37	0.5	7.9 ± 0.1	3.9	6.4 ± 0.1	20.8	170	53	-
	1.0	7.6 ± 0.1	-	5.3 ± 0.1	-	111	-	57.3
	2.0	7.3 ± 0.2	-3.9	5.1 ± 0.1	-3.8	100	-10	136.7
	3.0	6.8 ± 0.1	-10.5	4.8 ± 0.1	-9.4	84	-24	222.1
	4.0	5.9 ± 0.1	-22.4	4.0 ± 0.1	-24.5	49	-56	237.6

A3.7 Mean length, width and volume of *Asteromonas* strains after hyperosmotic shock

Table A1.3 shows the summary of the values and percent changes from 1.0 M NaCl of length, width and volume of *Asteromonas* strains after hyperosmotic shock

Table A1-3 Mean cell volume of *Asteromonas* strains exposed to different salinity stress for 20 min at pH 7.5, 25 ^{o}C and 45 μmol m^{-2} s^{-1} under light regime. Estimates are based on 5 independent measurements for each strain

Strain	Suspending Medium NaCl (M)	Length (μm)	% length change from 1.0M	Width (μm)	% width change from 1.0M	Volume (μm^3)	% Volume change from 1.0 M	Glycerol (pg/cell)
T33a	0.5	7.8 ± 0.2	4.0	6.8 ± 0.2	21.4	189	54	-
	1.0	7.5 ± 0.1	-	5.6 ± 0.1	-	123	-	58.4
	2.0	7.1 ± 0.1	-5.3	5.4 ± 0.1	-3.6	108	-12	105.9
	3.0	6.4 ± 0.1	-14.7	5.3 ± 0.1	-5.4	94	-24	136.5
	4.0	5.8 ± 0.2	-22.7	5.1 ± 0.1	-8.9	80	-35	133.1
T33b	0.5	8.4 ± 0.1	7.7	6.7 ± 0.1	-6.3	198	21	-
	1.0	7.8 ± 0.2	-	6.3 ± 0.1	-	163	-	68.4
	2.0	7.4 ± 0.2	-5.1	6.1 ± 0.1	-3.2	144	-12	108.0
	3.0	6.8 ± 0.1	-12.8	5.3 ± 0.1	-15.9	101	-38	143.7
	4.0	6.3 ± 0.1	-19.2	4.7 ± 0.2	-25.4	73	-55	128.7
T33c	0.5	8.6 ± 0.2	14.7	6.7 ± 0.1	21.8	205	77	-
	1.0	7.5 ± 0.1	-	5.5 ± 0.1	-	119	-	69.8
	2.0	7.2 ± 0.1	-4.0	5.2 ± 0.1	-5.5	103	-13	117.8
	3.0	6.7 ± 0.2	-10.7	4.9 ± 0.1	-10.9	84	-29	167.7
	4.0	6.0 ± 0.1	-20.0	4.2 ± 0.1	-23.6	56	-53	184.2

A3.8 Exposure of *Asteromonas* to hyperosmotic stress under light and dark regimes

The result presented in Table A1.4 shows that there is so significant different between light and dark on the amount of glycerol accumulated by *Asteromonas* strains

Table A1-4 Mean glycerol content of *Asteromonas* cells (T33a, T33b and T33c) cultured in 1.0 M NaCl and treated by hyperosmotic shock under continuous Light and dark. Cells were transferred from 1.0 M NaCl to fresh media of increased salt and glycerol content recorded after 240 min.

Strains	**Salinity (M)**	**Glycerol under constant light (pg/cell)**	**Glycerol under constant dark (pg/cell)**	**Number of cells x 10^6 /ml**
***Asteromonas* T33a**	1.0	58.4 ± 1.2	58.2 ± 1.5	2.33
	2.0	105.9 ± 3.1	103.0 ± 4.1	2.33
	3.0	136.5 ± 5.5	131.3 ± 7.1	2.33
	4.0	133.1 ± 3.8	130.5 ± 1.4	2.33
***Asteromonas* T33b**	1.0	68.4 ± 1.2	67.1 ± 1.8	2.95
	2.0	108.0 ± 1.0	105.3 ± 0.7	2.95
	3.0	143.7 ± 3.7	140.5 ± 0.7	2.95
	4.0	128.7 ± 1.2	125.7 ± 2.5	2.95
***Asteromonas* T33c**	1.0	69.8 ± 2.3	69.1 ± 0.9	2.79
	2.0	117.8 ± 9.9	114.7 ± 1.8	2.79
	3.0	167.7 ± 5.7	161.4 ± 2.5	2.79
	4.0	184.2 ± 2.6	176.2 ± 2.1	2.79

Appendix 4.0

A4.1 Supplementary metabolites and their possible roles in *Dunaliella* cells metabolism

Asparagine: Asparagine is non-essential amino acids that can be produced in microorganisms with various enzymes. Asparagine together with glutamine, glutamate and aspartate they called N-transport amino acids.

Asparagine is a metabolically reactive amino acid that serves as a nitrogen donor in numerous aminotransferase reactions; asparagine is relatively inert and serves primarily as nitrogen transport and storage compound. Asparagine and other N-transport amino acids are the major amino acids translocated in the phloem of most species such as corn, pea and Arabidopsis. The concentration of these transport amino acids are not static but are modulated by factors such as light (Buchanan *et al*., 2000). Molecular studies have shown that genes involved in asparagine synthesis are preferentially transcribed in the dark in several species such as Arabidopsis, confirming the initial discovery of light repression of asparagine synthesis reported in the 1800s (Buchanan *et al*., 2000). In this study the presence of asparagine was only detected in the cells exposed to 1.0 and 4.0 M NaCl in constant dark regime (Table 8.1). This result is also in support of the view that said genes involved in asparagine synthesis are preferentially transcribed in the cells exposed dark regime.

Pyroglutamic acid: Pyroglutamic acid (5-oxoproline) is the cyclic lactam (a cyclic amide formed from aminocarboxylic acids by elimination of water molecule) of glutamic acid. Its presence in living cells has been reported from archaebacteria to humans. Although it is considered as non-protein amino acid, pyroglutamic acid is found as an N-terminal modification in many neuronal peptides and hormones, antibodies, some enzymes and structural proteins. The modification in proteins has been shown to contribute to both the structural and activity-related properties of the proteins. Pyroglutamate also exists as a free metabolite in living cells. The proposed functions of free pyroglutamic acid include its role as an analogue or reservoir of glutamate, as well as a possible role in osmoprotection (Kumar and Bachhawat, 2012).

In this study pyroglutamic acid was detected in the cells exposed to 1.0 and 4.0 M NaCl in constant light/dark regime with highest amount in the cells exposed to 1.0 M NaCl in constant dark regime. The possible reasons for the production of pyroglutamic acid *Dunaliella* T35 could be a reservoir of glutamate as a precursor for the synthesis of δ-ALA (an initial substrate for chlorophyll biosynthesis) as mentioned in earlier. The osmoprotective role of pyroglutamic acid could be considered as negligible as this role is dominated by glycerol (see Table 8.1).

Tryptamine: Tryptamine is a monoamine alkaloid found with a wide spread occurrence in living organisms such as animals, fungi and plants. It contains an indole ring structure, and is structurally similar to the amino acid tryptophan, from which it derives its name, and is a well-known biological active compound. It results from the conversion of tryptophan by tryptophan decarboxylase, and is involved in indole alkaloid secondary metabolism as a precursor of diverse secondary metabolites (Facchini, 2001).

In mammals, it was known to forms the chemical backbone for neurotransmitters and hormones, such as serotonin and melatonin, and may also serve as a neuromodulator (Berry, 2004; Fantegrossi *et al*., 2008).

According to Buchanan *et al*., (2000) there was an evidences which indicates that plant can synthesize indole-3-acetic acid (IAA) from L-tryptophan by three different routes: the indole-3-pyruvic acid, the indole-3-acetaldoxime, and the tryptamine pathways (figure A1.8). A study conducted by Perley and Stowe (1966) shows that strain of *Bacillus cereus* can produce tryptamine when grown in a broth containing tryptophan. Although, the L-tryptophan was below the detection limit for all the samples investigated, tryptamine was detected in all the cells exposed to 1.0 and 4.0 M NaCl in both constant light and dark regime with highest amount in the cells exposed to 1.0 M NaCl concentrations (Table 8.1). The result suggested that possibly *Dunaliella* T35 produced tryptamine as an intermediate for the biosynthesis of IAA from L-tryptophan (figure A1.8).

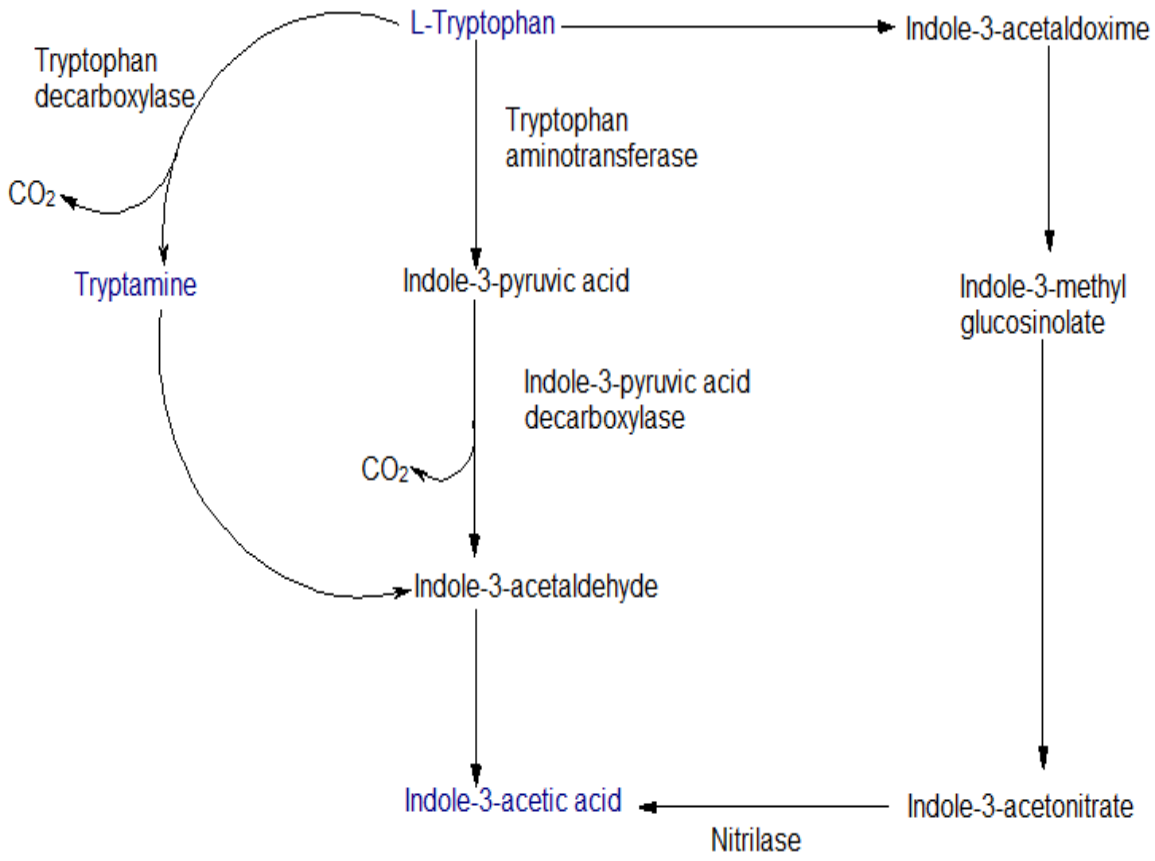


Fig. A1-8 L-tryptophan-dependent Indole-3-acetic acid biosynthesis pathway. Adopted from Buchanan *et al*. (2000)

Accumulation of aromatic and branched chain amino acids: L-tryptophan, L-phenylalanine, and L-tyrosine are aromatic amino acids (AAAs) which are used for the biosynthesis of proteins in plant and also serve as precursors of several natural products, such as pigments, alkaloids, hormones, and cell wall components. All three aromatic amino acids are derived from the shikimate pathway, to which ≥30 % of photosynthetically fixed carbon is directed in vascular plants (Maeda and Dudareva, 2012). Because their biosynthetic pathways have been lost in animal lineages, the AAAs are essential components of the diets of humans.

Valine, leucine and isoleucine form the small group of branched-chain amino acids (BCAAs) classified by their small branched hydrocarbon residues. Unlike animals, plants are able to de novo synthesize these amino acids from pyruvate, 2-oxobutanoate and acetyl-CoA (Binder, 2010). Bacteria, fungi and algae are also able to synthesize BCAAs. Apart from the

threonine independent citramalate pathway in some prokaryotes, in which isoleucine is formed from pyruvate and acetyl-CoA, the biosynthesis of BCAAs follows a common scheme, as described for plants (Wu *et al*., 2010; Binder, 2010). Like AAAs, BCAAs biosynthetic pathways have been lost in animal lineages.

Valine is the only BCAA detected in cells exposed to all experimental conditions (Table 8.1), L-Leucine was only detected in the cells exposed to 1.0 M NaCl in constant dark and it was below the detection level in other experimental conditions investigated. Leucine was detected in cells exposed to both 1.0 and 4.0 M NaCl under constant light and its level was below the detection limit in cells exposed to similar medium under constant dark regime. For AAAs, L-phenylalanine, and L-tyrosine are detected only on the cells exposed to 1.0 M NaCl in constant dark regime whereas L-tryptophan was below the detection limit for all the sample investigated (Table 8.1).

Although AAAs and BCAAs are synthesized as building blocks of proteins, Shimizu *et al*. (2010) found that the fungus *Aspergillus nidulans* excretes BCAAs into the culture medium under hypoxia. As a result of their aliphatic nature, BCAAs are predominantly are also found in membrane-spanning protein domains (Binder, 2010). Similarly, *Dunaliella* cells synthesized AAAs and BCAAs as building blocks of proteins and probably as membrane-spanning protein, as they are deficient of like cell walls.

Phenylalanine and L-DOPA: Phenylalanine and L-DOPA (3,4-dihydroxyphenyl-l-alanine) are compounds that both serve as precursors of dopamine and other catecholamines in plants. L-DOPA is extracted from the seeds of *Mucuna pruriens* (Misra and Wagner, 2007) and *Vicia faba* (Shetty *et al*., 2002). The L-DOPA production was reported in synthetic medium by the fungal species Acremonium rutilum (Krishnaveni *et al*., 2009) and Aspergillus oryzae (Ali and Haq, 2006). Bacterial species such as *Erwinia herbicola* have been reported to produce LDOPA using catechol as precursor (Koyanagi *et al*., 2005) whereas *Bacillus sp*. JPJ has also been reported to produce L-DOPA in a reaction mixture containing L-tyrosine as precursor (Surwase and Jadhav, 2011). A green microalga called *Ulvaria obscura* has been reported to produce dopamine (Van Alstyne *et al*. 2006). The production of L-DOPA in *D. salina* was first reported by Lamers (2011) when this microalga was growth under high-light stress and nutrient -starvation.

L-DOPA production from L-tyrosine is a one-step reaction catalyzed by tyrosinase (E.C.1.14.18.1), which is a copper-containing enzyme widely distributed in plants, animals and microorganisms (Claus and Decker, 2006). However, catetecholamine (dopamine, norepinephrine, and epinephrine) biosynthetic pathway can be initiated either from the hydroxylation of L-phenylalanine to l-tyrosine, or directly from l-tyrosine, which is then hydroxylated to l-DOPA which proceeds to variable multistep transformations until reaches its final product. Figure A1.9 represent the pathway for the biosynthesis of catetecholamine in plants; similar to that in mammals and is initiated by two equally active routes. In the first route tyrosine, the initial precursor for the biosynthesis of catecholamines is hydroxylated by tyrosine hydroxylase (TH) giving dihydroxyphenylalanine (L-DOPA). The second route is initiated by substrate decarboxylation driven by tyrosine decarboxylase (TD) and results in tyramine production. Finally, dopamine is produced via both the hydroxylation of tyramine or decarboxylation of L-DOPA figure A1.9 (Shetty *et al*., 2002).

The synthesis of catecholamines in many plants was found to be regulated by stress conditions; for example accumulation of catecholamines in potato plants, resulting from over expression of tyrosine decarboxylase (Swiedrych *et al*., 2004), was found to positively correlate with the levels of glucose, sucrose and fructose, suggesting a regulatory role of catecholamines in carbohydrate metabolism of plants (Kulma and Szopa 2007). In *D. salina*, high-light-stressed and nitrogen-starved was found simultaneous and significant increases in L-DOPA and sucrose, as well as an increase in glucose when exposed to high-light-stress and an increase in phenylalanine levels during nitrogen starvation. In this study, the cells exposed to 1.0 M NaCl in constant dark/light contained high amounts of L-DOPA, whereas glucose was only detected in cells exposed to 1.0 M NaCl in continuous illumination and the phenylalanine was only detectable in cells exposed to 1.0 M NaCl in constant dark (Table 8.1). The observed production of phenylalanine and L-DOPA in *Dunaliella* T35 may possibly reflect changes related to a metabolic switch towards energy storage (Lamers, 2011).

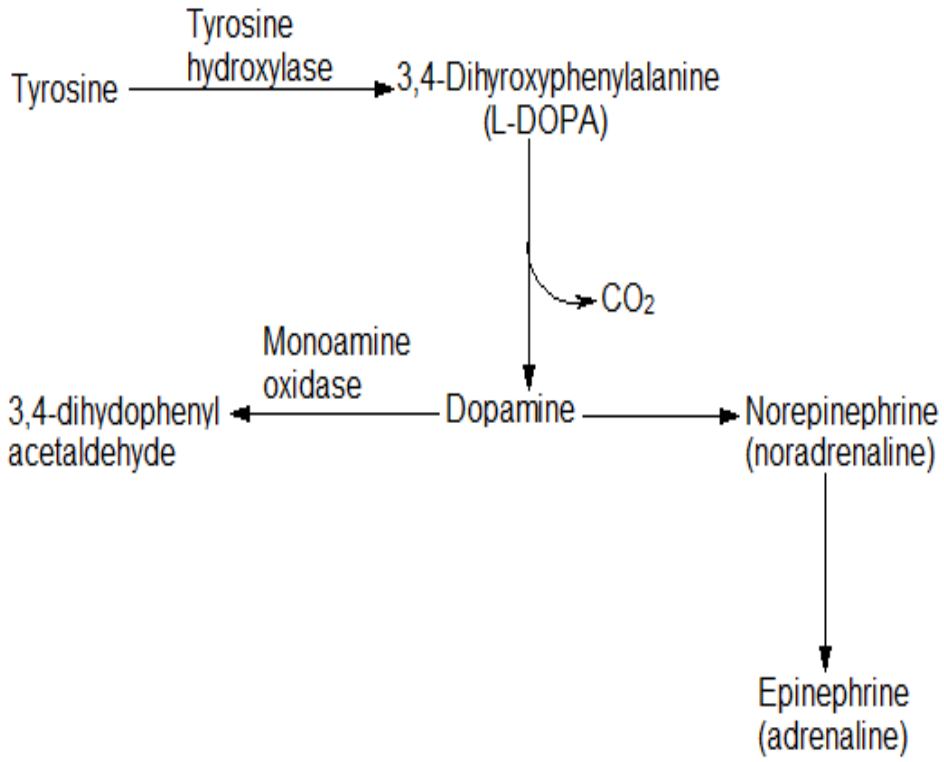


Fig. A1-9 Biosynthesis of dopamine form tyrosine via 3,4-dihydroxyphenyl-l-alanine. Adopted from Shetty *et al*. (2002)

Polyamines (Putrescine, spermidine and spermine): Polyamines, organic compounds having two or more primary amino groups, are ubiquitous compounds found in all organisms. Their concentrations correlate with cell division frequency, and they also stimulate many reactions involved in the synthesis of DNA, RNA and proteins. The significance of these compounds in plants has been recognized (Buchanan *et al*., 2000). In plants, polyamines elicit diverse physiological responses, inducing cell division, tuber formation, root initiation, embryogenesis, flower development and fruit ripening. The polyamines found most frequently in plants are putrescine, spermidine and spermine. Although cadaverine (1,5-diaminopentane) is present much less than the diamine putrescine, it is a common constituent of legumes. In this study only putrescine and spermidine were detected. Putrescine is synthesized from L-arginine by two routes, one involving L-ornithine, the other by way of agmatine and N-carbamoylputrescine (Buchanan *et al*., 2000).

Spermidine appears to be required for the G_1 and S (also called G_1 phase and S phase; the latter is the period in the cell cycle from the end of cell division to the beginning of DNA replication whereas the former is the period in the cell cycle during which DNA replication takes place) transition in cell division. The conversion of putrescine to spermidine is thought to be important in controlling the rate of cell division. Spermidine synthase catalyzes the conversion of putrescine to spermidine (Figure A1.10). Putrescine was detected in all the cells exposed to 1.0 and 4.0 M NaCl in both constant light and dark regime with highest amount in the cells exposed to 1.0 and 4.0 M NaCl concentrations in constant illumination (Table 8.1) whereas spermidine was below the detection limit for cell exposed in the cells exposed to 1.0 M NaCl in constant dark regime. The result suggested that possibly *Dunaliella* T35 produced polyamines (putrescine and spermidine) in relation to inducing cell division; as the result indicated that higher amount of putrescine was detected in the cells exposed to light (cells supported photosynthetically).

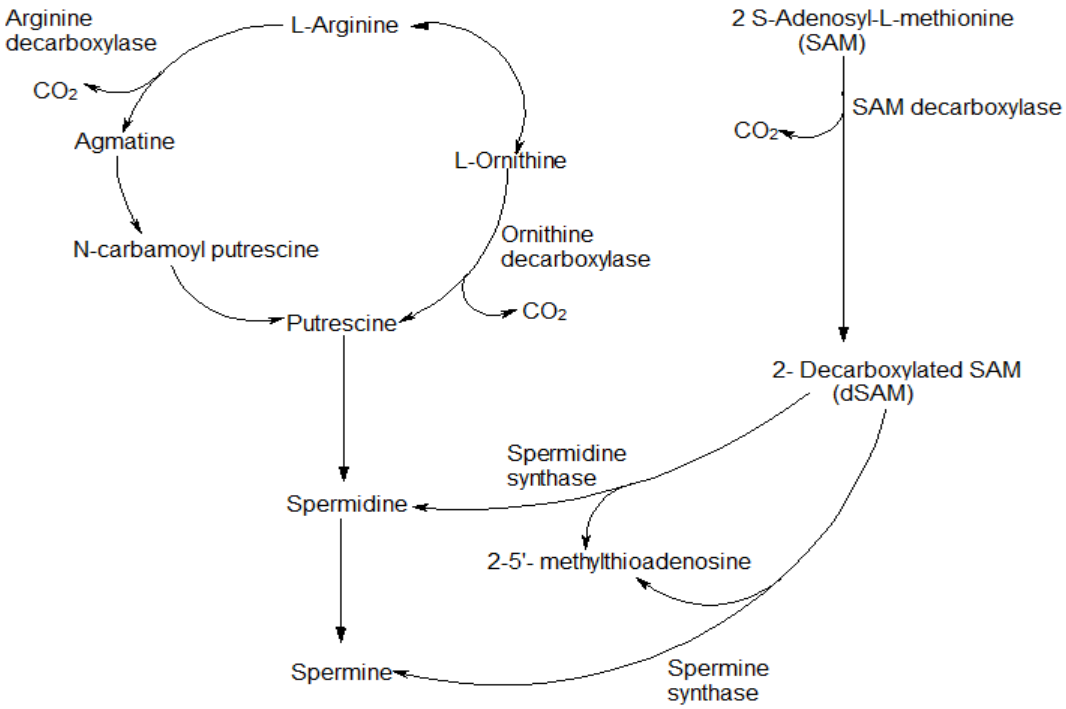


Fig. A1-10 Pathway for the biosynthesis of putrescine, spermidine and spermine from arginine. Adopted from Buchanan *et al*. (2000)

Pyrazine: Pyrazine is a heterocyclic aromatic organic compound, with the chemical formula $C_4H_4N_2$, found naturally in many vegetables. Some microorganisms are known to produce pyrazines as their primary or secondary metabolites (Murray *et al*., 1970; Murray *et al*., 1975; Besson *et al*., 1977). Pyrazines molecule have in their general structure the four hydrogen atoms at the 2-, 3-, 5- and 6-positions that may can be substituted by one to four alkyl, hydroxy or methoxy groups (Beck *et al*., 2003).

The commonly naturally occurring pyrazines are 2-methoxy-3-isopropylpyrazine, sec-butyl- and isobutyl-substituted pyrazines from green peas (*Pisum sativum*) ((Murray *et al*., 1970; Murray *et al*., 1975) 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-sec-butylpyrazine were detected in Pseudomonas spp. (Chen *et al*., 1991) as well as 2,3,5,6-tetramethylpyrazine and 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, isolated from *Bacillus species* (Owens *et al*., 1997; Leejeerajumnean *et al*., 2001). Hydroxylated pyrazines are rarely found in microorganism includes 2-hydroxy-3-isobutyl-6-sec-butyl pyrazine-1-oxide (aspergillic acid) and 2-hydroxy-3-isobutyl-6-(1-hydroxy-1-methylpropyl) pyrazine-1-oxide (hydroxyaspergillic acid) isolated from Aspergilli (MacDonald, 1973). Some pyrazines are known to exhibit bactericidal (antibiotic) or chemoprotective (antitumor) activities (MacDonald, 1973; Kim *et al*., 1997; Beck *et al*., 2003).

Pyrazine was detected in all the cells exposed to 1.0 and 4.0 M NaCl in both constant light and dark regime with highest amount in the cells exposed to 1.0 M NaCl concentrations in constant dark regime (Table 8.1). As microalgae are commonly known to growth with bacteria, this result may suggest that *Dunaliella* T35 produced pyrazine as a bactericidal agent. Unlike all the pyrazine produced by organisms mentioned earlier, 2-hydroxy-3-methyl-pyrazine is found to be the metabolite of *Dunaliella* T35.

Unknown metabolites: In addition to the metabolites described earlier 3 unknown metabolites were detected and found to either correlate positively or negatively with salinity or light/dark regime exposure. The three unknown metabolites; named UN001, UN002 and UN003 (retention times and mass spectra are given in figure A1.11 to A1.13), respectively.

UN001 was detected in all the cells exposed to 1.0 and 4.0 M NaCl in both constant light and dark regime having the larger amount in the cells exposed to 1.0 M NaCl concentrations in

constant dark regime (Table 8.1). The result shows that the synthesis of UN001 is favoured more in Dark regime.

UN003 correlate negatively with higher salinity. This metabolites was not detected in the cells exposed to 4.0 M NaCl in constant illumination and the amount detected in the cells exposed to 4.0 M NaCl in constant dark regime is negligible when compared to the cell exposed to 1.0 M NaCl concentrations in both constant light and dark regime (Table 8.1). UN002 was detected in all the experimental conditions and there is no difference in the amount of UN002 among all samples.

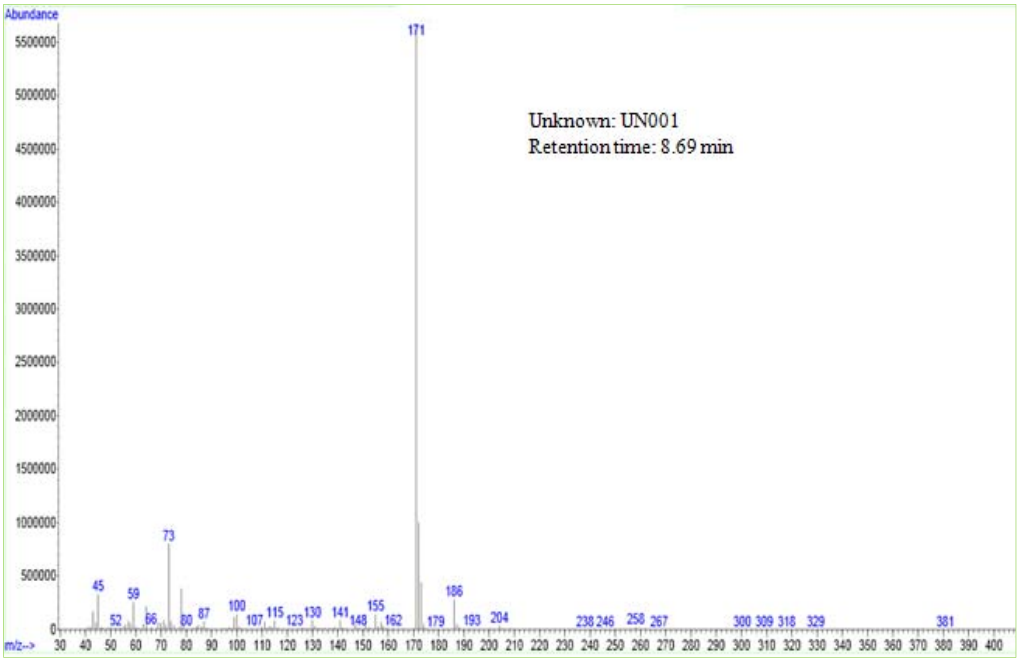


Fig. A1-11 Mass fragmentation pattern of UN001 (unidentified metabolite) that correlate highly with cells exposed to 1.0 M NaCl in constant dark

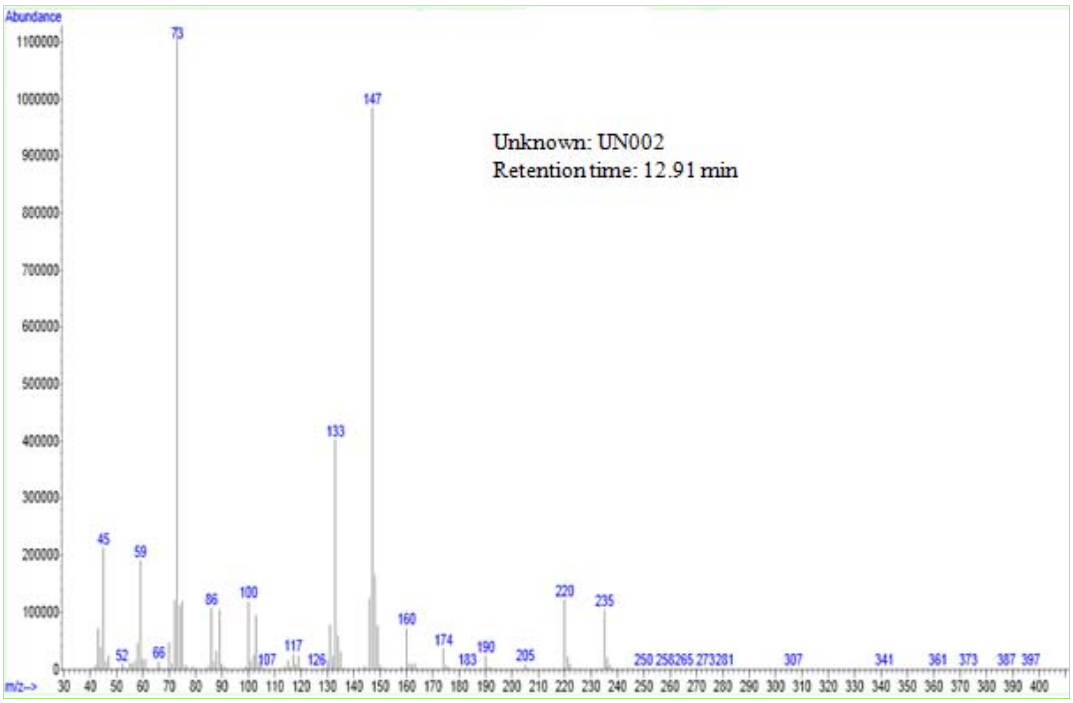


Fig. A1-12 Mass fragmentation pattern of UN002 (unidentified metabolite)

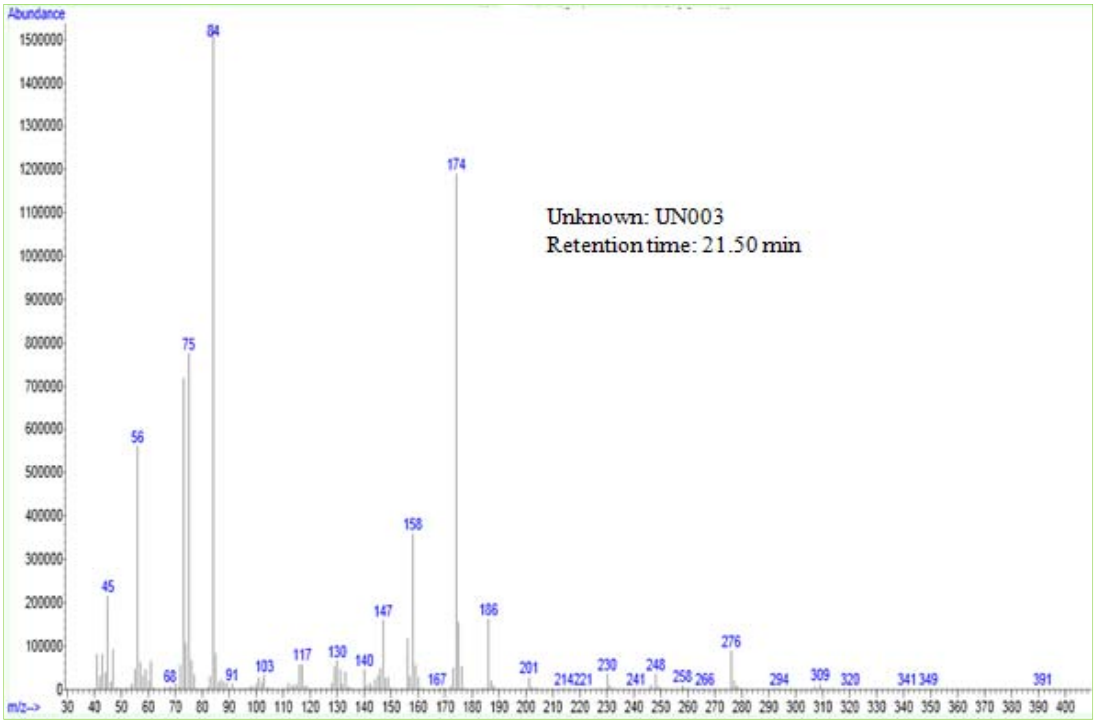


Fig. A1-13 Mass fragmentation pattern of UN003 (unidentified metabolite) that correlate negatively with cells exposed to hyperosmotic shock

Appendix 5.0

A5.1 Three way analysis of variance for the effect of pressure, temperature and time on glycerol extraction (addition of chloroform after SC-CO_2 extraction)

This experiment was conducted with the original harvested pellet (contains 60% aqueous medium w/w). Experimental methodology was applied to optimize the process variables pressure (100 to 200 bar), temperature (40-50 °C) and dynamic extraction time (15-90 min). The factors and levels investigated were pressure, temperature and time (3 x 3 x 4 x 2 = 72 runs).

Dependent Variable: Col 4 (Glycerol mg/g dry weight)
Normality Test (Shapiro-Wilk): Failed (P < 0.050)
Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Col 1 (Pressure)	2	183833.507	91916.753	17078.726	<0.001
Col 2 (Temperature)	2	5875.304	2937.652	545.835	<0.001
Col 3 (Time)	3	21584.809	7194.936	1336.866	<0.001
Col 1 x Col 2	4	3611.545	902.886	167.762	<0.001
Col 1 x Col 3	6	2365.972	394.329	73.269	<0.001
Col 2 x Col 3	6	3364.800	560.800	104.200	<0.001
Col 1 x Col 2 x Col 3	12	3747.309	312.276	58.023	<0.001
Residual	36	193.750	5.382		
Total	71	224576.997	3163.056		

There is a statistically significant interaction between Col 1, Col 2 and Col 3 (P = <0.001).This indicates that the effect of one factor is not consistent at all combinations of the two other factors; and, therefore, an unambiguous interpretation of the main effects is not possible.SigmaStat will evaluate significant interactions as appropriate.

Three Way Interaction Term Analyses:

Evaluated Col 1 x Col 2 across levels of Col 3:

The effect of the Col 1 x Col 2 interaction depends on what level of Col 3 is present.

There is a significant Col 1 x Col 2 interaction at level 15.000 of Col 3.(P = <0.001)

There is a significant Col 1 x Col 2 interaction at level 30.000 of Col 3.(P = <0.001)

There is not a significant Col 1 x Col 2 interaction at level 60.000 of Col 3.(P = 0.169)

There is a significant Col 1 x Col 2 interaction at level 90.000 of Col 3.(P = 0.001)

Simple Main Effects at level 15 min of Col 3:

The difference in the mean values among the different levels of Col 1 evaluated within level 40 of Col 2 and level 15 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 40-15**

Comparison	Diff of Means	p	q	P	P<0.05
200 vs. 100	73.125	3	44.577	<0.001	Yes
200 vs. 150	46.250	3	28.194	<0.001	Yes
150 vs. 100	26.875	3	16.383	<0.001	Yes

The difference in the mean values among the different levels of Col 1 evaluated within level 45 of Col 2 and level 15 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 45-15**

Comparison	Diff of Means	p	q	P	P<0.05
200 vs. 100	134.375	3	81.915	<0.001	Yes
200 vs. 150	100.625	3	61.341	<0.001	Yes
150 vs. 100	33.750	3	20.574	<0.001	Yes

The difference in the mean values among the different levels of Col 1 evaluated within level 50 of Col 2 and level 15 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 50-15**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
200 vs. 100	132.500	3	80.772	<0.001	Yes
200 vs. 150	89.375	3	54.483	<0.001	Yes
150 vs. 100	43.125	3	26.289	<0.001	Yes

The difference in the mean values among the different levels of Col 2 evaluated within level 100 of Col 1 and level 15 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 100-15**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
50 vs. 40	16.250	3	9.906	<0.001	Yes
50 vs. 45	6.250	3	3.810	0.028	Yes
45 vs. 40	10.000	3	6.096	<0.001	Yes

The difference in the mean values among the different levels of Col 2 evaluated within level 150of Col 1 and level 15 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 150-15**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
50 vs. 40	32.500	3	19.812	<0.001	Yes
50 vs. 45	15.625	3	9.525	<0.001	Yes
45 vs. 40	16.875	3	10.287	<0.001	Yes

The difference in the mean values among the different levels of Col 2 evaluated within level 200 of Col 1 and level 15 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 200-15**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
50 vs. 40	75.625	3	46.101	<0.001	Yes
50 vs. 45	4.375	3	2.667	0.157	No
45 vs. 40	71.250	3	43.434	<0.001	Yes

Simple, Simple Main Effects at level 30.000 of Col 3:

The difference in the mean values among the different levels of Col 1 evaluated within level 40 of Col 2 and level 30 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 40-30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
200 vs. 100	60.625	3	36.957	<0.001	Yes
200 vs. 150	15.000	3	9.144	<0.001	Yes
150 vs. 100	45.625	3	27.813	<0.001	Yes

The difference in the mean values among the different levels of Col 1 evaluated within level 45 of Col 2 and level 30 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 45-30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
200 vs. 100	131.250	3	80.010	<0.001	Yes
200 vs. 150	84.375	3	51.435	<0.001	Yes
150 vs. 100	46.875	3	28.575	<0.001	Yes

The difference in the mean values among the different levels of Col 1 evaluated within level 50 of Col 2 and level 30 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 50-30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
200 vs. 100	132.500	3	80.772	<0.001	Yes
200 vs. 150	84.375	3	51.435	<0.001	Yes
150 vs. 100	48.125	3	29.337	<0.001	Yes

The difference in the mean values among the different levels of Col 2 evaluated within level 100 of Col 1 and level 30 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = 0.113).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 100-30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
50 vs. 40	5.000	3	3.048	0.093	No
50 vs. 45	2.500	3	1.524	0.534	Do Not Test
45 vs. 40	2.500	3	1.524	0.534	Do Not Test

The difference in the mean values among the different levels of Col 2 evaluated within level 150 of Col 1 and level 30 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = 0.010).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 150-30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
50 vs. 40	7.500	3	4.572	0.007	Yes
50 vs. 45	3.750	3	2.286	0.252	No
45 vs. 40	3.750	3	2.286	0.252	No

The difference in the mean values among the different levels of Col 2 evaluated within level 200 of Col 1 and level 30 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 200-30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
50 vs. 40	76.875	3	46.863	<0.001	Yes
50 vs. 45	3.750	3	2.286	0.252	No
45 vs. 40	73.125	3	44.577	<0.001	Yes

Simple Main Effect Tests at level 60 of Col 3:

The difference in the mean values among the different levels of Col 1 evaluated within level 60 of Col 3 (averaging over levels of Col 2) is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 60**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
200 vs. 100	138.125	3	145.841	<0.001	Yes
200 vs. 150	92.292	3	97.447	<0.001	Yes
150 vs. 100	45.833	3	48.394	<0.001	Yes

The difference in the mean values among the different levels of Col 2 evaluated within level 60 of Col 3 (averaging over levels of Col 1) is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 60**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
50 vs. 40	8.958	3	9.459	<0.001	Yes
50 vs. 45	4.583	3	4.839	0.004	Yes
45 vs. 40	4.375	3	4.619	0.007	Yes

Simple, Simple Main Effects at level 90 of Col 3:

The difference in the mean values among the different levels of Col 1 evaluated within level 40 of Col 2 and level 90 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 40-90**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
200 vs. 100	132.500	3	80.772	<0.001	Yes
200 vs. 150	84.375	3	51.435	<0.001	Yes
150 vs. 100	48.125	3	29.337	<0.001	Yes

The difference in the mean values among the different levels of Col 1 evaluated within level 45 of Col 2 and level 90 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 45-90**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
200 vs. 100	131.875	3	80.391	<0.001	Yes
200 vs. 150	84.375	3	51.435	<0.001	Yes
150 vs. 100	47.500	3	28.956	<0.001	Yes

The difference in the mean values among the different levels of Col 1 evaluated within level 50 of Col 2 and level 90 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 1 within 50-90**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
200 vs. 100	120.625	3	73.533	<0.001	Yes
200 vs. 150	84.375	3	51.435	<0.001	Yes
150 vs. 100	36.250	3	22.098	<0.001	Yes

The difference in the mean values among the different levels of Col 2 evaluated within level 100 of Col 1 and level 90 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 100-90**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
50 vs. 40	12.500	3	7.620	<0.001	Yes
50 vs. 45	11.250	3	6.858	<0.001	Yes
45 vs. 40	1.250	3	0.762	0.853	No

The difference in the mean values among the different levels of Col 2 evaluated within level 150 of Col 1 and level 90 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = 0.953).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 150-90**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
45 vs. 40	0.625	3	0.381	0.961	No
45 vs. 50	0.000	3	0.000	1.000	Do Not Test
50 vs. 40	0.625	3	0.381	0.961	Do Not Test

The difference in the mean values among the different levels of Col 2 evaluated within level 200 of Col 1 and level 90 of Col 3 is greater than would be expected by chance. There is a statistically significant difference (P = 0.953).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Col 2 within 200-90**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
45 vs. 40	0.625	3	0.381	0.961	No
45 vs. 50	0.000	3	0.000	1.000	Do Not Test
50 vs. 40	0.625	3	0.381	0.961	Do Not Test

Power of performed test with alpha = 0.0500: for Col 1: 1.000

Power of performed test with alpha = 0.0500: for Col 2: 1.000

Power of performed test with alpha = 0.0500: for Col 3: 1.000

Power of performed test with alpha = 0.0500: for Col 1 x Col 2: 1.000

Power of performed test with alpha = 0.0500: for Col 1 x Col 3: 1.000

Power of performed test with alpha = 0.0500: for Col 2 x Col 3: 1.000

Least square means for Col 1:

Group	**Mean**
100	587.438
150	630.250
200	709.417

Std Err of LS Mean = 0.474

Least square means for Col 2:

Group	**Mean**
40	629.938
45	646.031
50	651.135

Std Err of LS Mean = 0.474

Least square means for Col 3:

Group	**Mean**
15	619.556
30	633.167
60	651.986
90	664.764

Std Err of LS Mean = 0.547

Least square means for Col 1 x Col 2:

Group	Mean
100 x 40	582.750
100 x 45	586.344
100 x 50	593.219
150 x 40	624.156
150 x 45	630.563
150 x 50	636.031
200 x 40	682.906
200 x 45	721.188
200 x 50	724.156

Std Err of LS Mean = 0.820

Least square means for Col 1 x Col 3:

Group	Mean
100 x 15	570.250
100 x 30	581.500
100 x 60	590.667
100 x 90	607.333
150 x 15	604.833
150 x 30	628.375
150 x 60	636.500
150 x 90	651.292
200 x 15	683.583
200 x 30	689.625
200 x 60	728.792
200 x 90	735.667

Std Err of LS Mean = 0.947

Least square means for Col 2 x Col 3:

Group	Mean
40 x 15	594.833
40 x 30	614.417

40 x 60	647.542
40 x 90	662.958
45 x 15	627.542
45 x 30	640.875
45 x 60	651.917
45 x 90	663.792
50 x 15	636.292
50 x 30	644.208
50 x 60	656.500
50 x 90	667.542

Std Err of LS Mean = 0.947

Least square means for Col 1 x Col 2 x Col 3:

Group	**Mean**
100 x 40 x 15	561.500
100 x 40 x 30	579.000
100 x 40 x 60	587.750
100 x 40 x 90	602.750
100 x 45 x 15	571.500
100 x 45 x 30	581.500
100 x 45 x 60	588.375
100 x 45 x 90	604.000
100 x 50 x 15	577.750
100 x 50 x 30	584.000
100 x 50 x 60	595.875
100 x 50 x 90	615.250
150 x 40 x 15	588.375
150 x 40 x 30	624.625
150 x 40 x 60	632.750
150 x 40 x 90	650.875
150 x 45 x 15	605.250
150 x 45 x 30	628.375
150 x 45 x 60	637.125
150 x 45 x 90	651.500
150 x 50 x 15	620.875
150 x 50 x 30	632.125
150 x 50 x 60	639.625
150 x 50 x 90	651.500

200 x 40 x 15	634.625
200 x 40 x 30	639.625
200 x 40 x 60	722.125
200 x 40 x 90	735.250
200 x 45 x 15	705.875
200 x 45 x 30	712.750
200 x 45 x 60	730.250
200 x 45 x 90	735.875
200 x 50 x 15	710.250
200 x 50 x 30	716.500
200 x 50 x 60	734.000
200 x 50 x 90	735.875

Std Err of LS Mean = 1.640

A5.2 Chromatogram from samples treated using SC-CO_2.

Figure A1.14 and A1.15 shows the chromatogram of samples treated using SC-CO_2 at 200 and 250 bar at 50 ^{o}C for 60 min.

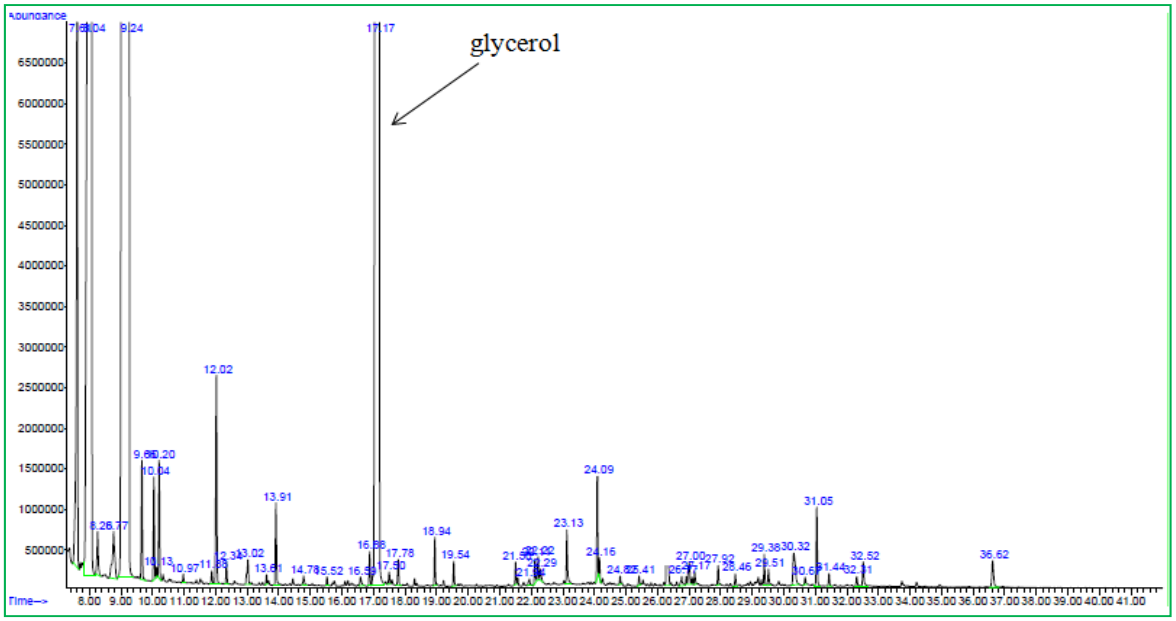


Fig. A1-14 Chromatogram of samples treated with SC-CO2 at 200 bar, 50 ^{o}C for 60 min.

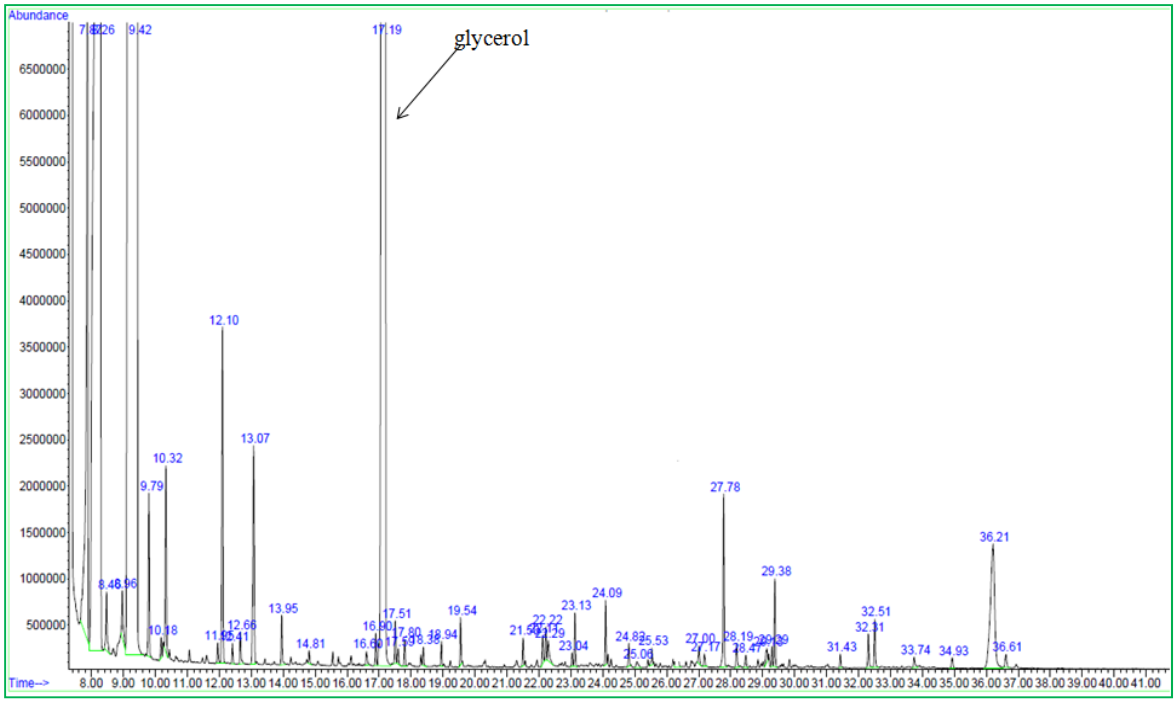


Fig. A1-15 Chromatogram of samples treated with SC-CO2 at 250 bar, 50 oC for 60 min.

REFERENCES

Abd El-Baky, H. H., El-Baz, F. K. and El-Baroty, G. S. (2004) 'Production of antioxidant by the green alga *Dunaliella salina*', *International Journal of Agriculture and Biology* **6**: 49-57

Agarwal, G. P. (1990) 'Glycerol', Advances in Biochemical Engineering and Biotechnology **41**: 95-128

Aguilar, C. P., Gonzalez, M., Cifuentes, A. S. and Silva, M. (2004) 'Growth and accumulation of total carotenoids in two strains of *Dunaliella salina* teod. (Chlorophyceae) from the northern and central coast of Peru', *Journal of the Chilean Chemical Society* **49**: 69-74

Ahmad, I. and Hellebust, J. A. (1986) 'The role of glycerol and inorganic ions in osmoregulatory responses of the euryhaline flagellate *Chlamydomonas pulsatilla* Wollenweber', *Plant Physiology* **82**: 406-10

Akiko, K. and Go, T. (1996) 'Photorespiration protects C3 plants from photooxidation', *Nature*, **384**: 557-560

Ali, S. and Haq, I. (2006) 'Kinetic basis of celite (CM 2:1) addition on the biosynthesis of 3,4-dihydroxyphenyl- L-alanine (L-DOPA) by *Aspergillus oryzae* ME2 using L-tyrosine as a basal substrate', *World Journal of Micro-biology & Biotechnology* **22**: 347-353

Allwood, J. W., Ellis, D. I. and Goodacre, R. (2008) 'Metabolomic technologies and their application to the study of plants and plant–host interactions', *Physiologia Plantarum* **132**: 117-135

Anderca, M. I., Furuichi, T., Pinontoan, R. and Muto, S. (2002) 'Identification of a mitochondrial nucleoside diphosphate kinase from the green alga *Dunaliella* tertiolecta', *Plant Cell Physiology* **43**: 1276-1284

Andrich, G., Nesti, U., Venturi, F., Zinnai, A. and Fiorentini, R. (2005) 'Supercritical fluid extraction of bioactive lipids from the microalgae *Nannochloropsis* sp. European', *Journal of Lipid Science and Technology* **107**:381-386

Antoni, D., Zverlov, V. V., and Schwarz W. H. (2007) 'Biofuels from microbes', *Applied Microbiology and Biotechnology* **77**: 23-35

Araneda, P., Jimenez, C., Gomez-Silva, B. (1992) 'Microalgae from Northerm Chile III. Growth and beta-carotene content of three isolates of *Dunaliella salina* from the Atacame Desert', *Revista de Biologia Marina de Valparaiso***27**: 157-162

Avron, M. (1992) 'Osmoregulation', In: Avron M, Ben-Amotz A, editors. *Dunaliella*: Physiology, biochemistry and biotechnology. Florida: CRC Press, Boca Raton. pp 135-164

Avron, M. and Ben-Amotz, A. (1978) 'Production of Glycerol from Algae' *US Patent* **4**: 115

Avron, M. and Ben-Amotz, A. (1992) In: Avron, M. and Ben-Amotz, A. Editors, *Dunaliella Physiology Biochemistry and Biotechnology*, CRC Press p. 240

Balat, M. (2006) 'Fuel characteristics and the use of biodiesel as a transportation fuel', *Energy sources* **28**: 855-864

Baldauf, S. L. (2008) 'An overview of the phylogeny and diversity of eukaryotes' *Journal of Systematics and Evolution* **46**: 263-273

Barsanti, L. and Gualtieri, P. (2006) 'Algae: Anatomy, Biochemistry, and Biotechnology'. CRC Press, Boca Raton, 301 pp.

Barsanti, L., Frassanito, A. M., Passarelli, V., Evangelista, V., Etebari, M., Paccagnini, E., Lupetti, P., Lenzi, P., Verni, F. and Gualtieri, P. (2013) '*Tetraflagellochloris mauritanica* GEN. ET SP. NOV. (Chlorophyceae), a new flagellated alga from the Mauritanian desert: morphology, ultrastructure, and phylogenetic framing', *Journal of Phycology* **49**: 178-193

Beale, S. I. and Castelfranco, P. A (1974) 'Biosynthesis of δ-aminolevulinic acid in higher plants. II. Formation of 4C-8-aminolevulinic acid from labeled precursors in greening plant tissues', *Plant Physiology* **53**: 297-303

Beck, H. C., Hansen, A. M. and Lauritsen, F. R. (2003) 'Novel pyrazine metabolites found in polymyxin biosynthesis by *Paenibacillus polymyxa*', *FEMS Microbiology Letters* **220**: 67-73

Becket, J., Dibiasio, D., Keough, P. A. and Chen, B. J. (1985) 'Algal Glycerol Production: Initial Glycerol Synthesis Kinetics', *Chemical Engineering Communications* **32**: 357-367

Ben-Amotz, A, Avron, M. (1983) 'Accumulation of metabolites by halo-tolerant algae and its industrial potential', *Annual Review of Microbiology* **37**: 95-119

Ben-Amotz, A. (1975) 'Adaptation of the unicellular alga *Dunaliella* parva to a saline environment', *Journal of Phycology* **11**: 50-54

Ben-Amotz, A. (1980) 'Glycerol Production in the alga *Dunaliella',* In Biochemical and photosynthetic aspects of energy production, A San Pietro (ed.), Academic Press, New York

Ben-Amotz, A. (2011) 'Biotechnology of the Algae *Dunaliella* and *Nannochloropsis* for Production of Commercial Products and Biofuel', *1st International Conference on Algal Biomass, Biofuels and Bioproducts* 7/17/2011 to 7/20/2011, Westin St Louis, United States

Ben-Amotz, A. and Avron, M. (1973) 'The role of glycerol in the osmotic regulation of the halophilic alga *Dunaliella parva'*, *Plant Physiology* **51**: 875-878

Ben-Amotz, A. and Avron, M. (1978) 'On the mechanism of osmoregulation in *Dunaliella*-In: Energetics and Structure of Halophilic Microorganisms', ed. Caplan, S. R. & Ginzburg, M. 529-541. Amsterdam: Elsevier/North Holland.

Ben-Amotz, A. and Avron, M. (1980) 'Osmoregulation in the halophilic algae *Dunaliella* and *Asteromonas*', In DW Rain, ed, Genetic Engineering of Osmoregulation: Impact on Plant Productivity for Food, Chemicals and Energy. Plenum Press, New York, pp 91-100

Ben-Amotz, A. and Avron, M. (1983) 'On the factors which determine massive, β-carotene accumulation in the halotolerant alga *Dunaliella* bardawil', *Plant Physiology*, **72**: 593-597

Ben-Amotz, A. and Avron, M. (1989) 'The Biotechnology of Mass Culturing *Dunaliella* for Products of Commercial Interest', In Algal and Cyanobacterial Biotechnology, RC Cresswell, T A V Rees and N Shah (eds.), Longmans, Harlow

Ben-Amotz, A. and Avron, M. (1989) 'The wavelength dependence of massive carotene synthesis in *Dunaliella* bardawil (Chlorophyceae)', *Journal of Phycology*, **25**: 175-178

Ben-Amotz, A. and Avron, M. (1990) 'The biotechnology of cultivating of the halotolerant alga *Dunaliella*', *Trends in Biotechnology* **8**: 121-126

Ben-Amotz, A. and Grunwald, T. (1981) 'Osmoregulation in the halotolerant algae *Asteromonas* gracilis', *Plant Physiology* **67**: 613-616

Ben-Amotz, A., Gressel, J. and Avron, M. (1987) 'Massive accumulation of phytoene induced by norflurazon in *Dunaliella bardawil* prevents recovery from photoinhibition', *Journal of Phycology* **23**: 176-181

Ben-Amotz, A., Katz, A. and Avron, M. (1982) 'Accumulation of β-carotene in halotolerant algae: purification and characterization of β-carotene-rich globules from *Dunaliella* bardawil (Chlorophyceae)', *Journal of Phycology* **18**: 529-537

Ben-Amotz, A., Lers, A. and Avron, M. (1988) 'Stereoisomers of b-carotene and phytoene in the alga *Dunaliella bardawil*', *Plant Physiology* **86**: 1286-1291

Ben-Amotz, A., Polle, J. E. W. and Rao, D. V. S. (2009) 'The Alga *Dunaliella*: Biodiversity, Physiology, Genomics and Biotechnology', *Science Publishers*, Enfield, USA

Ben-Amotz, A., Shaish, A. and Avron, M., (1991) 'The biotechnology of cultivating *Dunaliella* for production of b-carotene rich algae', *Bioresource Technology* 38: 233-235

Ben-Amotz, A., Sussman, I. and Avron, M. (1982) 'Glycerol production by *Dunaliella*', *Experientia* **13**: 49-52

Benemann, J. R. (1997) 'CO_2 mitigation with microalgal systems' *Energy Conversion and Management* **38**: 475-479

Bental, M., Pick, U., Avron, M. and Degani, H. (1990) 'The role of intracellular orthophosphate in triggering osmoregulation in the alga *Dunaliella salina*', *European Journal of Biochemistry*, **188**: 117-122

Berry, M. (2004) 'Mammalian central nervous system trace amines, Pharmacologic amphetamines, physiologic neuromodulators', *Journal of Neurochemistry* **90**: 257-271

Berube, K. A., Roessler, J., Jones, T. P. and Janes, S. (1994) 'The determination of volume of *Dunaliella* cell by transmission electron microscope and image analysis', *Annals of Botany* **73**: 481-491

Besson, I., Creuly, C., Gros, J. B. and Larroche, C. (1997) 'Pyrazine production by *Bacillus subtilis* in solid-state fermentation on soyabeans', *Applied Microbiology and Biotechnology* **47**: 489-495

Bhalla, R., Narasimhan, K. and Swarup, S. (2005) 'Metabolomics and its role in understanding cellular responses in plants', *Plant Cell Reproduction* **24**: 562–571

Bimakr, M., Rahman, R. A., Taip, F. S., Chuan, L. T., Ganjloo, A.,Selamat, J. and Hamid, A. (2009) 'Supercritical Carbon Dioxide (SC-CO2) Extraction of Bioactive Flavonoid Compounds from Spearmint (*Mentha spicata L.*) Leaves', *European Journal of Scientific Research* **33**: 679-690

Binder, S. (2010) 'Branched-Chain Amino Acid Metabolism in *Arabidopsis thaliana*', *American Society of Plant Biologists* 2010 10.1199/tab.0137

Bold, H. C., and Wynne, M. J. (1978) 'Introduction to the Algae Structure and Reproduction' PRENTICE-HALL, INC., Englewood Cliffs, New Jersey pp 1-28

Bolling, C. and Fiehn, O. (2005) 'Metabolite profiling of *Chlamydomonas reinhardtii* under nutrient deprivation', *Plant Physiology* **139**: 1995-2005

Borowitzka, L. J, Kessly, O. S, and Brown, A. D. (1977) 'The Salt Relations of *Dunaliella*', *Archives of Microbiology* **113**: 131-l38

Borowitzka, L. J. (1981) 'The microflora: Adaptation to life in extremely saline lakes', *Hydrobiologia*, **81**: 33-46

Borowitzka, M. A. (1988) 'Algal growth media and sources of cultures', In: Borowitzka M.A. and Borowitzka L.J. (eds.), Micro-algal Biotechnology. Cambridge University Press: Cambridge. pp. 456-465

Borowitzka, M. A. and Borowitzka, L. J. (1988) '*Dunaliella*. Microalgal biotechnology', page 27-58, *Cambridge University Press*. Cambridge, UK

Borowitzka, M. A. and Huisman, J. M. (1993) 'The ecology of *Dunaliella* salina (Chlorophyceae, Volvocales)-Effect of environmental conditions on aplanospore formation', *Botanica Marina* **36**:233-243

Borowitzka, M. A. and Siva, C. J. (2007) 'The taxonomy of the genus *Dunaliella* (Chlorophyta, *Dunaliella*les) with emphasis on the marine and halophilic species', *Journal Applied Phycology* **19**: 567-590

Borowitzka, M. A., Chiappino, M. L. and Volcani, B. E. (1977) 'Ultrastructure of a chain-forming diatom *Phaeodactylum tricornutum', Journal Phycology* **13**: 162-170

Borowitzka, M., Borowitzka, L. and Kessly, D. (1990) 'Effects of salinity increase on carotenoid accumulation in the green alga *Dunaliella* salina', *Journal of Applied Phycology* **2**: 111-119

Brown, A. D. and Borowitzka, L. J. (1979) 'Halotolerance of *Dunaliella'*. In: Biochemistry and Physiology of Protozoa, ed. Hutner, S. H. and Levandowsky, M. 2nd ed'. Vol. 1. Ch. 6. New York: Academic Press

Brunham, B. F. and Lascelles, J. (1963) 'Control of porphyrin biosynthesis through a negative-feedback mechanism. Studies with preparations of δ-aminolaevulate synthetase and δ-aminolaevulate dehydratase from Rhodopseudomonas spheroides', *Biochemical Journal* **87**: 462-472

Buchanan, B. B., Gruissem, W. and Jones, R. L. (2000) 'Biochemistry and molecular biology of plants', *American Society of Plant physiologist* ISBN 0-943088-37-2

Burki, F., Shalchian-Tabrizi, K., Minge, M., Skjaeveland. Å., Nikolaev, S. I., Jakobsen, K. S. and Pawlowski, J. (2007) 'Phylogenomics reshuffles the eukaryotic supergroups', *PLoS ONE* **8**: 790-795

Burns, B. D. and Beardall, J. (1987) 'Utilisation of inorganic carbon by marine microalgae', *Journal of Experimental Marine Biology and Ecology* **107**: 75-86

Calvin, M. and Taylor, S. E. (1989) 'Fuels from Algae. In Algal and Cyanobacterial Biotechnology, RC Cresswell, TAV Rees and N Shah (eds .)', Longmans, Harlow

Cardozo, K. H. M., Guaratini, T., Barros, M. P., Falcao, V. R., Tonon, A. P., Lopes, N. P., Campos, S., Torres, M. A., Souza, A. O., Colepicolo, P. and Pinto, E. (2007) 'Metabolites from algae with economical impact', *Comparative Biochemistry and Physiology* **146**: 60-78

Castor, T. P. and Hong, G. T. (1995) 'Supercritical Fluid Disruption of and Extraction from Microbial Cells', *US Patent* **5**: 380-826

Celekli, A. and Donmez, G. (2006) 'Effect of pH, light intensity, salt and nitrogen concentrations on growth and beta-carotene accumulation by a new isolate of *Dunaliella sp*.', *World Journal of Microbiology and Biotechnology* **22**: 183-189

Central Bureau of Statistics, (2010) 'An atlas of Namibia's population: monitoring and understanding its characteristics', *Central Bureau of Statistics*, Windhoek.

Chaumont, D. (1993) 'Biotechnology of algal biomass production: a review of systems for outdoor mass culture', *Journal of Applied Phycology* **5**: 593-604

Chen, B. J. and Chi, C. H. (1981) 'Process development and evaluation for algal glycerol production', *Biotechnology and Bioengineering* **23**: 1267-1288

Chen, C. Y. and Durbin, E. G. (1994) 'Effects of pH on the growth and carbon uptake of marine phytoplankton', *Marine Ecology Progress Series* **109**: 83-94

Chen, H. and Jiang, J. (2009) 'Osmotic Responses of *Dunaliella* to the Changes of Salinity', *Journal of Cellular Physiology* **219**: 251-258

Chen, H., Lao, Y. and Jiang, J. (2011) 'Effects of salinities on the gene expression of a (NAD+)-dependent glycerol-3-phosphate dehydrogenase in *Dunaliella salina*', *Science of the Total Environment* **409**: 1291-1297

Chen, T. B., Reineccius, G. A., Bjorklund, J. A. and Leete, E. (1991) 'Biosynthesis of 2-methoxy-3-isopropylpyrazine in *Pseudomonas perolens*', *Journal of Agricultural and Food Chemistry* **39**: 1009-1012

Chitlaru, E. and Pick, U. (1989) 'Selection and Characterization of *Dunaliella* salina Mutants Defective in Haloadaptation', *Plant Physiology* **91**: 788-794

Chitlaru, E and Pick, U. (1991) 'Regulation of Glycerol Synthesis in Response to Osmotic Changes in *Dunaliella*', *Plant Physiology*, **96**: 50-60

Chow, Y. Y. S., Goh, S. J. M., Su, Z., Ng, D. H. P., Lim, C. Y., Lim, N. Y. N., Lin, H., Fang, Lei. and Lee, Y. K. (2013) 'Continual production of glycerol from carbon dioxide by *Dunaliella* tertiolecta', *Bioresource Technology* **136**: 550-555

Cifuentes, A. S., Gonzalez, Mariela, A. and Parra, O. O. (1996) 'The effect of salinity on the growth and carotenogenesis in two Chilean strains of *Dunaliella salina* Teodoresco', *Biological Research* **29**: 227 – 236

Claus, H. and Decker, H. (2006) 'Bacterial tyrosinases', *Systematic and Applied Microbiology*, **29**: 3-14

Coesel, S. N., Baumgartner, A. C., Teles, L. M., Ramos, A. A., Henriques, N. M., Cancela, L., Varela, J. C. S. (2008) "Nutrient limitation is the main regulatory factor for carotenoid accumulation and for Psy and Pds steadystate transcript levels in *Dunaliella salina* (Chlorophyta) exposed to high light and salt stress", *Journal of Marine Biotechnology* **10**: 602-611

Colman, B. and Rotatore, C. (1995) 'Photosynthetic inorganic carbon uptake and accumulation in two marine diatoms', *Plant Cell and Environment* **18**: 919-924

Cook, D., Fowler, S., Fiehn, O. and Thomashow, M. F. (2004) 'A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis', *Proceedings of the National Academy of Sciences of the United States of America*', **101**: 15243-15248

Cui, L., Xue, L., Li, J. Zhang, L. and Yan, H. (2010) 'Characterization of the glucose-6-phosphate isomerase (GPI) gene from the halotolerant alga *Dunaliella salina*', *Mol. Biol. Rep.* **37**: 911-916

Davis, J. S. (1990) 'Biological management for the production of salt from seawater'. In: Akatsuka, I. (Ed.), Introduction to Applied Phycology. SPB Academic Publishing, The Hague, The Netherlands, pp. 479-488

Day, P., McNeil, J. and Sirovski, F. (2011) '(E)mission impossible? By Aquafuel Research Ltd', *Emission Control tce*, Issue 839

Degani, H., Sussman, I., Peschek, G. A. and Avron, M. (1985) '13C- and lH-NMR Studies of Osmoregulation in *Dunaliella*', *Biochimica et Biophysica Acta* **846**: 313-323

Demirbas, A. (2003) 'Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: A survey', *Energy Conversion and Management* **44**: 2093-2109

Demirbas, A. (2006) 'Biodiesel production via non-catalytic SCF method and biodiesel fuel characteristics', *Energy Conversion and Management*, **47**: 2271-2282

Dettmer, K., Aronov, P. A. and Hammock, B. D. (2007) 'Mass spectrometry-based metabolomics', *Mass Spectrometry Reviews* **26**: 51-78

Diaz, P., Borsani, O., Mirquez, A. and Monza, J. (2005) 'Nitrogen metabolism in relation to drought stress responses in cultivated and model Lotus species', *Lotus Newsl* **35**: 83-92

Dieter, A., Wolf-Gladrow, D., Riebesell, U., Burkhardt, S. and Jelle, B. (1999) 'Direct effects of CO2 concentration on growth and isotopic composition of marine plankton' *Tellus* **51B**: 461-476

Dimitriou, I. and Bridgwater, A. V. (2010) 'Syngas production for biomass-to-liquids (BTL) applications. Process analysis and optimisms' Edited by Bridgwater, A. V. (2010) Proceedings of the bioten conference on biomass, bioenergy and biofuels, CPL Press, UK p 244

Dittami, S. M., Gravot, A., Renault, D., Goulitquer, S. Eggert, A. Bouchereau, A., Boyen, C. and Tonon, T. (2011) 'Integrative analysis of metabolite and transcript abundance during the short-term response to saline and oxidative stress in the brown alga *Ectocarpus siliculosus*', *Plant, Cell and Environment* **34**: 629-642

Du, H., Wang, Z., Yu, W., Liu, Y. and Huang, B. (2011) 'Differential metabolic responses of perennial grass *Cynodon transvaalensis* × *Cynodon dactylon* (C4) and *Poa Pratensis* (C3) to heat stress', *Physiologia Plantarum* **141**: 251-264

Ehrenfeld, J. and Cousins, J. L. (1982) 'Ionic regulation of the unicellular green alga *Dunaliella* tertiolecta', *Journal of Membrane Biology* **70**: 47-57

Einspahr, K. J., Maeda, M and Thompson, Jr. G. A. (1988) 'Concurrent Changes in *Dunaliella salina* Ultrastructure and Membrane Phospholipid Metabolism after Hyperosmotic Shock', *The Journal of Cell Biology* **107**: 529-538

El-Mashad, H. M., Zhang, R., and Avena-Bustillos, R. J. (2007) 'A two-step process for biodiesel production from salmon oil', *Biosystem Engineering* **99**: 220-227

Eppley, R. W. (1972) 'Temperature and phytoplankton growth in the sea', *Fishery Bulleting* **70**: 1063 - 1085

Erongo Regional Council, (2005) 'Erongo regional survey on public participation', *National Survey in Public Participation*, Namibia

Facchini, P. (2001) 'Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications', *Annual Reviews in Plant Physiology and Plant Molecular Biology* **52**: 29-66

Fangrui, M. and Milford, A. H. (1999) 'Biodiesel production: a review', *Bioresources Technology* **70**: 1-15

Fantegrossi, W., Murnane, K. and Reissig, C. (2008) 'The behavioural pharmacology of hallucinogens', *Biochemical Pharmacology* **75**: 17-33

Fazeli, M. R., Tofighi, H. Samadi, N. and Jamalifar, H. (2006) 'Effects of salinity on b-carotene production by *Dunaliella* tertiolecta DCCBC26 isolated from the Urmia salt lake, north of Iran', *Bioresource Technology* **97**: 2453-2456

Foyer, C. H., Parry, M. and Noctor, G. (2003) 'Markers and signals associated with nitrogen assimilation in higher plants', *Journal of Experimental Botany* **54**: 585-593

Fu, W., Paglia, G., Magnusdottir, M., Steinarsdottir, E. A., Gudmundsson, Steinn., Palsson, B. O, Andresson, O. S. and Brynjolfsson, S. (2014) 'Effects of abiotic stressors on lutein production in the green microalga *Dunaliella salina*', *Microbial Cell Factories* **13**:3

Fujii, S. and Hellebust, J. A. (1992) 'Release of Intracellular Glycerol and Pore Formation in *Dunaliella tertialecta* exposed to hypotonic stress', *Canadian Journal of Botany* **70**: 1313-1318

Garcia, A. and Barbas, C. (2011) 'Gas chromatography-Mass spectrometry (GC-MS) - based metabolomics', *Methods in Molecular Biology* **708**: 191-204

Garcia, F., Freile-Pelegrin, Y and Robledo, D. (2007) 'Physiological characterization of *Dunaliella* sp. (*Chlorophyta, Volvocales*) from Yucatan, Mexico', *Bioresource Technology* **98**: 1359-1365

Gehron, M. J. and White, D. C. (1983) 'Sensitive Assay of Phospholipid Glycerol in Environmental Samples', *Journal of Microbiological Methods* **1**: 23-32

Gensemer, R. W., Smith, R. E. H., Duthie, H. C. and Schiff, S. L. (1993) 'pH tolerance and metal toxicity in population of the planktonic diatom Asterionella: influence of synthetic and natural dissolved organic carbon. *Canadian Journal of Fisheries and Aquatic Sciences* **50**: 121 – 132

Gilmour, D. J., Hipkins, M. F. and Boney, A. D. (1984) 'The effect of decreasing the external salinity on the primary processes of photosynthesis in *Dunaliella tertiolecta*', *Journal of Experimental Botany* **35**: 28-35

GimmIer, H. and Möller, E. (1981) 'Salinity-dependent Regulation of Starch and Glycerol Metabolism in *Dunaliella parva*', *Plant Cell and Environment* **4**: 367-375

Gimmler, H., Kuhnl, E. M., Carl G. (1978) 'Salinity-dependent resistance of *D. parva* against extreme temperatures. I. Salinity and thermoresistance', *Zeitschrift fuer Pflanzenphysiologie* **90**: 133-153

Ginzburg, M. (1987) '*Dunaliella*: a green alga adapted to salt', *Advances in Botanical Research* **14**: 93-183

Ginzburg, M and Ginzburg, B. Z. (1981) 'Interrelationships of light, temperature, sodium chloride and carbon source in growth of halotolerant and halophilic strains of *Dunaliella', British Phycology Journal,* **16***:*313-324

Ginzburg, M. and Ginzburg, B. Z. (1993) 'Effect of light intensity on adaptation of *Dunaliella* to very high salt concentrations', *Journal of Experimental Botany* **44**: 1561-1567

Giordano, M. (1997) 'Adaptation of *Dunaliella* salina (Volvocales, Chlorophyta) to growth on NH^{+}_{4} as the sole N source', *Phycologia*, **36**: 345-350

Goldman, J. C. and Carpenter, E. J. (1974) 'A kinetic approach to the effect of temperature on algal growth', *Limnology and Oceanography* **19**: 756-766

Golldack, D., Dietz, K. and Gimmler, H. (1995) 'The Effects of Sudden Salt Stress on Protein Synthesis in the Green Alga *Dunaliella parva*', *Journal of Plant Physiology* **146**: 508-514

Gomez, A. M. and de la Ossa, E. M. (2000) 'Quality of wheat germ oil extracted by liquid and supercritical carbon dioxide', *Journal of the American Oil Chemists Society* **77**: 969-974

Gonzalez, M. A., Gomez, P. I. and Montoya, R. (1998) 'Comparison of PCR-RFLP analysis of the ITS region with morphological criteria of various strains of *Dunaliella*', *Journal of Applied Phycology* **10**: 573-581

Gordillo, F. J. L., Goutx, M., Figueroa, F. L. and Niell, F. X. (1998) 'Effects of light intensity, CO2 and nitrogen supply on lipid class composition of *Dunaliella* viridis', *Journal of Applied Phycology* **10**: 135 - 144

Gouveia, L. and Oliveira, A. C. (2009) 'Microalgae as raw material for biofuels production', *Journal of Industrial Microbiology and Biotechnology* **36**: 269-274

Goyal, A. (2007) 'Osmoregulation in *Dunaliella*, Part II: Photosynthesis and starch contribute carbon for glycerol synthesis during a salt stress in *Dunaliella* tertiolecta', *Plant Physiology and Biochemistry* **45**: 705-710

Goyal, A., Brown, A. D. and Gimmler, H. (1987) 'Regulation of salt-induced starch degradation in *Dunaliella* tertiolecta', *Journal of Plant Physiology* **127**: 77-96

Gravot, A., Dittami, S. M., Rousvoal, S., Lugan, R., Eggert, A., Collen J., Boyen, C., Bouchereau, A. and Tonon, T. (2010) 'Diurnal oscillations of metabolite abundance and gene analysis provide new insights into central metabolic processes of the brown alga *Ectocarpus siliculosus*', *New Phytologist* **188**: 98-110

Guy, C., Kaplan, F., Kopka, J., Selbig, J. and Hincha, D. K. (2008) 'Metabolomics of temperature stress', *Physiologia Plantarum* **132**: 220-235

Hackett, J. D., Yoon, H. S., Li, S., Reyes-Prieto, A., Rummele, S. E. and Bhattacharya, D. (2007) 'Phylogenomic analysis supports the monophyly of Cryptophytes and Haptophytes and the Association of Rhizaria with Chromalveolates', *Molecular Biology and Evolution* **24**: 1702-1713

Hadi, M. R., Shariati M. and Afsharzadeh S. (2008) 'Microalgal biotechnology: carotenoid and glycerol production by *Dunaliella* sp. algae isolated from the Gave khooni salt marsh, Iran', *Biotechnology and Bioprocess Engineering* **13**: 540-544

Halim, R., Danquah, M. K. and Webley, P. A. (2012) 'Extraction of oil from microalgae for biodiesel production: A review', *Biotechnology Advances* **30**: 709-732

Hall, D. O. and Rao, K. K. (1999) 'Photosynthesis' Studies in Biology: Sixth edition, Cambridge University Press, ISBN: 0521 64257 4: p 4

Hamburger, C. (1905) 'Zur Kenntnis der *Dunaliella salina* und einer Amobe aus Salinenwasser von Cagliari', *Arch f Protistenkd* **6**: 111-131

Hardie, D. G. (2003) 'Minireview: The AMP-activated protein kinase cascade: The key sensor of cellular energy status', *Endocrinology* **144**: 5179-5183

Harries, D. and Rosgen, J. (2008) 'A Practical Guide on How Osmolytes Modulate Macromolecular Properties', *Methods in Cell Biology*, **84**: 679-735

Hartono, R., Mansoori, G. A. and Suwono, A. (2001) 'Prediction of solubility of biomolecules in supercritical solvents', *Chemical Engineering Science* **56**: 6949-6958

Harvey, P. J., Psycha, M., Kokossis, A., Abubakar, A. L., Trivedi, V., Swamy, R., Cowan, A. K., Schroeder, D., Highfield, A., Reinhardt, G., Gartner, S., McNeil, J., Day, P., Brocken, M., Varrie, J. and Ben-Amotz, A. (2012) 'Glycerol Production by Halophytic Microalgae: Strategy for Producing Industrial Quantities in Saline Water', *20th EU Biomass conference and Exhibition* 18-22 June, Milan Italy pp 85-90.

Hayat, M. A. (1986) 'Basic Techniques for Transmission Electron Microscopy', London: Academic Press Inc. ISBN: 0-12-333925-1 (alk. paper), ISBN: 0-12-333926-X

He, Q., Qiao, D., Bai, L., Zhang, Q., Yang, W., Li, Q. and Cao, Y. (2007) 'Cloning and characterization of a plastidic glycerol 3-phosphate dehydrogenase cDNA from *Dunaliella salina*', *Journal of Plant Physiology* **164**: 214-220

Hellebust, J. A. (1976) 'Osmoregulation', *Annual Review of Plant Physiology* **27**: 485-505

Herrero, M., Cifuentes, A. and Ibanez, E. (2006) 'Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review', *Food Chemistry,* **98**: 136-148

Hines, A., Oladiran, G. S., Bignell, J. P., Stentiford, G. D. and Viant, M. R. (2007) 'Direct sampling of organisms from the field and knowledge of their phenotype: Key recommendations for environmental metabolomics', *Environmental Science and Technology* **41**: 3375-3381

Hotos, G. N. (2003) "Growth, filtration and ingestion rate of the rotifer Brachionus plicatilis fed with large (*Asteromonas* gracilis) and small (Chlorella sp.) celled algal species" *Aquaculture Research* **34**: 793-0802

Huber, G. W., Iborra, S. and Corma, A. (2006) 'Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering', *Chemical Reviews* **106**: 4044-4098

Huertas, I. H., Colman, B., Espie, S. G. and Lubian, M. L. (2000) 'Active transport of CO_2 by three species of marine microalgae', *Journal of Phycology* **36**: 314-320

Humphrey, G. F. (1975) 'The photosynthesis:respiration ratio of some unicellular marine algae', *Journal of Experimental Marine Biology and Ecology* **18**: 111-119

Igamberdieva, A. U., Bykovaa, N. V., Leab, P. J. and Gardestrom, P. (2001) 'The role of photorespiration in redox and energy balance of photosynthetic plant cells: A study with a barley mutant deficient in glycine decarboxylase', *Physiologia Plantarum* **111**: 427–438

Ilknur, A. K., Cirik, S. and Goksan, T. (2008) 'Effects of Light Intensity, Salinity and Temperature on Growth in Camalti Strain of *Dunaliella viridis* Teodoresco from Turkey', *Journal of Biological Science* **8**: 1356-1359

Ishihara, A., Matsuda, F. and Miyagawa, H. and Wakasa, K. (2007) 'Metabolomics for metabolically manipulated plants: effects of tryptophan overproduction', *Metabolomics* **3**:319-334

Jahnke, L. S. and White, A. L. (2003) 'Long-term hyposaline and hypersaline stresses produce distinct antioxidant responses in the marine alga *Dunaliella tertiolecta'*, *Journal of Plant Physiology* **160**: 1193-1202

Jaime, L., Mendiola, J., Ibanez, E., Martin-Alvarez, P. J., Cifuentes, A., Reglero, G. and Senorans, F. (2007) 'β-carotene isomer composition of sub- and supercritical carbon dioxide extracts, Antioxidant activity measurement', *Journal of Agricultural and Food Chemistry* **55**: 10585-10590

Janssen, M., Slenders, P., Tramper, J., Mur, L. R. and Wijffels, R. H. (2001) 'Photosynthetic efficiency of *Dunaliella tertiolecta* under short light/dark cycles', Enzyme *and Microbial Technology* **29**: 298-305

Jayappriyan, K.R., Rajkumar, R., Sheeja, L., Nagaraj, S., Divya, S. and Rengasamy, R. (2010) 'Discrimination between the morphological and molecular identification in the genus *Dunaliella', International Journal of Current Research* **8**: 073-078

Jimenez, C. and Niell, F. X. (1991) 'Growth of *Dunaliella viridis* Teodoresco: effect of salinity, temperature and nitrogen concentration', *Journal of Applied Phycology* **3**: 319-327

Jimenez, C., Niell, F. X. and Fernandez, J. A. (1991) 'The photosynthesis of *Dunaliella parva* Lerche as a function of temperature, light and salinity', *Hydrophobia* **197**: 165-206

John, R. P., Anisha, G. S., Nampoothiri, K. M. and Pandey, A. (2011) 'Micro and macroalgal biomass: A renewable source for bioethanol', *Bioresource Technology* **102**: 186-193

Johnson, M. K., Johnson, E. J., Mac Elroy, R. D., Speer, H. L. and Bruff, B. S. (1968) 'Effects of salts on the halophilic alga *Dunaliella* viridis', *Journal of Bacteriology* **95**: 1461-1468

Kaçka, A. and Donmez, G. (2008) 'Isolation of *Dunaliella* spp. from a hypersaline lake and their ability to accumulate glycerol', *Bioresource Technology* **99**: 8348-8352

Kaplan, A., Schreiber, U. and Avron, M. (1980) 'Salt-Induced Metabolic Changes in *Dunaliella salina*', *Plant Physiology* **65**: 810-813

Katz, A. and Avron, M. (1985) 'Determination of Intracellular Osmotic Volume and Sodium Concentration in *Dunaliella*', *Plant Physiology* **78**: 817-820

Katz, A. and Pick, U. (2001) 'Plasma membrane electron transport coupled to Na+ extrusion in the halotolerant alga *Dunaliella*', *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1504**: 423-431.

Katz, A., Bental, M., Degani, H. and Avron, M. (1991) 'In vivo pH regulation by a Na^+/H^+ antiporter in the halotolerant alga *Dunaliella* salina', *Plant Physiology* **96**: 110-115

Katz, A., Kaback, H. R. and Avron, M. (1986) 'Na+/H+ antiport in isolated plasma membrane vesicles from the halotolerant alga *Dunaliella salina'*, *FEBS Letters* **202**: 141-144

Kazazi, H., Rezaei, K., Ghotb-Sharif, S. J., Emam-Djomeh, Z. and Yamini, Y. (2007) 'supercritical fluid extraction of flavours and fragrances from *Hyssopus officinalis* l. cultivated in Iran', Food *Chemistry* **105**: 805-811

Kempa, S., Hummel, J., Schwemmer, T., Pietzke, M., Strehmel, N., Wienkoop, S., Kopka, J. and Weckwerth, W. (2009) 'An automated GCxGC-TOF-MS protocol for batch-wise extraction and alignment of mass isotopomer matrixes from differential 13Clabelling experiments: a case study for photoautotrophic-mixotrophic grown *Chlamydomonas reinhardtii* cells', *Journal of Basic Microbiology* **49**: 82-91

Khalil, Z. I., Asker, M. M. S., El-Sayed, S. and Kobbia, I. A. (2010) 'Effect of pH on growth and biochemical responses of *Dunaliella* bardawil and Chlorella ellipsoidea', *World Journal of Microbiology and Biotechnology* **26**: 1225-1231

Khosravi-Darani, K., Vasheghani-Farahani, E., Shojaosadati, S. A. Yamini, Y. (2004) 'The effect of process variables on supercritical fluid disruption of Ralstonia eutropha cells for poly-(hydroxybutyrate) recovery', *Biotechnology Progress* **6**: 1757-1765

Kikuchi, G. Kumar, A., Taimage, P and Shemin, D. (1958) 'The enzymatic synthesis of δ-aminolevulinic acid', *Journal of Biological Chemistry* **223**: 1214-1219

Kim, N. D., Kwak, M. K. and Kim, S. G. (1997) 'Inhibition of cytochrome P450 2E1 expression by 2-(allylthio)pyrazine, a potential chemoprotective agent: Hepatoprotective effects', *Biochemical Pharmacology* **53**: 261-269

Kipe-Nolt, J. A. and Stevens, S. E. Jr. (1980) 'Biosynthesis of δ-Aminolevulinic Acid from Glutamate in *Agmenellum quadruplicatum*', *Plant Physiology* **65**: 126-128

Kleinegris, D. M. M., Van-Es, M. A., Janssen, M., Brandenburg, W. A. and Wijffels, R. H. (2010) 'Carotenoid fluorescence in *Dunaliella* salina', *Journal of Applied Phycology* **22**: 645-649

Kleinubing, S. J., Vieira, R. S., Beppu, M. M., Guibal, E. and da Silva, M. G. C. (2010) 'Characterization and Evaluation of Copper and Nickel Biosorption on Acidic Algae *Sargassum Filipendula'*, *Materials Research* **13**: 541-550

Kluender, C., Sans-Piche, F., Riedl, J., Altenburger, R., Hartig, C., Laue, G. and Schmitt-Jansen, M. (2009) 'A metabolomics approach to assessing phytotoxic effects on the green alga *Scenedesmus vacuolatus*', *Metabolomics* **5**: 59-71

Koek, M. M., Renger, H.. Jellema, R. H., Greef, J. V., Tas, A. C. and Hankemeier, T. (2011) 'Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives', *Metabolomics* **7**: 307-328

Kopka, J., Schauer, N., Krueger, S., Birkemeyer, C., Usadel, B., Bergmuller, E., Dormann, P., Weckwerth, W., Gibon, Y., Stitt, M., Willmitzer, L., Fernie, A. R. and Steinhauer, D. (2005) 'GMD@CSB.DB: The GOLM metabolome database', *Bioinformatics* **21**: 1635-1638

Korb, R. E., Saville, P. J., Johnston, A. M. and Raven, J. A. (1997) 'Source of inorganic carbon or photosynthesis by three species of marine diatoms', *Journal of Phycology* **33**: 433-440

Koyanagi, T., Katayama, T., Suzuki, H., Nakazawab, H., Yokozeki, K. and Kumagai, H. (2005) 'Effective production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) with Erwinia herbicola cells carrying a mutant transcriptional regulator TyrR' *Journal of Biotechnology*, **115**: 303-306

Krienitz, L., Ustinova, I., Friedl, T. and Huss, V. A. R. (2001) 'Traditional generic concepts versus 18S rRNA gene phylogeny in the green algal family Selenastraceae (Chlorophyceae, Chlorophyta)', *Journal of Phycology* **37**: 852-865

Krishnaveni, R., Rathod, V., Thakur, M. S. and Neelgund, Y. F. (2009) 'Transformation of L-tyrosine to L-dopa by a novel fungus, Acremonium rutilum, under submerged fermentation' Current Microbiology, **58**: 122-128

Kulma, A. and Szopa, J. (2007) 'Catecholamines are active compounds in plants' *Plant Science*, **172**: 433-440

Kumar, A. and Bachhawat, A. K. (2012) 'Pyroglutamic acid: throwing light on a lightly studied metabolite' *Current Science*, 102: 1-10

Kumar, R. (2009) 'Role of naturally occurring osmolytes in protein folding and stability', *Archives of Biochemistry and Biophysics',* **491**: 1-6

Lamers, P. P. (2011) 'Metabolomics of carotenoids accumulation in *Dunaliella salina',* PhD Thesis, Wageningen University, Wageningen, The NetherlandsISBN: 978-90-8585-852-2

Larkum, A. W. D. (2010) 'Limitations and prospects of natural photosynthesis for Bioenergy production', *Current Opinion in Biotechnology* **21**: 271-276

Latorella, A. H. and Vadas, R. L. (1973) 'Salinity adaptation by *Dunaliella tertiolecta*. I. Increases in carbonic anhydrase activity and evidence for a light-dependent Na+/H+ exchange', *Journal of Phycology* **9**: 273-277

Lee, D. and Fiehn, O. (2008) 'High quality metabolomic data for *Chlamydomonas reinhardtii*', *Plant Methods* **4**: 7

Leejeerajumnean, A., Duckham, S. C., Owens, J. D. and Ames, J. M. (2001) 'Volatile compounds in Bacillus-fermented soyabeans', *The Journal of the Science of Food and Agriculture* **81**: 525-529

Leliaert, F., Smith, D. R., Moreau, H., Herron, M., Delwiche, C. F. and Clerck, O. D. (2012) 'Phylogeny and molecular evolution of the green algae', *Critical Reviews in Plant Sciences* **31**: 1-46

Lendle, A, and Schaus, M. (2010) 'Sustainability Criteria in the EU Renewable Energy Directive:Consistent with WTORules?' *ICTSD information note No*. **2**: 1-52

Less, H. and Galili, G. (2008) 'Principal transcriptional programs regulating plant amino acid metabolism in response to abiotic stresses', *Plant Physiology* **147**: 316-330

Lewis, L. A. and Lewis, P. O. (2005) 'Unearthing the molecular phylodiversity of desert soil green algae (Chlorophyta)', *Systematic Biology* **54**: 936-947

Lewis, L. A. and McCourt, R. M. (2004) 'Green algae and the origin of land plants', *American Journal of Botany* **91**: 1535-56

Li, H., Dong, J., Chen, W., Wang, S., Guo, H., Man, Y., Mo, P., Li, (2006) 'Measurement of serum total glycerides and free glycerol by high-performance liquid chromatography' *Journal of Lipid Research* **47**: 2089-2096

Lichtenthaler, H. K. (1987) 'Chlorophylls and carotenoids: pigments of photosynthetic biomembranes', *Methods in Enzymology* **148**: 350-382

Lin, C. Y., Viant, M. R. and Tjeerdema, R. S. (2006) 'Metabolomics: Methodologies and applications in the environmental sciences', *Journal of Pesticide Science* **31**: 245-251

Lin, E. and Magasanik, B. (1960) 'The activation of glycerol dehydrogenase from *Aerobacter aerogenese* by Monovalent cations', *Journal of Biological Chemistry*, **234**: 1820

Lin, H. M. and Chen, L. F. (1994) 'Method for recovery of intracellular material by disruption of microbial cells with carbon dioxide under pressure', *US Patent* **5**: 306-637

Lin, H. M., Yang, Z. and Chen, L. F. (1992) 'An improved method for disruption of microbial cells with pressurized carbon dioxide', *Biotechnology Progress* **8**:165-166

Lin, H., Fang, L., Low, C. S., Chow, Y. and Lee, Y. K. (2013) 'Occurrence of glycerol uptake in *Dunaliella* tertiolecta under hyperosmotic stress', *Federation of European Biochemical Societies* (*FEBS Journal*) **280**: 1064 -1072

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L. and Fernie, A. R. (2006) 'Gas chromatography mass spectrometry-based metabolite profiling in plants', *Nature Protocols* **1**: 387-396

Liu, B. H, and Lee, Y. K. (2000) 'Secondary carotenoids formation by the green alga Chlorococcum sp.', *Journal of Applied Phycology* **12**: 301-307

Lopez-Bautista, J. M. and Chapman, R. L. (2003) 'Phylogenetic affinities of the Trentepohliales inferred from small-subunit rDNA', *International Journal of Systematic and Evolutionary Microbiology* **53**: 2099-2106

Lorence, A., Chevone, B. I., Mendes, P. and Nessler, C. L. (2004) 'myo-Inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis', *Plant Physiology* **134**: 1200-1205

Ma, F., and Hanna, M. A. (1999) 'Biodiesel production: A review', *Bioresource Technology* **70**: 1-15

MacDonald, J. C. (1973) 'Toxicity, analyses, and production of aspergillic acid and its analogues', *Canadian journal of biochemistry* **51**: 1311-1315

Macias-Sanchez, M. D., Mantell, C., Rodriguez, M., Martinez de la Ossa, E., Lubian, L. M., and Montero, O. (2009) 'Comparison of supercritical fluid and ultrasound-assisted extraction of carotenoids and chlorophyll a from *Dunaliella* salina', *Talanta* **77**: 948-952

Macias-Sanchez, M. D., Mantell, C., Rodriguez, M., Martinez de la Ossa, E., Lubian, L. M., and Montero, O. (2007) 'Supercritical fluid extraction of carotenoids and chlorophyll a from Synechococcus sp.', *The Journal of Supercritical Fluids* **39**: 323-329

Macias-Sanchez, M. D., Mantell, C., Rodriguez, M., Martínez de laOssa, E., Lubian, L. M. and Montero, O. (2005) 'Supercritical fluid extraction of carotenoids and chlorophyll a from Nannochloropsis gaditana', *Journal of Food Engineering* **66**: 245-251

Maeda, H. and Dudareva, N. (2012) 'The shikimate pathway and aromatic amino Acid biosynthesis in plants', *Annual Review of Plant Physiology*, **63**: 73-105

Maeda, M and Thompson, Jr. G. A. (1986) 'On the Mechanism of Rapid Plasma Membrane and Chloroplast Envelope Expansion in *Dunaliella salina* Exposed to Hypoosmotic Shock', *The Journal of Cell Biology* **102**: 289-297

Markovits, A., Gianelli, M.P., Conejeros, R., Erazo, S. (1993) 'Strain selection for beta carotene production by *Dunaliella*', *World Journal of Microbiology and Biotechnology,* **9**: 534-537

Masuda, T., Polle, J. E. W. and Melis, A. (2002) 'Biosynthesis and Distribution of Chlorophyll among the Photosystems during Recovery of the Green Alga *Dunaliella* salina from Irradiance Stress', *Plant Physiology* **128**: 603-614

Mata, T. M., Martins, A. A. and Caetano, N. S. (2010) 'Microalgae for biodiesel production and other applications: A review', *Renewable and Sustainable Energy Reviews* **14**: 217-233

Matsumoto, T., Shinozaki, F., Chikuni, T., Yabuki, A., Takishita, K., Kawachi, M., Nakayama, T., Inouye, I., Hashimoto, T., and Inagaki, Y. (2011) 'Green-colored Plastids in the Dinoflagellate Genus *Lepidodinium* are of Core Chlorophyte Origin', *Protist* **162**: 268-276

McKenzie, L. C., Thompson, J. E., Sullivan, R. and Hutchison, J. E. (2004) 'Green chemical processing in the teaching laboratory: a convenient liquid CO2 extraction of natural products', *Green Chemistry* **6**: 355-358

Mendes, R. L., Reis, A., Fernandes, H. L., Novais, J. M. and Palavra, A. F. (1999) 'Supercritical CO2 extraction of lipids from a GLArich *Arthrospira (Spirulina) maxima* biomass', In: *Proc. 5th It. Conference on Supercritical Fluids and Their Applications*. Garda (Verona, Italy) pp. 209-216

Mendes, R. L., Fernandes, H. L., Cygnarowicz-Provost, M., Cabral, J. M. S., Novais, J. M. and Palavra, A. F. (1994) 'Supercritical CO2 extraction of lipids from microalgae', In: *Proc. 3rd Int. Symposium on Supercritical Fluids*. Strasbourg (France) pp. 477-480

Mendiola, J. A., Jaime, L., Santoyo, S., Reglero, G., Cifuentes, A., Ibanez, E. and Senorans, F. J. (2007) 'Screening of functional compounds in supercritical fluid extracts from Spirulina platensis', *Food Chemistry* **102**: 1357-1367

Mendoza, A., Cesareo, R., Valdes, M., Meitin, J. J. Perez, R. and Lorente, Y. (1999) 'Energy dispersive X-ray fluorescence analysis of marine pollution indicators' *Journal of Radioanalytical and Nuclear Chemistry* **240**: 459-465

Mercer, R. and Armenta, R. E. (2011) 'Developments in oil extraction from microalgae', *European Journal of Lipid Science and Technology* **113**: 539-547

Mishra, A. Mandoli, A. and Jha, B. (2008) 'Physiological characterization and stress-induced metabolic responses of *Dunaliella salina* isolated from salt pan', *Journal of Indian Microbiology and Biotechnology* **35**: 1093-1101

Misra, L. and Wagner, H. (2007) 'Extraction of bioactive principles from Mucuna pruriens seeds', *Indian Journal of Biochemistry and Biophysics* **44**: 56-60

Mittova, V., Theodoulou, F. L., Kiddle, G., Gomez, L.,Volokita, M., Tal, M., Foyer, C. H. and Guy, M. (2003) 'Coordinate induction of glutathione biosynthesis and glutathione-metabolizing enzymes is correlated with salt tolerance in tomato', *FEBS Letters* **554**: 417-421

Moradi, M. and Ismail, A. M. (2007) 'Responses of Photosynthesis, chlorophyll fluorescence and ROS - Scavenging systems to salt stress. During seedling and reproductive stages of Rice', *Annals of Botany* **99**: 1161-1173

Munoz, J., Mudge, S. M. and Sandoval, A. (2004) 'Effects of ionic strength on the production of short chain volatile hydrocarbons by *Dunaliella* salina (Teodoresco)', *Chemosphere* **54**: 1267-1271

Muradyan, E. A., Klyachko-Gurvich, G. L., Tsoglin, L. N., Sergeyenko, T. V. and Pronina, N. A. (2004) 'Changes in lipid metabolism during adaptation of the *dunaliella salina* photosynthetic apparatus to high CO_2 Concentration', *Russian Journal of Plant Physiology* **51**: 53-62

Murray, K. E. and Whiteld, F. B. (1975) 'The occurrence of 3-alkyl- 2-methoxypyrazines in raw vegetables', *Journal of the Science of Food and Agriculture* **26**: 973-986

Murray, K. E., Shipton, J. and Whiteld, F.B. (1970) '2-Methoxypyrazines and the flavour of green peas (*Pisum sativum*)', *Chemistry and Industry* **4**: p897-898

Nakamura, K., Enomoto, A., Fukushima, H., Nagai, K. and Hakoda, M. (1994) 'Disruption of microbial cells by the discharge of high pressure carbon dioxide'. *Bioscience, Biotechnology and Biochemistry* **58**: 1297-1301

Nigam, P. S. and Singh, A. (2011) 'Production of liquid biofuels from renewable resources', *Progress in Energy and Combustion Science* **37**: 52-68

Niyogi, K. K. (1999) 'Photoprotection revisited: Genetic and molecular approaches', *Annual Review of Plant Physiology and Plant Molecular Biology* **50**: 333-359

Ooi, Y., Zakaria, R., Mohamed, A. and Bhatia, S. (2004) 'Catalytic conversion of palm oil-based fatty acid mixture to liquid fuel', *Biomass and Bioenergy* **27**: 477-484

Oren, A. (2005) 'A Hundred years of *Dunaliella* Research: 1905–2005. Saline Systems outdoor mass culture', *Journal of Applied Phycology* **5**: 593-604

Owens, J. D., Allagheny, N., Kipping, G. and Ames, J. M. (1997) 'Formation of volatile compounds during *Bacillus subtilis* fermentation of soya beans', *The Journal of the Science of Food and Agriculture* **74**: 132-140

Parra, O. O., Floyd, G. L. and Wilcox, L .W. (1990) 'Taxonomic identification and ultrastructural characterization of a Chilean strain of *Dunaliella*', *Revista Chilena de Historia Natural* **63**: 239-245

Patil, K. J., Patil, V. A., Mahajan, S. R. and Mahajan, R. T. (2011) 'Bio-activity of Algae Belonging to Bhusawal region, Maharashtra', *Current Botany* **2**: 29-31

Pauling, L., Robinson, A. B., Teranishi, R., Cary, P. (1971) 'Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography', *Proceedings of the National Academy of Sciences of the United States of America*', **68**: 2374–2376

Perley, J. E. and Stowe, B. B. (1966) 'The Production of Tryptamine from Tryptophan by Bacillus cereus (KVT)', *Biochemical Journal* **100**: 169-174

Perretti, G., Bravi, E., Montanari, L., Fantozzi, P. (2003) 'Extraction of PUFAs rich oil from algae with supercritical carbon dioxide', In: *Proc. 6th Int. Symposium on Supercritical Fluids.* Versailles (France) pp. 29-34

Perrut, M. (2001) 'Supercritical fluid application: industrial developments and economical issues', In: *Proc. 5th It. Conference on Supercritical Fluids and Their Applications.* Maiori (Italy) pp. 1-8

Peterfi, L. S. and Manton, I. (1968) 'Observations with the electron microscope on *Asteromonas* gracilis Artari emend. (Stephanoptera gracilis (Artari) Wisl.), with some comparative observations on *Dunaliella* sp.', *British Phycology Bulletin* **3**: 423-440

Phadwal, K. and Singh, P. K. (2003) 'Effect of nutrient depletion on β-carotene and glycerol accumulation in two strains of *Dunaliella* sp.' *Bioresource Technology*, **90**: 55-58

Pick, U. (1991) 'Regulation of Glycerol Synthesis in Response to Osmotic Changes in *Dunaliella*', *Plant Physiology* **96:** 50-60

Pick U. (1992) 'ATPases and ion transport in *Dunaliella*', In: Avron M, Ben-Amotz A, (editors). *Dunaliella*; physiology, biochemistry and biotechnology. *New-York: CRC Press*. pp 63-97

Pick, U., Karni, L and Avron, M. (1986) 'Determination of Ion Content and Ion Fluxes in the Halotolerant Alga *Dunaliella salina*', *Plant Physiology* **81**: 92-96

Polak, J. T., Balanan, M., Peplow, A. and Philips, A. J. (1989) '*S*FE of EPA rich lipid from *Ochronomas danica* and *Skeletonema costatum', ACS Symposium Series* **406**: 449-455

Pombert, J. F., Otis, C., Lemieux, C. and Turmel, M. (2004) 'The complete mitochondrial DNA sequence of the green alga *Pseudendoclonium akinetum* (*Ulvophyceae*) highlights distinctive evolutionary trends in the Chlorophyta and suggests a sister-group relationship between the Ulvophyceae and Chlorophyceae', *Molecular Biology and Evolution* **21**: 922-935

Pombert, J. F., Otis, C., Lemieux, C. and Turmel, M. (2005) 'Chloroplast genome sequence of the green alga *Pseudendoclonium akinetum* (Ulvophyceae) reveals unusual structural features and new insights into the branching order of chlorophyte lineages', Molecular Biology and Evolution **22**: 1903-1918

Porra, R. J., Thompson, W. A. and Kriedemann, P. E. (1989) 'Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy', *Biochimica et Biophysica Acta*, **975**: 384-394

Prescott, G.W. (1969) 'The Algae: Review', Butler and Tanner Ltd. Frome London pp 3-10

Rad, F. A., Aksoz, N and Hejazi, M. A. (2011) 'Effect of salinity on cell growth and β-carotene production in *Dunaliella* sp. isolates from Urmia Lake in northwest of Iran', *African Journal of Biotechnology* **10**: 2282-2289

Rai, A. K. (1990) 'Biochemical characteristics of photosynthetic response to various external salinities in halotolerant and fresh-water cyanobacteria. FEMS Microbiology Letters 69, 177-180

Rai, A. K. and Abraham, G. (1993) 'Salinity tolerance and growth analysis of the cyanobacterium *Anabaena doliolum*', Bulletin of Environmental contamination and Toxicology **51**: 724-731

Ramaraj, R., Tsai, D. D.W, and Chen, P. H. (2013) 'Chlorophyll is not Accurate Measurement for Algal Biomass', *Chiang Mai Journal of Science* **40**: 547-555

Ramos, A. A., Polle, J., Tran, D., Cushman, J. C., Jin**,** E. and Varela, J. C. (2011) 'The unicellular green alga *Dunaliella salina* Teod. as a model for abiotic stress tolerance: genetic advances and future perspectives', *Algae* **26**: 3-20

Randolph, T. W. (1990) 'Supercritical fluid extraction in biotechnology', *Trend in Biotechnology* **8**: 78-82

Raven, J. A. (1980) 'Nutrient transport in microalgae' In *Advances in Microbial Physiology* (Editors: Rose, A.H. and Morris, J.G., editors) **21**: 47-226, Academic Press, London

Rehm, H. (1988) 'Microbial production of glycerol and other polyols. In: Biotechnology', Weinheim: VCH, p. 205-27

Richmond, A. (1986) 'Cell Response to Environmental Factors. In Handbook of Microalgal Mass Culture', A Richmond (ed.), CRC Press, Inc., Baton Rouge, Florida

Richmond, A. (2004) 'Handbook of microalgal culture: biotechnology and applied phycology', Blackwell Science Ltd; Company, 2121 State Avenue, Ames, Iowa 50014-8300, USA

Riebesell, U. (2004) 'Effects of CO2 Enrichment on Marine Phytoplankton', *Journal of Oceanography* **60**: 719-729

Ritchie, R. J. (2010) 'Modelling photosynthetic photon flux density and maximum potential gross photosynthesis', *Photosynthetica* **48**: 596-609

Roessner, U., Wagner, C., Kopka, J., Tretheway, R. N. and Willmitzer, L. (2000) 'Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry', *Plant Journal* **23**: 131-142

Saha, P., Kunda, P. and Biswas, A. K. (2012) 'Influence of sodium chloride on the regulation of Krebs cycle intermediates and enzymes of respiratory chain in mungbean (*Vigna radiata* L. Wilczek) seedlings', *Plant Physiology and Biochemistry* **60**: 214-222

Schenk, P. M., R. S., Hall, T., Stephens, E., Marx. U. C., Mussgnug, J. H., Posten, C., Kruse, O. and Hankamer, B. (2008) 'Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production', *Bioenergy Research* **1**:20-43

Shariati, M. (2003) 'Characterization of three species of *Dunaliella salina, Dunaliella parva and Dunaliella pseudosalina* isolated from salt marsh of GaveKhoni of Isfahan-IRAN', *Iranian Journal of Science and Technology* **27**: 185-190

Shariati, M. and Lilley, R. M. (1994) 'Loss of intracellular glycerol from *Dunaliella* by electroporation at constant osmotic pressure: Subsequent restoration of glycerol content and associated volume changes', *Plant Cell Environment* **17**: 1295-1304

Shemin, D. and Russell, C. S. (1953) 'δ-Aminolevulinic acid, its role in the biosynthesis of porphyrins and purines', *Journal of the American Chemical* Society **75**: 4873-4874

Shen, B., Hohman, S., Jensen, R. G. and Bohnert, H. J. (1999) 'Role of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast', *Plant Physiology* **121**: 45-52

Shetty, P., Atallah, M. T. and Shetty, K. (2002) 'Effects of UV treatment on the proline-linked pentose phosphate pathway for phenolics and L-DOPA synthesis in dark germinated *Vicia faba*', *Process Biochemistry* **37**: 1285-1295

Shimizu, M., Fujii, T., Masuo, S. and Takaya, N. (2010) 'Mechanism of *De Novo* Branched-Chain Amino Acid Synthesis as an Alternative Electron Sink in Hypoxic *Aspergillus nidulans* Cells', *Applied and Environmental Microbiology* **76**: 1507–1515

Siegel, B. Z., Siegel, S. M, Speitel, T., Waber, J. and Stoeker, R. (1984) 'Brine organisms and the question of habitat-specific adaptation', *Origins of Life* **14**: 757-770

Singh, J. and Gu, S. (2010) 'Commercialization potential of microalgae for biofuels production', *Renewable and Sustainable Energy Reviews* **14**: 2596-2610

Stahl, E., Schutz, E. and Mangold, H. K. (1980) 'Extraction of Seed Oils with Liquid and Supercritical Carbon Dioxide', *Journal of Agricultural Food Chemistry* **28**: 1153-1157

Street, T. O., Bolen, D. W. and Rose, G. D. (2006) 'A molecular mechanism for osmolyte-induced protein stability', *Biophysics* **103**: 13997-14002

Surwase, S. N. and Jadhav, J. P. (2011) 'Bioconversion of L-tyrosine to L-DOPA by a novel bacterium *Bacillus sp*. JPJ', *Amino Acids* **41**: 495-506

Swiedrych, A., Stachowiak, J. and Szopa, J. (2004) 'The catecholamine potentiates starch mobilization in transgenic potato tubers', *Plant Physiology and Biochemistry* **42**: 103-109

Tafreshi, A. H and Shariati, M. (2009) '*Dunaliella* biotechnology: methods and applications', *Journal of Applied Microbiology* **107**: 14-35

Taherzadeh, M. J., Adler, L. and Liden, G. (2002) 'Strategies for enhancing fermentative production of glycerol-a review', *Enzyme and Microbial Technology* **31**: 53-66

Tartar, A. and Boucias, D. G. (2004) 'The non-photosynthetic, pathogenic green alga *Helicosporidium* sp. has retained a modified, functional plastid genome', *FEMS Microbiology Letters* **233**: 153-157

Tausz, M., Sircelj, H. and Grill, D. (2004) 'The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid?', *Journal of Experimental Botany* **55**: 1955-1962

Tazaki, K., Fyfe, W. S., Iizumi, S., Sampei, Y., Watanabe, H., Goto, M., Miyake, Y. and Noda, S. (1994) 'Clay aerosols and arctic ice algae' *Clays and Clay Minerals* **42**: 402-408

Thana, P., Machmudah, S., Goto, M., Sasaki, M., Pavasant, P. and Shotipruk, A. (2008) 'Response surface methodology to supercritical carbon dioxide extraction of astaxanthin from *Haematococcus pluvialis'*, *Bioresource Technology* **99**: 3110-3115

Tong, Y., Gao, L., Xiao, G. and Pan, X. (2011) 'Supercritical CO2 Extraction of Chlorophyll a from *Spirulina platensis* with a Static Modifier', *Chemical Engineering Technology* **34**: 241-248

Tran, D., Vo, T., Portilla, S., Louime, C., Doan, N., Mai, T., Tran, D. and Ho, T. (2013) 'Phylogenetic study of some strains of *Dunaliella*', *American Journal of Environmental Science* **9**: 317-321

Troxler, R. F. and Brown, A. S. (1975) 'Metabolism of 6-aminolevulinic acid in red and blue-green algae', *Plant Physiology* **55**: 463-467

Turmel, M., Brouard, J. S., Gagnon, C., Otis, C. and Lemieux, C. (2008) 'Deep division in the Chlorophyceae (Chlorophyta) revealed by chloroplast phylogenomic analyses', *Journal of Phycology* **44**: 739-750

Turmel, M., Otis, C., and Lemieux, C. (2003) 'The mitochondrial genome of *Chara vulgaris*: Insights into the mitochondrial DNA architecture of the last common ancestor of green algae and land plants', *Plant Cell* **15**: 1888-1903

Van Alstyne, K., Nelson, A., Vyvyan, J. and Cancilla, D. (2006) 'Dopamine functions as an antiherbivore defense in the temperate green alga *Ulvaria obscura*', *Oecologia* **148**: 304-311

Vergara-Fernandez, A., Vargas, G., Alarcon, N. and Velasco, A. (2008) 'Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system', *Biomass and Bioenergy* **32**: 338-344

Wackett, L., P. (2008) 'Microbial-based motor fuels: science and technology', *Microbial Biotechnology* **1**: 211-25

Wagner, L. (2007) 'Biodiesel form Algae Oil'. MORA Associates pp 1-8

Wang, Z., Zhuge, J., Fanga, F., and Prior, B. A. (2001) 'Glycerol production by microbial fermentation: A review', *Biotechnology Advances* **19**: 201-223

Watanabe, S. and Nakayama, T. (2007) 'Ultrastructure and phylogenetic relationships of the unicellular green algae *Ignatius tetrasporus* and *Pseudocharacium americanum* (Chlorophyta)', *Phycological Research* **55**: 1-16

Wegmann, K., Ben-amotz, A. and Avron, M. (1980) 'Effect of Temperature on Glycerol Retention in the Halotolerant Algae *Dunaliella* and *Asteromonas*', *Plant Physiology* **66**: 1196-1197

Weiss, M., Haimovich, G. A. L. and Pick, U. R. I. (2001) 'Phosphate and sulfate uptake in the halotolerant alga *Dunaliella* are driven by Na^+-symport mechanism', *Journal of Plant Physiology* **158**: 1519-1525

Wellburn, A. R. (1994) 'The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution', *Journal of Plant Physiology* **144**: 307-313

Williams, T. G. and Turpin, D. M. (1987) 'The role of external carbonic anhydrase in inorganic carbon acquisition by *Chlamydomonas reinhardtii*', *Plant Physiology* **80**: 92-96

Wolf-Gladrow, D. A., and Riebesell, U. (1997) 'Diffusion and reactions in the vicinity of plankton: a refined model for inorganic carbon transport'. *Marine Chemistry* **59**, 17-34.

Wu, B., Zhang, B., Feng, X., Rubens, J. R., Huang, R., Hicks, L. M,, Pakrasi, H. B., Tang, Y. J. (2010) 'Alternate isoleucine synthesis pathway in cyanobacterial species', *Microbiology* **156**: 596-602

Yancey, P. H. (2005) 'Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses', *The Journal of Experimental Biology*, **208**: 2819-2830

Yang, W., Cao, Y., Sun, X., Huang, F., He, Q., Qiao, D. and Bai, L. (2007) 'Isolation of a FAD-GPDH gene encoding a mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase from *Dunaliella salina*', *Journal of Basic Microbiology* **47**: 266-274

Zaidul, I. S. M., Nik Norulaini, N. A., Mohd Omar, A. K., and Smith Jr. R. L. (2007) 'Supercritical carbon dioxide (SC-CO2) extraction of palm kernel oil from palm kernel', *Journal of Food Engineering* **79**: 1007-1014

Zhang, X., Lin, C., Chen, H., Wang, H., Qu, Z., Zhang, H., Yao, J. and Shen, D. (2003) 'Cloning of a NaCl induced fructose-1, 6-diphosphate aldolase cDNA from *Dunaliella salina* and its expression in tobacco', *China Life Science* **46**: 49-57

Zhao, L., Gong, W., Chen, X. and Chen, D. (2013) 'Characterization of genes and enzymes in *Dunaliella salina* involved in glycerol metabolism in response to salt changes', *Phycological Research* **61**: 37-45

Zhuge, J., Fang, H. Y., Wang, Z. X., Chen, D. Z., Jin, H. R. and Gu, H. L. (2001) 'Glycerol production by a novel osmotolerant yeast Candida glycerinogenes', *Applied Microbiology and Biotechnology* **55**: 686-692

Ziska L. H., Runion G.B., Tomecek M., Prior S. A., Torbet H. A. and Sicher R. C. (2009) 'An evaluation of cassava, sweet potato and field corn as potential carbohydrate sources for bioethanol production in Alabama and Maryland', *Biomass and Bioenergy* **33**: 1503-1508