Physiological and Biotechnological Studies on the Microalga *Dunaliella*, the Bacterium *Halomonas*, and the Cyanobacteria *Arthrospira* and *Spirulina*

by

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Dedication

To

My Father "Abdullah"

My Mother "Bakhitah"

My Wife "Samiah"

My Sons "Nawwaf, Faisal and Bassel"

For Their Love and Support

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Summary

A bacterial isolate and a microalga were identified to the genus level using 16S and 18S rRNA gene sequences respectively and phylogenetic trees were constructed. The bacterial isolate belonged to the genus *Halomonas* and it was called *Halomonas* sp. NAH1, whereas the microalga was confirmed as belonging to the strain *Dunaliella salina* CCAP 19/30 which was the source of 18S rRNA gene. Glycerol production by three strains of the unicellular microalga *Dunaliella* (*D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30) was explored. The strains were grown in batch cultures in a range of salinities (0.1 – 4.0 M NaCl). Both intracellular and extracellular glycerol concentrations were measured. All three strains grew well over the range of salinities with optimum growth for all strains at 0.1 to 0.4 M NaCl. All strains leak significant amounts of glycerol in batch cultures. Significant leakage of glycerol into the growth medium was found to be an intrinsic property of the three strains tested. Two strains of *Dunaliella salina* (CCAP 19/18 and 19/30) were grown in batch cultures and aerated with different concentrations of CO_2. Strain 19/18 accumulated large amounts of β-carotene under nitrogen limitation whereas the strain 19/30 did not. *Halomonas* sp. NAH1 grew optimally at 1.0 M NaCl and utilised glucose, glycerol or betaine as the sole source of carbon. Glucose supported the most rapid growth rate. Sensitivity of NAH1 to antibiotics was determined and tetracycline had the most inhibitory effect on growth. The cyanobacteria *Arthrospira fusiformis* CCAP 1475/8 and *Spirulina platensis* UTEX LB 2340 were shown to be only slightly halotolerant with optimum growth for *S. platensis* at 0.1 M NaCl and for *A. fusiformis* at 0.5 M NaCl. Phycobiliprotein content was very low in both strains, but very high protein content (92.9% of the dry weight biomass) was obtained for *S. platensis*. This strain looks very promising for mass cultivation for food and/or feed purposes. Glucosyl-glycerol was found to be the compatible solute in both *A. fusiformis* and *S. platensis*.

Abbreviations

APC	Allophycocyanin
BLAST	Basic Local Alignment Search Tool
BM	Basal medium
BMA	Basal medium agar
bp	basepair(s)
BSA	Bovine serum albumin
CCAP	Culture Collection of Algae and Protozoa
Chl.	Chlorophyll
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
D_2O	Deuterated H_2O
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDTA	Ethylenediaminetetraacetic acid
g	G-force
g	Gram(s)
h	Hour(s)
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
Hz	Hertz
kb	Kilobase(s)
kBq	Kilo Becquerel
L / l	Litre(s)

m	Metre
M	Molar
mg	Milligram(s)
MHz	Megahertz
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimole
MUR	Murdoch University Microalgae Culture Collection
NCBI	National Centre for Biotechnology Information
NCIMB	National Collection of Industrial, Marine and Food Bacteria
ng	Nanogram(s)
nm	Nanometre
NMR	Nuclear Magnetic Resonance
OD	Optical density
PC	C-phycocyanin
PCR	Polymerase chain reaction
PE	C-phycoerythrin
pmoles	Picomole(s)
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
s	Second(s)
TCA	Trichloroacetic acid
THF	Tetrahydrofuran

Tris	Tris (hydroxymethyl) methylamine
U	Units
UTEX	The Culture Collection of Algae at the University of Texas at Austin
v/v	Volume per volume
w/v	Weight per volume
µg	Microgram(s)
µl	Microlitre(s)
µm	Micrometre(s)
µmol(es)	Micromole(s)
µ	Specific growth rate

Introduction

Chapter One

1.1. General Introduction

Environmental stresses (e.g. high or low temperature or high salinity) stop or severely inhibit the growth of many organisms. However, particularly in the microbial world, many organisms can adapt to grow in extreme environments e.g. in acidic hot springs with a pH of 3 and temperatures exceeding 80 °C (Rothschild & Mancinelli, 2001). These adaptations affect the physiology of the cells profoundly and often lead to novel enzymes being synthesised and some metabolites being overproduced.

In the present work the main environmental stress of interest is salinity i.e. the ability of microorganisms to grow under conditions of fluctuating salinity found in rock pools on the shore or under conditions of very high salinity found in hypersaline lakes such as the Dead Sea (Gilmour, 1990). In the latter case the microorganisms are often halophilic as opposed to halotolerant i.e. they require substantial levels of NaCl for optimum growth. There are two main methods utilized by microorganisms to grow at high salinity. The first method replaces NaCl with KCl inside the cell and therefore under high salinity conditions, there is a very high internal concentration of KCl (Gilmour, 1990). This method is mainly in the group of halophiles called the Halobacteriaceae, which are members of the domain Archaea, but some bacterial groups (e.g. anaerobic Gram-positive bacteria belonging to the *Haloanaerobiales*) also utilize KCl accumulation (Galinski & Truper, 1994). However, there is as yet no evidence for the KCl method being used by any halotolerant or halophilic eukaryotic species.

The second method utilized by eukaryotic halophiles, most bacterial halophiles and a few groups of Archaea is the synthesis or uptake of small molecular weight organic compounds to balance the external osmotic pressure exerted by high concentrations of NaCl (Gilmour, 1990). These organic compounds are called compatible solutes because they do not inhibit cellular

metabolism even when present in high concentrations (see Section 1.6.2). The possibility of utilizing halophiles to overproduce compounds of interest is based on the synthesis of compatible solutes and has led to a number of biotechnology companies being set up to exploit halophilic microorganisms.

In this introductory chapter the characteristics of four groups of halophilic/halotolerant microorganisms will be discussed so that experimental work on these organisms described in the rest of the thesis can be put in context. The microorganisms studied are the green microalga *Dunaliella*, the cyanobacteria *Arthrospira* and *Spirulina*, and the Gram-negative bacterium *Halomonas*. In the following Sections the habitats, morphology and taxonomy of each microorganism will be discussed. In the second half of the chapter, the physiological and biotechnological aspects of their responses to environmental stress will be discussed.

1.2. Habitats

1.2.1. *Dunaliella*

Dunaliella is a unicellular microalga which it is found in many habitats especially hypersaline environments such as the Dead Sea in Palestine, the Great Salt Lake in Utah, the Pink Lake and Hutt lagoon in Australia, and artificially produced hypersaline ponds (Avron, 1992). In these salt water bodies, especially in high light intensity niches, *D. salina* Teod. form natural unialgal cultures which tend to move phototactically and concentrate on the surface of these bodies causing films of typical orange to red colour (Figures 1.1 and 1.2) due to massive accumulation of β-carotene inside the cells (Ben-Amotz & Shaish, 1992).

Figure 1.1. Solar evaporation ponds at Great Salt Lake's northeast end (USA, Utah). The lake is dominated by some halophiles mainly *Dunaliella salina* and Haloarchaea. These microorganisms accumulate carotenoid pigments, e.g. β-carotene, and this gives the Lake an unusual reddish or purplish colour (www.wikipedia.org).

1.2.2. ***Arthrospira* and *Spirulina***

Arthrospira and *Spirulina* are planktonic filamentous cyanobacteria and their species are present in a wide range of habitats such as freshwater, brackish waters, tropical lagoons, and hot springs. Such diversity would suggest these species or at least some of them are capable of adaptation to extreme environmental conditions. Perhaps the most illuminating example of this adaptability is that of alkaliphilic species (e.g. *Arthrospira fusiformis*) that grow abundantly in waters whose alkalinity is so high the pH may reach up to 11 eliminating or highly reducing the density of other microorganisms (Ciferri & Tiboni, 1985; Hu, 2004; Kebede & Ahlgren, 1996). *Spirulina platensis* Geitler for example grows naturally in tropical and subtropical water bodies where the temperature and the levels of carbonate and bicarbonate are quite high (Tomaselli, 1997).

Figure 1.2. The large open ponds used for the culture of *Dunaliella salina* at Hutt Lagoon, Western Australia, by Congnis Nutrition and Health. The largest ponds are about 250 ha in area. The reddish colour is due to massive accumulation of β-carotene inside *D. salina* cells (http://wwwscieng.murdoch.edu.au/centres/algae/B-EAM-Net/BEAM-Appl4a.htm).

1.2.3. ***Halomonas***

Halomonas species are abundant in many habitats and they were isolated from different environments such as estuarine waters, sea water, salt lakes, hypersaline lakes, salty foods and sea ice (Lim *et al.*, 2004).

1.3. Morphology

1.3.1. *Dunaliella*

Dunaliella species vary in cell shape from ellipsoid, ovoid, cylindrical, pyriform, and fusiformis to almost spherical. Changes in cell shape and size were recognised in some species as a result of altering growth conditions. *Dunaliella* cells lack a rigid cell wall and have instead a distinctive mucilaginous cell coat which can be visualised under the light microscope with Indian ink. They have also two flagella which are equal in length and apically inserted (Figure 1.4 – A). Most of the cell body is occupied by a single cup-, dish-, or bell-shaped chloroplast which has a thickened basal portion containing a pyrenoid (Figure 1.3). The pyrenoid is usually surrounded by starch grains which may also be found at other places within the chloroplast. The chloroplast in some species, especially *D. salina*, may also accumulate large quantities of β-carotene in interthylakoid spaces within oily globules (Figure 1.3). This makes the cells appear orange-red rather than green (Figure 1.4 – A). The chloroplast has also an eyespot (stigma) which is barely visible under the light microscope. The nucleus occupies most of the anterior part of the cell and has a porous envelope and a single prominent nucleolus. Various parts of the cell contain mitochondrial profiles. Golgi bodies are situated between the anterior end of the nucleus and the basal bodies and occur in numbers of 2 to 4. *Dunaliella* species contain vacuoles of different types (Preisig, 1992).

The vegetative reproduction of the cells occurs by lengthwise division in the motile state. Aplanospores (asexual cysts) may be formed under extreme conditions (Figure 1.4 – B). Sexual reproduction occurs by isogamy in which gametic fusion occurs by flagellar agglutination and activation of special mating structures (Preisig, 1992).

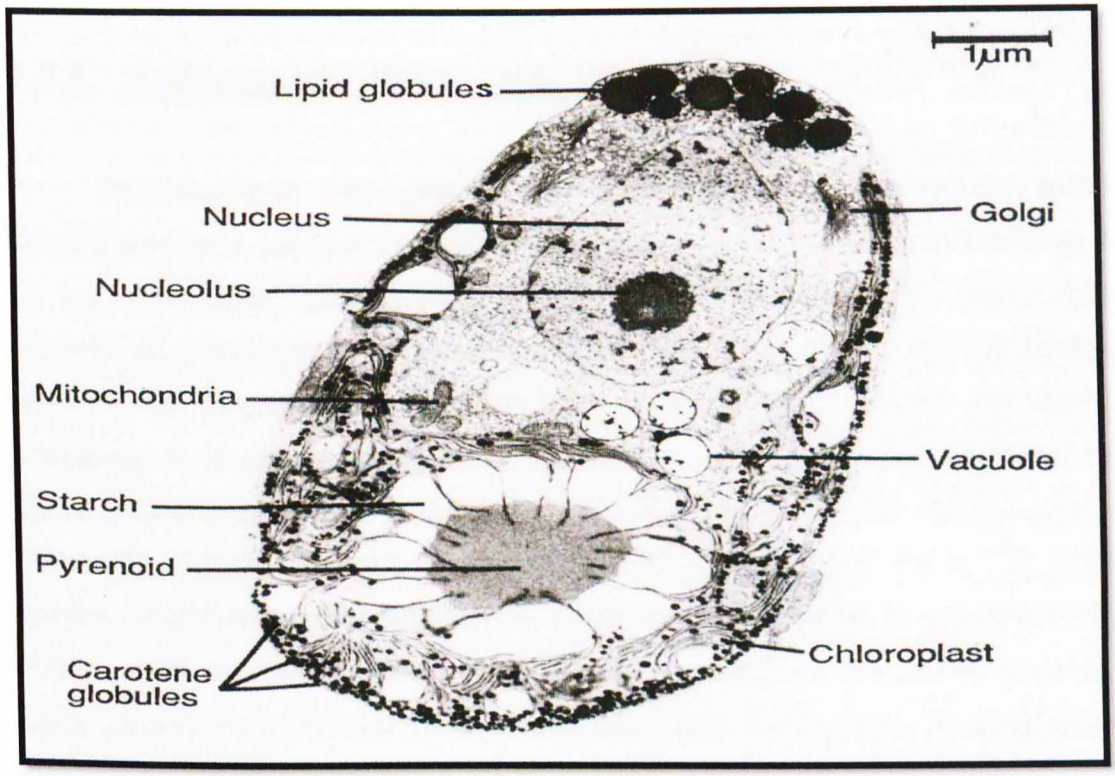

Figure 1.3. Electron micrograph of a section through β-carotene-rich *Dunaliella bardawil* (Ben-Amotz & Avron, 1990).

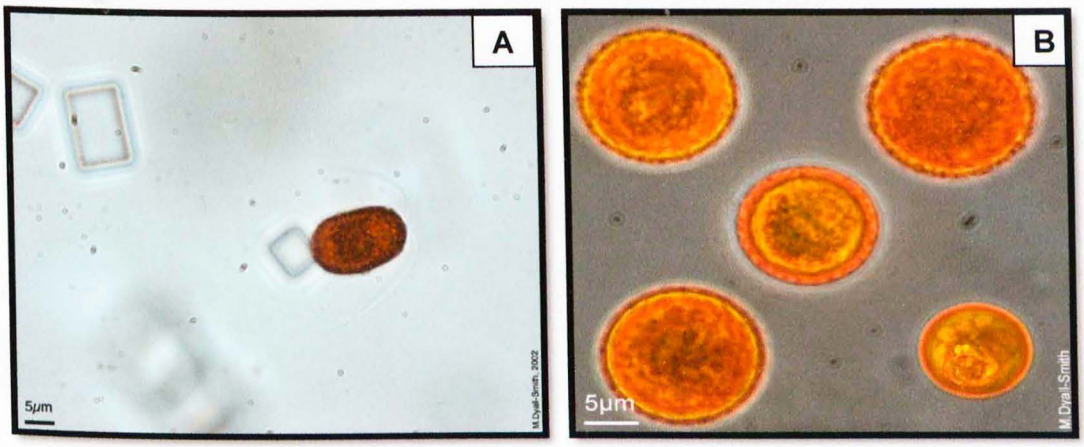

Figure 1.4. Photomicrographs of *Dunaliella salina* showing a cell swimming nearby salt crystals (**A**) and cysts (aplanospores) which form under extreme conditions (**B**) (http://www.microbiol.unimelb.edu.au/people/dyallsmith/research/salt_lakes/Salt_Lakes2002/SaltLakes2002MDS.html).

1.3.2. *Arthrospira* (Stizenberger 1852)

Cyanobacteria belonging to the genus *Arthrospira* consist of spiral shaped filaments called trichomes (Figure 1.5). The entire trichome is arranged as an open helix. Cross-walls may be seen in the trichome using light microscope, but when gas vesicles are abundant it is hard to see the cross-walls. Cells are generally shorter than broad or isodiametric and slight constrictions at cross-walls may be present or absent. The terminal cells in some strains may acquire a cap-like thickening of the outer cell wall (calyptra). Persistent sheaths are not produced and gliding motility is evident in most strains. Thylakoids are arranged in a radial form in trichome cross-section. In culture, trichome width varies from about 5-12 µm in numerous isolates, although smaller forms (3 µm) have been described from nature. The helix is an open spiral ranging from about 30 to 60 µm in length. The helix undergoes a transition to a flat spiral on solid medium (Castenholz *et al.*, 2001).

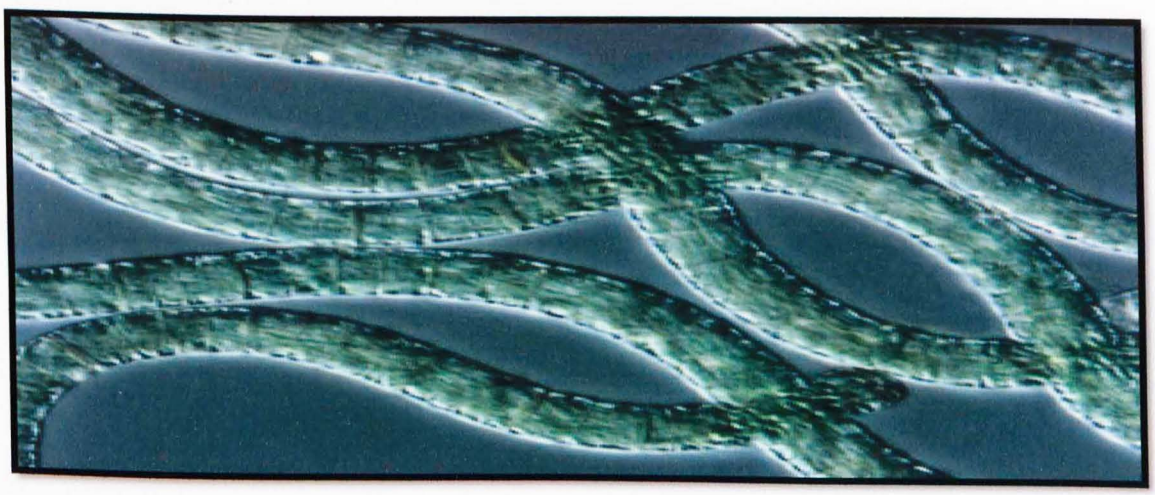

Figure 1.5. Photomicrograph of of *Arthrospira* (Stizenberger ex Gomont) showing cross walls between cells and thylakoid membranes (http://silicasecchidisk.conncoll.edu/LucidKeys/Carolina_Key/html/Arthrospira_Main.html).

1.3.3. *Spirulina* (Turpin 1829 ex Goment 1892)

Spirulina species are filamentous cyanobacteria that divide exclusively by binary fission in one plane and they grow in the form of a tightly coiled helix (right- or left-handed) (Figure 1.6). The helix may become partly unwound in some trichomes within a culture. Members of this genus can be distinguished from those of *Arthrospira* genus by having cross-walls that are thin and barely visible even by using light microscopy with phase contrast objectives. No sheath is visible under the light microscope. Healthy trichomes are in constant motion. Gliding motility occurs by "turning the screw" with great transverse movement and little forward motion. Motility occurs by rotation around the outer surface of the helix. The terminus of the trichome was found to be either blunt or pointed. The trichome diameter ranges from <1 μm to about 5 μm in size. The width of the whole helix may reach up to 12 μm when the diameter of the trichome is about 5 μm. Colour is variable among strains and it ranges from blue-green to red. The red strains are marine representatives containing large amounts of C-phycoerythrin which acts as the major light-harvesting pigment, but they contain relatively little phycocyanin and allophycocyanin. The trichome structure of *Spirulina* isolates seems to be stable, straight variants have not been observed even after maintenance in culture for more than 30 years (Castenholz *et al.*, 2001). This stability is another feature of *Spirulina* strains which helps in distinguishing between the two genera "*Arthrospira* and *Spirulina*".

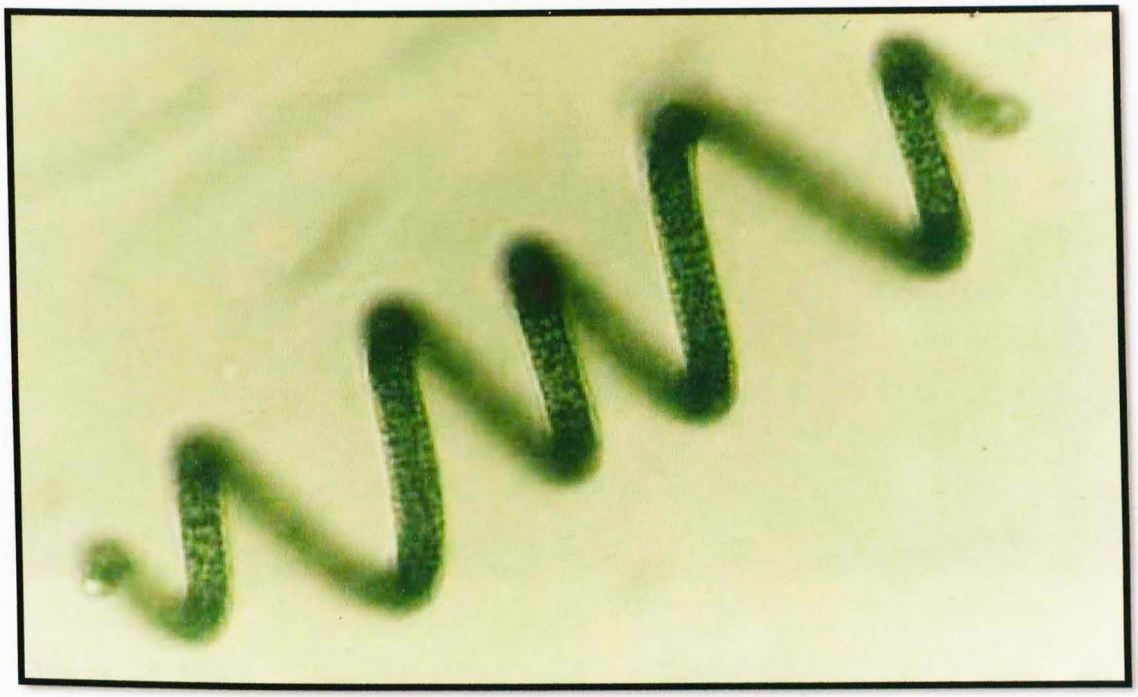

Figure 1.6. Photomicrograph of *Spirulina platensis* (http://www.spirulinasource .co m/cultivez1a.html).

1.3.4. ***Halomonas***

The genus *Halomonas* consists of Gram-negative rod-shaped bacteria about 0.6 µm wide by 2 µm long (Figure 1.7). Motile by lateral or polar flagella and they almost exclusively have a respiratory type metabolism with oxygen as the terminal electron acceptor. Colonies on plates are white to cream coloured. A range of carbon compounds can support growth (Vreeland, 1984).

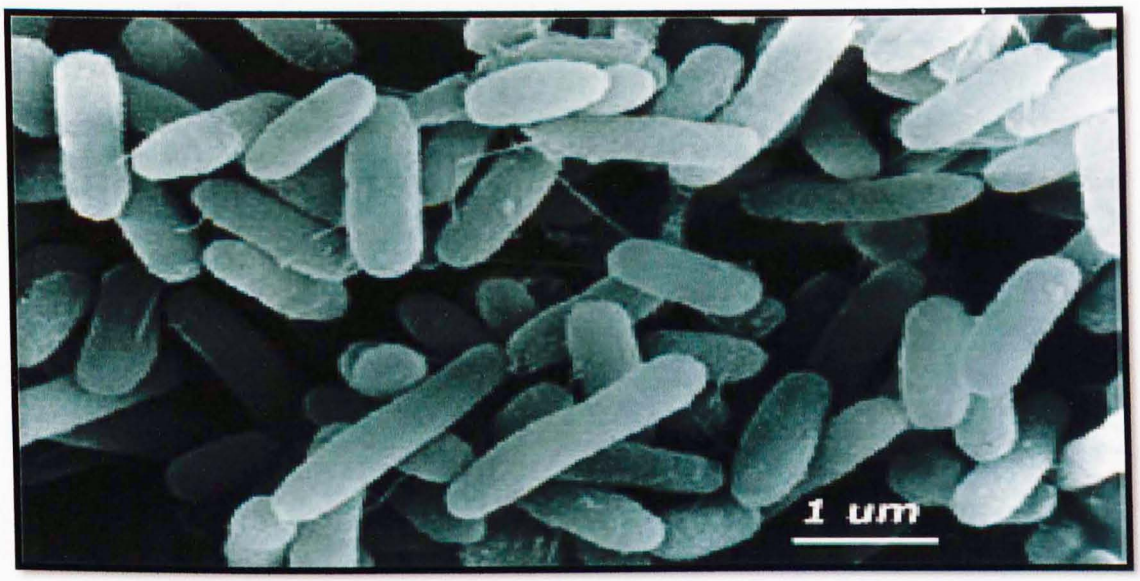

Figure 1.7. Scanning electron micrograph of *Halomonas* sp. (http://genome.jgi-p sf.org/draft_microbes/images/maraq.jpg).

1.4. Taxonomy

1.4.1. *Dunaliella*

The taxonomic placement of the genus *Dunaliella* is as follows: *Dunaliella*, Dunaliellaceae, Dunaliellales, Chlorophyceae, Chlorophyta (Borowitzka & Siva, 2007).

Dunaliella salina is the type species of the genus *Dunaliella* and it was originally described by Dunal in 1838 as *Haematococcus salinus*. However, later work by Teodoresco in 1905 showed that *D. salina* is different from both *Haematococcus* and the morphologically similar genus "*Chlamydomonas*". Further studies revealed 28 recognisable species of *Dunaliella* (Preisig, 1992). Most of these species are shown in Figure 1.8 (Borowitzka & Siva, 2007).

Taxonomy of *Dunaliella* species was defined primarily by morphological criteria which may vary depending on growth conditions and therefore many species were ill-defined. In addition, some described species may be found to be polymorphic forms of one species. Therefore, this has led to misnaming problems for *Dunaliella* species e.g. *D. salina* UTEX 200 (CCAP 19/3) is probably *D. viridis* (Preisig, 1992).

The taxonomic placement of *D. salina* Teod. is not certain yet e.g. a few strains were placed in this species despite the fact that they do not possess the ability to accumulate β-carotene. The ability to produce β-carotene in large amounts is normally used as a key characteristic in naming a strain *D. salina* (or *D. bardawil*) (Borowitzka & Borowitzka, 1988; Loeblich, 1982). *D. salina* Teod. and *D. bardawil* are morphologically similar to other strains of *Dunaliella*. Both species are motile, ovoid in shape, containing one large chloroplast, and other organelles that are similar to other Volvocales (Dunaliellales). However, there are several features that can be employed to differentiate these important species in terms of β-carotene productivity from other strains of *Dunaliella* that lack the ability to produce β-carotene or produce low quantities of it. The two species have a cell volume range from 300 to 1000 μm^3 which is larger than that of other strains of *Dunaliella* which range from 30 to 150 μm^3. Unfavorable growth conditions change the ellipsoidal shape of *D. salina* Teod. to a large reddish sphere of about 2000 μm^3 and it may lose its flagella. This species can be differentiated from *D. bardawil* based on the presence and the number of eye spots or lipid globules. *D. salina* has no eye spot at the exterior end of the chloroplast, whereas *D. bardawil* has a few distinguishing eye spots at the anterior part of the cytoplasm close to the basal part of the cell (Ben-Amotz & Shaish, 1992).

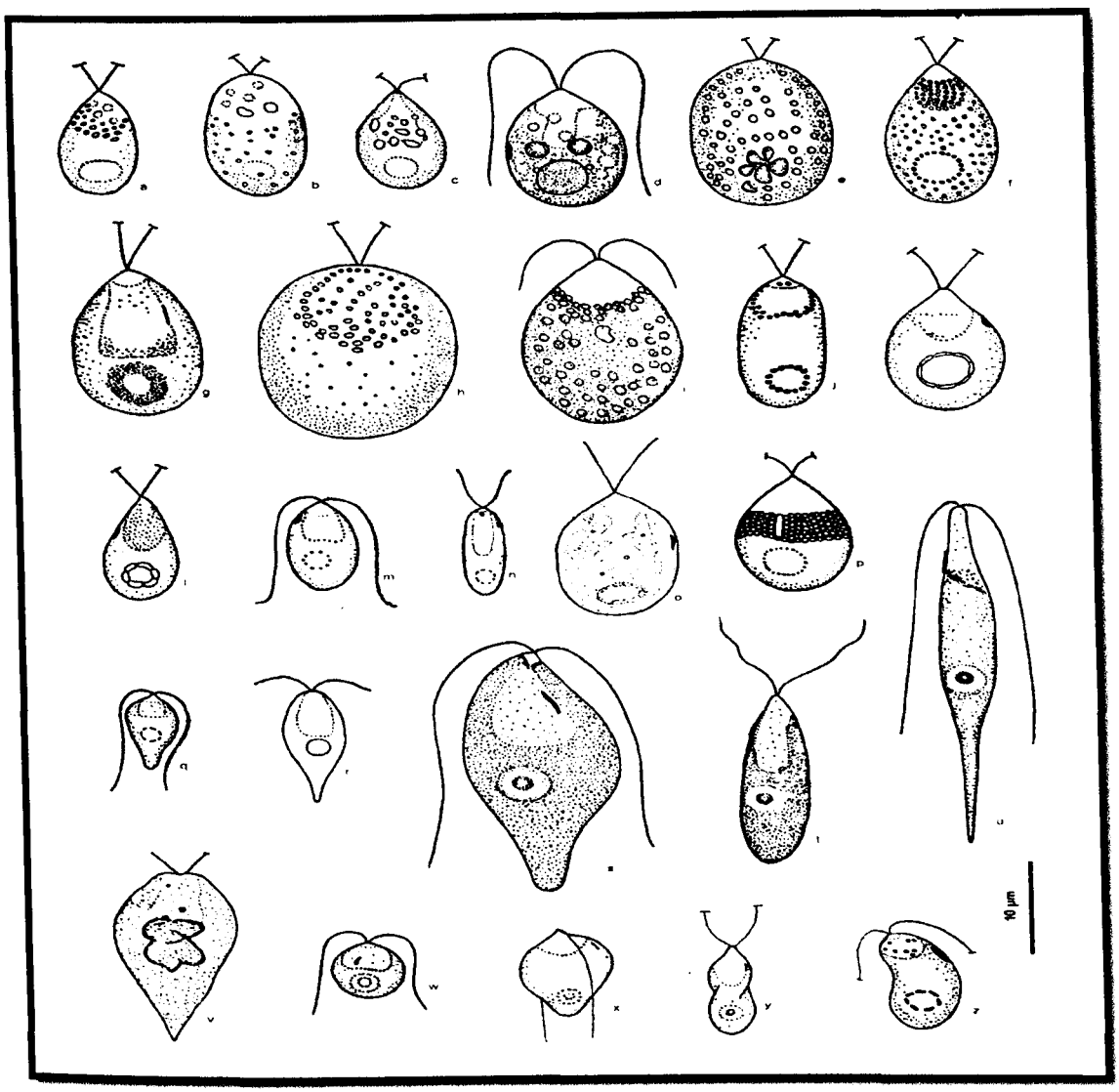

Figure 1.8. Selected illustrations of *Dunaliella* species taken from Massyuks monograph (Massyuk 1973c). (a) *D. tertiolecta* (2 M NaCl); (b) *D. tertiolecta* (5 M NaCl); (c) *D. primolecta*; (d) *D. quartolecta*; (e-g). *D. salina* ssp. *salina* f. *salina*; (h) *D. salina* spp. *salina* f. *magna*; (i) *D. salina* spp. *salina* f. *salina*; (j) *D. salina* spp. *salina* f. *oblonga*; (k, l) *D. viridis* var. *viridis* f. *viridis*; (m) *D. viridis* var. *viridis* f. *euchlora*; (n) *D. minuta*; (o) *D. carpatica*; (p) *D. granulata*; (q) *D. terricola*; (r) *D. media*; (s) *D. ruineniana*; (t) *D. baasbeckingii*; (u) *D. gracilis*; (v) *D. peircei*; (w) *D. minutissima*; (x) *D. turcomanica* ('surface' view); (y) *D. turcomanica* ('side' view); (z) *D. asymmetrica*. (d = originally from Butcher (1959b); r = originally from Lerche (1937); s - u = originally from Ruinen (1938); v = originally from Nicolai and Baas-Becking (1935)) (Borowitzka & Siva, 2007).

There are many factors which contribute to the ill-defined nature of species of *Dunaliella*. The species grow over wide environmental conditions in particular salinity. In addition, there is a great morphological variability between the species due to the fact that the cells lack a cell wall. Therefore, several strains in culture collections are misidentified and the given names have been perpetuated leading to difficulty in reconciling published information especially when there is inadequate information about the strain being studied. This confusion in the taxonomy of *Dunaliella* species is likely to increase because molecular techniques are being used without taking into account the current morphology-based taxonomy (Borowitzka & Siva, 2007).

Employing new features of *Dunaliella* species such as ultrastructural differences in the flagellar apparatus or physiological, biochemical, and molecular biological characteristics may lead to a better definition of these species (Preisig, 1992). Therefore, several molecular techniques have been used by some researchers to resolve the taxonomic placement of *Dunaliella* strains. These techniques include RAPD (Random Amplified Polymorphic DNA) band patterns and nuclear ribosomal DNA internal transcribed spacer (ITS-1 and ITS-2) sequences (Coleman & Mai, 1997; Gomez & Gonzalez, 2004; Gonzalez *et al.*, 2001), RFLP (Restriction Fragment Length Polymorphism) analysis, and the utilisation of conserved and species-specific primers (González *et al.*, 1998; Olmos-Soto *et al.*, 2002; Olmos *et al.*, 2000). RAPD is a DNA-fingerprinting method that allows the detection of multi-locus genetic variation. This technique has been used to identify microalgae down to the strain level. The nuclear ribosomal DNA internal transcribed spacer (ITS-1 and ITS-2) sequences were shown to be useful in assessing genetic relatedness in microalgae. This technique was used to resolve intra- and inter-specific relationships among a variety of organisms by employing some non-coding rapidly evolving sequences (Gomez & Gonzalez, 2004). RFLP is a simple and inexpensive molecular technique used to reveal DNA polymorphism in the ITS

region. This technique has been widely used in taxonomy and population biology (González *et al.*, 1998). Olmos-Soto *et al.* (2002) utilised sequence-specific-oligonucleotides analysis to identify three species of *Dunaliella* form hypersaline environments: *D. bardawil*, *D. salina*, and *D. parva* based on structural features of introns from their 18S rDNA genes. They found that *D. bardawil* and *D. salina* were harbouring 2 and 1 introns within their 18S rDNA genes respectively. However, *D. parva* was not found in the collected samples (Olmos-Soto *et al.*, 2002).

1.4.2. ***Arthrospira***

The taxonomic placement of the genus *Arthrospira* is as follows: genus *Arthrospira*, Subsection III (formerly Oscillatoriales Elenkin 1934), Cyanobacteria, *Cyanobacteria*, Bacteria (Garrity & Holt, 2001).

Arthrospira has frequently been included in the genus *Spirulina*. However, there are differences between the two genera on the basis of ultrastructural properties and the mean DNA base composition. Therefore, it is clear that the smaller coiled *Spirulina* strains which lack crosswalls discernible by light microscope constitute an independent taxonomic unit. This was confirmed by employing some molecular techniques such as 16S rDNA sequence data as well as comparison between Internally Transcribed Spacer (ITS) regions between 16S and 23S rDNA of about fifty strains of *Arthrospira* from four continents. The analyses revealed that only two main clusters were found without correlation to geographic origin or different specific assignments, and that one strain from each cluster have been shown to share 99.7% sequence similarity. Therefore, it is likely that all *Arthrospira* strains are representatives of a single nomen species (Castenholz *et al.*, 2001).

1.4.3. *Spirulina*

The taxonomic placement of the genus *Spirulina* is as follows: genus *Spirulina*, Subsection III (formerly Oscillatoriales Elenkin 1934), Cyanobacteria, *Cyanobacteria*, Bacteria (Garrity & Holt, 2001).

The genus *Spirulina* was used in the traditional literature to accommodate morphotypes conforming to the current definition and also harboured the larger coiled members assigned to *Arthrospira*. It was shown in a recent study on the physiology, morphology, and phylogeny of 11 strains of *Spirulina* that all strains were clustered phenotypically far from *Arthrospira*. However, the strains showed a considerable genetic diversity. Three strains were highly halotolerant and formed a separate cluster. Therefore, the genera *Spirulina* Turpin 1829 ex Gomont 1892 and *Arthrospira* Stizenberger 1852 ex Gomont 1892 constitute without doubt separate taxonomic units. As a consequence many strains have moved from *Spirulina* to *Arthrospira*. Therefore, based on these grounds the cyanobacteria referred to as *Spirulina* by both Jeeji-Bai (1985) and Lewin (1980) and also the commercially available "*Spirulina*" which is used as a protein supplement, should all be considered species or varieties of the genus *Arthrospira* (Castenholz *et al.*, 2001).

1.4.4. *Halomonas*

The taxonomic placement of the genus *Halomonas* is as follows: *Halomonas*, *Halomonadaceae*, Oceanospirillales, Gammaproteobacteria, *Proteobacteria* phy. nov., Bacteria (Garrity & Holt, 2001).

In a recent study based on 23S and 16S rDNA sequence comparisons and phylogenetic analysis of many species of *Halomonas*, it was found that the genus *Halomonas* is not monophyletic and two phylogenetic groups were distinguishable. The first group comprised *Halomonas elongata*, the type species, and four other species: *H. eurihalina*, *H. halmophila*, *H. halophila*, and *H. salina*. It was found that the mean 16S rDNA sequence similarity of this group is about 98.2% and same value was also obtained with 23S rDNA sequences. *H. meridiana*, *H. magadiensis*, *H. variabilis*, *H. venusta*, *H. halodurans*, and *H. subglaciescola* formed the second group with means of 16S and 23S rDNA sequence similarities of 97.4 and 97.6% respectively. The species *H. pacifica*, *H. halodentrificans*, *H. cupida*, *H. desiderata*, *H. campisalis*, and *H. pantelleriensis* did not fall clearly in one of the above groups nor did they form a group by themselves. These results were in agreement with the phenotypic heterogeneity reported for species of the genus *Halomonas*, e.g. it was found that the G+C content of *Halomonas* species range from 52 to 68 mol %. This range is too wide for a single genus taking into consideration the generally accepted rule that G+C content of members of the same genus should not differ by more than 10 mol % (Arahal *et al.*, 2002).

1.5. Osmoregulation

1.5.1. *Dunaliella*

1.5.1.1. Responses to salt stress

Several species of *Dunaliella* are able to grow in media containing an extremely wide range of salt concentrations ranging from 0.05 M NaCl (0.3 % (w/v) NaCl) to saturation at about 5.5 M NaCl (35%) (Avron, 1992). The ability of some species of *Dunaliella* to thrive and tolerate high concentrations of NaCl in the medium as well as rapid changes in those concentrations is due to the ability to accumulate high concentrations of the compatible solute "glycerol" as an osmoregulatory solute. Glycerol is an infinitely soluble polyhydric alcohol which was shown to cause little or no inhibition of metabolic processes (Borowitzka & Borowitzka, 1988).

Dunaliella cells respond rapidly to changes in osmotic pressure by changes in cell volume due to the cells lacking a cell wall and being enclosed solely by a thin elastic plasma membrane. The cell shrinks or swells in response to hyperosmotic or hypoosmotic stress respectively. It can withstand three- to fourfold increases or decreases in osmotic pressure and above this limit the cell may burst during hypoosmotic stress or shrink irreversibly during hyperosmotic stress. It was found that glycerol is the essential intracellular osmolyte which is responsible for balancing the extracellular osmotic stress. Its intracellular concentration is linearly related to the medium osmotic pressure. The intracellular glycerol concentration in cells grown at high salinity may exceed 50% of the algal dry weight and that is sufficient to account for essentially all of the osmotic pressure required to balance the external salinity (Avron, 1992).

The initial reaction of the cells after an osmotic shock is allowing water to enter or leave the cell osmotically depending on the type of osmotic pressure imposed on the cells. This happens within seconds and therefore brings the cell back into osmotic equilibrium with the medium. Thereafter, the cells start synthesising or eliminating glycerol via enzymatic pathways accompanied by water re-entry or efflux respectively. Thus, the cells regain approximately their original volume. That indicates that glycerol synthesis or reassimilation is induced and triggered by volume changes. The synthesis of glycerol in the cells or eliminating it from the cells depends on the direction and extent of the osmotic shock and also on the metabolic conditions of the cells. This transition process lasts 1 to 3 hours under optimal growth conditions and during this period cell division is arrested. It can occur in the light or in the dark and it is independent of new protein synthesis. The plasma membrane of *Dunaliella* is permeable to water but not to glycerol. Its permeability coefficient for water is around 2×10^{-3} cm s^{-1} whereas that for glycerol is much lower than 5×10^{-11} cm s^{-1} at around 20 °C. This unique property of the plasma membrane was found to be temperature independent. All intracellular glycerol content was leaked out immediately to the medium upon the exposure of *Dunaliella* cells for a few minutes to high temperature exceeding 45 °C (Avron, 1992).

Other responses of *Dunaliella* cells to salt stress include enhancing the elimination of Na^+ cation, accumulation of specific proteins (Katz & Pick, 2001), phosphorylation of light harvesting chlorophyll a/b proteins in thylakoid membranes (Liu & Shen, 2004), formation of salt containing vacuoles and changes in chloroplast ultrastructure (Stoynova-Bakalova & Toncheva-Panova, 2003).

1.5.1.2. Glycerol metabolism

Production of glycerol by *Dunaliella* can be either by photosynthetic CO_2 fixation or by starch degradation (Figure 1.9). The source of carbon for glycerol production depends on the availability of light, the starch reserve pool, and the size of the salt stress. The cells produce glycerol in the dark exclusively by degradation of starch and therefore the higher the starch reserve pool the higher the capacity of the cells to recover from hyperosmotic shock in the dark. However, in the light it was found that hyperosmotic shock stimulates the rate of glycerol production from CO_2 and at the same time enhances starch degradation. These findings indicate that starch degradation also has a significant contribution toward glycerol production in the light. It was shown by ^{13}C-NMR experiments that cells exposed to hyperosmotic shock started to produce glycerol immediately after the shock mainly by starch degradation, whereas after 45 min of the shock the glycerol production continued by photosynthetic CO_2 fixation. Production of glycerol from both photosynthesis and starch degradation upon the exposure of the cells to hyperosmotic shock indicates the immediate requirement for glycerol synthesis to balance the high salinity in the medium. In addition, it indicates that hyperosmotic shock inhibits both photosynthesis and starch synthesis. On the other hand, exposing *Dunaliella* cells to hypoosmotic shock induces a decrease in glycerol content as well as an increase in starch content. This indicates that the cells convert glycerol metabolically to starch. In addition, a transient inhibition of photosynthesis and a substantial inhibition of glycerol synthesis were reported when the cells exposed to hypoosmotic shock. In general, *Dunaliella* utilises a dynamic interconversion between the two major carbon pools, glycerol and starch (Avron, 1992).

The activation of glycerol synthesis does not involve *de novo* protein synthesis and the enzymes glycerol phosphate dehydrogenase (Belmans & Vanlaere, 1987) and phosphofructokinase (Chitlaru & Pick, 1991) are probably

the check point enzymes which control glycerol synthesis (see Figure 1.9). Moreover, the glycerol produced by *Dunaliella* is triggered by an unknown molecular mechanism. Osmotic shock of *Dunaliella* cells causes changes in plasma membrane structure, inorganic phosphate content, and internal pH. Therefore, it was suggested a plasma membrane sensor and also soluble metabolites are involved in the activation of glycerol synthesis (Avron, 1992).

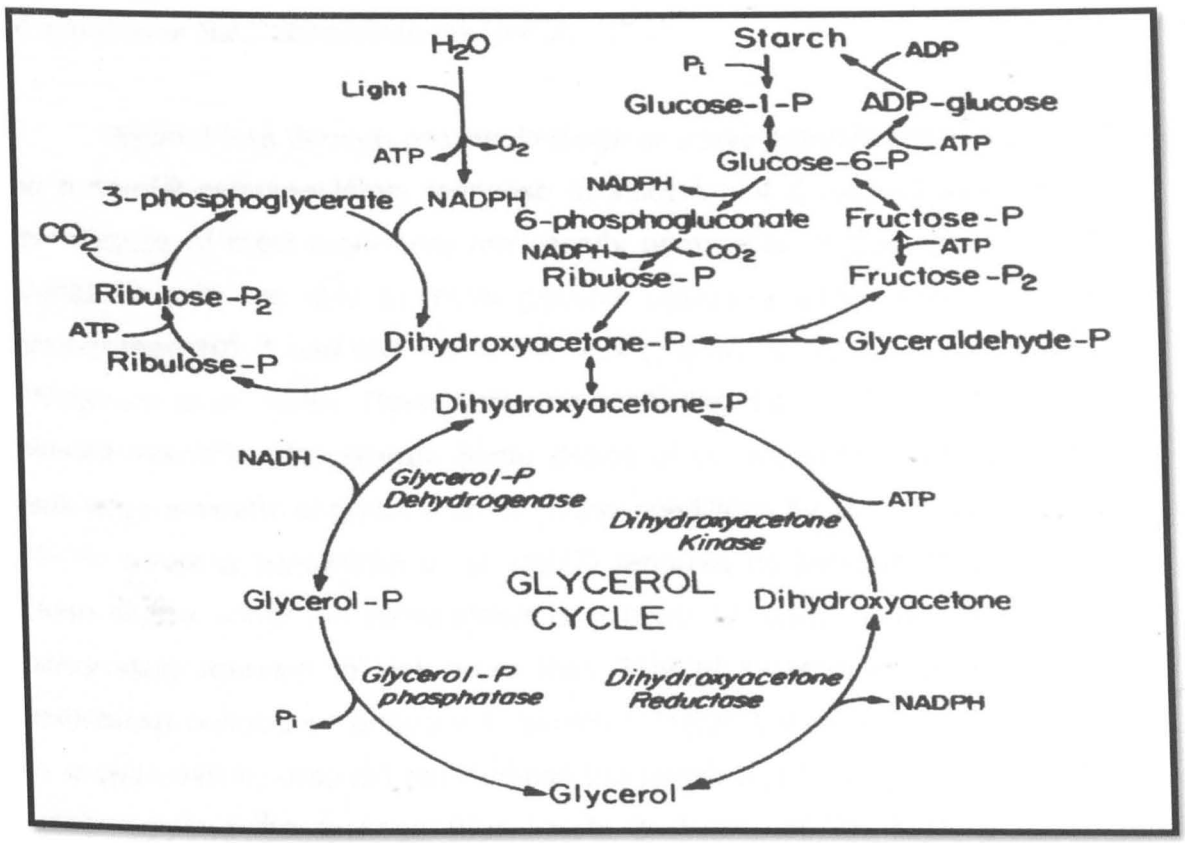

Figure 1.9. Glycerol cycle which shows that glycerol synthesis pathway could begin from CO_2 fixation in photosynthesis or from stored polysaccharides during growth following a hyperosmotic treatment. It also shows the synthesis of polysaccharides from glycerol following a hypoosmotic treatment. Glycerol cycle involves several novel enzymes to *Dunaliella*: glycerol-phosphate phosphatase, dihydroxyacetone reductase, and dihydroxyacetone kinase (Ben-Amotz & Avron, 1990).

1.5.1.3. Glycerol leakage to the medium

It was claimed that hypoosmotic shock induces leakage of glycerol from *Dunaliella* cells to the medium. This loss of glycerol might occur as a result of nonspecific permeabilisation of plasma membrane since glycerol leakage to the medium is only thought to occur in response to exceptionally large dilutions or at suboptimal NaCl concentrations (Avron, 1992).

Glycerol loss through passive leakage or active extrusion appears not to be a normal osmoregulatory response of salt-tolerant *Dunaliella* strains. Cell membranes of most organisms are usually permeable to glycerol. However, *Dunaliella* cells are able to retain glycerol against a concentration gradient greater than 10^4: 1 and this allows the cells to grow in high salt environments (Wegmann *et al.*, 1980). There were also conflicting data on the permeability of plasma membrane to glycerol. Some strains of *D. tertiolecta* were reported to leak large amounts of glycerol under stress conditions by Jones and Galloway (1979) whereas Borowitzka *et al.* (1977) reported no leakage of glycerol by those strains under the same stress conditions. Similarly, some strains of *D. parva* were reported to leak more than 33% of intracellular glycerol under unstressed conditions (Enhuber & Gimmler, 1980), but severe stress induced by a large salinity drop did not increase the percentage of glycerol leakage but rather stopped the leakage (Ben-Amotz & Avron, 1973). NMR studies of glycerol diffusional permeabilities showed that the plasma membrane of *D. salina* has an exceptionally low permeability to glycerol. However, increasing the growth temperature above 50 °C changed the membrane permeability and led to rapid leakage of glycerol and cell death (Borowitzka & Borowitzka, 1988).

It was reported that a mutant of *D. parva* CCAP 19/9 leaks large amounts of intracellular glycerol into the growth medium when compared with the wild type. The mutant leaked up to 75% of the intracellular glycerol into the

medium when grown at 1.5 M NaCl compared with the wild type strain which leaked only 17% of intracellular glycerol. At lower concentration (0.4 M NaCl) the mutant leaked up to 73% of intracellular glycerol into the medium while the wild type strain did not show any leakage of glycerol at all (Hard & Gilmour, 1991). Although the stability of the presumed mutant was not confirmed, this finding is interesting and further research in this line would reveal more features of *Dunaliella* strains especially those with potential for commercial applications.

1.5.2. ***Arthrospira* and *Spirulina***

It is known that high intracellular sodium concentrations are toxic to most biological systems and the development of mechanisms that limit the accumulation of sodium inside the cell is required in order for organisms to adapt to salinity. These mechanisms could be one or more of the following: low permeability of the plasma membrane to sodium, extrude the entering sodium ions by the Na^+/H^+ antiporter in which the extrusion of sodium from the cell is coupled to inwardly movement of the protons, and accumulation of organic compatible solutes in order to cope with unbalanced osmotic pressure. In *Spirulina platensis*, it was shown that the osmotic adjustment was achieved by intracellular accumulation of the carbohydrates glucosyl-glycerol and trehalose (see Figure 1.11) (Warr *et al.*, 1985). It was found that increasing external NaCl concentration in *S. platensis* medium seems to have no effect on cellular sodium content which was maintained at a constant low level. However, potassium content declined linearly versus increased external NaCl concentration (Vonshak *et al.*, 1988). In contrast, carbohydrate content of the cells increased exponentially with the increase in NaCl concentration. *S. platensis* responds to salt stress in two stages: a relatively short shock stage followed by an adaptation stage. The first stage occurred upon the exposure to high salt concentrations of 0.5 M NaCl and above where photosynthesis and respiration were inhibited and a complete cessation of growth was observed.

Energy-yielding processes restored activity and initiated the adaptation stage after a lag time. In this stage, at 0.5 and 1.0 M NaCl photosynthesis reached 80% and 50% of the control rate while respiration was enhanced by 140% and 200% respectively (Vonshak *et al.*, 1988). Exposing the cells to high concentrations of NaCl resulted in long lag times. Recovery of the respiration rate was found to be the key for the resumption of growth and the establishment of new steady state growth rates. There was an inverse linear relationship between the growth rates after adaptation and the increased NaCl concentrations (Vonshak *et al.*, 1988).

Reed and Stewart (1988) reported that *S. platensis* grown in a 150% sea water medium (0.6 M NaCl) accumulated glucosyl-glycerol as a primary organic osmolyte at concentrations in excess of 240 mM. Glucosyl-glycerol was the only organic solute present in osmotically significant quantities. That was confirmed by natural abundance ^{13}C NMR spectroscopy of cells grown in 100% sea water medium (0.4 M NaCl). However, trehalose was also detected in small amounts in cells subjected to salinity shock (48 hours incubation). This disaccharide was at its highest level when cells were grown in a 50% sea water medium (0.2 M NaCl), in contrast to glucosyl-glycerol which was at its highest level when cells were grown in a 150% sea water medium. Moreover, the intracellular concentration of trehalose in *S. platensis* was temperature-sensitive, increasing as the growth temperature was raised. These observations suggest that *S. platensis* cells do not only accumulate glucosyl-glycerol in response to changes in salinity, but they may also produce trehalose as a secondary organic osmoticum which could help the cells achieve osmotic adjustment (Reed & Stewart, 1988).

Warr *et al.*, (1985) estimated that *S. platensis* cells accumulate glucosyl-glycerol up to 5% of their dry weight when grown in 100% seawater medium and accumulate trehalose up to 1.0% of their dry weight when grown in 50% seawater medium. They found that the ratio of trehalose to glucosyl-glycerol

varied with temperature for cells grown in 100% seawater medium. 31% (w/w) of the low molecular weight carbohydrates was accounted for by trehalose at 37 °C. However, at 20 °C only 9% was accounted for by trehalose. Warr *et al.*, (1985) also found that exposing the cells to hypoosmotic shock decreased the intracellular concentration of glucosyl-glycerol and this was mirrored by an increase in the glycogen content of the cells.

Spirulina subsalsa, a marine cyanobacterium, was isolated from the hypersaline Bardawil lagoon in the Sinai Peninsula and it was found that it tolerates a wide range of salt concentrations from 0.3 to 2.5 M NaCl. It possesses potent mechanisms for salt adaptation and salt tolerance (Gabbayazaria *et al.*, 1992). One of these mechanisms was found to be the synthesis and accumulation of glycine-betaine (see Figure 1.11). This compatible solute protects the activity of glucose-6-phosphate dehydrogenase in the presence of salt. Since the biosynthesis of glycine-betaine is a slow process, it was suggested that it is not enough to provide protection against sudden changes in extracellular salt concentration. Therefore there must be a rapid response mechanism that can regulate the intracellular mineral ion concentration in *S. subsalsa* in order to adapt and live in the above range of NaCl concentrations. It was shown that respiration was involved directly in maintaining low intracellular sodium levels. An early phase of Na^+ and Cl^- influx occurred during the transition of *S. subsalsa* cells from saline to hypersaline medium. This phase was followed by an adaptation phase where both Na^+ and Cl^- ions were excluded from the cell. It was demonstrated that cytochrome oxidase was involved in the extrusion of sodium ions from the salt-tolerant cells of *S. subsalsa* (Gabbayazaria *et al.*, 1992).

Cyanobacteria, in general, can be divided into three groups based on the upper salinity limit for growth. First group includes those isolates that tolerate up to 0.7 M NaCl, this group is considered to be the least halotolerant and accumulate the disaccharides sucrose and trehalose (see Figure 1.11). The

second group includes intermediate halotolerant species that accumulate glucosyl-glycerol in response to osmotic stress. This group tolerates up to 1.8 M NaCl. The third group of cyanobacteria includes the most halotolerant species that accumulate betaine (either glycine betaine or glutamate betaine) in response to osmotic stress. This group can be grown in media containing up to 2.8 M NaCl (Reed *et al.*, 1986).

1.5.3. ***Halomonas***

Halophilic and halotolerant bacteria, in general, developed two main strategies of osmoadaptation: the halobacterial or KCl type and the compatible-solute or organic-solute type. The first mechanism was discovered in members of the family Halobacteriaceae (Archaea) and it is a typical mechanism for those members. Halophilic Archaea achieve osmotic equilibrium by maintaining a cytoplasmic concentration of KCl similar to the NaCl concentration of the surrounding medium. This strategy requires a considerable number of physiological changes such as adaptation of enzymes and cellular components to salt stress to safeguard all regulatory and metabolic functions at high salinity (Eisenberg & Wachtel, 1987). Some bacterial groups also use KCl as a compatible solute e.g. acetogenic anaerobes and sulphate reducers. In general, organisms employing the KCl strategy display a relatively narrow adaptation for a specific stable high salinity environment (Galinski & Truper, 1994).

The compatible-solute type seems to be wide-spread in bacterial halophiles, but it is also found in some methanogens, one of the major groups of Archaea. The advantage of using osmolytes is that bacteria can adapt to a wide range of salinities while normal salt-sensitive enzymatic machinery is preserved. Concentrations of compatible solutes in the cytoplasm were found to be well above 1 mol kg^{-1} water. These solutes are responsible for osmotic balance and compatible with the metabolic activities in the cells. In addition,

they are effective stabilisers of enzymes and protect against salinity, high temperature, freeze-thaw treatment, and drying (Galinski & Truper, 1994). Further information on the compatible solutes of *Halomonas* will be presented in Section 1.6.2.

1.6. Valuable Products form Microorganisms and Their Applications

1.6.1. β-Carotene

1.6.1.1. Sources

β-carotene can be found in many plants and its content varies considerably in the range of 0.01 to 10 mg 100 g^{-1}. Green leafy plants such as parsley, spinach, and broccoli; yellow orange fruits such as mandarins, mangos, peaches, and red palm; and certain vegetables such as carrots, sweet potatoes, and pumpkin are the most common β-carotene-rich plants. β-carotene can also be accumulated to a high level in few microorganisms such as the fungus *Phycomyces blakesleanus* and the yeast *Rhodotorula*. They accumulate up to 5 and 0.5 mg β-carotene g^{-1} dry weight respectively. It was shown that β-carotene rich organisms contain a mixture of different carotenoids, carotenoid esters, and carotenoid isomers, with a varying fraction being β-carotene.

Dunaliella is capable of producing large amounts of β-carotene within oily globules in the interthylakoid spaces of the chloroplast (Figure 1.3). *Dunaliella salina* Teodoresco and *D. bardawil* are the overproducers of β-carotene within *Dunaliella* genus and all other strains of *Dunaliella* lack this unique capability of cartenogenesis. Both species are green under noninducing conditions for β-carotene synthesis and their content of this pigment under these conditions was

estimated to be 0.3% of the algal dry weight (Ben-Amotz & Shaish, 1992). However, under proper inductive conditions for β-carotene synthesis they accumulate up to 14% of the algal dry weight as β-carotene. This content of β-carotene on a dry weight basis is the highest of any known alga, plant, or other microorganism. β-carotene accumulation and rate of synthesis depend on certain physiological growth parameters including high salinity, nitrogen deficiency, phosphorus deficiency, high light intensity, and high temperature (Ben-Amotz & Shaish, 1992; Borowitzka, 1992; Garcia-Gonzalez *et al.*, 2005; Lers *et al.*, 1990; Shaish *et al.*, 1992).

1.6.1.2. Chemical composition and characteristics

Ben-Amotz and Shaish (1992) reported the following general features of β-carotene: a chemical formula of C_{40} H_{56} (Figure 1.10), a molecular weight of 536.9, eleven conjugated double bonds, a typical violet-red crystalline colour, the colour varies from light yellow to orange in oily solution whereas in aqueous dispersion orange is the most common colour. Synthetic β-carotene (pure crystalline) has a stereogeometry of all-*trans* (Figure 1.10) which has the following characteristics: very low solubility in oil, low solubility in organic solvents, very high tendency to crystallise out of solution in the form of typical crystals, an absorption maxima in petroleum ether at 453 nm and 481 nm. β-carotene from *D. salina*, however, contains the following stereoisomers of β-carotene as percentages of total β-carotene: 15-*cis*-β-carotene, 10%; 9-*cis*-β-carotene, 41%; all-*trans*-β-carotene, 42% (Figure 1.10); other isomers, 6% (Borowitzka & Borowitzka, 1989). Therefore, the main two stereoisomers of the β-carotene accumulated by *D. salina* Teod. and *D. bardawil* are: all-*trans* and 9-*cis*. These stereoisomers are approximately equal in amounts and the 9-*cis* to all-*trans* ratio depends on the amount of light absorbed by the cell during one division cycle. Therefore the higher the light intensity and the lower the growth rate of the alga, the higher is the 9-*cis* to all-*trans* β-carotene ratio. In contrast to

all-*trans* β-carotene, the 9-*cis* β-carotene has many unique physicochemical properties: absorption maxima shift and *cis* peak in the ultraviolet region, oily nature, high solubility in hydrophobic solvents, and difficult to crystallise (Ben-Amotz & Shaish, 1992).

Most of the commercially available β-carotene is synthetic being produced in complex chemical processes developed by researchers of Hoffmann La Roche in 1950s. The synthetic β-carotene is sold for approximately US$600 kg^{-1} with a market volume of around US$200 million $year^{-1}$. On the other hand, *Dunaliella* β-carotene is sold for more than this value, but the market volume is much smaller (Ben-Amotz & Avron, 1990).

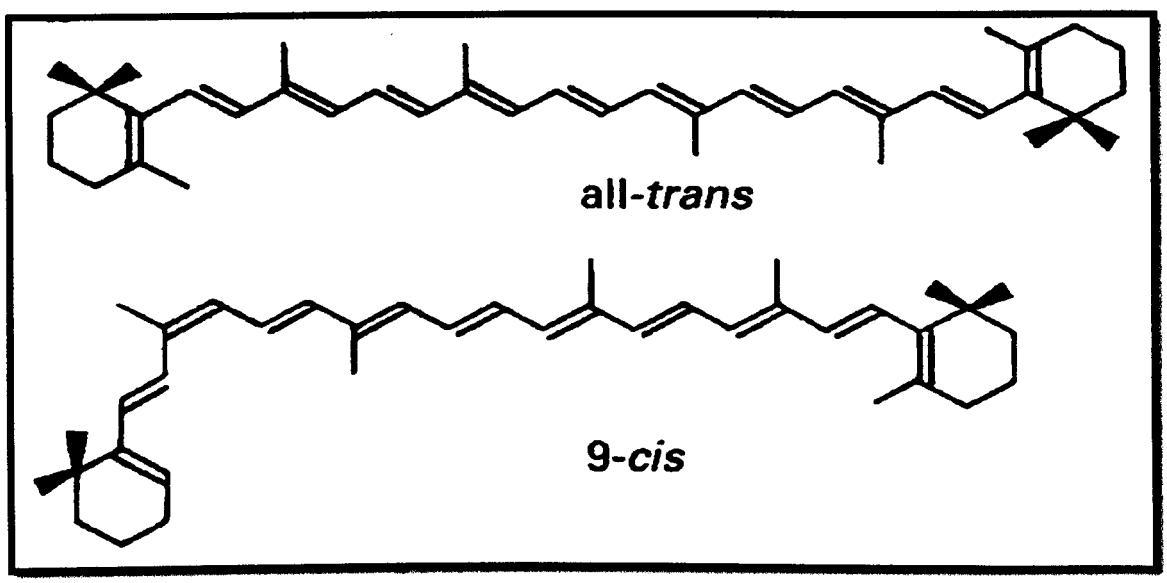

Figure 1.10. Isomeric forms of β-carotene accumulated in *Dunaliella bardawil* (Ben-Amotz & Avron, 1990).

1.6.1.3. Biological functions

There are many roles of carotenoids in nature such as provitamin A activity, absorption of light energy, triplet chlorophyll and single oxygen quenching, antioxidation activity, oxygen transport, and general coloration of many different organisms (Zamir, 1992).

It was hypothesised that *Dunaliella* accumulate and store β-carotene as an extra photosynthetic product for later use under growth rate limitations. β-carotene was shown to provide good protection for the cells against injury by high intensity radiation under limited growth conditions by absorbing excess radiation. The photoprotection function of β-carotene was supported by the fact that β-carotene accumulates within oily globules at the periphery of the cup shaped chloroplast and therefore this structure is most efficient for photoprotection purposes (Ben-Amotz & Shaish, 1992).

1.6.1.4. Induction of carotenogenesis

It is generally known that carotenogenesis is greatest when growth is least. High salinity, nitrogen deficiency, phosphorus deficiency, high light intensity, and high temperature are known factors that limit growth and stimulate β-carotene production in *D. salina* (Ben-Amotz & Shaish, 1992; Borowitzka & Borowitzka, 1990; Borowitzka, 1990; Phadwal & Singh, 2003; Shaish *et al.*, 1992; Zamir, 1992).

Many carotenogenesis studies on *D. salina* showed that the higher the light intensity and the slower the growth of the alga, the higher the β-carotene content. It was shown that growing the alga under nitrogen-limiting conditions and low light intensity resulted in a lower content of protein and chlorophyll compared with the same cells grown in a nondeficient medium. These nitrogen-

deficient cells when exposed to high light intensity for a short period of 1 to 2 days yielded very high β-carotene content per cell (Ben-Amotz & Shaish, 1992).

1.6.1.5. Applications

β-carotene is a valuable pigment and has many pharmaceutical and nutritional applications. It can be used as food colouring agent, pro-vitamin A (retinol) in food and animal feed, additive to multivitamin preparations and cosmetics (Garcia-Gonzalez *et al.*, 2005; Salguero *et al.*, 2003). Several studies claimed that β-carotene can act as anti-cancer agent, immune-system stimulator, and degenerative diseases preventive because of its protective ability against activated oxygen radical forms (Salguero *et al.*, 2003).

Normal to high levels of β-carotene in the body may protect it against cancer as suggested by many recent epidemiological and oncological studies. It was found that maintaining higher than average levels of serum β-carotene in humans and animals who were fed on a diet that was high in carotenoid-rich vegetables and fruits have a lower incidence of several types of cancer. This has increased the interest in a natural source of β-carotene especially after the observation that natural β-carotene, as found in *Dunaliella* and in most fruits and vegetables, contains a mixture of all-*trans* β-carotene and 9-*cis* β-carotene together with a few other stereoisomers. Therefore a new market for *Dunaliella* β-carotene was developed since the synthetic β-carotene contains only all-*trans* β-carotene (>99% of total β-carotene) (Ben-Amotz & Avron, 1990; Ben-Amotz & Shaish, 1992). This unique stereoisomeric composition of *Dunaliella* β-carotene may be of use as a pharmaceutical product because recent nutritional studies also indicate a marked preferential accumulation of natural *Dunaliella* β-carotene over that of the synthetic β-carotene in the liver of animal tissues and therefore it could be better in terms of disease prevention purposes (Ben-Amotz & Avron, 1990).

1.6.2. Compatible solutes

The intracellular ion concentrations measured in moderate halophiles are in general insufficient to provide osmotic balance with the external medium. Therefore, much effort has been dedicated to the search for the accumulated organic compounds in the cells. Employing techniques such as natural-abundance ^{13}C-NMR and high-pressure liquid chromatography has increased our knowledge about the intracellular environment of the moderate halophiles and led to the identification of the tetrahydropyrimidines ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) and hydroxyectoine (1,4,5,6-tetrahydro-2-methyl-5-hydroxy-4-pyrimidinecarboxylic acid) (Figure 1.11). It was shown that these compounds may be present inside the cells in molar concentrations to enable halophilic and halotolerant bacteria to cope with the osmotic stress and to maintain positive turgor pressure. They are called "compatible solutes" because they provide osmotic balance without interfering with the metabolic functions of the cells. They are polar, highly soluble molecules, uncharged at physiological pH, strong water structure formers, effective stabilisers of the hydration shell of proteins and therefore preventing the unfolding and denaturation of proteins caused by heating, freezing, and drying (Ventosa *et al.*, 1998).

1.6.2.1. Ectoine and hydroxyectoine

The most widespread compatible solutes found in the domain Bacteria are ectoine and its β-hydroxy derivative (Figure 1.11). High concentrations of ectoine were found in different *Halomonas* species and it is the dominant compatible solute in cells grown in defined medium lacking glycine betaine or its precursor (choline) (Cummings *et al.*, 1993). Intracellular ectoine concentrations

along with a combination of the intracellular ions and other dissolved organic compounds can be sufficiently high to balance the osmotic pressure of the medium. Growing *Halomonas elongata* in a medium containing 10% NaCl was shown to give an ectoine concentration of 2.25 M. However, *H. israelensis* showed a lower concentration of intracellular ectoine ranging from 0.1 to 0.2 M when grown in 1 to 2 M salt (Ventosa *et al.*, 1998).

It was shown that *Halomonas elongata* can produce large amounts of the compatible solutes ectoine and hydroxyectoine. The bacterium was allowed first to grow at high cell density up to 48 g cell dry weight per litre before subjecting the biomass to low osmotic shock to release the intracellular content of the compatible solutes to the medium. Then crossflow filtration techniques were used to harvest the compatible solutes. Reincubation of the biomass in a medium of higher salt concentration resulted in resynthesis of the compatible solutes. The above procedure was repeated up to nine times and it was called "bacterial milking". A productivity of 155 mg of ectoine per cycle per gram cell dry weight was achieved (Sauer & Galinski, 1998).

Ectoines and glycine (Figure 1.11) are used as stress protectants against high salinity, thermal denaturation, desiccation, and freezing. They also can be used as stabilisers of enzymes, nucleic acids, membranes, and whole cells. These compounds are also used in many industries such as enzyme technology, pharmaceuticals, and cosmetics. In addition, ectoine and hydroxyectoine are used as protectants for labile enzymes such as lactate dehydrogenase and phosphofructokinase. Genes that are involved in the synthesis of ectoine and its regulation in halophilic bacteria were isolated and this facilitated its overproduction. Also, transferring these genes to agricultural crops might help in increasing the tolerance of these crops to salt and drought and could enable them to grow in more saline soils (Ventosa *et al.*, 1998).

1.6.2.2. Glycerol

Glycerol (Figure 1.12) is used in many applications such as drugs and cosmetics manufacture, food and beverage industries, and in the production of urethane, cellophane, and explosives. Glycerol is produced mainly by petrochemical industry using propylene as the starting substance. It can also be produced as a by-product of fat and soap industry. Therefore its market cost is dependent on the price of oil (Gilmour, 1990).

Glycerol production by *Dunaliella* is an attractive possibility since CO_2 and light can be used as carbon and energy sources (Wang *et al.*, 2001). Intracellular glycerol concentration is linearly proportional to the salt concentration of the growth medium. Cultivation of *Dunaliella* in a growth medium containing high salt concentration yields more than 50% glycerol in terms of the algal dry weight (Ben-Amotz & Avron, 1989; Ben-Amotz & Avron, 1990). It was estimated that at 5 M NaCl in the medium, the intracellular glycerol concentration is around 7 M which is equivalent to a 56% glycerol solution (Ben-Amotz & Avron, 1983). On the other hand, high extracellular glycerol concentrations up to 5 g l^{-1} have been also reached using *D. tertiolecta* immobilised in calcium alginate in a hypersaline medium containing 4 M NaCl (Wang *et al.*, 2001). Despite this high productivity, glycerol has not yet proved economical to produce commercially form *Dunaliella* since it is currently produced mostly from petrochemical sources as well as from biodiesel industry as a by-product (Chapman & Gellenbeck, 1989; Chisti, 2007). The market price of glycerol is about US$5 kg^{-1} and this price fluctuates in relation to the price of crude oil (Ben-Amotz & Avron, 1990). The current (January 2008) price of crude oil is very high, however the large amounts of glycerol being produced by the biodiesel industry means that there is a large surplus of glycerol on the world market.

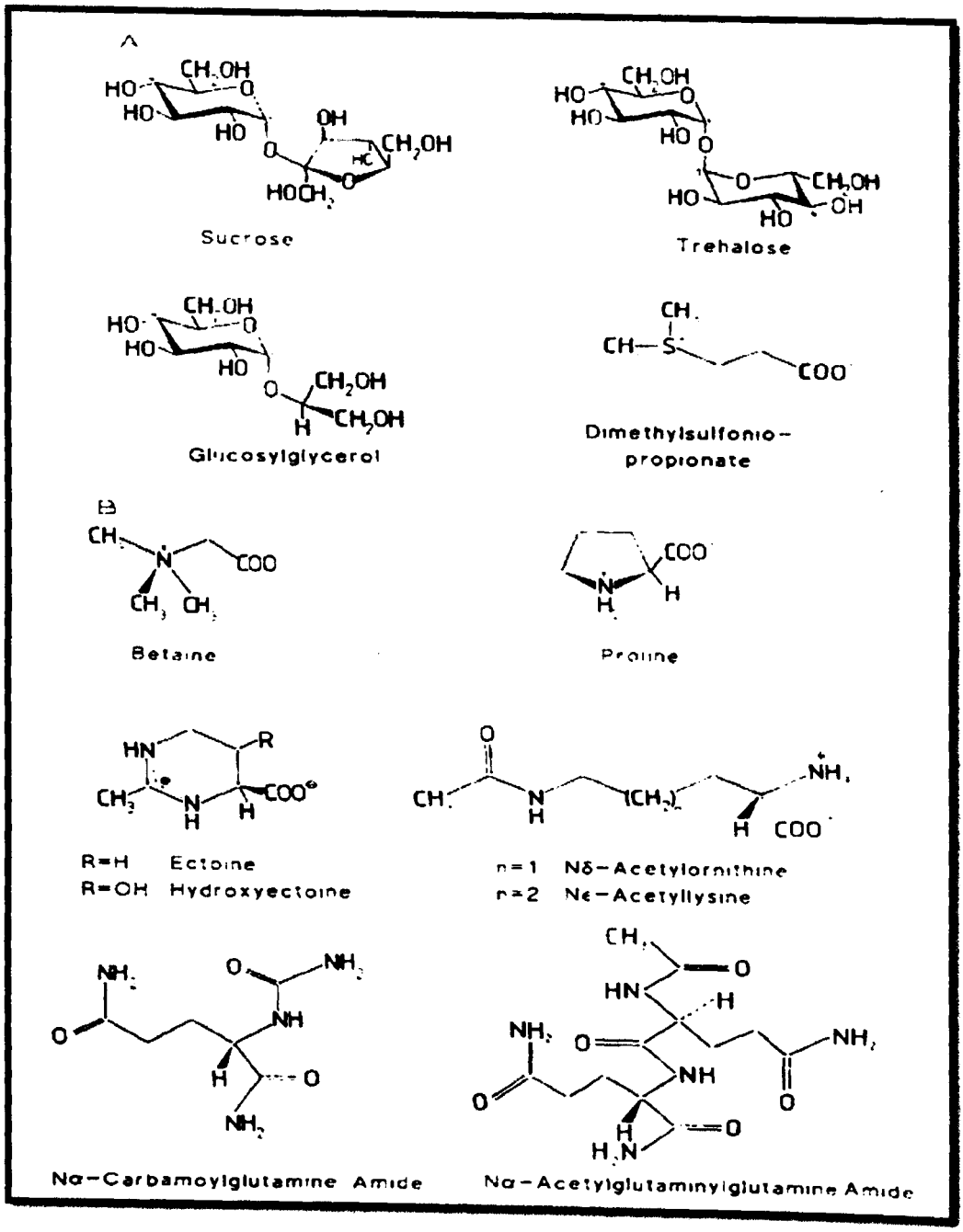

Figure 1.11. Compatible solutes of halophilic and halotolerant bacteria. (A) Non-reducing sugars and dimethysulfoniopropionate. (B) Amino acids and derivatives (Galinski & Truper, 1994).

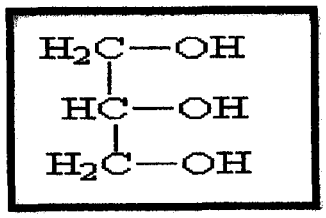

Figure 1.12. Chemical structure of glycerol [$C_3H_5(OH)_3$] (http://chemistry.about.com/library/graphics/blglycerol.htm).

1.6.2.3. Glycine betaine

Glycine betaine (Figure 1.11) was recognised as the most important organic osmotic solute in photosynthetic purple bacteria and halophilic cyanobacteria. Most halophilic bacteria accumulate glycine betaine or its precursor (choline) from the medium when grown in complex growth media. Under these conditions it was found that glycine betaine was the sole osmotic solute. However, halophilic or halotolerant heterotrophic bacteria seem not to be able to synthesise the compound *de novo* (Ventosa *et al.*, 1998).

1.6.3. Exopolysaccharides

Exopolysaccharides are produced by some bacterial strains and their production has important ecological and physiological functions. Moreover, these compounds have gained close attention because of their wide applications in many industries such as food, pharmaceutical, and petroleum industries. The traditional producers of exopolysaccharides are some strains belonging to *Xanthomonas campestris*, *Pseudomonas*, *Azotobacter*, *Sphingomonas*, and *Alcaligenes*. It was shown recently that 19 strains of the halophile *Halomonas eurihalina* are able to produce these polysaccharides and this could lead to the discovery of exopolysaccharides with better properties

than those of the existing polymers because of the wide diversity offered by microorganisms (Bejar *et al.*, 1998).

1.6.4. Proteins and phycobiliproteins

1.6.4.1. *Arthrospira (Spirulina)*

Arthrospira has been used in the human diet for at least 700 years in America and Africa. About 1300 AD, it was reported that the Indians in the Valley of Mexico harvested *Arthrospira* form Lake Texcoco and made a type of cake (called tecuitlatl) made of sun-dried *Arthrospira* biomass. Also, around the same time, the Africans in the vicinity of Lake Chad were in the habit of eating *Arthrospira* in a way that was very similar to that of the Mexican Indians and they called it *dihe* (Hu, 2004). The nutritional value of *Arthrospira* is great since it contains high quantities of proteins up to 66% of dry weight (Hu, 2004; Jassby, 1988), along with good amounts of essential acids (e.g. γ-linolenic acid [GLA]), polysaccharides, phycobiliproteins, carotenoids, vitamins (especially B12) and minerals. This unique composition and the presence of a cell wall that is more easily digestible than that of yeasts or eukaryotic algae make *Arthrospira* an attractive food source for both humans and animals (Ciferri & Tiboni, 1985; Hu, 2004).

1.6.4.2. Structure and characteristics of phycobiliproteins

Phycobiliproteins are coloured proteins with linear tetrapyrrole prosthetic groups, called bilins. They are found in Cyanophyceae, Rhodophyceae and Cryptophyceae only (Bermejo Roman *et al.*, 2002; Reis *et al.*, 1998). According to their structure and absorption properties they can be subdivided into three

main groups: phycocyanins (PCs, λ_{max}: 610-620 nm, blue)(Figure 1.13 - A), allophycocyanins (APCs, λ_{max}: 650-655 nm, blue), and phycoerythrins (PEs, λ_{max}: 540-570 nm, red)(Figure 1.13 - B) (Abalde *et al.*, 1998; Bermejo *et al.*, 2003; Sarada *et al.*, 1999). Phycocyanin and allophycocyanin are always present in Cyanophyceae and Rhodophyceae, whereas phycoerythrin may be present in Cyanophyceae, but is always present in Rhodophyceae. In order to maximize energy transfer to the chlorophyll-protein complexes which are located at the thylakoid membrane, phycobiliproteins are organized in supramolecular aggregates called phycobilisomes (Reis *et al.*, 1998). Phycobilisomes (Figure 1.14) contain allophycocyanin cores surrounded by phycocyanin at the periphery. The major constituent of phycobilisomes is phycocyanin whereas allophycocyanin functions as the bridging pigment between phycobilisomes and the photosynthetic lamellae (Sarada *et al.*, 1999).

Arthrospira is an excellent source of phycobiliproteins since it contains up to 17-20% of cell dry weight as phycocyanin (Chen *et al.*, 2006; Hu, 2004; Jassby, 1988). Phycobiliproteins have a number of unique properties such as high molar absorbance coefficients, fluorescence quantum yields, stokes shift, stable oligomers, and high photostability. Therefore they are used as fluorescence tags for cell surface markers for flow cytometry analyses of cell sorting, high throughput clinical analyses, and phycobiliprotein-based assays for reactive oxygen species. Phycobiliproteins have high free-radical scavenging capacity and this could make them useful as potent antitumor and anticancer drugs (Hu, 2004).

Since phycobiliproteins are water-soluble proteins, they can be easily isolated as pigment-protein complexes and also purified because they dissolve in water whereas other pigments do not (Abalde *et al.*, 1998). Unlike synthetic dyes, phycobiliproteins are not harmful to humans if applied externally or ingested and that makes them attractive compounds in the food and cosmetics industries (Niu *et al.*, 2007).

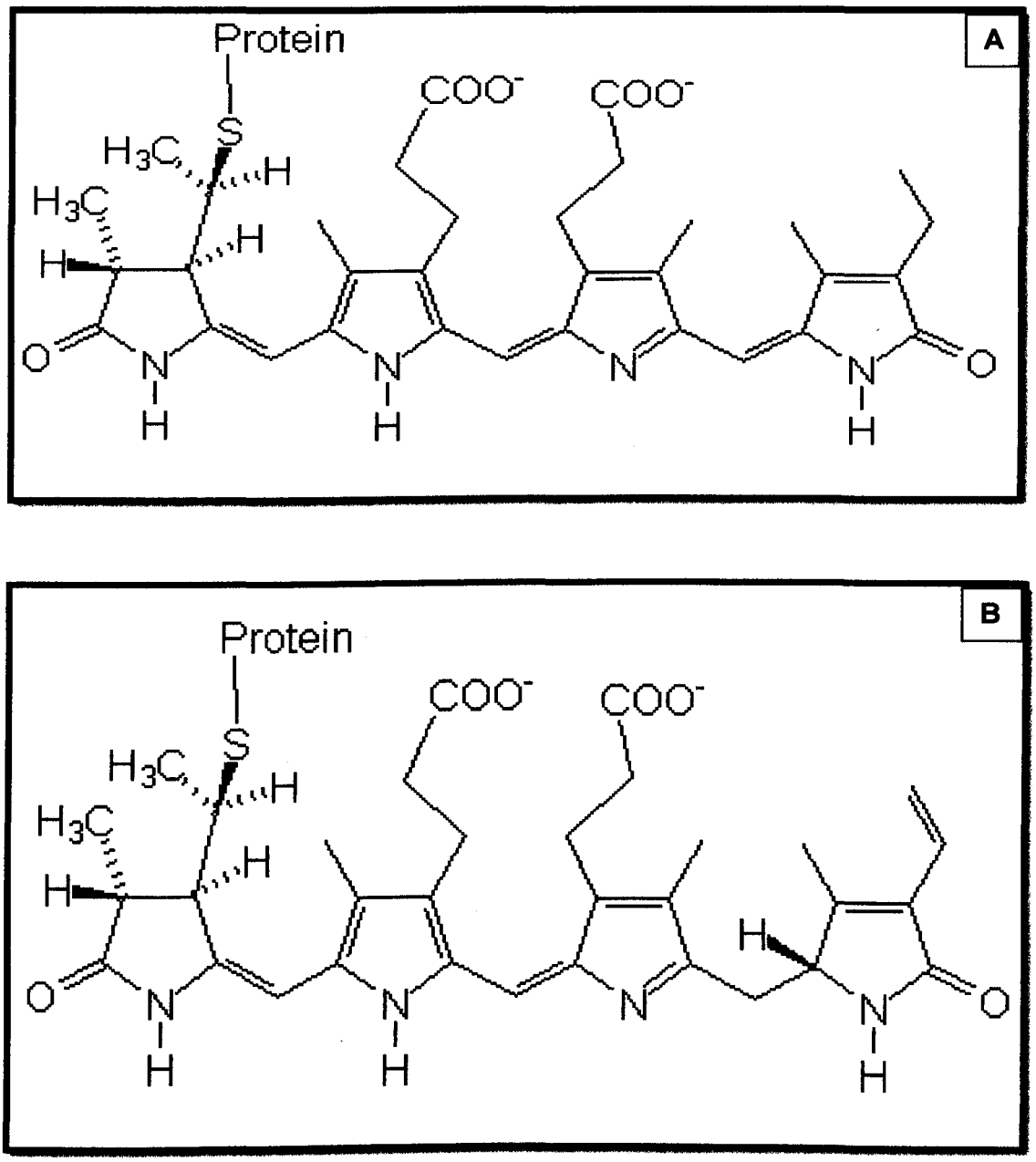

Figure 1.13. Structure of phycocyanin (A) and phycoerythrin (B) (http://www.mbari.or g/staff/conn/botany/reds/daisy/pigments.htm).

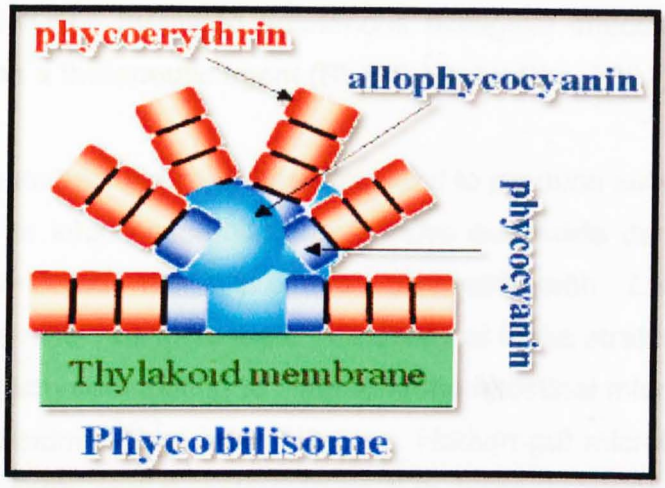

Figure 1.14. Structure of phycobilisome (http://hypnea.botany.uwc.any.uwc.ac .za/phylog eny/classif/images/phycobilisome.gif).

1.6.4.3. Applications and medicinal effects

Arthrospira (*Spirulina*) can be used as a healthy foodstuff, growth stimulator through thyroid hormone synthesis, wound treatment agent, malnutrition alleviator, cancer preventative agent, enhancer of milk secretion in mothers experiencing lactation problems (Ogbonda *et al.*, 2007), strong antioxidant agent, and anti-inflammatory agent (Chen *et al.*, 2006).

Phycobiliproteins can be used as natural protein dyes in the food industry and in the cosmetic industry, as tracers in fluorescence immunoassays, and in microscopy for diagnostic and biomedical research, due to their high absorbance and reddish fluorescence (Reis *et al.*, 1998).

It is known that peroxynitrite ($ONOO^-$) has the ability to inactivate important cellular targets and it also mediates oxidative damage to DNA. It has been shown that a phycocyanin isolated from *Spirulina platensis* has the ability

to inhibit the $ONOO^-$ - mediated deleterious biological effects and therefore it could be used as a therapeutic agent (Bhat & Madyastha, 2001).

Some cyanobacteria have been reported to produce substances that can either promote or inhibit microbial growth. The worldwide demand of yoghurt and other fermented dairy products prepared with *Lactobacillus* and *Streptococcus* strains has increased. The ability of these strains to colonize the intestinal epithelium contributing to stabilizing the intestinal microflora especially after antibiotic treatment has been reported. Human gut microbiota can play a major role in health and therefore there has been some interest in finding functional food ingredients that may stimulate beneficial lactic acid bacteria. It was demonstrated that biomass from *Spirulina platensis* increased the growth of *Lactococcus lactis* subsp. *lactis*. In addition, extracellular products (cell-free culture medium) from *Spirulina platensis* promoted significantly the growth of some lactic acid bacteria (*Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Lactobacillus bulgaricus*) (Parada *et al.*, 1998).

Spirulina contains significant amounts of omega-6 gamma linolenic acid (GLA) and it is the richest cyanobacterial source of GLA. This compound has anti-inflammatory properties (Jensen *et al.*, 2001) and also lowers low-density lipoproteins in hypercholestroemic patients, alleviates the symptoms of premenstrual syndrome, and treats atopical eczema (Hu, 2004).

Feeding *Spirulina* to fish and poultry has some beneficial effects such as increasing growth rate and colour enhancement (Belay *et al.*, 1996).

There were some studies on the biomodulatory effects of whole *Spirulina* on human and animal metabolism. In humans: it reduced body weight; in rats: it reduced cholesterol levels, increased activity of lipases, reduced glucose levels,

and inhibited maltase and sucrase; in mice: it modulated carcinogen metabolic enzymes as well as lead toxicity (Jensen *et al.*, 2001).

It was reported that aqueous extract of *Arthrospira platensis* contained antiretroviral activity since it inhibited HIV-1 replication in human T-cell lines, peripheral blood mononuclear cells, and Langerhans cells (Ayehunie *et al.*, 1998). Calcium Spirulan, a *Spirulina* product, was found to inhibit (selectively) penetration of many viruses (such as herpes simplex, human cytomegalovirus, measles, mumps, influenza A, and HIV-1) into host cells. Another product from *Spirulina* called "Cyanovirin-N" was found to irreversibly inactivate several strains of HIV since it inhibited cell-to-cell and virus-to-cell fusion (Jensen *et al.*, 2001).

Oral supplementation with *Spirulina fusiformis* to humans with tobacco-induced oral leukoplakia resulted in complete regression of 57% of the subjects with homogenous leukoplakia. It was found that after discontinuation of *Spirulina* supplementation, almost half of the complete responders developed recurrent lesions (Jensen *et al.*, 2001).

Phycobiliproteins are used in food industry as colorants for many products such as chewing gums, jellies, and dairy products. In Japan, Thailand, and China, they are used in cosmetic products like lipstick and eyeliners (Bermejo Roman *et al.*, 2002).

Spirulina platensis could be a good source of restriction endonucleases. It was shown that *S. platensis* subspecies siamese was capable of producing three restriction endonucleases named *Sp1*I, *Sp1*II, and *Sp1*III. Analyses on these enzymes showed that *Sp1*I has new specificity compared to known restriction endonucleases whereas *Sp1*II and *Sp1*III are isoschizomers of *Tth*111I and *Hae*III respectively (Kawamura *et al.*, 1986).

1.7. Mass Cultivation of Microalgae and Cyanobacteria

1.7.1. *Dunaliella*

The unicellular microalga *D. salina* has been cultivated in many countries around the world such as USA and Australia (e.g. Hutt Lagoon in Western Australia – Figure 1.2) for β-carotene production (Borowitzka, 1995). Several features have made *Dunaliella* a successful candidate for outdoor cultivation. First, mass cultivation of algae in open ponds requires considerable land where the environmental conditions such as high intensity solar light and moderate temperatures are available almost throughout the year. In such land freshwater is normally scarce but salty water including sea water is often available and therefore *Dunaliella* can be cultivated in such areas since it thrives in media containing about 6-12% (w/v) NaCl for optimal growth. Second, being halotolerant makes *Dunaliella* thrive in almost pure culture and minimises the number of predatory species. Third, the growth of non-photosynthetic organisms such as bacteria and fungi is limited in *Dunaliella* culture since the culture is composed mainly of inorganic constituents. Fourth, under the appropriate growth conditions *Dunaliella* accumulate valuable products such as β-carotene (up to 14% of the algal dry weight), glycerol (20-40% of the dry weight) (Ben-Amotz & Avron, 1989). The remainder of the algal cells after extracting these products is mainly protein (40%) which can be used as animal feed (Ben-Amotz & Avron, 1989; Ben-Amotz & Avron, 1990). Finally, *Dunaliella* is easily and fully digestible by animals and humans since it lacks a rigid cell wall (Ben-Amotz & Avron, 1989).

1.7.2. *Arthrospira (Spirulina)*

Arthrospira platensis has a long history of being utilized as a part of human diet and therefore it was the first cyanobacterium to be commercially cultivated using modern biotechnology (Hu, 2004). Growing *Spirulina* in mass outdoor cultures has increased in many parts of the world due to the increased awareness about the potential therapeutic effects of *Spirulina* and also due to its benefits for human and animal use (Belay, 1997; Sarada *et al.*, 1999). Earthrise Farms is the largest food-grade *Spirulina* plant (Figure 1.15) in the world and is the first *Spirulina* farm established in the USA. It produces more than 200,000 kg of food-grade *Spirulina* per year (Belay, 1997). The current annual worldwide production of *Arthrospira* (*Spirulina*) was estimated to be in the range 2000-3000 tonnes on a dry weight basis (Hu, 2004; Shimamatsu, 2004). It was estimated that the number of health food products that containing *Spirulina* is 50 or more. These products are in capsule, tablet, and powder forms (Reed *et al.*, 1985).

Figure 1.15. Raceway Ponds used for the culture of *Spirulina platensis* by Earthrise Farms in Calipatria, California (Courtesy A. Belay) (http://wwwscieng.murdoch.edu.au/centres/algae/BEAM-Net/BEAM-Appl4a.htm).

1.8. Aims of the Project

The following aims were addressed during the project.

- To identify halophilic microorganisms using 16S and 18S rRNA gene sequencing (Chapter 3).

- To examine the leakage of the compatible solute glycerol from several species of *Dunaliella* with a view to improving the economic viability of commercial glycerol production from this alga (Chapter 4).

- To examine the synthesis of β-carotene from *Dunaliella salina* to develop optimum conditions for commercial β-carotene production (Chapter 5).

- To further characterize a new strain of *Halomonas* isolated as a contaminant from a *Dunaliella* culture (Chapter 6).

- To carry out preliminary studies on the cyanobacteria *Arthrospira* and *Spirulina* with regard to their salt tolerance, cell content of phycobiliproteins and total protein, and compatible solute identification (Chapter 7).

Materials and Methods

Chapter Two

2.1. Sources of Microorganisms

2.1.1. *Dunaliella* strains

Four strains of *Dunaliella* (Chlorophyta, Dunaliellales) were obtained from the Culture Collection of Algae and Protozoa (CCAP), Oban, UK: *D. parva* CCAP 19/9, *D. parva* CCAP 19/10, *D. salina* CCAP 19/18, and *D. salina* CCAP 19/30.

Dunaliella salina MUR 9 was kindly provided by Professor Michael Borowitzka, School of Biological Sciences and Biotechnology, Murdoch University, Perth, Western Australia.

2.1.2. *Arthrospira fusiformis* and *Spirulina platensis*

Two strains of cyanobacteria were used: *Spirulina platensis* (Norst.) Geitler LB 2340 was obtained from The Culture Collection of Algae at the University of Texas at Austin (UTEX), USA, and *Arthrospira fusiformis* (Voronichin) Komarek & Lund 1990 CCAP 1475/8, was obtained from the Culture Collection of Algae and Protozoa, Oban, UK.

2.1.3. *Pseudomonas aeruginosa* NCIMB 12469

Pseudomonas aeruginosa strain was obtained from the National Collection of Industrial, Marine and Food Bacteria (NCIMB), Aberdeen, UK.

2.1.4. *Halomonas* sp. DSM 6507 and *Halomonas boliviensis* DSM 15516

Both strains were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Braunschweig, Germany.

2.1.5. *Halomonas* sp. NAH1

During the growth of *D. salina* CCAP 19/30 on modified Johnsons medium (J/2) (see Appendix A5), white pellets were noticed alongside the green algal pellets after centrifuging volumes of *D. salina* cultures grown at 0.1, 0.4, 1.5, and 2.5 M NaCl. These pellets represent a possible bacterial contaminant of *D. salina* cultures. The highest bacterial contamination was observed with *D. salina* culture grown at 1.5 M NaCl. 30 ml of this culture was centrifuged at 3000 *g* for 10 minutes. Supernatant was discarded and a large white pellet was observed alongside the green algal pellet. Nutrient broth tubes were prepared at 1.5 M NaCl (see Appendix A6) and nine tubes were inoculated each with a loopful of the white pellet. The tubes were incubated at three different temperatures 25, 30, and 37 °C (3 tubes at each temperature). Also, bacterial smears from the white pellet were streaked on nine nutrient agar plates containing 1.5 M NaCl (Appendix A7) and then incubated at the above temperatures (3 plates at each temperature).

2.2. Microscopy and Staining

In order to determine cell shape and its reaction to Gram's stain, the bacterial isolate (*Halomonas* sp. NAH1) was stained with Gram's stain and examined under a light microscope at 100X. Motility was examined by using a hanging drop slide. Colony shape was observed under binocular microscope (Nikon 71606, 40X) after growing the bacteria on BMA agar plates (Appendix A2) at 1.5 M NaCl.

2.3. Growth Conditions and Maintenance of Microalgal Strains

Dunaliella strains were grown in *Dunaliella* medium (Appendix A4) at 25 °C with different concentrations of NaCl in the medium. Cultures were illuminated with constant light of 53 µmol photons m^{-2} s^{-1} provided by cool white fluorescent lamps. Volumes of the medium were 100 ml each in 250 ml conical flasks inoculated with 5 ml each of an active inoculum from a culture grown for 7-10 days. The flasks were shaken manually, usually every two days.

Sub-culturing of the strains was performed routinely as above once every two weeks. *Dunaliella* cultures were also maintained by streaking three *Dunaliella* medium agar plates containing 1.5 M NaCl (Appendix A4) once every three months and when some single colonies have developed on the plates (after 2-3 weeks of incubation) they were used as an inoculum for 50 ml of *Dunaliella* medium.

The strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 were adapted to grow at different concentrations of NaCl (0.1, 0.4, 1.5, 2.5, 3.5, and 4.0 M) in *Dunaliella* medium by subculturing three times at the required levels of salinity before commencing salinity tolerance experiments.

Stock cultures of *D. salina* MUR 9 were maintained in 50 ml of modified Johnsons medium (J/2) (Appendix A5) at 12.5% (w/v) NaCl. Growth temperature was 25 °C and cultures were illuminated with 20-50 µmol photons m^{-2} s^{-1} provided by cool white fluorescent lamps on a 12 h light: 12 h dark cycle. Subculturing was performed at least once a month.

2.4. Growth Conditions and Maintenance of Cyanobacterial Strains

A. fusiformis and *S. platensis* strains were cultured in *Spirulina* medium (Appendix A8) at 37 °C with constant light of 60-80 µmol photons m^{-2} s^{-1}, provided by cool white fluorescent lamps. They were grown in batch cultures in a volume of 50 ml each in 250 ml conical flasks. The flasks were shaken on a rotary shaker at 130 rpm.

Routine subculturing of *A. fusiformis* and *S. platensis* strains was performed as follows: 50 ml of the medium was put into a 250 ml conical flask and then inoculated with 10 ml of an active inoculum (OD_{560} = 0.5 – 1.5). The flask was incubated at the above growth conditions. In addition, three *Spirulina* medium agar plates were used for keeping stocks of each strain. The plates were incubated for 7 days at 37 °C and then kept at room temperature. Routine streaking was performed once a month. Liquid media were also inoculated with single filaments (from agar plates) once a month to minimize contamination over time.

Cyanobacterial strains were adapted to grow over a wide range of NaCl concentrations (0.017, 0.1, 0.5, and 1.0 M) in *Spirulina* medium by subculturing three times at the required levels of salinity before commencing salinity tolerance experiments.

2.5. Growth Conditions and Maintenance of Bacterial Strains

2.5.1. *Halomonas* strains

BM defined medium (Appendix A1) containing 1.0 M NaCl was used for growing and routine subculturing of *Halomonas* strains. 50 ml of the medium was put into a 250 ml conical flask and then inoculated with 0.1 ml of an active inoculum (18-24 h old). The flask was incubated at 30 °C on a rotary shaker at 100 rpm. In addition, BMA plates (Appendix A2) containing 1.0 M NaCl were used for keeping stocks of the three strains of *Halomonas*. Each strain was streaked on three plates and then incubated for 3 days at 30 °C and then a piece of parafilm was stretched around each plate's edge and kept at room temperature. Routine streaking was performed once a month. Liquid media were also inoculated with single colonies at least once a month to minimize contamination over time.

Halomonas strains were adapted to grow over a wide range of NaCl concentrations (0.1, 0.5, 1.0, 2.0, and 3.0 M) in BM medium by subculturing three times at the required levels of salinity before commencing salinity tolerance experiments. Same approach was followed when using different carbon sources for *Halomonas* strains.

2.5.2. *Pseudomonas* strain

P. aeruginosa was grown in 250 ml conical flasks containing 30 ml of nutrient broth (Appendix A6) at 37 °C for 18-24 h. The flasks were shaken on a rotary shaker at 250 rpm. Single colonies were obtained by streaking a loopful of broth on a plate of nutrient agar (Appendix A7) and incubating the plate for 18-24 h at 37 °C.

2.6. Determination of Cell Number

Cell number was determined by direct counting using a light microscope (magnification ×400) with a 0.1 mm deep counting chamber (haemocytometer) (Neubauer Improved). 10 µl of Gram's iodine was added to each 1 ml of *Dunaliella* culture to kill the cells.

2.7. Determination of Chlorophyll Content

2.7.1. *Dunaliella* strains

The chlorophyll content of *Dunaliella* cultures was determined according to MacKinney (1941) and Powtongsook (1998). *Dunaliella* culture was mixed well and then a sample of 5 ml was put into a 5 inch glass test tube. The sample was centrifuged in a bench centrifuge for 10 min at 3900 *g* and supernatant was discarded immediately to prevent the motile algae from suspending themselves in the medium. Pellet was resuspended in 1 ml of distilled water and whirlimixed well. 4 ml of 100% (v/v) acetone was added into the test tube and therefore the final concentration of acetone was 80% (v/v). The tube was left for 10-15 min in dim light to allow chlorophyll extraction by acetone then the extract was centrifuged at 3900 *g* for 5 min. After centrifugation, the pellet should be white indicating that all pigments have been extracted. Optical density (OD) at 645 and 663 nm was measured using UNICAM Helios Alpha spectrophotometer and 1 ml glass cuvette. 80% (v/v) acetone was used as a blank. Chlorophyll concentration was calculated as follows:

$OD_{645} \times 202 = X$

$OD_{663} \times 80.2 = Y$

$(X + Y) / 2 = \mu g$ Chl. 5 $ml^{-1} = Z$

$Z / 5 = \mu g$ Chl. ml^{-1}

2.7.2. *Arthrospira* and *Spirulina* strains

The chlorophyll content of cyanobacterial strains was determined as described in Vonshak (1997a). *Arthrospira* and *Spirulina* cultures were mixed well and then a sample of 5 ml each was put into a glass 5 inch test tube then centrifuged for 5 min at 2000 *g*. Supernatant was discarded and pellet was kept. The pellet was resuspended in 5 ml methanol (absolute) and ground in a glass tissue homogenizer. Sample was incubated in a hot block at 70 °C for 2 min and then centrifuged for 5 min at 2000 *g*. After centrifugation supernatant has green colour (chlorophyll a) and pellet has dark blue colour (phycocyanin and other pigments). Optical density at 665 nm was measured for clear green supernatant and the pellet was kept for protein determination.

The chlorophyll concentration was calculated as follows: optical density at 665 nm × factor (derived from absorption coefficient which is 13.9 for *Spirulina*) = Chlorophyll concentration in $\mu g\ ml^{-1}$ or $mg\ l^{-1}$. When 5 ml sample was used as above, $OD_{665} \times 13.9 = \mu g$ chlorophyll 5 ml^{-1} and it was divided by 5 to obtain μg chlorophyll ml^{-1} or mg chlorophyll l^{-1}.

The standard curves relating chlorophyll contents ($mg\ l^{-1}$) of *A. fusiformis* and *S. platensis* to population densities (OD at 560 nm) were accomplished

using cultures in their late exponential growth phase (OD_{560} = 1.5) grown as described in Section 2.4 and at low salinity (0.017 M NaCl). Three replicates were used for each OD_{560} (0.1 - 1.5). Means of results and standard errors were used to plot the standard curves.

2.8. Determination of β-carotene Content

β-carotene was determined according to Hejazi (2002).

2.8.1. Concentration curve

In order to determine β-carotene content in *Dunaliella* samples, it was required first to make a standard β-carotene curve using a series of known concentrations of β-carotene. 30 mg of all-trans β-carotene (Sigma) was dissolved in 10 ml of tetrahydrofuran (THF) (Sigma) to make a concentrated stock solution of β-carotene (3 mg ml^{-1}). The stock solution was used to make a series of diluted solutions: 0.6, 1.2, 1.8, 2.4, and 3.0 µg β-carotene ml^{-1} using THF. Duplicates were made for each concentration of β-carotene. Optical density was measured at 457 nm in a spectrophotometer against the blank (THF) using a 3.0 ml glass cuvette. β-carotene standard curve was plotted using β-carotene concentrations (µg ml^{-1}) along abscissa (x) axis and OD_{457} along ordinate (y) axis. Means for two replicates were calculated and shown in Appendix B1.

2.8.2. Extraction of β-carotene

D. salina culture was mixed well and then a sample of 1 ml was taken and placed into a 15 ml plastic centrifuge tube. The sample was centrifuged at

3000 *g* for 5 min and then supernatant was discarded. 2 ml of THF was added to the algal pellet and vortexed for 2 min to reach complete extraction of β-carotene. The mixture was centrifuged at 3000 *g* for 5 min to separate the biomass (white colour) and the solvent phase (contains β-carotene). Two replicates were made for each culture of *D. salina*. Optical density was measured as described in Section 2.8.1. Chlorophyll content and cell number were also determined for the cultures as described in Sections 2.7.1 and 2.6 respectively.

2.9. β-carotene Production by *Dunaliella salina*

2.9.1. Effects of nitrogen and carbon concentrations in the medium on the growth and β-carotene production by *Dunaliella salina* 19/30

The concentration of β-carotene was determined after growing *D. salina* 19/30 in a cultivation system composed of four phases, each phase lasted for 7 days. In the first phase the algae were grown in batch cultures in 250 ml conical flasks containing *Dunaliella* medium (Appendix A4) at 1.5 M NaCl and 5 mM $NaNO_3$ and incubated at 30°C under continuous light of 53 µmol photons m^{-2} s^{-1}. Growth in 250 ml conical flasks was initiated by inoculating 100 ml of *Dunaliella* medium with 5 ml of a batch culture of the strain grown for 7 days at the above growth conditions. Two replicates (cultures) were used and chlorophyll content, β-carotene content, and cell number were determined for each culture at the end of each phase of cultivation. At the end of the first and second phases of cultivation, cultures were centrifuged at 3000 *g* for 10 min and then supernatants were discarded and algal pellets were treated as follows. Second phase, they were washed twice with 100 ml each of *Dunaliella* medium containing 1.5 M NaCl and 0.5 mM $NaNO_3$ (10 times less N than that used in

the first phase) and then resuspended in 100 ml of the same medium. Third phase, as in the second phase, except no nitrogen source was added to the medium (nitrogen-free medium). In the fourth phase, cultures were not centrifuged but provided with more $NaHCO_3$ at a concentration of 0.2 g 100 ml^{-1} (usual amount in *Dunaliella* medium was 0.1 g 100 ml^{-1}). For each culture, 0.2 g of $NaHCO_3$ was dissolved in 1 ml sterile Milli-Q water in a 1.5 ml Eppendorf tube. Where applicable the organic carbon source was sterilized by filtration using 0.2 µm filter and then added to the culture aseptically.

2.9.2. Effects of nitrogen concentration in the medium and bubbling CO_2 through cultures of two strains of *Dunaliella salina* on the growth and β-carotene production

Batch cultures of *D. salina* 19/18 and 19/30 (four cultures each) were initiated by inoculating 100 ml each of *Dunaliella* medium containing 1.5 M NaCl and 5 mM $NaNO_3$ with 5 ml each of a batch culture of *D. salina* 19/18 or 19/30 grown for 7 days in the same medium in 250 ml conical flasks. The flasks were incubated as described in Section 2.9.1 for 7 days. Initial cell number, chlorophyll content, and β-carotene content were determined. Then cultures were centrifuged at 3000 *g* for 10 min and supernatants were discarded and algal pellets were treated as follows for each strain: pellets in flasks 1 and 3 were washed twice with 100 ml each of nitrogen-free *Dunaliella* medium containing 1.5 M NaCl, whereas pellets in flasks 2 and 4 were treated as above but with *Dunaliella* medium containing 5 mM $NaNO_3$. All four flasks were resuspended in 100 ml each of an appropriate *Dunaliella* medium as in the washing steps above and placed into sterile fresh 250 ml conical flasks that were provided with sterile Pasteur pipettes and plastic tubes.

A large glass tank was filled with water to three quarters of its volume and then a temperature control unit was attached to the tank and immersed in

the water to fix the temperature at 30 °C. Four fluorescent cool white lamps were put outside the tank (two on each side) to provide a mean continuous light intensity of 44 μmol photons m^{-2} s^{-1}. Each flask was attached to a stand and immersed in the water bath (3 quarters of the flask was in the water). Cultures in flasks 1 and 2 were bubbled with filtered 5% (v/v) CO_2 provided by a CO_2 cylinder whereas cultures in flasks 3 and 4 were bubbled with filtered natural air (≈ 0.035% (v/v) CO_2). The air flow rates for both aeration systems were set at 100 cm^3 min^{-1}. Cultures were grown under the above growth conditions for 8 days and then cell number, chlorophyll content, and β-carotene content were determined for each culture.

2.9.3. Production of β-carotene by *Dunaliella salina* in an airlift fermenter

D. salina 19/18 was grown in a 2 L airlift fermenter (see Figure 5.2). The cultivation was in three phases. In the first phase 1.8 L of *Dunaliella* medium containing 1.5 M NaCl and 5 mM $NaNO_3$ was inoculated with 100 ml of a batch culture of the strain grown for 7 days under the growth conditions described in Section 2.3. The culture inside the fermenter was bubbled with filtered 5% (v/v) CO_2 at a flow rate of 200 cm^3 min^{-1}. Temperature was fixed at 30 °C by pumping water from a water bath set at 30 °C to a water jacket surrounding the fermenter. Fluorescent lamps were fitted in vertical position around the fermenter and the mean light intensity was 44 μmol photons m^{-2} s^{-1}. The working volume of the fermenter was 1.8 litres.

The first phase of cultivation lasted for 7 days until high cell number was achieved. Small volume samples (1-2 ml) were taken daily through the front valve of the fermenter to determine the cell number in the first phase of cultivation.

At the end of the first phase the whole volume of the culture (1,8 L) was centrifuged at 3000 *g* for 10 min. Supernatant was discarded and the algal pellet was resuspended in 1.8 L of nitrogen-free *Dunaliella* medium containing 1.5 M NaCl. The second phase of cultivation was started by returning the above suspension to the fermenter. The second phase lasted for 7 days until large amounts of β-carotene were accumulated in the cells. Small volume samples (2-4 ml) were taken daily as above to determine cell number and β-carotene content.

At the end of the second phase, 1.5 L of the culture was harvested and the remaining 300 ml of the culture was diluted with 1.5 L of *Dunaliella* medium containing 1.5 M NaCl and 5 mM $NaNO_3$ and then a third phase of cultivation was started and lasted for 7 days during which cell number and β-carotene content were monitored daily.

2.9.4. Large-scale culturing of *Dunaliella salina* in an outdoor raceway pond for β-Carotene production

Dunaliella salina MUR 9 was cultured outdoors in a 1 m^2 surface area fibreglass paddle wheel driven raceway pond located at Murdoch University, Perth, Western Australia and operated at 15-18 cm depth. The 4-paddle paddle wheel was operated at a rotation speed of about 28 rpm generated a flow rate of 20 cm s^{-1}.

Modified Johnsons medium (J/2) (Appendix A5) containing 12.5% (w/v) NaCl (2.14 M NaCl) was used for culturing the strain in the pond. Seawater (3.8% (w/v) NaCl) was used for preparing a large volume of the medium (>150 L). It was brought from Hilary's Beach (Perth, Western Australia) and stored in 10,000 L tanks at Murdoch University in the dark to prevent algal growth. Seawater was chemically sterilized before use with sodium hypochlorite

overnight and then the sodium hypochlorite was neutralised with sodium thiosulphate. Extra amounts of NaCl were dissolved in seawater in order to obtain 12.5% (w/v) NaCl. Pod salt was used instead of Analar NaCl because high amounts of NaCl were needed for preparing the medium. It was brought from Bunnings, Western Australia. All other nutrients were added as in Appendix A5 except micronutrients which were already available in seawater. The medium was chemically sterilized by adding 1 ml of 12.5% (w/v) sodium hypochlorite to every 20 L of the medium as above.

The inoculum for the pond was 15 L liquid culture which was used to inoculate 130 L of the medium in the pond to give an initial cell number of 2×10^4 cells ml^{-1}. Carboy photobioreactor (20 L) was used to produce *D. salina* inoculum for the raceway pond. The inoculum culture was grown at temperature of 25 ± 1 °C and illuminated with a continuous light intensity of 350 µmol photons m^{-2} s^{-1} provided by cool white fluorescent lamps. Humidified, sterile (sterilised using a Milex FG50 0.2 µm filter) air was provided at a flow rate of 200 cm^3 min^{-1}. The reactor was fitted with an inlet gas tube with an air stone, an outlet gas tube connected to a glass bottle containing 0.1 M HCl to prevent contamination, and a sampling tube. The photobioreactor was stirred with a 10 cm magnetic stir bar at the bottom of the reactor.

The pond's depth was initially operated at 15 cm (150 L culture) and then increased to 18 cm (180 L culture) by adding sterile modified Johnsons medium containing extra amounts of NaCl to increase salinity from 12.5 to 20% (w/v) NaCl (3.42 M NaCl). Salinity was increased to 20% NaCl over a period of 3 days (2.5% NaCl daily). Samples were taken every two days for counting cell number and monitoring the salinity in the pond. Pond temperature was recorded continuously using an underwater Tiny Tag TG-3110 temperature logger. Salinity in the pond was monitored using a digital hand-held refractometer (Atago Co. Ltd., Japan).

2.10. Determination of Intracellular and Extracellular Glycerol Content

Glycerol assay was performed chemically using acetylacetone method according to Powtongsook (1998).

2.10.1. Preparation of reagents

Two reagents were prepared to perform the assay: periodate reagent and acetylacetone reagent. Periodate reagent was prepared by dissolving 130 mg of sodium periodate ($NaIO_4$) in 180 ml of 2% (v/v) acetic acid containing 15.4 g ammonium acetate, and when the sodium periodate was dissolved completely 20 ml of glacial acetic acid was added. Acetylacetone reagent was prepared as follows: 1% (v/v) acetylacetone in isopropanol. The latter reagent was kept in the dark.

2.10.2. Concentration curve

In order to estimate intracellular and extracellular glycerol it was required first to make a standard glycerol curve using a series of known concentrations of glycerol. Concentrated glycerol solution (1.26 g ml^{-1}) was diluted with distilled water to obtain a stock solution with a concentration of 125 µg 100 $µl^{-1}$. The stock solution was used to make the following diluted solutions of glycerol: 10, 20, 30, 40, and 50 µg glycerol 100 $µl^{-1}$. Six fresh test tubes (15 ml centrifuge tubes) were set up as in the following table:

Tube	Blank	10 µg	20 µg	30 µg	40 µg	50 µg
Distilled water	100 µl	-	-	-	-	-
Glycerol solution	-	100 µl	100 µl	100 µl	100 µl	100 µl
Dunaliella* Medium	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
30% (w/v) TCA**	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl

Table 2.1. Components of test tubes which were needed to make a standard glycerol curve. *0.4 or 1.5 M NaCl medium buffered by HEPES. **Trichloroacetic acid.

1 ml of periodate reagent was added to each test tube above and then the tubes were whirlimixed and left on bench for 5 min. Acetylacetone reagent was then added (2.5 ml each) and the tubes were whirlimixed and placed in a water bath set at 45 °C for 15 min. The tubes were removed from the water bath and put on bench for few minutes to allow them to cool to room temperature. Optical density was measured at 410 nm in a spectrophotometer against the blank using a 3.0 ml glass cuvette. Three replicates were made for each test tube above. Glycerol standard curve was plotted using glycerol concentrations (µg) along abscissa (x) axis and OD_{410} along ordinate (y) axis. Standard errors for three replicates were calculated and the standard curve is shown in Appendix B2.

2.10.3. *Dunaliella* strains

In order to measure the intracellular glycerol concentrations in *Dunaliella* cells and the extracellular glycerol concentrations that leaked from the cells to the medium, a *Dunaliella* culture was mixed well and then four samples were taken. Two samples (6 ml each) were taken for extracellular glycerol determinations and they were put into fresh 15 ml centrifuge tubes. The tubes were centrifuged at 3000 *g* for 10 min in a bench centrifuge. The top 4.5 ml of each supernatant was transferred into a fresh 15 ml centrifuge tube and the remaining (1.5 ml supernatant plus algal pellet) was discarded. The tubes were labelled "medium only" A and B. Another two samples (4.5 ml each) from *Dunaliella* culture were taken and put into fresh 15 ml centrifuge tubes and labelled "cells plus medium" C and D. To all four tubes (A, B, C, and D), 0.5 ml of 30% (w/v) trichloroacetic acid (TCA) was added to each tube and the tubes were left on bench for 10 min. After this incubation time, the tubes which contained algae (C and D) turned from green to brown indicating that all the protein structures of the cell membranes have been denatured and the intracellular glycerol content has been released into the medium whereas the tubes which contained medium only (A and B) did not change in colour. All four tubes were centrifuged at 3000 *g* for 10 min to separate cell debris from the medium and supernatants were kept for glycerol assay. 110 µl from each supernatant was taken and put into a fresh 15 ml centrifuge tube and then 100 µl of distilled water was added to each tube. A blank was set up in a fresh 15 ml centrifuge tube which contained 100 µl distilled water, 10 µl 30% TCA and 100 µl of an appropriate *Dunaliella* medium. Glycerol concentration in each tube was determined by performing the procedure described in Section 2.10.2.

2.10.4. Calculation of intracellular and extracellular glycerol contents and percentage of glycerol leakage

2.10.4.1. Extracellular glycerol concentration

Extracellular glycerol concentration was determined by using OD_{410} readings of the tubes A and B (medium only) to read off the value from the glycerol standard curve (Appendix B2). The readings from the curve (µg 100 μl^{-1}) were multiplied by 10 and divided by 92.1 to give µmoles glycerol ml^{-1}. Four replicates (two *Dunaliella* cultures and two glycerol assays per culture) were used for each treatment and the standard error was calculated from these replicates.

2.10.4.2. Intracellular glycerol concentration

Readings of OD_{410} of the tubes A and B (Section 2.10.4.1) were subtracted from those of the tubes C and D (cells plus medium) and the resulting OD_{410} values were used to read off the amount of glycerol from the standard curve. The readings from the curve (µg 100 μl^{-1}) were multiplied by 10 and divided by 92.1 to give µmoles glycerol ml^{-1}. When intracellular glycerol content was expressed as µmoles mg^{-1} chlorophyll, values in µmoles glycerol ml^{-1} was multiplied by 1000 and divided by chlorophyll content (µg ml^{-1}). Similarly, when intracellular glycerol content was expressed as µmoles / 10^6 cells, values in µmoles glycerol ml^{-1} was divided by cell number (10^6 cells ml^{-1}). Four replicates (two *Dunaliella* flasks and two glycerol assays per culture) were used for each treatment and the standard error was calculated from these replicates.

2.10.4.3. Percentage of glycerol leakage

Percentage of glycerol leakage was determined by dividing the amount of glycerol (µg) that was found in the medium by the amount of glycerol (µg) that was found in cells plus medium multiplied by 100. Thus 100% leakage means equal amounts of glycerol was found inside and outside the cells.

2.11. Glycerol Production by *Dunaliella*

2.11.1. Batch cultures

Three strains of *Dunaliella* (*D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30) were adapted to grow in a wide range of salinities (0.1 - 4.0 M NaCl) as described in Section 2.3. Twelve cultures were used for each strain (two cultures for each salinity). The strains were grown for an incubation period of 14 days at the growth conditions described in Section 2.3. Samples for cell number, chlorophyll content, intracellular glycerol content, and extracellular glycerol content determinations were taken from each culture after 3, 7, 10, and 14 days of incubation. Cell number, chlorophyll content, intracellular glycerol content, extracellular glycerol content, and percentage of glycerol leakage were determined as described in Sections 2.6, 2.7.1, 2.10.3, and 2.10.4 respectively. Growth curves of *Dunaliella* strains across the range of salinities were plotted by using both cell number and chlorophyll content. In addition, intracellular and extracellular glycerol concentrations were used to plot curves showing these concentrations across the range of salinities.

2.11.2. Semi-batch culture

Dunaliella parva 19/10 was grown in a 2 L airlift fermenter under semi-batch culture conditions. The medium used was *Dunaliella* medium (Appendix

A4) containing 1.5 M NaCl. The initial inoculum of 1.8 L of the medium in the fermenter was 50 ml of a batch culture of *D. parva* 19/10 grown for 10 days in 1.5 M NaCl medium under the growth conditions described in Section 2.3. The semi-batch culture was aerated by either filtered natural air (≈ 0.035% (v/v) CO_2) or filtered CO_2 / O_2 mixture (0.1% (v/v) CO_2, 20% (v/v) O_2, balance N_2) at a flow air rate of either 100 or 200 cm^3 min^{-1}. Growth temperature was fixed at 25 °C as described in Section 2.9.3. Light intensity and the working volume of the fermenter were also as described in Section 2.9.3.

Samples were taken on regular basis (almost every day) for a period up to 22 days to determine chlorophyll content, intracellular glycerol concentration, extracellular glycerol concentration, and percentage of glycerol leakage as described in Sections 2.7.1, 2.10.3, and 2.10.4 respectively. Samples were collected through the front tap of the fermenter by allowing 100 ml of the culture to go out to clean the tube which was then discarded and then a sample of 100 ml was collected. The culture was then diluted with 200 ml of the growth medium which was supplied aseptically via an external 250 ml conical flask which was connected to the fermenter at the initial setting up processes of the culture.

2.11.3. Continuous culture (chemostat)

Dunaliella parva 19/10 was grown in a 2 L airlift fermenter (Figure 2.1) under continuous culture conditions. Growth medium used was as described in Section 2.11.2. Light intensity and working volume of the fermenter were as described in Section 2.9.3. Growth temperature was fixed at 25 °C as described in Section 2.9.3. The initial inoculum was 100 ml of a batch culture of *D. parva* 19/10 grown for 11 days in *Dunaliella* medium containing 1.5 M NaCl under the growth conditions described in Section 2.3. The culture was aerated by either filtered natural air or filtered CO_2 / O_2 mixture (0.1% CO_2, 20% O_2, balance N_2) at a flow air rate of 200 cm^3 min^{-1}. The flow rate of the medium was either 45 or

90 ml h^{-1}. Samples were taken to determine chlorophyll content, intracellular glycerol concentration, extracellular glycerol concentration, and percentage of glycerol leakage as described in Sections 2.7.1, 2.10.3, and 2.10.4 respectively. Samples were collected by putting the outlet tube (connected to waste reservoir) to a sterile 250 ml conical flask.

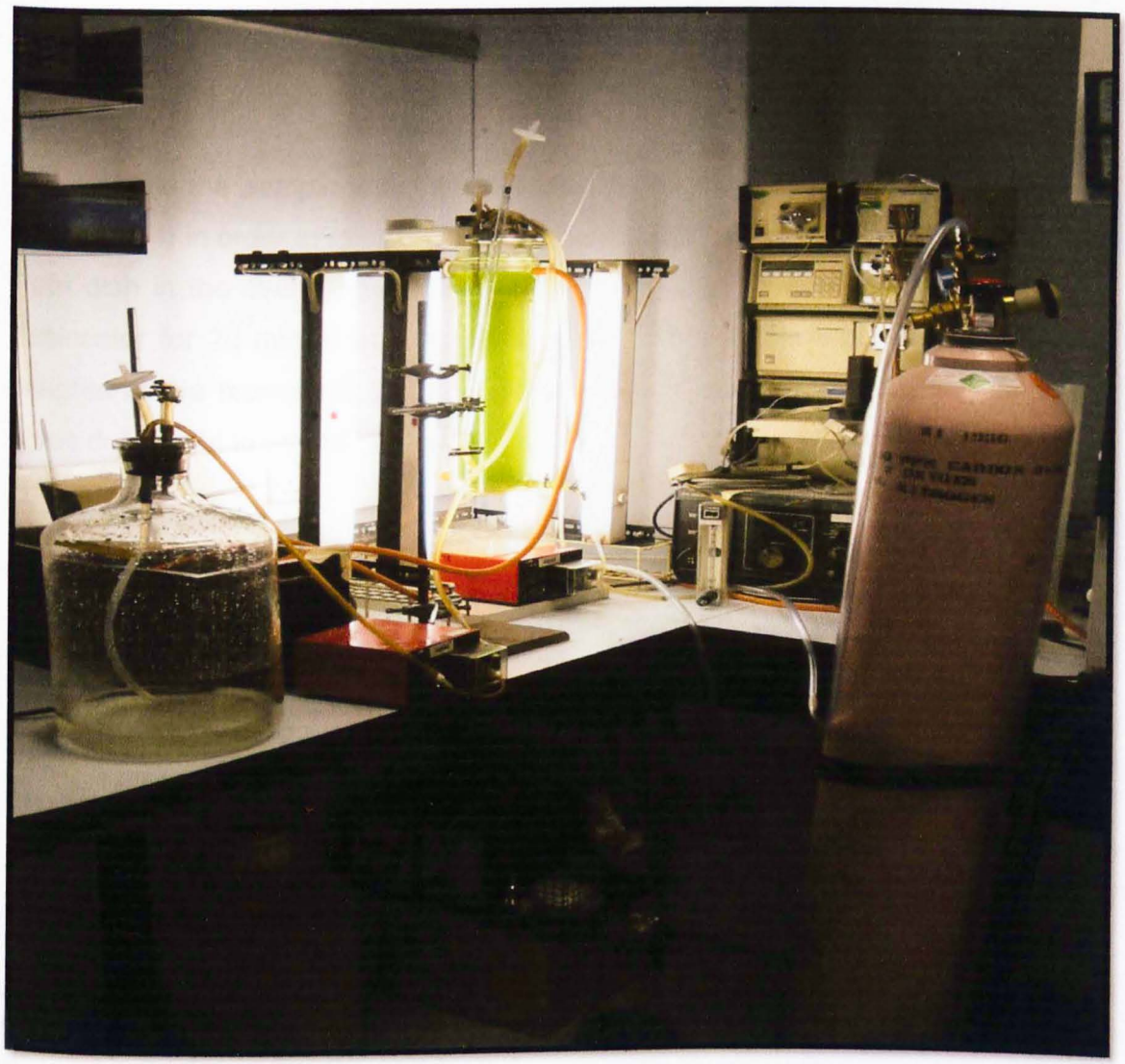

Figure 2.1. Photograph of a continuous culture (chemostat) of *Dunaliella parva* 19/10 showing the culture inside a 2 L airlift fermenter and the light source in the centre of the picture. Medium reservoir, water bath, medium automatic pump are to the left of the picture whereas a CO_2 / O_2 cylinder and air flow meter are to the right of the picture. The outflow tube to the waste reservoir and the waste reservoir are visible at the bottom of the picture.

2.12. Determination of Dry Weight

Dry weight was determined according to Vonshak (1997a). *Arthrospira* and *Spirulina* cultures were mixed well and then a sample of 25 ml each was filtered through a Whatman GF/C filter 47 mm (diameter) which was dried in an oven for 24 h or overnight at 70 °C or 2 h at 105 °C and weighed prior to the filtration.

While the sample was being filtered it was washed with 20 ml acidified water (pH 4) in order to remove insoluble salts. The filter then was put in a glass Petri dish in the oven under the above conditions. After cooling the filter in a desiccator for 20 min, it was weighed again. The difference between the two weights above represents the dry weight in 25 ml sample and dividing by 25 gives dry weight in mg ml^{-1} (or g l^{-1}).

Standard curves relating dry weights (g l^{-1}) of *Arthrospira fusiformis* and *Spirulina platensis* to population densities (OD at 560 nm) were accomplished using cultures in their late exponential growth phase (OD_{560} = 1.5) grown as described in Section 2.4 and at low salinity (0.017 M NaCl). Three replicates were used for each OD_{560} (0.1-1.5). Means of results and standard errors were used to plot the standard curves.

2.13. Determination of Protein Content by Bradford (1976) Assay

2.13.1. Concentration curve

A standard curve was made using Bovine Serum Albumin (BSA) in the range of 0 – 200 µg by dissolving 250 mg of BSA in 50 ml of distilled water. The final concentration of protein in the stock solution was 5 mg ml^{-1} (5 µg $µl^{-1}$).Then the stock solution was used to make a range of protein concentrations by a series of dilutions as in the following table:

Tube number	Volume of BSA stock solution (µl)	Volume of distilled water (µl)	Total volume in each tube (µl)	Protein concentration (µg)
1	0	100	100	0 (Blank)
2	2.5	97.5	100	12.5
3	5	95	100	25
4	7.5	92.5	100	37.5
5	10	90	100	50
6	15	85	100	75
7	20	80	100	100
8	25	75	100	125
9	30	70	100	150
10	35	65	100	175
11	40	60	100	200

Table 2.2. Components of test tubes which were needed to make a standard protein curve.

Three replicates were made for each test tube above. To each test tube, 3 ml of Bradford Reagent (Sigma, USA) was added then whirlimixed and left on bench for 5 min. Optical density was measured for each sample at 595 nm using a 3 ml glass cuvette. The blank was used to zero spectrophotometer.

Protein standard curve (Appendix B3) was plotted from which protein concentration for samples can be determined. Standard errors for three replicates for each sample were calculated and shown on the curve.

2.13.2. *Halomonas* sp. NAH1

Halomonas samples (1 ml each in 1.5 ml Eppendorf tubes) were kept in a freezer until analysis. When the analysis is due, they were thawed, whirlimixed, and 100 µl was taken from each sample and placed in a separate 15 ml plastic centrifuge tube (resistant to temperature up to 100 °C). 0.9 ml of 1 M NaOH was added to each sample and whirlimixed. Tubes were put in a hot block at 90 °C for 10 min, cooled on ice, dried outside, and centrifuged in a bench centrifuge at 3000 *g* for 10 min. A sample of 100 µl was taken from each supernatant and put into a fresh 15 ml centrifuge tube. Similarly, 100 µl of distilled water was put into another test tube to serve as a blank. To all test tubes, 3 ml of Bradford Reagent was added and then the protein determination was carried out as described in Section 2.13.1.

Protein content was determined by reading (µg protein) off the standard curve (Appendix B3), multiplying the reading by 10 to get µg protein ml^{-1}, and multiplying the result by 10 again to take into account the dilution by NaOH.

2.13.3. *Arthrospira* and *Spirulina* strains

Protein content in *A. fusiformis* and *S. platensis* samples was determined according to Vonshak (1997a). The pellet from chlorophyll determination (Section 2.7.2) was dried by blowing a gentle stream of air over it and then resuspended in 4 ml of 1 M NaOH by whirlimixing. The mixture was incubated for 20 min at 100 °C in a hot block (tubes were covered to avoid evaporation). Tube was cooled on ice, dried from outside and centrifuged at 2000 *g*. A sample of 0.1 – 0.5 ml was taken from the supernatant and put into a 15 ml plastic centrifuge tube and then 1 M NaOH was added to a final volume of 1 ml. Similarly, a sample of 0.1-0.5 ml of distilled water was put into another tube to serve as a blank and treated as the sample above. 3 ml of Bradford Reagent was added to each tube and then the protein determination was carried out as described in Section 2.13.1.

Protein concentration was calculated as follows: if 0.1 ml was used from the supernatant above, the reading (µg protein) from the standard curve (Appendix B3) was multiplied by 40 to get µg protein 5 ml^{-1} (because protein content was resuspended in 4 ml of NaOH and therefore 4 ml/0.1 ml sample = 40). Since the original cyanobacterial sample was the 5 ml used in chlorophyll determination (Section 2.7.2), the resulting value was divided by 5 to get µg protein ml^{-1}. However if another volume of supernatant was used, e.g. 0.3 ml instead of 0.1 ml, the reading (µg protein) from the standard curve was divided by 3 to get µg protein 0.1 ml^{-1} then multiplied by 40 to get µg protein 5 ml^{-1}. Again since the original cyanobacterial sample was the 5 ml used in chlorophyll determination, the resulting value was divided by 5 to get µg protein ml^{-1}.

Standard curves relating protein contents (g l^{-1}) of *Arthrospira fusiformis* and *Spirulina platensis* to population densities (OD at 560 nm) were accomplished using cultures in their late exponential growth phase (OD_{560} = 1.5) grown as described in Section 2.4 and at low salinity (0.017 M NaCl).

Three replicates were used for each OD_{560} (0.1-1.5). Means of results and standard errors were used to plot the standard curves.

2.14. Determination of Salinity Tolerance

2.14.1. *Dunaliella* strains

Salinity tolerance of three strains of *Dunaliella* was investigated when glycerol production by those strains was investigated in batch cultures (see Section 2.11.1).

2.14.2. *Arthrospira* and *Spirulina*

After adapting *Arthrospira* and *Spirulina* strains to grow in a salinity range of 0.017 - 1.0 M NaCl (Section 2.4), growth curve experiments were conducted at each concentration of NaCl for each strain. Growth conditions were as described in Section 2.4. Three flasks were used for each salinity. Initial OD_{560} after inoculation for both strains for all treatments was in the range 0.1 – 0.13.

Growth was monitored spectrophotometrically by measuring the population density at OD_{560} using UNICAM Helios Alpha spectrophotometer. Means and standard errors for three replicates were calculated and the growth curves were plotted. Moreover, specific growth rates and generation times were calculated for both strains at different levels of salinity.

2.14.3. *Halomonas* strains

BM complex medium (Appendix A3) containing 1.28 M NaCl was used to grow *Halomonas* sp. NAH1 in order to get a growth curve at this level of salinity. BM defined media (Appendix A1) containing 0.1, 0.5, 1.0, 2.0, and 3.0 M NaCl

were used to grow *Halomonas* sp. NAH1, *Halomonas* sp. DSM 6507, and *Halomonas boliviensis* DSM 15516 in order to get growth curves across this range of salinity. 50 ml of the appropriate medium were put into 250 ml conical flasks (three flasks were used for each treatment). They were then inoculated with 1 ml each of an appropriate active inoculum (cells in their exponential growth phase i.e. 18-48 h old depending on the salinity of the medium). Initial OD_{600} after inoculation (for each strain of *Halomonas*) were approximately 0.051, 0.051, 0.061, 0.051, 0.032, and 0.036 for BM media at 0.1, 0.5, 1.0, 2.0, and 3.0 M NaCl respectively. The flasks were then incubated at 30 °C on a rotary shaker at 100 rpm for 171 h when glucose (20 mM) was the carbon source in the medium or for 48 h when glycerol (20 mM) or betaine (20 mM) was the carbon source in the medium. Growth was monitored spectrophotometrically by measuring the population density at OD_{600}. Means and standard errors for three replicates were calculated and then growth curves were plotted. Moreover, specific growth rates and generation times were calculated for all three strains of *Halomonas* at different levels of salinity in the medium.

2.15. Measurement of Oxygen Uptake

Oxygen uptake was measured according to Howat (1997) using a modified Clark oxygen electrode of the type described by Delieu and Walker (1972). The reaction chamber (working volume 2 ml) was maintained at a constant 30 °C by circulating water from a temperature controlled water bath.

To allow the rate of oxygen uptake to be calculated, the amount of oxygen in 2 ml of air saturated medium had to be known. Oxygen solubilities in sea water (approximately 0.4 M NaCl) at 25 and 30 °C are 0.206 and 0.190 μmoles ml^{-1} respectively (www.engineeringtoolbox.com/oxygen-solubility-water-d_841.html). The difference between the two values of oxygen solubility is

0.016 µmoles ml^{-1}. Therefore increasing temperature by 5 °C decreases oxygen solubility by about 0.016 µmoles ml^{-1}. Since standard oxygen solubility at 25 °C and at 1.0 M NaCl in the medium is 0.19 µmoles ml^{-1} (Gilmour, 1982), it is expected to be lower than that by 0.016 µmoles ml^{-1} i.e. 0.19 – 0.016 = 0.174 µmoles ml^{-1}. Thus approximate standard of oxygen solubility at 1.0 M NaCl in the medium and at 30 °C = 0.174 µmoles ml^{-1}. Oxygen was removed from the chamber by adding sodium dithionite to allow calibration of the electrode.

Rate of oxygen uptake was measured in *Halomonas* sp. NAH1 cells grown in BM medium (Appendix A1) containing 1.0 M NaCl with glucose, glycerol, or betaine as a carbon and energy source at a concentration of 20 mM for each carbon source. Each culture (glucose, glycerol, or betaine) was grown until it reached mid exponential growth phase. 40 ml of the culture was centrifuged at 3000 *g* for 5 min in a bench centrifuge. The supernatant was discarded and bacterial pellet was washed twice with 20 ml each of the same growth medium but without carbon source (carbon-free medium) and then pellet was resuspended with 20 ml of an appropriate carbon-free medium.

A sample of the cells (2 ml in carbon-free medium) was placed in the electrode chamber and left for 3 min and then an appropriate carbon source (glucose, glycerol, or betaine) was added to the sample to give a final concentration of 20 mM and then the rate of oxygen uptake was measured for 3 min. Three treatments were made for each culture: one treatment with a carbon source on which the culture was grown and two treatments with the two other carbon sources. The total number of treatments for this experiment was nine, each of them was repeated three times. For each replicate, a culture sample of 1 ml was transferred into a 1.5 ml Eppendorf tube from which protein content was determined using the Bradford assay (Section 2.13.2).

Respiration rate was calculated using the following equation:

$$\text{Respiration rate} = \frac{\text{standard}}{\text{range}} \times \frac{\text{number of units}}{\text{time}} \times \frac{60}{\mu\text{g protein present in sample}}$$

Standard: oxygen solubility in 1.0 M NaCl medium at 30 °C = 0.174 μmoles ml^{-1} × 2 = 0.348 μmoles.

Range: units taken from calibration.

Number of units: number of units covered in 3 min.

Time: the time length in minutes for which the sample was measured (3 min).

60: this converts the time from minutes to hours.

μg protein present in sample: this relates to amount of protein in a sample of 2 ml.

After calculating respiration rate of all treatments, standard errors for three replicates for each treatment were also calculated.

2.16. Measurements of Uptake of ^{14}C-Glucose and ^{3}H-Glycerol

Halomonas sp. NAH1 was grown in BM medium (Appendix A1) containing 0.5 M NaCl and 20 mM glucose until they reached mid exponential growth phase and then they were concentrated five times in order to get a dense suspension of bacteria. D-[U-^{14}C] glucose (10 μl) (7.4 kBq μl^{-1}, Amersham Life Science) or 2 μl of [2-^{3}H]-glycerol (37 kBq μl^{-1}, Amersham Life Science) was added to 5 ml of the concentrated cells of *Halomonas* sp. NAH1 in a 15 ml centrifuge tube. Final radioisotope concentration of ^{14}C-glucose or ^{3}H-glycerol was the same at 14.8 kBq ml^{-1}.

Samples of 300 µl were added to 300 µl of silicone fluid (Dow Corning 550) in 1.5 ml Eppendorf tubes after 1, 5, 15, 30, and 60 min from the addition of either ^{14}C-glucose or ^{3}H-glycerol. Three replicates were made for each time interval. Samples were whirlimixed and then centrifuged for 10 min at 11,400 *g*. After centrifugation, components of each Eppendorf tube were as follows: medium (upper layer), silicone oil (lower layer), and bacterial pellet (bottom). 50 µl of the medium (the upper layer) was taken and added to a scintillation vial (Sarstedt, Germany) containing 5 ml of scintillation cocktail (FluoranSafe 2, BDH Scintran). The scintillation vial was mixed well by hand and then placed in a scintillation counter sample holder. The Eppendorf tube tip which contained the bacterial pellet was cut right in the middle of the silicone oil layer by using a sharp single edge blade. The tip was held by forceps and pressed against a tissue to get rid of as much silicone oil as possible and then the tip was put into an Eppendorf tube containing 300 µl of distilled water with the cut end of the tip face down. The Eppendorf tube was centrifuged briefly for 5 seconds to allow the bacterial pellet to pass into the water. The empty tip was discarded into solid radioactive waste. The bacterial pellet was resuspended fully in distilled water by whirlimixing and the mixture (300 µl) was taken and added to a scintillation vial containing 5 ml of scintillation fluid. The scintillation vial was mixed well by hand and then placed in a scintillation counter sample holder. The sample holder was placed in the scintillation counter and a ^{14}C or ^{3}H programme was used for counting.

The protein content (in 1 ml sample) (3 replicates) of the culture was determined by using Bradford assay (Section 2.13.2). ^{14}C-glucose and ^{3}H-glycerol uptake by *Halomonas* sp. NAH1 were calculated in pmoles mg^{-1} protein.

2.17. Extraction and Quantification of Phycobiliproteins

Extraction of phycobiliproteins was carried out as described by Silveria *et al.* (2007). *Arthrospira* and *Spirulina* strains were grown in batch cultures in *Spirulina* medium (Appendix A8) until they reached late exponential growth phase (OD_{560} = 1.5) and then harvested by filtration using Whatman GF/C filters 47 mm (diameter). The filters were pre-dried in an oven for 24 h or overnight at 40 °C and weighed prior to the filtration and then biomass was harvested by filtration and dried in an oven at 40 °C for 48 h. The filters were put in a desiccator for 20 min and weighed again to determine the biomass dry weight for both strains. The biomass was frozen at -18 °C overnight, detached from the filters, and 1 g of each biomass was weighed and put in a glass mortar and ground by pestle until a fine powder was produced. Each biomass sample was sieved using a sieve with perforation of 150 mesh and then 0.8 g of the sieved biomass was weighed and added to 10 ml of distilled water (containing 0.05 % (w/v) sodium azide) in a 100 ml conical flask to give a biomass – solvent ratio of 0.08 g ml^{-1}. The flask was incubated at 30 °C on a rotary shaker at 100 rpm for 24 h. Samples of 1 ml were collected at 0, 12, and 24 h of incubation. Three samples were taken at each time interval and they were put into 1.5 ml Eppendorf tubes (1 ml each) and then centrifuged at 5000 *g* for 15 min. Supernatants were transferred into 1 ml glass cuvettes and optical density was measured for each sample at 280, 562, 615, and 652 nm against distilled water (blank).

Phycobiliprotein content for each sample was quantified according to Abalde *et al.* (1998), Bennett and Bogorad (1973), Liu *et al.* (2005), and Silveira *et al.* (2007) as follows:

2.17.1. C-phycocyanin

$$\text{C-phycocyanin (PC) (mg ml}^{-1}) = \frac{OD615 - 0.474\,(OD652)}{5.34}$$

$$\text{Extract Purity of C-phycocyanin (EP – PC)} = \frac{OD615}{OD280}$$

OD_{615} indicates c-phycocyanin concentration, while OD_{280} indicates the total concentration of protein in the solution.

$$\text{Extraction Yield of C-phycocyanin (EY – PC) (mg phycocyanin g}^{-1}\text{ biomass)} = \frac{PC * V}{DB}$$

Where PC is the phycocyanin concentration (mg ml^{-1}), V is the volume of solvent (ml), and DB is dried biomass (g).

2.17.2. Allophycocyanin

$$\text{Allophycocyanin (APC) (mg ml}^{-1}) = \frac{OD652 - 0.208\,(OD615)}{5.09}$$

$$\text{Extract Purity of Allophycocyanin (EP – APC)} = \frac{OD652}{OD280}$$

OD_{652} indicates allophycocyanin concentration, while OD_{280} indicates the total concentration of protein in the solution.

Extraction Yield of Allophycocyanin (EY–APC) (mg allophycocyanin g^{-1} biomass) = $\frac{APC*V}{DB}$

Where APC is the allophycocyanin concentration (mg ml^{-1}), V is the volume of solvent (ml), and DB is dried biomass (g).

2.17.3. C-phycoerythrin

C-phycoerythrin (PE) (mg ml^{-1}) = $\frac{OD562 - 2.41\ (PC) - 0.849\ (APC)}{9.62}$

Extract Purity of C-phycoerythrin (EP – PE) = $\frac{OD562}{OD280}$

OD_{562} indicates c-phycoerythrin concentration, while OD_{280} indicates the total concentration of protein in the solution.

Extraction Yield of C-phycoerythrin (EY – PE) (mg c-phycoerythrin g^{-1} biomass) = $\frac{PE*V}{DB}$

Where PE is the c-phycoerythrin concentration (mg ml^{-1}), V is the volume of solvent (ml), and DB is dried biomass (g).

2.18. NMR Analysis of Compatible Solutes

A 5 ml sample of *Arthrospira* or *Spirulina* cells was put into a 15 ml centrifuge tube and centrifuged at 3000 *g* for 10 min and the supernatant was discarded. The cyanobacterial pellet was kept in a freezer until the analysis was due.

The pellet was resuspended in 10 ml of 80% (v/v) ethanol and vortexed for 8-10 minutes at room temperature. The mixture was allowed to stand for 72 hours at 90 °C in a hot block until dryness. The dried sample was dissolved in 2 ml Milli-Q water and it was allowed to stand for 3 nights with good whirlimixing twice a day to dissolve as much from the dried sample as possible. The sample was then centrifuged twice. First, at 3000 *g* for 10 minutes to precipitate the bulk of undissolved sample and then the supernatant were transferred into two 1.5 ml Eppendorf tubes. Second, the tubes were centrifuged at 11,000 *g* for 10 minutes to precipitate fine particles. Supernatants were combined together in a new tube and kept in a fridge until the analysis was due.

^{13}C NMR spectra were obtained from 0.5 ml aliquots of the concentrated cell suspension to which 50 µl of D_2O was added. NMR spectra were measured on a Bruker AMX-500 spectrometer operating at 125.8 MHz. Spectra were obtained at 10 °C and were collected into 16 K real points using a spectral width of 33,000 Hz (Cummings, 1991).

2.19. Sensitivity of *Halomonas* sp. NAH1 to Antibiotics

The sensitivity of *Halomonas* sp. NAH1 to four antibiotics was tested using standard disk method. Four antimicrobial susceptibility test disks were obtained from Oxoid, England: streptomycin – 25 µg, tetracycline – 50 µg, neomycin – 30 µg, and penicillin G – 10 units. Test was performed on 24 h old culture of *Halomonas* sp. NAH1 grown on BM medium (Appendix A1) containing 1.0 M NaCl and 20 mM glucose. 0.2 ml of the culture was spread on an agar plate of BMA medium (Appendix A2) containing 1.0 M NaCl and 20 mM glucose using a sterile glass spreader and then left for 10 min to dry. The four antimicrobial test disks were put on the bacterial film (one in each quarter of the plate). Three plates were used to perform the test and a plate without any antibiotics was used as a control. The plates were incubated (uninverted) for 4 days at 30 °C. Inhibition zones were measured by a ruler in mm and then the mean and standard error of the three inhibition zones for each antibiotic was calculated.

2.20. Biochemical Characteristics of *Halomonas* sp. NAH1

Biochemical characteristics of *Halomonas* sp. NAH1 were determined using API 20 NE identification system. The API 20 NE strips and other required reagents and materials were obtained from bioMérieux, Marcyl'Etoile, France.

API 20 NE is a standardized system for the identification of non-fastidious non-enteric Gram-negative rods, combining 8 conventional tests, 12 assimilation tests and a database. It consists of 20 microtubes containing dehydrated substrates. The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produces colour changes that are either spontaneous or are revealed by the addition of reagents. The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate. The reactions are read according to the Reading Table and the identification is obtained by referring to the identification software (bioMérieux).

Although *Halomonas* is not usually one of the organisms which can be identified by using this system, the kit was the best available to determine the biochemical characteristics of *Halomonas* sp. NAH1. The manufacturer's instructions were followed by incubating bacteria at 30 °C. Examination of the strips was conducted after 24 and 48 h of incubation. Results for each test were recorded as positive or negative and then a seven digit profile was ascribed. The profile was used to identify the bacteria according to the identification software (apiweb). *Pseudomonas aeruginosa* NCIMB 12469 was used as a control for the API 20 NE identification system.

2.21. DNA Extraction and Purification

DNA was extracted as described by Chen *et al.* (2001) and Syn & Swarup (2000) from either a culture (13-15 µg chlorophyll ml^{-1}) of *Dunaliella salina* CCAP 19/30 grown for 7 days at 25 °C in *Dunaliella* medium (Appendix A4) containing 1.5 M NaCl or a culture (OD_{600} = 1.2-1.6) of a bacterial isolate (*Halomonas* sp. NAH1) grown for 12 h at 30 °C in BM medium (Appendix A1) containing 0.5 M NaCl. 10 ml of the microalgal culture or 5 ml of the bacterial culture was centrifuged at 3000 *g* for 10 min. Supernatant was discarded and then the pellet was resuspended in 500 µl of CTAB buffer (Appendix C1) in a 1.5 ml Eppendorf tube. The mixture was thoroughly vortexed and then incubated at 65 °C for 1 h. DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and then centrifuged at 11,400 *g* for 10 min at room temperature. The aqueous layer was transferred into a fresh 1.5 ml Eppendorf tube and the DNA was precipitated with 2 volumes of 100% (v/v) cold ethanol and then pelleted by centrifugation at 11,400 *g* for 15 min at 4 °C. The DNA pellet was dried by air and then 500 µl of RNase A solution (10 µl of RNase [500 µg ml^{-1}, DNase free] in 500 µl elution buffer [Appendix C2]) was added to the pellet and incubated for 30 min at 37 °C. DNA was precipitated with 2 volumes of 100% cold ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and incubated at -18 °C for 30 min. DNA was pelleted by centrifugation at 11,400 *g* for 15 min at 4 °C. Supernatant was discarded and then the DNA pellet was washed with 1 ml of 70% cold ethanol and precipitated by centrifugation at 11,400 *g* for 15 min at 4 °C. Supernatant was discarded and the DNA pellet was dried by air. 400 µl of elution buffer (Appendix C2) was added to the DNA pellet and then the DNA was dissolved by placing the Eppendorf tube into a hot block at 50 °C for 1-2 h. Genomic DNA samples were purified with the DNeasy kit (Qiagen) according to the manufacturer's instructions.

2.22. Amplification of 16S and 18S rRNA genes

The 16S and 18S rRNA genes of *Halomonas* sp. NAH1 and *D. salina* 19/30 respectively were amplified by PCR using two sets of primers as described in Table 2.3.

Primer	Sequence (5' to 3')	Designed for:	Reference
fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	16S rRNA gene (forward)	(Weisburg *et al.*, 1991)
rP2	CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT	16S rRNA gene (reverse)	(Weisburg *et al.*, 1991)
NS1	GTAGTCATATGCTTGTCTC	18S rRNA gene (forward)	(Kong *et al.*, 2000)
18L	CACCTACGGAAACCTTGTTACGACTT	18S rRNA gene (reverse)	(Maddison *et al.*, 1999)

Table 2.3. Primers used to amplify 16S and 18S rRNA genes. The primers were synthesised by Dr Arthur Moir (Krebs Institute, Sheffield University) using DNA sequencing and synthesis facility.

The amplification reaction was carried out in a 50 µl reaction mixture containing 1 µl of a 1:10 dilution of extracted DNA, 5 µl of 10x PCR buffer (Invitrogen), 2.5 µl of 50 mM $MgSO_4$ (Invitrogen), 1 µl of 10 mM mixture of dNTPs (Invitrogen), 0.5 µl of *Taq* DNA Polymerase (5U μl^{-1}) (Invitrogen), 1 µl of each primer (80 ng μl^{-1}), and 38 µl of autoclaved MilliQ water.

PCR amplification was carried out on a MyCycler thermal cycler (Bio-Rad Laboratories, Inc., USA) using the following protocol: initial denaturation for 3

min at 94 °C followed by 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 60 °C (for 16S rRNA gene) or 50 °C (for 18S rRNA gene), and elongation at 72 °C for 2 min. A final elongation step at 72 °C for 5 min was included at the end of the protocol. A PCR product of approximately 1.5 or 1.8 kb was obtained for 16S or 18S rRNA gene respectively.

2.23. Purification and Cloning of PCR Products

The PCR products obtained were purified using a QIAquick PCR Purification Kit (Qiagen Ltd., UK) according to the manufacturer's instructions. The purified products were cloned into pCR 2.1 vectors with a TA Cloning Kit (Invitrogen) used according to the manufacturer's instructions. The vectors containing the PCR inserts were sent to Cogenics (Essex, UK) for DNA sequencing.

2.24. Phylogenetic analysis

For phylogenetic placement, 16S and 18S rRNA gene sequences were initially checked using Basic Local Alignment Search Tool (BLAST) available from the website of the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Nucleotide BLAST was used and it was optimised for highly similar sequences (megablast). Sequences that produced significant alignment (more than 98% identity) with the query sequence were downloaded from GenBank to the software MEGA version 4.0 (Tamura *et al.*, 2007). They were aligned using CLUSTALW in MEGA4 and then used to build a Neighbour-joining tree (Saitou & Nei, 1987) based on *p*-distance substitution model.

2.25. Determination of Specific Growth Rate (μ) and Generation Time (g)

Specific growth rate and generation time were determined according to Vonshak (1997b).

$$\mu = \frac{\ln x2 - \ln x1}{t2 - t1}$$

where x_1 and x_2 are biomass concentrations at time intervals t_1 and t_2.

$$g = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}$$

2.26. Statistics

Most of the experiments in this study were carried out in triplicate and error bars represent standard errors of the means. If no error bars are shown, they were smaller than the symbol used to represent the mean. For experiment carried out in duplicate, both values plus the average are shown.

Molecular Identification of Microorganisms Using 16S and 18S rRNA Gene Sequences

Chapter Three

3.1. Introduction

Bacteria in clinical microbiology laboratories are usually identified phenotypically by means of conventional techniques such as Gram staining, culturing, and biochemical characterisations. However, two major drawbacks have been reported for these methods. First, some microorganisms are non cultivable and therefore the conventional techniques cannot be used to identify such microorganisms. Second, some microorganisms have biochemical characteristics that do not fit into patterns of any known genus and species (Woo *et al.*, 2000).

Accurate identification of microorganisms is very important in both clinical microbiology and environmental microbiology. In clinical microbiology it is essential to identify correctly the microorganisms that are involved in a disease in order to gain better understanding of the disease initiation and progression and also to prescribe an effective antibiotic (Spratt, 2004). In environmental microbiology, accurate identification of microorganisms especially those that have the ability to produce valuable products is very useful to differentiate the hyper-producing strains of a valuable product from those that produce lesser amounts of the product or do not produce it at all.

The discovery of polymerase chain reaction (PCR) and automated sequencing (and associated database construction and searching software) revolutionised the detection and identification of microorganisms (Spratt, 2004). It was shown that 16S rRNA gene is highly conserved within a bacterial species and among species of the same genus. Therefore, this gene can be used for identifying prokaryotes (Woo *et al.*, 2000). Similarly, 18S rRNA gene is highly conserved within a species and among species of the same genus and can be used for identifying eukaryotes. Employing these genes in the identification of microorganisms has many advantages over using the conventional techniques. First, 16S and 18S rRNA gene are present in prokaryotes and eukaryotes

respectively and perform the same function. Second, their sequences are highly conserved, but contain regions of conserved, variable and hypervariable sequences. Third, their sizes (ca. 1500 bases for 16S rRNA gene and 1800 bases for 18S rRNA gene) are relatively easy to sequence but large enough to contain sufficient information for identification and phylogenetic analyses of organisms. Finally, the technique is straightforward and could be learned easily and the identification could be made within two weeks on a part time basis compared with about few months by an experienced microbiologist using conventional techniques of identification (Spratt, 2004).

In this chapter a bacterial isolate was identified to the genus level by 16S rRNA gene sequencing. The 16S bacterial rRNA gene was amplified by the polymerase chain reaction (PCR) and sequenced. The sequence of the PCR product (≈ 1.45 kb) was compared with known 16S rRNA gene sequences in the GenBank database by multiple sequence alignment as described in Section 2.24. Similarly, the 18S microalgal rRNA gene of the strain *Dunaliella salina* CCAP 19/30 was treated as above and the taxonomic placement of the alga was tested. Phylogenetic trees were constructed for both microorganisms to predict their genetic relatedness.

Sequences of 16S and 18S rRNA genes obtained in this project were deposited in GenBank under accession numbers EU239362 and EU239363 respectively.

3.2. Results and Discussion

3.2.1. 16S rRNA Gene Sequence of a Bacterial Isolate

The 16S rRNA gene sequence of the bacterial isolate was aligned with the highly similar sequences available from GenBank by using BLAST. All hits (100) belonged to the genus *Halomonas* indicating that the isolate belongs to this genus. In order to determine the species name, it was necessary to find out the most highly similar species to the isolate among the BLAST results and to determine how close they are to the isolate based on the 16S rRNA gene sequences. It was observed that only 15 out of 100 hits were assigned species names. The identity between the isolate and the 15 species of *Halomonas* was in the range of 99.3-98.3% (Table 3.1). This result indicates that it is not possible to assign a species name of the isolate based only on the comparison of 16S rRNA gene sequences and further molecular analyses are needed for that purpose. The isolate was called initially *Halomonas* sp. NAH1.

The 16S rRNA gene sequences of both *H.* sp. NAH1 and the 15 closely related species were treated as in Section 2.24 to construct a neighbour joining tree which could determine the phylogenetic placement of the strain. The tree (Figure 3.1) shows that the isolate did fall in a group of *Halomonas* species which comprises *H.* sp. NAH1 as well as two other species (*H. frigidi* DD 39, *H. variabilis* SW48, and *H. variabilis* SW32). However, *H.* sp. NAH1 formed a robust cluster including only *H.* sp. NAH1 and therefore this differentiates it from other species/strains in the same group. Figure 3.2 shows that there was only 10 bases difference between the sequences of 16S rRNA genes of *H.* sp. NAH1 and the strain *H. variabilis* SW48, but 12 bases difference between *H.* sp. NAH1 and both *H. taeheungii* and *H. alkantarctica* over 1383 bases of the sequences. These results suggest that *H.* sp. NAH1 could be a strain of the species *H. variabilis*. However, there is a possibility that the isolate could be a new species. Further analyses are needed to confirm that.

Query* (Accession Number)	Sequence Length (bp)	Match Species/ Strain	GenBank Accession Number	Sequence Length (bp)	Shared Base Pairs	Identity (%)
EU239362	1444	*Halomonas taeheungii*	AB354933	1511	1418/1428	99.30
		Halomonas alkantarctica CRSS	AJ564880	1518	1417/1427	99.30
		Halomonas variabilis SW48	HVU85873	1445	1375/1385	99.28
		Halomonas variabilis ANT-3b	AY616755	1495	1417/1428	99.23
		Halomonas neptunia Eplume1	AF212202	1454	1393/1404	99.21
		Halomonas variabilis SW32	HVU85872	1444	1371/1382	99.20
		Halomonas frigidi DD 39	AJ431369	1488	1416/1428	99.16
		Halomonas variabilis ANT9112	AY167282	1483	1415/1427	99.16
		Halomonas variabilis BSi20336	DQ520887	1411	1369/1381	99.13
		Halomonas variabilis GSP28	AY505526	1498	1400/1413	99.08
		Halomonas boliviensis LC1	AY245449	1441	1380/1394	98.99
		Halomonas boliviensis LC2	AY245450	1434	1377/1391	98.99
		Halomonas variabilis SW04	HVU85871	1446	1367/1381	98.98
		Halomonas variabilis DSM 3051	AJ306893	1528	1411/1426	98.95
		Halomonas variabilis HTG7	AY204638	1497	1406/1430	98.32

Table 3.1. Similarity between 16S rRNA gene sequences of the bacterial isolate* and 15 closely related species / strains based on BLASTN (see Section 2.24).

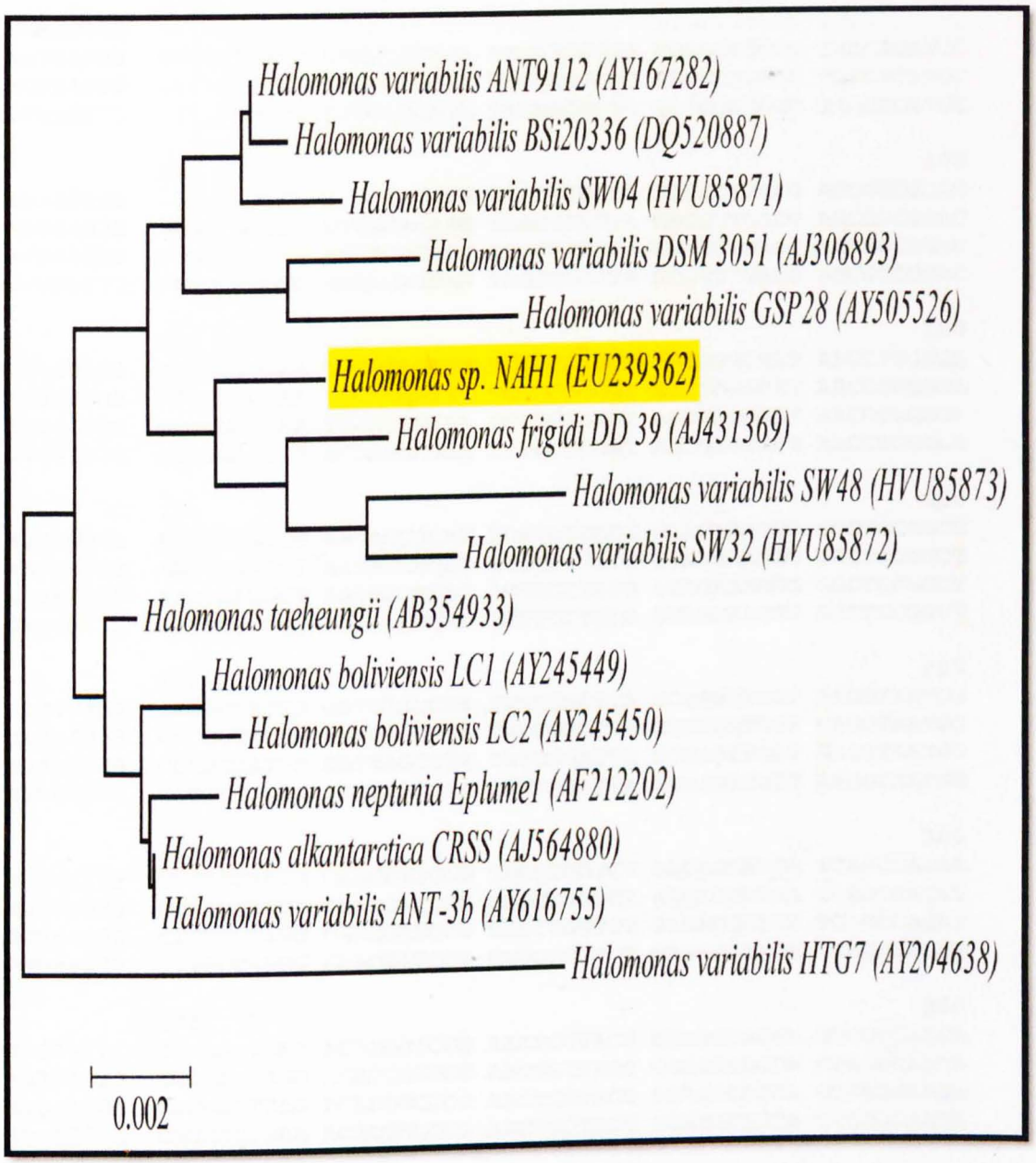

Figure 3.1. Neighbour-joining tree of *Halomonas* strains based on 16S rRNA gene sequences. The tree was built as described in Section 2.24. *Halomonas* sp. NAH1 is highlighted in yellow and GenBank accession numbers for all sequences are indicated after species or strain names. The bar below the tree indicates the number of nucleotide substitutions per site.

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            1                                                   50
EU239362    .......... .......... .......... .......... ..........
AB354933    GAGTTTGATC CTGGCTCAGA TTGAACGCTG GCGGCAGGCC TAACACATGC
AJ564880    ......GATC CTGGCTCAGA TTGAACGCTG GCGGCAGGCC TAACACATGC
HVU85873    .....TGATC CTGGCTCAGA TTGAACGCTG GCGGCAGGCC TAACACATGC

            51                                                  100
EU239362    .......... .......GGG TAGCTTGCTA CCCGCTGACG AGCGGCGGAC
AB354933    AAGTCGAGCG GTAACAGATG TAGCTTGCTA GACGCTGACG AGCGGCGGAC
AJ564880    AAGTCGAGCG GTAACAGATC TAGCTTGCTA GATGCTGACG AGCGGCGGAC
HVU85873    AAGTCGAGNG GNAACAGGGG TAGCTTGCTA CCCGCTGACG AGCGGCGGAC

            101                                                 150
EU239362    GGGTGAGTAA TGCATAGGAA TCTGCCCGGT AGTGGGGGAT AACCTGGGGA
AB354933    GGGTGAGTAA TGCATAGGAA TCTGCCCGGT AGTGGGGGAT AACCTGGGGA
AJ564880    GGGTGAGTAA TGCATAGGAA TCTGCCCGGT AGTGGGGGAT AACCTGGGGA
HVU85873    GGGTGAGTAA TGCATAGGAA TCTGCCCGAT AGTGGGGGAT AACCTGGGGA

            151                                                 200
EU239362    AACCCAGGCT AATACCGCAT ACGTCCTACG GGAGAAAGGG GGCTCCGGCC
AB354933    AACCCAGGCT AATACCGCAT ACGTCCTACG GGAGAAAGGG GGCTCCGGCT
AJ564880    AACCCAGGCT AATACCGCAT ACGTCCTACG GGAGAAAGGG GGCTCCGGCT
HVU85873    AACCCAGGCT AATACCGCAT ACGTCCTACG GGAGAAAGGG GGCTCCGGCT

            201                                                 250
EU239362    CCCGCTATTG GATGAGCCTA TGTCGGATTA GCTAGTTGGT AAGGTAATGG
AB354933    CCCGCTATTG GATGAGCCTA TGTCGGATTA GCTAGTTGGT GAGGTAATGG
AJ564880    CCCGCTATTG GATGAGCCTA TGTCGGATTA GCTAGTTGGT GAGGTAATGG
HVU85873    CCCGCTATTG GATGAGCCTA TGTCGGATTA GCTAGTTGGT AAGGTAATGG

            251                                                 300
EU239362    CTTACCAAGG CAACGATCCG TAGCTGGTCT GAGAGGATGA TCAGCCACAT
AB354933    CTCACCAAGG CAACGATCCG TAGCTGGTCT GAGAGGATGA TCAGCCACAT
AJ564880    CTCACCAAGG CAACGATCCG TAGCTGGTCT GAGAGGATGA TCAGCCACAT
HVU85873    CTTACCAAGG CAACGATCCG TAGCTGGTCT GAGAGGATGA TCAGCCACAT

            301                                                 350
EU239362    CGGGACTGAG ACACGGCCCG AACTCCTACG GGAGGCAGCA GTGGGGAATA
AB354933    CGGGACTGAG ACACGGCCCG AACTCCTACG GGAGGCAGCA GTGGGGAATA
AJ564880    CGGGACTGAG ACACGGCCCG AACTCCTACG GGAGGCAGCA GTGGGGAATA
HVU85873    CGGGACTGAG ACACGGCCCG AACTCCTACG GGAGGCAGCA GTGGGGAATA

            351                                                 400
EU239362    TTGGACAATG GGGGCAACCC TGATCCAGCC ATGCCGCGTG TGTGAAGAAG
AB354933    TTGGACAATG GGGGGAACCC TGATCCAGCC ATGCCGCGTG TGTGAAGAAG
AJ564880    TTGGACAATG GGGGCAACCC TGATCCAGCC ATGCCGCGTG TGTGAAGAAG
HVU85873    TTGGACAATG GGGGCAACCC TGATCCAGCC ATGCCGCGTG TGTGAAGAAG

            401                                                 450
EU239362    GCCCTCGGGT TGTAAAGCAC TTTCAGCGAG GAAGAACGCC TATCGGTTAA
AB354933    GCCCTCGGGT TGTAAAGCAC TTTCAGCGAG GAAGAACGCC TAGTGGTTAA
AJ564880    GCCCTCGGGT TGTAAAGCAC TTTCAGCGAG GAAGAACGCC TAGTGGTTAA
HVU85873    GCCCTCGGGT TGTAAAGCAC TTTCAGCGAG GAAGAACGCC TATCGGTTAA
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            451                                                       500
EU239362    TACCCGGTAG GAAAGACATC ACTCGCAGAA GAAGCACCGG CTAACTCCGT
AB354933    TACCCATTAG GAAAGACATC ACTCGCAGAA GAAGCACCGG CTAACTCCGT
AJ564880    TACCCATTAG GAAAGACATC ACTCGCAGAA GAAGCACCGG CTAACTCCGT
HVU85873    TACCCGGTAG GAAAGACATC ACTCGCAGAA GAAGCACCGG CTAACTCCGT

            501                                                       550
EU239362    GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG
AB354933    GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG
AJ564880    GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG
HVU85873    GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGGATTACTG

            551                                                       600
EU239362    GGCGTAAAGC GCGCGTAGGT GGCTTGATAA GCCGGTTGTG AAAGCCCCGG
AB354933    GGCGTAAAGC GCGCGTAGGT GGCTTGATAA GCCGGTTGTG AAAGCCCCGG
AJ564880    GGCGTAAAGC GCGCGTAGGT GGCTTGATAA GCCGGTTGTG AAAGCCCCGG
HVU85873    GGCGTAAAGC GCGCGTAGGT GGCTTGATAA GCCGGTTGTG AAAGCCCCGG

            601                                                       650
EU239362    GCTCAACCTG GGAACGGCAT CCGGAACTGT CAGGCTAGAG TGCAGGAGAG
AB354933    GCTCAACCTG GGAACGGCAT CCGGAACTGT CAGGCTAGAG TGCAGGAGAG
AJ564880    GCTCAACCTG GGAACGGCAT CCGGAACTGT CAGGCTAGAG TGCAGGAGAG
HVU85873    GCTCAACCTG GGAACGGCAT CCGGAACTGT CAGGCTAGAG TGCAGGAGAG

            651                                                       700
EU239362    GAAGGTAGAA TTCCCGGTGT AGCGGTGAAA TGCGTAGAGA TCGGGAGGAA
AB354933    GAAGGTAGAA TTCCCGGTGT AGCGGTGAAA TGCGTAGAGA TCGGGAGGAA
AJ564880    GAAGGTAGAA TTCCCGGTGT AGCGGTGAAA TGCGTAGAGA TCGGGAGGAA
HVU85873    GAAGGTAGAA TTCCCGGTGT AGCGGTGAAA TGCGTAGAGA TCGGGAGGAA

            701                                                       750
EU239362    TACCAGTGGC GAAGGCGGCC TTCTGGACTG ACACTGACAC TGAGGTGCGA
AB354933    TACCAGTGGC GAAGGCGGCC TTCTGGACTG ACACTGACAC TGAGGTGCGA
AJ564880    TACCAGTGGC GAAGGCGGCC TTCTGGACTG ACACTGACAC TGAGGTGCGA
HVU85873    TACCAGTGGC GAAGGCGGCC TTCTGGACTG ACACTGACAC TGAGGTGCGA

            751                                                       800
EU239362    AAGCGTGGGT AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
AB354933    AAGCGTGGGT AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
AJ564880    AAGCGTGGGT AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
HVU85873    AAGCGTGGGT AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA

            801                                                       850
EU239362    CGATGTCGAC CAGCCGTTGG GTGCCTAGAG CACTTTGTGG CGAAGTTAAC
AB354933    CGATGTCGAC CAGCCGTTGG GTGCCTAGAG CACTTTGTGG CGAAGTTAAC
AJ564880    CGATGTCGAC CAGCCGTTGG GTGCCTAGAG CACTTTGTGG CGAAGTTAAC
HVU85873    CGATGTCGAC CAGCCGTTGG GTGCCTAGAG CACTTTGTGG CGAATTTAAC

            851                                                       900
EU239362    GCGATAAGTC GACCGCCTGG GGAGTACGGC CGCAAGGTTA AAACTCAAAT
AB354933    GCGATAAGTC GACCGCCTGG GGAGTACGGC CGCAAGGTTA AAACTCAAAT
AJ564880    GCGATAAGTC GACCGCCTGG GGAGTACGGC CGCAAGGTTA AAACTCAAAT
HVU85873    GCGATAAGTC GACCGCCTGG GGAGTACGGC CGCAAGGTTA AAACTCAAAT
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            901                                                 950
EU239362    GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGATG
AB354933    GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGATG
AJ564880    GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGATG
HVU85873    GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGATG

            951                                                1000
EU239362    CAACGCGAAG AACCTTACCT ACCCTTGACA TCTACAGAAG CCGGAAGAGA
AB354933    CAACGCGAAG AACCTTACCT ACCCTTGACA TCTACAGAAG CCGGAAGAGA
AJ564880    CAACGCGAAG AACCTTACCT ACCCTTGACA TCTACAGAAG CCGGAAGAGA
HVU85873    CAACGCGAAG AACCTTACCT ACCCTTGACA TCTACAGAAG CCGGAAGAGA

            1001                                               1050
EU239362    TTCTGGTGTG CCTTCGGGAA CTGTAAGACA GGTGCTGCAT GGCTGTCGTC
AB354933    TTCTGGTGTG CCTTCGGGAA CTGTAAGACA GGTGCTGCAT GGCTGTCGTC
AJ564880    TTCTGGTGTG CCTTCGGGAA CTGTAAGACA GGTGCTGCAT GGCTGTCGTC
HVU85873    TTCTGGTGTG CCTTCGGGAA CTGTAAGACA GGTGCTGCAT GGCTGTCGTC

            1051                                               1100
EU239362    AGCTCGTGTT GTGAAATGTT GGGTTAAGTC CCGTAACGAG CGCAACCCTT
AB354933    AGCTCGTGTT GTGAAATGTT GGGTTAAGTC CCGTAACGAG CGCAACCCTT
AJ564880    AGCTCGTGTT GTGAAATGTT GGGTTAAGTC CCGTAACGAG CGCAACCCTT
HVU85873    AGCTCGTGTT GTGAAATGTT GGGTTAAGTC CCGTAACGAG CGCAACCCTT

            1101                                               1150
EU239362    GTCCTTATTT GCCAGCGAGT AATGTCGGGA ACTCTAAGGA GACTGCCGGT
AB354933    GTCCTTATTT GCCAGCGAGT AATGTCGGGA ACTCTAAGGA GACTGCCGGT
AJ564880    GTCCTTATTT GCCAGCGAGT AATGTCGGGA ACTCTAAGGA GACTGCCGGT
HVU85873    GTCCTTATTT GCCAGCACGT AATGGTGGGA ACTCTAAGGA GACTGCCGGT

            1151                                               1200
EU239362    GACAAACCGG AGGAAGGTGG GGACGACGTC AAGTCATCAT GGCCCTTACG
AB354933    GACAAACCGG AGGAAGGTGG GGACGACGTC AAGTCATCAT GGCCCTTACG
AJ564880    GACAAACCGG AGGAAGGTGG GGACGACGTC AAGTCATCAT GGCCCTTACG
HVU85873    GACAAACCGG AGGAAGGTGG GGACGACGTC AAGTCATCAT GGCCCTTACG

            1201                                               1250
EU239362    GGTAGGGCTA CACACGTGCT ACAATGGCCG GTACAAAGGG CTGCGAGCTC
AB354933    GGTAGGGCTA CACACGTGCT ACAATGGCCG GTACAAAGGG CTGCGAGCTC
AJ564880    GGTAGGGCTA CACACGTGCT ACAATGGCCG GTACAAAGGG CTGCGAGCTC
HVU85873    GGTAGGGCTA CACACGTGCT ACAATGGCCG GTACAAAGGG CTGCGAGCTC

            1251                                               1300
EU239362    GCGAGAGTCA GCGAATCCCT TAAAGCCGGT CTCAGTCCGG ATCGGAGTCT
AB354933    GCGAGAGTCA GCGAATCCCT TAAAGCCGGT CTCAGTCCGG ATCGGAGTCT
AJ564880    GCGAGAGTCA GCGAATCCCT TAAAGCCGGT CTCAGTCCGG ATCGGAGTCT
HVU85873    GCGAGAGTCA GCGAATCCCT TAAAGCCGGT CTCAGTCCGG ATCGGAGTCT

            1301                                               1350
EU239362    GCAACTCGAC TCCGTGAAGT CGGAATCGCT AGTAATCGTG AATCAGAATG
AB354933    GCAACTCGAC TCCGTGAAGT CGGAATCGCT AGTAATCGTG AATCAGAATG
AJ564880    GCAACTCGAC TCCGTGAAGT CGGAATCGCT AGTAATCGTG AATCAGAATG
HVU85873    GCAACTCGAC TCCGTGAAGT CGGAATCGCT AGTAATCGTG AATCAGAATG
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          1351                                                    1400
EU239362  TCACGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCATG
AB354933  TCACGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCATG
AJ564880  TCACGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCATG
HVU85873  TCACGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCATG

          1401                                                    1450
EU239362  GGAGTGGACT GCACCAGAAG TGGTTAGCCT AACGCAAGAG GGCGATCACC
AB354933  GGAGTGGACT GCACCAGAAG TGGTTAGCCT AACGCAAGAG GGCGATCACC
AJ564880  GGAGTGGACT GCACCAGAAG TGGTTAGCCT AACGCAAGAG GGCGATCACC
HVU85873  GGAGTGGACT GCACCAGAAG TGGTTAGCCT AACGCAAGAC GGCGCTCACC

          1451                                                    1500
EU239362  ACGGTGTGGT TCATGACTGG GGTGAAGTCG TAACAAGGTA GCCGTAAGCT
AB354933  ACGGTGTGGT TCATGACTGG GGTGAAGTCG TAACAAGGTA GCCGTAGGGG
AJ564880  ACGGTGTGGT TCATGACTGG GGTGAAGTCG TAACAAGGTA GCCGTAGG..
HVU85873  .......... .......... .......... .......... ..........

          1501                      1527
EU239362  TGGATCCCGG G......... .......
AB354933  AACCTGCGGC T......... .......
AJ564880  .GGAACCTGC GGCTGGATCA CCTCCTT
HVU85873  .......... .......... .......
```

Figure 3.2. Multiple sequence alignment of 16S rRNA gene sequences of *Halomonas* sp. NAH1 (GenBank Accession Number = EU239362, the query sequence), *H. taeheungii (AB354933), H. alkantarctica (AJ564880), and H. variabilis SW48 (HVU85873).* The yellow highlighted bases represent those in *H.* sp. NAH1 sequence that are different from the corresponding ones in the other strains of *Halomonas*.

3.2.2. 18S rRNA Gene Sequence of *Dunaliella salina* CCAP 19/30

The 18S rRNA gene sequence of *D. salina* 19/30 was treated as in Sections 2.24 and 3.2.1. A large number of species/strains of *Dunaliella* (27 database entries) were found to be very similar to the strain *D. salina* 19/30 with identity of 99.7-99.0% (Table 3.2). Although the first result of BLAST was the exact strain used in this project with similarity of 99.7%, the second result was *D. tertiolecta* with the same sequence length, shared base pairs, and identity (Table 3.2). This result suggests that 18S rRNA gene sequences can be used to define the genus name. Species name, however, is difficult to be certain of, especially when the sequences are very similar to each other and almost identical as it is the case with many strains of *Dunaliella*.

Figure 3.3 shows a neighbour-joining tree of *Dunaliella* strains based on 18S rRNA gene sequences. It shows the high similarity between *Dunaliella* species/strains and also shows that the strain *D. salina* 19/30 formed a distinguishable cluster among the major group of *Dunaliella* species. In addition, Figure 3.4 shows that there was only 4 bases difference between the 18S rRNA gene sequences of the strain *D. salina* 19/30 and the three other strains of *Dunaliella* (*D. salina* 19/30, *D. salina* 19/18, and *D. tertiolecta* 19/27) over 937 bases of the sequences. Moreover, the above three strains of *Dunaliella* were identical over 937 bases of 18S rRNA gene sequences.

These results indicate that the strain *D. salina* 19/30 more likely to be a strain of either the species *D. salina* or *D. tertiolecta*. Moreover, it suggests that the technique 18S rRNA gene sequencing is useful in revealing the taxonomic placement of unknown or doubtful strains as genus names but not at the species level, especially in some *Dunaliella* species that are known for taxonomic problems (see Section 1.4.1). Therefore, this justifies the use of other molecular techniques by some researchers to resolve the taxonomic

placement of *Dunaliella* strains. These techniques include RAPD (Random Amplified Polymorphic DNA) band patterns and nuclear ribosomal DNA internal transcribed spacer (ITS-1 and ITS-2) sequences (Coleman & Mai, 1997; Gomez & Gonzalez, 2004; Gonzalez *et al.*, 2001), RFLP (Restriction Fragment Length Polymorphism) analysis, and the utilisation of conserved and species-specific primers (González *et al.*, 1998; Olmos-Soto *et al.*, 2002; Olmos *et al.*, 2000) (see Section 1.4.1).

Query (Accession Number)	Sequence Length (bp)	Match Species/ Strain	GenBank Accession Number	Sequence Length (bp)	Shared Base Pairs	Identity (%)
EU239363	936	*Dunaliella salina* CCAP 19/30	EF473749	1750	931/934	99.68
		Dunaliella tertiolecta CCAP 19/27	EF473747	1750	931/934	99.68
		Dunaliella salina CCAP 19/18	EF473745	2151	931/934	99.68
		Dunaliella salina CCAP 19/3	EF473743	2128	931/934	99.68
		Dunaliella salina SAG 42.88	EF473740	1750	931/934	99.68
		Dunaliella salina SAG 19-3	EF473739	2128	931/934	99.68
		Dunaliella tertiolecta SAG 13.86	EF473737	1750	931/934	99.68
		Dunaliella salina Dsge	EF473731	1750	931/934	99.68
		Dunaliella tertiolecta Dtsi	EF473729	1750	931/934	99.68
		Dunaliella salina CCAP 19/30	DQ447648	2185	931/934	99.68
		Dunaliella salina UTEX LB 200	DQ009779	2065	931/934	99.68
		Dunaliella peircei UTEX LB 2192	DQ009778	2065	931/934	99.68
		Dunaliella bardawil UTEX LB 2538	DQ009777	2088	931/934	99.68
		Dunaliella tertiolecta UTEX LB 999	DQ009773	1687	931/934	99.68
		Dunaliella tertiolecta CCMP 364	DQ009772	1687	931/934	99.68
		Dunaliella tertiolecta CCMP 1302	DQ009771	1685	931/934	99.68
		Dunaliella primolecta UTEX LB 1000	DQ009764	1687	931/934	99.68
		Dunaliella parva SAG 19-1	DQ009763	DQ009763	931/934	99.68
		Dunaliella bioculata UTEX LB 199	DQ009761	1687	931/934	99.68
		Dunaliella tertiolecta CCMP 1320	EF537907	1698	930/934	99.57
		Dunaliella salina UTEX LB 1644	DQ009765	1687	930/934	99.57
		Dunaliella salina	DUNRDGAB	2182	930/934	99.57
		Dunaliella salina	EF195157	1780	929/934	99.46
		Dunaliella salina	AF506698	1787	929/934	99.46
		Dunaliella bardawil DB1	AF150905	2584	926/935	99.0
		Dunaliella viridis CONC002	DQ009776	2494	925/934	99.0
		Dunaliella parva	DUNRDEAA	2585	925/934	99.0

Table 3.2. Similarity between 18S rRNA gene sequences of the strain *Dunaliella salina* 19/30* and 15 closely related species / strains based on BLASTN (see Section 2.24).

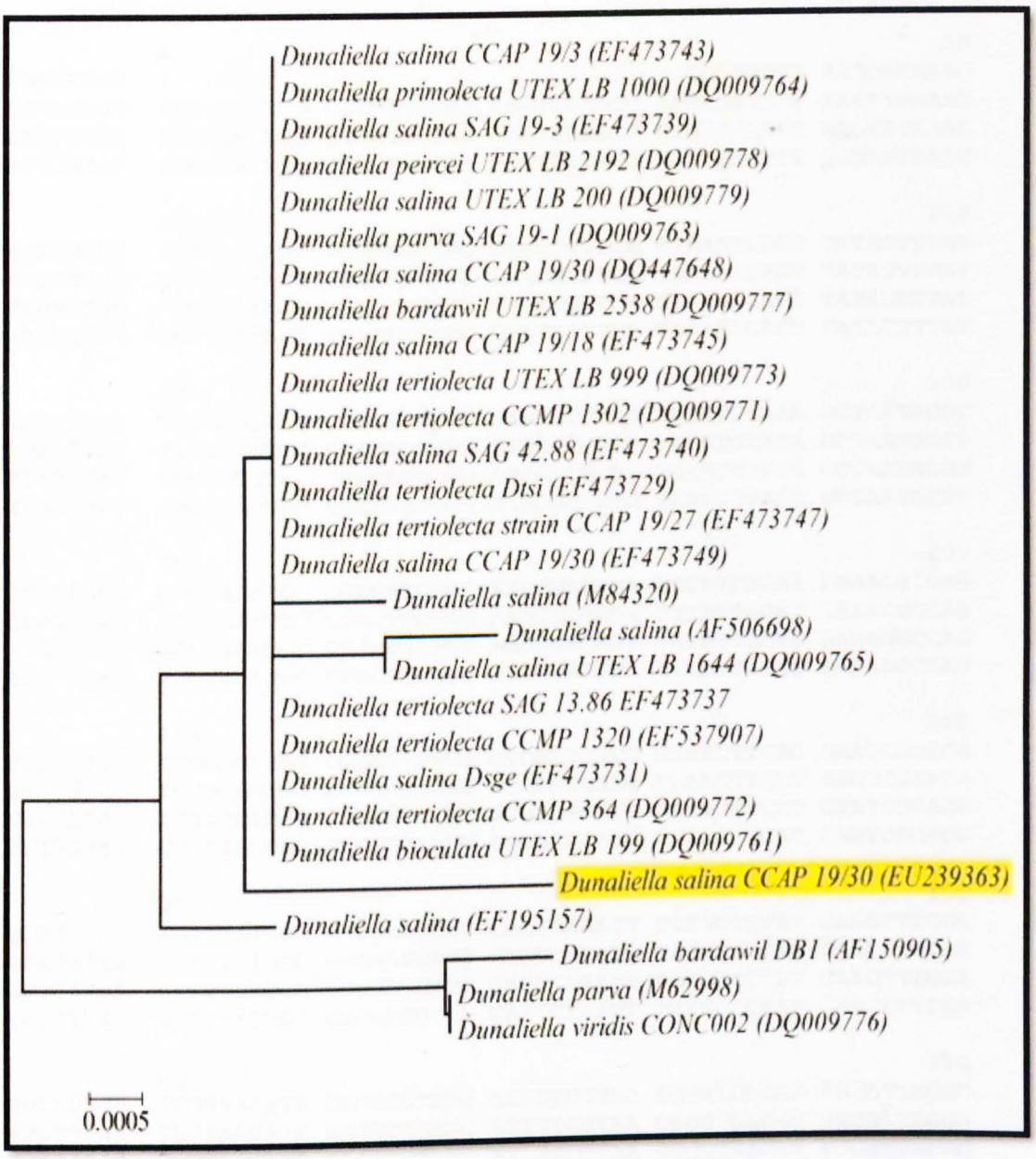

Figure 3.3. Neighbour-joining tree of *Dunaliella* strains based on 18S rRNA gene sequences. The tree was built as described in Section 2.24. *Dunaliella salina* CCAP 19/30 is highlighted in yellow and GenBank accession numbers for all sequences are indicated after species or strain names. The bar below the tree indicates the number of nucleotide substitutions per site.

```
           1                                                    50
EU239363   .......... .......... .......... ..GC.TGTCT AAGTATAAAC
EF473749   TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAC
EF473745   TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAC
EF473747   TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAC

           51                                                  100
EU239363   TGCTTATACT GTGAAACTGC GAATGGCTCA TTAAATCAGT TATAGTTTAT
EF473749   TGCTTATACT GTGAAACTGC GAATGGCTCA TTAAATCAGT TATAGTTTAT
EF473745   TGCTTATACT GTGAAACTGC GAATGGCTCA TTAAATCAGT TATAGTTTAT
EF473747   TGCTTATACT GTGAAACTGC GAATGGCTCA TTAAATCAGT TATAGTTTAT

           101                                                 150
EU239363   TTGATGGTAC CTTTACTCGG ATAACCGTAG TAATTCTAGA GCTAATACGT
EF473749   TTGATGGTAC CTTTACTCGG ATAACCGTAG TAATTCTAGA GCTAATACGT
EF473745   TTGATGGTAC CTTTACTCGG ATAACCGTAG TAATTCTAGA GCTAATACGT
EF473747   TTGATGGTAC CTTTACTCGG ATAACCGTAG TAATTCTAGA GCTAATACGT

           151                                                 200
EU239363   GCGTAAATCC CGACTTCTGG AAGGGACGTA TTTATTAGAT AAAAGGCCAG
EF473749   GCGTAAATCC CGACTTCTGG AAGGGACGTA TTTATTAGAT AAAAGGCCAG
EF473745   GCGTAAATCC CGACTTCTGG AAGGGACGTA TTTATTAGAT AAAAGGCCAG
EF473747   GCGTAAATCC CGACTTCTGG AAGGGACGTA TTTATTAGAT AAAAGGCCAG

           201                                                 250
EU239363   CCGGGCTTGC CCGACTCTTG GCGAATCATG ATAACTTCAC GAATCGCACG
EF473749   CCGGGCTTGC CCGACTCTTG GCGAATCATG ATAACTTCAC GAATCGCACG
EF473745   CCGGGCTTGC CCGACTCTTG GCGAATCATG ATAACTTCAC GAATCGCACG
EF473747   CCGGGCTTGC CCGACTCTTG GCGAATCATG ATAACTTCAC GAATCGCACG

           251                                                 300
EU239363   GCTTTATGCC GGCGATGTTT CATTCAAATT TCTGCCCTAT CAACTTTCGA
EF473749   GCTTTATGCC GGCGATGTTT CATTCAAATT TCTGCCCTAT CAACTTTCGA
EF473745   GCTTTATGCC GGCGATGTTT CATTCAAATT TCTGCCCTAT CAACTTTCGA
EF473747   GCTTTATGCC GGCGATGTTT CATTCAAATT TCTGCCCTAT CAACTTTCGA

           301                                                 350
EU239363   TGGTAGGATA GAGGCCTACC ATGGTGGTAA CGGGTGACGG AGGATTAGGG
EF473749   TGGTAGGATA GAGGCCTACC ATGGTGGTAA CGGGTGACGG AGGATTAGGG
EF473745   TGGTAGGATA GAGGCCTACC ATGGTGGTAA CGGGTGACGG AGGATTAGGG
EF473747   TGGTAGGATA GAGGCCTACC ATGGTGGTAA CGGGTGACGG AGGATTAGGG

           351                                                 400
EU239363   TTCGATTCCG GAGAGGGAGC CTGAGAAACG GCTACCACAT CCAAGGAAGG
EF473749   TTCGATTCCG GAGAGGGAGC CTGAGAAACG GCTACCACAT CCAAGGAAGG
EF473745   TTCGATTCCG GAGAGGGAGC CTGAGAAACG GCTACCACAT CCAAGGAAGG
EF473747   TTCGATTCCG GAGAGGGAGC CTGAGAAACG GCTACCACAT CCAAGGAAGG

           401                                                 450
EU239363   CAGCAGGCGC GCAAATTACC CAATCCCAAC ACGGGGAGGT AGTGACAATA
EF473749   CAGCAGGCGC GCAAATTACC CAATCCCAAC ACGGGGAGGT AGTGACAATA
EF473745   CAGCAGGCGC GCAAATTACC CAATCCCAAC ACGGGGAGGT AGTGACAATA
EF473747   CAGCAGGCGC GCAAATTACC CAATCCCAAC ACGGGGAGGT AGTGACAATA
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            451                                                      500
EU239363    AATAACAATA CCGGGCATTT TTGTCTGGTA ATTGGAATGA GTACAATCTA
EF473749    AATAACAATA CCGGGCATTT TTGTCTGGTA ATTGGAATGA GTACAATCTA
EF473745    AATAACAATA CCGGGCATTT TTGTCTGGTA ATTGGAATGA GTACAATCTA
EF473747    AATAACAATA CCGGGCATTT TTGTCTGGTA ATTGGAATGA GTACAATCTA

            501                                                      550
EU239363    AATCCCTTAA CGAGTATCCA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC
EF473749    AATCCCTTAA CGAGTATCCA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC
EF473745    AATCCCTTAA CGAGTATCCA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC
EF473747    AATCCCTTAA CGAGTATCCA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC

            551                                                      600
EU239363    GGTAATTCCA GCTCCAATAG CGTATATTTA AGTTGTTGCA GTTAAAAAGC
EF473749    GGTAATTCCA GCTCCAATAG CGTATATTTA AGTTGTTGCA GTTAAAAAGC
EF473745    GGTAATTCCA GCTCCAATAG CGTATATTTA AGTTGTTGCA GTTAAAAAGC
EF473747    GGTAATTCCA GCTCCAATAG CGTATATTTA AGTTGTTGCA GTTAAAAAGC

            601                                                      650
EU239363    TCGTAGTTGG ATTTCGGGTG GGTTGTAGCG GTCAGCCTTT GGTTAGTACT
EF473749    TCGTAGTTGG ATTTCGGGTG GGTTGTAGCG GTCAGCCTTT GGTTAGTACT
EF473745    TCGTAGTTGG ATTTCGGGTG GGTTGTAGCG GTCAGCCTTT GGTTAGTACT
EF473747    TCGTAGTTGG ATTTCGGGTG GGTTGTAGCG GTCAGCCTTT GGTTAGTACT

            651                                                      700
EU239363    GCTACGGCCT ACCTTTCTGC CGGGGACGAG CTCCTGGGCT TAACTGTCCG
EF473749    GCTACGGCCT ACCTTTCTGC CGGGGACGAG CTCCTGGGCT TAACTGTCCG
EF473745    GCTACGGCCT ACCTTTCTGC CGGGGACGAG CTCCTGGGCT TAACTGTCCG
EF473747    GCTACGGCCT ACCTTTCTGC CGGGGACGAG CTCCTGGGCT TAACTGTCCG

            701                                                      750
EU239363    GGACTCGGAA TCGGCGAGGT TACTTTGAGT AAATTAGAGT GTTCAAAGCA
EF473749    GGACTCGGAA TCGGCGAGGT TACTTTGAGT AAATTAGAGT GTTCAAAGCA
EF473745    GGACTCGGAA TCGGCGAGGT TACTTTGAGT AAATTAGAGT GTTCAAAGCA
EF473747    GGACTCGGAA TCGGCGAGGT TACTTTGAGT AAATTAGAGT GTTCAAAGCA

            751                                                      800
EU239363    AGCCTACGCT CTGAATACAT TAGCATGGAA TAACACGATA GGACTCTGGC
EF473749    AGCCTACGCT CTGAATACAT TAGCATGGAA TAACACGATA GGACTCTGGC
EF473745    AGCCTACGCT CTGAATACAT TAGCATGGAA TAACACGATA GGACTCTGGC
EF473747    AGCCTACGCT CTGAATACAT TAGCATGGAA TAACACGATA GGACTCTGGC

            801                                                      850
EU239363    TTATCTTGTT GGTCTGTAAG ACCGGAGTAA TGATTAAGAG GGACAGTCGG
EF473749    TTATCTTGTT GGTCTGTAAG ACCGGAGTAA TGATTAAGAG GGACAGTCGG
EF473745    TTATCTTGTT GGTCTGTAAG ACCGGAGTAA TGATTAAGAG GGACAGTCGG
EF473747    TTATCTTGTT GGTCTGTAAG ACCGGAGTAA TGATTAAGAG GGACAGTCGG

            851                                                      900
EU239363    GGGCATTCGT ATTTCATTGT CAGAGGTGAA ATTCTTGGAT TTATGAAAGA
EF473749    GGGCATTCGT ATTTCATTGT CAGAGGTGAA ATTCTTGGAT TTATGAAAGA
EF473745    GGGCATTCGT ATTTCATTGT CAGAGGTGAA ATTCTTGGAT TTATGAAAGA
EF473747    GGGCATTCGT ATTTCATTGT CAGAGGTGAA ATTCTTGGAT TTATGAAAGA
```

```
           901                                                        950
EU239363   CGAACTTCTG CGAAAGCATT TGCCAAGGAT GTTTTCATTA ACCCAAGAAC
EF473749   CGAACTTCTG CGAAAGCATT TGCCAAGGAT GTTTTCATTA ATC.AAGAAC
EF473745   CGAACTTCTG CGAAAGCATT TGCCAAGGAT GTTTTCATTA ATC.AAGAAC
EF473747   CGAACTTCTG CGAAAGCATT TGCCAAGGAT GTTTTCATTA ATC.AAGAAC

           951                                                       1000
EU239363   GAAAGTTTGG GGGCTCGAA. .......... .......... ..........
EF473749   GAAAGTT.GG GGGCTCGAAG ACGATTAGAT ACCGTCGTAG TCTCAACCAT
EF473745   GAAAGTT.GG GGGCTCGAAG ACGATTAGAT ACCGTCGTAG TCTCAACCAT
EF473747   GAAAGTT.GG GGGCTCGAAG ACGATTAGAT ACCGTCGTAG TCTCAACCAT
```

Figure 3.4. Multiple sequence alignment of 18S rRNA gene sequences of *Dunaliella salina* CCAP 19/30 (GenBank Accession Number = EU239363, the query sequence), *D. salina* CCAP 19/30 (EF473749), *D. salina* CCAP 19/18 (EF473745), and *D. tertiolecta* CCAP 19/27 (EF473747). The yellow highlighted bases represent those in the query sequence that are different from the corresponding ones in the other strains of *Dunaliella*.

3.3. Conclusions

The bacterial isolate (NAH1) was identified to the genus level by 16S rRNA gene sequencing. It belongs to *Halomonas* genus, but the species name could not be assigned due to the low level of diversity in 16S rRNA gene sequences of *Halomonas* species. Similarly, the strain *D. salina* 19/30 was identified to the genus level and only 4 bases difference between 18S rRNA gene sequences of the strain and the corresponding strain from the GenBank was observed. These results are in good agreement with the conclusion made by Fox *et al.*, (1992) who reported that effective identity (>99.5% identity) between 16S rRNA gene sequences is not necessarily a sufficient criterion to guarantee species identity.

Comparing 16S/18S rRNA gene sequences is a good molecular technique to distinguish and establish relationships between genera and well-resolved species. However, further phenotypic and phylogenetic analyses are needed when working with species that are very similar to each other. Therefore, combining molecular and conventional techniques in the identification of microorganisms seems to be a good solution for obtaining accurate identification for important strains.

Glycerol Production by *Dunaliella*

Chapter Four

4.1. Introduction

Unicellular green microalgae belonging to the genus *Dunaliella* (Dunaliellales, Chlorophyceae) are used for the commercial production of β-carotene (Curtain, 2000). A viable industry has been built in many parts of the world including Australia, USA, China, Japan, and India (Dufosse *et al.*, 2005). It is also known that *Dunaliella* species synthesise glycerol in response to increasing external salinity. In the 1970's, glycerol was thought to be the main product that could be commercially produced using *Dunaliella* cells (Ben-Amotz, 1981). However, the low cost of petroleum-derived glycerol coupled with the expense of harvesting glycerol meant that glycerol production from *Dunaliella* has not become an established commercial process (Gilmour, 1990).

Nevertheless, the capacity for glycerol production by *Dunaliella* is impressive. As the salinity is increased from 0.1 to 3 M NaCl, the level of intracellular glycerol increases in a linear fashion (Ben-Amotz & Avron, 1973). At NaCl concentrations approaching saturation (about 5 M NaCl) about 80% of the dry weight of *Dunaliella* cells is made up by glycerol. The physiology of the glycerol cycle has been fully worked out (see Figure 1.9) and the enzymes involved and their cellular location identified (Ben-Amotz & Avron, 1990; Gimmler & Lotter, 1982). However, glycerol is not normally retained within cells due to the ease by which it passes through most cell membranes. Clearly, *Dunaliella* species and a few other microalgae and a number of salt tolerant yeasts must have altered their membrane structure to retain several molar glycerol within their cells when subjected to high salt stress. The full basis of glycerol retention is not understood, but it appears to involve modifications in membrane lipids (Azachi *et al.*, 2002; Borowitzka, 1999) with the presence of sterol peroxides in the membrane being of particular interest (Sheffer *et al.*, 1986; Zelazny *et al.*, 1995).

Hard and Gilmour (1991) reported that under laboratory batch culture conditions, a mutant of *Dunaliella parva* 19/9 leaked large amounts of glycerol into the medium. Despite this apparently detrimental loss of glycerol, no decrease in growth rate was observed, although oxygen evolution rates were elevated in the mutant at high external salinity levels.

In this chapter, further evidence that *Dunaliella* strains leak glycerol into the medium on a continuous basis is described. This property is associated with growth in batch and continuous cultures. It does not depend on a mutant strain, but it is an intrinsic property of at least several strains of *Dunaliella*. This opens up a number of possibilities to improve the harvesting of glycerol and also improve the likelihood of a commercially successful method for glycerol production by *Dunaliella*.

4.2. Results and Discussion

4.2.1. Growth of *Dunaliella* strains

4.2.1.1. Monitoring growth by determining chlorophyll content

Three strains of *Dunaliella* (*D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30) were adapted to grow in batch cultures in salinities from 0.1 to 4.0 M NaCl in *Dunaliella* medium (Appendix A4) as described in Section 2.3. The growth of each strain was monitored by determining chlorophyll content (see Section 2.7.1) over an incubation period of 14 days (Figures 4.1 – 4.3).

All the strains showed optimum biomass production at 0.4 M NaCl with chlorophyll content of 24.6, 17.6, and 19.7 μg ml^{-1} for *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively after 14 days of incubation (Figures 4.1 – 4.3). The growth rates at 0.1 and 1.5 M NaCl were in general quite similar to that obtained at 0.4 M NaCl in all the strains. However, increasing salinity above 1.5 M NaCl (2.5 – 4.0 M) decreased the growth significantly especially at 3.5 and 4.0 M NaCl. Chlorophyll contents at the highest salinity (4.0 M NaCl) were very low at 3.1, 2.6, and 4.0 μg ml^{-1} for *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively after 14 days of incubation (Figures 4.1 – 4.3).

These results indicate that all the strains are halotolerant since they grew optimally at 0.4 M NaCl. In general, the higher the salinity the lower the growth in all the strains. The most halotolerant strain was *D. salina* 19/30 since its chlorophyll content at 4.0 M NaCl was the highest among the strains tested.

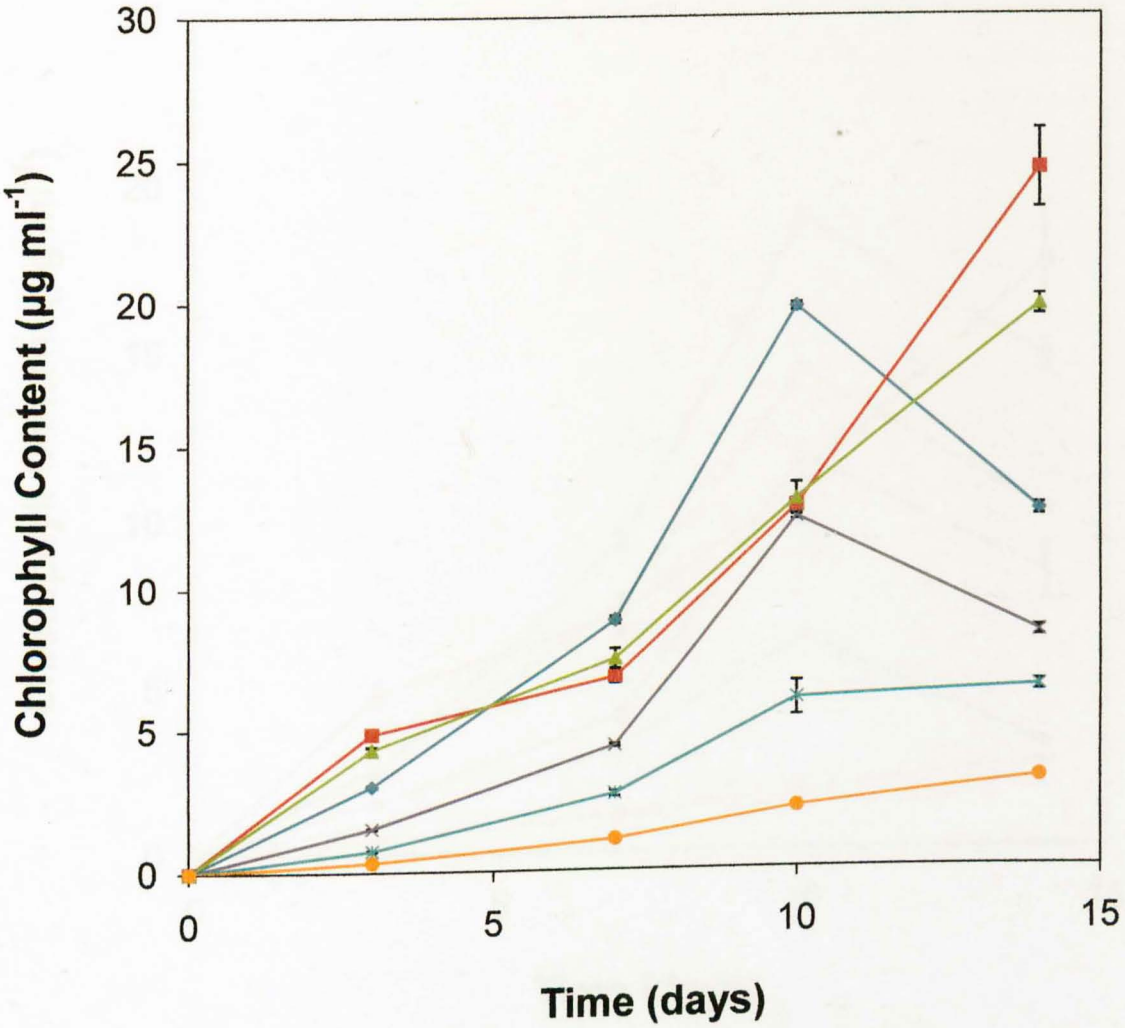

Figure 4.1. Growth of *Dunaliella parva* 19/9 in batch cultures at different salinities. The growth was monitored by determining the chlorophyll content. Each point represents the mean of four determinations plus or minus standard error.

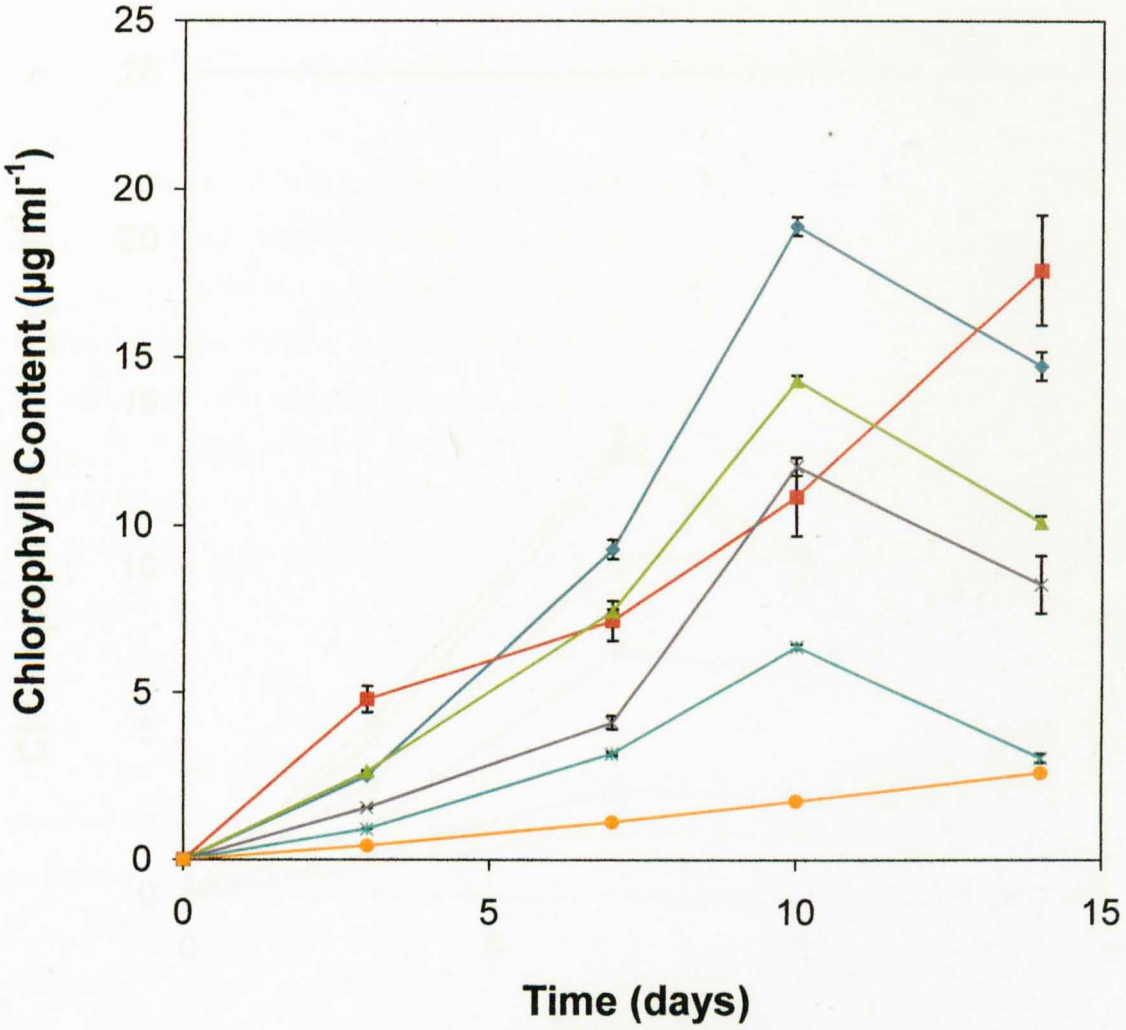

Figure 4.2. Growth of *Dunaliella parva* 19/10 in batch cultures at different salinities. The growth was monitored by determining the chlorophyll content. Each point represents the mean of four determinations plus or minus standard error.

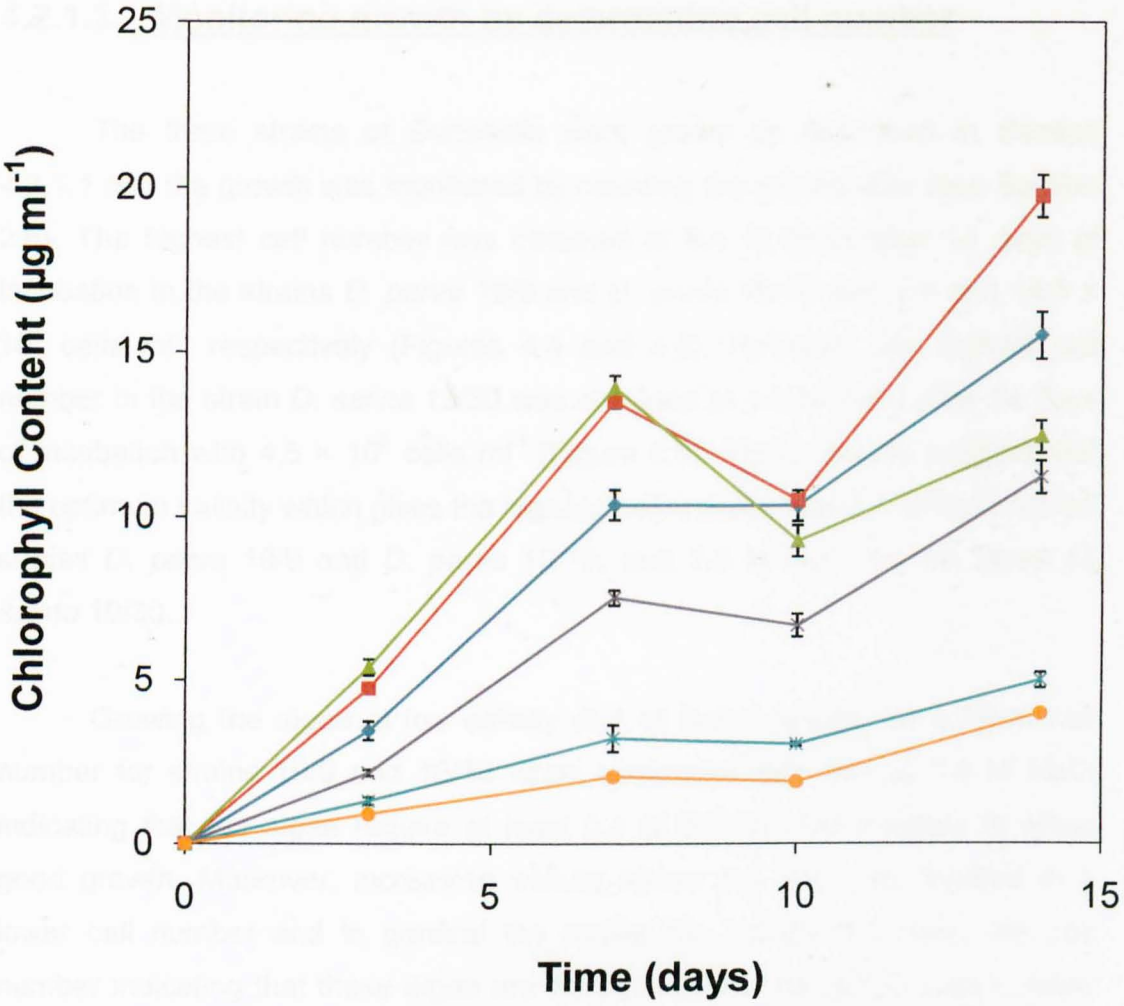

Figure 4.3. Growth of *Dunaliella salina* 19/30 in batch cultures at different salinities. The growth was monitored by determining the chlorophyll content. Each point represents the mean of four determinations plus or minus standard error.

4.2.1.2. Monitoring growth by determining cell number

The three strains of *Dunaliella* were grown as described in Section 4.2.1.1 and the growth was monitored by counting the cell number (see Section 2.6). The highest cell number was obtained at 0.4 M NaCl after 14 days of incubation in the strains *D. parva* 19/9 and *D. parva* 19/10 with 8.4 and 14.3 × 10^6 cells ml^{-1} respectively (Figures 4.4 and 4.5). However, the highest cell number in the strain *D. salina* 19/30 was obtained at 1.5 M NaCl after 14 days of incubation with 4.5 × 10^6 cells ml^{-1} (Figure 4.6). These results suggest that the optimum salinity which gives the highest cell number was 0.4 M NaCl for the strains *D. parva* 19/9 and *D. parva* 19/10, and 1.5 M NaCl for the strain *D. salina* 19/30.

Growing the algae at low salinity (0.1 M NaCl) resulted in a lower cell number for strains 19/9 and 19/10 when compared with that at 0.4 M NaCl indicating that the algae require at least 0.4 M NaCl in the medium to attain good growth. Moreover, increasing salinity above the optimum resulted in a lower cell number and in general the higher the salinity the lower the cell number indicating that these algae are halotolerant not halophilic. Cell number at the highest salinity (4.0 M NaCl) was low with 0.79, 0.76, and 1.12 × 10^6 cells ml^{-1} for the strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively after 14 days of incubation.

It can be concluded that similar growth patterns were in general obtained from both methods used to estimate the growth (chlorophyll determination in Section 4.2.1.1 and cell number in this Section).

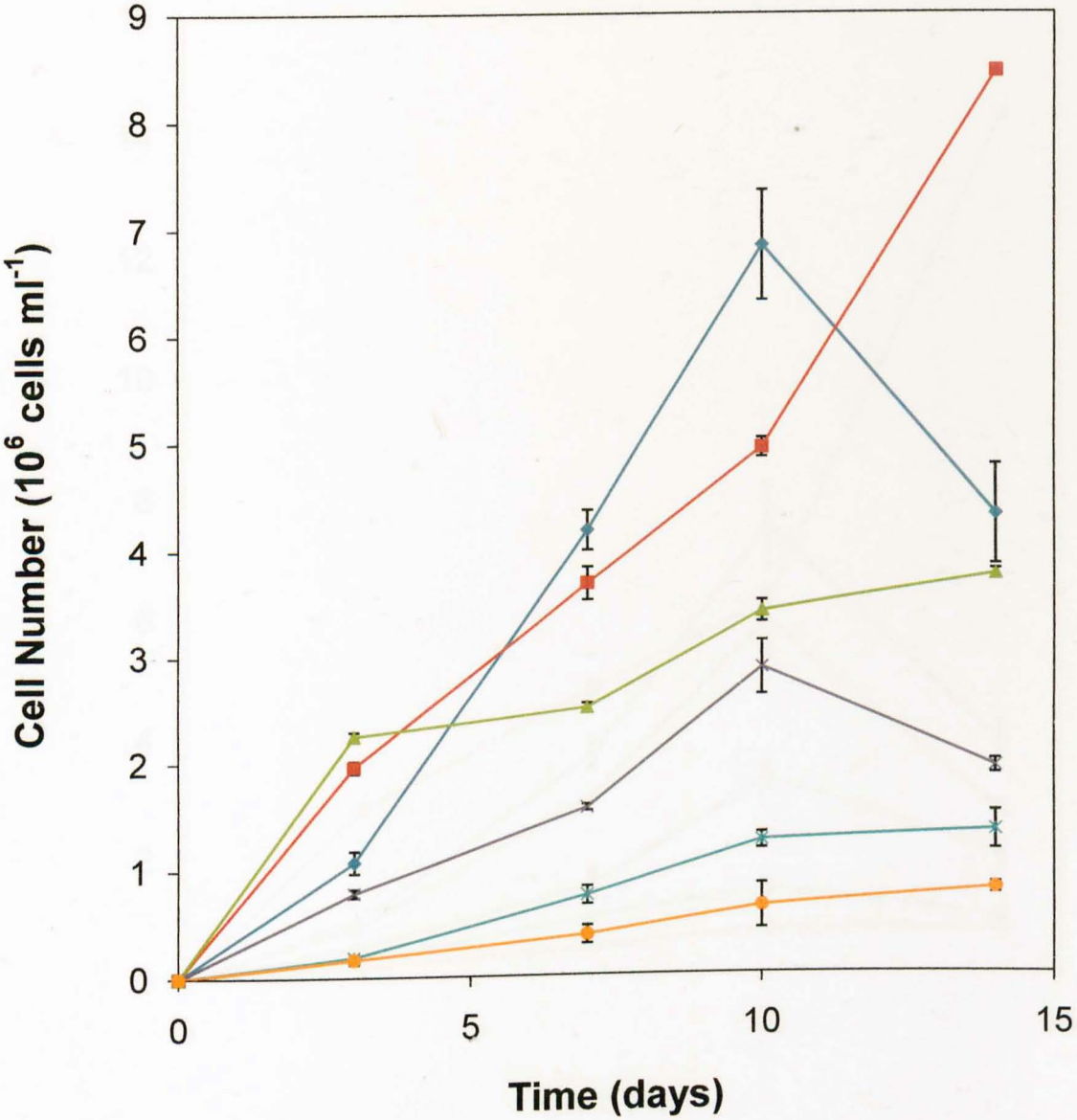

Figure 4.4. Growth of *Dunaliella parva* 19/9 in batch cultures at different salinities. The growth was monitored by counting the cell number. Each point represents the mean of ten determinations plus or minus standard error.

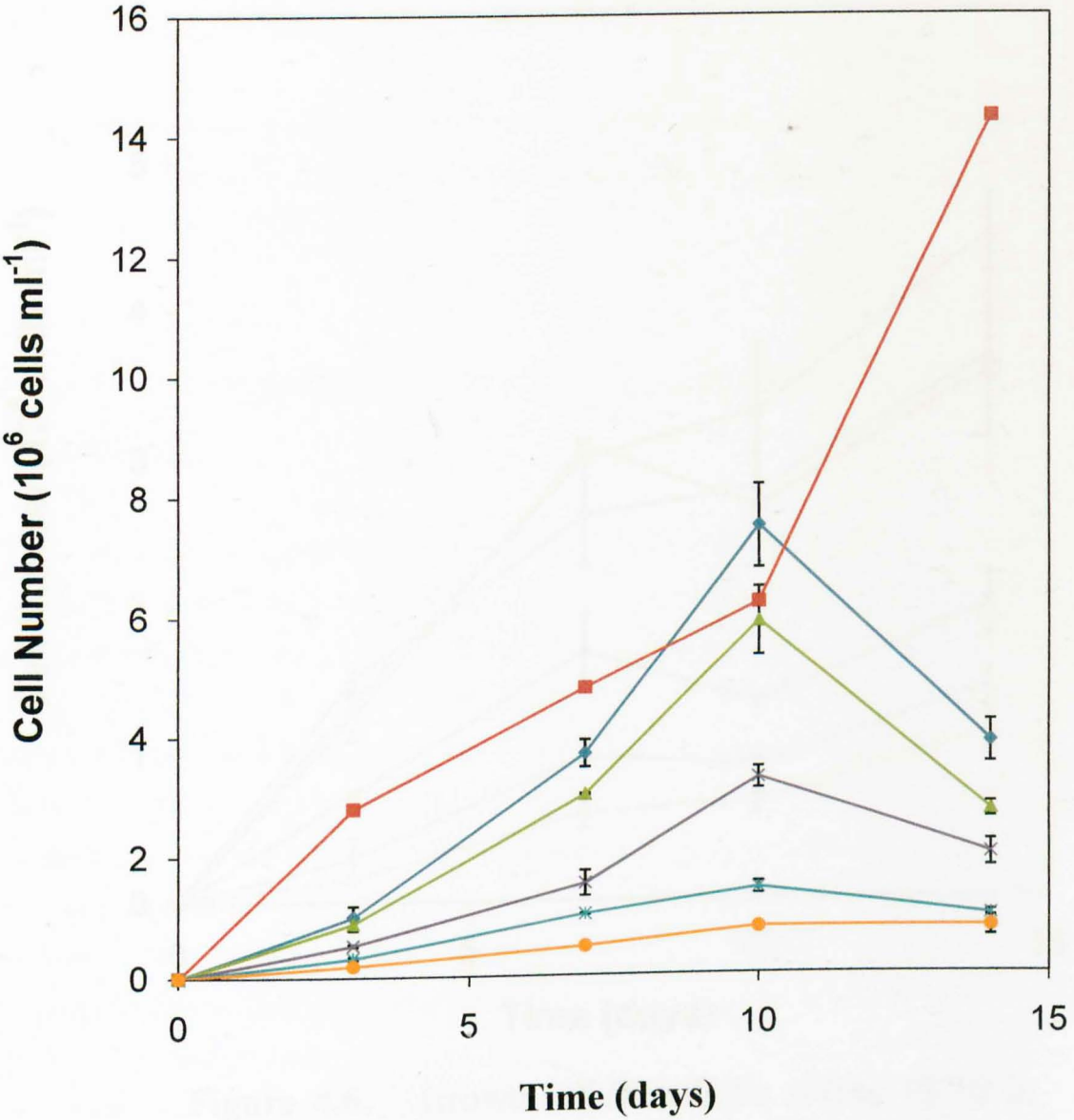

Figure 4.5. Growth of *Dunaliella parva* 19/10 in batch cultures at different salinities. The growth was monitored by counting the cell number. Each point represents the mean of ten determinations plus or minus standard error.

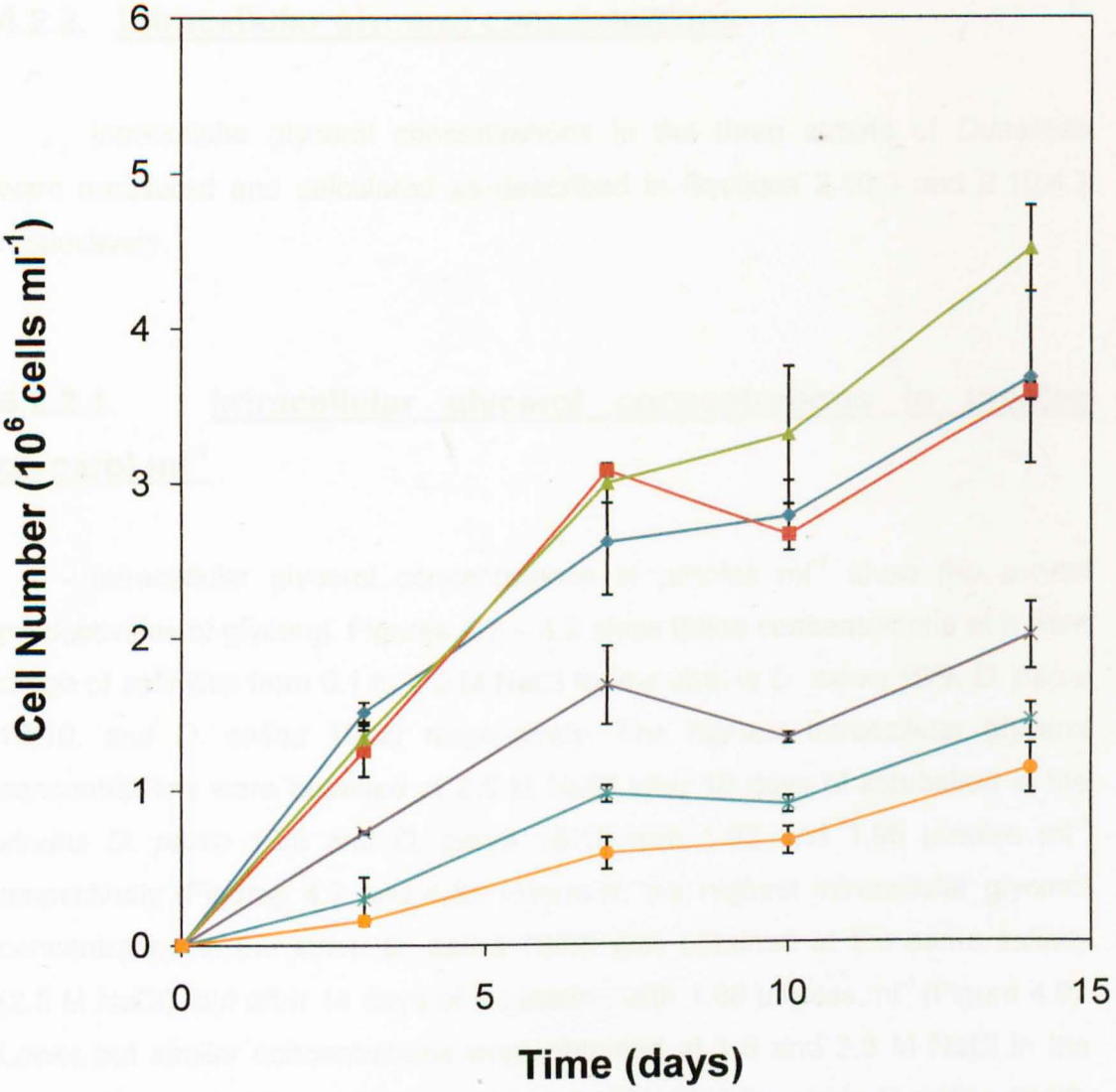

Figure 4.6. Growth of *Dunaliella salina* 19/30 in batch cultures at different salinities. The growth was monitored by counting the cell number. Each point represents the mean of ten determinations plus or minus standard error.

4.2.2. Intracellular glycerol concentrations

Intracellular glycerol concentrations in the three strains of *Dunaliella* were measured and calculated as described in Sections 2.10.3 and 2.10.4.2 respectively.

4.2.2.1. Intracellular glycerol concentrations in µmoles glycerol ml^{-1}

Intracellular glycerol concentrations in µmoles ml^{-1} show the overall productivities of glycerol. Figures 4.7 – 4.9 show these concentrations at a wide range of salinities from 0.1 to 4.0 M NaCl for the strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively. The highest intracellular glycerol concentrations were obtained at 2.5 M NaCl after 10 days of incubation in the strains *D. parva* 19/9 and *D. parva* 19/10 with 1.82 and 1.95 µmoles ml^{-1} respectively (Figures 4.7 and 4.8). However, the highest intracellular glycerol concentration in the strain *D. salina* 19/30 was obtained at the same salinity (2.5 M NaCl), but after 14 days of incubation with 1.68 µmoles ml^{-1} (Figure 4.9). Lower but similar concentrations were obtained at 1.5 and 3.5 M NaCl in the strains *D. parva* 19/9 and 19/10, and at 1.5 M NaCl in the strain *D. salina* 19/30. Moreover, the concentrations were very low at 0.1 M NaCl followed by 0.4 and 4.0 M in all the strains.

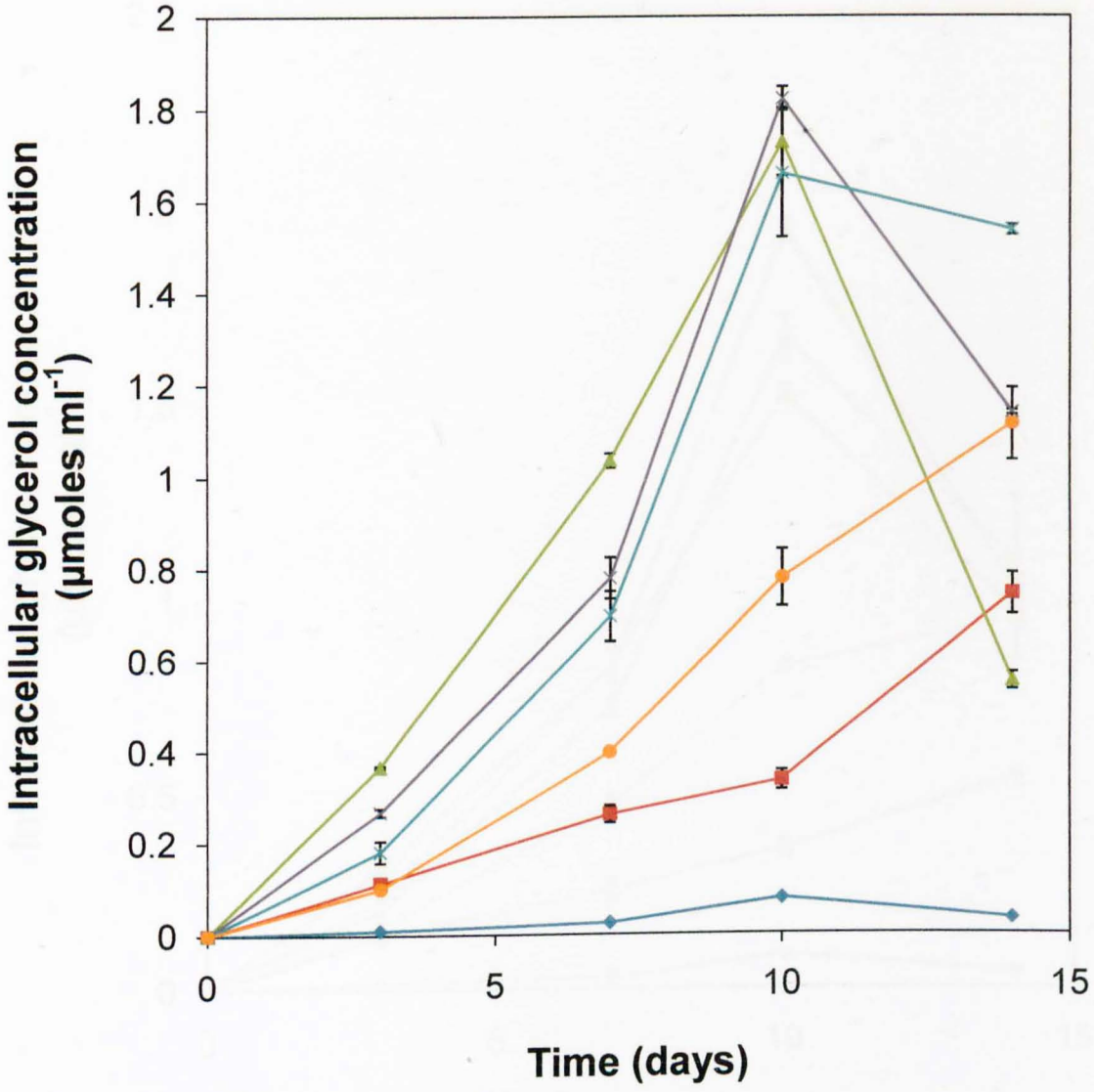

Figure 4.7. Intracellular glycerol concentrations (µmoles ml^{-1}) of *Dunaliella parva* 19/9 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus strandard error.

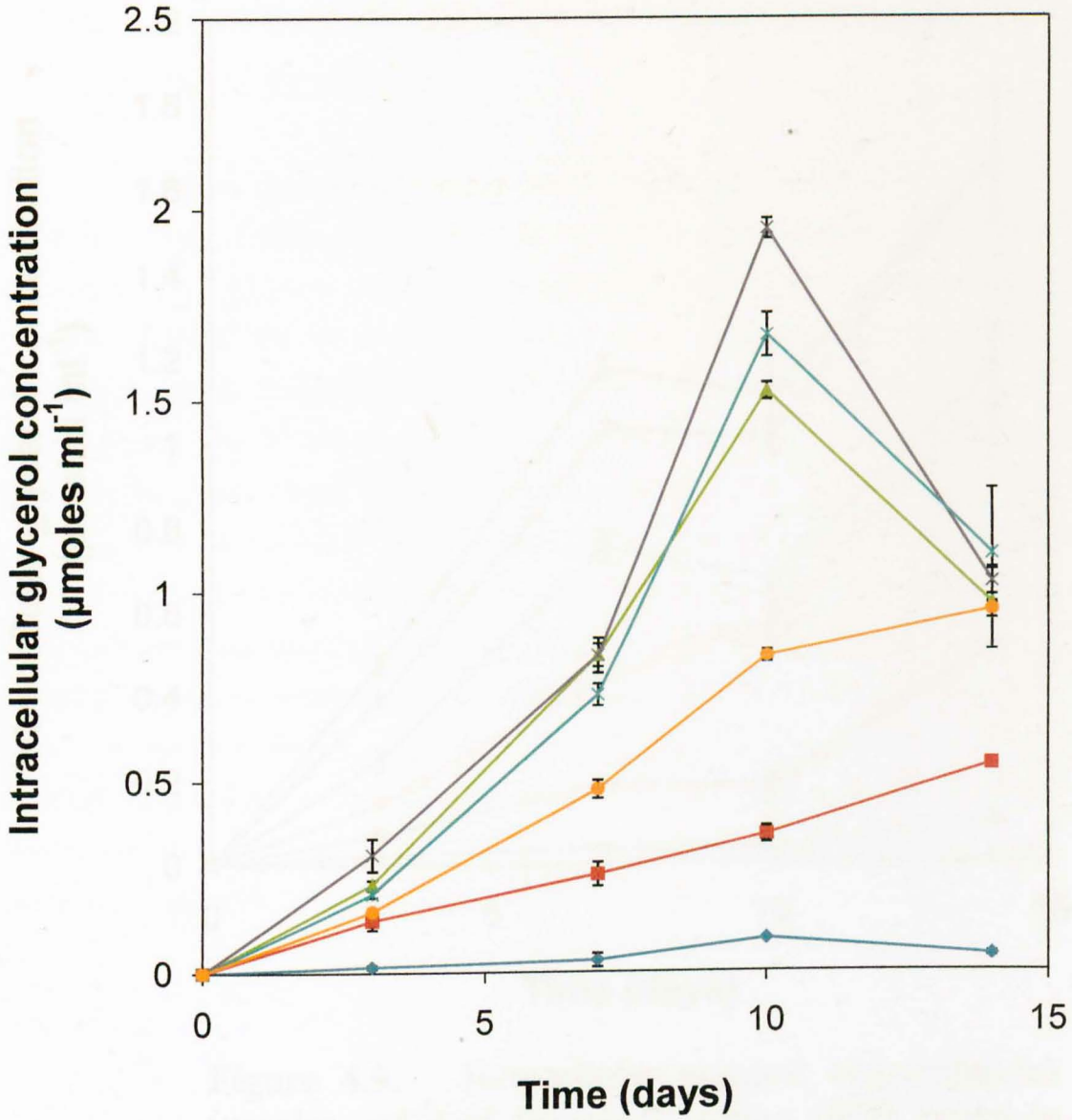

Figure 4.8. Intracellular glycerol concentrations (µmoles ml^{-1}) of *Dunaliella parva* 19/10 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

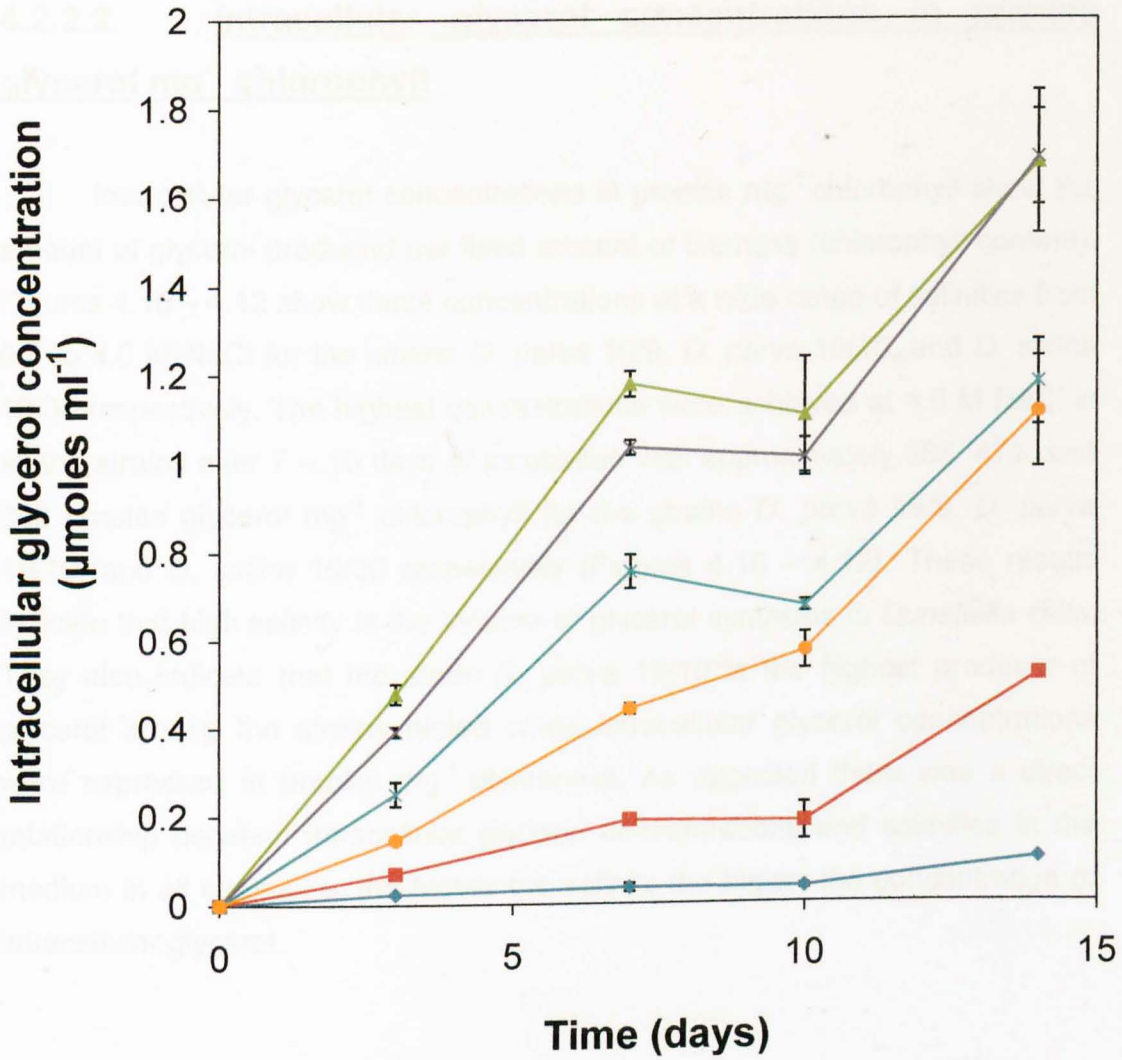

Figure 4.9. Intracellular glycerol concentrations (µmoles ml^{-1}) of *Dunaliella salina* 19/30 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

4.2.2.2. Intracellular glycerol concentrations in µmoles glycerol mg^{-1} chlorophyll

Intracellular glycerol concentrations in µmoles mg^{-1} chlorophyll show the amount of glycerol produced per fixed amount of biomass (chlorophyll content). Figures 4.10 – 4.12 show these concentrations at a wide range of salinities from 0.1 to 4.0 M NaCl for the strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively. The highest concentrations were achieved at 4.0 M NaCl in all the strains after 7 – 10 days of incubation with approximately 365, 471, and 302 µmoles glycerol mg^{-1} chlorophyll for the strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively (Figures 4.10 – 4.12). These results indicate that high salinity is the inducer of glycerol synthesis in *Dunaliella* cells. They also indicate that the strain *D. parva* 19/10 is the highest producer of glycerol among the strains tested when intracellular glycerol concentrations were expressed in µmoles mg^{-1} chlorophyll. As expected there was a direct relationship between intracellular glycerol concentrations and salinities in the medium in all strains i.e. the higher the salinity the higher the concentration of intracellular glycerol.

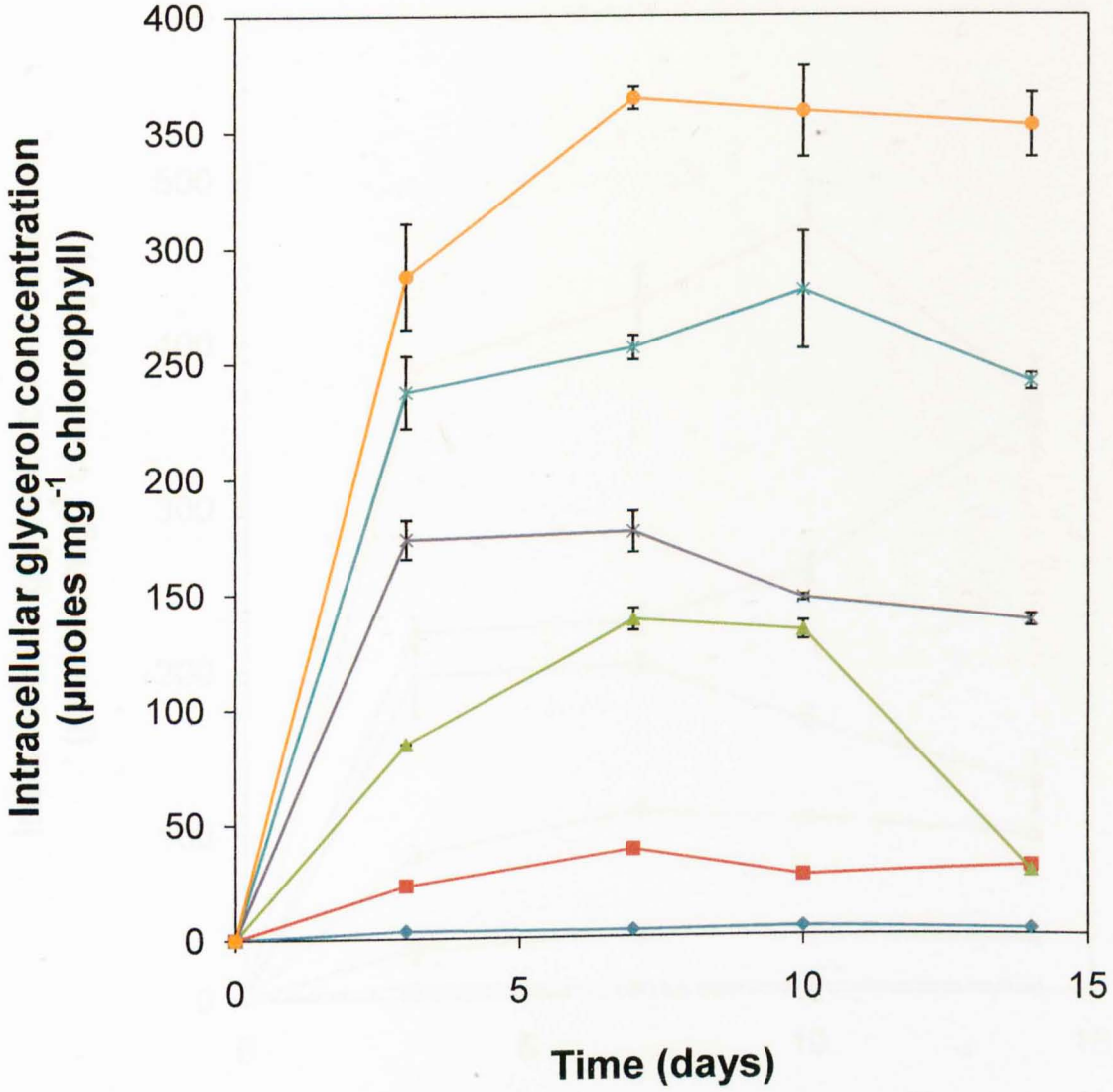

Figure 4.10. Intracellular glycerol concentrations (µmoles mg^{-1} chlorophyll) of *Dunaliella parva* 19/9 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

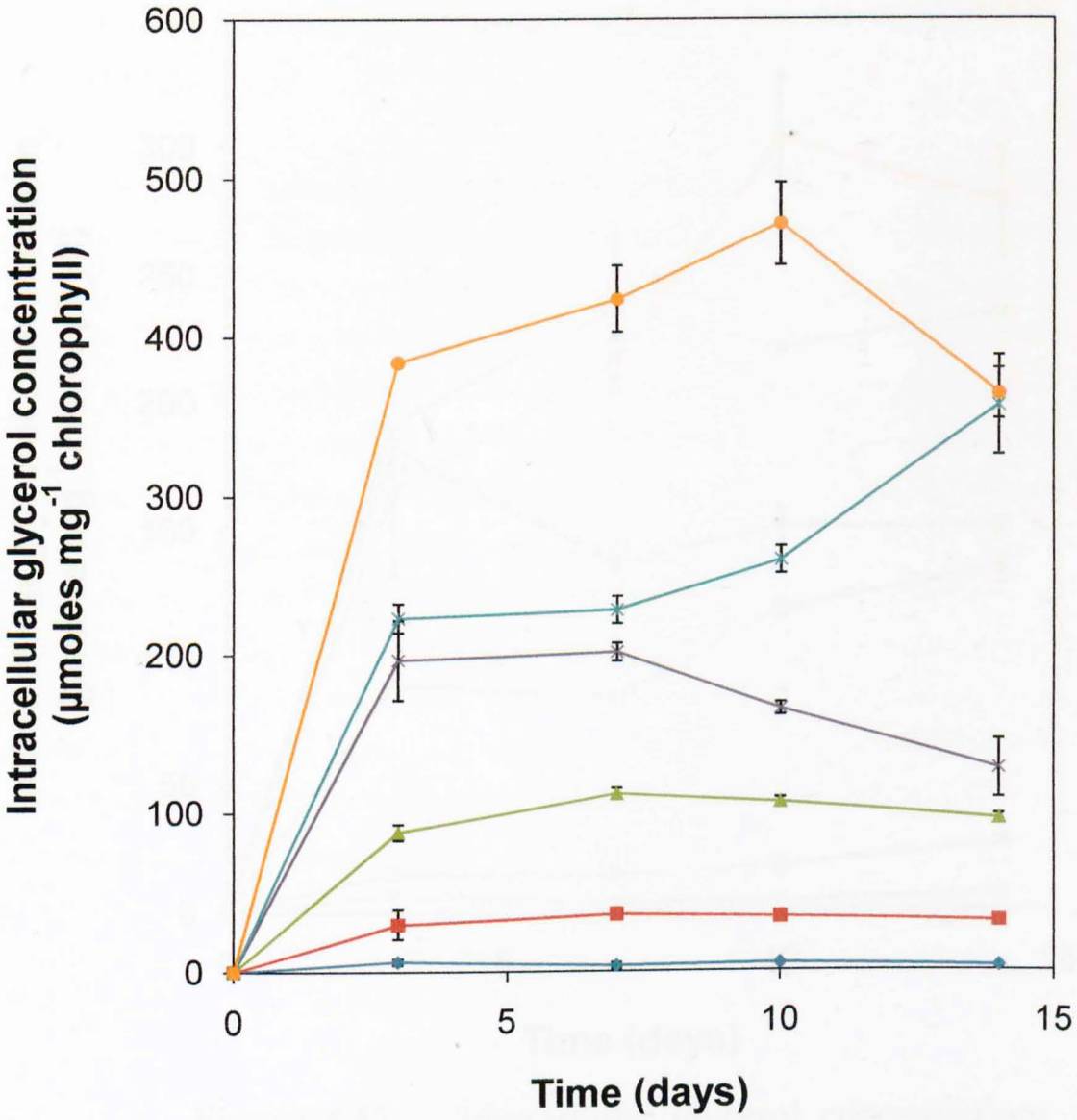

Figure 4.11. Intracellular glycerol concentrations (µmoles mg^{-1}chlorophyll) of *Dunaliella parva* 19/10 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

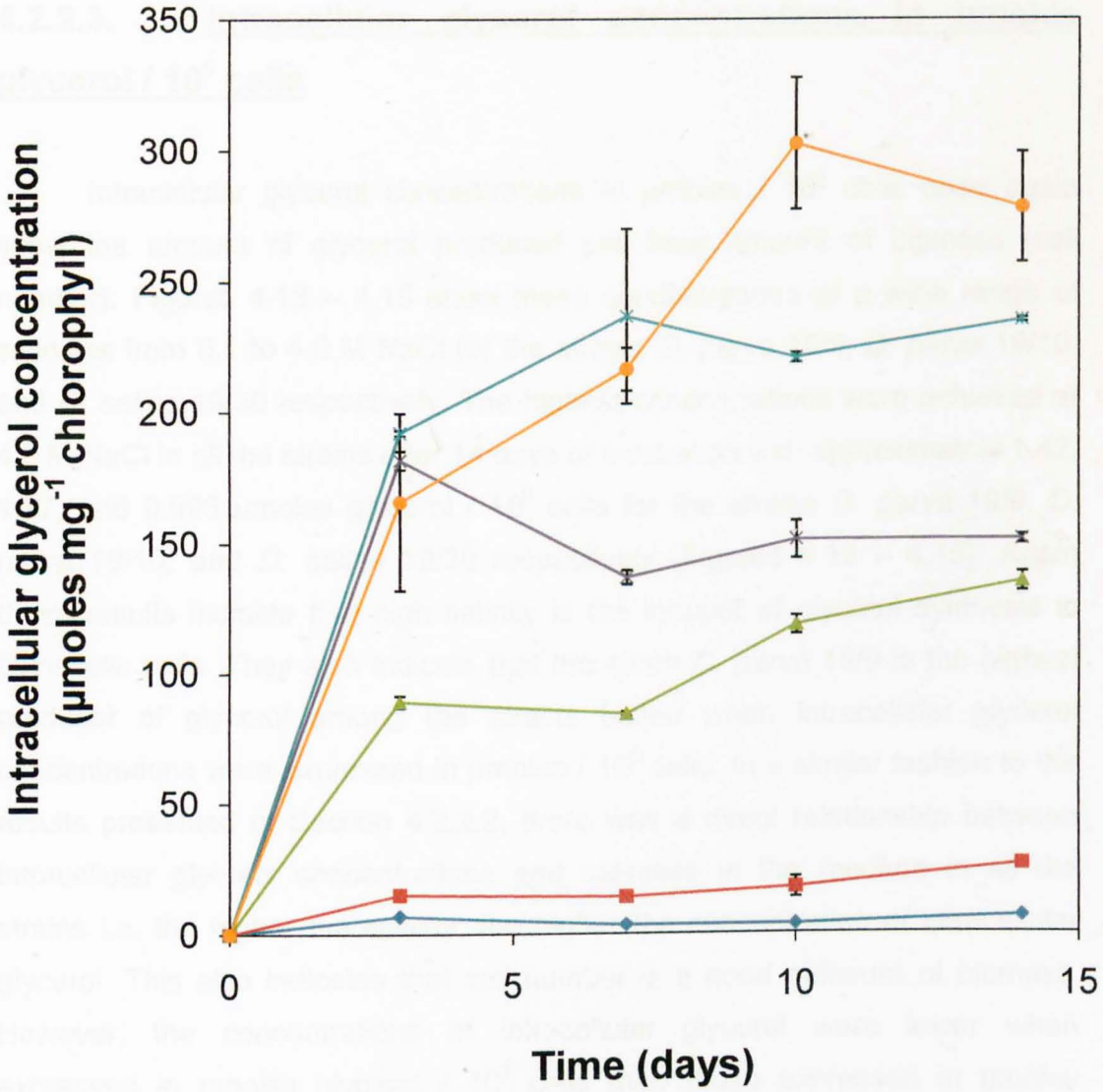

Figure 4.12. Intracellular glycerol concentrations (µmoles mg^{-1} chlorophyll) of *Dunaliella salina* 19/30 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

4.2.2.3. Intracellular glycerol concentrations in µmoles glycerol / 10^6 cells

Intracellular glycerol concentrations in µmoles / 10^6 cells once again show the amount of glycerol produced per fixed amount of biomass (cell number). Figures 4.13 – 4.15 show these concentrations at a wide range of salinities from 0.1 to 4.0 M NaCl for the strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively. The highest concentrations were achieved at 4.0 M NaCl in all the strains after 14 days of incubation with approximately 1.42, 1.27, and 0.995 µmoles glycerol / 10^6 cells for the strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively (Figures 4.13 – 4.15). Again these results indicate that high salinity is the inducer of glycerol synthesis in *Dunaliella* cells. They also indicate that the strain *D. parva* 19/9 is the highest producer of glycerol among the strains tested when intracellular glycerol concentrations were expressed in µmoles / 10^6 cells. In a similar fashion to the results presented in Section 4.2.2.2, there was a direct relationship between intracellular glycerol concentrations and salinities in the medium in all the strains i.e. the higher the salinity, the higher the concentration of intracellular glycerol. This also indicates that cell number is a good measure of biomass. However, the concentrations of intracellular glycerol were lower when expressed in µmoles glycerol / 10^6 cells than those expressed in µmoles glycerol mg^{-1} chlorophyll (Section 4.2.2.2) for the same strains grown under the same conditions and length of incubation. It is noteworthy that the amount of intracellular glycerol produced was the same for the strains under the same growth conditions; the observed difference in these amounts resulted from the way in which the data were expressed.

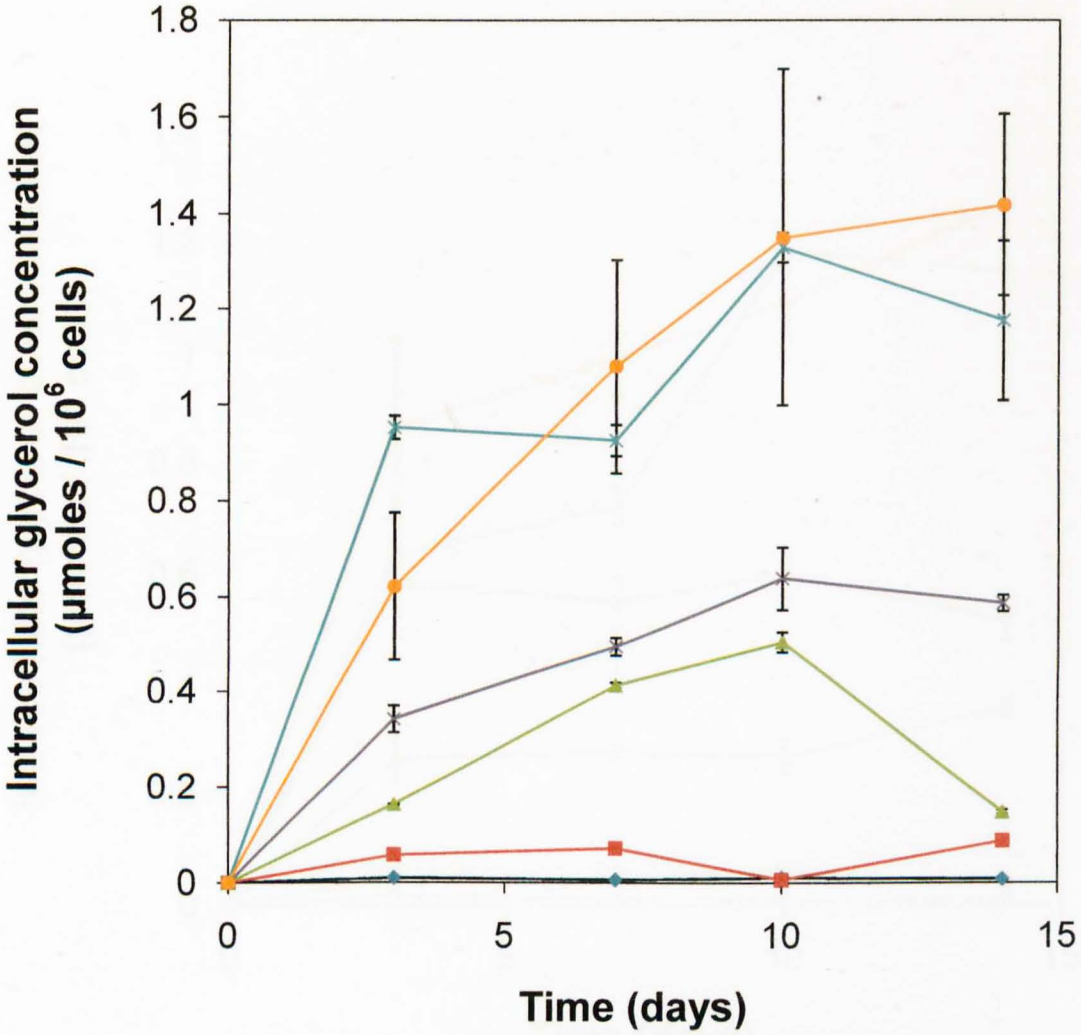

Figure 4.13. Intracellular glycerol concentrations (µmoles / 10^6 cells) of *Dunaliella parva* 19/9 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

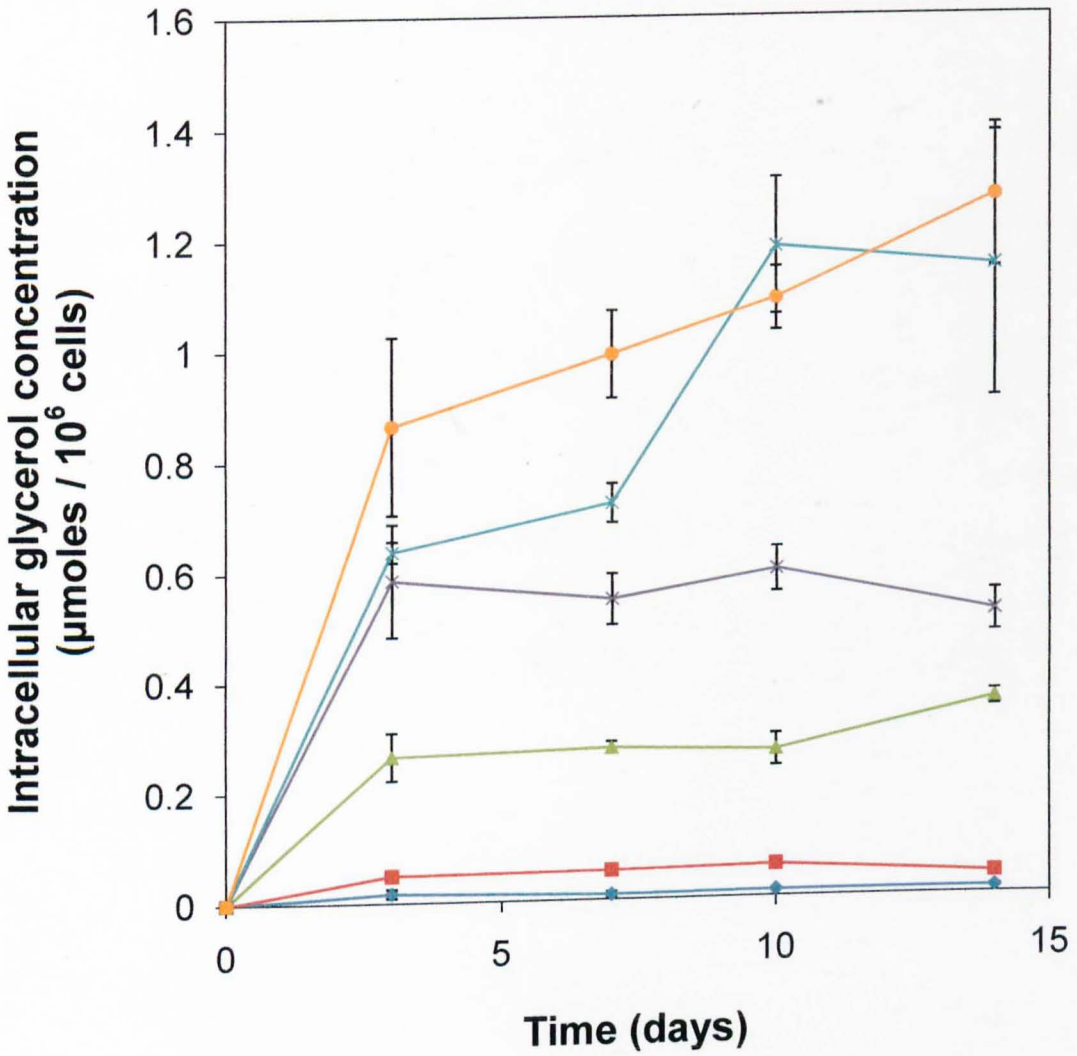

Figure 4.14. Intracellular glycerol concentrations (µmoles / 10^6 cells) of *Dunaliella parva* 19/10 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

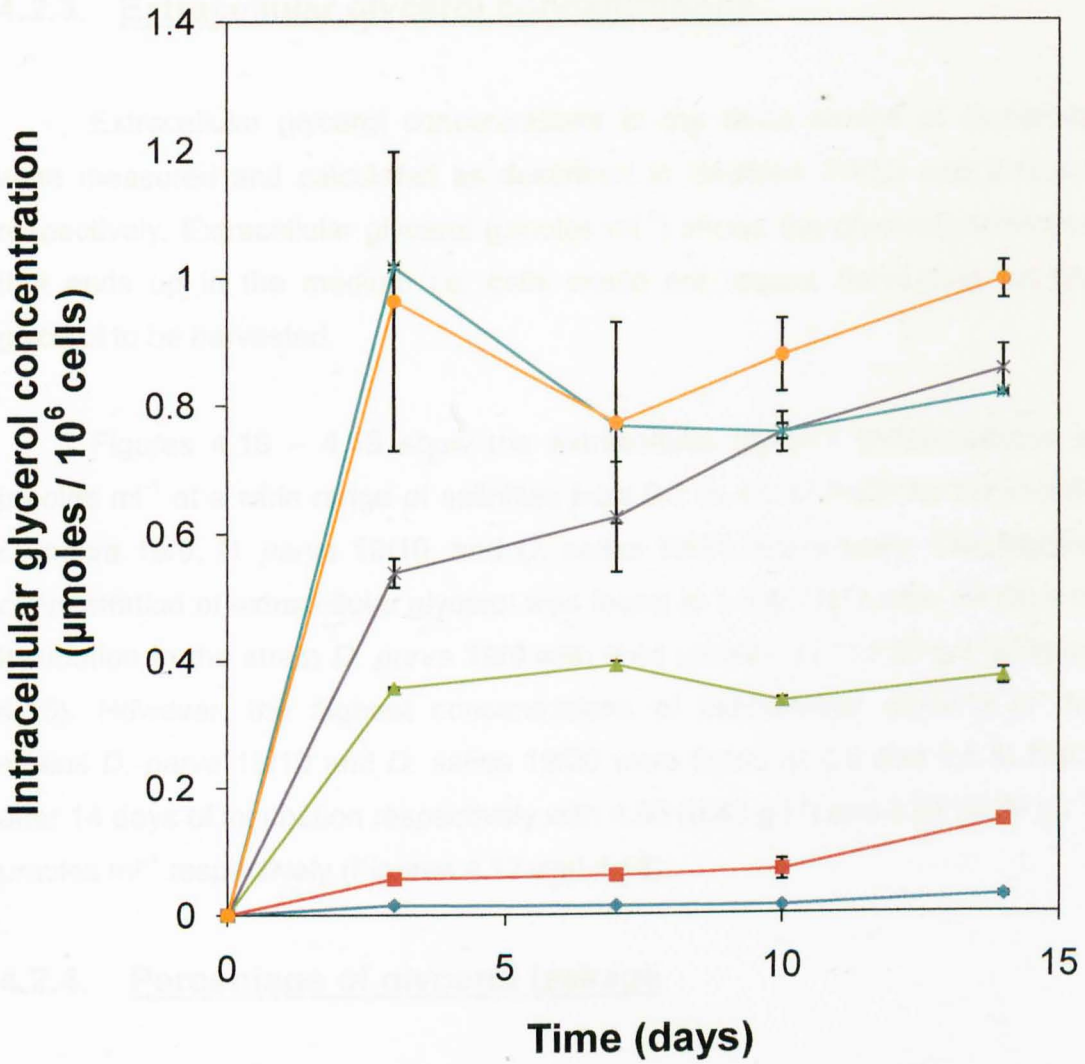

Figure 4.15. Intracellular glycerol concentrations (µmoles / 10^6 cells) of *Dunaliella salina* 19/30 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

4.2.3. Extracellular glycerol concentrations

Extracellular glycerol concentrations in the three strains of *Dunaliella* were measured and calculated as described in Sections 2.10.3 and 2.10.4.1 respectively. Extracellular glycerol (µmoles ml^{-1}) shows the glycerol production that ends up in the medium i.e. cells would not require harvesting for this glycerol to be harvested.

Figures 4.16 – 4.18 show the extracellular glycerol concentrations in µmoles ml^{-1} at a wide range of salinities from 0.1 to 4.0 M NaCl for the strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively. The highest concentration of extracellular glycerol was found at 1.5 M NaCl after 14 days of incubation in the strain *D. parva* 19/9 with 6.44 µmoles ml^{-1} (0.59 g l^{-1})(Figure 4.16). However, the highest concentrations of extracellular glycerol in the strains *D. parva* 19/10 and *D. salina* 19/30 were found at 2.5 and 3.5 M NaCl after 14 days of incubation respectively with 4.95 (0.45 g l^{-1}) and 2.87 (0.26 g l^{-1}) µmoles ml^{-1} respectively (Figures 4.17 and 4.18).

4.2.4. Percentage of glycerol leakage

Leakage of glycerol was determined for the three strains of *Dunaliella* as described in Section 2.10.4.3. Figures 4.19 – 4.21 show the percentages of leakage of glycerol in the strains *D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30 respectively. All the strains tested leaked very significant levels of glycerol into the medium. High levels of leakage were found at all salinities over the full 14 days time period of the experiment. The highest levels of leakage were shown by the strains *D. parva* 19/9 and *D. parva* 19/10 with several measurements exceeding 80% leakage (Figures 4.19 and 4.20). Lower levels of maximum leakage (around 70%) were found in *D. salina* 19/30 cells and the 19/30 cultures grown at 0.4 and 1.5 M NaCl showed much lower levels of leakage (Figure 4.21).

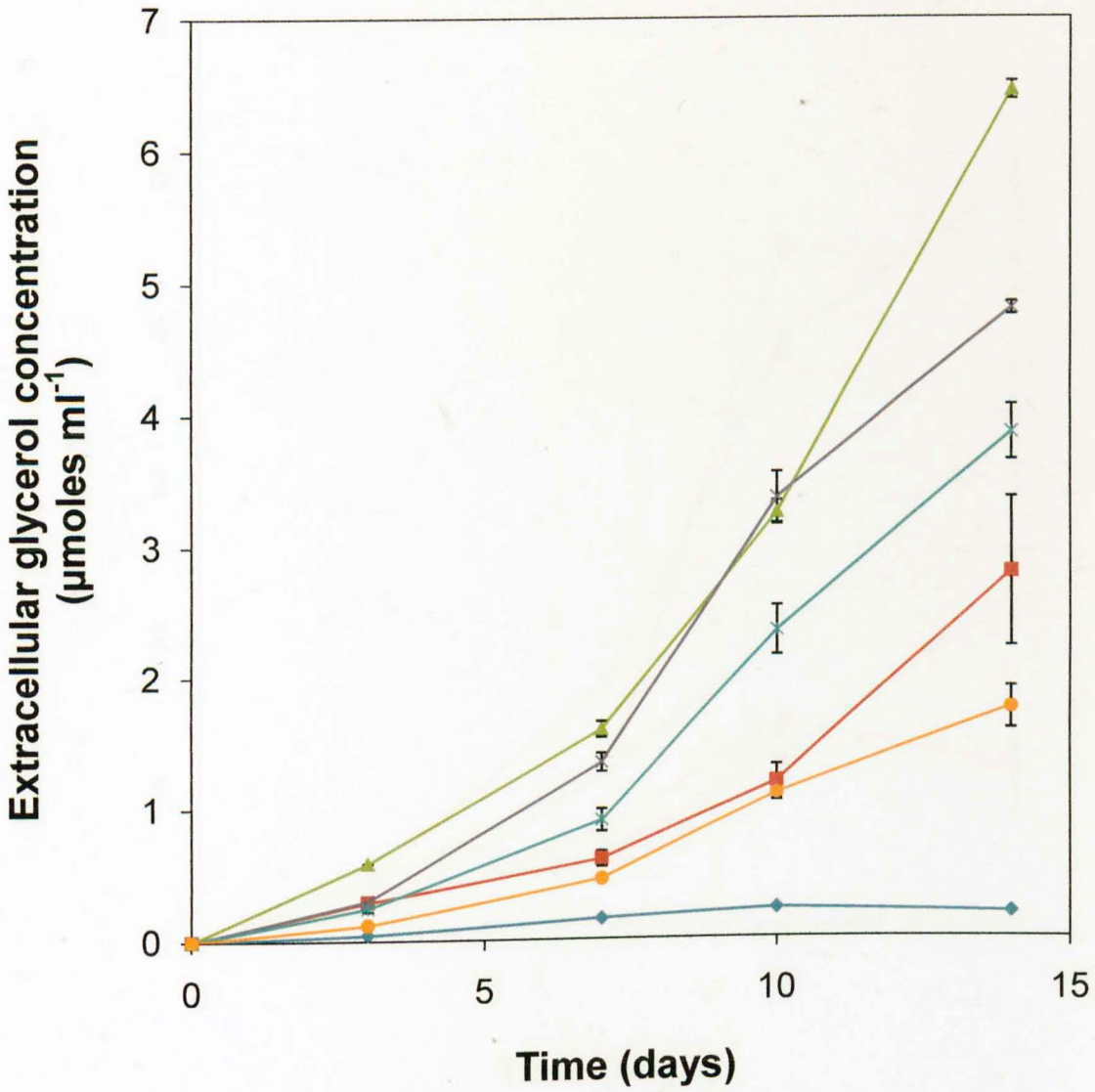

Figure 4.16. Extracellular glycerol concentrations (µmoles ml^{-1}) of *Dunaliella parva* 19/9 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

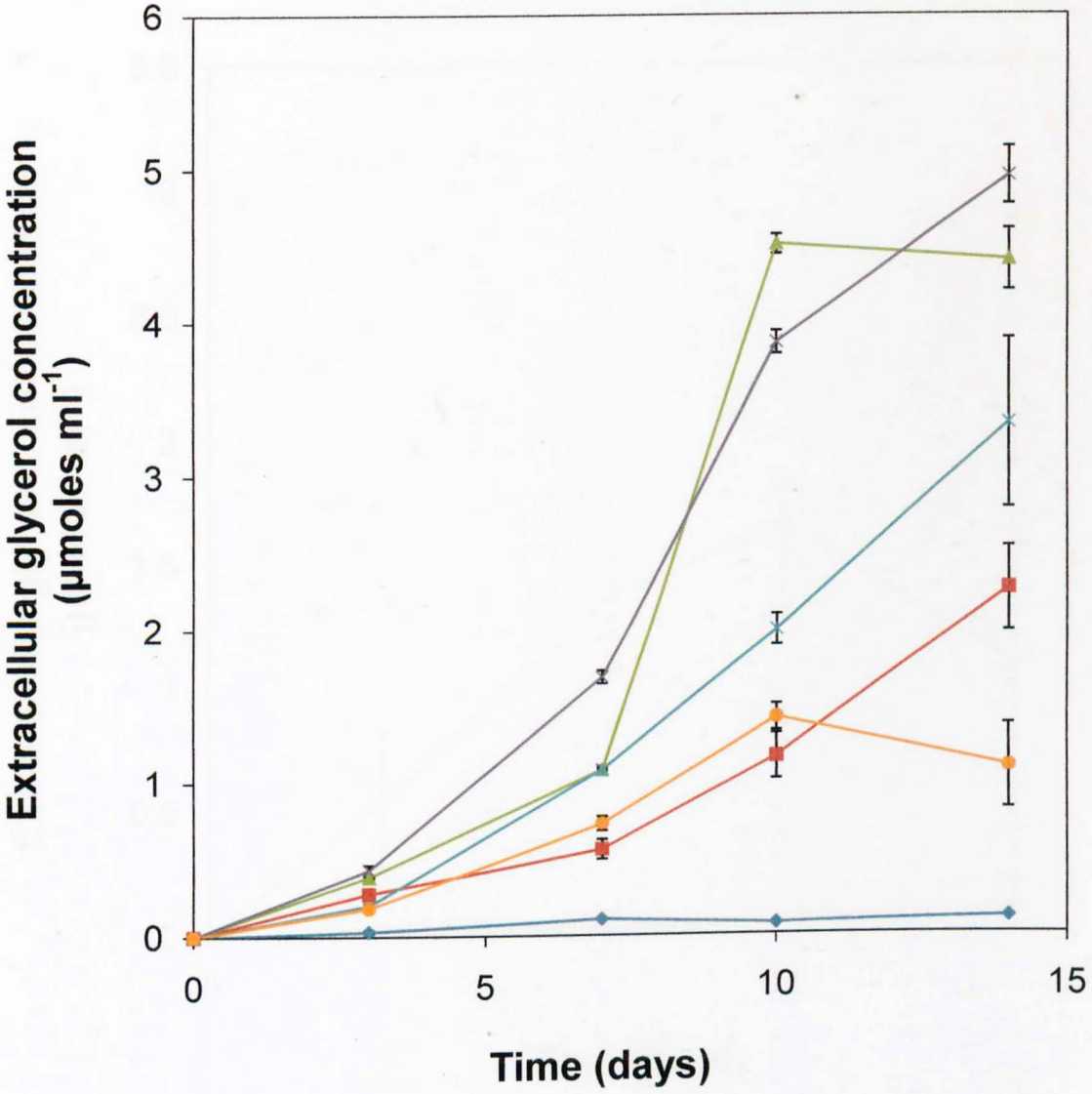

Figure 4.17. Extracellular glycerol concentrations (µmoles ml^{-1}) of *Dunaliella parva* 19/10 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

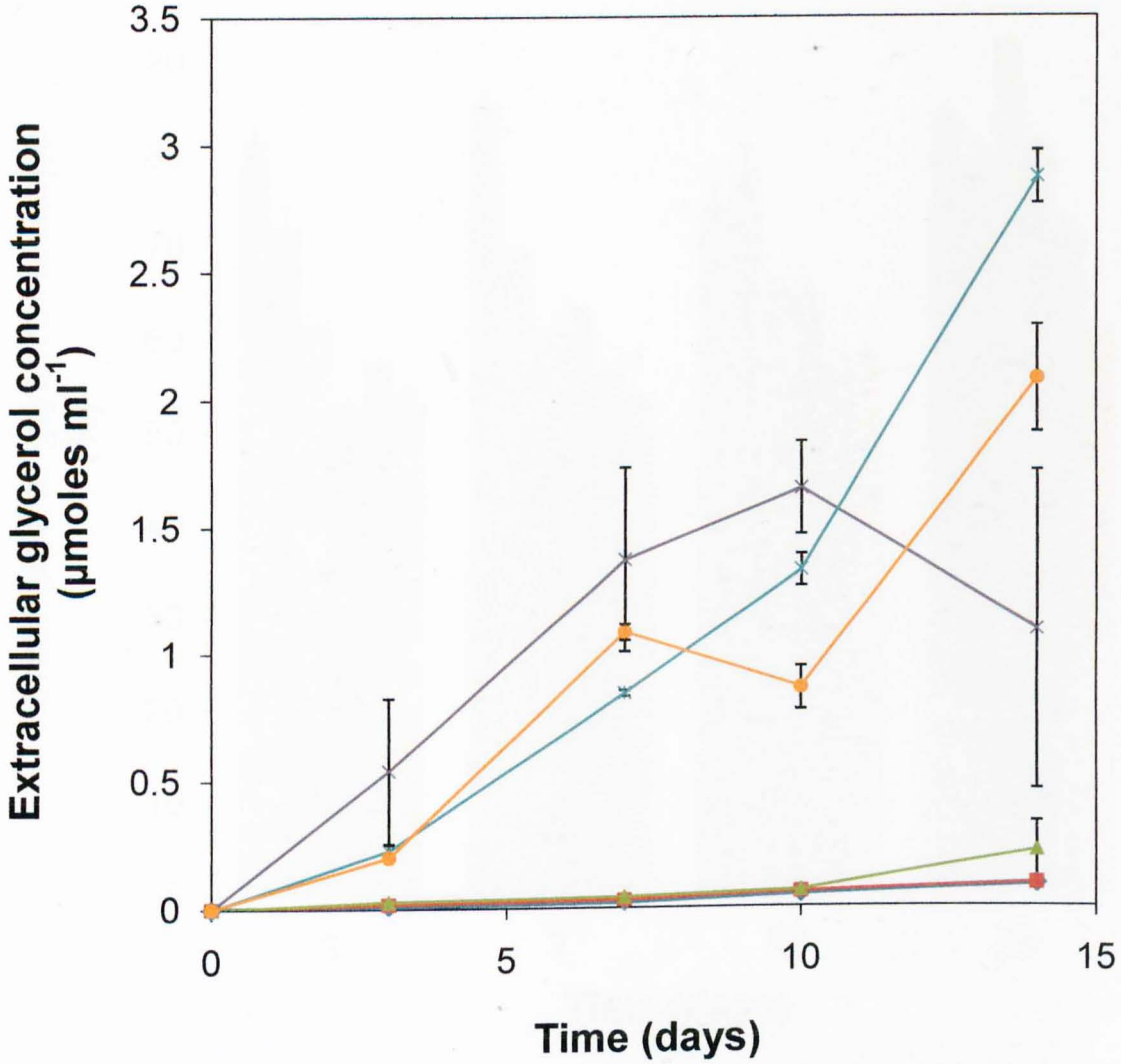

Figure 4.18. Extracellular glycerol concentrations (µmoles ml^{-1}) of *Dunaliella salina* 19/30 grown in batch cultures at different concentrations of NaCl. Each point represents the mean of four determinations plus or minus standard error.

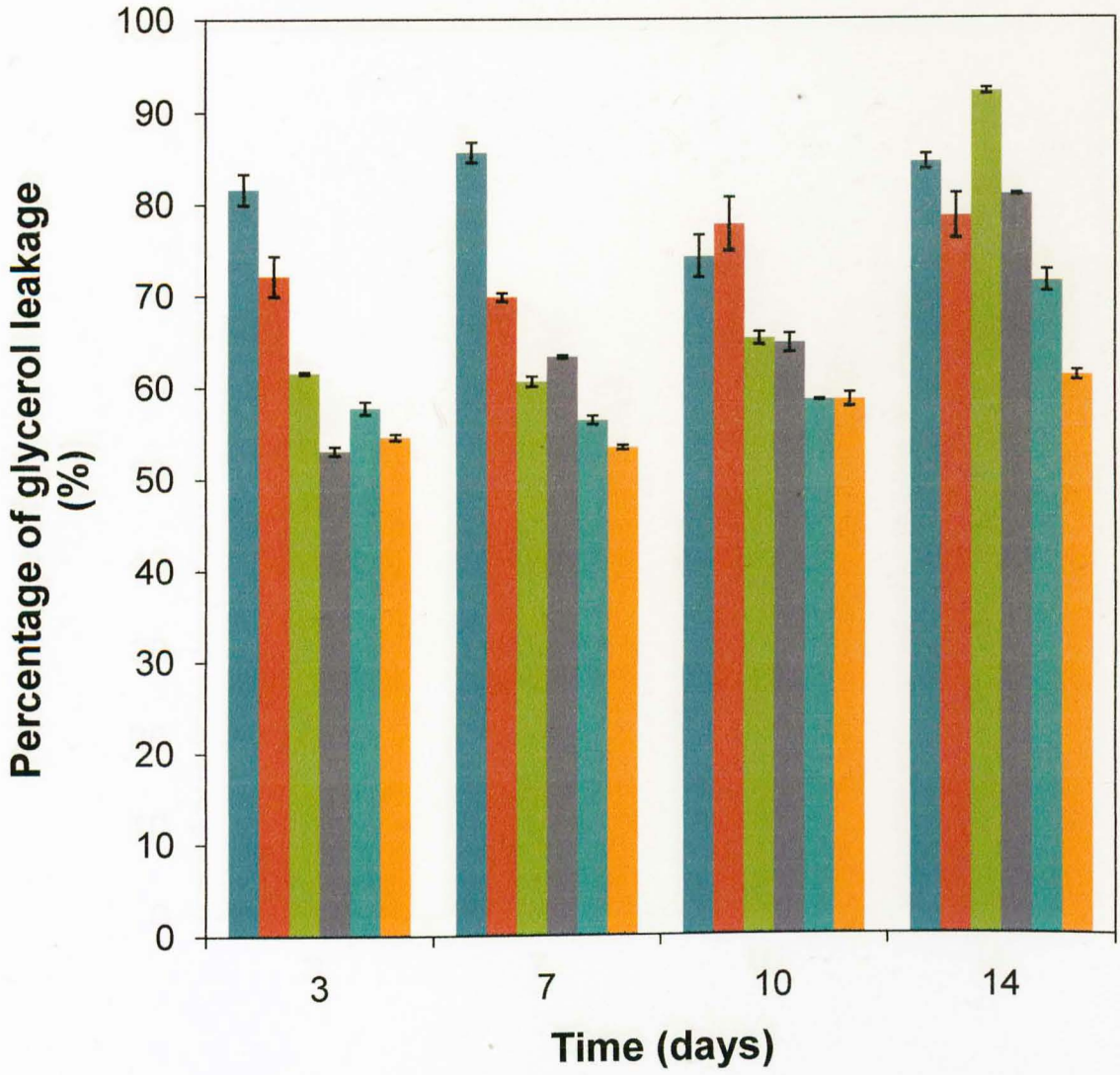

Figure 4.19. Percentage of glycerol leakage of *Dunaliella parva* 19/9 grown in batch cultures at different concentrations of NaCl. Each column represents the mean of four determinations plus or minus standard error.

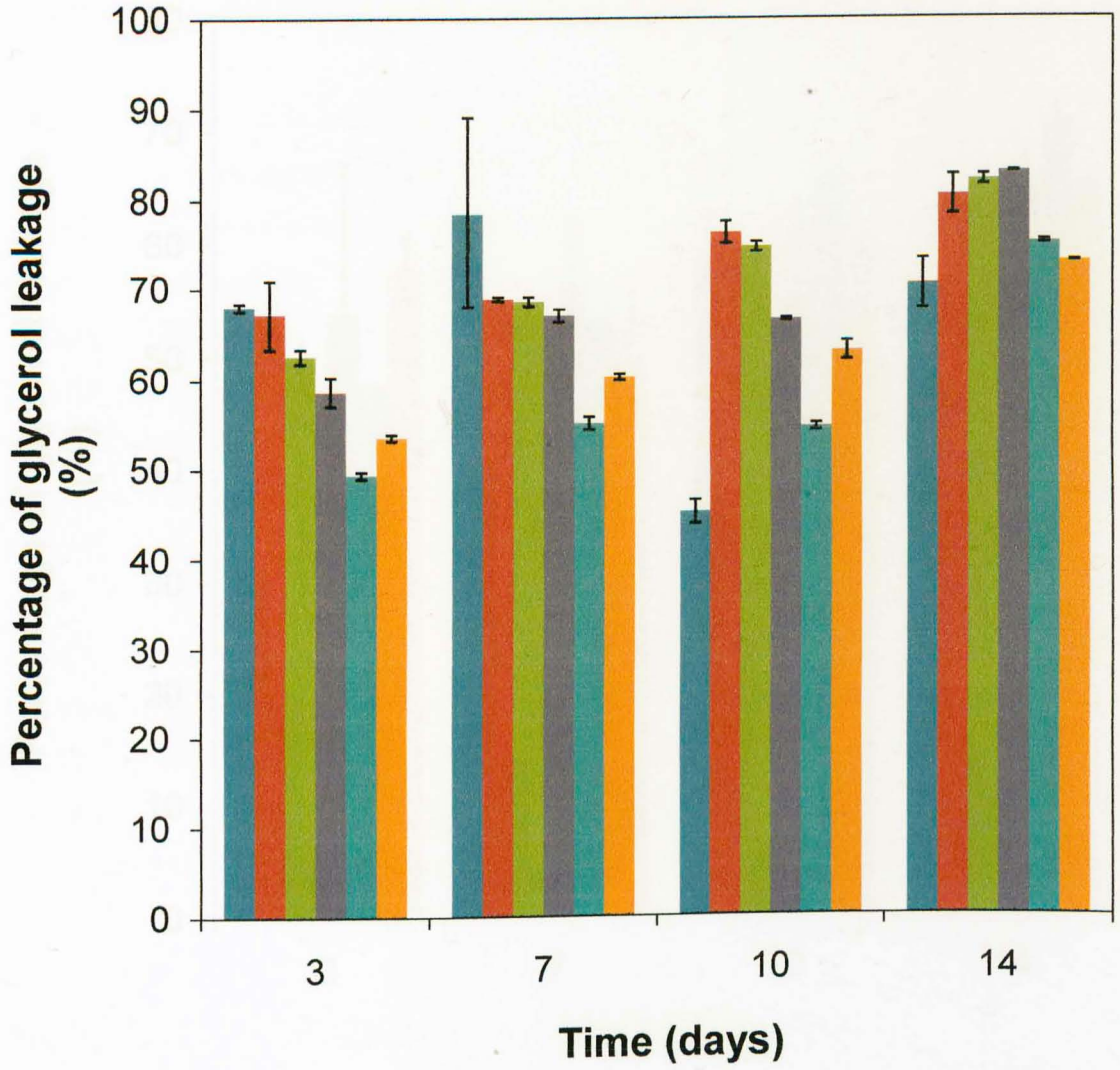

Figure 4.20. Percentage of glycerol leakage of *Dunaliella parva* 19/10 grown in batch cultures at different concentrations of NaCl. Each column represents the mean of four determinations plus or minus standard error.

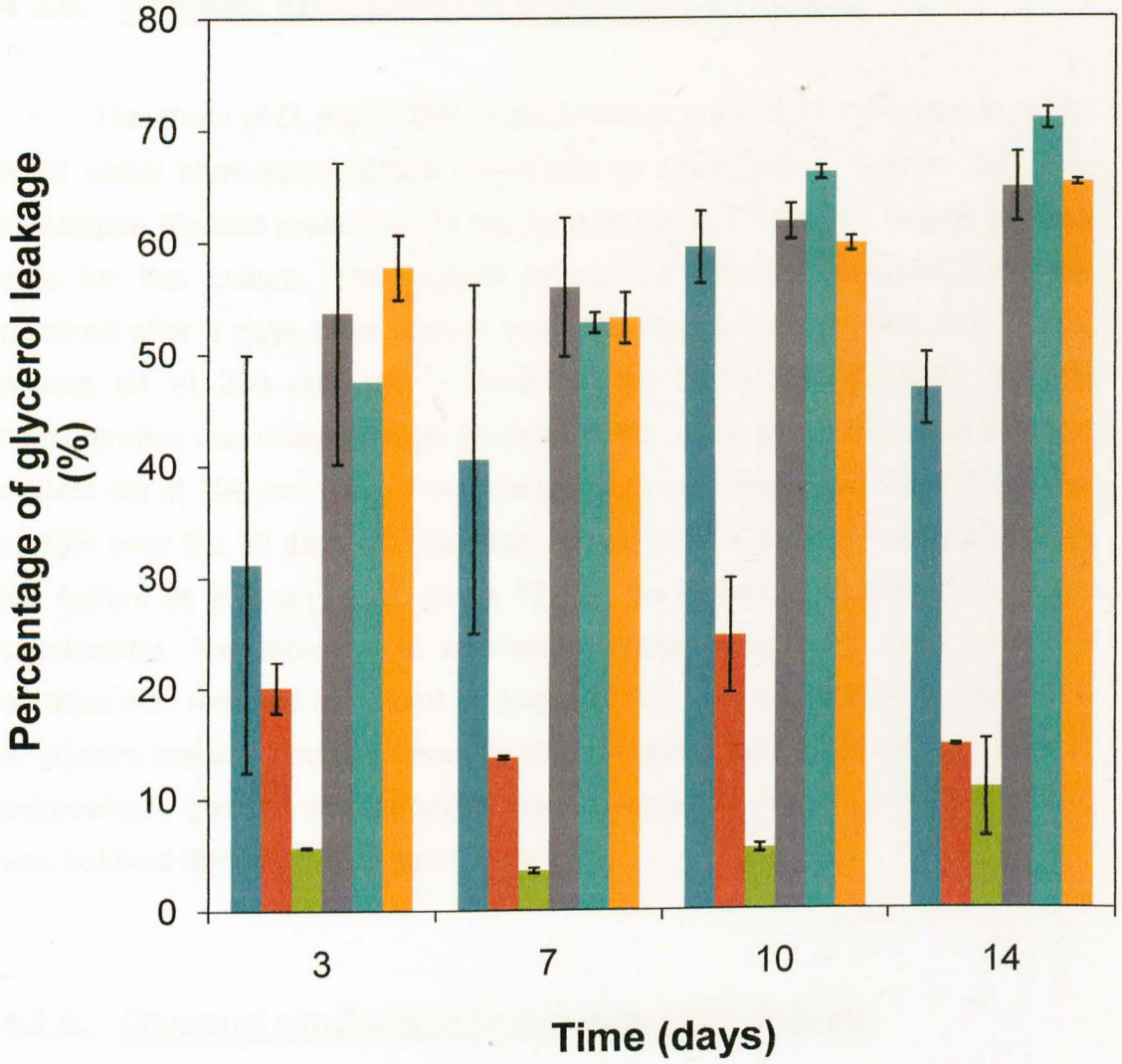

Figure 4.21. Percentage of glycerol leakage of *Dunaliella salina* 19/30 grown in batch cultures at different concentrations of NaCl. Each column represents the mean of four determinations plus or minus standard error.

4.2.5. Glycerol production in a semi-batch culture

The strain of *D. parva* 19/10 was grown in a 2 L airlift fermenter at 1.5 M NaCl under semi-batch culture conditions as described in Section 2.11.2 to investigate glycerol production in this type of culture. Table 4.1 shows glycerol data for this culture. The highest intracellular glycerol concentration was obtained after 9 days of incubation when the culture was aerated with filtered natural air at 200 cm^3 min^{-1}. However, the highest extracellular glycerol concentration was obtained after 20 days of incubation under the same aeration system but at 100 cm^3 min^{-1}. Percentages of glycerol leakage ranged from 61.3 to 88% over the 20 days of incubation. When 0.1% CO_2 was bubbled through the culture at 200 cm^3 min^{-1} (days 17-19), the chlorophyll content increased significantly. The increase in chlorophyll content continued even after the aeration was returned to natural air (days 20-22). The intracellular glycerol and % glycerol leakage remained broadly similar throughout the experiment, but the extracellular glycerol concentration was consistently higher when 0.1% CO_2 was bubbled through the culture (Table 4.1).

4.2.6. Glycerol production in a continuous culture

The strain of *D. parva* 19/10 was grown in a 2 L airlift fermenter at 1.5 M NaCl under continuous culture conditions as described in Section 2.11.3 to investigate glycerol production in this type of culture. Table 4.2 shows glycerol data for this culture. It shows that the level of glycerol leakage (around 67 – 76%) was similar to that found in batch cultures (Section 4.2.4) and a semi-batch culture (Section 4.2.5). The leakage was slightly higher when 0.1% CO_2 was supplied to the fermenter, but it decreased again when the flow rate of medium was increased from 45 to 90 ml h^{-1} (Table 4.2).

Time from onset of the semi-batch culture (day)	Type of aeration	Chlorophyll Content (μg ml^{-1})	Intracellular Glycerol Concentration (μmoles mg^{-1} chlorophyll)	Extracellular Glycerol Concentration (μmoles ml^{-1})	Percentage of glycerol leakage (%)
1	Filtered Natural Air (CO_2 ≈ 0.035%) at 100 cm^3 min^{-1}	0.46 ± 0.14	-	-	-
2		0.92 ± 0.12	-	-	-
3		1.54 ± 0.01	7.4 ± 0.00	0.35 ± 0.01	76.8 ± 1.8
5		2.9 ± 0.056	24.26 ± 0.92	1.21 ± 0.00	65 ± 0.95
6		2.64 ± 0.18	108.08 ± 2.04	4.72 ± 0.02	64.2 ± 0.45
7		3.03 ± 0.22	120.28 ± 8.86	5.32 ± 0.07	61.3 ± 1.4
8		2.83 ± 0.07	119.96 ± 12.73	5.75 ± 0.05	64.8 ± 3.1
9	Filtered Natural Air (CO_2 ≈ 0.035%) at 200 cm^3 min^{-1}	2.00 ± 0.17	128.75 ± 2.68	7.38 ± 0.02	75.6 ± 0.1
10		2.7 ± 0.05	95.7 ± 1.98	7.07 ± 0.08	74.8 ± 0.3
11		2.88 ± 0.07	104.9 ± 30.78	14.07± 0.1	83.5 ± 3.0
12		2.907 ± 0.12	90.85 ± 4.62	8.76 ± 0.06	78.2 ± 0.55
13		3.11 ± 0.08	-	-	-
14	Filtered 0.1% CO_2 plus 20% O_2 at 100 cm^3 min^{-1}	3.15 ± 0.19	-	-	-
15		2.9 ± 0.14	-	-	-
16		2.65 ± 0.14	58.07 ± 19.27	11.18 ± 0.12	88.7 ± 2.55
17	Filtered 0.1% CO_2 plus 20% O_2 at 200 cm^3 min^{-1}	4.4 ± 0.05	89.01 ± 3.67	11.8 ± 0.1	76.6 ± 0.8
18		4.67 ± 0.12	119.08 ± 6.32	13.56 ± 0.29	72.5 ± 0.95
19		5.9 ± 0.44	83.73 ± 0.9	14.12 ± 0.16	75.6 ± 0.2
20	Filtered Natural Air (CO_2 ≈ 0.035%) at 100 cm^3 min^{-1}	7.74 ± 0.26	110.89 ± 4.15	14.68 ± 0.33	65 ± 0.5
21		7.81 ± 0.32	-	-	-
22		6.34 ± 0.30	-	-	-

Table 4.1. Glycerol data of *Dunaliella parva* 19/10 under semi-batch culture conditions. Each figure represents the mean of three determinations. Standard errors values are shown.

Growth Conditions	Specific growth rate (h^{-1})	Chlorophyll content ($\mu g\ ml^{-1}$)	Intracellular glycerol concentration ($\mu moles\ mg^{-1}$ chlorophyll)	Extracellular glycerol concentration ($\mu moles\ ml^{-1}$)	Percentage of glycerol leakage (%)
A	0.025	2.94 ± 0.07	103.4 ± 7.63	5.61 ± 0.04	66.7 ± 1.28
B	0.025	3.48 ± 0.03	128.3 ± 4.37	13.19 ± 0.09	76.1 ± 0.73
C	0.05	4.14 ± 0.02	76.9 ± 1.14	7.38 ± 0.05	71.4 ± 0.13

Table 4.2. Glycerol production by *Dunaliella parva* 19/10 under continuous culture conditions. **A.** Culture was aerated by filtered natural air at 200 $cm^3\ min^{-1}$ and the flow rate of the medium was 45 ml h^{-1}. **B.** Culture was aerated by filtered CO_2 / O_2 mixture (0.1% CO_2, 20% O_2, balance N_2) at 200 $cm^3\ min^{-1}$ and the flow rate of the medium was 45 ml h^{-1}. **C.** Culture was aerated by filtered CO_2 / O_2 mixture (0.1% CO_2, 20% O_2, balance N_2) at 200 $cm^3\ min^{-1}$ and the flow rate of the medium was 90 ml h^{-1}. Each figure represents the mean of three determinations. Standard errors values are shown.

The three strains of *Dunaliella* used in this study come from different habitats. *D. salina* 19/30 (originally designated *D. bardawil*) was isolated form a salt pool in North Sinai, Egypt. The "bardawil strain" was transferred to *D. salina* and a diagnostic feature of strains of *D. salina* is their ability to overproduce β-carotene under conditions of high light intensity and low N content in the medium (Borowitzka, 1999). The other two strains are both designated *D. parva* and do not overproduce β-carotene, but they were isolated from very different environments. Strain 19/9 was isolated from a salt marsh in the south of England and strain 19/10 from the area around the Dead Sea (information from the Culture Collection of Algae and Protozoa, www.ccap.ac.uk).

D. salina strains are normally associated with the most saline environments and usually require high levels of NaCl for optimal growth. Figures 4.3 and 4.6 demonstrate that this is not true for *D. salina* 19/30, which showed optimum growth at 0.4 M NaCl in line with the two *D. parva* strains. Therefore, the designation of a strain as *D. salina* does not in itself guarantee halophilic growth characteristics. All three strains tested showed decreased growth above 0.4 M NaCl, but all three were capable of significant growth at 4 M NaCl (Figures 4.1 – 4.6). High concentrations of NaCl may reduce the growth due to inhibitory effect of maintaining a very high concentration of glycerol within the cells (Figures 4.10 – 4.12). Leakage of glycerol from the cells should be very detrimental at high salinities, yet it does not seem to decrease significantly (Figures 4.19 – 4.21).

In addition to batch culture experiments, *D. parva* 19/10 was grown in an airlift fermenter under semi-batch culture (Table 4.1) and continuous culture (Table 4.2) conditions and the level of glycerol leakage was similar despite the very different growth conditions. Bubbling CO_2 enriched air through the culture tended to increase the leakage. Therefore, it appears that high percentage leakage of glycerol is a robust phenomenon in at least three different strains of *Dunaliella* and it may become an important process in the commercial exploitation of *Dunaliella* for glycerol production.

The low cost of petroleum-derived glycerol coupled with the expense of harvesting glycerol from *Dunaliella* means that glycerol production from *Dunaliella* has not become an established commercial process (Gilmour, 1990). Despite that, there are several factors that encourage the re-examination of glycerol production from *Dunaliella*. 1) The price of crude oil has been consistently high for several years reaching 100US$ / barrel and that could change the economics of glycerol production by this route. 2) The rise of biodiesel has re-introduced the idea of renewable sources of energy and microalgae have many advantages over crop plants in biodiesel production. Not

least that microalgal cultivation does not utilise land suitable for food production (Chisti, 2007). 3) Over the last decade, new bioreactor types have been produced (Borowitzka, 1999) and harvesting methods for small microalgal cells such as *Dunaliella* are also much improved, but the cost of biomass recovery is still a very significant factor at up to 60% of the total production cost (Grima *et al.*, 2003). It would be possible to alleviate these harvesting problems by inducing the glycerol to leak form the cells where it could be directly harvested from the medium. The results presented in Section 4.2.3 show that up to 0.59 g glycerol l^{-1} can be harvested directly from the medium of the strain *D. parva* 19/9.

4.3. Conclusions

The three strains of *Dunaliella* are halotolerant since they grew optimally at 0.4 M NaCl. They were all capable of significant growth at 4 M NaCl. The highest biomass at this salinity was obtained in 10-14 days of incubation in all the strains. High concentrations of NaCl may reduce the growth due to inhibitory effect of maintaining a very high concentration of glycerol within the cells. Chlorophyll content and cell number are good measures of biomass since intracellular glycerol concentrations increased in a broadly linear fashion when the salinity was increased from 0.1 – 4 M NaCl.

Dunaliella species synthesise glycerol in response to increasing external salinity. As the salinity is increased from 0.1 to 4 M NaCl, the level of intracellular glycerol increases in a linear fashion. The three strains leak significant amounts of glycerol into the medium on a continuous basis, although this should be very detrimental at high salinities. This property is associated with growth in batch and continuous cultures. It does not depend on a mutant strain as suggested by Hard and Gilmour (1991), but it is an intrinsic property of

at least these three strains of *Dunaliella*. This opens up a number of possibilities to improve the harvesting of glycerol and also improve the likelihood of a commercially successful method for glycerol production by *Dunaliella*.

Production of β-Carotene by *Dunaliella salina*

Chapter Five

5.1. Introduction

Dunaliella salina is the most salt-tolerant eukaryote known and is commonly found in salt lakes, solar salt works and other hypersaline water bodies (Borowitzka, 1992). It was recognised as an efficient biological source of β-carotene which accumulates within oily globules in the interthylakoid spaces of the chloroplast. This pigment protects the microalga against damage by high irradiation due to its absorption of the blue region of the spectrum and it is composed mainly of two stereoisomers: 9-*cis* and all-*trans* (Shaish *et al.*, 1992).

β-carotene is a valuable pigment and has many pharmaceutical and nutritional applications. It can be used as food colouring agent, pro-vitamin A (retinol) in food and animal feed, additive to multivitamin preparations and cosmetics (Garcia-Gonzalez *et al.*, 2005; Salguero *et al.*, 2003). Several studies claimed that β-carotene can act as an anti-cancer agent, immune-system stimulator, and degenerative diseases preventive because of its protective ability against activated oxygen radical forms (Salguero *et al.*, 2003).

Under proper inductive conditions, *D. salina* accumulates up to 14% of its dry weight as β-carotene (Borowitzka, 1992; Garcia-Gonzalez *et al.*, 2005; Lers *et al.*, 1990; Shaish *et al.*, 1992). It is generally known that cartenogenesis is greatest when growth is least (Borowitzka, 1990). High salinity, nitrogen deficiency, phosphorus deficiency, high light intensity, and high temperature are known factors that limit growth, but stimulate β-carotene production in *D. salina* (Borowitzka & Borowitzka, 1990; Borowitzka, 1990; Phadwal & Singh, 2003; Shaish *et al.*, 1992).

Most of the biotechnological and physiological studies on β-carotene production in *D. salina* were carried out using laboratory cultures at relatively high irradiance (> 200 μmol photons m^{-2} s^{-1}). Therefore, the physiological stress

resulting from such light levels may have interfered with the effects of the parameter under study such as nutrient starvation (Marín *et al.*, 1998).

The aim of the work described in this chapter was to examine the effects of growing two strains of *D. salina* (CCAP 19/18 and 19/30) at relatively low constant irradiance of 44-53 µmol photons m^{-2} s^{-1} combined with different concentrations of nitrogen ($NaNO_3$) on the growth and β-carotene content of these strains. In addition, cultures were grown in batch cultures and aerated with different concentrations of CO_2 to study the effects of this treatment on growth and β-carotene content. Finally, the halophile *D. salina* MUR 9 was cultivated in an outdoor raceway pond located at Murdoch University, Perth, Western Australia to investigate β-carotene production in a large-scale culture.

5.2. Results and Discussion

5.2.1. Morphology of the strains

The cells of *Dunaliella salina* CCAP 19/18, *D. salina* CCAP 19/30 and *D. salina* MUR 9 were examined microscopically and they were similar morphologically (Figure 5.1, A – C respectively). At low salinity (0.4 M NaCl), the colour of the cells was green (Figure 5.1, A and B). At high salinity above 1.72 M NaCl, however, massive accumulation of β-carotene turned the colour of the cells from green to red (see Figure 5.1, C). Among the tested strains, only *D. salina* 19/18 and MUR 9 can change colour from green to red which is indicative of the ability to synthesise large amounts of β-carotene under the appropriate growth conditions.

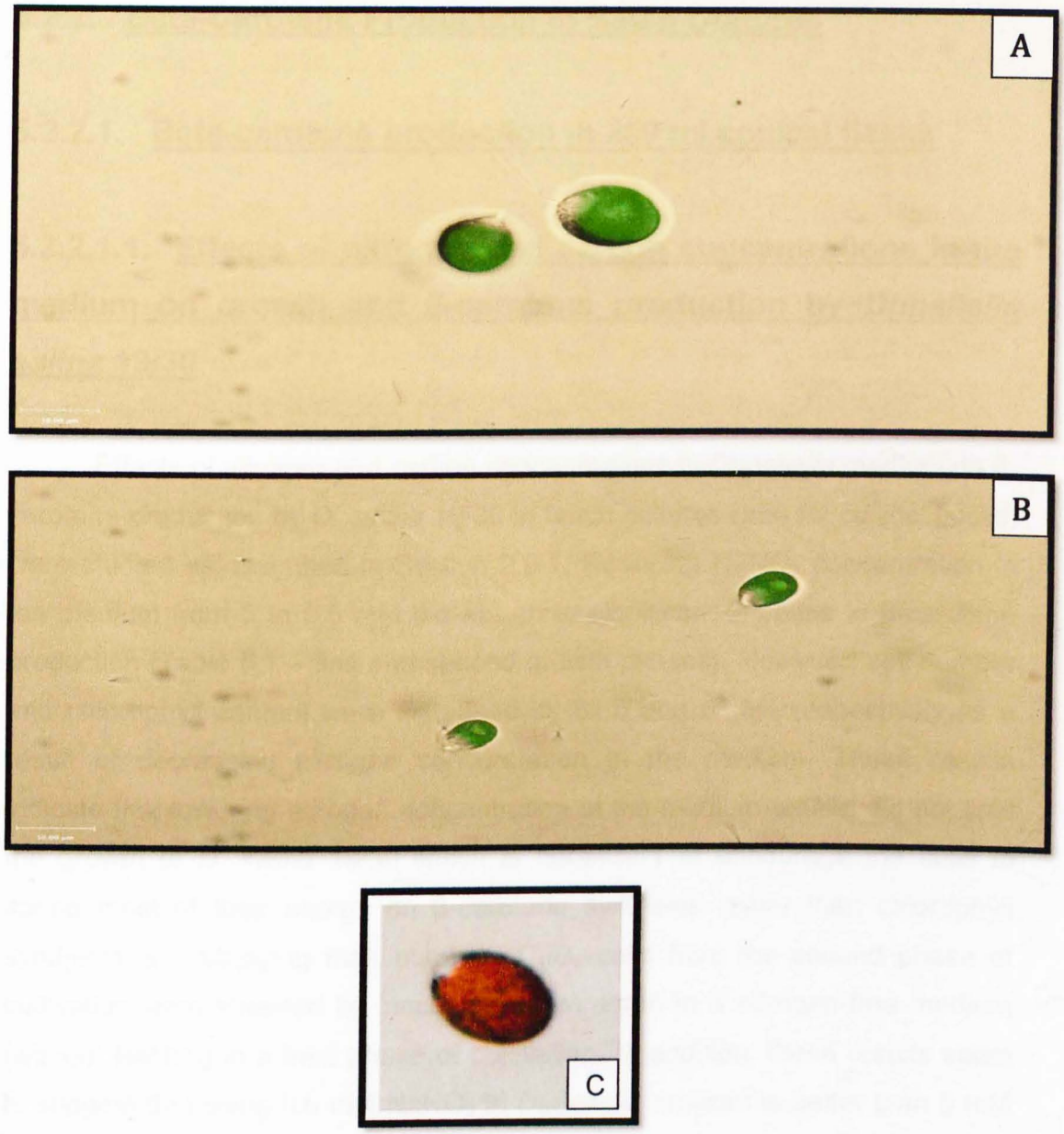

Figure 5.1. Light micrographs of *Dunaliella salina* CCAP 19/18 (A), *D. salina* CCAP 19/30 (B) and *D. salina* MUR 9 (C). The cells in A and B were grown at 0.4 M NaCl and the micrographs were taken by a fluorescence microscope (upright Olympus Bx-61) at 100x. The scale bar represents 10 µm. Micrograph C was adapted from Borowitzka and Siva (2007), the cells were grown in 5.17 M NaCl. Note the green colour of the cells and the two flagella for each cell in A and B, and the dark red colour of the cell in C due to the massive accumulation of β-carotene.

5.2.2. Beta-Carotene Production in Batch Cultures

5.2.2.1. Beta-carotene production in 250 ml conical flasks

5.2.2.1.1. Effects of nitrogen and carbon concentrations in the medium on growth and β-carotene production by *Dunaliella salina* 19/30

Effects of nitrogen and carbon concentrations in *Dunaliella* medium on β-carotene production by *D. salina* 19/30 in batch cultures (250 ml conical flasks) were studied as described in Section 2.9.1. Reducing $NaNO_3$ concentration in the medium from 5 to 0.5 mM did not show significant increase in β-carotene production (Table 5.1 – first and second growth phases). However, cell number and chlorophyll content were increased by 61.5 and 53.6% respectively as a result of decreasing nitrogen concentration in the medium. These results indicate that lowering nitrogen concentration in the medium tenfold did not limit the growth of *D. salina* 19/30 which is necessary to encourage the cells to spend most of their energy on β-carotene synthesis rather than chlorophyll synthesis or multiplying their number. Thus, cells from the second phase of cultivation were stressed by incubating them again in a nitrogen-free medium (without $NaNO_3$) in a third phase of cultivation. In addition, these results seem to suggest that using 0.5 mM $NaNO_3$ in *Dunaliella* medium is better than 5 mM for high biomass purposes.

Growing *D. salina* 19/30 in nitrogen-free medium did not induce β-carotene production. However, as expected cell number and chlorophyll content were decreased when the cells were grown in N-free medium (Table 5.1 – third growth phase); these decreases were small however.

The cultures used above were old (after three weeks of incubation) and therefore when no nitrogen source was available to the cells the CO_2 / HCO_3 concentration was low. Therefore supplying CO_2 to the cultures could increase β-carotene production because more carbon becomes available to the cells for growth and β-carotene production. Thus, in the fourth phase of cultivation (Table 5.1) the cultures were supplied with sterile 0.2 g $NaHCO_3$ 100 ml^{-1} culture which is double the amount used in normal subculturing of *D. salina*. There was little increase in β-carotene production after the addition of $NaHCO_3$. On the other hand, supplying the cultures with more $NaHCO_3$ resulted in a decrease in cell number and chlorophyll content (Table 5.1 – Fourth growth phase). That could be due to three reasons. First, more CO_2 in the medium could encourage the cells to photosynthesise more and as a result of that more O_2 accumulates in the culture and that could inhibit the growth (photoinhibition). Second, the cells were too old to grow and synthesise high amounts of β-carotene. Finally, the algae may prefer inorganic CO_2 over organic CO_2 ($NaHCO_3$).

Cultivation phase	Cell number (10^6 cells ml^{-1})	Chlorophyll content ($\mu g\ ml^{-1}$)	β-carotene content ($pg\ cell^{-1}$)
First	2.61, 2.76 Av. = 2.68	7.31, 7.8 Av. = 7.55	0.475, 0.476 Av. = 0.475
Second	4.30, 4.36 Av. = 4.33	11.45, 11.73 Av. = 11.6	0.526, 0.517 Av. = 0.521
Third	4.25, 4.21 Av. = 4.23	9.91, 9.62 Av. = 9.76	0.457, 0.436 Av. = 0.446
Fourth	3.42, 3.47 Av. = 3.44	8.72, 8.25 Av. = 8.48	0.616, 0.607 Av. = 0.611

Table 5.1. Growth and β-carotene production by *Dunaliella salina* 19/30 grown in batch cultures in a cultivation system composed of four phases of growth as follows. First, cells were grown at 5 mM $NaNO_3$; second, at 0.5 mM $NaNO_3$; third, N-free medium; fourth, 0.2 g $NaHCO_3$ was added to each culture (100 ml). Determinations were carried out in duplicate and therefore both values plus the average are shown for each treatment. Av. = average.

5.2.2.1.2. Effects of nitrogen concentration in the medium and bubbling CO_2 through cultures of two strains of *Dunaliella salina* on growth and β-carotene production

A new experiment was designed as described in Section 2.9.2 to study the effects of nitrogen concentration and bubbling CO_2 through fresh cultures of two strains of *D. salina*: 19/30 and 19/18 on growth and β-carotene production. Results are shown in Table 5.2 for *D. salina* 19/30 and in Table 5.3 for *D. salina* 19/18.

Growing *D. salina* 19/30 in nitrogen-free medium with bubbling of filtered 5% (v/v) CO_2 through the culture increased cell number by about 3.6 times in an incubation period of 8 days and decreased chlorophyll and β-carotene contents by approximately 0.18 and 1.3 times respectively (Table 5.2 – Treatment 1). However, growing the strain in 5 mM $NaNO_3$ medium with the same aeration system (5% CO_2) resulted in an increase in cell number and chlorophyll content by about 10.3 and 6.7 times respectively and a little decrease in β-carotene content (Table 5.2 – Treatment 2). These results indicate that nitrogen deficiency does limit the growth of this strain, but still no significant production of β-carotene was noticed.

When the strain *D. salina* 19/30 was grown in nitrogen-free medium with bubbling of filtered natural air (≈ 0.035% (v/v) CO_2) through the culture instead of filtered 5% CO_2, an increase of cell number by about 0.7 times and a decrease in chlorophyll and β-carotene contents by approximately 0.6 and 0.5 times respectively were observed (Table 5.2 – Treatment 3). However, growing the strain in 5 mM $NaNO_3$ medium with the same aeration system (natural air) resulted in an increase in cell number and chlorophyll content by about 7.9 and 4.3 times respectively and a decrease in β-carotene content by about 0.3 times (Table 5.2 – Treatment 4). In agreement with the results obtained in treatments 1 and 2 above, these results also indicate that nitrogen deficiency does limit the growth of this strain. Comparing the results obtained in all treatments (1-4) for the strain *D. salina* 19/30, it can be concluded that growing the strain in 5 mM $NaNO_3$ medium and bubbling filtered 5% CO_2 through the culture give maximum productivity of both growth and β-carotene. No overproduction of β-carotene was seen under N-limited conditions.

Treatment of batch culture	Cell number (10^6 cells ml^{-1})		Chlorophyll content (µg ml^{-1})		β-carotene content (pg $cell^{-1}$)	
	Initial	After 8 days of resuspension	Initial	After 8 days of resuspension	Initial	After 8 days of resuspension
1	1.43, 1.3 Av. = 1.36	4.68, 5.23 Av. = 4.95	5.36, 5.31 Av. = 5.33	4.52, 4.48 Av. = 4.5	0.662, 0.662 Av. = 0.662	0.291, 0.287 Av. = 0.289
2	1.5, 1.5 Av. = 1.5	15.23, 15.78 Av. = 15.5	5.68, 5.76 Av. = 5.72	39.31, 38.01 Av. = 38.66	0.566, 0.561 Av. = 0.563	0.521, 0.521 Av. = 0.521
3	1.75, 1.75 Av. = 1.75	3.05, 2.81 Av. = 2.93	5.82, 5.81 Av. = 5.81	3.58, 3.48 Av. = 3.53	0.486, 0.484 Av. = 0.485	0.329, 0.325 Av. = 0.327
4	1.22, 1.37 Av. = 1.29	10.15, 10.23 Av. = 10.19	5.21, 5.28 Av. = 5.24	22.81, 22.12 Av. = 22.46	0.550, 0.554 Av. = 0.552	0.418, 0.415 Av. = 0.416

Table 5.2. Growth and β-carotene production by *Dunaliella salina* 19/30 grown in batch cultures. The cultures were grown initially for 7 days under the growth conditions described in Section 2.9.2 and then four treatments were initiated as follows. **1**. Culture was resuspended in a N-free medium and bubbled with 5% (v/v) CO_2. **2**. Culture was resuspended in a medium containing 5 mM $NaNO_3$ and bubbled with 5% CO_2. **3**. Culture was resuspended in a N-free medium and bubbled with filtered natural air (≈ 0.035% (v/v) CO_2). **4**. Culture was resuspended in a medium containing 5 mM $NaNO_3$ and bubbled with filtered natural air. Determinations were carried out in duplicate and therefore both values plus the average are shown for each treatment. Av. = average.

Treatment of batch culture	Cell number (10^6 cells ml^{-1})		Chlorophyll content ($\mu g\ ml^{-1}$)		β-carotene content ($pg\ cell^{-1}$)	
	Initial	After 8 days of suspension	Initial	After 8 days of suspension	Initial	After 8 days of suspension
1	0.31, 0.27 Av. = 0.29	0.32, 0.34 Av. = 0.33	3.23, 3.17 Av. = 3.2	1.57, 1.61 Av. = 1.59	9.14, 9.14 Av. = 9.14	27.3, 27.1 Av. = 27.2
2	0.26, 0.24 Av. = 0.25	3.65, 3.59 Av. = 3.62	3.07, 3.01 Av. = 3.04	28.63, 28.89 Av. = 28.76	8.14, 8.06 Av. = 8.1	4.14, 4.14 Av. = 4.14
3	0.31, 0.3 Av. = 0.3	0.36, 0.29 Av. = 0.32	3.02, 3.1 Av. = 3.06	1.7, 1.76 Av. = 1.73	4.16, 4.01 Av. = 4.08	23.0, 22.68 Av. = 22.84
4	0.31, 0.21 Av. = 0.26	3.09, 3.2 Av. = 3.14	2.71, 2.83 Av. = 2.77	17.1, 17.04 Av. = 17.07	6.78, 6.86 Av. = 6.82	2.05, 2.01 Av. = 2.03

Table 5.3. Growth and β-carotene production by *Dunaliella salina* 19/18 grown in batch cultures. The cultures were grown initially for 7 days under the growth conditions described in Section 2.9.2 and then four treatments were initiated as follows. **1**. Culture was resuspended in a N-free medium and bubbled with 5% (v/v) CO_2. **2**. Culture was resuspended in a medium containing 5 mM $NaNO_3$ and bubbled with 5% CO_2. **3**. Culture was resuspended in a N-free medium and bubbled with filtered natural air (≈ 0.035% (v/v) CO_2). **4**. Culture was resuspended in a medium containing 5 mM $NaNO_3$ and bubbled with filtered natural air. Determinations were carried out in duplicate and therefore both values plus the average are shown for each treatment. Av. = average.

Growing *D. salina* 19/18 in nitrogen-free medium with bubbling of filtered 5% CO_2 through the culture increased cell number and β-carotene content by about 0.14 and 3 times respectively in an incubation period of 8 days and decreased chlorophyll content by approximately 50% (Table 5.3 – Treatment 1). However, growing the strain in 5 mM $NaNO_3$ medium with the same aeration system (5% CO_2) resulted in an increase in cell number and chlorophyll content by about 14.5 and 9.5 times respectively and a decrease in β-carotene content by approximately 50% (Table 5.3 – Treatment 2). These results indicate that the strain *D. salina* 19/18 is better than the strain 19/30 in terms of β-carotene production because it accumulates large amounts of β-carotene inside the cells with an excess of 94 times that accumulated in the strain 19/30 under nitrogen limitation (Table 5.3 – Treatment 1). These large amounts of β-carotene turned the colour of the cells from green to red (see Figure 5.1, C). In addition, the results obtained indicate that nitrogen deficiency does limit the growth of this strain and therefore enable more production of β-carotene.

When the strain *D. salina* 19/18 was grown in nitrogen-free medium with bubbling of filtered natural air (≈ 0.035% (v/v) CO_2) through the culture instead of filtered 5% (v/v) CO_2, cell number almost remained the same, chlorophyll content decreased by about 0.75 times, and β-carotene content increased by approximately 5.6 times (Table 5.3 – Treatment 3). However, growing the strain in 5 mM $NaNO_3$ medium with the same aeration system (natural air) resulted in an increase in cell number and chlorophyll content by about 12 and 6 times respectively and a decrease in β-carotene content by about 70% (Table 5.3 – Treatment 4). In agreement with the above results, these results also confirmed that nitrogen deficiency does limit the growth of this strain.

Comparing the results obtained in all treatments (1-4) for the strain *D. salina* 19/18, it can be concluded that limiting the growth by nitrogen deficiency and bubbling filtered natural air through the culture gives maximum productivity of β-carotene. In addition, maximum growth can be achieved by growing the strain in 5 mM $NaNO_3$ medium and bubbling filtered 5% CO_2 through the culture.

5.2.2.2. Beta-carotene production in a 2 L airlift fermenter

D. salina 19/18 was grown in a 2 L airlift fermenter in a cultivation system composed of three phases as described in Section 2.9.3. The first phase involved growing the cells in *Dunaliella* medium containing 5 mM $NaNO_3$ to allow the algae to thrive and increase in cell number. The purpose of the second phase was to limit the growth and induce the cells to accumulate large amounts of β-carotene by exposing them to nitrogen deficiency stress. The third phase of cultivation was to test the physiological ability of the cells to return to the first phase of cultivation i.e. the physiological state before the stress was imposed.

Results of growth and β-carotene production by *D. salina* 19/18 in the above system are shown in Table 5.4. It shows that cell number increased from 2.5×10^4 to 7.7×10^5 cells ml^{-1} in the first phase i.e. an increase of about 30 times over 7 days incubation (Table 5.4 – First phase). This result confirmed that growing the cells in a medium containing all necessary nutrients without stress does allow good growth. It is noteworthy that the cells in this phase of cultivation are green in colour indicating a healthy physiological state (Figure 5.2).

In the second phase cell number varied between 1 and 1.6 × 10^6 cells ml^{-1}, but no overall increase was seen (1.03 × 10^6 cells ml^{-1} at the beginning and 1.07 × 10^6 cells ml^{-1} after 7 days of incubation) (Table 5.4 – Second phase). This result confirms the results obtained when growing the algae in 250 ml conical flasks (Section 5.2.2.1.2) i.e. that nitrogen deficiency in the medium does limit the growth enabling the algae to spend the available energy towards β-carotene synthesis rather than growth. Beta-carotene content in the second phase increased from 2.91 to 19.86 pg $cell^{-1}$ over an incubation period of 7 days i.e. an increase of approximately 7 times (Table 5.4 – Second phase). This result suggests that nitrogen deficiency in the medium is a good inducer for β-carotene synthesis. It also confirmed the capability of the strain *D. salina* 19/18 to accumulate large amounts of β-carotene inside the cells when compared with the strain *D. salina* 19/30 (see Sections 5.2.2.1.1 and 5.2.2.1.2). This was confirmed by observing the change in the colour of *D. salina* 19/18 cells from green (first phase) to dark red (second phase) (Figures 5.2 and 5.3). It is noteworthy that the cell number at the beginning of the second phase was higher than that at the end of the first phase. That was because some cells did stick to the walls of the fermenter and when emptying the fermenter to prepare the culture for the second phase most of those cells were resuspended with the culture.

Incubation time (days)	First phase	Second phase		Third phase	
	Cell number (10^6 cells ml^{-1})	Cell number (10^6 cells ml^{-1})	β-carotene content (pg $cell^{-1}$)	Cell number (10^6 cells ml^{-1})	β-carotene content (pg $cell^{-1}$)
0	0.025	1.03	2.91	0.1	16.5
1	0.013	1.31	3.39	0.67	4.77
2	0.013	1.28	5.08	1.13	2.78
3	0.014	1.63	5.52	1.67	2.15
4	0.6	1.23	8.94	3.14	1.22
5	0.63	1.53	8.66	3.14	1.54
6	0.85	1.39	9.89	3.05	1.81
7	0.77	1.07	19.86	3.85	0.76

Table 5.4. Growth and β-carotene production by *D. salina* 19/18 in a 2 L airlift fermenter. Cultivation was carried out into three phases as described in Section 2.9.3.

Removing the stress imposed on the cells in the second phase of cultivation increased cell number from 1.0×10^5 to 3.85×10^6 cells ml^{-1} i.e. an increase by about 39 times over an incubation period of 7 days (Table 5.4 – Third phase). This result suggests that the cells can return to their first physiological state (first phase) after returning the nitrogen source to the medium. This was confirmed by observing the change of the colour of the cells from dark red (Figure 5.3) to greenish yellow (Figure 5.4). It is noteworthy that the cell number at the beginning of the third phase was very low when compared to that at the end of the second phase. That was because 1.5 L of the culture was harvested at the end of the second phase and replaced with the same volume of fresh medium (see Section 2.9.3). β-carotene content in the third phase decreased from 16.5 to 0.76 pg $cell^{-1}$ i.e. a decrease of about 22 times. The β-carotene content per cell has thus returned to approximately the same value at the beginning of the second phase. This again indicates that the cells have returned to their original physiological state.

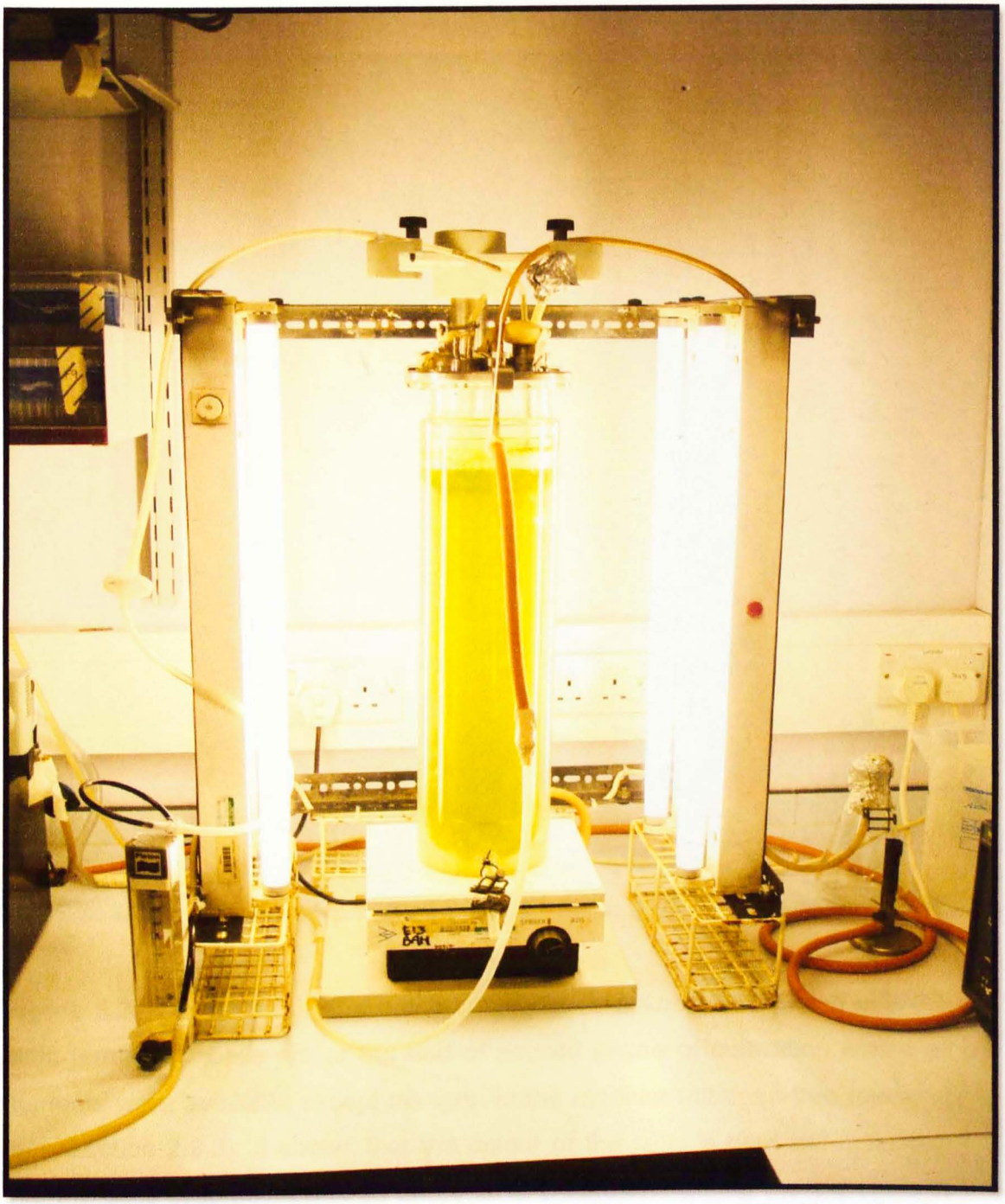

Figure 5.2. Photograph of a batch culture of *D. salina* 19/18 grown in a 2 L airlift fermenter. Cells are in the first phase of cultivation where all nutrients were available in the medium (see Section 2.9.3). It shows that the colour of the cells is green indicating a healthy state of the cells.

Figure 5.3. Photograph of a batch culture of *D. salina* 19/18 grown in a 2 L airlift fermenter. Cells are at the end of second phase of cultivation where all nutrients were available except nitrogen in the medium (nitrogen-free medium) (see Section 2.9.3). It shows that the colour of the cells is dark red indicating that the cells accumulated large amounts of β-carotene during the exposure of nitrogen deficiency stress. The fermenter and the light source are to the right of the picture whereas CO_2 / O_2 cylinder and water bath are to left of the picture.

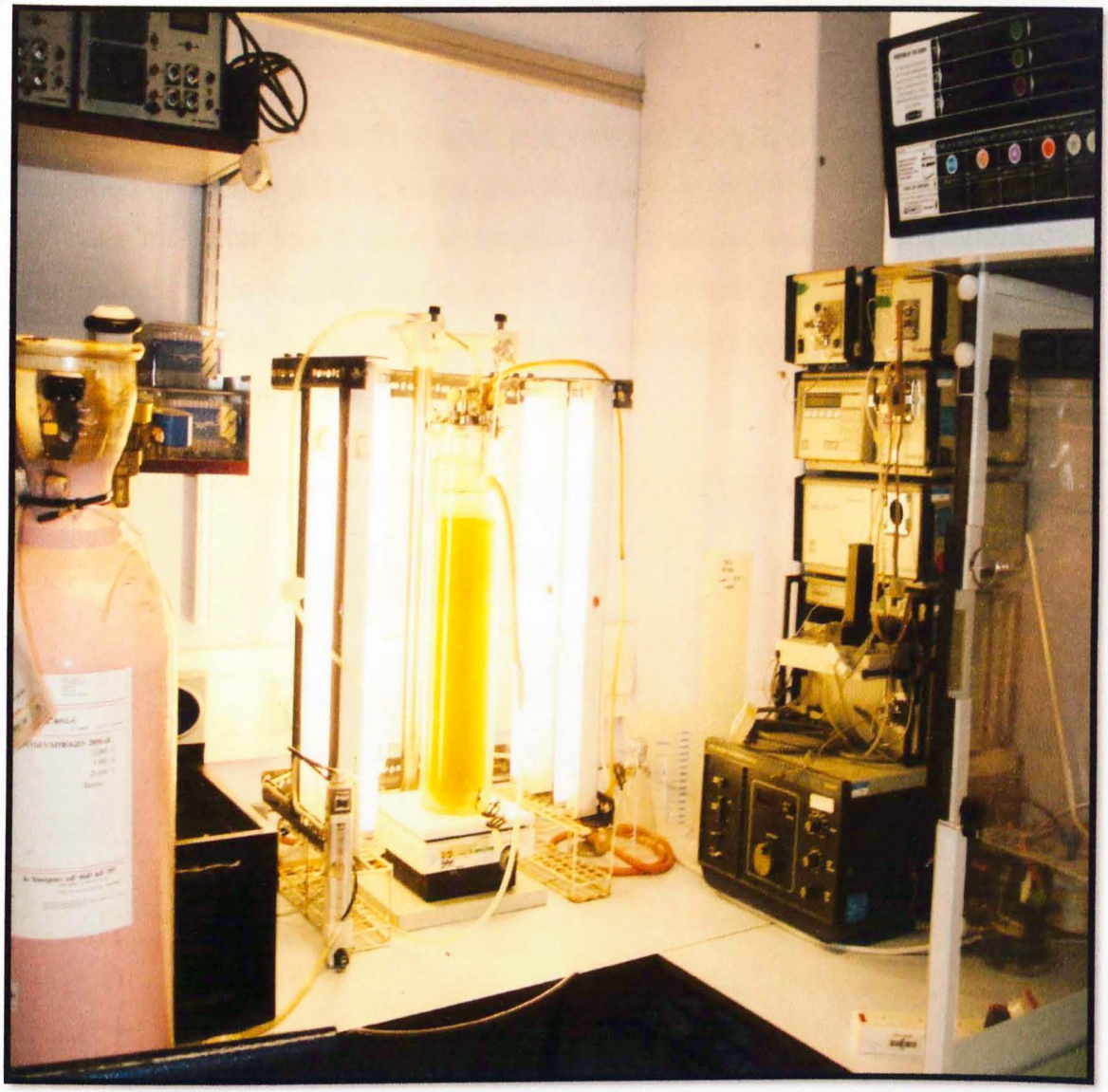

Figure 5.4. Photograph of a batch culture of *D. salina* 19/18 grown in a 2 L airlift fermenter. Cells are in the third phase of cultivation where all nutrients were available as in the first phase of cultivation (see Section 2.9.3). It shows that the colour of the cells is greenish yellow indicating that the cells are returning to normal physiological state before the exposure of nitrogen deficiency stress.

5.2.3. Large-Scale Culturing of *D. salina* in an Outdoor Raceway Pond for β-carotene Production

Dunaliella salina MUR 9 was cultured outdoors in a raceway pond as described in Section 2.9.4. Cell number increased from 2×10^4 cells ml^{-1} to 4×10^4 cells ml^{-1} after just 5 days incubation. Microscopic examinations showed that the cells were healthy, motile, and turned from light green to yellow. Beta-carotene synthesis in the cells was induced by increasing salinity in the pond from 12.5 to 20% (w/v) NaCl (see Section 2.9.4).

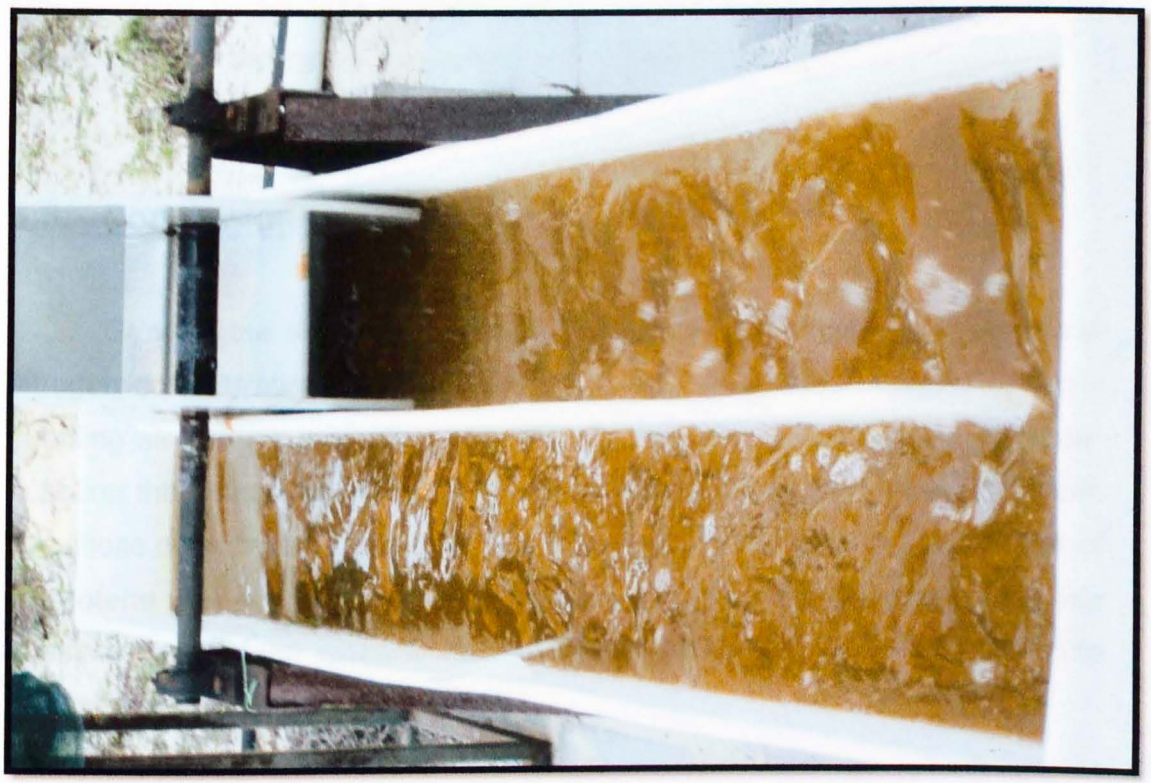

Figure 5.5. Increasing salinity from 12.5 to 20% NaCl resulted in changing of *D. salina* MUR 9 colour from green to dark yellow because of massive accumulation of β-carotene inside the cells.

Unfortunately, heavy rain and low water temperature (14-18 °C during day and 6-10 °C during night) at the time of the experiment resulted in loss of the culture in the pond and therefore hindered carrying out β-carotene production analyses in the pond. However, it was noticed that the cells started to accumulate more β-carotene once the salinity in the pond increased from 12.5 to 20% (w/v) NaCl (2.14 – 3.42 M NaCl) and that was detected indirectly by observing the change of the colour of the cells from green to dark yellow (see Figure 5.5). This was confirmed by microscopic examinations on samples from the pond which showed that the colour of the cells were dark yellow to red (data not shown). It is noteworthy that β-carotene levels in the cells should be confirmed by β-carotene analyses as in Section 5.2.2. However, it was not possible due to the loss of the culture as mentioned above.

5.3. Conclusions

Growing the strain *D. salina* 19/30 in *Dunaliella* medium containing low nitrogen concentration (0.5 mM $NaNO_3$) or in a nitrogen-free medium without bubbling air through the culture did not limit the growth of the strain. However, bubbling the culture with either 5% CO_2 or natural air under nitrogen limitation conditions does limit the growth of this strain but still no significant production of β-carotene was noticed. The highest growth and β-carotene production by this strain were obtained when it was grown in 5 mM $NaNO_3$ medium and the culture was bubbled with 5% (v/v) CO_2.

The strain *D. salina* 19/18 is better than the strain 19/30 in terms of β-carotene productivity because the former accumulated large amounts of β-carotene with an excess of 94 times that accumulated by the latter strain under nitrogen limitation conditions. Therefore, the colour of cells of the strain 19/18 changes from green under optimum growth conditions to dark red under inductive conditions for β-carotene. However, it is not the case with the strain

19/30 which changes colour from green to yellow under the above growth conditions. Nitrogen-free medium does limit the growth of the strain *D. salina* 19/18 and act as an inducer for β-carotene production.

Maximum productivity of β-carotene by the strain *D. salina* 19/18 was obtained under nitrogen limitation conditions and at the same time bubbling the culture with either filtered natural air in 250 ml flasks or filtered 5% CO_2 in a 2 L fermenter. In addition, maximum growth can be achieved by growing the strain in 5 mM $NaNO_3$ medium and bubbling filtered 5% CO_2 through the culture.

Characterization of an Isolate of *Halomonas* sp.

Chapter Six

6.1. Introduction

Many microalgae and cyanobacteria have been exploited commercially for food and animal feed as well as production of valuable products in many parts in the world (see Section 1.7). High biomass productivity of these microorganisms is required to meet market demands. However, the productivity of microalgae is sometimes reduced by contamination by other microorganisms particularly bacteria, which are well known as major contaminants of microalgae cultures. Moreover, consuming microalgae that are heavily contaminated with bacteria, particularly pathogenic bacteria, could be hazardous to human health.

Dunaliella salina is a unicellular microalga which has been cultivated commercially in many countries for production of β-carotene. Induction of β-carotene in this microalga can be performed by many factors, one of which is high salinity (2 – 5 M NaCl) in growth medium (see Section 1.6.1.4). Therefore, many predators especially bacteria are eliminated or at least minimised by such high salinity. However, laboratory physiological studies on microalgae at salinities below 2 M NaCl are often subject to bacterial contamination. Moreover, performing molecular techniques such as cloning and transformation on microalgae requires axenic cultures.

During the course of β-carotene production studies using *Dunaliella salina* (see Chapter 5), a bacterial contamination was observed in some cultures of *D. salina*. The bacterial contaminant was isolated and identified to the genus level by using 16S rRNA gene sequence of the isolate. The isolate belong to *Halomonas* genus and was named *Halomonas* sp. NAH1 (see Chapter 3). It was deposited in the National Collection of Industrial, Marine and Food Bacteria (NCIMB), Aberdeen, UK under accession number NCIMB 14402.

This chapter describes some characteristics of *Halomonas* sp. NAH1 including contamination levels of *D. salina* cultures by *Halomonas* sp. NAH1, growth on different media, salinity tolerance, carbon sources characterisation, biochemical characteristics, susceptibility to some antibiotics, uptake of ^{14}C-glucose and ^{3}H-glycerol, and respiration measurements.

6.2. Results and Discussion

6.2.1. Contamination level of *Dunaliella salina* CCAP 19/30 cultures by *Halomonas* sp. NAH1

Halomonas sp. NAH1 was found as a major bacterial contaminant of *Dunaliella salina* cultures grown at a wide range of NaCl concentrations (0.1 – 2.5 M NaCl) (see Section 2.1.5). The highest bacterial contamination was noticed in *D. salina* culture grown at 1.5 M NaCl (Table 6.1). This was an indication that the contaminant was a moderately halophilic bacterium. Therefore in order to isolate this contaminant bacterium, a suitable medium at this level of salinity was needed.

D. salina culture – conc. of NaCl in the medium (M)	Level of bacterial contamination
0.1	+
0.4	+
1.5	+++
2.5	+
3.5	-
4.0	-

Table 6.1. Assessment of *Halomonas* sp. NAH1 contamination of *D. salina* cultures grown at different salinities. (+) = the white bacterial pellet was smaller than the green algal pellet, (+++) = the white bacterial pellet was as big as the green algal pellet, (-) = no white pellet was noticed.

6.2.2. Growth on nutrient agar and broth media at different temperatures

Halomonas sp. NAH1 was grown initially on nutrient agar and broth media containing 1.5 M NaCl at 25, 30, and 37 °C (see Section 2.1.5). The results are shown in Table 6.2. These results suggest that the bacterial contaminant had an optimum growth temperature at 30 °C, but also grow at 25 and 37 °C. Therefore, 30 °C was routinely used to incubate this bacterium in this project.

Incubation temperature (°C)	Type of growth medium	Bacterial growth		
		24 h	48 h	72 h
25	agar	-	+	+++
	broth	-	+	+++
30	agar	++	+++	+++
	broth	++	+++	+++
37	agar	+	++	+++
	broth	+	++	+++

Table 6.2. Growth of *Halomonas* sp. NAH1 on nutrient agar and broth media (1.5 M NaCl) at different incubation temperatures. (–), (+), (++), and (+++) = level of bacterial growth on nutrient agar plates (density of colonies) or in nutrient broth tubes (turbidity) as follows: no, low, medium, and high growth respectively.

6.2.3. Morphology

Halomonas sp. NAH1 was examined microscopically as described in Section 2.2. The results revealed that the strain was a Gram-negative bacterium, rod shaped cells (see Figure 6.1), motile. Colonies were raised, round and entire, but small like pin prick.

Figure 6.1. Light micrograph of *Halomonas* sp. NAH1 which was taken by a dark field microscope.

6.2.4. Antimicrobial susceptibility test

Sensitivity to four antibiotics by the isolate *Halomonas* sp. NAH1 was tested using standard disk method as described in Section 2.19. The results are shown in Table 6.3 and indicate that tetracycline (50 µg) has the most effect on *Halomonas* sp. NAH1 growth when compared with other antibiotics used in this test. Therefore, *Halomonas* sp. NAH1 is susceptible to tetracyclin at a concentration of 50 µg and thus this antibiotic could be added to *Dunaliella* cultures to minimize or get rid of the bacterial contaminant (*Halomonas* sp. NAH1) especially when axenic cultures are required. However, more investigation is needed to find out the stability of this antibiotic in the presence of light.

Antibiotic disc	Inhibition zone (mm)
Streptomycin (25 µg)	0.90 ± 0.10
Neomycin (30 µg)	0.56 ± 0.07
Penicillin G (10 units)	1.10 ± 0.15
Tetracycline (50 µg)	2.03 ± 0.14

Table 6.3. Inhibition zones of *Halomonas* sp. NAH1 growth by four antimicrobial susceptibility test disks. Means and standard errors for three replicates are shown.

6.2.5. Growth in a complex medium

Halomonas sp. NAH1 was grown in BM complex medium (Appendix A3) containing 7.5% (w/v) NaCl (≈ 1.28 M NaCl) as described in Sections 2.5.1 and 2.14.3. Growth curve experiment was carried out at this level of salinity by monitoring population density (OD at 600 nm) (Figure 6.2). *Halomonas* sp. NAH1 grew well on the medium used with a specific growth rate of 0.212 and a generation time of 3.26 h (Figure 6.2).

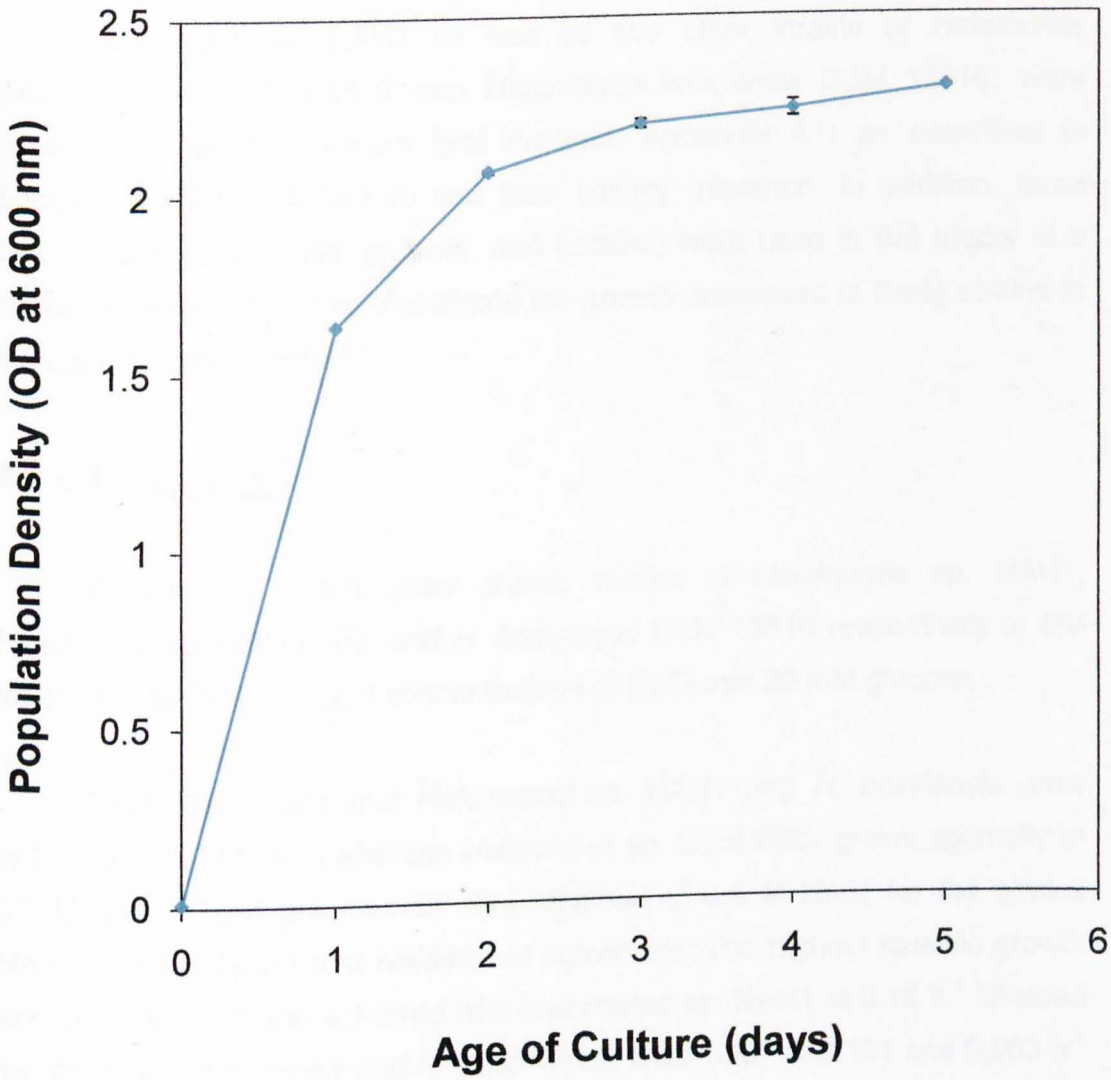

Figure 6.2. Growth curve of *Halomonas* sp. NAH1 in BM complex medium (Appendix A3) at 7.5 % (w/v) NaCl (≈ 1.28 M NaCl). Means and standard errors for three replicates are shown.

6.2.6. Salinity tolerance and characterization of carbon sources of three strains of *Halomonas*

Halomonas sp. NAH1 as well as two other strains of *Halomonas* (*Halomonas* sp. DSM 6507 and *Halomonas boliviensis* DSM 15516) were grown in a defined medium (BM medium, Appendix A1) as described in Sections 2.5.1 and 2.14.3 to test their salinity tolerance. In addition, three carbon sources (glucose, glycerol, and betaine) were used in BM media at a concentration of 20 mM to investigate the growth responses of these strains to the carbon sources used.

6.2.6.1. Glucose

Figures 6.3 – 6.5 show growth curves of *Halomonas* sp. NAH1, *Halomonas* sp. DSM 6507, and *H. boliviensis* DSM 15516 respectively in BM medium containing different concentrations of NaCl and 20 mM glucose.

Table 6.4 shows that *Halomonas* sp. NAH1 and *H. boliviensis* grow optimally at 1.0 M NaCl whereas *Halomonas* sp. DSM 6507 grows optimally at 0.5 M NaCl. Good growth was also obtained at 0.5 M NaCl for the strains *Halomonas* sp. NAH1 and *Halomonas boliviensis*. The highest specific growth rate at 2.0 M NaCl was achieved with *Halomonas* sp. NAH1 at 0.15 h^{-1} followed by *Halomonas boliviensis* and *Halomonas* sp. DSM 6507 at 0.131 and 0.063 h^{-1} respectively.

Growth at higher level of salinity in the medium (3.0 M NaCl) was variable among the tested strains. *Halomonas* sp. NAH1 was the most halotolerant among them, followed by *Halomonas* sp. DSM 6507 with generation times of 31.5 and 63 h respectively (Table 6.4). The growth of *H. boliviensis*, however, was completely inhibited by 3.0 M NaCl (Figure 6.5).

Therefore, this strain appears to be more sensitive to very high salinity in minimal medium.

Growth at lower salinity (0.1 M NaCl) was quite similar to that at 0.5 M NaCl for both *Halomonas* sp. NAH1 and *Halomonas* sp. DSM 6507 at a generation time of 2.97 and 3.0 h respectively (Table 6.4). In contrast, *H. boliviensis* showed a lag phase lasted for about 37 h after which the cells entered log phase (Figure 6.5) indicating that the utilization of glucose by this strain at this low level of salinity (0.1 M NaCl) is less efficient than that at 0.5 M NaCl. Therefore, it can be concluded that *H. boliviensis* does require at least 0.5 M NaCl in BM defined medium for efficient growth.

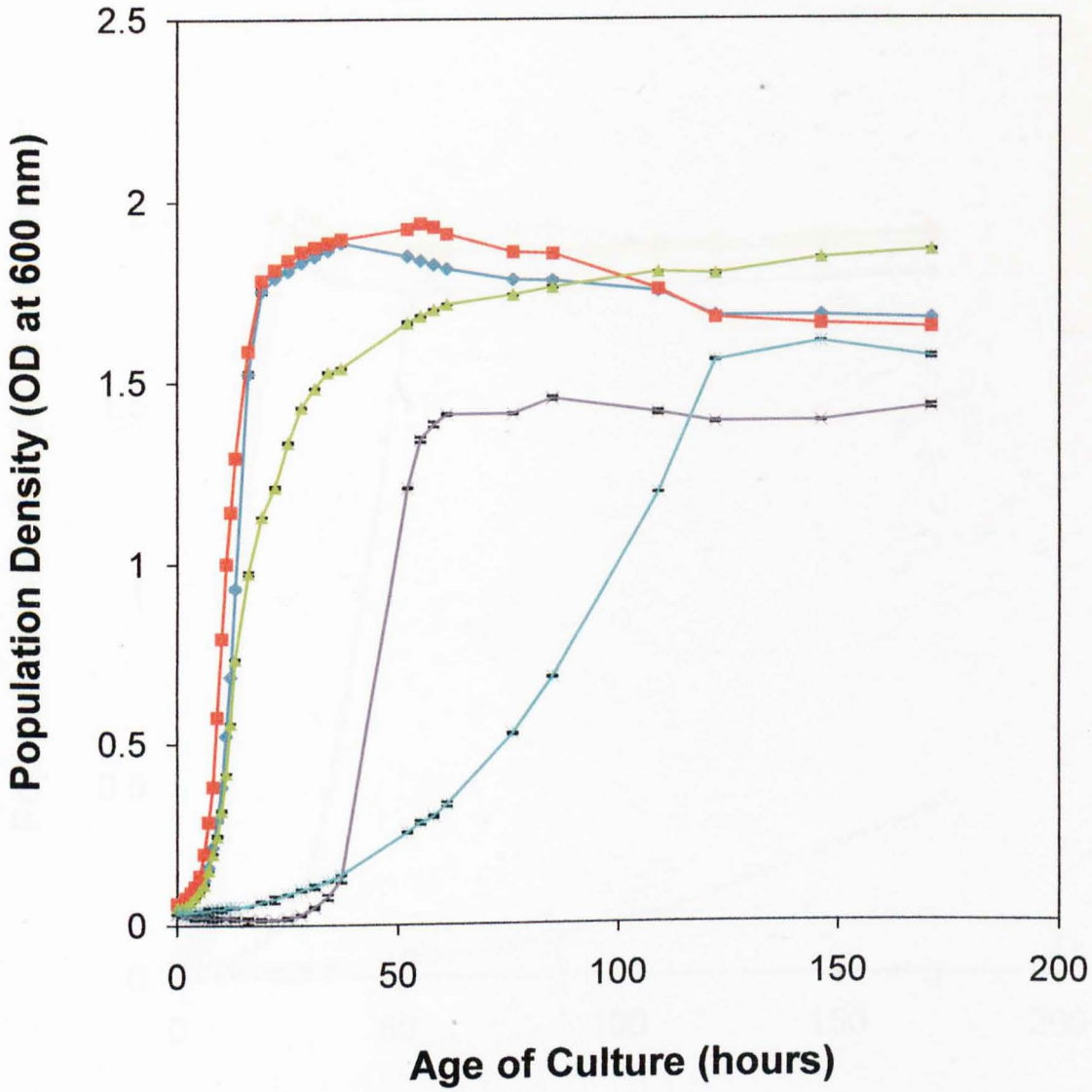

Figure 6.3. Growth curves of *Halomonas* sp. NAH1 grown in BM defined medium which contaning different concentrations of NaCl and 20 mM glucose as a carbon source. Means and standard errors for three replicates are shown.

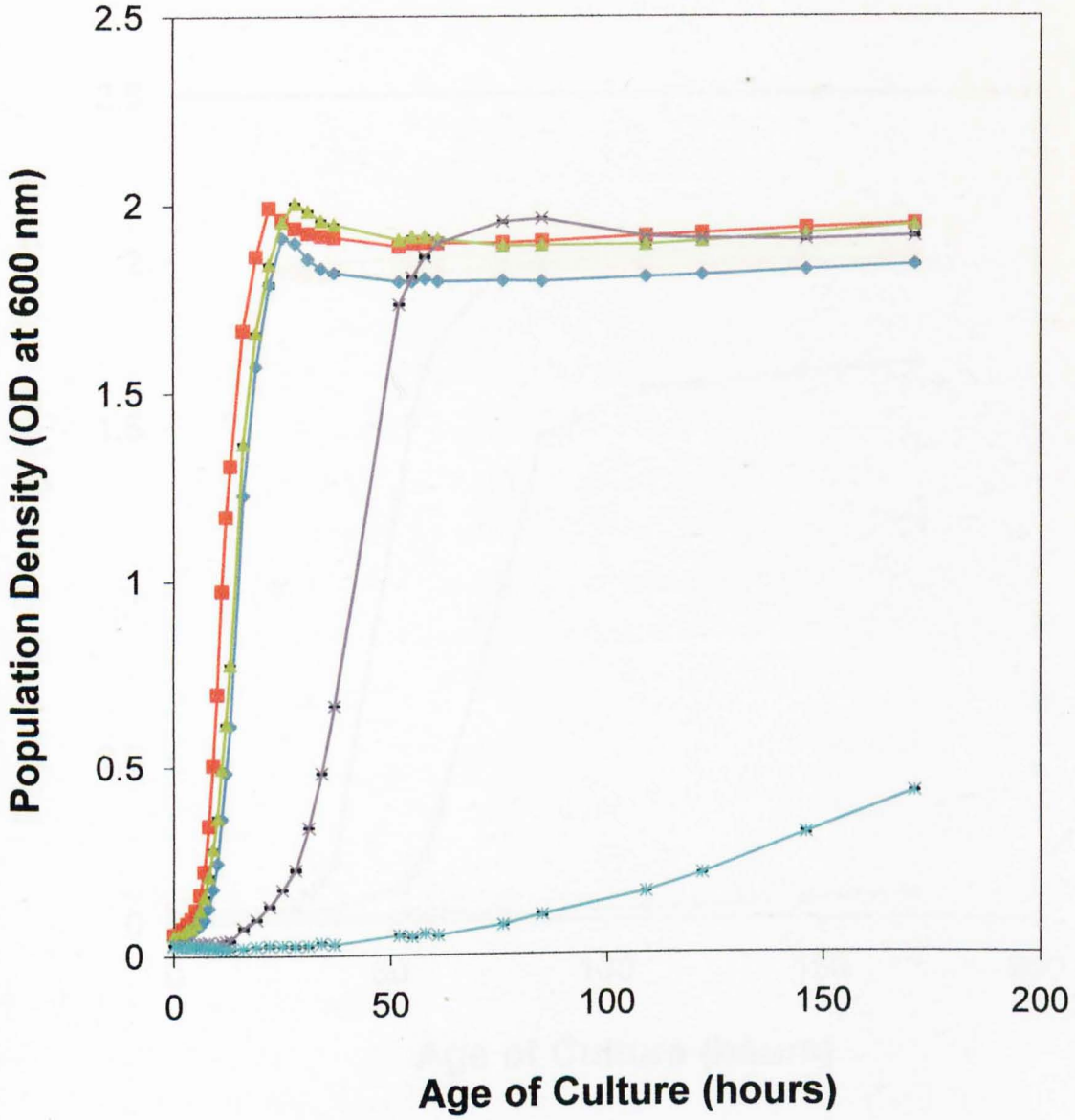

Figure 6.4. Growth curves of *Halomonas* sp. DSM 6507 grown in BM defined medium which containing different concentrations of NaCl and 20 mM glucose as a carbon source. Means and standard errors for three replicates are shown.

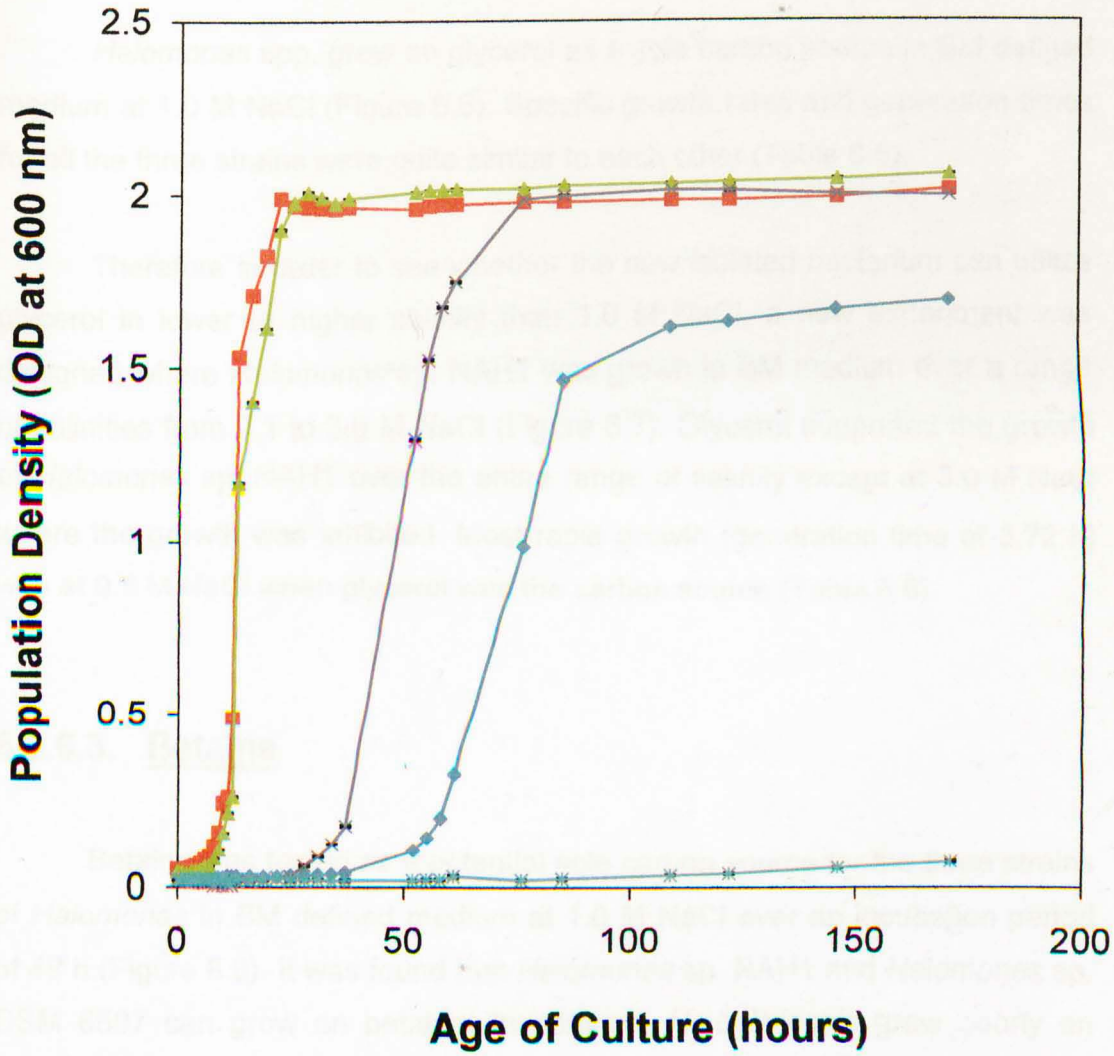

Figure 6.5. Growth curves of *Halomonas boliviensis* DSM 15516 grown in BM defined medium which containing different concentrations of NaCl and 20 mM glucose as a carbon source. Means and standard errors for three replicates are shown.

6.2.6.2. Glycerol

Halomonas spp. grew on glycerol as a sole carbon source in BM defined medium at 1.0 M NaCl (Figure 6.6). Specific growth rates and generation times for all the three strains were quite similar to each other (Table 6.5).

Therefore in order to see whether the new isolated bacterium can utilize glycerol in lower or higher salinity than 1.0 M NaCl, a new experiment was designed where *Halomonas* sp. NAH1 was grown in BM medium over a range of salinities from 0.1 to 3.0 M NaCl (Figure 6.7). Glycerol supported the growth of *Halomonas* sp. NAH1 over the entire range of salinity except at 3.0 M NaCl where the growth was inhibited. Most rapid growth (generation time of 3.72 h) was at 0.5 M NaCl when glycerol was the carbon source (Table 6.6).

6.2.6.3. Betaine

Betaine was tested as a potential sole carbon source for the three strains of *Halomonas* in BM defined medium at 1.0 M NaCl over an incubation period of 48 h (Figure 6.8). It was found that *Halomonas* sp. NAH1 and *Halomonas* sp. DSM 6507 can grow on betaine. In contrast, *H. boliviensis* grew poorly on betaine under the test conditions. *Halomonas* sp. DSM 6507 showed the highest specific growth rate (0.094 h^{-1}) and lowest generation time (7.37 h) (Table 6.7). In general, the growth on betaine was low when compared with glucose and glycerol.

A number of compounds were tested as potential sole carbon sources for *Halomonas* strains. Of the compounds examined, glucose supported the most rapid growth rate. Glycerol could also be utilized as the sole carbon source, although the growth was slower than with glucose. However, under the test conditions betaine could be utilized only by *Halomonas* sp. NAH1 and

Halomonas sp. DSM 6507 since *H. boliviensis* showed no noticeable growth when betaine was used as a sole carbon source. These results indicate the presence of catabolic pathways for glucose, glycerol and betaine with the above exception of *H. boliviensis* with betaine.

Glucose contains 6 carbon atoms whereas glycerol and betaine contain 3 and 5 carbon atoms respectively. This might explain the high specific growth rate which was obtained when glucose was the sole carbon source compared with that obtained with glycerol and betaine. This means the higher the carbon atoms in a carbon source the higher the bacterial growth within the constraints of this experiment because more energy will be available to bacteria to utilize and divide.

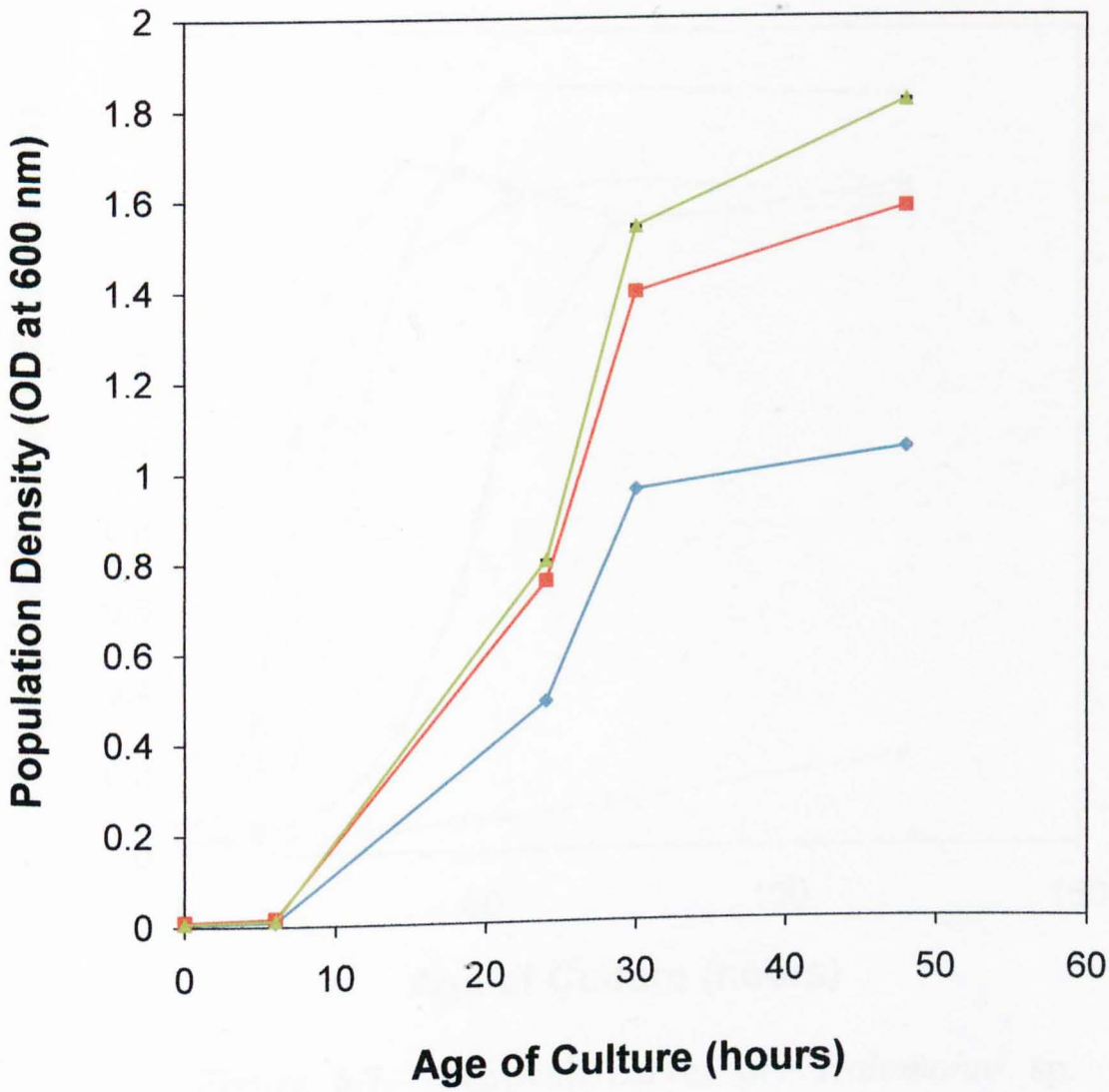

Figure 6.6. Growth curves of *Halomonas* spp. grown in BM defined medium containing 1.0 M NaCl and 20 mM glycerol as a carbon source. Means and standard errors for three replicates are shown.

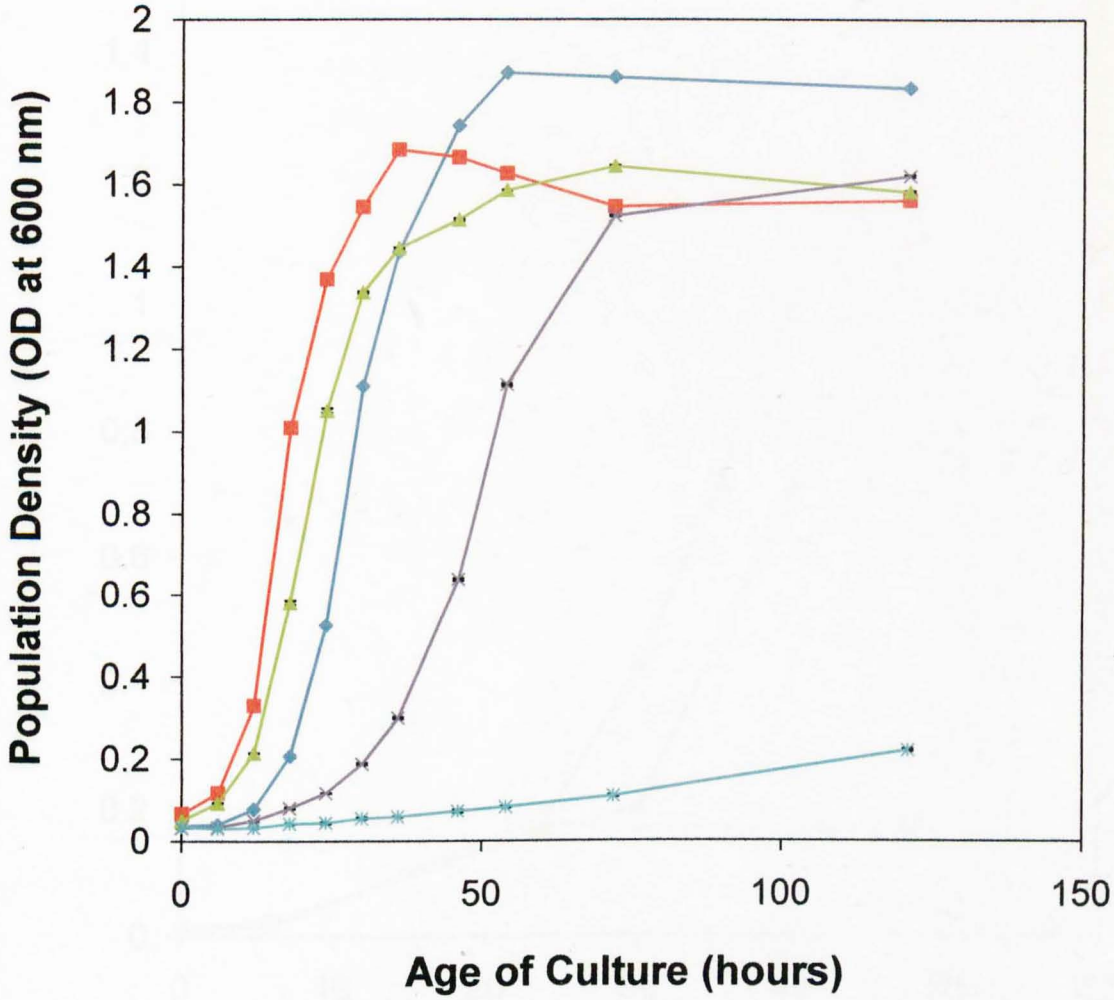

Figure 6.7. Growth curves of *Halomonas* sp. NAH1 grown in BM defined medium containing different concentrations of NaCl and 20 mM glycerol as a carbon source. Means and standard errors for three replicates are shown.

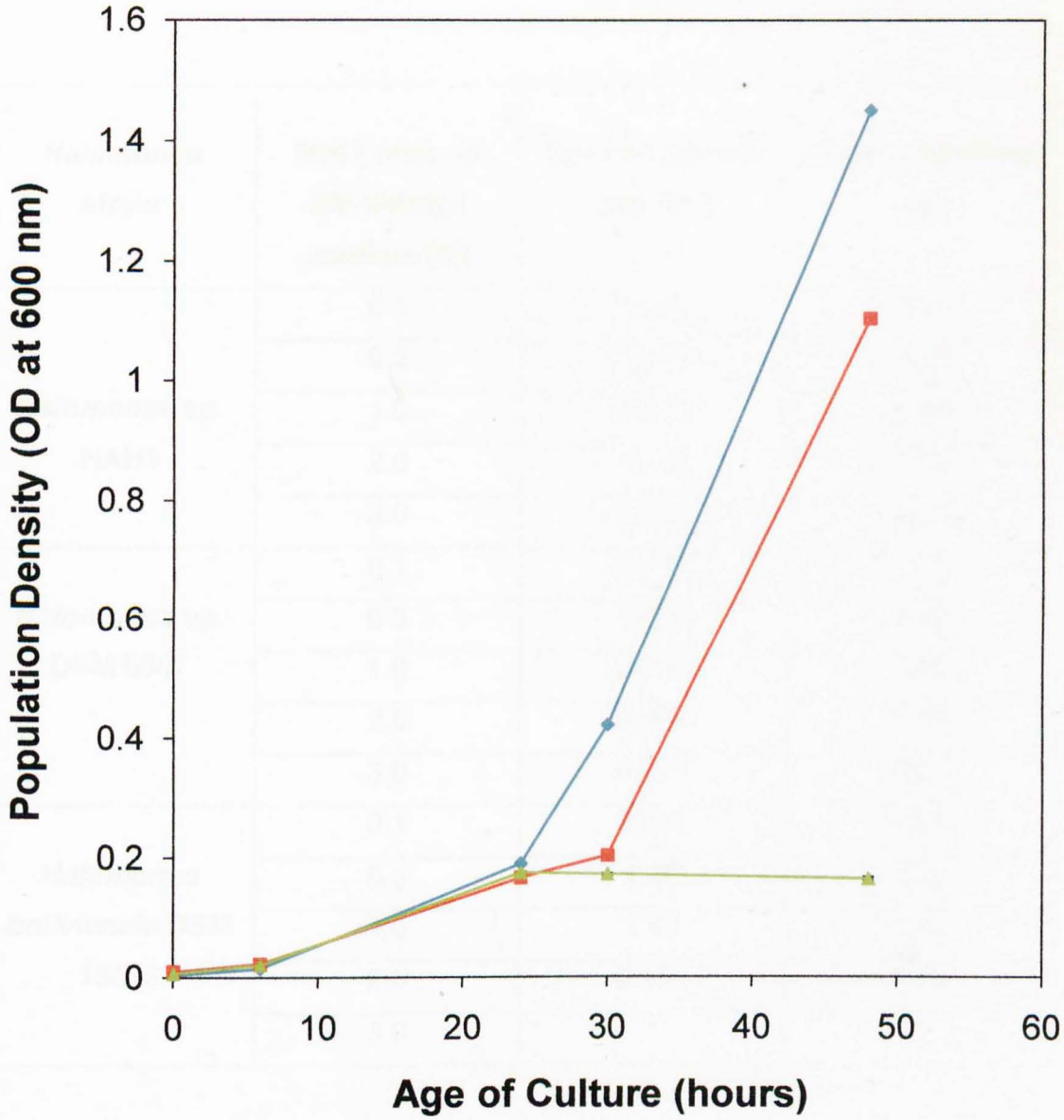

Figure 6.8. Growth curves of *Halomonas* spp. grown in BM defined medium containing 1.0 M NaCl and 20 mM betaine as a carbon source. Means and standard errors for three replicates are shown.

Halomonas strain	NaCl conc. in BM defined medium (M)	Specific growth rate (h^{-1})	Generation time (h)
Halomonas sp. NAH1	0.1	0.233	2.97
	0.5	0.243	2.85
	1.0	0.281	2.46
	2.0	0.15	4.62
	3.0	0.022	31.5
Halomonas sp. DSM 6507	0.1	0.231	3.0
	0.5	0.28	2.47
	1.0	0.188	3.68
	2.0	0.063	11.0
	3.0	0.011	63.0
Halomonas boliviensis DSM 15516	0.1	0.061	11.3
	0.5	1.15	0.6
	1.0	1.49	0.46
	2.0	0.131	5.29
	3.0	*	*

Table 6.4. Specific growth rates and generation times of *Halomonas* spp. grown in BM defined medium which containing different concentrations of NaCl and 20 mM glucose. * = No log phase.

***Halomonas* strain**	**Specific growth rate (h^{-1})**	**Generation time (h)**
Halomonas sp. NAH1	0.112	6.18
Halomonas sp. DSM 6507	0.101	6.86
Halomonas boliviensis DSM 15516	0.108	6.41

Table 6.5. Specific growth rates and generation times of *Halomonas* spp. grown in BM defined medium containing 1.0 M NaCl and 20 mM glycerol.

NaCl conc. in BM defined medium (M)	**Specific growth rate (h^{-1})**	**Generation time (h)**
0.1	0.124	5.58
0.5	0.186	3.72
1.0	0.132	5.25
2.0	0.072	9.62
3.0	*	*

Table 6.6. Specific growth rates and generation times of *Halomonas* sp. NAH1 grown in BM defined medium containing different concentrations of NaCl and 20 mM glycerol. * = No log phase.

Halomonas strain	Specific growth rate (h^{-1})	Generation time (h)
Halomonas sp. NAH1	0.068	10.19
Halomonas sp. DSM 6507	0.094	7.37
Halomonas boliviensis DSM 15516	*	*

Table 6.7. Specific growth rates and generation times of *Halomonas* spp. grown in BM defined medium which containing 1.0 M NaCl and 20 mM betaine. * = No log phase.

6.2.7. Uptake of ^{14}C-glucose and ^{3}H-glycerol

The uptake of ^{14}C-glucose and ^{3}H-glycerol by *Halomonas* sp. NAH1 were measured as described in Section 2.16. The results are shown in Figures 6.9 and 6.10. There was a rapid initial uptake of glucose, which quickly tailed off at around 15 pmoles mg^{-1} protein and only reached 20 pmoles mg^{-1} protein after 60 minutes (Figure 6.9). In contrast, the uptake rate of glycerol in the same medium above was more or less linear slow uptake to reach 40 pmoles mg^{-1} protein after 15 minutes. The glycerol uptake continued over the full 60 minutes ending at 60 pmoles mg^{-1} protein (Figure 6.10).

These results seem to suggest that glucose is taken up by *Halomonas* sp. NAH1 more rapidly, but glycerol is taken up to a higher concentration. This may indicate a slow metabolism of glycerol. The results are consistent with the data of growth curves, specific growth curves, and generation times of *Halomonas* sp. NAH1 cultures grown in BM defined media at same salinity (0.5 M NaCl) but in different carbon sources (either glucose or glycerol) at the same concentration in the media (20 mM). The highest biomass obtained with glucose after 36 h of incubation was about OD_{600} = 1.9 (Figure 6.3), whereas it was 1.68 (Figure 6.7) when glycerol was the carbon source in the medium. Similarly, specific growth rates and generation times were higher with glucose than with glycerol (Tables 6.4 and 6.6).

6.2.8. Respiration measurements

The rate of oxygen uptake was measured in *Halomonas* sp. NAH1 cultures as described in Section 2.15. Figure 6.11 shows the respiration rates of *Halomonas* sp. NAH1 cells grown in BM defined media at 1.0 M NaCl and at 20 mM of glucose, glycerol, or betaine. The highest rate of respiration was found in betaine grown cells and it was about three fold or more than that in glucose or glycerol grown cells.

Changing the carbon source in the same culture seems to have little effect on respiration rates in all three cultures of *Halomonas* sp. NAH1 because respiration rate values were quite similar to each other for the same culture. Moreover, there was no significant difference between respiration rates in cells grown in glucose and those grown in glycerol (Figure 6.11). This information supports the growth curve data (Figures 6.3 and 6.7) where biomass densities in cells grown in glucose or glycerol were not much different.

The growth of *Halomonas* NAH1 on betaine was much slower and only started after a long period of slow growth (Figure 6.8). This is in contrast to the respiration rate results in this section, since betaine grown cells showed the highest respiration rate. This apparent paradox may be resolved by assuming that the betaine grown cells are under stress and this is reflected in a higher respiration rate.

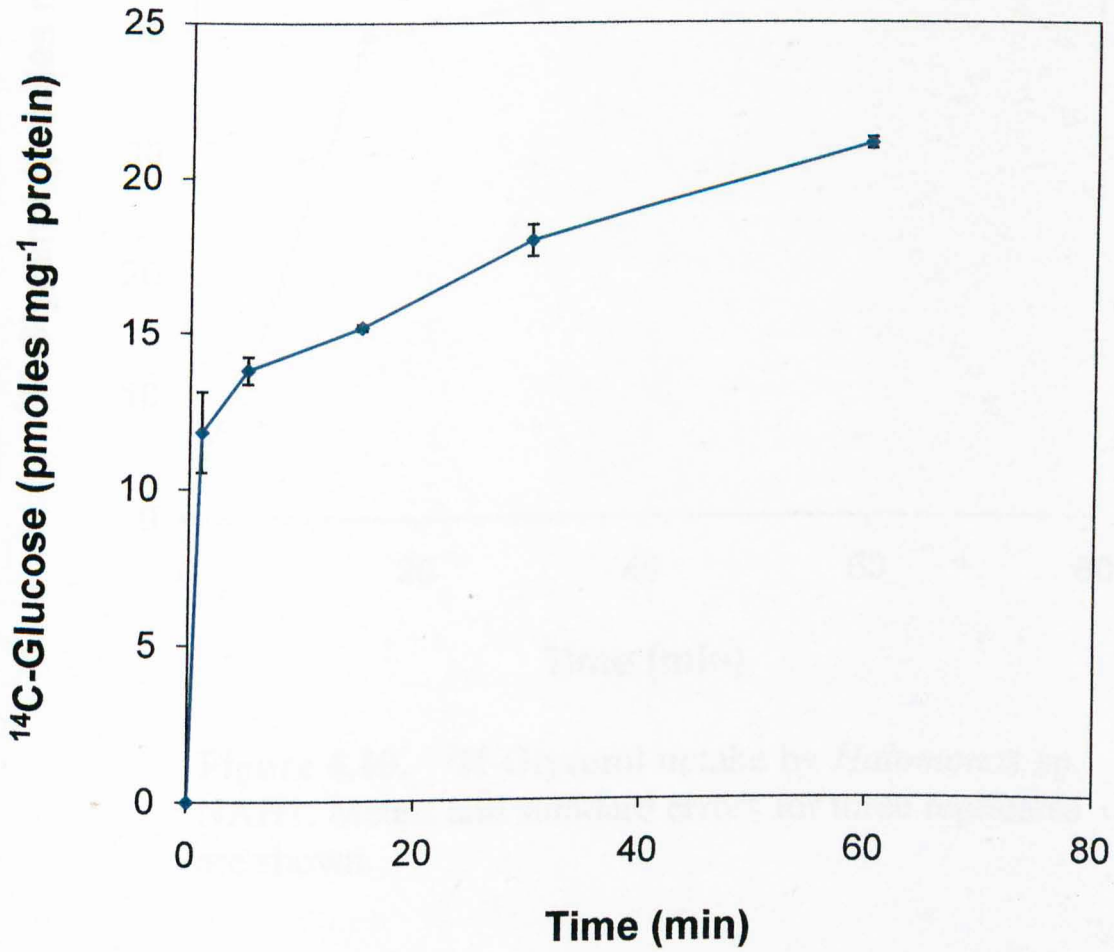

Figure 6.9. ^{14}C-Glucose uptake by *Halomonas* sp. NAH1. Means and standard errors for three replicates are shown.

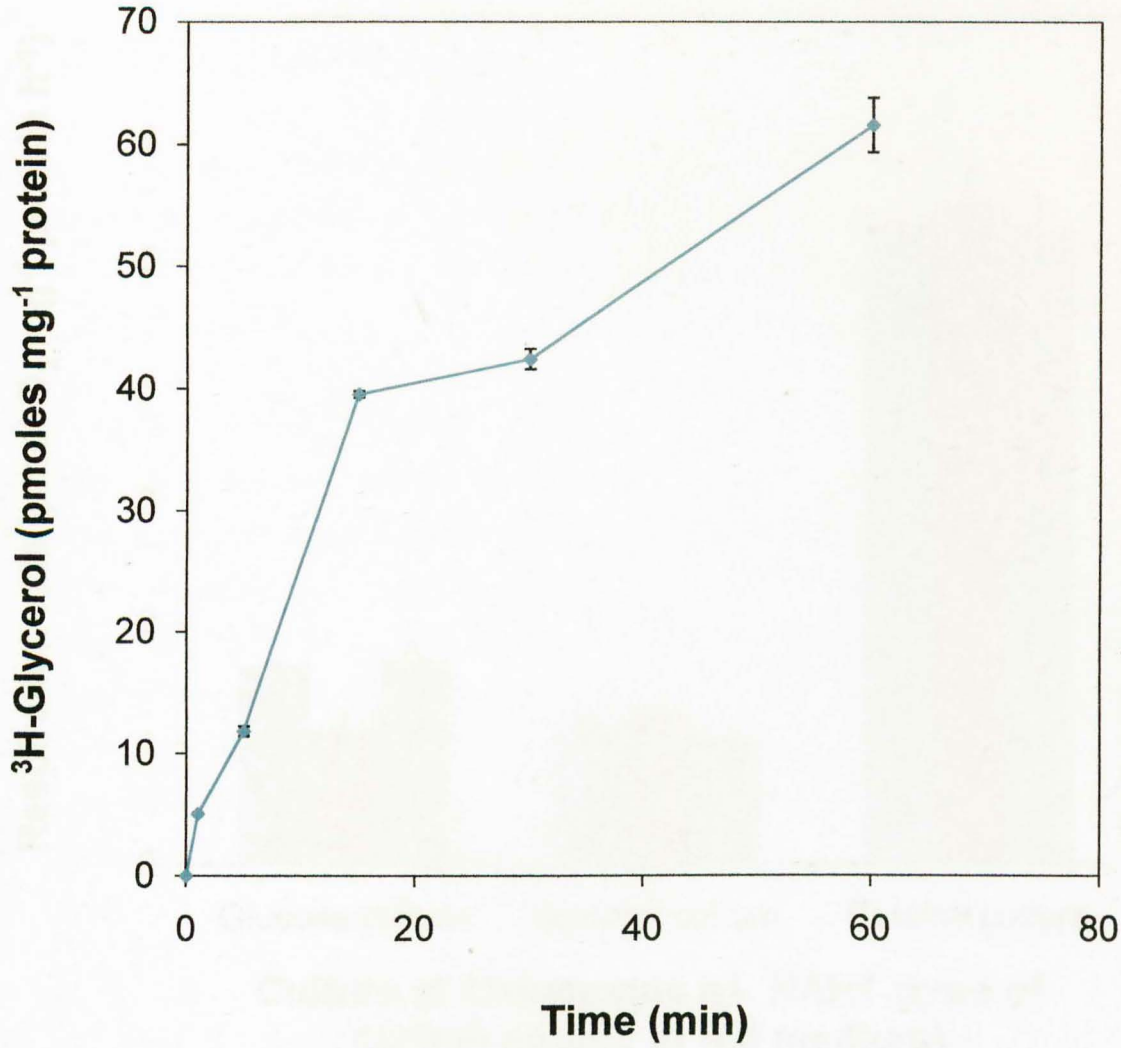

Figure 6.10. ^{3}H-Glycerol uptake by *Halomonas* sp. NAH1. Means and standard errors for three replicates are shown.

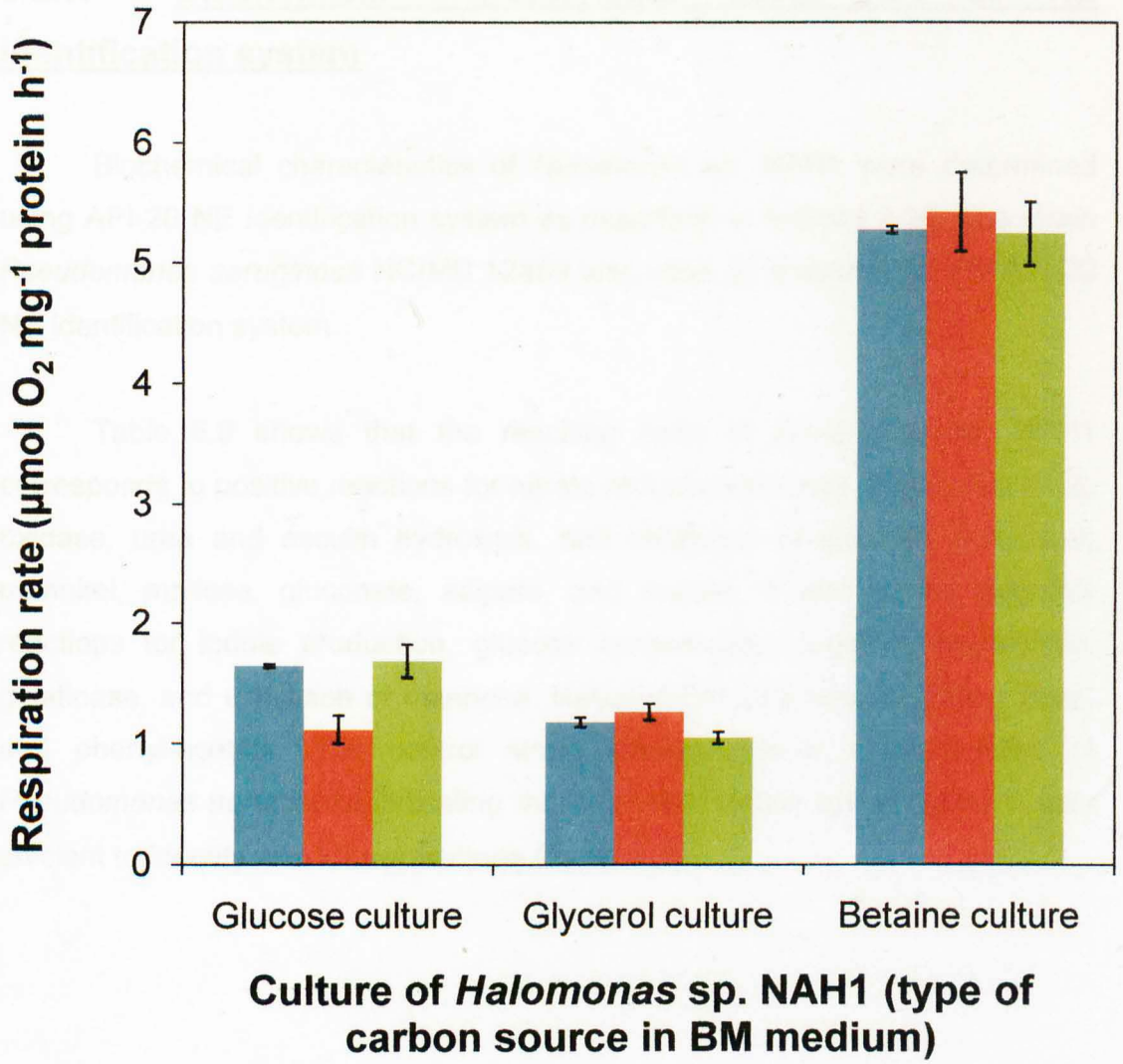

Figure 6.11. Respiration rates of *Halomonas* sp. NAH1 cells grown in BM defined media at 1.0 M NaCl and at 20 mM of glucose, glycerol, or betaine. Means and standard errors for three replicates are shown.

6.2.9. Biochemical characteristics using API 20 NE identification system

Biochemical characteristics of *Halomonas* sp. NAH1 were determined using API 20 NE identification system as described in Section 2.20. The strain *Pseudomonas aeruginosa* NCIMB 12469 was used as a control for the API 20 NE identification system.

Table 6.8 shows that the resulting code of *Halomonas* sp. NAH1 corresponds to positive reactions for nitrate reduction to nitrite, β-galactosidase, oxidase, urea and esculin hydrolysis, and utilization of glucose, arabinose, mannitol, maltose, gluconate, adipate, and malate. It also shows negative reactions for indole production, glucose fermentation, arginine dihydrolase, gelatinase, and utilization of mannose, N-Acetyl-Glucosamine, caprate, citrate, and phenyl-acetate. The control strain showed typical characteristics of *Pseudomonas aeruginosa* indicating that the identification system used is very efficient to identify well known bacteria (Table 6.8).

Number	Test	Results			
		P. aeruginosa		*H.* NAH1	
		24 h	48 h	24 h	48 h
1	Nitrate reduction	+	*	+	*
2	Indole production	-	*	-	*
3	Glucose fermentation	-	*	-	*
4	Arginine dihydrolase	+	+	-	-
5	Urease	-	-	+	+
6	Esculin hydrolysis	-	-	+	+
7	Gelatinase	+	+	-	-
8	β-galactosidase	-	-	+	+
Assimilation of					
9	Glucose	+	+	+	+
10	Arabinose	-	-	-	+
11	Mannose	-	-	-	-
12	Mannitol	+	+	-	+
13	N-Acetyl-Glucosamine	+	+	-	-
14	Maltose	-	-	+	+
15	Gluconate	+	+	-	+
16	Caprate	+	+	-	-
17	Adipate	+	+	-	+
18	Malate	+	+	+	+
19	Citrate	+	+	-	-
20	Phenyl-acetate	-	-	-	-
21	Oxidase	+	*	+	*
Resulting code		1154575		1665664	
Interpretation		very good identification		unacceptable profile	
Significant taxa (99.5 %)		*P. aeruginosa*		No significant taxa	

Table 6.8. Biochemical profiles of *Halomonas* sp. NAH1 and *Pseudomonas aeruginosa* NCIMB 24169 (control) after 24 and 48 h of incubation using API 20 NE identification system. Key: (-) = Negative, (+) = Positive, (*) = read only once at 24 h of incubation.

6.3. Conclusions

Halomonas sp. NAH1 was a major contaminant of *Dunaliella salina* cultures particularly those grown at 1.5 M NaCl. Optimum growth temperature of this bacterium was at 30 °C. The isolate grew well on BM complex medium at 1.28 M NaCl. It grew optimally at 1.0 M NaCl when compared to other concentrations of NaCl used. It was the most halotolerant among the strains tested.

The strain *Halomonas boliviensis* DSM 15516 appears to be more sensitive to very high salinity in minimal medium when compared to other strains tested. However, this strain does require at least 0.5 M NaCl for efficient growth.

Glucose supported the most rapid growth rate for all strains tested. Glycerol could also be utilized as the sole carbon source, although the growth was slower than with glucose. However, under the test conditions betaine could be utilized only by *Halomonas* sp. NAH1 and *Halomonas* sp. DSM 6507 since *H. boliviensis* showed no noticeable growth when betaine was used as a sole carbon source. These results indicate the presence of catabolic pathways for glucose, glycerol and betaine with the above exception of *H. boliviensis* with betaine.

The results of ^{14}C-glucose and ^{3}H-glycerol uptake seem to suggest that glucose is taken up by *Halomonas* sp. NAH1 quicker, but glycerol is taken up to a higher concentration. This may indicate a slow metabolism of glycerol.

The highest respiration rate in *Halomonas* sp. NAH1 was found in betaine grown cells and it was about three fold or more than that in glucose or glycerol grown cells possibly reflecting that the cells are stressed by growth on

betaine. There was no significant difference between respiration rates in cells grown in glucose and those grown in glycerol.

Tetracycline (50 µg) has the most effect on *Halomonas* sp. NAH1 growth when compared with other antibiotics used.

Although *Halomonas* is not usually one of the organisms which can be identified by using API 20 NE identification system, it is a useful kit to determine the biochemical characteristics of the isolate *Halomonas* sp. NAH1.

Growth and Chemical Composition of *Arthrospira fusiformis* and *Spirulina platensis* in Batch Cultures

Chapter Seven

7.1. Introduction

Arthrospira fusiformis and *Spirulina platensis* are filamentous cyanobacteria and cosmopolitan in distribution (Ciferri & Tiboni, 1985; Kebede & Ahlgren, 1996). They appear to be very promising sources of food or animal feed due to their unique composition: high protein content (up to 66% of the dry weight), low content of nucleic acids, high concentrations of vitamins and other growth factors, and the presence of a cell wall that is more easily digestible than that of yeasts or most eukaryotic algae (Ciferri & Tiboni, 1985; Jassby, 1988). In addition, many medicinal effects of *Arthrospira* (*Spirulina*) have been reported (Section 1.6.4.3). These unique features have led to mass cultivation of some species of *Arthrospira* (*Spirulina*) in many parts of the world. Therefore, the worldwide production of *Spirulina* may now exceed 3000 tons a year on a dry weight basis (Hu, 2004; Sarada *et al.*, 1999; Shimamatsu, 2004).

Selection of appropriate strains and providing growth conditions that favour more biomass and/or desirable products are very important for the success of the commercial production of *Arthrospira* (*Spirulina*). The work presented in this chapter describes some physiological and biotechnological features of *Arthrospira fusiformis* CCAP 1475/8 and *Spirulina platensis* UTEX LB 2340 in terms of growth, salinity tolerance, compatible solutes, biomass production, total protein content, phycobiliprotein content, and chlorophyll *a* content.

7.2. Results and Discussion

7.2.1. Growth at Different Concentrations of NaCl in the Medium

Growth curves for *S. platensis* and *A. fusiformis* at an incubation temperature of 37 °C and at a wide range of salinities (0.017-1.0 M NaCl) are shown in Figures 7.1 and 7.2 respectively. Also, specific growth rates and generation times were calculated from the growth curves and are shown in Tables 7.1 and 7.2. Optimum growth for *S. platensis* was found at 0.1 M NaCl with a specific growth rate of 1.19 day^{-1} and generation time of 0.58 day. *A. fusiformis*, however, showed optimum growth at 0.5 M NaCl with a specific growth rate and generation time of 1.35 day^{-1} and 0.51 day respectively. Growth was only slightly decreased at 0.017 and 0.5 M NaCl for *S. platensis*, and at 0.017 and 0.1 M NaCl for *A. fusiformis*. It was found that 1.0 M NaCl inhibited the growth of both strains, but some growth did take place over the 11 days of the incubation period (Figures 7.1 and 7.2). This indicates that the maximum salinity tolerance for both strains is in excess of 1.0 M NaCl. In general, both strains exhibited similar growth patterns, but *S. platensis* showed better growth at 1.0 M NaCl.

S. platensis was isolated from Natron Lake, Chad (http://web.biosci.utexas.edu/utex) whereas *A. fusiformis* was isolated from Lake Chitu (hypersaline), Ethiopia (http://www.ccap.ac.uk). The fact that they originated from different habitats would suggest that they might have different salinity ranges in terms of the major cation Na^+. However, it does not seem so at least under the experimental conditions applied here. Therefore, this would limit their mass cultivation to freshwater, brackish waters, or sea water. Further studies on the basic physiology of these two important strains are needed to shed light on their ionic requirements which could increase biomass production and therefore reduce the cost of commercial production of *Spirulina*.

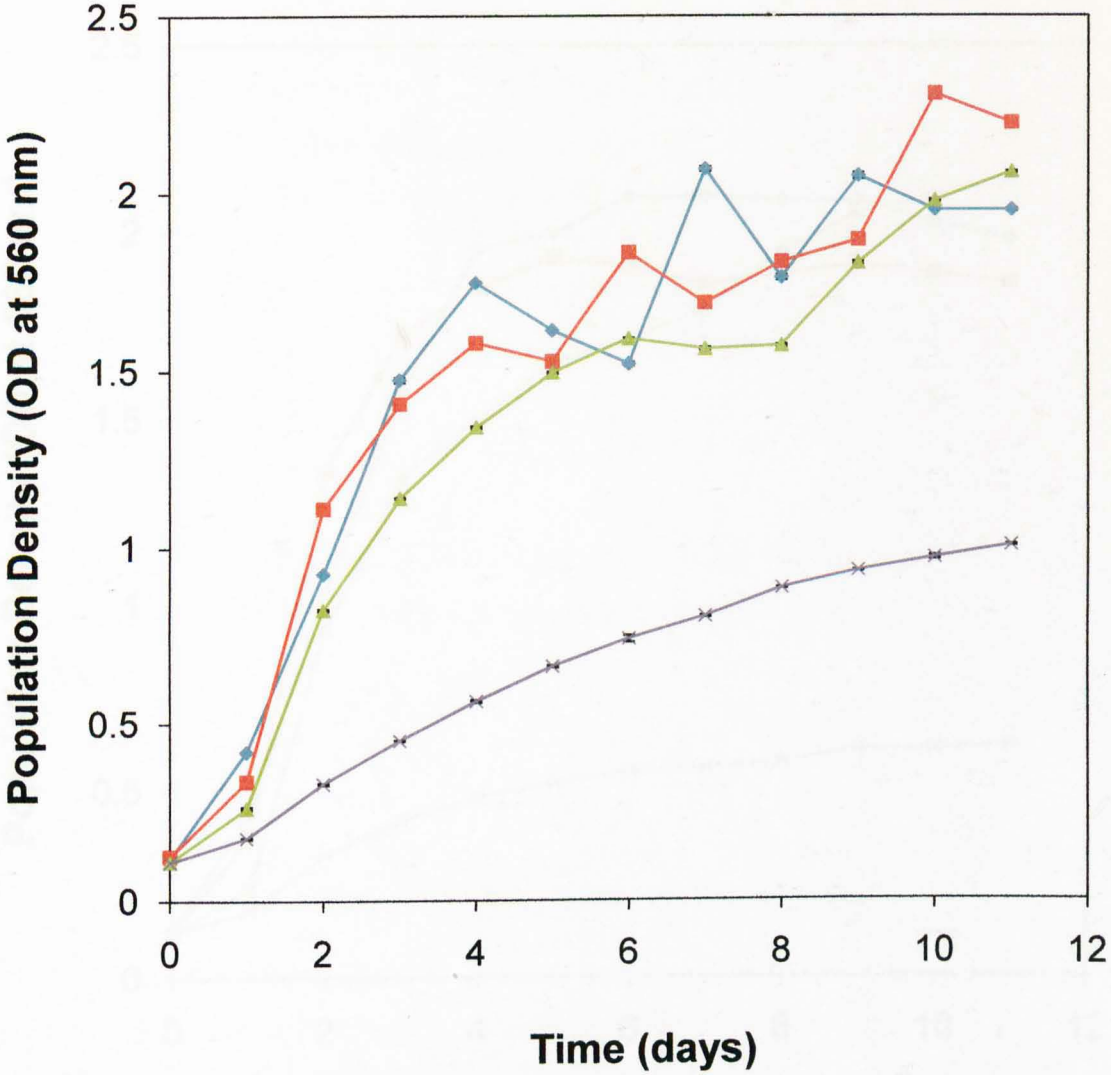

Figure 7.1. Growth curves of *Spirulina platensis* UTEX LB 2340 at 37 °C and different concentrations of NaCl. Means and standard errors for three replicates are shown.

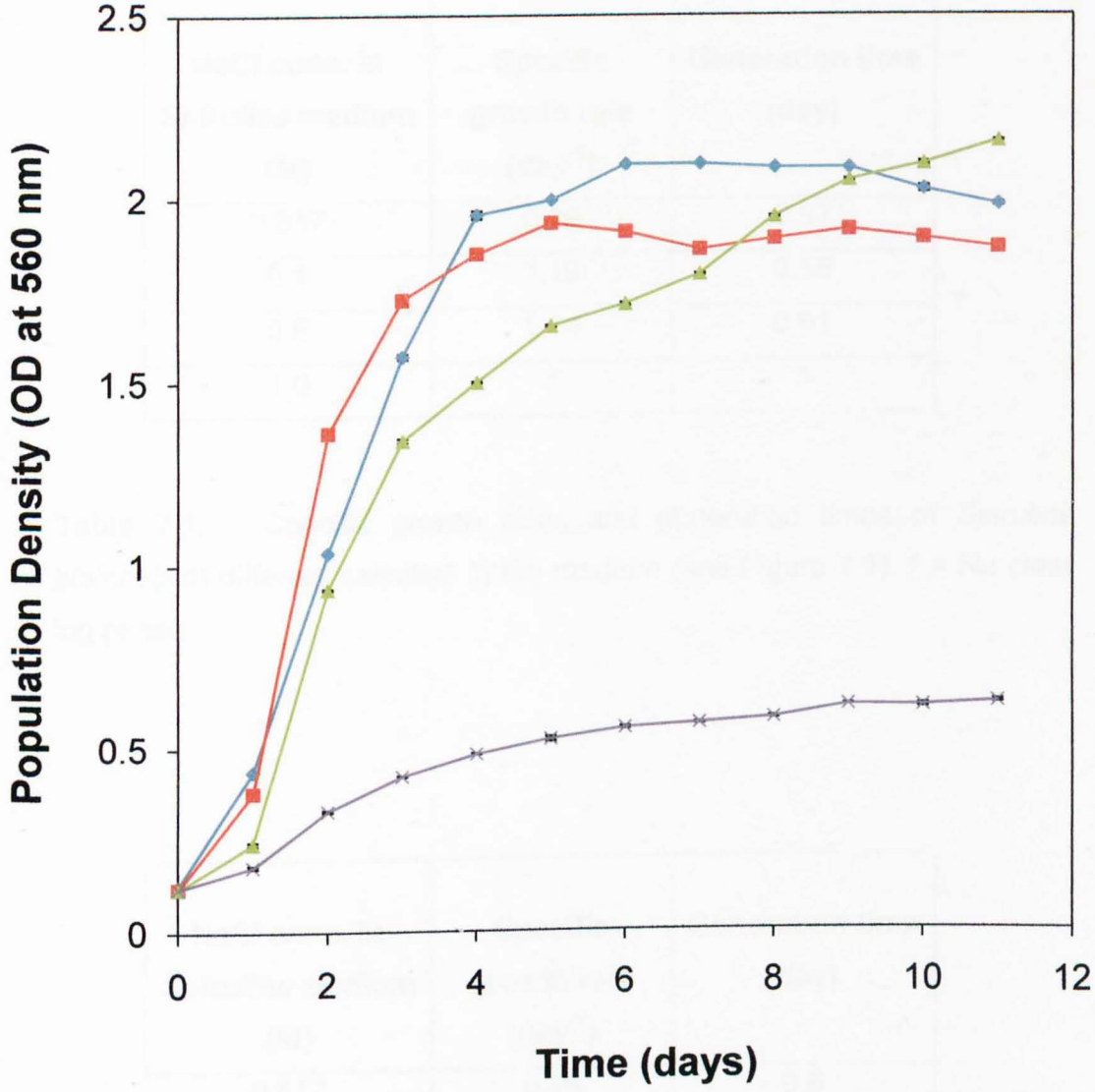

Figure 7.2. Growth curves of *Arthrospira fusiformis* CCAP 1475/8 at 37 °C and different concentrations of NaCl. Means and standard errors for three replicates are shown.

NaCl conc. in *Spirulina* medium (M)	Specific growth rate (day^{-1})	Generation time (day)
0.017	0.79	0.87
0.1	1.19	0.58
0.5	1.14	0.61
1.0	*	*

Table 7.1. Specific growth rates and generation times of *Spirulina platensis* at different salinities in the medium (see Figure 7.1). * = No clear log phase.

NaCl conc. in *Spirulina* medium (M)	Specific growth rate (day^{-1})	Generation time (day)
0.017	0.86	0.8
0.1	1.28	0.54
0.5	1.35	0.51
1.0	*	*

Table 7.2. Specific growth rates and generation times of *Arthrospira fusiformis* at different salinities in the medium (see Figure 7.2). * = No clear log phase.

7.2.2. Compatible Solutes

Compatible solutes in *Arthrospira fusiformis* and *Spirulina platensis* were determined by NMR as described in Section 2.18. Figures 7.3 and 7.4 show the presence of the compatible solute glucosyl-glycerol in *A. fusiformis* and *S. platensis* cells respectively at three different salinities (0.017, 0.75, and 1.0 M NaCl). There was a clear relationship between salinity in the medium and the concentration of glucosyl-glycerol. The concentration of glucosyl-glycerol increased as the salinity increased. It was barely detected at the lowest salinity (0.017 M NaCl) indicating that the synthesis of this compatible solute is induced by increasing salinity. There was no detectable trehalose in either *A. fusiformis* or *S. platensis*. These results are in agreement with those obtained by Reed and Stewart (1988) who reported that *S. platensis* accumulates glucosyl-glycerol in significant quantities as a primary organic osmolyte when grown in a high salt medium.

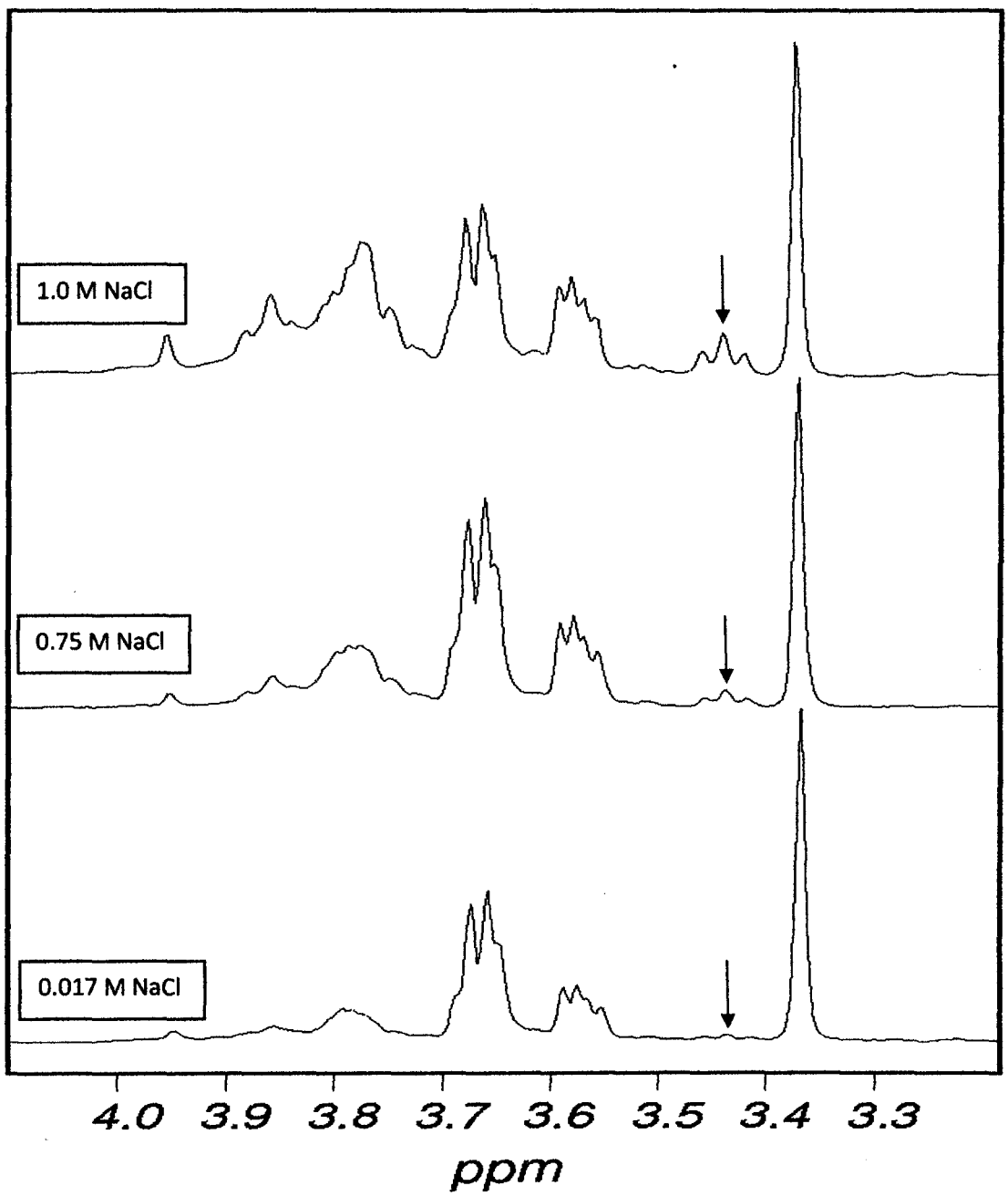

Figure 7.3. ^{13}C-NMR spectra of *Arthrospira fusiformis* cells grown at 0.017, 0.75, and 1.0 M NaCl. The arrows indicate to the glucosyl-glycerol peaks. Other peaks represent other cell metabolites.

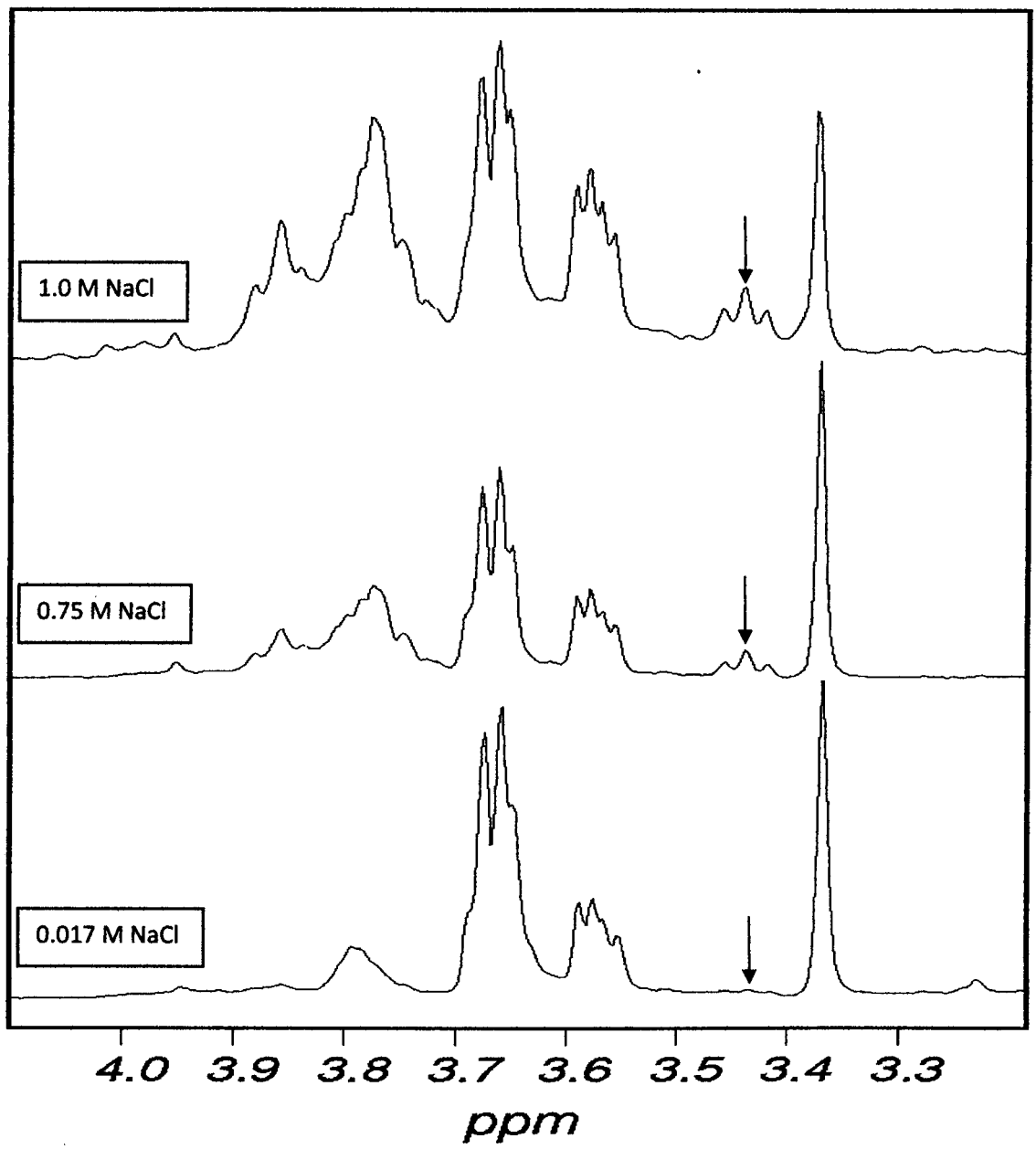

Figure 7.4. ^{13}C-NMR spectra of *Spirulina platensis* cells grown at 0.017, 0.75, and 1.0 M NaCl. The arrows indicate to the glucosyl-glycerol peaks. Other peaks represent other cell metabolites.

7.2.3. Phycobiliprotein Quantification

A. fusiformis and *S. platensis* cultures were grown in *Spirulina* medium (Appendix A8) at 0.017 M NaCl until they reached late exponential growth phase (OD_{560} = 1.5) (Figures 7.1 and 7.2) and then harvested to quantify the concentrations of three types of phycobiliproteins: c-phycocyanin, allophycocyanin, and phycoerythrin. These phycobiliproteins were extracted by a simple extraction method (see Section 2.17) using water as a solvent and therefore the extraction cost was very cheap. Concentrations of these phycobiliproteins over an extraction time up to 24 h are shown in Figures 7.5 - 7.7. It is noteworthy that some extraction of phycobiliproteins takes place almost immediately after adding the biomass to the solvent.

Spirulina platensis yielded more c-phycocyanin (0.293 mg ml^{-1}) than *A. fusiformis* (0.287 mg ml^{-1}) after just 12 h of extraction time. C-phycocyanin concentrations were already quite high at the beginning of the extraction and then increased to the maximum after 12 h of the extraction. They levelled off between 12 and 24 h of extraction indicating that 12 h is enough time to extract this pigment under the conditions applied (Figure 7.5).

Allophycocyanin concentrations were higher than c-phycocyanin concentrations for both strains. Their maximum concentrations were obtained at 24 and 12 h extraction time for *S. platensis* and *A. fusiformis* respectively. However, there were no significant differences between allophycocyanin concentrations obtained after 12 h extraction time and those obtained at the end of the extraction time (24 h) (Figure 7.6). It should also be noted that about 90% of the allophycocyanin was extracted immediately.

Phycoerythrin concentrations in both *A. fusiformis* and *S. platensis* were lower when compared with phycocyanin and allophycocyanin concentrations (Figure 7.7). This result is in agreement with the fact that phycoerythrin

commonly occurs in red algae, but it may be present in some cyanobacteria in low amounts (Cohen, 1997; Reis *et al.*, 1998). *S. platensis* content of phycoerythrin was decreased after 12 h of the extraction and then levelled off. However, *A. fusiformis* showed very little difference in phycoerythrin concentration over the extraction time applied (Figure 7.7).

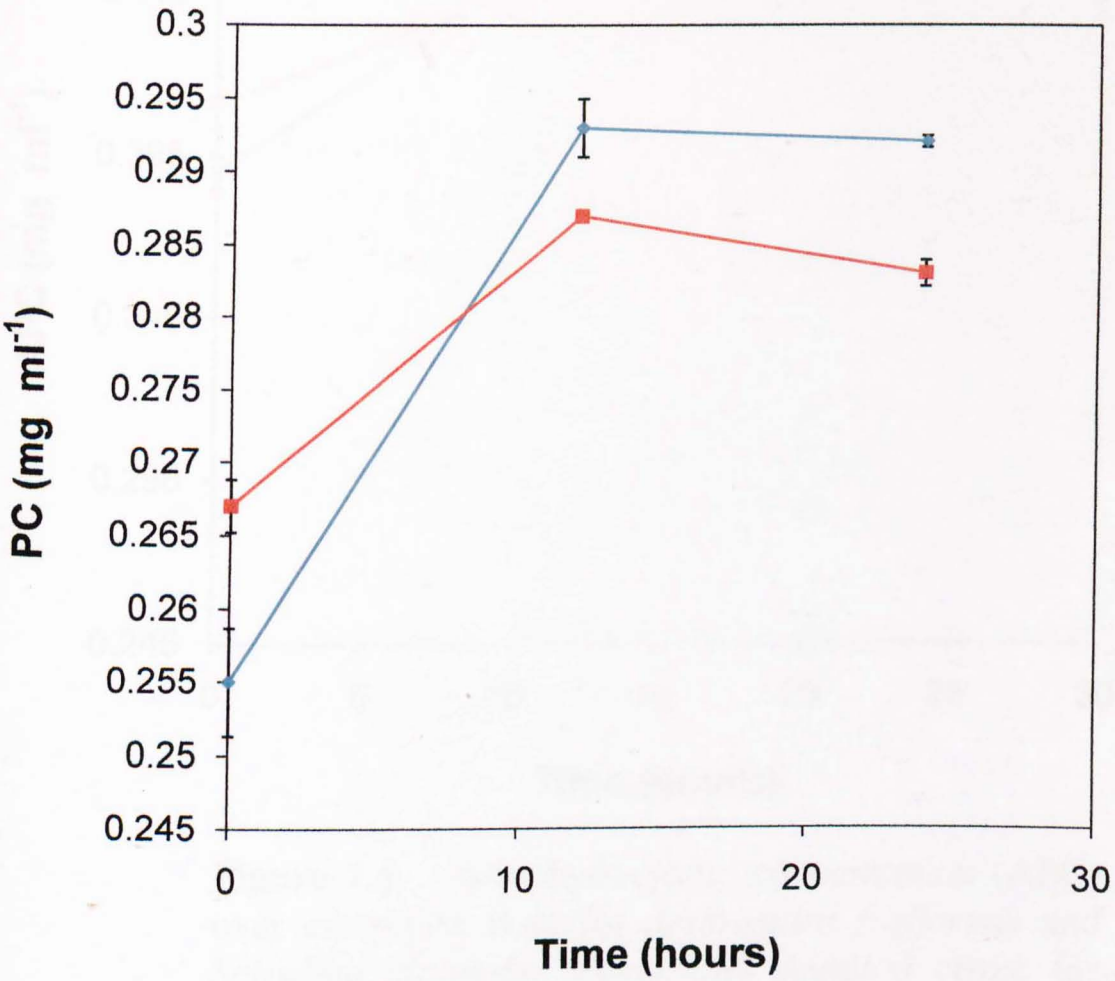

Figure 7.5. C-phycocyanin concentration (PC) over extraction time for *Arthrospira fusiformis* and *Spirulina platensis*. Means and standard errors for three replicates are shown.

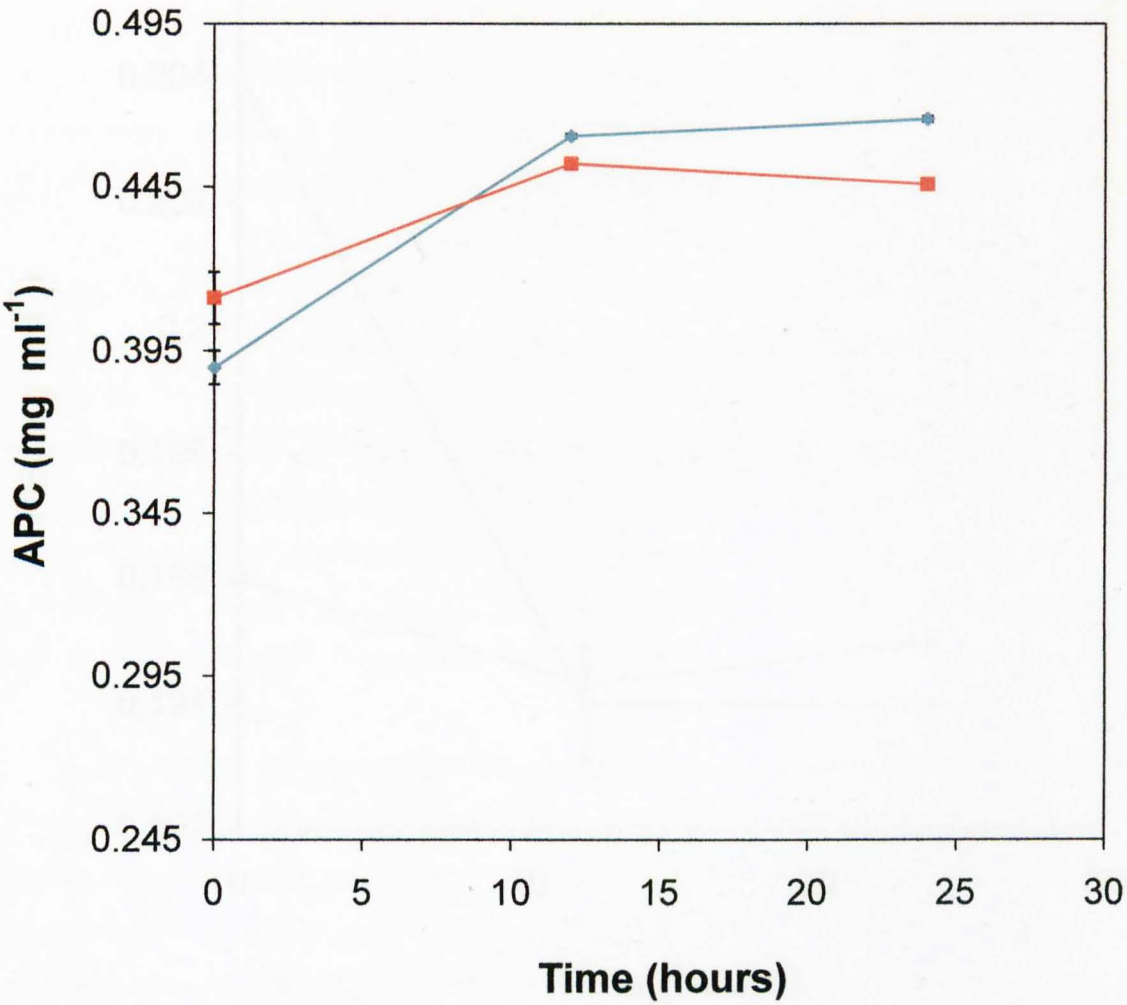

Figure 7.6. Allophycocyanin concentration (APC) over extraction time for *Arthrospira fusiformis* and *Spirulina platensis*. Means and standard errors for three replicates are shown.

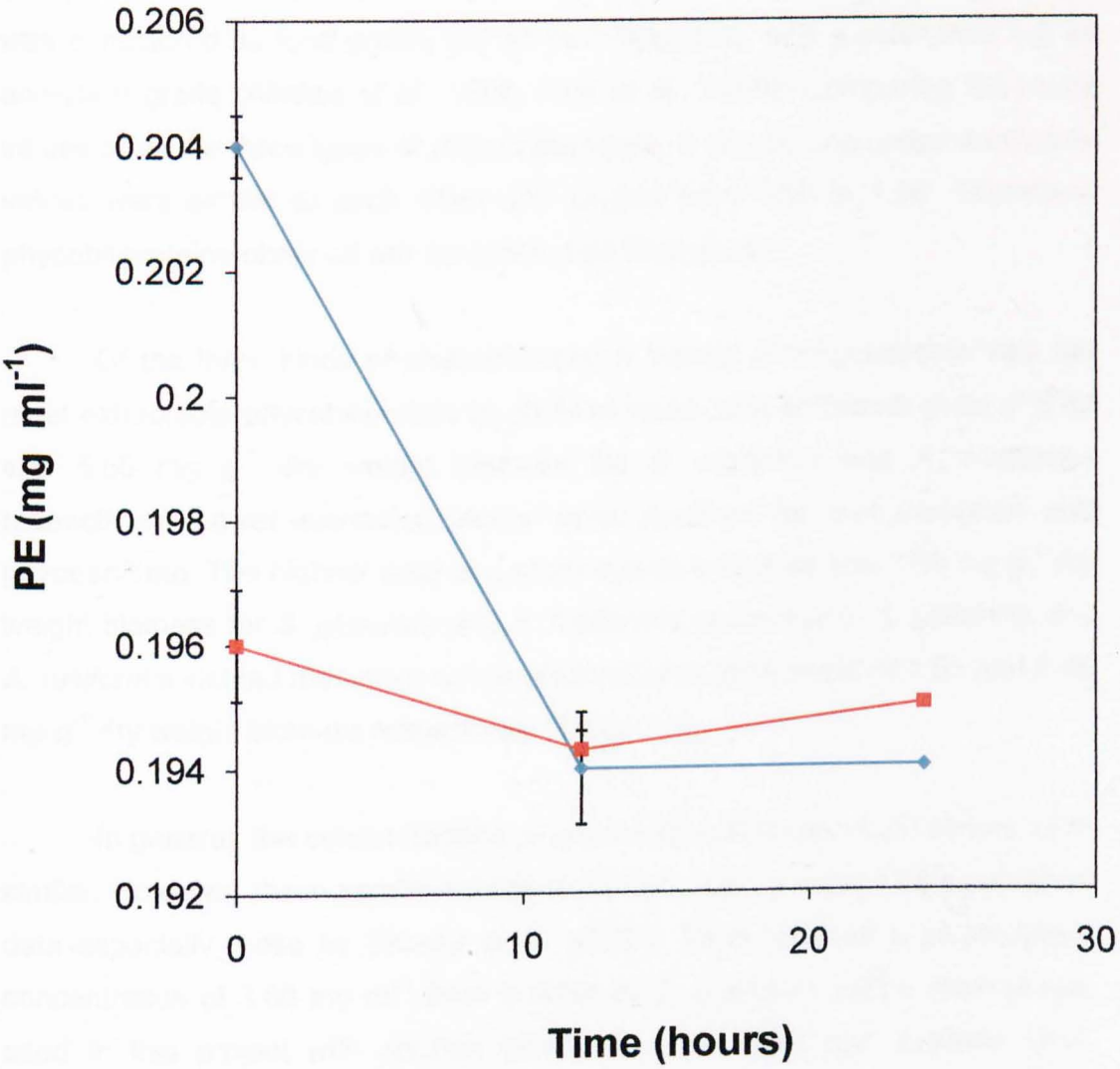

Figure 7.7. Phycoerythrin concentration (PE) over extraction time for *Arthrospira fusoformis* and *Spirulina platensis*. Means and standard errors for three replicates are shown.

Extraction purities of phycobiliproteins across the extraction times (up to 24 h) were calculated (Table 7.3) as described in Section 2.17. A purity of 0.7 was considered as food grade, 3.9 as reactive grade, and greater than 4.0 as analytical grade (Abalde *et al.*, 1998; Patil *et al.*, 2006). Comparing the purity values of all the three types of phycobiliproteins, it can be concluded that these values were similar to each other and ranged from 1.69 to 1.96. Therefore, phycobiliproteins obtained are considered as food grade.

Of the three kinds of phycobiliproteins tested, allophycocyanin was the most extractable phycobiliprotein by distilled water with extraction yield of 5.83 and 5.65 mg g^{-1} dry weight biomass for *S. platensis* and *A. fusiformis* respectively. Lower extraction yields were obtained for c-phycocyanin and phycoerythrin. The highest yield of c-phycocyanin was 3.66 and 3.59 mg g^{-1} dry weight biomass for *S. platensis* and *A. fusiformis* respectively. *S. platensis* and *A. fusiformis* yielded maximum concentrations of phycoerythrin of 2.55 and 2.45 mg g^{-1} dry weight biomass respectively (Table 7.4).

In general, the concentrations of phycobiliproteins from both strains were similar. However, these concentrations were very low compared with published data especially those by Silveira *et al.* (2007). They reported a phycocyanin concentration of 3.68 mg ml^{-1} from a strain of *S. platensis*. Same method was used in this project with another strain of *S. platensis* and a strain of *A. fusiformis* and the maximum phycocyanin concentration obtained was 0.293 and 0.287 mg ml^{-1} respectively (Figure 7.5) i.e. about 12 times less than that reported by Silveira et al. (2007). These figures are equivalent to 0.36% of the dry weight biomass of each strain (Table 7.5). They are very low when compared with published figures which range from 14 to 20% of the dry weight biomass of *Spirulina* (Belay, 1997; Cohen, 1997; Hu, 2004). Therefore although the extraction method used in this work is very simple and economic, it is not recommended for phycobiliprotein extractions. There are many reported methods for phycobiliprotein extraction and purification but most of them are

laborious, complex, time-consuming, and difficult to scale up. These methods include: sonication at 4 °C, French press, freezing in liquid N_2 and thawing at 4 °C three times, freezing at -21 °C and thawing at 4 °C three times, precipitation in ammonium sulfate, ion-exchange chromatography, gel-filtration, and chromatography on hydroxyapatite (Abalde *et al.*, 1998; Niu *et al.*, 2007). Moreover, an extraction and purification procedure that works well for a phycobiliprotein from one organism may not be the method of choice for the corresponding phycobiliprotein from another organism (Abalde *et al.*, 1998). Therefore, it is recommended to use one or more of the methods above instead of the method used in this project in order to investigate whether the low yield of phycobiliproteins was because of the ineffectiveness of the extraction method used or because of another factor. It was shown that drying *Spirulina* biomass by crossflow drying, spray drying, or oven drying resulted in approximately 50% loss of phycocyanin content, and that extracting phycocyanin from fresh wet biomass yielded 100% recovery of phycocyanin content (Sarada *et al.*, 1999). In the method used in this project, the biomass was dried in an oven at 40 °C for 48 h before extraction of phycobiliproteins. Thus, this might be one of the reasons for the low content of phycobiliproteins in both *A. fusiformis* and *S. platensis.*

Phycobiliprotein	*Spirulina platensis*			*Arthrospira fusiformis*		
	Extraction time (h)			Extraction time (h)		
	0	12	24	0	12	24
PC	1.72 ± 0.06	1.81 ± 0.09	1.79 ± 0.07	1.76 ± 0.04	1.79 ± 0.07	1.71 ± 0.01
APC	1.69 ± 0.05	1.81 ± 0.08	1.8 ± 0.12	1.74 ± 0.03	1.79 ± 0.07	1.7 ± 0.01
PE	1.96 ± 0.08	1.81 ± 0.09	1.79 ± 0.02	1.89 ± 0.06	1.81 ± 0.07	1.74 ± 0.01

Table 7.3. Extraction purities of phycobiliproteins (PC = C-phycocyanin, APC = Allophycocyanin, and PE = Phycoerythrin) from *Spirulina platensis* and *Arthrospira fusiformis*. Means and standard errors for three replicates are shown.

Phycobiliprotein	*Spirulina platensis*			*Arthrospira fusiformis*		
	Extraction time (h)			Extraction time (h)		
	0	12	24	0	12	24
PC	3.18 ± 0.04	3.66 ± 0.03	3.65 ± 0	3.34 ± 0.02	3.59 ± 0	3.53 ± 0.01
APC	4.87 ± 0.06	5.76 ± 0.01	5.83 ± 0.01	5.14 ± 0.1	5.65 ± 0.02	5.57 ± 0.01
PE	2.55 ± 0.01	2.43 ± 0.01	2.42 ± 0	2.45 ± 0.01	2.43 ± 0	2.44 ± 0

Table 7.4. Extraction yields (mg g^{-1} dry weight) of phycobiliproteins (PC = C-phycocyanin, APC = Allophycocyanin, and PE = Phycoerythrin) from *Spirulina platensis* and *Arthrospira fusiformis*. Means and standard errors for three replicates are shown.

7.2.4. Standard Curves for Protein, Chlorophyll, and Dry Weight Determinations

Figures 7.8 - 7.13 show standard curves that relate total protein content, chlorophyll content, and dry weight biomass to different population densities (OD at 560 nm) for both *S. platensis* and *A. fusiformis* as described in Sections 2.13.3, 2.7.2, and 2.12 respectively. Chlorophyll, total protein, and phycocyanin contents were calculated from these standard curves as % of the dry weight biomass, and the results are shown in Table 7.5. In addition, the curves could be used to identify the above cell contents for individual samples, during future work.

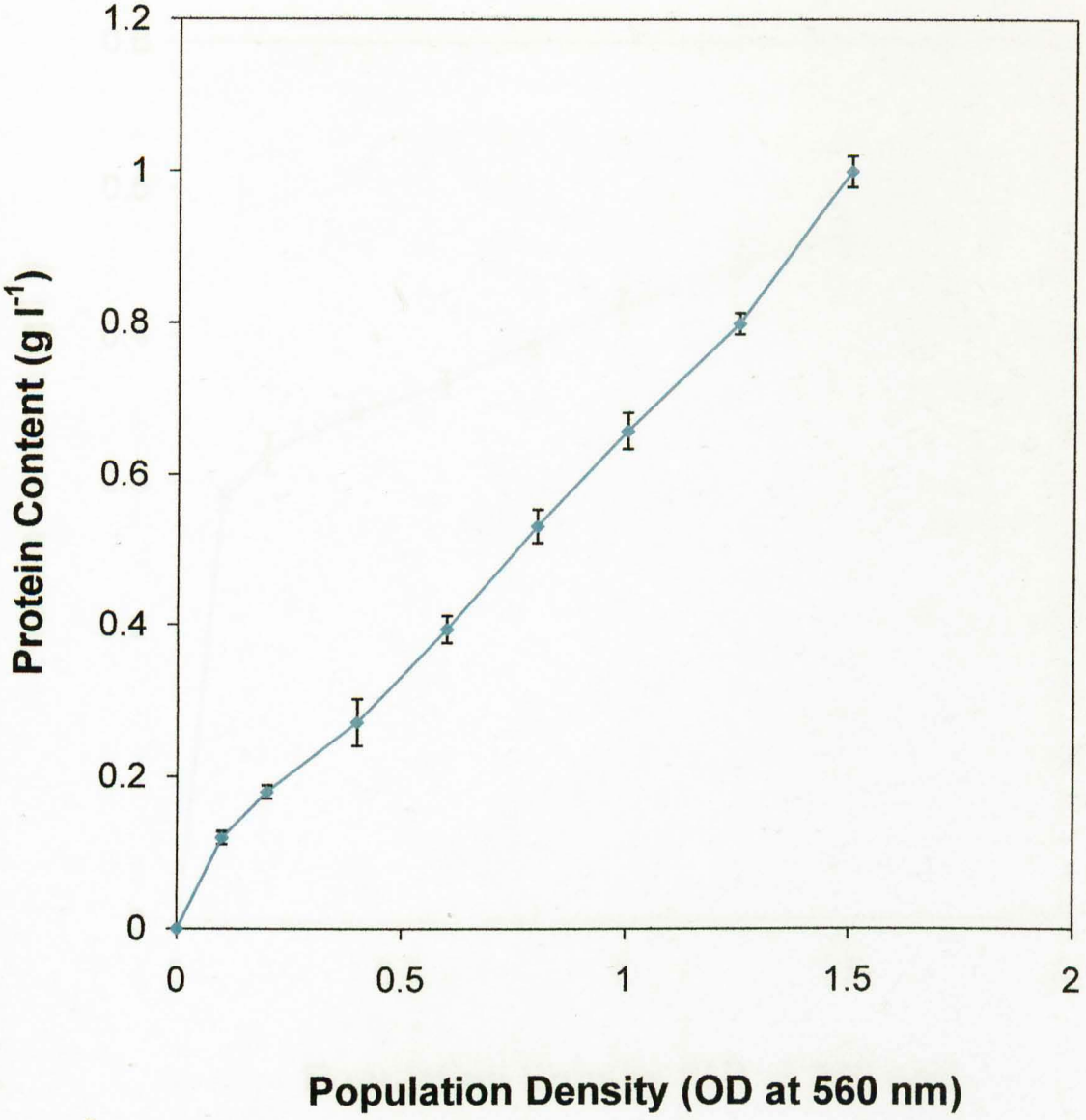

Figure 7.8. Standard curve for protein determination in *Spirulina platensis*. Means and standard errors for three replicates are shown.

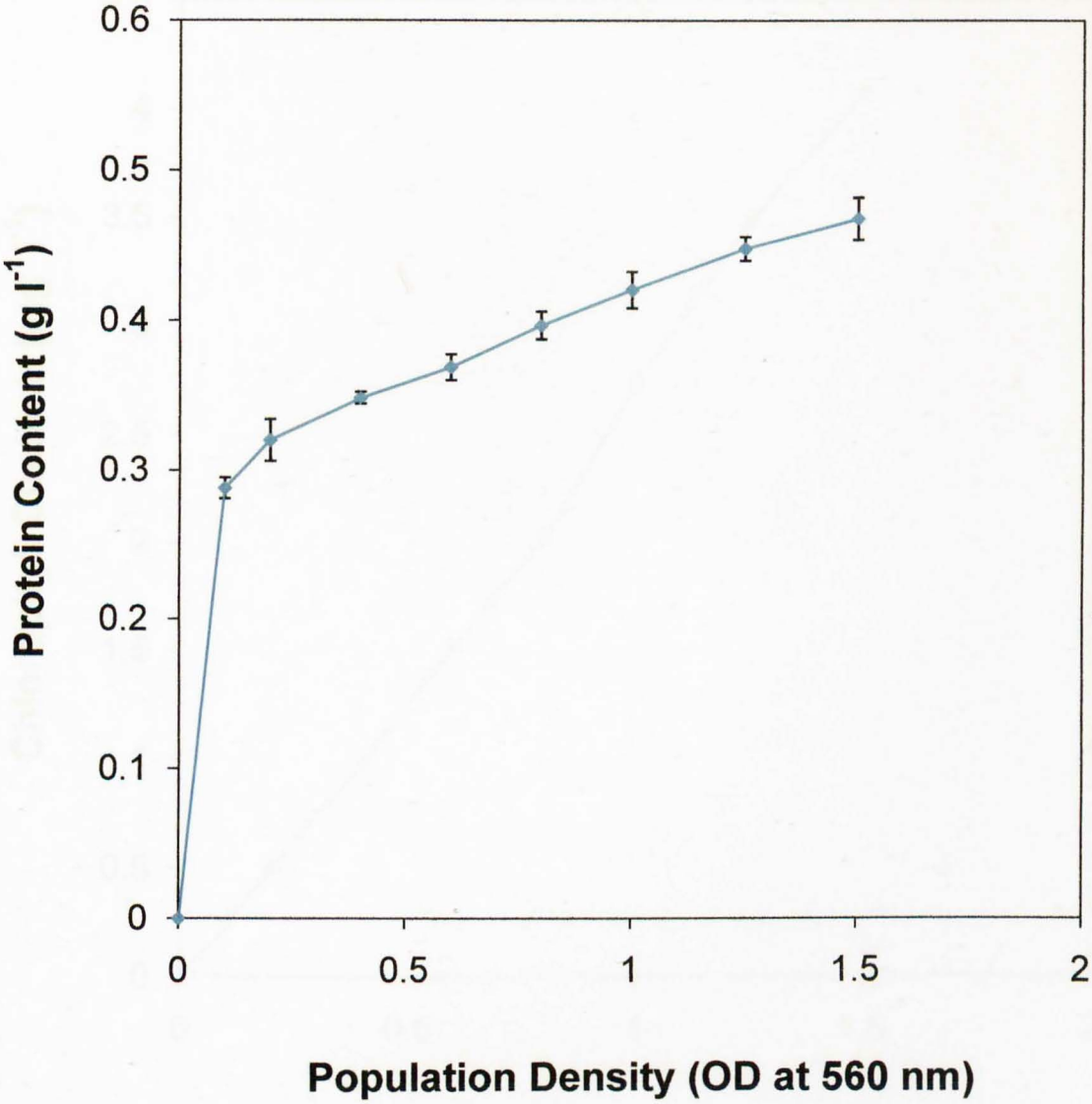

Figure 7.9. Standard curve for protein determination in *Arthrospira fusiformis*. Linear relationship only above OD_{560} = 0.25. Means and standard errors for three replicates are shown.

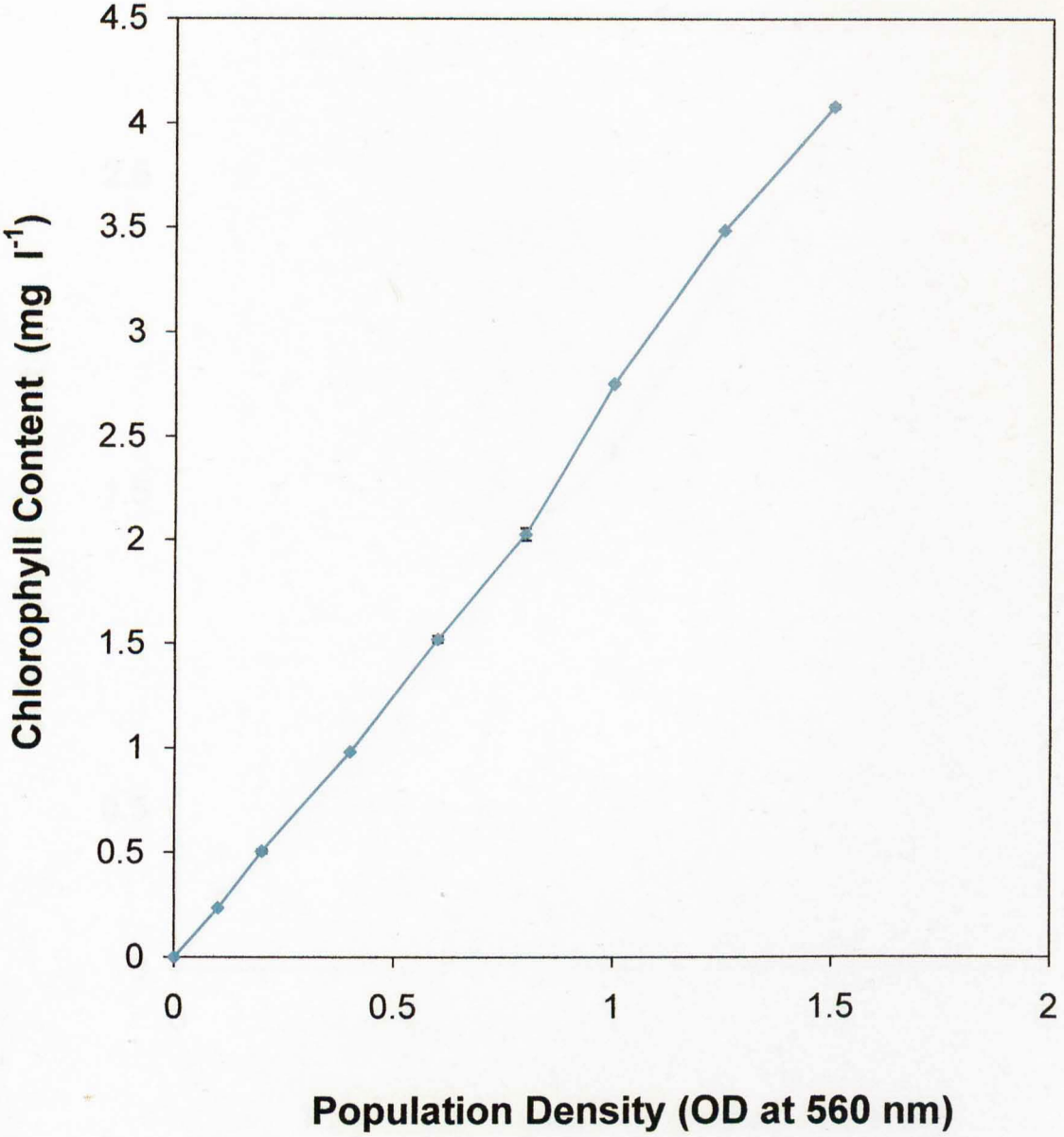

Figure 7.10. Standard curve for chlorophyll content determination in *Spirulina platensis*. Means and standard errors for three replicates are shown.

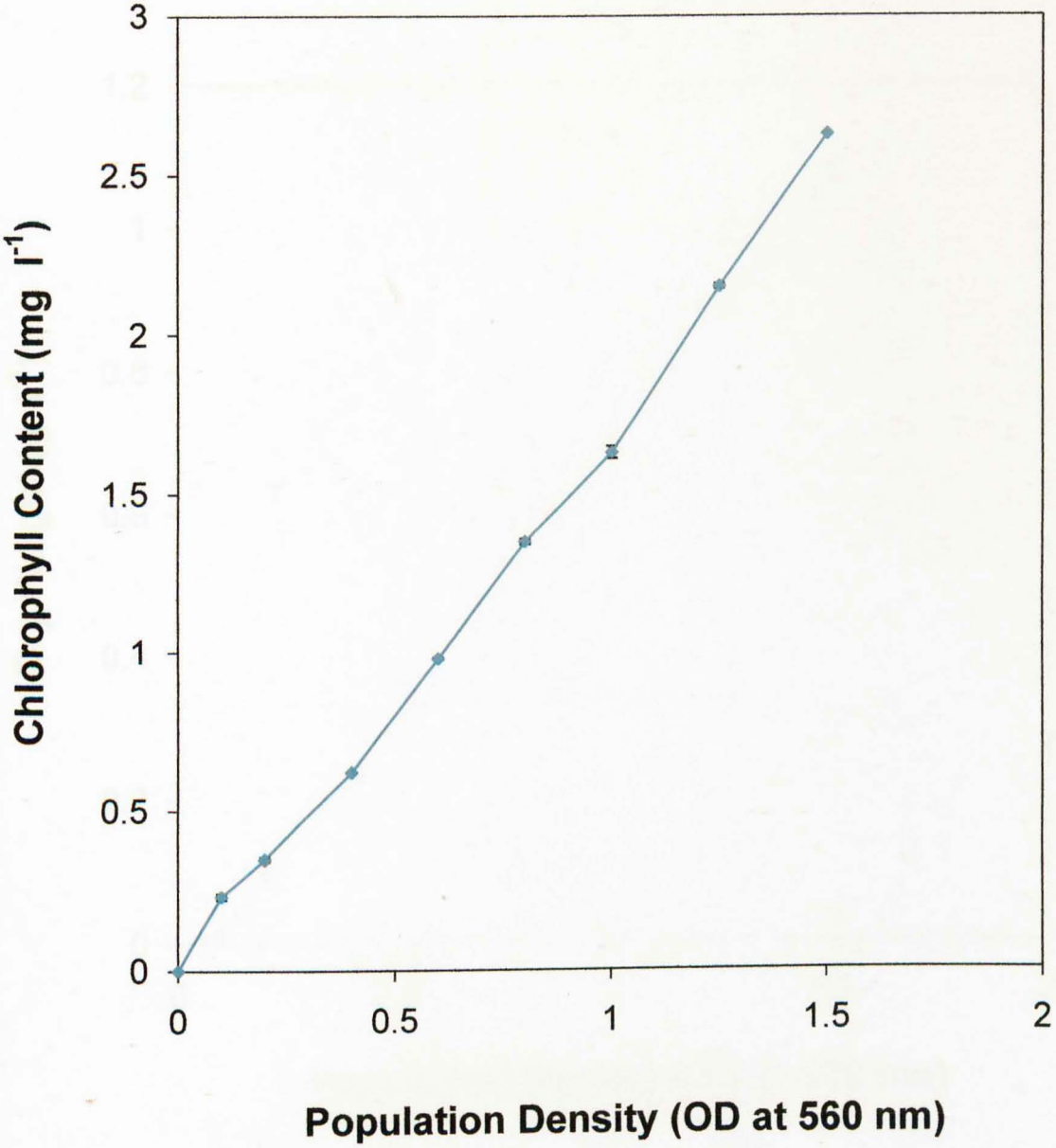

Figure 7.11. Standard curve for chlorophyll content determination in *Arthrospira fusiformis*. Means and standard errors for three replicates are shown.

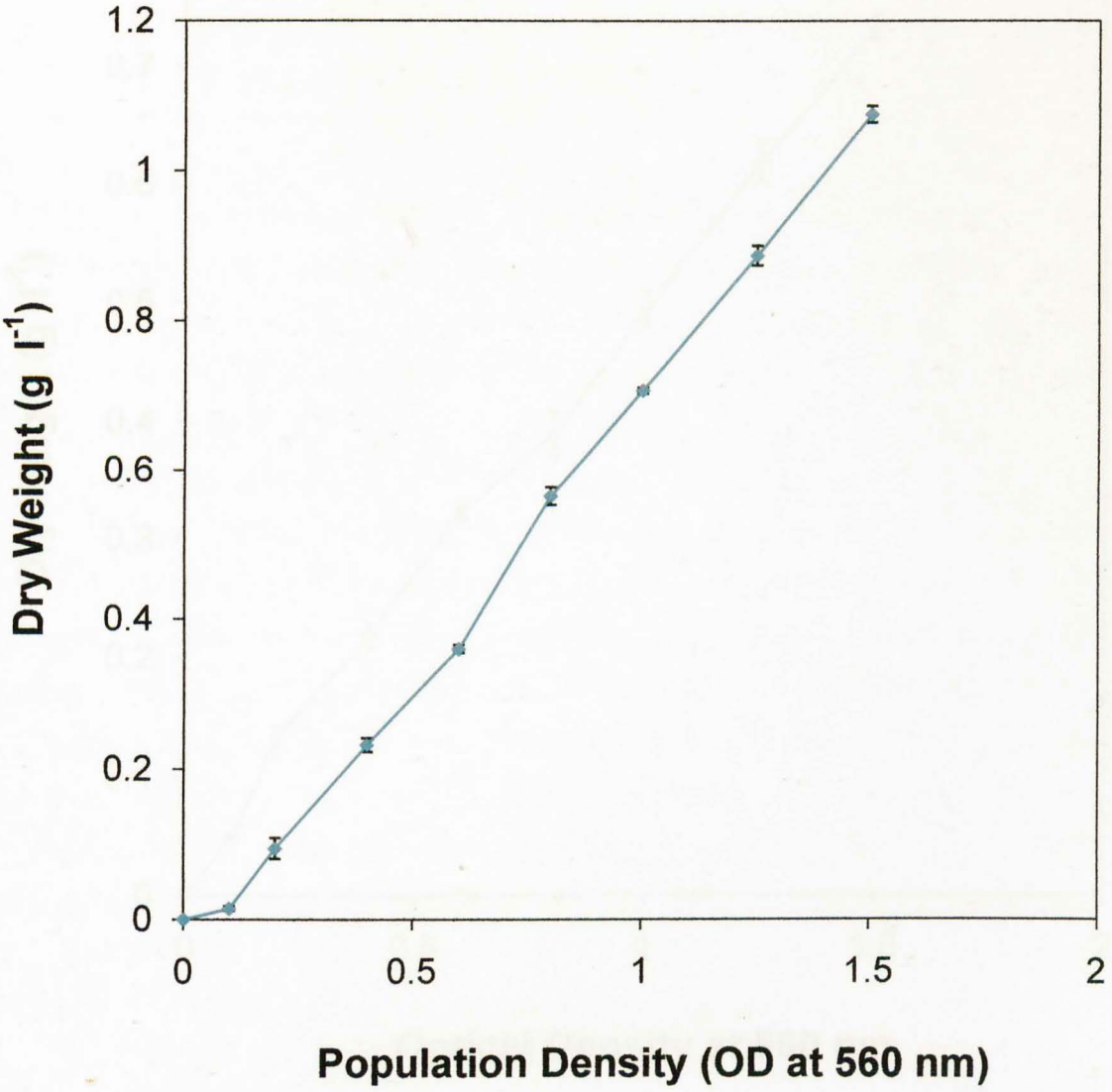

Figure 7.12. Standard curve for dry weight determination in *Spirulina platensis*. Means and standard errors for three replicates are shown.

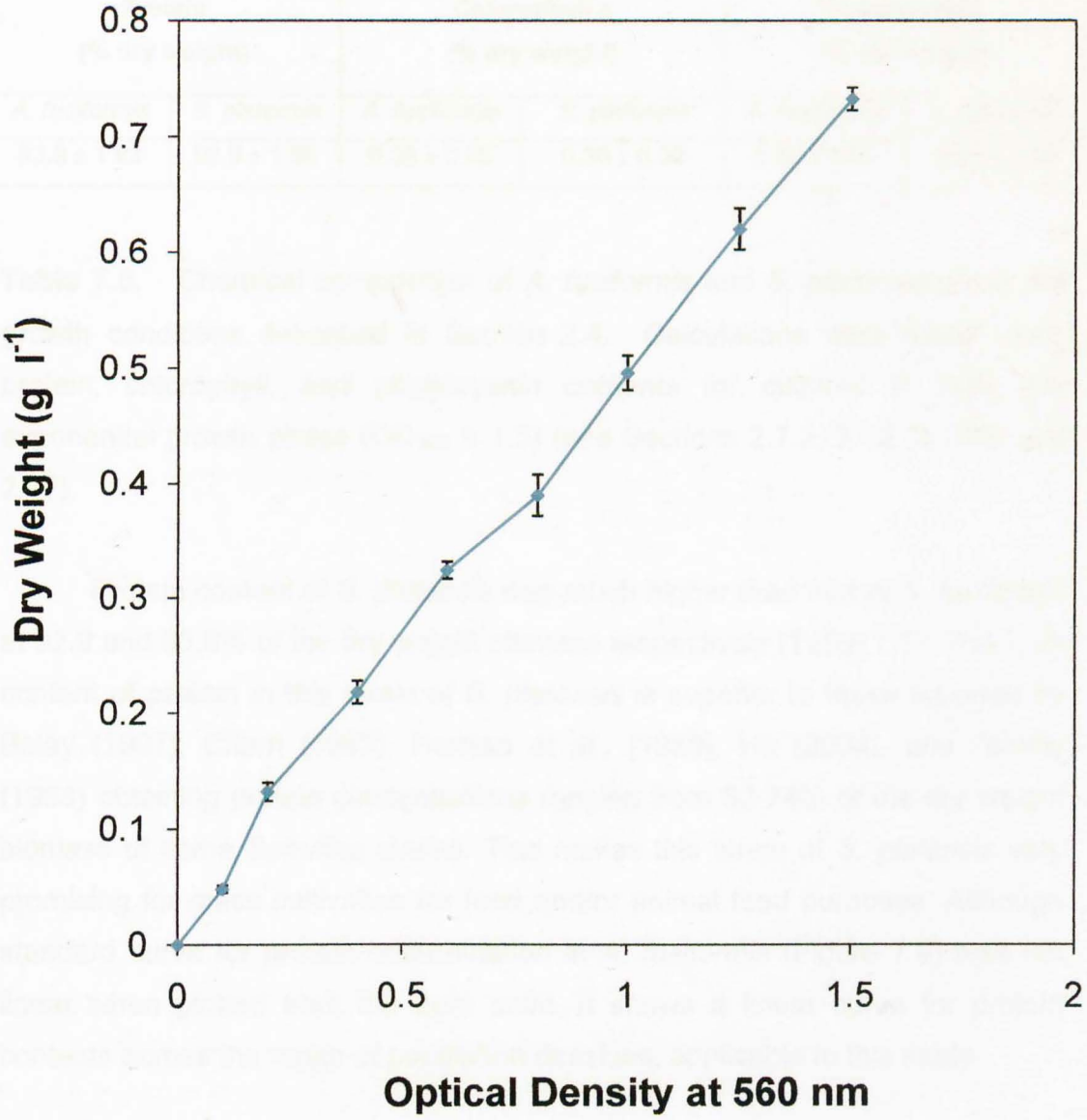

Figure 7.13. Standard curve for dry weight determination in *Arthrospira fusiformis*. Means and standards errors for three replicates are shown.

Protein (% dry weight)		Chlorophyll a (% dry weight)		Phycocyanin (% dry weight)	
A. fusiformis	*S. platensis*	*A. fusiformis*	*S. platensis*	*A. fusiformis*	*S. platensis*
63.8 ± 1.93	92.9 ± 1.95	0.36 ± 0.00	0.38 ± 0.00	0.36 ± 0.00	0.36 ± 0.00

Table 7.5. Chemical composition of *A. fusiformis* and *S. platensis* under the growth conditions described in Section 2.4. Calculations were made using protein, chlorophyll, and phycocyanin contents for cultures in their late exponential growth phase (OD_{560} = 1.5) (see Sections 2.7.2, 2.12, 2.13.3, and 2.17).

Protein content of *S. platensis* was much higher than that of *A. fusiformis* at 92.9 and 63.8% of the dry weight biomass respectively (Table 7.5). This high content of protein in this strain of *S. platensis* is superior to those reported by Belay (1997), Ciferri (1983), Herrera *et al.*, (1989), Hu (2004), and Jassby (1988) obtaining protein concentrations ranging from 52-74% of the dry weight biomass of some *Spirulina* strains. This makes this strain of *S. platensis* very promising for mass cultivation for food and/or animal feed purposes. Although standard curve for protein determination in *A. fusiformis* (Figure 7.9) was not linear when plotted from the zero point, it shows a linear curve for protein contents across the range of population densities, applicable to this study.

Chlorophyll *a* content, however, was low in both strains at 0.36 and 0.38% of the dry weight biomass of *A. fusiformis* and *S. platensis* respectively (Table 7.5) compared with published figures by Belay (1997) and Ciferri (1983) who reported a range of chlorophyll *a* content from 0.8 to 1.5% of the dry weight of *Spirulina*.

To avoid false protein or chlorophyll content as a result of dilutions of *A. fusiformis* or *S. platensis* cultures (see Section 2.7.2 and 2.13.3), chemical composition of both strains in Table 7.5 were calculated from figures of the actual cultures (undiluted) at OD_{560} = 1.5.

7.3. Conclusions

Optimum growth for *S. platensis* was found at 0.1 M NaCl, *A. fusiformis*, however, showed optimum growth at 0.5 M NaCl. 1.0 M NaCl was found to inhibit the growth of both strains.

The phycobiliprotein content was very low in both strains under the growth and extraction conditions applied. Therefore, it is recommended that other extraction methods be used under the same growth conditions in order to investigate the reasons for this reduction in phycobiliprotein content. Extracting phycobiliproteins from fresh wet biomass of *Arthrospira* (*Spirulina*) would be a good choice.

Under the growth conditions applied, a very high protein content (92.9% of the dry weight biomass) was obtained from the strain *S. platensis* UTEX LB 2340. This makes this strain very promising for mass cultivation for food and/or feed purposes. However, chlorophyll *a* content was low in both strains.

The compatible solute glucosyl-glycerol was found in *A. fusiformis* and *S. platensis* and its concentration increased as the salinity increased indicating that the synthesis of this compatible solute is induced by increasing salinity.

Conclusions and Future Work

Chapter Eight

8.1. Conclusions

1. A bacterial isolate and a microalga were identified to the genus level using 16S and 18S rRNA gene sequences respectively (Chapter 3). Phylogenetic trees were constructed for both microorganisms to predict their genetic relatedness to closest matches. The bacterial isolate belonged to the genus *Halomonas* and as a result it was called *Halomonas* sp. NAH1. It was deposited in the National Collection of Industrial, Marine and Food Bacteria (NCIMB), Aberdeen, UK under accession number NCIMB 14402. The microalga belonged to the genus *Dunaliella* and its closest match was the strain *D. salina* CCAP 19/30 which was the source of 18S rRNA gene. The 16S and 18S rRNA gene sequences of *Halomonas* sp. NAH1 and *D. salina* 19/30 were deposited in the GenBank under accession numbers EU239362 and EU239363 respectively.

2. In Chapter 4, glycerol productivity was investigated in three strains of *Dunaliella* (*D. parva* 19/9, *D. parva* 19/10, and *D. salina* 19/30). The strains were first adapted to grow in batch cultures in salinities from 0.1 to 4.0 M NaCl and the growth across the salinity range was monitored by determining chlorophyll content and by counting cell number. Despite their provenances, all three strains were shown to be halotolerant and not halophilic. Intracellular and extracellular glycerol concentrations were determined for all three strains. Leakage of glycerol into the medium was shown to occur for all three strains and is thus an inherent property of *Dunaliella* cells and not due to a mutation as originally proposed by Hard and Gilmour (1991). It was also demonstrated for one strain (*D. parva* 19/10) that glycerol leakage also occurred in an airlift fermenter culture grown under semi-batch and continuous culture conditions.

3. Two strains of *Dunaliella salina* (CCAP 19/18 and 19/30) were grown in batch cultures at relatively low constant irradiance of 44-53 µmol photons m^{-2} s^{-1} combined with different concentrations of nitrogen ($NaNO_3$) and the effect on

the growth and β-carotene content were investigated (Chapter 5). In addition, cultures were grown in batch cultures and aerated with different concentrations of CO_2 to study the effects of this treatment on growth and β-carotene content. Low concentration of nitrogen (0.5 mM $NaNO_3$) in the medium or nitrogen-free medium was shown not to limit the growth of the strain *D. salina* 19/30 and therefore β-carotene productivity by this strain was very low. However, this treatment was shown to limit the growth of the strain *D. salina* 19/18 and thus increasing β-carotene accumulation in the cells with an excess of 94 times that accumulated by *D. salina* 19/30. It was also found that bubbling the batch cultures of both strains with 5% CO_2 increased the growth to a higher level than that obtained when cultures were bubbled with natural air. The highest β-carotene production by *D. salina* 19/30 was obtained when the strain was grown in 5 mM $NaNO_3$ medium and the culture was bubbled with 5% CO_2. However, much higher concentration of β-carotene was obtained from *D. salina* 19/18 using same aeration system but under nitrogen limitation conditions.

4. In Chapter 6, the bacterial isolate *Halomonas* sp. NAH1, which was identified in Chapter 3, was characterised by examining morphological features, growth on different media, salinity tolerance, susceptibility to some antibiotics, uptake of ^{14}C-glucose and ^{3}H-glycerol, respiration rates, utilisation of several carbon sources, and biochemical characteristics. *Halomonas* sp. NAH1 cells are rod shaped and motile and grow optimally at 1.0 M NaCl in BM defined medium. Glucose supported the most rapid growth rate. Betaine and glycerol could also be utilised as sole carbon sources, although the growth was slower than with glucose. This indicates the presence of catabolic pathways for all three carbon sources. It was shown that glucose was taken up by the strain NAH1 quicker than glycerol, but glycerol was taken up to a higher concentration. This may indicate a slow metabolism of glycerol. In addition, there was no significant difference between respiration rates in cells grown in glucose and those grown in glycerol. However, higher respiration rates were found when the cells were grown on betaine possible indicative of a stress

response. Among four antibiotics used, tetracycline (50 µg) has the most effect on the growth of the strain NAH1.

5. Some physiological and biotechnological features of the cyanobacteria *Arthrospira fusiformis* CCAP 1475/8 and *Spirulina platensis* UTEX LB 2340 were investigated (Chapter 7). They included growth patterns, salinity tolerance, compatible solutes, biomass production, total protein content, phycobiliprotein content, and chlorophyll *a* content. Optimum growth for *S. platensis* was found at 0.1 M NaCl, *A. fusiformis*, however, showed optimum growth at 0.5 M NaCl. 1.0 M NaCl was found to inhibit the growth of both strains. Phycobiliprotein content was very low in both strains under the growth and extraction conditions applied. However, very high protein content (92.9% of the dry weight biomass) was obtained from the strain *S. platensis* UTEX LB 2340. This makes this strain very promising for mass cultivation for food and/or feed purposes. Chlorophyll *a* content was low in both strains. Glucosyl-glycerol was found to be the compatible solute in both *A. fusiformis* and *S. platensis* at a wide range of salinities (0.017-1.0 M NaCl). The concentration of glucosyl-glycerol increased as the salinity increased indicating that the synthesis of this compatible solute is induced by increasing salinity.

8.2. Future Work

It was found that the use of 16S and 18S rRNA gene sequence comparisons provide a good molecular technique to identify unknown prokaryotes and eukaryotes respectively to the genus level and possibly to species and/or strain level (see Chapter 3). However, species or strain identity is difficult to be certain of especially when there is low diversity in the sequences. Therefore, it would be a good idea to use other molecular techniques such as RAPD band patterns and nuclear ribosomal DNA internal transcribed spacer regions (see Section 1.4.1) for identification down to the

strain level in most cases. Moreover, employing conventional techniques in addition to molecular techniques in identifying microorganisms would confirm the identity obtained from the latter techniques.

Glycerol production by *Dunaliella* has been proved to be uneconomic and therefore focusing the research on β-carotene from *Dunaliella* could improve the existing yields of β-carotene. That can be done by various means such as selection of hyper-producer strains of β-carotene and mutating the current high producer strains of *Dunaliella salina* to produce larger amounts of β-carotene (greater than 14% of the dry weight).

Many strains of *Halomonas* have the capability to produce significant amounts of the compatible solutes ectoine and hydroxyectoine, and good quality exopolysaccharides. *Halomonas* sp. NAH1 could be a good candidate and therefore examining its capability to produce such products would be beneficial.

Some strains of *Arthrospira* and *Spirulina* are well known for their high productivity of protein, phycobiliproteins, and other valuable products. In this project, *Spirulina platensis* UTEX LB 2340 produced very high total protein content (92.9% of the dry weight) and very low phycocyanin content (0.36% of the dry weight) under the growth conditions applied. Therefore, studying the ratio between total protein content and phycobiliprotein content, and the growth conditions that increase or decrease phycobiliprotein are essential to optimize the yields of phycobiliproteins.

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Appendix A

Growth Media

A1. Basal Medium (BM)

BM was prepared according to Baumann and Baumann (1981). All solutions were prepared using distilled water and stored at room temperature. The chemicals were all "Analar grade".

Stock Soultions:

2.4 M $MgSO_4 . 7H_2O$

2.0 M KCl

1.0 M NH_4Cl

0.5 M Na_2SO_4

50 mg. ml^{-1} $FeCl_2 . 6H_2O$

67 mg ml^{-1} Na_2EDTA

0.5 M $K_2HPO_4 . 3H_2O$

Micronutrient Supplements (*Dunaliella* Medium, Appendix A4)

1.0 M Tris-HCl*

10 % Glucose

*Solution was adjusted to pH 7.6 before addition to the medium.

Volumes of stock solutions added to the medium (ml l^{-1}):

Stock Solution	Volume	Final Concentration (mM)
$MgSO_4$	20.83 ml	50
KCl	5.0 ml	10
NH_4Cl	19.0 ml	19
Na_2SO_4	200 µl	0.1
$FeCl_2$	135 µl	0.025
Na_2EDTA	139 µl	0.025
K_2HPO_4	660 µl	0.33
Micronutrient Supplements	1.0 ml	-
Tris-HCl	50 ml	50
Glucose*	36 ml	20

*Solution was added to the medium aseptically after autoclaving and cooling the medium to 25 °C. 5.84, 29.22, 58.44, 116.88, or 175.32 g l^{-1} was added to the medium to give 0.1, 0.5, 1.0, 2.0, or 3.0 M NaCl medium respectively.

pH of the medium was adjusted to 7.6 with either HCl or NaOH. Medium was made up to 964 ml and then autoclaved for 20 min at 121 °C. After cooling the medium to 25 °C, 36 ml of 10 % glucose stock solution was added to the medium. The medium was mixed and kept at room temperature. When glycerol was used as a carbon source instead of glucose, 18.4 ml of 10 % glycerol stock solution was added aseptically and therefore the final volume of the medium

before autoclaving was 981.6 ml ≈ 982 ml. Similarly, when betaine was used as a carbon source instead of glucose, the required amount (2.34 g) of betaine to give a final concentration of 20 mM l^{-1} was dissolved in 50 ml of distilled water and autoclaved and added aseptically to the medium after cooling to 25 °C. Therefore, the final volume of the medium before autoclaving was adjusted at 950 ml.

A2. Basal Medium Agar (BMA)

BMA was prepared according to Baumann and Baumann (1981). 1.6 % (w/v) of bacteriological agar (Oxoid) was added to BM (Appendix A1). The medium was then autoclaved for 20 min at 121 °C. After cooling to 45 °C, agar plates were prepared by pouring the medium into 9 cm^3 plastic Petri dishes.

A3. BM Complex Medium

BM complex medium was prepared as described in Baumann and Baumann (1981). BM (Appendix A1) was supplied with the following: peptone no.3 (Difco): 5 g l^{-1} and yeast extract (Difco): 10 g l^{-1}.

A4. *Dunaliella* Growth Medium

Dunaliella medium was prepared as described by Hard (1991). Stock solutions were prepared using distilled water and stored at room temperature. All chemicals were "Analar" grade and obtained from different companies mainly Sigma.

Stock solutions:

2.4 M $MgSO_4 . 7H_2O$

2.0 M $MgCl_2 . 6H_2O$

1.0 M $CaCl_2 . 2H_2O$

4.0 M $NaNO_3$

0.5 M Na_2SO_4

0.1 M NaH_2PO_4

2.0 M KCl

1.0 M HEPES*

1.5 mM FeEDTA*

* Both solutions were adjusted to pH 7.6 before adding them to the medium.

Micronutrient Supplements:

A stock solution of micronutrient supplement was prepared by combining the following.

185.0 mM H_3BO_3

7.0 mM $MnCl_2$. $4H_2O$

0.8 mM $ZnCl_2$

0.02 mM $CoCl_2$

0.0002 mM $CuCl_2$

0.02 mM $CoCl_2$ was prepared by adding 1 ml of a 2 mM stock solution to H_2O to a final volume of 100 ml.

0.0002 mM $CuCl_2$ was prepared by adding 0.1 ml of a 0.2 mM stock solution to H_2O to a final volume of 100 ml.

Volumes of Stock Solutions were added to the medium (ml l^{-1}):

Stock Solution	Volume (ml)	Final Concentration (mM)
KCl	5.0	10
$MgCl_2$	10.0	20
$CaCl_2$	10.0	10
$MgSO_4$	10.0	24
$NaNO_3$	1.25	5
Na_2SO_4	48.0	24
NaH_2PO_4	1.0	0.1
FeEDTA	1.0	0.0015
Micronutrients	1.0	-
Tris-HCl	20.0	20

Different amounts of NaCl were added to different media in order to get a wide range of salinities ranging from 0.1 – 4.0 M NaCl as in the following table.

NaCl concentration in the medium (M)	Required amount of NaCl (g l^{-1})
0.1	5.85
0.4	23.38
1.5	87.68
2.5	146.13
3.5	204.58
4.0	233.8

After dissolving NaCl, pH was adjusted to 7.8 with either HCl or NaOH and then the solution was made up to 1 liter. 1 g l^{-1} of solid $NaHCO_3$ was added and when it was dissolved, the medium was autoclaved for 20 min at 121 °C.

Solid Media:

In order to get solid media, 1% (w/v) of bacteriological agar (Oxoid) was added to the medium above and then the medium was autoclaved for 20 min at 121 °C. After cooling to 45 °C the plates were prepared by pouring the medium into 9 cm^3 plastic Petri dishes. For slopes the medium was poured into sterile 25 ml universal bottles or 5 or 6 inch test tubes with cotton bungs. The bottles and/or test tubes were rested at an angle so that the setting agar formed a slope.

A5. Modified Johnsons Medium (J/2)

Modified Johnsons medium (J/2) was prepared according to Borowitzka (1988). Stock solutions were prepared as in the following table using distilled water and stored in a fridge. All chemicals were "Analar" grade and obtained from different companies mainly Sigma.

Stock Solution	g l^{-1}	Volume of stock solution for 1 liter of medium (ml)
$MgCl_2 . 6H_2O$	150	10
$MgSO_4 . 7H_2O$	50	10
KCl	20	10
$CaCl_2 . 2H_2O$	20	10
KNO_3	100	5
$NaHCO_3$	4.3	10
KH_2PO_4	3.5	5
Trace elements:		
H_3BO_3	0.061	
$(NH_4)_6Mo_7O_{24} . 4H_2O$	0.038	
$CuSO_4 . 5H_2O$	0.006	10
$CoCl_2 . 6H_2O$	0.0051	
$ZnCl_2$	0.0041	
$MnCl_2 . 4H_2O$	0.0041	
Fe stock solution:		
Na_2EDTA	0.189	10
$FeCl_3 . 6H_2O$	0.244	

The required amount of NaCl was dissolved in 800 ml of distilled water. 12.5% (≈ 2.14 M) NaCl was used for subculturing and inoculum production whereas 20% (≈ 3.42 M) NaCl was used for β-carotene production.

The above nutrients were added except iron and phosphate stock solutions. The medium was made up to 985 ml with distilled water and then pH was adjusted at 7.5. The medium was autoclaved at 121 °C for 20 min. After cooling the medium, iron and phosphate stock solutions were added aseptically in a laminar flow cabinet as sterile solutions.

A6. Nutrient Broth Medium (Oxoid)

Ingredients (g l^{-1}):

Lab-lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
NaCl	5.0

According to the manufacturer's instructions, 13 g of the above formula was added to 1 liter of distilled water. The medium was stirred for a while until all components were dissolved and then pH was adjusted to 7.4. After making up the medium to its final volume, it was distributed into 5′ glass test tubes (10 ml each). Tubes were then autoclaved at 121 °C for 20 min. After cooling down to room temperature, tubes were kept in a fridge until use. When 1.5 M NaCl was needed in the medium, an amount of NaCl (82.75 g l^{-1}) was added to give a final concentration of 1.5 M in the medium.

A7. Nutrient Agar Medium (Oxoid)

Ingredients (g l^{-1}): as in nutrient broth medium (Appendix A6) plus 15.0 g agar. According to the manufacturer's instructions, 28 g of the above formula was added to 1 liter of distilled water. The medium was stirred for a while until all components were dissolved and then pH was adjusted to 7.4. After making up the medium to its final volume, it was autoclaved at 121 °C for 20 min. After cooling down to about 45 °C, the agar medium was distributed into plastic Petri dishes and allowed to solidify at room temperature and then kept in a fridge (inverted) until use. When 1.5 M NaCl was needed in the medium, an amount of NaCl (82.75 g l^{-1}) was added to give a final concentration of 1.5 M in the medium.

A8. *Spirulina* Medium

Spirulina medium was prepared according to UTEX (USA) in two solutions to prevent the formation of precipitates during autoclaving. Preparation method of each solution was as follows.

Spirulina Solution 1:

The following components were added in the order listed to approximately 450 ml of distilled water in a 1 L Duran bottle while stirring continuously.

No.	Component	Amount (g / 0.5 L of *Spirulina* Solution 1)	Final Concentration (mM / L of *Spirulina* Medium)
1	$NaHCO_3$	13.61	162
2	Na_2CO_3	4.03	38
3	K_2HPO_4	0.51	2.9

Total volume was made up to 500 ml with distilled water and then the solution was autoclaved for 20 min at 121 °C.

Spirulina Solution 2:

The following components (except vitamin B_{12}) were added in the order listed to approximately 450 ml of distilled water in a 1 L Duran bottle while stirring continuously.

No.	Component	Amount (g or ml / 0.5 L of *Spirulina* Solution 2)	Final Concentration (mM / L of *Spirulina* Medium)
1	$NaNO_3$	2.5 g	29.4
2	K_2SO_4	1 g	5.68
3	NaCl	1 g	17.1
4	$MgSO_4 \cdot 7H_2O$	0.2 g	0.81
5	$CaCl_2 \cdot 2H_2O$	0.04 g	0.27
6	P-IV Metal Solution	6 ml	-
7	Chu Micronutrient Solution	1 ml	-
8	Vitamin B_{12} (add after autoclaving)	1 ml	-

Total volume was made up to 500 ml with distilled water and then the solution was autoclaved for 20 min at 121 °C. After autoclaving and cooling to room temperature, 1 ml of vitamin B_{12} stock solution was added aseptically to the solution.

For 1 L total volume of *Spirulina* medium:

After sterilization of both solution 1 and 2, both solutions were allowed to cool down to room temperature and then the content of solution 2 was transferred aseptically into the 1 L Duran bottle which containing solution 1 and mixed thoroughly. The medium was stored in a fridge.

Preparation of P-IV Metal Solution

The following components were added in the order listed to approximately 950 ml of distilled water in a 1 L Duran bottle while stirring continuously (Na_2EDTA should be fully dissolved before adding other components).

No.	Component	Amount (mg / L)	Final Concentration (mM / L of P-IV Metal Solution)	Final Concentration (μM / L of *Spirulina* Medium)
1	$Na_2EDTA.\ 2H_2O$	750	2	12
2	$FeCl_3 \cdot 6H_2O$	97	0.36	2.16
3	$MnCl_2 \cdot 4H_2O$	41	0.21	1.26
4	$ZnCl_2$	5	0.037	0.222
5	$CoCl_2 \cdot 6H_2O$	2	0.0084	0.05
6	$Na_2MoO_4 \cdot 2H_2O$	4	0.017	0.102

Total volume was made up to 1 L with distilled water and then stored at refrigerator temperature.

Preparation of Vitamin B_{12}

50 mM of HEPES buffer was prepared by dissolving 2.39 g of HEPES into 200 ml of distilled water and then adjusted at pH 7.6. 27 mg of vitamin B_{12} (cyanocobalamin) was dissolved in the buffer to give a final concentration of 4 μM ml^{-1}. Vitamin B_{12} solution was sterilized by filtration using 0.45 μm Millipore filter and then stored in dark at freezer temperature.

Preparation of Chu Micronutrient Solution

The following components were added in the order listed to approximately 900 ml of distilled water in a 1 L Duran bottle while stirring continuously.

No.	Component	Amount (mg / L)	Final Concentration (mM / L of Chu Micronutrient Solution)	Final Concentration (μM / L of *Spirulina* Medium)
1	$CuSO_4 \cdot 5H_2O$	20	0.08	0.08
2	$ZnSO_4 \cdot 7H_2O$	44	0.15	0.15
3	$CoCl_2 \cdot 6H_2O$	20	0.084	0.084
4	$MnCl_2 \cdot 4H_2O$	12	0.06	0.06
5	$Na_2MoO_4 \cdot 2H_2O$	12	0.05	0.05
6	H_3BO_3	620	10	10
7	Na_2EDTA	50	0.13	0.13

Total volume was made up to 1 L with distilled water and then stored at refrigerator temperature.

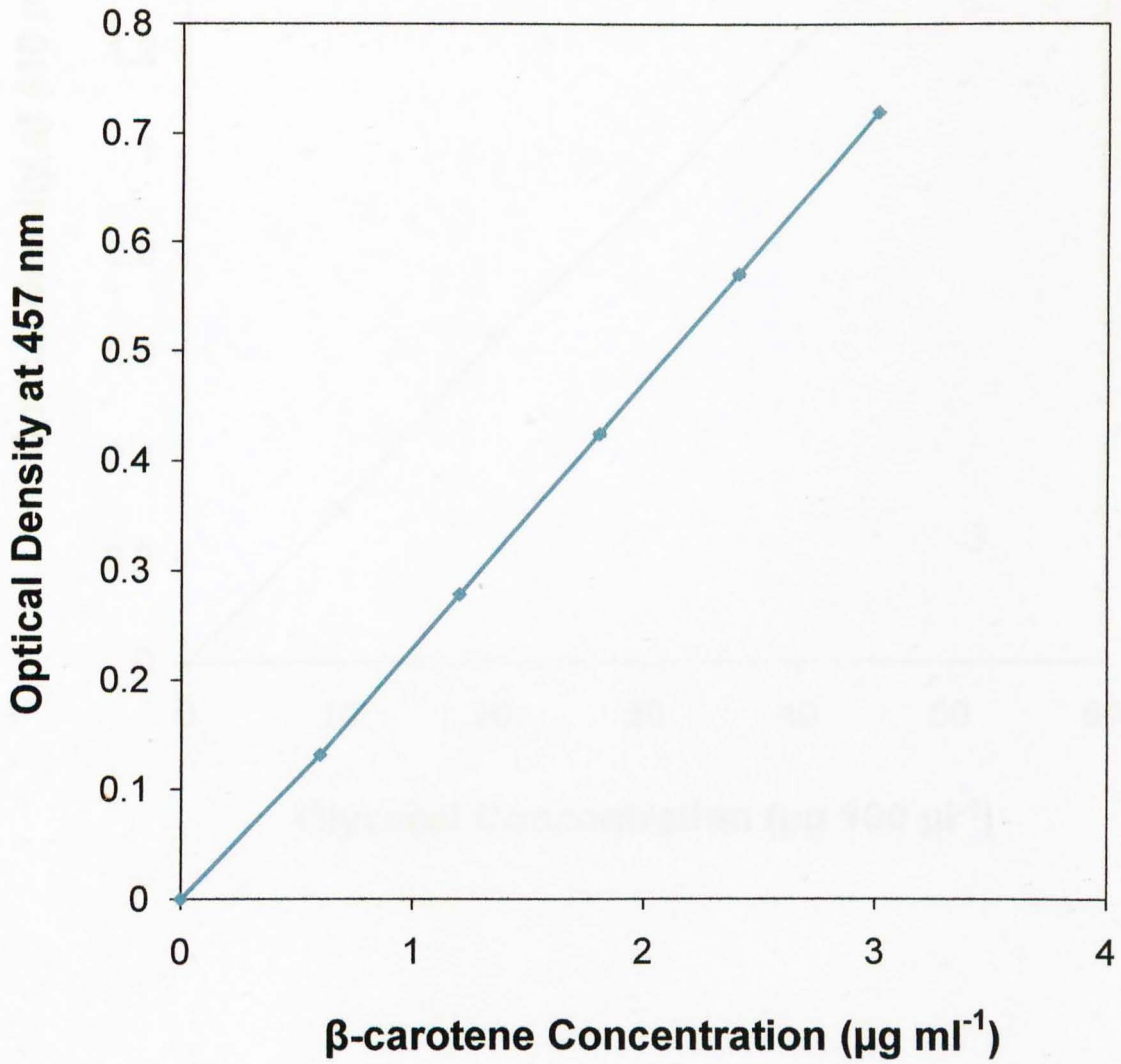
Spirulina Medium Agar

In order to get solid *Spirulina* medium, 1.6 % (w/v) of bacteriological agar (Oxoid) was added to solution 2. Solution 1 was prepared as above. Both solutions were autoclaved for 20 min at 121 °C. After cooling to 45 °C, 1 ml of vitamin B_{12} stock solution was added aseptically to the solution 2 and then the content of solution 2 was transferred aseptically into the 1 L Duran bottle which containing solution 1 and mixed gently. Agar plates were prepared by pouring the medium into 9 cm^3 plastic Petri dishes and then stored at refrigerator temperature.

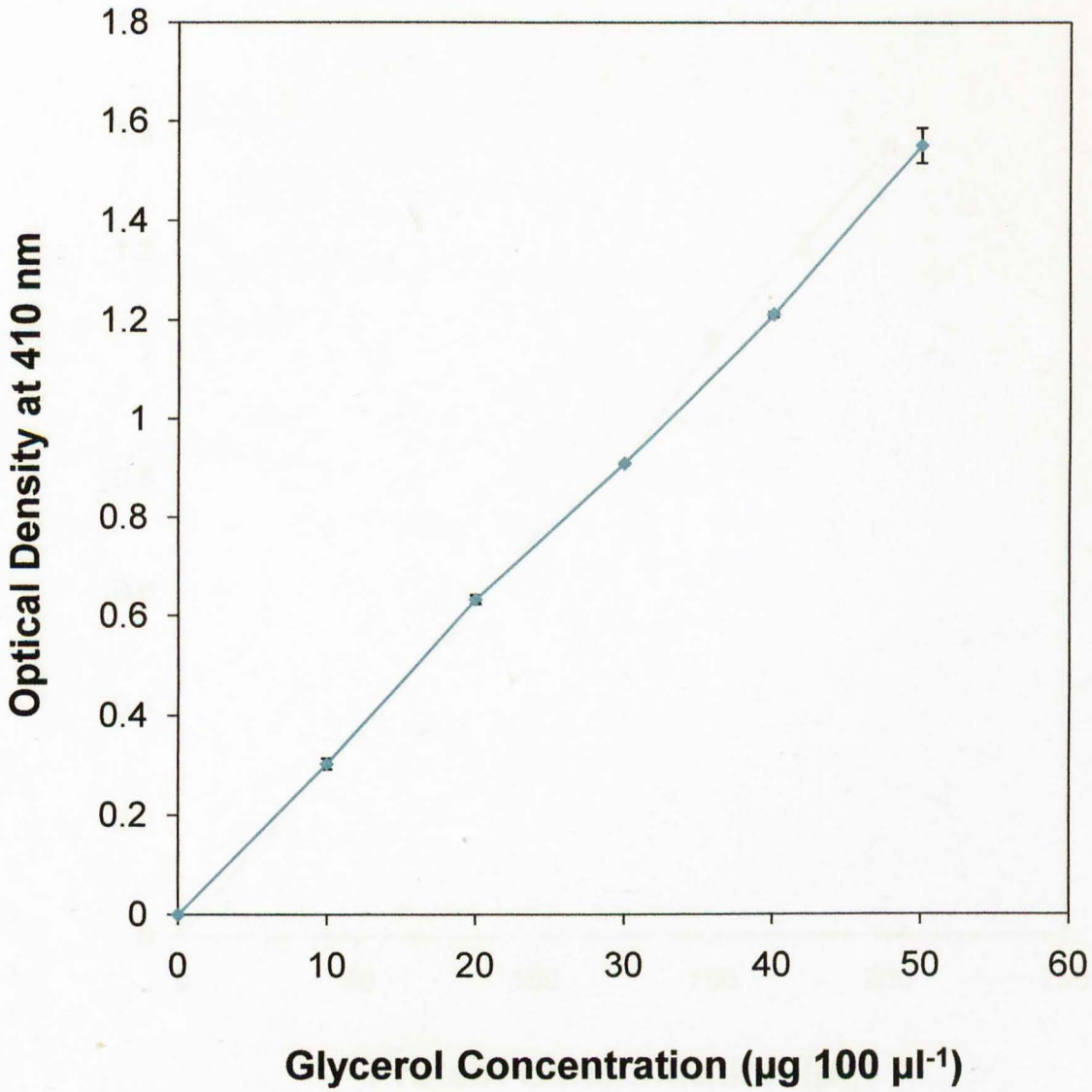
Appendix B

Standard Curves

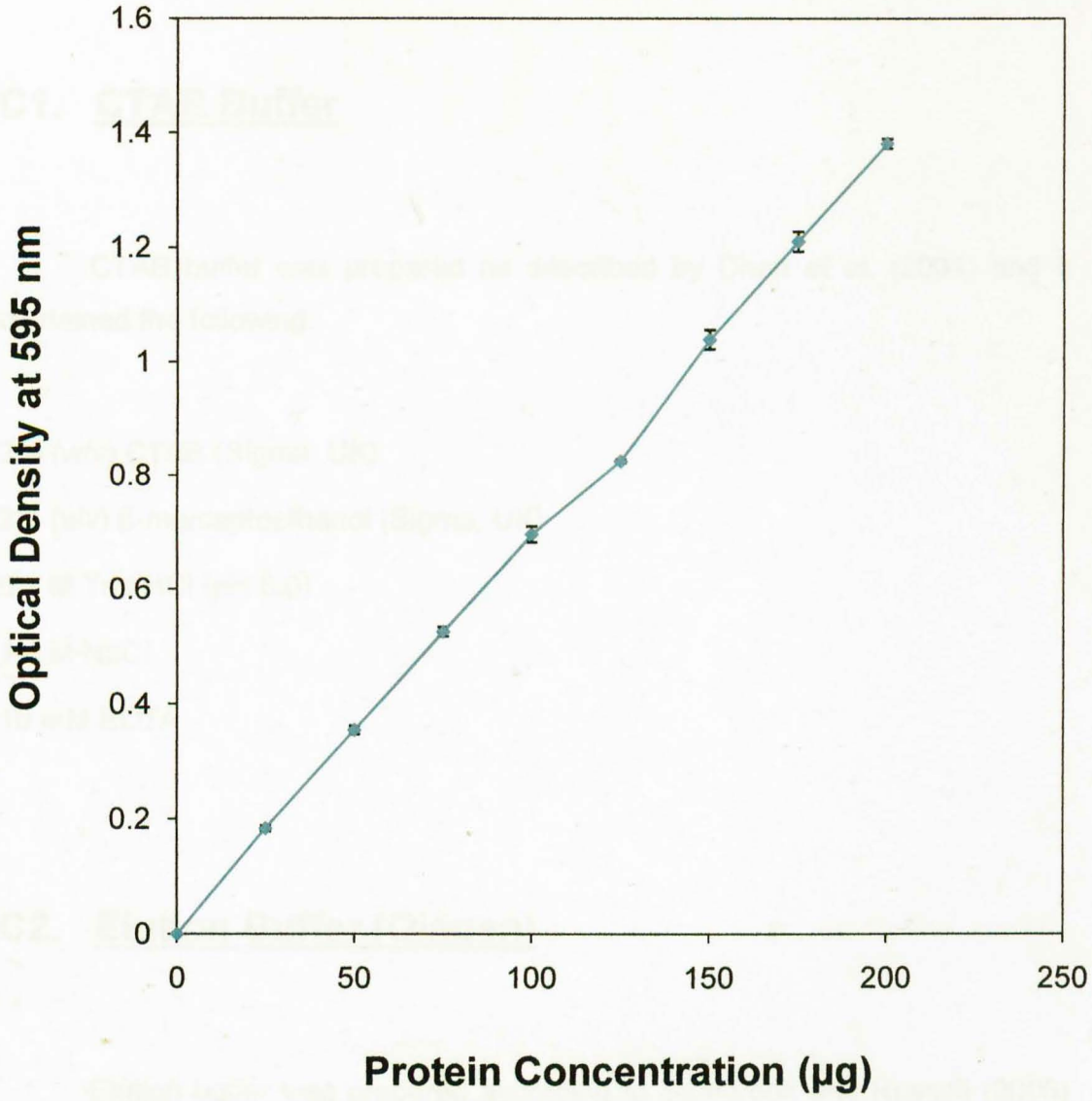
B1. β-carotene Standard Curve



B2. Glycerol Standard Curve



B3. Protein Standard Curve



Appendix C

Buffers

C1. CTAB Buffer

CTAB buffer was prepared as described by Chen *et al.* (2001) and it contained the following.

2% (w/v) CTAB (Sigma, UK)

2% (v/v) β-mercaptoethanol (Sigma, UK)

0.1 M Tris-HCl (pH 8.0)

1.4 M NaCl

10 mM EDTA

C2. Elution Buffer (Qiagen)

Elution buffer was prepared according to Sambrook and Russell (2006) and it contained the following.

50 mM Tris-HCl (pH 8.1 – 8.2)

1.4 M NaCl

15% (v/v) ethanol