Physiology and Biotechnology of Glycerol Production Using the Green Microalga *Dunaliella*

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In memory of my grandfather

Mr. Sangiem Powtongsook

1910 - 1998

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หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเล ณ จุฬาลงกรณ์มหาวิทยาลัย ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

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Dunaliella, a green microalga, accumulates intracellular glycerol as its osmoticum when exposed to high salinity. In this thesis, *Dunaliella parva* strain CCAP 19/9 was studied for the production of glycerol. *D. parva* cells, in 1.5 M NaCl salinity, were immobilised in calcium alginate beads. *D. parva* was found to grow well under entrapped conditions. Cells divided and formed sphere-shape cell clusters inside the beads. Growth of algal cells was demonstrated by measuring the increasing in chlorophyll content. Photosynthetic oxygen evolution and chlorophyll fluorescence indicated that immobilised *D. parva* cells were in a healthy condition for more than two months and glycerol was continuously released into the medium. Self shading from cells inside the beads and packing of beads themselves can reduce photosynthetic rate which affects glycerol production. Glycerol productivity could be enhanced by semi-continuous removal and replacement (harvesting) of the medium. The harvesting maintained a low glycerol concentration in the medium and induced more glycerol to be released from the cells. An improvement of the immobilisation technique using calcium alginate with spongy supporter or algal sheet showed good potential for future use.

However, glycerol was also found in the medium of free cells of *D. parva*. Previous publications suggested that *Dunaliella* cells do not leak glycerol when growing in medium containing more than 0.6 M NaCl. This study demonstrates leakage of glycerol without osmotic shock from *D. salina* as well as *D. parva* and the leakage was found even in 3.5 M NaCl. Dark conditions and metabolic inhibitors, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and sodium vanadate, did not affect glycerol leakage, therefore, glycerol leakage is possibly not an active process and may simply to be diffusion down a glycerol gradient. After 4-7 days, more than 70% of glycerol synthesised had leaked from *Dunaliella* cells and accumulated in the medium. This study confirms that glycerol leakage is a normal characteristic of *Dunaliella*.

endoplasmic reticulum can be found close to the nuclear membrane (Hoshaw and Maluf, 1981). An electron micrograph of *Dunaliella* is shown in Figure 1.1.

Figure 1.1: Electron micrograph of *Dunaliella* (Ben-Amotz and Avron, 1989).

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List of abbreviations

Abbreviations used in this thesis, not including elemental symbols and SI units are as follows:

Abbreviation	Full Designation
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
CCAP	Culture Collection of Algae and Protozoa
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
Chl	Chlorophyll
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetate
HEPES	n-[2-hydroxyethyl]pipperazine-N′-2-ethanesulfonic acid
GK	Glycerokinase
LDH	Lactate dehydrogenase
NAD	β-Nicotinamide Adenine Dinucleotide
NADH	Reduced NAD
NADP	β-Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced NADP
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PEP	Phosphoenolpyruvate
pH	- (log H^+ concentration in M)
Pi	Inorganic phosphate
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N′,N′-tetramethylene-ethylenediamine
Tris	Tris(hydroxymethyl) methylamine
UV	Ultraviolet

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction to *Dunaliella*

1.1.1 Morphology and Life History

Dunaliella is a unicellular green alga. It is unusual in the plant and bacterial kingdoms because of the lack of a cell wall (Ginzburg, 1978). Cell shape varies among species, from ellipsoid, ovoid, cylindrical, pyriform, fusiform to spherical with two equal flagella at the anterior end. Cell size ranges from 8-25 μm long and 5-15 μm wide. Ultrastructure of *Dunaliella* spp. is similar to *Chlamydomonas* (Volvocales, Chlamydomonadaceae) another alga in the same order, in structure and arrangement (Hoshaw and Maluf, 1981), but the most important characteristic of *Dunaliella* is the lack of a rigid polysaccharide cell wall. *Dunaliella* cells are enclosed within a flexible plasma membrane and a mucus "surface coat". The plasma membrane protects the cell from the external environment and the surface coat is possibly a cleaning system that removes contaminants from the cell surface (Oliveira *et al.*, 1980). Under the light microscope, *Dunaliella* can be observed and easily distinguished from *Chlamydomonas* by applying hypoosmotic shock to the cells. After the sudden salinity change, *Dunaliella* cells will swell into a ball shape due to water flowing into the cells.

Dunaliella has one large cup-shaped chloroplast which occupies most of the cell body and a single pyrenoid surrounded by polysaccharide granules. The amount and shape of pyrenoid starch granules were reduced/changed when cells were grown in high CO_2 concentration (Tsuzuki *et al.*, 1986). The eye spot (stigma) is reported in many species of *Dunaliella* but may hardly be visible under the light microscope in some species, especially *D. salina* (Preisig, 1992). The nucleus is located at the anterior end, surrounded by the anterior lobe of the chloroplast, and heterochromatin and a nucleolus are present. The nuclear envelope has many nuclear pores and

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endoplasmic reticulum can be found close to the nuclear membrane (Hoshaw and Maluf, 1981). An electron micrograph of *Dunaliella* is shown in Figure 1.1.
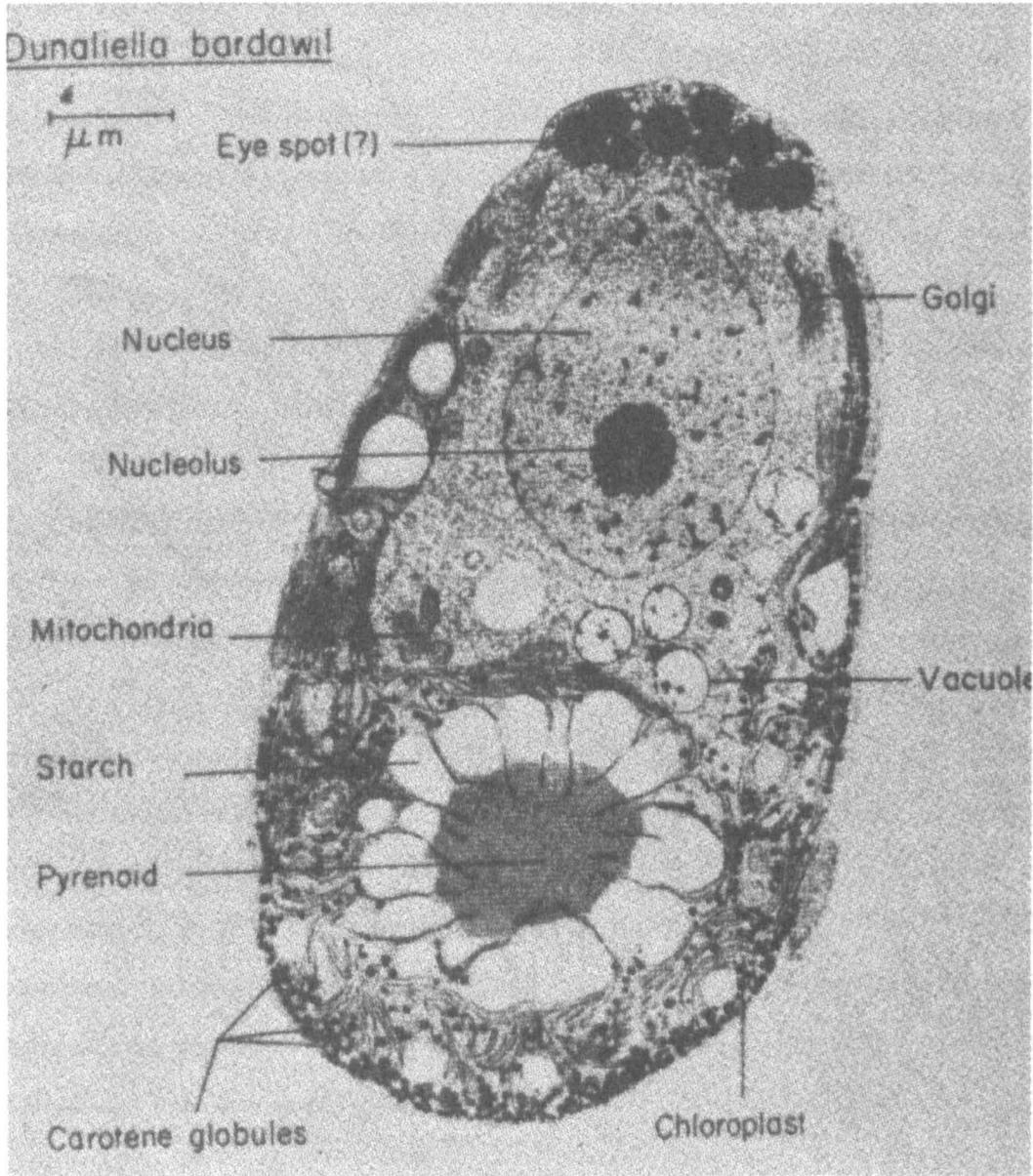
Figure 1.1: Electron micrograph of *Dunaliella* (Ben-Amotz and Avron, 1989).

Dunaliella is found in high salinity environments such as in the Dead Sea in Israel, the Great Salt Lake in Utah, seaside rock pools and in commercial salt production ponds in which the salinity is higher than normal seawater. It is the most important primary producer in hypersaline environments (Brown and Borowitzka, 1979). *D. salina* was found to be the dominant species, associated with *D. viridis*, in most of the high salinity lakes and salt ponds (Post, 1977; Montoya and Olivera, 1993; De Deckker, 1983; Powtongsook *et al.*, 1995). *D. parva* is the dominant species in the Dead Sea (Oren, 1993) and is also found in estuarine rockpools and other hypersaline lakes elsewhere (Borowitzka and Borowitzka, 1988). There is only one species of *Dunaliella*, *D. acidophila* (formerly *Spermatozopsis acidophila*), that is found in acidic freshwater lakes and does not occur in marine habitats (Gimmler and Weis, 1992). This species can tolerate extremely low pH values of between pH 0.3-1.

Reproduction cycle of *Dunaliella* is similar to that found in *Chlamydomonas* (Harris, 1989). The cell cycle consists of a vegative cell, aplanospore (cyst), isogamete and zygote. The apparent life cycle of *D. salina* in nature was described by Montoya and Olivera (1993) and is shown in Figure 1.2. In *D. salina*, sexual activity could be enhanced by low salt concentration [0.3-0.8 M NaCl or 2-5% (w/v) NaCl] and much less sexual activity was found in 5 M NaCl medium [30% (w/v) NaCl] (Martinez *et al.*, 1995). Aplanospore formation was found either when *D. salina* cells were subjected to a very dilute medium (low salinity) or when the habitat dried up (saturated salinity) as it is a resistant stage which allows cells to survive in unfavorable conditions (Montoya and Olivera, 1993). However, aplanospore formation has not been reported in other species of *Dunaliella* (Brown and Borowitzka, 1979).
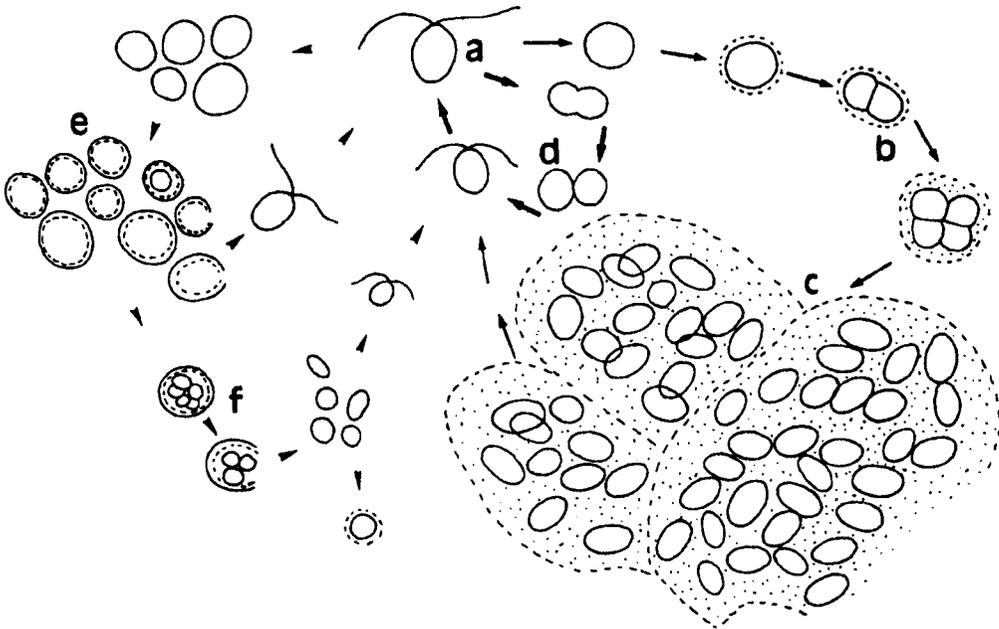
Figure 1.2: Apparent life cycle of natural populations of *Dunaliella salina* (Montoya and Olivera, 1993). a: biflagellate cells; b: successive cell divisions of non-motile rounded cell with gradual increase of colonial mucilage; c: palmelloid thalli formation with multicellular aggregated colonies within a common mucilage, and later cell release; d: binary fission of free cells; e: formation of aplanospores with thick cell walls; these produce motile cells directly or divide before release of small free cells (f).

1.1.2 Classification

Dunaliella has been classified to:

Division	Chlorophyta
Class	Chlorophyceae
Order	Volvocales
Family	Polyblepharidaceae
Genus	*Dunaliella*

The classification above is according to a key to families of Volvocales described by Dillard (1980). Although according to phylogenetic studies the class Chlorophyceae is heterogeneous and could possibly be split into two classes: Chlamydophyceae and Chlorophyceae. The order Volvocales is placed in Chlamydophyceae in this new classification (Kouwets, 1994 quoted by Hoek *et al.*, 1995). This idea is not yet widely accepted (Hoek *et al.*, 1995).

Genus *Dunaliella* was described by Teodoresco in 1905 and was named in honour of M.F. Dunal, who in 1837 was the first to recognise that the red colour of hypersaline reservoirs was caused by an alga (Borowitzka and Borowitzka, 1988). Teodoresco (1905) separated *Dunaliella* from *Haematococcus* and *Chlamydomonas* and established a new genus.

According to the key to the chief genera of the smaller Chlorophyceae by Butcher (1959), the important characteristics of genus *Dunaliella* were described as:
- Green free-living unicells
- Flagella originating at the anterior end
- Cells regular, ovoid to ellipsoid
- Chromatophore single, pyrenoid present

Butcher (1959) reported and classified *Dunaliella* from British coastal waters into 12 species. More recently, Masjuk (1972) quoted by Preisig (1992) devided the genus into two subgenera, *Pascheria* and *Dunaliella* in which *Pascheria* has contractile vacuoles in vegetative cells. According to Preisig (1992), species of *Dunaliella* were listed as follows:

Subgenus *Pascheria*
>   *D. acidophila, D. flagellata, D. lateralis, D. obliqua, D. paupera*

<u>Subgenus *Dunaliella*</u>

D. *maritima*, D. *polymorpha*, D. *primolecta*, D. *quartolecta*, D. *tertiolecta*, D. *parva*, D. *pseudosalina*, D. *salina*, D. *baas-beckingii*, D. *bioculata*, D. *carpatica*, D. *gracilis*, D. *granulata*, D. *media*, D. *minuta*, D. *minutissima*, D. *ruineniana*, D. *terricola*, D. *viridis*, D. *asymmetrica*, D. *jacobae*, D. *peircei*, D. *turcomanica*

Pictures of several species of *Dunaliella* are shown in Figure 1.3.
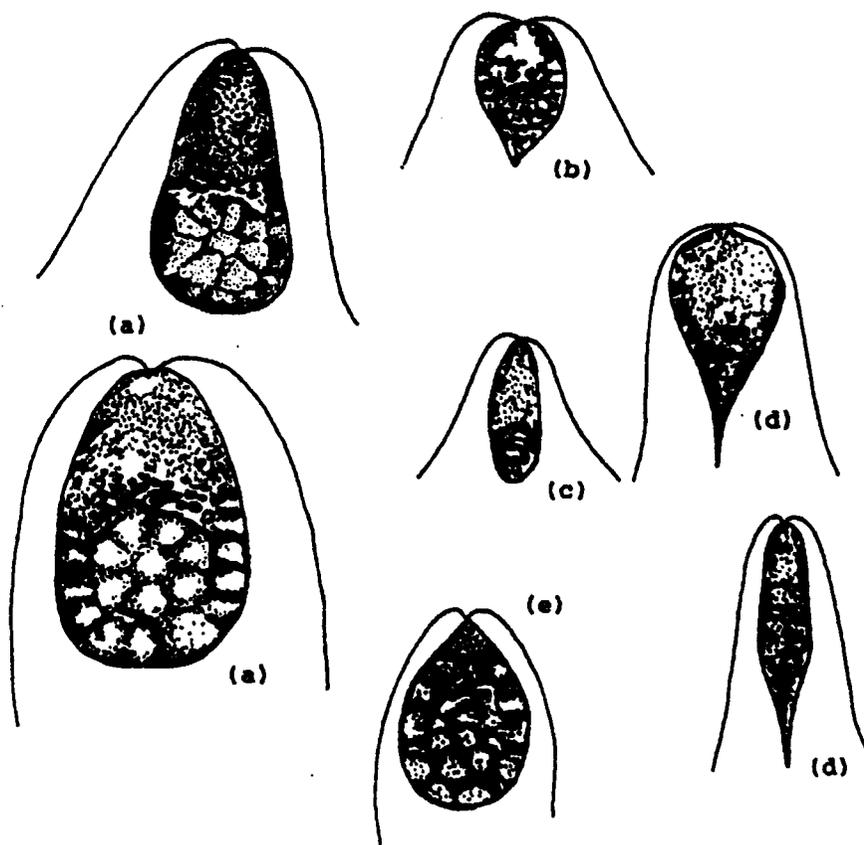
Figure 1.3: Pictures of some *Dunaliella* species (adapted from Lerche, 1937).

a: *D. salina*; b: *D. euchlora*; c: *D. minuta*; d: *D. piercii*; e: *D. parva*

However, several species such as *D. minor, D. piercii* and *D. euchlora* which were described in Butcher (1959) did not appear in the list of Preisig (1992) and *D. bardawil* (Ben-Amotz *et al.*, 1982a; Ben-Amotz and Avron, 1983a) was classified as a synonym of *D. salina.*

Among 31 species identified, only 7 species of *Dunaliella* have been regularly studied. For example, *D. acidophila* was studied to understand its capability to grow in extremely acidic conditions (Gimmler *et al.*, 1989; Geib *et al.*, 1996); *D. salina*, which can accumulate large amounts of β-carotene, has been used for mass production of β-carotene (Borowitzka and Borowitzka, 1988; Ben-Amotz, 1995); *D. parva*, a dominant algal species in the Dead Sea (Oren *et al.*, 1995), has been intensively studied in relation to osmoregulation, photosynthesis and glycerol synthesis pathway (Ben-Amotz and Avron, 1973; Ginzburg, 1991); *D. viridis* is a species associated with *D. salina* in nature and it has been used in ecological studies (Post, 1977, Moulton *et al.*, 1987) as well as in mass cultivation experiments (Moulton and Burford, 1990); *D. tertiolecta* has been used for osmoregulation and photosynthesis studies (Gilmour *et al.*, 1984a,b; Aoki *et al.*, 1986), study of the carbon dioxide concentrating mechanism (Aizawa *et al.*, 1986; Amoroso *et al.*, 1996) and used as feed in aquaculture (Fabregas *et al.*, 1986); *D. primolecta* has been studied as a source of antibiotics which are active against many strains of bacteria (Chang *et al.*, 1993, Ohta *et al.*, 1995); and *D. bioculata* has been used to study the plasma membrane ATPase (Smahel *et al.*, 1990; Raschke and Wolf, 1996) and for comparative studies of ultrastructure (Marano *et al.*, 1985, Hoshaw and Maluf, 1981).

Classical taxonomy of *Dunaliella* is based on morphology and organelles which can be seen under the light microscope. However, as *Dunaliella* does not have a rigid cell wall, clonal culture of a single species can still be found to vary in cell shape and size. This causes confusion in species descriptions and misnamed species have appeared in culture collections. For example, Borowitzka and Borowitzka (1988) suggested that all *Dunaliella* strains that appear red, due to accumulation of large amounts of β-

carotene under extreme environmental conditions, are *D. salina* and they explained that *D. bardawil* (Ben-Amotz and Avron, 1983a) is actually a strain of *D. salina* while *D. salina* of Ben-Amotz and Avron (1982) is probably *D. parva*. On the other hand, Ben-Amotz (personal communication) quoted that the major difference between *D. bardawil* and *D. salina* is the presence of distinguishing extra chloroplastic eye spots, and the related active phototaxis of *D. bardawil* in comparison to *D. salina*. Both species can accumulate large amounts of chloroplastic β-carotene when exposed to high light intensities which retard growth. Loeblich (1982) proposed that *D. salina* is an organism that has the capability of turning red with a carotenoid to chlorophyll ratio greater than 6:1. Some strains of *Dunaliella*, currently called *D. salina*, were not accepted if cells do not turn red (by accumulating β-carotene) at salinities up to 4.3 M NaCl [25% (w/v) NaCl]. The only way to be sure that an isolate of *Dunaliella* is *D. salina* is to grow it at a salinity greater than 2.5 M NaCl [15% (w/v) NaCl] under high light intensity and observe whether it is capable of turning red, or alternatively, to isolate single red cells from high salinity natural water samples. So, it is accepted now that *D. bardawil* was a technically invalid synonym of *D. salina* or a *nomen nudum*.

Dunaliella parva strain CCAP 19/9 [synonym: *D. maritima* Masjuk (1973)], which is the major strain used in this study, was isolated by R.W. Butcher from a salt mash on Northy Island, Essex, England. Salinity at the collection site was 24.31 parts per thousand (0.41 M NaCl). Cells of *D. parva* are 7-12 μm × 4-7 μm in size. It is usually green, but accumulates a small amount of β-carotene pigment under nutrient deficiency conditions (Brown and Borowitzka, 1979). This strain has been used in many publications, i.e., Ginzburg (1978), Ginzburg (1981), Ginzburg and Ginzburg (1981), Ginzburg and Richman (1985), Ginzburg and Ginzburg (1985), Ginzburg *et al.* (1995), Evans *et al.* (1982), Hajibagheri *et al.* (1986), Hard and Gilmour (1991) and Hard and Gilmour (1996). There is no comprehensive study on salinity tolerance of *D. parva*. However, Borowitzka and Brown (1974) suggested that *D. parva* has a salt tolerance intermediate between *D. tertiolecta* (marine species) and *D. viridis*,

(extremely halotolerant species). It has an optimum salinity of 0.1-2.0 M NaCl (Ginzburg and Ginzburg, 1981). Ginzburg (1978) mentioned that *D. parva* cells seem to lyse at 5 M NaCl.

The other strain used in the present study, *Dunaliella salina* strain DS91008, was isolated from salt ponds in Samut Songkhram Province, Thailand (Powtongsook *et al.*, 1995). *D. salina* has a larger cell size than *D. parva*. Cell dimensions range from 6-30 μm × 4-15 μm. Under high salinity, high light intensity and/or nutrient deficiency, *D. salina* accumulates β-carotene and turns orange. Optimum salinity for growth is from seawater salinity (~0.5 M) to 2 M NaCl, but it can tolerate salinity up to saturated salt (>5.5 M NaCl).

1.2 Physiology of salt tolerance in *Dunaliella*

1.2.1 Salt tolerance in *Dunaliella*

Dunaliella is possibly the most halotolerant organism of all single-celled eukaryotes (Brock, 1975). The mechanism of salt tolerance in *Dunaliella* differs from that of halophilic bacteria, therefore, *Dunaliella* is called a halotolerant not a halophilic alga. This means it can tolerate high salinity environments, up to saturated salt in some cases, but does not require high salt for growth and cellular metabolism as in extremely halophilic archaebacteria. Archaebacteria such as *Halobacterium*, *Halococcus*, *Natronobacter* and *Natronococcus*, which are extremely halophilic species, require at least 1.5 - 3.0 M NaCl to survive so they are irreversibly adapted to high salinity (Gilmour, 1990). In halophilic bacteria, enzymes, especially those associated with the cell membrane and ribosomes, can maintain their activity in high salt concentrations and their optimum salinity for activity can be as high as 4 M NaCl (Brown and Borowitzka, 1979), so, halophilic bacteria cannot grow at low salinity. *Dunaliella*, however, can adapt to various salinities from seawater to extremely high salinity. *Dunaliella* can be trained to grow in higher or lower salt concentration media by serial transfers. Some species such as *D. viridis* can survive after being

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transferred from 3.4 to 0.5 M NaCl in a single step (Brown and Borowitzka, 1979), but normally *Dunaliella* withstands up to three- to four-fold increases or decreases in osmotic pressure (Avron, 1992). For example, *D. salina* can grow after a salinity increase from 1.5 to 5 M NaCl, but it needs a long lag period (S. Powtongsook, unpublished observation).

In general, there are two mechanisms for balancing osmotic pressure in algae. First, ion transport through the cell membrane using transport proteins (ionic pumps) associated with contractile vacuoles which remove water and, second, the synthesis (or degradation) of an osmolyte which is normally a low molecular weight metabolite (Wegmann, 1986). *Dunaliella* uses the second mechanism because it accumulates glycerol to balance the external osmotic pressure when it is exposed to high salinity. The first report on glycerol accumulation in *Dunaliella* was presented by Craigie and McLachlan (1964). Thereafter, Ben-Amotz and Avron (1973) confirmed the role of glycerol in the osmotic regulation by *Dunaliella* in which glycerol is produced and accumulated in large amounts when cells are grown in high salinity. They suggested that approximately 2.1 M intracellular glycerol is needed to balance 1.5 M external NaCl concentration (another figure reported by Degani *et al.* (1985) was that 1.9 M glycerol is osmotically equivalent to 1.25 M NaCl). Up to 7 M glycerol is found in *Dunaliella* cells growing in saturated salt i.e. approximately 5.5 M NaCl (Avron, 1992). Borowitzka and Brown (1974) suggested that glycerol is not only an osmotic regulator but that its physiological role is to act as a "compatible solute" in which it actively protects enzyme activity from inactivation and inhibition.

Various organic compounds act as osmoregulators in different species of algae. Examples of different osmotic metabolites found in algae are shown in Table 1.

Table 1: Accumulation of osmotic metabolites in some halotolerant algae (adapted from Ben-Amotz and Avron, 1983b).

Organisms	Osmotic range (M NaCl)	Intracellular osmoregulator
Synechoccocus (Cyanophyceae)	0.03-1.20	Glucosylglycerol
Porphyra (Rhodophyceae)	0.30-0.60	Floridoside, isofloridoside
Fucus (Phaeophyceae)	0.04-0.55	Mannitol
Navicula (Bacillariophyceae)	0.25-2.75	Proline
Cylindrotheca (Bacillariophyceae)	0.57-0.70	Mannose
Chlamydomonas (Chlorophyceae)	0.34-1.71	Glycerol
Dunaliella (Chlorophyceae)	0.10-5.50	Glycerol
Stichococcus (Chlorophyceae)	0.39-0.60	Sorbitol, proline

1.2.2 Glycerol and glycerol metabolism in *Dunaliella*

Glycerol, propane-1,2,3-triol or 1,2,3-propanetriol ($C_3H_8O_3$) is also known as glycerin or glycerine. It has a molecular weight of 92.09, density of 1.2613 $g.cm^{-3}$, melting point at 18.2°C and boiling point at 290°C. It is a clear, colourless, oily liquid which is soluble in water and alcohol, slightly soluble in ethyl ether but insoluble in benzene (Lide and Frederikse, 1994). The chemical structure of Glycerol is shown in Figure 1.4.

$$H_2C - OH$$
$$|$$
$$HC - OH$$
$$|$$
$$H_2C - OH$$
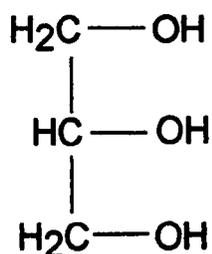
Figure 1.4: Structural formula of glycerol

Glycerol is a three carbon alcohol with a hydroxyl group on each carbon. It is a backbone for lipid in which fatty acid molecules are linked to two of these hydroxyl groups. Examples of lipid molecules are triglycerides (fat and oil) and phosphoglyceride (found in cell membranes). Glycerol is produced from dihydroxyacetone phosphate via glycerol phosphate. The process is carried out mostly within the chloroplast and partly in the cytosol. In *Dunaliella*, glycerol is produced either by photosynthetic CO_2 fixation or by starch degradation (Brown and Borowitzka, 1979; Avron, 1992). A diagram of glycerol metabolism in *Dunaliella* is shown in Figure 1.5.
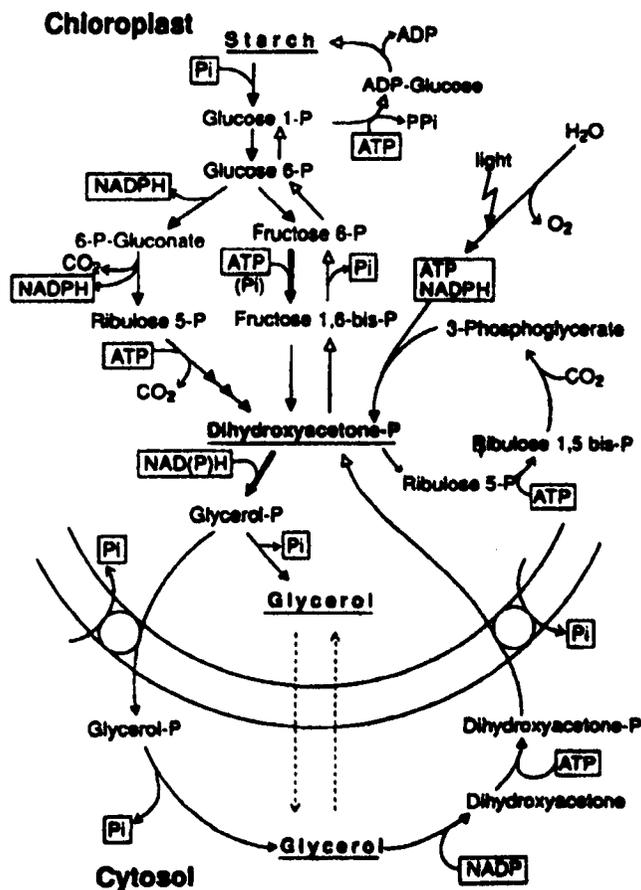
Figure 1.5: A proposed metabolic pathway for glycerol metabolism in *Dunaliella* (Avron, 1992)

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1.2.3 Energetic requirements of glycerol synthesis

Kaplan *et al.* (1980) described the energy requirements of glycerol synthesis as follows:

Glycerol synthesis by the photosynthesis pathway:

$$6\ CO_2 + 8\ H_2O + 18\ ATP + 14\ NADPH + 14\ H^+ \rightarrow$$

$$2\ C_3H_8O_3\ (glycerol) + 7\ O_2 + 18\ ADP + 18\ Pi + 14\ NADP^+$$

$$P/2e = 1.3;\ O_2/CO_2 = 1.2$$
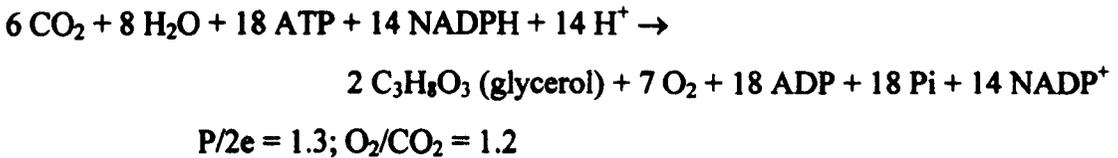
Glucose (starch) synthesis by photosynthesis pathway:

$$6\ CO_2 + 6\ H_2O + 18\ ATP + 12\ NADPH + 12\ H^+ \rightarrow$$

$$(CH_2O)_6\ (glucose) + 6\ O_2 + 18\ ADP + 18\ Pi + 12\ NADP^+$$

$$P/2e = 1.5;\ O_2/CO_2 = 1.0]$$
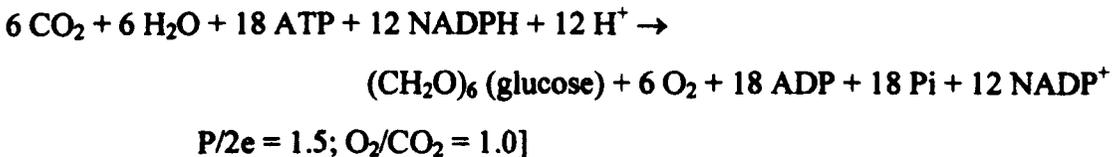
Therefore, in glycerol photosynthesis, 9 ATP and 7 NADPH are needed for each molecule of glycerol produced.

Glycerol synthesis from starch degradation via the glycolytic pathway:

$$(CH_2O)_6 + 2\ H_2O + ATP + 2\ NADPH + 2\ H^+ \rightarrow$$

$$2\ C_3H_8O_3 + O_2 + ADP + Pi + 2\ NADP^+$$

$$P/2e = 0.5;\ O_2/CO_2 = \infty$$
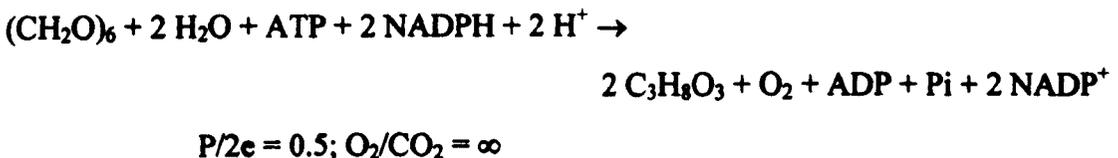
In this process, the energy cost amounts to 0.5 ATP and 1 NADH for one molecule of glycerol produced. However, starch degradation can possibly happen by a combination of glycolytic and pentose phosphate pathways (Degani *et al.*, 1985; Avron, 1992). The energy requirement for this mixed pathway can be stated as:

$$3 \ (CH_2O)_6 + \frac{1}{2} \ O_2 + 2 \ H_2O + 2 \ ATP \rightarrow 5 \ C_3H_8O_3 + 3 \ CO_2 + 5 \ ADP + 5 \ Pi$$
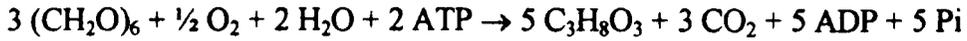
This require at least 0.4 ATP/glycerol.

On the other hand, energy is also needed in order to convert or reassimilate glycerol into starch (Avron, 1992). In this case, 1.5 ATP and 1 $NADP^+$ are need for one molecule of glycerol. The energetic equation can be shown as:

$$2 \ C_3H_8O_3 + 2 \ NADP^+ + 3 \ ATP \rightarrow (CH_2O)_6 + 3 \ ADP + 3 \ Pi + 2 \ NADPH + 2 \ H^+$$
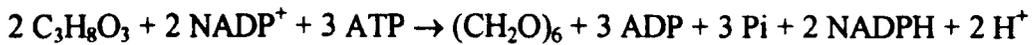
1.2.4 Cellular response to salt

Dunaliella cells respond immediately to osmotic shock by morphological change. Cells can shrink to give elongated necks or expand to a spherical shape when subjected to upward osmotic shock (hyperosmotic) or downward osmotic shock (hypoosmotic) respectively. An electron microscope study of morphological ultrastructure after osmotic shock was performed by Weiss *et al.* (1991). The results of this study are summarised diagramatically in Figure 1.6.

Avron (1992) suggested that glycerol synthesis and reassimilation in *Dunaliella* are strictly controlled by volume changes which result from cell shrinkage or swelling in response to changing osmotic pressure. This process is a rapid response and does not require *de novo* protein synthesis. Although there are several long-term effects found after cells are adapted to different salinities such as induction of carbonic anhydrase activity (Brown *et al.*, 1987) and new plasma membrane proteins (Sadka *et al.*, 1991; Fisher *et al.*, 1994 and Golldack *et al.*, 1995). These have been called secondary adaptations and are not directly linked to glycerol metabolism.

1 to 0.5 M NaCl

1 to 2 M NaCl

1 minute   30 minutes
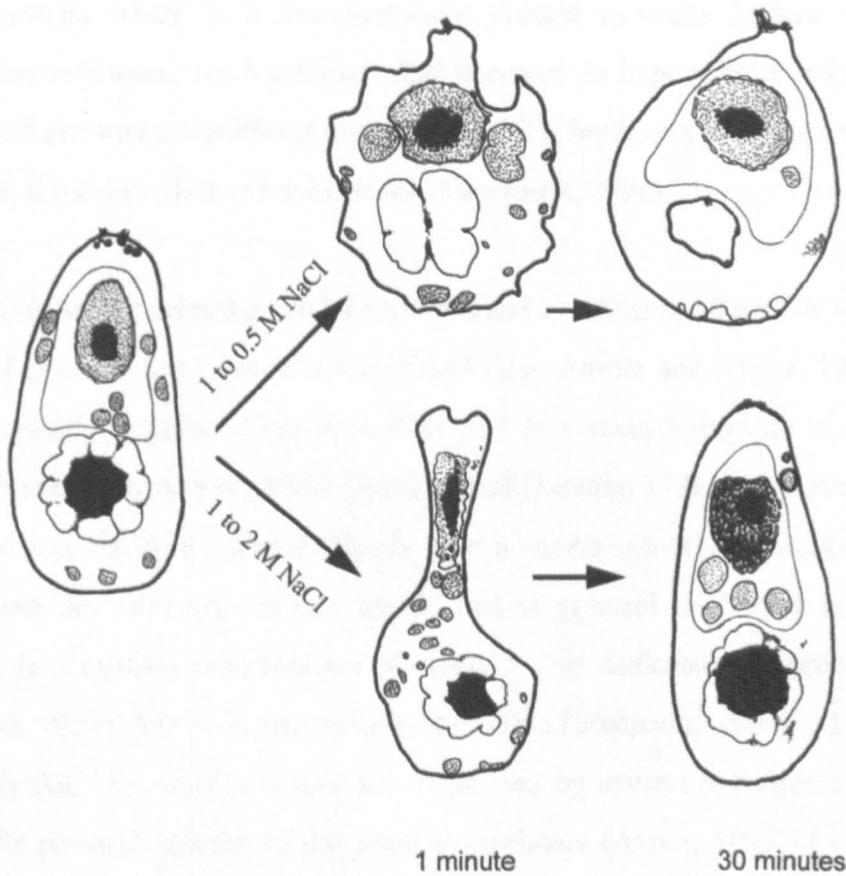
Figure 1.6: Diagram which illustrates morphological change of *Dunaliella* cells after osmotic shocks.

1.2.4.1 Cell response to hypoosmotic shock

After a sudden reduction in the salinity of the medium to half its original value, *Dunaliella* cells round up and swell by approximately 1.8 times their original volume (Maeda and Thompson, 1986). The swelling usually appears with a half-time of 1-2 seconds to 30 seconds and then cells can shrink back to normal size with an apparent half-time of 40-50 minutes (Ginzburg, 1978). During swelling, the surface area of the cell increases by 50 to 60% and the number of small cytoplasmic vesicles is reduced. Maeda and Thompson (1986) suggest that this happens because a large population of

small vesicles (~ 0.15 μm in diameter) are fused with the plasma membrane and allow cell surface to expand. This was further confirmed by tracing a 20-30 kDa GTP-binding protein, which is a low molecular protein normally located within the endoplasmic reticulum, for 8 minutes after exposure to hypoosmotic stress. It was found that there was a significant migration of GTP-binding activity into the plasma membrane, the chloroplast and the cytosol (Thompson, 1994).

In *Dunaliella*, under normal growth conditions and at salinities above 0.6 M NaCl, no leakage of glycerol from the cells was found (Ben-Amotz and Avron, 1973). This low permeability to glycerol has been discussed as a special property of *Dunaliella* cell membrane (Brown *et al.*, 1982; Gimmler and Hartung, 1988). However, glycerol is released into the medium immediately after a severe osmotic downshock. Then, within 5 minutes after hypoosmotic shock, rate of glycerol production is decreased and there is a parallel accumulation of starch. This indicates a bioconversion of glycerol to starch which is osmotically inactive (Thompson, 1994). Leakage of glycerol during hypoosmotic shock was explained by several hypotheses such as a nonspecific permeabilization of the plasma membrane (Avron, 1992), by temporary small nonspecific pores in the membrane which allow glycerol and K^+ to leak out (Fujii and Hellebust, 1992) or by an energy driven plasma membrane ATPase in which glycerol leakage could be reduced by the uncoupler CCCP (Zidan *et al.*, 1987). After hypoosmotic shock, photosynthesis rate is inhibited for at least 100 minutes and larger salinity decreases cause higher levels of inhibition (Gilmour *et al.*, 1984b).

1.2.4.2 Cell response to hyperosmotic shock

During normal growth conditions in light and without osmotic shift, glycerol was found to be the main product of photosynthesis in *Dunaliella*: 50-60% of ^{14}C is fixed into glycerol in 3 minutes (Wegmann, 1969). Normally, after 10 minutes, up to 90% of fixed CO_2 from photosynthesis can be converted to glycerol with only minor amounts of other intermediates being produced (Frank and Wegmann, 1974).

Adaptation of *Dunaliella* cells to hyperosmotic stress has been intensively studied. It was found that, after hyperosmotic shock, *Dunaliella* synthesized additional glycerol to osmotically balance the external salinity. Glycerol production increases for the first 60-90 minutes after a hyperosmotic shock (Ben-Amotz, 1975), and the cells will reach a new osmotic equilibrium after 3-5 hours. Glycerol is known to be synthesized from the photosynthetic carbon reduction cycle, however, its biosynthesis can also be a degradation process from starch. ^{13}C-NMR studies indicated that *Dunaliella* use starch degradation to produce glycerol to allow the initial rapid response to hyperosmotic shock. Then glycerol production from starch gradually decreases and photosynthetic production of glycerol takes over. Finally, approximately 45 minutes after hyperosmotic stress was imposed, glycerol production comes mainly from photosynthesis (Degani *et al.*, 1985).

Mechanism of glycerol regulation in *Dunaliella* is based on an assumption that synthesis and reassimilation of glycerol are controlled by volume changes (Avron, 1992). However, the process that senses the volume change is not fully understood (Zelazny *et al.*, 1995). Topics of recent investigations were mainly concerned with three stages: (1) checkpoint enzymes controlling glycerol synthesis and reassimilation which possibly are glycerol phosphate dehydrogenase or phosphofructokinase; (2) the volume-sensor mechanism; and (3) the signal transduction mechanism that communicates between the sensor and the target enzyme(s) (Avron, 1992).

Many hypotheses have been suggested to explain the sensor mechanism for glycerol synthesis and two prime candidates are a plasma membrane sensor and a change in the intracellular concentration of a critical metabolite (Avron, 1992). Oren-Shamir *et al.* (1989) concluded that the plasma membrane ATPase plays an important role in the recovery of *Dunaliella* after hypertonic shock because this ATPase is activated in response to an osmotic shock. Zelazny *et al.* (1995) reported that, after hyperosmotic shock, sterol:phospholipid or protein:lipid ratio in the plasma membrane changed and there was a correlation between glycerol synthesis and synthesis of sterols in the

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plasma membrane. Finally, they proposed a hypothetical model of osmosensing in *Dunaliella* as shown in Figure 1.7. This model consists of a latent hypothetical sensor in the plasma membrane layer in which sterols are involved in the activation of glycerol synthesis through this sensor molecule.
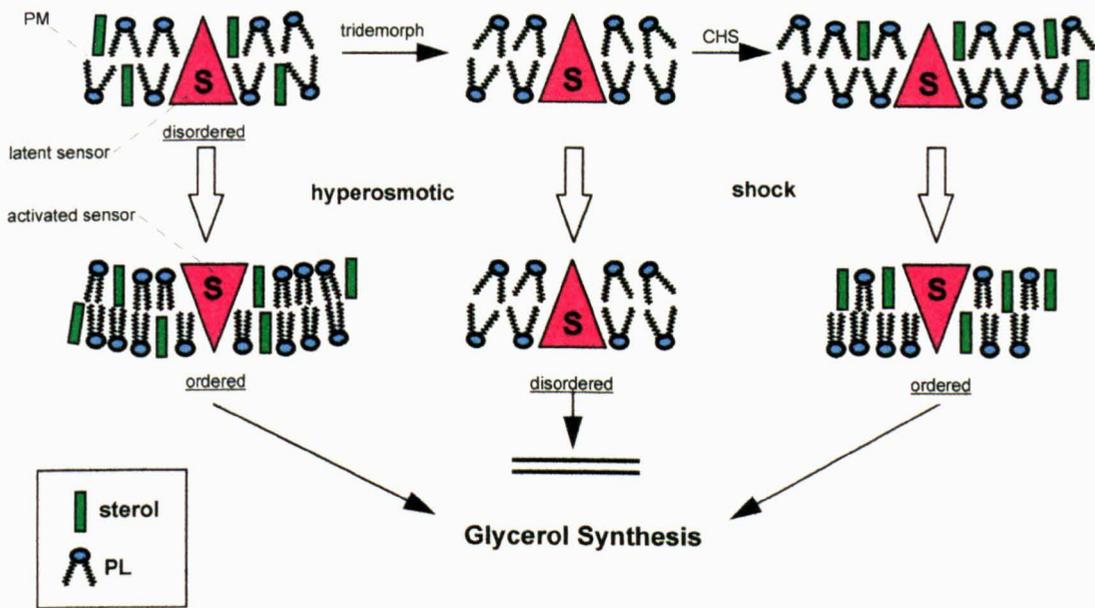
Figure 1.7: Hypothetical model of osmosensing in *Dunaliella* proposed by Zelazny *et al.* (1995).

        [S] Osmosensor

        [PL] phospholipid

        [PM] plasma membrane

        [====] blocked response or no response)

        [Tridemorph] specific inhibitor of steroid biosynthesis

        [CHS] cholesterol hemisuccinate (exogenous sterol)

1.2.4.3 *Dunaliella* response to salt: the summary

Salt responses in *Dunaliella* can be summarised as follows (modified from Avron (1992) with the addition of more recent data):

Plasma membrane phenomena

- Hypoosmotic

    Fusion with cytoplasmic vesicles (Maeda and Thompson, 1986)

    Intracellular translocation of a 28-kDa GTP-binding protein

        (Memon *et al.*, 1993)

    Inositol phospholipid turnover

        (Oren-Shamir *et al.*, 1989; Chitlaru and Pick, 1991)

- Hyperosmotic

    Infoldings (Weiss *et al.*, 1991)

    Lipid ordering (Ginzburg, 1981)

    Permeability changes (Fontana and Haug, 1982)

    Na^+ influx (Weiss and Pick, 1990; Ginzburg, 1981)

    Hyperpolarization (Oren-Shamir *et al.*, 1990)

    Inositol phospholipid turnover

        (Oren-Shamir *et al.*, 1989; Chitlaru and Pick, 1991)

    Choline phospholipid turnover

        (Oren-Shamir *et al.*, 1989; Chitlaru and Pick, 1991)

    Phosphorylation, 45-kDa polypeptide (Avron, 1992)

    Changes in plasma membrane sterols (Zelazny *et al.*, 1995)

    Activation of two 40-kDa protein kinases (Yuasa and Muto, 1996)

    Accumulation of a 13-kDa protein (Lu *et al.*, 1996)

    Activation of 74-kDa plasma membrane protein kinase

        (Chitlaru *et al.*, 1997)

- High salt (slow adaptation)

> Synthesis of 150-kDa polypeptide (Sadka *et al.*, 1991)
>
> Synthesis of 60-kDa polypeptide (Fisher *et al.*, 1994)
>
> Expression of 36 and 170-kDa polypeptides (Golldack *et al.*, 1995)
>
> Enhanced carbonic anhydrase (Brown *et al.*, 1987)

Internal factors

- Hypoosmotic and hyperosmotic

> Polyphosphate metabolism (Fontana and Haug, 1982; Bental *et al.*, 1991)
>
> ATP, NAD(P) changes (Belmans and Van Laere, 1987; Goyal *et al.*, 1988)

- Hyperosmotic

> pH drop (Weiss and Pick, 1990)
>
> pH rise (Goyal *et al.*, 1987; Bental *et al.*, 1990)
>
> Pi rise (Avron, 1992)

1.3 Leakage of cellular metabolites from *Dunaliella* and other microorganisms

The loses of intracellular metabolites from algal cells is a normal occurrence during phytoplankton photosynthesis in both the natural environment and laboratory cultures. Up to 50% of the carbon fixed per unit area in lakes or the sea may be released as extracellular products (Fogg *et al.*, 1965). In laboratory culture, both the proportion and composition of photosynthetic products released into the medium differ in relation to age of culture and species. The accumulation of organic substances occurs in the culture medium due to a lack of reutilization or because release rates exceed utilization rates (Nalewajko and Lean, 1972).

Examples of the extracellular products from phytoplankton are polysaccharides, glycolate and amino acids. Glycolate is the most abundant extracellular product of natural phytoplankton populations (Fogg *et al.*, 1965). Watt (1966) stated that glycolate, a derivative of a key intermediate in the phytosynthetic assimilation of

carbon dioxide via the Calvin cycle, was released from *Chlorella pyrenoidosa*. Its release was dependent on the relative growth rate, population density, light intensity and carbon dioxide supply.

Glycerol is an osmoregulator in many microorganisms and there have been many investigations into glycerol leakage from growing cells. Lucas *et al.* (1990) suggested that the halotolerant yeast *Debaryomyces hansenii* maintains a glycerol gradient by using a sodium-glycerol co-transport mechanism which kept sodium and glycerol on the opposite sides of the plasma membrane. However, in high salinity, Gustafsson and Norkrans (1976) reported leakage of glycerol from *D. hansenii* growing at 2.7 M NaCl and the amount of extracellular glycerol reached up to 80% of total glycerol.

Ben-Amotz and Avron (1978) mentioned that, in most membranes, there is no facilitated glycerol transport system because glycerol is not a very polar molecule and most membranes are already permeable to glycerol. However, glycerol transport systems have been found in many microorganisms. For example, in *E. coli*, glycerol enters the cells by facilitated diffusion rather than by active transport or vectorial phosphorylation (Lin, 1986). Other examples of facilitated diffusion of glycerol are found in the fungus *Fusarium oxysporum* (Castro and Loureiro-Dias, 1991) and human erythrocyte cells (Stein, 1958). On the other hand, the nature of glycerol transport systems in yeast was found to vary among species. As noted above, *D. hansenii* used active sodium-glycerol co-transport (Lucas *et al.*, 1990), while in another species of halotolerant yeast, *Pichia sorbitophila*, glycerol/H^+ symport plays an important role. *P. sorbitophila* survives in high salinity by accumulating glycerol inside the cell along with protons. This process compensates for the leakage of glycerol due to its liposoluble character. Another species of yeast that was found to actively transport glycerol is *Zygosaccharomyces rouxii* (van Zyl *et al.*, 1990 quoted by Lages and Lucas, 1995).

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In *Dunaliella*, glycerol transport system is not yet fully understood. Zidan *et al.* (1987) suggested that glycerol is excreted from *D. tertiolecta* under hypoosmotic shock by active transport. Meanwhile, Fujii and Hellebust (1992) commented that glycerol is leaked through nonspecific transient pores in plasma membrane in response to hypoosmotic shock. However, under normal growth conditions without any change in osmotic potential, glycerol is always retained in the cells. This character is defined as an unusual property of the plasma membrane leading to a very low permeability for glycerol and it has been concluded that glycerol is not leaked from *Dunaliella* cells under normal isoosmotic growth conditions (Ben-Amotz and Avron, 1973; Ben-Amotz, 1975; Ben-Amotz and Avron, 1978; Brown and Borowitzka, 1979; Brown *et al.*, 1982; Gimmler and Hartung, 1988; Avron, 1992).

It is important to state that, although *Dunaliella* cells have only a plasma membrane without a rigid cell wall, they are not particularly fragile. Cells were not found to be damaged by most experimental treatments such as centrifugation or vigorous shaking (Ginzburg, 1978). Glycerol leakage after hypoosmotic shock is not due to physical cell damage (Fujii and Hellebust, 1992).

Nevertheless, conflicting data for glycerol leakage have been reported, for example, Jones and Galloway (1979) reported a significant loss of glycerol from *D. tertiolecta* without osmotic stress, while Borowitzka *et al.* (1977) did not detect glycerol leakage from the same species. Until now, most of published papers on *Dunaliella* osmoregulation have concluded that there is no leakage of glycerol from this alga under isoosmotic conditions.

Meanwhile, some reports still do suggest that there is the leakage of glycerol in *Dunaliella*. Wegmann (1969) observed the leakage of ^{14}C-labelled photosynthetic products from *Dunaliella* cells. He reported that 11% of this labelled organic material was found in the medium after 3 minutes under isoosmotic conditions. Release of glycerol into the medium was found in both dark and light conditions. Huntsman

(1972) reported up to 66 percent of ^{14}C being excreted from *D. tertiolecta* under various growth conditions. The leakage can reach levels of 20% in 120 minutes and glycerol was the only ^{14}C product found in the medium (Frank and Wegmann, 1974). They also assumed that glycerol is lost by diffusion into the medium. In 1978, Wegmann concluded that glycerol can still maintain a balanced osmotic pressure in *D. tertiolecta*, even when it was lost into the medium in considerable amounts.

Giordano *et al.* (1994) reported that soluble organic compounds were released from actively growing *D. salina*. However, although glycerol is a good candidate because it is the main osmoticum and the major carbon species fixed through photosynthesis, they assumed, based on the previous publications, that glycerol is never leaked. Nevertheless, it has to be noted that they did not analyse the composition of the organic carbon released from the cells.

A mutant of *D. parva* which leaked large amounts of glycerol into the medium was reported by Hard and Gilmour (1991). This mutant was isolated from a wild type culture grown in continuous culture in a chemostat. In this mutant, up to 75% of glycerol was found in the culture medium after 15 days of culture. This suggests the possibility of commercial glycerol production from *Dunaliella* cells and a study of the characteristics of glycerol leakage forms part of the work described in this thesis.

1.4 Biotechnology of *Dunaliella*

Dunaliella is probably the most successful microalga for outdoor cultivation (Ben-Amotz and Avron, 1989). The following characteristics of *Dunaliella* make it an attractive candidate for commercial mass cultivation (Vonshak, 1990; Ben-Amotz and Avron, 1989):

- Its ability to accumulate relatively high concentrations of β-carotene, known to be in high demand and have a high commercial value.

- Its ability to thrive under extreme conditions such as 1-2 M NaCl provides a selective advantage, because development of other algae or predators in open raceway ponds is prevented.

- Under suitable growth conditions, *Dunaliella* accumulates massive amounts of highly priced product, β-carotene (6-10% of the algal organic weight), in addition to glycerol (around 20-40%) and most of the remaining dry weight is protein.

- As it lacks a cell wall, dried *Dunaliella* is easily and fully digestible by animals and humans.

However, one of the most important technical problems for mass culture of *Dunaliella* is the harvesting step (Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989; Vonshak, 1990). A small cell size (smaller than 20 μm) and the lack of a rigid cell wall make filtration an impractical technique for harvesting the biomass because a microscreen filter with approximately 1 μm pore size is needed (Kormanik and Cravens, 1979). During filtration, algae clogged the filter rapidly by forming a layer of mucus material which prevents further filtration unless backwashing is performed frequently. Several harvesting techniques have been tried such as high pressure filtration through sand filters, cellulose fibres, diatomaceous earth, the use of salinity-dependent buoyancy properties in stationary or moving salt gradients, alkaline flocculation, exploitation of the phototactic and gyrotactic responses of the algae (Borowitzka and Borowitzka, 1988), but the most suitable techniques appear to be flocculation and centrifugation. Unfortunately, flocculation by chemical agents cannot be used for food grade algal product unless the flocculant is safe or is completely removed from the algae prior to utilization. Hence, centrifugation is the best technique and has been used for commercial production of *Dunaliella* in many countries such as Israel, Australia and China. The disadvantage of continuous centrifugation is that it requires high energy inputs and high production costs.

Dunaliella have been cultured for use in the processes described below:

1.4.1 Aquaculture

D. tertiolecta is one of the most popular microalgal species for aquaculture. For example, *D. tertiolecta* CCMP1320 (DUN) is one of 14 algal strains used for aquaculture in the CCMP-Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Andersen *et al.*, 1997). *Dunaliella* spp. has been used as feed for bivalve molluscs and shrimp larvae as well as for marine rotifers or brine shrimp which are then supplied for fish larviculture (De Pauw and Persoone, 1988).

1.4.2 Feed and single cell protein

Because *Dunaliella* does not have a rigid cell wall and the algal meal contains a relatively high protein content (24-55% protein), it has the potential to be used as a protein source for animal and human consumption. Dried biomass of *Dunaliella* was tested to be used as a protein supplement in bread by blending with wheat flour, but it was essential to remove salt content as *Dunaliella* were grown in high salt medium (Finney *et al.*, 1984). After extracting glycerol and β-carotene from algal biomass, remaining algal meal contains up to 70% protein. This high protein content, along with the amino acid composition shown in Table 1.2, indicates that it is suitable as food for animals in aquaculture and poultry industries (Ben-Amotz and Avron, 1980).

1.4.3 β-carotene

β-carotene is the most important product from *Dunaliella* mass culture. It is a carotenoid pigment widely used as food colouring agent (yellow-orange colour), pro-vitamin A or retinol in animal feed (Ben-Amotz *et al.*, 1986), as an additive to cosmetic sunscreen products, multivitamin preparations and health food products (Ben-Amotz and Avron, 1990). Safety of *Dunaliella* for human consumption was tested (Mokady *et al.*, 1989) and it was accepted to be used as a food ingredient. Recently, *Dunaliella* biomass has been used as a natural β-carotene source in many anti-oxidant health food products being sold in many countries.

Table 1.2: Amino acid content of *Dunaliella bardawil* (Ben-Amotz and Avron, 1980)

Amino acid	Dried algae pellet (40% protein) g/100 g protein	Dry material after glycerol and β-carotene removal (70% protein) g/100 g protein
Alanine	7.3	6.8
Arginine	7.3	7.5
Aspartic acid	10.4	10.6
Cysteine	1.2	1.3
Glutamic acid	12.7	12.6
Glycine	5.5	5.9
Histidine	1.8	1.7
Isoleucine	4.2	4.0
Leucine	11.0	11.1
Lysine	7.0	7.6
Methionine	2.3	1.7
Phenylalanine	5.8	5.7
Proline	3.3	2.8
Serine	4.6	4.9
Threonine	5.4	5.5
Tryptophan	0.7	0.4
Tyrosine	3.7	3.9
Valine	5.8	5.7

Natural β-carotene from *Dunaliella* is composed of two dominant stereoisomers, i.e. 9-cis β-carotene and all-trans β-carotene (Shaish *et al.*, 1990). The proportion of 9-cis to all-trans isomers can reach 50% in *Dunaliella* while the synthetic β-carotene is composed of all-trans isomer only (>99%). The physicochemical properties of 9-cis β-carotene differs from those of the all-trans isomer as all-trans β-carotene is practically insoluble in oil and is easily crystallized. 9-cis β-carotene is much more soluble in hydrophobic solvents, very difficult to crystallize, and is generally an oil in its concentrated form (Ben-Amotz and Avron, 1990). Takenaka *et al.* (1993) suggested that, in a rat model, *Dunaliella* β-carotene with its isomer mixture was found to accumulate in higher amounts than synthetic all-trans β-carotene. They then concluded that natural β-carotene significantly decreased gastric mucosal lesions or

water-immersion-induced stress in rats. Structure of two isomers of β-carotene is shown in Figure 1.8.

all trans - Beta carotene

9-cis-Beta carotene
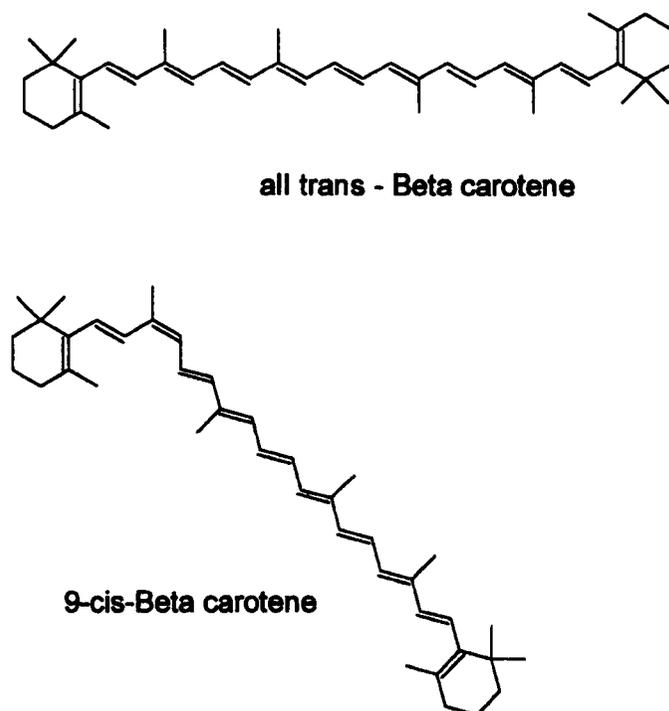
Figure 1.8: Molecular structure of the two major isomers of β-carotene.

Most of the commercially available β-carotene is synthetic. The synthetic product is sold for approximately US$ 600 per kilogram. The natural β-carotene is, however, sold for more than twice this price (Ben-Amotz and Avron, 1990) and there is still increasing demand (M.A. Borowitzka, 1997 personal communication).

1.4.4 Glycerol

As already described in the previous section, *Dunaliella* accumulates large amounts of glycerol to balance the external salinity. This leads to the potential of producing glycerol from *Dunaliella* biomass. Glycerol is used in the chemical and food industries especially in drug, cosmetics and beverage production. It is also used in the production of urethane, cellophane and explosives (Gilmour, 1990). Currently,

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glycerol is produced mainly by petrochemical industry with some from by-products of fat and soap manufacture. As the compatible solute for osmoregulation, glycerol can be produced from *Dunaliella* cells growing in high salinity. Chen and Chi (1981) proposed a process of glycerol production from *Dunaliella* citing three advantages:

1. The raw material carbon dioxide is a renewable resource.
2. Solar illumination can be used as a major energy source.
3. Animal feed protein and β-carotene can be recovered as co-products.

Chen and Chi (1981) suggested that increasing oil prices will in turn increase the glycerol production costs more rapidly than the costs for the algal process. The economic analysis indicated the possibility of a large scale algal cultivation system for glycerol with dramatically high cell concentrations (8 $g.l^{-1}$) based on their laboratory cultivation experiments. Nevertheless, this cell concentration has never been reported in any large scale culture of *Dunaliella*. The probable maximum *Dunaliella* cell concentrations in an open raceway pond, calculated according to the maximum cell concentration reported by Ben-Amotz (1995) and Grobbelaar (1995), is usually only around 0.2-0.8 $g.l^{-1}$.

Ben-Amotz *et al.* (1982b) quoted the figure of approximately 40% glycerol on dry weight basis and a yield of 16 g $glycerol.m^{-2}.day^{-1}$ or 4.5 g $glycerol.m^{-2}.day^{-1}$ for long term production at 3.5 M NaCl. *Dunaliella*, since 1980s, has been cultivated for β-carotene production (Ben-Amotz and Avron, 1989, Borowitzka and Borowitzka, 1988), glycerol which approaches approximately 30-50% of dry weight can be harvested as a by-product.

1.5 Immobilisation of living microorganisms

1.5.1 Immobilisation techniques and applications

Technology of immobilised enzymes has been reported since 1960s and has expanded rapidly. This method was then modified for use with whole cells in many industrial microbiology processes (Brodelius and Vandamme, 1987). There are four main procedures available for cell and enzyme immobilisation which are adsorption, entrapment in gels or polymers, covalent coupling, and cross linking to insoluble matrices (Brouers *et al.*, 1989). Variety of matrices are now commercially available such as polyacrylamide, agar, carrageenan, calcium alginate gels, glass, ceramic and silica beads, and polyurethane and polyvinyl foams. In general, the physical processes involved in cell immobilisation can be divided in to four catagories, *i.e.* attachment, entrapment, containment and aggregation which are shown in Figure 1.9.

Attachment: Cells are bound to the surface of a solid support, particles normally less than 1 mm in diameter such as sand, glass beads, chitosan flakes, etc.

Entrapment: Cells are entrapped within a variety of porous structures which are either preformed or formed *in situ* around the cells. The popular choice of entrapment matrix are calcium alginate, kappa-carrageenan, agar and porous biomass supports such as stainless steel spheres or polyurethane foam cubes.

Containment: Cells are kept behind a barrier, either preformed or formed *in situ*. The barriers include semi-permeable membranes or an interface between two immiscible liquids.

Aggregation: Cells are aggregated by natural flocculation or by adding artificial flocculants. In some organisms such as fungi, mycelia can form aggregates in spherical pellet shape.

Attachment

Entrapment

Containment

Aggregation
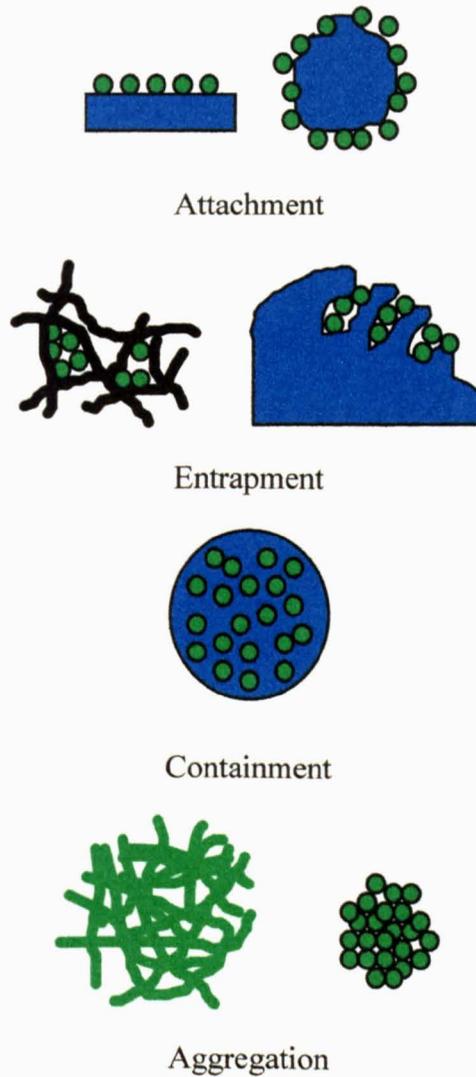
Figure 1.9: Immobilisation processes (modified from Webb, 1987)

1.5.2 Immobilisation matrix

In order to immobilise a motile organism such as *Dunaliella*, entrapment in a gel is considered to be the most suitable technique. Immobilisation technique could be modified from that used for plant or microbial cells. The most common entrapment medium involves a polymeric matrix, such as alginate, polyacrylamide, carrageenan or agar. Each matrix has different characteristics and can be described as follows:

1.5.2.1 Cell immobilisation in calcium alginate

Alginic acid is a compound found in the brown algae of Phaeophyceae. Commercial alginic acid is produced from giant kelp such as *Macrocystis pyrifera*, *Laminaria* spp. and *Ascophylum* spp. (Bucke, 1987). Alginate is an unbranched binary copolymer of 1-4-linked β-D-mannuronic acid and α-L-guluronic acid with widely varying composition and sequence, depending on the organism and tissue they are isolated from (Smidsrod and Skjåk-Bræk, 1990). Sodium alginate from different sources can provide different gel strengths, this depends on high L-guluronate content. For example, alginate from *Laminaria hyperborea*, which has a high gel strength, contains around 70% L-guluronate whereas low gel strength alginate from *Microcystis* contains less than 30% L-guluronate (Bucke, 1987).

Use of alginate gel is the most common method for immobilisation (Hulst and Tramper, 1989) as it is very simple to carry out. Although alginic acid has a capability to form weak gels, its acidic nature makes it unsuitable for cell entrapment. Gel formation can be performed by an interaction of alginate with divalent ions such as Ca^{2+}, Sr^{2+} and Ba^{2+}. Among these, calcium alginate is the most popular for alginate immobilisation due to ease of operation and a good stability.

Structure of alginate and a diagram showing calcium alginate gel formation are shown in Figure 1.10. The procedure for forming calcium alginate beads is described in Section 2.5.1.

Calcium alginate gel can be dissolved in the presence of Ca^{2+}-chelating agents such as phosphate ions, citrate and EDTA (Brodelius and Vandamme, 1987).

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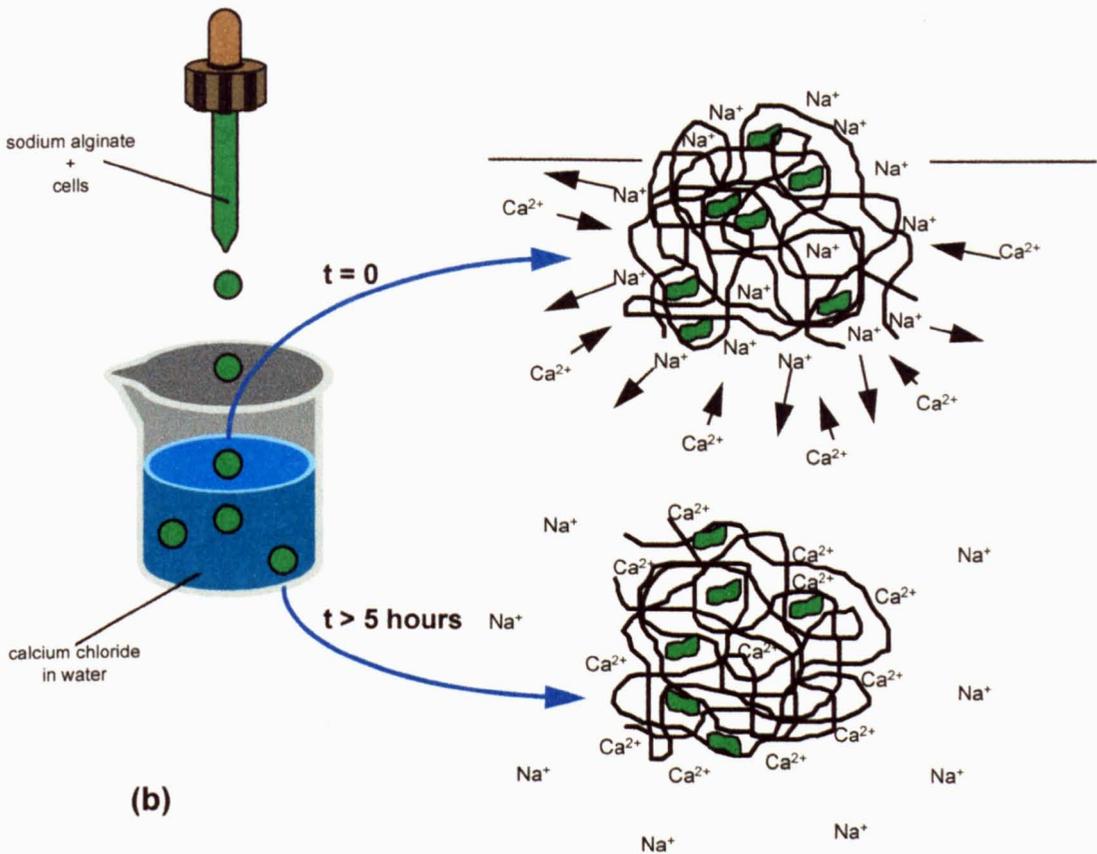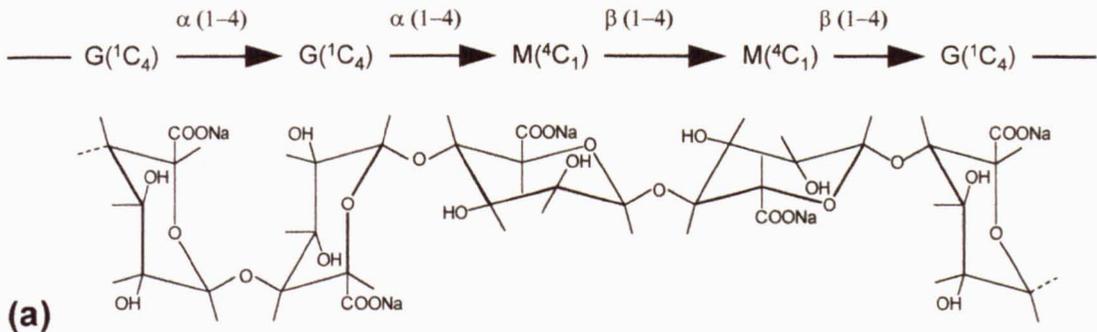
Figure 1.10: (a) Chemical structure of sodium alginate.

G, guluronic acid; M, mannuronic acid

(b) Calcium alginate gel formation

[modified from Smidsrod and Skjåk-Bræk (1990) and Hulst and Tramper, (1989)]

1.5.2.2 Cell immobilisation in polyacrylamide

Polyacrylamide gel, a polymeric network of the monomers acrylamide and N,N′-methylenebis-acrylamide (cross-linker), has been widely used for enzyme and cell immobilisation (Skryabin and Koshcheenko, 1987; Brodelius and Vandamme, 1987). It is one of the universal carriers for living microbial cells. Immobilisation in polyacrylamide can be performed by adding plant cells to a solution of acrylamide monomer and N,N′-methylenebis-acrylamide in Tris-HCl buffer. Then, after adding ammonium persulphate and N-N-N′-N′-tetramethylethylenediamine (TEMED) which act as the polymerization initiators, beads can be obtained by the moulding technique (Hulst and Tramper, 1989). However, sensitive plant cells cannot be entrapped in polyacrylamide unless an adaptation using cross-linked prepolymerized polyacrylamide-hydrazide is used (Nilsson *et al.*, 1987; Freeman, 1987). Immobilisation in polyacrylamide gels must be carefully investigated for each particular strain because of the toxicity of the monomers and initiator compounds involved (Brodelius and Vandamme, 1987).

1.5.2.3 Cell immobilisation in carrageenan

Carrageenan is a polysaccharide extracted from the red seaweeds *Eucheumi cottonii*, *Chondrus crispus* and *Gigartina stellata*. Carrageenan polymer consists of a backbone of alternating 1,3-linked β-D-galactose and 1,4 linked α-D-galactose (Guiseley, 1989). Six types of carrageenan are known, depending on the sulphate content of the polymer, but the most usable form for immobilisation is kappa-carrageenan (Phillips and Poon, 1988). Cell immobilization in kappa-carrageenan can be performed in a similar way to alginate. However, microbial cells must be mixed with carrageenan (1 to 20 %) at high temperature (37 to 50 °C). Solidification of the gel occurs when the solution is cooled to the appropriate gelification temperature and stability of the gel can be improved by treatment with cations, usually K^+ (Brodelius and Vandamme, 1987; Hulst and Tramper, 1989). Nevertheless, in most plant cell media no extra addition of potassium is required

because there is usually sufficient concentration of potassium already present (Brodelius, 1985).

There are several publications on carrageenan immobilisation with microalgae (Chevalier and de la Noue, 1985; Karube *et al.*, 1986; Mallick and Rai, 1994). Unfortunately, carrageenan gel is easily dissolved in saline solutions (Yang and Wang, 1990). This makes carrageenan immobilisation useless for marine species like *Dunaliella*.

1.5.2.4 Cell immobilisation in agar and agarose

Agar and agarose (purified agar) are polysaccharides extracted from marine red algae. Polymer is composed of a repeating agarobiose unit consisting of alternating 1,3 - linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose (Brodelius, 1985). Agar forms gels according to temperature which modifies its structure. Normally, a gel that solidifies between 30 and 40°C is used. Preparation for immobilisation is performed by mixing cells and agar at high temperature followed by cooling to allow gel formation. However, a major disadvantage of agar and agarose is the relatively high cost of this polymer (Brodelius and Vandamme, 1987).

1.5.2.5 Cell immobilisation in chitosan

Chitosan became an alternative method for cell immobilisation because of several perceived advantages. It is stable in phosphate solution and the immobilisation process can be performed at room temperature (Vorlop and Klein, 1987). Chitosan is a partially deacetylated chitin. It is produced by the reaction of chitin with a concentrated alkali. The molecule consists of glucosamine in a 1-4 linkage as shown in Figure 1.11.
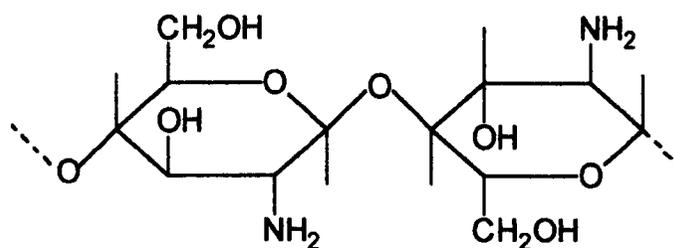
Figure 1.11: Chemical structure of chitosan.

Chitosan can form gels by an ionotropic mechanism similar to alginate. Chitosan solution (pH < 6), consisting of chitosan dissolved in a dilute organic acid such as acetic acid, can be dropwise added into a cross-linker solution to form gels. Examples of cross-linked solutions are ferricyanide, ferrocyanide, polyphosphate, poly(aldehydocarbonic acid) and poly(1-hydroxy-1-sulfonate-propene-2) (Brodelius and Vandamme, 1987). Vorlop and Klein (1981) described that a real ionotropic gel is formed only in a cross-linking solution with a pH < 6. Bead formation in a cross-linking solution with a pH > 7.5 is possible, but ionotropic gel can form only in an early stage then, when the pH of the chitosan gel is more than 7.5, it will precipitate.

1.5.3 Immobilisation of living algal cells

In general, immobilised algae have been used for two reasons, water treatment and, chemical or secondary metabolite production. For example, Robinson *et al.* (1989) reported that orthophosphate phosphorus (PO_4-P), in both synthetic growth medium and secondary treated effluent, was rapidly removed by immobilised *Chlorella* in batch and continuous-flow culture. In this experiment, small packed-bed reactors containing 10 ml gel were able to remove up to 240 μmol PO_4-P from 4-5 l of medium within 10-12 days. Travieso *et al.* (1992) also used alginate immobilised *Chlorella* in a fluidized bed reactor for domestic sewage treatment. The chemical oxygen demand (COD) and nutrients (Ortho-P, Total-P, Organic-N and Ammonia-N) were removed efficiently. Proulx and de la Noue (1988) suggested that carrageenan

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"micronised" *Phormidium* (filamentous algae) could be used effectively for the removal of macronutrients from waste water. Another report of the success of immobilized algae for waste water treatment is the use of kappa-carragenan immobilised *Spirulina* for swine waste treatment (Cañizares *et al.*, 1993). In most cases, using immobilised algae improves biological waste water treatment because using free microscopic algae is quite costly and inefficient in harvesting biomass. Instead, entrapped algal cells in beads could be easily separated from water by sedimentation or simple filtration.

Immobilised algae can also be used for metal recovery. Generally, living algal cells can accumulate trace nutrient heavy metals (such as Co, Mo, Ca, and Mg) by active transport. However, toxic heavy metals may be removed from water due to at least one of three ways: (1) intracellular chelation by biological polymers, (2) accretion or precipitation of the heavy metals on the cell wall surface, or (3) by adsorptive surface binding to various cell wall chemical functional groups (Greene and Bedell, 1990). Immobilised *Chlorella* has been successfully used for metal biosorption of many metals such as mercury (Wilkinson *et al*, 1989), cadmium, zinc and gold (Da Costa and Leite, 1991).

There are several reports on metabolite production or bioconversion by immobilised algae. Examples are ammonia production by alginate immobilised *Anabaena* (Musgrave *et al.*, 1982); formation of α-keto acids from amino acids by immobilised *Chlorella* and *Anacystis* and by co-immobilisation of *Chlorella* and bacteria (Wikström *et al.*, 1982); H_2 production by glass bead immobilised *Anabaena* (Lambert *et al.*, 1979), polyurethane immobilised cyanobacteria *Chlorogoea*, *Nostoc* and *Mastigocladus*; and the increase of hydrogenase and nitrogenase activities and ammonia excretion from polyurethane and polyvinyl foam immobilised cyanobacterium, *Anabaena azollae* (Shi *et al.*, 1987),

1.5.4 Physiology of immobilised algae

Division of algal cells occurs within the gel after immobilisation (Dainty *et al.*, 1986). In an alginate immobilised *Euglena gracilis*, oxygen evolution was decreased by 50% during the first 15 days and then remained constant at approximately 50 μmol O_2.mg $Chl^{-1}.hr^{-1}$ for 85 days of the experiment (Tamponnet *et al.*, 1985). It was concluded that immobilisation in a calcium alginate gel can fix and maintain ultrastructure and the physiological activities of *E. gracillis* cells in the state existing when immobilisation occurred. After two years entrapped in beads, *Euglena* cells were still active after transfer into fresh medium and, moreover, no difference in growth and physiological activities were observed in comparison to control cells. Similarly, Hertzberg and Jensen (1989) studied the long term entrapment of seven species of the marine microalgae using calcium alginate beads. Algal beads could be stored under low light intensity at 4°C for 6 to 18 months. The result demonstrated the possibility of using immobilisation techniques for marine microalga stock culture management. Brouers and Hall (1985) reported the enhancement of photosynthetic activity of immobilised cyanobacterium *Phormidium laminosum* by drying pretreatment. Beads, after gel solidified, were allowed to dry for 24 hours at room temperature in the dark and were then resuspended in growth medium. This stabilized the photosystem II water splitting activity for at least 3 months.

Although calcium alginate is the most conventional method for cell immobilisation, it has at least two disadvantages. Leakage of cells produced by division of the entrapped cells (Cheetham *et al.*, 1979) and the chelating compounds such as phosphate, citrate and lactate or anti-gelling cations such as Na^+ or Mg^+ in the medium has been reported to affect calcium alginate gel strength (Smidsrod and Skjåk-Bræk, 1990). Unfortunately, phosphate is an essential nutrient for algae and must be added to the algal culture medium and it is always found in most polluted waters. Due to the problem with phosphate, the use of calcium alginate for algal immobilisation has to be carefully considered. An additional step of washing algal cells with phosphate depleted medium before forming beads was recommended by

Robinson *et al.* (1985). This technique could reduce phosphate content in beads and has been proved to be an effective approach. However, alginate immobilistion is not recommended to be used for waste water treatment (de la Noüe *et al.*, 1990). Vorlop and Klein (1981) proposed chitosan to overcome the disadvantage of the Ca-alginate matrix due to its instability in more concentrated electrolytes and buffer solutions. Chitosan beads are stable in phosphate containing solution and cheap to produce, so it may be one of the most suitable immobilisation techniques for waste water treatment.

1.6 Glycerol production, from petrochemical Industry to an alternative "green" technology

Currently, environmental problems such as global warming from CO_2 emissions and air pollution from fuel and industries are inducing the development of more sustainable technologies which utilise renewable natural resources. This "green" technology means it can minimise resource useage and, at the same time, minimise waste and pollution.

At the moment, glycerol is commercially produced from the petrochemical industry. Process of glycerol synthesis uses propylene and intermediates such as allylchloride, acrolein or propylene oxide are obtained. A diagram showing glycerol synthesis is shown in Figure 1.12. However, during glycerol synthesis, large amounts of chlorinated by-products are produced and this can cause some environmental problems (Rehm, 1996). Thus, although glycerol production from a biological process still cannot compete with glycerol from petroleum industry which is relatively cheap (Gilmour, 1990), it could be considered as a possibility that might be of biotechnological interest in the future when the cheap petroleum based sources become scarce.
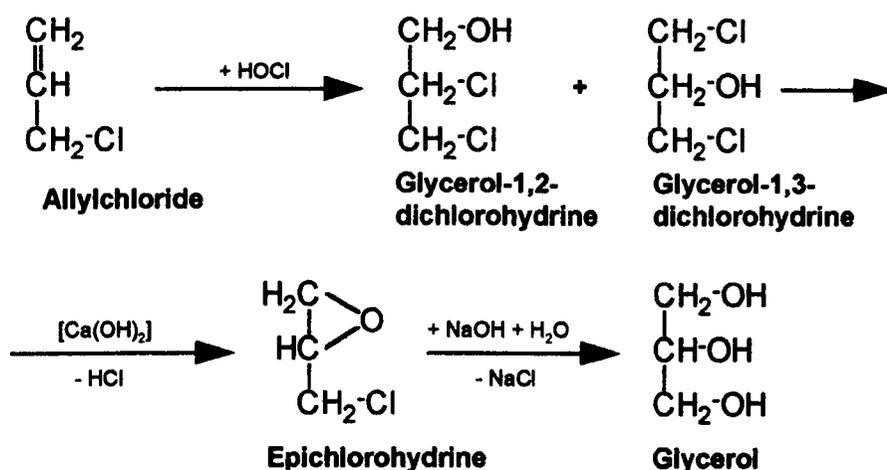
Figure 1.12: Synthesis of glycerol from allylchloride (Rehm, 1996).

The biotechnological process of glycerol production was invented in 1915 in Germany and was patented in the United States in 1919 by yeast growing in alkaline conditions. Examples of microorganisms that were reported by Rehm (1996) as glycerol producers are osmotolerant yeasts such as *Zygosaccharomyces acidifaciens*, *Saccharomyces rouxii*, *S. mellis*, *Pichia etchelsii*, *P. farinosa*, *Hansenula*, *Debaryomyces*, *Candida*, *Torula*, and bacteria, *Bacillus subtilis* and *Lactobacillus lycopersici*. However, the problem of glycerol recovery from the crude fermented mash was discussed by Rehm (1996) as 40-50% percent of glycerol can be lost during extraction process.

An early idea for the commercial production of glycerol from *Dunaliella* was proposed by Chen and Chi (1981) and Ben-Amotz and Avron (1982). Chen and Chi suggested a two step process of algal production using different salinities and nutrient concentrations. Algae are firstly grown in low salinity (1.5 M NaCl) with nutrient enrichment to maximise biomass, then one third of the batch is removed and mixed with very high salinity (4.0 M NaCl) medium. This second step increases salinity and allows *Dunaliella* to accumulate glycerol and β-carotene. Two step or two phase

culture is now successfully used for an intensive mass production of *Dunaliella* for β-carotene synthesis in Israel (Ben-Amotz, 1995).

Ben-Amotz and Avron (1982) pointed out the potential of *Dunaliella* mass cultivation for glycerol and β-carotene production. Now, β-carotene from *Dunaliella* is commercially produced but there are no reports of glycerol production even as a by-product after β-carotene extraction. This might be because most of the *Dunaliella* product on sale is a dried crude algal powder (containing 4-5% β-carotene/dry weight) rather than β-carotene extract in oil.

In the process described by Chen and Chi (1981), the problem step is the separation of algal cells from culture medium. Normally, continuous centrifugation is the most effective method to harvest *Dunaliella* biomass. Hence, an immobilisation of *Dunaliella* could help solve this problem especially with a strain that can release large amounts of glycerol into the medium such as the mutant strain of *D. parva* reported by Hard and Gilmour (1991). This strain leaked up to 75% of glycerol produced. As glycerol can be obtained directly from the culture medium, two complicated processes, cell harvesting and cell extraction, could be skipped. Grizeau and Navarro (1986) reported a preliminary result of glycerol production from immobilised *D. tertiolecta* in calcium alginate beads. They found a significant amount of glycerol was released into the medium. León and Galván (1994) also reported the possibility of glycerol production from immobilised *Chlamydomonas reinhardtii* . Therefore there is a great opportunity to use immobilised algae for glycerol production.

Moreover, glycerol can be converted biologically to other higher value products. Williams *et al.* (1979) proposed several ideas of using glycerol-rich algal lysate from *Dunaliella*, such as to support yeast (*Pichia* sp.) growth in order to produce single cell protein, to produce alcohol by an anaerobic yeast (*Candida utilis*) or to use bacteria (*Klebsiella* sp. or *Bacillus macerans*) that metabolize glycerol to ethanol, and the production of other chemicals for example, acetone, butanol, propanol and several

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orgainc acids from glycerol. One of the fermentation products of glycerol is dihydroxyacetone which is used in cosmetic and suntan preparations. Dihydroxyacetone can be produced oxidatively from glycerol by *Gluconobacter suboxydans* (Williams *et al.*, 1979) or *Acetobacter xylinum* (Nabe *et al.*, 1979). However, they probably requires pure glycerol as substrate.

Nakas *et al.* (1983) reported the bioconversion of glycerol from biomass of several *Dunaliella* species (*D. bardawil, D. primolecta, D. tertiolecta, D. parva* and *D. salina*) by a fermentation process using the bacterium *Clostridium pasteurianum*. The fermentation end products were *n*-butanol, 1,3-propanediol, ethanol and acetate. 1,3-propanediol, a high value chemical, has been considered to be a potential new product in biotechnology. By using facultative anaerobic bacteria, glycerol can be converted to 1,3-propanediol, which can be used as a monomer for producing plastics with special properties, i.e. polycondensates like polyesters, polyethers and polyurethanes (Deckwer, 1995).

Bioconversion of glycerol to 1,3-propanediol was reported by several researchers. Streekstra *et al.* (1987) studied the mechanism of glycerol conversion to 1,3-propanediol in an anaerobic culture of *Klebsiella aerogenes*. They found that the conversion is maximal under phosphate limitation and in low potassium environments which interact with the enzyme glucose dehydrogenase. Fermentation of 1,3-propanediol can be performed by several strains of bacteria such as *K. pneumoniae* (Zeng *et al.*, 1993, Menzel *et al.*, 1997), *Klebsiella oxytoca, Citrobacter freundii* (Homann *et al.*, 1990), *Clostridium butyricum* (Günzel *et al.*, 1991), and *Enterobacter agglomerans* (Barbirato *et al.*, 1996). In *Clostridium*, fermentation process was successfully scaling up to 2 m^3 (Günzel *et al.*, 1991).

1.7 Plan of thesis

The aim of this study was to investigate cellular physiology of immobilised *Dunaliella* cultured for glycerol production and to characterise glycerol leakage from this alga. The following experiments were conducted in this thesis:

Growth, photosynthesis and glycerol production from calcium alginate immobilised *Dunaliella* (Chapter 3).

Enhancement of glycerol production from immobilised *Dunaliella* (Chapter 4).

An attempt to isolate a mutant strain which had different glycerol leakage characteristics (Chapter 5).

Investigation of glycerol leakage from *Dunaliella* (Chapter 6).

This will be followed by conclusions in Chapter 7.

CHAPTER 2: MATERIALS AND METHODS

2.1 Algal and bacterial strains

Dunaliella parva CCAP 19/9 was obtained from the Culture Collection of Algae and Protozoa, Scottish Marine Biological Association, Oban, UK. The mutant strain of *D. parva*, which leaks large amounts of glycerol into the medium, was isolated by Hard and Gilmour (1991) from a CO_2-limited continuous culture.

Dunaliella salina DS91008 was isolated from a salt pond in Samut-Songkhram Province, Thailand (Powtongsook *et al.*, 1995). *D. salina* has a larger cell size than *D. parva* and has the ability to accumulate β-carotene when it is exposed to high light intensity and high salinity.

A moderately halophilic eubacterium *Halomonas* sp. strain DUNCON was initially isolated as a contaminant of a laboratory culture of *D. parva* CCAP 19/9 by Cummings (1991). The strain was isolated and checked for purity by streaking on nutrient agar.

2.2 Growth conditions

Algal strains were grown in artificial sea water medium (see Appendix A), as described by Hajibagheri *et al.* (1986) and Blackwell and Gilmour (1989), containing different NaCl concentrations. In some experiments, culture medium was modified by using 20 mM HEPES buffer instead of Tris buffer. HEPES buffer medium allowed the acetylacetone method to be used for glycerol analysis (see section 2.8). All algal strains were normally kept in the growth room at 25°C constant temperature, illuminated with white fluorescence lamps at 65-70 $\mu E.m^{-2}.s^{-1}$.

Algal cultures in 1 to 2 l flasks with 150 ml.min^{-1} air bubbling were used for mass production of algal cells for immobilisation (see section 2.5). The culture flasks were incubated in a glass water bath (aquarium) with temperature control unit and illumination from four fluorescent tubes, two to each side of the water bath, giving a light intensity of approximately 100 μE m^{-2}.s^{-1}.

Halomonas was cultured in 1.5 M NaCl *Dunaliella* medium with the addition of 0.1 g.l^{-1} glycerol as a carbon source. The cultures were incubated at 25°C without shaking.

2.3 Growth analysis

2.3.1 Cell counting using haemacytometer

Improved Neubauer type haemacytometer (0.1 mm depth with 1/400 mm^2 smallest grid area) from Weber Scientific International Ltd. was used for cell counting. Algal cells from a well mixed culture were fixed by adding 0.1 ml of Gram's iodine (1.0 g iodine and 2.0 g potassium iodide in 300 ml of distilled water) to 0.9 ml of algal cells. The counting unit area can be varied depending on the cell density in the sample, however, individual counts of at least 30 cells per unit area with 7-10 replicate counts was recommended by Schoen (1988). Cell number per ml was calculated as follows:

a) Mean number of cells/unit area × area of entire grid/unit area counted = number of cells/grid

b) Number of cells/grid × ml/volume of sample on the grid = number of cells/ml

The number of cells/ml was finally multiplied by 1.1 to compensate for the amount of Gram's iodine added.

2.3.2 Chlorophyll determination

2.3.2.1 Chlorophyll determination in free *Dunaliella* cells

This method was described by Mackinney (1941). 5 ml of algal sample from a well mixing flask was centrifuged at 3000 g for 10 minutes and supernatant was immediately discarded. Pellet was resuspended in 1 ml of distilled water and mixed well. After mixing, 4 ml of acetone was added into the test tube to obtain a final concentration of 80% acetone (v/v). The extraction was performed in dim light for 10-15 minutes or may be kept overnight in the refrigerator. Before measurement in the spectrophotometer, the acetone extract was centrifuged at 3000 g for 5 minutes. At this stage the pellet should be white, indicating that all pigments have been extracted. Then optical density (OD) was measured spectrophotometrically at 645 and 663 nm.

Chlorophyll concentration calculation:

$$OD_{645} \times 202 = Y$$

$$OD_{663} \times 80.2 = Z$$

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

2.3.2.2 Chlorophyll determination in immobilised cells

The same procedure of chlorophyll analysis using 80% acetone was applied to immobilised cells. However, instead of algal culture in liquid medium, 5 large beads (4-5 mm diameter) or up to 30 small beads (2-3 mm), depending on chlorophyll concentration in beads, were simply placed into the test tube. After an addition of 1 ml distilled water and 4 ml acetone, mechanical homogenising by a tissue grinder was performed in order to break up bead structure. Chlorophyll in broken beads must be extracted overnight in the refrigerator in order to achieve complete extraction.

2.3.3 Evan's blue staining for testing cell survival

0.5% (w/v) Evan's blue (Sigma Chemical Co.) staining, which has been used for testing the survival of plant cells (Gaff and Okong'O-Ogala, 1971; Taylor and West, 1980), was applied for testing the survival of *Dunaliella*. Staining is based on the loss of semi-permeability of the cell membrane on death. Dead cells which cannot exclude Evan's blue pigment will change colour from green to blue after 5 minutes staining. Survival percentage was calculated from Evan's blue stained cells against total cell number present.

2.4 Strain isolation using double-layer agar plates and axenic culture preparation

It is necessary to obtain bacteria free cultures for most of the experiments because bacteria can use glycerol produced by the algae as a carbon source. Combination of methods such as Percoll gradient centrifugation, followed by double-layer agar plates were used. A clean algal colony was then picked up by a single colony isolation technique.

2.4.1 Percoll gradient centrifugation

Percoll gradient centrifugation was carried on in 5-inch sterile test tubes with metal caps. Two ml of 4 different sterile Percoll solutions, 100%, 75%, 50%, and 25% (v/v) respectively, were gently pipetted into the test tube to form layers of different Percoll concentrations. 2 ml of *D. parva* culture, treated with 1 mM sodium vanadate to stop flagella movement (Gilmour *et al*, 1985), was placed on top of the Percoll solution and the test tube was centrifuged at 2000 *g* for 60 minutes. Algal cells which appeared as a green band in the Percoll gradient were removed using a small Pasteur pipette. The bacteria, due to their smaller cell size, were found to have passed through the Percoll solution to the bottom of the test tube.

2.4.2 Double-layer agar plates

This technique has been modified from Brown *et al.* (1987) as *Dunaliella* cells cannot grow well on 1.5% (w/v) agar. Therefore, double-layer agar, consisting of soft agar (0.9%) on top of the hard agar base (1.5%) was used instead. The agar media were prepared by adding Oxoid No.3 agar into the culture medium and then sterilized by autoclaving (20 minutes at 15 lbs.in^{-1}). Petri dishes, 3.5 inch diameter, were poured conventionally with 25 ml of 1.5% agar medium and left at room temperature for the agar to solidify. Then, warm 0.9% agar medium was immediately poured on top of the 1.5% agar base. With this double layer of agar, *Dunaliella* cells can be streaked directly on the top layer in order to obtain colonies. Alternatively, 9 ml of 0.9% agar medium, which was kept molten at 43°C, could be mixed with 1 ml of diluted liquid cultures of *Dunaliella* (100 cells.ml^{-1}) and then poured on top of the 1.5% agar medium base. This latter method was used for strain isolation.

2.4.3 Single colony isolation

This technique used a glass micropipette made by stretching a Pasteur pipette in a flame. A small single *Dunaliella* colony without surrounding bacteria, was picked out from an agar plate under a stereo microscope in a laminar air flow cabinet. The colony was allowed to grow in fresh medium. Bacterial contamination was assessed by adding a sample of the algal culture into nutrient broth medium (*Dunaliella* medium with 13 g.l^{-1} Oxoid nutrient broth) and leaving it for three days.

2.5 Immobilisation of *Dunaliella* cells

2.5.1 Immobilisation of *Dunaliella* cells in calcium alginate beads

Dunaliella cells, normally cultured in 1.5 M NaCl medium, were concentrated by centrifugation at 1000 *g* for 15 minutes and washed with phosphate free medium before being mixed with 2.5% (w/v) sodium alginate in 1:3 (algae:alginate) proportion. Sodium alginate (alginic acid, sodium salt: viscosity = 14,000 cps in 2% solution at 25°C) used in the experiment was an extract from Kelp, *Macrocystis*

pyrifera, supplied by Sigma Chemical Co. Algal beads were formed after dropping the alginate/algal mixture into 0.1 M CaCl₂ (Figure 2.1). Beads were left in CaCl₂ solution for 1-2 hours before being transferred to culture medium. The algal beads were usually left overnight in the growth room before starting any experiments. A photograph of algal beads is shown in Figure 2.2.

2.5.2 Immobilisation of *Dunaliella* cells in calcium alginate with spongy sheet supporter

The "algal sheet" has been developed as an alternative to algal beads. It was made by casting calcium alginate entrapped algae in a 0.5 mm thick plastic sponge sheet with pore diameter of approximately 3 mm. The porous structure of the sponge sheet can be a supporter for immobilised algae by holding the solidified calcium alginate. Photograph of an algal sheet is shown in Figure 2.3.

2.5.3 Immobilisation of *Dunaliella* cells in agar beads

Agar and agarose (a purified preparation of agar) are polysaccharides extracted from marine red algae. Entrapment of living cells in agar has not been used in industry due to the high cost of this polymer. In this study, agar beads were used only for comparison of glycerol leakage with calcium alginate beads.

Immobilisation of *Dunaliella* cells in agar beads was performed according to the method described by Nilsson *et al.* (1987). 2% (w/v) agar, a technical grade, high strength Oxoid No.3 which gives a firm gel at 1.2% (w/v), was added to normal 1.5 M NaCl culture medium and sterilised by autoclaving. After autoclaving, the solution was kept molten in a water bath at 45°C until used. To obtain agar beads, 2% agar/1.5 M NaCl culture medium was mixed with concentrated algal cells in a 87.5:12.5 ratio (the final agar concentration was 1.75%). The mixture was then dispersed by magnetic stirring in warm (45°C) sterilized vegetable cooking oil. Size of agar beads could be controlled by adjusting stirring speed. Thereafter, the mixture was cooled down in an ice bath under continuous stirring until agar solidified (at

approximately 15°C). Agar beads were separated from oil by adding culture medium which caused the beads to be deposited into the medium phase. This procedure was repeated several times until all the beads were free from oil phase.

2.5.4 Immobilisation of *Dunaliella* cells in chitosan beads

Chitosan immobilisation was modified from the original method described by Vorlop and Klein (1981). 1.5 g chitosan (Sigma Chemical Co.) were dissolved in 100 ml distilled water containing 0.25 ml glacial acetic acid and stirred overnight. This chitosan-acetate solution was then mixed with concentrated algal cells in 3:1 (chitosan:algae) proportion. The mixture was dropped into crosslinking solution which is 1.5% (w/v) sodium-tri-polyphosphate ($Na_5P_3O_{10}$) solution pH 5.5. After 30 minutes beads were transferred into 1.5% (w/v) sodium-tri-polyphosphate solution pH 8.5 for a shrinking and stabilization procedure. After treatment for 3 hours, beads were finally transferred into the culture medium.

2.6 Morphological observations

Dunaliella cell clusters in calcium alginate beads can be observed under light microscope after dissecting a bead into thin pieces of gel, placing on a microscope slide and pressing with cover slip. For electron microscope, free and immobilised algal cells were fixed with 3% (v/v) glutaraldehyde in 1.5 M NaCl growth medium before being observed by scanning and transmission electron microscope.

Bacteria contaminating *Dunaliella* cultures could be observed under a phase-contrast microscope at 40× objective lens.
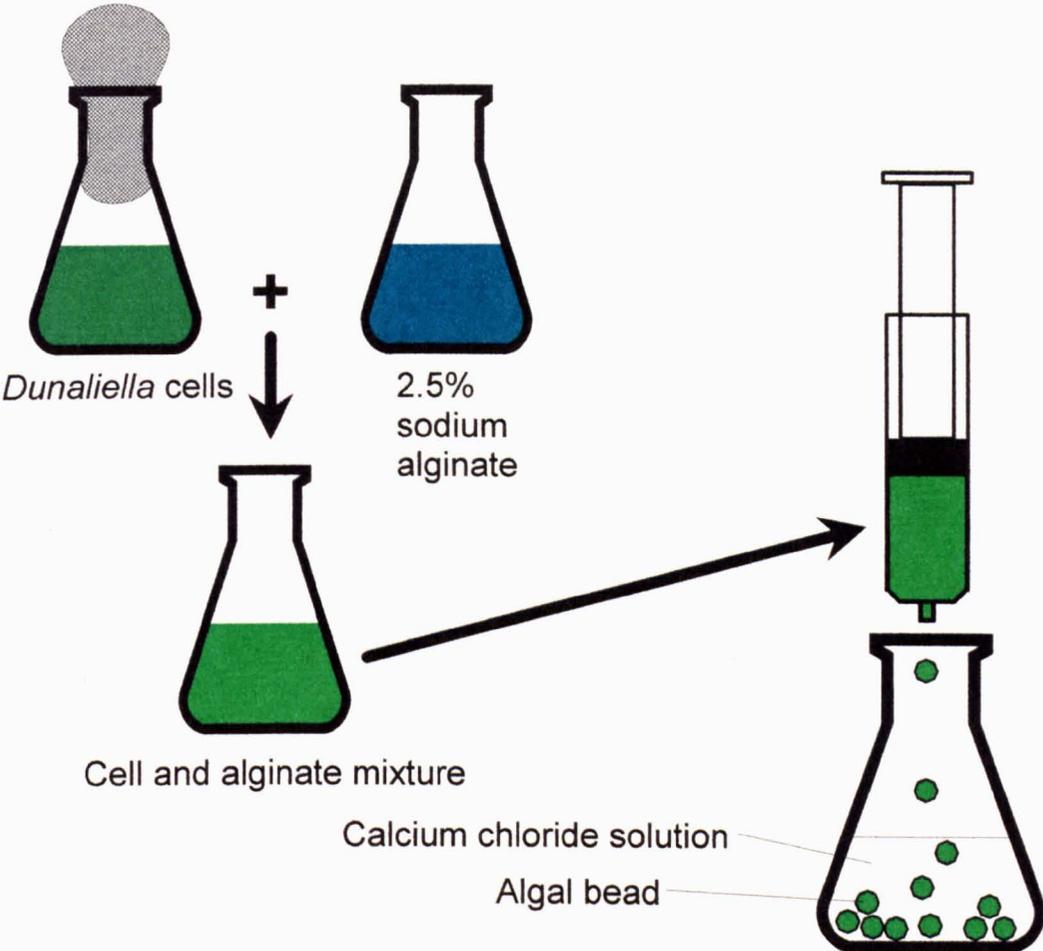
Figure 2.1: Immobilisation of *Dunaliella* cells in calcium alginate.

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Figure 2.2: Living algal cells immobilised in calcium alginate (algal beads).

Figure 2.3: Immobilised algae in calcium alginate with spongy sheet supporter (algal sheet). Top picture shows pouring algal and alginate mixture on spongy sheet and picture below shows "algal sheet" after it has solidified.

2.7. Photosynthesis

2.7.1. Oxygen evolution

Oxygen evolution from *Dunaliella* was measured in a Clarke-type oxygen electrode (Hansatech Scientific Instruments Ltd., Kings Lynn, UK). Details of the oxygen electrode principle were described by Delieu and Walker (1972) and Walker (1993). Diagram of the oxygen electrode is shown in Figure 2.4. The electrode, consisting of platinum cathode and silver anode linked by a KCl bridge, is separated from the medium by an oxygen permeable membrane. When a small voltage (0.5-0.8 V) is applied across the two electrodes the platinum becomes negative and the silver becomes positive.

At cathode (Pt) oxygen is reduced at the platinum surface, initially to hydrogen peroxide.

$$O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^-$$
$$H_2O_2 + 2e^- \rightarrow 2OH^-$$
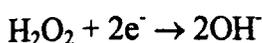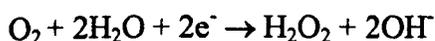
At anode (Ag) the silver is oxidized and silver chloride is deposited.

$$4Ag \rightarrow 4Ag^+ + 4e^-$$
$$4Ag^+ + 4Cl^- \rightarrow 4AgCl$$
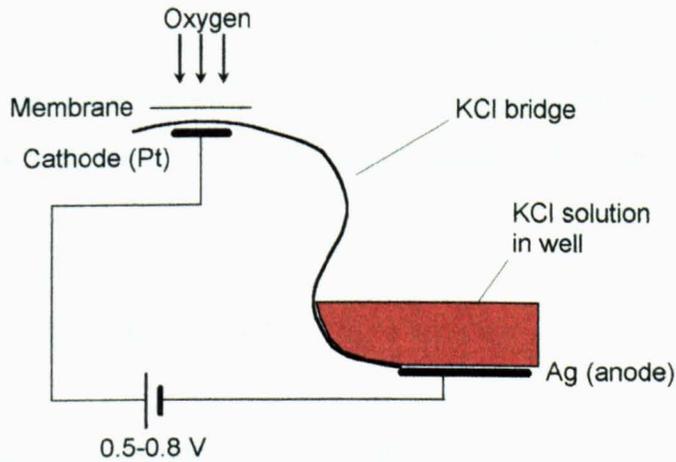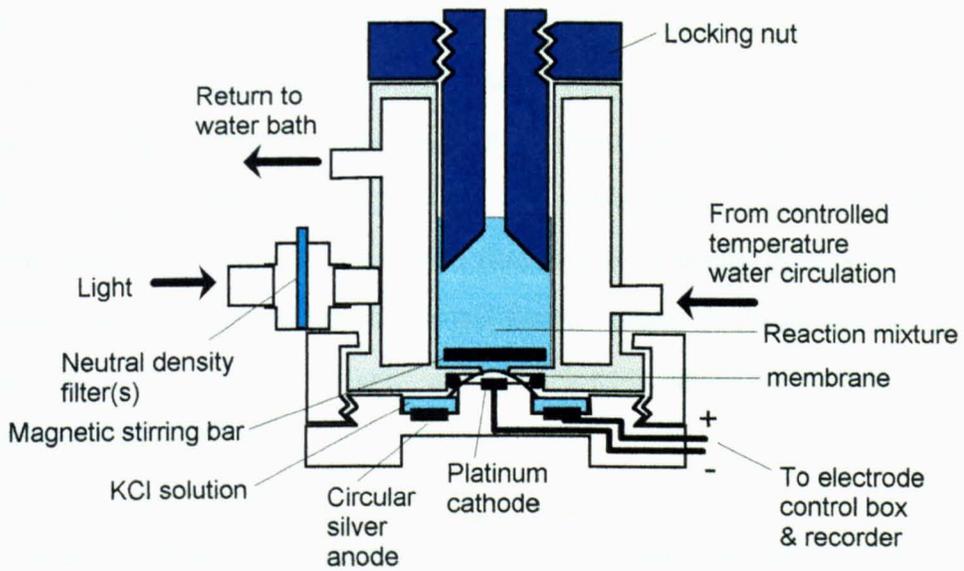
Figure 2.4: Diagrams of the oxygen electrode.

Electrons from platinum cathode which flow to silver anode have been stoichiometrically related to oxygen consumed at the cathode. In a well-stirred solution the oxygen diffusion across the membrane is related to the activity of the oxygen in solution. The current flowing in the electrode was monitored by a sensitive potentiometric chart recorder. The reaction chamber used in the experiments was kept at a constant temperature (25°C) by circulating water from a temperature-controlled water bath. Light was supplied from a high intensity light box with fibre optic attachment. A combination of neutral density filters could be used to control light intensity in the chamber. Maximum light intensity inside the oxygen electrode chamber was 600 $\mu E.m^{-2}.s^{-1}$.

It is necessary to calibrate the oxygen electrode before any measurements are taken. The calibration was carried out by measuring saturated oxygen solution and zero oxygen solution. The saturated oxygen solution (air bubbled water) gives 100% oxygen value to the scale while zero oxygen solution could be achieved by adding a small amount of sodium dithionite (sodium hydrosulphate) into the chamber. Sodium dithionite reacts with dissolved oxygen and removes it from the solution as shown below.

$$Na_2S_2O_4 + O_2 + H_2O \longleftrightarrow NaHSO_4 + NaHSO_3$$
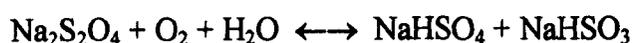
Oxygen evolution was calculated by the equation:

$$O_2 \text{ evolution rate } (\mu \text{mol } O_2 . \text{mg Chl}^{-1}.h^{-1}) = \frac{O_2 \text{ content of 2 ml medium at } 25°C}{\text{range in chart units } (0-100\%)}$$

$$\times \frac{60}{\text{mg chl}^{-1}} \times \frac{\text{No. of chart units change}}{\text{time (min)}}$$

The following is the concentration of saturated oxygen at different salinities:

Salinity (M NaCl)	O_2 concentration at 25°C (μmoles.ml^{-1})
0	0.2202
0.4	0.2084
0.8	0.1835
1.0	0.1776
1.4	0.1633
1.5	0.1598
1.8	0.1468
2.0	0.1397
2.5	0.1231

2.7.2. Potential yield of the photochemical reaction of PSII (Fv/Fm ratio)

When a chlorophyll molecule is exposed to light (photon energy), an electron in ground state is pushed to an energy-rich excited state. This energy of the unstable excited chlorophyll molecule can then be transferred to photosynthetic electron transport, or released as heat (thermal dissipation), or emitted as a photon of lower energy content (fluorescence). Chlorophyll fluorescence emission always has a longer wavelength than the absorbed energy spectrum, and is in the red region of spectrum. This phenomenon is called Stoke's shift (Hall and Rao, 1994).

The "quenching analysis" is based on the photosynthesis quench state after all photosystem II (PSII) centres are closed (by a process of dark adaptation) prior to a pulse of strong light. The variable fluorescence (Fv) or photochemical fluorescence quenching is the difference in fluorescence between maximum fluorescence (Fm), when all the Q_A molecules [a quinone-type electron acceptor of photosystem II] are fully reduced (reaction centres are closed), and minimum fluorescence (F$_0$), when all the Q_A molecules are in the oxidized state (reaction centres are open). An example of

the quench curve is shown in Figure 2.5. The ratio of Fv/Fm can be used as a probe for energy transfer and stress physiology in photosynthesis.

To evaluate Fv/Fm, beads or cells must be kept in the dark for 30 minutes before being placed under an optical fibre probe. This dark adaptation period reverses all non-photochemical fluorescence quenching as recommended by Bolhàr-Nordenkampf and Öquist (1993) and Q_A is fully oxidized. 1 to 5 beads or 1 ml of free cell culture could be used in each measurement. Instrument consisted of a fibre optic probe connected to the Walz Chlorophyll Fluorimeter (Walz Mess-und Regeltechnik, Heinz Walz GmbH, Germany), 655 nm LED measuring beam source and flash trigger controlled unit which provided saturating light of 2000 $\mu E.m^{-2}.s^{-1}$. The measurement is recorded by a chart recorder connected to the fluorimeter. Diagram of the instrument is shown in Figure 2.6. During measurement, beads (or culture) were exposed to the measuring beam at 3 $\mu E.m^{-2}.s^{-1}$ and, at this stage, minimum fluorescence (F_0) was recorded. This was followed by a one second pulse of saturating light. The maximum fluorescence (Fm) is the peak of fluorescence recorded on the chart scale. The variable fluorescence (Fv) is the chart scale value of Fm - F_0.

2.8 Glycerol assay

Two methods for glycerol assay were used:

a) Enzymatic glycerol test kit from Boehringer-Mannheim UK (Diagnostics and Biochemicals) Ltd.

b) Acetylacetone method (Ben-Amotz and Avron, 1978). This method provides fast, accurate, and economical measurement. However, Tris buffer in the culture medium can interfere with this method so HEPES buffer has to be used instead.
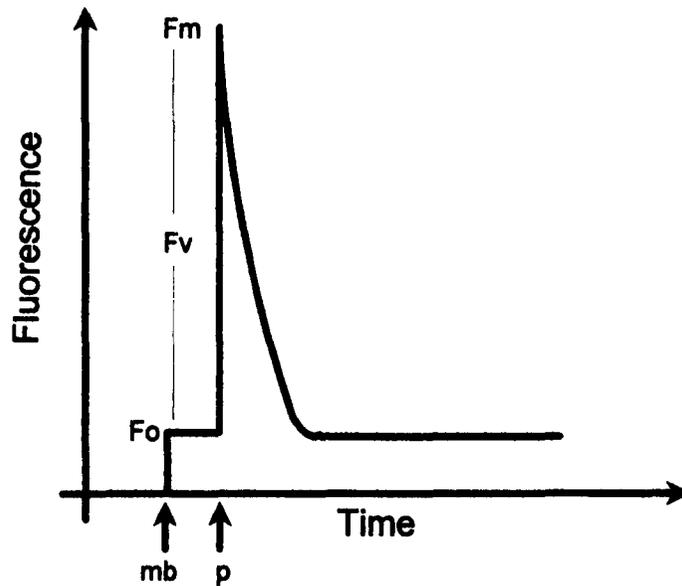
Figure 2.5: Fluorescence quenching measurement of the potential yield of the photochemical reaction of PSII (Fv/Fm ratio).

mb = measuring beam

p = pulse of saturating light

Fluorescence detector unit

Actinic light source

Shuttered saturation pulse illumination

Fluorometer

0.04

Fibre optic

Algal bead(s)

Chart recorder
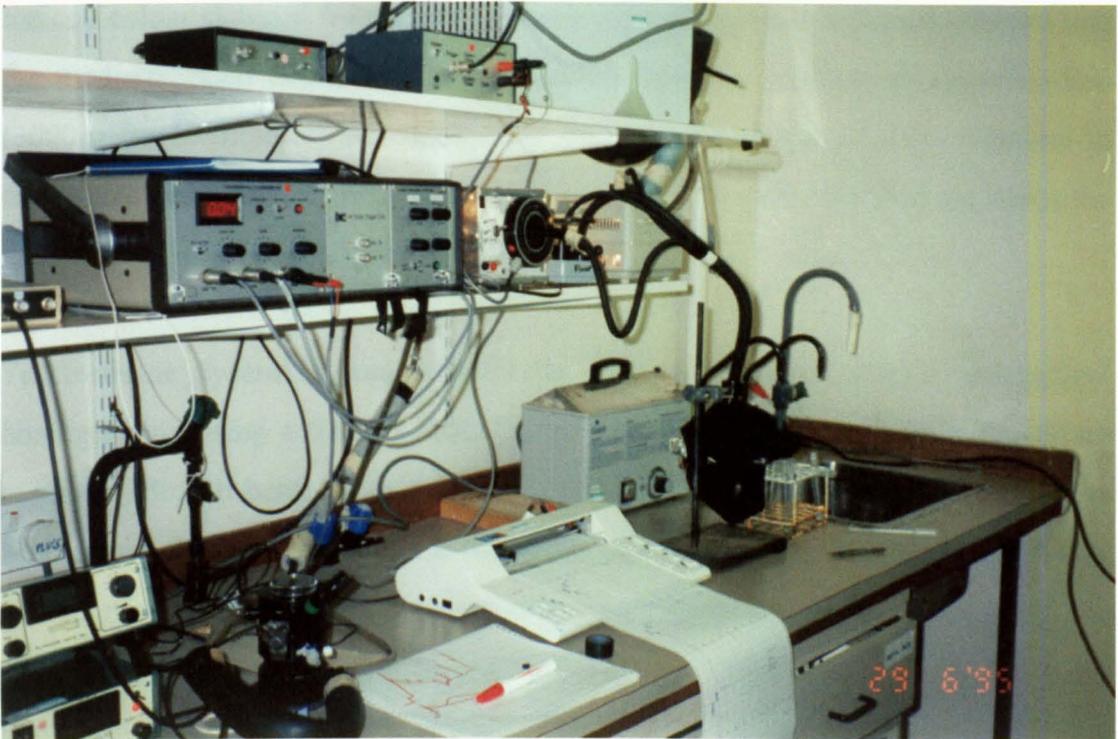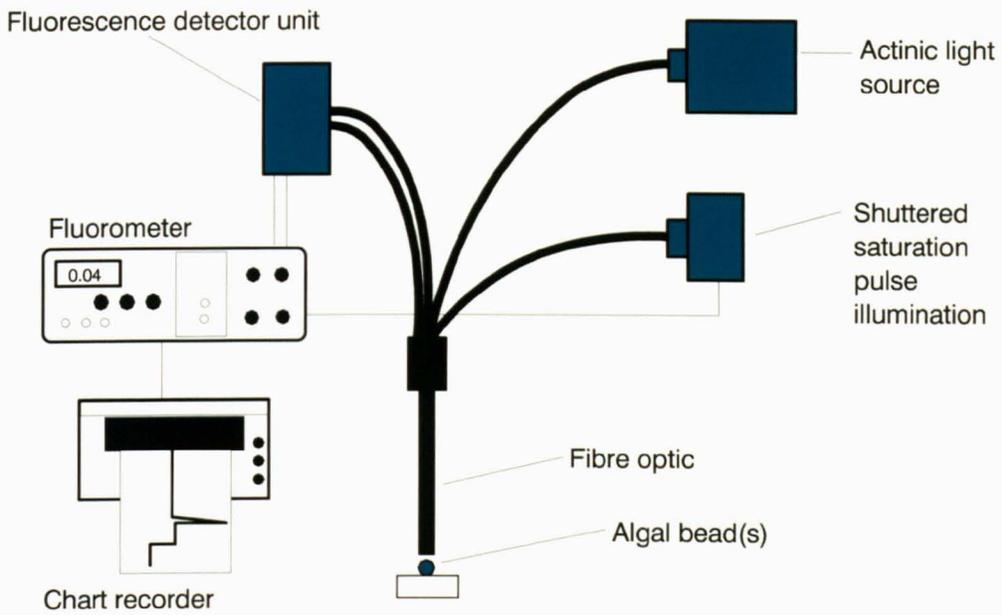
Figure 2.6: Diagram and photograph of the chlorophyll fluorescence instrument.

2.8.1 Sample preparation for glycerol assay

To determine glycerol concentration in the medium, 5 ml of culture medium were placed in 5 inch test tubes. Algal cells were separated from the medium by centrifugation at 1500 g for 15 minutes which is the speed recommended by Enhuber and Gimmler (1980). This speed did not result in cell breakage nor in incomplete sedimentation. The supernatant was sampled and kept in the freezer at -20°C until analysed. If the sample was collected for glycerol leakage analysis, 0.9 ml of supernatant was mixed with 0.1 ml of 30% trichloroacetic acid (TCA).

Total glycerol in cells plus medium was determined by adding 0.5 ml of 30% TCA into 4.5 ml of culture samples and incubating for at least 10 minutes. TCA denatures protein structure of the cell membrane and releases glycerol into the medium. When the cell colour changed from green to brown then all glycerol had been released into the medium. Cell debris was then separated from the medium by centrifugation (3000 g for 10 minutes) and supernatant was kept for glycerol assay. Glycerol in cells could be calculated from the amount of glycerol in cells plus medium minus the amount of glycerol in medium only.

To determine glycerol in immobilised cells, beads were broken down by mechanical homogenising using a tissue grinder in the presence of 3% (w/v) TCA in culture medium. Broken beads were left in 3% TCA for 1-1.5 hours, during this time the bead colour changed from green to brown indicating denaturation of protein by TCA. The solution was then centrifuged and sampled for glycerol analysis.

Percent of glycerol leakage was calculated by:

$$\% \text{ glycerol leakage} = \frac{(\text{glycerol in the medium} \times 100)}{\text{glycerol in cells and medium}}$$

2.8.2 Determination of glycerol by enzymatic method

The enzymatic test kit for glycerol determination was obtained from Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd.. In this method, glycerol is phosphorylated by ATP to L-glycerol-3-phosphate in the reaction catalyzed by glycerokinase (GK).

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{L-glycerol-3-phosphate} + \text{ADP}$$
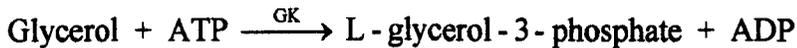
The ADP from the above reaction is reconverted by phosphoenolpyruvate (PEP) with the aid of pyruvate kinase (PK) into ATP with the formation of pyruvate.

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$
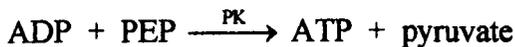
In the presence of the enzyme lactate dehydrogenase (L-LDH), pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the oxidation of NADH to NAD.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+$$
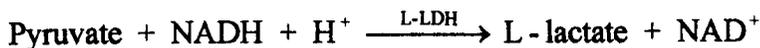
The amount of NADH oxidized in the above reaction is stoichiometric with the amount of glycerol. NADH is determined by means of its absorption at 334, 340 or 365 nm.

Reagents in Boehringer Mannheim glycerol test kit:

Solution 1: (Coenzyme buffer) glycylglycine buffer, pH 7.4; NADH, 7mg; ATP, 22 mg; PEP, 11 mg; magnesium sulphate; stabilizers. This solution is prepared by adding 11 ml of distilled water and is stable for 4 days at +4°C, it needs to be warmed to room temperature before use.

Solution 2: (Enzyme suspension) pyruvate kinase, approx. 240 U; lactate dehydrogenase, approx. 220 U.

Solution 3: Glycerokinase suspension approx. 34 U. These enzyme suspensions (Solution 2 and 3) are stable for 1 year at +4°C.

Solution 4: Standard glycerol (0.389 g.l^{-1}) solution.

The following are placed in glass cuvettes: (volume = 4 ml)

Pipette into cuvettes	Blank	Standard	Sample
Solution 1	1.0 ml	1.0 ml	1.0 ml
Distilled water	1.9 ml	1.8 ml	1.9 ml
Culture medium	0.09 ml	0.09 ml	-
30% TCA	0.01 ml	0.01 ml	-
Glycerol standard	-	0.1 ml	-
Sample	-	-	0.1 ml
Solution 2	0.01 ml	0.01 ml	0.01 ml

Mix the solution using a small plastic spatula (or by gentle swirling after closing the cuvette with Parafilm®) and wait for 5-10 minutes and read absorbances of the solutions at 340 nm (A$_1$) followed by addition of solution 3 to start the reaction

	Blank	Standard	Sample
Solution 3	0.01 ml	0.01 ml	0.01 ml

Mix, wait for completion of the reaction (5-10 minutes) and read the absorbances of blank and sample immediately one after another (A$_2$). If the reaction has not stopped after 15 minutes, continue to read the absorbance at 2 minute intervals until the absorbance decreases constantly over 2 minutes.

Determine the absorbance difference (A_1 - A_2) for blank, standard and samples. Subtract the absorbance difference of the blank from the absorbance difference of the samples.

$$\Delta A = \Delta A_{sample} - \Delta_{blank}$$

Calculation:

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A$$

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of the substance to be assayed

d = light path (cm)

ε = absorption coefficient of NADH at 340 nm = 6.3 ($1 \times mmol^{-1} \times cm^{-1}$)

It follows for glycerol:

$$\text{concentration of glycerol (g.l}^{-1}\text{)} = \frac{3.02 \times 92.1}{6.3 \times 1 \times 0.1 \times 1000} \times \Delta A = \frac{2.781}{6.3} \times \Delta A$$

Nevertheless, the commercial glycerol test kit is expensive (£1 per sample). In some experiments, enzymes and other chemicals were ordered to prepare a custom-made test kit with some modifications as follows:

(all enzymes were ordered from Boehringer Mannheim)

Solution 1 was prepared freshly before each test by mixing 11 ml of a solution containing 100 mM glycylglycine pH 7.4 and 100 mM $MgSO_4$ with 22 mg ATP, 11 mg PEP, and 7 mg NADH.

Solution 2 was prepared by mixing 0.403 ml pyruvate kinase (400 U/ml) with 0.097 ml L-lactate dehydrogenase (3000 U/2ml). With this custom-made solution, 0.02 ml was used instead of 0.01 ml in the commercial test kit.

Solution 3 was prepared by adding 1.0 ml ammonium sulphate (3.2 M) into 500 U (13 mg) solid glycerokinase. Thereafter, 0.1 ml of this solution was diluted by mixing with 0.5 ml of 3.2 M ammonium sulphate.

For the custom-made glycerol test, NaOH is needed to neutralize acidic pH from trichloroacetic acid (TCA) and concentration of enzymes in solution 2 is different from the commercial test kit. Therefore, the following are placed in glass cuvette:

Pipette into cuvettes	Blank	Standard	Sample
Solution 1	1.0 ml	1.0 ml	1.0 ml
Distilled water	1.9 ml	1.8 ml	1.9 ml
1 M NaOH	0.02 ml	0.02 ml	0.02 ml
Culture medium	0.09 ml	0.09 ml	-
30% TCA	0.01 ml	0.01 ml	-
Glycerol standard	-	0.1 ml	-
Sample	-	-	0.1 ml
Solution 2	0.02 ml	0.02 ml	0.02 ml

wait for 5 min			
Solution 3	0.01 ml	0.01 ml	0.01 ml

2.8.3. Determination of glycerol by acetylacetone method

The reaction can be described by the following equations:

1. $CH_2OH\text{-}CHOH\text{-}CH_2OH \xrightarrow{HIO_4} 2HCHO + HCOOH$

64

2. $HCHO + 2CH_3COCH_2COCH_3 + NH_4 \longrightarrow$

(reaction product structure shown)
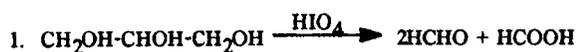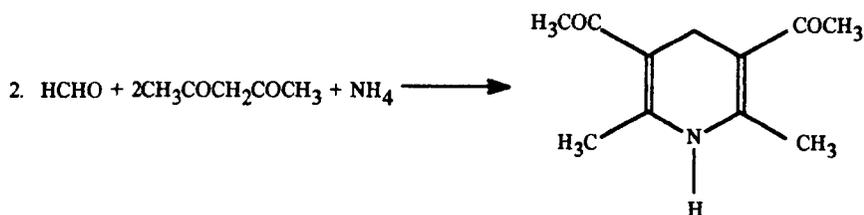
From these reactions, the final compound has a yellowish colour which can be detected by spectrophotometer at 410 nm.

Reagents:

1. Periodate reagent: 130 mg of $NaIO_4$ was dissolved in 180 ml of 2% (v/v) acetic acid containing 15.4 g ammonium acetate. After mixing, 20 ml of glacial acetic acid was subsequently added.

2. Acetylacetone reagent: 1% (v/v) acetylacetone in isopropanol.

Procedure:

0.1 ml of samples or glycerol standards (0-500 µg glycerol.ml^{-1}) were added in to test tubes containing 1.9 ml distilled water. 1 ml of periodate reagent was then added and, after mixing, the tubes were incubated for 5 minutes at room temperature. Thereafter, 2.5 ml of acetylacetone reagent were added and vortex mixed. The test tubes were incubated at 40-50°C for 20 minutes. After cooling down to room temperature, the absorption was measured spectrophotometrically at 410 nm against the reagent blank. The standard curve for glycerol analysis by the acetylacetone method is shown in Appendix B.

Notes: High blank value can result from "old" acetylacetone and other compounds such as Tris, mannitol, sorbitol, and glucose may interfere with the reaction. HEPES is an appropriate buffer and has been used instead of Tris for all experiments which used acetylacetone method for glycerol determination.

2.9 Starch assay

Starch was assayed by the method described by Takeda and Hirokawa (1978).

Reagents:

1. KI reagent (0.03 M KI and 0.01% (w/v) I_2)

2. 17.5% (v/v) perchloric acid

Procedure:

2 to 5 ml of algal culture were centrifuged at 3000 g for 10 minutes and the medium (supernatant) was discarded. Starch in the pellets was extracted by adding 1.5 ml 17.5% perchloric acid and incubating at 45°C for 50 minutes. Thereafter, 1.5 ml of KI reagent were added and the pellet was centrifuged at 3000 g for 5 minutes. Absorbance of the supernatant was measured spectrophotometrically at 560 nm against 17.5% perchloric acid as blank. The standard curve was prepared with 0 - 250 μg soluble strach solution in 17.5% perchloric acid (see Appendix C).

2.10 Uptake and release of radiolabelled substances from *Dunaliella* in culture

2.10.1 Separation of algal cells from culture medium by silicone oil technique

An appropriate density of silicone oil allows only algal cells to penetrate through silicone layer by centrifugal force while leaving culture medium on top. In this technique, the density of silicone oil used must be relevant to the density of culture medium which is determined by salt concentration. To discover the proper oil concentration, a series of silicone oil densities must be tested for each salinity. The test can be done by mixing different oil densities (e.g. Dow Corning 710 with Dow Corning 550 or 200) and checking that cells can pass through during centrifugation. For cultures grown in 1.5 M NaCl medium, Dow Corning 710 oil provides a good

separation. However, lower density Dow Corning 550 must be used instead for 0.5 M NaCl grown cells. Figure 2.7 illustrates the principle of silicone oil density test.

Procedure:

1. In 1.5 ml Eppendorf tube, add 300 µl of silicone oil, followed by gently adding 300 µl algal culture on top.

2. Centrifuge at 13,000 g for 3 minutes in a microfuge (MSE, Micro-Centaur).

3. After centrifugation, Eppendorf tube must be re-checked to ensure complete separation (see Fig. 2.7). 100 µl of culture medium, which has been separated from algal cells, was sampled and placed in a scintillation vial containing 5 ml of scintillation fluid (Safe Fluor S, Lumac LSC B.V., The Netherlands).

4. Remove algal pellet at the bottom tip of the Eppendorf by cutting the tube within the oil layer using a razor-blade. This piece of tip was placed cut end down on a tissue paper to remove silicone oil. Then, the tip was placed in another Eppendorf tube containing 300 µl distilled water.

5. Centrifuge at 13,000 g for 30 seconds. After centrifugation, pellet was moved from the tip into the new Eppendorf tube.

6. Discard the empty tip, all algae and water were finally transferred into a scintillation vial containing 5 ml of scintillation fluid.

2.10.2 Uptake of NaH^{14}CO$_3$ by *Dunaliella*

Aqueous solution of NaH^{14}CO$_3$ with specific activity 1.92 GBq.mmol^{-1} (52.0 mCi.mmol^{-1}) from Amersham Life Science was used in all bicarbonate uptake experiments. The stock radioactive solution was diluted to 0.1 µCi.µl^{-1} with sterile distilled water. Algal cells were always incubated in white illumination (approximately 50 µE.m^{-2}.s^{-1}) for 10 minutes before adding radioactive substance. 50 µl of the diluted NaH^{14}CO$_3$ solution was then added into 20 ml algae (1 × 10^7 cells.ml^{-1}) in a 100 ml flask. The culture was left in a fume cupboard at room temperature under white fluorescent illumination throughout the experiment. During experimental period, three replicates of 300 µl of algal culture were sampled each

time and algal cells were separated from culture medium by silicone oil technique. Radioactivity in culture medium and in cells was counted in a Beckman LS 1801 Liquid Scintillation Counter.
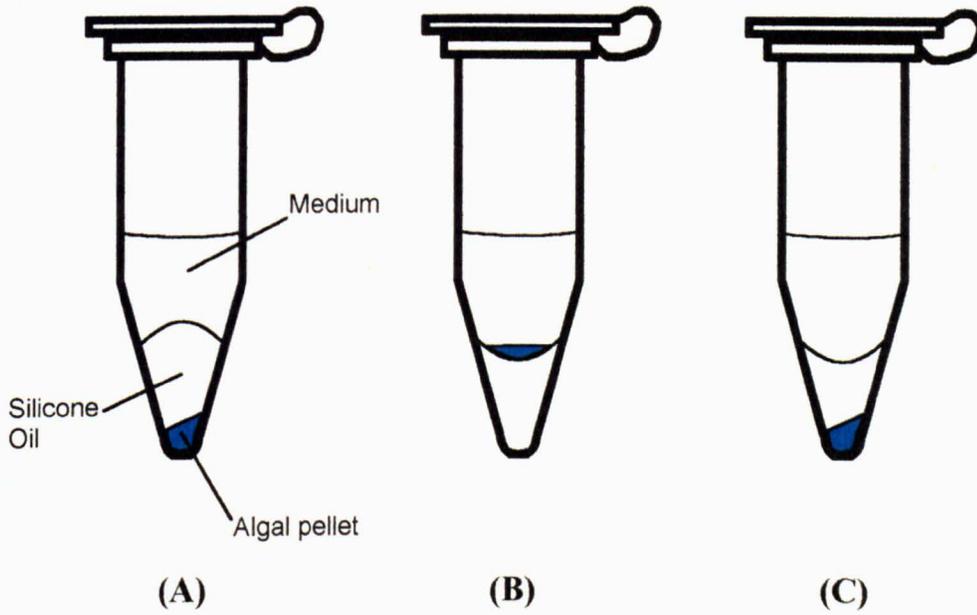
Figure 2.7: Separation of algal cells from culture medium by silicone oil technique. Pictures show the Eppendorf tubes after centrifugation.

       (A) Oil is not dense enough

       (B) Oil is too dense

       (C) Oil is in the correct density

2.10.3 Release of ^{14}C from *Dunaliella*

To study ^{14}C release from algal cells, *Dunaliella* culture (1×10^7 cells.ml^{-1}) in 1.5 M NaCl medium during exponential growth phase was centrifuged and resuspended in fresh 1.5 M NaCl medium without NaHCO$_3$. 20 ml of this culture in 100 ml flask were left under white illumination for 10 minutes before adding 50 µl of 0.1 µCi.µl^{-1} NaH^{14}CO$_3$. Cells were incubated for 6 hours at room temperature with approximately 50 µE.m^{-2}.s^{-1} illumination, to allow ^{14}C to be taken up and metabolised.

After incubation, 1 ml of culture was placed in a 1.5 ml Eppendorf tube and washed with 1.5 M NaCl fresh medium (normal medium) or with different salinity medium if it was an osmotic shock experiment. Samples were then incubated by attaching the Eppendorf tubes to a piece of foam floating on a temperature controlled water bath at 25°C under white illumination (50 µE.m^{-2}.s^{-1}). Release of ^{14}C could be determined after a period of time when cells were separated from the medium by silicone oil technique.

2.10.4 Determination of organic carbon fixed by *Dunaliella*

^{14}C radioactivity in algal pellet represents both organic and inorganic ^{14}C associated with the cells. In order to measure only the amount of organic carbon which has been fixed into cell structure and metabolites, inorganic carbon must be removed. This can be done by an addition of 3% TCA into algal pellet after being separated from culture medium by silicone oil technique. Under acidic conditions, major inorganic carbon species will be in the CO$_2$ form which will be released to the atmosphere. After drying, radioactivity in the pellet portion was assumed to be organic carbon associated with the cells.

Procedure:

300 µl of algal pellet, after separation by silicone oil technique, in 3% TCA solution was transferred into a scintillation vial. Vial was left overnight in the fume cupboard

to allow water to evaporate and $^{14}CO_2$ to be released. After drying, 5 ml of scintillation fluid was then added and radioactivity was counted in the scintillation counter.

2.10.5 ^{3}H-glycerol uptake by *Dunaliella*

Stock solution of [2-^{3}H] glycerol (37.0 GBq.mmol^{-1}, 1.0 Ci.mmol^{-1}) in ethanol was obtained from Amersham Life Science and it was diluted to 0.1 μCi.μl^{-1} with sterilized distilled water. 50 μl of diluted ^{3}H-glycerol was added into 20 ml (1 $\times$ 10^7 cells.ml^{-1}) *Dunaliella* culture. Culture was sampled at intervals and cells were separated from medium by silicone oil technique. The experimental procedure was similar to NaH^{14}CO$_3$ uptake experiment (section 2.10.2).

2.11 UV mutagenesis of *Dunaliella*

For UV mutation, 4 ml of 5×10^6 cells.ml^{-1} *D. parva* CCAP 19/9 wild type strain from nonsynchronized, log-phase culture, were transferred into 9.5 cm Petri dish and exposed to UV irradiation (λ = 260 nm). However, to obtain appropriate mutagenesis conditions, a UV dose-response curve was conducted. Unlike bacteria, algal cells have a slower growth rate. At least 4 weeks is needed for algal colonies to become visible on agar plates (Ginzburg *et al.*, 1995). So, in this study, the number of surviving cells was determined by direct counting of living cells with Evan's blue staining (section 2.3.3) instead of colony counting. At a constant UV intensity of 800 μW.cm^{-2}.s^{-1} (8,000 ergs.cm^{-2}.s^{-1}), exposure time was varied from 0 to 180 seconds. A UV dose-response curve was then plotted as percent survival against exposure time. The exposure time that resulted in 1% survival (99% death) was chosen for mutagenesis. After UV irradiation, algal cells were kept in the dark for 24 hours to avoid DNA photorepair processes.

2.12 Selection of high salinity tolerant mutants and organic carbon leaking mutants

2.12.1 High salinity tolerant mutants

For high salinity tolerant mutants, a mixture of mutant strains after UV mutagenesis was transferred into saturated salt (approximately 5.5 M NaCl) culture medium. Algal strains which could grow in saturated salt medium were transferred into 1.5 M NaCl medium and isolated by single colony isolation on double layer agar plates as the isolation could not be done in very high salinity medium because of very slow growth rate. After isolation, mutant strains were kept in saturated salt medium.

2.12.2 Organic carbon leaking mutants

The idea to isolate organic carbon leaking mutants (which in the case of *Dunaliella* most of the organic carbon product is glycerol) was derived from a comment by Lewin (1988). To detect glycerol leaking mutants, a bacterium *Halomonas* sp. was used as an indicator. Normally, *Halomonas* sp. cannot grow in *Dunaliella* medium which has only $NaHCO_3$ as a carbon source. For these experiments, *Halomonas* cultures were routinely maintained in 1.5 M *Dunaliella* medium with 0.1 $g.l^{-1}$ glycerol and then were washed twice with *Dunaliella* medium without glycerol before being mixed with the algal culture.

Procedure:

1. *Dunaliella* cells after UV mutation were grown in normal medium with 1.5 M NaCl. Cells were washed twice with fresh medium to remove glycerol from the culture medium and diluted to 1,000 $cells.ml^{-1}$.

2. Wash *Halomonas* culture twice with fresh *Dunaliella* medium (without glycerol added). Thereafter, 1 ml of *Halomonas* culture (OD_{600} > 1.0) was then added to 20 ml of 1,000 $cells.ml^{-1}$ mutant *Dunaliella* cells.

3. 1 ml of algae and bacteria mixture was added into 9 ml of warm, sterile, 1.5 M *Dunaliella* medium with 0.9% agar (this agar medium was kept in a water bath at 43°C since autoclaving).

4. Pour the mixture on top of 25 ml freshly solidified 1.5% agar medium in 9.5 cm plastic Petri dish and incubate in 25°C growth room with illumination. Green algal colonies should be seen after 3-4 weeks.

5. Algal colonies were observed under stereo microscope. Colonies which were surrounded by white bacterial colonies (assumed to be metabolite leaking mutants) and colonies without bacteria (assumed to be non-leaking mutants) were picked up by single colony isolation technique and grown in 1.5 M NaCl medium.

6. All mutant strains were separated from bacteria using double layer agar plates and single colony isolation technique.

2.13 Analysis of protein profile in *Dunaliella* using SDS-Polyacrylamide Gel Electrophoresis

Sample preparation method was modified from Fisher *et al.* (1994). *Dunaliella* cells from exponential phase culture were centrifuged and resuspended in fresh medium (containing 20 mM HEPES buffer) at 1×10^7 cells.ml^{-1}. 1 ml of cells was transferred into 1.5 ml Eppendorf tube and cells were separated from medium by centrifugation at 13,000 g for 3 minutes. Supernatant were discarded. Algal pellet was then mixed with 100 µl of 1 mM phenylmethylsulfonyl fluoride (PMSF), immediately followed by the addition of 2× volume of loading buffer from Sigma Co. (Laemmli, 1970) consisting of 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromphenol blue and 0.125 M Tris HCl pH 6.8 and boiled at 100°C for 3 minutes. The sample, after boiling, can be kept at -20°C for weeks but repeated freeze-thawing may lead to protein degradation (Bollag *et al.*, 1996).

Protein separation was performed in the Bio-Rad Mini-Protean II apparatus. Running protocol was according to Mini-Protean II instruction manual. Polyacrylamide separating gel (7.5% gel) was prepared by mixing 9.7 ml Milli-Q water with 5 ml of 1.5M Tris-HCl pH 8.8, 200 μl of 10% (w/v) sodium dodecyl sulphate (SDS) solution, and 5 ml of acrylamide/bis solution (Easigel, Scotlab Co.; consisted of 30% (w/v) acrylamide/0.8% (w/v) bis acrylamide in 37.5:1 ratio). This mixture was degassed by vaccuum for 20 minutes before being mixed with 100 μl of 10% (w/v) ammonium persulphate (prepare fresh daily) and 10 μl of N,N,N',N'-tetramethylene-ethylenediamine (TEMED). Thereafter, acrylamide solution was gently poured into the gel sandwich using Pasteur pipette. Isopropanol was poured as an overlay on top to protect gel surface from drying and keep the gel surface flat. Gel was allowed to polymerise for 45 min. The gel can be kept overnight by replacing isopropanol with a buffer/distilled water mixture (1:4 1.5 M Tris buffer solution pH 8.8: distilled water).

Stacking gel was prepared by mixing 6.1 ml of Milli-Q water with 2.5 ml of 0.5 M Tris-HCl pH 6.8, 100 μl of 10% (w/v) SDS, 1.33 ml acrylamide/bis solution. The mixture was degassed for 15 minutes before being mixed with 50 μl of fresh 10% (w/v) ammonium persulphate and 10 μl TEMED. This 10 ml stock monomer is enough for casting two stacking gels over the separating gel.

SDS-PAGE running condition was at constant 200 V setting. Running time was approximately 45 minutes. Standard protein markers (Sigma Co.) were used as references for protein molecular weight. After running, gels were stained by coomassie blue.

2.14 Statistics

In all figures and tables, error bars show standard error from experiments with at least three replicates. If no errors are shown, then the data are from one representative experiment, in this case the experiment will have been repeated on at least two occasions with the same trends evident. All sampling has been regulated to be less than 10% of total culture volume. The significant difference of means was evaluated by Student's t statistic or by analysis of variance (ANOVA), if there are more than two means. Linear regression analysis, with r^2 of at least 0.95, was used for all standard curve calculations for glycerol and starch analysis (see Appendices B and C).

CHAPTER 3:
PHYSIOLOGY OF IMMOBILISED *Dunaliella parva*

3.1 Introduction

Dunaliella accumulates glycerol as its osmoticum when growing in high salinity. Many previous reports suggested the idea of producing glycerol from this alga such as Chen and Chi (1981) and Ben-Amotz *et al.* (1982b). In 1986, Grizeau and Navarro reported the results from a study of glycerol production using immobilised *D. tertiolecta*. To date, this is the only paper published on immobilising *Dunaliella* with cell entrapment. This chapter will report results on growth, photosynthesis and glycerol release from *D. parva* CCAP 19/9 immobilised in calcium alginate beads.

It needs to be emphasised that if a *Dunaliella* culture is contaminated, bacteria could consume glycerol as a carbon source and this will reduce the amount of glycerol in the medium down to a non-detectable level. At an early stage of this research, glycerol was not found in *Dunaliella* cultures in preliminary experiments, because it was later found that the cultures were contaminated by halophilic bacteria. These bacteria can grow with *Dunaliella* in medium containing more than 4.5 M NaCl. The contaminant was not obvious with routine microscope observation, but it can be seen under phase contrast microscope at high magnification. Therefore, both mutant and wild type strains of *D. parva* were then separated from bacterial contaminants by a combination of Percoll centrifugation and single colony isolation on double layer agar plates. Cultures were checked by phase contrast microscopy and by inoculating nutrient broth until it was assured to be a bacteria free culture.

In most experiments in this chapter, *D. parva* was immobilised using calcium alginate (see section 2.5 in Materials and Methods). Algal cells from culture were harvested by centrifugation and then mixed with 2.5% (w/v) sodium alginate before

being dropped into 0.1 M CaCl₂ solution. After gel formation, beads were left in CaCl₂ solution for 1-2 hours before being transferred to culture medium. Entrapment of algal cells in calcium alginate prevents movement of the cells. Previous studies reported that chlorophyll concentration of immobilised algae can increase (Dainty *et al.*, 1986). This indicates that entrapped algae can grow inside the beads. The experiments described in this chapter aims to study the physiology of immobilised *Dunaliella* by examining growth and photosynthesis.

3.2 Results and Discussion

3.2.1 Growth of *Dunaliella* cells in calcium alginate beads

Concentrated *Dunaliella* cells were mixed in 2.5% sodium alginate in 1:3 proportion and then dropped into calcium chloride solution to form algal beads (Section 2.5). By this procedure, initial cell concentrations can be varied, depending on the concentration of *Dunaliella* cells being mixed with sodium alginate. Two initial chlorophyll concentrations, 3.2 ± 0.1 µg.5 beads^{-1} for low concentration and 7.07 ± 0.3 µg.5 beads^{-1} for high concentration, were used in this experiment. Average bead size was 3.9 ± 0.2 mm.

Figure 3.1 shows chlorophyll concentrations of immobilised *Dunaliella* cells increasing in 1.5 M NaCl medium. In this experiment, medium was changed at day 21 (indicated by an arrow in the figure) to prevent any effects of nutrient deficiency. The results indicated that the higher initial chlorophyll concentration produced the higher final chlorophyll concentration after a period of culture. Final chlorophyll concentration in high chlorophyll beads was approximately twice the amount of low chlorophyll beads.
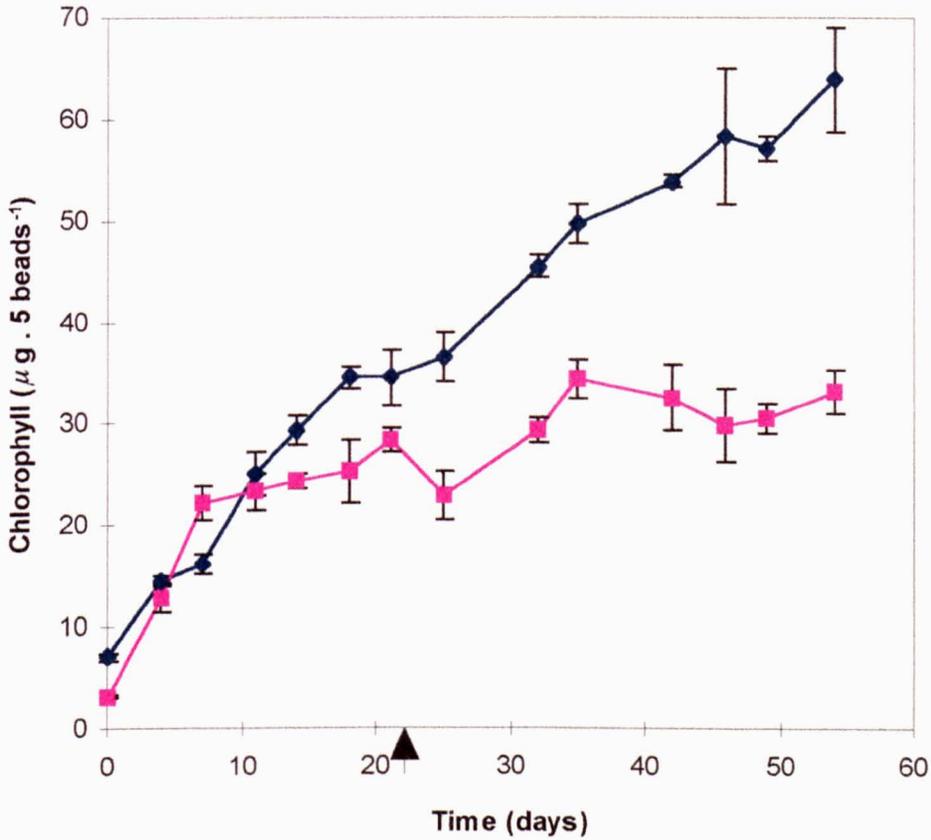
Figure 3.1: Growth of calcium alginate immobilised *Dunaliella* in 1.5 M NaCl
medium.

        (♦) High initial chlorophyll concentration

        (■) Low initial concentration

        Arrow indicates medium changed.

To examine cellular morphology of the entrapped cells, beads were cut into thin
pieces of calcium alginate gel, placed on a microscope slide and then squeezed by a
cover slip. A photograph of *Dunaliella* cells in a bead is shown in Figure 3.2. It was

found that entrapped cells divided and formed ball-shaped clusters. A diagram illustrating cell growth in a bead is shown in Figure 3.3. This diagram proposes the idea that the maximum chlorophyll concentration is, however, limited by the maximum number of cells in each cluster and the size of a cluster is limited by calcium alginate polymer structure. This concept can be used to explain the result shown in Figure 3.1 i.e. that the higher initial cell concentration could provide a higher final cell concentration.
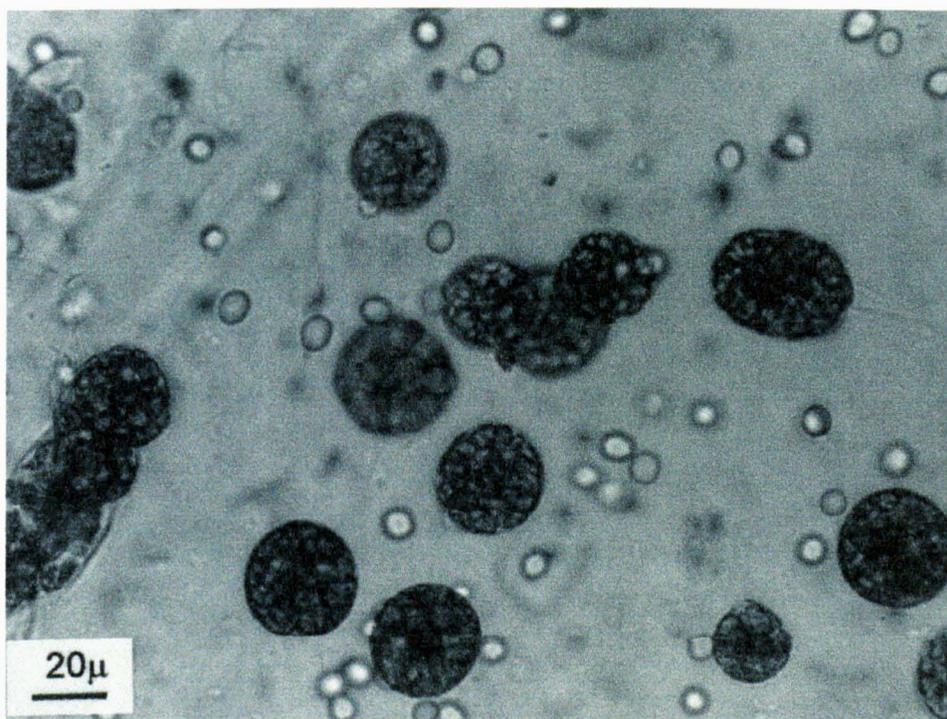
Figure 3.2: Photograph of *Dunaliella* cell clusters found inside calcium alginate bead.
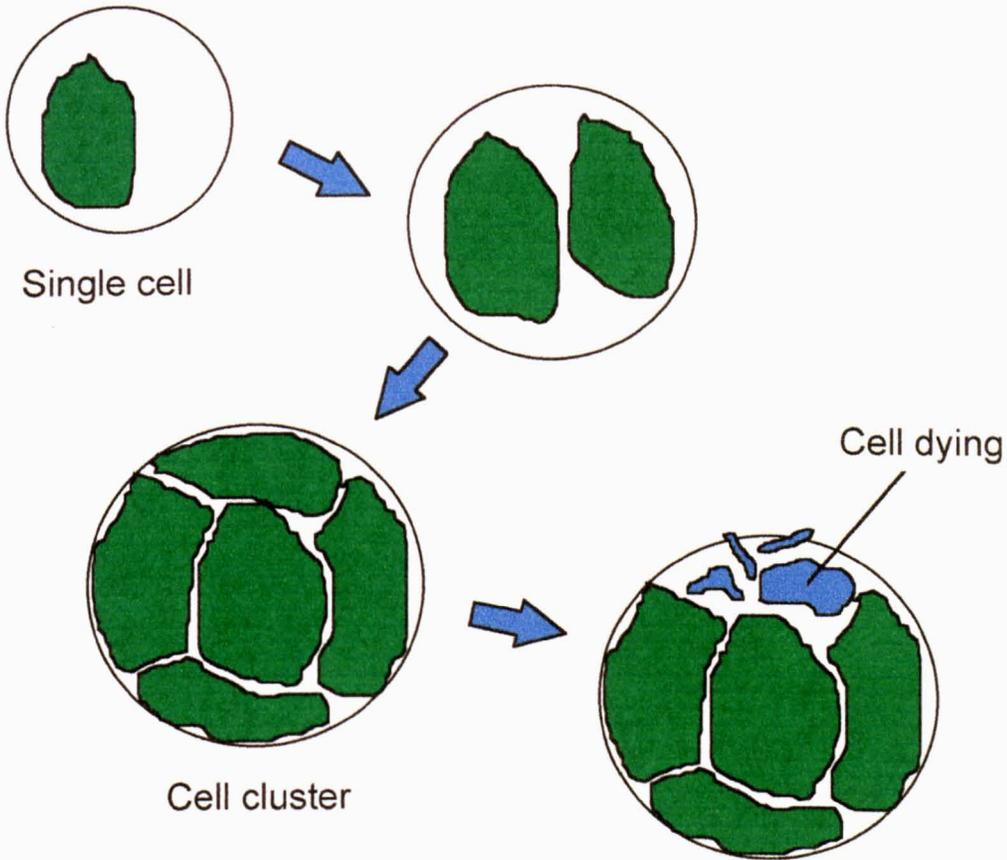
Figure 3.3: Diagram to illustrate growth of immobilised *Dunaliella* by formation of cell clusters.

Scanning electron microscope (Figure 3.4) shows *Dunaliella* cells attached to calcium alginate structure. This polymer structure entrapped the cells and, at the same time, limited the expansion of algal clusters. Limitation of cell cluster expansion is clearly seen under transmission electron microscope. Figure 3.5 shows a newly immobilised *Dunaliella* cell in a bead. At this stage, flagella were still apparent and there was some space between algal cell and calcium alginate structure.

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This space, in combination with the flexible structure of calcium alginate, allowed the algal cluster to expand in size during cell division. Once immobilised, the cells lose their flagella (Figure 3.6A). As *Dunaliella* does not has a rigid cell wall, it can be relatively easily damaged so not all cells can survive the immobilisation process. An electron micrograph showing a dead cell that burst during the immobilisation process is shown in Figure 3.6B.
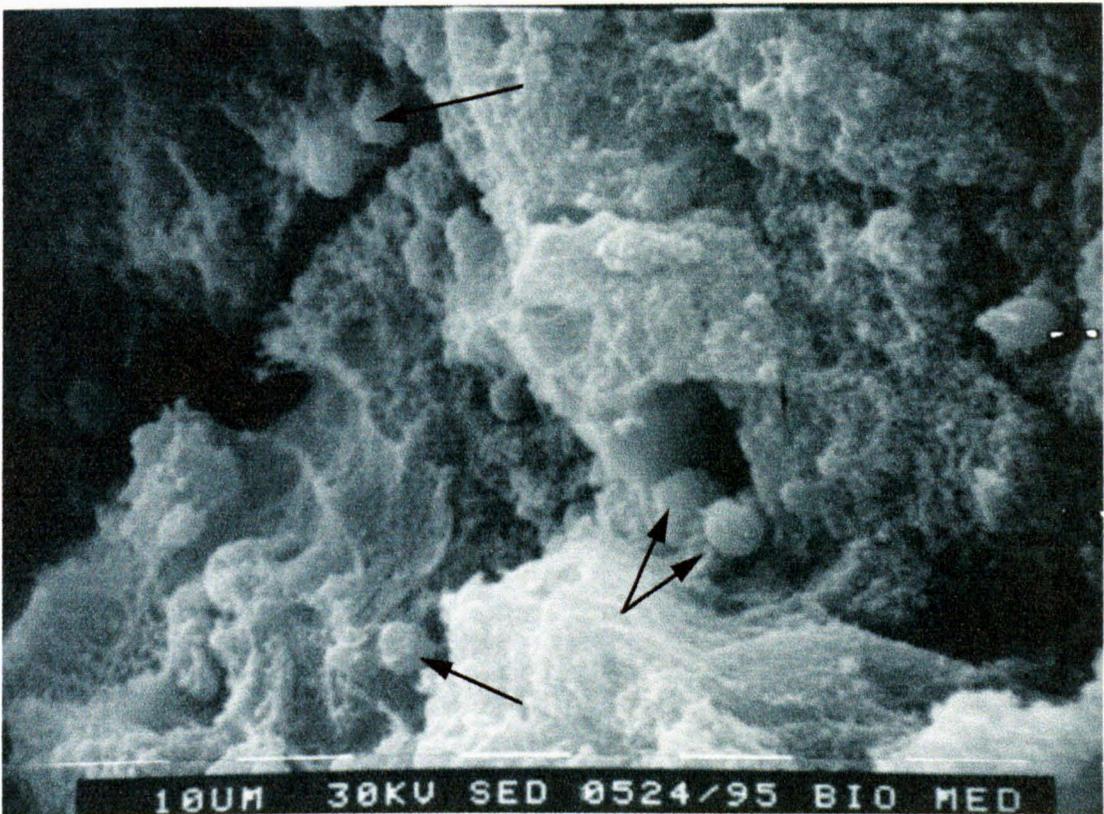
Figure 3.4: Scanning electron micrograph of immobilised *Dunaliella* in calcium alginate bead. The arrows indicate *Dunaliella* cells.
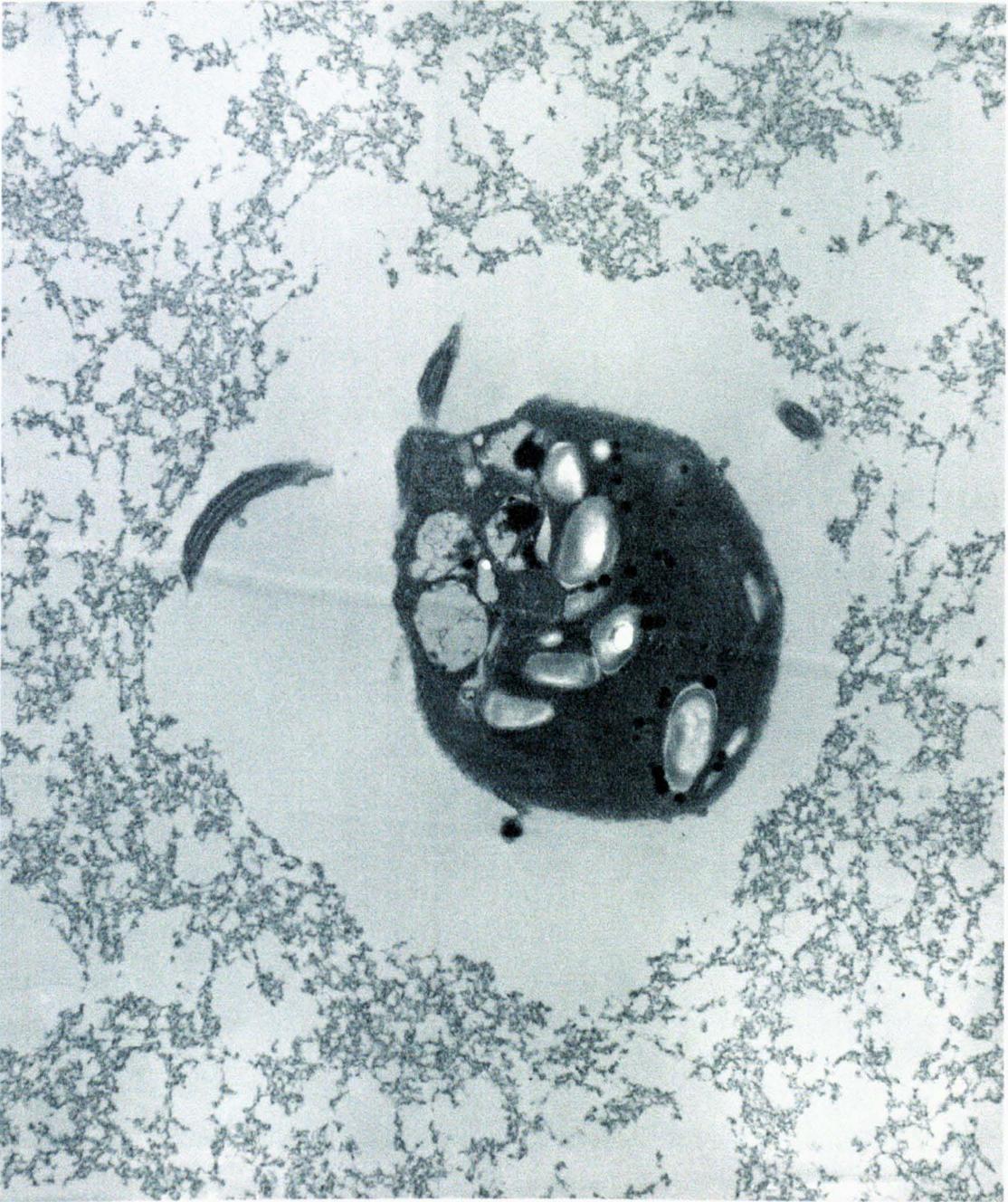
Figure 3.5: Transmission electron micrograph of a newly immobilised *Dunaliella* cell in a calcium alginate bead. At this stage, flagella are still present. (magnification ×14,000)

(A)

(B)
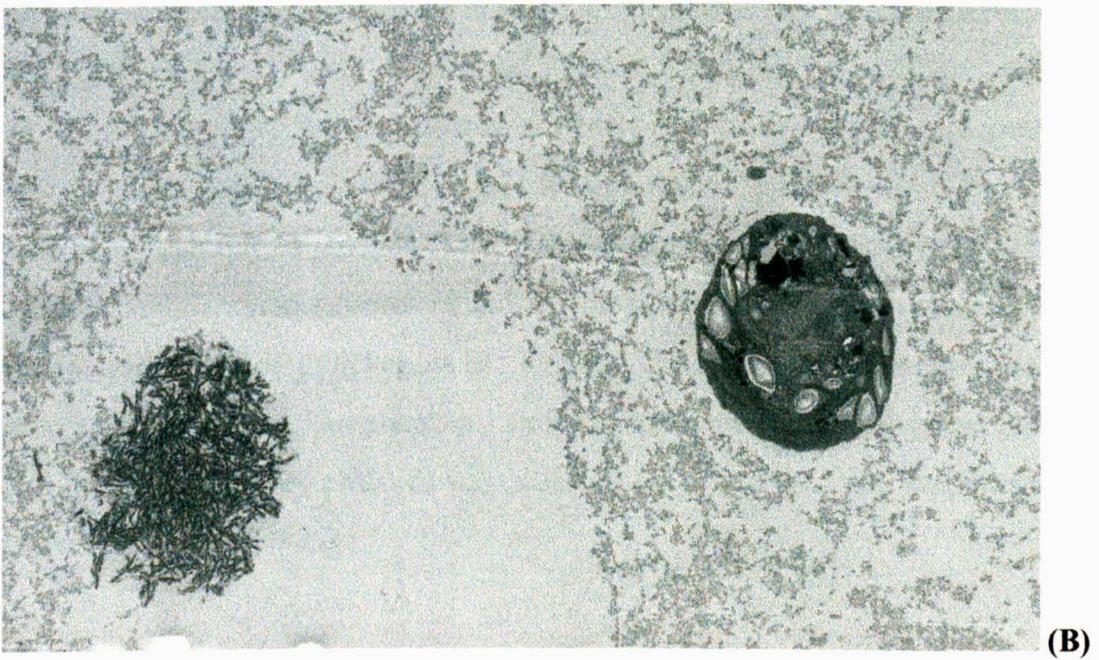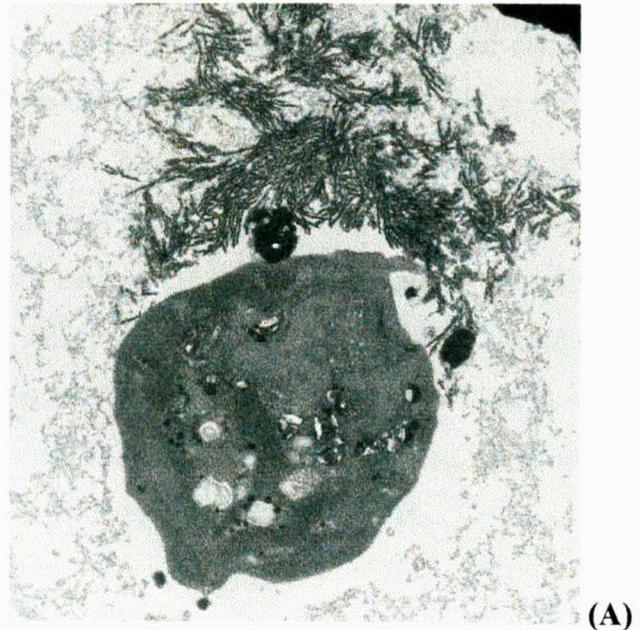
Figure 3.6: **(A)** Possible loss of flagella in newly immobilised *Dunaliella*.
(magnification ×5,700)

(B) Some cells (left) disappear during the immobilisation process. This left a space in calcium alginate structure On the right is a healthy cell.
(magnification ×5,500)

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During culture, growth of *Dunaliella* is seen by increasing cell number and chlorophyll concentration in each cluster. Since *Dunaliella* lacks a rigid cell wall, cells can densely pack together and form ball-shaped clusters. Transmission electron micrograph of a cluster is shown in Figure 3.7. Due to the limitation on the size of a cluster, maximum chlorophyll concentration for each bead was limited too. When algal cells in each cluster divided to the limit, some cells died. Dead cells then burst and finally disappeared (Figure 3.8). This provided some space for a newly divided cell. At this stage, chlorophyll concentration was constant as the dead cells were replaced with new cells. This is one possible explanation of how the immobilised algae can remain metabolically active over a very long culture period.

In other immobilised algal species, rigid cell wall structure kept algal cell morphology normal when growing in beads, this was true for *Chlorella* (Robinson *et al.*, 1986) and *Scenedesmus* (Jeanfils and Thomas, 1986). So, each cell divided into a group of cells rather than a ball-shaped cluster like *Dunaliella* and no report has described cell breakage in beads. In *Chlamydomonas* immobilised in agar beads, cells were found to accumulate close to the surface of the beads after three days of growth. Cellular division occurred under a thin skin of polymer and caused bubbling and a rough surface (Vilchez *et al.*, 1997). If cell growth continued until the stationary phase, the *Chlamydomonas* cells will penetrate through the bead and leak into the medium. This evidence was observed under scanning electron microscope (Santos-Rosa *et al.*, 1989).
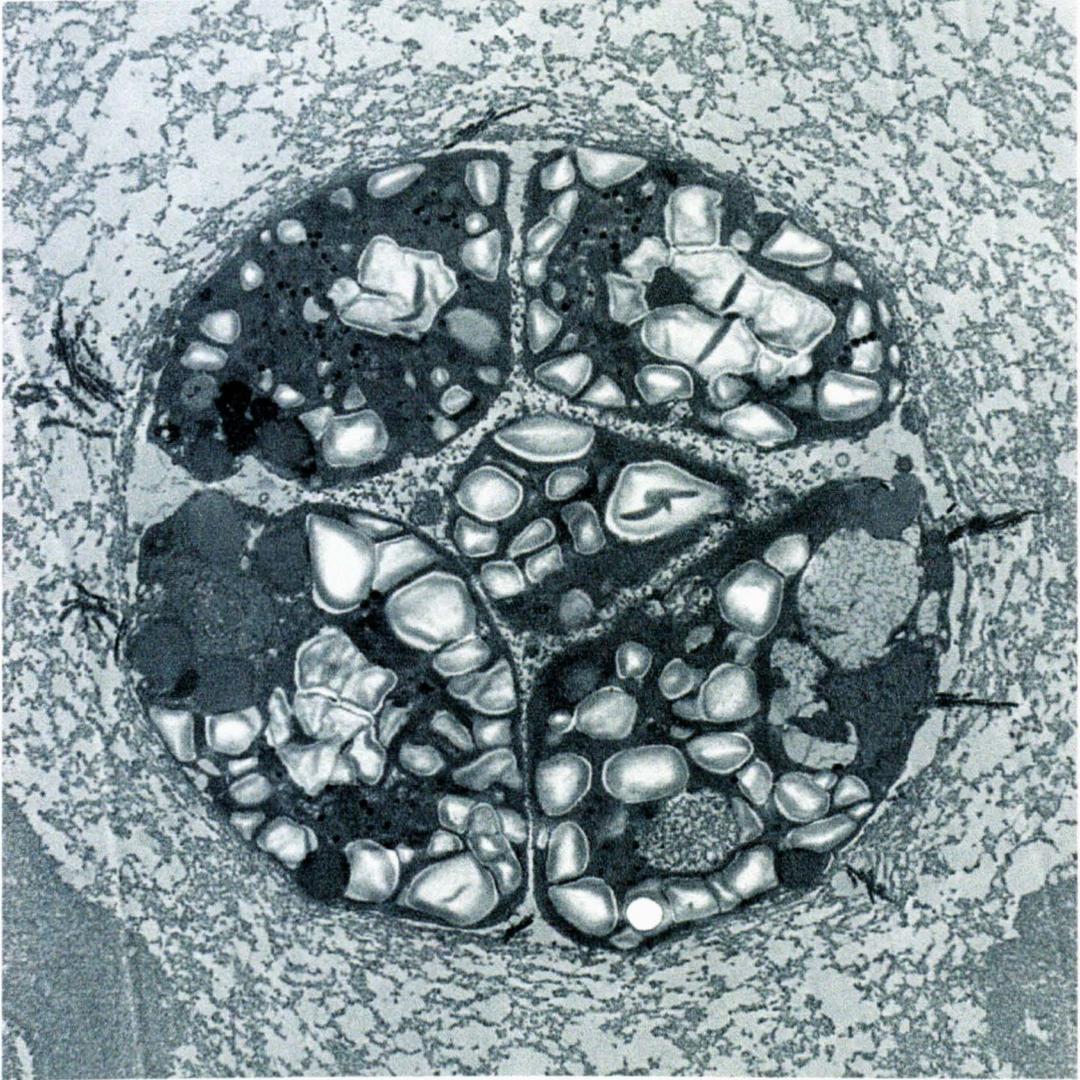
Figure 3.7: Transmission electron micrograph of *Dunaliella* cell cluster.

(magnification ×7,600)
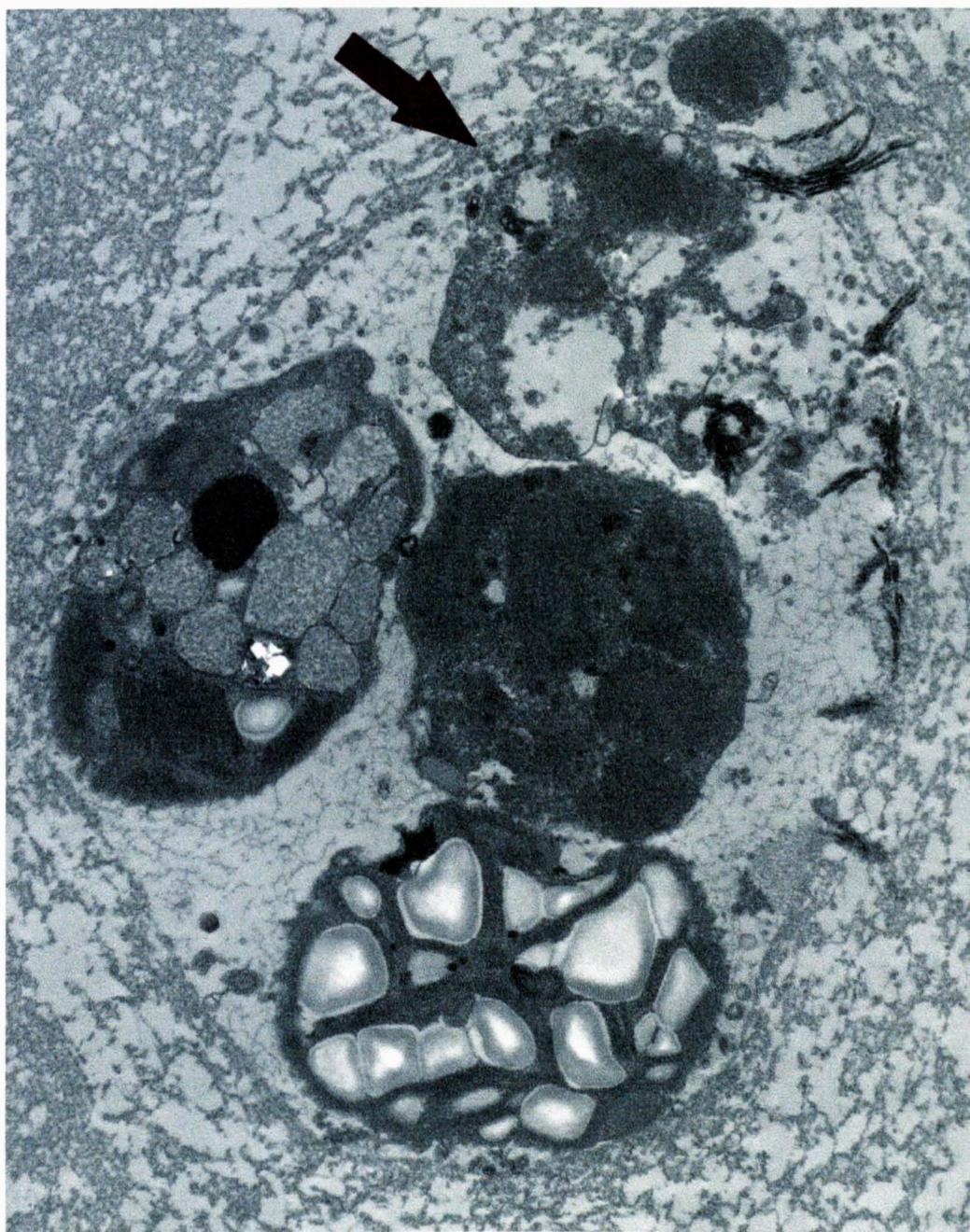
Figure 3.8: Transmission electron micrograph showing cell breakage in the cluster.
The arrow indicates the broken cell.
(magnification ×14,000)

Photosynthetic oxygen evolution and dark oxygen uptake were used to examine cellular activity in the beads during the culture period. In this experiment, 5 beads suspended in 2 ml medium were used instead of the normal 2 ml of liquid culture. Oxygen evolution and oxygen uptake, from two different initial cell concentrations, are shown in Figures 3.9 and 3.10 respectively. It was found that there was no difference in the pattern of photosynthesis for different cell densities. Oxygen evolution rate was high during the early period of cultivation then gradually decreased until day 20, it then remained constant to the end of the experiment (day 55). This is in contrast with chlorophyll concentration which increased during the first 20 days (see Figure 3.1). Oxygen uptake (Figure 3.10) showed a different pattern, it remained relatively constant through out the culture period. Reduction of oxygen evolution indicates a decrease in photosynthesis efficiency. The explanation is that cell clusters near the surface can cause self shading by reduced light passing to inner clusters in the middle of the bead. This causes a reduction of photosynthesis per chlorophyll unit. Vilchez and Vega (1995) also reported the reduction of photosynthesis in immobilized *Chlamydomonas*. They found that entrapped *Chlamydomonas* cells in calcium alginate beads accumulated at the surface of the beads.

Oxygen evolution rate from immobilised *Dunaliella* in this experiment was lower than that reported in other papers such as Tamponnet *et al.* (1985) and Day and Codd (1985). This was because the light intensity in oxygen electrode chamber, normally used for free cell cultures, did not reach the saturation light intensity for beads. Hence, various light intensities in the oxygen electrode chamber were tested using a combination of neutral density filters to determine a light saturation curve (Figure 3.11). Light intensity of 600 $\mu E.m^{-2}.s^{-1}$, which is the maximum intensity achieved from fibre optic light source (maximum intensity of 750 $\mu E.m^{-2}.s^{-1}$ can be achieved without fibre optic) gave an acceptable saturation point and was then used for the following experiments.
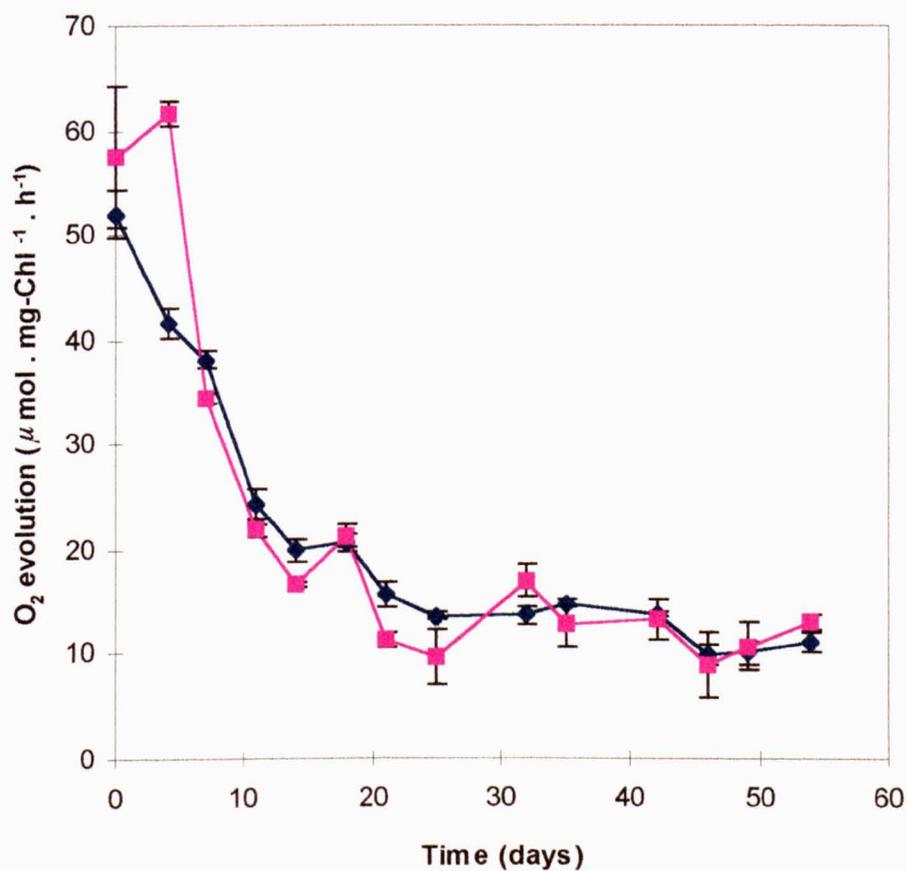
Figure 3.9: Oxygen evolution from calcium alginate immobilised *Dunaliella*.

($\blacklozenge$) High initial chlorophyll concentration (7.07 ± 0.3 μg.5 beads^{-1})

($\blacksquare$) Low initial chlorophyll concentration (3.2 ± 0.1 μg.5 beads^{-1})
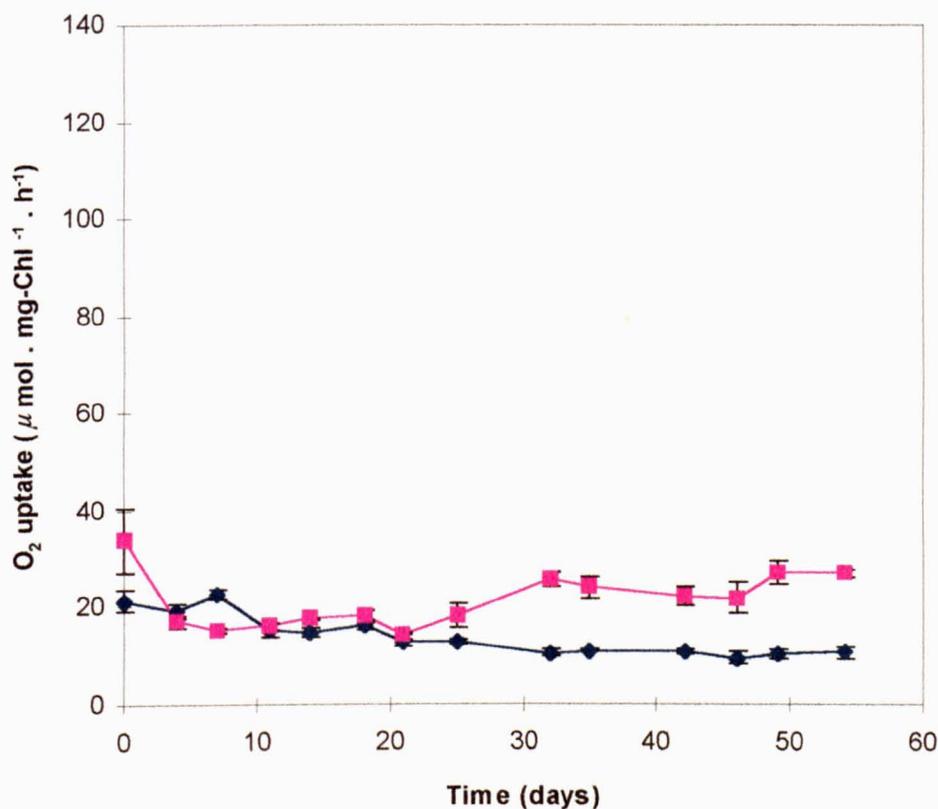
Figure 3.10: Oxygen uptake from calcium alginate immobilised *Dunaliella*.

($\blacklozenge$) High initial chlorophyll concentration (7.07 ± 0.3 µg.5 beads^{-1})

($\blacksquare$) Low initial chlorophyll concentration (3.2 ± 0.1 µg.5 beads^{-1})
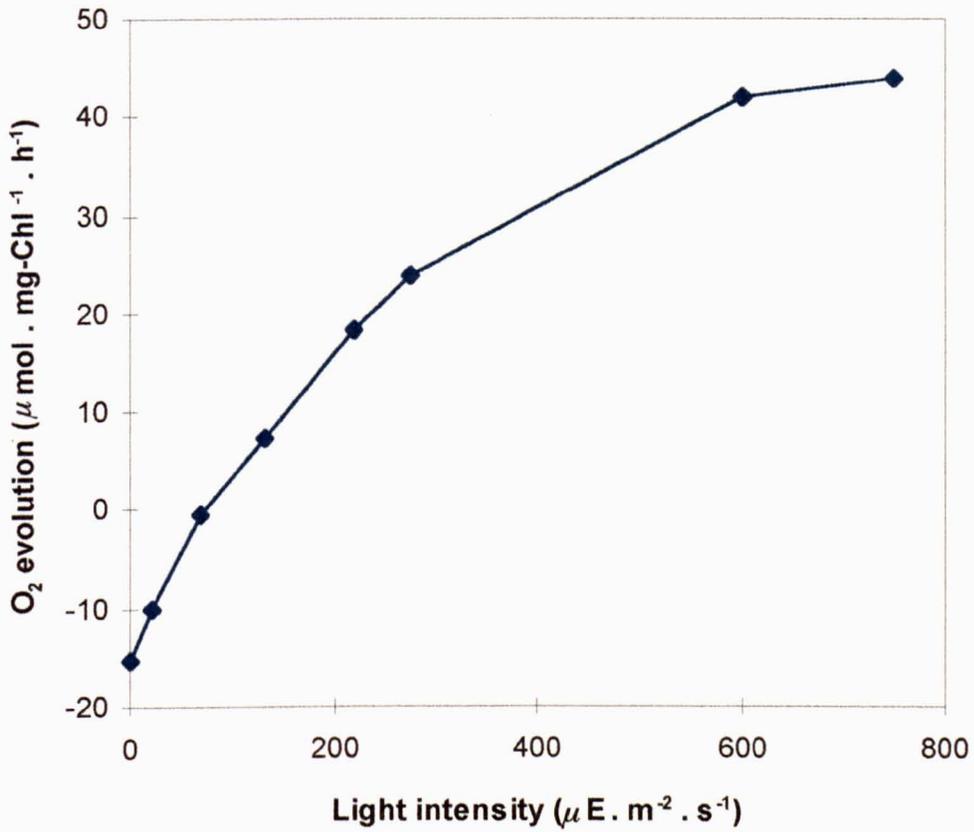
Figure 3.11: Light saturation curve for oxygen evolution from immobilised *Dunaliella* in an oxygen electrode chamber. Five beads were resuspended in 2 ml of 1.5 M NaCl medium.

3.2.2 Growth, photosynthesis and glycerol production from free and immobilised *Dunaliella*

These experiments aim to compare basic physiology and glycerol production from free cells and immobilised cells. Growth of both free cells and immobilised cells was determined by chlorophyll concentration. Initial chlorophyll concentrations were 5.27 ± 0.6 $\mu g.5$ beads^{-1} (5 beads.ml^{-1} medium) for immobilised cells and 4.16 ± 0.1 $\mu g.ml^{-1}$ for free cells culture. During the experiment, photosynthetic oxygen evolution, oxygen uptake, chlorophyll fluorescence and glycerol concentration in culture medium were measured.

The experiment was originally planned to be a comparison of glycerol production from wild type strain and glycerol leaking mutant strain of *D. parva* isolated by Hard and Gilmour (1991). However, after the experiment was completed, it was found that there was no difference in growth and other physiological parameters between the two strains. Therefore, raw data from both wild type and mutant strains were combined and the results are reported as a comparison of free cells and immobilised cells.

Figure 3.12 shows the growth curve of *Dunaliella*. It was found that immobilised *Dunaliella* had much higher chlorophyll concentration. Free cells culture seemed to enter stationary growth phase after 10 days while immobilised cells kept growing for up to 40 days. In fact, immobilised *Dunaliella* can be loaded with much higher density per unit volume by increasing cell concentration in each bead and increasing number of beads per ml of the medium. For example, this experiment used only 5 beads per ml while it is possible to load up to 15-20 beads per ml. For free cells, however, it must be remarked that growth curve of free cell culture did not show a rapid increase in chlorophyll because the initial chlorophyll concentration used in this experiment is almost the maximum chlorophyll level found in normal culture grown without mixing.
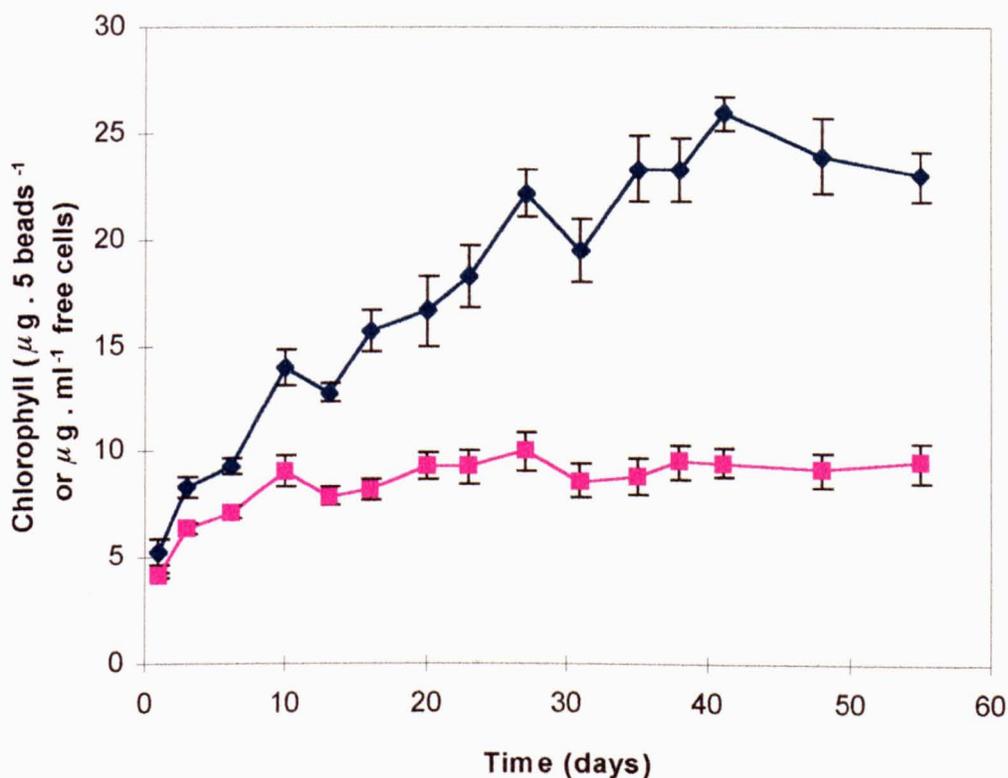
Figure 3.12: Chlorophyll concentration in immobilised and free cells of *Dunaliella*.

($\blacklozenge$) Immobilised cells

($\blacksquare$) Free cells

Self shading in beads reduced the amount of light that passed through to the growing cells inside. This can decrease the amount of photosynthetic oxygen evolution. To investigate this the rate of oxygen evolution was plotted against light intensity (Figure 3.13). At the highest light intensity (approximately 600 $\mu E.m^{-2}.s^{-1}$), immobilised

Dunaliella have lower oxygen evolution than free cells which had not yet reached the saturation point. Unfortunately, it was not possible to obtain any higher light intensity from this light source so 600 $\mu E.m^{-2}.s^{-1}$ had to be used as the saturating light intensity for both free and immobilised cells.
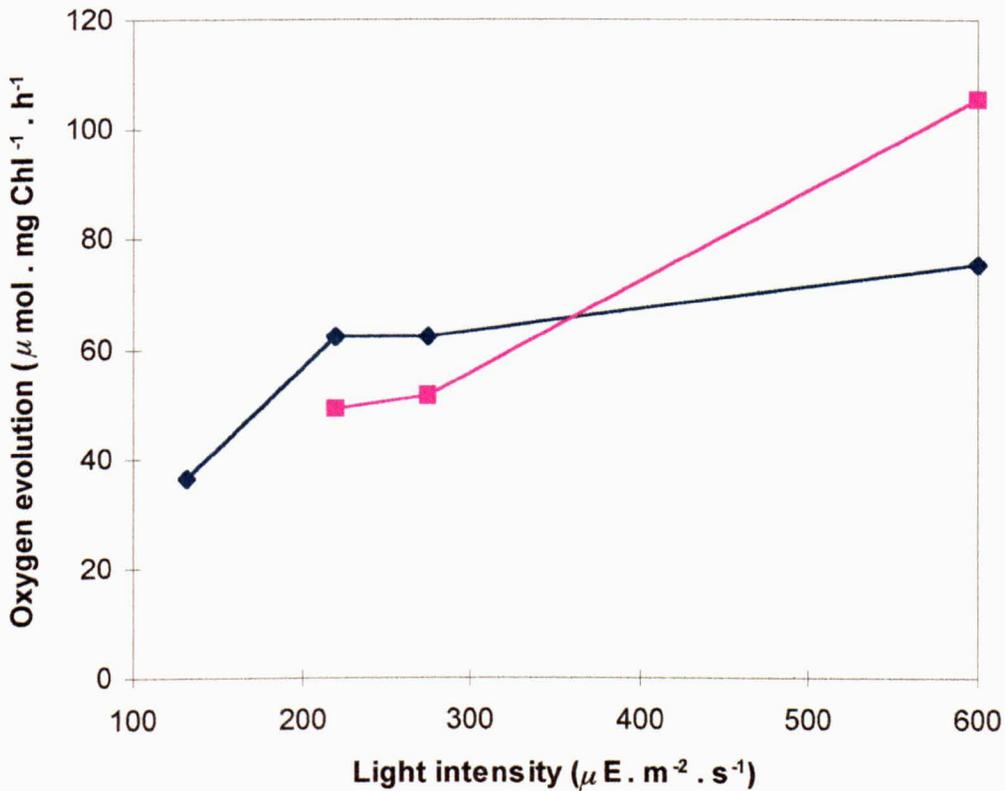
Figure 3.13: Oxygen evolution from immobilised and free cells of *Dunaliella* in different light intensities.

(◆) Immobilised cells

(■) Free cells

Comparison of photosynthetic oxygen evolution from free cells and immobilised cells is shown in Figure 3.14. Free cells had higher oxygen evolution during the initial period of culture. When they reached stationary phase, after approximately 10 days, oxygen evolution rate was constant. In contrast, immobilised cells showed reduced oxygen evolution as the chlorophyll concentration increased. This indicated that old beads, which showed a lower photosynthetic rate, seemed to have some problems with self shading. In calcium alginate immobilised *Chlorella*, oxygen production rate varied with cell density and it can be enhanced by increasing light intensity (Adlercreutz and Mattiasson, 1982; Robinson *et al.*, 1986). This also suggests that immobilised cell culture systems need higher light intensity than normal free cell cultures.

On the other hand, during the first 30 days of the experimental period, oxygen uptake rate was constant and there was no difference between free cells and immobilised cells (Figure 3.15). After 30 days, free cells had higher oxygen uptake rate indicating higher cell activity. This also happened with *Chlorella* (Robinson *et al.*, 1985), where it was found that free cells had greater respiration rate than immobilised cells.

Potential yield of the photochemical reaction of photosystem II (Fv/Fm), measured by chlorophyll fluorescence, can be used to indicate stress in *Dunaliella* cells. Figure 3.16 shows that Fv/Fm ratio declined during the growth period for both free and immobilised cells. It was found that free cell culture had less stress indicated by higher Fv/Fm ratio. For immobilised cells, Fv/Fm ratio of approximately 0.56 at day 55 was much lower than the ratio found in healthy plant leaves which is around 0.83 (Hall and Rao, 1994). However, Fv/Fm ratio in microalgae is usually found to be lower than in higher plants. For example, Gilmour *et al.* (1984b) stated that Fv/Fm in *D. tertiolecta* varied from 0.60 - 0.71. Reduction of Fv/Fm reveals significant stress in cellular physiology of aging beads and the result shows the same trend as photosynthetic oxygen evolution (see Figure 3.14). Although Fv/Fm ratio can be used as an indicator for stress physiology, these results can only indicate stress from the

cells growing near the surface of the beads where chlorophyll fluorescence was emitted.
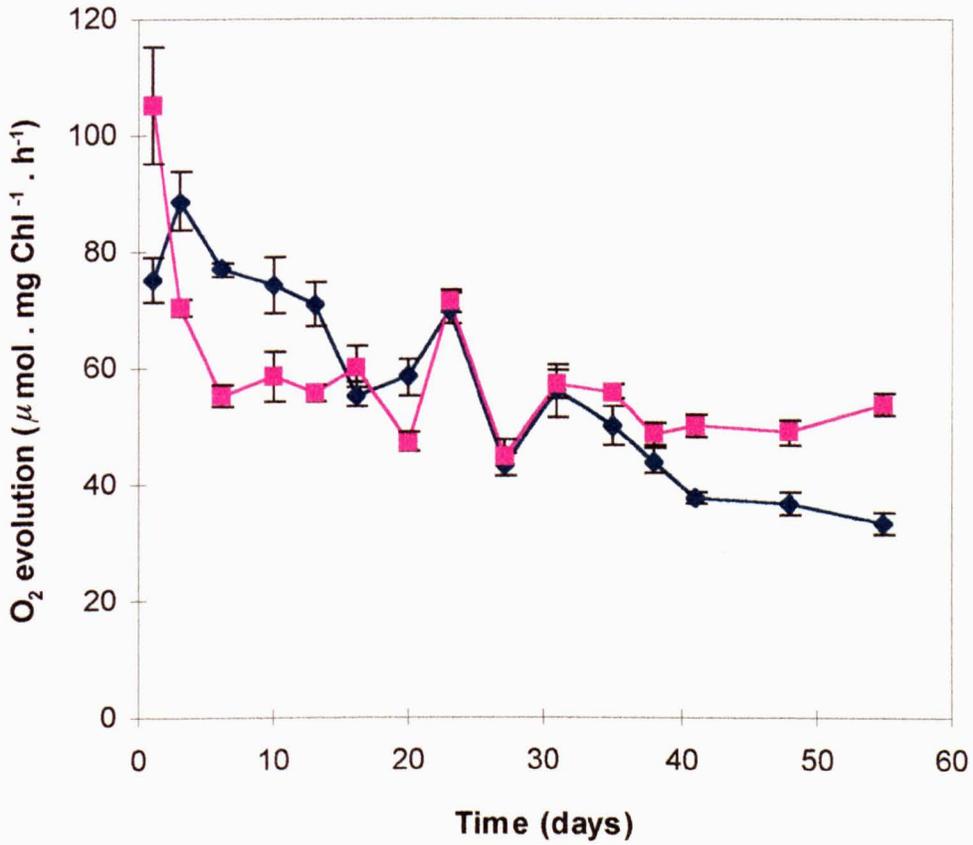
Figure 3.14: Oxygen evolution from immobilised and free cells of *Dunaliella*.

      (◆) Immobilised cells
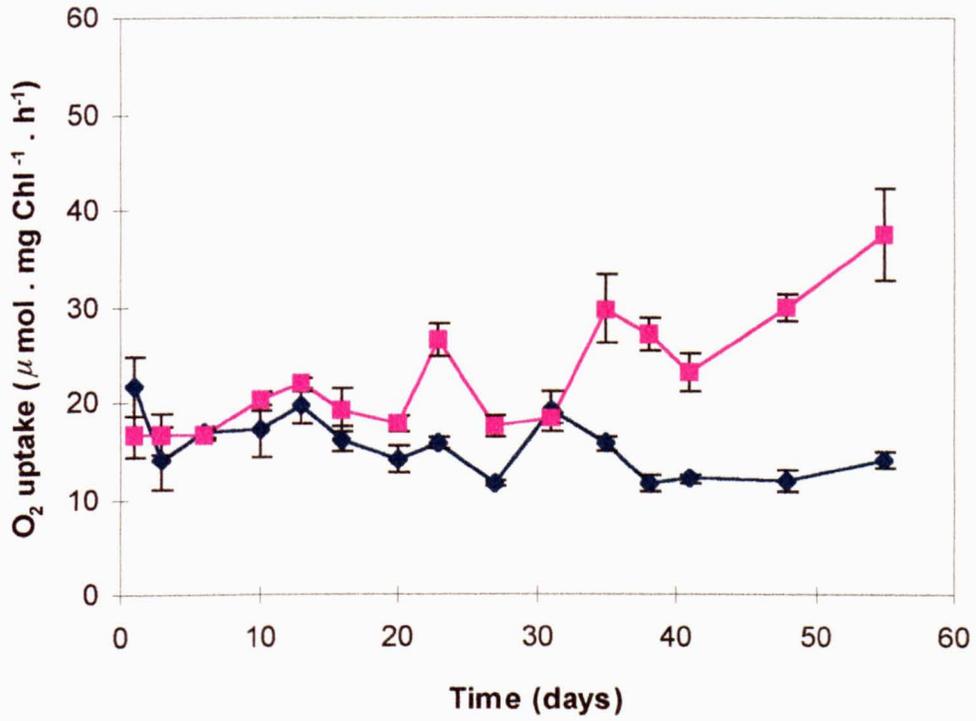
      (■) Free cells

Figure 3.15: Oxygen uptake by immobilised and free cells of *Dunaliella*.

      (◆) Immobilised cells
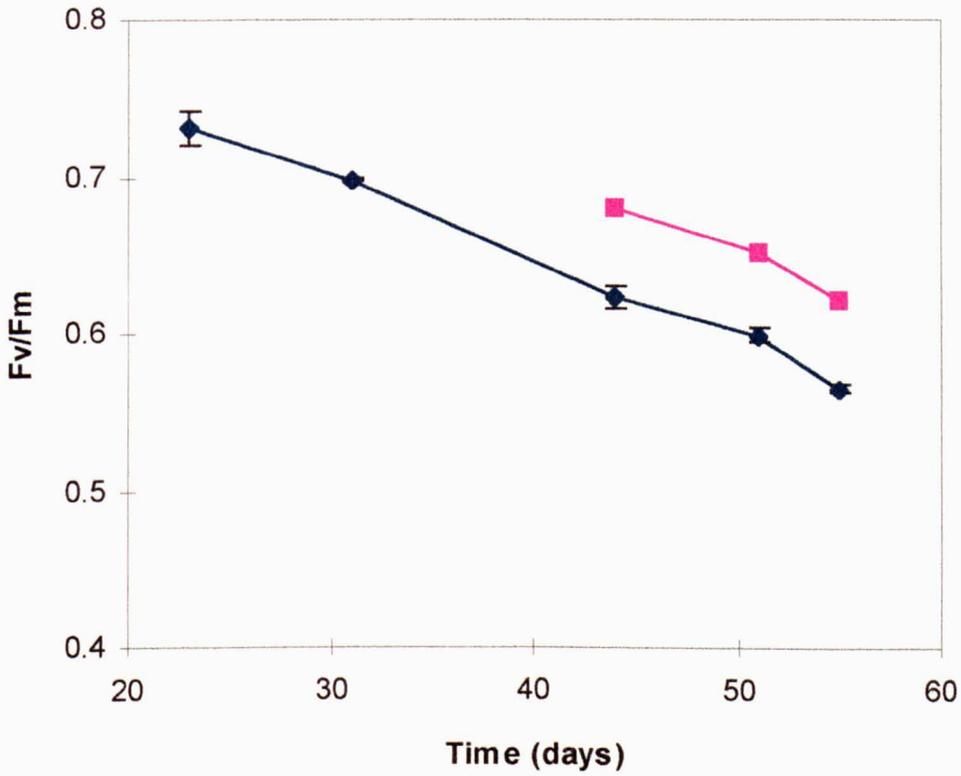
      (■) Free cells

Figure 3.16: Potential yield of the photochemical reaction of photosystem II (Fv/Fm ratio) from immobilised and free cells of *Dunaliella*.

(◆) Immobilised cells

(■) Free cells

Glycerol was released into the medium from both free and immobilised cells (Figure 3.17), but immobilised cells released more glycerol than free cells. Glycerol concentration was high during stationary growth phase indicating that it might be released from dead cells or be released as a excess product when most energy from photosynthesis was not used for growth. Nevertheless, immobilised cells which had three times higher chlorophyll concentration than free cells (Figure 3.12) had only double the glycerol concentration in the medium. The explanation might involve the glycerol gradient between cells and medium, the higher the glycerol concentration in the medium the lower the amount of glycerol released.

Excretion of glycerol from free and immobilised *Dunaliella* showed the same pattern as *Chlamydomonas* (León and Galván, 1994) although the results from the present experiment show higher glycerol excretion presumably due to the different organism used and higher salinity culture. León and Galván (1994) suggested that *Chlamydomonas* was unable to retain very high glycerol concentration in the cells and the majority of glycerol produced was excreted. They quoted the study from Wegmann *et al.*(1980) who suggested that *Dunaliella* does not excrete glycerol because its unusual membrane characteristics retain glycerol in the cells. The present work shows that *Dunaliella* excreted large amounts of glycerol in to the medium and it has the same excretion characteristic as *Chlamydomonas*. This indicates a possibility of producing glycerol from living *Dunaliella* cells.

3.2.3 Effect of bead size, self shading and bead packing on glycerol production

3.2.3.1 Effect of bead size on cellular physiology and glycerol release

Cellular physiology of immobilised *Dunaliella* cells is strongly affected by light intensity. Cells growing inside the bead receive less light intensity than cells growing near the surface. One possibility to reduce the effect from self shading is to reduce

the size of beads. This can be done by using a smaller nozzle diameter during beads formation step (see section 2.5). Smaller beads are also expected to have better oxygen, carbon and nutrient exchange between immobilised cells and culture medium.
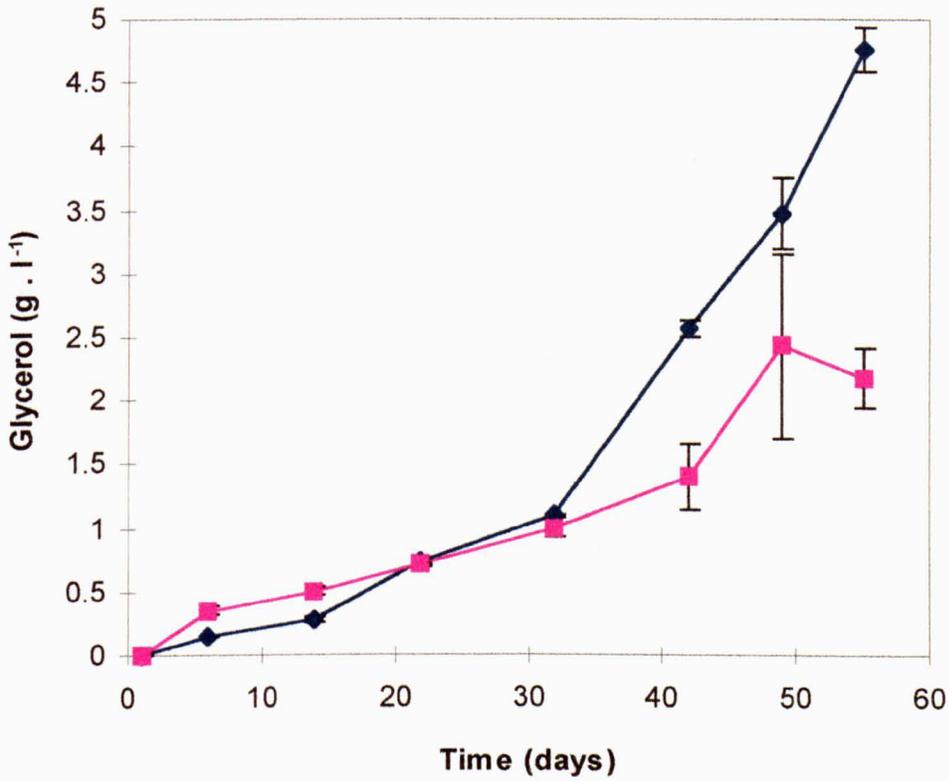
Figure 3.17: Glycerol concentration in the medium of immobilised and free cells of *Dunaliella*.

    (♦) Immobilised cells

    (■) Free cells

Another consideration is that, when beads are placed in a flask or test tube without mixing, small beads will pack densely and this factor may reduce light passing to some beads in the middle. This is the "bead packing effect". In theory, to minimise bead packing, a thin layer of beads is the best solution but it may not be practical in a real culture situation when surface area is limited by size of the reactor.

In order to compare basic physiology of *Dunaliella* immobilised in large and small beads, 200 large beads for each replicate (4.34 ± 0.4 mm in diameter) or 900 small beads (2.89 ± 0.27 mm in diameter) were placed in 20 ml of 1.5 M NaCl *Dunaliella* medium in 125 ml flasks. The experiment was conducted in 25°C growth room with 65 $\mu E.m^{-2}.s^{-1}$ light intensity. Chlorophyll, photosynthesis, and glycerol release were measured throughout 42 days of the experimental period.

Figure 3.18 shows the growth curves of large and small beads measured by chlorophyll concentration of 5 large beads or 25 small beads. It was found that no difference in growth was detected. Increasing chlorophyll concentration showed the same pattern for large and small beads. On the other hand, for photosynthetic oxygen evolution, both large and small beads showed a reduction in oxygen evolved, but small beads had higher oxygen evolution rate during the first 22 days (Figure 3.19). However, after day 22, large beads seemed to have higher photosynthesis activity. Figure 3.20 shows that there was no difference in oxygen uptake rate between large and small beads.

Light intensity used in the oxygen electrode system was assumed to provide a saturated intensity for large beads (see Figure 3.11). Significant difference in oxygen evolution from large and small beads was the predicted result, but this was not evident from the results (Figure 3.19). This might be an effect of light intensities during oxygen evolution measurement which, in this experiment, 5 large beads or 25 small beads were placed in the chamber for each test. 25 small beads seemed packed in the chamber and magnetic bar stirrer might not work so effectively. It was not

possible to reduce the number of small beads used for oxygen evolution measurements because of the need for a sufficient chlorophyll concentration for chlorophyll analysis.
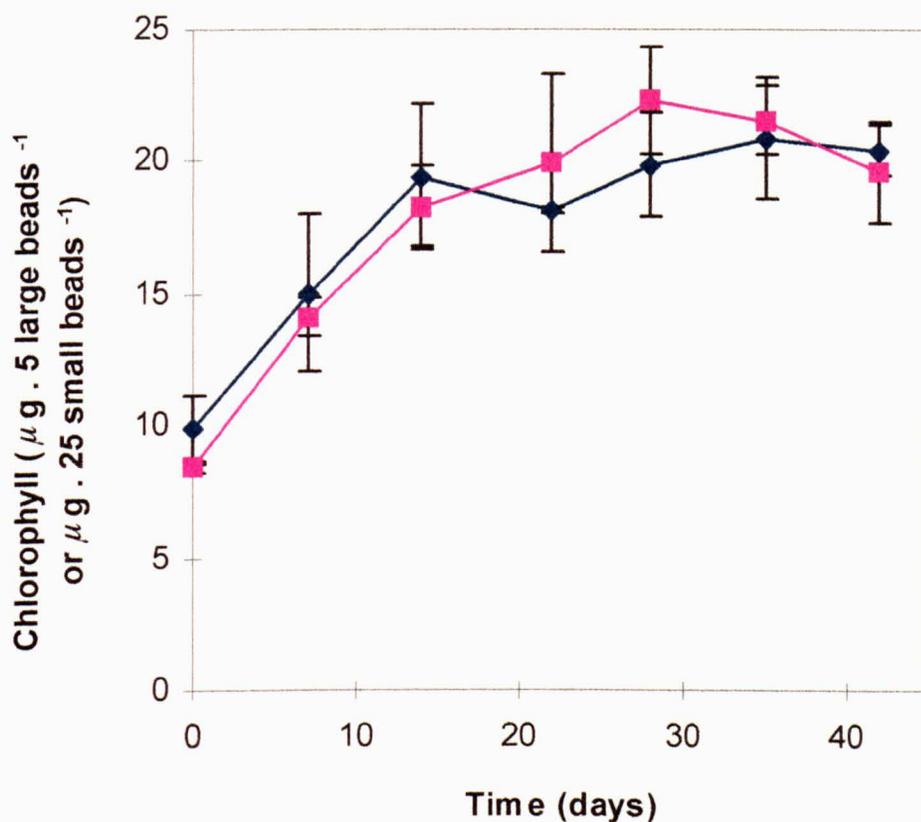
Figure 3.18: Chlorophyll concentration of *Dunaliella* immobilised in large and small calcium alginate beads.

(◆) Large beads

(■) Small beads
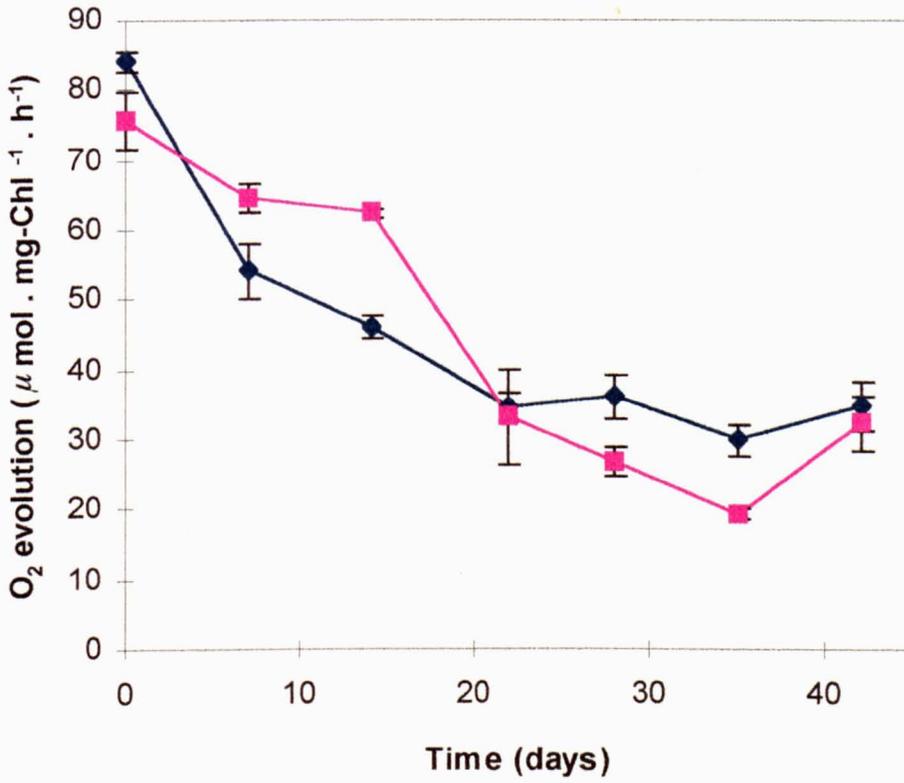
Figure 3.19: Oxygen evolution from large and small beads of immobilised

Dunaliella.

(◆) Large beads
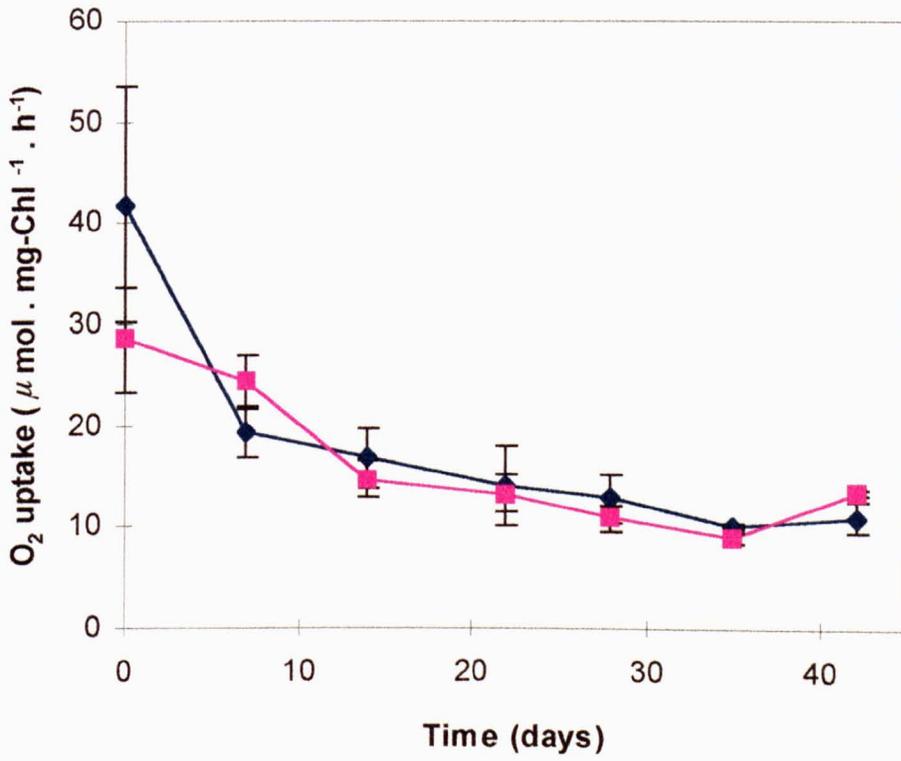
(■) Small beads

Figure 3.20: Oxygen uptake in large and small beads of immobilised *Dunaliella*.

（◆）Large beads

（■）Small beads

Figure 3.21 shows chlorophyll fluorescence from large and small beads. No difference in Fv/Fm ratio for large and small beads was found in this experiment. Decreasing Fv/Fm suggests that cells became more stressed after a period of time. However, Fv/Fm was in the range of 0.7 - 0.8 which suggests that cells were in a healthy condition throughout the experiment.
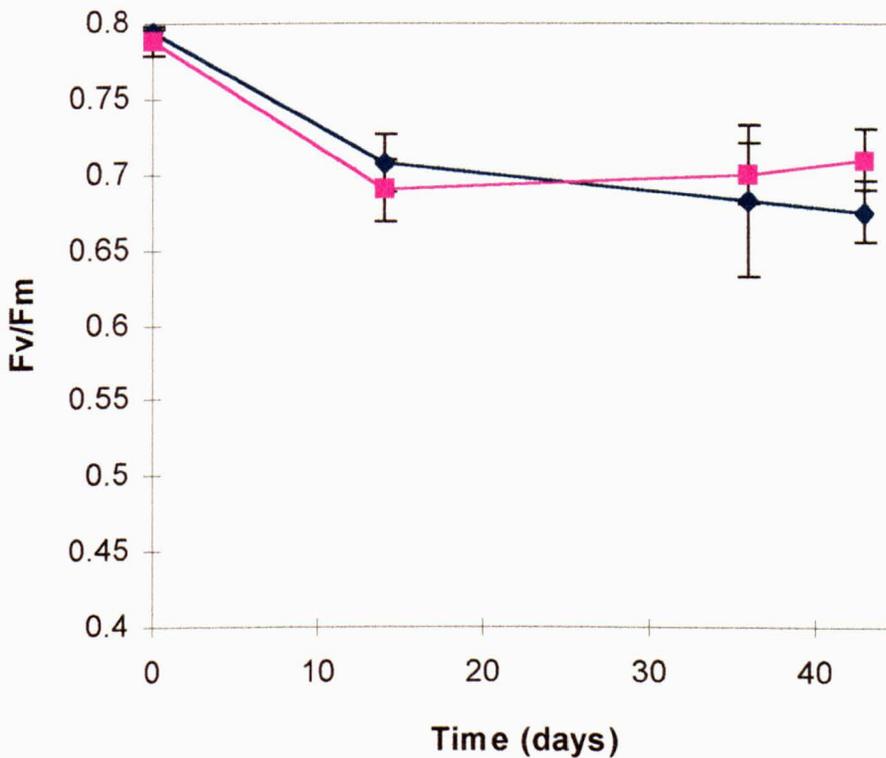
Figure 3.21: Potential yield of the photochemical reaction of photosystem II (Fv/Fm ratio) from large and small beads of immobilised *Dunaliella*.

($\blacklozenge$) Large beads

($\blacksquare$) Small beads

Excretion of glycerol into the medium (Figure 3.22) was not different for large and small beads. This means that bead sizes ranging from 2.8 to 4.3 mm do not exhibit any difference in cellular physiology and glycerol excretion. Nevertheless, it cannot be stated that there is no effect of packing or self shading. The following experiment was carried out to study the effect of packing density on glycerol production.
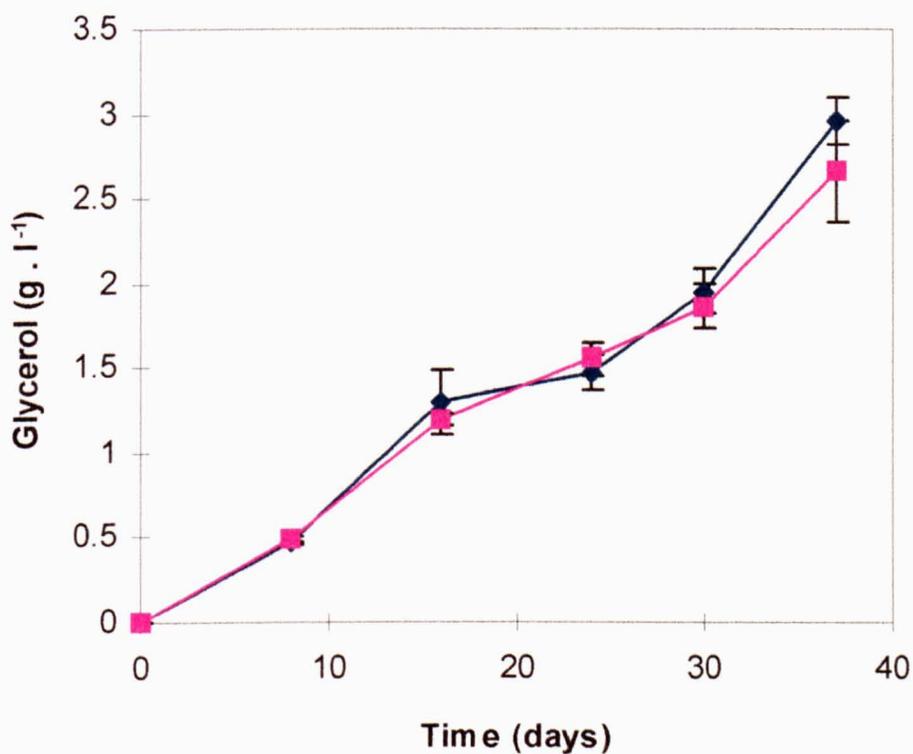
Figure 3.22: Glycerol concentration in the medium of large and small beads of immobilised *Dunaliella*.

      (◆) Large beads

      (■) Small beads

3.2.3.2 Effect of bead packing on glycerol release

20 g wet weight of large and small beads (4.56 ± 0.26 mm and 2.66 ± 0.17 mm in diameter respectively) were placed in 2.4 cm diameter × 25 cm length test tubes containing 15 ml of 1.5 M NaCl *Dunaliella* medium with HEPES buffer. Test tubes was placed at a slant under a white fluorescent lamp (65 $\mu E.m^{-2}.s^{-1}$) at 25°C. Glycerol in the medium was measured by acetylacetone method (see section 2.8.3). To reduce the effect of nutrient depletion, medium was replaced with fresh medium at day 21, 28, 35 and 42. From day 0 to day 35, culture tubes were placed on the rack with an approximately 30 degree slant. Thereafter, packing effect was changed by tilting the tubes to a different angle (almost horizontal) this increased the possibility of beads being exposed to light i.e. the bead packing effect was reduced. Diagram illustrating this procedure is shown in Figure 3.23.

It was found that large beads excreted significantly higher amounts of glycerol into the medium during the first 35 days of culture (Figure 3.24). Semi-continuous harvesting at day 21 and 28 showed the same trend that significantly (P<0.05) higher amounts of glycerol were found in large beads culture. However, at day 35, culture tubes were tilted to approximately 10 degrees from horizontal. This made bead layers became thinner and packing effect for small beads was reduced. Glycerol production was found to increase for both sizes and now there was no difference in glycerol excretion from large and small beads (Figure 3.24). This result suggests that, as glycerol is produced by photosynthetic process, bead packing can reduce photosynthesis and cause less glycerol to be produced. When packing effect was reduced after day 35, small beads could produce glycerol in the same amount as large beads. Chlorophyll fluorescence results in Figure 3.25 show that there is no difference in Fv/Fm for both large and small beads during the experimental period, indicating that *Dunaliella* cells were in the same stress condition.
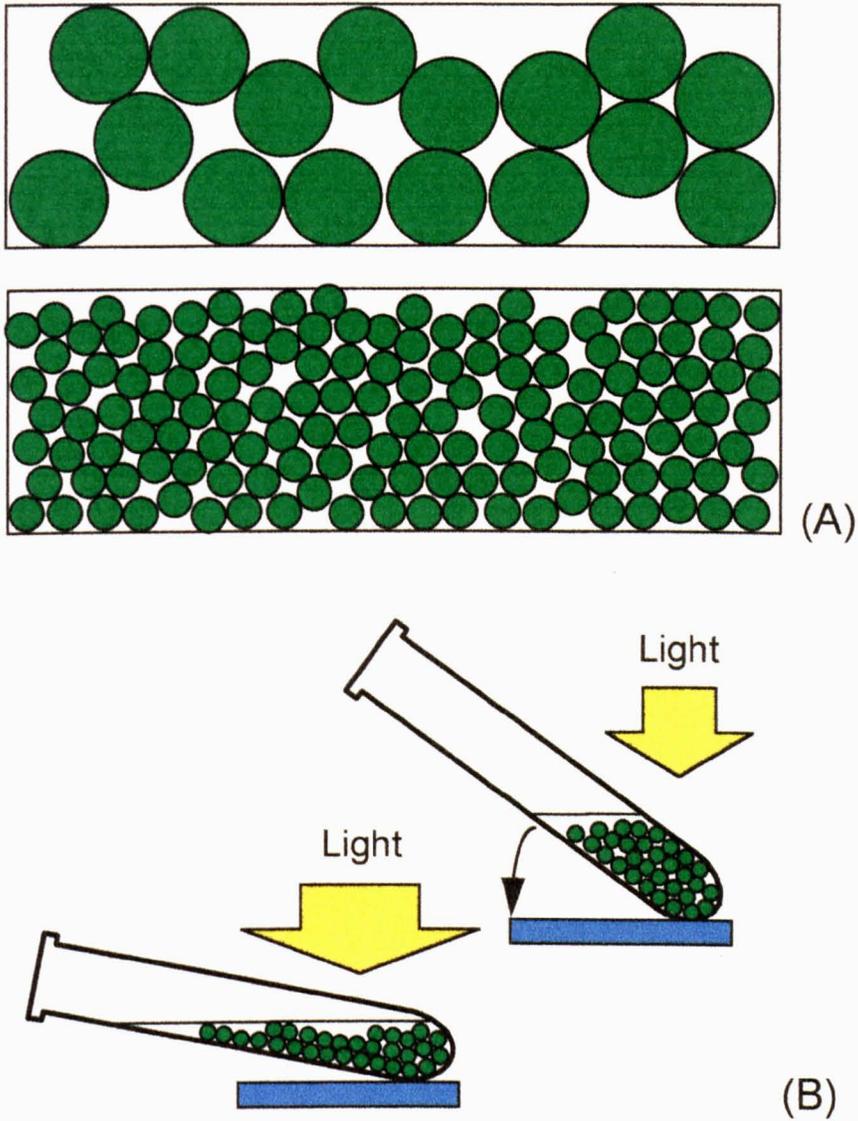
Figure 3.23: **(A)** Packing of large and small beads. Small beads were found densely packed and this reduces light passing through to the inside. **(B)** Reduction of packing effect by tilting the test tubes to a different angle.
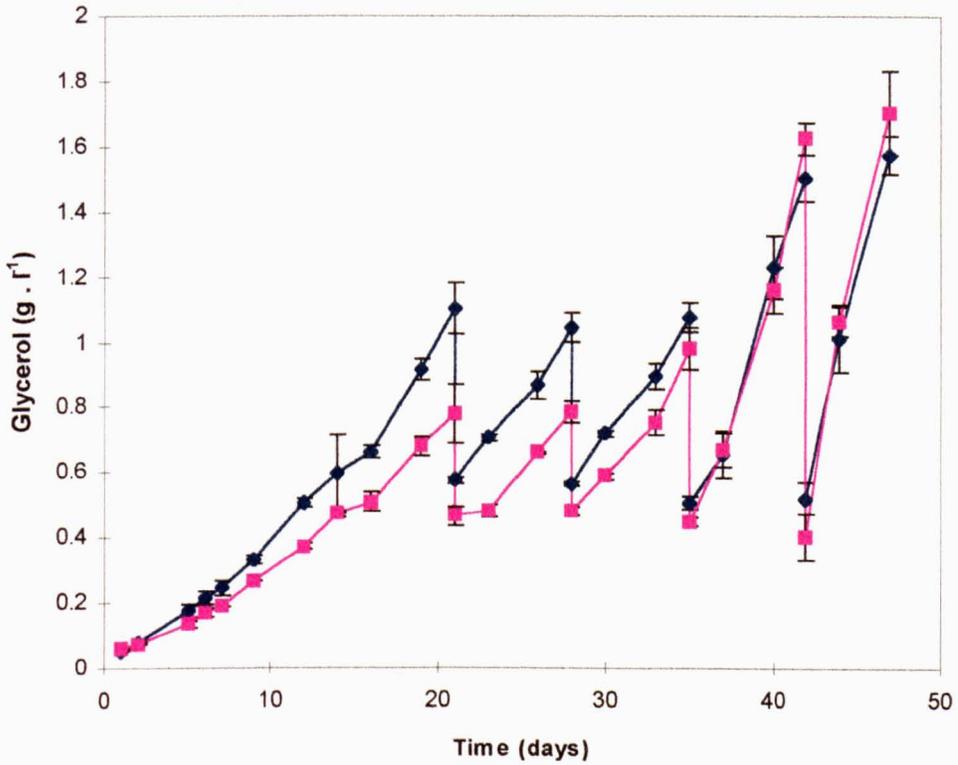
Figure 3.24: Glycerol concentration in culture medium of large and small beads of immobilised *Dunaliella*. Medium was semi-continuously harvested on day 21, 28, 35 and 42. After day 35, slant angle of the culture tubes was changed from 30 degrees to 10 degrees to reduce packing effect.

    (◆) Large beads

    (■) Small beads
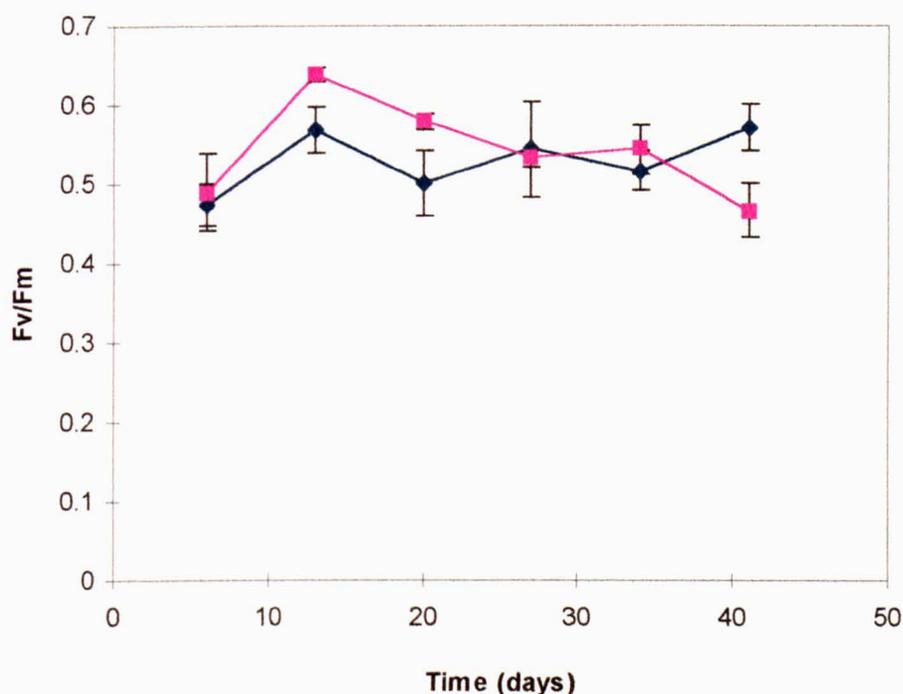
Figure 3.25: Potential yield of the photochemical reaction of photosystem II (Fv/Fm

ratio) from large and small beads of immobilised *Dunaliella* during semi-

continuous harvesting.

(◆) Large beads

(■) Small beads

In this experiment, large beads had a greater variation in chlorophyll concentration (Figure 3.26). This may be due to the sampling error because only 5 large beads (compared to 25 small beads) were sampled each time for chlorophyll determination. However, oxygen evolution and oxygen uptake rate showed the same trends for both large and small beads (Figures 3.27 and 3.28, respectively). It needs to be stated that the results from photosynthesis studies cannot directly represent the photosynthetic

activity of immobilised algae growing *in situ*. This is because beads had to be taken out from culture tubes to measure oxygen evolution and oxygen uptake in an oxygen electrode. Therefore, photosynthesis results can be used only to indicate that *Dunaliella* cells entrapped in calcium alginate beads are still metabolically active throughout the experimental period.
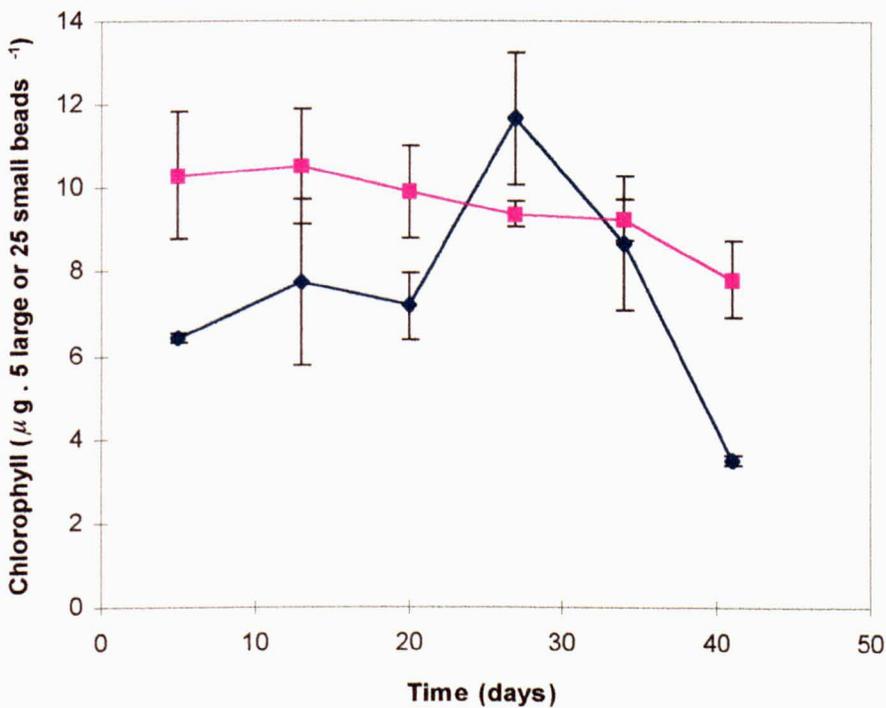
Figure 3.26: Chlorophyll concentration in large and small beads of immobilised
Dunaliella during semi-continuous harvesting.

(♦) Large beads
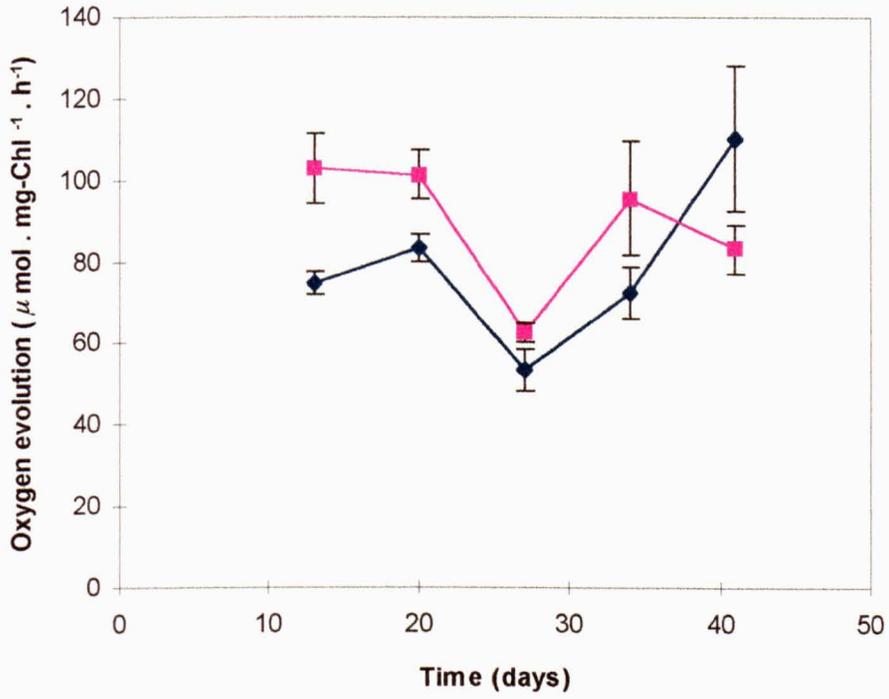
(■) Small beads

Figure 3.27: Oxygen evolution from large and small beads of immobilised

Dunaliella during semi-continuous harvesting.

(♦) Large beads
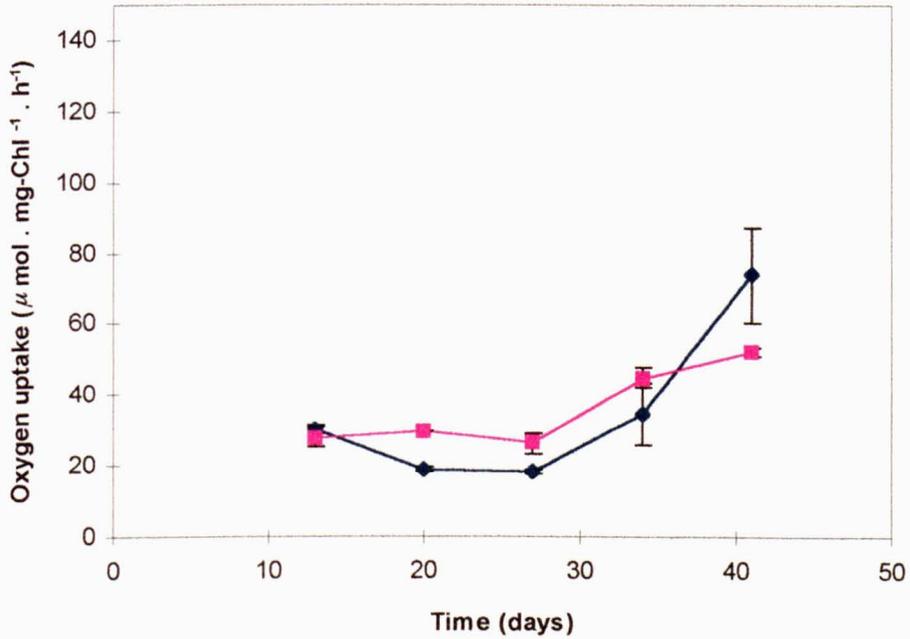
(■) Small beads

Figure 3.28: Oxygen uptake in large and small beads of immobilised *Dunaliella*
during semi-continuous harvesting.

     (◆) Large beads

     (■) Small beads

3.2.4 Immobilisation of *Dunaliella* in chitosan beads

Calcium alginate bead structure can be degraded by phosphate present in the medium. Although this effect can be reduced by resuspending cells in phosphate free medium during bead formation, it cannot be avoided as beads have to be placed in algal medium which contains phosphate. Chitosan beads, which are inexpensive to produce and tolerate high salinity and high phosphate medium (Vorlop and Klein, 1987), were tested as an alternative to calcium alginate beads.

Chitosan beads were formed after dropping the algal-chitosan mixture into polyphosphate solution. However, *Dunaliella* cells in chitosan beads died after a short period when colour of the beads changed from clear to white. Chlorophyll fluorescence was applied to study cellular condition during immobilisation process and the results are shown in Figure 3.29. It was found that *Dunaliella* cells can survive in acidic chitosan solution as well as in sodium alginate solution. However, after chitosan beads were solidified in polyphosphate solution, Fv/Fm ratio suddenly dropped and no fluorescence was detected after 10 minutes. Meanwhile, Fv/Fm ratio of an algal-alginate mixture was unchanged during calcium alginate bead formation (Figure 3.29).

This problem was also reported by Beaumont and Knorr (1987) who studied the viability of celery (*Apium graveolens*) cells. Chitosan immobilised *A. graveolens* had low viability compared to alginate immobilised and free cells. They reported that, chitosan beads lose viability as determined by respiration and plasmolysis within the first 24 hours (there was no measurement between 0 and 24 hours). Only carrageenan/chitosan copolymer beads can maintain cell viability for 2 weeks of the experiment. Unfortunately, carrageenan is not suitable for saline environments (Chibata *et al.*, 1987) so it cannot be applied to *Dunaliella*.

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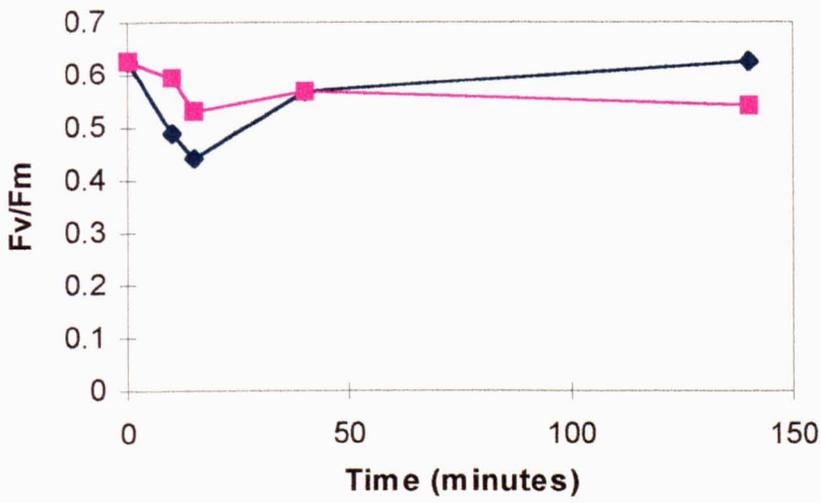
(A)
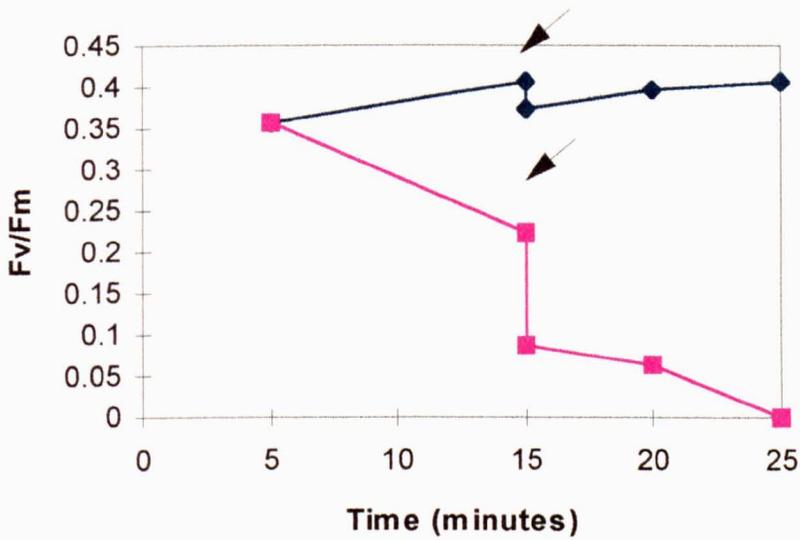
(B)

Figure 3.29: **(A)** Potential yield of the photochemical reaction of photosystem II (Fv/Fm ratio) from *Dunaliella* cells in (◆) sodium alginate solution and (■) chitosan solution.

(B) Fv/Fm ratio from Dunaliella cells after (◆) cells in sodium alginate were dropped into calcium chloride solution and (■) cells in chitosan solution were dropped into polyphosphate solution. Arrows indicate dropping time.

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Immobilisation by cell attachment to chitosan flakes was reported for filamentous cyanobacteria *Phormidium* (de la Noüe and Proulx, 1988), *Anabaena* and green microalga *Chlorella* (Mallick and Rai, 1994). However, as *Dunaliella* is a motile single cell alga, this technique is not suitable because *Dunaliella* cells were always found to swim freely in the medium rather than attach to substrates.

3.2.5 Comparison of glycerol leakage from calcium alginate and agar immobilised *Dunaliella*

It has been reported by Fujii (1994) that Ca^{2+} can affect the retention of intracellular glycerol and amino acids in *D. tertiolecta*. Lack of Ca^{2+} in culture medium causes leakage of glycerol into the medium within 30 minutes. Thus, as immobilisation of *Dunaliella* using calcium alginate will have exposed cells to a high concentration of Ca^{2+}, this might have some effect on glycerol excretion. In this experiment, immobilisation of *Dunaliella* cells in agar beads was carried out to be allow comparison with calcium alginate beads for glycerol excretion.

Approximately 4 g wet weight of calcium alginate and agar immobilised *Dunaliella* were placed in test tubes containing 5 ml of 1.5 M NaCl *Dunaliella* medium. Average chlorophyll contents of the beads were 8.43 ± 1.11 and 5.83 ± 0.94 μg.5 beads^{-1} and average size was 3.22 ± 0.69 and 2.89 ± 1.21 mm for calcium alginate and agar beads respectively.

Glycerol in the medium was monitored and the results are shown in Figure 3.30. It was found that agar and calcium alginate beads released glycerol into the medium and there was no significant difference in glycerol concentration found in the medium. This indicated that these two immobilisation matrices show the same level of glycerol release.
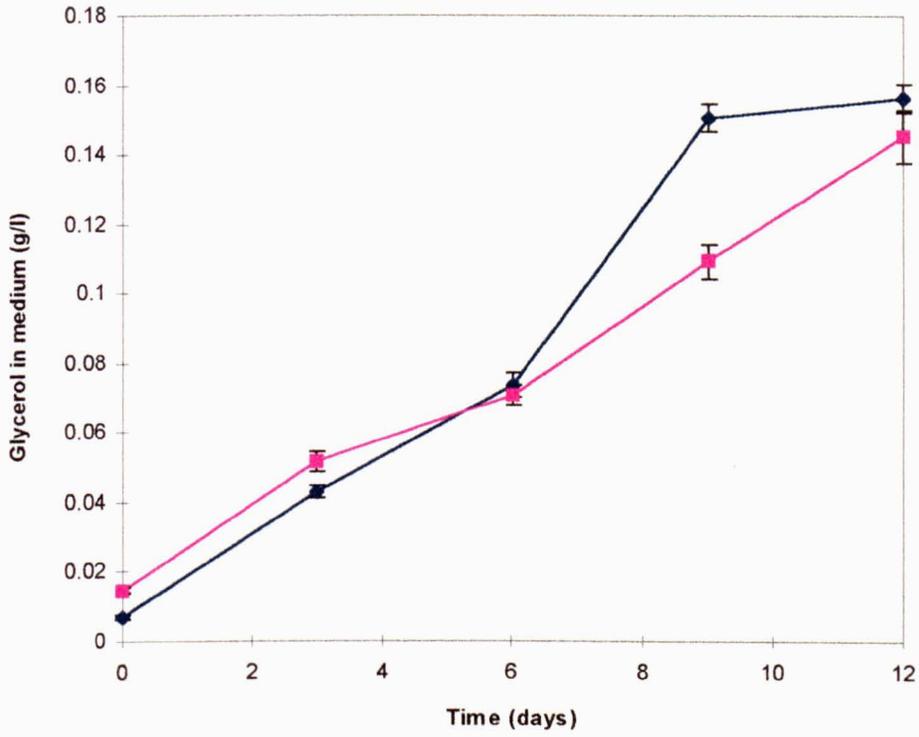
Figure 3.30: Glycerol concentration in the medium of agar and calcium alginate

immobilised *Dunaliella*.

(◆) *Dunaliella* immobilised in agar.

(■) *Dunaliella* immobilised in calcium alginate.

3.3 Conclusions

This chapter mainly considered activities of immobilised cells inside the beads. It appears that *Dunaliella* cells can maintain their basic physiology especially reproduction and photosynthesis, when they were entrapped in calcium alginate (Section 3.2.1). Light is a very important factor for photosynthetic algae. Self shading of cells inside the beads and packing of beads themselves can reduce photosynthetic rate which affects glycerol production (Section 3.2.3), because glycerol is a product of photosynthesis. This must be a serious concern when scaling up to production scale in a photobioreactor. Beads must be arranged as a thin film to maximise light efficiency usage by entrapped algae.

The idea of industrial production of glycerol from *Dunaliella* has been proposed by Ben-Amotz and Avron (1982) and process was also evaluated by Chen and Chi (1981). However, harvesting of glycerol from *Dunaliella* needs sophisticated techniques in order to separate cells from the medium and cells have to be broken to release glycerol. Immobilisation of *Dunaliella* cells demonstrates an alternative to using free cells for glycerol production. It has two advantages, firstly, cells can be grown to very high density and kept active for a long period and, secondly, it does not require cell separation as glycerol is released directly into the medium.

In 1991, Hard and Gilmour found a mutant strain of *D. parva* which leaks large amounts of glycerol into the medium. This mutant strain was used in this study in order to compare glycerol excretion with wild type strain (*D. parva* CCAP 19/9). However, the raw data showed that there was no difference between glycerol release from wild type and mutant strain in both immobilised and free cell culture, so the results were then finally combined as if there was only one strain (section 3.2). Hence, it could be stated that *Dunaliella* cells (free and immobilised) leak or excrete glycerol into the medium. The results presented here argue against the idea that *Dunaliella* has a special plasma membrane property which retains intracellular

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glycerol. That theory was proposed in many publications such as Ben-Amotz and Avron (1973), Wegmann *et al.* (1980) or Gimmler and Hartung (1988). Results from section 3.2 suggest that the glycerol release from *Dunaliella* is similar to that from *Chlamydomonas*, which is a species that does not retain intracellular glycerol and has never been reported as having special membrane properties.

When glycerol leakage was found in wild type strain as well as mutant, another original culture of *D. parva* 19/9 was ordered from Culture Collection of Algae and Protozoa to re-check the glycerol leakage. The new *D. parva* culture from the culture collection, in bacteria free environment, was found to continuously release glycerol into the medium. More investigations were then performed and the results are shown in Chapter 6.

Living cells immobilised in chitosan beads are a good option to be used in industrial immobilisation. Chitosan beads have good stability in high Na^+ and high phosphate. However, *Dunaliella* immobilisation in chitosan was not successful and this has been reported for other plant cells (Beaumont and Knorr, 1987). Cells died during bead formation (Figure 3.29). It is possible that when the colour of beads changed from clear to matted white, light was blocked out and algal cells cannot photosynthesise.

Agar immobilised *Dunaliella* grew well and showed no difference in glycerol release compared to calcium alginate immobilised cells (Figure 3.30). Thus glycerol release is not an effect of a specific immobilisation matrix. Agar has better stability in high phosphate medium which is a disadvantage of calcium alginate beads. Unfortunately, immobilisation in agar was considered too expensive and has never been used in industry (Brodelius and Vandamme, 1987).

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CHAPTER 4:
ENHANCEMENT OF GLYCEROL PRODUCTION FROM
IMMOBILISED *Dunaliella* CELLS

4.1 Introduction

As described in the previous chapter, *Dunaliella* was successfully immobilised using calcium alginate beads. The basic physiology such as growth, photosynthesis and release of glycerol from immobilised *Dunaliella* cells was studied. This chapter will examine the possibility of using several techniques to improve the glycerol productivity from immobilised *Dunaliella* cells.

When a *Dunaliella* cell is exposed to hypoosmotic stress, cell volume will increase (as it does not have a rigid cell wall) and glycerol will be immediately released from the cell (Ben-Amotz and Avron, 1973). So, hypoosmotic shock by replacing the existing culture medium with a lower salinity medium or even with distilled water could possibly induce more glycerol to be released into the medium. In this chapter, the effect of hypoosmotic shock (downshock) on photosynthesis and glycerol release in immobilised *Dunaliella* cells was studied.

Another way to improve glycerol production is to optimise the harvesting system. The advantage of growing immobilised *Dunaliella* instead of free cell culture is that algal beads can be easily separated from culture medium. Therefore, the complex and expensive step of harvesting by centrifugation is not necessary. Harvesting of the medium can be done simply by pouring out the medium and replacing it with fresh medium. As most of the experiments in the previous chapter were done by using batch culture, the experiments in this chapter will examine the possibility of semi-continuous harvesting to increase glycerol productivity.

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The final part of this chapter will evaluate an alternative immobilisation technique that does not use beads. This may be useful because algae entrapped in small calcium alginate beads are not easy to manipulate on a large scale. Therefore, a novel algal sheet with spongy plastic backbone to support calcium alginate structure was produced (see section 2.5.2). Algal sheet is very easy to maintain and can be removed from the growing system by simply lifting out the whole sheet at once. By combining the algal sheet with a specially designed growth chamber, it could possibly be an alternative choice for a cell immobilisation system.

4.2 Results and Discussion

4.2.1 Effect of hypoosmotic shock on glycerol release from free cells of *Dunaliella*

Hypoosmotic shock, which can induce glycerol release in *Dunaliella* cells, was applied to free cells of *Dunaliella* in order to increase glycerol production. Figure 4.1 shows glycerol concentration in the medium after cells were centrifuged and the medium was then replaced with distilled water (0 M NaCl), 0.75 M NaCl medium or 1.5 M NaCl medium (control). The first sampling point was approximately 30 seconds after cells were resuspended in fresh medium. It was found that glycerol was released immediately after hypoosmotic shock. In 1.5 to 0 M NaCl (distilled water) hypoosmotic shock, cells burst and released all glycerol into the medium. Hypoosmotic shock from 1.5 to 0.75 M NaCl induced more glycerol release than control (Figure 4.1).
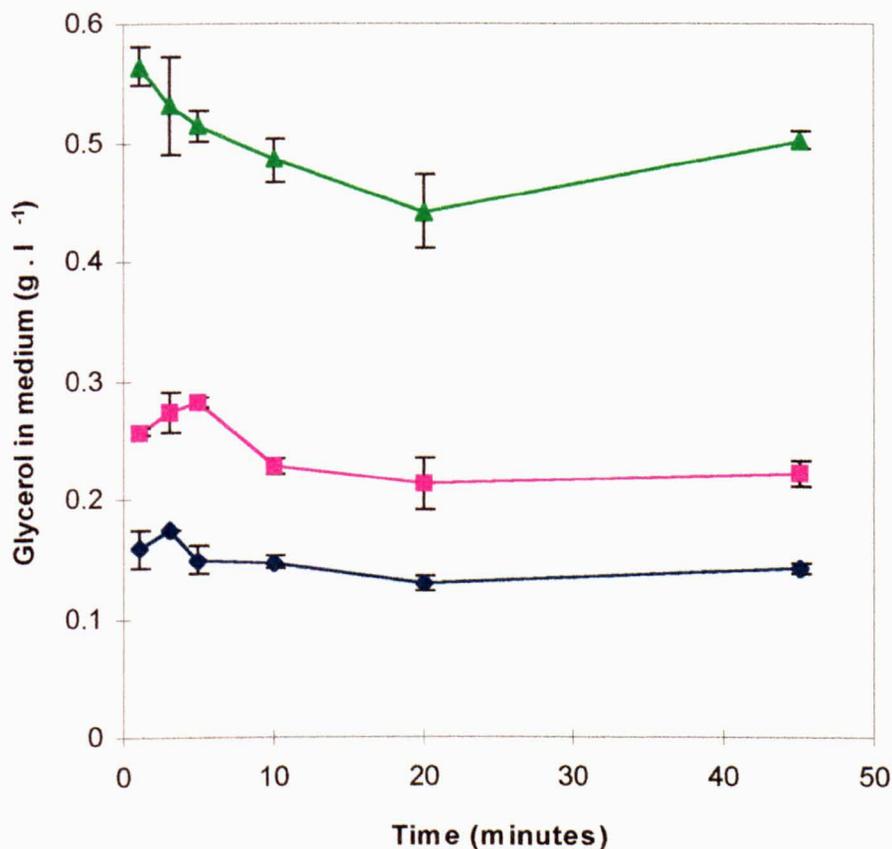
Figure 4.1: Effect of hypoosmotic shock on glycerol release from free cells of *Dunaliella*. Cells were grown in 1.5 M NaCl medium to a chlorophyll concentration of 5.86 µg Chl.ml^{-1}. 5 ml of cells were then centrifuged and resuspended in 1.5 M (control, ◆), 0.75 M (■) and 0 M NaCl (▲).

4.2.2 Effect of hypoosmotic shock on glycerol release from immobilised *Dunaliella*

To study the effect of hypoosmotic shock on glycerol release from immobilised *Dunaliella*, two different sizes of calcium alginate beads (large beads, 4.48 ± 0.11 mm in diameter with 4.17 ± 0.14 μg Chl.bead^{-1}, and small beads, 2.78 ± 0.18 mm in diameter with 0.81 ± 0.02 μg Chl.bead^{-1}) were prepared for this experiment. The experiment was carried out in test tubes containing 0.9 g of large or small beads and 1 ml of 1.5 M NaCl medium. Culture medium was replaced with fresh medium at different salinities (0 M NaCl distilled water, 0.75 M NaCl medium and 1.5 M NaCl medium for control) and the medium was then sampled for glycerol determination. Figures 4.2 and 4.3 show that, within three minutes, glycerol was released in all treatments including control. Immobilised *Dunaliella* exposed to distilled water showed the fastest glycerol release during first three minutes following by 0.75 M NaCl and 1.5 M NaCl control respectively. Size of the beads did not significantly affect glycerol release rate and it appears that the amount of glycerol released was mainly dependent on the amount of *Dunaliella* biomass present.

Pattern of glycerol release from immobilised *Dunaliella* was different from free cells (Figures 4.1 - 4.3). With free cells, glycerol was immediately released after hypoosmotic shock and then the amount of glycerol in the medium was fairly stable over the next 45 minutes. Immobilised cells, however, gradually released glycerol into the medium and release rate depended on external salinity. This indicates that the calcium alginate structure moderated the effect of hypoosmotic shock by reducing the diffusion rate of medium into the cells and, at the same time, delaying the diffusion rate of glycerol from entrapped cells to the medium. Moreover, the rigid calcium alginate structure should protect cells from bursting when beads were suspended in distilled water.

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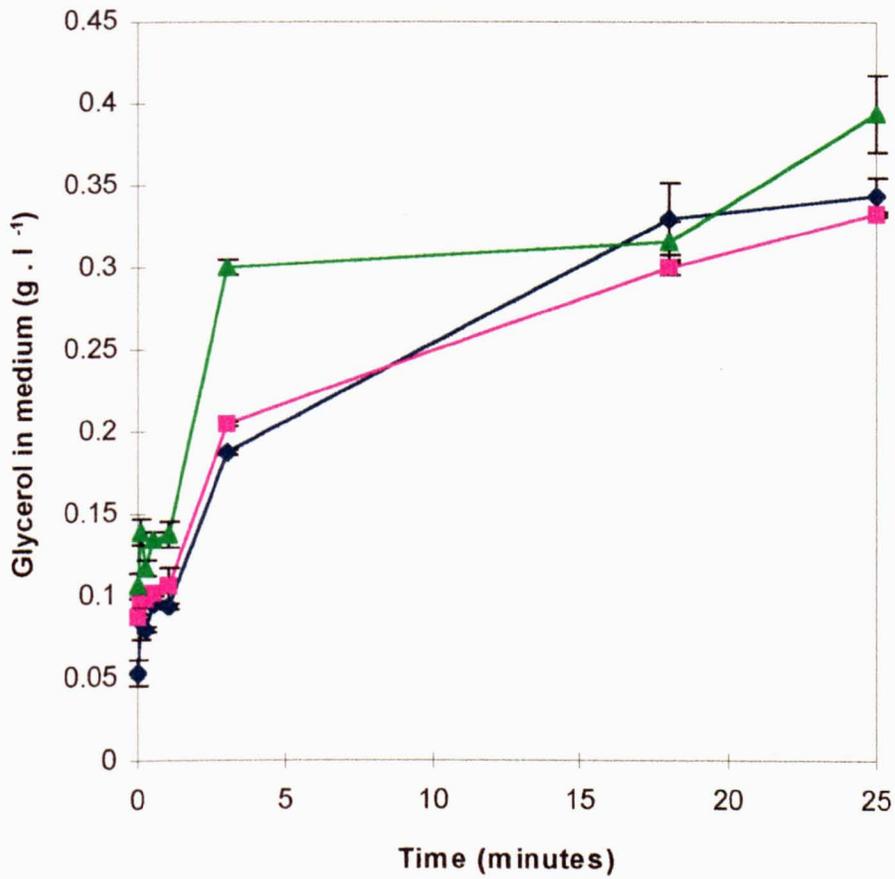
Figure 4.2: Effect of hypoosmotic shock on glycerol production from *Dunaliella* cells immobilised in large calcium alginate beads ($\varnothing$ = 4.5 mm). 0.9 g of beads from a culture in 1.5 M NaCl medium were resuspended in 1 ml of 1.5 M NaCl (control, ◆), 0.75 M (■) or 0 M NaCl (▲).
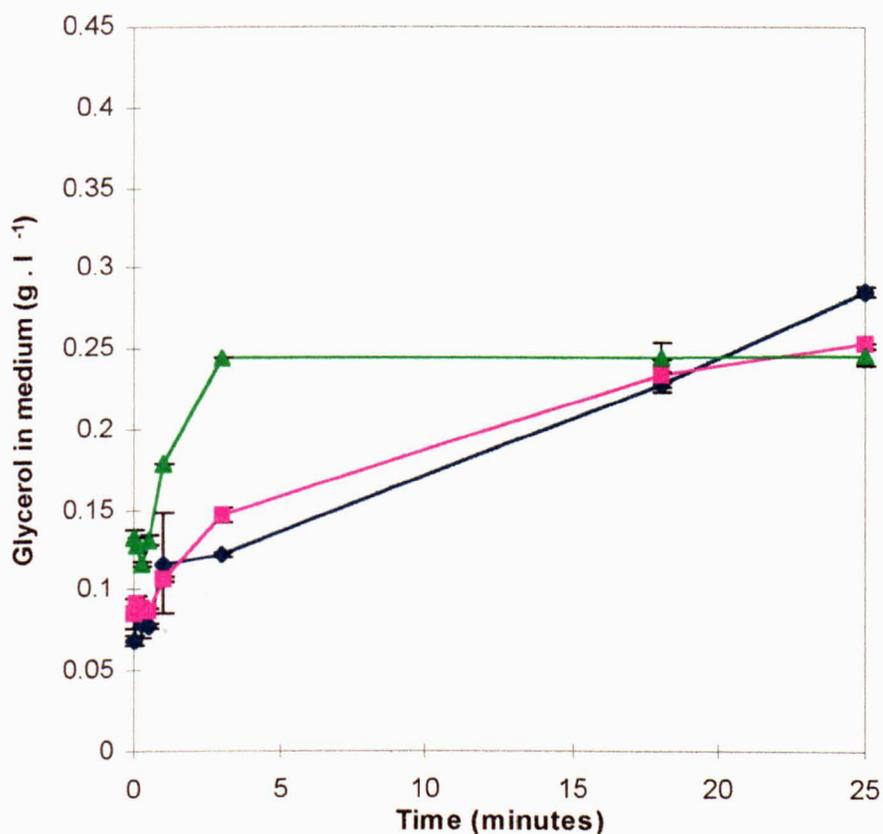
Figure 4.3: Effect of hypoosmotic shock on glycerol production from *Dunaliella* cells immobilised in small calcium alginate beads ($\varnothing$ = 2.8 mm). 0.9 g of beads from a culture in 1.5 M NaCl medium were resuspended in 1 ml of 1.5 M NaCl (control, ◆), 0.75 M (■) or 0 M NaCl (▲).

To study glycerol release in response to osmotic shock (hypo- and hyper-osmotic) over a longer time period, immobilised *Dunaliella* were exposed to various salinities for 3 hours. In this experiment, algal beads were exposed to several salinities, i.e. 0 M (distilled water), 0.75 M, 1.5 M (control), 2.5 M and 3.5 M NaCl medium. Glycerol was monitored as glycerol released into the medium and glycerol content in entrapped cells. The results are shown in Figure 4.4 and 4.5 respectively.

In Figure 4.4, osmotic shock from 1.5 to 0 M NaCl induced the highest glycerol release. For up to one hour, all treatments were found to release glycerol into the medium including 1.5 to 2.5 M and 1.5 to 3.5 M NaCl hyperosmotic shock treatments. However, the amount of glycerol in the medium did slightly decrease in all treatments between one and three hours after the osmotic shock.

Decreasing glycerol content in the immobilised cells (Figure 4.5) corresponded with glycerol release data. Beads exposed to osmotic shock from 1.5 M to 0 M NaCl, which released the highest amount of glycerol into the medium, were found to have the lowest glycerol content. This was followed by osmotic shock from 1.5 M to 0.75 M NaCl, control (replaced with fresh 1.5 M NaCl medium) and hyperosmotic shock from 1.5 to 2.5 M and 1.5 to 3.5 M NaCl. Although glycerol concentration in the medium (Figure 4.4) was found to decrease after the first hour of rapid release, all treatments and control showed a continuous reduction of glycerol content in the cells over the three hour experimental period (Figure 4.5). This indicates that there was another factor that reduced glycerol concentration in the medium and it was possibly bacterial activity as the experiment was not performed under good sterile conditions due to the large number of samples taken.
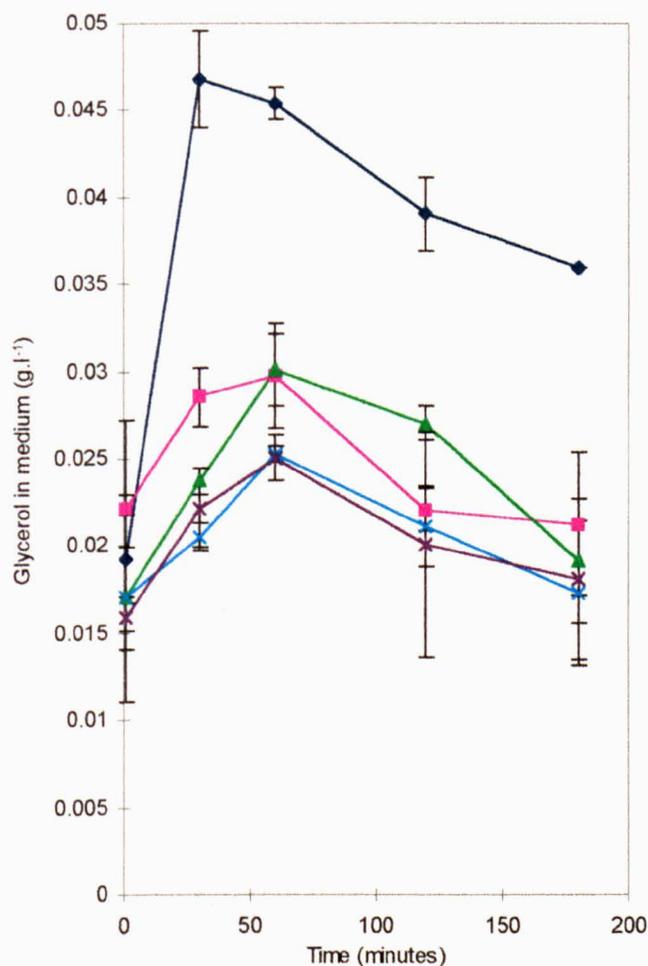
Figure 4.4: Effect of osmotic shock on glycerol release from immobilised *Dunaliella*. Large beads ($\varnothing$ = 4.5 mm) were cultured in 1.5 M NaCl medium and reached a chlorophyll content of 2.19 ± 0.042 μg Chl. bead^{-1}. The beads were then resuspended as follows.

($\blacklozenge$) hypoosmotic shock from 1.5 M to 0 M NaCl

($\blacksquare$) hypoosmotic shock from 1.5 M to 0.75 M NaCl

($\blacktriangle$) control: replaced with fresh 1.5 M NaCl medium

($\times$) hyperosmotic shock from 1.5 M to 2.5 M NaCl

($*$) hyperosmotic shock from 1.5 M to 3.5 M NaCl

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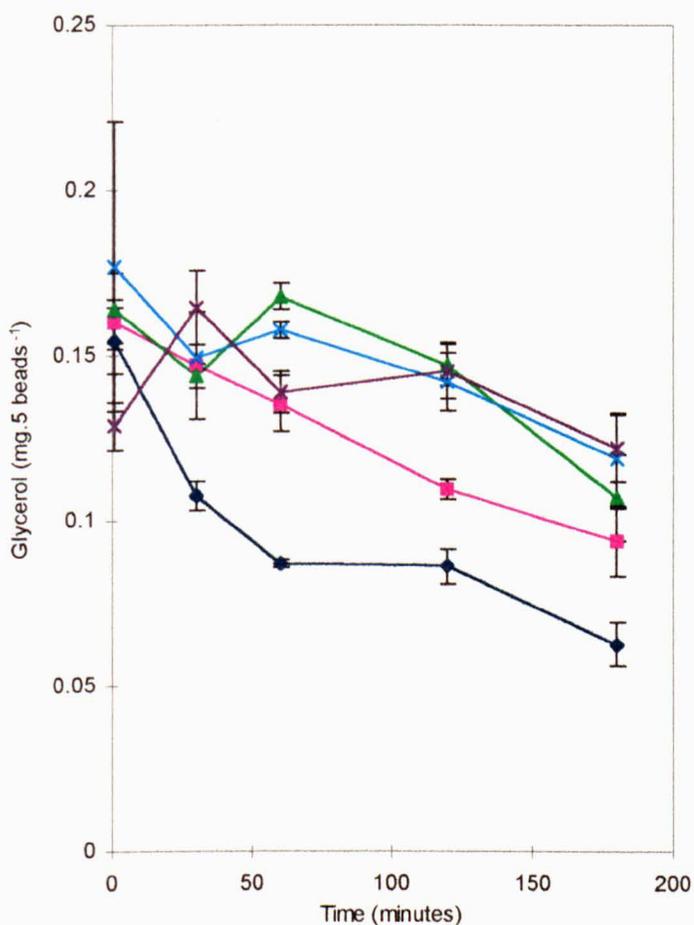
Figure 4.5: Effect of osmotic shock on glycerol concentration in the cells of immobilised *Dunaliella*. Large beads ($\varnothing$ = 4.5 mm) were cultured in 1.5 M NaCl medium and reached a chlorophyll content of 2.19 ± 0.042 µg Chl.bead^{-1}. The beads were then resuspended as follows.

($\blacklozenge$) hypoosmotic shock from 1.5 M to 0 M NaCl

($\blacksquare$) hypoosmotic shock from 1.5 M to 0.75 M NaCl

($\blacktriangle$) control: replaced with fresh 1.5 M NaCl medium

($\times$) hyperosmotic shock from 1.5 M to 2.5 M NaCl

($\ast$) hyperosmotic shock from 1.5 M to 3.5 M NaCl

4.2.3 Effect of osmotic shock on photosynthetic oxygen evolution in immobilised *Dunaliella*

One problem of measuring oxygen evolution from immobilised *Dunaliella* is that the calcium alginate structure of the bead is a barrier between cells and external medium. This prolongs oxygen release into the medium and can delay the detector response of the oxygen electrode. Therefore, measuring oxygen evolution from immobilised *Dunaliella* in beads needs a longer period of measurement in the oxygen electrode chamber than that for free cells. This might lead to the carbon source supplied in the medium (which in *Dunaliella* medium is 11.9 mM sodium bicarbonate) becoming exhausted. To test this effect, extra sodium bicarbonate was added into the chamber (2 ml in volume) while measuring oxygen evolution. So, 50 μl of medium in the oxygen electrode chamber was taken out and replaced with 50 μl of sodium bicarbonate solution (1.68 mg $NaHCO_3.50\mu l^{-1}$) which made the final concentration of additional sodium bicarbonate equal to 10 mM.

Figure 4.6 shows that an addition of $NaHCO_3$ after 10 minutes of oxygen evolution measurement did not increase the rate of oxygen evolution compared with control (Figure 4.7). Hence, it appeared that there was no bicarbonate deficiency in the oxygen electrode chamber during 20 minutes measurement, therefore, in the following experiments oxygen evolution was measured over time periods no longer than 20 minutes.

Relative oxygen evolution in immobilised *Dunaliella* after osmotic stress is shown in Figure 4.7. It was found that hypoosmotic shock from 1.5 M NaCl to 0 M (distilled water) obviously reduced oxygen evolution. On the other hand, hyperosmotic shocks from 1.5 M to 2.5 M and 1.5 M to 3.5 M NaCl slightly increased photosynthesis, which indicated that cells needed more energy to produce more glycerol in order to balance the osmotic pressure.
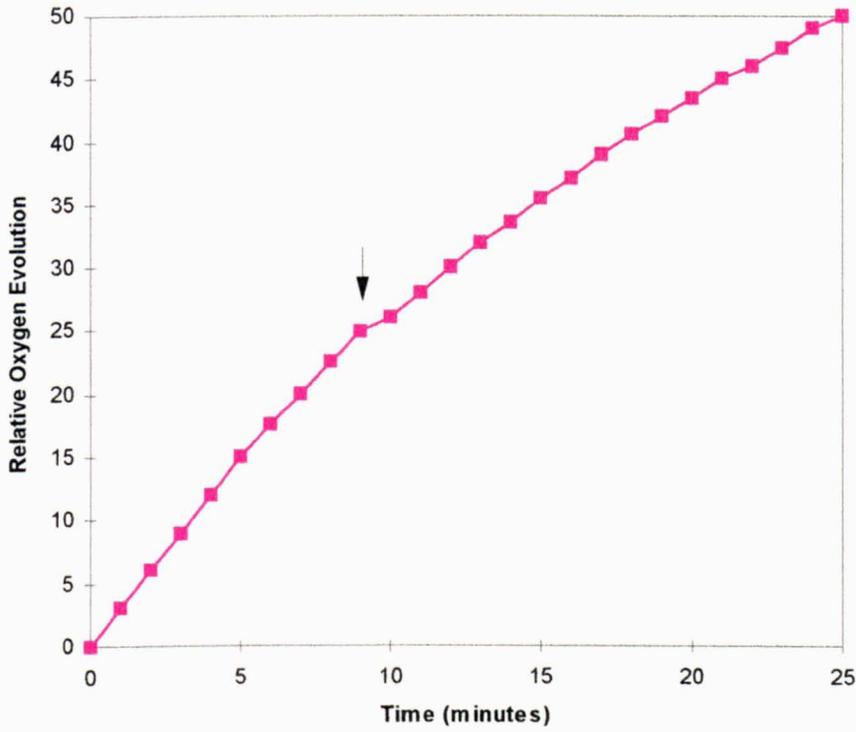
Figure 4.6: Effect of carbon dioxide deficiency on the oxygen evolution of large algal beads ($\varnothing$ = 4.5 mm). An arrow indicates the point that additional sodium bicarbonate was injected into the chamber during oxygen evolution measurement. The chlorophyll concentration was 1.01 ± 0.02 μg.bead^{-1}.
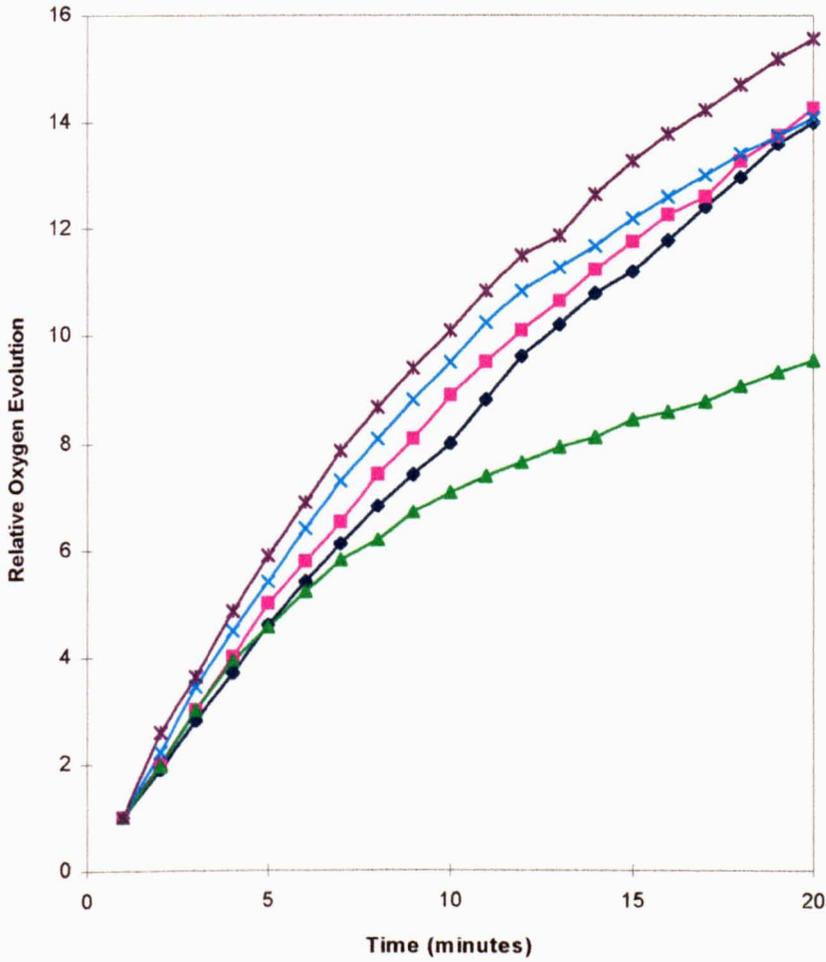
Figure 4.7: Relative oxygen evolution from immobilised *Dunaliella* after osmotic shock in different salinities. Algal beads ($\varnothing = 4.5$ mm) were grown in 1.5 M NaCl medium to a chlorophyll content of 1.97 µg.bead^{-1} and then resuspended in different salinity medium as indicated below.

($\blacklozenge$) control: replaced with fresh 1.5 M NaCl medium

($\blacksquare$) hypoosmotic shock from 1.5 M to 0.75 M NaCl

($\blacktriangle$) hypoosmotic shock from 1.5 M to 0 M NaCl

($\times$) hyperosmotic shock from 1.5 M to 2.5 M NaCl

($*$) hyperosmotic shock from 1.5 M to 3.5 M NaCl

As the photosynthesis rate of immobilised *Dunaliella* was reduced after exposure to 0 M NaCl, this would be a problem if a further hypoosmotic shock was applied to induce more glycerol production from immobilised *Dunaliella*. Therefore, it is necessary to provide a recovery period after hypoosmotic stress that lets photosynthesis rate return to normal. Photosynthesis recovery from osmotic stress in immobilised *Dunaliella* was studied at 20 minutes intervals, as shown in Figure 4.8. In this experiment, the first 20 minutes was oxygen evolution under normal growth conditions in 1.5 M NaCl medium. After 20 minutes, culture medium was replaced by distilled water. At this stage (20-40 minutes), oxygen evolution decreased. However, cells showed a rapid recovery after 40 minutes when distilled water was replaced with fresh 1.5 M medium and oxygen evolution returned to normal rate after the second medium change (60 - 80 minutes). And finally, repeating the hypoosmotic shock after 80 minutes again reduced oxygen evolution.

During recovery period when photosynthesis rate returned to normal, cells have to accumulate glycerol to compensate for glycerol lost during osmotic shock. This process requires time and can be monitored by measuring glycerol content in the cells. Therefore, glycerol concentration in the medium and in *Dunaliella* cells was measured after hypoosmotic shock in distilled water compared with control (1.5 M NaCl fresh medium). The results are shown in Figure 4.9. It was found that during the first 30 minutes, both hypoosmotic shock and control released glycerol into the medium but a higher release rate was found after hypoosmotic shock. Thereafter, culture medium for both control and hypoosmotic shock treatment was replaced with fresh 1.5 M NaCl medium and glycerol concentration was monitored for approximately 24 hours. During this period, glycerol content in *Dunaliella* cells slightly increased indicating that cells were accumulating glycerol to compensate for the glycerol lost. However, at the same time, glycerol concentration in the medium was also rising (Figure 4.9). This suggests that glycerol is continuously released from *Dunaliella* cells at all times.
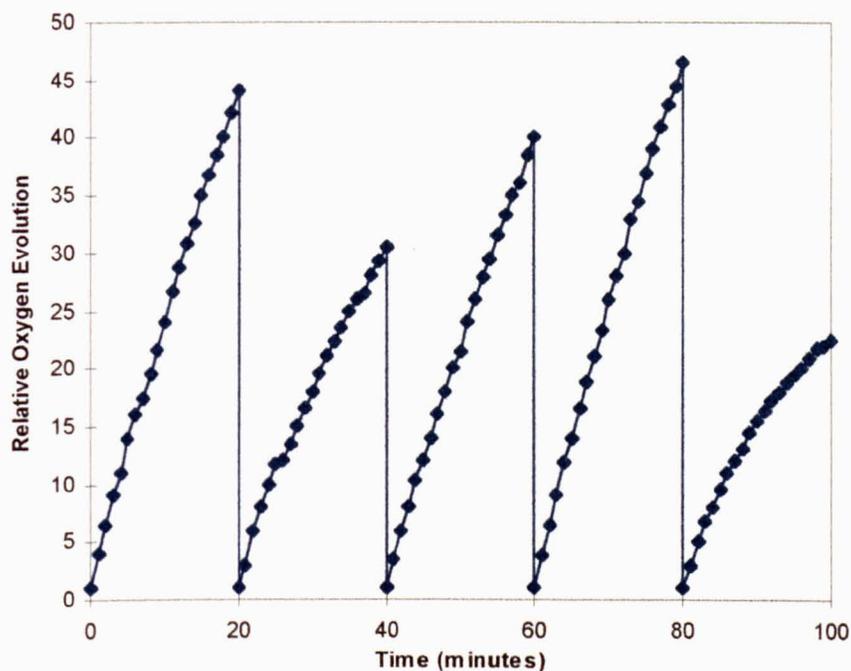
Figure 4.8: Recovery of photosynthetic oxygen evolution of the immobilised *Dunaliella* after changing salinity of the culture medium. Large beads were grown in 1.5 M NaCl medium to a chlorophyll content of 1.1 μg. bead^{-1} and then treated as shown below.

0 - 20 minutes = 1.5 M NaCl (normal growth)

20 - 40 minutes = 0 M NaCl (osmotic shock)

40 - 60 minutes = 1.5 M NaCl (recovery)

60 - 80 minutes = 1.5 M NaCl (recovery)

80 - 100 minutes = 0 M NaCl (repeated osmotic shock)
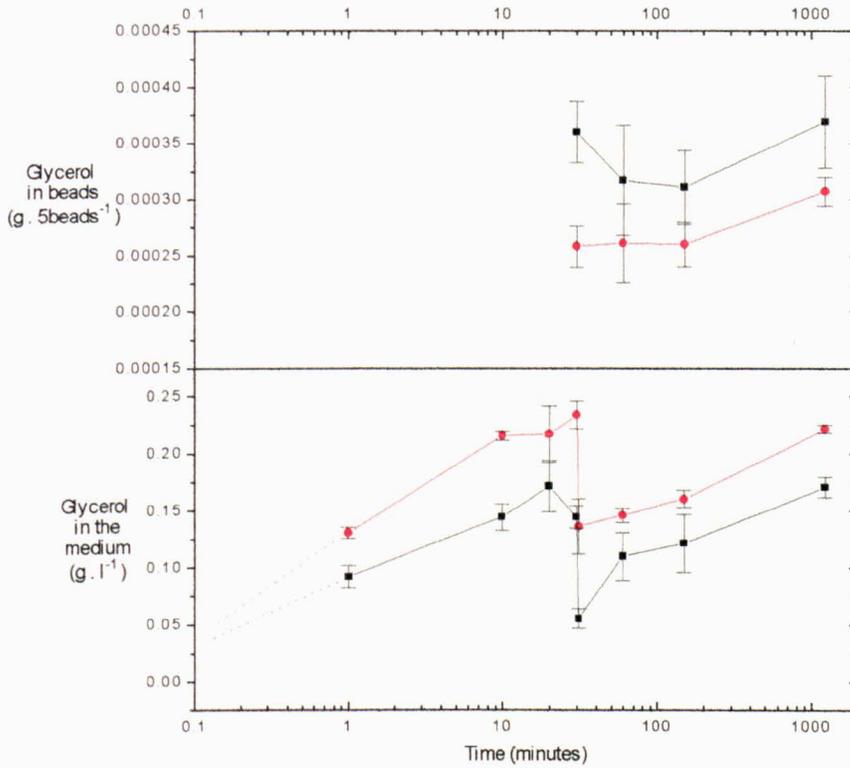
Figure 4.9: Glycerol accumulation and release from immobilised cells after hypoosmotic shock. Picture on the top shows glycerol content in the beads after 30 minutes hypoosmotic shock (1.5 to 0 M NaCl) and control. The bottom picture shows glycerol concentration in the culture medium. In this experiment, medium was changed at the time = 30 minutes. ($\bullet$) represents treatment group with hypoosmotic shock and ($\blacksquare$) represents control group which the medium was replaced with fresh 1.5 M NaCl medium.

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4.2.4 Glycerol productivity from immobilised *Dunaliella* after repeated hypoosmotic shock

The following experiment was designed to evaluate the possibility of using osmotic shock for glycerol production from immobilised *Dunaliella*. Algal beads were exposed to 30 minutes hypoosmotic shock with distilled water (or 1.5 M NaCl medium for control) followed by 24 hours recovery in fresh 1.5 M NaCl medium. Results are shown in Figure 4.10 in which the lower part of the bars represents glycerol in the medium after 24 hour recovery period and the upper part of the bars is the concentration of glycerol in the medium after 30 minute hypoosmotic shock. It was found that total glycerol production over the 24 hour period after hypoosmotic shock was higher than control only in the first two days, then the amount of glycerol produced was not significantly different. This suggests that hypoosmotic shock may not be necessary in order to increase glycerol production. Replacing culture medium with the same salinity fresh medium could give the same result and this idea was applied by using daily semi-continuous harvesting.

4.2.5 Effect of semi-continuous harvesting on glycerol production from immobilised *Dunaliella*

A comparison of glycerol production from immobilised *Dunaliella* in batch culture and daily semi-continuous harvesting was carried out using 4.5 mm diameter beads (0.93 µg Chl.bead^{-1}) growing in 1.5 M NaCl medium. Figure 4.11a shows that, during the first day of culture, glycerol was rapidly released. For batch culture, rate of glycerol released declined after 2 days, but there was a continuous release of glycerol throughout the 14 days of the experiment. On the other hand, semi-continuous harvesting showed much higher glycerol production compared to batch culture. This can be seen when the same data were plotted as cumulative glycerol production as shown in Figure 4.11b.
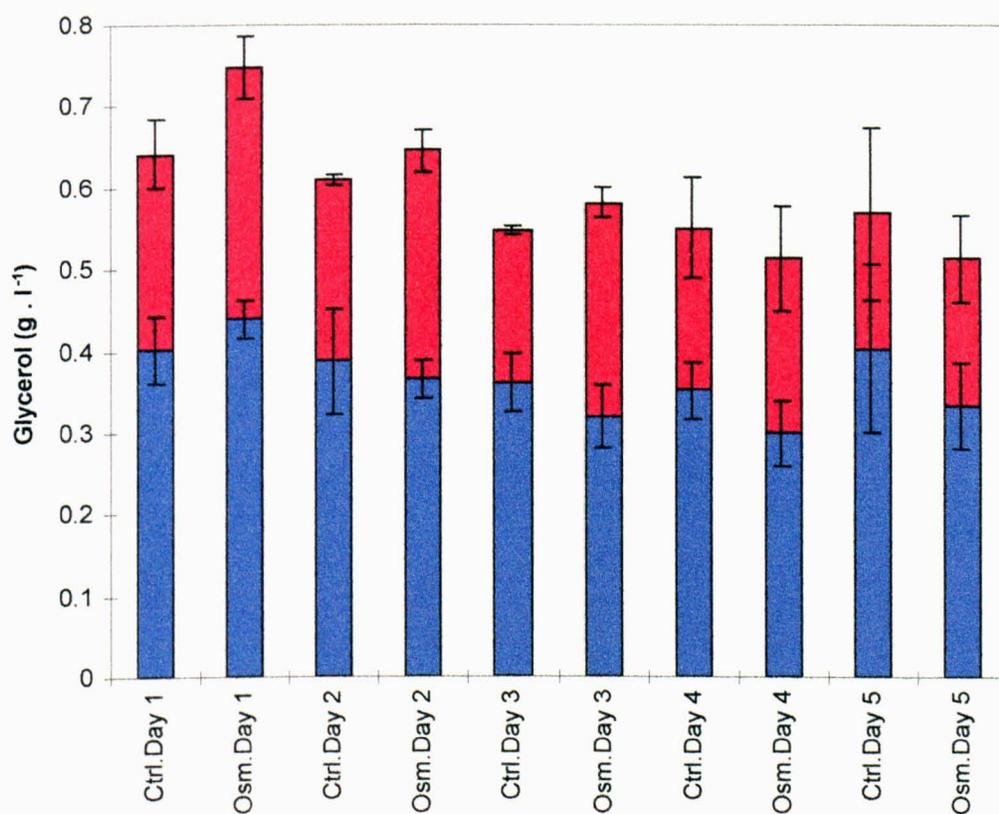
Figure 4.10: Glycerol production from immobilised *Dunaliella* after hypoosmotic shock (Osm) or control (Ctrl). Upper part of the bars represents glycerol in the medium after 30 minutes hypoosmotic shock from 1.5 M to 0 M NaCl (distilled water) and 1.5 M NaCl medium was used instead of distilled water for control group. The lower part of the bars represents glycerol in the medium after 24 hour recovery period in 1.5 M NaCl medium. The experiment was carried out in test tubes containing 70 large beads plus 2 ml of 1.5 M NaCl medium, immobilised cells were grown at 25°C with approximately 70 μE. $m^{-2}.s^{-1}$ continuous illumination.
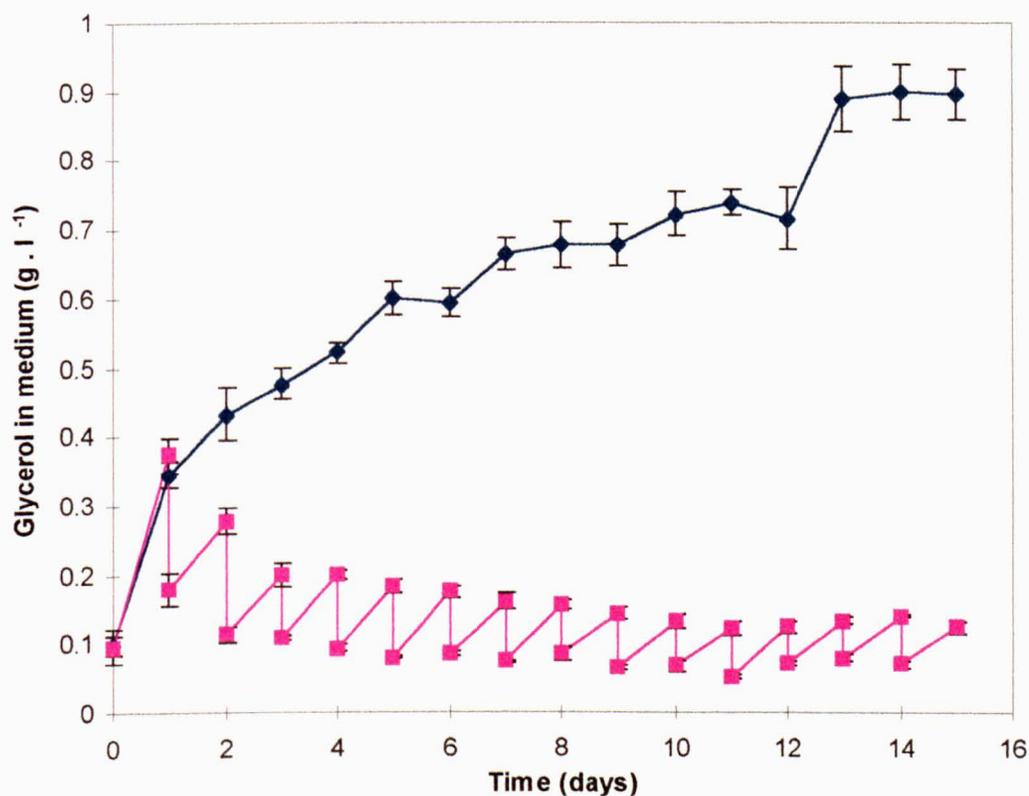
Figure 4.11a: Comparison of glycerol production from batch (♦) and semi-continuously harvested (■) immobilised *Dunaliella*. Large (4.5 mm diameter) beads (0.93 µg Chl.bead^{-1}) were grown in 1.5 M NaCl medium. Immobilised cells were kept at 25°C with continuous illumination (70 µE.m^{-2}.s^{-1}). Semi-continuous harvesting was performed by removing all medium and replacing with fresh medium. Samples were collected for glycerol measurement before and after harvesting.
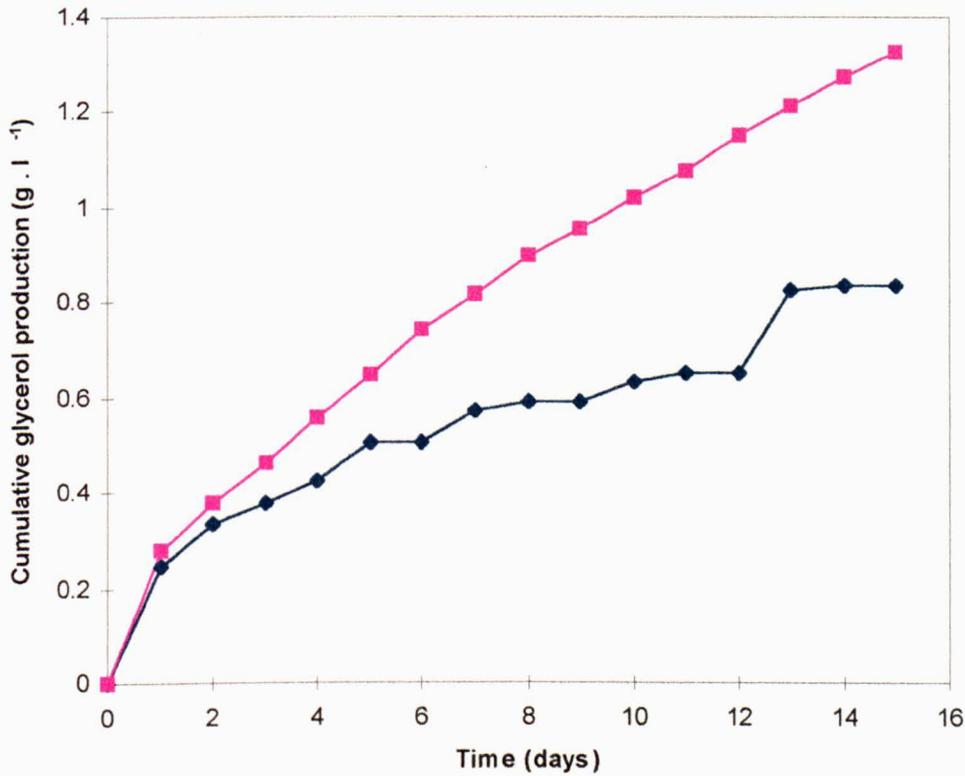
Figure 4.11b: Cumulative plotting of glycerol production from batch (♦) and semi-continuously harvested (■) immobilised *Dunaliella* using the data shown in Figure 4.11a.

4.2.6 Effect of harvesting frequency on glycerol productivity from immobilised *Dunaliella*

Previous experiments have indicated that semi-continuous harvesting is an efficient way of maximising glycerol production. The next step was to compare glycerol production from different harvesting cycles.

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Beads of immobilised *Dunaliella* were grown in 1.5 M NaCl medium in the 25°C growth room. Glycerol concentration in the medium was measured using three different protocols, batch culture, daily semi-continuous harvesting and every four days semi-continuous harvesting. It was found that glycerol was continuously released from all treatments (Figure 4.12a). However, daily semi-continuous harvesting induced significantly higher glycerol production as seen in Figure 4.12b when plotting is cumulative. Cumulative glycerol production was not different in batch culture and semi-continuous harvesting every 4 days. This suggests that release of glycerol from entrapped cells is dependent on glycerol concentration in the medium. By changing culture medium every day, the concentration of glycerol in the medium was minimised, thus, cells released more glycerol. Daily harvesting also reduced the limitation of nutrients and carbon source as they are known to be rapidly taken up by immobilised algae.

4.2.7 Limitation of *Dunaliella* cell concentration in the beads

Another feasible way to increase glycerol production is by increasing the cell concentration in the beads. However, for immobilised algae, very high cell density can reduce light penetration into the inner part of the bead (self shading) and this can affect photosynthesis of the entrapped algae. To examine the limitation of cells entrapped in calcium alginate beads, very high cell density beads (2.56 ± 0.16 and 6.47 ± 0.68 µg Chl.bead^{-1}) were prepared (Figure 4.13a). Unfortunately, it was found that *Dunaliella* cells could not survive in entrapped conditions with too high cell density (Figure 4.13b). Glycerol production (Table 4.1.) was found to be high in the early culture period, thereafter, glycerol productivity declined.

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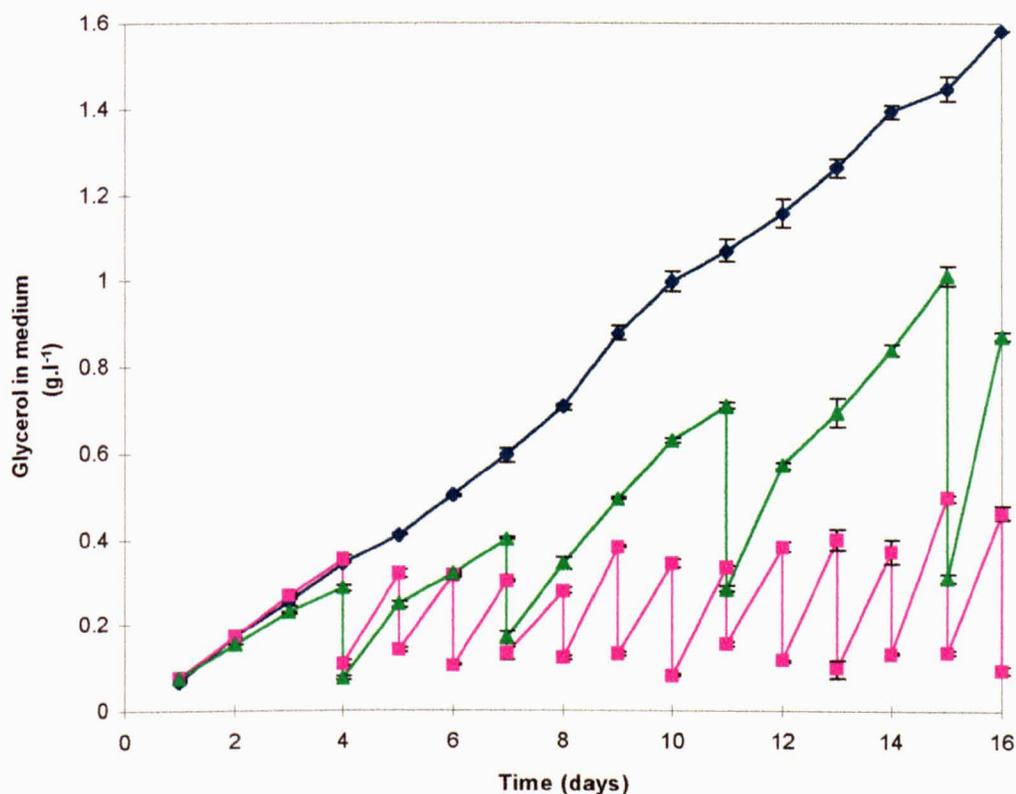
Figure 4.12a: Effect of harvesting frequency on glycerol production from batch culture (◆), daily semi-continuously harvested (■) and every 4 days semi-continuously harvested (▲) immobilised *Dunaliella*. Beads (average diameter 2.78 ± 0.28 mm, average weight 12.8 mg.bead^{-1}, 1.13 µg Chl.bead^{-1}) were grown in 1.5 M NaCl medium with 68 µE. m^{-2}.s^{-1} continuous illumination at 25°C. Semi-continuous harvesting was performed by removing all medium and replacing with fresh medium. Samples were collected for glycerol measurement before and after harvesting.
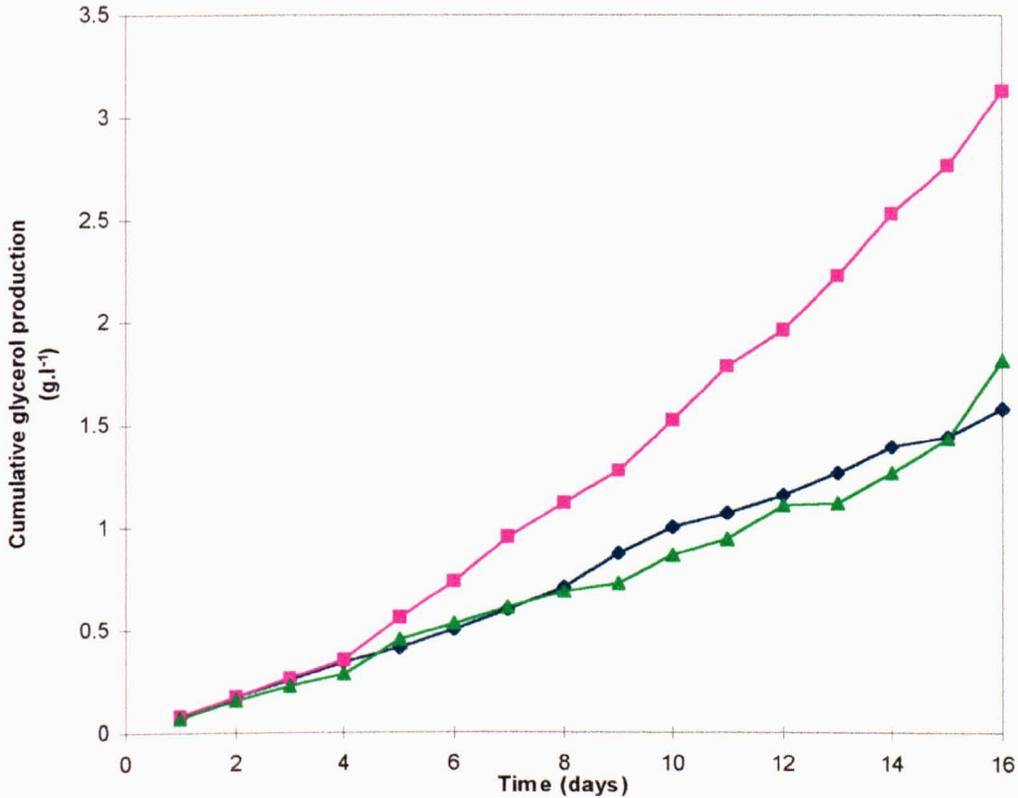
Figure 4.12b: Cumulative plotting of glycerol production from batch culture (♦), daily semi-continuously harvested (■) and every 4 days semi-continuously harvested (▲) immobilised *Dunaliella* using the data shown in Figure 4.12a.

Table 4.1 Glycerol productivity from very high density immobilisation of *Dunaliella*

Day	High density immobilisation (Glycerol productivity $g.l^{-1}.day^{-1}$)	Very high density immobilisation (Glycerol productivity $g.l^{-1}.day^{-1}$)
2	0.251 ± 0.004 [3]	0.608 ± 0.009 [3]
5	0.080 ± 0.002 [3]	0.036 ± 0.007 [3]
7	0.092 ± 0.0004 [3]	0.033 ± 0.002 [3]

The number of replicates are shown in [ ].
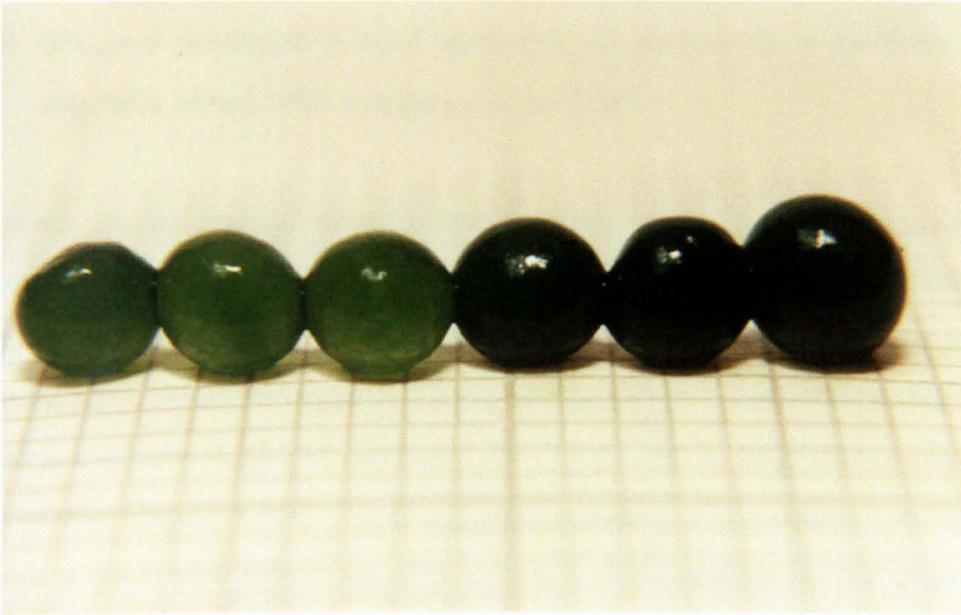
Figure 4.13a: Picture of immobilised *Dunaliella* alginate beads loaded with high cell density (2.56 µg Chl.bead^{-1}, 2.68 ± 0.11 mm diameter: three from left) and very high cell density (6.47 µg Chl.bead^{-1}, 2.57 ± 0.16 mm diameter : three on the right).
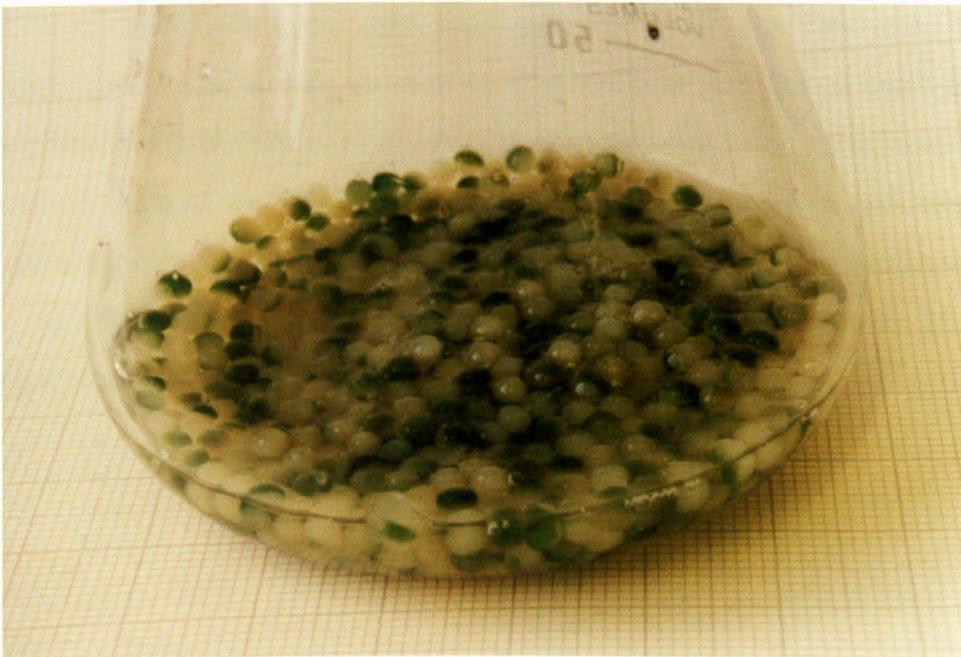
Figure 4.13b: Immobilised *Dunaliella* beads with very high cell density loading after 4 days. 13 g of beads were placed in 125 ml flasks with 5 ml of 1.5 M NaCl medium. Flasks were placed in 25°C growth room under continuous white light (68 µE.m^{-2}.s^{-1}).

4.2.8 Glycerol production from immobilised *Dunaliella* in calcium alginate sheet with a spongy supporter

The novel calcium alginate sheet was prepared by casting 128 g of an algae-alginate mixture in a square plate with 0.1 M $CaCl_2$ under clean conditions in a laminar air flow hood (section 2.5.2). In this experiment, algal beads (control) were prepared using the same amount of algae-alginate mixture (128 g). Algal sheet or beads were placed in a custom made glass chamber, $15 \times 15 \times 1$ cm in dimensions, with 250 ml total volume (Figure 4.14a). Sampling and harvesting of the culture medium were through a glass tube which was attached in a fixed position to the edge of the chamber. To keep the chamber sterile, the open end of the chamber was covered with spongy foam and the chamber was sterilised before use (Figure 4.14a). The experiment was performed in a water bath illuminated from both sides and beads or algal sheet were placed in the chamber with 70 ml of fresh 1.5 M NaCl medium.

Glycerol production from the algal sheet and algal beads was monitored for two months (Figure 4.15). During the first 11 days, cultures were left as batch cultures. During this period, glycerol concentration in beads chamber was approximately twice the concentration of the sheet chamber. After day 12, medium for both treatments was replaced with fresh medium every day until day 17 (semi-continuous harvesting). After day 18, the medium was changed every second day and after day 28, it was changed every fourth day.
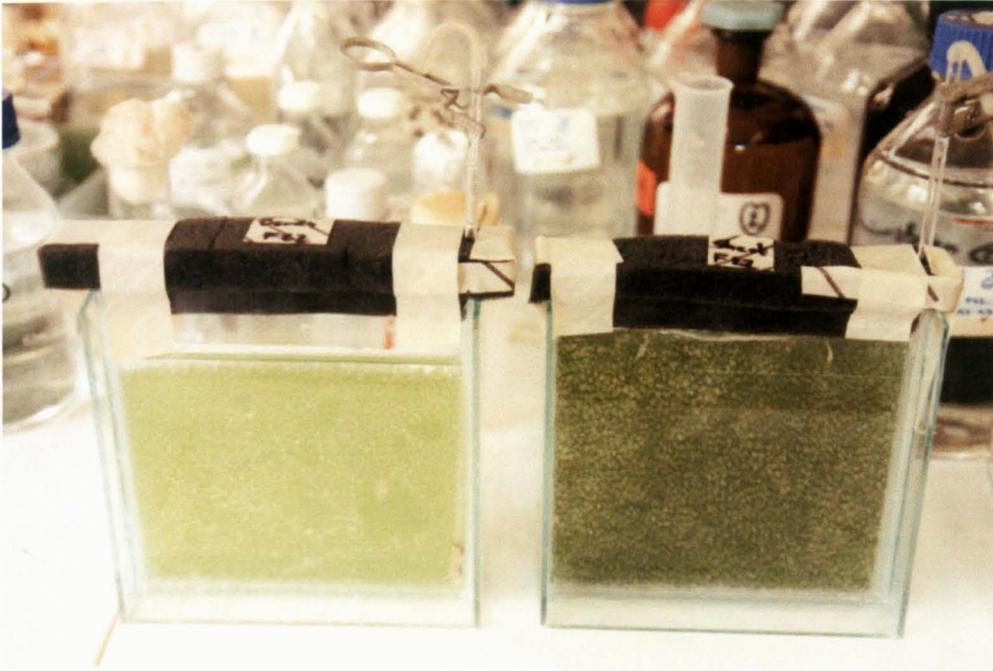
Figure 4.14a Picture shows immobilised *Dunaliella* within the ordinary beads (left) or within a spongy sheet supporter or algal sheet (right) in a flat glass chamber.
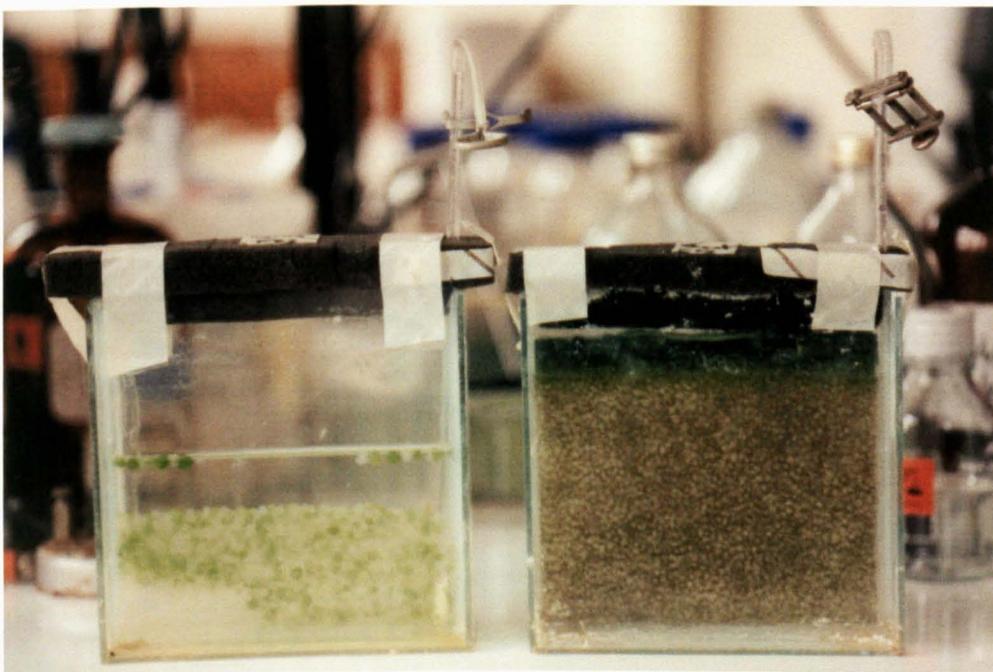
Figure 4.14b: Immobilised *Dunaliella* after 2 months. Beads (left) had been lost during medium harvesting while the algal sheet (right) was still in a good condition.
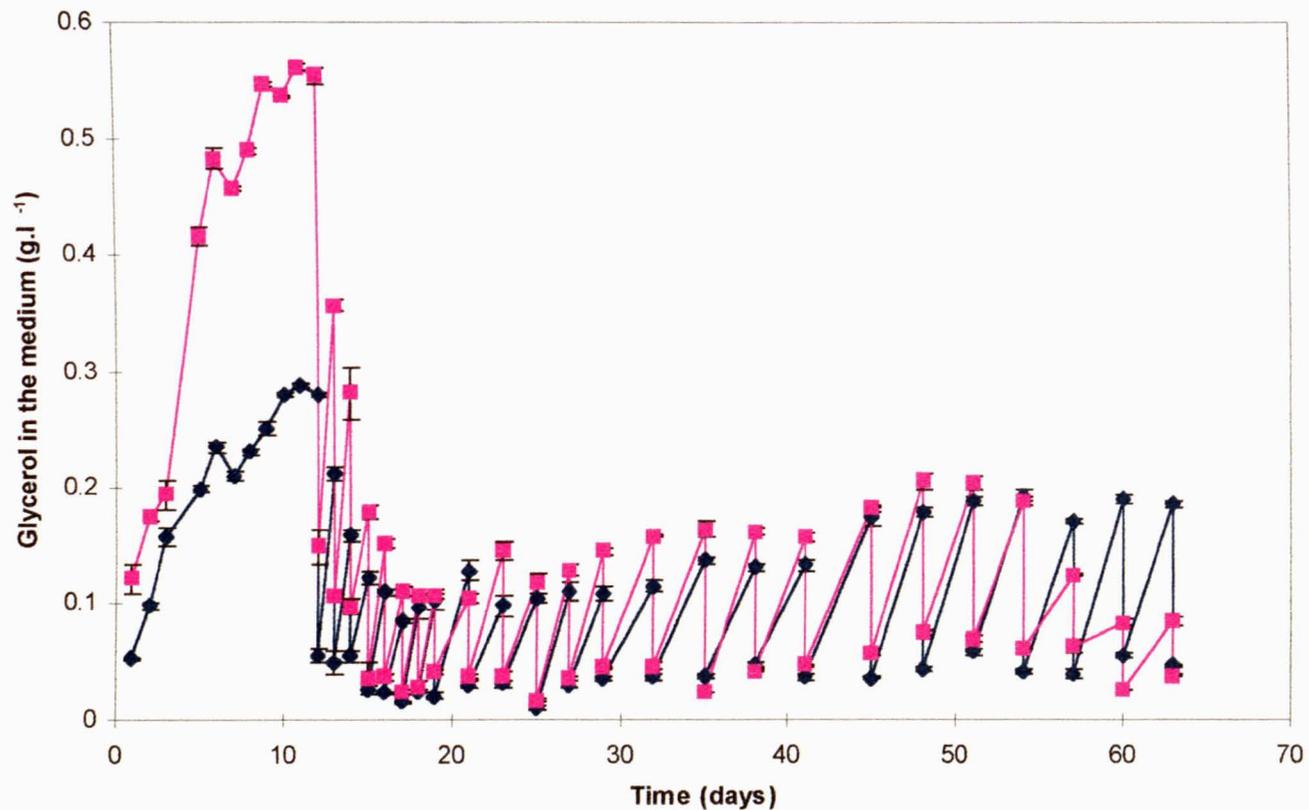
Figure 4.15: Glycerol production from *Dunaliella* cells immobilised in beads (■) and algal sheet (◆). Bead size was 2.73 ± 0.6 mm diameter and the chlorophyll concentration was 2.24 ± 0.24 mg.g^{-1} gel for both beads and algal sheet. No fresh medium was added until day 12 and then semi-continuous harvesting was carried out until the end of the experiment.

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After day 17, bead gel seemed soften and some beads were found to be broken. Then, after approximately 25 days, bead gel was very soft and always broke during sampling. Therefore, the medium had to be centrifuged in order to separate algal cells which leaked into the medium during sampling. This caused loss of gel biomass every time semi-continuous harvesting was carried out. On the other hand, the algal sheet had a longer working life and did not show significant loss of the gel mass. This can be seen in Figures 4.14a and b which show photographs of the experimental units at the start day and two months later. It was found that algal beads produced higher glycerol than algal sheet until approximately day 40 then glycerol productivity decreased (Figure 4.15). Glycerol production from the algal sheet, on the other hand, was stable throughout the experiment.

Nevertheless, the glass chamber used in this experiment might not be a good design for growing immobilised algae. Although it has a large area for illumination there is only a small surface area for exchanging oxygen and carbon dioxide between the medium and air as the experiment was conducted without medium circulation. This can cause a high oxygen content in the medium which might inhibit growth or photosynthesis of the entrapped algae.

4.3 Conclusions

Hypoosmotic shock can increase glycerol release by immobilised cells as well as free cells (Figures 4.1-4.3) and calcium alginate structure of the bead can protect entrapped cells from osmotic shock down to fresh water from 1.5 M NaCl. It may be that the calcium alginate structure, which retains a volume of culture medium inside the bead, can delay the ingress of distilled water into the beads. So, it reduced the sudden effect of hypoosmotic shock and, at the same time, calcium alginate structure can limit cell expansion because of limited space for each cell cluster (see Figures 3.7 and 3.8). On the other hand, when distilled water was replaced with fresh 1.5 M NaCl medium, the reverse effect may occur because salinity inside the bead will be

lower than salinity in the culture medium. Therefore, repeated hypoosmotic shock can reduce the production of glycerol because entrapped cells were always in contact with lower salinity than it was in the culture medium. Unfortunately, therefore repeated osmotic shock did not show any advantage for glycerol productivity over the control group in which culture medium was replaced with fresh medium at the same salinity (Figure 4.10). This is another reason to support the idea that glycerol leakage is a normal characteristic of *Dunaliella* which will be investigated in detail in Chapter 6.

In biotechnological processes, improvement of product yield can be achieved by many procedures such as strain improvement and modification of culture technique. Apart from strain improvement which is described in the next chapter, continuous or semi-continuous harvesting is a common procedure for biological processes especially in fermentation (van Dam-Mieras, 1992). Product removal from culture medium by harvesting means reducing negative feedback control of cellular biosynthesis and can increase product yield. In this chapter, an enhancement of glycerol production by product removal seems to be a successful technique, whereas hypoosmotic shock was found to be unnecessary (Figure 4.11).

Since glycerol production can be enhanced by maintaining low glycerol in the medium during semi-continuous harvesting, this means that glycerol leakage from *Dunaliella* could possibly be a diffusion process. Moreover, there are also other factors that can induce an increase in glycerol leakage after a change of medium e.g. nutrients may play a role. In immobilisation systems, algal cells are entrapped at very high densities which are much higher than those found in normal culture conditions. Therefore, nutrients can be another limiting factor for cellular function which can affect product yield. This was demonstrated by many reports on nutrient uptake by immobilised algae and this was applied for using immobilised algae to reduce nutrient concentration in waste water treatment processes (Chevalier and de la Noüe, 1985; Mallick and Rai, 1994; Kaya *et al.*, 1995).

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Limitation of light and nutrients are the main factors that are of concern when high density of algal cells were loaded in the beads. This was shown in the result that algal cells could not survive when they were entrapped with too high density (Figure 4.13b and Table 4.1). However, only increasing light intensity and nutrient supply cannot ensure success in the immobilisation process as many factors still need to be taken into account such as packing of beads (see Chapter 3), which can reduce light efficiency, or softness of the calcium alginate beads after a long period of culture. Thus, a new modification of the immobilisation system, an algal sheet, was designed and tested in this chapter.

Although the algal sheet has some disadvantages e.g. small surface area compared to beads, it is easier to control the thickness of the gel when all of the gel mass is in one piece and the thickness can be adjusted by the thickness of the spongy plastic supporter. The culture chamber can be designed to have a maximum area for illumination. In this study, the algal sheet showed good stability and it was easy to maintain (Figures 4.14a,b and 4.15). Unfortunately, the small area for gas exchange can be a problem when there is no movement of the medium, therefore, this flat chamber design must be used with a modification of medium circulating or with a continuous flow system. Algal sheet in a flat chamber could also be very useful for other aspects especially in waste water treatment by immobilised algae.

CHAPTER 5:
SELECTION OF HIGH SALINITY TOLERANT MUTANTS AND GLYCEROL LEAKING MUTANTS OF *D. parva*

5.1 Introduction

Mutation is a very useful technique to study basic processes in microbial genetics and biochemical pathways (Miller, 1992). In this study, ultraviolet light (UV) mutagenesis was applied with two aims in mind, first, to enhance the rate of glycerol release from *Dunaliella* cells by selecting glycerol leaking mutants and, second, by comparing normal cells with mutants, it is hoped to better understand the molecular basis of glycerol leakage from *Dunaliella*.

To obtain the mutants in this study, UV irradiation was used. Mutation by UV has been successfully used with microalgae especially in *Chlamydomonas* (Loppes, 1970; Harris, 1989), but also in *Dunaliella* (Ginzburg *et al.*, 1995). UV damages DNA so that specific pairing of bases is no longer possible and the replication machinery stalls at these nonpairing lesions. The SOS regulatory system, which is a special system found in many bacteria but whose function is not clear, is turned on by a series of reactions that starts with DNA damage. SOS system bypasses the blocking lesions and results in frequent mutations across from the damaged bases when the replication machinery can no longer recognise the correct base. UV mutation greatly favours pyrimidine-pyrimidine sequences as mutational sites (Miller, 1992; Zubay and Marmur, 1993). However, UV mutants are likely to revert to the wild-type condition by the activity of a light-dependent repair enzyme. Therefore, UV irradiated cells must be kept in darkness for a few hours after mutagenesis (Lewin, 1988).

In order to increase glycerol productivity from immobilised *Dunaliella*, UV was used to induce mutations and the cells were screened for high glycerol leaking strains. The wild type of *D. parva* CCAP 19/9 was used and two characters were attempted to be produced by mutation, firstly, high salinity tolerance and, secondly, mutants which can leak large amounts of organic carbon, which is assumed to be 70-90% glycerol, into the medium.

The high salinity mutant was expected to retain glycerol in the cells in order to tolerate high salinities up to saturated NaCl concentration. These mutants were simply isolated from UV-treated cells by growing them in saturated NaCl medium (> 5.5 M NaCl). On the other hand, leaky mutants were assumed to have different membrane properties which allow glycerol to pass through the plasma membrane. The procedure for isolating glycerol leaking mutants was modified from Lewin (1988) who suggested the use of bacteria to indicate metabolite leakage from mutant cells. In this experiment, a culture of *Halomonas* sp. strain DUNCON, which was originally found as a contaminant of a laboratory *Dunaliella* culture (Cummings, 1991), was mixed with mutant cells. The colonies surrounded by bacteria were then selected.

5.2 Results and Discussion

5.2.1 UV-mutation and selection of high salinity tolerant and glycerol leaking mutants

It is essential that bacteria used for the detection of glycerol leakage can not grow in normal *Dunaliella* medium which has sodium bicarbonate as the sole carbon source. To test this *Halomonas* was grown in 1.5 M *Dunaliella* medium with or without addition of 0.1 g.l^{-1} glycerol. Figure 5.1 shows that *Halomonas* can not grow in *Dunaliella* medium without the addition of glycerol.
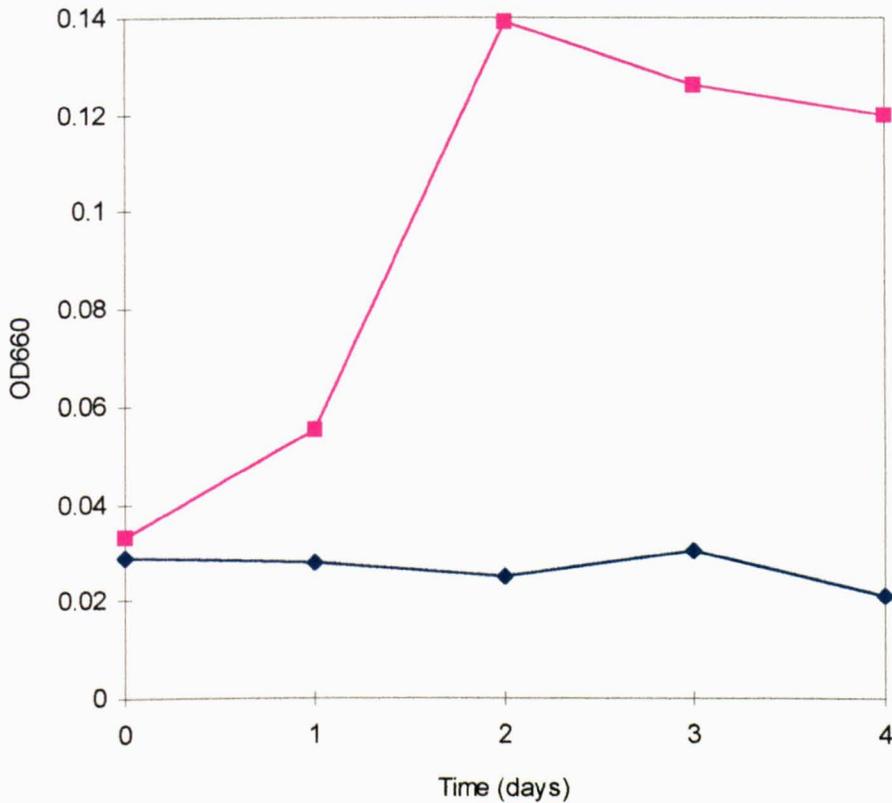
Figure 5.1: Growth of *Halomonas* in 1.5 M NaCl *Dunaliella* medium (◆)and 1.5 M NaCl *Dunaliella* medium supplemented with 0.1 g . l⁻¹ glycerol (■).

UV-mutation was performed as described in section 2.11. The UV intensity was 800 $\mu W.cm^{-2}.s^{-1}$ and a duration of 2.5 minutes was selected for all UV-mutation experiments. These conditions led to a 99% death rate (1% survival as measured by Evan's blue staining). A UV dose-response curve is shown in Figure 5.2. After UV exposure, *D. parva* cells were transferred from Petri dishes into a small flask and kept in the dark at 25°C for 24 hours. The mutants were then allowed to grow under normal growth conditions (1.5 M NaCl, 65 $\mu E.m^{-2}.s^{-1}$ illumination) until there were enough cells for the selection process to be initiated.
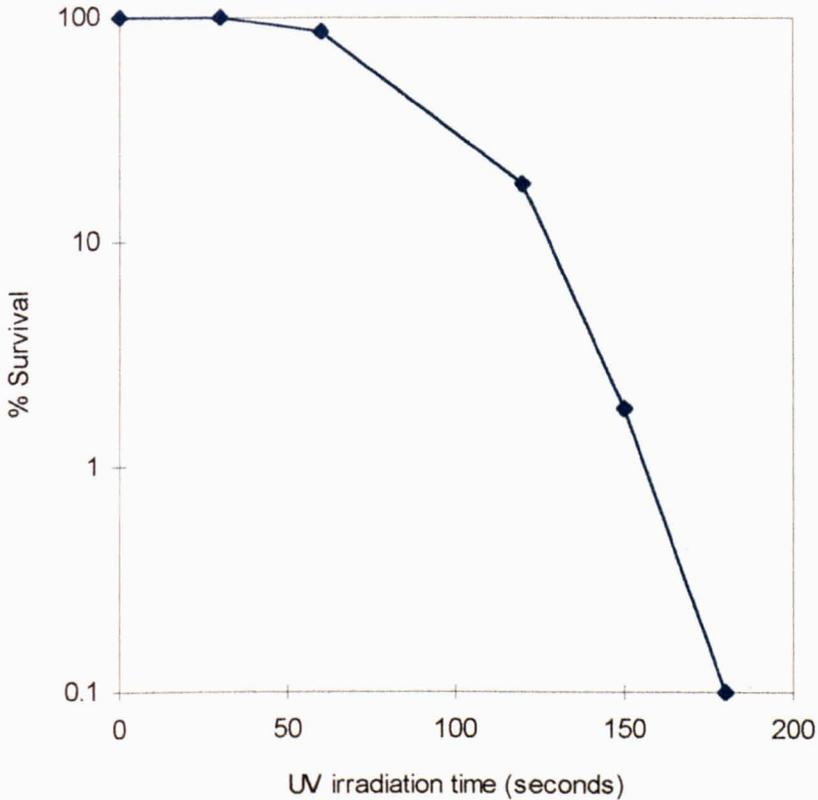
Figure 5.2:. UV-dose response curve for *Dunaliella* mutagenesis. The UV intensity was 800 $\mu W.cm^{-2}.s^{-1}$.

This is the first report of the use of Evan's blue staining to test cell survival in conjunction with UV-dose. In bacteria, which have much faster growth rates than algae, survival of mutants can be easily measured by growing cells on agar plates. Bacterial colonies will normally appear within one or two days. However, to do the same procedure with *Dunaliella*, it needs at least three to four weeks for colonies to be seen under the microscope. Other reports involving mutation in *Dunaliella* such as Ginzburg *et al.*(1995) and Chitlaru and Pick (1989), did not state the dose response

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curve in their experiments. So, Evan's blue staining gives an advantage in allowing the direct counting of cell death and give a UV dose-response curve which can be generated quickly.

After UV mutagenesis, organic carbon leaking mutants were screened by mixing with molten agar and *Halomonas* culture and then poured to make the top layer of a double layer agar plate (see Section 2.12.2). Colonies usually appeared within three to four weeks after plates were inoculated (Figure 5.3). At this stage, under a stereo microscope, the algal colonies could be easily seen as greenish spots surrounded by white bacterial colonies. By estimation, approximately 95% of all colonies were surrounded by bacteria. Figure 5.4 shows a close up photograph of an algal colony with bacteria while Figure 5.5 shows an algal colony without bacteria. Colonies, both with bacteria and without bacteria surrounding them, were picked up by a heat-stretched micropipette under the microscope and were transferred into normal 1.5 M NaCl medium. The colonies with bacteria were assumed to be leaking glycerol while the colonies without bacteria were assumed to be non-leaking mutants.

After isolation, all strains were observed under the microscope and were found to be heavily contaminated with *Halomonas*. Therefore, all strains were diluted with fresh medium and re-isolated on double-layer agar plates by single colony isolation. Cultures of all mutants were then checked until they were free from bacteria. Only clones that were successfully cleaned from bacterial contamination were then used for further experiments.

On the other hand, for high salinity tolerance selection, mutants were selected in saturated salt medium and finally isolated by streaking on double layer agar plates to obtain a clonal culture. However, as *Dunaliella* has a very slow growth rate in high salinity, so the salinity was reduced to 1.5 M NaCl before performing the clone isolation on the agar plates.
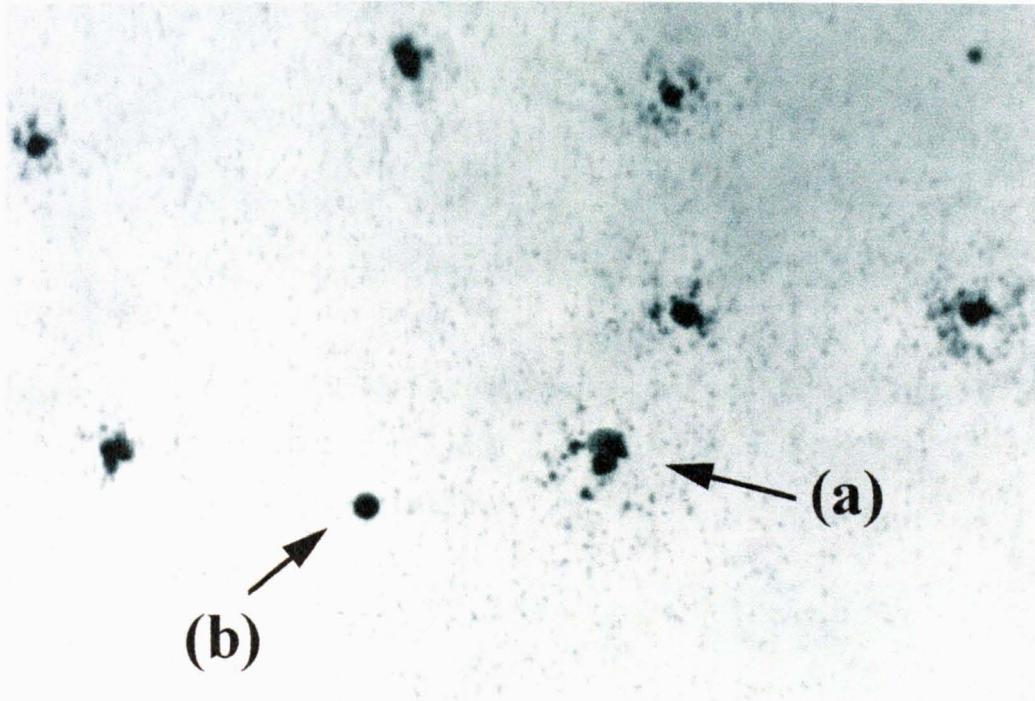
Figure 5.3: A photograph of *Dunaliella* colonies in a double layer agar plate. **(a)** indicates a colony surrounded by bacteria and **(b)** is a colony without bacteria.
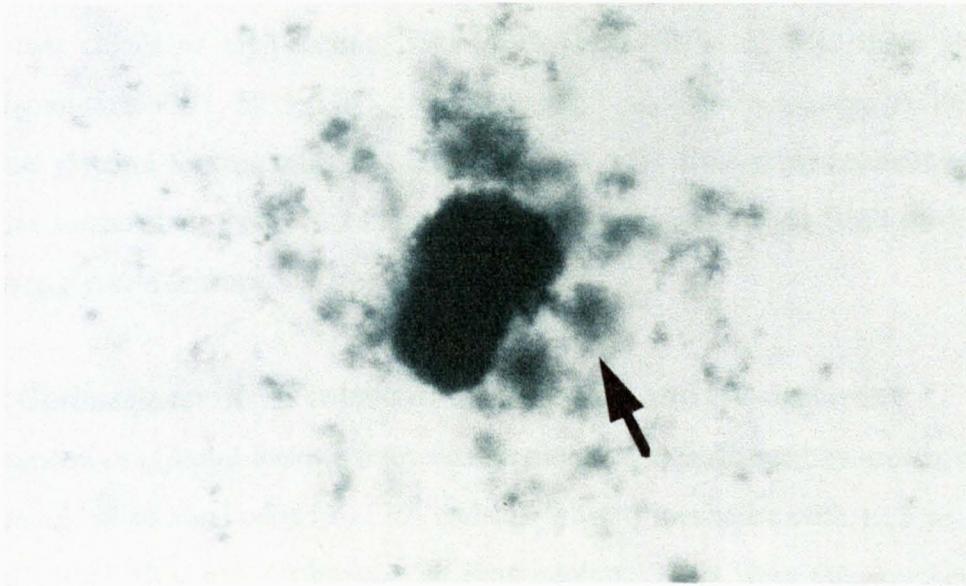
Figure 5.4: Close up of *Dunaliella* colony surrounded with bacterial colonies. This was then isolated as a high glycerol leakage mutant (HG). The arrow indicates bacterial colonies.
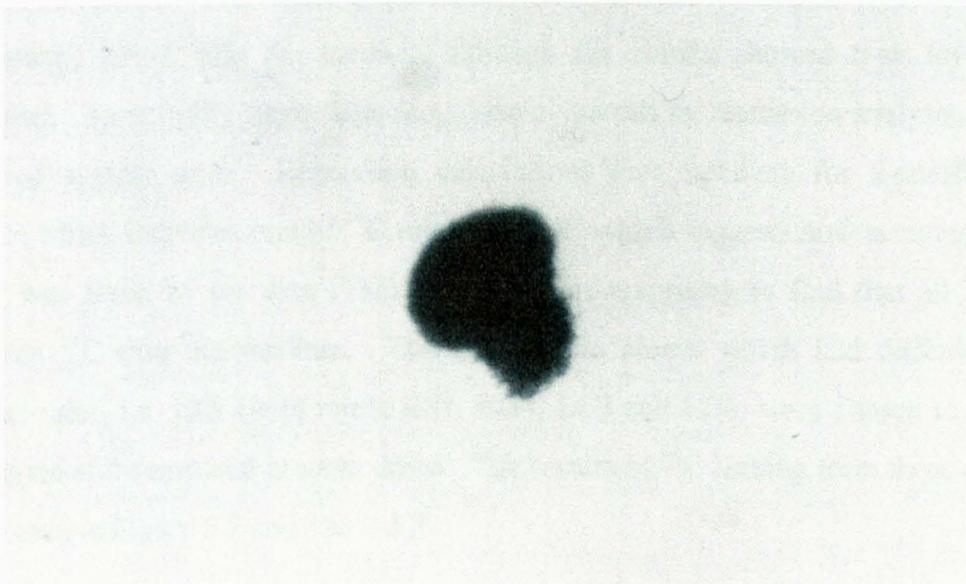
Figure 5.5: Close up of *Dunaliella* colony without surrounding bacteria. This was then isolated as a low glycerol leakage mutant (LG).

Finally, several single clone mutants in *Halomonas* free culture were achieved. There were two clones of high salinity tolerant mutants; HS1 and HS2, high glycerol leaking mutants HG1, HG3, HG4, HG6 from algal colonies surrounded by bacteria, and low glycerol leaking mutants LG1, LG3, LG4, LG6 from algal colonies without bacteria surrounding them. All clones were then grown in 1.5 M NaCl medium to compare glycerol leakage.

5.2.2 Comparison of ^{14}C released by wild type and UV-mutants

Comparison of glycerol leaking from various clones was performed by measuring ^{14}C release. 5 ml of algal cells (1×10^7 cells.ml^{-1}) were incubated with 12.5 μl of 0.1 μCi.μl^{-1} NaH^{14}CO$_3$ for 16 hours with illumination. Cells were then washed with fresh medium and radioactivity in the medium from released ^{14}C was measured after separating cells from medium by silicone oil centrifugation (Section 2.10.3).

Figure 5.6 shows the amount of ^{14}C released from various clones. It was found that all clones released ^{14}C, which was assumed to be at least 80% glycerol (Frank and Wegmann, 1974), into the medium although the results showed high levels of variation. To simplify these data, they were evaluated by regression analysis of ^{14}C released against time. Regression calculations give numbers for x-coefficients (slope) which indicates rate of ^{14}C release and R^2 which suggests how accurately the slope was fitted by the data (Table 5.1). It was surprising to find that all clones released ^{14}C into the medium. Therefore, some clones which had different ^{14}C release rates, i.e. 19/9 (wild type), HS1, HG4, LG3 and LG6, were chosen to be re-examined and compared in more detail. The results of ^{14}C leaking form those clones are shown in Figure 5.7 and Table 5.2.

Unfortunately, the results in Figure 5.7 and Table 5.2 do not exactly correspond to data shown in Figure 5.6 and Table 5.1. This was due to variation in the amount of ^{14}C in samples. However, all results suggest that there was leakage of cellular metabolite(s) from *D. parva* and *D. salina*.
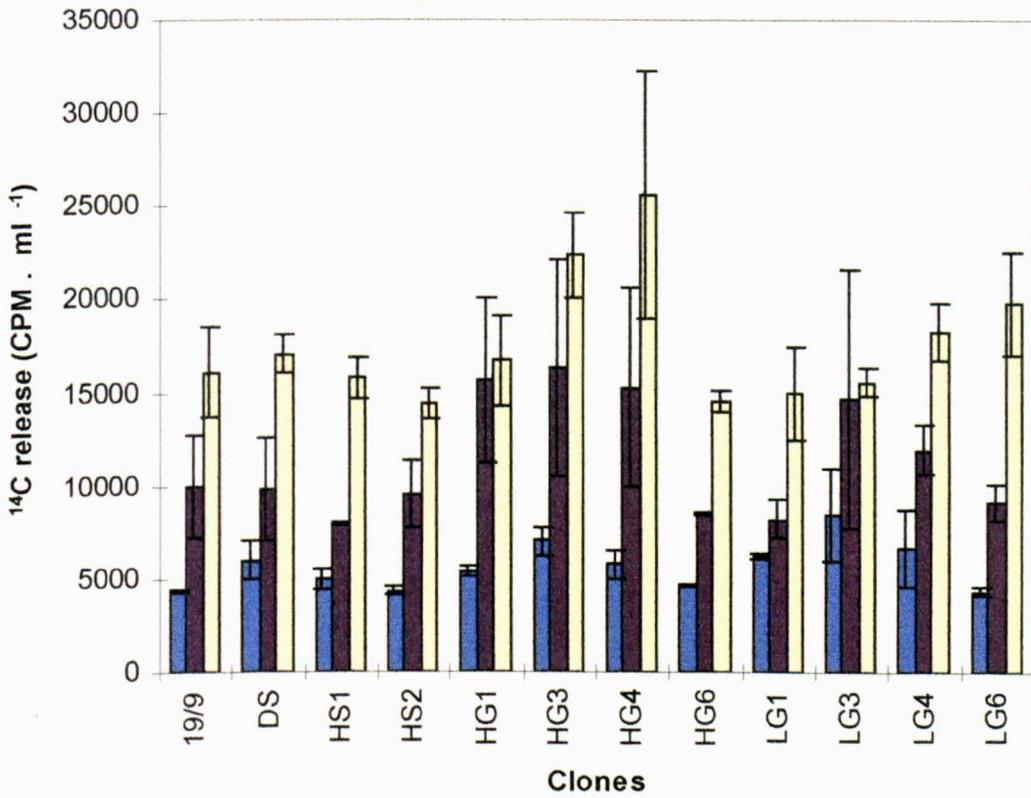
Figure 5.6: ^{14}C release from various clones of *Dunaliella*. The first bar was 3 minutes after resuspension in fresh non-radioactive 1.5 M NaCl medium, the second bar was after 6 hours and the third bar was after 24 hours.

Table 5.1: Regression analysis of ^{14}C release from various clones of *D. parva* wild type and mutants. Wild type *D. salina* strain DS91008 was also included in this experiment.

Clones	X-coefficients (slope)	R^2
D. parva (wild type)	7.58	0.67
D. salina DS91008	7.45	0.75
HS1	7.46	0.95
HS2	6.15	0.76
HG1	5.49	0.27
HG3	9.51	0.51
HG4	12.85	0.54
HG6	6.57	0.96
LG1	6.09	0.83
LG3	3.96	0.13
LG4	7.53	0.75
LG6	10.52	0.87

Table 5.2: Regression analysis of ^{14}C releasing from some clones of *D. parva* wildtype and mutants clones.

Clones	X-coefficients (slope)	R^2
D. parva (wild type)	17.02	0.78
HS1	5.62	0.96
HG4	6.74	0.97
LG3	7.19	0.85
LG6	6.57	0.78
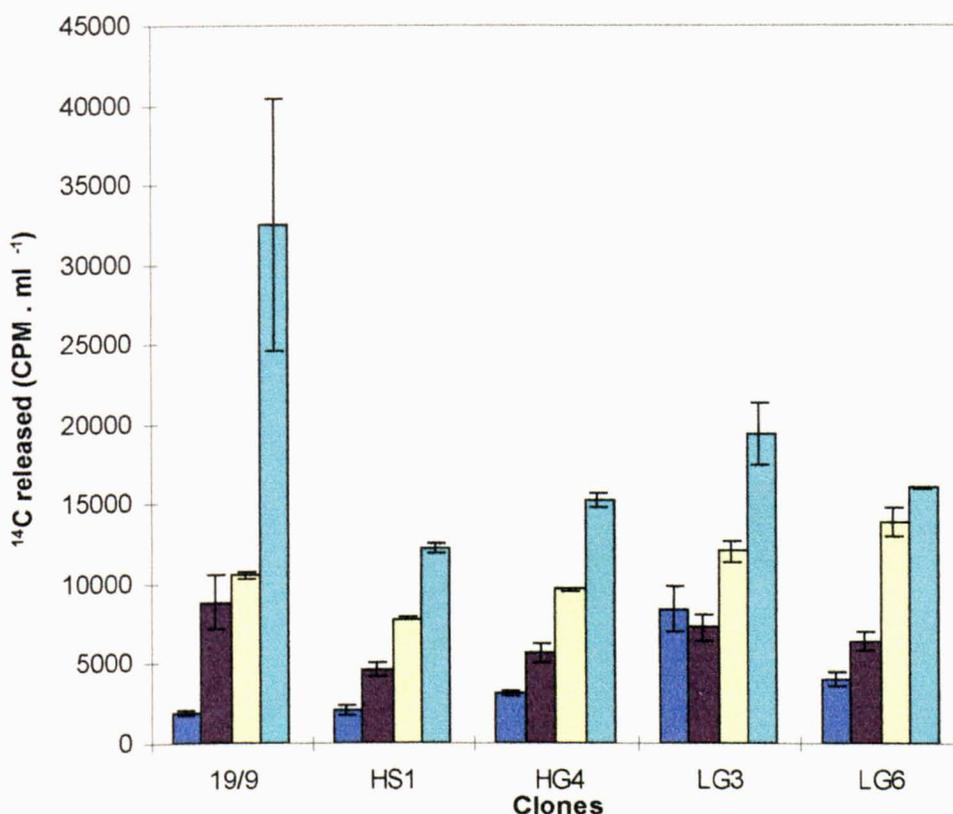
Figure 5.7: A repeat experiment showing ^{14}C release from some clones of *Dunaliella*. The first bar was 3 minutes after resuspension in fresh medium (1.5 M NaCl), the second bar was after 4.5 hours, the third bar was after 12 hours and the fourth bar was after 29 hours.

5.2.3 Comparison of growth between wild type and UV-mutant strains in different salinities

Comparison of growth rates of mutant and wild type strains in different salinities, i.e. 0.5, 1.5, and 3.5 M NaCl, are shown in Figures 5.8, 5.9 and 5.10 respectively. It was found that, in all salinities, there was no significant difference in growth rate (as indicated by slope of the growth curve) of mutants and wild type strains.
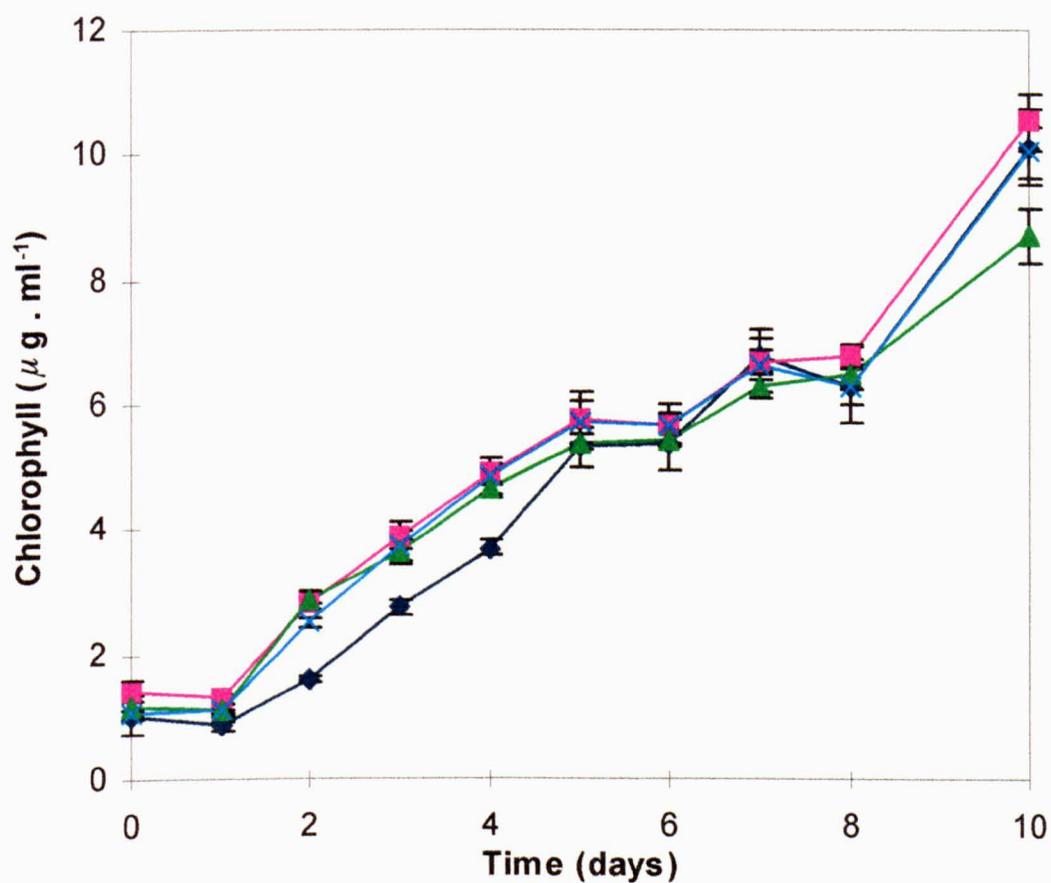
Figure 5.8: Growth of various clones of *Dunaliella* in 0.5 M NaCl medium.

(◆) *D. parva* (wild type)

(■) HS1 (mutant)

(▲) HG4 (mutant)
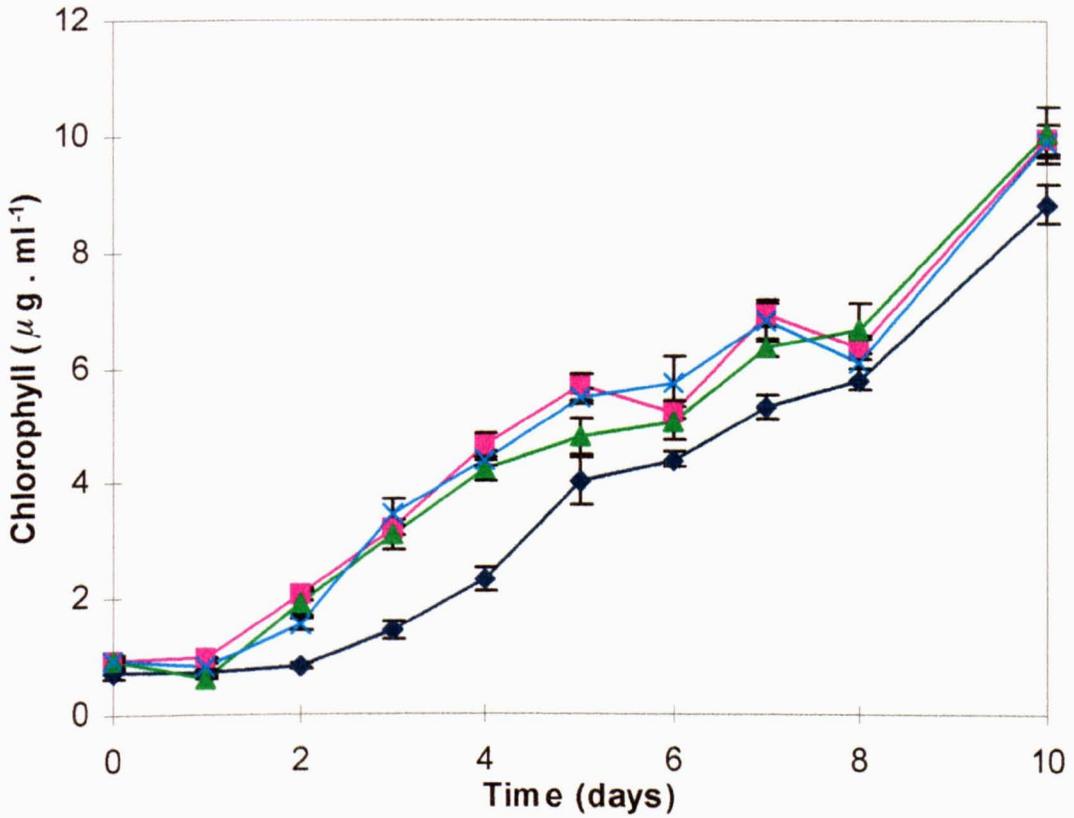
(✕) LG4 (mutant)

Figure 5.9: Growth of various clones of *Dunaliella* in 1.5 M NaCl medium.

(◆) *D. parva* (wild type)

(■) HS1 (mutant)

(▲) HG4 (mutant)
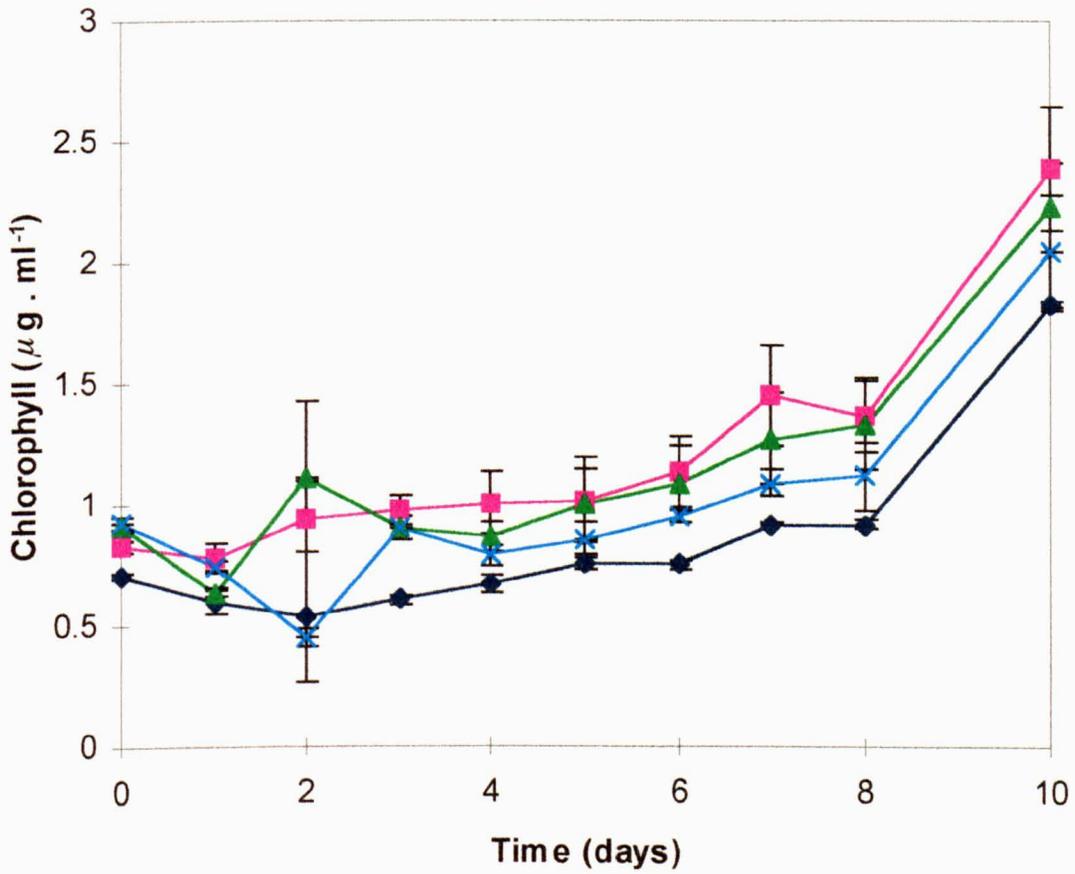
(✗) LG4 (mutant)

Figure 5.10: Growth of various clones of *Dunaliella* in 3.5 M NaCl medium.

(◆) *D. parva* (wild type)

(■) HS1 (mutant)

(▲) HG4 (mutant)

(✕) LG4 (mutant)

In designing this experiment, it was assumed that *D. parva* shows optimum growth at a moderate to high salt concentration i.e. 1.5-2.0 M NaCl. Brown and Borowitzka (1979) stated that there is no comprehensive study of salinity tolerance in this species and they referred to Ben-Amotz and Avron (1973) who had cultured *D. parva* within a range of 4-12% NaCl (0.6 - 2 M).

Ginzburg *et al.* (1995) found no growth of *D. parva* 19/9 in a medium containing 2.5 M NaCl. Oren and Shilo (1985) observed a bloom of *D. parva* in an experimental pond containing water from Dead Sea. They observed that *D. parva* bloomed in natural Dead Sea water of specific gravity between 1.20-1.22 (approximately 3.4-3.7 M NaCl). However, at the same salt concentration, *D. parva* could not be grown in the experimental ponds. Therefore the evidence in the literature suggested that *D. parva* is not as tolerant to salinity as *D. salina*.

However, in the present study, it was found that not only *D. salina*, but also *D. parva* wild type and clones can grow in saturated NaCl medium. Nevertheless, growth rate of *D. parva* in saturated salt is very slow and has been reported as no growth by previous publications. Growth rate of *D. parva* in saturated NaCl medium is shown in Table 5.3

Table 5.3: Growth rate of *D. parva* in saturated NaCl medium.

	D. parva (WT)	HS1	HG4	LG3	*D. salina* (DS91008)
Specific growth rate (day)	0.071	0.045	0.060	0.084	0.0650
Doubling time (day)	9.7	15.4	11.6	8.3	10.6

Therefore the use of high salinity to isolate mutants is not feasible because *D. parva* can also grow in saturated salt. From Table 5.3, *D. salina* did not appear to show a faster growth rate than *D. parva* in saturated NaCl medium. In natural environments, *D. salina* becomes the dominant species in hypersaline lakes in conjunction with *D. viridis*. *D. viridis* will be virtually absent in salinities higher than 300 g.l^{-1} or 5 M NaCl (Moulton *et al.*, 1987). These two species can be easily distinguished as *D. salina* has a larger cell size and has a deep orange colour due to β-carotene accumulation. On the other hand, *D. parva* is a dominant species in the Dead Sea and sometimes has been confused with *D. salina*. For example, Moulton *et al.* (1987) and Borowitzka and Borowitzka (1988) suggested that *D. salina* used in work reported by Ben-Amotz and Avron (1983a) is probably *D. parva*. They suggest the capability to accumulate some β-carotene and turn "yellowish" (rather than orange or red in *D. salina*) is a good criterion for identifying *D. parva*. However, *D. parva* has never been reported as a dominant species in hypersaline lakes anywhere else except in the Dead Sea.. Although there are clear differences in physiology and morphology among *Dunaliella* species, Nakas *et al.* (1983) reported that there was no significant difference in glycerol accumulation from five species of *Dunaliella* (*D. parva*, *D. salina*, *D. bardawil*, *D. tertiolecta* and *D. primolecta*) grown in 2 M NaCl.

In this research, *D. salina* DS91008 changed its colour to orange when grown in a medium containing 4.5 M NaCl at 25°C and 66 μE.m^{-2}.s^{-1} illumination. Under the same conditions, *D. parva* CCAP 19/9 does not change its colour so it remains green and it has a smaller cell size than *D. salina* at all salinities. These characteristics indicate that these two species are different.

5.3 Conclusions

It has to be noted that one problem in making *Dunaliella* mutant is that there are not yet available any auxotrophic mutant selection protocols for this alga. In *Chlamydomonas*, which has been intensively studied, inhibitors such as penicillin, bromouracil or 8-azaguanine were used to isolate auxotrophs which did not grow in minimal media (Harris, 1989). Auxotroph mutants are used to confirm mutagenesis and indicate the frequency of mutation occurring after UV irradiation. However, the *Chlamydomonas* technique can not be applied to *Dunaliella* and it was ignored in the present experiments.

Mutant and wild type strains of *D. parva* did not show any difference in morphology. Although a determination of frequency of mutation was not performed in this study, it could reasonably be assumed that mutant strains obtained after UV mutagenesis must receive some effect from UV exposure. Unfortunately, no mutations showed an effect on either salt tolerance (Figure 5.8 - 5.10) or glycerol leakage (Figure 5.6 and 5.7).

As both mutant and wild type strains of *D. parva* exhibited the same capability to grow in saturated NaCl medium and leak glycerol into the medium, it would appear that the attempt to produce different levels of glycerol leakage by mutagenesis was not a success. The results show that glycerol is always released from *Dunaliella* as a normal characteristic of growth. This idea was supported by many experimental results in this chapter and also in previous chapters which demonstrated glycerol leakage from *Dunaliella* cells in both normal growth conditions or entrapped in calcium alginate beads.

Attempts to select cells by growing mutant cells in saturated NaCl medium is not the correct procedure. This is because *D. parva* was later found to be capable of growth in saturated NaCl medium. On the other hand, a selection technique using bacteria as

an indicator is an interesting technique. Unfortunately, because all *Dunaliella* cells leaked glycerol into the medium, this meant that most colonies were surrounded by bacteria. The failure of this technique, in which there were some 5% of the colonies without bacterial encirclement, might be because of poor mixing of algal cells with bacterial cells in the upper layer of the agar plate. However, this technique has a high potential to be applied for other purposes in the future.

In general, although there have been intensive studies concerning glycerol leakage after hypoosmotic shock in *Dunaliella*, the mechanism of glycerol transport in *Dunaliella* is not well understood. However, if there is no specific transport system for glycerol in *Dunaliella* plasma membrane, glycerol might pass through the membrane driven by a gradient between glycerol concentration outside and inside the cells. This can cause glycerol leakage after cells are resuspended in fresh medium of the same salinity. So, it is interesting that glycerol leakage from *Dunaliella* might not be an active transport process. This area is investigated in more detail in the next chapter.

CHAPTER 6:
INVESTIGATION OF GLYCEROL LEAKAGE FROM *Dunaliella*

6.1 Introduction

All species of *Dunaliella* accumulate glycerol as their compatible solute when grown in high salinity. There are several reports that have investigated the leakage of glycerol from *Dunaliella* and most of these studies suggested that no glycerol was released from growing cells without hypoosmotic shock, due to special properties of the cell membrane (Ben-Amotz and Avron, 1973; Brown and Borowitzka, 1979; Gimmler and Hartung 1988; Avron, 1992). However, the results shown in all three previous chapters in this study suggest that large amounts of glycerol are present in *Dunaliella* culture medium during normal growth (exponential phase) for both immobilised cells and free cell cultures. Therefore, further investigations into the characteristics of glycerol release or leakage in *Dunaliella* species are described in this chapter.

6.2 Results and Discussion

6.2.1 Effect of HEPES Buffer on Glycerol Leakage

Concentration of glycerol in this study was determined by acetylacetone method (Ben-Amotz and Avron, 1978) which is more practical and economical than the enzymatic method. However, as Tris buffer in normal culture medium can interfere with the acetylacetone method, HEPES buffer was used in the growth medium instead of Tris (see Section 2.2 and Appendix A). Hence, a preliminary experiment was carried out to determine the effect of different buffers on glycerol leakage (Table 6.1). In this experiment, glycerol was determined by enzymatic method (Section 2.8.2). The initial pH was 7.38, 7.39 and 7.27 for no buffer, 20 mM HEPES and 20 mM Tris (control), respectively.

Table 6.1: Effect of buffers on glycerol leakage from *D. parva*

Day	no buffer		20 mM HEPES		20 mM Tris	
	% leakage	pH	% leakage	pH	% leakage	pH
4	69.3 ± 8.90	9.41	53.1 ± 0.03	9.43	54.9 ± 7.10	8.76
7	58.8 ± 10.1	9.41	58.3 ± 6.33	9.25	47.3 ± 2.76	8.78

It was found that when 20 mM Tris was used in the medium, the pH of the culture medium was kept closer to neutrality. However, leakage of glycerol was found in all experimental groups as, after 4 days, over 50% of glycerol produced was detected in the medium. Hence, as there was no significant difference in glycerol leakage, HEPES was used instead of Tris in all of the following experiments.

In addition, as 20 mM HEPES was found to be not as good as Tris in controlling the pH of culture medium (Table 6.1), it was decided that higher concentrations of HEPES were required. Therefore, another experiment was carried out to examine the effect of HEPES concentrations on pH of the medium and glycerol leakage from *D. parva* (Table 6.2).

Table 6.2: Effect of HEPES concentrations on glycerol leakage from *D. parva*

Day	no buffer		20 mM HEPES		50 mM HEPES	
	% leakage	pH	% leakage	pH	% leakage	pH
1	39.85 ± 1.09	8.50	34.30 ± 0.41	7.89	38.06 ± 1.63	7.63
3	51.69 ± 0.78	9.62	39.29 ± 1.88	9.53	41.58 ± 1.38	8.08

A concentration of 50 mM HEPES gave better pH control and did not affect the glycerol leakage compared to 20 mM HEPES. Therefore 50 mM HEPES was used in the following experiments.

6.2.2 Glycerol Leakage from *D. parva* Cells Growing in Dialysis Tube

This experiment aimed to confirm the leakage of glycerol from *Dunaliella* without any physical impact to the cells during sampling. *D. parva* culture, 24.1 ± 1.25 µg Chl.ml^{-1}, was centrifuged and resuspended in fresh 1.5 M NaCl medium with 50 mM HEPES. Thereafter, 5 ml of culture was placed in a dialysis tube and then the tube was suspended in a 100 ml flask containing 50 ml of fresh 1.5 M NaCl medium. Culture was kept in 25°C growth room with continuous illumination (70 µE.m^{-2}. s^{-1}). Medium was sampled for glycerol analysis and the results are shown in Table 6.3.

Table 6.3: Glycerol concentration in the medium of *D. parva* growing in dialysis tube.

Hour	Glycerol (g.l^{-1})
0	0.038 ± 0.002
23	0.386 ± 0.001
64	0.421 ± 0.007
92	0.445 ± 0.003

As glycerol was found to accumulate in the medium, the result confirmed that the glycerol released from *Dunaliella* was not due to breakage of cells during centrifugation.

6.2.3 Glycerol Leakage from *D. parva* under Normal Growth Conditions

Normal growth conditions in most of the experiments in this chapter were as follows:

- *Dunaliella* medium [(Hajibagheri *et al.* (1986); Blackwell and Gilmour (1989) composition of the culture medium is shown in Appendix A] with 1.5 M NaCl and 50 mM HEPES buffer.
- Temperature = 25°C
- Continuous illumination by white fluorescence lamps at 65-70 µE.m^{-2}.s^{-1}

- Static culture with daily mixing by hand.

- Growth was monitored by measuring chlorophyll concentration.

Growth of *D. parva* and glycerol concentration in the medium are shown in Figure 6.1. It was found that glycerol accumulated in the medium in parallel with cell growth in batch culture. During this period, no dead cells were detected by Evan's blue staining. During medium sampling for glycerol analysis, cell breakage was kept to a minimum by centrifugation at 1500 *g* for 15 minutes which is the speed recommended by Enhuber and Gimmler (1980). Cell viability was observed before and after centrifugation and no adverse effects of centrifugation were detected. Supernatant was then centrifuged repeatedly to ensure that there are no cells left in the medium before sampling for glycerol analysis. Therefore, it seems clear that glycerol was released from *D. parva* under normal culture conditions (Figure 6.1).

Hard and Gilmour (1991) isolated a mutant strain of *D. parva* from a chemostat running with a high rate of CO_2 bubbling. This was reported as an environmental induced mutant which leaked large amounts of glycerol into the medium. In the present study, an experiment was set up to evaluate the effect of CO_2 on glycerol leakage. Wild type strain of *D. parva* CCAP 19/9 was purchased again from Culture Collection of Algae and Protozoa specially for this experiment. This was to prevent any possibility that an environmental mutation had occurred with the wild type culture which had been kept in this laboratory for many years.

In this experiment, *Dunaliella* cells was grown in glass bottles containing 500 ml of 1.5 M NaCl medium with 50 mM HEPES. Each bottle was sealed with a rubber plug assembly with two glass tubes, the first long tube reaching to the bottom of the bottle was for air (or CO_2) inlet and another short tube with a cotton wool filter was for the air outlet. Air or 5% CO_2 + 95% N_2 was bubbled into the bottles at 100 ml min^{-1}. The experiment was carried out in a temperature controlled water bath at 25°C with continuous illumination (approximately 100 $\mu E.m^{-2}.s^{-1}$) from both sides.
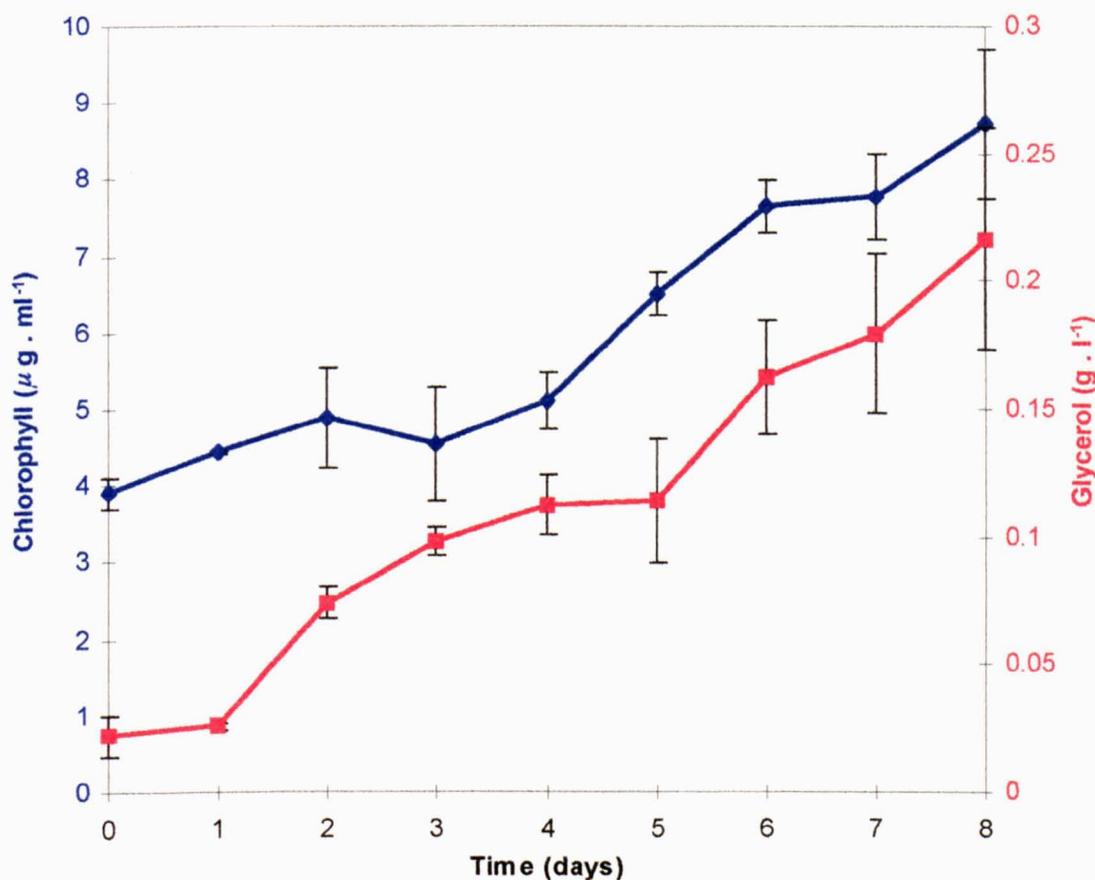
Figure 6.1: Growth of *D. parva* and glycerol release into the medium under normal culture conditions (1.5 M NaCl, 25°C with 65 μE.m^{-2}.s^{-1} illumination).

Growth of *D. parva*, represented by chlorophyll concentration, and glycerol leakage from the culture are shown in Figures 6.2 and 6.3 respectively. Figure 6.2 shows that *D. parva* grew rapidly in the presence of CO_2 bubbling and the chlorophyll concentration was more than 3 fold the level found with air bubbling. Consequently, glycerol concentration in culture medium and in cells plus medium was correspondently higher in CO_2 enriched culture with high cell biomass (Figure 6.3).

During the first day of culture, there was some glycerol present in the inoculum so this was possibly the reason that the leakage appeared too high. The effect of the inoculum declined as more glycerol was produced along with cell growth. Therefore, the most reliable data for glycerol leakage was taken after day 4 when glycerol concentration in both cells and medium was high enough. The result show that glycerol leakage varied from 35-90% during growth and that up to day 11 there was no difference between air and CO_2 cultures. However, at day 12, CO_2 culture was found to be leaking up to 90% of glycerol produced into the medium while the air bubbled culture had around 70% glycerol leakage (Figure 6.3). It was evident that higher glycerol leakage was found when cells reached the exponential phase.
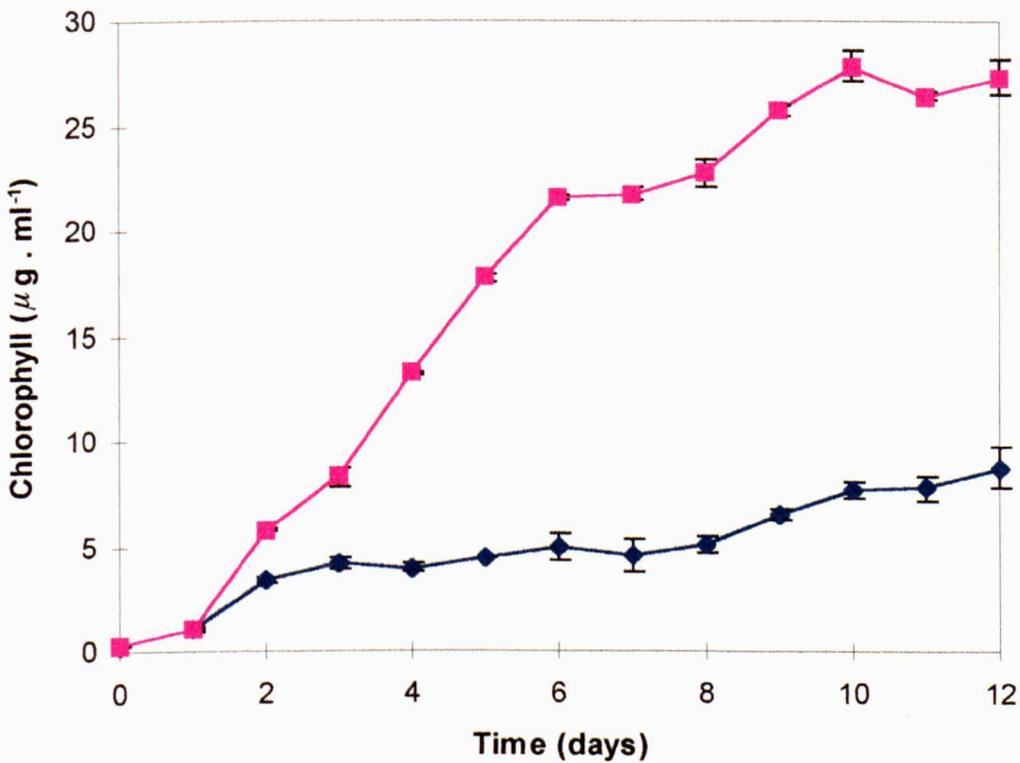
Figure 6.2: Growth of *D. parva* when bubbled with 5%CO_2 + 95% N_2 (■)
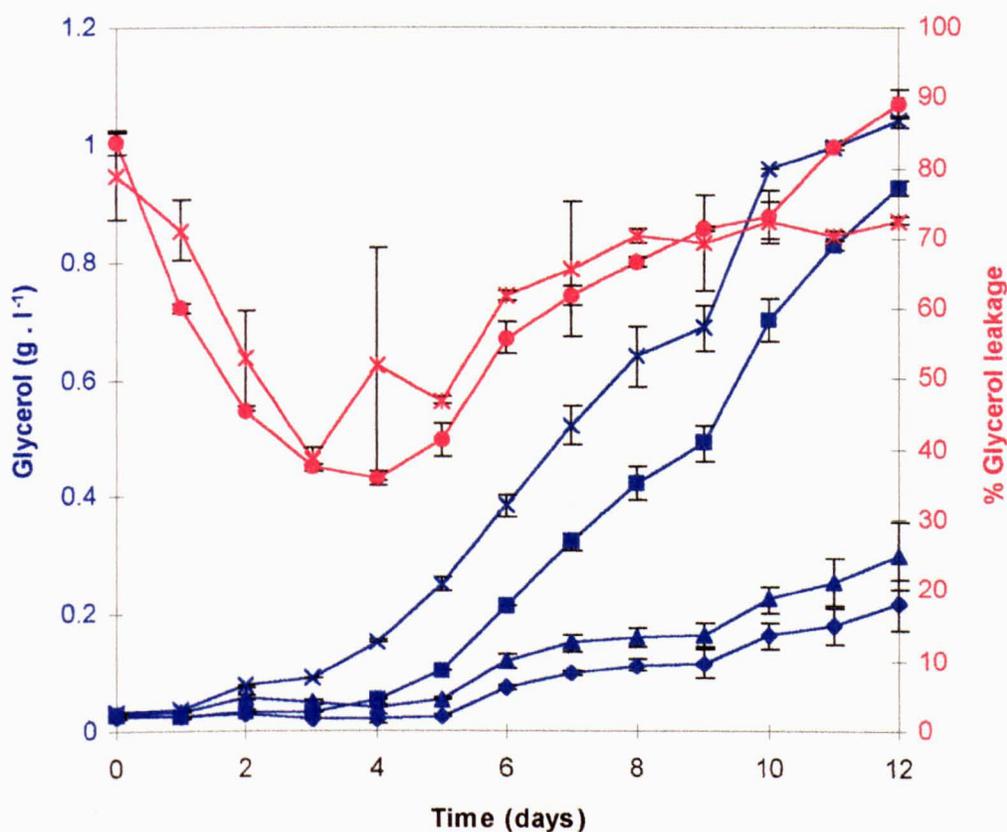
or air (◆).

Figure 6.3: Glycerol leakage from *D. parva* bubbled with 5% CO_2 + 95% N_2 or air.

Blue line and the left Y-axis indicate amount of glycerol while the red line with the right Y-axis are glycerol leakage

    (■) glycerol in the medium of CO_2 enriched culture

    (✗) glycerol in the cells plus medium of CO_2 enriched culture

    (◆) glycerol in the medium of air bubbled culture

    (▲) glycerol in the cells plus medium of air bubbled culture

    (●) percent of glycerol leakage from CO_2 enriched culture

    (✱) percent of glycerol leakage from air bubbled culture

Concentration of CO_2 in culture medium could affect the ultrastructure of the cells especially pyrenoid starch granules and the chloroplast (Tsuzuki *et al.*, 1986). However, as air bubbled cultures of the wild type strain of *D. parva* 19/9 could release more than 70% of its glycerol into the medium, there was no clear evidence to support an effect of CO_2 on glycerol leakage. Pattern of protein composition on SDS PAGE gel (Figure 6.4) illustrates a band of a 60 kDa protein found only in air grown culture. However, as there was no clear difference in glycerol leakage between air and CO_2 culture, this protein was presumably not involved in glycerol transport. Therefore, it was assumed to be a protein connected with the CO_2 concentrating mechanism (CCM) induced by low CO_2 and somehow related to carbonic anhydrase enzyme and plasma membrane ATPase (Aizawa and Miyachi, 1986; Badger and Price, 1992). Several CCM polypeptides with different molecular weights were reported in many algal species such as *Scenedesmus* (Thielmann *et al.*, 1990), *Chlorella* (Satoh and Shiraiwa, 1996), *Chlamydomonas* (Villarejo *et al.*, 1996; Geraghty and Spalding, 1996) and also in *D. tertiolecta* (Ramazanov *et al.*, 1995).

It is a strong possibility that the 60 kDa protein found in this study might be the same as the 60 kDa membrane protein induced in low CO_2 and high salt conditions in *D. salina* as reported by Fisher *et al.* (1996). This 60 kDa protein was found in *D. salina* when growing in CO_2 limited culture and it was identified as a highly salt-resistant form of carbonic anhydrase that helps to optimise CO_2 uptake in cells growing in high salinity. Fisher *et al* (1996) suggested that it is an internally duplicated carbonic anhydrase with each repeat homologous to animal and *Chlamydomonas reinhardtii* carbonic anhydrase, but exceptional in the excess of acidic over basic residues.
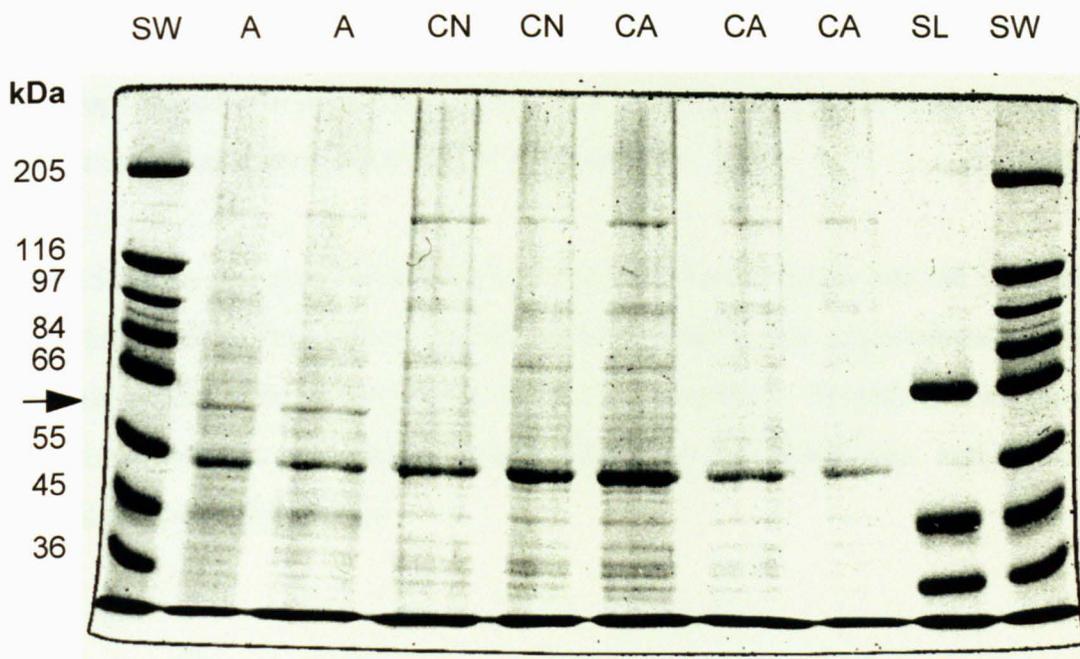
Figure 6.4: SDS PAGE of crude protein extract from *D. parva* growing in 1.5 M

NaCl medium with different types of CO_2 supplement. An arrow

indicates a 60 kDa protein found only in air grown culture.

       A = Air bubbled

       CN = 5% CO_2 + 95% N_2

       CA = 5% CO_2 + 95% air

       SW = Standard molecular weight (wide range)

       SL = Standard molecular weight (low range)

6.2.4 Comparison of Glycerol Leakage from *D. parva* and *D. salina*

A mutant that leaked large amounts of glycerol into the medium, reported by Hard and Gilmour in 1991, was a mutant of *D. parva* CCAP19/9. However, as shown in the previous section the wild type strain of *D. parva* 19/9 also released large amounts of glycerol. Therefore, it was decided to compare the glycerol leakage pattern of *D. parva* 19/9 with another species, *D. salina*. These two species have different morphology and cellular physiology which can be distinguished easily by observation under the microscope (see section 1.1 for more details).

Figure 6.5 shows that glycerol leakage was also found in *D. salina* and the glycerol leakage followed the same pattern in both species. After 4 days, approximately 40% of glycerol produced by each alga was found in the medium. Therefore, it appears that glycerol leakage is a normal characteristic for *Dunaliella* spp. and is not restricted to a particular species.
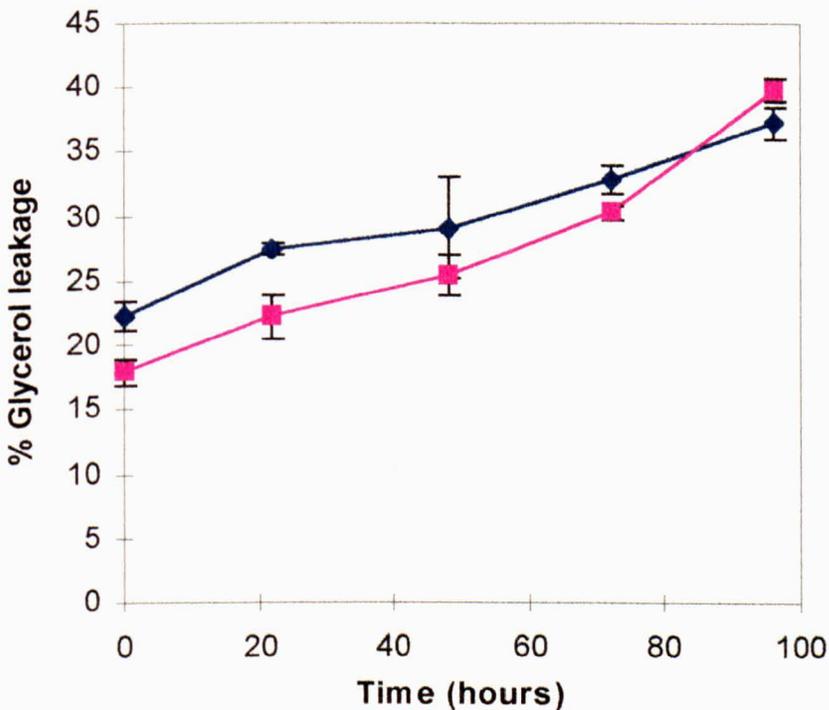
Figure 6.5: Glycerol leakage from *D. parva* (♦) and *D. salina* (■).

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6.2.5 Effect of Salinity on Glycerol Leakage from *D. parva*

As *Dunaliella* accumulates glycerol to balance the osmotic potential of external salinity, controlling glycerol leakage may be vital for cells grown in high salinity. When *D. parva* cells were cultured at two different salinities, *i.e.* 1.5 M and 3.5 M NaCl, as expected less glycerol was released from 3.5 M NaCl grown culture (Figure 6.6). This confirms the vital role of glycerol in maintaining the osmotic balance. However, as glycerol leakage was also found in high salinity culture (3.5 M NaCl) it could be suggested that glycerol is always overproduced, even in high salinity, and *Dunaliella* cells leaked excess glycerol into the medium.
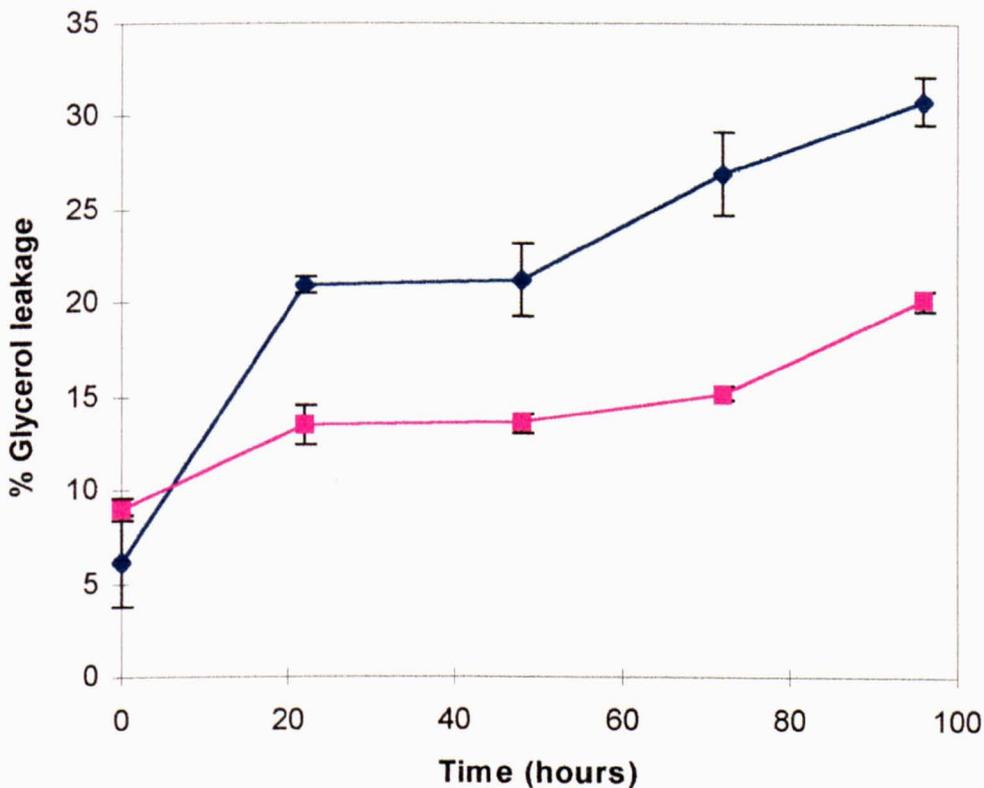
Figure 6.6: Glycerol leakage from *D. parva* grown at different salinities.

(◆) 1.5 M NaCl

(■) 3.5 M NaCl

6.2.6 Effect of Osmotic Shock on Glycerol Leakage from *D. parva*

6.2.6.1 Hypoosmotic Shock Compared to Resuspension in Fresh Medium of Same Salinity

It is well known that *Dunaliella* release glycerol when exposed to hypoosmotic shock (Ben-Amotz and Avron, 1973; Brown *et al.*, 1982). However, the experiments in the previous chapter (Section 5.2.2) showed that glycerol was also released from *Dunaliella* cells after resuspension in fresh medium with the same salinity. This experiment was, therefore, conducted in order to examine the characteristics of glycerol leakage from *D. parva* after resuspension in fresh medium (with or without hypoosmotic shock) by direct measurement of glycerol concentration in the cells and in the medium.

Figure 6.7 shows that hypoosmotic shock from 1.5 M to 0.75 M NaCl induced a large leakage of glycerol within the first minute. When a large amount of glycerol was already present in the medium at the first minute, percentage glycerol leakage was found to remain constant at approximately 30% throughout the 8 hour experimental period. On the other hand, *D. parva* cells resuspended in fresh 1.5 M NaCl medium also released glycerol but at a lower concentration. In this treatment, glycerol leakage increased from 6.5% to 15% in 8 hours. This indicated that glycerol was being accumulated in the medium.

6.2.6.2 Accumulation of Fixed ^{14}C in *D. parva* after Incubation with NaH^{14}CO$_3$

To study glycerol leakage over a short time period, especially less than 1 hour after replacing medium, direct glycerol analysis by acetylacetone method did not seem to be sensitive enough when there was a low concentration of glycerol in the medium. Therefore, radioactive carbon labelling experiments were applied instead. This was performed by incubating *D. parva* culture with NaH^{14}CO$_3$ under illumination and then measuring ^{14}C release. However, it is necessary to ensure that ^{14}C was fixed into

organic carbon by determining the amount of organic ^{14}C and the amount of ^{14}C remaining as $H^{14}CO_3^-$ associated with the cells. So, a preliminary experiment was needed to examine the amount of radioactive organic carbon fixed by *Dunaliella* cells.
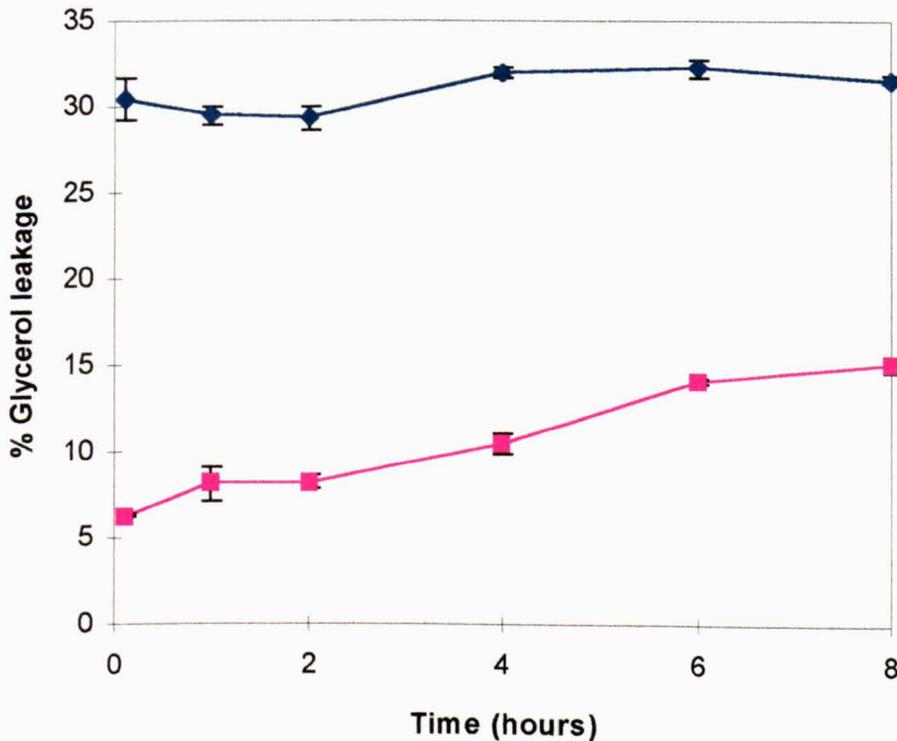
Figure 6.7: Glycerol leakage from 1.5 M NaCl culture of *D. parva* after exposure to fresh medium at the same salinity (■) and hypoosmotic shock with 0.75 M NaCl medium (◆)

To determine the organic ^{14}C fixation in *Dunaliella*, algal cells (20 ml, 1×10^7 cell. ml^{-1}), after being incubated with 5µCi NaH^{14}CO$_3$ for 6 hours, were separated from the culture medium by silicone oil centrifugation. Percentage organic ^{14}C fixation was calculated from the total ^{14}C counted in pellet and the amount of organic-^{14}C

released from the pellet after being treated with 3% TCA and drying overnight in the fume hood. A diagram of the TCA treatment process is shown in Figure 6.8 and the results are shown in Table 6.4.

medium

silicone oil
pellet

scintillation vial

(a)   (b)   (c)
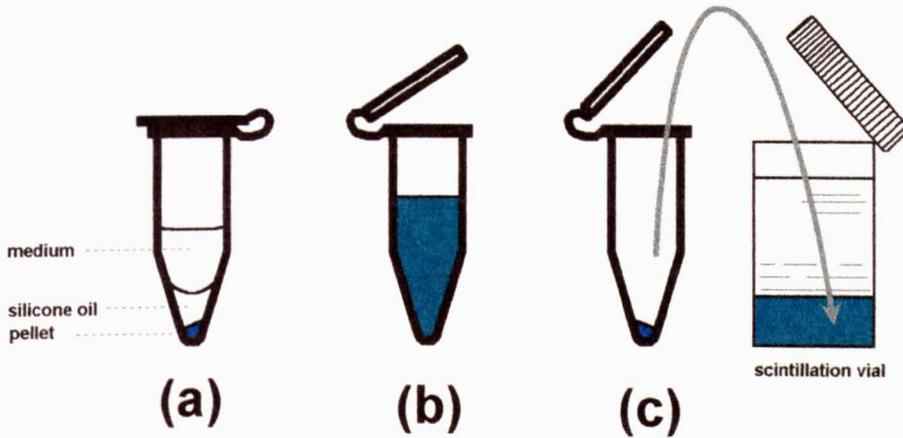
Figure 6.8: Diagram illustrates the addition of TCA for organic ^{14}C determination.

    (a) After silicone oil centrifugation, algal cells (pellet) were separated from medium and radioactivity in the medium was counted.

    (b) Algal pellet was resuspended in 3% TCA, all water soluble ^{14}C was released from the cells. Algal debris was then separated from TCA solution by centrifugation.

    (c) After centrifuge, supernatant containing water soluble ^{14}C was transferred into scintillation vial and dried overnight to release $^{14}CO_2$ (which was converted from $H^{14}CO_3$ under the acidic conditions). Radioactivity that remained in the scintillation vial after drying was counted as water soluble organic-^{14}C. Radioactivity in the algal debris pellet (in Eppendorf tube) after TCA treatment was assumed to be the rest of the organic carbon and insoluble ^{14}C associated with cellular structure.

Table 6.4: Accumulation of ^{14}C in *Dunaliella* cells after incubation with $NaH^{14}CO_3$.

Time	Control (^{14}C DPM)		Treatment with 3% TCA (^{14}C DPM)		
	Medium	Cells	Medium	Organic-C	Algal pellet
2 minutes	105956±1747	373.09±22.5	no data	no data	no data
6 hours	8336.85±1214	11795.8±716.	8498±142	6646.79±26.3	5079.9±105.0

After $^{14}CO_2$ was released from the pellet by TCA extraction, organic carbon portion was approximately 56% of total ^{14}C in the cells. In addition, a combination of water soluble organic carbon part and the radioactivity remaining in algal pellet was equal to total ^{14}C found in cells without TCA treatment. This indicates that most of ^{14}C incorporated into the cells was fixed into an organic form, of which at least 70-80% of that carbon was assumed to be ^{14}C-glycerol (Wegmann, 1969; Frank and Wegmann, 1974).

6.2.6.3 Release of Fixed ^{14}C from *D. parva*

After incubating 20 ml of 1×10^7 cell.ml^{-1} *D. parva* cells in 1.5 M NaCl medium containing 50 μl of 0.1 μCi.μl^{-1} $NaH^{14}CO_3$ for 6 hours in light, cells were resuspended in fresh 1.5 M NaCl medium or in 0.5 M NaCl medium (hypoosmotic shock). The amount of ^{14}C in the medium and in the algal pellet was counted after separation of algal cells from culture medium by silicone oil centrifugation to monitor the release of ^{14}C.

The results are shown in Figure 6.9 a,b and 6.10 a,b. For both figures, graphs on the top (Figure 6.9a and 6.10a) indicate ^{14}C concentration in the medium after cells were resuspended in fresh 1.5 M NaCl medium or 0.5 M NaCl (hypoosmotic shock), respectively. Similarly, graphs on the bottom (Figure 6.9b and 6.10b) illustrate ^{14}C in algal pellet after resuspension of the cells in fresh medium. It was found that ^{14}C was released immediately after hypoosmotic shock and the amount of ^{14}C at the first data

point in Figure 6.10a was much higher than the control (Figure 6.9a). However, the ^{14}C release pattern was exactly the same for both conditions and, moreover, the reduction of ^{14}C in algal pellets (Figure 6.9b and 6.10b) showed a relationship with ^{14}C release into the medium.
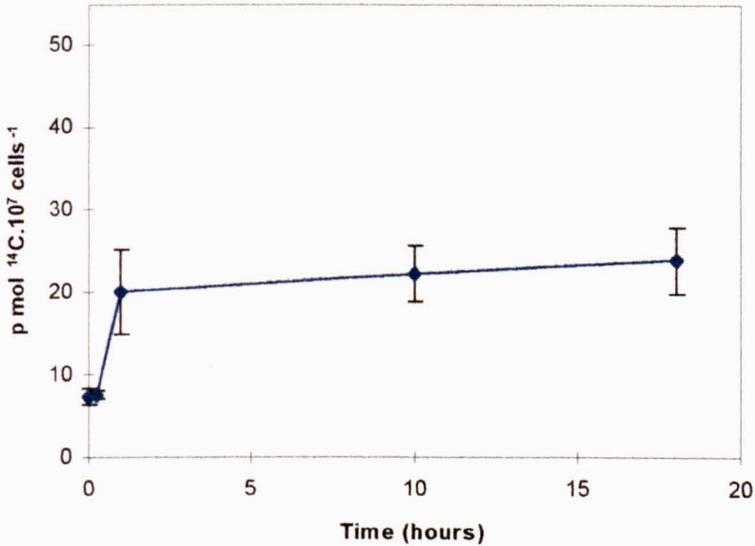
Figure 6.9a: Release of ^{14}C from *D. parva* after exposure to fresh medium at the same salinity (1.5 M NaCl)
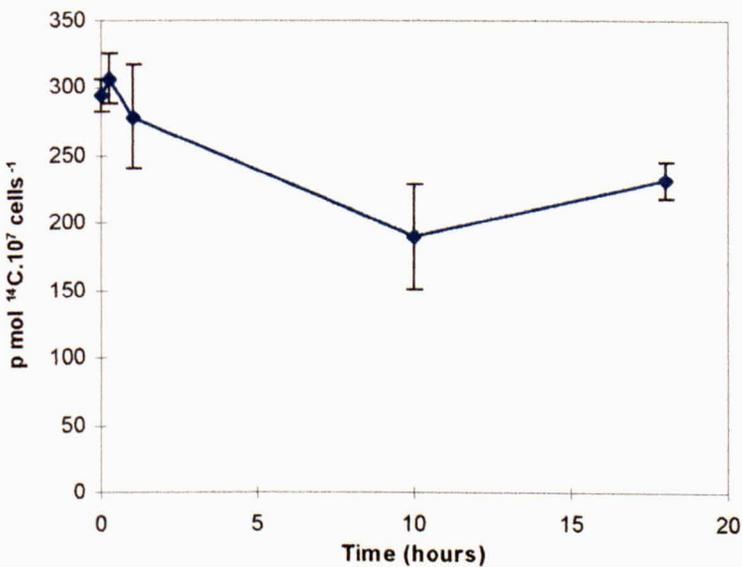
Figure 6.9b: ^{14}C content in the cells of *D. parva* after exposure to fresh medium at the same salinity (1.5 M NaCl)
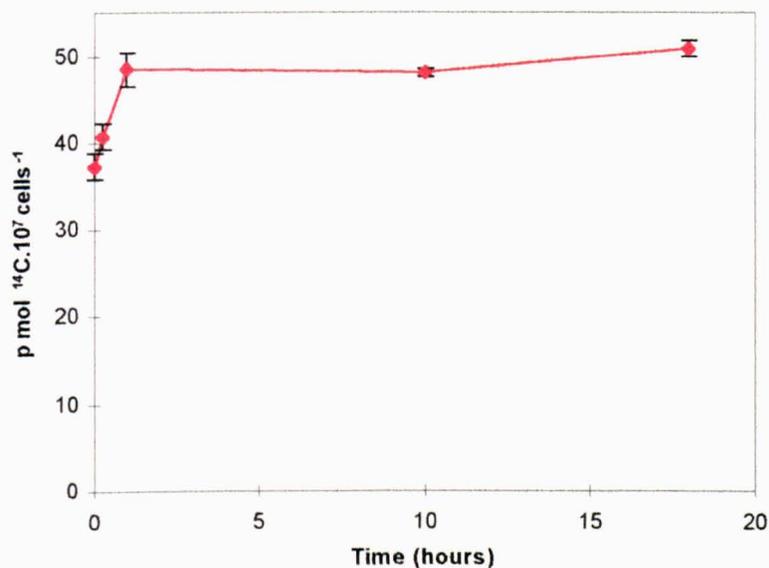
Figure 6.10a: Release of ^{14}C from *D. parva* after exposure to hypoosmotic shock from 1.5 M to 0.5M NaCl
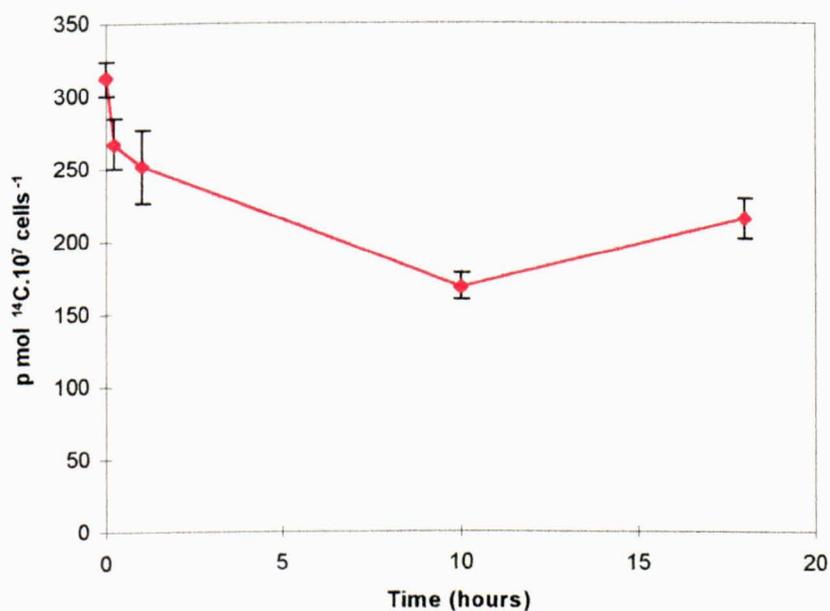
Figure 6.10b: ^{14}C content in the cells of *D. parva* after exposure to hypoosmotic shock from 1.5 M to 0.5 M NaCl

The result from this experiment suggests that there is a leakage of ^{14}C, which is assumed to be composed of at least 70-80% ^{14}C-glycerol, from *Dunaliella* cells after resuspension in fresh medium (with or without hypoosmotic shock) and the results confirm the role of glycerol leakage found in previous experiments with direct glycerol measurement.

6.2.7 Effect of Inhibitors on Glycerol Leakage in *D. parva*

From previous experiments, it could be concluded that there is a leakage of glycerol from *Dunaliella*. In this experiment, two inhibitors, CCCP and vanadate, were used to test whether or not an active transport system for glycerol was present in *D. parva*. This was performed by measuring glycerol leakage from *D. parva* cells in the presence of the inhibitor.

CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) is a powerful inhibitor and uncoupler of photosynthetic and oxidative phosphorylation and can inhibit ATP production in *Dunaliella* (Zidan *et al.*, 1987). Another inhibitor, vanadate (sodium orthovanadate), can strongly inhibit ATPases associated with plasmalemma or the flagella, but does not affect the activity of the ATPases of mitochondria or thylakoids in *D. parva* (Gilmour *et al.*, 1985).

Figure 6.11 shows that CCCP could inhibit growth of *D. parva*. When the concentration of CCCP in culture medium increased from 0 μM (control) to 25 μM, growth of *D. parva*, as indicated by chlorophyll concentrations, was decreased. However, as shown in Figure 6.12, different concentrations of CCCP did not induce obviously lower glycerol leakage when compared with control.

In Figure 6.13, a higher concentration of CCCP (50 μM) or Gram's iodine (2% v/v) were applied to 100 ml of 20 μg Chl.ml^{-1} *D. parva* culture. Gram's iodine immediately killed most cells in the culture and all glycerol was found to be rapidly released from the cells. Under these conditions, morphology of the dead cells was

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unchanged when observed under the microscope. Release of glycerol was found to show the same pattern as when the cells were exposed to hypoosmotic shock, but without morphological change (cell expansion). 50 µM CCCP treatment, which is a concentration that strongly inhibits cellular metabolism, gave only a slightly higher glycerol leakage than control.
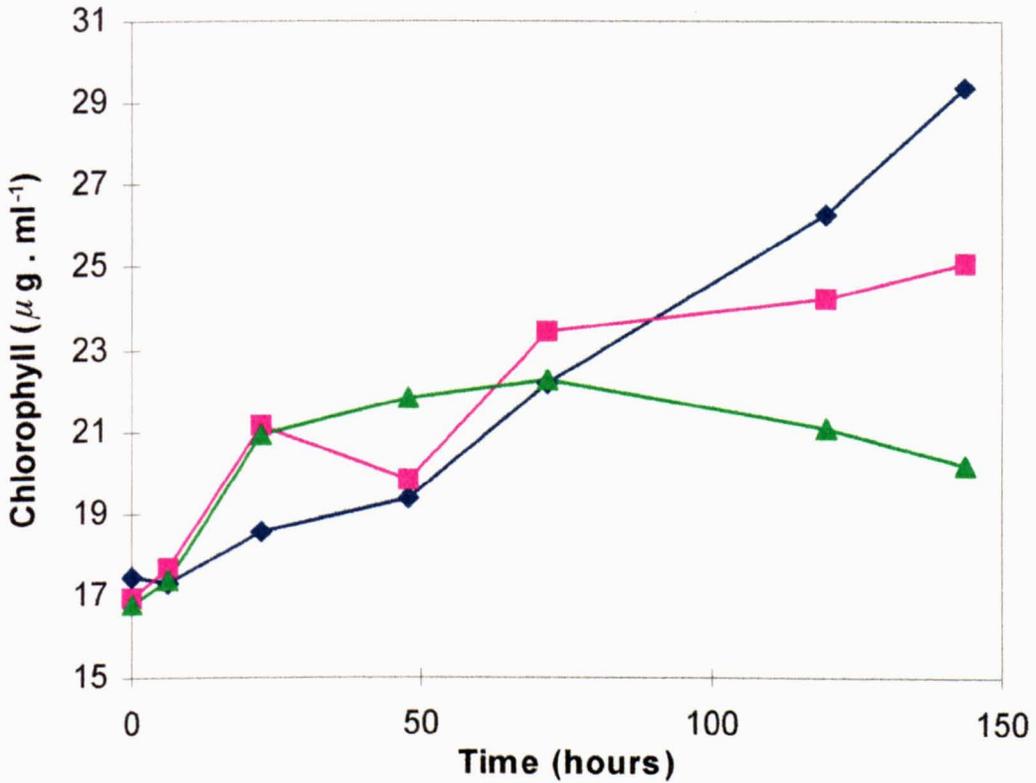
Figure 6.11: Effect of different concentrations of CCCP on growth of *D. parva*. The inhibitor (or an equivalent volume of ethanol for control) was added to exponentially growing cultures and growth was followed for the next 6 days.

        (◆) 0 µM CCCP (control)

        (■) 12.5 µM CCCP
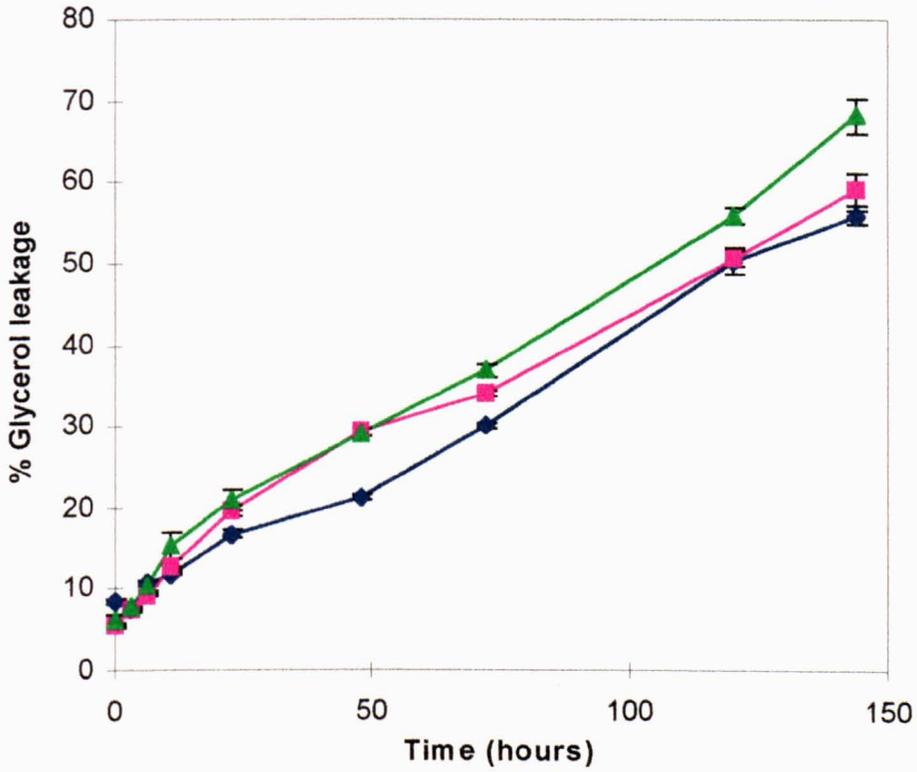
        (▲) 25 µM CCCP

Figure 6.12: Effect of different concentrations of CCCP on glycerol leakage from *D. parva*. Samples were taken at the same time as growth measurements in Figure 6.11.

> (◆) 0 μM CCCP (control)
>
> (■) 12.5 μM CCCP
>
> (▲) 25 μM CCCP
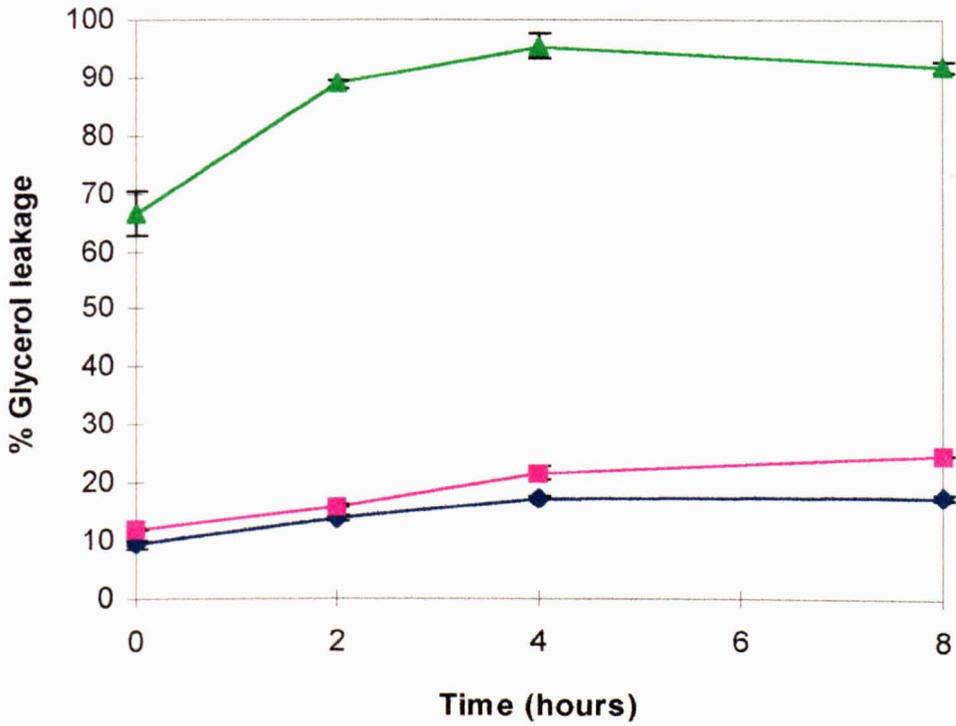
Figure 6.13: Effect of high concentration of CCCP and Gram's iodine on glycerol

leakage from *D. parva*

(◆) control without CCCP or Gram's iodine

(■) 50 μM CCCP

(▲) 2% (v/v) Gram's iodine

The effect of vanadate on glycerol leakage was tested by adding sodium vanadate (freshly prepared) into 100 ml of *D. parva* culture to obtain a final concentration of 10 mM. The results, as shown in Figure 6.14, indicate that the leakage of glycerol was not different between the control and sodium vanadate treatment.

As the inhibitors, CCCP and vanadate, did not show any clear effect on glycerol leakage, it appears that glycerol leakage in *Dunaliella* is not due to an active transport system.
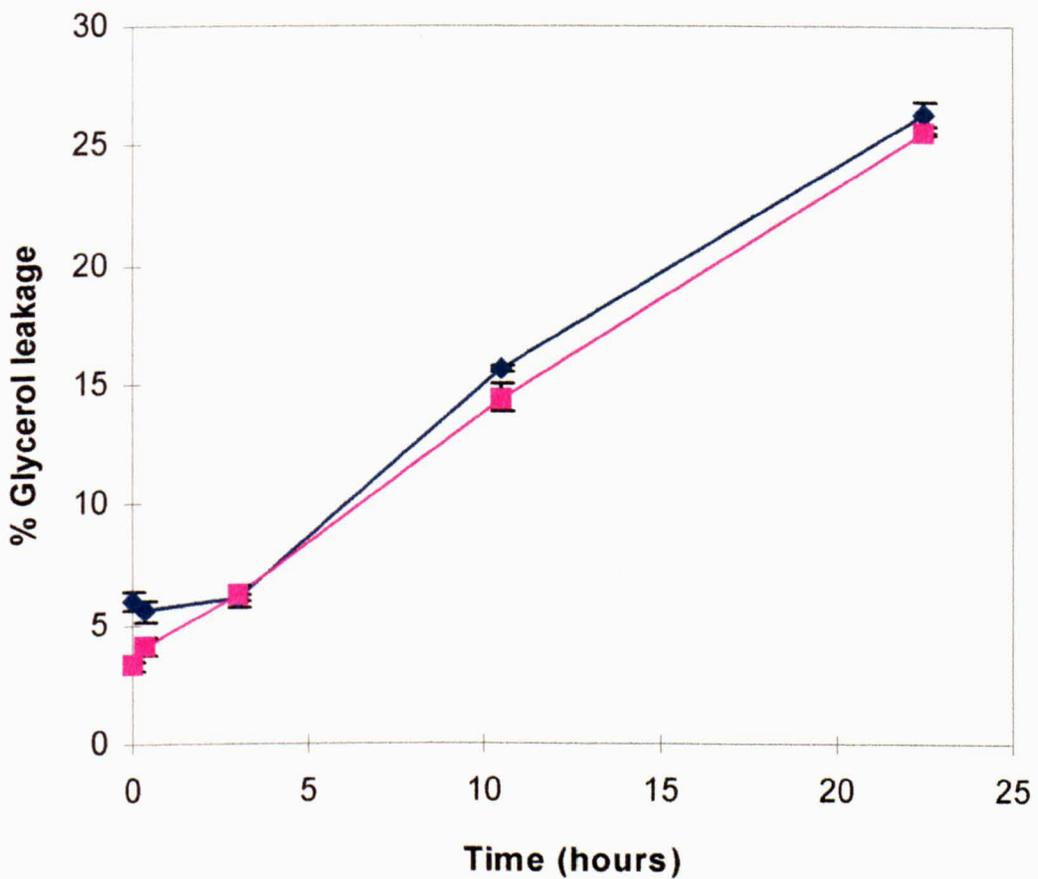
Figure 6.14: Effect of sodium vanadate on glycerol leakage from *D. parva*

($\blacklozenge$) 0 mM sodium vanadate (control)

($\blacksquare$) 10 mM sodium vanadate

6.2.8 Effect of darkness on glycerol leakage from *D. parva*

In *Dunaliella*, glycerol is produced by either photosynthetic CO_2 fixation or by starch degradation when there is no light available. Untill now, release of glycerol has been measured under illuminated conditions, therefore this experiment was performed in order to compare the pattern of glycerol leakage from *D. parva* cells in light and dark environments.

The experiment was carried out by growing 100 ml of *D. parva* in 250 ml flasks. All treatments were grown under continuous illumination (70 $\mu E.m^{-2}.s^{-1}$) in 25°C growth room for 24 hours before covering the flasks with aluminium foil to provide darkness. Control cultures, without aluminium foil covering, were monitored in parallel. Chlorophyll concentration, starch content and glycerol leakage were analysed.

It was found that *Dunaliella* cells stop growing when they were kept in darkness. This was indicated by no further increase in chlorophyll concentration compared to control (Figure 6.15). At this time, when growth was inhibited in the dark, glycerol was still found to leak from the cells (Figure 6.16). Nevertheless, the leakage of glycerol was somewhat lower in the dark than in the light.

The results suggested that glycerol leakage from *Dunaliella* was not dependent on photosynthesis as it was also found in the dark. Therefore, as the cells were continuously releasing glycerol into the medium, more glycerol needed to be produced. Table 6.5 shows that the starch content of the cells rapidly decreased in the dark. This indicated that *Dunaliella* was converting starch into glycerol but it may also indicate that starch was being broken down to provide energy for the cells.

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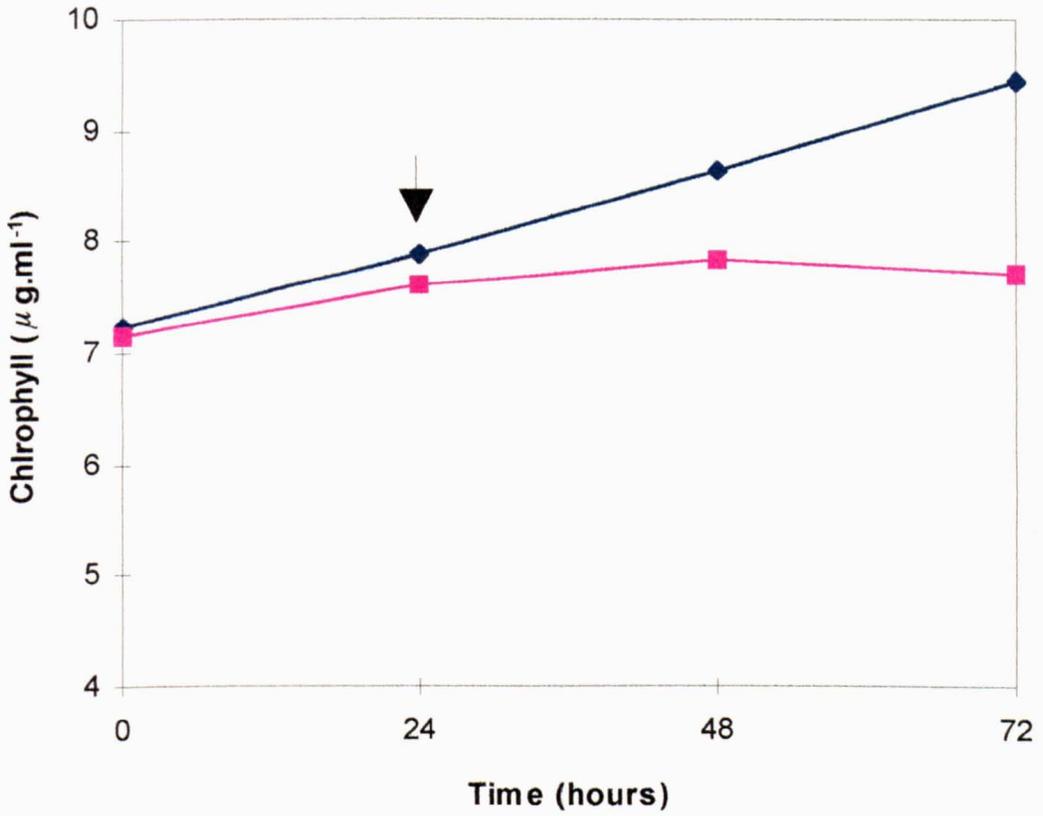
Figure 6.15: Growth of *D. parva* in light or in dark environment. Both control (◆) and treatment (■) groups were illuminated for 24 hours (indicated by an arrow), thereafter, the dark treatment was provided by covering the culture flasks with aluminium foil.
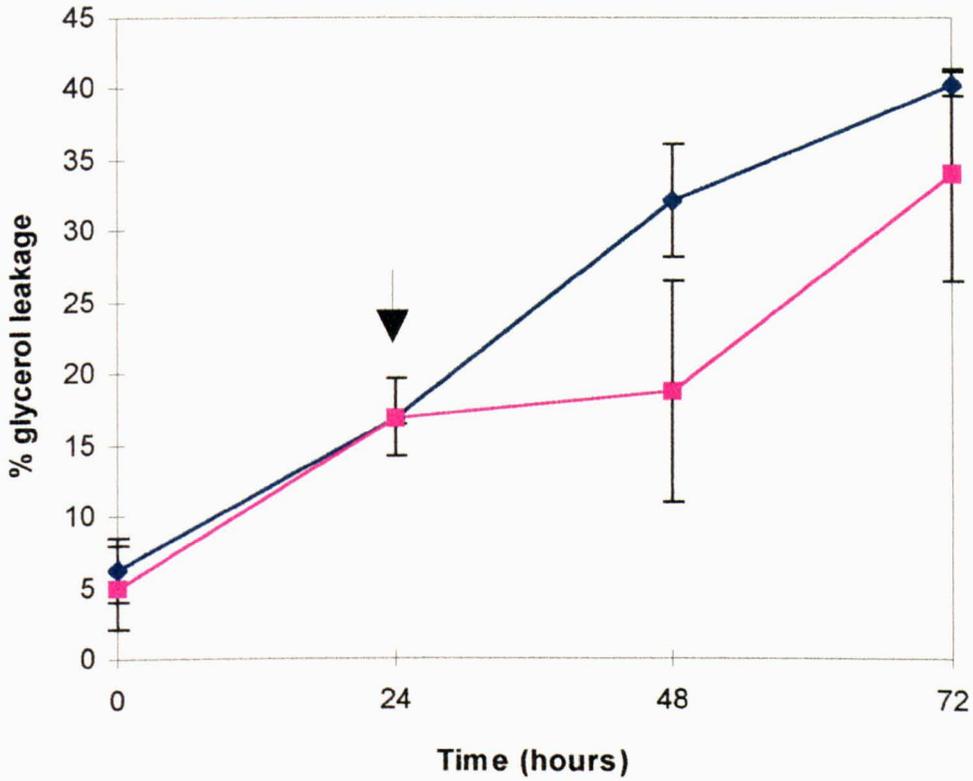
Figure 6.16: Glycerol leakage from *D. parva* in light or in dark environment. Both control (♦) and treatment (■) groups were illuminated for 24 hours (indicated by an arrow), thereafter, the dark treatment was provided by covering the culture flasks with aluminium foil.

Table 6.5: Starch content in *D. parva* cells in light and dark cultures.

Hour	Starch content ($\mu g.\mu g$ Chl^{-1})	
	Control (light)	Treatment (dark)
28	19.30 ± 0.01	14.8 ± 0.01
48	21.00 ± 8.70	7.25 ± 0.30
72	10.87 ± 2.12	1.93 ± 0.35

However, if the leakage of glycerol is the result of a diffusion process not active transport, it should not made any difference to glycerol leakage whether the cells are in the light or dark as long as there was a sufficient amount of glycerol accumulated in the cells. Therefore, the experiment was repeated with more sampling during 55 hours of darkness. The results are shown in Figure 6.17 and 6.18a,b.

Figure 6.17 shows that starch content decreased when cells were kept in the dark. This suggested a conversion of starch into glycerol. In Figure 6.18a, accumulation of glycerol in the medium, indicated by increasing glycerol concentration, was found to be slightly higher in light (control) than in the dark treatment. Glycerol in the cells, on the other hand, was found to decrease in dark condition (Figure 6.18a). This indicates that cells released intracellular glycerol into the medium. However, pattern of glycerol leakage, as shown in Figure 6.18b, was found not to be different in both light and dark environments.
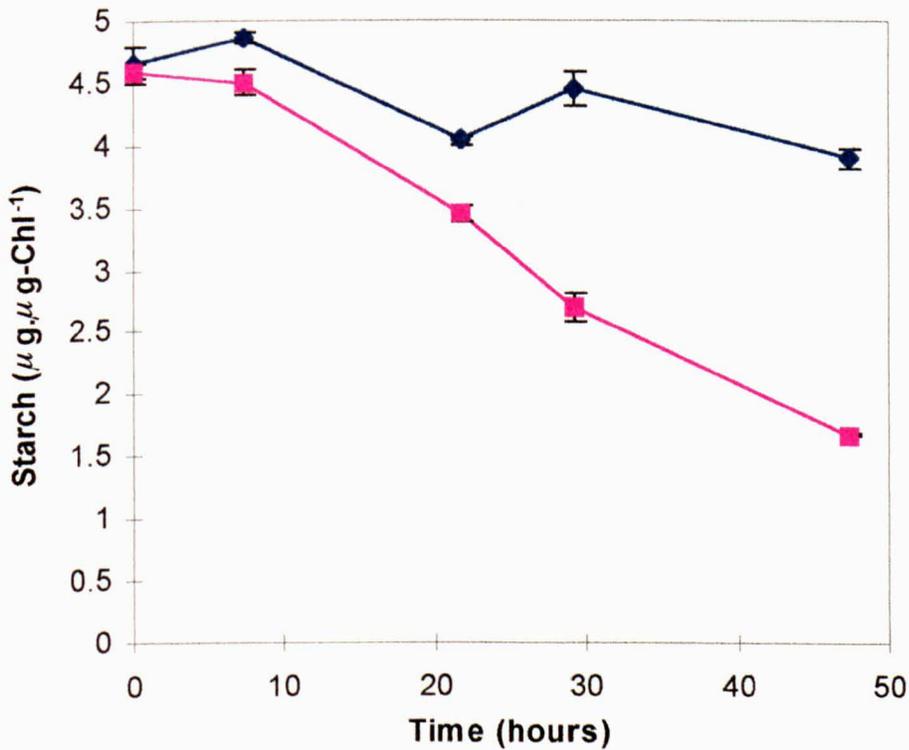
Figure 6.17: Starch content of *D. parva* in light (◆) and dark (■) environment
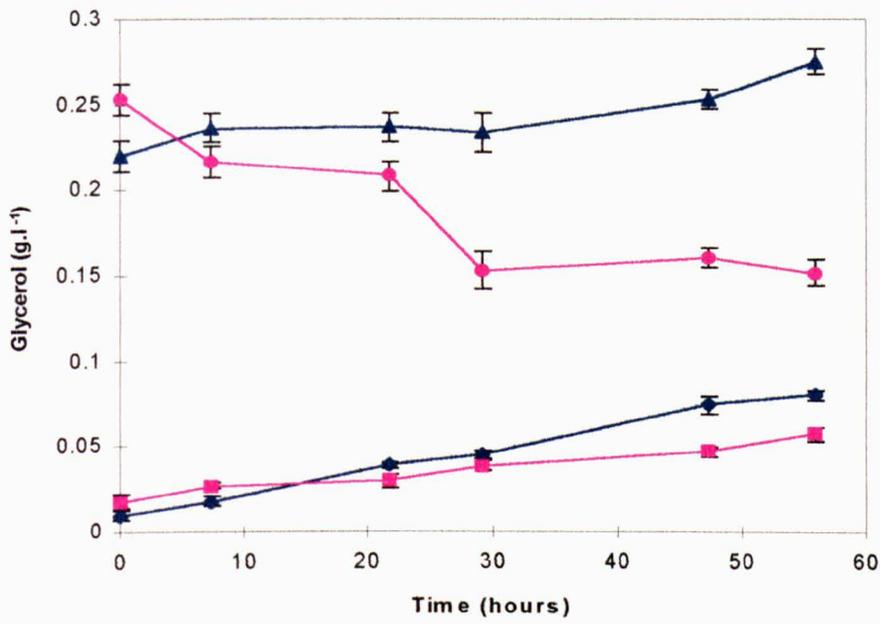
Figure 6.18a: Glycerol concentration in *D. parva* culture in light and dark environments.

(♦) glycerol in the medium of *D. parva* culture with light

(■) glycerol in the medium of *D. parva* culture without light

(▲) glycerol in the cells of *D. parva* culture with light

(●) glycerol in the cells of *D. parva* culture without light
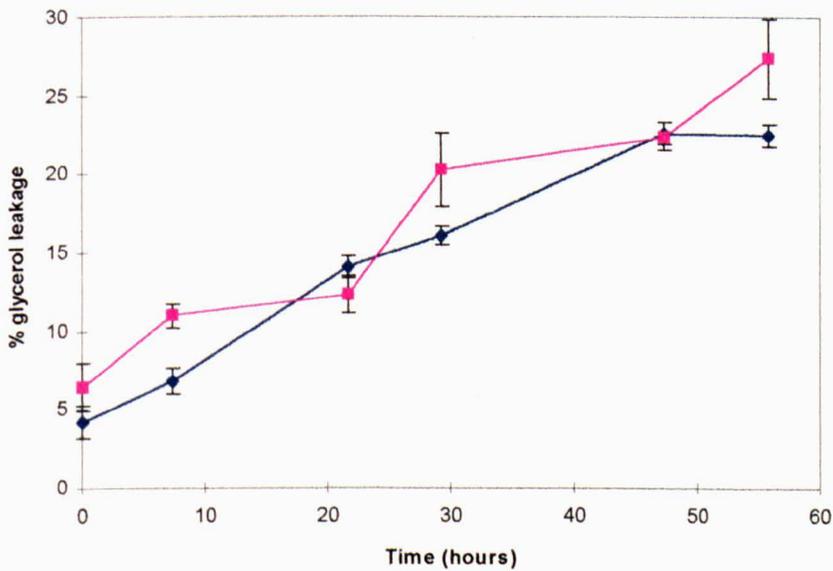
Figure 6.18b: Glycerol leakage from *D. parva* culture in light (♦) and dark (■) environment

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6.2.9 ³H-glycerol uptake by *D. parva*

The uptake of radioactively labelled glycerol by *D. parva* was carried out by addition of 50 μl of 0.1 μCi.μl^{-1} [2-³H] glycerol in to 20 ml of *D. parva* culture (1×10^7 cells.ml^{-1} in 1.5 M NaCl medium). The experiment was carried out under illumination (70 μE.m^{-2}.s^{-1}) at approximately 25°C. Culture was sampled and the amount of radioactive glycerol was counted after separating algal cells from the culture medium by silicone oil centrifugation.

It was found that, as shown in Figure 6.19, ³H-glycerol was taken up by *D. parva*, but at a very low concentration (0.2-1.2 pmol-glycerol.10^{7}cells^{-1}) when compared with the concentration of ³H-glycerol in the medium (0.2 nmol-glycerol.10^{7}cells^{-1}. Therefore, no significant amount of glycerol was taken up by *D. parva*.
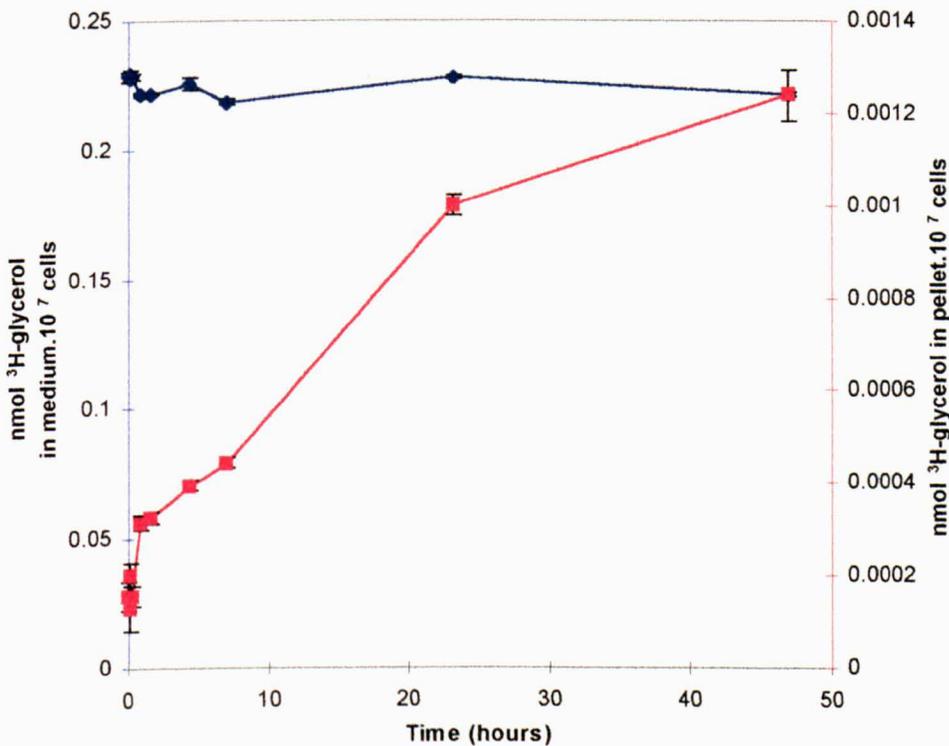
Figure 6.19: ³H-glycerol uptake by *D. parva*. (◆) with the left Y-axis is the concentration of ³H-glycerol in the medium and (■) with the right Y-axis is the concentration of ³H-glycerol taken up by cells.

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Figure 6.20 shows the effect of inhibitors, CCCP and Gram's iodine, on ^{3}H-glycerol uptake in *D. parva*. The results suggest that CCCP did not affect glycerol uptake. However, the concentration of ^{3}H-glycerol in the cells treated with the Grams's iodine was increased slightly. This might be because all cells were dead, therefore, glycerol could pass freely through the cell membrane. However, as noted above, there was only a very low level of ^{3}H-glycerol uptake by *D. parva* cells in these experiments.
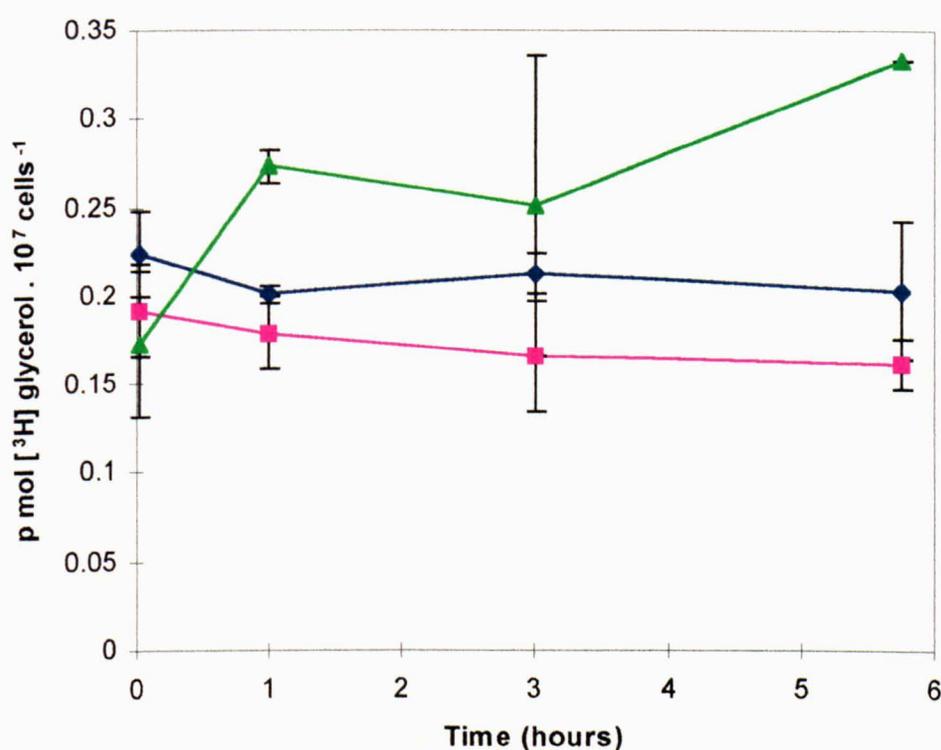
Figure 6.20: Effect of CCCP and Gram's iodine on ^{3}H-glycerol uptake by *D. parva*

($\blacklozenge$) control without CCCP or Gram's iodine

($\blacksquare$) 50 μM CCCP

($\blacktriangle$) 2% (v/v) Gram's iodine

6.2.10 Glycerol in natural *Dunaliella* habitat: a field study

In addition to experiments in the laboratory, a survey of glycerol concentration in a natural habitat of *Dunaliella* was performed. Saline water from salt ponds in Chon Buri Province, along the Eastern coast of Thailand, was sampled. Algal cells were counted using haemacytometer and glycerol was analysed by acetylacetone method.

It was found that, in water sample (200 ppt salinity / 3.5 M NaCl)) containing 1.1×10^4 cell.ml^{-1} *D. salina* and 1.3×10^4 cell.ml^{-1} *D. viridis*, glycerol could not be detected by acetylacetone method. However, by observing water samples with the microscope at high magnification, halophilic bacteria were found to be abundant in the samples. These bacteria could possibly be taking up all of the glycerol leaked from *Dunaliella*.

6.3 Conclusions

In this chapter, several experiments were carried out in order to study the characteristics of glycerol leakage from *Dunaliella*. It is necessary to be sure that glycerol leaking from algal cells is not due to cell breakage. In this chapter, 1500 *g* centrifugation for 15 minutes was used as recommended by Enhuber and Gimmler (1980). This speed did not result in cell breakage nor in incomplete sedimentation. Silicone oil centrifugation also provides a good separation without damaging cells and it was used for determining ^{14}C release from cells. Moreover, by growing a culture in a dialysis tube (Section 6.2.2), glycerol release from *Dunaliella* was confirmed not to be due to cell breakage caused by centrifugation.

Both direct glycerol measurement and ^{14}C leakage experiments showed that glycerol was released or leaked from *Dunaliella* cells. This characteristic was found in both *D. parva* and *D. salina* (Figure 6.5), but it was dependent on salinity as cells need glycerol to balance the osmotic pressure (Figure 6.6). CCCP and sodium vanadate did not show a clear effect on glycerol leakage (Figures 6.13 and 6.14). This might

indicate that glycerol leakage did not require energy or is not driven by active transport. This conclusion is also supported by the data showing that glycerol was leaked from cultures in the dark. Hence, it appears that glycerol leakage is a normal characteristic of *Dunaliella* and is not an active process.

The previous studies showing no glycerol leakage from *Dunaliella* were mostly done over short time periods. The result from an NMR study by Brown *et al.* (1982) showed that glycerol leakage can reach approximately 20% after 7 hours at 25°C. Ben-Amotz (1975) and Ben-Amotz and Avron (1973) reported that glycerol was not excreted into the medium for salinities above 0.6 M NaCl or with the temperature below 40°C (Wegmann *et al.*, 1980). However, in the papers mentioned, no experimental data showing glycerol excretion versus time over a range of several hours are reported.

It could probably be said that *Dunaliella* has "low" rather than "no" permeability to glycerol. Although the rate of glycerol leakage is low during the first hour, after several days in normal growth conditions, more than 70% of glycerol produced can be accumulated in the medium. As up to 90% of carbon fixed from photosynthesis can be converted into glycerol (Frank and Wegmann, 1974), this glycerol released from *Dunaliella* could play an important role in natural environments as a carbon source for halophilic bacteria (Oren, 1995). However, glycerol is present in natural hypersaline environments in very low concentrations and could not be detected in water samples containing *Dunaliella* and halophilic bacteria (Section 6.2.10) or even in a *Dunaliella* laboratory culture with bacterial contamination (see Section 3.1). This might indicate that bacteria immediately take up glycerol to use as a carbon source.

In natural hypersaline environments such as salt ponds, growth rate of *Dunaliella* can be very slow because it is exposed to very high light intensity. Under these conditions, oxygen evolution could reach many hundreds of percent over oxygen

saturation concentration without significant photoinhibition (Powtongsook *et al.*, 1994). Releasing glycerol into the environment is one possible way to reduce the excess energy coming from photosynthesis. Moreover, in hypersaline lakes with high evaporation rates, glycerol can protect *Dunaliella* cells found in completely dry salt crust. In this way, cells have a much higher survival chance than if intracellular glycerol was absent (Neimax, 1971 cited by Frank and Wegmann 1974).

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

The original rationale of the project was to study the physiology and biotechnology of the glycerol leaky mutant of *Dunaliella parva* CCAP 19/9 isolated by Hard and Gilmour (1991). However, the original aim of the project had to be altered to take into account the finding that wild type strains of *D. parva* and *D. salina* also showed glycerol leakage. Therefore, the project was then split into two parts, firstly, the physiology and biotechnology of glycerol production from immobilised *Dunaliella* and, secondly, the characteristics of glycerol leakage in *Dunaliella*.

In Chapter 3, immobilisation techniques were applied to *Dunaliella* cells and glycerol production from *Dunaliella* cells immobilised in calcium alginate beads was investigated. This technique provides advantages over free cell culture because the algal cells can be grown to very high densities and the product, glycerol, can be easily obtained as cells release glycerol directly into the medium. This simplifies the otherwise complex cell harvesting process and therefore reduces costs. In this study, *Dunaliella* cells grew well when immobilised and during growth, cells were found to be dividing to form cell clusters (Section 3.2.1). The number of initial cells at the start of the immobilisation process and the maximum number of cells per cluster are the factors that limit the maximum chlorophyll concentration of the beads (Figure 3.1). Photosynthetic oxygen evolution rate was high during the first period of culture then the amount of oxygen evolved decreased by more than 60% and the rate was stable at this level for a long period of time (Figure 3.9). Chlorophyll fluorescence measurements showed that immobilised cells had high Fv/Fm ratio and the ratio gradually decreased over a period of culture (Figure 3.21). High Fv/Fm ratio indicated that entrapped cells in calcium alginate were in a healthy condition because Fv/Fm ratio is proportional to the rate of photosynthesis and could be affected by stress factors (Hall and Rao, 1994).

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However, the experiment that compared the leakage of glycerol from immobilised wild type and original mutant strain of *D. parva* showed that there was no difference in glycerol production between these wild type and mutant strains (Section 3.2.2). The results for glycerol leakage from both immobilised strains were therefore combined in this chapter and this led to a more detailed investigation of glycerol leakage from *Dunaliella* in Chapter 6.

Many physiological aspects of immobilised *Dunaliella* have been investigated in Chapter 3 and 4. Most were attempts to find out the factors that could effect glycerol leakage from calcium alginate immobilised *Dunaliella*. The results suggested that bead size, bead packing and cells density in the beads are among the most important factors that need to be considered when scaling up the process. Yield of glycerol production was found to be higher with semi-continuous harvesting than with osmotic shock, hence, glycerol production and leakage could be induced by maintaining a low concentration of glycerol in the culture medium by addition of fresh medium of the same salinity (Figures 4.10 and 4.11a,b). The amount of glycerol leakage from immobilised *Dunaliella* was not dependent on a particular immobilisation matrix as agar beads produced as much glycerol as calcium alginate beads (Figure 3.30).

A novel "algal sheet" technique in Chapter 4 showed several advantages over the algal beads, especially in the ease of maintenance and better stability. This algal sheet also has potential to be applied to several biotechnological aspects, not only for metabolite production, but also for waste water treatment. Algal sheet is very easy to maintain, but the culture chamber for this system needs to be re-designed with modifications to the water circulating system (Figures 4.14a,b and 4.15).

In Chapter 5, a mutation technique using UV was applied to *D. parva* CCAP 19/9 wild type strain in an attempt to produce mutants that had different glycerol leakage characteristics from the wild type strain. Unfortunately, no mutant strains from this

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experiment exhibited a different glycerol leakage pattern when compared to the wild type. This failure may be due to two possibilities. The first is that there was unfortunately no mutants obtained because of the inefficient selection technique. Another possibility is based on the hypothesis that glycerol leakage is a basic character of *Dunaliella*. If *Dunaliella* cells always leak glycerol into the medium, altering the genotype should not have any effect.

In this study, glycerol leakage was found in both free and immobilised *Dunaliella*. Several publications such as Ben-Amotz and Avron (1973), Ben-Amotz and Avron (1978), Brown and Borowitzka (1979), Gimmler and Hartung (1988) and Avron (1992) claimed that there is no leakage of glycerol from this algal species in salinities higher than 0.6 M NaCl. The work described in Chapter 6 set out to investigate this anomaly. The results confirmed that glycerol leakage always happens in *Dunaliella* spp., as it was found in both *D. parva* and *D. salina*. Leakage of glycerol could occur even in high salinity (3.5 M NaCl) without osmotic shock (Figure 6.6). The experiments with the inhibitors, CCCP and sodium vanadate, suggested that glycerol leakage is not an active process (Figures 6.11-6.14), therefore, it was assumed to be diffusion down a glycerol gradient.

The results from this study provide important data to help us understand the physiology of immobilised *Dunaliella*, especially for glycerol production, and the physiology of glycerol leakage from this alga. To enhance glycerol production from *Dunaliella*, several aspects must be considered. It is known that glycerol is a product of photosynthesis, therefore, maximising photosynthetic efficiency could be the best way to achieve higher glycerol production. In practice, it might make more sense to improve immobilisation techniques and culture systems by designing a proper culture chamber with a maximum illumination area rather than trying to improve strains genetically to get higher glycerol leakage. This is because the leakage of glycerol is confirmed to be a normal characteristic of *Dunaliella*.

Recommendations for future research

1. Several designs of photobioreactors could be tested to be used for glycerol production from immobilised *Dunaliella*. Moreover, long term production of glycerol by immobilised *Dunaliella* in an automatic controlled photobioreactor with semi-continuous harvesting would be a good model system for research into scaling up glycerol production.

2. Bioconversion of glycerol produced by *Dunaliella* to a high value product, e.g. 1,3-propanediol, is another interesting prospect. This might be done by either separate cultures of *Dunaliella* and the appropriate bacteria or by co-immobilisation of *Dunaliella* and a specific strain of bacteria.

3. Calcium alginate immobilised algae on a spongy plastic supporter (algal sheet) has been successfully used for glycerol production in this project. Modifications of the culture chamber to provide the maximum light efficiency and good water circulation are still needed. Moreover, this algal sheet might also be used in waste water treatment systems with immobilised algae.

4. Chitosan immobilisation is one of the alternative techniques for entrapping living microorganisms. However, the immobilisation of *Dunaliella* in chitosan beads was not successful in this study. More research could be performed in order to immobilise algal cells in chitosan beads as chitosan has better stability properties than calcium alginate. Low cost semi-purified chitosan from shrimp or crab shell could be a good source of chitosan instead of an expensive purified form.

5. More experiments on the glycerol transport system in *Dunaliella* are required to confirm the mechanism of glycerol transport. This includes the study of plasma membrane properties.

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6. A better understanding is required of the role of glycerol produced from *Dunaliella* on the halophilic bacteria communities in natural hypersaline environments. A survey of glycerol leakage from natural populations of *Dunaliella* and the interaction between *Dunaliella* and halophilic bacteria is an interesting topic.

7. Glycerol leakage is a process that could be considered to be a loss of energy. Therefore, an intensive study of the energy balance between photosynthesis and glycerol leakage might explain the role of this phenomenon.

REFERENCES

Adlercreutz, P. and Mattiasson, B. (1982) Oxygen supply to immobilized cells: 1. Oxygen produciton by immobilized *Chlorella pyrenoidosa*. *Enzyme and Microbial Technology* 4: 332-336.

Aizawa, K. and Miyachi, S. (1986) Carbonic anhydrase and CO_2 concentrating mechanisms in microalgae and cyanobacteria. *FEMS Microbiology Reviews* 39: 215-233.

Aizawa, K., Tsuzuki, M., and Miyachi, S. (1986) Kinetic studies on the active species of inorganic carbon absorbed by the cells of *Dunaliella tertiolecta*. *Plant and Cell Physiology* 27: 37-48.

Amoroso, G., Weber, C., Sultemeyer, D. and Fock, H. (1996) Intracellular carbonic anhydrase activities in *Dunaliella tertiolecta* (Butcher) and *Chlamydomonas reinhardtii* (Dangeard) in relation to inorganic carbon concentration during growth: Further evidence for the existence of two distinct carbonic anhydrases associated with the chloroplasts. *Planta* 199: 177-184.

Andersen, R.A., Morton, S.L. and Sexton, J.P. (1997) CCMP-Provasoli-Guillard National Center for Culture of Marine Phytoplankton. a supplement to Journal of Phycology volume 33, December 1997.

Aoki, K., Ideguchi, T., Kakuno, T., Yamashita, J., and Horio, T. (1986) Effects of sodium chloride and glycerol on photosynthetic oxygen-evolving activity with thylakoid membranes from halophilic green alga *Dunaliella tertiolecta*. *Journal of Biochemistry* 100: 1223-1230.

Avron, M (1992) Osmoregulation. In Avron, M. and Ben-Amotz, A. (eds) *Dunaliella: Physiology, Biochemistry, and Biotechnology*. CRC Press, Boca Raton, Florida, pp. 135-164.

Badger, M.R. and Price, G.D. (1992) The CO_2 concentrating mechanism in cyanobacteria and microalgae. *Physiologia Plantarum* 84: 606-615.

Barbirato, F., Soucaille, P. and Bories, A. (1996) Physiologic mechanisms involved in accumulation of 3-hydroxypropionaldehyde during fermentation of glycerol by *Enterobacter agglomerans*. *Applied and Environmental Microbiology* 62: 4405-4409.

Beaumont, M.D. and Knorr, D. (1987) Effects of immobilizing agents and procedures on viability of cultured celery (*Apium graveolens*) cells. *Biotechnology Letters* 19: 377-382

Belmans, D. and Van Laere, A. (1987) Glycerol cycle enzymes and intermediates during adaptation of *Dunaliella tertiolecta* cells to hyperosmotic stress. *Plant Cell and Environment* 10: 185.

Ben-Amotz, A. (1975) Adaptation of the unicellular alga *Dunaliella parva* to a saline environment. *Journal of Phycology* 11: 50-54

Ben-Amotz, A. (1995) New mode of *Dunaliella* biotechnology: Two-phase growth for beta-carotene production. *Journal of Applied Phycology* 7: 65-68.

Ben-Amotz, A. and Avron, M. (1973) The role of glycerol in the osmotic regulation of the halophilic alga *Dunaliella parva*. *Plant Physiology* 51: 875-878.

Ben-Amotz, A. and Avron, M. (1978) On the mechanism of osmoregulation in *Dunaliella*. In Caplan, R.S. and Ginzburg, M. (eds.) *Energetics and structure of halophilic microorganisms*. Elsevier/ North-Holland Biomedical Press, pp. 529-541.

Ben-Amotz, A. and Avron, M. (1980) Glycerol, B-carotene and dry algal meal production by commercial cultivation of *Dunaliella*. In Shelef, G. and Soeder, C.J. (eds) *Algae Biomass*, Elsevier/North-Holland Biomedical Press, pp.605-609.

Ben-Amotz, A. and Avron, M. (1982) The potential use of *Dunaliella* for the production of glycerol, β-carotene and high-protein feed. In San Pietro, A. (ed) *Biosaline Research: A Look to the Future*. Plenum Publishing Co. pp.207-214

Ben-Amotz, A. and Avron, M. (1983a) On the factors which determine massive β-carotene accumulation in the halotolerant alga *Dunaliella bardawil*. *Plant Physiology* 72: 593-597.

Ben-Amotz, A. and Avron, M. (1983b) Accumulation of metabolites by halotolerant algae and its industrial potential. *Annual Review in Microbiology* 37: 95-119.

Ben-Amotz, A. and Avron, M. (1989) The biotechnology of mass culturing *Dunaliella* for products of commercial interest. In Cresswell, R.C., Rees, T.A.V., Shah, N. (eds) *Algal and Cyanobacterial Biotechnology*, Longman Scientific and Technical Press, pp. 90-114.

Ben-Amotz, A. and Avron, M. (1990) The biotechnology of cultivating the halotolerant alga *Dunaliella*. *Trends in Biotechnology* 8: 121-126.

Ben-Amotz, A., Katz, A. and Avron, M. (1982a) Accumulation of Beta-carotene in halotolerant algae: purification and characterization of beta-carotene-rich globules from *Dunaliella bardawil* (Chlorophyceae). *Journal of Phycology* 18: 529-537.

Ben-Amotz, A., Sussman, I. and Avron, M. (1982b) Glycerol production by *Dunaliella*. *Experientia* 38: 49-52

Ben-Amotz, A., Edelstein, S. and Avron, M. (1986) Use of the β-carotene rich alga *Dunaliella bardawil* as a source of retinol. *British Poultry Science* **27**: 613-619.

Bental, M., Pick, U., Avron, M. and Degani, H. (1990) Metabolic studies with NMR spectroscopy of the alga *Dunaliella salina* trapped within agarose beads. *European Journal of Biochemistry* **188**: 111-116.

Bental, M., Pick, U., Avron, M. and Degani, H. (1991) Polyphosphate metabolism in the alga *Dunaliella salina* studied by ^{31}P-NMR. *Biochemica et Biophysica Acta* **1092**: 21-28.

Blackwell, J. R. and Gilmour, D. J. (1989) Determination of volume of *Dunaliella* cells by lithium dilution measurements and derivation of internal solute concentrations. *Journal of Experimental Botany* **40**: 795-802.

Bolhàr-Nordenkampf, H.R. and Öquist, G. (1993) Chlorophyll fluorescence as a tool in photosynthesis research. In Hall, D.O., Scurlock, J.M.O., Bolhàr-Nordenkampf, H.R., Leegood, R.C., Long, S.P. (eds.) *Photosynthesis and Production in a Changing Environment: a field and laboratory manual*. Chapman & Hall, London pp.193-206.

Bollag, D.M., Rozycki, M.D. and Edelstein, S.J. (1996) *Protein Methods* (2nd ed.) John Wiley & Sons, Inc., New York, p.123.

Borowitzka, L.J. and Brown, A.D. (1974) The salt relations of marine and halophilic species of the unicellular green alga, *Dunaliella*: The role of glycerol as a compatible solute. *Archives of Microbiology* **96**: 37-52.

Borowitzka, L.J., Kessly, D.S. and Brown, A.D. (1977) The Salt relations of Dunaliella. Further observations on glycerol production and its regulation. *Archives of Microbiology* **113**: 131-138.

Borowitzka, M.A. and Borowitzka, L.J. (1988) *Dunaliella*. In Borowitzka, M.A. and Borowitzka, L.J. (eds.), *Microalgal Biotechnology*, Cambridge University Press, Cambridge, pp. 27-58.

Brock, T.D. (1975) Salinity and the ecology of *Dunaliella* from Great Salt Lake. *Journal of General Microbiology* **89**: 285-292.

Brodelius, P. (1985) Immobilized plant cells. In A.I. Laskin (ed) *Enzymes and Immobilized Cells in Biotechnology*, The Benjamin/Cummings Publishing Company, Inc., California, 109-148.

Brodelius, P. and Vandamme, E.J. (1987) Immobilized cell systems. In Rehm, H.J. & Reed, G. (eds) *Biotechnology Volume 7a (Enzyme Technology)*, VCH Verlagsgesellschaft mbH, Weinheim, FRG: 405-464

Brouers, M. and Hall, D.O. (1985) Drying pretreatment enhances the photosynthetic stability of alginate immobilised *Phormidium laminosum*. *Biotechnology Letters* **7**: 567-572.

Brouers, M., deJong, H., Shi, D.J. and Hall, D.O (1989) Immobilized cells: An appraisal of the methods and applications of cell immobilization techniques. In R.C. Cresswell, T.A.V. Rees and N. Shah (eds.) *Algal and Cyanobacterial Biotechnology*, Longman Group UK Limited, Essex, England. pp. 272-293.

Brown, A.D. and Borowitzka, L.J. (1979) Halotolerance of *Dunaliella*. In Levandowsky, M. and Hunter, S.H. (eds.) *Biochemistry and Physiology of Protozoa* (2nd ed.), Academic Press Inc., pp. 139-190.

Brown, A.D., Goyal, A., Larsen, H. and Lilley, R.McC. (1987) A salt-sensitive mutant of *Dunaliella tertiolecta*. *Archives of Microbiology* **147**: 309-314.

Brown, F.F., Sussman, I., Avron, M. and Degani, H. (1982) NMR studies on glycerol permeability in lipid vesicles, erythrocytes and the alga *Dunaliella*. *Biochimica et Biophysica Acta* **690**: 165-173.

Bucke, C. (1987) Cell immobilization in calcium alginate. *Method in Enzymology* **135**: 175-189.

Butcher, R.W. (1959) An introductory account of the smaller algae of British coastal waters. I. Introduction and Chlorophyceae. *Fish. Invest. Lond.*, Ser.4.

Cañizares, R.O., Domínguez, A.R., Rivas, L., Montes, M.C. Travieso, L. and Benítez, F. (1993) Free and immobilized cultures of *Spirulina maxima* for swine waste treatment. *Biotechnology Letters* **15**: 321-326.

Castro, I.M. and Loureiro-Dias, M.C. (1991) Glycerol utilization in *Fusarium osysporum* var. *lini*: regulation of transport and metabolism. *Journal of General Microbiology* **137**: 1497-1502.

Chang, T., Ohta, S., Ikegami, N., Miyata, H., Kashizmoto, T. and Kondo, M. (1993) Antibiotic substances produced by a marine green alga, *Dunaliella primolecta*. *Bioresource Technology* **44**: 149-153.

Cheetham, P.S.J., Blunt, K.W. and Bucke, C. (1979) Physical studies on cell immobilization using calcium alginate gels. *Biotechnology and Bioengineering* **21**: 2155-2168.

Chen, B.J. and Chi, C.H. (1981) Process development and evaluation for algal glycerol production. *Biotechnology and Bioengineering* **23**: 1267-1287.

Chevalier, P. and de la Noüe, J. (1985) Waste water nutrient removal with microalgae immobilized in carrageenan. *Enzyme and Microbial Technology* **7**: 621-624

Chibata, I., Tosa, T., Sato, T. and Takata, I. (1987) Immobilization of cells in carrageenan. *Methods in Enzymology* **135**: 189-198.

Chitlaru, E. and Pick, U. (1989) Selection and characterization of *Dunaliella salina* mutants defective in haloadaptation. *Plant Physiology* **91**: 788-794.

Chitlaru, E., and Pick, U. (1991) Regulation of glycerol synthesis in response to osmotic changes in *Dunaliella*. *Plant physiology* **96**: 50-60.

Chitlaru, P., Seger, R., and Pick, U. (1997) Activation of a 74 kDa plasma membrane protein kinase by hyperosmotic shocks in the halotolerant alga *Dunaliella salina*. *Journal of Plant Physiology* **151**: 429-436.

Craigie, J.S., McLachlan, J. (1964) Glycerol as a photosynthetic product in *Dunaliella tertiolecta* Butcher. *Canadian Journal of Botany* **42**: 777-778

Cummings, S.P. (1991) Physiological adaptations to increasing salinity of a novel eubacterial halophile. PhD thesis, University of Sheffield.

Da Costa, A.C.A. and Leite, S.G.F. (1991) Metals biosorption by sodium alginate immobilized *Chlorella homosphaera* cells. *Biotechnology Letters* **13**: 559-562.

Dainty, A.L., Goulding, K.H., Robinson, P.K., Simpkins, I. and Trevan, M.D. (1986) Stability of alginate-immobilized algal cells. *Biotechnology and Bioengineering* **28**: 210-216.

Day, J.G. and Codd, G.A. (1985) Photosynthesis and glycollate excretion by immobilized *Chlorella emersonii*. *Biotechnology Letters* **7**: 573-576.

Deckwer, W-D (1995) Microbial conversion of glycerol to 1,3-propanediol. *FEMS Microbiology Reviews* **16**: 143-149.

De Deckker, P. (1983) Australian salt lakes: their history, chemistry, and biota - a review. *Hydrobiologia* **105**: 231-244.

Degani, H., Sussman, I., Peschek, G.A. and Avron, M. (1985) ^{13}C- and ^{1}H-NMR studies of osmoregulation in *Dunaliella*. *Biochimica et Biophysica Acta* **846**: 313-323.

De la Noüe, J. and Proulx, D. (1988) Biological tertiary treatment of urban wastewaters with chitosan-immobilized *Phormidium*. *Applied Microbiology and Biotechnology* **29**: 292-297.

De la Noüe, J., Chevalier, P. and Proulx, D. (1990) Effluent treatment with immobilized microalgae and cyanobacteria: a critical assessment. In Tyagi, R.D. and

Vembu, K. (eds.) *Waste water treatment by immobilized cells*. CRC Press, USA, pp. 143-152.

Delieu, T. and Walker, D. A. (1972) An improved cathode for the measurement of photosynthetic oxygen evolution by isolated chloroplasts. *New Phytologists* **72**: 201-225.

De Pauw, N. and Persoone, G. (1988) Micro-algae for aquaculture. In Borowitzka, M.A. and Borowitzka, L.J. (eds) *Micro-Algal Biotechnology*, Cambridge University Press, pp.197-221

Dillard, G.E. (1980) Freshwater Chlorophycean genera of the Southeastern United States. I. Introduction and Volvocales (Polyblepharidaceae). *Transactions of Kentucky Academy of Science* **41**:126-131.

Enhuber, G. and Gimmler, H. (1980) The glycerol permeability of the plasmalemma of the halotolerant green alga *Dunaliella parva* (Volvocales). *Journal of Phycology* **16**: 524-532.

Evans, R.W., Kates, M., Ginzburg, M. and Ginzburg, B.Z. (1982) Lipid composition of halotolerant algae, *Dunaliella parva* Lerche and *Dunaliella tertiolecta*. *Biochimica et Biophysica Acta* **712**: 186-195.

Fabregas, J., Herrero, C., Abalde, J., Liaño, R., Cabezas, B. (1986) Biomass production and biochemical variability of the marine microalga *Dunaliella tertiolecta* (Butcher) with high nutrient concentrations. *Aquaculture* **53**: 187-199.

Finney, K.F., Pomeranz, Y. and Bruinsma, B.L. (1984) Use of algae *Dunaliella* as a protein supplement in bread. *Cereal Chemistry* **61**: 402-406.

Fisher, M., Pick, U. and Zamir, A. (1994) A salt-induced 60-kilodalton plasma membrane protein plays a potential role in the extreme halotolerance of the alga *Dunaliella*. *Plant Physiology* **106**: 1359-1365.

Fisher, M., Gokhman, I., Pick, U. and Zamir, A. (1996) A salt-resistant plasma membrane carbonic anhydrase is induced by salt in *Dunaliella salina*. *Journal of Biological Chemistry* **271**: 17718-17723.

Fogg, G.E., Nalewajko, C. and Watt, W.D. (1965) Extracellular products of phytoplankton photosynthesis. *Proceedings of the Royal Society of London* (Series B. Biological Science) **162B**: 517-534.

Fontana, D.R. and Haug, A. (1982) Effects of sodium chloride on the plasma membrane of halotolerent *Dunaliella primolecta*: an electron spin resonance study. *Archives of Microbiology* **131**: 184-190.

Frank, G. and Wegmann, K. (1974) Physiology and biochemistry of glycerol biosynthesis in *Dunaliella. Biologisches Zentralblatt* **93**: 707-723.

Freeman, A. (1987) Gel entrappment of whole cells in cross-linked prepolymerized polyacrylamide-hydrazide gels. *Methods in Enzymology* **135**: 216-222.

Fujii S. (1994) Effect of Ca-2+ on the retention of glycerol and amino acids in *Dunaliella tertiolecta. Plant Cell and Environment* **17**: 227-230.

Fujii, S. and Hellebust, J.A. (1992) Release of intracellular glycerol and pore formation in *Dunaliella tertiolecta* exposed to hypotonic stress. *Canadian Journal of Botany* **70**: 1313-1318.

Gaff, D. F. and Okong'O-Ogola, O. (1971) The use of non-permeating pigments for testing the survival of cells. *Journal of Experimental Botany* **22**: 756-758.

Geib, K.; Golldack, D. and Gimmler, H. (1996) Is there a requirement for an external carbonic anhydrase in the extremely acid-resistant green alga *Dunaliella acidophila*? *European Journal of Phycology* **31**: 273-284.

Geraghty, A.M. and Spalding, M.H. (1996) Molecular and structural changes in *Chlamydomonas* under limiting CO_2: A possible mitochondrial role in adaptation. *Plant Physiology* **111**: 1339-1347.

Gilmour, D.J. (1990) Halotolerant and halophilic microorganisms. In Edwards, C.A. (ed) *Microbiology of Extreme Environments*. Open University Press, Milton Keynes, U.K., pp.147-177.

Gilmour, D.J., Hipkins, M.F. and Boney, A.D. (1984a) The effect of osmotic and ionic stress on the primary processes of photosynthesis in *Dunaliella tertiolecta. Journal of Experimental Botany* **35**: 18-27.

Gilmour, D.J., Hipkins, M.F., Boney, A.D. (1984b) The effect of decreasing the external salinity on the primary processes of photosynthesis in *Dunaliella tertiolecta. Journal of Experimental Botany* **35**: 28-35.

Gilmour, D. J., Kaaden, R., Gimmler, H. (1985) Vanadate inhibition of ATPase of *Dunaliella parva in vitro* and *in vivo. Journal of Plant Physiology* **118**: 111-126.

Gimmler, H. and Hartung, W. (1988) Low permeability of the plasma membrane of *Dunaliella parva* for solutes. *Journal of Plant Physiology* **133**: 165-172.

Gimmler, H. and Weis, U. (1992) *Dunaliella acidophila* - life at pH 1.0. In Avron, M. and Ben-Amotz, A. (eds) *Dunaliella: Physiology, Biochemistry, and Biotechnology*. CRC Press, Boca Raton, Florida, pp. 100-133.

Gimmler, H., Weis, U., Weiss, C.; Kugel, H., Treffny, B., (1989) *Dunaliella acidophila* (Kalina) Masyuk: An alga with a positive membrane potential. *New Phytologist* **113**: 175-184.

Ginzburg, B.Z. (1978) Regulation of cell volume and osmotic pressure in *Dunaliella*. In Caplan, S.R., Ginzburg, M. (eds) *Energetics and Structure of Halophilic Microorganisms*, Elsevier/North-Holland Biomedical Press, pp 543-560

Ginzburg, M. (1981) Measurements of ion concentrations in *Dunaliella parva* subjected to hypertonic shock. *Journal of Experimental Botany* **32**: 333-340.

Ginzburg, M. (1991) Processes of adaptation of different cell-lines of *Dunaliella* to widely differing salt concentrations. In Rodriguez-Valera, F. (ed) *General and Applied Aspects of Halophilic Microorganisms*, Plenum Press, New York: 81-88.

Ginzburg, M. and Ginzburg, B.Z. (1981) Interrelationships of light, temperature, sodium chloride and carbon source in growth of halotolerant and halophilic strains of *Dunaliella*. *British Phycological Journal* **16**: 313-324.

Ginzburg, M., Ginzburg, B.Z. (1985) Influence of age of culture and light intensity on solute concentrations in 2 *Dunaliella parva* strains. *Journal of Experimental Botany* **36**: 701-712.

Ginzburg, M., Richman, L. (1985) Permeability of whole *Dunaliella* cells to glucose. *Journal of Experimental Botany* **36**: 1959-1968.

Ginzburg, B.Z., Ginzburg, M., Cohen, M. (1995) A salt-sensitive *Dunaliella* mutant I. Growth and osmoregulatory responses to increases in salt concentration. *Journal of Experimental Botany* **46**: 1505-1513.

Giordano, M, Davis, J.S. and Bowes, G. (1994) Organic carbon release by *Dunaliella salina* (Chlorophyta) under different growth conditions of CO_2, nitrogen, and salinity. *Journal of Phycology* **30**: 249-257.

Golldack, D., Dietz, K.J. and Gimmler, H. (1995) The effects of sudden salt stress on protein synthesis in the green alga *Dunaliella parva*. *Journal of Plant Physiology* **146**: 508-514.

Goyal, A., Brown, A.D. and Gimmler, H. (1987) Regulation of salt-induced starch degradation in *Dunaliella tertiolecta*. *Journal of Plant Physiology* **127**: 77-96.

Goyal, A., Brown, A.D., Lilley, R.M. (1988) The response of the green halotolerant alga *Dunaliella* to osmotic stress: effects on pyridine nucleotide contents, *Biochimica et Biophysica Acta* **936**: 20-28.

Greene, B. and Bedell, G.W (1990) Algal gels or immobilized algae for metal recovery. In I.Akatsuka (ed) *Introduction to Applied Phycology*, SPB Academic Publishing bv, Netherlands, pp.137-149.

Grizeau, D. and Navarro (1986) Glycerol production by *Dunaliella tertiolecta* immobilized within Ca-alginate beads. *Biotechnology Letters* **8**: 261-264.

Grobbelaar, J.U. (1995) Influence of areal density on beta-carotene production by *Dunaliella salina. Journal of Applied Phycology* **7**: 69-73.

Guiseley, K.B. (1989) Chemical and physical properties of algal polysaccharides used for cell immobilization. *Enzyme and Microbial Technology* **11**: 706-716.

Günzel, B., Yonsel, S. and Deckwer, W-D (1991) Fermentative production of 1,3 propanediol from glycerol by *Clostridium butyricum* up to a scale of 2 m^3. *Applied Microbiology and Biotechnology* **36**: 289-294.

Gustafsson, L. and Norkrans, B. (1976) On the mechanism of salt tolerance. Production of glycerol and heat during growth of *Debaryomyces hansenii. Archives of Microbiology* **110**: 177-183.

Hajibagheri, M. A., Gilmour, D. J., Collins, J. C. and Flowers, T. J. (1986) X-ray microanalysis and ultrastructural studies of cell compartments of *Dunaliella parva. Journal of Experimental Botany* **37**: 1725-1732.

Hall, D.O. and Rao, K.K. (1994) *Photosynthesis* (5th ed.). Cambridge University Press, Cambridge.

Hard, B. C. and Gilmour, D. J. (1991) A mutant of *Dunaliella parva* CCAP19/9 leaking large amounts of glycerol into the medium. *Journal of Applied Phycology* **3**: 367-372.

Hard, B.C. and Gilmour, D.J. (1996) The uptake of organic compounds by *Dunaliella parva* CCAP 19/9. *European Journal of Phycology* **31**: 217-224.

Harris, E.H. (1989) Genetic analysis. In Harris, E.H. (ed) *The Chlamydomonas Sourcebook*, Academic Press, Inc., San Diego, 399-446.

Hertzberg, S. and Jensen, A. (1989) Studies of alganate-immobilized marine microalgae. *Botanica Marina* **32**: 267-273.

Hoek, C. van den, Mann, D.G. and Jahns, H.M. (1995) Note added in proof, Subdivision of the Chlorophyta. In Van den Hoek, C., Mann, D.G. and Jahns, H.M. (eds.) *Algae: An introduction to Phycology*. Cambridge University Press, p. 341.

Homann, T., Tag, C., Biebl, h., Deckwer, W-D and Schink, B. (1990) Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. *Applied Microbiology and Biotechnology* **33**: 121-126.

Hoshaw, R.W. and Maluf, L.Y. (1981) Ultrastructure of the green flagellate *Dunaliella tertiolecta* (Chlorophyceae, Volvocales) with comparative notes on three other species. *Phycologia* **20**: 199-206.

Hulst, A.C. and Tramper, J. (1989) Immobilized plant cells: a literature survey. *Enzyme and Micribial Technology* **11**: 546-558.

Huntsman, S.A. (1972) Organic excretion by *Dunaliella tertiolecta*. *Journal of Phycology* **8**: 59-63.

Izawa, S. and Good, N.E. (1972) Inhibition of photosynthetic electron transport and photophosphorylation. *Methods in Enzymology* **24b**: 355-377.

Jeanfils, J. and Thomas, D. (1986) Culture and nitrite uptake in immobilized *Scenedesmus obliquus*. *Applied Microbiology and Biotechnology* **24**: 417-422.

Jones, T.W. and Galloway, R.A. (1979) Effect of light quality and intensity on glycerol content in *Dunaliella tertiolecta* (chlorophyceae) and the relationship to cell growth/osmoregulation. *Journal of Phycology* **15**: 101-106.

Kaplan, A., Schreiber, U. and Avron, M. (1980) Salt-induced metabolic changes in *Dunaliella salina*. *Plant Physiology* **65**: 810-813

Karube, I., Ikemoto, H., Kajawara, K., Tamiya, K. and Matsuoka, H. (1986) Photochemical energy conversion using immobilized blue-green algae. *Journal of Biotechnology* **4**: 73-80

Kaya, V.M., de la Noüe, J. and Picard, G. (1995) A comparative study of four systems for tertiary waste water treatment by *Scenedesmus bicellularis*: New technology for immobilization. *Journal of Applied Phycology* **7**: 85-95.

Kormanik, R.A. and Cravens, J.B. (1979) Removal of algae through microscreening. *Water and Wastes Engineering*. August 1979: 31-35.

Kouwets, F. (1994) The cell cycle in multinucleate coccoid green algae: Ultrastructure and systematics. Dissertation, University of Leiden.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lages, F. and Lucas, C. (1995) Characterization of a Glycerol/H^+ symport in the halotolerant yeast *Pichia sorbitophila*. *Yeast* **11**: 111-119.

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Lambert, G.R., Daday, A. and Smith, G.D. (1979) Hydrogen evolution from immobilized cultures of the cyanobacterium *Anabaena cylindrica* B629. *FEBS Letters* **101**: 125-128.

León, R. and Galván, F. (1994) Halotolerance studies on *Chlamydomonas reinhardtii*: glycerol excretion by free and immobilized cells. *Journal of Applied Phycology* **6**: 13-20.

Lerche, W. (1937) Untersuchungen über Entwicklung und Fortpflanzung in der Gattung *Dunaliella*. *Archiv für Protistenkunde* **88**: 236-268.

Lewin, R.A. (1988) Making mutants and influencing genes - the genetic exploitation of algae and cyanobacteria. In Rogers, L.J. and Gallon, J.R. (eds.) *Biochemistry of the Algae and Cyanobacteria*, Clarendon Press, Oxford, pp.351-359.

Lide, D.R. and Frederikse, H.P.R. (1994) *CRC Handbook of Chemistry and Physics* (75th ed.), CRC Press, Boca Raton, Florida, section 3 page 278.

Lin, E.C.C. (1986) Glycerol facilitator in *Escherichia coli*. *Methods in Enzymology* **125**: 467-473.

Loeblich, L.A. (1982) Photosynthesis and pigments influenced by light intensity and salinity in the halophile *Dunaliella salina* (Chlorophyta). *Journal of the Marine Biological Association, UK* **62**: 493-508.

Loppes, R. (1970) Selection of Arginine-requiring mutants in *Chlamydomonas reinhardi* after treatment with three mutagens. *Experientia* **26**: 660-661.

Lu, S., Zhang, X-N, Li, P-Y, Liu, Z-L and Xia, Z-H (1996) A salt-induced chloroplast protein in *Dunaliella salina* (Chlorophyta). *Journal of Phycology* **32**: 983-986.

Lucas, C., Da Costa, M. and Van Uden, N. (1990) Osmoregulatory active sodium-glycerol co-transport in the halotolerant yeast *Debaryomyces hansenii*. *Yeast* **6**: 187-191.

Mackinney, G. (1941) Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry* **140**: 315-322.

Maeda, M. and Thompson, G.A. Jr (1986) On the mechanism of rapid plasma membrane and chloroplast envelope expansion in *Dunaliella salina* exposed to hypoosmotic shock. *Journal of Cell Biology* **102**: 289-297.

Mallick, N. and Rai, L.C. (1994) Removal of inorganic ions from wastewaters by immobilized microalgae. *World Journal of Microbiology and Biotechnology* **10**: 439-443.

Marano, F., Santa-Maria, A. and Krishnawamy, S. (1985) The flagellar apparatus of *Dunaliella*: Isolation of basal body-flagellar root complex. *Protoplasma* **127**: 82-92

Martinez, G., Cifuentes, A.S., Gonzalez, M.A. and Parra, O.O. (1995) Effect of salinity on sexual activity of *Dunaliella salina* (Dunal) Teodoresco, strain CONC-006. *Revista Chilena de Historia Natural* **68**: 131-138

Masjuk, N.P. (1972) On phylogeny and taxonomy of the genus *Dunaliella* Teod.. *Ukrayins'kyi Botanichnyi Zhurnal* **29**: 744.

Memon, A.R., Herrin, D.L. and Thompson, G.A. Jr (1993) Intracellular translocation of a 28 kDa GTP-binding protein during osmotic shock-induced cell volume regulation in *Dunaliella salina*. *Biochimica et Biophysica Acta* **1179**: 11-22.

Menzel, K, Zeng, A.P. and Deckwer, W.D. (1997) High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by *Klebsiella pneumoniae*. *Enzyme and Microbial Technology* **20**: 82-86.

Miller, J.H. (1992) A short course in bacterial genetics: A laboratory manual and hand book for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, USA pp. 83-129.

Mokady, S., Abramovici, A. and Cogan, U. (1989) The safety evaluation of *Dunaliella bardawil* as a potential food supplement. *Fd Chem. Toxic.* **27**: 221-226.

Montoya, T.H. and Olivera, G.A. (1993) *Dunaliella salina* from saline environments of the central coast of Peru. *Hydrobiologia* **267**: 155-161

Moulton, T.P. and Burford, M.A. (1990) The mass culture of *Dunaliella viridis* (Volvocales, Chlorophyta) for oxygenated carotenoids: laboratory and pilot plant studies. *Hydrobiologia* **204/205**: 401-408.

Moulton, T.P., Sommer, T.R., Burford, M.A., Borowitzka, L.J. (1987) Competition between *Dunaliella* species at high salinity. *Hydrobiologia* **151/152**: 107-116.

Musgrave, S.C., Kerby, N.W., Codd, G.A. and Stewart, W.D.P. (1982) Sustained ammonia production by immobilized filaments of the nitrogen-fixing cyanobacterium *Anabaena* 27893. *Biotechnology Letters* **4**: 647-652.

Nabe, K., Izuo, N., Yamada, S. and Chibata, I. (1979) Conversion of glycerol to dihydroxyacetone by immobilized whole cells of *Acetobacter xylinum*. *Applied and Environmental Microbiology* **38**: 1056-1060.

Nakas, J.P., Schaedle, M., Parkinson, C.M., Coonley, C.E. and Tanenbaum, S.W. (1983) System development for linked-fermentation production of solvents from algal biomass. *Applied and Environmental Microbiology* **46**: 1017-1023.

Nalewajko, C. and Lean, D.R.S. (1972) Growth and excretion in planktonic algae and bacteria. *Journal of Phycology* **8**: 361-366.

Niemax, F. (1971) Die Schutzfunktion des Glycerins bei *Dunaliella tertiolecta*. Dipl.-Thesis Tübingen.

Nilsson, K., Brodelius, P., Mosbach, K. (1987) Entrapment of microbial and plant cells in beaded polymers. *Methods in Enzymology* **135**: 222-230.

Ohta, S., Shiomi, Y., Kawashima, A., Aozasa, O., Nakao, T., Nagate, T., Kitamura, K., Miyata, H. (1995) Antibiotic effect of linolenic acid from *Chlorococcum* strain HS-101 and *Dunaliella primolecta* on methicillin-resistant *Staphylococcus aureus*. *Journal of Applied Phycology* **7**: 121-127.

Oliveira, L., Bisalputra, T. and Antia, N.J. (1980) Ultrastructural observation of the surface coat of *Dunaliella tertiolecta* from staining with cationic dyes and enzyme treatments. *New Phytologist* **85**: 385-392.

Oren, A. (1993) The Dead Sea - alive again. *Experientia* **49**: 518-522

Oren, A. and Shilo, M. (1985) Factors determining the development of algal and bacterial blooms in the Dead Sea: a study of simulation experiments in outdoor ponds. *FEMS Microbiology Ecology* **31**: 229-237.

Oren, A. (1995) The role of glycerol in the nutrition of halophilic archaeal communities: A study of respiratory electron transport. *FEMS Microbiology Ecology* **16**: 281-290.

Oren-Shamir, M., Pick, U. and Avron, M. (1989) Involvement of the plasma membrane ATPase in the osmoregulatory mechanism of the alga *Dunaliella salina*. *Plant Physiology* **89**: 1258-1263.

Oren-Shamir, M., Pick, U. and Avron, M. (1990) Plasma membrane potential of the alga *Dunaliella*, and its relation to osmoregulation. *Plant Physiology* **93**: 403-408.

Oren, A., Gurevich, P., Anati, D.A., Barkan, E., and Luz, B. (1995) A bloom of *Dunaliella parva* in the Dead Sea in 1992: Biological and biogeochemical aspects. *Hydrobiologia* **297**: 173-185.

Phillips, C.R. and Poon, Y.C. (1988) Properties of immobilized cell system. In Phillips, C.R. and Poon, Y.C. (eds) *Immobilization of Cells*. Springer-Verlag, Berlin, pp.91-131.

Post, F.J. (1977) The microbial ecology of the Great Salt Lake. *Microbial Ecology* **3**: 143-165.

Powtongsook, S., Wisessang, S. and Menasveta, P. (1994) Mass cultivation of *Dunaliella salina* in open raceway pond. *Thai Journal of Aquatic Science* 1: 60-66.

Powtongsook, S., Kittakoop, P., Menasveta, P. and Wisessang, S. (1995) Isolation and characterization of *Dunaliella salina* from Thailand. *Journal of Applied Phycology* 7: 75-76.

Preisig, H.R. (1992) Morphology and taxonomy. In Avron, M. and Ben-Amotz, A. (eds) *Dunaliella: Physiology, Biochemistry, and Biotechnology*. CRC Press, Boca Raton, Florida, pp.2-15.

Proulx, D. and de la Noue, J. (1988) Removal of macronutrients from wastewaters by immobilized microalgae. In M. Moo-Young (ed) *Bioreactor Immobilized Enzymes and Cells: Fundamentals and Application*, Elsevier Applied Science, London, 301-310.

Ramazanov, Z., Sosa, P.A., Henk, M.C., del Rio, M.J., Gómez-Pinchetti, J.L. and Reina, G.G. (1995) Low-CO_2-inducible protein systhesis in the green alga *Dunaliella tertiolecta*. *Planta* 195: 519-525.

Raschke, B.C. and Wolf, A.H. (1996) Molecular cloning of a P-type Ca^{2+}-ATPase from the halotolerant alga *Dunaliella bioculata*. *Planta* 200: 78-84.

Rehm, H.J. (1996) Microbial production of glycerol and other polyols. In Rehm, H-J and Reed, G. (eds) *Biotechnology Vol.6* (2nd ed) Products of Primary Metabolism, VCH Verlagsgesellschaft mbH, Weinheim, FRG 206-227.

Robinson, P.K., Dainty, A.L., Goulding, K.H., Simpkins, I. and Trevan, M.D. (1985) Physiology of alginate-immobilized *Chlorella*. *Enzyme and Microbial Technology* 7: 212-216.

Robinson, P.K., Goulding, K.H., Mak, A.L. and Trevan, M.D. (1986) Factors affecting the growth characteristics of alginate-entrapped *Chlorella*. *Enzyme and Microbial Technology* 8: 729-733.

Robinson, P.K., Reeve, J.O. and Goulding, K.H. (1989) Phosphorus uptake kinetics of immobilized *Chlorella* in batch and continuous-flow culture. *Enzyme and Microbial Technology* 11: 590-596.

Sadka, A., Himmelhoch, S. and Zamir, A. (1991) A 150-kilodalton cell surface protein is induced by salt in the halotolerant green alga *Dunaliella salina*. *Plant Physiology* 95: 822-831.

Santos-Rosa, F., Galván, F. and Vega, J.M. (1989) Biological viability of *Chlamydomonas reinhardtii* cells entrapped in alginate beads for ammonium photoproduction. *Journal of Biotechnology* 9: 209-220.

Satoh, A. and Shiraiwa, Y. (1996) Two polypeptide inducible by low levels of CO_2 in soluble protein fraction from *Chlorella regularis* grown in low or high pH. *Plant and Cell Physiology* 37: 431-437.

Schoen, S. (1988) Cell counting. In Lobban, C.S., Chapman, D.J. and Kremer, B.P. (eds.) *Experimental Phycology: A Laboratory Manual.* Cambridge University Press, Cambridge pp.16-22.

Shaish, A., Avron, M. and Ben-Amotz, A. (1990) Effect of inhibitors on the formation of stereoisomers in the biosynthesis of β-carotene in *Dunaliella bardawil.* *Plant and Cell Physiology* 31: 689-696.

Shi, D.-J., Brouers, M., Hall, D.O. and Robins, R.J. (1987) The effects of immobilization on the biochemical physiological and morphological features of *Anabaena azollae*. *Planta* 172: 298-308.

Skryabin, G.K. and Koshcheenko, K.A. (1987) Immobilization of living microbial cells in polyacrylamide gel. *Methods in Enzymology* 135: 198-216.

Smahel, M., Hamann, A. and Gradmann, D. (1990) The prime plasmalemma ATPase of the halophilic alga *Dunaliella bioculata*: purification and characterization. *Planta* 181: 496-504.

Smidsrod, O. and Skjåk-Bræk, G. (1990) Alginate as immobilization matrix for cells. *Trends in Biotechnology* 8: 71-78.

Stein, W.D. (1958) N-Terminal histidine at the active centre of a permeability mechanism. *Nature* 181: 1662-1663.

Streekstra, H., Teixeira de Mattos, M.J., Neijssel, O.M. and Tempest, D.W. (1987) Overflow metabolism during anaerobic growth of *Klebsiella aerogenes* NCTC 418 on glycerol and dihydroxyacetone in chemostat culture. *Archives of Microbiology* 147: 268-275.

Takeda, H. and Hirokawa, T. (1978) Studies on the cell wall of *Chlorella* I. Quantitative changes in cell wall polysaccharides during the cell cycle of *Chlorella ellipsoidea*. *Plant and Cell Physiology* 19: 591-598.

Takenaka, H., Takahashi, H., Hayashi, K. and Ben-Amotz, A. (1993) Protective effect of *Dunaliella bardawil* on water-immersion-induced stress in rats. *Planta Medica* 59: 421-424.

Tamponnet, C., Costantino, F., Barbotin, J-N., and Calvayrac, R. (1985) Cytological and physiological behaviour of *Euglena gracillis* cells entrapped in a calcium altinate gel. *Physiologia Plantarum* 63: 277-283.

Taylor, J. A. and West, D. W. (1980) The use of Even's blue stain to test the survival of plant cells after exposure to high salt and high osmotic pressure. *Journal of Experimental Botany* **31**: 571-576.

Teodoresco, E.C. (1905) Organisation et déveleppement du *Dunaliella*, nouveau genre de Volvocacée-Polyblépharidée, *Botanisches Zentralblatt, Beihefte* **18**: 215-232.

Thielmann, J., Tolbert, N.E., Goyal, A. and Senger, H. (1990) Two systems for concentrating CO_2 and bicarbonate during photosynthesis by *Scenedesmus*. *Plant Physiology* **92**: 622-629.

Thompson, G.A. (1994) Mechanisms of osmoregulation in the green alga *Dunaliella salina*. *Journal of Experimental Zoology* **268**: 127-132.

Travieso, L., Benitez, F. and Dupeiron, R. (1992) Sewage treatment using immobilized microalgae. *Bioresource Technology* **40**: 183-187.

Tsuzuki, M., Gantar, M., Aizawa, K. and Miyachi, S. (1986) Ultrastructure of *Dunaliella tertiolecta* cells grown under low and high CO_2 concentrations. *Plant and Cell Physiology* **27**: 737-739.

van Dam-Mieras, M.C.E. (1992) An introduction to bioreactor design and product yield. In James, J.W. (ed) *Bioreactor Design and Product Yield*. Butterworth-Heinemann Ltd., Oxford, pp.1-7.

van Zyl, P.J., Kilian, S.G. and Prior, B.A. (1990) The role of an active mechanism in glycerol accumulation during osmoregulation by *Zygosaccharomyces rouxii*. *Applied Microbiology and Biotechnology* **34**: 231-235.

Vilchez, C. and Vega, J.M. (1995) Nitrite uptake by immobilized *Chlamydomonas reinhardtii* cells growing in airlift reactors. *Enzyme and Microbial Technology* **17**: 386-390.

Vilchez, M.J., Vigara, J., Garbayo, I., Vilchez, C. (1997) Electron microscopic studies on immobilized growing *Chlamydomonas reinhardtii* cells. *Enzyme and Microbial Technology* **21**: 45-47.

Villarejo, A., Reina, G.G. and Ramazanov, Z. (1996) Regulation of the low-CO_2-inducible polypeptides in *Chlamydomonas reinhardtii*. *Planta* **199**: 481-485.

Vonshak, A. (1990) Recent advances in microalgal biotechnology. *Biotechnology Advances* **8**: 709-727.

Vorlop, K.D. and Klein, J. (1981) Formation of spherical chitosan biocatalysts by ionotropic gelation. *Biotechnology Letters* **3**: 9-14.

Vorlop, K.D. and Klein, J. (1987) Entrapment of microbial cells in chitosan. *Methods in Enzymology* **135**: 259-268.

Walker, D.A. (1993) Polarographic measurement of oxygen. In Hall, D.O., Scurlock, J.M.O., Bolhàr-Nordenkampf, H.R., Leegood, R.C., Long, S.P. (eds.) *Photosynthesis and Production in a Changing Environment: a field and laboratory manual.* Chapman & Hall, London pp.168-180.

Watt, W.D. (1966) Release of dissolved organic material from the cells of phytoplankton populations. *Proceeding of the Royal Society of London* **164B**: 521-551

Webb, C. (1987) Cell immobilisation. In Forster, C.F. & Wase, D.A.J. (eds) *Environmental Biotechnology*, Ellis Horwood Limited, Chichester, England, pp. 347-376.

Wegmann, K. (1969) On the pathways of CO_2 fixation in *Dunaliella*. *Progress in Photosynthesis Research* **3**: 1559-1564.

Wegman, K. (1978) Osmotic regulation in Dunaliella - Facts and questions. In Caplan, S.R. and Ginzburg, M. (eds.) *Energetics and structure of halophilic microorganisms*, Elsevier/North-Holland Biomedical Press, pp.615-618

Wegmann, K. (1986) Osmoregulation in eukaryotic algae. *FEMS Microbiology Reviews* **39**: 37-43.

Wegmann, K., Ben-Amotz, A. and Avron, M. (1980) Effect of temperature on glycerol retention in the halotolerant algae *Dunaliella* and *Asteromonas*. *Plant Physiology* **66**: 1196-1197.

Weiss, M. and Pick, U. (1990) Transient Na^+ flux following hyperosmotic shock in the halotolerant alga *Dunaliella salina*. *Journal of Plant Physiology* **136**: 429-438.

Weiss, M., Bental, M. and Pick, U. (1991) Hydrolysis of polyphosphates and permeability changes in response to osmotic shocks in cells of the halotolerant alga *Dunaliella*. *Plant Physiology* **97**: 1241-1248

Wikström, P., Szwajcer, E., Brodelius, P., Nilsson, K. and Mosbach, K. (1982) Formation of α-keto acids from amino acids using immobilized bacteria and algae. *Biotechnology Letters* **4**: 153-158.

Wilkinson, S.C., Goulding, K.H. and Robinson, P.K. (1989) Mercury accumulation and volatilization in immobilized algal cell systems. *Biotechnology Letters* **11**: 861-864.

Williams, L.A., Foo, E.L., Foo, A.S., Kuhn, I. and Heden, C.G. (1979) Solar bioconversion systems based on algal glycerol production. *Biotechnology and Bioengineering* **8**: 115-130.

Yang, P.Y. and Wang, M.L. (1990) Entrapment of microbial cells for wastewater treatment. In Tyagi, R.D. & K. Vembu (eds) *Waste Water Treatment by Immobilized Cells*, CRC Press, pp.45-53.

Yuasa, T. and Muto, S. (1996) Activation of 40-kDa protein kinases in response to hypo- and hyperosmotic shock in the halotolerant green alga *Dunaliella tertiolecta*. *Plant and Cell Physiology* **37**: 35-42.

Zelazny, A.M., Shaish, A. and Pick, U (1995) Plasma membrane sterols are essential for sensing osmotic changes in the halotolerant alga *Dunaliella*. *Plant Physiology* **109**: 1395-1403.

Zidan, M.A., Hipkins, M.F. and Boney, A.D. (1987) Loss of intracellular glycerol from *Dunaliella tertiolecta* after decreasing the external salinity. *Journal of Plant Physiology* **127**: 461-470.

Zeng, A.P., Biebl, H., Schlieker, H. and Deckwer, W.D. (1993) Pathway analysis of glycerol fermentation by *Klebsiella pneumoniae*: Regulation of reducing equivalent balance and product formation. *Enzyme and Microbial Technology* **15**: 770-779.

Zubay, G. and Marmur, J. (1993) DNA replication, repair, and recombination. In Zubay, G. (ed.) *Biochemistry* (3rd ed.), Wm. C. Brown Publishers, Iowa, U.S.A. p. 750.

Appendix A

Dunaliella Growth Medium

(Hajibagheri *et al.*, 1986; Blackwell and Gilmour, 1989)

In 750 ml distilled water, add:

	Amount	Final Concentration
NaCl[1]	87.66 g	1.5 M
2.0 M MgCl$_2$.6H$_2$O	10 ml	20 mM
1.0 M CaCl$_2$.2H$_2$O	10 ml	10 mM
2.4 M MgSO$_4$.7H$_2$O	10 ml	24 mM
4.0 M KCl	2.5 ml	10 mM
0.5 M Na$_2$SO$_4$	48 ml	24 mM
4.0 M NaNO$_3$	1.25 ml	5 mM
0.1 M NaH$_2$PO$_4$	1 ml	0.1 mM
1.0 M Tris-HCl pH 7.6[2]	20 ml	20 mM
1.5 mM FeEDTA pH 7.6	1 ml	1.5 μM
Supplements[3]	1 ml	

adjust pH to 7.5 with 0.1 M HCl or 0.1 M NaOH

NaHCO$_3$	1 g	11.9 mM

adjust final volume to 1 l, autoclave for 20 minutes at 15 lbs.in^{-1}

[1]Amount of NaCl can be varied when needed.

[2]1.0 M HEPES was used instead of Tris when the medium was needed for glycerol determination using acetylacetone method.

[3]Supplements stock solution: 185 mM H$_3$BO$_3$; 7.0 mM MnCl$_2$.4H$_2$O; 0.8 mM ZnCl$_2$; 0.02 mM CoCl$_2$; 0.0002 mM CuCl$_2$

Appendix B

Standard curve for glycerol determination by acetylacetone method

($R^2 = 0.99$)
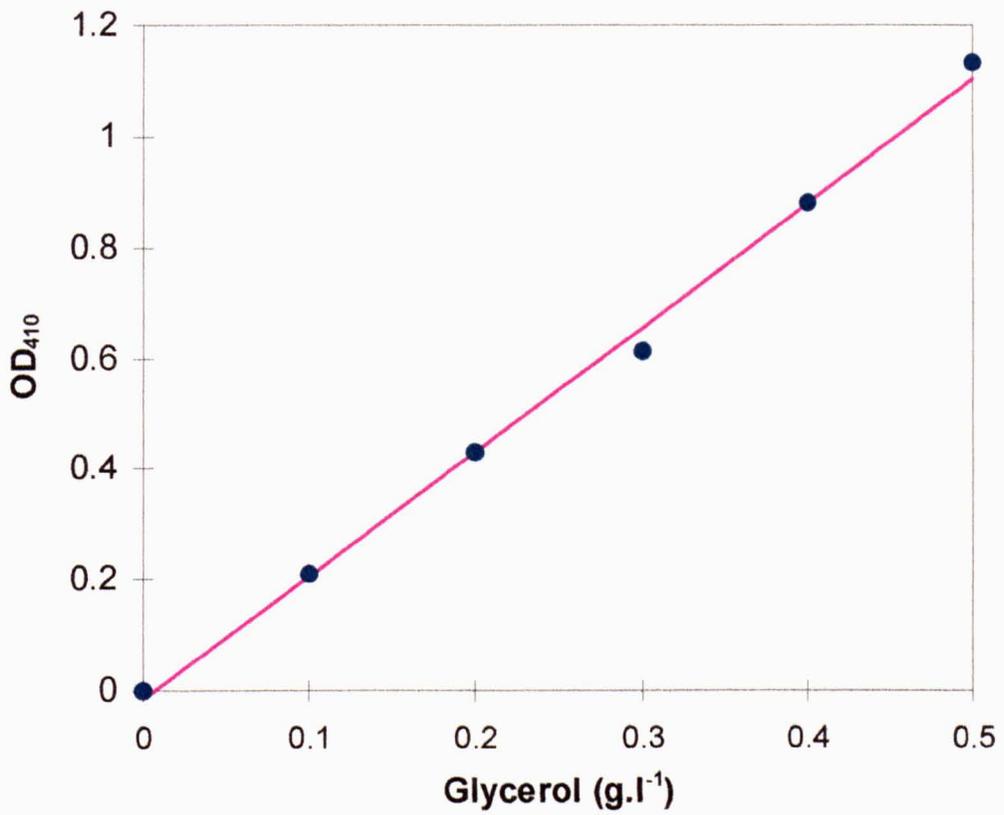
Appendix C

Standard curve for starch analysis

$(R^2 = 0.98)$