

**GLYCEROL PRODUCTION BY  
*DUNALIELLA* SPECIES IN SALINE  
WASTE WATER TREATMENT**

**THESIS**

Submitted in fulfilment of the  
requirements for the Degree of  
MASTER OF SCIENCE  
of Rhodes University

by

**ROBYN ANGELA EMMETT**

March 1995

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## ABSTRACT

In recent years, much research has focused on Algal High Rate Oxidation Ponds as both an economic means for wastewater treatment and as a system for the mass production of algae. With the advent of these systems for the treatment of saline organic effluents, the extreme halophile, *Dunaliella salina* was considered. In this study, the growth and productivity of a number of *Dunaliella* species (and strains thereof) was evaluated in hide soak liquor tannery effluent.

Hide soak liquor, diluted to 20% with water, proved to be highly suitable as a growth medium for the majority of the *Dunaliella* species under study and in some instances, resulted in enhanced growth rates and higher biomass yields compared to those obtained in defined inorganic medium. A few *Dunaliella* species failed to grow in this effluent. A correlation was observed between the lack of growth displayed by these species in this organic-rich medium and their failure to utilise organic compounds. Glycine, a major component of this effluent, possibly stimulates the growth of *Dunaliella*. Studies on the mechanism of growth stimulation by glycine revealed that an algal-bacterial relationship existed whereby the bacteria mineralised the amino acid, releasing ammonia which was then utilised by the alga.

Results of this work revealed significant variations in the intracellular glycerol content amongst the *Dunaliella* species under study. Large differences were also observed between the glycerol contents of effluent-grown and control *Dunaliella* cells, where the effluent-grown cells were characterised by greatly reduced intracellular glycerol content. These reduced glycerol levels are assumed to have arisen from the glycine-induced stimulation of glycerol release which was observed in this study, where the high glycine content of the hide soak liquor is proposed to have induced glycerol release. This enhanced glycerol release in tannery effluent could play a central role in the function of *Dunaliella*-based High Rate Oxidation Ponding systems, by stimulating bacterial activity. Observed glycerol productivities were therefore proposed to be a function of the type and concentration of the organic constituents of the medium. A similar medium-induced phenomenon was observed in the starch content of *Dunaliella* cells.

## ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisor, Prof. Peter Rose for his guidance in this project. I would also like to thank the staff of the Department of Microbiology, and in particular, the technical staff, for assistance rendered throughout this research. My special thanks to my colleagues and friends for their support.

Thanks also to Dr Les Parolis and the School of Pharmaceutical Sciences at Rhodes University for use of laboratory equipment and assistance.

I would like to express my sincere gratitude to the Water Research Commission and The Foundation of Research and Development for their financial support.

Thank you to my Parents for assisting in the financing of a portion of my studies. A special thank you to my Husband for his continued support throughout the duration of this research and for his belief in me and my abilities. Also for his computer expertise.

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## ABBREVIATIONS

ADP	=	adenosine diphosphate
ATCC	=	American Type Culture Collection
ATP	=	adenosine triphosphate
BOD	=	biological oxygen demand
CCAP	=	Culture Collection of Algae and Protozoa
CE	=	constant environment
CFU	=	colony forming units
COD	=	chemical oxygen demand
DFAA	=	dissolved free amino acids
DHA	=	dihydroxyacetone
DHA-kinase	=	dihydroxyacetone kinase
DHAP	=	dihydroxyacetone phosphate
DHA-RD	=	dihydroxyacetone reductase
DO	=	dissolved oxygen
DOC	=	dissolved organic carbon
dpm	=	decay per minute
EDTA	=	ethylene diamine tetraacetic acid
GC	=	gas chromatography
glycerol-P	=	glycerol phosphate
GOGAT	=	glutamine oxo-glutarate aminotransferase/glutamate synthetase
GPase	=	glycerol phosphate phosphatase
GP-DH	=	glycerophosphate dehydrogenase
GS	=	glutamine synthetase
HPLC	=	high performance liquid chromatography
HROP	=	high rate oxidation pond
HS	=	hide soak liquor tannery effluent
LDH	=	lactate dehydrogenase
LIRI	=	Leather Industries Research Institute
M	=	molar
NADH	=	nicotinamide adenine dinucleotide (reduced)
NADPH	=	nicotinamide adenine dinucleotide phosphate (reduced)

NH <sub>3</sub>	=	ammonia
NH <sub>4</sub> <sup>+</sup>	=	ammonium
NMR	=	nuclear magnetic resonance
PEP	=	phosphoenolpyruvate
Pi	=	inorganic phosphate
PK	=	pyruvate kinase
PS	=	photosystem
TCA	=	trichloroacetic acid
Triose-P	=	triose-phosphate

# CHAPTER 1

## INTRODUCTION

### 1.1 Solar Bioconversions and Mass Algal Culture

Utilisation of the photosynthetic machinery for the production of energy, chemicals and food has a particular appeal because it is the most abundant energy-storing and life-supporting process on Earth (Ben-Amotz *et al*, 1982a; Ben-Amotz and Avron, 1989). Exploration for new approaches to the utilisation of solar energy through biological systems focused one line of interest on commercial utilisation of marine algae.

Micro-algae may be grown in outdoor mass cultures at the expense of light energy, mineral nutrients, CO<sub>2</sub> and mechanical energy. Provided that the nutrient supply is adequate, productivity depends primarily on irradiance and temperature (Soeder, 1986). Actual biomass yields in commercial algal ponds tend to be low relative to the theoretical maxima (Richmond, 1986e). With improved technology algal yields have, however, risen dramatically over the past decade. Early efforts in mass algal culture resulted in yields of only 10 to 15g dry weight.m<sup>-2</sup>.day<sup>-1</sup> for short periods. To date however sustained yields have reached 20 to 25g dry weight.m<sup>-2</sup>.day<sup>-1</sup> (Goldman, 1980). There have also been reports of daily yields of 46g dry weight.m<sup>-2</sup>.day<sup>-1</sup> obtained over a one-month period (Richmond, 1986e). Sizeable autotrophic yields can only be obtained in suitable areas of warmer countries. According to Soeder (1986), the highest productivities to date for autotrophic mass cultures of micro-algae under field conditions were obtained in Peru and South Africa.

Under optimal conditions, algal production can compete with that of sugar cane, the most productive of the land plants, where both can generate biomass at a similar rate (Ben-Amotz and Avron, 1989). Algal growth, in addition, is not sensitive to seasonal variations in biomass yield, as are terrestrial plants (Goldman, 1980). Some micro-algae have the ability to utilise highly saline waters (both seawater and inland saline waters) found in arid or semi-arid zones, which are unsuitable for conventional agriculture. The presence of saline waters therefore implies that competition for land with agriculture will be minimised (Richmond and Preiss, 1980; Richmond *et al*, 1980; Ben-Amotz *et al*, 1982a; Benemann, 1989). In addition, algal biomass production in arid zones has the advantages of buffered temperature variations (due to the high heat capacity of

water) and abundant light availability (Ben-Amotz and Avron, 1989). Oswald (1980) has described algal growth as the most efficient way known to fix solar energy in the form of biomass.

The first applications of algal biomass production were aimed at single cell protein production. In recent years however many other potential applications for large-scale algal cultures have been developed, some of which include the production of extractable chemicals of commercial interest, waste-water treatment and the production of food and feed (Ben-Amotz and Avron, 1983b; Benemann, 1989).

### **1.1.1 Mono-algal Mass Culture**

Maintaining a mono-algal culture is perhaps the most formidable challenge facing the producer of algal biomass. An organically rich suspension, such as an open outdoor mass algal culture, is subject to contamination by bacteria, fungi, viruses and protozoa. These contaminants have been shown to reduce the quality as well as the overall yield of the algal products (Richmond, 1986a). According to Soeder (1986), micro-algal biomass production in fresh water systems has proved unsuccessful. Besides the difficulties involved in maintaining a mono-algal culture, this is due to the lack of adequate fresh inland water (Soeder, 1986).

### **1.1.2 Halotolerant Algae - A Solution**

One of the most promising approaches to maintaining a mono-algal mass culture is to propagate algae that have the ability to tolerate extreme environmental conditions and can thereby avoid competition and contamination from other less tolerant organisms (Ben-Amotz and Avron, 1983b). Halotolerant algae are very attractive candidates due to their ability to grow in saline water on arid land (as described above) which may result in reduced land and water costs (Ben-Amotz, 1980).

Three species of algae have been identified as showing the best potential for practical exploration. These include *Dunaliella*-, *Spirulina*- and *Porphyridium spp.* (Cohen, 1986; Benemann, 1989; Chaumont, 1993).

## 1.2 *Dunaliella* spp.

*Dunaliella* spp. are highly suitable for biotechnology exploitation due to their ability to adjust to a changing environment. Besides being able to withstand extreme fluctuations in the salinity of its growth medium (Borowitzka and Borowitzka, 1988; Avron, 1992), they display a wide tolerance to other environmental conditions such as pH and temperature (Avron and Ben-Amotz, 1978; Ben-Amotz, 1980; Ben-Amotz and Avron, 1989; Borowitzka and Borowitzka, 1988).

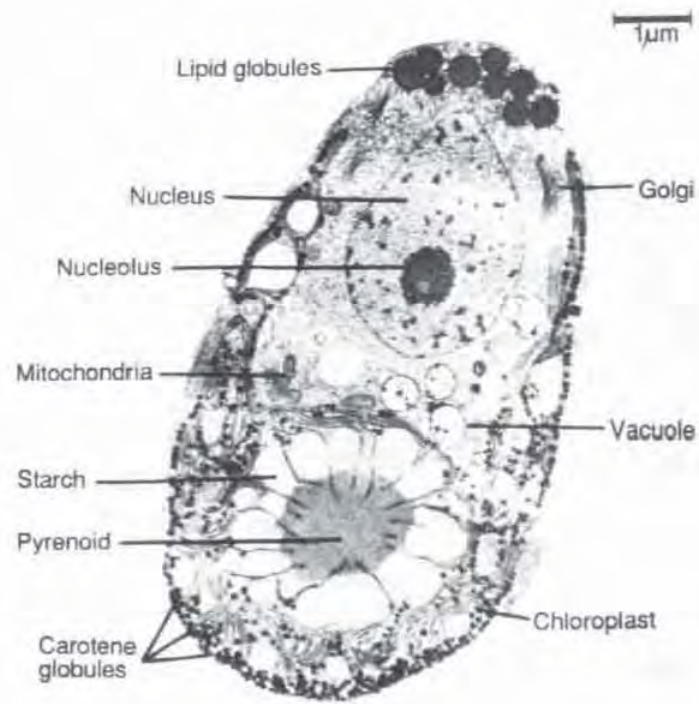
### 1.2.1 Physiology

The Chlorophyte *Dunaliella* is classified under the order Volvocales, which includes a variety of ill-defined unicellular algal species (Richmond, 1986e; Ben-Amotz and Avron, 1990). The cells are generally ovoid or ellipsoid in shape with each cell containing one large cup-shaped chloroplast, which occupies about half of the 50-1000  $\mu\text{m}^3$  volume (Ben-Amotz and Avron 1980; 1990). The basal portion of the chloroplast contains a large pyrenoid surrounded by the storage product, starch (Figure 1.1).

The chief morphological characteristic of *Dunaliella*, in contrast with other members of the Chlorophyta, is the lack of a rigid polysaccharide wall. The cell is a natural protoplast enclosed by a thin, elastic, plasma membrane covered by a glycoprotein "surface coat" (Ben-Amotz and Avron, 1983b; Ben-Amotz, 1993). This permits rapid cell volume changes in response to extracellular osmotic changes (Ben-Amotz and Avron, 1990). *Dunaliella* thus demonstrates a remarkable degree of environmental adaptation to salinity and is widely distributed in natural habitats (Richmond, 1986e), thriving in media ranging from 0,05M (0,2%) to 5,5M (35%) NaCl (Katz and Avron, 1985; Avron, 1992). *Dunaliella* species thus qualify as the most salt-tolerant eukaryotes known, having a growth range broader than the most salt-tolerant prokaryotes, the halobacteria, with which they share extremely saline environments (Borowitzka and Borowitzka, 1988).

The ability of *Dunaliella* species to tolerate high solute concentrations in the environment, as well as rapid changes in those concentrations, is brought about by the unique ability of this alga to produce (by photosynthesis) very high concentrations of intracellular glycerol (Ben-Amotz and Avron, 1980; Borowitzka and Borowitzka, 1988). As a result, an osmotic balance with the extracellular salt concentration is maintained (Ben-Amotz, 1975; Ben-Amotz and Avron, 1980), where the intracellular glycerol concentration varies in direct proportion to the extracellular salt concentration (Ben-Amotz *et al*, 1982a). In this way, under highly saline conditions, glycerol is

reported to accumulate to approximately 85% of the algal dry weight (Avron and Ben-Amotz, 1978; Ben-Amotz, 1980; Calvin and Taylor, 1989).



**Figure 1.1** An Electron Micrograph of a section through *D. salina* var. *hardawii*.  
(From Preisig, 1992).

### 1.2.2 Other Biochemicals and Natural Products

In addition to glycerol production, *Dunaliella* yields a number of commercially valuable products. It is the eukaryote most enriched with  $\beta$ -carotene. Under appropriate cultivation conditions, more than 10% of the dry weight can be attributed to  $\beta$ -carotene (Ben-Amotz and Avron, 1989;1990; Ben-Amotz, 1993), the function of which appears to be protection from the intense solar radiation in the areas where cultivation is practical, for example in arid regions (Ben-Amotz, 1993; Ben-Amotz *et al*, 1982b; Borowitzka *et al*, 1984).  $\beta$ -carotene produced by *Dunaliella* has reached the stage of commercial production. Current market research has indicated a world  $\beta$ -carotene market of approximately 50t, with selling prices of up to \$US 1000 kg<sup>-1</sup>, to specialist customers who market the 'natural' aspects to the food and health food industries (Borowitzka and Borowitzka, 1989).

Since protein constitutes fifty percent of its dry weight (Ben-Amotz, 1980; Borowitzka and Borowitzka, 1988) *Dunaliella* has proved successful as a feed for aquaculture, poultry and other animals (Ben-Amotz and Avron, 1983b). Other minor by-products of *Dunaliella* include thiamine and vitamin B<sub>12</sub> (Ben-Amotz, 1980).

### 1.2.3 Osmoregulation

When *Dunaliella* cells are subjected to a hypo- or hyperosmotic shock, the cells react within seconds, swelling or shrinking, respectively, due to rapid water fluxes (Ben-Amotz, 1980; Grizeau *et al*, 1983; Bental *et al*, 1990). This initial reaction, which is physical in nature, brings the cell back into osmotic equilibrium with the medium (Avron, 1992). This is followed by a metabolic phase of approximately a two hour duration (Bental *et al*, 1990) during which time glycerol is eliminated or synthesised via temperature-dependent enzymatic pathways, accompanied by water efflux and re-entry, respectively, so that the cells regain approximately their original volume (Ben-Amotz, 1980; Grizeau *et al*, 1983; Avron, 1992; Zamir, 1992).

Other osmolytes (beside glycerol) have been shown to play a role in osmoregulation in *Dunaliella* although glycerol has been shown to be the major osmoregulatory solute (Ben-Amotz and Avron, 1973; Frank and Wegmann, 1974). Ehrenfeld and Cousin (1984) described a Na<sup>+</sup>/K<sup>+</sup> exchange mechanism which helped regulate the ionic composition of *Dunaliella* over a wide range of salinities. They reported that the Na<sup>+</sup> and K<sup>+</sup> ionic effect is particularly important in the initial phases of shock.

### 1.2.3.1 Glycerol - Starch Interconversions

Glycerol is synthesised in *Dunaliella* from the photosynthetic product of CO<sub>2</sub> fixation through the Calvin Cycle or via starch degradation (Fujii and Hellebust, 1992). The contribution of these metabolic pathways to glycerol synthesis depends on the availability of light, the starch reserve pool and the size of the salt stress (Avron, 1992).

In the dark, *Dunaliella* produces glycerol exclusively by degradation of starch. The cell's recovery from hyperosmotic shock is therefore dependent on the starch reserve pools (Avron, 1992). In the light, hyperosmotic shock greatly stimulates the rate of glycerol production (Gimmler and Möller, 1981) and gives rise to a corresponding degradation of starch. This indicates that starch degradation also has a significant contribution to glycerol production in the light (Avron, 1992). Starch breakdown has, in fact, been shown to account for seventy percent of glycerol synthesis in the light following hyperosmotic stress (Borowitzka and Borowitzka, 1988). Hyperosmotic shock, in turn, strongly inhibits starch synthesis (Avron, 1992 and Gimmler and Möller, 1981). On the other hand, hypo-osmotic shock induces a decrease in glycerol content and a parallel increase in starch content (Gimmler and Möller, 1981). This indicates a metabolic conversion of glycerol to starch. A transient inhibition of photosynthesis is also induced under hypo-osmotic shock and a substantial reduction (although not inhibition) of glycerol synthesis (Avron, 1992). *Dunaliella*, therefore, appears to utilise a dynamic interconversion between the two major carbon pools (glycerol and starch), as well as photosynthesis, to meet the osmotic requirements set by the external salt concentration (Borowitzka *et al*, 1977; Avron, 1992). The regulation of carbon flow is thus integrated with the normal pathways of carbon metabolism prevalent in these cells (Bental *et al*, 1990). The exact enzymology and regulation of the pathway for CO<sub>2</sub> incorporation into glycerol is not completely known.

Glycerol production in *Dunaliella* is carried out mostly within the chloroplast and partly in the cytoplasm (Avron, 1992). Dihydroxyacetone phosphate (DHAP), produced either by photosynthesis or glycolysis and the oxidative pentose phosphate pathway, directs carbon flow in *Dunaliella* towards glycerol or starch (Goyal *et al*, 1986; Belmans and Van Laere, 1987; Bental and Degani, 1992). Subjecting the algae to a hyperosmotic shock activates a pathway converting stored polysaccharides or Calvin Cycle intermediates to DHAP, followed by reduction to glycerol phosphate and hydrolysis by the specific phosphatases. This results in the observed accumulation of glycerol (Ben-Amotz and Avron, 1983b). On being subjected to hypo-osmotic shock, a pathway leading to the oxidation of glycerol to dihydroxyacetone, its phosphorylation to DHAP

and the conversion of the latter to non-osmotically active polysaccharides is thought to be activated (Ben-Amotz and Avron, 1983b).

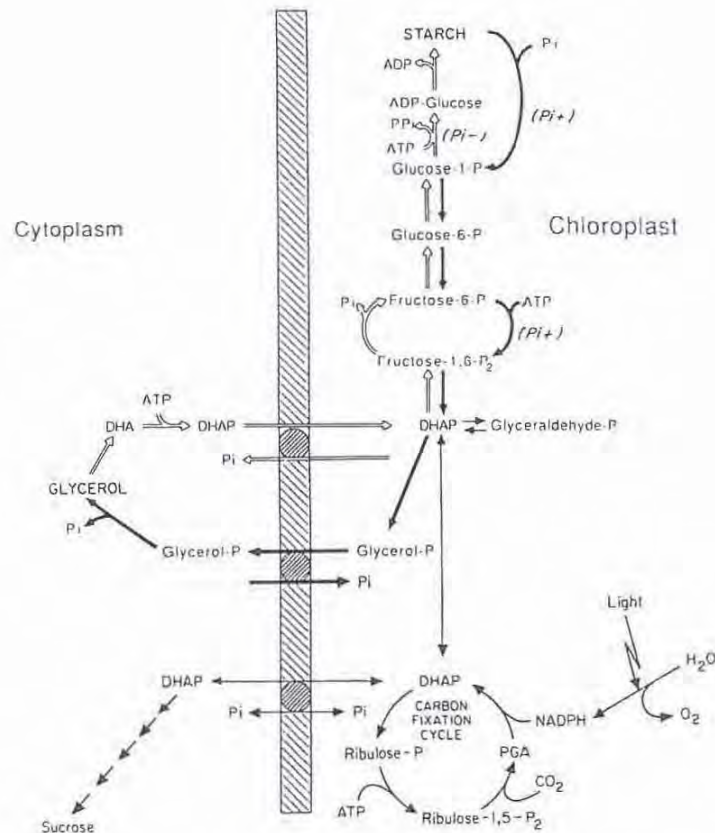
### 1.2.3.2 The Glycerol Cycle and the Pi/triose-P Translocator

Several authors have proposed a metabolic pathway for glycerol metabolism in *Dunaliella* based mainly on the identification of several enzymes which are unique to *Dunaliella* (Gimmler and Möller, 1981; Ben-Amotz *et al*, 1982a). Four enzymes catalyse the interconversions between DHAP and glycerol in *Dunaliella*: two reversible steps, viz. a glycerophosphate dehydrogenase (GP-DH) and NADPH-specific dihydroxyacetone reductase (DHA-RD) and two irreversible steps, viz. a glycerol phosphate phosphatase (GPase) and a dihydroxyacetone kinase (DHA-kinase). DHA-RD catalyses the conversion of glycerol to DHA, DHA-kinase mediates the phosphorylation of DHA by ATP, GPase specifically dephosphorylates DL-glycerol-3-P and GP-DH catalyses the conversion of DHAP to glycerol-3-P (Gimmler and Lotter, 1982; Bental and Degani, 1992). The enzymes involved in the osmoregulatory process in *Dunaliella* are presented schematically in figure 1.2

These same unique enzymes were isolated in another halotolerant alga, *Asteromonas gracilis*, which also osmoregulates with glycerol (Ben-Amotz *et al*, 1982a). These enzymes comprise the so-called glycerol cycle originally proposed by Wegmann in 1979, who recognised the pivotal role for DHAP (Gimmler and Möller, 1981).

DHAP can either remain in the chloroplast and be used as a substrate for starch synthesis, or leave the chloroplast via the Pi/triose-P translocator to the cytoplasm, where it will be converted to cytoplasmic carbon metabolites - mainly glycerol (Bental and Degani, 1992). The Pi/triose-P translocator is a key enzyme determining the partitioning of DHAP between the chloroplast and the cytoplasm. The direction of transport via the translocator is controlled by the relative concentrations of its substrates in the chloroplast and cytoplasm viz. Pi, phosphoglycerate, triose phosphates as DHAP or glycerol-P (Bental and Degani, 1992) (Figure 1.2). The role of Pi described above and the validity of this proposed glycerol cycle is supported by the hypotheses presented by Gimmler and Möller (1981) and Bental *et al* (1990) who suggested the role of Pi as a trigger in controlling the carbon distribution between starch and glycerol. The osmotic response is therefore thought to be initiated by differential volume changes of the cytoplasm and chloroplast, which alter the cytoplasmic orthophosphate concentration, triggering the Pi/triose-P translocator. This would activate the chloroplast enzymes in the direction of either the starch or

glycerol synthesis (Bental *et al*, 1990). Oren-Shamir *et al* (1990), however, suggested that activation of the plasma membrane ATPase may be the trigger for osmoregulation in *Dunaliella*.



**Figure 1.2** A hypothetical pathway of glycerol to starch interconversion following osmotic shocks. The heavy arrows represent pathways activated by hyperosmotic shocks, the empty arrows represent pathways activated following hypo-osmotic shocks and the thin arrows represent other pathways of carbon metabolism prevalent in the cell. The circles in the chloroplast membrane represent the Pi/triose-phosphate translocator. Fructose-6-P = fructose 6-phosphate; fructose-1,6-P<sub>2</sub> = fructose 1,6-diphosphate; glucose-1-P = glucose 1-phosphate; glucose-6-P = glucose 6-phosphate; ribulose-P = ribulose 5-phosphate; ribulose-1,5-P<sub>2</sub> = ribulose 1,5-diphosphate; Glycerol-P = glycerol-3-phosphate; PGA = 3-phosphoglycerate; Pi = inorganic phosphate; DHA = dihydroxyacetone; DHAP = dihydroxyacetone-phosphate. (From Bental and Degani, 1992)

#### 1.2.4 Glycerol Leakage

Glycerol is a solute to which cell membranes are usually readily permeable (Brown *et al*, 1982a). *Dunaliella* cells are, however, able to maintain an extreme concentration gradient ( $>10^4$ ) between the intracellular fluid and the external medium (Wegmann *et al*, 1980). This is necessary for osmoregulation in environments of high salinity. NMR spectroscopy revealed that the cell membrane of *Dunaliella* is unique in exhibiting an exceptionally low permeability towards glycerol (Brown *et al*, 1982b). This low permeability is, however, disturbed when cells are subjected to high temperatures (Wegmann *et al*, 1980), abrupt and intense hypo-osmotic stress (Gilmour *et al*, 1984a; Zidan *et al*, 1987; Fujii and Hellebust, 1992) and ageing or death (Borowitzka and Borowitzka, 1988).

Fujii and Hellebust (1992) reported that *D. tertiolecta* has the ability to osmoregulate by releasing intracellular glycerol into the medium in response to a very abrupt hypo-osmotic shock. They concluded that the release of intracellular glycerol is mainly due to the formation of small, transient, non-specific pores induced by the hypo-osmotic shock and that the pore size (and possibly pore duration) is related to the degree of the applied down shock. Further research performed by Fujii (1994) substantiated these findings.

There is conflicting data on the amount of glycerol leakage from stressed and unstressed, healthy *Dunaliella* cells (Ben-Amotz and Avron, 1973; Jones and Galloway, 1979; Borowitzka and Borowitzka, 1988). Strains of *Dunaliella parva* have been shown to leak more than thirty-three percent of the cell content of glycerol per day under unstressed conditions (and more when the cells were stressed) (Enhuber and Gimmler, 1980; Borowitzka and Borowitzka, 1988). The considerable amount of glycerol released by *Dunaliella* into the medium is of importance for the heterotrophs growing in association with *Dunaliella* in saline waters (Hellebust, 1965; Sharp, 1977; Oren, 1993; Giordano *et al*, 1994).

### 1.3 The Economics of Glycerol Production by *Dunaliella* spp.

At present glycerol is mainly produced by chemical synthesis from the petrochemical industry (Chen and Chi, 1981; Ben-Amotz and Avron, 1983b) or as a by-product of the soap industry (Chen and Chi, 1981). Limitations on oil reserves and the replacement of soaps with other detergents have, however, raised interest in the commercial prospects of a biological process for glycerol production. Attention of researchers was therefore focused on the ability of algae to achieve this objective. The potential production of glycerol by *Dunaliella* is thus of obvious

economic benefit and has been reviewed by Chen and Chi (1981) and Ben-Amotz and Avron (1980).

### 1.3.1 A Two-Stage Process for Algal Glycerol Production

Chen and Chi (1981) proposed and developed a process for large scale algal production of glycerol based on a two-stage process which was patented by Avron and Ben-Amotz (1978). The first stage of the two-stage process involves growth in a medium of 1,5M NaCl to achieve maximum cell yield and the second stage involves the transfer of the algal biomass to a 3M NaCl medium for maximum glycerol accumulation.  $\beta$ -carotene and animal feed protein were recovered as by-products, solar energy was utilised in glycerol synthesis (as well as in product recovery) and CO<sub>2</sub> was utilised as a stack gas (Chen and Chi, 1981). The process is extremely labour and capital intensive but total production cost was found to be competitive with existing petroleum-based glycerol processes (Chen and Chi, 1981). The cultivation strategy used in the Koor Food Ltd process (Israel) is very similar to that outlined above, varying only in the cell harvesting and product recovery steps (Chen and Chi, 1981).

### 1.3.2 Cost Analysis

Williams *et al* (1978) noted that the production of glycerol from organic and biological sources requires purification from crude dilute aqueous solutions. This process constitutes a major cost. The production of pure glycerol from these crude solutions normally involves evaporation and steam-vacuum distillation and the total steam requirement is reported to be 5-10 kg steam.kg<sup>-1</sup> pure glycerol. The purification energy required is thus approximately equal to the energy content of the glycerol produced.

Chen and Chi (1981) calculated that glycerol from *Dunaliella parva* would cost (in 1980 prices) US\$ 0,752.kg<sup>-1</sup>, compared to a cost of US\$ 0,880.kg<sup>-1</sup> for glycerol produced by a petrochemical process. Increasing prices for crude oil would further improve the cost differential between these two processes in favour of the algal process. With present techniques of *Dunaliella* cultivation and current relatively depressed petroleum prices, algal glycerol production on a commercial scale has not, however, proved economically feasible (Ben-Amotz and Avron, 1990).

### 1.3.3 Integration of Algal Glycerol Production with Wastewater Treatment

The use of wastewater as a source of nitrate and phosphate for algal cultivation has been proposed. The integration of wastewater treatment with algal glycerol production offers the advantage of reducing not only the cost of raw materials, but also the total production cost. This is due to the benefits arising from the wastewater treatment stage of this two-fold process (Chen and Chi, 1981). Wastewater systems differ from "clean systems" (well defined mineral media) with respect to their dissolved organic carbon (DOC) levels and their mixed populations. The harvested mass from a DOC loaded system is a mixture of algae, organic residues at different stages of decomposition and bacteria, which may amount to one quarter of the harvested product (Richmond, 1986a;e). The biomass is collectively referred to as the "albazod" (algae, bacteria, zooplankton and detritus) (Richmond, 1986e; Soeder, 1986).

There are a number of benefits which could arise from the integration of algal glycerol production and wastewater treatment, some of which include:

- (a) The initial cost outlay of ponds and associated infrastructure is a major factor limiting development in algal mass cultivation. The use of existing infrastructure (at wastewater works) would give rise to benefits of operating economies of scale (Chen and Chi, 1981).
- (b) Techniques for the supply of carbon dioxide constitute a significant portion of the running costs incurred in the large-scale cultivation of algae. Benemann (1989) estimated this to amount to approximately one-third of the cost for large-scale systems. Bacterial respiration produces carbon dioxide as an end-product of oxidation in wastewater treatment works and would thus significantly reduce this cost (Chen and Chi, 1981; Richmond, 1986e).
- (c) Mechanical aeration contributes significantly to the operating costs. Algae serve an important function in the production of oxygen which is required for the bacterial oxidation of organic waste. This photosynthetic oxygenation therefore offers significant savings (Richmond, 1986e; Oswald, 1988a).
- (d) The cost of nutrients contributes to a large portion of the running costs (approximately 30%) of large-scale cultivation of algae in "clean" systems (Chen and Chi, 1981; Richmond, 1986d). The end-products of bacterial oxidation e.g. ammonia and phosphate are utilised by the algae. This photosynthetic reduction of the oxidised nutrients accomplishes a further degree of tertiary wastewater treatment (Oswald, 1988b). In addition, *Dunaliella* is reported to utilise organic compounds as nitrogen sources (Williams *et al*, 1978; Rose, 1992) which would aid in reducing the organic load of the wastewater.

- (e) In "clean" algal cultures, dissolved oxygen (DO) may be accumulated to toxic concentrations, reducing the photosynthetic rate. In wastewater systems, however, the oxygen produced by photosynthesising biomass is taken up by the heterotrophic micro-organisms (Richmond, 1986a;e).
- (f) Glycerol released by *Dunaliella* enhances the treatment of organic wastes (Rose, 1992).
- (g) *Dunaliella* yields a number of commercially valuable products. The production of any by-product would be beneficial to the waste treatment process, which normally operates without recovery of value.

To date, however, there is little indication in the literature that algae are harvested, processed and utilised from wastewater treatment plants. This is due mainly to toxicological considerations (Behr and Soeder, 1981). Oswald (1988b) stated that growth of algae in wastewater for specific products other than oxygen and biomass is unlikely. Williams *et al* (1983) then focused attention on additional possibilities based on algal glycerol production, such as the linked fermentation production of high value chemicals from algal biomass.

#### **1.4 The Linked Fermentation Production of Chemicals from Algal Biomass**

In the past there was great interest in developing the concept of energy bioconversion by growing, harvesting and fermenting micro-algae and in this way, producing an environmentally and economically feasible alternative to fossil fuels (Goldman, 1980; Cohen, 1986; Benemann 1989). Most of the world's energy supply is derived from non-renewable fossil-fuels (Oswald *et al*, 1977; Calvin and Taylor, 1989). While there is much debate on the quantity of these fuels still available, and on their longevity, it is unarguable that these resources are finite (Aaronson *et al*, 1980). It is therefore essential that alternative sources of energy be identified and developed. There are also economic and environmental problems associated with the use of fossil fuels (Oswald *et al*, 1977). One attractive alternative to petroleum and coal is photosynthetically generated biomass. It is renewable, the technology for its production and utilisation is within our grasp, and its use has no effect on the CO<sub>2</sub> concentration of the atmosphere. Because of the limitations of available geographical areas, low yields, and lack of investment capital, it is, however, doubtful that algal-derived fuels can totally substitute for either petroleum fuels or natural gas. By using such a system it is, however, possible to make an HROP self-sufficient in energy. According to Oswald (1988a), waste-grown algal biomass can be fermented to methane with a fifty percent efficiency and converted to electrical energy with a twenty-five percent efficiency. In this way, each kilogram of micro-algae can yield approximately 1 kW-h of

electrical energy. Because of the capital costs and costs involved in the fermentation system, this process is, however, only applicable to areas where electrical energy is very costly or not available at all (Oswald, 1988a;b).

#### 1.4.1 *Dunaliella* Biomass as a Substrate

Due to the fact that *Dunaliella* can accumulate glycerol up to eighty-five percent of its dry weight (Avron and Ben-Amotz, 1978; Ben-Amotz, 1980), Williams *et al* (1978) suggested that this glycerol-rich biomass could be ideal as a substrate for a variety of bioconversion processes.

The genus *Dunaliella* possesses unique characteristics which can be exploited in linked fermentation's, viz.

- (a) Members of this genus lack a cell wall and can be lysed in dilute media (Ben-Amotz and Avron, 1981), thereby eliminating elaborate process development for cell wall breakage (Nakas *et al*, 1983; Aizawa and Miyachi, 1992).
- (b) These micro-organisms accumulate high intracellular concentrations of glycerol during their osmoregulatory activities (Ben-Amotz and Avron, 1980; Borowitzka and Borowitzka, 1988).
- (c) They thrive in saline wastewater (such as tannery effluent), where they aid in the breakdown of organics. High biomass yields can therefore be produced in this inexpensive waste product and the wastewater can be treated simultaneously (Laubscher, 1991; Rose, 1992).
- (d) Following the extraction of the crude glycerol as a fermentation substrate, the remaining residue may be used as a proteinaceous feed or for the extraction of  $\beta$ -carotene, a valuable chemical (Richmond and Preiss, 1980).

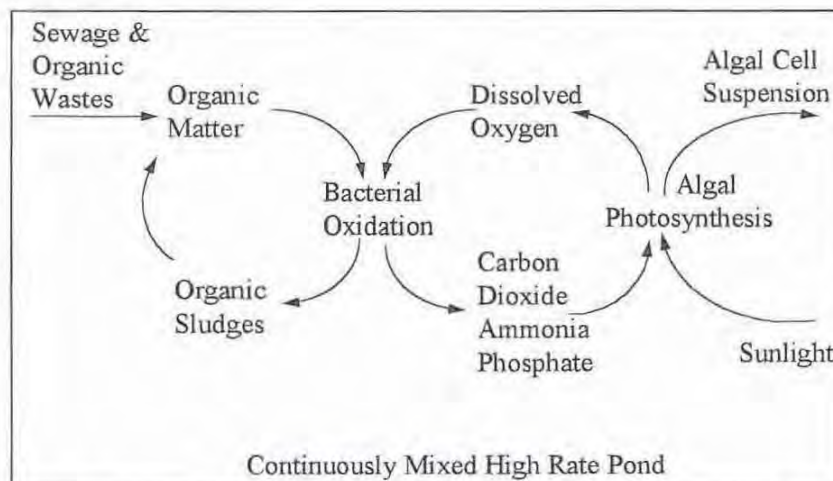
Nakas *et al* (1983) explored the possibility of using the glycerol-rich biomass of *D. tertiolecta*, *D. primolecta*, *D. parva* and *D. salina* as a feedstock for fermentation to solvents such as n-butanol, 1,3-propanediol and ethanol using *Clostridium pasteurianum*. The fermentation of the algal biomass was rapid and a glycerol utilisation efficiency of over 93% was reported. This was assumed to be due to the high protein and nutrient content of the *Dunaliella* biomass.

## 1.5 Algal Culture and Wastewater Treatment

### 1.5.1 Photosynthetic Wastewater Treatment

The development of large scale algal culture for wastewater treatment by Oswald and co-workers introduced a new concept in algal mass cultivation (Richmond and Preiss, 1980; Behr and Soeder, 1981). In this process, which is termed "photosynthetic wastewater treatment" (Soeder, 1986; Oswald, 1988b), an association is established between the high population of algae and the residing heterotrophic bacteria. The oxygen providing role of micro-algae in photosynthetic wastewater treatment is linked to an interaction with the bacteria responsible for the eventual aerobic degradation of the wastewater organic compounds (Soeder, 1986). Photosynthesising according to light energy input, micro-algae make use of re-mineralised end products of bacterial metabolism ( $\text{CO}_2$ , ammonium *etc.*). The algae in turn supply the bacteria with the  $\text{O}_2$  required for full breakdown of degradable organic compounds (Soeder, 1986; Oswald, 1988b). The process therefore involves biological oxidation and photosynthetic reduction of oxidised nutrients.

The role of algae as oxygen generators is shown in figure 1.3. Waste oxidation can be greatly enhanced in such systems because of the interaction depicted in this cycle. Such a combination of algae and bacteria for a controlled and intensive process of waste treatment is known as an Algal High Rate Oxidation Pond (HROP) (Abeliovich, 1986; Richmond, 1986e).



**Figure 1.3** The interaction between algae and bacteria in High Rate Oxidation Ponds (HROP) (From Oswald, 1988a; b)

Abeliovich and Weisman (1978), however, considered the small residing bacterial population in HROP to be insufficient to account for the extent of degradation observed and suggested that bacteria played only a minor role in the Biological Oxygen Demand (BOD) reduction in HROP systems. They postulated that the role of bacteria was probably confined to degradation of biopolymers, such as protein and starch, rendering them available for algal uptake. Abeliovich and Weisman (1978) have estimated that 25-50% of algal carbon could be directly incorporated without first being oxidised and then photoassimilated. Other authors have supported these findings (Williams *et al*, 1978; Kaplan *et al*, 1986). This would result in further reductions in the organic load of the wastewater.

### **1.5.2 High Rate Oxidation Ponds for Wastewater Treatment**

High Rate Oxidation Ponds are very simple in design, requiring only a shallow pond twenty to forty centimetres in depth and constructed in the form of a raceway. Continuous flow mixing, usually via paddlewheels, ensures that aerobic conditions are maintained (Abeliovich, 1986; Oswald, 1988b). These algal-bacterial systems are among the most economical systems available for the treatment of domestic sewage and industrial organic liquid waste (Oswald, 1988b). Where climate permits, algal HROP systems can equal or exceed the performance of conventional (high cost activated sludge) waste treatment processes and do so more economically (Soeder, 1986; Oswald, 1988b). These low technology systems require practically no energy inputs since the oxygen for decomposing the organic wastes is provided by algal photosynthesis (Abeliovich, 1986). According to Oswald (1988b), properly designed and operated HROPs are capable of removing in excess of ninety percent of the carbonaceous BOD and up to eighty percent of the Nitrogen and Phosphorus over a period of days. This aerobic wastewater treatment process yields water of good bacteriological quality which is suited for unrestricted use in irrigation (Behr and Soeder, 1981).

### **1.5.3 The HROP as a Multi-purpose System**

HROP systems at present involve parallel programmes for the separation of the algal biomass (Abeliovich, 1986). This low value proteinaceous matter has been assessed in animal feeding studies and was found to be suitable as a protein source for various animals and fish (Behr and Soeder, 1981; Herrero *et al*, 1993). In addition, heavy metals and organic pollutants were not accumulated above permissible levels (Soeder, 1986). The chemical composition of the mixed

albazod material does not differ significantly from that of pure micro-algal biomass and comprises fifty to fifty-five percent crude protein (Soeder, 1986). Other major albazod components include carbohydrates, lipids, vitamins and nucleic acids (Soeder, 1981).

Attention was then drawn to the potential of the HROP as a multi-purpose system (Soeder, 1986). Oswald and his co-workers developed such a system incorporating algal biomass production in the treatment of municipal effluents (Richmond and Preiss, 1980; Richmond, 1986e), thereby achieving the dual aim of proteinaceous biomass production and water reclamation. Such systems have a promising economic future since the value of treated water compensates for the overall cost of algal production (Richmond and Preiss, 1980; Richmond, 1986e). The application of algal HROP in wastewater treatment, if exploited fully as a multi-purpose system, appears to offer the greatest potential of all biotechnologies based on micro-algae (Soeder, 1986).

## **1.6 Algal-Bacterial Interrelations**

Many researchers have suggested that algal-bacterial interrelations are more complex than the simplistic view of the mutualistic co-existence of algae and bacteria described by Oswald (1988) above. Dor (1980) researched these algal-bacterial symbiotic associations said to occur in HROPs and questioned the mutual synergism described on the basis of competition for nutrients (including organic compounds) and the production of bacterial inhibitors by algae (Cloete and Toerien, 1981). This thinking lead to a postulate that the relationship between algae and bacteria is antagonistic, not synergistic and that where planktonic algae and bacteria co-exist in the same aquatic environment, their influence on each other might be neutral, synergistic or antagonistic (Cloete and Toerien, 1981).

### **1.6.1 Algal Control over Bacterial Populations**

Bacterial inhibition by algal activity has been known since the 1940's (Cloete and Toerien, 1981). Dor (1980) reported the inhibition of bacteria through soluble organic compounds excreted by algae, which accumulate in the surrounding media. Such antibacterial substances have been identified in old cultures of *Dunaliella* species (Cloete and Toerien, 1981; Lustigmann, 1988). According to Lustigmann (1988), antibiotic production alters the micro-environment surrounding the cell, providing it with enhanced space and light. Dor (1980) emphasised the importance of antibiotic production by showing that only those species capable of producing specific antibacterial inhibitors were able to grow in wastewater in the presence of a specific bacterial flora. Exfoliation of *Dunaliella* cell coat material has been identified by Oliveira *et al*

(1980). This process was intensified in cultures contaminated with bacteria, suggesting that it could provide *Dunaliella* with a cleaning system by removing contaminants from the cell surface, thereby providing a protective role.

Ammonia is generated by the bacterial reduction of nitrate and nitrite and by the deamination of organic nitrogenous compounds (Antia *et al*, 1991). Rose (1992) identified a possible positive control mechanism whereby *Dunaliella* may use its glycerol release to regulate ammonia availability via control of its associated bacterial population. *Dunaliella* displays a preferential uptake of ammonia (Williams *et al*, 1978; Antia *et al*, 1991) which is incorporated at very high rates regardless of the nitrogen status of the cell (Flynn and Butler, 1986). This is thought to be related to lower energy expenditure by the cells since other nitrogen-sources require reduction to ammonia prior to incorporation into amino acids using products of CO<sub>2</sub> fixation (Flynn and Butler, 1986). Algal photosynthesis is however under the control of ammonia. Ammonia is an uncoupler of photosynthesis (Abeliovich, 1986) and, in high concentrations, is toxic to the algae. An algal inhibitory control mechanism over the bacterial population would therefore control excessive deamination and the resultant production of toxic ammonium. Rose (1992) stressed the importance of the deamination rate in the treatment of proteinaceous wastes, such as those from the tanning industry.

### **1.6.2 Bacterial Control over Algal Populations**

Numerous authors have suggested that algae may be unsuccessful in competition with bacteria for certain nutrients. Bacteria are thought to outcompete phytoplankton for most organic (Wheeler and Kirchman, 1986) and inorganic substrates (Antia *et al*, 1991) in marine ecosystems. Dor and Svi (1980) examined the effect of sewage bacteria on algal growth and concluded that only bacteria growing in the presence of an organic substrate had an adverse effect on algal growth, decreasing the rate of divisions and/or yields. In addition to outcompetition of nutrients, bacteria are reported to adversely affect algal growth by the excretion of toxic by-products (Dor, 1980; Cloete and Toerien, 1981) and by the removal of organics necessary as chelating agents (Dor and Svi, 1980). These findings are, however, contrary to the findings of Richmond (1986a,e) who stated that there is no evidence that bacteria directly control the size of algal populations in a well managed, rapidly growing mass culture of algae. This was partly attributed to the extracellular substances excreted by many algae (Richmond, 1986a). Research performed by Vaqué *et al* (1990) also suggested such a role for young healthy algae in algal-bacterial relationships. They stated that actively growing phytoplankton supported a smaller load of attached bacteria than senescent ones. Flynn and Fielder's studies (1989) on the predation of

*Dunaliella* by the heterotrophic dinoflagellate *Oxyrrhis marina* indicated that dead *Dunaliella* cells were selectively removed by *Oxyrrhis*, enabling the *Dunaliella sp.* to dominate the culture.

### 1.6.3 Mutualistic/Synergistic Algal-Bacterial Interactions

Under certain conditions, such as high stress and ageing (stationary phase), *Dunaliella* cells are known to excrete organic matter into the surrounding medium (Huntsman, 1972 and Sharp, 1977; Giordano *et al*, 1994). Oliveira *et al* (1980) estimated that the organic carbon excreted by *Dunaliella* accounts for as much as 66% of the CO<sub>2</sub> fixed in photosynthesis. Huntsman (1972) reported lower values of 4-18% of total assimilation. Glycerol is the major excretory product in *Dunaliella* (as discussed previously). Hellebust (1965) postulated that the glycerol released by algae was of significance in heterotrophic growth of bacteria. Flynn and Butler (1986) reported the release of a number of amino acids by *Dunaliella*, some of which include alanine, leucine, threonine and phenylalanine. The exfoliation or sloughing of *Dunaliella's* glycocalyx has been reported to result in the release of protein and amino sugars (Antia *et al*, 1991). Sharp (1977) observed a correlation between organic accumulation in media and bacterial numbers where old cultures of algae (which showed large accumulations of organic carbon) supported large bacterial populations.

The amino acids released by algae are readily utilised by bacteria, resulting in the regeneration of ammonium which is subsequently used by the algae (Flynn and Butler, 1986). Flynn and Fielder (1989) observed a clustering effect between *Dunaliella* and a heterotroph which was especially apparent under nitrogen deprived conditions for *Dunaliella*. The attraction of motile *Dunaliella* cells to ammonia has been suggested. It is thought to aid them in locating aggregates of heterotrophs which are actively feeding and thus regenerating ammonia. Conversely, the release of free amino acids by *Dunaliella* may be detected by the heterotrophs (Flynn and Fielder, 1986).

## 1.7 Utilisation of Organic Nitrogen by *Dunaliella*

Much controversy exists in the literature regarding the uptake of organics by micro-algae, which have until recently been viewed as photoautotrophic organisms. Species of the genera *Chlorella* and *Scenedesmus* have been found to have the ability to shift rapidly and reversibly between growth in darkness on organic substrates and growth in the light on CO<sub>2</sub> (Kaplan *et al*, 1986). Photosynthetic organisms are able to utilise L-amino acids as a source for growth. This takes place either by means of energy-dependent transport systems (Flynn and Butler, 1986; Antia *et*

*al*, 1991) or by deamination mechanisms occurring on the cell surface with the subsequent uptake and assimilation of the ammonia produced (Muñoz-Blanco *et al*, 1990).

Neilson and Larsson (1980) reported an extensive range of substrates utilised by algae. These include amino acids, urea, acetamide, ureate and some nucleotides. The growth yields with organic nitrogen compounds were generally comparable to those obtained with nitrate or ammonia, although the growth rate varied considerably.

The uptake of amino acids by *Dunaliella* is species specific (Williams *et al*, 1978). Flynn and Fielder (1989) reported that *Dunaliella* has a minimal ability to take up and use free amino acids for growth. Rose (1992), however, reported the uptake of glycine in *D.salina*, which reportedly resulted in enhanced growth. Borowitzka and Borowitzka (1988) reported glutamine utilisation in *D.salina*. Phototrophic growth of *D.tertiolecta* on purine derivative hypoxanthine or allantoate has been reported by Oliveira and Huyhn (1989). These organic Nitrogen compounds were used as efficiently as nitrate for growth. The uptake and utilisation of organic compounds holds important implications for the integration of algal production and organic waste treatment in saline systems.

Pinocytotic uptake of lectins (Antia *et al*, 1991), hypoxanthine and allantoic acid (Oliveira and Huyhn, 1989) have been reported in *D.tertiolecta*. The formation of ligand-receptor complexes occurs at the anterior end of the glycocalyx-covered *D.tertiolecta* cells. Following their internalisation they accumulate within acid-phosphatase-positive vacuoles (presumably lysosomes) (Antia *et al*, 1991). Research performed by Rose (1992) has indicated the presence of a similar endocytotic uptake mechanism in *D.salina*.

Muñoz-Blanco *et al* (1990) described a general deaminating system for L-amino acids in the unicellular green alga, *Chlamydomonas reinhardtii*. This novel mode of amino acid utilisation occurs extracellularly without uptake of the amino acids. It involves a non-specific, inducible cell surface deaminase, which converts L-amino acids to  $\text{NH}_4^+$ . In this way, *C.reinhardtii* was shown to grow at the expense of ammonium supplied by twelve different L-amino acids (Muñoz-Blanco *et al*, 1990). A similar system has been described in *Monodus subterraneus*, some prymnesiophytes and dinoflagellates (Antia *et al*, 1991).

## 1.8 The Treatment of Saline Wastewater

### 1.8.1 The Development and Potential of the Saline HROP

Another advance was made when a saline algal HROP process, similar to that described by Abeliovich (1986) and Oswald (1988a) was developed for the removal of organics from highly saline effluents (Rose *et al*, 1991). This process is based on a near-monoculture of the halotolerant alga *Dunaliella* in tannery effluent over a wide range of salinities (Richmond, 1986; Rose *et al*, 1992). The addition of a salinity factor offers advantages not available in fresh water algal HROPs. These include reduced contamination and regulation of predation and the yield of a predictable algal biomass. The development of saline HROP systems, together with the use of saline organic effluent as growth media for algae, has provided incentives to adequately deal with the treatment of these tannery wastes (Rose *et al*, 1992).

### 1.8.2 Tannery Effluent

Wastewater generated by tannery processes is very pollutive and requires considerable treatment (prior to discharge) to comply with local authority and Department of Water Affairs discharge limits (LIRI Technologies, 1990). Tanneries have been described by Tsotsos (1986) as producing the most polluting waste of any industry. Processing methods have changed toward shorter processing times, lower water usage and more intensive chemical usage, all resulting in far more concentrated effluents. The reduction in water usage is especially relevant to a water-scarce country like South Africa, where the need to conserve and recycle water is becoming increasingly urgent (LIRI Technologies, 1990). Salting is traditionally the most common type of curing method. Each twenty-five kilogram hide cured will produce an outflow of six to seven litres of saturated brine (LIRI Technologies, 1990). It has been estimated that South African tanneries use approximately 600 000 m<sup>3</sup> water, almost all of which becomes effluent (Rose, 1992).

Organic animal wastes typically contain all the necessary nutrients and can therefore support the growth of large concentrations of algae (Shelef *et al*, 1980; Oswald, 1988b). Laubscher (1991) reported the growth of *D.salina* in a range of tannery effluents, indicating their suitability as a growth medium. Saline hide soak liquor supported the best algal growth, producing enhanced biomass yields compared to those obtained in defined inorganic media. Furthermore, hide soak liquor was the only effluent tested which did not require the addition of further enrichment, vitamins or trace elements (Richmond, 1986a). Hide soak liquor is a highly proteinaceous saline

solution and comprises blood, manure, dirt, salt, protein and emulsifiers (Tsotsos, 1986; LIRI Technologies, 1990). The skins consist mainly of the protein collagen and elastin (Tsotsos, 1986). Glycine, a major protein degradation product of collagen, therefore occurs in high concentrations in this effluent (Rose *et al*, 1991).

### 1.8.3 The Role of Glycerol Leakage in Wastewater Treatment

The mechanism by which *Dunaliella* copes with its hypersaline environment involves the accumulation of large concentrations of intracellular glycerol. As stated previously, *Dunaliella* cells are susceptible to a certain amount of glycerol leakage, which over time accumulates in the tannery effluent. The “leakage” of glycerol and its role in heterotroph production has been an area of much controversy dating back to research done by Hellebust and others in the 1960’s. Rose *et al* (1991) suggested that glycerol release offers the possibility of using saline waters and brines for the disposal of refractory organic wastes where C:N ratios do not favour degradative bacterial growth. The proteinaceous nature of tannery effluent results in very low C:N ratios. This causes suppressed bacterial activity, since the C:N ratios are too low for normal biodegradation. Photosynthate leakage (together with the other organics reported to be excreted by *Dunaliella*) elevates the C:N ratio, thereby aiding the residing heterotrophs in the treatment of refractory organic wastes (Rose, 1992). The positive correlation that Sharp (1977) observed between bacterial numbers and organic accumulation in media further substantiates the function of glycerol release in aiding wastewater treatment.

Rose (1992) demonstrated rates of organic load reduction in these systems similar to those observed in the fresh water HROP equivalent. Reduction in the organic load was simultaneous with the growth of *Dunaliella*.

## 1.9 Research Objectives

Given the work reported above, a number of questions arise concerning the practical application of *Dunaliella*-based HROP technology for the treatment of saline effluents. The role of heterotrophic nutrition and glycerol release require both confirmation and quantification in order to understand the operation of these systems. A further potential exists to recover the glycerol reserve in some form and utilise it as a feedstock for the production of Biotechnology products. The research objectives of this study were focused on aspects of the above and were identified as follows:

- 1) To develop an accurate and reproducible means of quantitating glycerol accumulated in and released by *Dunaliella* species in a saline medium.
- 2) To investigate the suitability of bovine salted hide soak liquor - tannery effluent as a culture medium for a variety of *Dunaliella* species and strains.
- 3) To investigate the nature of the reported stimulatory effect of glycine on *Dunaliella* growth.
- 4) To investigate the effect of medium composition on glycerol and starch productivity in *Dunaliella* species.
- 5) To estimate glycerol release by *Dunaliella spp.* and to investigate the effect of medium composition on glycerol release.

## CHAPTER 2

### A COMPARISON OF METHODOLOGIES FOR THE QUANTIFICATION OF GLYCEROL ACCUMULATED AND RELEASED BY *DUNALIELLA* SPECIES.

#### 2.1 INTRODUCTION

The presence of high concentrations of photosynthetically produced glycerol in *Dunaliella* species was first demonstrated by Craigie and McLachlan (1964) in *D. tertiolecta* grown under conditions of high salinity. Photosynthetic production of glycerol has subsequently been confirmed in other species of *Dunaliella* (Ben-Amotz and Avron, 1973; Ben-Amotz *et al*, 1982).

The unique ability of *Dunaliella sp.* to survive in highly saline waters is dependent on the production and accumulation of high intracellular concentrations of glycerol. Central to this is the tolerance by the genus of virtually the entire solubility range of NaCl (Ben-Amotz and Avron, 1973; Frank and Wegmann, 1974; Ben-Amotz *et al*, 1982a). The intracellular glycerol concentration under conditions of constant osmotic environment is the result of a balance between synthesis and metabolic removal of glycerol. The increase or decrease in intracellular glycerol concentration, responding to changes in external NaCl concentration, is due to a change in the balance between these two metabolic processes (Jones and Galloway, 1979; Borowitzka and Borowitzka, 1988). When grown at high salinity (>4M), the intracellular glycerol concentration exceeds 50% of the total dry weight of the cell (Ben-Amotz *et al*, 1982a; Avron, 1992). Several authors have reported glycerol accumulation to 85% of the algal dry weight (Avron and Ben-Amotz, 1978; Ben-Amotz, 1980; Calvin and Taylor, 1989). The accumulation of glycerol by *Dunaliella* has led to a special interest in this alga and its industrial potential (Ben-Amotz, 1980; Chen and Chi, 1981; Ben-Amotz and Avron, 1983a).

Leakage of glycerol from cells has been reported in many different species of the genus *Dunaliella* (Craigie and McLachlan, 1964; Hellebust, 1965; Huntsman, 1972; Ben-Amotz and Avron, 1973; Frank and Wegmann, 1974; Jones and Galloway, 1979). The reported data, however, varies greatly and is not comparable with respect to rates and conditions of glycerol loss. A number of authors have stated that since glycerol concentration is under tight metabolic control, little or no release of glycerol occurs under normal conditions (Borowitzka and Borowitzka, 1988; Goyal, 1989). Ben-Amotz (1975) and Borowitzka *et al* (1977) reported virtually no leakage of glycerol from selected species, except at salt concentrations below 0.6M NaCl.

Glycerol leakage has been observed from cells subjected to an abrupt hypo-osmotic shock (Frank and Wegmann, 1974; Jones and Galloway, 1979; Gilmour *et al*, 1984a). Fujii and Hellebust (1992) attributed this to the formation of transient, non-specific pores in the cell membrane through which intracellular glycerol is released. Wegmann *et al* (1980) reported that glycerol leakage was highly temperature dependent. Essentially no glycerol leakage was observed below 40°C, while exposure to temperatures above 60°C resulted in the release of all intracellular glycerol. Fifty percent release occurred around 50°C. Ben-Amotz and Avron (1989) attributed this to the effect of temperature on membrane organisation. Sharp (1977) observed no appreciable accumulation of glycerol during exponential growth. The onset of the stationary phase, however, resulted in increased rates of excretion. This is in agreement with observations by Hellebust (1965). A high rate of release has also been observed at high salinity corresponding to high intracellular glycerol concentration (Jones and Galloway, 1979; Enhuber and Gimmler, 1980).

There is also little agreement regarding the permeability of the cell membrane to glycerol. Enhuber and Gimmler (1980), who reported the continuous diffusion of significant quantities of glycerol into the medium (following the concentration gradient between the cells and the medium), stated that the cell membrane was not exceptionally impermeable to glycerol. Brown *et al* (1982b), however, using NMR Spectroscopy, found that the cell membrane has a very low permeability to glycerol.

Glycerol efflux rates, varying from 0,1 - 2  $\mu\text{moles glycerol.mg}^{-1}$  chlorophyll.  $\text{h}^{-1}$  have been observed in *Dunaliella spp.* (Enhuber and Gimmler, 1980; Richmond, 1986c). The glycerol accumulated over time is reported to reach concentrations comparable with that of intracellular glycerol (Jones and Galloway, 1979).

The release of organic matter (such as glycerol) is of importance to the economy of ecosystems, since it stimulates the growth of heterotrophic organisms, which enrich the environment with inorganic nutrients (Hellebust, 1965; Enhuber and Gimmmler, 1980; Giordano *et al*, 1994). Ben-Amotz and Avron (1989) have observed an association between glycerol leakage in *Dunaliella* and bacterial blooms, if the organic load is sufficient to support their heterotrophic needs. The quantification of these glycerol levels is therefore of importance to aid in enhancing the function of HRQP, which are based on the action of bacteria. The release of glycerol is also of practical importance in hypersaline ponds of solar salt-works (or similar production plants) since the presence of organic matter is detrimental to the formation of the final product and necessitates increased processing costs (Giordano *et al*, 1994).

### **2.1.1 Research Objectives**

The controversy in the literature regarding the range of concentrations reported for both glycerol release and accumulation in *Dunaliella* species, as well as the need to predict glycerol production, has necessitated an investigation into this matter. Many different methods for the quantification of glycerol have been reported. High NaCl levels in the medium however present a particular analytical challenge. The objective of this study was to compare a number of methods reported in the literature and to develop some which could accurately quantify these levels. A comparison between various methods is presented. The validation of the procedure is described in terms of linearity, accuracy and suitability.

## 2.2 METHODS AND MATERIALS

### 2.2.1 *Dunaliella* Culture

The *Dunaliella* cultures were obtained from the Culture Collection of Algae and Protozoa, Oban, United Kingdom.

### 2.2.2 Culture Medium

The defined inorganic medium, described by Ben-Amotz and Avron (1983a), adjusted to 1.5M NaCl, was used for the culture of *Dunaliella* in this study. It was prepared as outlined below:

All quantities are expressed in g. ℓ<sup>-1</sup>

Stock solution A: 0.401g FeCl<sub>2</sub>, 2.233g EDTA, 11.433g H<sub>3</sub>BO<sub>4</sub>,  
1.382g MnCl<sub>2</sub>, 0.109g ZnCl<sub>2</sub>

Stock solution B: 2.596g CoCl<sub>2</sub>, 0.0342g CuCl<sub>2</sub>

Growth medium: 87.660g NaCl (for 1.5M NaCl), 4.200g NaHCO<sub>3</sub>,  
0.510g KNO<sub>3</sub>, 1.230g MgSO<sub>4</sub>, 0.044g CaCl<sub>2</sub>,  
0.027g KH<sub>2</sub>PO<sub>4</sub>

Working solution: Add 1mℓ stock solution B to 1ℓ stock solution A (this may be stored at 4°C). Add 1mℓ of this stock solution per 1ℓ growth medium.

The medium was made up with distilled water, adjusted to 8.7% NaCl (1.5M) by means of a hand-held salinometer (Atago) and filtered under vacuum through Whatman No.1 filter paper. The medium was sterilised by autoclaving at 121°C and 1bar for 10 minutes. Autoclaving for a longer period results in precipitation of the salts.

### 2.2.3 Culture Conditions

Media were dispensed in cotton wool stoppered 100mℓ conical flasks and, after inoculation, incubated at 26°C in a Constant Environment (CE) room under conditions of continuous illumination of 167 - 250 μmol.m<sup>-2</sup>.sec<sup>-1</sup>.

## 2.2.4 THE QUANTIFICATION OF EXTRACELLULAR GLYCEROL

A culture of a *Dunaliella* species was centrifuged at 1 700g x 10 minutes. The glycerol concentration in the growth medium was determined by several methods.

### 2.2.4.1 Chemical Analysis

#### 2.2.4.1.1 Gas-Liquid Chromatography

Two methods of glycerol derivatisation were evaluated in an attempt to quantitate extracellular glycerol levels by gas-liquid chromatography, viz. the Pyridine-Acetic Anhydride Acetylation Procedure (Parolis Pers. Comm.) and the 1-Methylimidazole Acetylation Procedure (Blakeney, *et al.*, 1983).

##### The Pyridine-Acetic Anhydride Acetylation Procedure

An equal volume (usually 5mℓ) of the glycerol sample and the internal standard, erythritol (0.05g ℓ<sup>-1</sup>) were freeze-dried to dryness in a Virtus™ Freezemobile Model 6. Pure analytical grade pyridine (1mℓ) and acetic anhydride (1mℓ) were added to the glycerol residue for the acetylation process. A round bottomed flask containing the reaction mixture was connected to an oven-dried condensor and incubated at 100°C x 1hr in an oil bath which was placed on the heating plate of a magnetic stirrer. The acetylation process was assisted by continuous stirring. The derivatives were then added to a separating funnel containing a small amount of ice. Following a 10 minute incubation, the derivatised glycerol was extracted with an amount of chloroform equal in volume to that of the ice. The lower chloroform layer (organic phase) was then eluted into a second separating funnel where it underwent a series of washing steps to 'clean up' the sample, i.e. washed with distilled water, then acid (10% H<sub>2</sub>SO<sub>4</sub>), distilled water, then base (saturated NaHCO<sub>3</sub>), followed by a final wash with distilled water. The chloroform-containing organic phase was then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and filtered (through Whatman No. 1 filter paper) into a round bottomed flask. The chloroform was evaporated off to approximately a 1mℓ volume under reduced pressure in a rotary evaporator and transferred to a small vial. The sample was then reduced to dryness under nitrogen. Prior to analysis, the sample was re-suspended in 50μℓ chloroform.

The derivatised sample was analysed with a Hewlett Packard 5890A Gas Chromatograph, fitted with a Flame-Ionisation Detector and a 3392A Recording Integrator, using Helium as carrier gas. A 1 $\mu$ l sample volume was injected into a J&W Scientific fused-silica DB-17 bonded-phase capillary column (30m x 0.25mm; having a film thickness of 0.25  $\mu$ m). The temperature of the injection port, column and detector were 250 °C, 180°C and 250°C respectively. Known quantities of glycerol (together with internal standard, erythritol) were acetylated as described to determine whether a linear relationship could be established.

#### The 1-Methylimidazole Acetylation Procedure

This process, as described by Blakeney *et al* (1983), is a simple and rapid method which involves only one phase separation step.

The glycerol samples (and standards) were prepared and freeze-dried as described for the pyridine-acetic anhydride acetylation procedure. 1-Methylimidazole (0.1ml), followed by acetic anhydride (1ml), were added to the glycerol residue which contained the internal standard erythritol. The reaction components were mixed and then briefly sonicated. After a 10 minute incubation at room temperature, water (2.5ml) was added to decompose the excess acetic anhydride. When cool, the reactants were transferred to a separating funnel. Dichloromethane (1ml) was added and the mixture was agitated. After the phases had separated, the lower one was removed and dripped through a small funnel plugged with glass wool, containing a small amount of anhydrous Na<sub>2</sub>SO<sub>4</sub> to remove any remaining water. The eluant was collected and reduced to dryness under nitrogen. Immediately prior to analysis, the residue was re-suspended in 50 $\mu$ l chloroform. Pure analytical grade chemicals were used in all cases. The derivatised sample was analysed as described for the pyridine-acetic anhydride acetylation procedure.

#### **2.2.4.1.2 High Performance Liquid Chromatography**

Optimal growth media for *Dunaliella* species comprises 1.5M NaCl. If not reduced, the eluting salt peak masks all subsequent peaks, preventing accurate integration of the eluting compounds, adversely affecting the assay. The salt could also be detrimental to the HPLC column. This may be overcome by desalting, which in this case was achieved through interaction of the sample with a mixed bed ion-exchange resin, prior to HPLC analysis. Amberlite monobed resin MB-1 was used for this purpose. It comprised IR-120 (H<sup>+</sup>), a strongly acidic exchanger and IR-400 (OH<sup>-</sup>), a strongly basic exchanger.

The following procedure yielded optimal desalting:

The glycerol to be quantitated (1.5mℓ) was added to 2g of Amberlite Monobed Resin, which had been washed with distilled water (until a neutral pH had been reached) and then drained to remove all excess water. The sample was then agitated gently for 5 minutes, during which time ion-adsorption was occurring. A 1mℓ aliquot of the sample was then removed and analysed by chromatography as described below.

The desalted glycerol sample (1mℓ) was removed and filtered through a 0.22 micron filter, together with 100μℓ of the internal standard glucitol (0.36 g.ℓ<sup>-1</sup>). This was analysed by HPLC using a Spectra Physics IsoChrom LC pump and a 300 x 7.8mm Supelcogel™ C-611 column. Chromatography was conducted at 65°C with a 10<sup>-4</sup> M NaOH mobile phase at a flow rate of 0.5mℓ.min<sup>-1</sup>. The injection volume was 20μℓ. Glycerol was detected by using an ERC-7515A Refractive Index Detector. Known quantities of glycerol in the range of 0.01 - 0.1 μg.mℓ<sup>-1</sup>, together with internal standard (glucitol), were also analysed by this method.

#### **2.2.4.2 Enzymatic Analysis**

The extracellular glycerol content was determined enzymatically with the Biochemica Test Kit 'Glycerol UV Method' (Boehringer Mannheim). The assay, which followed the amount of NADH oxidised, was read at a wavelength of 340 nm by means of a Shimadzu UV-Vis Spectrophotometer UV-160A.

#### **2.2.5 THE QUANTIFICATION OF INTRACELLULAR GLYCEROL**

The following procedure was developed and optimised during the course of this study:

In order to extract the intracellular glycerol, algal cells (20mℓ), cultured as described in 2.2.3, were pelleted by centrifugation at 1 700g x 10 minutes. The sedimented cells were then re-suspended in 5mℓ re-distilled water, which contained the internal standard, galactose (200 μg.mℓ<sup>-1</sup>). If the glycerol content of a culture of low cell density (less than 40 x 10<sup>4</sup> cells.mℓ<sup>-1</sup>) was to be determined, a 40mℓ volume of *Dunaliella* culture was centrifuged. The re-suspended algal solution was then vortex mixed for approximately 3 minutes to ensure cell lysis. The glycerol extract was centrifuged at 12 000g x 15 minutes to remove insoluble material. If not analysed immediately, the extracted material was frozen to prevent possible enzymatic or bacterial degradation of the glycerol.

### **2.2.5.1 Chemical Analysis**

#### **High Performance Liquid Chromatography**

Prior to analysis, the sample was filtered through a 0.45 $\mu$ m cellulose acetate membrane (Lida) to remove particulate contaminants which could clog the column. The glycerol extract was analysed by HPLC, using a Spectra Physics IsoChrom LC pump and a 300 x 7.8mm RHM-Monosaccharide Rezex Column (Phenomenex). The packing material was in the hydrogen ionic form and the primary separation mechanisms were ligand exchange and ion-exclusion. A Phenomenex guard column was used to protect the analytical column. Chromatography was conducted at 65°C, with a mobile phase of re-distilled water at a flow rate of 0.5ml.min<sup>-1</sup>. The injection volume was 20 $\mu$ l. Glycerol was detected by means of an ERC-7515A Refractive Index detector. A series of 6 standard solutions of glycerol (15.63 - 500 $\mu$ g.ml<sup>-1</sup>), each containing internal standard (galactose) (200 $\mu$ g.ml<sup>-1</sup>), were analysed under the above conditions to determine the reliability of the method.

#### **2.2.5.2 Enzymatic Analysis**

The intracellular content was determined enzymatically with the Biochemica Test Kit "Glycerol UV Method" (Boehringer Mannheim).

## **2.3 RESULTS**

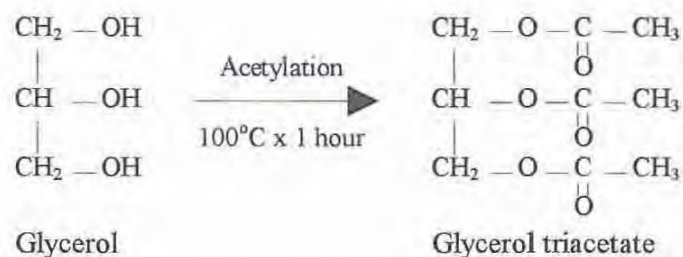
### **2.3.1 THE QUANTIFICATION OF EXTRACELLULAR GLYCEROL**

#### **2.3.1.1 Chemical Analysis**

##### **2.3.1.1.1 Gas-Liquid Chromatography**

###### The Pyridine-Acetic Anhydride Acetylation Procedure

During the acetylation process, glycerol triacetate (more commonly referred to as tri-O-acetyl glycerol) is formed when acetic anhydride donates acetyl groups which replace the hydroxyl groups on glycerol.



This acetylation process yielded a very clean acetylation product and traces indicated that 100% acetylation had occurred (results not shown). To correct for possible losses during the extraction procedure and to correct for errors due to volume changes, the internal standard, erythritol was used. The glycerol- and erythritol triacetate retention times were 4.09 and 9.20 minutes, respectively, under the given conditions. The assay proved to be adequately sensitive and could detect glycerol over the expected range of glycerol, i.e. 10 - 100  $\mu\text{g} \cdot \text{m}^{-1}$ .

A standard curve covering the expected range of glycerol concentrations did not indicate linearity over the concentration range used. Each acetylation attempt yielded a significantly different ratio of the acetylated glycerol and erythritol product. This method was therefore considered inappropriate.

The following are given as possible reasons for the failure of this method:

- i) This method comprises a number of washing steps (phase separations) between the organic chloroform- and aqueous phase. A certain amount of glycerol is thought to be lost in each washing step, resulting in poor recovery of the derivatised glycerol.
- ii) During certain stages in growth, e.g. logarithmic growth, *Dunaliella* is characterised by low rates of glycerol release (Huntsman, 1972; Sharp, 1977). The potential for error would be proportionately more significant in these samples of low glycerol concentration following repeated washing steps, leading to inconsistent results.
- iii) This method required sample concentration by a rotary evaporator prior to GC analysis. This is a very harsh process and much glycerol loss was thought to have occurred here.

Owing to the inconsistencies prevalent in this assay and especially due to the fact that they are particularly noticeable in the range of low glycerol concentration dealt with in this study, it was necessary to investigate another method for the quantification of glycerol.

### The 1-Methylimidazole Acetylation Procedure

This method was developed in an attempt to improve the current acetylation methods which involve relatively long acetylation times at elevated temperatures (100 - 120°C) (Blakeney *et al*, 1983). This method differs from the pyridine-acetic anhydride acetylation method, since it involves only one phase separation (washing step) and requires an incubation of only 10 minutes at room temperature. A potential site for glycerol loss is eliminated in this method since it does not require sample volume reduction via a rotary evaporator.

1-Methylimidazole functions as the catalyst in the acetylation of glycerol and erythritol. Blakeney *et al* (1983) reported this compound to be a highly efficient catalyst for the acetylation of hydroxy compounds. The acetylated product obtained using this method is, however, the same as that described in the pyridine-acetic anhydride acetylation procedure. Although the consistency of this technique was marginally better than the former, linearity could not be proved over a range of standard concentrations. For this reason, the remaining potential site for glycerol loss was eliminated, viz. volume reduction under nitrogen. This method has the advantage that no evaporation of the sample is necessary. This obviates selective losses of more volatile acetylated products, which could otherwise affect the ratio of the glycerol and erythritol triacetates, thereby introducing inconsistency to the method. The selective loss of components during evaporation limits the quantitative recovery of components in acetylation processes. Selective loss of monosaccharides during evaporation to dryness has been reported by Blakeney *et al* (1983). The author attributed this to adsorption on glass.

Although the elimination of all evaporation steps greatly improved the reproducibility of this method, linearity could still not be established. The only remaining explanation for the described inconsistencies appear to be that such small concentrations of glycerol cannot be accurately quantitated using this method. This effect was enhanced by the elimination of the evaporation step, since it resulted in an extremely dilute sample.

#### **2.3.1.1.2 High Performance Liquid Chromatography**

Due to the sample loss experienced by the volatile acetylated products during the Gas Chromatographic quantification of glycerol, attention was redirected towards High Performance Liquid Chromatography (HPLC). The major distinguishing characteristic regarding this method is that it does not involve derivatisation of the glycerol to be quantitated.

The inability of this method to accurately quantitate glycerol in the presence of the high salt concentration in which *Dunaliella* thrives (0.05 - 5.5M NaCl) was its only shortfall. Desalting was therefore required prior to the HPLC analysis. Using the desired sample volume of 1.5mℓ, 2g of Amberlite Monobed Resin was shown to achieve optimal NaCl reductions of up to 82.76%, yielding a final salinity of 1.5% NaCl (as determined by salinometer). When analysed by HPLC, the samples yielded good, well separated peaks. The glycerol and glucitol (internal standard) had retention times of 20.60 and 26.50 minutes, respectively. The salt eluted over approximately 9 - 17.50 minutes. If untreated, at 1.5M NaCl, the salt peak would completely mask both the glycerol and glucitol peaks. Glycerol and glucitol were, however, retained by the ionic exchange resin's highly reactive active groups. For this reason, the internal standard was added after desalting the glycerol sample (a number of internal standards were tested, all of which were retained by the resin). This enabled the percentage loss of glycerol to be calculated. The percentage glycerol loss during the desalting was calculated to range between 6% and 20% (results not shown). Since the range was so great, it could not be taken into account in the quantification of each glycerol sample. For this reason, the method did not yield reproducible results.

In the absence of salt or under low salinity (under 0.75M NaCl) this method proved to be very sensitive and reproducible. A standard curve plotting glycerol in the range of 10 - 100µg.mℓ<sup>-1</sup> demonstrated linearity. An alternative method of desalting the sample may increase the feasibility of this method.

### **2.3.1.2 Enzymatic Analysis**

This method involves three enzyme catalysed reactions. Glycerol is phosphorylated by adenosine-5-triphosphate (ATP) to L-glycerol-3-phosphate in the reaction catalysed by glycerokinase. A product of this reaction, adenosine-5-diphosphate (ADP), is reconverted by phosphoenolpyruvate (PEP) with the aid of pyruvate kinase (PK) to ATP with the formation of 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. The amount of NADH oxidised is stoichiometric with the amount of glycerol present in the sample. This assay detects glycerol concentrations between 30 and 400 µg.mℓ<sup>-1</sup>.

Results were reproducible and linearity was established. The only problems experienced concerned the range of this assay. As mentioned previously, *Dunaliella* cultures under certain conditions, such as logarithmic growth, are reported to release very small amounts of glycerol (Sharp, 1977; Huntsman, 1972). The extracellular glycerol would thus be very low in these cultures (below 30  $\mu\text{g.m}^{-1}$ ) and would not be accurately quantitated using this method. The effect of salt on the assay was not tested. It is possible that it inhibited enzyme activity to some extent.

### **2.3.2 THE QUANTIFICATION OF INTRACELLULAR GLYCEROL**

The extraction procedure employed in the quantification of intracellular glycerol yielded a 100% recovery of the glycerol. Neither HPLC analysis, nor enzymatic analysis of the cell debris (remaining after glycerol extraction), detected traces of glycerol.

#### **2.3.2.1 Chemical Analysis**

##### **High Performance Liquid Chromatography**

HPLC analysis of the extracted glycerol yielded three well separated peaks. These represented the salt, galactose and glycerol peaks at retention times of approximately 11.40, 18.18 and 20.40 minutes, respectively. The elevated temperature (65°C) used in this method reduced the retention times (to the above values) and increased column efficiency.

Desalting of the sample was not required prior to HPLC analysis. The salt peak was greatly reduced compared to that obtained in the determination of extracellular glycerol by HPLC, since NaCl is not accumulated intracellularly in *Dunaliella* (Calvin and Taylor, 1989). The salt content was less than 1% (as determined by salinometer).

This method enabled the analysis of the intracellular glycerol content of *Dunaliella* grown in highly proteinaceous solutions, such as tannery effluent. This was beneficial to the content of this study. The quantification of intracellular glycerol by HPLC proved to be an extremely sensitive and accurate method within the range of glycerol tested (15.63 - 500  $\mu\text{g.m}^{-1}$ ) (a greater range was not tested). All reported intracellular glycerol levels fall within this range.

### 2.3.2.2 Enzymatic Analysis

A similar level of accuracy was shown in the quantification of intracellular glycerol to that exhibited by the quantification of extracellular glycerol (under the influence of salinity). As previously reported, the detection range for this assay is 30 - 400  $\mu\text{g.m}^{-1}$ . The enzyme assay was tested for correct performance (gross errors) and for the presence of interfering substances, by using a standard solution of glycerol as an internal standard. This yielded a recovery of 0.950. Since this is  $\cong 1$ , this proves that the glycerol samples assayed were basically free from interfering substances and that the assay is accurate for these samples.

## 2.4 DISCUSSION

The above data showed that enzymatic analysis was the most appropriate method for the quantification of extracellular glycerol, whereas both enzymatic and HPLC analysis were suitable for intracellular glycerol quantification.

The large variation in glycerol levels reported has given rise to some controversy. Enhuber and Gimmler (1980) reported that extracellular glycerol concentrations ( $<1\text{mmol.l}^{-1}$ ) are negligibly small compared with the intracellular glycerol concentrations (0.2 - 2.8  $\text{mol.l}^{-1}$ ). Jones and Galloway (1979), however, reported that the values for extracellular glycerol are high relative to intracellular content, since the cells continuously leak glycerol, which accumulates in the medium. The accurate quantification of extracellular glycerol requires a wide-ranging assay, which can quantify both low initial glycerol levels, as well as higher final levels of accumulated glycerol. The necessity for accurate measurement of low glycerol levels in the presence of high salt concentrations in the growth medium, limits the choice of technique.

Several techniques have been described for the quantification of glycerol. Enzymatic analysis appears to be the more commonly used method in the quantification of both intra- and extracellular glycerol (Ben-Amotz and Avron, 1973; Frank and Wegmann, 1974; Borowitzka *et al*, 1977; Enhuber and Gimmler, 1980; Ehrenfield and Cousin, 1984; Gilmour *et al*, 1984b; Becket *et al*, 1985; Goyal *et al*, 1986; Belmans and van Laere, 1987; Fujii and Hellebust, 1992; León and Galván, 1994). Such enzymatic assays may be conducted by means of commercially produced enzyme kits such as that used in this study. Enzymatic analysis is however costly, not as sensitive as other methods (such as HPLC) and permits a range of analysis (30 - 400  $\mu\text{g.m}^{-1}$ ) considered to be inadequate for the purpose of this study. For general estimation purposes it is, however, a simple and rapid technique.

High Performance Liquid Chromatography proved to be ideal for the quantification of intracellular glycerol. It was sensitive, reproducible and could quantitate glycerol in the required range (15.63 - 500  $\mu\text{g.m}^{-1}$ ). This method is cost-effective and generally more reliable than enzymatic analysis (enzymes are thermolabile and subject to denaturation). A comparison of glycerol levels obtained from enzymatic and HPLC analysis yielded different levels (results not shown). Values obtained with HPLC were in all cases significantly higher.

Several authors have reported the use of Gas-Chromatography in the quantification of both intra- and extracellular glycerol (Gehron and White, 1983; Jones and Galloway, 1979; Kessly and Brown, 1981; Borowitzka *et al*, 1977). Gehron and White (1983) described a method which could accurately analyse  $10^{-11}$  moles of glycerol. This acetylation procedure is similar to that adopted in this chapter of study (acetic-anhydride pyridine) but involves an incubation at 60°C for 2 hours (other minor differences were also observed). Jones and Galloway (1979) described a derivatisation method which involved the silylation of glycerol by hexamethylsilazane and trimethylchlorosilane, to form tri-methyl-silyl glycerol. This method involved a total incubation time of 2 hours at room temperature. Gas Chromatography is usually the method of choice for the qualitative and quantitative analysis of a wide range of compounds, because of its extreme sensitivity (Williams and Wilson, 1979). It is reported to be particularly suitable for sugar analysis, since it combines great sensitivity with high resolution (Whittaker, 1993). The described inaccuracies caused by the repeated phase separations of the derivatised glycerol and by sample concentration under reduced pressure, however, necessitated the use of an alternative method.

Oren (1993) has recently developed a sensitive assay for the detection and quantification of both intra- and extracellular glycerol. It is based on periodate oxidation to formaldehyde, followed by colorimetric determination of formaldehyde with 3-methyl-2-benzothiazolone hydrazone (MBTH). This method was reported to detect glycerol in concentrations between 0.25 and 20 $\mu$ M and the absorption was linear with concentration. Provided precipitate formation was avoided, the author found that high salt concentrations did not interfere in the procedure (highly saline solutions required dilution). This colorimetric assay has been reported to have a much greater sensitivity than any of the existing chemical or enzymatic assays for glycerol (Oren, 1993). This, together with the minimal inhibition by high salt concentrations, make this method an attractive alternative for glycerol (particularly extracellular glycerol) quantification. While this method was not evaluated in this study, since the literature only became available on completion of this work, it would seem worth noting here for consideration by subsequent workers in the field.

## CHAPTER 3

### THE ROLE OF A BACTERIAL ASSOCIATION IN THE UTILISATION OF GLYCINE BY *D. SALINA*

#### 3.1 INTRODUCTION

It was initially assumed that algae played a secondary role in wastewater treatment systems, primarily by removing CO<sub>2</sub> produced by heterotrophic bacteria and providing O<sub>2</sub> for the growth of aerobic organisms. Increasing evidence, however, suggests that their role is an active one and that they contribute significantly to the degradation of low molecular weight compounds (Abeliovich and Weisman, 1978; Rose, 1992). Laubscher (1991) and Rose *et al* (1991) observed the stimulated growth of *D.salina* in hide soak liquor tannery effluent. This alga exhibited a 100% increase in its growth rate when cultured in tannery effluent compared to that in defined inorganic growth media. Since hide soak liquor comprises glycine-rich collagen hydrolysate, the effect of glycine on the growth of *Dunaliella* was studied. The authors reported that nearly 50% of the observed stimulatory effect on the growth of *Dunaliella* was attributed to glycine alone. This indicates that in a *D.salina* based HROP, the organic removal function extends beyond a mutualistic association between the heterotrophic bacteria and the algae.

##### 3.1.1 Uptake of Organics

Although *Dunaliella* has been classified as a photoautotroph, certain species have been shown to utilise organic substrates (Borowitzka and Borowitzka, 1988; Rose, 1992; Oliveira and Huyhn, 1989; Antia *et al*, 1991). This attribute is, however, species specific (Williams *et al*, 1978) and inducible (Flynn and Butler, 1986).

Several algae utilised some component of dissolved free amino acids (DFAA) for growth, following an incubation period (Neilson and Larsson, 1980; Flynn and Butler, 1986; Muñoz-

Blanco, 1990; Antia *et al*, 1991). Despite Flynn and Fielder's (1989) report of the minimal uptake of amino acids by *Dunaliella*, there are numerous reports of uptake and utilisation of amino acids in the literature. Rose (1992) reported that *D.salina* utilises glycine as its sole nitrogen source. An enhanced growth of over 50% was observed when glycine was used as the sole nitrogen source (as compared to nitrate and ammonia) and 67% where it was used in combination with nitrate. Enhanced growth using amino-nitrogen is in agreement with earlier observations by Flynn and Butler (1986). Glycine has been described as one of the most favourable amino acid nitrogen sources (Kaplan *et al*, 1986; Neilson and Larsson, 1980). Flynn and Butler (1986) have reported that, for at least two different algal species, glycine is a better source of nitrogen than inorganic nitrogen.

The rate of glycine uptake is, according to Flynn and Butler (1986), inversely related to cellular nitrogen content. The development and operation of micro-algal amino acid uptake systems is reported to be different to that of nitrate and ammonia in that control appears to be at the level of the intracellular amino acid pool. Cells exposed to high levels of illumination in the presence of dissolved inorganic nitrogen take up amino acids at a low rate because the intracellular synthesis of amino acids inhibits their transport. The substrate itself therefore limits transport (by a process called transinhibition) preventing an accumulation beyond the immediate needs of the cell (Flynn and Butler, 1986). Glycine is one of the first amino acids to be derived from photosynthetic incorporation of ammonia or nitrate and could therefore give rise to low uptake rates (Flynn and Butler, 1986).

Some algae have a novel mode of amino acid utilisation. This occurs on the cell surface without uptake and involves a cell surface deaminase which converts L-amino acid to  $\text{NH}_4^+$  and oxo-acids (Muñoz-Blanco, 1990). The ammonia is subsequently utilised by the algae as a nitrogen source for growth. Muñoz-Blanco (1990) observed a general deaminating system in *Chlamydomonas reinhardtii* which enabled this alga to utilise a wide range of L-amino acids as the sole carbon source. The deaminase activity was non-specific, sensitive to repression by high ammonium concentrations and required an organic carbon source for its *de novo* synthesis. A similar system has been observed in *Monodus subterraneus*. This alga was reported to deaminate glutamine by a highly specific extracellular deaminase (glutaminase) with the accumulation of glutamate and

the liberation of  $\text{NH}_4^+$  (Antia *et al*, 1991; Muñoz-Blanco, 1991). A cell surface deaminase has also been reported in some prymnesiophytes and dinoflagellates (Antia *et al*, 1991).

Both bacteria and algae are therefore able to utilise either organic or inorganic forms of nitrogen as sole nitrogen source. Wheeler and Kirchman (1986) have however made a general observation that bacteria primarily use amino acids as a nitrogen source, while algae primarily use ammonia and nitrate. According to Flynn and Butler (1986), bacteria respond rapidly to increased levels of DFAA by an increased rate of uptake. In this way, bacteria are thought to outcompete algae for most organic substrates (Wheeler and Kirchman, 1986).

### 3.1.2 Algal-Bacterial Interrelations

There is much controversy in the literature concerning the nature of algal-bacterial relationships. Cloete and Toerien (1981) reviewed reports of such interactions and concluded that where micro-algae and bacteria co-exist in the same aquatic environment, their influence on each other may be neutral, synergistic or antagonistic (refer to section 1.6). Kaplan *et al* (1986), however, stated that algae form symbiotic associations with bacteria on a community scale and that bacteria have been shown to be essential, or at least beneficial, for algae in a number of studies. *Dunaliella* species, nonetheless, display an important self-defence mechanism which enables species survival in highly competitive situations. This is performed through the release of a broad spectrum antibiotic substance into the surrounding media (Lustigman, 1988).

Algal-bacterial interactions in algal ponds are seen to be more complex than the simplistic view of the mutualistic co-existence of algae and bacteria. In order to achieve the full potential of algal ponding systems in the treatment of wastewater and the production of a protein-rich algal-bacterial biomass, a thorough knowledge of algal-bacterial interactions will be necessary.

### 3.1.3 Research Objectives

Due to the reported enhancement in growth displayed by *Dunaliella spp.* in bovine salted hide soak liquor - tannery effluent, a study was undertaken to investigate the effect of glycine, which occurs in high concentrations in this effluent. The following research objectives were identified:

1. To determine whether glycine has a stimulatory role in glycerol excretion in *D.salina*.
2. To determine whether *D.salina* utilises glycine as a nitrogen source for growth.
3. To investigate the role of heterotrophic bacteria in these functions.

## 3.2 METHODS AND MATERIALS

### 3.2.1 *Dunaliella* Culture

*Dunaliella salina* (Teodoresca) var. *bardawil* was used in this study. This alga, originally isolated by Ben-Amotz and Avron, was obtained from the Culture Collection of Algae and Protozoa, Oban, United Kingdom, and is designated as CCAP 19/30 (CCAP Strain Data). This strain has been referred to as *D. bardawil* by several authors. Borowitzka and Borowitzka (1988), however, refer to this as a *nomen nudum* and considered *bardawil* to be a strain of *D.salina*. It is reported as being halophilic and carotenogenic in character (CCAP Strain Data). Unless otherwise indicated, pure non-axenic cultures of this strain were used.

### 3.2.2 Culture Medium

The defined inorganic medium, described by Ben-Amotz and Avron (1983a), was used for the culture of *D.salina* in this study. It was prepared as outlined in section 2.2.2. Unless otherwise indicated, the medium contained 1.5M NaCl.

### **3.2.3 Algal Cell Counts**

Algal growth was monitored by direct cell counts using an improved Neubauer haemocytometer. The cells were immobilised by brief exposure to iodine crystals. This also served to stain the cells, facilitating their counting. All cell counts were performed in triplicate.

### **3.2.4 Fluorescence Quenching Study**

Fluorescence quenching studies by continuous flow fluorometry were performed to determine the effect of glycine addition on photosynthetic and related pathways. It is well established that the emission of Chlorophyll a fluorescence provides an indicator of the primary photochemistry of photosynthesis, as well as providing information on the carbon reduction cycle (Ögren, 1990). Continuous flow fluorometry on algal cultures has been described by Rose (1992).

#### **3.2.4.1 Chlorophyll Fluorescence**

A Sequoia-Turner model 112 Digital Fluorometer was adapted for chlorophyll measurement by the installation of a blue lamp (390 - 450nm excitation range), a 405nm excitation filter (Corning J-62) and a 680nm emission filter (Corning Z-60).

#### **3.2.4.2 Culture conditions**

A New Brunswick 5ℓ Bioflow III Reactor was operated as a photobioreactor and was illuminated by means of a bank of cool-white fluorescent lights arranged around the reactor. The *Dunaliella* culture was maintained at log phase throughout the duration of the experiments. A minimum cell density of  $1.0 \times 10^5$  cells.mℓ<sup>-1</sup> was required before commencing the experiment. The temperature was maintained at 28°C (±2°C). The pH was measured continuously.

#### **3.2.4.3 Experimental Layout**

A peristaltic pump maintained a controlled flow of culture from the photobioreactor, through the fluorometer (fitted with a flow-through cell) and returning to the photobioreactor. Clear tubing

was used to connect the photobioreactor and the peristaltic pump. A chart recorder, linked to the fluorometer, detected the changes in fluorescence. The fluorometer was set at a value of 50.0% and allowed to equilibrate for at least 30 minutes. This value represents the base-level. The equipment layout is illustrated in plate I.

#### **3.2.4.4 Sensitivity Testing**

In order to ensure measurable fluorescence levels, CO<sub>2</sub> and O<sub>2</sub> were bubbled through the culture and NaCl added to the photobioreactor. The subsequent quenching of fluorescence on CO<sub>2</sub> addition and the increase in fluorescence on O<sub>2</sub> addition was used as an indication that an adequate level of sensitivity prevailed. NaCl addition up to a 3.5M concentration (hyperosmotic stress) provided a further test of the sensitivity of this system.

#### **3.2.4.5 The Effect of Glycine Addition**

After equilibrium had been regained in the culture, glycine was added to the *Dunaliella* culture to achieve a final 5mM concentration in order to monitor its effect on fluorescence. This study was performed in quadruplicate.



**Plate I** The experimental equipment used in the fluorescence-quenching study.  
1=Bioflow III Reactor; 2=Peristaltic Pump; 3=Fluorometer; 4=Chart Recorder

### 3.2.5 The Effect of Glycine on Glycerol Excretion

In this experiment the tests were performed in duplicate and the results averaged, using either an axenic or a non-axenic culture of *D.salina* in defined inorganic media (as described in 2.2.2). The study was repeated and the results of the second experiment were reported.

#### 3.2.5.1 The Preparation of an Axenic Culture of *D. salina*

An axenic culture of *D.salina* was obtained following two transfers through sterile medium containing both penicillin (novopen) and chloramphenicol at concentrations of  $100\mu\text{g.m}^{-1}$  and  $50\mu\text{g.m}^{-1}$ , respectively. Plate counts confirmed that axenic conditions had been achieved (i.e. the absence of heterotrophs).

#### 3.2.5.2 Experimental Procedure

Stock axenic and non-axenic cultures of *D.salina* were dispensed (using sterile technique) into four 100mℓ conical flasks. Cell counts of stock cultures were made prior to dispensing. Glycine and Chloramphenicol were aseptically added to the algal cultures at the outset of the experiment. The experimental flasks contained 5mM Glycine, 5mM Glycine +  $50\mu\text{g.m}^{-1}$  Chloramphenicol,  $50\mu\text{g.m}^{-1}$  Chloramphenicol and the control (axenic/non-axenic culture). At certain time intervals (viz.  $t = 0$ ,  $t = 12$ ,  $t = 24$ ,  $t = 48$  and  $t = 72$  hours) a 1.5mℓ volume of sample was microfuged for 2 minutes and the supernatant fluid assayed for glycerol content.

#### 3.2.5.3 Culture Conditions

The experiment was conducted in a Constant Environment (CE) room under continuous illumination at a light intensity of  $167 - 250\mu\text{mol.m}^{-2}\text{sec}^{-1}$  (as measured by a LX101 Luxmeter (Lutron) and at a temperature of  $26^{\circ}\text{C} (\pm 2^{\circ}\text{C})$ .

#### 3.2.5.4 Glycerol Assay

The extracellular glycerol content was determined enzymatically using the Biochemica Test Kit “glycerol UV method” (Boehringer Mannheim). NADH oxidised in a chemical reaction was measured in a Shimadzu UV-Vis Spectrophotometer UV-160A at 340 nm.

#### 3.2.5.5 Quantification of Heterotrophs

Bacterial cell numbers in the axenic and non-axenic cultures were counted prior to aliquotting the stock cultures and then again at the completion of the experiment ( $t = 72$  hours). This was done by spread plates of various dilutions of algal culture on 1.5M NaCl Nutrient Agar, incubated at 37°C for 48 hours. The plate counts establish (a) whether axenic conditions have been reached and maintained throughout the experiment and (b) whether any change in bacterial count has occurred over the duration of the experiment.

#### 3.2.6 Glycine Binding Studies

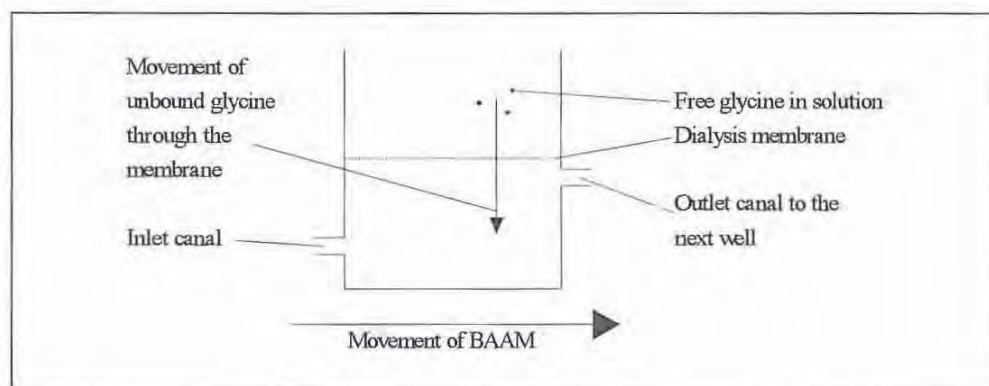
Extracellular deaminase activity in *D.salina* was determined by equilibrium dialysis using radio-labelled glycine.

##### 3.2.6.1 Determination of the [ $^{14}\text{C}$ ]-Glycine : [ $^{12}\text{C}$ ]-Glycine Ratio

A glycine [ $^{14}\text{C}$ ] : [ $^{12}\text{C}$ ] ratio was required with a resultant count of approximately 100 000 dpm on transfer of 100 $\mu\text{l}$  to 5m $\ell$  of aqueous scintillant. The scintillation cocktail was a 1:1 mixture of toluene and Triton X-100 (alkylaryl polyether alcohol) with 8g. $\ell^{-1}$  of PPO (2,5-diphenyl oxazole). A 20 $\mu\text{l}$  volume of undiluted [ $^{14}\text{C}$ ]-glycine added to 2m $\ell$  of 2mM [ $^{12}\text{C}$ ]-glycine was found to yield the desired count for use in this experiment i.e. 1 $\mu\text{Ci.m}\ell^{-1}$ .

### 3.2.6.2 Microdialysis

A microdialysis system based on a flow-through technique was used in this study. The microdialysis system comprised a number of wells which are separated into an upper and lower compartment by a differentially permeable dialysis membrane. The lower compartments are interlinked via small canals which enable the continuous flow of liquid through the system, while the upper compartments contain the samples to be tested. A peristaltic pump ensured the continuous flow of a “washing” liquid through the microdialysis system to remove unbound glycine. In this experiment, algal growth medium was used for this purpose. Figure 3.1 is a diagrammatic representation of a well comprising this system. The experimental equipment used is illustrated in plate II.



**Figure 3.1** A diagrammatic representation of a well comprising the microdialysis system which was used to investigate the presence of extracellular deaminase activity in *D.salina*.

### 3.2.6.3 Determination of the Required Cut-off Time

A cut-off time was determined, representing the time taken for unbound glycine to be dialysed out of the system. This enabled the approximate reaction time to be established. A 2ml volume of  $1\mu\text{Ci.ml}^{-1}$  glycine was placed in each of the wells of the microdialysis system and the decrease in radioactivity measured over a 150 minute period. A decrease from 94 000 to 40 000 dpm over this period was considered to be a sufficient decrease to determine whether binding of any sort had occurred.

#### 3.2.6.4 Experimental Procedure

Glycine binding studies were performed on the following samples:

- 1) An axenic culture of *D.salina* + glycine
- 2) A glycine - control
- 3) A non-axenic culture of *D.salina* + glycine
- 4) Bacteria from the non-axenic culture + glycine
- 5) Algal growth medium - control

A 1.5ml volume of the axenic culture, non-axenic culture or bacteria were added to three separate wells and subsequently made up to 2ml with the radio-labelled  $1\mu\text{Ci.ml}^{-1}$  glycine solution referred to in 3.2.6.1 above. The bacteria from the non-axenic sample were contained in the supernatant fluid obtained from centrifugation of the non-axenic algal culture (1 700g x 10 minutes). The two controls were placed in the fourth and fifth wells and contained 2ml of the radiolabelled glycine solution and 2ml of growth medium, respectively. An algal count of  $30 - 50 \times 10^4 \text{ cells.ml}^{-1}$  was required for both the axenic and non-axenic cultures and the cultures were diluted or concentrated accordingly. Samples of  $100\mu\text{l}$  were taken at 30 minute intervals over a 210 minute period and added to 5ml of aqueous scintillant. The radioactivity was determined using a Beckman LS 3150T Scintillation Counter.

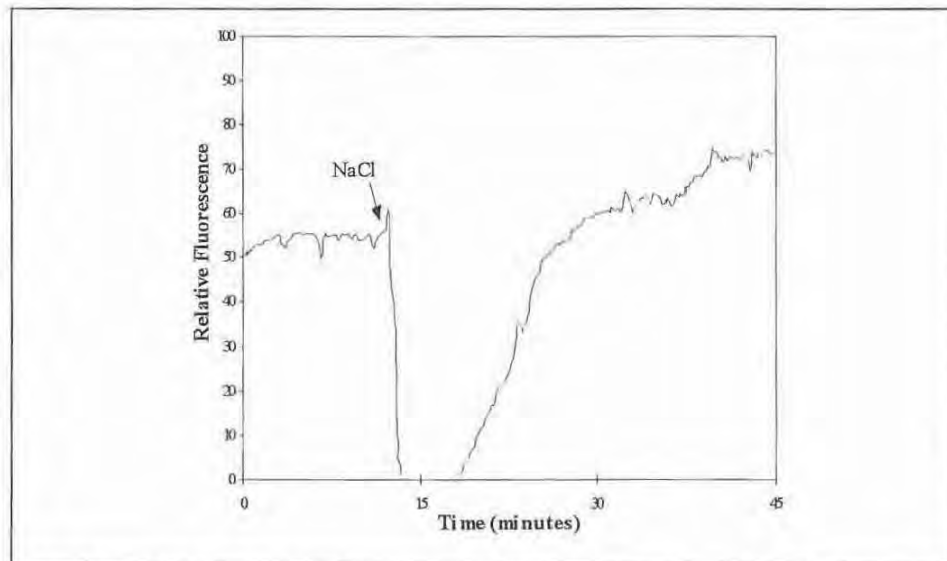


Plate II The experimental equipment used in the Glycine Binding Study.  
1=Microdialysis System; 2=Peristaltic Pump

### 3.3 RESULTS

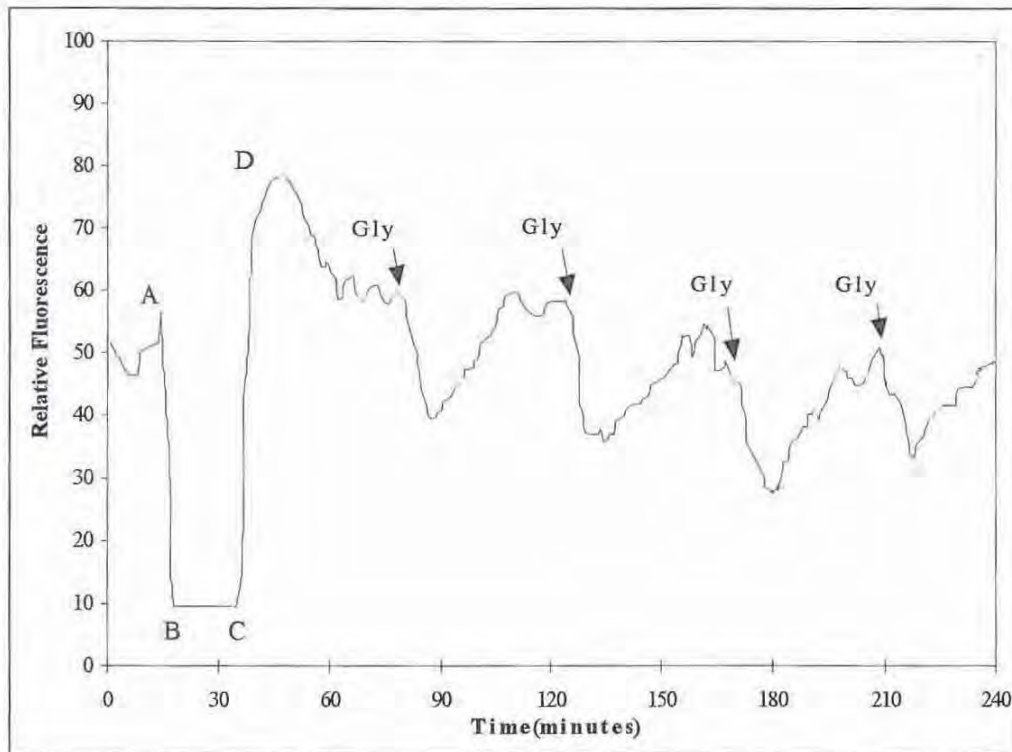
#### 3.3.1 Fluorescence Quenching Study

The effect of ionic stress on chlorophyll fluorescence was used to provide an indication of the sensitivity of the system to external influences (Figure 3.2). The substantial quenching of fluorescence observed after salt-stressing the *D.salina* culture from 1.5M - 3M NaCl indicates the sensitivity with which changes in the cell environment are reflected by the photosynthetic apparatus. The relative fluorescence value decreased to zero from an equilibrium base-level value of 50.



**Figure 3.2** The fluorescence induction response in *D.salina* subjected to a salinity shock. Salt was added to a final concentration of 3.5M NaCl.

The fluorescence response upon glycine addition to the growth medium demonstrates its effect on photosynthesis and related pathways in *D.salina*. Prior to commencing the experiment, CO<sub>2</sub> and O<sub>2</sub> were introduced, serving to further confirm the sensitivity of the system. A dramatic quenching of fluorescence occurred on bubbling CO<sub>2</sub> through the algal culture. Oxygen, however, reversed this effect by dramatically increasing the fluorescence again. Glycine, like NaCl, resulted in a quenching of fluorescence within seconds of its addition, although the effect was not as extreme in this case.



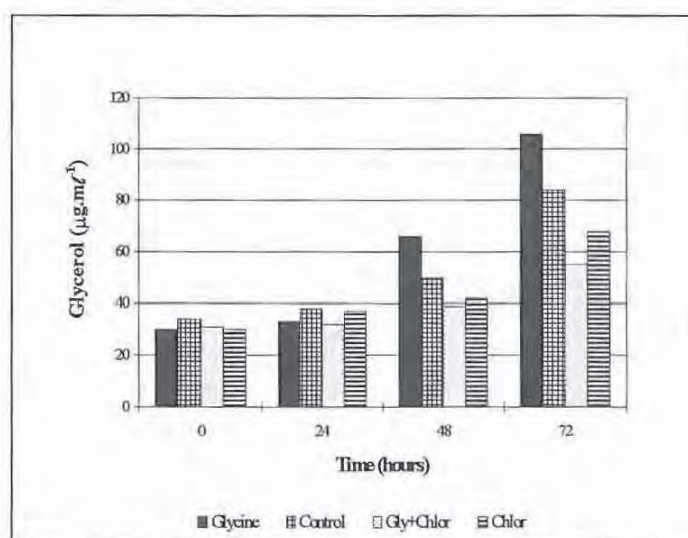
**Figure 3.3** The fluorescence induction response in *D.salina* subjected to 5mM glycine addition. This study was performed in quadruplicate as indicated. A=CO<sub>2</sub> on; B=CO<sub>2</sub> off; C=O<sub>2</sub> on; D=O<sub>2</sub> off.

This rapid quenching of fluorescence provides an indication that glycine addition at low concentrations induces a sudden demand for reducing power supplied by photosystems II (PSII) and, in this way, exercises a fairly direct effect on the primary photochemistry of the alga. Subsequent additions of glycine mimicked this quenching effect.

### 3.3.2 The Effect of Glycine on Glycerol Excretion

In order to determine whether the glycine effect recorded above results in the stimulation of C-metabolism, and specifically resulting in glycerol production and excretion, changes in the glycerol concentration in the surrounding medium were measured. Chloramphenicol was added to eliminate bacterial growth.

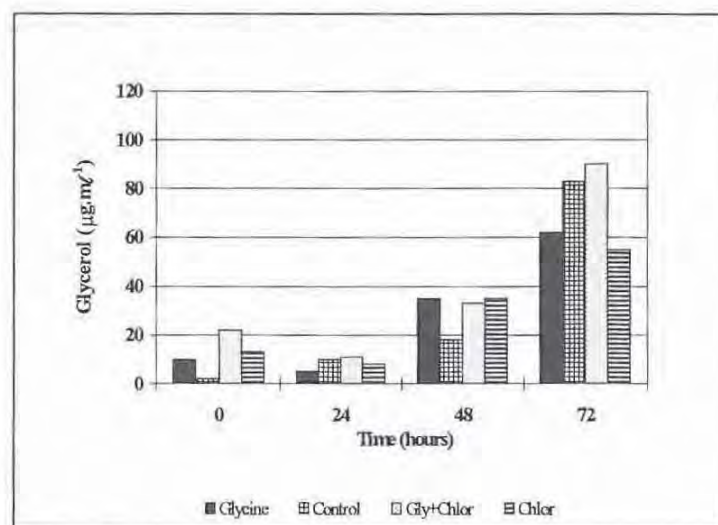
Results indicate a stimulatory effect by glycine on glycerol release in the axenic culture of *D.salina*, which was especially noticeable after 24 hours of exposure (Figure 3.4). Bacterial plate counts confirmed that sterile conditions had been maintained throughout the experimental procedure. Chloramphenicol + glycine did not, however, induce the same stimulatory effect. The cultures containing chloramphenicol appeared to have inhibited algal growth to some extent. A minor toxic effect of chloramphenicol was observed during the preparation of the axenic cultures of *D.salina* where, on average, only one in three cultures were able to grow in the presence of chloramphenicol (results not shown). This is because chloroplasts are sensitive to most of the antibiotics which prevent bacterial growth. Algal cell counts were, however, not performed to verify this.



**Figure 3.4** Glycerol release in an axenic culture of *D. salina* to investigate the possible stimulatory function of glycine (5mM). This was achieved through comparison with controls, some of which contained chloramphenicol ( $50\mu\text{g.ml}^{-1}$ ).

The non-axenic cultures of *D.salina* displayed a similar glycine-enhanced glycerol release (Figure 3.5). On comparison of the glycine induced glycerol release with that of the control, an obvious stimulatory effect is observed at time intervals 0 and 48 hours. The glycine-enhanced glycerol release is more consistent in the chloramphenicol + glycine-treated cultures, where it is not under bacterial influence. This indicates a bacterial role in the utilisation of glycerol. Bacterial plate

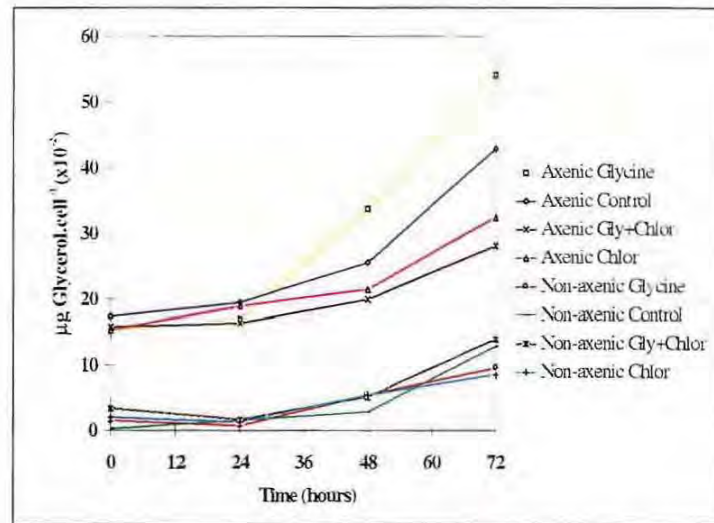
counts confirmed that the chloramphenicol-treated cultures were free of bacterial contaminants. A reduction in the bacterial numbers was, however, observed in the glycine-treated and control algal cultures, from  $8 \times 10^6$  cells.m $\ell^{-1}$  (stock culture) to  $1,2 \times 10^4$  and  $2,2 \times 10^3$  cells.m $\ell^{-1}$  respectively. Some factor present in the algal cultures appears to have inhibited the bacteria and controlled their activity. These findings are in agreement with the observations of Lustigmann (1988) who reported the production of an antibiotic substance by *Dunaliella*. The large increase in glycerol observed between 24 and 48 hours could be associated with bacterial death due to the excretion and accumulation of such antibacterial agents.



**Figure 3.5** Glycerol release in a non-axenic culture of *D.salina* to investigate the possible stimulatory function of glycine (5mM). This was achieved through comparison with controls, some of which contained chloramphenicol ( $50\mu\text{g.m}\ell^{-1}$ ).

A comparison between the abovementioned glycerol levels obtained in the axenic and non-axenic cultures of *Dunaliella* is depicted in Figure 3.6. The glycerol is represented as  $\mu\text{g glycerol.cell}^{-1}$  to facilitate a comparison between cultures of differing cell numbers. The glycerol concentration of the axenic culture is in all cases at least five-fold greater than that of the non-axenic culture indicating that the glycerol is largely removed by the activity of the bacterial population. Had the chloramphenicol not had an inhibitory effect on the algae, the glycerol release in the

chloramphenicol and glycine + chloramphenicol-treated non-axenic cultures could have been in the range experienced by the axenic cultures of *D.salina*.

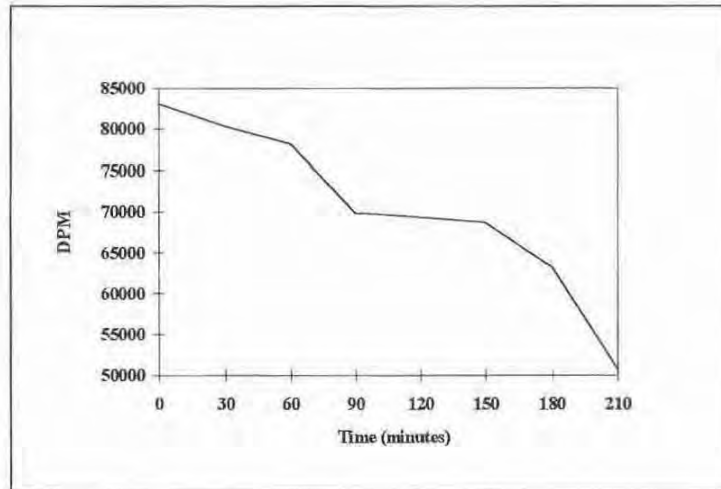


**Figure 3.6** A comparison of the extracellular glycerol levels in axenic and non-axenic cultures of *D.salina* indicating the effect of bacteria on these levels.

### 3.3.3 Glycine Binding Studies

Studies were undertaken in order to determine whether *D.salina* possesses glycine binding sites on its surface. Transient binding of the amino acid glycine is thought to be associated with deamination and may provide an indication of deaminase activity. Because glycine is a small molecule, it passes freely through the dialysis membrane. Its movement is enhanced by the maintenance of a concentration gradient along the membrane, provided by the continuous flow of algal growth medium through the lower wells of the apparatus used. (See Figure 3.1 and plate II). The algae and bacterial cells (due to their larger size) are maintained in the upper compartment of the microdialysis system. Glycine binding is indicated where, compared to the control, a reduced rate of decreasing radioactivity (in the case of reversible binding) or a plateau (in the case of irreversible binding) is recorded. The algal growth media control contained negligible levels of radioactivity (48 - 209 dpm) which were constant over time.

The dialysis of glycine in the control (no cells present) is recorded in figure 3.7.1. The decrease in glycine in the axenic, non-axenic and bacterial systems are recorded in Figures 3.7.2, 3.7.3 and 3.7.4, respectively.



**Figure 3.7.1** The dialysis of [ $^{14}\text{C}$ ]-glycine from the control (glycine [ $^{12}\text{C}$ ]) measured in DPM over 210 minutes.

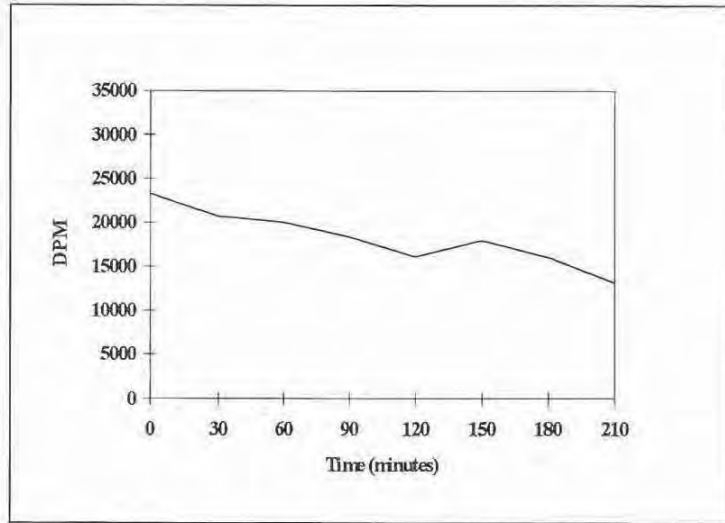


Figure 3.7.2 The dialysis of [ $^{14}\text{C}$ ]-glycine from axenic cultures of *D. salina* measured in DPM over 210 minutes.

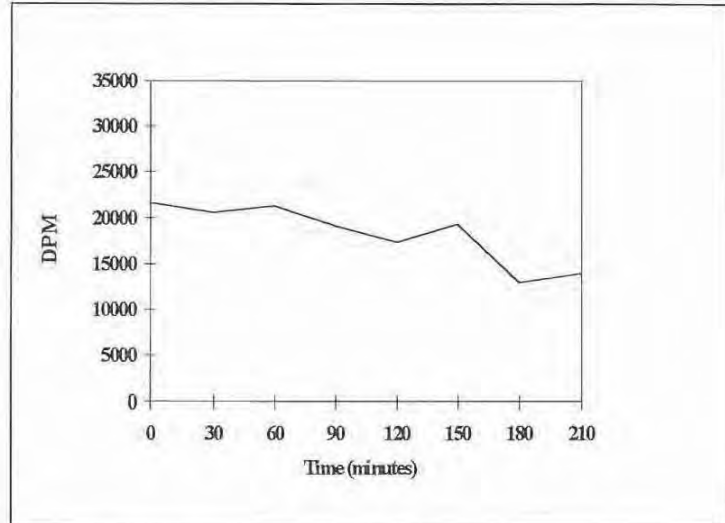
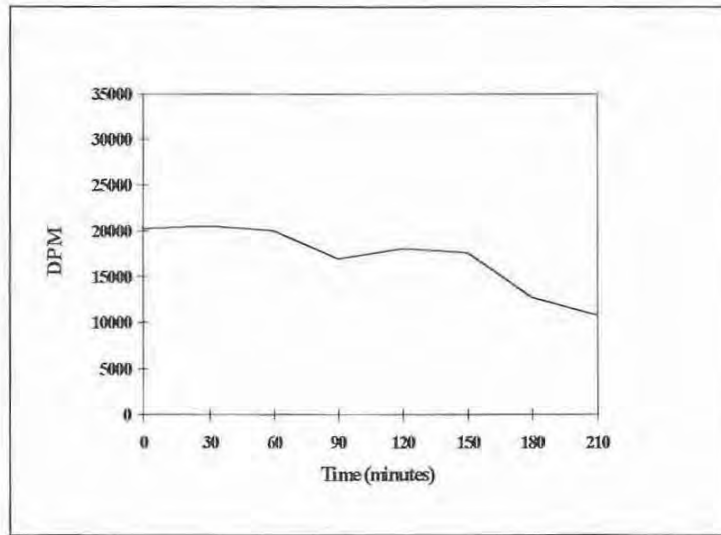
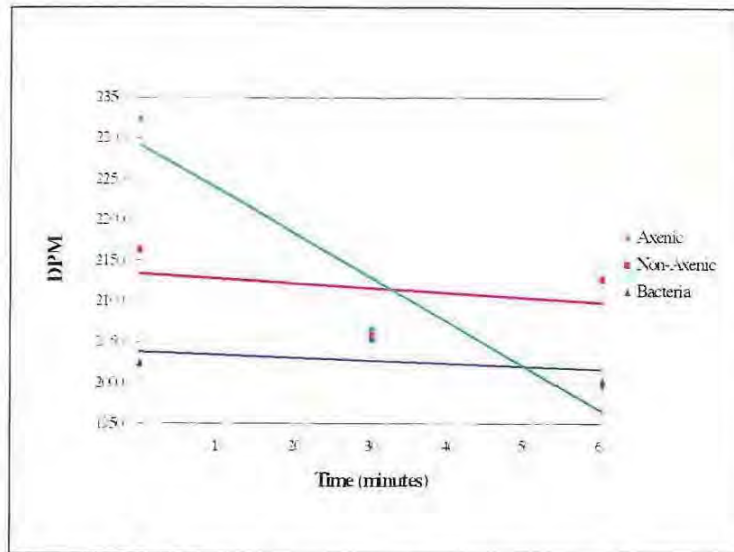


Figure 3.7.3 The dialysis of [ $^{14}\text{C}$ ]-glycine from non-axenic cultures of *D. salina* measured in DPM over 210 minutes.



**Figure 3.7.4** The dialysis of [ $^{14}\text{C}$ ]-glycine from the bacterial systems (derived from non-axenic algal cultures) measured in DPM over 210 minutes.

Figure 3.8 illustrates the trends exhibited by the axenic and non-axenic cultures of *Dunaliella* and bacteria present in the non-axenic cultures. The dpm's of the axenic cultures decreased substantially with a slope of -54,6. Also apparent from Figure 3.8, is the similarity of the trends exhibited by the non-axenic culture of *D.salina* and the bacteria, where the dpm's in both cases remained fairly constant, suggesting binding. The slopes of these plots were -6 and -3,6 respectively, which represents a 15-fold difference to that of the axenic cultures over a 60 minute period. This indicates the role of heterotrophic bacteria in the binding of glycine. Given the results over 210 minutes, it may however be assumed that this is either a reversible process, or that labelled carboxylic acids were released after deamination.



**Figure 3.8** The decreasing trend of glycine dialysis indicated by dpm for the bacteria, axenic and non-axenic cultures of *D. salina* over the first 60 minutes of the binding study

## 3.4 DISCUSSION

### 3.4.1 Fluorescence Quenching Study

The outdoor production of algal mass culture involves a continuous response of the cell to a changing environment. Changes in salinity of the medium are common problems facing outdoor systems. The response to increased salinity involves some distinct physiological changes. Following exposure to the stress, photosynthesis is temporarily inhibited (Gilmour *et al.*, 1985; Kessly and Brown, 1981). Gilmour *et al.* (1985) stated that there is an increased flux of ions ( $\text{Na}^+$  or  $\text{Cl}^-$ ) across the thylakoid membrane when algae are subjected to salt stress. Recovery from inhibition by salt stress is accompanied by a marked increase in respiratory activity, which appears to supply the energy needed for correction of the  $\text{Na}^+/\text{K}^+$  balance and/or for the synthesis of glycerol to maintain osmotic equilibrium (Richmond, 1986b). Gilmour *et al.* (1985) reported that the large quenching of fluorescence induced by ionic stress (as observed in figure 3.2) is caused by a pH gradient across the thylakoid membrane. This large change in pH (which has

been observed in algae) could produce the ATP that contributes to the production of glycerol from starch. Although photosynthetic O<sub>2</sub> production may be suppressed immediately after a salt stress, respiration is not suppressed and neither is the degradation of starch, from which glycerol is derived under such conditions (Kessly and Brown, 1981).

The quenching of fluorescence observed in *D.salina* upon addition of 5μM glycine (Figure 3.3) is similar to the study of Rose (1992). A similar response has also been reported on the addition of casamino acids (Rose, 1992). This quenching effect represents a transient inhibition of photosynthesis and is thought to indicate a demand for energy and reducing power supplied by photosystem II (PSII). The energy is presumably required for glycerol synthesis or for the active uptake of glycine. Glycerol synthesis, either by photosynthetic CO<sub>2</sub> fixation or by degradation of starch, is dependant on metabolic energy (Ben-Amotz and Avron, 1973; Borowitzka and Borowitzka, 1988; Avron, 1992). Avron (1992) reported that the energy cost in the photosynthetic pathway amounts to 9 ATP + 7 NADPH/glycerol while that provided via starch degradation amounts to an energy cost of 0.5 ATP + 1 NADH/glycerol.

A variety of non-ionic compounds may inhibit photosynthesis in a similar manner to that of NaCl, viz. glucose and glycine (Ben-Amotz and Avron, 1980) and sucrose (Gilmour *et al*, 1984b; Fujii *et al*, 1984) (which has also been shown to induce glycerol synthesis). Gilmour *et al* (1984b) investigated the nature of the transient inhibition of photosynthesis and concluded that osmotic stress alone inhibits photosynthesis to a certain extent, but that additional inhibition occurred due to ions.

#### **3.4.2 The Effect of Glycine on Glycerol Excretion in *D.salina***

Glycine stimulated glycerol release (Figures 3.4 and 3.5). It is postulated that this enhanced glycerol release was brought about by glycine, which in turn served to increase the osmotic pressure of the medium, thereby causing a further accumulation of glycerol within the cell. This in turn is thought to result in enhanced glycerol release, due to the increased concentration gradient between the cell content and surrounding medium. Jones and Galloway (1979) and Enhuber and Gimmler (1980), similarly observed a high rate of glycerol release in a medium of high osmotic pressure, corresponding to high intracellular glycerol concentration. Fujii *et al*

(1984) and Gilmour *et al* (1984b) likewise found that glycerol formation occurs in response to increased osmotic pressure in the growth medium rather than ionic strength.

The glycine stimulated glycerol release (Figures 3.4 and 3.5) is in agreement with the findings of Rose (1992), who observed a two-fold increase in glycerol release on addition of glycine to a chloramphenicol-treated non-axenic culture of *D.salina*. In this case, however, the enhanced glycerol release was reported to be transient since the glycerol had been removed by the increased activity of the bacterial population (which had been stimulated on addition of glycine to the media). Rose (1992) observed a direct correlation between glycine addition and bacterial numbers. Sharp (1977) also reported that old cultures of algae, which showed large accumulations of organic carbon, supported large bacteria populations. The abovementioned enhancement of bacterial activity by glycine is contrary to the results reported in this study, which in fact suggest an inhibition of bacterial activity, presumably by some factor released by the algal culture.

#### **3.4.3 The Role of Algal-Bacterial Interrelations in the Deamination of Glycine**

Richmond (1986a,e) reported that the bacterial count in healthy, rapidly growing cultures of the cyanobacterium, *Spirulina* in outdoor ponds is usually relatively small, ranging between 1 and 5 x 10<sup>3</sup> bacteria.m<sup>-1</sup>. The small size of this population was attributed to algal-bacterial interrelations. Only on deterioration of the algal culture did the bacterial population increase by an order of magnitude. The observed repression of the bacterial population is substantiated by Lustigman (1988) who reported that a broad spectrum antibiotic substance is produced by strains of *Dunaliella*. According to Lustigman (1988) this substance is only produced in highly polluted waters (for example wastewaters). The addition of glycine, together with the glycerol released by *D.salina*, would produce an environment rich in organic compounds. This may have stimulated the release of an antibiotic substance by this alga. Lustigman (1988) and Dor (1980) stated that the successful growth of algae in a highly competitive situation (such as occurs in wastewater) is connected with the repression of bacterial activity. The concentration of glycerol is seen to treble between 24 and 48 hours in the non-axenic culture of *D.salina* (Figure 3.5). This increase could correspond with the initial release of an antibacterial substance by *D.salina*.

A comparison of the glycerol released by the axenic- and non-axenic cultures of *D.salina* (Figure 3.6) illustrates the bacterial utilisation of the glycerol. Hellebust (1965) and Giordano *et al* (1994) have reported on the significance of glycerol to the growth of heterotrophic bacteria. Glycerol is readily utilised as a carbon and energy source by members of the halobacteriaceae that live in association with *Dunaliella* in hypersaline brines (Oren, 1993).

The active uptake of glycine could also have been responsible for the energy demand and thus the quenching of fluorescence observed in figure 3.3. Rose *et al* (1991) observed an accumulation of ammonia in *Dunaliella* cultures grown on synthetic media with glycine as sole nitrogen source and suggested the active deamination of glycine by *Dunaliella*. The results documented in section 3.3.3 tentatively refute this possibility and suggest the role of bacteria in this regard. This is substantiated by the fact that deaminase, the enzyme responsible for this process, is prevalent in most bacteria. The heterotrophic bacteria therefore appear to undertake the mineralisation of glycine and release the free ammonia, which in turn is available for energy dependent uptake by the algae as a nitrogen source for growth. The quenching of fluorescence observed in figure 3.3 therefore appears to represent the sudden demand for NADH and ATP by the GS-GOGAT ammonia assimilation system.

It is thus feasible to postulate the following sequence of events:

On addition of glycine to the non-axenic culture of *D.salina*, the residing heterotrophic bacteria bind the glycine temporarily, enabling its deamination. The ammonia thus released is then rapidly assimilated by the algal GS-GOGAT system, the primary pathway for ammonia assimilation in algae (Kaplan *et al*, 1986, Antia *et al*, 1991). This system comprises two enzymes, viz. Glutamine synthetase (GS) and Glutamine oxo-glutarate aminotransferase (GOGAT or glutamate synthetase) (Lea, 1987). GS, which is involved in the assimilation of newly fixed ammonia into the amide position and GOGAT, which is involved in its transfer to the 2-amino position, are both energy dependent processes. GOGAT requires reducing power which is either supplied as reduced ferredoxin or NADH (Lea, 1987). The energy demand created on glycine addition therefore causes a shunting of electrons into photosynthetic and related pathways and in so doing, reduces the background of electrons lost as fluorescence. This is indicated by the quenching effect observed on glycine addition. This postulate may be substantiated by the fact that ammonium assimilation can be regarded as a photosynthetic process, since it occurs in the chloroplast, where ATP and reduced ferredoxin are generated from light energy (Lea, 1987;

Kaplan *et al*, 1986). According to Lea (1987), ammonia is assimilated rapidly due to its toxicity. This could explain the rapid quenching of fluorescence observed within seconds of glycine addition to the culture of *Dunaliella*.

The proposed bacterial deamination is substantiated by reports from Antia *et al* (1991) and Ietswaart *et al* (1994) that ammonia is generated by the bacterial deamination of organic nitrogenous compounds. Flynn and Butler (1986) reported that amino acids are readily utilised by bacteria, resulting in the regeneration of ammonia, which is subsequently used by the algal population.

The ability of micro-algae to utilise amino acids is highly variable between groups, species and even isolates of the same species (Flynn and Butler, 1986). The results documented in this study do not, however, rule out the presence of an external deaminase system in this particular alga. The extracellular deamination of L-amino acids has been identified in a number of algae, viz. *Chlamydomonas reinhardtii*, *Monodus subterraneus* and some prymnesiophytes and dinoflagellates. Muñoz-Blanco (1990) reported that the deaminase activity was induced by L-amino acids and that cells grown with ammonium required a 10-30 hour incubation in the presence of the amino acid prior to the appearance of the deaminating system. This was omitted in the present study. Muñoz-Blanco (1990) also reported a sensitivity of the deaminase system to ammonium, where added ammonium prevented cells from deaminating amino acids to an extent proportional to the external ammonium concentration. It is possible that, since bacteria out-compete algae for nutrients (Wheeler and Kirchman, 1986) the rapid uptake and deamination of glycine by bacteria yielded NH<sub>3</sub>, which could serve to inhibit the algal deamination system (if it were present). Muñoz-Blanco (1990) reported that in *Chlamydomonas reinhardtii*, a preformed carbon source was required to induce deaminase activity. This was also omitted in the present study. Further research is therefore required in this area to confirm the presence or absence of an algal deaminase on the surface of *D.salina*.

Due to the apparent absence of an extracellular deaminase in *Dunaliella*, the uptake of glycine, which was observed by Rose (1992), could have occurred through active transport as pinocytosis. This is substantiated by Antia *et al* (1991) who stated that in the absence of deaminase activity, amino acids are taken up, assimilated and often utilised as a nitrogen source for growth.

## CHAPTER 4

### A COMPARISON OF THE GLYCEROL AND STARCH CONTENT OF EFFLUENT-GROWN *DUNALIELLA* SPP.

#### 4.1 INTRODUCTION

Since Teodoresco's initial description of *D.salina*, many different species from a wide range of habitats have been described. According to Preisig (1992) and Borowitzka and Borowitzka (1988), 28 species of *Dunaliella* are presently recognised, some of which are subdivided into a number of varieties and forms. The genus *Dunaliella* has been divided into two subgenera, *Pascheria* and *Dunaliella*. The subgenus *Pascheria* is relatively rare and it comprises 5 species, all of which occur in freshwater. Their inclusion within the genus is uncertain. The remaining species, which are of the subgenus *Dunaliella*, occur in saline environments (Preisig, 1992). This chapter deals specifically with species of the *Dunaliella* subgenus.

The most widely known *Dunaliella* species are *D.salina*, *D.tertiolecta*, *D.parva*, *D.primolecta* and *D.viridis* (Ben-Amotz, 1980). *Dunaliella salina* and *D.tertiolecta* are the most studied species (Nakas *et al*, 1983; Borowitzka and Borowitzka, 1988).

All *Dunaliella* species produce and accumulate large amounts of glycerol in response to increased salinity of the environment (Jones and Galloway, 1979; Borowitzka and Borowitzka, 1988). There are, however, reported differences between species and among species in measurement of these values.

Glycerol and starch, the two major carbon pools in *Dunaliella*, may be inter-converted (Beckett *et al*, 1985; Gimmler and Möller, 1981; Degani *et al*, 1985). Glycerol may be synthesised from intermediates of the photosynthetic pathway via the glycerol cycle, via starch degradation, or both (Gimmler and Möller, 1981; Fujii and Hellebust, 1992). The contribution of these metabolic pathways to glycerol synthesis depends on the availability of light, the starch reserve pool and the

extent of the salt stress (Avron, 1992). Starch breakdown accounts for 70% of the glycerol synthesis in the light and for all the glycerol synthesis in the dark (Borowitzka and Borowitzka, 1988). Avron (1992), however reported that immediately following hyperosmotic shock in *D. tertiolecta* cultures, starch degradation provides over 90% of glycerol production.

Starch (alpha-1,4-glucosan) constitutes the major storage product in *Dunaliella* (Gimmler and Möller, 1981; Degani *et al*, 1985; Preisig, 1992). It has an unusual composition in that it contains a low amylose content (12-14%) and a high amylopectin content, which has a high degree of branching (Gimmler and Möller, 1981; Degani *et al*, 1985). Starch grains usually surround the pyrenoid (Ben-Amotz and Avron, 1980; Richmond, 1986e), but may occur elsewhere in the chloroplast, especially in older cultures (Preisig, 1992).

The major elements required for growth of *Dunaliella* are carbon, nitrogen, phosphorous, sulphur, calcium, potassium, sodium, chloride and trace elements (Ben-Amotz, 1980). Municipal, agricultural and many industrial wastes are reported to contain all, or most of the nutrients required to maintain algal growth (Oswald, 1980; Shelef *et al*, 1980). A number of authors have reported on the practical and economic uses of wastewater as a medium for the mass culture of algae (Richmond and Preiss, 1980; Shelef *et al*, 1980; Behr and Soeder, 1981; Abeliovich, 1986; Richmond, 1986e; Oswald, 1988a; Rose *et al*, 1992). Tannery wastewater is a highly pollutive effluent which contains a high concentration of organic matter, suspended particles and salt (Tsotsos, 1986). For this reason, it requires extensive treatment prior to its discharge (LIRI Technologies, 1990). Laubscher (1991) identified a possible use for these saline effluents turning a problematic waste product into a potentially valuable resource. The author tested a range of tannery effluents for their suitability in the mass cultivation of *D. salina* (var. *bardawil*). Hide soak liquor was identified as the ideal culture medium, since it was the only effluent tested which did not require the addition of a chemically defined enrichment to support enhanced algal growth (Laubscher, 1991).

Algal growth in hide soak liquor, diluted to 50% or less, has been reported to produce a substantial enhancement of cell growth compared to that in an inorganic growth medium control (Laubscher, 1991; Rose, 1992). Hide soak liquor is a product of the curing process (the classical international method of hide preservation), generated in the rehydration of salt cured hides (Cooper *et al*, 1984). Apart from the high salt content, hide soak liquor also contains a high

organic load (Tsotsos, 1986). Antiseptic agents, such as naphthalene and antibiotics, may be used in hide preservation, especially during transport (LIRI Technologies, 1990). Hide soak liquor obtained from the washing and rinsing of such hides would therefore contain low concentrations of these agents. Laubscher (1991) proposed a 2-stage system for  $\beta$ -carotene production in this effluent, where hide soak liquor was used for cell propagation and nitrogen deficient medium used for induction of  $\beta$ -carotene production. Using such a system, the author reported significantly raised biomass yields, while  $\beta$ -carotene production remained constant. The effect of hide soak liquor on glycerol production in *Dunaliella spp.* is, however, unknown.

#### 4.1.1 Research Objectives

There is no known account in the literature of an extensive screening of a range of *Dunaliella* species with regard to glycerol accumulation, starch content and growth rate. There is also no known account of a comparison of these values with effluent grown equivalents. A study was therefore undertaken to investigate the above in eight different *Dunaliella* species (and a variety of strains thereof) and three unknown *Dunaliella* species. The following research objectives were identified:

1. To investigate the suitability of 20% bovine salted hide soak tannery effluent (1,5M NaCl) as a culture medium for the *Dunaliella* species under study.
2. To screen a number of effluent-adapted *Dunaliella* species for optimal intracellular glycerol and starch content when cultured in both defined inorganic algal growth medium and hide soak liquor.
3. To investigate the effect of medium composition on intracellular carbon production (glycerol and starch).
4. To estimate glycerol release by *Dunaliella* species and to investigate the effect of medium composition on glycerol release.
5. To select a *Dunaliella* biomass from those under study which exhibits:
  - a) the greatest yield of potentially fermentable carbon and
  - b) the most favourable cell yieldsin hide soak liquor, to serve as a potential substrate in the linked fermentation production of a high value product.

## 4.2 METHODS AND MATERIALS

### 4.2.1 *Dunaliella* Culture

A variety of *Dunaliella* species, and strains thereof, were investigated in this study. The majority of these species were acquired from the Culture Collection for Algae and Protozoa, Oban, United Kingdom. The remainder were supplied by the University of Natal, Pietermaritzburg, South Africa. The collection sites and characteristics of these species are given in table 4.1. All experiments performed in this study involve the use of pure cultures or tannery effluent-adapted cultures of these strains.

**Table 4.1** The *Dunaliella* species (and strains) investigated.

No.	Species	Place of Collection	Characteristics
1	<i>D.salina</i> 19/30 (PMB)	Supplied by University of Natal,Pietermaritzburg	Unknown
2	<i>D.salina</i> 19/3 (PMB)	Supplied by University of Natal,Pietermaritzburg	Unknown
4	<i>D.tertiolecta</i> 19/27 (PMB)	Supplied by University of Natal,Pietermaritzburg	Unknown
5	<i>D.tertiolecta</i> (PMB)	Supplied by University of Natal,Pietermaritzburg	Unknown
6	<i>D.primolecta</i> 11/34 (PMB)	Supplied by University of Natal,Pietermaritzburg	Unknown
7	<i>D.parva</i> 19/10 (PMB)	Supplied by University of Natal,Pietermaritzburg	Unknown
8	<i>Dunaliella</i> sp. P.R.R.	Supplied by University of Natal,Pietermaritzburg	Unknown
9	<i>D.salina</i> CCAP 19/3	Brackish; a salt lake, USSR	Axenic culture
10	<i>D.salina</i> CCAP 19/18	Hypersaline brine; Hutt Lagoon, Western Australia	Axenic culture, carotenogenic
11	<i>D.salina</i> CCAP 19/20	Marine	Axenic culture
12	<i>D.salina</i> CCAP 19/25	Evaporating salt ponds, Punta Colorado Salina, Mexico	Axenic culture
13	<i>D.salina</i> ( <i>bardawil</i> ) CCAP 19/30	Marine, salt pond near Bardawil Lagoon, N-Sinai, Egypt	Non-axenic culture, carotenogenic, halophilic
14	<i>D.salina</i> CCAP 19/31	Unknown	Non-axenic culture (bacteria)
15	<i>D.peircei</i> CCAP 19/2	Brackish pool in the Marina area, California, USA	Axenic culture
16	<i>D.tertiolecta</i> CCAP 19/6B	Marine, Oslo Fjord, Norway	Non-axenic culture (bacteria/other organisms)
17	<i>D.polymorpha</i> CCAP 19/7B	Marine, Isle of Sheppey, Kent, UK	Non-axenic culture (bacteria/other organisms)
18	<i>D.quartolecta</i> CCAP 19/8	Marine, Southampton, UK	Axenic culture
19	<i>D.parva</i> CCAP 19/10	Marine, Dead Sea, Israel	Non-axenic culture (bacteria/other organisms)
20	<i>Dunaliella</i> sp. CCAP 19/19	Hypersaline brine; Hutt Lagoon, Western Australia	Non-axenic culture (bacteria)
21	<i>Dunaliella</i> sp. CCAP 19/21	Saline water, Wad al Neifur, Egypt	Non-axenic culture (bacteria)
23	<i>D.minuta</i> CCAP 19/5	Sand and sea water sample, Per Haridy, France	Non-axenic culture (bacteria)

The species designated CCAP were obtained from the Culture Collection of Algae and Protozoa, United Kingdom

#### **4.2.2 Maintenance of the Algal Culture Collection**

*Dunaliella* subcultures were maintained by periodic transfer in artificial algal growth medium (1.5M NaCl). Following inoculation, the cultures were incubated at 26°C ( $\pm 2^\circ\text{C}$ ) on a light-bench at approximately 200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  illumination. Once good growth had been obtained, the cultures were maintained at 18°C ( $\pm 2^\circ\text{C}$ ) at a lower light intensity, for slower growth and storage. Stock cultures were maintained on 1.5M NaCl agar plates under the conditions described above. The algal spread plates were sealed with parafilm to prevent desiccation.

#### **4.2.3 Culture Medium**

##### **4.2.3.1 Tannery Effluent Medium**

Bovine salted hide soak liquor, sourced from LIRI Experimental Tannery (Grahamstown), was used for this purpose. The growth medium was made up by dilution of this effluent to 20% by the addition of tap water. Unless otherwise indicated, the final salinity was adjusted to 1.5M with NaCl (8.7%, as read on a salinometer). Unsterilised, raw hide soak tannery effluent was used in all cases.

##### **4.2.3.2 Defined Inorganic Algal Growth Medium**

The growth of *Dunaliella* in hide soak liquor (HS) was compared to that in the defined inorganic algal growth media described by Ben-Amotz and Avron (1983a). This is outlined in section 2.2.2.

#### **4.2.4 Adaptation to Hide Soak Liquor - Tannery Effluent**

In adapting the algal species to growth in hide soak liquor, the algae were first transferred to fresh inorganic algal growth medium. Once significant growth had been observed, the algae were transferred to media of increasingly higher concentrations of hide soak liquor, until adapted to growth in full strength effluent (1.5M NaCl).

A number of *Dunaliella* cultures were adapted to grow in hide soak liquor following a direct inoculation (10% inoculum) into this medium viz. *D.primolecta* 11/34 (PMB), *D.polymorpha* CCAP 19/7B, *D.quartolecta* CCAP 19/8 and all the *D.tertiolecta* strains. Other strains/species of *Dunaliella* viz. *D.salina* strains required a gradual adaptation process to enable growth in this medium. This was performed by sequential subculturing in defined algal media, which contained increasing concentrations of hide soak liquor (of the same molarity). Other species of *Dunaliella* were not able to adapt to grow in this organic tannery effluent, viz. the two *D.parva* strains, two strains of *D.salina* (CCAP 19/20 and 19/25) and two unidentified *Dunaliella* species (CCAP 19/19 and 19/21).

#### **4.2.5 Culture Conditions**

Media were dispensed in cotton wool stoppered 500ml conical flasks and, after inoculation, incubated at 26°C ( $\pm 2^\circ\text{C}$ ) in a Constant Environment (CE) room under continuous illumination of 167-250  $\mu\text{mol.m}^{-2}.\text{sec}^{-1}$ . The cultures were shaken continuously without additional aeration.

#### **4.2.6 Determination of Growth**

Algal growth was monitored both directly by cell counts and indirectly by chlorophyll content.

#### 4.2.6.1 Cell Counts

Algal cell counts were performed by means of a haemocytometer as outlined in section 3.2.3. Cell counts were performed in triplicate.

The growth rate of each *Dunaliella* species (doublings per day) was calculated during the exponential phase of growth according to the procedure described by Fabregas *et al* (1986). Doublings per day were calculated according to the formula:

$$\text{Dbls / day} = \frac{\ln.f(t_n) - \ln.f(t_i)}{\ln 2(t_n - t_i)}$$

where  $f(t)$  is the cellular density and  $t_i$  and  $t_n$  are the initial and final time of the logarithmic phase (both are expressed in days).

#### 4.2.6.2 Chlorophyll Assay

The chlorophyll content of *Dunaliella* was determined by the procedure described by Ben-Amotz and Avron (1983a). *Dunaliella* cells (10mℓ) were pelleted by centrifugation at 1 700g x 10 minutes and the sedimented cells re-suspended in approximately 7mℓ 100% acetone. The acetone served to extract the chlorophyll from the pellet during a 2 hr incubation period (in the dark). Cell debris was removed by centrifugation at 1 700g x 5 minutes, after which the supernatant was made up to 8mℓ with 100% acetone. The acetone-chlorophyll mix was thereafter diluted to 80% by the addition of distilled water to a final volume of 10mℓ. The chlorophyll was assayed on a Shimadzu UV-160A double-beam recording spectrophotometer at 666nm. The chlorophyll content was determined in  $\text{mg.mℓ}^{-1}$  by the formula:

$$\frac{A_{666} \times 10 \times \text{dilution factor}}{890}$$

#### **4.2.7 Cell Volume Determination**

Average cell volume was determined with a Coulter Counter.

#### **4.2.8 The Screening of *Dunaliella* species for Maximised Glycerol and Starch Content**

The *Dunaliella* species were screened during logarithmic growth for optimal intracellular glycerol and starch content. Cell counts and chlorophyll assays were performed at the time of sampling to enable comparison of these values amongst the different species and strains of *Dunaliella* under study.

##### **4.2.8.1 The Quantification of Intracellular Glycerol**

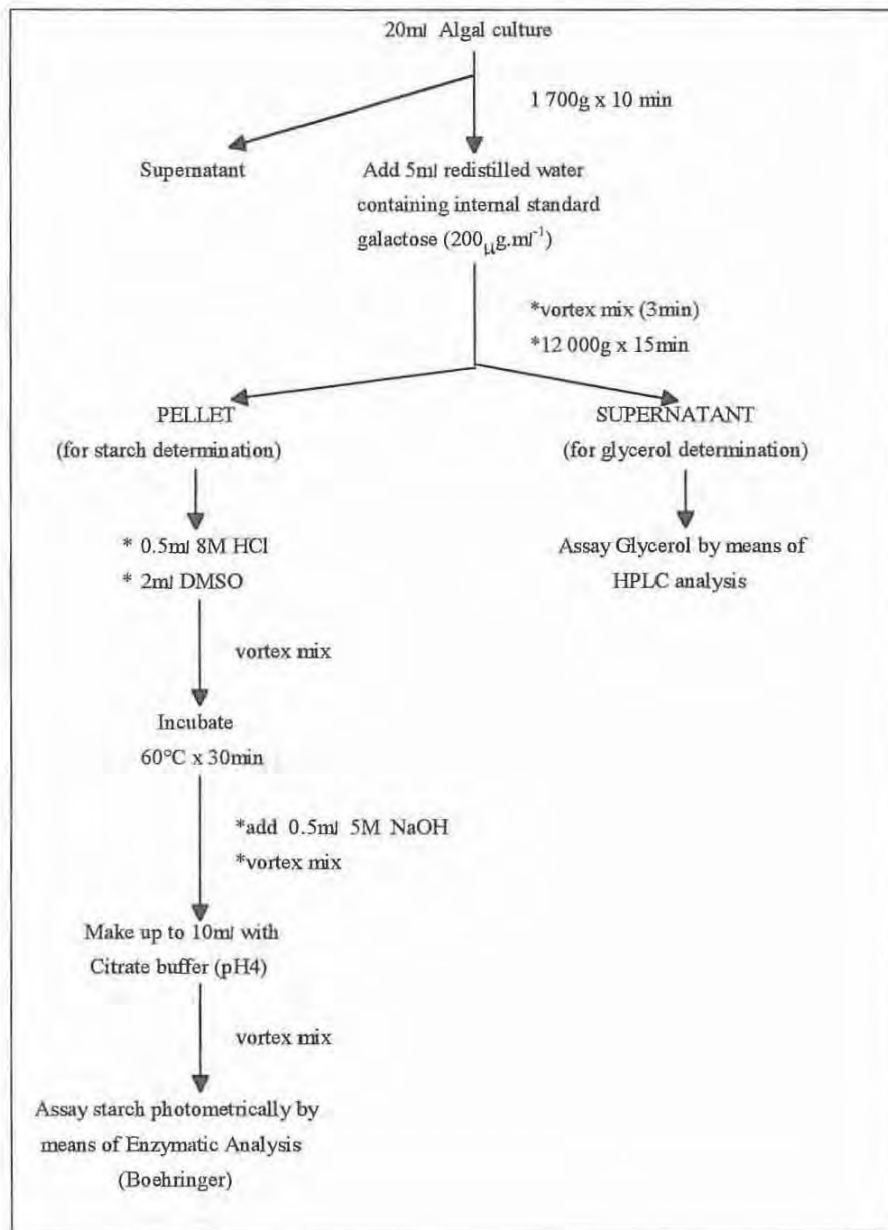
The intracellular glycerol content of the *Dunaliella* species was determined by HPLC. The methodology of the extraction procedure and the parameters used in HPLC analysis are as outlined in section 2.2.5. Due to the nature of the extraction procedure, the high protein content of the hide soak liquor in which the algae grew did not interfere with the quantification of intracellular glycerol by HPLC. Folin-Lowry assays indicated an acceptable level of protein in these samples.

##### **4.2.8.2 The Quantification of Starch Content**

The starch content of the *Dunaliella* species was determined in conjunction with the intracellular glycerol content and the preliminary stages of this extraction procedure are common with that of glycerol extraction. The flow diagram of the glycerol and starch extraction procedures is depicted in figure 4.1.

The pellet resulting from the final spin of 12 000g x 15 minutes was then re-suspended in 0.5ml 8M HCl, to which 2ml DMSO is added (to aid membrane disruption). This was vortex mixed and incubated in a waterbath at 60°C for 30 minutes. The reaction was stopped by the addition of cold 5M NaOH (0.5ml), vortex mixed, decanted into a 10ml volumetric flask and made up to

a 10ml volume with citrate buffer (pH 4). The starch content was assayed immediately by means of an enzyme assay kit 'Starch UV Method' (Boehringer, Mannheim).



**Figure 4.1** A Flow diagram of the extraction procedure for the determination of intracellular glycerol and starch.

#### **4.2.9 The Quantification of Potential Glycerol Reserves**

*Dunaliella* cells were stressed by the addition of 3M NaCl. This caused a flow of carbon from starch into glycerol, enabling the use of the accumulated glycerol as an osmolyte in the more saline environment. In this way, it was possible to determine the maximum glycerol yield potential of each of the *Dunaliella* species.

Glycerol and starch assays were performed (as outlined above) over a period of 12 days, at 4 day intervals. The *Dunaliella* cultures were salt stressed at  $t = 4$  days by the addition of NaCl to the medium at a final concentration of 3M NaCl. Glycerol and starch content was determined both prior to the salt stress and following a 2 hour incubation. Cell counts and chlorophyll assays were performed at the time of sampling.

#### **4.2.10 Glycerol Release by *Dunaliella* sp.**

The glycerol content of the media (1.5M NaCl) was determined enzymatically (Boehringer) as outlined in section 2.2.4 and 2.2.4.2.

##### **4.2.10.1 The Quantification of Extracellular Glycerol**

Random extracellular glycerol assays were performed in selected *Dunaliella* species to estimate glycerol release in defined inorganic media. Two *D.salina* strains, *D.salina* CCAP 19/18 and *D.salina* (*bardavil*) CCAP 19/30, and *D.quartolecta* CCAP 19/8 were used to represent glycerol release by this genus. Glycerol assays were performed during early exponential phase and late stationary phase. Culture conditions are as outlined in section 4.2.5.

##### **4.2.10.2 The Effect of Media Composition on Glycerol Release**

In order to investigate the effects of different media on glycerol release in *Dunaliella*, the extracellular glycerol levels of *D.salina* were determined in both defined inorganic algal growth medium and hide soak liquor. These values are expressed as pg glycerol excreted per cell. Bacterial cell counts were performed to investigate the relationship between bacterial number and

extracellular glycerol levels. Bacterial cell numbers were determined as CFU on 1,5M NaCl agar. Extracellular glycerol concentration and bacterial number was determined at time 0, 5 and 10 days. Culture conditions are as outlined in section 4.2.5.

## 4.3 RESULTS

### 4.3.1 Hide Soak Liquor as an Algal Growth Medium

Appropriately diluted hide soak liquor proved to be highly suitable as a growth medium for most species and in some instances resulted in enhanced growth rates and higher biomass yields. This is reflected in table 4.2, which reports a comparison of the growth rates of the 15 effluent-adapted *Dunaliella* cultures in hide soak liquor and in defined medium. Most species exhibited a 2-fold enhancement of growth in hide soak liquor, while the remaining species appear to display slightly retarded growth in this medium (*D.polymorpha* and *D.minuta*).

It is possible that the cultures of *Dunaliella* which failed to grow in hide soak liquor could be correlated with their inability to use organic compounds present in this effluent or to tolerate certain inhibitory substances which may be present.

Tannery effluent containing antimicrobial agents was unsuitable as a growth medium for all the strains tested. Aerobic digestion of such effluent may remove persistent antimicrobial agents, thereby enabling algal growth (Laubscher, 1991).

**Table 4.2** A comparison of growth rates for 15 different species/strains of *Dunaliella* grown in hide soak liquor and defined inorganic algal growth medium. The growth rate was determined during logarithmic growth at 26°C ( $\pm 2^\circ\text{C}$ ), under continuous illumination ( $170\text{-}250 \mu\text{mol.m}^{-2}.\text{sec}^{-1}$ ), without aeration and is represented as doublings per day. Hide soak media refers to 20% bovine salted hide soak liquor (1.5M NaCl). Def. media refers to the defined inorganic algal growth media (1.5M NaCl) used in this study.

No.	Species and strains	Growth Rate (Doublings.day <sup>-1</sup> )	
		Def. inorg. media	Hide Soak media
1	<i>D.salina</i> 19/30 (PMB)	0.600	1.377
2	<i>D.salina</i> 19/3 (PMB)	0.616	1.257
4	<i>D.tertiolecta</i> 19/27 (PMB)	0.484	0.687
5	<i>D.tertiolecta</i> (PMB)	0.697	0.661
6	<i>D.primolecta</i> 11/34 (PMB)	0.474	0.663
8	<i>D.spp</i> P.R.R.	0.796	0.785
9	<i>D.salina</i> CCAP 19/3	0.650	0.833
10	<i>D.salina</i> CCAP 19/18	0.839	1.213
13	<i>D.salina</i> ( <i>bardawil</i> ) CCAP 19/30	0.656	0.951
14	<i>D.salina</i> CCAP 19/31	0.816	0.886
15	<i>D.peircei</i> CCAP 19/2	0.624	0.808
16	<i>D.tertiolecta</i> CCAP 19/6B	0.767	0.942
17	<i>D.polymorpha</i> CCAP 19/7B	1.147	0.739
18	<i>D.quartolecta</i> CCAP 19/8	0.897	0.982
23	<i>D.minuta</i> CCAP 19/5	0.899	0.448

The results indicate a correlation between *Dunaliella* species cell volume and its ability to display enhanced growth in hide soak liquor. The cell volumes of selected *Dunaliella* species in defined media and hide soak liquor are given in table 4.3.

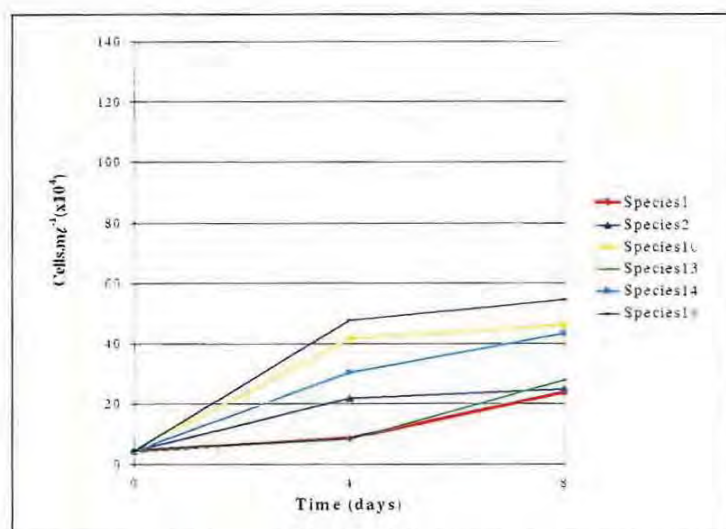
**Table 4.3** The variation in cell volume induced by a change in growth medium (hide soak liquor and defined inorganic growth medium) in selected *Dunaliella* species. HS refers to 20% bovine salted hide soak liquor (1.5M NaCl). Def. media refers to the defined inorganic algal growth media (1.5M NaCl).

No.	Species	Cell volume ( $\mu\text{m}^3$ )	
		Def. media	HS
1	<i>D.salina</i> 19/30 (PMB)	1003	636
2	<i>D.salina</i> 19/3 (PMB)	707	596
5	<i>D.tertiolecta</i> (PMB)	247	523
6	<i>D.primolecta</i> 11/34 (PMB)	180	465
8	<i>D.spp</i> P.P.R.	110	552
9	<i>D.salina</i> CCAP 19/3	389	601
10	<i>D.salina</i> CCAP 19/18	772	522
13	<i>D.salina</i> ( <i>bardawil</i> ) CCAP 19/30	965	459
14	<i>D.salina</i> CCAP 19/31	225	398
15	<i>D.peircei</i> CCAP 19/2	445	690
16	<i>D.tertiolecta</i> CCAP 19/6B	242	461
17	<i>D.polymorpha</i> CCAP 19/7B	231	354
18	<i>D.quartolecta</i> CCAP 19/8	196	401
23	<i>D.minuta</i> CCAP 19/5	181	467

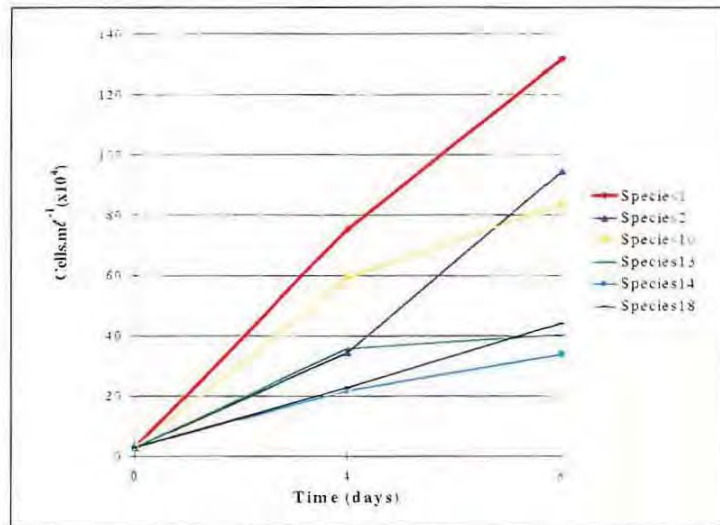
Large species, such as most *D.salina* strains ( $>700 \mu\text{m}^3$ ), which displayed such an enhanced growth, exhibited a decrease in cell volume on growth in hide soak liquor. Small species ( $<250 \mu\text{m}^3$ ), which displayed negligible growth enhancement in hide soak liquor (or on some occasions were even suppressed), exhibited an increase in cell volume on growth in this medium. Further research is however necessary to confirm these trends which are at present only tentatively correlated.

*Dunaliella salina* strains yielded the highest growth rates of all the species tested. *Dunaliella salina* 19/30 (PMB)(sp.1) yielded optimal growth of 1.377 doublings per day in effluent, a 2.3-fold enhancement from that obtained in defined medium. Such enhanced cell yields have been confirmed by Laubscher (1991) and Rose (1992), who dealt with *D.salina* strains. This indicates that hide soak liquor contains the complete nutritional requirements and provides an adequate alternative medium for *Dunaliella* cultivation.

*Dunaliella* growth responses are complicated interactions of a number of variables such as temperature, salinity and nitrogen content (Borowitzka *et al.*, 1977; Jiménez and Niell, 1992). From the species/strains listed in table 4.2, six were chosen that displayed the most favourable growth rates under the given conditions in hide soak liquor and which yielded a high degree of adaptability to growth in this effluent. The selected *Dunaliella* species include five *D.salina* strains and one *D.quartolecta* strain. The growth curves of these species in defined medium and hide soak liquor are presented in figure 4.2 and figure 4.3, respectively. These figures indicate that those species which exhibit high biomass yields in defined inorganic media differ from those which exhibit such yields in the tannery effluent. Figure 4.3 shows that growth in hide soak liquor resulted in sustained exponential growth for the majority of the *Dunaliella* species. Also apparent from figure 4.3 is the significantly raised biomass yield (compared to those in figure 4.2).



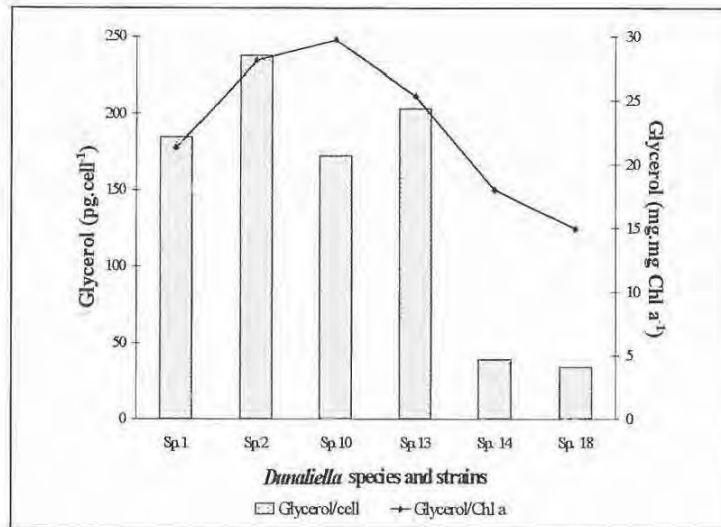
**Figure 4.2** Growth of the 6 selected species/strains of *Dunaliella* in defined inorganic growth medium. (*Dunaliella* species are as designated in table 4.1).



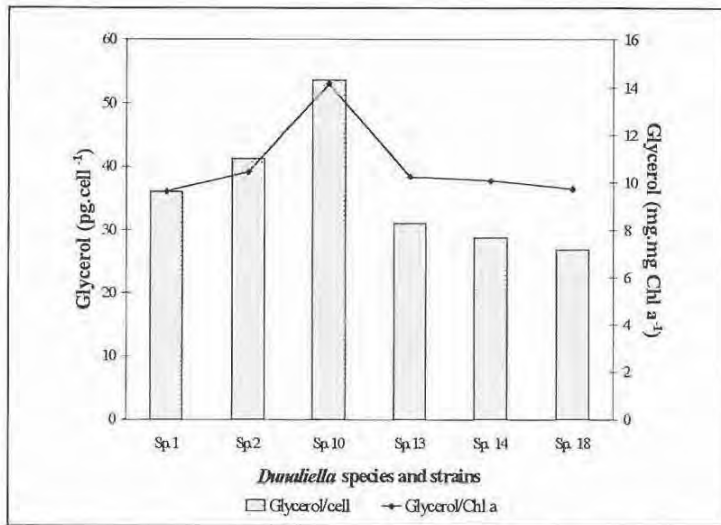
**Figure 4.3** Growth of the 6 selected species/strains of *Dunaliella* in hide soak liquor. Effluent adapted strains were used in each case. (*Dunaliella* species are as designated in table 4.1).

#### 4.3.2 The Screening of *Dunaliella* species for Optimal Glycerol Content

The intracellular glycerol content of the selected *Dunaliella* species was evaluated in both defined inorganic growth medium and hide soak liquor during logarithmic growth. The results of this are depicted graphically in figure 4.4 and figure 4.5. Glycerol content was expressed both per cell and as a function of chlorophyll a content.



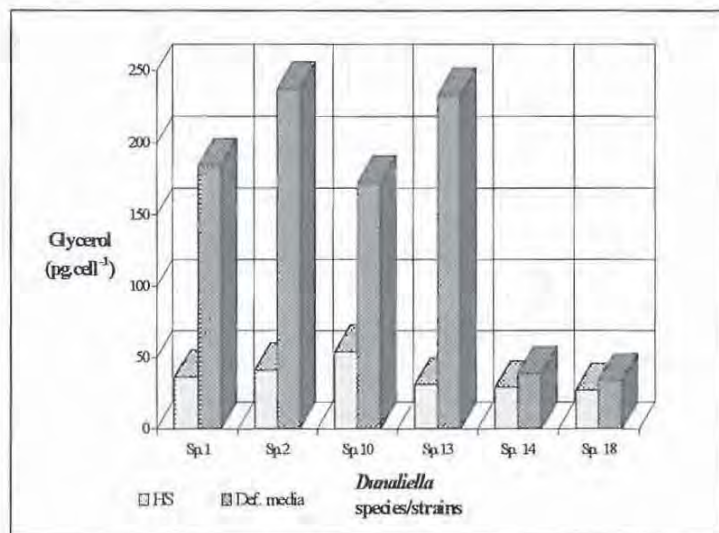
**Figure 4.4** The intracellular glycerol content of selected *Dunaliella* species in defined inorganic growth medium (1.5M NaCl). Experimental results represent the quadruplicate mean of exponentially growing cells. (Species are as designated in Table 4.1)



**Figure 4.5** The intracellular glycerol content of selected *Dunaliella* species in hide soak liquor (1.5M NaCl). Experimental results represent a quadruplicate mean of exponentially growing cells. (Species are as designated in Table 4.1)

The results show that these *Dunaliella* species accumulate high concentrations of intracellular glycerol. This accounts for their growth and survival in media containing high salt concentrations. A significant difference in glycerol content is, however, observed between different *Dunaliella* species/strains for each medium. For instance, an 8-fold difference is observed between the mean glycerol content per cell in sp.18 (*D.quartolecta* CCAP 19/8) and sp.2 (*D.salina* CCAP 19/3 (PMB)) in defined media, which equates with a distribution in glycerol content of 263.17 pg.cell<sup>-1</sup> (figure 4.4). This represents a standard deviation about the mean of 56%. This variation between species is in agreement with observations by Degani *et al* (1985). The fluctuations in glycerol content are not as extreme in hide soak liquor-grown cultures, the maximum variation being a 2-fold difference between sp.18 (*D.quartolecta* CCAP 19/8) and sp.10 (*D.salina* CCAP 19/18). This equates with a distribution in glycerol content of 26.79 pg.cell<sup>-1</sup>, which represents a standard deviation about the mean of 30% (figure 4.5). The variation in glycerol content amongst *Dunaliella* species could be partially or wholly attributed to cell volume, since the species which exhibit low glycerol content (sp.14, *D.salina* CCAP 19/31 and sp.18, *D.quartolecta* CCAP 19/8) are characterised by small cell volume (table 4.3). Such a relationship between intracellular glycerol content and cell volume has been observed by Ginzburg and Ginzburg (1985). The reduced variation amongst *Dunaliella* species in hide soak liquor could also be attributed to the equalising effect of this effluent on cell volume, i.e. bigger cells are reduced in size while smaller cells are enlarged in hide soak liquor. Whether the variation in glycerol content is due entirely to cell volume remains to be investigated. An alternative explanation for the inter-species glycerol variation could be the selective use of an additional osmolyte, besides glycerol, in osmoregulation.

Also apparent from figure 4.4 and figure 4.5 is the large difference in glycerol content displayed by *Dunaliella* species in the different media under study. This is further illustrated in figure 4.6 which reflects the glycerol content per cell in both defined media and hide soak liquor.



**Figure 4.6** A comparison of the intracellular glycerol content in selected *Dunaliella* species when grown in hide soak liquor and in defined inorganic growth medium (expressed per algal cell). Species are as designated in Table 4.1.

The medium-induced variation in glycerol content was not anticipated, since the glycerol content was expected to be constant at a given molarity, regardless of the external environment. The difference in glycerol content again appears to be partially correlated with cell volume. Cells which are large in defined media, such as *D.salina* CCAP 19/30 (PMB), 19/3, 19/18 and 19/30, exhibit a large variation in glycerol content between the media. This appears to be due to the fact that these species are reduced in size when grown in effluent. Cells which are small in defined media, such as *D.salina* CCAP 19/31 and *D.quartiolecta* CCAP 19/8, however, increase in size when grown in hide soak liquor and therefore exhibit very similar glycerol content between the media. The use of an alternative osmolyte could also play a role in the above variation. It is proposed that the increased cellular metabolism associated with the enhanced growth rate in hide soak liquor could lead to an accumulation of metabolites, which could function as osmolytes.

As reported in section 3.3.2, glycine appears to have a stimulatory role in glycerol excretion in *D.salina*. The high glycine content of hide soak liquor would therefore serve to induce the release of glycerol in this medium. This apparent increased glycerol release exhibited by effluent-grown *Dunaliella* cells (as depicted in table 4.4) is also considered to have had an influence on the media-induced variation in glycerol content. Significantly higher extracellular glycerol levels are

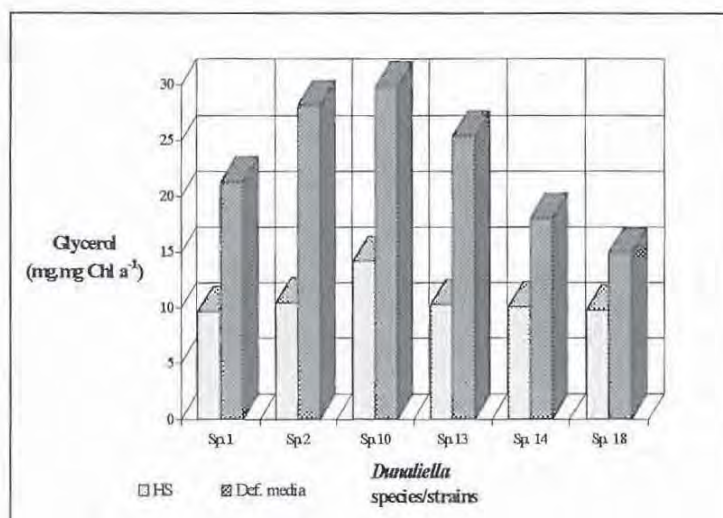
found in hide soak liquor-grown *D.salina* cultures than those cultured in inorganic growth medium. This could explain the lower intracellular glycerol levels observed in effluent-grown algal cultures.

**Table 4.4** Media-induced variation in glycerol excretion by *D.salina* species. HS refers to 20% bovine salted hide soak liquor. Def. media refers to the defined inorganic algal growth medium (1.5M) used in this study.

Time (in days)	Extracellular glycerol		Bacterial cell count	
	(pg excreted.cell <sup>-1</sup> )		(x 10 <sup>6</sup> cells.mℓ <sup>-1</sup> )	
	Def.media	HS	Def. media	HS
0	121.85	311.80	0.16	1.11
5	109.96	464.40	3.60	6.83
10	147.30	366.00	6.57	11.13

Under such conditions, an additional osmolyte would be required to maintain iso-osmotic conditions with the external environment. Also apparent from table 4.4, is the elevated bacterial count observed in hide soak liquor-grown *D.salina* cultures. This is assumed to be due to the high organic content of this medium, induced by the enhanced glycerol release observed above.

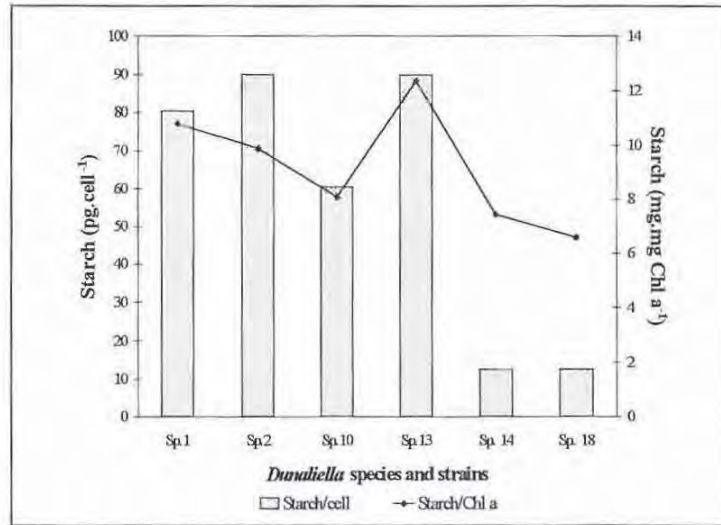
Figure 4.7 expresses the variation in glycerol content in defined medium and hide soak liquor as a function of chlorophyll a content. This reflects a similar trend to that reported above (figure 4.6), although the difference between glycerol levels is less extreme.



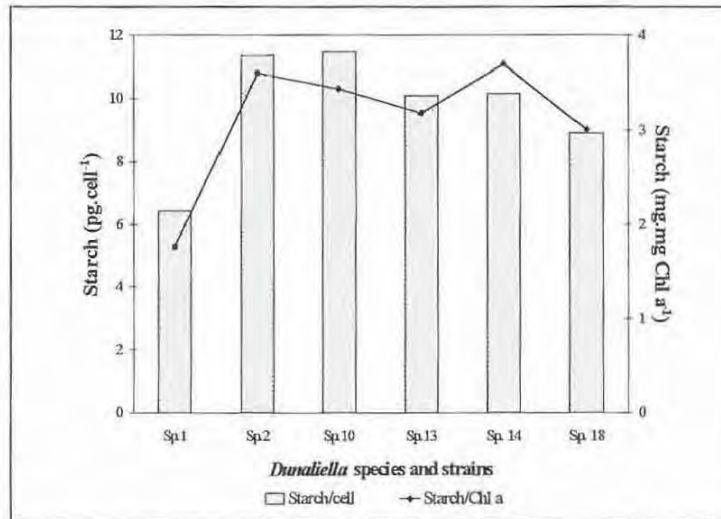
**Figure 4.7** A comparison of the intracellular glycerol content in selected *Dunaliella* species when grown in hide soak liquor and in defined inorganic growth medium (expressed as a function of the chlorophyll a content). Species are as designated in Table 4.1.

#### 4.3.3 The Screening of *Dunaliella* species for Optimal Starch Content

The starch content of the selected *Dunaliella* species was investigated during exponential growth in both defined inorganic algal growth media and hide soak liquor. The results of this are depicted graphically in figure 4.8 and figure 4.9. The starch content is in each case expressed per cell and as a function of chlorophyll a content.

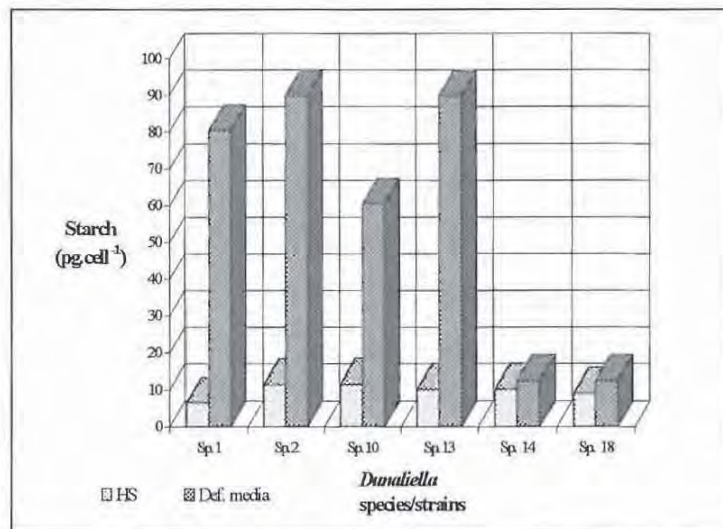


**Figure 4.8** The starch content of selected *Dunaliella* species in defined inorganic growth medium (1.5M NaCl). Experimental results represent the quadruplicate mean of exponentially growing cells. (Species are as designated in Table 4.1)

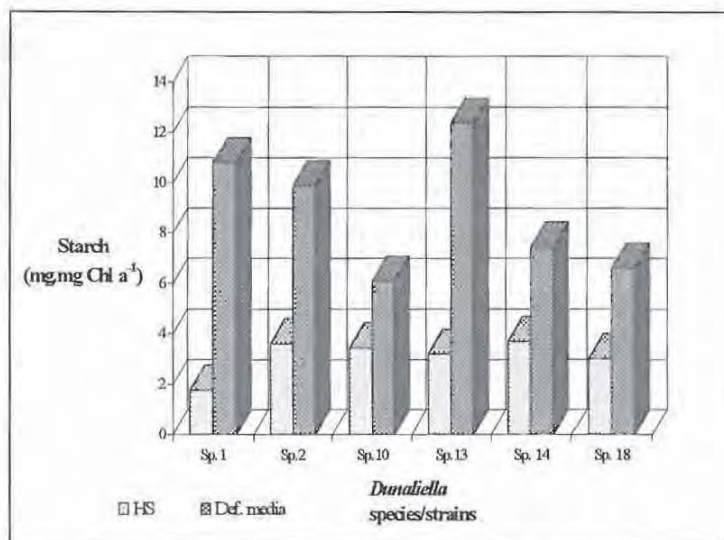


**Figure 4.9** The starch content of selected *Dunaliella* species in hide soak liquor (1.5M NaCl). Experimental results represent the quadruplicate mean of exponentially growing cells. (Species are as designated in Table 4.1).

Starch content varied among the *Dunaliella* species investigated. This variation is greatly exaggerated in defined medium and is of the same order of magnitude as that exhibited for glycerol content, encompassing a distribution of starch content values of 77.61  $\text{pg}\cdot\text{cell}^{-1}$ . This represents a standard deviation about the mean of 65%. Hide soak liquor-grown cultures, however, exhibit a distribution of 5.04  $\text{pg}\cdot\text{cell}^{-1}$ , which represents a standard deviation about the mean of 17%. The large difference in starch content displayed by *Dunaliella* species when grown in defined media and hide soak liquor is reflected in figure 4.10 (per cell) and figure 4.11 (per mg Chl a).



**Figure 4.10** A comparison of the starch reserves in selected *Dunaliella* species when grown in hide soak liquor and in defined inorganic algal growth medium (expressed per algal cell). Species are as designated in Table 4.1.



**Figure 4.11** A comparison of the starch reserves in selected *Dunaliella* species when grown in hide soak liquor and in defined inorganic algal growth medium (expressed as a function of Chlorophyll a content). Species are as designated in Table 4.1.

The variation in starch content displayed by a particular species in different media, as well as that displayed among the different *Dunaliella* species, is partially attributed to cell volume. An accumulation of starch in *Dunaliella* has been observed under nutrient limiting conditions (Preisig, 1992). The inverse could possibly also hold true, with the depletion of starch reserves under nutrient-rich conditions. This could, in part, account for the low starch content observed in *Dunaliella* cultured in nutrient-rich hide soak liquor.

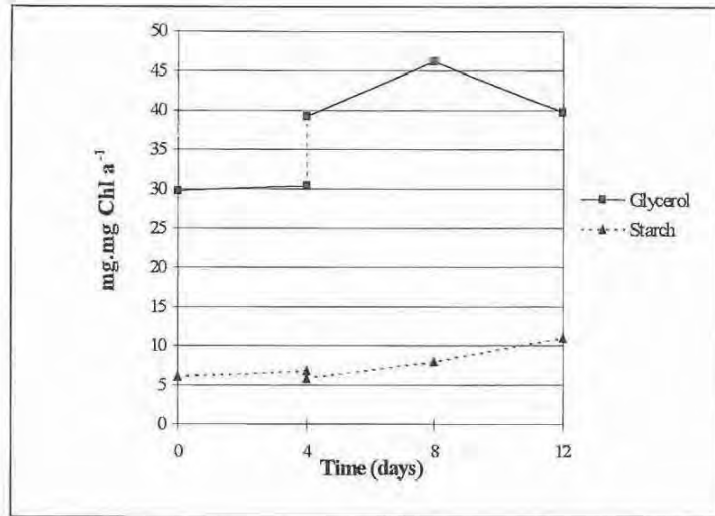
#### 4.3.4 The Screening of *Dunaliella* species for Maximum Glycerol Yield Potential

The maximum glycerol yield potential of the selected *Dunaliella* species was determined by salt stressing the 1,5M culture to 3M NaCl and monitoring the subsequent elevation of the glycerol content. Table 4.5 reflects the yield potential (established for both defined media and hide soak liquor) and expresses it per cell and as a function of chlorophyll a content.

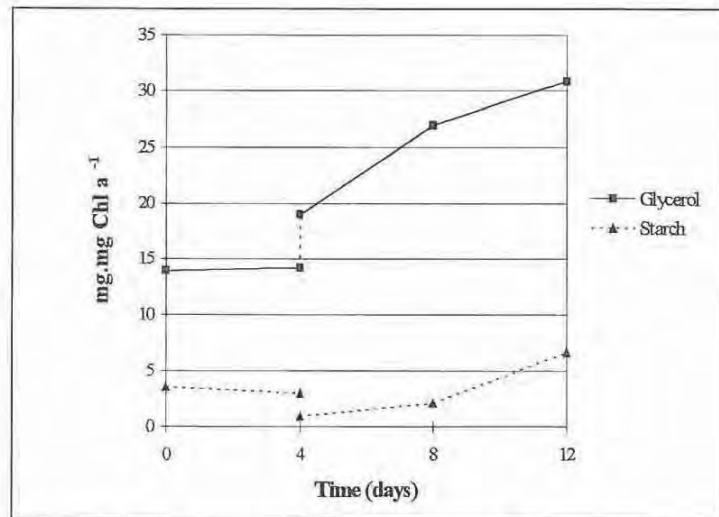
**Table 4.5** The maximum glycerol yield potential of selected *Dunaliella* species. This was performed by salt stressing the cells by the addition of salt to a final concentration of 3M NaCl. HS refers to 20% bovine salted hide soak liquor. Def. media refers to the defined inorganic algal growth media used in this study. The maximum glycerol yield potential is expressed as glycerol per cell and glycerol per mg chlorophyll a.

No.	Species	Maximum glycerol potential			
		Def. media (pg/cell)	Def. media (mg/mg Chl a)	HS (pg/cell)	HS (mg/mg Chl a)
1	<i>D.salina</i> 19/30 (PMB)	249.20	34.98	82.45	27.08
2	<i>D.salina</i> 19/3 (PMB)	299.75	36.35	93.75	25.04
10	<i>D.salina</i> CCAP 19/18	215.70	46.14	90.70	26.96
13	<i>D.salina</i> ( <i>bardawil</i> ) CCAP 19/30	283.15	44.66	60.30	22.44
14	<i>D.salina</i> CCAP 19/31	83.25	27.05	51.85	21.49
18	<i>D.quartolecta</i> CCAP 19/8	64.20	25.01	43.00	21.28

The impact of salt stress on the glycerol and starch content in *Dunaliella* species is illustrated using sp.10 (*D.salina* CCAP 19/18) which overall displayed a favourable glycerol yield potential and cell growth in hide soak liquor. The fluctuations in glycerol and starch content in defined inorganic media and hide soak liquor are represented in figure 4.12 and figure 4.13, respectively, expressed as a function of chlorophyll a.



**Figure 4.12** The impact of salt stress on a culture of *D.salina* CCAP 19/18 (sp.10) grown in defined inorganic growth medium (1,5M NaCl). The culture was stressed at t = 4 days by the addition of salt to 3M NaCl. Glycerol and starch levels are expressed in mg per mg Chlorophyll a.



**Figure 4.13** The impact of salt stress on a culture of *D.salina* CCAP 19/18 (sp.10) grown in hide soak liquor (1,5M NaCl). The culture was stressed at t = 4 days by the addition of salt to 3M NaCl. Glycerol and starch levels are expressed in mg per mg Chlorophyll a.

*Dunaliella* species utilise a dynamic interconversion between glycerol and starch, the two major carbon pools, as well as photosynthesis, to meet the osmotic requirements which are set by the

external salinity (Gimmler and Möller, 1981; Beckett *et al*, 1985; Avron, 1993). As shown in figure 4.12 and figure 4.13, salt stressing caused a flow of carbon from the starch reserve into glycerol, serving as an osmolyte in the more saline environment, enabling the intracellular glycerol content to be comparable with that of the ambient solution. This reinforces the fact that glycerol is the major component for osmoregulation in *Dunaliella* species, as previously reported by Ben-Amotz and Avron (1973) and Frank and Wegmann (1974).

A new osmotic equilibrium is reached at the new salinity (3M) which is basically double that observed at 1.5M NaCl (figure 4.4 and figure 4.5). A number of authors have reported that such an equilibrium is accomplished after approximately 90 minutes (Ben-Amotz and Avron, 1973; Borowitzka and Borowitzka, 1988). Fujii *et al* (1985) and Grizeau *et al* (1983), however, reported that this readjustment may take up to 2 hours. A further increase in glycerol content was observed in this study following that induced by the salt stress (figure 4.12 and figure 4.13). This could signify that steady-state had not yet been accomplished during the 2 hour incubation. Alternatively, this could be attributed to the accumulation of glycerol in stationary phase cells. This is in agreement with earlier observations by Jones and Galloway (1979), who showed that cells grown to stationary phase ( $\pm 11$  days) exhibit a 40% higher intracellular glycerol content than equivalent cells in exponential phase. A decrease in glycerol content was, however, observed after the eighth day of growth in defined medium. A similar trend was observed in the majority of the *Dunaliella* species investigated (when grown in defined media). This was consistent when glycerol levels were expressed per cell (results not shown).

The effect of salinity stress was far less extreme on the starch content in *Dunaliella*, as observed in figure 4.12 and figure 4.13. Starch breakdown has been reported to account for approximately 70% of the glycerol synthesis in the light following an increase in external salinity (Borowitzka and Borowitzka, 1988). A decrease larger than this was therefore expected on salt stress. This inaccuracy in starch content was attributed to the method employed in its quantification (enzymatic analysis), since the values were out of the range of sensitivity. Following the salt stress, an increase in starch content was observed over time. This accumulation of starch was assumed to be the result of nutrient limitation in the medium (Preisig, 1992) or ageing of cells (Oliveira and Huynh, 1989).

#### 4.3.5 The Quantification of Extracellular Glycerol

Glycerol release was observed in all the *Dunaliella* species under study. The extracellular glycerol concentrations of the different species were comparable and appeared to fall within the range 10 - 100 $\mu\text{g.m}^{-1}$  (0.01 - 0.1 $\text{mg.m}^{-1}$ ) (results not shown). Restrictions were, however, imposed by the method of quantification. A slight increase in extracellular glycerol was observed in the culture medium between the exponential and stationary phase samples (results not shown). This increased excretion with the onset of stationary phase has been confirmed by Huntsman (1972) and Sharp (1977). The extracellular glycerol concentrations observed in the *Dunaliella* sp. during exponential growth were very low and, although they could be detected, they could not be accurately quantitated. The given range of extracellular glycerol concentrations is therefore an estimate. A more sensitive method would be required to confirm these values.

#### 4.3.6 The Selection of an Algal Biomass

A summary of the glycerol and starch content displayed by the effluent-grown *Dunaliella* species investigated in this study is reported in table 4.6.

**Table 4.6** A summary of the intracellular glycerol- and starch content of the effluent-grown *Dunaliella* species under study. The glycerol potential was determined by salt stressing from 1.5M - 3M NaCl.

No.	Species	Glycerol				Starch	
		Intracellular Glycerol		Glycerol Potential		pg/cell	per mg Chl a
		pg/cell	per mg Chl a	pg/cell	per mg Chl a		
1	<i>D.salina</i> 19/30 (PMB)	36.043	9.625	82.450	27.080	6.424	1.763
2	<i>D.salina</i> 19/3 (PMB)	41.196	10.427	93.750	25.040	11.360	3.603
10	<i>D.salina</i> CCAP 19/18	53.653	14.167	90.700	26.960	11.468	3.435
13	<i>D.salina (bardawil)</i> CCAP 19/30	21.041	10.239	60.300	22.440	10.098	3.181
14	<i>D.salina</i> CCAP 19/31	28.791	10.067	51.850	21.490	10.152	3.700
18	<i>D.quartolecta</i> CCAP 19/8	26.856	9.729	43.000	21.280	8.918	3.010

*Dunaliella salina* CCAP 19/18 (sp.10) was selected as the algal biomass to be used as a substrate in the linked fermentation production of a high value product. This species appeared to display the greatest yield of potentially fermentable carbon from all the *Dunaliella* species under study. Although not optimal in defined media, this species displayed favourable yields in hide soak liquor, in which the algae are proposed to be cultured. This species exhibits a high cell yield

(in hide soak liquor) as well as a large cell volume, resulting in maximal glycerol yields. The carotenogenic nature of this particular *Dunaliella* strain increased its attractiveness as the species of choice in linked fermentation, since this would enable the recovery of the high value by-product,  $\beta$ -carotene.

## 4.4 DISCUSSION

### 4.4.1 Tannery Effluent as a Culture Medium

According to Dubinsky *et al* (1980), carbon, nitrogen and phosphorous are the elements most commonly in short supply and soon become growth limiting in dense algal cultures. These nutrients, as well as others, can be supplied to the algae in wastewater, thus avoiding a dependence upon costly substrates. The culture of *Dunaliella* in saline tannery effluents would therefore provide a saving in the cost of the medium. In such systems, heterotrophic bacteria release nutrients, such as ammonia from the effluent, which are then utilised by the algae and the photosynthetic oxygen evolution by the algae accelerates the bacterial mineralisation process. The algae have also been shown to directly contribute to the reduction in the organic load of the wastewater by the uptake of organic compounds (Abeliovich and Weismann, 1978).

*Dunaliella* species have been reported to utilise an extensive range of organic substrates. This ability is however inducible (Flynn and Butler, 1986) and species specific (Williams *et al*, 1978), which could explain the lack of growth in hide soak liquor exhibited by some *Dunaliella* species. According to Flynn and Butler (1986), some *Dunaliella* species require a period of incubation in an organic medium during which time transport and assimilatory systems are presumed to be induced. It is assumed that those *Dunaliella* species which readily adapted to growth in hide soak liquor could easily initiate amino acid uptake systems or deaminase activity. Those that required a gradual adaptation may have required such an incubation to initiate the pathways necessary for the uptake of organics, in order to enable growth in this organic effluent. The species of *Dunaliella* which were unable to grow in hide soak liquor appeared to be deficient in this ability and are therefore purely photoautotrophic in character. *Dunaliella* species which were unable to grow in hide soak liquor did not lyse immediately on transfer to this effluent. The

lysis, which was observed after 2-3 days, was probably due to nutrient starvation in the organic-rich media.

The enhanced growth rates and cell yields observed in this study have been reported by a number of authors (Abeliovich and Weisman, 1978; Laubscher, 1991; Rose, 1992). Dubinsky *et al* (1978) reported that algal yields of  $40\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$  have repeatedly been obtained in experimental oxidation ponds. Such high production rates have been partially attributed to heterotrophic growth (Abeliovich and Weisman, 1978).

#### 4.4.2 A Comparison of Intracellular Glycerol Levels

A search of the available literature has indicated a vast variation in the intracellular glycerol values for given *Dunaliella* species. The values reported in this study are, however, in general agreement with those observed by Zidan *et al* (1987) and Ben-Amotz (1987).

In this study, intracellular glycerol values were expressed per cell and as a function of chlorophyll content. Both of these, however, vary depending on the growth conditions of the study, such as temperature (which leads to an increased growth rate, decreasing cell size and cell contents) (Jones and Galloway, 1979).

Chlorophyll content is reported to vary significantly with nitrogen concentration, light intensity, temperature and salinity (Ginzburg and Ginzburg, 1985; Jiménez and Niell, 1991; Aizawa and Miyachi, 1992). Intracellular glycerol content is also a function of the variation in any of the environmental or experimental conditions. Ben-Amotz and Avron (1973) reported enhanced intracellular glycerol levels in *Dunaliella* cultures sparged with  $\text{CO}_2$ . An increase in glycerol content with cellular ageing has also been observed in *Dunaliella* species (Jones and Galloway, 1979; Jiménez and Niell, 1991). Reduced intracellular glycerol levels have, however, been observed when cultured under high light intensity or under blue light (Jones and Galloway, 1979).

Since the glycerol content of *Dunaliella* is a function of the cell volume, and bearing in mind that both cell volume and chlorophyll levels vary greatly, it is imperative to determine the intracellular glycerol content for cells grown and harvested under identical conditions. Only then can an accurate comparison of intracellular glycerol concentration be achieved.

The misclassification of several *Dunaliella* species (Borowitzka and Borowitzka, 1988; Preisig, 1992) further compounds the difficulty of a comparison of reported glycerol levels.

#### **4.4.3 The Effect of Hide Soak Liquor on Intracellular and Extracellular Glycerol levels**

The reduced intracellular glycerol levels observed in effluent-grown *D.salina* cultures (compared to those grown in defined media) were partially attributed to increased glycerol excretion (as suggested by the significantly higher extracellular glycerol concentrations observed in hide soak liquor). The high concentration of glycine in the hide soak liquor may have induced glycerol release. Alternatively, or in addition, the larger population of bacteria observed in the effluent (as compared to the control) could have resulted in the degradation of the algal cell membrane. Oliveira *et al* (1980) identified an active dispersal of *Dunaliella* cell coat material (which was especially evident in bacteria-contaminated cultures) and suggested this to be a protective cleaning function which removes contaminants from the cell surface. It is possible that above a certain threshold of bacteria, this protective function is no longer effective, resulting in damage to the algal cell membrane and the release of the intracellular glycerol. The fact that hide soak liquor exhibited enhanced algal yields, however, suggests that, if such a phenomenon was responsible for glycerol release, the damage would only be transient.

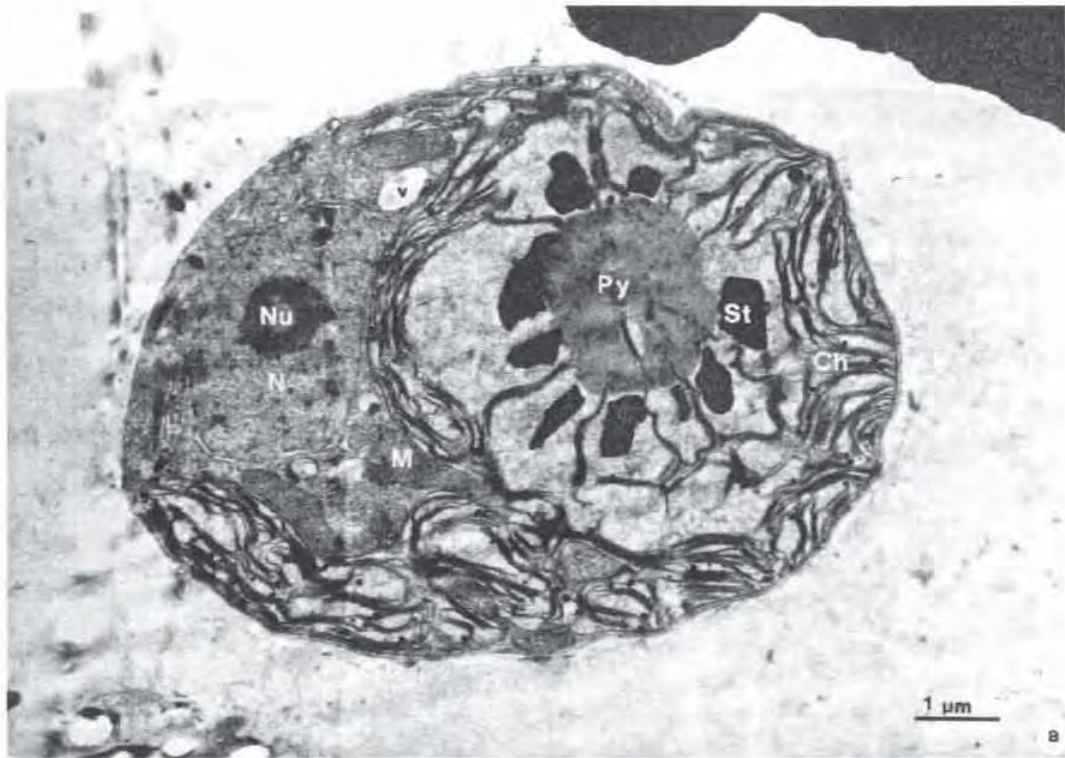
#### **4.4.4 An Ultrastructural Study as an Explanation**

An ultrastructural study of effluent-grown *Dunaliella* species could serve to explain the reduced intracellular glycerol levels (or the elevated extracellular glycerol levels) observed in this medium. On comparing the ultrastructure of hide soak liquor-grown *D.salina* with those cultured in defined inorganic growth media, Rose (1992) reported that growth in effluent was associated with the extensive accumulation of multi-component vacuoles in the cytoplasm and a proliferation of the dictyosome/endoplasmic reticulum apparatus. Rose (1992) suggested that this signified the presence of an uptake and release process. The cell surface of effluent-grown cells is also reported to have many pit-like invaginations of the cytoplasmic membrane (when compared to that of the control cells) which are often associated with small vesicles massed beneath it (Dunn, 1991; Rose, 1992). This is assumed to indicate the release of organic matter, such as glycerol, by an exocytotic extrusion process (described by Rose, 1992) whereby assimilated vacuolar

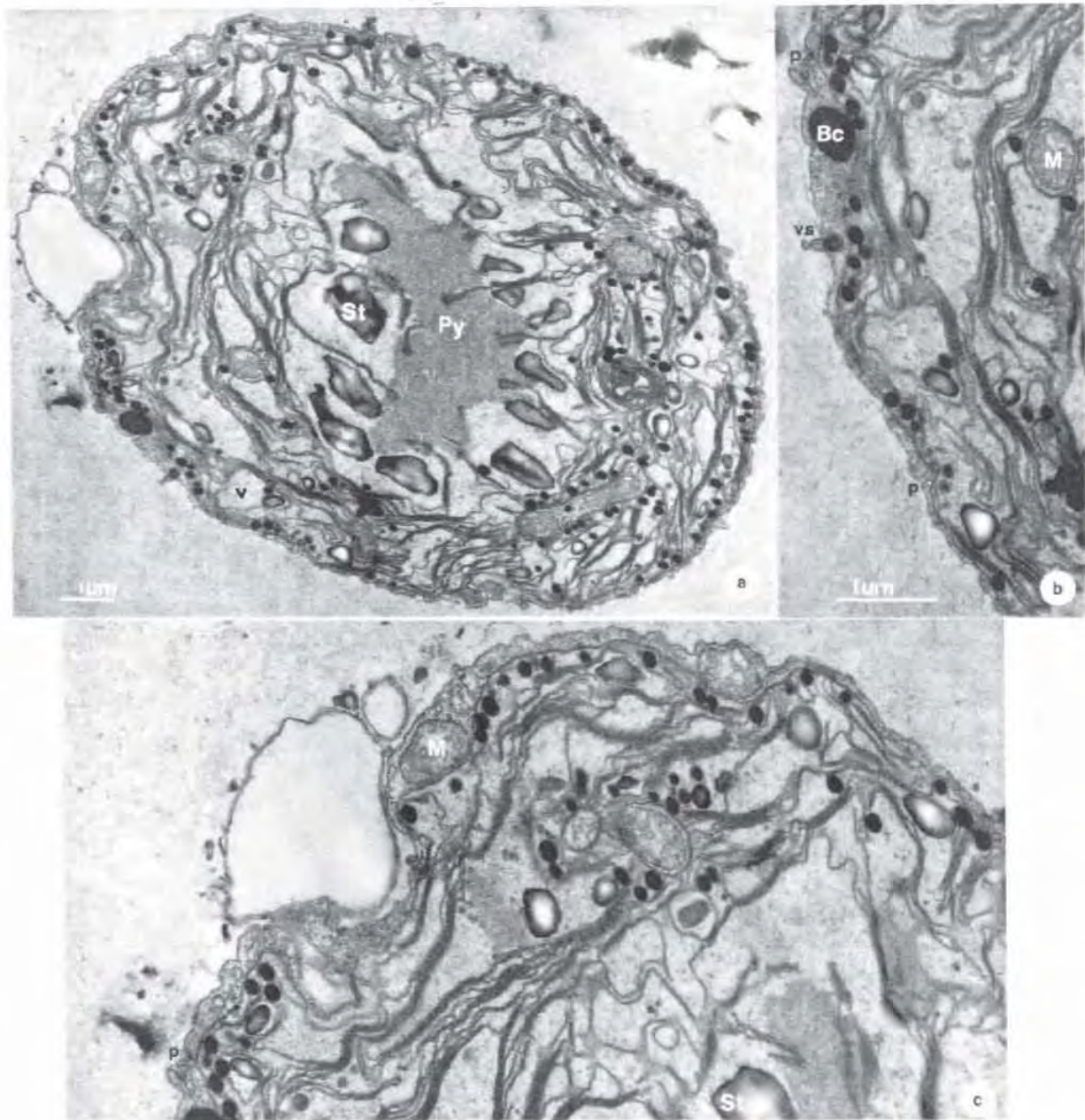
contents are released to the cell's external environment. These extrusion structures, which are more abundant when cultured in hide soak liquor, are assumed to account for the substantial release of glycerol that has been observed in this effluent.

Plate III and IV illustrate the ultrastructural differences between effluent-grown and control *D.salina* cells. Plate III represents cells grown in defined inorganic media. Plate IV, which represents cells cultured in hide soak liquor, clearly illustrates the increased vacuolar content as well as the pit-like invaginations observed in *D.salina* cells. Plate IV also clearly illustrates the exocytotic extrusion process by which glycerol is proposed to be released.

Ultrastructural studies could also serve to explain the reduced starch levels observed in effluent-grown *Dunaliella* cells. This can be deduced from the observations of Dunn (1991) and Rose (1992) who reported a reduced pyrenoid area in this medium, with a disturbance in the neat circular arrangement of starch grains surrounding the pyrenoid (which is observed in the control cells). This is also illustrated in Plate IV.



**Plate III** An Electronmicrograph of *D. salina* cultured in defined inorganic growth medium (1.5M NaCl).  
Magnification = 7 200 (From Dunn, 1991).



**Plate IV** An Electronmicrograph of *D.salina* cultured in hide soak liquor - Tannery effluent (1.5M NaCl). Magnification A = 5 800; B = 10 000; C = 10 000. Bc =  $\beta$ -carotene; M = mitochondria; p = pit; Py = pyrenoid; St = starch granules; v = vacuole; vs = vesicle. (From Dunn, 1991)

#### 4.4.5 Additional Osmolytes in Osmoregulation

The use of an additional osmolyte (besides glycerol) could serve to explain the osmoregulatory mechanisms behind the reduced intracellular glycerol levels observed in hide soak liquor-grown *Dunaliella* species. Under such conditions of reduced glycerol, the algal cell would otherwise not survive these saline environments. It is proposed that the increased cellular metabolism associated with the enhanced growth in hide soak liquor could lead to an accumulation of metabolites, which could function as osmolytes.

A number of authors have reported on the role of  $\text{Na}^+$  (Ehrenfield and Cousin, 1984; Ginzburg and Ginzburg, 1985),  $\text{K}^+$  (Ehrenfield and Cousin, 1984; Ginzburg and Ginzburg, 1985; Avron, 1986),  $\text{Cl}^-$  and  $\text{Mg}^{2+}$  (Ginzburg and Ginzburg, 1985) and Phosphate (Ginzburg and Ginzburg, 1985; Avron, 1986) in the osmotic adaptation of *Dunaliella*. Ehrenfield and Cousin (1984) described a  $\text{Na}^+/\text{K}^+$  exchange mechanism which helped to regulate the ionic composition in *Dunaliella* over a wide range of salinities. This involved a  $\text{Na}^+$  influx, which served as a temporary osmolyte prior to the accumulation of glycerol. Weiss and Pick (1990), however, refuted the argument that this transient  $\text{Na}^+$  influx served an osmoregulatory function.

Ginzburg and Ginzburg (1985) reported that the relative concentration of intracellular glycerol and  $\text{Na}^+$  varied according to the cell volume, where smaller cells contained more glycerol and less  $\text{Na}^+$  than larger cells. This could justify the interspecies variation in glycerol content observed at a constant molarity. The selective use of an additional osmolyte could therefore also explain the extensive variation observed among the *Dunaliella* species in a given medium in this study.

#### 4.4.6 Salt Relations

Besides illustrating the flow of carbon between the starch and glycerol pools in *D.salina*, salt stressing illustrated the strong positive relationship between extracellular NaCl concentration and final intracellular glycerol concentration where, as expected, the glycerol levels increased with increased medium salinity. This concept has been applied in algal-glycerol production systems. Due to the different optimal NaCl concentrations required for the production of biomass (1.5M) versus the production of intracellular glycerol (3 - 4M), a 2-stage cultivation system was

proposed (as mentioned previously) by Avron and Ben-Amotz (1978) and Chen and Chi (1981) for large scale algal-glycerol production. The algae were therefore first cultured at 1.5M NaCl to obtain a maximum biomass yield and subsequently transferred to a medium of at least 3M NaCl to favour maximum glycerol accumulation. Section 3.3.1 and 3.4.1 gives an indication of the effect of salt stressing on the primary photochemistry of photosynthesis, as established by Fluorescence Quenching Studies.

#### **4.4.7 Glycerol Release and its Function in HROP**

There is contradictory evidence in the literature regarding the release of organic compounds by *Dunaliella* (section 1.2.4; 1.6.3 and 2.1). An understanding of glycerol release is, however, essential to the efficient operation of a *Dunaliella*-based HROP process for the treatment of saline tannery effluents.

Contrary to the observations of several authors (Ben-Amotz, 1975; Borowitzka *et al*, 1977; Borowitzka and Borowitzka, 1988; Goyal, 1989), glycerol release was observed throughout growth in the *Dunaliella* species under study (at 1.5M NaCl). These findings are, however, in agreement with those of Craigie and McLachlan (1964), Hellebust (1965), Jones and Galloway (1979) and Enhuber and Gimmler (1980), who reported a continuous diffusion of glycerol along the concentration gradient between the cells and the medium.

The quantification of the extracellular glycerol levels was of interest due to the importance of glycerol release to the heterotrophic bacteria living in association with *Dunaliella* species in the saline waters. As reported by numerous authors (Hellebust, 1965; Sharp, 1977; Ben-Amotz and Avron, 1989; Rose, 1992; Oren, 1993; Giordano *et al*, 1994), an association was observed in this study between glycerol leakage in *D.salina* and bacterial number. The hide soak liquor-grown *D.salina* cultures (which exhibited enhanced glycerol leakage) supported a bacterial count approximately 2-fold higher than that observed in defined media. It is therefore assumed that the increased organic load in the media, brought about by glycerol leakage, stimulated the residing heterotrophs. It is, however, possible that the bacteria could then in turn have further stimulated algal glycerol release by causing transient damage to the algal cell membrane (as described in 4.4.3). Due to the function of heterotrophs in the wastewater treatment process, the advantages of glycerol release by *Dunaliella* species are obvious. The benefit of glycerol release is, however,

increased in the treatment of saline tannery effluents. The proteinaceous nature of this effluent results in a low organic carbon content (low C:N ratio), causing a suppression of bacterial activity. Rose (1992) proposed that this photosynthate release, together with the other organics reported to be excreted by *Dunaliella*, would elevate the C:N ratio, thereby aiding the residing heterotrophs in the treatment of this saline refractory organic waste.

#### 4.4.8 The Selection of an Algal Biomass

*D.salina* CCAP 19/18 (sp.10) was selected as the algal biomass to be used as a potential substrate for subsequent bacterial fermentations, since it appeared to exhibit the greatest yield of potentially fermentable carbon when grown in hide soak liquor (the media in which the algae are proposed to be cultured). Nakas *et al* (1983), in a similar study, also selected the biomass of a variant of *D.salina* (*D.bardawil*) for the linked fermentation production of solvents from algal biomass, since it was reported to serve as the best unamended substrate.

*D.salina* (and strain CCAP 19/18 in particular) displayed a good tolerance of environmental conditions and adapted readily to changes in effluent strength and salinity. Borowitzka and Borowitzka (1988) reported that the productivity of *D.salina* was better than that of most other algae. This was observed to some extent in this study, since five of the six effluent-grown *Dunaliella* species chosen for optimal productivity were *D.salina* strains. This effect was, however, not as obvious when cultured in defined media. The high productivities exhibited by *D.salina* have also been observed by Ben-Amotz (1980), who reported yields of 60g dry weight.m<sup>-2</sup>.day<sup>-1</sup>, at least for short periods. This illustrates the great potential of this species.

*Dunaliella salina* species, in general, also yielded high intracellular glycerol concentrations in defined media, relative to those of the other *Dunaliella* species under study. This comparison of *D.salina* species with other *Dunaliella* species (besides *D.quartolecta*) is, however, not shown. The higher glycerol levels are mostly attributed to their large size (which reflects increased glycerol concentrations per cell volume). Effluent-grown *D.salina* species (which exhibited comparable cell volumes to those of other species), however, still yielded higher intracellular glycerol concentrations.

The variation in the intracellular glycerol content observed amongst the *Dunaliella* species in this study was of sufficient significance to necessitate the maintenance of a pure culture of the selected species, *D.salina* CCAP 19/18. The glycerol contents of the other *D.salina* strains were, however, almost comparable. Contamination of the selected culture with another *D.salina* strain under outdoor conditions would therefore appear to be acceptable, especially if it is also a highly carotenogenic strain, e.g. *D.salina (bardawil)* CCAP 19/30, which would also enable recovery of a valuable by-product.

## CHAPTER 5

### GENERAL DISCUSSION

Although research into algal mass cultivation systems in South Africa is still in its infancy, Toerien and Grobelaar (1980) have reported that sufficient evidence has been obtained to indicate that considerable potential exists for commercial exploitation. Ben-Amotz and Avron (1989) have described the unicellular algae, *Dunaliella spp.* as being the most successful micro-algae described for outdoor cultivation. They are able to thrive in media containing very high salt concentrations, accumulating large amounts of commercially important chemicals, such as  $\beta$ -carotene and glycerol (Borowitzka and Borowitzka, 1988; Ben-Amotz, 1993). Their success is also attributed to their ability to adjust to a changing environment, where, besides being able to withstand extreme fluctuations in the salinity of its growth medium (Borowitzka and Borowitzka, 1988; Avron, 1992), it displays a wide tolerance to other environmental conditions, such as pH (Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989) and temperature (Ben-Amotz, 1980; Borowitzka and Borowitzka, 1988).

There are a multitude of potential applications for micro-algae, including single-cell protein production (Goldman, 1980; Ben-Amotz and Avron, 1983b), wastewater treatment (Abeliovich, 1986; Richmond, 1986; Oswald, 1988b), aquaculture (Ben-Amotz and Avron, 1983b; DePauw *et al.*, 1984), chemical extraction (Benemann, 1989; Ben-Amotz, 1993) and bioconversion of energy (Calvin and Taylor, 1989; Benemann, 1989). Moreover, there is demonstrated potential for combining several of these applications into one overall process (Goldman, 1980).

In recent years, much attention has focused on HROPs as both an economic means for wastewater treatment and as a substrate for the mass production of algae, where protein could serve as animal fodder (Soeder, 1986). These systems therefore have great potential for the combined water and nutrient recycling via the recovery of microbial biomass.

Growth of algae on tannery effluent converts a problematic waste product into a potentially valuable resource. Nutrients are supplied to the algae in wastewater, thereby avoiding a

dependence upon costly substrates. The culture of *Dunaliella spp.* on tannery effluent would therefore provide a savings in the cost of the medium. Appropriately diluted hide soak liquor proved to be highly suitable as an algal growth medium for *Dunaliella spp.* and in some instances resulted in enhanced growth rates and higher biomass yields, indicating its suitability as an alternative growth medium in the cultivation of *Dunaliella*. Most species exhibited a 2-fold enhancement of growth in this effluent. The remaining species, however, appeared to display slightly retarded or no growth in this medium. It is postulated that the inability of some *Dunaliella* species to grow in this hide soak liquor is correlated with an inability to utilise organic compounds present in the medium. This is in agreement with Williams *et al* (1978), who described the uptake of organics in *Dunaliella* as being species-specific.

Glycine, a major component of hide soak liquor, is reported to be responsible for the observed stimulatory effect on *Dunaliella* growth (Rose, 1992). The nature of the effect of glycine on the growth of *Dunaliella* was thus investigated in this study. A fluorescence quenching study revealed that glycine addition induces a sudden demand for reducing power supplied by photosystem II. The possibility of this energy being required in the active uptake of glycine (as a nitrogen source) was thus investigated by performing a glycine binding study, where transient binding was thought necessary prior to uptake. This study, however, tentatively refuted this possibility and suggested the role of bacteria in this regard. An algal-bacterial interrelationship was proposed as being responsible for the deamination of glycine, where the bacteria appear to undertake the mineralisation of this amino acid and release the free ammonia. This, in turn, is available for the energy-dependent uptake by the algae as a nitrogen-source for growth. The glycine-induced quenching of fluorescence observed in this work therefore appears to represent the sudden demand for NADH and ATP required by the GS-GOGAT ammonia assimilation system, the primary pathway for ammonia assimilation in algae. This work therefore appeared to dispute the presence of an algal-deaminase in this particular *Dunaliella* species, *D.salina*. Due to the apparent absence of an extracellular deaminase in *Dunaliella*, the uptake of glycine, which was observed by Rose (1992) and others, could have occurred through pinocytosis.

In spite of numerous reports concerning the tight metabolic control over intracellular glycerol concentration in *Dunaliella spp.* (Borowitzka *et al*, 1977; Borowitzka and Borowitzka, 1988; Goyal, 1989), the high extracellular glycerol levels observed in this study indicated that a large amount of this glycerol is excreted at a given salinity, particularly when grown in hide soak liquor. Since glycine was shown to have a stimulatory role in glycerol excretion by *D.salina*, it is assumed that the high glycine content of hide soak liquor induced glycerol excretion, which

was particularly prevalent in effluent-grown *Dunaliella* cultures. The presence of short chain carboxylic acids, resident in the hide soak liquor, or the elevated bacterial population characteristic of this effluent, may also have contributed to the enhanced glycerol release observed in effluent-grown *Dunaliella* cultures. Ultrastructural studies, performed by Dunn (1991) and Rose (1992), have enabled the identification of morphological differences displayed by effluent-grown *Dunaliella* cells, which suggest the release of organic matter (such as glycerol) by exocytosis or leakage. The observed extrusion structures, which were more abundant in effluent-grown *Dunaliella* cells, are assumed to account for the substantial release of glycerol that has been observed in this effluent.

The demonstration that glycine has a stimulatory effect on glycerol production indicates an important role for the algal-bacterial association in the functioning of a *Dunaliella*-based HROP system. The heterotrophic nature of bacteria does not permit their proliferation unless the medium contains an organic load of sufficient quantity. Since the proteinaceous nature of the hide soak liquor tannery effluent results in a low organic carbon content (low C:N ratio), this limits bacterial activity. The release of glycerol by *Dunaliella spp.*, however, serves to elevate the C:N ratio (by increasing the organic load), thereby aiding the residing bacteria in the treatment of this saline refractory organic waste. This positive correlation between bacterial number and organic compound accumulation, as observed by Sharp (1977), has been substantiated by the findings of this study, and serves to confirm the function of glycerol release in facilitating wastewater treatment.

Due to the large differences observed between the intracellular glycerol contents of effluent-grown and control *Dunaliella* cells, it is apparent that glycerol productivities are a function of the growth medium, and of the organic constituents of the medium in particular. The greatly reduced intracellular glycerol content observed in effluent-grown *Dunaliella* species was therefore postulated to be attributable to significantly enhanced glycerol excretion, where the glycine content of the hide soak liquor is assumed to have contributed to the movement of intracellular glycerol through the *Dunaliella* cell membrane. This results in the observed medium-induced variation in intracellular glycerol content. This phenomenon is contrary to the expected results, since the intracellular glycerol content related to osmoregulation functions may be expected to remain constant at a given salinity, regardless of the external environment. A similar medium-induced phenomenon was observed in the starch content of *Dunaliella* cells. Lower carbon-fixation rates (into glycerol and starch), due to the utilisation of organic compounds (amino acids) in the protein-rich hide soak liquor, could also have attributed to the

reduced intracellular glycerol content. Medium composition is therefore important in the consideration of function. Hide soak liquor offers a useful medium since it enables the cultivation of *Dunaliella sp.*, where the glycine content in turn promotes glycerol release by these species, and thus facilitates the function of HROPs, by stimulating bacterial growth. If the production of a glycerol-rich algal biomass had been the primary objective of this study, then hide soak liquor would not have been the ideal culture medium (since the organic content of this effluent would have resulted in reduced intracellular glycerol content, thereby reducing its potential as a growth medium) but, since the algal-biomass is a by-product of the wastewater treatment process, it is an acceptable outcome.

The study has confirmed previous findings relating the effects of glycine in tannery effluent to the enhanced growth of *Dunaliella spp.* in this medium. It has furthermore elucidated aspects of the mechanism regulating glycerol release and the contribution of this mechanism to the operation and control of High Rate Oxidation Ponds used for the treatment of hypersaline organic effluents.

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