STRESS MANIPULATION IN *DUNALIELLA SALINA* AND DUAL-STAGE ß-CAROTENE PRODUCTION

THESIS

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CONTENTS

ABSTRACT

The alga *Dunaliella salina* accumulates large quantities of ß-carotene in response to certain environmental and physiological stresses. This hyper-accumulation process has been commercially exploited. However, the currently employed averaging or single-stage process produces ß-carotene yields well below the genetic potential of the organism due to the inverse relationship between growth and secondary metabolite production. A dual-stage process, which separates the distinctive growth and secondary metabolite production stages of the alga, has been proposed. The broad aim of the research programme was to evaluate the practicality, scale-up and economic viability of a dual-stage ß-carotene production process from *D. salina*.

Preliminary laboratory studies showed that although stress factors such as high salinity and a range of nutrient limitations enhance ß-carotene accumulation in *D. salina*, high light intensity is the single most important factor inducing ß-carotene hyper-accumulation in the alga. Furthermore, the preliminary studies indicated that ß-carotene production could be successfully manipulated by the imposition of stress.

The stress response of *D. salina* to high light stress was examined at a fundamental level. The relative partitioning of ß-carotene between thylakoid membrane and interthylakoid globular ß-carotene has revealed two responses to high light stress. The first is a response in which the alga adapts to the photoinhibitory effects of high light stress by the rapid accumulation and the peripheral localisation of ß-carotene to the outer extremities of the chloroplast. This is followed by a maintenance response which is characterised by the recovery of the photosynthetic rate and cell growth. A possible interrelationship between the extent of the photoinhibitory response and the amount of ß-carotene hyper-accumulation has been noted.

An outdoor evaluation of the growth stage of the dual-stage system has demonstrated that *D. salina* can be grown in a relatively low salinity, nutrient sufficient medium for extended periods without overgrowth by small non-carotenogenic *Dunaliella* species. In addition, biomass productivities of three times greater than those obtained in the currently employed averaging system were achieved.

The role of high light intensity in ß-carotene hyper-accumulation was confirmed in outdoor scale-up stress pond studies. The studies demonstrated the feasibility of stress induced ß-carotene production in outdoor cultures of *D. salina* and ß-carotene yields three times greater than those obtained in the currently employed averaging process were achieved.

The dual-stage process imposes the specific requirement of viable cell separation on the harvesting system employed. A flocculation-flotation process and an air-displacement crossflow ultrafiltration system were developed and successfully evaluated for the separation of *D. salina* from the brine solution in a viable form.

The extraction of ß-carotene from *D. salina* was evaluated. Supercritical fluid extraction studies showed that the use of a co-solvent mixture of carbon dioxide and propane could effectively reduce the high extraction pressures associated with supercritical carbon dioxide extraction. In addition, a novel hydrophobic membrane assisted hot oil extraction process was developed which separates the complex oil-water emulsions produced during hot oil extraction of ß-carotene from wet *D. salina* biomass.

Process design and economic evaluation studies were undertaken and showed that the economics of the dual-stage process offer significant advantages over the currently employed averaging process.

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To my parents, thank you both for believing in me. Your faith has been vindicated.

This thesis is dedicated to my wonderful wife Lesley who's love and patience has been a constant source of strength.

FIGURES

TABLES

ABBREVIATIONS

ABA - abscisic acid

ATCC - American Type Culture Collection

Bq - Becquerel

CCAP - Culture Collection for Algae and Protozoa

CFF - cross-flow filtration

CFMF - cross-flow microfiltration

CFUF - cross-flow ultrafiltration

EM - electron microscope

HPLC - high performance liquid chromatography

IRR - Internal Rate of Return

LMH - litres per square meter per hour

NPV - Net Present Value

UOFS - University of the Orange Free State

PAC - polyaluminium chloride

PFD - photon flux density

ROI - Return on Investment

rpm - revolutions per minute

TEM - transmission electron microscopy

10E - exponential

CHAPTER 1

INTRODUCTION

ALGAL BIOTECHNOLOGY AND THE COMMERCIAL PRODUCTION OF ß-CAROTENE FROM *DUNALIELLA SALINA*

Summary.

The role of the alga *Dunaliella salina* in the development of algal biotechnology is noted together with its unique ability to accumulate large quantities of ß-carotene. The limitations of existing *D. salina* cultivation practices are discussed and have led to the proposal of a dual-stage process for the separate optimization of cell growth and metabolite accumulation phases of production. The feasibility and economic viability of the implementation of a dual-stage *D. salina* ß-carotene production process forms the basis of this investigation.

1.1 INTRODUCTION.

1.1.1 Historical perspective.

Algal biotechnology is a relatively new field of biotechnology and its roots can be traced to work carried out in the late 1940s by several groups in the USA and other countries (Burlew, 1953). Primary interest was focused on producing single cell protein from the freshwater green alga *Chlorella* (Burlew, 1953; Tamiya, 1957). However, the relatively high cost of producing algae as a protein source, compared to less expensive protein sources such as soybean meal, was further complicated by uncertainty regarding the acceptability of algal meal in the human diet (Benemann, 1989). Since then, many other potential applications for large-scale algal culture have been advanced, including the production of alternative food sources (Soeder and Binsack, 1978), the production of fine chemicals (Aaronson *et al.* 1980), aquaculture (Persoon and Claus, 1980), life support systems (Oswald *et al.* 1965), wastewater treatment (Oswald and Golueke, 1968) and health promoting algal preparations (Kawaguchi, 1980).

Much of the current production of microalgae has been generated for use as health foods. Production for the health food market has however, shown swings in consumer demand, making this a very high risk commercial activity (Benemann, 1989). Two high value, speciality products, which are not health foods, are currently being produced in significant amounts from microalgae: "linablue", a phycobiliprotein concentrate from *Spirulina*, which is used as a colouring agent for foods such as ice-cream and yoghurt; and ß-carotene produced from *Dunaliella*, which is used as a food colorant, pro-vitamin A and therapeutic anti-oxidant (Benemann, 1989; Ben-Amotz and Avron, 1989). Natural ß-carotene commands a market price of more than double that of synthetically produced ß-carotene (Ben-Amotz and Avron, 1990). Furthermore, clinical and experimental studies have shown that ß-carotene may be effective as an anticancer agent, particularly the naturally derived ß-carotene because of increased biological activity (Peto *et al.* 1981; Shklar and Schwartz, 1988; Ben-Amotz *et al.* 1989a; Nagasawa *et al.* 1989a; Nagasawa *et al.* 1989b). The increasing demand for natural food colouring agents and the recent findings that only natural ß-carotene, containing both *cis*- and *trans*-isomers, is likely to be effective as an anticancer agent, provide a bright future for both products (Benemann, 1989).

Although the potential of ß-carotene production from *Dunaliella* was first recognised by the Russian researchers in the early 1960s (Musyuk, 1966, cited in Moulton *et al.* 1987), the commercial production of ß-carotene from *Dunaliella* was only developed over the last decade (Moulton *et al.* 1987, Schlipalius, 1991). Much of the preliminary studies were done by groups in Israel (Ben-

Amotz and Avron, 1980a; Ginzburg and Ginzburg, 1985) and Australia (Borowitzka and Brown, 1974; Borowitzka, *et al.* 1977) and to date there are at least five companies actively engaged in cultivating *Dunaliella* for commercial purposes.

1.1.2 The alga *Dunaliella*.

The unicellular green algae of the genus *Dunaliella* belong to the class Chlorophyceae and the order Volvocales, of which there are several ill-defined species (Ben-Amotz and Avron, 1989). Members of the genus *Dunaliella* are all ovoid biflagelates with cell volumes in the range of 50-1000 μm^3. *Dunaliella* posses cellular organelles typical of other green algae. The chloroplast is cup-shaped with a single pyrenoid embedded in the basal portion. Unlike other algae, *Dunaliella* is characterised by the lack of a rigid cell wall and the cell is enclosed by a thin elastic plasma membrane covered by a mucus "surface coat" or glycocalix (Ben-Amotz and Avron, 1989).

Despite comprehensive taxonomic studies of the genus, many of the strains in culture collections are misnamed, and some unnecessary species names have arisen (Borowitzka and Borowitzka, 1988). An example of this is *D. bardawil* (Ben-Amotz and Avron, 1983). This strain was originally isolated from a salt pond near Bardawil Lagoon, North Sinai, by Ben-Amotz and Avron in 1976. The strain was identified by them as being a high ß-carotene producing strain and was subsequently patented (US Patent #4,199,895) and deposited with the American Type Culture Collection (ATCC 30861). Borowitzka and Borowitzka (1988) maintain that *D. bardawil* is a *nomen nudum* and is actually a strain of *D. salina* Toed, as are all strains that appear red due to ß-carotene hyper-accumulation.

Vegetative reproduction is by lengthwise division in the motile state (Lerche, 1937; Ben-Amotz, 1980) and under specific conditions the cells may form palmellae with a thick layer of mucilage or rarely cysts with a thick, rough wall (Borowitzka and Borowitzka, 1988). Cysts are formed in response to extreme conditions, such as dehydration. Sexual reproduction is by isogamy and the zygotes produced are surrounded by a thick, smooth wall. After a resting stage the zygote nucleus divides, forming up to 32 cells, which are released through the ruptured wall of the mother cell (Lerche, 1937).

Dunaliella is widely distributed, and is found in many aquatic marine habitats, such as oceans, brine lakes, salt marshes and salt water ditches near the sea (Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1990). *Dunaliella* is a minor component of the marine phytoplankton (Borowitzka and Borowitzka, 1988), however, in hypersaline water bodies *Dunaliella* is often the major primary producer (Post, 1977; Borowitzka and Borowitzka, 1988).

1.1.3 Osmoregulation.

One of the most remarkable characteristics of *Dunaliella* is its ability to withstand changes in the osmotic concentration of its environment (Ben-Amotz, 1980). *Dunaliella* osmoregulates by varying the intracellular concentration of glycerol in response to the extra cellular osmotic pressure (Ben-Amotz and Avron, 1973; Ben-Amotz, 1975)

When grown in medium containing different salt concentrations, the intracellular glycerol concentration is directly proportional to the extracellular salt concentration and is sufficient to account for most of the required osmotic pressure (Ben-Amotz and Avron, 1973, 1990). The lack of a rigid cell wall allows the algae to shrink or swell rapidly when exposed to hypertonic or hypotonic conditions, respectively. Synthesis and breakdown of glycerol, in response to changes in extracellular solute concentration, occurs both in the light and in the dark and the time taken to achieve equilibrium is approximately 90 minutes (Ben-Amotz and Avron, 1973). Osmoregulation does not require protein synthesis. Starch breakdown accounts for all glycerol synthesis in the dark and 70 % of glycerol synthesis in the light, following an increase in medium salinity (Brown and Borowitzka, 1979). A hypothesis for the mechanism of metabolic response during osmoregulation in the alga *D. salina* is presented by Bental *et al.* (1990).

The high glycerol content of *Dunaliella* under hypersaline conditions, up to 85 % dry wt. (Ben-Amotz, 1980), prompted research into using the alga as an industrial producer of glycerol (Ben-Amotz, 1980; Chen and Chi, 1981), since the algae represents a model system for biosolar energy conversion. However, the algal technology was not viable due to the large scale synthetic production of glycerol and its low price (Borowitzka and Borowitzka, 1989).

1.1.4 ß-carotene production.

Approximately 570 carotenoid pigments have been identified in plants. These pigments provide the yellow, orange, red and purple colours of many foods, including carrots, tomatoes, pumpkins and citrus fruits (Pfander *et al.* 1987; Mordi, 1993). Carotenoids have dual functions in the photosynthetic membrane and are associated with the absorption and transfer of light energy to the chlorophyll molecules in the reaction centre thereby protecting the organism against photodynamic destruction (Goodwin, 1980a, 1980b). The most common carotenoid in all green plants is ß-carotene, which is normally located within the thylakoid membrane of the chloroplast (Goodwin, 1980a, 1980b).

In hypersaline lakes, which are generally nitrate limited and exposed to high solar irradiation, the *Dunaliella* strains that predominate are usually red in colour rather than green, due to ß-carotene hyper-accumulation. Of the many strains belonging to the genus *Dunaliella*, two have been shown to produce large amounts of ß-carotene when cultivated under specific conditions; *D. salina* Toed. and *D. salina* var *bardawil* (Ben-Amotz and Avron, 1989).

ß-carotene accumulation of up to 10-13 % dry wt. (Ben-Amotz and Avron, 1989) and 14 % dry wt. (Borowitzka *et al.* 1986) has been reported. This contrasts strongly with the ß-carotene content of plant leaves and other algae, which is approximately 0.3 % dry wt. (Ben-Amotz and Avron, 1990). The ß-carotene accumulated in *Dunaliella* is localised within oily globules in the interthylakoid spaces of the chloroplast (Ben-Amotz *et al.* 1982). ß-carotene hyper-accumulation in *Dunaliella* is thought to have a photoprotective function (Ben-Amotz, 1987; Ben-Amotz and Avron, 1989). It is thought to protect against the high intensity irradiation to which the organism is exposed in its natural habitat by absorbing energy in the blue region of the spectrum (Ben-Amotz *et al.* 1989). *Dunaliella* strains which are unable to accumulate ß-carotene die when exposed to high irradiation. In contrast, the ß-carotene rich *D. salina* flourishes because of its ability to accumulate ß-carotene (Ben-Amotz and Avron, 1989).

In addition to light, ß-carotene accumulation in response to nutrient limitation (Mil'ko, 1963a; Semenenko and Abdullaev, 1980; Ben-Amotz and Avron, 1983; Ben-Amotz, 1987; Lers *et al.* 1990) and high salinity (Mil'ko, 1963a; Ben-Amotz and Avron, 1980a; Borowitzka *et al.* 1984; Ben-Amotz, 1986; Borowitzka *et al.* 1990) has been reported. Generally, ß-carotene production is greatest under conditions which retard the growth rate and the amount of ß-carotene produced is a function of the integral amount of light absorbed by the cell during one division cycle - the higher the light intensity and the lower the growth rate of the alga, the greater the ß-carotene content of the cell (Ben-Amotz *et al.* 1988).

ß-carotene accumulated in *Dunaliella* is composed of mainly two isomers, all-*trans* ß-carotene and 9-*cis* ß-carotene, in approximately equal amounts (Ben-Amotz *et al.* 1988). The ratio of 9-*cis* to all-*trans* ß-carotene increases under conditions of high light intensity (Ben-Amotz *et al.* 1988). The physiochemical properties of the two isomers is markedly different; all-*trans* ß-carotene is practically insoluble in oil and is readily crystallized, while 9-*cis* ß-carotene is much more soluble in hydrophobic solvents, very difficult to crystallize, and is generally an oil in a concentrated form (Ben-Amotz and Avron, 1990).

Most of the commercially available ß-carotene is produced synthetically by companies such as Hoffman La Roche. Recent nutritional studies (Ben-Amotz *et al.* 1986; Ben-Amotz *et al.* 1989a) indicate a marked preferential accumulation of the *Dunaliella* sterioisomeric mixture of ß-carotene over that of the synthetic all-*trans* ß-carotene in livers of animal tissue. The requirement for cancer prevention of better ß-carotene absorbtion (Peto *et al.* 1981; Shklar and Schwartz, 1988; Nagasawa *et al.* 1989a; Nagasawa *et al.* 1989b) may create a new market for the *Dunaliella* ß-carotene sterioisomeric mixture (Ben-Amotz and Avron, 1990).

The extraction of ß-carotene from *D. salina* is relatively easy due to the absence of a cell wall (Moulton *et al.* 1987), however, it forms one of the major costs associated with commercial ß-carotene production (Borowitzka, 1992). Several organic solvent extraction methods ((Ruane, 1974b; Ruegg, 1984) and a hot oil extraction process (Nomomura, 1987) have been patented. Due to industrial secrecy, little extraction information is available in the public domain.

1.1.5 Commercial production of ß-carotene from *Dunaliella*.

D. salina is a highly suitable alga for mass cultivation and is probably the most successful microalga for outdoor cultivation described so far (Ben-Amotz and Avron, 1989). The ability of the alga to thrive in media with high salt concentrations enables it to be grown outdoors in relatively pure culture, with few potential predators (Ben-Amotz and Avron, 1989, 1990). In addition, the high ß-carotene content of *D. salina* protects it from high solar irradiation in areas in which it is usually cultivated, such as arid or desert areas with access to brackish water or seawater (Ben-Amotz and Avron, 1990).

The large-scale cultivation of *D. salina* is based on autotrophic growth in media containing inorganic nutrients such as nitrates, phosphates, chlorides, sulphates, magnesium, calcium, and iron (Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989). The alga can utilize either carbon dioxide or carbonate as an inorganic carbon source, however, in large scale cultivation, carbon dioxide is the preferential carbon source (Ben-Amotz and Avron, 1990). The solubility of inorganic carbon is limited at high salinities (Borowitzka and Borowitzka, 1988) and therefore, *Dunaliella* cultures have a relatively high demand for carbon dioxide; approximately 37 g $CO_2.m^{-2}.day^{-1}$ (Ben-Amotz and Avron, 1989).

The pH of the medium in which the cells grow affects many processes associated with algal growth and metabolism, including the availability of carbon dioxide and the availability and uptake of ions (Borowitzka and Borowitzka, 1988). *D. salina* has a wide pH optimum between pH 7 and 9, and for commercial cultivation the pH of the cultures is maintained at about pH 8 by sparging carbon dioxide gas into the culture (Ben-Amotz and Avron, 1989). There are two main advantages in using carbon dioxide gas as a pH controlling acid; firstly, it is safe in that it does not cause dangerously low pH levels, and secondly, it maintains the inorganic carbon concentration in the growth medium (Ben-Amotz and Avron, 1989).

The effect of temperature on the physiology of microalgae, in general, has been summarised by Richmond (1986). *D. salina* has a wide range of temperature tolerance, from below freezing to around 45 °C (Wegmann *et al.* 1980), with a temperature optimum of around 32 °C (Ben-Amotz and Avron, 1989). Temperature has a profound affect on glycerol retention in *Dunaliella*. Below 25 °C little glycerol is found in the medium. Above 25 °C the rate of glycerol loss to the medium gradually increases and above 40 °C, it increases dramatically. By 50 °C most of the glycerol has been released to the medium (Wegmann *et al.* 1980). Glycerol release has been related to temperature induced alterations of the cell membrane integrity, as has been demonstrated by changes in the phospholipid composition of the membrane by Lynch and Thomson (1982).

Bacteria may proliferate in *Dunaliella* cultures due to excessive glycerol leakage into the medium (Ben-Amotz and Avron, 1989). Under normal conditions, the heterotrophic nature of the bacteria does not allow excessive growth, however, an increase in the organic load of the medium can induce bacterial blooms (Ben-Amotz and Avron, 1989).

There are two cultivation systems currently employed for ß-carotene production from *D. salina*, termed intensive and extensive cultivation. In the more common, intensive mode, *Dunaliella* are grown in mixed, open, raceway ponds, in a defined medium, under controlled conditions. The concentration of nutrients, the pH, rate of mixing, salt concentration and cell density are monitored and adjusted as necessary (Ben-Amotz and Avron, 1989). Algal biomass and ß-carotene productivities of about 4-10 g dry wt.m^{-2}.day^{-1} and 250-500 mg ß-carotene.m^{-2}.day^{-1}, respectively, are typical for intensive modes of cultivation (Ben-Amotz and Avron, 1989; Mohn and Condero-Contreras, 1990). Examples of such systems are those operated by Koor Foods, Eilat, Israel; Microbio Resources, Inc., Calipatria, USA; and Cyanotech Corp., Woodinville, USA.

In the extensive mode, there is no mechanical mixing and minimal control of the culture environment (Moulton *et al.* 1987). The extensive mode exploits natural populations of *D. salina* in wind mixed ponds by the addition of specific nutrients (Schlipalius, 1991, Borowitzka, 1991). Algal productivity under extensive cultivation is, however, low, averaging 0.1 g algal dry wt.m^{-2}.day^{-1} or 10 mg ß-carotene.m^{-2}.day^{-1} (Ben-Amotz and Avron, 1990). The low productivity of the extensive system is off-set by the relatively low operating costs of such facilities (Curtain *et al.* 1987) and the ideal choice of location (Schlipalius, 1991). Examples of extensive systems are those operated by Betatene Ltd, Cheltenham, Victoria, Australia and Western Biotechnology, Perth, Australia. Borowitzka and

Borowitzka (1989) record their experience with the scale-up sequence of their algal biotechnology program at Hutt Lagoon, Western Australia.

1.1.6 Production dilemma.

The basic dilemma underlying the commercial production of ß-carotene from *D. salina* is that ß-carotene accumulation is greatest when the growth rate is limited (Borowitzka *et al.* 1990). Ben-Amotz and Avron, (1989) state that *Dunaliella* represents a special case in optimizing productivity. The aim is not to increase the productivity of algal biomass but to maximize the production of ß-carotene even at the expense of algal biomass. ß-carotene accumulation in *Dunaliella* increases with higher light intensities and slower growth rates. Consequently, commercial production facilities are located in areas where solar light output is maximal (Ben-Amotz and Avron, 1990). The growth rate of the algae can be limited in two ways, by controlling the supply of essential nutrients in the cultivation medium, such as nitrate, or by adjusting a limiting physiological parameter, such as the salinity of the cultivation medium (Ben-Amotz and Avron, 1989, 1990). With the current commercial system, *D. salina* is cultured under conditions both sub-optimal for growth and for ß-carotene accumulation, but optimized for the best overall ß-carotene yield over time. This approach has been termed the "averaging production process" or the "single stage process" and is used in all existing ß-carotene production systems. The averaging process relies on careful manipulation to ensure the maximum compromise is achieved between growth and ß-carotene production, as any deviation in operating conditions will shift the final ß-carotene productivity off this optimum or "knife-edge" on which the system is balanced.

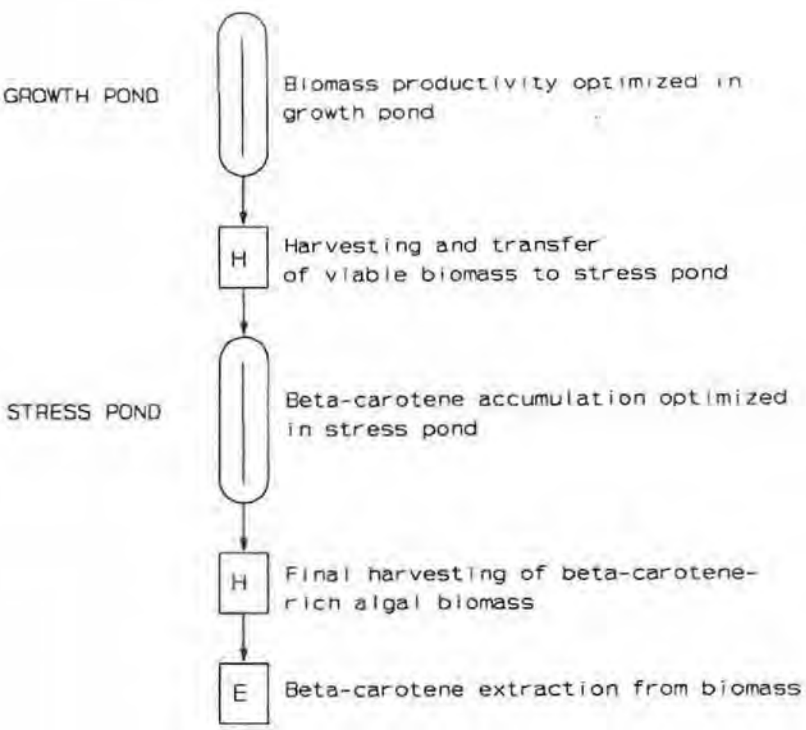


Figure 1.1. Flow chart of a hypothetical dual-stage ß-carotene production system.

An alternative approach to the averaging process is to use a dual-stage process, whereby algal biomass production is separated from ß-carotene accumulation. In this way maximum biomass production can be obtained in optimal growth medium, followed by transfer to a medium optimized for ß-carotene production (Figure 1.1). Such a process has been proposed for the production of glycerol (Avron and Ben-Amotz, 1978; Chen and Chi, 1981), for a dual-stage batch process for ß-carotene production (Borowitzka and Borowitzka, 1988; Rose and Cowan, 1991a) and for the recovery of both ß-carotene and glycerol from effluent grown algae (Rose *et al.* 1992).

Figure 1.2 depicts a flow chart of the algal high rate oxidation process for the treatment of saline organic effluent described by Rose *et al.* (1992). The process proposes to both treat saline organic effluent and produce products of commercial value such as ß-carotene from *D. salina*. The conflicting requirements for biomass production and metabolite induction observed in this alga are accommodated in separate units and cell separation is undertaken primarily by crossflow ultrafiltration (CFUF) which has been shown to produce algal concentrates in a viable state (Figure 1.2)(Rose *et al.* 1992).

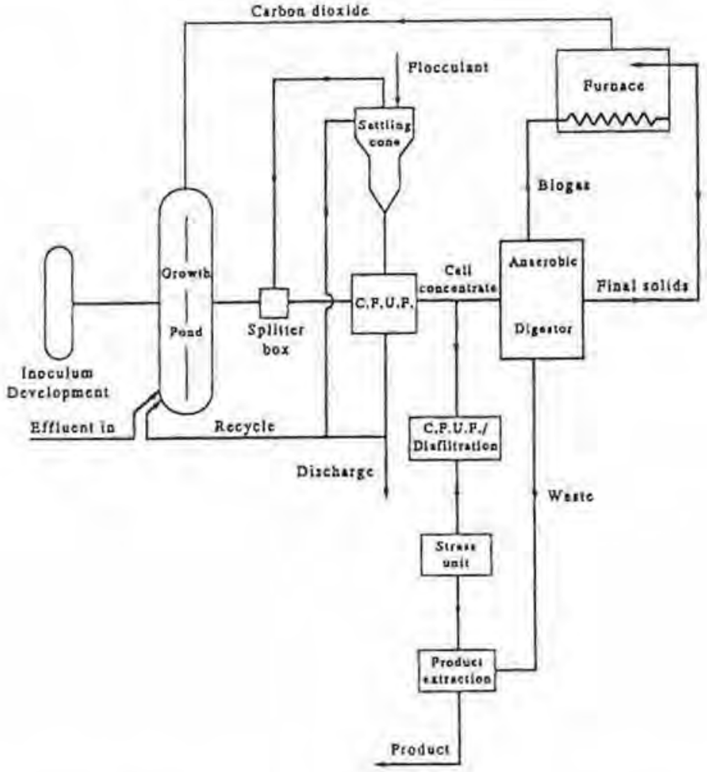


Figure 1.2 Flow chart of the algal high rate oxidation ponding process proposed for the simultaneous treatment of saline organic effluent and the production of ß-carotene and glycerol from *D. salina* (Rose *et al.* 1992).

The separation of growth and metabolite production stages is not novel in biotechnology and a number of bacterial and fungal secondary metabolite fermentation processes employ dual-stage production processes (Atkinson and Mavituna, 1991). However, no detailed outdoor evaluations of an algal dual-stage process have been undertaken, although Borowitzka *et al.* (1984) have evaluated a dual-stage system in a laboratory study. In this study, the algae were first grown in a low salinity medium (15 % NaCl w/v) to optimize biomass production, and then the salinity was increased to 25 % NaCl (w/v) in order to induce ß-carotene production. No indication of the ß-carotene production potential, compared to conventional processes, was reported.

Numerous laboratory studies have shown that ß-carotene hyper-accumulation can be induced by the imposition of various stress factors such as high intensity irradiation, high salinities and nutrient limitation for a range of compounds (Ben-Amotz and Avron, 1983; Borowitzka *et al.* 1984; Ben-Amotz, 1987; Borowitzka *et al.* 1990; Lers *et al.* 1990). However, it has not been determined whether these stress factors can be used to manipulate ß-carotene accumulation in a controlled environment such as that proposed in the stress ponds of the dual-stage system (Figure 1.1).

Borowitzka and Borowitzka (1988) maintain that the dual-stage production scheme has inherent draw backs. Firstly, at low salinities and high nitrate concentrations, which would be encountered in the growth ponds of a dual-stage system (Figure 1.1), predatory species and non-carotenogenic *Dunaliella* species may reduce the biomass production potential of *D. salina*. Secondly, the additional labour and retention time required for a dual-stage batch process would not compare favourably with the continuous averaging process.

In an extensive ß-carotene production system it is unlikely that a dual-stage system would be feasible because of the relatively uncontrolled cultivation strategy. The extensive systems are not unialgal and in addition to *D. salina*, the ponds contain a number of non-carotenogenic *D. salina* species, such as *D. viridis* and *D. parva*, which have lower salinity optima than *D. salina* (Borowitzka *et al.* 1984). This requires the use of higher salt concentrations, in order for *D. salina* proliferation (Ben-Amotz and Avron, 1990). A dual-stage ß-carotene production system would be more suited to the intensive mode of cultivation, where greater control of the system may allow the maintenance of unialgal cultures.

Borowitzka (1992) has examined the affects of various parameters on the economic modelling of an intensive ß-carotene production process. The analysis demonstrated that an increase in biomass productivity of 50 % can reduce the final production costs by 19 %, furthermore, a 50 % increase in the ß-carotene content of the biomass can decrease the final production costs by 50 % (Borowitzka, 1992). It is unlikely that the averaging ß-carotene production process can obtain these goals, since an increase in algal biomass productivity would compromise cellular ß-carotene accumulation and visa versa.

The dual-stage ß-carotene production system has the potential to increase both the biomass productivity and the ß-carotene content of the biomass, by separating the mutually exclusive growth and ß-carotene production stages of *D. salina*. When grown under conditions optimal for growth, maximum *D. salina* productivities of 20 to 60 g dry wt.m^{-2}.day^{-1} have been reported (Ben-Amotz, 1980; Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989). Under conditions which promote ß-carotene accumulation, ß-carotene contents of greater than 10 % dry wt. have been reported (Borowitzka *et al.* 1986; Ben-Amotz and Avron, 1989). These values are significantly higher than the biomass productivities and ß-carotene contents reported for the averaging process; 5-10 g dry wt.m^{-2}.day^{-1} and about 5 % ß-carotene dry wt., respectively (Ben-Amotz and Avron, 1989, 1990).

The separation of the algal biomass from the growth medium and the subsequent transfer to a ß-carotene production medium (stress medium) (Figure 1.1), forms a crucial component of a dual-stage system, as the separated biomass will have to be maintained in a viable form. Without the development of a suitable harvesting technique the potential of a dual-stage process would remain an academic curiosity.

Harvesting of microalgae represents a significant component of the total capital and operating costs of a mass culture system (Borowitzka, 1992). The techniques employed for harvesting *Dunaliella* have recently been reviewed (Ben-Amotz and Avron, 1989) and include centrifugation, flocculation, filtration and hydrophobic binding. The choice of harvesting technique depends on a number of factors such as medium recycle (Ben-Amotz and Avron, 1989; Borowitzka, 1992), the final product desired and the production strategy used (Borowitzka, 1992). A dual-stage ß-carotene production process will require a gentle harvesting process which does not affect the viability of the fragile cells. Since the cultivation mode is likely to be intensive, medium recycle will be necessary. Rose *et al.* (1992) have demonstrated that CFUF is a suitable technique for the recovery of *D. salina* cells in a viable form and offers additional advantages with respect to medium recycle.

1.1.7 Development of a Dual-stage ß-carotene production process.

The alga *D. salina* has been identified as one of the most suitable and successful alga for outdoor cultivation. Furthermore, it produces large amounts of commercially valuable ß-carotene when cultured under appropriate conditions. A collaborative research program was developed between Rhodes University, Grahamstown, and Sastech, the research and development wing of Sasol Pty., Sasolburg, to evaluate the commercial potential of ß-carotene production from the alga *D. salina*. Market surveys (Sasol confidential studies) have indicated that natural ß-carotene commands a substantially higher price than synthetic ß-carotene, due to an up-swing in the market for natural products. The current ß-carotene market size is greater than 100 tons.year^{-1}, however, only a small proportion of the market is composed of natural ß-carotene and the natural market is dominated by a few producers (Borowitzka, 1992). In order to gain entry into such a market, a competitive advantage is necessary which can buffer the potential entrant against possible price drops.

The need for an economically superior ß-carotene production system was perceived from these requirements. Rose and Cowan (1991a,1991b) of Rhodes University in collaboration with the Water Research Commission have patented a proposed multi-stage process for the recovery of ß-carotene from *D. salina*, using either effluent grown algae or artificial sea water grown algae. The patent claims are based on laboratory studies and no outdoor scale-up evaluation studies were undertaken. Furthermore, no indication as to the economic viability of the processes are given. The research programme of this thesis forms part of the verification of the patents in an on-going research programme at Rhodes University.

1.2 RESEARCH HYPOTHESIS.

Single stage processes currently used for ß-carotene production by *D. salina* do not utilize the full genetic potential of the organism, given the conflicting requirements for maximum growth and secondary metabolite production. The separation of the distinctive growth and ß-carotene accumulation phases into two engineerable stages may allow for greater physiological control of the organism, with the possibility of significantly higher ß-carotene yields.

1.3 RESEARCH OBJECTIVES.

The primary objective of the research programme was to evaluate dual-stage ß-carotene production processing and to determine the economic viability compared to the conventional averaging process. A research programme was formulated with the objective of addressing issues pertaining to the research hypothesis.

1. It is well established that certain environmental and physiological stresses are responsible for hyper-accumulation of ß-carotene in *D. salina* (Ben-Amotz and Avron, 1989; Borowitzka and Borowitzka, 1988). The question that arises is whether these stresses can be used to induce and control ß-carotene accumulation in *D. salina* in the stress pond stage of a dual-stage

process (Figure 1.1) and what the maximum ß-carotene yields in response to such stresses would be under outdoor conditions.

2. Conventional commercial ß-carotene production processes employ either salinity or nutrient stress to retard the growth rate of *D. salina* in order to simultaneously optimize both growth and ß-carotene accumulation. Consequently, the biomass productivities reported from such systems are lower than if the algae were cultivated under conditions optimal for biomass production. Short term maximum biomass productivities of between 20-60 g dry wt.m^{-2}.day^{-1} have been reported for *D. salina* cultured under optimal outdoor conditions (Ben-Amotz, 1980; Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989), however, no reports of longer-term maximum biomass productivities are available in the literature reviewed. As biomass yields in the growth stage of a dual-stage process (Figure 1.1) will be crucial to the whole process it is essential to determine what maximum long-term biomass productivity of *D. salina* may be anticipated.

3. Borowitzka and Borowitzka (1988) maintain that growth of *D. salina* under optimal growth conditions (ie. low salinity, high nitrate concentrations) allows for contamination of cultures by non-carotenogenic *Dunaliella* species. The extent of this threat and its possible control requires evaluation.

4. The extraction of ß-carotene from the separated algal biomass forms one of the major costs associated with the production of ß-carotene from *D. salina* (Borowitzka, 1992) and an investigation of currently used and alternative techniques requires evaluation in order to improve the costing of the extraction process.

5. The separation and transfer of the fragile *D. salina* cells from the growth pond into the stress ponds in a viable state is a crucial step without which the dual-stage process would not function (Figure 1.1). It is necessary to evaluate cost effective harvesting processes which maintain the cells in a viable state.

6. From a biotechnological perspective, perhaps the most important question that requires answering is how the economics of a dual-stage ß-carotene production process compare to that of the currently used single-stage averaging process and whether a commercial advantage can be derived from dual-stage processing.

CHAPTER 2

STRESS INDUCED ß-CAROTENE PRODUCTION IN *D. SALINA* - A LABORATORY STUDY

Summary.

A comparison of various stress factors that promote the induction of ß-carotene production confirmed the primary role of high light intensities in obtaining maximal ß-carotene yields. Optimal stress conditions were determined and an inverse relationship between *D. salina* growth and ß-carotene hyper-accumulation in high light stress systems was observed. Maximum ß-carotene yields under optimal stress conditions were achieved in 3-5 days. Furthermore, it has been demonstrated that continuous high light illumination is not necessary in order to achieve high ß-carotene yields.

2.1. INTRODUCTION.

It is well established that factors such as nutrient limitation, high salinities and high light intensities are associated with the hyper-accumulation of ß-carotene in *D. salina* (Lerche, 1937; Mil'ko, 1963a, 1963b; Ben-Amotz, 1987; Ben-Amotz and Avron, 1983, 1989; Loeblich, 1982; Lers *et al.* 1990; Borowitzka *et al.* 1990). Furthermore, Pace *et al.* (1977) have shown that sub-lethal concentrations of copper and lead can also increase ß-carotene hyper-accumulation.

Ben-Amotz and Avron (1983) have demonstrated an inverse relationship between the ß-carotene content and the specific growth rate of *D. salina*. In general, ß-carotene accumulation is greatest under conditions which reduce the growth rate, such as nutrient limitation, high salinity, high temperatures and high light intensities. The inverse relationship between growth and ß-carotene production is the main cause of sub-optimal ß-carotene yields in the currently employed averaging ß-carotene production process (Borowitzka *et al.* 1990). The separation of the distinctive growth and secondary metabolite production stages, as proposed by the dual-stage process, may allow for greater manipulation of stress-induced hyper-accumulation of ß-carotene in *D. salina*. Factors which influence ß-carotene accumulation are thus discussed below.

2.1.1. Salt stress.

Salt stress was recognised early as a major factor in the hyper-accumulation of ß-carotene in *D. salina* (Labbe, 1921) and a number of studies have further substantiated these findings (Mil'ko, 1963a; Ben-Amotz and Avron, 1980a; Ben-Amotz, 1986; Borowitzka *et al.* 1984; Borowitzka *et al.* 1990). Cellular ß-carotene content has been shown to be directly related to the growth medium salinity and at high salinities cellular ß-carotene levels are greatest. An inverse relationship exists between both the specific growth rate and cellular chlorophyll content of *D. salina*, and the growth medium salinity (Ben-Amotz and Avron, 1983; Ben-Amotz, 1986; Borowitzka *et al.* 1990).

A study of the kinetics of growth and carotenogenesis in hypersaline stressed *D. salina* isolates has revealed two distinctive ß-carotene accumulation phases (Borowitzka *et al.* 1990). Firstly, a lag phase in carotenoid accumulation, which is consistent with transition to a stationary phase and secondly, a rapid ß-carotene accumulation phase after about two days. It was shown that the duration of the lag phase is dependent on the final salinity and the magnitude of the salinity change. The rate of carotenogenesis and the final carotenoid content achieved was shown to be dependent on the final salinity (Borowitzka *et al.* 1990). The results, however, do not explain why *D. salina* synthesises more ß-carotene at higher salinities when compared to the non-carotenogenic *Dunaliella* species. Borowitzka *et al.* (1990) suggest that the increase in carotenoid synthesis may be due to enhanced gene expression under conditions of stress, since environmental stresses have been shown to alter gene expression in higher plants (Sachs and Ho, 1986).

2.1.2. Nutrient Stress.

Nutrient composition of growth medium often has a dramatic effect on the biochemical profile of microalgae (Rhee, 1978). Metabolite induction by nitrogen stress has been demonstrated for a number of microalgae, including astaxanthin from *Haematococcus pluvialis* (Renstrom *et al.* 1981), eicosapentaenoic acid (EPA) from *Isochrysis galbana* (Ben-Amotz *et al.* 1985) and lipids and hydrocarbons from *Botryococcus braunii* (Singh and Kumar, 1992). The alga *D. salina* is no exception and nitrate, sulphate and phosphate limitation have all been reported to significantly increase ß-carotene accumulation (Mil'ko, 1963a; Semenenko and Abdullaev, 1980; Ben-Amotz and Avron, 1983; Ben-Amotz, 1987; Borowitzka and Borowitzka, 1988; Lers *et al.*,1990).

Ben-Amotz and Avron (1983) have shown that maximum ß-carotene accumulation occurs under conditions of nitrate limitation (approximately 1 mM NO_3^-) as opposed to complete nitrate deficiency. The converse exists for sulphate and phosphate where maximum accumulation is obtained under conditions of complete deficiency (Ben-Amotz and Avron, 1983; Ben-Amotz, 1987).

The kinetics of ß-carotene accumulation in cells grown under low light in sulphate free medium have been studied (Lers *et al.*, 1990). Transfer of *D. salina* cells to sulphate free medium results in a termination of cell growth and a progressive increase in ß-carotene. No lag phase in ß-carotene production is present under these conditions in contrast to that reported for salinity stress (Borowitzka, 1990).

2.1.3 Light stress.

The major inducing factor of ß-carotene accumulation is light intensity (Ben-Amotz, 1986) and the photoinduction of massive ß-carotene accumulation is well documented (Mil'ko, 1963b; Ben-Amotz and Avron, 1983, 1989; Ben-Amotz, 1986, 1987; Lers *et al.* 1990; Loeblich, 1982). Loeblich (1982) has demonstrated a linear relationship between ß-carotene accumulation and light intensity over a photon flux density (PFD) of 10 to 396 μmol. $m^{-2}.s^{-1}$. Ben-Amotz and Avron (1983) have shown that the extent of ß-carotene accumulation is correlated with the integral irradiance received by the alga during a division cycle. The higher the integral amount of light per division cycle, the lower the cellular chlorophyll content, the higher the cellular ß-carotene content, and therefore the higher the ß-carotene-to-chlorophyll ratio (Ben-Amotz and Avron, 1983).

An examination of the wavelength dependence of ß-carotene accumulation in *D. salina* has shown that ß-carotene biosynthesis and accumulation is dependent upon light intensity but is independent of light quality within the photosynthetically active radiation region (Ben-Amotz and Avron, 1989).

The hyper-accumulated ß-carotene in *D. salina* is concentrated in oily globules in the interthylakoid space of the chloroplast (Ben-Amotz and Avron, 1982). Intrathylakoid ß-carotene is generally believed to protect plants by quenching damaging reagents such as singlet oxygen produced by excessive excitation of chlorophyll (Goodwin, 1980b). However, since the massively accumulated ß-carotene in *D. salina* is located outside the thylakoid, Ben-Amotz and Avron (1983, 1989) have hypothesised a photoprotective function for ß-carotene as a screen absorbing excess irradiance and thus limiting photoinhibition.

The induction of massive ß-carotene accumulation in response to salinity and nutrient stress can also be explained in accordance with this hypothesis. Any environmental stress factor that causes a decrease in the photosynthetic rate of an organism will have the effect of increasing the ratio of PFD to photosynthesis, even if PFD remains constant (Demmig-Adams and Adams, 1992). Under such circumstances continued absorption of light by chlorophyll leads to an increased level of excitation of the chlorophyll (Demmig-Adams and Adams,1992). The imposition of salinity or nutrient stress has been shown to decrease the photosynthetic rate of *D. salina* (Loeblich, 1982; Ben-Amotz and Avron, 1983). The subsequent increase in ß-carotene production in response to these conditions may serve to reduce excess excitation of the chlorophyll and therefore prevent photo-damage. The observed ecological dominance of high ß-carotene producing strains of *D. salina* in natural environments such as salt lakes has been attributed to the photoprotective function of massive ß-carotene accumulation (Ben-Amotz and Avron, 1983; Moulton *et al.* 1987).

Another possible reason for the increased ß-carotene accumulation in *D. salina* under conditions of stress may be that it functions as a carbon sink when one or more metabolic pathways are inhibited by substrate limitation (Borowitzka and Borowitzka, 1988). Under conditions of stress photosynthesis continues at a reduced rate and the excess photosynthetically produced organic carbon must be either excreted or stored. ß-carotene is a suitably "neutral" molecule that could serve as a storage compound.

Lers *et al.* (1990) have demonstrated two clearly separate ß-carotene accumulation stages in response to high light intensities. The first ß-carotene accumulation stage, lasting for about 24 hours, starts shortly after exposure, whereas the second stage starts concomitantly with the onset of the stationary phase and persists until the cells die. The kinetics of ß-carotene production in response to high light intensities contrasts with the kinetics of production in response to salinity (Borowitzka *et al.*, 1990) and sulphate stress (Lers *et al.*, 1990). It seems likely that the different environmental stresses may induce different stress responses in *D. salina*.

2.1.4 Synergistic stress.

There is little mention of the effect of multiple stress induction of ß-carotene in the literature reviewed. Mil'ko (1963b) has shown that combined temperature and light stress can produce up to 5 $mg.l^{-1}$ ß-carotene in 12 days. Borowitzka *et al.* (1986) report that 14 % dry wt. carotenoids can be obtained with a combination of nutrient limitation and high salinity (25 % w/v), whereas nutrient limitation with 10 % (w/v) salt results in only 3.9 % dry wt. carotenoids. Ben-Amotz and Avron (1983) investigated the interaction between salt concentration and light intensity on the ß-carotene-to-chlorophyll ratio and have shown that the maximum ß-carotene-to-chlorophyll ratio is obtained at high salinities and light intensities.

2.2 RESEARCH OBJECTIVES.

Although the literature reviewed contains numerous specific references to the effects of cellular ß-carotene accumulation in response to a variety of individual and combined stresses, a comprehensive comparison of the rates and final ß-carotene yields for the different individual and synergistic stresses has not been undertaken. The implementation of a dual-stage ß-carotene production process relies on the successful manipulation of the stress response in *D. salina*. A comparative evaluation of single and multiple stress induction of ß-carotene was necessary in order to assess the use of various stress factors in the dual-stage process.

The following experimental objectives were thus identified:

1. To determine the minimum nitrate concentration for ß-carotene production in *D. salina*.
2. To determine the production time for maximum ß-carotene yields.
3. To compare the effects of individual stresses on the cellular pigment content and biomass yield, and to determine the magnitude of these stresses.

4. To determine the degree to which multiple stress synergism can enhance ß-carotene production in *D. salina*.
5. To determine the quantity of light required for maximum ß-carotene yields.

2.3 MATERIALS AND METHODS.

2.3.1 *Dunaliella* Culture.

Dunaliella salina (Teodoresco) var *bardawil* was acquired from the Culture Collection for Algae and Protozoa, Oban, United Kingdom (CCAP 19/30). All experiments reported involve the use of pure cultures, unless otherwise indicated, and all references to *D. salina* pertain to *D. salina* var *bardawil*.

2.3.2 Growth medium.

The defined culture medium described by Ben-Amotz and Avron (1983) was used in this study (Table 1). The individual components were dissolved separately, then combined and made up to the appropriate volume with distilled water. The medium was then filter-sterilized through 0.45 µm cellulose acetate membrane filters (Millipore).

Table 2.1 Constituents of Growth Medium (Ben-Amotz and Avron, 1983).

NaCl M	1.5	H_3BO_4 µM	185
$NaHCO_3$ mM	50	$MnCl_2$ µM	7
KNO_3 mM	5	EDTA µM	6
$MgSO_4$ mM	5	$FeCl_2$ µM	1.5
$CaCl_2$ mM	0.3	$ZnCl_2$ µM	0.8
KH_2PO_4 mM	0.2	$CoCl_2$ µM	0.02
		$CuCl_2$ µM	0.2

2.3.3 Stress medium.

Nutrient limitation and high salinity have been identified as appropriate means of inducing ß-carotene production in *D. salina* (Ben-Amotz and Avron, 1983; Borowitzka and Borowitzka, 1988; Borowitzka *et al.*, 1990). Consequently, the induction and production of ß-carotene in *D. salina* was determined by transferring the algae to high salinity media (3 M NaCl), media limited in nitrate (the determination of the minimum medium NO_3^- concentration is discussed in section 2.3.8), medium deficient in phosphate or sulphate (Ben-Amotz and Avron, 1983, 1987); or combinations of these media.

2.3.4 Algal Growth Conditions.

Algae were incubated in a constant environment chamber at 30 °C under continuous illumination. Growth and ß-carotene studies were undertaken in 500 ml tissue culture flasks (to optimise light penetration) and agitation was supplied by placing the flasks on an orbital shaker. Low light intensity studies were conducted at a light intensity of 90 μmol.m.$^{-2}$s.$^{-1}$ and high light intensity studies were conducted at a light intensity of 1000 μmol.m^{-2}.s^{-1}. Low light intensity was provided by a bank of four cool white fluorescent tubes, supplemented with far red light from incandescent bulbs. High light intensity was provided by a 400 W high pressure sodium lamp (Lascon Lighting, RSA).

2.3.5 Light Intensity Measurement.

Light intensity was measured with a Skye four channel light sensor Model SDL2580.

2.3.6 Cell Counts.

Algal cell counts were made using an improved Neubauer haemocytometer. Results reflect a triplicate mean.

2.3.7 ß-carotene and Chlorophyll Determination.

D. salina cells (10 ml) were separated from the medium by centrifugation at 2000 g for 10 minutes. Chlorophyll and ß-carotene were extracted from the algal pellet with 100 % acetone, diluted to 80% acetone with distilled water (v/v) and assayed according to Arnon (1949) and Jensen (1978). $E^{1\%}_{1cm}$ of 2273 at 480 nm and $E^{1\%}_{1cm}$ of 890 at 666 nm was used to calculate ß-carotene and chlorophyll concentrations respectively.

ß-carotene production was expressed as cellular ß-carotene content (pg.cell^{-1}) and as total ß-carotene concentration (mg.l^{-1}). Total ß-carotene concentration reflects the total ß-carotene production as it takes both cellular ß-carotene accumulation and cell concentration into account.

2.3.8 Determination of minimum nitrate concentration for maximum ß-carotene accumulation.

Ben-Amotz and Avron (1983) and Ben-Amotz (1987) have shown that maximum ß-carotene accumulation in response to both sulphate and phosphate nutritional stress occurs when these compounds are totally absent from the culture medium. For this reason total deficiency was used in the ß-carotene induction studies. However, in the case of nitrate nutritional stress, Ben-Amotz and Avron (1983) have shown that total nitrate deficiency compromises ß-carotene accumulation and that a concentration of approximately 1 mM NO_3^- is optimal for maximum ß-carotene accumulation. The experiment described by the authors was performed with nitrate starved *D. salina* cells and consequently, the results cannot be extrapolated to the dual-stage process since the dual-stage process will require the transfer of cells grown in nitrate excess into nitrate limited stress media. Thus, an experiment was performed to determine the optimal nitrate concentration for maximum ß-carotene accumulation in cells previously exposed to nitrate excess conditions.

A log phase culture of *D. salina* cells cultured in growth medium (5 mM NO_3^-) was centrifuged at 2000 g for 10 minutes. The algal pellet was washed twice with 1.5 M KCl and the washed cells were transferred to 250 ml volumes of growth medium containing the following NO_3^- concentrations: 0 mM, 0.1 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM, respectively. The cultures were incubated at 30 °C under constant illumination of 1000 μmol.m^{-2}.s^{-1} for 6 days.

2.3.9 Determination of production time for maximum ß-carotene yields.

Once the optimal nitrate concentration was determined, an experiment was conducted to obtain an indication as to the optimum retention time for maximum ß-carotene production, since this was likely to be an important factor in the evaluation of the dual-stage process. Synergistic stress has been shown to result in greater ß-carotene accumulation (Ben-Amotz and Avron, 1983; Borowitzka *et al.* 1986; Rose, 1992) and for this reason a multiple stress medium was used for this study.

A log phase culture of *D. salina* cells cultured in growth medium was harvested by centrifugation at 2000 g for 10 minutes and washed with 1.5 M KCl. The washed cells were resuspended in a high salinity (3 M NaCl), nitrate limited medium (0.2 mM NO_3^- as determined in section 2.3.8) and incubated under high light intensity (1000 μmol.m^{-2}.s^{-1}) for 10 days. The cellular ß-carotene content (pg.cell^{-1}) as well as the culture ß-carotene concentration (mg.l^{-1}) were monitored over the 10 day period. Of particular interest was the ß-carotene concentration as this is indicative of the total ß-carotene yield. Experiments were conducted in triplicate.

2.3.10 ß-carotene induction studies.

Algae were cultivated in defined culture medium (growth medium) at 30 °C under constant illumination (90 μmol.m^{-2}.s$^{-1)}$ as previously described. Single stress ß-carotene accumulation studies were conducted by transferring washed log phase cultures of *D. salina* (cell density approximately 40 x 10^4 cells.ml^{-1}) into 250 ml of the following media: nitrate limited (0.2 mM NO_3^- as determined in section 2.3.8), high salinity (3 M NaCl), phosphate deficient, sulphate deficient and growth medium under high light intensity (1000 μmol.m^{-2}.s^{-1}). Multiple stress studies were conducted under high light intensity in combinations of the single stress media. Cultures were incubated under constant illumination of 90 μmol.m^{-2}.s^{-1} or 1000 μmol.m^{-2}.s^{-1} at a temperature of 30 °C for a duration of 5 days. Experiments were conducted in triplicate.

2.3.11 Effect of photoperiod on ß-carotene yield.

A series of experiments were conducted to determine the effect of photoperiod on ß-carotene production. Log phase *D. salina* cultures (cell count approximately 40 x 10^4 cells.ml^{-1}) were centrifuged at 2000 g for 10 min. The algal pellet was washed twice with a 1.5 M KCl solution and the washed cells were resuspended to the same cell concentration in 250 ml of a 3 M NaCl, nitrate limited, sulphate and phosphate deficient stress medium. Cultures were incubated at 30 °C for 5 days and subjected to the following photoperiods:

A - continuous illumination at 1000 μmol.m^{-2}.s^{-1}.

B - diurnal cycle, comprised of 12 hours of illumination at 1000 μmol.m^{-2}.s^{-1} and 12 hours of complete darkness.

C - diurnal cycle, comprised of 12 hours of various illumination and 12 hours of complete darkness. The 12 hour illumination period was varied as follows: day 1 and 5, continuous illumination at 1000 μmol.m^{-2}.s^{-1}; day 2-4, cycle of 1 hour high light intensity (1000 μmol.m^{-2}.s^{-1}) and 1 hour darkness.

D - diurnal cycle, comprised of 12 hours various illumination and 12 hours darkness. The 12 hour illumination period was varied as follows: day 1-5, cycle of 1 hour high light intensity (1000 μmol.m^{-2}.s^{-1}) and 1 hour darkness. The major difference between C and D is that no continuous illumination was supplied on day 1 and 5 in D.

E - diurnal cycle, comprised of 12 hours of various illumination and 12 hours of complete darkness. The 12 hour illumination period was varied as follows: day 1 and 5 , continuous illumination at 1000 μmol.m^{-2}.s^{-1}; day 2-4, cycle of 1 hour high light intensity (1000 μmol.m^{-2}.s^{-1}) and 1 hour of low light intensity (90 μmol.m^{-2}.s^{-1}).

F - diurnal cycle, comprised of 12 hour various illumination and 12 hours darkness. The 12 hour illumination period was varied as follows: day 1-5, cycle of 1 hour high light intensity and 1 hour low light intensity. The major difference between E and F is that no continuous high light intensity illumination was supplied on day 1 and 5 in F.

2.4 RESULTS.

2.4.1 Effect of nitrate concentration on the pigment content of *D. salina*.

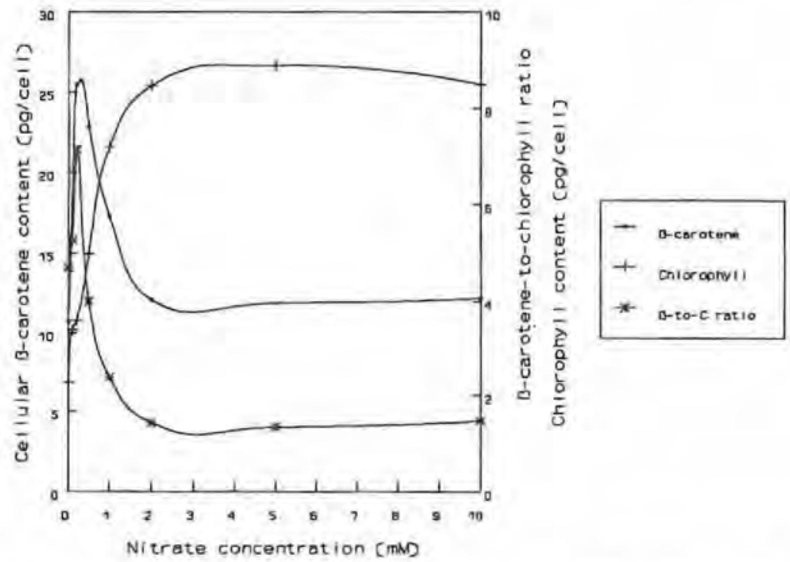


Figure 2.1 Effect of nitrate concentration on the pigment content of *D. salina* cells. Cells were previously exposed to nitrate sufficient conditions.

ß-carotene production is not significantly stimulated under conditions of complete nitrate deficiency, unlike sulphate and phosphate deficiency where this is found to be the case (Ben-Amotz and Avron, 1983, Ben-Amotz, 1987). Figure 2.1 illustrates the effect of nitrate concentration on the pigment content of algae previously grown in conditions of nitrate excess. It is apparent from Figure 2.1 that maximum ß-carotene accumulation occurred at nitrate concentrations of between 0.2 and 0.3 mM nitrate. ß-carotene accumulation does not appear to be stimulated by complete nitrate deficiency even though the ß-carotene-to-chlorophyll ratio was relatively high (4.8:1). However, the amount of chlorophyll per cell decreases with the decreased availability of nitrate leading to a marked increase in the ß-carotene-to-chlorophyll ratio in the nitrate starved cells. A nitrate concentration of 0.2 mM was thus used in the following experiments.

2.4.2 Determination of the production time for maximum ß-carotene yields.

Figure 2.2 shows the cell count, cellular ß-carotene content and ß-carotene yield (culture ß-carotene concentration) of *D. salina* grown in high salinity, nitrate limited medium under high light intensity. It is evident from Figure 2.2 that after a transient increase in cell number over the first 2 days, there was a gradual decrease in cell number until day 6. After this, there was a rapid decline in cell number to day 10. The cellular ß-carotene content increased steadily over the first 5 days, however, after day 6 there was a gradual decrease in the ß-carotene yield. The decrease in ß-carotene yield coincided with a rapid fall in cell number.

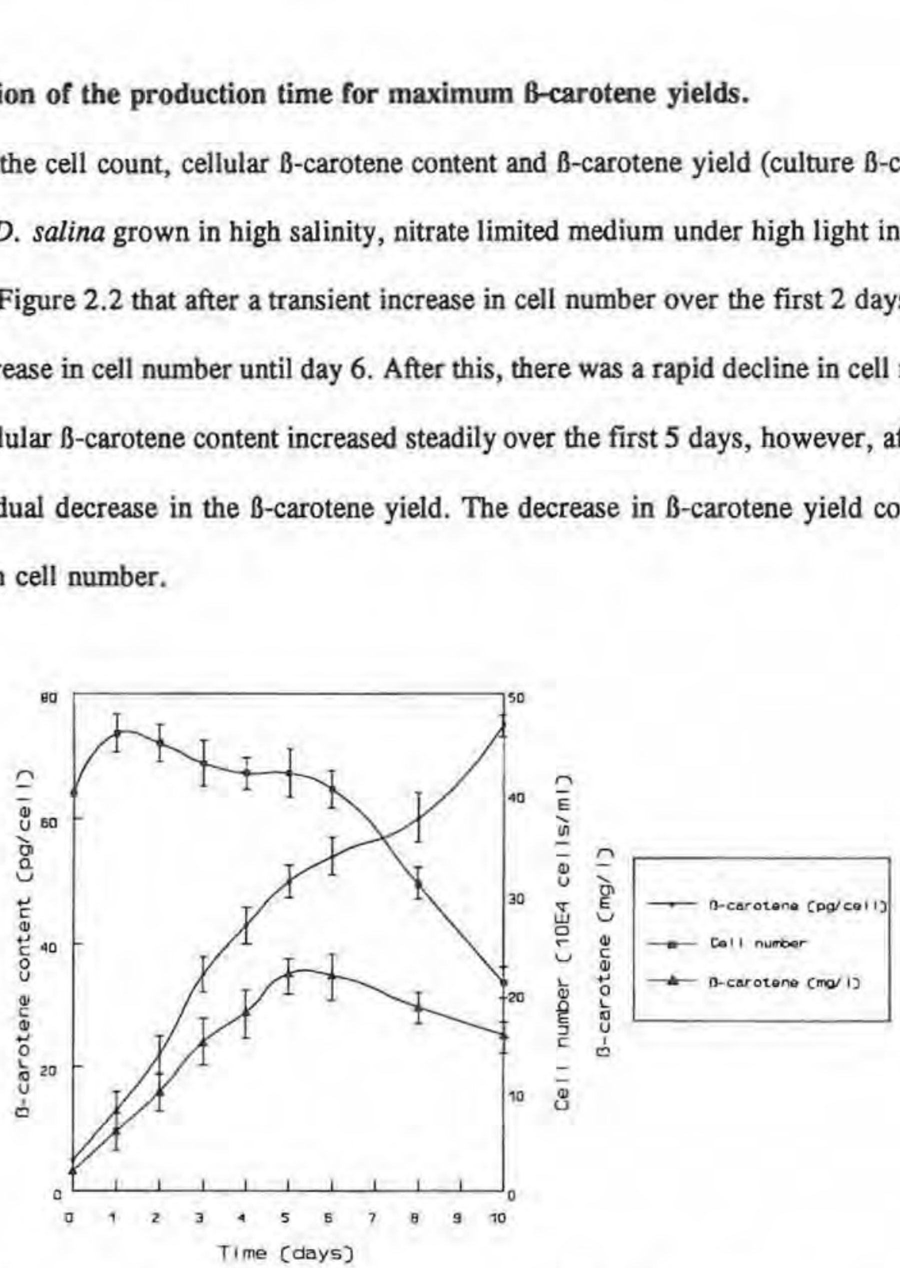


Figure 2.2 Cell growth, ß-carotene content and ß-carotene yield of *D. salina* cells grown in high salinity, nutrient limited medium under high light intensity.

The results of this experiment have shown that under high intensity stress, although there was a steady increase in cellular ß-carotene accumulation, the ß-carotene yield reached a maximum by day 5 of the experiment (Figure 2.2). The sharp fall in cell number was responsible for the concomitant decrease in ß-carotene yield. Based on these results a retention time of 5 days was used for further experiments.

2.4.3 Effect of single stress on the growth and pigment content of *D. salina*

The effect of the following single stresses on *D. salina* growth and pigment content were examined; nitrate limitation (0.2 mM nitrate), phosphate deficiency, sulphate deficiency, high salinity (3 M NaCl) and high light intensity (1000 μmol.m^{-2}.s^{-1}).

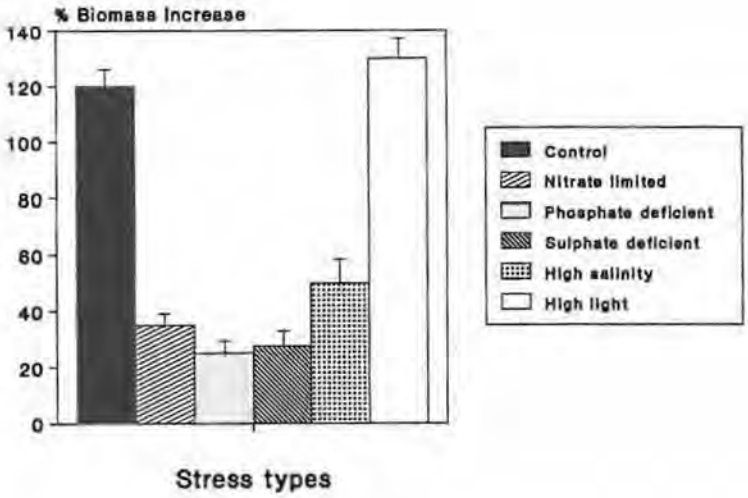


Figure 2.3 Effect of single stress factors on *D. salina* growth. Results reflect day 5 values.

The effect of the various stresses on algal growth is illustrated in Figure 2.3. High salinity, nitrate limitation, phosphate deficiency and sulphate deficiency have a marked impact on *D. salina* growth with a reduction in growth of over 50 %. Phosphate and sulphate deficiency appear to be the most detrimental to *D. salina* growth, whereas high light intensity does not appear to affect algal growth since the percentage biomass increase of algae grown under high light intensity was marginally higher than that of the control (growth medium at 90 μmol.m^{-2}.s^{-1})(Figure 2.3).

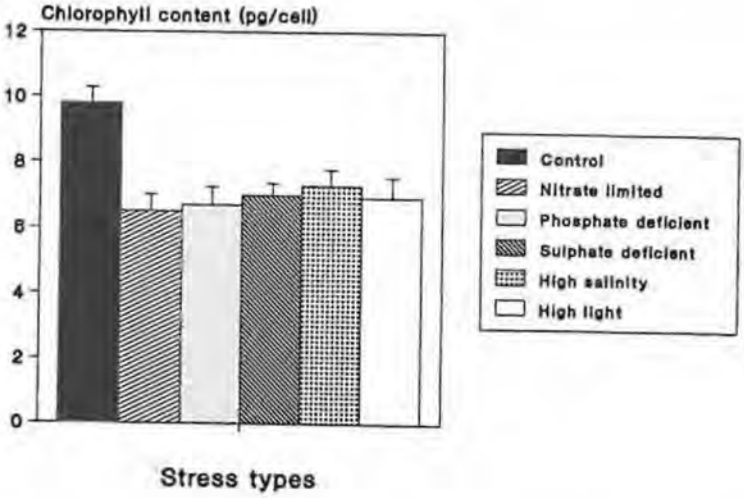


Figure 2.4 Effect of single stress factors on the chlorophyll content of *D. salina* cells. Results reflect day 5 values.

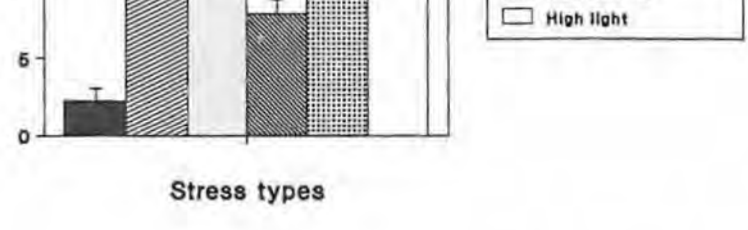


Figure 2.5 Effect of single stress factors on the ß-carotene accumulation in *D. salina*. Results reflect day 5 values.

Figure 2.4 illustrates the effect of the single stresses on the chlorophyll content of *D. salina*. The imposition of single stress on *D. salina* gave rise to a reduction in the chlorophyll content of approximately 3.2 pg.cell^{-1}. What is noteworthy is that the magnitude of the reduction in chlorophyll content was similar for all the stresses examined.

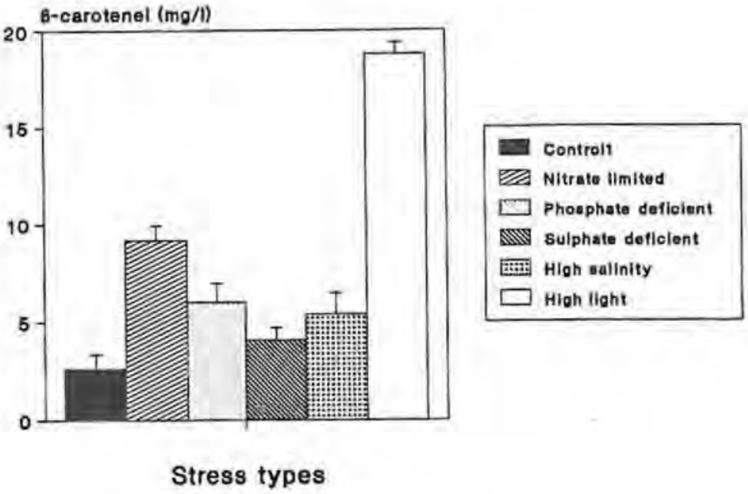


Figure 2.6 Effect of single stress factors on the ß-carotene yield of *D. salina* cells. Results reflect day 5 values.

There was a marked difference in cellular ß-carotene accumulation over the five day stress period in response to the various stresses examined (Figure 2.5). All the stresses examined promoted an increase in the cellular ß-carotene levels when compared to the control, however, high light intensity was clearly the major factor inducing ß-carotene accumulation in *D. salina*, followed by nitrate limitation, phosphate deficiency, high salinity and sulphate deficiency (Figure 2.5). The final day ß-carotene yields, expressed as mg.l^{-1} ß-carotene (Figure 2.6), followed a similar trend with high light intensity being responsible for the highest ß-carotene yield and sulphate deficiency for the lowest yield. A high light intensity of 1000 μmol.m^{-2}.s^{-1} resulted in a final day ß-carotene yield of 18.8

mg.l^{-1}, which is 5 times higher than the control and more than double that of the other stresses. This was due to the high cellular ß-carotene content (Figure 2.5) and to the relatively high biomass productivity (Figure 2.3) recorded under high light intensity conditions.

2.4.4 Effect of multiple stress on the growth and pigment content of *D. salina*.

The effect of multiple stress synergism was examined by monitoring the effect of cumulative stress on *D. salina*. High light intensity was used as the basic stress, as it is the major inducing factor of ß-carotene accumulation in *D. salina*, additional stresses were consecutively applied as follows:

A - high light intensity (control).

B - high light intensity and nitrate limitation.

C - high light intensity, nitrate limitation and high salinity.

D - high light intensity, nitrate limitation, high salinity and phosphate deficiency.

E - high light intensity, nitrate limitation, high salinity, phosphate and sulphate deficiency.

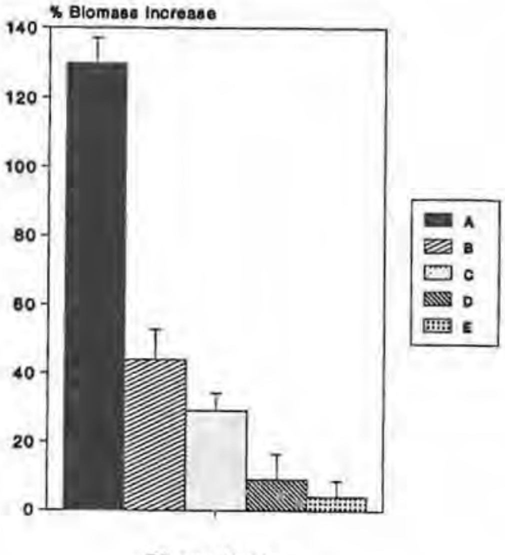


Figure 2.7 Effect of cumulative stress on *D. salina* growth. A - high light intensity (control). B - high light intensity and nitrate limitation. C - high light intensity, nitrate limitation and high salinity. D - high light intensity, nitrate limitation, high salinity and phosphate deficiency. E - high light intensity, nitrate limitation, high salinity, phosphate and sulphate deficiency. Results reflect day 5 values.

Figure 2.7 illustrates the effect of cumulative stress on *D. salina* growth. A sequential decrease in algal productivity was noted (Figure 2.7). This trend was also evident in the cellular chlorophyll content of *D. salina* in response to cumulative stress (Figure 2.8); as increasing stress factors were applied the cellular chlorophyll content dropped from 7 pg.cell^{-1} to 3.6 pg.cell^{-1}. An inverse trend was observed for cellular ß-carotene accumulation (Figure 2.9) and an increase in cellular ß-carotene content from 20 pg.cell^{-1} to 58 pg.cell^{-1} could be observed as additional stresses were applied to high light intensity stress.

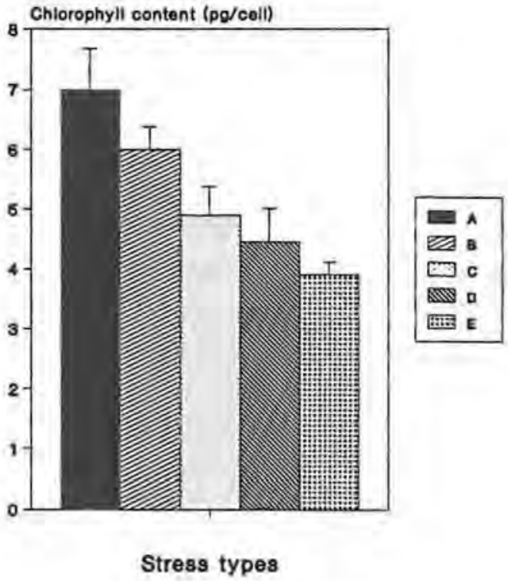


Figure 2.8 Effect of cumulative stress on the chlorophyll content of *D. salina* cells. A - high light intensity (control). B - high light intensity and nitrate limitation. C - high light intensity, nitrate limitation and high salinity. D - high light intensity, nitrate limitation, high salinity and phosphate deficiency. E - high light intensity, nitrate limitation, high salinity, phosphate and sulphate deficiency. Results reflect day 5 values.

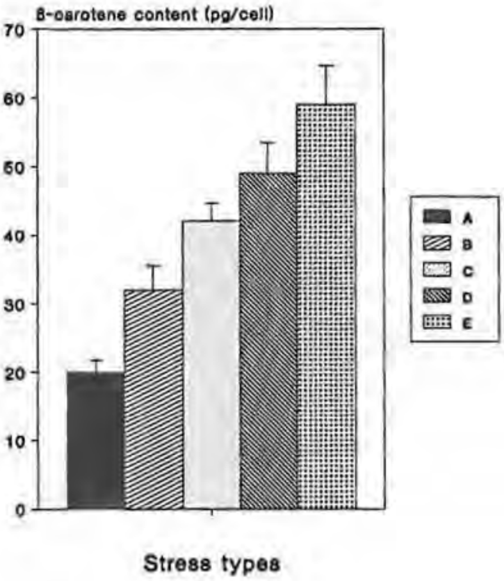


Figure 2.9 Effect of cumulative stress on ß-carotene accumulation in *D. salina*. A - high light intensity (control). B - high light intensity and nitrate limitation. C - high light intensity, nitrate limitation and high salinity. D - high light intensity, nitrate limitation, high salinity and phosphate deficiency. E - high light intensity, nitrate limitation, high salinity, phosphate and sulphate deficiency. Results reflect day 5 values.

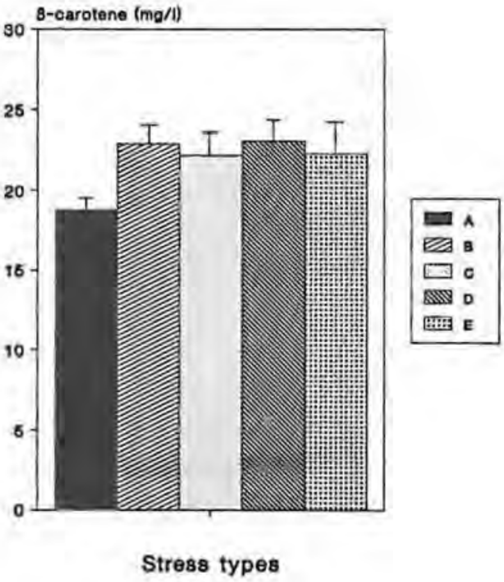


Figure 2.10 Effect of cumulative stress on the ß-carotene yield. A - high light intensity (control). B - high light intensity and nitrate limitation. C - high light intensity, nitrate limitation and high salinity. D - high light intensity, nitrate limitation, high salinity and phosphate deficiency. E - high light intensity, nitrate limitation, high salinity, phosphate and sulphate deficiency. Results reflect day 5 values. Bonferroni multiple range test shows A vs B, C, D, E, $p < 0.001$ and B vs C vs D vs E, $p > 0.05$.

Figure 2.10 shows the final day ß-carotene yields of the various cumulative stress systems. What is noteworthy is that the final day ß-carotene yields were largely similar and were approximately 22 % higher than the single high light stress. A Bonferroni multiple range test showed that the ß-carotene yield in response to high light stress alone was significantly lower than when cumulative stress factors were also applied to high light stress (Figure 2.10). An inverse relationship between algal growth (Figure 2.7) and cellular ß-carotene accumulation (Figure 2.9) in response to cumulative stress can be observed and gave rise to similar net ß-carotene yields.

In the absence of high light stress the final day ß-carotene yield was substantially lower (15 $mg.l^{-1}$) than in the same stress medium (high salinity, nitrate limited, phosphate and sulphate deficient) under high light intensity (22.4 $mg.l^{-1}$) (Figure 2.11). Under low illumination the percentage biomass increase was higher than under high light intensity, however, cellular ß-carotene hyper-accumulation did not occur under low light intensity which accounts for the lower ß-carotene yield (Figure 2.11).

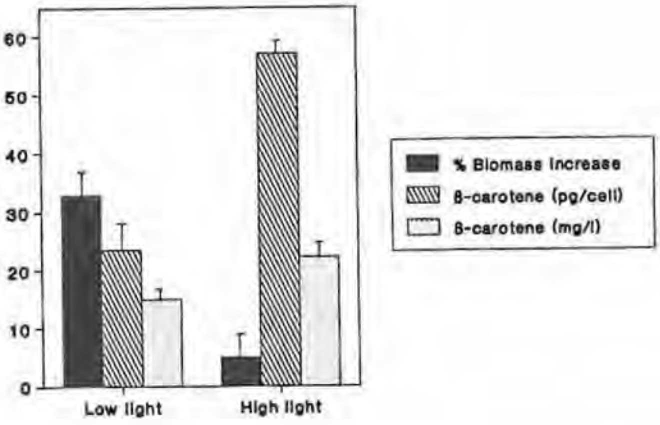


Figure 2.11 Cell growth, ß-carotene accumulation and ß-carotene yield in multiple stress media in response to low and high light intensities. Results reflect day 5 values.

Since the final day ß-carotene yields in response to high light intensity and nitrate limitation were similar to the ß-carotene yield under the full stress complement, the rates of ß-carotene production were compared to determine whether there is an advantage to using multiple stresses. It is apparent from Figure 2.12 that over the second and third days of the experiment the ß-carotene production rate (mg ß-carotene.l^{-1}.day^{-1}) was substantially higher with multiple stress than with high light intensity and nitrate limitation. This resulted in a ß-carotene concentration of 21 mg.l^{-1} being achieved by day 3 with multiple stresses, in comparison to a ß-carotene concentration of 12.6 mg.l^{-1} on the same day for the dual high light intensity and nitrate limitation stress (Figure 2.13).

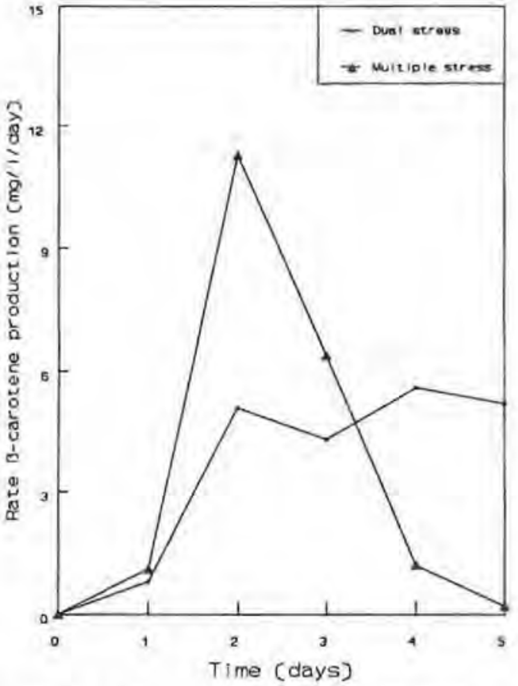


Figure 2.12 Rate of ß-carotene production in dual (high light and nitrate limited) and multiple (high light, high salinity, total nutrient limited) stress systems.

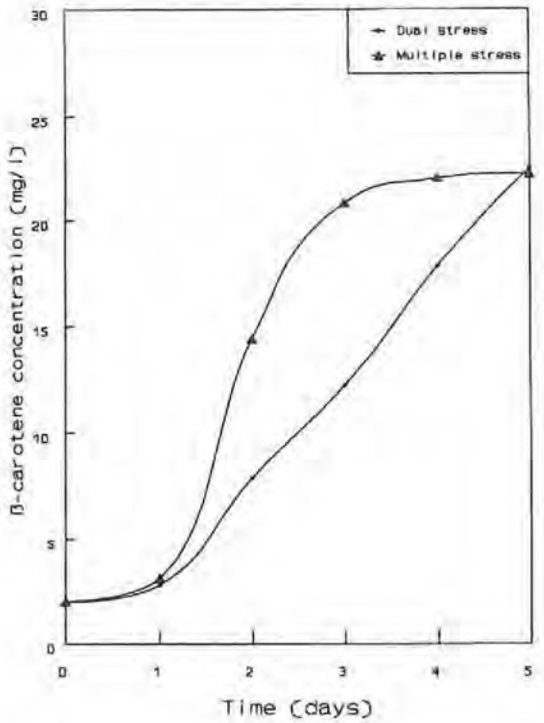


Figure 2.13 ß-carotene production in response to dual (high light and nitrate limited) and multiple (high light, high salinity, total nutrient limited) stress.

2.4.5 Effect of photoperiod on ß-carotene yield.

Previous results have indicated that high light intensity is the major ß-carotene inducing stress. Furthermore, both cellular ß-carotene hyper-accumulation and ß-carotene yields can be increase by the addition of cumulative stresses. The question that remains unresolved is: what is the minimum quantity of high light necessary for maximum ß-carotene yields?

Figure 2.14 shows the effect of various photoperiods on cellular ß-carotene accumulation in multiple stress media (high salinity, nitrate limited, phosphate and sulphate deficient). It is evident from Figure 2.14 that maximum ß-carotene accumulation occurred under continuous illumination (Photoperiod A). The imposition of diurnal cycles with varying photoperiods (Photoperiod B-F) gave rise to lower cellular ß-carotene accumulation than continuous illumination, with the highest ß-carotene accumulation having occurred where high light was maximized in the 12 hour light cycle (Photoperiod

B). A reduction in ß-carotene accumulation was also evident where culture did not receive 12 hours of continuous illumination during the first and last days of the diurnal light cycle (Photoperiod E and F) (Figure 2.14). In cultures where the photoperiod was fluctuated between high and low light intensity intensities (Photoperiod D and F), ß-carotene accumulation was marginally higher than in cultures where the photoperiod was fluctuated between high light intensity and complete darkness (Photoperiod C and D) (Figure 2.14).

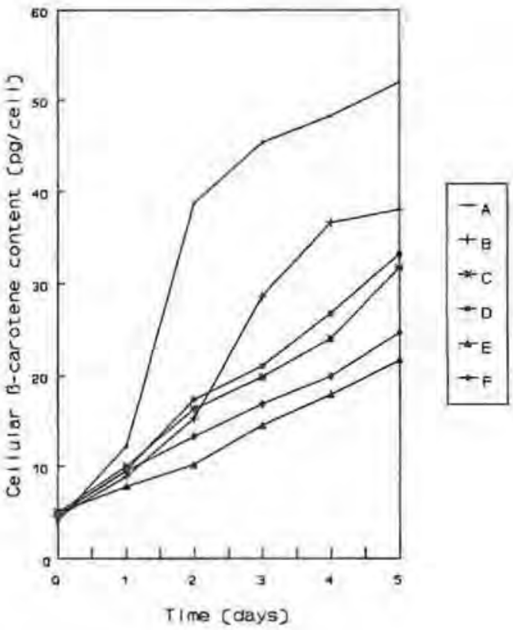


Figure 2.14 Effect of photoperiod on ß-carotene accumulation. A - continuous illumination. B - diurnal cycle, comprised of 12 hours high light intensity and 12 hours darkness. C - diurnal cycle. The 12 hour illumination period was varied as follows: day 1 and 5, continuous illumination; day 2-4, cycle of 1 hour high light intensity and 1 hour darkness. D - diurnal cycle. The 12 hour illumination period was varied as follows: day 1-5, cycle of 1 hour high light intensity and 1 hour darkness. E - diurnal cycle. The 12 hour illumination period was varied as follows: day 1 and 5 , continuous illumination; day 2-4, cycle of 1 hour high light intensity and 1 hour low light intensity. F - diurnal cycle. The 12 hour illumination period was varied as follows: day 1-5, cycle of 1 hour high light intensity and 1 hour low light intensity.

Cell growth (Figure 2.15) followed an opposite trend to that observed for cellular ß-carotene accumulation (Figure 2.14) and was most severely retarded under continuous illumination. The highest cell growth could be observed in cultures which did not receive 12 hours of continuous illumination over the first and last days of the diurnal cycle (Photoperiod E and F)(Figure 2.15).

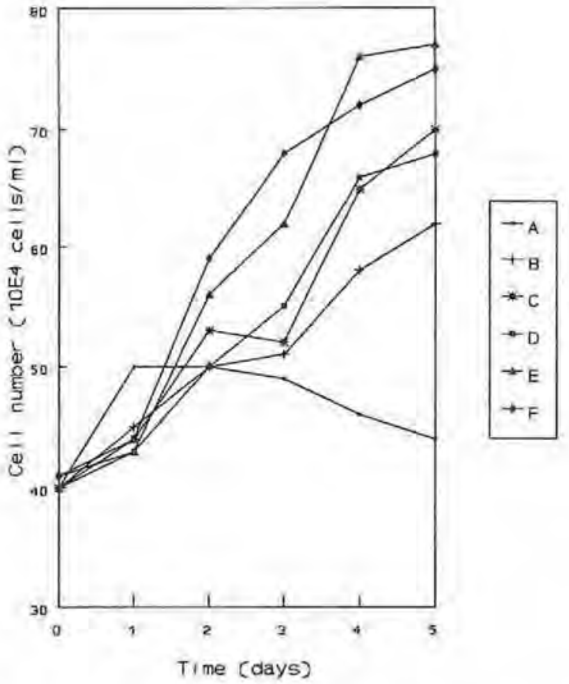


Figure 2.15 Effect of photoperiod on cell growth. A - continuous illumination. B - diurnal cycle, comprised of 12 hours high light intensity and 12 hours darkness. C - diurnal cycle. The 12 hour illumination period was varied as follows: day 1 and 5, continuous illumination; day 2-4, cycle of 1 hour high light intensity and 1 hour darkness. D - diurnal cycle. The 12 hour illumination period was varied as follows: day 1-5, cycle of 1 hour high light intensity and 1 hour darkness. E - diurnal cycle. The 12 hour illumination period was varied as follows: day 1 and 5 , continuous illumination; day 2-4, cycle of 1 hour high light intensity and 1 hour low light intensity. F - diurnal cycle. The 12 hour illumination period was varied as follows: day 1-5, cycle of 1 hour high light intensity and 1 hour low light intensity.

The inverse relationship observed between cellular ß-carotene accumulation (Figure 2.14) and cell growth (Figure 2.15) gives rise to similar day 5 ß-carotene yields (approximately 22-23 $mg.l^{-1}$) in the continuously illuminated culture (Photoperiod A) and in diurnal cultures that received continuous illumination over the first and last 12 hours of their diurnal cycle (Photoperiod B, C, D)(Figure 2.16). The rates of ß-carotene production were, however, vastly different, with continuous illumination producing 93.3 % of the maximum ß-carotene yield by day 3 of the experiment and the 12 hour high light intensity- 12 hour dark photoperiod (B) producing 93 % of the maximum by day 4 of the experiment. ß-carotene yields in cultures which did not receive 12 hours of continuous illumination during the first and last 24 hours of their diurnal cycle were substantially lower than in cultures which received continuous illumination over the first and last 24 hours.

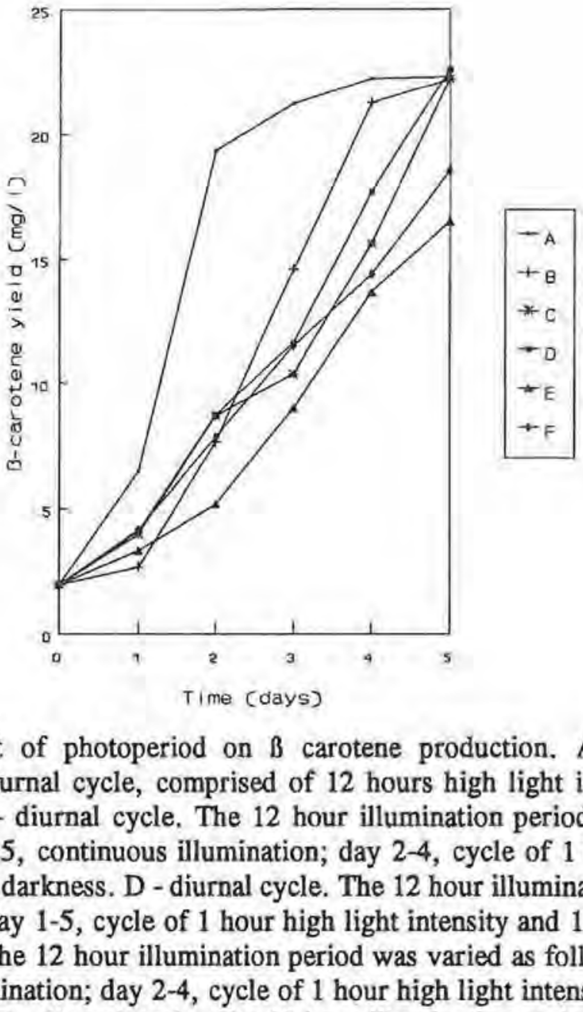


Figure 2.16 Effect of photoperiod on ß carotene production. A - continuous illumination. B - diurnal cycle, comprised of 12 hours high light intensity and 12 hours darkness. C - diurnal cycle. The 12 hour illumination period was varied as follows: day 1 and 5, continuous illumination; day 2-4, cycle of 1 hour high light intensity and 1 hour darkness. D - diurnal cycle. The 12 hour illumination period was varied as follows: day 1-5, cycle of 1 hour high light intensity and 1 hour darkness. E - diurnal cycle. The 12 hour illumination period was varied as follows: day 1 and 5 , continuous illumination; day 2-4, cycle of 1 hour high light intensity and 1 hour low light intensity. F - diurnal cycle. The 12 hour illumination period was varied as follows: day 1-5, cycle of 1 hour high light intensity and 1 hour low light intensity.

Figure 2.17 shows the relationship between the total hours of light received and cell growth and cellular ß-carotene accumulation. It is evident from Figure 2.17 that above 40 hours of high light there is an inverse relationship between cell growth and cellular ß-carotene accumulation. Below 40 hours of high light the inverse relationship does not hold as cellular ß-carotene hyper-accumulation did not occur (Figure 2.17). The lack of ß-carotene hyper-accumulation below 40 hours of high light may explain why cultures which did not receive 12 hours of continuous illumination over the first and last days of the experiment (Photoperiod E and F) did not achieve the 22 mg.l^{-1} yield as did the other cultures (Figure 2.17).

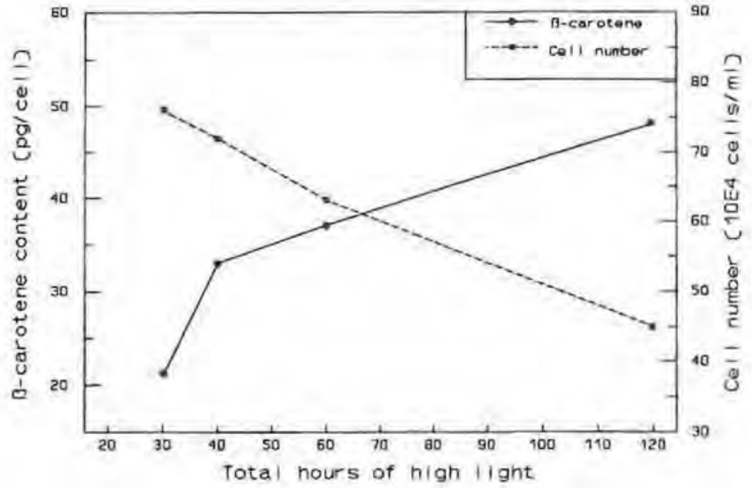


Figure 2.17 Relationship between cell growth, ß-carotene accumulation and the total hours of light received. Values reflect day 5 results.

2.5 DISCUSSION.

A nitrate concentration of 0.2 mM was demonstrated to be optimal for ß-carotene accumulation in *D. salina*. This value is significantly lower than the 1 mM nitrate optimum reported by Ben-Amotz and Avron (1983), however, the experiment described here was undertaken with cells previously exposed to conditions of nitrate sufficiency as would be anticipated in a dual-stage system, whereas the experiment described by Ben-Amotz and Avron (1983) was performed with nitrate-starved cells. The discrepancy between the results may be explained by the fact that when deprived of external nitrogen supply, higher plants have been shown to increase their nitrogen absorbtion potential (Chapin, 1991). In addition, Laubscher (1992) has demonstrated luxury uptake of nitrate in *D. salina*. The luxury uptake of nitrate may alter the nitrate absorption potential of cells previously exposed to high nitrate concentrations.

In a dual-stage system the retention time of biomass in the stress ponds (stress unit) is likely to be a critical factor as it will have a direct bearing on the ß-carotene productivity and the size of the stress ponds. The results reported in Section 2.4 have shown that in multiple stress medium, a peak in ß-carotene yield occurs over day 5 and 6 (Figure 2.2). No increase in ß-carotene yield can be obtained by prolonging the retention time past day 5, due to a rapid fall in cell number, which has a negative impact on the ß-carotene yield.

The impact of a variety of single stresses on the growth and pigment composition of *D. salina* confirms a number of the previously reported findings of other investigators (Mil'ko, 1963a, 1963b; Ben-Amotz and Avron, 1980a, 1983, 1989, Borowitzka, *et al.* 1990, Loeblich, 1982; Lers *et al.* 1990). With the exception of high light intensity, the application of other stresses factors gives rise to a significant reduction (over 50 %) in the growth rate of *D. salina*. The results showed that sulphate and phosphate deficiency were the most detrimental to algal growth. This may be due to their entire absence from the medium when compared to nitrate which was only limited (0.2 Mm NO_3^-). The growth rate was not adversely affected by a light intensity of 1000 μmol.m^{-2}.s^{-1}. The apparent lack of growth inhibition in response to a wide range of non-limiting light intensities has been previously observed (Ben-Amotz and Avron, 1983; Loeblich, 1982).

The results have also shown that the application of all the single stresses examined resulted in a similar decrease in cellular chlorophyll content and a varied increase in the cellular ß-carotene content. The impairment of cellular chlorophyll content in response to stress is well established (Ben-Amotz, 1986; Ben-Amotz and Avron 1982,1983; Borowitzka and Borowitzka, 1988). It is generally accepted that high light intensities promote a smaller chlorophyll antenna size of both Photosystem 1 (PSI) and Photosystem 2 (PSII)(Smith *et al.* 1990). The decrease in chlorophyll content in response to salinity and nutrient stress is probably related to the decreased photosynthetic rates under such stress conditions which warrant smaller light harvesting complexes (Ben-Amotz and Avron, 1983; Jimenez *et al.* 1990).

Ben-Amotz (1986) has shown that light stress is the most important stress associated with ß-carotene accumulation, however, the order of magnitude of other stresses has not been described. The study of various single stresses showed that high light intensity is the major ß-carotene inducing stress in *D. salina*, followed by nitrate limitation, phosphate deficiency, high salinity and sulphate deficiency. The final day ß-carotene yields followed a similar trend to that of the cellular ß-carotene content. Although there was only a 4 pg. cell^{-1} difference in cellular ß-carotene content between nitrate limitation and high light stress (Figure 2.5), the apparent lack of growth inhibition by high light resulted in a superior final day ß-carotene yield. Consequently, the final day ß-carotene yield in response to high light intensity was 88 % higher than that obtained for nitrate stress and 422 % higher than unstressed cells.

Ben-Amotz and Avron (1983) have demonstrated an inverse relationship between the specific growth rate and the cellular ß-carotene content of *D. salina*. The results presented here substantiate this observation and have further shown that the application of additional stresses to high light stress gives rise to an inverse relationship between algal growth and cellular ß-carotene content. The implications of this inverse relationship are that in the presence of high light stress the day 5 ß-carotene yields were similar irrespective of the number of cumulative stress factors acting in conjunction with high light intensity. However, in the absence of high light intensity, ß-carotene yields in response to multiple stress were substantially lower than when high light intensity was present (Figure 10). The reason for this is that cellular ß-carotene hyper-accumulation was not induced in the absence of high light intensity. This study highlights the need for high light intensities in order to achieve maximal ß-carotene yields.

The application of cumulative stress factors to high light stress has been shown to increase the ß-carotene yield by 22 % (Figure 2.10). Furthermore, multiple stress as opposed to dual stress, resulted in an accelerated initial rate of ß-carotene production, which has the potential to reduce the retention time of biomass in the stress ponds. A possible pay-off between increased retention time and higher ß-carotene yields or reduced retention times and marginally lower ß-carotene yields must be considered. The use of multiple stress has an additional advantage in that where specific nutrients are omitted from the stress medium (eg. nitrate, phosphate, and sulphate) the absence of these compounds will reduce the cost of the stress medium.

The results presented in the photoperiod study have highlighted a number of interesting considerations. The results observed with cultures subjected to high light intensities supports claims that ß-carotene hyper-accumulation may have a photoprotective function (Ben-Amotz and Avron, 1983, 1989; Lers *et al.* 1990), as ß-carotene accumulation was shown to be directly proportional to the number of hours of high light to which the culture was exposed (Figure 2.17). More importantly, in terms of the mass culture of the alga, continuous high light is not essential for maximum ß-carotene yields, although the initial rate of ß-carotene production is faster in cultures which receive a greater quantity of high light. The results have demonstrated that less than a third of the total hours of high light used in continuous illumination were necessary for similar net ß-carotene yields (Figure 2.17). However, below a critical point the ß-carotene yield falls rapidly.

It has also been demonstrated that cellular ß-carotene hyper-accumulation was greater with prolonged exposure to high light, however, compromised algal growth was probably due to the more sustained stress. The inverse relationship between algal growth and cellular ß-carotene accumulation observed under these conditions confirms the inverse relationship observed with cumulative stress as similar ß-carotene yields (approximately 22-23 mg ß-carotene.l^{-1}) were obtained with cumulative stress and varying photoperiod (Figure 2.16).

The fact that long photoperiods are not necessary for high ß-carotene yields raises the question as to whether mass culture systems should be located in desert or semi-desert regions like most of the existing commercial ß-carotene production systems (Ben-Amotz and Avron, 1989). The photoperiod study has shown that the initial ß-carotene production rates are faster where stressed cultures are subjected to a greater quantity of high light. If retention time is compromised it may be possible to locate mass culture systems in more temperate regions which on average have fewer hours of sunshine due to cloud cover. The major benefit of locating a ß-carotene production in a more temperate region is that in South Africa these regions are usually not as isolated with more sophisticated infrastructure than the semi-desert regions. However, the effect of fewer hours of sunshine on biomass productivity under optimal growth conditions should be taken into account, as this forms a crucial stage in a dual-stage system. The effect of environmental conditions on biomass productivity is discussed in Chapter 4.

Concluding remarks.

The stress induced ß-carotene accumulation study has shown that the application of both single and multiple stress increases ß-carotene hyper-accumulation in *D. salina*. However, what is relevant in this study is the ß-carotene yield. It appears that high light intensity in conjunction with additional stress factors is necessary for maximum ß-carotene yields. Of particular significance is the observed inverse relationship between cellular ß-carotene accumulation and cell growth which persists throughout the study. The implication of this inverse relationship is that a maximum ß-carotene yield of approximately 22-23 $mg.l^{-1}$ exists under these stress conditions. The application of additional stresses to the system is unlikely to increase ß-carotene yields because, although greater ß-carotene accumulation will take place, cell growth is likely to be further inhibited. This indicates that a probable maximum production knife edge exists in the system, similar to that described for the averaging process where changes in optimal conditions may result in reduced yields. Therefore in order to further manipulate the stress system, future research should be focused on increasing the rate of ß-carotene production in order to decrease the retention time of cultures in the stress ponds. This may be the only way to further improve the productivity of the second stage of the dual-stage system.

CHAPTER 3

MONITORING THE PHYSIOLOGICAL RESPONSE OF *D. SALINA* TO HIGH LIGHT STRESS

Summary.

The physiological response of *D. salina* to high light stress was monitored with particular reference to the intracellular partitioning of ß-carotene between thylakoid membrane-bound and globular ß-carotene pools. Two responses to high light stress were observed, the first response is primarily a photoadaptive response and is characterised by increased globular ß-carotene accumulation and the peripheral localization of the ß-carotene globules. The second response appears to be a maintenance response and is characterised by a stabilization of the photosynthetic rate and sustained globular ß-carotene accumulation, albeit at a slower rate. A link between the extent of the initial photoinhibitory response and the degree of ß-carotene hyper-accumulation has been postulated.

3.1 INTRODUCTION.

Light intensity has been shown to have a distinct affect on the carotenoid content of both higher plants and algae (Goodwin, 1980a). The results obtained in the laboratory studies reported in Chapter 2 have unequivocally confirmed that light intensity is the primary factor influencing ß-carotene accumulation in *D. salina*. In order to improve the ability to manipulate ß-carotene accumulation in the alga, a clearer understanding of the physiological responses associated with light-induced ß-carotene hyper-accumulation is required.

Carotenoids can be divided into the oxygen-free carotenes (eg. ß-carotene) and the xanthophylls, which contain oxygen in different forms, such as one or several hydroxy or epoxy groups (eg. zeaxanthin, violaxanthin, antheraxanthin)(Lichtenthaler, 1987). Carotenoids are present in the thylakoid membranes of all eukaryotes and it is generally believed that all carotenoids are synthesised in the chloroplast stroma and are thereafter attached to the thylakoid membrane (Grumbach, 1979). Carotenoids are synthesised via the terpenoid pathway from mevalonic acid (Figure 3.1)(Goodwin, 1980a; Creelman, 1989). The terpenoid pathway is also thought to be responsible for the biosynthesis of the plant stress hormone abscisic acid (ABA), which is ubiquitous in higher plants, and is also produced by algae (Hirsch *et al.* 1989) and fungi (Creelman, 1989). The controversy as to whether ABA is produced directly from mevalonic acid or indirectly from the xanthophylls remains unresolved (Figure 3.1)(Creelman, 1989).

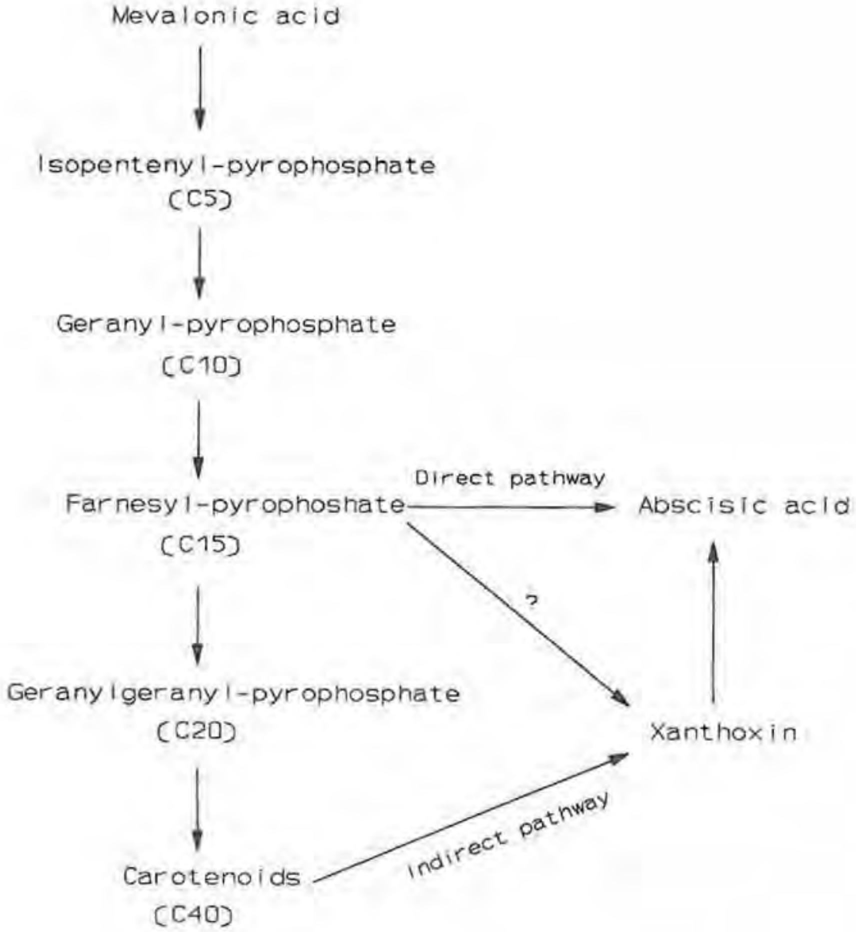


Figure 3.1 Terpenoid pathway and the theoretical biosynthetic pathways for abscisic acid (Creelman, 1989).

The well-established functions of carotenoids in plants are all related to their ability to absorb visible light. It has been suggested for many years that they protect the photosynthetic apparatus against the destructive effects of light and oxygen (Goodwin, 1980b). One line of evidence supporting this role is found in mutants of algae and higher plants which are deficient in carotenoid biosynthesis and are only viable when maintained in low light intensities (Krinsky, 1971). Exposure of photosynthetic tissue to excess light results in photoinhibition and a consequent reduction in photosynthetic activity, due primarily to a sustained reduction in the photochemical efficiency of PSII (Demmig and Bjorkman, 1987). Photoinhibition may result from two different processes operating singly or simultaneously: (a) decreased photosynthetic activity caused by direct damage to PSII reaction centres and (b) an increase in the rate constant for non-radiative dissipation of excitation energy (Demmig and Bjorkman, 1987). Recent evidence (Demmig *et al.* 1987) has implicated the xanthophyll cycle (Figure 3.2), especially the xanthophyll zeaxanthin, in the dissipation of non-radiative energy produced by excess excitation of the PSII. The operation and function of the xanthophyll cycle in photoprotection has recently been reviewed (Demmig-Adams and Adams, 1992). Although ß-carotene may partially serve as a light-harvesting pigment, its main function seems to be the protection of chlorophyll *a* from photooxidation in or near the reaction centre (Lichtenthaler, 1987). The singlet oxygen oxidation products of ß-carotene have recently been isolated and identified *in vitro* by Stratton *et al.* (1993).

Biosynthetic studies have revealed the presence of two ß-carotene pools in chloroplasts from both the alga *Chlorella pyrenoidosa* and the seedlings of radish *Raphanus sativus* (Grumbach, 1979). The smaller pool of ß-carotene is available for the biosynthesis of other carotenoids, while the larger pool serves to protect the photosynthetic apparatus of the chloroplast from damage by light (Harrison, 1986). In addition to their functional role in light, carotenoids are also structural elements essential for the assembly of the photosystems (Plumley and Schmidt, 1987).

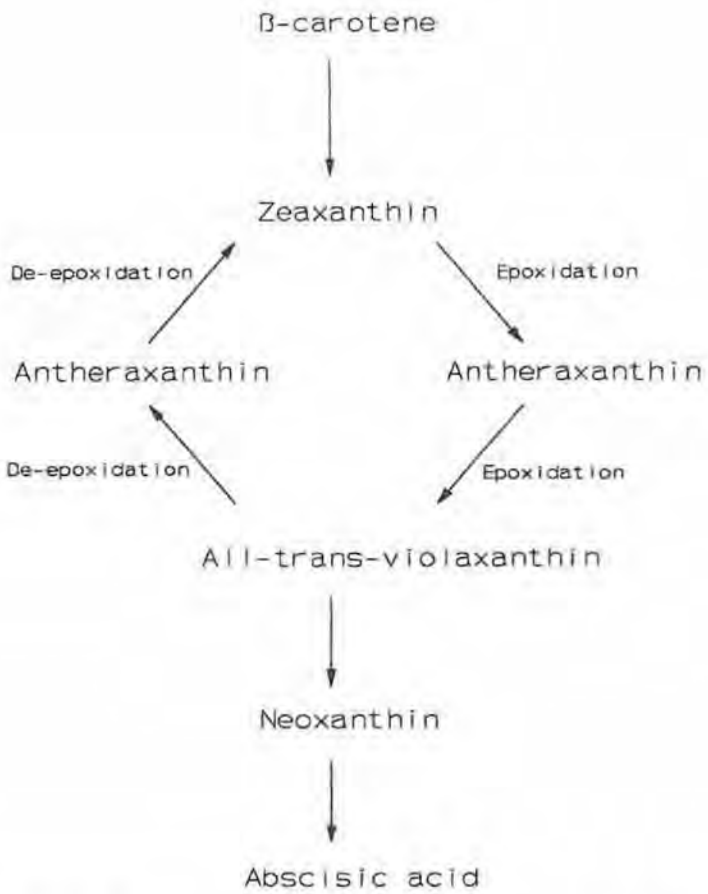


Figure 3.2 Xanthophyll cycle and proposed pathway to ABA (Demmig *et al.* 1987).

Unlike most higher plants and green algae, the alga *D. salina* accumulates ß-carotene within oily globules in the interthylakoidal spaces of the chloroplast (Ben-Amotz *et al.* 1982). Three different hypotheses have been proposed for the function of the ß-carotene globules (Ben-Amotz *et al.* 1982). The first suggests that ß-carotene accumulation protects against chlorophyll catalysed singlet oxygen production under high light intensities, however, the large distance between ß-carotene globules and the thylakoid located chlorophyll is unlikely to allow efficient quenching of singlet oxygen, or any other chlorophyll generated free-radical. The second potential role of the globular ß-carotene is as a carbon-sink under conditions of growth limitation (Borowitzka and Borowitzka, 1988). However, as pointed out by Ben-Amotz *et al.* (1982), the alga does not utilize the accumulated ß-carotene when transferred to darkness or to a carbon dioxide free medium. The third and most widely supported hypothesis is that the ß-carotene globules protect the cell against injury by high intensity irradiation under limited growth conditions by acting as a screen to absorb excess irradiation (Ben-Amotz *et al.* 1982; Lers *et al.* 1990; Ben-Amotz and Avron, 1990).

This hypothesis is further supported by studies with strains of *D. salina* which do not accumulate ß-carotene (Ben-Amotz *et al.* 1989) and studies where carotenogenic *D. salina* has been photobleached with herbicides (Ben-Amotz *et al.* 1987). The inability to over-accumulate ß-carotene prevents recovery from photoinhibition and leads to cell death. Furthermore, Ben-amotz *et al.* (1989) and Gomez-Pinchetti *et al.* (1992) have shown that *D. salina* cells with high ß-carotene contents exhibit higher photosynthetic rates on exposure to high light intensities, than cells with low ß-carotene contents. The results indicate that high levels of ß-carotene imply more resistance to photoinhibition at high light intensities (Ben-Amotz *et al.* 1989; Gomez-Pinchetti *et al.* 1992).

The regulation of carotenoid metabolism in both higher plants and algae is not fully understood, however, it has recently been proposed that carotenoid biosynthesis may be regulated by the plant hormone ABA (Cowan and Rose, 1991; Cowan *et al.* 1992). The evidence is almost conclusive that ABA is produced by a process involving oxidative cleavage of epoxy-carotenoids to yield xanthoxin which is then sequentially metabolized to ABA-aldehyde and ABA (Indirect pathway, Figure 1) (Li and Walton, 1990; Parry and Horgan, 1991; Rock and Zeevaart, 1990; and Zeevaart *et al.* 1989). Furthermore, recent reports have provided evidence that zeaxanthin is the major precursor (Duckham *et al.* 1991; Gilmore and Yamamoto, 1991).

Activation of the carotene pathway in the fungi *Blakeslea trispore* by ABA has been demonstrated (Dendekar *et al.* 1980) and recently, Cowan and Rose (1991) have linked increased ABA biosynthesis with the onset of ß-carotene accumulation in *D. salina* in response to salinity stress. A possible interrelationship between ß-carotene hyper-accumulation in *D. salina* and ABA production has been suggested. Cowan *et al.* (1992) have proposed an integrated stress response in *D. salina* based on the "stretched activated ion channel model" (Kirst, 1990) and mediated by the intracellular partitioning of ABA within the cell. However, a similar ABA response to other physiological stress (eg. high light intensities, nutritional limitation) is necessary to provide a clear interrelationship between ß-carotene accumulation and ABA production.

Recently, Shaish *et al.* (1993) have suggested that photosynthetically produced oxygen radicals are involved in triggering ß-carotene hyper-accumulation in *D. salina* var *bardawil*. The addition of promoters of oxygen radicals or of azide (an inhibitor of catalase and superoxide dismutase) under conditions which are sub-optimal for cellular ß-carotene hyper- accumulation was shown to greatly enhance ß-carotene synthesis, photodegredation of chlorophyll and inhibition of photosynthesis.

3.2 RESEARCH OBJECTIVES.

It is well established that in *D. salina* the accumulated ß-carotene is localised in oily globules within the interthylakoid spaces of the chloroplast and that the globules are thought to protect against high intensity irradiation (Ben-Amotz *et al.* 1982; Ben-Amotz *et al.* 1989; Ben-Amotz and Avron, 1989, 1990; Borowitzka and Borowitzka, 1988). However, the relative partitioning between thylakoid membrane bound ß-carotene and globular ß-carotene in response to stress has not been examined. Significant factors regarding this response are the timing and magnitude of the relative partitioning and the flow of carbon to the separate ß-carotene pools. Insight into ß-carotene partitioning in *D. salina* may allow for further control of ß-carotene production via the manipulation of various metabolic pathways or by direct manipulation of the stress source (ie. light intensity and duration). Furthermore, a clearer understanding of the high light stress response may provide a link between the stress retention time and ß-carotene productivity and may provide an explanation as to why continuous high light illumination is not necessary for high ß-carotene yields.

The following research objectives were identified to address the above issues:

1. To determine photosynthetic rates under low and high light intensities and to examine the effect of cumulative stress on the photosynthetic rate.
2. To examine the storage of starch under low and high light intensities.
3. To monitor the partitioning of ß-carotene between the two ß-carotene pools - membrane bound and globular ß-carotene.
4. To determine the carbon source of the two ß-carotene pools.

3.3 MATERIAL AND METHODS.

3.3.1 Algal cultivation medium.

D. salina was cultivated in 1.5 M NaCl growth medium (Ben-amotz and Avron, 1983) as detailed in Chapter 2. Photosynthetic oxygen evolution studies were also conducted in high salinity, nitrate limited (0.2 mM NO_3^-), phosphate and sulphate deficient medium or nitrate limited medium (see Chapter 2). Cell counts and total chlorophyll were determined as previously described in Chapter 2.

3.3.2 Photosynthetic carbon fixation.

The rate of carbon fixation was measured to monitor the effects of high light stresses on photosynthesis. Log phase cultures of *D. salina* grown under a light intensity of 90 $\mu mol.m^{-2}.s^{-1}$ were centrifuged at 2000 g for 10 minutes, washed with 1.5 M KCl and resuspended in growth medium. Cultures were placed into 1 l beakers at a depth of 6 cm. The cultures were incubated under either low illumination (90 $\mu mol.m^{-2}.s^{-1}$) or under high illumination (1000 $\mu mol.m^{-2}.s^{-1}$) at 30 °C. Representative samples (40 ml) of these cultures were taken each day and placed in narrow 100 ml beakers at a depth of 6 cm. Radiolabelled $NaH[^{14}C0]_3$ (Amersham) was added to each representative sample and 1 ml sub-samples were taken at various time intervals. The sub-samples were placed in a solution containing 0.5 ml 20 % (v/v) formaldehyde and 1 ml 1 M HCl and were incubated at 20

°C overnight. Aqueous scintillant (Hewlett Packard 299) was added (7 ml) and the samples were counted using a Beckman LS 315 OT scintillation counter. The amount of carbon fixed was determined according to Gocke and Hoppe (1978).

3.3.3 Determination of relative photosynthetic oxygen evolution.

The relative rate of oxygen evolution was monitored to determine the effect of high light on photosynthesis. The effect of nitrate limitation (0.2 mM NO_3^-) and of multiple stress (high salinity nitrate limitation, phosphate and sulphate deficiency) in conjunction with high light was also examined.

A log phase *D. salina* culture grown in growth medium was harvested by centrifugation at 2000 g for 10 minutes. Cells were washed with 1.5 M KCl and resuspended in growth medium, nitrate limited medium, and multiple stress medium. Cultures were allowed to equilibrate for 30 minutes at a light intensity of 90 μmol.m^{-2}.s^{-1}. The control culture (growth medium) was maintained at the same light intensity and the other cultures were then placed under a light intensity of 1000 μmol.m^{-2}.s^{-1}. Samples (1.5 ml) were taken at 0, 4, 8, 24, 48, 72 and 96 hours and immediately placed in a Hansatech oxygen microprobe illuminated at the respective light intensities. The oxygen evolution rate was monitored once a linear response was observed. The photosynthetic oxygen evolution rate was expressed as relative (to starting) oxygen evolution rate per mg chlorophyll per hour.

3.3.4 Starch determination.

Starch was determined enzymatically using starch determination kits (Boehringer Mannheim, Cat. No. 207748). Algal cells (10 ml) were recovered by centrifugation and resuspended in 10 ml distilled water. The cell lysate was agitated vigorously for 5 minutes and the algal pellet was recovered by centrifugation at 5000 g for 10 minutes. This step is essential to remove soluble mono and oligosaccharides. The lysed algal pellet was then assayed for starch content by enzyme analysis.

3.3.5 Dual-labelling study of ß-carotene partitioning.

A 4 l *D. salina* culture (50-60 x 10^4 cells.ml^{-1}) was centrifuged at 2000 g for 15 minutes. The cell pellet was resuspended in 400 ml of growth medium containing 1.5 mCi NaH[^{14}CO]$_3$. The enriched culture was incubated for 24 hours under a light intensity of 90 μmol.m^{-2}.s^{-1}. The cells were separated by centrifugation (2000 g for 10 min), washed twice with 1.5 M KCl and resuspended in 4 l cold growth medium. The cultures were left for 1 hour under the same light intensity in order to stabilize.

Tritiated (^{3}H) mevalonic acid lactone (1.5 μCi) was added and the culture was incubated for 1 hour. The culture was then split into 2 l fractions and incubated at 28 °C for 96 hours under light intensities of 90 μmol.m^{-2}.s^{-1} and 1000 μmol.m^{-2}.s^{-1}, respectively. Samples were taken at various time intervals for the first 12 hours and then daily.

3.3.6 Partitioning of ß-carotene between thylakoid membrane and interthylakoidal globules.

Membrane associated and globular ß-carotene was separated using a modified technique for the purification of ß-carotene globules (Ben-Amotz *et al.* 1982). Samples (10 ml) were centrifuged at 2000 g for 10 minutes. The cell pellet was resuspended in 4 ml distilled water and mixed vigorously for 15 minutes. This step induces osmotic lysis and the release of ß-carotene globules from the membranes. The mixing time was pre-determined and microscopic examination revealed that 15 minutes of vigourous mixing was sufficient to release the ß-carotene globules from the membrane fraction. The cell lysate was then subjected to centrifugation at 12 000 g for 15 minutes and the supernatant containing the ß-carotene globules was recovered. All operations were performed at 4 °C, as this was essential to maintain the integrity of the membranes. The membrane (pellet) and the globular (supernatant) ß-carotene fractions were then separated and quantified using HPLC.

3.3.7 HPLC determination and radio-label quantification of ß-carotene partitioning

The membrane and globular ß-carotene fractions were extracted for total carotenoids in ice-cold acetone (-20 °C) containing 2,6-Di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene, BHL; 20 mg.l^{-1}), added as an antioxidant, and stored at -20 °C under nitrogen in the dark until purified.

For purification of carotenoids, acetone extracts were reduced to dryness *in vacuo*, redissolved in 70 % methanol and applied to Sep-pak C_{18} cartridges. The total carotenoids were eluted with 100 % acetone and reduced to dryness *in vacuo*. The dried extracts were then resuspended in a small volume of 100 % acetone, filtered through 0.2 μm centrifuge filters (Separations) and reduced to dryness under nitrogen. The dried extracts were then stored in this state until analyses.

Dried extracts were resuspended in 60 μl aliquot of isopropanol/dichloromethane (9:1 v/v) and ß-carotene was quantified by reverse-phase HPLC using 0-100 % ethyl acetate in acetonitrile/water (9:1 v/v) each containing 0.1 % triethylamine. ß-carotene was quantified by peak integration and calibration with ß-carotene standards (Sigma). The liquid chromatography apparatus consisted of a Beckman Systems Gold 126 programmable solvent module, a Rheodyne 7125 injector (50 μl loop) and a 5 μm C_{18} Nucleosil column (250 x 4.6 mm i.d.)(Macherey-Nagel). The flow rate was set at 0.8 ml.min^{-1} and peaks were monitored at 410 nm with a Beckman Systems Gold 168 diode array detector coupled to a PC driven by Beckman System Gold system driver software.

The ß-carotene peak was collected with a LKB Bromma 2112 Redirac fraction collector and fractions were pooled and transferred to aqueous/non-aqueous scintillant (Hewlet Packard 299). ^{3}H and ^{14}C was quantified (cpm) using a Beckman LS 315 OT scintillation counter.

3.3.8 Ultrastructure of low and high light grown *D.salina* cells - transmission electron microscopy.

A time course ultrastructure study of low and high light grown *D. salina* cells was undertaken to monitor the partitioning of ß-carotene globules under high light. Samples were prepared for electron microscopy following the methods of Cross (1979).

Cells were centrifuged at 2000 g for 10 minutes, the cell pellet was immediately entrapped in 5 % agarose gel (Sigma, low melting point) held at 36 °C and then rapidly cooled in an ice bath. The solid agarose pellet was cut into 2 mm by 2 mm cubes and fixed overnight in 2.5 % glutaraldehyde in 0.1 M phosphate buffer. Samples were then washed in 0.1 M phosphate buffer followed by post-fixation in 1 % phosphate buffered osmium tetroxide. Following further washing with 0.1 M phosphate buffer, the samples were dehydrated by transfer through a series of ascending concentrations of ethanol (30 % to 100 %). This was followed by two 15 minute washes of propylene oxide and transition to a resin medium through three propylene oxide: epoxy resin mixtures (75:25, 50:50, 25:75) and finally pure epoxy resin. Samples were then transferred to fresh epoxy resin in embedding moulds, and polymerization was allowed to take place over 36 hours at 60 °C.

Ultra-thin sections were cut with a LKB 111 ultramicrotome and collected onto alcohol-washed grids. Sections were then post-stained with 5 % aqueous uranyll acetate for 30 min, followed by Reynold's lead citrate for 90 minutes. Grids were examined by transmission electron microscopy (TEM) using a JEOL JEM 100 CK11 transmission electron microscope.

3.4 RESULTS.

3.4.1 Algal growth in response to high light stress.

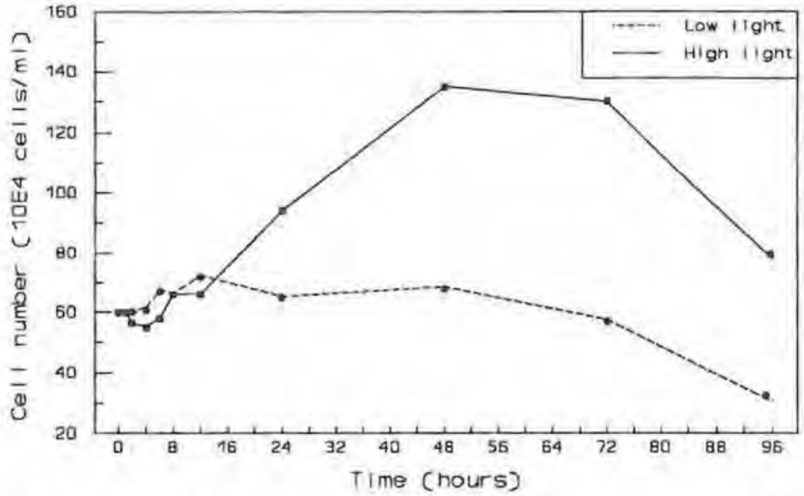


Figure 3.3 Growth curves of *D. salina* cells exposed to low and high light intensities in growth medium.

The growth curves of *D. salina* grown under low and high light intensities are depicted in Figure 3.3. It is apparent from Figure 3.3 that high light intensity stimulated *D. salina* growth to a greater degree than low light intensity. *D. salina* cells cultured under high light intensities exhibited an initial decrease in cell number over the first 6 hours, and thereafter, cell numbers increased (Figure 3.3). After 48 hours cell growth ceased in both low and high light illuminated cultures and there was a marked decline in cell numbers by 96 hours.

3.4.2 Photosynthetic carbon fixation in response to high light stress.

The results depicted in Figure 3.4 show changes in the carbon fixation rate of *D. salina* on exposure to low and high light intensity. The results show that over the first 12 hours the overall carbon fixation rate of high light stressed cells was substantially lower than in cells grown under low light intensities. The reduction in the carbon fixation rate may indicate an initial reduction in the photosynthetic rate. This possible high light induced inhibition of photosynthesis coincided with the initial reduction in cell number observed in Figure 3.3.

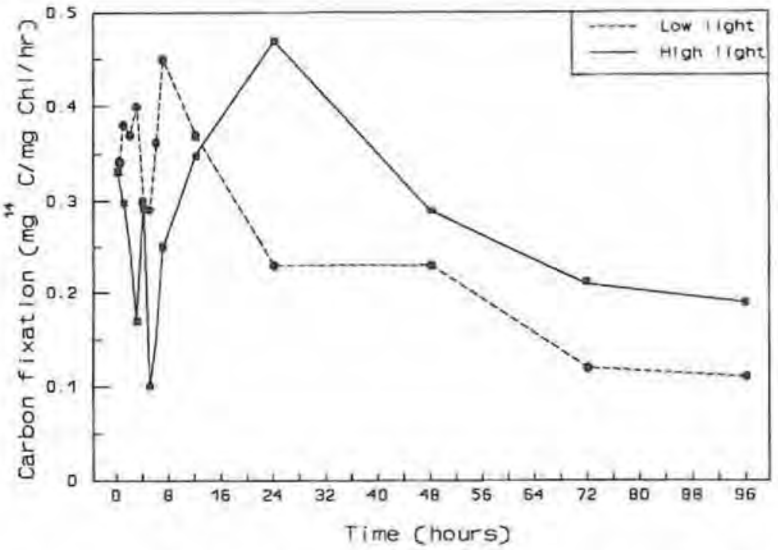


Figure 3.4 Carbon fixation rates of *D. salina* cells exposed to high and low light intensities in growth medium.

By 24 hours, the carbon fixation rate of high light grown cells recovered and remained consistently higher than the carbon fixation rate in the low light grown cells (Figure 3.4). There was a gradual decline in the carbon fixation rate after 24 hours in both the low light and the high light cultures (Figure 3.4).

3.4.3 Photosynthetic oxygen evolution in response to high light stress.

The results depicted in Figure 3.5 show changes in the relative oxygen evolution rates of cultures subjected to various stress systems. It is apparent from Figure 3.5 that there was a sharp reduction in the oxygen evolution rate of high light stressed cultures over the first 4 hours. The reduction in the oxygen evolution rate was more pronounced in cultures where additional stress factors such as nitrate limitation and multiple stress (high salinity, nitrate limited, phosphate and sulphate deficiency) were imposed. The initial reduction in the oxygen evolution rate in response to high light stress (Figure 3.5) was consistent with the decline in photosynthetic carbon fixation observed in Figure 3.4.

Shaish *et al* (1993) have also shown a decrease in the oxygen evolution rate of low light adapted cells over the first 8 hours of high light stress. Furthermore, Ben-Amotz *et al.* (1989) and Gomez-Pinchetti (1992) have shown that low light adapted cells (green cells) have reduced photosynthetic rates on exposure to high light. This initial reduction in both the carbon fixation rate and the oxygen evolution rate indicates an initial photoinhibitory response to high light stress.

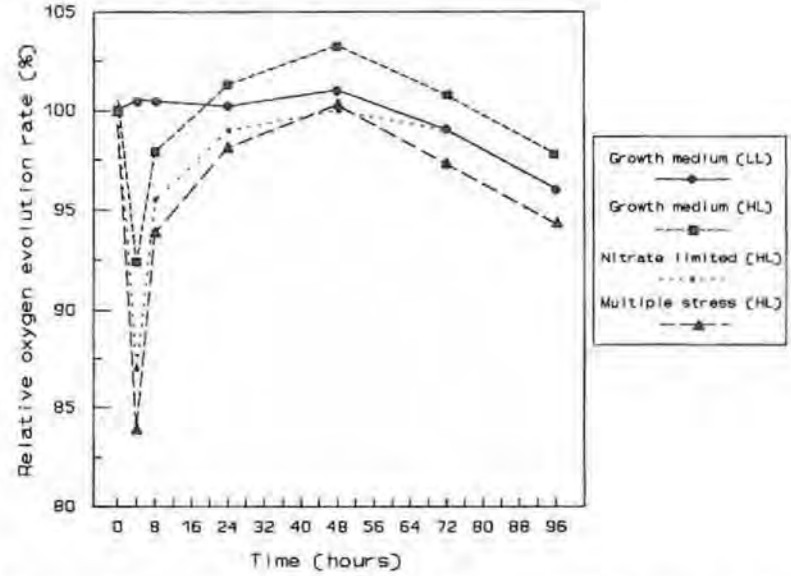


Figure 3.5 Relative oxygen evolution rate of *D. salina* cells grown in various stress media under high light intensity. Control cells grown in growth medium under low light intensity.

A steady recovery in the oxygen evolution rate in all stress cultures was observed from 8 to 24 hours (Figure 3.5). In the high light stress culture (growth medium) the oxygen evolution rate recovered to a marginally higher level than the low light grown cells, whereas recovery of the oxygen evolution rates in cultures where additional stress factors were acting was substantially slower. By 48 hours there was a gradual decline in the oxygen evolution rate in all cultures (Figure 3.5) which coincided with the cessation of algal growth (Figure 3.3).

3.4.4 Starch accumulation in response to high light stress.

Starch forms one of the major storage pools of carbon in photosynthetic organisms. Ben-Amotz (1987) has shown that under conditions of nutritional and high light stress there is a massive increase in the total carbohydrate content of *D. salina*. The starch content of *D. salina* was monitored to ascertain whether starch accumulation is responsible for the increase in carbohydrate content of stressed cells (Ben-Amotz, 1987) and to determine whether it forms an integral part of the stress response.

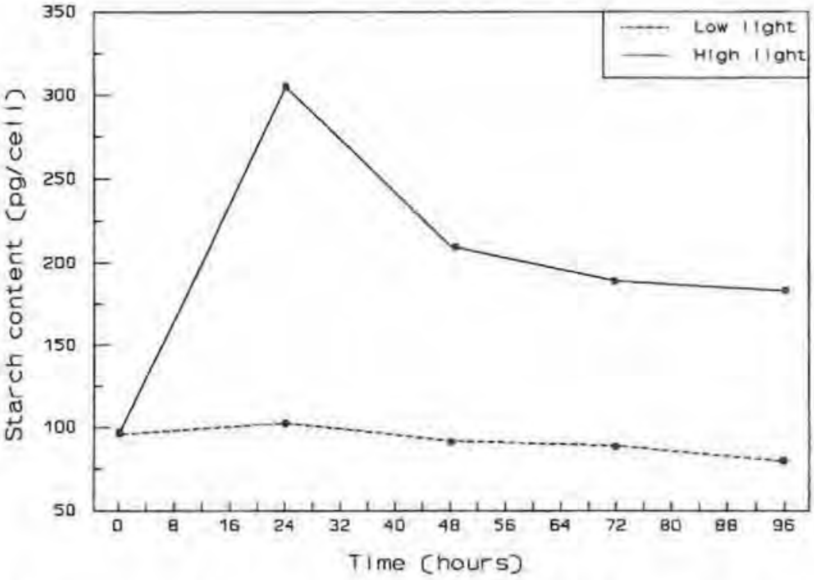


Figure 3.6 Starch content of *D. salina* cells exposed to low and high light intensities.

Figure 3.6 shows the starch content of *D. salina* cells on exposure to high and low light intensities. The starch content of low light grown cells remained relatively constant over the growth period (Figure 3.6). However, the starch content of high light stressed cells increased dramatically over the first 24 hours and then decreased sharply during the next 48 hours. After this, the starch content remained relatively constant (Figure 3.6).

The marked increase in the starch content over the first 24 hours in the high light stressed cells was consistent with the high photosynthetic carbon fixation and oxygen evolution rates observed at 24 hours in high light stressed *D. salina* cells (Figure 3.4 and 3.5). The decrease in starch content over the 24 to 48 hours period (Figure 3.6) coincided with a decrease in the photosynthetic rate of the cells (Figure 3.4 and 3.5) and an increase in cell growth (Figure 3.3). During rapid cell growth an increase in the photosynthetic carbon fixation is anticipated, however, the results indicate that metabolism of stored carbon (ie. starch) may compensate for the decrease in photosynthetic rate during active cell growth.

3.4.5 Pigment content in response to high light stress.

Figure 3.7 depicts the chlorophyll content of *D. salina* in response to low and high light intensities. The chlorophyll content of low light grown cells remained relatively constant over the experimental period, whereas, a sharp decrease in the chlorophyll content of cells grown under high light intensities was observed over the first 24 hour period. The chlorophyll content stabilized between 24 to 72 hours and subsequently decreased from 72 to 96 hours (Figure 3.7).

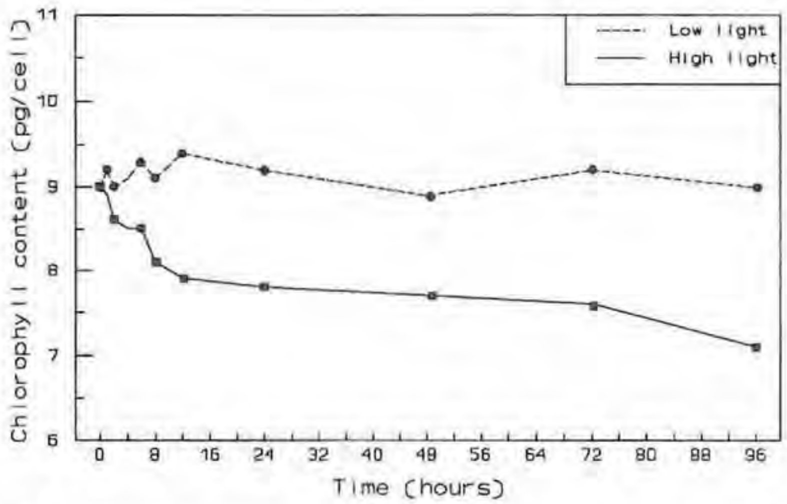


Figure 3.7 Chlorophyll content of *D. salina* cells exposed to low and high light intensities in growth medium.

The ß-carotene content of cells grown under low and high light intensities is depicted in Figure 3.8. The ß-carotene content of low light grown cells decreased over the first 24 hours and then gradually increased over the remainder of the experiment (Figure 3.8). This contrasts strongly with the ß-carotene content of high light stressed cells. The results showed two clearly defined ß-carotene accumulation stages; the first stage occurred during the first 24 hour period, followed by a 48 hour period when the ß-carotene content remained constant; the second accumulation stage occurred over the final 24 hours of the experiment (Figure 3.8).

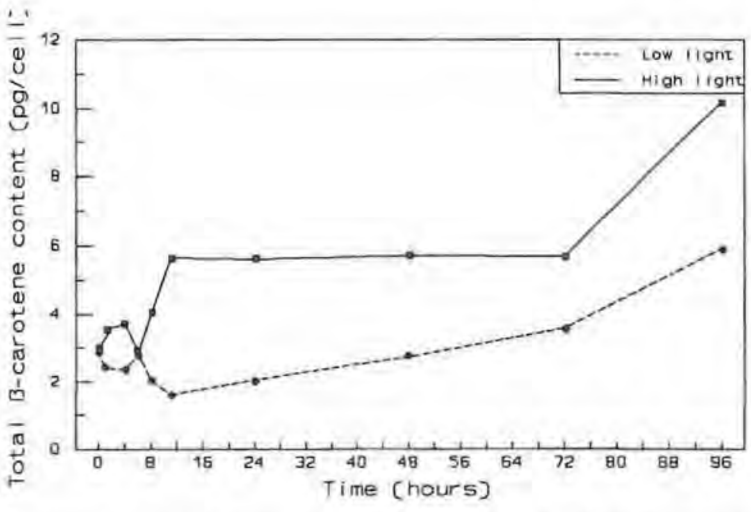


Figure 3.8 ß-carotene content of cells exposed to low and high light intensities in growth medium.

Lers *et al.* (1990) have also noted two ß-carotene accumulation stages in cells exposed to high light intensities, however, they report that the second ß-carotene accumulation stage occurs 3-4 days after the initial response.

The ß-carotene content of high light stressed cells (Figure 3.8) exhibits the opposite trend to that of the chlorophyll content of the algae (Figure 3.7). As the ß-carotene content increased there was a concomitant decrease in the chlorophyll content. In addition, the first stage of ß-carotene accumulation (Figure 3.8) took place when the photosynthetic carbon fixation rate (Figure 3.4) and the oxygen evolution rate (Figure 3.5) of *D. salina* was severely inhibited.

3.4.6 ß-carotene partitioning in response to hight light stress.

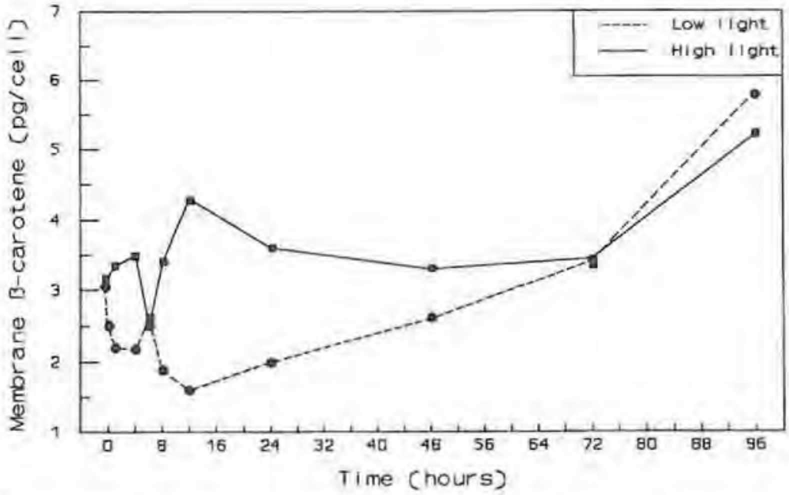


Figure 3.9 Membrane ß-carotene content of *D. salina* cells exposed to low and high light intensities in growth medium.

The partitioning of ß-carotene between thylakoid membrane bound ß-carotene and interthylakoidal globular ß-carotene was monitored under low and high light intensities. Figure 3.9 shows the ß-carotene content of the thylakoid membranes. It is evident that under low light intensities there was an overall decrease in the ß-carotene content of the thylakoid membranes up until 24 hours and thereafter the ß-carotene content steadily increased until 96 hours (Figure 3.9). At 6 hours there was a small peak in the ß-carotene content of the thylakoid membranes, which is also evident in the total ß-carotene content of the cells grown under low light intensity (Figure 3.8). In contrast, at 6 hours, high light stressed cells exhibited a sharp drop in the total ß-carotene (Figure 3.8) as well as in membrane bound ß-carotene (Figure 3.9). The thylakoid membrane ß-carotene content in response to high light stress showed three stages of ß-carotene accumulation (Figure 3.9); the first stage occurred over the first 4 hours and was followed by a sharp drop in ß-carotene content at 6 hours; the second stage occurred between 8 and 12 hours and was followed by a gradual decrease in membrane ß-carotene content; the third accumulation stage occurred over the last 24 hours of the experiment.

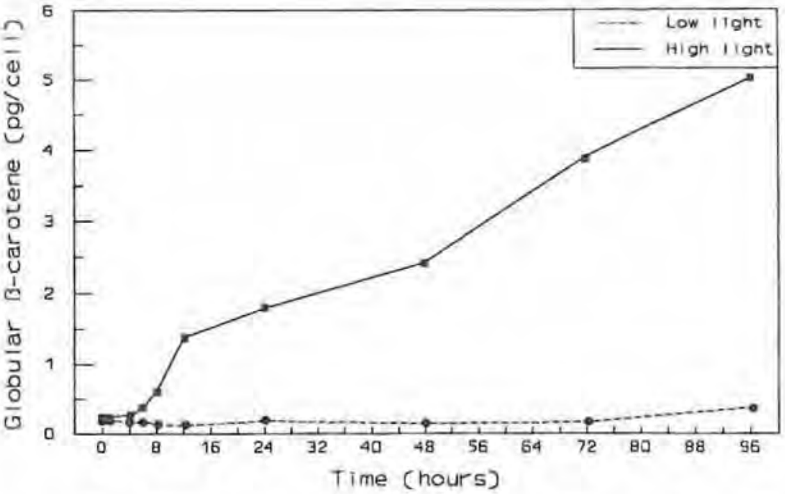


Figure 3.10 Globular ß-carotene content of *D. salina* cells exposed to low and high light intensities in growth medium.

The globular ß-carotene content of cells exposed to low and high light intensities is depicted in Figure 3.10. The globular ß-carotene fraction of the low light grown cells remained static over the whole experimental period (Figure 3.10). A totally different response was evident in high light stressed cells and after 6 hours a marked increase in the globular ß-carotene content could be observed (Figure 3.10). After 12 hours the globular ß-carotene content increased linearly over the remainder of the experiment, albeit at a slower rate than the initial increase at 6 hours. The onset of globular ß-carotene hyper-accumulation (at approximately 6 hours) coincided with a sharp decrease in membrane-bound ß-carotene (Figure 3.9) and it appears as if there may be a thylakoidal demand for ß-carotene from the chloroplast stroma, the site of ß-carotene synthesis. In addition, the initial increase in thylakoid membrane bound ß-carotene over the first 4 hours indicates that membrane-bound ß-carotene may be responsible for the primary photoprotective response to photoinhibition (Figure 3.4 and 3.5).

3.4.7 Ultrastructure of cells in response to high light stress.

A time-course ultrastructure study of high light stressed cells was undertaken to observe changes in the ß-carotene partitioning of interthylakoidal globules. Figure 3.11 and Figure 3.12 shows electron micrographs of *D. salina* cells grown under low and high light intensities, respectively. It is clear from Figure 3.12 that by 12 hours there was a localisation of ß-carotene globules at the periphery of high light stressed cells, which became more apparent after 24 hours of light stress. The peripheral localisation of ß-carotene globules was not evident in low light grown cells after 96 hours and the ß-carotene globules were relatively evenly distributed throughout the chloroplast of the cell (Figure 3.11). An examination of the high magnification insets also shows that the globules of the high light grown cells (Figure 3.14) appeared to increase in size in comparison to the low light grown cells (Figure 3.13). In addition, the thylakoid membranes of the high light stressed cells appeared less condensed than those in the low light exposed cells. This reduction in thylakoid stacking in *D. salina* in response to high light intensities has previously been noted by Lers *et al.* (1990).

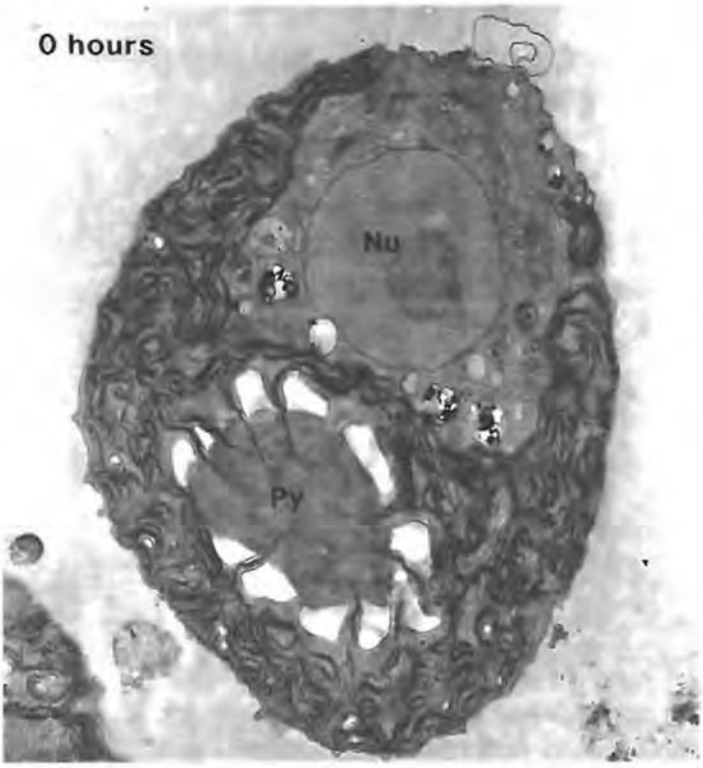
Explanation of Figures 3.11-3.14

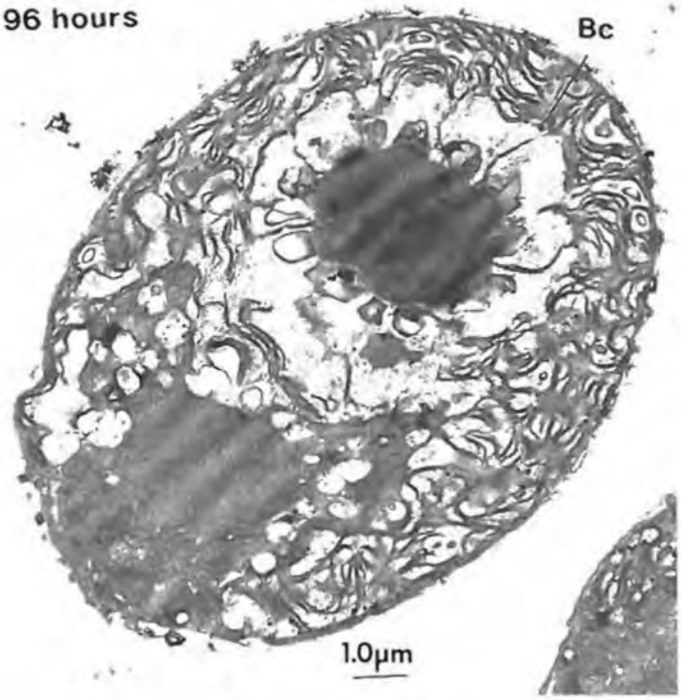
Page 70, Figure 3.11 Electron micrographs of low light illuminated cells at Time 0 and Time 96 hours. Note the even distribution of ß-carotene globules (B-c) throughout the chloroplast at both time points. Py - pyrenoid, Nu - nucleus.

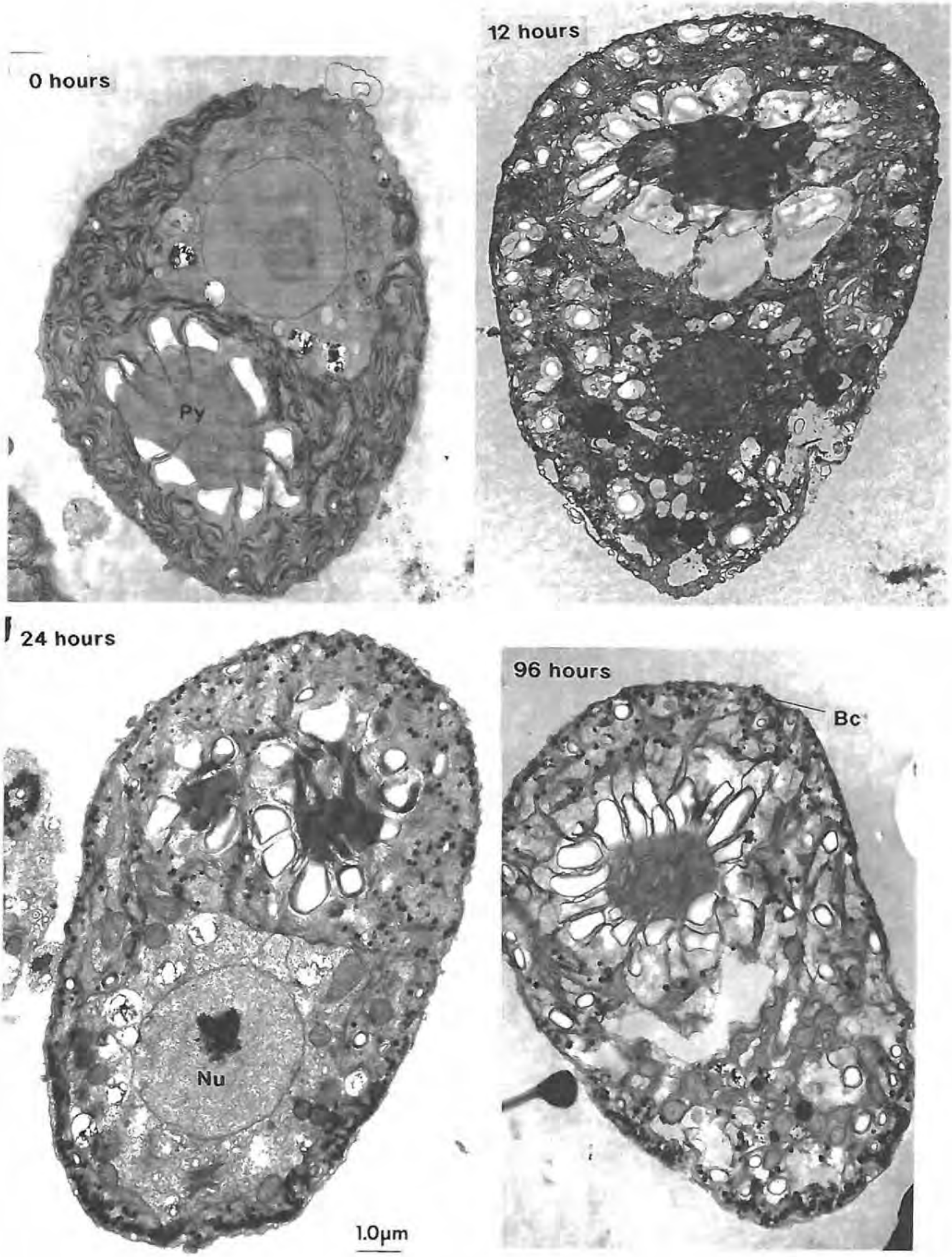
Page 71, Figure 3.12 Electron micrographs of high light illuminated cells at Time 0, 12, 24 and 96 hours. Note the increase in ß-carotene globules (B-c) and the peripheral localisation of the globules after 12 hours of high light illumination.

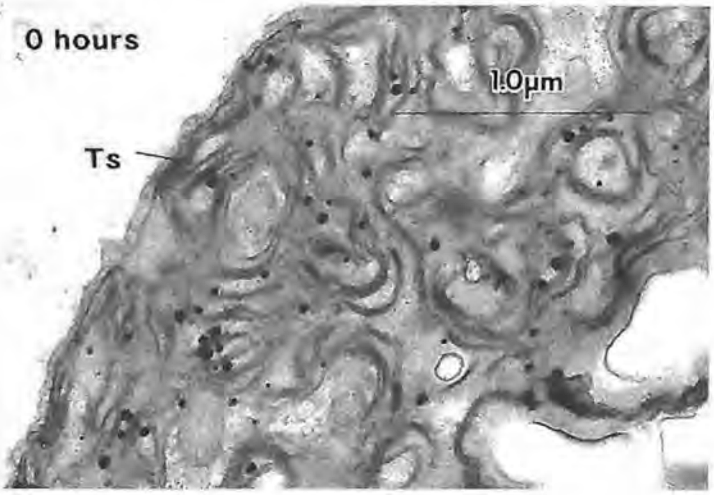
Page 72, Figure 3.13 High magnification insets of the outer portion of the chloroplast in low light illuminated cells. Ts - thylakoid membrane stacks.

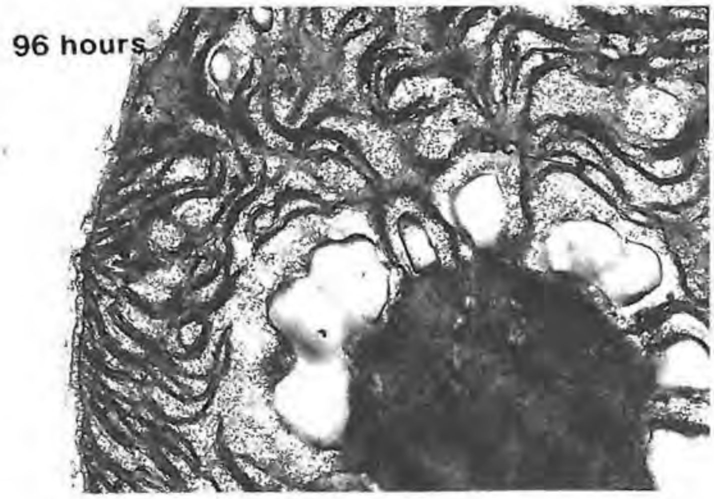
Page 73, Figure 3.14 High magnification insets of the outer portion of the chloroplast in high light illuminated cells. Note the increase in size and number of ß-carotene globules (B-c). A decrease in the thylakoid membrane stacking (Ts) is also evident after 12 hours of illumination.


0 hours
Nu
Py


96 hours
Bc
1.0µm


0 hours
Py
12 hours
24 hours
Nu
1.0µm
96 hours
Bc


0 hours
1.0µm
Ts


96 hours

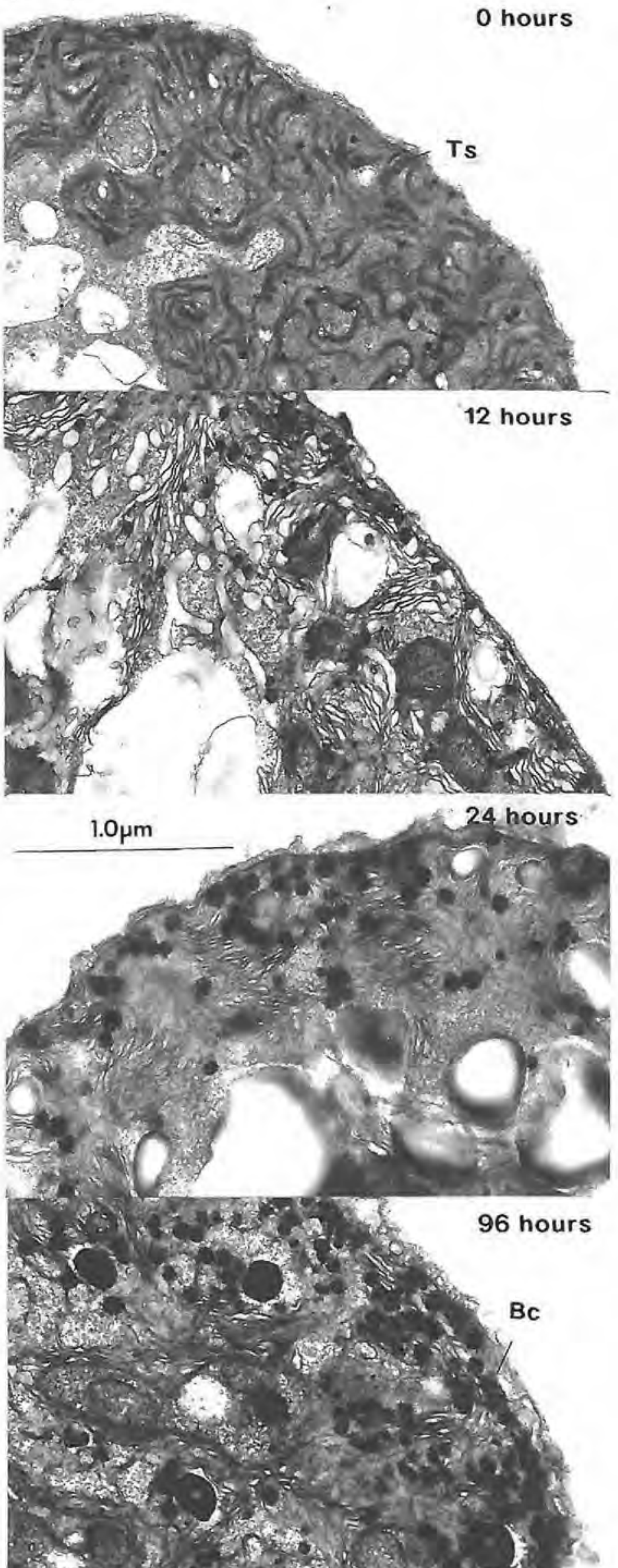

0 hours
Ts
12 hours
1.0μm
24 hours
96 hours
Bc

Table 3.1. ß-carotene globule distribution within the chloroplast of cells exposed to low and high light intensities. Results represent the average of three electron micrographs at each time point.

TIME (hrs)	LOW LIGHT			HIGH LIGHT		
	OUTER* glob.μm^{-2}	INNER** glob.μm^{-2}	TOTAL glob.μm^{2}	OUTER* glob.μm^{-2}	INNER** glob.μm^{-2}	TOTAL glob.μm^{2}
0	4.25	5.3	4.93	4.25	5.3	4.93
12	-	-	-	7	3.7	4.60
24	-	-	-	7.9	4	5.30
96	4.3	5.1	4.63	8.2	3.9	5.40

* outer refers to the 1 μm outer border of the chloroplast

** inner refers to the inside of the 1 μm outer border.

\- not determined

Table 3.1 shows the distribution of ß-carotene globules throughout the chloroplast of low and high light grown cells as determined by a 1 μm^2 grid analysis. It is evident from Table 3.1 that in low light grown cells the distribution of ß-carotene globules remained even over the experimental period, whereas, in high light stressed cells there was a definite increase in the localization of ß-carotene globules to the outer extreme of the chloroplast. There was also a slight reduction of ß-carotene globules in the inner portion of the chloroplast of high light stressed cells (Table 3.1), however, the total number of globules in high light stressed cells was only slightly higher than in low light grown cells.

Table 3.2 shows the average ß-carotene globule size over the experimental period. What is noteworthy is that compared to the low light grown cells, the globule size in high light stressed cells increased approximately 6-fold by 24 hours of high light illumination. The slight increase in globule number and the huge increase in globule size may account for the relatively large ß-carotene increase observed in Figure 3.8.

Table 3.2. ß-carotene globule size in response to low and high light intensities. Results reflect the average globule diameter of three electron micrographs.

TIME (hours)	LOW LIGHT AVE. GLOBULE DIAMETER (μm)	HIGH LIGHT AVE. GLOBULE DIAMETER (μm)
0	0.09	0.09
12	-	0.13
24	-	0.62
96	0.1	0.62

- not determined.

3.4.8 Dual-labelling study.

A dual labelling study was undertaken to determine the source and flow of carbon into the two ß-carotene pools of *D. salina*. Cells were grown in medium containing NaH[^{14}CO]$_3$ in order to pre-load the cells with ^{14}C and then they were transferred to medium containing ^{3}H mevalonic acid lactone. Chloroplasts of higher plants are relatively impermeable to mevalonic acid (Goodwin, 1980a), however, chloroplasts are permeable to mevalonic acid lactone. The basic aim of the dual-label study was that the flow of ^{14}C through the ß-carotene pools would represent the utilization of stored carbon, whereas the flow of ^{3}H would represent newly fixed carbon, given that mevalonic acid is the immediate precursor of the terpenoid pathway.

Figure 3.15 shows the flux of ^{14}C through the membrane and globular ß-carotene pools in response to high and low light intensities. Over the first 4 hours of exposure to high light intensities there was a marked increase in the ^{14}C specific activity of both membrane associated and globular ß-carotene (Figure 3.15). The increase in ^{14}C specific activity was more pronounced in the globular ß-carotene fraction (Figure 3.15) which may indicate a stronger draw on the stored ^{14}C pool by globular ß-carotene. Thereafter, the ^{14}C specific activity decreased and remained relatively constant over the remainder of the experiment (Figure 3.15), indicating a constant flow of ^{14}C to both ß-carotene pools.

Under low light intensities there was a massive increase in the ^{14}C specific activity of globular ß-carotene over the first 8 hours, followed by a transient decrease up to 24 hours (Figure 3.15). Following this, the ^{14}C specific activity increased again at 48 hours and remained relatively constant. The ^{14}C specific activity of thylakoid membrane ß-carotene was substantially lower than ^{14}C specific activity of globular ß-carotene, however, there was a small peak in membrane ß-carotene ^{14}C specific activity at 48 hours (Figure 3.15). The increase in the ^{14}C specific activity of globular and membrane associated ß-carotene at 48 hours (Figure 3.15) was coincident with the cessation of cell growth (Figure 3.3).

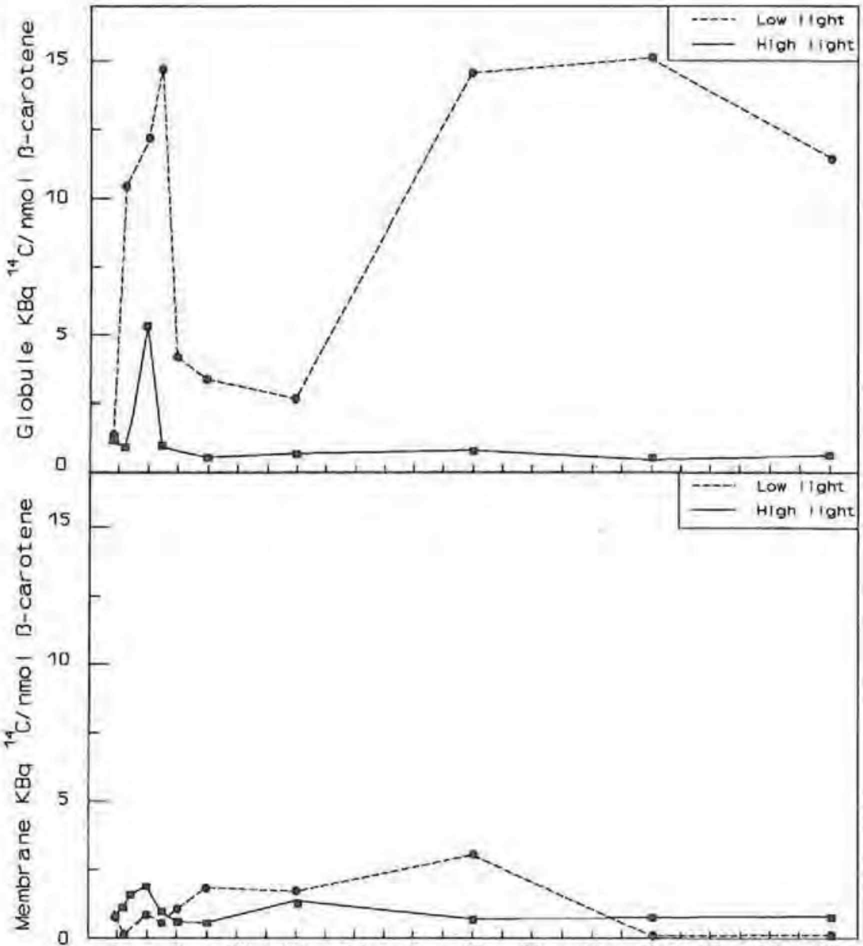


Figure 3.15 ^{14}C specific activity of thylakoid membrane bound and globular ß-carotene in cells exposed to low and high light intensities.

Under low light conditions the ^{14}C specific activity of globular ß-carotene was substantially higher than in high light stressed cells (Figure 3.15). The lower ^{14}C specific activity in response to high light stress is indicative of a greater turnover of carbon through the terpenoid pathway, whereas the high ^{14}C specific activity in response to low illumination is indicative of relatively static partitioning to globular ß-carotene.

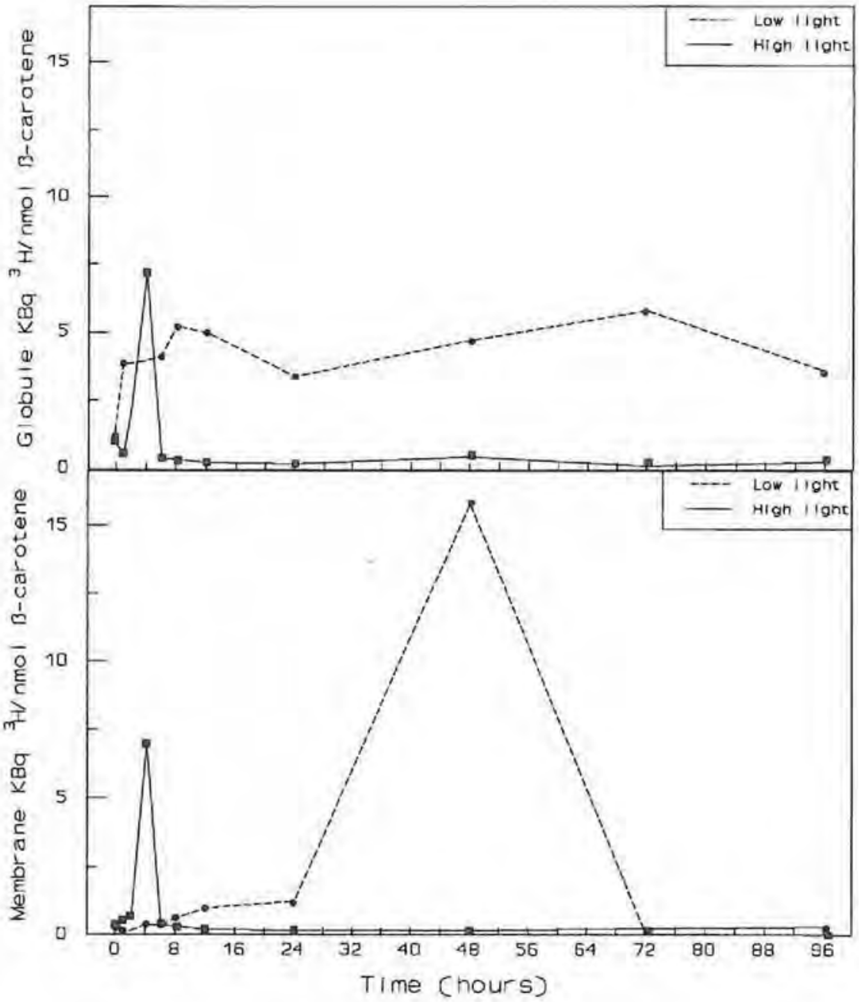


Figure 3.16 ^{3}H specific activity of thylakoid membrane bound and globular ß-carotene in cells exposed to low and high light intensities.

Figure 3.16 shows the flux of ^3H to the thylakoid membrane and globular ß-carotene pools. In high light stressed cells there was a peak in ^3H specific activity in both the membrane bound and globular ß-carotene pools at 4 hours, followed by a sharp reduction in ^3H specific activity (Figure 3.16). What is important is that the peak in ^3H specific activity of globular ß-carotene at 4 hours (Figure 3.16) is higher than that of ^{14}C specific activity (Figure 3.15). This indicates that more newly fixed carbon is partitioned into the ß-carotene globules at this point.

The peaks in the ^{14}C and the ^3H specific activity of membrane associated and globular ß-carotene at 4 hours (Figure 3.15 and 3.16 respectively) also indicates that the partitioning of ß-carotene to the two ß-carotene pools occurs via the same pathway.

The ^3H specific activity of globular ß-carotene for low light illuminated cells showed an initial increase followed by a stabilization in ^3H specific activity over the remainder of the experiment (Figure 3.16). The ^3H specific activity (Figure 3.16) was however, consistently lower than the ^{14}C specific activity (Figure 3.15) of globular ß-carotene, indicating a higher accumulation of ^{14}C in ß-carotene globules under low light illumination.

The ^3H specific activity of membrane associated ß-carotene in low light illuminated cells remained low over the experimental period except for a large peak at 48 hours (Figure 3.16). This peak coincided with the small peak in ^{14}C specific activity of membrane associated ß-carotene for low light illuminated cells and both the peaks in ^{14}C and ^3H specific activities were coincident with the cessation of cell growth (Figure 3.3).

3.5 DISCUSSION.

Physiological response to high light stress.

The results have shown that *D. salina* responds to high light stress via a number of integrated responses which can be clearly divided into an early and late response.

Early response.

The early response takes place over approximately the first 16 hours and appears to be primarily a photoadaptive response in which the alga adjusts to the higher PFD. The initial exposure to high light intensities is characterised by the following cascade of events:

Exposure of photosynthetic tissues to light in excess of that which can be utilized in photosynthesis results in photoinhibition, a reduction in photosynthetic activity, due primarily to a sustained reduction in the photochemical efficiency of PSII (Demmig *et al.* 1987). The sharp fall in the photosynthetic rate of the alga observed over the first 3-4 hours, as judged by the carbon fixation rate and the oxygen evolution rate, is consistent with the uncoupling of photosynthesis and the onset of photoinhibition (Ben-Amotz *et al.* 1989; Gomez-Pinchetti *et al.* 1992; Shaish *et al.* 1993). Over the same period there was a small increase in the thylakoid membrane ß-carotene content and a sustained fall in the chlorophyll content of the cells. In addition, the cell numbers declined slightly. This first series of events indicates an inadequate photoprotective response focused within the thylakoid membrane pigment bed, mediated by increased thylakoidal ß-carotene synthesis and photodegradation of chlorophyll.

The second series of responses occurred from approximately 4 to 16 hours and were characterised by increase globular ß-carotene partitioning. At 4 hours there was a sharp increase in both the ^{14}C and the ^{3}H specific activity in globular ß-carotene (Figure 3.15 and 3.16 respectively) indicating a surge of carbon into globular ß-carotene from both stored and newly fixed carbon. The higher ^{3}H specific activity indicates that more newly fixed carbon was directed into globular ß-carotene at this point. This is further substantiated by a peak in carbon fixation rate at 4 hours (Figure 3.4). At 6 hours there was a rapid fall in the thylakoid membrane ß-carotene content (Figure 3.9) which was coincident with increased partitioning to globular ß-carotene (Figure 3.10). The fall in thylakoid membrane ß-carotene content and the concomitant increase in globular ß-carotene suggests that there was a shift in the relative partitioning to the two ß-carotene pools at this point. It is unclear whether the surge of carbon to the globular ß-carotene pool at 4 hours was associated with the increased globular ß-carotene accumulation at 6 hours. The difference in timing may implicate a lag in the flow of carbon and the subsequent ß-carotene accumulation. Alternatively, the surge of carbon to ß-carotene at this point may indicate the passage of a large flux of carbon through the ß-carotene pool to the xanthophyll cycle, as the xanthophyll cycle is responsible for the dissipation of non-radiative energy caused by over excitation of PSII (Demmig-Adams and Adams, 1992). Cowan and Rose (1991) have observed a peak in ABA production in response to salinity stress within the first 8 hours of stress. Recently, Cowan *et al.* (1993) have reported a similar peak in ABA in response to high light stress. It is therefore possible that a surge of carbon through the ß-carotene and xanthophyll pools may culminate in the peak in ABA production reported under high light stress (Cowan *et al.* 1993). To further support this hypothesis, the results have shown that during the early response the ^{14}C and the ^{3}H specific activity was generally lower in high light stressed cells than in low light illuminated cells, indicating a greater turnover of carbon through the globular ß-carotene pool, and possibly into the xanthophyll cycle and ABA.

During the remainder of the early high light stress response the globular ß-carotene content of the cells increased and localisation of ß-carotene globules to the periphery of the chloroplast was evident by 12 hours (Figure 3.12 and Table 3.1). The increase in globular ß-carotene and the peripheral localisation of these globules appears to enable a more sustained photoprotective response. This is supported by an increase in the photosynthetic rate during this period. The electron micrographs of cells exposed to high light stress for 12 hours also show a reduction in the proportion of thylakoid membrane stacking (Figure 3.12), which can be attributed mainly to the decreased size of PSII antenna chlorophyll (Pick *et al.* 1987). The decreased thylakoid stacking can also be related to a continued decline in the chlorophyll content of the alga during the early response.

Ben-Amotz (1987) have shown that *D. salina* cells exposed to high light and nutrient stress show an increase in the total carbohydrate content. The results indicate that the increase in total carbohydrate content reported by the authors may be due to massive starch accumulation over the first 24 hours of high light stress. The increase in starch accumulation in response to high light stress appears to be an integral part of the photoadaptive response.

In summary, it appears that during the early response the initial photoprotective response is located in the thylakoid membrane where there is accelerated photodegradation of chlorophyll and an increase in the membrane ß-carotene content. Following this a repartitioning of ß-carotene to globular ß-carotene occurs and these globules are localised at the periphery of the chloroplast acting as a screen to the excess irradiation. The photosynthetic rate subsequently increases and the chlorophyll content stabilizes. The early response thus appears to be a photoadaptive response and the rapid turnover of carbon through the globular ß-carotene pool implicates the xanthophyll cycle in this response. In addition, the regulation of the response may be mediated by increased ABA biosynthesis (Cowan *et al.* 1992; Cowan *et al.* 1993).

Late response.

During the late response the alga appeared to have adapted to the high light intensity as judged by the recovery of the photosynthetic rate and cell growth. Ben-Amotz *et al.* (1989) and Gomez-Pinchetti *et al.* (1992) have shown that *D. salina* cells with high ß-carotene contents have higher photosynthetic rates than cells with low ß-carotene contents on exposure to high light intensities. The results obtained in this study confirm these observations as exposure of cells with a low ß-carotene content initially gave rise to low photosynthetic rates, however, once ß-carotene had been accumulated (during early response) the photosynthetic rate recovered.

During the late response the chlorophyll content of the cells remained relatively constant, however, there was a reduction in the starch content of the high light stressed cells. The total ß-carotene content of the alga remained constant from 24 to 72 hours (Figure 3.8) however, there was sustained partitioning to globular ß-carotene over this period, albeit at a lower rate than during the early response. The low ^{14}C and ^{3}H specific activities in both thylakoid membrane and globular ß-carotene indicate a rapid turnover of carbon within these pools compared to cells cultured under low light intensities. It thus appears that during this period there is a sustained photoprotective response via globular ß-carotene partitioning and possibly by continued de-epoxidation of the xanthophyll cycle which is less pronounced than during the early response.

The high ^{14}C and ^{3}H specific activities of globular ß-carotene in low light illuminated cells (Figure 3.15 and 3.16 respectively) may indicate that under low light conditions globular ß-carotene may act as a carbon-sink because the globular ß-carotene pool remains relatively static in comparison to high light stressed cells.

In both the low light and high light cultures, cell growth ceased at approximately 48 hours and from 72 hours there was a marked decrease in both the cell number and the photosynthetic rate. During the last 24 hours of the experiment an increase in the total ß-carotene content of both low and high light illuminated cells was observed. The fact that an increase in ß-carotene accumulation occurred in both low and high light illuminated cells suggests that nutrient limitation may have been responsible for this increase in ß-carotene accumulation. The increase in ß-carotene accumulation was, however, higher in the high light stressed cells which indicates a possible interaction between nutrient limitation and high light stress.

In summary the late response appears to be a less intense response than the early response and is focused at maintaining photoprotection at a level suitable for cell growth and normal physiological functioning. The increased ß-carotene accumulation during the final stages of the late response may be due to the combined effects of nutrient exhaustion and the high light stress. The globular ß-carotene hyper-accumulation and the peripheral localisation of the globules during the early and late responses support the photoprotective screening hypothesis of Ben-Amotz *et al.* (1982).

Effect of cumulative stress on photosynthesis.

Prolonged illumination of cyanobacteria or plants with visible light of high intensity leads to the phenomenon of photoinhibition, as observed by a decrease of the oxygen evolution capacity (Critchley, 1988). Shaish *et al.* (1993) have observed a photoinhibitory response in *D. salina* cells over the first 8 hours of high light stress. The results presented here (Figure 3.5) have shown that cumulative stress factors greatly exacerbate the photoinhibitory effect of high light stress in *D. salina*. The interaction between high light stress and additional stress factors had a compounding effect on the degree of photoinhibition, as more stress factors were applied to high light stress there was a sequential increase in the degree of photoinhibition (Figure 3.5). Furthermore, the rate of recovery from photoinhibition was slower in cells where cumulative stress factors were operating on the high light system. The imposition of additional stress factors throughout exposure to high light stress has previously been shown to have a cumulative effect on the photoinhibitory response in higher plants (Powles, 1984).

The results presented in Chapter 2 have shown that ß-carotene hyper-accumulation also increases with cumulative stress. It would appear that there may be a direct correlation between the extent of ß-carotene hyper-accumulation and the degree of photoinhibition experienced by the cells during the early response. Such a link would explain why cells accumulate larger quantities of ß-carotene in response to multiple stress and why the rate of ß-carotene production is higher in multiple stress systems. Additionally, it may explain why continuous high light illumination is not essential for high ß-carotene yields (see Chapter 2) since the early response is accountable for the initiation of ß-carotene hyper-accumulation.

Concluding remarks.

It was concluded at the end of Chapter 2 that due to the inverse relationship between cell growth and ß-carotene hyper-accumulation, which results in similar ß-carotene yields, the only alternative to improving the efficiency of a stress system would be to reduce the retention time of the biomass within the system in an attempt to improve the ß-carotene productivity. The results reported here have attempted to explain why high light and multiple stress are essential for ß-carotene hyper-accumulation in *D. salina*. The physiological study has shown that the alga has the remarkable ability to adapt to high intensity irradiation primarily via the hyper-accumulation of ß-carotene and the localization of this accumulated ß-carotene into oily globules at the periphery of the chloroplast. ß-carotene accumulation is initiated within the first 24 hours of high light stress and appears to be in response to the onset of photoinhibition and the photodegradation of chlorophyll. The results have indicated that the extent of the photoinhibitory response to combinations of stress is directly proportional to the degree of ß-carotene hyper-accumulation during the early photoprotective response. A greater degree of photoinhibition would reduce the cell growth, but, ß-carotene hyper-accumulation would increase. Conversely, a lesser degree of photoinhibition would allow for greater cell growth, however, ß-carotene hyper-accumulation would be compromised. This response may partly explain the inverse relationship between cell growth and ß-carotene accumulation in *D. salina*.

Attempts to increase the rate of ß-carotene production (ie. with multiple stress or a long photoperiod) are likely to increase the initial rate of ß-carotene production as demonstrated in Chapter 2, as the early photoprotective response is enhanced. After this the interaction of stress is likely to impair cell growth, resulting in similar day 5 ß-carotene yields. If retention time is sacrificed by relieving a stress element (ie. by reducing the photoperiod, see Chapter 2) high yields are still possible, albeit at a slower ß-carotene production rate. This may allow for the future development of elegant culture systems which attempt to maximise stress over the first 24 hours, followed by stress relief in order to promote the recovery of cell growth. Maximum stress induction over the first 24 hours could be applied in shallow ponds or in cascade (Setlik *et al.* 1970) or tubular photobioreactor systems (Berlew, 1953), which can increase the light environment of the culture. Once ß-carotene accumulation has been induced cultures could be transferred to conventional ponds for the remainder of the stress period. Such systems may offer the potential to reduce pond area, which forms one of major capital costs of any mass cultivation system (Borowitzka, 1992).

CHAPTER 4

OUTDOOR STUDY OF THE OPTIMAL GROWTH AND BIOMASS PRODUCTIVITY OF *D. SALINA* - SCALE-UP I (GROWTH PONDS)

Summary.

The biomass productivity potential of *D. salina* cultured under nutrient sufficient conditions was examined. The results have demonstrated that if outdoor growth pond cultures are maintained at an average areal density of 45 g dry wt.m^{-2}, infection by small non-carotenogenic *Dunaliella* species such as *D. viridis* can be maintained at a low level for extended periods. The manipulation of the light environment in the culture can both induce or eliminate high levels of infestation by *D. viridis*. A long-term average biomass productivity of 16.6 g dry wt.m^{-2}.day^{-1} was obtained over the three and a half month experimental period and short term peaks of up to 31 g dry wt.m^{-2}.day^{-1} were obtained when climatic conditions were most favourable. The productivity of *D. salina* cultured under nutrient sufficient conditions was found to be about three times greater than the productivities reported for the averaging ß-carotene production system where nutrients are limited.

4.1 INTRODUCTION.

D. salina represents an unusual case in optimizing productivity given the demonstrated inverse relationship between ß-carotene accumulation and biomass production. Current extensive ß-carotene production systems employ high salinities in order to limit the growth rate and thus induce ß-carotene production, whereas in current intensive ß-carotene production systems, ß-carotene production is manipulated by controlling the availability of the nitrogen supply (Ben-Amotz and Avron, 1990). Both strategies compromise biomass productivity. The dual-stage process proposes to separate the distinct growth and ß-carotene accumulation stages in an attempt to separately maximise both biomass yields and ß-carotene production. The production of *D. salina* biomass under optimal growth conditions forms the first stage of the dual-stage ß-carotene production process (Chapter 1, Figure 1.1). Consequently, the determination of outdoor biomass productivities under optimal culture conditions is essential for the accurate evaluation of the process.

The fundamental driving-force in the commercial cultivation of photoautotrophic algae is the sustained harvesting of solar energy at high efficiencies (Richmond, 1992). The more efficient the process, the higher the biomass productivity and consequently, the sounder the economic basis of the process. A theoretical photosynthetic conversion of solar irradiance to chemical energy at 2 % efficiency on a year-round basis implies annual net yields averaging between 30-40 g dry wt.m^{-2}.day^{-1} (Richmond, 1992). This productivity goal is some 2-3 times higher than long term practical productivities reported for a number of algal species (Thomas *et al.* 1984; Laws *et al.* 1988). The reason for this anomaly is that algal productivity in outdoor culture is limited by a wide range of factors; these include nutrient supply, sunlight availability, culture density, temperature, climate and the design and operation of the bioreactor (Richmond, 1988b, 1992).

A number of bioreactor designs have been developed for the cultivation of photoautotrophic algae which utilize the principle of high surface area to volume ratios in order to maximize the exploitation of photon flux. Designs include tubular systems (Burlew, 1953), sloping cascade systems (Setlik *et al.* 1970), circular ponds (Goldman and Ryther, 1975), horizontal raceways (Shelef *et al.* 1978) and U-shaped ponds (Ben-Amotz and Avron, 1989). Mixing is essential to avoid cell sedimentation, thermal stratification and to improve mass transfer of nutrients and gases between the suspended algae and their immediate environment (Oswald, 1988; Sukenik *et al.* 1991). By far the most common system used in industry is the open raceway in which stirring is provided by a paddle wheel (Dodd, 1986). There is however, no clear consensus on an optimal bioreactor design (Robinson and Toerien, 1982).

Most large-scale commercial ponds of *Dunaliella* consist of oblong raceways constructed of repeated units which are stirred by paddle wheels (Ben-Amotz and Avron, 1989). Systems such as sloping cascades and tubular bioreactors where circulation is provided by continuous mechanical pumping of the algal culture are not suitable for *Dunaliella* culture due to the fragility of the cells. This has been confirmed in studies where monopump circulated tubular photobioreactors were evaluated for the cultivation of *D. salina* (Jarvis *pers comm.* 1993). Extremely low biomass productivities were experienced due to cell damage caused by the continuous pumping of the cultures. Furthermore, Silva *et al.* (1987) have demonstrated that even a simple gas-bubbling dispersion and broth mixing system is harmful to *D. salina* cells.

Nutrient supply has a marked effect on algal productivity (Toerien *et al.* 1987). The most important nutrients for photoautotrophic algae are carbon, nitrogen and phosphorous (Ben-Amotz and Avron, 1989). In photoautotrophic systems the high carbon content of algae (approximately 50 % ash-free dry weight) necessitates a carbon dioxide supply of about 45 g $CO_2.m^{-2}.day^{-1}$ depending on the yield achieved (Soeder, 1980). In photoautotrophic systems inorganic carbon is most commonly supplied in the form of gaseous CO_2. The maximal amount of inorganic carbon that can be dissolved in an algal growth medium is a function of the pH, the concentration of CO_2 in the gas phase, the salt concentration and the temperature (Ben-Amotz and Avron, 1989). In practice, special equipment is required to achieve high transfer rates through long residence times and small bubble size (Ben-Amotz and Avron, 1989). It has been shown that the supply of nitrogen and phosphorous to mass cultures of photoautotrophic algae should be in the range of 1.6 to 6.5 g N. $m^{-2}.day^{-1}$ and 0.2 to 0.83 g P. $m^{-2}.day^{-1}$ (Robinson and Toerien, 1982). In the case of *Dunaliella* certain other nutrients are required for optimal growth. These include sulphate, magnesium, potassium and calcium.

Net photosynthesis in an algal culture is determined by the light regime to which the average cell in the culture is exposed (Richmond, 1987). The optimal light regime cannot be defined only in terms of the incident PFD, but usually depends on the culture density (areal density) (Grobbelaar, 1982). The concept of areal biomass density was first suggested by Soeder (1980) as an important factor in determining productivity in algal mass culture. Grobbelaar and co-workers (Richmond and Grobbelaar, 1986; Hartig *et al.* 1988; Grobbelaar *et al.* 1990) further exploited the concept by performing outdoor experiments and designing a simulation model that describes the experimental data. Other models based on areal density have been developed (Sukenik *et al.* 1991) and research has shown that the maintenance of optimum areal density is essential for maximal biomass productivities (Richmond, 1992). Grobbelaar *et al.* (1990) report that an areal density of 38-41 g dry wt.m^{-2} is optimum for maximal productivity of *Scenedesmus obliquus*. Sukenik et al (1991) calculated an optimal areal density of between 64 to 120 g dry wt. m^{-2} for the marine prymnesiophyte *Isochrysis galbana* . The difference in optimal areal density reported by Grobbelaar *et al.* (1990) and Sukenik *et al.* (1991) is attributed to differences in physiological and biophysical properties of the different algal species.

When nutrients are supplied in excess the two main environmental factors affecting microalgal outdoor culture are solar irradiance and temperature (Richmond, 1992). Castillo *et al.* (1980) reported a linear relationship between biomass yields and culture temperature and in addition, they present evidence for interrelationships of algal productivity with both light and temperature. Other works have shown that a rise in temperature greatly increases the saturating light intensity constant (E.m^{-2}.s^{-1}) and reduces the maintenance energy coefficient (KJ.g^{-1} biomass. hr^{-1}) (Collins and Boylen, 1982; Lee *et al.* 1985). Richmond (1992) maintains that the interaction between light and temperature forms the basis of unsatisfactory performance of the open raceway ponds, because maximum utilization of solar energy can be achieved only when the temperature is optimal. Diurnal variations in temperature result in the optimal range of temperature being maintained in the culture during only a portion of the light period. This problem is further compounded by seasonal temperature variations. Consequently the full potential present in the PFD for the photosynthetic reaction cannot be utilized (Richmond, 1992).

The interaction between light and temperature has an affect on physiological processes such as photooxidation, photoinhibition and dark respiration at night. When temperature falls below the optimum temperature by 15 to 18 °C (usually during winter), algal cultures may be damaged by photooxidation when exposed to high irradiance (Richmond, 1992). In contrast, at high temperatures and light intensities (usually during mid summer) photoinhibition may severely impede biomass productivity (Richmond, 1992). Torzillo *et al.* (1991) have shown that at high irradiance or sub-optimal temperatures an excess of carbohydrates are produced during the day resulting in respiratory losses during the night.

Outdoor mass cultures of algae are subject to infections by foreign algae, invertebrates (eg. rotifers and protozoa), bacteria and fungi (Grobbelaar, 1981). Due to the selective salt environment employed for the culture of *D. salina*, infection by foreign algae does not present a major problem (Ben-Amotz and Avron, 1989). However, occasional overgrowth by non-carotenogenic *Dunaliella* species does occur (Borowitzka and Borowitzka, 1988). The heterotrophic nature of bacteria does not allow for their proliferation in *D. salina* ponds unless the medium contains a substantial organic load (Ben-Amotz and Avron, 1989). Glycerol leakage from the algal cells due to high temperatures and mechanical damage of cultures may induce bacterial blooms. Few amoebae and zooplankton ciliates tolerate salinities of 2 M NaCl and above (Post *et al.* 1983).

Richmond, (1987) maintains that the low biomass-output rates which are currently obtained in large scale outdoor algal cultures are due to the open horizontal raceway systems, with paddlewheel stirring, which are presently used in most commercial culture systems. Such systems are not suitable for obtaining high photosynthetic efficiencies due to ineffective mixing, lack of temperature control, easy contamination and the interactions between light and temperature (Richmond, 1987, 1992). Closed systems (eg. tubular photobioreactors) offer many advantages; greater control of temperature and culture density, higher yields due to the improved light environment, greatly reduced evaporation, greater control of infections and elimination of insects and dirt (Richmond, 1987, 1988b, 1992). In the case of *Dunaliella*, unless gentle and effective pumping systems are developed the use of open raceway type ponds stirred with paddlewheels remains the only alternative and the choice of suitable geographical location is of prime importance.

4.2 RESEARCH OBJECTIVES.

Ben-Amotz and Avron (1989) report that *D. salina* can attain maximal biomass productivities of 20 g dry wt.$m^{-2}.s^{-1}$ when grown under optimal growth conditions. In order to determine the full economic potential of a dual-stage ß-carotene production process it is necessary to determine longer-term biomass productivities, since short-term maxima such as those reported by Ben-Amotz and Avron (1989) may be misleading.

Borowitzka and Borowitzka (1988) state that the growth of *D. salina* at low salinities and high nitrate concentrations is conducive to infection by non-carotenogenic *Dunaliella* species. The prevention and control of such infections is of prime importance to the dual-stage ß-carotene production process since the contamination of the growth ponds will undoubtably reduce the productivity of the whole system.

The following research objectives were thus identified:

1. Determination of the maximum long-term biomass productivities under conditions of nutrient sufficiency.
2. To determine whether the growth ponds can be operated without encouraging infection.
3. To investigate the possible eradication of infections once established.

4.3 MATERIALS AND METHODS.

4.3.1. Outdoor *D.salina* productivity in optimal culture medium.

4.3.1.1 Culture Medium.

The culture medium described by Ben-Amotz and Avron (1983) (previously detailed in Chapter 2) was used for the outdoor evaluation. Carbon was supplied as sodium hydrogen carbonate ($NaHCO_3$). The salinity was increased from 1.5 M to 2 M NaCl to reduce the chance of infection by protozoans and non-carotenogenic *Dunaliella* species. *D. viridis* and *D. minuta* have lower salinity optima than *D. salina* (Borowitzka and Borowitzka, 1988). The medium was prepared in 200 l containers and the nutrients were dissolved with a drum pump.

4.3.1.2 Culture Units.

Figure 4.1 Culture units used for the determination of *D. salina* biomass productivities. Note the dark green colours of the algal cultures.

The algae were cultivated in circular fibreglass ponds with a surface area of 2 m^2 (Figure 4.1). Mixing was supplied with a central stainless steel blade (length 30 cm), which was driven by an electric motor to facilitate a rotation speed of 62 rpm. Turbulence was created by four 20 cm baffles situated on the pond walls (Figure 1). A depth gauge was fitted to each pond and the cultures were run at a depth of 12 cm (volume 240 l). Evaporative losses were made up each day with tap water before any measurements were made.

4.3.1.3 Preparation of inoculum.

A non-axenic *D. salina* culture was obtained from the University of the Orange Free State (UOFS) courtesy of Professor J.U. Grobbelaar. The 2 m^2 ponds were inoculated from laboratory cultures, which were first pre-cultured in 5 l flasks. On transfer, the outdoor ponds were covered with shadecloth so as to prevent photooxidation of the low light adapted cultures. The shade cloth was removed after 3-4 days.

4.3.1.4 Operation of outdoor ponds.

Culture density (g dry wt.l^{-1}) was determined daily using a Coulter Multisizer with a 70 μm orifice tube. The use of the Coulter Multisizer enabled the quantification of different size related peaks, so that various populations (ie. bacteria, *D. salina*, contaminating algae) could be quantified separately by size. ß-carotene and chlorophyll concentrations were determined daily as previously described in Chapter 2.

Hartig *et al.* (1988) have shown that maximum biomass productivity of green algae occurs at an areal density of 40 to 45 g dry wt.m^{-2}. Based on this, the *D. salina* cultures were maintained at an average areal density of 45 g dry wt.m^{-2}. This was achieved by operating the culture as a continuous culture and oscillating the areal density between approximately 35 to 55 g dry wt.m^{-2} by harvesting and refilling the culture.

Specific culture volumes were harvested and from this the net productivity (PROD) was determined as follows:

$$\text{PROD (g dry wt.m}^{-2}\text{.day}^{-1)} = (\text{C.H})/(\text{t.A})$$

where C = culture density (g dry wt.l^{-1}) at time of harvest, H = harvest volume (l), t = time taken to reach the specific culture density (days) and A = area of pond (m^2). The retention time varied between 1 to 2 days depending on the ambient climatic conditions.

4.3.1.5 Nitrate Determination.

The nitrate concentration of media samples was determined with Merck Spectroquant Nitrate test cells (Cat. No. 14556) for the determination of NO_3^- in high salinity solutions. Samples were pre-filtered through 0.4 μm nylon filters to remove cells and cell debris.

4.3.1.6 Location and climatic conditions of the experimental site.

Experimentation took place from December 1992 to mid-March 1993 at Sastech (Ltd), Sasolburg. This is typically mid-to-late summer in this region which is situated 27° 50 S and 26° 48 E at an altitude of 1500 m above sea level. Table 4.1 shows the general climatic conditions of this region. What is noteworthy is that the average hours of sunshine (cloud free) is higher in winter, than in summer. This is due to excessive cloud cover experienced during summer, in the rainy season.

Climatic data for this region over the experimental period was obtained from the South African Weather Bureau.

Table 4.1. Yearly climatic data for Sasolburg, South Africa.

	SUMMER November-February	WINTER May-August
AVERAGE MAXIMUM TEMPERATURE	27.3	19.4
AVERAGE MINIMUM TEMPERATURE	14.8	2
AVERAGE HOURS SUNSHINE (CLOUDLESS)	8.2	9.1

4.3.2 Control of infection by small non-carotenogenic *Dunaliella* species.

All the *Dunaliella* culture obtained from the UOFS algal ponding facility was found to be contaminated with a small *Dunaliella* species (approximate size range of 7 μm in diameter). Axenic cultures of the contaminant were derived from single colonies grown on agar plates as follows: 100-200 cells were suspended in 6 ml melted 0.6 % agar in growth medium, at 37-40 °C, and overlayed on solid 1.5 % agar in growth medium. The contaminant was cultured in a high salinity nitrate limited medium in an attempt to induce ß-carotene accumulation. The contaminant was provisionally identified by morphological characteristics using the key provided in Ben-Amotz and Avron, (1992).

An outdoor pond was inoculated with the infected culture and was run at an average areal density of 60 g dry wt.m^{-2} in an attempt to encouraged overgrowth of the contaminant for treatment studies. The Coulter Multisizer was used to determine the extent of infestation as it can differentiate between different sized population groups. Infestation was expressed as percentage total algal dry weight.
Once a high level of infestation was obtained (approximately 40 % dry wt.) the average areal density of the culture was reduced to 20 g dry wt.m^{-2} to increase the light environment within the culture and the salinity was increased to 3 M NaCl. The percentage infestation and *D. salina* productivity were monitored before, during and after this treatment period and compared to the growth pond maintained at an average areal density of 45 g dry wt.m^{-2} where high levels of infestation were not induced.

4.4 RESULTS.

4.4.1 *D. salina* productivity in optimal culture medium.

Figure 4.2 shows the daily biomass productivity of a growth pond from the 1st December 1993 to 16th March 1993. The ambient maximum temperatures during this period are also plotted. It is apparent from Figure 4.2 that biomass productivity fluctuated over the experimental period following a similar pattern to that of the ambient temperature. The average biomass productivity during this period was 16.6 g dry wt.m^{-2}.day^{-1}, however, peaks as high as 31.4 g dry wt.m^{-2}.day^{-1} were obtained in January. A minimum productivity of 7.4 g dry wt.m^{-2}.day^{-1} was also obtained in January.

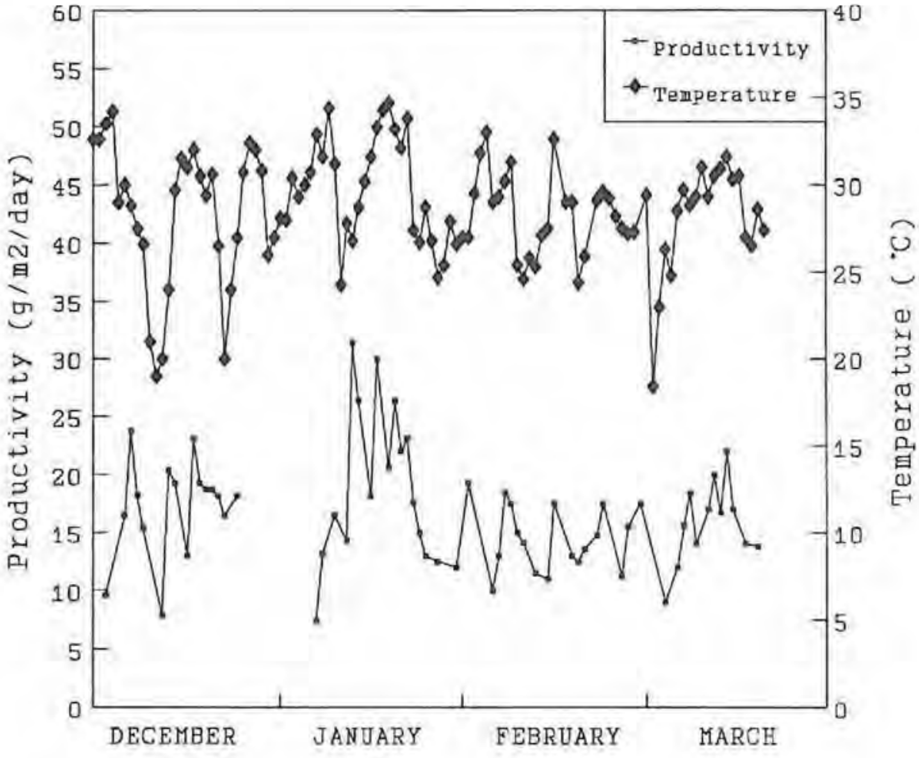


Figure 4.2. Daily biomass productivity of *D.salina* growth in low salinity, nutrient sufficient medium. The ambient maximum temperatures are also depicted. Biomass productivity was not recorded from 26.12.92 to 4.1.93.

Table 4.2. Ambient climatic conditions during experimental period.

MONTH	AVERAGE HOURS SUNSHINE	AVE. MAXIMUM TEMPERATURE (°C)	AVE. MINIMUM TEMPERATURE (°C)
DECEMBER	7.7	28.3	16.5
JANUARY	8.84	29.7	16
FEBRUARY	7.3	26.92	15.6
MARCH[1]	7.8	28.7	14

1 - averages refer only to first half of March.

The climatic conditions over the experimental period are summarised in Table 4.2. Generally, pond temperatures were 0.5 °C lower than ambient temperatures. The average hours of sunshine and the average maximum temperature were greatest during January, whereas the lowest average hours of sunshine and average maximum temperatures were experienced during February (Table 4.2) Correspondingly, the highest average biomass productivity was recorded in January and the lowest average biomass productivity in February (Table 4.3).

Table 4.3. *D. salina* biomass productivities.

MONTH	AVE. BIOMASS PRODUCTIVITY ($g.m^{-2}.day^{-1}$)	MAX. BIOMASS PRODUCTIVITY ($g.m^{-2}.day^{-1}$)	MIN. BIOMASS PRODUCTIVITY ($g.m^{-2}.day^{-1}$)
DECEMBER	17.3	23.8	7.9
JANUARY	18.9	31.4	7.4
FEBRUARY	14.33	18.5	10
MARCH	15.8	22	9

* - average productivity over experimental period
16.6 g dry $wt.m^{-2}.day^{-1}$.

A peak in biomass productivity of 31.4 g dry wt.m^{-2}.day^{-1} occurred in January when climatic conditions were most favourable (Table 4.3). During this period the lowest minimum productivity of 7.4 g dry wt.m^{-2}.day^{-1} was also recorded (Table 4.3).

The biomass productivity during December was 2 g dry wt.m^{-2}.day^{-1} higher than during March (Table 4.3) although the average hours of sunshine and the average maximum temperature during March were marginally higher than during December (Table 4.2). One would have expected similar productivity values, however, the explanation for this may be related to the fact that the average minimum temperature during March was 2.5 °C lower than during December (Table 4.2).

ß-carotene productivity was monitored over the experimental period and a summary of the results is given in Table 4.4. ß-carotene productivity seems to be a function of biomass productivity as the average monthly ß-carotene productivities (Table 4.4) followed a similar trend to that of the average monthly biomass productivities (Table 4.3). As with biomass productivity, the highest ß-carotene productivity occurred in January and the lowest occurred in February. A long term average ß-carotene productivity of 170.6 mg.m^{-2}.day^{-1} was obtained, however, peaks of up to 331 mg.m^{-2}.day^{-1} were obtained in January (Table 4.4).

Table 4.4. ß-carotene productivities.

MONTH	AVERAGE ß-CAROTENE PRODUCTIVITY (mg.m^{-2}.day^{-1})	MAXIMUM ß-CAROTENE PRODUCTIVITY (mg.m^{-2}.day^{-1})	MINIMUM ß-CAROTENE PRODUCTIVITY (mg.m^{-2}.day^{-1})
DECEMBER	182.3	327	79.9
JANUARY	200	331	73.5
FEBRUARY	144	291	93.2
MARCH	159.1	307.3	81.8

* - Average ß-carotene productivity over experimental period 170.6 mg.m^{-2}.day^{-1}.

Table 4.5 depicts the average monthly ß-carotene content of *D. salina* cells. There is no marked difference in the ß-carotene content of *D. salina* cells during the different months and a long term average content of 1.03 % dry wt. or 18.15 pg.cell^{-1} was obtained (Table 4.5). The similarity in monthly ß-carotene content explains the relationship between the maximum monthly biomass productivities and the maximum ß-carotene productivities. It follows that if the ß-carotene content of cells is similar than the greater the biomass productivity the greater the ß-carotene productivity.

Table 4.5. ß-carotene content of *D. salina* grown under optimal culture conditions.

MONTH	AVE. ß-CAROTENE CONTENT (pg.cell^{-1})	AVE. ß-CAROTENE CONTENT (% dry wt.)
December	18.2	1.05
January	18.3	1.06
February	18.1	1.0
March	18.0	1.0
AVERAGE	18.15	1.03

The nitrate content of the growth pond was monitored for a five day period to determine whether nitrate exhaustion was occurring during the usual operation of the ponds (Figure 4.3). The sharp drop in culture density and the sharp increases in nitrate concentration indicate harvesting events where 100 l of the culture was removed in order to maintain the optimum areal density. During operation of the pond the nitrate concentration fluctuated between 2 to 3.5 mM (Figure 4.3) indicating that the culture was run with an excess of nitrate and growth inhibition due to nutrient limitation was unlikely.

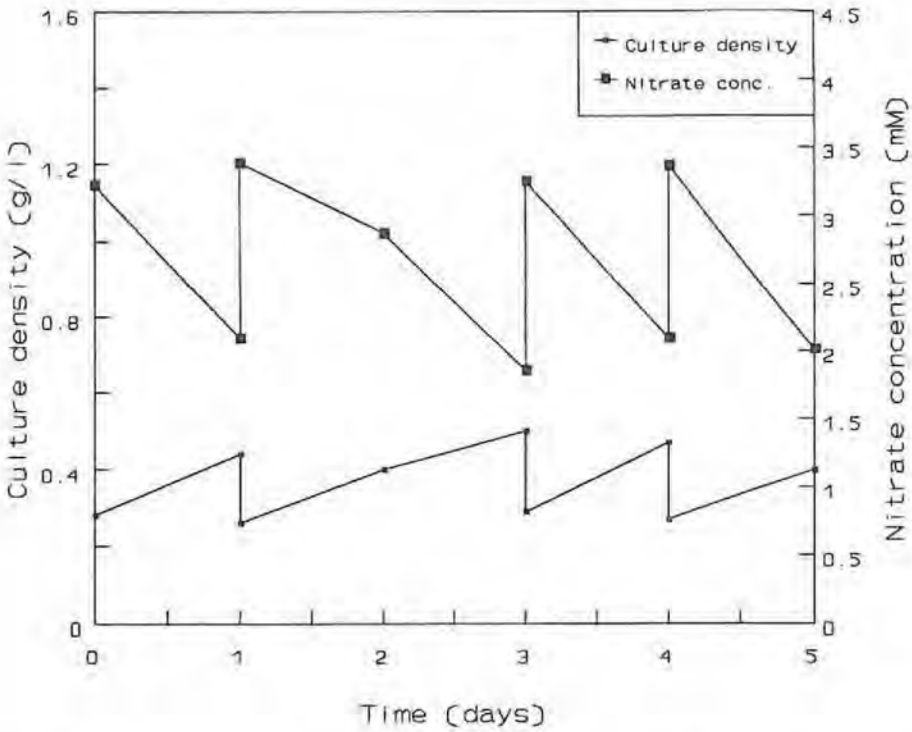


Figure 4.3. Nitrate concentration and culture density during the normal operation of a growth pond. The sharp drop in culture density and the sharp increase in nitrate concentration indicate harvesting and refill events.

4.4.2 Control of infections.

ß-carotene hyper-accumulation could not be induced in the small contaminating *Dunaliella* species and a maximum ß-carotene content of 0.25 % dry wt., compared to a maximum ß-carotene content of 10 % dry wt. in *D. salina*, was obtained over a ten day growth period. Based on morphological characteristics the isolate was provisionally identified as *D. viridis* (Ben-Amotz and Avron, 1992).

Post (1977) observed that in the Great Salt Lake, natural populations of *D. viridis* were not prevalent in the plankton, but were generally most commonly found on the underside of wood, rocks or debris where they are out of the direct sunlight. These observations indicate that *D. viridis* is more sensitive to light than *D. salina*, which is abundant in the plankton of the Great Salt Lake (Post, 1977). The light sensitivity of *D. viridis* was used to both encourage and eradicate infestation of *D. salina* cultures with *D. viridis*.

Over the three and a half month experimental period the level of infestation in the growth pond operated at an average areal density of 45 g dry wt.m^{-2} remained below 4 %. However, in the pond where the light environment of the culture was deliberately decreased by increasing the average areal density, overgrowth of the culture by *D. viridis* occurred. After 20 days, 41 % of the culture had become infected (Figure 4.4, Period A). At this point, the salinity of the medium was increased to 3 M NaCl and the operating areal density was decreased to 20 g dry wt.m^{-2}.

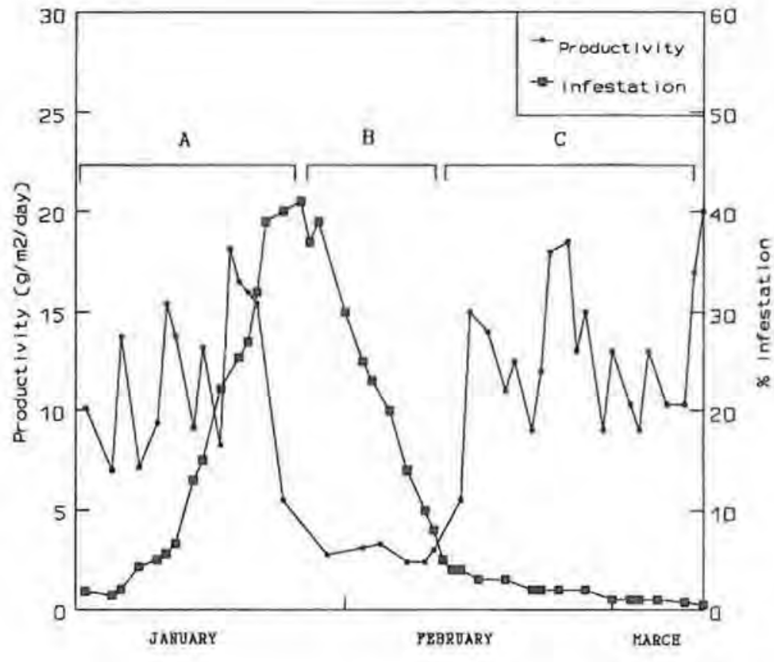


Figure 4.4. Biomass productivity of an infected pond before (period A), during (Period B) and after (Period C) treatment. Treatment comprised of increasing the salinity and decreasing the areal density.

Table 4.6. Biomass productivities of an infected pond during the various time periods depicted in Figure 4 as compared to a low infected pond over the same time periods.

TIME PERIOD	AVERAGE PRODUCTIVITY OF HIGH-INFECTED POND ($g.m^{-2}.day^{-1}$)	AVERAGE PRODUCTIVITY OF LOW-INFECTED POND ($g.m^{-2}.day^{-1}$)
PERIOD A	11.9	19.3
PERIOD B	2.8	15.3
PERIOD C	13.44	14.1

During treatment the biomass productivity decreased to below 4 g dry $wt.m^{-2}.day^{-1}$ and the level of infestation decreased rapidly (Figure 4.4, Period B). After 16 days the infestation dropped to 4 %, at this point treatment was terminated and the pond was operated at normal salinity and areal density. During this period (period C, Figure 4.4) the biomass productivity increased and the infection gradually decreased to less than 1 %.

A comparison of *D. salina* biomass productivity in the heavily infected growth pond and in the growth pond where infestation was maintained at a low level is shown in Table 4.6. As the infection persisted (Period A) the average biomass productivity was 38.3 % lower than in the pond where infestation was maintained at a low level. During treatment (Period B) the productivity of the infected pond decreased to 2.8 g dry $wt.m^{-2}.day^{-1}$. This represents a reduction in productivity of 81.7 %. Once the infestation level had dropped to 4 % and treatment had been terminated, the average biomass productivity of the heavily infected pond increased to 13.44 g dry $wt.m^{-2}.day^{-1}$. Over the same time period the average biomass productivity of the pond where infestation had been maintained at a low level was 14.1 g dry $wt.m^{-2}.day^{-1}$(Table 4.6). During Period C the average biomass productivity of the infected pond was 4.7 % lower than in the pond where infestation had not been allowed to reach a high level.

4.5 DISCUSSION.

Outdoor *D. salina* productivity in optimal culture medium.

Long-term biomass productivity of between 15 to 25 g dry wt.m^{-2}.day^{-1} are typical for most fresh water and marine microalgae species grown under conditions of nutrient sufficiency (Goldman, 1980; Thomas *et al.* 1984; Toerien *et al.* 1987; Borowitzka and Borowitzka, 1988; Laws *et al.* 1988). Maximum yields attained over short periods are in the range of 30 to 40 g dry wt.m^{-2}.day^{-1} (Goldman, 1980) but yields of up 54 g dry wt.m^{-2}.day^{-1} have been reported (Grobbelaar, 1982). Both the long-term average *D. salina* productivity of 16.6 g dry wt.m^{-2}.day^{-1} and the short term maximum yields of up to 31.4 g dry wt.m^{-2}.day^{-1} obtained at Sasolburg (Table 4.3) compare well with the productivities reported by other investigators.

Ben-Amotz and Avron (1980a, 1989) report that for commercial intensive culture systems operated using the averaging process, maximum peaks in *D. salina* productivity of around 10 g dry wt.m^{2}.day^{-1} can be attained for short periods, with yearly long-term average yields of around 5 g dry wt.m^{-2}.day^{-1} being typical. Mohn and Condero-Contreras (1990) report a similar long-term *D. salina* biomass productivity of 4 g dry wt.m^{-2}.day^{-1}. It is apparent that the long-term average biomass productivity recorded here for *D. salina* grown under nutrient sufficiency is approximately three-fold higher at 16.6 g dry wt.m^{-2}.day^{-1}. It may be argued that since the long-term biomass productivity obtained does not represent a yearly average, a direct comparison with reported biomass productivities is not applicable. However, a more accurate reflection of the potential biomass productivities of the two systems may be gained by examining the short-term biomass productivity maxima. As with the long-term biomass yields, *D. salina* grown under optimal conditions has a maximum short-term biomass productivity (31.4 g dry wt.m^{-2}.day^{-1}) of more than three-fold higher than cells grown using the averaging process (10 g dry wt.m^{-2}.day^{-1}).

The influence of climatic conditions on algal productivity is clearly illustrated in the results. The highest average monthly biomass productivity (18.9 g dry wt.m^{-2}.day^{-1}) was obtained when the ambient maximum temperature and hours of sunshine were highest. Conversely, during February, when the ambient maximum temperature and hours of sunshine were at a minimum, the lowest monthly biomass productivity of 14.33 g dry wt.m^{-2}.day^{-1} was obtained.

The average productivity in March was 1.5 g dry wt.m^{-2}.day^{-1} lower than in December, despite the fact that during March the average maximum temperature and average hours of sunshine were marginally higher than during December. The reason for this may be two fold. Firstly, during December PFD strikes the earth at a higher intensity than during March in the Southern Hemisphere. The influence of light intensity on biomass productivity has been discussed by Goldman (1980); generally when light intensity does not exceed the saturation light intensity, biomass productivity is greatest at high light intensities. Secondly, during March the average minimum temperature was 2.5 °C lower than in December. Diurnal variations in temperature results in the optimal temperature range being maintained in the culture during only a fraction of the light period (Richmond, 1987). The effect is more pronounced as diurnal variations increase and the net outcome is a reduction in biomass productivity.

The choice of geographical location determines the light and temperature regimes which will be experienced. The climatic conditions in Sasolburg during summer are not totally suited to algal growth, since this period falls within the rainy season and productivities may be marred by excessive cloud cover. *D. salina* has a temperature optimum of 32 °C (Ben-Amotz and Avron, 1989) and in addition to excessive cloud cover, the average maximum temperature was approximately 2-6 °C lower than optimum. The long-term average biomass productivity obtained at Sasolburg is probably an under estimation of the potential biomass productivities as higher *D. salina* productivities may be anticipated in hot arid regions (Ben-Amotz and Avron, 1989).

Average monthly ß-carotene productivities followed a similar pattern to that of the average monthly biomass productivities, with maximum ß-carotene productivity occurring in January and a minimum productivity occurring in February. The reason for this is that the ß-carotene content of the biomass remained relatively constant during the experimental period (about 1.03 % or 18.15 $pg.cell^{-1}$)(Table 4.5) . Consequently, any change in the biomass productivity results in a similar change in ß-carotene productivity.

The need to manipulate stress in *D. salina*, in order to achieve high ß-carotene productivities, is clearly demonstrated when the ß-carotene productivities of cells grown under nitrate excess (optimal growth conditions) are compared to cells grown under nitrate limitation (current commercial systems). The averaging process used by most intensive ß-carotene production processes produces maximum ß-carotene productivities of between 400 to 600 $mg.m^{-2}.day^{-1}$ (Ben-Amotz and Avron, 1980a, 1989, 1990) with long-term averages of about 250 $mg.m^{-2}.day^{-1}$. The ß-carotene productivity in such systems where nitrate is limited is significantly higher than the ß-carotene productivities obtained in the growth ponds (nitrate sufficient). A long-term average ß-carotene productivity of 170 $mg.m^{-2}.day^{-1}$ and a short term peak of 331 $mg.m^{-2}.day^{-1}$ (Table 4.4) was obtained.

Although, the biomass productivities obtained in the growth ponds are significantly higher than those reported by Ben-Amotz and Avron (1989), the ß-carotene content of cells grown under optimal conditions (approximately 1.03 % dry wt.)(Table 4) is far lower than cells grown where the nitrate supply is manipulated (about 5 % dry wt.)(Ben-Amotz and Avron, 1989,1990), resulting in inferior ß-carotene yields. Ideally, in order to improve current ß-carotene yields, high biomass productivities in conjunction with high cellular ß-carotene levels (over 5 %) are necessary. The dual-stage ß-carotene production process has the potential to produce this effect. The productivity results reported in this Chapter indicate that long-term productivities of more than triple that obtained with the averaging process are attainable. What remains to be determined is whether high levels of ß-carotene can be induced in the biomass produced in this first stage of the dual-stage process.

Control of infections.

D. viridis was present at low levels (< 4% dry wt.) in all the outdoor algal cultures used. The results have shown that overgrowth of *D. salina* cultures by small non-carotenogenic *Dunaliella* species severely affects *D. salina* productivity, probably due to competition for nutrients and light. During the three and a half month growth season the level of infection in the growth pond maintained at an average areal density of 45 g dry wt.m^{-2}. did not exceed 4 %. This indicates that if algal cultures are operated at areal densities (culture density) of about 45 g dry wt.m^{-2}.day^{-1}, contamination of *D. viridis* can be maintained at a low level.

Borowitzka and Borowitzka (1988) maintain that growth of *D. salina* at low salinities under conditions of nitrate excess leads to the occasional overgrowth by the non-carotenogenic *D. viridis* and *D. minuta*, which have lower salinity optima than *D. salina*. Research by Post (1977) on natural populations of *D. viridis* suggests that *D. viridis* is more sensitive to light due to its low levels in the plankton. These observations were used to control the extent of infestation by *D. viridis*.

The results have shown that overgrowth and eradication of *D. viridis* is possible by the manipulation of the culture light environment and culture salinity. Overgrowth of *D. salina* cultures with *D. viridis* was induced by reducing the light environment within the culture, this in turn was done by increasing the areal density of the culture. Conversely, eradication of *D. viridis* was accomplished by raising the salinity and decreasing the areal density thus increasing the light environment within the culture.

During the treatment period the biomass productivity was severely retarded due to the unfavourable salinity and high light environment. The duration of the treatment period was 16 days and during this period the biomass productivity was 81.7 % lower than that of the pond where infestation was maintained at a low level. In a commercial plant, a loss in productivity of this proportion for this length of time would severely affect the economics of the process. However, in a practical situation, it is unlikely that the extent of the infestation would be allowed to persist to a level of 41 %. treatment would be initiated at an earlier stage of infestation, thus reducing the duration and possibly the intensity of the treatment.

Concluding remarks.

The results of the outdoor biomass production evaluation have shown that high *D. salina* biomass productivities can be achieved for extended periods without the overgrowth of non-carotenogenic *Dunaliella* species. In the event of possible overgrowth by a small, non-carotenogenic *Dunaliella* species, a treatment process has been developed which can successfully eradicate the smaller contaminating species.

The long-term biomass productivities obtained in the evaluation compare well with those of other algal species, despite the sub-optimal climatic conditions. Significantly higher short-term and long-term biomass productivities than those obtained with the current commercial *D. salina* systems were obtained. The results reported in Chapter 2 have indicated that continual high light intensities are not necessary over the full stress period for optimizing ß-carotene productions in the stress pond stage of the dual-stage system, however, biomass productivities are dependant on longer hours of cloud free sunshine. Clearly, further research is necessary to determine the optimum photoperiod required for maximum biomass productivities. Such information will allow for the selection of an ideal location for a dual-stage system.

CHAPTER 5

OUTDOOR STUDY OF STRESS INDUCED ß-CAROTENE PRODUCTION - SCALE-UP II (STRESS PONDS)

Summary.

A scale-up study was undertaken to determine whether ß-carotene accumulation and production could be manipulated under outdoor conditions. It was demonstrated that biomass produced in the growth pond stage of the dual-stage process could be induced to accumulate large quantities of ß-carotene. The light environment within the outdoor stress cultures was shown to be the most important factor influencing ß-carotene accumulation and production. An inverse relationship between growth and ß-carotene accumulation was identified and quantified. A possible maximum ß-carotene yield of 22-23 $mg.l^{-1}$ was observed under the specified stress conditions. Factors such as the retention time of biomass in the stress ponds and the starting culture density require careful consideration in the process design and economic evaluation.

5.1 INTRODUCTION.

The results reported in Chapter 4 have demonstrated that *D. salina* biomass productivity under low salinity, nutrient sufficient conditions, representing the first stage of the dual-stage system (Figure 1.1), is approximately three times greater than the biomass productivity of the averaging system. The ß-carotene content (about 1 % dry wt.) of algae cultivated under such conditions is substantially lower than the ß-carotene content of algae produced in the averaging system (about 5 % dry wt.) (Ben-Amotz and Avron, 1989). The question then posed is: can ß-carotene accumulation be sufficiently manipulated under outdoor conditions in the second stage of the dual-stage process in order to utilize the high biomass productivity obtained in the first stage of the dual-stage system? Maximum ß-carotene accumulation of up to 10-13 % dry wt. (Ben-Amotz and Avron, 1989) and 14 % dry wt. (Borowitzka *et al.* 1990) have been reported in laboratory studies. These values indicate the ß-carotene accumulation potential of the organism. The second stage of the a dual-stage system requires the optimization and maximization of ß-carotene accumulation in the stress ponds (Figure 1.1).

Ben-Amotz (1986) has shown that the major inducing factor of ß-carotene hyper-accumulation in *D. salina* is high light intensity. The results reported in Chapter 2 and 3 have further substantiated this observation. One of the main practical problems with the induction of ß-carotene accumulation in outdoor cultures of *D. salina* is the manipulation of the light intensity, since PFD strike the earth at fixed intensities at different times of the day and year. One of the most obvious solutions is to vary the culture density (areal density) of the shallow ponds in which the algae are cultured. This will not alter the ambient light intensity, but it will alter the light environment within the culture, either by increasing or decreasing the extent of self-shading of the algae (Richmond, 1992).

Economically, it would be most beneficial to run the stress ponds at high culture densities as this would have the desired effect of reducing the size of the stress ponds. Furthermore, it would reduce the culture volumes required for processing. A reduction in pond size would have a marked affect on the capital investment of a production plant and it is well established that the harvesting of dense algal cultures is more efficient (Ben-Amotz and Avron, 1990). The results reported in Chapter 2 showed that although, stresses such as nutrient limitation and high salinity significantly increase ß-carotene accumulation in *D. salina*, the extent of accumulation is substantially lower in comparison to high light stress. One can only speculate that, although not ideal, the requirement for high light intensities, may warrant the use of low culture densities in order to achieve maximum ß-carotene hyper-accumulation in the outdoor stress ponds. The determination of the optimal culture density required for the induction of ß-carotene hyper-accumulation is thus of prime importance to the process.

The results reported in Chapter 2 also showed that the initial rate of ß-carotene accumulation in response to multiple stress is far greater than for single or double stresses, giving rise to high ß-carotene yields by the third day of stress induction. The determination of the optimal retention time of the biomass in the stress ponds is therefore an important factor requiring attention, as it is likely to have a marked affect on the ß-carotene productivity of the system.

The wet biomass harvested from the high nitrate growth ponds contains a certain amount of growth medium. The nitrate content of the wet algal biomass may have the undesirable effect of repressing ß-carotene accumulation within the stress pond stage of the dual-stage system. Rose *et al.* (1992) have shown that CFUF diafiltration can be used to wash the high nitrate medium from the concentrated algal biomass. However, this process would require large volumes of nitrate-free medium and would thus increase the operating cost of the separation process. Furthermore, other biomass separation techniques such as flocculation may not be conducive to a washing process, as this may disrupt the integrity of the fragile flocs and result in poor separation efficiencies. The results reported in Chapter 2 have shown that a low nitrate concentration of 0.2 mM is optimal for ß-carotene hyper-accumulation. A more cost effective alternative would be to carryover the high nitrate growth medium associated with the wet algal biomass into the stress ponds in order to obtain a stress medium nitrate concentration of around 0.2 mM.

If the cellular ß-carotene content of the algae can be increased to above that currently obtained in the "averaging" process (about 5 %), this, coupled with the high biomass productivities reported in Chapter 4, implies that greater ß-carotene yields can be achieved by the implementation of a dual-stage ß-carotene production system.

5.2 RESEARCH OBJECTIVES.

The second stage of the dual-stage ß-carotene production process involves the manipulation of stress in order to achieve maximum ß-carotene accumulation in the biomass transferred from the growth ponds. The fundamental question that requires answering is: can biomass produced in the growth pond stage of the dual-stage system (Figure 1.1) be transferred to stress ponds and produce ß-carotene at anticipated maximum yields under outdoor conditions?

The following research objectives were identified to answer this question:

1. To examine the effect of growth medium carry over on ß-carotene accumulation in the stress ponds.
2. To determine the optimum stress medium for ß-carotene accumulation based on the laboratory investigation (Chapter 2).
3. To determine the optimal culture density required for maximum ß-carotene accumulation.
4. To examine whether a similar inverse relationship to that observed in Chapter 2, exists between ß-carotene accumulation and cell growth under outdoor conditions and to determine its effect on the ß-carotene yield.
5. To determine whether similar ß-carotene yields can be obtained by a scale-up of the stress system.

5.3 MATERIAL AND METHODS.

5.3.1 Location and experimental duration.

As with the outdoor optimal growth pond study (Chapter 4), experimentation took place from December 1992 to mid-March 1993 at Sastech (Ltd), Sasolburg.

5.3.2 Separation of biomass from the growth ponds.

Algal biomass produced in the growth ponds (Chapter 4) was harvested at a culture density of 0.4 g dry wt.l^{-1} for all experiments by the flocculation-flotation process described in Chapter 6. The pH of the growth ponds was pre-adjusted to pH 8 by sparging the culture with carbon dioxide prior to the addition of the K300 polyaluminium chloride (PAC) flocculant. Flotation of the flocculated biomass was induced by sparging the culture with carbon dioxide through a 30 cm long sintered stainless steel tube positioned at the bottom of the flocculation vessel. The algal float was recovered and the separated biomass was immediately transferred to the stress ponds. The results reported in Chapter 6 have shown that the presence of the PAC flocculant has no affect on *D. salina* growth and ß-carotene accumulation.

5.3.3 Culture units (Stress ponds).

ß-carotene induction studies were conducted in 0.2 m^2 circular ponds operated at a depth of 12 cm (Figure 5.1). A multi-stirring unit, driven by a single motor, was used to simultaneously mix four 0.2 m^2 ponds (Figure 5.1).

Figure 5.1 Multi-stirring unit used to mix the 0.2 m^2 stress ponds. Note the bright orange colour of the stressed cultures.

Mixing was provided by stainless steel blades (20 cm x 5 cm) rotated at a speed of approximately 30 rpm. Equal mixing was thus supplied to each 0.2 m^2 pond. Once ß-carotene accumulation conditions had been optimised, selected experiments were conducted in the 2 m^2 fibreglass ponds described in Chapter 4.

5.3.4 Cell counts, chlorophyll and ß-carotene determinations.

Cell counts were determined using a haemocytometer and chlorophyll and ß-carotene were determined as previously described in Chapter 2.

5.3.5 Determination of optimal growth medium carryover for ß-carotene production.

The results reported in Chapter 4 (Figure 4.4) have shown that at the time of harvest (culture density approximately 0.4 g dry wt.l^{-1}) the nitrate concentration in the growth ponds was typically about 2.0 mM NO_3^-. Therefore, in order to obtain a nitrate concentration of approximately 0.2 mM NO_3^- in the stress ponds, 10 % (v/v) of the stress medium should consist of growth medium carryover.

The effect of a range of growth medium carryovers on ß-carotene accumulation was examined. *D. salina* biomass was separated from the growth ponds (culture density 0.4 g dry wt.l^{-1}) and inoculated into the stress ponds at the same culture density so that the stress pond medium consisted of the following growth medium carryovers: 2.5 %, 5 %, 10 % and 15 % (v/v). A high salinity, nitrate, sulphate and phosphate deficient medium was used in order to achieve the various growth medium carryovers.

5.3.6 Determination of optimal stress medium for ß-carotene production.

Biomass was harvested from the growth ponds (culture density 4 g dry wt.l^{-1}) and resuspended to the same culture density in the following media:

- 2 M NaCl growth medium described in Chapter 4 (Control)
- 3 M NaCl growth medium (high salinity).
- 2 M NaCl nitrate deficient medium.
- 3 M NaCl nitrate deficient medium.
- 3 M NaCl nitrate, phosphate and sulphate deficient multiple stress medium (medium constituents, 3 M NaCl and 50 mM $NaHCO_3$).

A growth medium carryover of 5 % (v/v) was used for this study.

5.3.7 Determination of optimal starting culture density for ß-carotene production.

Biomass produced in the growth ponds was harvested at a culture density of 0.4 g dry wt.l^{-1} and resuspended into the optimal stress medium determined in Section 5.3.5 at the following starting culture densities: 0.1, 0.2, 0.3, 0.4, 0.8, 1.2, 1.6 g dry wt.l^{-1}. A growth medium carryover of 5 % (v/v) was used for all studies.

5.4 RESULTS.

5.4.1 Determination of optimal growth medium carryover for ß-carotene production in the stress ponds.

The results reported in Chapter 2 demonstrated that a nitrate concentration of about 0.2 mM NO_3^- is optimal for ß-carotene accumulation. At the time of harvest the nitrate concentration in the growth medium was typically about 2.0 mM NO_3^- (Chapter 4). Therefore, a growth medium carryover into the stress medium of 10 % (v/v) produced a starting nitrate concentration of about 0.225 mM NO_3^- (Table 5.1). The effect of both reducing and increasing the growth medium carryover below or above the 10 % (v/v) growth medium carryover optimum was examined.

Table 5.1. Comparison of *D. salina* growth, pigment content and ß-carotene yield in response to different growth media carryover levels. Results reflect day 5 values and the mean of two experiments. Average starting cell number 28 x 10^4 cells.ml^{-1}, average starting chlorophyll content 9.3 pg.cell^{-1}, average starting ß-carotene content 18.3 pg.cell^{-1} and average starting ß-carotene concentration 5.2 mg.l^{-1}.

PARAMETER	2.5 % CARRYOVER	5 % CARRYOVER	10 % CARRYOVER	15 % CARRYOVER
STARTING NO_4^- CONC. (mM)	0.056	0.118	0.225	0.485
CELL NUMBER 10^4 Cells.ml^{-1}	49	50	49	48
ß-CAROTENE* (pg.cell^{-1})	53.1	51.2	52.8	52.5
CHLOROPHYLL (pg.cell^{-1})	6.9	6.8	6.71	6.85
ß-CAROTENE (mg.l^{-1})	26	25.6	25.9	25.2

Figure 5.2 depicts the nitrate utilization in high salinity, nutrient limited stress medium over the 5 day experimental period. It is apparent from Figure 5.2 that after 48 hours massive depletion of the available nitrate occurred and by day 4 the nitrate concentration was less than 0.01 mM in all the cultures. Despite the different starting nitrate concentrations, growth and ß-carotene accumulation over the first four days of the experiment was similar for all growth medium carryovers. Table 5.1 depicts the cell number, pigment content and ß-carotene yield of *D. salina* on day 5 of the experiment. There was no marked difference between the final day cell numbers and ß-carotene yields (mg.l^{-1}) for the different growth medium carryovers (Table 5.1). Similarly, cellular ß-carotene and chlorophyll contents differed slightly for the different growth media carryovers (Table 5.1), indicating the same degree of stress was present in all cultures.

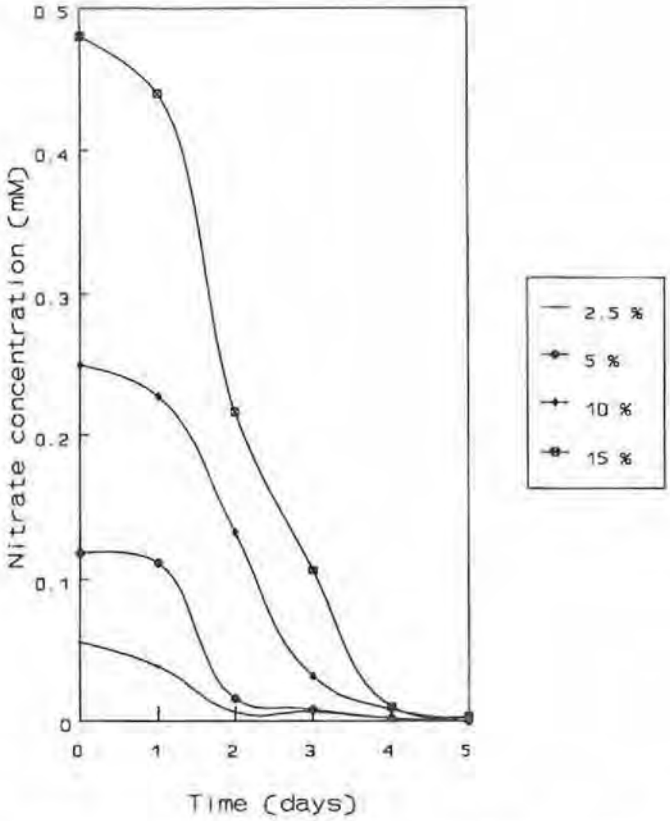


Figure 5.2 Nitrate utilization at different growth medium carryovers.

5.4.2 Determination of optimal growth medium for ß-carotene production.

Figures 5.3 to 5.6 show the effect of various stress media on the growth (Figure 5.3), cellular chlorophyll content (Figure 5.4), cellular ß-carotene content (Figure 5.5) and ß-carotene concentration (Figure 5.6) of *D. salina* cultures.

As anticipated from the laboratory study (Chapter 2), the highest cell growth was observed in the control medium (growth medium) (Figure 5.3), however, due to the lack of cellular ß-carotene hyperaccumulation (Figure 5.5), the ß-carotene concentration within the control culture was substantially lower than in all the stress media examined (Figure 5.6). The cellular chlorophyll content remained relatively constant over the experimental period in the control culture (Figure 5.4).

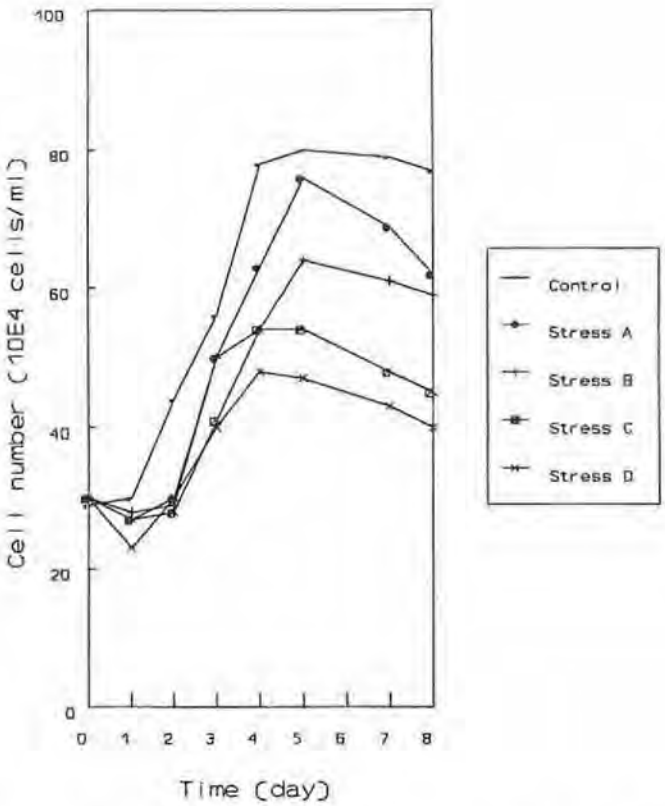


Figure 5.3 Effect of different stress medium on cell growth. Control- growth medium. Stress A - high salinity (3 M NaCl). Stress B - nitrate limited. Stress C - high salinity and nitrate limited. Stress D - High salinity and total nutrient limited.

In the single stress media (nitrate limited or high salinity), cell growth was less inhibited than in the dual (high salinity, nitrate limited) and multiple stress media (high salinity, nitrate, phosphate and sulphate limited)(Figure 5.3). It is apparent that salinity stress affected cell growth the least and high salinity, total nutrient limitation affected growth the most (Figure 5.3). A decrease in cellular chlorophyll content was observed for both single, dual and multiple stresses, however, multiple stress caused a greater degree of chlorophyll degradation (Figure 5.4).

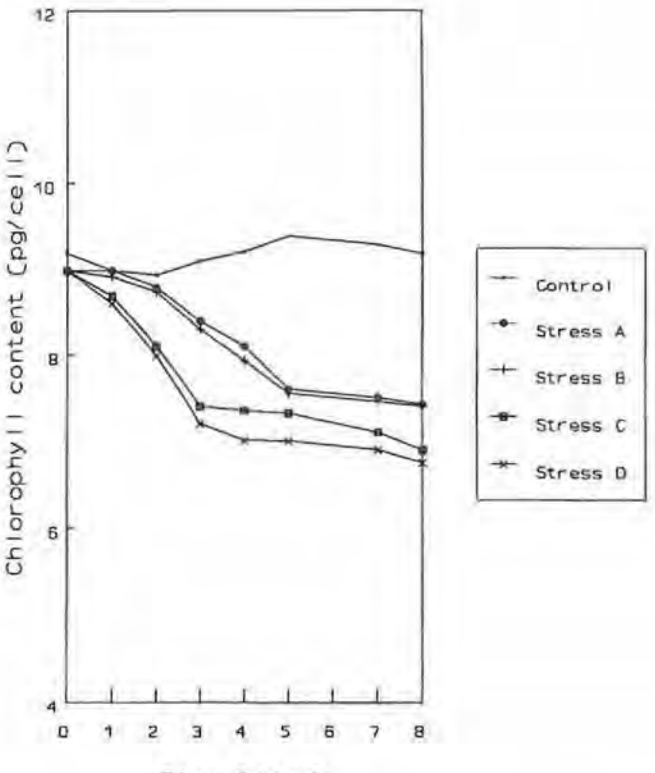


Figure 5.4 Effect of different stress media on the cellular chlorophyll content. Control- growth medium. Stress A - high salinity (3 M NaCl). Stress B - nitrate limited. Stress C - high salinity and nitrate limited. Stress D - High salinity and total nutrient limited.

Cellular ß-carotene hyper-accumulation was greatest in the high salinity, total nutrient limited medium, followed by the high salinity, nitrate limited medium (Figure 5.5). Of the single stresses, nitrate limitation resulted in greater ß-carotene accumulation than salinity stress (Figure 5.5). Similar trends were previously observed in Chapter 2.

Despite the lower ß-carotene accumulation observed for the single stresses (Figure 5.5), the improved growth obtained in these systems (Figure 5.3), gave rise to similar maximum ß-carotene concentrations for both single, dual and multiple stresses (Figure 5.6). A similar pay-off between cell growth and ß-carotene hyper-accumulation to that observed in the laboratory study (Chapter 2) thus exists under outdoor cultivation conditions. The major difference in ß-carotene production for all the stresses examined, is that the initial rate of ß-carotene production in response to dual and multiple stresses was more rapid than for the single stresses (Figure 5.6). The higher initial ß-carotene production rate observed for the dual and multiple stress systems resulted in a peak in ß-carotene concentration on day 5 of the experiment, as opposed to a peak in ß-carotene concentration on day 7, for the single stresses (Figure 5.6). It appears that under outdoor conditions there is an accelerated shift in the ß-carotene production rate of the dual and multiple stress systems with ß-carotene yields of greater than 20 mg.l^{-1} being obtained by day 3 compared to day 4.5 for the single stresses and maximum levels achieved by day 5 compared to day 7.

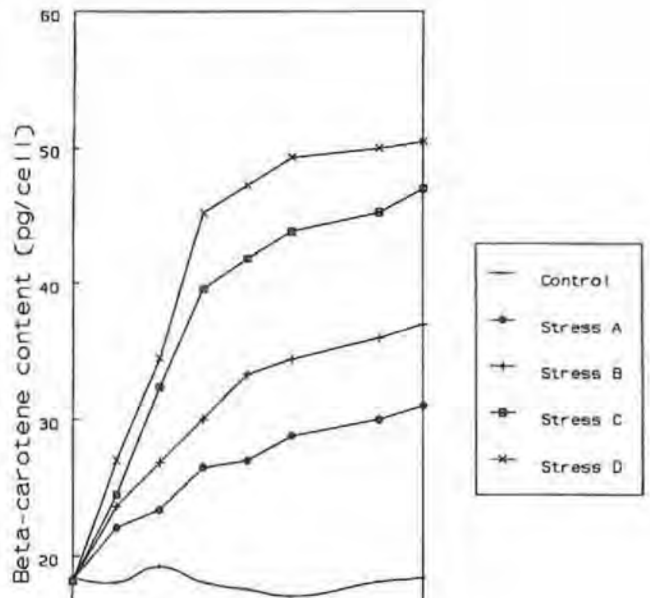


Figure 5.5 Effect of different stress media of the cellular ß-carotene content. Control-growth medium. Stress A - high salinity (3 M NaCl). Stress B - nitrate limited. Stress C - high salinity and nitrate limited. Stress D - High salinity and total nutrient limited.

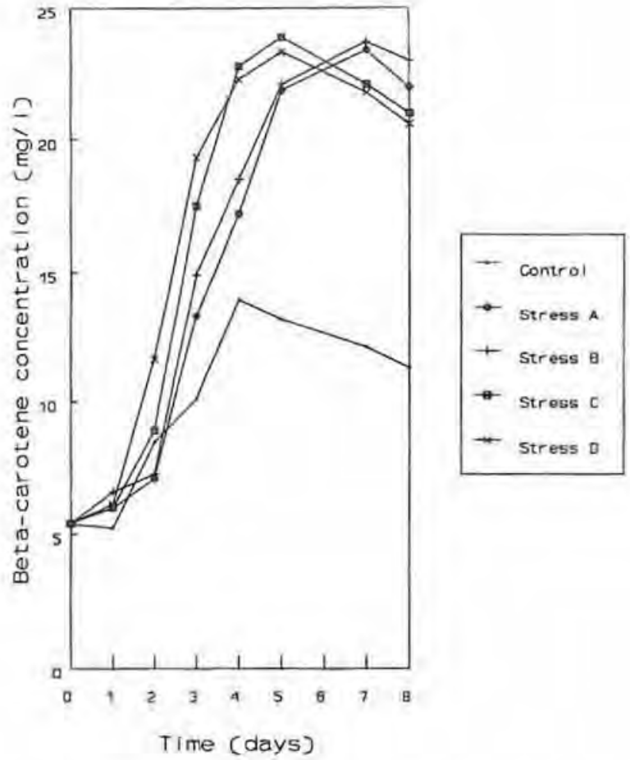


Figure 5.6 Effect of different stress media on ß-carotene production. Control- growth medium. Stress A - high salinity (3 M NaCl). Stress B - nitrate limited. Stress C - high salinity and nitrate limited. Stress D - High salinity and total nutrient limited.

The results obtained in this section indicate that both the high salinity, nitrate limited medium and the high salinity, total nutrient limited medium are suitable for ß-carotene production in outdoor stress cultures of *D. salina* due to higher initial rates of ß-carotene production (Figure 5.6). The high salinity, total nutrient limited media was chosen for further evaluation because of its simple constituent make up (ie. NaCl and $NaHCO_3$) and potential of reducing the medium costs.

5.4.3 Determination of optimal starting culture density for ß-carotene production in *D. salina*.

Starting culture densities between 0.1 to 1.6 g dry wt.l^{-1} were evaluated to determine the optimal culture density at which to operate the stress ponds. Experiments were run for 5 days as results presented in Section 5.4.2 have demonstrated a peak in ß-carotene production on the fifth day in the high salinity, total nutrient limited media which was used in this study.

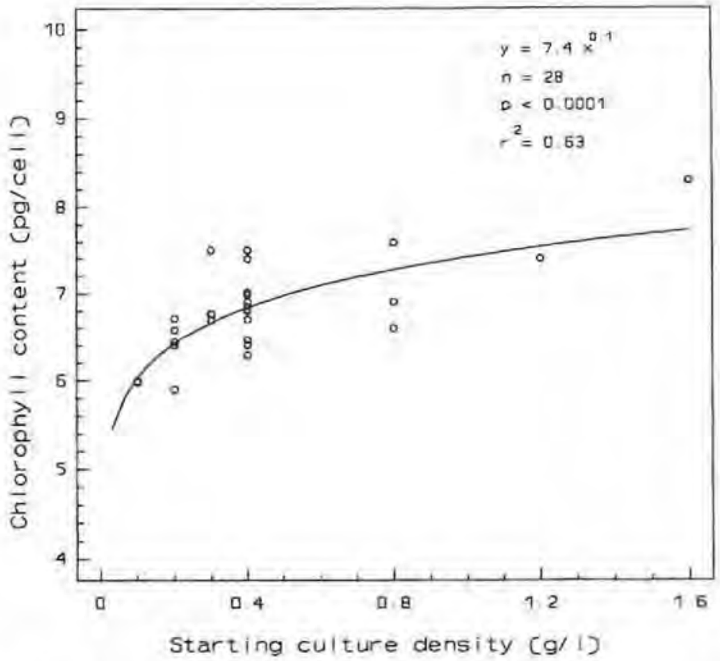


Figure 5.7 Relationship between starting culture density and cellular chlorophyll content. Results reflect day 5 values. Average starting chlorophyll content 9 pg.cell^{-1}.

Figure 5.7 shows the relationship between starting culture density and the cellular chlorophyll content of *D. salina* cells on the final day of the experiment. A weak multiplicative relationship ($r^2 = 0.63$) exists between the cellular chlorophyll content and starting culture density. As the starting culture density decreased there was a gradual decrease in the chlorophyll content of *D. salina*, however, below 0.4 g dry wt.l^{-1} the cellular chlorophyll content dropped rapidly.

The converse relationship exists for the cellular ß-carotene content (Figure 5.8) and a strong negative multiplicative relationship ($r^2 = 0.95$) exists between the starting culture density and the cellular ß-carotene content on day 5 of the experiment. It is apparent from Figure 5.8, that below a starting culture density of approximately 0.4 g dry wt.l^{-1} there was a sharp rise in the ß-carotene content of *D. salina* from about 50 pg.cell^{-1} at 0.4 g dry wt.l^{-1} to about 100 pg.cell^{-1} at 0.1 g dry wt.l^{-1}. This coincides with the sharp decrease in the chlorophyll content of *D. salina* below a starting culture density of 0.4 g dry wt.l^{-1} (Figure 5.7).

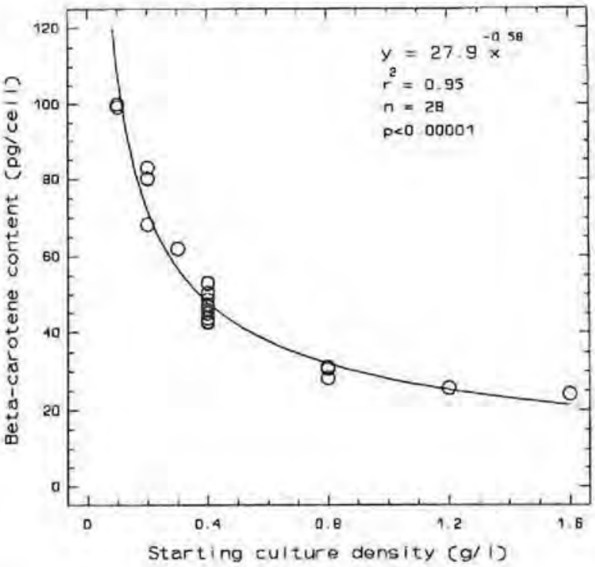


Figure 5.8 Relationship between starting culture density and cellular ß-carotene accumulation. Results reflect day 5 values. Average starting ß-carotene content 18 pg.cell^{-1}.

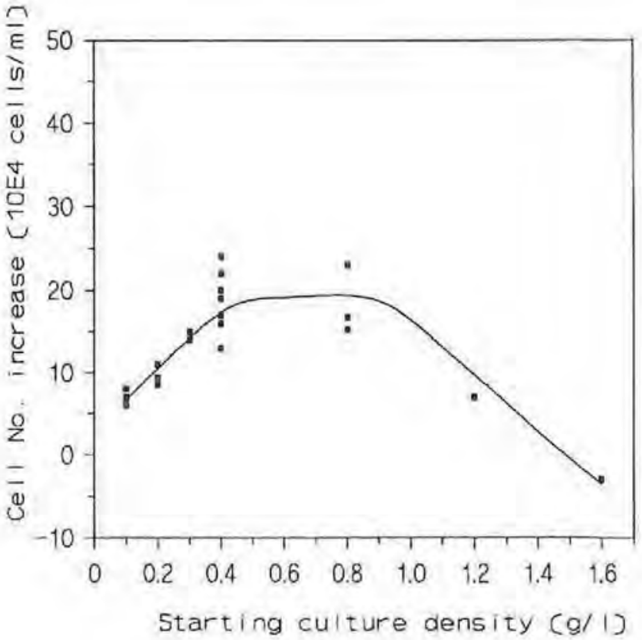


Figure 5.9 Relationship between starting culture density and algal growth. Results reflect day 5 values.

A complex relationship exists between the starting culture density and algal growth (Figure 5.9). At low starting culture densities (<0.4 g dry wt.l^{-1}) and at high starting culture densities ($>$ 0.8 g dry wt.l^{-1}) *D. salina* growth was limited, whereas between 0.4 to 0.8 g dry wt.l^{-1} growth remained relatively constant. It appears that at starting culture densities of above 0.8 g dry wt.l^{-1}, light may be severely limited which would explain the inhibition in cell growth. At starting culture densities below 0.4 g dry wt.l^{-1} it appears that light may be in excess, resulting in a decrease in algal growth (Figure 5.9) and cellular chlorophyll content (Figure 5.7), and an increase in ß-carotene hyper-accumulation (Figure 5.8).

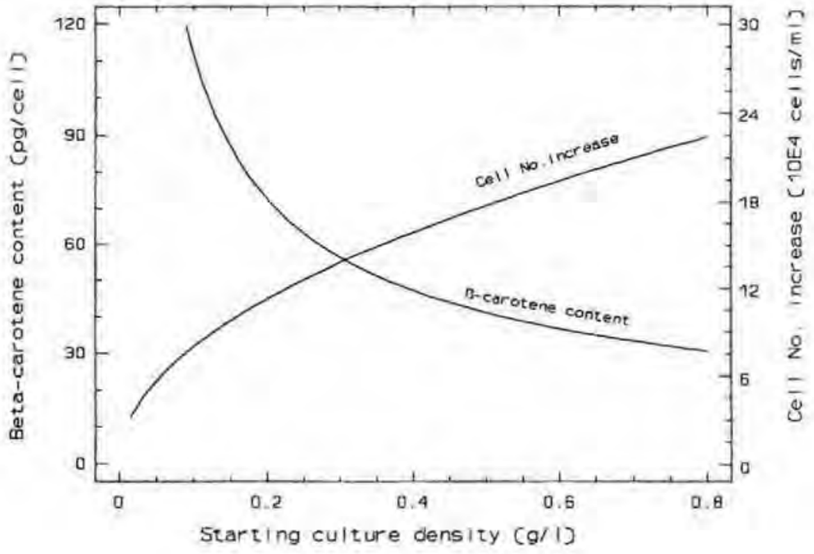


Figure 5.10 Inverse relationship between growth and ß-carotene accumulation at starting culture densities below 0.8 g dry wt.l^{-1}.

The shape of the curve of starting culture density versus algal growth , up to a starting culture density of 0.8 g dry wt.l^{-1} (Figure 5.9), appears to be the direct inverse of the culture density versus cellular ß-carotene content curve (Figure 5.8). Figure 5.10 depicts the multiplicative relationships between starting culture density and cell number increase, and starting culture density and the cellular ß-carotene content up to 0.8 g dry wt.l^{-1}. It is clear from Figure 5.10 that a inverse relationship exists between the cellular ß-carotene content and cell growth at culture densities below 0.8 g dry wt.l^{-1}. This is further substantiated when the equations of the two curves are examined (Table 5.2).

Table 5.2. Regression analysis equations and probability coefficients for Figure 5.10.

REGRESSION vs CULTURE DENSITY	EQUATION OF CURVE	R SQUARED	MODEL PROBABILITY
CELLULAR ß-CAROTENE CONTENT	$Y = 26.5\ X^{-0.6}$	0.94	<0.00001
CELL NUMBER INCREASE	$Y = 25\ X^{0.5}$	0.70	<0.00001

The equations of the two curves are almost identical, the primary difference being that the slope of the cellular ß-carotene content versus starting culture density is negative, indicating an inverse relationship between *D. salina* growth and ß-carotene production over this culture density range (Table 5.2).

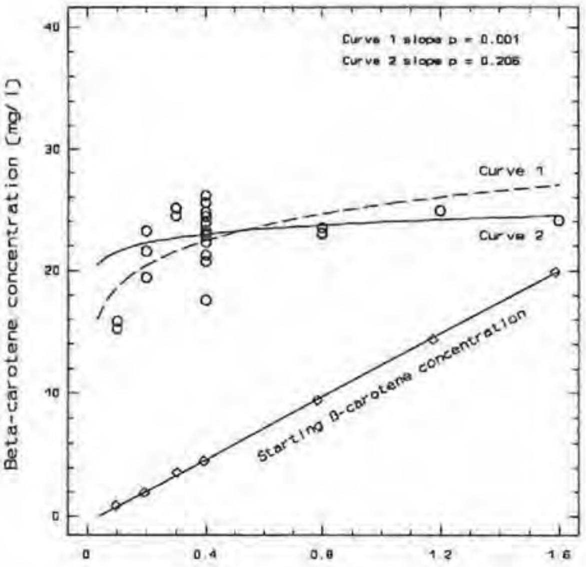


Figure 5.11 Relationship between final ß-carotene yield, starting culture density and the starting ß-carotene concentration of the innoculum. Curve 1 includes 0.1 g dry wt.l^{-1} data points, whereas Curve 2 excludes 0.1 g dry wt.l^{-1} data points. Starting ß-carotene concentration represents the average starting ß-carotene concentration at each starting culture density point. Results reflect day 5 values.

The effect of starting culture density on the day 5 ß-carotene yield is depicted in Figure 5.11. If the day 5 ß-carotene yields are compared to the starting ß-carotene concentration, it is apparent that more ß-carotene was produced in cultures inoculated at lower starting culture densities than at higher culture densities (Figure 5.11). Practically, it is unlikely that a 0.1 g dry wt.l^{-1} starting culture will be used due to the complications associated with harvesting such a dilute culture, however, what is noteworthy about the relationships depicted by Curve 1 and Curve 2 is that if the 0.1 g dry wt.l^{-1} ß-carotene yields are omitted, then Curve 2 is almost horizontal (slope probability 0.206), indicating similar ß-carotene yields of about 23 mg.l^{-1} were obtained irrespective of starting culture density. However, if the 0.1 g dry wt.l^{-1} ß-carotene concentration results are included, then this model does not hold, as the slope becomes significant (slope probability 0.001) and a gradual decline in ß-carotene concentration is apparent at lower starting culture densities.

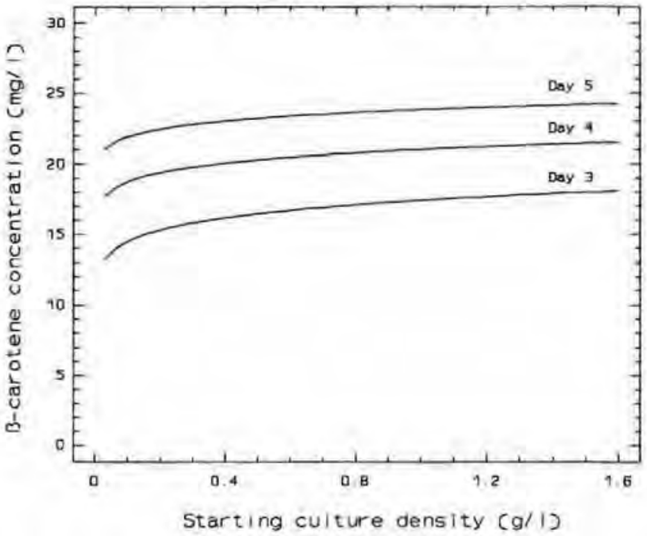


Figure 5.12 Effect of retention time on ß-carotene yield. Results exclude 0.1 g dry wt.l^{-1} data.

Figure 5.12 shows ß-carotene concentration at different retention times within the culture (0.1 g dry wt.l^{-1} data omitted). It is evident that a similar relationship exists for day 3 and 4, as for the final day culture ß-carotene yield. However, there were progressively lower ß-carotene yields at decreased retention times (Figure 5.12).

The results reported in Chapter 4 have shown that the percentage ß-carotene composition of the biomass produced in the growth ponds is consistently about 1 % (w/w). In order to determine the relative ß-carotene production at the various culture densities and to compare them to those obtained in the growth ponds, the percentage ß-carotene composition was determined for day 5. Ignoring the contribution of algal growth to the day 5 ß-carotene yield within the stress ponds, the percentage composition was calculated as follows:

$$\% \text{ ß-CAROTENE COMPOSITION} = X.100/Y$$

Where, X = ß-carotene concentration (g.l^{-1}) on day 5, and Y = starting culture density (g dry wt.l^{-1}). The values obtained are not an accurate reflection of the ß-carotene percentage composition of the biomass on day 5, however, in terms of the dual-stage process, they reflect the ß-carotene increase incurred by the biomass during the stress period ie. both cellular ß-carotene accumulation (Figure 5.8) and growth (Figure 5.9) are taken into account.

Table 5.3. Relative ß-carotene composition of biomass inoculated into the stress ponds. Values reflect means for each starting culture density for day 5 values. Average starting ß-carotene content 1 % dry wt.

START CULTURE DENSITY (g dry wt.l^{-1})	0.1	0.2	0.3	0.4	0.8	1.2	1.6
PERCENTAGE COMPOSITION (% dry wt.)	15.5	11.2	8.2	5.57	2.94	2.08	1.5

It is clear from Table 5.3 that more ß-carotene per unit biomass obtained from the growth ponds was produced at the lower starting culture densities. If one takes into account that the ß-carotene content of the biomass on transfer from the growth ponds was 1 % dry wt., then it becomes apparent that greater ß-carotene hyper-accumulation occurs at the lower culture densities.

Upscale of stress ponds from the 0.2 m^2 to 2 m^2 ponds.

In order to determine the effect of a 10-fold upscale of the stress ponds a comparison of ß-carotene production in the 0.2 m^2 ponds and the 2 m^2 ponds was made. Biomass produced in the stress ponds was inoculated into both the 0.2 m^2 and 2 m^2 pond at a starting culture density of 0.2 g dry $wt.l^{-1}$. Table 5.4 shows the cell number, cellular pigment composition and ß-carotene concentrations within the different ponds on day 5.

Table 5.4 Comparison between 0.2 m^2 and 2 m^2 ponds. Results reflect day 5 values. Starting cell count 15 x 10^4 $cells.ml^{-1}$, starting chlorophyll content 9 $pg.cell^{-1}$, starting ß-carotene content 18 $pg.cell^{-1}$ and starting ß-carotene concentration 2.5 $mg.l^{-1}$.

POND SIZE	CELL No. ($10^4.ml^{-1}$)	CHLOROPHYL ($pg.cell^{-1}$)	ß-CAROTENE ($pg.cell^{-1}$)	ß-CAROTENE ($mg.l^{-1}$)
0.2 m^2	27	6.51	80.1	21.63
2 m^2	25	6.30	85.3	21.33

* results reflect the mean of two experiments.

It is apparent from Table 5.4 that the cell number and the cellular chlorophyll content were slightly lower for cultures grown in the larger 2 m^2 ponds. The cellular ß-carotene content of 85.3 $pg.cell^{-1}$ in the 2 m^2 was however, higher than the ß-carotene content of cells grown in the smaller ponds. What is relevant to the study, is that the ß-carotene yields in both the large and the small ponds were similar (Table 5.4).

5.5 DISCUSSION.

The results presented here have clearly demonstrated that ß-carotene hyper-accumulation can be induced in the biomass obtained from the growth pond stage of the dual-stage system. Furthermore, ß-carotene yields comparable to those obtained in the laboratory studies (approximately 22-23 $mg.l^{-1}$)(Chapter 2) were obtained by the manipulation of environmental and physiological stress factors without compromising the biomass retention time in both the 0.2 and 2 m^2 stress ponds.

The removal of high nitrate containing growth media from flocculated biomass is unlikely to be feasible. The results have shown that the optimal nitrate concentration for ß-carotene accumulation (determined in Chapter 2) can be obtained by the carryover of a specific quantity of growth medium into the stress ponds (Table 5.1). The evaluation of a range of growth medium carryovers, above and below the nitrate optimum, did not reveal any marked difference in the growth and pigment composition of *D.salina* (Table 5.1). Consequently, the final day ß-carotene yields were similar. A difference in algal growth and pigment content at the different nitrate concentrations was anticipated, however, this was not demonstrated. The reason for this may be two-fold, firstly, it is possible that the nitrate concentrations covered by the different carryovers falls within the range required for maximum ß-carotene accumulation. Secondly, it is possible that under outdoor conditions other stress factors such as the high incident PFD may override any apparent differences.

In a closed production system (ie.medium is recycled after harvesting), such as that anticipated for the dual-stage system, media purge will have a significant affect on the economics of the process (Borowitzka, 1992). Ideally, medium purge should be maintained at a minimum. If the dual-stage system is operated on the basis of growth medium carryover, the growth medium carryover to the stress ponds, will constitute a media purge from the growth ponds. Therefore, a minimum growth medium carryover to the stress ponds would be most economically beneficial to the system. The results indicate that a growth medium carry over of as low as 2.5 % can be used without compromising ß-carotene production.

A direct comparison between outdoor and laboratory values (Chapter 3) for the different stresses examined is not possible due to the different light regime experienced by the outdoor culture (ie. higher light intensities, and diurnal light cycle). However, a comparison of the trends in each system is possible.

As demonstrated in the laboratory studies (Chapter 2), it was shown that some form of stress is necessary for increased ß-carotene accumulation. The imposition of all the stresses examined results in growth inhibition, an impaired chlorophyll content and increased cellular ß-carotene accumulation. The extent of this response is more pronounced as more stresses are applied to the system (eg. multiple stress). Consequently, as observed in the laboratory study (Chapter 2), the high salinity, total nutrient limited multiple stress medium resulted in the highest degree of growth inhibition, the lowest cellular chlorophyll content and the highest cellular ß-carotene content. Of the single stresses examined, nitrate limitation was shown to result in higher cellular ß-carotene accumulation and a greater decrease in the cell growth. This trend is consistent with results obtained in the laboratory studies (Chapter 2).

The inverse relationship observed between cell growth and ß-carotene accumulation in response to cumulative stress was previously shown to result in similar day 5 ß-carotene yields (Chapter 2). Under outdoor conditions a shift in the maximum ß-carotene yields was observed, and a peak in ß-carotene yield in the dual and multiple stress systems occurred a day earlier (day 5) than in the single stress systems (day 6). The reason for the shift in the peak in ß-carotene yields appears to be a function of the initial rapid ß-carotene production rates observed in the dual and multiple stress systems, which gives rise to high ß-carotene yields (>20 mg.l^{-1}) by day 3. The accelerated rate of ß-carotene production observed under outdoor conditions may be a function of the high incident PFD.

The primary objective of the stress ponds in the dual-stage process is to maximise ß-carotene production in the shortest time period possible. The use of a multiple stress medium shows clear advantages in this respect. Additional economic benefits may be incurred by the use of the high salinity, total nutrient limited medium because of its limited constituent make-up (NaCl and $NaHCO_3$).

The determination of the optimal starting culture density for the operation of the stress ponds is possibly the most important prerequisite for the optimization of massive ß-carotene accumulation in *D. salina* as it determines the quality and quantity of light each cell within the culture receives. Previous research has shown that light intensity is the most important factor influencing ß-carotene hyper-accumulation in *D. salina* (Mil'ko, 1963b; Ben-Amotz and Avron, 1983, 1989, 1990, Ben-Amotz, 1986,1987; Lers *et al.* 1990, Loeblich, 1982). The results reported in Chapter 2 and 3 have further confirmed these observations.

The outdoor studies have established that high levels of ß-carotene accumulation only occurred at culture densities below 0.4 g dry wt.l^{-1} (Figure 5.8). In addition, there was a sharp decrease in cellular chlorophyll content (Figure 8) and in cell growth (Figure 10) below 0.4 g dry wt.l^{-1}. It is well established that high light intensities promote a smaller chlorophyll antenna (Smith *et al.* 1990) and increase the cellular ß-carotene content of *D. salina* (Ben-Amotz and Avron, 1983, 1990). The drastic change in the pigment composition of the cells indicates that light was in excess at culture densities of below 0.4 g dry wt.l^{-1}, resulting in a progressive increase in both cellular ß-carotene accumulation and chlorophyll degradation as the culture density decreased. The increase in growth inhibition experienced at culture densities below 0.4 g dry wt.l^{-1} appeared to be a response to the additional stress subjected to the system by the increasing light intensity.

At culture densities above 0.8 g dry wt.l^{-1} a gradual decrease in the cellular ß-carotene content and a gradual increase in the chlorophyll content of *D. salina* was exhibited by *D. salina*. It appears that light was limiting at these high starting culture densities since growth was severely inhibited at culture densities above 0.8 g dry wt.l^{-1}. An increase in the cellular chlorophyll content and a decrease in the cellular ß-carotene content is anticipated when light becomes limited (Ben-Amotz and Avron, 1983). However, the additional stress factors such as high salinity and total nutrient limitation may have provided a certain degree of stress within the light limited cultures which maintained the relatively high cellular ß-carotene content and the relatively low cellular chlorophyll content.

A direct inverse relationship between cell growth and ß-carotene accumulation was demonstrated for starting culture densities below 0.8 g dry wt.l^{-1} (Figure 5.10). Previously published results have shown that *D. salina* accumulates ß-carotene under conditions which retard cell growth (Ben-Amotz and Avron, 1983, Borowitzka and Borowitzka, 1988). However, no quantitative analysis of the inverse relationship has been reported. The inverse relationship observed between growth and ß-carotene production forms the basic dilemma underlying the current commercial process of ß-carotene production from *D. salina*, since current processes attempt to simultaneously optimise both cell growth and ß-carotene production (Borowitzka and Borowitzka, 1988, Borowitzka *et al.* 1990). The inverse relationship between growth and ß-carotene production is unlikely to impose as great a limitation on ß-carotene production in the dual-stage system because of the separation of the distinct growth and ß-carotene production stages.

The inverse relationship between cell growth and ß-carotene hyper-accumulation had a marked impact on the ß-carotene yields obtained within the stress cultures. If the 0.1 g dry wt.l^{-1} starting culture density data is omitted, then similar day 5 ß-carotene yields (approximately 22-23 mg.l^{-1}) were obtained irrespective of starting culture density (Figure 5.11). This is demonstrated by the "horizontal curve" obtained for ß-carotene yields at starting culture densities above 0.1 g dry wt.l^{-1} (Figure 5.11, Curve 2). The nature of the relationship between starting culture density and ß-carotene yield may indicate a "ceiling" in ß-carotene yield over this starting culture density range. The proposed "ceiling" in ß-carotene yield in response to starting culture density forms a strong link with the maximum ß-carotene yield reported under laboratory conditions (Chapter 2). The postulation of a maximum ß-carotene yield seems plausible due to the inverse relationship between cell growth and ß-carotene accumulation which has been observed throughout both the laboratory (Chapter 2) and the outdoor study.

The significance of the "ceiling" in ß-carotene yield to the dual-stage ß-carotene production system is that more ß-carotene per unit biomass inoculated into the stress ponds is produced at lower culture densities, in order to achieve a day 5 ß-carotene yield of approximately 22-23 $mg.l^{-1}$. This is clearly evident if the day 5 ß-carotene yields obtained at the different starting culture densities are compared to their starting values (Figure 5.11).

If the day 5 ß-carotene yields are expressed as a percentage of the starting culture density (Table 5.3) this provides an indication of the ß-carotene content of the biomass inoculated into the stress ponds after a 5 day stress period. The ß-carotene content of biomass produced in the growth ponds was consistently around 1 % dry wt. At high culture densities ß-carotene production was marginal compared to the starting ß-carotene content of 1 % dry wt. (Table 5.3). However, ß-carotene contents of up to 15.5 % dry wt. were obtained at the lower starting culture densities.

Ben-Amotz and Avron (1989, 1990) report that for the current commercial ß-carotene production process (averaging process) the average ß-carotene content of the biomass is typically about 3-5 % dry wt. When one compares these values to the relative ß-carotene content of biomass produced in the stress ponds (Table 5.4), the full potential present in the dual-stage ß-carotene process is realised because, the relative ß-carotene content of biomass obtained at low starting culture densities (below 0.3 g.l^{-1}) is 2 to 3 times greater than that produced by the averaging process.

Concluding remarks.

The outdoor stress pond evaluation has clearly established that the biomass produced in the high productivity growth ponds can be successfully separated and transferred into the stress ponds. Furthermore, ß-carotene hyper-accumulation can be induced in this biomass by the manipulation of both environmental and physiological stress factors.

As observed in the laboratory studies (Chapter 2), the role of high light intensity has been shown to be possibly the most important stress associated with ß-carotene hyper-accumulation. The strong multiplicative relationship observed between starting culture density and ß-carotene accumulation (Figure 5.8) unequivocally demonstrates its influence on ß-carotene hyper-accumulation. Another factor associated with ß-carotene production which was previously highlighted in Chapter 2 is the inverse relationship between cell growth and ß-carotene accumulation which persists throughout the indoor and outdoor studies. Its effect on the ß-carotene yield is clearly illustrated by the "ceiling' in ß-carotene production above a starting culture density of 0.1 g dry wt.l^{-1}.

The results presented in Chapter 2 suggested the presence of a maximum ß-carotene yield of about 22-23 mg.l^{-1}. The results reported here confirm this observation. It is unlikely that higher ß-carotene yields will be obtained by further manipulation of either growth or ß-carotene accumulation, given the demonstrated inverse relationship between growth and ß-carotene accumulation.

As speculated at the onset of this study, the results have shown that low starting culture densities are necessary for maximum ß-carotene accumulation. ß-carotene accumulation per gram biomass inoculated into the stress ponds is greatest at the lower culture densities due to the a reduction in self shading at lower culture densities which improves the light penetration of the culture. If one considers that the growth ponds would be harvested at a culture density of about 0.4 g dry wt.l^{-1} (Chapter 4) this implies that in order to obtain starting culture densities of below 0.4 g dry wt.l^{-1} in the stress ponds, dilution of the biomass would be necessary. The implication of this is that if low starting culture densities are employed, larger stress ponds will be necessary in order to compensate for the dilution factor. A possible compromise between ß-carotene production and pond size is therefore noted. Another important aspect in terms of the design of a dual-stage system is the retention time of the biomass in the stress ponds. The results have shown that as the retention time decreases there is a progressive reduction in ß-carotene yield. It is therefore concluded, that the influence of retention time and starting culture density require careful consideration in the process design and economic evaluation of the dual-stage system. This study was undertaken and is reported in Chapter 8.

CHAPTER 6

AN EVALUATION OF *D. SALINA* CELL SEPARATION

Summary.

D. salina separation with flocculation and crossflow ultrafiltration (CFUF) was examined with the specific aim of maintaining cell viability during separation. A flocculation/flotation process using a polyaluminium chloride flocculant, K300, was shown to be highly suitable for the dual-stage system. The process produces a wholly viable cell concentrate which can be inoculated into the stress pond stage of the dual-stage system. Pump driven CFUF was not suitable for *D. salina* separation due to a high degree of cell damage experienced during cell concentration. An air-displacement CFUF system was developed and a preliminary study was undertaken. The process appears to alleviate the cell damage caused by mechanical pumping of *D. salina* cultures.

6.1 INTRODUCTION.

The commercial production of algal biomass depends on two main biotechnological processes; firstly, a reliable cultivation system and secondly, an effective and economical separation and harvesting system (Borowitzka, 1986). Of all the technical aspects of algal production, harvesting the biomass from the relatively dilute suspensions is one of the most crucial steps, both from an economical and a technological view (Bilanovic *et al.*,1988). Although significant advances in the field have been made (Moraine *et al.* 1980; Mohn, 1978, 1980; Curtain *et al.* 1987), the separation of microalgae has developed into a technological bottleneck for algal biotechnology and the development of appropriate technologies is open to further improvement.

D. salina grown outdoors achieves the similar low level of biomass (around 0.5 g dry wt.l^{-1}) as do other algae. In addition *D. salina* possess a number of other features which makes separation a major limiting factor in their commercial utilization (Ben-Amotz and Avron, 1989). These factors include:

- the cells are small and therefore simple cost effective processes such as microscreens can not be successfully employed for separation.
- the cells are motile and have a density only slightly greater than that of the growth medium and thus natural sedimentation does not occur spontaneously.
- the high salinity and ionic strength of the media interferes with chemical coagulation and flocculation (Sukenik *et al.* 1988; Bilanovic *et al.*, 1988).
- the cells lack a rigid cell wall and are therefore fragile. This has implications for down-stream processing as damage to the cells leads to the loss of the intercellular contents and renders the ß-carotene susceptible to oxidation. Furthermore, as stated previously, the dual-stage ß-carotene production process depends implicitly on the implementation of an effective separation technology which maintains viable cells.

There are a number of separation processes available for the harvesting of *D. salina*. These include centrifugation, flocculation combined with sedimentation and flotation, filtration through diatomaceous earth (Ben-Amotz and Avron, 1989; Ruane, 1974a) and hydrophobic adsorption (Curtain *et al.* 1987). However, the choice of an appropriate separation technology depends on a number of factors such as the intended use of the harvested biomass, the production strategy employed and whether media recycle is necessary.

Algae grown specifically for consumption, whether as a dried whole algae feed or as a protein supplement after carotenoid extraction, can not be harvested by flocculation with toxic chemical flocculants due to contamination of the biomass (Ben-Amotz and Avron, 1989).

The cost of medium in the intensive commercial production of *D. salina* constitutes a major portion of the total production costs and thus, recycling of the medium is important in the reduction of production costs (Borowitzka, 1992). Ideally, the separation technique employed should remove bacteria, undesirable algal species and protozoans and it should not damage the cells as this leads to cytoplasm leakage and the build-up of organic compounds in the growth media which favours bacterial growth. Failure of the separation technology to achieve these criteria would mean that additional costs would have to be incurred in processing the media for recycle (eg. by activated sludge treatment). Media recycle in the extensive systems is not as crucial due to the vast reserves of natural media at the disposal of such systems.

Centrifugation has been the most widely used technique for harvesting microalgae (Mohn, 1980). It is highly efficient and simple to operate but it involves a relatively high initial capital investment and high energy and labour cost for operational maintenance (Ben-Amotz and Avron, 1989; Mohn, 1980). Part of Sastech's research program has been the evaluation of a continuous-flow centrifuge at the UOFS, Bloemfontein, South Africa. It was found that the system was effective for harvesting *D. salina* cells however, massive cell damage was experienced and consequently, the quality of the clarified media was impaired by significant amounts of organic material (Jarvis *pers comm,* 1993). A high degree of cell destruction has been reported by other researchers for continuous flow automatic discharge centrifuges (Ben-Amotz and Avron, 1989). The cell damage experienced during centrifugation obviously disqualifies it as a suitable separation technology for the dual-stage system.

The centrifugation process is specific for particles with a size and density similar to the algae for which the system has been designed. In systems where media is recycled, this can lead to the selective accumulation of smaller particles including dirt, dust and contaminating microorganisms (i.e. small algal species, fungi, halophilic bacteria and their spores)(Moraine *et al.*,1980). The accumulation of dust and dirt in the growth medium may be an important contributing factor to the sub-optimum yields reported for open pond algal cultures due to a reduction in light penetration (Richmond, 1988a).

It has been established that chemical flocculation followed by sedimentation or flotation is the most practical and economical way of harvesting microalgae and it has been extensively used in waste water treatment (Friedman *et al.* 1977; Golueke and Oswald, 1965; McGary, 1970). *Dunaliella* cells flocculate on the addition of flocculants such as aluminium sulphate, ferrous and ferric sulphate, ferric chloride, lime, and a number of commercial polyelectrolytes (Ben-Amotz and Avron, 1989). However, owing to the high salinity of the growth media, conventional flocculating agents are effective only at high concentrations because of the electrostatic shielding effects that occur at high salt concentrations (Curtain *et al.* 1987).

One of the main drawbacks to the use of chemical flocculation is that flocculated algae cannot be used directly for the food market unless the flocculant is safe or completely removed from the algal biomass. However, ß-carotene can be extracted from the flocculated algae, leaving behind the contaminated cell debris (Ben-Amotz and Avron, 1989).

Chitosan, a non-toxic biopolymer has been successfully tested on a number of freshwater and marine microalgal species and due to its digestibility and lack of toxicity it can used to flocculate algal biomass intended for use as a feed (Nigam *et al.* 1980; Shelef *et al*, 1980; Lubian, 1989; Morales *et al.* 1985).

Besides the economic advantages, flocculation has other features that make it an attractive separation technology. Flocculation can be used in *Dunaliella* ponds which operate in a closed system (i.e. medium is recycled to the pond following harvesting) as long as care is taken to prevent a carryover of flocculant back into the growth ponds (Ben-Amotz and Avron, 1989). Flocculation is a comparatively gentle process and cell destruction due to mechanical shear is not a consideration. This makes flocculation an attractive candidate technology for the dual-stage process.

Conventional filtration of *Dunaliella* through sand filters, cellulose fibres and other filterable materials has not proved practical (Ben-Amotz and Avron, 1989). The algae clog the filters by the formation of a mucous layer which further impedes filtration. Repeated backwashing of the system causes cell damage and the release of glycerol and organic matter into the medium (Ben-Amotz and Avron, 1989).

Traditionally, cross-flow (tangential flow) filtration (CFF) has been used in the water treatment field (eg. the removal suspended solids from sewage, the removal of organic material from river water) (Bindoff,1987; Teffry-Goatly, 1987) and in the food industry (eg. clarification of fruit juices, the removal of whey solids from milk) (Tutunjian, 1985). However, more recently CFF has been successfully applied to a number of cell separation and concentration processes in biotechnology (Tutunjian, 1985). These include the concentration of fragile mammalian cells (Bailey *et al.* 1990) and the concentration of yeast and other microorganisms in continuous fermentation cell recycle systems (McGregor, 1986; Cheryan and Mehaia, 1983; Ohleyer *et al.* 1985). CFF can also be used as a cell washing or diafiltration process (Gabler, 1985). The process incorporates the combined effects of dialysis and filtration to remove solutes and retain particulates, at a constant volume and hence particulate concentration, by the addition of fresh medium.

Ruane (1974a) has patented a method for the recovery of *Dunaliella* using diatomaceous earth as a cross-flow microfiltration (CFMF) filter aid. The filtered algae are extracted from the diatomaceous earth with an organic solvent, yielding an extract containing ß-carotene which can be further concentrated and purified.

Ben-Amotz and Avron (1989) report that CFF has been unsuccessfully evaluated for *Dunaliella* separation with a number of commercially available filtration apparatuses (eg. hollow fibres, porous tubing, and filter trays). The authors noted that shortly after pressure filtration is initiated, the cells clog the filter , possibly due to cell lysis, and only temporary relief is obtained by backwashing.

Rose (1992) evaluated both CFMF, using diatomaceous earth as a filter aid, and cross-flow ultrafiltration (CFUF), using a 9 mm diameter polyethersulphone membrane tubular ultrafiltration unit with 80 000 MW cut-off (Membratek) as a cell separation and a cell washing (diafiltration) process for *Dunaliella*. CFMF was not found to be a successful technique for the separation of *Dunaliella* in a viable form due to the massive cell damage that occurred during filtration. However, CFUF was demonstrated to be an effective cell separation and diafiltration process with minor cell losses of between 0.5-5% being reported for the various small scale ultrafiltration trials. In addition, the 80 000 MW cut-off of the polyethersulphone membrane produces a sterile permeate which is highly suitable for recycling (Rose, 1992).

The microfiltration system requires a high outlet pressure for the continuous maintenance of the diatomaceous earth dynamic membrane pre-coating which results in a low differential between inlet and outlet pressures (1.6:1). This produces a sharp pressure drop at the outlet point, to atmospheric pressure, to which has been attributed the cause of cell damage. By contrast, the pressure fall across the CFUF system is less severe (35:1), with a low outlet pressures of 10-15 KPa (Rose, 1992).

One of the main drawbacks to CFF is membrane fouling (Le and Howell, 1985: Tutunjian, 1985). A gradual reduction in membrane flux is expected due to the formation of a polarized layer which forms an intimate part of the membrane filtration effect. The regeneration of flux is imperative and is used to determine the suitability of an application. Rose (1992) has shown the fall in flux rate experienced during CFUF of *Dunaliella* cells can be fully restored by washing the membrane with clean water after the ultrafiltration run. The CFUF results reported by Rose (1992) indicate that the CFUF system is a suitable separation technology for the dual-stage ß-carotene production process and warrants further investigation.

6.2 RESEARCH OBJECTIVES.

The separation of algal biomass from the growth ponds and the transfer of viable cells into the stress ponds forms a critical step in the dual-stage process (Chapter 1, Figure 1.1). The evaluation of cost effective separation techniques which maintain the cells in a viable state is essential for the implementation of a dual-stage system. Furthermore, factors such as the suitability of the separation technique for medium recycle should be addressed since medium recycle is imperative for low operating costs (Borowitzka, 1992).

The following research objectives were identified to address the above requirements:

1. To evaluate flocculation and CFUF for the production of viable cell concentrates in the dual-stage system.
2. To evaluate a flocculant removal and recovery process.
3. To determine the effect of flocculant carry-over into the growth and stress environment of the dual-stage system and thus, to determine the effectiveness of flocculation for media recycle.

6.3 MATERIALS AND METHODS.

6.3.1 Algal culture medium and growth conditions.

D.salina cells used in the laboratory flocculation studies were cultured in 20 l glass containers in the growth medium described in Chapter 2. Cultures were incubated at 28 °C under constant illumination of 90 μmol.m^{-2}.sec^{-1}.

CFUF and flocculation scale-up studies were conducted using both biomass produced in the growth ponds (Chapter 2) or with that obtained from the UOFS algal ponding facility.

6.3.2 Aluminium determination.

Aluminium was determined using atomic absorbtion spectroscopy. The aluminium present in the medium was measured directly without making any prior adjustments. The determination of aluminium in algal biomass was performed as follows: a known mass of algal biomass (dry wt) was boiled in a solution containing 5 ml concentrated nitric acid and 15 ml sulphuric acid/perchloric acid (1:3) in a fume cupboard. Once acid digestion was complete the solution was cooled and diluted to a specific volume in a volumetric flask. Aluminium was then determined by atomic absorption spectroscopy.

6.3.3 Laboratory flocculation study.

A range of flocculants were assayed in 1 litre flocculation tests. The method used was an adaptation of the method described by Morales *et al.* (1985):

1. The algal culture was homogenised by mixing gently on a magnetic stirrer in a 1 litre beaker.
2. The pH was adjusted by drop-wise addition of 0.1 M HCl or 0.1 M NaOH and the flocculant was added.
3. The culture was stirred at 100 rpm for 1 min and 40 rpm for 4 min and transferred to a 1 litre plastic cone (cone height 30 cm) in order to induce sedimentation or flotation.

The flocculation rate was established by the time taken for the flocculated biomass to settle or float completely. The flocculation efficiency (%) was determined by comparing the starting culture cell count with the cell count of the clarified portion, taken at a constant depth of 15 cm with a pasteur pipette.

A 0.5 % chitosan (Sigma C3646) in 1 % acetic acid solution (pH 3.5) was used for the flocculation evaluations (Lubian, 1989).

6.3.4 Bacterial counts.

Bacterial counts were determined by plate count colony forming units or using a Coulter Multisizer. Results reflect a triplicate mean.

6.3.5 Effect of flocculants on *D. salina* physiology - a laboratory study.

Experiments were conducted to determine the effect of large amounts of flocculants on the growth and pigment composition of *D. salina*. The effect of the flocculants was monitored in both growth medium and a high salinity nitrate limited medium. Log phase *D. salina* cultures grown in growth medium were harvested by flocculation with 500 mg.l^{-1} aluminium chloride, 100 mg.l^{-1} chitosan and 100 $\mu l.l^{-1}$ K300, respectively. The algal flocs were then transferred to either growth medium or high salinity (3 M NaCl) nitrate limited (0.2 mM NO_3^-) stress medium. Cultures were incubated at 28 °C under a high light intensity of approximately 1000 $\mu mol.m^{-2}.s^{-1}$ for 5 days. Algal cell counts were made using an improved Neubauer haemocytometer. Results reflect a triplicate mean. ß-carotene and chlorophyll were determined as previously described.

6.3.6 Removal of aluminium from flocculated biomass.

An acid washing process was evaluated for the removal of aluminium from biomass separated with aluminium chloride. A 1 l algal culture (0.8 g dry wt.l^{-1}) was harvested with 1000 mg.l^{-1} aluminium chloride (Al^{3+} concentration 112 mg.l^{-1}). The clarified portion of the medium after flocculation and cell separation was analyzed for aluminium. The flocculated biomass was resuspended in 500 ml of fresh growth medium at pH 1, at which value both aluminium oxide and aluminium phosphate are soluble (Moraine *et al.* 1980). The cell suspension was mixed for 2 minutes at 100 rpm. Following this, the cell suspension was centrifuged at 2000 g for 10 minutes. The supernatant was examined for aluminium. The algal pellet was resuspended in growth medium at pH 1 and the washing process was repeated twice.

A scale-up evaluation of the acid washing process was undertaken using CFUF diafiltration (cell washing) to simultaneously recover the aluminium-containing solution and to concentrate the algal biomass. The CFUF test rig (Figure 6.1) was fitted with a 0.8 m^2 Memtuf membrane module (Membratek, Ltd). A 150 l culture of *D. salina* cells (culture density 0.2 $g.l^{-1}$) was separated with 1000 $mg.l^{-1}$ aluminium chloride (Al^{3+} concentration 112 $mg.l^{-1}$). The separated algal floc was diluted to 50 l with fresh growth medium and the pH was lowered to pH 1 by the addition of 1.5 l concentrated HCl. The cell suspension was placed in the holding tank of the CFUF test rig (Figure 6.1) and the system was operated on a non-concentrating mode for 5 minutes to effectively mix the cell suspension (operating pressure 400 KPa, feed rate 730 $l.hour^{-1}$). The permeate stream and the concentrated cell biomass were monitored for aluminium.

6.3.7 Scale-up evaluation of laboratory flocculation studies.

Two of the most successful flocculants determined in the laboratory study were evaluated in a scale-up trial. Sedimentation studies were conducted in a 50 l sedimentation cone fitted with an outlet tap situated at the bottom of the cone. Flotation studies were conducted in a 200 l drum fitted with an outlet valve at the bottom of the drum. Flotation of the cultures was induced by sparging with carbon dioxide gas at a pressure of 100 KPa for 10 seconds through a 30 cm by 1.5 cm sintered stainless steel tube.

Culture volumes of 50 l were used for the scale-up evaluations (culture density 0.4 g dry $wt.l^{-1}$). The pH of the cultures was preadjusted with either 1 M HCl or by sparging the cultures with carbon dioxide gas. After sedimentation or flotation the separated algal biomass was resuspended in growth medium in the 0.2 m^2 ponds described in Chapter 5 and the viability of the cultures was monitored.

6.3.8 Crossflow Ultrafiltration evaluation.

CFUF trials were conducted using the Rhodes University cross-flow filtration semi-technical scale unit (Figure 6.1) fitted with 9 mm diameter polyethersulphone tubular ultrafiltration modules (Memtuf modules, Membratek Ltd) of various total membrane area. The feed rate and thus membrane inlet pressures were controlled by a variable speed positive displacement pump (Monopump). Cultures to be filtered were added to tank T1 (Figure 6.1). The operational feed rates, inlet and outlet pressures and permeate flow rates were recorded. The permeate flux was calculated as $l.m^{-2}.hr^{-1}$ (LMH).

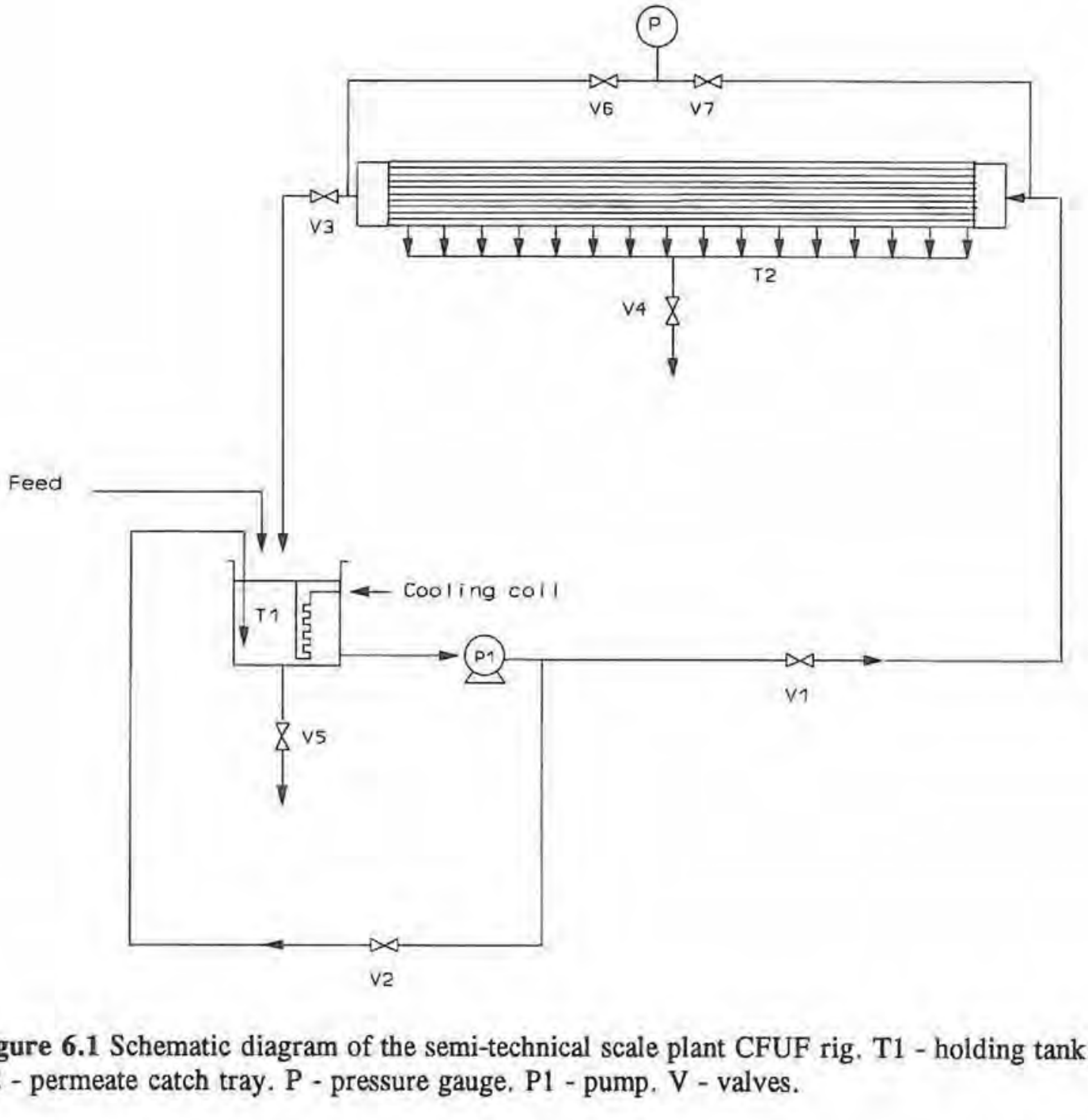


Figure 6.1 Schematic diagram of the semi-technical scale plant CFUF rig. T1 - holding tank. T2 - permeate catch tray. P - pressure gauge. P1 - pump. V - valves.

Scale-up CFUF trials were conducted using an ultrafiltration unit of a similar basic design as the Rhodes University unit (Figure 6.1). The system was driven by a larger variable speed Monopump capable of delivering 2.8 $m^3.hr^{-1}$.

Cell damage was determined microscopically by counting three fields of 100 cells and expressing damaged cells as a percentage of the total counted. The algal dry weight was determined using a Coulter Multisizer.

6.3.9 Evaluation of a pressure driven CFUF system (Air-displacement CFUF).

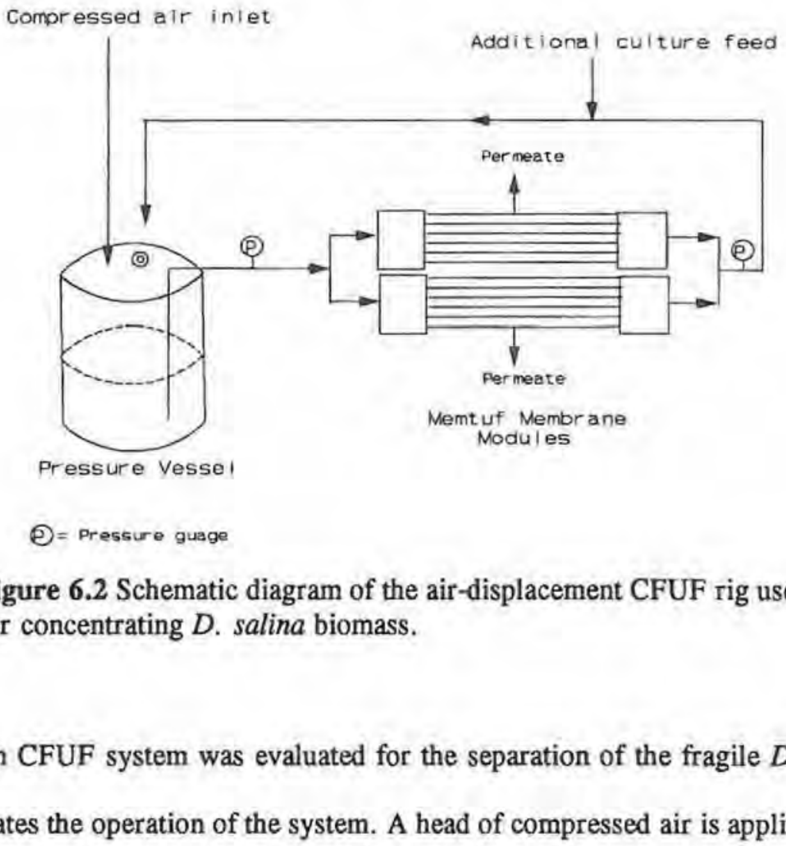


Figure 6.2 Schematic diagram of the air-displacement CFUF rig used for concentrating *D. salina* biomass.

A pressure driven CFUF system was evaluated for the separation of the fragile *D. salina* cultures. Figure 6.2 illustrates the operation of the system. A head of compressed air is applied to the pressure vessel, this forces the culture through the membrane modules where clarified permeate is removed from the system, the cell concentrate (reject volume) is recovered and if desired is returned to the pressure vessel for further filtration and concentration.

The system was fitted with either a single 1.2 m^2 Memtuf membrane module or two 1.2 m^2 Memtuf membrane modules configured in parallel (Figure 6.2). The membrane inlet pressure was adjust by controlling the compressed air inlet (Figure 6.2)

6.3.10 Comparison of ß-carotene production in cultures separated using flocculation/flotation or air-displacement CFUF.

A 20 l volume of algal culture (culture density 0.4 g dry wt.l^{-1}) obtained from the growth ponds (Chapter 4) was separated using either flocculation/flotation or air-displacement CFUF. The separated algal biomass was resuspended in a high salinity, total nutrient limited medium in the 0.2 m^2 ponds described in Chapter 5. Algal growth and ß-carotene accumulation were monitored.

6.4 RESULTS.

6.4.1. Laboratory flocculation study.

Inorganic flocculants as well as organic polymers were evaluated for *Dunaliella* harvesting. The importance of pH in flocculation processes has been demonstrated by several authors (Tenney and Stumm, 1965; Moraine *et al.* 1980; Morales *et al.* 1985; Lubian, 1989). The influence of medium pH and flocculant dosage on flocculation efficiency was examined.

Figure 6.3 shows the dosage/pH response curve of the polyvalent metal ion flocculant aluminium chloride (as $AlCl_3.6H_20$), over a pH range of 5-10 and a dosage range of 50-500 mg/l. It is apparent from Figure 6.3 that optimal flocculation efficiencies were obtained at neutral pH and at flocculant dosages of 250 mg/l and above. The deleterious effect of low (<pH 6) and high pH (>pH 8) on flocculation efficiency is clearly illustrated at the lower aluminium chloride concentrations. At high flocculant dosages (>250 mg/l) the effect of pH on flocculation efficiency was less severe and good efficiencies were obtained over a wider pH range (pH 6-9) (Figure 6.3).

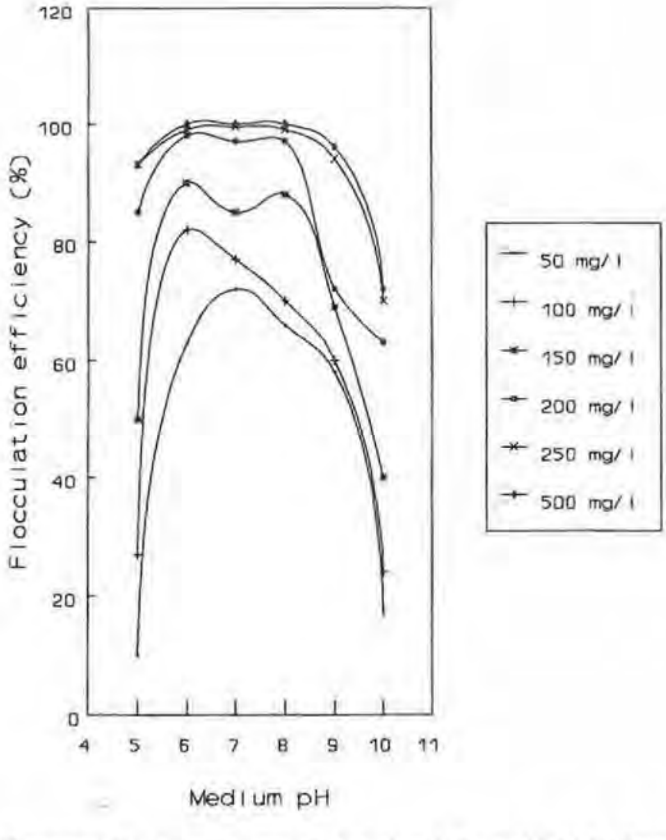


Figure 6.3 Dosage/pH response curve for aluminium chloride flocculation.

Dosage/pH response experiments were conducted with a number of additional flocculants; aluminium sulphate (as $Al(SO_4)_3.18H_2O$), ferric chloride (as $FeCl_3$), two polyaluminium chloride (PAC) flocculants, K300 and K19P, and chitosan. Table 6.1 gives a summary of the experiments showing the minimum flocculant dosage required for a flocculation efficiency greater than 98 %, the effective pH range and the time taken for complete sedimentation.

It is apparent from Table 6.1 that the two polyvalent metal ions, aluminium chloride and aluminium sulphate have similar minimum dosages and effective pH ranges (250 $mg.l^{-1}$; pH 6-8). However, it was observed that at this dosage an orange-yellow discolouration to the clarified portion of the medium was obtained during aluminium sulphate flocculation.

Table 6.1. Minimum flocculant dosages required for flocculation efficiencies greater than 98 %, showing effective pH range and time taken for complete sedimentation.

Flocculant type	Effective pH range	Minimum dosage	Sedimentation time (min.)
$AlCl_3.6H_2O$	6-8	250 $mg.l^{-1}$	45
$AlSO_4.18H_2O$	6-8	250 $mg.l^{-1}$	42
*$FeCl_3$	5.5-6.5	450 $mg.l^{-1}$	49
**PAC (K300)	6.5-7.5	100 $\mu l.l^{-1}$	5
**PAC (K19P)	6.5-7.5	100 $\mu l.l^{-1}$	4:30
Chitosan	> 8	80 $mg.l^{-1}$	44

* - maximum flocculation efficiency obtained for ferric chloride was 83 % over a dosage range of 50-500 $mg.l^{-1}$.

**- flotation of the flocculated biomass occurs.

Poor *D. salina* separation was obtained with the polyvalent metal ion, ferric chloride over the dosage range tested (Table 6.1). A maximum flocculation efficiency of 83 % was obtained at a dosage of 450 $mg.l^{-1}$. An orange-yellow discolouration of the clarified portion of the medium, similar to that obtained with aluminium sulphate, was also observed with ferric chloride flocculation.

Two commercially available Polyaluminium chloride (PAC) flocculants were tested, K19P and K300 (Floccotan, Ltd). The PAC flocculants consist of aluminium ions cross-linked with hydroxychloride dimethyl alylamine chloride polymers of varying chain length. The extended polymer network enables relatively smaller dosages of the flocculants to be used in comparison to conventional polyvalent metal ion flocculants.

It is evident from Table 6.1 that a minimum dosages of 100 $\mu l.l^{-1}$ was required to obtain high flocculation efficiencies for both PAC flocculants. Their effective pH range of 6.5-7.5 was marginally lower than the effective pH range of aluminium chloride and aluminium sulphate, pH 6-8. However, what distinguishes these flocculants from the others is firstly, that flotation of the flocculated biomass occurred as opposed to sedimentation and secondly, the time taken for complete flotation (<6 min) was considerably more rapid than the sedimentation times of the other flocculants evaluated (>40 min) (Table 6.1).

Chitosan has been used as a flocculant in the depuration of urban and industrial wastes (Bough *et al.* 1978; Wu *et al.* 1978) and due to its lack of toxicity and digestibility, it has been used for the harvesting of algal biomass intended for use as a feed (Morales *et al.* 1985; Lubian, 1989). A relatively low chitosan dosage of 80 mg.l^{-1} was demonstrated to be effective for *D. salina* separation at pH greater than 8 (Table 6.1). The flocculation time obtained for chitosan was similar to those obtained for the polyvalent metal ions aluminium chloride, aluminium sulphate and ferric chloride.

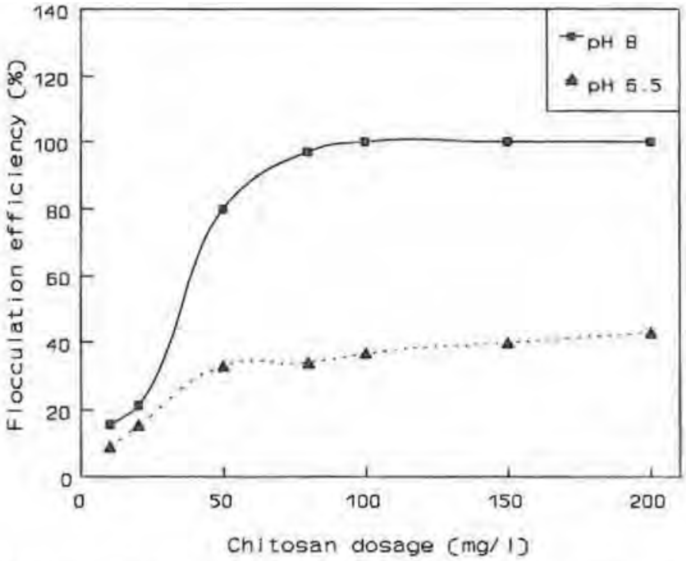


Figure 6.4 Effect of chitosan dosage on the flocculation efficiency at starting pH of pH 6.5 and pH 8

Morales *et al.* (1985) and Lubian (1989) describe the influence of pH adjustment of algal cultures prior to chitosan addition and after chitosan addition. Lubian (1989) reports that a pH preadjustment of the algal culture to pH 6.5 significantly improves flocculation at low chitosan dosages (<40 mg.l^{-1})

for a number of marine microalgal species. Figure 6.4 shows the effect of different chitosan dosages on flocculation efficiency with pH preadjustment to pH 8 and to pH 6.5 as described by Lubian (1989). It is evident from Figure 6.4 that no improvement to flocculation efficiency was obtained by pre-adjusting the pH to 6.5 and superior flocculation efficiencies were obtained at pH 8 over the whole dosage range tested.

6.4.2. Bacterial Removal

While the reuse of medium is important in reducing the running costs, a build up of contaminants in the recycled medium is undesirable. Bacterial removal was used as an indication of the effectiveness of flocculation for the removal of small contaminants such as small non-carotenogenic algae and predators.

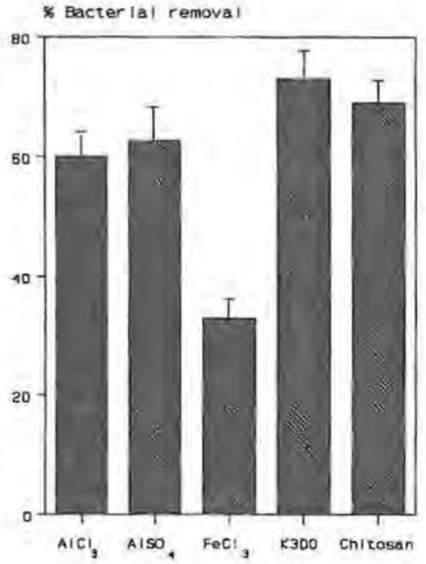


Figure 6.5 Percentage bacterial removal of the various flocculants evaluated.

Figure 6.5 illustrates the percentage bacterial removal obtained with the various flocculants at dosages and pH described in Table 6.1. Over 60 % bacterial removal was obtained for most of the flocculants evaluated except for ferric chloride which removed only 33 % of the bacterial population (Figure

6.5). The low bacterial removal efficiency obtained with ferric chloride may be related to its poor performance as a flocculant in saline medium.

6.4.3 Aluminium removal.

The major disadvantage of harvesting algae with chemical flocculants is the presence of residues in the separated biomass. For algae grown specifically for ß-carotene extraction this may not present a problem because the ß-carotene can be extracted from the algae leaving behind the contaminated biomass (Ben-Amotz and Avron, 1989; Borowitzka *pers com.* 1993). However, for algae grown for direct consumption, the flocculant should be "safe" or it should be removed from the separated biomass.

Dietary aluminium is known to be harmful to animals, probably by binding phosphates (Moraine *et al.* 1980). Moraine *et al.* (1980) describe a process for removing aluminium from aluminium sulphate separated freshwater algal biomass. Aluminium, being amphoteric, is soluble in acid and basic solutions, but forms insoluble hydroxides in neutral aqueous solutions. This amphoteric property is utilized during flocculation by operating at pH 6-7, on the border of insolubility. The aluminium may be subsequently dissolved by lowering the pH of the flocculated solids removing most of the aluminium (Moraine *et al.* 1980). This process was evaluated for removing aluminium from aluminium chloride separated *D. salina* biomass in hypersaline conditions.

It is apparent from Table 6.2 that after three washes at pH 1 the majority of the aluminium was removed from the separated biomass. At an initial aluminium dosage of 112 $mg.l^{-1}$, 93.5 % of the aluminium could be accounted for in the acid wash solutions.

The viability of the cells after acid washing was examined microscopically by resuspending the separated cells in fresh growth medium. Microscopic examination showed that the cells were apparently intact, however, the cells had shrunk in size and the outer membrane had a convoluted appearance. Acid washed cells that had been resuspended in fresh growth medium (pH 8) were all dead within 24 hours.

Table 6.2. Recovery of aluminium from separated *D. salina* biomass after acid washing at pH 1.

STARTING CONCENTRATION (112 mg.l^{-1} Al^{3+})	
SAMPLE	Al^{3+} PRESENT IN 500 ml
Clarified portion	1.7 mg
Acid wash 1	76.25 mg
Acid wash 2	21.0 mg
Acid wash 3	5.8 mg
Total recovered	104.75 mg
Percentage Recovery = 93.5%	

Although the acid wash process does not lend itself to the first cell separation step in the dual stage process, due to the total loss in cell viability, it may be incorporated after the final cell separation step to remove aluminium from the separated algae. The findings of the laboratory aluminium removal studies thus, warranted a scale-up evaluation of the process. The scale-up system incorporated the use of CFUF diafiltration (cell washing) to simultaneously remove the aluminium and concentrate the algal biomass.

Figure 6.6 shows the CFUF membrane flux rates during the CFUF acid wash trial. Flux rates ranged between 25 and 50 l.m^{-2}.h^{-1} (LMH) with a steady state flux of approximately 36 LMH, which is comparable to flux rates reported by Rose *et al.* (1992) for the separation of *D. salina* from saline effluent wastes.

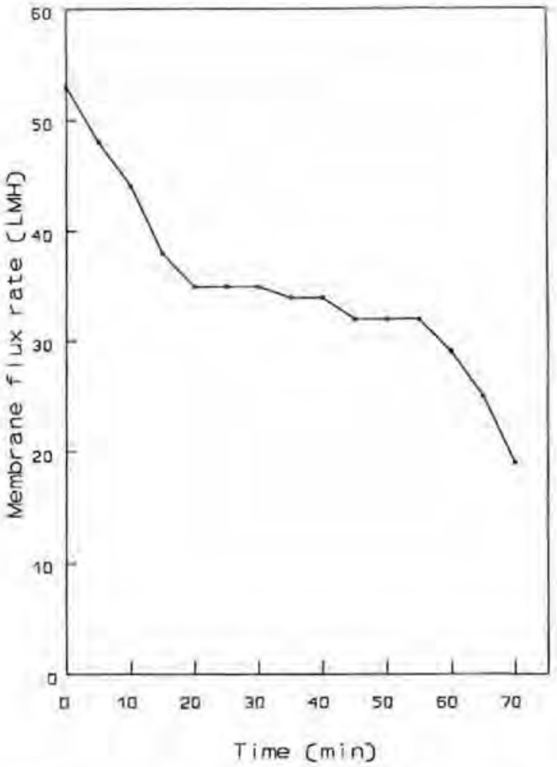
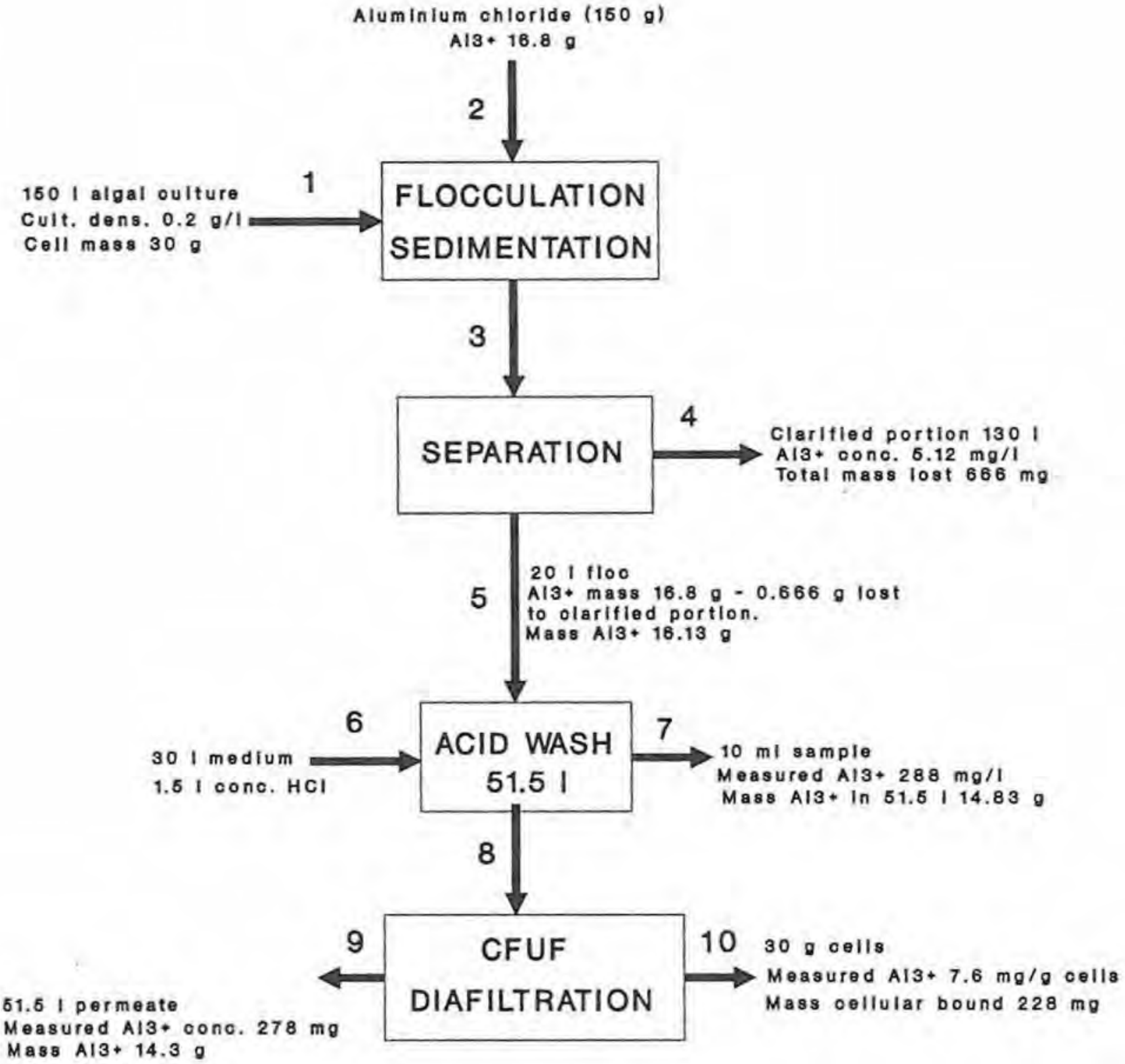


Figure 6.6 Permeate flux rates obtained during CFUF acid washing of aluminium chloride flocculated algal biomass.

Figure 6.7 shows a mass balance flow chart of the experiment. An initial mass of 16.8 g aluminium was added to the system. Aluminium present in the clarified portion of the medium (Stream 4) was measured, giving a concentration of 5.12 $mg.l^{-1}$ and therefore, a total loss of 666 mg to this stream (Figure 6.7). The aluminium present in the CFUF permeate (Stream 9) and in the biomass (Stream 10) was determined, giving total masses of 14.3 g and 0.228 g respectively (Figure 6.7). Theoretically, the mass of aluminium that can be accounted for, is the sum of the aluminium present in Stream 4, 9 and 10. This gives a total mass of 15.25 g which is 90.75 % of the aluminium added. The loss of 9.2 % of the aluminium in the mass balance may be due to a reduction in sensitivity of the analytical technique due to the high sodium and chloride ion content in the samples or to the hold up volumes in the different stages of the process. However, it is important to note that the 14.3 g aluminium measured in the CFUF permeate (Stream 9) is firstly, 88.7 % of the theoretical aluminium mass in the acid wash and secondly, when compared to the actual measured aluminium mass in the acid wash (14.83 g Stream 7) this represents a 96.4 % recovery of aluminium in the CFUF permeate (Figure 6.7). Aluminium therefore, appears to move easily across the CFUF membrane in the permeate.

The 7.6 mg aluminium.g biomass^{-1} represents 1.4 % of the total potential contamination of the biomass by aluminium. The reason that only one wash was sufficient to obtain aluminium removals of this magnitude is possibly due to the duration of the acid wash and the high degree of turbulence experienced in the CFUF system.



	STREAM NUMBER											
	1	2	3	4	5	6	7	8	9	10	Total	% Accounted
T	-	Starting conc. 16.8 g	16.8 g		16.13 g	-	16.13 g	16.13 g	16.13 g	0 g	16.13 g	100 %
M	-	Starting conc. 16.8 g		Loss 0.66 g		-	14.8 g		14.3 g	0.28 g	15.25 g	90.8 %

T = Theoretical M = Measured

Figure 6.7 Mass balance flow chart of the CFUF diafiltration acid wash evaluation.

6.4.4 Physiological Effects of Flocculants.

Effect of flocculants in the growth medium.

As stated previously the recycling of the saline medium in an intensive culture system is vital for reducing the running costs of the system. If flocculation is used as the separation technique it is important to ascertain the effects of the flocculant on the physiology of the algae in the event of accidental carryover of the flocculant into the culture medium or in case of the gradual accumulation of the flocculant in the culture medium. Aluminium sulphate and ferric chloride were not evaluated due to the discolouration of the medium experienced with these flocculants. The PAC flocculant K300 was used for the evaluation due to its easy availability.

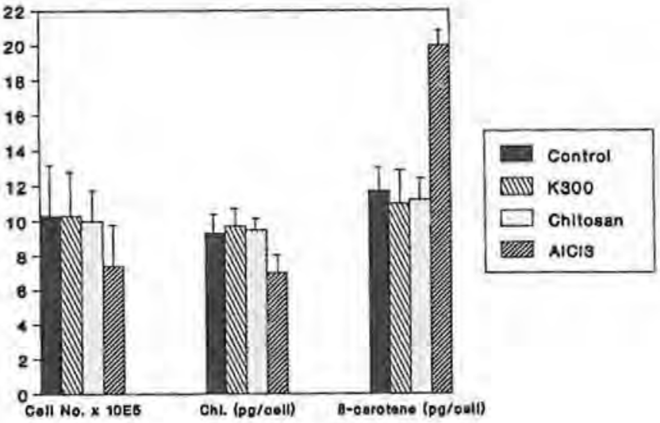


Figure 6.8 Effect of various flocculants on the growth and pigment content of *D. salina* cells in growth medium. Results reflect day 5 values.

Figure 6.8 illustrates the influence of the various flocculants on growth and the cellular ß-carotene and chlorophyll content of *D. salina*. Control experiment cells and cells grown in the presence of chitosan and the PAC flocculant, K300, had similar final day cell counts and cellular chlorophyll and ß-carotene contents. However, the final day cell number and chlorophyll content of cells grown in the presence of aluminium chloride were considerably lower than the cell number and cellular chlorophyll content of the control experiment, 28 % and 25 % lower, respectively (Figure 6.8). It is well established that factors that adversely affect the growth of *D. salina* are associated with an increase in ß-carotene production (Chapter 2). It is clear from Figure 6.8 that ß-carotene production was enhanced by the presence of aluminium chloride in the growth medium, with 53 % more ß-carotene being produced per cell than in the control experiment.

Effect of flocculants in stress medium.

Since the removal of flocculants from the flocculated biomass is likely to cause a significant loss in biomass viability, as with the aluminium acid washing process, the separated biomass will have to be transferred to the stress medium together with the specific flocculant used. It is essential to determine the physiological effects of flocculants under conditions that stimulate ß-carotene production (ie. in the stress ponds of the dual-stage system).

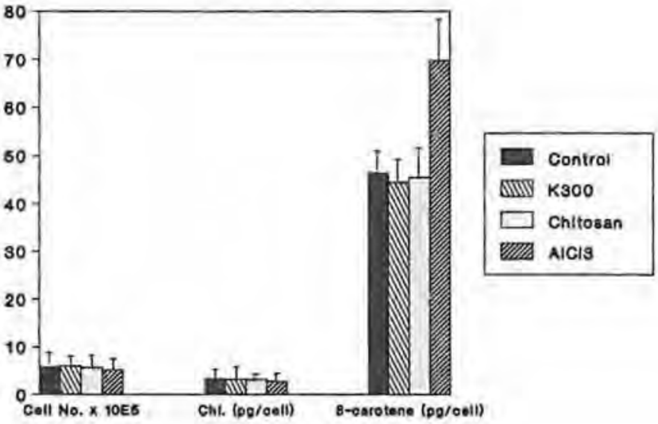


Figure 6.9 Effect of various flocculants on the growth and pigment content of *D. salina* cells in stress medium. Results reflect day 5 values.

The influence of the various flocculants in stress medium on *D. salina* growth and cellular ß-carotene and chlorophyll content is depicted in Figure 6.9. It is evident that in stress medium (Figure 6.9) lower final day cell numbers and cellular chlorophyll contents, and higher cellular ß-carotene levels were obtained than in experiments conducted in growth medium (Figure 6.8). The final day cellular chlorophyll content and cell numbers of all the different flocculants were similar to the control experiment, however, the ß-carotene content of cells grown in the presence of aluminium chloride was 53 % higher than in the control experiment.

An experiment was conducted using a range of aluminium chloride concentrations to determine at what concentration aluminium chloride affects cell physiology. Growth medium was used so that the synergistic effects of the additional stresses (high salinity and nitrate limitation) would not obscure the results.

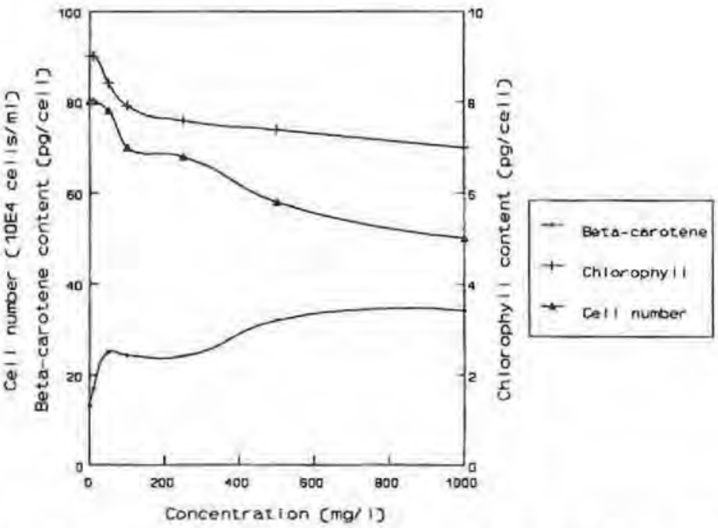


Figure 6.10 Effect of aluminium chloride concentration on cell growth and pigment content. Results reflect day 5 values.

Figure 6.10 illustrates the effect of aluminium chloride concentration on the growth and pigment content of *D. salina* cultivated under a light intensity of 1000 μmol.m^{-1}.s^{-1}. It is apparent from Figure 6.10 that as the concentration of aluminium chloride increased, the cell yield (day 5) decreased. As the aluminium chloride concentration increased there was a progressive increase in the ß-carotene-to-chlorophyll ratio (Figure 6.10). This was due to a marked increase in ß-carotene content and a marked decrease in chlorophyll content (Figure 6.10). It appears that aluminium chloride adversely affects cell function, even at low concentrations and the affect becomes greater in magnitude as the concentration increases.

6.4.5 Scale-up evaluations of laboratory flocculation studies.

The laboratory flocculation studies identified a number of suitable flocculants for the separation of *D. salina* biomass. Of the flocculants tested in the laboratory evaluations, aluminium chloride and the polyaluminium chloride (PAC) flocculant, K300, were chosen for the outdoor harvesting of biomass produced in the growth ponds. The reasons being, firstly, aluminium chloride has the additional advantage of stimulating ß-carotene production and secondly, low dosages combined with rapid separation times were observed for the PAC flocculant K300. The dosage and pH range determined in the laboratory study (Table 6.1) were used for the outdoor evaluation. The viability of cultures, after separation, was evaluated by placing the flocculated biomass into growth medium in the outdoor 0.2 m^2 ponds.

It was observed that pH pre-adjustment with 1 M HCl was unsuitable for outdoor flocculation evaluations as all cultures that had been resuspended in growth medium in the 0.2 m^2 ponds were dead after 24 hours, irrespective of the flocculation dosage or pH. Carbon dioxide sparging was thus used for pH adjusting the cultures prior to flocculation.

It is apparent from Table 6.3 that similar flocculation efficiencies and flocculation times to those obtained in the laboratory study were obtained in the outdoor scale-up evaluation. Flotation of the aluminium chloride floc could not be induced with the flotation system employed, whereas flotation of the K300 floc was rapid and effective (Table 6.3). What is noteworthy is that, irrespective of the flocculation pH, the percentage survival of cultures separated with aluminium chloride was unacceptably low. A maximum percentage survival of 17 % was obtained at pH 8.5 (Table 6.3). The percentage survival of cells separated with the K300 flocculant was strongly pH dependent and only at a pH of 7.5 were cells viable (Table 6.3).

Table 6.3 Summary of outdoor flocculation studies. The percentage survival was determined by resuspending the flocculated biomass into fresh growth medium in the 0.2 m^2 ponds.

	FLOC pH	FLOC EFF. (%)	FLOC TIME (min)	FLOTATION/ SEDIMENT	% SURVIVAL AFTER 24 HR
$AlCl_3$	6.5	94	3	sediment	2
	7	95.5	36	sediment	6
	7.5	94.5	40	sediment	5
	8	94	40	sediment	15
	8.5	95	41	sediment	17
K300	6.5	97.5	4	flotation	30
	7	96.5	6	flotation	49
	7.5	98	5	flotation	>100
	8	97.5	4	flotation	>100
	8.5	97	4	flotation	>100

It is unclear why cells separated with aluminium chloride are not viable under outdoor conditions. The results presented in Section 6.4.4 have demonstrated that aluminium chloride imposes stress on *D. salina* cells as was determined by the reduction in the growth rate and elevated ß-carotene content. It is possible that under outdoor conditions the high incident PFD may be responsible for the loss in cell viability.

6.4.6 Monopump driven CFUF.

CFUF was evaluated as a separation technique for harvesting *D. salina* in a viable form. The Rhodes University (RU) ultrafiltration unit and a larger unit built at Sastech were used for evaluations (Figure 6.1) and were fitted with Memtuf Ultrafiltration modules (Membratek, Ltd) with molecular weight cut-offs of 40 000.

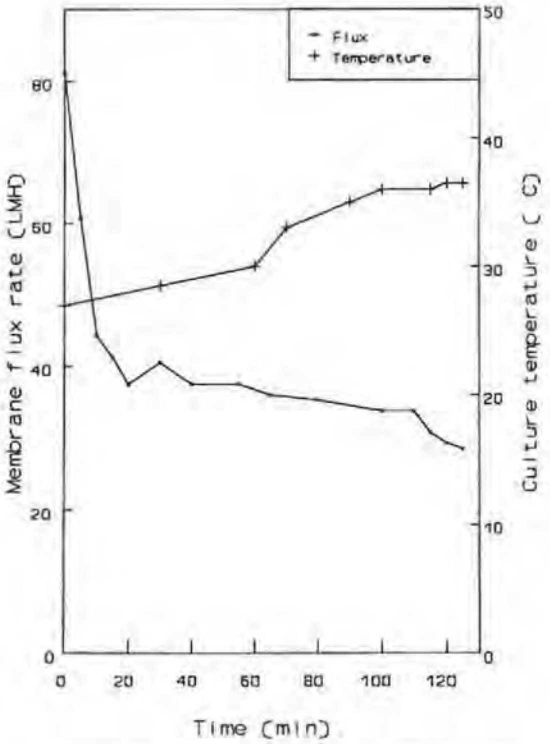


Figure 6.11 CFUF separation of 50 l *D. salina* culture. The system was configured with a single 0.8 m^2 membrane module and the algal biomass was concentrated to 12 g dry wt.l^{-1}.

The CFUF system, fitted with a single 0.8 m^2 membrane module, was evaluated for the separation of outdoor grown *D. salina* cells at the UOFS algal ponding facility. Figure 6.11 illustrates the permeate flux rates and reject volume temperature during a twenty-fold concentration of 50 l pond culture; feed rate 400 l.hr^{-1}, inlet pressure 300 KPa and outlet pressure 10 KPa. The permeate flux rate fell rapidly before stabilizing at approximately 36 LMH. The initial fall in permeate flux rate on initiation of filtration is consistent with the establishment of a polarized layer of cells (Tutunjian, 1985). During the latter stage of the run, a rapid drop in permeate flux was observed. On completion of this ultrafiltration run it was found that 38 % of the harvested cells had been damaged.

The temperature of the reject volume rose by 10 °C, from 27.5 to 37.5 °C (Figure 6.11). The rise in temperature may have been partly responsible for the high cell damage experienced. Consequently, the system was adapted in order reduce the temperature increase. This was achieved by placing a cooling coil in the culture holding tank (Figure 6.1) and increasing the culture volume.

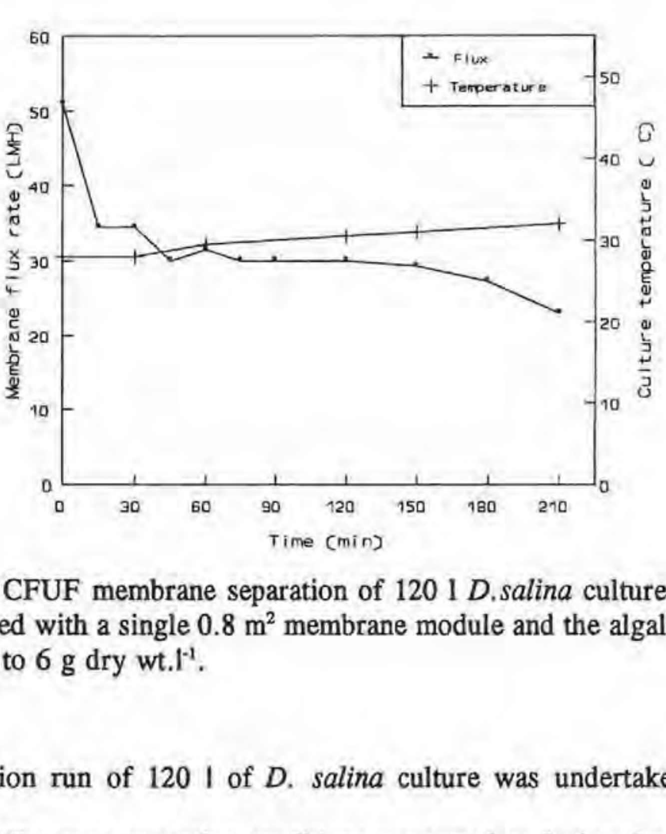


Figure 6.12 CFUF membrane separation of 120 l *D.salina* culture. The system was configured with a single 0.8 m^2 membrane module and the algal biomass was concentrated to 6 g dry wt.l^{-1}.

A ten-fold concentration run of 120 l of *D. salina* culture was undertaken using the modified ultrafiltration system. The same operating conditions were used as during the first ultrafiltration run and the permeate flux rates and culture temperature are illustrated in Figure 6.12. Again, there was a sharp fall in permeate flux at the onset of filtration, however, the rate stabilized at approximately 30 LMH which was lower than during the earlier CFUF run (Figure 6.11). The temperature during the run only increased by 4 °C from 28 °C to 32 °C (Figure 6.12). The lower operating temperature may have been responsible for the reduction in permeate flux rate.

Figure 6.13 illustrates the increase in solids level and the increase in cell damage during the ten-fold concentration of 120 l of *D. salina* culture. It is apparent from Figure 6.13 that at 180 minutes there was a sharp rise in solids level. The sharp rise in solids level coincided with a rapid increase in cell damage. The sharp rise in cell damage may have been directly related to the increase in solids level and/or to the increase in the number of passes of the reject volume through the ultrafiltration system. Jarvis (1993), has shown a linear relationship between *D. salina* cell damage and the number of passes of a culture volume through a positive displacement pump. Since relatively large culture volumes were used in the CFUF evaluations in conjunction with a small membrane area of 0.8 m^2, the number of passes of the culture volume through the system was high and may have been responsible for the high level of cell damage.

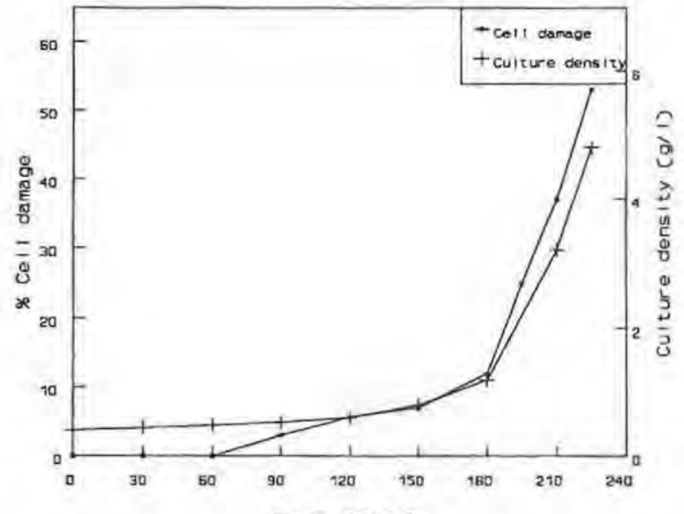


Figure 6.13 Cell damage in response to increasing culture density (solids level) during the CFUF run depicted in Figure 6.12.

The RU rig fitted with two 1.2 m^2 Memtuf modules configured in parallel was used to separate a mixed culture of outdoor grown *Dunaliella* species. Figure 6.14 shows the permeate flux rates and culture density (solids level) during a twenty-fold concentration run of 50 l of culture; feed rate 650 $l.hr^{-1}$, inlet pressure 300 KPa, outlet pressure 10 KPA. The permeate flux stabilized at about 30 $l.m^2.h^{-1}$ (LMH) and gradually decreased to 25.5 LMH due to a rising solids level in the reject volume.

A clear permeate was obtained and analysis showed no evidence of particulate material, ß-carotene or chlorophyll. A bacterial count reduction of 99.54 % was determined as plate count CFUs. It was found that during this ultrafiltration run less than 1 % cell damage occurred.

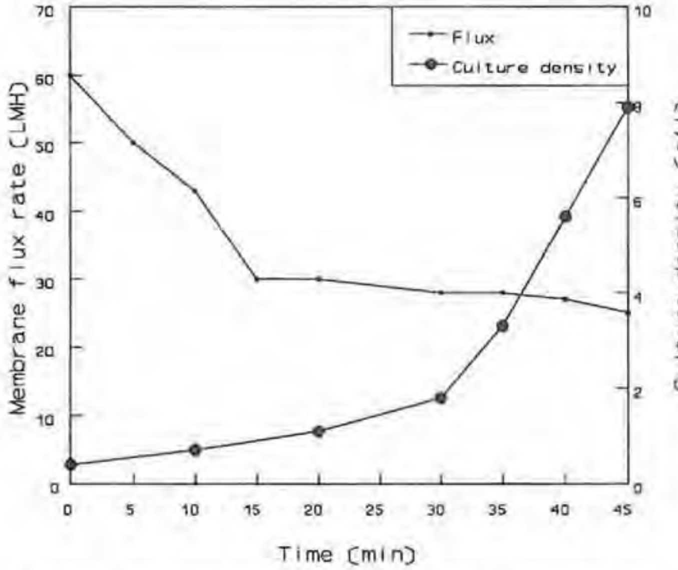


Figure 6.14 CFUF membrane separation of 50 l mixed *Dunaliella* culture. The CFUF system was configured with two 1.2 m^2 membrane modules configured in parallel.

A larger ultrafiltration system comprising of four 1.5 m^2 Memtuf ultrafiltration modules configured in parallel was constructed (total membrane area 6 m^2) in an attempt to reduce the number of passes of the culture volume through the system by maintaining similar culture volumes as used previously and increasing the membrane area. Figure 6.15 shows the results of four different experiments during which 100 l of outdoor grown *D. salina* culture obtained from the growth ponds (Chapter 4) was concentrated ten-fold. Various operating parameters were measured to determine a possible relationship with cell damage.

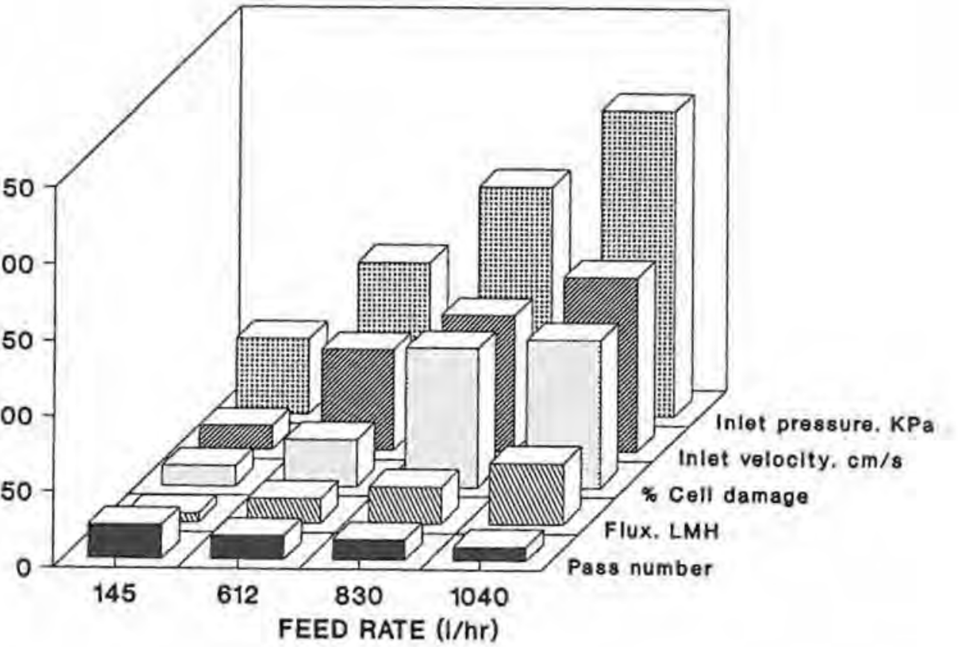


Figure 6.15 Results of the scale-up CFUF evaluations.

It is apparent from Figure 6.15 that for this system, with the membrane modules configured in parallel, greater feed rates were necessary to obtain inlet pressures in excess of 100 KPa than in the system configured with a single membrane module. As the feed rate increased there was an increase in the inlet pressure and the membrane inlet velocity which coincided with an increase in permeate flux rate; since permeate flux is dependent on inlet velocity and pressure (Tutunjian, 1985). The percentage cell damage increased to 98 % as the feed rate was increased to 1040 $l.hr^{-1}$, even though there was a significant reduction in the number of passes of the culture through the system (Figure 6.15). At a feed rate of 145 $l.hr^{-1}$, 13.11 % cell damage occurred, however, a steady state flux of only 6 LMH was obtained. The high level of cell damage caused by this system may stem directly from system configuration or indirectly from the high feed rates used in the system which resulted in increased shear forces on the algal cells.

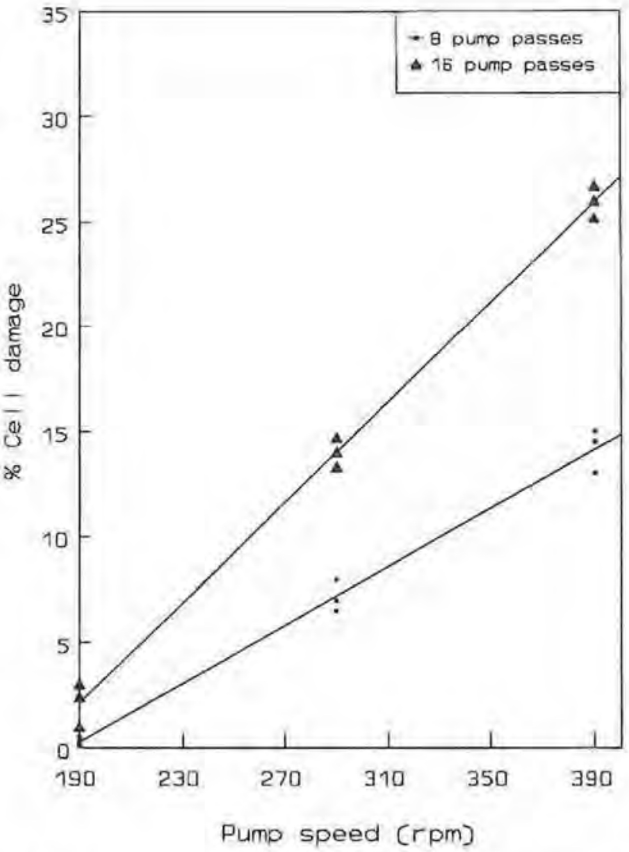


Figure 6.16 Effect of pump speed and number of passes on cell damage.

An experiment was conducted to determine the effect of pump speed (rpm) on cell damage. A 30 l volume was circulated through the monopump used in the larger ultrafiltration system for a fixed number of pump passes at various pump speeds (membrane modules excluded from system). A linear relationship exists between pump speed and cell damage at 8 and 16 passes of the culture volume through the system (Figure 6.16). As the pump speed increased above 290 rpm significant cell damage occurred at 8 and 16 passes. What is also apparent from Figure 6.16 is that higher cell damage levels occurred during the experiment with 16 passes of the culture volume through the system.

The results of the experiment demonstrate that cell damage is caused by the continual circulation of culture through the monopump and greater cell damage can be expected at higher pump speeds. However, the high levels of cell damage (>90 %) that occurred in the large CFUF system at feed rates of 830 $l.hr^{-1}$ and 1040 $l.hr^{-1}$ (pump speeds 290 and 360 rpm respectively) cannot solely be attributed to pump damage as cell damage reported in Figure 6.16 is substantially lower than that obtained in the large CFUF system (Figure 6.15). The massive cell damage occurring in the CFUF system may be a combination of mechanical shear caused by the pump system and other shear inducing factors.

6.4.7 Air-displacement CFUF.

In order to eliminate the cell damage caused by the circulation of the *D. salina* culture through positive displacement pumps a system was developed which employs air pressure to displace the culture through the membrane modules (Figure 6.2). This was termed air-displacement CFUF. Figure 6.17 illustrates the permeate flux rates and solids levels during ten-fold concentrations of 30 l outdoor grown *D. salina* culture at inlet pressures of 50 KPa, 100 Kpa, 200 KPa and 300 Kpa. The system was configured with a single Memtuf membrane module with a total membrane area of 1.2 m^2. It is apparent from Figure 6.17 that there was a slight drop in flux rate at the onset of filtration at all inlet pressures, however, the fall in flux rate was less severe than in the positive displacement pump driven CFUF. What should also be noted is that at an inlet pressure of 300 KPa the stabilized flux rate of 48 LMH was substantially higher than the stabilized flux rates of 30 and 36 LMH reported for the conventional CFUF system at an equivalent inlet pressure (Figure 6.12 and 6.13). The small fall in flux rate at the onset of filtration and the higher flux rates obtained with the system are probably due to the constant depressurization and refill of the pressure vessel which destabilizes the formation of a polarized layer of cells. As the solids level in the system rose above 1 g dry $wt.l^{-1}$ there was a progressive reduction in permeate flux rates at this point which coincided with a sharp increase in the solids level (Figure 6.17).

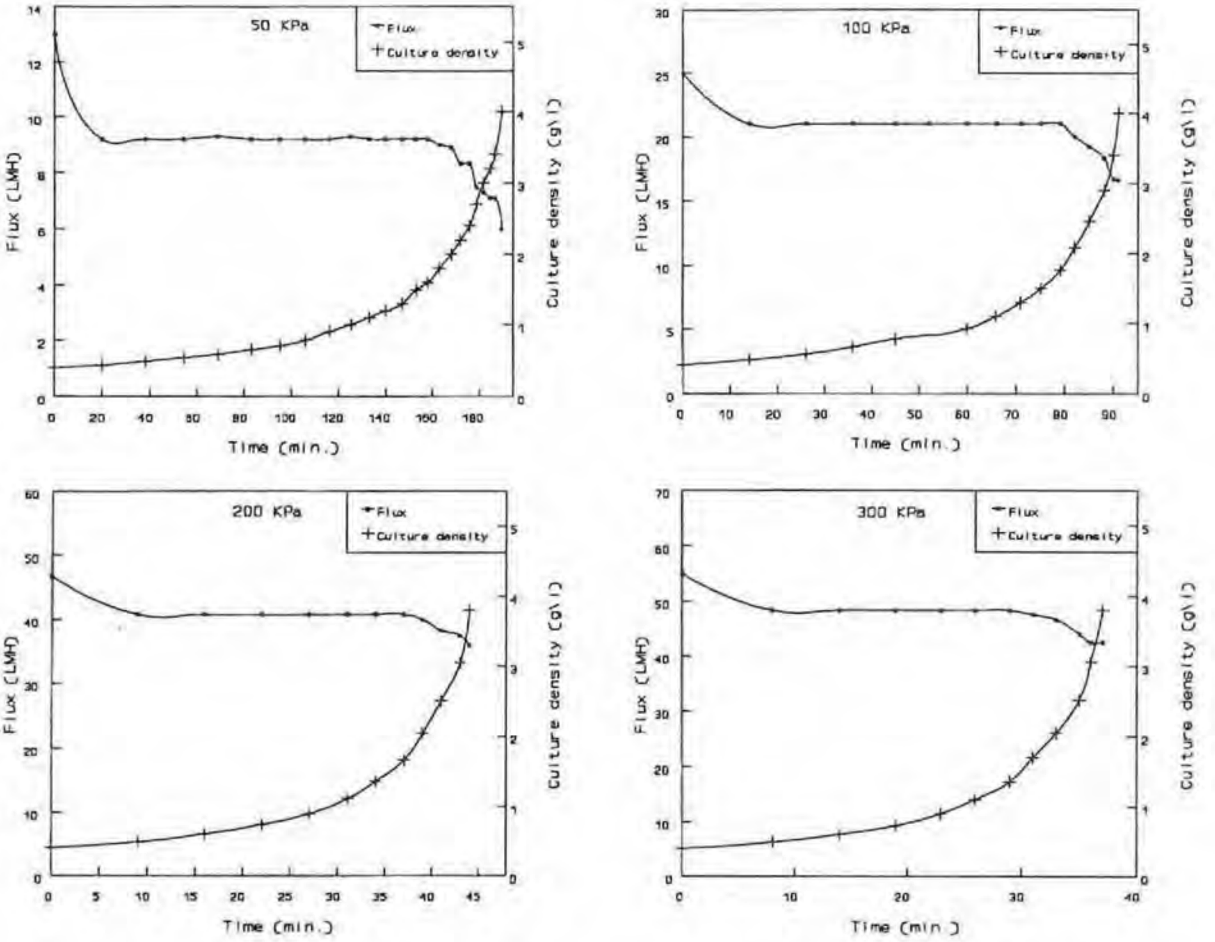


Figure 6.17 Permeate flux and culture concentration obtained with the air-displacement CFUF system at different inlet pressures.

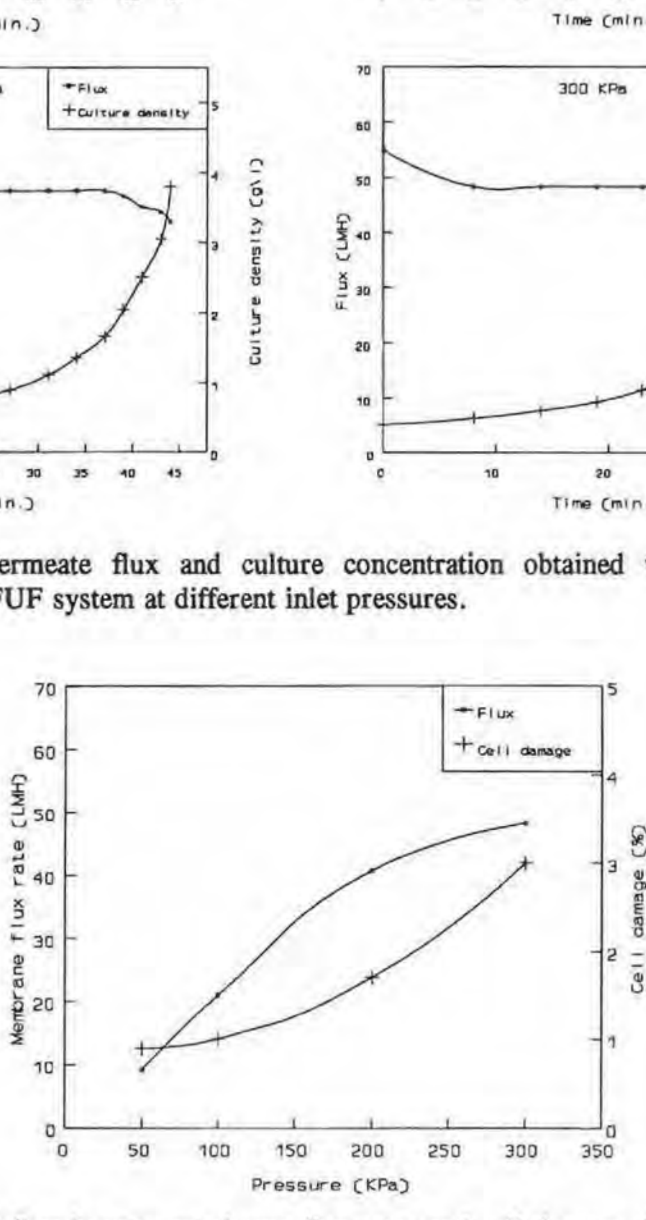


Figure 6.18 Steady state membrane flux rates and cell damage during the air-displacement CFUF runs depicted in Figure 6.17.

The steady state flux rates and percentage cell damage obtained at the different inlet pressures are recorded in Figure 6.18. As the inlet pressure increased there was an increase in the steady state permeate flux rates from 9 LMH at 50 KPA to 48 LMH at 300 KPA, as permeate flux is directly proportional to inlet pressure (Tutunjian, 1985). There was a slight increase in cell damage from 1 to 3 %, however the cell damage experienced in the air-displacement system was an order of magnitude less severe than in the conventional system. Microscopic examination of the concentrated cells revealed motile cells.

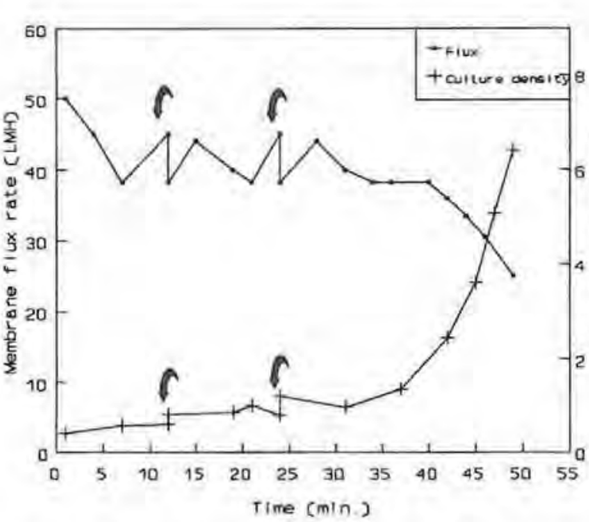


Figure 6.19 Air-displacement CFUF run of 80 l *D. salina* culture. Arrows indicate pressure vessel refill.

An experiment was undertaken to determine whether system configuration increases cell damage. The air-displacement CFUF unit was configured with two 1.2 m^2 Memtuf modules configured in parallel; total membrane area 2.4 m^2. A pure *D.salina* culture volume of 80 l was concentrated 16-fold (inlet pressure 200 LMH), however, because the pressure vessel volume was only 40 l the reject volume was topped up twice with 20 l culture volumes (indicated with arrows in Figure 6.19). As filtration was initiated there was a fall in flux rate from 50 LMH to 38 LMH, when the concentrate was diluted by the addition of fresh culture (marked by arrows) there was a partial restoration of the flux rate and a decrease in solids level (Figure 6.19). Once the solids level reached approximately 1 g dry wt.l^{-1}

the flux rate decreased rapidly (Figure 6.19). Figure 6.20 shows the culture before and after concentration, and the permeate obtained with the system. The clear permeate was found to contain no ß-carotene or chlorophyll and a bacterial load reduction of 99.2 % was measured using a Coulter Multisizer.

Figure 6.20 Air-displacement CFUF concentrate (4.3 g dry wt.l^{-1}) and permeate. Starting culture density 0.4 g dry wt.l^{-1}.

Microscopic examination of the algal concentrate revealed that 5 % of the cells had been damaged. Although, this is still considerably lower than in the pump driven CFUF system it represents more than double the cell damage experienced at 200 KPa with the system configured with a single membrane module.

6.4.8 Comparison of ß-carotene production in the stress ponds for cells separated with flocculation flotation and air-displacement CFUF.

The flocculation/flotation process produces an algal float, which consists of *D. salina* cells entrapped within the extended chemical matrix of the flocculant. On transfer to the stress ponds (Chapter 5), microscopic examination showed that the algal flocs almost completely disintegrate within 24 hours releasing the individual cells from the chemical flocculant. This is probably due to the continuous mixing within the ponds. However, approximately 20 % of the cells remain bound to the flocculant in flocs of between about 50-150 μm diameter.

An experiment was conducted to determine whether the presence of the K300 flocculant affects algal growth and ß-carotene production, by reducing the light environment within the culture. Biomass separated with a non-chemical harvesting process, air displacement CFUF, and biomass separated with the flocculation/flotation process was transferred to multiple stress medium, at the same starting culture density.

Table 6.4. Comparison of *D. salina* growth, pigment content and ß-carotene production obtained with flocculation/flotation and air displacement CFUF. Results reflect day 5 values. Starting cell count 33 x 10^4 cells.ml^{-1}, starting chlorophyll content 9.1 pg.cell^{-1}, starting ß-carotene content 18.4 pg.cell^{-1} and starting ß-carotene concentration 5.3 mg.l^{-1}.

PARAMETER	FLOCCULATION/ FLOTATION (K300)	AIR DISPLACEMENT CFUF
CELL NUMBER (10^4 cells.ml^{-1})	50	50.8
Chl CONTENT (pg.cell^{-1})	6.41	6.37
ß-CAROTENE CONTENT (pg.cell^{-1})	46.1	45.21
ß-CAROTENE (mg.l^{-1})	23.1	23.05

The initial growth and ß-carotene production rates were similar and Table 6.4 depicts the cell number, pigment composition and ß-carotene yield on the final day of the experiment. The presence of the flocculant in the stress medium did not appear to effect the physiology of *D. salina* as the algal growth and the cellular ß-carotene and chlorophyll contents were almost identical (Table 6.4). It follows that the day 5 ß-carotene yields were also similar (Table 6.4).

6.5 DISCUSSION.

Separation of *D. salina* with chemical flocculation.

The implementation of a dual-stage ß-carotene production system relies fundamentally on the ability to separate the algal biomass from the high nitrate growth medium in a viable form. Restrictions on the choice of separation technology are further complicated by the high ionic strength of the saline cultivation medium and the need for medium recycle.

The laboratory flocculation studies have confirmed the suitability of flocculation as a separation technique for the harvesting of *D. salina* biomass from high salinity media. However, the specific requirements for sustained cell viability and medium recycle disqualify a number of the flocculants evaluated as candidates for the dual-stage system.

Aluminium sulphate has been reported to be the most effective flocculant for the harvesting of all microalgal species (Richmond, 1986; Ben-Amotz and Avron, 1989) at dosages in the range of 70-200 $mg.l^{-1}$ and at pH between 5-7 (Golueke *et al*, 1965; Van Vuuren and Van Vuuren, 1965; McGary, 1971; Moraine *et al*, 1980). Aluminium sulphate flocculation linked to dissolved air flotation (DAF) was successfully used for the harvesting of *D. salina* by Western Biotechnology Ltd (Australia) in their extensive ß-carotene production system for a number of years (Borowitzka *pers comm.* 1993). The laboratory flocculation studies indicate that aluminium sulphate is not, however, suitable where medium recycle is required, because it imparts an orange-yellow discolouration to the medium which interferes with the light penetration properties of the medium. The build up of excess sulphates during medium recycle may also present a problem.

The use of aluminium chloride as a flocculant for microalgae has received little consideration. The laboratory flocculation studies have shown that its performance in a saline environment is similar to that of aluminium sulphate. Aluminium chloride imparts no colour to the culture medium and it is unlikely that the presence of excess chloride ions in medium containing a high NaCl concentration will affect the quality of recycled medium. The main drawback to its use as a flocculant appeared to be its presence in the flocculated biomass.

The aluminium removal study showed that aluminium can be removed from aluminium chloride flocculated *D. salina* biomass by an acid washing process similar to the one described by Moraine *et al.* (1980) for aluminium sulphate. The incorporation of CFUF diafiltration into the acid washing process was subsequently shown to be a feasible process with 98.6 % of the aluminium being removed from the flocculated biomass in one acid washing step. Although the acid washing process causes a loss in cell viability it could be linked to the second and final harvesting step in the dual-stage process (Chapter 1, Figure 1.1). The acid washing process also provides a means of recovering the aluminium for reuse (Moraine *et al.* 1980).

The study on the effect of flocculants in both growth and stress media demonstrated that aluminium chloride enhances ß-carotene hyper-accumulation. Aluminium is known to bind phosphates in solution forming insoluble aluminium phosphate ($AlPO_4$) at pH above 1 (Moraine *et al.* 1980). Phosphate deficiency in *D. salina* reduces the growth rate and increases ß-carotene production (Ben-Amotz, 1987). Therefore, ß-carotene hyper-accumulation may be indirectly stimulated by aluminium chloride through the precipitation of phosphates. The role of the heavy metal stress factor should also be considered since other heavy metals such as copper and lead have been reported to induce ß-carotene hyper-accumulation in *D. salina* (Pace *et al.* 1977).

Despite the potential demonstrated with aluminium chloride flocculation in the laboratory studies, the outdoor scale-up evaluation showed that aluminium was an unsuitable flocculant for the dual-stage system. A loss in cell viability was observed under outdoor conditions for *D. salina* biomass that had been separated with aluminium chloride and reinoculated into fresh growth medium. The results of the outdoor study conflict with results obtained in the laboratory study, however, they highlight the need for outdoor scale-up evaluations.

Chitosan, a biopolymer formed by the de-acelation of chitin was shown to be an effective flocculant for *D. salina* at relatively low concentrations. Furthermore, the laboratory studies indicated that its presence in the growth and stress medium does not effect *D. salina* growth or pigment content, as was observed with aluminium chloride. The main limitation to its use in a mass culture system is its relatively high price. Unless it can be acquired as a waste product it is likely to significantly increase the operating costs in a commercial mass culture system.

The PAC flocculants K300 and K19P were shown to be the most effective for separating *D. salina* cells. Low flocculant dosages (100 $\mu l.l^{-1}$) in conjunction with relatively rapid flotation times (approximately 5 minutes) were observed for high flocculation efficiencies. The flotation observed with the PAC flocculants in the laboratory study may be associated with photosynthetic oxygen evolution since this phenomenon has been observed for other algae and flocculants (Moraine *et al.* (1980).

The results obtained in both the laboratory and outdoor scale-up evaluation of K300 have demonstrated that its presence in the growth and stress medium does not affect algal growth and ß-carotene accumulation. The comparison between algal growth and ß-carotene production in biomass separated with the flocculation flotation process or the gentle air-displacement CFUF showed that equivalent cell growth and ß-carotene accumulation occurs in the K300 flocculated biomass. The results also indicate that the carryover of the flocculant into the stress medium does not affect the light environment within the algal cultures.

Flocculation/flotation with K300 is an attractive separation technique for the dual-stage system. In addition to its effectiveness as a harvesting technique, the results indicate that the clarified portion of the medium is suitable for recycle. Vigorous harvesting processes such as centrifugation can promote the occurrence of bacterial blooms in recycled medium by damaging the algal biomass and thus releasing glycerol and cell debris to the recycled medium (Ben-Amotz and Avron, 1989). The flocculation/flotation process is gentle and removes about 70 % of the bacterial population. This may minimize the occurrence of bacterial blooms which can reduce the biomass productivity in an outdoor system by decreasing the light penetration properties of the culture (Richmond, 1986).

Economically, flocculation followed by sedimentation or flotation is one of the most cost effective means of harvesting microalgae (Golueke *et al.* 1976; Moraine *et al.* 1980; Mohn *pers comm.* 1993). The apparent economic advantages of flocculation, coupled with the gentleness and effectiveness of the process make it an ideal potential separation technology for a dual-stage system.

Separation of *D. salina* with Crossflow Ultrafiltration (CFUF).

The scale-up evaluation of pump driven CFUF showed that pump driven CFUF was not successful for the separation of *D. salina* cells in a viable form as significant cell damage was obtained during all CFUF runs. Cell damage remained low during the earlier stages of ultrafiltration, however, as the solids level increased sharply there was a concomitant increase in cell damage. The cell damage obtained with the CFUF system configured with a single membrane module was lower than that obtained with the large CFUF unit configured with four membrane modules. The cell damage experienced with the CFUF system configured with a single membrane module, may have been due to the multiple cycle of relatively large volumes of culture through the small membrane system. In addition, cultures were concentrated to a relatively high density with this system.

The concentration of a mixed culture of *Dunaliella* species consisting predominantly of small non-carotenogenic *Dunaliella* species gave rise to 1 % cell damage. The smaller *Dunaliella* species appear to be less fragile and more resistant to mechanical shear than the larger, ß-carotene-rich *D. salina* cells. Another factor which may have contributed to the lower cell damage was the larger membrane area to culture volume ratio used in this system which resulted in fewer passes of the culture through the system. This is unlikely, considering the high degree of cell damage experienced with the large CFUF system configured with four membrane modules. It is possible that, with membranes configured in parallel, the disruption of linear flow at the junction between membranes modules may be responsible for the increase in cell damage because of increased turbulence and shear at these points. Furthermore, with membrane modules configured in parallel, higher feed rates are necessary to achieve similar inlet pressure than in systems configured with a single membrane module. This may also contribute to increased cell damage through greater shear forces acting on the algal cells due to higher flow velocities. If either of these factors are responsible for cell damage they could be detrimental to the scale-up of the process where higher feed rates and parallel configuration of membrane modules will be essential.

It is well established that mechanical pumping of *D. salina* can induce cell damage (Moulton *et al.* 1987; Ben-Amotz and Avron, 1989; Gudin and Chaumont, 1991). The results have indicated that a large degree of cell damage is related to pump speed and the number of passes through the pump. The air-displacement CFUF system was developed to determine whether pump damage was the major factor associated with cell damage.

Air-displacement CFUF was effective for the separation of *D. salina* cells and higher flux rates were achieved with the system than obtained with mechanical pumping. This is probably due to the destabilization of the polarized layer caused by the continual depressurization of the system when the algal concentrate was returned for subsequent filtration. Stabilized flux rates of between 9-50 LMH were achieved depending on the inlet pressure; higher flux rates were obtained at higher pressures. These flux rates compare well with flux rates considered acceptable for industrial processes (Kalke and Langlykke, 1986).

Cell damage obtained with a single membrane module ranged between 0.8-3 %, however, when the system was fitted with two 1.2 m^2 membrane modules configured in parallel the cell damage increased to 5 %. This represents a doubling in cell damage compared to the damage obtained at an equivalent inlet pressure with a single membrane module. Nevertheless, the cell damage obtained with the air-displacement CFUF system is considerably less than obtained with the pump driven system.

Figure 6.21 illustrates a possible design for the upscale of the process. The membranes would be positioned between two pressure vessels which would consecutively be pressurized or depressurized through their respective valves, depending on the flow direction of the culture (Figure 6.21). Such a system would significantly increase the capital costs of ultrafiltration due to the need for two pressure vessels and a large compressor. However, the inherent benefits of using ultrafiltration for the separation of *D. salina* such as the excellent medium quality for recycle and the diafiltration cell washing potential (Rose *et al.* 1992) may warrant further investigation of an industrial-sized scale-up trial.

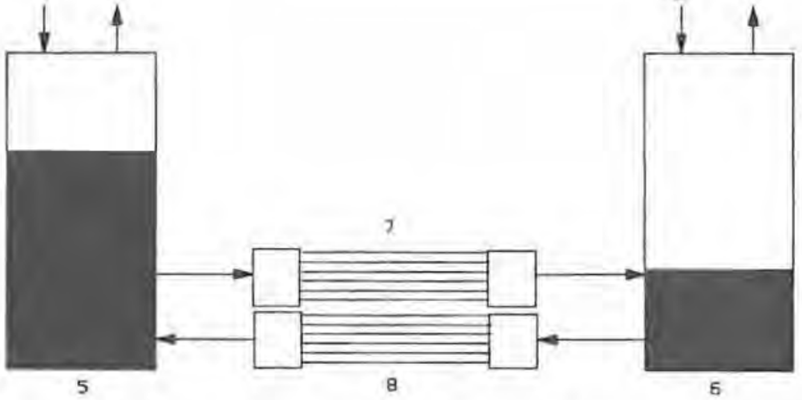


Figure 6.21 Schematic diagram of a potential industrial scale air-displacement CFUF system. 1 and 2 - pressure inlet valves. 3 and 4 - pressure outlet valves. 5 and 6 - pressure vessels. 7 and 8 -membrane modules.

The scale-up experimental evaluation of pump driven CFUF did not demonstrate its suitability for the first harvesting step of the dual-stage process due to the requirement for wholly viable cells. However, as a separation process for the final harvesting step or in a conventional single stage process, pump-driven CFUF offers many advantages not present in other technologies. Even with cell damage occurring the molecular weight cut-off and the hydrophillic nature of the membrane retains most of the organic material (including ß-carotene, bacteria and particulate matter) and produces a "sterile" permeate highly suitable for recycle (Rose *et al.* 1992). The results have shown that air-displacement CFUF alleviates the cell damage induced by mechanical shear and may provide an alternative harvesting process suitable for the dual-stage process.

CHAPTER 7

EXTRACTION OF ß-CAROTENE FROM *D. SALINA*

Summary.

A preliminary investigation of ß-carotene extraction using supercritical fluid extraction was undertaken. It was demonstrated that a co-solvent mixture of carbon dioxide and propane reduced the extraction pressure by about two-fold and significantly improved the extraction efficiency of the process compared to pure supercritical carbon dioxide.

A hot oil extraction process was developed and evaluated. The process incorporated the use of hydrophobic membranes for the separation of oil:water emulsions produced in the ß-carotene extraction step. Given the high capital and operating costs of centrifugation, the hydrophobic membrane process has the potential to replace centrifugation as a means of separating biphasic solutions. A promising patent application has been based on this study.

7.1 INTRODUCTION.

The extraction of ß-carotene from the separated algae forms one of the major operating costs associated with the production of ß-carotene from *D. salina* (Borowitzka, 1992). Unlike other algae, *D. salina* is characterised by the absence of a cellulosic cell wall, which simplifies the extraction of ß-carotene from the cells (Moulton *et al.* 1987).

Several methods for the extraction of the ß-carotene from *D. salina* have been described. Ruane (1974b) patented a method whereby algal biomass harvested with crossflow microfiltration, using diatomaceous earth as a filter aid, is directly subjected to solvent extraction using non-polar hydrocarbon solvents such as toluene or hexane. Ben-Amotz and Avron (1980b) patented a similar extraction process, however, the biomass is first suspended in an apolar solvent, followed by ß-carotene extraction with a polar solvent such as hexane or benzine. This process reduces the co-extraction of chlorophyll and other algal pigments. Ruegg (1984) patented a process for the isolation of pure ß-carotene from algae, using a saponification step to precipitate the chlorophyll, followed by solvent extraction with methylene chloride or hexane.

There has been a shift away from the use of organic solvents for extraction of food and pharmaceutical grade ß-carotene due to potential solvent residues in the final product which may contravene health regulations. ß-carotene is marketed as either a ß-carotene rich spray dried product or as 1.5 % to 30 % extracts in edible oils, such as vegetable oil, soybean oil or peanut oil (Curtain *et al.* 1987; Moulton, *et al.* 1987; Schlipalius, 1991; Ben-amotz and Avron, 1990). The commercial natural ß-carotene producer, Betatene Ltd. (Australia), claim a market advantage because no synthetic chemicals or petroleum solvents are employed during their secret extraction process. While the currently used extraction processes are shrouded in industrial secrecy (Borowitzka *pers comm.* 1993), it appears as though most of the commercial natural ß-carotene producers use some form of hot oil extraction process based on the hot oil extraction patent of Nonomura (1987).

One of the main problems associated with the extraction of ß-carotene from *D. salina* is the rapid oxidation of ß-carotene on exposure to air (Borowitzka, 1992). The extraction of ß-carotene into vegetable oils may have a stabilizing effect on ß-carotene, due to the presence of relatively large quantities of anti-oxidants, such as tocopherol (up to 2800 $mg.l^{-1}$), within the oil, (Grayson, 1983). The main problem associated with the extraction of ß-carotene into oil is that all-*trans* ß-carotene has a low solubility in vegetable oil 0.05-0.08 g.100 ml^{-1}, while the 9-*cis* isomer is apparently more soluble (Ben-Amotz *et al.* 1989a).

The extraction of ß-carotene from wet algal biomass with hot oil produces a complex slurry consisting of cell debris, oil, water, salts and high concentrations of the flocculant used for cell separation. Passive phase separation is inadequate for the separation of oil from the water/oil emulsion because the oil phase is likely to be contaminated with small amounts of cell debris, water and possibly the flocculant used for harvesting the algal biomass. High speed centrifugation is commonly used to separate the aqueous and the oil phases (Nonomura, 1987), however, the high capital and operating costs associated with centrifugation make it an unattractive candidate technology.

The use of membrane technology provides a tool for the separation of oil-water emulsions. Traditionally, oily waste waters have been separated using hydrophillic ultrafiltration membranes, where the clean water phase passes through the membrane and the oil is retained (Cheryan, 1986). This approach is not suitable for hot oil extraction of ß-carotene because in addition to the oil being retained by the membrane, the cell debris will be retained as well. The use of a hydrophobic membrane system would be more relevant as the oil phase could be recovered as the sole permeate, retaining the water phase together with the cell debris, flocculant and salts. A hydrophobic membrane system could easily be engineered into existing hot oil extraction processes and may provide a novel and potentially more cost effective technology compared to centrifugation.

Carbon dioxide, at a pressure and temperature above its critical point can be used as a non-toxic solvent for hydrophobic compounds (Wells *et al.* 1990). Curtain and Snook (1982) in their patent "Methods for Harvesting Algae" propose the use of liquid supercritical carbon dioxide for ß-carotene extraction from *D. salina.* Supercritical carbon dioxide has a number of properties which make it an attractive alternative to other means of commercial extraction. The low boiling point of liquid carbon dioxide has the advantage of minimizing the decomposition of the extracted thermo-labile caroteniferous material, it is non-toxic, non-flammable, non-corrosive, low priced and is easily available. Furthermore, it is also favoured as a food and pharmaceutic grade solvent because it leaves no unacceptable residues in the product (Willson, 1985; Fong, 1989). Currently, supercritical carbon dioxide extraction is employed for a number of natural products, including the decaffeination of coffee (Roselius *et al.* 1974), the extraction of flavour essences from hops (Willson, 1985), and the extraction of essential oils from citrus peels (Kassim *et al.* 1990).

Bruno (1990) has patented a process for the extraction of ß-carotene from biological material using supercritical carbon dioxide. The process involves firstly, extracting the ß-carotene from the material with supercritical carbon dioxide and then passing the supercritical extract through oil, thus forming an oil/ß-carotene suspension. The process, however, suffers from extremely high extraction pressures, in excess of 1000 bar, which reduces the economic viability of the process compared to conventional hot oil extraction processes.

The solubility of many substances in supercritical fluids have been found to be increased by the addition of small quantities of a component intermediate in volatility between the supercritical fluid and the compound to be extracted (Brunner, 1983). Such compounds are known as entrainers. Cygnarowicz *et al.* (1990) measured the equilibrium solubilities of pure all-*trans*-ß-carotene in supercritical carbon dioxide at temperatures ranging from 313 to 343 K and pressures up to 439 bar. The highest solubilities were reported at 343 K and 405 bar. The addition of a 1 % (g/g) ethanol entrainer increased the solubility of ß-carotene considerably, with a mole fraction increase from 25.4 x 10^{-7} (moles ß-carotene/ mole solvent) in pure supercritical carbon dioxide to 37.5 x 10^{-7} in the supercritical carbon dioxide/ethanol binary mixture. The degradation of ß-carotene was examined up to extraction temperatures of 353 K (80 °C) and it was found that little thermo-degradation occurred during extraction (Cygnarowicz *et al.* 1990).

Cygnarowicz and Seider (1990) maintain that supercritical extraction can be competitive for the recovery of high-value products, like ß-carotene, at low production rates. Extraction pressures in the vicinity of 300-500 bar entail considerable capital and running costs for compression (Wells *et al.* 1990). It is unlikely that with a multi-functional process like the production of ß-carotene from *D. salina* that pressures in excess of 300 bar will be cost effective, in view of the other costs associated with biomass production.

The use of solvent mixtures of similar volatility to the supercritical fluid extractant can also be used so that the operating temperature and pressure of a process can be tailored, for instance with thermo-labile products. In the case of ß-carotene extraction, a reduction in extraction pressure could possibly improve the operating costs of a supercritical fluid extraction process. This requires further investigation.

7.2 RESEARCH OBJECTIVES.

The extraction of ß-carotene from the ß-carotene-rich biomass produced in the stress stage of the dual-stage system forms the final stage of the process outlined in Figure 1.1. Two ß-carotene extraction processes were examined, supercritical fluid extraction and hot oil extraction. The following research objectives were identified:

1. To determine the extraction potential of pure supercritical carbon dioxide and to evaluate the influence of ethanol entrainers on ß-carotene extraction and solubility.
2. To examine the use of a co-solvent mixture for reducing the operating pressure.
3. To determine the extraction potential and solubility of natural ß-carotene in vegetable oil.
4. To determine the potential of a hydrophobic membranes hot oil extraction process.

7.3 MATERIALS AND METHODS.

7.3.1 Preliminary study of supercritical fluid extraction of ß-carotene from *D. salina*

7.3.1.1 Preparation of algal biomass.

A culture of *D. salina* was harvested by centrifugation in a Sigma S10 low speed centrifuge (6000 rpm for 10 minutes) and the cellular biomass was freeze dried using a Modulyo Edwards 8 port manifold freeze drier for 12 hours. The dried biomass was homogenised so as to obtain a powder of uniform consistency. The homogenised biomass was stored under nitrogen at 4^0 C. Dry biomass was used for supercritical fluid extractions so as to prevent possible solvent interference by the water content in the wet biomass.

7.3 1.2 Extraction equipment.

Supercritical extractions were conducted using a Sitec Phase equilibria apparatus (Sieber Engineering AG, Zurich). Figure 7.1 depicts a schematic representation of this apparatus.

The following supercritical fluids were used for the extractions of the ß-carotene:

a) Pure carbon dioxide.

b) Carbon dioxide with ethanol entrainers of 1 %, 5 % and 10 % (g/g).

c) Propane:carbon dioxide mixtures in the following ratios - 35:65 and 40:60 (g/g).

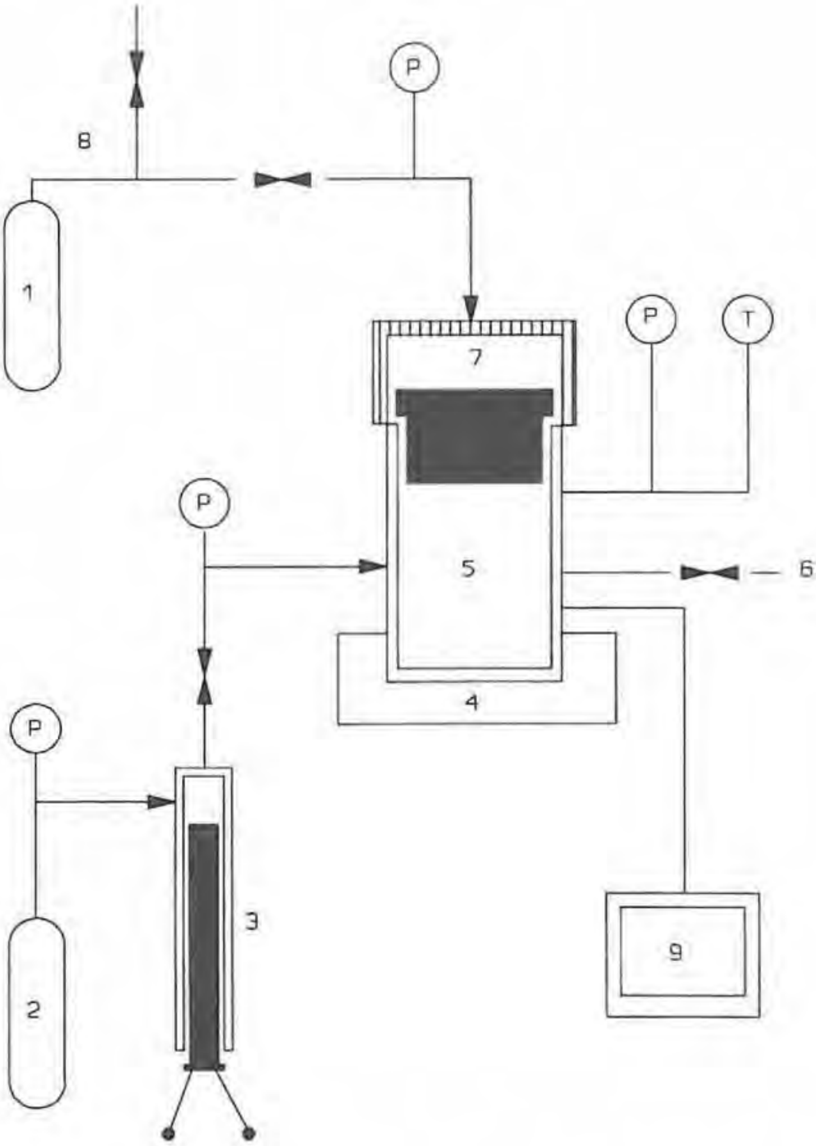


Figure 7.1 Schematic diagram of the Sitec Phase Equilibria apparatus. 1 - counter-balance gas supply. 2 - pressure generator gas supply. 3 - pressure generator. 4 - heating block. 5 - extraction chamber. 6 - sampling port. 7 -counter-balance piston. 9 - counter-balance release valve. P - pressure gauge. T - temperature gauge.

7.3.1.3 Extractions with supercritical carbon dioxide (70°C, 450 bar).

Cygnarowicz *et al.* (1990) have shown that the equilibrium solubility of ß-carotene is greatest at supercritical carbon dioxide temperatures and pressures in excess of 70 °C (343 K) and 405 bar respectively. Consequently, an extraction temperature of 70 °C and an extraction pressure of 450 bar was chosen. Known amounts of algal biomass were placed within the extraction chamber (Figure 7.1; 5). The following amounts were used:

1) 0.02 g dry wt.

2) 0.2 g dry wt.

3) 0.4 g dry wt.

The mass of carbon dioxide used for the extractions was determined as follows; the counterbalance head-space (Figure 7.1; 7) was evacuated by filling the extraction chamber (Figure 7.1; 5) with carbon dioxide. The extraction temperature was selected, and once obtained the extraction chamber was filled with carbon dioxide to a pressure of 200 bar using the pressure generator (Figure 7.1; 3). The density of carbon dioxide at the known temperature and pressure was determined using a temperature-entropy chart. Since the volume of the extraction chamber is known (25.4 cm^3), the mass of carbon dioxide could be determined as follows:

$$C = P_{co2}.v \qquad (1)$$

C = mass of carbon dioxide

P_{co2} = density of carbon dioxide at a certain temperature and pressure

v = volume of extraction chamber

The extraction chamber was further pressurised to 450 bar, by applying pressure to the counterbalance piston (Figure 7.1; 10). The extraction process was monitored through a fibre optic camera linked to a video screen.

Samples were extracted for one hour. Prior to sampling, the extraction chamber volume was calculated by measuring the counterbalance piston travel. The volume of the extraction chamber was calculated from the dimensions of the extraction chamber as follows:

$$y = -0.4267x + 25.4 \tag{2}$$

y = volume of the extraction chamber

x = distance travelled by the counterbalance piston

Representative samples of the extraction chamber were taken using pressure-stable sample bombs of a known capacity.

7.3.1.4 Extractions with Carbon dioxide/ethanol entrainer mixtures (70° C; 450 bar).

Cygnarowicz *et al.* (1990) have reported that equilibrium solubilities of pure, crystalline ß-carotene in carbon dioxide are increased by the addition of entrainers which have volatility properties intermediate to those of the ß-carotene and the supercritical solvent used in the extraction. It was reported that a 1 % (g/g) ethanol entrainer provided the greatest enhancement of the equilibrium solubility of ß-carotene. The following entrainer systems were used: 1 %, 5 % and 10 % (g/g) ethanol. The extraction procedure was similar to that used for the pure carbon dioxide extractions with the exception of the addition of a known amount of the entrainer to the extraction chamber. Dried algal biomass samples of 0.4 g dry wt. were used for all extractions.

7.3.1.5 Extractions with Propane:Carbon dioxide mixtures.

Owing to the high pressures used in the extraction of ß-carotene with supercritical carbon dioxide, a propane:carbon dioxide mixture was evaluated for extraction with the aim of reducing the extraction pressures and therefore, possibly increasing the cost effectiveness of the process. It is evident from

Table 7.1 that the critical temperature of propane is higher than that of carbon dioxide, however, its critical pressure is substantially lower. By employing a mixture of these two solvents in the extraction chamber it is possible to obtain extraction temperatures and pressures, intermediate between these two solvents. Two Propane:Carbon dioxide mixture ratios were used - 35:65 and 40:60 at 336 and 215 bar respectively. A mass of 0.4 g algal biomass was used for extractions.

Table 7.1 Critical points of carbon dioxide and propane.

Solvents	Critical temperature (°C)	Critical pressure (bar)
Carbon dioxide	31.2	73.7
Propane	97	42

The mass of propane added was determined by filling the pre-heated extraction chamber (chamber volume set at 12.6 cm^3) with propane to a specific pressure. The density of propane at the specific temperature and pressure was determined using a temperature-entropy chart for propane and the mass of propane in the chamber was calculated using Equation 3.

$$M_p = P_p . v \qquad (3)$$

M_p = mass of propane

P_p = density of propane at known temperature and pressure

v = volume of extraction chamber

The counterbalance piston was raised to double the chamber volume, the temperature was set to 75 ° C and the extraction chamber was pressurized to the respective extraction pressures of 336 or 215 bar with carbon dioxide.

The density of the propane:carbon dioxide mixture in the sample bomb was determined by measuring the mass of the mixture in the sample bomb (sample bomb volume 1.5 cm^3). From this the total mass of the mixture was calculated using Equation 4.

$$M_m = P_m . v \tag{4}$$

M_m = mass of the mixture

P_m = density of mixture

v = extraction chamber volume (2)

The mass of carbon dioxide was calculated by difference:

$$C = M_m - M_p \tag{5}$$

C = mass of carbon dioxide

M_p = mass of propane

The mixture ratio could then be determined.

7.3.1.6 Sample analysis.

ß-carotene was washed from the sample bomb with 100 % acetone and the ß-carotene concentration in the sample bomb was determined as previously described. Extracts were assayed for chlorophyll using the method previously described in Chapter 2. However, no chlorophyll was detected. Figure 7.2 shows a UV scan from 200 nm to 700 nm, of a supercritical extract and a whole algal extract. The absence of a peak between 600 and 700 nm, for the supercritical extract, indicates the absence of chlorophyll.

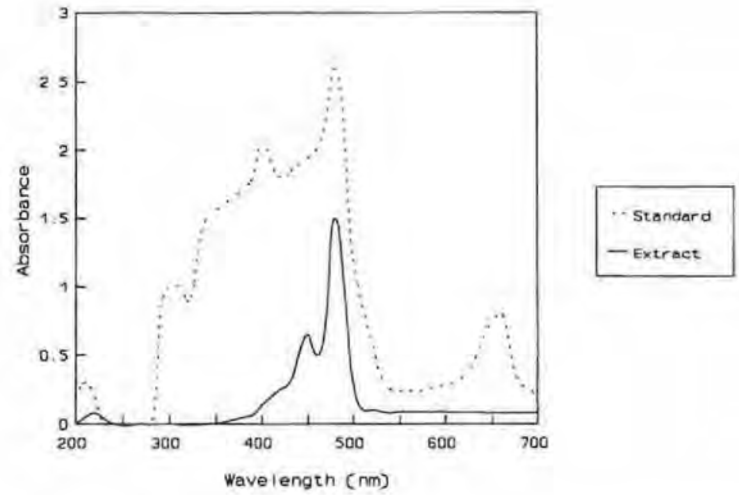


Figure 7.2 Wavelength spectral scan of a biomass standard and a supercritical carbon dioxide extract. Note the absence of a peak at 666 nm in the extract.

The total ß-carotene extracted into the supercritical fluids was determined using equation 6.

$$T_{B.C} = \frac{z.v}{v_{sb}} \qquad (6)$$

$T_{B.C}$ = total ß-carotene extracted

v = extraction chamber volume on sampling.

z = ß-carotene extracted into the sample bomb

v_{sb} = sample bomb volume (1.5 cm^3)

The extraction efficiency was calculated by determining the ß-carotene mass in an equivalent mass of algae and using this as a standard:

$$\%E = \frac{T_{B.C}}{q} \qquad (7)$$

%E = extraction efficiency

q = ß-carotene concentration in a equivalent mass of biomass

The solvent loading was calculated as mole fractions of ß-carotene in carbon dioxide, as follows:

$$L = \frac{T_{B.C}}{C} \cdot \frac{T_{CO\ mw}}{T_{B.Cmw}} \qquad (8)$$

L = mole fraction loading of carbon dioxide

$T_{B.C}$ = mass of ß-carotene

C = mass of carbon dioxide loaded (equation 1)

$T_{B.Cmw}$ = molecular weight of ß-carotene

$T_{CO\ mw}$ = molecular weight of carbon dioxide

7.3.2 Development of a hydrophobic membrane assisted hot oil extraction process for ß-carotene from *D. salina*.

7.3.2.1 Determination of ß-carotene solubility in vegetable oil.

A ß-carotene rich *D. salina* culture (200 l), produced in the stress ponds, was harvested using the flocculation/flotation process described in Chapter 6. The cell concentrate from the flocculation/flotation separation was further concentrated by centrifugation (2000 g for 15 min), producing a 500 ml concentrate of 160 g dry wt.l^{-1} with a ß-carotene content of 10 % dry wt. The concentrate was stored at -20 °C, under nitrogen.

Aliquots of 25 ml (ß-carotene concentration 0.4 g.25 ml^{-1}) were used to determine the solubility of natural ß-carotene in oil. The 25 ml aliquots were diluted with distilled water to a volume of 50 ml and mixed using a magnetic stirrer at approximately 200 rpm for 10 min. Cell lysis was induced due to the osmotic shock and the vigorous agitation. Pre-heated commercial grade sunflower oil (20 ml) was added at the following temperatures: 25 °C, 40 °C, 50 °C, 60 °C and 70 °C. The slurry was mixed at 100 rpm for 20 min, at the respective extraction temperatures and samples were taken every 5 minutes. Extraction temperatures in excess of 70 °C were not evaluated due to the potential degradation of ß-carotene at these temperatures. After extraction, the oil was separated from the slurry by centrifugation at 5000 g for 15 min. ß-carotene was quantified by diluting the samples with fresh oil and reading against a standard curve by absorption measurement at 450 nm. The all-*trans* ß-carotene standard (Sigma) was prepared in pure sunflower oil.

The solubility of natural ß-carotene in oil was compared to that of synthetic all-*trans* ß-carotene (Sigma). The solubility of synthetic ß-carotene was determined by adding 20 ml of oil, at the respective temperatures, to 0.4 g crystalline all-*trans* ß-carotene and mixing at 100 rpm for 20 min. Undissolved crystals were centrifuged out of solution at 5000 g for 15 min. ß-carotene was quantified using the standard curve of ß-carotene in oil.

7.3.2.2 Incorporation of a hydrophobic microfiltration membrane system for the separation of oil from the extraction slurry.

Two hydrophobic polypropylene hollow fibre membrane modules consisting of 40 hollow fibres (outer membrane area 0.0036 m^2 and 0.038 m^2) with a pore size of 0.1 μm, were obtained courtesy of Dr. R. Sanderson, Stellenbosch University. Figure 7.3 is a schematic representation of the system used for the evaluation of hydrophobic membrane assisted hot oil extraction. The system was operated on a tangential flow mode (crossflow filtration), with the oil-water emulsion passing along the outside of the hollow fibres. The flow was directed on the outside of the membrane because the narrow bore of the hollow fibres presented high flow resistance to the flow of the viscous emulsions. Operating pressures were adjusted using a back pressure valve (Figure 7.3; 6). Separation of the oil-water emulsion occurs across the polypropylene membrane and the clarified oil is recovered as the permeate (Figure 7.3; 5).

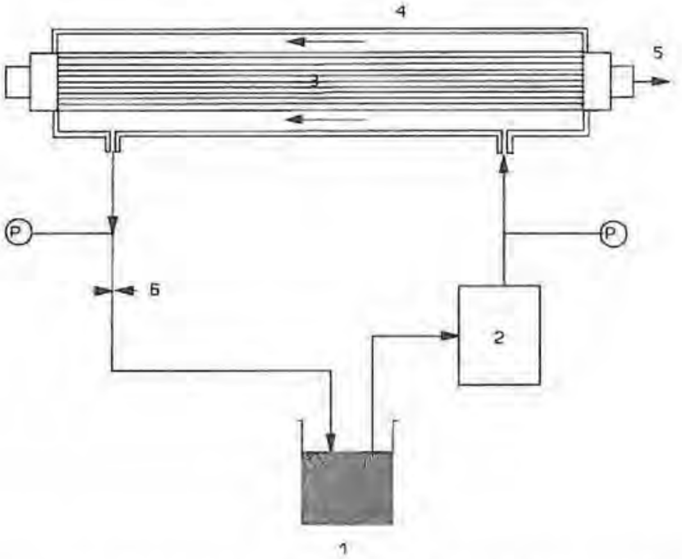


Figure 7.3 Schematic diagram of hydrophobic membrane assisted hot oil extraction process. 1 - extraction slurry. 2 - peristaltic pump. 3 - hydrophobic membrane. 4 - membrane housing. 5 - oil permeate. 6 - back pressure valve. 7 - magnetic stirrer.

The following membrane criteria were examined for the 0.0037 m^2 and 0.038 m^2 polypropylene microfiltration membranes, using pure sunflower oil-water emulsions:

(a) The water breakthrough pressure of the system, which is the minimum pressure at which the system could be operated without co-permeation of water with the oil. This was calculated by operating the system at different pressures using a 1:1 water-to-oil emulsion and monitoring the oil permeate for the presence of water. Samples of permeate (10 ml) were taken at the different operating pressures and centrifuged at 5000 g for 15 min. Any water present was recovered with a 100 μl Hamilton syringe and quantified.

(b) The effect of temperature on water breakthrough pressure was determined as described above, except that oil-water emulsions at various temperatures were tested.

(c) The affect of water-to-oil ratio on membrane flux rates. A 1:1 water-to-oil emulsion was passed through the system until 95 % of the oil had been recovered. The oil flux rates were monitored as the water-to-oil ratio increased.

Whole extract slurries were passed through the system to determine the effect of the presence of cell debris on the membrane flux rate of the system. The 0.038 m^2 polypropylene microfiltration membrane was used for these evaluations. A 250 ml algal concentrate of 160 g dry $wt.l^{-1}$ was extracted with 250 ml of sunflower oil at an extraction temperature of 60 °C in a beaker agitated on a magnetic stirrer at 100 rpm for 15 min. The extraction slurry was cooled to 25 °C and passed through the hydrophobic microfiltration system. An identical extraction was repeated, however, passive phase separation of the oil and aqueous phases was induced by ceasing agitation of the slurry, and the oil phase was passed through the hydrophobic microfiltration system.

7.3.2.3 Contamination of oil with flocculants.

In order to determine whether the flocculant contaminates the oil phase during ß-carotene extraction, aluminium chloride was used as a test flocculant due to its relatively simple detection. A *D. salina* culture (1 l) was harvested using 2 $g.l^{-1}$ aluminium chloride (224 $mg.l^{-1}$ Al^{3+}). The flocculated biomass (100 ml) was extracted with 50 ml sunflower oil and the slurry was subjected to hydrophobic membrane oil separation (Figure 7.3) at an inlet pressure of 90 KPa.

A 20 ml volume of the oil recovered from the membrane separation process was acid digested with a 1:3 sulphuric acid/perchloric acid solution in a fume cupboard. Once acid digestion was complete, the solution was cooled and diluted to a specific volume in a volumetric flask. Aluminium was quantified by Atomic Absorption spectrophotometry.

7.3.2.4 Qualitative analysis of ß-carotene extract in oil.

A qualitative analysis of the ß-carotene oil extract was undertaken to determine the specificity of the extraction process. A 100 μl volume of the 70 °C ß-carotene extract was dissolved in a 10 ml solution of isopropanol/dichloromethane (9:1) and pigments were separated by reverse-phase HPLC as previously described in Chapter 3. Results were compared to a whole cell extract containing approximately the same quantity of ß-carotene. The ß-carotene isomers were identified from spectral scans using the Beckman Systems Gold 168 diode array detector. Xanthophylls and chlorophylls were identified from spectral scans by Phillips (*pers comm.* 1993).

7.4 RESULTS.

7.4.1 Preliminary study of supercritical fluid extraction of ß-carotene from *D. salina*

7.4.1.1 Extraction with pure carbon dioxide.

Table 7.2 Extraction efficiency and solvent loading of pure carbon dioxide. Extraction pressure 450 bar and extraction temperature 70 °C.

Sample mass	Extraction efficiency	Solvent loading (mole/mole)
0.02 g dry wt.	97 %	11.5×10^{-7}
0.2 g dry wt.	81.7 %	129×10^{-7}
0.4 g dry wt.	59.5 %	175×10^{-7}

Table 7.2 shows the extraction efficiency (% extracted of total) and solvent loading (mole fraction loading) of supercritical carbon dioxide for three different algal masses. As the algal mass was

increased there was an increase in the solvent loading from 11.5 x 10^{-7}, for 0.02 g dry wt., to 175 x 10^{-7}, for 0.4 g dry wt.. However, there was a progressive decrease in the extraction efficiency (Table 7.2).

Although a ten-fold increase in algal biomass from 0.02 to 0.2 g dry wt., gave rise to an approximate ten-fold increase in the solvent loading of supercritical carbon dioxide from 11.5 x 10^{-7} at 0.02 g to 129 x 10^{-7} at 0.2 g, doubling the biomass from 0.2 to 0.4 g dry wt. did not result in a doubling in the solvent loading of supercritical carbon dioxide. Figure 7.4 depicts the relationship between solvent loading and algal biomass. The shape of the curve indicates that at an algal mass of 0.4 g dry wt., solvent loading is close to maximum.

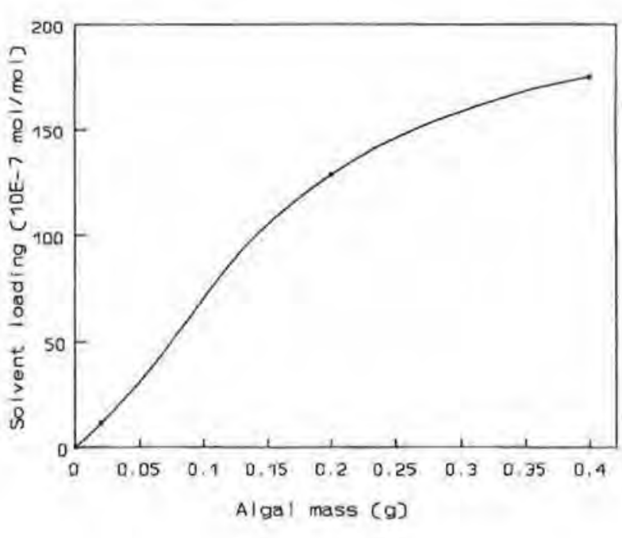


Figure 7.4 Effect of increasing algal mass on the solvent loading of supercritical carbon dioxide.

An algal mass of 0.4 g dry wt. was used for subsequent experimentation to determine the affects of entrainers and co-solvent mixtures on the extraction efficiency and solvent loading of supercritical carbon dioxide.

7.4.1.2 Extraction with carbon dioxide/ethanol entrainer mixtures.

Table 7.3 Extraction efficiency and solvent loading of carbon dioxide/ethanol entrainer mixtures. Extraction pressure 450 bar, extraction temperature 70 °C, algal mass 4 g dry wt.

Entrainer system	Extraction efficiency (%)	Solvent loading (mole/mole)
Pure CO_2	59.5	175×10^{-7}
1 % ethanol	58.1	171×10^{-7}
5 % ethanol	68.3	201×10^{-7}
10 % ethanol	74.5	219×10^{-7}

The effect of using ethanol entrainers on the extraction efficiency and solvent loading of ß-carotene is shown in Table 7.3. The addition of a 1 % entrainer did not improve the extraction efficiency and solvent loading of ß-carotene and resulted in a slight reduction in extraction efficiency and solvent loading when compared to pure carbon dioxide. However, both the 5 % and 10 % entrainer systems increased the extraction efficiency and the solvent loading of ß-carotene. Maximum extraction efficiency and solvent loading was obtained with the 10 % ethanol entrainer system and a 28.2 % increase in both solvent loading and extraction efficiency was obtained with this system, compared to pure carbon dioxide (Table 7.3).

7.4.1.3 Extractions with propane:carbon dioxide mixtures

The use of propane:carbon dioxide mixture has a marked impact on the extraction efficiency and solvent loading of ß-carotene (Table 7.4). At an extraction pressure of 336 bar, the 35:65 propane:carbon dioxide mixture increased the solvent loading and thus the extraction efficiency by

16.6 %. However, at an extraction pressure of less than half that used for pure carbon dioxide (215 bar), an extraction efficiency of 95.54 % was obtained with the 40:60 propane:carbon dioxide mixture (Table 7.4). This translates into an increase in solvent loading and extraction efficiency of 60.57 %.

Table 7.4 Extraction efficiency and solvent loading of propane:carbon dioxide co-solvent mixture at different extraction pressures.

Propane:CO_2 mixture	Extraction efficiency	Solvent loading ($mol.mol^{-1}$)
Pure CO_2 (70 °C, 450 bar)	59.5 %	175 x 10^{-7}
35:65 Propane:CO_2 (70 °C, 336 bar)	69.4 %	204 x 10^{-7}
40:60 propane:CO_2 (70 °C, 215 bar)	95.54	281 x 10^{-7}

7.4.2 Development of a hydrophobic membrane assisted hot oil ß-carotene extraction process from *D. salina.*

7.4.2.1 ß-carotene solubility in vegetable oil.

Table 7.5 Solubility of natural ß-carotene and synthetic all-*trans* ß-carotene in oil at various temperatures after 15 min extraction.

TEMPERATURE	NATURAL ß-CAROTENE (g.100 ml or % w/v)	SYNTHETIC ß-CAROTENE (g.100 ml or % w/v)
25 °C	1.31	0.080
40 °C	1.41	0.085
50 °C	1.48	0.088
60 °C	1.53	0.090
70 °C	1.69	0.092

The solubility time study showed that maximum ß-carotene solubilities were obtained after 15 minutes irrespective of the extraction temperature. Table 7.5 shows the maximum ß-carotene solubility of both synthetic and natural ß-carotene in sunflower oil. It is clear from Table 7.5 that the natural isomeric mixture of ß-carotene is more soluble than the synthetic all-*trans* ß-carotene. For both the natural and the synthetic ß-carotene, greater solubilities were obtained at higher temperatures. Over the temperature range evaluated the solubility of natural ß-carotene was approximately 16-18 fold higher than that of the synthetic ß-carotene. Maximum solubility of both the natural and synthetic ß-carotene occurred at 70 °C (Table 7.5). Figure 7.5 shows the difference in colour of the maximum synthetic and natural ß-carotene extracts. It is apparent from Figure 7.5 that the solubility of the natural ß-carotene is far greater than that of the synthetic ß-carotene.

Figure 7.5 Difference in solubility of synthetic all-*trans*-ß-carotene (A) and of the *D. salina* sterioisomeric mixture (B).

By determining the ß-carotene content of biomass prior to extraction and extracting with an appropriate volume of oil, 98.9 % of the ß-carotene can be consistently extracted from the *D. salina* biomass.

7.4.2.2 Incorporation of a hydrophobic microfiltration membrane system for the separation of oil from the extraction slurry.

Water breakthrough occurred for both the 0.0036 m^2 and the 0.038 m^2 membranes at operating pressures of greater than 90 Kpa (Figure 7.6). Above 90 Kpa there was a linear increase in water breakthrough as the pressure increased. Water breakthrough was slightly greater in the larger membrane system (0.038 m^2) at operating pressures above 100 Kpa.

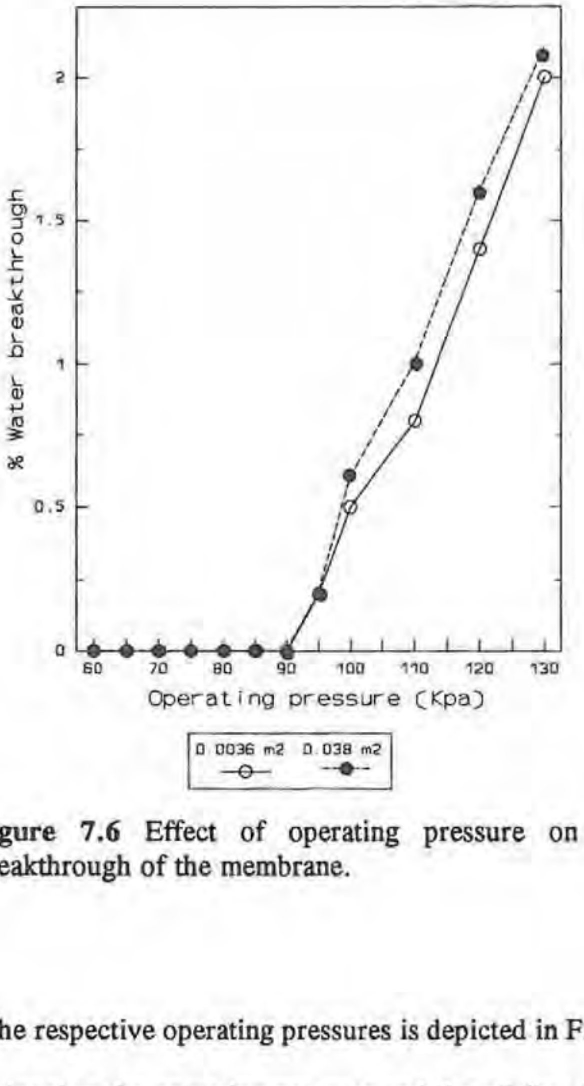


Figure 7.6 Effect of operating pressure on the water breakthrough of the membrane.

The oil flux rates at the respective operating pressures is depicted in Figure 7.7. Oil flux rate appears to be directly proportional to the operating pressure and the oil flux increases as operating pressure increases. At optimal operating pressure of 90 Kpa (ie. no water breakthrough) flux rates of 23.1 and 24.7 $l.m^{-2}.hr^{-1}$ (LMH) were obtained for the 0.0036 m^2 and the 0.038 m^2 membranes, respectively.

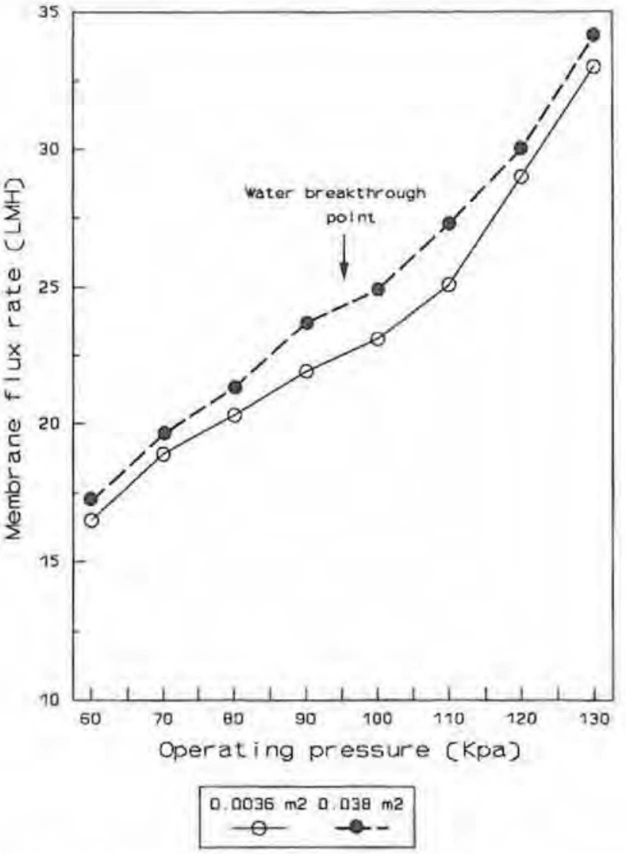


Figure 7.7 Effect of operating pressure on oil flux rates.

Table 7.6 Effect of temperature on water breakthrough pressure.

TEMPERATURE (°C)	0.0036 m² MEMBRANE		0.038 m² MEMBRANE	
	PRESSURE (Kpa)	FLUX (LMH)	PRESSURE (Kpa)	FLUX (LMH)
25	95	25.2	95	27.4
30	90	24.7	90	25.0
35	75	23.9	80	23.1
40	60	22.2	55	22.7

The effect of temperature on water breakthrough pressure is shown in Table 7.6. Similar water breakthrough pressures were obtained for both membranes over the temperature range evaluated. As the temperature of the oil-water emulsion increased there was a marked decrease in the water breakthrough pressure of the membranes, however, a slight decrease in oil flux rate was observed. Oil flux rates of above 21 LMH were obtained despite a reduction in operating pressure of approximately 35-40 Kpa at 40 °C.

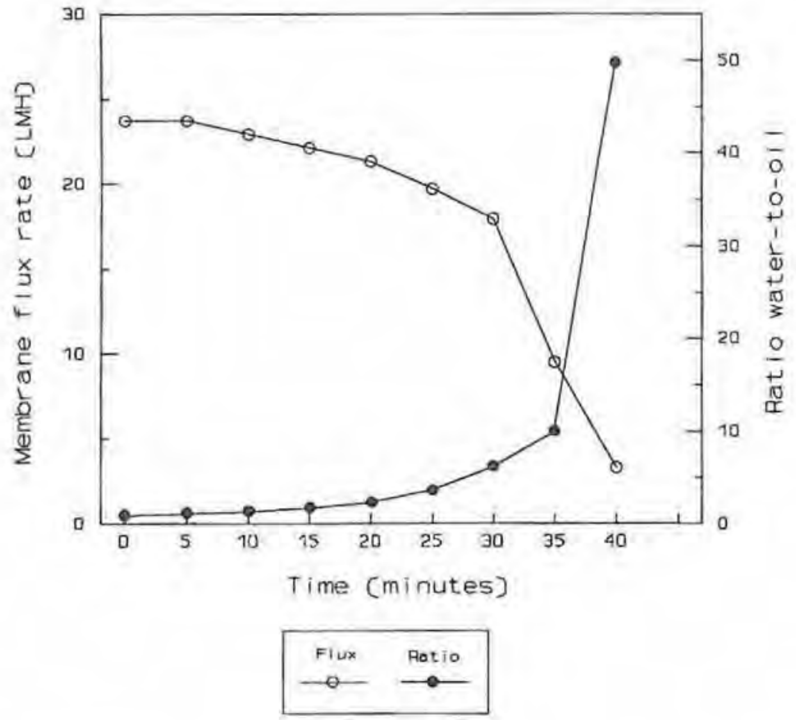


Figure 7.8 Oil flux rate in response to increasing water-to-oil ratio (0.038 m^2 membrane).

The effect of the water:oil ratio on membrane flux rate, at an operating pressure of 90 Kpa and an operating temperature of 25 °C, is illustrated in Figure 7.8. A gradual decrease in the oil flux was evident over water:oil ratios of 1 to 7, however, beyond this point a sharp decrease in oil flux occurred (Figure 7.8). At a water:oil ratio of 50:1, the oil flux rate fell to 2.6 LMH (Figure 7.8).

Figure 7.9 shows a comparison of operating the system with a whole extract slurry (ie. cell debris, flocculant, salts, oil and water) or with the passively separated oil phase. It is clear from Figure 7.9 that due to the high organic load of the extract slurry, oil flux rates were lower than with the oil phase. Consequently, oil recovery was compromised by the extract slurry, and after 20 minutes only 210 ml of oil was recovered (55 % recovery) compared to 280 ml with the oil phase (80 % oil recovery)(Figure 7.9). This represents a 25 % decrease in recovery for the whole extract slurry.

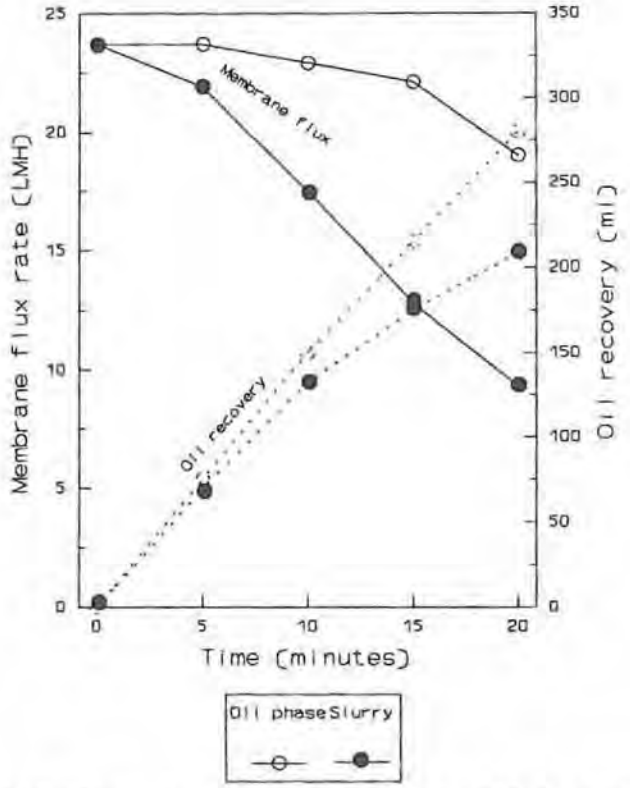


Figure 7.9 Membrane flux rates and oil recovery with the membrane system operated with either the whole extract slurry or the passively separated oil phase.

7.4.2.3 Contamination of oil with flocculants.

Analysis of the oil recovered from a ß-carotene extraction of *D. salina* biomass harvested with 2000 mg.l^{-1} aluminium chloride showed no detectable Al^{3+} using atomic absorption spectroscopy.

7.4.2.4 Qualitative analysis of ß-carotene oil extract.

Figure 7.10 shows the HPLC profiles of the ß-carotene oil extract (A) and a whole cell extract (B). It is clear from Figure 7.10 that hot oil extraction of *D. salina* is specific for ß-carotene. Chlorophyll a, chlorophyll b and the various xanthophylls were undetectable in the hot oil ß-carotene extract using HPLC analysis, whereas it is apparent from Figure 7.10 that these compounds were largely present in the whole cell extract.

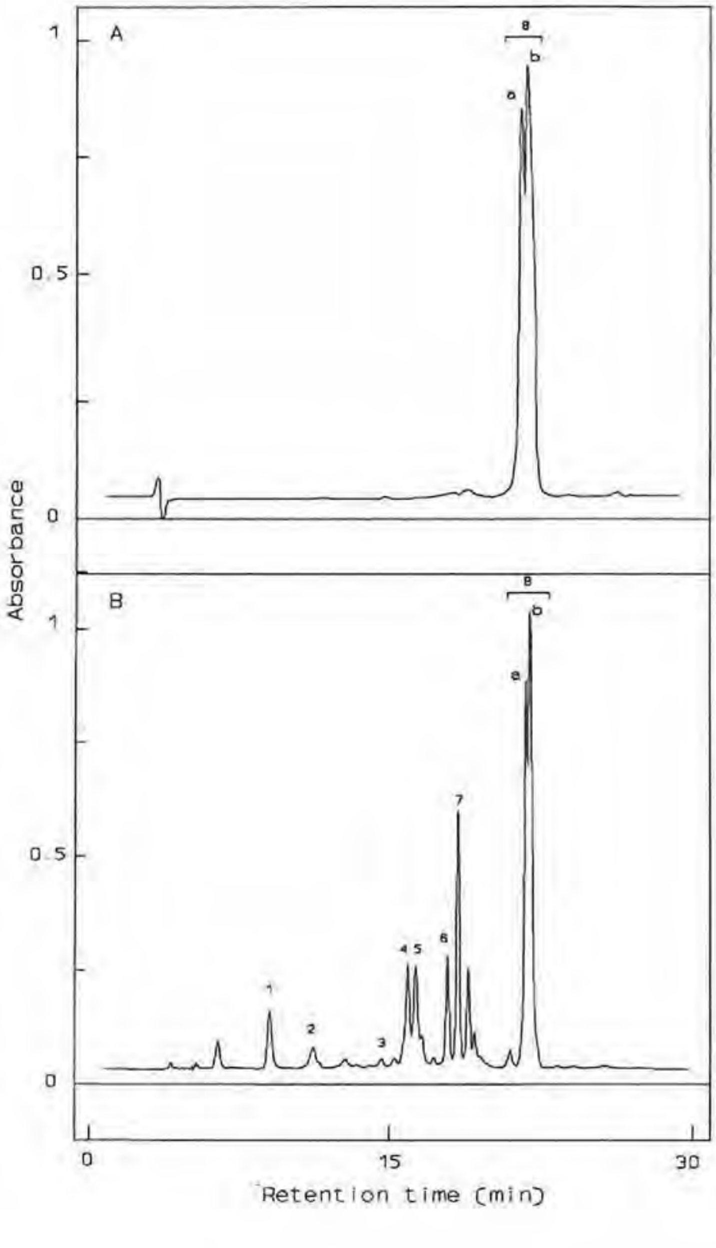


Figure 7.10 HPLC profile of the ß-carotene oil extract (A) and a whole cell extract (B). 1 - neoxanthin. 2 - violaxanthin. 3 - antheraxanthin. 4 - lutien. 5 - zeaxanthin. 6 - chlorophyll b. 7 - chlorophyll a. 8a - all-*trans* ß-carotene. 8b - 9-*cis* ß-carotene.

7.5 DISCUSSION.

Preliminary study of supercritical fluid extraction of ß-carotene from *D. salina*

The preliminary study has shown that supercritical fluid extraction is feasible for the extraction of ß-carotene from *D. salina* and high extraction efficiencies can be obtained by manipulating the supercritical fluid composition.

Cygnarowicz *et al.* (1990) report maximum solvent loadings of 25.4×10^{-7} mol.mol^{-1} for pure carbon dioxide, at a temperature of 70 °C and a pressure of 439 bar. A solvent loading of 37.5×10^{-7} mol.mol^{-1} was obtained with a 1 % ethanol/ carbon dioxide entrainer mixture at a temperature of 70 °C and a pressure of 374 bar. These values are significantly lower than the values obtained in this study, where a solvent loading of 175×10^{-7} mol.mol^{-1} was obtained with pure carbon dioxide at an extraction temperature of 70 °C and an extraction pressure of 450 bar. A 10 % ethanol/carbon dioxide entrainer mixture produced a solvent loading of 219×10^{-7} mol.mol^{-1} however, the extraction pressure was substantially higher (450 bar) than that used by Cygnarowicz *et al.* (1990).

It appears that the algal ß-carotene mixture is more soluble than the crystalline all-*trans*-ß-carotene used by Cygnarowicz *et al.* (1990), since the maximum solvent loading obtained with pure carbon dioxide at similar extraction temperatures and pressures is 6.9 times higher than that obtained by the authors.

The use of 5 and 10 % ethanol/carbon dioxide entrainer mixtures substantially increases the solvent loading of the system. However, contrary to the results obtained by Cygnarowicz *et al.* (1990) no increase in solvent loading was obtained with a 1 % ethanol/carbon dioxide entrainer mixture. It is possible that the relatively small quantity of ethanol used for the 1 % entrainer mixture may have reacted with or been absorbed by the cellular material thus reducing its effectiveness.

The most promising results of the study were obtained with the propane:carbon dioxide co-solvent mixtures. High extraction efficiencies and solvent loadings were obtained at relatively low extraction pressures with the propane:carbon dioxide mixtures. The 40:60 propane:carbon dioxide mixture increased the extraction efficiency of pure carbon dioxide from 59.5 % to 95.54 %. Furthermore, the extraction pressure of the propane:carbon dioxide mixture was less than half of that used for pure carbon dioxide. A reduction in operating pressure has the potential to substantially reduce the running costs of a supercritical fluid extraction process (Fong, 1989). Willson (1985) maintains that for biological processes propane is a suitable component for co-solvent mixtures, in addition it is approved by the World Health Organisation as a solvent for food products.

The preliminary study has highlighted the potential of using supercritical fluid extraction for ß-carotene extraction from *D. salina*. Supercritical fluid extraction has a number of advantages over conventional liquid/liquid extraction processes: such as non-toxic inexpensive solvents, high energy efficiencies, high diffusibility and adjustable solvent power. Further research is, however, needed to optimise the extraction of ß-carotene from *D. salina*. The results indicate that co-solvent mixtures of carbon dioxide and propane have the potential to significantly reduce the extraction pressure and future research should be focused on determining the optimal co-solvent mixture, the optimal extraction temperature and pressure, and whether the extraction from wet biomass is feasible.

Development of a hydrophobic membrane assisted hot oil ß-carotene extraction process from *D. salina*.

Synthetic all-*trans* ß-carotene has a relatively low solubility in vegetable oil and Ben-Amotz *et al.* (1989a) report solubilities of 0.05-0.08 g.100 ml^{-1} at 20 °C. These values compare well with the solubility of synthetic ß-carotene at 25 °C obtained in this study (0.08 g. 100 ml^{-1}). An increase in oil temperature to 70 °C raises the solubility to 0.092 g.100 ml^{-1}, however, the low solubility of synthetic all-*trans* ß-carotene contrasts the high solubilities obtained with the natural isomeric ß-carotene mixture. A maximum solubility of 1.69 g.100 ml^{-1} was obtained at 70 °C which is approximately 18 times greater than that obtained for the synthetic ß-carotene at the same temperature.

The superior solubility of the natural isomeric mixture may be related to the higher fat solubility of the 9-*cis* isomer (Goodwin, 1980a), and to the tendency of the all-*trans* isomer to crystallize at relatively low concentrations. The 9-*cis* isomer may also serve as a good solvent for the all-*trans* isomer, thus explaining the higher solubility of the isomeric mixture. No report on the solubility of natural *D. salina* derived ß-carotene was found in the literature reviewed and this probably relates to the technological secrecy shrouding the industry.

If one examines the documentation of the commercialization process undertaken by Western Biotechnology at Hutt Lagoon, Western Australia, (Borowitzka *et al.* 1984; Moulton *et al.* 1987; Borowitzka and Borowitzka, 1989, 1990), the first marketable product was a 1.5 % w/v solution of ß-carotene in vegetable oil, which has a variety of purposes, including direct incorporation into margarine and encapsulation as a nutritional supplement (Moulton *et al.* 1987). Following this, ß-carotene was marketed as 1.6-4 % solution in vegetable oil and a 30 % suspension of crystalline ß-carotene in oil (Borowitzka and Borowitzka, 1990). One can only speculate that the first product, the 1.5 % w/v solution of ß-carotene in oil, is equivalent to the ß-carotene solutions obtained in this study (1.31-1.69 % w/v, Table 5), since further concentration of ß-carotene in oil is performed by some physical, post extraction process (Curtain, *et al.* 1987)

The incorporation of hydrophobic membrane assisted oil separation into hot oil extraction of ß-carotene from *D. salina* provides a novel and practical solution to separating the oil phase from the extraction slurry. Passive oil phase separation is inadequate and produces a cloudy oil suspension contaminated with small quantities of both the aqueous extraction phase (about 5 % v/v) and cell debris. The hydrophobic membrane oil separation is characterised by low operating pressures, and flux rates which are comparable with other filtration processes (reviewed in Cheryan, 1986). The oil recovered by this process is of brilliant clarity and analysis has shown that it is not contaminated with the flocculant used to harvest the algal biomass.

High reproducibility was demonstrated with the two polypropylene membrane modules evaluated (0.0036 m^2 and 0.038 m^2) and water breakthrough pressures of 95 Kpa were obtained for both membranes. Oil flux rates were similar over operating pressures 60-130 Kpa, however, flux rates obtained with the larger membrane module were marginally higher than with the 0.0036 m^2 membrane module. The reason for this is unclear, but could be the result of inconsistency in membrane manufacture or a difference in mass transfer characteristics caused by different membrane housing geometries.

An increase in operating temperature of the hydrophobic membrane system results in water breakthrough occurring at lower operating pressures. This is probably due to the decreased viscosity of oil at elevated temperatures. Despite the decrease in operating pressures at increased temperatures, oil flux rates remains relatively high, again, this is possibly due to the decrease in oil viscosity. However, trans-membrane oil flux rates at elevated temperatures were consistently lower than for unheated oil, and no apparent advantage can be obtained by operating the hydrophobic membrane system at elevated temperatures.

The separation of oil from whole extract slurries is detrimental to membrane flux, the reason for this is two-fold. Firstly, the study has shown that membrane flux is compromised as the water:oil ratio increases, probably due to a decrease in the volume of oil being presented to the membrane surface. Secondly, the high solids level of the whole extract clogs the filter pores thus reducing membrane flux.

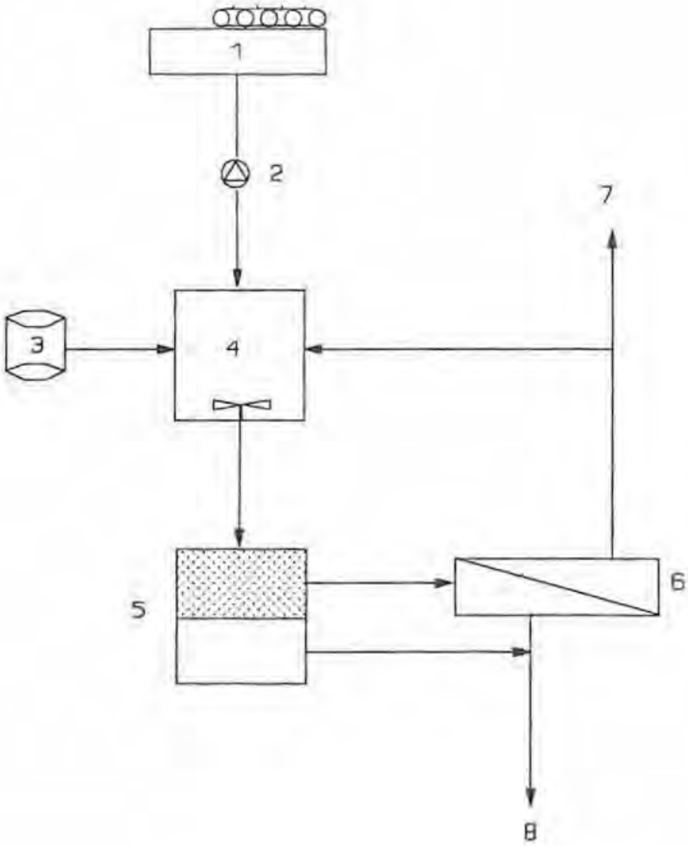


Figure 7.11 Schematic diagram of a potential hydrophobic membrane assisted hot oil extraction process. 1 - harvesting unit. 2 - high pressure pump. 3 - hot oil. 4 - extraction vessel. 5 - passive phase separation. 6 - hydrophobic membrane. 7 - oil recovery. 8 - waste stream.

The results indicate that the extraction slurry should undergo passive phase separation of the aqueous and oil phase and only the oil phase should be purified by separation through the hydrophobic membrane system. Relatively high flux rates can be maintained for longer periods by using only the oil phase, consequently improving the oil recovery rate. Figure 7.11 illustrates a potential hot oil extraction process, incorporating a hydrophobic membrane system for oil separation from extract slurry. Biomass harvested by the flocculation process would be pumped under pressure through a high pressure nozzle (2), thus inducing mechanical cell shattering (Figure 7.11). Hot oil extraction would take place in the extraction vessel (4) and the passive phase separation and cooling would be induced in a phase separation tank (5)(Figure 7.11). The oil phase could then be clarified by passing the oil phase through the hydrophobic membrane (6)(Figure 7.11). The clarified oil could possibly be returned for further ß-carotene extraction, in order to achieve supersaturation of the oil.

In addition to the incorporation into hot oil extraction of ß-carotene from *D. salina*, the hydrophobic membrane separation of oil from oil-water emulsions offers additional applications in the fields of oil soluble metabolite extraction of other biological materials and the treatment and recovery of oil from oily wastewater, such as those produced in the petrochemical industry. Oil separation is currently achieved with hydrophillic membrane systems. Further research should be focused at evaluating other types of hydrophobic membranes and the affect of membrane configuration (ie. hollow fibre, tubular) on the operating parameters of the process should be examined. Membrane life is of prime importance although, in this study, membranes stored in oil for periods of up to 3 months exhibited no reduction in membrane flux rates or water breakthrough pressure.

This study has resulted in a provisional patent application by Rose *et al.* (1993)(see Postscript).

CHAPTER 8

PROCESS DESIGN AND ECONOMIC EVALUATION.

Summary.

Process design and economic evaluation of the dual-stage system was undertaken using the results obtained in Chapter 4 and Chapter 5. A preliminary evaluation of the dual-stage system has shown that the stress ponds should be operated at a starting culture density of 0.2 g dry wt.l^{-1} and a retention time of 5 days. A comparative economic evaluation of both the averaging process and the dual-stage process has demonstrated that a dual-stage system is far superior to the current averaging process and would offer a marked competitive advantage.

8.1 INTRODUCTION.

In its widest context Biotechnology may be understood as referring to the commercialization of all biological processes (Hacking, 1986). The ultimate aim of algal biotechnology is therefore, to take an idea from the laboratory to the production plant and the market place (Borowitzka and Borowitzka, 1989). In order to achieve this the economic viability of a potential process must be carefully examined.

The results of the outdoor growth ponds (Chapter 4) have indicated that a long-term average biomass productivity of 16.6 g dry wt.m^{-2}.day^{-1} can be achieved by cultivating *D. salina* under conditions suitable for biomass production. This value is approximately three-fold greater than the long-term average biomass productivities reported in the literature for the averaging process; 5 g dry wt.m^{-2}.

day^{-1} (Ben-Amotz and Avron, 1989) and 4 g dry wt.m^{-2}.day^{-1} (Mohn and Condero-Contreras, 1990). In addition, the outdoor stress pond evaluation (Chapter 5) has shown that ß-carotene hyper-accumulation in *D. salina* can be achieved by cultivating the alga in a high salinity, total nutrient limited medium, particularly at low culture densities. ß-carotene contents as high as 15 % dry wt. were obtained in the outdoor evaluation which are approximately three-fold higher than the ß-carotene content of algae produced in the averaging process (about 5 % dry wt.)(Ben-Amotz and Avron, 1989). However, factors such as the extra ponding area required for the stress ponds, the additional harvesting step and the retention time in the stress ponds, do not allow for a direct comparison of the productivities of the two systems. A more detailed economic evaluation which addresses the above is necessary for an accurate comparison.

Before one can embark on an economic evaluation a clear understanding of the basic operation of the proposed process design for a dual-stage system is necessary. Figure 8.1 is a schematic representation of the proposed operation of a dual-stage ß-carotene production system, assuming a stress pond retention time of 5 days. The first stage of the system involves maximum biomass production in the continuous culture growth ponds (Figure 8.1). Continuous culture permits increased productivity by eliminating the starting up and stopping of batch cultures (Hacking, 1986). The biomass produced in the growth ponds is harvested and transferred to consecutive stress ponds daily. The stress ponds function as batch cultures and form the second stage of the system (Figure 8.1). After five days, all the stress ponds would have been inoculated and would contain biomass at various retention times. On the sixth day, after a biomass retention time of 5 days, pond A would be harvested and refilled, similarly, on the seventh day, pond B would be harvested and refilled after the biomass had been in stress pond B for five days (Figure 8.1). The cyclical operation of the system would continue for the remaining ponds, until the newly inoculated biomass in pond A has had a retention time of 5 days and the cycle would repeat itself until the end of the production season.

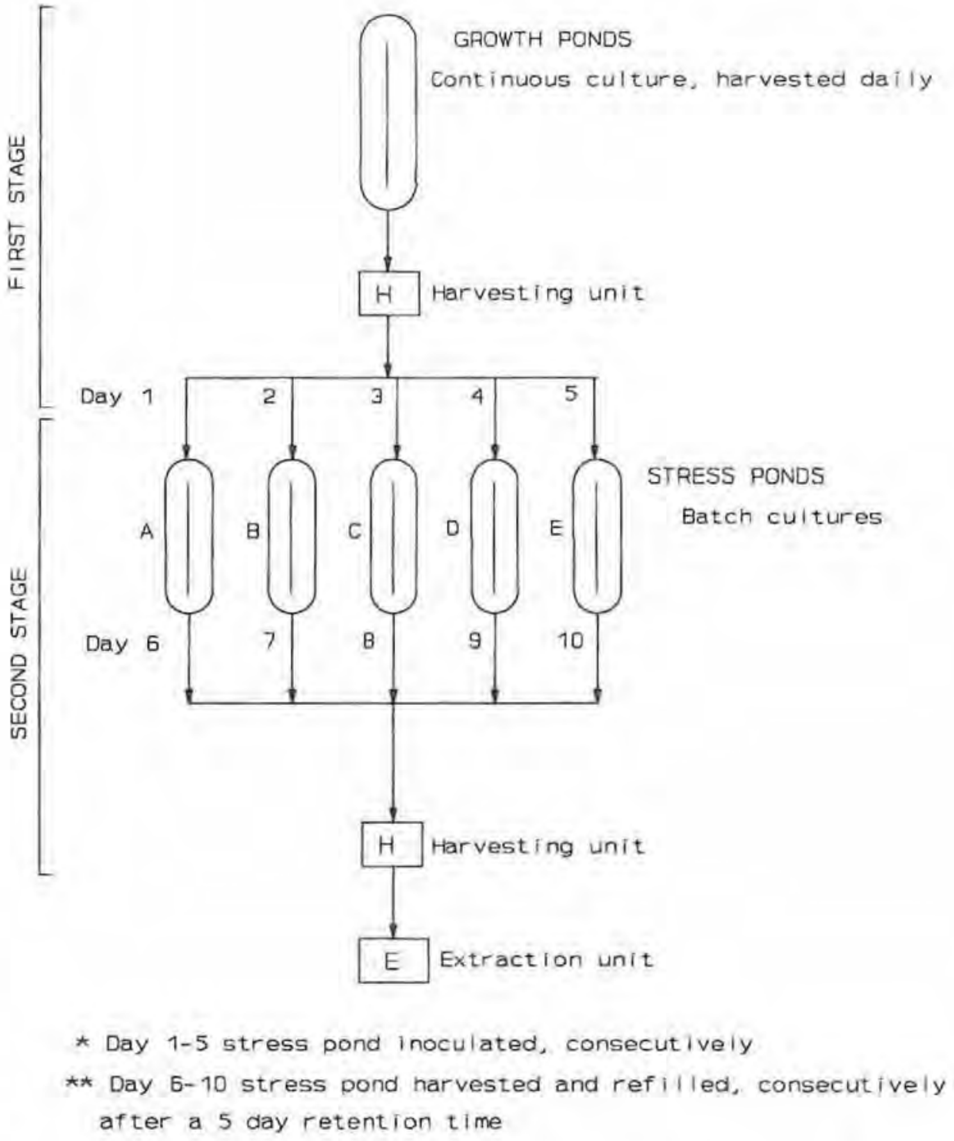


Figure 8.1 Flow chart of a potential dual-stage ß-carotene production system.

With this system two noticeable factors emerge. Firstly, the number of stress ponds is directly proportional to the retention time (in days) of the biomass in the stress ponds, and secondly, with the system operated in this fashion, the only loss in production time would be at the beginning of the production season, when the stress ponds are inoculated for the first time. After this, ß-carotene rich biomass would be produced daily from one of the stress ponds. Thus, the major difference between the dual-stage system and the averaging system is the requirement for additional stress pond area.

Biotechnological processes require specialized equipment and are therefore, highly capital intensive (Hacking, 1986). The recovery of capital costs is one of the most significant factors in project analysis. Several methods of varying complexity can be employed to determine a project's acceptability in terms of the capital expenditure. The four most useful capital budgeting techniques are the payback period, the net present value (NPV), the return on investment (ROI) and the internal rate of return (IRR) (Hacking, 1986; Gidman, 1988).

Although widely use, the payback period is generally viewed as an unsophisticated capital budgeting technique as it does not explicitly consider the time value of money by discounting cash flows to find present values (Gidman, 1988). Of all the capital budgeting techniques, the IRR is probably the most commonly used sophisticated budgeting technique. For conventional projects, NPV and IRR will always generate the same accept-reject decision, but differences in their underlying assumptions can cause them to rank projects differently (Gidman, 1988). For this reason, both NPV and IRR were used in the following economic evaluation.

8.2 PRELIMINARY ECONOMIC ANALYSIS AND PROCESS DESIGN.

When considering algal biotechnological processes, the area of large-scale production systems can be used as an indication of the total capital and operating costs of the process (Ben-Amotz, 1980). The area of a production system dictates the pond size, number of ponds and the culture volumes required for processing and thus provides an approximate indication as to capital and operating costs of the system. Production area was used in preliminary economic analysis to determine the optimal starting culture density for operating the system. Once this had been determined the influence of retention time on pond area was examined.

8.2.1 Pond area calculation.

The total production area was calculated using the long-term average biomass productivity of 16.6 g dry wt.m^{-2}.day^{-1} as obtained in Chapter 4 and the relative ß-carotene content of biomass produced in the growth ponds after a retention time of 5 days in the stress ponds (Table 5.3, Chapter 5). The total growth pond and stress pond area required to produce 5 tons of ß-carotene per annum, with a 240 day production season, was calculated as follows:

Firstly, the annual biomass production was calculated using Equation 1:

$$T\ (kg) = ß \times 100\ /\ C \tag{1}$$

Where T = total *D. salina* biomass (kg) required to produce 5 tons ß-carotene, ß = mass ß-carotene required (5000 kg) and C = relative ß-carotene content (% dry wt) of biomass transferred to stress ponds after a retention time of 5 days in the stress ponds (These values were obtained from Table 4, Chapter 5). The daily biomass production (D) required can then be calculated.

$$D\ (kg.day^{-1}) = T\ /\ 240\ days \tag{2}$$

Using the daily biomass production (D), the growth pond area (G) can be calculated using the long-term average biomass productivity of 16 g dry wt.m^{-2}.day^{-1} obtained in Chapter 4.

$$G\ (m^2) = D\ /\ 16.6 \tag{3}$$

Assuming the growth ponds are harvested at an areal density of 48 g dry wt.m^{-2} (culture density of 0.4 g.l^{-1}), then a biomass productivity of 16.6 g dry wt.m^{-2}.day^{-1} represents 34.5 % of the total biomass in the growth ponds. If the harvested biomass is resuspended to its original concentration in the stress ponds (ie. starting culture density 0.4 $g.l^{-1}$), then the area of the stress ponds would be 34.5 % of that of the growth ponds, assuming that the depth in both the growth and stress ponds depth is 12 cm. However, since the retention time is 5 days, there would be five ponds of the same area. Thus the total stress pond area (S) can be calculated as follows:

$$S\ (m^2) = G \times 0.35 \times 5 \times F \tag{4}$$

Where F is the concentration or dilution factor, depending whether the starting culture density is greater or less than 0.4 $g.l^{-1}$, respectively.

The pond area required for the averaging process was calculated as above for the growth pond area, using a long-term biomass productivity of 5 g dry $wt.m^{-2}.day^{-1}$ and a ß-carotene content of 5 % dry wt. (Ben-Amotz and Avron, 1989)

8.2.2 Determination of optimal stress pond starting culture density.

The results reported in Chapter 5 have shown that the major factor influencing ß-carotene production is the starting culture density of the stress ponds. More ß-carotene per unit biomass is produced at lower starting culture densities, however, dilution of the biomass produced in the growth ponds would be necessary. The possible pay-off between improved ß-carotene production and increased stress pond area or decreased ß-carotene production and reduced stress pond area was examined.

Table 8.1 shows the growth, stress and total pond area required to produce 5 tons of ß-carotene per year at different stress pond starting culture densities. Except for the 0.1 g dry $wt.l^{-1}$ starting culture density, the stress pond area remains relatively constant, whereas, the growth pond area increases as the starting culture density becomes greater (Table 8.1). The reason for this is that as the starting culture density of the stress ponds increases, ß-carotene accumulation is impaired, therefore, greater biomass production is required to compensate for the reduced ß-carotene accumulation. Consequently, larger growth pond areas are necessary for increased biomass production.

The reason that the 0.1 g dry wt.l^{-1} stress pond requires a relatively large stress pond area (Figure 8.1) is related to the massive dilution necessary to obtain a starting culture density of 0.1 g dry wt.l^{-1} and also because ß-carotene production at this starting culture density does not achieve the 23 mg.l^{-1} ceiling in ß-carotene production described in Chapter 5.

Table 8.1 Effect of stress pond starting culture density on the pond area required to produce 5 tons of ß-carotene per annum. Pond areas are corrected to the nearest thousand m^2. Calculations are based on a 5 day retention time.

START. CULTURE DENSITY (g.l^{-1})	GROWTH POND AREA (m^2)	STRESS POND AREA (m^2)	TOTAL AREA (m^2)
0.1	8000	56000	64000
0.2	11000	39000	50000
0.3	15000	39000	54000
0.4	22000	39000	61000
0.8	43000	37000	80000
1.2	63000	36000	99000
1.6	84000	36000	120000
AVERAGING PROCESS	83000		83000

An examination of the total production area required for 5 tons of ß-carotene per annum indicates that the optimal stress pond starting culture density is 0.2 g dry wt.l^{-1} (Table 8.1). The total production area at 0.2 g dry wt.l^{-1} (50000 m^2) is also substantially lower than the total production area required for the averaging process (83000 m^2)(Table 8.1). The smaller production area necessary for the dual-stage process, in comparison to the averaging process, can be attributed to the high biomass productivities obtained in the growth ponds (Chapter 4) and the high degree of ß-carotene hyper-accumulation induced in the stress ponds (Chapter 5).

It is unlikely that dual-stage ß-carotene production processes operated at starting stress pond culture densities of 0.8 g dry wt.l^{-1} and higher would offer any competitive advantages over the averaging process, as the total production areas are similar or greater than the area required for the averaging process (Table 8.1).

8.2.3 Effect of retention time on production area.

Table 8.2 Effect of stress pond retention time on total production area required to produce 5 tons ß-carotene per annum at a starting culture density of 0.2 g dry wt.l^{-1}.

RETENTION TIME (DAYS)	RELATIVE ß-CAROTENE CONTENT (%)	TOTAL POND AREA (m^2)
2	4.5	67000
3	7.4	52000
4	9	52000
5	11.2	50000

With the proposed operation of the dual-stage ß-carotene production process the number of stress ponds required is directly proportional to the retention time (Figure 8.1). The possible pay-off between reduced retention time, thus decreased ß-carotene yield, and fewer stress ponds, or increased retention time, thus enhanced ß-carotene yield and more stress ponds was examined for the 0.2 g.l^{-1} starting stress pond culture density (Table 8.2).

It is clear from Table 8.2 that a five day retention time results in the optimal production area and no apparent benefit can be obtained by operating the dual-stage ß-carotene production system at a reduced retention time as judged by total production area.

8.2.4 Process design.

A dual-stage ß-carotene production process was designed using the growth and stress pond area calculated for the 0.2 g dry wt.l^{-1} starting culture density system operated on a retention time of 5 days as this requires the smallest total pond area necessary to produce 5 tons of ß-carotene per annum. A single stage process (averaging process) was also designed for comparison.

Most commercial *Dunaliella* and *Spirulina* mass-cultivation systems employ oblong, shallow (10-30 cm) raceway ponds, stirred with paddlewheels and lined with plastic sheeting, usually polyvinylchloride (PVC) (Richmond, 1988; Bubrick, 1991). Pond sizes are typically between 500 m^2 and 5000 m^2 (Richmond, 1988; Bubrick, 1991). Figure 8.2 depicts a diagrammatic representation of the design of the dual-stage process using oblong, shallow raceway ponds, stirred with paddlewheels. The first stage of the system consists of two 5500 m^2 growth ponds (Figure 8.2) operated at a depth of 12 cm. The second stage of the process, consists of ten 3900 m^2 stress ponds also operated at a depth of 12 cm (Figure 8.2). Each of the growth ponds supplies biomass, daily, to one of a series of five stress ponds.

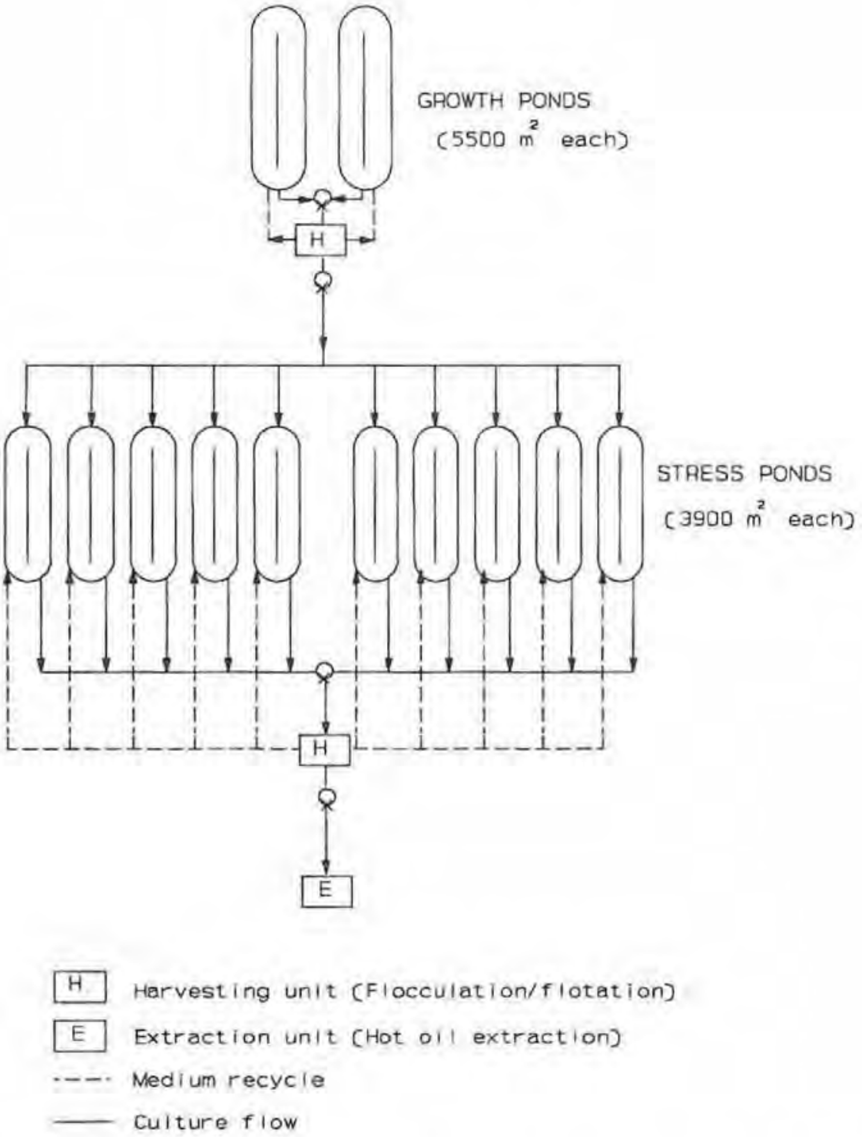


Figure 8.2 Schematic diagram of the proposed dual-stage ß-carotene production system.

Biomass produced in both the growth and the stress ponds is harvested using the flocculation/flotation process described in Chapter 6. The results obtained in Chapter 6 indicate that flocculation with the PAC flocculant K300 produces a medium suitable for recycle. Both growth and stress medium would be recycled using a minimum medium purge. The results obtained in Chapter 5 have demonstrated that a growth medium carryover of 2.5 % into the stress ponds does not adversely affect ß-carotene production. This would constitute a media purge from the growth ponds and would be supplemented with fresh media. Stress pond medium purge would be of the same magnitude. ß-carotene-rich biomass produced in the stress ponds would be extracted with the hydrophobic membrane assisted hot oil extraction process described in Chapter 7.

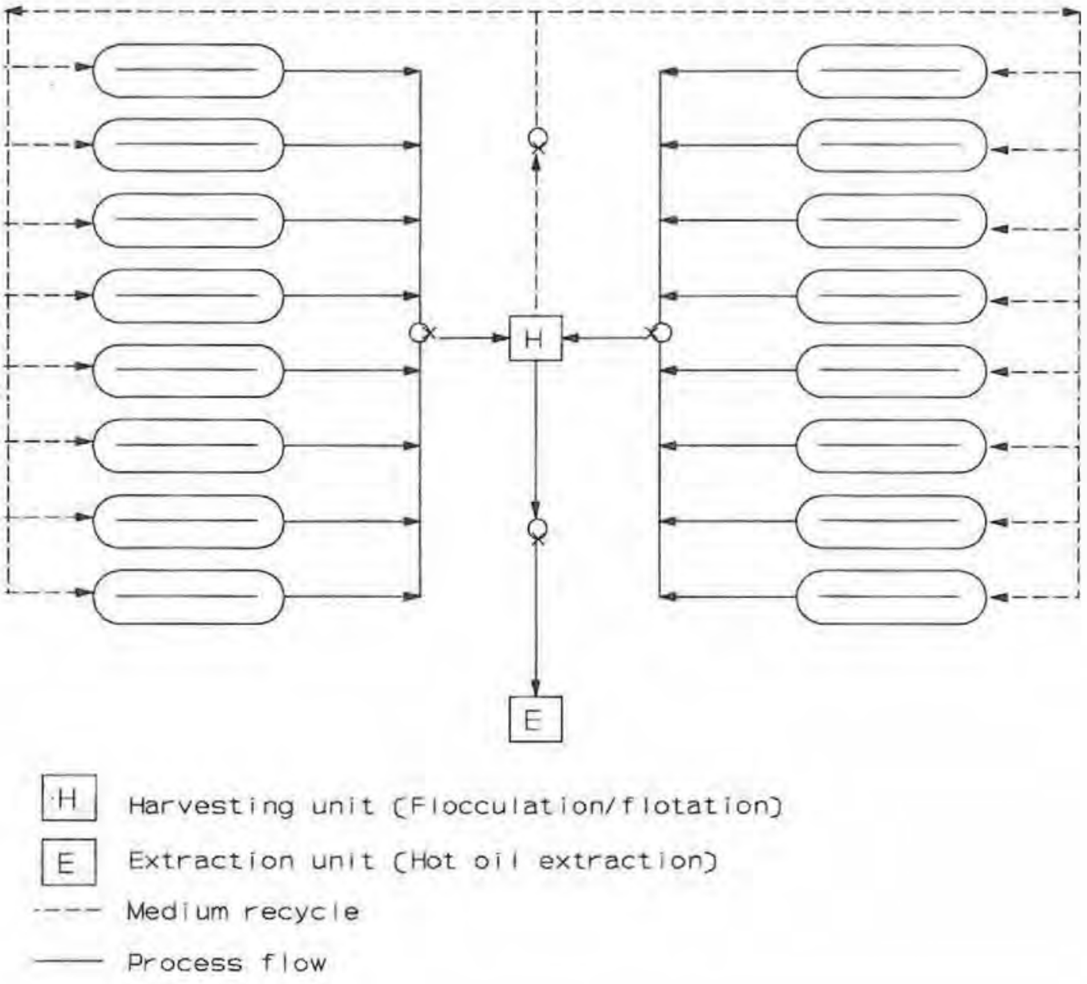


Figure 8.3 Schematic diagram of a averaging (single-stage) ß-carotene production system. The system consists of 16 ponds of 5200 m^2 each.

Figure 8.3 shows a diagrammatic representation of a single stage (averaging) process designed with the same harvesting and extraction processes. The system consists of sixteen 5200 m^2 ponds operated at a depth of 12 cm (Figure 8.3). Medium recycle would operate with a medium purge of 2.5 %.

Table 8.3 Comparative summary of averaging (single-stage) and dual-stage ß-carotene production processes.

PROCESS PARAMETER	SINGLE-STAGE	DUAL-STAGE
ß-carotene production per annum ($kg.yr^{-1}$)	5000	5000
Production period (days)	240	240
Biomass productivity (g dry $wt.m^{-2}.day^{-1}$)	5	16.6
ß-carotene content (%)	5	11.2
Pond area required (m^2)	83200	50000
Total number of ponds required	16	12
Total harvest volume/day (m^3)	1697	1391
Mass flocculant require/day (kg)	169.7	139.1
Total media purge ($m^3.day^{-1}$)	42.4	11.6
Biomass extracted/ day (kg)	509	281

The summary of the dual-and single-stage ß-carotene production processes (Table 8.3) shows that due to the smaller size of the dual-stage process, smaller volumes of culture would be processed each day. This has significant economic implications, because, although the dual-stage process requires two harvesting steps, the total capacity of the harvesting units would be approximately 18 % smaller than the harvesting unit required for the single stage system. Furthermore, the daily flocculant requirement (Table 8.3) is substantially less than that required for the single-stage system, as smaller volumes of culture would be harvested. Similarly, the capacity of the extraction unit required for the dual-stage process is significantly smaller than that required for the single-stage system.

The daily media purge in the dual-stage system is 72 % less that in the single-stage system because of the smaller size of the system. Medium purged from the stress ponds (2.5 % per day) would be made up by the growth medium carryover into the stress ponds, therefore, only the medium purged from the growth ponds would constitute the total purge in the dual-stage system.

Factors such as the smaller ponding area, the smaller process volumes and the minimum medium purge will ultimately reduce both the capital and running costs of the dual-stage process in comparison to the single-stage process.

8.3 COMPARATIVE ECONOMIC EVALUATION.

A detailed economic evaluation of both the averaging and dual-stage processes described above was conducted to determine the comparative costing of both the processes. IRR, ROI and NPV for both the processes were calculated using a Lotus 1-2-3 spreadsheet supplied by Sastech Process Design. Capital estimates were supplied courtesy of Sastech Engineering Services (see Appendix A for a breakdown of the estimated capital expenditure). The economic evaluations were calculated using the growth medium recipe described by Ben-Amotz and Avron (1983) and the high salinity, total nutrient limited stress medium described in Chapter 5. The economic evaluations were initially based on a ß-carotene sales price of R 5000.kg^{-1}. An extract of the discounted cash flow (DFC) spreadsheet for both processes can be found in Appendix A.

Table 8.4 A summary of the economic evaluations of the single and dual-stage ß-carotene production processes, based on a ß-carotene sales price of R5000.kg^{-1}

	SINGLE-STAGE SYSTEM	DUAL-STAGE SYSTEM
Total pond area required to produce 5 tons.annum^{-1}	83 200	50 000
Total capital cost estimate (R million)	37	27.5
Total operating costs (R million)	8.866	3.692
Cash cost to produce 1 kg ß-carotene (R.kg^{-1})	1773	738
Net Present Value (NPV) at 15 % (R million)	10.51	30.49
Return on Investment (ROI) before tax (%)	40	77
Internal Rate of Return (IRR) (%)	20.9	36.35

It is evident from Table 8.4 that the economic aspects of the dual-stage ß-carotene production process are far superior to those of the single-stage system and provide a clear competitive advantage. The total capital costs of the single-stage system is R 9.5 million more than that of the dual-stage process due to the larger size of the production plant. In addition, the total operating costs of the single-stage system are R 5.174 million higher due to the larger process volumes. Consequently, irrespective of the capital budgeting technique employed, the NPV, ROI and IRR all indicate that the dual-stage system is a economically more attractive and provides a greater return on investment.

Borowitzka (1992) provides an inflation adjusted summary of the biomass production costs reported over the last decade for a variety of microalgal species. The biomass production costs vary between A$ 0.30 to A$ 60.00 kg^{-1} dry wt., however, Borowitzka (1992) maintains that a biomass production cost of A$ 15-25 kg^{-1} is more appropriate. A direct comparison with these figures is not possible since the production costs reported here are per kg ß-carotene. The only cost estimate for ß-carotene production available in the literature reviewed is reported by Mohn and Cordero-Contreras (1990) who estimate a total production cost of US$ 92.23.kg ß-carotene. If this value is inflation adjusted at 5 %.year^{-1} it would be equivalent to US$ 111.4.kg ß-carotene in 1993 which is equivalent to approximately R 345.kg^{-1} ß-carotene, based on current exchange rates. This value is substantially smaller than that obtained for both the single and dual-stage ß-carotene production processes, however, it does not include extraction and marketing costs which may account for the difference in cost estimates.

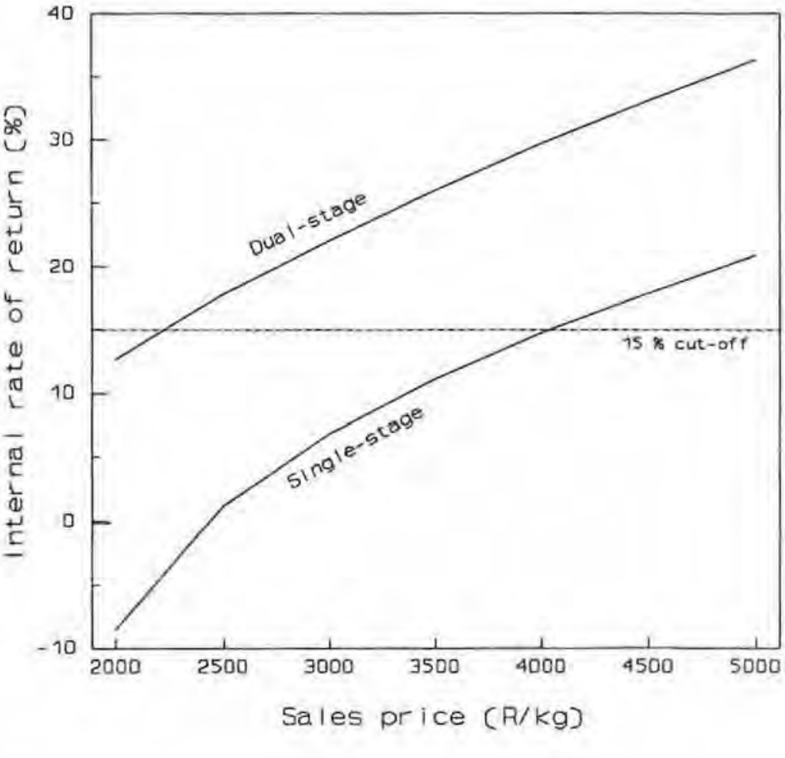


Figure 8.4 Sensitivity analysis of the cost effectiveness of the dual-stage and the averaging processes to changes in the ß-carotene sales price.

Ben-Amotz and Avron (1990) report that synthetic ß-carotene is sold for approximately US$ 600.kg^{-1} and that naturally derived ß-carotene is sold for more than double this value. If the synthetic price is adjusted for inflation by using a 5 %.year^{-1} inflation factor (Borowitzka, 1992) then the current synthetic price is approximately US$ 694.6.kg^{-1}, which is roughly R 2000 - 2300.kg^{-1}, depending on the exchange rate. Figure 8.4 shows a sensitivity analysis of the single and dual-stage ß-carotene production systems to a change in ß-carotene sales price. Using an IRR of 15 % as a decision criterion for accepting or rejecting a project, it is evident from Figure 8.4 that the dual-stage system could tolerate a drop in the sales price to about R 2250 while still maintaining economic viability, whereas the cut-off sales price for the single stage-process is at approximately R 4000. The implications of this are that the dual-stage process could possibly compete with the synthetic process, while the single-stage process must rely on the inflated natural ß-carotene sales price.

A recent report in the Chemical Marketing Reporter (August, 1993) has indicated a falling market trend in the world price of natural ß-carotene. This confirms the need for more economically sound *D. salina* ß-carotene production systems than the currently available single-stage system.

8.4 CONCLUSION.

The comparative economic evaluation of the single and dual-stage ß-carotene production processes has dispelled a number of the previous misconceptions of a potential dual-stage process. The high biomass productivities in conjunction with a high degree of ß-carotene hyper-accumulation, obtained by separating the distinctive growth and ß-carotene accumulation stages of *D.salina*, leads to higher net ß-carotene productivities. The implication of this is that the overall production size of a dual-stage process is significantly smaller than that required to produce an equivalent mass of ß-carotene with a single-stage process. Factors such as the additional harvesting step and possible increased labour costs are negated by the smaller process volumes required in the dual-stage system. In addition, by operating the system as described in Figure 1, no significant loss in retention time occurs as ß-carotene rich biomass is produced daily from the stress pond chains.

In summary, the implementation of a dual-stage process provides a definite competitive advantage over the existing technology and may allow the process to compete at a reduced sales price, possibly equivalent to that of synthetic ß-carotene.

CHAPTER 9

CONCLUSION

The development of ß-carotene production from *D. salina* over the last decade has been one of the few algal biotechnological processes to proceed to full scale commercial production (Benemann, 1989). The currently employed averaging process, however, has severe limitations given the demonstrated inverse relationship between growth and ß-carotene accumulation, and consequently, both biomass and secondary metabolite yields are well below the genetic potential of the organism. This is compounded by the high production costs, a falling market price and fierce competition from synthetic ß-carotene.

Hacking (1986) describes the classic technological or product life cycle theory in which a technology or product proceeds from conception to maturity and then declines. During the maturation phase of a technological life cycle, commercial survival may depend on a further phase of innovation and development. The currently employed ß-carotene production technology appears to have reached the level of maturity described by Hacking (1986) and further innovation and development may be required to sustain the concept of commercial algal ß-carotene production. The research hypothesis advanced in this thesis proposed to separate the distinctive growth and secondary metabolite accumulation stages of *D. salina* in an attempt to increase the overall productivity and hence commercial viability of the system.

The laboratory and outdoor scale-up studies largely confirmed that manipulation and control of ß-carotene production using physiological and environmental stress factors is possible. The laboratory studies confirmed the major role of high light intensity in ß-carotene hyper-accumulation in *D. salina*. However, it was demonstrated that higher ß-carotene yields were obtainable when cumulative stress factors were operating in conjunction with high light stress. Maximum ß-carotene yields of around 22-23 $mg.l^{-1}$ were achievable within 5 days within the stress stage. The photoperiod study established that less than a third of the light used in continuous illumination is necessary for inducing these high ß-carotene yields. This finding has important implications for stress pond design and the location of mass culture systems in isolated semi-desert or desert regions (Ben-Amotz and Avron, 1989). However, the effect of photoperiod on biomass productivity should also be considered.

The examination of the physiological response of *D. salina* to high light stress confirmed the ß-carotene photoprotective screening hypothesis advanced by Ben-Amotz and Avron (1983). The hyper-accumulation and the peripheral localisation of ß-carotene interthylakoidal globules was shown to correlate with the recovery of cell growth and photosynthesis, following the photoinhibitory effects of high light intensity. A possible correlation between the initial photoinhibitory response to cumulative stress and the extent of ß-carotene hyper-accumulation was also noted.

The outdoor growth pond study demonstrated that *D. salina* could be cultured in a relatively low salinity, nutrient sufficient medium for extended periods without overgrowth by small non-carotenogenic *Dunaliella* species. Furthermore, biomass productivities of approximately three times greater than those obtained in the averaging process were obtained.

The role of high light intensity in ß-carotene hyper-accumulation was exploited in the outdoor stress pond study by the manipulation of culture density. Greater ß-carotene accumulation was obtained at low starting culture densities, however, the inverse relationship between growth and ß-carotene accumulation which persisted throughout the laboratory and the outdoor study resulted in similar ß-carotene yields irrespective of the starting culture density. The major implication of this is that more ß-carotene per unit biomass inoculated into the stress ponds was produced at low starting culture densities. The demonstrated inverse relationship between growth and ß-carotene accumulation also indicates that a maximum ß-carotene yield of 22-23 $mg.l^{-1}$ exists for the stress system. The relative ß-carotene content of the biomass inoculated into the stress ponds was up to three times greater than the ß-carotene content of biomass produced in the averaging process.

An essential feature of the dual-stage system is the separation of biomass from the growth ponds in a viable form for transfer into the stress pond stage. Of the separation techniques evaluated, flocculation-flotation with the K300 flocculation and air-displacement CFUF were demonstrated to fulfil the requirement of maintaining cell viability. A scale-up evaluation of the air-displacement CFUF is however, necessary to determine whether cell shear caused by increased flow rates and parallel membrane configuration significantly contribute to cell damage in scale-up systems.

The high extraction pressures associated with supercritical carbon dioxide extraction of ß-carotene from biological material were addressed. A co-solvent mixture of carbon dioxide and propane was shown to reduce the extraction pressure by approximately 50 % and to increase both the extraction efficiency and the solvent loading of the co-solvent mixture. Further research should be focused at optimizing the solvent ratio and extraction pressure.

The separation of oil water emulsions has traditionally been accomplished with centrifugation or by the use of hydrophillic membranes. The unsuitability of hydrophillic membrane separation and the high costs associated with centrifugation in down stream processing (Hacking, 1986) lead to the development of the novel hydrophobic membrane assisted hot oil extraction process. The practical demonstration of the process as an effective technique for the separation of oil water emulsions is likely to contribute to more cost effective ß-carotene extraction.

In summary, the three-fold increase in biomass productivity and two to three-fold increase in the ß-carotene content of the biomass indicated a potential overall ß-carotene yield advantage of six to ninefold greater than that obtained in the averaging process. However, factors such as the additional pond area required for the stress pond stage of the system and the retention time of the biomass in the stress ponds significantly alter the practical economic advantages of the process. An IRR of 36.35 % was obtained for the dual-stage system, compared to an IRR of 20.9 % for the averaging process. The significance of this is that the dual-stage system has the potential to compete with synthetic ß-carotene at an equivalent sales price, whereas the averaging process must rely on the inflated natural ß-carotene price.

Although the concept of dual-stage processing is not novel in fermentation processes, the integration of environmental and physiological stress factors as manipulative tools in secondary metabolite production and the practical demonstration and subsequent economic evaluation of the dual-stage process indicates sufficient potential to warrant industrial pilot scale studies for the process. Dual-stage processing may have applications in other algal secondary metabolite production processes such as the production of astaxanthin from *Haematococcus* (Bubrick, 1991) and as such, presents a production method offering greater control in manipulating algal mass cultures in general.

POSTSCRIPT

The following is a list of publications and reports emanating from the research presented in this study:

1. Phillips, T.D., Rose, P.D. (1991). Separation of *Dunaliella salina.* Sasol Symposium, RAU.

2. Phillips, T.D., Rose, P.D. (1992). Combined stress induction of ß-carotene accumulation in *Dunaliella salina.* Proc Seventh Congress SA Soc. Microbiol., Bloemfontein.

3. Rose, P.D., Phillips, T.D., Sanderson, R. (1992). A process for membrane based ß-carotene extraction. RSA Patent Application 93/0953.

4. Rose, P.D., Maart, B.A., Phillips, T.D., Tucker, S.L., Cowan, A.K., and Rowswell, R.A. (1992). Cross-flow ultrafiltation used in algal high rate oxidation pond treatment of saline organic effluents with the recovery of products of value. *Wat. Sci. Tech.* **25(10):** 319-327.

As a result of the favourable economic evaluation of the dual-stage process described in this thesis, Sasol Pty. has commissioned the construction of an industrial-scale pilot plant. The industrial-scale evaluation of the dual-stage process will commence during 1994.

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APPENDIX

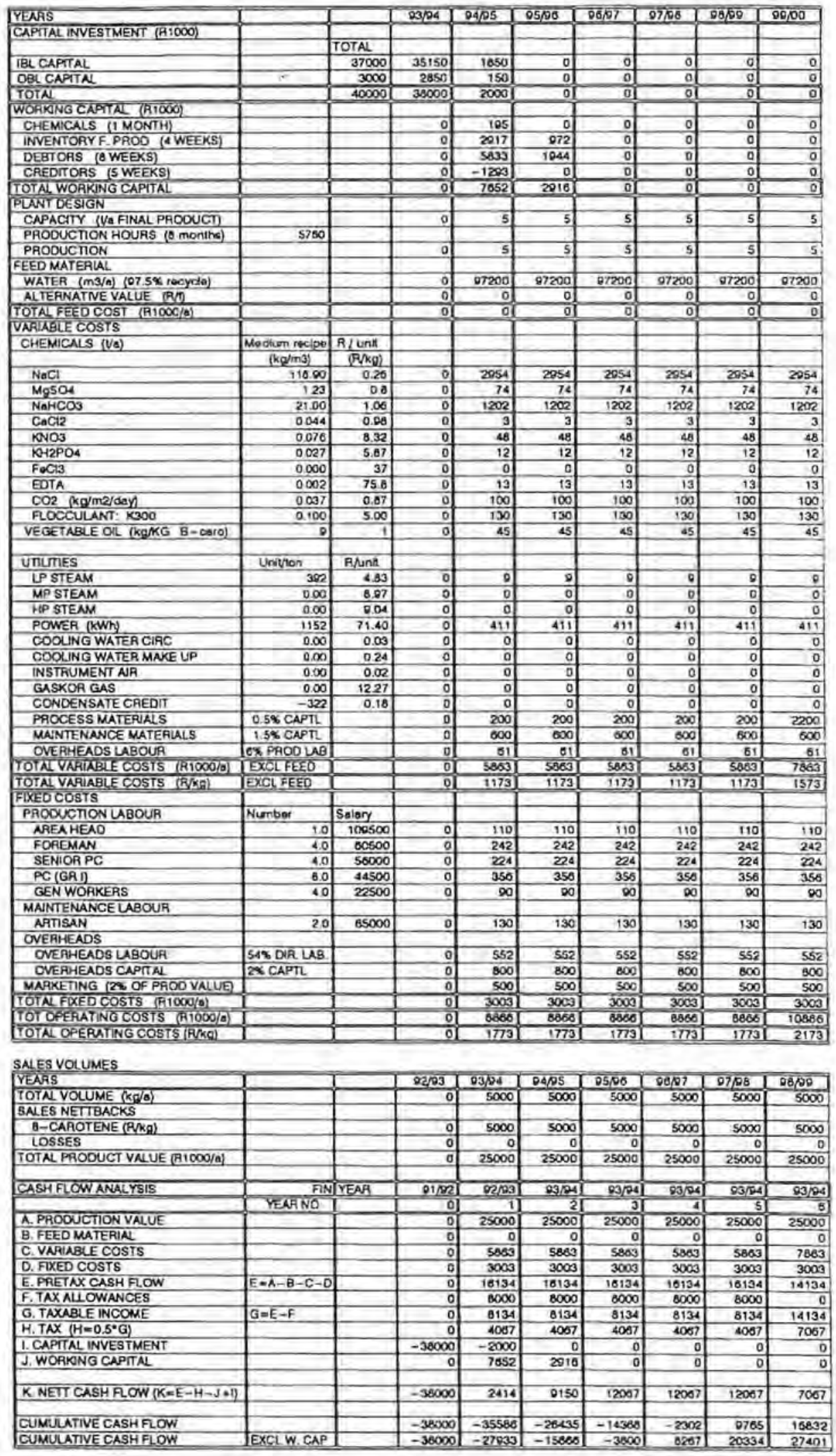
Table A1 Capital estimate for a single-stage ß-carotene production process (Sastech Engineering Services).

DESCRIPTION	AMOUNT (R)
Ponding (83200 m^2)	8 420 000
16 Paddle Mixers	860 800
1 Mixing Tank (3x4 m)	45 000
1 Blade mixer	80 000
4 Large Monopumps	98 000
2 Small Monopumps	24 600
1 Harvesting Unit	180 000
1 Extraction Unit	400 000
1 Air Compressor	9 000
TOTAL MATERIAL COST	10 117 800
DIRECT FIELD COST	15 330 000
PROJECT COST	31 794 420
CONTINGENCY	5 205 580
TOTAL PROJECT COST	**37 000 000**

Table A2 Capital estimate for dual-stage ß-carotene production process (Sastech Engineering Services).

DESCRIPTION	AMOUNT (R)
Ponding (50000 m^2)	5 173 440
12 Paddle Mixers	645 600
2 Mixing Tank (2x2 m)	30 000
2 Blade mixer	80 000
4 Large Monopumps	98 000
2 Small Monopumps	24 600
2 Harvesting Unit	160 000
1 Extraction Unit	250 000
1 Air Compressor	9 000
TOTAL MATERIAL COST	6 471 040
DIRECT FIELD COST	9 804 606
PROJECT COST	20 334 753
CONTINGENCY	4 165 247
TOTAL PROJECT COST	**24 500 000**

Figure A3 Discounted cash flow spreadsheet for the single-stage process.



YEARS			93/94	94/95	95/96	96/97	97/98	98/99	99/00
CAPITAL INVESTMENT (R1000)									
		TOTAL							
IBL CAPITAL		37000	35150	1850	0	0	0	0	0
OBL CAPITAL		3000	2850	150	0	0	0	0	0
TOTAL		40000	38000	2000	0	0	0	0	0
WORKING CAPITAL (R1000)									
CHEMICALS (1 MONTH)			0	195	0	0	0	0	0
INVENTORY F. PROD (4 WEEKS)			0	2917	972	0	0	0	0
DEBTORS (8 WEEKS)			0	5833	1944	0	0	0	0
CREDITORS (5 WEEKS)			0	−1293	0	0	0	0	0
TOTAL WORKING CAPITAL			0	7652	2916	0	0	0	0
PLANT DESIGN									
CAPACITY (t/a FINAL PRODUCT)			0	5	5	5	5	5	5
PRODUCTION HOURS (8 months)	5760								
PRODUCTION			0	5	5	5	5	5	5
FEED MATERIAL									
WATER (m3/a) (97.5% recycle)			0	97200	97200	97200	97200	97200	97200
ALTERNATIVE VALUE (R/t)			0	0	0	0	0	0	0
TOTAL FEED COST (R1000/a)			0	0	0	0	0	0	0
VARIABLE COSTS									
CHEMICALS (t/a)	Medium recipe (kg/m3)	R / unit (R/kg)							
NaCl	118.90	0.26	0	2954	2954	2954	2954	2954	2954
MgSO4	1.23	0.8	0	74	74	74	74	74	74
NaHCO3	21.00	1.06	0	1202	1202	1202	1202	1202	1202
CaCl2	0.044	0.98	0	3	3	3	3	3	3
KNO3	0.076	8.32	0	48	48	48	48	48	48
KH2PO4	0.027	5.87	0	12	12	12	12	12	12
FeCl3	0.000	37	0	0	0	0	0	0	0
EDTA	0.002	75.8	0	13	13	13	13	13	13
CO2 (kg/m2/day)	0.037	0.87	0	100	100	100	100	100	100
FLOCCULANT: K300	0.100	5.00	0	130	130	130	130	130	130
VEGETABLE OIL (kg/KG B−caro)	9	1	0	45	45	45	45	45	45
UTILITIES	Unit/ton	R/unit							
LP STEAM	392	4.83	0	9	9	9	9	9	9
MP STEAM	0.00	8.97	0	0	0	0	0	0	0
HP STEAM	0.00	9.04	0	0	0	0	0	0	0
POWER (kWh)	1152	71.40	0	411	411	411	411	411	411
COOLING WATER CIRC	0.00	0.03	0	0	0	0	0	0	0
COOLING WATER MAKE UP	0.00	0.24	0	0	0	0	0	0	0
INSTRUMENT AIR	0.00	0.02	0	0	0	0	0	0	0
GASKOR GAS	0.00	12.27	0	0	0	0	0	0	0
CONDENSATE CREDIT	−322	0.18	0	0	0	0	0	0	0
PROCESS MATERIALS	0.5% CAPTL		0	200	200	200	200	200	2200
MAINTENANCE MATERIALS	1.5% CAPTL		0	600	600	600	600	600	600
OVERHEADS LABOUR	6% PROD LAB		0	61	61	61	61	61	61
TOTAL VARIABLE COSTS (R1000/a)	EXCL FEED		0	5863	5863	5863	5863	5863	7863
TOTAL VARIABLE COSTS (R/kg)	EXCL FEED		0	1173	1173	1173	1173	1173	1573
FIXED COSTS									
PRODUCTION LABOUR	Number	Salary							
AREA HEAD	1.0	109500	0	110	110	110	110	110	110
FOREMAN	4.0	60500	0	242	242	242	242	242	242
SENIOR PC	4.0	56000	0	224	224	224	224	224	224
PC (GR I)	8.0	44500	0	356	356	356	356	356	356
GEN WORKERS	4.0	22500	0	90	90	90	90	90	90
MAINTENANCE LABOUR									
ARTISAN	2.0	65000	0	130	130	130	130	130	130
OVERHEADS									
OVERHEADS LABOUR	54% DIR. LAB.		0	552	552	552	552	552	552
OVERHEADS CAPITAL	2% CAPTL		0	800	800	800	800	800	800
MARKETING (2% OF PROD VALUE)			0	500	500	500	500	500	500
TOTAL FIXED COSTS (R1000/a)			0	3003	3003	3003	3003	3003	3003
TOT OPERATING COSTS (R1000/a)			0	8866	8866	8866	8866	8866	10866
TOTAL OPERATING COSTS (R/kg)			0	1773	1773	1773	1773	1773	2173

SALES VOLUMES

YEARS			92/93	93/94	94/95	95/96	96/97	97/98	98/99
TOTAL VOLUME (kg/a)			0	5000	5000	5000	5000	5000	5000
SALES NETTBACKS									
B−CAROTENE (R/kg)			0	5000	5000	5000	5000	5000	5000
LOSSES			0	0	0	0	0	0	0
TOTAL PRODUCT VALUE (R1000/a)			0	25000	25000	25000	25000	25000	25000

CASH FLOW ANALYSIS	FIN	YEAR	91/92	92/93	93/94	93/94	93/94	93/94	93/94
	YEAR NO		0	1	2	3	4	5	6
A. PRODUCTION VALUE			0	25000	25000	25000	25000	25000	25000
B. FEED MATERIAL			0	0	0	0	0	0	0
C. VARIABLE COSTS			0	5863	5863	5863	5863	5863	7863
D. FIXED COSTS			0	3003	3003	3003	3003	3003	3003
E. PRETAX CASH FLOW	E=A−B−C−D		0	16134	16134	16134	16134	16134	14134
F. TAX ALLOWANCES			0	8000	8000	8000	8000	8000	0
G. TAXABLE INCOME	G=E−F		0	8134	8134	8134	8134	8134	14134
H. TAX (H=0.5*G)			0	4067	4067	4067	4067	4067	7067
I. CAPITAL INVESTMENT			−38000	−2000	0	0	0	0	0
J. WORKING CAPITAL			0	7652	2916	0	0	0	0
K. NETT CASH FLOW (K=E−H−J+I)			−38000	2414	9150	12067	12067	12067	7067
CUMULATIVE CASH FLOW			−38000	−35586	−26435	−14368	−2302	9765	16832
CUMULATIVE CASH FLOW	EXCL W. CAP		−38000	−27933	−15866	−3800	8267	20334	27401

ECONOMIC CRITERIA	
ROI (before tax)	40
IRR (%)	20.9
NPV @ 15% (R1000)	10511

Figure A4 Discounted cash flow spread sheet for the dual-stage process.

YEARS			93/94	94/95	95/96	96/97	97/98	98/99	99/00
CAPITAL INVESTMENT (R1000)									
		TOTAL							
IBL CAPITAL		24500	23275	1225	0	0	0	0	0
OBL CAPITAL		3000	2850	150	0	0	0	0	0
TOTAL		27500	26125	1375	0	0	0	0	0
WORKING CAPITAL (R1000)									
CHEMICALS (1 MONTH)			0	195	0	0	0	0	0
INVENTORY F. PROD (4 WEEKS)			0	2917	972	0	0	0	0
DEBTORS (8 WEEKS)			0	5833	1944	0	0	0	0
CREDITORS (5 WEEKS)			0	−538	0	0	0	0	0
TOTAL WORKING CAPITAL			0	8407	2916	0	0	0	0
PLANT DESIGN									
CAPACITY (t/a FINAL PRODUCT)			0	5	5	5	5	5	5
PRODUCTION HOURS (8 months)	5760								
PRODUCTION			0	5	5	5	5	5	5
FEED MATERIAL									
WATER (m3/a) (97.5% recycle)			0	6480	6480	6480	6480	6480	6480
ALTERNATIVE VALUE (R/t)			0	0	0	0	0	0	0
TOTAL FEED COST (R1000/a)			0	0	0	0	0	0	0
VARIABLE COSTS									
CHEMICALS (t/a)	Medium recipe (kg/m3)	R / unit (R/kg)							
NaCl	116.90	0.26	0	197	197	197	197	197	197
MgSO4	1.23	0.8	0	45	45	45	45	45	45
NaHCO3	21.00	1.06	0	529	529	529	529	529	529
CaCl2	0.044	0.96	0	2	2	2	2	2	2
KNO3	0.076	8.32	0	29	29	29	29	29	29
KH2PO4	0.027	5.87	0	7	7	7	7	7	7
FeCl3	0.000	37	0	0	0	0	0	0	0
EDTA	0.002	75.8	0	8	8	8	8	8	8
CO2 (kg/m2/day)	0.037	0.87	0	11	11	11	11	11	11
FLOCCULANT: K300	0.100	5.00	0	130	130	130	130	130	130
VEGETABLE OIL (kg/KG B−caro)	9	1	0	45	45	45	45	45	45
UTILITIES	Unit/ton	R/unit							
LP STEAM	392	4.83	0	9	9	9	9	9	9
MP STEAM	0.00	8.97	0	0	0	0	0	0	0
HP STEAM	0.00	9.04	0	0	0	0	0	0	0
POWER (kWh)	1152	71.40	0	411	411	411	411	411	411
COOLING WATER CIRC	0.00	0.03	0	0	0	0	0	0	0
COOLING WATER MAKE UP	0.00	0.24	0	0	0	0	0	0	0
INSTRUMENT AIR	0.00	0.02	0	0	0	0	0	0	0
GASKOR GAS	0.00	12.27	0	0	0	0	0	0	0
CONDENSATE CREDIT	−322	0.18	0	0	0	0	0	0	0
PROCESS MATERIALS	0.5% CAPTL		0	138	138	138	138	138	2138
MAINTENANCE MATERIALS	1.5% CAPTL		0	413	413	413	413	413	413
OVERHEADS LABOUR	6% PROD LAB		0	20	20	20	20	20	20
TOTAL VARIABLE COSTS (R1000/a)	EXCL FEED		0	1993	1993	1993	1993	1993	3993
TOTAL VARIABLE COSTS (R/kg)	EXCL FEED		0	399	399	399	399	399	799
FIXED COSTS									
PRODUCTION LABOUR	Number	Salary							
AREA HEAD	1.0	109500	0	110	110	110	110	110	110
FOREMAN	1.0	60500	0	61	61	61	61	61	61
SENIOR PC	1.0	56000	0	56	56	56	56	56	56
PC (GR I)	2.0	44500	0	89	89	89	89	89	89
GEN WORKERS	1.0	22500	0	23	23	23	23	23	23
MAINTENANCE LABOUR									
ARTISAN	2.0	65000	0	130	130	130	130	130	100
OVERHEADS									
OVERHEADS LABOUR	54% DIR. LAB.		0	182	182	182	182	182	182
OVERHEADS CAPITAL	2% CAPTL		0	550	550	550	550	550	550
MARKETING (2% OF PROD VALUE)			0	500	500	500	500	500	500
TOTAL FIXED COSTS (R1000/a)			0	1700	1700	1700	1700	1700	1700
TOT OPERATING COSTS (R1000/a)			0	3692	3692	3692	3692	3692	5692
TOTAL OPERATING COSTS (R/kg)			0	738	738	738	738	738	1138

SALES VOLUMES

YEARS			92/93	93/94	94/95	95/96	96/97	97/98	98/99
TOTAL VOLUME (kg/a)			0	5000	5000	5000	5000	5000	5000
SALES NETTBACKS									
B−CAROTENE (R/kg)			0	5000	5000	5000	5000	5000	5000
LOSSES			0	0	0	0	0	0	0
TOTAL PRODUCT VALUE (R1000/a)			0	25000	25000	25000	25000	25000	25000
CASH FLOW ANALYSIS	FIN	YEAR	91/92	92/93	93/94	93/94	93/94	93/94	93/94
	YEAR NO		0	1	2	3	4	5	6
A. PRODUCTION VALUE			0	25000	25000	25000	25000	25000	25000
B. FEED MATERIAL			0	0	0	0	0	0	0
C. VARIABLE COSTS			0	1993	1993	1993	1993	1993	3993
D. FIXED COSTS			0	1700	1700	1700	1700	1700	1700
E. PRETAX CASH FLOW	E=A−B−C−D		0	21308	21308	21308	21308	21308	19308
F. TAX ALLOWANCES			0	5500	5500	5500	5500	5500	0
G. TAXABLE INCOME	G=E−F		0	15808	15808	15808	15808	15808	19308
H. TAX (H=0.5*G)			0	7904	7904	7904	7904	7904	9654
I. CAPITAL INVESTMENT			−26125	−1375	0	0	0	0	0
J. WORKING CAPITAL			0	8407	2916	0	0	0	0
K. NETT CASH FLOW (K=E−H−J+I)			−26125	3622	10487	13404	13404	13404	9654
CUMULATIVE CASH FLOW			−26125	−22503	−12016	1388	14792	28196	37850
CUMULATIVE CASH FLOW	EXCL W. CAP		−26125	−14096	−692	12711	26115	39519	49173

ECONOMIC CRITERIA	
ROI (before tax)	77
IRR (%)	36.35
NPV @ 15% (R1000)	30490