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THE CULTURE OF *DUNALIELLA SALINA* AND THE PRODUCTION OF ß-CAROTENE IN TANNERY EFFLUENTS

THESIS

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ABBREVIATIONS AND SYMBOLS

A	= area
AA	= acetic acid
ANOVA	= analysis of variance
BA	= butyric acid
C	= Celsius
CA	= caproic acid
COD	= chemical oxygen demand
cm	= centimetre
EDTA	= ethylene diamine tetraacetic acid
F*	= sample value of F test statistic
fig	= figure
g	= gram
ha	= hectare
i.d.	= internal diameter
l	= litre
LIRI	= Leather Industries Research Institute
M	= molar
MM	= molecular mass
m	= metre
mg	= milligram
min	= minute
ml	= millilitre
mm	= millimetre
N	= normal
N^+	= nitrogen-containing
N^-	= nitrogen-deficient
P	= probability
PA	= propionic acid
pg	= picogram (10^{-12} g)
pH	= negative logarithm of the hydrogen ion concentration
PV	= permanganate value
Rf	= sample distance : solvent distance
rpm	= revolutions per minute
s	= second
SG	= specific gravity
SS	= suspended solids

t*	= sample value of *t* test statistic
TDIS	= total dissolved inorganic solids
TDS	= total dissolved solids
V	= volume
VA	= valeric acid
var.	= variety
VFA	= volatile fatty acid
ß	= beta
$	= dollar
10E	= exponential
%	= percent
μmol	= micromole
μg	= microgram
μm	= micrometre

ABSTRACT

The problems of waste disposal in the tanning industry are unique in that the effluents are highly saline, have a high organic loading and contain heavy metals. Methods are available for the safe treatment and disposal of the latter two components, but the saline component requires the expensive outlay of evaporation ponds. This study has identified a possible use for the saline effluents, turning a problematic waste product into a potentially valuable by-product.

A range of tannery effluents were identified and tested for their suitability for the mass cultivation of *Dunaliella salina* (bardawil strain). The bardawil strain was preferred over a local isolate because of its higher production of ß-carotene. Ponded tannery effluents and combined processes effluent proved unsuitable for realistic propagation of the alga. Anaerobic digestion of combined processes effluent did not improve its suitability significantly. Anaerobic digestion of hide-soak effluent may remove persistent antimicrobial agents which influence algal growth, but its contribution to enhancing algal growth is equivocal. Undigested hide-soak effluent lacking in persistent antimicrobial agents was found to be an ideal culture medium, as no additional nutrients needed to be added. Significantly higher biomass was obtained in this effluent compared to chemically defined media.

Induction of ß-carotene was achieved in nitrogen-deficient defined media after culture in tannery effluent. This suggests that a two-stage system using hide-soak effluent for cell propagation and nitrogen deficient media for ß-carotene induction, could be possible for the mass cultivation of *D. salina* for ß-carotene production.

CHAPTER 1

INTRODUCTION

Dunaliella salina var. bardawil (Teodoresco), is a unicellular, biflagellate green alga (Chlorophyta, Chlorophyceae). Its unique features are its lack of a cell wall (Gibbs and Duffus, 1976) and its extreme halotolerance (Ginzburg and Ginzburg, 1985). It is also able to tolerate a wide pH range (Borowitzka and Borowitzka, 1988). The bardawil strain was described by Ben-Amotz and Avron (1980) and isolated from saline pools in the Sinai Peninsula.

The alga yields a number of useful by-products which makes it ideal for mass cultivation (Ben-Amotz and Avron, 1983a). These products are glycerol (Beckett *et al*, 1985), ß-carotene (Borowitzka *et al*, 1984) and protein (Fabregas and Herrero, 1985). The bioconversion of *Dunaliella* into neutral solvents has also been proposed (Nakas *et al*, 1983). *Dunaliella* is mass-cultured extensively in some parts of the world, namely Australia (Borowitzka *et al*, 1984) and Israel (Weiner, 1985). Most of these enterprises utilise lagoons or large ponds supplied by saline aquifers. One of the main drawbacks of large-scale aquaculture of *Dunaliella* is the initial cost outlay of the ponds and associated infrastructure. The cost of nutrient medium and CO_2 supply can also contribute to a large portion of the plant's running costs (Chen and Chi, 1981).

The culture of *Dunaliella* on saline tannery effluents could provide a saving in the cost of medium. The leather tanning industry in South Africa generates large quantities of saline effluent which contains high concentrations of organic and inorganic wastes from the hide treatment process. Furthermore, should this effluent prove itself satisfactory for *Dunaliella* culture, a further saving could be made if *in situ* culture in pre-existing effluent ponds were possible. Tanneries have large outdoor ponds which are used as holding dams to facilitate the evaporation of the liquid component of the effluent and the concentration of salts. These are facultative ponds which allow for the microbial degradation of organic matter into nitrates and phosphates, which in turn are converted into useful biomass by the algae. The algae also serve an important function in the production of oxygen required for the bacterial degradation of organic waste (Oswald, 1988). The first attempt at the culture of *Dunaliella* on tannery effluent has been reported recently (Laubscher *et al*, 1990; Rose *et al*, 1991).

The tanning industry generates diverse effluents from the various activities of the tanning process, from the processing of wet, salted hides to the final finished leather product. Each of these effluents are also a possible source of culture medium. A brief outline of the tanning process is as follows (Rowswell *et al*, 1984):

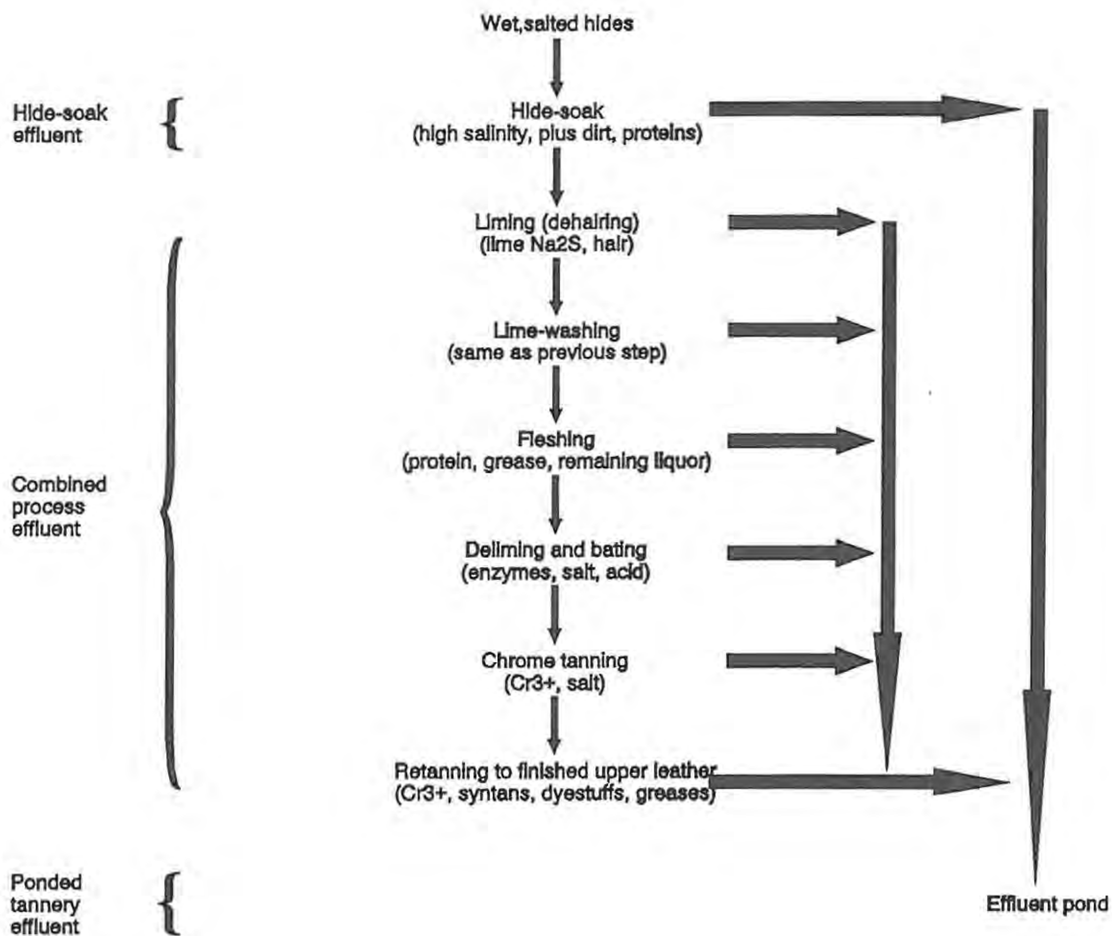

Fig. 1.1 Flow diagram of the leather tanning process.

The hide-soak effluent generated from soaking salt-preserved hides raises the salinity of the final effluent. Antimicrobial agents, such as naphthalene and antibiotics used in hide preservation, can also occur in the effluent. Effluents generated in the leather tanning processes contain organic pollutants such as protein and grease, and inorganic pollutants such as sulphides, salts and chromium ions.

The ponded tannery effluent is probably the most variable of all, as this has been subjected to microbial degradation and exposure to the elements. Decomposition of nitrogenous organic matter (protein from skin

and hair) releases ammonia into solution (Jackson-Moss, 1991). Aerobic bacteria in the ponds oxidise ammonia to nitrite and subsequently nitrate (Keeny *et al*, 1971), which is utilized by algae. However, under anaerobic conditions nitrate is reduced to nitrogen gas and released into the atmosphere (Oswald, 1988). Sulphide is introduced into the effluent from the liming liquor and through the biological reduction of sulphate by *Desulfovibrio* (McKinney, 1962) under anaerobic conditions. Effluents can be aerated to convert sulphide to sulphate, but should there be a reversion to anaerobic conditions, the reverse process occurs. Further reduction of sulphide to gaseous hydrogen sulphide also occurs in anaerobic conditions. The pH of the pond effluent ranges between 8.0 and 8.6. The ponds contain substantial numbers of bacteria, fungi, algae and protozoa, of which bacteria are by far the most important. The ponds are arranged in a cascade and increase in salinity the further away they are situated from the effluent inlet. Salinity variations are a function of the prevailing climatic conditions (sunshine, humidity, wind and rain) as well as the rate of effluent discharge from the tannery and the retention time of the preceding pond in the series. An example of a leather tannery in the Eastern Cape consists of four operational ponds connected in series, having a surface area of 1.4, 1.6, 1.3 and 2.2ha, a total of 6.5 ha (Rowswell *et al*, 1984). Their average depth is 1.58m which indicates that they are facultative ponds. The effluent is pumped into the first pond and any overflow is passed on to the next pond in the series.

An example of a mass algal culture system is a model proposed by Chen and Chi (1981) for *Dunaliella* cultivation in 133ha and 400ha ponds. The cost of the capital outlay was budgeted at $53 million. The actual land and infrastructure accounted for some 8% of the total capital investment, while the water and CO_2 feed system accounted for 72%. The remaining amount was budgeted for harvesting and processing the algae for glycerol. Benemann *et al* (1987) investigated the cost outlay for a 10 ha and a 100 ha pond system, for which capital costs were estimated at $3.3 million and $7.5 million respectively. Forty three percent of the final cost is attributed to ponds and infrastructure in the 10 ha system, and 31% in a 100 ha system. Media supply accounts for 9% and 10 % respectively. Tapie and Bernard (1988) have recently reviewed a number of proposed large-scale microalgae production systems. By far the greatest cost is attributed to pond and infrastructure construction, CO_2 supply and nutrient addition. The remaining cost covers the harvesting processes.

Should the medium prove suitable, the use of tannery ponds for *in situ Dunaliella* culture could mean a saving of up to 80% of the above costs. Using tannery effluents and the pre-existing pond infrastructure could considerably reduce outlay costs. CO_2 would be provided by the bacterial degradation of organic matter. The alkaline pH of the ponded effluent (>8.0) also provides increased CO_2 solubility. Thus the high cost of CO_2 supply can be avoided. Improved harvesting technologies will also reduce the overall cost of the operation.

RESEARCH OBJECTIVES

This project sets out to answer certain basic questions pertaining to the hypothesis that tannery effluents and the ponding treatment infrastructure could serve as a suitable environment for the cultivation of *D. salina*. These are:

1) Which is the most suitable effluent of the various wastes generated by the tannery for the culture of *D. salina*?

2) What are the optimum concentrations of these effluents that will give the highest yields of *D. salina*?

3) How do these yields compare with those in conventional defined media such as Provasoli's and M11-enriched sea-water medium?

4) Is the bardawil strain more suitable for cultivation on tannery effluents than the local strain of *D. salina*?

5) Is ß-carotene yield affected by growth in tannery effluent and do yields differ in different effluent media?

6) Does exposure to medium deficient in nitrogen induce ß-carotene production after cells have been grown in tannery effluents?

7) Can the separation of the cultivation process into two stages, namely growth in effluent and then transfer to medium deficient in nitrogen, improve the overall ß-carotene production rate?

8) Is pre-treatment of the effluent necessary for the successful culture of *D. salina*?

CHAPTER 2

MATERIALS AND METHODS

1. **Algal strains.**

The initial experiment (Experiment 1) was carried out using a local isolate of *Dunaliella salina*. This strain was isolated from the Couga Salt Pans outside Port Elizabeth in 1987 and identified by Dr Mark Aken of the Department of Plant Sciences, Rhodes University. This isolate had previously been shown to be a poor producer of ß-carotene (Laubscher, 1987) and subsequently a second strain was obtained from the Culture Collection of Algae and Protozoa in the United Kingdom. This strain, *Dunaliella salina* (var. bardawil Teod.) CCAP 19/30, is referred to as *Dunaliella bardawil* by some authors. It is reported as being a halophilic and highly carotenogenic strain (Avron and Ben-Amotz, 1980). This alga was used from Experiment 2 onwards.

2. **Culture media.**

2.1. Preparation of sea-water.

Sea-water was obtained from near the mouth of the Kowie River, Port Alfred, at high tide. The sea-water was stored in 25l polyethylene drums and aged for approximately two weeks. Prior to use, it was mixed with activated charcoal (Merck) at 2g/l to adsorb organic compounds, and stood overnight with occasional agitation. Following this treatment, the sea-water was filtered twice through Whatmans GF/A filter paper and then diluted 1:10 with de-ionised water prior to autoclaving. This step was necessary to prevent precipitation of salts during autoclaving. The filtrate was then autoclaved at 121°C and 15 bar for 20 minutes. All experiments were conducted in media made up to 1.5M NaCl using commercial grade coarse sea-salt. Molarity was calculated by measuring the refractive index of the medium from each flask using an Otago hand-held salinometer, and then extrapolating the molarity from a standard curve of refractivity vs NaCl molarity. NaH_2CO_3 was used as a source of CO_2, and 0.125g was added to each flask.

2.2. Enriched sea-water medium.

The local isolate of *D. salina* was cultured in Provasoli's enriched sea-water medium. The bardawil strain however, grew very poorly in Provasoli's medium and was subsequently cultured in M11-enriched sea-water medium (hereafter referred to as M11 medium) which proved to be very suitable. Formulation of both enriched media is given below.

2.2.1. Provasoli's Enriched Sea-water Medium (Provasoli, 1957).

a) Buffered salts solution: 5.61g/l $NaNO_3$, 0.78g/l $Na_2C_3H_5(OH)PO_4.5H_2O$, 0.26g/l Fe EDTA and 7.99g/l Tris buffer.

b) Trace element solution: 230mg/l $ZnSO_4.7H_2O$, 163 mg/l $MnSO_4.4H_2O$, 5.0 mg/l $CoSO_4.7H_2O$, 1.14g/l H_3BO_3, 1.0 g/l Na_2 EDTA and 60mg/l Fe citrate.

c) Vitamin solution: 1.6mg/l cyanocobalamin, 0.8mg/l biotin and 20mg/l thiamine·HCl.

d) Working solution: 10ml buffered salt solution, 10ml trace element solution and 1ml vitamin solution added to 979ml treated sea-water. The pH is adjusted to 7.5 with either 1M HCl or NaOH, and sterilised by filtering through a 0.45μm Nucleopore membrane filter.

2.2.2. M11 Enriched Sea-water Medium (CCAP data sheet).

a) Extra salts stock solution: 30.0 g/l $NaNO_3$, 1.2 g/l Na_2HPO_4 and 1.0 g/l K_2HPO_4. The solution is sterilized by autoclaving at 121°C and 15 bar for 20 min.

b) Vitamin stock solution: 0.2 mg/l biotin, 20.0 mg/l calcium pantothenate, 4.0 mg/l cyanocobalamin, 0.4 mg/l folic acid, 1000.0 mg/l inositol, 20.0 mg/l nicotinic acid, 100.0 mg/l thiamine, and 600.0 mg/l thymine. The solution is sterilized by filtering through a 0.45μm Nucleopore membrane filter.

c) Soil extract: air dried soil and twice its volume of distilled water were autoclaved together. The autoclaved soil suspension is then filtered through Whatmans No. 1 filter paper and the filtrate is sterilized by autoclaving.

d) Working solution: 3.75 ml/l extra salt solution, 2.5ml/l vitamin stock solution, 50 ml soil extract and 0.5 g Tris to 1000 ml of aged filtered sea-water and adjusted to pH 7.6-7.8 with 1N NaOH or HCl.

2.3. Preparation of tannery effluents for growth experiments.

Four different types of tannery effluent were tested: ponded tannery effluent, combined processes effluent, hide-soak effluent and anaerobically digested hide-soak effluent. These were diluted with the appropriate amounts of sea-water to make the final volume up to 250ml. The various dilutions of tannery effluent and the controls were prepared in 500ml Pyrex conical flasks stoppered with cotton wool and capped with tin foil for autoclaving. They were autoclaved for 20 minutes at 121°C and 15 bar. When the flasks had cooled, they were enriched with a working solution of Provasoli's or M11 medium. All of the tannery effluents were initially enriched with one of the two enriched sea-water media. This was because the early experiments were concerned primarily with investigating the toxicity of tannery effluents rather than their nutritional value. In every experiment a control was prepared containing effluent-free sea-water enriched with a working solution of Provasoli's or M11 media. The exact details of dilutions and types of tannery effluent used will be clarified in Chapters 3 to 7.

3. **Environmental conditions.**

The flasks were placed on a light table in a controlled environment room. The cultures were grown in a 16 hour light and 8 hour dark cycle at an average of 21°C (maximum fluctuations were 4°C above and below the average). The light intensity of the light table was measured at 165.4 $\mu mol/m^2/s^1$ at the surface of the table. All treatments were carried out in triplicate.

4. **Inoculum.**

The algal inoculum was grown up in enriched sea-water medium prior to the start of each experiment. A 10% inoculum of *Dunaliella* in logarithmic growth was added to each flask.

5. **Quantification of algal biomass.**

Cell growth was monitored by direct cell counts using an improved Neubauer haemocytometer. The mean cell count and standard deviation of the three replicates were recorded. Cell counts were carried out on a daily basis with a few exceptions. Dry weights were of no use where effluents were used, as suspended solids present in these effluents contributed to erroneous readings.

6. **The assay of ß-carotene.**

6.1. Concentration of cells.

a) Centrifugation method.

This method was used where the number of samples to be removed for ß-carotene analysis was small. Ten millilitres of an agitated culture were removed, centrifuged and pelleted at 6000rpm for 10 min at 5°C in a Sorval refrigerated centrifuge. The supernatant was discarded and the pellets refrigerated in the centrifuge tubes at -20°C until they could be chromatographed. The centrifuged pellets were dried down under a stream of air to remove as much of the interstitial water as possible to facilitate loading onto chromatography paper. Nitrogen drying was precluded by cost. The pellet was resuspended in 5ml of methanol to dissolve the cell membrane and to release ß-carotene. All steps were carried out under diffuse light to prevent photo-oxidative degradation of ß-carotene. The methanol was dried down until about 1ml remained and was then loaded onto strips of chromatography paper. A further 1 ml was added to the centrifuge tubes to remove any residual ß-carotene.

b) Filtration method.

This method was preferred for the assay of ß-carotene from a large number of samples. Ten millilitres of culture were removed from each flask and filtered through Whatmans GF/A 2.5cm glass microfilter discs. Initially 2.5cm Millipore nitrocellulose filters were used, but they proved too costly and were slower to filter. The filter discs and filtrate were stored separately in small glass screw-capped bottles at -20°C. About 5ml of methanol or acetone were added to the bottles containing the filters before

freezing, in order to remove the ß-carotene from the cells. The methanol was soon replaced by acetone, which evaporated more rapidly during the application of the eluted ß-carotene to paper chromatography strips. The nitrocellulose filters were soluble in both solvents which was originally thought to be advantageous as there was no retention of ß-carotene on the filter discs. However these filters showed no improvement over the glass microfilter filters in terms of higher ß-carotene extraction. Later, acetone was only added to the bottles immediately before chromatography as it corroded the rubber seals of the bottle caps during storage. The filter extracts were assayed for ß-carotene and the filtrate for nutrient concentrations.

6.2. ß-carotene assay by paper chromatography.

Paper chromatography strips 40mm wide and 460mm long were cut from sheets of Whatmans chromatography paper. The samples were loaded onto the paper with a Pasteur pipette, 500mm from one end. A stream of warm air was blown over the surface of the paper during application of the sample in order to facilitate the evaporation of the solvent. All the manipulations were carried out under low light to lessen the possibility of photo-oxidation of ß-carotene. Descending paper chromatography was carried out in a glass chromatography tank that had been equilibrated for a few hours with the developing solvent (mobile phase) of 10% acetone in petroleum ether. A black cloth was draped over the tank in order to shut out light. After the strips were developed, they were removed and the zone containing the ß-carotene (apparent by its intense orange coloration and its characteristic Rf value determined from a ß-carotene standard) was cut out and eluted in petroleum ether. It was stored at -20°C until the ß-carotene concentration was determined by measuring the absorption at 450nm and reading off a previously prepared standard curve.

This assay was designed to compare ß-carotene production between treatments and not to determine maximum ß-carotene yields. It is assumed that a certain amount of ß-carotene was lost during the assay as not all the precautions aimed at preventing its oxidation were taken. This was justified in terms of time-saving and cost. As the method was consistently applied for each set of experiments it can safely be assumed that differences between ß-carotene concentrations in the samples are due to experimental conditions and not experimental error.

7. **Nutrient analysis.**

Nutrient analyses were carried out to determine the nitrate, nitrite, ammonia and phosphate content of the filtrates from 10 ml samples filtered through Whatmans GF/A 2.5cm glass microfilter filter discs. The filtrates were stored at -20°C until they could be analyzed on a Technicon Autoanalyzer II. The method is described by Mostert (1983).

8. **Nitrogen limitation induction of ß-carotene synthesis.**

ß-carotene production could be induced by allowing the culture to age or by transferring the cells to nitrogen-free culture medium. The latter procedure was carried out by centrifuging cultures at early stationary phase at 6000rpm for 10 minutes in a refrigerated Sorval centrifuge. The cells were resuspended in sterile 1.5M saline and then re-centrifuged as above to remove old medium from the resuspended cells. The cells were then resuspended in nitrogen-free M11 medium.

When the experiment required that the treatments be split into nitrogen-containing (N^+) and nitrogen-free (N^-) M11 medium for purposes of comparison, the culture was divided into two equal volumes. After washing to remove old medium, they were transferred to either N^+ or N^- M11 medium. Samples for measuring ß-carotene production were taken at regular intervals.

9. **Anaerobic and aerobic pre-treatment of tannery effluents.**

Where digestion of hide-soak effluent was appropriate, it was carried out in a 15l New Brunswick Microferm fermenter. The fermenter was covered in tin foil to prevent the growth of purple photosynthetic bacteria. Temperature was maintained at 35°C and the fermenter was agitated by a magnetic stirrer. The volume of tannery effluent added depended on the requirements of the experiment and is discussed under the headings of the experiments. An inoculum of anaerobic bacteria was obtained from an anaerobic digester at the Grahamstown sewage treatment plant. Gas production was monitored by water displacement in a reservoir connected to the headspace of the reactor. Headspace gas samples and effluent samples were removed from the reactor at weekly intervals. The gas samples were analyzed for CO_2 and methane. A decrease in the rate of CO_2 and methane production indicated the near completion of anaerobic digestion. When gas production ceased and the chemical oxgen demand (COD) remained constant the fermenter was aerated for a few days to assist aerobic digestion. Thereafter, the effluent was used in algal growth trials. The effluent was dispensed into various dilutions with sea-water and then autoclaved.

9.1. Gas chromatography.

Gas chromatography of headspace gas and volatile fatty acids were undertaken on a Hewlett-Packard 5830A gas chromatograph (Jackson-Moss, 1991).

9.1.1. Analysis of headspace gas for CO_2 and methane.

Headspace gas was analyzed by injecting a 200µl sample onto a 2m glass column (2mm i.d.) packed with Poropak N (Waters Associates). The flow rate of the carrier gas (helium) was 35ml/min. The temperatures of the injection port, column and detector were 80°C, 60°C and 80°C respectively. The detector used was a thermal conductivity detector. The CO_2 and methane peaks were identified using

a standard of known composition (Supelco). Concentration of the two gases were calculated as relative percentages as follows:

1) $$\% CH_4 = \frac{A\ CH_4}{A\ CH_4 + (0.57 \times A\ CO_2)} \times 100$$

where: $A\ CH_4$ = integrated area of the CH_4 peak in the sample
$A\ CO_2$ = integrated area of the CO_2 peak in the sample

2) $\% CO_2 = 100 - \% CH_4$

It was accepted by Jackson-Moss (1991) that the biogas generated consists almost entirely of CO_2 and CH_4.

9.1.2. Analysis of volatile fatty acids (VFA's) using gas chromatography.

Volatile fatty acids were analyzed by injecting 0.5µl of effluent sample onto a 2m glass column (2mm i.d.) packed with Chromosorb W AW, 100-120 mesh, coated with 10% SP-1000 and 3% H_3PO_4 (Supelco). The flow rate of the carrier gas (nitrogen) was 35ml/min. The temperatures of the injection port, column and detector were 170°C, 150°C and 180°C respectively. The detector used was a flame ionization detector. Two millilitres of the sample was acidified with 6N HCl and centrifuged at 3000g for 5min. Individual fatty acids were identified and concentrations determined by comparisons with known standards and the concentrations calculated as follows:

$$\text{VFA (mg/l)} = \frac{\text{A sample} \times \text{V sample} \times \text{SG} \times 1000}{\text{A standard} \times \text{V standard}}$$

where: A = integrated area of the standard or sample peak
V = volume (ml) of the standard or sample
SG = specific gravity of the individual VFA

Each VFA is expressed as acetic acid (after Jackson-Moss, 1991) as follows:

$$\text{VFA acetic (mg/l)} = [\text{VFA}] \times \frac{\text{MM VFA}}{\text{MM AA}}$$

where: VFA acetic = concentration (mg/l) of the individual VFA expressed as acetic acid.
[VFA] = concentration (mg/l) of the individual VFA
MM VFA = molecular mass of the individual VFA
MM AA = molecular mass of acetic acid

$$\text{Total VFA as acetic} = [\text{AA}] + [\text{PA}] \times \frac{\text{MM PA}}{\text{MM AA}} + [\text{BA}] \times \frac{\text{MM BA}}{\text{MM AA}} + [\text{VA}] \times \frac{\text{MM VA}}{\text{MM AA}}$$

$$+ [CA] \times \frac{MM\ CA}{MM\ AA}$$

where: [AA] = concentration of acetic acid (mg/l)

MM AA = molecular mass of acetic acid

[PA] = concentration of propionic acid (mg/l)

MM PA = molecular mass of propionic acid

[BA] = concentration of butyric acid (mg/l)

MM BA = molecular mass of butyric acid

[VA] = concentration of valeric acid (mg/l)

MM VA = molecular mass of valeric acid

[CA] = concentration of caproic acid (mg/l)

MM CA = molecular mass of caproic acid

10. **Statistical analyses.**

The Paired Sample Test was used to compare growth rates of the various cultures, 95% confidence limits being applied. For accurate results at least 15 pairs per test needed to be analyzed. ANOVA tests were carried out on ß-carotene production and nutrient utilisation measurements. Ninety-five percent confidence limits were also set. The statistical analyses were carried out using Statgraphics, a statistical and graphics software programme supplied by the Statistical Graphics Corporation (Statistical Graphics System, 1989). All graphs in this thesis were generated using Statgraphics. Prior to the use of Statgraphics, a few statistical tests were carried out by hand using the Paired Sample Test and ANOVA tests as outlined in Byrkit (1987). These were cross-checked with similar tests using Statgraphics and were found to be in good agreement.

CHAPTER 3

GROWTH IN PONDED TANNERY EFFLUENT

1. **Introduction.**

The investigation of tannery effluent as a culture medium for *Dunaliella* started with ponded tannery effluents. This effluent is already present in what amounts to a large open bioreactor which is exposed to solar irradiance, and in which large populations of heterotrophic organisms exist. A number of problems must be addressed before this effluent can be considered as a suitable culture medium. These are: 1) the maximum strength of effluent that can be tolerated by *Dunaliella*, 2) the correct choice of an economically useful *Dunaliella* strain, and 3) the effect of the effluent on growth and production of useful products such as ß-carotene.

The local isolate of *Dunaliella salina* was used to test the suitability of ponded tannery effluent as possible growth medium for algal culture and to find the maximum concentration of this effluent that could be tolerated. Provasoli's medium was added to all the dilutions of ponded tannery effluent so that possible nutrient limitation would not mask the possible toxic effects of concentrated effluent.

The first experiment was designed to compare the effects of effluent strength on cell growth and ß-carotene production. The second experiment compared the effects of ageing over 24 days and the effects of nitrogen deficiency in the medium. The latter experiment was designed to see how best ß-carotene production can be influenced by manipulating the medium.

2. **Methods.**

The first experiment was the only experiment to use the local isolate of *D. salina*.

2.1. Growth of the local isolate (Experiment 1).

Full strength, 50% and 25% strength ponded tannery effluent and an effluent-free control were enriched with Provasoli's medium and used as culture medium. The cells were grown for 11 days and counted daily.

It was decided at this point to introduce the ß-carotene-producing bardawil strain of *Dunaliella* into the experimental program because of its well documented economic potential relating to the production of ß-carotene.

2.2. Growth of the bardawil strain (Experiment 2).

This experiment was designed to investigate whether the bardawil strain differed from the local isolate in terms of growth performance. Effluent strengths of 25% and 33% were used. One notable difference between the two strains was that the bardawil strain could not be cultured in Provasoli's medium initially so a change was made to the use of M11 medium which was used in all subsequent experiments. Cells were counted daily for 14 days.

2.3. Optimal effluent strengths for growth (Experiment 3a).

The following experiment was set up to determine both the highest effluent strength that could be tolerated and that which was optimal for the culture of the bardawil strain. The effluent strengths used were 10%, 20%, 25%, 30%, 35%, 40% and 50%, together with an effluent-free control. All effluent strengths were made up with M11 medium. The experiment was run over 14 days and cells counted daily. Samples for ß-carotene analysis were taken on the final day of growth.

2.4. Effect of effluent strength on ß-carotene production (Experiment 3b).

The aim of this experiment was to determine the effects of effluent strength on ß-carotene production in cells transferred to fresh M11 medium, as well as the effects of ageing and nitrogen deficiency on ß-carotene production. Each flask of the previous experiment, with the exception of the 40% and 50% strength effluent treatments (which were excluded because of very low cell yields), was divided into two equal volumes, spun down and washed as previously described. One volume was resuspended in N^+ M11 medium and the other in N^- M11 medium. After 24 days cells were counted and samples for ß-carotene analysis were taken.

3. Results.

The characteristics of the ponded tannery effluent are shown in table 3.1.

ph	4.40
conductivity	4240mS/cm
PV	0.285g/l
COD	1.330g/l
TDS	34.100g/l
TDIS	31.600g/l
SS	0.112g/l
nitrate	0.177g/l
ammonia	0.163g/l
phosphate	0.011g/l
sodium	0.454M
chloride	0.485M
sulphate	1.390g/l
chromium	0.001g/l

Table 3.1. Characteristics of East Cape Tannery's ponded tannery effluent.

3.1 Growth of the local isolate (Experiment 1).

The growth curves of the local isolate are shown in fig. 3.1.

	Cell counts (cell/ml)	
Treatment	Experiment 1	Experiment 2
Control	$146.83x10^4$ $\pm 14.27x10^4$	$77.33x10^4$ $\pm 28.18x10^4$
25% effluent	$307.83x10^4$ $\pm 125.29x10^4$	$94.33x10^4$ $\pm 23.91x10^4$
33% effluent	not carried out	$3.52x10^4$ $\pm 5.61x10^4$

Table 3.2. Cell concentrations at day 11 in Experiments 1 and 2.

It can be seen that growth was very poor in 50% strength effluent and was non-existent in 100% strength effluent (omitted from the graph). The 25% strength effluent had twice the cell yield of the

control after 11 days (307.83×10^4 cell/ml vs 146.83×10^4 cell/ml; table 3.2) and this was found to be significant ($t^* = 2.533$, $P < 0.05$; table 3.3).

Treatments tested	t*	Signif.
Control vs 25% strength effluent (Experiment 1a)	2.533	*
Control vs 25% strength effluent (Experiment 2)	1.643	n.s.

Table 3.3. Statistical analyses of growth rates in Experiments 1 and 2 using the Paired Sample Test.
Key: * * * = P<0.0005; * * = P<0.005; * = P<0.05; n.s. = no significance

The differences in growth rates between the control and 50% strength effluent, and between 25% and 50% strength effluent over the 11 days (fig. 3.1) are equally significant.

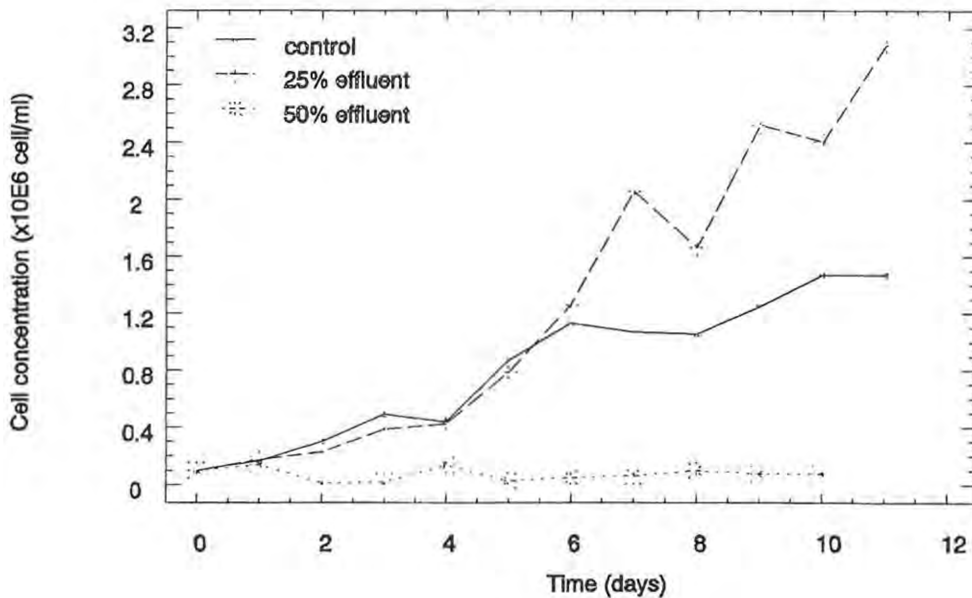

Figure 3.1. Experiment 1: Growth of the local isolate of *Dunaliella salina* in ponded tannery effluent.

3.2. Growth of the bardawil strain (Experiment 2).

This was the first experiment to use the bardawil strain. The comparative growth profiles in different effluent strengths are shown in fig. 3.2. The cell concentrations at day 14 are shown in table 3.2. Cell counts are the highest in the 25% strength effluent (94.33×10^4 cells/ml), which is 1.25 times that of the

control (77.33×10^4 cells/ml), and the lowest in 33% strength effluent (3.52×10^4 cells/ml), which is 22 times lower than the control. There is no statistically significant difference between the growth rates of the control and 25% strength effluent using the Paired Sample Test ($t^* = 1.643$, $P > 0.05$; table 3.3). Because of poor growth in the 33% strength effluent, statistical tests comparing the growth rates with the other treatments were considered unnecessary.

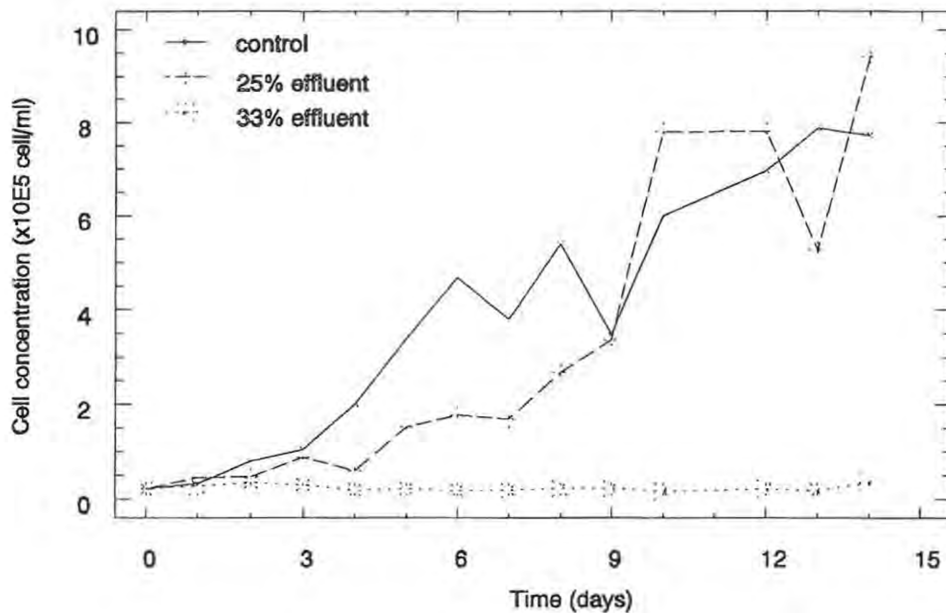

Figure 3.2. Experiment 2: Growth of the bardawil strain of *Dunaliella salina* in ponded tannery effluent.

3.3 Optimal effluent strengths for growth (Experiment 3a).

This experiment focused more closely on the strength of ponded tannery effluent which was optimal for the culture of the bardawil strain. The growth rates in each effluent strength over 14 days are shown in fig. 3.3 (day 1 in N^+ M11 medium) and the cell counts in table 3.4.

	Treatment	Cell count (cell/ml)	ß-carotene/cell (pg/cell)	ß-carotene/ml of culture (pg/ml)
Day 1 in N^+ M11 medium	Control	67.50×10^4 $\pm 17.76 \times 10^4$	0.059 ±0.070	0.161 ±0.200
	10%	62.17×10^4 $\pm 15.33 \times 10^4$	0.064 ±0.035	0.145 ±0.040
	20%	82.83×10^4 $\pm 21.55 \times 10^4$	0.027 ±0.008	0.085 0.000
	25%	70.17×10^4 $\pm 17.22 \times 10^4$	0.039 ±0.006	0.118 0.000
	30%	55.33×10^4 $\pm 15.45 \times 10^4$	0.167 ±0.141	0.312 ±0.169
Day 24 in N^+ M11 medium	Control	117.33×10^4 $\pm 9.39 \times 10^4$	0.525 ±0.076	2.452 ±0.243
	10%	128.67×10^4 $\pm 1.26 \times 10^4$	1.318 ±0.086	6.788 ±0.509
	20%	118.00×10^4 $\pm 10.15 \times 10^4$	0.970 ±0.125	4.708 ±1.014
	25%	101.17×10^4 $\pm 32.52 \times 10^4$	1.473 ±0.522	5.515 ±0.764
	30%	131.00×10^4 $\pm 31.83 \times 10^4$	1.105 ±0.391	5.636 ±1.117
Day 24 in N^- M11 medium	Control	38.67×10^4 $\pm 10.52 \times 10^4$	35.867 ±6.540	53.827 ±7.995
	10%	44.50×10^4 $\pm 12.58 \times 10^4$	38.016 ±10.539	64.130 ±3.483
	20%	60.33×10^4 $\pm 18.54 \times 10^4$	26.732 ±9.717	65.867 ±3.402
	25%	58.83×10^4 $\pm 35.20 \times 10^4$	36.472 21.946	59.827 ±3.402
	30%	52.50×10^4 $\pm 16.90 \times 10^4$	22.127 ±5.593	44.157 ±8.241

Table 3.4. Cell counts and ß-carotene yield calculated per cell and per ml of culture for day 1, and for day 24 in both N^+ and N^- M11 media (35% and 40% effluent strengths omitted due poor growth).

The highest cell counts were obtained in the 20% strength effluent (82.83×10^4 cells/ml), which is 1.25 times more than that of the control, and 25% strength effluent (70.17×10^4 cells/ml), the latter being almost equal to the yield of the control. The lowest cell counts were obtained in the 40% strength effluent (2.85×10^4 cells/ml, data not shown), 24 times lower than the control. No growth was detected in 50% strength effluent after the first few days and so its measurement was terminated. The Paired Sample Test was carried out for each permutation of compared effluent strengths and the results are shown in table 3.5.

	Control	10%	20%	25%	30%	35%	40%
Control		n.s.	- -	n.s.	+	+ + +	+ + +
10%			- -	n.s.	+	+ + +	+ + +
20%				+	+ + +	+ + +	+ + +
25%					+ +	+ + +	+ + +
30%						+ +	+ +
35%							+ +
40%							

Table 3.5. Paired Sample Tests for comparisons of growth rates in different strengths of ponded tannery effluent. The Null hypothesis is rejected if there is a difference between the growth rates. (The table is read from left to right), where - denotes significantly lower growth rate (- - = $P<0.005$), + denotes significantly higher growth rate (+ + + = $P<0.0005$; + + = $P<0.005$; + = $P<0.05$) and n.s. denotes no significant difference in growth rates

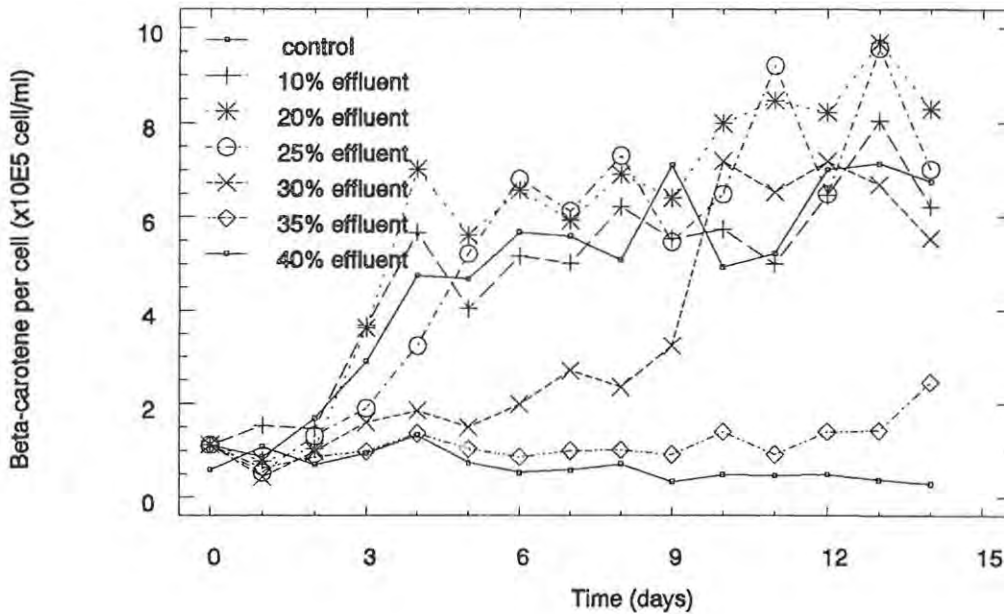

Figure 3.3. Experiment 3a: Growth of the bardawil strain of *Dunaliella salina* in a variety of strengths of ponded tannery effluent.

It can be seen that only cultures grown in the control, 10% and 25% strength effluents showed equivalent growth rates, while significantly higher growth rates are achieved in 20% strength effluent compared to all the other effluent concentrations evaluated.

3.4. Effect of effluent strength on ß-carotene production (Experiment 3b).

This experiment described the effects of the ponded tannery effluent strengths on ß-carotene production. The ß-carotene values at day 1 reflect the influence of the tannery effluents on ß-carotene production during the previous 14 days of growth (i.e. Experiment 3a) prior to splitting the cultures into N^+ M11 and N^- M11 media. The concentration of ß-carotene per cell at day 1 is shown in table 3.4 and fig. 3.4 (the 35% strength effluent has been omitted, because of its low cell yield). It is highest in cells grown in 30% strength effluent (0.167 pg/cell), an almost 3 times greater yield than that of the control (0.059 pg/cell), and lowest in cells grown in 20% strength effluent (0.027 pg/cell), half the control's yield. When the ß-carotene yield is calculated per ml of culture medium (table 3.4 and fig. 3.5), it can be seen that cells grown in 30% strength effluent and in the control show the greatest concentration of ß-carotene (0.312 pg/ml and 0.161 pg/ml, respectively), while the lowest yield occurs in cells grown in 20% strength effluent (0.085 pg/ml). Thus the yield in 30% strength effluent was twice that of the control, and the 20% strength effluent produced half the yield of the control.

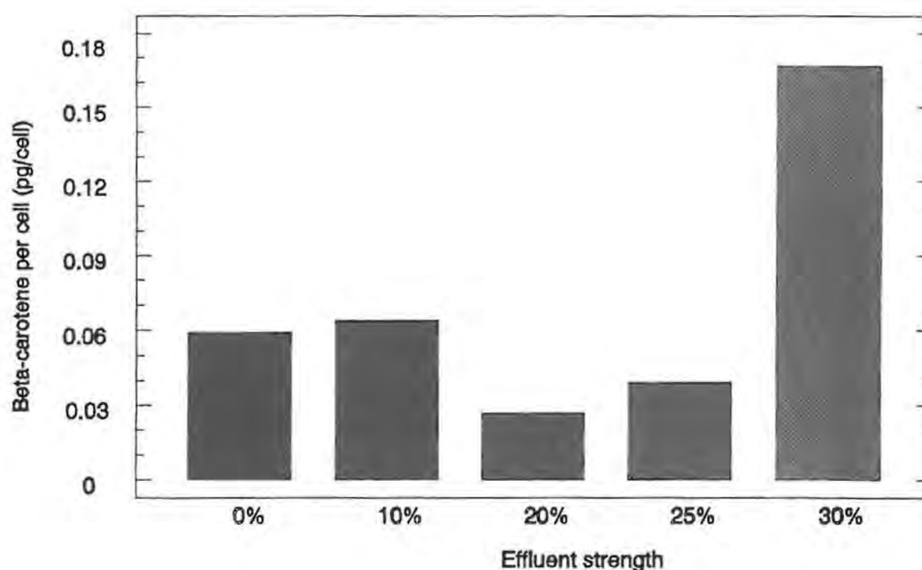

Figure 3.4. Experiment 3b: ß-carotene per cell in cultures grown in ponded tannery effluent at day 1.

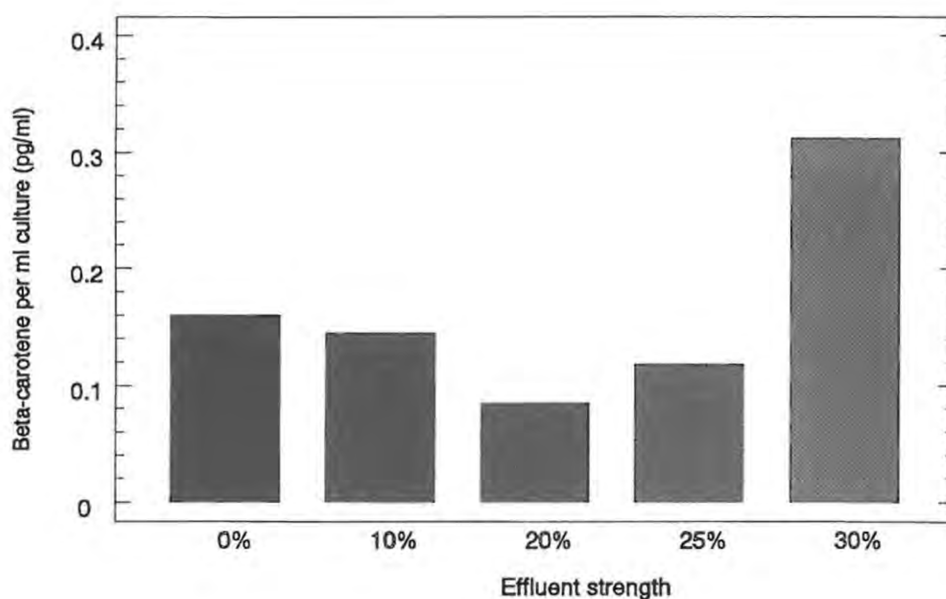

Figure 3.5. Experiment 3b: ß-carotene per ml of culture in cultures grown in ponded tannery effluent at day 1.

3.5. ß-carotene production after transfer to N^+ M11 medium.

After 24 days in N^+ M11 medium (table 3.4), ß-carotene per cell (fig. 3.6) was highest in cells that had been exposed to 10% and 25% strength effluent (1.318 pg/cell and 1.473 pg/cell, respectively). This is twice the yield of cells grown in the control (0.525 pg/cell). ß-carotene per ml of culture was also considerably higher in the effluent treatments (fig. 3.7). The 10% strength effluent had the highest yield (6.788 pg/ml) and the 20% strength effluent the lowest yield (4.708 pg/ml) of all the effluent treatments. This is three times and twice the concentration of that of the control, (2.452 pg/ml) respectively.

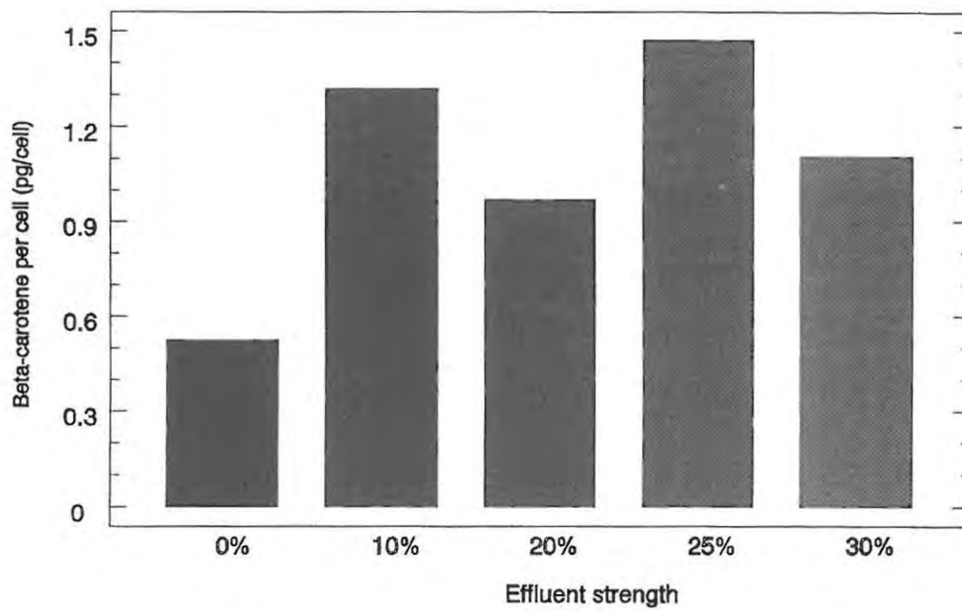

Figure 3.6. Experiment 3b: ß-carotene per cell in cultures grown in ponded tannery effluent 24 days after being transferred to fresh N^+ M11 medium.

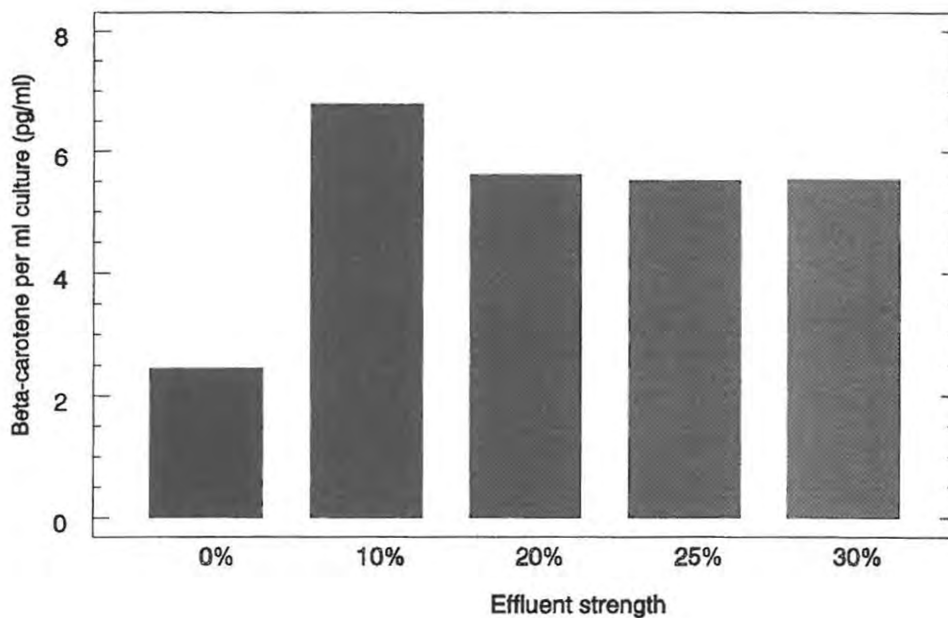

Figure 3.7. Experiment 3b: ß-carotene per ml of culture in cultures grown in ponded tannery effluent 24 days after being transferred to fresh N^+ M11 medium.

3.6. <u>ß-carotene production after transfer to N⁻ M11 medium.</u>

After 24 days in N^- M11 medium (table 3.4) the ß-carotene concentration per cell (fig. 3.8) is higher in cultures originating from 10% strength effluent (38.016 pg/cell) and 25% strength effluent (36.472 pg/cell), which is nearly equivalent to that found in the control (35.867 pg/cell). The lowest concentrations of ß-carotene per cell are found in the 30% and 20% strength effluents (22.127 pg/cell and 26.732 pg/cell, respectively), which is about 1.5 times lower than that of the control. The higher ß-carotene per ml of culture (fig. 3.9) is found in cultures originating from 10% and 20% strength effluents (64.130 pg/ml and 65.867 pg/ml, respectively), about 1.25 times that of the control (53.827 pg/ml). The lowest concentration of ß-carotene per ml of culture is found in the 30% effluent strength (44.157 pg/ml) which is about 1.25 times lower than the control.

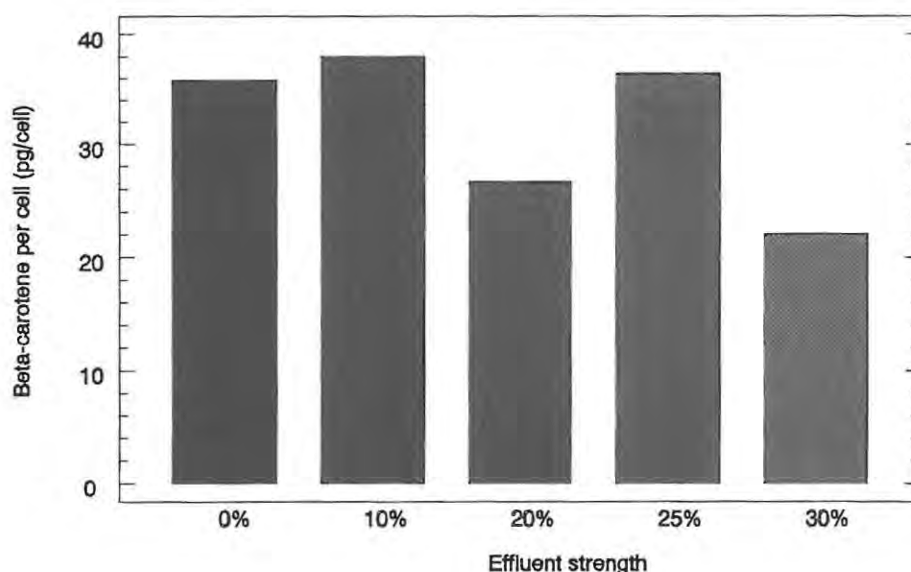

Figure 3.8. Experiment 3b: ß-carotene per cell in cultures grown in ponded tannery effluent 24 days after being transferred to fresh N^- M11 medium.

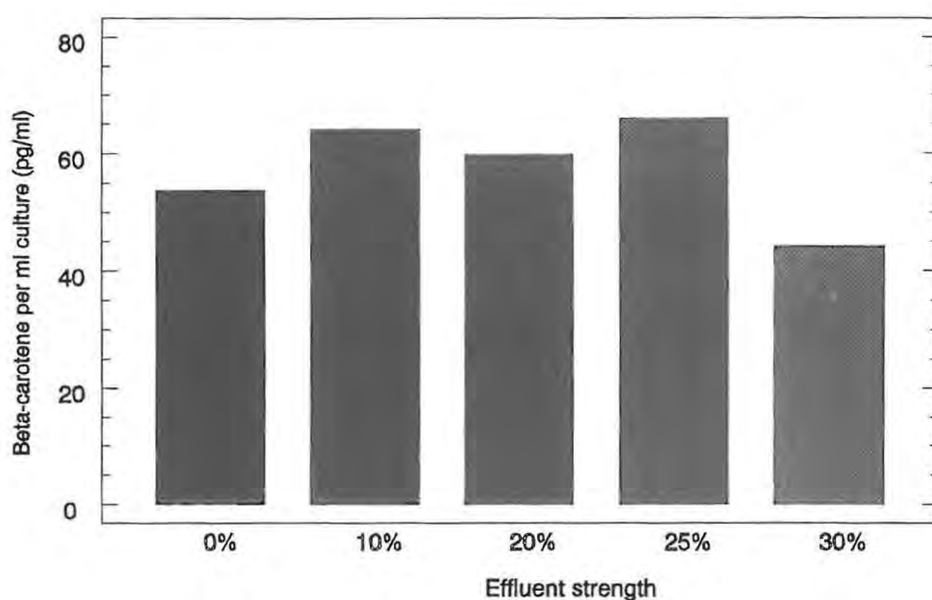

Figure 3.9. Experiment 3b: ß-carotene per ml of culture in cultures grown in ponded tannery effluent 24 days after being transferred to fresh N⁻ M11 medium.

3.7. Factors influencing ß-carotene production.

The hypothesis that the cultivation of the algae in different strengths of ponded tannery effluent will have an effect on ß-carotene production *after* it is transferred into effluent-free medium, can be tested by using ANOVA tests (table 3.6).

		Concentration of ß-carotene/cell		Concentration of ß-carotene/ml culture	
Treatments	Factor	F*	Signif.	F*	Signif.
Effects of ageing and effluent strength on ß-carotene production between cultures at day 1 and 24	Ageing	8.853	* * *	533.650	* * *
	Effluent strength	93.939	* * *	12.033	* * *
Effects of nitrogen starvation on ß-carotene production between cultures grown in N^+ and N^- M11 media	Nitrogen availability	94.646	* * *	978.363	* * *
	Effluent strength	0.913	n.s.	6.323	* *

Table 3.6. Effects of ageing and nitrogen-deficiency on ß-carotene production in cultures derived from various strengths of ponded tannery effluent (Analysis of variance).

Key: * * * = P<0.0005; * * = P<0.005; * = P<0.05; n.s. = no significance

This also takes into account the effects of ageing of the culture and nitrogen deprivation. ANOVA tests show that various strengths of tannery effluents significantly influence ß-carotene production per cell between day 1 and day 24 ($F^* = 93.939$; $P<0.05$), as does the age of culture ($F^* = 8.853$; $P<0.05$). A similar trend is observed for the ß-carotene per ml of culture. There is a significantly higher concentration of ß-carotene per ml of culture in cultures at day 24 than at day 1 ($F^* = 533.650$; $P<0.05$), and between cultures originating from different strengths of tannery effluent and the control, from day 1 to day 24 ($F^* = 12.033$; $P<0.05$).

When the effects of nitrogen deprivation on ß-carotene per cell are examined in cultures from different effluent strengths, it is found that the nitrogen deficiency definitely promotes the production of ß-carotene per cell ($F^* = 94.646$, $P<0.05$). However, the effects of effluent strength on the production of ß-carotene per cell is negligible ($F^* = 0.913$; $P>0.05$). The amount of ß-carotene per ml of culture measured at day 24 in N^- M11 medium, is significantly higher than that in N^+ medium ($F^* = 978.363$; $P<0.05$). Statistically, effluent strength is seen to effect ß-carotene production per ml of culture ($F^* = 6.323$; $P<0.05$).

In plates 3.1 and 3.2 the effects of nitrogen starvation are well illustrated in the cultures derived from the control and split into N^+ and N^- M11 media.

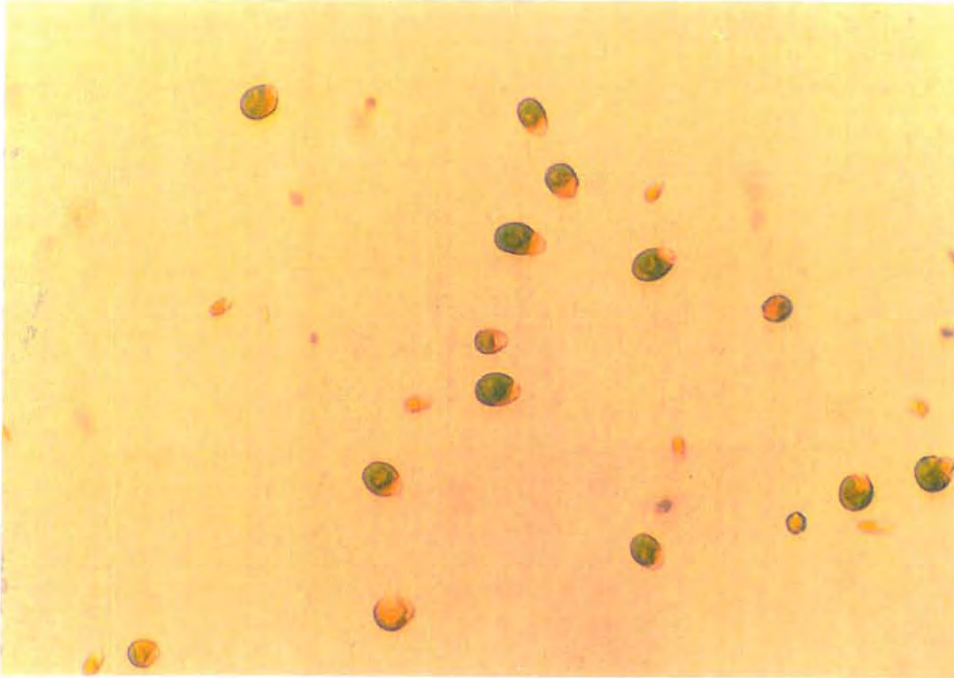

Plate 3.1. *Dunaliella* (bardawil strain) 24 days after being transferred from the control to N^+ M11 medium. Note the green colour.

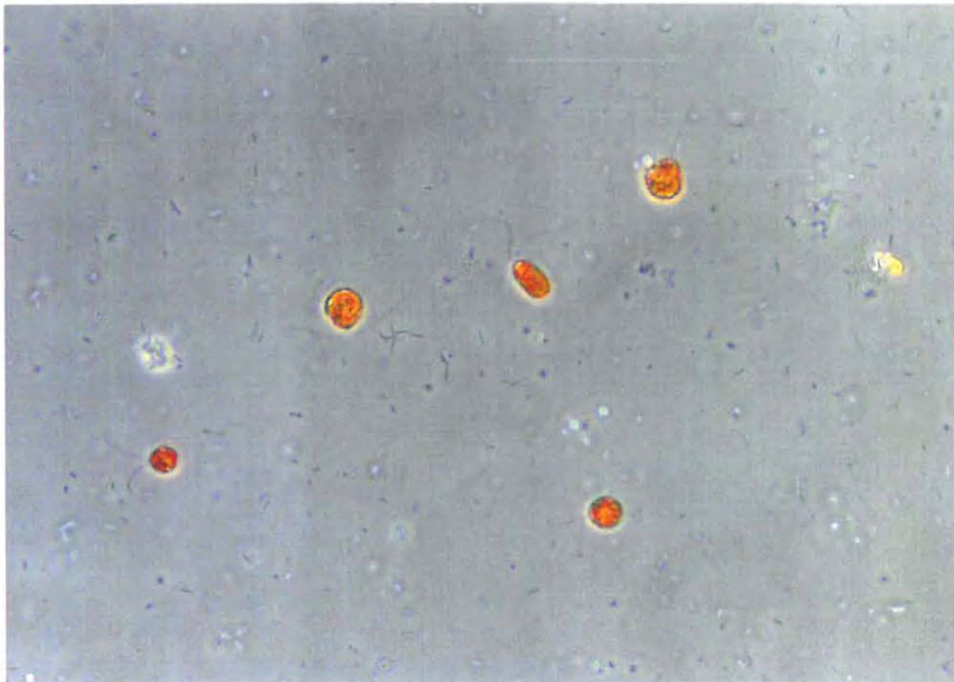

Plate 3.2. *Dunaliella* (bardawil strain) 24 days after being transferred from the control to N^- M11 medium. Note the orange colour.

4. Discussion.

4.1. Local isolate versus bardawil strain.

The choice of a suitable strain of *Dunaliella* was made on the merit of its commercial exploitability. Two strains of *Dunaliella* are considered here. The first, a local strain of *Dunaliella salina* isolated from a nearby salt pan, had previously been investigated for its ability to produce glycerol and ß-carotene under salinity and light stress (Laubscher, 1987). The second species, *Dunaliella salina* var. bardawil, is a halophilic and high ß-carotene producing species (Avron and Ben-Amotz, 1980).

Comparisons of the growth rates between the local isolate and the bardawil strain could not be made, as different enriching media were used. However, the local isolate is seen to yield higher cell concentrations than the bardawil strain in both the control and 25% strength effluent. Considering that the local isolate had been repeatedly cultured under the same environmental conditions for over a year as opposed to the bardawil strain, which was only received from the supplier some 6 weeks prior to the beginning of Experiment 2, it is perhaps not unexpected that higher cell yields were obtained with the local strain.

A factor that was not taken into consideration was the period of adaptation necessary for the bardawil strain to acclimatize to the environmental conditions used. At this point it could be suggested that either algal strain could possibly be adapted to tolerate higher effluent strengths by selection and exposure to increasing effluent strengths over a period of time.

4.2. Ponded tannery effluent as a growth medium.

Ponded tannery effluent can be used as a growth medium for the culture of *Dunaliella* provided it is diluted to 25% strength. This is shown by the results obtained from Experiment 1 and 2 which indicate that 25% strength effluent is the least toxic dilution to both the local isolate and bardawil strain. The growth of the local isolate in this effluent strength is better than in the effluent-free Provasoli's-enriched control, but the growth of the bardawil strain in 25% strength effluent is comparable to the M11-enriched control. Higher effluent concentrations such as the 50% and 100% strengths in Experiment 1 and 2 do not support growth or show very poor growth. All the treatments were equivalent in salinity, were enriched with either Provasoli's or M11 medium, and CO_2 and light were not limiting. Therefore the poor performances could probably be due to as yet unidentified growth inhibitory factors present in the effluent and not to growth-limiting concentrations of nutrients.

At 25% strength, the concentration of the putative toxic component is insufficient to prevent the algae from equalling the growth rate of the control over the 14 days observed. However, an apparent retardation of the bardawil strain's growth appears to occur in the 25% strength effluent from day 4 to day 8 (fig. 3.2), but is subsequently overcome over the following days.

The high concentrations of Na^+ and Cl^- ions (0.454M and 0.485M) in the undiluted effluent are well below the optimal concentration of 1.5M NaCl. Additional NaCl had to be added to the various effluent dilutions to bring the molarity of the effluent dilution up to 1.5M. The maximum tolerable concentration of NaCl for the bardawil strain is 5.5M (Brown *et al*, 1982), while that of the local isolate was found to be 5M (Laubscher, 1987). The sulphate concentration of the concentrated effluent was found to be 1.39g/l which is far below that of the 48g/l reported to be the maximum tolerable concentration for *D. parva*, and above that of the sulphate limiting condition of 0.02g/l for the same species (Gimmler and Weiss, 1987). The minimum sulphate requirement for the bardawil strain is 4.80×10^{-3} g/l (Ben-Amotz, 1987). Borowitzka and Borowitzka (1988) report that the highest inhibitory concentrations of phosphate are in excess of 0.110g/l and for ammonia are over 42.6g/l. The maximum concentration of these two nutrients were 0.011g/l and 0.163g/l, respectively. Therefore, the identical constituents of the effluent (table 3.1.) do not appear to be responsible for retardation of growth in the higher concentrations of ponded tannery effluent.

4.3. Optimal effluent strength.

The growth performances in ponded tannery effluent were investigated more thoroughly in Experiment 3a, optimal growth being shown to occur in the 20% strength effluent. This effluent strength also gave the highest cell yields, surpassing that of the control as well as all the other effluent concentrations. Higher concentrations of effluent show progressive retardation of growth. The growth in 35% strength effluent is considerably retarded, while that of the 30% strength effluent shows limited growth until about day 9, whereafter it rises dramatically to about the same cell concentrations as the higher dilutions of effluent. This may be due to adaptation of the culture to this concentration of effluent, or else to a modification of the effluent, possibly the toxic component, by the algae and/or the coexisting bacterial population.

For the culture of the bardawil strain, the 20% strength effluent could be a compromise between lower toxicity and higher nutrient availability. The lower algal growth in the 10% strength effluent compared to the 20% strength effluent could be explained by lower nutrient availability. Even though the culture would be exposed to a lower toxicity owing to a higher dilution of the effluent, nutrient availability is reduced and this effect seems to surpass that of any toxic effects. Likewise, it can be said that the increased effects of effluent toxicity are responsible for limiting algal growth in higher strengths of ponded tannery effluent, even though nutrients would be more abundant.

For a more economical system, the 25% strength effluent should be considered for culture purposes as it would have a greater dilution economy. This means that a lesser volume of water would need to be added, an important consideration in effluent management.

4.4. ß-carotene production in ponded effluent.

Firstly, it should be noted that in the case of the higher effluent strengths, a certain amount of effluent solids are present which adds to the weight in biomass assessments. Therefore, ß-carotene per dry weight measurements were replaced by measurements of ß-carotene per ml of culture to exclude this inaccuracy. After 14 days of growth in a range of effluent strengths, 30% strength effluent showed the highest concentration of ß-carotene per cell as well as per ml of culture. This effluent strength also shows the highest degree of growth retardation (fig. 3.3). The combination of a high ß-carotene concentration and low growth rate appear to be in response to stress. Stress responses referred to in the literature are manifested by the high ß-carotene per cell and per dry weight ratios (Mil'ko, 1963a; Ben-Amotz and Avron, 1983b). Growth in 30% effluent strength clearly stimulates ß-carotene production at the expense of cell growth.

The local isolate is also recorded to produce ß-carotene in response to stress. Laubscher (1987) was able to induce the local isolate to produce between 0.05 and 0.1 pg/cell of ß-carotene at 5M NaCl and 344.25 $\mu mol/m^2/s^1$ after 20 days of culture. This was far less than the 0.167 pg/cell produced by the bardawil strain in 30% effluent after 14 days. The higher ß-carotene per cell and per ml of culture in the control and 10% strength effluent compared to the 20% and 25% strength effluents (fig. 3.4 and fig. 3.5, respectively), could be due to the same factors discussed for Experiment 3a. These are the additional nutrients provided by the effluent which delay the ageing process, thereby delaying induction of ß-carotene synthesis, and secondly the toxic effects of some compounds present in the effluent which at high concentrations stress the cell and induce ß-carotene synthesis and accumulation. Therefore, at the mid-range effluent strengths of 20% and 25% it is possible that the higher nitrate levels and lower effluent toxicity are less stressful and are able to delay the ageing process of the culture.

In terms of biomass production, the use of 20% strength effluent would be favoured as higher cell numbers are obtained. In terms of economy, the 25% effluent would be preferred as less dilution of effluent is needed and ß-carotene per cell is some 1.5 times higher at the end of 14 days. ß-carotene analysis of algae grown in the 35% and 40% effluent was not considered as growth was too poor.

4.5. Effect of ageing and effluent strength on ß-carotene production in N^+ M11 medium.

Twenty four days after the cells from all the treatments were transferred to fresh M11 medium, the amount of ß-carotene per cell was significantly higher than at day 1. This trend is repeated for the ß-carotene per ml of culture measurements. Over the 24 days, a significant amount of nitrogen exhaustion must have taken place to initiate ß-carotenogenesis. The relationship between nitrogen exhaustion and ß-carotene production is investigated in greater detail in the following chapter.

It appears that previous exposure to different effluent strengths also influences ß-carotene production, even after transfer to fresh N^+ M11 medium. ANOVA tests indicated significantly higher ß-carotene yields per cell and per ml of culture between cultures originating from the different effluent strengths and the control (fig. 3.6 and fig. 3.7). Final cell counts are more or less equivalent throughout the treatments and control on day 24. Whatever factors have promoted ß-carotene production and accumulation have not compromised cell yields. As no cell counts or ß-carotene assays were carried out over the 24 days of growth in N^+ M11 medium, it is difficult to speculate as to how the exposure to the effluent may have have influenced the process of ß-carotene production.

4.6. Effect of nitrogen-deficiency and effluent strength on ß-carotene production N^- M11 medium.

After 24 days, significantly higher concentration of ß-carotene per cell and ß-carotene per ml of culture occur in cultures in N^- M11 medium compared to those in N^+ M11 medium. It appears that higher levels of ß-carotene in the N^- M11 cultures are influenced by a deficiency of assimilable nitrogen, as reported in the literature (Mil'ko, 1963a). This deficiency results in the slowing down of the growth rate with the concomitant accumulation of ß-carotene, is also supported by Ben-Amotz (1986).

Exposure to different effluent strengths is not a significant factor in influencing ß-carotene concentrations per cell when comparisons are made between cells grown in N^+ and N^- M11 media, whereas it does contribute significantly to the differences in ß-carotene concentration per ml of culture. This can be explained when final cell yields in both N^+ and N^- M11 media are taken into consideration (table 3.4). Effluent-grown cultures have higher cell yields than control-grown cultures in N^- M11 medium, therefore even if there is little variation in ß-carotene per cell concentrations between all the treatments and control, final ß-carotene per ml of culture will be higher in those treatments with the highest cell yields.

5. **Conclusion.**

The bardawil strain of *Dunaliella salina* has been selected over the local isolate for future investigation, as it is a higher producer of ß-carotene. Ponded tannery effluent does influence the growth of *Dunaliella*. Effluent strengths above 25% retard growth, while 20% strength effluent enhances growth. ß-carotene production is also affected by the effluent. High effluent strengths stress the cell and enhance ß-carotene accumulation at the expense of cell growth. Mid-range effluent strengths may delay ageing and therefore ß-carotene production. Ageing is observed to promote ß-carotene accumulation, and nitrogen deprivation enhances its production. Higher ß-carotene concentration per cell and per ml of culture occur in nitrogen-stressed cultures than in non-stressed cultures where nutrient exhaustion is allowed to take place of over a period of time. This event is covered in greater detail in the following chapter.

In summary, this series of experiments has served to find the optimal dilution of ponded tannery effluent for maximum biomass accretion as well as the lowest dilution of this effluent required for satisfactory growth of the bardawil strain. Production of ß-carotene through the manipulation of the nitrogen content in the medium is possible.

CHAPTER 4

ß-CAROTENE PRODUCTION IN PONDED EFFLUENTS.

1. **Introduction.**

The potential of *Dunaliella salina* (bardawil) as a ß-carotene producer has been demonstrated by Ben-Amotz and Avron (1980) and confirmed for ponded effluent in the studies reported in the previous chapter. The relationship of ß-carotene synthesis and nutrient utilization in this medium needs to be investigated more thoroughly if ß-carotene production is to be manipulated. Previously it was shown that the effluent strength of the ponded tannery effluent influenced ß-carotene production as well as cell growth. In the previous chapter, it was shown that the 25% strength was the best dilution for cultivating *Dunaliella* in ponded tannery effluent. The rates of nutrient utilization were investigated in this effluent strength and in N^+ and N^- M11 media. The effects of nitrogen deficiency and ageing on ß-carotene production were investigated over an extended period of time (84 days), as this allowed realistic comparisons to be made regarding the maximisation of ß-carotene production.

The nutrients investigated were the three most common forms of inorganic nitrogen (namely nitrate, nitrite and ammonia) and phosphate. Nitrogen deficiency is well documented as an inducer of ß-carotene synthesis (Mil'ko, 1963a; Ben-Amotz and Avron, 1983b), and thus the effects of progressive nitrogen depletion by ageing as well as its immediate deprivation were to be examined. The depletion of nutrients was followed regularly over a period of time and coordinated with cell counts and assays of ß-carotene. This allowed a composite picture of the effects of nitrogen depletion to be formulated. Experiment 4a and b were set up to investigate whether a two-stage system would optimise ß-carotene production. In the first stage ponded effluent could be used to provide nutrients for biomass production. Transfer to a second stage consisting of N^- M11 medium would then induce ß-carotene production. This would be compared to the natural ageing of cultures in M11 medium.

A further question that needed to be answered was whether cell growth in ponded tannery effluent possibly had any adverse or other influence on subsequent ß-carotene production once cells were transferred into fresh effluent-free medium. The results were statistically analyzed using the Paired Sample Test for comparison of growth rates and ANOVA for testing the effects of ageing and effluent on nutrient utilization and ß-carotene production.

2. **Methods.**

2.1. Growth in 25% effluent (Experiment 4a).

Previous experiments had established that 25% strength is the best dilution of ponded tannery effluent for *Dunaliella* culture. This experiment was designed to relate ß-carotene production to nutrient

depletion. *D. salina* was inoculated into M11-enriched 25% strength effluent medium and an effluent-free M11-enriched sea-water control. Cells were counted daily and ß-carotene production and nutrient utilization were monitored every second day to determine the effects of ageing and of the effluent on the culture.

2.2. Transfer to N^+ and N^- M11 media (Experiment 4b).

After 16 days the cultures in the effluent and control were spun down, washed, and split into N^+ M11 and N^- M11 media. Cells were counted and ß-carotene and nutrient concentrations were assayed. The cultures in N^+ M11 medium served as a control, while the cultures in N^- M11 medium served to illustrate the effects of nitrogen starvation on ß-carotene production. The effects of pre-exposure to 25% strength ponded effluent on cell growth and production, after transfer to effluent-free fresh M11 medium, were investigated by comparing the performances of effluent-exposed and control cultures in the respective N^+ and N^- M11 media.

3. **Results**.

3.1. Growth in 25% effluent (Experiment 4a).

A 1.2 times greater cell yield is obtained in the effluent treatment than in the control over the 16 days ($96.00x10^4$ cell/ml vs. $69.00x10^4$ cell/ml, table 4.1).

	Treatment	Cell count (cell/ml)	ß-carotene/cell (pg/cell)	ß-carotene/ml culture (pg/ml)
Prior to split into N^+ and N^- M11 media	Control	$69.00x10^4$ $\pm 3.46x10^4$	0.917 ± 0.180	2.545 ± 0.447
	Effluent	$96.00x10^4$ $\pm 9.17x10^4$	0.460 ± 0.100	1.757 ± 0.331
After split of control and effluent into N^+ and N^- M11 media	Control N^+ M11	$104.67x10^4$ $\pm 13.43x10^4$	12.683 ± 11.254	50.449 ± 40.773
	Effluent N^+ M11	$128.33x10^4$ $\pm 11.34x10^4$	6.725 ± 1.298	34.152 ± 4.030
	Control N^- M11	$21.83x10^4$ $\pm 4.25x10^4$	11.293 ± 2.525	9.818 ± 2.832
	Effluent N^- M11	$37.67x10^4$ $\pm 11.88x10^4$	13.193 ± 5.467	20.436 ± 12.806

Table 4.1. Cell counts, ß-carotene per cell and ß-carotene per ml of culture at day 16 prior to split of the control and effluent treatment into N^+ and N^- M11 media, and at day 83 after the split.

The growth rates in fig. 4.1 indicate better growth in effluent than in the control. This is confirmed by the Paired Sample Test at 95% confidence limits ($t^* = 2.083$; $P < 0.05$; table 4.2). By referring to

fig. 4.1, it can be seen that initially higher growth occurs in the control until day 7, whereafter higher growth occurs in the effluent.

Treatments tested	t*	Signif.
Control vs effluent treatment prior to split	2.083	* * *
Control split into N^+ and N^- media	6.271	* * *
Effluent split into N^+ and N^- M11 media	7.156	* * *
Control vs effluent treatment in N^+ M11 media	8.738	* * *
Control vs effluent treatment in N^- M11 media	7.661	* * *

Table 4.2. Statistical analyses of growth rates using the Paired Sample Test.
Key: * * * = $P<0.0005$; * * = $P<0.005$; * = $P<0.05$; n.s. = no significance

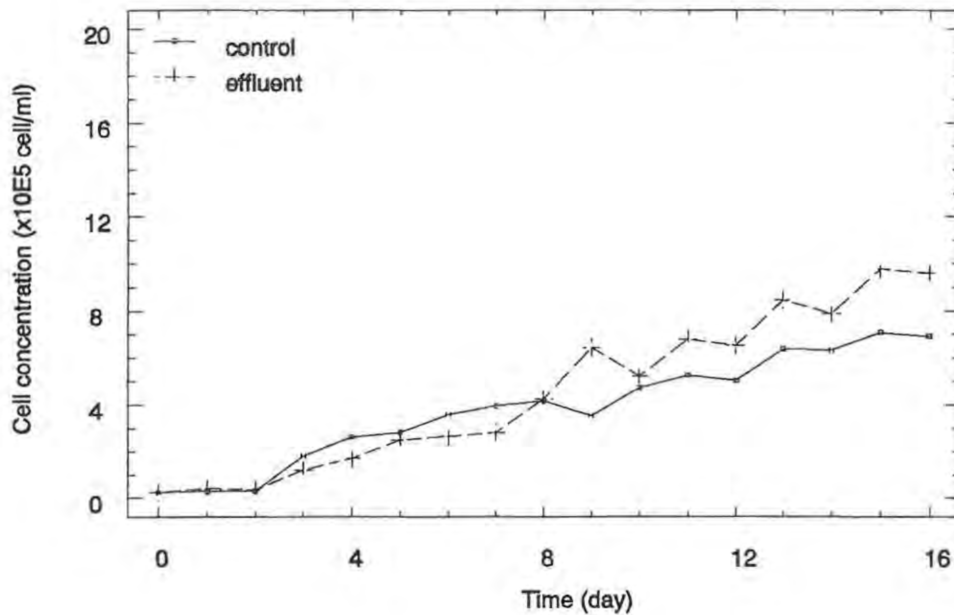

Figure 4.1. Experiment 4a: Growth in M11 control and effluent for 16 days.

ANOVA tests were used to determine whether effluent treatment or ageing of the cultures influenced nutrient utilisation. Nitrate levels (fig. 4.2) were significantly higher in the effluent treatment (F* =

161.165, $P < 0.05$; table 4.3) but decreased significantly over time in both the effluent treatment and control ($F^* = 5.204$, $P < 0.05$; table 4.3). This decrease was preceded by a sharp increase in nitrate concentration after day 1. Nitrite levels (fig. 4.3) were also found to be significantly higher in the effluent treatment ($F^* = 53.836$, $P < 0.05$; table 4.3), and a significant decrease over time is apparent for both the effluent treatment and control. There is also a significant increase in nitrite levels after day 1, followed by a gradual decline over time ($F^* = 51.277$, $P < 0.05$; table 4.3). Phosphate levels (fig. 4.4) do not differ significantly between the two treatments ($F^* = 2.212$, $P > 0.05$; table 4.3), but a significant decrease in phosphate concentration over time, in both effluent treatment and control is evident until day 6 ($F^* = 34.877$, $P < 0.05$; table 4.3). Ammonia levels (fig. 4.5) are significantly higher in the effluent treatment ($F^* = 104.424$, $P < 0.05$; table 4.3) which decreases significantly over time ($F^* = 18.685$, $P < 0.05$; table 4.3). On the contrary, ammonia levels in the control remain low throughout the growth period.

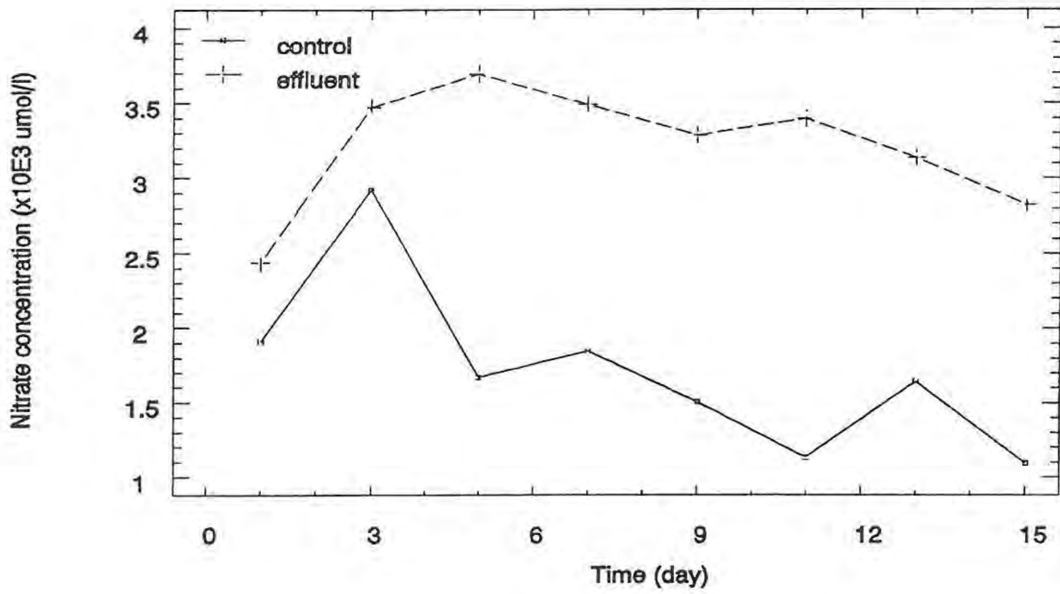

Figure 4.2. Experiment 4a: Nitrate concentration in the M11 control and effluent treatment grown over 16 days.

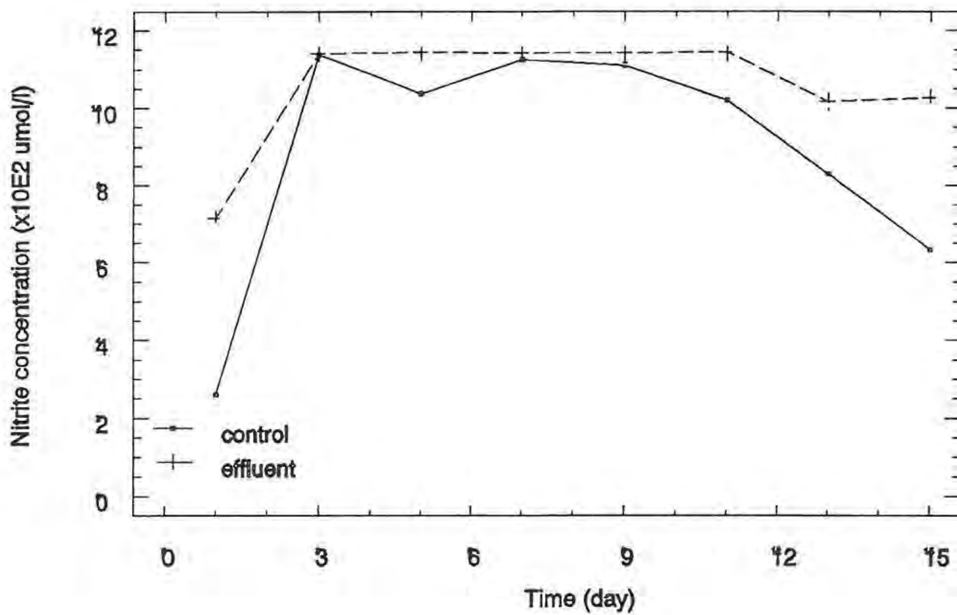

Figure 4.3. Experiment 4a: Nitrite concentration in the M11 control and effluent treatment grown over 16 days.

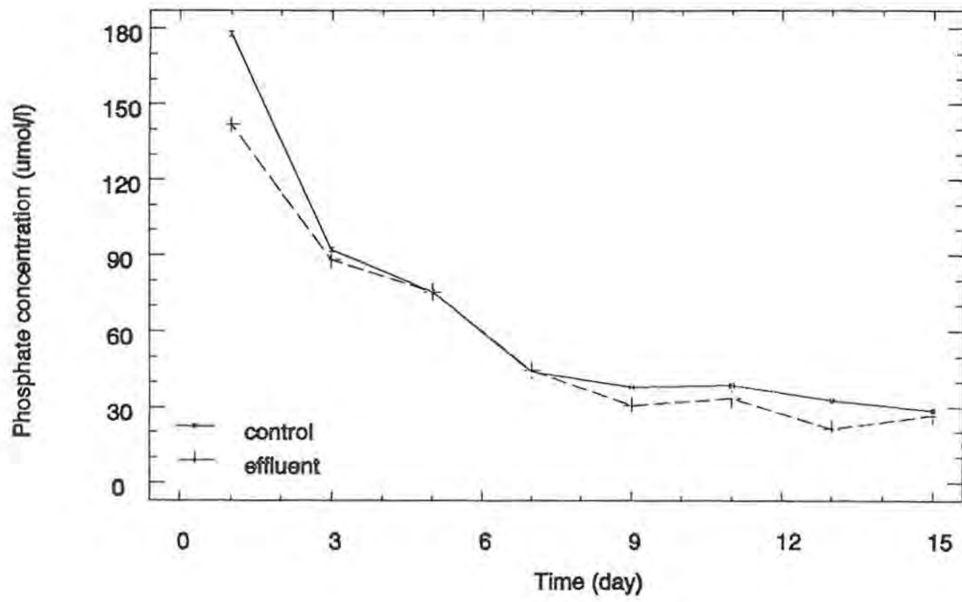

Figure 4.4. Experiment 4a: Phosphate concentration in the M11 control and effluent treatment grown over 16 days.

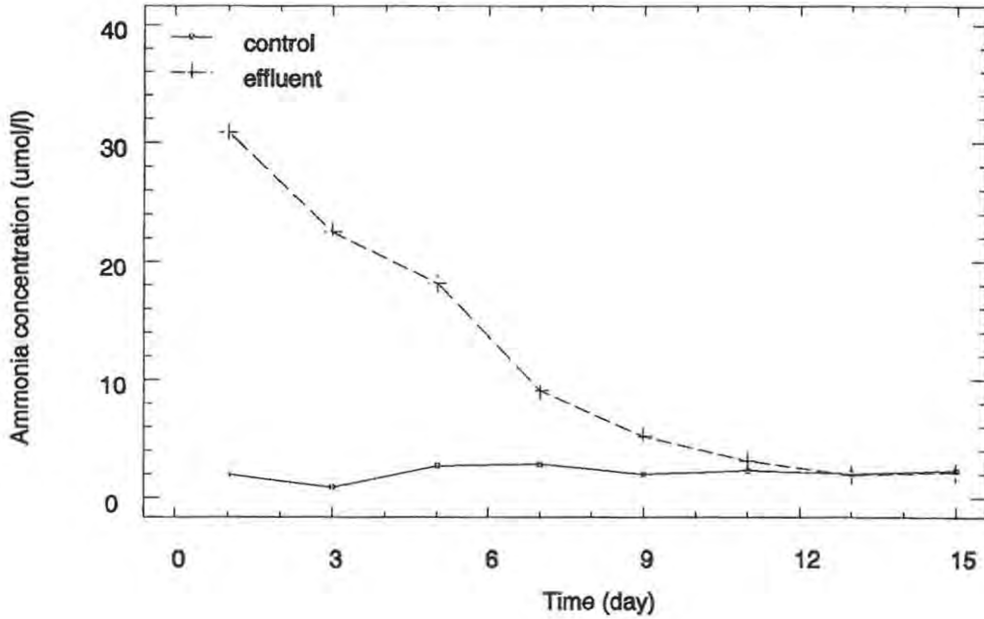

Figure 4.5. Experiment 4a: Ammonia concentration in the M11 control and effluent treatment grown over 16 days.

	Nutrient	Factor	F*	Signif.
Control vs effluent prior to split	Nitrate	Treatment	161.165	* * *
		Days	5.204	* * *
	Nitrite	Treatment	53.836	* * *
		Days	51.277	* * *
	Phosphate	Treatment	2.212	n.s.
		Days	34.877	* * *
	Ammonia	Treatment	104.424	* * *
		Days	18.685	* * *
Control after split into N^+ and N^- M11 media	Nitrate	Treatment	227.588	* * *
		Days	14.028	* * *
	Nitrite	Treatment	166.842	* * *
		Days	27.593	* * *
	Phosphate	Treatment	23.654	* * *
		Days	9.098	* * *
	Ammonia	Treatment	1.788	n.s.
		Days	1.824	n.s.
Effluent treatment after split into N^+ and N^- M11 media	Nitrate	Treatment	385.495	* * *
		Days	18.453	* * *
	Nitrite	Treatment	444.989	* * *
		Days	18.926	* * *
	Phosphate	Treatment	20.149	* * *
		Days	9.248	* * *
	Ammonia	Treatment	6.120	*
		Days	2.476	*

Table 4.3. Analyses of variance of nutrients for control vs effluent treatment prior to split between N^+ and N^- M11 media (continued on next page).

	Nutrients	Factor	F*	Signif
Control and effluent treatment transferred to N^+ M11 media	Nitrate	Treatment	2.736	n.s.
		Days	31.765	* * *
	Nitrite	Treatment	18.419	* * *
		Days	28.816	* * *
	Phosphate	Treatment	12.572	* *
		Days	15.589	* * *
	Ammonia	Treatment	5.046	*
		Days	2.697	* *
Control and effluent treatment transferred to N^- M11 media	Nitrate	Treatment	0.005	n.s.
		Days	1.674	n.s.
	Nitrite	Treatment	1.288	n.s.
		Days	14.538	* * *
	Phosphate	Treatment	192.522	* * *
		Days	6.393	* * *
	Ammonia	Treatment	2.374	*
		Days	1.715	n.s.

Table 4.3. continued.

Key: * * * = $P<0.0005$; * * = $P<0.005$; * = $P<0.05$; n.s. = no significance

There is no significant difference in ß-carotene concentration per cell (fig. 4.6) between the effluent treatment and control over 16 days ($F^* = 0.530$, $P>0.05$; table 4.4). Neither is there any significant increase over that time ($F^* = 0.543$, $P>0.05$; table 4.4). At day 16, ß-carotene concentration per cell in the control is double that of the effluent treatment (0.917 pg/cell vs. 0.460 pg/cell, table 4.1). However, ß-carotene concentrations per cell are relatively low at this point and there is great variation in cellular concentrations in both treatments (fig. 4.6). No significant differences in ß-carotene concentrations per ml of culture were found between the two treatments ($F^* = 0.576$, $P>0.05$; table 4.4 and fig. 4.7), but a significant increase occurs over time ($F^* = 23.968$, $P<0.05$; table 4.4 and fig. 4.7) as the cell concentration increases. There is a 1.5 times greater yield of ß-carotene per ml of culture in the control than in the effluent treatment (2.545 pg/ml compared to 1.757 pg/ml; table 4.1).

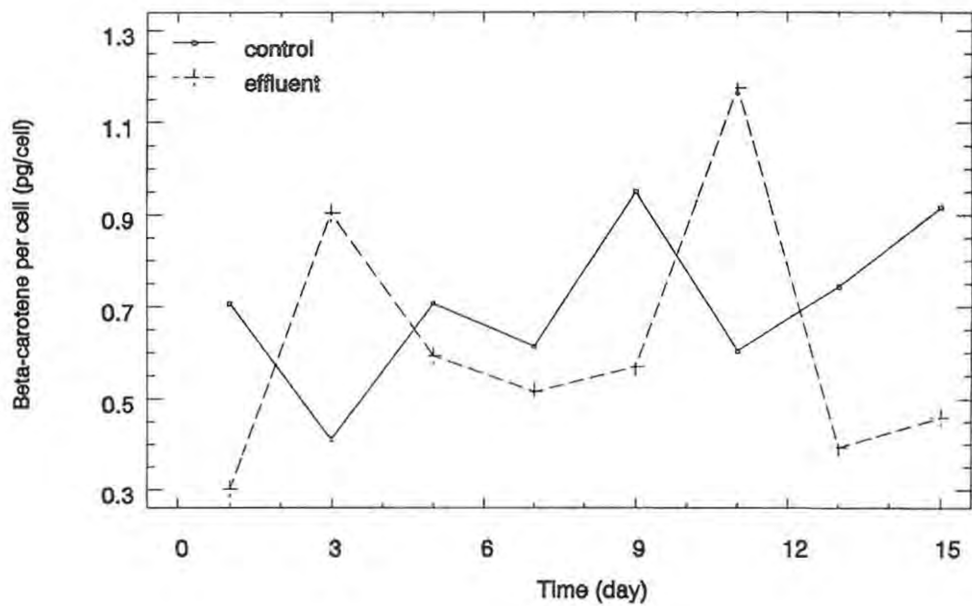

Figure 4.6. Experiment 4a: ß-carotene per cell in the M11 control and effluent treatment grown over 16 days.

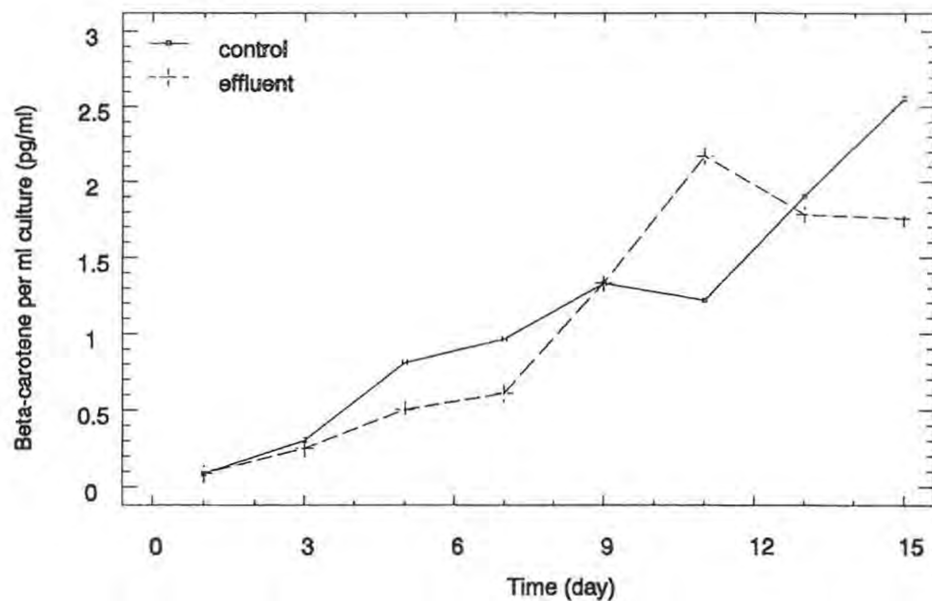

Figure 4.7. Experiment 4a: ß-carotene per ml of culture in the control and effluent treatment grown over 16 days.

	Concentration	Factor	F*	Signif.
Control vs effluent prior to split	ß-carotene per cell	Treatment	0.530	n.s.
		Days	0.543	n.s.
	ß-carotene per ml of culture	Treatment	0.576	n.s.
		Days	23.968	* * *
Control after split into N^+ and N^- M11 media	ß-carotene per cell	Treatment	50.543	* * *
		Days	10.984	* * *
	ß-carotene per ml of culture	Treatment	0.615	n.s.
		Days	8.058	* * *
Effluent treatment after split into N^+ and N^- M11 media	ß-carotene per cell	Treatment	185.360	* * *
		Days	19.400	* * *
	ß-carotene per ml of culture	Treatment	7.273	* *
		Days	40.849	* * *
Control and effluent treatment transferred to N^+ M11 media	ß-carotene per cell	Treatment	2.408	n.s.
		Days	8.474	* * *
	ß-carotene per ml of culture	Treatment	0.836	n.s.
		Days	17.689	* * *
Control and effluent treatment transferred to N^- M11 media	ß-carotene per cell	Treatment	4.945	*
		Days	17.837	* * *
	ß-carotene per ml of culture	Treatment	4.686	*
		Days	10.118	* * *

Table 4.4. Analyses of variance for ß-carotene per cell and per ml of culture for control and effluent treatment prior to and after split between N^+ and N^- M11 media.

Key: * * * = $P<0.0005$; * * = $P<0.005$; * = $P<0.05$; n.s. = no significance

3.2. Transfer to N^+ and N^- M11 media (Experiment 4b).

3.2.1. The control cultures split into N^+ M11 and N^- M11 media.

3.2.1.1. Cell growth.

In fig. 4.8, it can be seen that cells grown in N^+ M11 medium have significantly higher growth than cells grown in N^- M11 medium ($t^* = 6.271$, $P<0.05$; table 4.2). The differences in cell counts between the two media are 104.67×10^4 cell/ml vs. 21.83×10^4 cell/ml, respectively (table 4.1). This is an almost 5 times difference in cell concentration between the two treatments.

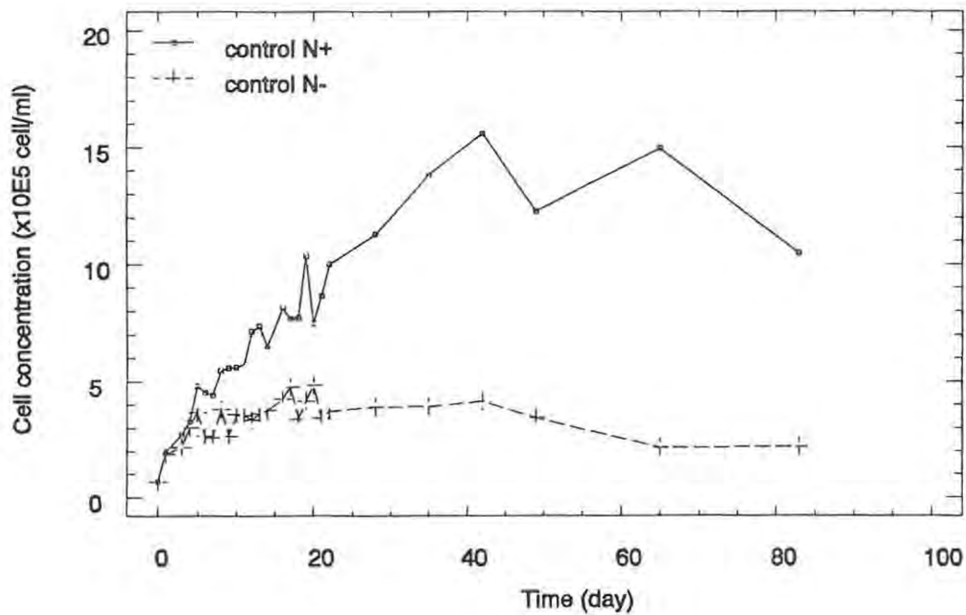

Figure 4.8. Experiment 4b: Growth in the M11 control split into N^+ and N^- M11 media and grown over 83 days.

3.2.1.2. Nutrient utilization.

In fig. 4.9 and 4.10 respectively, nitrate and nitrite are seen, as expected, to be significantly higher in N^+ M11 medium, compared to N^- M11 medium ($F^* = 227.588$, $P<0.05$ for nitrate, and $F^* = 166.842$, $P<0.05$ for nitrite; table 4.3). The concentration of nitrate and nitrite declines significantly over time as the cultures age ($F^* = 14.028$, $P<0.05$ for nitrate, and $F^* = 27.593$, $P<0.05$ for nitrite; table 4.3). Phosphate uptake (fig. 4.11) is seen to differ significantly between the two media ($F^* = 23.654$, $P<0.05$; table 4.3), although it is difficult to discern in which medium phosphate utilization is higher. However, there is a significant decrease in phosphate concentration over time until day 40 in N^+ M11 medium and after day 65 in N^- M11 medium ($F^* = 9.098$, $P<0.05$; table 4.3), before an increase in phosphate concentration is detected in either medium. The phosphate levels in the N^+ M11 medium show a more rapid decline initially and earlier release of phosphate back into the medium than in the N^- M11 medium. Ammonia levels (fig. 4.12) are low and erratic in both medium over the 83 days and no significant difference is detected between treatments or over the duration of the experiment ($F^* = 1.788$, $P>0.05$, and $F^* = 1.824$, $P>0.05$; table 4.3).

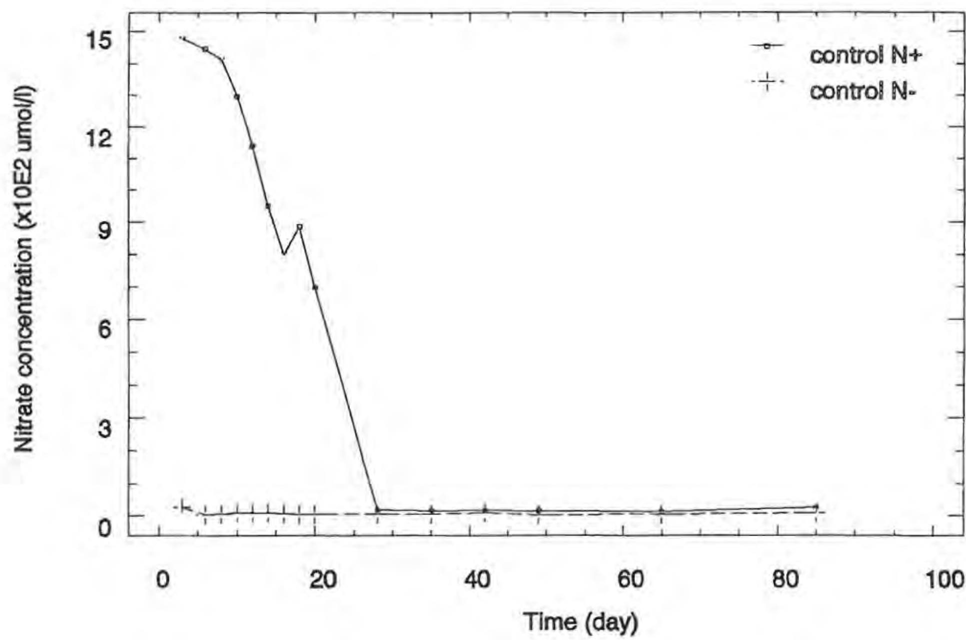

Figure 4.9. Experiment 4b: Nitrate concentration in the M11 control split into N^+ and N^- M11 media and grown over 83 days.

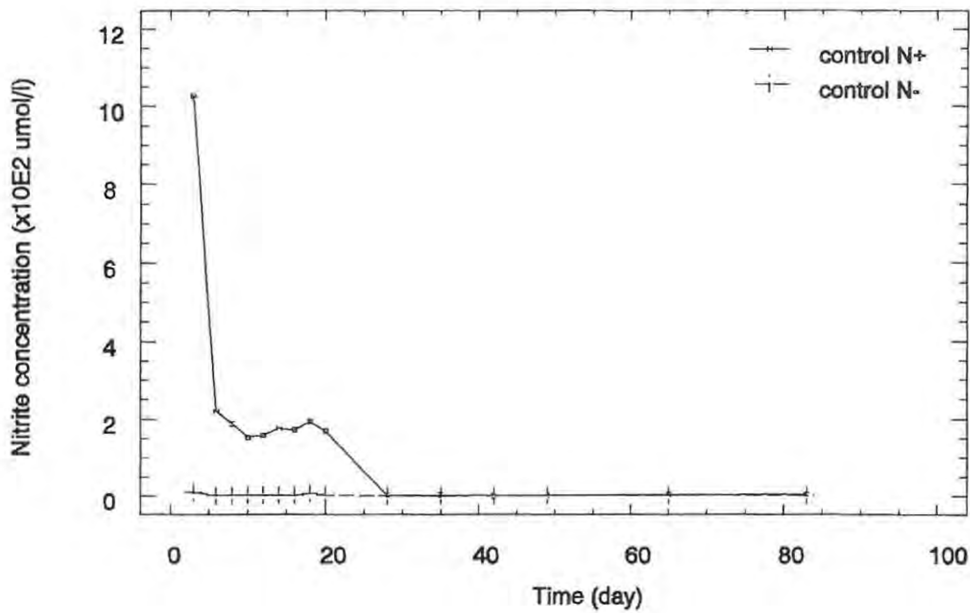

Figure 4.10. Experiment 4b: Nitrite concentration in the M11 control split into N^+ and N^- M11 media and grown over 83 days.

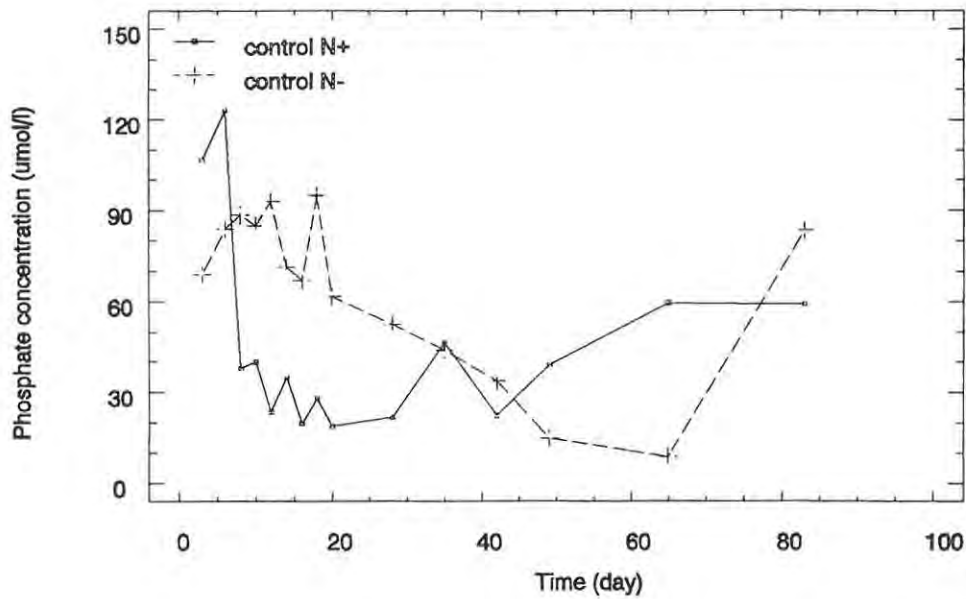

Figure 4.11. Experiment 4b: Phosphate concentration in the M11 control split into N^+ and N^- M11 media and grown over 83 days.

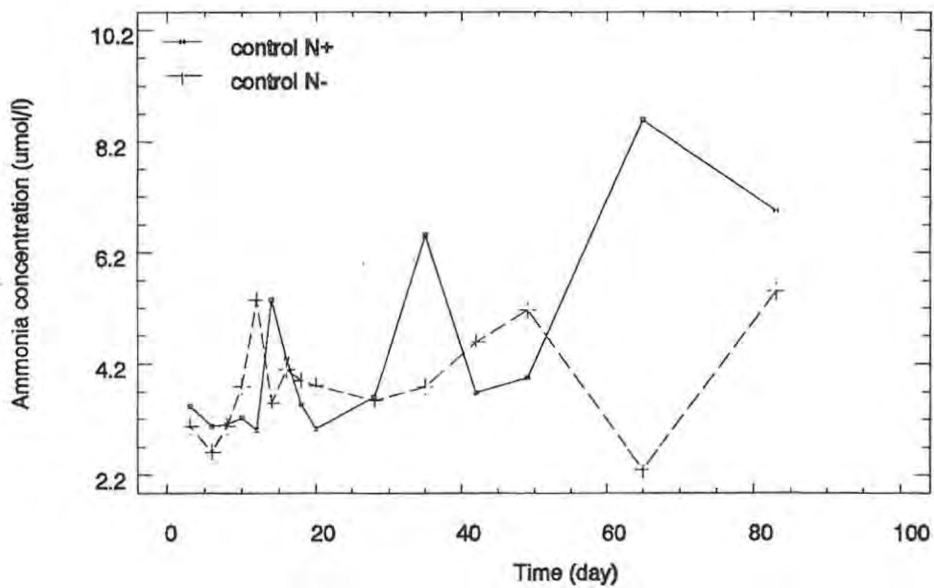

Figure 4.12. Experiment 4b: Ammonia concentration in the M11 control split into N^+ and N^- M11 media and grown over 83 days.

3.2.1.3. ß-carotene production.

Significantly higher concentrations of ß-carotene per cell (fig. 4.13) are present in the N^- M11 medium compared to in the N^+ M11 medium ($F^* = 50.543$, $P < 0.05$; table 4.4), and it increases significantly in both media over time ($F^* = 10.984$, $P < 0.05$; table 4.4). On day 83 there is very little difference between the two treatments (12.683 pg/cell in the control vs. 11.293 pg/cell in the effluent treatment; table 4.1), but on day 65, a 3 times higher yield is obtained in the N^- M11 medium.

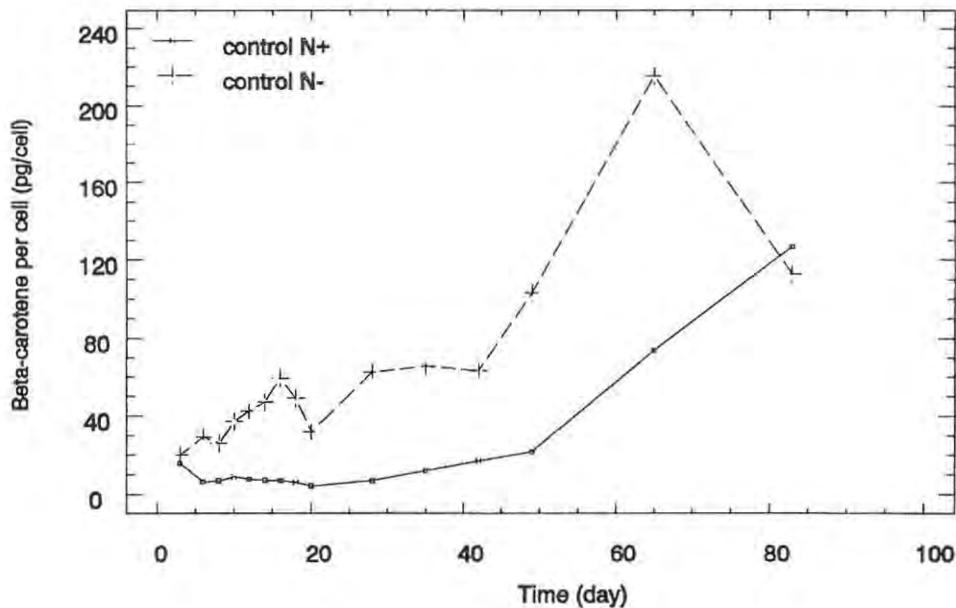

Figure 4.13. Experiment 4b: ß-carotene per cell in the M11 control split into N^+ and N^- M11 media and grown over 83 days.

In fig. 4.14, it can be seen that initially, higher concentrations of ß-carotene per ml of culture are present in the N^- M11 medium until day 49, whereafter a dramatic increase in ß-carotene per ml of culture occurs in the N^+ M11 medium. However, the differences between the two media are statistically insignificant over the 83 days of growth ($F^* = 0.615$, $P > 0.05$; table 4.4), but the increase over time is significant ($F^* = 8.058$, $P < 0.05$; table 4.4). In real terms, the final yields are 50.449 pg/ml in the N^+ M11 medium and 9.818 pg/ml in the N^- M11 medium (table 4.1), a 5 times greater yield in the N^+ M11 medium. Even though the differences in ß-carotene per ml of culture are far greater between the two treatments after day 49 compared to before day 49, there are far more data points in the latter case which can compromise the statistical test. It is therefore prudent to note the limitations of the statistical test for this case.

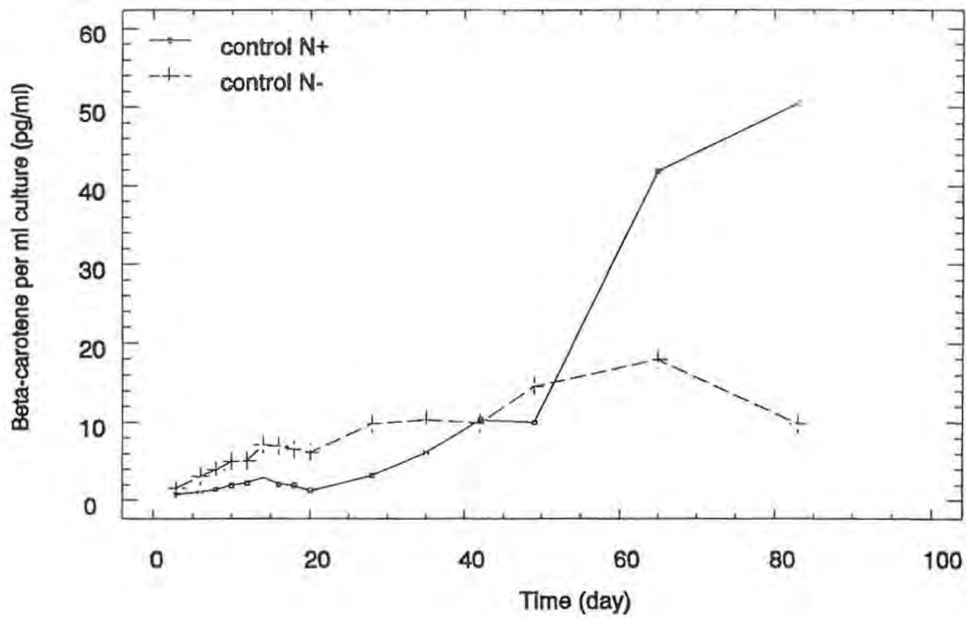

Figure 4.14. Experiment 4b: ß-carotene per ml of culture in the M11 control split into N^+ and N^- M11 media and grown over 83 days.

3.2.2. The effluent treatment split between N^+ and N^- M11 media.

3.2.2.1. Growth.

Cells grown in effluent and then split into the N^+ and N^- M11 media (fig. 4.15) show significantly higher growth rates in N^+ M11 medium ($t^* = 7.156$, $P < 0.05$; table 4.2). Cell counts at the end of 83 days are 128.33×10^4 cell/ml in N^+ M11 medium and 37.67×10^4 cell/ml in N^- M11 medium (table 4.1). This is almost a 3½ times greater cell concentration in the N^+ M11 medium.

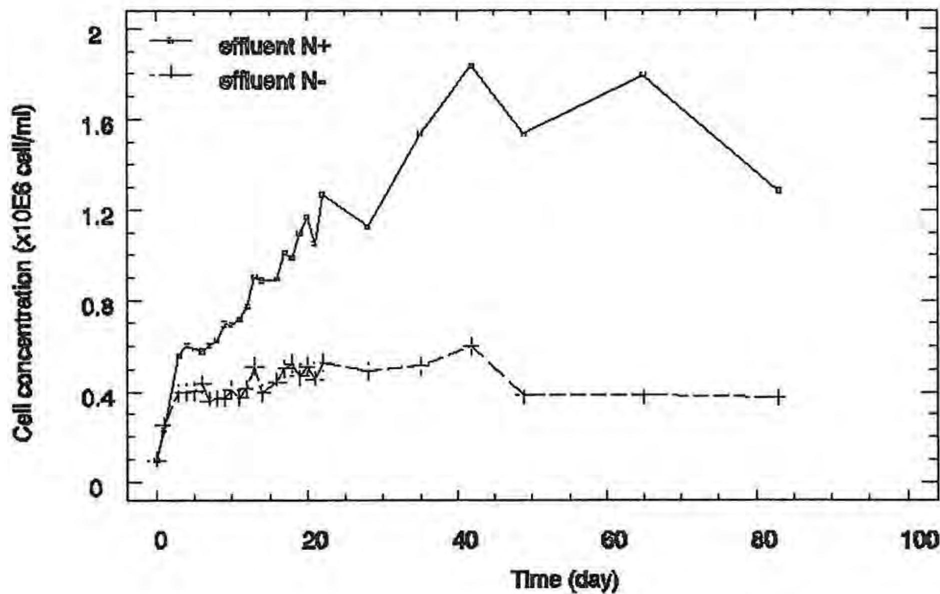

Figure 4.15. Experiment 4b: Growth curves for the effluent treatment split into N^+ and N^- M11 media and grown over 83 days.

3.2.2.2. Nutrient utilization.

In figs. 4.16 and 4.17 respectively, nitrate and nitrite are seen to be significantly higher in N^+ M11 medium as expected ($F^* = 385.495$, $P<0.05$ for nitrate, and $F^* = 444.989$, $P<0.05$ for nitrite; table 4.3). The concentration of nitrate and nitrite is seen to decline significantly over time ($F^* = 18.453$, $P<0.05$ for nitrate, and $F^* = 18.926$, $P<0.05$ for nitrite; table 4.3). In fig. 4.18 phosphate levels are seen to be significantly higher in N^+ M11 medium ($F^* = 20.149$, $P<0.05$; table 4.3), although both media show a similar pattern of phosphate utilization up to day 49. There is also a significant variation in the phosphate concentration over time for both treatments ($F^* = 9.248$, $P<0.05$; table 4.3), although greater release of phosphate into the medium is seen in N^+ M11 medium after day 49. Erratic fluctuations in ammonia occur in both media (fig. 4.19), which makes it difficult to determine trends in ammonia utilization. Significant differences in ammonia concentrations are however detected between the media ($F^* = 6.120$, $P<0.05$; table 4.3) and over time ($F^* = 2.476$, $P<0.05$; table 4.3).

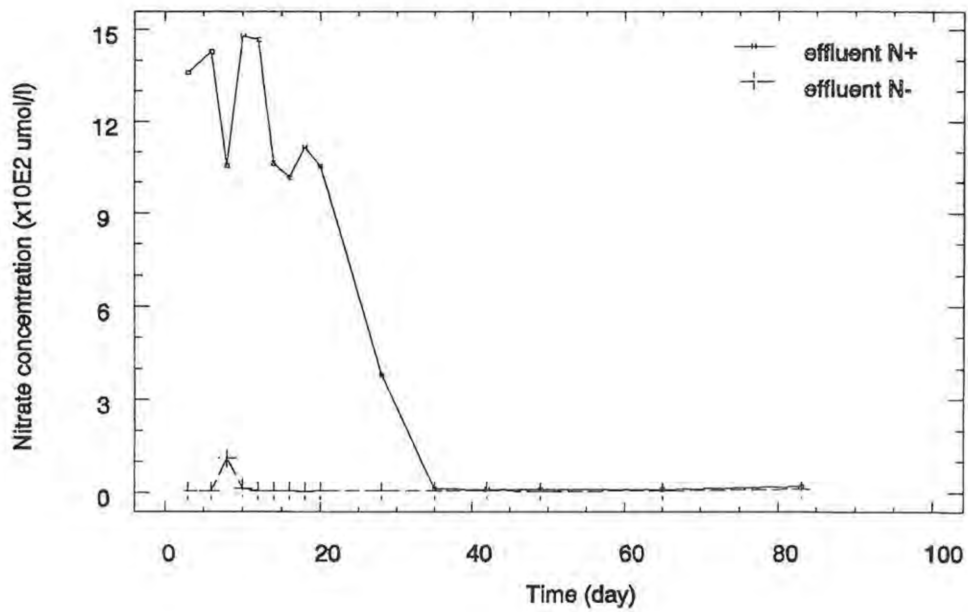

Figure 4.16. Experiment 4b: Nitrate concentration for the effluent treatment split into N^+ and N^- M11 media and grown over 83 days.

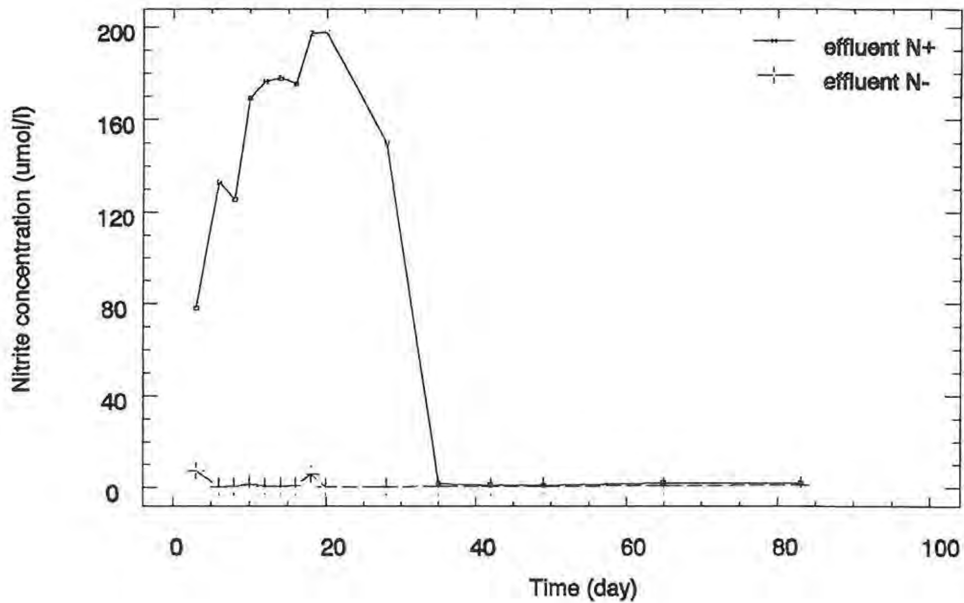

Figure 4.17. Experiment 4b: Nitrite concentration for the effluent treatment split into N^+ and N^- M11 media and grown over 83 days.

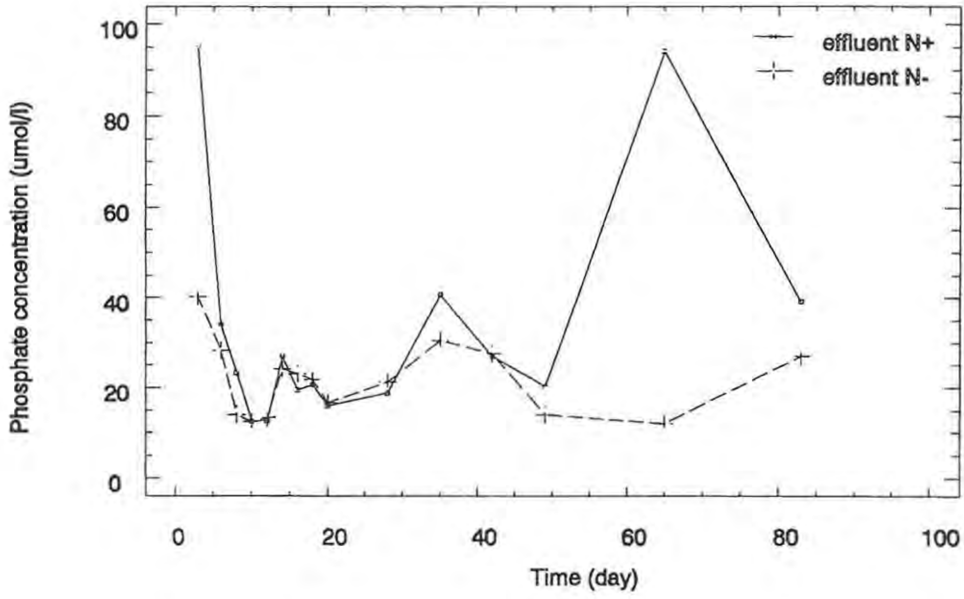

Figure 4.18. Experiment 4b: Phosphate concentration for the effluent treatment split into N^+ and N^- M11 media and grown over 83 days.

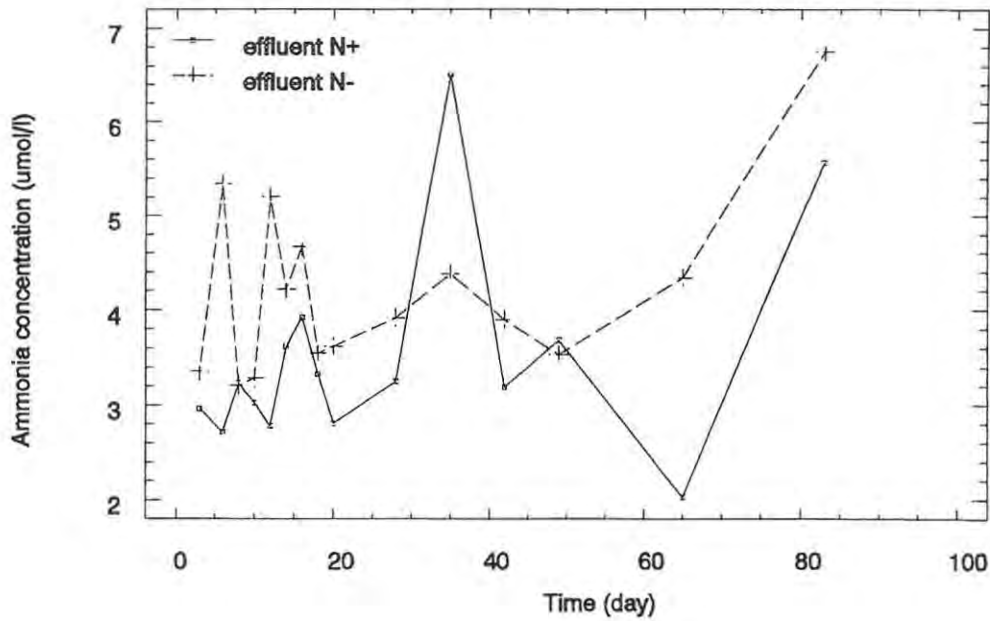

Figure 4.19. Experiment 4b: Ammonia concentration for the effluent treatment split into N^+ and N^- M11 media and grown over 83 days.

3.2.2.3. ß-carotene production.

In fig. 4.20, ß-carotene concentration per cell is seen to be significantly higher in N^- M11 medium ($F^* = 185.360$, $P < 0.05$; table 4.4) and to increase significantly over time in both media ($F^* = 19.400$, $P < 0.05$; table 4.4). The final ß-carotene per cell concentrations in the N^+ and N^- media are 6.725 pg/cell and 13.193 pg/cell respectively (table 4.1), a two-fold increase in the N^- M11 medium. A similar trend is seen initially in the concentration of ß-carotene per ml of culture (fig. 4.21) where it is significantly higher in N^- M11 up to day 49. Thereafter, the concentration of ß-carotene in N^+ M11 medium greatly exceeds that in the N^- M11 medium ($F^* = 7.273$, $P < 0.05$; table 4.4). The final concentrations of ß-carotene per ml of culture on day 85, are 34.152 pg/ml in the N^+ M11 medium and 20.436 pg/ml in the N^- M11 medium (table 4.1). This is over a 1.5 times increase in the N^+ M11 medium. A significant increase in the ß-carotene concentration in both media is also seen over time ($F^* = 40.849$, $P < 0.05$; table 4.4).

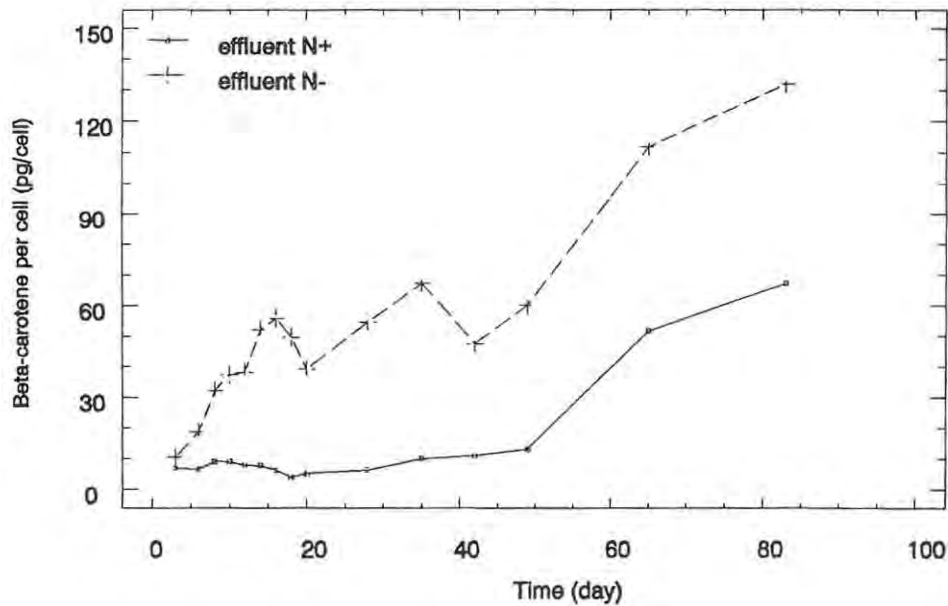

Figure 4.20. Experiment 4b: ß-carotene per cell for the effluent treatment split into N^+ and N^- M11 media and grown over 83 days.

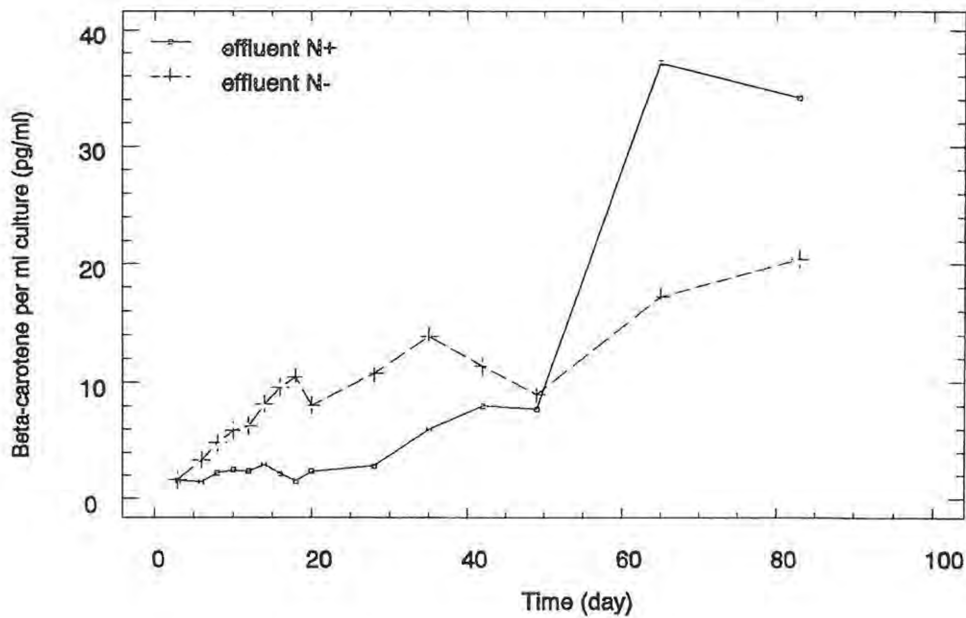

Figure 4.21. Experiment 4b: ß-carotene per ml of culture for the effluent treatment split into N^+ and N^- M11 media and grown over 83 days.

Having compared the control and effluent-grown cultures separately in N^+ and N^- M11 media, the following sections compare the control and effluent grown cultures in either N^+ or N^- M11 media.

3.2.3. The control and effluent treatment transferred to N^+ M11 medium.

3.2.3.1. Cell growth.

When the control and effluent-grown cultures are transferred to fresh N^+ M11 medium (fig. 4.22), significantly higher growth occurs in the cultures derived from the effluent treatment ($t^* = 8.738$, $P<0.05$; table 4.2). After 83 days, the cell concentration in the effluent-derived cultures was 128.33×10^4 cell/ml vs. 104.67×10^4 cell/ml in the control-derived cultures (table 4.1). This is only a 1.25 times greater yield than in the control.

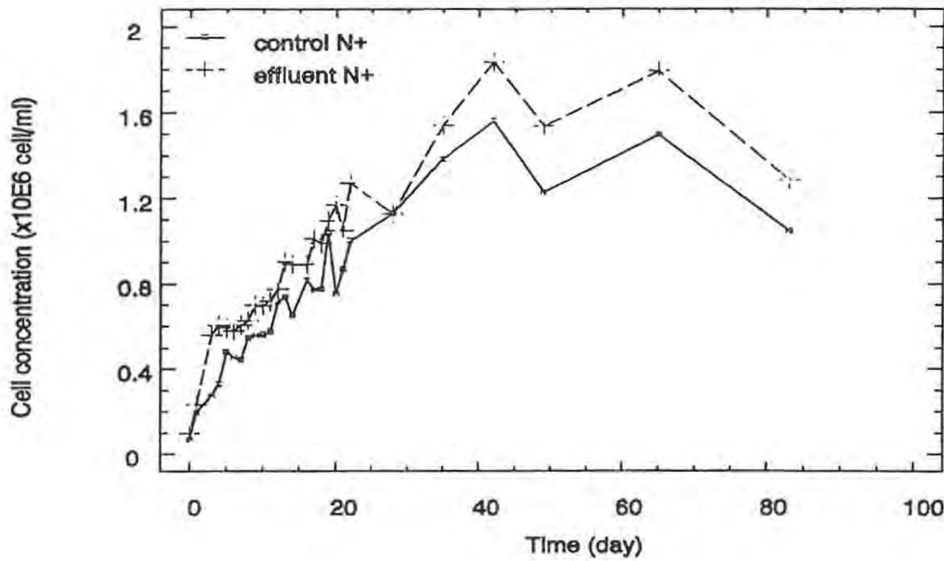

Figure 4.22. Experiment 4b: Growth in M11 control and effluent treatment transferred to fresh M11 medium and grown over 83 days.

3.2.3.2. Nutrient utilization.

In fig. 4.23, no significant difference in nitrate utilization is discernible between the two treatments ($F^* = 2.736$, $P > 0.05$; table 4.3). There is also a decrease of significant proportions in nitrate concentration over time ($F^* = 31.765$, $P < 0.05$; table 4.3), particularly over the first 20 days. Nitrite concentration (fig. 4.24) is significantly higher in the control-derived treatment ($F^* = 18.419$, $P < 0.05$; table 4.3), and in both treatments the concentration decreases significantly over time ($F^* = 28.816$, $P < 0.05$; table 4.3). Phosphate concentrations (fig. 4.25) differ significantly in both treatments, with the control-derived treatments showing the greatest concentration ($F^* = 12.572$, $P < 0.05$; table 4.3). However, both treatments show a similar and significant decrease of phosphate over the first 20 days followed by an increase in the medium ($F^* = 15.589$, $P < 0.05$; table 4.3). Ammonia concentrations (fig. 4.26) are very low, but show significant variation between the two treatments ($F^* = 5.046$, $P < 0.05$; table 4.3) and over time ($F^* = 2.697$, $P < 0.05$; table 4.3). The pattern of ammonia utilization is too erratic to interpret.

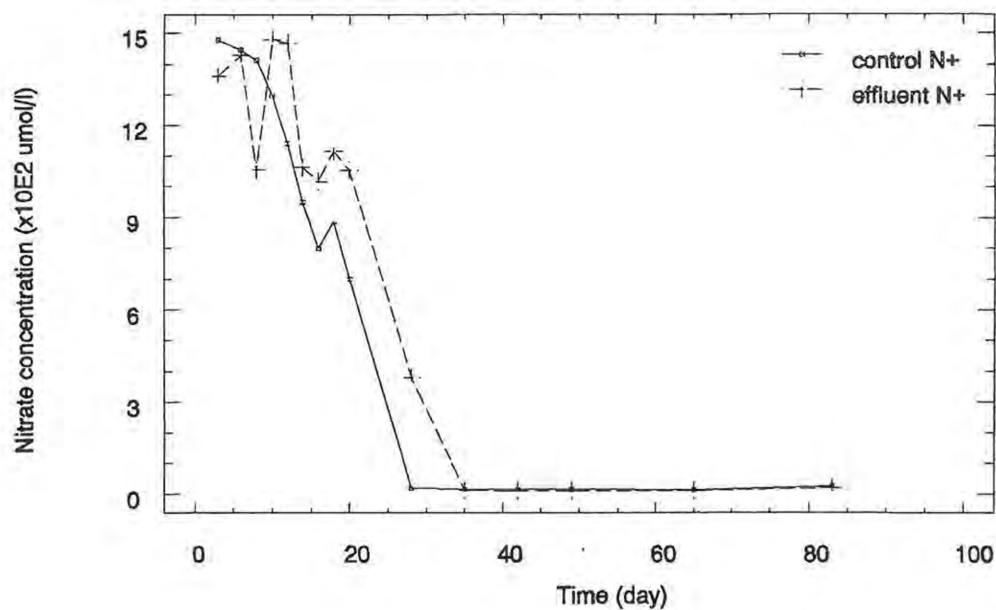

Figure 4.23. Experiment 4b: Nitrate concentration for the M11 control and effluent treatment transferred to fresh M11 medium and grown over 83 days.

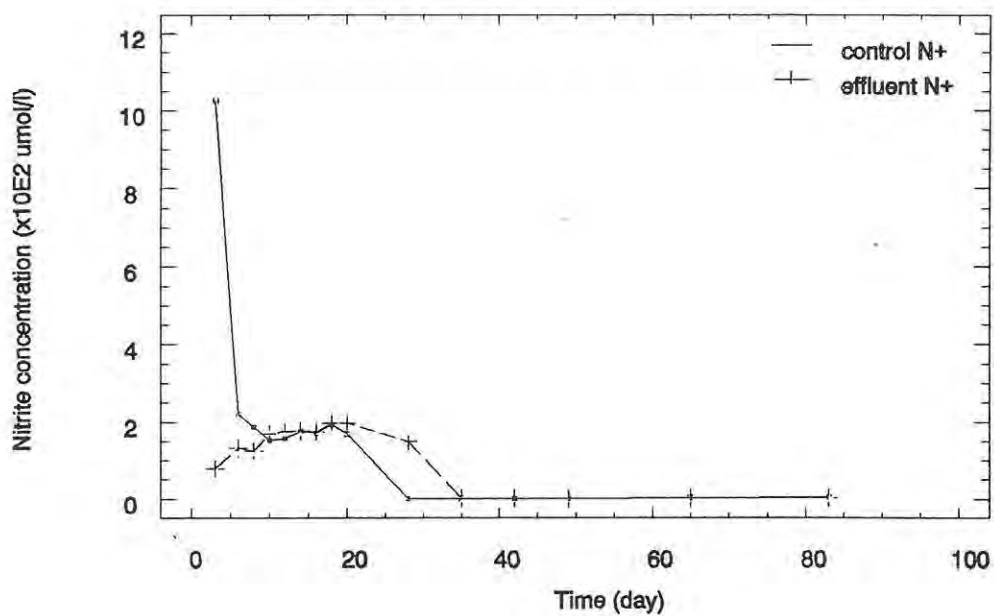

Figure 4.24. Experiment 4b: Nitrite concentration for the M11 control and effluent treatment transferred to fresh M11 medium and grown over 83 days.

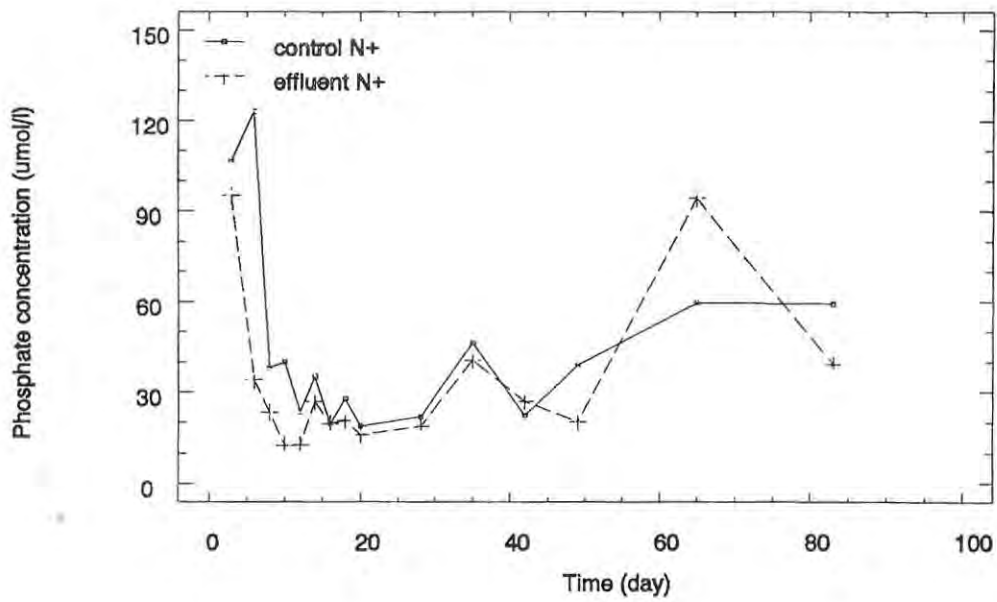

Figure 4.25. Experiment 4b: Phosphate concentration for the M11 control and effluent treatment transferred to fresh M11 medium and grown over 83 days.

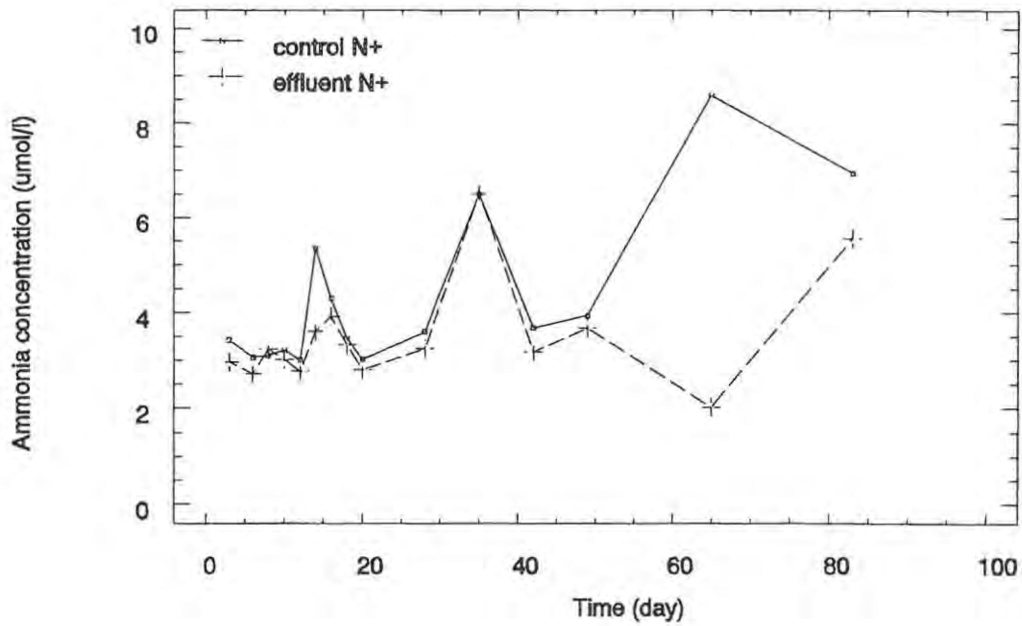

Figure 4.26. Experiment 4b: Ammonia concentration for the M11 control and effluent treatment transferred to fresh M11 medium and grown over 83 days.

3.2.3.3. ß-carotene production.

In fig. 4.27, no significant difference in ß-carotene concentration per cell between the two treatments is apparent ($F^* = 2.408$, $P < 0.05$; table 4.4), but significant increases occur in both treatments over time ($F^* = 8.474$, $P < 0.05$; table 4.4). The concentration after 83 days is 12.683 pg/cell in the control-derived treatment compared to 6.725 pg/cell in the effluent-derived treatment (table 4.1). This is a 2-fold increase over the effluent-derived treatment. A similar trend is observed in ß-carotene concentrations per ml of culture (fig. 4.28), where there is no significant difference between the two treatments ($F^* = 0.836$, $P > 0.05$; table 4.4), but a significant increase is detected in both treatments over time ($F^* = 17.689$, $P < 0.05$; table 4.4). The concentration of ß-carotene per ml of culture for the control- and effluent-derived cultures are 50.449 pg/ml and 34.152 pg/ml respectively (table 4.1). This is a 1.5 times greater yield in the control.

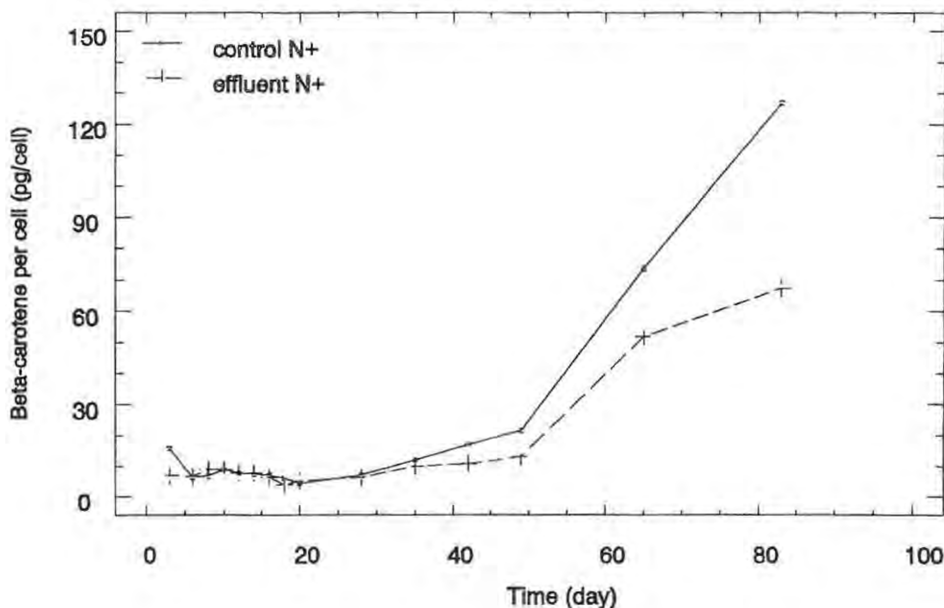

Figure 4.27. Experiment 4b: ß-carotene per cell for the M11 control and effluent treatment transferred to fresh M11 medium and grown over 83 days.

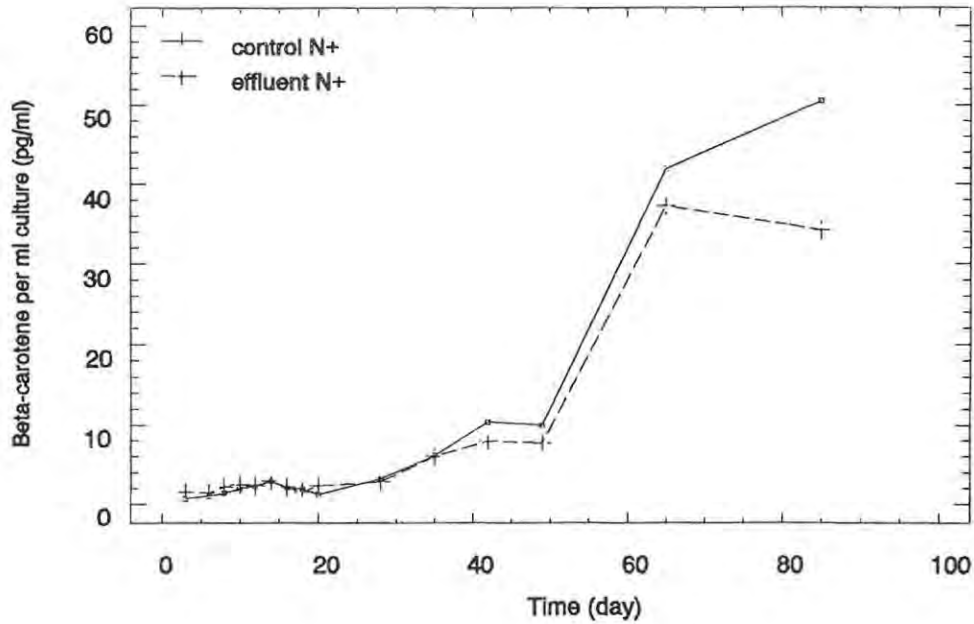

Figure 4.28. Experiment 4b: ß-carotene per ml of culture for the M11 control and effluent treatment transferred to fresh M11 medium and grown over 83 days.

3.2.4. The control and effluent treatments transferred to N^- M11 medium.

3.2.4.1. Cell growth.

In fig. 4.29, the culture originating from the effluent is seen to experience marginally better growth than the culture originating from the control ($t^* = 7.661$; $P < 0.05$; table 4.2). The respective cell counts are 37.67×10^4 cell/ml vs. 21.83×10^{-4} cell/ml (table 4.1). This is a 1.5 times greater yield in the effluent-derived treatment.

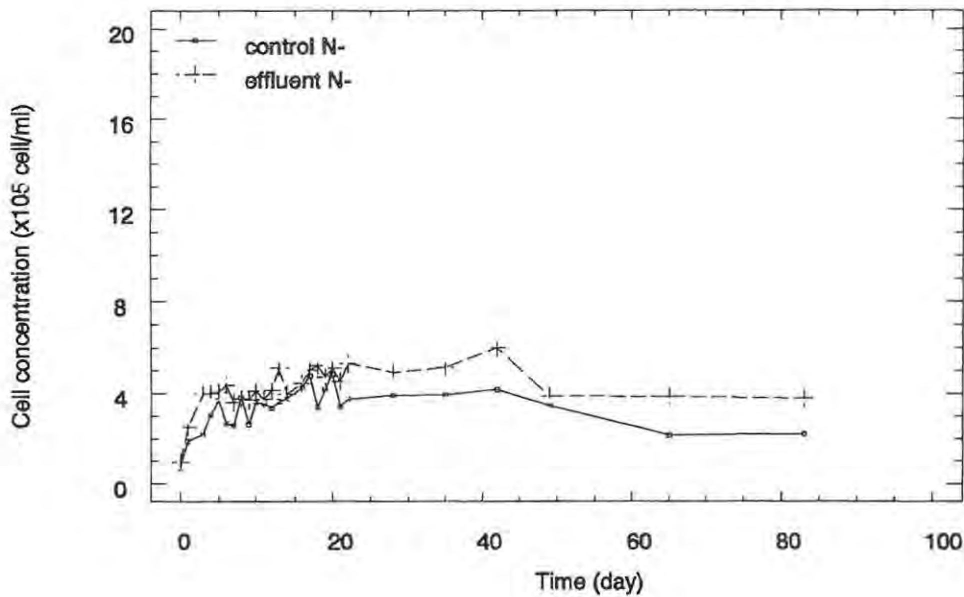

Figure 4.29. Experiment 4b: Growth curves for the M11 control and effluent treatment transferred to N^- M11 medium and grown over 83 days.

3.2.4.2. Nutrient utilization.

In fig. 4.30 and 4.31 respectively, there is no significant difference in nitrate and nitrite utilization between the two treatments ($F^* = 0.005$, $P > 0.05$ for nitrate, and $F^* = 1.288$, $P > 0.05$ for nitrite; table 4.3). No significant variation in nitrate is evident over time ($F^* = 1.674$, $P > 0.05$; table 4.3), although a significant decrease in nitrite from day 3 is observed ($F^* = 14.538$, $P < 0.05$; table 4.3). Significantly higher concentrations of phosphate (fig. 4.32) are detected in the control-derived cultures ($F^* = 192.522$, $P < 0.05$; table 4.3), and a significant decrease over time is evident up to day 65 ($F^* = 6.393$, $P < 0.05$; table 4.3), particularly in the control-derived treatment. After day 65 phosphate is seen to increase in the medium. The low ammonia concentrations (fig. 4.33) differ significantly between the two treatments ($F^* = 2.374$, $P < 0.05$; table 4.3). Although erratic, they do not differ over time ($F^* = 1.715$, $P > 0.05$; table 4.3).

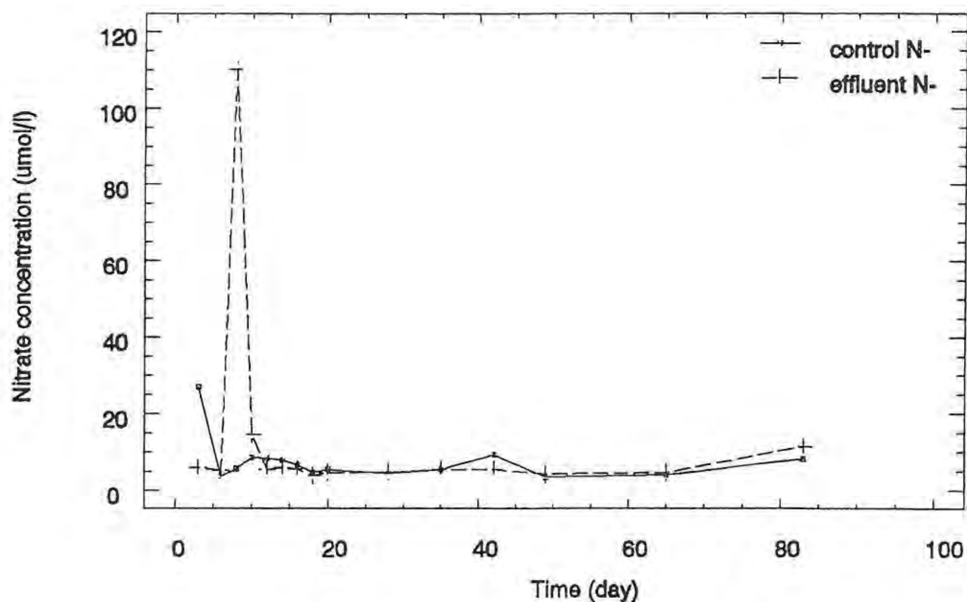

Figure 4.30. Experiment 4b: Nitrate concentration for the M11 control and effluent treatment transferred to N⁻ M11 media and grown over 83 days.

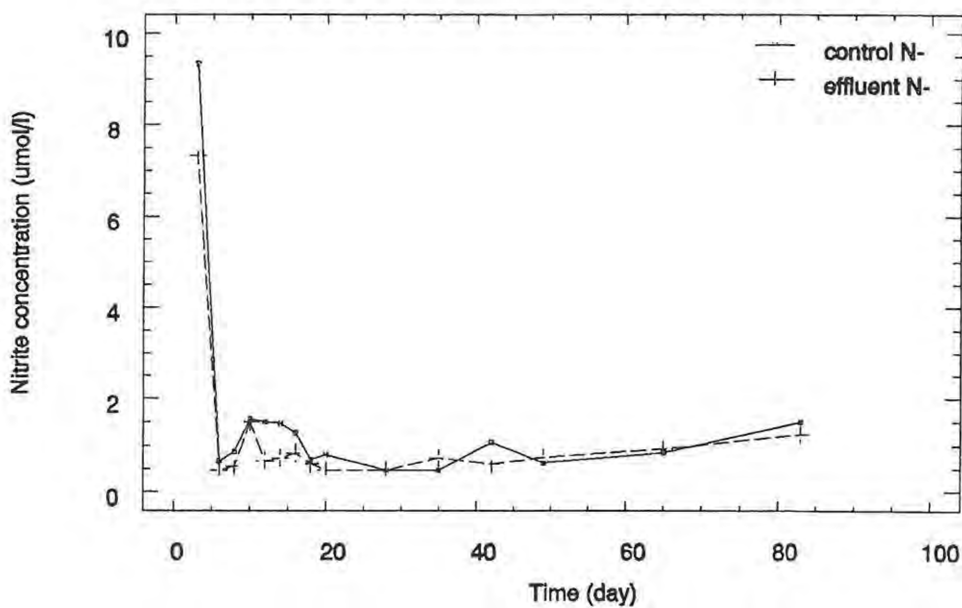

Figure 4.31. Experiment 4b: Nitrite concentration for the M11 control and effluent treatment transferred to N⁻ M11 media and grown over 83 days.

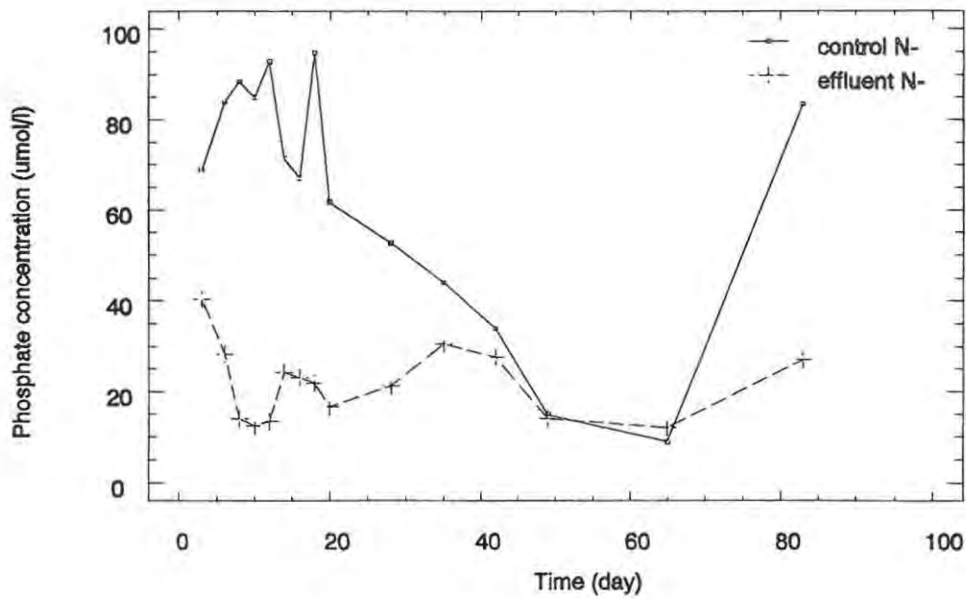

Figure 4.32. Experiment 4b: Phosphate concentration for the M11 control and effluent treatment transferred to N⁻ M11 medium and grown over 83 days.

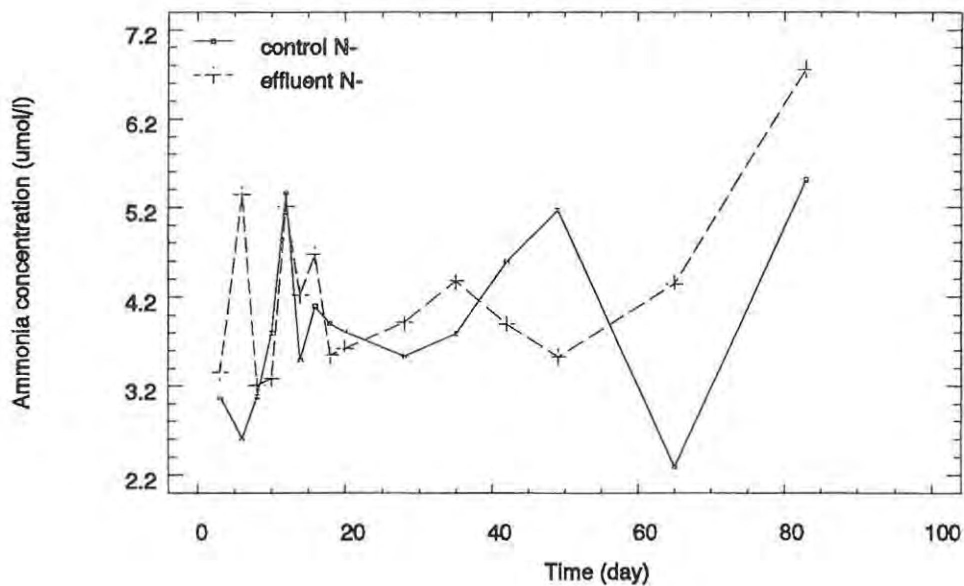

Figure 4.33. Experiment 4b: Ammonia concentration for the M11 control and effluent treatment transferred to N⁻ M11 medium and grown over 83 days.

3.2.4.3. ß-carotene production.

The increase in ß-carotene concentration per cell (fig. 4.34) is significantly higher in the control-derived cultures ($F^* = 4.945$, $P < 0.05$; table 4.4), but there is very little difference between the two treatments on day 83 (11.293 pg/cell for the control-derived treatment compared to 13.193 pg/cell for the effluent-derived treatment; table 4.1). Both treatments show a significant increase in ß-carotene per cell over time ($F^* = 17.837$, $P < 0.05$; table 4.4). However, ß-carotene per ml of culture (fig. 4.35) is significantly higher in the effluent-derived treatment ($F^* = 4.686$, $P < 0.05$; table 4.4). This is a two-fold greater yield (20.436 pg/ml compared to 9.818 pg/ml; table 4.1). There is also a significant increase in ß-carotene per ml of culture over time for both treatments (F^* 10.118, $P < 0.05$; table 4.4).

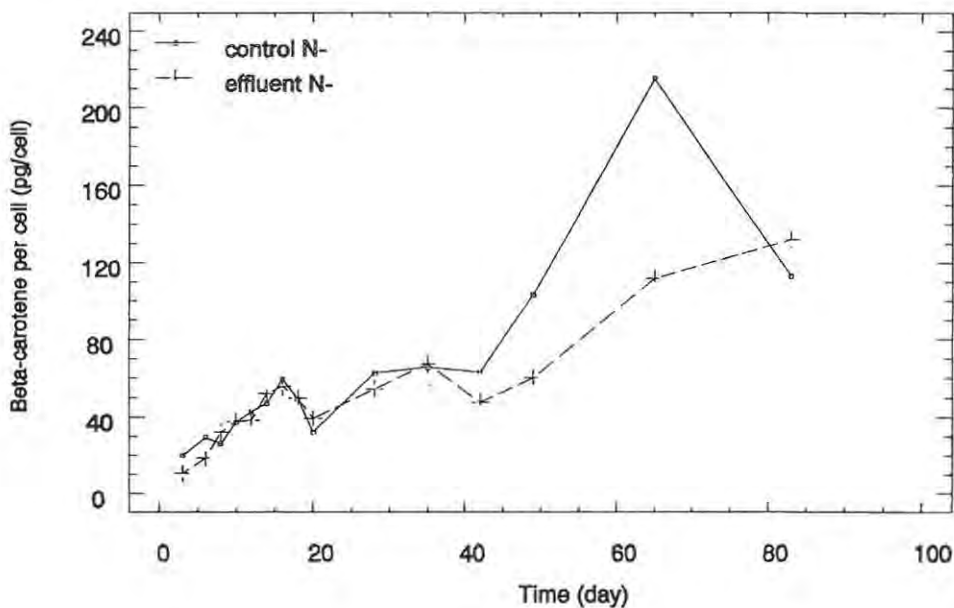

Figure 4.34. Experiment 4b: ß-carotene per cell for the M11 control and effluent treatment transferred to N^- M11 media and grown over 83 days.

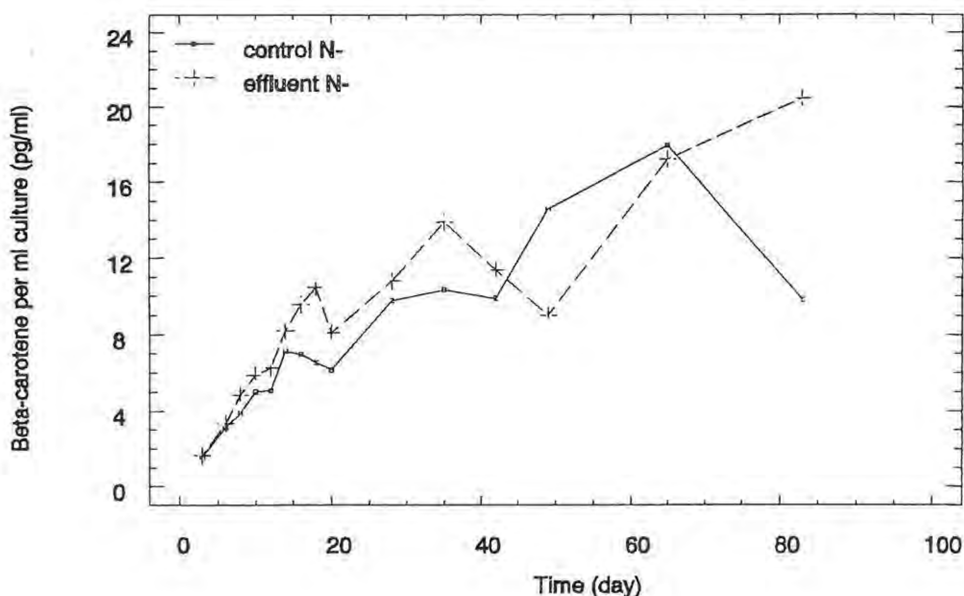

Figure 4.35. Experiment 4b: ß-carotene per ml of culture for the M11 control and effluent treatment transferred to N^- M11 medium and grown over 83 days.

4. Discussion.

4.1. Control and effluent-grown cultures prior to transfer to N^+ and N^- M11 media.

4.1.1. Cell growth.

Cells grown in the 25% ponded tannery effluent showed better growth than the control. Initially better growth was obtained in the control cultures, but after day 7, growth in the effluent exeeds that of the control (Experiment 4a). It would appear that the growth of the cells in the effluent are retarded over the first few days, but this is compensated for by much better growth later.

The lower initial growth rate in the effluent cultures may possibly be due to the presence of growth inhibitors in the effluent. The role of bacteria in the decomposition of the effluent needs to be elucidated as they may be responsible for a reduction or inactivation of any growth inhibitors over a period of time. Furthermore, bacterial action may release additional nutrients from the effluent.

4.1.2. Nutrient utilization.

Decreases in all nutrients in both media occurs over time (with the exception of ammonia in the control medium), which correlates with increases in cell concentrations. This indicates that incorporation into cellular biomass is occurring. Nitrate levels were found to be higher in the effluent medium culture and this can be ascribed to additional nitrate present in the effluent (table 1.1). Both

treatments show a steady decline in nitrate levels over the 16 days (fig. 4.2), which is matched by a steady increase in cell number (fig. 4.1). Nitrite levels were an order of magnitude lower than nitrate levels. Higher nitrite levels occur in the effluent-grown cultures and both cultures show a depletion in nitrite over time (fig. 4.3), although less markedly than in the case of the nitrate. It would appear that nitrate is preferentially taken up over nitrite as a source of nitrogen and this would seem to be most likely, given the higher concentrations of nitrate available. Preferential uptake of nitrate over nitrite has previously been recorded at high nitrate concentrations (McCarthy, 1980). Phosphate levels appear to be identical in both treatments at the start of the experiment, indicating that the effluent is poor in phosphate (table 3.1). No significant differences in phosphate utilisation is apparent between the two cultures over the 16 days, but a significant reduction in phosphate is recorded for both treatments over this period (fig. 4.4).

Ammonia levels are initially higher in the effluent medium than in the control, and this appears to be due to the effluent input. As a result, there is a significant difference in ammonia levels between the two treatments. Although ammonia is generally low in the 25% strength effluent ($\pm 6x10^{-4}$g/l) it is known to be an inhibitor of photosynthesis at low concentrations (Turpin, 1983). However, Turpin (1983) has shown ammonia concentrations higher than 10^{-4}g/l to suppress photosynthesis in *D. tertiolecta* initially and then to enhance it in the long term. At day 7, ammonia levels in the effluent treatment drop below $2x10^{-4}$g/l (fig. 4.5) and cell concentrations begin to exceed those in the control (fig. 4.1). Whether ammonia is responsible for the growth performance in the effluent treatment remains to be determined. No apparent change in ammonia seems to occur in the control cultures, which remain at low levels. Bacterial decomposition of effluent, or even dead algal cells, may release sufficient quantities of ammonia into the medium which either adversely effects or enhances the algal growth rate. The relationship between bacteria and *Dunaliella* is unclear.

4.1.3. ß-carotene production.

ß-carotene production and accumulation does not seem to be influenced by the effluent or nutrient depletion within this time period when comparisons are made between ß-carotene concentration per cell and cell concentrations. However, ß-carotene concentration per ml of culture rises significantly over time. This indicates that synthesis of ß-carotene is occurring during this time and its increase in the culture is related to increasing cell concentration. The similarity in ß-carotene concentration per cell and per ml of culture in the control and effluent treatments is statistically significant and indicates that no induction or repression of ß-carotene synthesis has been caused by the effluent treatment.

4.2. Transfer of cultures to N^+ and N^- M11 media.

4.2.1. Cell growth.

Both the cultures derived from the effluent treatment and control have a higher growth rate in N^+ M11 medium than in N^- M11 medium. This indicates the importance of nitrogen, largely in the form of nitrate, as a growth limiting factor. The effluent-derived cultures, whether they were grown in N^+ or N^- M11 media, are shown to have better growth rates than the control-derived cultures in equivalent media. It could be speculated that a higher amount of luxury uptake of nitrate (which entails a greater consumption of nitrates from the medium than is needed) possibly occurs in the effluent treatment cultures than the control. It has previously been shown that the effluent medium has higher nitrate levels than in the control medium, and the higher the nitrate in the culture, the greater the luxury uptake (Wikfors, 1986). Both treatments were washed twice in sterile 1.5M saline after centrifugation, so carry over of interstitial nutrients during cell resuspension can largely be ruled out.

4.2.2. Nutrient utilization.

Comparisons of nitrate and nitrite levels between the control-derived cultures transferred to N^+ and N^- M11 media, shows that the nitrogen level in the N^+ culture reaches parity with the nitrogen deficient culture after 28 days of growth. Cell growth in the N^+ M11 cultures seems to continue until day 42 before a decline is seen, whereas growth in the N^- M11 cultures reaches stationary phase at about day 20. It appears that a certain amount of growth can still occur in the presence of very low quantities of nitrate and nitrite, and enhancement of carbohydrate synthesis occurs during this phase (Semenenko and Abdullaev, 1980). It is possible that release of assimilable nitrogen from dead cells through bacterial action, may be a source of nutrients to maintain the cell populations at this time. The cultures in N^+ and N^- M11 media derived from the effluent treatment show similar trends in nitrite utilisation as the cultures derived from the controls. However, nitrogen levels (in the form of nitrate and nitrite) in N^+ M11 medium, only reach parity with N^- M11 medium on day 35. Although there is no significant difference in nitrate and nitrogen utilization between the control and effluent-derived cultures in either N^+ or N^- M11 media, the growth rates of cells in effluent-derived cultures were significantly higher than the controls. Luxury consumption of nitrogen has been reported in *D. tertiolecta* (Wikfors, 1986), which may explain why effluent exposed cells grow better than the control cells when placed in fresh N^- M11 medium. The role of nitrogen in cell growth has been established here, and the better growth in effluent exposed cultures may be due to higher nitrate concentrations or to the presence of a possible growth factor or factors.

In all the treatments phosphorous declines with cell growth, but is released into the media as the cultures begin to senesce. A possible explanation is that during the initial growth stages, luxury uptake of phosphate by the cells occurs. Luxury uptake of phosphorous in freshwater algae has been

documented (Fogg, 1966). The surplus is stored as polyphosphates (Nalewajko and Lean, 1980) and can be drawn upon when the supply in the medium is exhausted, thereby maintaining growth in culture when phosphate levels have been depleted. *Dunaliella* has a low requirement for phosphate, less than 0.025 g/l (Borowitzka and Borowitzka, 1988). During the decline of the cell population, bacterial action converts organic phosphates into inorganic orthophosphate (Cole, 1979), which becomes available for algal metabolism. A decline in cell numbers in N^+ M11 medium is observed after day 42, which is accompanied by an increase in phosphate in the medium. Cell numbers are generally low throughout the 83 days of growth, so a marked decrease in cell number is not evident with the release of phosphate in N^- M11 cultures after day 65. As to why there is no concomitant increase in nitrate or nitrite in all the media, it may be speculated that a loss of nitrogen from the medium in the form of ammonia may occur during the decomposition of organic nitrogen via ammonia to nitrate and nitrite. Alternatively, bacterial uptake of organic nitrogen may also be occurring.

Ammonia levels are difficult to interpret because of the great flux experienced over time. Their concentrations in the medium are very low and because of their low values, their importance as a nitrogen source is questionable. However, the presence of an ammonium-assimilating enzyme, glutamine synthetase, was suggested by Turpin (1983) in *D. tertiolecta*. It is possible that ammonia may have a role in sustaining growth immediately after nitrate and nitrite exhaustion. Their presence may also be due to activity of bacteria in the culture, especially during the breakdown of organic material (McCarthy, 1980).

It can be concluded from these experiments, that nitrogen in the form of nitrate and nitrite appear to be the primary nutrients in determining growth of the cultures, as they are steadily removed from the medium. Phosphate utilisation appears to be determined by nitrogen utilisation, as uptake of phosphates is retarded in N^- M11 cultures, and its release into the medium seems to be linked to decreases in cell concentration. Ammonia levels show a high degree of flux throughout their monitoring, and are present at very low levels.

4.3. Effects of ageing and nitrogen stress on ß-carotene production.

ß-carotene synthesis is a normal function of growing cells as can be seen from its presence in non-nutrient-stressed cells. However, the availability of nitrogen in the medium plays an important part in its synthesis (Mil'ko, 1963a). All nitrogen-stressed cultures, whether they originate from the effluent treatment or from the control, show earlier accumulation of ß-carotene per cell compared to unstressed cultures. This inverse relationship between ß-carotene content in *D. salina* and its specific growth rate has previously been documented (Ben-Amotz *et al*, 1982). Rapid accumulation of ß-carotene in N^+ M11 cultures begins once nitrogen has been depleted from the medium. The lack of nitrogen retards growth, and this is manifested by a lower cell division rate, and hence a greater

accumulation of ß-carotene per cell than the more rapidly dividing cultures (Ben-Amotz and Avron, 1983b). This is accompanied by a change in colour of the growth-retarded culture from green to orange, which was also observed in these experiments. Semenenko and Abdullaev (1980) have shown that nitrogen deficiency leads to more than a tripling of the ß-carotene content in *D. salina* cells under conditions of extensive culture. In this experiment, a three-fold higher ß-carotene yield was obtained in nitrogen-stressed cells originating from the control (at day 65), while a two-fold higher yield was obtained in nitrogen-stressed effluent-derived cultures.

Overall, final ß-carotene yield is seen to be higher in unstressed cultures. This is because of a higher cell growth and therefore a larger biomass, which when aged, will ultimately produce more ß-carotene than nitrogen-stressed cultures with lower cell concentrations. This finding is supported by Semenenko and Abdullaev (1980) who found that ß-carotene per cell is higher in nitrogen-limited cells, but that the total yield of ß-carotene per unit of suspension volume is dependent on the abundance of cells with the usual content of ß-carotene in them.

4.4. Effects of effluent on ß-carotene production.

Where the control and effluent treatment cultures are transferred to fresh N^+ M11 medium, no difference in ß-carotene production per cell or per ml of culture is evident. This indicates that under non-nitrogen-stressed conditions, pre-exposure to effluent treatment does not influence yields in ß-carotene significantly, although higher cell growth is attained after effluent treatment. However, when cells from the control and effluent treatment are nutrient stressed, the control shows a higher ß-carotene concentration per cell than the effluent-treated cells. A reversal of these trends occurs when ß-carotene per ml of culture is examined. This may be explained by the fact that effluent-treated cultures show a significantly better growth than the control under nutrient limitation. Accumulation of ß-carotene per cell in the control-derived cultures is higher due to the lower rate of cell division. However, because of the higher biomass produced by the effluent-treated cultures, the ß-carotene per ml of these cultures is higher.

5. Conclusion.

In conclusion, it appears that 25% ponded tannery effluent actually enhances growth, even after the culture is transferred to fresh M11 medium. There is no evidence here that the effluent directly initiates or adversely effects production ability of ß-carotenogenesis, although indirectly it influences final ß-carotene yields by enhancing cell growth. Nitrogen in the form of nitrate and nitrite is a growth-limiting factor that is important for cell growth and influences ß-carotene production. M11 medium enriched with 25% ponded tannery effluent is a better culture medium for cultivating *Dunaliella* than M11 medium alone. If a system for the maximum production of ß-carotene per volume of medium in ponded tannery effluent were to be suggested, transfer to a nitrogen-deficient

medium is not recommended, as lower cell yields are obtained and ultimately, lower ß-carotene yields. Cultures allowed to age naturally ultimately have higher ß-carotene yields as indicated by the ageing experiments.

CHAPTER 5

GROWTH IN COMBINED PROCESSES EFFLUENT

1. Introduction.

Combined processes effluent is composed of the entire effluent stream generated from the start of the tanning process, from the soaking of preserved hides, through to the final wastes of hide processing (tanning and dyeing). Fresh effluent direct from the tanning house of the Western Tanning Company was investigated for its suitability as a medium for *Dunaliella* culture. The difference between fresh effluent and the ponded tannery effluent, is that the latter is diluted by the inflow of water from rainfall as well as being altered by the resident pond microflora. This allows potentially harmful compounds such as antimicrobial agents to be broken down and other toxic compounds, such as various forms of sulphur and heavy metals, to be volatilised or precipitated.

Fresh combined processes effluent was found to be highly toxic to *Dunaliella*, even down to 25% strength. This lead to the investigation of anaerobic digestion as a form of pre-treatment to attempt to reduce the toxicity of this effluent. Anaerobic digestion converts organic nitrogen to ammonia, reduces sulphates to sulphides (which can be removed later by aeration) and precipitates heavy metal ions (Jackson-Moss, 1991).

2. Methods.

2.1. Growth on fresh effluent (Experiment 5a).

This experiment was designed to test whether different dilutions of the fresh combined processes effluent could be used to support the growth of *Dunaliella salina*. The dilutions used here were full strength, 50%, 33% and 25% strength effluent enriched with M11 media, as well as an effluent-free M11-enriched sea-water control.

2.2. Growth on anaerobically digested effluent (Experiment 5b).

The same effluent as Experiment 5a was used, except that an anaerobic digestion step was included before algal culture to degrade any toxic compounds present in the effluent. The digestion step took 10 weeks and took place in an air-tight plastic 25l drum. Gas production was monitored by the displacement of a fixed volume of water in a reservoir connected by a gas line to the headspace of the drum. Once gas production had halted, anaerobic digestion was terminated. Thereafter the digested effluent was diluted and used for growth media. The effluent strengths used were full strength, 50%, 33%, 25% and 10%, and were all enriched with M11 media. An effluent-free M11-enriched sea-water control was included. Cells were counted over 27 days.

3. **Results.**

3.1. Growth on fresh effluent (Experiment 5a).

The composition of the fresh combined processes effluent is shown in table 5.1. No growth was recorded in either the full strength, 50% or 25% effluent. Cells were seen to be ruptured and no viable cells were detected 24 hours after inoculation. The cultures were checked the following two days to see if any cells had survived, and thereafter the experiment was terminated.

	Fresh	Digested
pH	7.20	7.30
conductivity	1383mS/cm	1220mS/cm
PV	1.463g/l	0.705g/l
COD	6.240g/l	10.280g/l
TDS	8.200g/l	7.700g/l
TDIS	5.300g/l	6.100g/l
SS	0.230g/l	5.486g/l
nitrate	0.637g/l	0.666g/l
ammonia	0.721g/l	0.799g/l
phosphate	9.6×10^{-4}g/l	0.096g/l
sodium	0.947M	0.722M
chloride	1.148M	0.036M
sulphate	0.312g/l	2.750g/l
sulphide	0.771g/l	0.170g/l
chromium	4×10^{-4}	0.048g/l

Table 5.1. Characteristics of Western Tanning Company's combined processes effluent prior to and after anaerobic digestion.

3.2. Growth on anaerobically digested effluent (Experiment 5b).

The composition of the anaerobically digested combined processes effluent is shown in table 5.1. Anaerobic digestion has lowered the chemical oxgen demand (COD) and in particular, the suspended solids. Nitrate and ammonia levels do not differ markedly between the fresh and digested effluent, but phosphate seems to have been reduced to a large extent. A decrease in sulphate and sulphide occurs in the digested effluent. Most noticeably, there is a great increase in sodium, and especially chlorides, in the digested effluent. No growth was obtained with cells grown in the anaerobically digested combined processes effluent above 10% strength.

Only the 10% strength effluent supported growth (fig. 5.1). However, no significant difference in growth rates is evident between the control and 10% strength digested effluent over the 27 days of

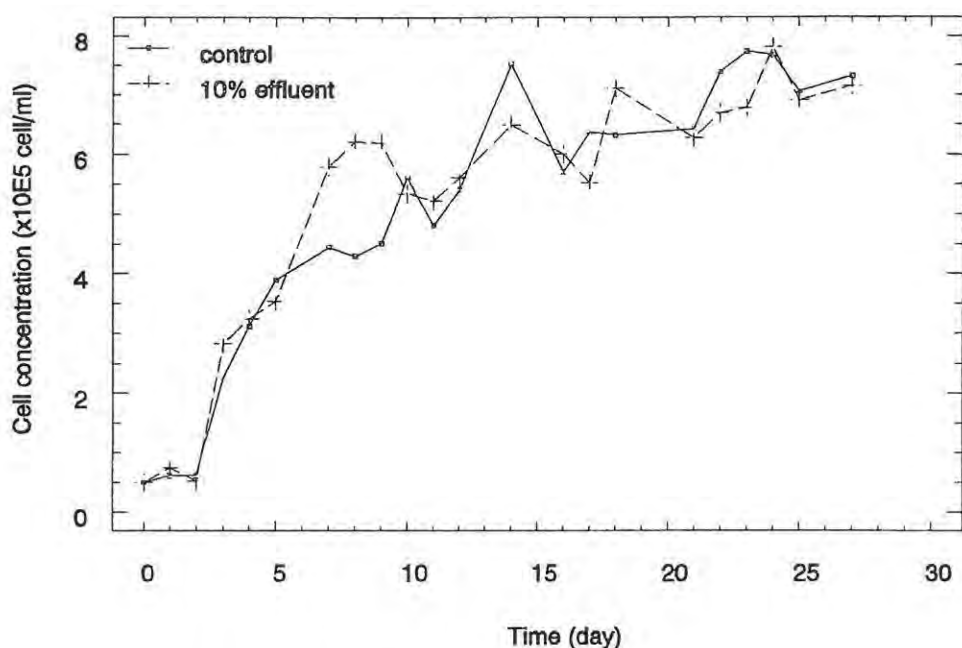

Figure 5.1. Growth in anaerobically-digested combined processes effluent.

	Treatment	Cell count (cell/ml)	Control vs 10% strength effluent	
			t*	Signif.
Cell count (cell/ml)	Control	73.17×10^4 $\pm 4.51 \times 10^4$	0.791	n.s.
	10% strength effluent	71.50×10^4 $\pm 9.66 \times 10^4$		
ß-carotene per cell (pg/cell)	Control	15.000 ±3.437	—	—
	10% strength effluent	19.814 ±2.053		
ß-carotene per ml culture (pg/ml)	Control	48.191 ±2.660	—	—
	10% strength effluent	62.518 ±3.693		

Table 5.2 Cell counts, ß-carotene concentrations and Paired Sample Test of growth rates for control and 10% anaerobically-digested combined processes effluent.

growth ($t^* = 0.791$, $P > 0.05$; table 5.2). Cell counts on day 27 (table 5.2) are comparable in both the control (73.17×10^4 cell/ml) and effluent cultures (71.50×10^4 cell/ml).

Plate 5.1. *Dunaliella* grown in 10% combined processes effluent (left), shows earlier onset of ß-carotenogenesis than the control (right).

ß-carotene concentrations were examined on the last day. The concentration of ß-carotene per cell was 15.000 pg/cell in the control compared to 19.814 pg/cell in the 10% strength effluent, which is a 1.33 times increase in the latter. The control culture contained 48.191 pg/ml of ß-carotene and the 10% strength effluent, 62.518 pg/ml of ß-carotene. The latter treatment showed a 1.25 times increase of ß-carotene per ml. It could be seen visually that the 10% strength effluent treatment appeared more yellow than the control (plate 5.1), indicating that an accumulation of ß-carotene was occurring.

4. Discussion.

The freshly generated combined processes effluent appears to be too strong for use as a culture medium in its undiluted form. Even anaerobic digestion does not improve its suitability to any large degree. The factors influencing growth in the effluent do not appear to be any of the constituents listed in table 5.1. The maximum concentration of sulphate tolerated by *D. salina* is 48g/l (Gimmler and Weiss, 1987). This is far above the levels measured in both effluents (0.312g/l and 2.750g/l for fresh and digested effluent, respectively; table 5.1). Phosphate is reported to be inhibitory at concentrations of 111.168 g/l (Borowitzka and Borowitzka, 1988). In fresh effluent it is 0.096g/l and in digested effluent, 9.6×10^{-5}g/l. The ammonia levels in both effluents were far below the 42.6g/l inhibitory concentration reported by Borowitzka and Borowitzka, 1988). Although not statistically tested, the 10% strength of anaerobically digested combined processes effluent seems to show a higher yield of ß-carotene per cell and per ml of culture than the control. In order to culture *D. salina* on

yield of ß-carotene per cell and per ml of culture than the control. In order to culture *D. salina* on this effluent, large amounts of water would have to be added as well as large quantities of salt, which is a seemingly pointless exercise when it comes to waste management in tanneries. The ponded tannery effluent is more suitable for *Dunaliella* culture than fresh combined processes effluent.

5. **Conclusion.**

The combined processes effluent is found to be unsuitable as a culture medium, either fresh or anaerobically digested. Where growth was attained, the concentration of effluent was too low to be of any practical significance, even though it appears that the ß-carotene production may be enhanced. If this effluent were to be used as a culture medium, large quantities of water would be required for dilution.

CHAPTER 6

GROWTH IN HIDE-SOAK EFFLUENT

1. **Introduction.**

The first step in tanning, is the soaking of the hides in fresh water. This removes the salt and antimicrobial agents used in hide preservation. The effluent from this operation is saline and contains fats and proteins washed from the hides, and may offer a potential culture medium for a halophilic alga such as *Dunaliella*. The value of the fresh effluent as a culture medium was tested by comparing growth in M11-enriched and M11-deficient fresh hide-soak effluent.

There is wide variability in the preservation treatment of hides from tannery to tannery. The effluent used was obtained from African Hides in Port Elizabeth. This company uses a hydrolysable antimicrobial agent, Busan[R], which is inactivated when the hide is soaked. The absence of antimicrobial agents in this effluent improves its potential as a culture medium for *Dunaliella*, while the presence of antimicrobial agents on the hides may adversely affect algal growth. Any antimicrobial agents present in hide-soak effluent will have to be removed before it can be used as a culture medium, possibly via anaerobic digestion. However, the products of anaerobiosis may also adversely influence the growth of *Dunaliella* (Abeliovich, 1980). A short aerobic step immediately following the anaerobic step, was included to determine whether the quality of the anaerobically digested effluent could be improved for *Dunaliella* culture.

The following experiments were planned to determine the highest concentrations of fresh, anaerobically digested and combined anaerobically and aerobically digested hide-soak effluent that could support growth of *Dunaliella*.

2. **Methods.**

2.1. Nutritional potential of fresh hide-soak effluent (Experiment 6a).

This experiment was intended to show whether the fresh hide-soak effluent could be used as a suitable algal culture medium without the addition of M11 medium. The optimal effluent strength for algal growth was also investigated. This experiment was composed of two components: one in which the diluted effluent was enriched with M11 medium, and the other which lacked M11 medium. Undiluted, 75%, 50% and 25% strength fresh hide-soak effluent was used together with M11-enriched sea-water as a control. Cells were counted daily for 22 days.

2.2. ß-carotene production and nutrient utilization (Experiment 6b).

The purpose of this experiment was to determine whether the fresh hide-soak effluent continued to affect ß-carotene production after the cultures were transferred to N^- M11 medium. After 22 days, cultures from each unenriched hide-soak effluent treatment were transferred into N^- M11 medium after washing in sterile 1.5M saline. Cell counts, ß-carotene and nutrient concentrations in the medium were analyzed weekly to see if the effects of prior culture in hide-soak effluent influenced cell and ß-carotene yields. The experiment was terminated after 31 days.

2.3. Optimal concentration of fresh hide-soak effluent (Experiment 7).

This experiment used a higher range of effluent strengths than Experiment 6a, namely 75%, 80%, 85%, 90% and 95% strength effluent. No M11 medium was added to any of the dilutions, except for the effluent-free control. This experiment was intended to more accurately determine the highest possible dilution of fresh hide-soak effluent suitable for *Dunaliella* culture. Cells were counted daily for 28 days.

2.4. Anaerobically digested hide-soak effluent (Experiment 8a).

The same effluent used in Experiment 6a was used here, with the addition of an anaerobic digestion step to digest the hide-soak effluent and determine the effects of the products of anaerobic digestion on *Dunaliella* culture. Headspace gas and VFAs were sampled weekly and analyzed on a gas chromatograph. The effluent strengths used as culture medium were 25%, 50%, 75% and undiluted effluent. An M11-enriched sea-water medium was included as a control. No M11 medium was added to any of the hide-soak treatments so that the growth could be compared directly against that in defined M11 medium. Cells were counted daily for 27 days.

2.5. Aerobic digestion of anaerobically digested hide-soak effluent (Experiment 8b).

The remaining anaerobically digested hide-soak effluent that was prepared for Experiment 8a was digested aerobically for a further week to see whether any improvement was effected. The experimental layout was identical to the previous experiment. No M11 medium was added to any of the treatments, except for the effluent-free control. Cells were counted daily for 28 days.

3. **Results**.

The constituents of the three hide-soak treatments are shown in table 6.1.

	Fresh	Anaerobic digested	Anaerobic and aerobic digested
pH	7.20	7.00	7.10
conductivity	6360mS/cm	6330mS/cm	6300mS/cm
PV	0.115g/l	0.488g/l	0.482g/l
COD	9.950g/l	5.952g/l	1.632g/l
TDS	57.100g/l	52.090g/l	52.670g/l
TDIS	48.240g/l	49.440g/l	49.200g/l
SS	4.752g/l	1.616g/l	1.350g/l
nitrate	0.143g/l	0.314g/l	0.291g/l
ammonia	0.170g/l	0.326g/l	0.296g/l
phosphate	0.012g/l	0.017g/l	0.018g/l
sodium	1.392M	1.392M	1.392M
chloride	0.870M	0.820M	0.840M
sulphate	1.559g/l	0.451g/l	0.792g/l
sulphide	0.005g/l	0.069g/l	0.018g/l
chromium	0.001g/l	0.003g/l	0.018g/l

Table 6.1. Characteristics of fresh, anaerobically, and combined anaerobically and aerobically-digested hide-soak effluent from African Hides tannery.

3.1. Nutritional potential of fresh hide-soak effluent (Experiment 6a).

3.1.1. Comparisons between fresh effluent and control.

In fig. 6.1 and 6.2 it can be seen that both the enriched or unenriched 50% strength fresh effluent gave the highest cell yields. In the enriched treatments (fig. 6.1), all the dilutions of fresh effluent had significantly better growth than the control ($P<0.05$; table 6.2). The full strength enriched fresh effluent showed significantly poorer growth than the control ($t^* = 10.898$, $P<0.05$; table 6.2). Of the unenriched treatments (fig. 6.2), only the 25% and 50% strengths of fresh effluent gave significantly better growth than the control ($P<0.05$; table 6.2). The 75% strength effluent showed similar growth to that of the control ($t^* = 0.983$, $P>0.05$; table 6.2) and the full strength, significantly poorer growth than the control ($t^* = 10.720$; table 6.2). No significant difference in growth between enriched and unenriched hide-soak effluent was found in effluent strengths greater than 25% ($P<0.05$; table 6.2).

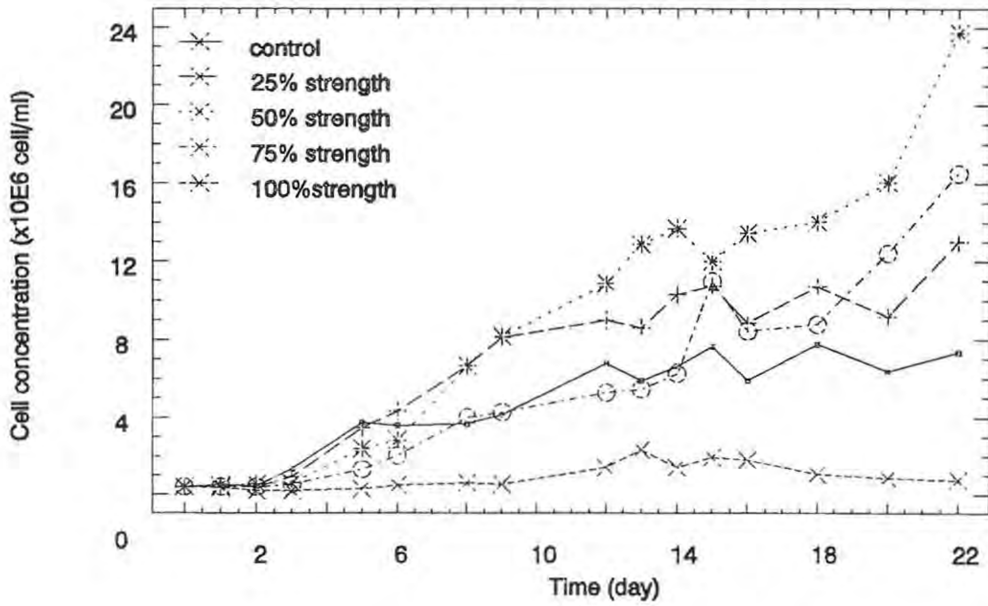

Figure 6.1. Experiment 6a: Cell concentrations in various strengths of fresh M11-enriched hide-soak effluent.

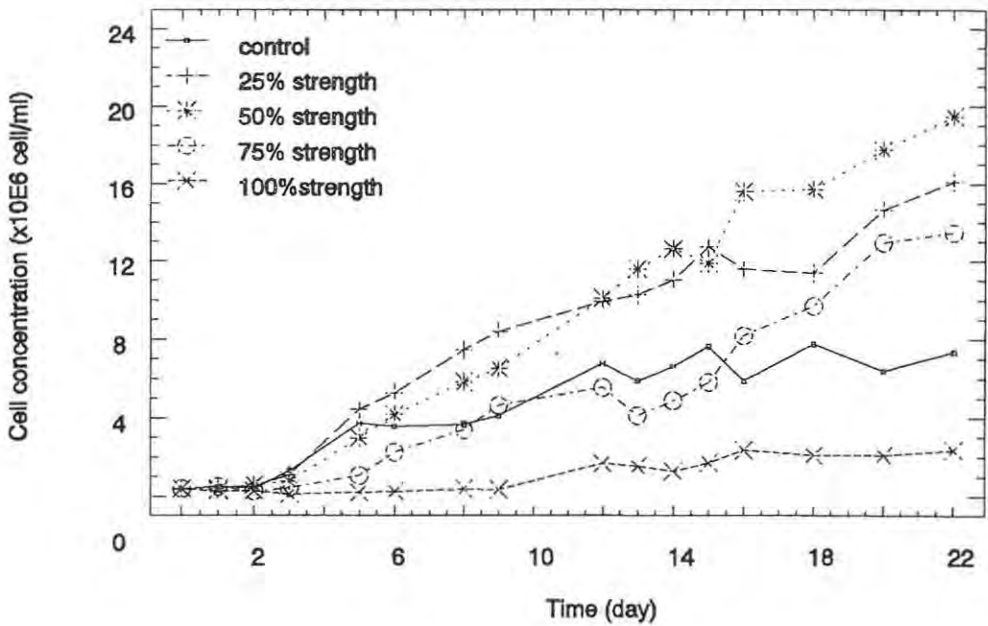

Figure 6.2. Experiment 6a: Cell concentrations in various strengths of fresh unenriched hide-soak effluent.

Nutrient status	Treatments tested	t*	Signif.
Enriched effluent treatments	Control vs 25% strength effluent	6.768	* * *
	Control vs 50% strength effluent	5.979	* * *
	Control vs 75% strength effluent	2.085	*
	Control vs 100% strength effluent	10.898	* * *
Unenriched effluent treatment	Control vs 25% strength effluent	7.956	* * *
	Control vs 50% strength effluent	4.754	* * *
	Control vs 75% strength effluent	0.983	n.s.
	Control vs 100% strength effluent	10.720	* * *
Comparisons of enriched and unenriched effluent treatment	25% strength effluent	3.616	* *
	50% strength effluent	0.344	n.s.
	75% strength effluent	1.566	n.s.
	100% strength effluent	1.209	n.s.

Table 6.2. Paired Sample Test of growth rates in the control and diluted hide-soak effluent treatments with and without the addition of M11 medium.

Key: * * * = $P<0.0005$; * * = $P<0.005$; * = $P<0.05$; n.s. = no significance

The highest cell yields on day 22 came from both the enriched and unenriched 50% strength fresh effluents (237.33x10^4 cell/ml and 194.75x10^4 cell/ml respectively; table 6.3), which show a 2.5 to 3 times higher yield than the control (73.50x10^4 cell/ml; table 6.3). All of the enriched and unenriched 25% (130.00x10^4 cell/ml and 161.17x10^4 cell/ml, respectively) and 75% strength fresh effluents (165.00x10^4 and 134.50x10^4, respectively) show a 1.75 to 2.25 times higher cell yield than the control (table 6.3). The 100% strength enriched effluent (7.50x10^4 cell/ml) shows a 10 times lower cell yield than the control, while the unenriched effluent (23.67x10^4 cell/ml) only shows a 3 times lower cell yield than the control (table 6.3).

	Treatments	Cell count (cell/ml)
Enriched effluent	25% strength	$130.00 \times 10^4 \pm 14.26 \times 10^4$
	50% strength	$237.33 \times 10^4 \pm 18.95 \times 10^4$
	75% strength	$165.00 \times 10^4 \pm 15.72 \times 10^4$
	100% strength	$7.50 \times 10^4 \pm 5.68 \times 10^4$
	Control (N^+ M11)	$73.50 \times 10^4 \pm 8.05 \times 10^4$
Unenriched effluent	25% strength	$161.17 \times 10^4 \pm 13.99 \times 10^4$
	50% strength	$194.75 \times 10^4 \pm 1.77 \times 10^4$
	75% strength	$134.50 \times 10^4 \pm 20.79 \times 10^4$
	100% strength	$23.67 \times 10^4 \pm 15.49 \times 10^4$

Table 6.3. Cell counts at day 22 in the control, enriched and unenriched fresh hide-soak effluent.

The effect of various concentrations of hide-soak effluent on *Dunaliella* culture is illustrated in plate 6.1. The greenest cultures are those grown in 50% and 75% strength hide-soak effluent.

3.1.2. Comparisons between unenriched and enriched fresh effluent.

The 25% strength unenriched effluent showed significantly better growth than the enriched equivalent ($t^* = 3.616$, $P<0.05$; table 6.2, fig. 6.3). In figs. 6.4, 6.5 and 6.6, it can be seen that there is no significant difference in growth rates between the respective enriched and unenriched 50%, 75% and full strength effluents ($P<0.05$; table 6.2).

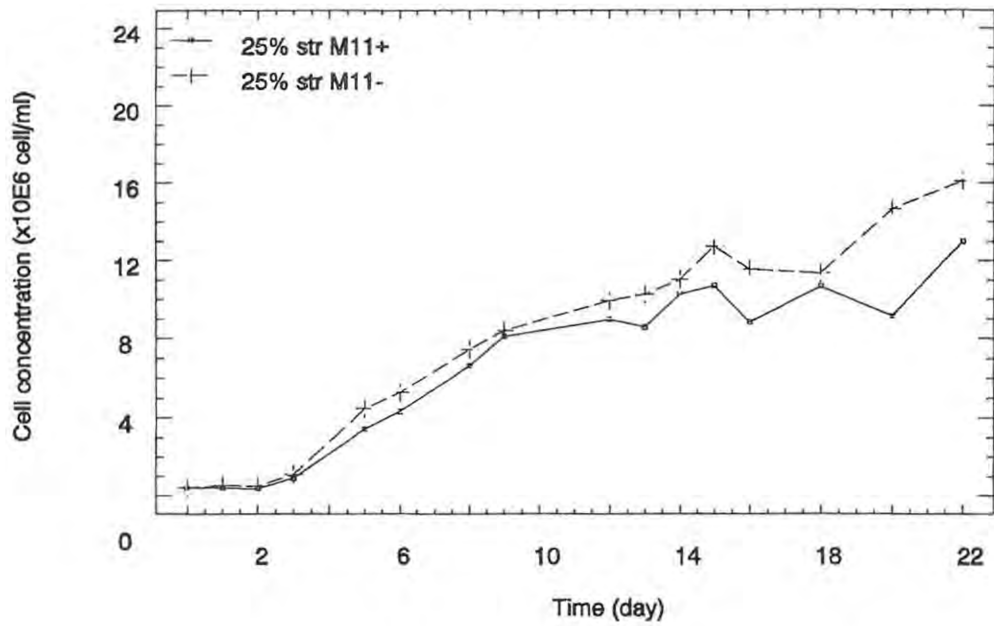

Figure 6.3. Experiment 6a: Cell concentrations in M11-enriched and unenriched 25% strength fresh hide-soak effluent (M11+ = M11 medium added; M11- = M11 medium not added)

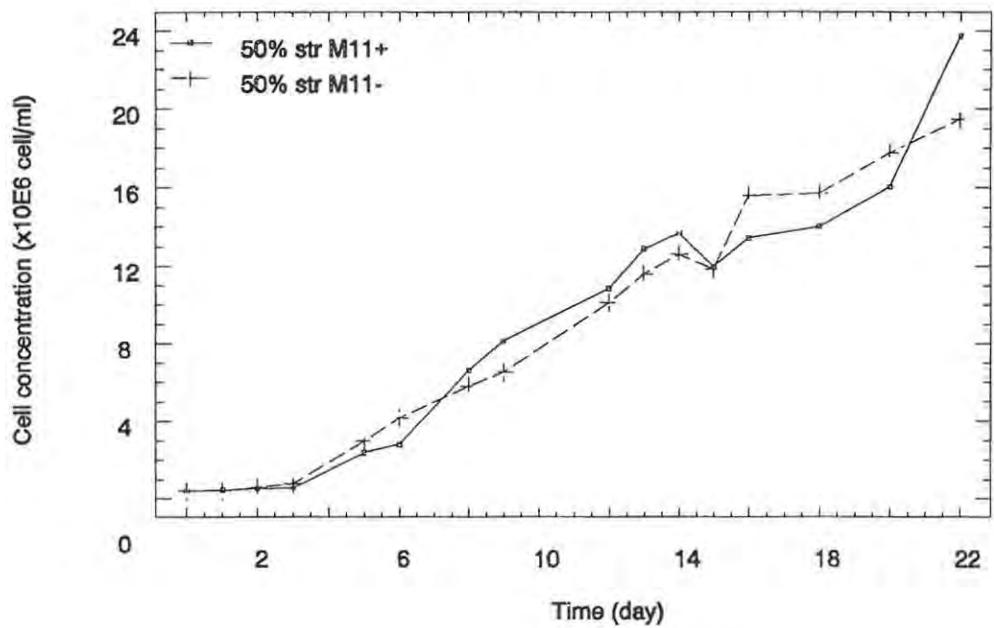

Figure 6.4. Experiment 6a: Cell concentrations in M11-enriched and unenriched 50% strength fresh hide-soak effluent (M11+ = M11 medium added; M11- = M11 medium not added).

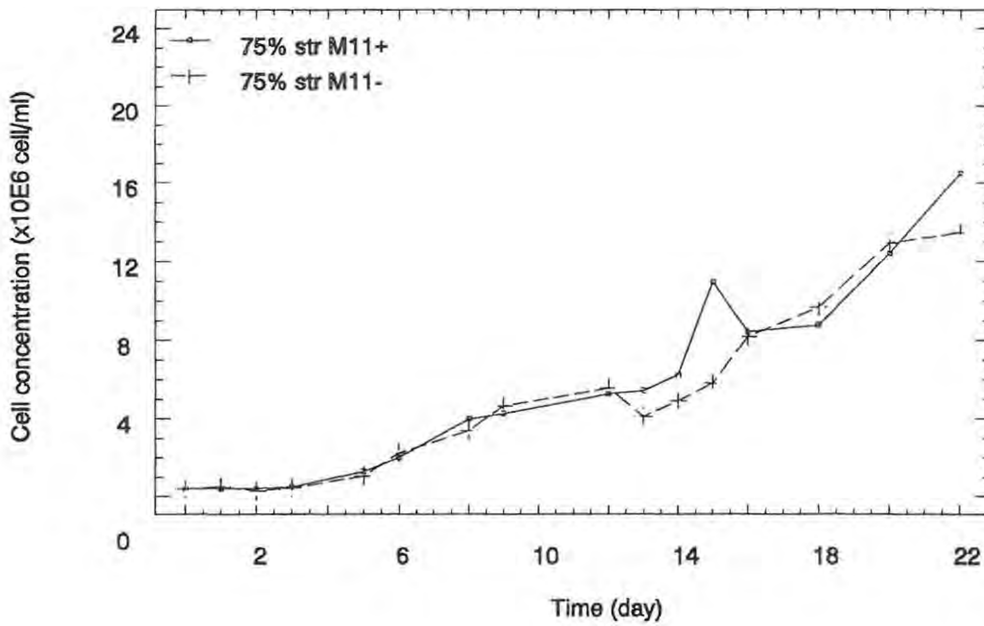

Figure 6.5. Experiment 6a: Cell concentrations in M11-enriched and unenriched 75% strength fresh hide-soak effluent (M11+ = M11 medium added; M11- = M11 medium not added).

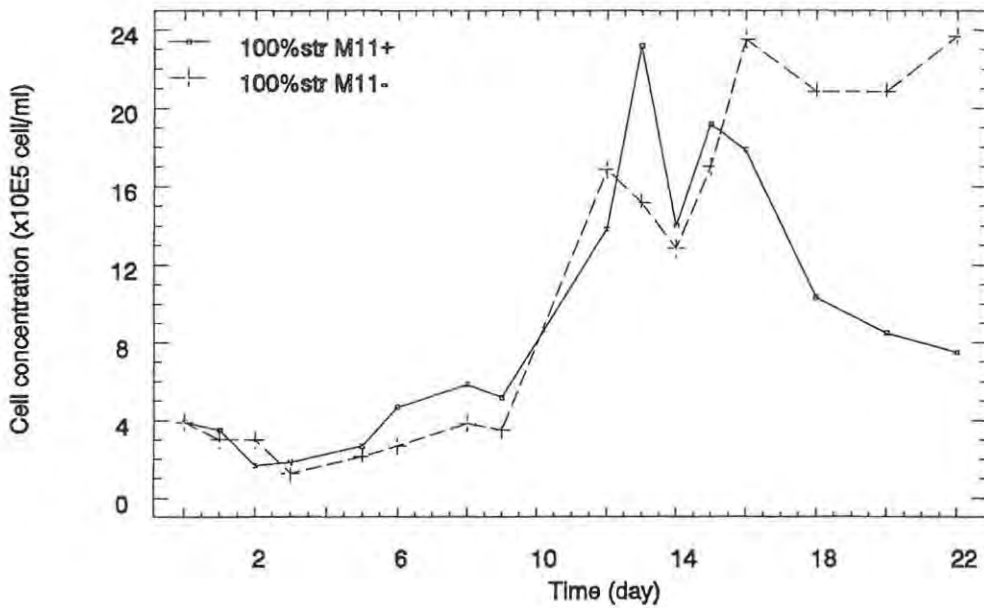

Figure 6.6. Experiment 6a: Cell concentrations in M11-enriched and unenriched 100% strength fresh hide-soak effluent (M11+ = M11 medium added; M11- = M11 medium not added).

Plate 6.1. Growth of *Dunaliella* in a range of hide-soak effluent strengths (from left to right: 100%, 75%, 50%, 25% and control).

3.2. ß-carotene production and nutrient utilization (Experiment 6b).

3.2.1. ß-carotene production.

Final cell yields and ß-carotene concentrations per cell and per ml of culture of the control and effluent treatments transferred to N^- M11 medium, are shown in table 6.4. On day 31, the highest cell yields are obtained in the 75% strength effluent. The final cell yields were influenced significantly by the strength of the effluents from which they originated ($F^{*} = 90.377$, $P < 0.05$; table 6.5). The highest ß-carotene concentration per cell was obtained in the control, and the highest ß-carotene concentration per ml of culture, in the 50% strength effluent. The 75% strength effluent showed over a 2.5 times increase in cell yield compared to the control, while it had a 2.5 times lower yield in ß-carotene per cell than the control. Total ß-carotene yield (ß-carotene per ml of culture) only varied by 1.25 times between the highest and lowest yield.

Treatments	Cell count (cell/ml)	ß-carotene/cell (pg/cell)	ß-carotene/ml culture (pg/ml)
Control	70.33×10^4 $\pm 10.30 \times 10^4$	14.766 ±2.686	4.083 ±0.218
25% effluent	93.67×10^4 $\pm 3.01 \times 10^4$	11.431 ±1.536	4.272 ±0.680
50% effluent	150.00×10^4 $\pm 13.44 \times 10^4$	7.549 ±0.120	4.532 ±0.478
75% effluent	181.50×10^4 $\pm 1.50 \times 10^4$	5.490 ±0.363	3.986 ±0.265

Table 6.4. Cell counts, ß-carotene per cell and ß-carotene per ml of culture in cultures grown in the control and various strengths of fresh hide-soak effluent and then transferred to N^- M11 medium.

The higher the original effluent strength, the higher the final cell yield (fig. 6.7). All treatments showed a significant increase in cell concentration over the 31 days that the cells were counted ($F^* = 8.910$, $P < 0.05$; table 6.5).

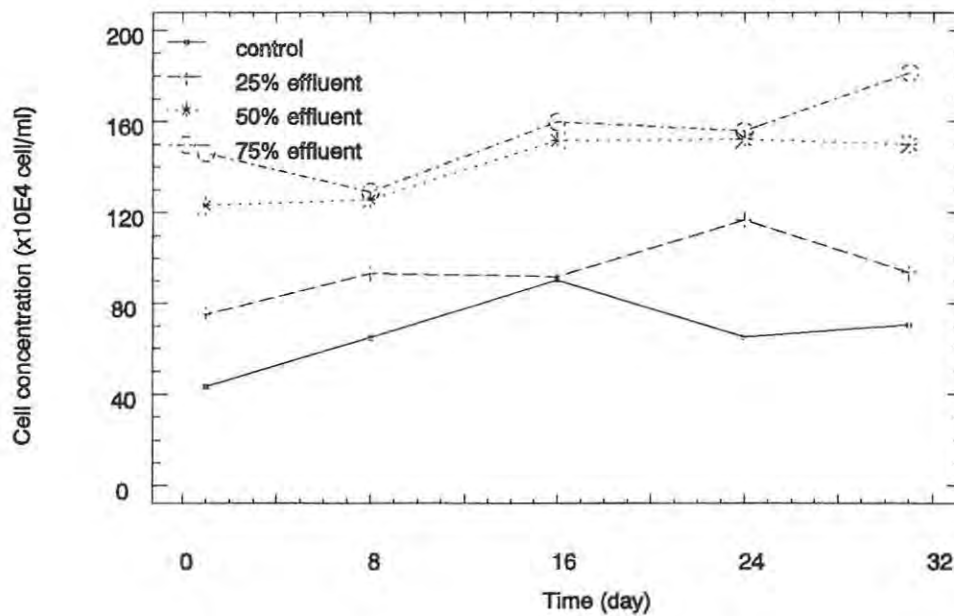

Figure 6.7. Experiment 6b: Cell concentrations in N^- M11 medium in cultures originating from various strengths of fresh hide-soak effluent.

Treatment	Factor	F*	Signif.
Control vs effluent strength	Ageing	8.910	* * *
	Effluent strength	90.377	* * *

Table 6.5. Effects of ageing and effluent strength on cell growth in cultures originating from various strengths of fresh hide-soak effluent and transferred to N⁻ M11 medium.

There is no significant increase or decrease in cellular ß-carotene (fig. 6.8) through ageing in the control ($F^* = 2.766$, $P > 0.05$; table 6.6). However, there is a significant increase in the cultures originating from effluent treatments with age ($P < 0.05$; table 6.6). Additionally, there is a significant decrease in cellular ß-carotene content on exposure to higher effluent strengths ($P < 0.05$; table 6.6). On the final day, ß-carotene concentration per cell is 1.25 times higher in the control (14.766 pg/cell; table 6.4) than the 25% strength effluent treatment (11.431 pg/cell; table 6.4), twice higher than the 50% strength effluent treatment (7.549 pg/cell; table 6.4) and 2.75 times higher than the 75% strength effluent treatment (5.490 pg/cell).

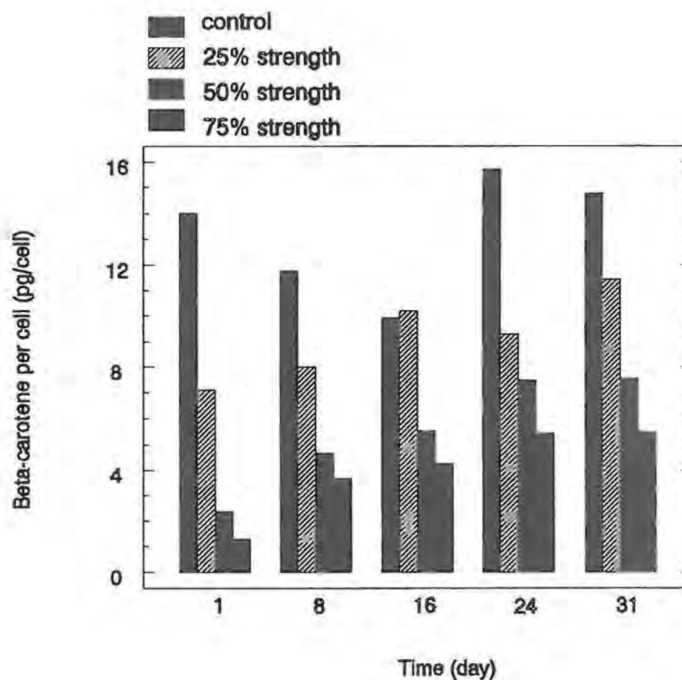

Figure 6.8. Experiment 6b: ß-carotene per cell in N^- M11 medium in cultures originating from various strengths of fresh hide-soak effluent.

		Concentration of ß-carotene/cell		Concentration of ß-carotene/ml culture	
Treatments	Factor	F*	Signif.	F*	Signif.
Control vs 25% strength effluent	Ageing	2.766	n.s.	52.444	* * *
	Effluent strength	25.843	* * *	1.549	n.s.
Control vs 50% strength effluent	Ageing	2.630	n.s.	36.461	* * *
	Effluent strength	69.403	* * *	6.910	*
Control vs 75% strength effluent	Ageing	3.533	*	110.187	* * *
	Effluent strength	148.231	* * *	96.111	* * *
25% strength effluent vs 50% strength effluent	Ageing	16.795	* * *	97.339	* * *
	Effluent strength	129.103	* * *	9.554	*
25% strength effluent vs 75% strength effluent	Ageing	26.766	* * *	158.060	* * *
	Effluent strength	330.639	* * *	150.109	* * *
50% strength effluent vs 75% strength effluent	Ageing	81.277	* * *	288.317	* * *
	Effluent strength	39.119	* * *	78.899	* * *

Table 6.6. Effects of ageing and effluent strength on ß-carotene production in cultures originating from various strengths of fresh hide-soak effluent and transferred to N^- M11 medium.
Key. * * * = $P<0.0005$; * * = $P<0.005$; * = $P<0.05$; n.s. = no significance

The ß-carotene concentration per ml of culture (fig. 6.9) is significantly lower in the 75% strength effluent treatment than all the remaining effluent treatments in the N^- M11 ($P<0.05$; table 6.6). The 50% strength effluent treatment (fig. 6.9) also has a significantly lower ß-carotene concentration than the 25% strength effluent and the control ($P<0.05$; table 6.6), although towards the end of the experimental period it is shown to have the highest concentration of ß-carotene of all treatments.

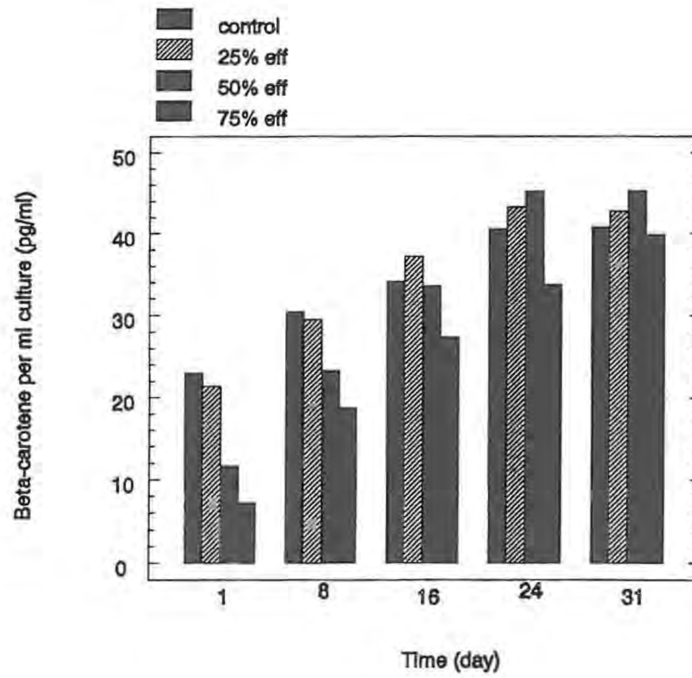

Figure 6.9. Experiment 6b: ß-carotene per ml of culture in N⁻ M11 medium in cultures originating from various strengths of fresh hide-soak effluent.

3.2.2. Nutrient utilization.

Nutrient values were monitored in all of the enriched and unenriched fresh hide-soak effluent treatments and the control after transfer to N^- M11 medium (table 6.7).

Treatments	Factor	F*	Signif.
Nitrate	Ageing	1000.000	* * *
	Effluent strength	1000.000	* * *
Nitrite	Ageing	585.574	* * *
	Effluent strength	626.455	* * *
Phosphate	Ageing	1000.000	* * *
	Effluent strength	1000.000	* * *
Ammonia	Ageing	1000.000	* * *
	Effluent strength	1000.000	* * *

Table 6.7 Effects of ageing and effluent strength on nutrient levels in the control and effluent treatments transferred to N^- M11 medium.

The weekly level of nitrate concentrations in N^- M11 medium can be followed in fig. 6.10. Nitrate values vary significantly between the effluent treatments, but generally, the higher the effluent concentration from which the cultures originated, the higher the nitrate concentration present in the medium over time ($F^* = 1000.000$, $P < 0.05$). All treatments vary significantly over time ($F^* = 1000.000$, $P < 0.05$). An interesting trend is observed, namely the increase of nitrate in the medium of the 50% and 75% strength effluents from its lowest levels on day 16. This trend is also observed in nitrite ($F^* = 585.574$, $P < 0.05$) and phosphate ($F^* = 1000.000$, $P < 0.05$) levels on day 16 for the same treatments (figs. 6.11 and 6.12). Nitrite and phosphate concentrations vary between the effluent treatments ($F^* = 626.455$, $P < 0.05$, and $F^* = 1000.000$, $P < 0.05$; respectively), with higher concentrations appearing in the medium of the higher strength effluents. Ammonia show the highest concentrations of all the nutrients over the 31 days (fig. 6.13). There concentration varies over time ($F^* = 1000.000$, $P < 0.05$) for all treatments, but no clear trend can be identified. Likewise, the concentration of ammonia varies significantly between the treatments ($F^* = 1000.000$. $P < 0.05$), but again no trend can be identified.

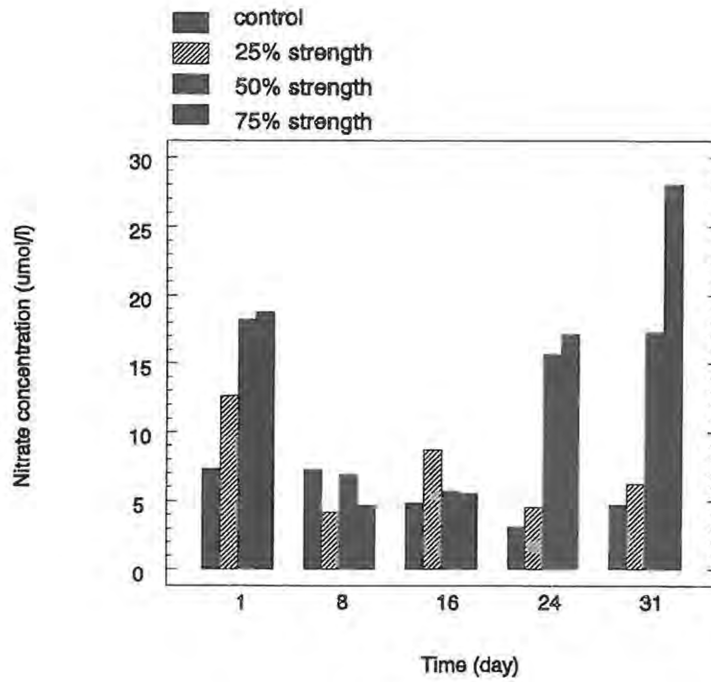

Figure 6.10. Experiment 6b: Nitrate concentrations in N⁻ M11 medium in cultures originating from various strengths of fresh hide-soak effluent.

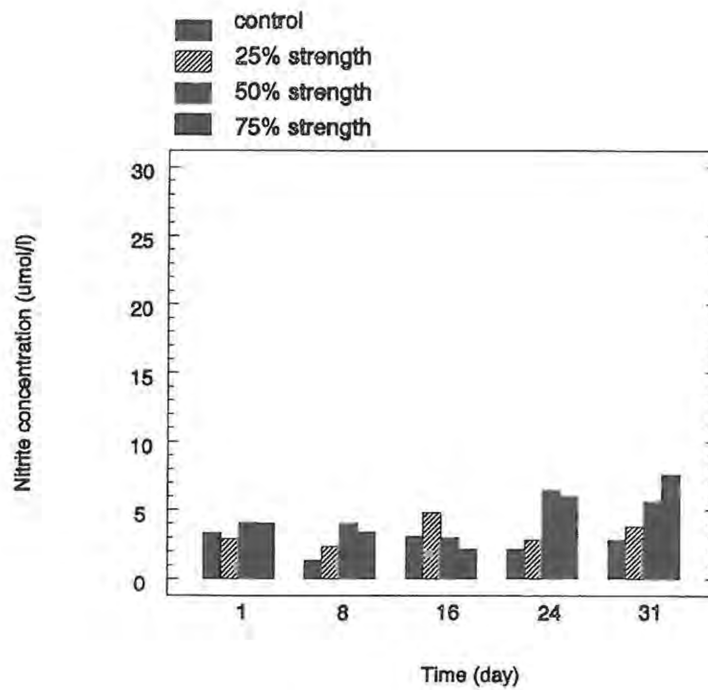

Figure 6.11. Experiment 6b: Nitrite concentrations in N⁻ M11 medium in cultures originating from various strengths of fresh hide-soak effluent.

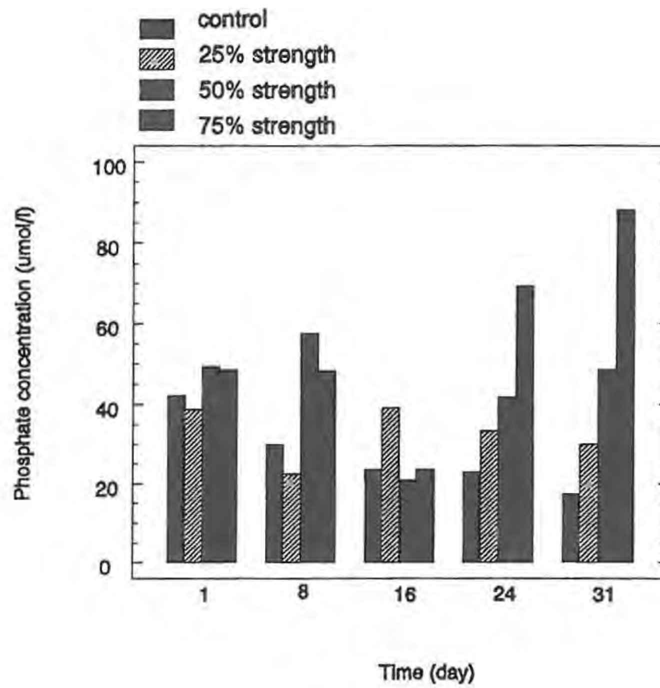

Figure 6.12. Experiment 6b: Phosphate concentrations in N⁻ M11 medium in cultures originating from various strengths of fresh hide-soak effluent.

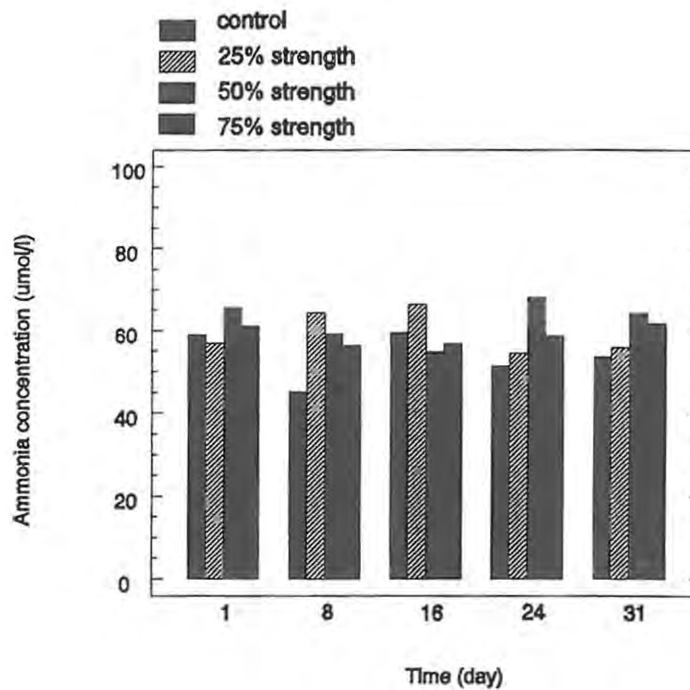

Figure 6.13. Experiment 6b: Ammonia concentrations in N⁻ M11 medium in cultures originating from various strengths of fresh hide-soak effluent.

3.3. Optimal concentration of hide-soak (Experiment 7).

From fig. 6.14 it can be seen that *Dunaliella* grows better in the 75% fresh hide-soak effluent than in the control ($t^* = 2.275$, $P<0.05$; table 6.8). Cell yields in this effluent strength are over 1.5 times greater than in the control. Statistically the 80% and 85% strength effluents show equivalent growth to the control ($P>0.05$; table 6.8), although cell yields in these two effluent strengths are 1.75 times greater than the control on the final day (table 6.8). The 90% and 95% strength effluents are seen to have significantly inferior growth to the control ($P<0.05$; table 6.8). However, on the final day, the 90% strength digested effluent has a slightly higher cell yield than the control, but the 95% strength fresh effluent has a 1.5 times lower cell yield than the control (table 6.8).

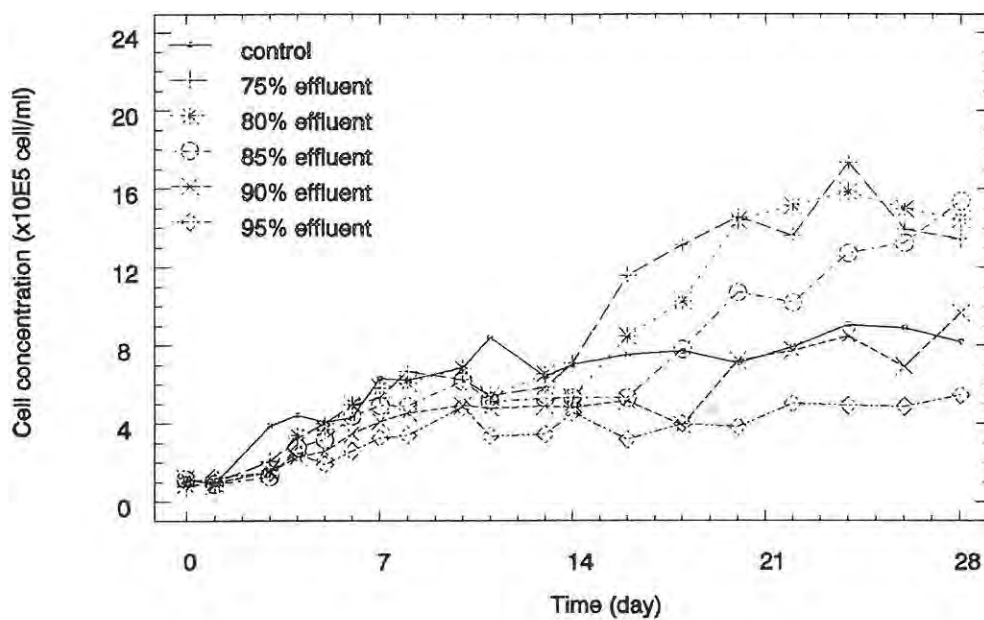

Figure 6.14. Experiment 7: Cell concentrations in unenriched high concentrations of fresh hide-soak effluent.

Treatment	Cell count (cell/ml)	Fresh effluent vs control	
		t*	Signif.
Control	$81.83x10^4$ $\pm 5.75x10^4$	———	———
75% strength	$134.50x10^4$ $\pm 13.94x10^4$	2.275	*
80% strength	$144.50x10^4$ $\pm 14.53x10^4$	1.919	n.s.
85% strength	$153.83x10^4$ $\pm 49.45x10^4$	0.375	n.s.
90% strength	$96.67x10^4$ $\pm 30.23x10^4$	4.644	* * *
95% strength	$54.50x10^4$ $\pm 29.51x10^4$	8.851	* * *

Table 6.8. Cell counts on the final day and Paired Sample Test of growth rates between the control and various strengths of fresh hide-soak effluent treatments.

3.4. Anaerobically digested hide-soak effluent (Experiment 8a).

The effects of anaerobic digestion on the composition of the hide-soak effluent can be seen in table 6.1. Anaerobic digestion has lowered the COD by more than 1.5 times and the suspended solids by almost three times. Nitrate levels have increased by about 2.25 times, ammonia levels by almost twice and phosphate levels by just less than 1.5 times. Sulphates have decreased 3.5 times, but sulphide, although very low in both fresh and anaerobically digested effluent, increased by about 13.5 times in the latter. No significant changes in pH are recorded. Analysis of headspace gas (table 6.9) revealed that the CO_2 component was greater than methane, which only achieved its highest proportion by the third week (14.9% of the headspace gas).

Week	COD (g/l)	CO_2	CH_4
1	9.950	100.0%	0.0%
2	———	———	———
3	68.640	85.1%	14.9%
4	7.776	89.9%	10.2%
5	6.508	96.2%	3.8%
6	5.952	100.0%	0.0%

Table 6.9. Weekly analysis of COD and headspace gas composition of the anaerobic digestion of hide-soak effluent.

When gas samples were analyzed on the final day (week 6), methane was absent from the sample as it had been on the first day. The concentrations of volatile fatty acids (VFAs) are shown in table 6.10. VFAs show the highest concentrations in the fourth week, but in the sixth week, total VFA concentrations are still 5 times higher than the starting concentrations.

	Volatile fatty acids (VFA) mg/l					
Week	acetic	propionic	butyric	valeric	caproic	total*
1	210.0	23.6	54.0	10.6	6.4	256.0
2	———	———	———	———	———	———
3	708.7	184.4	263.9	153.0	5.4	1315.4
4	1283.3	283.4	357.2	265.5	29.0	2218.4
5	740.5	114.4	284.7	204.8	13.9	1358.3
6	657.9	80.1	314.0	237.8	12.2	1302.0

Table 6.10. Weekly analysis of VFAs in the anaerobic digester (* total VFAs reported as acetic acid).

After anaerobic digestion of the hide-soak effluent, no growth of *Dunaliella* was obtained in the 50% and 75% strength digested effluent. These growth rates were omitted from further statistical analyses and graphic representation. In fig. 6.15, the 25% strength digested effluent shows a far higher growth rate than the control. This is verified using the Paired Sample Test ($t^* = 4.796$, $P < 0.05$; table 6.11). On the final day of cell growth, this effluent had a 1.75 times greater cell yield than the control (124.12×10^4 cell/ml vs. 71.50×10^4 cell/ml; table 6.1).

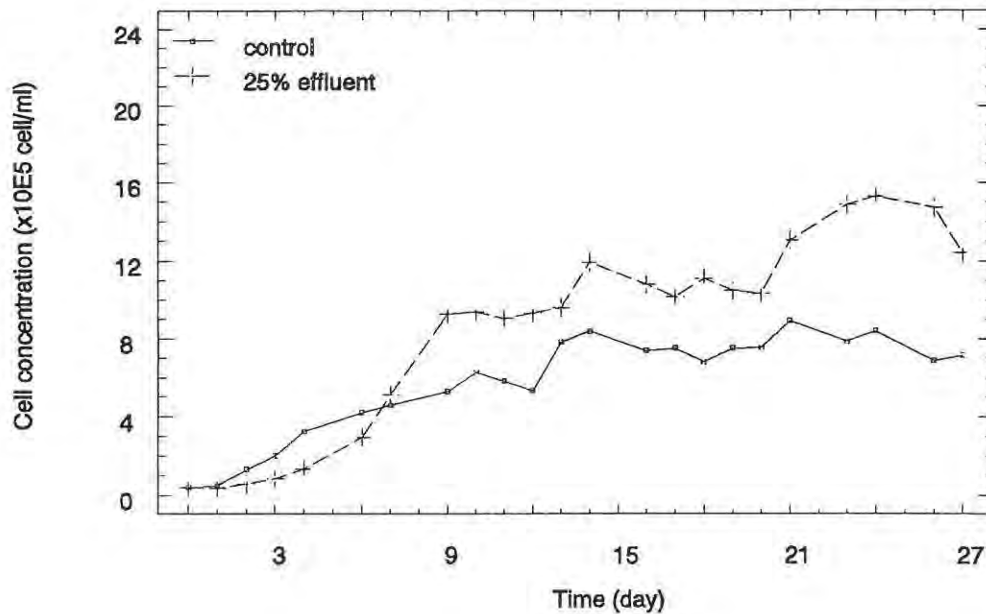

Figure 6.15. Experiment 8a: Cell concentrations in unenriched 25% strength anaerobically-digested hide-soak effluent.

Treatment		Cell count (cell/ml)	Treated effluent vs control	
			t*	Signif.
Anaerobic digestion	Control	71.50×10^4 $\pm 9.37 \times 10^4$	4.769	* * *
	25% strength effluent	124.12×10^4 $\pm 6.43 \times 10^4$		
Anaerobic and aerobic digestion	Control	81.83×10^4 $\pm 5.75 \times 10^4$	2.417	*
	25% strength effluent	97.83×10^4 $\pm 14.87 \times 10^4$		

Table 6.11. Cell counts on the final day, Paired Sample Test of growth rates between the controls, and anaerobically and combined anaerobically and aerobically digested effluents.

3.5. Aerobic digestion of anaerobically digested hide-soak effluent (Experiment 8b).

The change in the composition of the anaerobically digested effluent after further aerobic digestion, can be observed in table 6.1. COD values are further reduced by over 3.5 times, from those of the anaerobically digested effluent. Suspended solids are decreased by only 1.25 times. The concentrations of nitrate, ammonia and phosphate are little changed, although a slight increase is seen in all three. Sulphates, however, show a 1.75 times increase, but sulphides show a 3.75 times decrease, over the levels present in the anaerobically digested effluent prior to aerobic digestion.

Aerobic digestion following anaerobic digestion of the effluent is seen to further decrease the suitability of the hide-soak effluent as a culture medium for *Dunaliella*. The 50% and 75% strength effluents of this treatment show no growth. As can be seen from fig. 6.16, significantly poorer growth is seen in the 25% strength effluent compared to the control ($t^* = 2.417$, $P<0.05$; table 6.6). There is very little difference in cell yield between these two (81.83×10^4 cell/ml vs. 97.83×10^4 cell/ml; table 6.11).

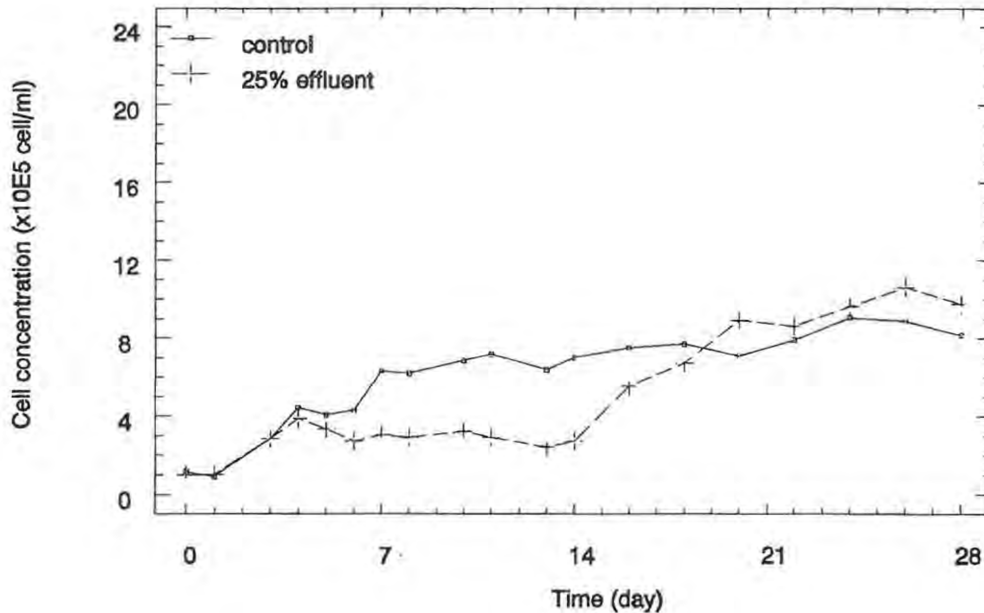

Figure 6.16. Experiment 8b: Comparisons of cell concentrations in unenriched 25% strength anaerobically and aerobically-digested hide-soak effluent.

4. **Discussion.**

4.1. Growth in hide-soak effluent.

It appears that this effluent contains all the necessary nutrients for the growth of *Dunaliella*. No difference is detected in the growth rates between the dilutions of the same effluents split into enriched and unenriched fractions (with the exception of the 25% strength effluent). This latter case shows better growth in the unenriched effluent than in the same M11-enriched effluent. This is contrary to what is expected, as additional nutrients should favour the growth of the enriched effluent culture, particularly when the nutrient levels are as low as they are in the 25% effluent strength. No explanation can be given here, and this result could be regarded as an artifact. The 50% strength fresh hide-soak effluent is optimal for the culture of *Dunaliella*, giving the best cell yield. Higher

strengths of effluent, such as 75% strength, can also be tolerated with good cell yields, but undiluted effluent appears to be too strong for algal culture.

Higher effluent strengths also have higher turbidities which reduce light penetration and enhance light scattering, both of which reduce the amount of total light which is available to the algal cell. This has the effect of lowering the growth rate as light becomes a limiting resource, and as a consequence, stationary phase is attained later, even though nutrients are in abundance. This has been observed where pig manure was used for the culture of *Chlorella* (Groeneweg *et al*, 1980). *Dunaliella* growth was only monitored over 22 days. Longer exposure to this effluent could produce different results, such as possibly even better yields in 75% strength effluent. Nevertheless, the 22 days were sufficient to illustrate the lower toxicity of this effluent compared to other effluent types evaluated.

4.2. ß-carotene production and nutrient utilization.

Cellular concentrations of ß-carotene in cultures transferred to N^- M11 medium, seem to indicate that the cells grown in 50% and 75% effluent strength were less nutrient-stressed than cells grown in the 25% strength effluent and the effluent-free control. This gives an indication of the nutrient potential of higher concentrations of effluent. Total yields of ß-carotene per ml of culture are initially higher in the control and 25% strength effluent, but as the cultures age the concentration of ß-carotene in the higher effluent concentrations increases as a result of increased cell concentrations. It can be speculated that if the experiment had been extended for another few weeks, an even higher yield of ß-carotene may have been obtained in the higher effluent strength cultures.

One important phenomenon which is difficult to explain is the reappearance of nitrate, nitrite and phosphate in the N^- M11 medium of the higher strength effluents from day 16. This is unexpected considering the high cell concentrations in these effluent strengths. Although luxury uptake of nutrients has been documented for nitrate and phosphate, release of these nutrients into the medium is difficult to justify, particularly since it seems to occur so late after transfer of cultures into the N^- M11 medium. Any luxury uptake of nitrate would be converted into protein (Wikfors, 1986). However, the amounts present in the medium are low in comparison to the levels of nutrients in the original effluent treatments (table 6.1).

The high concentration of ammonia in all treatments is noteworthy and would seem to indicate bacterial activity. This could be associated with cell death and the release of nutrients into the medium. It is assumed that bacterial activity would be much higher in the latter days of the experiment when cell death becomes a normal phenomenon. Also the onset of ß-carotenogenesis is usually associated with a decrease of nitrate in the medium (Ben-Amotz, 1987), but here ß-carotene accumulation occurs simultaneously with the appearance of increasing amounts of nitrate, nitrite and

phosphate in the medium. One would expect an increase in ammonia levels from day 1. This is, however, not the case, although significant variation does occur over time. The results therefore appear somewhat anomalous.

In previous experiments with ponded tannery effluents, it was found that ß-carotene increased in cells and in the culture at the expense of nitrate. This does not seem evident here. ß-carotene is also induced by other stress related factors (Cowan and Rose, 1991) which could account for this observation. It should be noted that although the experiment was monitored over 31 days, this may not be enough time to gain a clear insight into the utilization of nitrate and ß-carotene production. The experiment is also limited by the fact that it was carried out in flasks as opposed to a larger volume bioreactor.

4.3. The effects of anaerobic digestion on hide-soak effluent's suitability as a culture medium.

Anaerobic digestion is seen to have an adverse effect on the suitability of hide-soak effluent for algal culture. This is emphasised by the fact that no growth occurred in the 50% and 75% strength effluents, whereas it did in the same strength equivalents of the fresh effluent. Further aerobic digestion produces an even greater deterioration in the effluent suitability. Although anaerobic and aerobic digestion lowers the COD and suspended solids, and increases the concentrations of nutrients, the toxicity of the effluent is also increased. Anaerobic digestion can also result in the loss of nitrogen from the system by the process of denitrification, whereby organic nitrogen is transformed into nitrate and then quickly reduced to nitrogen gas. Up to 50% to 75% of organic nitrogen can be lost this way (Oswald, 1988). The concentrations of these nutrients do not reach the toxic levels as published by Borowitzka and Borowitzka (1988). The influence of pathogenic organisms can also be ruled out, as the effluent was sterilized by autoclaving during preparation of the media. A possible factor that could perhaps have an adverse effect on *Dunaliella* growth, could be the accumulation of fatty acids in toxic concentrations. VFAs are 5 times higher in the anaerobically digested effluent than in the fresh effluent. It is possible that one or more of these VFAs may exert some concentration-mediated influence on *Dunaliella*. Uptake of VFAs by the alga is uncertain, but it has been reported in *Scenedesmus* (Soeder and Hegewald, 1988). The nutrient or toxic influences of VFAs would seem to need more detailed evaluation.

Anaerobiosis in large scale cultivation may be beneficial if carefully controlled. Apart from breaking down organics, it also prevents the establishment of a significant food chain based on the algae and the infestation by the cytopathic, but oxygen-sensitive *Chytridium sp* (Abeliovich, 1986). Although this has been observed in outdoor mass culture of *Scenedesmus* culture, no incidences of contamination were observed in the laboratory cultures of *Dunaliella* reported here. Presumably the high salt concentrations also have a role in discouraging algal predators and pathogens.

4.4. Growth in high concentration fresh hide-soak effluent.

The repetition of the growth experiments in higher concentrations of hide-soak effluent reinforces the finding that fresh hide-soak effluent is superior as a growth medium compared to the digested hide-soak effluent. Although in Experiment 6a the 75% strength effluent was shown to give equivalent growth to the control, in Experiment 8 the 75% strength effluent gave significantly better growth than the control. The effluents used in both experiments were from the same source, except that in the latter case the effluent had been stored at 4°C for some weeks. Possibly the settling of some of the suspended solids had reduced the turbidity of the effluent so allowing greater light penetration into the medium. However, it is shown that very little dilution or modification of the medium is required (disregarding any effects caused by autoclaving) to make it a suitable medium for algal culture.

5. **Conclusion.**

Hide-soak is the most suitable medium for *Dunaliella* culture of all the effluents examined. Although combined anaerobic and aerobic digestion of this effluent appears to enhance its nutritional potential, the toxic effects are harmful to *Dunaliella*. The optimal concentration for use as a culture medium depends on the criteria that need to be satisfied. Fifty percent is the most suitable strength of fresh effluent to use where rapid growth and high algal biomass is required. However, higher strength effluents, such as 85% to 90%, are suitable where economy of dilution is required for effective waste management and conservation of fresh water, as well as reasonable returns in biomass yield.

CHAPTER 7

ANAEROBIC TREATMENT OF ANTIMICROBIAL-CONTAMINATED HIDE-SOAK EFFLUENT

1. Introduction.

In the previous chapter, hide-soak effluent was shown to be the most suitable of all the effluents tested for *Dunaliella* culture. Since variability exists within the tanning industry as to how hides are preserved, hide-soak effluent containing antimicrobial agents detrimental to algal growth will be encountered. One of these agents is naphthalene which is added to the salt cure to protect the hides from halotolerant bacteria such as *Halobacterium* species, which produces a discolouration of cured hides called "red heat". The effluent used in these experiments came from the Leather Industries Research Institute Technologies (LIRI Technologies) in Grahamstown, and contained naphthalene. An earlier attempt to grow *Dunaliella* on various dilutions of this fresh effluent had failed.

In the previous chapter, anaerobic digestion was shown to severely compromise the potential of the hide-soak effluent to support algal growth. However, because of the variability of this type of medium, it was decided to reattempt an anaerobic step with hide-soak effluent from a different source. As hide-soak effluents have been shown to be the most favourable tannery effluent for *Dunaliella* culture, the problem of effluent variability and toxicity needs to be resolved. It was decided to investigate whether the inclusion of an anaerobic digestion step to degrade naphthalene would make this effluent more suitable for *Dunaliella* culture. The purpose of this investigation is to accommodate hide-soak effluents which contain antimicrobial agents into a system exploiting hide-soak effluents as a culture medium. While anaerobic digestion has been shown to contribute growth limiting conditions as seen in the previous chapter, the process nevertheless also breaks down complex organic compounds such as proteins and fats into assimilable nutrients. Two questions needed to be answered: Can anaerobic digestion modify the effluent making it more suitable for *Dunaliella* culture, and does it affect ß-carotene production?

2. Methods.

2.1. Growth in anaerobically-digested hide-soak effluent (Experiment 9a).

The effluent used here was hide-soak effluent generated from the LIRI Technologies experimental tannery. Eight litres of hide-soak effluent were digested over 68 days in an anaerobic digester, the experiment being terminated once gas production stopped and COD removal remained constant. The effluent was dispensed into full strength, 50% and 25% strength dilutions with sea-water and enriched

with M11 medium after autoclaving. An M11-enriched sea-water control was included. Cells were counted daily.

2.2. ß-carotene production (Experiment 9b).

The control, 25% and 50% effluent strength cultures from Experiment 9a were split between N^+ and N^- M11 media after washing in sterile 1.5M saline. Cells were counted every second day and ß-carotene samples were taken every week. The resulting samples were analyzed to determine the effect of effluent strength, ageing and nitrogen deficiency on ß-carotene production.

3. **Results.**

3.1. Growth in anaerobically-digested hide-soak effluent (Experiment 9a).

The compositions of fresh and digested hide-soak effluent are shown in table 7.1.

	Fresh	Anaerobic digestion
pH	6.90	7.80
conductivity	6160mS/cm	6010mS/cm
COD	2.944g/l	2.616g/l
nitrate	0.014g/l	0.056g/l
ammonia	0.056g/l	0.068g/l
phosphate	0.100g/l	0.068g/l
sodium	0.643M	not measured
chloride	0.856M	0.823M
sulphate	1.560g/l	2.785g/l
sulphide	0.001g/l	not measured
chromium	$8x10^{-4}$g/l	not measured

Table 7.1. Effects of anaerobic digestion on hide-soak effluent from the LIRI Technologies' tannery.

Anaerobic digestion reduced the COD insignificantly from 2.944g/l to 2.616 g/l. Nitrate, ammonia and sulphate increased in the digested effluent. Nitrate increased from 0.014g/l to 0.056g/l, ammonia from 0.056g/l to 0.068g/l, and sulphate from 1.560g/l to 2.785g/l. Phosphate levels dropped from 0.100g/l to 0.068g/l in the digested effluent. The pH increased from 6.9 to 7.8.

In fig. 7.1 it can be seen that the 25% strength anaerobically digested hide-soak effluent shows the best growth rate. There is significantly better growth in the 25% strength effluent than in the control (t^* = 6.373, $P<0.05$; table 7.3). Cell concentrations on the final day were $118.00x10^4$ cell/ml in the 25% effluent compared to $83.50x10^4$ cell/ml in the control (table 7.2). This is almost a 1.5 times higher yield in the 25% effluent. Although cell concentrations (fig. 7.1) are higher in 50% strength effluent

compared to the control on the final day ($108.50x10^4$ cell/ml vs. $83.50x10^4$ cell/ml; table 7.2), statistically there is no significant difference between the growth rates ($t^* = 1.966$, $P > 0.05$; table 7.3). In fig. 7.1 it can be seen that the control has a higher growth rate than the 50% strength effluent until day 8, whereafter greater growth occurs in the latter. Although growth in the 25% strength effluent is higher than that in the 50% strength effluent prior to day 8 (fig. 7.1), growth in the two effluent strengths are comparable by the final day ($t^* = 1.891$, $P > 0.05$; table 7.3). No growth was obtained in the full strength effluent.

	Treatment	Cell count (cell/ml)	ß-carotene/cell (pg/cell)	ß-carotene/ml culture (pg/ml)
Prior to split into N^+ and N^- M11 media	Control	$83.50x10^4$ $\pm 5.27x10^4$	—	—
	25% effluent	$118.00x10^4$ $\pm 5.00x10^4$	—	—
	50% effluent	$108.50x10^4$ $\pm 3.50x10^4$	—	—
After transfer of control and effluent into N^+ M11 media	Control N^+ M11	$121.67x10^4$ $\pm 11.73x10^4$	1.564 ± 0.246	7.533 ± 0.544
	25% effluent	$175.17x10^4$ $\pm 37.82x10^4$	1.414 ± 0.305	9.624 ± 0.533
	50% effluent	$152.00x10^4$ $\pm 21.86x10^4$	1.512 ± 0.292	9.048 ± 0.890
After transfer of control and effluent into N^- M11 media	Control N^- M11	$82.17x10^4$ $\pm 7.69x10^4$	5.926 ± 0.860	19.315 ± 1.456
	25% effluent	$84.67x10^4$ $\pm 13.77x10^4$	5.437 ± 0.840	18.115 ± 1.000
	50% effluent	$70.65x10^4$ $\pm 10.69x10^4$	7.034 ± 0.805	19.612 ± 0.846

Table 7.2. Cell counts, ß-carotene per cell and per ml of culture in control and diluted hide-soak effluent, 23 days prior to transfer and 21 days after transfer to N^+ and N^- M11 media.

Treatments tested	t*	Signif.
Control vs 25% effluent strength prior to split	6.373	* * *
Control vs 50% effluent strength prior to split	1.966	n.s.
25% effluent vs 50% effluent strength prior to split	1.891	n.s.
Control vs 25% effluent strength in N^+ M11 media	11.863	* * *
Control vs 50% effluent strength in N^+ M11 media	15.435	* * *
25% effluent vs 50% effluent strength in N^+ M11 media	1.017	n.s.
Control vs 25% effluent strength in N^- M11 media	4.690	* * *
Control vs 50% effluent strength in N^- M11 media	2.727	*
25% effluent vs 50% effluent strength in N^- M11 media	0.689	n.s.
Control split into N^+ and N^- M11 media	17.796	* * *
25% effluent split into N^+ and N^- M11 media	3.672	* *
50% effluent split into N^+ and N^- M11 media	3.886	* *

Table 7.3. Paired Sample Test of growth rates in the control and diluted effluent treatments, before and after split between N^+ and N^- M11 media.

Key: * * * = $P<0.0005$; * * = $P<0.005$; * = $P<0.05$; n.s. = no significance.

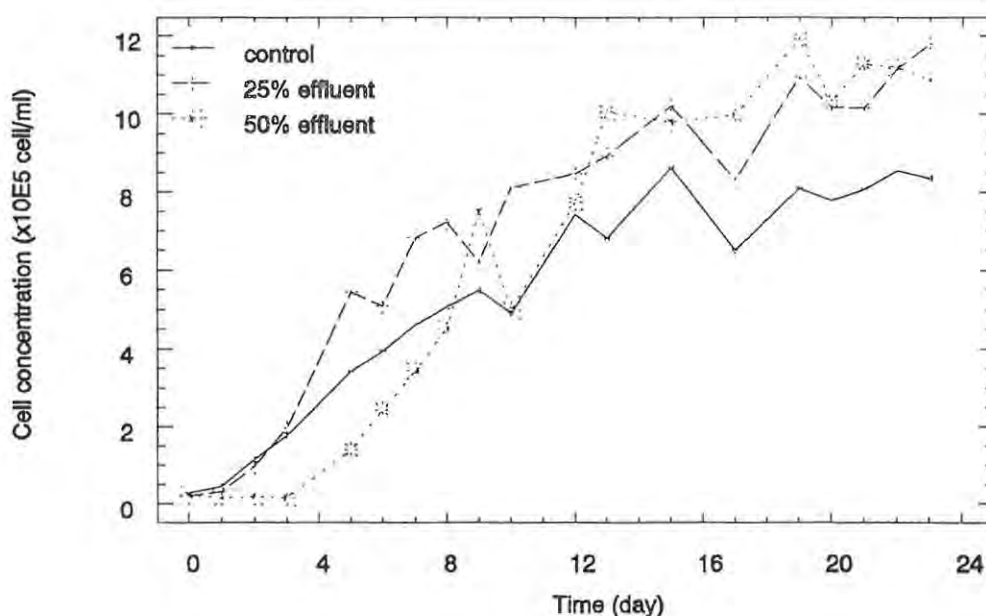

Figure 7.1. Experiment 9a: Growth in various strengths of anaerobically-digested hide-soak effluent.

3.2. ß-carotene production (Experiment 9b).

3.2.1. Growth in N^+ and N^- M11 media.

When the control and effluent cultures are transferred to fresh N^+ M11 medium (fig. 7.2), both effluent cultures show significantly higher growth rates than the control ($t^* = 11.863$, $P < 0.05$ for the 25% strength effluent and $t^* = 15.435$, $P < 0.05$ for the 50% strength effluent; table 7.3). Cell counts (table 7.2) on the last day were highest in the 25% strength effluent (175.17×10^4 cell/ml), followed by the 50% strength effluent (152.00×10^4 cell/ml) and finally the control (121.67×10^4 cell/ml). The 25% strength effluent has a 1.5 times greater yield, and the 50% strength effluent a 1.25 times greater yield than the control. As can be seen from fig. 7.2, there is very little difference between the growth rates of the two effluent strengths ($t^* = 1.017$, $P > 0.05$; table 7.3). The same trend is evident when comparing the growth rates of the effluents and the control transferred to N^- M11 medium (fig. 7.3). Growth in the 25% strength effluent is significantly higher than in the control ($t^* = 4.690$, $P < 0.05$; table 7.3) as it is in the 50% strength effluent ($t^* = 2.727$, $P < 0.05$; table 7.3). There was no significant differences in the growth rates between the two effluent strengths ($t^* = 0.689$, $P > 0.05$; table 7.3). Cell counts in all of the treatments are very similar on the last day (table 7.2).

Comparisons of the growth rates between cultures grown in N^+ and N^- M11 media reveal that growth is restricted in the latter medium. In fig. 7.4, the control in the N^+ M11 medium is shown to have a significantly higher growth rate than its equivalent in N^- M11 medium ($t^* = 17.796$, $P < 0.05$; table 7.3). Cell counts on the final day are 121.67×10^4 cell/ml vs. 82.17×10^4 cell/ml (table 7.2). This is a 1.5 times higher yield than the control grown in the N^- M11 medium. In fig. 7.5, it can be seen that growth in the 25% strength effluent transferred to N^+ M11 medium, compared to in the N^- M11 medium is significantly higher ($t^* = 3.627$, $P < 0.05$; table 7.3). There is a twice higher cell yield in the N^+ than the N^- M11 medium on the final day (175.17×10^4 cell/ml ± vs. 84.67×10^4 cell/ml; table 7.2). Fig. 7.6 shows that the growth rate in the 50% strength effluent transferred to N^+ M11 medium is significantly higher than in N^- M11 medium ($t^* = 3.886$, $P < 0.05$; table 7.3). On the final day the cell counts in the N^+ M11 medium are double those in the N^- M11 medium (152.00×10^4 cell/ml vs. 70.65×10^4 cell/ml; table 7.2).

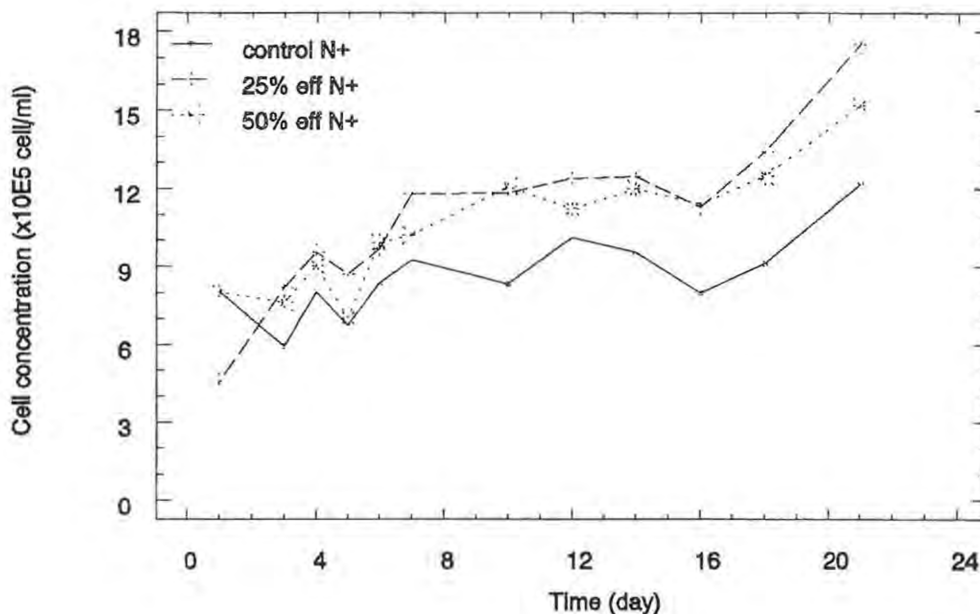

Figure 7.2. Experiment 9b: Growth of cultures originating from various strengths of anaerobically digested hide-soak effluent in N^+ M11 medium.

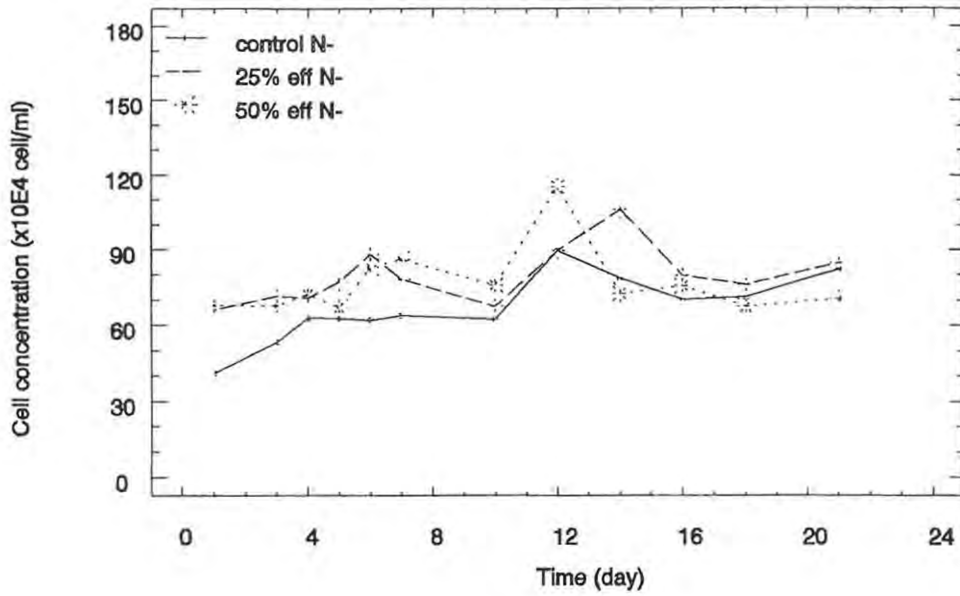

Figure 7.3. Experiment 9b: Growth of cultures originating from various strengths of anaerobically-digested hide-soak effluent in N^- M11 medium.

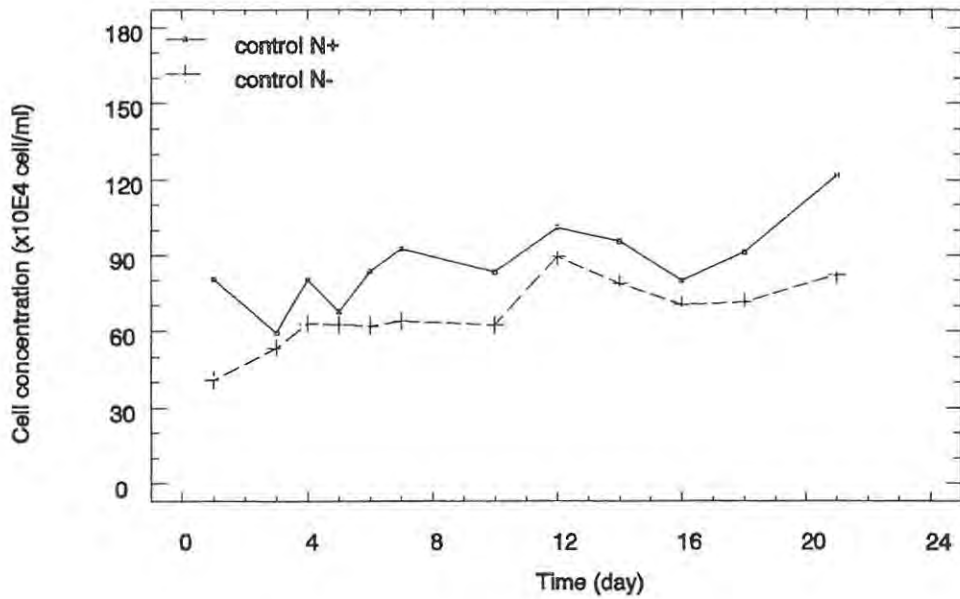

Figure 7.4. Experiment 9b: Growth of cultures originating from the control and split between N^+ and N^- M11 media.

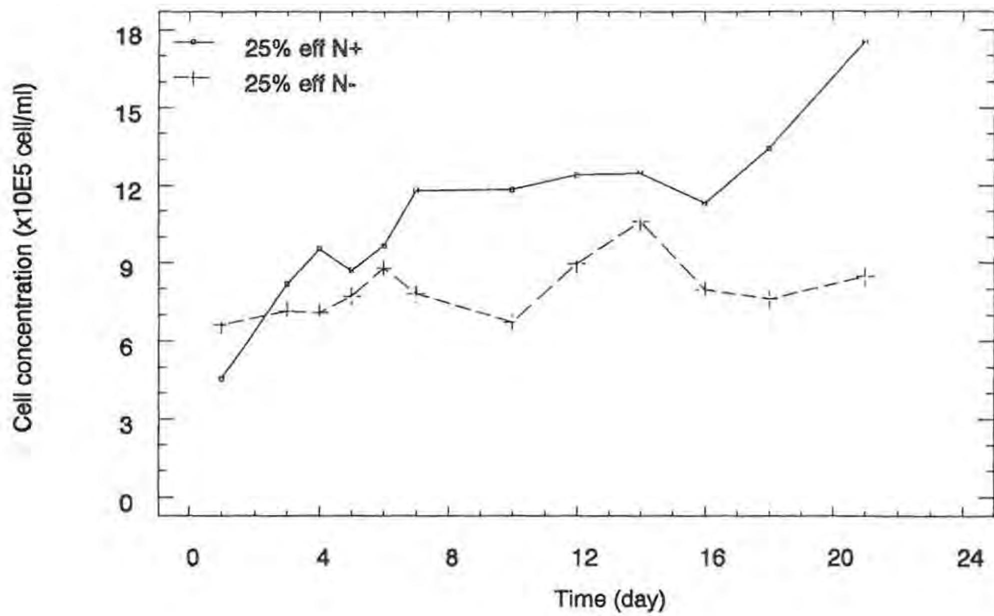

Figure 7.5. Experiment 9b: Growth of 25% strength effluent cultures originating from anaerobically-digested hide-soak effluent and split between N^+ and N^- M11 media.

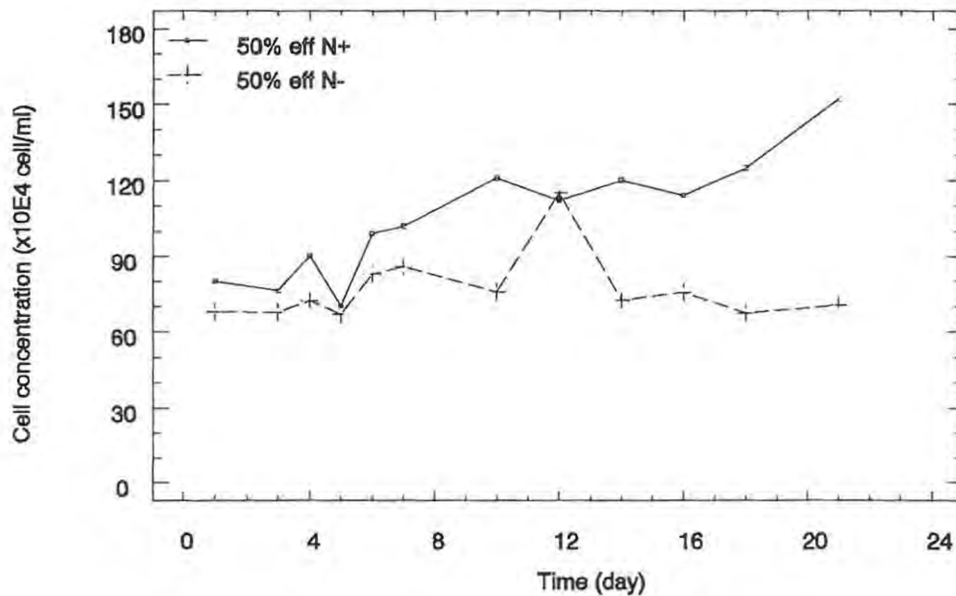

Figure 7.6. Experiment 9b: Growth of 50% strength effluent cultures originating from anaerobically-digested hide-soak effluent and split between N^+ and N^- M11 media.

3.2.2. ß-carotene production in N^+ M11 medium.

ß-carotene concentration per cell in the control and the effluent treatments are shown in fig. 7.7. Generally, the ß-carotene production per cell is similar for all three treatments over 21 days ($P > 0.05$; table 7.4), although the 25% strength effluent treatment shows an almost 2.5 times increase over the control, and a 2-fold increase over the 50% strength effluent treatment on day 1. All treatments are shown to increase their ß-carotene yield per cell over time ($P < 0.05$; table 7.4), with little difference between the treatments on the final day (table 7.2). The ß-carotene concentration per ml of culture is shown in fig. 7.8. The ß-carotene production is higher in the effluent treatments than in the control ($P < 0.05$; table 7.4), but there is no difference between the two effluent treatments ($F^* = 0.037$, $P > 0.05$; table 7.4). A significant increase in ß-carotene per ml of culture over time occurs in all three treatments ($P < 0.05$; table 7.4).

			Concentration of ß-carotene/cell		Concentration of ß-carotene/ml culture	
	Treatments	Factor	F*	Signif.	F*	Signif.
Control and effluent treatments transferred to N^+ M11 media	Control vs 25% strength effluent	Ageing	13.824	***	233.353	***
		Effluent strength	0.912	n.s.	26.975	***
	Control vs 50% strength effluent	Ageing	14.995	***	94.101	***
		Effluent strength	0.010	n.s.	11.063	**
	25% strength vs 50% strength effluent	Ageing	9.682	**	97.955	***
		Effluent strength	8.178	n.s.	0.037	n.s.
Control and effluent treatment transferred to N^- M11 media	Control vs 25% strength effluent	Ageing	120.673	***	564.167	***
		Effluent strength	8.178	*	0.244	n.s.
	Control vs 50% strength effluent	Ageing	183.313	***	731.091	***
		Effluent strength	2.610	n.s.	4.777	*
	25% strength vs 50% strength effluent	Ageing	190.380	***	808.304	***
		Effluent strength	22.974	***	9.007	*

Table 7.4. Effects of ageing and effluent strength on ß-carotene production in cultures derived from various strengths of anaerobically-digested hide-soak effluent and split between N^+ and N^- M11 media.

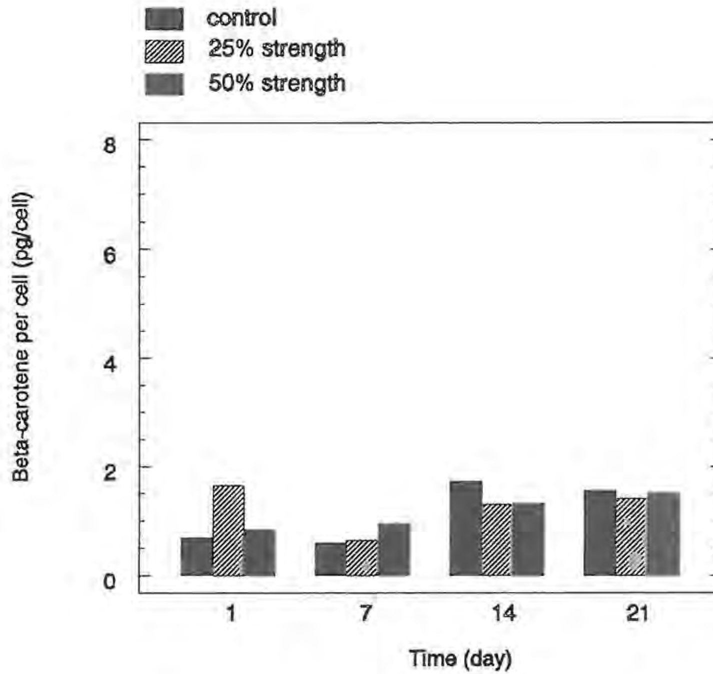

Figure 7.7. Experiment 9b: ß-carotene production per cell in cultures originating from various strengths of anaerobically-digested hide-soak effluent, in N^+ M11 medium.

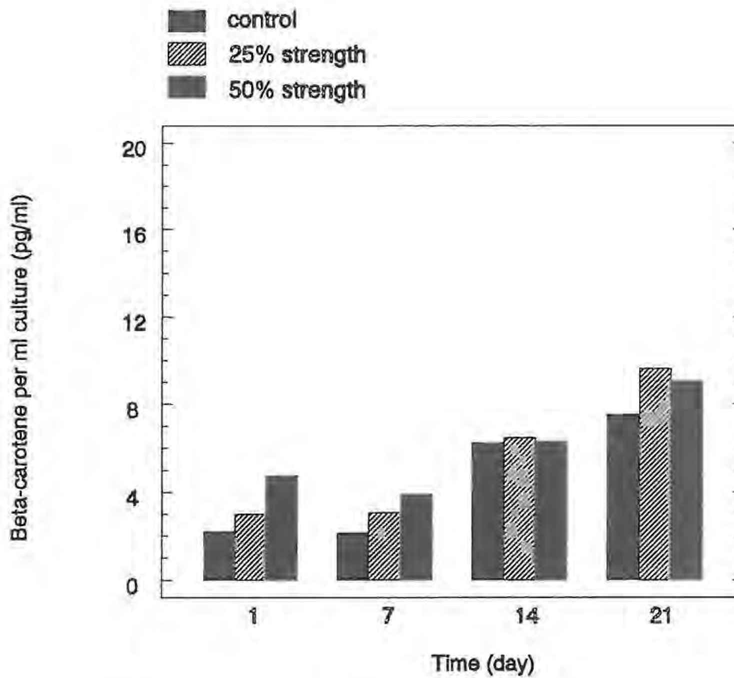

Figure 7.8. Experiment 9b: ß-carotene production per ml of culture in cultures originating from various strengths of anaerobically-digested hide-soak effluent, in N^+ M11 medium.

3.2.3. ß-carotene production in N^- M11 medium.

The ß-carotene concentrations per cell in the control and effluent treatments are shown in fig. 7.9. The ß-carotene yield per cell of the control over 21 days is significantly higher than in the 25% strength effluent treatment ($F^* = 8.178$, $P < 0.05$; table 7.4), but similar in yield to the 50% strength effluent ($F^* = 2.610$, $P > 0.05$; table 7.4). The ß-carotene per cell concentration is significantly higher in the 50% strength effluent treatment than in the 25% strength effluent treatment ($F^* = 22.974$, $P < 0.05$; table 7.4). All three treatments increase in ß-carotene per cell over 21 days of growth ($P < 0.05$; table 7.4). The control, 25% strength and 50% strength effluent treatments increase their cellular ß-carotene by 3, 4 and 5 times respectively, over the 21 days. There is no difference in yield of ß-carotene per ml of culture (fig. 7.10) between the control and 25% strength effluent treatment ($F^* = 0.244$, $P > 0.05$; table 7.4), whereas it is significantly higher in the 50% strength treatment than the 25% strength treatment ($F^* = 9.007$, $P < 0.05$; table 7.4) or the control (4.777, $P < 0.05$; table 7.4). All three treatments show significant increases in ß-carotene per ml of culture over the 21 days ($P < 0.05$; table 7.4). Both effluent treatments show a 5 times increase in ß-carotene per ml of culture by day 21, while the control shows a 6 times increase over the same time.

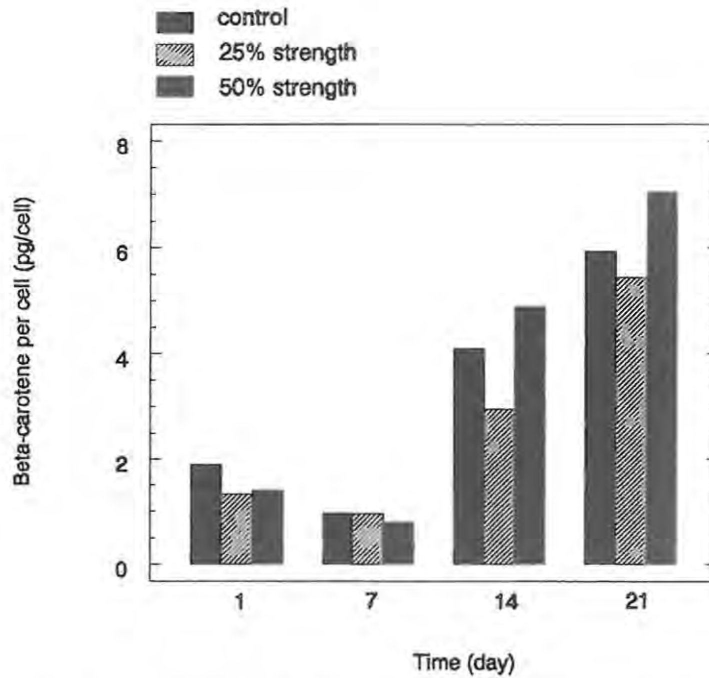

Figure 7.9. Experiment 9b: ß-carotene production per cell in cultures originating from various strengths of anaerobically-digested hide-soak effluent, in N⁻ M11 medium.

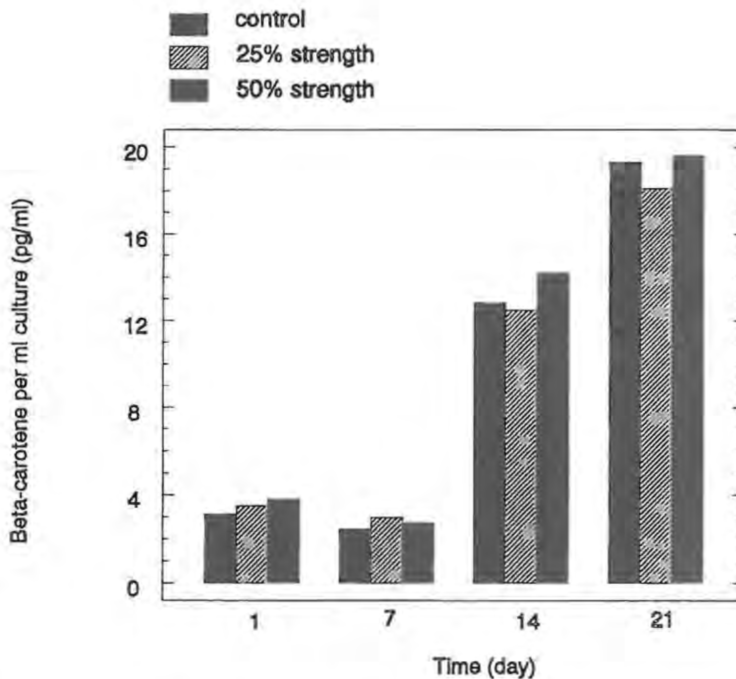

Figure 7.10. Experiment 9b: ß-carotene production per ml of culture in cultures originating from various strengths of anaerobically-digested hide-soak effluent, in N⁻ M11 medium.

3.2.4. Comparison of ß-carotene production in N^+ and N^- M11 media.

It can be clearly seen by comparing fig. 7.7 with fig. 7.8 and fig. 7.9 with fig. 7.10, that ß-carotene production is significantly higher in N^- than N^+ M11 cultures ($P<0.05$; table 7.5), and that it is also significantly increased as the cultures age ($P<0.05$; table 7.5). The cellular content of ß-carotene in all of the effluent strengths is 4 times higher in the N^- M11 medium, and the ß-carotene per ml of culture is twice higher in N^- M11 medium.

		Concentration of ß-carotene/cell		Concentration of ß-carotene/ml culture	
Treatments	Factor	F*	Signif.	F*	Signif.
Controls in N^+ and N^- M11 media	Ageing	65.261	* * *	403.236	* * *
	Nitrogen status	147.270	* * *	202.326	* * *
25% strength effluent in N^+ and N^- M11 media	Ageing	60.705	* * *	307.892	* * *
	Nitrogen status	97.581	* * *	217.822	* * *
50% strength effluent in N^+ and N^- M11 media	Ageing	131.817	* * *	394.237	* * *
	Nitrogen status	279.370	* * *	318.497	* * *

Table 7.5. Effects of ageing and nitrogen availability on ß-carotene production in cultures derived from various strengths of anaerobically-digested hide-soak effluent and split between N^+ and N^- M11 media.

4. **Discussion**.

4.1. The effects of anaerobic digestion on hide-soak effluent's suitability as a culture medium.

Anaerobic digestion of hide-soak effluent raises the pH and lowers the COD. The optimal pH for *D. salina* is pH 9.0, but the algae tolerates a pH range from 5.5 to 10.0 (Borowitzka and Borowitzka, 1988). The drop in COD indicates that a breakdown of organic compounds into their constituents is occurring. Ammonia is produced by the deamination of proteins, and the carbon skeleton is respired, introducing CO_2 into the medium. Nitrification of ammonia can produce nitrate, but some ammonia is probably also volatilised. Nitrate and ammonia do increase in the digested hide-soak effluent, indicating that they are being released during COD destruction. Sulphate also increases and is probably the product of the decomposition of sulphur-containing proteins. The chloride concentration remains constant after digestion and this seems to indicate that very little loss of water has taken place, so the increases in concentration of the three above-mentioned nutrients are due to release by microbial action and are therefore not an artifact of concentration. Phosphate is shown to decrease after anaerobic digestion. It is possible that it may have been precipitated out into an

insoluble phosphate form. None of the nutrients mentioned here were below the concentrations required for growth. The cultures grown in 25% strength digested hide-soak effluent show the fastest initial growth and a superior cell yield compared to the control after day 3. This could be due to the presence of additional growth stimulants such as nutrients in the form of nitrate and ammonia. The initial growth rate in the 50% strength digested effluent is inferior to both the 25% strength digested effluent and the control. Factors which may retard the growth in this culture, are growth inhibitors in concentrations sufficiently high as to affect the growth rate. However, sufficient recovery occurs after day 8 at which the biomass increment surpasses that of the control. It appears that the negative effects of growth inhibitors are overcome after a few days, and that growth enhancement by additional nutrients in the effluent occurs which increases cell concentrations to above that of the control. Pace *et al* (1977) also found that the onset of logarithmic growth in *Dunaliella* is retarded by sub-lethal concentrations of copper. Nevertheless, the earlier inhibition of the 50% strength digested effluent nullifies the later gains when statistically comparing this culture medium to that of the control. No difference in growth rates is therefore detected using the Paired Sample Test. The same observation is made when comparing the growth rates of the 25% and 50% strength digested effluent using the Paired Sample Test.

4.2. Growth in N^+ and N^- M11 media.

When the cultures from Experiment 9a are transferred to N^+ M11 medium, it can be seen that the growth rates differ significantly between cultures originating from the control and digested effluents (fig. 7.2). It would seem that pre-exposure to this effluent enhances growth when the culture is transferred to effluent-free M11 medium. No significant difference is detected between the growth rates of the cultures originating from the two digested effluent strengths. This seems to support the idea that the anaerobically digested hide-soak effluent contains certain useful growth factors. The transfer of these cultures to N^- M11 medium shows exactly the same trends as in the N^+ M11 medium and the same conclusions can be drawn. Comparisons of the growth of the three treatments transferred to N^+ and N^- M11 media, reveal that higher growth rates are obtained in the N^+ M11 medium. This reinforces the role of nitrogen as a limiting factor in algal growth (Mil'ko, 1963a).

4.3. ß-carotene production.

ß-carotene production per cell and per ml of culture is significantly greater in the N^- than the N^+ M11 media. The production and accumulation of ß-carotene is inversely related to the presence of nitrogen in the medium. Cellular ß-carotene is significantly lower in the 25% strength than in the 50% strength digested effluent and control transferred to N^- M11 medium. It would appear that cultures derived from the 25% strength effluent are under less stress than the 50% strength effluent and control. It may be speculated that the control is under higher nutrient stress than the effluent treatments, as prior to transfer into N^- M11 medium, there was less scope for luxury uptake of nutrients than in the

effluent treatments. The 50% strength effluent treatment may be stressed as a result of exposure to concentrated toxic components in the effluent medium prior to transfer to N^- M11 medium. Either way, the nitrogen deficient medium further stresses all the cultures so that the previous effects are still manifested. Cultivation in N^+ M11 medium allows the cells to outgrow their previous exposure to stress. Whatever ß-carotene accumulation occurred in the previous media, will be diluted out by the increasing cell concentrations brought about by renewed growth in the fresh medium. When ß-carotene concentrations increase, it will be as a result of stress caused by ageing in the medium.

It therefore appears that the strength of the digested hide-soak effluent can influence the ß-carotene concentration in *Dunaliella*. This is more of an enhancement effect, as the main influence on ß-carotene production is nitrogen deficiency in the medium. The effluent concentration therefore has a synergistic effect on the production of ß-carotene. A two-stage system is proposed here, where cell concentrations are grown up in 50% strength anaerobically-digested hide-soak, and then transferred into N^- M11 medium. This should obtain the maximum number of cells in the smallest dilution of effluent and then produce the maximum amount of ß-carotene.

5. Conclusion.

Anaerobically digested hide-soak requires dilution to 25% strength to give optimal cell growth. However, good cell growth, which exceeds that of the control, is also obtained in the 50% strength effluent. Toxicity proved to be a problem in an earlier attempt to grow *Dunaliella* on fresh hide-soak effluent containing naphthalene. Anaerobic digestion may be the only way of ridding the effluent of the effects of antimicrobial agents. An alternative is to use hide-soak liquor which has not been preserved with antimicrobial agents, as was attempted in the previous chapter. Anaerobic digestion does appear to influence ß-carotene production, but only when cells are nitrogen-starved.

CHAPTER 8

GENERAL DISCUSSION

1. Choice of algal strains.

It appears that many carotenogenic varieties of *Dunaliella salina* appear throughout the world. High ß-carotene producers are reported in the warmer, southern parts of the USSR (Mil'ko, 1963b), in salt marshes of the Sinai Peninsula (Ben-Amotz and Avron, 1980) and southern Kuwait (Al-Hasan et al, 1987), and in Western Australia (Borowitzka *et al*, 1988). The local isolate used here is probably one of the non-carotenogenic strains of *D. salina* (non-carotenogenic in terms of producing only small amounts of ß-carotene when under stress) (Laubscher, 1987).

The bardawil strain of *Dunaliella salina* is the preferred strain for the purpose of cultivation in tannery effluents. Previous studies with the local strain (Laubscher, 1987) showed smaller increments of ß-carotene than the bardawil strain when this alga was placed under stress. The deciding factor in selecting the bardawil strain over the local isolate, was its ability to produce high amounts of ß-carotene and not its ability to produce high cell yields.

Comparisons in growth performances are tenuous, as both strains had preferences for different defined media used to enrich the tannery effluents. Nevertheless, the local isolate showed a higher biomass production when grown in 25% strength ponded tannery effluent. It must be mentioned that this isolate had been maintained under the same environmental conditions in Provasoli's medium for over a year, while the bardawil isolate was received 6 weeks before experimentation was commenced, and thus did not have the advantage of adapting to the prevailing environmental conditions that the local isolate had. However, towards the end of this project, culminating in the use of fresh hide-soak effluent, very high cell counts of the bardawil strain were obtained. Adaptation of the bardawil strain to the prevailing environmental conditions may have played a part in the higher cell counts.

The local isolate could be of some economic importance in the future of mass cultivation of carotenogenic strains of *D. salina*, in that they are potential contaminants. The possibility arises for further studies in the culture of this local strain in the same range of effluents attempted for the bardawil strain, so that comparisons of growth performances between the two strains can be made, as well as responses to nitrogen deficiency in the medium. It is anticipated that the bardawil strain could be more tolerant to nitrogen limitations under high light intensities, in view of the fact that high levels of ß-carotene are produced which afford protection to the light harvesting mechanisms (Ben-Amotz, 1986; Ben-Amotz, 1987). This has been found in pilot plant studies by Borowitzka *et al* (1984).

2. <u>Choice of tannery effluents.</u>

Identifying suitable tannery effluent for *Dunaliella* culture poses a significant methodological problem. Firstly, the tanning process varies throughout the tanning industry, especially in the preservation of green hides. Secondly, composition of the tannery effluent is very complex, and is influenced by a number of physical, chemical and biological interactions. Chemical composition of all of the raw effluents have been comprehensively assayed to determine as many of the interactive components as possible. The use of defined media to simulate tannery effluent could allow selective exclusion of effluent components in order to determine their effects on *Dunaliella* culture, but variability of effluent composition makes this largely irrelevant.

The first criterion that had to be satisfied is whether the alga would grow on effluent. This involved dilution of the effluent to a level that would sustain growth. The next step was to identify effluent types within the tanning industry that showed the greatest potential for algal culture.

The most successful tannery effluent for the culture of *Dunaliella* appears to be the fresh hide-soak effluent which is uncontaminated by active anti-microbial agents. The least successful were the fresh LIRI Technologies hide-soak effluent and fresh combined processes effluent, followed by the anaerobically digested combined processes effluent. Although significant stimulation of ß-carotene production was detected in the latter effluent, biomass production was low.

In ponded tannery effluents the best growth was achieved in 20% strength, which was superior to that in effluent-free M11 medium. Satisfactory growth could still be obtained in 25% strength and was equivalent to that in effluent-free M11 medium. Higher or lower strengths of this effluent gave poorer growth. This effluent was always enriched with M11 medium (or in the case of Experiment 1, with Provasoli's medium), therefore its limits as a culture medium have not yet been fully elucidated, as growth in unenriched ponded tannery effluent has not been investigated. Subsequently, better growth has been obtained in hide-soak effluent, which has served to divert further attention away from ponded tannery effluent. Nevertheless, it may still be feasible to utilise these ponded effluents on an opportunistic basis where they exist. It may even be possible to adapt the bardawil strain to tolerate higher strengths of ponded effluent.

By far the best results were obtained on fresh hide-soak effluent from African Hides, where very good growth was possible in up to 85% strength effluent. This was the only effluent type to be tested as a growth medium without the addition of a chemically defined enrichment to the medium such as M11. High effluent strengths gave better growth results than the control. No addition of vitamins or trace elements were found to be necessary. The effluent is a cost effective nutrient source as it is generated as a waste product of the tannery industry.

3. Anaerobic digestion of tannery effluent.

Where anaerobic digestion is unnecessary, it should be avoided. Cell growth was noticeably lower in African Hide hide-soak effluent that had been anaerobically digested, compared to the fresh effluent of the same strength. However, anaerobic digestion of hide-soak effluent gave two equivocal results. In digested hide-soak effluent from LIRI Technologies, growth in up to 50% effluent strengths was obtainable, whereas no growth was obtained in digested hide-soak from African Hides at the same dilution. It is possible that M11-enrichment of the medium may have promoted growth in the LIRI Technologies effluent. This variability could also stem from the origin of the hide-soak effluents and the differing methods of hide preservation used by the two institutions, which makes comparative assessment of the effluents difficult. This also serves to show the variability of the effluents dealt with in the tanning industry.

It was also shown that further aerobic digestion of the anaerobically-digested hide-soak effluent further decreased its suitability as a culture medium. In certain cases, anaerobic digestion may be unavoidable if hide-soak effluent is to be used. The case in point is the LIRI Technologies hide-soak effluent which was unusable prior to anaerobic digestion because of the presence of toxic amounts of naphthalene.

The causative agents of growth inhibition in anaerobic-mediated toxicity, may be the accumulation of volatile fatty acids (VFA) which increase ten-fold in the anaerobically-digested effluent. Acetic acid is the major component of the VFAs and would seem to be the most logical VFA to investigate. Suspended solids have been dramatically reduced in anaerobically-digested effluent and could be an important factor in reducing turbidity in the hide-soak effluent. Clearly, the anaerobic process and its effects on *Dunaliella* growth needs to be more thoroughly investigated.

It is unlikely that the quality of hide-soak effluent can be improved by dictating the choice of hide preservatives to the tanning industry, but a positive development is that degradable antimicrobial agents such as Busan[R] are available. Nevertheless, the investigation into anaerobic digestion is justified in terms of extending *Dunaliella* culture to tanneries where anaerobic treatment of contaminated hide-soak effluent is the only option. This strategy may be quite feasible, as anaerobic digestion is being investigated by the tanning industry for the treatment and disposal of tannery effluents (Jackson-Moss, 1991).

4. The role of nitrogen in *Dunaliella* culture.

Dunaliella is able to utilise many forms of nitrogen (Antia *et al*, 1991). Nitrate is preferentially taken up over ammonia when it is in abundance, particularly at high light intensities. Nitrate uptake is linked to the synthesis rate of organic nitrogenous compounds, while that of ammonia is governed by

the rate of transport across the cell membrane (Kanda *et al*, 1990). Ammonia is also utilised by *Dunaliella*, but its levels were never high enough to be considered as a main nitrogen source in the initial growth stages. However, ammonia is liberated by bacteria during the degradation of organic materials and so organic nitrogen assimilated from nitrate during the early growth phase can be recycled to and taken up as ammonium (Kanda *et al*, 1990). This could perhaps sustain algal cells during the stationary phase.

Nitrate has been shown to be a growth-limiting nutrient in *Dunaliella* culture. Cessation of cell division has been observed on depletion of available assimilable nitrogen (Ben-Amotz and Avron, 1983b). Subsequently this leads to a reduction of biomass in algal cultures (Mil'ko, 1963a) and a decrease in cellular levels of protein (Ben-Amotz, 1987). It was noted in this investigation that cell division did not cease with the depletion of assimilable nitrogen in the medium, and this leads us to believe that an internal pool of nitrogen may exist. The origin of this pool may arise from luxury uptake of nitrate from the medium and its immediate conversion into protein, which has been recorded for *D. tertiolecta* (Wikfors, 1986). Ben-Amotz (1987) noted that the bardawil strain continued to divide after six days of cultivation in nitrogen-deficient medium.

In this investigation, growth was also seen to continue for six days after cultivation in nitrogen-deficient medium, albeit at a lower rate than similar cultures in nitrogen-enriched medium. It would appear that nitrogen availability influences protein synthesis (Wikfors, 1986), and termination of protein synthesis eventually leads to the termination of cell division and finally, cessation of growth.

Cells which are grown on ponded tannery and fresh hide-soak effluents, and then transferred to N^- M11 medium, show degrees of nitrogen starvation related to the effluent strength from which they originated. The higher the effluent strength, the longer the time taken for the effects of nitrogen starvation to take effect. This leads to the speculation that the algal cultures in high effluent strengths are saturated with nitrate and other assimilable forms of nitrogen, and take in an excess of that required for normal growth. Once they are nitrogen-stressed, they can draw on these reserves and delay the cessation of cell division. This conclusion is further substantiated by delayed ß-carotenogenesis in cultures derived from high strengths of fresh hide-soak, which will be discussed below.

The method of ammonia analysis used here needs to be discussed. Samples were frozen at -20°C prior to analysis, but the volatility of ammonia in solution can lead to incorrect results. It is recommended that ammonium be read within 3hrs of sampling (Mostert, pers comm). Other forms of nitrogen, such as urea and organic forms of nitrogen, were not considered in this project. Urea is a product of bacterial decomposition of organic matter, which includes senescent cells. Its

utilization has been recorded in *Dunaliella primolecta* (Oliviera and Huynh, 1989). *Dunaliella tertiolecta* is known to take up proteins such as bovine serum albumin through pinocytosis, but no evidence of proteolytic digestion has been observed, nor any significant contribution to the alga's growth (Klut, 1985; cited in Antia *et al*, 1991). The influence of the above mentioned organic nitrogen sources on *Dunaliella salina* (bardawil strain) need to be further elucidated. This may help to explain the observed lag in ß-carotene production in cultures derived from 25% strength ponded effluent in N^+ M11 media, after nitrate exhaustion.

5. ß-carotene production in effluents.

There seems to be no enhancement of total cellular ß-carotene production by the ponded tannery effluent, although at high effluent strengths, production is increased at the expense of cell growth. This has been observed in *Dunaliella tertiolecta* exposed to increasing levels of copper ions (Lustigman, 1986). In 10% anaerobically-digested combined process effluent, ß-carotene production per cell and per ml is significantly higher than in the effluent-free control, without any noticeable adverse effect on cell concentration. How stimulation of ß-carotene occurs without a compromise in cell growth, remains to be further elucidated. However, a system utilising this effluent would be impractical as high dilution is required. This detracts from the main purpose of waste management, which is conservation of fresh water and reduction of the volume of effluent.

ß-carotene production is a symptom of stress (Semenenko and Abdullaev, 1980), therefore high effluent strengths subject *Dunaliella* to stress. This is not the case with fresh hide-soak effluent. In fact, exposure to higher effluent strengths actually delays the onset of ß-carotenogenesis when cells are transferred to nitrogen-deficient medium. Further cell growth in nitrogen-deficient medium is also observed, as was discussed above. As cell concentrations increase in cultures exposed to higher effluents, so the final ß-carotene in the cultures increases.

Manipulation of ß-carotene production is somewhat enigmatic. ß-carotene production is known to increase when cell division decreases (Borowitzka *et al*, 1984), so although cellular ß-carotene concentrations are higher in stressed cultures of *Dunaliella*, final ß-carotene yields can be much higher in unstressed cultures allowed to age. This is because unstressed cultures will continue to grow and therefore have more cells available to accumulate ß-carotene. The trade off is: moderate yields of ß-carotene per volume of culture medium in a shorter period of time, versus higher yields of ß-carotene per volume of culture medium over a longer period of time. What is clear is that high cell yields should be achieved before ß-carotene production is induced. This could be achieved by cultivating *Dunaliella* at fresh hide-soak effluent strengths that give the highest cell yield, followed by transfer to nitrogen-free medium for maximum ß-carotene production once stationary phase has been achieved.

Other stressful factors that influence ß-carotene production and can be manipulated in an outdoor pond system should be investigated. Both high light intensity and temperature have been shown to increase cellular ß-carotene (Mil'ko, 1963b; Semenenko and Abdullaev, 1980), but manipulation of these factors are impractical. One practical way of manipulating ß-carotene, may be by increasing the salinity of the effluent, after the desired cell concentrations have been obtained. Salinity-mediated ß-carotene increase has been reported by Al-Hasan *et al* (1987) and Borowitzka and Borowitzka (1984). Increase in ß-carotene production in the local isolate under salinity stress, has been reported in this laboratory (Laubscher, 1987). Manipulation of ß-carotene production by salt addition needs to be further investigated before a model can be proposed.

6. The relationship between *Dunaliella* and bacteria.

Tannery effluents have their own microbial flora which are eliminated during autoclaving. It is uncertain what effect this natural flora would have on *Dunaliella* growth in unautoclaved effluent. Nevertheless, bacteria were observed in growing cultures of *Dunaliella* in tannery effluents. What the role of bacteria in nutrient recycling and on algal mortality in tannery effluents is, remains to be clarified. *D. salina* has been shown to produce a broad spectrum antibiotic substance effective against a range of bacteria (Lustigman, 1988). Rapidly growing algae are normally found to have less epialgal bacteria than slower growing algae which have had their surfaces exposed to colonization for a far greater time (Vaque, 1990). A glycocalyx cell envelope is thought to confer protection from epialgal bacteria in actively growing *Dunaliella*. The coat is known to slough off providing it with a cleaning system which removes contaminants from the cell surface (Oliviera *et al*, 1980). Scanning electron microscopy of *Dunaliella* at various stages of growth may give further insight into algal-bacterial relationships.

Synergistic relationships between algae and bacteria are well documented. Algae supply O_2 for the bacterial oxidation of organic wastes (Oswald, 1988), high rates of photosynthesis producing high oxygen concentrations (Abeliovich, 1980). However, the fact that bacteria reciprocate by suppling CO_2 is often questioned. Much controversy exists as to whether the numbers of bacteria present in these systems can produce sufficient CO_2 to support algal cells (Abeliovich, 1980). Nevertheless, the ammonium and urea which is produced during bacterial decomposition of organics can only be beneficial to algal nutrition (Oswald, 1988)

7. Recommendation and proposals.

From the results obtained in this project, it is recommended that the saline hide-soak effluent should be segregated from the rest of the tannery-generated effluent, in order to confine salt problems. Other effluents can be disposed of by a number of processes, without the problem of salt pollution. Hide-preserving agents should be restricted to hydrolysable antimicrobials, otherwise a costly anaerobic digestion step could be required.

The establishment of a two-stage system is recommended. The first stage would be used to produce a high biomass of *Dunaliella* in fresh hide-soak effluent. The second stage would require the removal of the algal biomass once stationary phase has been reached, to a nitrogen-deficient medium to encourage ß-carotene production. A cost effective harvesting system is required for the transfer of the biomass into nitrogen-deficient medium for the propagation of ß-carotene, as well as for the final harvest. Work on cross-flow filtration in this laboratory has been shown to be very successful in harvesting *Dunaliella* without a significant loss in cell numbers (Maart, 1989; Maart *et al*, 1990).

A flow diagram can be used to summarise a proposed two-stage system:

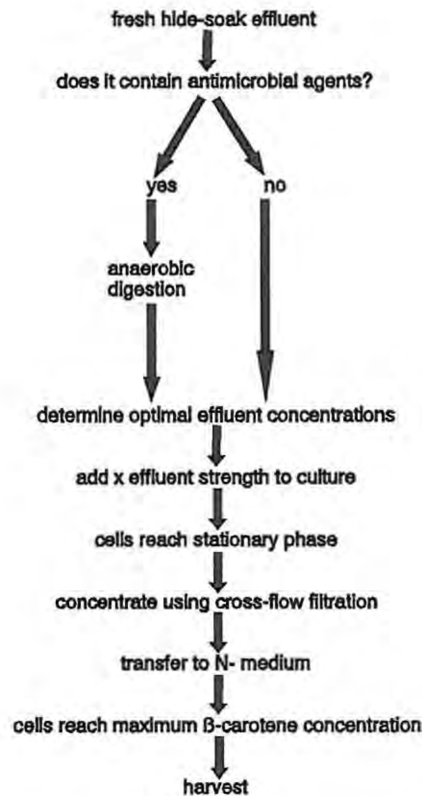

Figure 8.1. Flow diagram of a proposed two-stage system for the culture of *Dunaliella* on fresh hide-soak effluent.

Alternatively, the biomass can remain in the tannery effluent until all of the nitrogen is exhausted. The latter option requires a much longer time to accomplish and may not be practical where a high turnover of product is required.

A pilot-plant study, utilising a two-stage system described above is necessary in order to determine the effects of fluctuating environmental conditions on the bardawil strain's growth performance, and to compare yields with laboratory yields. The geographic location is important, tanneries situated in areas of low rainfall and humidity, and high insolation being recommended. One of the limiting factors suggested by laboratory studies, is the effect of turbidity caused by suspended solids in the

effluent, as well as cell densities. One way of circumventing this would be to gently agitate the cells. This also prevents sedimentation, thermal stratification, and anaerobic conditions from developing, as well as keeping the cells in contact with nutrients (De Pauw and Persoone, 1988). Contamination by non-carotenogenic *D. salina* could be reduced by inoculating the culture medium with a large monospecific inoculum of the bardawil strain in logarithmic growth. Grazers may be a serious problem at low salinities (Borowitzka *et al*, 1984) when precipitation is high and evaporation low, and may be anticipated seasonally. Salinity can also be controlled by the judicious addition of fresh saline effluent.

8. Summary.

The bardawil strain of *Dunaliella salina* is considered to be a better economical prospect in large scale algal cultivation than the local isolate. Fresh hide-soak effluent has been shown to be a simple and effective culture medium, and pre-treatment of this effluent has been shown to be unnecessary, provided it is uncontaminated by antimicrobial agents. It is cheaper and more effective than defined medium, and is available in large quantities. ß-carotene production cannot be induced by hide-soak effluent, so transfer to nitrogen-deficient medium is required. The timing of this transfer appears to be critical for optimal ß-carotene production. Ageing also increases ß-carotene concentrations, but requires a lengthy period of time. A two-stage system using fresh hide-soak effluent as a medium in the first stage, and nitrogen-deficient medium in the second stage, has been proposed as a model for obtaining high cell yields and consequently, high ß-carotene yields. A pilot plant stage would be the next logical step in any further feasibility studies.

CONCLUSION

1. It has been established that the bardawil strain of *Dunaliella salina* is a more suitable strain than the local isolate, for the potential commercial cultivation on tannery effluents on the grounds of its higher ß-carotene producing potential.

2. Fresh hide-soak is the most suitable tannery effluent for mass culture of *Dunaliella*, provided the hides have not been treated with a persistent antimicrobial agent.

3. Half strength fresh hide-soak effluent gives the highest algal biomass, but yields equal to defined media are obtained in 90% strength hide-soak effluent.

4. Pre-treatment of the hide-soak effluent by anaerobic digestion reduces the suitability of the effluent as a culture medium. Anaerobic treatment may be effective if the effluent contains a persistent antimicrobial agent.

5. A two-stage system is recommended requiring the mass culture of *Dunaliella* in fresh hide-soak effluent, followed by the transfer into nitrogen-deficient medium for the induction of ß-carotenogenesis.

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APPENDIX

The following posters were presented at the Sixth Biennial Congress of the South African Society for Microbiology at Stellenbosch University, March 1990:

SALINE TANNERY EFFLUENTS AS GROWTH MEDIA FOR THE HALOPHILIC ALGA Dunaliella salina.

LAUBSCHER, R.K., P.D. ROSE & M.E. AKEN

Department of Biochemistry and Microbiology, Rhodes University, Grahamstown.

Tanneries generate highly saline liquid wastes which are rich in organic and inorganic nutrients, which, if suitably processed, can be used as growth media for the commercial cultivation of the halophilic alga, Dunaliella salina (var. bardawil). This alga is a rich source of glycerol, Beta-carotene, proteins and lipids.

This study examined three different effluent streams produced by the tanning industry. High cell yields occurred in hide soak liquors produced in the rehydration of salt-cured hides. This contains fats, proteins, blood and excrement washed from the hides which adds to the nutritional potential of these wastes. Also present in these wastes are anti-microbial agents which are added to preserve hides during transport and storage. These anti-microbial agents have a marked influence on the performance of algal growth on these wastes and could be reduced by anaerobic and aerobic digestion processes.

It was shown in the study that biomass yields were significantly raised while Beta-carotene production remained constant. The overall yield potential of this system has a significant advantage over defined laboratory growth medium.

TUBULAR ULTRAFILTRATION - A PROCESS FOR THE SEPARATION OF FRAGILE ALGAL CELLS

MAART, B.A., P.D.ROSE & R.K.LAUBSCHER

Department of Biochemistry and Microbiology, Rhodes University, Grahamstown.

Dunaliella salina (var. bardawil) is a fragile halotolerant microalga that does not possess a cell wall. It produces commercially significant quantities of β-carotene and glycerol. A two-stage cultivation process has been proposed to facilitate the differing requirements for growth and β-carotenogenesis. To implement this, an efficient cell recovery system is needed. Numerous cell separation methods have been examined in this laboratory and found to be unsuitable.

This study was undertaken to determine the efficiency of tubular ultrafiltration for cell recovery and recycle. Optimization of this process showed that : 1. Concentrated cells suffered no permanent physiological damage. All cells recovered full motility and continued growing logarithmically. Cells that had been subjected to the concentration process produced similar amounts of β-carotene as the control cells. 2. Both vegetative and β-carotene-producing cells were concentrated up to four times, with the potential to recover cell concentrations of up to 4% solids. 3. High cell concentrations can be viably maintained for many months.