Spirulina Production in Brine Effluent from Cooling Towers

A DISSERTATION SUBMITTED IN COMPLIANCE WITH THE REQUIREMENTS FOR THE MASTERS DEGREE IN TECHNOLOGY IN THE DEPARTMENT OF BIOTECHNOLOGY, DURBAN UNIVERSITY OF TECHNOLOGY, DURBAN

2007

Spirulina Production

in Brine Effluent from

Cooling Towers

by

BILKIS BANU CHOONAWALA

I hereby declare that the dissertation represents my own work. It has not been submitted for any diploma/degree or examination at any other Technikon/University.

_____

Bilkis Banu Choonawala

I hereby approve the final submission of the following dissertation.

_____

Mr. F.M. Swalaha

MSc. Microbiology (UDW)

this _____ day of _____, 2007, at Durban University of Technology.

Dedication

For my Creator, for my family, for their love, for my existence.

Meditation in God is my capital.

Reason and sound logic are the root of my existence.

Love is the foundation of my existence.

Enthusiasm is the vehicle of my life.

Contemplation of Allah is my companion.

Faith is the source of my power.

Sorrow is my friend.

Knowledge is my weapon.

Patience is my clothing and virtue.

Submission to the Divine Will is my pride.

Truth is my salvation.

Worship is my habit.

And in prayer lies the coolness of my eye and my peace of mind.

Hazrath Ali (RA)

Acknowledgements

1. *Acquire knowledge, because he who acquires it, in the way of the Lord, performs an act of piety; who speaks of it praises the Lord; who seeks it, adores God, who dispenses instruction in it, bestows alms; and who imparts it to its fitting objects, performs an act of devotion to God. With knowledge the creatures of Allah rises to the heights of goodness and to noble position, associates with the sovereigns in this world and attains the perfection of happiness in the next (Prophet Muhammed SAW).*

For your guidance, assistance, undying support and friendship and most importantly, for the passing of boundless knowledge, not just in the completion of my research, but in completing my life, I give sincere thanks to my supervisor, mentor and friend, Feroz Mahomed Swalaha.

2. Eddie Davies and Partrick Chamane, Mechanical Engineering, Durban University of Technology

3. Gerald Tutt and Steven Smith, Mossop Western Leathers.

4. Eugene Wellum, CSIR, Uppington

5. Ruben Mudaly, CSIR, Durban

6. Steve Moonsamy, Buying Department, Durban University of Technology

7. Skynet Couriers

8. Gerard Gericke and Carl Woodhouse, Eskom, Vereeniging

9. Vee Dilraj (Technician) and Rose Scott-Briggs (Masters Student), Food Technology Laboratory, Department of Biotechnology and Food Technology, DUT

10. Imam Sadiq said: '*Be careful to have truthful friends and try to obtain them, for they are your support when you are in welfare, and your advocator when you have misfortune'* (*Bihar-ul-Anwar, vol. 74, p. 187)*

For your undying support , assistance and encouragement I sincerely thank my friends, Rena Brijlal, Sharon Govender and Lawrence Shange, Microbiology Laboratory staff, at the Steve Biko Campus.

11. *'Knowledge enables its possessor to distinguish what is forbidden from what is not; lights the way to Heaven; it is our friend in the desert, our companion in solitude, our companion, when bereft of friends; it guides us to happiness; it sustains us in misery; it is our ornament in the company of friends; it serves as an armour against our enemies'. [Prophet Muhammed (SAW)]*

For the opportunity to acquire this knowledge I give sincere thanks to my parents and sister. I love you all.

Preface

10th April 2006: Oral presentation ***Spirulina platensis* Production in Laboratory Scale Bioreactors using Optimised Brine Effluent from Cooling Towers** at the14th Biennial Congress of the South African Society for Microbiology (SASM) held in Pretoria at the CSIR Convention Centre, 9-12th April 2006.

22nd May 2006: Oral Presentation **Optimisation of *Spirulina platensis* Production in Brine Effluent from Cooling Tower Water** at the WISA Biennial Conference and Exhibition held in Durban at the ICC, 20th -25 May 2006.

Comment [Comment1]: I meant where the brine effluent is from e.g. Cooling Water Towers??????? (is this correct)
Don't mention the company's name as we do not have permission to do so

20th September 2006: Poster Presentation ***Spirulina platensis* Production in Laboratory Scale Bioreactors using Optimised Brine Effluent from Cooling Towers** at the Faculty of Engineering, Science and the Built Environment Research Day, Durban University of Technology, 21st September 2006.

Abstract

Spirulina *is a blue-green, multicellular, filamentous cyanobacterium that can grow to sizes of 0.5 millimetres in length. It is an obligate photoautotroph and has a pH growth range from 8.3 to 11.0. The large-scale production of Spirulina biomass depends on many factors, the most important of which are nutrient availability, temperature and light. These factors can influence the growth of Spirulina and the composition of the biomass produced by changes in metabolism. Brine effluent from cooling towers of electricity generating plants may provide an ideal growth medium for Spirulina based on its growth requirements, i.e. high alkalinity and salinity. The aim of this research was to optimise brine effluent from cooling towers by supplementing it with salts, in order to use this optimised effluent in a small open laboratory raceway pond in an attempt to increase the biomass production of Spirulina. Since brine effluent has been previously shown to be a suitable medium for the growth of Spirulina, it was used in laboratory experiments using two cultures of Spirulina, viz. Spirulina maxima and Spirulina platensis. Both cultures were grown in Synthetic Spirulina medium (SSM), Zarrouks Medium (ZM) and Brine Effluent (BE) to determine which grew faster, to choose an organism to use in subsequent experiments. Flasks, each containing 250 ml SSM, ZM and BE respectively, were inoculated with 1.00 x 10^7 ml Spirulina maxima or Spirulina platensis and incubated for 10 days to determine the growth rate and amount of Spirulina produced in each culture flask. Measurement of turbidity, quantification of chlorophyll produced and protein were three parameters that were used to estimate the algal biomass produced. It can be concluded that both Spirulina maxima and Spirulina platensis are capable of growing on brine effluent. Spirulina platensis produced 103 mg.l^{-1} biomass when grown in BE. The t_d of Spirulina platensis (15 h)was shorter indicating that this organism grew faster in BE. Overall, Spirulina platensis performed better and was the organism of choice for*

optimisation experiments. Statistical experimental design comprising half factorial, resolution IV experiments were used to optimise abiotic factors and biotic supplements to be added to BE in order to maximise biomass production. The variables tested were pH, temperature and light (abiotic optimisation) and the salts present in Zarrouks medium, ie $NaNO_3$, NaCl, K_2SO_4 and $NaHCO_3$ (Macronutrient optimisation) and K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2$, $FeSO_4.7H_2O$, NaEDTA and a trace metals solution (Micronutrient optimisation). All experiments were conducted within the 95% confidence interval with $p<0.005$. Abiotic optimisation indicated that light was required for the growth of Spirulina platensis and the pH should be 10 and the temperature at 50^oC for maximum production. Macronutrient and micronutrient optimisation screened for the effects of the 10 factors above with either the supplement being present or absent in a specific flask. Concentrations of Spirulina platensis produced were measured daily and each combination was assessed at 172 hours when maximum concentrations were reached. After 172 hours a maximum of 197 $mg.l^{-1}$ of Spirulina was found in brine effluent which was supplemented with NaCl and $NaNO_3$. This was a 90% increase as compared to growth in unsupplemented brine effluent. μ_{max} was calculated to be 0.08 h^{-1} and t_d was 9 h. A maximum concentration of 193 $mg.l^{-1}$ was obtained in brine effluent supplemented with $FeSO_4.7H_20$, $CaCl_2$ and trace metals. The μ_{max} was 0.03 h^{-1} and t_d was 27 h. This was a 86% increase as compared to growth in unsupplemented brine effluent. NaCl, $NaNO_3$, $FeS0_4.7H_2O$, $CaCl_2$ and trace metals had the largest positive effect on the growth of Spirulina platensis in brine effluent. These experiments show that effluent medium needs to be supplemented in order to obtain higher yields. A final optimised media was formulated for mass production of Spirulina platensis in an open raceway pond, by incorporating the selected nutrients from the macronutrient and micronutrient optimisation studies in brine effluent. This medium contained $NaNO_3$, NaCl, $FeSO_4.7H_2O$, $CaCl_2$ and Trace metals. A maximum concentration of 287 $mg.l^{-1}$ of Spirulina platensis was produced (178% increase as compared to growth in unsupplemented brine effluent). Spirulina platensis grown in the presence of these salts had a μ_{max} of 0.037 and t_d of 18 h. This optimised effluent was used in a bioreactor kept in the laboratory and on the roof of the University. The maximum concentration produced was greater in the bioreactor exposed to sunlight (1086 $mg.l^{-1}$) as compared to the bioreactor indoors (944 $mg.l^{-1}$), however the culture took longer to grow (t_d = 35 h) when outdoors.

Nutritional analysis and feasibility studies indicated that there is potential to produce Spirulina platensis on a large scale with a profit of 248%.

Contents Page

List of Figures

List of Tables

Abbreviations

PBR - Photobioreactor

DOE - Design of Experiments

ANOVA -Analysis of Variance

μ - specific growth rate

μ_{max} - maximum specific growth rate

t_d - doubling time

λ - lambda - A lambda value of 1 indicates that no transformation is required. ie the model falls within the 95% confidence interval.

δ - sigma - standard deviation

SSM - Synthetic Spirulina Medium

ZM- Zarrouks Medium

BEM -Brine Effluent Medium

BE- Brine Effluent (unsupplemented)

Definitions

ANOVA	*A statistical technique which subdivides the total variation of a set of data into component parts associated with specific sources of variation for the purpose of testing a hypothesis on the parameters of a model (Design Expert 7, 2006).*
aquaculture	*cultivation of the natural produce of water (such as fish or shellfish, algae and other aquatic organisms) (http://en.wikipedia.org/wiki/Aquaculture*)

centre points *Numerical factor levels set at the midpoint of each factors high and low settings (Design Expert 7, 2006).*

DOE *Abbreviated form of 'Design of Experiments', is the statistical technology through which processes can be scientifically improved (Design Expert 7, 2006).*

endorheic *terminal or closed basin—is a watershed from which there is no outflow of water, either on the surface as rivers, or underground by flow or diffusion through rock or permeable material (http://en.wikipedia.org/wiki/Endorheic)*

factor *A controllable (input) variable of which two or more values (levels) are selected for study (Design Expert 7, 2006).*

Main effect *The change in response by changing a single factor (Design Expert 7, 2006).*

mixotrophically *Organisms that simultaneously grow on light and glucose*

myzotrophy *type of feeding where the predatory cell pierce the cell wall and/or cell membrane of the prey cell with a feeding tube, sucks the out cellular contents and digests it (http://en.wikipedia.org/wiki/Myzocytosis)*

osmotrophy *uptake of dissolved organic compounds by osmosis for nutrition (http://en.wikipedia.org/wiki/Osmotrophy)*

p-value *Is the risk of falsely rejecting a given hypothesis (Design Expert 7, 2006).*

phagotrophy *enveloping by the cell membrane (http://www.newworldencyclopedia.org/preview/Algae)*

photolysis *chemical reaction in which a chemical compound is broken down by photons (http://en.wikipedia.org/wiki/Photolysis)*

response *Measureable reaction of each experimental unit to every treatment in the design space (Design Expert 7, 2006).*

Interaction	*The effect that occurs when the combined change in two factors produces a/an in/significant effect (Design Expert 7, 2006).*
Run	*A treatment that is applied to an experimental unit (Design Expert 7, 2006).*
screen	*Process of sifting through a large number of factors with the fewest number of trials in a given design (Design Expert 7, 2006).*
ubiquitous	*existing or being everywhere at the same time (http://www.webster.com/cgi-bin/dictionary?book=Dictionary&va=ubiquitous)*

CHAPTER ONE

1. INTRODUCTION

Humans have taken advantage of natural populations of microalgae for centuries (Nostoc in Asia and *Spirulina* in Africa and North America), it is only recently that the potential for microalgal biotechnology has been realised. Microalgal biotechnology has the potential to produce a vast array of products including foodstuffs, industrial chemicals, compounds with therapeutic applications, bioremediation solutions (Olaizola, 2003), production of natural pigments, biofuels and dietary supplements

(Andrade and Costa, 2007), from a virtually untapped source, the primary goal being to establish profitable business ventures (Olaizola, 2003).

Algae are a diverse group of simple, plantlike organisms that photosynthesise to store energy. Although algae have been traditionally regarded as simple plants, they span more than one domain, including both Eukaryota and Bacteria. Algae range from single-celled to multicellular organisms, some with fairly complex differentiated forms and (if marine) called seaweeds. All lack leaves, roots, flowers, and other organ structures that characterise higher plants. They are distinguished from other protozoa in that they are photoautotrophic although this is not a hard and fast distinction as some groups contain members that are mixotrophic, deriving energy both from photosynthesis and uptake of organic carbon either by osmotrophy, myzotrophy or phagotrophy Some unicellular species rely entirely on external energy sources and have reduced or lost their photosynthetic apparatus (Bold and Wynne, 1978, http://en.wikipedia.org/wiki/Algae).

Cyanobacteria have been used as a food source and as a nutritional supplement (BEAM, 2005). Cyanobacteria are prokaryote-single-celled organisms with characteristics that cause biologists to debate whether they are really algae or bacteria. Cyanobacteria are ubiquitous, occurring in typical aquatic and terrestrial habitats as well as in extreme environments such as hot springs with temperatures as high as 71° C and dry crevices of desert rocks. Their colours vary from blue-green to red or purple and this is determined by the proportions of two secondary pigments, *c-phycocyanin* (blue) and *c-phycoerythrin* (red), that tend to mask the green chlorophyll

present in the thylakoids (Becker, 1984).

The cyanobacterium, *Spirulina* was consumed by the Aztecs in 16th century Mexico (http://www.answers.com/topic/spirulina-food-supplement), around Lake Texcoco and has long been a traditional food in Chad. It is now farmed in many countries such as the USA, Thailand, China, India and Australia and sold as a health food and dietary supplement. Commercial algal production is still restricted to very few plants producing high value food or pigments.

A common feature of most of the algal species currently produced commercially (i.e. *Chlorella, Spirulina and Dunaliella*) is that they grow in highly selective environments which means that they can be grown in open air cultures and still remain relatively free of contamination by other algae and protozoa. Thus, *Chlorella* grows well in nutrient-rich media, *Dunaliella salina* grows at very high salinity *and Spirulina* requires a high pH, salinity and bicarbonate concentration (BEAM, 2005).

Spirulina requires a high salinity medium in order to grow and reject brine from desalination plants is high in salts (Eskom, 2006) and would therefore be a suitable medium to grow *Spirulina*. *Spirulina* can be grown on wastewater to couple protein production to recycling of nutrients, removal of inorganic pollutants and disposal of wastes (Ciferri, 1983). Other factors that need to be considered are the biology of the area, the cost of the land, labour, energy, water, nutrients, climate and the type of final product. Various large scale culture systems also need to be compared on their basic properties such as light utilisation, temperature and the ability to maintain a uni-

algal culture (Borowitzka, 1999).

Existing commercial microalgae culture systems are of three types: large open ponds, circular ponds with a rotating arm to mix the cultures, and raceway ponds. The cultivation of any alga, especially under outdoor conditions involves a complex system that depends on the interaction of various external and internal factors. Under natural conditions, most algae grow as mixed communities that include various species and genera. The isolation of a desired species depends on the provision of a suitable environment for its growth (Becker, 1984).

Effluents require supplementation in order to maximise biomass production and supplements can be included from various growth media. Abiotic factors affect the growth and may require optimisation as well. Traditional methods employed one factor-at-a-time (OFAT) design and optimisation whereas factors often interact and can provide better results if these interactions are investigated.

Design of experiments (DOE) provides powerful statistical problem solving tools that can be used to maximize the efficiency and productivity of empirical problem solving (Haaland, 1989). Design of experiments uses data to make decisions in each of its experimental designs, which is a collection of predetermined settings of the process variables. Statistical methods provide an advantage over traditional problem solving methods such as OFAT experimentation because statistical methods focus on small, well designed experiments which answer questions about the process being studied and results in a steeper learning curve and faster progress toward problem solving

(Haaland, 1989).

This research was carried out to determine whether brine effluent from a desalination plant would support the growth of *Spirulina* and whether additional supplementation of the effluent with various salts would increase the productivity. Nutritional analysis and feasibility studies were also conducted to determine whether *Spirulina* could be produced on a large scale with a profitable income.

1.1 AIM AND OBJECTIVES

1.1.1 Aim

To cultivate *Spirulina* in cooling tower brine effluent and statistically optimise the growth in an open tank bioreactor with brine effluent medium, in order to assess the feasibility of producing a food-grade quality of *Spirulina* in marketable form.

Comment [Comment2]: ...assess the feasability of producing a food-grade quality of Spirulina in marketable form.

1.1.2 Objectives

1. To select a fast-growing, non-genetically modified strain of *Spirulina* which offers a promising potential for large-scale production of *Spirulina.*

2. To assess the growth kinetics of *Spirulina* in laboratory scale batch processes to determine the suitability of the medium to support the growth of *Spirulina*.
3. To determine, statistically, the effect of abiotic factors and biotic supplements on the growth of *Spirulina* in brine effluent.
4. To construct and operate a suitable open bio-reactor for the cultivation and production of *Spirulina* in an optimised growth medium.
5. To determine the nutritional composition *of Spirulina platensis* and assess the feasibility of producing *Spirulina platensis* on a large scale.

CHAPTER TWO

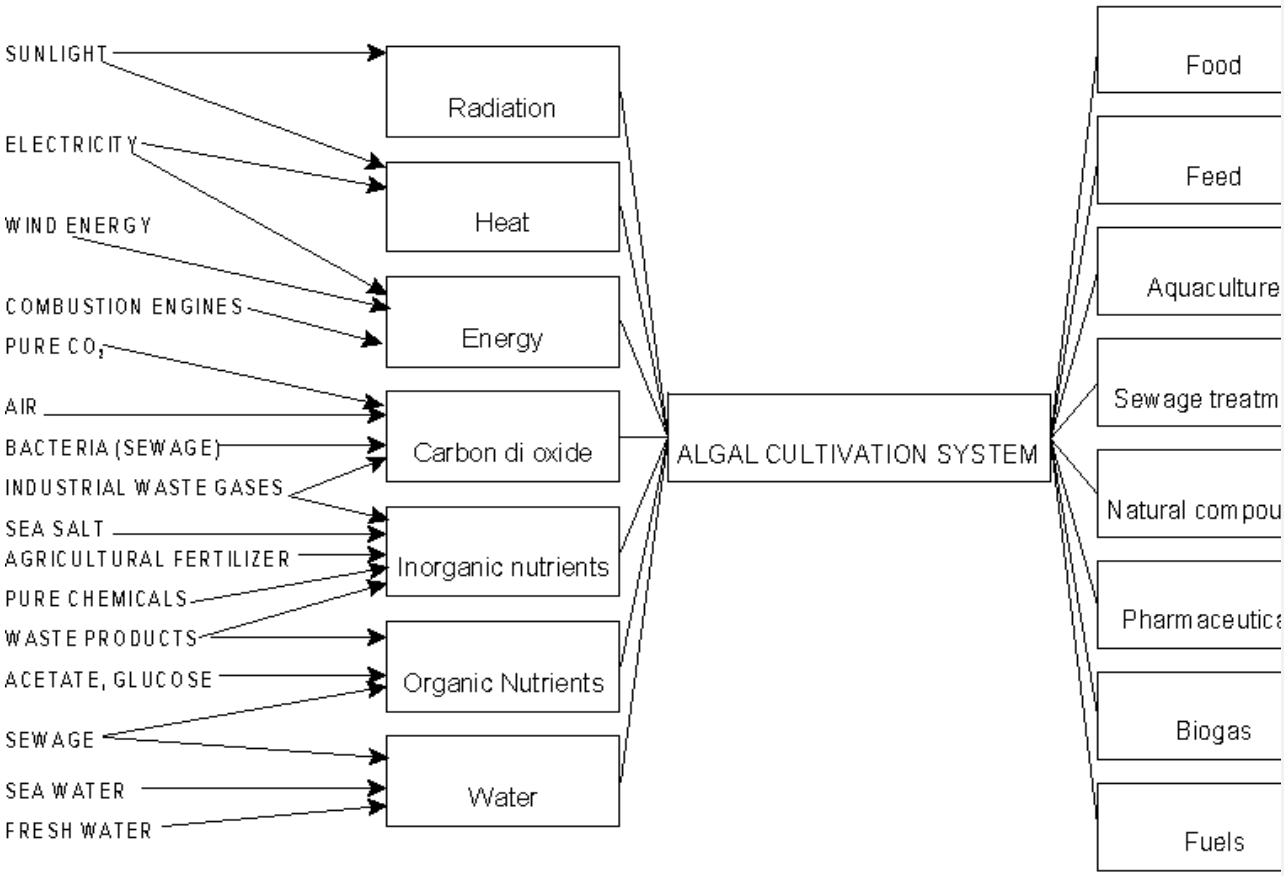
2. LITERATURE REVIEW

The large scale cultivation of microalgae and the use of this biomass as a source of certain food constituents was considered as early as the 1950's. In the past decades, scientists in several countries have made great strides in the production and utilisation of algae and large scale processes have been successfully developed. Algal biomass can be used for various applications such as animal feed, bio-fertilizers, soil conditioner and as feed in aquaculture (Figure 2.1). It may also help solve public health problems by means of biological purification of waste-waters of a

fast developing society (Becker, 1994). Today about 30 000 algal species are known and their classification has changed over the years due to the recognition of previously unknown chemo-taxonomic and submicroscopic characteristics (Soeder, 1986).

In the USA, the pioneer in the production of *Spirulina* is Earthrise farms. In 1985, food-grade *Spirulina* prices ranged between US $ 15,000 to US $ 30,000 per metric ton . In the late 1980s, the worldwide production of *Spirulina* for food was 850 metric tons produced on 10 farms with a total of 35.6 hectares in cultivation. Today, the commercial production is about 2500 metric tons per year (Perret *et al*, 2000).

Figure 2.1 Potential uses of algal biomass: raw material, energy sources, products and applications



Spirulina is a blue-green, ubiquitous organism (Ciferri, 1983) with its name being derived from the latin word for ‘helix’ or ‘spiral’ denoting the physical configuration of the organism when it forms swirling microscopic strands (Anon*,* 2005). (Figure 2.2).

Spirulina production is mainly for speciality items such as vitamins, pigments, pharmaceutical products and the human health food market. Production of *Spirulina* by Earthrise Farms, one of the largest *Spirulina* farms, is over 500 tons a year (Hendrikson, 1998). The continuous cultivation of algae under partially or fully controlled systems has become an important development with various economic possibilities (Becker, 1984).

2.1 ECOLOGY AND HABITAT

Spirulina is a ubiquitous organism and can be found in widely differing environments such as soils, marshes, fresh, brackish and sea water and thermal springs (Richmond, 1986). The fresh-water ponds and lakes that it favours are notably more alkaline - in the range of pH 8 to 11 than ordinary lakes and do not encourage any other form of microorganism. The ability of *Spirulina* to grow in hot and alkaline environments ensures its hygienic status as no other organisms can survive to pollute

the waters in which this alga thrives. Unlike the stereotypical association of microorganisms with 'germs' and 'scum', *Spirulina* is one of the cleanest, most naturally sterile foods in nature. In addition, *Spirulina* thrives in very warm waters of 32 to 45^{o}C and has even survived in temperatures of 60^{o}C. *Spirulina platensis* is found in waters containing from 70 to 85 g.l^{-1} salts. (Ciferri, 1983). Certain desert related species will survive when their pond habitats evaporate in the intense sun, drying to a dormant state on rocks as hot as 70^{0}C. In this dormant condition, the naturally blue-green alga turns a frosted white and develops a sweet flavour (Anon, 2005).

Its adaptation to heat also allows *Spirulina* to retain its nutritional value when subject to high temperatures during processing and shelf storage, unlike many plant foods that rapidly deteriorate at high temperatures (Anon, 2005). Comparative measurements of pH, salinity and alkalinity in several lakes have indicated that the higher the concentration of these factors, the higher the predominance of *Spirulina* (Richmond, 1988).

2.2 MORPHOLOGY AND GROSS STRUCTURE

Algae are differentiated according to their predominating colourations, and are divided into blue-green, green, red and brown types. *Spirulina*, one of the blue-green algae, is a multicellular, filamentous cyanobacterium (Richmond, 1986). Even though it is single-celled, individual filaments of *Spirulina* are relatively large, attaining sizes of 0.5 millimetres in length, which is about 100 times the size of other algae (Anon, 2005).

Under the microscope, *Spirulina* appears as a blue-green filament composed of cyclindrical cells arranged in unbranched trichomes. The filaments are motile, gliding along their axis. Heterocysts are absent. The diameter of the cells ranges from 1 to 3 μm in the smaller species and from 3 to 12 μm in the larger species. The prolific reproductive capacity of the cells and their proclivity to adhere in colonies makes *Spirulina* a large and easily gathered biomass (Richmond, 1986).

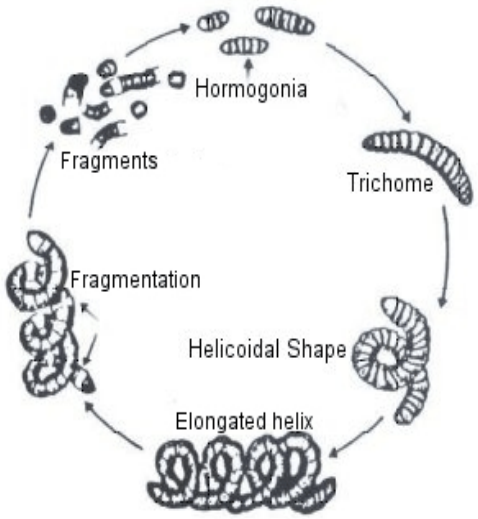
2.3 CELLULAR STRUCTURE

Spirulina are Gram-negative, with soft cell walls that consist of complex sugars and protein. They are undifferentiated and filamentous. Their main photosynthetic pigment is phycocyanin, which is blue in color. These cyanobacteria also contain chlorophyll a and carotenoids. Some contain the pigment phycoythrin, giving them a red or pink colour. *Spirulina* also have gas vesicles, giving them bouyancy in the aquatic environments they inhabit. *Spirulina* are photosynthetic, and therefore autotrophic (http://microbewiki.kenyon.edu/index.php/Spirulina). The photosynthetic apparatus that they possess is similar to that of higher plants. However, since they are prokaryotic, the photosynthetic apparatus is not organised in the chloroplast, but dispersed throughout the cell (Ciferri and Tiboni, 1985).

Many cyanobacteria demonstrate a considerable tolerance to salinity stress. This ability depends on several mechanisms including the accumulation of inorganic and organic osmo-regulators. Research has indicated that *Spirulina platensis* had adapted to salinity stress by increasing carbohydrate metabolism in cells (Zeng and Vonshak, 1998).

2.4 LIFE CYCLE

The life cycle of *Spirulina* is shown in Figure 2.3. A mature trichome is broken into several pieces through the formation of specialised cells, called necridia that undergo lysis, giving rise to biconcave separation disks. The fragmentation of the trichome at the necridia produces gliding, short chains (the hormogonia) (two to four cells), that give rise to a new trichome. Cells in the hormogonium lose attached portions of the



necridial cells, becoming rounded at the distal ends with little or no thickening of the walls. During this process the cytoplasm appears less granulated and the cells assume a pale green colour. The number of cells in hormogonium increases by cell fission while the cytoplasm becomes less granulated and the cells assume a brilliant blue-green colour. The trichome increases in length and assumes the typical helicoidal shape. Random and spontaneous breakage of the trichome and the formation of necridia assures growth and dispersal of the organism (Richmond, 1988).

2.5 CHEMICAL AND NUTRITIONAL COMPOSITION

The blue-green algae in general, and *Spirulina* in particular, have a primitive structure with few starch storage cells and cell membrane proliferation, but rich amounts of ribosomes. This particular arrangement of cellular components allows for rapid photosynthesis and formation of proteins. The lack of hard cellular walls assures that *Spirulina* protein is rapidly and easily assimilated by consuming organisms (http://microbewiki.kenyon.edu/index.php/Spirulina).

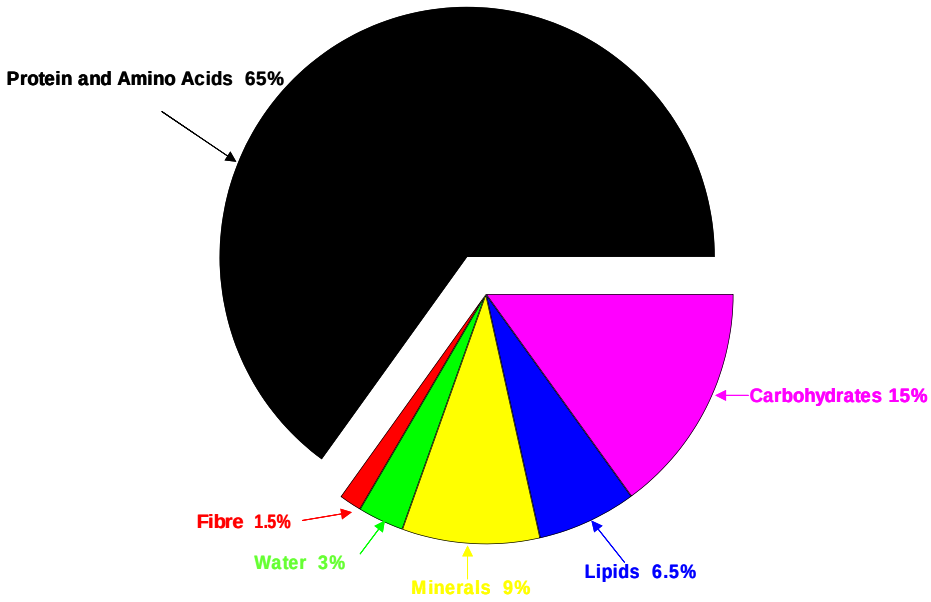
The chemical composition of *Spirulina* reflects its potential as human food, animal feed and as a source of natural products (Richmond, 1988). Rich in protein, β-carotene and γ-linolenic acid, *S. platensis* is a good source of complementary nutrition to prevent malnutrition in developing countries (Kim, 1990). Analysis of *Spirulina* confirms that protein represents more than 60% of the dry weight of *Spirulina* (Richmond, 1988, Singh *et al*, 2002). These proteins are biologically complete, which means they provide all eight essential amino acids in the proper ratios (http://www.naturalways. com/spirulina-analysis.htm).

The amino acid spectrum of *Spirulina* protein is similar to that of other microorganisms**,** and when compared to proteins of eggs or milk, it is deficient in methionine, cysteine and lysine (Ciferri, 1983). Although the amino acid content of *Spirulina* is generally well balanced, it is low in the sulphur containing amino acids and in tryptophan (Richmond, 1988). RNA has been reported to represent 2.2 to 3.5% of the dry weight, whereas DNA represents 0.6 to 1%. The total nucleic acid content is therefore 4% of the dry weight. Algae are single celled organisms rich in nucleic acid so they not only provide a lot of protein, but large amounts of uric acid

which could result in kidney stones or gout (True *et al*, 1982).

About 9% of the total dry weight is composed of essential minerals, potassium, magnesium, calcium, zinc and iron. The waters *Spirulina* favors are saturated with minerals deposited from ancient soils and mountains and because *Spirulina* thrives in such alkaline waters, it incorporates and synthesizes many minerals and derivative compounds into its cell structure. Around 15% is in hydrocarbons, primarily in the form of rhamnose and glycogen. These two polysaccharides are easily absorbed by human cells with minimal intervention by insulin. Hence, *Spirulina* sugars provide speedy energy, without taxing the pancreas or precipitating hypoglycemia (http://www.naturalways.com/ spirulina-analysis.htm).

About 6.5% is composed of lipids (Figure 2.4), including 2.6% of the omega-3 and omega-6 non-saturated essential fatty acids plus an average of 0.17% of beta-carotene and the B vitamins including four micrograms of non-analog vitamin B12 in each 10-gram daily dose of *Spirulina*. The required daily dose of B12 for adults is 2.4 ug/day.



Pigments found in *Spirulina* belong to three classes:

- Chlorophyll *a* which comprises 1.7% of the organic cell weight
- Carotenoids and xanothophylls which comprise 0.5% of the organic weight
- Two phycobiliproteins: c-phycocyanin and allophycocyanin, which normally comprise about 29% of the cellular protein and are

quantitatively the dominant pigments in *Spirulina*.

The dominant lipids in *Spirulina* are mono, di- and higher galactosyl-diglycerides and phosphate-idyl-glycerol. The dominant components in the non-polar lipid fraction were free fatty acids. The lipopolysaccharides comprised 1.8% of the dry weight. The carbohydrate content of the lipolysaccharide consisted of hexose, heptose and glucosamine. The carbohydrate content of *Spirulina* was about 15% of the dry weight and hydrolysis yields glucose, sucrose, levulose, glycerol and several polyols (Singh, 2005).

Intact cells of other algae that are fed to animals or humans are poorly utilised. Various methods for processing and drying the algae to improve digestibility have been extensively studied. In general, *Spirulina* are unique in that they are highly digestible and do not require special processing (Richmond, 1988).

2.6 USES OF *SPIRULINA*

Spirulina is being developed as the ‘food for the future’ because of its remarkable ability to synthesise high-quality concentrated food more efficiently than any other alga. Most notably *Spirulina* is 65 to 71% percent complete protein, with all essential amino acids in perfect balance. In comparison beef is only 22% protein. *Spirulina*

provides a high concentration of many other nutrients, chelated minerals, pigments, rhamnose sugars (complex natural plant sugars) trace elements and enzymes in an easily assimilable form (Richmond, 1986).

The nutritive value of *Spirulina* is amplified in that it has a relatively low percentage of nucleic acids (4%) as compared with the high percentage of nucleic acids content in bacteria. It is high in Vitamin B_{12}, the muco-protein cell walls are easily digestible unlike the cellulose cell wall found in other nutritional algae, it is non-toxic and has lipids that are made up of unsaturated fatty acid that do not form cholesterol. This makes *Spirulina* a potential treatment for people suffering from coronary illness and obesity (Richmond, 1986).

Pre-clinical and clinical studies suggest that *Spirulina* has certain therapeutic effects such as reduction in blood cholesterol, protection against some cancers, enhancement of the immune system, increase of intestinal lactobacilli, reduction of nephrotoxicity by heavy metals and drugs, radiation protection, reduction of hyperlipidemia and obesity (Jimenez *et al*, 2003a). Besides *Spirulina* pills and capsules, there are also pastries, blocks, and *Spirulina* containing chocolate bars, marketed as health food. Other *Spirulina* products are formulated for weight loss and as an aid for quitting drug-addictions (Li and Qi, 1997).

The use of *Spirulina's* pigments as colourants has been explored by the pharmaceutical and food industries. The blue pigment, phycocyanin is used by the Japanese as a food colourant. The trend is to substitute artificial colourants with

natural ones and since microalgae are the highest source of chlorophyll, the idea is to exploit chlorophyll for this purpose (Pelizer *et al*, 2003).

Algae have been used for wastewater treatment and water quality improvement. They use solar energy while absorbing nutrients and other chemicals including heavy metals from wastewater. Although the removal of individual metal species has been extensively studied, there is little information concerning the mixtures of metals which are present in real effluents. The greatest progress towards the industrial application of microbial species for metal removal has been the use of non-living biomass. *S. platensis* has been found to be an effective biosorbent and bioaccumulant of solutions of Cr^{3+}, Cd^{2+} and Cu^{2+} heavy metal ions (Chojnacka *et al*, 2004, Chojnacka *et al*, 2005). Lead biosorption using intact and pre-treated biomass of *S. maxima* indicated that 84% of lead from aqueous solutions of varying concentrations, was removed by intact biomass and up to 92% by the pre-treated biomass (Gong *et al*, 2005) and a *S. platensis* strain was capable of removing 78% cadmium (Rangsayatorn *et al*, 2002).

2.7 LABORATORY CULTIVATION METHODS

The manner in which algae are cultivated varies widely, depending not only on the organism, but also on the use to be made of the culture (Vonshak *et al*, 1988). Just like all plants, each specie of microalga grows best under certain conditions. There is

an ideal combination of temperature, nutrient mix, pH and other factors for each species.

2.7.1 Small Scale Culture Vessels

Various methods have been developed to isolate and cultivate microalgae in the laboratory as well as outdoors on a large scale. Culture vessels that are made out of glass are preferred and are stoppered by means of cotton plugs wrapped in cotton gauze and covered by wax paper or aluminium foil to exclude dust and reduce evaporation. For routine cultivation of small volumes of algal culture, test tubes are sufficient, however if larger quantities of algae are required, 200-500 ml Erlenmeyer flasks or flat bottomed depressed flasks (Figure 2.5a) are normally employed and filled to approximately 30% of their volume with the algal suspension. The 'Penicillium flask' (Figure 2.5b) is suitable for growing cultures up to one litre under ordinary laboratory conditions and the 'Roux' flask (Figure 2.5c) achieves higher irradiance (Becker, 1984).

Figure 2.5 Different types of vessels for laboratory culture of algae

2.8 MEDIA OPTIMISATION

The primary aim in developing culture media is ensuring that the required nutrients are present in an appropriate form and at non-inhibitory concentrations. This aim can be challenging when one considers the diversity of microorganisms in nature as well as their diverse nutritional requirements. (Greasham and Herber, 1997).

After initial cultivation is achieved, the media can then be subjected to optimisation

studies in order to find the conditions, appropriate nutrients, nutrient concentration, temperature, aeration etc, that will best support the growth of the organism (Greasham and Herber, 1997).

2.8.1 Design of Experiments

Design of Experiments (DOE) provides a powerful statistical problem solving tool that can be used to maximise the efficiency and productivity of problem solving. Statistical methods provide an advantage over traditional problem solving methods (Haaland, 1989). Design Expert 7 ® (Statease, Minneapolis, USA) allows one to screen for effects of factors and the responses obtained can be analysed statistically using the appropriate software.

An optimisation strategy that is commonly used in the laboratory is the one -variable-at-a-time method in which an independent variable is tested and optimised while all other variables are kept constant. Each variable is in turn tested in the same way and an optimum determined, provided that the variables do not interact with each other. Most often there is an interaction between the variables making this method of optimisation ineffective. When a large number of variables are presented, valuable time is also wasted. An alternative strategy for optimisation is to then use statistically designed experiments that allow an investigation of more that one independent variable at a time (Haaland, 1989, Greasham and Herber, 1997).

A full factorial experiment covers all combinations of factors and provides valuable information on interactions (Anderson and Whitcomb, 2000). It is a statistical study in which each observation is categorised according to more than one factor. Such an experiment allows studying the effect of each factor on the response variable, while requiring fewer observations than by conducting separate experiments for each factor independently. It also allows studying the effect of the interaction between factors on the response variable (http://en.wikipedia.org/wiki/Factorial_experiment).

With these experiments, the number of experimental runs increases rapidly, with increasing factor number, so it is better to use a fractional factorial experiment which allows one to study many factors, but keep experiments at a reasonable size. A fractional factorial design is a fraction of a full factorial design which decreases the number of experiments but still allows for optimisation (Anderson and Whitcomb, 2000).

Two level fractional factorial designs are used as the initial step for optimisation of media supplements and the designs include centre points. Centre points are runs for which all factors have an intermediate value that occurs in the middle of the high and low value of each variable. They give a better estimate of the experimental error and also indicate whether the best conditions are in the middle of the design and suggests the direction of progress towards optimisation (Haaland, 1989).

FF0408 and FF0616 are Resolution IV designs. This implies that every main effect

is aliased or confounded with a three factor interaction and all two factor interactions are aliased with each other. A Resolution IV design also represents a good compromise between minimal runs and maximum information on the main effects (Anderson and Whitcomb, 2000). FF0308 and FF0516 are Resolution V designs, which indicates aliasing of at least one main effect with one or more four factor interactions, and/or at least one two factor interactions with one or more three factor interactions.

FF0308, FF0616 and FF0516 have centre points added to the design. These are created by setting all factors at their midpoints. The centre points act as a measure of process stability and variability in the experiment and to check for curvature. The remedy for dealing with significant curvature in a two level factorial design is to add more points and by locating these points along the axes of the factor space, a central composite design is created (Anderson and Whitcomb, 2000).

2.9 CULTURE MEDIA FOR THE GROWTH OF ALGAE

Under natural conditions most algae grow as mixed communities, which include various species and genera. The isolation and growth of a particular species will depend on the provision of a suitable environment for its growth. For the successful growth of an alga in a culture, all intrinsic parameters of that organism must be met in the environment. Environmental factors may be physical eg. temperature and light, or

chemical that provides all the raw material used for structural and protoplasmic synthesis of the algal cell. Culture media that are used for algae can be broadly grouped into three major categories, namely

- Complete synthetic media
- Those that are based on natural waters enriched by mineral supplementation and
- Liquid wastes such as effluents from fermented wastes or from waste water treatment plants, industries, etc (Becker, 1984).

2.9.1 Mineral Supplementation

Phosphorus is a major nutrient required for normal growth of all algae and is essential for almost all cellular processes, ie the biosynthesis of nucleic acids and energy transfer. The phosphorus concentration is often growth limiting in the natural aqueous habitat, where it occurs as orthophosphate and in organic combinations. The major form in which algae require phosphorus is as inorganic phosphate either as $H_2PO_4^-$ or HPO_3^{2-}. Like phosphorus, sulphur is also vital to all cells because it is a constituent of the essential amino acids, methionine, cysteine and cystine, vitamins and sulpholipids. It is generally provided as inorganic sulphate in the culture medium (Becker, 1984).

Calcium ions may play a role in the maintenance of cytoplasmic membranes, salt

formation with colloids and the precipitation of $CaCO_3$, however, the absolute requirement for algal growth is in disputable. Sodium is essential for cyanobacteria only and is required by some algae and not by others. Higher concentrations of sodium might be toxic to cyanobacteria, which may account for the lack of these algae in marine environments, but the abundant growth of *Spirulina* and other cyanobacteria in inland saline lakes suggests that sodium is necessary for all marine and halophilic algae. Since sodium and potassium have similar chemical characteristics, it is assumed that sodium may replace potassium which is a requirement for all algae (Becker, 1984).

Magnesium is an absolute requirement for all algal species because of the strategic position it occupies in the photo synthetic apparatus as the cental atom (Mg) of the chlorophyll molecule. Iron is a key element in metabolism, being a constituent of cytochromes, it plays an important role in nitrogen assimilation and affects the synthesis of phycocyanin and chlorophyll. Bleaching and yellow colouring of algae are indicative of an iron deficiency. Phosphate, magnesium and calcium cannot be increased much without precipitating magnesium or calcium phosphate, possibly leading to imbalances in the solution (Becker, 1984).

2.9.2 Trace Elements

No culture medium is considered adequate unless it is provided with several micronutrients required in micro-, nano- or even picogram quantities per litre. Trace elements are those elements that:

- Influence growth in a representative number of species,
- Have a positive effect on total growth,
- Show a direct physiological effect on algal growth,
- Cannot be replaced by another element, and
- Show reversible signs of deficiency in cultures lacking these elements.

The primary aim of recipe formulation is to reduce the high amount of bicarbonate provided in the standard medium described by Zarrouk (Fox, 1996). Effluent from cow dung biogas digesters has been used as a substrate to replace certain chemicals in *Spirulina* culture media. Bone meal, rich in phosphate and calcium, can be used as a substitute for these salts in *Spirulina* cultures that are normally provided with K_2HPO_4 and $CaCl_2$ (Becker, 1984).

2.9.3 pH

Zarrouk studied the effect of salinity on cultures grown under laboratory conditions and reported that *Spirulin*a can tolerate approximately 7 g of NaCl and 50 g $NaHCO_3$ per litre without any measurable ill effects. This tolerance reflected the conditions prevalent in its natural habitat. Alkalinity is mandatory for the growth of this microalga as reflected in its pH optimum, which according to Zarrouk, ranges from 8.3 to 11. *Spirulina* can readily tolerate changes in pH, but abrupt changes could cause the culture to deteriorate quickly (Richmond, 1986).

2.9.4 Light

Growth only takes place in light, but illumination for a full 24 hours is not recommended. During the dark phase, chemical reactions like synthesis of proteins and respiration take place. Respiration decreases the mass of *Spirulina* and its rate is much greater at high temperature. Individual *Spirulina* filaments are destroyed by prolonged strong illumination, in a process called photolysis. Therefore it is necessary to agitate the culture in order to minimise the time it is exposed to full sunlight. Artificial light and heating may be used to grow *Spirulina* although it is not economical. Flourescent tubes and halogen lamps are both convenient since they illuminate and heat at the same time (Jordan, 2003).

2.9.5 Temperature

Temperature is the most important climatic factor influencing the rate of growth of *Spirulina*. Growth below 20^{o}C is practically nil, but *Spirulina* does not die. The optimum temperature is 35^{o}C but above 40^{o}C, the organism is in danger (Becker, 1984; de Oliveira *et al.* 1999*)*. In winter, *Spirulina* does not grow significantly in open tanks, resulting in lower yields due to the absence of sunlight or low temperatures. *Spirulina* manufacturers normally keep their ponds covered with transparent polyethylene to keep the medium warmer and free of contamination (de Oliveira *et al.* 1999).

2.10 MEASUREMENT OF ALGAL GROWTH

One of the parameters for monitoring the growth of algae is the estimation of the algal biomass produced. The growth of algal cultures can be expressed as the increment of biomass, number of cells, amount of pigments and proteins over a given period of time (Becker, 1984).

2.10.1 Turbidity

Measurement of the turbidity, either visually or with a spectrophotometer, is the most general method for estimating the algal concentration of suspended solids in the culture. Spectrophotometric analysis is the measurement of the optical density at a given wavelength. This method is based on the application of the photometric law which states that each elementary layer of a dispersed system scatters the same relative portion of the passing monochromatic light, the magnitude of the scatter being proportional to the dispersion concentration. A wavelength of 550 nm is recommended by Becker (1984).

2.10.2 Dry Weight

Gravimetric estimation of algal dry weight is one of the most direct ways to determine biomass production. This parameter only characterises the general physico-chemical state of the given sample and cannot always be applied as the sole method for biomass estimation because it does not differentiate between the actual algal biomass and suspended non-biological solids. Collected algae should be dried by a method avoiding excessive heat, thereby assuring good reproducibility and identical

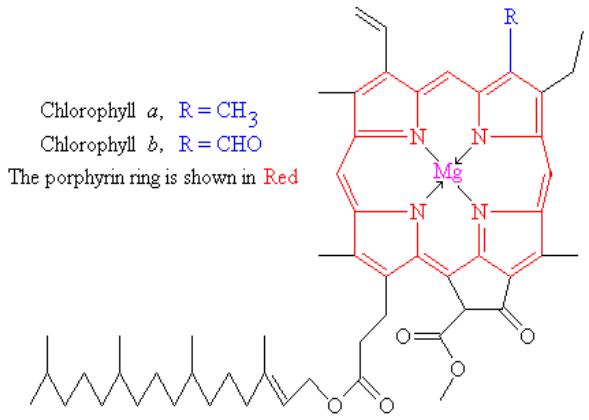
weights obtained for given samples read at the same hourly intervals. Temperatures commonly applied are between 70 and 110^{o}C (Vonshak,1986).

2.10.3 Microscopic Examination

Microscopic examination is often used for algal quantification. Different models of counting chambers are available commercially, the choice depending on algal cell size and concentration. Reproducibility of the counts is the main problem with this procedure so attention must be given to sampling, dilution of the medium and filling of the chamber (Vonshak, 1986).

2.10.4 Chlorophyll

Algae contain chlorophyll so the accurate estimation and quantification of this pigment is a reliable method in algal biomass computation (Simon and Helliwell, 1998). Chlorophylls are cyclic tetrapyrroles, that contain a fifth isocyclic ring which is biosynthetically derived from the C-13 propionic acid side chain of protoporphyrin, and generally a central magnesium atom and a long chain terpenoid alcohol at C-17 (Sandman and Scheer, 2000).

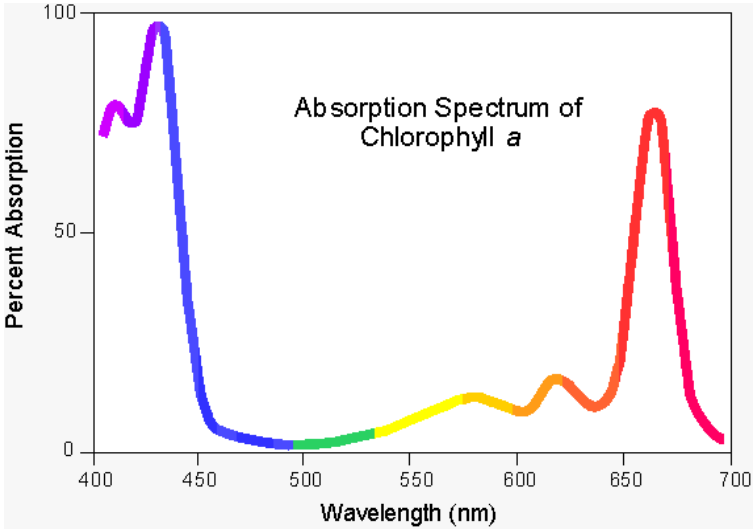


The basic structure of a chlorophyll molecule is a porphyrin ring, co-ordinated to a central atom. This is very similar in structure to the heme group found in haemoglobin, except that in heme, the central atom is iron, whereas in chlorophyll it is magnesium (Figure 2.6).

The different side groups in chlorophyll a and b, absorb light at slightly different

wavelengths, so that light that is not significantly absorbed by chlorophyll *a*, at 460 nm, will instead be captured by chlorophyll *b*, which absorbs strongly at that wavelength. Thus these two kinds of chlorophyll complement each other in absorbing sunlight.

Plants can obtain all their energy requirements from the blue and red parts of the spectrum, however, there is still a large spectral region, between 500-600nm, where very little light is absorbed. This light is in the green region of the spectrum, and since it is reflected, this is the reason plants appear green. Chlorophyll absorbs so strongly that it can mask other less intense colours. Some of these more delicate colours (from molecules such as carotene and quercetin) are revealed when the chlorophyll molecule decays in the Autumn, and the woodlands turn red, orange, and golden brown. (May, ND).



2.10.5 Microalgal Protein Sources

A pre-requisite for the isolation, characterisation and/or utilisation of any protein is the identification of a suitable protein source. In some cases the desired protein may only be unique to a specific species (Walsh, 2002). The blue-green alga (cyanobacteria), *Spirulina* spp, is traditionally harvested from natural lakes as a protein source by the Aztec Indians and some North American tribes. Another microalgae used as food is

the green algae *Chlorella* spp. In the 1950's the Carnegie Institute's research program studied the potential for mass culturing of *Chlorella* to feed the hungry people of the world. The alga has high growth rates and yields, approximately 30 tons of dried cell powder is produced from a one acre pond. Chlorella contains an impressive 60+% bioavailable protein; providing 19 amino acids including all essential aminos in excellent proportions. *Spirulina* contains 65-75% easily digested complete protein, the highest amount of any food. This is 3 to 4 times more than the poorly assimilable protein found in fish, chicken and beef. It has 18 amino acids including the auxotrophic eight. (http://www.botanicalpreservationcorps.com/microalgae.htm)

2.10.5.1 Protein determination

Different methods are available for the estimation of protein. The three methods of choice are:

- Biuret reaction,
- Estimation after Lowry *et al* (1951), and
- Estimation after Bradford (1976)

All three methods required the preparation of a standard curve with known concentrations of bovine serum albumin (BSA).

a. Biuret Reaction method

The biuret reaction is a method that can be used to determine the amount of soluble protein in a solution. The biuret reagent (copper sulphate in a strong base) reacts with peptide bonds and changes colour when it does so

(http://www.hamline.edu/depts/biology/courses/biocon2/biuret.html)

b. Folin-Lowry method

The principle of the method is that a copper tartrate complex is allowed to react with a protein in alkaline solution. The protein-copper complex can reduce phospho-molybdate to develop a blue colour with a broad spectrum absorption peak around 750 nm. The Lowry method is sensitive to low concentrations of protein. Dunn (1992) suggests concentrations ranging from 0.10 - 2 mg of protein per ml while Price (1996) suggests concentrations of 0.005 - 0.10 mg of protein per ml.

c. Bradford method

The Bradford assay is less time consuming and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford method is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis. Assay materials including colour reagent, protein standard, and instruction booklet are available from Bio-Rad Corporation. The method is used for a 100 μl sample volume using five ml colour reagent. It is sensitive to about five to 200 micrograms protein, depending on the dye quality. In assays using five ml colour reagent prepared in the lab, the sensitive range is closer to five to 100 μg protein.

The assay is based on the observation that the absorbance maximum for an acidic

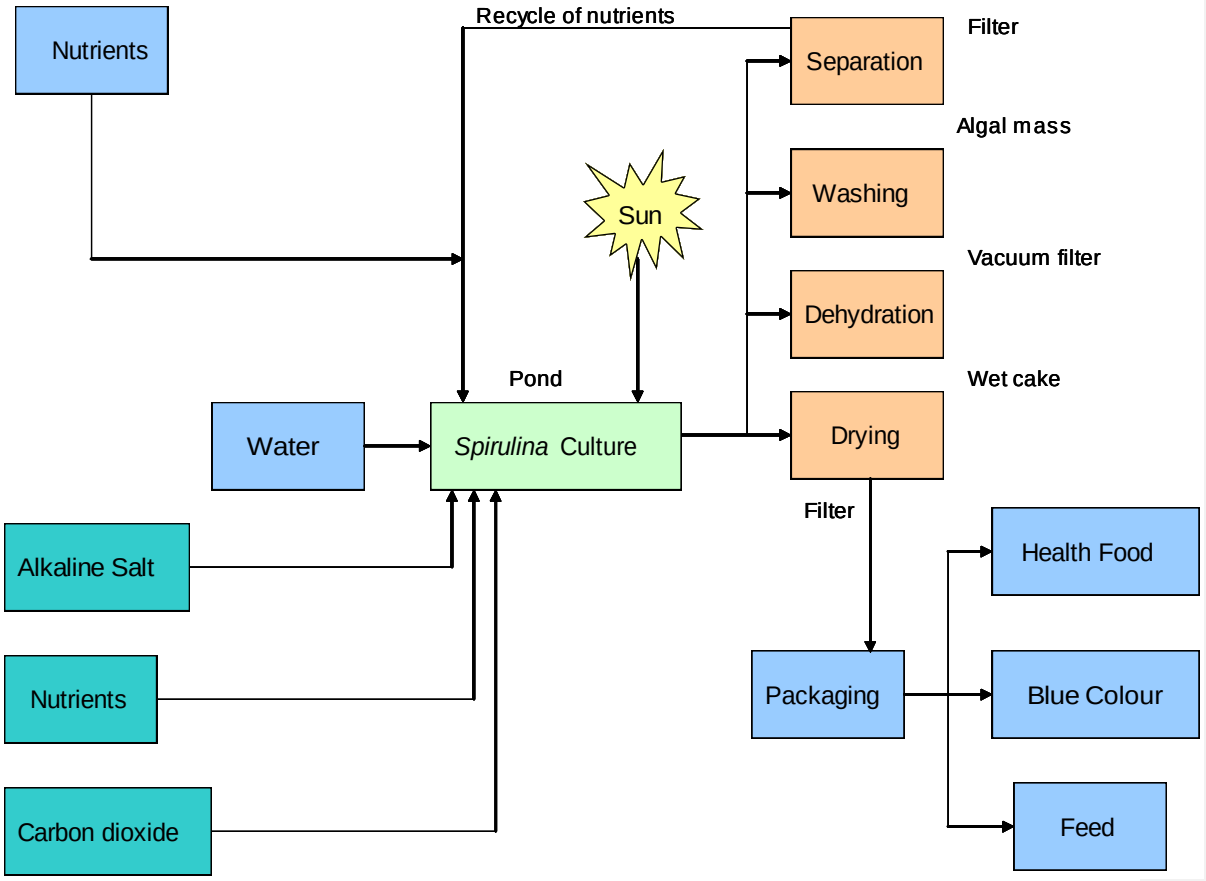
solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range (Bradford, 1976).

The Folin Lowry method was chosen due to its sensitivity to low concentrations of proteins.

2.11 MASS CULTIVATION

Microalgae have long been grown under controlled conditions in the laboratory, where temperature, pH and other important variables can be well controlled, usually with large amounts of researcher and technician time spent doing it. However, these systems are too small – just a few litres – and the amount of time and effort needed to keep them optimized is too costly. The first attempts to produce microalgae on a very large scale were done in "open ponds." A schematic diagram of a typical production system of *Spirulina* is presented in Figure 2.8 The broad, shallow channels in the open ponds

minimised the shading effect and could be affordably built at a substantial scale. The pond is a rectangular open channel with a paddle wheel that generates a circular flow, giving a uniform speed and uniform mixing. Harvesting is done through a series of filtration steps including an inclined gravity screen and vibrating screens. The wet



algal cake is dried by spray drier. Successful outdoor cultivation requires the construction of suitable basins that should be efficient, easy to operate, durable and cheap. The size, shape and material used for construction and the type of agitation employed vary depending on the local conditions, raw material available and the final utilisation of the algal biomass (Becker, 1984).

2.12 COMMERCIAL PRODUCTION OF *Spirulina*

A basic issue in the production of photoautotrophic organisms in general, and *Spirulina spp*. in particular, is to maintain a continuous culture with an optimal population density. Successful maintenance of *Spirulina* sp. in an outdoor mass culture requires a constant vigil ie. up-to-the minute information from which to assess the state of the culture. The pH should be maintained as high as possible to create exclusive surroundings for *Spirulina* without limiting growth. The most crucial challenge in the commercial production of *Spirulina* is to maintain a monoalgal culture throughout the year. The basic demand in this respect is to provide growth conditions that will not be too different from the optimal conditions for *Spirulina.* Nutritional deficiencies can be easily controlled, so it is then important to regulate the temperature. The greater the temperature decline from the 35 to 37^{o}C optimum for *Spirulina*, the slower the growth rate and the easier for contaminants to take over the culture (Richmond, 1988).

Commercial production of *Spirulina* today takes place in Mexico, Taiwan, Thailand, California, Japan and Israel (Ciferri, 1983, Jimenez *et al*, 2003a) and the dried product is a valuable food supplement that is rich in proteins, vitamins and minerals (Jimenez *et al*, 2003a). In the 1970s, a Mexican company realized the algae in Lake

Texcoco clogging the extraction of soda brines from the lake was *Spirulina mexicana*. The world's first large plant was built here (Figure 2.9). Lake Texcoco had a larger potential capacity than any other farm. In 1979 Mexican *Spirulina* was first exported to the U.S. for use in health food products, but in 1982 it was blocked by U.S. authorities due to quality problems. Steps were taken to improve product quality, including heat sterilization to reduce bacteria levels in the lake. Much of the product was sold as animal and aquaculture feed. Daily production of the plant has been reported to have approached 2 tons dry weight, with a yield of 28 tons of protein/ha per year (Ciferri, 1983). *Spirulina mexicana* production has been halted for several years (http://www.spirulinasource.com/earthfoodch6c.html#farms). The reason for this is that the lake was formed within a closed basin, with no outlet and subject to

evaporation which decreased the lake volume. It was then referred to as an endorheic lake (http://en.wikipedia.org/wiki/Lake_Texcoco)

2.13 TYPES OF BIOREACTORS EMPLOYED

Mass production of *Spirulina* requires an inexpensive yet reliable enclosure for growing the culture. The design of these enclosures represents a compromise between the cost of the investment in relation to the expected yield (Richmond, 1988).

The basic requirements for algal growth are solar energy, water, carbon dioxide and inorganic nutrients. Three major technical problems must be considered in developing commercial systems for the cultivation of microalgae:

- The construction of a suitable culture system. The design will reflect the need to balance the biological requirements of the algae with the physical characteristics of the engineered system.
- The maintenance of a monoalgal culture. Contaminants can reduce the quality and the overall yield of the algal product.
- The most suitable method for separation of the biomass from the medium (Chaumont, 1993, Chaumont *et al*, 1987).

During the short history of algal mass cultivation, different kinds of ponds have been

developed and operated at experimental, pilot and industrial scale. The two approaches to the design of such ponds is as follows:

- Open units which are easy to maintain in order to compete with other methods for the production of either proteinaceous matter or cell constituents
- Closed bioreactors for the cultivation of specialised algal strains for the production of specific biochemicals such as enzymes and toxins (Becker, 1984).

The basic design that is presently used consists of an oblong, shallow raceway stirred with paddles and lined with some plastic sheeting, usually polyvinylchloride (PVC). (Figure 2.10) Commercial pond areas range between 500 and 5000 m^2. These ponds may be open and exposed to the atmosphere or covered with a transparent material (Richmond, 1988).

2.13.1 Open Systems

The culture of *Spirulina* and *Chlorella* require a well mixed raceway system and the greater susceptibility to contamination by other algae means that these two organisms must be grown in batch or semi-batch mode with periodic re-seeding of the pond (Borowitzka, 1999).

Open ponds most closely resemble the natural milieu of microalgae (Pulz, 2001). All very large scale commercial systems used today are open air systems. The reason for this is that closed systems are very expensive and difficult to scale up. Also, closed systems that are operated indoors require artificial lighting that results in high

energy costs. Open air systems can utilise sunlight (Borowitzka, 1999) and resemble most closely the natural surroundings of microalga (Pulz, 2001). An advantage of closed systems is the reduction of dirt and insects that would contaminate the algal product. The disadvantages are reduced light penetration, dust accumulation on the outer surfaces and water condensation on the inner surfaces (Richmond, 1988).

The most common design for open ponds are raceway cultivators driven by paddle wheels and usually operate at water depths of 15-20 cm (Pulz, 2001). The culture of *Spirulina* requires a well mixed system such as a raceway system to achieve high growth rates and to minimise the risk of overgrowth of other algae. The **'Caracol'** used at Lake Texcoco, where *Spirulina* grows naturally, was a giant spiral shaped solar evaporator with a surface area of 900 ha and a diameter of 3200 metres. This system

was abandoned due to contamination from urban and industrial development. The problems with open air pond systems are that the productivity achieved is less than that theoretically possible and that it is difficult to control the culture environment which is exposed to the elements (Borowitzka, 1999).

Three different principles of open algal pond designs have been developed:

- Circular ponds with agitation provided by a rotating arm, (Figure 2.11a)
- Oblong forms (raceways) which are constructed singularly (Figure 2.11 b) or joined together to form several units (meander - Figure 2.11c). Agitation is by means of a paddle wheel propeller or an air-lift pump.
- Sloping meander-like constructions, where mixing is achieved by pumping and gravity flow (Figure 2.11d).

Figure 2.11 Schematic outline of major algal pond designs, **a**.

The selection of a particular system is also influenced by intrinsic properties of the alga as well as local climatic conditions and the cost of land and water. Betatene Ltd an Australian producer of *Dunaliella salina* use very large ponds that are unmixed other than by wind and convection. This is made possible since land costs are low and seawater is freely available (Borowitzka, 1999).

2.13.1.1 Circular ponds

Circular ponds (Figure 2.11a) with diameters of 45 m were initially used in the large scale cultivation of algae in Taiwan and Japan. These systems require extensive concrete constructions and high energy consumption for stirring. Other disadvantages of these systems are mechanical problems in controlling the stirring device and the use of land is inefficient (Becker, 1984).

2.13.1.2 Oblong ponds

The majority of ponds employed today are of the oblong type, either in the form of a

single unit or the ‘raceway’ type (Figure 2.11a) with a central dividing wall, or as a meandering channel (Figure 2.11b) obtained by joining individual raceways (Becker, 1984). In this shallow pan, the culture volume is spread over a large area, providing as large a volume-to-area ratio as is practically feasible (Gitelson *et al*, 1996).

The most simple of oblong ponds are no more than a hole dug into the ground. These consist of shallow ditches dug into the ground and covered with thin plastics sheets. While the construction of such ponds might be cheap and easy to complete without technical skill, factors such as wrinkling of the plastic, puncture and other damage to the thin material, and bubbles, water and gas accumulation under the sheets are a few of the major problems (Becker, 1984).

Almost all commercial reactors for production of *Spirulina* are based on shallow raceways in which algal cultures are mixed in a turbulent flow sustained by a paddle wheel. Stirring is accomplished in all ponds with a paddle wheel, the design of which varies from large wheels with a diameter of up to 2 m to small wheels 0.7 m in diameter (Vonshak and Richmond, 1988).

2.13.2 Closed Systems

Irrespective of the success achieved with an open system, closed photobioreactors (PBRs) are now widely used in microlagal biotechnology (Borowitzka, 1999). Closed PBRs are characterised by the regulation and control of nearly all of the biotechnologically important parameters as well as by the following fundamental benefits:

- A reduced contamination risk,
- No CO_2 losses,
- Reproducible cultivation conditions,
- Controllable hydrodynamics and temperature, and
- Flexible technical design.

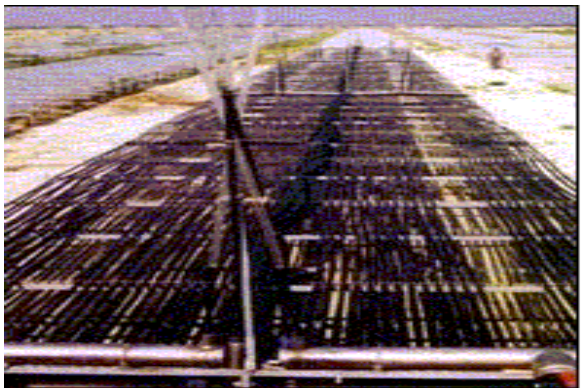
While open air systems are more advantageous, future advances in microalgal mass culture will require closed systems as the algal species do not grow in highly selective environments. Also the final product needs to be free of contaminants such as heavy metals and microorganisms. The two basic designs in closed systems are the flat plate reactors and the tubular reactors. The fundamental principle in these reactors is to reduce the light path and increase the amount of light available to the cells. The closed reactors have several advantages such as ‘clean’ algal culture, high light utilisation efficiency, high productivities, temperature control and the ability to be used outdoors in natural daylight. Contamination is avoided (Borowitzka, 1999). They also allow for better control of growth and have higher photosynthetic efficiencies, but the

technology is capital-intensive even-though it may be justified with economics of scale (Jimenez *et al*, 2003b).

Recent years have shown several major advances in the design of closed PBRs for algal culture. The two basic designs are a flat plate reactor (Figure 2.12) and a tubular PBRs (Figure 2.13) (Borowitzka, 1999).

2.13.2.1 Closed tubular systems

The high value of algal products and the need for good manufacturing practices as well as the necessity for sterile conditions are reasons for the use of a closed bioreactor. The first attempts to grow algae in an outdoor tubular system was performed by the A. D. Little Company on the roof of their building. They encountered leaks in the plastic that they used, the algal mass settled and the medium overheated. In recent years improved designs of tubular photobioreactors have been proposed and realised in France, Italy and the USA (Becker, 1984).



A tubular pilot plant v owth of *Spirulina*. The reactor was made fr with a 14 cm diameter and 0.3 mm thickne lume of 8000 litres. Due to their inadequa ubes were replaced by Plexiglass tubes v ckness. Each tube was joined by a PVC ng Torzillo *et al* (1986).

The BIOCOIL ^{TM} is a helical tubular photobioreactor consisting of a photostage of small diameter plastic tubing wound helically around a tower (Figure 2.14), which allow

Spirulina sp. and *Chlorella sp.* to be grown reliably outdoors at high densities in semi-continuous cultures (Borowitzka, 1999). BIOCOIL originally was a batch reactor and its productivity tends to decrease when nutrients are consumed (Travieso *et al*, 2001). The BIOCOIL^{TM} is the most promising design of tubular PBRs as it ensures uniform mixing and minimises adhesion of the algal cells to the inside of the tubes. BIOCOIL can also be easily scaled up and the whole process automated which minimises labour costs (Borowitzka, 1999).

Figure 2.14 1000L pilot-scale helical tubular photobioreactor of the 'BIOCOIL' type. (http://wwwscieng.murdoch.edu.au/centres/algae/BEAM-Net/BEAM-Appl4a.htm).

2.13.2.2 Cylindrical bioreactors

Cylindrical reactors can be made of stainless steel and have a cylindrical shape with diameters between 20 and 60 cm and heights of between 35 and 100 cm with a volume of 20-250 litres. The top is covered by a flat steel lid and the bottom has a double steel wall for the circulation of water for temperature control. Most of these reactors have an internal illumination system. The algal suspension is agitated by

means of a stirrer. Sterile air with CO_2 is passed into the reactor. After a sufficient growth period, the system is switched off and the alga is allowed to settle to the bottom where it is suctioned out for further processing (Becker, 1984).

2.14 MATERIALS USED IN THE DESIGN OF AN ALGAL POND

The material that is to be used for the construction of the bottom, walls and sides as well as for the surface lining is very important when constructing an algal pond. The materials used for the construction of the sides of the pond can range from sand or clay embankments, brick or cement constructions up to more expensive plastics as UV light resistant PVC glass fibre or polyurethane. The pond lining material is equally important since it is this that comes into contact with the medium and determines seepage, erosion and turbidity (Becker, 1984).

The material used for algal pond linings should be durable and uniform, it should prevent loss of media and entrainment of dirt during mixing, be resistant to chemicals and UV light, be tough but elastic at various temperatures, non-toxic and easy to seam. Plastic sheaths made from UV resistant polyvinylchloride (PVC) or white reinforced UV resistant polyethylene sheets are the most common materials that are used for lining of algal ponds. Concrete has also been used and even though it requires initial high expenses, it lasts for many years and it a more economic than cheaper options. (Becker, 1984).

All materials have an advantage and a disadvantage that affects their stability. A combination of different materials can provide the most suitable solution to meet the needs at different areas of the pond (Becker, 1984).

2.15 ADVANTAGES AND DISADVANTAGES OF OPEN AND CLOSED ALGAL CULTIVATION PLANTS

The design of the technical and technological basis for photobioreactors is the most important issue for economic success in the field of microalgal biotechnology. For future applications, open pond systems for large scale production seem to have a lower potential than closed systems. Various parameters (Table 1.1) have to be considered and these have gained some importance in the choice of a PBR (Pulz, 2001).

Table 2.1 Advantages and disadvantages of open and closed algal cultivation systems

PARAMETER	OPEN PONDS SYSTEMS	CLOSED SYSTEMS
Contamination risk	Extremely high	Low
Space required	high	Low

Water losses	Extremely high	Almost none
CO_2 losses	High	Almost none
Biomass quality	Not susceptible	susceptible
Variability as to cultivatable species	Not given, restricted to a few varieties	High, all varieties can be cultivated

Flexibility of production	Nearly impossible	Change without any problems
Reproducibility of production parameters	not given	Possible with certain tolerances
Process control	not given	Given
Standardisation	not possible	Possible

Weather dependance	Absolute, impossible during rain	Insignificant, production during bad weather possible
Period until net production is reached after start or interruptions	Long, approx. 6-8 weeks	Short, approx 2-4 weeks
Biomass concentration during production	Low, approx. 0.1-0.2 $g.l^{-1}$	High, approx. 2-8 $g.l^{-1}$
Efficiency of treatment process	Low, time consuming	High, short time

2.16 FACTORS AFFECTING MASS CULTIVATION

2.16.1 pH

The pH optimum for *Spirulina* ranges from 8.3 to 11.0, therefore a high alkalinity is mandatory for its growth. *Spirulina* can readily tolerate progressive changes in pH. However, it could quickly deteriorate when the pH is changed abruptly, as may happen in a growth medium which is not well buffered. Buffering can be provided with the use of 0.2 M $NaHCO_3$. *Spirulina* can tolerate high concentrations of NaCl and $NaHCO_3$. Nitrates are the main nitrogen source assimilated by *Spirulina*. If ammonium salts are to be used, then the ammonium concentration must be less than 100 mg $N.l^{-1}$. Like most cyanobacteria, *Spirulina* is an obligate photo-autotroph i.e. it cannot grow in the dark in media containing organic sources of carbon. However in light it may utilise carbohydrates (Richmond, 1988).

The pH of the medium determines the solubility of the carbon dioxide and minerals in the medium. Algae exhibit a clear dependancy on the pH of the medium and different species vary greatly in their response to the pH. In unaerated *Spirulina* cultures, the pH remains stable at 9.5. If adjustments are needed they can be done with the addition of NaOH or sodium bicarbonate (Becker, 1984).

2.16.2 Temperature

Spirulina is a mesophilic organism, ie. the optimal temperature for its growth is relatively high. An optimum temperature of between 35 and 37^0C has been reported and up to 40^0C has been found to be injurious. The minimum temperature that will

still permit growth of *Spirulina* is 18^0C (Richmond, 1988).

2.16.3 Light

Light as energy source for photoautotrophic life is the principal limiting factor in photobiotechnology if the nutrients supplied and the temperature are not limiting the growth of *Spirulina sp.* (Richmond, 1988) and (Pulz, 2001). Phycobilisomes (PBSs) play the most important role of light harvesting in photosynthesis for a cyanobacterium, that is able to adapt its photosynthetic apparatus not only to light intensity, but also to spectral composition, temperature and availability of CO_2 or other essential nutrients (Li *et al*, 2003). The rate of photosynthesis is directly proportional to light intensity if the illumination intensity is above the light compensation point (Pulz, 2001).

2.16.4 Agitation

Agitation is one of the most important requirements for obtaining consistently high yields of biomass, provided that the environmental conditions are favourable and the supply of nutrients adequate. Agitation allows for the uniform dispersion of the algal biomass to ensure sufficient exposure to light and avoidance of settling and continuous mixing of nutrients. Another important effect achieved by mixing is the movement of the algal cells from the illuminated upper zone of the pond to the lower, darker one and back again to the surface (Becker, 1984).

The various agitation methods that are used in pilot and commercial algal plants are:

- A paddle wheel driven by an electrical motor, currently in use for most existing algal projects.

- A combination of pumps and gravity flow used in some large scale production units.

- Air-lift system, and

- Experimental devices eg. free propellers, injectors, manual agitation with simple pumps or brooms and paddle wheels or vertical stirrers driven by wind mills, solar cells, clock-work, animals and man.

When temperature and nutrients do not limit the growth, ie. when light is the sole limiting growth factor, the productivity will depend on the irradiance impinging on the culture surface and on how efficiently radiant energy is converted into chemical energy. It is common experience that cultures attain a higher productivity on sunny days than on cloudy ones. This is related to the fact that outdoor algal cultures are maintained at high cell densities so that they may absorb all the light intercepted and so maximise productivity (Tredici and Zittelli, 1998).

In mass *Spirulina* production using open ponds, maintaining a unialgal culture for a long time is a major challenge (Shimamatsu, 2004). Contamination in outdoor culture are inevitable in view of the non-aseptic conditions. Unwanted algae, mould, yeast

and bacteria are common contaminants. Outdoor cultures of *Spirulina* had an initial bacterial contamination of 2.0 $x10^4$ $CFU.ml^{-1}$ and this increased after 10 days, however growth of the culture in biogas decreased the bacterial load, possibly due to the high alkalinity or the inhibitory role of leaching from the algae (Becker, 1984).

2.17 COMPARISON OF PRODUCTION METHODS

Morist *et al* (2001) have carried out research in order to develop life support systems for men in outer space. In these missions, continuous food resupply is not envisageable thus making it necessary to develop life support systems. *Spirulina platensis* was grown in a photobioreactor and was used to design a process for its recovery to be used as a food source.

Godia *et al* (2002) reported on the development of a loop of interconnected continuous bioreactors, which also aims to provide life support in space. Micro

Ecological Life Support System Alternative (MELISSA) is a model system for an advanced life support system based on different microbial species and higher plants. It consists of a loop of

interconnected bioreactors and a higher plant chamber, each one with a specific biotransformation task to fulfil. The third chamber which consists of an external loop gas-lift photobioreactor is for the culture *Spirulina platensis*.

2.18 HARVESTING OF *Spirulina*

Developing processes for removing cells from the growth medium is a major difficulty. With *Spirulina sp*, however, the filaments are long enough to be removed by filtration (Figure 2.15). A vibrating screen can also be used, however, it can cause cell damage by bruising delicate cells. Microstraining is another method that can be used for *Spirulina*. When harvesting is accompanied by a small amount of cell

breakage, the returning flow is enriched in organic matter to provide an advantage to mixed autotrophic and heterotrophic competitors. Thus, the method of harvesting that is going to be used should remove all the biomass with no cell breakage. Such a system is not yet available for *Spirulina sp* (Richmond, 1988).

The economics of microalgal production depends on the technology employed for harvesting and concentrating the algal suspension in order to render a slurry suitable for further processing (Becker, 1984).

2.18.1 Gravity Filtration

The simplest and cheapest method for separating suspended solids of sufficient size from liquid is by gravity filtration such as screening or straining. The process does become difficult if the particles are compressible or display a certain plasticity like the unicellular algae, *Chlorella* or *Scenedemus*. It is therefore restricted to filamentous or colony forming algae such as *Spirulina* and *Oscillatoria.* It is possible to filter *Spirulina* with ordinary cloth material.

2.18.2 Microstrainer

Microstrainers were initially designed for the elimination of particulate matter from effluents of sewage-treatment plants, but have now also found use in microalgal technology. The device is restricted in its use for filamentous microalgae and not for the removal of smaller algae that cannot be retained on the 20 μm fabric used. This

may result in an enrichment of the medium with small contaminating algal species (Becker, 1984).

2.18.3 Centrifugation

Centrifugation, the most direct method for removal of all types of algae, is used for both filamentous and non-filamentous algae.

2.19 Drying

Drying constitutes about 30% of the production cost. The various systems available for the drying of *Spirulina* differ in both the extent of capital investment and in energy requirements. These factors then affect the food value and the taste of the product. In almost all applications where the harvested and concentrated algal biomass is to be utilised further, a product with a water content of less that 10% is required. Moisture affects spoilage so drying is one of the most important steps in algal production.

2.19.1 Sun-drying

Sun-drying is the simplest and least expensive way of drying, however the method is weather dependant and involves the risk of spoilage. The method is currently restricted for use with *Spirulina* since it does not rupture the cellulosic algae cell walls, which is necessary if the algae is used for feed.

The concentrated *Spirulina* slurry is spread on trays lined with plastic sheets and exposed, in a dust protected place, to the sun. The dried algal flakes are then easy to remove from the fabric. For sun-drying to be effective the layer of the slurry must be no more than 0.75 cm thick. The method is restricted to small cultivation units, since on a larger scale, sun-drying would entail costs for setting up drying beds lined with plastic.

The *Spirulina* slurry should be rinsed in acid water to remove adsorbed carbonates. It can then be stored at -18 ^{0}C for an indefinite time. The usual method for drying *Spirulina* is spray-drying, which yields a very good product from which tablets can be readily formed. Sun-drying (Figure 2.16) is not feasible since sun dried *Spirulina* has an unpleasant odour and might exhibit a larger bacterial count. It is an option if the *Spirulin*a sp. is to be used as animal feed (Richmond, 1988).

Figure 2.16 Sun drying of *Spirulina*

(http://www.auroville.org/health/images/spiru_4.jpg)

2.19.2 Solar Heating

Solar heaters shorten the drying time as compared with direct sun-drying. They consist of a wooden chamber with the inside surface painted black. Temperatures reach an average of 65^{o}C.

2.19.3 Drum Drying

Drum drying, although requiring a high cost for investment, produces a fully digestible and bacteriologically safe product. The method involves placing the wet slurry onto a rotating chromium plated heated drum. The material to be dried is heated for a few seconds and the ensuing dehydration causes the cell wall to open. There is no uniform distribution of the slurry and clogging of the spraying device may occur. The major disadvantage, however, is the large amounts of energy required. Nutritional studies have shown that drum-drying yields a highly digestible algae powder (Becker, 1984). Drum-drying also yields a useful product in the form of flakes (Richmond, 1988).

2.19.4 Spray-drying

The efficiency of spray-drying is comparable to drum-drying but requires a less concentrated paste and give a fine uniform powder. The product can be less digestible because of the temperatures reached within the sprayed algal slurry.

2.20 EFFLUENTS AND STRAINS USED IN THE CULTIVATION OF *Spirulina*

Algae are used to purify and treat wastewater by stripping it of its waste material and products. The difficulty in this is the removal of the algae from the waste water. *Spirulina*, however, can be separated from the medium quite easily, and since it has a

high nutritive value, it is a promising organism for waste water treatment. Canizares and Dominguez (1993) used *Spirulina* sp and *Spirulina maxima* for secondary treatment of swine waste from which methane was generated. The use of animal waste to produce algal biomass as a means to recycle its feed value has been investigated in recent years. *Spirulina* has been grown outdoors in a medium containing sodium chloride and nutrients originating solely from solid cattle waste in order to optimise a medium based on animal waste for economic production (Mitchell and Richmond, 1998).

An integrated system that uses untreated sea-water that was supplemented with anaerobic effluents from pig waste is an attractive process which serves two purposes. One, is the production of *Spirulina* at a low cost, and two, the treatment of

animal waste (Olguin *et al*, 2001),

Effluent from a domestic sewage treatment plant has been shown together with sea water, to enhance the growth of unicellular microalgae. The waste effluent from the National Fertilizer Company of Nigeria has been shown to support the growth of *Chlorella* and *Spirulina* spp (Anaga and Abu, 1996).

Wastewater arising from the production of sago starch had a high carbon to nitrogen ratio, which was improved with anaerobic fermentation in an upflow packed bed digester and supported the growth of *S. platensis (Arthrospira*) (Phang, *et al*, 2000).

2.21 GROWTH OF *Spirulina* IN BRINE

Desalination plants are usually installed in areas where there is a shortage of good quality water. Measures of prevention of disposal of waste brine into the aquatic environment is inadequate (Buckley, *et al*, 1987). The culture of *Spirulina* depends

on a suitable growth medium such as saline water. In some salty and high aqueous environments, *Spirulina* strains may form, at a given time, a bloom representing more than 90% of the total phytoplankton biomass (Vonshak*, et al* 1988). Parker and Bates (2003) have shown that oilfield brine, a wastewater from the petroleum industry contains high levels of dissolved salts and residual hydrocarbons. Treatment of the brine with hydrocarbon-degrading bacteria and dilution to desired salinity, produces a diluent that is suitable for preparation of a growth medium for *Spirulina sp.* The brine to be used in this study will be obtained from Lethabo power station, Eskom (Figure 2.17) and is the result of the cooling tower blow-down at the power station.

Figure 2.17 Lethabo Power Station.

(www.eskom.co.za)

South Africa has a large number of coal-fired power stations in the Mpumulanga area. Lethabo power station located close to the Vaal River near Vereeniging, is owned and operated by Eskom, a state-owned utility that supplies 95 percent of South Africa's electricity, as well as exporting power to neighbouring countries (http:// www.africanenergy.co.za/ magazines/ evol1no3/ story11.htm)Vol 1 NO 3). This 3.6 GW plant is built adjacent to the opencast coal mine which supplies its fuel and the main source of water for cooling is the Vaal River (Brouckaert and Buckley, 2003), highly concentrated underground water from the New Vaal Coal Mine and treated sewage water as "make-up" water for the cooling water. The mine and river water could introduce approximately 27 tons of salts per day into the cooling water systems and these must be removed on a continuous basis to prevent above acceptable salt thresholds, otherwise corrosion damage will cause premature failure of the condenser and associated plant. Using reverse osmosis, a pump forces approximately 80% of the water through membranes, leaving behind approximately 20% of the water containing most of the salts. The advantage of this process is that any volume of dirty, unusable water can be reduced by 80% leaving a residual volume, called brine, to be disposed of in an environmentally acceptable manner, ie. by mixing it with ash on the dry ash dump. The cementing properties of the ash are used to encapsulate the salts contained in the brine preventing them from polluting the environment (Eskom, 2006).

2.22 SUMMARY

Starvation and malnutrition are still widespread in vast portions of the world. Present day agriculture is unable to satisfy the basic human need i.e. adequate nutrition as evidenced by the large number of starving humans. Agribusiness farming practices have externalized many production costs, and relentlessly destroy natural resources (http://www.spirulinasource.com/earthfoodch7b.html#agricultural). Hailed as the 'greatest food on earth' by various international health organisations, *Spirulina* (Phylum, Cyanobacteria) is an ideal nutritional supplement and can be the answer to malnutrition problems in developing countries (Vendan and Rajeshwari, 1998).

Spirulina, composed of 60% protein, is one of the most extensively used microalgae for animal and human nutrition. It is rich in amino acids, natural pigment such as chlorophyll and beta carotene and γ-linoleic acid (Jimenez *et al*, 2003). It can be cultivated on marginal, unusable and non-fertile land. Growing algae for food has become more attractive since it does not compete with needs for drinking water or agriculture for land, it is less dependent on favorable weather conditions and its yield is much higher than that of agriculture. (http://www.spirulinasource.com/ earthfoodch7b.html#agricultural).

Spirulina grows naturally in alkaline lakes and has a long history of being used as a human food (Shimamatsu, 2004). The growth of *Spirulina* on wastewater is an attractive possibility. Research has indicated that brine effluent from desalination plants has a high salinity and is suitable for the growth of *Spirulina*. This study aims to

explore this substrate as a possible growth medium for *Spirulina* spp.

Many producers of *Spirulina* are faced with the problem of how to achieve the high productivity (Shimamatsu, 2004). Cultivation of algae using natural and man-made open ponds is technologically simple, but not necessary cheap due to the high down-stream processing costs. The need to achieve higher productivity and to maintain a monoculture of algae led to the development of closed photobioreactors. However, volumetric productivity and cost of production are much better in open-pond culture (Lee, 2001). Most commercial reactors used in large scale productions of algae are of the open, shallow, raceway type, in which the algal culture is mechanically mixed.

Productivity can be dramatically improved by statistical experimental design (DOE) where more than one factor can be assessed and optimised in a short series of experiments. It is especially suited to industrial product development as yields are

improved at each iteration (Haaland, 1989). Brine effluents can be supplemented with traditional media components in order to increase *Spirulina* yield and DOE can be used to select and optimise these components. This study aims to achieve this.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 CULTURE CONDITIONS

Two *Spirulina* cultures, *S. maxima* and *S. platensis* were used in this study. The first culture was obtained from University of Texas Algal Culture Collection (UTEX) and the second, from CSIR in Upington. *S. maxima* was grown in batch culture in Synthetic Spirulina Medium (SSM), as described by Schlosser (1982) (Appendix 1) and *S. platensis*, in Zarrouks Medium (ZM) (Appendix 2). Cultures were kept under continuous light (0.1522 Lux) and agitation (160 rpm) and were re-inoculated into a new medium every month in order to maintain viability.

3.2 INOCULUM PREPARATION

Inoculum was prepared in 250 ml Erlenmeyer flasks containing 100 ml of each of the media and agitated at 180 rpm in a rotary shaking incubator. Temperature was kept constant at 30^0C and illumination was provided by flourescent lamps at an intensity of

0.1522 Lux for 10 days. Fifty millilitres of each culture was centrifuged at 3500 rpm for 10 minutes. Supernatants were discarded and the remaining pellets were re-suspended in distilled water. The number of cells present in 1 ml of inoculum of *S. maxima* and *S. platensis* was counted using a Nebauer counting chamber and was found to be 1.16×10^7 cells per ml and 1.51×10^7 cells per ml respectively (Appendix 3). This was adjusted to a concentration of 1.00×10^7 cells per ml. Five ml of the adjusted cell concentration was used as inocula for further experiments.

3.3 *SPIRULINA* PRODUCTION IN BATCH EXPERIMENTS

Both *S. maxima* and *S. platensis* growth kinetics were studied to determine the one that produced more biomass. Laboratory shake flask experiments comprised of twenty four 500 mL conical glass flasks containing 250 mL Synthetic Spirulina Medium (SSM), Zarrouks Medium (ZM), and Brine Effluent (BE). (Appendix 4). Flasks were divided equally between the two microalgae, with 12 designated to *S. maxima* and the other twelve to *S. platensis*. Nine of the twelve flasks represented the three growth media used in triplicate experiments and the remaining three flasks represented the controls, which contained growth media only. One set of twelve flasks was inoculated with 5 ml of *S. maxima* and another set was inoculated with 5 ml *S. platensis*. Illumination was provided by a florescent light source at an intensity of 0.1522 Lux. All flasks were incubated in a rotary shaking incubator at room temperature for 10 days.

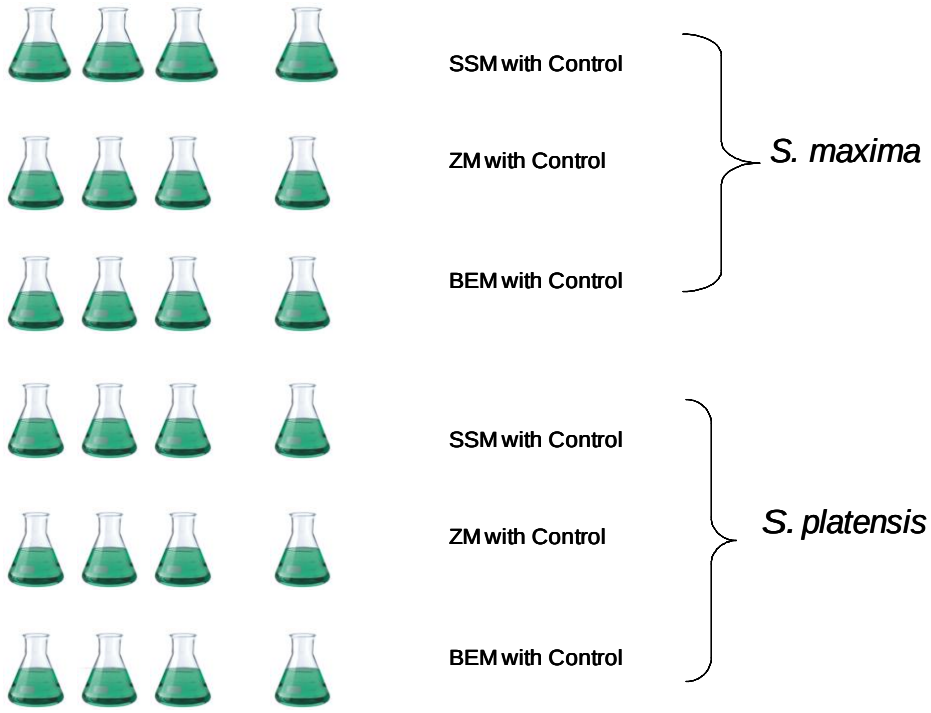


Figure 3.1 Schematic diagram of distribution of flasks for laboratory batch production of *Spirulina*

3.4 GROWTH ESTIMATION OF *Spirulina*

3.4.1 Concentration of *Spirulina*

Five ml aliquots from each set of triplicate flasks were removed every twenty four hours and the turbidity was measured at 550 nm using a WPA S2000 Lightwave Diode Array Spectrophotometer to determine cell density. This was compared to standard curves

(Appendix 8.8.1 to 8.8.6) to determine quantities of biomass produced. pH was also determined using a Beckman 50 pH Meter.

3.4.2 Kinetic Parameters and Calculation

The growth rate of *Spirulina* spp follows the common pattern of many other microorganisms that undergo a simple cell division without any sexual or differential step (Bhattacharya and Shivaprakash, 2005).

The specific growth rate of the microalgae was estimated with the formula (Carlile and Watkinson, 1997):

$$u = \frac{2.3(\log x - \log x_o)}{t}$$

where

μ = specific growth rate

x = biomass concentration at a specific time

x_0 = concentration at the initial time (T_0) and

t = time (hrs)

The equation that combines the specific growth rate and the doubling time is given by:

$$t_d = \frac{\ln 2}{\mu}$$

where

t_d = doubling time and

μ = specific growth rate as calculated above

3.4.3 Chlorophyll Extraction and Determination from *Spirulina* spp

Chlorophyll was extracted from samples collected daily using the Standard Method for extraction (Appendix 5). The collected supernatant absorbance was read at 630, 647, 664, and 750 nm to determine the amount of chlorophyll *a* in each sample. The

first three readings were subtracted from the absorbance reading at 750 nm. These represented the corrected absorbance readings. The concentration of Chlorophyll a in the extract is calculated by inserting the correct optical densities in the following equation:

$$C_a = 11.85\ (OD_{664}) - 1.54\ (OD_{647}) - 0.08\ (OD_{630})$$

Where:

C_a = concentrations of chlorophyll a in mg.l^{-1} and

OD_{664}, OD_{647}, OD_{630} = corrected optical densities (with a 1 cm light path) at the respective wavelengths.

No standard curve is necessary for chlorophyll determination since the equation converts the optical densities and presents the values in $mg.l^{-1}$.

3.4.4 Folin Lowry Method of Protein Measurement from *Spirulina* spp

3.4.4.1 Sample preparation

The remaining pellet from chlorophyll extraction was re-suspended in 10 ml distilled water and subject to the protocol for protein determination (Appendix 8.6).

3.4.4.2 Reagent preparation

The reagents that were required were prepared according to the method outlined in Appendix 8.6.

3.4.4.3 Protein measurement

The pellet was solubilised in 3 ml of 0.1 M NaOH and 0.5% alkaline copper sulphate solution, mixed in a ratio of 1:50. An aliquot of 0.3 ml diluted Folin-Ciocalteau reagent was then added. The mixture was allowed to stand at room temperature for half an hour, and the absorbance was read at 720 nm in a WPA S2000 Lightwave Diode Array Spectrophotometer. The readings are converted to $mg.ml^{-1}$ of protein using a BSA Standard Curve (Appendix 7).

3.5 MEDIA OPTIMISATION

A fractional factorial experiment FF0308 (Table 3.1) was used to determine the effects of biotic factors, pH, temperature and light, on the growth of *Spirulina*, The parameters obtained were then used for all remaining experiments. The 10

components of ZM, ($NaNO_3$, NaCl, $NaHCO_3$, K_2SO_4, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2$, $FeSO_4.7H_2O$, EDTA, Trace metals) were used to supplement the BE in 2 fractional factorial experiments, the first of which consisted of 8 runs, Macronutrient Optimisation (Table 3.2) and the second 19 runs, Micronutrient Optimisation (Table 3.3). Salts that were present in larger quantities in the original Zarrouks medium were grouped together in the first fractional factorial (Macronutrient Optimisation) and those salts present in lower quantities were grouped together in the second fractional factorial (Micronutrient Optimisation). The factors designated A, B, C etc represent the medium supplements. After analysis of the results obtained from these two fractional factorials, those salts that had a positive effect of the production of *Spirulina* sp were grouped together in another fractional factorial FF0516 (Table 3.4). This represented the optimised media containing the best salts from the two fractional factorials. Brine does not contain these components and they were added to determine their effect on the productivity of *Spirulina platensis.*

The first two numbers (FF**04**08) indicate the number of components used in the experiment and the last two numbers are the number of experiments (FF04**08**).

Table 3.1 Abiotic Factor Optimisation - Fractional Factorial Experimental Design (FF0308) for screening of three abiotic factors, pH, Temperature and Light. The design consists of 8 runs and is a Resolution V design.

Run	Factor*

	A	B	C
1	-	-	-
2	-	-	+
3	-	+	-
4	-	+	+
5	+	-	-
6	+	-	+
7	+	+	-
8	+	+	+

*(A), pH at a low value of 8 and a high value of 10; (B), temperature at a low value of 25^{o}C and a high value of 30^{o}C; (C), absence or presence of light. +, is the high value of the factor and - is the low value of the factor.

Table 3.2 Macronutrient Optimisation - Fractional Factorial Experimental Design (FF0408) for screening of four factors, $NaNO_3$ (A), NaCl (B), K_2SO_4 (C) and $NaHCO_3$ (D) for *Spirulina platensis* production. The design consists of 8 runs and is a Resolution IV design. The table indicates the presence and absence of the four factors in $g.l^{-1}$ that were used in the experiment. These values are representative of the amounts of salt present in ZM.

Run	Factors*			
	A	B	C	D
1	-	-	-	-
2	-	-	+	+
3	-	+	-	+
4	-	+	+	-
5	+	-	-	+
6	+	-	+	-
7	+	+	-	-
8	+	+	+	+

* (A), $NaNO_3$ at a concentration of 2.5 $g.l^{-1}$; (B), NaCl at a concentration of 1 $g.l^{-1}$; (C), K_2SO_4 at a concentration of 1 $g.l^{-1}$ and (D), $NaHCO_3$ at a concentration of 16.8 $g.l^{-1}$. + , is the presence of the factor and - is the absence of the factor.

Table 3.3 Micronutrient Optimisation - Fractional Factorial Experimental Design (FF0616) for screening of six factors, $FeSO_4 7H_2O$ (A), $CaCl_2$ (B), $MgSO_4 7H_2O$ (C), K_2HPO_4 (D), Na.EDTA (E) and Trace metals (F) for *Spirulina platensis* production. The design consists of 16 runs and 3 centre points and is a Resolution IV design. The table indicates the presence and absence of the six factors in $g.l^{-1}$ and mls for trace metals, that were used in the experiment. These values are representative of the amounts of salt present in ZM.

Run	Factors					
	A	B	C	D	E	F
1	-	-	-	-	-	-
2	+	-	-	-	+	-
3	-	+	-	-	+	+
4	+	+	-	-	-	+
5	-	-	+	-	+	+
6	+	-	+	-	-	+
7	-	+	+	-	-	-
8	+	+	+	-	+	-
9	-	-	-	+	-	+
10	+	-	-	+	+	+
11	-	+	-	+	+	-
12	+	+	-	+	-	-
13	-	-	+	+	+	-
14	+	-	+	+	-	-

15	-	+	+	+	-	+
16	+	+	+	+	+	+
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0

* (A), $FeSO_4.7H_2O$ at a concentration of 0.01 $g.l^{-1}$; (B), $CaCl_2$ at a concentration of 0.04, (C), $MgSO_4.7H_2O$ at a concentration of 0.2 $g.l^{-1}$, (D), K_2HPO_4 at a concentration of 0.5 $g.l^{-1,}$ (E) Na.EDTA at a concentration of 0.08 $g.l^{-1}$ and (F), Trace metals at a concentration of 4 $ml.l^{-1}$. + , is the presence of the factor and - is the absence of the factor. 0 is the average between the high and low concentrations.

Table 3.4 Optimised BEM - Fractional Factorial Experimental Design (FF0516) for screening of 5 factors, $NaNO_3$ (A), NaCl (B), $FeSO_4.7H_2O$ (C), $CaCl_2$ (D) and Trace metals (F) for *Spirulina platensis* production. The design consists of 16 runs and 3 centre points and is a Resolution V design. The table indicates the high and low values of the salts that were added to BE.

Run	**Factors**				
	A	**B**	**C**	**D**	**E**
1	-	-	+	-	+
2	+	-	+	-	-
3	-	+	+	-	-
4	+	+	+	-	+
5	-	-	+	-	-
6	+	-	+	-	+

7	-	+	+	-	+
8	+	+	+	-	-
9	-	-	+	+	-
10	+	-	+	+	+
11	-	+	+	+	+
12	+	+	+	+	-
13	-	-	+	+	+
14	+	-	+	+	-
15	-	+	+	+	-
16	+	+	+	+	+
17	0	0	0	0	0
18	0	0	0	0	0
19	0	0	0	0	0

* (A), $NaNO_3$ at a high concentration of 2.5 $mg.l^{-1}$ and a low concentration of 1 $g.l^{-1}$; (B), NaCl, at a high concentration of 2 $mg.l^{-1}$ and a low concentration of 1 $g.l^{-1}$; (C), $FeSO_4.7H_2O$ at a high concentration of 0.002 $mg.l^{-1}$ and a low concentration of 0.01 $g.l^{-1}$; (D), $CaCl_2$, at a high concentration of 0.06 $mg.l^{-1}$ and a low concentration of 0.04 $g.l^{-1}$ and (E), Trace metals at a high concentration of 6 $ml.l^{-1}$ and a low concentration of 4 $ml.l^{-1}$. + , the high concentration of the factor and -, the low concentration of the factor. 0 is the average between the high and low concentrations.

This design is a combination of the Macronutrient Optimisation and Micronutrient Optimisation. The salts from these two experiments with the highest effect on the

growth of *Spirulina platensis* are combined here in order to complete final optimisation studies.

3.6 DESIGN EXPERT ANALYSIS

3.6.1 Evaluation of Model Effects

A Pareto Chart ranked the effects of the supplements according to the greatest effect on the final response. There were two different *t* limits plotted on the graph, based on the Bonferroni corrected *t* and a standard *t*. When effects are selected, those that are above the Bonferroni Limit are almost certainly significant. Effects that were above the *t*-Value Limit are possibly significant and should be added if they are not already selected. Effects that are below the *t*-Value limit are not likely to be significant. Positive effects were indicated by the orange bars and negative effects by the blue bars.

3.6.2 ANOVA and statistical evaluation

The analysis of variance (ANOVA) tests the difference between the means of two or more groups. By default, Design Expert 7® (Statease, Minneapolis, USA) considers values of 0.05 and below as significant. This value can change depending on which factors are selected for the analysis. Analysis of variance (ANOVA) is employed for

the determination of significant variables. ANOVA consists of classifying and cross-classifying statistical results and was tested by the means of a specified classification difference, which was carried out by Fisher's statistical test (*F*-test). The *F*-value is defined as the ratio of the mean square of regression to the error, representing the significance of each controlled variable on the tested model (Zhi *et al*, 2005).

3.6.3 Validation of the Model

The Box Cox plot is a tool to help determine the most appropriate power transformation to apply to response data. A transformation is needed if the error (residuals) is a function of the magnitude of the response (predicted values). The blue line indicates the current transformation, if any, the green line represents the best model and the red lines are the confidence interval surrounding it. Lambda (λ) is 1- δ (Standard deviation). A lambda value of 1 indicates that no transformation is required, this means that if the blue line falls within the red lines, then no transformation is required. The power transformation allows transformation to any power in the range –3 to +3, provided the data are positive.

3.6.4 Main Effects

Cube plots are useful for representing the effects of three factors at a time. They show the predicted values from the coded model for the combinations of the –1 and +1 levels of any three factors that you select. The contour and 3D plots are a two-

dimensional representation of the response for selected factors. Cube plots are useful for
representing the effects of three factors at a time.

3.7 CONSTRUCTION OF A PHOTOBIOREACTOR

A raceway algal photobioreactor was constructed especially for microalgal culture (Figure 3.2a), with uniform artificial lighting provided by a 20 W florescent tube (4.28 Lux). The reactor consisted of a paddle that was turned using an electric source (Figure 3.2 b), consisting of an AC/DC output and a rheostat to control the voltage supplied. The speed at which the paddle turned was controlled by the electrical output device. The total volume of the bioreactor was 10 litres with a depth of 179 mm and a length of 668 mm. The working volume was 5 litres with a depth of 40 mm for sufficient exposure to light (Figure 3.3). Light transmission distance was 300 mm from light source to the bioreactor and was focussed on the bioreactor surface to provide homogenous illumination. The bioreactor was covered to prevent dust from entering and contamination occurring. All measurements were performed in triplicate and the corresponding data were expressed as mean concentration values.

Figure 3.2 Bioreactor used for the growth of *Spirulina platensis*. The paddle is controlled by an electric power supply and the speed can be adjusted.

Figure 3.3 Schematic view of the bioreactor used for the production of *Spirulina platensis*

3.7.1 Measurement of *Spirulina platensis*

The inoculum was a sample of *Spirulina platensis* cultured in the laboratory and a total volume of 250 ml was used. Five ml aliquots from the bioreactor was removed every twenty four hours and the optical density was measured at 550 nm using a WPA S2000 Lightwave Diode Array Spectrophotometer to determine growth. A volume of fresh medium was added to the culture to maintain a constant volume.

3.8 DRYING OF *Spirulina platensis* POWDER AND MICROBIOLOGICAL TESTING

After a suitable period of cultivation had passed (20 days), the alga from the remaining volume of media was recovered. The wet slurry was sun-dried and blended, in a pestle and mortar, into a powder for further testing. *S. platensis* wet biomass was tested for bacterial and fungal contamination by pipetting one ml of sample into nine ml sterile distilled water. Serial dilutions were carried out up to 10^{-6}. One ml from each dilution was plated on Plate Count Agar (PCA) for bacteria and Sabaraud Dextrose Agar (SDA) for yeast and fungi. Plates were incubated at 37^{o}C overnight. The number of colony forming units were counted for each dilution and expressed as CFU/ml.

3.9 NUTRITIONAL ANALYSIS OF *Spirulina platensis* POWDER (Pearson, 1976).

3.9.1 Moisture

$$\% \ Moisture = \frac{Sample\,mass}{Initial\,sample\,mass} x\,100$$

Samples were dried in a pre-weighed mortar at 65^{o}C overnight.

3.9.2 Ash

After the moisture content of the *S. platensis* powder had been determined, the sample was incinerated at 650^{o}C, and weighed.

3.9.3 Fat Content

The *S. platensis* powder was weighed on a Whatman Filter No 41 and transferred to a mortar. 200 ml of petroleum ether (solvent), was added and the sample was ground using the pestel. The mixture was transferred to a pre-weighed, Soxhlet round bottom flask on a filter paper in a funnel. The sample was distilled for 20-30 minutes and reflux back into the Soxhlet flask was allowed for at least 4 times. The flask was topped up with petroleum ether. Once the solvent had evaporated, the flask was left in a desiccator.

$$\%\ Fat = \frac{Extract\ mass(\ flask\ -extract\ -flask)}{Sample\ mass} x\,100$$

3.9.4 Protein - Modified Macro Kjeldahl Method

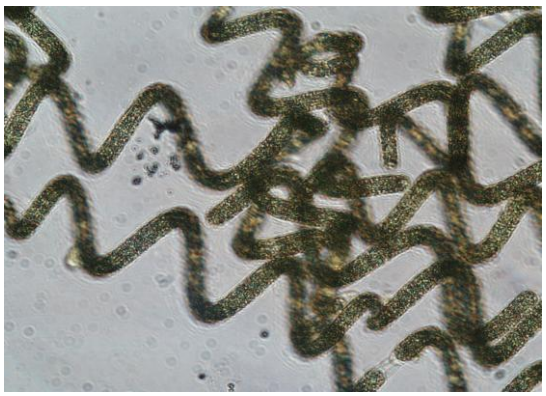
1 g of sample was transferred to a Kjeldahl digestion flask and 8 g of a catalyst mixture

(96% anhydrous sodium sulphate, 3.5% copper sulphate and 0.5% selenium dioxide) and 20 ml concentrated sulphuric acid is added. The digest was washed with ammonia free water and allowed to distill into 6% boric acid and a screened methyl red indicator until a colour change from clear to light pink was observed. The digest was treated with 35% NaOH and titrated with 0.1 N Sulphuric acid.

$$\% \, Nitrogen = \frac{Titre - 0.14(factor)}{Samplemass} x\,100$$

to convert to % protein,

% Nitrogen x 6.25 (general factor)



CHAPTER FOUR

4. RESULTS

4.1 MICROSCOPIC EXAMINATION OF *Spirulina*

Slides of *S. maxima* and *S. platensis* were cultured, prepared as wet mounts and viewed at a 1000 x magnification under a (make and model) light microscope. The following photomicrographs illustrate the physical differences between the two cultures.

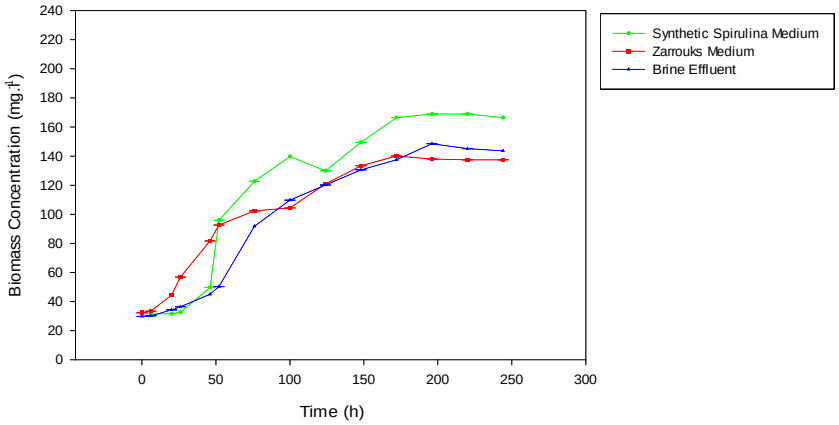


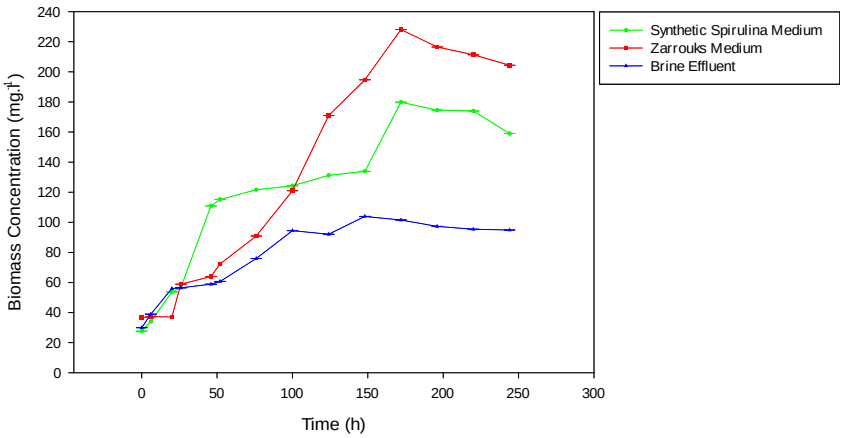
Figure 4.3 Growth curve of *S. maxima* in Synthetic Spirulina Medium, Zarrouks Medium and Brine Effluent.

4.2 *Spirulina* PRODUCTION IN BATCH EXPERIMENTS

4.2.1 Growth Curves

Samples were collected daily from triplicate flasks, for 10 days and growth curves of *S. maxima* and *S. platensis* were constructed and are shown below. Specific growth rates and doubling times were and were calculated from the growth curves.

Figure 4.4 Growth curve of *S. platensis* in Synthetic Spirulina Medium,



The highest biomass concentration (166 $mg.l^{-1}$) was obtained for *S. maxima* grown in SSM. This organism took a shorter time to adapt to ZM and BE (5 h), but grew at a slower rate (0.6 $g.h^{-1}$) than when grown in SSM (0.8 $g.h^{-1}$). Maximum concentrations of 140 $mg.l^{-1}$ and 148 $mg.l^{-1}$ were obtained for ZM and BE respectively (Figure 4.3). *S. platensis* showed a better tolerance to the salts present in SSM and ZM. A high concentration of 179 $mg.l^{-1}$ and 228 $mg.l^{-1}$ were observed for the organism grown in the two media respectively (Figure 4.4).

4.2.2 Growth Kinetics

Tables 4.1-4.3 indicate the μ and t_d for *S. maxima* and *S. platensis* grown in SSM, ZM and BE. Table 4.3 is a summary of the μ and t_d for *S. maxima* and *S. platensis.* Data presented in this table was used to determine which organism was to be used in further experiments.

Table 4.1 μ and t_d for *Spirulina maxima* and *Spirulina platensis* grown in SSM for a period of 244 hours.

Time (h)	**μ (h^{-1})**		**t_d (h)**	
	S. maxima	*S. platensis*	*S. maxima*	*S. platensis*
0	0	0	0	0

6	0	0.035	0	19.962
20	0.001	0.033	957.826	21.306
26	0.005	0.009	148.768	81.097
46	0.021	0.034	33.03	20.603

52	0.110*	0.006	6.324*	109.961
76	0.01	0.002	67.634	307.534
100	0.005	0.001	128.019	763.951
124	-0.003	0.002	0	306.062

148	0.006	0.001	119.289	826.851
172	0.004	0.012	154.214	56.438
196	0.001	-0.001	1149.156	0
220	0	0	0	0

244	-0.001	-0.004	0	0

* indicates the highest μ and shortest t_d

Table 4.2 μ and t_d for *Spirulina maxima* and *Spirulina platensis* grown in ZM for a period of 244 hours.

Time (h)	**μ (h^{-1})**		**t_d (h)**	
	S. maxima	*S. platensis*	*S. maxima*	*S. platensis*

0	0	0	0	
6	0.005	0.003	131.861	270.37
20	0.02	0	34.178	0
26	0.041	0.077*****	16.936	9.032*

46	0.018	0.004	38.361	166.026
52	0.021	0.02	33.076	34.013
76	0.004	0.01	166.978	72.781
100	0.001	0.012	835.27	58.169

124	0.006	0.014	113.321	48.166
148	0.004	0.005	170.661	128.165
172	0.002	0.007	333.674	105.415
196	0	-0.002	0	0

220	0	-0.001	0	0
244	0	-0.001	0	0

* indicates the highest μ and shortest t_d

Table 4.3 μ and t_d for *Spirulina maxima* and *Spirulina platensis* grown in BE for a period of 244 hours.

Time (h)	**μ (h^{-1})**		**t_d (h)**	
	S. maxima	*S. platensis*	*S. maxima*	*S. platensis*
0	0	0	0	0
6	0.002	0.044*****	367.123	15.807*****

20	0.009	0.026	75.163	26.695
26	0.01	0.001	71.071	487.74
46	0.011	0.002	65.062	337.575
52	0.018	0.005	38.168	131.009

76	0.025	0.009	27.644	74.656
100	0.007	0.009	92.997	76.332
124	0.004	-0.001	184.242	0
148	0.003	0.005	201.092	137.432

172	0.002	-0.001	322.255	0
196	0.003	-0.002	214.915	0
220	-0.001	-0.001	0	0
244	-0.001	0	0	0

* indicates the highest μ and shortest t_d

Table 4.4 Summary of μ_{max} and t_d of *Spirulina maxima* and *Spirulina platensis*.

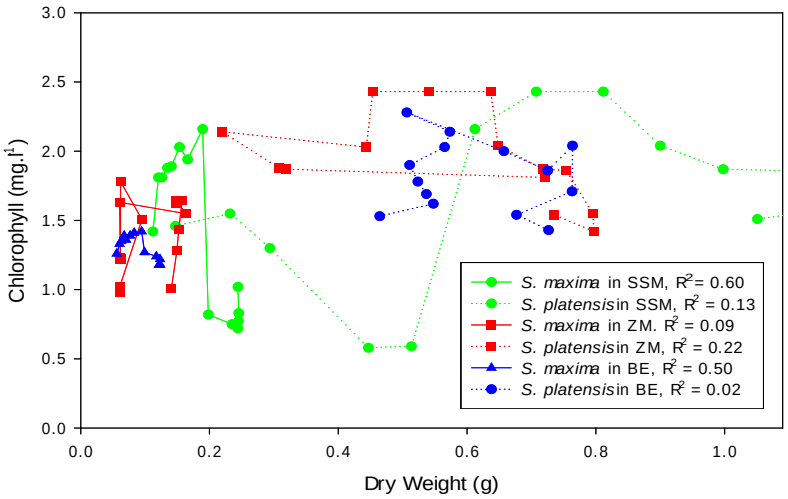
	μ_{max} (h^{-1})			t_d (h)		
	SSM	**ZM**	**BE**	**SSM**	**ZM**	**BE**
Concentration						
S. maxima	0.11	0.041	0.025	6.324	16.936	27.644
S. platensis	0.04	0.077*	0.044	19.962	9.032*	15.807
Chlorophyll a						
S. maxima	0.04	0.04	0.01	17.157	17.244	77.007
S. platensis	0.064*	0.029	0.011	10.721*	23.149	62.91
Protein						
S. maxima	0.137	0.041	0.025	5.034	16.936	27.644
S. platensis	0	0.077*	0.044	74.923	9.032*	15.807

* indicates the highest μ and shortest t_d

μ_{max} and t_d were calculated for each of the parameters that were used to determine growth. *S. platensis* showed a higher growth rate when grown in ZM when concentration and protein were used as deciding factors of the growth. The t_d was also much shorter for this organism for all three growth parameters. Maximum concentration was also higher in ZM. It was therefore concluded that this organism would be used in the optimisation studies.

4.2.3 Chlorophyll *a*

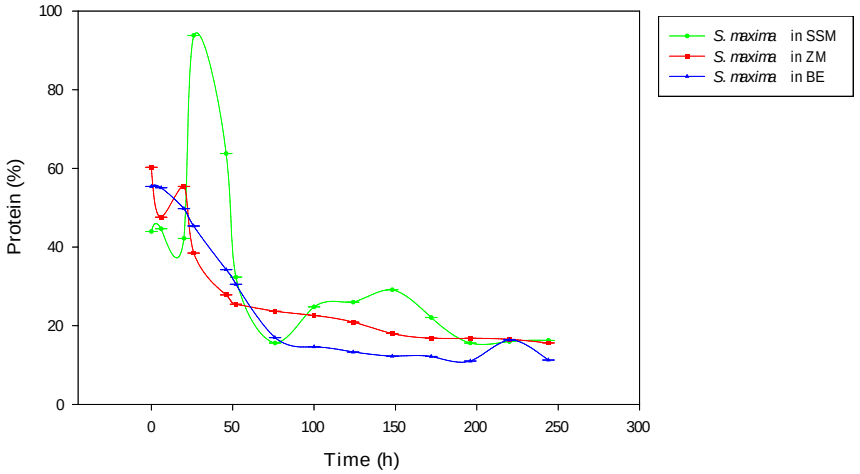
The following graphs show the correlation between the amount of chlorophyll extracted from *S. maxima* and *S. platensis* and dry weight of biomass. Squares of Pearson's least square correlation coefficients were used to determine the relationship between chlorophyll and dry weight.



A greater amount of chlorophyll was extracted from *Spirulina maxima* grown in SSM and this increased until a maximum was reached at 100 hours (2.16 $mg.l^{-1}$). Thereafter the concentration decreased. No marked increased was noted for *S. maxima* grown in ZM and BE (Figure 4.5). Larger amounts were extracted from *S.*

platensis grown in ZM (Figure 4.5) and these values correlate well with the growth *S. platensis* in ZM (Figure 4.4).

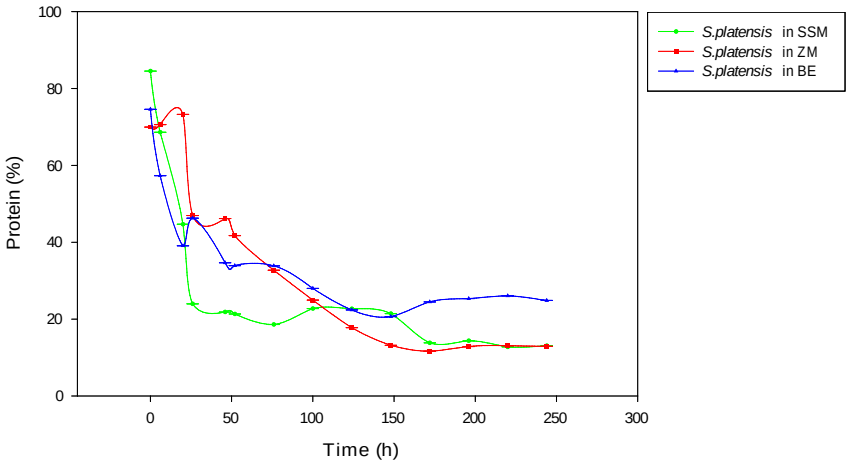
Figure 4.6a Percentage protein measured from *S. maxima* grown in



4.2.4 Protein Determination

The amount of protein produced from *S. maxima* and *S. platensis* was determined and converted to a percentage.

Figure 4.6b Percentage protein measured from *S. platensis* grown in



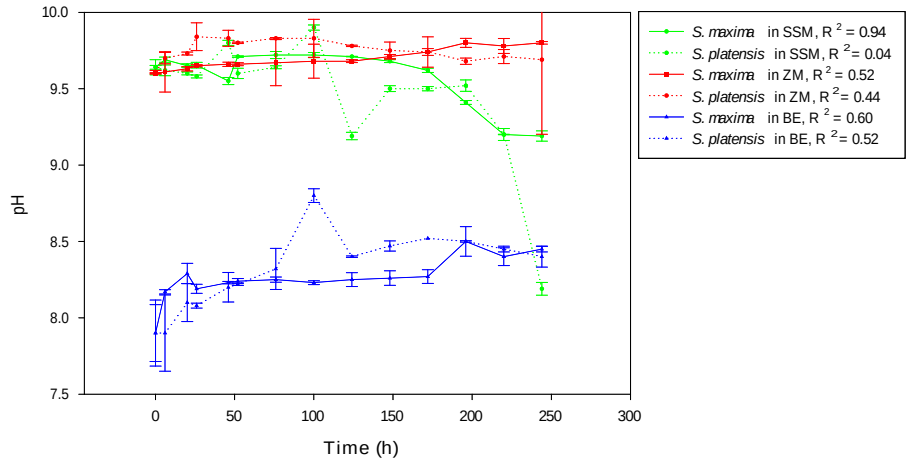
The highest percentage of protein in

measured from *S. maxima* grown in SSM was 93% and *S. platensis* in ZM was 68% (Figures 4.6a,b). These figures indicated a sharp, initial increase in the percentage of protein measured which was presumed to be the presence of induced enzymes that are used by the organisms. The average percentage of protein measured after a 100 h from *S. platensis* grown in BE was greater (23.76%) than *S. maxima* grown in BE (17.29%).

4.2.5 pH

The change in pH for *S. maxima* and *S. platensis* grown in each of the growth media was monitored daily for 10 days and illustrated below.

Figure 4.7 pH changes of *S. maxima* and *S. platensis* grown in Synthetic



The initial pH of SSM was 9.6, ZM, 9.64 and BEM, 7.9. Similar trends in pH changes were observed for the tow species of *Spirulina* grown in ZM and BEM. Fluctuations in pH of SSM were noted when this media was used for growth of *S. platensis* (Figure 4.7).

4.3 ABIOTIC FACTOR OPTIMISATION

Response values were assigned to the design screen using the software Design Expert 7® . The data was analysed and interpreted through the use of various statistical tools, including the Pareto Chart, Analysis of Variance Report, Cube plot and contour graphs. Analysis of the data is presented below.

Figure 4.8 Concentration of *S. platensis* produced when grown in varying pH,

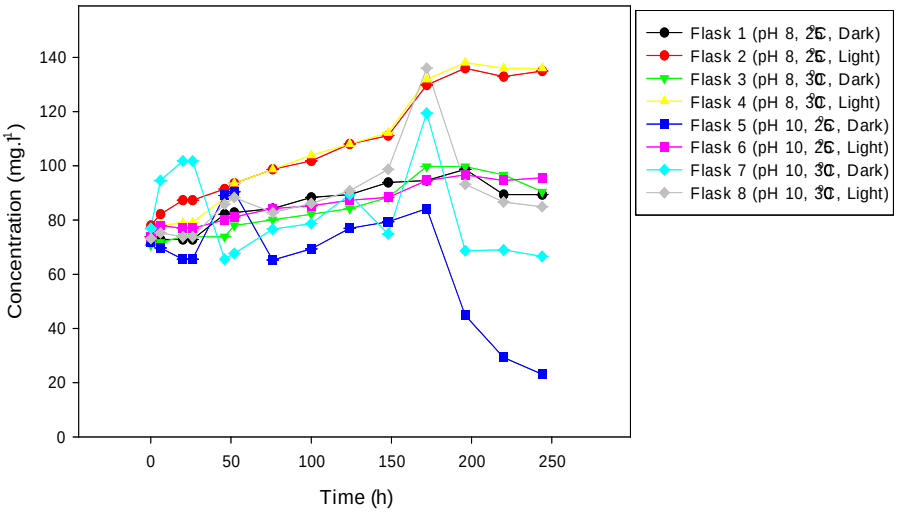
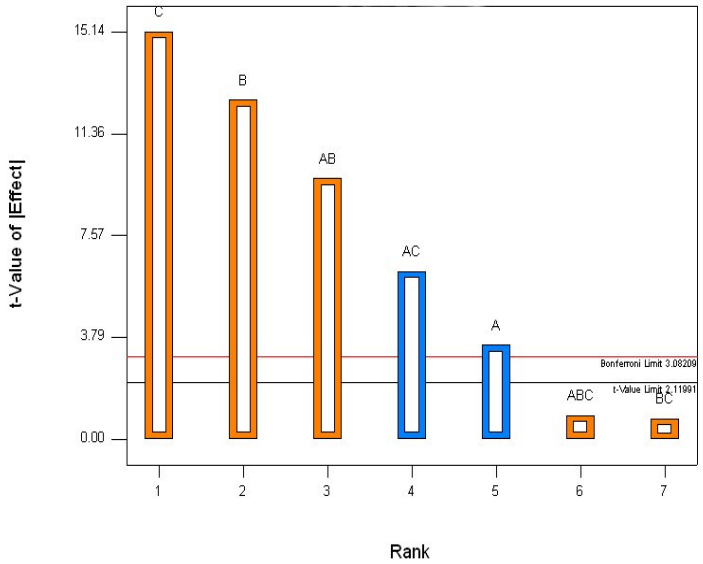


Figure 4.9 Pareto Chart showing the most significant factors,



Runs (1, 3, 5 and 7) kept in the dark produced small amounts of *S. platensis*. Growth in Run 3 increased gradually up to 150 hours and then decreased. The increase in the number of cells could have been due to the high temperature of incubation.

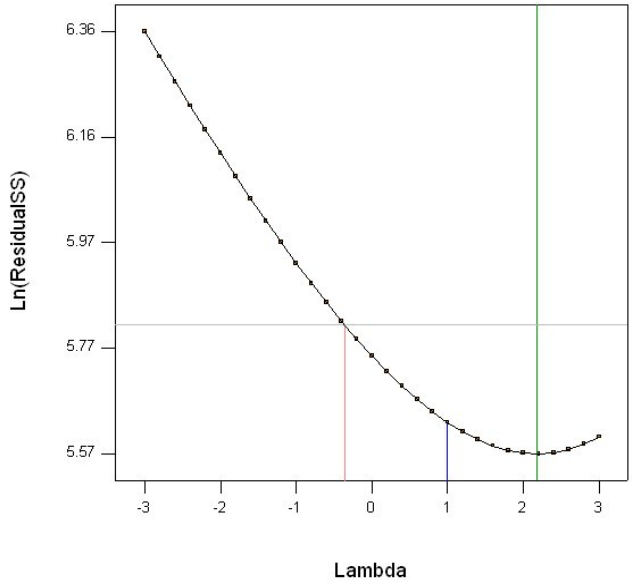
4.3.1 Design Expert Analysis

4.3.1.1 Evaluation of the Model Effects

The effects of each individual components were expressed in a Pareto chart and are ranked according to the greatest effect, on the production of biomass. Light had the largest positive effect followed by temperature. Factors that were above the t-limit are significant and those above the Bonferroni limit are most certainly significant and had to be included. In this case the interaction of AC and A had to be considered as a positive effect. All three factors combined contributed positively to the growth of *S. platensis*. Interactions of each factor also contributed positively to *S. platensis* production.

4.3.1.2 Validation of the model

Figure 4.10 Box Cox plot for power transformations of the



The *Box-Cox Plot for power transforms* suggests that no transformation of the model is required, with the transformation being set on “Lambda = 1” (blue line) and positioned only 2.18 away from the best transformation (green line). This transformation also lies neatly within the optimal zone, i.e. between the 95% confidence interval limits (red lines), making it a satisfactory model for further analysis and forthcoming hypothesis testing.

4.3.1.3 ANOVA and statistical analysis

The Model F-value

Figure 4.11 Cube plot showing the effect of pH, temperature and light on the growth of *S. platensis*.

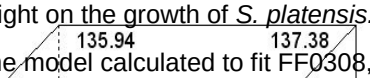


Table 4.5 Analysis of variance of the model calculated to fit FF0308, abiotic

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	9304.69	7	1329.24	76.33	< 0.0001	significant
A-pH	*211.80*	*1*	*211.80*	*12.16*	*0.0030*	
B-Temperature	*2768.46*	*1*	*2768.46*	*158.98*	*< 0.0001*	
C-Light	*3992.71*	*1*	*3992.71*	*229.29*	*< 0.0001*	
AB	*1634.14*	*1*	*1634.14*	*93.84*	*< 0.0001*	
AC	*674.55*	*1*	*674.55*	*38.74*	*< 0.0001*	
BC	*9.83*	*1*	*9.83*	*0.56*	*0.4633*	
ABC	*13.19*	*1*	*13.19*	*0.76*	*0.3971*	
Pure Error	278.62	16	17.41			
CorTotal	9583.31	23				

of 76.33 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC are significant model terms. Values greater than 0.0500 indicate the model terms are not significant. Inferences from the graphs drawn subsequently can be made at 99.99% probability.

Final Equation in Terms of Coded Factors:

Biomass (mg) = 112.38 - 2.97A + 10.74B +12.90C + 8.25 AB - 5.30AC + 0.64BC + 0.74ABC

4.3.1.4 Main effects resolution

All three factors, pH, temperature and light contribute positively to the growth of *S. platensis*. A maximum of 137 mg.l^{-1} is produced when the pH is 10, temperature is 30^{0}C and light is supplied to the culture. If the pH is decreased to 8, and temperature and light kept constant, 135 mg.l^{-1} of *S. platensis* is produced. If the temperature is decreased to 25^{0}C and pH to 8 but light supplied, 131 mg.l^{-1} of *S. platensis* is produced (Figure 4.11).

Figure 4.12 Contour graph showing the effect of temperature

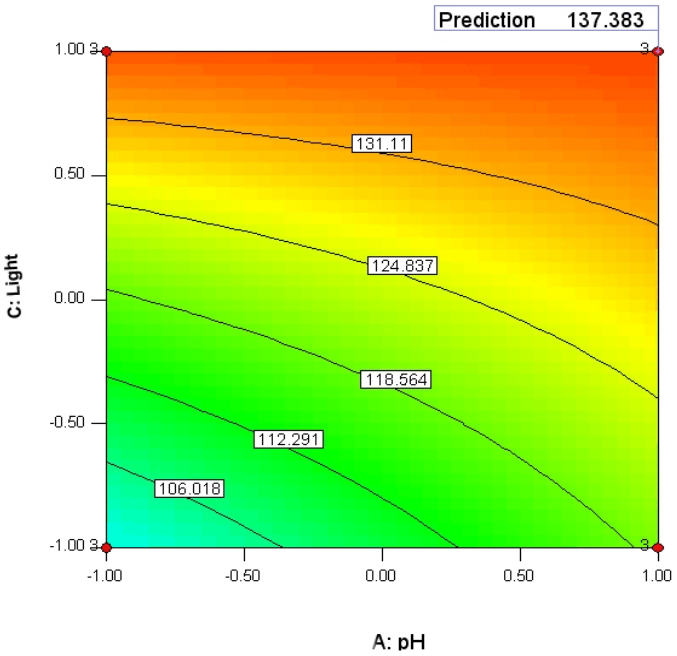
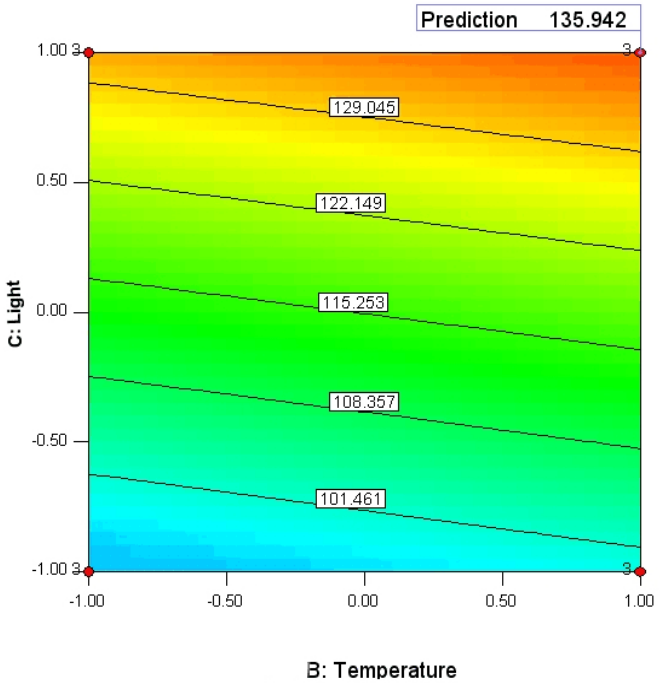


Figure 4.13 Contour graph showing the effect of pH on the

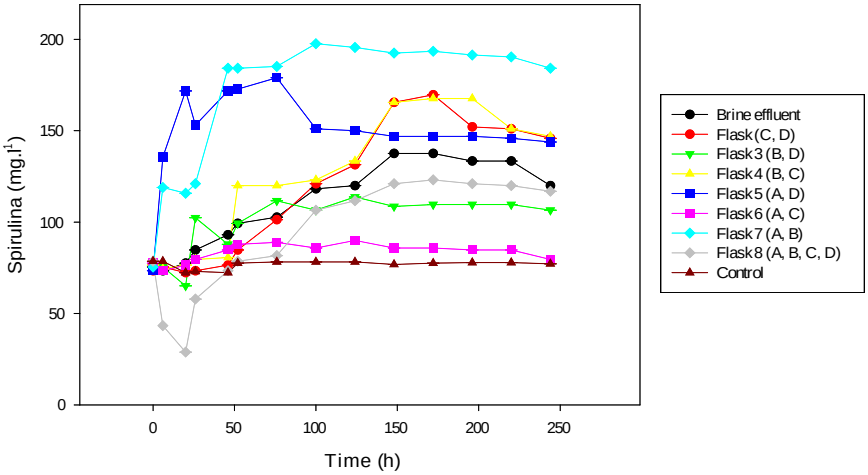


At a temperature of 30^{o}C and pH 11, 131 mg.l^{-1} *S. platensis* is produced (Figure 4.12) and a value of 137 mg.l^{-1} is predicted At a lower pH, 129 mg.l^{-1} *S. platensis* is produced (Figure 4.13). The accuracy of the predicted value is based on Table 4.6 with $p<0.0001$.

4.4 MACRONUTRIENT OPTIMISATION

BE was supplemented with 4 salts, $NaNO_3$ (A), NaCl (B), K_2SO_4 © and $NaHCO_3$ (D).

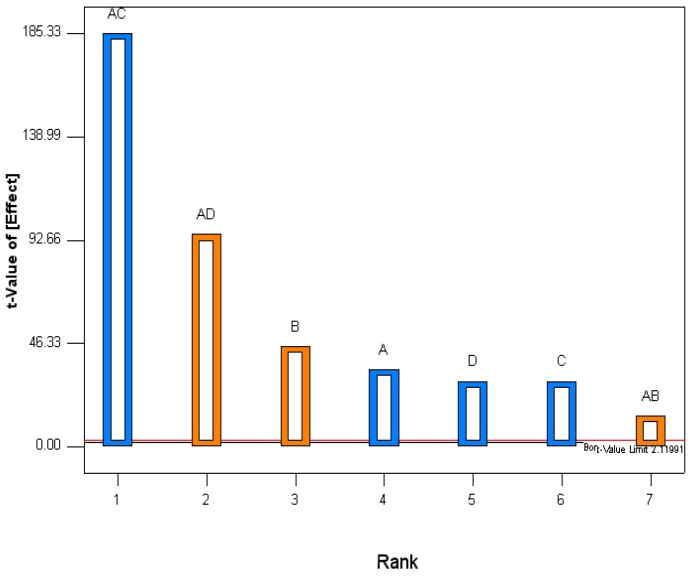
Figure 4.14 Concentrations of *Spirulina platensis* grown in BE



Run 7 (Figure 4.14) supplemented with $NaNO_3$ and NaCl resulted in the highest amount of biomass of *S. platensis*. (197 $mg.l^{-1}$ after 100 hours). The μ_{max} for this run was calculated to be 0.03 with a doubling time of 23 hours. A decline in growth was noted between the initial inoculation and 24 hours of incubation for Run 8 which contained all four salts. This was attributed to *S. platensis* initially, being unable to

adapt to or regenerate from the starting medium, however growth occurred thereafter, suggesting later adaptation.

Figure 4.15 Pareto Chart showing the effects of $NaNO_3$ (A),

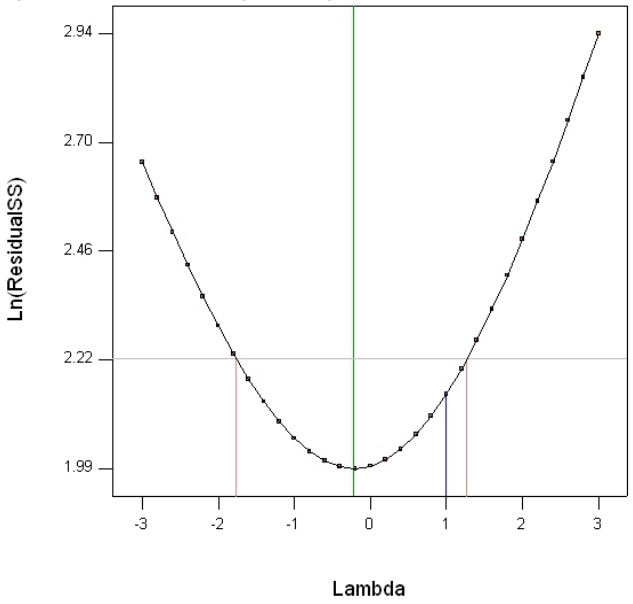


4.4.1 Design Expert Analysis

4.4.1.1 Evaluation of model effects

The effects of each individual components are expressed in the Pareto chart and are ranked according to the greatest effect, on the production of biomass. The mixture of $NaNO_3$ (A) and NaCl (B) had the greatest effect on the growth of *S. platensis*. This effect was also clearly seen in the large amount of biomass produced over the 10 day period in Run 7. NaCl also had a positive effect on the growth of *S. platensis* when used as an individual component and was the only individual component to have a positive effect on the growth of *S. platensis*. The order of positive effects for a mixture of components was ($NaNO_3$, NaCl) > ($NaNO_3$, $NaHCO_3$). The Bonferroni line and t-limit are close to zero (Figure 4.15).

Figure 4.16 Box Cox plot for power transformations for



4.4.1.2 Validation of the model

The *Box-Cox Plot for Power Transformation* (Figure 4.16) suggested that no transformation of model was required, with the transformation being set on “Lambda” = 1 (blue line - transformation parameter) and positioned only 1.20 away from the best transformation (green lines). This transformation also lay within the optimal zone, i.e. between the 95% confidence interval limits (red lines), which made it a satisfactory model for further analysis and forthcoming hypothesis testing (Figure 4.16).

4.4.1.3 ANOVA and statistical analysis

The above model is significant. This significance is determined by an F-value of 6939.43. This implies that the model fits the design appropriately with only a 0.01% chance that this value

could have occurred due to noise. Inferences from the graphs drawn subsequently can be made at 99.99% probability.

If the p-values are very small (less than 0.05), then the terms in the model have a significant effect on the response, in this case factors *A*, *B, C and D.* If the p-values are very small (less than 0.05) then the individual terms in the model have a significant effect on the response, ie. *AB, AC and AD.*

Table 4.6 Analysis of variance of the model calculated to fit FF0408, macronutrient optimisation.

Analysis of variance table [Partial sum of squares Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	26016.61	7	3716.66	6938.54	< 0.0001	significant
A-NaNO3	*642.53*	*1*	*642.53*	*1199.52*	*< 0.0001*	
B-NaCl	*1085.68*	*1*	*1085.68*	*2026.84*	*< 0.0001*	
C-K2SO4	*463.94*	*1*	*463.94*	*866.11*	*< 0.0001*	
D-NaHCO3	*464.64*	*1*	*464.64*	*867.43*	*< 0.0001*	
AB	*102.75*	*1*	*102.75*	*191.83*	*< 0.0001*	
AC	*18397.24*	*1*	*18397.24*	*34345.36*	*< 0.0001*	
AD	*4859.83*	*1*	*4859.83*	*9072.70*	*< 0.0001*	
Pure Error	8.57	16	0.54			
CorTotal	26025.18	23				

Final Equation in Terms of Coded Factors:

Biomass (mg) = 141.70 - 4.40A + 6.73B - 5.18C - 4.40D + 14.23AB - 27.69AC - 2.07AD

The model was based on the effects of individual and combinations of components and are given by the equation above. ANOVA indicated that the model was statistically significant with $p < 0.0001$ and cube plots and contour graphs were drawn based on the significance of the model. The above equation was used to forecast the concentration of *S. platensis* based on the mathematical model chosen.

The above equation may be used to forecast the concentration of *S. platensis* at a predicted confidence of 99.99%.

4.4.1.4 Main effects resolution

Figure 4.17 Cube plot showing the effects of $NaNO_3$ (A),

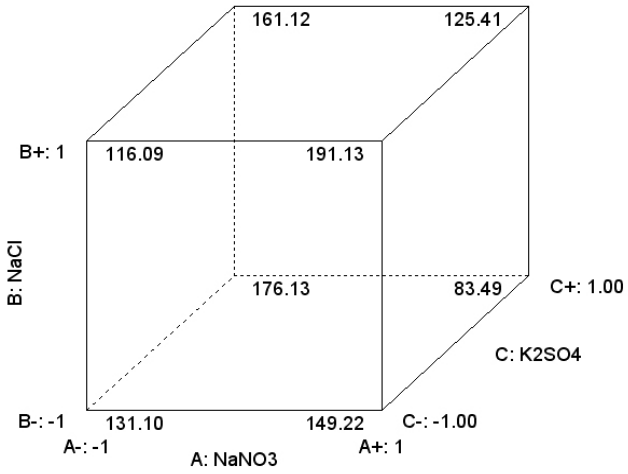
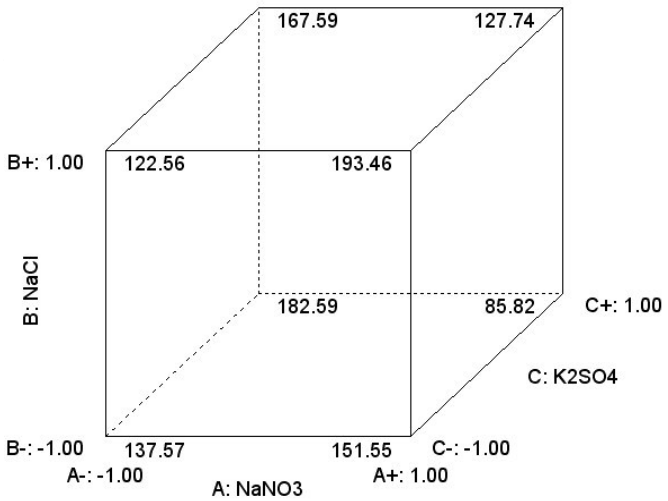


Figure 4.18 Cube plot showing the effect of $NaNO_3$ (A)



When $NaNO_3$ and NaCl were added to BE, and K_2SO_4 was absent, a maximum of 191 $mg.l^{-1}$ of *S. platensis* biomass was obtained. Addition of K_2SO_4 to BE decreased the *S. platensis* biomass produced to 125 $mg.l^{-1}$ (Figure 4.17). If $NaNO_3$ and NaCl were added to brine effluent, and K_2SO_4 (C) and $NaHCO_3$ (D) were omitted, then 193 $mg.l^{-1}$ of *S. platensis* was produced (Figure 4.18).

Figure 4.19 Contour graph showing the effect of K_2SO_4

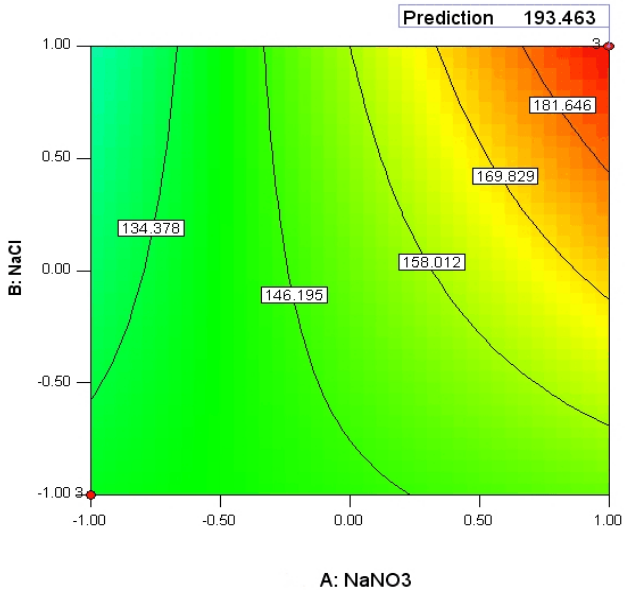
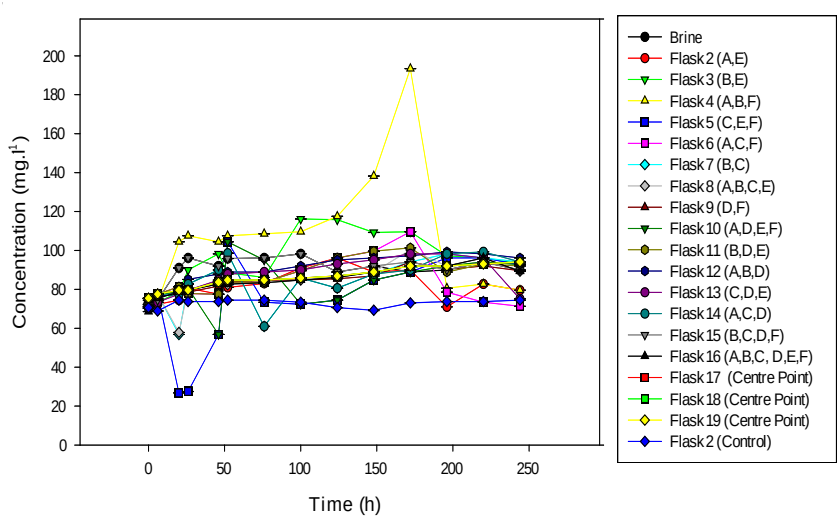


Figure 4.20 Concentration of *S. platensis* grown in BE supplemented



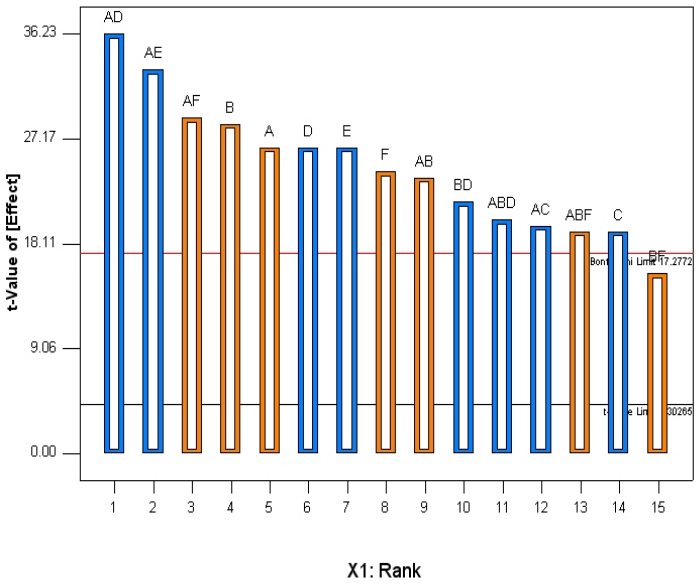
A greater production of *S. platensis* biomass (181 mg.l^{-1}) was noticed if the K_2SO_4 and $NaHCO_3$ were absent from BE and an even higher value of 193 mg.l^{-1} was obtained (Figure 4.19). The accuracy of the predicted value was based on Table 4.6 with $p<0.0001$.

4.5 MICRONUTRIENT OPTIMISATION

BE was supplemented with various salts, $FeSO_4.7H_2O$ (A), $CaCl_2$ (B), $MgSO_4.7H_2O$ (C),KH_2PO_4 (D), Na.EDTA (E) and Trace metals (F). Macronutrients from the previous experiment were not added to this in order to determine the effects of the micronutrients themselves without interference from the macronutrients.

Run 4 which contained $FeSO_4 7H_2O$, $CaCl_2$ and Trace metals produced the largest amount of *S. platensis* (193 $mg.l^{-1}$ at 172 hours). A decline of growth having occurred

between the initial inoculation and 24 hours of incubation rate was noted in Run 5 which contains $MgSO_4.7H_2O$, Trace metals and Na.EDTA. This may be attributed to *S. platensis* being unable to adapt to or regenerate from the starting medium. *S. platensis* grown in the presence of these salts had a specific growth rate of 0.02 doubling time of 34 hours.



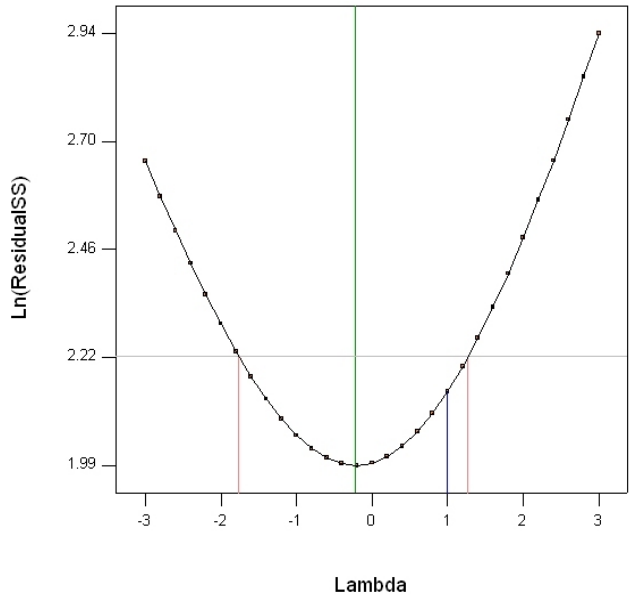
4.5.1 Design Expert Analysis

4.5.1.1 Evaluation of model effects

Figure 4.21 Pareto chart showing the effects of $FeSO_4.7H_2O$ (A), $CaCl_2$ (B), $MgSO_4.7H_2O$ (C), K_2HPO_4 (D), NaEDTA (E) and Trace metals (F). Positive effects are indicated by the orange bars and negative effects by the blue bars. Factors that are above the t-limit are significant and those that are above the Bonferroni limit are definitely significant and have to be included.

The effects of each individual and combination of components are expressed in the

Pareto chart and are ranked according to the greatest effect on the production of biomass. The Pareto chart indicated that $FeSO_4.7H_20$, $CaCl_2$ and Trace metals had positive effects on the production of *S. platensis* biomass. This was in keeping with the trend observed in Run 4 (Figure 4.20). The order of positive effects for a combination of components were ($FeSO_4.7H_20$ and Trace metals) > ($FeSO_4.7H_20$ and $CaCl_2$) > ($FeSO_4.7H_20$, $CaCl_2$ and Trace metals) > ($CaCl_2$ and Trace metals). $FeSO_4.7H_20$, $CaCl_2$ and Trace metals as individual components of the media also had a positive effect on biomass production (Figure 4.21).



4.5.1.2 Validation of the model

Figure 4.22 Box Cox plot for power transformations for micronutrient optimisation. The blue line shows the current transformation of the model. Since lambda = 1, no transformation is required as the model falls within the 95% confidence intervals.

The *Box-Cox Plot for Power Transforms* suggested that no transformation of the model was required, with the transformation being set on “Lambda” (blue line) and positioned only 0.2 away from the best transformation (green lines). This transformation also lay within the optimal zone, i.e. between the 95% confidence interval limits (red lines), making it a satisfactory model for further analysis and forthcoming hypothesis testing.

4.5.1.3 ANOVA and statistical analysis

The Model F-value of 63660000.00 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Inferences

Table 4.7 Analysis of variance of the model calculated to fit FF0416, micronutrient

Analysis of variance table [Partial sum of squares - Type III]

Source		Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model		9548.10	15	636.54	6.366E+007	< 0.0001	significant
A-FeSO47H2O		*696.56*	*1*	*696.56*	*6.366E+007*	*< 0.0001*	
B-CaCl2		*810.11*	*1*	*810.11*	*6.366E+007*	*< 0.0001*	
C-MgSO47H2O		*366.44*	*1*	*366.44*	*6.366E+007*	*< 0.0001*	
D-K2HPO4		*696.56*	*1*	*696.56*	*6.366E+007*	*< 0.0001*	
E-NaEDTA		*696.56*	*1*	*696.56*	*6.366E+007*	*< 0.0001*	
F-Trace metals		*591.58*	*1*	*591.58*	*6.366E+007*	*< 0.0001*	
AB		*566.80*	*1*	*566.80*	*6.366E+007*	*< 0.0001*	
AC		*386.81*	*1*	*386.81*	*6.366E+007*	*< 0.0001*	
AD		*1312.43*	*1*	*1312.43*	*6.366E+007*	*< 0.0001*	
AE		*1096.77*	*1*	*1096.77*	*6.366E+007*	*< 0.0001*	
AF		*839.70*	*1*	*839.70*	*6.366E+007*	*< 0.0001*	
BD		*472.52*	*1*	*472.52*	*6.366E+007*	*< 0.0001*	
BF		*241.10*	*1*	*241.10*	*6.366E+007*	*< 0.0001*	
ABD		*407.33*	*1*	*407.33*	*6.366E+007*	*< 0.0001*	
ABF	*366.82*	*1*	*366.82*	*6.366E+007*	*< 0.0001*		
Curvature	207.11	1	207.11	6.366E+007	< 0.0001	significant	
Pure Error	0.000	2	0.000				
CorTotal	9755.21	18					

from the graphs drawn subsequently can be made at 99.99% probability.

If the P-values are very small (less than 0.05), then the terms in the model have a significant effect on the response. In this case A, B, C, D, E, F, AB, AC, AD, AE, AF, BD, BF, ABD, ABF are significant model terms.

The "Curvature F-value" of 63660000.00 implies there is significant curvature (as measured by difference between the average of the center points and the average of the factorial points) in the design space. There is only a 0.01% chance that a "Curvature F-value" this large could occur due to noise. Curvature implies that a point of optimisation can be reached based on the model.

Final Equation in Terms of Coded Factors:

Biomass (mg) = 101.08 + 6.60A + 7.12B - 4.79C - 6.60D -6.60E + 6.08F + 5.95AB x 4.92AC x 9.06AD - 8.28AE +7.24AF - 5.43BD +3.88BF - 5.05AC + 4.79CEF

The above equation may be used to forecast the concentration of *Spirulina platensis* at a predicted confidence of 99.99%.

4.5.1.4 Main effects resolution

Figure 4.23 Cube plot showing the effects of

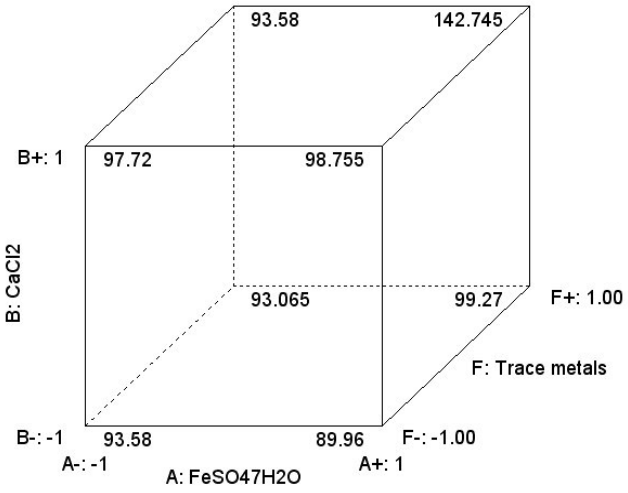
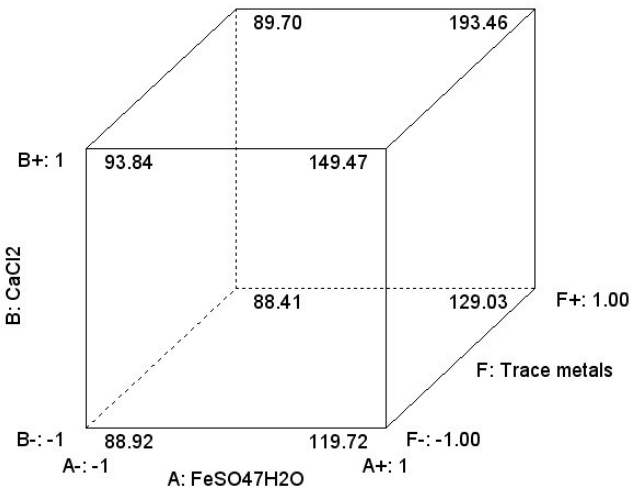
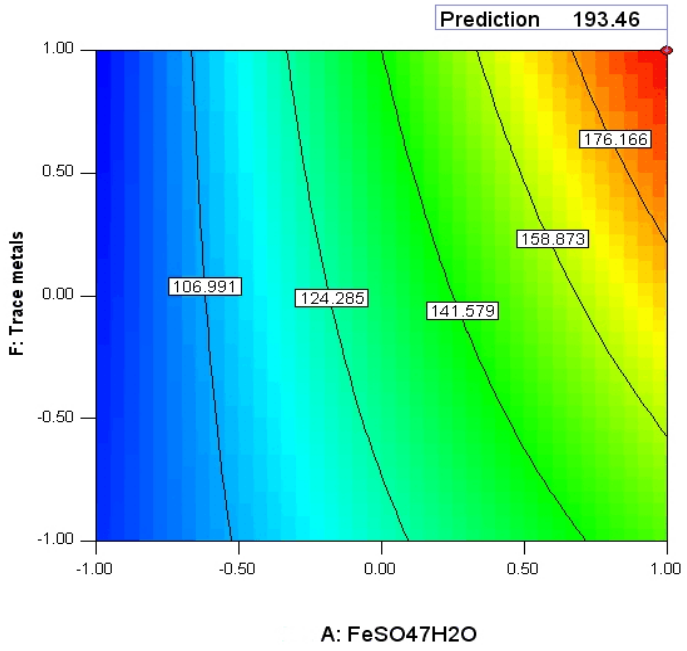


Figure 4.24 Cube plot showing the effects of $MgSO_4.7H_2O$ (C), K_2HPO_4 (D) and NaEDTA (E). When



The presence of $FeSO_4.7H_2O$, $CaCl_2$ and Trace metals in BE produced 142 $mg.l^{-1}$ of *S. platensis* biomass (Figure 4.23). If these three supplements were included in BE, and $MgSO_4.7H_2O$ ©, K_2HPO_4 (D) and NaEDTA (E) were excluded then a larger amount (193 $mg.l^{-1}$) of biomass was produced (Figure 4.24). The accuracy of the predicted value was based on Table 4.7 with $p<0.0001$.

Figure 4.25 Contour plot showing the effect of $CaCl_2$, $MgSO_4.7H_2O$, K_2HPO_4 and NaEDTA. If $MgSO_4.7H_2O$, K_2HPO_4 and NaEDTA are excluded as supplements and the concentration of $CaCl_2$ is increased, then the actual amount of 176 $mg.l^{-1}$ is produced. Prediction shown is based on actual values.



$FeSO_4.7H_2O$ and Trace metals had a positive effect on the growth of *S. platensis* (Figure 4.25). The effect of the remaining salts was negligible since excluding them from BE produced a larger quantity of *S. platensis* (193 $mg.l^{-1}$) (Figure 4.25). The accuracy of the predicted value was based on Table 4.7 with $p<0.0001$.

Based on the evaluation of the main effects of micro and macro nutrient optimisation, it was concluded that the following salts have a positive effect on the growth of *S. platensis*:

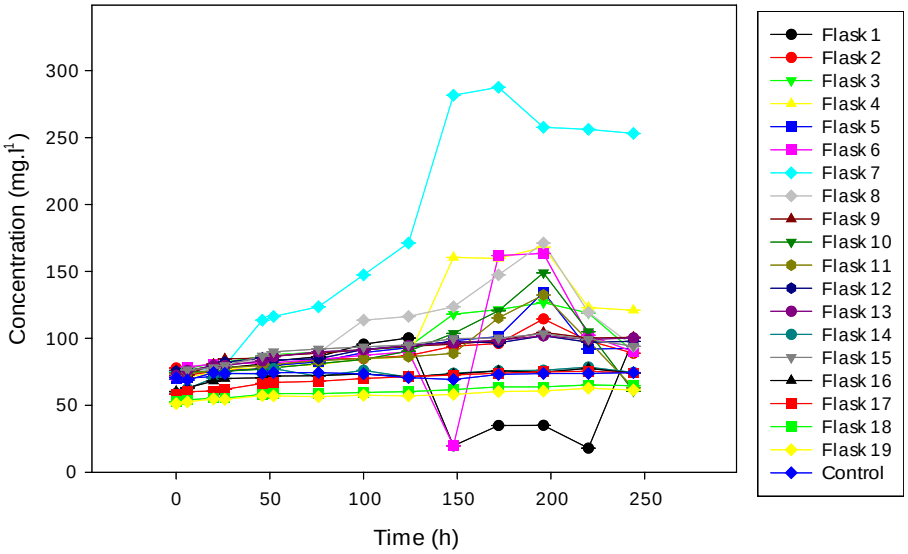
Table 4.8 Salts and concentrations that were selected from the two optimisation experiments and used as supplements of BE for final optimisation.

Macronutrient Optimisation			Micronutrient Optimsiation		
Salt	Concentration		Salt	Concentration	
	Old Value	New Value		Old Value	New Value
$NaNO_3$	2.5	2.5	$FeSO_4.7H_2O$	0.01	0.01
NaCl	1	2	$CaCl_2$	0.04	0.06
			Trace metals	4	6

4.6 ANALYSIS OF FINAL OPTIMISED MEDIA

Salts were selected from the macronutrient and micronutrient optimisation (Table 4.8) and combined to form the optimised medium that was used for *Spirulina* production.

Figure 4.26 Concentration of *S. platensis* grown in BE supplemented with $NaNO_3$ (A), NaCl (B), $FeSO_4.7H_2O$ (C), $CaCl_2$ (D) and Trace metals (E). The combination of salts present in each flask is given in Table 3.4.

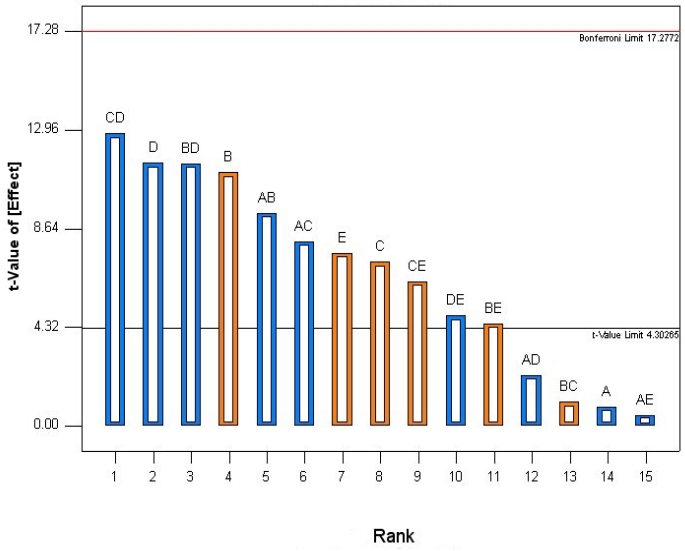


Run 7 contained large amounts of NaCl, $FeSO_4.7H_2O$ and Trace metals and produced the greatest amount of *S. platensis* biomass (287 $mg.l^{-1}$).

4.6.1 Design Expert Analysis

4.6.1.1 Evaluation of the model effects

Figure 4.27 Pareto chart showing the effects of $NaNO_3$ (A), NaCl (B), $FeSO_4.7H_2O$ (C), $CaCl_2$ (D) and Trace metals (E). Positive effects are indicated by the orange bars and negative effects by the blue bars. Effects that are above the t-limit are significant and those that are above the Bonferroni limit are definitely significant and must be included.

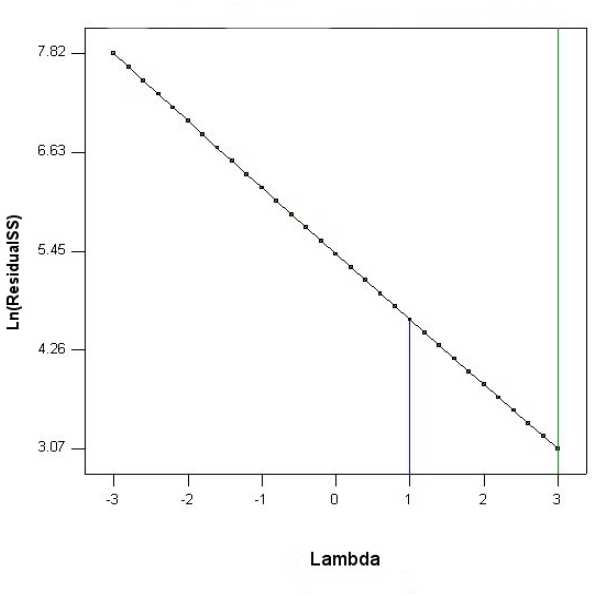


The effects of each individual components are expressed in the Pareto chart and are ranked according to the greatest effect, on the production of biomass. The mixture of $NaNO_3$ (A) and NaCl (B) which in FF0408 had a positive effect on the growth of *S. platensis* now had a negative effect on the growth of biomass. This could be due to the use of these salts in conjunction with other chemicals. NaCl had a positive effect on the growth of *Spirulina platensis* when used as an individual component.

Other positive effects of individual components were Trace metals (E) and $FeSO_4.7H_2O$ (C). The order of positive effects for a mixture of components was ($FeSO_4.7H_2O$, Trace metals) > (NaCl, Trace metals) (Figure 4.27).

4.6.1.2 Validation of the model

Figure 4.28 Box Cox plot for power transformations. The blue line shows the current transformation of the model. Since lambda = 1, no transformation is required as the model falls within the 95% confidence intervals.



The *Box-Cox Plot for Power Transforms* suggested that no transformation of the model was required, with the transformation being set on “Lambda” =1 (blue line) and positioned 2 away from the best transformation (green lines) (Figure 4.28).

4.6.1.3 ANOVA and statistical analysis

Table 4.9 Analysis of variance of the model calculated to fit FF0516, final optimised media.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	46034.65	15	3068.98	60.24	0.0164	significant
A-NaNO3	*37.24*	*1*	*37.24*	*0.73*	*0.4826*	
B-NaCl	*6279.58*	*1*	*6279.58*	*123.27*	*0.0080*	
C-FeSO47H2O	*2628.25*	*1*	*2628.25*	*51.59*	*0.0188*	
D-CaCl2	*6755.49*	*1*	*6755.49*	*132.61*	*0.0075*	
E-Trace Metals	*2929.50*	*1*	*2929.50*	*57.51*	*0.0169*	
AB	*4422.00*	*1*	*4422.00*	*86.80*	*0.0113*	
AC	*3330.40*	*1*	*3330.40*	*65.38*	*0.0150*	
AD	*250.91*	*1*	*250.91*	*4.93*	*0.1567*	
AE	*11.61*	*1*	*11.61*	*0.23*	*0.6801*	
BC	*56.78*	*1*	*56.78*	*1.11*	*0.4018*	
BD	*6717.68*	*1*	*6717.68*	*131.87*	*0.0075*	
BE	*1027.51*	*1*	*1027.51*	*20.17*	*0.0462*	
CD	*8358.76*	*1*	*8358.76*	*164.08*	*0.0060*	
CE	*2039.70*	*1*	*2039.70*	*40.04*	*0.0241*	
DE	*1189.23*	*1*	*1189.23*	*23.34*	*0.0403*	
Curvature	6886.79	1	6886.79	135.19	0.0073	significant
Pure Error	101.88	2	50.94			
Cor Total	53023.33	18				

The Model F-value of 60.24 implies the model is significant. There is only a 1.64% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, C, D, E, AB, AC, BD, BE, CD, CE, DE are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The "Curvature F-value" of 135.19 implies there is significant curvature (as measured by difference between the average of the center points and the average of the factorial points) in the design space. There is only a 0.73% chance that a "Curvature F-value" this large could occur due to noise. Curvature implies that a point of optimisation can be reached based on the model.

Final Equation in Terms of Coded Factors:

Biomass (mg) = 118.22 - 1.53A + 19.81 B + 12.82 C x 20.55D + 13.53 E - 16.62AB - 14.43AC - 3.96AD - 0.85 AE + 1.88BC - 20.49BD + 8.01BE - 22.86CD +11.29CE - 8.62DE

The above equation may be used to forecast the concentration of *S. platensis* at a predicted confidence of 98.36.

4.6.1.4 Main effects resolution

Figure 4.29 Cube plot showing the effects of NaCl (B),

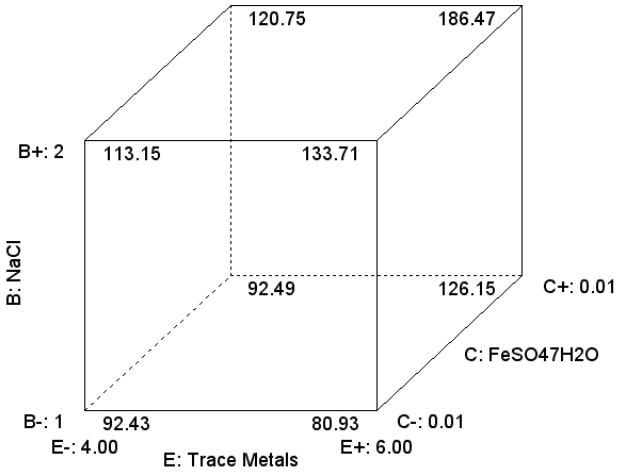
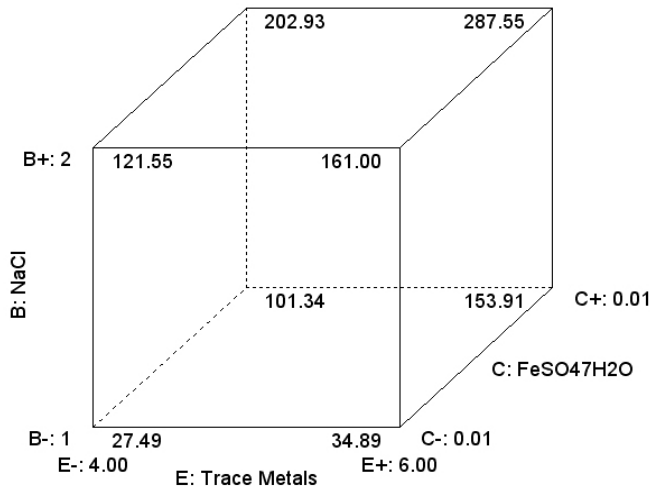
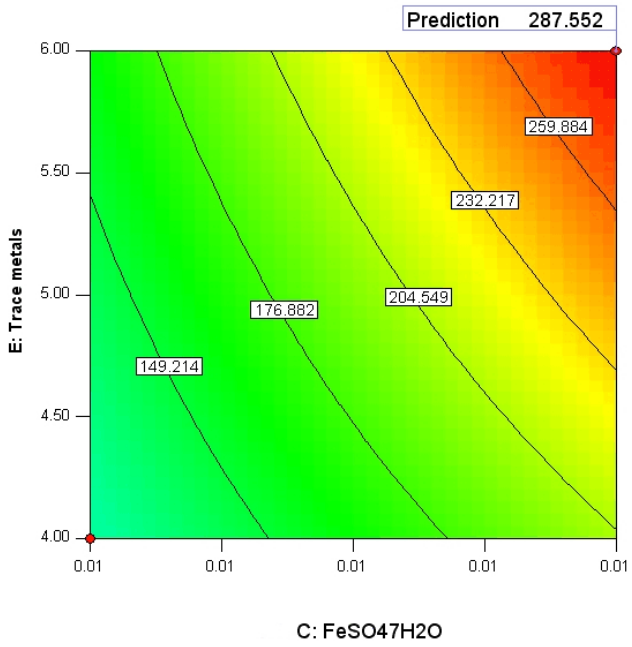


Figure 4.30 Cube plot showing the effect of decreasing the concentration of supplements $NaNO_3$ (A) and $CaCl_2$ (D). 287 $mg.l^{-1}$ *S. platensis* is produced.

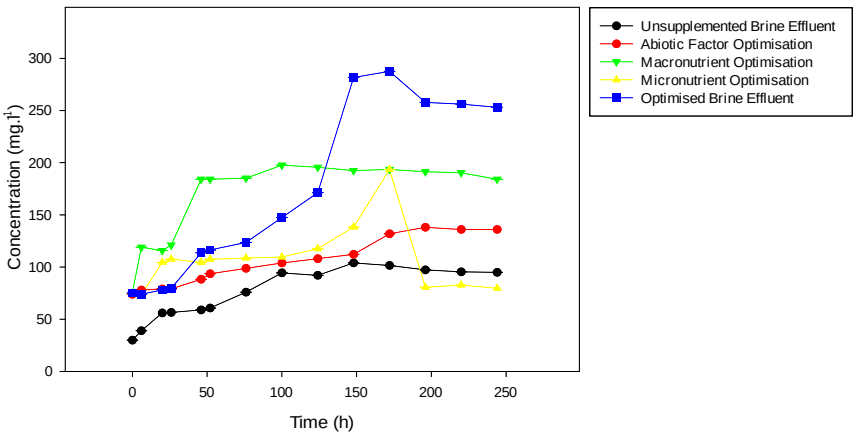


Supplements that showed a positive effect on the growth of *S. platensis* were NaCl, $FeSO_4.7H_2O$ and Trace metals. 189 $mg.l^{-1}$ of *S. platensis* was produced when BE was supplemented

Figure 4.31 Contour graph showing the effects of $NaNO_3$ (A), NaCl (B) and $CaCl_2$ (D). The concentration of NaCl (B) was increased and $NaNO_3$ (A) and $CaCl_2$ (D) decreased to produce 287 $mg.l^{-1}$ of *S. platensis*. Prediction shown is based on actual values.

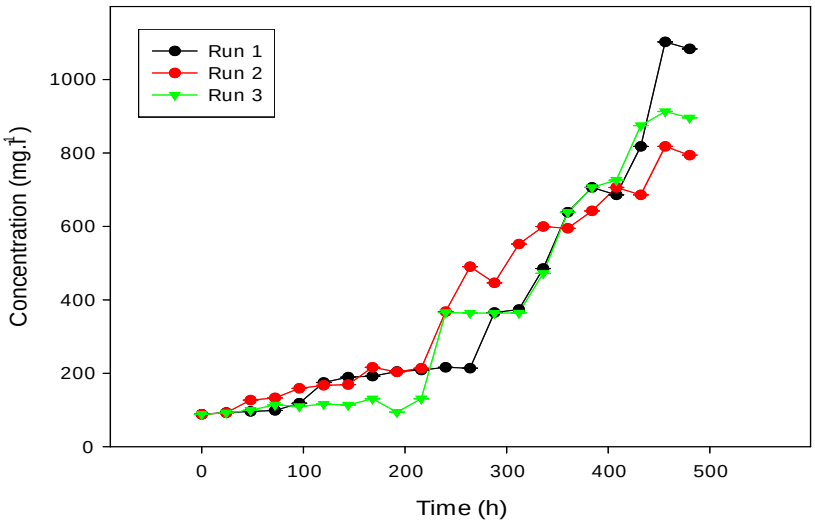


with these salts and the remaining salts, $CaCl_2$ and $NaNO_3$ are at 0.05 and 1.75 $g.l^{-1}$ respectively (Figure 4.29). Decreasing these concentrations to 1 and 0.04 $g.l^{-1}$ resulted in a greater biomass production (287 $mg.l^{-1}$) (Figure 4.30).



NaCl showed a positive effect when used as an individual component and $FeSO_4.7H_2O$ and Trace metals showed a positive, combined interaction on the production of *S. platensis* (Figure 4.27). If these salts are kept as supplements, and the concentrations of $CaCl_2$ (D) and $NaHCO_3$ are decreased, 256 $mg.l^{-1}$ *S. platensis* biomass was produced (Figure 4.31). The accuracy of the predicted value was based on Table 4.9 with $p<0.0001$.

Figure 4.33 Changes in *Spirulina platensis* biomass in the raceway pond

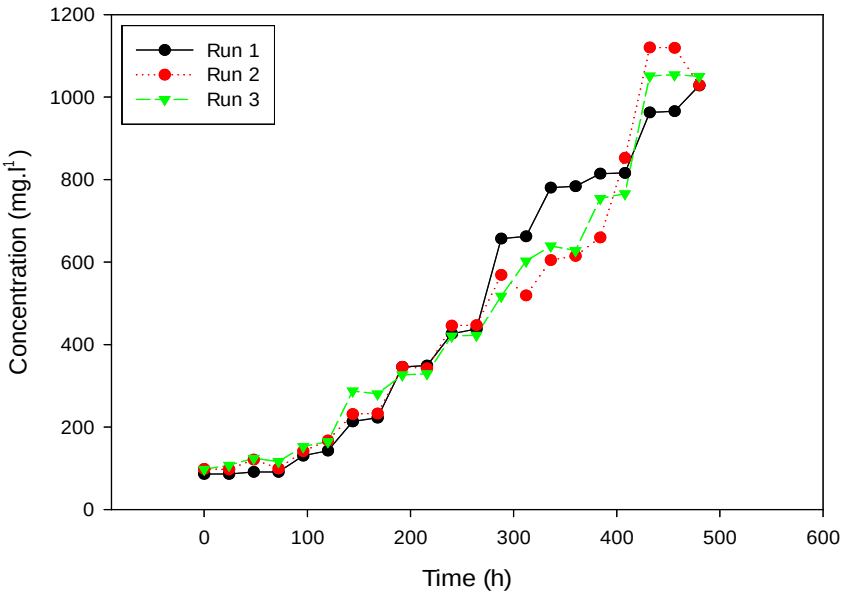


The progressive increase in *S. platensis* biomass from unsupplemented BE to the optimised BE was clearly seen in Figure 4.32. Optimised BE produced the largest amount of *S. platensis* (287 $mg.l^{-1}$) as compared to the 103 $mg.l^{-1}$ produced from growth in unsupplemented BE.

4.7 Laboratory Scale Bioreactor

S. platensis was grown in a laboratory scale bioreactor of 5 L working volume for 20 days and biomass was monitored daily. The experiment was run thrice and biomass production is shown below.

Figure 4.34 Changes in *Spirulina platensis* biomass in the raceway pond over



A steady microalgal growth was seen during the experiment up to 456 hours after which the culture entered the stationary phase. Lag phase lasted 24 hours. The average over the 3 runs was 944 $mg.l^{-1}$. μ_{max} was 0.02 and the t_d was 31 h (Figure 4.33).

A steady microalgal growth was seen during the experiment up to 456 hours after which the culture entered the stationary phase. Decreases were noted in the early parts of the morning, however, once exposed to natural light, concentrations of *S. platensis* increased. Lag phase lasted 24 hours. The average over the 3 runs was 1086 $mg.l^{-1}$. μ_{max} was 0.02 and the t_d was 35 h (Figure 4.34).

Table 4.10 indicates a comparison of parameters of *S. platensis* for all experiments carried out.

Table 4.10 Comparison of maximum concentrations, % increase, μ_{max}, t_d and rate of production for *S. platensis* for all experiments.

	Maximum concentration ($mg.l^{-1}$)	**% increase**	**μ_{max} (h^{-1})**	**t_d (h)**	**Rate of production ($g.h^{-1}$)**

Unsupplemented BE	103.93	-	0.04	15.81	1.28
Abiotic Factor	138.07	33	0.01	73.26	0.7
Macronutrient	197.6	90	0.02	38.97	1.22
Micronutrient	193.46	86	0.03	27.53	1.16
Optimised	287.55	177	0.06	11.69	1.29
Indoor Bioreactor	944.35	809	0.02	31.01	2.22
Outdoor Bioreactor	1086.9	944	0.02	35.81	2.51

S.platensis wet biomass was tested for bacterial and fungal contamination on Plate Count Agar and Saborauds Dextrose Agar respectively. There was no fungal contamination found and the table below shows only the bacterial contamination found.

Table 4.11 Colony forming units of bacteria present in *S. platensis* powder on PCA.

	***Spirulina platensis* powder**						
Dilution	Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
CFU/ml	TNTC*	TNTC*	TNTC*	428	56	31	8

*TNTC = Too numerous to count

Table 4.12 Nutritional Analysis of *S. platensis* powder.

	Moisture	**Ash**	**Fat**	**Protein**

Spirulina platensis	3.49%	1.12%	12.19%	18%

4.8 FEASIBILITY STUDIES AND COST ANALYSIS

4.8.1 Growth of Microorganism

S. platensis was obtained at no cost from CSIR in Upington, South Africa and maintained in Zarrouks Medium. A one litre volume of ZM was prepared and 250 ml used for inoculation of the pilot raceway pond. The total cost of preparing one litre is R5.00 and therefor 250 ml of ZM costs R1.25.

4.8.2 Cost of Bioreactor and Peripherals

The bioreactor was constructed from perspex and a motor for running the paddle. The total cost was R2000.00. A power supply was purchased to run the paddlewheel and this cost R1200.00.

4.8.3 Effluent and Supplements

The brine effluent was obtained at no cost from Lethabo Power Station, Eskom, Vereeniging, however transportation costs of the brine were incurred. The solution to

overcome this would be to build the pond at the site. The brine was supplemented with five salts, $NaNO_3$, NaCl, $FeSO_4.7H_2O$, $CaCl_2$ and Trace metals and the cost of each was included.

4.8.5 Summary of the costing

Table 4.13 Summary of the cost of producing *Spirulina platensis* in laboratory scale.

	Spirulina platensis
Fixed Cost	
Bioreactor	R2000.00
Power supply	R1200.00
Consumables	
Chemicals	R5 per litre (5 litre working volume)
Brine effluent	R0
Supplements	R1 per litre
Harvesting and Drum drying	R1200.00
Total Cost	**R4406.00**

Table 4.14 Summary of the cost of producing *S. platensis* on a large scale

Capital Costs	**R12 000.00**
400 m^2 Concrete Raceway	R7000.00*
Building Costs	R5000.00*
Running Expenses	**R27 920.00**
Chemical supplements	R16920.00
Brine Effluent if located on site	R0.00

Labour 1 month	R3000.00
Processing (harvesting and drying)	R4000.00**
Mixing by air current and convection	R0.00
Packaging per batch	R1083.00
Marketing per batch	R650.00
Total	**R28 053.00**
Cost per gram	**R2.15**

*(Cost calculated by Kidson, 2007)
**(Cost calculated by Dilraj, 2007)

Breakdown of Calculations:
Selling price = Total/(Amount produced*1000)
= R28053.00/(13.04292 *1000)
= R2.15

Amount produced = Average yield x volume / 1 million
= 1086.90 x 12000/100000
= 13.04292 kg

No of tablets produced = 13.04292 kg x 1000000 / 300 mg
= 43333 tablets

No of tablets produced = 43333 tablets / 40 (no of tablets in one pack)
= 1083 packs

To package 1083 packs would cost R1083 if the cost of packaging was R1.00 and it

would cost R650.00 to market 1083 packs.

Percentage Profit = (Selling price - Cost price) / Cost price
= R7.50 - R2.15 / R2.15
= 248%

CHAPTER FIVE

5. DISCUSSION

5.1 LABORATORY BATCH EXPERIMENTS

Lethabo Power Station currently has the largest local multimembrane desalination plant in South Africa, and allows it to operate as a zero-liquid-effluent discharge facility (http://www.engineeringnews.co.za/eng/features/tempcontrol/?show=35033). The sources where water is drawn from, can introduce 27 tons of salts per day into the cooling water systems and these salts must be removed daily. Desalination technologies developed in recent years, especially the reverse osmosis process, enable massive amounts to be produced with a moderate cost (Arnal *et al*, 2005). Reverse osmosis (RO) technology, is the extraction of salts while the solution is in the liquid phase (Ahmed, 2000). The generation of brine effluent must be properly managed in order to avoid environmental contamination (Arnal *et al*, 2000). When brine is dumped into the sea, sent to wastewater plants, injected into saline aquifers, or injected into oil

wells, it threatens sea life, particularly those at the lower end of the food chain and injected, it runs the risk of polluting groundwater (http://desertgardens.suite101.com/ blog.cfm/is_desalination_answer_to_water).

This study was carried out to assess the use of brine effluent from cooling towers as an economic, alternative growth medium that could decrease the production costs of *Spirulina* as well as utilise the saline brine effluent. This type of study is relevant because lower production costs derived from the use of a low cost effluent could lead to a competitive process (Costa *et al*, 2004).

Spirulina are free-floating filamentous cyanobacteria characterized by cylindrical, multicellular trichomes in an open left-hand helix. In liquid media the cells are helical in shape and this changes to a complete spiral on solid media (Ciferri, 1983). The cells appear green due to the predominant presence of the pigment chlorophyll. The microorganisms that were used in this study were two, non genetically modified cultures of *Spirulina*, viz., *Spirulina maxima* (Figure 4.1), obtained from UTEX Culture Collection and *Spirulina platensis* (Figure 4.2), obtained from CSIR, Upington in order to determine which culture grew at a faster rate. *Spirulina platensis* occurs in Africa, Asia and South America, whereas *S. maxima* is confined to Central America (http://en.wikipedia.org/wiki/ Spirulina_dietary_supplement). Non genetically modified cultures were selected as the aim was to produce *Spirulina* which could be used as a potential food source and there are no regulatory protocols governing their use. The Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) have regulatory laws that govern the use of genetically modified organisms and the

substances that they produce (http://pewagbiotech.org/ resources/issuebriefs/1-regguide.pdf). The culture that grew at a faster rate on the effluent was then used in further experiments to optimise the brine effluent for use as a substrate for the growth of *Spirulina*. Zarrouks Medium (ZM) and Synthetic Spirulina Medium (SSM) were used as comparative media in the laboratory scale experiments. Brine Effluent (BE) from Lethabo Power Station was included as a baseline as this became the base medium for the growth of *Spirulina* and was supplemented with salts from Zarrouks Medium.

Pelizer *et al* (2003) showed that inoculum age as well as concentration of the inoculum had an influence on cell growth. Their research showed that a 10 and 14-day old inoculum showed low cell growth while a three-day inoculum grew better, however the inoculum volume required for cultivation would be high. Further research by them concluded that an inoculum of 6 days with 50 $mg.l^{-1}$ of starter biomass concentration was therefore preferable (Pelizer *et al,* 2003). For the preparation of an inoculum for this study, a 50 $mg.l^{-1}$ (wet biomass) concentration of *Spirulina* was obtained after three days of growth for both cultures and it was considered unnecessary to cultivate for six days. This 3-day inoculum of 1.16×10^7 cells per ml of *S. maxima* (Figure 4.1) and 1.51×10^7 cells per ml of *S. platensis* (Figure 4.2) was adjusted to a concentration of 1.00×10^7 cells per ml and inoculated into the three media, ie, Synthetic Spirulina Medium (SSM), Zarrouks Medium (ZM) and Brine Effluent (BE). This was carried out to determine which of the two organisms had better growth potential and would therefore be used in all remaining experiments.

Batch cultures have been frequently used to study the kinetics of microbial growth (Chojnacka and Norworyta, 2004). When organisms are grown in a homogenous batch

culture, for example, mixed liquid media in an Erlenmeyer flask, three phases of growth could be distinguished – the lag, exponential and stationary phases (Carlile and Watkinson, 1994). These phases represent microbial patterns of growth. Growth curves of *S. maxima* (Figure 4.3) and *S. platensis* and (Figure 4.4) cultured at a temperature of 25^{o}C and on the different growth media were plotted to determine growth kinetics of the two organisms and to select the better of the two for other experiments. *S. maxima* showed typical growth curves in SSM, ZM and BE (Figure 4.3). The organism grew best in SSM (168 mg.l^{-1}). This concentration in SSM is expected of this culture grown in this medium since it is the standard medium of cultivation (Schlosser, 1982). The lag phase of *S. maxima* in SSM lasted 20 hours after which the culture adapted very well to its environment and a progressive increase in concentration was noted until 172 hours when the stationary phase commenced (Figure 4.3).

S. platensis showed a better affinity to the salts present in ZM and SSM than in BE. Concentrations of 179 mg.l^{-1} and 228 mg.l^{-1} were observed for the organism grown in the two media respectively. Research by Soletto *et al* (2005) showed biomass production after 2 days was 130 -140 mg.l^{-1} and after 5 days this increased to between 200-240 mg.l^{-1} and 1200 mg.l^{-1} of biomass was obtained after 7 days. *S. platensis* grown in ZM was the only organism to show a similar trend after 5 days of incubation (228 mg.l^{-1}) as compared to the maximum of 179 mg.l^{-1} of *S. maxima* produced only after 7 days of incubation. The high concentration of the organism in ZM could be as a result of its ability to tolerate high saline environments and utilise the salts present. A slight decline in growth rate was noted at the start of the shake flask experimentation for *S. platensis* grown in BE, with the decline of cells having occurred between the initial

inoculation and 26 hours of incubation. This may be attributed to a small quantity of cells that were unable to adapt to or regenerate from the starting medium. However, after this time, the organism adapted to its environment and progressive increases in biomass were noted (103 $mg.l^{-1}$) (Figure 4.4). It can be concluded that both *S. maxima* and *S. platensis* are capable of growing on BE. Although *S. maxima* produced higher quantities of biomass, the doubling time of *S. platensis* was shorter indicating that this organism grew faster in BE. ZM was the medium of choice to use as a supplement base.

The specific growth rates (μ) for *S. maxima* and *S. platensis* grown in SSM, ZM and BEM for each time interval are given in Tables 4.1, 4.2 and 4.3. The t_d varied for the different media. *S. maxima* had the shortest t_d (6.32) when grown in SSM and this correlates with the high concentrations obtained (Figure 4.3). μ_{max} when grown in BE was 0.025 h^{-1} and in ZM, 0.077 h^{-1} and the organism doubled within 9 hours (Table 4.2). Bhattacharya and Shivaprakash (2005) showed that *S. laxissima* had a t_d of 26.4 h and *S. platensis*, 12.05h. *S. platensis* used in this research had a shorter t_d than that showed by Bhattacharya and Shivaprakash, (2005). It was observed that the μ for *S. platensis* (0.044 h^{-1}) was greater than that obtained for *S. maxima* (0.024 h^{-1}) when grown in BE. *S. platensis* had a shorter t_d in BE (15 h) than *S. maxima* (27 h) (Table 4.3). Lower t_d are preferable because as the t_d increases, the speed of cell duplication decreases and the cultivation would become economically unfeasible. This observation led to the choice of *S. platensis* to be used in the optimisation experiments because of its high μ_{max} and lower t_d on BE.

Table 4.4 is a summary of μ_{max} of the different methods used to measure growth of the *S. maxima* and *S. platensis*. From the table it can be seen that *S. platensis* grew better and was therefore used in all further experiments using BE as the base medium, supplemented with salts from ZM to optimise with the aid of statistical experimental design.

Accurate quantification of Chlorophyll *a* is an important step in estimating phytoplankton biomass. Most commonly, acetone, methanol or ethanol have been used as solvents for extraction followed by quantification by either spectrophotometry (Simon and Helliwell, 1998). Acetone was used as a solvent to extract Chlorophyll *a* from *S. maxima* and

S. platensis (Figure 4.5). The highest amount of Chlorophyll *a* extracted from *S. maxima* was when grown in SSM (2.16 $mg.l^{-1}$). (Figure 4.5). *S. platensis* produced more when grown in ZM (2.43 $mg.l^{-1}$)(Figure 4.5) and these values correlate well with the growth curve (Figure 4.4). The graph indicates that there is a very small correlation between biomass produced and the amounts of Chlorophyll *a* extracted (Figure 4.5). R^2 of 0.50 was obtained for *S. maxima* in BE and 0.02 for *S. platensis* in BE. Chlorophyll *a* as an indicator of biomass was therefore not used in the optimisation experiments. Biomass concentration and the growth kinetics were used as deciding factors.

Spirulina contains all the essential amino acids required by the body and intake of this protein means that is does not have to be produced by the body (http://spirulina.org.uk/protein_supplement.htm). The only single, natural source providing the highest amount of protein is *Spirulina* which contains 71% protein. The

protein content in *Spirulina* is three times that of soybean and five times that of meat. The protein yield per unit area per year is the highest compared to other protein yielding crops (http://www.nrdcindia.com/pages/sprulina.htm). The highest percentage of protein measured from *S. maxima* grown in SSM was 93% and *S. platensis* in ZM was 68% (Figures 4.6a,b). These figures indicate a sharp, initial increase in the percentage of protein measured which is presumed to be the production of induced enzymes that are used by the organisms. Average protein produced by the two microorganisms from 100 h up to the end of the experiment was the highest for *S. maxima* grown in SSM (32.72%) and highest for *S. platensis* grown in ZM (27.79%). The increase and subsequent decrease in the percentage of protein present in *S. platensis* could indicate a possible error in the time of the growth of the organism in which protein is determined, resulting in some researchers quoting up to 60% protein for *S. platensis* (Richmond, 1988, Singh *et al*, 2002). The results indicate that protein should be determined at the end of growth rather than during the early log phase. The percentage of protein measured from *S. platensis* (23.76%) grown in BE was greater than *S. maxima* (17.29%) grown in BE (Figures 4.6a,b).

Changes in pH of the media are shown in Figure 4.7. A drop is noted for *S. maxima* grown in SSM but this increased after 52 hours, indicating possible by-product release that decreases the pH. Growth in ZM and BE continues to increase the pH. *S. platensis* grew effectively in Schlosser medium containing bicarbonate and carbonate, leading to a progressive pH increase from 9.6 to 10.8 (Binaghi *et al*, 2003). These findings are in accordance with the results presented here, of growth in SSM and ZM, where the pH ranged from 9.6 to 9.8. Larger amounts of biomass are produced in these

growth media as compared to BE (Figures 4.3 and 4.4) which has a pH range of 7.9 to 8.4. Although high in salinity, the pH has to be increased in order to facilitate better growth of *Spirulina*. The drop in pH corresponds to the sharp, initial increase in the protein measured. Possible production of enzymes could lead to the decrease in pH (Figure 4.7).

5.2 ABIOTIC FACTOR OPTIMISATION

Light and temperature are the main factors that influence the growth of photoautotrophic organisms. Even though *Spirulina* can grow both heterotrophically and mixotrophically, it depends on light as an energy source (Costa *et al*, 2004). If the levels of light and temperature are not limiting, other factors such as nutrient concentration, pH and salinity can be altered in order to increase biomass production of *Spirulina* (Costa *et al*, 2004). Light, temperature and pH were thus the first 3 components to be optimised before supplementation optimisation.

Three abiotic factors, light, temperature and pH were evaluated in order to determine the best parameters that should be used in optimising the production of *Spirulina*. Growth decreases when illumination levels are too low or too high (Andrade and Costa, 2007). In the absence of light, small amounts of *S. platensis* were produced (Runs 1, 3, 5 and 7) with no noticeable changes in the concentration (Figure 4.8). Runs exposed to light (0.1522 Lux), Runs 2 (136 $mg.l^{-1}$), 4 (138 $mg.l^{-1}$), 6 (96 $mg.l^{-1}$) and 8 (104 $mg.l^{-1}$), showed an average increase in growth after 6 hours of incubation (Figure 4.8).

Spirulina is an alkalophile with an optimum pH range of 9.5 -10.5 and can, but does not grow well at pH 8 and below (Jimenez, 2003b, Romano *et al*, 2000). Ciferri (1983) mentioned that in laboratory cultures *Spirulina* showed a wide range of optimum pH (8 to 11), but growth was also evident at pH values close to 7 and as high as 11. Runs 1 (pH 8, 25^{o}C, dark) and 3 (pH 8, 35^{o}C, dark) follow this trend where low concentrations in biomass were obtained (Figure 4.8). Run 2 (pH 8, 25^{o}C, light) and 4 (pH8, 35^{o}C, light), showed a higher productivity of *S. platensis* and this could be attributed to the flasks being exposed to light. Richmond (1988) reported that a pH of 11.0 is limiting to growth of *Spirulina.* It was further added that *Spirulina* can tolerate progressive changes in pH but not an abrupt change which could deteriorate the culture. This observation is clearly seen in Runs 5 (pH 11, 25^{o}C, dark) and 7 (pH 11, 35^{o}C, dark).

Spirulina is a mesophilic microorganism with an optimal growth temperature of 30-37^{0}C. (Jimenez *et al*, 2003b, de Oliveira *et al*, 1999). The growth curves for 30^{o}C are shown in Figure 4.8. The biomass concentrations achieved were, Run 4 (pH 8, 30^{o}C, Light) (138 mg.l^{-1}) and Run 8 (pH 11, 30^{o}C, Light) (104 mg.l^{-1}). The high pH of Run 8 could contribute to the smaller concentration of biomass produced. Run 3 at 30^{o}C, even though not exposed to light, produced high concentrations of biomass (99.74 mg.l$^{-1)}$ after 172 hours of incubation. The preference for high temperature was also evident in Run 7 (pH 11, 30^{o}C, Dark) since growth was not initially inhibited, however light then becomes the limiting factor and after 172 hours, a decline in growth is noted (Figure 4.8). Romano *et al* (2000) isolated a new strain of Cyanobacterium, '*Pantelleria*' in Italy. It was found that this strain grew in a temperature range of 20 to 35^{o}C with and optimum growth at 30^{o}C. In respect to increase in biomass, the best

response for the current study was obtained at 30^{o}C (Run 4). Sanchez Luna *et al* (2004) showed that temperature influences cell concentration, cell metabolism, nutritional needs of the cell and biomass composition. Their research indicated that 28^{o}C was the best temperature for cell growth. The low culture temperature is an important factor affecting productivity of *Spirulina* and could have resulted in a slower growth rate in the cultures. When the temperature was increased from 20 to 30^{0}C, the activity of *Spirulina* increased four fold (Chanawongse *et al,*1994). Here the general trend is that light has to be present, temperature needs to be at 30^{o}C and pH at 10 to produce more *S. platensis*.

Although pH, temperature and light have been assessed individually, they were part of an experiment that assesses them as mixed components as well. Since this is the situation that *Spirulina* is exposed to in the environment, assessing these factors in combination allows one to make better decisions about their effects on each other. Here, the tool of statistical experimental design is most useful in determining interaction of effects. The effects of each individual component and a combination of components are expressed in the Pareto chart (Figure 4.9) and are ranked according to the largest effect on the production of biomass. Light ©) has the largest positive effect followed by temperature (B). All three factors contribute positively to the growth of *S. platensis*. Interactions of factors (AB and ABC) also contribute positively to *S. platensis* production. The interaction of pH and temperature had a negative effect on the growth of *S. platensis*, however these factors cannot be disregarded since they lie above the Bonferroni limit which states that these factors contribute to the experiment and should be regarded as significant (Figure 4.9).

Figure 4.10 is the Box-Cox plot for power transformations and this graph validates the model given by Table 4.5. The plot suggests that no transformation of the model was required, with the transformation being set on “Lambda” (blue line) and positioned only 0.2 away from the best transformation. This transformation also lies within the optimal zone, i.e. between the 95% confidence interval limits (red lines), making it a satisfactory model for further analysis and forthcoming hypothesis testing.

A model was developed based on the effects of individual and combinations of the components and is given by the equation accompanying Table 4.5. ANOVA indicates that the model (F-value of 76.33) is statistically significant with $p< 0.0001$. There is only a 0.01% chance that an F-Value this large could occur due to noise. Model terms, A, B, C, AB, AC, BC, ABC are also statistically significant (Table 4.5) with $p<0.0001$. This implies that the equation is significant and all inferences and predictions made from the graphs drawn based on the model, are significant to 99.99%.

All three factors, pH, temperature and light contribute positively to the growth of *S. platensis*. A Cube plot was used to assess the interaction of these three factors. A maximum of 137 $mg.l^{-1}$ is produced when the pH is 10, temperature is 30^0C and light is supplied to the culture. If the pH was decreased to 8, temperature kept at 30^oC and light kept constant, 135 $mg.l^{-1}$ of *S. platensis* is produced. If the temperature is decreased to 25^0C and pH to 8 but light supplied, 131 $mg.l^{-1}$ of *S. platensis* is produced (Figure 4.11).

The data presented by the Contour graphs indicated that light was definitely required for the growth of *S. platensis* and for optimum production, the pH should be 10 and the temperature, 30^{o}C. At a temperature of 30^{o}C and pH 10, 137 mg.l^{-1} *S. platensis* was produced (Figure 4.12). At a lower pH, 135 mg.l^{-1} *S. platensis* was produced (Figure 4.13).

The maximum concentration that was produced when *S. platensis* was grown in BE in laboratory experiments was 103 mg.l^{-1} (Figure 4.3). By adjusting the pH, temperature and light, biomass increased to 138 mg.l^{-1} (Figure 4.8). This indicates a 33% increase in the biomass production without supplementation. Also, significant reductions in the production costs can be achieved by selecting a strain that grows at temperatures between 25 and 30^{o}C, since production can operate all year around (Olguin *et al,* 1997). These parameters (light, pH 10, temperature at 30^{o}C) were used for all further optimisation experiments.

5.3 MACRONUTRIENT OPTIMISATION

Once cultivation of the microrganism was achieved and abiotic parameters for growth had been optimised, the development of a suitable culture medium became important. The required nutrients were added as supplements in concentrations that are non-inhibitory to the microorganism. Brine effluent was shown to grow *Spirulina* and media optimisation studies were conducted in order to find the appropriate concentrations of nutrients that may be added to it to maximise the growth of *S. platensis*.

Salts present in ZM in larger quantities were grouped together in a macronutrient optimisation experiment to determine their effect on the growth of *S. platensis.* *S. platensis* was able to grow in the presence of various salts, $NaNO_3$ (A) NaCl (B), K_2SO_4 ©) and $NaHCO_3$ (D). Run 7 (Figure 4.14), supplemented with 2.5 $g.l^{-1}$ $NaNO_3$ and 1 $g.l^{-1}$ NaCl resulted in 197 $mg.l^{-1}$ of *S. platensis* biomass being produced after 100 hours. The μ_{max} for this run was calculated to be 0.08 and the t_d of *S. platensis* was 9 h. When compared to the concentration of biomass obtained when grown in BE, these values represent a 91% increase in the amount of *S. platensis* produced. The μ for this run was calculated to be 0.03 with a t_d of 23 hours. Runs 3, 4 and 7 (all containing NaCl) showed higher productivity of biomass. Research has indicated that growth rate of a marine *Spirulina* was enhanced in hypersaline medium (seawater supplemented with 0.5 M NaCl) thus suggesting that the organism is halophilic rather than halotolerant (Gabbay and Tel-Or, 1985).

Run 5, containing $NaNO_3$ and $NaHCO_3$ also produced high amounts of *S. platensis* (Figure 4.14). *Spirulina*, being a non-diazotrophic cyanobacterium requires nitrogen in the medium (Raoof *et al*, 2006), which is required for synthesis of the amino acids which make up proteins and other cellular components such as phycocyanin (Colla *et al*, 2007). Since this is an important nutritional source for the growth of *S. platensis* and is not supplied to the culture, the nitrogen is assimilated from the $NaNO_3$ (Costa *et al*, 2001). This could be seen by the positive effect of this salt on the growth of the microalga. Raoof *et al*, 2006 showed that a reduction in the amount of $NaNO_3$ supplied to the culture led to a considerable decrease in dry weight and other parameters therefore the concentration of 2.5 $g.l^{-1}$ is essential for the growth of

Spirulina. *Spirulina's* nutrient composition is a reflection of the substances that have been artificially added to it in the form of mineral (and other) supplements (Raoof *et al*, 2006).

More than one nutrient is required for different metabolic processes that occur with *Spirulina*. These nutrients do not occur individually in the environment, neither do they function on their own, so interactions of components have to be assessed. A fractional factorial experiment assesses more than one factor at a time, hence the use of a Pareto chart to rank and then develop models in each experiment to determine individual effects of the salts. The effects of each individual component and a combination of components are expressed in the Pareto chart (Figure 4.15) and are ranked according to the greatest effect on the production of biomass. The order of positive effects for components was:

- ($NaNO_3$ + NaCl) > (NaCl) > ($NaNO_3$ + $NaHCO_3$) (Figure 4.15).

NaCl also has a positive effect on the growth of *S. platensis* when used as an individual component and was the only individual component to have a positive effect on the growth of *S. platensis*. Lu and Vonshak (2002) have shown that *S. platensis* is capable of adapting to high concentrations of NaCl. $NaNO_3$ as an individual component has a negative effect on the growth of *S. platensis* and has to be used together with NaCl in order for its effect to be positive (Figure 4.15). Had this experiment been conducted with single factors, $NaNO_3$ may have been wrongly discarded as a negative factor.

A model was developed based on the effects of individual and combinations of components and is given by the equation accompanying Table 4.6. The equation may be used to forecast the concentration of *S. platensis* based on the mathematical model described. ANOVA indicated that the model was statistically significant with $p< 0.0001$. This implies that the equation is significant and all inferences and predictions made from the graphs drawn based on the model, are significant to 99.99%. Effects of individual components as well as combination of components were also statistically significant with $p<0.0001$. If the P-values are very small (less than 0.05), then the terms in the model have a significant effect on the response, in this case factors *A*, *B, C* and D which are

$NaNO_3$, NaCl, K_2SO_4 and $NaHCO_3$ (Table 4.6). This significance is determined by an F-value of 6939.34. This implies that the model fits the design appropriately with only a 0.01% chance that this value could have occurred due to noise. All inferences based on this model can be made at a confidence interval of 99.99%.

Figure 4.16 is the Box-Cox plot for power transformations and this graph validates the model given by Table 4.6. The plot suggests that no transformation of the model is required, with the transformation being set on “Lambda” (blue line) and positioned only 1.20 away from the best transformation (green lines). This transformation also lies within the optimal zone, i.e. between the 95% confidence interval limits (red lines), making it a satisfactory model for further analysis and forthcoming hypothesis testing.

The effect of variables $NaNO_3$ (A) and NaCl (B) was greater when BE is supplemented with only these supplements (191 $mg.l^{-1}$). If K_2SO_4 ©) was added to BE, the amount of *S. platensis* produced decreased to 125 $mg.l^{-1}$ (Figure 4.17). This data corresponds to

Run 6 containing $NaNO_3$ (A) and K_2SO_4 ©) (Figure 4.17). Both $NaNO_3$ (A) and K_2SO_4 ©) had a negative effect on the growth of *S. platensis*. This trend was also seen in the data presented by the Pareto Chart (Figure 4.15). Although K_2SO_4 ©) as an individual component had a negative effect on *S. platensis* production, this salt combined with NaCl (B) produced 161 $mg.l^{-1}$ of *S. platensis* biomass (Figure 4.17). This finding was in keeping with Run 4 which contained only NaCl (B) and K_2SO_4 ©) (Figure 4.14).

From the data presented, it can clearly be seen that NaCl and $NaNO_3$ had a positive effect on the production on *S. platensis* and the presence of K_2SO_4 with these salts decreased productivity. However, if BE was supplemented with these salts and if the fourth supplement, $NaHCO_3$ (D) was omitted from the supplementation, then 193 $mg.l^{-1}$ of *S. platensis* is produced (Figure 4.18) with a predicted maximum of 197 $mg.l^{-1}$. This represents an 91% increase as compared to *S. platensis* grown on unsupplemented BE and a 43% increase as compared to the maximum produced after abiotic optimisation. Batch experiments by others using *S. platensis* and ammonium sulphate and urea as nitrogen sources produced 240 $mg.l^{-1}$ of biomass after 5 days with a higher value of nitrogen (Soletto *et al*, 2005). The presence of $NaNO_3$ and NaCl and absence of K_2SO_4 and $NaHCO_3$ produced 193 $mg.l^{-1}$ of *S. platensis* (Figure 4.19). The salts that showed the greatest increase in *S. platensis* biomass were NaCl and $NaNO_3$.

5.4 MICRONUTRIENT OPTIMISATION

Even though it was previously determined that NaCl and $NaNO_3$ had a positive effect

on the growth of *Spirulina platensis*, these salts were not included in the micronutrient optimisation because this batch of experiments were carried out to assess the effect of the salts from Zarrouks Medium that are present in micro-quantities. Micronutrient optimisation comprised of the remaining salts from Zarrouks medium and are $FeSO_4.7H_20$ (A), $CaCl_2$ (B), $MgSO_4.7H_20$ ©), K_2HPO_4 (D), Na.EDTA (E) and Trace metals (F). When the concentration of these variables ie. $FeSO_4.7H_2O$ (A), $CaCl_2$ (B) and Trace metals (F) is at a positive level (Run 4), *S. platensis* biomass was 193 $mg.l^{-1}$ (Figure 4.20). This is an 87% increase in the production of biomass as compared to *S. platensis* grown in unsupplemented BE and a 40% increase as compared to the maximum produced after abiotic optimisation. *S. platensis* grown in the presence of these salts had a specific growth rate of 0.03 and a doubling time of 27 h. $CaCl_2$ is essentially required for cell membrane activity and also as a catalyst in enzymatic reactions (Raoof *et al*, 2006). Run 3 which contains $CaCl_2$ produced the second highest concentration of biomass (109 $mg.l^{-1}$). A decline of growth having occurred between the initial inoculation and 24 hours of incubation rate is noted in Run 5 which contains $MgSO_4.7H_2O$, NaEDTA and trace metals (Figure 4.20). This may be attributed to *S. platensis* biomass being unable to adapt to or regenerate from the starting medium.

The effects of each individual and combination of components is expressed in the Pareto chart and are ranked according to the greatest effect on the production of biomass. The order of positive effects for a combination of components are:

◆($FeSO_4.7H_20$ and Trace metals) > ($FeSO_4.7H_20$ and $CaCl_2$) > $FeSO_4.7H_20$, $CaCl_2$ and

Trace metals) > ($CaCl_2$ and Trace metals).

$FeSO_4.7H_20$, $CaCl_2$ and Trace metals are the only individual supplements that have a positive effect on the production of *S. platensis* biomass (Figure 4.21). These effects can be clearly seen in Runs 4 and 8 (Figure 4.20) containing these salts. Supplements $MgSO_4.7H_2O$ ©), K_2HPO_4 (D) and Na.EDTA (E) showed a negative effect on the growth of *S. platensis*, however all values that fall above the t-limit are significant and those that lie above the Bonferroni line are definitely significant. Microalgal phosphate assimilation depends on other factors such as pH, sodium concentration, potassium concentration, magnesium concentration and the presence of heavy metals (Costa *et al,2001*) .

The *Box-Cox Plot for Power Transforms* suggests that no transformation of the model is required, with the transformation being set on “Lambda” (blue line) and positioned only 0.2 away from the best transformation (green lines). This transformation also lies neatly within the optimal zone, i.e. between the 95% confidence interval limits (red lines), making it a satisfactory model for further analysis and forthcoming hypothesis testing. (Figure 4.22).

A model was developed based on the effects of individual and combinations of components and is given by the equation accompanying Table 4.7. ANOVA (Model F-value of 63660000.00) indicates that the model is statistically significant with $p< 0.0001$. This implies that the equation is significant and all inferences and predictions made from the graphs drawn based on the model, are significant to 99.99%. The terms

in the model also have a significant effect on the response. In this case A, B, C, D, E, F, AB, AC, AD, AE, AF, BD, BF, ABD, ABF are significant model terms (Table 4.7).

Figures 4.23-4.25 indicated the main effects and were drawn based on the significance of the model. Since the Pareto Chart indicated the positive effect of $FeSO_4.7H_2O$, $CaCl_2$ and Trace metals on biomass production, the Cube plot, Figure 4.23 confirmed this with a maximum of 142 $mg.l^{-1}$ of biomass being obtained when all three supplements were present in the BE. When the concentration of the variables, $MgSO_4.7H_2O$ ©), K_2HPO_4 (D) and NaEDTA (E) is at the negative level, *S. platensis* production increases (193 $mg.l^{-1}$)(Figure 4.24). $FeSO_4.7H_2O$, $CaCl_2$ and Trace metals had a positive effect on the growth of *S. platensis*. The effect of the remaining salts was negligible since excluding them from BE produced a larger quantity of *S. platensis* (193 $mg.l^{-1}$).(Figure 4.25). This is an 87% increase as compared to *S. platensis* grown in unsupplemented BE and 40% as compared to biomass produced in abiotic factor optimisation.

5.5 OPTIMISATION OF BRINE EFFLUENT MEDIUM (BEM)

A new medium was formulated for mass production of *S. platensis* by incorporating the selected nutrients from the macronutrient and micronutrient optimisation studies in brine effluent. This medium contained $NaNO_3$ (A), NaCl (B), $FeSO_4.7H_2O$ ©), $CaCl_2$ (D) and Trace Metals (E).

$NaNO_3$, NaCl, $FeSO_4.7H_2O$, $CaCl_2$ and Trace Metals had a marked effect on the

growth of *S. platensis*. These salts at varying concentrations were used to supplement the brine effluent in order to assess the feasibility of their use as supplements (Table 4.8). Run 7 containing large amounts of NaCl, $FeSO_4.7H_2O$ and Trace metals produced the highest amount of *S. platensis* biomass (287 $mg.l^{-1}$) (Figure 4.26). *S. platensis* grown in the presence of these salts had a specific growth rate of 0.037 and a doubling time of 18 h. The effects of each individual components are expressed in the Pareto chart and are ranked according to the greatest effect, on the production of biomass (Figure 4.27). The mixture of $NaNO_3$ (A) and NaCl (B) which had the greatest effect on the growth of *S. platensis* in the macronutrient optimisation studies, now had a negative effect on the growth of biomass. Supplements that show a positive effect on the growth of *S. platensis* are NaCl, $FeSO_4.7H_2O$ and Trace metals. The maximum positive effect was shown by NaCl and runs having a higher concentration of this supplement shower greater biomass production (Figure 4.29). Other positive effects of individual components are Trace metals (E) and $FeSO_4.7H_2O$ ©) as indicated in the micronutrient optimisation experiments. The order of positive effects for a mixture of components was ($FeSO_4.7H_2O$ + Trace metals) > (NaCl + Trace metals). The maximum negative effect was shown by the $CaCl_2$ supplement and all experimental runs containing a higher concentration of this supplement showed lower *S. platensis* productivity ie Runs 9-16.

The *Box-Cox Plot for Power Transforms* suggests that no transformation of the model was required, with the transformation being set on “Lambda” (blue line) and positioned 2 away from the best transformation (green lines).(Figure 4.28)**.**

A model was developed based on the effects of individual and combinations of components and is given by the equations accompanying Table 4.9. This implies that the equation is significant and all inferences and predictions made from the graphs drawn based on the model, are significant to 98.36%. The Model F-value of 60.24 implies the model was significant. There was only a 1.64% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, C, D, E, AB, AC, BD, BE, CD, CE, DE were significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Curvature F-value" of 135.19 implies there was significant curvature (as measured by difference between the average of the centre points and the average of the factorial points) in the design space. Curvature implies that a point of optimisation can be reached based on the model. There was only a 0.73% chance that a "Curvature F-value" this large could occur due to noise (Table 4.9).

189 $mg.l^{-1}$ of *S. platensis* is produced when BEM is supplemented with these salts and the remaining salts, $CaCl_2$ and $NaNO_3$ are at concentrations of 0.05 and 1.75 $g.l^{-1}$ respectively (Figure 4.29). Decreasing these concentrations to 1 and 0.04 $g.l^{-1}$ results in much greater biomass production (287 $mg.l^{-1}$) (Figure 4.30).

When $NaNO_3$, NaCl, $FeSO_4.7H_2O$ and Trace metals were added to brine effluent, a maximum of 189 $mg.l^{-1}$ of *S. platensis* biomass was obtained (Figure 4.29). If BE was supplemented with $NaNO_3$, NaCl, $FeSO_4.7H_2O$ and trace metals and the concentration of $CaCl_2$ (D) was decreased, 287 $mg.l^{-1}$ of *S. platensis* was produced (Figure 4.30). NaCl showed a positive effect when used as an individual component and $FeSO_4.7H_2O$

and Trace metals showed a positive, combined interaction on the production of *Spirulina platensis* (Figure 4.27). Keeping these salts as supplements, and decreasing the concentrations of $CaCl_2$ (D) and $NaHCO_3$ resulted in 256 $mg.l^{-1}$ *S. platensis* biomass being produced (Figure 4.31).

Overall, supplementation of the BEM with these salts and optimisation of the abiotic factors, resulted in a 178% increase as compared to *S. platensis* grown in unsupplemented BE, a 107% increase as compared to biomass obtained in the abiotic factor optimisation, a 46% increase when compared to the macronutrient optimisation alone and 48% increase when compared to the micronutrient optimisation alone.

Figure 4.32 clearly indicates the progressive increase in the production of biomass from unsupplemented BE to the final optimised effluent. The data presented here indicated that BE needs to be supplemented in order for larger amounts of *S. platensis* to be produced.

The salts that showed the highest increase in *S. platensis* biomass were NaCl, $NaNO_3$, $FeSO_4.7H_2O$, $CaCl_2$ and trace metal mixture. The concentrations of these salts were optimised and used to supplement the BE to be used as the final medium for the growth of *S. platensis* in a laboratory scale bioreactor.

5.6 LABORATORY SCALE BIOREACTOR

A 10 litre bench-top laboratory scale raceway bioreactor with a paddle for stirring

(Figures 3.2 and 3.3) was operated under steady state conditions in the laboratory, for *S. platensis* production. Light was supplied to the reactor (4.28 Lux) for the entire duration of the 20 days. The highest concentration of *S. platensis* biomass in Run 1 (1102 $mg.l^{-1}$) can be explained on the basis of the combination of the highest temperature (30^{o}C) and light intensity (4.28 Lux)(Figure 4.33). The lag phase lasted 24 hours. This was longer than that taken in laboratory scale batch experiments (6 h).

In the commercial production of *Spirulina*, the two main advantages of open ponds are the small capital investment and the use of a free energy source, the solar radiance (Jimenez, 2003b). When exposed to the sun and at a daily temperature of over 30^{o}C, the system (Figure 4.34) reached the maximum biomass concentration (1120 $mg.l^{-1}$) in Run 2. This was a 2% increase as compared to the maximum obtained with the reactor running indoors (1102 $mg.l^{-1}$) and exposed to 4.28 Lux (Figure 4.33). A decrease in the amount of biomass is noted in the early parts of the morning and this is due to the raceway located outdoors and not receiving any natural light. Converti *et al* (2006) produced 1.5 $g.l^{-1}$ *S. platensis* in 15 days from a pond that was 0.13 m^2 in size while Costa *et al* (2002) produced 0.78 $g.l^{-1}$ *Spirulina* grown in marine lagoon water. The μ_{max} of this organism was reported to be 0.13 h^{-1}.

The high temperature experienced during this period of cultivation was suitable for the growth of *S. platensis*. The increase in the biomass concentration observed in the bioreactor demonstrated that exposure to sunlight allows for the better performance of *S. platensis* in the pond. When comparing the two experiments it can be seen that although greater amounts of *S. platensis* were produced outdoors (average = 1086 m

g.l^{-1}), the organism took a longer period to grow (t_d = 35 h) than when the kept indoors (t_d = 31 h) (Table 4.10).

S. platensis had a shorter t_d of 11 h when grown in optimised BE, than compared to the reactor run indoors (31 h) and outdoors (35 h) (Table 4.10). The culture took a longer time to grow, however greater concentrations were achieved in the reactor kept outdoors (1086 m g.l^{-1}). This was a 278% increase in the production of *S. platensis* biomass from optimised BE used in batch experiments to the outdoor raceway pond and 944% increase from unsupplemented BE. The rate of production of *Spirulina platensis* was the highest in the reactor kept outdoors (2.51 mg.h^{-1}) than when compared to the reactor kept indoors (2.22 mg.h^{-1}) and in the batch experiment (1.28 mg.h^{-1}) (Table 4.10).

Microbiological analysis of the powder indicated only bacterial contamination and the number of bacteria counted per 1 ml of sample is given in Table 4.11. The greatest number of cells that were counted was in the 10^{-3} dilution and this was 4.28 x 10^2 CFU/ml. Costa *et al* (2002) showed that microbiological analysis of *S. platensis* grown in an open raceway pond had < 1.6 x 10^4 CFU/ml). The number of bacteria identified per ml of sample in this study was far less. It should be remembered that the counts were taken before drying of the *S. platensis* culture and that the drying process could probably decrease the level of contamination. A lower count of bacterial contamination was observed in this study since the raceway pond was covered and this decreased exposure to contamination.

After sufficient drying of the powder, a nutritional analysis was carried out to determine the properties of the *S. platensis* powder to be used as a food supplement. It was found that the powder contained a moisture content of 3.49%, ash, 1.12%, lipid content of 12% and the percentage protein was 17%. (Table 4.12). These findings are in keeping with some literature regarding the nutritional composition of *Spirulina.* Olguin *et al* (1997), showed that *Spirulina* grown outdoors in seawater supplemented with an anaerobic effluent from digested pig waste and no pH control produced a minimum of 17% protein. The results presented in this research is similar to that obtained by Olguin *et al* (1997). Other researchers (Richmond, 1988, Singh *et al*, 2002) indicated that *Spirulina* is 60% protein. The findings in this study disprove this and a possible explanation could be that previous researchers measured protein at a particular time, possibly in the early log phase (Figures 6a, 6b). Measurement should be done on the final product.

A cost analysis was carried out to determine the feasibility of producing *S. platensis* on a large scale. The major concern in microalgal biotechnology is the low efficiency and yield of biomass cultures outdoors, so there is a need for improvements in the growth performance of microalgae in these conditions (Jimenez *et al*, 2003b).

The most common design for open ponds are raceways with a paddle for stirring (Pulz, 2001). The 10 litre raceway bioreactor with a paddle for stirring built with perspex, cost R2000. The bioreactor has a working volume of 5 litres. It would cost between R25 000 to R30000 if it were to be built with fibre glass and R7000 if it were to be built with cement (Kidson, 2007).

The total cost for the laboratory-scale bioreactor is R4406.00 (Table 4.13). 1086 $mg.l^{-1}$ of *S. platensis* was produced from the bioreactor (Table 4.10), therefore, approximately, one gram of *S. platensis* is produced for every litre of supplemented BE. This would cost R881.00 to produce one gram of *S. platensis*, however the capital costs are fixed and will be excluded from the estimation for the use of the bioreactor in the laboratory. This implies that it would cost R6.00 to produce one gram of *S. platensis*. The market value of *Spirulina platensis* in a bottle containing 40 tables, each weighing 300 mg is R7.50 per gram.

The total cost involved in building a concrete, 400 m^2 open bioreactor with a working volume of 120 000 litres would cost R12 000. The total running expenses cost is R27 920. The total cost of the entire production process is R28 053.00 (Table 4.14). The average biomass concentration in the raceway pond outdoors is 1086 $mg.l^{-1}$. If the volume of BE utilised is taken into consideration, it would cost R2.15 to produce one gram of

S. platensis. This is much less than the current market price (R7.50) and laboratory scale production (R6.00). The summary presented in Table 4.16 and the percentage profit calculation of 248% indicates that the production of *S. platensis* in supplemented BE is a very attractive business proposition on a large-scale

6. Conclusions and Recommendations

Conclusions:

1. This research has indicated that it is possible to grow *Spirulina* on brine effluent and increased yields were obtained on supplemented brine effluent.

2. Statistical optimisation increased the productivity of the system in the following ways:

- Elimination of 5 of the 10 supplements as media components resulting in a decreased cost of the final product.
- A 33% increase in production after abiotic factor optimisation as compared to biomass obtained in unsupplemented BE.
- A 90% increase in production after macronutrient optimisation as compared to biomass obtained in unsupplemented BE.
- A 86% increase in production after micronutrient optimisation as compared to biomass obtained in unsupplemented BE.
- A 178% increase in production in optimised BE as compared to biomass

obtained in unsupplemented BE.

3. Final concentrations were optimised in a laboratory raceway operated under synthetic light and natural conditions and produced 944 and 1086 $mg.l^{-1}$ of *S. platensis* biomass. The total increase from batch experiments in unsupplemented BE to indoor laboratory cultivation was 809% and from batch experiments to outdoor laboratory cultivation, was 944%.

4. Cost analysis indicates that it would be feasible to produce *S. platensis* on a large scale at an estimated profit of 248%.

Recommendations:

1. Continuation of optimisation of BE in the outdoor raceway. Factors to be optimised would be supplements, pH and agitation.

2. A large scale bioreactor should be constructed to test the predications made in this research.

3. A feasible method of harvesting and drying should be investigated.

7. References

Ahmed, M., Shayya, W.H., Hoey, D., Mahendran, A., Morris, R. and Al-Handaly, J. 2000. Use of evaporation ponds for brine disposal in desalination plants. *Desalination*, 130: 155-168.

Anaga, A. and Abu, G.O. 1996. A laboratory scale cultivation of *Chlorella* and *Spirulina* using waste effluent from a fertilizer company in Nigeria. *Bioresource Technology*, **58**: 93-95.

Anderson, M.J., Whitcomb, P.J., 2000. *DOE Simplified*. *Practical Tools for effective Experimentation*. Productivity Inc.: New York

Andrade, M.R. and Costa, J.A.V. 2007. Mixotrophic cultivation of *Spirulina platensis* using molasses as organic substrate. *Aquaculture*, Article in Press.

Anon. 2005. *What is Spirulina?* [Online]
Available from: http://www.naturalways.com/spirul1.htm. [Accessed 02 August 2002].

Arnal,J.M., Sancho, M., Iborra, I., Goza´ lvez, J.M., Santafe´, A. and Lora, J. 2005. Concentration of brines from RO desalination plants, by natural evaporation. *Desalination*. **182**: 435–439.

Beam Biotechnological and Environmental Applications, An Australian Research Network [Online]. Available from: www.scieng.murdoch.edu.au/centres/algae/BEAM-Net/BEAM-Appl3.htm [Accessed 25th October 2005].

Becker, E.W. 1994. *Microalgae: Biotechnology and Microbiology*. Cambridge studies in Biotechnology, No 10. Cambridge University Press.

Bhattacharya, S. and Shivaprakash, M.K. 2005. Evaluation of three *Spirulina* species grown under similar conditions for their growth and biochemicals. *Journal of Science and Food and Agriculture*, **85**: 333-336.

Binahgi, L., Del Borghi, A., Lodi, A., Converti, A. and Del Borghi, M. 2003. Batch and fedbatch uptake of carbon dioxide by *Spirulina platensis*. *Process Biochemistry*, **38**: 1341-1346.

Bold, H.C. and Wynne, M.J. 1978. *Introduction to the Algae, Structure and Reproduction*. Prentice Hall Inc, Englewood Cliffs, New Jersey, 1-5.

Borowitzka, M.A. 1999. Commercial production of microalgae: ponds, tanks, tubes and fermentors. *Journal of Biotechnology*, **70**: 313-321.

Buckley, C.A., Simpson, A.E., Kerr, C.A., and Schutte, C.F. 1987. The treatment and disposal of waste brine solutions. *Desalination*, **67**: 431-438.

Bradford, D. 1976. *Bradford Protein Assay*. [Online] Available from: http://www.ruf.rice.edu/~bioslabs/methods/protein/bradford.html [Accessed June 2006].

Brouckaert, C.J. and Buckley, C.A. 2003. *The Application of Pinch Analysis For the Rational Management of Water and Effluent in an Industrial Complex*. [Online]. Available from: http://www.nu.ac.za/department/data/WRC851.pdf [Accessed January 2007].

Canizares, R.O. and Dominguez, A.R. 1993. Growth of *Spirulina maxima* on swine waste. *Bioresource Technology*, **45**(1): 73-75.

Carlile, M.J., and Watkinson, S.C. 1997. *The Fungi*. Academic Press Inc: New York, 102-109.

Chanwongse, L., Lee, Y.K., Bunnag, B. and Tanticharoen, M. 1994. Productivity of the cyanobacterium *Spirulina platensis* in cultures using sunlight. *Bioresource Technology,* **48**: 143-148.

Chaumont, D. 1993. Biotechnology of algal biomass production: a review of systems for outdoor mass culture. *Journal of Applied Phycology*, **5**: 593-604.

Chaumont, D., Thepenier, C., Gudin, C., and Junjas, C. 1987. Scaling up a photobioreactor for continuous culture of *Porphyridium cruentum* from laboratory to pilot plant (1981-1987). *In*: Stadler, T., Mollion, J., Verdus, M. C., Karamonos, Y., Morvan, H. and Christiaen, D. (eds) *Algal Biotechnology*, London: Elsevier Applied Science, 199-208.

Chojnacka, K., Chojnacki, A., and Gorecka, H. 2005. Biosorption of Cr^{3+}. Cd^{2+} and Cu^{2+} ions by blue green algae *Spirulina* sp: kinetics, equilibrium and the mechanism of the process, *Chemosphere*, **59**: 75-84.

Chojnacka, K., Chojnacki, A., and Gorecka, H. 2004. Trace removal by *Spirulina* sp from copper smelter and refinery effluents. *Hydrometallurgy*, **73**: 147:153.

Chojnacka, K. and Nororyta, A. 2004. Evaluation of *Spirulina* sp growth in photoautotrophic, hetertrophic and mixotrophic cultures. *Enzyme and Microbiological Technology,* **34**: 461-465.

Ciferri, O. 1983. *Spirulina*, the Edible Organism. *Microbiological Reviews***, 47**(4): 551-578.

Ciferri, O. and Tiboni, O., 1985. The Biochemistry and Industrial Potential of *Spirulina*. *Annual Review of Microbiology*, **39**: 503-526.

Clesceri, L.S. 1998. Standard Methods for the examination of Water and Wastewater,Amercian Public Health Association, 1-10.

Colla, L.M., Reinehr, C.O., Teichert, C. and Costa, J.A.V. 2007. Production of biomass and nutritional compounds by *Spirulina platensis* under different temperature and nitrogen regimes. *Bioresource Technology*, **98**: 1489-1493.

Converti, A., Lodi, A., Del Borghi, A. and Solisio, C. 2006. Cultivation of *Spirulina platensis* in a combined airlift-tubular reactor system. *Journal of Biochemical Engineering*, **32**: 13-18.

Costa. J.A.V., Colla, L.M. and Filho, P.F.D. 2004. Improving *Spirulina platensis* biomass yield using a fed-batch process. *Bioresource Technology*, **92**: 237-241.

Costa. J.A.V., Colla, L.M. and Filho, P.F.D. 2002. *Spirulina platensis* growth in open raceway ponds using fresh water supplemented with Carbon, Nitrogen and metal Ions. [Online]. Available from: www.znaturforsch.com. [Accessed 28 February 2007].

Costa, J.A.V., Colla, K.L., de Oliviera, L. and Magagnin. 2001. Different nitrogen sources and growth responses of *Spirulina platensis* in microenvironments, *World Journal of Microbiology and Biotechnology,* **17**: 439-442.

de Oliveira, M.A.C.L., Monteiro, M.P.C., Robbs, P.G. and Leite, S.G.F. 1999. Growth and chemical composition of *S. maxima* and *S. platensis* biomass at different temperatures, *Aquaculture International*, **7**: 261-275.

Dilraj, V. 2007. Personal Communication to B.B Choonawala, 2nd March 2007. Technician. Department of Biotechnology and Food Technology, Durban University of Technology.

Dunn, M.J., 1992. Protein determination of total protein concentration. *In*: Harris, E.L.V. and Angal, S., (Eds), *Protein Purification Methods*. Oxford: IRL Press.

Design Expert 7. 2006. Glossary of Terms [software program] Available from: Design Expert 7P/Help/Contents/Glossary of Terms [Accessed 30 October 2006].

Eskom, 2006. [Online]. Availaible from: www.eskom.co.za [Accessed 09 February 2007].

Fox, R. D. 1996. *Spirulina, Production and Potential*. Editions Edisud: France.

Gabbay, R. and Tel-Or, E. 1985. Cyanobacterial biomass production in saline media. *Plant and Soil*, **89**: 107-116.

Gitelson, A., Qiuang, H. and Richmond, A. 1996. Photic volume in photobioreactors supporting ultrahigh population densities of the photoautotroph *Spirulina platensis*. *Applied and Environmental Microbiology*, **62**(5): 1570-1573.

Godia, F., Albiol, J., Montesinos, J. L., Perez, J., Creus, N., Cabello, F., Mengual, X., Montras, A. and Lasseur, C.H. 2002. MELISSA: a loop of interconnected bioreactors to develop life support in space. *Journal of Biotechnology*, **99**: 319-330 .
Gong, R., Ding, Y., Liu, H., Chen, Q., and Liu, Z. 2005. Lead biosorption and desorption by intact and pretreated *Spirulina maxima* biomass. *Chemosphere*, **58**:123-130.

Greasham, R.L. and Herber, W.K. 1997. Design and Optimisation of growth media. *In* Rhodes, P.M. and Stanbury, P.F (eds). *Applied Microbial Physiology, A Practical Approach*. Oxford University Press: Oxford, 53-57.

Guide to U.S. Regulation of Genetically Modified Food and Agricultural Biotechnology Products.[Online].
Available from: http://pewagbiotech.org/resources/issuebriefs/1-regguide.pdf [Accessed 22nd February 2007].

Haaland, P.D. 1989. *Experimental Design in Biotechnology*. Marcel Dekker, Inc. New York and Basel. 1-249.

Hendrikson, 1998. *Spirulina: Health Discoveries From The Source Of Life* [Online].
Available from: http://www.spirulina.com/SPLNews98.html [Accessed 20 July 2005].

[Online]. Available from:
http://desertgardens.suite101.com/blog.cfm/is_desalination_answer_to_water
[Accessed January 2007].

[Online]. Available from: http://en.wikipedia.org/wiki/Algae [Accessed 13th June 2006].

[Online]. Available from: http://en.wikipedia.org/wiki/Aquaculture [Accessed January 2007].

[Online]. Available from: http://en.wikipedia.org/wiki/Endorheic [Accessed January 2007].

[Online]. Available from: http://en.wikipedia.org/wiki/Factorial_experiment) [Accessed January 2007].

[Online]. Available from: http://en.wikipedia.org/wiki/Lake_Texcoco [Accessed January 2007].

[Online]. Available from: http://en.wikipedia.org/wiki/Spirulina_(dietary_supplement)
[Accessed January 2007].

[Online]. Available from: http://en.wikipedia.org/wiki/Osmotrophy [Accessed January 2007].

[Online]. Available from: http://microbewiki.kenyon.edu/index.php/Spirulina [Accessed January 2007]

[Online]. Available from: http://spirulina.org.uk/protein_supplement.htm [Accessed: June 2006].

[Online]. Available from: http://www.africanenergy.co.za/magazines/evol1no3/story11.htm)Vol 1 NO 3 [Accessed January 2007].

[Online]. Available from: http://www.answers.com/topic/spirulina-food-supplement [Accessed 26 February 2007].

[Online]. Available from: http://www.auroville.org/health/images/spiru_4.jpg [Accessed January 2007].

[Online]. Available from: http://www.botanicalpreservationcorps.com/microalgae.htm [Accessed 27th May 2005]

[Online]. Available from: http://www.engineeringnews.co.za/eng/features/tempcontrol/?show=35033 [Accessed January 2006].

[Online]. Available from: http://www.hamline.edu/depts/biology/courses/biocon2/biuret.html) [Accessed January 2007].

[Online]. Available from: http://www.naturalways.com/spirulina-analysis.htm [Accessed 21st August 2002].

[Online]. Available from: http://www.newworldencyclopedia.org/preview/Algae [Accessed January 2007].

[Online]. Available from:http://www.parrynutraceuticals.com/spirulina/prod_spiphoto.jpg [Accessed January 2007].

[Online]. Available from: http://wwwscieng.murdoch.edu.au/centres/algae/BEAM-Net/BEAM-Appl4a.htm [Accessed 25th October 2005].

[Online]. Available from: http://www.spirulinasource.com/earthfoodch6c.html#farms). [Accessed January 2007].

[Online]. Available from: http://www.webster.com/cgi-bin/dictionary?book=Dictionary&va=ubiquitous [Accessed January 2007].

[Online]. Available from: http://spirulina-program.org/3compos.htm [Accessed January

2007]

[Online]. Available from: http://spirulina-program.org/10harvest.htm) [Accessed January 2007]

Jimenez, C., Cossio, B.R., Labella, D. and Niell, F.X. 2003a. The feasibility of industrial production of *Spirulina* (Arthrospira) in Southern Spain. *Aquaculture*, 217: 179-190.

Jimenez, C., Cossio, B.R. and Niell, F.X. 2003b. Relationships between physichochemical variables and productivity in open ponds for the production of *Spirulina*: a predictive model of algal yield. *Aquaculture,* **221**: 331-345.

Jordan, J.P. 2003. *Grow your own Spirulina*. [Online] Available from: www.antenna.ch/manuel/grow.htm. [Accessed January 2005].

Kidson, G. 2007. Personal Communication to B.B Choonawala, 2nd March 2007. Engineer. Department of Mechanical Engineering, Durban University of Technology.

Kim, D. 1990. Outdoor mass culture of *Spirulina platensis* in Vietnam. *Journal of Applied Phycology*, **2**: 179-181.

Lee, Y.K. 2001. Microalgal mass culture systems and methods: Their limitations and potential, *Journal of Applied Phycology*, **13:** 307-315.

Li, D. M. and Qi, Y. Z. 1997. *Spirulina* industry in China: Present status and future prospects. *Journal of Applied Phycology*, **9**: 25-28.

Li, D., Xie, J., Zhao, Y. and Zhao, J. 2003. Probing connection of PBS wioth the photosystem in intact cells of *Spirulina* platensis by temperature-induced flourescence fluctuation. *Biochimica ET Biophysica Acta,* **1557**: 35-40.

Lowry, L.O.H., Rosebrough, N.J., Farr,A.L. and Randall, R.J. 1951. Protein measurement with the Folin-Phenol reagents. *Journal of Biological Chemistry*, **193**: 265-275.

Lu, C. and Vonshak, A. 2002. Effects of salinity stress on photsystem II function in cyanobacterial *Spirulina platensis* cells. *Physiologica Plantarum,* **114**: 405-413.

May,P. ND.
Available from: http://www.chm.bris.ac.uk/motm/chlorophyll/chlorophyll_h.htm)
[Accessed 13th July 2006].

Mitchell, S.A. and Richmond, A. 1988. Optimisation of a growth medium for *Spirulina* based on cattle waste. *Biological Wastes*, **25** :41-50.

Morist, A., Montesinos, J.L, Cusida, J.A. and Godia, F. 2001. Recovery and treatment

of *Spirulina platensis* cells cultured in a continuous photobioreactor to be used as food. *Process Biochemistry*, **37**: 535-547.

Olaizola, M. 2003. Commercial development of Microalgal Biotechnolgy: from the test tube to the market place. *Biomolecular Engineering,* **20**(4-6): 459-466.

Olguin, E.J., Galacia, S., Angulo-Guerrero, O., Hernandez, E. 2001. The effect of low light and nitrogen deficiency on the chemical composition of *Spirulina* sp (Arthrospira) grown on digested pig waste. *Bioresource Technology,* **77**: 19-24.

Olguin, E.J., Galacia, S., Camacho, R., Mercado, G. and Perez, T.J. 1997. Production of *Spirulina* sp. in sea water supplemented with anaerobic effluents in outdoor raceways under temperature climatic conditions. *Applied Microbiology and Biotechnology*, **48**: 242-247.

Parker, N.C. and Bates, M.C. 2003*. Integrated aquaculture based on Spirulina, livestock wastes, brine and power plant by products*.
[Online]. Available from: http://www.trcu.ttu.edu/tcru/kc/pubs/parker/p80.htm
[Accessed 29 July 2002].

Pearson, D. 1976. *The Chemical Analysis of Food*. Churchill Livingston: Edinburgh, London and New York, 6-11.

Pelizer, L.H., Danesi, E.D.G., de O Rangel, C., Sassano, C.E.N., Carvalho, J.C.M., Sato, S. and Maraes, I.O. 2003. Influence of innoculum age and concentration in *Spirulina platensis* cultivation. *Journal of Food Engineering*, **56**: 371-375.

Perret, J., Claereboudt, M. and Jup, B. 2000. *Spirulina* culture using reject brine water from desalination plants.

[Online]. Availabel from: http://jperret.trip[od.com/research_johan/spirulina.html.
[Accessed 2 July 2002].

Phang. S.M., Miah, M.S., Yeoh, B.G. and Hashim, M.A. 2000. *Spirulina* cultivation in digested sago starch factory wastewater. *Journal of Applied Phycology*, **12**: 395-400.

Price, N.C., 1996. *Proteins*, Labfax, Oxford: Academic Press.

Pulz, O. 2001. *Photobioreactors: Production systems for phototrophic microorganisms*. [Online] Availabel from: http://link.springer.de. [Accessed June 2005].

Raoof, B., Kaushik, B. D. and Prasanna, R. 2006. Forumlation of a low-cost medium for mass production of *Spirulina*. *Biomass and Bioenergy*, **30**: 537-542.

Rangsayatorn, N., Upatham, E.S. Kruatrachue, M. Pokethitiyook, P. and Lanza, G.R. 2002. Phytoremediation potential of *Spirulina (Arthrospira) platensis* biosorption and

toxicity studies of cadmium. *Environmental Pollution*, **119**: 45-53.

Richmond, A. 1988. Microalgae of economic potential. *In: CRC handbook of Microalgal mass culture*. CRC Press, Inc: Boca Raton, Florida, 199-244.

Richmond, A. 1986. Spirulina. In: Borowitzka, M.A. and Borowitzka, L.J. (eds.) *Micro-algal Biotechnology*. Cambridge: Cambridge University Press, 85-121.

Romano, I. Bellitti, M.R., Nicolaus, B., Lama, L., Manca, M.C, Pagnotta, E., Gambacorta, A. 2000. Lipid proflie: a useful chemotaxonomic marker for classification of a new cyanobacterium in *Spirulina* genus. *Phytochemistry,* **54**: 289-294.

Sanchez-Luna, L.D., Converti, A., Tonini, G.C., Sato, S., and Carvalho, J.C.M. 2004. Continuous and pulse feedings of urea as a nitrogen source in fed batch cultivation of *Spirulina platensis*. *Aquacultural Engineering*, **31**: 237-245.

Schlosser. 1982. *Media Recipes*. [Online] Available from: http://www.bio.utexas.edu/research/utex/old/medrec.htm#spir [Accessed 2^{9th} April 2002].

Shimamatsu, H. Mass production of *Spirulina*, an edible microalga. 2004. *Hydrobiologia*, 512: 39-44

Simon, D. and Helliwell, S. 1998. Extraction and quantification of chlorophyll A from freshwater green algae. *Water Research*, **32**(7): 2220-2223.

Singh, Y. 2005. *Spiruling - A wonder vegetarian protein source*. [Online].[Accessed 12th August 2004]

Singh, S.C., Sinha, R.P. and Hader, D.P. 2002. Role of light and fatty acids in stress tolerance in cyanobacteria. *Acta Protozoological,* **41**: 297 - 308.

Soeder, C. 1986. *CRC handbook of Microalgal mass culture*. CRC Press, Inc: Boca Raton, Florida, 25-29.

Soletto, D., Binaghi, L., Lodi, A., Carvalho, J.C.M., and Converti, A. 2005. Batch and fed batch cultivations of *Spirulina platensis* using ammonium sulphate and urea as nitrogen sources. *Aquaculture*, **243**: 217-224.

Steer, J. 2006. *Structure and Reactions of Chlorophyll* [Online]. Available from: http://www.ch.ic.ac.uk/local/projects/steer/chloro.htm [Accessed June 2006].

Torzillo, G., Pushparaj, B., Bocci, F., Balloni, W., Materassi, R. and Florenzano, G. 1986. Production of *Spirulina* in closed photbioreactors. *Biomass,* **11**: 61-74.

Travieso, L., Hall, D.O., Rao, K.K., Benitez, F. Sanchez, E. and Borja, R. 2001. A helical tubular photobioreactor producing *Spirulina* in a semicontinuous mode. *International Biodeterioration and Biodegradation*, **47**: 151-155.

Tredici. M.R. and Zittelli, G.C. 1998. Efficiency of sunlight utilization: tubular versus flat photobioreactors. *Biotechnology and Bioengineering*, 57(2):187-197.

True, G.N., Willis, J. and Nair, P.R. 1982. Blue-green algae: *Spirulina geitler, Spirulina maxima, Spirulina platensis* [Online]
Available from: http://www.geocities.com/nutriflip/Naturopathy/Spirulina.html.
[Accessed 31 July 2002].

Vendan, R.T. and Rajeshwari, T. 1998. *Spirulina* - the wonder algae, Kisan World. **25**: 29.

Vonshak, A. 1986. Laboratory Techniques for the cultivation of microalgae, *In*,*CRC handbook of Microalgal mass culture*. CRC Press, Inc: Boca Raton, Florida, 117-128.

Vonshak, A., Guy, R. and Guy, M. 1988. The response of the filamentous cyanobacterium *Spirulina platensis* to salt stress. *Archives of Microbiology*, **150**(5): 417-420.

Vonshak, A., and Richmond, A. 1988. Mass production of the blue-green Alga *Spirulina*: an overview. *Biomass,* 233-247.

Walsh,G. Proteins, Biochemistry and Biotechnology. John Wiley and Sons, 1-10.

Zeng. M.T. and Vonshak, A. 1998. Adaptation of *Spirulina platensis* to salinity stress. *Comparitive Biochemistry and Physiology Part A,* **120**: 113-118.

Zhi, W., Song, J., Ouyang, F. and Bi, J. 2005. Application of response surface methodology to the modeling of α-amylase purification by aqueous two-phase systems. *Journal of Biotechnology*, **118**: 157–165.

8. Appendices

Appendix 8.1

Synthetic *Spirulina* Medium

Preparation: In order to prevent the formation of precipitates during autoclaving, two solutions are prepared as follows:

Solution A

glass distilled water	500 ml
$NaHCO_3$	13.61 g

$Na2CO_3$	4.03 g
K_2HPO_4	0.50 g

Solution B

glass distilled water	500 ml
$NaNO_3$	2.50 g
K_2SO_4	1.00 g
NaCl	1.00 g

$MgSO_4.7H_2O$	0.20 g
$CaCl_2.2H_2O$	0.04 g
P-IV Metal Solution	6 ml
Chu micronutrient solution	1 ml
vitamin B12 (15 ug/100 mL H2O)	1 ml

Solutions A and B are autoclaved separately and combined aseptically after cooling.

To 1000 ml of glass distilled water, add 0.750 g of NaEDTA and dissolve dully. Add the following salts in the amounts indicated:

P-IV Metal Solution

	mg/l
$FeCl_3.6H_2O$	97
$MnCl_2.4H_2O$	41
$ZnCl_2$	5
$CoCl_2.6H_2O$	2
$Na_2MoO_4.2H_2O$	4

To 1000 ml of autoclaved glass-distilled water, add:

Chu Micronutrient Solution

	mg/l
NaEDTA	50
H_3BO_3	61.8
$CuSO_4.5H_2O$	19.6
$ZnSO_4.7H_2O$	44
$CoCl_2.6H_2O$	20
$MnCl_2.4H_2O$	12.6
$Na_2\ MoO_4.2H_2O$	12.6

Appendix 8.2

Zarrouks Medium

Ingredients for Zarrouks Medium

	g/l
$NaNO_3$	2.5
NaCl	1

$NaHCO_3$	16.8
K_2SO_4	1
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
$CaCl_2$	0.04

$FeSO_4.7H_2O$	0.01
$EDTA.Na_2$	0.08
Trace metals	4 ml

Trace metals

	g/l
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.13
$ZnSO_4.7H_2O$	0.222
$NaMoO_4.5H_2O$	0.39
$Co(NO_3)_2.6H_2O$	0.049
$CuSO_4.5H_2O$	0.079

Appendix 8.3

Calculation of the amount of *Spirulina maxima* cells in 1 ml

232 cells per 0.02 ul

232 x 50 x 1000 cells per ml

11600000 cells per ml

1.16 x 10^7 cells per ml

Calculation of the amount of *Spirulina platensi*s cells in 1 ml

302 cells per 0.02 ul

302 x 50 x 1000 cells per ml

15100000 cells per ml

1.51 x 10^7 cells per ml

Cells were adjusted by dilution to 1.00 x 10^7 cells per ml.

Appendix 8.4

Brine Effluent Analysis

Chemical Determined	Analysis Result

Colour (Hazen Units)	94
Turbidity	0.38
pH	5.69
Conductivity (mS/m)	1108
Ammonia (mg/l)	0.47
Chloride	1330
Sulphate	3250
Flouride	8.4
Nitrate	55.2
TDS	8938
TSS	0.11
TKN	5634
TP	1233

Appendix 8.5

Chlorophyll Determination (Clesceri, 1998)

as per Standard Methods for Water and Wastewater Determination with a few changes.

A. Equipment and reagents

1. Tissue grinder/ Sonicator
2. Centrifuge
3. 15 ml graduated, screw cap centrifuge tubes
4. aqueous acetone solution - mix 90 parts acetone with 10 parts distilled water.

B. Pigment Extraction Procedure

1. Concentrate the sample by centrifuging or filtering as soon as possible after collection.
2. Place sample in a tissue grinder, cover with 2 to 3 ml 90% aqueous acetone and macerate at 500 rpm for 1 minute.
3. Transfer to a screw cap centrifuge tube and adjust the volume to 10 ml with 90% aqueous acetone.
4. Steep samples in the dark at least 2 hours at $4^{O}C$.
5. Clarify by centrifugation and decant supernatant into a clean, calibrated centrifuge tube and measure the volume.

C. Spectrophotometric Determination of Chlorophyll - Trichromatic Method

1. Transfer extract to a 1 cm cuvette and measure optical density (OD) at

750, 664, 647 and 630 nm.

2. The OD reading at 750 nm is a correction for turbidity. Subtract this reading from each of the pigment OD values of the other wavelengths before using them in the equation below.

$C_a = 11.85 (OD_{664}) - 1.54 (OD_{647}) - 0.08 (OD_{630})$

where, C_a = concentration of chlorophyll *a* in $mg.l^{-1}$ and

OD_{664}, OD_{647} and OD_{630} = corrected optical densities at the respective wavelengths.

Appendix 8.6

Folin Lowry Protein Determination in Cells (Lowry, 1951).

Principle

In a two step reaction, Folin's reagent reacts with the aromatic amino acids in protein, after treatment with alkaline copper to give a blue colour

Outline

The sample is solubilised in 0.1 M NaOH, alkaline copper solution is added and then diluted Folin reagent. Absorbance is read at 720 nm in a spectrophotometer.

Reagents

A: 2 % Na_2CO_3 in 0,1 M NaOH

B: 0,5 % $CuSO_4.5H_2O$ in 1 % KNa $C_4H_4O_4.4H_2O$

C: 1 ml of reagent B mixed with 50 ml reagent A

D: Folin-Ciocalteau reagent diluted 1:1 in distilled water

E: Bovine Serum Albumin (BSA) standard solution

Reagent D is unstable and should be discarded after 24 hrs.

METHOD

1. Dispense 3 ml Reagent C into each test tube.
2. Dispense 0,6 ml of the sample to 6 ml Reagent C.

3. Control: Add 0,6 ml of distilled water instead of sample to 6 ml Reagent C.
4. Add 0,3 ml of Reagent D (diluted Folin reagent) to each tube and mix immediately after addition.
5. Allow tubes to stand for 30 min at room temperature.
6. Read absorbance at 720 nm.
7. Protein concentration, determined from a standard curve is expressed as $mg.l^{-1}$ protein

Appendix 8.7

Bovine Serum Albumin Standard Curve for Protein Determination

A standard stock solution of 1000 mg BSA was prepared by dissolving 1 g BSA in 1000 ml distilled water.

Working stock solutions of 10, 20, 40, 60, 80, 100, 150 and 200 mg.l0-1 was prepared as using the equation:

$C_1V_1 = C_2V_2$

C_1 - initial concentation

C_2 = final concentration

V_1 = final volume

V_2 = volume required

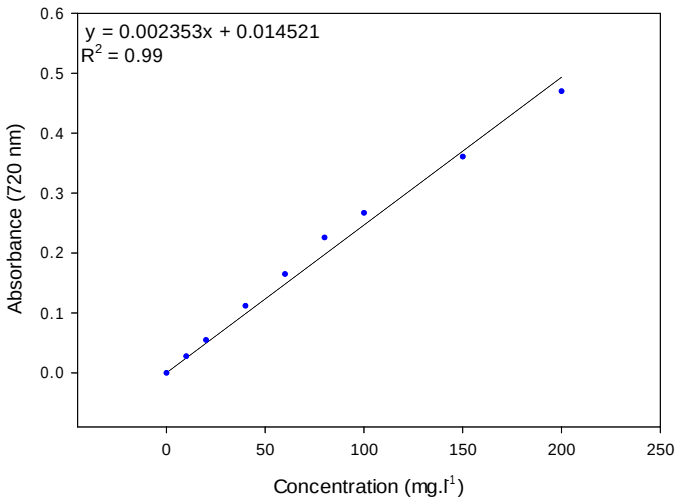
Using the equation, to prepare a 10 ml working stock solution, 10 ml from the stock solution was made up to a volume of 100 ml with distilled water.

The same procedure is followed for the other concentrations.

Each concentration is measured at 720 nm and an absorbance vs concentration graph is used to calculate the amount of protein present in a sample.

Bovine Serum Albumin Standard Curve for Protein Determination

Concentration	Absorbance
0	0
10	0.028
20	0.055
40	0.112
60	0.165
80	0.226
100	0.267
150	0.361
200	0.47



Appendix 8.8.1

Standard Curve of *Spirulina maxima* in Synthetic Spirulina Medium

Absorbance of *Spirulina maxima* in Synthetic Spirulina Medium

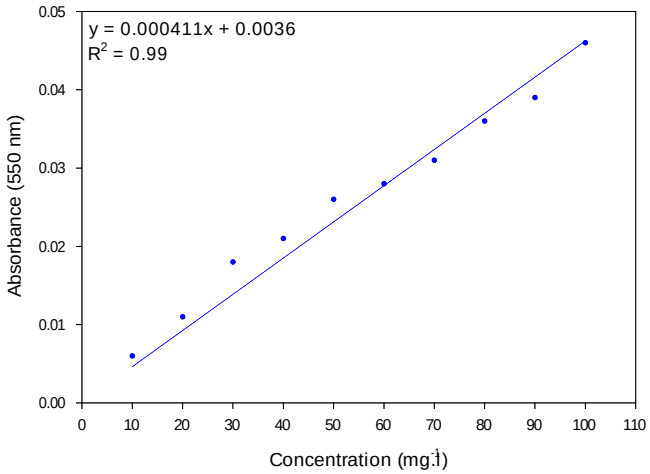
Concentration	Absorbance
10	0.006
20	0.011
30	0.018
40	0.021
50	0.026
60	0.028
70	0.031
80	0.036
90	0.039
100	0.046

Appendix 8.8.2
Standard Curve of *Spirulina maxima* in Zarrouks Medium

Absorbance of *Spirulina maxima* in Zarrouks Medium

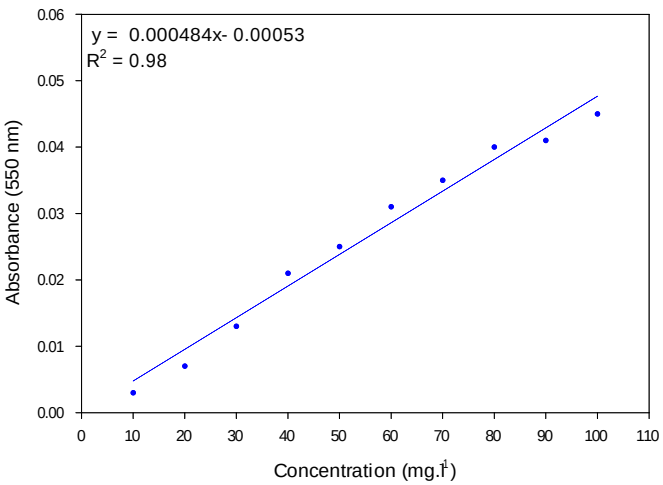
Concentration	Absorbance

Standard curve of *Spirulina maxima* in Synthetic Spirulina Medium.



10	0.003
20	0.007
30	0.013
40	0.021
50	0.025
60	0.031
70	0.035
80	0.04
90	0.041
100	0.045

Standard curve of *Spirulina maxima* in Zarrouks Medium.



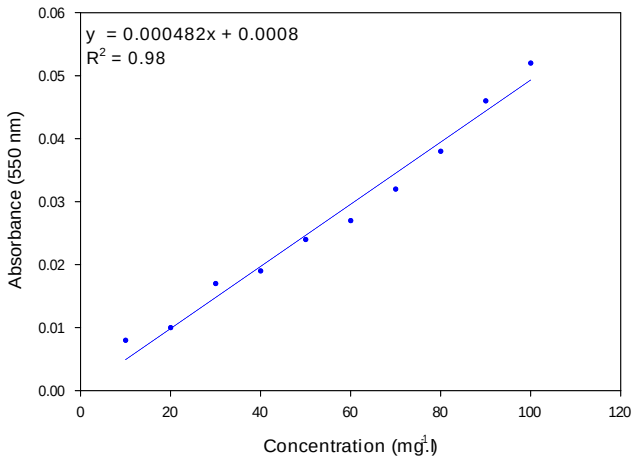
Appendix 8.8.3 Standard Curve of *Spirulina maxima* in Brine

Effluent

Absorbance of *Spirulina maxima* in Brine Effluent

Concentration	Absorbance
10	0.008
20	0.01
30	0.017
40	0.019
50	0.024
60	0.027
70	0.032
80	0.038
90	0.046
100	0.052

Standard curve of *Spirulina maxima* in Brine Effluent

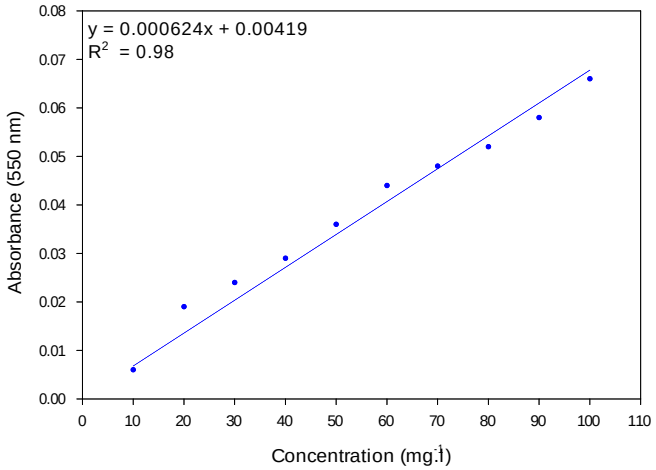


Appendix 8.8.4
Standard Curve of *Spirulina platensis* in Synthetic Spirulina Medium

Absorbance of *Spirulina platensis* in Synthetic Spirulina Medium

Concentration	Absorbance
10	0.006
20	0.019
30	0.024
40	0.029
50	0.036
60	0.044
70	0.048
80	0.052
90	0.058
100	0.066

Standard curve of *Spirulina platensis* in Synthetic Spirulina Medium.



Appendix 8.8.4
Standard Curve of *Spirulina platensis* in Zarrouks Medium

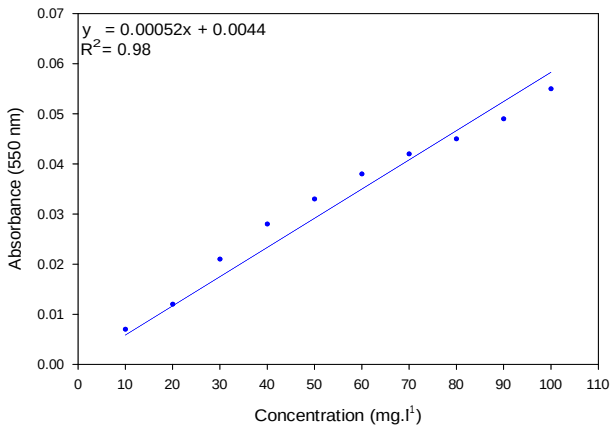
Absorbance of *Spirulina platensis* in Zarrouks Medium

Concentration	Absorbance
10	0.007
20	0.012

30	0.021
40	0.028
50	0.033
60	0.038
70	0.042

80	0.045
90	0.049
100	0.055

Standard curve of *Spirulina platensis* in Zarrouks Medium.



Appendix 8.8.4
Standard Curve of *Spirulina platensis* in Brine Effluent

Absorbance of *Spirulina platensis* in Brine Effluent

Concentration	Absorbance
10	0.01
20	0.023
30	0.028
40	0.037
50	0.041
60	0.046
70	0.054
80	0.063
90	0.069
100	0.075

Standard curve of *Spirulina platensis* in Brine Effluent