PROPAGATION OF ALGAE MIXOTROPHICALLY USING GLUCOSE AS SUBSTRATE FOR BIOMASS PRODUCTION

BY

CHUNG, DUSU ISAAC

U14/NAS/BTG/016

DEPARTMENT OF BIOTECHNOLOGY AND APPLIED BIOLOGY, FACULTY OF NATURAL AND APPLIED SCIENCES,

GODFREY OKOYE UNIVERSITY, UGWUOMU-NIKE,

ENUGU STATE.

JULY, 2018

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF BIOTECHNOLOGY AND APPLIED BIOLOGY, FACULTY OF NATURAL AND APPLIED SCIENCES,

GODFREY OKOYE UNIVERSITY, UGWUOMU-NIKE,

ENUGU STATE.

IN PARTIAL FULFILLMENT OF THEREQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.SC) HONOURS DEGREEIN BIOTECHNOLOGY

SUPERVISOR: PROFESSOR JAMES CHUKWUMA OGBONNA

JULY, 2018

APPROVAL PAGE

This project has been approved by the department of biotechnology and applied biology, faculty of natural and applied science, Godfrey Okoye University, Enugu State.

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CHUNG, DUSU ISAAC	DATE
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PROF. JAMES OGBONNA PROJECT SUPERVISOR	DATE
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DR. CHRISTIE ONYIA HEAD OF DEPARTMENT	DATE

DEDICATION

This work is dedicated to God Almighty for His sufficient grace, kindness and favour towards a successful completion of my project.

ACKNOWLEDGEMENT

My sincere gratitude is to God Almighty for the exceeding love He has shown me, wisdom granted to guide and directs me throughout my stay in the tertiary institution and for the successful completion of this project.

With joy in my heart, I appreciate the Vice Chancellor (Rev. Prof. Christian Anieke) of Godfrey Okoye University, Enugu for the opportunity given to me to study and for his words of wisdom and encouragement that have been a guide during this process of learning. I equally appreciate the Dean of Natural and Applied Science (Prof. Chidi Uhuegbu), the Head of Department Biotechnology and Applied Biology (Dr. Christie Onyia) and all the staff of the department for their care and unweaving commitment they showed toward me. My Supervisor (Prof. James Ogbonna) was more than helpful during the course of this research and I am ever grateful for the gift of having him supervise me. I also would like to acknowledge the contribution of Industrial Training coordinator of Godfrey Okoye university (Dr. Micheal Ozofor)), members of the Nigerian Fellowship of Evangelical Students ((NIFES), Uncle Obih Chosen, Uncle Okoro Micheal, Uncle Collins Best for their love and support.

My profound gratitude goes out to my departmental colleagues and friends: Miss. John Christabel, Mr David, Miss. Drisu Victoria, Miss. Nome Melody Ifunanya, thank you all for contributing towards the success of this project.

And my family, I couldn't have been able to succeed without you al. God bless every one of you for always been there for me. Am really grateful, thank God, we made it.

Abstract
Algae are large and diverse group of microorganisms that can carry out photosynthesis since they are able to capture energy from sun light. Algae may range in size from single cells as small as one micrometer to large seaweeds that grow to over 50 meters. Algae grow in the wide and are prompt to attack by predators and can easily be invaded which could result to competition that will eventually lead to low production of biomass which are important to organisms and the ecosystem. *Chlorella viriabilis* recently renamed C*hlorella viriabilis*NC64A that is a bona fide member of the true Chlorella genus, belonging to the Trebouxiophyceae was used in this present research. *Chlorellaviriabilis* was propagated in BG11 media enriched with 0.5g/L of glucose for mixotrophic growth and in autotrophic growth condition. The cell culture was monitored using the hemocytometer for increase in cells concentration. At the end of three weeks, the cells were harvested after centrifugation and dried in the oven. The mixotrophic dried biomass weighed 0.5g/L and that of the autotrophic weighed 0.1g/L. The results for protein analyses for both mixotrophic and autotrophic yielded 1.118g/L &0.07g/L respectively. Also, the results for the glucose was obtained using the Mercz protocol, the mixotrophic had higher glucose content than the autotrophic with 0.0564g/L & 0.0266g/L respectively. The cell concentration was more in the autotrophic than in mixotrophic but the mixotrophic cell culture had bigger cell size which showed the presence of accumulated materials. Glucose enhanced the production of algal biomass.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Smith (1955) defined algae based on characters of the sex-organs. He said-in algae the sex organs are usually unicellular and when they are multicellular as in most brown algae, all cells are fertile (Smith, 1995). There are approximately 1800 genera with 21,000 species which are highly diverse with respect to habitat, size, organization, physiology, biochemistry and reproduction (Pandey, 2009).

Algae may range in sizes from single cell as small as one micrometer to large seaweeds that may grow to over fifty meters (Vymagal, 1995). Algae are ubiquitous, they occur in almost every habitable environment on earth, soil, permanent ice, snow fields, hot and cold desert. Biochemically and physiologically, algae are similar in many aspects to other plants. Furthermore, algae are the major primary producers of organic compounds and play a central role as the base of the food chain in aquatic systems. Besides forming the basic food source for these food chains, they also produce oxygen necessary for the metabolism of the consumer organism (Lee,*et al.,* 1989).

Algal biomass is always made up of these three main components: Carbohydrates, Protein and Natural oils. The most important component for biodiesel production is the natural oils that can be converted to biodiesel. The percentage lipid composition varies and so the fatty acid

composition varies according to the algae strain within a range of 10 to 40% under natural conditions. The lipids present are mainly made up of polyunsaturated lipids (John,*et al*., 1998). The algae *Spirulina* has been considered for use as a supplementary protein (Raja,*et al.*, 2008),it is a blue green algae having strong antioxidant activity and provokes a free radical scavenging enzyme system.

In addition, the presence of algae leads to reduced erosion by regulating the water flow into soils. Similarly, they play a role in soil fertility, soil reclamation, and bio-controlling of agricultural pest, formation of microbiological crust, agricultural wastewater treatment and recycling of treated water. Human civilization depends on agriculture for its existence.

They are aquatic, both marine and fresh water, and occur on or within soil and on moist stones and woods as well as in association with fungi and certain animals. The algae are of great importance as primary producers of energy rich compounds which form the basis for this purpose, the planktonic algae are of special importance, since they serve as food for many animals. It is thought that 90% of the photosynthesis on earth is carried on by aquatic or by aquatic plants, the planktonic (suspended) algae are chiefly responsible this while photosynthesizing, they oxygenate their habitat, thus increasing the level of dissolved oxygen in their environment. Certain blue-green algae like some bacteria can use gaseous nitrogen from the atmosphere in building their protoplasm and in this way; they increase the nitrogenous compounds in water and soils of their habitat.

Light conditions affect directly the growing and photosynthesis of microalgae (duration and intensity). Microalgae needs a light/dark regime for productive photosynthesis, it needs light for a photochemical phase to produce Adenine triphosphate(ATP), Nicotinamide adenine

dinucleotide phosphate oxidase (NADPH) and also needs dark for biochemical phase to synthesize essential molecules for growth (Belcher, 1982).

1.2 RELEVANCE OF STUDY

The relevance of this project was to grow algae using glucose in a sterile bioreactor for better yield of protein which can be used for nutritional enrichment of cereals, pharmaceutical, animal feeds and other purposes.

1.3 STATEMENT OF PROBLEM

World Health Organization (WHO) report in 2012 states that malnutrition is the underlying contributory factor in over one third of all child deaths, making children more vulnerable to severe diseases. The increasing world deficiency of protein is becoming a main problem of humankind. Since the early fifties, intense efforts have been made to explore new, alternate and unconventional protein. Research has shown that the chance of infection with HIV virus might be reduced in individuals who have good nutrition status with micro nutrients (Egal & Valstar, 1999).

Algae in the oceans, rivers, and lakes of the world are thought to produce about half of all the oxygen produced on the planet. Given that the total biomass of the world's algae is but a tenth of the biomass of all the other plants, the efficiency of the algae is impressive and of interest in terms of producing biofuels. Cyanobacteria currently cultivated in large scale systems are economically viable sources of protein used in food because they often meet the requirements of nutrient in the diets. Moreover, through them you can get other human consumer products

(Kuhad *et al.,* 1997). A cyanobacterium as a source of single-cell protein has certain advantages over the use of other microorganisms because of its rapid growth and quantity and quality of protein (Molina *et al.,* 2002). Among the microalgae, the genus *Spirulina* contains about 60 to 70% of proteins, nucleic acids and amino acids recommended by the Food and Agriculture Organization (Pelizer,*et al.,* 2003). It also contains betacarotene and absorbable iron, and other minerals and high levels of vitamins, phenolic compounds, gammalinolenic acid and other essential fatty acids (Belay*et al.*, 1993: Von *et al.*, 2000).

The protein content of *Spirulina* varies between 50% and 70% of its dry weight. These levels are quite exceptional, even among microorganisms. Moreover, the best sources of vegetable protein achieve only half these levels; for example, soya flour contains "only" 35% crude protein. However, the protein content varies by 10-15% according to the time of harvesting inrelation to daylight. The highest values being obtained at early daylight (Association française pour l'a1gologie appliquée (AFAA) (1982).

1.4 AIM AND OBJECTIVES

The broad aim of this research was to grow algae mixotrophically using glucose as organic substrate with the following objectives:

- To culture and compare the algal growth using glucose
- To harvest and purify algal biomass.
- To evaluate the nutritional content of the algal biomass.

1.5 HYPOTHENSIS

- There is no significant difference in the amount of algal biomass cultivated in glucose and ethanol.
- There is a significant difference in the nutritional content of algae cultivated in glucose and ethanol.

1.6 SCOPE OF THE STUDY

The scope of this study was governed around *Chlorellaviriabilis*,a strain of *Chlorella* spp isolated from our local environment

CHAPTER TWO

LITERATURE REVIEW

2.1 ALGAE

Members of the kingdom plantae are not the only organisms that are photosynthetic. Photosynthetic organisms not in the plant kingdom, traditionally referred to as "algae" are typically aquatic and members of the traditionally organized eukaryotic kingdom protista (where all simple eukaryotic organisms go) or the Domain Bacteria.

Algae (or singular "alga") is historically a term applied to an unnatural assemblage of photosynthetic organisms that are usually morphologically simple than members of the plant kingdom (the plant kingdom including bryotophytes and seed plants). Some algae are unicellular, but others are larger and multicellular organisms that are called seaweeds. Within the algae there is clear trend in evolution, changing from unicellular to multicellular as reflected in the diversity of organismal forms i.e. unicellular >> filamentous (exhibiting ID multicellular growth) >>planar/ thalloid (exhibiting 2D multicellular growth) >> 3D forms that begin to resemble

plants in their complexity. This is important because it is this evolutionary trend that gave rise to the kingdom plantae from amongst the green algae in particular.

2.2 THE ORIGIN AND EVOLUTION OF GREEN ALGAL AND PLANT ACTIN

Actin is one of the most highly conserved and best studied eukaryotic proteins. Actin shares a common evolutionary history with actin related proteins (ARPS) (Clark*et al*., 1992) and in the more distant post with other ATPases such as hexokinase and the 70-KDa heat-shock protein common to all eukaryotes (Bork*et al*., 1992: Sheterline*et al*., 1995: Bhattacharya & Weber, 1997). Actin generally occurs in complex families in multicellular organisms (e.g. animals, land plants) and as single copies in many protists (e.g. ciliates, fungi, red algae, most green algae, diplomonads (Bhattacharya & Ehlting, 1995, Drouin*et al*., 1995).

The origin and phylogeny of actin genes and their role in the evolution of the angiosperms are therefore largely unresolved. In addition, virtually all existing studies on land plant actin gene origin and evolution (e.g. Hightower & Meagher, 1986: McDowell*et al*., 1996: Moniz & Drouin, 1996) have focused on analyses of angiosperms and have not included enough members of earlier-diverging lineage within the streptophyta (Sensu, 1995). The streptophyta contain the angiosperms and gymnosperms within a larger clade that includes the charophyte algae, bryophytes and ferns (Kenrick & Crane, 1997). Molecular phylogenetic studies show that the origin of viridiplantae can be interpreted as a set of evolutionary “steps” from a single-celled, scaly, biflagellate ancestor that gave rise on the one side to the green algae of the chlorophyta and on the other side to streptophyta (Melkonian & Surek, 1995: Graham, 1996: Huss & Kranz,

1997; Bhattacharya,*et al.,* 1998). Recent analyses of actin sequences identify the single-celled prasinophyte mesostigma viride as the earliest divergence within the streptophyta (Bhattacharya,*et al.,* 1998), rDNA sequence analyses are consistent with this result (Melkonian & Surek, 1995).

2.3 ALGAL PHYLOGENY AND THE ORIGIN OF LAND PLANTS

The green algae and plants form a monophyletic lineage (the chlorophytes) that contains both protists and higher taxa (Graham, 1996). An important issue regarding the evolution of this green lineage that still remains in question is the identity of the green algae (i.e. flagellate) ancestor of land plant. Modern molecular phylogenetic data provide the framework for reconstructing this evolutionary history and for asking deeper questions about the origin of the genetic inventions that have played a role in the radiation of the green lineage, a group that contains nearly all levels of vegetative morphology, from single cells to filaments to well organized colonies to complex terrestrial plants.

The green lineage is however, only one example of photosynthetic taxa that have successfully colonized our planet. A much greater diversity of plastid-containing organism is defined by the various other forms of algae. The algae include the green algal relatives of land plants and a diverse collection of single- celled and multicellular taxa such as the heterokonts, rhodophytes (red algae), cryptophytes, chlorarachniophytes, dinoflagellates and haptophytes. Understanding the interrelationship and origin of these lineages is an interesting problem in evolutionary biology, not only because the algae contain the dominant primary producers on this planet, but also because uncovering the ancestry of their plastids offers the possibility to gain insights into

the many factors of endosymbiosis, such as endosymbiont genome reduction and gene transfer to the host nucleus (Gilson & McFadden, 1996).

2.4 **SINGLE CELL PROTEIN (SCP)**

The global population is expected to increase by over a third (2.3 billion people) by 2050, requiring an estimated 70% increase in food production (Godfray *et al*., 2010). A combination of improved agricultural food production methods and an increase of average per capita income have led to a decrease in global hunger over the last half-century, despite a doubling of the world's population (Godfray *et al.,* 2010). However, worldwide food production is now facing a greater challenge than ever before. Previously utilized methods of intensifying agriculture will soon no longer be an option due to the high impact trade-offs they have on the environment, including fragmenting natural habitats and threatening biodiversity, production of greenhouse gases from land clearing, fertilizers and animal livestock production, and nutrient run-off from fertilizer damaging marine, freshwater and terrestrial ecosystems (Tilman*et al.,* 2011)

The increasing world deficiency of protein is becoming a main problem of humankind. Microbial biomass has been considered an alternative to conventional sources of food or feed. Large-scale processes for single cell protein production show interesting features including:

- The wide variety of methodologies, raw material and microorganisms that can be used for this purpose.
- High efficiency in substrate conversion.
- High productivity, derived from the fast growth of microorganisms.

- Independence of seasonal factors (Roth, 1980: Parajo*et al.*, 1995)

Table 1: Average Different Composition of the Main Groups of Microorganisms (% Dry Weight) source: Miller & Listsky (1976).

Composition	Fungi	Algae	Yeast	Bacteria
Protein	30-45	40-60	45-55	50-65
Fat	2-8	7-20	2-6	1-3
Ash	9-14	8-10	5-10	3-7
Nucleic acid	7-10	3-8	6-12	8-12

2.5 NEW PHYLOGENETIC CLASSFICATION

The classification of algae is complex and somewhat controversial, especially concerning the blue-green (cyanobacteria) which are sometimes known as blue-green bacteria or (cyanobacteria) cynophyta and sometimes include in the chlorophyta**.** Veriag (1993, 1997) in his book "algae" and, Hoek*et al.*, (1995) in their book "algae: An introduction of phycology" compiled the different phylogenetic classification of algae considering observations on ultra-structural studies and molecular genetics. The broad outline of classification of algae is as follows

1 Kingdom – Eubacteria

Division 1 – Cynophyta (cyanobacteria)

Division 2 –Prochylorophyta (chloroxybacteria)

2 Kingdom – Eukaryota

Division 1 – Glaucophyta

Class 1 – Glaucophyceae

Division 2 – Rhodophyta

Class 1 – Bangiophyceae

Class 2 – Florideophyceae

Division 3 – Heterokontophyta

Class 1 – Chrysophyceae

Class 2 – Parmophyceae

Class 3 – Saracinochrysidophyceae

Class 4 – Xanthophyceae

Class 5 – Eustigmatophyceae

Class 6 – Bacillariophyceae

Class 7 – Raphidophyceae

Class 8 – Dictyochophyceae

Class 9 – Phaeophyceae

Division 4- Haptophyta

Class 1 – Haptophyceae

Division 5 – Cryptophyta

Class 1 – Cryptophyceae

Division 6 – Dinophyta

Class 1 – Dinophycea e

Division 7 – Euglenophyta

Class 1 – Euglenophyceae

Division 8 – Chlorarachniophyta

Class 1 – Chlorarachniophyceae

Division 9 – Chlorophyta

Class 1 –Prasinophyceae

Class 2 – Chlorophyceae

Class 3 – Ulvophyceae

Class 4 – Cladophorophyceae

Class 5 – Bryopsidophyceae

Class 6 – Zygnematophyceae

Class 7 – Trentipohiliophyceae

Class 8 – klebsormidiophyceae

Class 9 –Charophyceae

2.6 LIFE CYCLE PATTERNS FOUND IN THE ALGAE

Many life cycle patterns are found in algae. However, there is no regular and fixed alternation of generations as found in higher plants. In blue-green algae and certain Chlorophyceae (e.g. *Protococus, Scenedesmus,* etc) which reproduce asexually, there is no alternate of generations. The following are the life-cycle patterns found in algae.

2.6.1 HAPLONTIC

This is the simplest and most primitive type of life-cycle. The other patterns of life cycle have originated from this type. It is found in all chlorophyceae except a few. Sometimes, this is called Ulothrix or chlamydomonad. The somatic phase (plant) is haploid (gametophyte) while the diploid phase (sporophyte) is represented by zygote. During germination, the zygote (2n) divides meiotically producing haploid (n) zoospores, which develops into individual plants. The unicellular (e.g. *Chlamydomonas*) or filamentous (e.g. *Ulothrix, Spirogyra, Oedogonium, Chara,* etc) gametophyte (n) alternates with a one celled zygote or sporophyte (2n).

2.6.2 DIPLONTIC

This pattern is reverse of Haplontic. In this case, somatic phase (plant) is diploid (sporophyte 2n) while the haploid phase (gametophyte n) is restricted to gamates which are produced by meiotic division. After gametic union, a diploid zygote is formed, which develops into a diploid (sporophyte 2n) plant by mitotic division. Example includes *Codium, Bryopsis,* and *Saryassum*.

2.6.3 ISOMORPHIC

In this type, there are two exactly similar (morphologically identical) somatic phase. The somatic phase is diploid (gametophyte n). The zygote develops into a diploid multicellular plant (sporophyte by postponement of meiosis. Prior to zoospore (meiospore) formation there is meiosis. These zoospores (n) develop into haploid plants (gametophyte n). The haploid plant produce gametes (n) which after fusion develop into zygotes (2n)

2.6.4 HETEROMORPHIC

This pattern of life cycle is exactly like that of preceding one (isomorphic) only with the difference that the alternating haploid (n) and diploid (2n) somatic phase (plant) are morphologically different. In such cases, the diploid multicellular sporophytic plant produces haploid zoospores (meiospores) by meiosis. These zoospores develop into gametophytes. Each gametophytic plant (n) produces gametes which after their union form a zygote and the water develops into a diploid sporophytic plant by mitotic divisions.

2.6.5 HAPLOBIONTIC

In this pattern, there are three phases in the life cycle. Out of three, two phases are haploid (n) and one diploid (2n). The examples are found among Nemailionates (e.g., *Batrachospermum*) of rhodophyceae and coleochaete of chlorophyceae.

2.6.6 DIPLOBIONTIC

This type of life cycle is found in almost all rhodophyceae except Nemailionates. The most common example is polysiophonic of order ceramiales. Here, the life-cycle is triphasic and involves an alternation of two diploid (2n) or sporophyte and tetrasporophyte with one haploid (n) or gametophytic. Thus, there are two diploid phases and one haploid phase. The gametophyte produces gametes which unite and form a zygote (2n). Now the zygote divides mitotically

forming a corposhoropyte (2n) bearing diploid (2n) corpospres. On germination, these diploid corpospores form another diploid plant, the tetrasporophyte.

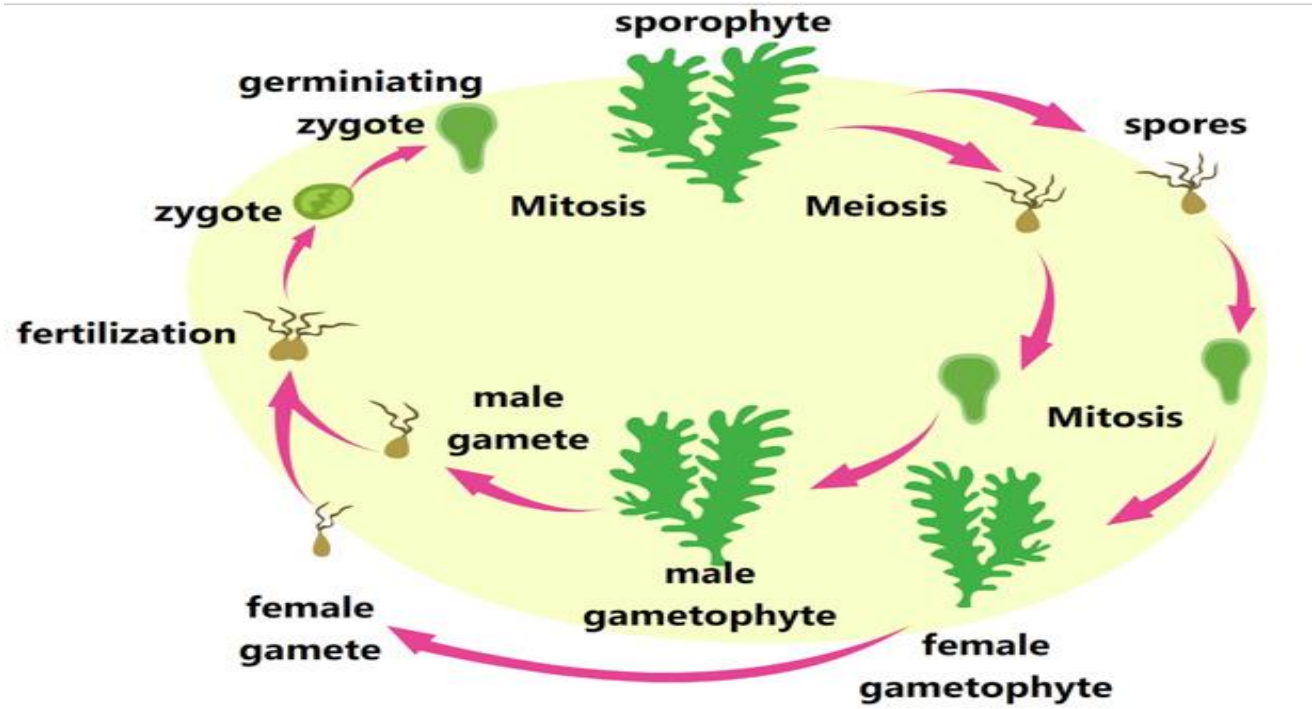


Figure 1: A Diagram Showing the Life Cycle of Algae

2.7 HABIT AND HABITAT

Most parts of the land are covered either by fresh water or sea water. Besides, several other algae are found in somewhat drier conditions. They are found on the trunk of trees, on telephone wires on rocks, on walls, in hot springs and in several other habitats. Here some of the algae have been classified according to their habitats. Special emphasis has been given on the occurrence of fresh water algae.

2.7.1 HYDROPHYTES

These are more or less completely submerged or free floating on the surface of the water. The hydrophytes may be subdivided into the following

I. BENTHOPHYTES: Several fresh water and marine algae are found in attached condition. The fresh water such as chara, nitella, cladophora, gongrosira, and chaemosiphon are found attached to some substratum in the bottom of the water. Almost all brown algae (phaeophyceae) are found in attached condition to some substrata in the sea.

II. EPACTIPHYTES: Such algae grow along the shores of lakes and ponds and may be delimited from benthophytes with some difficulty. The most important fresh water forms are *Oedogonium*, *Chaetophora*, some species of *Spirogyra*, *Maugeodia*, some diatoms, *Scytonema* and *Rivalaria*.

III. THERMOPHYTES: These are microorganisms that grow at very high temperatures, as 90^{o}c. Their optimum temperature is usually above 60oc. They have thermostable ribosomes, membranes and various enzymes. At low temperatures, they lose membrane fluidity and thus are unable to grow (Ogbonna, 2013).

IV. PLANKTOPHYTES: The algae which float on the surface of the water are "planktophytes". They may be of two types

Euplanktophytes: They are never attached and from the very beginning are free floating e.g. diatoms, *Cosmorium*, *Closterium*, *Microcystis*, *Sphaeroptea*, *Scenedesmus*, *Pediastrum*, *Chlamydomonas*, *Volvox*, other volvocales and some members of chroococcales. The above given forms are fresh water in habitat.

Tychoplanktophytes: In the beginning, such algae are attached but later on they become detached and free floating e.g. some species of *Spirogyra, Zygnema, Cladophora, Oedogonium, Rhizoclonium, Mougeotia, Tribonema, Microspora, Cyclindropermum, Tetraspora, Rivuloria, Nostoc, Gloeotrichia, Sargassum etc*

V. HALOPHYTES: The algae occur in saline waters and are known as ‘halophytes”. Themost striking examples are *Dunalielia* and *Chlamydomonas* which occur in salt lakes, the species of *Scenedesmus, Aphanocepsa, Pediastrum, Aphanothece, Oscillatoria* are found in saline water.

VI. EPIPHYTES: Many algae are found upon other plants and bigger species of algae. Aphanochaete, bulbochaete, *Oedogonium* and *Microspora* are found as epiphytes upon larger species of *Oedogonium, Cladophora, Rhizoclonium, Voucheria* and *Hydrodictyan* species

VII. EPIZOOPHYTES: Certain algae are found living on aquatic animals such as turtles, mollusk shells, and fishes.Species of *Cladophiora* grow upon mollusk shells. *Protoderma* and *Basicladia* occur on the back of turtles. *Choraciopsis* and *Characium* occur on the posterior and anterior legs of *Branchipus* respectively.

2.7.2 EDAPHOPHYTES

Algae in this category are called terrestrial algae. They are found upon or inside the surface of the earth. They can be:

I. SAPHOPHYTES. They are surface algae. Most of the species of myxophyceae are found upon the surface of the soil.

II. CRYPTOPHYTES. Such algae are subterranean in habit and occur inside the soil. The species of myxophyceae are found in the soil. The species of *Nostoc*, *Anabaena* and *Euglena* have been reported from paddy fields where they also fix the atmospheric nitrogen in the soil to enrich the fertility of the soil.

2.7.3 AEROPHYTES

Such algae are aerial in habitat. They are found on the truck of trees, walls, fencing wire, rocks and animals and so many others.

I. EPIPHYLLOPHYTES. Such algae are epiphytic upon leaves of trees. Species of trentepollia are commonly found upon the bark of trees.

II. EPITHLOEPHYTES. These algae grow on the bark of trees mixed with many mosses and liverworts

III. EPIZOOPHYTES: These algae are found even on the bodies of land animals. Certain chaetophorates are found even on the hairs of sloth

IV. LITHOPHYTES: Many algae grow on the rocks and walls. The species of scytonema grow on the walls in rainy season and the whole wall becomes black spotted.

2.7.4 CRYOPHYTES

These algae are found on ice and snow. These algal forms cause red snow, green snow, yellow snow, yellowish green snow and violet snow.

2.7.5 SYMBIONTS OR ENDOPHYTES

Many algae grow in symbiotic association with other plants. The most striking example of symbiosis is lichens, here the algae are found in symbiotic association of fungi

2.7.6 ENDOZOOPHYTES

Certain algae occur inside the body of animals. *Zooxanthella* is found inside fresh water sponges

2.7.7 PARASITES

Certain algae are parasites upon other plants. Example is *Cephaleoros virescens* which causes the havoc of tea. Foliage in Assam and neighboring areas called red rust of tea.

2.7.8 FLUVIATILE ALGAE

Such algae are found in rapidly flowing waters; *Ulothrix* occurs in mountains falls. *Stigeocloniu*mand *Batrachospermum* are reported from the swift running of Dehradum and other hilly tracts.

2.8 FRESHWATER ALGAE

Freshwater algae include a wide range of organisms that float in the water or grow on submerged surfaces and have the ability to photosynthesize (using sunlight energy), CO_2 and water to manufacture organic matter and O_2.

Freshwater algae are made up of:

- The green and red algae (plant kingdom) multicellular
- The bacteria blue (blue- green algae)
- Protozoa (single- celled swimming groups)
- Chromista e.g. diatom.

2.8.1 GREEN ALGAE

Green algae often look like strands of green hair flowing in the current. *Spirogyra* is common green algae. Under a microscope its chloroplast are clearly seen as spirals.

2.8.2 RED ALGAE

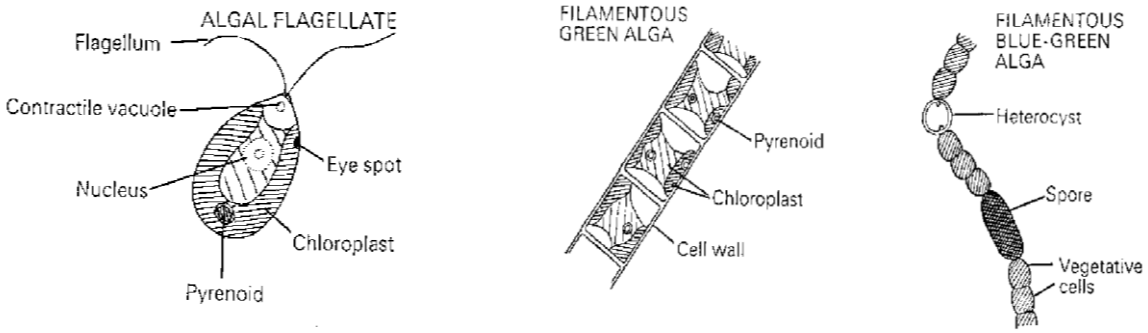
Red algae , such as *Audouinella*, uses a different part of the light spectrum it is able to grow in places where the other algae cannot, so tends to be found in shaded places such as under rocks or banks.

2.8.3 BLUE-GREEN ALGAE

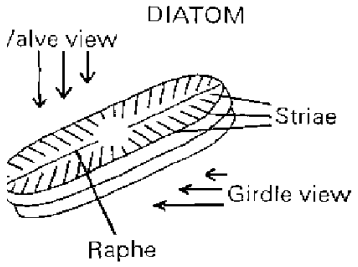
The chlorophyll in the algae cyanobacteria is not in chloroplasts but diffused throughout the cell. Pigments other than chlorophyll contribute to their colouration so cyanobacteria are not usually bright green. *Nostoc* is another cyanobacteria that is often conspicuous in stream. It looks like bubbles of firm jelly attached to the rocks. The bubbles are masses of small chains of cells.

2.8.4 DIATOMS

Mats of brown growth, fluffy masses or slimy layers on rocks are some of the ways diatoms appear to the naked eye. Gomphoneis forms thick, glistening, light-brownish mats on river substances and is often mistaken for didymo. However, didymo has much larger cells and grows in tougher, more fibrous mats (Dee Bewers *et al.,* 2018)



2a) Chlamadomonas 2b) Spirogyra



2c) Diatom

Figure 2: Diagrammatic Structure of Some Algae revealing some vital parts. (2a) Chlamadomonas (2b) Spirogyra (2c) Diatom

2.9 FACTORS REQUIRED FOR CULTIVATION OF ALGAE

2.9.1 WATER

Being photosynthetic organisms, algae have comparatively simple requirements for growth. Water, containing the accurate amounts of salts and minerals are particularly essential components needed for algal cultivation. Based on the need for water, algae are basically categorized into aquatic or semi aquatic species. The standard quantity of water required for effective farming of aquatic algae is approximately 1.5 L/ha (Misbah*et al.*, 2014). This figure is valid considering the fact that growth occurs in an open pond and roughly 7–11 million liters of water is evaporated from that region annually. Algal production can be linked to the remediation of wastewater from both domestic and industrial sources. The wastewater, containing the essential elements, can be directly supplied to the algal culture. This allows nourishment of algae while simultaneously treating wastewater.

2.9.2 CARBON

Algae require very high amount of carbon for efficient growth. Procuring carbon for algal growth costs up to 60 % of the total nutrients budget. Carbon can be obtained from multiple sources, which include (1) CO_2 from the atmosphere (2) CO_2 contained within industrial smoke (3) CO_2 from soluble carbonates. For each kilogram of algae that is grown, approximately 1.65 kg of CO_2 is used.

Although heterotrophic and mixotrophic microalgae can use a wide range of carbon and nitrogen sources, from a commercial perspective, the most economic organic substrates for heterotrophic cultivation are glucose, glycerol, and acetate (Perez-Garcia *et al.*, 2011; Barclay*et al.*, 2013).

However many carbon sources, such as arabinose, citrate, fructose, malate, lactic acid, lactose, peptone, urea, fulvic acids, ethanol, methanol, and sucrose, have been tested for heterotrophic cultivation of microalgae. *Chlorella vulgaris* cultures on the above-mentioned carbon sources reached significantly lower biomass concentration in comparison to cultures on acetate or glucose (Perez-Garcia *et al*., 2011).

2.9.3 LIGHT

The key ingredient to initiate photosynthesis is light as it is involved in the conversion of carbon dioxide to carbohydrates. As compared to higher plants, algae require relatively low intensity of light for proper development. Solar waves are the primary source of light. The light source in the cultivation system can be either natural, artificial, or combination of different light sources. The cheapest source is the solar energy, which is utilized in open pond systems, which require a large area for construction and have a higher contamination risk. In closed systems, fiber optics and solar concentrators can be used to maximize the effect of sunlight. Khoeyi*et al*,(2011) used three algae samples placed in different light conditions (photoperiod, intensity) and reported that there was a huge difference in biomass concentrations between them. The maximum biomass was recorded with about 62.5umol photons $m^{-1}s^{-1}$ for a 16:8 hrs light/dark photoperiod duration, while the maximum percentage of total saturated fatty acids (SFA) was 33.38% at 100umol photons $m^{-1}s^{-1}$ for 16:8 hrs light/dark photoperiod duration

2.9.4 NITROGEN

Being the main constructing element of proteins and nucleic acids, nitrogen plays a significant role in algal metabolism.

2.9.5 PHOSPHOROUS

This element is used in the form of phosphates because if it is present in any other state, it may become unavailable to the algae due to its ability to combine with other metallic ions, which results in precipitation.

2.9.6 ADDITIONAL NUTRIENTS

Apart from the above-mentioned nutrients, trace amount of vitamins and metals like sodium, calcium, magnesium, manganese, zinc, copper, iron, and molybdenum are also required for efficient growth of algal culture.

2.9.7 SPACE

Unlike other organisms, algae are very versatile and do not require arable land for productive growth. They can be cultivated in ponds, water bodies, and even reactors. Issue of appropriate space is not a concern and does not put a strain on the budget or available resources.LED lights are shown to be more economical and stable than fluorescent lamps.

2.10 METHODS FOR CULTIVATION OF ALGAE

Some of the techniques used for biomass cultivation are:

2.10.1 PHOTOAUTOTROPHIC PRODUCTION

This form of cultivation takes place when algae utilize an energy source (light) and a carbon source (inorganic carbon) to form carbohydrates through a process termed as photosynthesis. This is the most general method used for cultivating algae and results in the formation of algal cells with lipid content ranging from 5 to 68 % depending on the algal specie being cultivated. If algae are cultivated for oil production, then the prime advantage of using this cultivation technique is to utilize carbon dioxide to meet the carbon requirement.

2.10.2 HETEROTROPHIC PRODUCTION

In this method, the algal specie is grown on a carbon substrate like glucose thus eliminating the need for light energy. This process can be performed in a reactor with a small surface to volume ratio. A much higher degree of growth control is achieved and harvesting budget is lowered due to production of high-density cells. The set-up cost is negligible but more energy is used as compared to the process utilizing light energy because photosynthetic processes are utilized to form the carbon source on which the algae are grown. Studies have shown that heterotrophic method of biomass production has a higher yield and cells have higher lipid content (55 % as compared to 15% in autotrophic cell (Misbah *et al.*, 2014)

2.10.3 MIXOTROPHIC PRODUCTION

Some algae have the capability to obtain nutrition by both autotrophic and heterotrophic methods. This means light energy is not a primary need for mixotrophs as cell growth can occur by digesting organic material. These cultures are shown to lessen photo inhibition with enhanced growth rates as compared to autotrophic and heterotrophic cultures. This is because cultivation of mixotrophs utilizes both photosynthetic and heterotrophic elements, which reduces loss of biomass and reduces the quantity of organic substrate consumed.

Glucose is the most commonly used carbon source for heterotrophic cultivation of microalgae, as is the case for many other microbial species. Far higher rates of growth and respiration are obtained with glucose than with any other substrate, such as other simple sugars, sugar alcohols, sugar phosphates, organic acids, and monohydric alcohols (Griffiths *et al., 1960*). This happens because glucose possesses more energy content per mol, compared with other substrates. For example, glucose produces 2.8 kJ mol −1 of energy compared to 0.8 kJ/mol for acetate (Boyle & Morgan, 2009).

2.10.4 PHOTOHETEROTROPHIC CULTIVATION

Unlike mixotrophs, photo-organotrophs cannot grow without light energy. Although this process can enhance the production of certain useful light-regulated metabolites, this mode of cultivation is not preferred in case of procedures like biodiesel production.

Table 2: Growth Mode of Algae (Microalgae) Cultivation. Source: Wang *et al*., 2014

Growth mode	Energy source	Carbon source	Light availability requirement	Metabolism variability
Photo-autotrophic	Light	Inorganic	Obligatory	No switch between sources
Heterotrophic	Organic	Organic	No requirements	Switch between sources
Photoheterotrophic	Light	Organic	Obligatory	Switch between sources
Mixotrophic	Light/organic	Inorganic/organic	No obligatory	Simultaneous utilization

2.10.5 COMPARISON OF DIFFERENT CULTIVATION TECHNIQUES

In case of heterotrophic cultivation, the culture has a high chance of getting contaminated especially in open pond cultivations. Apart from this, carbon source is also purchased at a high cost. Photoautotrophic system of algae cultivation is the most frequently used method for biomass growth. It is easy to scale up and can easily take carbon dioxide from the surface air.

2.11 HARVESTING OF ALGAL BIOMASS

The main step after the bulk cultivation of algae is its harvesting, which performs a very vital part in shaping the process budget of algal biofuel. Despite the excessive presence of algal biomass, the harvesting of macro-algal biomass is considered as simpler and less costly as compared to the harvesting of algal biomass. Due to the diluted nature of algal culture cells and small size, the operating expenses of dewatering and harvesting of algal biomass is high. The typical size of single-celled eukaryotic algae is measured around 3–30μm (Grima *et al*., 2003), and the range of cyanobacteria is 0.2–3μm (Chorus & Bartram, 1999). The improvement and wide-scale application of different technologies for energy generation is currently a great challenge and of a significance to the scientists and the machinists of active systems. It is generally believed that numerous roots, properties, and active transformation of biomass are the mainbases of renewable energy (McKendry, 2002: Goyal *et al*., 2008). A number of procedures including chemical as well as mechanical can be performed, which includes centrifugation,

flotation, flocculation, filtration, screening and gravity sedimentation, and electrophoresis for harvesting of algal biomass (Uduman *et al.,* 2010). There are critical parameters to consider for the selection process of algae for harvesting. Such parameters include density, size, and value of the desired products. Two-step processes are usually used for the harvesting of algae: 1. Bulk harvesting: This step is performed for the separation of algal biomass from the bulk suspension. The techniques that can be used to complete this process are flocculation, flotation, or gravity sedimentation. 2. Thickening: the second step required for harvesting of algae is thickening which is performed to thicken the slurry by filtration or centrifugation (Brennan & Owende, 2010). The most important and the most operative method used for the separation of algal biomass is by centrifugation technique in algae harvesting, but it is only done on high-valued products due to high operational and functional cost (Grima *et al.*, 2003).

2.11.1 FLOCCULATION

This is the process in which circulated algae cells are combined together to form bulky biomass collection for settling. The precipitate of carbonates with algal cells at high pH, due to CO2 ingestion by the algae, results in auto-flocculation (Sukenik & Shelef, 1984).

2.11.2 CHEMICAL COAGULATION

Chemical coagulation is performed by making the mixture of chemicals for initiation of flocculation in the fusion of algae. The mixture of chemicals includes inorganic flocculants and organic flocculants or poly-electrolyte flocculants. The activity of two predictable chemical coagulants ($FeCl_3$ and Fe_2 $(SO_4)_3$) and five commercial polymeric flocculants (Drewfloc 447, Flocudex CS/5000, Flocusol CM/78, Chemifloc CV/300, and Chitosan) was matched by de Godos *et al*. (2011) to check their capability to eliminate bacterial biomass in algae from the discharge of a photosynthetically oxygenated piggery wastewater biodegradation process. Ferric

salts achieved the uppermost biomass elimination (66–98 %) at the absorption of 150–250 mg/L. Polymer flocculants were considered sufficient for the similar elimination efficacies and eliminated the bacterial biomass at lower concentration (25–50 mg/L), though the efficiency reduced at upper polymer flocculants amount.

2.11.3 COMBINED FLOCCULATION

Combined flocculation is a multi-step process, which consists of the use of more than one type of flocculants and electro-flocculation or electrocoagulation (Chen *et al.,* 2011). For harvesting of marine and fresh water algae, Vandamme *et al.,* (2011) has examined the method of electrocoagulation–flocculation. Continuous- flow electrocoagulation has also been examined by Azarian *et al.,* (2007) for the separation of algae from industrial wastewater. Throughout electrocoagulation, no sulphate chlorine anions are produced. It is also noted that unlike centrifugation process, power consumption is less in electrocoagulation–flocculation (Vandamme *et al.,* 2011). These are the advantageous reasons, due to which electrocoagulation–flocculation is considered as a convenient procedure, which can be used for harvesting of algae. There are certain shortcomings such as inconsistency in speciation of metal hydroxides as well as disturbances by pH, chemical configuration and conductivity of water required to be considered and addressed.

2.11.4 GRAVITY SEDIMENTATION

Gravity sedimentation is a simple process used for the separation of algae in water and wastewater treatment, which is often supported by flocculation to upsurge the effectiveness of gravity sedimentation (Chen *et al.,* 2011). Another model of gravity sedimentation procedure is flotation, which is considered more effectual and advantageous as compared to sedimentation and can capture the bits with thickness of less than 500μm (Yoon & Luttrell, 1989). The

operative and efficient methods for harvesting of algal biomass include centrifugation and chemical precipitation (Chen *et al.,* 2011). These procedures are not economically practicable for harvesting of algae due to high procedure charge of centrifugation or chemical flocculants for the production of biogas. Filtration also appears to have great prospective for condensing algal biomass from bulk culture, integration of different techniques such as flocculation, gravity sedimentation, or flotation can also be done. For biogas production, concentrated slurry is considered as a good substrate for anaerobic digestion (Prajapati *et al.,* 2012, 2013). The consumption of wet algal biomass reduces the water necessity, which is required in excessive amount for the digestion of conventional biomass, for biogas production.

2.12 PROTEIN EXTRACTION METHOD

2.12.1 CONVENTIONAL PROTEIN EXTRACTION METHODS

Seaweed and microalgae have poor protein digestibility in their raw, unprocessed form and it isfor this reason that great emphasis has been placed on developing improved methods for algalprotein extraction in order to improve their bioavailability. Algal proteins and their extractionis a relatively poorly studied topic compared to proteins from other crops (Barbarino, *et al*., 2005). Algal proteinsare conventionally extracted by means of aqueous, acidic, and alkaline methods, followed by several rounds of centrifugation and recovery using techniques such as ultrafiltration, precipitation, or chromatography (Kadam,*et al.,* 2016). Chemical extraction methods, such as two-phase acid and alkali treatments, have been especially efficient for extracting proteins from A. *nodosum*, *Ulva* spp. and L. *digitatal*

However, the successful extraction of algal proteins can be greatly influenced by the availability

of the protein molecules, which can be substantially hindered by high viscosity and anionic cell-wall polysaccharides, such as alginates in brown seaweed and carrageenan in red seaweed (Fleurence*et a*l., 1999).Cell disruption methods and the inclusion of selected chemical reagents are therefore used in orderto improve the efficiency of algal protein extraction. Some examples of conventional methodsthat are commonly utilised include mechanical grinding, osmotic shock, ultrasonic treatment, andpolysaccharidases-aided hydrolysis.

2.12.2 PHYSICAL PROCESSES

Barbarino and Lourenço (2005) reported that physical grinding with the use of a Potterhomogeniser significantly increased protein extraction yield from *Porphyra acanthophora* var.Alternatively, osmotic stress has also been reported to improve extraction of algal proteins Efficiency (Wong*et al.,* 2001; Marrion*et al.,* 2003). Osmotic shock was reported to yield a significantly higher concentration of watersoluble proteins from P. *palmata* (1.02 - 0.07 g/100 g) compared to high shear force with anUltra-turrax® T25 Basic tool (IKA®, Staufen, Germany) (0.74 _ 0.02 g/100 g) (Harnedy, *et al*, 2013). However, there wasno significant difference in the amount of total protein extracted between the two methods (6.77 versus6.92 g/100 g). Alternatively, the use of polysaccharidases was reported to be a more promising methodof protein extraction, with a concentration of 11.57 -0.08 g/100 g P. palmata, equating to a yield of 67%.

2.12.3 ENZYMATIC HYDROLYSIS

Seaweed is rich in several types of polysaccharides, including cellulose, galactans, xylans,fucoidan, laminarin, alginates, carrageenans, and floridean starch (Holdt*et al.,* 2011). These polysaccharides canreduce the availability of algal proteins and decrease protein extraction efficiency (Barbarino,*et al.,* 2005). Enzymessuch as polysaccharidases can therefore

be applied as a cell disruption treatment prior to protein extraction in order to increase protein yield.

2.12.4 CURRENT PROTEIN EXTRACTION METHODS

Protein extraction methods used on algae to date are limited for commercial use due to concerns with up-scaling. Conventional mechanical and enzymatic methods for protein extraction may alsoaffect the integrity of extracted algal proteins due to the release of proteases from cytosolic vacuoles (Ganeva, et *al*., 2003).Furthermore, these methods are also laborious and time consuming (Kadam, et al., 2016). Improved extraction methods of cell disruption and extraction are therefore required. Pre-treatment with cell-disruption technique said the breakdown of the tough algal cell wall, increasing the availability of proteins and other high-value components for later protein extraction. Some examples of novel protein extraction methods include ultrasound-assisted extraction, pulsed electric field, and microwave-assisted extraction (Kadam *et al*., 2016)

2.12.5 ULTRASOUND-ASSISTED EXTRACTION

Ultrasound-assisted extraction (UAE) can be applied to food sources for a number of applications, Including modification of plant micronutrients to improve bioavailability, simultaneous extraction and encapsulation, quenching radical sonochemistry to avoid degradation of bioactives, and increasingbioactivity of phenolics and carotenoids by targeted hydroxylation (Vilkhu*et al.*, 2008). The degradative effect of radical sonochemistry, which is the most relevant aspect in terms of improving bioavailability of algal proteins, is not produced by the ultrasound waves, but rather by the formation, growth, and implosion of bubbles formed by what is known as acoustic cavitation (Ashokkuma, *et al.*, 2008).

2.12.6 PULSED ELECTRIC FIELD

Pulsed electric field (PEF) has been used as a cell disruption technique in microalgae, although its primary use has thus far been for the extraction of lipids for conversion to biofuel (Ashokkumar*et al*., 2008). PEF involves applying high electric currents in order to perforate a cell wall or cell membrane, causing reversible or irreversible electroporation. Electroporation enables the introduction of various foreign components to cells, including DNA, proteins, and drugs (Fox, *et al*., 2006) PEF is a fast and green technology for inactivating microorganisms by irreversible electroporation and aiding the release of intracellular contents of plant cells.

2.13 IMPORTANCE OF ALGAE

Man's uses of algae, particularly marine algae, are far more diverse and economically important than generally realized (Abbott & Cheney, 1982). They are used as human food, in agriculture (fertilizer, manure, fodder and aquaculture), medicine, textile, paper and paint industries, chemical extracts from larger marine algae (example alginic acid, carrageenan or agar) are used in the food industry, and diatomaceous earth (deposits of diatom frustules) is widely used as filtration and polishing materials (Abbott& Cheney1982).

CHAPTER THREE

MATERIALS AND METTHOD

3.1 **CHLORELLA VIRIABILIS**

Algae originally included in the genus chlorella are among the most widely distributed and frequently encountered algae in freshwaters (Fott & Novakova, 1969). *Chlorella viriabilis* recently renamed *chlorella viriabilis NC64A* (Ryo *et al.,* 2010), that is a bona fide member of the true chlorella genus, belonging to the Trebouxiophyceae. The true chlorella species, including NC64A, are characterized by glucosamine as a major component of their rigid cell walls (Takeda, 1991; Chuchird *et al*., 2001).

3.2 **SAMPLE COLLECTION**

The strain, *Chorella viriabilis* was collected from the microbiology laboratory, University of Nigeria Nsukka (UNN). It was isolated from the soil using BG11 media, a selective media that is suitable for the growth of green algae. About 10×10^6 per mile was collected with a sterile 1000um micropipette and transferred into 200 ml fresh sterile media of BG11.

PROTOCOL FOR BG11 (BLUE-GREEN MEDIUM) STOCK PREPARATIO

Stocks per 500ml

1. $NaNO_3$……..75.0g
2. K_2HPO_4…….2.0g
3. $MgSO_4.7H_2O$……....3.75g
4. $CaCl_2.2H_2O$…….....1.80g
5. Citric acid……....0.30g
6. Ammonium ferric citrate green……...0.30g

7. $EDTANa_2$……...0.05g
8. Na_2CO_3…….....1.00g
9. Trace metal solution: per liter

   H_3BO_3…….2.86g

   $MnCl_2.4H_2O$……...1.81g

   $ZnSO_4.7H_2O$……...0.22g

   $Na_2MoO_4.2H_2O$……..…0.39g

   $CuSO_4.5H_2O$……...0.08g

   $CO(NO_3)_2.6H_2O$……..0.05g

Medium per liter

Stock solution 1-8……....10.0 ml each

Stock solution 9…….. 1.0 ml

Make up to 1 litre with deionized water. Adjust PH to 7.1 with 1M NaOH or HCl. For agar add 15.0g per litre of Bacteriological Agar. Autoclave at 15 psi for15 minutes (Stanier*et al.,* 1971).

3.3 CULTIVATION OF *CHORELLA VIRIABILIS*

MATERIALS/EQUIPMENTS

Conical flasks 250 ml; Bunsen burner; Ethanol 5 ml; Glucose 5g; Distilled water; Light; Autoclave; Micro pipette 1000 ul; Light microscope; Heamocytometer; BG11 (nutrient media); Micro pipette tips; Foil paper; Analytical weighing balance; Pure *Chlorella viriabili*s culture; Laminar flow hood.

PROCEDURE

The algal cells were counted using a neubauer heamocytometer to know the initial cell concentration. The pre-culture (starter) was grown in 200 ml of the sterilized medium. About 5.120×10^5 per ml of the innoculum was transferred into a BG11 medium containing glucose using a 100ul micropipette and allowed to grow for two weeks mixotrophically. An innoculum of about 4.096×10^5 per ml was also inoculated in the medium and allowed to grow autotrophically. The cell concentrations were measured at an interval of 48 hours. The inoculation was done in the laminar flow hood after wiping with 70% ethanol. All the materials and equipments used were sterilized to avoid contamination. The autotrophic and mixotrophic growth were cultivated in the presence of light (fluorescent tube) for a period of two weeks. The pH of the media was about 7.2 which support growth of *Chlorella variabilis*. The formula for calculating specific growth rate used was: Specific growth = $\ln(x_2\text{-}x_1) / t_2\text{-}t_1$

Where x2 is the cell concentration at time t2, and x1 is the cell concentration at time t1.

Number of cells per ul volume = counted cells / counted surface (mm^2) × chamber depth (mm)

3.4 HARVESING OF CELLS

The algal cells were harvested using the principle of centrifugation. About 300ml of culture volume was used. The cell culture was dispensed into eppendorf tubes and centrifuged at 100rpm for 10 minutes. The supernatant was discarded while the pellet which is the mass that settled at the bottom of the tube was transferred into glass Petri dishes and transferred to the oven for drying.

3.5 DRYING

Drying was done using an oven at a temperature of 60^{o}c for 24 hours to obtain a moisture free (powdered) algal biomass. Since algal biomass is hydrophobic (can easily absorb moisture), it was kept in a dried air tide container until when ready to use.

3.6 PROTOCOL FOR CHLOROPHYLL QUANTIFICATION

The dried cells were crushed in a test tube with a glass rod to break the cell wall of the algal biomass for the release of its contents. Chlorophyll was extracted in 100% ethanol. The supernatant was collected into an eppendorf tube and centrifuged at 1000rpm for 10 minutes to get rid of any particle. The supernatant was dispensed into a sterile tube. The absorbance was read on the spectrophotometer at 664 and 647 nm wavelengths and the following equations were used to calculate for the various chlorophyll (Jeffrey& Humphrey, 1975).

For green algae which contain chlorophylls *a* and *b*

chlorophyll *a* = 11.93 E664 - 1.93 E647

chlorophyll *b* = 20.36 E647 - 5.50 E664

3.7 PROTOCOL FOR CARBOHYDRATE QUANTIFICATION (Mercz, 1994)

This method used concentrated acid and phenol.

Reagents

Glucose standard solution: 0.1 g L-1

Phenol stock solution: 50 g L-1

1M H_2SO_4

Concentrated H_2SO

Table 3: Glucose Standard Curve

Glucose (ug)	0	40	80	120	160	200
Standard glucose solution	0	0.4	0.8	1.2	1.6	2
dH_2O	2	0.16	1.2	0.8	0.4	0

The standard curve was used to determine the concentration (amount) of carbohydrate present in the cells. An amount of biomass was weighed in the weighing balance, crushed with a glass rod before adding 0.5 ml 1M H_2SO_4 in a 10 ml acid resistant plastic test tube with screw lid for homogeneity. The volume was made up to 5 ml with 1M H_2SO_4. The lid was tightened and incubated in a 100°C water bath for 60 min. It was then cooled to room temperature (~30min) and centrifuged at 1000 – 2000 x *g* for 5-10 min. 2 ml of the supernatant was pippetted into another acid resistant test tube. Inside a fume hood, 1 ml of phenol solution was added and mixed rapidly using a Vortex stirrer. Inside a fume hood, 5 ml of concentrated H_4SO_2 was added rapidly and the test tube lid was closed tightly and mixed well by vortexing. The test tubes were cooled for 30 min at room temperature and mixed again. The absorbance was read at 485 nm and the carbohydrate content was calculated from the standard curve using the equation:

$$\text{Carbohydrate yield (mg/L)} = \frac{\text{carbohydrate value from standard curve}}{\text{Volume digested material} \times \text{culture volume}}$$

3.8 PROTOCOL FOR PROTEIN QUANTIFICATION (Lowrey,*el at.*, 1951)

REAGENTS

1. Bovine serum albumin (BSA) Fraction V -stock solution: 2.5 BSA /L

2. Stock solutions for Biuret reagent

(a) Na2CO3 = 200 g/L

(b) NaOH = 40 g/L

(c) NaK tartrate = 200 g/L

(d) CuSO4.4H2O = 50 g/L

3. Biuret reagent preparation:

From the above stock solutions, 20 mL of (a), 20 mL of (b), and 160 mL of deionized water were properly mixed followed by addition of 2mL of (c) and 2 mL of (d).

4. Folin-phenol reagent preparation: Dilute Folin reagent 1:1 with deionized water.

Table4: Values for Protein Curve

Protein (µg)	0	50	100	150	200	250	300	350
BSA V (mL)	0.00	0.02	0.04	0.06	0.8	0.10	0.12	0.14
dH_2O (mL)	0.14	0.12	0.10	0.8	0.06	0.04	0.02	0.00

To determine the amount of protein in the algal cells, there was need to draw a protein standard curve from which the protein concentration was determined at a particular wavelength. These steps include: A 1ml Biuret reagent was added to an amount of cell biomass and mixed well with a glass rod. After mixing, the contents were carefully transferred to a 10 ml centrifuge tube. Another1ml Biuret reagent was added over the glass rod into the 4mL glass tube, mixed well, and the content transferred to a 10 ml centrifuge tube. Another 3 ml Biuret reagent was added to the 10mL centrifuge tube and 5 ml Biuret reagent was added to each centrifuge tube. The sample and protein standard tubes were placed in a 100ºC water bath for 60 min. Clean glass marbles

were placed over the opening of the centrifuge tubes to prevent losses from splattering. The tubes were removed from the water bath and immediately.5ml Folin-phenol reagent was added while mixing in a vortex. The tubes were placed in a 10-15°C water bath for 20 min and then allowed to equilibrate to room temperature for another 15 min. They were centrifuged at 1000-2000 x *g* for 5-10 min, the supernatant was removed carefully and the absorbance read at 660 nm. The protein content of the samples was determined using a standard curve as:

$$\text{Protein content (mg/L)} = \frac{\text{Protein value from standard curve}}{\text{Volume of digested material} \times \text{culture volume}}$$

CHAPTER FOUR

RESULTS

4.1 **Growth of *Chlorella variabilis***

The growth of *Chlorella variabilis*in a sterilized BG11medium was evaluated in batch cultures. There was no additional nutrient added into the culture media for the period of three weeks when the cells were harvested. Cell growth in autotrophic condition increased more than the cells in mixotrophic condition even though the size of inoculums introduced in mixotrophic was more at the beginning as shown in table 5.

Table5: Growth of *Chlorella* under three growth conditions

Time (hour)	AUTOTROPHIC PER MILE	MIXOTROPHIC PER MILE
0	4.096×10^5	5.120×10^5
48	8.000×10^5	3.376×10^6
96	1.760×10^6	3.760×10^6
144	2.240×10^6	3.792×10^6
192	3.072×10^6	3.820×10^6
240	3.488×10^6	3.968×10^6
480	1.283×10^7	6.528×10^6

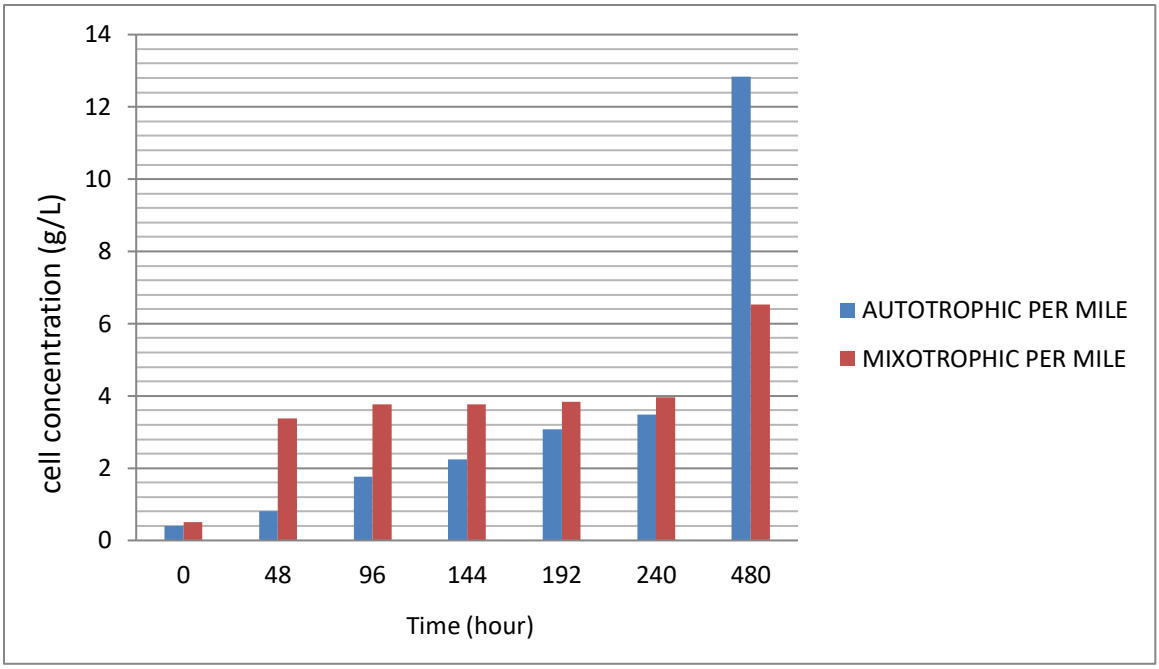


Figure 3: Changes in the concentration of *Chlorella variabilis* cells over a period of time. The cells were monitored by counting using the hemocytometer at an interval time of 48 hrs. There was a magnificent increase in cells number.

4.2 Growth rate of *Chlorella variabili*

Growth rates of algae cultures are usually given as either the doubling time (i.e.d2- the time for cell number or biomass to double) or the specific growth rate i.e. (u; time -1 –the proportion increased in cell number or biomass per time. The growth rate is usually expressed as an increase in cell concentration over a given period of time.

Table 6: Comparison between the growth rates of *Chlorella variabilis*

At early stage of this research, cells growing mixotrophically increased faster in the presence of glucose but decrease with time while the cells growing autographically continued to increase exponentially as shown in Table 6.

TIME (HOURS)	AUTOTROPHIC	GLUCOSE + MIXOTROPHIC
48	8,113	59,666
96	20,000	8,000
144	10,000	666
192	17,333	583
240	8,666	3, 083
480	38,925	10,666

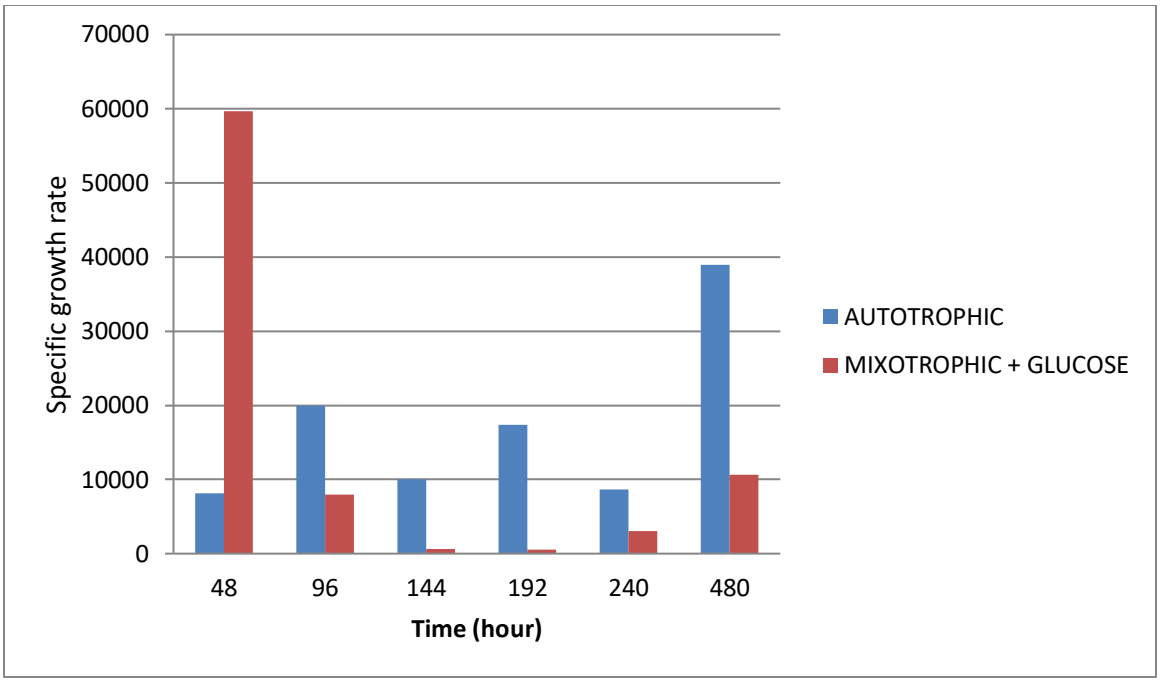


Figure 4: A bar chart showing the specific growth of *Chlorella variabilis* under different growth conditions. During the cultivation, growth rate varied as the cultivation progressed due to various reasons such as depletion of nutrients, and accumulation of products. As shown in figure 4, it is possible that as the glucose level decreased with time the growth rate slowed down as well.

4.3 Determination of chlorophyll content of the cell biomass

Chlorophyll a concentration has been used widely as a method for measuring the growth and abundance of algae and it can be determined using the 90% acetone extraction method of Jeffrey and Humphrey (1975) as shown in Table 7

Table 7: Result of Chlorophyll Content Obtained from the Biomass Produced

		WAVELENGTH 664 nm	WAVELENGTH 647 nm
AUTOTROPHIC	chlorophyll a & b	0.317	0.337
MIXOTROPHIC	chlorophyll a & b	0.555	0.606

4.4**A glucose standard curve for glucose determination**

The protocol of Mercz (1994) was used to calculate the glucose content of the cell biomass. The values in Table 8 were used to plot a standard curve which was used to trace the value of glucose mass based on the value of absorbance obtained from spectrophotometer.

Table 8: Glucose Standard Curve

Glucose (ug)	0	400	800	1200	1600	2000
Standard glucose solution	0	0.4	0.8	1.2	1.6	2
dH_2O	2	0.16	1.2	0.8	0.4	0
Absorbance at 485 nm	0.000	0.029	0.0707	0.104	0.136	0.177

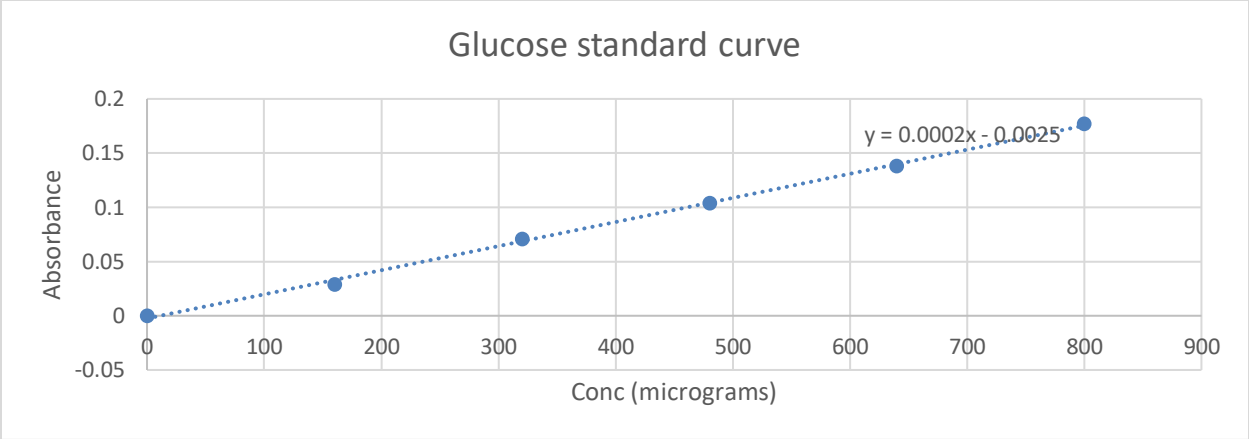


Figure 5: Glucose Standard Curve. Absorbance was plotted against concentration. From this graph, an equation to calculate the quantity of glucose in ug/L was used.

Table 9: Glucose Content obtained from the standard curve for both autotrophic and mixotrophic growth.

	Total concentration (g/l)	% composition
Autotrophic	0.0266	21.25
Mixotrophic	0.054	42.9

Table 9 shows that cells from the mixotrophic culture have more glucose content than the cell from autotrophic culture.

4.5**A protein standard curve for glucose determination**

Protein standard curve makes use of values obtained from the absorbance by varying the concentration of BSA V (Ml) with water. The absorbance was plotted against protein mass in nanogram.

Table 10: Data obtained for Protein Standard Curve

Protein (µg)	0	500	1000	1500	2000	2500
BSA V (mL)	0.00	0.02	0.04	0.06	0.8	0.10
dH_2O (mL)	0.14	0.12	0.10	0.8	0.06	0.04
Absorbance at 660 nm	0	0.12	0.31	0.512	0.695	0.916

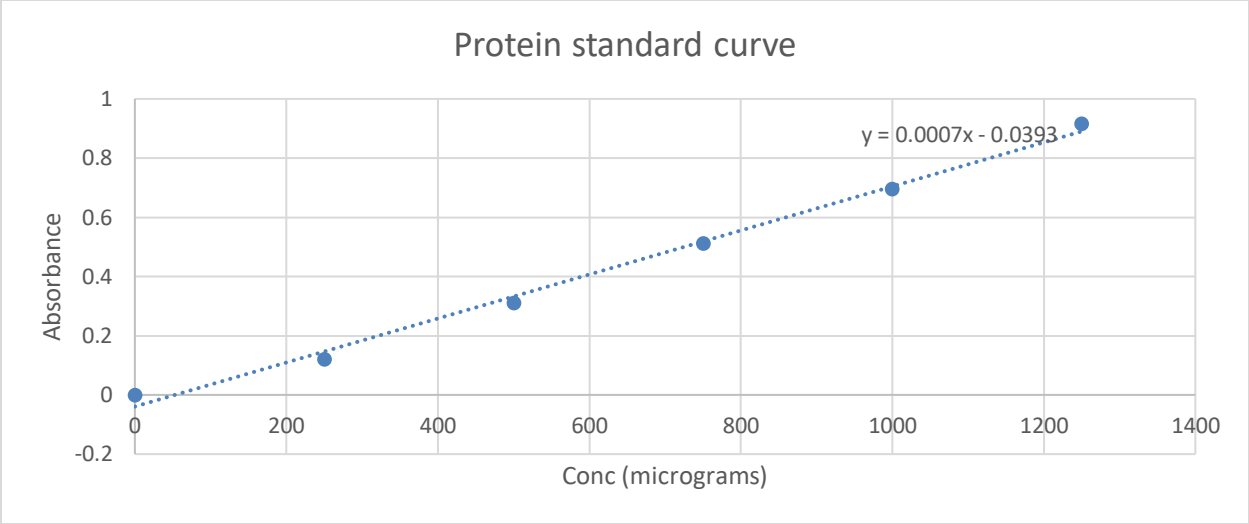


Figure 6: Protein Standard Curve. The standard curve was plotted based on line of best fit. Absorbance on the y-axis and concentration (ug) on the x-axis. Linear graph was used to calculate the value of x.

Table 11: Protein Content obtained from algal biomass.

	Total concentration (g/l)	% composition

Autotrophic	0.07	24
Mixotrophic	1.118	40.60

The autotrophic growth yielded less protein than the quantity produced by mixotrophic growth.

CHAPTER FIVE

DISCUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Researchers have determined optimal temperatures, light conditions and nutrients to obtain high growth rates for many strains (Chinnasamy*et al.*, 2010; Price, Yin & Harrison, 1998). The growth of microalgae is generally slow compared to bacterial, yeast and fungi. Neubauer haemocytometer is a special, rather thick, microscope slide originally developed for counting blood cells but which has proven excellent for counting single celled algae. There are various types of haemocytometer with different depths but the improved Neubauer haemocytometer is the version used mainly in phycology (Guillard, et al., 2005).Glucose serves as a source of organic carbon which can easily be assimilated into the cell wall of organism. As seen from the results, glucose helped to shorten the lag phase and prolong the log phase which was the period for exponential growth. The result also demonstrated that the addition of glucose actually increased the energy conversion efficiencies over autotrophic growth. It was considered that growth was maximum at log phase before it begins to decline as substrate was depletedby cells. In comparison between autotrophic and mixotrophic cell culture, the autotrophic cell concentration was more than the cells number in mixotrophic culture but the mixotrophic had more biomass and bigger cells size. This means that biomass was not really as a result of increase in cell number but due to increase in the size of cells.

Dry weight is the most reliable and accurate method for quantitative measurement of cell growth. After centrifugation or filtering the culture broth, the cells are washed and dried to a constant weight (Ogbonna, 2013).The harvested algal biomass was centrifuged, dried in the oven at 60^{o}c overnight and weighed. The mixotrophic cell biomass had a mass of 0.5g/L while the autotrophic cell biomass weighed 0.1g/L. Biomass production was dependent upon cultivation methods for harvesting, followed by quantification of biochemical composition of biomass. A major

challenge is the small size of algal cells and their subsequent low concentration in the culture medium ranging between0.5 and 2.0 g/L approximately (Prajapati*et al*.,2013; Christenson and Sims 2011;Vandamme*et al*., 2011).

Total carbohydrates are usually measured by the phenol-sulphuric acid method developed by Kochert (1978) and Ben-Amotz *et al*.,(1985). Mercz (1994) further optimised this method. The protein content of microalgae can be measured by either colorimetric dye-binding methods or by methods that measure the concentration of elemental nitrogen. The most widely used method for total protein determination in microalgae the Lowry method (Lowery *et al*., 1951).

At the end of glucose and protein extraction process, their absorbances were taken; glucose had OD of 0.04 while protein had 0.261. Readings were obtained from the photospectrometer at wavelength of 485 nm and 660 nm for glucose and protein respectively. The concentration of glucose was obtained from glucose standard curve and the protein concentration was traced on the protein standard curve graph. Glucose yielded 0.0266 (g/L) autotrophically while mixotrophic biomass yielded 0.054 (g/L). By composition %, autotrophic yielded 21.25% and mixotrophic yielded 42.9%. The protein concentration was 0.07 (g/L) for autotrophic while mixotrophic yielded 1.118 (g /L). The % composition of protein autotrophically was 24 % while mixotrophic yielded 40.6. Proper plotting of the graph is required to obtain an accurate result. Biomass composition range obtained in photo-autotrophic, heterotrophic and mixotrophic cultivation of microalgae.

5.2 CONCLUSION

Cultivation of C*hlorella variabilis* was enhanced by glucose addition to the medium. Addition of glucose made it possible for C*hlorella* v*ariabilis* to synthesis some compounds like protein and carbohydrate better. The cells grown mixotrophically increased in size which reveals accumulation of material and also weighed more after drying. The number of cells does not determine the cell biomass but the sized of cells. The more biomass produced the more single cell protein (scp). Since there is need and a high demand for protein, large scale production of algae can be used to meet up that demand.

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