AMERICAN UNIVERSITY OF BEIRUT

POTENTIAL OF LOCAL MICROALGAE CULTURES FOR BIODIESEL PRODUCTION AND FOR DISEASE MANAGEMENT

by
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AN ABSTRACT OF THE THESIS OF

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Microalgae are prokaryotic or eukaryotic photosynthetic plant like microorganisms that have received a lot of attention in the last 50 years due to the increased demand on environmentally safe alternatives for diesel production. The taxonomy of these microalgae relied on microscopic and chemical analysis, however, due to difficulties and inaccuracy of these methods, molecular identification is gaining importance. Several primary and secondary markers have been developed targeting different genomic regions. In this study 25 local isolates, unialgal or mixed algal blooms, were isolated and cultured in either seawater or fresh water. Twelve isolates were identified to the genus level and twelve to the species level using primers targeting the LSU, ITS and Tuf A region. The following genera and/or species were identified: *Acutodesmus obliquus, Amphora sp., Chlorella sp., Chloromonas sp., Cyanobacterium aponinum, Micractinium reisseri, Microcoleus sp., Neochloris conjuncta, Scenedesmus sp., Scherffelia dubia, Tetraselmis marina,Tetraselmis sp.,* and *Tetraselmis striata.* The ability of microalgae to accumulate high levels of lipids under certain culture conditions motivated researchers to explore the possibilities of using them as a renewable biosource to produce biodiesel. The lipid content was determined for 21 microalgal isolates. Under our experimental conditions, total lipid content varied between 5% DW in *Tetraselmis marina* and 21.85% DW in PS isolate. However, higher lipid content can be induced through manipulation of culturing conditions by imposition of environmental and nutritional stress. Three of the local microalgae isolates contained about 50% protein. Microalgae are also significant sources for bioactive secondary metabolites. The antimycotic activity of methanolic and water extracts from five microalgae isolates was tested in-vitro against five different plant pathogenic fungi. None of the isolates showed an antimycotic activity, but better sporulation and growth was recorded in the methanolic and crude water extracts. However, when, the same methanolic exctrats were also tested against four pathogenic bacteria, namely, *Escherichia coli, Salmonella enteritidis, Streptococcus sp.* and *Staphylococcus aureus,* they all showed an inhibitory activity, with NK2 Bloom expressing the highest activity. Two out of five microalgal water extracts tested, resulted in significant plant growth stimulation relative to their respective controls. These preliminary results showed that local microalgae isolates may have a potential for use in agriculture as feed supplements, antibacterial agents or as plant growth stimulants, in addition to their use as feedstock for biofuel.

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ABBREVIATIONS

±	Plus or minus
°C	Degrees Celsius
L	Litter
mL	Millilitter
µg	Micrograms
µl	Microliter
µm	Micrometer
bp	Base pair
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
SAR	Systemic acquired resistance
FA	Fatty Acids
g	gram (s)
hr	hour (s)
min	Minutes (s)
PCR	Polymerase chain reaction
DW	Dry weight
PBRs	Photo bioreactors
FAO	Food and Agriculture Organization
DCM	Dichloromethane
TAG	Triacylglycerol
FAME	Fatty Acid Methyl Ester

JBS	Jbeil
JOS	Jounieh
PM	Portemilio
CM	*Chlorella marina*
PALS	Palace Café (manara)
DAS	Dawra
CAS	Casino Port
EM	Ein Mreiseh
PS	Batroun Port
BR2/3	Brumana
JS	Jiyyeh
D	Dbaye
DPS	Dbaye Port
UNS	Beirut Port

CHAPTER I

INTRODUCTION

The waters of the world, seas, oceans, rivers, lakes, creeks and even ice, host a wide variety of organisms that are able to use light as the only energy source for their metabolic processes; these organisms are known as phytoplankton or algae. Microalgae are a very heterogeneous group comprising prokaryotic organisms similar to bacteria known as cyanobacteria (Cyanophyta) and eukaryotic organisms that include green algae (Chlorophyta), diatoms (Bacillariophyta) and few others. Algae exhibit a large biodiversity, with an estimated number of species exceeding one million (Guiry, 2012).

Microalgae contribute largely to the global oxygen production (between 50 to 87%) due to the fact that they use sunlight and nutrients from their surroundings to convert CO_2 to sugars and oxygen. Based on their simple structures and photosynthetic ability, microalgae are capable of rapidly generating biomass. This biomass consists of important primary and secondary metabolites such as lipids, proteins, carbohydrates and antioxidants, from which high value products including food and feed supplements, industrial chemicals, pharmaceuticals and biofertilizers are produced.

The use of microalgae as fuel feedstock was first proposed over 50 years ago for the production of methane gas (Meier 1955), after the rising need for energy in developing nations. Nerveless, microalgae can provide several different types of renewable biofuel, these include methane produced by anaerobic digestion of the algal biomass (Spolaore *et al*. 2006), biodiesel derived from microalgal oil (Roessler *et al*. 1994) and photobiologically produced biohydrogen (Ghirardi *et al*. 2000).

The idea of using microalgae as a source of fuel is not new but has gained more importance nowadays, due to the escalating price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning fossil fuels (Gavrilescu and Chisti, 2005). Besides, new research focusing on the use of microalgae for the production of high value products such as protein, vitamins, animal feed additives, biofertilizers and biopesticides has increased in the last 15 years.

The specific objectives of this research were:

1. Isolation and purification of local microalgae species collected from seawater and freshwater sources and their molecular characterization.
2. Biomass production.
3. Analysis of their lipid content and their fatty acid profiles, for evaluation of their potential use as biodiesel feedstocks.
4. Analysis of their protein content.
5. Evaluation of selected microalgal isolates for their efficacy as plant growth stimulator.
6. Evaluation of selected microalgal isolates for their in-vitro and in-vivo antifungal and antibacterial activity.

CHAPTER II

LITERATURE REVIEW

A. Microalgae

Algae are plant-like chlorophyll containing organisms that are typically photosynthetic and aquatic, but do not have true roots, stems, leaves, vascular tissue and have simple reproductive structures. Algae are responsible for half of the photosynthesis that occurs on earth despite the fact that their photosynthetic biomass represents only about 0.2% of that on land (Falkowski *et al*. 1998). They comprise macroalgae and microalgae.

Seaweed or macroalgae are multicellular large algae that look like plants. They are divided into Chlorophyta, Chrysophyta and Rhodophyta based on their colors that are mainly due to the green, brown, and red pigments within the macroalgae. Phytoplankton or microalgae are microscopic plant- like organisms ranging from 1-50 micrometers in diameter; they are chlorophyll a containing organisms with a thallus not differentiated into roots, stems and leaves (Lee, 1989). They produce around half of the atmospheric oxygen and simultaneously use the greenhouse gas (CO_2) to grow photoautotrophically. Although they are mostly found in aquatic habitat, they are also established in terrestrial habitats, on soil surfaces and on rocks adapting to a wide range of environmental conditions (Lee and Lerner, 2008).

B. Microalgae Classification

Traditionally, microalgae were classified according to their color as a result of their pigments content. Three major classes of photosynthetic pigments occur among the microalgae: chlorophylls, carotenoids, and phycobilins. Though all microalgae contain chlorophyll, other photosynthetic pigments usually mask the green color of chlorophyll.

The ability to see minute differences in microalgal cells with the electron microscope has changed classifications substantially since the 1960s. Molecular techniques based on sequencing of polymorphic genes are gaining more importance for distinguishing between closely related genera, species or strains. Classifications of these minute phytoplanktons are changing as new differentiators are being discovered. Different features such as cell division, flagellar apparatus and organelle structure and function in addition to similarities and differences among algal, fungal and protozoan groups have led scientists to propose major taxonomical changes.
Pigmentation, structure, cell wall composition, shape, flagella characteristics, products stored and method of propagation are the new characteristics to distinguish between major algal groups. Major classification criteria are discussed below.

1. ***Microscopic and physiological characters***

a. Prokaryotes

i. Cyanophyta

Blue-green algae, or cyanobacteria, are photosynthetic organisms that resemble the eukaryotic algae in many characteristics; however, due to their lack of a membrane-bound nucleus, mitochondria, golgi apparatus, chloroplasts, and endoplasmic reticulum, they are classified as prokaryotic- like organisms.

Cyanobacteria are the largest prokaryotic organisms; they are either unicellular or filamentous. The name blue–green has been given because of the presence of phycocyanin and phycoerythrin, which usually masks the chlorophyll pigmentation (Tomaselli, 2004). In anaerobic conditions they are known to assimilate atmospheric nitrogen through special structures called heterocysts - the heterocysts are thick walled cell inclusions that are impermeable to oxygen; they provide the anaerobic conditions necessary for the function of the nitrogen-fixing enzymes. Cyanobacteria reproduce asexually, either by fragmentation and spore formation in filamentous species or by means of binary or multiple fission in unicellular and colonial forms.

Under favorable conditions, cyanobacteria can reproduce at explosive rates forming dense concentrations called blooms that are especially common in waters that have been polluted by nitrogen wastes; in such cases, the overgrowth of cyanobacteria can consume so much of the water's dissolved oxygen that fish and other aquatic organisms may perish. Likewise, some cyanobacteria produce toxins known as cyanotoxins, which are of two types: neurotoxins and hepatotoxins. However, some strains are utilized as human food supplements, animal feed and biofertilizers.

About 2,000 species are estimated to belong to this group, yet, none of them has been found to produce significant quantities of lipids (Sheehan *et al.* 1998).

b. Eukaryotes

ii. Chlorophyta

Green algae are the most diverse group of algae found in freshwater, seawater and terrestrial environment. Most Chlorophyta are unicellular, but some are multicellular, colonial or coenocytic. Their green color is a result of the present of

chlorophyll a and b, in the same proportions as vascular plants, in addition to a variety of beta-carotenes and xanthophylls that act as accessory pigments. Reproduction occurs asexually via fragmentation, fission, budding or by the production of spores that develop directly into new individuals, or sexually by the union of two gametes. Starch (α-1,4-linked glucan) is their primary storage component found in their chloroplasts; their cell walls generally contain cellulose (Richmond, 2004).

In certain species, nitrogen deficiency has been found to boost lipid accumulation (Sheehan *et al*. 1998). Approximately 13,000 species are estimated to be in existence (Guiry, 2012).

iii. Bacillariophyta

Bacillariophyta, or the diatoms, are among the most common groups of algae that dominate the phytoplankton and serve as primary sources of food for zooplankton in both marine and freshwater habitats. They are photosynthetic microalgae but some species that lack chlorophyll live heterotrophically among decaying marine algae.

Diatoms are mostly unicellular but some are colonial or filamentous. Bacillariophyta lack flagella except for their male gametes; instead many diatoms achieve locomotion from controlled secretions in response to outside physical and chemical stimuli. Diatoms have unique shells that serve as their cell wall which are made from silica. Their golden-brown color is due to the presence of fucoxanthin and β-carotene that mask the green color, chlorophyll a and chlorophyll c (Tomaselli, 2004). The main storage compounds of diatoms are lipids and a β-1, 3-linked carbohydrates known as chrysolaminarins.

iv. Rodophyta

The red algae are one of the eldest groups in eukaryotic algae; they include multicellular and filamentous forms, whereas unicellular species are less represented. The red color of this group results from the pigments phycocyanin and phycoerthrin that mask other pigments like chlorophyll a, beta-carotene and xanthophylls.

Their main reserves are floridoside and floridean starch (α-1,4-linked glucan) as storage products accumulated in the cytoplasm outside the chloroplast (Sze, 1998).

Rhodophyta lack flagellated cells. Their cell walls are made of micro-fibrillar layer of cellulose or xylan and amorphous polysaccharidic mucilages (agar or carrageenan).

Red algae represent the majority of seaweeds distributed mostly in temperate and tropical regions (Richmond, 2004).

v. Dinoflagellates

Dinoflagellates are a diverse group of flagellate eukaryotic microalgae that are mostly found in marine habitats; however, they are also common in freshwater systems. All dinoflagellates possess two flagella; the transverse flagellum that is confined in a groove-like structure around the equator of the organism (the cingulum) providing forward motion and spin to the dinoflagellate, and the longitudinal flagellum which is trailing behind providing little propulsive force mainly acting as a rudder. In addition, the dinoflagellate nucleus is very distinctive; it displays an unusual combination of prokaryotic and eukaryotic characteristics.

Chlorophyll a & c, beta-carotene, neoperidinin, xanthophyll, fucoxanthin, dinoxanthin, peridinin, neodinoxanthin, and diatoxanthin are found in dinoflagellates. Dinoflagella cysts or dinicysts are resting spores produced by some dinoflagellaltes as

part of their life cycles.

Some heterotrophic dinoflagellates are potentially harmful organisms releasing deadly toxins in water bodies to harm other marine organisms.

vi. Chrysophyta

Chrysophyta or golden-brown algae are mostly found in freshwater habitats, especially in oligotrophic waters low in calcium. The chloroplasts contain chlorophylls a, c1, and c2, fucoxanthin and β-carotene that are responsible of the golden brown color (Sze, 1979). Their storage product is chrysolaminarin (β-1, 3-linked glucan), which is accumulated in a cytoplasmic vesicles.

Most of the species are unicellular or colonial; cells usually have two different apical flagella, one smooth while the other is hairy. Cells contain parietal chloroplasts with an eyespot (Richmond, 2004). The cell wall is often lacking or composed of cellulose. A characteristic of the chrysophyta is the formation of special resting spores, statospores, enclosed in a silicified wall. Some species require additional vitamins and growth substances (Richmond, 2004).

Table 1. Characteristics of selected phyla of algae

	Brown Algae	**Red Algae**	**Green Algae**	**Diantoms**	**Dinoflagellates**
Phylum	Phaeophyta	Rhodophyta	Chlorophyta	Bacillariophyta	Dinoflagellate
Color	Brownish	Reddish	Green	Brownish	Brownish
Cell Wall	Cellulose nd alginic acid	Cellulose	Cellulose	Pectin and silica	Cellulose in membrane
Cell Arrangement	Multicellular	Most are multicellular	Unicellular and multicellular	Unicellular	Unicellular
Photosynthetic Pigments	Chlorophyll a and c, xanthophylls	Chlorophyll a and d, phycobiliproteins	Chlorophyll a and b	Chlorophyll a and c, carotene, xanthophylls	Chlorophyll a and c, carotene, xanthophylls
Sexual Reproduction	Yes	Yes	Yes	Yes	In a few
Storage Material	Carbohydrates	Glucose polymer	Glucose polymer	Oil	Starch

Source: Pearson Education, 2004.

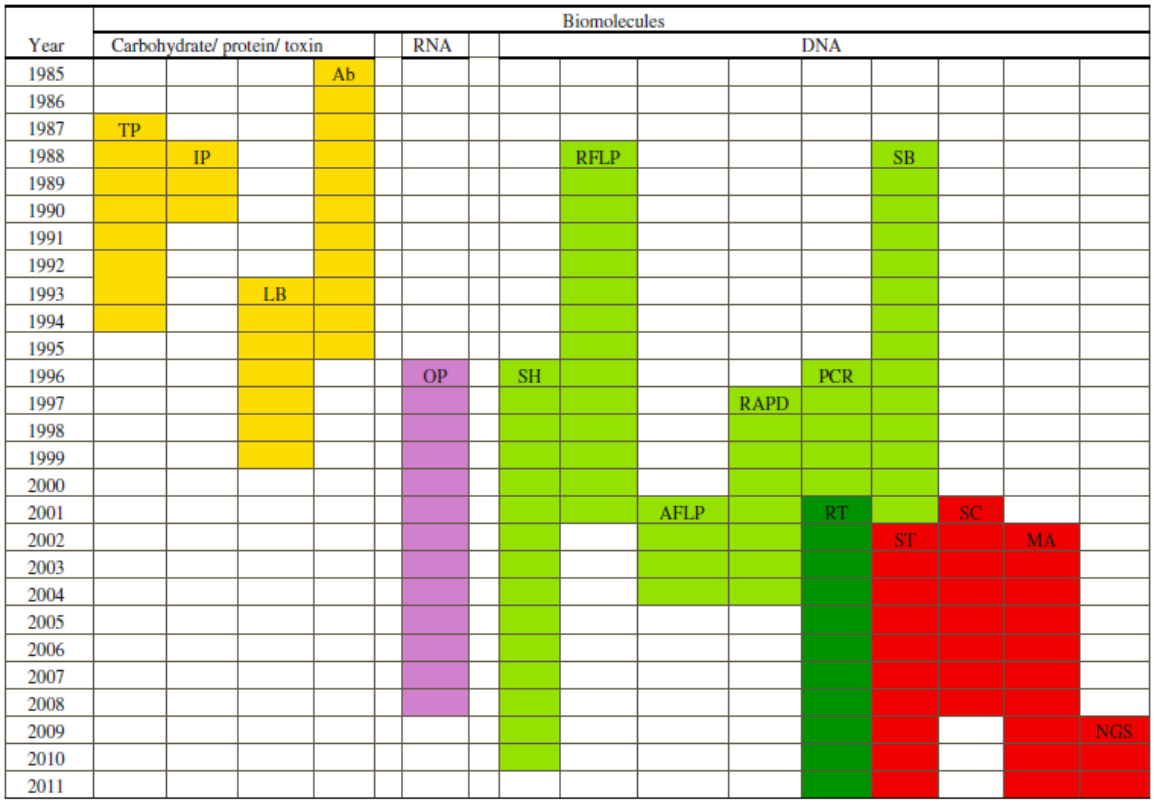
2. Molecular identification

Traditionally, identification of microalgae was based on microscopic observations of morphological characters and on the pigment content of the algae. However, due to their microscopic size and minute structures, morphological identification was difficult and sometimes inaccurate. The advent use of molecular technology in phycology began in 1970s. Since that time, phycologists developed molecular techniques as accurate diagnostic methods for closely related genera and species (Table 2).

DNA based identification are gaining importance and are very effective, especially for the pico-sized fractions that have very few morphological features that can be used for identification (Not *et al.* 2007). Several studies has been undertaken lately to identify microalgae using molecular tools. Primers targeting both short subunit (SSU) and large subunit (LSU) of the 16S ribosomal DNA are being employed to identify microalgae belonging to different genera and species. Several other genes have been used for diagnostic purposes including 18S rDNA.

Today, the description of new species, creation of new genera, or rearrangement of a species to a different genus is usually supported by molecular data in addition to morphological structures, ultrastructure, and information on biogeographic distribution (Fraga *et al*. 2008).

Table 2. Indirect methods for the detection, quantification, and diversity of microalgae based on the published papers (different time scale).



Source: Ebenezer *et al*. 2011.

* Colored cells in each column represent individual technologies based on corresponding years of publications between first and the latest. TP toxin profile, IP isozyme pattern, LB lectin binding, Ab antibody, OP oligonucleotide probe, including FISH, SH sandwich hybridization, RFLP restriction fragment length polymorphism, AFLP amplified fragment length polymorphism, RAPD random amplification of polymorphic DNA, PCR polymerase chain reaction, RT real-time PCR, SB sequence-based discrimination, ST sequencing typing, SC single-cell PCR, MA microarray, NGS next generation sequencing.

C. Culturing Parameters of Microalgae

Microalgae are exposed to a variety of environmental conditions that influence the growth rate and cellular composition in both natural and engineered systems. The amounts of carbon fixed in lipids and carbohydrates are highly affected by environmental factors and nutrient availability; hence, to develop a suitable high productivity bio-algal system, understanding synergistic interactions between multiple nutritional factors and environmental variables is essential.

1. Temperature

Temperature is a major influencing factor for optimal growth of microalgae as temperature can go below or above the optimal for each species. Microalgae can tolerate a temperature ranging from 18-24°C depending on the species and culture media; however, some microalgae can survive beyond that range. Temperatures lower than 16°C will slow down growth, while those higher than 35°C are lethal to many species. The effect temperature exerts on biochemical reactions and how it affects the biochemical composition of algae makes temperature one of the most important environmental factors (Hu, 2004).

Temperatures lower than the optimum influence the lipid profile of microalgae species. Unsaturated fatty acids, which help to protect the photosynthetic machinery from photo inhibition, are synthesized by the cells to adapt to lower temperatures. The impact of low temperature is more directed to the type of lipid rather than enhancing the total content of lipid (Hu, 2004). Temperature also has a direct correlation to the nutrient concentration and cell constituents. The algae cell volume and biochemical content will increase if the temperature is below or above the optimal level, thus more

nutrients are required to grow microalgae under temperature stress compared to growth at optimal temperature (Hu, 2004). Therefore, the choice for temperature suitable for a microalgae culturing system needs to be decided strictly based on the original aim of algae cultivation.

2. Light

Light is one of the most important factors for microalgae growth, as it is the primary energy source for photosynthesis. Efficient utilization of light remains one of the major challenges in microalgal biotechnology, especially when an increase in the biomass yield is desired (Barbosa *et al*. 2001). Usually natural light is sufficient for outdoor production, however, additional light can be provided by fluorescent lamps (cool white or day light lamps) in the laboratory.

Light intensity requirements vary according to the culture volume and type or species of the microalgae, though, 2500-5000 lux is considered optimum. Increasing light intensity and duration is often associated with faster division, better growth and increase in photosynthesis.

For outdoors systems, natural sun is used to take advantage of the provided natural light, thus reducing the cost of production, however availability of sunlight is extremely dependent on regional climate, with variation in the light intensity both daily and seasonally. On the other hand, in an indoor system, artificial light is supplied in light and dark cycles usually 16:8 hrs light/dark.

Microalgae become light saturated when the photosynthetic rate is at its maximum. Prior to reaching saturation, when light is limiting, the photosynthesis rate is linearly dependent on the light irradiance, meaning algae cells grow faster as the light

intensity increases. Once the light saturation point is reached, the cells reduce the number of photosynthetic units leading to photo inhibition, oxidative damage of PUFA and a decrease in the cellular lipid and PUFA content.

Light intensity affects the cellular composition of microalgae. For example, *Dunaliela tertiolecta* exhibits an increase in the lipid content and a decrease in protein fraction when light intensity increases to saturation; whereas, low light intensity will result in higher protein content while high photon flux density (PFD) results in increased extracellular polysaccharide content.

3. pH

pH is an environmental factor that affects the microalgal growth and is balanced by the interplay between photosynthesis and respiration. Microalgae have the capacity to grow under a pH range from 7 to 9 but the majority has an optimum range between 8.2 and 8.7 (Lavens and Sorgeloos, 1996). For instance, some species like *Spirulina platensis* are tolerant to higher pH (pH 9) (Hu *et al*. 1998) whereas lower pH can be tolerated by *Chlorococcum littorale* (pH 4) (Kodama *et al.* 1993).

Failure to maintain appropriate pH levels can affect algal growth in many ways; it can change the distribution of carbon dioxide species and carbon availability, inhibit photosynthesis, alter the availability of trace metals and essential nutrients, and at extreme pH levels potentially cause direct physiological effects.

A complex relationship exists between pH and CO_2 levels in the bioreactor systems due to the underlying chemical equilibrium among CO_2, HCO_3^-, H_2CO_3, and CO_3^{2-}. Increasing CO_2 concentration can lead to higher biomass productivity, but will also decrease the pH, which can have an undesirable effect on the microalgal physiology. By

contrast, microalgae have been shown to cause a rise in pH to 10-11 in open ponds due to carbon dioxide uptake. This increase in pH can be beneficial for inactivation of pathogens in microalgal wastewater treatment, but can also inhibit microalgae growth. Similarly, the speciation of NH_3 and NH_4^+ in microalgal bioreactors is strongly dependent on pH – NH3 uncouples electron transport in the microalgal photosystem and competes with water molecules in oxidation reactions, thus leading to release O_2.
Two strategies used to avoid pH increase due to CO_2/HCO_3^- imbalance: to bubble CO_2 and to appropriately mix all elements (which is also achieved by CO_2 bubbling) in the tank (Lavens and Sorgeloos, 1996).

4. Salinity

Marine microalgae grow best at salinity between 20-24 g of salt /L, however, some species like *Dunaliella salina* can tolerate very saline environment. Most species grow better at a salinity level lower than that of their initial habitat. Salinity between 20-25 g/L is generally best for the growth of diatoms while 25-30 g/L for the culture of flagellates (Laing and Britain, 1991).
Lipids, carbohydrates and proteins appear to be slightly affected by a wide range of salinity for most microalgae species. However, in some species, increase in lipid and ash content were observed at higher salinity.

D. Culturing Media of Microalgae

In general, microalgae require inorganic nutrients, light, and favorable temperatures to grow (Fogg 1975, Bold and Wynne, 1978). The primary inorganic nutrients are nitrogen and phosphorus.

Microalgae growth, lipid content and composition can be altered by the culturing media composition. Guillard's F/2 medium and Walne medium are the two enriched media extensively used for the growth of most marine microalgae while Bold Basal medium and BG-11 medium have been extensively used to culture fresh water microalgae. Most marine cyanobacteria cannot live in Walne or Guillard's F/2 because they have elevated requirements for Na^{+}, $C1^{-}$, Mg^{2+} and Ca^{2+} (Stanier and Cohen-Bazire, 1977) rather, most of them are maintained in MN medium , which has a natural seawater base and is supplemented with the minerals of BG-11 medium at half strength. Some strains grow poorly on MN and are maintained in a synthetic seawater medium, ASN-111(Ripka *et al.* 1978).

For large-scale production, agricultural grade fertilizers (Table 3) such as urea, ammonium sulfate and calcium superphosphate are used as a cost effective alternative chemicals for the expensive culturing media. When compared with the standard Zarouk's media, maximum growth rate in terms of dry biomass, protein and chlorophyll where recorded in some of the media formulated from these fertilizers.

Table 3. Various combinations of fertilizers that can be used for mass culture of marine algae

Fertilizers	Concentration ($mg.l^{-1}$)					
	A	B	C	D	E	F
Ammonium sulfate	150	100	300	100	-	-
Urea	7.5	5	-	10-15	-	12-15
Calcium superphosphate	25	15	50	-	-	-
Clewat 32	-	5	-	-	-	-
N:P 16/20 fertilizer	-	-	-	10-15	-	-
N:P:K 16-20-20	-	-	-	-	12-15	-
N:P:K 14-14-14	-	-	-	-	-	30

Source: FAO 1994.

1. Nitrogen

After carbon, the most important nutrient needed for the production of good biomass is nitrogen. Ammonium, nitrate, nitrite and many organic nitrogen compounds such as urea, free amino acids and peptides are the major nitrogen source utilized even though several inorganic compounds can be utilized by microalgae. When both ammonium and nitrate are available in a culture, ammonium is often preferred over nitrate as a nitrogen source since ammonium does not need to be reduced prior to amino acid synthesis. However, ammonium concentrations greater than 25μM are reported to be toxic to phytoplankton. When ammonium is used as the only nitrogen source, a drop in pH can be observed especially during active growth due to the release of H^+ ions (Grobbelaar, 2004). On the other hand, an increase in pH is observed when nitrate is used as the only N source (Grobbelaar, 2004).

When nitrogen is limited in the culture, discolorations of the cells occur frequently due to the decrease in chlorophyll content and an increase in carotenoids. Nitrogen starvation results in a growth limitation, but also in a higher proportion of lipids in every cell; the accumulation of organic carbon compounds such as polysaccharides and certain oils like polyunsaturated fatty acids (PUFAs) is enhanced (Becker, 1994b). Nevertheless, nitrogen deficiency might not always result in an increase in total lipid content in microalgae but a change in lipid composition.
It was reported that the green algae *Botryococcus braunii* contained high content (28.4-38.4%) of oleic acid under nitrogen starvation, but the content of total lipids and triglycerides did not change (Huang *et al.* 2010). However, lower dry weights are common in nitrogen starvation conditions. On the other hand, nitrogen addition is

associated with higher dry weight, increase in protein content and increase in chlorophyll.

2. Phosphorus

Phosphorus is another essential nutrient for microalgal growth, as it is an active participant in many metabolic processes, like energy transfer and different biosynthetic pathways. Phosphorus is mostly added to the culture media as inorganic orthophosphate (PO_4^{3-}, $H_2PO_4^-$ and HPO_4^{2-}) although organic glycerol phosphate is sometimes used, mainly when precipitation of phosphate is anticipated.

Phosphorus, generally as orthophosphate (PO_4^{2-}), is taken up by the algae in an energy dependent way. Although a low amount of phosphorus (20 to 50 μg/l) is required for microalgae growth compared to C and N, phosphorus uptake can be limiting because orthophosphates can easily bind to other ions present in the culture (carbonate and iron) and precipitate, thus it will become unavailable for algae uptake (Grobbelaar, 2004). pH also mediates precipitation of phosphate, which re-solubilizes when the pH drops. A general ratio of 6:1 N to P is preferentially used in culture media.

The supply of phosphorus also influences the composition of the produced biomass. Similar effects to the ones obtained in microalgae grown under nitrogen limitation. A tendency to accumulate large amount of lipids, with a decreased amount of proteins, chlorophyll and nucleic acids content has been reported on phosphate deficient cultures (Becker, 1994a).

3. Other nutrients

Hydrogen and oxygen are also necessary for algal growth, but water provides an abundance of those elements. Many other micronutrients are needed for microalgal growth. For example, silica is needed by diatoms as a structural component for their rigid cell walls. Silicon limitation can also lead to the accumulation of secondary metabolites, such as lipids (Grobbelaar, 2004).

Other micronutrients required for growth and enzymatic activity include calcium, magnesium, sodium, potassium, iron, manganese, sulfur, zinc, copper, and cobalt (Goldman and Horne, 1983). Vitamins such as B1, B12 and sometimes biotin can be added (FAO, 1994). All tested microalgal species have been shown to have a requirement for vitamin B12, whereas around 20% need B1, and less than 5% need biotin. B12 appears to be important in transferring methyl groups and methylating toxic elements such as arsenic, tin, mercury, thallium, gold, platinum and tellurium (Brand, 1986).

E. Growth Kinetics

The growth of an axenic culture of microalgae is characterized by five phases (Figure 1):

1. Lag phase:

During this phase little increase in cell density occurs. The lag in growth is attributed to the physiological adaptation of the cell metabolism to growth, such as the increase of the levels of enzymes and metabolites involved in cell division and carbon fixation. The condition of the inoculum has a strong bearing on the duration of the lag

phase (Spencer, 1954). A lag phase may occur if the inoculum is transferred from one set of growth conditions to another; if an inoculum is taken from an exponentially growing culture, it is unlikely to have any lag phase when transferred to fresh medium under similar growth conditions of light, temperature and salinity.

2. Exponential phase:

In this phase, the cell numbers increase at a fast rate as a function of time t according to a logarithmic function: $C_t = C_0.e^{mt}$; where C_t and C_0 being the cell concentrations at time t and 0, respectively, and m = specific growth rate.
The duration of exponential phase depends upon the size of the inoculum, the growth rate, the capacity of the medium, temperature, pH and light intensity.

3. Phase of declining growth rate:

During this phase, the cell division slows down when either a specific requirement for cell division is limiting or something else is inhibiting the reproduction. The biomass is often very high and exhaustion of a nutrient salt, limiting carbon dioxide or light limitation becomes the primary causes of declining growth.

4. Stationary phase:

When net growth is zero, the culture enters the stationary phase and within a short period of time the cells may undergo dramatic biochemical changes. The nature of the changes depends upon the growth-limiting factor.

5. Death phase:

During this phase, the cell density decreases rapidly and the culture eventually collapses. The water quality deteriorates and nutrients are depleted to a level incapable of sustaining growth. Some species will lose their pigmentation and appear washed out or cloudy, whereas cells of other species may lyse but the culture color will be maintained.

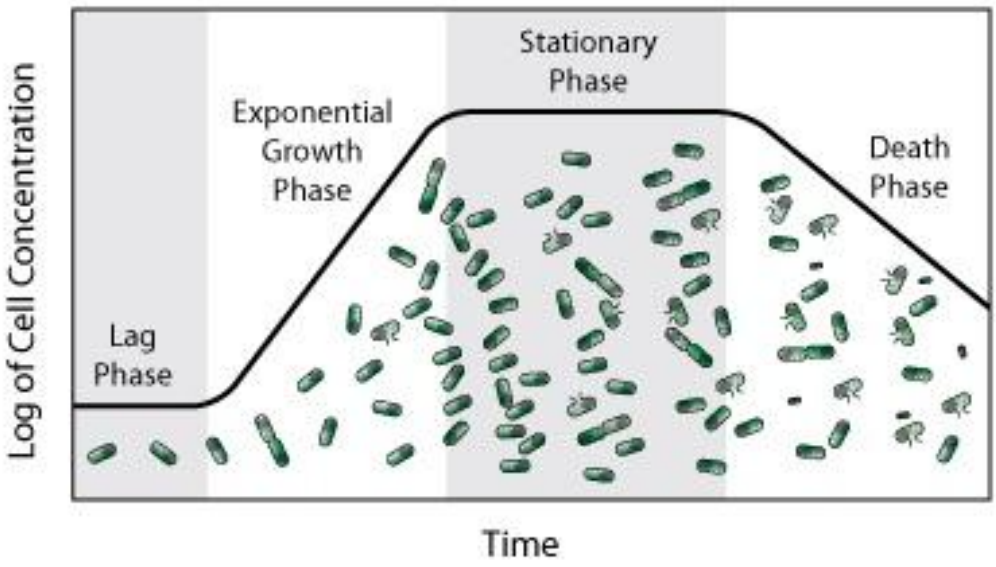


Figure 1. Growth phases of microalgae (Microalgae and Bioremediation 2014).

F. Chemical Composition

Since 1950s, there is a growing interest in algae as biomass producing organisms because they contain several high-value molecules such as lipids, protein, carbohydrates and carotenoids in varying proportions. The percentages of these molecules vary between the species and between the different families of microalgae (Table 4). Nevertheless, these proportions can be changed according to the grower purpose by adjusting the culturing parameters.

Table 4: Biomass composition of microalgae expressed on a dry matter basis

Strain	Protein	Carbohydrates	Lipid
Anabaena cylindrica	43-56	25-30	4-7
Botryococcus braunii	40	2	33
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	41-58	12-17	10-22
Dunaliella bioculata	49	4	8
Dunaliella salina	57	32	6
Dunaliella tertiolecta	29	14	11
Euglena gracilis	39-61	14-18	14-20
Porphyridium cruentum	28-39	40-57	9-14
Prymnesium parvum	28-45	25-33	22-39
Scenedesmus dimorpinus	8-18	21-52	16-40
Scenedesmus obliquus	50-56	10-17	12-14
Scenedesmus quadricauda	47	-	1.9
Spirogyra sp.	6-20	33-64	11-21
Spirulina maxima	60-71	13-16	6-7
Spirulina platensis	42-63	8-14	4-11
Synechoccus sp.	63	15	11
Tetraselmis maculate	52	13	3

Source: Um and Kim 2009; Sydney *et al.* 2010.

1. Lipids and FAME

Microalgae contain three main components: proteins, carbohydrates, and lipids. Because of the high lipid content in the form of triacylglycerol or triglycerides, which is the right type of oil for producing biodiesel, microalgae represent the new trend in the algae-to-biofuel arena (Danielo, 2005). The lipid profile of the microalgae is critical for the quality of the biodiesel which is mainly controlled by the FAME profile of the algae and the levels of saturated and monounsaturated lipids.

Determination of carbon chain length and the degree of saturation is a key to ensuring high quality biodiesel that delivers optimum performance during engine combustion; for instance, the higher the percentage of saturated lipids, the better the biodiesel quality obtained.

Lipids are divided into polar or neutral based on their chemical structures. Polar lipids include the phospholipids and the glycolipids, while non-polars comprise mono-, di-, and triglycerides, waxes and isoprenoid type lipids. Lipid composition in microalgae is dependent on the species; each lipid type is present in a certain position inside the cell. For example, glycerolipids are mainly present in the chloroplasts while, phosphoglycerides are found in the plasma membrane and endoplasmic membrane systems.

The fatty acids linked to the triglycerides within the algal cells can be both short and long chain hydrocarbons. The shorter chain length acids are ideal for the production of biodiesel, and some of the longer chains can have other beneficial uses. Fatty acids generally contain even numbers of carbon atoms in straight chains normally in the range of C14 to C24, with a carboxyl group at one end. Unsaturated fatty acids occur when there are double bonds of the *cis* configuration in specific positions. Fatty

acids from microalgae have been found to contain combinations of zero to five *cis* double bonds (Thompson, 1996).

Larger amounts of even-numbered saturated fatty acids (12:0, 14:0, 16:0,18:0) are mostly produced by microalgae, with only little quantities of odd numbered fatty acids (13:0 to 19:0) (Pohl, 1982).

Different reports exist on biodiesel production from microalgae, but the majority of the data refers only to fatty acids, most of which are C14 to C20 (Benemann and Weissman, 1984). It is known that pure cultures of green algae have primarily C16 and C18 fatty acids with a high degree of unsaturation (Thompson, 1996). Small amounts of free fatty acids can be found in microalgae.

Extraction of lipids from microalgal cells has been performed physically or chemically through the use of solvent mixtures or by the combination of both. Chemically, many methods have been recommended such as Bligh and Dyer (1959), Soxhlet method and Folch methods (Folch *et al.* 1957; Bligh and Dyer, 1959).

As for biodiesel production, four primary methods can be used: blending, microemulsion, pyrolysis and transesterification. The most common is by transesterification (Figure 2) of microalgal triglycerides with an alcohol in the presence of a catalyst to produce monoesters termed as biodiesel (fatty acid methyl or ethyl ester FAMEs). During the transesterification reaction, fatty acids should be derivatized into their FAMEs, followed by a GC-MS analysis for an efficient separation, quantification and identification of the profile of fatty acid methyl esters present in the biomass.

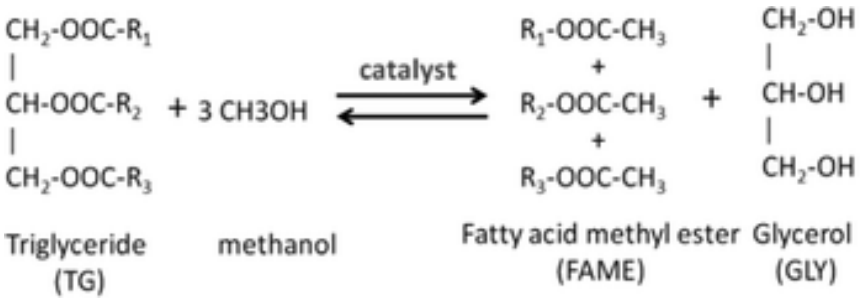


Figure 2. Transesterification reaction.

Lipid compositions of microalgae change with the environmental conditions. Many studies highlighted the importance of nutrient deficiencies on the lipid accumulation in microalgae. Media deficient in nitrogen, phosphorus, silicon, and sulfur were found to favor the lipid accumulation (Griffiths *et al.* 2009). An increase of 138% of lipids under nitrogen deficiency and of 168% under silicon deficiency when compared to nutrient sufficient medium were reported. In a study conducted by Piorreck *et al.* (1984), it was shown that microalgae have the tendency to synthesize neutral lipids and fatty acids with low degrees of unsaturation at low nitrogen levels, while high levels favor the synthesis of polar lipids.

Besides nutrient availability, other factors affect lipid composition of microalgae such as temperature and light intensity. According to Rodolfi *et al.* (2008), triglycerides accumulation has been stimulated by high irradiances while under low irradiances, phospholipid and glycolipid contents were higher. An increase of unsaturated fatty acids was observed when the temperature decreased, whereas a higher temperature induced an increase in saturated FAME (Hu *et al.* 2008).

A good microalgal processing is based on a good microalgal choice. This was observed by Pulz and Gross (2004) who stated that "successful algal biotechnology mainly depends on choosing the right alga with relevant properties for specific culture

conditions and products".

2. Proteins

Due to the increased research focusing on the production of biodiesel from lipids, production of proteins from microalgae was under-estimated even though they make up a large fraction of microalgal biomass that can reach up to 60-70 % in some filamentous blue green algae.

Since 1950, research focusing on the production of protein from algae as a feed source for animals and farm fish is increasing due to the growing world population and the economics of production as compared to biofuel production. For instance, some microalgae such as *Chlorella, Spirulina* and *Dunaliella* have very high protein contents compared to traditional sources of protein (meat, eggs, milk, soybeans, etc.). According to FAO, protein content in microalgae range from 12 to 35%. In general, blue green algae contain a higher percentage of protein (18 to 54%) as compared to green algae (8-34%). For example, *Spirulina* have low lipid content and a high protein percentage; therefore, it is used as a food rich in proteins comprising all essential amino acids needed.

Growing the microalgae in a medium rich in nitrogen can increase the protein percentage. The protein quality is also important and can be determined by determining their amino acid composition since green plants are mainly deficient in lysine, phenylalanine, tryptophan and methionine, with lysine being present at a much lower concentration in major food plants than in animal foods.

Protein in microalgae can be determined either by colorimetry that measures the change in color caused by the oxidation of amino acid residues by the folin reagent or by Kjeldahl method that measure the concentration of elemental nitrogen.
If all the nitrogen in the biomass is associated with protein, then nitrogen mass fraction measured by the Kjeldahl method can be unambiguously converted to the protein content by multiplying by a nitrogen-to-protein conversion factor value of 6.25. Though, algal biomass usually contains a proportion of nitrogen that is not associated with proteins, but with compounds such as DNA, pigments such as chlorophyll and inorganic nitrogen (Fujihara *et al*. 2001; Lourenço *et al.* 1998; Wallace and Fox, 1998). Consequently, the commonly used multiplier of 6.25 causes the protein content to be overestimated (Ezeagu *et al.* 2002) and biomass specific multipliers have to be determined. Therefore, a nitrogen-to-protein conversion factor of 5.95 is used for Kjeldahl method and 4.44 for elemental analysis.

3. Carbohydrates

Carbohydrates are one of the most important cell components; they are used as cellular structures and as storage of energy for many cell functions. Microalgal carbohydrates are complex and consist of a mixture of amino sugars, neutral sugars and uronic acids and the composition vary across species and growth conditions. Carbohydrates represent the smallest proportions in microalgal compositions constituting only 4.6-23% of their dry weights (FAO, 1997). They are mainly constituted from cellulose in the cell wall, and starch in the plastids. The net accumulation of carbohydrates and starch can be affected by several physical and chemical factors, such as medium composition, the type of light and light intensity, and

growth conditions, which cause the microalgae to accumulate carbohydrates. Despite their relatively small concentrations, their effectiveness in bio-ethanol and methane gas productions by fermentation was proven.

4. Pigments

Pigments are divided into three major classes: chlorophylls (green pigments), carotenoids (yellow or orange pigments) and phycobilins. The first two pigments are lipophilic while the latter is hydrophilic.

Different types of chlorophylls that differ from each other in their side-group substituent are found in microalgae.

Chlorophylls have two major absorption ranges: blue or blue-green (450-475 nm) and red (630-675nm). Carotenoids are either hydrocarbons or oxygenated hydrocarbons; they have an absorption range between 400 and 550nm. Phycobilins are water-soluble; they absorb blue- green, yellow, or orange light. They are considered a secondary group of carotenoids like xanthophylls, astaxanthin and canthaxanthin, and these pigments are overproduced in microalgal cells grown under stress conditions.

Pigments can be extracted using methanol, ethanol or acetone, and their identification and quantification can be performed either by spectrophotometric analysis or by high liquid performance chromatography (HPLC).

Previously, classification of microalgae was based on their pigment content (Table 5).

Table 5. Pigments in major algae taxonomic groups

Taxonomic group	Chlorophyll	Carotenoids
Bacillariophyta (diatoms)	a, c	β-carotene ± -carotene rarely fucoxanthin
Chlorophycophta (green algae)	a, b	β-carotene, ± -carotene rarely carotene and lycopene, lutein
Chrysophycopyta (golden algae)	a, c	β-carotene, fucoxanthin
Cyanobacteria (blue green algae)	a, c	β-carotene, phycobilins
Phaecophycophta (brown algae)	a, c	β-carotene, fucoxanthin, violaxanthin
Dinophyta (dinoflagellates)	a, c	β-carotene, peridinin, neoperididnin, neodinoxanthin
Rhodophycophta (red algae)	a, rarely d	β-carotene, zeaxanthin ± β carotene

5. Ash

The ash content in microalgae vary between the different groups of microalgae; for instance green algae have higher ash content ranging from just under 12 % to over 44, whereas it ranges between 3-11% in blue-green algae (FAO, 1997). High ash content negatively affects the production of biofuels.

G. Mode of Cultures

A variety of microalgal culture modes has been reported; however, all of them are variations of two basic systems: batch and continuous cultures (Table 6).

1. Batch culture:

Due to their simplicity and flexibility, batch culture systems are considered as the most reliable method and are widely adopted. However, batch culture systems are not the most efficient culturing method. The batch culture consists of a single inoculation of cells into a container of fertilized seawater followed by a growing period of several days and finally harvesting when the algal population reaches its maximum or near-maximum density. Furthermore, the quality of the harvested cells may be less predictable than that in continuous systems because of the timing of the harvest that may vary between the cycles. Batch cultures require a lot of labor to harvest, clean, sterilize, refill, and inoculate the containers.

Despite the limitations of batch culturing, it is widely used for the commercial cultivation of microalgae for its ease of operation and simple culture system as well as it reduces the probability of contamination.

2. Continuous culture:

In continuous systems, fresh culture medium is continuously added so that algal cells stay close to their maximum growth rate. The culture is removed continuously or intermittently. To sustain cell growth, the growth limiting factor must be replenished and the growth inhibitory products need to be removed or diluted by adding fresh culture media (FAO, 1996). Continuous culture system is reliable, automated, require

less labor as well as the quality of algae produced is predictable. Nevertheless, they are more complex and have a relatively high cost.

The requirements for constant illumination and temperature control mostly restrict continuous systems to indoors and this is only feasible for relatively small production scales (FAO, 1996). The success of a commercial project will depend on many factors including the selection of an adapted species and the selection of an economical and reliable cultivation system.

H. Cultivation Systems

1. Open system:

Large scale commercial production of microalgae is performed in open systems. However, a small number of microalgal species can succeed in such conditions due to the high possibility of contamination. Open ponds may be categorized into natural waters such as lakes, lagoons, ponds or artificial ponds and containers. The most commonly used systems include shallow big ponds, tanks, circular ponds and raceway ponds.

Open ponds are easy to construct, operate and clean, require low energy input, have low production and operating costs and have the potential to produce net energy. Nonetheless they have many drawbacks: requirement of large areas of land, light utilization by cells might be poor, diffusion of CO_2 to the atmosphere, high evaporation which necessitates large amounts of water which might lead to low productivity or even inconsistencies in production and most importantly they are subject to contamination by fungi, bacteria or other microalgae.

A variation of the basic open-pond system is to cover the system with a greenhouse. While this usually results in a smaller system, it does take care of many of the problems associated with an open pond system such as contamination. Raceway ponds equipped with paddle wheels are most commonly used in commercial plants.

2. Closed system:

Closed systems are alternatives to open ponds where the control over the environment is better. Choosing a closed system for microalgal mass production is a way to overcome limitations of open systems because light is produced evenly, pollution and contamination risks are highly minimized with a real chance of producing single- species of microalgae. However, production costs are still higher compared to open systems.

a. Flat photobioreactor:

Flat photobioreactors have received much attention due to their large illumination surface area and relatively low cost. Flat photobioreactors are made from transparent materials for maximum utilization of solar light energy. Accumulation of dissolved oxygen concentrations in flat photobioreactors is relatively low compared to horizontal tubular photobioreactors. It has been reported that with flat photobioreactors high photosynthetic efficiencies can be achieved (Hu *et al.* 1996; Richmond, 2000). This system is very suitable for mass cultures of algae but still some limitations should be considered such as the possibility of hydrodynamic stress to some algal strains, difficulty to upscale and to control the culture temperature.

b. Tubular photobioreactor

Due to the their ability to completely isolate the culture from potentially contaminating external environments, large illumination surface area and their efficient outputs, the tubular photobioreactors represent one of the most suitable types for outdoor mass cultures.

The latter have limitations related to the culture temperature, the poor mass transfer, the adherence of the cells on the walls of the tubes and the gradients of oxygen and CO_2 transfer. However, tubular bioreactors are often recommended for large scale production of high value products since they are less prone to contamination and due to their ability to be mounted vertically, horizontally or at an angle, indoors or outdoors, thus saving space.

The advantages and disadvantages of the various culturing are summarized in Table 6

Table 6. Advantages and disadvantages of various algal culturing techniques

Culture Type	**Advantages**	**Disadvantages**
Indoors	A high degree of control (predictable)	Expensive
Outdoors	Cheaper	Little control (less predictable)
Closed	Contamination less likely	Expensive
Open	Cheaper	Contamination more likely
Axenic	Predictable, less prone to crashes	Expensive, difficult
Non-axenic	Cheaper, less difficult	More prone to crashes
Continuous	Efficient, provides a consistent supply of high-quality cells, automation, highest rate of production over extended periods	Difficult, usually only possible to culture small quantities, complex, equipment expenses may be high
Semi-continuous	Easier, somewhat efficient	Sporadic quality, less reliable
Batch	Easiest, most reliable	Least efficient, quality may be inconsistent

Source: Modified from Anonymous, 1991

I. Biodiesel Production

Global warming and excessive use of fossil fuels along with their effects on climate change have led to a growing demand to reduce the dependence on fossil fuel and substitute them with renewable and environmentally friendly biofuels; as a result, a lot of attention has been given to deriving oil from biomass that can be used for production of biofuel. Biofuels are any hydrocarbon produced by or from organic matter over a short period.

In 2013, world biofuel production exceeded 116 billion liters (Figure 3). North central and South American countries were the major producers of ethanol, while the European countries were the major producers of biodiesel (Figure 4). As compared to 2012, global biofuels production in 2013 grew by a 6.1% (80,000 b/doe), driven by increases in the two largest producers: Brazil (+16.8%) and the US (+4.6%). Increased biofuels output in North America, South and Central America and Asia Pacific outweighed declines in Europe and Eurasia (Figure 5). Global ethanol production increased 2.4 million tons (mtoe) (+6.1%), the first increase in two years. Biodiesel production increased 1.2 mtoe (+6.2%), despite the declines in South and Central America, Europe and Eurasia (Figure 4).

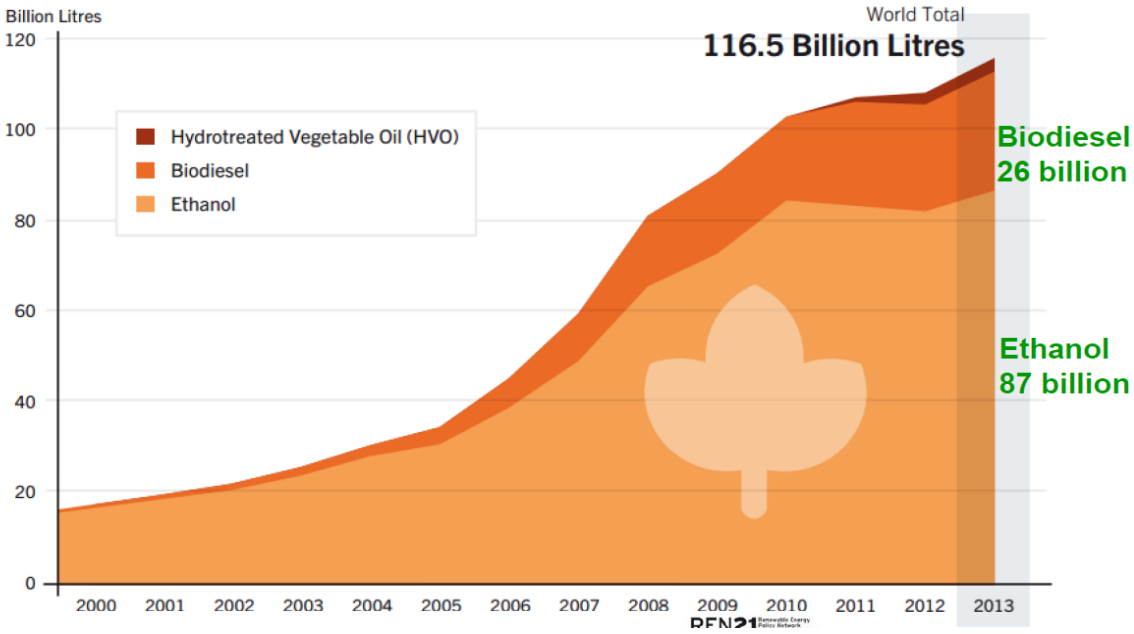


Figure 3. Ethanol, Biodiesel and HVO Global Production from 2000-2013 (REN21. 2014)

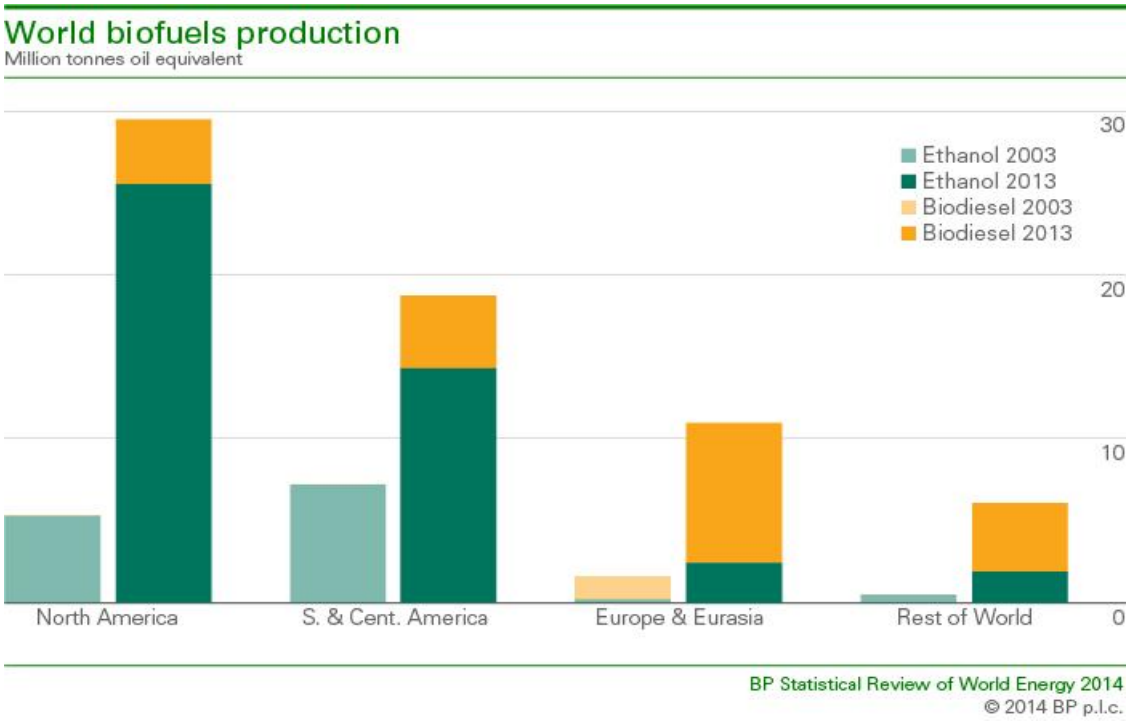


Figure 4. World biodiesel and bioethanol production in 2003 - 2013 (BP Statistical Review of World Energy 2014).

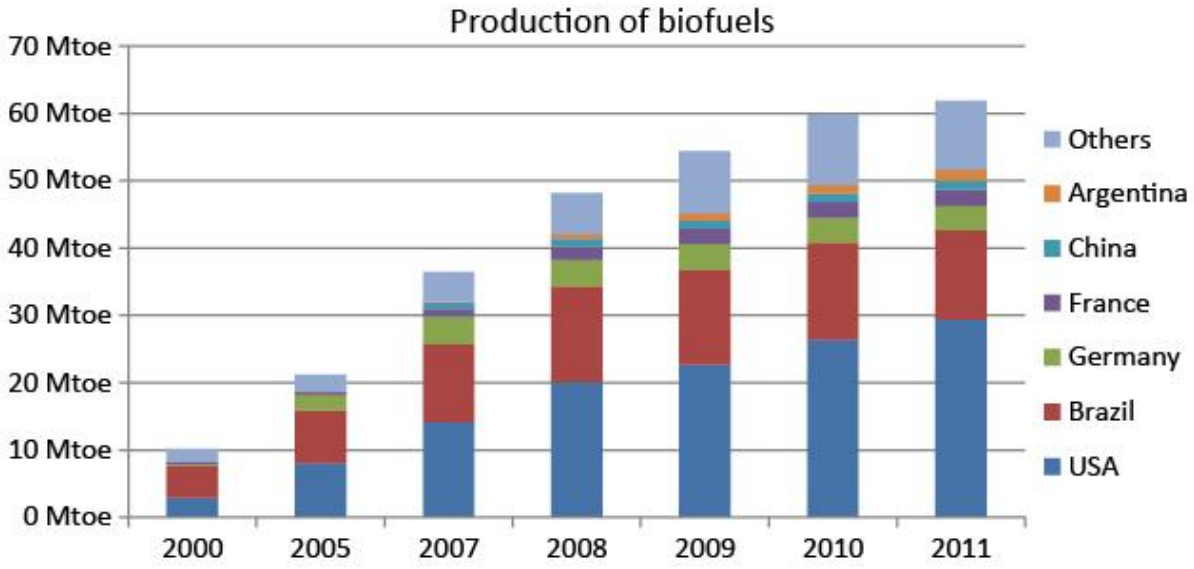


Figure 5. Production of biofuels 2000 - 2011

Depending on feedstock used for the production, three generations are available: first, second, and third generation biofuels.

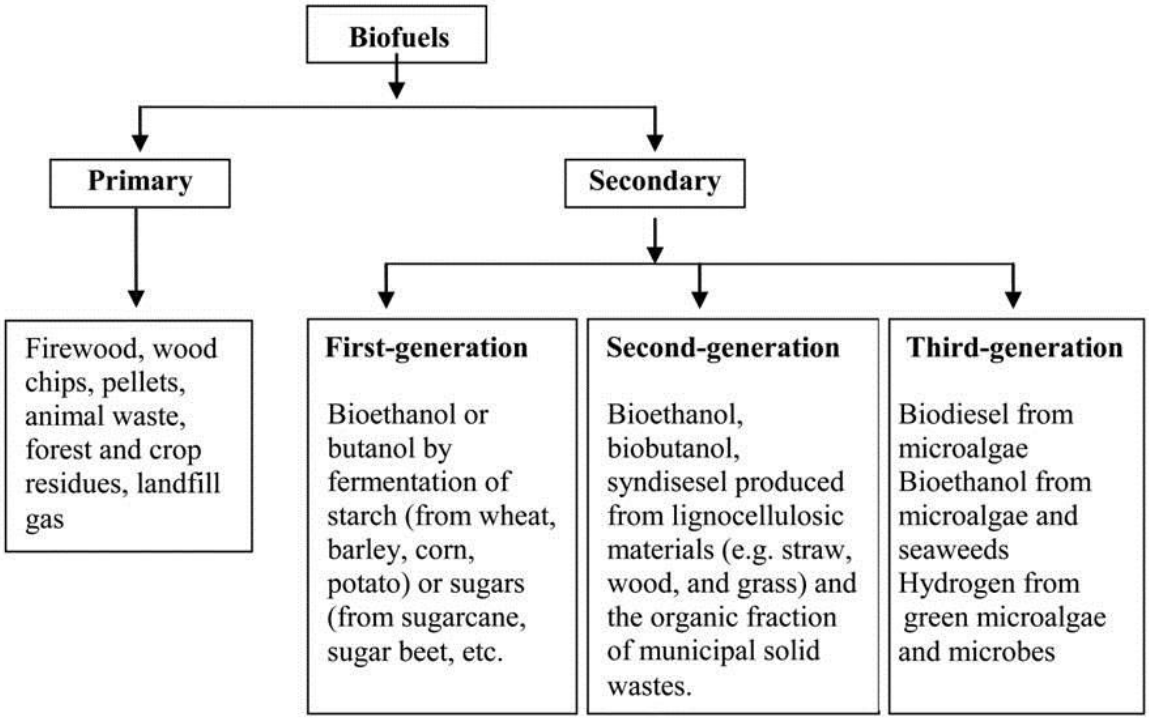


Figure 6. Biofuels generations.

First generation biofuels are the dominating biofuels in the current market. This category includes both bioethanol and biodiesel produced from sugars, grains or seeds, where starch and sugar crops are used for bioethanol and vegetable oils are used as feedstock for biodiesel production. Conventional methods of fermentation and esterification are used in the production of first generation biofuel. First-generation biofuels are being produced in significant commercial quantities in a number of countries. The US has become the largest producer having recently overtaken Brazil. The sustainability of the first-generation biofuels is questionable because of the conflict with food supply (Giselrød *et al.* 2008) since they compete with food, food crops, cultivated land and water resources, as well as it have a high cost of production and indirect utilization of fossil fuels. These limitations favored the search for non-edible biomass for the production of biofuels, hence the emergence of the second generation.

Second generation biofuels are a more promising alternative that uses more advanced technologies than first generation biofuels and are usually produced from either non-edible residues of food crops or non-edible plant biomass such as grasses and trees grown on poor lands usually not suitable for food crops and are processed through biological or thermochemical processing. The second generation limits the direct competition for food, increases the land use efficiency when compared to first generation fuel and opens the door for genetically modified organisms that can be specifically bred for energy purposes, thus increasing production per unit land area and increasing availability of plant material to be converted to biofuel. Although second generation biofuels hold more potential than first generation biofuel, it is evident that the second generation biofuels require more sophisticated processing production

equipment, more investment per unit of production and larger-scale facilities to conform with the economics of large scale production (Nigam and Singah, 2011).

The third generation is directed toward biofuels derived from algae. Algae were initially studied as a potential replacement fuel source for fossil fuels in the 1970s during the gas scarcity period (Barkley *et al.* 1987), but the high cost of production and other limitations discouraged the commercial development of algae-based fuel production. However, in the recent decade, drastic rises in oil price and climate concerns have rejuvenated the attention towards algae research.

Biodiesel has arisen as a possible alternative for petroleum diesel because of the similarities that biodiesel has with petroleum diesel. Since most of the natural oil produced by microalgae is in the form of triacylglycerol, which is the essential type of oil for producing biodiesel, microalgae are the exclusive focus in the algae-to-biofuel arena (Danielo, 2005). When compared to fossil diesel, biodiesel has advantages in that it is renewable, biodegradable, and produces less SO_2 and particulate emissions when burned besides not requiring engine modification for its usage (Sheehan *et al*. 1998). In the first and second generation biofuels, only a fraction of the crop was used for biofuel production; thus the third generation biofuels give further value to the process and reduce waste.

Currently, algal biofuel production has not been commercialized due to the high costs associated with production, harvesting, preparation for traditional extraction methods and transesterification into fatty acid methyl esters (FAMEs). Once the lipids from the algae are extracted, the transesterification reaction to make biodiesel is the same as when using vegetable oils, which has been in use for a number of years (Apt

and Behrens, 1999). However, most methods used to extract the oil from algal biomass rely on a dry biomass product.

Microalgae possess a simple structure and can accumulate carbohydrates and lipids under certain conditions; therefore, they are a potential feedstock to be used in bio- refineries. Microalgae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass (Sharif *et al.* 2007), biodiesel derived from microalgal oil (Thomas, 2006), and photobiologically produced biohydrogen (Gavrilescu and Chisti, 2005).

As a potential feedstock for biofuels, microalgae provide several advantages compared to terrestrial plants. The most important is that algae are non- competitive with the food resources. The annual productivity and oil content of microalgae is far greater than seed crops because of their ability of all year round production, hence oil productivity of microalgae cultures exceeds the yield of the best oilseed crops (Table 7). Moreover, it should be noted that the biochemical composition of the microalgal biomass can be modulated by varying growth conditions, hence, the oil yield may be significantly enhanced (Qin, 2005).

Table 7. Comparison of some sources of biodiesel.

Row Material	Oil Content (% in Dry Weight Biomass)	Output (L oil/ha year)	Land Used (m^2 year/kg Biodiesel)	Water Footprint (m^3/ton)	Production Cost (US$/L)	Acid Value of Oil	Biodiesel Yield (%)
Soybean	18	636	18	4200	0.40 – 0.60	0.2	90
Rapeseed	41	974	12	4300	0.99	2.0	87
Sunflower	40	1070	11	6800	0.62	0.1	90
Oil Palm	36	5366	2	5000	0.68	6.1	95
Castor	48	1307	9	24700	0.92 – 1.56	4.6	89
Microalgae*	50	97800	0.1	591 - 3276	3.96 – 10.56	8.9	60

Source: Gonzalez & Kafarov, 2001.

Although they grow in aqueous media, microalgae need less water than terrestrial crops, therefore reducing the load on freshwater sources (Dismukes *et al.* 2008). Microalgae require relatively less light intensity, can tolerate a wide variety of waters, and have high biomass productivity due to their fast growth; generally, the maximum doubling time during exponential growth for microalgae can reach up to 3.5 hrs.

Oil contents of microalgae vary usually between 20-50% of the dry weight (Table 8). In some strains, oil content can reach as high as 80% (Metting, 1996).

Table 8. Lipid content of several species of marine and freshwater microalgae.

Strains of marine and freshwater microalgae	**Total lipid content (% in dry weight biomass)**
Ankistrodesmus sp.	24 – 31
Botriococcus braunii	25 – 75
Chaetoceros calcitrans	14.6 – 39.8
Chlorella emersonii	25 - 63
Chlorella protothecoides	14.6 – 57.8
Chlorella vulgaris	5 – 58
Chlorella sp.	10 – 48
Chlorococcum sp.	19.3
Dunaliella sp.	17.5 – 67
Dunaliella tertiolecta	16.7 – 71.0
Hemmatococcus pluvialis	25
Isochrysis galbana	7 – 40
Nannochloris sp.	20 – 56
Nannochloropsis oculata	22.7 – 29.7
Nannochloropsis sp.	12 – 53
Neochloris oleabundans	29 – 65
Pavlovalutheri	35.5
Phaeodactylum tricornutum	18 – 57
Porphyridium cruentum	9 – 60.7
Scenedesmus obliquus	11 – 55
Spirulina maxima	4 - 9

Source: modified from Mata *et al.,* 2010.

Microalgae are considered a sustainable, environmental friendly alternative source for diesel because they are naturally carbon neutral and can clean the environment by taking CO_2 from the atmosphere as part of their growth requirements;

according to Chisti (2008), approximately 183 tons of CO_2 is required to produce 100 tons of algae biomass.

If fresh water is used without recycling, in order to achieve 1 kg of biodiesel from microalgae biomass, it would require about 3726 kg water, 0.33 kg of nitrogen and 0.71 kg of phosphate, however these nutrients (especially nitrogen and phosphorus) can be obtained from wastewater; therefore, apart from providing growth medium, there is dual potential for treatment of organic effluent from the agri-food industry (Cantrell, 2008).

Although algae biofuel promises sustainability relative to the first and second generations of biofuels and ethanol, this biotechnology is still at its infancy due to the high expenses regarding construction material for cultivation system, proper agitation, CO_2 administration, and supply of large amount of water and nutrient resources. Indeed, microalgal oil cost is currently considerably higher than soybean oil cost (table 7), due to the existing infrastructure for terrestrial crops and the lack of investment in the culture of large scale microalgal aquatic organisms (Eriksen, 2008).

Currently only around 5,000 to 10,000 tons of algal biomass is commercially produced worldwide. Most of the experts believe that production of microalgae biofuels will not achieve its full commercial scale until 2020, and that from 2021 until 2030 it could represent from 1% to 5% of the worldwide fuel consumption (Ribeiro *et al.* 2015). Analysis by Chisti (2007) concludes that the use of photobioreactors and genetic and metabolic engineering to improve the algae biology are necessary to achieve economical production of biodiesel from microalgae.

J. Potential Uses of Microalgae Biomass

1. Commercial microalgae production

Though commercial utilization of algae biomass dates back to the fifteenth century, the first use of microalgae as food supplement dates back to 2000 years ago when people ate edible algae like *Nostoc* to survive. Over the years, with the growing knowledge on algae, microalgae biomass has been commercialized for a wide array of uses including: human food or food supplements, feed additives, fish meal in aquaculture, manure and fertilizer in agriculture, in paint, in paper textile industries, in the food industry (extracts from marine algae like carrageenan, alginic acid, agar) and as filtration and polishing materials from diatomaceous earth (deposits of diatom frustules). Moreover, they are being used in cosmetics, pharmaceuticals, antifungal, antibacterial or antiviral compounds, etc.

a. Food and feed additives

Microalgae are often used as food for people and feed for animals due to their nutritional value. Microalgae are a rich source of protein, carbohydrates, lipids including omega 3 FA, fiber and enzymes. Besides, many vitamins and minerals like vitamin A, C, B1, B2, B6, niacin, potassium, iodine, iron, calcium and magnesium are abundantly found in microalgae. Nevertheless, many species are known to have double the protein content (up to 60%) when compared to the traditional protein supplements like meat or eggs, and contain essential amino acids that are responsible of the major metabolic processes such as energy and enzyme production, high amounts of simple and complex carbohydrates which provide the body with a source of additional energy, an extensive fatty acid profile, including Omega 3 and Omega 6 as well as an abundance

of vitamins, minerals, and trace elements, hence, they are being cultivated to be used as food or food supplements. For instance, *Spirulina* is often used commercially as a nutritional supplement because it has high protein content and other nutrients used as a food supplements to treat for malnutrition.

Microalgae are also used as animal feed or feed supplements. Improvement in growth rates, carcass quality and coloration, increase in survival rates, reduction in the requirement for medication and higher immunity are the main benefits associated with the use of feed containing microalgae biomass. However, the high cost of most of these algae may limit their commercial uses to few applications. Algae additives can be used for poultry, pets, and ruminants however, the main application of microalgae as feed additives is in aquaculture.

The results of several research studies showed that microalgae as dietary additives contribute to an increase in growth and feed utilization of cultured fish due to efficacious assimilation of dietary protein, improvement in stress response, physiological activity, disease resistance, starvation tolerance, and carcass quality. Moreover, several microalgae such as *Chlorella* and *Spirulina* are being used in chicken feed in low amounts (around 5% only). Chicken fed a diet supplemented with microalgae are reported to have a better health due to the enhancement of the immune function (Belay *et al*. 1996). Egg yolk of chickens fed with algae tended to have reduced cholesterol levels (by 10%) and increased linoleic acid and arachidonic acid levels (by 29% and 24%, respectively). In addition, the color of egg yolk became darker, indicating that higher carotenoid content (2.4 fold higher).

Prior to commercialization, microalgae must be tested for the presence of toxic compounds; some microalgae may contain toxins and heavy-metal components that might constrain microalgae incorporation as dietary supplements.

b. Pharmaceuticals

Algae biomass is a rich source of biologically active primary and secondary metabolites that are difficult to produce by chemical synthesis and that can be used commercially. The use of microalgae and especially the cyanobacteria for pharmacologically active compounds and antibiotics is receiving much interest; some of the pharmaceutical products derived from algae include drugs, therapeutic proteins, neuroprotective products, antimicrobials, etc. Different important biomolecules, such as antioxidants, has been isolated from cyanobacteria (Patel *et al.* 2006). The antioxidant activity of phycocyanin isolated from three cyanobacterial species *Lyngbya*, *Phormidium* and *Spirulina* sp. was studied and the importance of antioxidants to prevent or inhibit cancer in humans and animals was reported as in the case of *Arthrospira platensis*.

c. Anticancer and antiviral agents

Microalgae have received attention as potential antiviral and anticancer agent suppliers due to their primary and secondary metabolites. Several studies claimed the importance of marine algae as antiviral and antioxidant compounds.

As a result of drug resistance and mutations and the oxidative damage to living cells, there is an increasing interest in discovering new and safe antioxidants and antiviral compounds from natural sources such as plant material. Microalgae that contain

biologically active compounds with different modes of action, such as, anti-microtubule, anti-proliferative, antiviral and antioxidant activities are being screened as potential anti-viral and anti-cancer compounds. These chemicals are predicted to be produced under stressful conditions and low growth rate and released at concentrations large enough to be effective.

Lately, research on the antiviral activities of marine natural products and especially marine polysaccharides are gaining importance and attention. These polysaccharides along with their oligosaccharide derivatives has been proven to have a wide range of bioactivities such as antiviral, antitumor, antioxidant, anticoagulant, immune inflammatory effects and other medicinal properties.

Cyanovirin (CV-N, cyanoviorin-N), a 101 amino acid protein extracted from *Nostoc ellipsosporum* was found to have potent activity against all human immunodeficiency viruses such as HIV-1, M and T tropic strains of HIV-1, HIV-2, SIV (Simian), and FIV (Feline) (Burja, 2001). Mohd Syahril *et al.* (2011) have proved in many studies that *Chlorella vulgaris* and *Spirulina platensis* display anticancer properties towards several kinds of cancers. Moreover, *Chlorella* sp._PR1 was found to be potent against murine melanoma B16F10 cell line at 5.5µg/ml (Pratibha *et al*. 2014).

d. Antimicrobial agents

The infectious diseases caused by bacteria, fungi, viruses and parasites are still the major threat to public health and universal economies despite all the progress in the field of human medicine. Since the emergence of antibiotic resistance by the pathogenic microorganisms and the high cost of synthetic chemicals, the need to develop alternatives for antibiotics arose. Plant-based antimicrobials are considered a potential

substitute as they can serve the purpose with lesser side effects than that associated with the use of synthetic antimicrobials.

Phytoplankton serve as a rich source of several novel biologically active compounds but a very few species have been investigated for their medicinal properties. Recently, microalgae extracts are being considered as potential antimicrobial agents effective against a wide range of important bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*, *Streptococcus pneumonia* and many others. Medina-Jaritz (2006) demonstrated that the aqueous extract of *Arthrospira maxima* was antagonistic to *Proteus vulgaris, Staphylococcus aureus, and Escherichia coli* but not to *Bacillus subtilis*; though, the methanol extracts showed better antimicrobial activity against all bacteria used. It was also proven that cell extracts and extracts of the growth media of various unicellular algae (e.g. *Chlorella vulgaris* and *Chlamydomonas pyrenoidosa*) have in-vitro antibacterial activity against both Gram-positive and Gram-negative bacteria.

2. Major uses in agriculture

Microalgae play a major role in agriculture by acting as bio-control for agricultural pests, building of microbiological crust, treatment of agricultural wastewater, and reducing erosion by regulating the water flow into soils. In addition, after the death and decay of algae, they can be incorporated in the soil acting as a source of organic matter and as binding agent for soil texture, thus increasing the humus content and making it more habitable for other plants (Marathe and Chandhari, 1975). Moreover, these microscopic organisms improve the growth and development of the crops because they produce a variety of biologically active compounds, contribute to

soil fertility in many ecosystems as well as they have a higher efficiency in bio-absorption of heavy metals (bioremediation) (Ibraheem, 2007).

Like other organisms, algae which are found in different soil types, may help to improve the soil characteristics such as, carbon content, texture, aeration (Ibraheem, 2007) and also nitrogen fixation (Hamed, 2007). A quantity of 2 and 3 grams dry biomass per kg soil can improve soil fertility, provides plant nutrients required for obtaining good yields and leads to less environmental pollution (Faheed and Abd-El Fattah, 2008). However, the heavy application of several toxic agrochemicals, especially herbicides, acts as inhibitors of cyanobacterial diazotrophic growth, and in some cases act as mutagenic agents.

This review will focus on the use of microalgae for plant health management

a. Antifungal activity

Cyanobacteria have the ability to produce intracellular and extracellular metabolites. Secondary metabolites from cyanobacteria are coupled with toxic, hormonal, antimicrobial and anti-neoplastic effects (Shweta *et al.* 2011). Cyanobacteria can be promising biocontrol agents for the control of plant pathogenic fungi as they can be easily cultured, can be produced at a lower cost than synthetic fungicides and are eco-friendly as they can improve soil fertility and promote plant growth. Cyanobacteria had been reported as an important agent for the control of many pathogens (Hewedy *et al.* 2000). Kim (2006) reported that *Oscillatoria, Nostoc, Anabaena, Calothrix and Nodularia* exhibited antifungal activity against seven phytopathogenic fungi causing diseases in hot pepper. The fungal inhibitory properties were not specific to cyanobacteria. Green algae as well as other groups of microalgae

showed similar activities. *Chlorella salina*, *Tetraselmis chuii* and *Nannochloropsis oculata* exudates reduced the growth of *Fusarium solani*. The purified active coumpounds of *Spirulina maxima* showed a broad spectrum of antifungal activity against *Penicillium oxalicum* (91%), *Fusarium solani* (65%) and *Rhizoctonia solani* (20%) compared to the tested antifungal drugs (Battah, 2014).

b. Nematicidal

Besides their wide activity as antifungal, antiviral and antibacterial compounds, microalgae showed an important potential as nematicidal biological control agents. The nematicidal effects of algae can be used to prevent or at least reduce the plant root damage caused by nematodes, reducing the serious losses to crop plants, thus positively affecting the gardens and agricultural economy.

Cyanobacteria may produce neurotoxins that induce a change in nematode activity and even in their life cycle by interfering with their mobility, hatching processes and by causing mortality of second stage juveniles (Holajjer *et al.* 2013).

Shawky *et al.* (2009) studied the nematicidal effect of nine culture filtrates of algae against the root knot nematode, *Meloidogyne incognita* in cucumber. High percentage of juvenile mortality was reported during all exposure periods of all treatments, the best results were after 72 hrs of exposure.

c. Plant growth promotion

Blue green algae have the ability to secrete plant growth hormones including cytokinin-like substances, auxins like substances and gibberellic like substances. These microalgae have been reported to profit plants by producing growth-promoting

regulators, amino acids, vitamins, polypeptides, antifungal, and antibacterial substances that exert phytopathogen bio-control and polymers, especially exopolysaccharides, that improve soil structure and exoenzyme activity.

A gibberellin-like substance was isolated from the cyanobacterium *Phormidium foveolarum* (Gupta and Agarwal, 1973). Moreover, an auxinic activity and characteristics similar to IAA were identified in an exudate from *Nostoc muscorum* (Caire *et al.* 1979). Likhitkar and Tarar (1995) found that the germination rate along with total length of seedlings and radicels were increased after soaking cotton seeds in different concentrations of exudates of *Nostoc muscorum*; this cyanobacterium had a positive effect on seed germination of sorghum, wheat, maize and lentils in both live algal filtrate (exogenous) and boiled algal extract (endogenous) (Adam, 1999).

d. Biofertlizers

Algae play a major role in agriculture where they are used as biofertilizer and soil stabilizers. One way to positively influence soil structure and nutrient content is by adding cyanobacteria (Rogers and Burns, 1994). Application of algal biofertilizers is also used for the reclamation of marginal soils such as calcareous and saline-alkali soils (Hedge *et al.* 1999).

Biofertilizers add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus and enhancing plant growth through the synthesis of growth promoting substances. Seaweeds are utilized as biofertilizers, resulting in less nitrogen and phosphorous runoff compared to that from the use of livestock manure; this in turn, improves the quality of water flowing into oceans and rivers. Algae excrete extracellular phosphatases almost immediately upon the onset of P limited conditions (Healy, 1973);

they can excrete substances to enhance nutrient availability. Moreover, algae can excrete other compounds and change the pH of their surroundings, which in turn can turn adsorbed P available (Grobbelaar, 1983). In addition, algae can store resources like phosphorus in excess of their immediate needs.

Algae, mainly cyanobacteria, might be the most important nitrogen-fixing agents in many agricultural soils (Rodrigo and Eberto, 2007). Diazotrophic cyanobacteria are known to have the ability to reduce atmospheric nitrogen to ammonia.
Blue-green algae are environmentally friendly biofertilizers for rice based cropping systems, being the main components of rice ecosystems, which are easily available and serve as the cheapest sources of natural biofertilizers (Omar, 2000; Ladha and Reddy, 2003). The utilization of cyanobacteria as a biofertilizer for rice fields is very promising but still limited due to fluctuations in quantity and quality of inoculum and in its physiological characteristics in diverse agro ecological regions.

K. Wastewater Treatment

Since the beginning of industrial revolution water pollution has become a problem to humankind especially the industrial and agro-industrial waste that are released in the oceans, rivers and lakes leading to water pollution, affecting the ecosystem and polluting the ground water. Therefore, wastewater treatment plays a major role in living in a healthy environment and ensures the security of water supply for human race. Chemical, physical and biological treatments are used to treat wastewater from the industrial and agricultural effluents.

Secondary treatment of domestic and industrial wastewater still release large amount of nitrogen and phosphorus; these elements play a major role in eutrophication

of oceans, rivers, and lakes and disposal of partially treated wastewater can deteriorate freshwater resources on a global scale (Lau *et al.* 1997). This issue can be solved by growing microalgae in wastewater that serve as feed for the microalgal growth and reduce the cost of production of biomass as feedstock for biofuels.

Research targeting the use of microalgae to treat wastewater have gained importance recently because microalgae need the nutrients available in the wastewater to grow and produce biomass, hence biofuel production. Microalgae have the capacity to reduce chemical oxygen demand (COD), heavy metals, nitrates, phosphate levels and other substrate value (organic matter or nutrients in wastewater, that are converted during biological treatment or that may be limiting in biological treatment), thus treated wastewater can be released in the sea or rivers without causing pollution. In addition, microalgae will accumulate biomass that can be used for the production of biofuels or other products such as bio-fertilizer, animal feed etc.

Wastewaters provide not only water medium but also most of the necessary nutrients needed for cultivation of microalgae evidenced by high algae growth rate and productivity and nutrient removal efficiency. The significant reduction of water and fertilizers costs are expected to tip the economic equation in favor of wastewater based algae-to-fuel approach. Therefore, coupling wastewater treatment with algae cultivation may offer an economically viable and environmentally friendly way for sustainable renewable algae-based biofuel and bio-based chemicals production as well as credits for wastewater treatment since large quantities of freshwater and nutrients required for algae growth could be saved and the associated life cycle burdens could be reduced significantly.

L. Genetic Engineering

Large-scale production of biodiesel is hampered by the availability of few algal strains that can be selectively optimized for both high biomass productivity and high triacylglycerol (TAG) content. Recent research have focused on applying genetic methods and metabolic engineering to microalgae in order to develop organisms optimized for high productivity and energy value, in order to achieve their full processing capabilities. For instance, high oil-yielding microalgal strains are being genetically engineered based on the crucial enzymes that can be targeted for triacylglycerol (TAG) accumulation and molecular understanding of lipid metabolic pathway in microalgae. Moreover, molecular engineering can be used to enhance biomass growth rate, increase photosynthetic efficiency, increase oil content, improve tolerance to extreme temperatures, reduce photo inhibition that actually reduces growth rate at midday light intensities that occur in temperate and tropical zones and reduce susceptibility to photo oxidation that damages the cells.

Genetically engineered microalgae are nearing commercial release for biofuels production.

CHAPTER III

MATERIALS AND METHODS

A. Water Sampling

Seawater samples, 2 L each, were collected from 16 sites across the Lebanese coastal area in summer 2013 and 2014 (Table 9). Four fresh water samples were also collected. The samples collected from "stagnant" water were directly filtered using a 100µm filter to remove any macroalgae or zooplankton. Once in the lab, the samples were transferred to clean 1L Erlenmeyer flasks, amended with either Guilliards F/2 medium (Appendix I) for seawater samples or Bold Basal medium (Appendix I) for freshwater samples. The flasks were closed with hydrophobic cotton plugs and placed next to a window for acclimatization. After observing slight blooms, the water samples were transferred to a growth chamber equipped with daylight lamps with a photoperiod of 16/8 hrs.

Table 9. Site and date of water samples collection.

Site	Date	Type
Ein El Mreiseh (EM)	9/4/2013	Sea Water
Olympic Stadium - Tripoli	10/6/2013	Sea Water
Qalamun - Tripoli	10/6/2013	Sea Water
Amchit	3/7/2013	Sea Water
Maameltein	3/7/2013	Sea Water
Tabarja	3/7/2013	Sea Water

Safra	3/7/2013	Sea Water
Okaibe	3/7/2013	Sea Water
Bachata	3/7/2013	Sea Water
Monsef	3/7/2013	Sea Water
Sour Port	19/5/2014	Sea Water
Saida Port	19/5/2014	Sea Water
Jiyyeh Marina Port	19/5/2014	Sea Water
Nakoura Port	19/5/2014	Sea Water
Jbeil Port	26/5/2014	Sea Water
Batroun Port	26/5/2014	Sea Water
Broumana	26/6/2013	Fresh Water
Nahr El Kalb	3/7/2013	Fresh Water
Borak Ras El Ein	19/5/2014	Fresh Water
Nahr Ibrahim	26/5/2014	Fresh Water

B. Isolation

1. Non-flagellates

Subcultures were made by inoculating 50 µl of the algal bloom suspension onto either Guilliards F/2 or Bold Basal agar plates. After microalgal growth, the colonies were further streaked and purified by plate streaking technique. The colonies were checked for purity under the microscope; once a unialgal strain was successfully isolated, it was transferred to a 5 ml of enriched liquid media.

2. Flagellates

Tenfold serial dilutions technique was used for the isolation of flagellates. Tubes showing unialgal growth were up-scaled.

3. Water sterilization

Water was filtered using a 0.22µm filter or autoclaved at 121°C and 15 psi for 15 min or longer depending on the volume. The seawater was cooled directly to avoid the formation of precipitates and was left for 24 hrs to equilibrate.

For larger volumes especially those for photobioreactors and raceway ponds, water was chlorinated using 5 ml of Clorox per 20 L of seawater and then de-chlorinated using sodium thiosulfate at a ratio of 0.2 g per 20 L of water.

4. Up-scaling

The up-scaling of pure strains of selected species of microalgae started from the 5 ml enriched tubes. This small volume was increased to 20 ml→ 100 ml → 500 ml →2L flasks →10L gallons. In culture up-scaling, the new vessels should be inoculated with a sufficiently high microalgae density in order to ensure a rapid growth and to limit the risk of contamination with different algae or other micro-organisms. Therefore, an algal inoculum of 15-20% was usually added. The new flasks containing the cultures were stoppered with sterile hydrophobic cotton and placed in a growth chamber at a photoperiod of 16/8 hrs.

5. Stock cultures

To maintain microalgae stocks, an inoculum of 1 ml of microalgal suspension was transferred under aseptic conditions, to 12 ml sterile tubes containing 9 ml culture media. Tubes were stored in an environmentally controlled refrigerator at low light intensity (50 lux) and a temperature of 15-17°C. Sub-culturing was performed twice every month.

6. Microscopic observations

Microalgae cells were observed under a microscope (Carl Zeiss AxioLab A1) at 400X or 1000X magnification. Diameter of the microalgal cells was recorded based on the average of 10 cells.

C. Molecular Diagnosis

1. DNA extraction

An aliquot of 15 ml of microalgal suspension was harvested at mid to late exponential phase by centrifugation at 5000 rpm for 5 min and the pellet was retained. Cells were disrupted with liquid nitrogen. 800 µl extraction buffer (Tris–HCl buffer, 2 % acetyl trimethylammonium bromide (CTAB), 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, 1 % polyvinylpyrrolidone (PVP), 2 % β-mercaptoethanol) were added and incubated at 60°C for 20 min. The debris and impurities were separated using 600µl iso-amylalcohol : chloroform (24:1). Following vortexing and centrifugation at 10,000 rpm, the supernatant was transferred to a new tube and an equal volume of isopropanol was added and the mixture was kept at -20°C for 1 hr and then centrifuged.

The obtained pellet was then rinsed with 75 % ethanol and eluted in 50 µl of molecular grade water (Sigma-Aldrich, MO, USA).

Extracted DNA was analyzed for purity by electrophoresis in 1 % agarose gel and by NanoDrop 2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2. PCR

The DNA extracts were amplified by PCR using several primers targeting different genes or gene loci (table 10). A 20 µl PCR reaction mixture was prepared using 10 µl of ReadyMixTM PCR Reaction Mix with MgCl2, 0.5µl of each primer (10µM) and 2µl nucleic acid template. The thermal cycling parameters were performed as described in the literature for each primer pair. A normal PCR consists of initial denaturation at 95°C for 3 min, followed by 35 cycles of 30 sec at 94°C, annealing temperature varied with the primer pair (Table 10), the extension step was at 72°C for 30-60 sec with a final extension step at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 1% agarose gels prepared with 0.5x TAE buffer. Amplicons were visualized under UV light. The PCR products were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). Sequencing of the purified amplicons in both forward and reverse directions was performed at the University of Washington High-Throughput Sequencing facility; BLASTN analysis (http://www.ncbi.nim.nih.gov/BLAST/) was performed to determine the homology to the most closely related sequences.

Table 10. List of primer pairs used in this study showing their sequences, target gene or gene locus, annealing temperature and the expected amplicon size.

	Sequence	Target Gene	Purpose	Expected Size	Annealing temperature (°C)	Reference
T16N	AMAAGTACCRYGAGGGAAAG	Nuclear D2/D3 region of the large ribosomal subunit (LSU D2/D3)	Universal	567	50	Harper and Saunders 2001
T24U	SCWCTAATCATTCGCTTTACC					
tufGF4	GGNGCNGCNCAAATGGAYGG	Gene tufA (encodes the elongation factor Tu)	Chlorophyta	807	45	Fama et al. 2002
tufAR	CCTTCNCGAATMGCRAAWCGC					
ITS 1	AGGAGAAGTCGTAACAAGGT	Internal transcribed spacer of the ribosomal cistron	Chlorophyta	NR*	52	White et al., 1990 (modified)
ITS 4	TCCTCCGCTTATTGATATGC					

NR*: not reported

D. Growth Kinetics

Microalgae cultures were grown in 3L photobioreactors, 5L gallons or 25L flat photobioreactors. The cultures were incubated in a growth chamber at room temperature, with an illumination for 16hr light at 450 lux and 8 hrs of darkness. Samples were collected every day and their optical density was measured at 560 nm. . Samples for the dry matter were also collected at 0.2, 0.4, 0.6 and 0.8

E. Chemical and Biochemical Profiling

1. Determination of total lipid content

Microalgae were harvested at late exponential stage by centrifugation (5000 rpm for 10 min). The harvested cells were placed at -20°C for some time and then freeze dried overnight (LABCONCO lyophilizer, USA). Total lipid content was determined gravimetrically using the Folch method (Folch *et al.* 1956). A solvent mixture of dichloromethane: methanol (2:1) was added to the freeze dried algal cells. Tubes were placed in an ultrasonic bath for an hour (at 40ºC) then vortexed well. Homogenates were then filtered into new screw capped tubes and 8 ml of water were added for 20 ml of solvent mixture. After vortexing for few seconds, the mixtures were centrifuged for 10 minutes at 4000 rpm to allow phase separation. The upper phase was discarded while the lower phase representing the lipids was recovered. To the lower phase, 3 g of magnesium sulfate were added on the filter for moisture absorption. The mixture was then filtered into a new tube and the solvent was allowed to evaporate under normal atmospheric pressure for 24 hrs.

Dry weights of lipids were measured and lipid contents (% in biomass) were determined using the following formula:

Lipid content = (weight of lipids in g/ dry weight of sample in g) x 100

After the extraction processes, the resulting algal oil product was converted into biodiesel through a process called transesterification. The resulting fatty acid methyl esters (FAMEs) were extracted and analyzed by GC-MS.

2. FAME extraction

Lipids previously extracted were dissolved in 2 ml dichloromethane. Methanol (6:1) was added to the sample along with concentrated H_2SO_4. Sample tubes were left in a shaker incubator (Kem lab ®) reactor for 24 hrs at 65°C, with continuous shaking and then were kept at room temperature for cooling. After cooling, 2 ml DCM and 2 ml distilled water were added depending on the sample volume then mixtures were vortexed and left to stand. This allowed the separation of the medium into two layers, the lower layer being the dichloromethane part containing the fatty acid methyl esters obtained during the reaction. The upper layer was discarded while the lower was washed for three times by distilled water to remove any remaining byproduct obtained during the reaction. After washing, 3g of $MgSO_4$ were added to the organic dichloromethane layer in order to remove moisture. After filtering the solution with Whatmann n°1 filter, dichloromethane layer was then transferred to a new pre-weighed vial and was subjected to evaporation under vacuum in a rotary evaporator.

3. GC analysis

GC-MS was used for an efficient separation, identification and quantification of the particular fatty acid methyl esters present in the sample.

The injector of the GC was set at a temperature of 260ºC. 1µL sample volume was injected into the split injector with a dilution split ratio of 100:1. Helium was used as a carrier and was injected in the DB 23 column (RESTEK, U.S.A) at a rate of 30cm / sec (0.54 ml/ min). Initial column temperature was set at 50ºC for 2 minutes, then temperature was increased to 250ºC at a rate of 4ºC / min. Temperatures of the transfer line as well as of the MS were set at 250ºC.

F. Protein Analysis

Protein analysis consists of three main steps: digestion, distillation and titration. The Kjeldahl method was followed: a sample of 0.25 g of dried microalgae were weighed on a nitrogen free filter paper and placed in digestion tube to which 50 ml of concentrated sulfuric acid and catalyst were added. The content of the digestion tube was digested for about 60 min until observing a green color. Tubes were left to cool for 5 min and then 50 ml of distilled water were gently added. In the second step, about 60 ml of NaOH were added to the digested sample to allow the separation of nitrogen from the mixture and obtain ammonia that was trapped in 50 ml boric acid. To quantify the amount of ammonia trapped, titration was performed. A blank and a reference were used as a control. The solution was back titrated with 0.10491 N sulfuric acid until a color change was observed. The volume of acid needed in the titration was used to calculate the crude protein percentage.

Since not all of the nitrogen in microalgae is associated with protein, the nitrogen-to-protein conversion factor value for microalgae is 5.95 for Kjeldahl method and 4.44 for elementary analysis instead of the 6.25 conversion factor.

$$Protein\% = (ml\ H2SO4 - blank) \times 5.95 \times 14 \times normality\ H_2SO_4 \times 0.1 / sample\ weight$$

G. Pigment Analysis

To 20 mg of dried algae, 5 ml of distilled water were added followed by vortexing. The mixture was then subjected to disruption by freezing and defreezing for three times using liquid nitrogen. To ensure a complete disruption, cultures were incubated in the ultrasonic bath for an hour at 40ºC and then freeze dried overnight.

10 ml of 90% acetone or 100% methanol were added to the culture tubes and then vortexed to ensure pigment extraction. Extracts were then filtered over a 0.2 μm cellulose acetate filter (SIGMA) and 3 ml of the extracted pigments were used to measure the absorbance at fixed wavelengths specific for each solvent.

Quantification of pigment concentration was determined based on spectrophotometric readings of methanol or acetone algal extracts at 3 or 4 specific wavelengths depending on solvent used and pigment to be quantified. Then empirical correlations, reported in the literature, were used to calculate the concentration of chlorophylls and carotenoids (Strickland and Parsons, 1968; Jeffrey and Humphrey, 1975; Porra and Lichtenthaler, 1989).

Calculations of pigment concentrations were obtained based on the following formulas:

For extractions based on acetone as a solvent, two correlations for chlorophyll concentrations were followed:

Jeffrey and Humphrey (1975)

$\mu g\ Chlorophyll/\ ml\ medium = (11.85A_{664}-1.54A_{647}-0.08A_{630})\ v/\ (l*V)$

Strickland and Parsons (1968)

$\mu g\ Chlorophyll/\ ml\ medium = (11.66A_{665}-1.31A_{645}-0.14A_{630})\ v/\ (l*V)$

*where *A* is the absorbance (nm), *v* means the volume of solvent used (mL), *l* is the spectrophotometric cell length (1cm) and *V* is the sample volume (mL).

For methanol-based solvents, two correlations were followed to get concentrations for both chlorophylls and carotenoids:

Porra et al. (1989)

$\mu g\ Chlorophyll/\ ml\ medium = (16.29A_{665}-8.54A_{652})\ v/\ (l*V)$

Lichtenthaler (1989)

$\mu g\ Total\ Carotenoids/\ ml\ medium = (1000A_{470} - 44.76A_{666})/221$

*where *A* is the absorbance (nm), *v* means the volume of solvent used (mL), *l* is the spectrophotometric cell length (1cm) and *V* is the sample volume (mL).

In order to obtain the chlorophyll composition following acetone extraction, formulas of Parsons and Strickland (1968) were followed for chlorophyll a, b and c:

$mg\ chl\ a/\ m^3 = 11.6\ A_{665}-1.31\ A_{645} - 0.14A_{630}$

$mg\ chl\ b/\ m^3 = 20.7\ A_{645}- 4.34A_{665} - 4.\ 42A_{630}$

$mg\ chl\ c/\ m^3 = 55\ A_{630}- 4.64A_{665} - 16.3A_{645}$

H. Effect of N and P Supplementation of Culture Media

Microalgae isolates were grown in 3L photobioreactors. Cultures were supplemented with F/2 medium in addition to Ammonium Chloride and Sodium Phosphate Tribasic to reach the same levels of nitrogen and phosphorus present in wastewater (Bashour 2014 ,personal communication) (40 ppm and 3 ppm , respectively). The cultures were incubated in a growth chamber at room temperature, with an illumination for 16hr light at 450 lux and 8 hrs of darkness. Samples were collected every day and their optical density was measured at 560 nm. Samples for the dry matter were also collected at OD readings close to 0.2, 0.4, 0.6 and 0.8

I. Antifungal Activity

The antimycotic activity of different extracts were tested against five plant pathogenic fungi.

1. In vitro experiment

a. Plant pathogens

All plant pathogens used in this study were isolated from infected tomato, eucalyptus, cucumber and apples at the plant pathology laboratory, American University of Beirut (Table 11).

Table 11. List of plant pathogens used and their hosts.

Plant pathogen	**Host**
Fusarium oxysporum	Eucalyptus
Alternaria solani	Tomato
Botrytis cinerea	Tomato
Verticillium sp.	Cucumber
Rosellinia sp.	Apple

b. Microalgae

All microalgae isolates used in this study were isolated from Lebanon. Some are single isolates and some are blooms (Table 12).

Table 12. List of microalgae used for the antimycotic tests

#	Name	Culture Type	Composition	Type
1	Brumana	Single	Chlorophyta (*Acutodesmus obliquus)*	Freshwater
2	DAS	Bloom	Cyanobacteria + Chlorella + Diatom	Seawater
3	II	Bloom	Cyanobacteria + Tetraselmis	Seawater
4	NK2	Bloom	Tetraselmis + Chlorella +Diatoms	Seawater
5	PM	Bloom	Cyanobacteria + Chlorella	Seawater
6	PALS	Bloom	Cyanobacteria + Tetraselmis + Chlorella	Seawater
7	D	Bloom	Cyanobacteria + Tetraselmis + Chlorella	Seawater
8	Spirulina	Bloom	Cyanobacteria + Chlorophyta	Seawater

c. Methanolic extracts

A sample of 1g of freeze dried algae was extracted with 100 ml of methanol for 24 hrs at room temperature. The extract was separated from the cell residues by filtration through Whatman filter paper no.1. The filtrate was then evaporated to dryness in a rotary evaporator (Buchi rotavapor r-114). The dried extract was then weighed and re-suspended in 95% ethanol. The concentration of the extracts was recorded as mg/ml.

Two test methods were used:

1) PDA was prepared and poured into 90 mm Petri dishes. Shortly after solidification, a 100 µl volume of methanolic extract (dissolved in 95% ethanol) was spread uniformly on the plate. Then 20 µl of spore suspensions (10^6 spores/ml) were added at the center. PDA plates amended with 100 µl of 95% ethanol served as negative controls.
2) Agar diffusion test: A volume of 100 µl of spore suspension (10^6 spores/ml) was spread uniformly on PDA plates. Sterile filter discs were placed on the plate and 20 µl of methanolic extracts were added to each disc.

All plates were incubated at 28± 0.5°C. After 4 days, the inhibition zones of fungal growth were recorded.

d. Non methanolic water soluble extract

The residues of microalgal cells that were not dissolved in methanol were collected and dissolved in previously autoclaved and cooled distilled water. 100 µl of spore suspension was spread uniformly on PDA plates. Sterile filter discs were placed on the plate and 20 µl of methanolic extracts were added to each disc. Plates were then incubated at 28± 0.5°C.

e. Crude water extract

Samples equal to 1000 ml of microalgae cultures at the early stationary growth stage (Table 13) were centrifuged at 5000 rpm for 10 minutes. Algal cells were collected and dissolved in 1000 ml of distilled water to which water agar 1.5% (w/v)

was added. The mixture was autoclaved for 5 minutes at 121^0C and then poured in petri dishes at 10 ml/plate. A 20 µl of a spore suspension (10^6 spores/ml) of each of five plant pathogenic fungi was inoculated at the middle of each petri-dish and the dishes were incubated at 28± 0.5°C.

Table 13. Microalgae used for the crude water extracts.

Microalgae	**O.D**	**Dry Matter**
PM	1.32	0.7
DPS	1.65	0.8

f. Cell extracts

Seawater single isolates were used in this experiment (Table 14). 1000 ml of the cultures were centrifuged. Algal cells were collected and dissolved in autoclaved distilled water at a ratio of 1:10 (w/v). Then the extracts were ultrasonicated (in order to rupture the cell wall) until a color change was observed. 100 µl of spore suspension was spread on the agar. Sterile filter discs were inoculated with 20 µl of the cell extracts.

Table 14. Microalgae species used for the cell extracts.

Isolate	**Name**
CM	*Nannochloropsis sp.*
Amshit	*Chlorella sp.*
Damour	*Dunaliella sp.*
JBS	*Tetraselmis sp.*
PM	*Cyanobacterium aponinum*

2. In vivo experiments

Two types of tests were performed to evaluate the effect of microalgal extracts on plant growth promotion: seed treatments and foliar sprays.

a. Seed treatments

i. Microalgae used

Beit Alpha cucumber seeds were soaked in different microalgae extracts and concentrations for 24 hrs (Table 15). The effect of seed treatment was evaluated based on root elongation and on aerial growth.

Table 15. List of microalgae used for the seed treatments.

#	**Name**	**Culture Type**	**Type**
1	Brumana	Single	Freshwater
2	CM	Single	Seawater
3	Amshit	Single	Seawater
4	JBS	Single	Seawater
5	JOS	Single	Seawater
6	PS	Single	Seawater
7	PM	Bloom	Seawater

ii. Root elongation test

Previously soaked seeds were placed on a blotter paper pre-soaked with distilled water and placed in a petri dish. The Petri dishes were incubated at 25 ± 0.5°C for few days. Seeds soaked in water were used as a control. The germination rate and root

length were recorded for all the treatments. Five replicates with five seeds per replicate were used.

iii. Vegetative growth

Pre-soaked Beit Alpha cucumber seeds for 24 hrs were sown in trays and kept at 28°C for two weeks. The percent germination, fresh and dry weights were recorded.

b. Foliar sprays

Two trial were conducted, one on tomato and the second on cucumber.

i. Tomato

Four-week old tomato seedlings (Seminis) grown in trays containing half soil + sand and half potting mix were sprayed with microalgae cell extracts. The sprays were repeated after one week and the plants were harvested 10 days later. Data on fresh weight, dry weights and length were collected.

ii. Cucumber

Beit Alpha cucumber seeds were first planted in trays and then transplanted to 30 cm diameter pots (half soil, half potting mix). When seedlings reached 2-leaf stage, they were sprayed with cell extracts or methanolic extracts of Brumana isolate. Two weeks later, a second spray was applied. The plants were harvested after 2 weeks. The length, fresh and dry weight as well as deficiency symptoms were recorded. Water served as a control for the cell extract whereas ethanol was the control for the methanolic extract.

J. Antibacterial tests

a. Microalgae used

The same microalgae methanolic exctrats used for the antifungal tests were also used for the antibacterial activity (Table 12).

b. Tested bacteria

Escherichia coli, Salmonella enteritidis, Staphylococcus aureus, and *Streptococcus sp*. isolated at the animal science laboratory, American University of Beirut, were used in these tests (Table 16).

Table 16. Concentration of bacteria used (CFU/mL).

Bacteria	**Concentration**
Escherichia coli	29×10^{10} CFU/mL
Salmonella enteritidis	25×10^{11} CFU/ mL
Staphylococcus aureus	15×10^{10} CFU/mL
Streptococcus sp.	5×10^{12} CFU/ mL

c. Extracts

An aliquot of 100 µl of bacterial suspension in the log phase (table 16) was spread on Müller-Hinton agar plates. Sterile filter discs were placed were treated with 20 µl of methanolic extracts. The plates were incubated at 25°C overnight. After 24 hrs, the inhibition zones were recorded. 95% ethanol served as a control.

Chapter IV

RESULTS

A. Microalgae Isolation

Freshwater and seawater samples were collected from different locations across the Lebanese coastal area and were kept near the window under direct light. The time that was required to observe the initial microalgal bloom varied greatly among the collected samples and ranged between one week to two months. The blooms of freshwater samples appeared faster than those of seawater samples.

When clear blooms were observed, 50 µl of algal suspension were streaked on agar plates. Clear growth of the algal colonies appeared within 3-4 weeks. The colonies were re-streaked until a monoculture was established. Serial dilution technique was also used to separate the flagellates. Later, a colony from the plate or 1 ml of the unialgal tube were transferred to a 5 ml nutrient enriched seawater or freshwater, from which the cultures were further upscaled.

A total of twenty five local microalgal isolates (13 seawater and 12 freshwater) along with 3 mixed blooms were successfully isolated and cultured. Microscopic observations at 400x or 1000x magnification are illustrated in Figures 7 to 30.

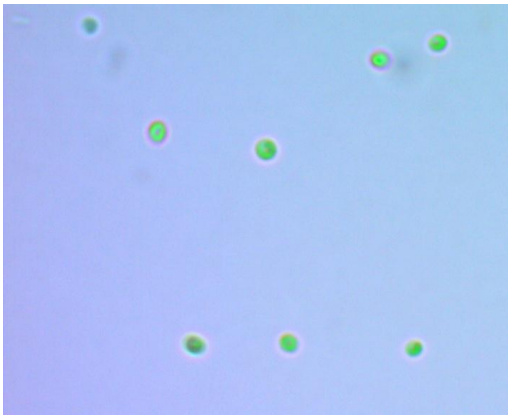


Figure 7. Microscopic observation of Amshit isolate magnified 400x (*Chlorella sp*., 2µm)

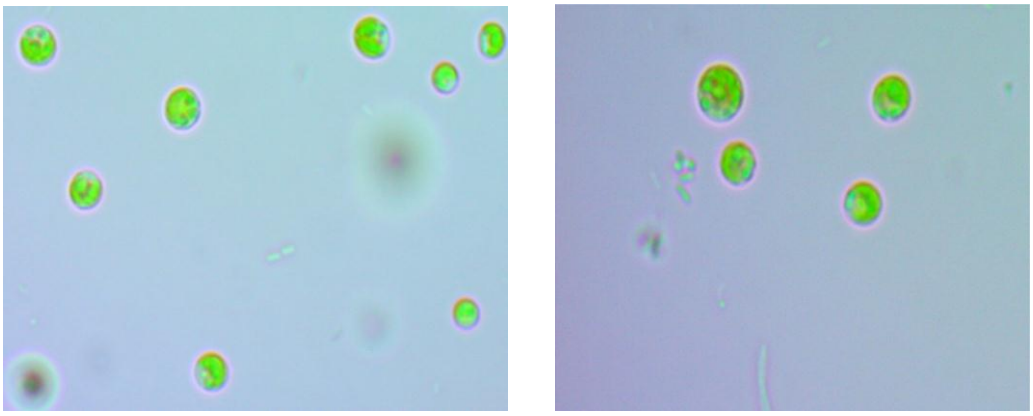


Figure 8. Microscopic observation of PS isolate magnified 1000x (Not identified, 2.5µm)

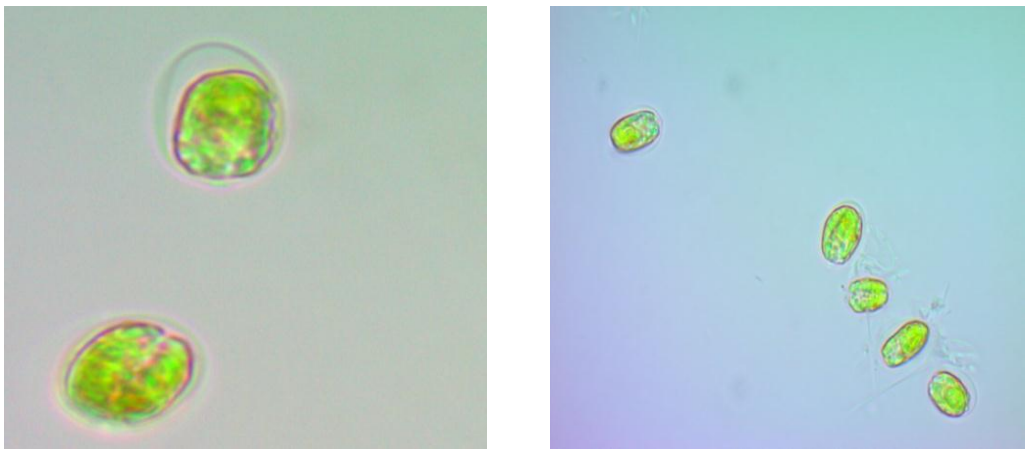


Figure 9. Microscopic observation of JOS isolate magnified 1000x (*Tetraselmis sp.*, 11µm)

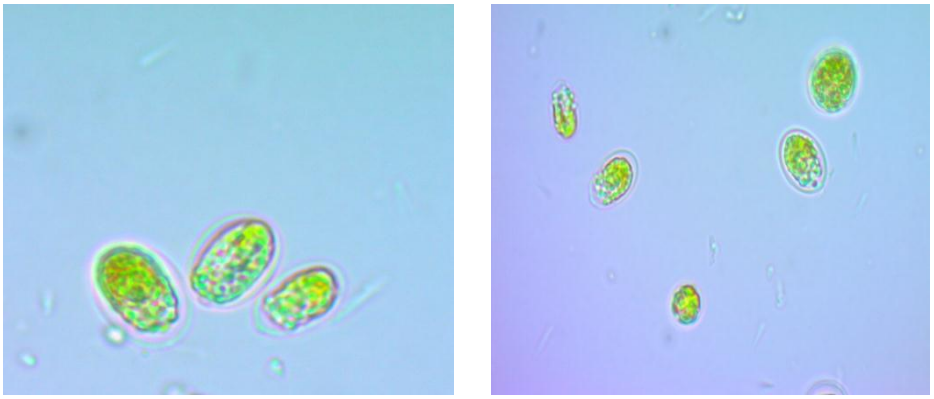


Figure 10. Microscopic observation of JBS isolate magnified 1000x (Not identified, 10-12µm)

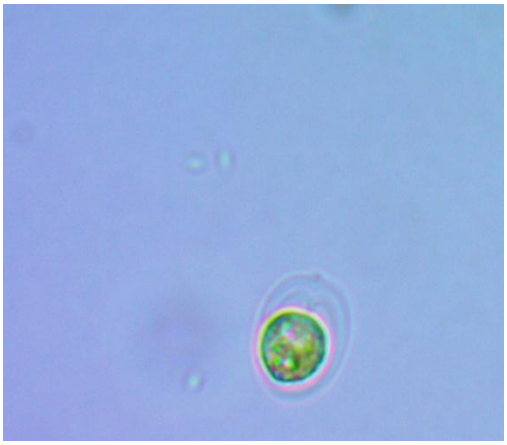


Figure 11. Microscopic observation of UNS isolate magnified 1000x (*Tetraselmis marina*, 7µm)

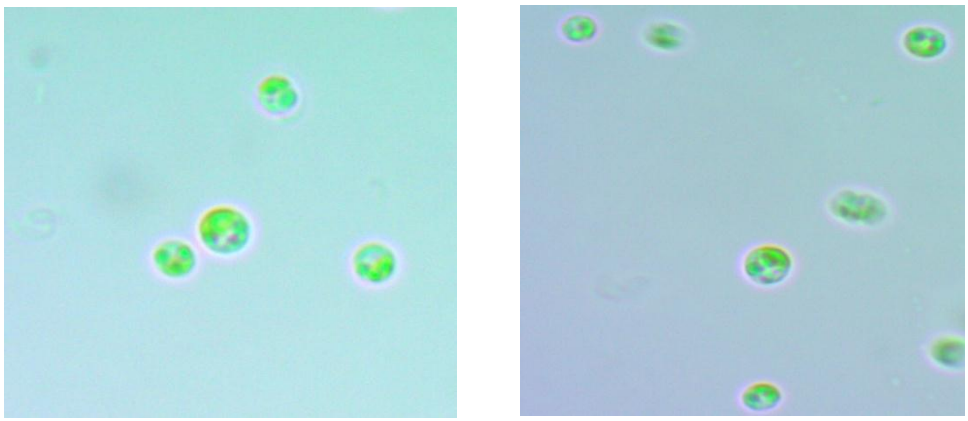


Figure 12. Microscopic observation of NS isolate magnified 1000x (*Chlorella sp*., 2-3µm)

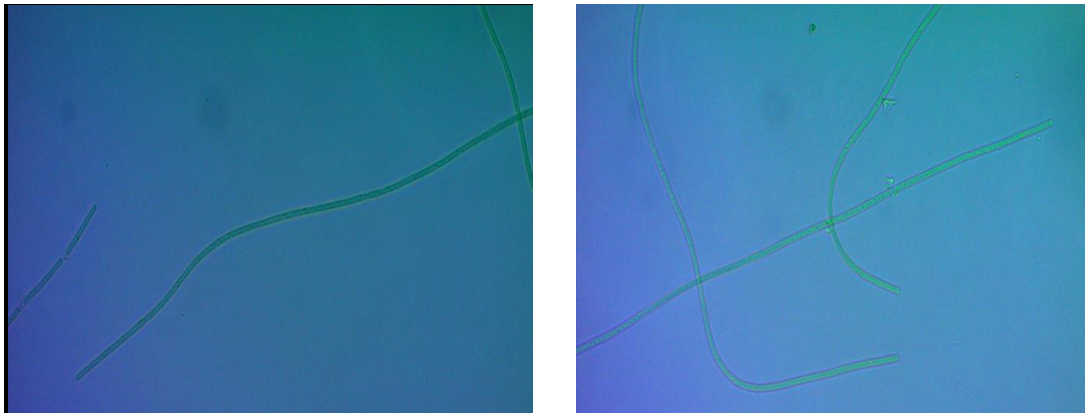


Figure 13. Microscopic observation of CAS isolate magnified 400x

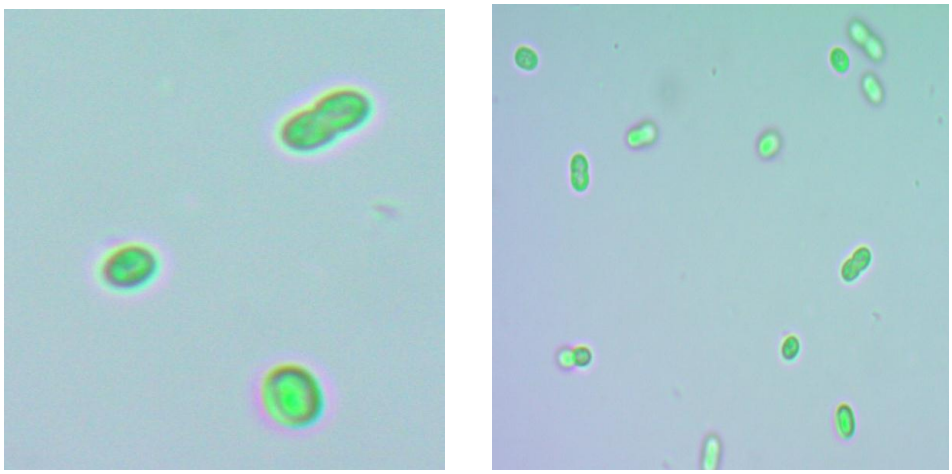


Figure 14. Microscopic observation of DAS isolate magnified 1000x (Not identified, 5-6 μm)

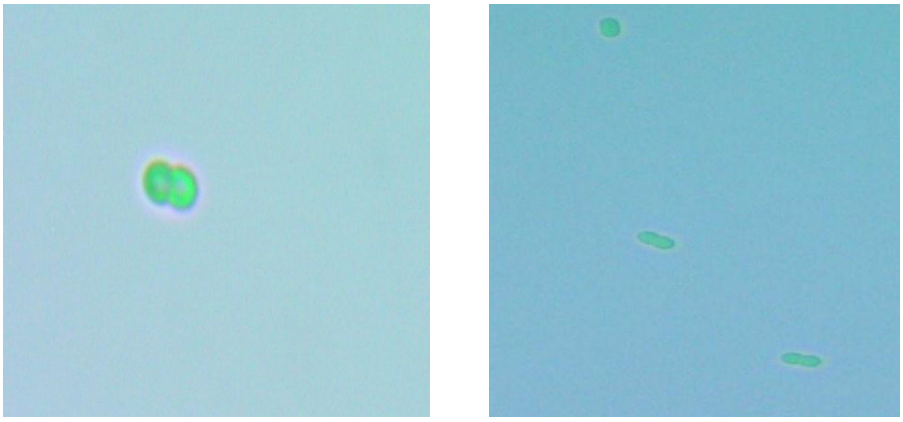


Figure 15. Microscopic observation of EMS isolate magnified 1000x and 400x (*Cyanobacterium aponinum*, 5-6 μm)

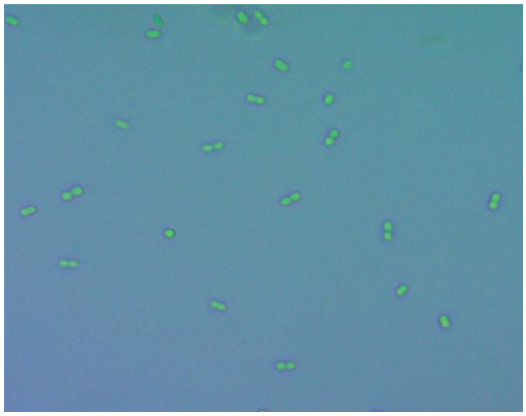


Figure 16. Microscopic observation of PALS isolate magnified 400x (Not identified, 5µm)

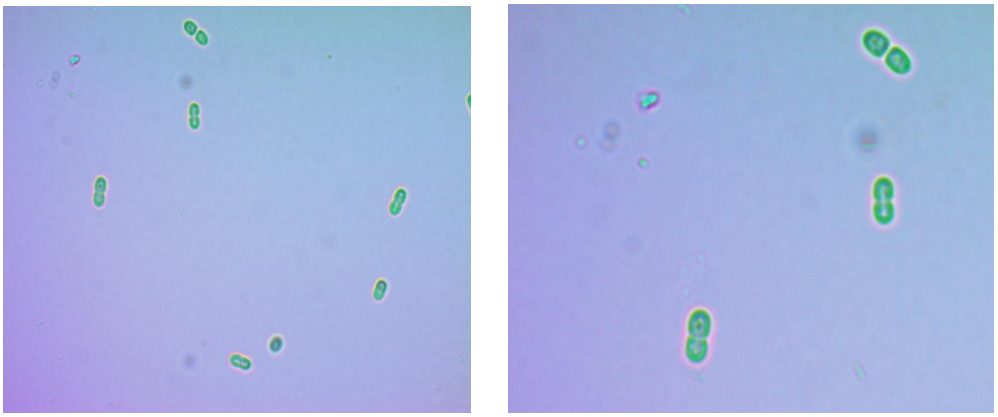


Figure 17. Microscopic observation of JBS 2 isolate magnified 400x and 1000x (Not identified, 5-6 µm)

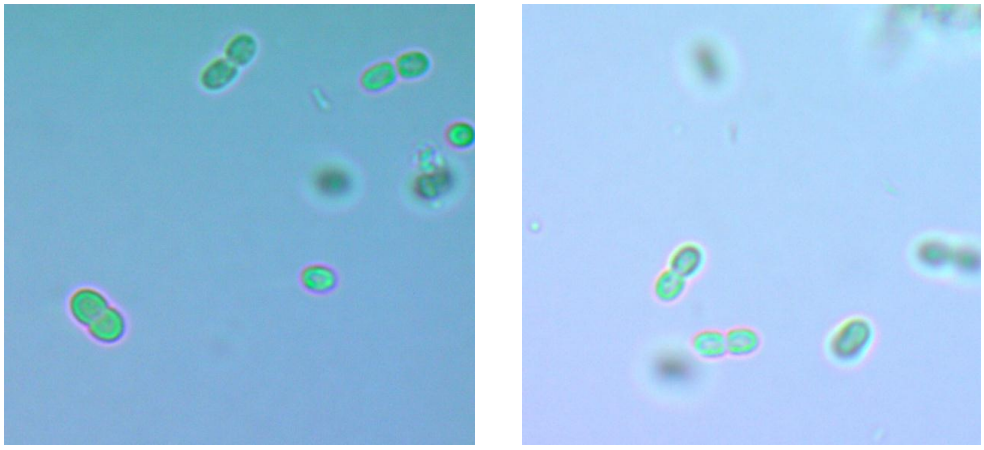


Figure 18. Microscopic observation of PM isolate magnified 1000x (Not identified, 5-6µm)

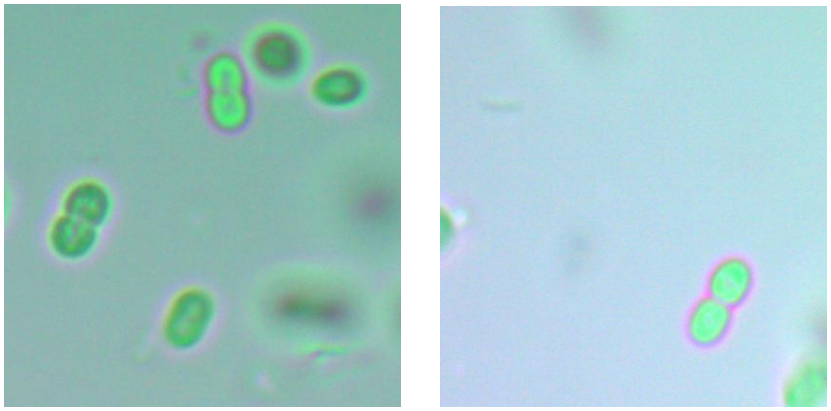


Figure 19. Microscopic observation of II isolate magnified 1000x (*Cyanobacterium aponinum*, 7µm)

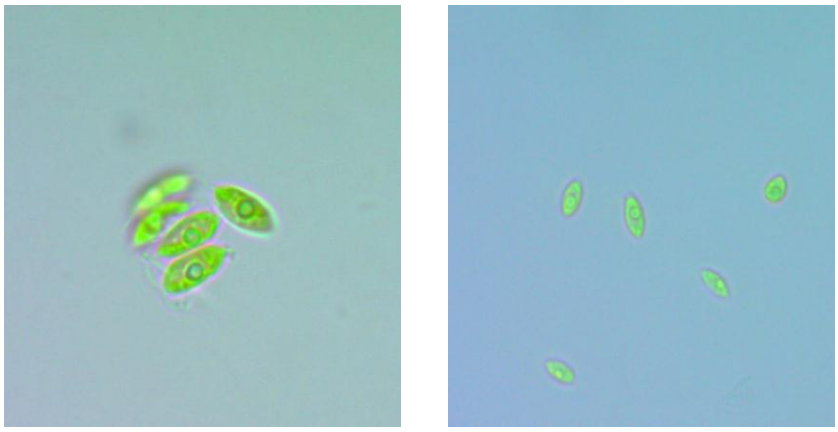


Figure 20. Microscopic observation of BR2 isolate magnified 1000x and 400x (*Acutodesmus obliquus*, 10-12µm)

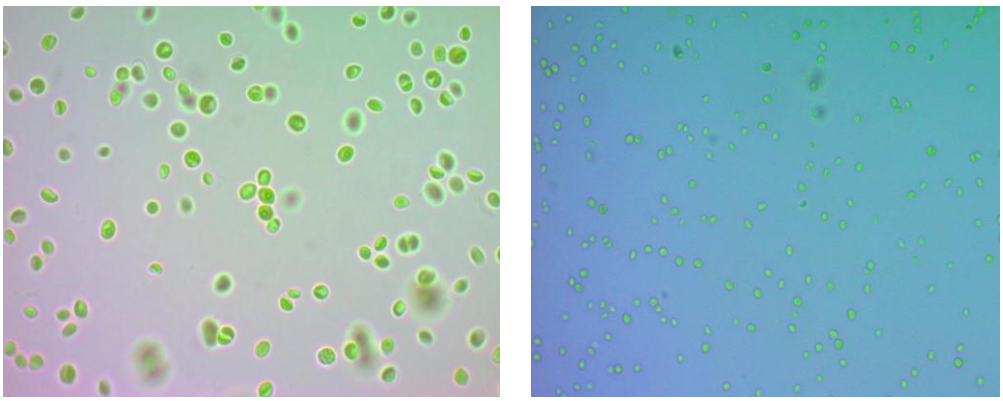


Figure 21. Microscopic observation of BR3 isolate magnified 1000x and 400x (*Micractinium reisseri*, 3µm)

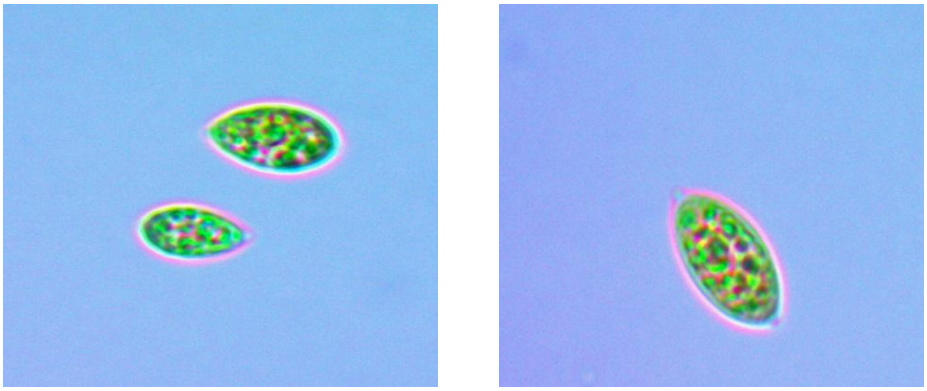


Figure 22. Microscopic observation of Brumana isolate magnified 1000x (*Acutodesmus obliquus*, 10-12µm)

Figure 23. Microscopic observation of BS isolate magnified 1000x (Not identified, 12µm)

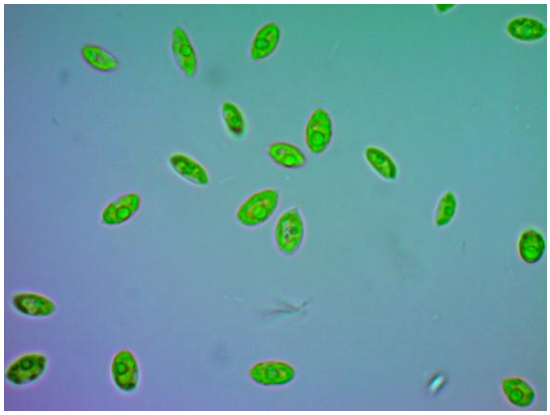


Figure 24. Microscopic observation of NHS isolate magnified 400x (*Scenedesmus sp.*, 10µm)

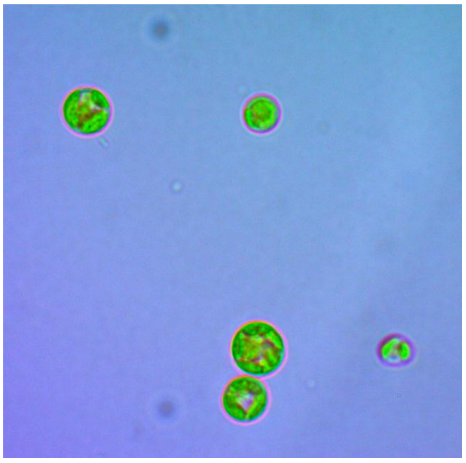


Figure 25. Microscopic observation of MAZ isolate magnified 1000x (*Chlorella sp*., 4µm)

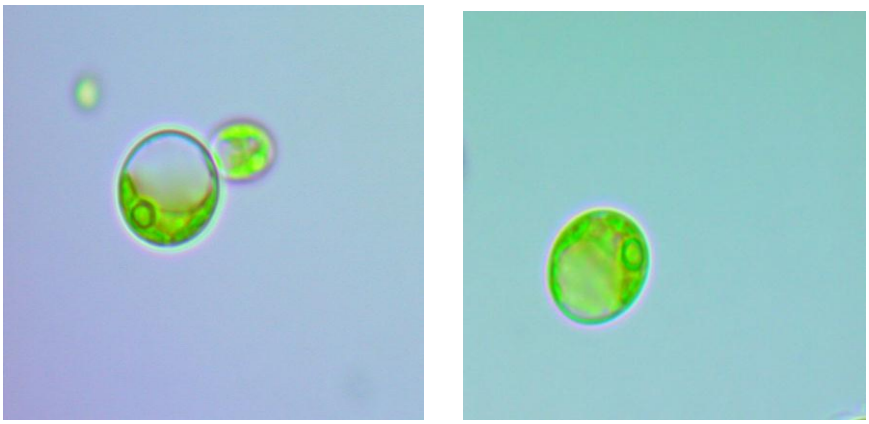


Figure 26. Microscopic observation of RS isolate magnified 1000x (Not identified)

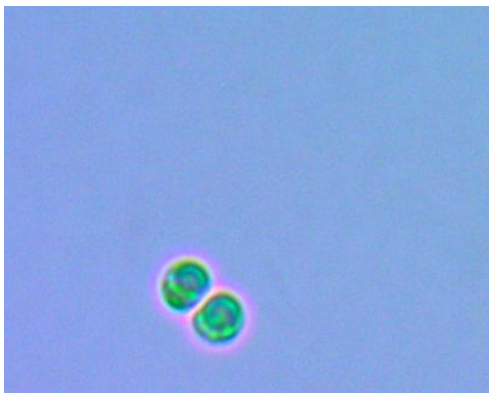


Figure 27. Microscopic observation of M2 isolate magnified 1000x (Not identified, 3-4µm)

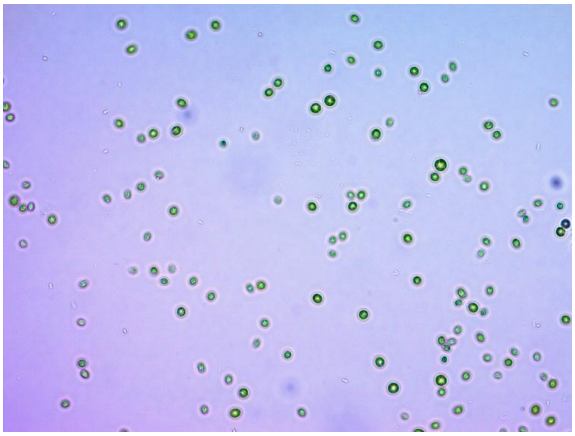


Figure 28. Microscopic observation of MAS isolate magnified 400x (*Chlorella sp*., 2µm)

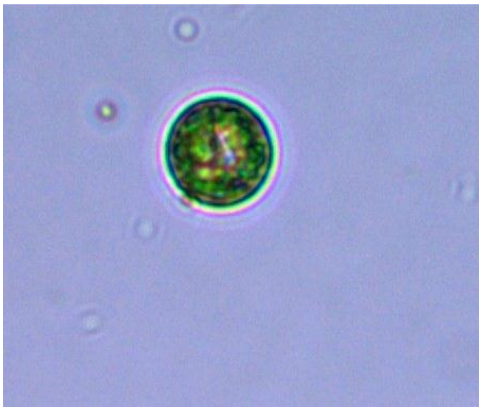


Figure 29. Microscopic observation of M1 isolate magnified 1000x *(Chlorella sp,* 4 µm**)**

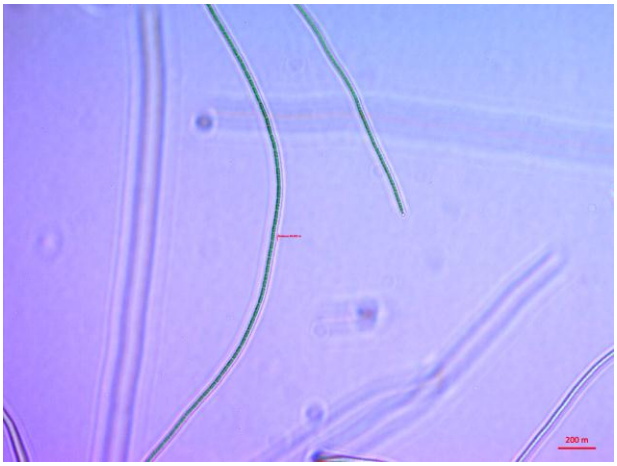


Figure 30. Microscopic observation of EM isolate magnified 400x

B. Molecular Identification

1. Molecular detection

The microalgal isolates (single and blooms) were identified using primary (taxon specific) and secondary (universal) barcode markers. One universal and two taxon specific primers were used in this study.

Positive results were obtained for most isolates when the LSU region was amplified using the primer pair T16N/ T24U with an amplicon size different from the expected size of 567bp. Instead, the amplicons obtained ranged between 700bp and 750bp with an additional nonspecific amplicon (1100bp) from DPS and II isolates (Figure 31). Amplification products were obtained from 19 out of 30 isolates tested.

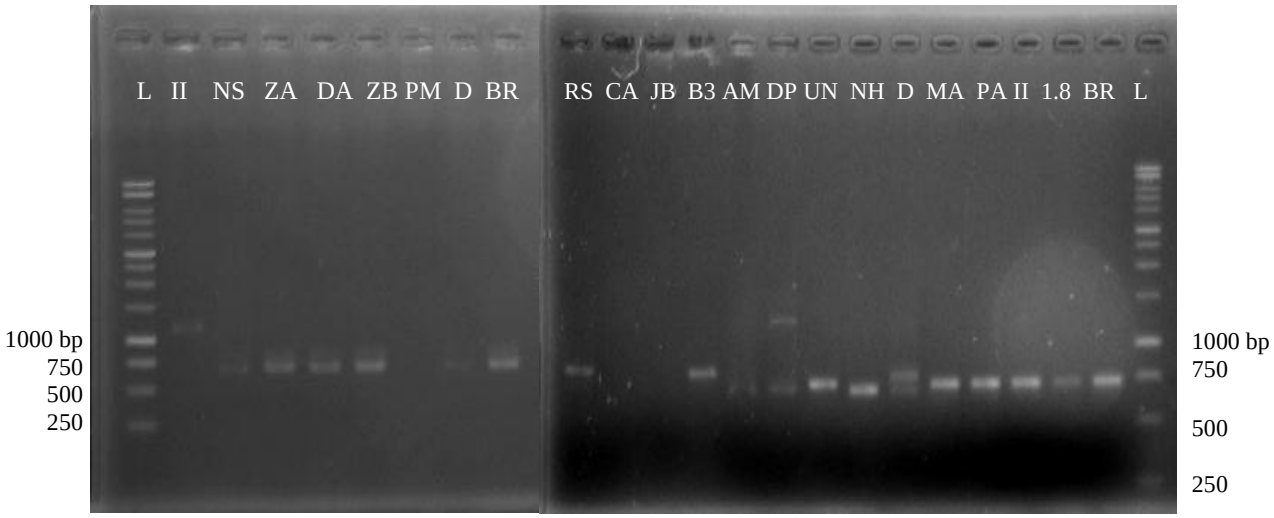


Figure 31. UV visualization in 1% agarose gel following electrophoresis of PCR amplified products using the universal primers T16N and T24U to amplify nuclear D2/D3 region of the large ribosomal subunit (LSU D2/D3). L: 1 Kbp molecular ladder, II to BR: acronyms of selected microalgae isolates.

All the isolates were tested with the secondary specific primers ITS1 and ITS4 amplifying the internal transcribed spacer of their ribosomal cistron. The amplicon sizes were around 800bp (Figure 32). Amplification products were obtained from 22 out of 30 isolates.

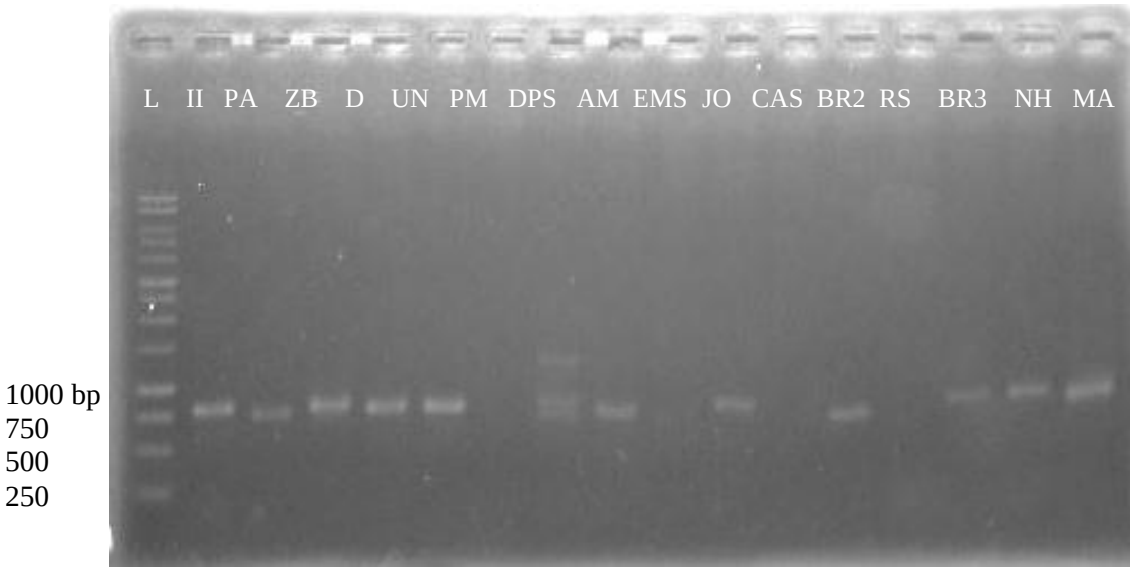


Figure 32. UV visualization in 1% agarose gel following electrophoresis of PCR amplified products using the taxon specific primers ITS1 and ITS4 to amplify internal transcribed spacer of the ribosomal cistron. L: 1 Kbp molecular ladder, II to MA: acronyms of selected microalgae isolates.

The taxon specific primers tufGF4 and tufAR were used in PCR to amplify a 900bp sequence from the *tuf A* gene from DNA extracts of all of the microalgal isolates, however only 8 isolates showed amplification products (Figure 33).

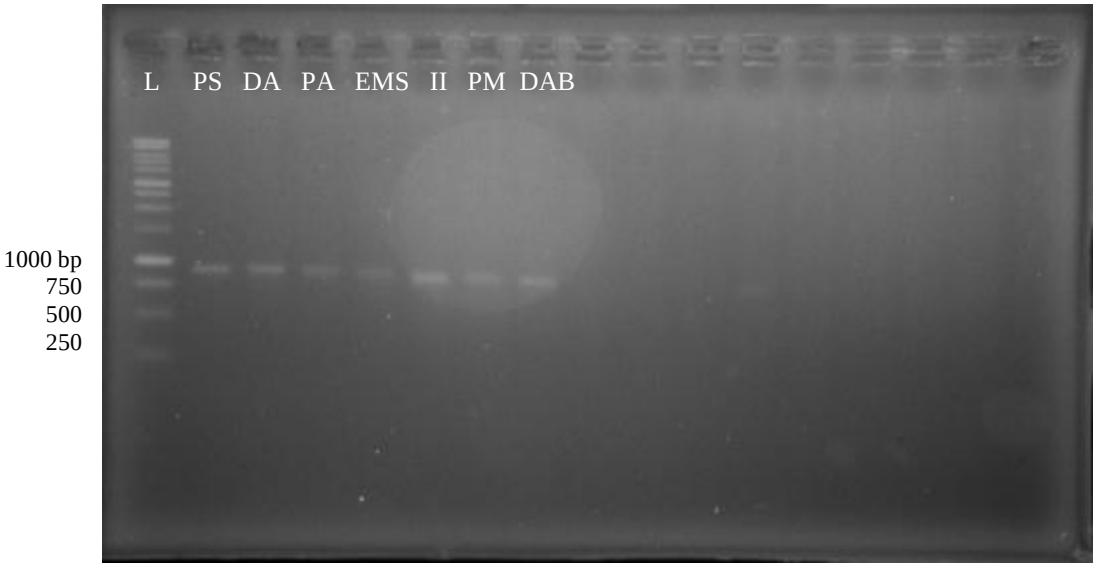


Figure 33. UV visualization in 1% agarose gel following electrophoresis of PCR amplified products using the taxon specific primers tufGF4 and tufAR to amplify gene *tuf A* that encodes the elongation factor Tu. L: 1 Kbp molecular ladder, PS to DAB: acronyms of selected microalgae isolates.

2. Sequence analysis

Sequencing success rates from the PCR purified amplicons were high to moderate in the case of the LSU and ITS markers with a pairwise identity ranging between 95 and 99% in all isolates (Table 17). However, the success rate with the other genomic marker (tufA) was low.

The following genera were identified in this study: *Tetraselmis sp., Chlorella sp., Amphora sp., Chloromonas sp., Microcoleus sp. and Scenedesmus sp.*

Twelve isolates were identified to the species level: *Tetraselmis marina, Tetraselmis striata, Micractinium reisseri, Scherffelia dubia, Neochloris conjuncta, Acutodesmus obliquus and Cyanobacterium aponinum* (Table 17).

Table 17. Genera of microalgae identified in this study, their maximum scores and their highest sequence identities with related isolates, using three barcode markers.

Acronym	Microalgae name	Markers					
		LSU		ITS		TUF	
		Max score	Pairwise identity (%)	Max score	Pairwise identity (%)	Max score	Pairwise identity (%)
JOS	*Tetraselmis sp.*	--	--	1129	99	--	--
UNS	*Tetraselmis marina*	1002	98	614	85	--	--
Amshit	*Chlorella sp.*	1365	98	1009	97	1132	98
PALS 2	*Amphora sp*	725-686	94-92	--	--	--	--
NS	*Chlorella sp.*	887	92	--	--	--	--
SPI	*Micractinium reisseri*	898-833	96-93	--	---	--	--
DP	*Chloromonas sp*	--	--	841	98	--	--
D	*Scherffelia dubia*	924-924	96	--	--	--	--
JS	*Neochloris conjuncta*	--	--	1083	98	--	--
EMS	*Cyanobacterium aponinum*	--	--	--	--	1493	98
MSG (fw)	*Chlorella sp.*	--	--	780-529	86	--	--
M1 (fw)	*Chlorella sp.*	--	--	697	87	--	--
MAZ (fw)	*Chlorella sp*	--	--	745	86	--	--
Brumana (fw)	*Acutodesmus obliquus*	791	96	1212	100	--	--
MAS (fw)	*Chlorella sp.*	787	98	--	--	--	--

BR3 (fw)	*Micractinium reisseri*	776-608	99	--	--	--	--
BR2 (fw)	*Acutodesmus obliquus*	1085-907	99	1166-1160	99	--	--
NHS (fw)	*Scenedesmus sp.*	--	--	1160-1155	99	--	--
AZ (fw)	*Chloromonas sp.*	--	--	337-337	95	--	--
Zalka Bloom	*Microcoleus sp.*	--	--	--	--	650	90
Zalka Bloom	*Micractinium reisseri*	894-833	95	--	--	--	--
DPS Bloom	*Cyanobacterium aponinum*	--	--	--	--	1390	96
II Bloom	*Tetraselmis striata*	1053	99	1081-422	99	--	--
II Bloom	*Cyanobacterium aponinum*	--	--	--	--	1258	97

Table 18. Taxonomic groups of microalgae isolates identified in this study.

Acr.	Kingdom	Phylum	Class	Order	Family	Genus	Species	Genomic region
Amshit	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Chlorella*	*sp*	LSU-ITS-TUF
JOS	Plantea	Chlorophyta	Chlorodendrophyceae	Chlorodendrales	Chlorodendraceae	*Tetraselmis*	*sp*	ITS
UNS	Plantea	Chlorophyta	Chlorodendrophyceae	Chlorodendrales	Chlorodendraceae	*Tetraselmis*	*Marina*	LSU-ITS
NS	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Chlorella*	*sp*	LSU
PALS 2	Chromista	Orchrophya	Bacillariophyceae	Thalassiophysales	Catanulacea	*Amphora*	*sp*	LSU
SPI	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Micractinium*	*reisseri*	LSU
DP	Plantea	Chlorophyta	Chlorophyceae	Chlamydomonadales	chlamydomonadaceae	*Chloromonas*	*sp*	ITS
D	Plantea	Chlorophyta	Prasinophyceae	Chlorodendrales	Chlorodendraceae	*Scherffelia*	*dubia*	LSU

EMS	Eubacteria	Cyanobacteria	Oscillariophycideae	Chroococcales	--	*Cyanobacterium*	*aponinum*	TUF
JS	Plantea	Chlorophyta	Chlorophyceae	Sphaeropleales	Neochloridaceae	*Neochloris*	*conjuncta*	ITS
MSG (fw)	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Chlorella*	*sp*	ITS
M1 (fw)	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Chlorella*	*sp*	ITS
MAZ (fw)	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Chlorella*	*sp*	ITS
MAS (fw)	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Chlorella*	*sp*	LSU
Brumana (fw)	Plantea	Chlorophyta	Chlorophyceae	Sphaeropleales	Scenedesmaceae	*Acutodesmus*	*obliquus*	LSU-ITS
BR3 (fw)	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Micractinium*	*reisseri*	LSU
BR2 (fw)	Plantea	Chlorophyta	Chlorophyceae	Sphaeropleales	Scenedesmaceae	*Acutodesmus*	*obliquus*	LSU-ITS
NHS (fw)	Plantea	Chlorophyta	Chlorophyceae	Sphaeropleales	Scenedesmaceae	*Scenedesmus*	*sp*	ITS
AZ (fw)	Plantea	Chlorophyta	Chlorophyceae	Chlamydomonadales	Chlamydomonadaceae	*Chloromonas*	*sp*	ITS
Zalka Bloom	Eubacteria	Cyanobacteria	Oscillariophycideae	Oscillatoriales	--	*Microcoleus*	*sp*	TUF

Zalka Bloom	Plantea	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Micractinium*	*reisseri*	LSU
DPS Bloom	Eubacteria	Cyanobacteria	Oscillariophycideae	Chroococcales	--	*Cyanobacterium*	*aponinum*	TUF
II Bloom	Plantea	Chlorophyta	Chlorodendrophyceae	Chlorodendrales	Chlorodendraceae	*Tetraselmis*	*striata*	LSU-ITS
II Bloom	Eubacteria	Cyanobacteria	Oscillariophycideae	Chroococcales	--	*Cyanobacterium*	*aponinum*	TUF

C. Growth Kinetics

Experiments on growth kinetics were conducted for twenty two unialgal isolates and blooms grown in 23 L or 3 L flat bioreactors with a light path of 5 cm or in 5L gallons. Below are graphs representing optical density readings taken at 560nm and the corresponding maximum growth rate µ/day (Figures 34 to 56).

From previous experience, the lag phase may be reduced if a more concentrated initial inoculum is used to reach an A_{560nm} reading between 0.05 and 0.1.

The lag period varied among isolates, however most isolates had an exponential growth rate that ranged between 4-6 and 8 days. The maximum growth rate (µ/day) ranged between 1.69 and 0.4 with the highest µ recorded for isolates Brumana (1.69) and C.LEB (1.62).

The growth kinetics in the flat photobioreactors (23 L) for isolates CM, C.LEB, C.CAP, NK2, Damour, Saida, and Jiyyeh are represented in Figures 34 to 40.

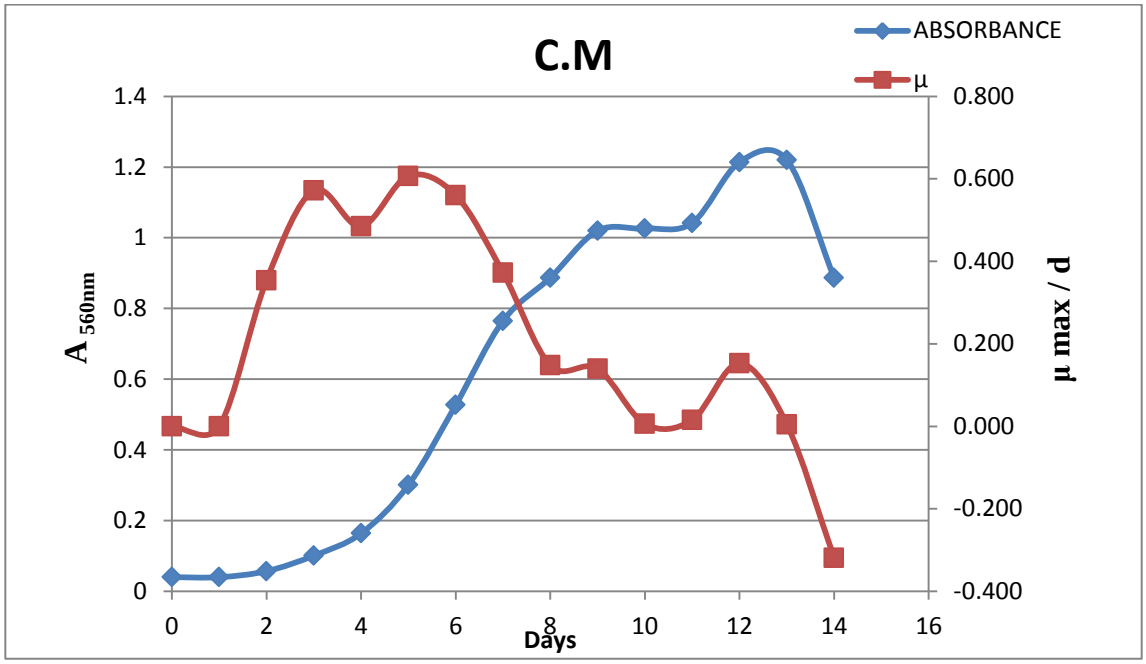


Figure 34. Growth kinetics and daily exponential specific growth rate (μmax d-1) for CM isolate.

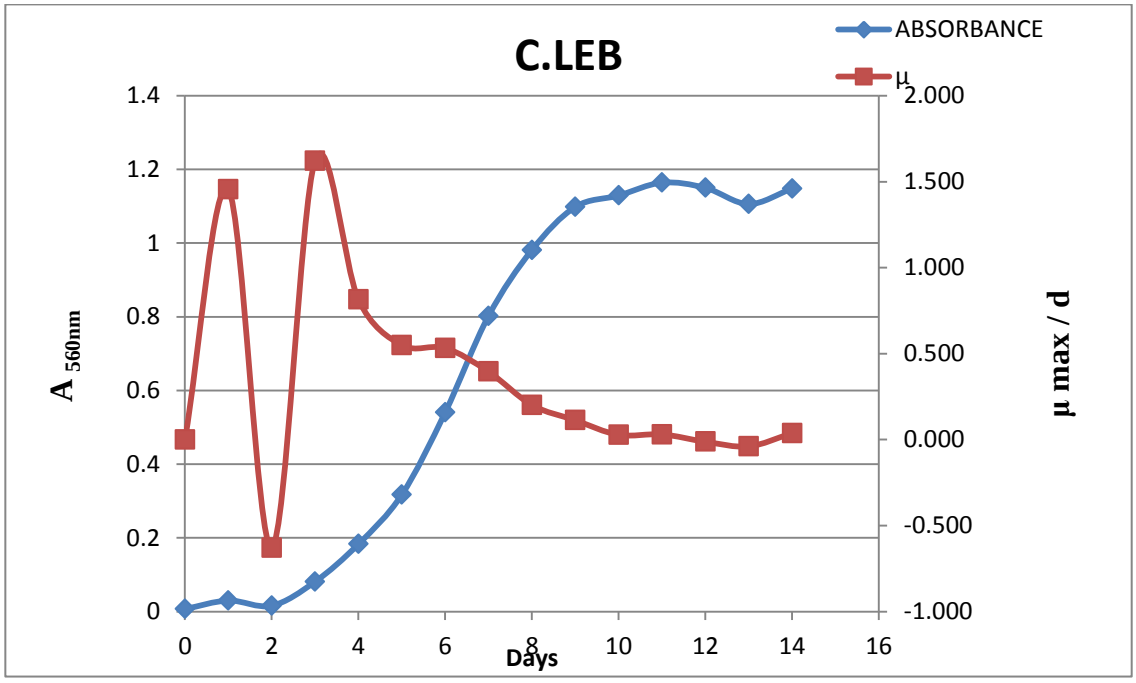


Figure 35. Growth kinetics and daily exponential specific growth rate (μmax d-1) for C.LEB isolate.

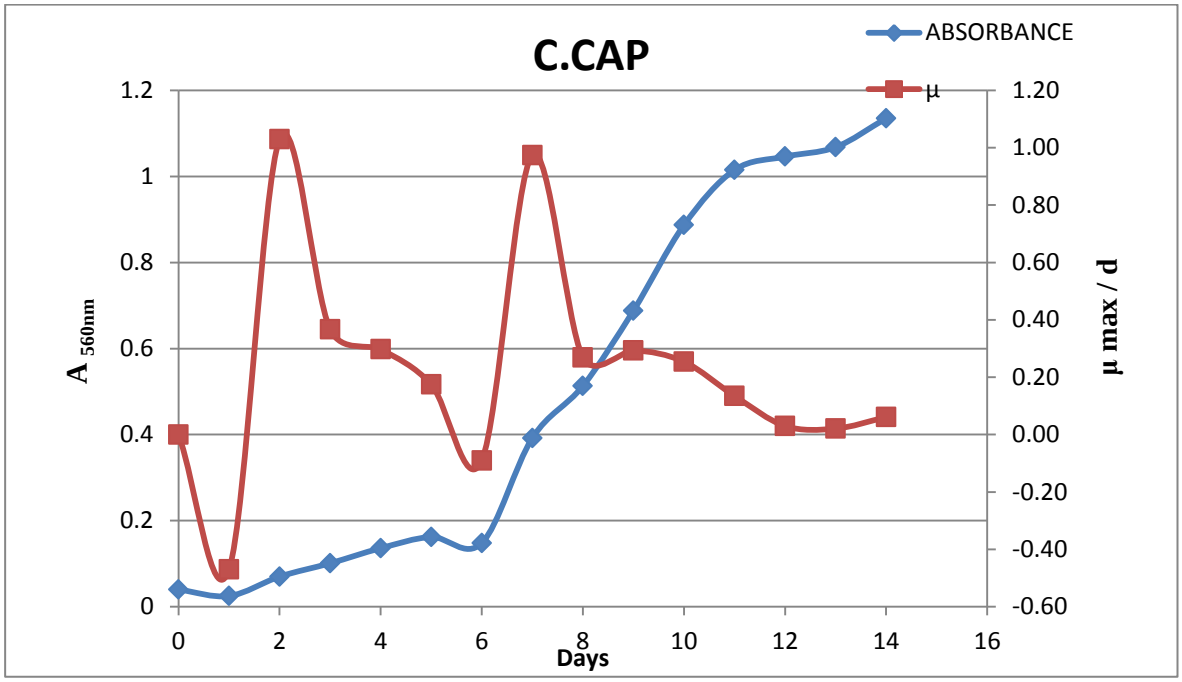


Figure 36. Growth kinetics and daily exponential specific growth rate (μmax d-1) for C.CAP isolate.

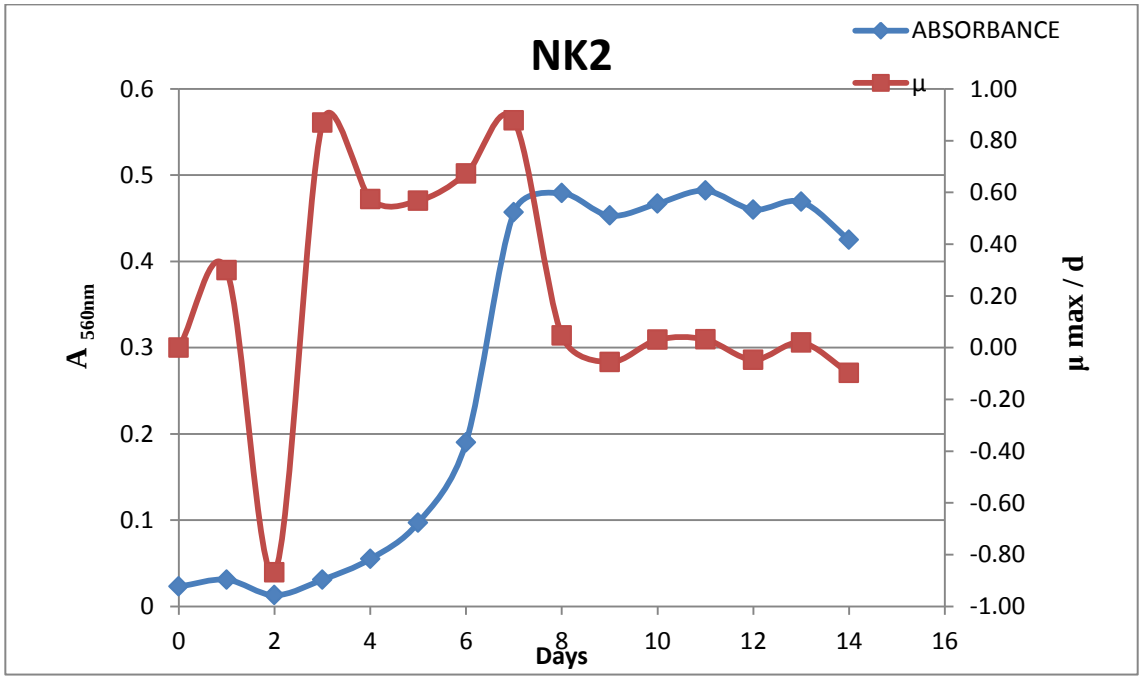


Figure 37. Growth kinetics and daily exponential specific growth rate (μmax d-1) for NK2 isolate.

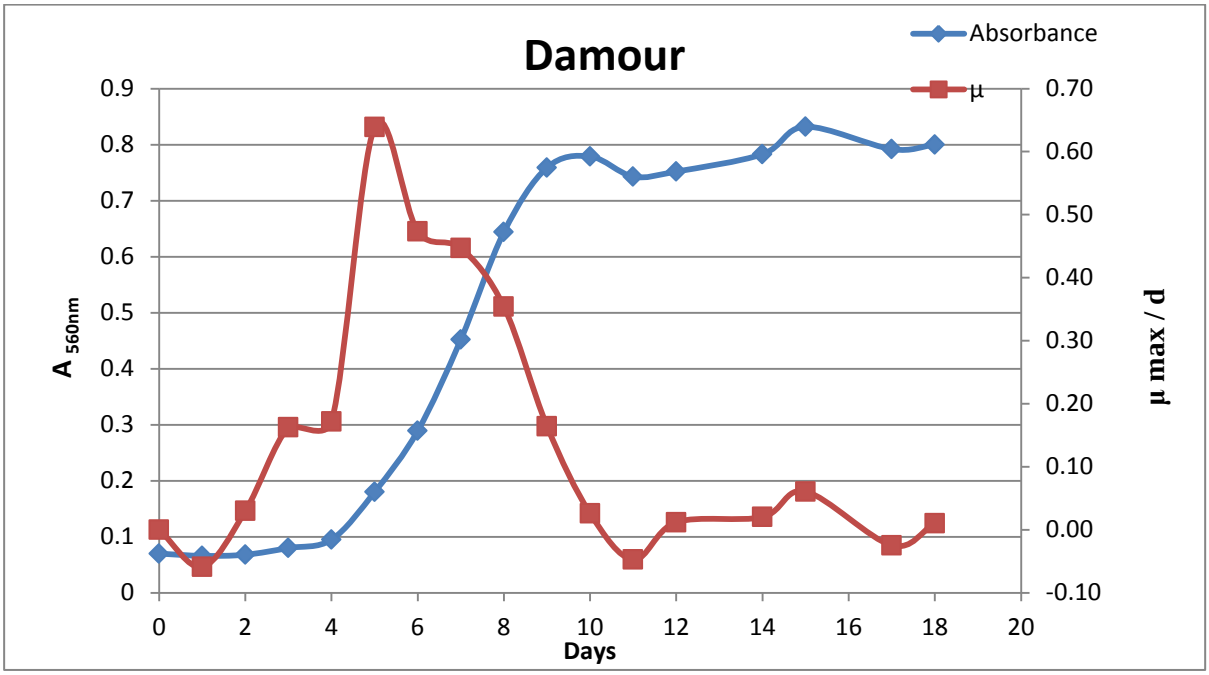


Figure 38. Growth kinetics and daily exponential specific growth rate (μmax d-1) for Damour isolate.

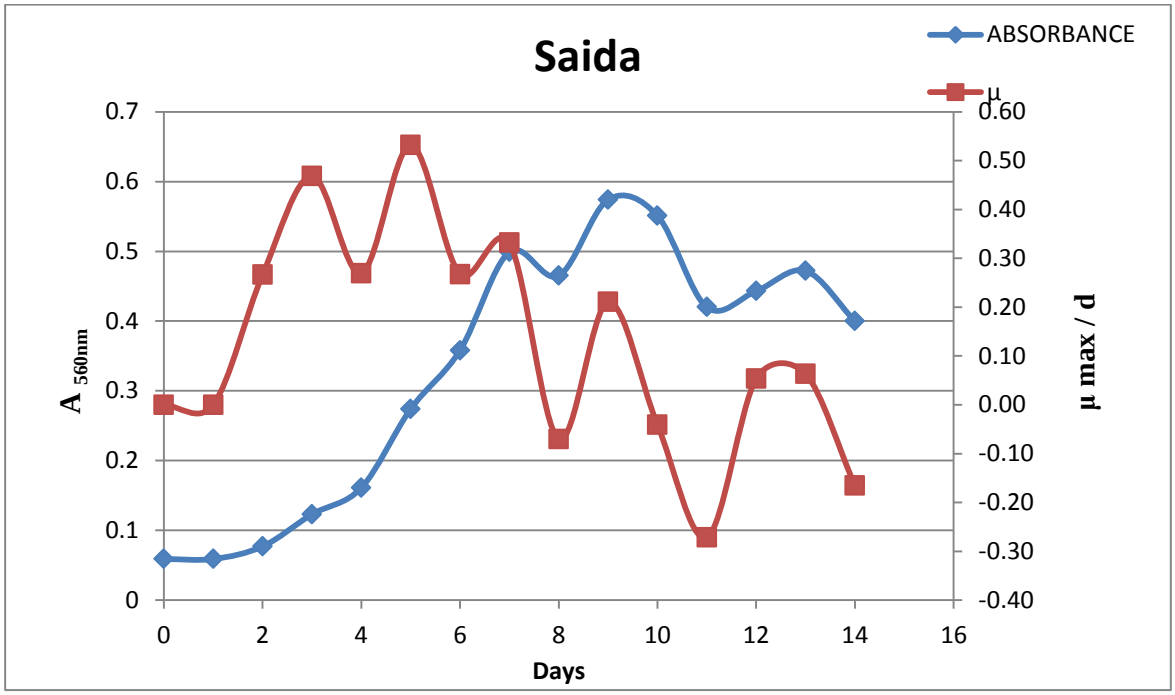


Figure 39. Growth kinetics and daily exponential specific growth rate (μmax d-1) for Saida isolate.

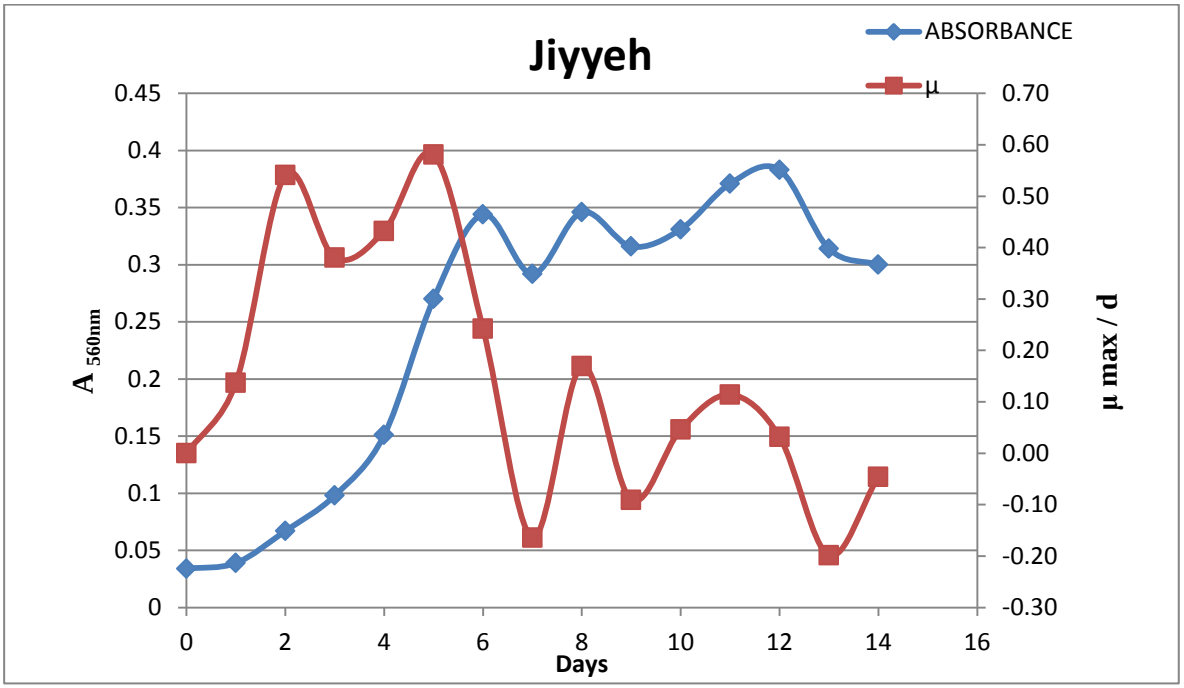


Figure 40. Growth kinetics and daily exponential specific growth rate (μmax d-1) for Jiyyeh isolate.

The growth kinetics in the small photobioreactors (3 L) for isolates Amshit, PS, Brumana (fw), RNS (fw), BS (fw) and MSG (fw) are represented in Figures 41 to 46.

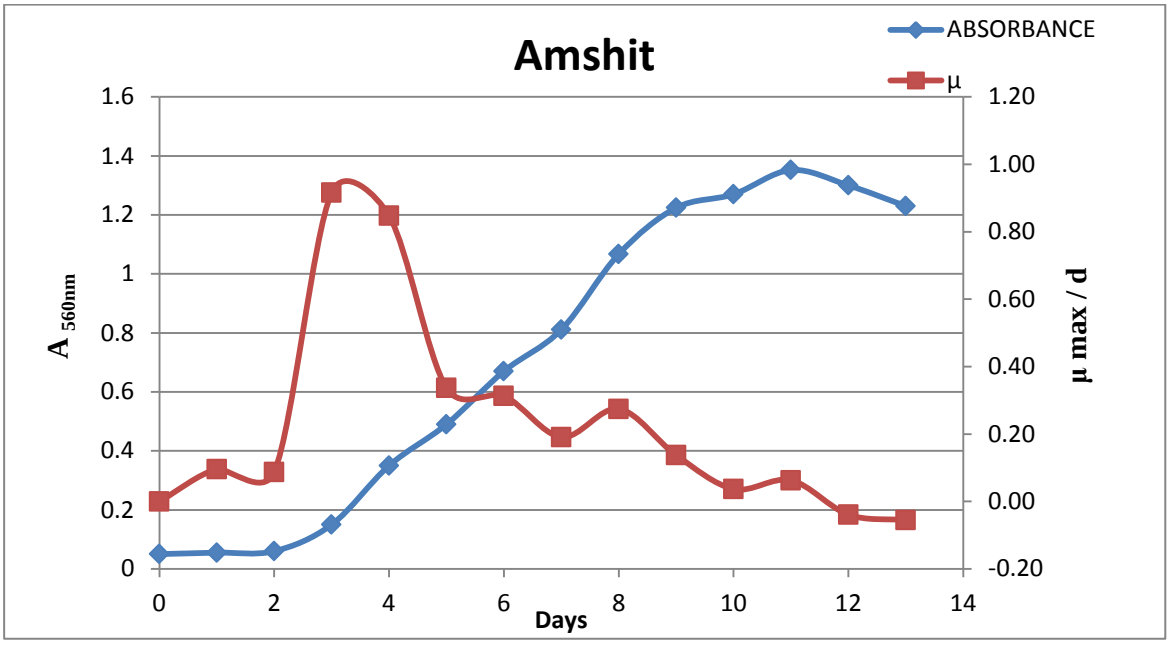


Figure 41. Growth kinetics and daily exponential specific growth rate (μmax d-1) for Amshit isolate.

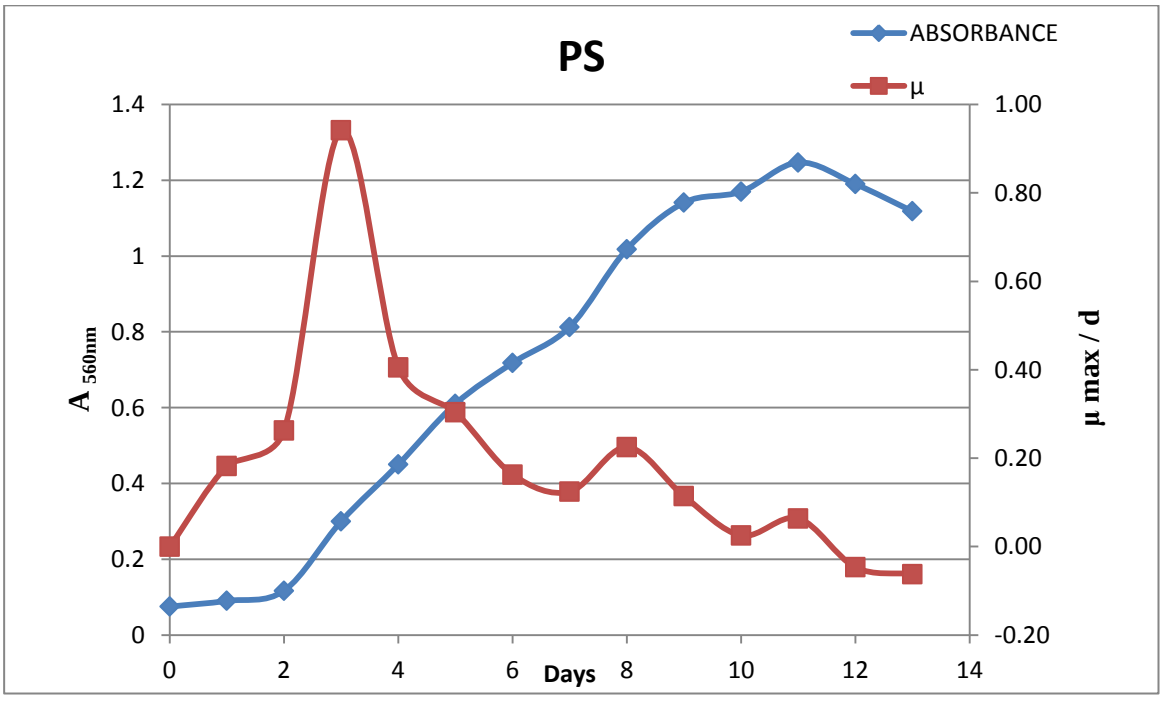


Figure 42. Growth kinetics and daily exponential specific growth rate (μmax d-1) for PS isolate.

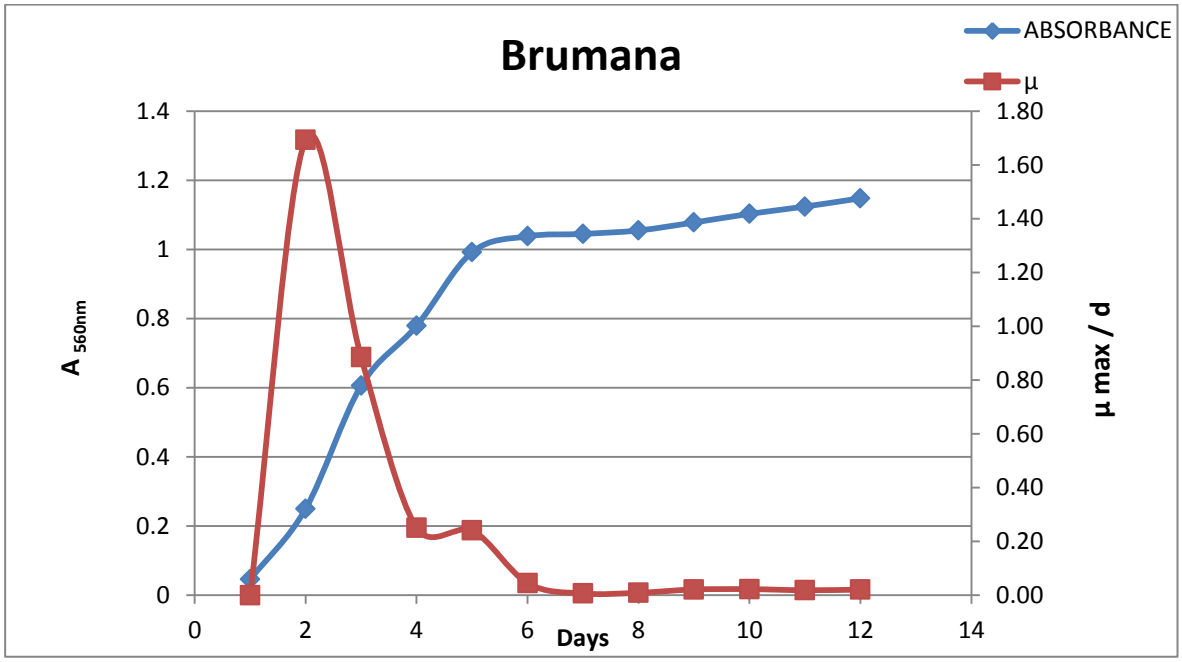


Figure 43. Growth kinetics and daily exponential specific growth rate (μmax d-1) for Brumana (fw) isolate.

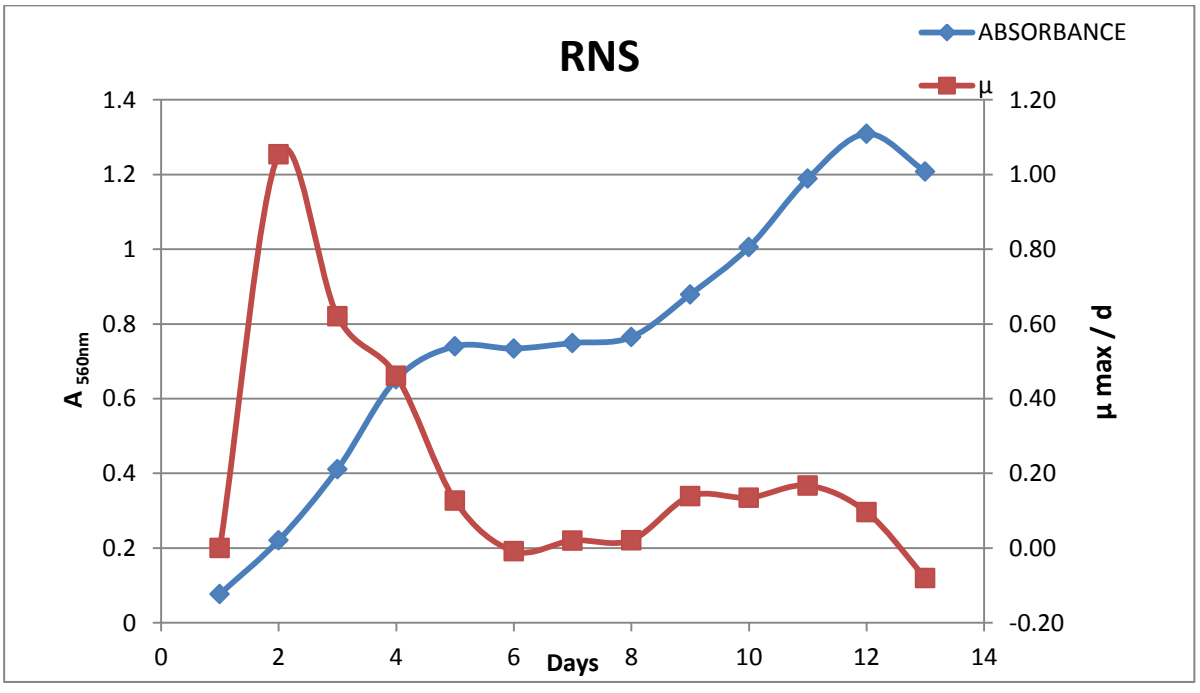


Figure 44. Growth kinetics and daily exponential specific growth rate (μmax d-1) for RNS (fw) isolate.

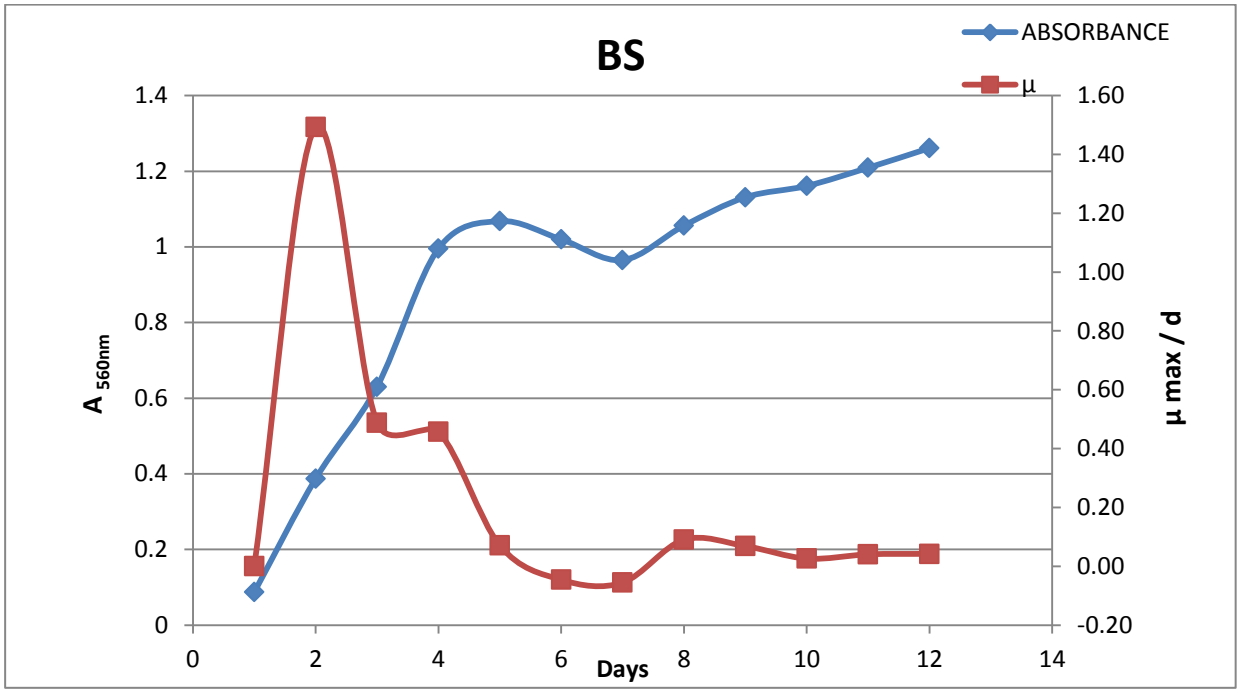


Figure 45. Growth kinetics and daily exponential specific growth rate (μmax d-1) for BS (fw) isolate.

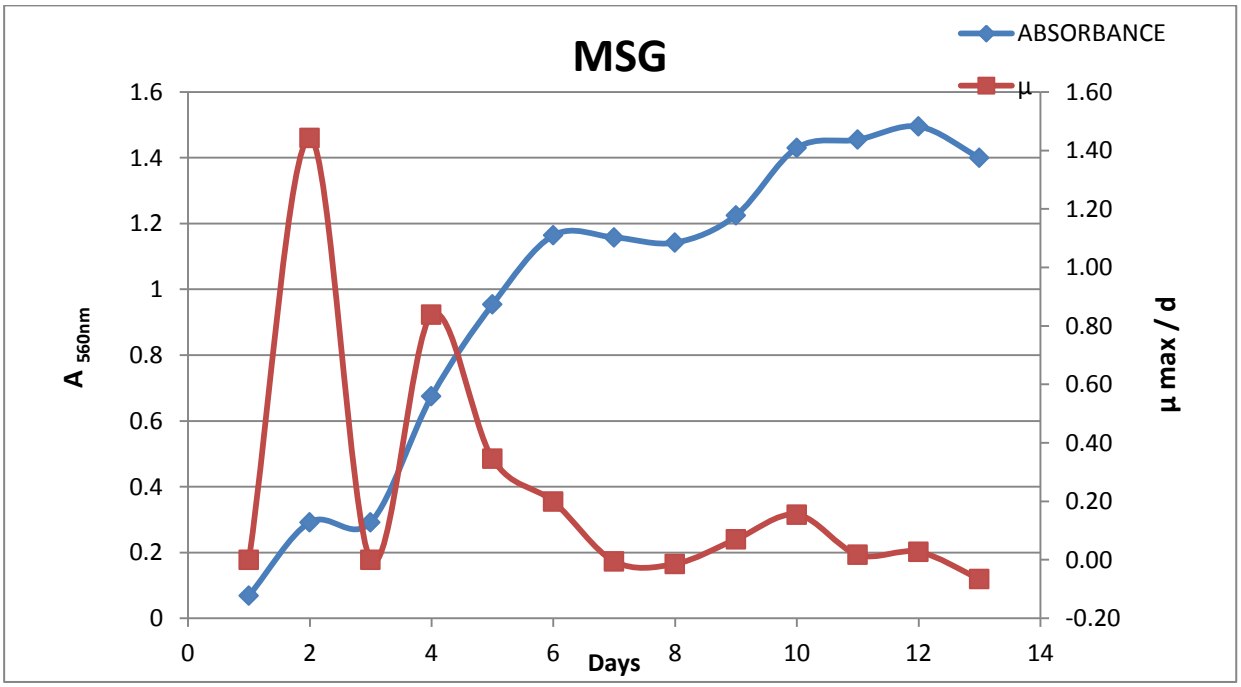


Figure 46. Growth kinetics and daily exponential specific growth rate (μmax d-1) for MSG (fw) isolate.

The growth kinetics in the gallons (5 L) for isolates JOS, JBS, PM, PM Bloom, PALS Bloom, DPS Bloom, D Bloom, DAS Bloom, II Bloom and SPIRULINA Bloom are represented in figures 47 to 56.

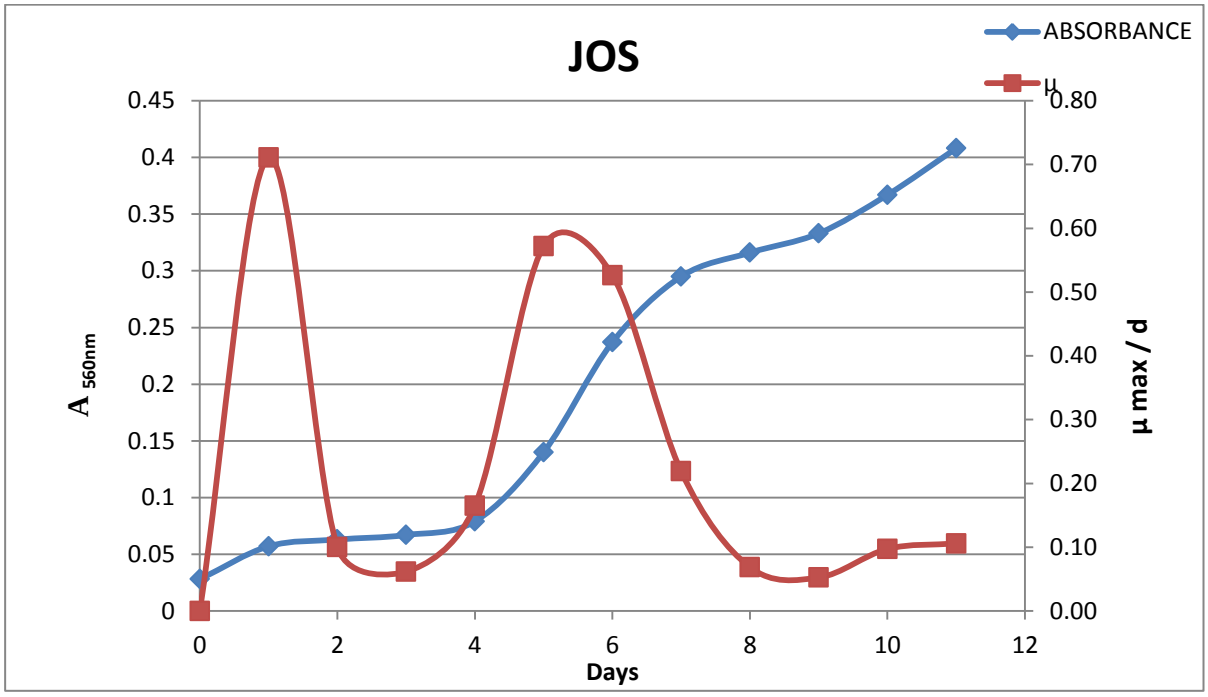


Figure 47. Growth kinetics and daily exponential specific growth rate (μmax d-1) for JOS isolate.

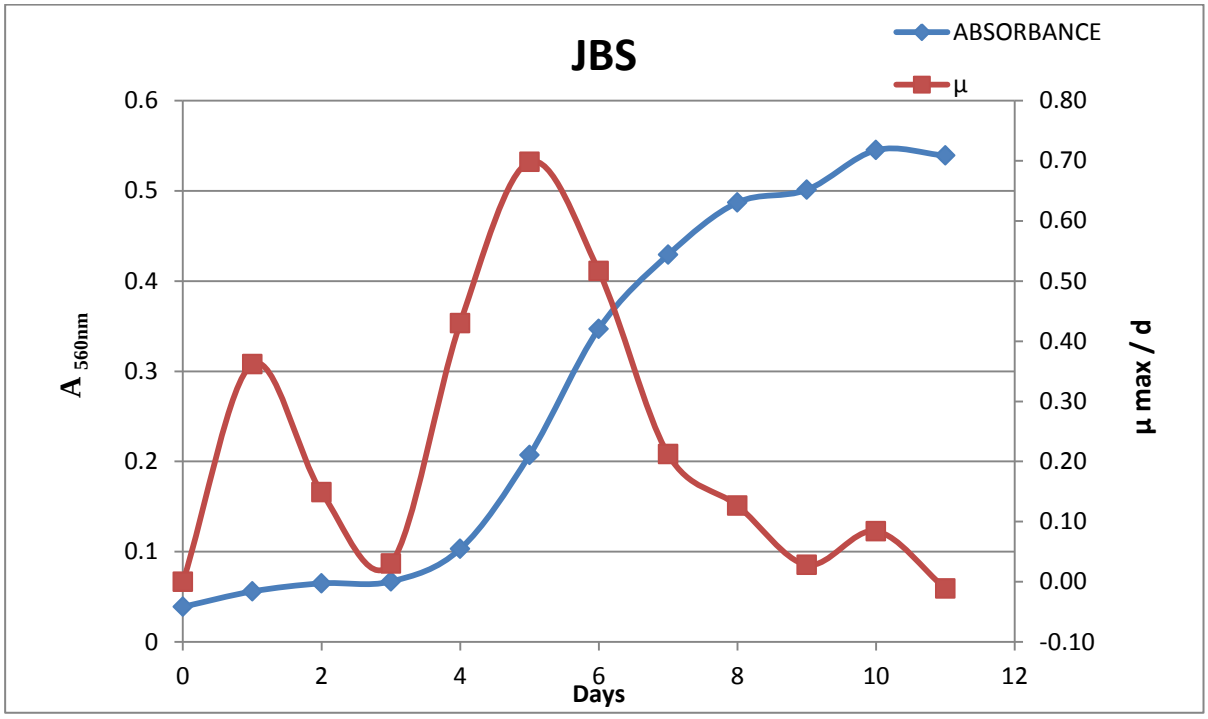


Figure 48. Growth kinetics and daily exponential specific growth rate (μmax d-1) for JBS isolate.

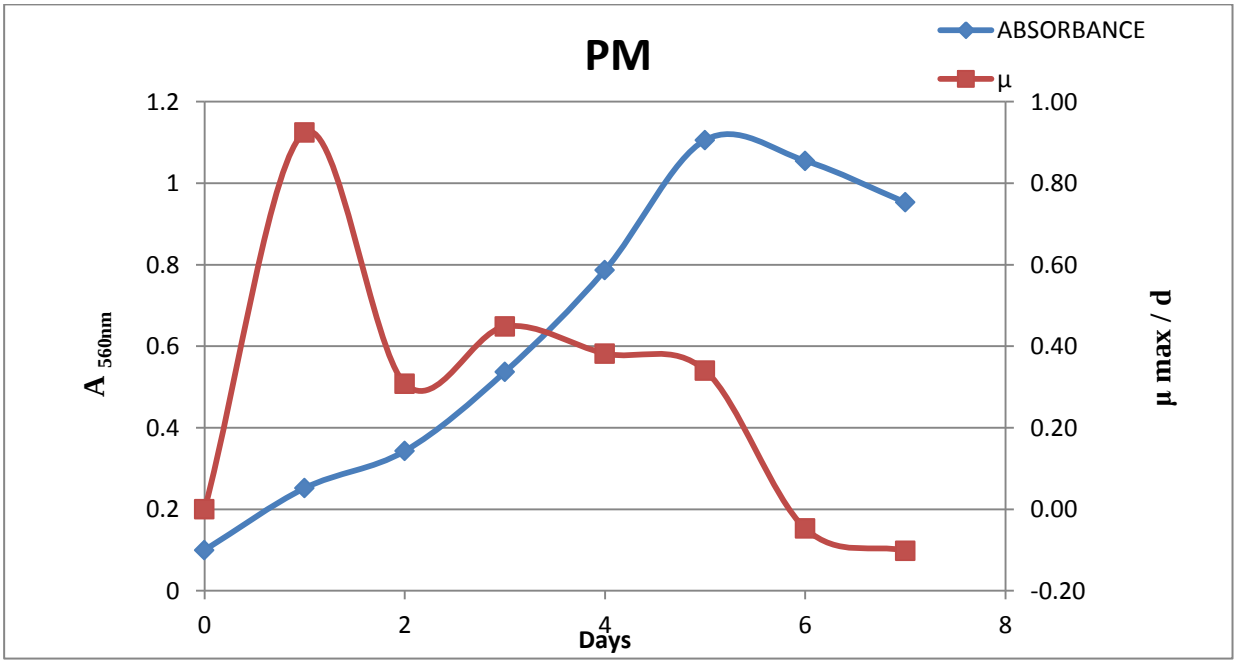


Figure 49. Growth kinetics and daily exponential specific growth rate (μmax d-1) for PM isolate.

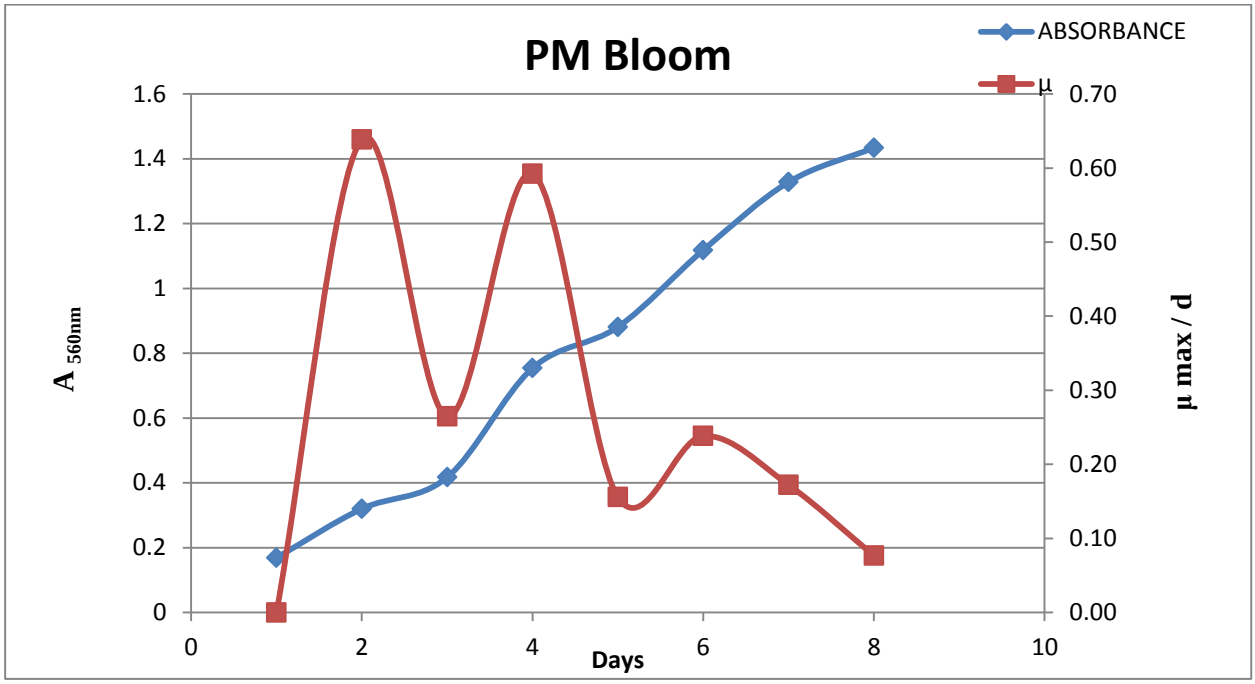


Figure 50. Growth kinetics and daily exponential specific growth rate (μmax d-1) for PM Bloom.

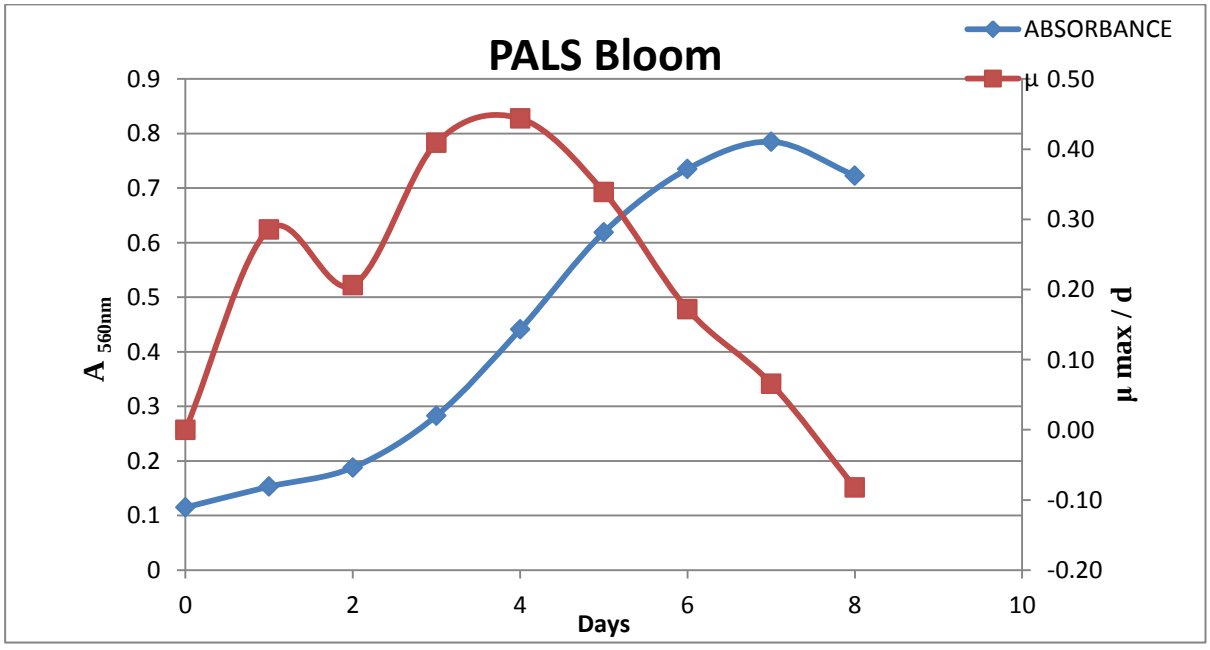


Figure 51. Growth kinetics and daily exponential specific growth rate (μmax d-1) for PALS Bloom.

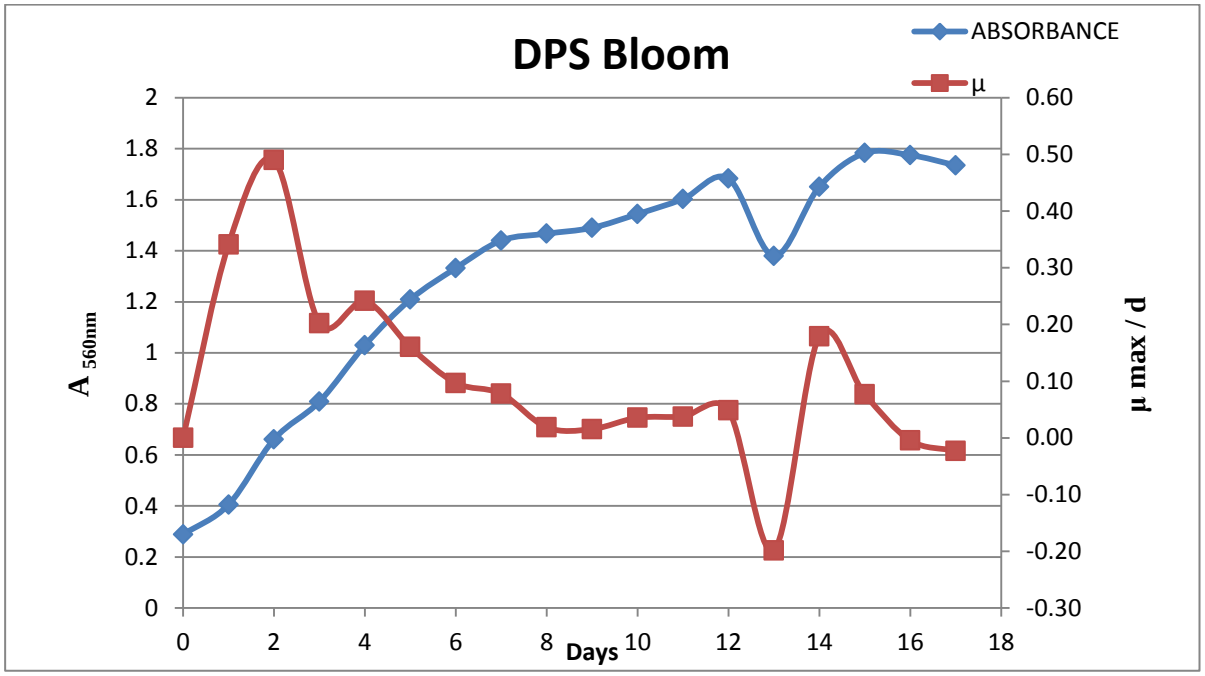


Figure 52. Growth kinetics and daily exponential specific growth rate (μmax d-1) for DPS Bloom.

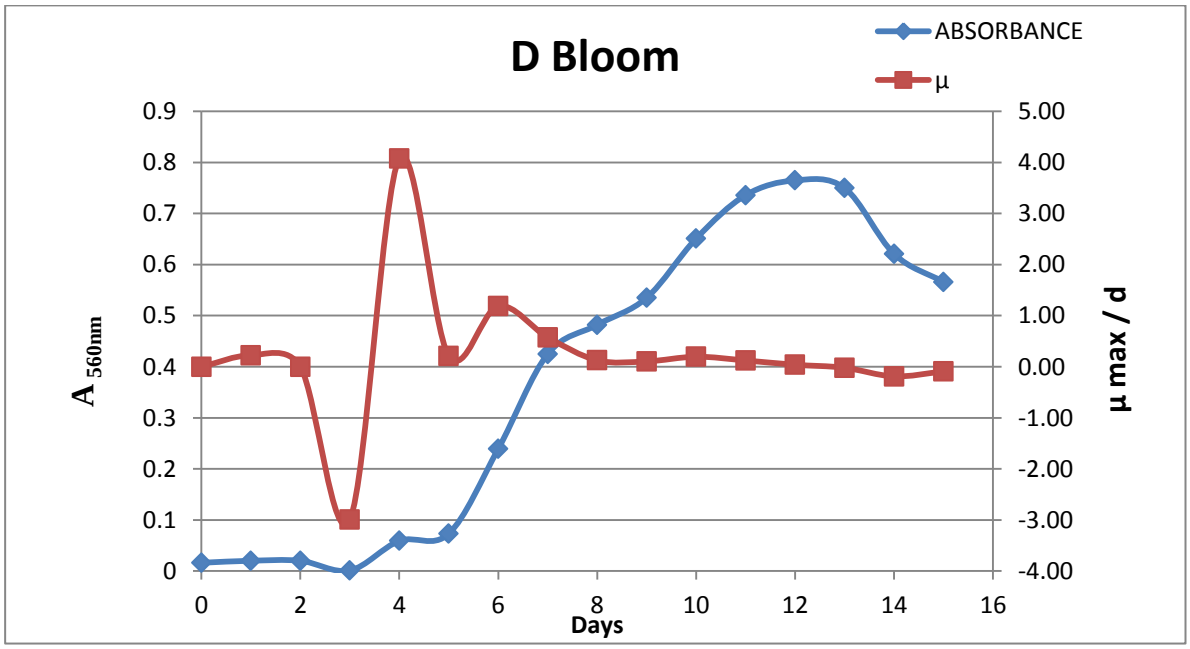


Figure 53. Growth kinetics and daily exponential specific growth rate (μmax d-1) for D Bloom.

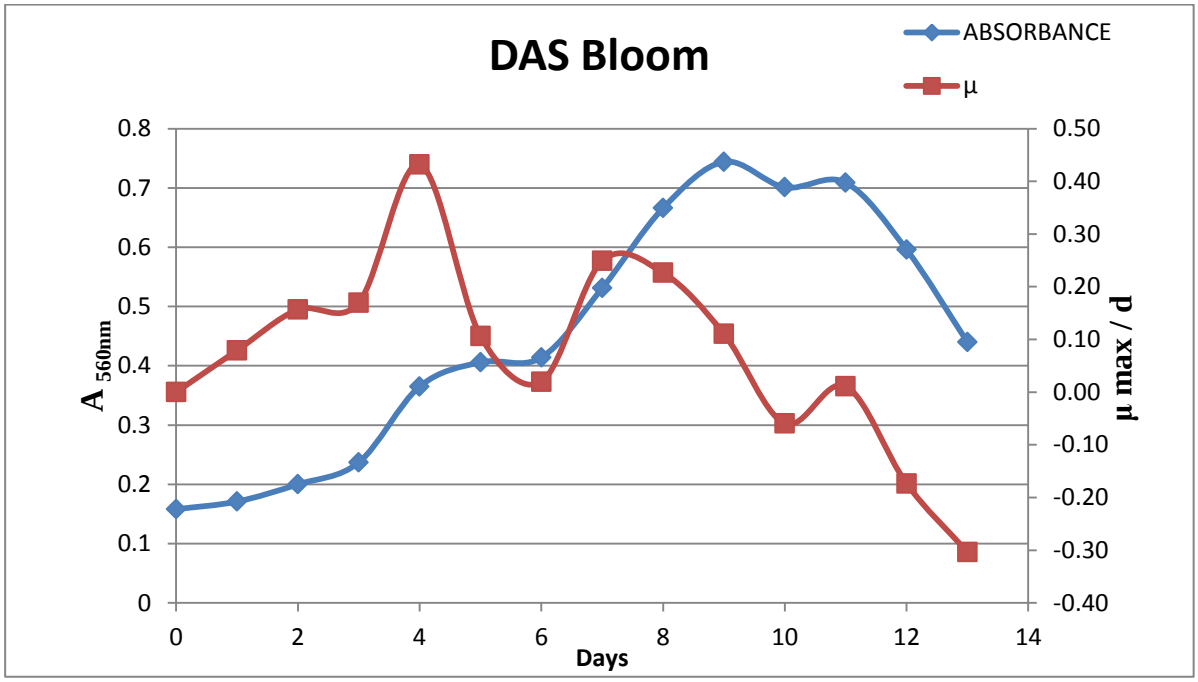


Figure 54. Growth kinetics and daily exponential specific growth rate (μmax d-1) for DAS Bloom.

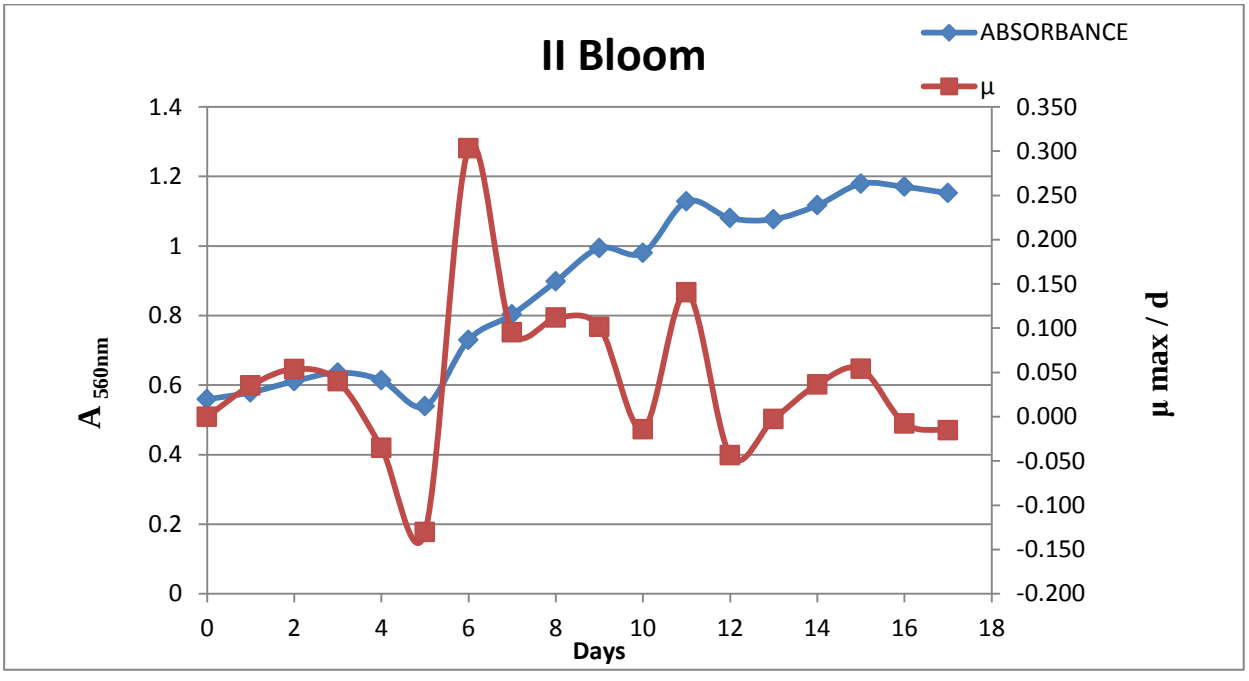


Figure 55. Growth kinetics and daily exponential specific growth rate (μmax d-1) for II Bloom.

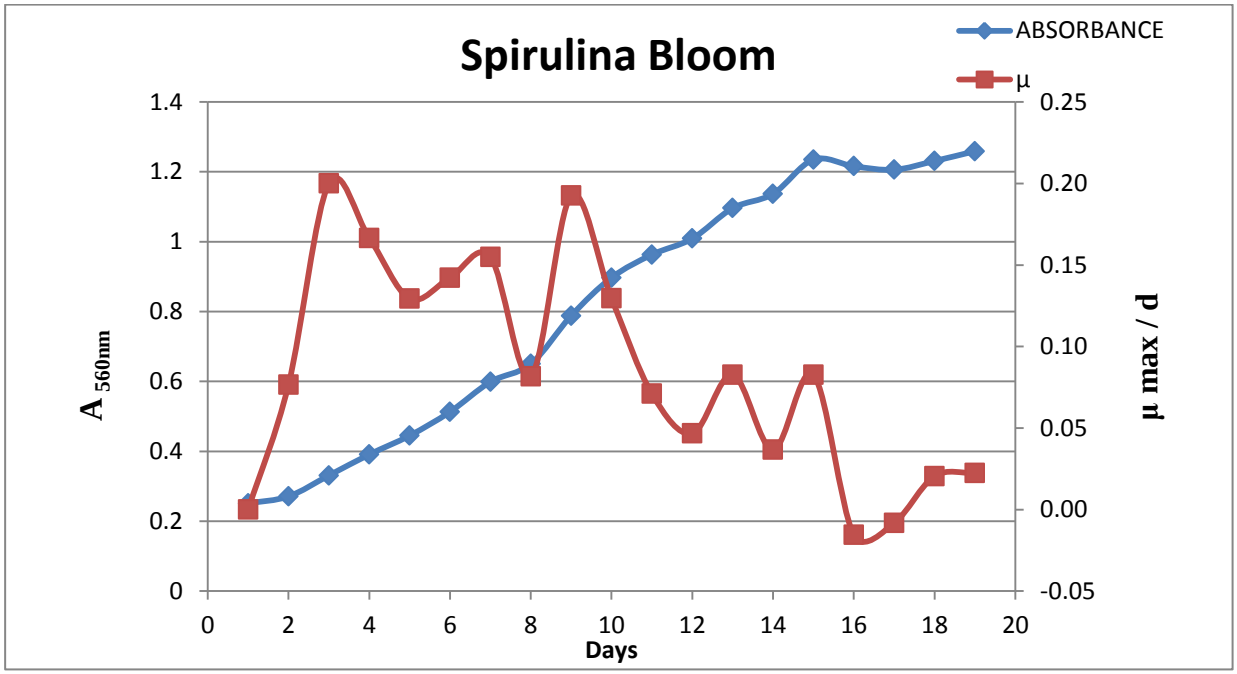


Figure 56. Growth kinetics and daily exponential specific growth rate (μmax d-1) for Spirulina Bloom.

D. Biomass Production

1. Dry weights

One or two days after reaching the stationary phase the microalgae were harvested and the biomass yield was determined as dry matter production/L of culture. Under the laboratory growth conditions, the dry matter yield ranged between 300 and 850 mg/L with an average around 500 mg/L (Table 19). The following isolates/blooms produced 700mg/L or more, MGS (850 mg/L), Brumana (750 mg/L), JOS (700 mg/L) and DPS bloom (700mg/L).

Table 19. Dry weight (mg/L) of microalgae at early stationary phase.

Isolate	**Genus**	**Dry Weight (mg/L)**
C.LEB	*Chlorella sp.*	300
C.CAP	*Nannochloropsis sp.*	450
C.M	*Nannochloropsis sp.*	480
NK2	*Tetraselmis sp.*	650
Damour	*Dunaliella sp.*	400
Saida	*Tetraselmis sp.*	600
Jiyyeh	*Tetraselmis striata*	615
Amshit	*Chlorella sp.*	450
PS	--	480
JOS	*Tetraselmis sp.*	700
JBS	--	650
UNS	*Tetraselmis marina*	550
PM	--	480
NS	*Chlorella sp*	650
II	*Cyanobacterium aponinum*	450
EMS	--	520
Brumana (fw)	*Acutodesmus obliquus*	750
RN (fw)	--	580
BS (fw)	--	610

BR2 (fw)	*Acutodesmus obliquus*	550
MSG (fw)	*Chlorella sp.*	850
DPS Bloom	*Cyanobacterium aponinum*	700
DAS Bloom	--	540
D Bloom	--	600
Spirulina Bloom	--	300
PALS Bloom	--	475
Zalka Bloom	*Microcoleus sp. and Micractinium reisseri*	500
NK Bloom (fw)	--	350

2. Lipid content

The gravimetric determination method was used to determine the percent lipid content based on dry weight for different microalgae species. The lipid content varied between 5% in DPS bloom to 21-22% in PS (Table 20). The following three cultures: Damour, Zalka Bloom and PS and had the highest lipid contents (19.8, 20.46 and 21.85%, respectively) which had similar lipid content to the reference isolate CM (20.72%). However, the marine isolates (JOS) and the DPS bloom that gave the highest dry matter yield were among the isolates that had the lower lipid content (5 - 9.7%). On the other hand, the Brumana (fw) isolate had about 14% lipid content (Table 20).
In terms of total lipid production per liter of culture, the following three isolates were the most promising: Brumana (104.2 mg/L), PS (103.44 mg/L), Zalka Bloom

(102.3 mg/L) and NS (91.4 mg/L) and with a level of production more or less similar to the reference isolate CM (99.46%) (Table 21).

Table 20. Total lipid content (%) in the different microalgae isolates or blooms tested.

Isolate	**Genus**	**Lipid content (% DW)**
CM	*Nannochloropsis sp.*	20.72
DAM	*Dunaliella sp.*	19.8
Amshit	*Chlorella sp.*	13.2
PS	--	21.85
JOS	*Tetraselmis sp.*	7.64
JBS	--	7.29
NS	*Chlorella sp*	14.07
CAS	--	6.17
UNS	*Tetraselmis marina*	4.43
PM	--	9.5
SPI	*Micractinium reisseri*	10.6
DPS bloom	*Cyanobacterium aponinum*	5
Zalka bloom	*Microcoleus sp. and Micractinium reisseri*	20.46
DAS bloom	--	7.04
D bloom	--	7.95
Brumana (fw)	*Acutodesmus obliquus*	13.9
BR2 (fw)	*Acutodesmus obliquus*	17.14
MSG (fw)	*Chlorella sp.*	9.7
M1 (fw)	*Chlorella sp.*	11.1
MAS (fw)	*Chlorella sp.*	11.6
NK Bloom (fw)	--	15.4

Table 21. Potential for biodiesel production of different isolates and blooms.

Isolate	Genus	Biodiesel Production (mg/L)
CM	*Nannochloropsis sp*	99.46
C.LEB	*Chlorella sp.*	38.7
Saida	*Tetraselmis sp.*	81.6
Jiyyeh	*Tetraselmis striata*	78.1
DAM	*Dunaliella sp.*	79.2
Amshit	*Chlorella sp.*	59.4
PS	--	103.44
JOS	*Tetraselmis sp.*	53.48
JBS	--	47.39
PM	--	45.6
NS	*Chlorella sp*	91.4
UNS	*Tetraselmis marina*	24.37
Brumana (fw)	*Acutodesmus obliquus*	104.2
BR2 (fw)	*Acutodesmus obliquus*	94.27
MSG (fw)	*Chlorella sp.*	82.45
DPS Bloom	*Cyanobacterium aponinum*	35
DAS Bloom	--	38.1
D Bloom	--	47.7
Zalka Bloom	*Microcoleus sp. and Micractinium reisseri*	102.3
NK Bloom (fw)	--	53.9

3. Fatty acid profile

The fatty acids profile is reported in Table 22. The percentage of saturated fatty acids ranged between 21 and 75% with the JOS Isolate and the Zalka bloom containing over 60%. The percentage of PUFA varied between 2 and 67% with the following isolates containing over 40% PUFA, listed in decreasing order: SPI, Brumana (fw), PS, Amshit and MAS (fw). Interestingly the Zalka bloom seems the most promising for

good quality biodiesel production since it contains a relatively high lipid content with a high percentage of saturated and monounsaturated fatty acids. While the isolates that have a high % of polyunsaturated fatty acids may be suitable for nutritional purposes.

Table 22. The fatty acid profile of some local microalgae expressed as percentages of saturated and unsaturated FA.

Isolate	**Total Lipid Content (%DW)**	**%Saturated FA**	**%Unsaturated fatty acid**			
			Total	**Mono**	**Di**	**Poly**
JOS	7.64	75.06	24.94	10.92	1.04	12.98
JBS	7.29	44.81	55.19	21.19	0.84	33.16
Amshit	13.2	32.7	67.3	11.28	11.40	44.62
PS	21.85	31.95	68.05	9.91	11.56	46.58
Damour	19.8	22.98	77.02	9.1	32.27	35.65
SPI	10.6	21.7	78.3	10.68	0.41	67.2
Zalka bloom	20.46	60.17	39.83	38	-	1.83
MSG (fw)	9.7	32.68	67.32	7.6	31.18	28.54
Brumana (fw)	13.9	28.2	71.8	4.11	3.78	63.91
MAS (fw)	11.6	40.34	59.66	5.8	11.08	42.78
M1 (fw)	11.1	38.52	61.48	8.25	27.07	26.16

E. Protein Content

The Kjeldahl method was used. Using N to protein conversion factor of 5.95, the protein % ranged from 10% in Zalka bloom to 57% in cyanobacteria blooms (Table 23).

Table 23. Total protein content (%) in the different microalgae isolates or blooms tested.

Isolate	**Genus**	**Protein content (% DW)**
CM	*Nannochloropsis sp.*	14.1
Amshit	*Chlorella sp.*	20.83
PS	--	14.00
JOS	*Tetraselmis sp.*	12.52
JBS	--	13.5
NS	*Chlorella sp.*	21.85
UNS	*Tetraselmis marina*	21.92
CAS	--	49.36
SPI	*Micractinium reisseri*	28.33
DPS bloom	*Cyanobacterium aponinum*	32.5
Zalka bloom	*Microcoleus sp. and Micractinium reisseri*	10.98
PM bloom	--	21.49
II bloom	*Cyanobacterium aponinum and Tetraselmis striata*	38.43
PALS bloom	--	56.24
DAS bloom	--	43.13
D bloom	--	47.37
Spirulina bloom	--	38.03

F. Ash

Ashing was performed at 575°C. Calculations were based on a dry weight basis. The ash content of the tested microalgae ranged from 11.64% for NK Bloom (fw) to 48.88% for the reference isolate CM (Table 24).

Table 24. Ash % of selected cultures.

Isolate	**Genus**	**Ash %** **(% DW)**
JOS	*Tetraselmis sp.*	25%
JBS	--	18%
CM	*Nannochloropsis sp.*	48.88%
PM	--	31%
II bloom	*Aponinum sp. and Tetraselmis striata*	29.2%
DPS bloom	*Aponinum sp.*	32%
Amshit	*Chlorella sp.*	45.86%
PS	--	37.19%
Spirulina Bloom	--	28.93%
DAS Bloom	--	38.7%
BR 2 (fw)	*Tetraselmis sp.*	30.9%
NK Bloom (fw)	--	11.64%

G. Effect of N and P Supplementation of Culture Media on Growth of Microalgae

Figure 57. Flat bioreactors used to study the effect of N and P on the growth of Amshit isolate experiment.

1. Growth kinetics

The Amchit isolate was grown in f/2 medium with or without amendment with N and/or P. The optical density readings at 560 nm were recorded for all the treatments (Figure 58). Three replicates were used. The faster growth occurred in the f/2 +N and f/2 +N+P with a growth rate μ= 1.04 and 1.10 respectively as compared to F alone or f/2 + P with a μ= 0.8 and 0.94

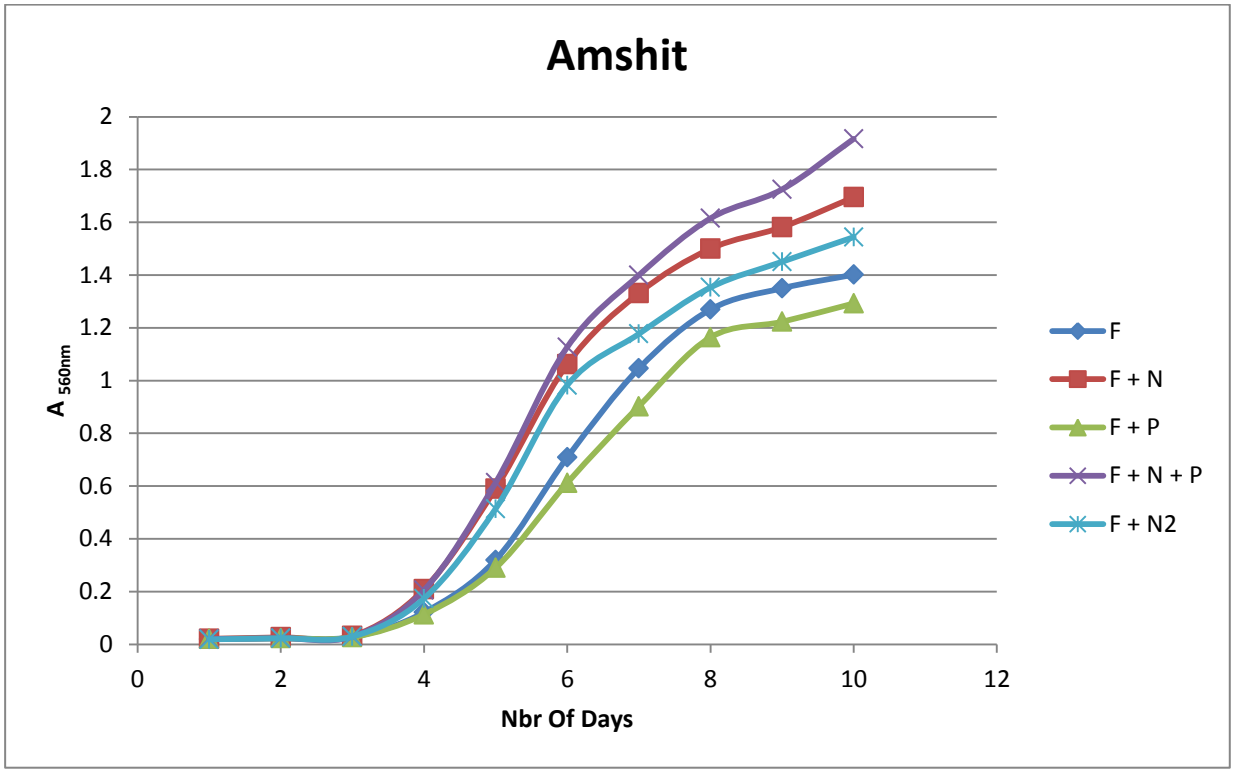


Figure 58. Growth kinetics of Amshit isolate grown in Guillard f/2 medium with or without N and P amendment.

2. Biomass production (Dry weight)

Dry weights of the different treatments were recorded from day 6 until the end of the experiment (Figure 59). After 10 days of growth the highest biomass production was obtained with the f/2+N+P followed by the f/2+N treatment with a dry matter content of 1.08 and 0.85 g/L respectively, which were significantly higher than in the basic medium (0.7 g/L). P amendment alone did not have any positive effect, while in combination with Nitrogen, it allowed to get a better yield. Doubling the Nitrogen amendment to 60 ppm did not improve biomass yield as compared to 30 ppm.

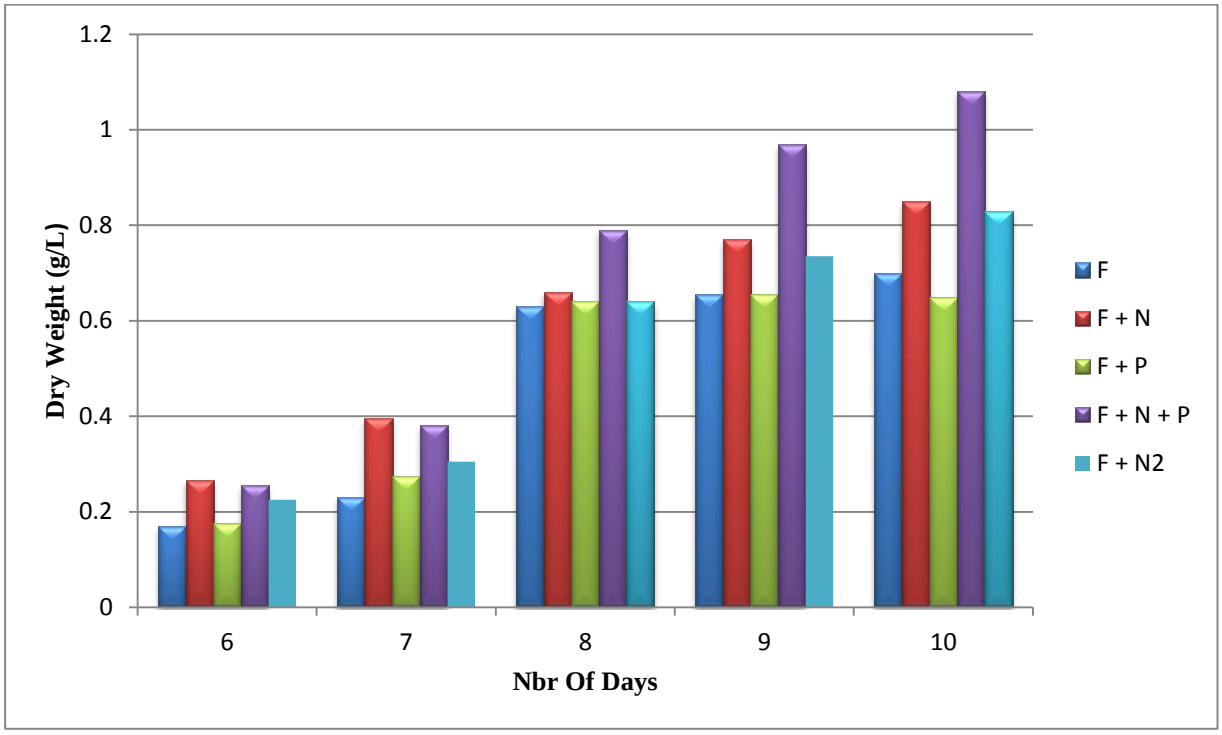


Figure 59. Dry weights for the different treatments of Amshit isolate (mg/L).

3. Lipid content

The effect of N and P amendment of f/2 nutrient medium on lipid content of the Amshit isolate was evaluated (Table 25). Total lipid content ranged between 13.35% in the reference treatment to 15.3% for the treatment supplemented with nitrogen. There was no statistically significant difference in the lipid content between the different treatments (Table 25, Figure 60).

Table 25. Lipid content of Amshit isolate grown in different media composition.

Treatment	% Lipid
f/2	13.35
f/2 +N	15.45
f/2 +N2	14.95
f/2 +P	14.4
f/2 +N+P	15.3

4. Protein content

The kjeldahl method was used to determine the protein content. The protein content ranged between 19.5 and 19.8% in the two treatments without nitrogen supplementation and reached 27.14 to 33.35% with nitrogen supplementation. A statistically significant increase in protein content, of an average of around 50% (19 to 34%), was observed upon nitrogen supplementation (Table 26, Figure 60).

Table 26. Protein content of Amshit isolate grown in different media.

Treatment	% Protein
f/2	19.5
f/2 +N	27.14
f/2 +N2	33.35
f/2 +P	19.8
f/2 +N+P	30.59

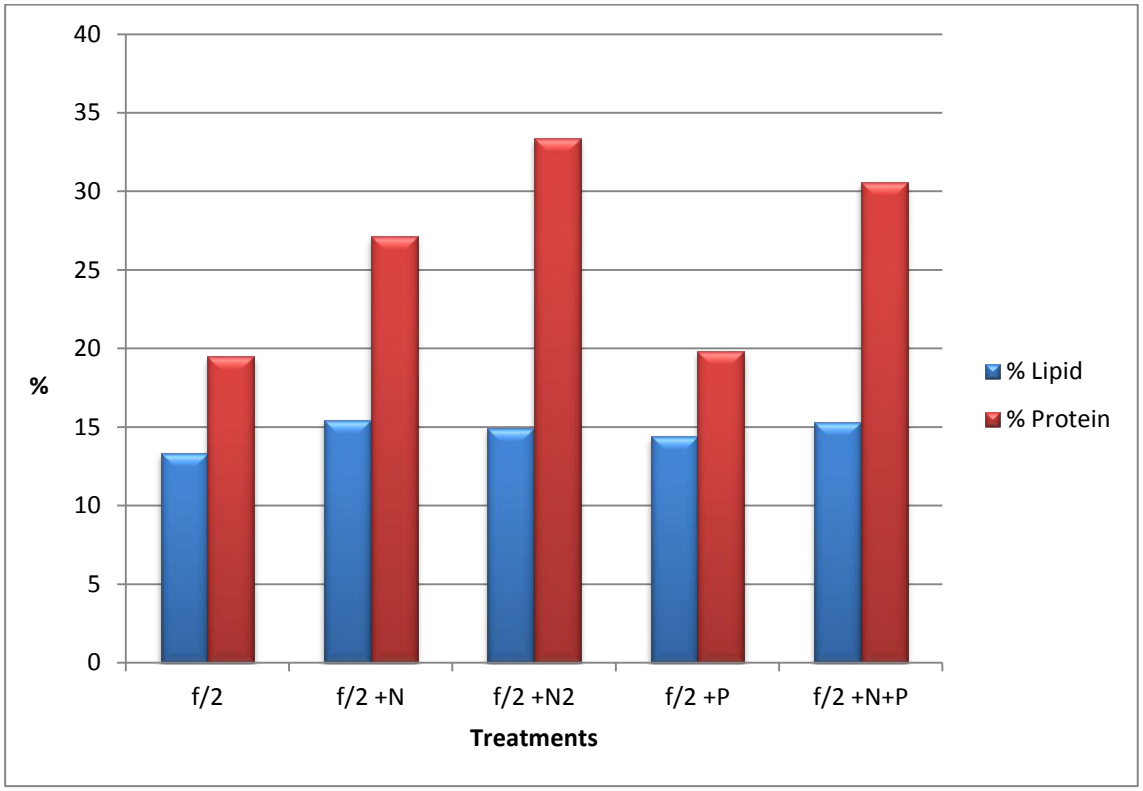


Figure 60. Lipid and protein percentage for Amshit isolate grown in different media.

5. Pigment content

The effect of N and P amendment on chlorophyll and carotenoid content were also determined using methanol and acetone extraction.

The optical density readings for the various treatments are reported in Table 27 and 29. Two equations were used to determine the concentration: Jeffrey and Humphrey (1975) and Strickland and Parsons (1968).

a. Acetone as solvent to extract pigments

Table 27. Optical density reading of acetone extracts from the various treatments.

No.	665.0nm	664.0nm	647.0nm	645.0nm	630.0nm	/ Abs
F2	0.7408	0.7626	0.4858	0.4586	0.3175	
F2+P	0.8398	0.8692	0.5078	0.4729	0.2695	
F2+N	1.1178	1.0327	1.0278	1.0037	1.1292	
F2+N+P	0.6928	0.7173	0.3607	0.3294	0.1816	

Table 28. Chlorophyll a quantification using acetone

Amshit	**f/2 medium**	**f/2 +P**	**f/2 +N**	**f/2 +N+P**
µg Chlorophyll (J. & H.)	3.27	4.57	8.44	5.67
µg Chlorophyll (S. & P.)	3.15	4.38	8.76	5.44

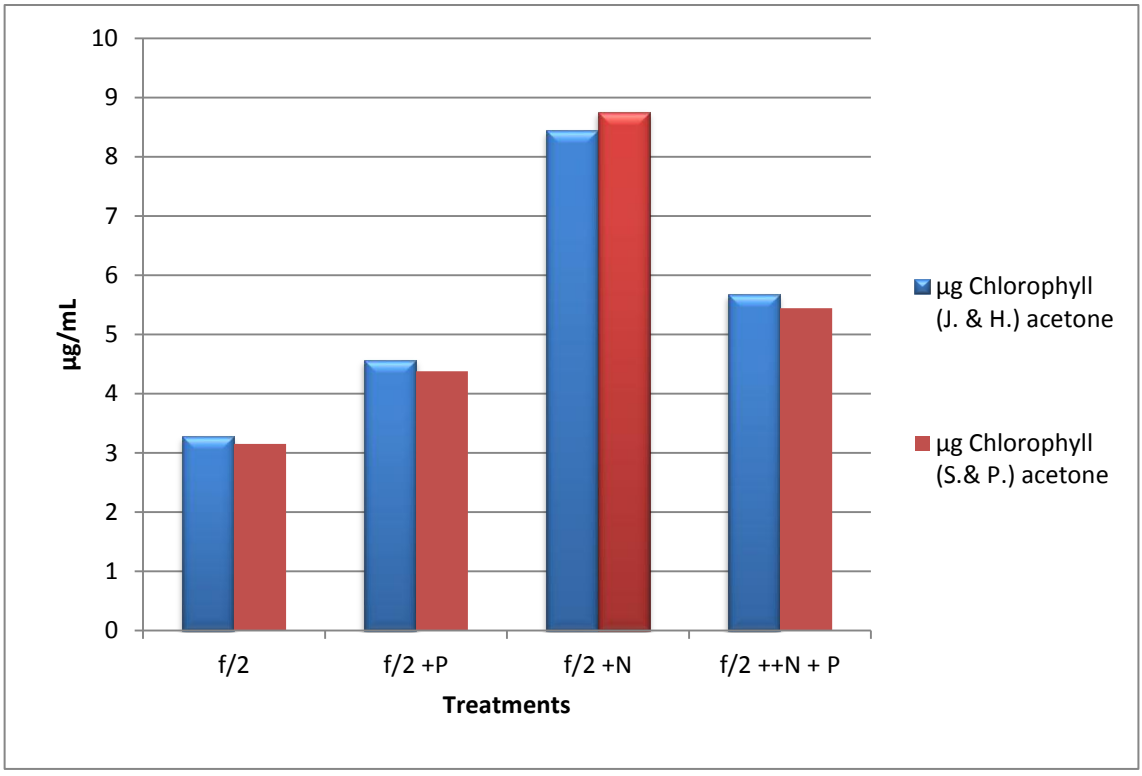


Figure 61. Chlorophyll a quantification using acetone.

b. Methanol as solvent to extract pigments

Table 29. Optical density reading of methanol extracts from the various treatments.

No.	428.0nm	666.0nm	665.0nm	652.0nm	470.0nm	/ Abs
F2	2.4821	2.2515	2.2664	1.8028	2.5575	
F2+P	2.5240	2.5953	2.5954	2.5009	2.5761	
F2+N	2.6540	2.6000	2.6011	2.4336	2.6958	
F2+N+P	2.3468	2.0432	2.0512	1.5087	2.4575	

Table 30. Chlorophyll a and carotenoids quantification using methanol.

Amshit	**f/2 medium**	**f/2+P**	**f/2+N**	**f/2+N+P**
µg Chlorophyll (P. et al)	4.25	5.03	8.57	7.33
µg Carotenoids (mL)	11.12	11.13	11.67	10.71

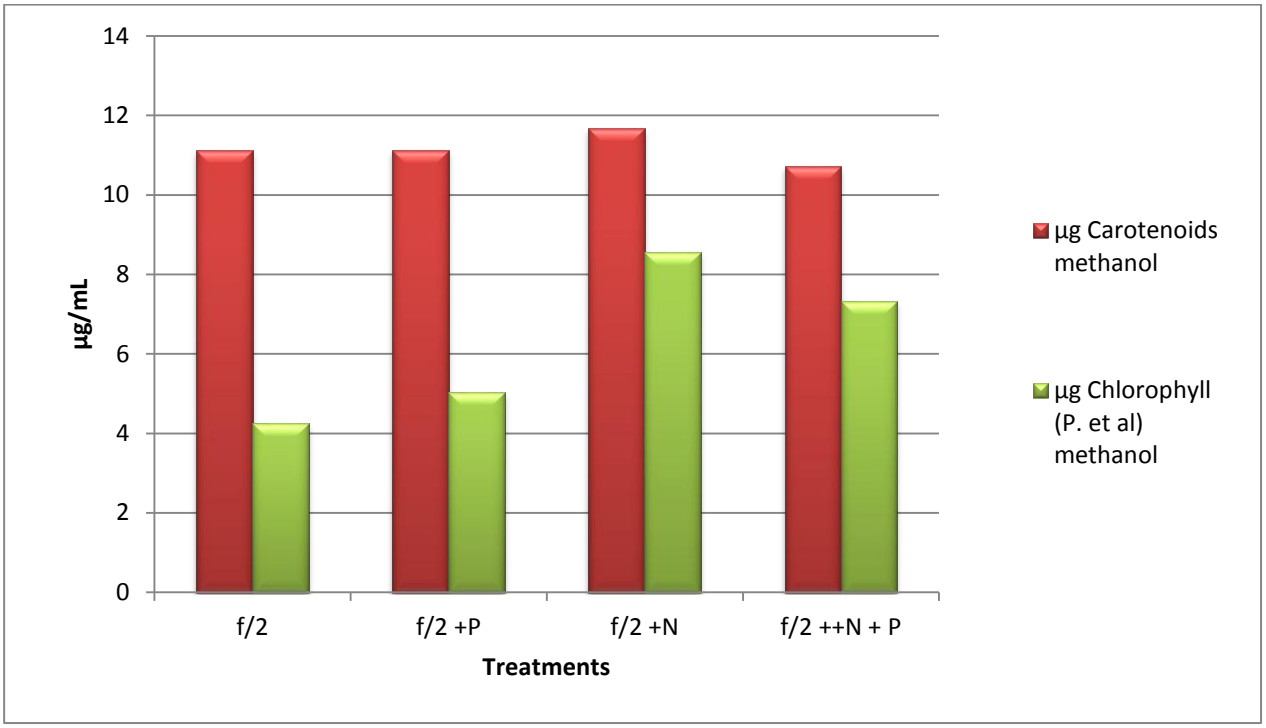


Figure 62. Chlorophyll a and Carotenoids quantification using methanol.

The chlorophyll concentration ranged between 3.1 and 8.7 µg/mL in the acetone extracts (Table 28) and 4.2 and 8.5 µg mL in the methanol extracts of the various treatments (Table 30), showing slight differences between the two methods specially for the f/2+N+P; however, the same trend for relative concentrations were maintained. Determination of chlorophyll content using either acetone or methanol as solvent showed clearly a significant increase in chlorophyll content in the treatments with nitrogen supplementation, with the highest concentration recorded in the nitrogen supplementation alone 8.56 µg/mL which was roughly the double of that determined in the basic medium, 4.25 µg/mL. Phosphorus supplementation increased the chlorophyll content but significantly less than that of nitrogen.

The carotenoid content ranged between 10.70 and 11.12 µg/mL in all treatments, and does not seem to have been affected by N or P supplementation.

H. Antifungal Activity

None of the microalgal extracts tested showed any in-vitro antifungal activity against the five plant pathogens. When compared to the control, better fungal sporulation was observed in the methanolic and cell extracts (Figure 63-64).

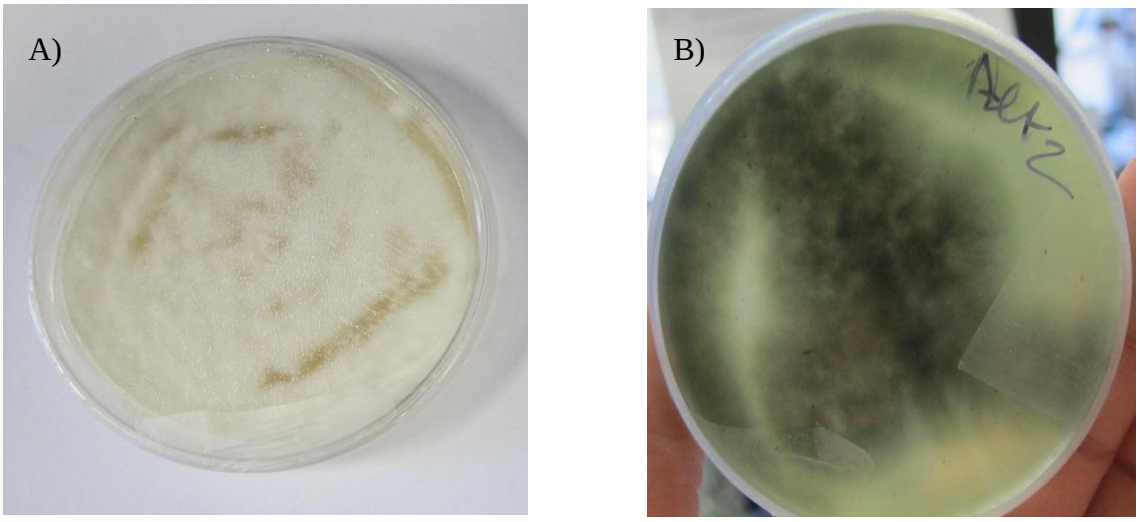


Figure 63. (A) The activity of methanolic extracts of II culture on the growth of *Fusarium oxysporum*, (B) The activity of PM crude water extracts on the growth of *Alternaria solani.*

The growth was similar to that of the respective control.

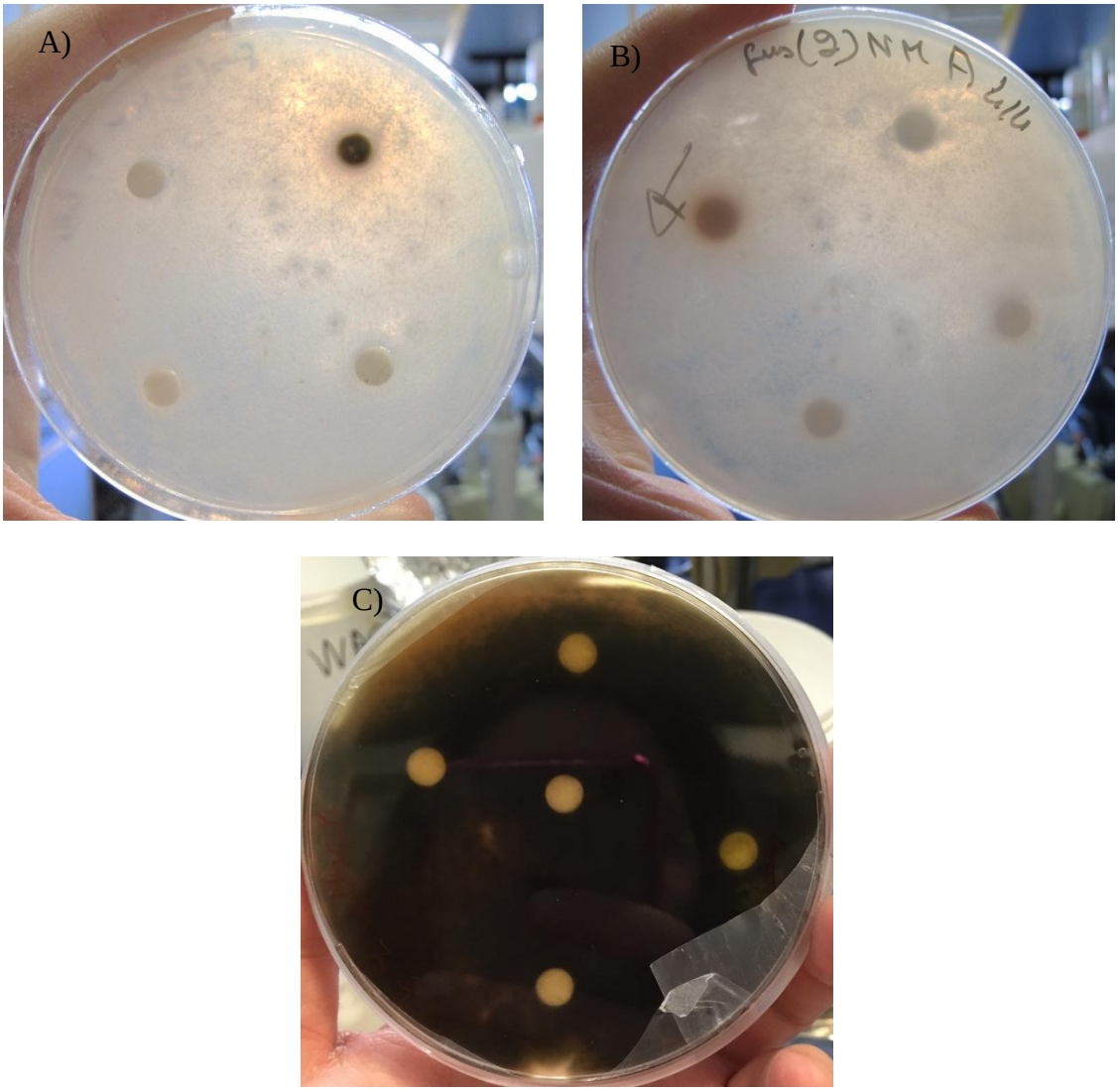


Figure 64. (A) The activity of cell extracts of D culture on the growth of *verticillium sp.;* (B) The activity of DAS non methanolic extracts on the growth of *Fusarium oxysporum;* (C) The activity of methanolic extracts of PALS culture on the growth of *Botrytis cinerea*

I. Antibacterial Activity

Most microalgal methanolic extracts showed significant antibacterial activity (table 31), with NK2 methanolic extracts showing the highest activity with an average inhibiton zone of 5-6 mm against *Streptococcus sp*., 4 mm against *Escherichia coli* and

Salmonella enteritidis and 3 mm against *Staphylococcus aureus* (Table 31). The lowest antibacterial activity was shown by isolate II, which showed either no inhibition for *E. coli* or low inhibition zone that did not exceed 2 mm for the other three bacteria tested. In most cases, the inhibition zones at 10 mg/L were similar to those observed at 20mg/L.

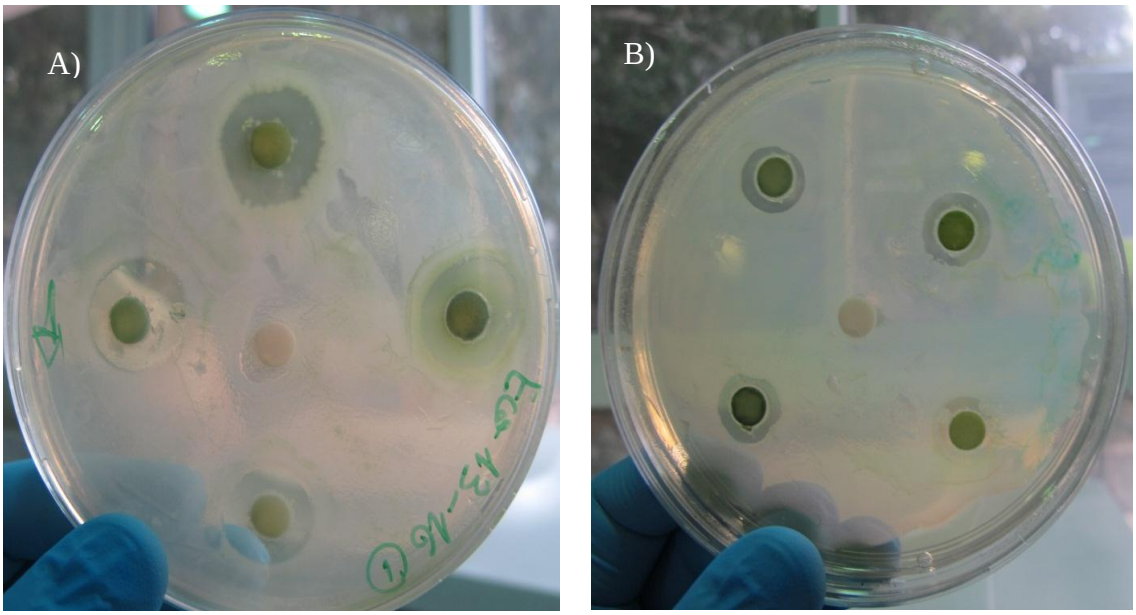


Figure 65. Two cultures of bacterial cells showing growth inhibition zones upon treatment with microalgal methnolic extracts. The four treatments on the periphery represent the microalgal extracts and the one in the center represent the ethanol negative control. A= NK2 and DPS extracts on *E.coli* ; B=DAS and D extracts on *Salmonella enteritidis*

Table 31. Inhibition zones of the microalgae methanolic extracts against four animal pathogenic bacteria.

Bacteria / Algae	Escherichia coli	Salmonella enteritidis	Streptococcus	Staphylococcus aureus
DAS (20 mg/mL)	3 mm	2 mm	3 mm	2 mm
DAS (10 mg/mL)	3 mm	3 mm	3 mm	2 mm
D (20 mg/mL)	1 mm	3 mm	3 mm	2 mm
D (10 mg/mL)	1 mm	3 mm	2 mm	2 mm
PALS (20 mg/mL)	3 mm	3 mm	3 mm	2 mm
PALS (10 mg/mL)	2 mm	4 mm	2 mm	2 mm
II (20 mg/mL)	0	1 mm	2 mm	2 mm
II (10 mg/mL)	0	1 mm	2 mm	1 mm
Spirulina (20 mg/mL)	4 mm	2 mm	4 mm	1 mm
Spirulina (10 mg/mL)	4 mm	2 mm	3 mm	2 mm
PM (20 mg/mL)	3 mm	4 mm	0	1 mm
PM (10 mg/mL)	3 mm	2 mm	0	1 mm
DPS (20 mg/mL)	4 mm	2 mm	4 mm	3 mm
DPS (10 mg/mL)	3 mm	3 mm	4 mm	3 mm
NK2 (20 mg/mL)	4 mm	4 mm	5 mm	3 mm
NK2 (10 mg/mL)	4 mm	3 mm	6 mm	2 mm
Ethanol (95%)	1 mm	0	0	0

J. Growth Promotion

1. Seed treatment

a. Root elongation

Soaking the cucumber seeds in different concentrations of PS, CM, PM, JBS or Brumana cell extracts did not have any significant root elongation as compared to the water control (Tables 32, 35, 36, 37, and 38). However, three concentrations of Amshit extract (10^{-2}, 10^{-4} and 10^{-6}) showed a significant increase in root length by 23.97%, 30.94% and 27.02% respectively as compared to the control (Table 33). Moreover, JOS (10^{-10}) enhanced the root elongation by 39.3% as compared to the control (Table 34). Seeds soaked in Brumana extracts had longer roots as compared to JBS and CM.

Table 32. Effects of various concentrations of PS isolate on the root length of cucumber seeds.

Treatment	**Root Length**
Water	$9.2^{a} \pm 1.43$
PS (10^{-2})	$10.6^{a} \pm 2.94$
PS (10^{-4})	$11.6^{a} \pm 0.98$
PS (10^{-6})	$10.9^{a} \pm 1.64$
PS (10^{-8})	$9.4^{a} \pm 1.16$
PS (10^{-10})	$10.8^{a} \pm 1.38$

[a] Means in a column followed by different alphabetical superscripts are significantly different ($P<0.05$)

Table 33. Effects of various concentrations of Amshit isolate methanolic extracts on root elongation of cucumber seeds.

Treatment	**Root Length**
Water	$9.2^{a} \pm 1.43$
Amshit (10^{-2})	$11.4^{b} \pm 1.28$
Amshit (10^{-4})	$12.0^{b} \pm 1.18$
Amshit (10^{-6})	$11.7^{b} \pm 1.33$
Amshit (10^{-8})	$10.8^{a,b} \pm 1.20$
Amshit (10^{-10})	$11.2^{a,b} \pm 1.04$

^{a,b} Means in a column followed by different alphabetical superscripts are significantly different ($P<0.05$).

Table 34. Effects of various concentrations of JOS isolate on the root length of cucumber seeds.

Treatment	**Root Length**
Water	$9.2^{a} \pm 1.43$
JOS (10^{-2})	$11.4^{a,b} \pm 2.49$
JOS (10^{-4})	$11.2^{a,b} \pm 2.61$
JOS (10^{-6})	$11.8^{a,b} \pm 1.61$
JOS (10^{-8})	$12.2^{a,b} \pm 1.20$
JOS (10^{-10})	$12.8^{b} \pm 2.16$

^{a,b} Means in a column followed by different alphabetical superscripts are significantly different ($P<0.05$)

Table 35. Effects of various concentrations of CM isolate on the root length of cucumber seeds.

Treatment	Root Length (cm)
Water	$5.4^{a} \pm 0.84$
CM (10^{-2})	$4.8^{a} \pm 0.61$
CM (10^{-4})	$4.3^{a} \pm 0.64$
CM (10^{-6})	$4.9^{a} \pm 0.29$
CM (10^{-8})	$5.0^{a} \pm 0.95$
CM (10^{-10})	$4.4^{a} \pm 0.65$

[a] Means in a column followed by different alphabetical superscripts are significantly different ($P<0.05$)

Table 36. Effects of various concentrations of JBS isolate on the root length of cucumber seeds.

Treatment	Root Length (cm)
Water	$5.4^{b,c} \pm 0.84$
JBS (10^{-2})	$3.8^{a,b} \pm 0.81$
JBS (10^{-4})	$3.4^{a} \pm 0.65$
JBS (10^{-6})	$5.6^{c} \pm 1.01$
JBS (10^{-8})	$4.9^{a,b,c} \pm 0.85$
JBS (10^{-10})	$5.3^{b,c} \pm 1.20$

[a,b,c] means in a column with different alphabetical superscripts are significantly different ($P<0.05$).

Table 37. Effects of various concentrations of PM isolate on the root length of cucumber seeds.

Treatment	Root Length (cm)
Water	$5.4^{a} \pm 0.84$
PM (10^{-2})	$4.6^{a} \pm 0.80$
PM (10^{-4})	$5.4^{a} \pm 1.13$
PM (10^{-6})	$5.2^{a} \pm 0.84$
PM (10^{-8})	$4.7^{a} \pm 1.03$
PM (10^{-10})	$5.2^{a} \pm 1.05$

[a] Means in a column followed by different alphabetical superscripts are significantly different ($P<0.05$)

Table 38. Effects of various concentrations of Brumana isolate on the root length of cucumber seeds.

Treatment	Root Length (cm)
Water	$5.4^{a,b} \pm 0.84$
Brumana (10^{-2})	$5.5^{a,b} \pm 0.21$
Brumana (10^{-4})	$5.4^{a,b} \pm 0.82$
Brumana (10^{-6})	$4.4^{a} \pm 0.75$
Brumana (10^{-8})	$5.4^{a,b} \pm 1.58$
Brumana (10^{-10})	$6.4^{b} \pm 1.20$

[a,b] Means in a column followed by different alphabetical superscripts are significantly different ($P<0.05$).

b. Seedling growth

Spraying the cucumber seedlings with some microalgae extracts had a statistically significant positive effect on the growth and weight of the seedlings compared to the control. For instance, an increase of 61.22% and 128.57% was observed with the CM (10^{-4}) and CM (10^{-8}) treatments respectively (Table 39; Figure 66) and an increase of 150% and 175% was recorded in the case of the JBS (10^{-8}) and JBS (10^{-10}) respectively (Table 40; Figure 67).

As for the differences between the concentrations of the same algae, there was an increase of 41.77% in the growth of the cucumber seeds treated with CM (10^{-8}) as compared to CM (10^{-4}).

Table 39. Effects of various concentrations of CM extracts on the weight of cucumber seedlings.

Treatments	**Weight (g)**
Water	$2.5^{a} \pm 0.73$
CM (10^{-4})	$4.0^{b} \pm 1.11$
CM (10^{-8})	$5.6^{c} \pm 1.34$

^{a,b,c} means in a column with different alphabetical superscripts are significantly different ($p<0.1$)

Figure 66. Cucumber seedlings treated with different concentrations of CM isolate.

Table 40. Effects of various concentrations of JBS isolate on the weight of cucumber seedlings.

Treatments	**Weight (g)**
Water	$2.4^{a} \pm 0.53$
JBS (10^{-2})	$4.8^{a,b} \pm 1.73$
JBS (10^{-4})	$4.8^{a,b,c} \pm 1.30$
JBS (10^{-6})	$3.5^{a,b} \pm 0.36$
JBS (10^{-8})	$5.9^{b,c} \pm 2.07$
JBS (10^{-10})	$6.5^{c} \pm 0.25$

^{a,b,c} means in a column with different alphabetical superscripts are significantly different ($p<0.1$)

Figure 67. Cucumber seedlings treated with different concentrations of JBS isolate.
1: JBS (10^{-10}), 2: JBS (10^{-8}), 3: control

2. Foliar sprays

a. Tomato seedlings

Visual observations showed that spraying different microalgae extracts (JBS and PM) at two concentrations (10^{-3} and 10^{-7}) seemed to enhance seedling growth. However, only JBS (10^{-3}) showed a statistically significant increase in weight from 2.86g for the control to 4.01g for the treated seedlings, an increase of 39.86% (Table 41).

As for the length, no statistical variations were recorded between the treatments and the control (Table 41) although the plants sprayed with microalgae seemed bigger and taller (Figure 68).

Table 41. Effects of two microalgal extracts, each used at two concentrations on the weight and length of tomato seedlings.

	Weight (g)	**Length (cm)**
Water	$2.9^{a} \pm 0.73$	$25.9^{a} \pm 3.81$
JBS (10^{-3})	$4.0^{b} \pm 1.19$	$27.8^{a} \pm 4.16$
JBS (10^{-7})	$2.6^{a} \pm 0.61$	$22.3^{a} \pm 4.83$
PM (10^{-3})	$3.4^{a,b} \pm 0.71$	$27.3^{a} \pm 2.98$
PM (10^{-7})	$3.6^{a,b} \pm 1.45$	$26.5^{a} \pm 6.87$

^{a,b} means in a column with means in a column with different alphabetical superscripts are significantly different ($p<0.05$).

Figure 68. Tomato seedlings sprayed with JBS and PM isolates.

b. Cucumber seedlings

i. Cell extracts

No statistical differences were found between all the concentrations of Brumana cell extracts and between the control (Table 42; Figure: 69).

Table 42. Effect of different concentration of cells extracts of Brumana isolate on the dry weight of cucumber seedlings.

Treatments (cells)	**Weight (g)**	**Length (cm)**
Control (water)	$8.5^{a} \pm 3.22$	$13.3^{a} \pm 3.88$
BRUM (10^{-2})	$11.4^{a} \pm 5.91$	$20.4^{a} \pm 7.30$
BRUM (10^{-4})	$9.7^{a} \pm 6.32$	$11.8^{a} \pm 4.50$
BRUM (10^{-6})	$11.1^{a} \pm 5.84$	$19.0^{a} \pm 5.55$
BRUM (10^{-8})	$9.9^{a} \pm 1.96$	$16.1^{a} \pm 2.79$

[a] means in a column with means in a column with different alphabetical superscripts are significantly different ($p<0.05$)

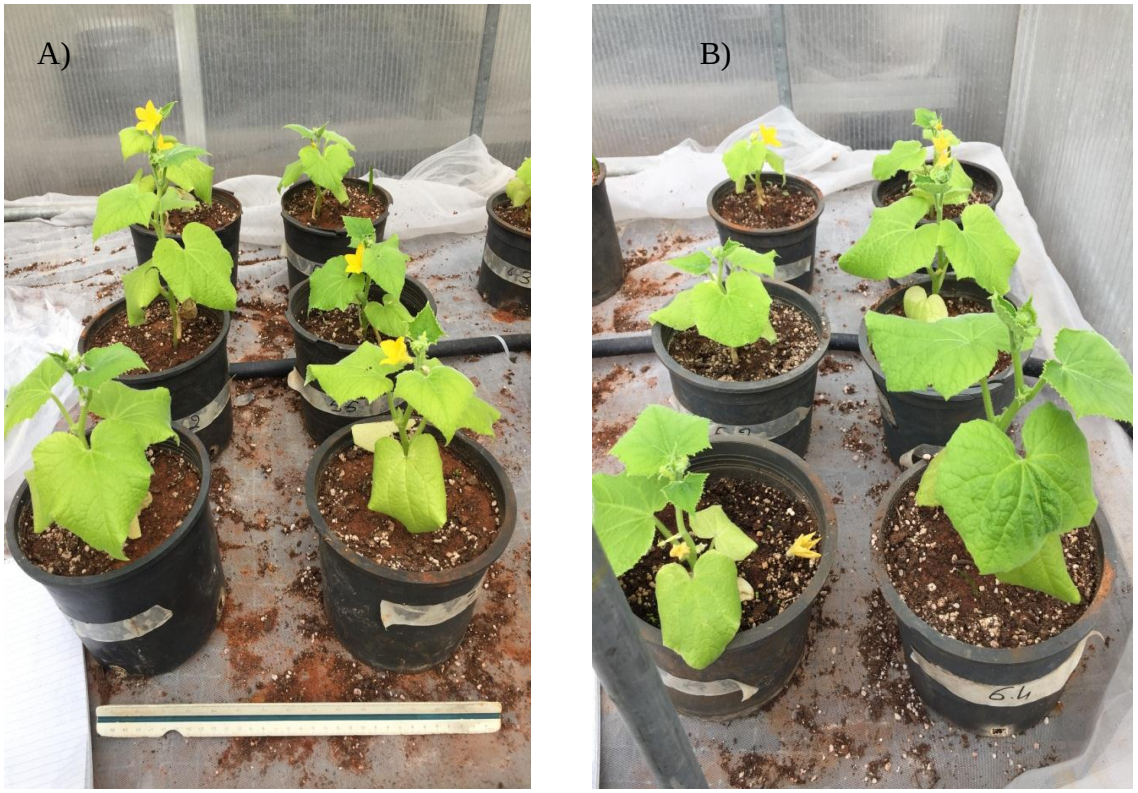


Figure 69. A) Brumana (10^{-2}) treatment; B) control treatment (water)

ii. Methanolic extracts

No statistical differences were found between all the concentrations of the methanolic extracts as compared to the control (10% ethanol) in the weight or length of the cucumber plants (Table 43; Figure 70).

Table 43. Effect of different concentrations of methanolic extracts of Brumana isolate on the dry weight and length of cucumber seedlings.

Treatments (methanolic)	**Weight (g)**	**Length (cm)**
Control (ethanol)	$5.8^{a} \pm 1.48$	$10.2^{a} \pm 5.46$
BRUM (1:1)	$6.2^{a} \pm 1.66$	$10.2^{a} \pm 1.60$
BRUM (10^{-1})	$9.0^{a} \pm 5.73$	$14.5^{a} \pm 5.96$
BRUM (10^{-2})	$8.2^{a} \pm 4.00$	$12.5^{a} \pm 3.78$
BRUM (10^{-3})	$8.9^{a} \pm 3.99$	$13.5^{a} \pm 5.21$

[a] Means in a column followed by different alphabetical superscripts are significantly different ($P<0.05$)

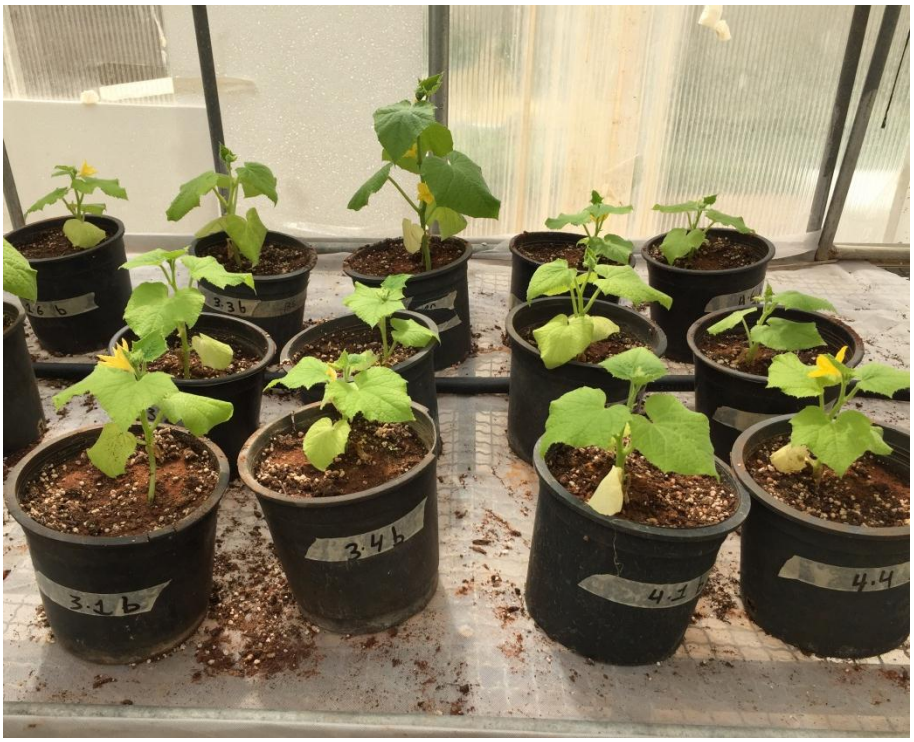


Figure 70. Methanolic extracts treatments; right: BRUM (10^{-2}); left: BRUM (10^{-3})

CHAPTER V

DISCUSSION

Microalgae are single or multi-celled microorganisms characterized by their efficient photosynthetic activity, high rate of reproduction and capacity to survive in different environments. The ability of microalgae to accumulate high quantities of biomass within a relatively short time has attracted the attention of researchers since several decades as potential source of food, feed and feedstock for renewable bioenergy (Sheehan *et al.* 1998). Since microalgae can grow in seawater or wastewater, they do not compete with cultivated land nor with fresh water resources needed for production of food and feed crops. In addition the pollution generated by burning fossil fuels and their contribution to global warming has led many developed countries to issue legislations pertaining to reduction of CO_2 emissions and the search for renewable sustainable sources of clean energy has become a priority (Chisti, 2007). Some reports showed that during their growth, microalgae can fix 1.83 g of CO_2 for each g of dry matter produced, therefore they can be used to trap CO_2 produced by factories. Some reports showed that microalgae represent the only feedstock capable of producing enough biodiesel to cover the world needs.

This research focused on exploring the potential use of local microalgae species as resources of renewable bioenergy as well as sources of bioactive substances that can be used in agriculture as antimicrobial agents, plant growth biostimulants or feed supplements. Therefore, local isolates were cultured, their proximate analysis was conducted to evaluate the potential uses as biofuel feedstock or feed supplements and

their extracts were used in laboratory trials to test their antimicrobial and growth promotion activities.

A. Isolation and Molecular Identification

Microalgae samples were collected from seawater all along the sea coast or from freshwater sources. The isolation procedure followed in this study may have resulted in the predominance of some species of microalgae like *Tetraselmis* and *Chlorella* belonging to the green microalgae. It would be recommended to use two or more isolation media including two specific media that favor the growth of diatoms and cyanobacteria, due to the importance of these organisms in the production of bioactive substances.

A total of twenty five local microalgae isolates were successfully grown in culture media. The microscopic nature of microalgae and the difficulty in finding suitable differentiating morphological characteristics lead many researchers to focus on molecular tools for the proper identification of microalgae species. In this research we used three genetic markers, two based on the 18S rDNA (LSU and ITS) and the third one on *tufA* gene. Twelve isolates were identified to the genus level and twelve to the species level. Four of these genera were not reported previously in Lebanon: *Scherffelia dubia*, *Neochloris conjuncta*, *Acutodesmus obliquus* and *Cyanobacterium aponinum*. Few other genetic markers will be needed to identify the isolates to the species levels. While the LSU , ITS and *tufA* gene loci are suitable for Chlorophyta, other specific markers were reported for the other groups of microalgae, like the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene for diatoms (Hamsher *et*

al. 2011), the cytochrome c oxidase subunit I (COI) for red and brown algae (Saunders 2001) and CYA for cyanobacteria (Nubel *et al.* 1997).

B. Growth Kinetics

The growth rate of microalgae depends on several parameters including the microalgae isolate and several environmental factors including temperature, light: dark cycles, light intensity, salinity, pH, CO_2 availability and nutrient availability. The growth rate of sixteen single isolates and seven blooms was studied. The highest growth rate was directly after the lag phase and lasted for two to four days only. After that stage, the growth rate dropped sharply. The major limitations leading to this slowdown in growth rate may be due to the decrease in nutrient availability or due to the accumulation of toxins and inhibitory products; therefore, it would be recommended to try different growth media or to supplement nutrients two to three days after the initiation of the exponential growth rate. Other limiting factors that may be taken into consideration are the light intensity and the temperature, which may improve the growth rate when optimized.

A higher growth rate (μmax) was obtained in the freshwater isolates (1.69 to1.05) compared to the seawater isolates (1.622 to 0.53). The highest growth rates were 1.69 for the Brumana freshwater isolates and 1.622 for the C.LEB marine isolate. As for the blooms, the μmax was 0.64 for PM Bloom.

It was also observed that the cyanobacteria isolates had lower growth rates (μmax) than those of isolates belonging to the green algae family.

C. Dry Matter

Under the experimental conditions used, namely lighting for 16 hrs at 450 lux at room temperature using Guillard's f/2 medium for seawater and Bold Basal for freshwater, the production of dry matter in flat photobioreactor varied significantly among isolates and ranged between 0.30-0.85 g dry matter/L with nine isolates and two blooms yielding over 0.6 g/L namely: NK2, Saida, Jiyyeh, JOS, JBS, NS, Brumana (fw), BS (fw), MSG (fw), DPS Bloom and D Bloom.

The level of production of the local microalgae isolates is of the same order of magnitude reported in many recent publications; Zhang (2014) reported that the biomass production of *Chlorella sp.* is 300 mg/L, which is similar to what we obtained for C.LEB (300 mg/L).

The dry matter produced in the systems used in this trial was of the same order reported in several articles, i.e. about 500-600 mg/L/harvest or 33- 40 mg/ L/ day(Devi et al. 2012; Gris, 2013). However, the dry matter yields of the Lebanese isolates are considerably lower than those reported under optimized growth conditions in flat bioreactors (green wall photobioreactors), where the dry matter yield reached about 300 mg/L/day (Rodolfi *et al.* 2009).

The biomass yield obtained from the Lebanese isolates does not probably represent the maximum potential production since the growth conditions were not optimized to meet the growth requirements of each isolate. This suggests that under our experimental conditions, there may be one or more limiting factor(s).

The growth rate of microalgae depends on various parameters including species and several environmental factors such as temperature, light duration and intensity, CO_2,

salinity, pH, nutrient availability and toxic materials excreted into the medium (Richmond, 2004).

D. Total Lipid Content

The total lipid content also varied greatly between isolates and ranged between 5 -7% in CAS, UN and DPS bloom and about 20- 22% in CM, PS and Zalka bloom. This lipid content is similar to that reported for most green algae (table 8). However, it is less than that reported for *Scenedesmus dimorphus* (16-40%) and *Botryococcus braunii* (25-75%) (Becker, 1994; Mata *et al.* 2010.)

Nitrogen starvation has been reported to increase the lipid content, for example the lipid content in *Chorella pyrenoidosa* increased by 26% after nitrogen starvation (Nigam *et al*. 2011).

For biodiesel production, the total lipid production expressed in mg lipids/L/day is the most important parameter, in addition to the quality of the FAs. Unfortunately, the isolates that produced the highest quantity of biomass were among those that contained the lower lipid percentage, for example the MGS (fw) isolate which gave the highest biomass yield (850 gm/L) contained only 9.7 % lipids. This negative relationship between high biomass production and high lipid production was often reported in the literature (Chisti, 2008). Under our experimental conditions two isolates and one bloom (PS: 103.44 mg/L, Brumana: 104.2 mg/L and Zalka bloom: 102.3 mg/L) gave a similar lipid yield/L compared to the reference isolate C.M (99.46 mg/L) provided by the European partners.

For a good quality biodiesel production, a high content of saturated and mono unsaturated fatty acids is preferred, while for feed supplements a high PUFA content is

preferred. Therefore, the JOS and Zalka bloom isolates may be more suited for biodiesel production although JOS has a low lipid content, while Brumana, Amshit and PS may be suited for nutritional purposes especially that Brumana isolate contains 13% (of its total lipids) as Docosahexaenoic Acid (DHA), the most important omega 3 FA. The lipid content of microalgae and the composition of their saturated and unsaturated fatty acids may be affected by several factors including the light conditions, nutrient composition of the medium, pH, salinity and mainly nitrogen starvation etc. Therefore, optimization of culturing conditions may improve the total biomass production, the total lipid content and/or the FA profile.

E. Protein Content

The protein content in microalgae varies greatly, in some *Spirulina* isolates it can reach over 60% (Sydney et al. 2010); some of these isolates are being commercialized as food supplements. Most of the Lebanese isolates had a protein content ranging between 10 and 40%; however, three blooms and one unialgal isolate had a high protein content exceeding 40% namely PALS bloom (56.24%), CAS (49.36%), D Bloom (47.37%) and DAS Bloom (43.13%); thus they may be evaluated as feed or food supplements. However, further studies are needed to analyze their amino acid content; those that may have a relatively high content of lysine and leucine may be preferred since these amino acids are deficient in most cereals and vegetables (vegetarian foods).

When water use efficiency is taken as a factor in protein production, microalgae cultivation may represent a very interesting alternative to beef meat protein. To produce a kg of beef meat, which contains about 20% protein (200g), an average of 15,000-

17,000 L of water are needed. While if we assume that the protein of the Lebanese microalgae cultures PALS and CAS blooms is of high quality, and at the current level of biomass production without the optimization of growth conditions to improve biomass production and/or protein content, a protein production of 266 g (0.475g/L × 56% × 1000L) to 288 g (0,60 g/L × 47% × 1000) of protein are produced per m^3 of water; a 20× higher water use efficiency over beef protein , without considering that for microalgae production, seawater may be used not fresh water.

F. Effect of N and P

The secondary effluent of sewage water treatment plants contains high levels of N and P which may be used as nutrient source for microalgae thus reducing pollution and at the same time reducing cost of microalgae production. When the f/2 medium was supplemented with 30ppm ammonium and 2 ppm phosphate, the levels found in sewage water effluent in Lebanon (Bashour, 2014 personal communication), the protein content of the Amchit isolate was increased from about 20 to 30% ie about 50%, while P amendment alone did not increase protein content significantly. Nitrogen amendments also lead to a significant increase in chlorophyll content, which nearly doubled as compared to that in the reference medium. Our results are in general agreement with several reports that showed that growth conditions, especially nitrogen levels in the nutrient medium might affect significantly the chlorophyll and protein content (Piorreck *et al*, 1984). N and P supplementation also lead to about 54% increase in biomass yield but did not seem to have affected the total lipid content nor the carotenoid content.

G. Antimicrobial Activity

When the antifungal activity of 12 selected microalgae isolates was evaluated in *in-vitro* trials, no activity was recorded for any of the extracts used, against the five plant pathogenic fungi tested. However, the same methanolic extracts showed a significant antibacterial activity against four animal pathogens, namely *Escherichia coli, Salmonella enteritidis*, *Staphylococcus aureus,* and *Streptococcus sp.* The highest activity was observed for NK2 bloom, which resulted in bacterial growth inhibition zones ranging from 6 mm for *Streptococcus sp.* to 3 mm for *Staphylococcus aureus* at the two concentrations used 10 and 20 mg/mL.

H. Growth Stimulation

For evaluation of plant growth stimulation/ promotion, two types of tests were performed: seed treatment by soaking in serial dilutions of various microalgae extracts or by foliar sprays with these extracts. Out of seven microalgae tested by seed treatment, only Amshit isolate, with extract concentrations ranging between 10^{-2} and 10^{-6} g/mL, gave a statistically significant increase in root elongation of about 30% compared to water. Similar results were previously reported for squash, tomato and cucumber seeds soaked in *Anabaena vaginicola, Nostoc sp*. and Nodularia *harveyana* microlgal extracts which resulted in a faster germination and an increase in fresh and dry weight (Shariatmari *et al*. 2011).

Foliar sprays of cucumber and tomato seedlings with either microalgae cell extracts or methanolic extracts were also tried. While visual observations seemed to indicate an improved growth of tomato seedlings, these observations were not confirmed by the statistical analysis of data collected on fresh weight and seedling

length. Since the sprayed seedlings were greener in color, determination of chlorophyll content may give a better indication than length.

However, upon treatment of cucumber seedlings with water extracts, two microalgae cell extracts (CM and JBS) gave significant growth promotion with 62 - 128% increase in weight for CM extracts and 150 - 175% for JBS extracts.

Our results are in line with those reported by Shariatmari *et al*. (2011) where an increase of at least 60% in fresh weight of cucumber treated with microalgae was observed.

CHAPTER VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

This study focused on the isolation and molecular characterization of local microalgal isolates, determination of their lipid content, fatty acids profile, and protein content as a preliminary evaluation of their potential use for biodiesel production and for food or feed supplements. Trials were also conducted to evaluate their potential use for the control of plant pathogenic fungi and animal bacteria as well as for plant growth promotion.

Although light and electron microscopy have been traditionally used to identify microalgae, molecular techniques are considered as the preferred tool in the identification of microalgae. In Lebanon, no efforts have been conducted regarding the identification of local microalgal isolates using molecular tools.

A total of 25 local isolates were cultured. Amplification and sequencing of the 18S rDNA genomic regions LSU and ITS proved very useful. The following genera and species were identified: *Acutodesmus obliquus, Amphora sp., Chlorella sp., Chloromonas sp., Cyanobacterium aponinum, Micractinium reisseri, Microcoleus sp., Neochloris conjuncta, Scenedesmus sp., Scherffelia dubia, Tetraselmis marina, Tetraselmis sp.* and *Tetraselmis striata.*

Microalgae have been long recognized as potentially good sources for biofuel production because of their high oil content and rapid biomass production. In recent years, use of microalgae as an alternative biodiesel feedstock has gained renewed interest especially since most of the lipid content of microalgae is in the form of TAG

which is the form needed to produce biodiesel. The lipid content of the isolates tested ranged between 5 and 22% DW in UNS and PS isolates respectively. Out of the twenty four isolates and blooms tested, only PS, Damour and Zalka bloom had lipid content similar or higher to that of the reference isolate CM. Four local microalgae isolates seem to have a good potential for biodiesel production namely PS, Brumana (fw), Zalka Bloom and NS. As for the protein content, the unialgal isolate CAS and three blooms (PALS, D and DAS) had a protein content of about 50% DW.

Under our experimental conditions, the increase of nitrogen and phosphorus levels seemed to have a positive effect on the dry weight, protein and chlorophyll content when compared to the control.

Microalgae are significant resources for bioactive metabolites. Several secondary metabolites were found to have antimicrobial activities. In our lab none of the 12 different microalgal isolates showed any in-vitro antimycotic activity against any of the five plant pathogenic fungi tested. When compared to the control, better sporulation and growth was observed in all the treatments containing microalgae especially in the methanolic and crude water extract respectively. This may be justified by the fact that most microalgal isolates used are highly nutritious and are used as supplements in human and animal nutrition. However, methanolic extracts of most microalgae tested showed a good antibacterial activity against four animal pathogenic bacteria.

The growth promotion activity of extracts from few microalgae cultures were evaluated. Water extracts from three local microalgae cultures promoted root length or seedling growth.

In conclusion, the available land area on the coastal plain, close to seawater is very limited to consider large scale biodiesel production facilities. However, our results

show that locally adapted isolates may be used for production of microalgae biomass for the extraction of high value bioactive substances, such as natural antimicrobial compounds, plant growth promotors and feed supplements that can be used for sustainable agricultural and economic development. The byproducts may be used for biofuel production. The preliminary data obtained so far look promising, it is highly recommended to concentrate research on promising local microalgae as sources of bioactive substances and to conduct replicated, multi-location trials to confirm the efficacy of bioactive substances, to improve microalgae yield, reduce the cost of production and evaluate economic/commercial viability of production systems.

Recommendations:

i. To further explore the local microalgae diversity: identification and proximate analysis of more isolates to assess their potential uses and prospects for commercialization.
ii. To search for specific molecular markers for identification of each group of microalgae.
iii. To perform further tests to determine the minimum inhibitory concentration (MIC) in antibacterial tests.
iv. To determine the best concentration for growth stimulation.
v. To conduct in vivo experiments, since some microalgal extracts were reported to stimulate the plant defense system against pests.
vi. Optimization of growth conditions for maximizing production of compounds of interest: TAG, carotenoids, omega-3 and proteins, etc.

vii. To focus search on high value bioactive compounds and to consider biodiesel as by-product.

viii. To acquire appropriate facilities and equipment to conduct growth kinetic studies under controlled conditions in order to determine the optimal growing conditions.

ix. To create a new Master degree in Agriculture Biotechnology at FAFS that may have some focus on Marine biotechnology.

x. To expand areas of research in cooperation with other faculties: antioxidant, antimicrobial, anticancer activities, nutritional value for use as food and/or feed supplements and medicinal properties

APPENDIX I

CULTURING MEDIA

A. Guillard F/2 Media (Guillard and Ryther 1962, Guillard 1975)

A common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, termed "f Medium" (Guillard and Ryther 1962), has been reduced by half.

To prepare, begin with 950 mL of filtered natural seawater and add the following components. Bring the final volume to 1 liter with filtered natural seawater. If the alga to be grown does not require silica, then it is recommended that the silica be omitted because it enhances precipitation. Autoclave.

Table 44: Stock solutions used for preparation of Guillard f/2 medium.

Component	Stock Solutions (g/L dH_2O)	Quantity (mL)	Molar Concentration in Final Medium (M)
NaNO3	75	1	8.82×10^{-4}
$NaH_2PO_4.H_2O$	5	1	3.62×10^{-5}
$Na_2SiO_3.9H_2O$	30	1	1.06×10^{-4}
Trace metal solution	(see recipe below)	1	-
Vitamin solution		0.5	-

F/2 trace metal solution: to prepare, begin with 950 mL of dH2O, add the components and bring final volume to 1L with dH_2O. Autoclave.

Table 45: Composition of micro nutrient stock solutions used in preparation of Guillard f/2 medium

Component	Stock Solutions (g/L dH_2O)	Quantity (mL)	Molar Concentration in Final Medium (M)
$FeCl_3$ $6H_2O$	-	3.15	1.17×10^{-5}
Na_2EDTA $2H_2O$	-	4.36	1.17×10^{-5}
$CuSO_4$ $5H_2O$	9.8	1	3.96×10^{-8}
Na_2MoO_4 $2H_2O$	6.3	1	2.60×10^{-8}
$ZnSO_4$ $7H_2O$	22.0	1	7.65×10^{-8}
$CoCl_2$ $6H_2O$	0.0	1	4.20×10^{-8}
$MnCl_2$ $4H_2O$	180.0	1	9.10×10^{-7}

F/2 Vitamin Solution: first, prepare primary stock solutions. To prepare final vitamin solution, begin with 950 mL of dH_2O, dissolve the thiamine, add the amounts of the primary stocks as indicated in the quantity column below, and bring final volume to 1 liter with dH_2O.

Table 46: Composition of vitamin stock solution used in preparation of Guillard f/2 medium

Component	Primary Stock Solution (g/L dH2O)	Quantity	Molar Concentration in Final Medium (M)
Thiamine-HCl (vit. B1)	-	200 mg	2.96×10^{-7}
Biotin (vit. H)	0.1	10 mL	2.05×10^{-9}
Cyanocobalamin (vit. B12)	1.0	1 mL	3.69×10^{-10}

B. Basal Medium – BBM (Bold, 1949)

This is a widely used artificial freshwater medium, especially for growing green algae. The medium lacks vitamins and some of the trace metal concentrations are relatively high, making the medium unacceptable for growth of many non-green algae. Six macronutrient stock solutions, an alkaline EDTA solution, an acidified iron solution, a boron solution and a trace metals solution are individually prepared. The usual recipe is based upon 400 mL stock solutions, but for comparison with other media, we have included 1 liter stock solutions.

To prepare the final medium, begin with 940 mL of dH2O and add 10mL of the first six stock solutions. Add 1 mL each of the alkaline EDTA, acidified iron, boron and trace metals solutions. Autoclave. The final pH should be 6.6.

Table 47: Composition of stock solutions used in preparation of Basal medium.

Major stock solution				
Component	400 mL Stock Solution (g/L dH2O)	1 Liter Stock Solution (g/L dH2O)	Add quantity below/ liter of medium (mL)	Molar Concentration in Final Medium (M)
$NaNO_3$	10	2	10	2.94×10^{-3}
$CaCl_2 \cdot 2H_2O$	1	2.5	10	1.70×10^{-4}
$MgSO_4 \cdot 7H_2O$	3	7.5	10	3.04×10^{-4}
K_2HPO_4	3	7.5	10	4.31×10^{-4}
KH_2PO_4	7	17.5	10	1.29×10^{-3}

NaCl	1	2.5	10	4.28 X 10^{-4}
Alkaline EDTA Stock (Add 1 mL of this solution/1 L medium)				
EDTA anhydrous		50		4.28 x 10^{-4}
KOH		31		1.38 x 10^{-3}
Acidified iron solution (Add 1 mL of this solution/1 L medium)				
$FeSO_4 \cdot 7H_2O$		4.98		4.48 x 10^{-5}
H_2SO_4			1	
Boron stock solution(Add 1 mL of this solution/1 L medium)				
H_3BO_3		11.42		62 x 10^{-4}
Trace Metal solution(Add 1 mL of this solution/1 L medium)				
$ZnSO_4 \cdot 7H_2O$		8.2		7.67 x 10^{-5}
$MnCl_2 \cdot 4H_2O$		1.44		1.82 x 10^{-5}
MoO_3		0.71		1.23 x 10^{-5}
$CuSO_4 \cdot 5H_2O$		1.57		1.57 x 10^{-5}
$Co(NO_3)_2 \cdot 6H_20$		0.49		4.21 x 10^{-5}

BIBLIOGRAPHY

Abdel-Raouf, N., Al-Homaidan, A. A., & Ibraheem, I. B. M. (2012). Agricultural importance of algae. *African Journal of Biotechnology*, 11, 11648-11658.

Ahmad, F., Khan, A. U., & Yasar, A. (2013). Transesterification of oil extracted from different species of algae for biodiesel production. *African Journal of Environmental Science and Technology*, *7*(6), 358-364.

Algae as a Source of Pharmaceuticals & Nutraceuticals - Oilgae Blog (Oilgae Blog RSS).

Algae Cultivation - Flat-plate Photobioreactor - Oilgae - Oil from Algae (Algae Cultivation - Flat-plate Photobioreactor - Oilgae - Oil from Algae).

Allègre M., Héloir M. C., Trouvelot S., Daire X., Pugin A., Wendehenne D., & Adrian M. (2009). *Molecular Plant Microbe Interactions*. 22, 977-986.

Apt, K. E., & Behrens, P. W. (1999). Commercial developments in microalgal biotechnology. *Journal of Phycology*, *35*(2), 215-226.

Arumugam, M., Agarwal, A., Arya, M. C., & Ahmed, Z. (2013). Influence of nitrogen sources on biomass productivity of microalgae Scenedesmus bijugatus. *Bioresource technology*, *131*, 246-249.

ASTM (2008).Standard specification for biodiesel fuel (B100) blend stock for distillate fuels. In: Annual Book of ASTM Standards, ASTM International, West Conshohocken, Method D6751-08.

Battah, M., Hassan, I., & El-Naggar, M. (2014). Antifungal Agent from Spirulina maxima: Extraction and Characterization. *Global Journal of Pharmacology*, 8 (2), 228-236. doi: 10.5829/idosi.gjp.2014.8.2.8369.

Becker, E. W. (1994a). Culture Media. In: Microalgae: Biotechnology and microbiology. (Ed Becker, E. W.), pp. 9-41. Cambridge University Press. Cambridge, UK.

Becker, E. W. (1994b). Chemical Composition: Lipids. In: Microalgae: Biotechnology and microbiology. (Ed Becker, E. W.), pp. 179-183. Cambridge University Press. Cambridge, UK.

Bhagavathy, S., & Sumathi, P. (2012). Evaluation of antigenotoxic effects of carotenoids from green algae Chlorococcum humicola using human lymphocytes. *Asian Pacific journal of tropical biomedicine*, 2(2), 109-117.

Bhaud, Y., Guillebault, D., Lennon, J., Defacque, H., Soyer-Gobillard, M. O., & Moreau, H. (2000). Morphology and behaviour of dinoflagellate chromosomes during the cell cycle and mitosis. *Journal of cell science*, 113(7), 1231-1239.

Bi, Z., & He, B. B. (2013). Characterization of microalgae for the purpose of biofuel production. *Trans. ASABE*, 56(4), 1529-1539.

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification.*Canadian Journal of Physiology and Biochemistry*, 37, 911-917.

Bligh, E.G., Dyer, W.J., (1959) "Extraction of Lipids in Solution by the Method of Bligh and Dyer. *Canadian Journal of Biochemical Physiology*. 37**:** pp. 911-917.

Blue-green algae. (2015). In *Encyclopædia Britannica*. Retrieved from http://www.britannica.com/EBchecked/topic/70231/blue-green-algae

Blunt J.W., Copp B.R., Hu W.P., Munro M.H., Northcote P.T., & Prinsep M.R. (2008) Marine natural products. Natural Product Reports, 25:35-94.

Bold, H. C., & Wynne, M. J. (1978). Introduction to the algae: structure and reproduction, 706.

Brennan, L., & Owende, P. (2010). Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and sustainable energy reviews*, 14(2), 557-577.

Burja, A. M., Banaigs, B., Abou-Mansour, E., Burgess, J. G., & Wright, P. C. (2001). Marine cyanobacteria—a prolific source of natural products.*Tetrahedron*, *57*(46), 9347-9377.

Cantrell K.B., Ducey T., Ro K.S., & Hunt P.G. (2008). Livestock waste to bioenergy generation opportunities. *Bioresource Technology*, 99(17),7941–7953.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology advances*, *25*(3), 294-306.

Chisti, Y. (2008). Biodiesel from microalgae beats bioethanol. *Trends in biotechnology*, *26*(3), 126-131.

Classification of Algae - Oilgae - Oil from Algae (Classification of Algae - Oilgae - Oil from Algae) http://www.oilgae.com/algae/cla/cla.html#sthash.Rb7NXN51.dpu

Cultivation of Algae - Open Pond - Oilgae - Oil from Algae (Cultivation of Algae - Open Pond - Oilgae - Oil from Algae) http://www.oilgae.com/algae/cult/op/op.html

Danielo, O. (2005). An algae-based fuel. *Biofuture*, 255, 33-36.

Dismukes G.C., Carrieri D., Bennette N., Ananyev G.M., & Posewitz M.C. (2008). Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Current Opinion in Biotechnology*, 19(3), 235–40.

Edwards, M. (2010, June 24). Algal Classification. *Algae Industry Magazine*.

El Seblani, R. G., & American University of Beirut. Faculty of Agricultural and Food Sciences.Department of Agricultural Sciences. (2013). *Molecular characterization of local microalgae and evaluation of their potential use for biodiesel production and as plant disease control agents.*

Faheed, F. A., & Abd-El Fattah, Z. (2008). Effect of Chlorella vulgaris as bio-fertilizer on growth parameters and metabolic aspects of lettuce plant. *Journal of Agrulture and Social Science*, *4*, 165-169.

Falkowski, P.G., Barber, R.T., & Smetacek V. (1998). Biogeochemical controls and feedbacks on ocean primary production. *Science*, 281, 200–206.

FAO (1996).Manual on the production and use of Live Food for Aquaculture. Intergovernmental panel on climate change, the physical science basis: human and natural drivers of climate change.

Folch J., Lees M., & Stanley G.H.S. (1956). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chem*istry, 226, 497–509.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of bioogicall chemistry*, *226*(1), 497-509.

Galal, H. R. M., Salem, W. M., & El-Deen, F. N. (2011). Biological control of some pathogenic fungi using marine algae extracts. *Research Journal of Microbiology*, 6(8), 645-657.

Gavrilescu, M. & Chisti Y. (2005). Biotechnology-a sustainable alternative for chemical industry. *Biotechnology Advances*, 23, 471-99.

Ghirardi, M. L., Zhang, L., Lee, J. W., Flynn, T., Seibert, M., Greenbaum, E., & Melis, A. (2000). Microalgae: a green source of renewable H 2. *Trends in biotechnology*, *18*(12), 506-511.

Giselrød H.R., Patil V., & Tran K. (2008).Towards sustainable production of biofuels from microalgae. *International Journal of Molecular Science*, 9, 1188-95.

Griffiths, M. J., & Harrison, S. T. L. (2009). Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*, 21(5), 493.

Gris, L. R. S., Paim, A. C., Farenzena, M., & Trierweiler, J. O. (2013). Laboratory apparatus to evaluate microalgae production. *Brazilian Journal of Chemical Engineering*, *30*(3), 487-497.

Grobbelaar, J. (2004). Mineral Nutrition. In: Handbook of microalgal culture (ed. Richmond A.), pp. 104-106. Blackwell Publishing Company, 2121 State Avenue, Ames, Iowa, USA.

Grobbelaar, J. Algal Nutrition: Mineral Nutrition. [book auth.] Amos Richmod. *Hand Book Of Microalgal Culture: Biotechnology and Applied Phycology.* s.l. : Blackwell Science Ltd., 2005, pp. 97-115.

Guillard, R.R.L. (1975). Culture of phytoplankton for feeding marine invertebrates.pp 26 60. *In* Smith W.L. and Chanley M.H (Eds.) Culture of Marine Invertebrate Animals.Plenum Press, New York, USA.

Guillard, R.R.L., & Ryther, J.H. (1962).Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. *Canadian* Journal of Microbiology ,8, 229-239.

Guiry, M. D. (2012), How many species of algae are there. *Journal of Phycology*, 48: 1057–1063. doi: 10.1111/j.1529-8817.2012.01222.x

Gupta, P., Sinha, D., & Bandopadhyay, R. (2014). Isolation and Screening of Marine Microalgae Chlorella sp. _pr1 for Anticancer Activity. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6(10), 517-519.

Hakalin, N., Paz, A., Aranda, D. & Moraes, L. (2014). Enhancement of Cell Growth and Lipid Content of a Freshwater Microalga *Scenedesmus* sp. by Optimizing Nitrogen, Phosphorus and Vitamin Concentrations for Biodiesel Production. *Natural Science*, 6, 1044-1054. doi: 10.4236/ns.2014.612095.

Herrero, M., Ibañez, E., Cifuentes, A., Reglero, G., Santoyo, S. (2006). Dunaliella salina microalga pressurized liquid extracts as potential antimicrobials. J*ournal of Food Protection*, 69:2471-2477.

Hewedy M.A., Rahhal M.M.H., & Ismail I.A. (2000). Pathological studies on soybean damping-off disease. Egypt. *Journal of Applied Sciences*, 15, 88-102.

Hii, Y. S., Soo, C. L., Chuah, T. S., Mohd-Azmi, A., & Abol-Munafi, A. B. (2011). Interactive effect of ammonia and nitrate on the nitrogen uptake by Nannochloropsis sp. *Journal of Sustainability Science and Management*, 6(1), 60-68.

Holajjer, P., Kamra, A., Gaur, H. S., & Manjunath, M. (2013). Potential of cyanobacteria for biorational management of plant parasitic nematodes: A review. *Crop Protection*, *53*, 147-151.

Hu, Q. (2004). Environmental Effects on Cell Composition. *Handbook of microalgal culture: biotechnology and applied phycology*, 83.

Hu, Q. Environmental Effects on Cell Composition. [book auth.] Amos Richmond. *Hand Book Of Microalagl Culture: Biotechnology and Applied Phycology.* s.l. : Blackwell Science Ltd., 2004, pp. 83-93.

Hu, Q., Guterman, H., & Richmond, A. (1996). A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs. *Biotechnology and Bioengineering*, *51*(1), 51-60.

Hu, Q., Miyashita, H., Iwasaki, I., Kurano, N., Miyachi, S., Iwaki, M., & Itoh, S. (1998). A photosystem I reaction center driven by chlorophyll d in oxygenic photosynthesis. *Proceedings of the National Academy of Sciences*, *95*(22), 13319-13323.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal*, *54*(4), 621-639.

Huang, G., Chen, F., Wei, D., Zhang, X., & Chen, G. (2010). Biodiesel production by microalgal biotechnology. *Applied energy*, *87*(1), 38-46.

Huang, X., Huang, Z., Wen, W., & Yan, J. (2013). Effects of nitrogen supplementation of the culture medium on the growth, total lipid content and fatty acid profiles of three microalgae (Tetraselmis subcordiformis, Nannochloropsis oculata and Pavlova viridis). *Journal of Applied Phycology*, *25*(1), 129-137.

Jeffrey, S. T., & Humphrey, G. F. (1975). New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochem Physiol Pflanz BPP*.

Juneja, A., Ceballos, R. M., & Murthy, G. S. (2013). Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: a review. *Energies*, 6(9), 4607-4638.

Kim, J., & Kim, J. D. (2008). Inhibitory effect of algal extracts on mycelial growth of the tomato-wilt pathogen, Fusarium oxysporum f. sp. lycopersici.*Mycobiology*, *36*(4), 242-248.

Laing, I. and Britain, G. (1991). Cultivation of marine unicellular algae. Ministry of Agriculture, Fisheries and Food.

Laungsuwon, R., & Chulalaksananukul, W. (2013). Antioxidant and anticancer activities of freshwater green algae, Cladophora glomerata and Microspora floccosa, from Nan River in northern Thailand. *Maejo International Journal of Science and Technology*, *7*(2), 181-188.

Lavens, P. & Sorgeloos, P. (1996). Manual on the production and use of live food for aquaculture.

Lee L.K & Lerner W.L. (2008). Algae. Gale encyclopedia of science. 4th ed. Detroit: Thomson Gale, 899.

Lee R.E. (1989). Phycology, Second edition, Cambridge University Press.

López, C. V. G., García, M. D. C. C., Fernández, F. G. A., Bustos, C. S., Chisti, Y., & Sevilla, J. M. F. (2010). Protein measurements of microalgal and cyanobacterial biomass. *Bioresource technology*, *101*(19), 7587-7591.

Lourenço, S. O., Barbarino, E., Lavín, P. L., Lanfer Marquez, U. M., & Aidar, E. (2004). Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors. *European Journal of Phycology*, *39*(1), 17-32.

Marathe, K. V., & Chaudhari, P. R. (1975). An example of algae as pioneers in the lithosere and their role in rock corrosion. *The Journal of Ecology*, 65-69.

Markou, G., Angelidaki, I., & Georgakakis, D. (2012). Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. *Applied microbiology and biotechnology*, *96*(3), 631-645.

Medina-Jaritz, D. R. Perez-Solis, S. L. Ruiloba de Leon F. & Olvera R. Antimicrobial activity of aqueous and methanolic extracts from Arthrospira maxima.

Meier, R. L. (1955). Biological cycles in the transformation of solar energy into useful fuels. *Solar energy research*, *23*, 179-183.

Metting, F. B. (1996). Biodiversity and application of microalgae. *Journal of Industrial Microbiology & Biotechnology.*, 17(5-6), 477-489.

Miyamoto, K., & Food and Agriculture Organization of the United Nations. (1997). *Renewable biological systems for alternative sustainable energy production*. Rome: Food and Agriculture Organization of the United Nations. http://www.fao.org/docrep/w7241e/w7241e00.HTM

Mostafa, S. S. (2012). *Microalgal biotechnology: prospects and applications*. INTECH Open Access Publisher.

Nazari, F., & Raheb, J. (2015). Genetic engineering of microalgae for enhanced biodiesel production suitable fuel replacement of fossil fuel as a novel energy source. *American Journal of Life Sciences*, 3(1), 32-41. doi: 10.11648/j.ajls.20150301.17
Nigam, P. S., & Singh, A. (2011). Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science*, *37*(1), 52-68.

Omar, H. H. (2000). Nitrogen-fixing abilities of some Cyanobacteria in sandy loam soil and exudates efficiency of rice grain germination. *Egyptian Journal of Phycology*, 1: 157-167.

Ördög, V., Stirk, W. A., Lenobel, R., Bancířová, M., Strnad, M., Van Staden, J. & Németh, L. (2004). Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. *Journal of applied phycology*, *16*(4), 309-314.

Parmar, A., Singh, N. K., Pandey, A., Gnansounou, E., & Madamwar, D. (2011). Cyanobacteria and microalgae: a positive prospect for biofuels. *Bioresource technology*, *102*(22), 10163-10172.

Patel, A., Mishra, S., & Ghosh, P. K. (2006). Antioxidant potential of C-phycocyanin isolated from cyanobacterial species lyngbya, phormidium and spirulina spp. *Indian Journal of Biochemistry & Biophysics*, *43*(1), 25.

Piorreck, M., Baasch, K. H., & Pohl, P. (1984). Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. *Phytochemistry*, *23*(2), 207-216.

Pittman, J. K., Dean, A. P., & Osundeko, O. (2011). The potential of sustainable algal biofuel production using wastewater resources. *Bioresource technology*, *102*(1), 17-25.

Pohl, P. (1982). Lipids and fatty acids of microalgae. In: CRC handbook of biosolar resources. (Ed. Zaborsky, O. R.), pp. 383-404. 1. CRC Press Inc., Boca Raton, FL, USA.

Priyadarshani, I., & Rath, B. (2012). Commercial and industrial applications of micro algae–A review. *Journal of Algal Biomass Utilization*, *3*(4), 89-100.

Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied microbiology and biotechnology*, *65*(6), 635-648.

Qin J. (2005). Bio-hydrocarbons from algae—impacts of temperature, light and salinity on algae growth. Barton, Australia: Rural Industries Research and Development Corporation.

Richmond, A. (2000). Microalgal biotechnology at the turn of the millennium: a personal view. *Journal of Applied Phycology*, *12*(3), 441-451.

Richmond, A. (2004). Handbook of microalgal culture biotechnology and applied phycology. Oxford, OX, UK: Blackwell Science.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General microbiology*, *111*(1), 1-61.

Rizvi, M. & Shameel, M. (2006). In vitro nematicidal activities of seaweed extracts from Karachi coast. *Pakistan Journal of Botany*, *38*(4), 1245.

Rodolfi L., Zittelli G.C., Bassi N., Padovani G., Biondi N., Bonini G. (2008). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering*, 102(1), 100–112.

Rodrigo, V. and Eberto, N. (2007). Seasonal changes in periphyton nitrogen fixation in a protected tropical wetland. *Biology and Fertililty of Soils*, 43: 367–372.

Roessler, P. G., Brown, L. M., Dunahay, T. G., Heacox, D. A., Jarvis, E. E., Schneider, J. C., & Zeiler, K. G. (1994, January). Genetic engineering approaches for enhanced production of biodiesel fuel from microalgae. In *ACS symposium series* (Vol. 566, pp. 255-270). Washington, DC: American Chemical Society,[1974].

Rogers, S. L., & Burns, R. G. (1994). Changes in aggregate stability, nutrient status, indigenous microbial populations, and seedling emergence, following inoculation of soil with Nostoc muscorum. *Biology and Fertility of Soils*, *18*(3), 209-215.

Sahu, D., Priyadarshani, I., & Rath, B. (2012). Cyanobacteria–as potential biofertilizer. *CIBTech Journal Microbiology, 1*, 20-26.

Samios D., Pedrotti F., Nicolau A., Reiznautt Q.B., Martini D.D., & Dalcin F.M. (2009). A transesterification double step process- TDSP for biodiesel preparation from fatty acids triglycerides. *Fuel Processing Technology*, 90,599-605.

Sanmukh, S., Bruno, B., Ramakrishnan, U., Khairnar, K., Swaminathan, S., & Paunikar, W. (2014). Bioactive compounds derived from microalgae showing antimicrobial activities. *Journal of Aquaculture Research and Development*,*5*(3), 224.

Santoyo, S., Jaime, L., Plaza, M., Herrero, M., Rodriguez-Meizoso, I., Ibañez, E., & Reglero, G. (2012). Antiviral compounds obtained from microalgae commonly used as carotenoid sources. *Journal of Applied Phycology*, *24*(4), 731-741.

Saunders, G. W., & McDevit, D. C. (2012). Methods for DNA barcoding photosynthetic protists emphasizing the macroalgae and diatoms. *Methods in Molecular Biology (Clifton, N.J.), 858*, 207.

Shanab, S. M., Mostafa, S. S., Shalaby, E. A., & Mahmoud, G. I. (2012). Aqueous extracts of microalgae exhibit antioxidant and anticancer activities. *Asian Pacific Journal of Tropical Biomedicine, 2*(8), 608–615. doi:10.1016/S2221-1691(12)60106-3.

Shariatmadari, Z., Riahi, H., & Shokravi, S. (2011). Study of soil blue-green algae and their effect on seed germination and plant growth of vegetable crops.*Rostaniha*, *12*, 101-110.

Sharif, A.H, Nasrulhaq A.B., Majid H.A.M., Chandran S., & Zuliana R., (2007). Biodiesel production from waste cooking oil as environmental benefits and recycling process.A Review.Asia Biofuel Conference Book.

Shawky, S. M., Mostafa, S. S., & El-All, A. A. M. A. (2009). Efficacy of some algal species, azolla and compost extract in controlling root knot nematode and its reflection on cucumber growth. *Bulletin of Faculty of Agriculture, Cairo University*, *60*(4), 443-459.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P. (1998). A look back at the U.S. Department of Energy‘s Aquatic Species Program. National Renewable Energy Laboratory.

Shenbaga Devi, A., Santhanam, P., Rekha, V., Ananth, S., Prasath, B. B., Nandakumar, R., & Kumar, S. D. Culture and biofuel producing efficacy of marine microalgae Dunaliella salina and Nannochloropsis sp.

Spolaore P., Joannis-Cassan C., Duran E., & Isambert A. (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering, 101*(2),87–96.

Sukenik, A., Carmeli, Y., & Berner, T. (1989). Regulation of fatty acid composition by irradiance level in the eustigmatophyte Nannochloropsis sp. *Journal of Phycology*, *25*(4), 686-692.

Sze, P. (1998). A biology of the algae. 3rd ed. Boston, Mass.: WCB/McGraw-Hill.

Talyshinsky, M. M., Souprun, Y. Y., & Huleihel, M. M. (2002). Anti-viral activity of red microalgal polysaccharides against retroviruses. *Cancer cell international*,*2*(1), doi:10.1186/1475-2867-2-8.

Thomas, F.R., (2006). Algae for liquid fuel production Oakhaven Permaculture center. Permaculture Activist, 59,1-2.

Thompson, G. A. (1996). Lipids and membrane function in green algae.*Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *1302*(1), 17-45.

Tiwari, A., & Sharma, A. (2013). Antifungal activity of anabaena variabilis against plant pathogens. *International Journal of Pharma and Bio Sciences*, 4(2), 1030 – 1036.

Tomaselli, L. (2004). The Microalgal cell. In: Handbook of microalgal culture (Ed. Richmond A.) pp. 11-17. Blackwell Publishing Company, 2121 State Avenue, Ames, Iowa, USA.

Van Leeuwe, M. A., Villerius, L. A., Roggeveld, J., Visser, R. J. W., & Stefels, J. (2006). An optimized method for automated analysis of algal pigments by HPLC. *Marine Chemistry*, *102*(3), 267-275.

Vigani, M., Parisi, C., & Cerezo, E. R. (Eds.). (2014). *Microalgae-based products for the food and feed sector: an outlook for Europe*. Publications Office.

Vonshak, Avigad and Torzillo, Giuseppe. Environmental Stress Physiology. [book auth.] Amos Richmond. *Hand Book of Microalgal Culture: Biotechnology and Applied Phycology*. s.l. : Blackwell Science Ltd., 2004, pp. 57-82.

Widjaja, A., Chien, C.C., & Ju Y.H. (2009) Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*.J. Taiwan Institute of *Chemical Engineering Journal*, 40, 13–20.

Yeh, K.L. Chang, J.S. (2011). Nitrogen starvation strategies and photobioreactor design for enhancing lipid production of a newly isolated microalga *Chlorella vulgaris*: Implications for biofuels. *Biotechnology Journal*, 6, 1358–1366.

Zhang, Q., Wang, T., & Hong, Y. (2014). Investigation of initial pH effects on growth of an oleaginous microalgae Chlorella sp. HQ for lipid production and nutrient uptake. *Water Science and Technology*, 70(4), 712-719. doi: 10.2166/wst.2014.285.