

AMERICAN UNIVERSITY OF BEIRUT

EVALUATING THE POTENTIAL OF LOCAL MICROALGAE
SPECIES FOR USE AS ENERGY SOURCES, NUTRITIONAL
SUPPLEMENTS AND BIOLOGICAL CONTROL OF PLANT
PATHOGENS

by

DINA MAHMOUD SALEH

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Agriculture Sciences
of the Faculty of Agriculture and Food Sciences
at the American University of Beirut

Beirut, Lebanon
May 2014

AMERICAN UNIVERSITY OF BEIRUT

EVALUATING THE POTENTIAL OF LOCAL
MICROALGAE SPECIES FOR USE AS ENERGY
SOURCES, NUTRITIONAL SUPPLEMENTS AND
BIOLOGICAL CONTROL OF PLANT PATHOGENS

by

DINA MAHMOUD SALEH

Approved by:

Youssef Abou- Jawdah, Professor
Agricultural Sciences


Advisor

Dr. Mustapha Haidar, Professor
Agricultural Sciences


Member of Committee

Dr. Imad Saoud, Associate Professor
Biology


Member of Committee

Date of thesis defense: May 7, 2014

ACKNOWLEDGMENTS

Foremost, I would like to express my gratitude to the sweet family I have. I am so blessed and grateful to be a member of yours. Thanks to you, I never knew the meaning of loneliness and fear, I never felt weak, you were always by my side with your absolute love, patience, trust and support. All I reached wouldn't have become real without you, I love you all.

My sincere thanks to my mentor, Dr. Youssef Abou-Jawdah for his continuous support of the research, his motivation, enthusiasm and immense knowledge. I would also like to thank my committee members, Dr. Imad Saoud and Dr. Mustapha Haidar for their insightful comments and meticulous questions. You made me change to the better.

Special thanks to Ms. Lucia Hanna. I know you might not like to be thanked, but you should also know how speechless I am in front of your care, motherhood, cuteness, perseverance, support, your ability to listen, laugh and always find solutions no matter how hard it is and what it costs. Thanks to you, I was able to overcome lots of the obstacles; you were like my mother at AUB. Things wouldn't have been easier without you.

My sincere thanks to my friend Sara Mekkawi and to my fellow labmate Patil Tavitian. Completing this work would have been harder without you giving extra hands whenever needed. I thank you for stimulating discussions, for working together and having fun, you made my masters unforgettable. Our friendship is a great asset to my personal life.

I appreciate the assistance of Maan El Jawhari and Ouais Abd El Kader. Your contribution was very important to the flow of the research, thank you. A special note of thanks to our molecular lab team: Hana and Samia, I learned a lot from you.

AN ABSTRACT OF THE THESIS OF

Dina Mahmoud Saleh for Master of Science
Major: Plant Pathology

Title: Evaluating the potential of local microalgae species for use as energy sources, nutritional supplements and biological control of plant pathogens

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that have received lots of attention since the Second World War for their use potential as nutritional and renewable bioenergy sources. Their identification relied mainly on microscopy and chemical analysis. Recently, molecular identification is gaining importance and several primary and secondary barcode markers are being developed. In this study, 11 local isolates were isolated and successfully grown in culture media. Using primer pairs targeting the LSU and ITS of the ribosomal DNA, *tufA* gene and mitochondrial cytochrome c oxidase 1 gene, the following genera were identified: *Amphidinium carterae*, *Amphora* sp., *Chlorella vulgaris*, *Chloroidium saccharophilum*, *Leptolyngbya* sp., *Micractinium reisseri* and *Tetraselmis marina*. Four selected isolates were studied for their lipid, pigments and protein contents. *Micractinium* sp, green algae contained the highest levels of all compounds tested. Its oil content varied between 14 and 16.5% DW, 4.2% DW omega FAs, with a chlorophyll content of 8µg/ml and a protein content ranging between 18.26 and 19.31%. Among the isolates tested, *Tetraselmis marina* and the Jbd isolate *Micractinium* sp had the highest lipid content, saturated and monounsaturated fatty acids and thus seem most promising for a good quality biodiesel production. The antimycotic activity of four different microalgal strains were tested in-vitro using four different extracts against four different plant pathogenic fungi. None of the isolates showed promising antimycotic activity; however, better sporulation and growth was recorded in the non-methanolic and crude water extracts. Extracts of the Jbd microalgal isolate were also tested for their ability to induce resistance in cucumber seedlings against powdery mildew fungus, *Sphaerotheca fuliginea*. At the concentrations used, the extracts did not result in any significant control of powdery mildew fungus nor in growth improvement of cucumber plants. Further studies are recommended to isolate a larger number of local strains, to determine their composition and to focus on evaluation of the agricultural and medicinal properties of their bioactive substances as well as their suitability for biodiesel production.

CONTENTS

ACKNOWLEDGMENTS	v
ILLUSTRATIONS.....	xii
TABLES	xvi
ABBREVIATIONS.....	xix

Chapter

I. INTRODUCTION.....	1
II. LITERATURE REVIEW.....	3
A. The microalgal cell	3
1. Meaning of microalgae.....	3
2. Microalgal structure and organization.....	3
3. Nutritional value of microalgae.....	3
B. Microalgal classification and taxonomy.....	4
1. Microscopic and physiological characters.....	4
a. Prokaryotes.....	4
b. Eukaryotes.....	5
i. Rhodophyta.....	6
ii. Chlorophyta.....	7
iii. Dinophyta.....	7
iv. Cryptophyta.....	9
v. Bacillariophyta.....	9
2. Molecular Identification.....	10
C. Physical and chemical factors affecting microalgal growth.....	11
1. Nutrients.....	11
2. Light.....	12
3. pH.....	12

4. Salinity	12
5. Temperature	13
D. Basic isolation and culturing techniques ..	13
1. Isolation of microalgae.....	13
2. Enrichment of culture media.....	13
3. Growth kinetics	14
a. Lag or induction phase.....	14
b. Exponential phase.....	15
c. Phase of declining relative growth.....	15
d. Stationary phase.....	15
e. Death phase.....	15
E. Cultivation systems and modes of culture.....	16
1. Open pond systems	16
2. Closed systems	17
a. Batch cultures.....	18
b. Continuous cultures.....	18
F. Microalgal composition.....	19
1. Lipids	19
2. Proteins	21
3. Carbohydrates	22
4. Pigments.....	23
5. Ash.....	25
G. Potential uses of microalgae biomass	25
1. Commercial microalgae production.....	25
2. Commercial products derived from microalgae.....	26
a. Food and feed.....	26
b. Pharmaceuticals.....	26
c. Pest management.....	26
i. Antifungal activity.....	27
ii. Antibacterial activity.....	27
iii. Antiviral activity.....	28
iv. Nematicidal activity.....	28

v. Induced resistance.....	29
3. Biofuel.....	30
a. Worldwide fuel market.....	30
b. Environmental and economical aspects of biofuel production.....	31
4. Wastewater treatment.....	32
III. MATERIALS AND METHODS.....	35
A. Isolation and culturing of local microalgae.....	35
1. Water Sampling.....	35
2. Microalgae Isolation	36
3. Water Sterilization	36
4. Up-scaling and growth kinetics.....	36
5. Microalgae stock cultures	37
6. Microscopic Observations.....	38
B. Molecular Diagnosis	38
1. DNA Extraction	38
2. PCR.....	39
C. Chemical and biochemical profiling.....	41
1. Determination of the total lipid content	41
2. Fatty acid profiling.....	42
3. Pigment analysis	43
4. Protein analysis	45
a. Kjeldahl method.....	45
b. Digesdahl method.....	46
D. Antimycotic activity.....	47
1. In-vitro experiments.....	47
a. Plant pathogens.....	47
b. Tested microalgae.....	48
c. Extracts	48
i. Methanolic extract.....	49
ii. Non-methanolic water soluble extract	49
iii. Crude water extract.....	50

iv. Culture filtrates	50
2 .In-vivo assays.....	50
a. Crop maintenance	51
b. Plant pathogens.....	51
c. Microalgal extracts	52
i. Methanolic extract.....	52
ii. Non-methanolic extract.....	52
E. Statistical Analysis	53
IV. RESULTS	54
A. Isolations.....	54
B. Molecular identifications	60
1. Molecular Detection.....	60
2. Sequence Analysis	64
C. Growth kinetics.....	66
D. Lipid and fatty acids content.....	71
E. Pigment concentrations.....	75
F. Protein Content.....	76
G. Antimycotic or antifungal activity.....	77
1. In- vitro experiment	77
a. Jbd extract.....	77
b. Jid extract.....	78
c. Ozd extract.....	79
d.Khd extract	80
2. In-vivo experiment.....	82
a. Effect of microalgal extracts on cucumber length.....	82
b. Effect of microalgal extracts on cucumber infection of cucumber plants by the powdery mildew fungus	83
V. DISCUSSION	85

A. Isolation and molecular identification	85
B. Growth Kinetics	86
C. Lipid analysis	87
D. Pigment Analysis	89
E. Protein analysis	911
F. Antimycotic activity	92
G. Induced Resistance	93
VI. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS	95
Appendix	
I. FORMULATION FOR MARINE GROWTH MEDIA	99
II. FUNGAL GROWTH, IN-VITRO	103
III.LIPID ANALYSIS.....	107
BIBLIOGRAPHY	119

ILLUSTRATIONS

Figure	Page
1. Cyanophyta : A: <i>Merismopedia</i> sp.; B: <i>Gomphosphaeria</i> sp.;C : <i>Eucapsis</i> sp.; D: <i>Gloeotrichia</i> sp; E: <i>Nostoc</i> sp; F: <i>Spirulina</i> sp.	5
2. Rhodophyta: A: <i>Asterocystis</i> sp (Chroodactylon); B: <i>Lemanea</i> sp; C: <i>Porphyridium crenutum</i>	6
3. Chlorophyta: A: <i>Stichococcus bacillaris</i> ; B: <i>Tetraselmis</i> sp.; C: <i>Zygonium</i> sp.....	7
4. Dinophyta: A: <i>Gambierdiscus toxicus</i> ; B: <i>Protoberidinium conicum</i> ; C: <i>Ceratium</i> sp.	8
5. Schematization of characteristics of organisms from dynophyta.	8
6. Cryptophyta. A: <i>Mesodinium rubrum</i>	9
7. Bacillariophyta. A: <i>Amphipleura</i> sp. B: <i>Tabellaria</i> sp.	10
8. Five growth phases of microalgal cultures.	16
9. Microscopic view of isolate Aqd magnified 1000x (Not identified, 4.9-7.8µm)	55
10. Microscopic view of Dad isolate magnified 400x (<i>Chlorella variabilis</i> , 2-2.4µm) ..	55
11. Microscopic view of Had isolate magnified 1000x (<i>Tetraselmis marina</i> , 8.3µm)....	56
12. Microscopic observation of Had2 mixed bloom magnified 1000x showing diatom (<i>Amphidinium carterae</i> ,10x5µm) and two filamentous cyanobacteria (<i>Spirulina</i> sp., 1.2 µm).....	56
13. Microscopic observation of Jbd isolate magnified 1000x (<i>Micractinium</i> sp, 3.5µm)	57

14. Microscopic observation of Jid isolate magnified 1000x (<i>Amphora coffeaeformis</i> , 13x3.5µm).....	57
15. Microscopic observation of Jod isolate magnified 1000x (<i>Micractinium reisseri</i> , 3µm).....	58
16. Microscopic view of Khd tube isolate magnified 1000x (<i>Tetraselmis marina</i> , 5x3.5µm).....	58
17. Microscopic observation of Khd plate isolate magnified 1000x (<i>Tetraselmis marina</i> , 5x3.5µm).....	59
18. Microscopic observation of Ozd green isolate magnified 1000x (<i>Chloroidium saccharophilum</i> , 8.35µm)	59
19. Microscopic observation of Ozd Red mixed bloom showing a diatom (<i>Amphora coffeaeformis</i> , 13x3.5µm) and a filamentous cyanobacterium (<i>Leptolyngbya sp.</i> , 1.2 µm).	60
20. UV visualization in 0.7% 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, AQU to DAM: differ.....	61
21. UV visualization in 0.7% agarose gel following electrophoresis of PCR amplified products using the taxon specific primers tufGF4 and tufAR to amplify gene tufA that encodes the elongation factor Tu. L: 1 Kbp molecular ladder, KP to OR: different microalgae.....	62
22. UV visualization in 0.7% 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. M: 1 Kbp molecular ladder, Ouz10 to KhP: different. .63	
23. UV visualization in 1% agarose gel following electrophoresis of PCR amplified products using the taxon specific primers GWSRx and GWSFn for the mitochondrial cytochrome c oxidase 1 gene. L: 1 Kbp molecular ladder, OR and HAM2: different microalgal isolates.	63

24. Growth kinetics of Jbd isolate as measured by optical density readings ($A_{560\text{nm}}$). The number of cells/ml is provided at specific dates and O.D. values.	67
25. Daily exponential specific growth rate ($\mu_{\text{max}} \text{d}^{-1}$) of Jbd isolate.	67
26. Growth kinetics of Had isolate as measured by optical density readings ($A_{560\text{nm}}$). The number of cells/ml is provided at specific dates and O.D. values.	68
27. Daily exponential specific growth rate ($\mu_{\text{max}} \text{d}^{-1}$) of Had isolate.	68
28. Growth kinetics of Jod isolate as measured by optical density readings ($A_{560\text{nm}}$). The number of cells/ml is provided at specific dates and O.D. values.	69
29. Daily exponential specific growth rate ($\mu_{\text{max}} \text{d}^{-1}$) of Jod isolate.	69
30. Growth kinetics of Ozd isolate as measured by optical density readings ($A_{560\text{nm}}$). The number of cells/ml is provided at specific dates and O.D. values.	70
31. Daily exponential specific growth rate ($\mu_{\text{max}} \text{d}^{-1}$) of Ozd isolate.	70
32. Total lipid content of microalgal isolates in both exponential and stationary phases.	73
33. Biodiesel production of four local microalgal isolates measured at the stationary stage	74
34. Effect of Khd extracts on growth and sporulation of <i>Alternaria</i> sp. grown in vitro.	81
35. Effect of microalgal methanolic extract (of the Jbd isolate) on the average length of cucumber seedlings (cm).	82
36. Effect of microalgal non-methanolic extract (of the Jbd isolate) on the average length of cucumber seedlings (cm).	83
37. Chromatogram of lipid components of Jbd isolate in the exponential phase.	107

38. Chromatogram of lipid components of Jbd isolate in the stationary phase.	107
39. Chromatogram of lipid components of Had isolate in the exponential phase.	110
40. Chromatogram of lipid components of Had isolate in the stationary phase.	110
41. Chromatogram of lipid components of Ozd isolate in the exponential phase.	113
42. Chromatogram of lipid components of Ozd isolate in the stationary phase.	113
43. Chromatogram of lipid components of Jod isolate in the exponential phase.	116
44. Chromatogram of lipid components of Jod isolate in the stationary phase.	116

TABLES

Table	Page
1. Pigment distribution according to taxonomical groups.	24
2. Sites, dates and isolate acronym of collected water samples.	35
3. List of primer pairs used in PCR assays for amplification and sequencing of microalgae genes.	40
4. Plant pathogenic fungi used in this study.	48
5. Microalgae species used in this study.	48
6. Genera of microalgae identified in this study, their maximum scores and their highest sequence identities with related isolates, using three barcode markers.	64
7. Taxonomic groups of microalgae isolates identified in this study.	65
8. Dry weights of four microalgal isolates at corresponding optical densities during exponential and stationary growth phases.	71
9. Total lipid content (%) and percentage saturated and unsaturated FA in four local microalgal isolates measured at two growth stages.	72
10. Content of omega FA in four local microalgal isolates observed during two growth stages (% of total lipids).	74
11. Chlorophyll and carotenoid content in four microalgal isolates ($\mu\text{g} / \text{ml}$).	75
12. Content of chlorophylls a, b, c in four local microalgal isolates (mg / m^3).	76
13. Crude protein content (%DW) for four microalgal isolates using Kjeldahl and Digesdahl methods.	76

14. Effect of various extracts of Jbd isolate on the in-vitro growth of four fungal species: Mean diameter of fungal colony (cm).	77
15. Effect of various extracts of Jid isolate on the in-vitro growth of four fungal species: Mean diameter of fungal colony (cm).	78
16. Effect of various extracts of Ozd isolate on the in-vitro growth of four fungal species: Mean diameter of fungal colony (cm).	79
17. Effect of various extracts of Khd isolate on the in-vitro growth of four fungal species: Mean diameter of fungal colony (cm).	80
18. Effect of microalgal extracts sprays on cucumber infection by powdery mildew, ten and twenty days post inoculation, expressed as % leaf area infected. 18a and 18b are two repetitions of the same experiment at one month interval.	84
19. Composition of Walne mineral medium (1000x).	99
20. Composition of the trace metal solution specific for Walne medium.	100
21. Composition of the vitamin solution specific for Walne medium.....	100
22. Composition of f/2 mineral medium.....	101
23. Composition of f/2 trace metal solution.....	101
24. Composition of f/2 vitamin solution	102
25. Effect of various extracts of Jbd isolate on the percentage growth change (+ or-) of fungi relative to the control.....	103
26. Effect of various extracts of Jid isolate on the percentage growth change (+ or-) of fungi relative to the control.....	104

27. Effect of various extracts of Ozd isolate on the percentage growth change (+ or-) of fungi relative to the control.....	105
28. Effect of various extracts of Khd isolate on the percentage growth change (+ or-) of fungi relative to the control.....	106
29. Chemical components of Jbd FAMES with their corresponding retention time and percentage peak area in both exponential and stationary phases.	108
30. Chemical components of Had FAMES with their corresponding retention time and percentage peak area in both exponential and stationary phases.	111
31. Chemical components of Ozd FAMES with their corresponding retention time and percentage peak area in both exponential and stationary phases.	114
32. Chemical components of Jod FAMES with their corresponding retention time and percentage peak area in both exponential and stationary phases.....	117

ABBREVIATIONS

± Plus or minus

°C Degrees Celsius

µg Micrograms

µl Microliter

µm Micrometer

bp Base pair

DNA Deoxyribonucleic acid

RNA Ribonucleic acid

SAR Systemic acquired resistance

FA Fatty Acids

g gram

hr hour

min Minutes

PCR Polymerase chain reaction

DW Dry weight

FAO Food and Agriculture Organization

DCM Dichloromethane

CHAPTER I

INTRODUCTION

Microalgae, more appropriately called phytoplankton, are photosynthetic free floating organisms, mainly unicellular algae and cyanobacteria. They are recognized as one of the eldest life forms on earth. They are divided into prokaryotes and eukaryotes. Eukaryotes are categorized into a variety of classes (Chlorophyta, Bacillariophyta and others), mainly characterized by their pigmentation, life cycle and cellular structure; while prokaryotes, restricted to cyanobacteria, are more similar to bacteria than algae. Microalgae cover unicellular and simple multi-cellular microorganisms, they are autotrophic and produce a large biomass since they have the capacity to produce all year long, leading to continuous supply of lipids, proteins, carbohydrates, vitamins and antioxidants [49]. Microalgae have been elected by the United Nations Advisory Committee on International Action as an alternative potential for food and feed products due to the sharp shortage in food commodities, particularly protein, toward the end of the Second World War [60]. Species of microalgae occur virtually in every type of habitat, including the most extremes, such as walls of urban buildings, biotic crusts in hot desert, soils, saline, brackish, fresh and sea water [58].

Under natural growth conditions phototrophic algae absorb sunlight, and assimilate carbon dioxide from the air and nutrients from the aquatic habitats. Based on microalgal characteristics, biodiesel production came into investigation when a shortage of petroleum reserves was expected in the foreseeable future; but it has not

been given much attention due to the uneconomical high cost of production. Biodiesel from algae has been considered as a promising solution to solve air pollution problems, reduce CO₂ and SO₂ gas emissions, and therefore ensure environmental and possibly economical sustainability [61]. It is non-toxic, biodegradable and considered as a CO₂ neutral energy source [62].

Cyanobacteria and algae produce a large number of antibacterial and antifungal products too. Many can be grown in mass cultures without being a threat to the environment [50].

Our research covered the objectives listed below:

1. Isolation and purification of local strains of microalgae from sea water sources.
2. Identification of the local strains using microscopic techniques, chlorophyll analysis and molecular tools.
3. Studying the chemical profile including fatty acids.
4. Determination of the growth kinetics of selected microalgae.
5. Evaluation of the antifungal activity of microalgal extracts, in-vitro against four plant pathogenic fungi and in-vivo for the management of powdery mildew fungus.

CHAPTER II

LITERATURE REVIEW

A. The microalgal cell

1. Meaning of microalgae

According to phycologist definitions, microalgae belong to the thallophytes which are a group of organisms known for being plants lacking roots, stems and leaves, possessing the primary photosynthetic pigment chlorophyll a, and also including cyanobacteria which are oxygenic photosynthetic bacteria [23].

2. Microalgal structure and organization

Microalgal cells have many different types of cellular organizations. They can be unicellular, colonial and filamentous. Some are motile due to the presence of flagella, others are not. They might be prokaryotic or eukaryotic, uninucleate or multinucleate.

3. Nutritional value of microalgae

The nutritional value of microalgae varies according to the algal species, composition of the medium and growth conditions. Protein is considered to be the major constituent of microalgae followed by lipids and then carbohydrates. Data published by FAO show that proteins represent 12-35%, lipids 7.2-23% and carbohydrates 4.6-23% of microalgal dry weights. Microalgae are also considered to be a rich source of ascorbic acid [35] vitamins such as pro-vitamin A, vitamin B12,

B6, biotin and carotenoids [48]. The composition of microalgae may vary depend on the composition of the medium. A commercial medium Algal-1, mentioned in FAO report showed the best results for *T. suecica*, *D. tertiolecta*, *I. galbana*, and *P. tricornutum* in terms of cellular density and proximate composition when compared to other known media such as Walne and f/2 (APPENDIX I).

B. Microalgal classification and taxonomy

Microalgal cells are divided into two basic types: prokaryotic and eukaryotic. They can be identified by their microscopic and physiological characters or by molecular techniques.

1. Microscopic and physiological characters

a. Prokaryotes

Organisms whose cell types are known for lacking a true nucleus and other membrane bound organelles such as plastids and mitochondria. Cyanobacteria belonging to the taxonomic group cyanophyta are grouped within prokaryotic microalgae (Figure 1).

According to Singh et al. (2005) [3], cyanobacteria are prokaryotic photosynthetic microorganisms known as blue green algae that have no nuclear membranes, internal organelles and histone proteins associated with chromosomes. They are surrounded by a gelatinous sheath, some are unicellular others live in colonies or filaments. Cyanobacteria are diazotrophs having the enzyme nitrogenase, able to fix atmospheric nitrogen into ammonium using ATP as a source of energy.

Some cyanobacteria produce toxins known as cyanotoxins, which are basically of two types: neurotoxins and hepatotoxins. The neurotoxins are compounds that interfere with transmission of the signal from neuron to neuron in animals and men. While hepatotoxins are inhibitors of protein phosphatases which affect the animal by causing bleeding of the liver. Furthermore, some cyanobacteria are utilized as human food or animal feed, that's why their production was developed by the industry and at present are produced at a large scale and sold as pills, capsules as well as powders for pasteries and chocolate blocks [44].

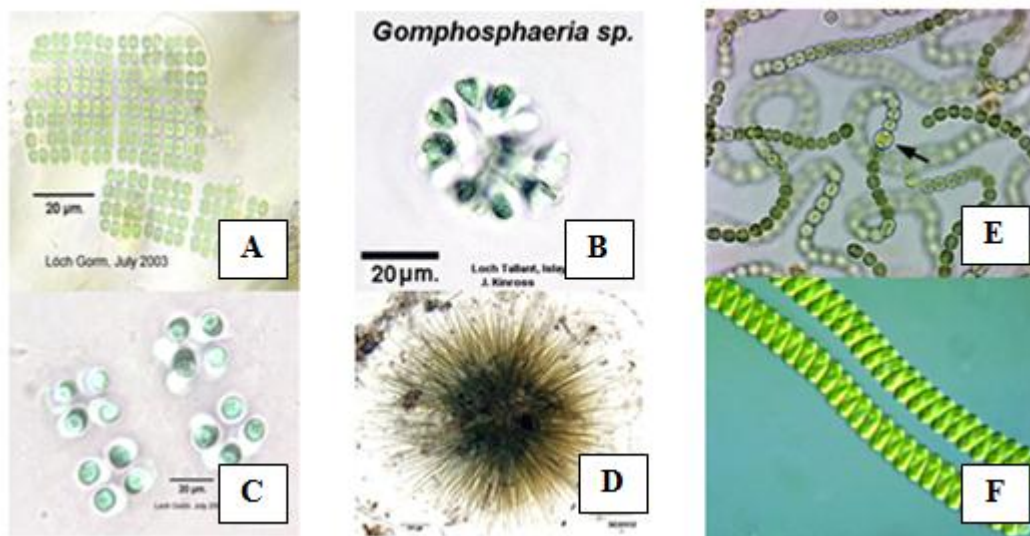


Figure 1. Cyanophyta : A: *Merismopedia* sp.; B: *Gomphosphaeria* sp.; C : *Eucapsis* sp.; D: *Gloeotrichia* sp; E: *Nostoc* sp; F: *Spirulina* sp.

b. Eukaryotes

Eukaryotes are single celled or multicellular organisms whose cells contain a distinct membrane bound nucleus; they include plants, animals, fungi and protists.

The review below focuses on major taxa within microalgae.

i. Rhodophyta

Rhodophyta division represents the red algae and comprises only one class known as rhodophyceae (Figure 2). They are probably one of the eldest groups in eukaryotic algae. They lack flagellated cells, have chlorophyll a, phycobiliproteins, floridean starch as a storage product and thylakoids occurring singly in the chloroplast. Red algal mucilages have been commercially utilized. Agar and carrageenan are two important polysaccharides derived from the Rhodophyceae. Agar is composed of two polysaccharides agarose and agarpectin, it is only soluble in hot water, forms a gel and can be used in food preparation and technology as well as in the pharmaceutical industry. On the other hand, carrageenan is similar to agar but with a higher ash content and requires higher concentrations to form a gel. That's why it is currently used for similar purposes as agar but also for stabilization of emulsions in paints, cosmetics and some pharmaceutical preparations [44].

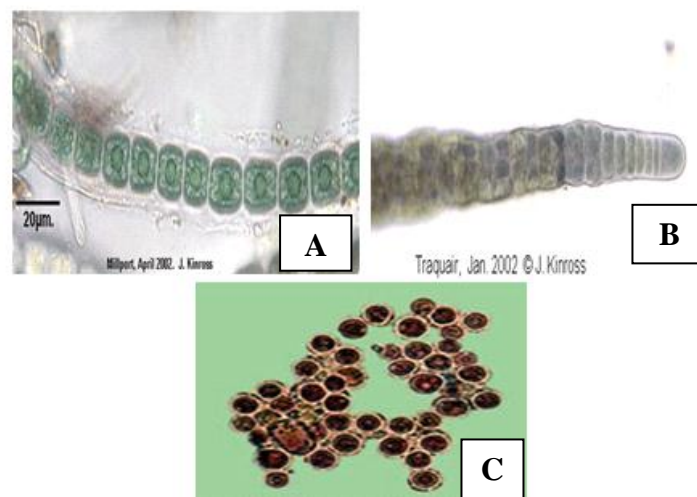


Figure 2. Rhodophyta: A: *Asterocystis sp* (Chroodactylon); B: *Lemanea sp*; C: *Porphyridium crenatum*

ii. Chlorophyta

They are the green algae (Figure 3) that have chlorophylls a and b capable of forming starch within the chloroplast. They differ from the rest of the eukaryotic algae by forming the storage product in the chloroplast instead of in the cytoplasm. Their cell walls have usually cellulose as the main structural polysaccharide although xylans and mannans might replace cellulose in certain cases. Accumulation of carotenoids occurs under conditions of nitrogen deficiency, high irradiance or high salinity [45].

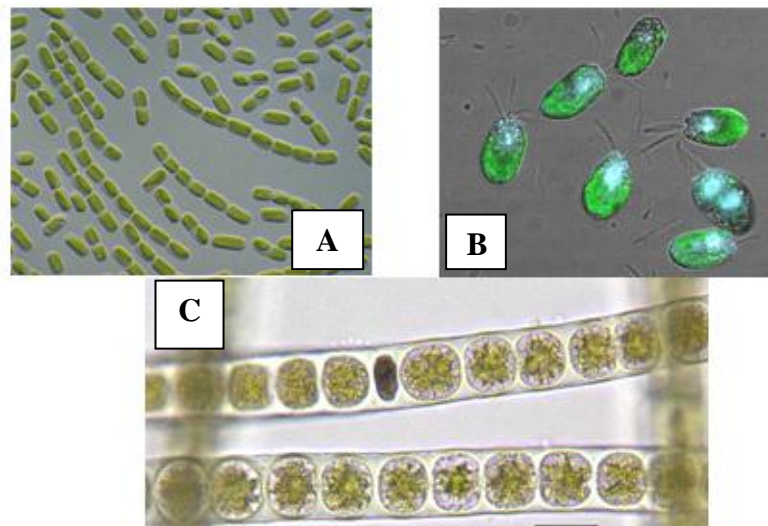


Figure 3. Chlorophyta: A: *Stichococcus bacillaris*; B: *Tetraselmis sp.*; C: *Zygonium sp.*

iii. Dinophyta

These organisms are important members of the plankton in both fresh and marine waters although a much greater variety of forms is present in marine environment. The cells can be photosynthetic or colorless and heterotrophic. Chlorophylls a and c_2 are present in the chloroplast with peridinin and neoperidinin being the main carotenoids. The storage product is starch as observed in higher plants and is found in the cytoplasm. A typical dinoflagellate consists of an epicone and hypocone separated from each other by the transverse girdle or cingulum which is

perpendicular to the longitudinal sulcus (Figure 4, Figure 5¹). Some dinophyceae have the ability to produce toxins which cause the death of fish and shellfish during dinoflagellate blooms and might be translocated to humans if shellfish are consumed [44][45]. Many dinophyceae exhibit rhythmic processes and produce light via bioluminescence by forming its own dinoflagellate-specific luciferin and luciferase.

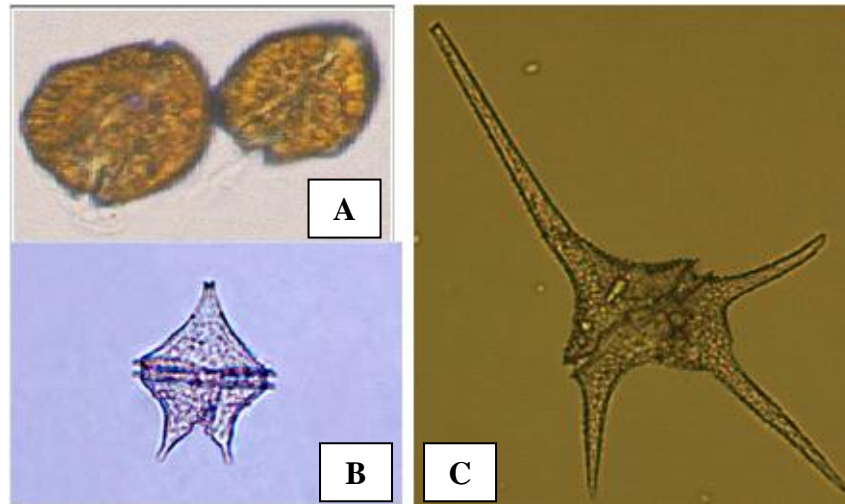


Figure 4. Dinophyta: A: *Gambierdiscus toxicus*; B: *Protoperidinium conicum*; C: *Ceratium sp.*

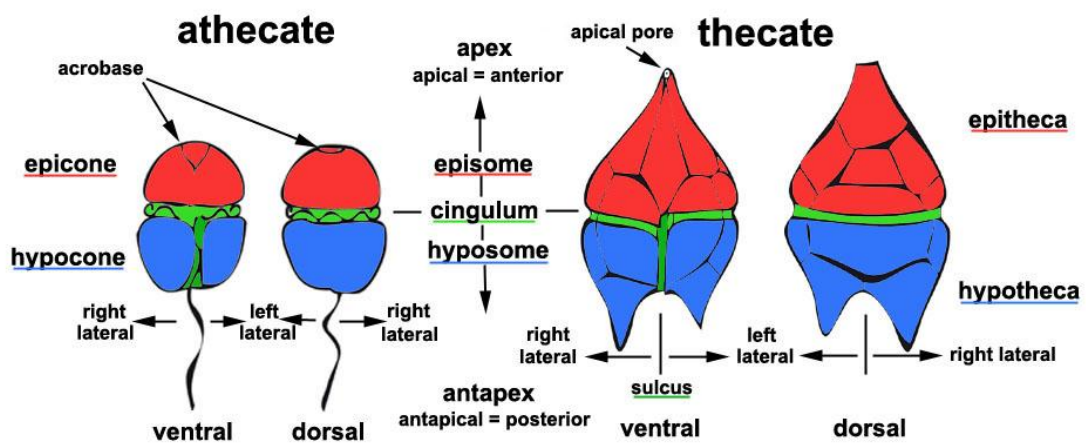


Figure 5. Schematization of characteristics of organisms from dinophyta.

¹ <http://tolweb.org/tree/ToLImages/dinoorientationtol.jpg>

iv. Cryptophyta

This group is primarily composed of flagellates (Figure 6) that occur both in marine and fresh waters. The cells contain chlorophylls a and c₂ and phycobiliproteins that occur inside the thylakoids of the chloroplast. The cell body is asymmetric with a clearly defined dorso-ventral/ right-left sides which results in peculiar swaying motion during swimming [45].

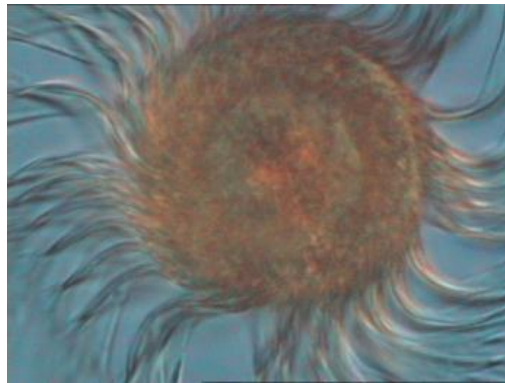


Figure 6. Cryptophyta. A: *Mesodinium rubrum*

v. Bacillariophyta

This is the division of the diatoms. The diatoms are unicellular, sometimes colonial algae found in every aquatic habitat as free-living photosynthetic autotrophs, colorless heterotrophs, or photosynthetic symbiotes (Figure 7). The cells are surrounded by shell made of silica, called the frustule. The chloroplasts contain chlorophylls a, c₁ and c₂ with the major carotenoid fucoxanthin. Diatoms are preferred food for invertebrates such as copepods. Their cells contain large quantities of highly unsaturated fatty acids such as eicosanoid acid [47].

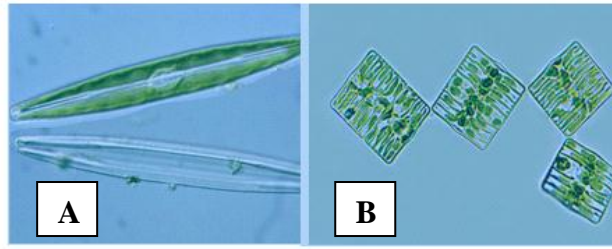


Figure 7. Bacillariophyta. A: *Amphipleura* sp. B: *Tabellaria* sp.

2. Molecular Identification

Many algal species cannot be identified based on their anatomy using light or electron microscopy. Several studies have been undertaken lately to identify microalgae using molecular tools. Several sets of primers have been widely employed targeting both short subunit (SSU) and large subunit (LSU) genes. The D2/D3 region of the LSU normally used as an eukaryote wide barcode marker [44] is known to be variable at the species level. It has been used to identify new species in various taxonomic groups including animals, plants and algae [63] which made of it a reasonable universal primer that can be used as a primary barcode marker. But LSU has limited sequence data available outside D2/D3 region which directed the design of a new primer combination T16N and T24U [44]. Moreover, LSU primer was successful in identifying a variety of diatoms. Cytochrome Oxidase subunit 1 5' Region (COI-5P) barcode marker was also developed mainly targeting brown and red algae. Likewise, it may be used to identify diatoms [64]. Other primers as elongation factor Tu (*tuf A*) and ribulose-bisphosphate carboxylase gene (*rbcL*) are also advocated as primary barcode markers for eukaryotic identifications. According to Saunders et al. (2001), *tuf A* has the highest universality (about 94%) and lowest levels of contamination (1%). The *tuf AR* and *tuf GF4* primers were designed and

proved efficient when tested. The *rbcL* -3P primers were used successfully for identification of diatoms.

C. Physical and chemical factors affecting microalgal growth

This reflects everything related to quality and quantity of nutrients, light, pH, salinity and temperature.

1. Nutrients

Microalgae need a variety of macro and micro nutrients, each required at certain concentrations depending on the genus and species. In general, the mostly needed nutrients are nitrogen and phosphorus. Trace elements and vitamins such as B1, B12, as well as biotin are also needed by many microalgal cells; diatoms for example have silicates as a special requirement for the formation of their external siliceous shell [35]. All the macro and micronutrients can be found in commercial solutions like f/2 or Walne media (APPENDIX I). The presence or absence of any of them will certainly make a difference in the algal growth system. For example, deficiencies in nitrogen, phosphorus and some metal elements were proven to affect the lipid content. The deprivation of *Chlorella vulgaris* from nitrogen increased its lipid content from 20% to 40% [19]. In addition, the form in which those nutrients are available is of critical importance. Zheng et al. (2013) reported that ammonium was the most preferred nitrogen source for algae due to the fact that its uptake requires less energy while nitrate consumption consumed more energy [4].

2 .Light

Light is a very important factor for growth since microalgae and cyanobacteria have the capacity to catch light and transform it to energy. This is done through the process of photosynthesis that converts the inorganic substances into simple sugars [1]. Light intensity requirements vary according to the culture type. For example, erlenmeyer flasks might not need more than 1,000 luxes, but 5,000 to 10,000 luxes might be needed for larger volumes. Low light conditions reduce photosynthesis and high light conditions enhance photo-inhibition [35].

3. pH

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. However, many algae are acid tolerant such as the green alga *Chlamydomonas* that can survive at pH 2.5 while other blue-green algae resist alkaline environment up to a pH of 11 [6]. Gehl and Colman (1990) proved that the internal pH of microalgae is affected by the external pH of the surrounding environment. This work has been proven on *Chlorella* whose internal pH was maintained at 7.3 while the external pH ranged from 5 to 7.5. Once the external pH dropped below 5 a significant decrease was also observed internally with a pH reaching 6.3 [20].

4. Salinity

Marine microalgae are used to grow in a saline environment. Studies have shown that it would be better for isolated microalgae to grow in slightly lower saline conditions than the ones they lived in. The optimum algal growth reported by FAO

ranged between 20-24 g of salt /L. But some isolated microalgae have the capacity to overcome the salinity range and survive in a very saline environment such as *Dunaliella salina* capable of living at 310 g of salt/L [65].

5. Temperature

Phytoplankton cultures require a temperature range from 20 to 24°C. However; some microalgae can survive beyond that range. For example, Ogbonda (2006) reported that *Spirulina platensis* gave the highest production at a temperature of 30°C, while researchers have reported that the optimum growth temperature of *Spirulina* ranges from 35 to 37°C [21].

D. Basic isolation and culturing techniques

1. Isolation of microalgae

Isolating microalgae is not a simple procedure to be performed since they normally live with other epiphytic species. Techniques of dilution, single cell isolation by micropipettes, and agar streaking can be performed to get a pure microalgal culture. Also, bacteria can be eliminated by the use of antibiotics [46] if the culture grown does not contain cyanobacteria in the medium.

2. Enrichment of culture media

To ensure the appropriate growth of microalgae, their culture medium should be enriched in a way that enhances their growth and inhibits or decreases nontarget

organisms. Microalgal growth requires the presence of three main macronutrients: nitrogen, phosphorus and silicate especially in the case of diatoms.

Numerous studies have shown that lipids accumulate in algae when they are deprived from nitrogen [46]. But on the other side, it has been noticed that some microalgal species increased their carbohydrate content under nitrogen deficiency [31]. This means that nitrogen absence or presence in the culture medium will definitely affect the growth of microalgae. In addition, phosphorus has proved its importance in cellular processes related to energy transfer and nucleic acid synthesis of microalgae. Also, microalgae are affected by phosphorus presence or absence. It has been found that phosphorus supply induces the activity of alkaline phosphatase in microalgae [76].

3. Growth kinetics

Microalgal culturing is characterized by five different phases (Figure 8).

a. Lag or induction phase

This is the first step of growth and could be considered as the acclimatization phase during which the microorganism adapts its physiological metabolism to the new growth conditions it is placed in. The length of this phase varies depending on the initial inoculum state. If cultures inoculated are in the exponential phase, lag phases would be short but if algal culture is transferred from plates to liquid, the process would take longer time.

b. Exponential phase

That is the period of time when cell numbers increase at a fast rate following a logarithmic function. Growth rate is dependent upon many variables such as the algal species, light intensity medium composition and temperature.

c. Phase of declining relative growth

After an unlimited growth has occurred in the exponential phase, cell density reaches a point of inflection where the culture becomes limited in nutrients, light, pH, carbon dioxide and other physical and chemical factors. Then, most cells stop division and this is shown as a rapid deceleration of the population growth.

d. Stationary phase

Cell density becomes stable due to limiting factors such as the depletion of essential nutrients or the formation of inhibitory products, or due to growth vs. death rates that become balanced.

e. Death phase

After a prolonged growth, cells start dying and the culture crashes since water quality deteriorates and nutrients become depleted to a level that growth becomes impossible. Furthermore, many factors might cause that cell death such as oxygen deficiency, overheating, pH disturbance or contamination [35].

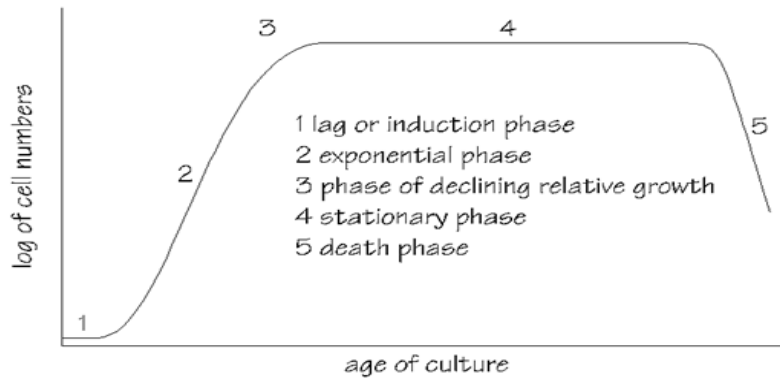


Figure 8. Five growth phases of microalgal cultures.

E. Cultivation systems and modes of culture

There are several factors to consider when growing microalgae as to which culture system to use. Considerations should focus on the biology of the microalgae, the cost of land and labor, the climate and the end product planning to obtain. 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. Mass production of microalgae is normally conducted either in open or closed systems using either batch or continuous production.

1. Open pond systems

Most of the microalgal work on a large commercial scale is performed in open systems. However, a small number of microalgal species can succeed in such conditions. For example, commercial production of *Dunaliella sp* and *Chlorella sp* in raceway ponds is currently performed with minimum risk of contamination [22]. The most common strains used for the open pond systems are *Anabaena sp.*, *Chlorella sp.*, *Dunaliella sp.*, *Haematococcus sp.* and *Nostoc sp* [25]. There are different designs of open-air system ponds such as shallow big ponds like lakes and natural ponds, tanks,

circular ponds (not highly appreciated since they require concrete construction and high energy) .[23] Raceway ponds equipped with paddle wheels, are the most commonly used in commercial plants [24].

Open ponds are easy to construct, operate and clean, require low energy input, and have the potential to produce net energy [24] but at the same time they have many drawbacks. Light utilization by cells might be poor, evaporation is high which necessitates large amounts of water, and might lead to low productivity or even inconsistencies in production and most importantly are subject to contamination by fungi, bacteria or other microalgae [1].

2 .Closed systems

Choosing closed systems for microalgal mass production is a way to overcome limitations of open systems. This is because light is produced evenly, pollution and contamination risks are highly minimized, with a real chance of producing single-species of microalgae [1]. However, production costs are still higher compared to open systems. Closed systems include flat, tubular and column photobioreactors. Flat bioreactors have received attention due to their capacity to catch light irradiance [23] accumulate low amounts of dissolved oxygen and perform high photosynthesis as compared to the tubular system [24]. The latter has limitations related to the length of the tubes, to their form which might affect respiration and gas concentrations. However, they are often recommended for large scale operation as in Hawaii plants. Vertical or column reactors are simple systems whose agitation is achieved by the injection of compressed air.

A variety of microalgal culture modes have been reported; however all of them are variations of two basic systems: batch and continuous cultures.

a. Batch culture

This is considered to be the most common culture system followed worldwide. Microalgal growth occurs in a culture vessel or a bioreactor by inoculating a small inoculum of the microalgae to a large culture medium. The process might take time depending on the microorganisms' population, their physiological and metabolic rates; then cultures are harvested before reaching to the stationary phase. Fed batch cultures on the other hand are used to reach a higher cell density as compared to the batch culture. This is because the system allows the constant addition of nutrients to compensate for any loss which is not considered in the batch culture. Despite the limitations of batch culturing, this method is considered reliable, inexpensive, and simple to maintain [19].

b. Continuous culture

In that system, fertilized sea water is continuously added to the culture so that algal cells stay close to the maximum growth rate. Two categories of continuous culture can be distinguished:

- The turbidostat culture in which the microalgal concentration is kept at a preset level by constantly diluting the culture with fresh medium by means of an automatic system.
- The chemostat culture in which fresh medium is constantly added at a predetermined rate. In this system, nutrients are added at a fixed rate leading to a constant growth rate of the culture.

Both categories have the capacity to produce a good quality of microalgae, require technological control which reduces labor interference, but at the same time, both share the same drawbacks since they are relatively costly, require constant illumination and temperature [19], [35].

F. Microalgal composition

1. Lipids

The interest in microalgae for oil production is due to its high lipid content. Lipids are normally divided based on their chemical structures into two main groups: polar and neutral. Polar lipids include the phospholipids and the glycolipids, while non-polars govern tri-, di-, and monoglycerides, waxes and isoprenoid type lipids. Various lipids are produced in microalgae depending on their species [29]. Each lipid type is present in microalgae 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.

Microalgae are also able to synthesize various fatty acids including medium chain (C10– C14), long-chain (C16–18) and very-long-chain (C20) which are either saturated or unsaturated [31]. However, lipid compositions of microalgae change under environmental conditions. Furthermore, hydrocarbons are accumulated in microalgae making less than 5% of their dry weight.

Many studies stressed on the importance of the effect of nutrient deficiencies on the lipid accumulation in microalgae. This is because media deficient in nitrogen, silicon, phosphorus and sulfur were found to favor the accumulation of lipids [26].

Reports have shown an increase of 138% of lipids under nitrogen deficiency and of 168% under silicon deficiency when compared to nutrient sufficient medium. Also, microalgal fatty acid composition varied according to the level of nutrient feeding. A study conducted by Piorreck et al. (1984) showed that microalgae have the tendency to synthesize neutral lipids and fatty acids with low degrees of unsaturation at low nitrogen levels, while high levels favour the synthesis of polar lipids [32]. Besides nutrient availability, other factors affected lipid composition of microalgae such as temperature and irradiance [27]. According to Rodolfi et al. (2008), triglycerides accumulation has been stimulated by high irradiances while under low irradiances, phospholipid and glycolipid contents were higher [27]. Temperature decrease, increased fatty acids unsaturation while its increase led to an increase in saturated lipids [31]. PUFA content of the marine diatom *Phaeodactylum tricornutum* has been shown to be higher at lower temperatures within the range of 10–25°C. The highest yields of PUFA and EPA (omega-3) per unit dry mass were 4.9 and 2.6%, respectively, when temperature was shifted from 25 to 10°C for 12 h, with both being raised by 120% compared to the control [47]. A good microalgal processing is based on a good microalgal choice. This was observed by Pulz and Gross (2004) who said that “successful algal biotechnology mainly depends on choosing the right alga with relevant properties for specific culture conditions and products” [26].

Various methods for effective lipid extraction have been tried so far.

Extraction of lipids from microalgal cells have been performed chemically through the use of solvent mixtures or physically, or by the combination of both. Chemically, many methods have been recommended such as Bligh and Dyer (1959), Soxhlet method and Folch methods [70]. Physical techniques, include microwaving algal cells, sonication and bead-beating [69]. Also, supercritical carbon dioxide (SCCO₂)

extraction is a promising green technology that can potentially displace the use of traditional methods [70], however, a final method has not been settled yet.

Microalgae have been selected for biodiesel production. Four primary methods can be used for biodiesel production: blending, microemulsion, pyrolysis and transesterification [71]. [71] The most commonly used method is transesterification of microalgal triglycerides with an alcohol in the presence of a catalyst to produce mono esters termed as biodiesel (fatty acid methyl or ethyl ester FAME) in a reaction known as transesterification. Transesterification is catalyzed by acids, alkalis and lipase enzymes. Alkali- based transesterification is considered the best, 4000 times faster than acid based catalysis commonly used at 1% of the weight of oil [28]. Strong bases such as sodium hydroxide (NaOH), potassium hydroxide (KOH) or methoxide (MeOH) are commonly used as catalysts in large scale production of biodiesel. During the transesterification reaction, fatty acids should be derivatised into their methyl ester fatty acids (FAMES), followed by a GC-MS analysis for an efficient separation, quantification and identification of the profile of fatty acid methyl ester present in the biomass.

2. Proteins

Proteins make up a large fraction of microalgal biomass. Although they have been so undervalued when compared to fatty acid production and omega 3 synthesis, it is currently more plausible to focus on microalgal protein production for animal feed due to the economics of production as compared to biofuel production. Currently, the main species used in animal feed are *Spirulina*, *Chlorella* and *Scenedesmus* [1]. According to FAO, protein content in microalgae often occupies

the highest percentages of their dry weights ranging from 12 to 35% [35]. Some microalgae are known to have higher concentrations of protein. This is the case of most blue green algae containing 18 to 54% as compared to green algae 8-34% [32]. For instance *Spirulina* is known to be useful as a food rich in proteins comprising all essential amino acids needed. Heterocystous cyanobacteria have that privilege over others since they possess a thick cell wall that protects their nitrogenase from the inhibitory effect of oxygen and at the same time have a heterocyst system capable of producing ammonia and oxygen from their surrounding environment [36]. In order to obtain high concentrations of protein, algae must be grown in high nitrogen concentrations.

Protein content of microalgal biomass can be quantified using two common procedures: by colorimetry [75] or by methods that measure the concentration of elemental nitrogen such as in Kjeldahl method. The colorimetric method is based on the measurement of color change caused by oxidation of amino acid residues by the Folin reagent while measurements of nitrogen using Kjeldahl's method and Hach techniques [86] [75] require acid digestion accomplished by exposing the sample material to a combination of mineral acids or acid and hydrogen peroxide at an elevated temperature. Once the substance under analysis has been dissolved, chemical methods can be applied to measure the concentration of each element.

3. Carbohydrates

They represent the smallest proportions in microalgal compositions constituting only 4.6-23% of their dry weights [35]. They are mainly constituted from cellulose in the cell wall, and starch in the plastids [70]. Their levels increase with

culture aging. For example, carbohydrate levels reached 53.10% and 48.35% in the last stages of growth of *P. lutheri* microalgae [43]. Despite their relatively small concentrations, their effectiveness in bio-ethanol and methane gas productions by fermentation was proven [1].

4. Pigments

Microalgae have organic pigments capable of harvesting energy. Pigments are divided into three major classes: chlorophylls (green pigments), carotenoids (yellow or orange pigments) and phycobilins. While the first two pigments are lipophilic, the latter is hydrophilic.

Chlorophyll molecules consist of a tetrapyrrol ring containing a central magnesium atom and a long chain terpenoid alcohol. In microalgae we can find different types of chlorophylls which differ from each other in their side-group substituent. Chlorophylls have two major absorption bands: blue or blue-green (450-475 nm) and red (630-675nm).

Carotenoids have an absorption range between 400 and 550nm. They are made from two hexacarbon rings joined by an 18 carbon conjugated double chain. They are either hydrocarbons or oxygenated hydrocarbons.

Phycobilins are water soluble linear terapyrroles, not associated with a magnesium atom. They absorb blue- green, yellow, or orange light.

Some pigments on the other hand do not transfer excitation energy. They are considered a secondary group of carotenoids like xanthophylls, astaxanthin and

canthaxanthin, and those are overproduced in algal cells if under unfavourable conditions [23].

Identification and quantification of pigments may be preferred using methanol, ethanol or acetone extraction, followed either by spectrophotometric analysis or by high liquid performance chromatography (HPLC).

Pigments have been used as a tool for microalgal identification. Literature reports specific pigmentation for each microalgal group (Table 1).

Table 1. Pigment distribution according to taxonomical groups.

Taxonomic Group	Chlorophyll	Carotenoids
Bacillariophyta (diatoms)	a, c	β -carotene \pm -carotene rarely fucoxanthin,.
Chlorophycophyta (green algae)	a, b	β -carotene, \pm -carotene rarely carotene and lycopene, lutein.
Chrysophycophyta (golden algae)	a,c	β -carotene, fucoxanthin
Cyanobacteria (blue green algae)	a,c	β -carotene, phycobilins
Phaeophycophyta (brown algae)	a,c	β -carotene, \pm fucoxanthin, violaxanthin
Dinophyta (dinoflagellates)	a,c	β -carotene, peridinin, neoperididnin dinoxanthin, neodinoxanthin.
Rhodophycophyta (red algae)	a, rarely d	β -carotene, zeaxanthin $\pm \beta$ carotene

5. Ash

The ash content of filamentous blue-green algae ranged from 3–11 % but those of filamentous green algae were generally much higher, ranging from just under 12 % to over 44 % in one sample of *Cladophora sp* [82].

G. Potential uses of microalgae biomass

1. Commercial microalgae production

Several microalgae have been produced commercially including *Chlorella*, *Spirulina* and *Dunaliella sp*.

Chlorella has been used successfully on an industrial scale for the production of human nutritional supplements. A diversity of production systems are used including circular ponds, paddle wheel mixed raceway ponds and tubular photobioreactors.

Spirulina, traditional food for Chad society, has also been produced on a large scale in many countries. It has been considered more promising than *Chlorella* due to its capacity to grow in a high bicarbonate medium and to its filamentous structure that makes it's harvesting easier.

Dunaliella salina is also produced for its high carotenoids value, beta-carotene and astaxanthin. It is capable of living in very harsh conditions, in a medium three times more saline than sea water which reduces the chances of contamination and makes it a strong candidate for commercial production [12].

2. Commercial products derived from microalgae

Microalgae are attractive as natural sources of bioactive molecules due to their potential to produce a variety of compounds difficult to produce by chemical synthesis [48][48].

a. Food and feed

The production of high value food, animal feed products, such as lutein for chicken feeds, beta carotene from *Dunaliella*, or fish meal replacement are considered promising features of microalgae [12].

b. Pharmaceuticals

Several uses of microalgae for medicinal purposes have been studied for many years. This is because marine photoautotrophic microorganisms are able to produce a wide range of secondary metabolites with allelopathic properties capable of inhibiting the growth of competitors or predators [8]. Various important biomolecules have been isolated such as antioxidants [1] found heavily in cyanobacteria as reported by Patel et al. (2006). The latter studied the antioxidant activity of phycocyanin isolated from three cyanobacterial species *Lyngbya*, *Phormidium* and *Spirulina* sp. and reported the importance of antioxidants to prevent or inhibit cancer in humans and animals as in the case of *Arthrospira platensis* [49].

c. Pest management

Currently, plant pests and pathogens are suppressed using chemical pesticides which lead to several health and environmental problems. Therefore, the search for safer alternative methods of plant pest control became a must. Biological control organisms are generally considered safe for the environment, and represent one of the

important potential strategic methods to be investigated. An environmentally sound approach to induce systemic resistance of plants against plant pathogens or other pests would be to use some microorganisms or safe natural compounds. Microalgae have shown antifungal, antibacterial [11], [14], [50] antiviral and nematicidal properties. This wide range of control is the result of the chemical composition of a large number of cyanobacteria in which researchers found polyketides, amides, alkaloids, lactones, peptides and lipopeptides [16], [51].

i. Antifungal activity

Microalgal extracts have shown activity against a variety of plant pathogens. The crude methanolic extract of the cyanobacterium *N. commune* gave a significant inhibition of *F. oxysporum* f. sp. *lycopersici* in tomato seeds similar to the commercial fungicide mancozeb [13]. Extracts of cyanobacterial isolates exhibited antifungal activity against several fungi including: *Aspergillus oryzae*, *Candida albicans*, *Penicillium* sp. and *Saccharomyces cerevisiae* [50]. Those fungal inhibitory properties were not specific to cyanobacteria. Green algae as well as others showed similar activities. *Chlorella salina*, *Tetraselmis chuii* and *Nannochloropsis oculata* exudates reduced the growth of *Fusarium solani* [8].

ii. Antibacterial activity

Growth of cyanobacterial blooms lead to the decrease of the gram negative bacteria like *Escherichia coli* found in water lakes [51]. This activity found in cyanobacteria was also highlighted in green algae. *Chlorella vulgaris* was considered to exhibit an antibiotic activity since it produces a mixture of fats and hydrocarbons called chlorellin [8]. Fatty acids detected in *Chlorella* were also detected in cyanobacteria and found to be efficient since they affected the permeability of the cell

membrane, interacted with proteins and lipids of the cell membrane and inhibited special enzymes [51]. Kellam et al. (1989) reported in-vitro antibacterial activity in the organic solvent extracts of *Tetraselmis sp.*, Prasinophyceae and Bacillariophyceae microalgal classes which were mainly effective against *Staphylococcus aureus* bacteria.

iii. Antiviral activity

Several studies claimed the importance of marine algae as antiviral compounds. *Ceramum rubrum* algal extracts were able to suppress the influenza viruses in vitro and in vivo. Also, sulfated polysaccharides of the red microalgae *Porphyridium sp.* were shown to be effective against *Herpes simplex* viruses types 1 and 2 (HSV 1, 2) and *Varicella zoster* virus (VZV). Furthermore, algal polysaccharide known as carrageenan inhibited the viral infection inside host cells. This occurs at a very early stage in the viral infection cycle during adsorption of the viral particle. This is explained by Huleihel (2000) as due the interaction between the negatively charged polysaccharides and the positively charged viral particles [17].

iv. Nematicidal activity

In addition to their wide range activity as antifungal, antiviral and antibacterial organisms, microalgae showed an important potential as a nematicidal biological control. 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 [15]. This was also proved by other studies where culture filtrates of the blue green algae *M. vaginatus* killed the juveniles of *Meloidogyne incognita* nematode [55]. Furthermore, scientists tested the effect of cyanobacterial endospores on nematodes and found that

endospores were able to penetrate the nematode's body within 10 days which affected their feeding activity and made them sluggish in their appearance [51].

v. Induced Resistance

Plants have the ability to develop resistance to diseases. This is done through two different processes known as local resistance or systemic resistance. Local acquired resistance takes place normally two to three days post infection; however, systemic acquired resistance (SAR) necessitates one week to be activated. These types of defense reactions followed by plants are induced through various types of elicitors secreted by microorganisms or derived from cell walls of fungi, bacteria or host plants. Laminarin derived from brown algae *Laminaria digitata* is a "potent elicitor of defense reactions in grapevine cells" since it showed a significant protection against *Botrytis cinerea* and *Plasmopara viticola* [7]. This is because it has the capacity to induce the expression of defense genes associated with different pathways. H₂O₂ concentrations associated with local induced resistance, changed after laminarin application. As concentrations of laminarin increased, production of H₂O₂ increased and became saturated between 0.5 and 1g/liter. Laminarin also induced a rapid calcium uptake in cultured grapevine cells followed by SAR through a higher expression of defense genes, the accumulation of phytoalexins as well as an increase in the activities of chitinases and β -1, 3-glucanases [7].

The extracts of the brown alga *Ascophyllum nodosum* induced jasmonic acid dependent systemic resistance in *Arabidopsis thaliana* against different pathogens [9]. *A. nodosum*, the most widely used seaweed in agriculture and horticulture production, has also been used due to its laminaran content which elicits both plant growth and defense responses by the induction of antimicrobial phytoalexins [9].

But induced resistance is not limited to brown algae. Green algae were also found to induce natural defense in tomato seedlings. This was accompanied by salicylic acid systemic acquired resistance as well as bioelicitors such as ulvans and oligoulvans [52]. The treatment of tomato seedlings by *Ulva Lactuca* polysaccharides and oligosaccharides lead to the suppression of *Fusarium oxysporum f. sp. lycopersici* by 44 and 54% compared to the control plants [16]. Ulvan of *U.lactuca* was even considered to be more effective than other polysaccharides of marine algae such as laminarin, carragenan and alginate. This high efficiency of elicitors is due to a sulfate group involved in many biological activities and physiological processes.

3. Biofuel

a. Worldwide fuel market and biofuel candidates

Based on the current statistics, fuels make up 70% of the global energy market [19]. Fuel energy demands were increasing since decades and nowadays debates are taking place on alternative feedstocks for fuel production. Higher plants like sugarcane, soybean, oil palm as well as microalgae are currently studied for biofuel production. Although energy crops have been commercially used as feedstocks for biofuel production, they are still not favored for long term fuel energy production since they compete with food and feed production [19]. In addition they do not have the capacities to replace petroleum oil in the foreseeable future. *Jatropha* crop capable of growing in non agricultural lands has also been proposed for fuel production. But, its oil is high in unsaturated fatty acids constituting more than 75% [72][72] and its yield varies according to environmental conditions which are hard to control. On the other hand, microalgae can provide several different types of renewable biofuels

including biodiesel, methane and biohydrogen [2]. It has been stated that microalgae are the highest yielding feedstock for biodiesel since they can produce 250 times more oil when compared to soybean, and 7 to 31 times more when compared to palm oil [2]. Statistics have shown that converting 1 to 3% of the US area for microalgal production would be enough to satisfy 50% of the country's fuel transport needs [27].

Microalgae and cyanobacteria have gained the privilege of producing biofuel for various reasons:

- 1) Do not need productive arable land based sources, they can be grown in marginal areas such as arid lands and therefore reduce competition with food crops and fresh water [1], [5], [26].
- 2) Can be produced in a variety of water sources such as fresh, brackish, sea water and waste water [1], [5].
- 3) Perform efficient photosynthesis and thus they are efficient converters of sun energy into chemical energy [1], [5].
- 4) Grow in high densities leading to a high production if mass cultivation is well performed [1], [5].
- 5) Their cultivation does not require herbicides or pesticides; and their residuals after oil extraction can be used as feed or fertilizers [50], and/or fermented to produce ethanol or methane [27].

b. Environmental and economical aspects of biofuel production

Economics have always been the main driver in fuel processing and studies. Indeed, the upcoming rise of biofuel topic is mainly related to economic reasons. The

last decade has known rises in fuel prices unexpectedly. The price of a fuel barrel reached \$140 in 2008 while it used to be \$20 in the 1990s. This has made the search for new fuel candidates (with high biomass, lipid content, easy extraction and harvesting, and low cost) a must [29]. Microalgae having the characteristics mentioned above have been studied for fuel production. It is a renewable resource that can be sustainably supplied with no limited reserves, thus will not be subjected to fluctuations in prices.

Environmentally speaking, pollution problem will be reduced [1]. Microalgae capture and use CO₂ during photosynthesis compensating for the CO₂ released during biodiesel burning [2]. Microalgal biodiesel contains no sulfur and performs as well as petroleum diesel, while reducing emissions of particulate matter, CO, hydrocarbons, and SO_x [37][37] which decreases 90% of air toxicity and 95% of cancers compared to common diesel source. In addition, microalgae having the capacity to convert 3-8% of solar energy to biomass which is an asset when compared to terrestrial plants that are capable of 0.5% conversion only [4]. Nevertheless, despite the promising future that microalgae is offering towards biodiesel production, the cost is still considered high [82][84]. Reports indicate the necessity of reducing the cost of microalgal oil from \$2.80/L to \$0.48/ L in order to eliminate our dependence on petroleum oil [27].

4. Wastewater treatment

Nowadays, industrialization and many human activities have made pollution issue a hot subject. Dumping large quantities of organic and inorganic substances into soil, water and the environment due to various agricultural, domestic and industrial practices aggravated the pollution problem [18]. Agricultural nitrates and animal

wastes are polluting surface and ground water. To resolve that problem, microalgae have been highlighted as a possible promising solution.

Microalgal interference in wastewater treatment comes in the secondary phase where sewage water is mixed with heterotrophic bacteria and microalgae to reduce the biochemical oxygen demand (BOD). Biochemical and chemical oxygen demand were found to be reduced by 68.4% and 67.2% respectively after algal treatment for domestic wastewater. Designing a system for wastewater treatment using microalgae requires the presence of either waste stabilization ponds or high rate algal ponds [53]. Microalgae use organic substances present in the sewage system for energy and growth production. Organic ions such as ammonium, nitrate and phosphate are removed [18].

A list of microalgae that tolerate organic pollutants includes 240 genera, 725 species and 125 varieties and forms. The most tolerant genera include eight green microalgae, five blue-greens, six flagellates and six diatoms. Removing nitrate, ammonia and phosphate by incorporating microalgae is an elegant solution especially that these minerals are essential for their lifecycle and also available in their inorganic forms. *Chlorella vulgaris* has shown a high efficiency in removal of 86% of inorganic N and 78% of inorganic P [54]. Furthermore, microalgae were efficient in removal of 99% of heavy metals, toxic organic compounds, and in reduction of coliform bacteria, and both the chemical and the biochemical oxygen demand. For example, marine cyanobacterium *Lyngbya tailorii* adsorbed Cd, Ni, Pb, and Zn [23] and *Dunaliella bioculata* was reported to degrade the pyrethroid insecticide Deltamethrin.

In the present work we isolated nine pure local microalgal strains, identified them using molecular tools, determined their protein, lipid and pigment

content, assessed their potential use for biodiesel production and evaluated their activity as antimicrobial compounds for the management of plant pathogenic fungi.

CHAPTER III

MATERIALS AND METHODS

A. Isolation and culturing of local microalgae

1. Water Sampling

Water samples for microalgae isolations were collected from stagnant water systems in 13 sites along the Lebanese coast (Table 2). Samples were directly filtered using a 100 µm filter to discard macroalgae and zooplanktons. The sea water samples were enriched with nutrients using Guillard f/2 medium (APPENDIX I).

Table 2 . Sites, dates and isolate acronym of collected water samples.

Site	Date	Isolate Acronyms
Jbeil	15/3/2013	Jbd
Jounieh	15/3/2013	Jod
Jiyeh	21/6/2013	Jid
Hammam El Askari	20/6/2013	Had
Saida	21/6/2013	Sad
Khaldeh	21/6/2013	Khd
Damour	21/6/2013	Dad
Tyre	25/8/2013	Tyd
Bourghoulyeh	25/8/2013	Bod
Aquamarina	15/3/2013	Aqd
Rmaileh	21/6/2013	Rmd
Ouzaii	21/6/2013	Ozd
Sarafand	21/6/2013	Sard

Once in the lab, samples were transferred to clean, previously acid washed (10% HCl) erlenmeyer flasks and were stoppered with hydrophobic cotton and cheese cloth. Flasks were left for acclimatization near window (low light intensity) until observing the beginning of bloom formation. Next, they were transferred to a growth chamber where 250 lux light from fluorescent lamps was supplied for 16hours / day.

2 .Microalgae Isolation

a. Flagellates

A ten-fold serial dilution of water samples was used to isolate each microalga in "pure" cultures.

b. Non-flagellates

Subcultures were made by inoculating 50-60µl of the algal bloom suspension onto Guiliards f/2 agar plates. After microalgal growth, the colonies were further purified by plate streaking techniques, using a microbial loop aseptically. Colonies were checked for purity under the microscope. Once a unialgal strain was successfully isolated, it was transferred into 5 ml of enriched liquid medium.

3. Water Sterilization

Water was filtered using a 0.22µm filter or autoclaved at 121°C at 15 psi for 15 min or longer depending on the volume. After autoclaving, the sea water was directly cooled in the refrigerator to avoid the formation of precipitates and was next left for 24 hrs to equilibrate. For larger volumes especially those for photobioreactors, water was chlorinated using 5ml of Clorox per 20L of sea water and then de-chlorinated using sodium thiosulfate at a ratio of 0.2g per 20L of water.

4. Up-scaling and growth kinetics

After isolating a pure culture, it was transferred to 5ml sterilized medium. This small volume was increased to 20 ml, then to 100 ml, 500 ml, then 2L flasks, 10L gallons.

20m → 100ml → 500ml → 2L → 10 L → 20L

7- 14 days → 3-7 days → 3- 4 days → 3-4 days → 2-3 days → 2-3days.

All containers were appropriately sanitized with 10% HCl for 24 hrs and were stoppered with hydrophobic cotton. They were then placed in a growth chamber and were supplied with artificial light using daylight fluorescent lamps to ensure growth of the cultures.

Growth kinetics

When cultures reached exponential phase, they were transferred to a 20L photobioreactor, where all factors like light intensity (450 luxes) and duration (16 hours light/ 8 hours dark), salinity (28g/L), pH (8-8.3) and average temperature (21°C) were controlled. Microalgal growth was estimated by measuring optical density of the culture suspension on a daily basis at 560nm until reaching the stationary phase. Data on cell number and dry matter content were recorded at different periods of time.

5. Microalgae stock cultures

To maintain microalgae stocks, under "aseptic conditions", an inoculum of 1 ml of microalgal suspension was transferred into 12ml 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

The isolated microalgal cells were observed under a microscope (Carl Zeiss AxioLab A1) at 400x or 1000x magnification. The average diameter of the microalgal cells was recorded based on the average of 10 cells.

B. Molecular Diagnosis

1. DNA Extraction

An aliquot (7-10ml) of microalgal suspension was harvested at mid to late exponential phase (10-14 days) by centrifugation at 5000 rpm for 5 min at 4°C and the pellet was retained. 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 to the precipitate thoroughly mixed by vortexing and the entire suspension was incubated at 60°C for 20-30 min. The debris and impurities were separated using 600µl iso-amylalcohol: chloroform (24:1) and moderate vortexing followed by low speed centrifugation were performed. The supernatant was then transferred to a clean microfuge tube, precipitated with an equal volume of isopropanol and stored at -20°C for 1hr. The suspension was then centrifuged at 14,000 rpm for 8minutes. 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 by electrophoresis in 0.7 % agarose gel and NanoDrop 2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA samples were stored at -20⁰C until use.

2. PCR

The DNA extracts were amplified by PCR using several primers (Table 3). A 20 µl PCR reaction mixture was prepared using 10 µl of ReadyMix™ PCR Reaction Mix with MgCl₂ (Sigma-Aldrich, St. Louis, MO, USA) 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 3Table 3), 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 facility² The nucleotide sequence data were assembled by employing the Contig Assembling program of the sequence analysis software BIOEDIT, version 7.0.³ BLASTN analysis was performed to determine the homology to the most closely related sequences.

² <http://www.htseq.org>

³ (<http://www.mbio.ncsu.edu/Bioedit/bioedit.html>).

Table 3 . List of primer pairs used in PCR assays for amplification and sequencing of microalgae genes.

Primer Pairs	Sequence	Target Gene	Purpose	Expected size (bp)	Annealing Temperature (°C)	Reference
T16N	5'AMAAGTACCRYGAGGGAAAG3'	Nuclear D2/D3 region of the large ribosomal subunit (LSU D2/D3)	Universal	567	50	Harper and Saunders 2001
T24U	5'SCWCTAATCATTTCGCTTTACC3'					
tufGF4	5'GGNGCNCAAATGGAYGG3'		Chlorophyta	807	45	Fama et al. 2002
tufAR	5'CCTTCNCGAATMGCRAAWCGC3'					
ITS1	5'AGGAGAAGTCGTAACAAGGT3'	Internal transcribed spacer of the ribosomal cistron	Chlorophyta	NR*	52	White et al., 1990 (modified)
ITS4	5'TCCTCCGCTTATTGATATGC3'					
GWSRx	5'ACTTCTGGRTGICCRAARAAYCA3'		Mitochondrial cytochrome c oxidase	Rhodophyta	NR*	46.5
GWSFn	5'TCAACAAAYCAYAAAGATATYGG3'	1 gene	Phaeophyceae			

*NR: Not reported.

C. Chemical and biochemical profiling

1. Determination of Total Lipid Content

Microalgae were harvested at two stages, during the exponential phase and the beginning of the stationary phase by centrifugation (4000 rpm for 12 min). The harvested cells were re-suspended in distilled water to remove any residual salt. The homogenate was recentrifuged two successive times for 10 minutes at 4000rpm. After decanting water, microalgal cells were placed at -20°C to freeze and then were incubated in a freeze drier overnight (LABCONCO lyophilizer).

Total lipid content was determined gravimetrically using the Folch method [73]. A solvent mixture of dichloromethane:methanol (2:1) was added to a known mass of freeze dried algae. The mixture was then homogenized to a volume 20 times the volume of tissue sample (1 g in 20 ml of solvent mixture). Tubes were placed in the ultrasonic bath for an hour and a half (at 40°C) then vortexed well. Homogenates were then filtered into new screw capped tubes and 0.4 volumes of water were added (8 ml of water for 20 ml of solvent mixture). After vortexing for few seconds, mixtures were centrifuged for 10 minutes at 4000 rpm to allow phases separation. Upper phases were discarded while the lower phases representing the DCM containing lipids were left. To the lower phase, were added 5g of sodium sulfate for moisture absorption. The mixture was then decanted in a new tube and the solvent allowed to evaporate under vacuum in a rotary evaporator. Dry weights of lipids were measured and lipid contents (%in biomass) were determined by the following formula,

$$\text{Lipid content} = (\text{weight of lipid in g} / \text{dry weight of sample in g}) \times 100.$$

After the extraction processes, the resulting algal oil product was converted into biodiesel through a process called transesterification.

2. Fatty acid profiling

Fatty acids were transformed into their methyl ester fatty acids (FAMES) by transesterification reaction and were subjected to GC analysis.

Lipids previously extracted were dissolved in dichloromethane. To the mixture was added methanol (6:1) in which was dissolved CH_3ONa catalyst making 1% of the solution volume. Sample tubes were left in a shaker incubator (Kem lab ®) reactor for 2 hours at 60°C with continuous shaking and were next kept for cooling. After cooling, dichloromethane and a saturated solution of NaCl (2:1) were added and mixtures were vortexed. This allowed the separation of the medium into two layers, the lower layer being the dichloromethane part containing the fatty acid methyl ester obtained during reaction. The upper layer was discarded while the lower was washed for three times by distilled water enriched with NaCl (2:1) to remove any remaining byproduct obtained during the reaction. After washing, MgSO_4 was added to the organic dichloromethane layer in order to remove moisture. After filtering the solution with Whatmann n°1 filter, dichloromethane layer was then moved into a newly pre-weighed vial and was subjected to evaporation under vacuum in a rotary evaporator. GC-MS was used for an efficient separation, identification and quantification of the particular fatty acid methyl ester present in the sample.

The injector of the GC was set at a temperature of 260°C. 1 µL 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 at a rate of 30cm / sec (0.54 ml/ min). Initial column temperature was set at 50°C for 2 minutes, then temperature was increased to 220°C at a rate of 4°C / min. Temperatures of the transfer line as well as of the MS were set at 260°C.

3. Pigment analysis

To 20 mg of dried algae, 5ml 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 40°C and then freeze dried overnight. Ten milliliters 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 and 3ml of the extracted pigments were used to measure the absorbance at fixed wavelengths specific for each solvent [38,39].

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: Porra and Lichtenthaler, 1989; Jeffrey and Humphrey, 1975; Strickland and Parsons, 1968 [39].

Calculations of pigment concentrations were obtained based on the following formulas [39].

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

Jeffrey and Humphrey (1975)

$$\mu\text{g Chlorophyll/ ml medium} = (11.85A_{664} - 1.54A_{647} - 0.08A_{630}) v / (lV)^4$$

Strickland and Parsons (1968)

$$\mu\text{g Chlorophyll/ ml medium} = (11.66A_{665} - 1.31A_{645} - 0.14A_{630}) v / (lV)^5$$

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

Porra et al. (1989)

$$\mu\text{g Chlorophyll/ ml medium} = (16.29A_{665} - 8.54A_{652}) v / (lV)^6$$

Lichtenthaler (1989)

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

In order to obtain the chlorophyll composition following acetone extraction, formulas of Parsons and Strickland (1968) were followed [74],

For chlorophyll a, b and c,

^{4,5,6}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).

*A: Absorbance (nm)

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

$$\text{mg chl b/ m}^3 = 20.7 A_{645} - 4.34 A_{665} - 4.42 A_{630}^*$$

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

4 .Protein analysis

Protein content was determined based on two methods: The classic Kjeldahl and Digestdahl. For conversion of % nitrogen into % protein, a conversion factor of 5.95 was used instead of 6.25 normally used for plant proteins [77].

a. Kjeldahl method

0.3 g of dried microalgae were weighed on a nitrogen free filter paper and placed in digestion flask to which 50ml of concentrated sulfuric acid and catalyst⁷ were added. The content of the digestion tube was boiled for about 60 minutes until observing a green color. Tubes were left to cool for 5 minutes and then 50ml of distilled water were gently added. This is known as the digestion step. In the second step known as distillation, about 60 ml of NaOH (0.122N) were added to the digested sample to allow the separation of nitrogen from the mixture and obtain ammonia which was trapped in 50ml boric acid. To quantify the amount of ammonia trapped, titration was performed. The solution was back titrated with 0.001 N sulfuric acid until a color change was recorded. The volume of acid titrated was placed in a formula to measure the crude protein content.

⁷ K315-1000 Fisher Tab TT-35 Kjeldahl Tablets: 94 % K₂SO₄, 2% TiO₂, N<0.005%, the rest is filler.

*A: Absorbance (nm)

% Protein

$$= \frac{[(ml \text{ standard acid} - ml \text{ blank}) \times NH_2SO_4 \times 14 \times \text{correction factor} \times 100]}{\text{Dry weight of the sample} \times 1000}$$

b. Digestdahl method

The sample (0.1 g) was weighed and transferred to a flat – bottom volumetric flask to which 4 ml of concentrated sulfuric acid were added. The sample was then heated for 5 to 6 minutes at 440⁰C using the Digestdahl apparatus. Next, 10ml of 50% hydrogen peroxide were added and the sample was heated for one more minute after all hydrogen peroxide has entered the digestion flask. Digestion flask was removed from the apparatus and the content diluted to 100ml using double distilled water, then mixed gently. 500 μ L were then transferred to 25ml cylinders to which was added 1 drop of TKN⁸ indicator , few drops of 1 N KOH, 3 drops of mineral stabilizer⁹, 3 drops of polyvinyl alcohol dispersing agent¹⁰. Mixing was performed after each addition. Cylinders were filled till 25ml with double distilled water and 1ml of Nessler reagent¹¹ was added, and solution mixed. The reaction was allowed to rest for 2 minutes and O.D. readings were taken spectrophotometrically (at a wavelength of 460nm) using HACH spectrophotometer using the 399 Nitrogen TKN program.

⁸ Indicator solution for pH adjustment in Total Kjeldahl Nitrogen determination by the Nessler Method.

⁹ For determination of Ammonia and Total Kjeldahl Nitrogen by the Nessler Method. 50 mL Self-Contained Dropping Bottle.

¹⁰ For determination of Ammonia and Total Kjeldahl Nitrogen by the Nessler Method. 50 mL Self-Contained Dropping Bottle.

¹¹ For ammonia nitrogen determination by the Nessler method (Hach method 8038). EPA accepted for wastewater analysis (distillation required). Range: 0.02-2.50 mg/L NH₃-N. 500 mL bottle.

Calculation for the nitrogen content of microalgae was obtained following the equation below [77, 86]

$$\frac{A \times 75}{B \times C} = \text{mg/L TKN}^{12}.$$

The TKN figure obtained was adjusted by multiplying by a correction factor of 1.0739 obtained from calibration of Digesdahl apparatus using glycine as a standard reference. For calculation of the protein content, a conversion factor of 5.95 was used as reported for microalgal proteins [77].

D. Antimycotic activity

In in-vitro experiment, four microalgae were tested on four fungi, each one having four treatments and three replicates per treatment. For in-vivo experiments, there were four treatments, five replicates each. The experiment was repeated three times at monthly intervals under controlled conditions and results of two trials were reported.

1. In- vitro experiments

a. Plant pathogens

The four plant pathogenic fungi used in this study were isolated at the American University of Beirut from infected crops (Table 4).

¹²Method 8075, Where A = mg/L reading from instrument, B = g sample amount from table, C = mL analysis volume from table.

Table 4. Plant pathogenic fungi used in this study.

Plant pathogen	Source
<i>Alternaria solani</i>	Tomato
<i>Botrytis cinerea</i>	Tomato
<i>Fusarium oxysporum</i>	Eucalyptus
<i>Rhizoctonia solani</i>	Potato

b. Tested microalgae

All microalgae used in this study were isolated from the Lebanese coastal region (Table 5).

Table 5. Microalgae species used in this study.

Isolate	Isolate Acronym
<i>Tetraselmis marina</i>	Khd
<i>Chloroidium saccharophilum</i>	Ozd
<i>Amphora</i> sp.	Jid
<i>Micractinium</i> sp	Jbd

c. Extracts

Erlenmeyer flasks (2L) were half filled with sterile enriched sea water and inoculated with 100 ml of microalgae. In the mid to late exponential phase (almost 10-14

days) the microalgae were extracted in three different methods. Four types of extracts were obtained.

i. Methanolic extract

A sample of 60 mg of freeze dried algae was extracted twice with 6 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 re-suspended in 2ml of 99.9% ethanol. Water agar was prepared at 1.5% (w/v) and poured into 70 mm petri dishes at 10ml/plate. Shortly after solidification; a 20 μ l volume of the methanolic extract was applied and spread uniformly at the center of the petri dish. The plates were inoculated at their centers with 100 μ l of 10⁶ spore suspension.

Un-amended water agar plates and plates amended with 99.9% ethanol, inoculated with 10⁶ spore suspensions served as controls. All plates were incubated at 28 \pm 0.5 $^{\circ}$ C.

ii. Non methanolic water soluble extract

Microalgal cells that were not dissolved in methanol were collected and dissolved in previously autoclaved and cooled distilled water (2ml volume was used). The filtrate was added directly to the center of water agar 1.5% (w/v) and then spread on its surface by a clean loop. Later, all plates were inoculated at their centers with a volume of 20 μ l of 10⁶ spore suspensions and were incubated at 28 \pm 0.5 $^{\circ}$ C.

iii. Crude water extract

250 ml of the cultures were centrifuged at 5000 rpm for 7 minutes. Algal cells that rested at the bottom of tubes were collected and dissolved in 250 ml of distilled water to which water soluble agar 1.5% (w/v) was added. The mixture was autoclaved for 5 minutes at 121⁰C and then poured in petri dishes at 10ml/plate. Corresponding pathogens were inoculated at the middle of each petri-dish at a rate of 10⁶ spores and incubated at 28± 0.5°C.

iv. Culture filtrates

Water collected from the centrifuged cultures was heated to 40°C and then added to autoclaved agar (1:1, v/v) and poured in petri-dishes (10ml/plate). Corresponding pathogens were inoculated at the middle of each petri-dish at a rate of 10⁶ spores and incubated at 28± 0.5°C. Four and eight days post inoculation (dpi), the antimycotic activity of each of the four microalgal extracts was recorded, based on the diameter expansion of each fungus tested.

2 .In-vivo assays

The aim of this experiment was to test the capacity of microalgal extracts to induce resistance of cucumber seedlings against infection by the powdery mildew fungus attacking plants.

a. Crop maintenance

Beit Alpha cucumber seeds were chosen because of their susceptibility to powdery mildew infection. They were first planted in trays and then transplanted to 30 cm diameter pots. When seedlings reached 3-4 leaf stage, 20-20-20 fertilizers were applied once weekly at 5g per 20L of water.

At the 4- 5 leaf stage, seedlings were sprayed with microalgal extracts. A week later, they were inoculated with powdery mildew. Plants were periodically observed for development of symptoms. Readings regarding the percentage of leaf area infected with powdery mildew were taken at seven to ten days post inoculation (dpi). A second microalgal spray took place 20 days after the first spray. A week after the second spray, % leaf area infected was recorded.

b. Plant pathogen

The powdery mildew fungus *Sphaerotheca fuliginea* was obtained from local infected areas and maintained on cucumber plants by periodic transfer to new plants. The transfer of the fungus occurred by tapping an infected leaf showing adequate fungal sporulation over leaves of the seedling to be inoculated. Newly inoculated plants were placed in humid conditions for 24 hours. The greenhouse compartment where cucumber seedlings were placed was environmentally controlled at a temperature range between 16-30°C (± 2) to allow multiplication of the fungus. Once powdery mildew inoculum was high, new cucumber seedlings dedicated for spray with microalgal extracts were inoculated by tapping. Source leaves were shaken 24 hours before harvest of the conidia to dislodge old conidia and ensure high viability of the inoculum. The conidia were

inoculated onto the adaxial surfaces of the leaves seven days after spraying the seedlings with the corresponding microalgal extract. The inoculated plants were then incubated in the greenhouse. When symptoms of powdery mildew became visible, the percentage leaf area covered by the fungus was measured using APS Assess Software.

c. Microalgal Extracts

The following four treatments were evaluated: methanolic extract, non methanolic extract and two controls, distilled water and 10% ethanol.

Each treatment had 5 replicates. In the first spray, each replicate was sprayed with 5ml of one of the extracts on both the adaxial and abaxial leaf surfaces. While in the second spray, 10ml volume was sprayed on each crop.

i. Methanolic extract

A sample of 1g of freeze dried algae was extracted twice in 30 ml of methanol for 24 hrs at room temperature. The extract was separated from the cell residue by filtration through Whatman filter paper no.1. The filtrate was then dried in a rotary evaporator. The dried extract was re-suspended in 10ml of 99.9% ethanol. Distilled water was added to the volumetric flask containing the extract until reaching the 100 ml mark line. This makes a 10% ethanol soluble extract.

ii. Non methanol water soluble extract

Microalgal cells captured on the filter were collected and mixed in 100ml autoclaved distilled water. The extract was used to spray the seedlings at five to ten ml volume depending on the crop leaf stage.

E. Statistical Analysis

Statistical analyses were conducted using SPSS 21 Software. One way ANOVA was performed to analyze data from in-vivo and in-vitro. Variation among means was investigated using the Tukey's HSD test ($P < 0.05$).

CHAPTER IV

RESULTS

A. Isolations

Several sea water samples were collected along the Lebanese coast. Blooms took some time to appear this varied between samples and ranged between few days to more than two months. Once clear blooms were observed, isolations on petri dishes allowed the growth of several algal colonies. Further purification of cultures from bacterial and fungal colonies occurred by re- streaking on new agar plates or by serial dilution. The whole process of microalgal colony purification required about two to three months. Later on, clean and pure colonies were transferred to 5 ml nutrient enriched sea water placed in 10ml plastic tubes from which scaling up began.

Nine isolates and two mixed cultures were successfully purified and observed microscopically under magnification 400x and 1000x. Below are their morphological appearances under the microscope.

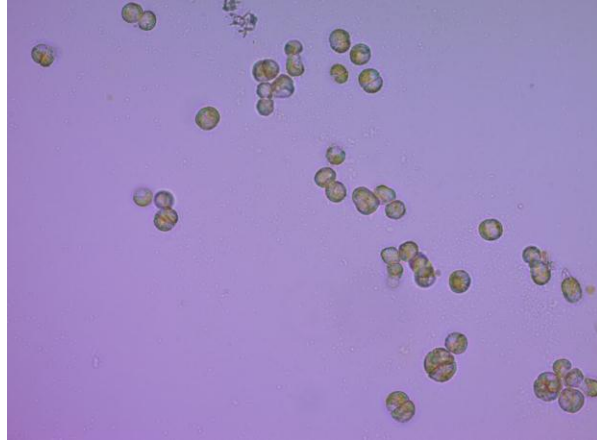


Figure 9. Microscopic view of isolate Aqd magnified 1000x (Not identified, 4.9-7.8 μ m)

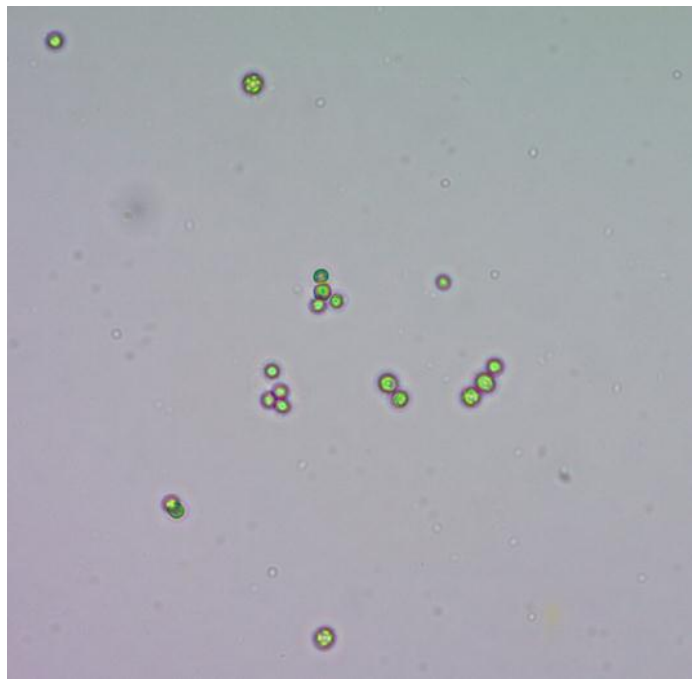


Figure 10. Microscopic view of Dad isolate magnified 400x (*Chlorella variabilis*, 2-2.4 μ m)

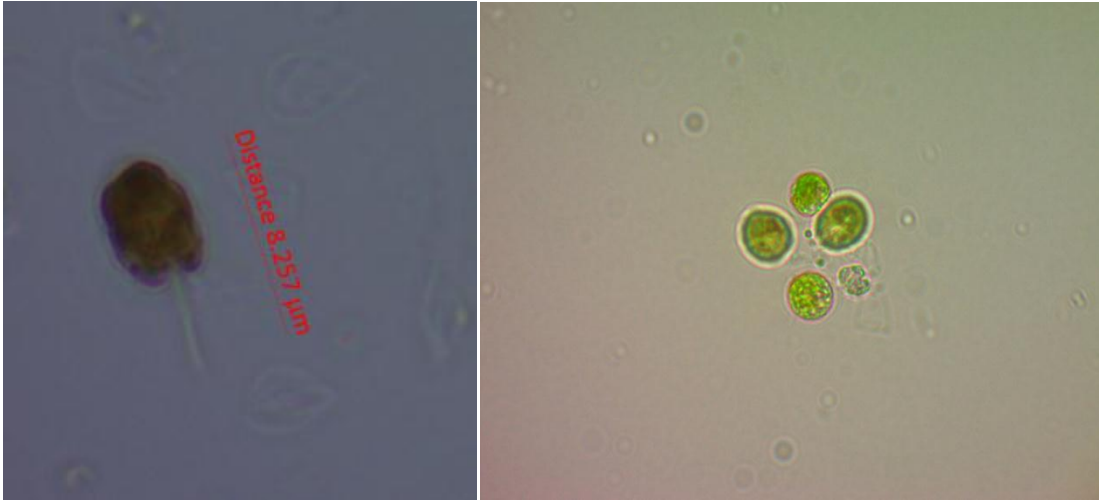


Figure 11. Microscopic view of Had isolate magnified 1000x (*Tetraselmis marina*, 8.3μm)

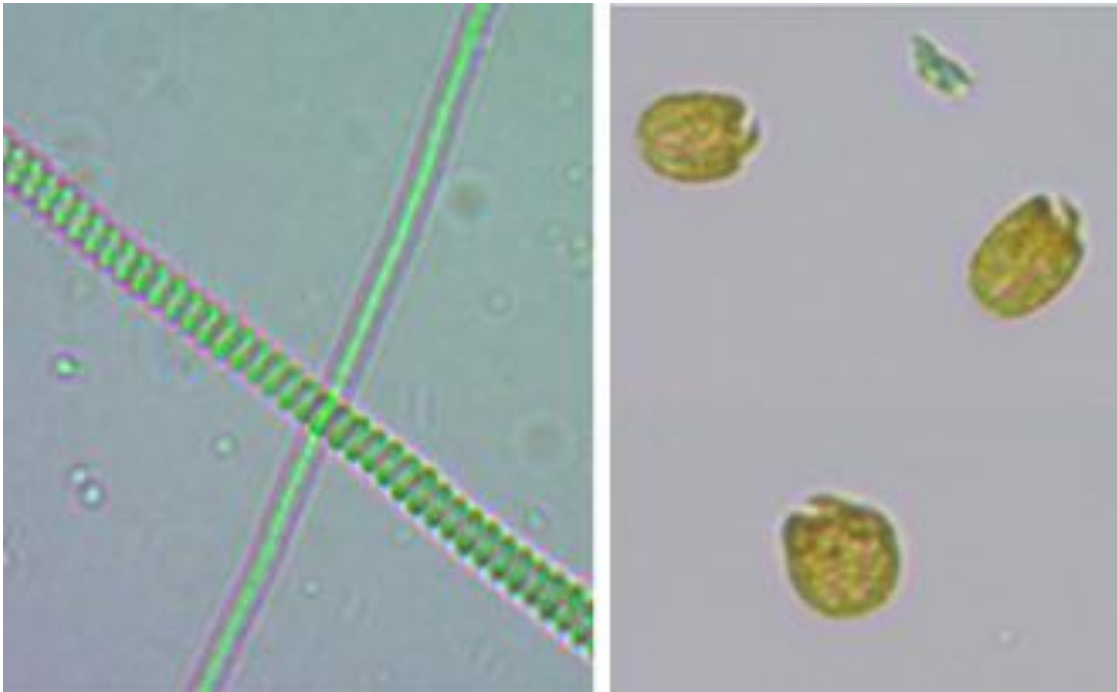


Figure 12. Microscopic observation of Had2 mixed bloom magnified 1000x showing diatom (*Amphidinium carterae*, 10x5μm) and two filamentous cyanobacteria (*Spirulina* sp., 1.2 μm).

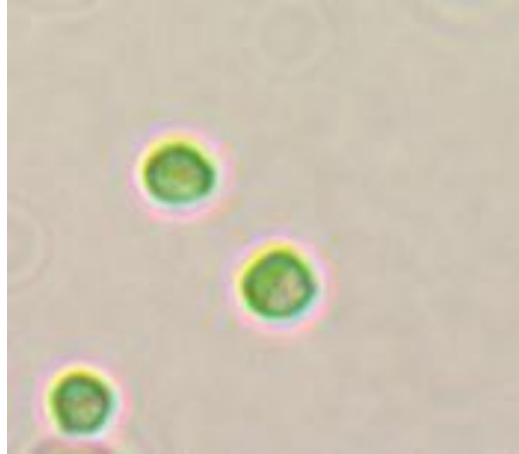


Figure 13. Microscopic observation of Jbd isolate magnified 1000x (*Micractinium* sp, 3.5 μ m)

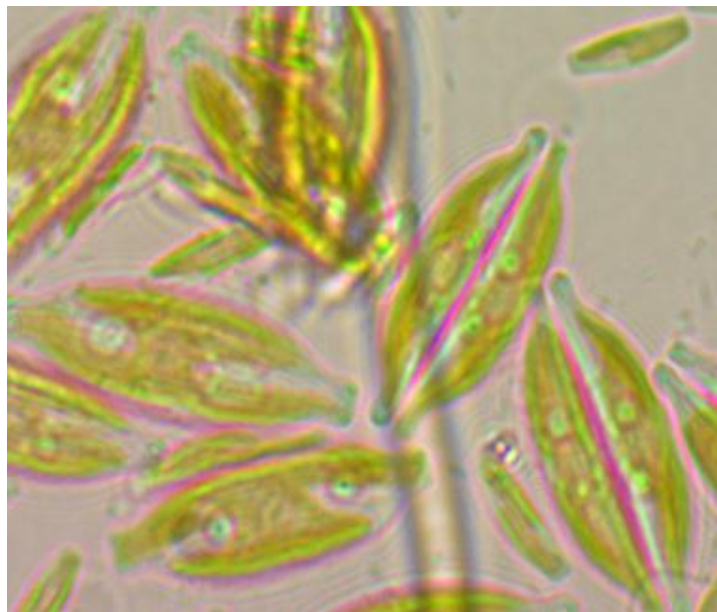


Figure 14. Microscopic observation of Jid isolate magnified 1000x (*Amphora coffeaeformis*, 13x3.5 μ m)



Figure 15. Microscopic observation of Jod isolate magnified 1000x (*Micractinium reisseri*, 3µm)



Figure 16. Microscopic view of Khd tube isolate magnified 1000x (*Tetraselmis marina*, 5x3.5µm)

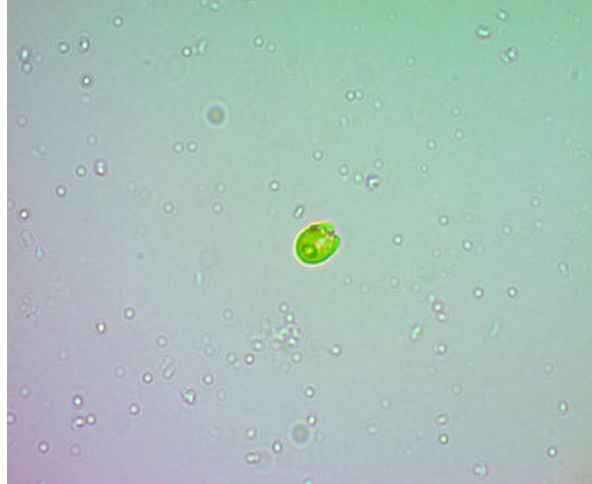


Figure 17. Microscopic observation of Khd plate isolate magnified 1000x (*Tetraselmis marina*, 5x3.5 μ m)

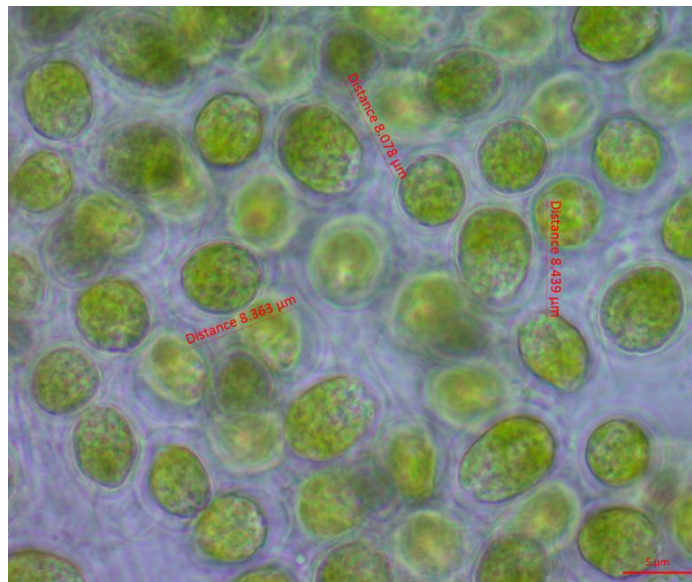


Figure 18. Microscopic observation of Ozd green isolate magnified 1000x (*Chloroidium saccharophilum*, 8.35 μ m)

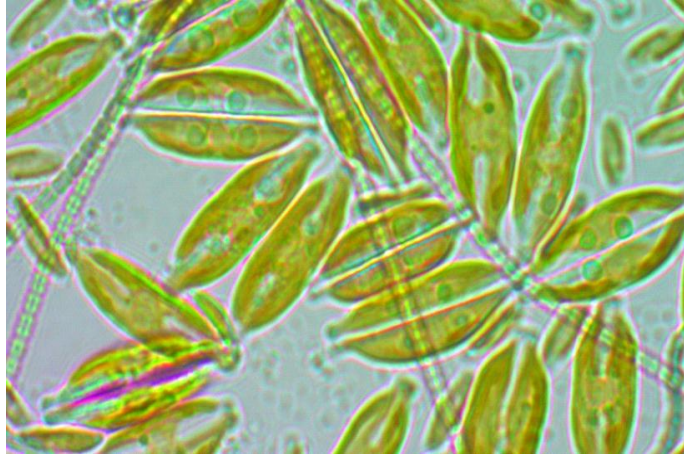


Figure 19. Microscopic observation of Ozd Red mixed bloom showing a diatom (*Amphora coffeaeformis*, 13x3.5µm) and a filamentous cyanobacterium (*Leptolyngbya sp.*, 1.2 µm).

B. Molecular identifications

1. Molecular Detection

The microalgal isolates were identified using primary (taxon specific) and secondary (universal) barcode markers. One universal primer was used while the three remaining were taxon specific.

The universal primer pair T16N/ T24U was used in PCR to amplify a partial region of The LSU from the eleven isolates collected. The size of the observed amplicons was different from the expected size of 567 bp. Instead, our amplicons ranged between 700bp and 750bp with an additional nonspecific amplicon (1000bp) from Ozd isolate (Figure 20).

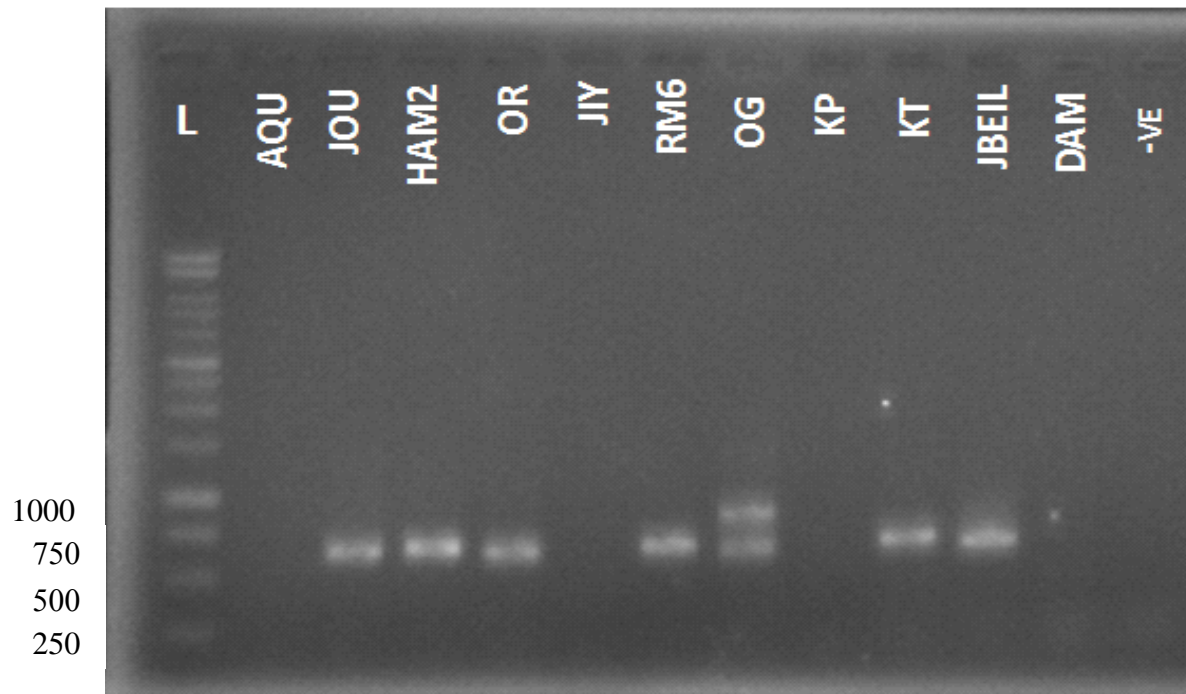


Figure 20. UV visualization in 0.7% agarose gel following electrophoresis of PCR amplified products using the universal primers T16N and T24U to amplify nuclear D2/D3 region of the large ribosomal subunit (LSU D2/D3). L: 1 Kbp molecular ladder, AQU to DAM: differ

The taxon specific primers *tufGF4* and *tufAR* were used in PCR to amplify a 807bp sequence from the *tuf A* gene from DNA extracts of the 11 microalgal isolates (Figure 21). Positive results were obtained in all isolates tested with an amplicon size of 750bp close to the expected size (807bp).

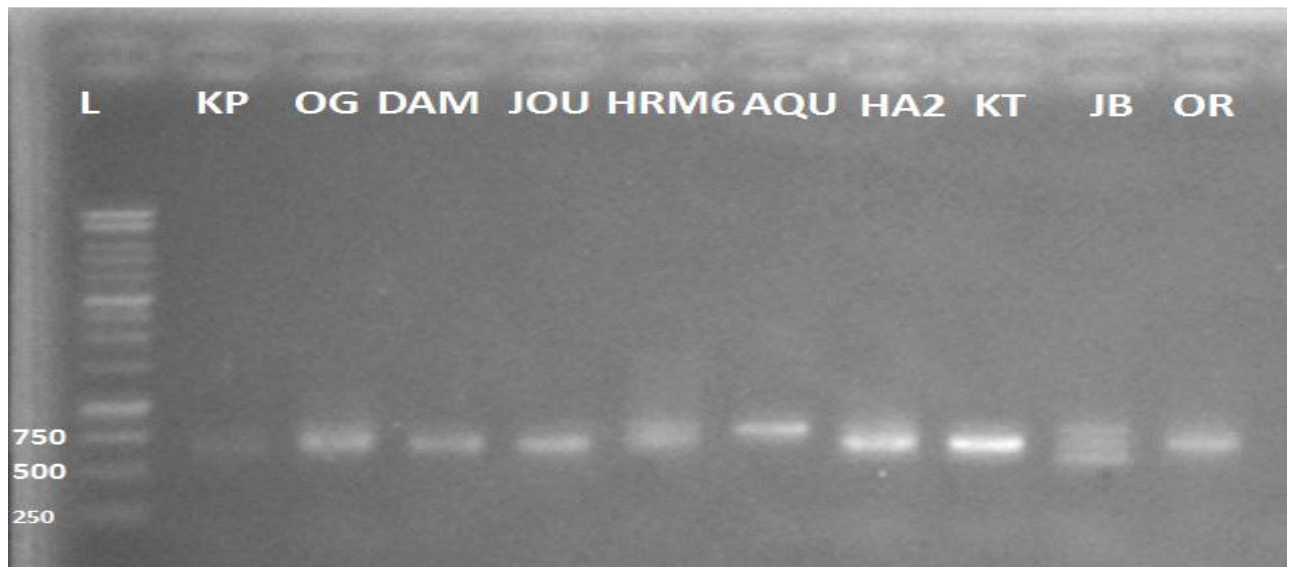


Figure 21. UV visualization in 0.7% agarose gel following electrophoresis of PCR amplified products using the taxon specific primers *tufGF4* and *tufAR* to amplify gene *tufA* that encodes the elongation factor Tu. L: 1 Kbp molecular ladder, KP to OR: different microalgae

All DNA extracts of the isolates were also tested with the chlorophyta taxon specific primers ITS1 and ITS4 amplifying the internal transcribed spacer of their ribosomal cistron. Considerable variation in amplicon sizes were observed depending on the isolates tested. They ranged between 600bp and 1000bp (Figure 22).

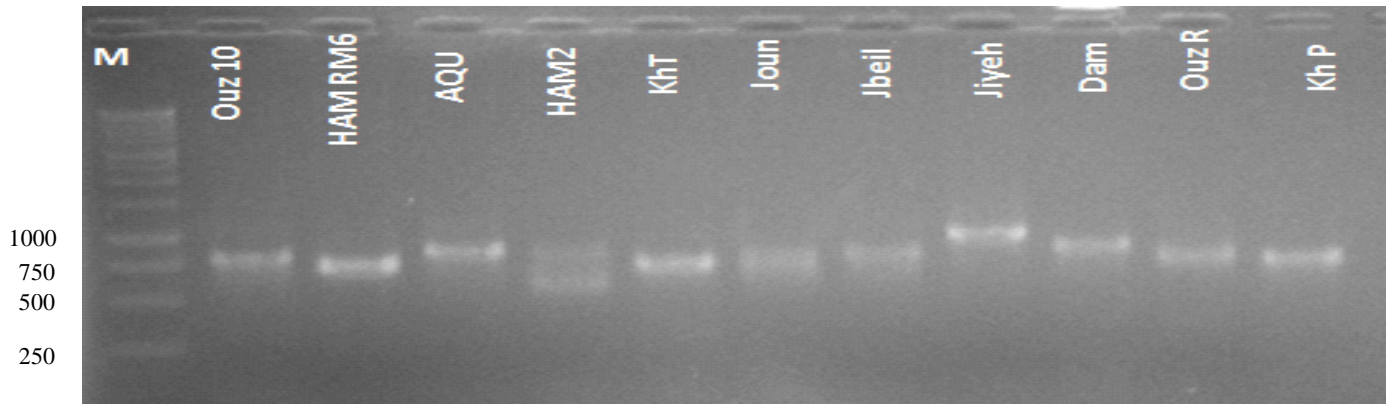


Figure 22. UV visualization in 0.7% agarose gel following electrophoresis of PCR amplified products using the taxon specific primers ITS1 and ITS4 to amplify internal transcribed spacer of the ribosomal cistron. M: 1 Kbp molecular ladder, Ouz10 to KhP: different.

The taxon specific primers for red and brown microalgae GWSRx and GWSFn which amplify the mitochondrial cytochrome c oxidase 1 (COI-5P) gene gave amplicons of about 500bp with the two isolates tested (Figure 23).

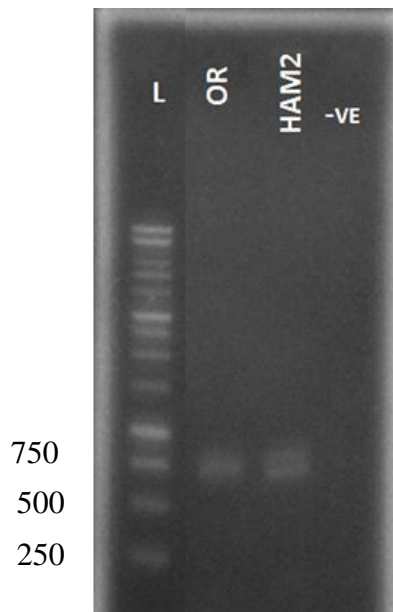


Figure 23. UV visualization in 1% agarose gel following electrophoresis of PCR amplified products using the taxon specific primers GWSRx and GWSFn for the mitochondrial cytochrome c oxidase 1 gene. L: 1 Kbp molecular ladder, OR and HAM2: different microalgal isolates.

2. Sequence Analysis

Sequencing success rates from the PCR purified amplicons were high in the case of the LSU marker with a pairwise identity ranging between 93 and 99% in all isolates (Table 6). However, the success rate with the two other genomic markers was moderate to low. Among the eleven locally purified microalgal isolates, seven were sequenced with one barcode marker, two with two barcode markers and two with three barcode markers (Table 7)

Table 6. 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	Marker					
		LSU		ITS		Tuf A	
		Max. score	Pairwise Identity (%)	Max. score	Pairwise Identity (%)	Max. score	Pairwise Identity (%)
Dad	<i>Chlorella variabilis</i>	966	96	-	-	-	-
Had 2	<i>Amphidinium carterae</i>	1075	99	809	100	-	-
Had	<i>Tetraselmis marina</i>	1033	99	593	87	1070	99
Jbd	<i>Micractinium</i> sp???	592	-	-	-	-	-
Jod	<i>Micractinium reisseri</i>	911	95	-	-	-	-
Khdt	<i>Tetraselmis marina</i>	1000	97	614	84		
Khdp	<i>Tetraselmis marina</i>	1059	97	628	85	1100	99
Ozd green	<i>Chloroidium saccharophilum</i>	907	95	-	-	-	-
Ozd Red	<i>Leptolyngbya</i> sp.	-	-	-	-	881	86
Ozd Red	<i>Amphora coffeaeformis</i>	745	94	-	-	-	-
Jid	<i>Amphora coffeaeformis</i>	677	93	-	-	-	-

Table 7. Taxonomic groups of microalgae isolates identified in this study.

Acr.	Kingdom	Phylum	Class	Order	Family	Genus	Species	Genomic region
Had ₂	Chromista	Dinophyta	Dinophyceae	Gymnodiniales	Gymnodiniaceae	<i>Amphidinium</i>	<i>carterae</i>	LSU-ITS
Had	Plantae	Chlorophyta	Chlorodendrophyceae	Chlorodendrales	Chlorodendraceae	<i>Tetraselmis</i>	<i>marina</i>	LSU-ITS-tuf A
Dad	Plantae	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	<i>Chlorella</i>	<i>variabilis</i>	LSU
Jbd	Plantae	Chlorophyta	<u>Trebouxiophyceae</u>	Chlorellales	Chlorellaceae	<i>Micractinium</i>	<i>reisseri</i>	LSU
Jod	Plantae	Chlorophyta	<u>Trebouxiophyceae</u>	Chlorellales	Chlorellaceae	<i>Micractinium</i>	<i>reisseri</i>	LSU
Ozd green	Plantae	Chlorophyta	Trebouxiophyceae	Chlorellales	Oocystaceae	<i>Chloroidium</i>	<i>saccharophilum</i>	LSU
Ozd red	Eubacteria	Cyanobacteria	Cyanophyceae	Oscillatoriales	Oscillatoriothricaceae	<i>Leptolyngbya</i>	<i>sp</i>	Tuf A
Ozd red	Chromista	Ochrophyta	Bacillariophyceae	Thalassiophysales	Catenulaceae	<i>Amphora</i>	<i>coffeaformis</i>	LSU
Jid	Chromista	Ochrophyta	Bacillariophyceae	Thalassiophysales	Catenulaceae	<i>Amphora</i>	<i>coffeaformis</i>	LSU
Khd	Plantae	Chlorophyta	Chlorodendrophyceae	Chlorodendrales	Chlorodendraceae	<i>Tetraselmis</i>	<i>marina</i>	LSU-ITS-tuf A

C. Growth kinetics

Experiments on growth kinetics were conducted for four isolates: Jbd (Figure 24), Had (Figure 26), Jod (Figure 28), and Ozd (Figure 30). Below are graphs representing readings of optical density taken at 560nm for a period of time. From previous experience, the lag phase may be reduced if a more concentrated initial inoculum is used to reach an $A_{560\text{nm}}$ reading between 0.05 and 0.1.

After an initial lag phase of about two days, exponential growth started and lasted for a period of two to four days in three out of four isolates (Figures 24, 26, 30). On the other hand, Jod isolate had a very short exponential phase of one day (Figure 28). The relatively high initial growth rate of Had, Jod, and Jbd was reflected by their specific exponential growth rate ($\mu_{\text{max}}/\text{d}$) 1.58, 1.50, 1.46, respectively (Figures 27, 29 and 25) as compared to μ_{max} of 0.885 for Ozd (Figure 31). The highest optical density readings were obtained with the Had isolate with $A_{560\text{nm}}$ value of 0.8 corresponding to about 4×10^6 cells and a dry matter of 406 mg/ml in the stationary phase (Table 8) while the lowest reading was recorded with Jbd isolate which reached an A_{560} of 0.2 with 0.8×10^6 cells/ml and a dry matter of 445mg /ml in the stationary phase however, since growth stopped at this O.D., the number of cells declined to 0.45×10^6 cells/ml after few days.

At an O.D. of $A_{560\text{nm}}$ reading close to 0.4, the number of cells reached close to 3×10^6 cells/ml in the Ozd and Had isolates. In the Jod isolate, the cell number recorded at the same absorbance was about 5×10^5 cells/ml and reached later 2.5×10^6 cells/ml at an O.D. of 0.55.

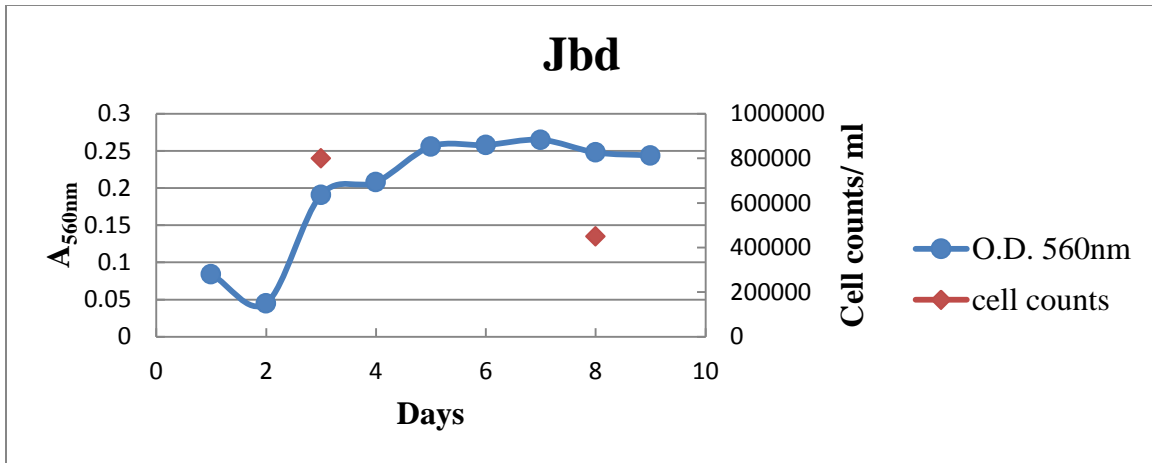


Figure 24. Growth kinetics of Jbd isolate as measured by optical density readings (A_{560nm}). The number of cells/ml is provided at specific dates and O.D. values.

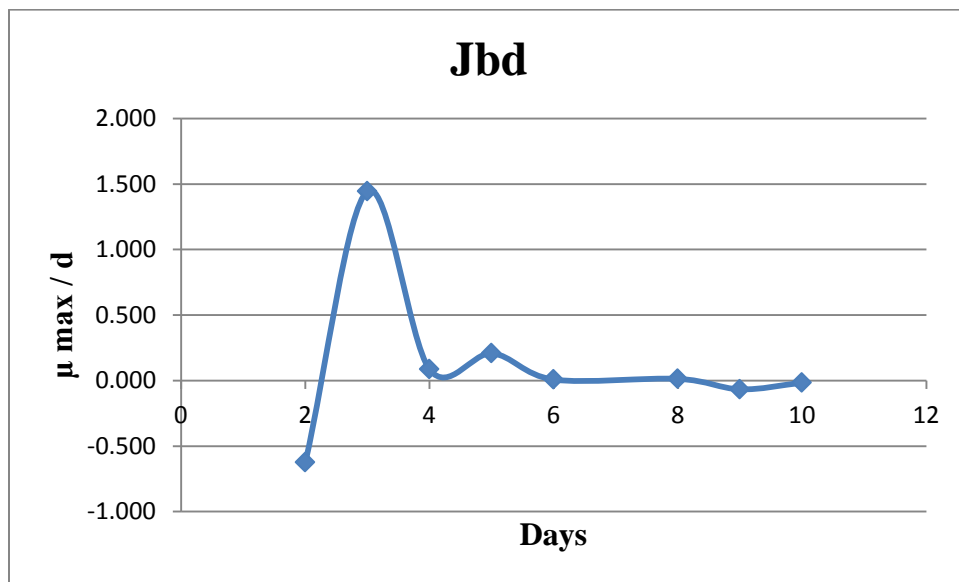


Figure 25. Daily exponential specific growth rate ($\mu_{max} d^{-1}$) of Jbd isolate.

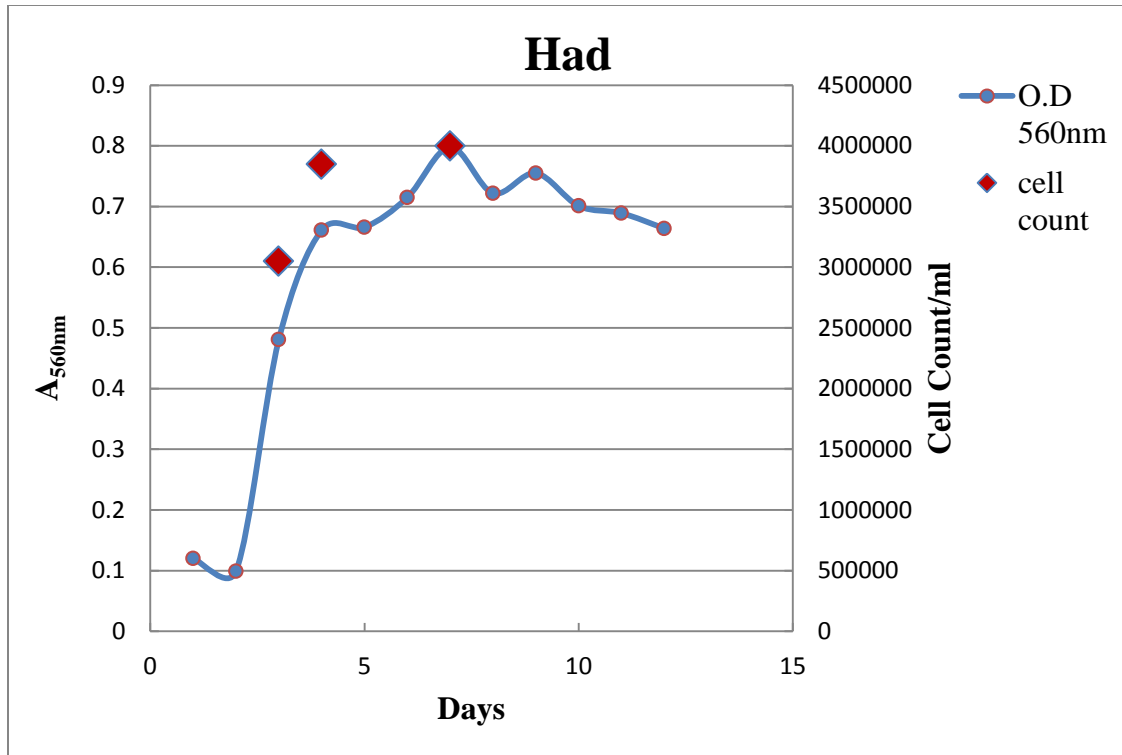


Figure 26. Growth kinetics of Had isolate as measured by optical density readings (A_{560nm}). The number of cells/ml is provided at specific dates and O.D. values.

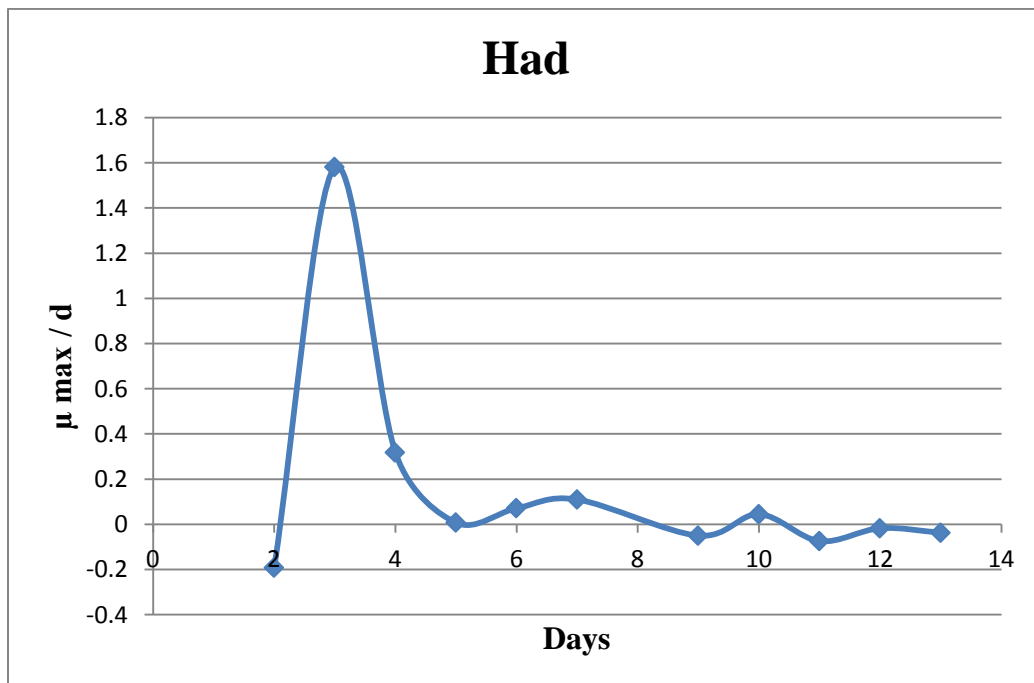


Figure 27. Daily exponential specific growth rate ($\mu_{max} d^{-1}$) of Had isolate.

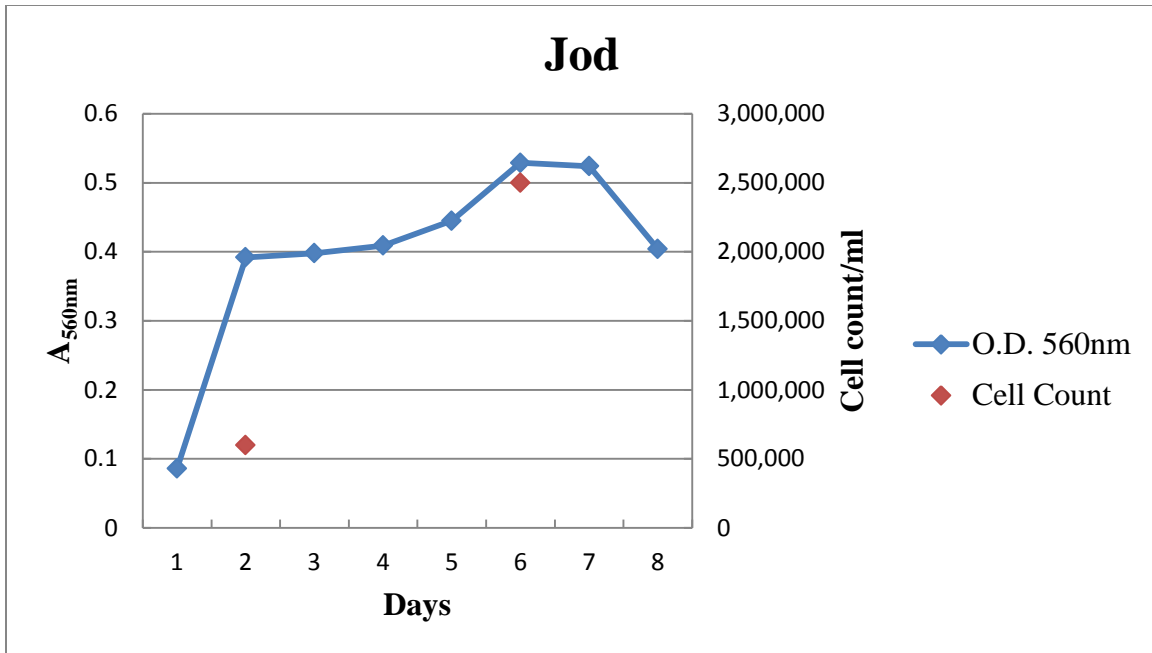


Figure 28. Growth kinetics of Jod isolate as measured by optical density readings (A_{560nm}). The number of cells/ml is provided at specific dates and O.D. values.

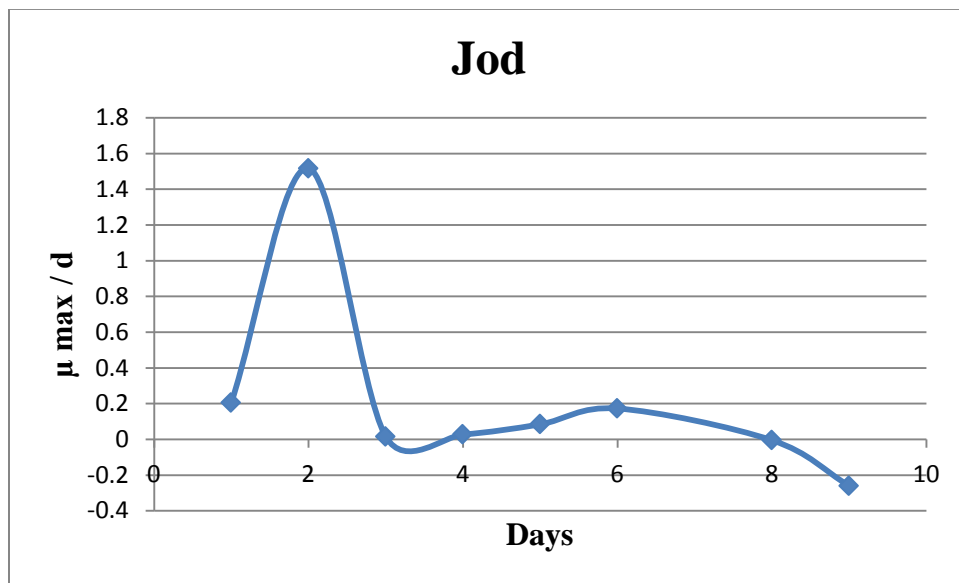


Figure 29. Daily exponential specific growth rate ($\mu_{max} d^{-1}$) of Jod isolate.

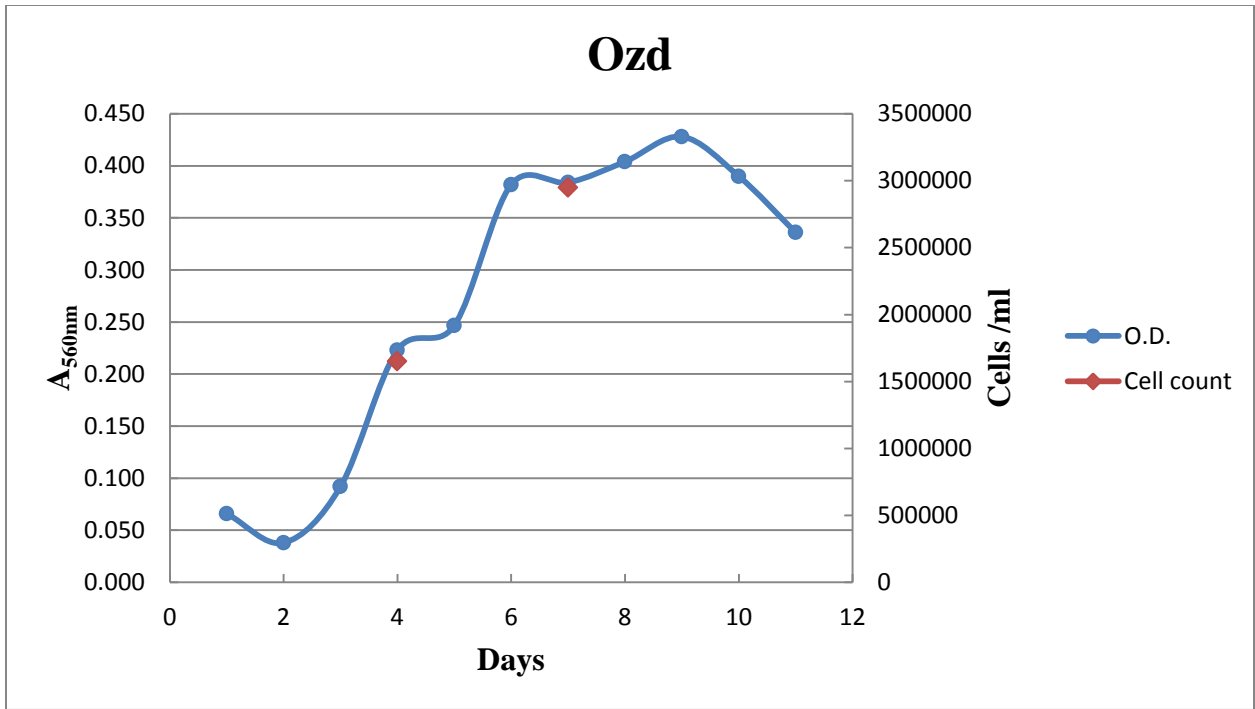


Figure30 . Growth kinetics of Ozd isolate as measured by optical density readings (A_{560nm}). The number of cells/ml is provided at specific dates and O.D. values.

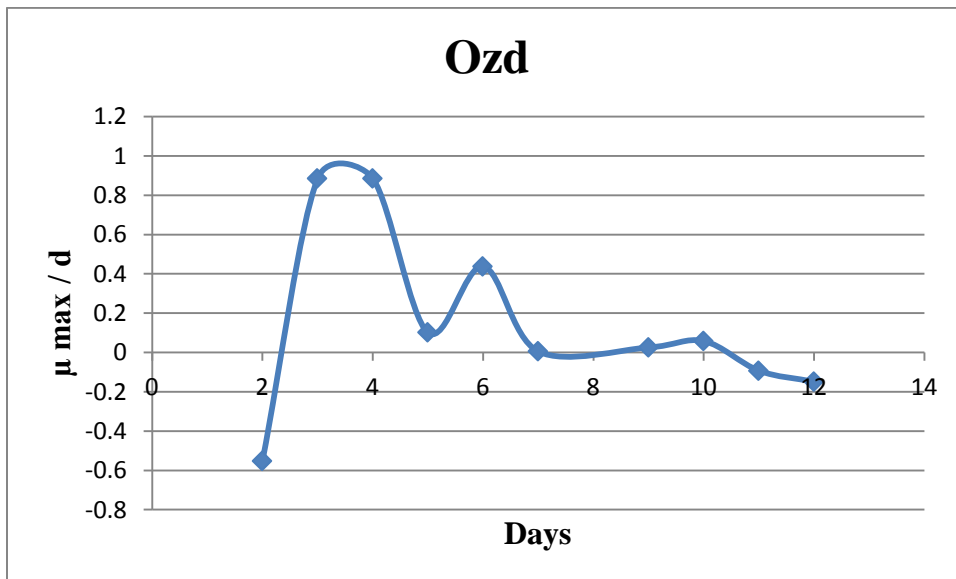


Figure 31. Daily exponential specific growth rate ($\mu_{max} d^{-1}$) of Ozd isolate.

Table 8. Dry weights of four microalgal isolates at corresponding optical densities during exponential and stationary growth phases.

Isolate	Exponential phase		Stationary phase	
	A 560	(mg / L)	A 560	(mg / L)
Jbd	0.191	42.8	0.244	445
Jod	0.392	62	0.404	443
Had	0.481	129.8	0.664	406
Ozd	0.247	104.4	0.336	495

D. Lipid and fatty acids content

The gravimetric determination method was used to determine the percent lipid content based on dry weight for different microalgal species. Saturated and unsaturated fatty acids were also determined for each isolate (Table 9). The highest total lipid content was recorded during the stationary phase. It reached about 16% in Jbd and Had isolates and about 14.5% in Jod and Ozd isolates. In the exponential phase, the lipid content was 1-2% lower than as compared to the stationary phase (Table 9, Figure 32, Figure 33). The proportions of saturated and monounsaturated FAs were greater in the stationary phase of the growth period as whilst polyunsaturated FA were considerably more in microalgae during the exponential phase of growth.

Table 9. Total lipid content (%) and percentage saturated and unsaturated FA in four local microalgal isolates measured at two growth stages.

Phase	Isolate	O.D./ no. of days	Total Lipid Content (%DW)	%Saturated FA	%Unsaturated fatty acid			
					Total	Mono	Di	Poly
Exponential	Jbd	0.191/3	14*	27.98	70.61	36.28	7.95	26.38
	Jod	0.398/3	12.17*	33.14	66.85	35.87	6.67	24.31
	Had	0.661/4	15*	31.24	67.49	37.68	4.81	25
	Ozd	0.247/5	13.33*	30.71	68.19	29.68	14.39	24.12
Stationary	Jbd	0.244/9	16.5*	34.8	65.19	48.25	6.59	10.35
	Jod	0.404/8	14.63*	31.92	68.07	38.32	8.87	20.88
	Had	0.710/10	16.03*	36.25	62.84	45.25	5.72	11.87
	Ozd	0.336/11	14.53*	35.89	64.13	47.16	7.5	9.47

* Average of three replicates.

In all four microalgal isolates, two types of omega FA were detected: omega 3 and 6. In the exponential phase, the content of omega 3 was greater than that of omega 6 with proportions of total lipid content ranging between 22.51 and 24.1% for omega 3 as compared to 3-14% for omega 6 (Table 10).

In the stationary phase, levels of omega 3 dropped and ranged between 8 and 17% while the concentration of omega 6 varied between 6.88 and 9.68% of total lipids. The highest content of omega FA (both 3 and 6) was detected in the Ozd isolate during the exponential growth and reach 38.5% of total lipids. While during the stationary phase the Jod isolate showed the highest total content of FA (Table 10).

Among the four tested isolates, Jbd, Jod and Had had a biodiesel production of 49.84, 42.8 and 48.72 mg/liter of media culture respectively while Ozd had a biodiesel production of 52.6mg/liter of media culture (Figure 33).

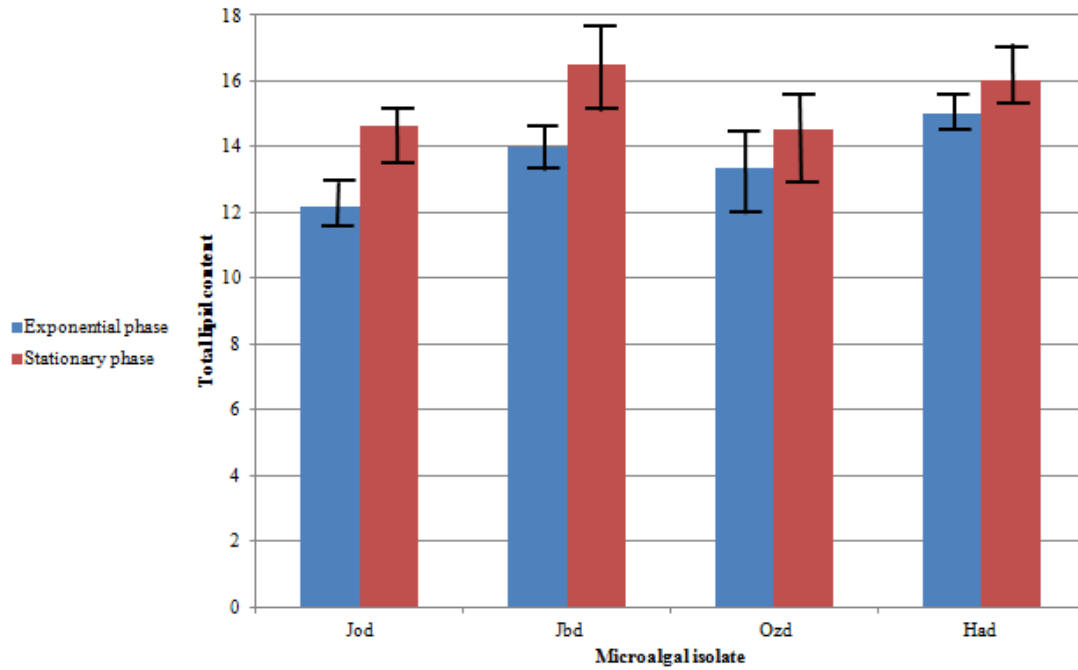


Figure 32. Total lipid content of microalgal isolates in both exponential and stationary phases.

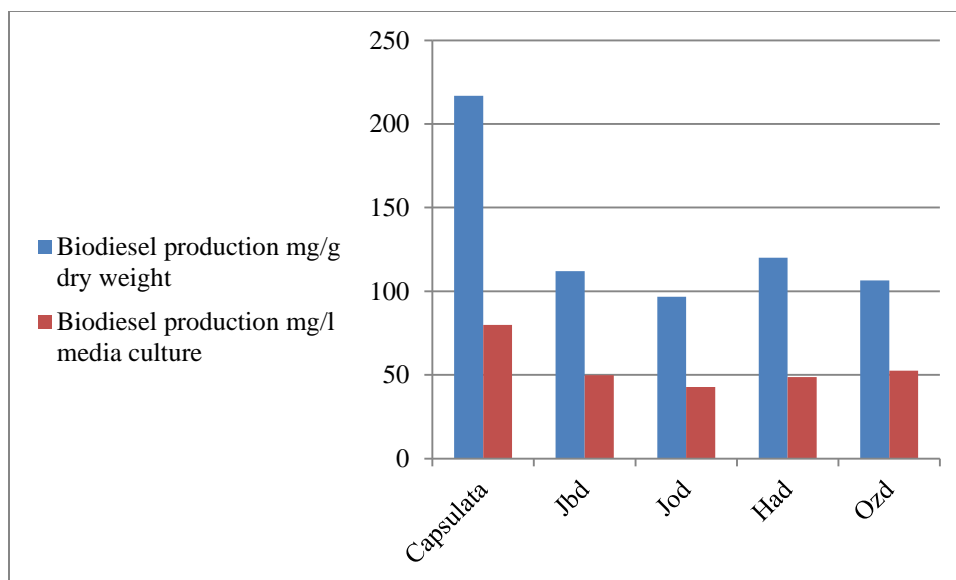


Figure 33. Biodiesel production of four local microalgal isolates measured at the stationary stage¹³.

Table 10. Content of omega FA in four local microalgal isolates observed during two growth stages (% of total lipids).

Phase	Omega type	% omega FA in total lipids			
		Isolates			
		Jbd	Jod	Had	Ozd
Exponential	Omega 6	3.03	7.62	5.38	14.39
	Omega 3	23.8	23.36	22.51	24.12
	Total	26.83	30.98	27.89	38.51
Stationary	Omega 6	7.87	9.68	6.88	8.58
	Omega 3	8.02	17.13	10.24	8.39
	Total	15.89	26.81	17.12	16.97

¹³ Capsulata is a reference obtained from a previous study.

E. Pigment concentrations

The chlorophyll content ranged between 2.166 and 8.2 $\mu\text{g} / \text{ml}$ of microalgal culture media using the acetone extraction protocol [39]. The Jbd had the greatest content while the Ozd had the least content. The methanol extraction method for chlorophyll quantification gave similar figures to those of the acetone extraction in two samples but lower figures in the remaining two samples. The Ozd isolate had the least content of carotenoids (3.3 $\mu\text{g} / \text{ml}$) while the other three isolates had a greater content ranging between 9 and 11 $\mu\text{g} / \text{ml}$ (Table 11).

Table 11. Chlorophyll and carotenoid content in four microalgal isolates ($\mu\text{g} / \text{ml}$).

Solvents		Isolates	Jbd	Had	Jod	Ozd
Acetone	$\mu\text{gChlorophyll}/ \text{ml}$ medium (Jeffrey & Humphrey) ¹⁴		8.17	4.83	3.28	2.166
	$\mu\text{gChlorophyll}/ \text{ml}$ medium (Strickland & Parsons) ¹⁴		7.981	4.658	3.0027	1.99
Methanol	$\mu\text{g Chlorophyll}/ \text{ml}$ medium (Porra et al) ¹⁴		4.19	5.06	3.46	1.17
	$\mu\text{g Carotenoids}/ \text{ml}$ medium (Lichtenthaler) ¹⁴		9.149	11.12	8.939	3.337

¹⁴ [38][39]

The four isolates showed considerable variations in their chlorophyll content with Jbd isolate containing Chl a, b and c, Had and Jod having only Chl a and b and Ozd probably having mainly Chl a with a low concentration of chlorophyll b. The Jbd isolate had the highest content of chlorophyll (Table 12).

Table 12. Content of chlorophylls a, b, c in four local microalgal isolates (mg /m³).

		Chlorophyll		
		Chl a	Chl b	Chl c
Isolates	Jbd	17.83	16.63	30.88
	Had	11.39	5.20	-0.50
	Jod	6.89	3.23	-2.11
	Ozd	4.00	0.74	-5.84

F. Protein Content

In general the two methods for quantification of proteins gave more or less similar results except for the Jod isolate for which the Digesdahl procedure gave a lower value as compared to Kjeldahl procedure. Using a N to protein conversion factor of 5.95, the Jbd isolate had the highest protein content (18-19%) while the other isolates contained between 7 to 9% based on Kjeldahl method (Table 13).

Table 13. Crude protein content (%DW) for four microalgal isolates using Kjeldahl and Digesdahl methods.

Samples	% proteins using	
	Kjeldahl method	Digesdahl method
Jbd	19.31	18.26
Jod	8.71	4.03
Had	7.42	6.84
Ozd	8.9	7.58

G. Antimycotic or antifungal activity

1. In- vitro experiment

a. Jbd extracts

All in the Jbd extracts did not show any significant inhibition of *Alternaria* and *Fusarium* growth. However, the crude water extract resulted in a significant inhibition of *Rhizoctonia* growth, observed at four and eight days post inoculation. This extract also resulted in a delayed growth of *Botrytis* (Table 14).

Table 14. Effect of various extracts of Jbd isolate on the in-vitro growth of four fungal species: Mean diameter of fungal colony (cm).

	<i>Alternaria</i>		<i>Rhizoctonia</i>		<i>Fusarium</i>		<i>Botrytis</i>	
	T1	T2	T1	T2	T1	T2	T1	T2
Control	3.10 ^{a,1}	4.85 ^{a,2}	4.50 ^{a,1}	5.20 ^{a,1}	4.15 ^{a,1}	5.00 ^{a,1}	2.30 ^{a,1}	3.00 ^{a,1}
Methanolic extract	3.43 ^{a,1}	5.20 ^{a,2}	3.73 ^{a,1}	5.03 ^{a,2}	4.53 ^{a,1}	5.20 ^{a,1}	3.63 ^{b,1}	4.67 ^{c,1}
Non methanolic water soluble extract	3.17 ^{a,1}	4.47 ^{a,2}	3.93 ^{a,1}	4.90 ^{a,1}	4.17 ^{a,1}	4.80 ^{a,1}	2.53 ^{ab,1}	3.40 ^{ab,1}
Crude water extract	3.30 ^{a,1}	5.03 ^{a,2}	0.77 ^{b,1}	1.53 ^{b,1}	4.00 ^{a,1}	5.07 ^{a,1}	0.97 ^{c,1}	2.40 ^{a,2}
Culture filtrate water Extract	4.20 ^{a,1}	5.20 ^{a,1}	4.47 ^{a,1}	5.20 ^{a,1}	4.10 ^{a,1}	5.20 ^{a,1}	2.50 ^{ab,1}	4.33 ^{bc,1}

^{a-c} Means in a column followed by different alphabetical superscripts are significantly different (P<0.05)

or

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

or

¹ Means in a row followed by different numerical superscripts are significantly different (P<0.05)

or

^{1,2} Means in a row followed by the same numerical superscript are not significantly different (P>0.05)

b. Jid extracts

None of the Jid extracts showed a significant inhibition effect on any of the four fungi tested except for the culture filtrate which resulted in a significant delay in *Rhizoctinia* growth at four days post inoculation (Table 15).

Table 15. Effect of various extracts of Jid isolate on the in-vitro growth of four fungal species: Mean diameter of fungal colony (cm).

	<i>Alternaria</i>		<i>Rhizoctonia</i>		<i>Fusarium</i>		<i>Botrytis</i>	
	T1	T2	T1	T2	T1	T2	T1	T2
Control	4.43 ^{a,1}	5.20 ^{a,1}	3.10 ^{a,1}	3.60 ^{a,1}	4.87 ^{a,1}	5.20 ^{a,1}	2.97 ^{a,1}	3.57 ^{a,1}
Methanolic extract	4.17 ^{a,1}	5.00 ^{a,1}	4.50 ^{a,1}	5.00 ^{a,1}	4.50 ^{a,1}	4.97 ^{a,1}	4.17 ^{a,1}	4.17 ^{a,1}
Non methanolic water soluble extract	4.40 ^{a,1}	4.80 ^{a,1}	3.83 ^{a,1}	4.60 ^{a,1}	4.80 ^{a,1}	4.80 ^{a,1}	4.07 ^{a,1}	4.50 ^{a,1}
Crude water extract	5.00 ^{a,1}	5.20 ^{a,1}	3.27 ^{a,1}	3.63 ^{a,1}	5.00 ^{a,1}	5.20 ^{a,1}	3.50 ^{a,1}	4.60 ^{a,1}
Culture filtrate water Extract	5.00 ^{a,1}	5.20 ^{a,1}	0.77 ^{b,1}	2.17 ^{a,1}	5.00 ^{a,1}	5.20 ^{a,1}	3.00 ^{a,1}	3.33 ^{a,1}

^{a-c} Means in a column followed by different alphabetical superscripts are significantly different (P<0.05)

or

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

or

¹ Means in a row followed by different numerical superscripts are significantly different (P<0.05)

or

^{1,2} Means in a row followed by the same numerical superscript are not significantly different (P>0.05)

c. Ozd extracts

Although observations showed that the non methanolic extracts of Ozd isolates retarded the growth of the four fungi evaluated; the difference was not statistically significant. Only culture filtrates resulted in significant inhibition of *Rhizoctonia* growth (Table 16).

Table 16. Effect of various extracts of Ozd isolate on the in-vitro growth of four fungal species: Mean diameter of fungal colony (cm).

	<i>Alternaria</i>		<i>Rhizoctonia</i>		<i>Fusarium</i>		<i>Botrytis</i>	
	T1	T2	T1	T2	T1	T2	T1	T2
Control	3.83 ^{a,1}	5.00 ^{a,1}	4.57 ^{a,1}	5.00 ^{a,1}	4.23 ^{a,1}	5.00 ^{a,1}	2.87 ^{a,1}	4.50 ^{a,1}
Methanolic extract	3.33 ^{a,1}	5.00 ^{a,1}	4.60 ^{a,1}	4.83 ^{a,1}	4.07 ^{a,1}	5.00 ^{a,1}	3.43 ^{a,1}	4.40 ^{a,1}
Non methanolic water soluble extract	2.07 ^{a,1}	2.50 ^{a,1}	3.07 ^{a,1}	3.33 ^{a,1}	3.33 ^{a,1}	4.57 ^{a,1}	1.40 ^{a,1}	2.17 ^{a,1}
Crude water extract	4.50 ^{a,1}	5.20 ^{a,1}	4.33 ^{a,1}	5.20 ^{a,1}	4.73 ^{a,1}	5.20 ^{a,1}	2.07 ^{a,1}	3.90 ^{a,1}
Culture filtrate water Extract	3.83 ^{a,1}	5.00 ^{a,1}	0.87 ^{a,1}	1.90 ^{a,1}	3.73 ^{a,1}	5.00 ^{a,1}	1.73 ^{a,1}	3.67 ^{a,1}

^{a-c} Means in a column followed by different alphabetical superscripts are significantly different (P<0.05)

or

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

or

¹ Means in a row followed by different numerical superscripts are significantly different (P<0.05)

or

^{1,2} Means in a row followed by the same numerical superscript are not significantly different (P>0.05)

d. Khd extracts

Most extracts of the Khd isolate did not show any significant effect on the growth of the four species of fungi tested, except for the culture filtrates which resulted in a significant delay of *Botrytis* growth and a nonsignificant delay in *Rhizoctonia* growth (Table 17).

Table 17. Effect of various extracts of Khd isolate on the in-vitro growth of four fungal species: Mean diameter of fungal colony (cm).

	<i>Alternaria</i>		<i>Rhizoctonia</i>		<i>Fusarium</i>		<i>Botrytis</i>	
	T1	T2	T1	T2	T1	T2	T1	T2
Control	3.00 ^{a,1}	5.20 ^{a,2}	3.25 ^{a,b,1}	5.20 ^{a,2}	3.23 ^{b,1}	5.20 ^{a,2}	3.33 ^{a,1}	5.20 ^{a,2}
Methanolic extract	4.50 ^{a,1}	5.20 ^{a,1}	3.83 ^{a,1}	5.20 ^{a,1}	4.33 ^{a,b,1}	5.20 ^{a,1}	4.33 ^{a,1}	5.20 ^{a,1}
Non methanolic water soluble extract	3.66 ^{a,1}	5.20 ^{a,1}	2.50 ^{a,b,1}	5.20 ^{a,2}	3.66 ^{a,b,1}	5.20 ^{a,1}	3.66 ^{a,1}	5.20 ^{a,1}
Crude water extract	4.43 ^{a,1}	5.20 ^{a,1}	3.66 ^{a,b,1}	5.20 ^{a,1}	4.86 ^{a,c,1}	5.20 ^{a,1}	4.33 ^{a,1}	5.20 ^{a,1}
Culture filtrate water Extract	4.00 ^{a,1}	5.20 ^{a,1}	2.16 ^{b,1}	3.7667 ^{a,2}	3.50 ^{a,b,1}	5.00 ^{a,2}	0.80 ^{b,1}	2.66 ^{b,2}

^{a-c} Means in a column followed by different alphabetical superscripts are significantly different (P<0.05)

or

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

or

¹ Means in a row followed by different numerical superscripts are significantly different (P<0.05)

or

^{1,2} Means in a row followed by the same numerical superscript are not significantly different (P>0.05)

Alternaria grew in all extracts of Khd isolate (Figure 34). However, sporulation varied from extract to extract. Crude and non methanolic extracts favored sporulation as compared to other extracts types. By observations, levels of sporulation of the methanolic extracts were very close to those present in the control whereas culture filtrates were more effective in suppressing fungal sporulation.

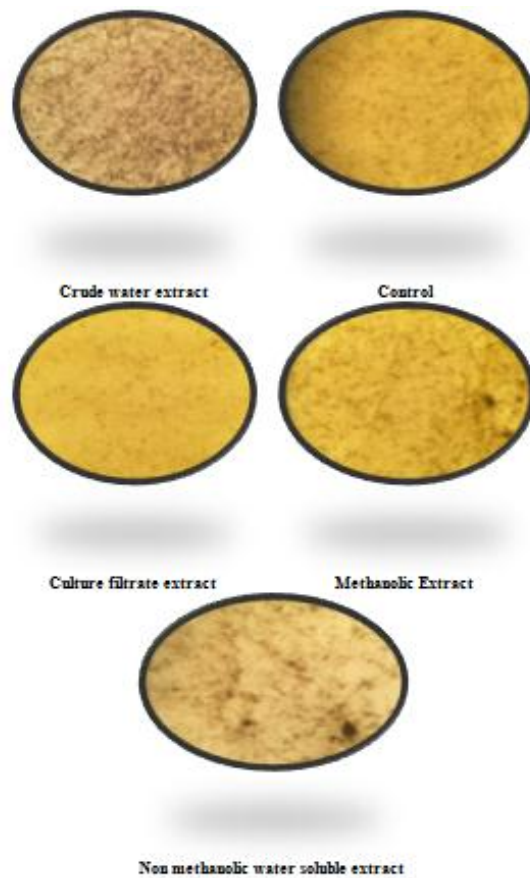


Figure 34. Effect of Khd extracts on growth and sporulation of *Alternaria* sp. grown in vitro.

2. In-vivo experiment

a. Effect of microalgal extracts on cucumber length

Sprays with the Jbd methanolic and non-methanolic water soluble extracts did not show a significant effect on the growth of cucumber seedlings (Figure 35 and 36).

as measured at 6, 10, 12 and 20 days post spraying respectively.

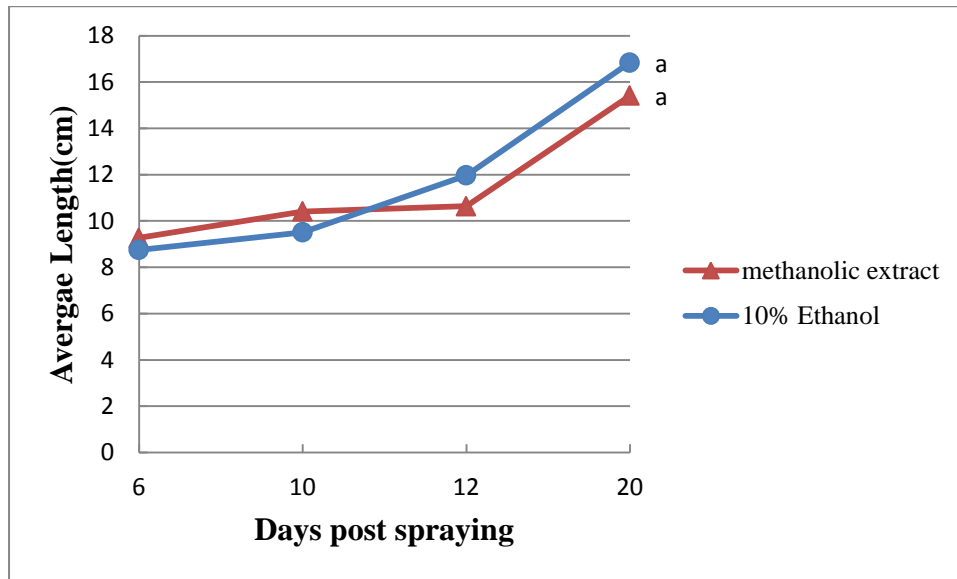


Figure 35. Effect of microalgal methanolic extract (of the Jbd isolate) on the average length of cucumber seedlings (cm).

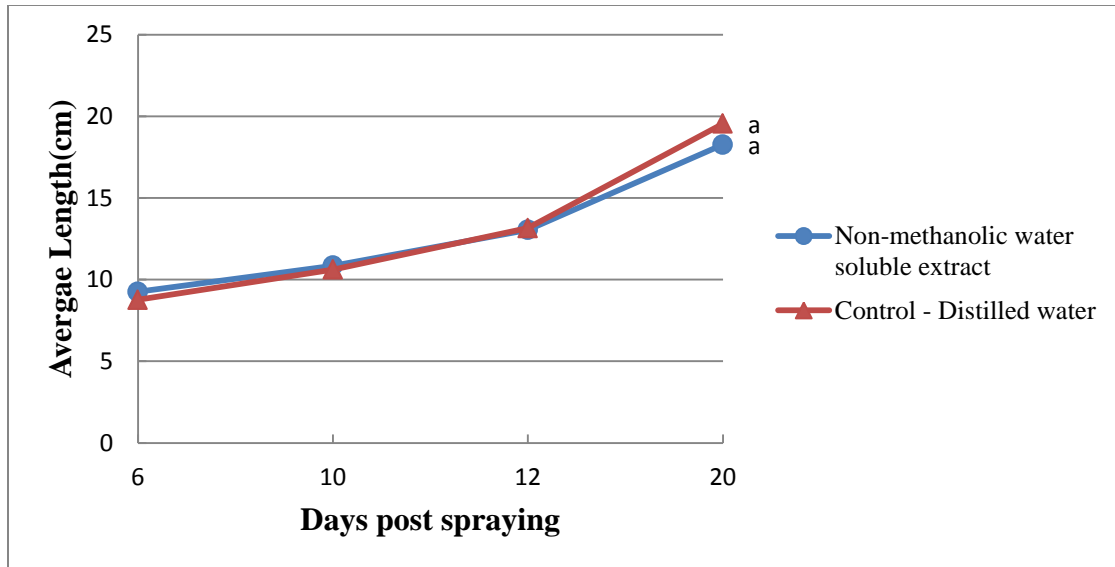


Figure 36. Effect of microalgal non-methanolic extract (of the Jbd isolate) on the average length of cucumber seedlings (cm).

b. Effect of microalgal extracts on cucumber infection of cucumber plants by the powdery mildew fungus

Spraying cucumber seedlings with methanolic or non methanolic water soluble extracts of the Jbd isolate resulted in a slight decrease in symptoms severity, but statistical analysis did not show a significant difference between treatments (Table 18). Therefore, no significant protection against infection and spread of powdery mildew fungus could be inferred.

Table 18. Effect of microalgal extracts sprays on cucumber infection by powdery mildew, ten and twenty days post inoculation, expressed as % leaf area infected. 18a and 18b are two repetitions of the same experiment at one month interval.

18a

Treatment	% leaf area infection at days post inoculation	
	Mean±SD	Mean±SD
	10	20
Methanolic extract	24.705±31.18 ^a	83.8± 26.01 ^a
Non-methanolic extract	25.78±36.58 ^a	86.07± 27.83 ^a
Control-Distilled water	42.82±29.477 ^a	98.17± 6.14 ^a
Control-10%ethanol	27.4±24.3069 ^a	88.21± 26.72 ^a

18 b

Treatment	% leaf area infection at days post inoculation	
	Mean±SD	Mean±SD
	10	20
Methanolic extract	81.09±20.14 ^a	100±0 ^a
Non-methanolic extract	89.9 ±18.13 ^a	98.76 ±5.093 ^a
Control-Distilled water	73.05 ±26.84 ^a	95.4 ±10.17 ^a
Control-10%ethanol	79.72 ±29.46 ^a	91.95 ±17.628 ^a

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

CHAPTER V

DISCUSSION

A. Isolation and molecular identification

The isolation procedure followed in this study may have resulted in the predominance of some species of microalgae, belonging mainly to the green microalgae and to the diatoms. It would be recommended to use two or more isolation media including a specific medium that favors the growth of cyanobacteria, due to the importance of these organisms in the production of bioactive substances.

Microalgae are mostly unicellular organisms; making identification based on morphological characteristics tedious and often not possible. Accordingly, molecular tools based on gene sequencing are gaining acceptance as identification tools accurate to the species level [83]. However, molecular characterization of microalgae still needs refinement and identification to species level may require sequencing of two or more genes.

In preliminary evaluations, many of the primers pairs reported in the literature failed to give amplification products from our isolates or gave amplification products whose sequences were non-specific and did not allow identification to the species level (data not reported). In the present thesis, we tried several primer pairs targeting the LSU and ITS of the ribosomal DNA, as well as *tufA* gene and mitochondrial cytochrome c oxidase 1 gene. Among the primer pairs tested, the one that targets the LSU D2/ D3

region was the most important in identifying many microalgae to the genus level. Four local isolates were identified based on at least two genomic regions. Had and Khd as *Tetraselmis marina* (LSU, ITS, Tuf A), Had 2 as *Amphidium carterae* (LSU, ITS, Tuf A), Ozd red isolate contained the cyanobacterium *Leptolyngbya* sp. (Tuf A) and *Amphora coffeaeformis* (LSU). The remaining isolates were identified based on one genomic region. Jid was identified as *Amphora* sp. (LSU), Dad as *Chlorella vulgaris* (LSU), Jod as a *Micractinium reisseri* and Ozd green a *Chloroidium saccharophilum* (Tuf A), noting that this *Chloroidium* species was also known as *Chlorella saccharophyla*, phylum chlorophyta [80]. It is worth mentioning that the obtained sequence for Jbd Isolate was not considered good enough for proper identification. However, careful analysis of molecular data in combination with the pigment analysis which showed that this culture contained chlorophylls a, b and c, suggests that the Jbd isolate is a mixture of *Micractinium reisseri* and another microalga genus belonging to a different phylum. It is recommended to further purify this isolate and repeat the sequencing. With reference to the previous study conducted in Lebanon [83], this study has identified the following new genera or species: *Amphidium carterae*, *Chlorella variabilis*, *Micractinium reisseri*, *Chloroidium saccharophilum*, *Leptolyngbya* sp and *Amphora* sp.

B. Growth Kinetics

The growth rate of microalgae depends on several parameters including microalgae isolate and several environmental factors including temperature, light duration and intensity, CO₂, salinity, pH, and nutrient availability. Under our

experimental conditions, the growth rate of the four isolates was highest directly after the lag phase and lasted for two to four days only. Later on, the growth rate dropped sharply. Since the CO₂ supply was adequate and the pH was stable, the major limitation may be due to nutrient availability or due to accumulation of toxic or 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 especially in the case of Ozd isolate. On the other hand, the early growth rate may be considered acceptable, fast and good in the remaining three isolates. It reached a μ_{\max} around 1.5 considered high since it reflects the doubling of microalgal cells each 11 hours [79].

C. Lipid analysis

Total lipid content varied from 12 to 16% of the microalgal dry weight (Table 9). *Tetraselmis marina* i.e. Had isolate, recorded 15% of its content as lipids which was also reported in the literature where *Tetraselmis* sp. had 14.7% lipids in its biomass. While the remaining green microalgal isolates tested reported in the literature had wider ranges from 19 to 21% [81]. Significant differences in total lipids and the FA profiles were observed depending on the growth phase. The closer to the stationary phase the higher was their total lipid content however the highest content of PUFA and omega FAs were observed during the exponential phase.

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 etc. Our data shows that the proportion of unsaturated fatty acids was greater than that of saturated FAs in both exponential and stationary phases (Table 9).

The relative percentage of saturated fatty acids increased in the stationary phase of three isolates out of four, as compared to their content during the exponential phase. On the other hand, the relative percentage of unsaturated FA and omega FA was higher in the exponential phase. This was also reported in the literature, where microalgae *P. tricornutum* and *P. cruentum* had higher percentages of omega FAs in the exponential phase [87]. This change in the percentage of unsaturated FA of the stationary phase might be due to the effect of nutrient starvation reached since continuous nutrient supply during culturing was not performed [28][29]. Furthermore, it has been noticed that saturated and monounsaturated fatty acids predominated all microalgae tested (Table 9). The higher the percentage of saturated and monounsaturated FA, the better they are suitable for biodiesel production. Proportions of the omega group of fatty acids varied from 25 to 57% of the unsaturated fatty acids (Table 10). The proportions of unsaturated FA are dependent on variations in temperatures as highlighted before with an increase in their content as temperature increases. On the other hand, there have been studies pointing the importance of lighting conditions since low light intensities favored the formation of membrane specific polyunsaturated fatty acids, while high light intensities favored neutral lipids [31].

The four isolates were also evaluated for their biodiesel potential and showed a biodiesel productivity around 50mg/l of culture media in three isolates (Figure 33). According to the literature, a biodiesel production less than 80mg/l of microalgal media culture is considered non- competitive and therefore non-economical as compared to current diesel produced for a mixed algal culture, *R. hieroglyphicum* and *C. vulgaris*. Ahmad et al (2013) reported a biodiesel productivity of 91, 92 and 94% respectively [85].

D. Pigment Analysis

Several methods can be found in the literature, most of them are related to spectrophotometric analysis for chlorophyll and carotenoids evaluations. Several factors were tested including the extraction solvent to be used, the cell wall disruption technique as well as several empirical correlations.

According to our results, acetone extraction proved the best for quantification of chlorophyll content. Using this method Jbd isolate recorded the highest quantities of chlorophyll about of 8.17 or 7.981 $\mu\text{g}/\text{ml}$ medium based on Jeffrey and Humphrey (1975), Strickland and Parsons (1968) correlations respectively [39] (Table 11).

Our results are in disagreement with those of Henriques et al (2007) [5][6] who reported methanol as the best extraction solvent to quantify chlorophyll a. In the Jbd isolate, the chlorophyll content obtained with acetone extraction was twice more compared to methanol extraction. Therefore, according to the above referred data, the best method for the evaluation of chlorophyll content in microalgal cells make use of acetone as extraction solvent with Jeffrey and Humphrey (1975) correlation.

Schumann et al (1998) considered that acetone is a suitable solvent for extraction of several algal components, because the extractability is related to the chemical and mechanical properties of microalgae [40]. Leeuwe et al (2005) proved that pigment extracts were unstable in methanol with recorded losses of chlorophyll a ranging from 10 to 60% per day; while those extracted with acetone lost less than 1% of their content. Other reports suggest that methanol activates chlorophyllase causing the loss of chlorophyll a [38, 42]. In addition to a good choice of solvent with suitable properties, a good method for mechanical cell disruption is needed. Probe sonication proved efficient in extraction of chlorophyll a [40] so did the freezing / defreezing technique. This was confirmed with our results due to the effect of combining both techniques of cell disruption as well as using the proper solvent for extraction. Joining acetone and freeze drying for pigment extraction would be a suitable combination since freeze drying technique will break the protein matrix of microalgal cells making solvent accessibility easier [38].

The Jbd isolate contained significant higher chlorophyll concentrations than the Had, Jod and Ozd isolates. All isolates contained chlorophylls a and b while chlorophyll c was present only in Jbd isolate at higher levels than chlorophyll a and b. Our results are in agreement with the characteristics of green algae that generally have photosynthetic pigments including chlorophylls a and b and zeaxanthin [41] which confirms our molecular identification in three out of four isolates belonging to the chlorophyta algae (Table 1). Only Jbd isolate had chlorophylls a, b and c, showing that the Jbd isolate is probably a mixture of two microalgae belonging to two different taxonomic groups, Chlorophyta and another undetermined phylum. This may explain the difficulty we got in

sequencing the amplicons obtained from the Jbd isolate (Table 6). Cloning of the amplicons prior to sequencing may help in proper identification of the two microalgae genera present in this culture.

On the other hand, to extract more polar pigments, such as carotenoids, methanol solvent was used. The empirical formula of Lichtenthaler specific for carotenoid concentrations was followed and large concentrations of the pigment were obtained.

According to our results, the isolate Had had the highest carotenoid content. Several studies have been undergone on the possible beneficial health effects of carotenoids isolated from microalgae. A study conducted on the green algae *C. humicola*, showed an activity of carotenoids against environmental genotoxic agents [39].

E. Protein analysis

Several methods have been reported for protein analysis including colorimetric and elemental analysis. Microalgal biomass usually contains a proportion of nitrogen not associated with proteins but with compounds like DNA and chlorophyll a, thus the commonly used Kjeldahl multiplier (6.25) for estimating plant proteins overestimates the protein content of microalgae. Therefore, a suitable conversion factor would be 5.95 [33]. This has been also stressed by Barbarino et al. (2005) who gave a cofactor range for 12 different microalgae [34].

In our experiments, protein content has been determined by the classic Kjeldahl method and by a modification of the latter known as Digestdahl method. In 3 out of 4 isolates, both methods showed similar results with a protein content ranging from 5.9 to

18.6% of the microalgal dry weight (Table 14). Jbd isolate had high protein double or triple the amount in the other isolates. Based on the Kjeldahl method, it has about 18% of its dry weight as protein. According to FAO reports the protein content in microalgae usually ranges from 12 to 35% [35]. However, the protein content of our isolates had low concentrations as compared to other green algae. According to Becker (1994), green algae have protein content ranging between 8-58% of the % dry matter basis as for *Scenedesmus*, *Chlorella* and *Tetraselmis sp* [80].

We conclude that Jbd isolate might be promising to be further studied in order to increase its protein content and therefore use it as a feedstock for animals.

F. Antimycotic activity

Marine microalgae are rich sources of vitamins, minerals, proteins, polysaccharides, lipids and fibers. Many substances obtained from microalgae are currently tested due to their promising content. Several reports suggested their potential as antibacterial, antifungal and antiviral agents. Four extracts of our microalgal isolates were extracted and tested in vitro against plant pathogenic fungi: *Alternaria solani*, *Fusarium oxysporum*, *Botrytis cinerea*, and *Rhizoctonia solani*. In general, the extracts of the microalgae tested did not show a promising antimycotic activity. There was no significant difference between the extracts tested and their controls. Culture filtrates of Jid (Table 15), Ozd (Table 16) and Khd (Table 17) were able to suppress the growth of *Rhizoctonia*, and *Botrytis*. But still the effect of the latter extract is questionable since it contains a high salt concentration that might have affected the growth of the fungus. In the future, an appropriate control should be included in such tests. Our results disagree with Omar et al. (2012) who recorded efficiency of crude microalgal extracts against

fungi [56] as well as other articles claiming the suppression of *Fusarium solani* after being subjected to three algal exudates [8]. On the other hand, researchers have reported the anti-sporulation effect of cyanobacteria *Nostoc muscorum* against *Fusarium verticilloides* [10, 13]. However, our microalgae did not show any inhibitory effect on sporulation of fungi (Figure 34). On the contrary, sporulation increased in crude water extract as well as in the non-methanolic water soluble extract.

G. Induced Resistance

Induced resistance is a secondary response of some plants that arises after being attacked by a pathogen or a non-virulent organism, or if the plants are subjected to chemical or physical treatment. It can be either local appearing two to three days after infection or systemic requiring a longer time by triggering the activation of different signals inside the plant. For example, the green algae *Ulva sp* with a relatively high content of a sulfated polysaccharide known as Ulvan, was shown to be effective in activating plant immunity through the jasmonic acid signaling pathway [66]. Also carrageenans, mainly λ - carrageenan, high in sulfate content proved efficient in inducing signaling and defense gene expression in tobacco leaves [68].

The two tested extracts of the microalgae *Micractinium sp.* failed to induce systemic resistance to powdery mildew. For future experiments, it is recommended to conduct preliminary screening tests with a large number of isolates and at a minimum of two host pathogen systems. Microalgal isolates may help in selection of isolates with

high laminarin, of xyloglucans content which may have a higher probability to induce resistance.

CHAPTER VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Microalgae are efficient photosynthetic organisms grow rapidly producing large biomass containing lipids, proteins, carotenoids and other important secondary metabolites. The main objectives of this thesis were to isolate local strains of microalgae, to identify them using molecular techniques, and to determine their lipid content, fatty acid profile, including saturated and unsaturated FA with a special attention to their omega FA content and their protein content as a preliminary evaluation for their potential use for biodiesel production, for food or feed supplements or for the control of plant pathogenic fungi. A total of 11 microalgal isolates were purified. Since microalgal identification based on anatomy is often difficult and sometimes impossible, their identification based on molecular techniques was attempted. Amplification and sequencing of the genomic regions LSU, ITS and partial Tuf A gene proved very useful. The following genera and species were identified: *Amphora coffeaeformis*, *Amphidinium carterae*, *Chlorella variabilis*, *Chloroidium saccharophilum*, *Micractinium reisseri*, *Tetraselmis marina*, and the cyanobacterium *Leptolyngbya sp.* The growth kinetics and the chemical composition of the selected isolates were analyzed. In general, the total lipid content was higher during the stationary phase while PUFA and omega fatty acids were higher during the exponential growth phase. The four tested isolates had total lipid

content ranging between 14.5 and 16.5% DW, the crude protein content ranged between 9 and 19%.

The Had isolate, *Tetraselmis marina* contained chlorophylls a and b, a low protein content (7.4% DW), 2.5% of omega FA and a total lipid content of 16% (DW) with 81% polyunsaturated and monounsaturated FAs.

The Jbd isolate, most probably a mixture of *Micractinium* sp. and another microalgae genus, contained the highest levels of chlorophylls a, b and c, a total lipid content of 16.5% DW with 82% polyunsaturated and monounsaturated FA, about 2.5% omega FA, and the highest level of protein (19.3% DW). This makes this isolate promising for use as feed supplement, for biodiesel production as well as for chlorophyll production.

The Jod isolate, *Micractinium* sp. contained chlorophylls a and b, 14.6% total lipids, the highest level of PUFA and the highest level of omega FA about 3.9% DW during the stationary phase, a lower percentage of unsaturated and monosaturated FA (70%) and 8.7% DW protein. Therefore, this isolate would be the best source for omega 3 FA among the isolates tested.

The Ozd isolate, *Chloroidium saccharophilum* contained chlorophyll a and b, 14.5% total lipids of which 82% are unsaturated and monounsaturated, and had the highest level of omega 3 and 6 FAs during the exponential growth phase. Its protein content is about 9%.

None of the four isolates mentioned above showed promising in- vitro activity against the four fungi tested even though the non-methanolic extracts of Ozd isolate retarded the development of three of the four fungi tested.

Under the conditions of the tests, none of the studied four isolates looks very promising for biodiesel production unless culturing conditions may be modified to increase the lipid content. However, their content of bioactive substances with interesting biological properties deserves further investigations. It would be interesting to study more local isolates trying to identify those with high growth rates coupled with over 30% total lipids for biodiesel production or with high protein or omega FAs content for their nutritional value.

The in-vivo antimycotic activity of only one isolate was evaluated. Sprays of *Micractinium* sp. extracts on cucumber leaves did not lead to improvement in plant growth nor did they give a significant protection against the powdery mildew fungus.

Recommendations:

Microalgae innovation is a hot and flourishing topic that requires a lot of studies to be undertaken. This requires adequate facilities as well as a multidisciplinary research project in order to reach success. The following recommendations may be suggested:

- i. To identify more Lebanese isolates to the species level, based on at least three genetic markers.
- ii. To analyze the chemical composition profile of the Lebanese isolates in order to assess their potential uses.

- iii. To evaluate the efficacy of microalgae bioactive substances for their medicinal properties including antioxidant, antimicrobial, and anticancer activities as well as their nutritional value for use as human food supplements or animal feed.
- iv. To screen the microalgal extracts for their potential use in agriculture for the management of plant pests as well as for use as organic fertilizers.
- v. To acquire appropriate facilities and equipments to conduct growth kinetic studies under controlled conditions in order to determine their optimum growth.

APPENDIX I

FORMULATION FOR MARINE GROWTH MEDIA

A. Walne medium (Walne 1970)

This enriched seawater medium was designed for mass culture of marine phytoplankton used as feed for shellfish.

Sodium metasilicate ($40 \text{ mg} \cdot \text{L}^{-1}$) should be added for the growth of diatoms. The vitamins solution is extremely dilute and lacks biotin. To prepare, pasteurize 1 liter of filtered natural seawater; after cooling, aseptically add 1 mL of the nutrient solution and 100 mL of the vitamins solution.

1. Nutrient Solution

Into 900 mL of high quality dH₂O, dissolve the components. Bring final volume to 1 liter with high-quality dH₂O, filter sterilize, and store at 4°C.

Table 19. Composition of Walne mineral medium (1000x).

Component	Stock Solution (g · L⁻¹ dH₂O)	Quantity Used	Concentration in Final Medium (M)
NaNO ₃	-	100.0 g	1.18×10^{-3}
H ₃ BO ₃	-	33.6 g	5.43×10^{-4}
Na ₂ EDTA (anhydrous)	-	45.0 g	1.54×10^{-4}
NaH ₂ PO ₄ · H ₂ O	-	20.0 g	1.28×10^{-4}
FeCl ₃ · 6H ₂ O	-	1.3 g	4.81×10^{-6}
MnCl ₂ · 4H ₂ O	-	0.36 g	1.82×10^{-6}
Trace metals solution	(See following recipe)	1 mL	-

2. Trace Metals Solution

Into 900 mL of high quality dH₂O, dissolve the components. This solution is normally cloudy. Acidify with a few drops of concentrated HCl to give a clear solution. Bring final volume to 1 liter with high-quality dH₂O, filter sterilize, and store at 4°C.

Table 20. Composition of the trace metal solution specific for Walne medium.

Component	Stock Solution (g · L ⁻¹ dH ₂ O)	Quantity Used	Concentration in Final Medium (M)
ZnCl ₂	-	21.0 g	1.54X 10 ⁻⁷
CoCl ₂ · 6H ₂ O	-	20.0 g	8.41 X10 ⁻⁸
(NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O	-	9.0 g	7.28 X10 ⁻⁹
CuSO ₄ · 5H ₂ O	-	20.0 g	8.01 X 10 ⁻⁸

3. Vitamins Solution

Into 950 mL of dH₂O, dissolve the thiamine · HCl and cyanocobalamin. Bring final volume to 1 liter, filter sterilize, and freeze.

Table 21. Composition of the vitamin solution specific for Walne medium

Component	Stock Solution (g · L ⁻¹ dH ₂ O)	Quantity Used	Concentration in Final Medium (M)
Thiamine · HCl (vitamin B1)	-	1.0 g	2.96X 10 ⁻¹⁰
Cyanocobalamin (vitamin B12)	-	50 mg	3.69 X10 ⁻¹²

B. f/2 medium

This is 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

(Guillard 1975). Into 950 mL of filtered natural seawater, add the following components.

Bring the final volume to 1 liter with filtered natural seawater. Autoclave.

Table 22. Composition of f/2 mineral medium.

Component	Stock Solution (g · L ⁻¹ dH ₂ O)	Quantity Used	Concentration in Final Medium (M)
NaNO ₃	75	1 mL	8.82X 10 ⁻⁴
NaH ₂ PO ₄ · H ₂ O	5	1 mL	3.62 X10 ⁻⁵
Na ₂ SiO ₃ · 9H ₂ O	30	1 mL	1.06 X 10 ⁻⁴
Trace metals solution	(See following recipe)	1 mL	-
Vitamins solution	(See following recipe)	0.5 mL	-

1. f/2 Trace Metals Solution

Into 950 mL of dH₂O, dissolve the EDTA and other components. Bring the final volume to 1 liter with dH₂O.

Table 23. Composition of f/2 trace metal solution

Component	Stock Solution (g · L ⁻¹ dH ₂ O)	Quantity Used	Concentration in Final Medium (M)
FeCl ₃ · 6H ₂ O	-	3.15 g	1.17 X 10 ⁻⁵
Na ₂ EDTA · 2H ₂ O	-	4.36 g	1.17 X 10 ⁻⁵
MnCl ₂ · 4H ₂ O	180.0	1 mL	9.10 X 10 ⁻⁷
ZnSO ₄ · 7H ₂ O	22.0	1 mL	7.65 X 10 ⁻⁸
CoCl ₂ · 6H ₂ O	10.0	1 mL	4.20 X 10 ⁻⁸
CuSO ₄ · 5H ₂ O	9.8	1 mL	3.93 X10 ⁻⁸
Na ₂ MoO ₄ · 2H ₂ O	6.3	1 mL	2.60 X 10 ⁻⁸

2. f/2 Vitamins Solution

Into 950 mL of dH₂O, dissolve the thiamine · HCl, and add 1 mL of the primary stocks.

Bring final volume to 1 liter with dH₂O. Filter-sterilize and store frozen.

Table 24. Composition of f/2 vitamin solution

Component	Stock Solution (g · L⁻¹ dH₂O)	Quantity Used	Concentration in Final Medium (M)
Thiamine · HCl (vitamin B1)	-	200 mg	2.96 X 10 ⁻⁷
Biotin (vitamin H)	1.0	1 mL	2.05X 10 ⁻⁹
Cyanocobalamin (vitamin B12)	1.0	1 mL	3.69 X10 ⁻¹²

APPENDIX II

FUNGAL GROWTH, IN-VITRO

Tables below represent the statistical analysis of experiments done in vitro using four microalgal extracts against four plant pathogenic fungi. Measures were undertaken considering the percentage increase of the fungus as compared to the control.

Table 25. Effect of various extracts of Jbd isolate on the percentage growth change (+ or-) of fungi relative to the control.

	<i>Alternaria</i>		<i>Rhizoctonia</i>		<i>Fusarium</i>		<i>Botrytis</i>	
	T1	T2	T1	T2	T1	T2	T1	T2
Control	0.00 ^{a,1(15)}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}
Methanolic extract	10.75 ^{a,1}	7.22 ^{a,1}	- 17.04 ^{a,1}	-3.20 ^{a,1}	9.24 ^{a,1}	4.00 ^{a,1}	57.97 ^{b,1}	55.56 ^{a,1}
Non methanol water soluble extract	2.15 ^{a,1}	-10.22 ^{a,1}	-12.59 ^{a,1}	-6.49 ^{a,1}	0.40 ^{a,1}	4.00 ^{a,1}	10.14 ^{a,1}	13.33 ^{a,1}
Crude water extract	6.45 ^{a,1}	3.77 ^{a,1}	- 82.96 ^{b,1}	- 70.51 ^{b,1}	-3.61 ^{a,1}	1.33 ^{a,1}	- 57.97 ^{c,1}	-20.00 ^{a,1}
Culture filtrate extract	35.48 ^{a,1}	7.22 ^{a,1}	-0.74 ^{a,1}	0.00 ^{a,1}	-1.20 ^{a,1}	4.00 ^{a,1}	8.69 ^{a,1}	44.45 ^{a,1}

⁽¹⁵⁾^{a-c} Means in a column followed by different alphabetical superscripts are significantly different (P<0.05)

or

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

or

¹ Means in a row followed by different numerical superscripts are significantly different (P<0.05)

or

^{1,2} Means in a row followed by the same numerical superscript are not significantly different (P>0.05)

Table 26. Effect of various extracts of Jid isolate on the percentage growth change (+ or-) of fungi relative to the control.

	<i>Alternaria</i>		<i>Rhizoctonia</i>		<i>Fusarium</i>		<i>Botrytis</i>	
	T1	T2	T1	T2	T1	T2	T1	T2
Control	0.00 ^{a,1(16)}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}
Methanolic extract	-5.94 ^{a,1}	0.00 ^{a,1}	45.16 ^{a,1}	38.89 ^{b,1}	-7.41 ^{a,1}	-4.49 ^{a,1}	40.77 ^{a,1}	17.04 ^{a,1}
Non methanol water soluble extract	-0.68 ^{a,1}	-7.69 ^{a,1}	23.65 ^{a,1}	27.77 ^{a,1}	-1.23 ^{a,1}	-7.7 ^{a,1}	37.39 ^{a,1}	26.40 ^{a,1}
Crude water extract	12.87 ^{a,1}	0.00 ^{a,1}	5.38 ^{a,1}	0.92 ^{a,1}	2.88 ^{a,1}	0.00 ^{a,1}	18.24 ^{a,1}	29.21 ^{a,1}
Culture filtrate extract	12.87 ^{a,1}	0.00 ^{a,1}	-75.27 ^{b,1}	-39.82 ^{a,1}	2.88 ^{a,1}	0.00 ^{a,1}	1.35 ^{a,1}	-6.36 ^{a,1}

⁽¹⁶⁾ ^{a-c} Means in a column followed by different alphabetical superscripts are significantly different (P<0.05)

or

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

or

¹ Means in a row followed by different numerical superscripts are significantly different (P<0.05)

or

^{1,2} Means in a row followed by the same numerical superscript are not significantly different (P>0.05)

Table 27. Effect of various extracts of Ozd isolate on the percentage growth change (+ or-) of fungi relative to the control.

	<i>Alternaria</i>		<i>Rhizoctonia</i>		<i>Fusarium</i>		<i>Botrytis</i>	
	T1	T2	T1	T2	T1	T2	T1	T2
Control	0.00 ^{a,1(17)}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}
Methanolic extract	-12.96 ^{a,1}	0.00 ^{a,1}	0.87 ^{a,1}	-3.33 ^{a,1}	-3.86 ^{a,1}	0.00 ^{a,1}	-9.09 ^{a,1}	-2.22 ^{a,1}
Polysaccharide extract	-46.04 ^{a,1}	-50.00 ^{b,1}	-32.01 ^{a,1}	-33.33 ^{ab,1}	-21.20 ^{a,1}	-8.66 ^{a,1}	-51.04 ^{a,1}	-56.66 ^{a,1}
Crude water extract	17.49 ^{b,1}	4.00 ^{a,1}	-4.97 ^{a,1}	4.00 ^{a,1}	11.90 ^{a,1}	4.00 ^{a,1}	-27.74 ^{a,1}	-13.33 ^{a,1}
Sea water extract	0.08 ^{a,1}	0.00 ^{a,1}	-80.99 ^{b,1}	-62.00 ^{b,2}	-11.74 ^{a,1}	0.00 ^{a,1}	-49.88 ^{a,1}	-18.51 ^{a,1}

(17) ^{a-c} Means in a column followed by different alphabetical superscripts are significantly different (P<0.05)

or

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

or

¹ Means in a row followed by different numerical superscripts are significantly different (P<0.05)

or

^{1,2} Means in a row followed by the same numerical superscript are not significantly different (P>0.05)

Table 28. Effect of various extracts of Khd isolate on the percentage growth change (+ or-) of fungi relative to the control.

	<i>Alternaria</i>		<i>Rhizoctonia</i>		<i>Fusarium</i>		<i>Botrytis</i>	
	T1	T2	T1	T2	T1	T2	T1	T2
Control	0.00 ^{a,1} (18)	0.00 _{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}	0.00 ^{a,1}
Methanolic extract	8.61 ^{a,1}	0.00 _{a,1}	0.36 ^{a,1}	0.00 ^{a,1}	9.80 ^{a,1}	-2.56 ^{a,1}	6.82 ^{a,1}	0.00 ^{a,1}
Polysaccharide extract	7.12 ^{a,1}	0.00 _{a,1}	-11.83 _{a,1}	0.00 ^{a,1}	9.80 ^{a,1}	0.00 ^{a,1}	18.18 ^{a,1}	0.00 ^{a,1}
Crude water extract	16.85 _{a,1}	0.00 _{a,1}	15.34 ^{a,1}	0.00 ^{a,1}	22.35 ^{a,1}	0.00 ^{a,1}	18.18 ^{a,1}	0.00 ^{a,1}
Sea water extract	7.12 ^{a,1}	0.00 _{a,1}	-50.54 _{b,1}	-60.95 _{b,1}	2.84 ^{a,1}	-3.85 ^{a,1}	-65.15 _{b,1}	-48.72 _{b,1}

(18) ^{a-c} Means in a column followed by different alphabetical superscripts are significantly different (P<0.05)

or

^a Means in a column followed by the same alphabetical superscript are not significantly different (P>0.05)

or

¹ Means in a row followed by different numerical superscripts are significantly different (P<0.05)

or

^{1,2} Means in a row followed by the same numerical superscript are not significantly different (P>0.05)

APPENDIX III

LIPID ANALYSIS

Separation, identification and quantification of lipid compounds in microalgal isolates were performed using GC-MS.

For Jbd isolate (*Micractinium* sp.),

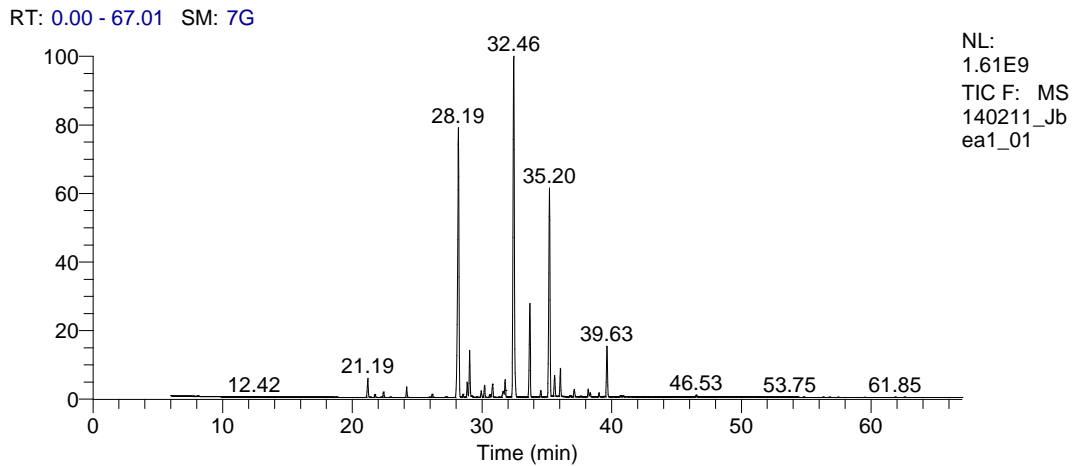


Figure 37. Chromatogram of lipid components of Jbd isolate in the exponential phase.

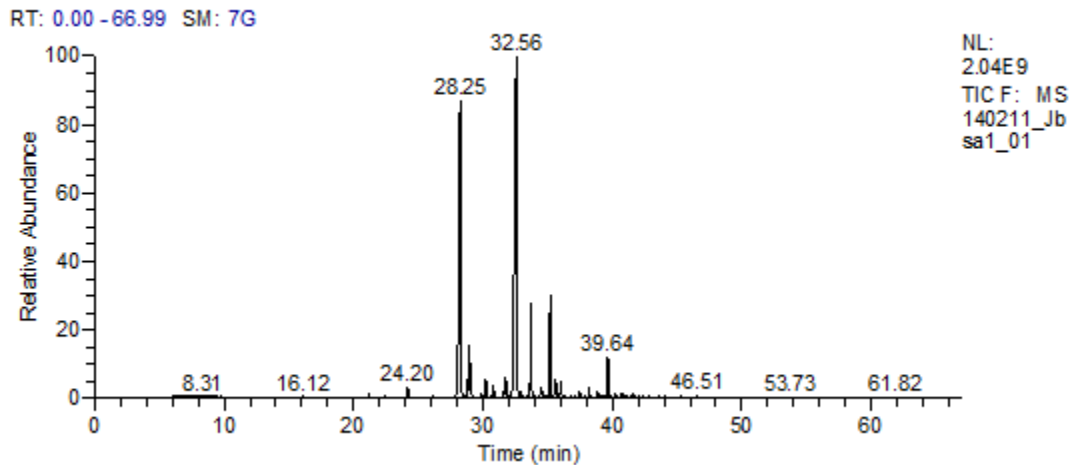


Figure 38. Chromatogram of lipid components of Jbd isolate in the stationary phase.

Table 29. Chemical components of Jbd FAMES with their corresponding retention time and percentage peak area in both exponential and stationary phases.

Exponential phase					Stationary phase				
RT	Area %	RSI	Chemical compound	Peak Area	RT	Area %	RSI	Chemical compound	Peak Area
21	1	911	From chlorophyll	513794618	24	1	919	Methyl tetradecanoate	305029256
24	1	923	Methyl tetradecanoate	243810403	28	32	937	Hexadecanoic acid, methyl ester	18853853930
28	27	946	Hexadecanoic acid, methyl ester	9970147561	29	5	935	9-Hexadecenoic acid, methyl ester, (Z)-	2887334005
29	4	945	9-Hexadecenoic acid, methyl ester, (Z)-	1626358735	30	1	921	7,10-Hexadecadienoic acid, methyl ester	559664422
30	1	924	7,10-Hexadecadienoic acid, methyl ester	461388777	31	1	917	4,7,10-Hexadecatrienoic acid, methyl ester	438597879
31	1	914	4,7,10-Hexadecatrienoic acid, methyl ester	412289292	32	2	927	Octadecanoic acid, methyl ester	947230748
32	2	908	7,10,13-Hexadecatrienoic acid, methyl ester	651383655	33	42	944	9-Octadecenoic acid, methyl ester, (E)-	25369217146

32	30	807	8-Octadecenoic acid, methyl ester	11040416923	34	6	921	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	3382878320
34	7	919	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	2444077252	35	1	808	Tetratetracontane	422602677
35	16	886	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	5964715399	35	6	886	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	3551516053
36	2	942	11-Eicosenoic acid, methyl ester	575582399	36	1	943	11-Eicosenoic acid, methyl ester	616132967
36	2	855	Methyl eicosa-5,8,11,14,17-pentaenoate	762982556	36	1	737	Z,Z,Z-4,6,9-Nonadecatriene	631265569
37	1	918	4,7,10,13,16,19-Docosaheptaenoic acid, methyl ester, (all-Z)-	234230934	37	0	886	Tetratetracontane	295725688
38	1	887	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	295469224	38	1	894	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	330462191
40	4	939	Methyl eicosa-5,8,11,14,17-pentaenoate	1309656522	40	2	940	Methyl eicosa-5,8,11,14,17-pentaenoate	1248978494

For Had isolate (*Tetraselmis marina*)

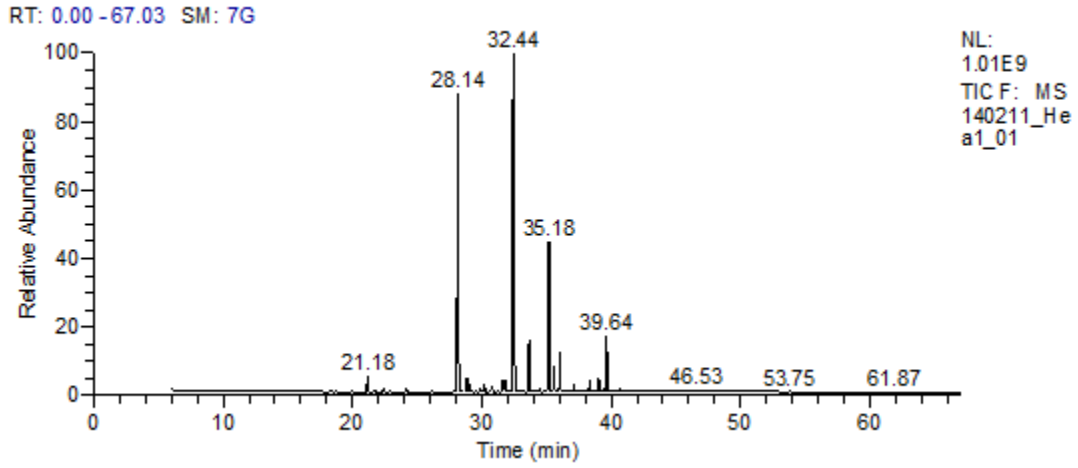


Figure 39. Chromatogram of lipid components of Had isolate in the exponential phase.

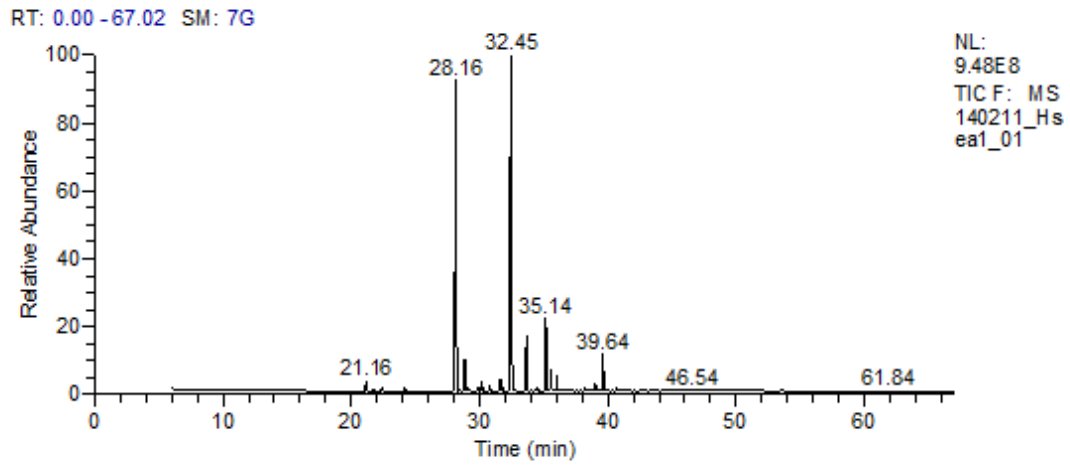


Figure 40. Chromatogram of lipid components of Had isolate in the stationary phase.

Table 30. Chemical components of Had FAMES with their corresponding retention time and percentage peak area in both exponential and stationary phases.

Exponential phase					Stationary phase				
RT	Area %	RSI	Chemical Compound	Peak Area	RT	Area %	RSI	Chemical Compound	Peak Area
21	1	922	Decomposed from chlorophyll	257673097	21	1	923	Decomposed from chlorophyll	157618203
28	31	932	Hexadecanoic acid, methyl ester	6280591652	28	34	931	Hexadecanoic acid, methyl ester	6027395612
29	2	913	9-Hexadecenoic acid, methyl ester, (Z)-	402664377	29	3	933	9-Hexadecenoic acid, methyl ester, (Z)-	551034906
30	1	913	7,10-Hexadecadienoic acid, methyl ester	178112289	30	1	926	7,10-Hexadecadienoic acid, methyl ester	203447792
31	1	919	4,7,10-Hexadecatrienoic acid, methyl ester	114103172	31	1	918	4,7,10-Hexadecatrienoic acid, methyl ester	100969467
32	2	832	7,10,13-Hexadecatrienoic acid, methyl ester	386333091	32	2	932	Octadecanoic acid, methyl ester	266279902
32	34	846	8-Octadecenoic acid, methyl ester	6752256320	32	40	916	8-Octadecenoic acid, methyl ester	7082976641

34	4	917	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	787740986	34	5	915	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	802350220
35	12	892	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	2440164258	35	6	885	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	1055605344
36	2	934	11-Eicosenoic acid, methyl ester	422597739	36	2	933	11-Eicosenoic acid, methyl ester	318351884
36	3	850	Methyl eicosa-5,8,11,14,17-pentaenoate	614982271	36	1	859	Methyl eicosa-5,8,11,14,17-pentaenoate	216684087
37	1	916	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	148150542	38	0	826	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	83337270
38	1	853	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	245549701	39	1	787	8,11,14-Eicosatrienoic acid, methyl ester, (Z,Z,Z)-	103684552
39	1	903	Methyl (Z)-5,11,14,17-eicosatetraenoate	192730769	40	3	942	Methyl eicosa-5,8,11,14,17-pentaenoate	526651628
40	4	935	Methyl eicosa-5,8,11,14,17-pentaenoate	882056957	41	0	789	Tetracosanoic acid, methyl ester	79847927

For Ozd isolate (*Chloroidium saccharophilum*)

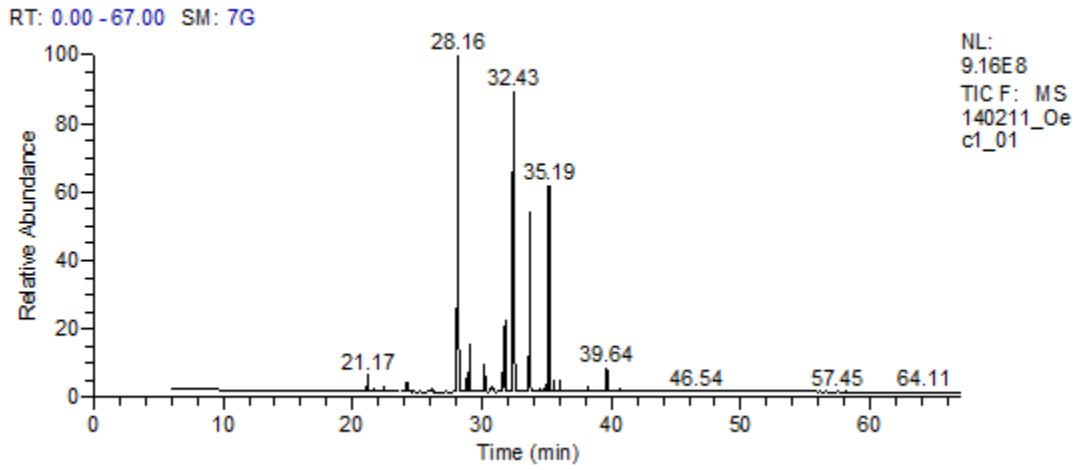


Figure 41. Chromatogram of lipid components of Ozd isolate in the exponential phase.

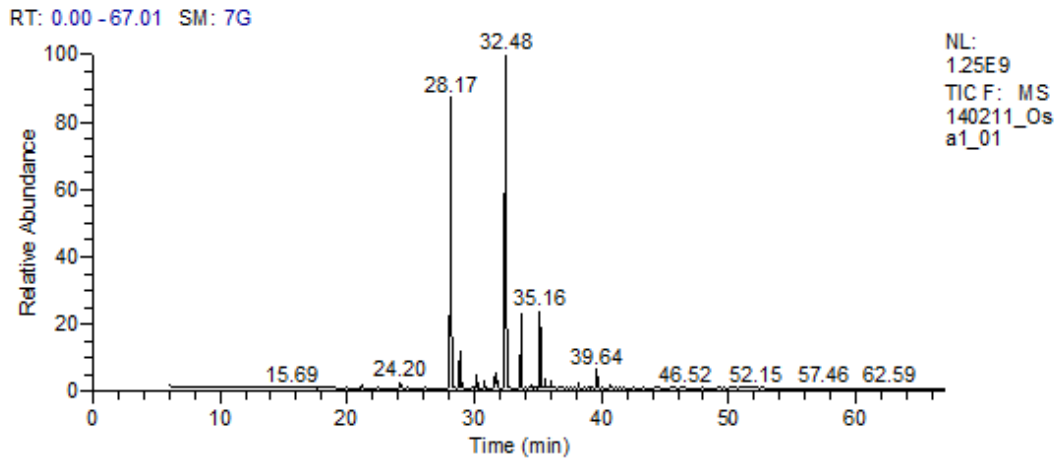


Figure 42. Chromatogram of lipid components of Ozd isolate in the stationary phase.

Table 31. Chemical components of Ozd FAMES with their corresponding retention time and percentage peak area in both exponential and stationary phases.

Exponential phase					Stationary phase				
RT	Area %	RSI	Chemical compound	Peak Area	RT	Area %	RSI	Chemical compound	Peak Area
21	1	924	Decomposed from chlorophyll	238523109	8	0	653	Canthaxanthin	114615105
22	0	614	Canthaxanthin	74326415	24	0	884	Methyl tetradecanoate	115247231
24	1	889	Methyl tetradecanoate	138822009	28	33	932	Hexadecanoic acid, methyl ester	7669169038
28	29	927	Hexadecanoic acid, methyl ester	6152934010	29	3	936	9-Hexadecenoic acid, methyl ester, (Z)-	805184459
29	4	944	9-Hexadecenoic acid, methyl ester, (Z)-	962339149	30	1	929	7,10-Hexadecadienoic acid, methyl ester	312872825
30	2	934	7,10-Hexadecadienoic acid, methyl ester	430215769	31	1	918	4,7,10-Hexadecatrienoic acid, methyl ester	163645315
31	1	660	Canthaxanthin	151979071	32	2	941	Octadecanoic acid, methyl ester	494910654

32	6	903	7,10,13-Hexadecatrienoic acid, methyl ester	1323803893	32	43	935	9-Octadecenoic acid, methyl ester, (E)-	10130539000
32	24	874	8-Octadecenoic acid, methyl ester	5234566257	34	6	923	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	1455215972
34	12	924	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	2661954866	35	6	883	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	1484498910
35	16	888	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	3363454269	36	1	928	11-Eicosenoic acid, methyl ester	193651367
36	1	918	11-Eicosenoic acid, methyl ester	180044330	36	1	851	Methyl eicosa-5,8,11,14,17-pentaenoate	126981951
36	1	857	Methyl eicosa-5,8,11,14,17-pentaenoate	159233978	38	0	850	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	92897667
38	0	670	Canthaxanthin	78567365	40	2	935	Methyl eicosa-5,8,11,14,17-pentaenoate	368015003
40	2	931	Methyl eicosa-5,8,11,14,17-pentaenoate	337431733	41	0	737	Pentacosanoic acid, methyl ester	72876209

For Jod isolate (*Micractinium reisseri*)

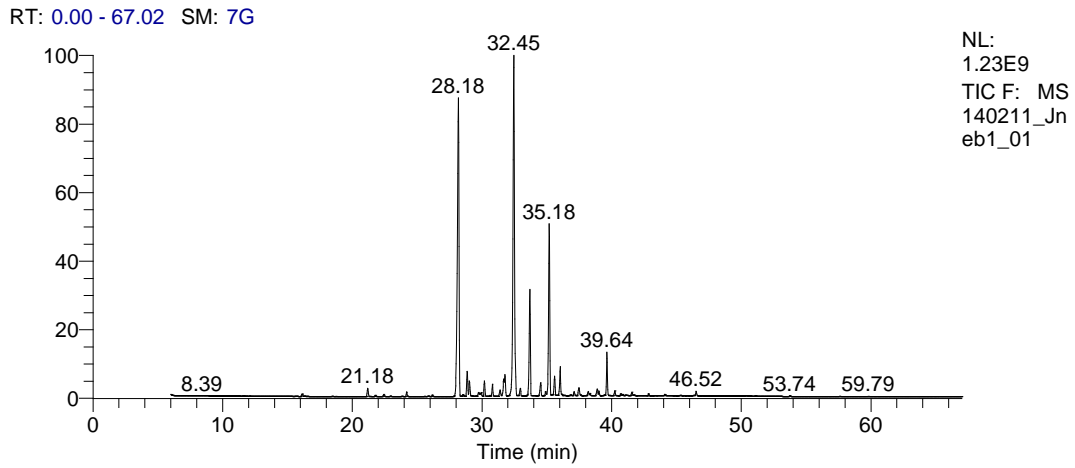


Figure 43. Chromatogram of lipid components of Jod isolate in the exponential phase.

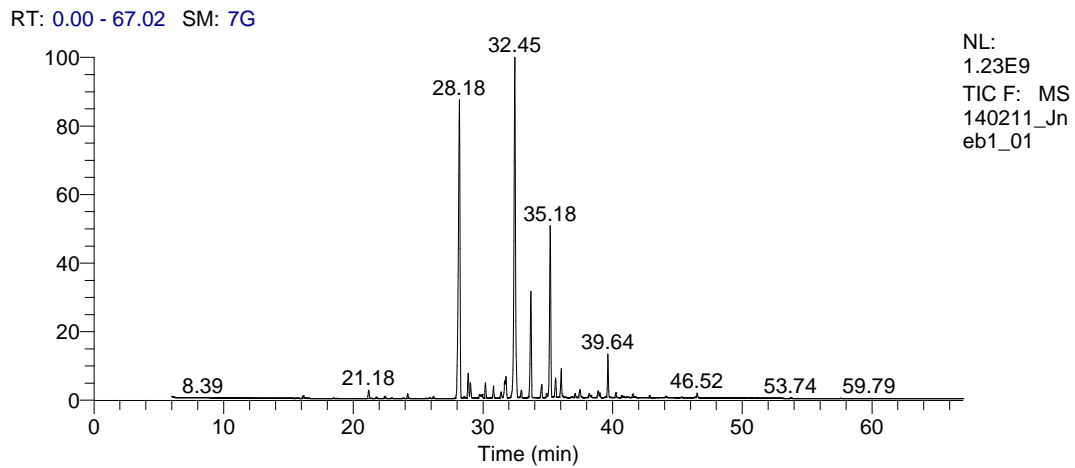


Figure 44. Chromatogram of lipid components of Jod isolate in the stationary phase.

Table 32. Chemical components of Jod FAMES with their corresponding retention time and percentage peak area in both exponential and stationary phases.

Exponential phase					Stationary phase				
RT	Area %	RSI	Chemical compound	Peak Area	RT	Area %	RSI	Chemical compound	Peak Area
28.16	28.92	934	Hexadecanoic acid, methyl ester	9021852617	28	28.92	934	Hexadecanoic acid, methyl ester	9021852617
28.88	2.64	934	9-Hexadecenoic acid, methyl ester, (Z)-	825150711	29	2.64	934	9-Hexadecenoic acid, methyl ester, (Z)-	825150711
30.19	1.63	924	7,10-Hexadecadienoic acid, methyl ester	508312724	30	1.63	924	7,10-Hexadecadienoic acid, methyl ester	508312724
30.82	0.81	910	4,7,10-Hexadecatrienoic acid, methyl ester	251653312	31	0.81	910	4,7,10-Hexadecatrienoic acid, methyl ester	251653312
31.72	2.94	808	8,11,14-Eicosatrienoic acid, methyl ester	918103517	32	2.94	808	8,11,14-Eicosatrienoic acid, methyl ester	918103517
32.45	34.34	851	8-Octadecenoic acid, methyl ester	10714776519	32	34.34	851	8-Octadecenoic acid, methyl ester	10714776519

32.95	0.58	861	Tetratetracontane	181364623	33	0.58	861	Tetratetracontane	181364623
33.69	7.24	926	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	2259809152	34	7.24	926	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	2259809152
34.51	0.99	802	Tetratetracontane	308589901	35	0.99	802	Tetratetracontane	308589901
35.18	12.41	893	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	3871935510	35	12.41	893	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	3871935510
35.6	1.34	944	11-Eicosenoic acid, methyl ester	416842401	36	1.34	944	11-Eicosenoic acid, methyl ester	416842401
36.03	2.05	805	Methyl eicosa-5,8,11,14,17-pentaenoate	640470347	36	2.05	805	Methyl eicosa-5,8,11,14,17-pentaenoate	640470347
37.48	0.67	882	Tetratetracontane	209819412	37	0.67	882	Tetratetracontane	209819412
38.89	0.76	869	Tetratetracontane	235675523	39	0.76	869	Tetratetracontane	235675523
39.64	2.67	935	Methyl eicosa-5,8,11,14,17-pentaenoate	833459522	40	2.67	935	Methyl eicosa-5,8,11,14,17-pentaenoate	833459522

BIBLIOGRAPHY

- [1]. 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.
- [2]. Hossain, A. B. M. S., Salleh, A., Boyce, A. N., chowdhury, P., & Naquiuddin, M. (2008). Biodiesel fuel production from algae as renewable energy. *American Journal of Biochemistry and Biotechnology*, *4*(3), 250-254. doi:10.3844/ajbbbsp.2008.250.254
- [3]. Singh, S., Kate, B. N., & Banerjee, U. C. (2005). Bioactive compounds from cyanobacteria and microalgae: an overview. *Critical reviews in biotechnology*, *25*(3), 73-95.
- [4]. Zheng, Y., Li, T., Yu, X., Bates, P. D., Dong, T., & Chen, S. (2013). High-density fed-batch culture of a thermotolerant microalga *Chlorella sorokiniana* for biofuel production. *Applied Energy*, *108*, 281-287.
- [5]. Mutanda, T., Ramesh, D., Karthikeyan, S., Kumari, S., Anandraj, A., & Bux, F. (2011). Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. *Bioresource Technology*, *102*(1), 57-70. doi:10.1016/j.biortech.2010.06.077
- [6]. Henriques, M., Silva, A., & Rocha, J. (2007). Extraction and quantification of pigments from a marine microalga: a simple and reproducible method. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology Formatex*, 586-593.
- [7]. Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bézier, A., Lambert, B., Pugin, A. (2003). Laminarin elicits defense responses in grapevine and induces protection against botrytis cinerea and plasmopara viticola. *Molecular Plant-Microbe Interactions : MPMI*, *16*(12), 1118

- [8]. El-Kassas, H. Y., & Khairy, H. M. (2009). A trial for biological control of a pathogenic fungus (*Fusarium solani*) by some marine microorganisms. *Am-Euras J Agric Environ Sci*, 5, 434-440
- [9]. Subramanian, S., Sangha, J. S., Gray, B. A., Singh, R. P., Hiltz, D., Critchley, A. T., & Prithiviraj, B. (2011). Extracts of the marine brown macroalga, *ascophyllum nodosum*, induce jasmonic acid dependent systemic resistance in *arabidopsis thaliana* against *pseudomonas syringae* pv. *tomato DC3000* and *sclerotinia sclerotiorum*. *European Journal of Plant Pathology*, 131(2), 237-248.
doi:10.1007/s10658-011-9802-6
- [10]. Hussien, M. Y., Abd El-All A., M., & Mostafa S. S. M. (2009). Bioactivity of algal extracellular byproducts on *Cercospora* leaf spot disease, growth performance and quality of sugar beet. *Recent Technologies in Agriculture in Egypt*, 4 th Conference.
- [11]. Volk, R. B., & Furkert, F. H. (2006). Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth. *Microbiological Research*, 161(2), 180-186.
- [12]. Lundquist, T. J., Woertz, I. C., Quinn, N. W. T., & Benemann, J. R. (2010). A realistic technology and engineering assessment of algae biofuel production. *Energy Biosciences Institute*, 1.
- [13]. 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.
- [14]. Provan, J., Murphy, S., & Maggs, C. A. (2004). Universal plastid primers for Chlorophyta and Rhodophyta. *European Journal of Phycology*, 39(1), 43-50.
- [15]. 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.

- [16]. Alwathnani, H. A., & Perveen, K. (2014). Biological control of fusarium wilt of tomato by antagonist fungi and cyanobacteria. *African Journal of Biotechnology*, 11(5), 1100-1105
- [17]. Huleihel, M., Ishanu, V., Tal, J., & Arad, S. (. (2001). Antiviral effect of red microalgal polysaccharides on herpes simplex and varicella zoster viruses. *Journal of Applied Phycology*, 13(2), 127-134. doi:10.1023/A:1011178225912
- [18]. Abdel-Raouf, N., Al-Homaidan, A. A., & Ibraheem, I. B. M. (2012). Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences*, 19(3), 257-275.
- [19]. Anonymous. (2011), Biotechnology; Elsevier science and technology notes report - comprehensive biotechnology, 2nd edition. edition no. 2. *Biotech Business Week*, pp. 28.
- [20]. Gehl, K. A., Colman, B., & Sposato, L. M. (1990). Mechanism of inorganic carbon uptake in *Chlorella saccharophila*: the lack of involvement of carbonic anhydrase. *Journal of experimental botany*, 41(11), 1385-1391.
- [21]. Ogbonda, K. H., Aminigo, R. E., & Abu, G. O. (2007). Influence of temperature and pH on biomass production and protein biosynthesis in a putative *Spirulina* sp. *Bioresource technology*, 98(11), 2207-2211.
- [22]. Borowitzka, M. A. (1999). Commercial production of microalgae: ponds, tanks, and fermenters. *Progress in industrial microbiology*, 35, 313-321.
- [23]. Richmond, A. (Ed.). (2008). *Handbook of microalgal culture: biotechnology and applied phycology*. John Wiley & Sons.
- [24]. 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.
- [25]. Chisti, Y. (2008). Biodiesel from microalgae beats bioethanol. *Trends in biotechnology*, 26(3), 126-131

- [26]. 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.
- [27]. Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., & Tredici, M. R. (2009). 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.
- [28]. Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology advances*, 25(3), 294-306.
- [29]. Greenwell, H. C., Laurens, L. M. L., Shields, R. J., Lovitt, R. W., & Flynn, K. J. (2010). Placing microalgae on the biofuels priority list: a review of the technological challenges. *Journal of the Royal Society Interface*, 7(46), 703-726.
- [30]. Huang, G., Chen, F., Wei, D., Zhang, X., & Chen, G. (2010). Biodiesel production by microalgal biotechnology. *Applied energy*, 87(1), 38-46.
- [31]. 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.
- [32]. 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.
- [33]. González López, C. V., 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.
- [34]. Barbarino E. and Louren S.O. (2005) "An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae" *Journal of Applied Phycology* Vol. 17, pp. 447–460
- [35]. Lavens, P., & Sorgeloos, P. (1996). *Manual on the production and use of live food for aquaculture* (No. 361). Food and Agriculture Organization (FAO).

- [36]. Benemann, J. R. (1979). Production of nitrogen fertilizer with nitrogen-fixing blue-green algae. *Enzyme and Microbial Technology*, 1(2), 83-90.
- [37]. Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews*, 14(1), 217-232.
- [38]. 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
- [39]. 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.
- [40]. Simon, D., & Helliwell, S. (1998). Extraction and quantification of chlorophyll a from freshwater green algae. *Water research*, 32(7), 2220-2223.
- [41]. 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.
- [42]. Hosikian, A., Lim, S., Halim, R., & Danquah, M. K. (2010). Chlorophyll extraction from microalgae: A review on the process engineering aspects. *International journal of chemical engineering*, 2010.
- [43]. Fernández-Reiriz, M. J., Perez-Camacho, A., Ferreiro, M. J., Blanco, J., Planas, M., Campos, M. J., & Labarta, U. (1989). Biomass production and variation in the biochemical profile (total protein, carbohydrates, RNA, lipids and fatty acids) of seven species of marine microalgae. *Aquaculture*, 83(1), 17-37.
- [44]. 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.
- [45]. Lee R.E. (2008). *Phycology*, 4th edition, Cambridge University Press, UK.

- [46]. Andersen, R. A. (Ed.). (2005). *Algal culturing techniques*. Academic press.
- [47]. Arts, M. T., Brett, M. T., & Kainz, M. J. (2009). Lipids in aquatic ecosystems.
- [48]. Borowitzka, M. A. (1995). Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of Applied Phycology*, 7(1), 3-15.
- [49]. 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.,
- [50]. Kulik, M. M. (1995). The potential for using cyanobacteria (blue-green algae) and algae in the biological control of plant pathogenic bacteria and fungi. *European Journal of Plant Pathology*, 101(6), 585-599.
- [51]. Kumar, A.C., Dhanam, M., & Sowjanya. (1993). Pathogenicity of *Microcoleus* spp. (Oscillatoriaceae), to plant and soil nematodes. *Indian Journal Nematol.*, 23, 8-9.
- [52]. Cai, T., Park, S. Y., & Li, Y. (2013). Nutrient recovery from wastewater streams by microalgae: Status and prospects. *Renewable and Sustainable Energy Reviews*, 19, 360-369
- [53]. FAO (2010). *Oilgae Guide to Algae-based Wastewater Treatment: A Sample Report*. Retrieved from < http://www.fao.org/uploads/media/0911_Oilgae_-_Wastewater_Treatment_Using_Algae_Report_Preview.pdf>
- [54]. Khan, Z., Kim, Y. H., Kim, S. G., & Kim, H. W. (2007). Observations on the suppression of root-knot nematode (*Meloidogyne arenaria*) on tomato by incorporation of cyanobacterial powder (*Oscillatoria chlorina*) into potting field soil. *Bioresource technology*, 98(1), 69-73.
- [55]. Omar, H. H., Gumgumji, N. M., Shiek, H. M., El-Kazan, M. M., & El-Gendy, A. M. (2012). Inhibition of the development of pathogenic fungi by extracts of some marine algae from the red sea of Jeddah, Saudi Arabia. *African Journal of Biotechnology*, 11(72), 13697-13704.

- [56]. Iqbal S. & Fatima B. (1999). Studies on aqueous extract of three green algae as an elicitor for plant defense mechanism. *Pakistan Journal*, 31(1), 193-198.
- [57]. 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.
- [58]. Romay, C., Armesto, J., Ramirez, D., González, R., Ledon, N., & García, I. (1998). Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. *Inflammation Research : Official Journal of the European Histamine Research Society ... [Et Al.]*, 47(1), 36-41. doi:10.1007/s000110050256
- [59]. Tredici, M. (2000). Edited by ZVI COHEN (editor). chemicals from microalgae. taylor & francis, london, 1999, 419 pp. *European Journal of Phycology*, 35(3), 305-305. doi:10.1017/S0967026200002791
- [60]. Mundt, S., Kreitlow, S., Nowotny, A., & Effmert, U. (2001). Biochemical and pharmacological investigations of selected cyanobacteria. *International Journal of Hygiene and Environmental Health*, 203(4), 327-334. doi:10.1078/1438-4639-00045
- [61]. Demirbas, A. (2011). Biodiesel from oilgae, biofixation of carbon dioxide by microalgae: A solution to pollution problems. *Applied Energy*, 88(10), 3541-3547.
- [62]. Gaurav, K., Srivastava, R., & Singh, R. (2013). Exploring Biodiesel: Chemistry, Biochemistry, and Microalgal Source. *International Journal of Green Energy*, 10(8).
- [63]. Saunders, G. W., & Kucera, H. (2010). An evaluation of rbcL, tufA, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. *Cryptogamie. Algologie*, 31(4), 487-528.
- [64]. Hall, J. D., Fucikova, K., Lo, C., Lewis, L. A., & Karol, K. G. (2010). An assessment of proposed DNA barcodes in freshwater green algae. *Cryptogamie. Algologie*, 31(4), 529-555

- [65]. Moulton, T. P., Sommer, T. R., Burford, M. A., & Borowitzka, L. J. (1987, January). Competition between *Dunaliella* species at high salinity. In *Twelfth International Seaweed Symposium* (pp. 107-116). Springer Netherlands.
- [66]. Jaulneau, V., Lafitte, C., Jacquet, C., Fournier, S., Salamagne, S., Briand, X., ... & Dumas, B. (2010). Ulvan, a sulfated polysaccharide from green algae, activates plant immunity through the jasmonic acid signaling pathway. *BioMed Research International*, 2010.
- [67]. Mercier, L., Lafitte, C., Borderies, G., Briand, X., Esquerré-Tugayé, M.-T. and Fournier, J. (2001), The algal polysaccharide carrageenans can act as an elicitor of plant defence. *New Phytologist*, 149: 43–51.
- [68]. Lee, J., Yoo, C., Jun, S., Ahn, C., & Oh, H. (2010). Comparison of several methods for effective lipid extraction from microalgae. *Bioresource Technology*, 101(1, Supplement), S75-S77.
- [69]. Surendhiran, D., & Vijay, M. (2012). Microalgal biodiesel-A comprehensive review on the potential and alternative biofuel. *Research Journal of Chemical Sciences* ISSN, 2231, 606X.
- [70]. Chen, C., Zhao, X., Yen, H., Ho, S., Cheng, C., Lee, D. (2013). Microalgae-based carbohydrates for biofuel production. *Biochemical Engineering Journal*, 78(0), 1-10.
- [71]. Koh, M. Y., & Mohd. Ghazi, T. I. (2011). A review of biodiesel production from *Jatropha curcas* L. oil. *Renewable and Sustainable Energy Reviews*, 15(5), 2240-2251.

- [72]. Achten, W. M. J., Verchot, L., Franken, Y. J., Mathijs, E., Singh, V. P., Aerts, R. (2008). Jatropha bio-diesel production and use. *Biomass and Bioenergy*, 32(12), 1063-1084.
- [73]. Axelsson, M., & Gentili, F. (2014). A single-step method for rapid extraction of total lipids from green microalgae. *PLoS One*, 9(2)
doi:<http://dx.doi.org/10.1371/journal.pone.0089643>
- [74]. Strickland, J. D. H., & Parsons, T. R. A practical handbook of seawater analysis, 1972. *Fisheries Research Board of Canada, Ottawa*, 310.
- [75]. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J biol chem*, 193(1), 265-275.
- [76]. Kellam, S. J., & Walker, J. M. (1989). Antibacterial activity from marine microalgae in laboratory culture. *British Phycological Journal*, 24(2), 191-194.
- [77]. 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
- [78]. Fitzgerald, G. P., & Nelson, T. C. (1975). Extractive and enzymatic analyses for limiting or surplus phosphorus in algae. *Journal of Phycology*, 11(s1), 32-37.
- [79]. Fogg, G. E. (1987). *Algal cultures and phytoplankton ecology*. Univ of Wisconsin Press <http://www.marine.csiro.au/microalgae/methods/Growth%20rate.htm>
- [80]. Hoshina, R., Iwataki, M., & Imamura, N. (2010). *Chlorella variabilis* and *Micractinium reisseri* sp. nov. (Chlorellaceae, Trebouxiophyceae): Redescription of the endosymbiotic green algae of *Paramecium bursaria* (Peniculia, Oligohymenophorea) in the 120th year. *Phycological research*, 58(3), 188-201.

- [81]. Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., & Tredici, M. R. (2009). 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.
- [82]. 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>
- [83]. 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*.
- [84]. Lardon, L., Hélias, A., Sialve, B., Steyer, J., & Bernard, O. (2009). Life-cycle assessment of biodiesel production from microalgae. *Environmental Science & Technology*, 43(17), 6475.
- [85]. 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.
- [86]. Anonymous (1999). Digesdahl Digestion Apparatus. Models 23130-20, -21. Instrument manual. Retrieved from < <http://www.hach.com/digesdahl-digestion-apparatus-115-vac/product-downloads?id=7640450051>>.
- [87]. Robles Medina, A., Esteban Cerdán, L., Giménez Giménez, A., Camacho Páez, B., Ibáñez González, M. J., & Molina Grima, E. (1999). Lipase-catalyzed esterification of

glycerol and polyunsaturated fatty acids from fish and microalgae oils. *Journal of Biotechnology*, 70(1), 379-391. doi:10.1016/S0168-1656(99)00091-7.