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Chlorella sp. as a Source of Biodiesel and By-Products: An Integral Study of Med-Algae Project; Part A

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Abstract: An integral lab study of Med-algae project was done on Chlorella sp. to examine its potential as a feedstock for biodiesel production, and the feasibility to use the delipidated cell ruminants for by-products as well. Chlorella sp. was cultivated in 25L Flat Panel Photobioreactor under best growth conditions. The highest growth activity was observed during the period from July to September, 2014, when the algal oil and many other valuable compounds were also extracted. The maximum cell dry wt. was 4g/l and the growth rate was 2/day at pH, 8.5; nitrate, 0.4g/l; salinity 45g/l; and dilution, 30%. The given results showed that ashes content was 4 mg/l, chlorophyll a was 10.47 mg/l, and the carotenoids were 6.12 mg/l. In addition, lipid content was 20% of the dry wt. Oil characterization was studied based on its acid value, saponification value, and the molecular weight. Detailed analysis of fatty acid composition and characterization of biodiesel are provided. However, in delipidated algal cells, the amounts of protein and carbohydrate were 100.50 mg/l and 15.5mg/l respectively. Analysis of amino acid pool indicated the presence of 13 fractions, mostly are essential fractions with appropriate amounts. This study represents a trend driving Chlorella sp.-based bio-refinery into economical production.
*This study has been organized on the basis of a submitted lab scale research component of Med-algae project.
Key Words: Biodiesel, By-Products, *Chlorella* sp.

Introduction

The global problems associates with the depletion of oil are searched by many authors[1, 2, 3, 4]. Egypt, as a developing country, needs to increase the national economy, individual's income, as well as new technologies for energy demands. Biofuels are renewable energies and recently they have received a considerable attention since they are non-toxic, biodegradable, and environmentally friendly[5]. Therefore practice of biofuel as an alternative energy could be a good strategy for our nation, if it was economically-based solution.

Biodiesel, a common alternative fuel, is a convenient energy carrier compound. Chemically, biodiesel is composed of a mixture of methylated, or ethylated, fatty esters[6, 7]. Biodiesel could be produced by a conversion process of vegetable and microbe's oils, animal fats, or waste cooked oil [6, 7, 8]. However, biodisel derived from algal lipid has been got a great attention in the present century[9].

Microalgae are tiny photosynthetic microbes, either being prokaryotic or eukaryotic organisms, that can convert solar energy into valuable compounds [3, 10, 11]. Unlike other oil crops, microalgae are characterized by having a high photosynthetic efficiency [12] and short doubling time[13, 14]. They are also able to store large amounts of lipids[15]. These reasons collectively make algae an ideal source for biodiesel production. Furthermore, algal biomass can be converted into high valuable compounds for food and animal feeding [16, 17].

Microalgae have high added value food such as polyunsaturated fatty acids[18], pigments and pharmaceutical products. In addition, algae can play an important role in aquaculture business[3,19. 20].

Utilization of microalgal biomass contributes in food security, facilitates cost-effective biofuel production, and reduces greenhouse gas production as well [21, 22].

Chlorella is a widespread genus that found in almost habitats [23]. These microalgae belong to the class Chlorophyceae and have good potential to accumulate more than 20% lipids, such as C 18:1, C 16:0 and C 18:3[24]. *Chlorella* algae are well known for its high nutritional value [25, 26], as they are employed in a variety of biotechnological applications as well [27, 16, 28].

The project "Production of biodiesel from microalgae in selected Mediterranean Countries" Med-algae project (http://www.med-algae.eu) is a new technology project which can contribute to the goals of the EU strategy on "Climate change and energy. The project funded by the Programme ENPI European Neighbourhood and Partnership Instrument (ENPI) - Mediterranean Sea Basin Joint Operational Programme. The consortium is consisted of 12 organisations: research organizations, academic institutions, energy agencies, private organizations from 6 countries: Cyprus, Greece, Italy, Malta, Lebanon and Egypt, table 1. Alexandria University, represented by the Faculty of Science, is a structural component of this international Mediterranean project.

Chlorella sp. has been chosen to be the experimental organism in each participated country of Med-algae project. The aim was to examine its potential as a source of biodiesel and other by-products in different Mediterranean regions. The objectives were to optimize and up-scaling the culture of the alga to recognize the region (s) with best results for these applications.

In the present study, *Chlorella* sp. was cultivated in 25L Flat Panel Photobioreactor to maximize biodiesel production and to examine the feasibility to refine the algal cell for other applications, at lab scale production.

Table 1: MED-ALGAE Project partners

Institute	Country
Agricultural Research Institute (ARI) COORDINATOR	Cyprus
Cyprus Energy Agency (CEA)	Cyprus
Malta Intelligent Energy Management Agency (MIEMA)	Malta
Fondazzjoni Temi Zammit (FTZ)	Malta
Studio Sardo (SS)	Italy
National & Kapodistrian University of Athens (NKUA)	Greece
National Research Centre (NRC)	Egypt
The Lebanese Association for Energy Saving & for Environment (ALMEE)	Lebanon
Faculty of Science, Alexandria University (ALEX)	Egypt
American University of Beirut (AUB)	Lebanon
National Technical University Of Athens (NTUA)	Greece
Universita' Mediterranea Di Reggio Calabria (UMRC)	Italy

Materials and Methods

Preliminary experiments: *Chlorella* sp. was kindly provided by Dr. I. Tzovenis, NKUA, originally was isolated from Crete, Greece. A lot of lab experiments were carried out on the strain to examine the effect of different variables, with different concentrations, of pH, salinity, nitrate and dilution - under the influence of different seasons of 2013-2014 - in order to examine the best season and growth promoting concentration on the density of *Chlorella* sp. cultivated in one liter flask.

Maintenance of *Chlorella* sp. culture: based on the given results of the previous experiments, *Chlorella* sp. culture was up-scaled in 25L Flat Panel Photobioreactor (FPP) with f2 growth medium. The dimension of FPP was 100 cm x 50 cm x 5 cm. The starter algae inoculum was 2.5 L. Aeration was supplied by bubbling air at constant pressure. Fluorescent lamps with light intensity of 2500 Lux were placed on one side of FPP, and were measured by the light meter. The light/ dark cycle was 12/12 h. The culture was maintained with the best previously examined conditions of pH: 8.5; nitrate conc: 4mg/l; salinity conc: 45g/l; and dilution of 30%, during the summer season (July-September), with temperature of 24 ± 1°C.

Growth measurements: cells of *Chlorella* sp. were counted daily by the hemocytometer, and the growth rate was calculated. At the maximum growth rate, dry weight of algal cells was determined gravimetrically using oven at 60°C.

Light microscopy examination: 0.5 ml culture sample was provided for light microscopy (Optika, 4083.B5) examination.

Ashes Determination: ash content of the algal dry weight was obtained by burning the cells in a muffle furnace at 550 °C.

Pigments analyses: chlorophyll *a* and total carotenoids content were determined spectrophotometrically 29, 30).

Lipid content: cells of *Chlorella* sp. were harvested at the late exponential growth phase using the centrifuge (Hermle 2300). Cell pellets were extracted in a separator funnel with chloroform/methanol (2/1:v/v) (31). After complete extraction, the chloroform layer containing the lipids was evaporated by rotary evaporator and the total crude lipids were determined gravimetrically. The cell residues (delipidated cell ruminants) were separated and washed twice by distilled water and kept in the refrigerator for further analyses of carbohydrate, protein, and amino acids.

Oil extraction and characterization: dried algal cells were extracted for its oil (crude triglycerides) using soxhlet with *n*-hexane at 60 °C for 8h. Both acid and Saponification values were determined (32), and the molecular weight of the oil was calculated (33).

Analysis of fatty acid methyl esters (FAMEs) composition: algal oil was estrified (34). The FAMEs were analyzed using GC equipped with Flame Ionization detector and HP (Hewlett Packard) 6890GC Model. The carrier gas was nitrogen at a flow rate 1 ml/ min and the injector and detector temperatures were 220°C and 280°C respectively. The FAMEs were identified by comparing their retention times with those of the standards.

Estimation of Carbohydrate: total carbohydrate of delipidated cells was determined, using UV spectrophoto meter, at 490 nm (35).

Estimation of protein and amino acids: total protein of delipidated cells was estimated according to (36). Amino acid composition was determined by acid hydrolysis method (37), and analyzed by amino acid analyzer (LC 3000, Hamburg, Germany).

Results

Chlorella sp. was cultivated in the FPP under the best growth promoting conditions of pH: 8.5; nitrate conc: 4mg/l; salinity conc: 45g/l; and dilution of 30%, during the summer season (July-September), with f2 growth medium, Fig. 1. The strain was studied microscopically and the image showed the unicellular globular none flagellated microalga with one parietal chloroplast, Fig. 2.

Fig. 1: *Chlorella* sp. cultivated in FPP under best growth promoting conditions of pH: 8.5, Salinity: 45 g/l, Nitrate: 0.4 g/l, and 30% dilution.

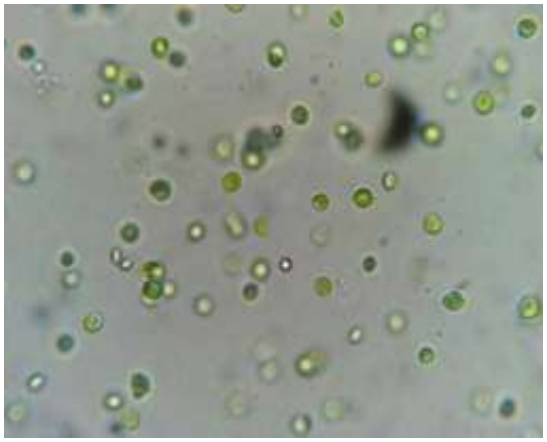

Fig. 2: Light micrograph of *Chlorella* sp. showed a unicellular globular green alga, without flagella. Magnification: x60 at 35mm size.

Results recorded in table 2 showed that the maximum biomass yield, cell growth rate and ashes content of complete cells of *Chlorella* sp. were 4 g/l, 2/day and 4mg/l respectively. The amount of chlorophyll a was 10.47 mg/l, carotenoids were 6.12mg/l, while the crud lipid content was 0.8 g/l of the complete cells of *Chlorella* sp.

Table 2: biomass and bio-analyses of complete and delipidated *Chlorella* sp.

Dry Weight	Growth Rate	Carbohydrate	Protein	Lipid	Chl. a	Carotenoid	ashes
4 g/l	2 / day	15.5 mg/l	100.50 mg /l	0.8 g/l	10.47mg/l	6.12mg/l	4mg/l

Table 3 showed some oil characterization of the examined strain. Acid value was 0.449; saponification value was 50.5, while the molecular weight was 336.26.

Table 3: Oil Characterization of *Chlorella* sp.

Acid Value	Saponification Value	M.W
0.449	50.5	336.26

Analysis of fatty acid methyl esters (biodiesel) demonstrated the presence of saturated, monounsaturated and polyunsaturated fractions, with carbon chain lengths ranged between C6 – C20. The most amounts were concentrated in C16:0 and C18:0 which represent more than 61% of FAMEs pool. C14:1, C16:1, C18:1 and C18:2 were also detected. The ratio of saturated/ unsaturated fatty ester fractions was 3.8, while MUFA/ PUFA was 3:1 (table 4).

Table 4: FAMEs comprising biodiesel of *Chlorella sp.*

Fatty acid	Dry wt %
Saturated	
C 6:0	0.3
C 8:0	4.0
C 10:0	0.6
C 11:0	1.8
C 12:0	0.4
C 13:0	0.4
C 14:0	1.6
C 16:0	41.1
C 17:0	1.1
C 18:0	20.7
C 20:0	7.3
Monounsaturated	
C 14:1	1.1
C 16:1	4.8
C 18:1ω9c	9.0
Polyunsaturated	
C18:2ω6c	5.8
Total saturated	79.3
Total unsaturated	20.7

On the other hand, the analyses of delipidated cell ruminants showed that the amount of crude protein and carbohydrate were 100.50 mg/l and 15.5mg/l respectively, table 2. Furthermore, the amino acid pool was composed of thirteen amino acids in delipidated cell (table 5). The highest concentration was found in isoleucine (15.75%), followed by arginine (14.74%). Lysine, glycine, serine, and tyrosine are found in more than 9%. The glutamic acid was 8.61%, followed by aspartic acid (7.21%). The concentration of alanine, valine, proline, and cyctine is more than 3%. The least amount was threonine; 1.16%.

Table 5: Amino acid composition of delipidated *Chlorella* sp.

Amino acid	Dry wt %
Arginine	14.74
Lysine	9.53
Alanine	3.31
Threonine	1.16
Glycine	9.61
Valine	3.46
Serine	9.83
Proline	3.66
Isoleucine	15.75
Glutamic	8.61
Aspartic	7.21
Cystine	3.92
Tyrosine	9.21

Discussion

Although microalgae are promising candidates for biodiesel production, the high cost associated with algal biomass production is not economically good strategy for this purpose. Hence, there is an urgent need to refine the algal cell to be utilized for further applications. In this study we have tried to convert the biomass of *Chlorella* sp. into lipids, biodiesel, and lipid associates products such as chlorophyll a and carotenoids. On the other hand, we have also tried to convert the delipidated cell ruminants into compounds with high nutritional values - like carbohydrates, proteins, and amino acids - to achieve full benefits of the studied strain on an economically-based concept.

The results showed that both growth rate and biomass production are found in relatively high values compared to other studies carried on different *Chlorella* strains [26, 38, 39, 40]. As we have mentioned above, the studied strain was cultivated and optimized under the best growth promoting conditions - of pH, nitrate, salinity, and dilution - which are collectively led to increase the growth of *Chlorella* sp. However, the results of ashes, chlorophyll a and total carotenoids are relatively agree with those obtained by [26, 41].

In this study, total lipid content represents 20% of the cell dry weight; a result agrees with [39, 42] for different *Chlorella* algae. For biodiesel production, algae must be characterized by having high biomass productivity and high lipid yield [43]. In this connection, the studied strain appeared a good chose for this purpose.

Concerning the specification of biodiesel, Whyte et al. [44] demonstrated that the ideal length of fatty acid comprising biodiesel is ranged from C10 to C18. In addition, biodiesel properties are connectedly with the degree of unsaturation and the alcohol content of composed fatty esters [1]. Compared with the results here, the chain lengths of FAMEs are ranged from C6 to C20, and the saturated, monounsaturated and polyunsaturated fatty ester fractions were detected.

Several types of fatty acids with different degree of un-saturation have been reported for various microalgae [43, 45, 46, 47, 48]. About 50–65% of FAMEs of microalgae *Chlorella vulgaris*, *Spirulina maxima*, *Nannochloropsis oleabundans*, *Scenedesmus obliquus* and *Dunaliella tertiolecta* are mainly composed of unsaturated fatty acids [49]. However, in the present study fatty acids are mostly saturated; more than 61% of total fatty esters are composed of both Palmitic acid (C16:0) and stearic acid (C18:0), which are known as the most common fatty acids contained in biodiesel [48]. Both acids give good cetane number and oxidative stability to biodiesel [49]. *Chlorella* sp. MCCS 040 is characterized by high saturated fatty acids production, and for this reason the authors have recommended the strain for biodiesel production applications [2].

In the present study, the proportion of sat/unsat is 3.8, while MUFA/ PUFA is 3:1; a mixture gives good viscosity to biodiesel [49, 50]. Moreover, both capric (C10) and myristic (C14) fatty acids – which are known to improve the quality of biodiesel [47] - are detected. Biodiesel must have the right kinds of FAMEs content for a high quality [51].

In this study we have tried to maximize utilization of the examined strain for simultaneous biodiesel production and other applications as well. Therefore, lipid extracted ruminants of the investigated alga was examined for the possibility to be used as a row material of nutrition.

Protein content of complete cell of *Chlorella* sp. (data not shown) was 118.50 mg/l. The results of protein content of delipidated cell is 100.50 mg/l. In other words, the percentage of protein in delipidated cell to the complete one is 84.8%. The de-fatted biomass of *Staurospira* sp. with 19% crude protein replaced 7.5% of corn and soybean meal without affecting the growth performance or health status of broiler chickens [(52)]. Furthermore, carbohydrate content of complete cell of *Chlorella* sp. (data not shown) was 20.6mg/l, while in delipidated cell is15.5mg/l. Consequently the percentage of carbohydrate in lipid extracted ruminant of *Chlorella* sp. is 75.0 % of the complete cell. Based on the given results, lipid extracted biomass of the studied strain is characterized by relatively high amounts of protein and carbohydrate and could be used for other applications like nutrition and animal feeding. Both protein and carbohydrate are molecules that would play an important role in sustainable microalgae-based bio-processes at large scale [(53)]. These highly valuable nutritional compounds are serving in human health [(19, 21)], animal feeding, and aquaculture industry [(54)].

The results revealed that the delipidated *Chlorella* sp. ruminants are richening with a variety of amino acids, with the presence of essential fractions that found with appropriate amounts. The same amino acid fractions was detected in the complete cells of *Chlorella* vulgaris [(21)]. It is well known that amino acids have an important role in human and animal nutrition [(19, 21)]. In addition, amino acids are considered in pharmaceutical, nutraceutical and cosmeceutical applications [(55)].

Conclusions

Based on the given results, *Chlorella* sp. showed good cultivation response in FPP, with high growth rate and high biomass production. Biodiesel-sourced *Chlorella* sp. is characterized by variety of carbon chain lengths, suitable amount of saturated fractions of C16 and C18, besides the presence of C10 and C14 acids; a lot of characters which are collectively lead to produce good quality biodiesel. The results of analyses of delipidated cell ruminants are promising and demonstrate the feasibility to utilize it for further applications like nutrition, animal feeding, and other industries.

For a future work, we need an economical evaluation study to figure out a life cycle assessment of *Chlorella* sp. for a large-scale cultivation and biorefinery process to reduce the cost of the biomass Production. Finally, we can put forward that *Chlorella* sp. could be utilized for simultaneous food and energy security.

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