GRASAS Y ACEITES, 61 (4),
OCTUBRE-DICIEMBRE, 416-422, 2010,
ISSN: 0017-3495
DOI: 10.3989/gya.021610

Enhancement of biodiesel production from different species of algae

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RESUMEN

Mejora de la producción de biodiesel a partir de diferentes especies de algas.

Ocho especies de algas (4 Rhodo, 1 cloro y 1 macroalgas *phaeophycean*, 1 cianobacteria y 1 microalga verde) fueron utilizados para la producción de biodiesel utilizando dos sistemas de extracción con disolventes (hexano/éter (1:1, v/v)) y (Cloroformo / metanol (2:1, v/v)). La evaluación bioquímica de las especies de algas se llevó a cabo mediante la estimación de los porcentajes de biomasa, de lípidos, de biodiesel y de sedimento (glicerina y pigmentos). El sistema extracción con el disolvente hexano/éter (1:1, v/v) produjo una bajada de los porcentaje de lípidos (2.3-3.5% de peso seco), mientras que el sistema de extracción con el disolvente cloroformo/metanol (2:1, v/v) demostró ser más eficaz en la extracción de los lípidos y del biodiésel (2,5 - 12,5% de peso seco) dependiendo de las especies de algas. Los extractos de microalgas verde *Dictyochloropsis splendida* produjeron los mayores porcentaje de lípidos y de biodiésel (12,5 y 8,75%, respectivamente), seguida de la cianobacteria *Spirulina platensis* (9,2 y 7,5% respectivamente). Por otra parte, las macroalgas (rojo, marrón y verde) produce los porcentajes más bajos de biodiesel (0,25 - 3,8%). Los ácidos grasos del biodiesel de *Dictyochloropsis splendida* Geitler se determinaron mediante cromatografía de gases. La producción de lípidos, de glicerol y de biodiesel con microalga verde *Dictyochloropsis splendida* Geitler (el alga más prometedora) fueron notablemente mejorada cuando se aumentó la concentración de sal o en ausencia de nitrógeno, durante su cultivo, con una producción máxima (26,8, 18,9 y 7,9%, respectivamente) en ausencia de nitrógeno.

PALABRAS CLAVE: Biodiesel – Glicerina – Macroalgas – Microalgas – Lípidos totales

SUMMARY

Enhancement of biodiesel production from different species of algae.

Eight algal species (4 *Rhodo*, 1 *chloro* and 1 *phaeophycean* macroalgae, 1 *cyanobacterium* and 1 green microalga) were used for the production of biodiesel using two extraction solvent systems (Hexane/ether (1:1, v/v)) and (Chloroform/methanol (2:1, v/v)). Biochemical evaluations of algal species were carried out by estimating biomass, lipid, biodiesel and sediment (glycerin and pigments) percentages. Hexane/ether (1:1, v/v) extraction solvent system resulted in low lipid recoveries (2.3-3.5% dry weight) while; chloroform/methanol (2: 1, v/v) extraction solvent system was proved to be more efficient for lipid and biodiesel extraction (2.5 – 12.5% dry weight) depending on algal species. The green microalga *Dictyochloropsis splendida* extract produced the highest lipid and biodiesel yield (12.5 and 8.75% respectively) followed by the cyanobacterium *Spirulina platensis* (9.2 and 7.5 % respectively). On the other hand, the macroalgae (red, brown and green) produced the lowest biodiesel yield. The fatty acids of *Dictyochloropsis splendida* Geitler biodiesel were determined using gas liquid chromatography. Lipids, biodiesel and glycerol production of *Dictyochloropsis splendida* Geitler (the promising alga) were markedly enhanced by either increasing salt concentration or by nitrogen deficiency with maximum production of (26.8, 18.9 and 7.9 % respectively) at nitrogen starvation condition.

KEY-WORDS: Biodiesel – Glycerin – Macroalgae – Microalgae – Total lipid.

1. INTRODUCTION

The basic sources of energy are petroleum, natural gas, coal, hydroelectrical and nuclear. The need of energy is increasing continuously due to the increase in population and industrialization. The continued use of petroleum sourced fuels is now widely recognized as unsustainable because of the depletion supplies and the contribution of these fuels to the accumulation of carbon dioxide in the environment leading to increase of global warming. In the last ten years, many studies have been conducted on biofuels for substituting fossil fuels and reduce the greenhouse gas emission (Bastianoni *et al*., 2008). Biodiesel from oil crops, waste cooking oil and animal fat cannot realistically satisfy even a small fraction of the existing demand for transport fuels. Recent researches involved not only the existing renewable sources available from land plants, but also those coming from aquatic systems. Algae (macro and micro) have been taken in consideration as a residual biomass ready to be used for energy purposes. Algae, especially microalgae, were found to be the only source of renewable biodiesel that is capable of meeting the global demand for transport fuels (Chisti, 2007 and 2008). The idea of using algae as a source of fuel is not new (Chisti, 1980 – 1981; Nagle and Lemke, 1990; Sawayama *et al*., 1995), but it is now being taken seriously because of the increasing price of petroleum and more significantly, the emerging concern about global warming that is associated with burning fossil fuels (Gavrilescu and Chisti, 2005). Microalgae can provide several different types of

renewable biofuels which include, methane, biodiesel (methyl esters) and biohydrogen (Gavrilescu and Chisti, 2005; Kapdan and Kargi, 2006; Spolaore *et al*., 2006). Oil productivity of many microalgae greatly exceeds the oil productivity of the best producing oil crops (Shay, 1993).

This work aimed to investigate, estimate and compare the potentiality and sustainability of the use of different algal species belonging to different divisions (macro and microalgae and cyanobacterium) for biodiesel production, using two different extraction solvent systems and comparing the biodiesel content produced by each algal species in both systems. Then, increase biodiesel production from the promising alga using salt stress and nitrogen deficiency conditions.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Pure hexane, chloroform, ethanol, ether, acetone and methanol were purchased from E.Merch Co. (Germany), and distilled before use.

2.2. Algal samples

Macroalgal collection

Four red macroalgal species were used in this investigation: *Jania rubens* (L) lamouroux (Intertidal zone, 5-8 cm), *Galaxaura oblongata* (Ellis et solander) lamouroux (Intertidal zone, 8-11 cm), *Gelidium latifolium* (Grev.) Bornet ex Bornet et Thuret (intertidal zone, 5-10 cm), were collected from the Mediterranean sea at Abu Quir beach, Alexandria, Egypt, during summer season (July, 2009), while *Asparagopsis taxiformis* (Delile) Trevisan (Supralitoral and intertidal zones, 10-19 cm) was collected from El Garam beach, Marsa Matrouh, Egypt, during spring season (April, 2009). Another two macroalgal species, the green *Ulva lactuca* Linnaeus (Intertidal zone, 10-20 cm) and the brown *Colpomenia sinuosa* (Mertens ex Roth) Derbes et Solier (Intertidal zone, 11-19 cm) species were collected from Abu Quir beach, Alexandria, Egypt, during summer season (July, 2009).

The collected algal species (4 red, 1 green and 1 brown macroalgae) once returned to the laboratory (Botany Depart. Fac. of Science, Cairo Univ.) were cleaned from sand and epiphytes, washed with fresh water then air dried, ground and kept in labeled glass bottles till use.

Microalgal cultures

One green microalga, *Dictyochloropsis splendida* Geitler, was isolated from Ain Helwan, Cairo, Egypt, during spring season (March, 2009), identified by Prof. Dr. Sanaa M. M. Shanab, Botany Department, Faculty of Science, Cairo University and cultured on Bold's basal medium (Bischoff and Bold, 1963) at 20°C under 16/8 light/dark cycles and light intensity of 40 µE / m²/s**.** The cyanobacterium, *Spirulina platensis* (Setch. et Gard) Geitler, was cultured on Zarrouk medium (Zarrouk, 1966) and obtained from the Culture Collection of Botany Department, Faculty of Science, Cairo University, Egypt.

Each microalgal species was harvested at the stationary phase (22 day for *Dictyochloropsis* splendida and 25 day for *Spirulina* platensis) by centrifugation (3000 rpm) pellets were finally dried at 60°C for 20 min before extraction

2.3. Stress conditions

Dictyochloropsis splendida Geitler was cultured on Bolds basal medium (Bischoff and Bold, 1963) containing different salt concentrations (2.5, 5, 7.5 and 10 g/l) and nitrate concentrations (25, 12.5, 6.25 and 0.0 g/l). A volume of 10 ml of concentrated algal suspension was mixed with 90 ml of culture media containing sodium chloride or nitrate concentrations. Algal cultures were aerated and incubated at optimal growth conditions (16-8 light/dark cycles, light intensity of 40 µE/m^2/s at 20±1°C) for 22 days. The harvested alga was centrifuge, filtered and dried at low temp. (Less than 60°C) to release water content.

2.4. Algal growth rate

The growth rate of the alga under different salt and nitrate concentrations were determined as chlorophyll and carotenoids content (mg/g dry weight) using the method described by Holden (1965). In briefly, the fresh sample (1.0 g) was grinded in a mortar with acetone (80%) in presence of calcium carbonate (0.1g). The residue was reextracted for several times with acetone, until the solvents were colorless. The combined extract and washings were made up to a known volume (25 ml) and the absorbance was measured at 663, 645 and 452 nm in 1cm quartz cell, against blank (80% aqueous acetone).

2.5. Extraction of oil

Extraction of oil was carried out using two extraction solvent systems to compare the oil content in each case and select the most suitable solvent system for the highest biodiesel yield.

Chloroform /methanol (2:1, v/v) method

A known weight of each ground dried algal species (10 g dry weight) was mixed separately with the extraction solvent mixture; chloroform/methanol (100 ml, 2:1, v/v) for 20 min. using shaker, followed by the addition of mixture of chloroform/water (50 ml, 1:1, v/v) for 10 min. filter and the algal residue was extracted three times by 100 ml chloroform followed by filtration (Fig.1) according to Bligh and Dayer (1959).

Hexane/ether (1:1, v/v) method

A known weight of each ground dried algal species (10 g dry weight) was mixed with the extraction solvent mixture, hexane/ether (100 ml, 1:1, v/v), kept to settle for 24 hrs, followed by filtration (Fig. 1) according to Hossain and Salleh (2008).

2.6. Transesterification and biodiesel production

The extracted oil was evaporated under vaccum to release the solvent mixture solutions using rotary evaporator at 40- 45 °C. Then, the oil produced from each algal species was mixed with a mixture of catalyst (0.25g NaOH) and 24 ml methanol (a process called transesterification, with stirring properly for 20 min. The Mixture was kept for 3hrs in electric shaker at 3000 rpm. (National Biodiesel Board, 2002). After shaking the solution was kept for 16 hrs to settle the biodiesel and the sediment layers clearly. The biodiesel layer was separated from sedimentation by flask separator carefully. Quantity of sediments (glycerin, pigments, etc) was measured. Biodiesel was washed by 5% water many times until it becomes clear then Biodiesel was dried by using dryer and finally kept under the running fan for 12 h. the produced biodiesel was measured (using measuring cylinder), pH was recorded and stored for analysis.

2.7. Analysis of fatty acids in the produced biodisel from *Dictyochloropsis splendida* Geitler using GLC

The GLC analysis was carried out with a Pro-GC gas chromatography, with a dual flame ionization detector. The glass column (1.5 m x 4 mm) was packed with 1% OV-17. Temperatures of injector and detector were 250°C and 300°C, respectively. The column was hold at 200°C for 3min. then Programmed from 200 to 240°C (at rate of 10°C/min). Nitrogen was

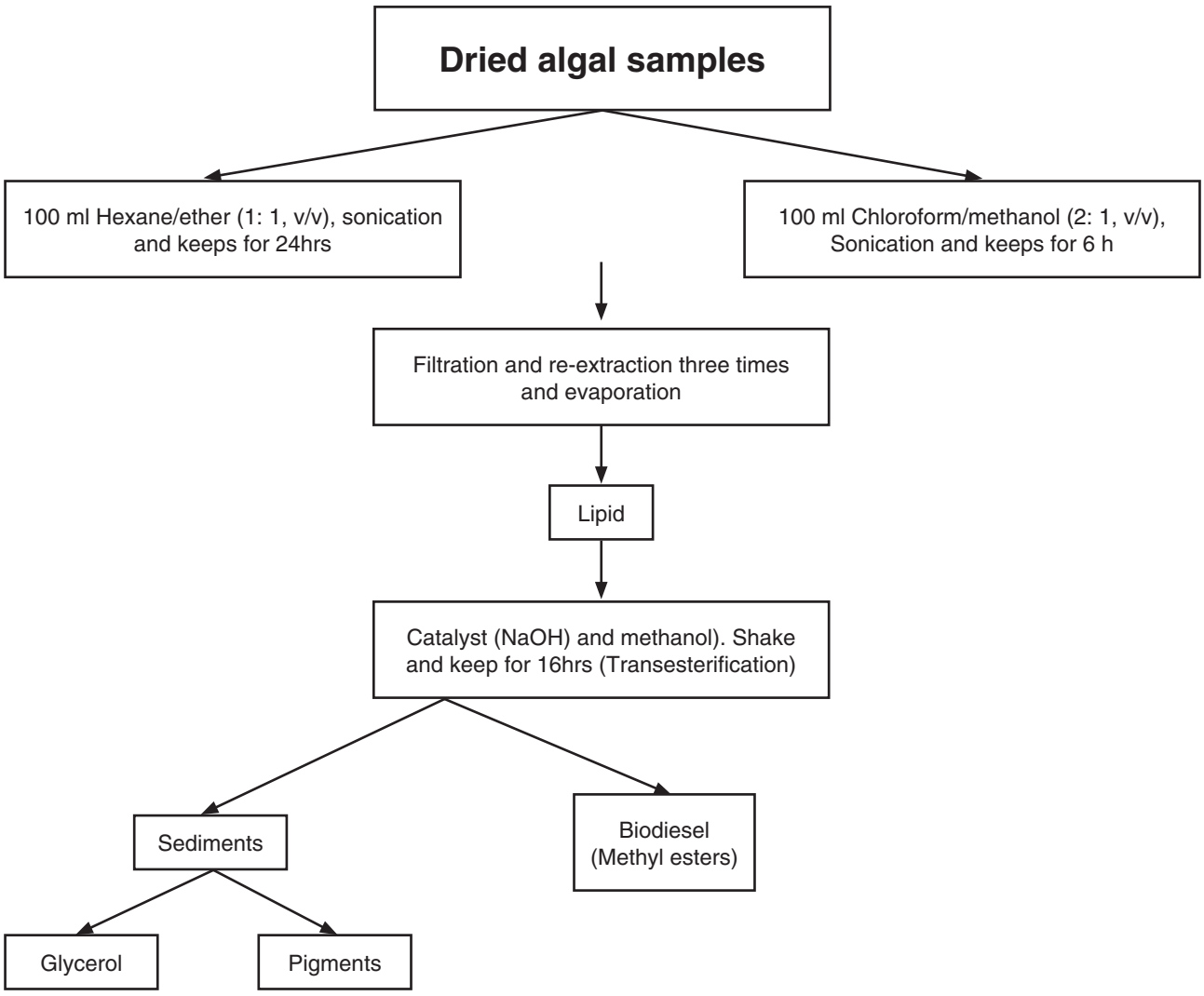

Figure 1.
Procedure (steps) of lipid and biodiesel production from algal sample using two solvent systems (hexane/ ether (1:1, v/v) and chloroform/ methanol (2:1, v/v)).

Table 1
Comparison between lipid percentages (%) produced by eight algal species using hexane/ether (1:1, v/v) and chloroform/methanol (2:1, v/v) extraction systems.

Algal species	Chloroform/methanol (2:1, v/v)	Hexane/ether (1:1, v/v)
Jania rubens	4.4±0.12	2.8±0.04
Galaxaura oblongata	2.5±0.09	2.4±0.01
Gelidium latifolium	3.0±0.0	3.1±0.02
Asporagopsis taxiformis	4.1±0.08	3.4±0.05
Ulva lactuca	4.2±0.1	3.5±0.1
Colpomenia sinuosa	3.5±0.05	2.3±0.03
Dictyochloropsis splendida	12.5±0.23	2.4±0.14
Spirulina platensis	9.2±0.25	3.0±0.10
LSD	0.3261	0.3261

Each value is presented as mean of triplet treatments, LSD: Least significant difference at $P \leq 0.05$ according to Duncan's multiple range test.

Table 2
Total lipid, biodiesel, sediments percentage and biodiesel color of different algal species using the extraction solvent system Chloroform/methanol (2:1, v/v)

Algal sp.	Lipid %	Biodiesel%	Sediment %	Biodiesel color
Jania rubens	4.4±0.12	0.25±0.01	4.2^{a} ± 0.05	Light brown
Galaxaura oblongata	2.5±0.09	2.06±0.02	0.08±0.0	Light green
Gelidium latifolium	3.0±0.0	1.3±0.0	1.6±0.01	yellow
Asporagopsis taxiformis	4.1±0.08	3.64^{c} ± 0.10	0.40±0.01	Dark green
Ulva lactuca	4.2±0.1	3.8±0.12	0.44±0.0	Light green
Colpomenia sinuosa	3.5±0.05	3.1±0.05	0.31±0.05	yellow
Dictyochloropsis splendida	12.5±0.23	8.75±0.24	3.75±0.08	colorless
Spirulina platensis	9.2±0.25	7.5±0.30	1.66±0.06	Light green
LSD	0.3261	0.3314	0.1786	

Each value is presented as mean of triplet treatments, LSD: Least significant difference at $P \leq 0.05$ according to Duncan's multiple range test.

a carrier gas, hydrogen and air gases were used at flow rates of 30, 33 and 330 ml/min, respectively. The identification of fatty acids was accomplished by comparing the peaks of retention times with those of the corresponding standards. The quantity of individual compounds was determined by comparing the produced peak area by known weight of the algal material with standard curve of the authentic substances which expressed the relation between the different concentrations and their peak area.

2.8. Statistical analysis

Data were subjected to an analysis of variance, and the means were compared using the Least Significant Difference (LSD) test at the 0.05 and 0.01 levels, as recommended by Snedecor and Cochran (1982).]

3. RESULTS AND DISCUSSION

Results in table 1 showed the lipid amounts extracted from eight algal species by the two extraction methods described in the experimental section. The red macroalga *Jania rubens* produced 2.8% lipid when extracted using hexane/ ether (1:1, v/v) system whereas the recovery was doubled (4.4%) using the extraction system chloroform/ methanol (2:1, v/v), No significant difference in the total lipid content was obtained from the red algae *Galaxaura* and *Gelidium* using both systems for extraction (2.4, 3.1 and 2.5, 3.0 respectively). The red seaweed *Asparagopsis taxiformis* and the green *Ulva lactuca* produced 1.2 fold increase in extracted lipid percentages and the brown macroalga *Colpomenia sinuosa*, produced a 1.52-fold increase in lipids when extracted by chloroform/methanol (2:1, v/v) system,

Table 3
Pigments contents (Chlorophyll a, b and carotenoids) of *Dictyochloropsis splendida* cultivated under stress conditions of NaCl, nitrogen depletion and starvation (mg/g fresh weight)

Sample culture conditions	Chlorophyll b	Chlorophyll a	Total Chlorophyll	Total carotenoids
Control (2.5 g/l NaCl and 25g/l $NaNO_3$)	0.171±0.02	11.84±0.07	12.01	2.58±0.05
NaCl stress				
5g/l	0.170±0.01	11.30±0.06	11.47	2.81±0.0
7.5 g/l	0.150±0.01	12.50±0.03	12.65	2.30±0.01
10 g/l	0.120±0.0	10.40±0.05	10.52	4.60±0.5
Nitrogen stress				
12.5g/l	0.120±0.02	10.40±0.1	10.52	0.67±0.01
6.25g/l	0.064±0.0	6.03±0.11	6.09	0.57±0.02
0.0g/l	0.08±0.0	5.43±0.06	5.51	5.60±0.2
LSD	0.0521	0.4321	0.4214	0.2241

Each value is presented as mean of triplet treatments, LSD: Least different significantly at $P \leq 0.05$ according to Duncan's multiple range test.

Table 4
Lipid, biodiesel and glycerol contents (%) of the green microalga *Dictyochloropsis splendida,* ulticated under salt stress, nitrogen depletion and starvation conditions

Sample culture conditions	Lipid content (%)	Biodiesel content (%)	Glycerol+ pigments content (%)	Biodiesel color
Control (2.5 g/l NaCl and 25g/l $NaNO_3$)	12.50±0.36	8.75±0.25	3.75±0.12	Colorless
NaCl stress				
5 g/l	14.50±1.2	8.90±0.62	5.60±0.18	Colorless
7.5 g/l	17.00±0.53	11.94±0.98	5.06±0.22	Light green
10 g/l	17.50±0.36	11.38±0.80	5.11±0.24	Light green
Nitrogen stress				
12.5 g/l	15.40±2.10	8.90±0.36	6.50±0.30	Yellowish green
6.25g/l	16.20±1.8	10.01±1.0	6.19±0.12	Light Yellow
0.0g/l	26.80± 2.12	18.90±1.2	7.9±0.50	Yellow
LSD	0.3643	0.1681	0.1431	

Each value is presented as mean of triplet treatments, LSD: Least different significantly at $P \leq 0.05$ according to Duncan's multiple range test.

as illustrated clearly in tables 1 and 2 . Moreover, the microalga *Dictyochloropsis splendida* showed 2.4 % of total lipid when extracted with hexane/ether (1:1, v/v) system, but on using chloroform/methanol (2:1, v/v) as extraction mixture, the percentage of total lipids increased 5.2 timed to reach 12.5% showing at the same time the highest biodiesel production (8.75 %) of the eight algal species used in this investigation. The cyanbacterium *Spirulina platensis* produced a 3-folds increase in lipid content using the chloroform/ methanol-based method (tables 1 and 2)

The obtained results illustrated in tables 1 and 2 revealed that the solvent mixture hexane/ether was not the most suitable system for lipid biodiesel extraction from algae because these solvents were unable to extract polar lipids. On the contrary chloroform/ methanol system extracted greater percentage of lipid (non polar and polar lipids) and consequently to higher biodiesel yields by transesterification (Fig. 1, Table 1). The lowest biodiesel production was observed in the red seaweed *Jania rubens* (0.25%) followed in ascending order by *Gelidium latifolium* (1.3%), *Galaxaura oblongata* (2.06%) and *Asparagopsis taxiformis* (3.64%). While the green macroalga *Ulva lactuca* and the brown seaweed *Colpomenia sinuosa* produced comparable biodiesel percentages (3.8 and

Table 5
Analysis of fatty acids of the obtained biodiesel from the promising green microalga *Dictyochloropsis splendida* using GLC

Fatty acids	[a]RT	Fatty acids percentage	
		Algae cultivated under normal conditions	Algae cultivated under free nitrogen media
C10:0 (Capric acid)	1.223	0.0	1.26
C14:0 (Myristic acid)	2.437	13.04	13.88
C16:0 (Palmitic acid)	2.860	81.14	69.59
C17:0 (Margeric acid)	3.240	1.01	1.21
C18:0 (stearic acid)	4.335	0.0	0.38
C18:1 (Oleic acid)	4.667	0.26	1.11
C18:2 (Linoleic acid)	5.333	4.39	12.14
C18:3 (linolenic acid)	6.948	0.15	0.42
Total saturated fatty acids		95.19	86.33
Total unsaturated fatty acids		4.81	13.67
TU/TS		0.05	0.16

[a]Retention time; TU/TS: total unsaturated/ total saturated fatty acids ratio.

3.1% respectively). The greatest yield of biodiesel was achieved by the green microalgae *Dictyochloropsis splendida* (8.75%) followed in descending order by the cyanbacterium *Spirulina platensis* (7.5%) as illustrated in table 2. Using chloroform/methanol (2:1, v/v) solvent system we were able to produce not only biodiesel in large percentage but also a sediment containing glycerin and pigments, the produced biodiesel have slightly alkaline pH values ranged 7.5-8.5 in all preparations. Our results, concerning the green microalga *Dictyochloropsis splendida* (produced 12.5% lipids), agreed with those obtained by Hossain and Salleh (2008) who reported that the green filamentous alga *Oedogonium* sp produced higher lipid percentage (9.2%) than *Spirogyra* sp (7.3%). The highest biodiesel production from *Dictyochloropsis splendida* observed in this investigation was in good agreement with data reported by Chisti (2007 and 2008) who demonstrated that, the biodiesel from microalgae seems to be the only renewable biofuel that has the potential to completely displace petroleum derived transport fuels. The author added that oil productivity of many microalgae greatly exceeds the oil productivity of the best producing oil crops. The obtained data from the present investigation, using stressed culture conditions (salt stress, nitrogen depletion and starvation), illustrated that the microalgal growth, pigments production, lipid as well as biodiesel contents (Table 3 and 4) were affected. Table 3, showed that algal growth rate (under both salt concentrations and nitrate depletion) was inhibited by increasing salt concentration and decreasing nitrate ones.

It seemed that nitrate starvation (0.0 g/l) produced the maximum decrease in algal growth represented as chlorophyll a content (mg/g dry weight). Also, chlorophyll b content was negativity affected by both stress conditions and the decrease in pigments contents under nitrate starvation was greater than those under salt stress conditions.

In both cases of stresses a remarkable increase in carotenoids contents were recorded at highest salt concentration (10 g/l) and nitrate starvation (0.0g/l). These may be produced as a result of metabolic alteration under these stress conditions giving a protective means against the associated oxidative stress process which might occur. Under stress conditions. Generally, under both high salt conc. and nitrate starvation, the algal lipid contents were higher than in control conditions (under normal growth condition).

Maximum lipid and biodiesel contents were recorded in complete absence of nitrate (nitrate starvation) from the nutritive medium (26.8 and 18.9% respectively) as illustrated in Table 4. These results may be explained by the fact that, under nitrate starvation, all the carbon structures produced during metabolic process might be directed towards lipid production which in turn converted to biodiesel by transesterification process. While in presence of nitrogen, most of the carbon structures was incorporated in nitrogenous compounds as amino acids, protein, nucleic acids or alkaloids. Under salt stress conditions (with normal nitrate conc. in culture media) the algal metabolism was altered with over production of carbon skeleton which were partly directed towards the production of substances with beneficial role in algal tolerance or defense mechanism as polyols, carbohydrate, methylated

amino acids and protein in addition to the nitrogenous compounds and partly to form lipids and biodiesel. This explained the relatively lower lipid and biodiesel contents under salt stress conditions. The data obtained in this investigation were in good agreement with results published by Widjaja (2009) who reported that the green microalga *Chlorella vulgaris* accumulated high lipid content when cultivated in nitrogen depletion condition (0.02 mg/l nitrate). Our results also went parallel with those obtained by Lardon *et al*. (2009) who found that, the control of nitrogen stress during the culture and optimization of wet extraction led to maximum biodiesel production from the microalgal culture *Chlorella vulgaris*.

Analysis of the produced biodiesel from the promising alga *Dictyochloropsis splendida* (Table 5) showed that the unsaturated fatty acids percentage was increased in alga cultivated in nitrogen free media (0.0g/l N) two times more than normal conditions (13.67, 4.81% respectively). However, the composition of fatty acids were different in these cases depending on its growth condition as showed in Table 5. These results were in agreements with those reported by Wood (1974) relative to *Chlorophycean* species. Furthermore Ramos *et al*. (2009) reported that monounsaturated, polyunsaturated and saturated methyl esters were built in order to predict the critical parameters of European standard for any biodiesel, composition. The extent of unsaturation of microalgae oil and its content of fatty acids with more than four double bonds can be reduced easily by partial catalytic hydrogenation of the oil (Jang *et al*., 2005, Dijkstra, 2006). Concerning the fatty acids contents of the produced biodiesel from microalgae, Chisti (2007) reported in his review that, microalgal oils differ from vegetable oils in being quite rich in polyunsaturated fatty acids with four or more double bonds (Belarbi *et al*., 2000) as eicosapentanoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) which occurred commonly in algal oils. The author added that, fatty acids and fatty acid methyl esters with four and more double bonds are susceptible to oxidation during storage and this reduces their acceptability for use in biodiesel especially for vehicle use (European standard EN 14214 limits to 12%) while no such limitation exists for biodiesel intended for use as heatling oil. In addition to the content of unsaturated fatty acids in the biodiesel also its iodine value (represented total unsaturation) must be taken in consideration (not exceeded 120 g iodine/100g biodiesel according to the European standard).

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Recibido: 8/2/10
Aceptado: 29/3/10