ISSN(PRINT) : 2320 -1967
ISSN(ONLINE) : 2320 -1975

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CHEMXPRESS 5(2), 48-55, (2014)

Investigation of factors affecting lipid accumulation in fresh water microalgae for enhancement of biofuel production

Sanaa Abo El-Enin[1], Entesar Ahmed[3], Sayeda M.Abdo[2], Rawheya S.El Din[3], Guzine El Diwani[1], Gamila Ali[1,2]*

[1]Chemical Engineering and Pilot Plant Department, National Research Centre, Cairo, (EGYPT)
[2]Water Pollution Research Department, National Research Centre, Post code, 12311, Cairo, (EGYPT)
[3]Botany Department, Faculty of Science, Al-Azhar University (Girls Branch), Cairo, (EGYPT)
E-mail: gamilaali2003@gmail.com

Received : 27th March, 2014 ; Revised : 26th May, 2014 ; Accepted : 01st June, 2014

Abstract : Lipid accumulation is limited by several factors, nutrient composition, light type and illumination period. Three algal strains isolated from River Nile of Egypt were used *Chlamydomonus variabills, Haematococcus pluvialis* (green algae) and *Microcystis aeruginosa* (blue green algae). The species were grown in BG11 medium under continuous illumination (24hr) with white fluorescent light intensity ≈2500 Lux and harvested at stationary phase. The factors studied were $NaNO_3$ starvation, NaCl stress, different color of Light Emitting diodes, with different light intensity and the effect of illumination period. GC-analysis were done to detect the fatty acids present at optimum condition for each strain to know which is more suitable for biofuel production. *Microcystis aeruginosa,* recorded the maximum lipid content (30%) at control and at NaCl stress 2.5g/l. *Chlamydomonus variabills*, showed maximum lipid content 21% at control condition. *Haematococcus pluvialis* given total lipid content 10% at control and nitrate starvation 0.075 and 0.0 g/l while at salt stress 0.5g/l the lipid content increased to reach 25%. In addition the results showed that continues illumination period is better with white light florescent, while, the light / dark 16/8 cycle illumination is better for selected isolates with LED light. **© Global Scientific Inc.**

Keywords : Microalgae; Biodiesel; Salinity; LED lights (Light Emitting Diodes).

INTRODUCTION

The ability of algae to survive or proliferate over a wide range of environmental conditions is reflected in the tremendous diversity and sometimes unusual pattern of cellular lipids as well as the ability to modify lipid metabolism efficiently in response to changes in environmental conditions[1-3,13,33]. Under optimal conditions of growth, algae synthesize fatty acids principally for esterification into glycerol-based membrane lipids, which constitute about 5–20% of their dry cell weight (DCW). Under unfavorable environmental or stress conditions for growth, however, many algae alter their lipid biosynthetic pathways towards the formation and accu-

mulation of neutral lipids (20–50% DCW), mainly in the form of triacylglycerol (TAG). Unlike the glycolipids found in membranes, TAGs do not perform a structural role but instead serve primarily as a storage form of carbon and energy. However, there is some evidence suggesting that, in algae, the TAG biosynthesis pathway may play a more active role in the stress response, in addition to functioning as carbon and energy storage under environmental stress conditions.. The major chemical stimuli are nutrient starvation, salinity and growth-medium pH. The major physical stimuli are temperature and light intensity. Moreover, chemical and physical factors, growth phase and/or aging of the culture also affects TAG content and fatty acid composition[33]. Nitrogen limitation in the nutrients is the most critical factor affecting lipid metabolism in algae. A general trend towards accumulation of lipids, particularly TAG, in response to nitrogen deficiency has been observed in numerous species or strains of various algal taxa[2,4-6].

Other types of nutrient deficiency that promote lipid accumulation include phosphate and sulfate limitation[7,30]. Salinity is a factor that can influence in lipid content of microalgae[8,9]. Salinity, in both open and closed systems, can affect the growth and cell composition of microalgae. Salinity changes normally affect phytoplankton in three ways[30]: (1) osmotic stress (2) ion (salt) stress; and (3) changes of the cellular ionic ratios due to the membrane selective ion permeability. The easiest way for salinity control is by adding fresh water or salt as required[31].

Algae grown at various light intensities exhibit remarkable changes in their gross chemical composition, pigment content and photosynthetic activity[10,11,32]. Based upon the algal species/strains examined[12], it appears, with a few exceptions, that low light favors the formation of polyunsaturated fatty acids (PUFAs), which are incorporated into membrane structures.

The present study aimed to investigate the factors affecting on lipid accumulations in three local isolated microalgae strains under different growth conditions of nitrate starvation, salinity stress, and light source, intensity and the illumination period. Fatty acids profile recorded by GC-analysis at both controlled and stress growth conditions for comparing between fractions of fatty acids and defined which growth conditions producing fatty acids with high efficiency for biofuel production.

MATERIAL AND METHODS

Algal isolations and cultures

The algal species used in this study were isolated from River Nile water using BG11 media[14] as mentioned in[15]. The isolated algal species are, *Chlamydomonus variabills, Haematococcus pluvialis* (green algae) *and Microcystis aeruginosa* (blue green algae). Normal condition for cultivation since isolation was carried out in sterilized 1 liter conical shoulder flasks containing 600 ml of the corresponding culture medium under continuous illumination (24hr) with white fluorescent light intensity ≈2500Lux. The cultivation time differed from one strain to another depending on the optimum growth rate till reaching stationary phase which always ranged between (15-20) days. Each strain was subjected to different conditions in order to study its effect on increasing lipid production.

Chlorophyll a content measurement

The fresh Sample (25ml) of each strain was taken every 48 h and filtered through 0.45µm membrane filter and extracted with hot methanol after the addition of 0.5ml magnesium carbonate solution (1%) in order to prevent chlorophyll degradation. The concentration of chlorophyll *a* was calculated according to the equation sited in (Standard Method, 1998).

$$C_a = 11.85(OD664) - 1.54(OD647) - 0.08(OD630)$$

Chlorophyll a µg/L = C_a X extract volume, L/ volume of sample, L

Where: OD664, 647 and 630 are the absorbance at 664, 647 and 630.

Effect of different factors on lipid production

To study factors affecting lipid production, the algal isolates exposed to adaptation period on stress conditions. The adaptation period involved the re- culture of the algal isolates on the selected factors e.g. NO_3 stress, NaCl stress and light conditions until the algal isolates reach the stationary phase then transfer the algal cells to anew culture with the stress condition in order to collect algal biomass and evaluate the lipid content.

Effect of nitrate concentrations

The algal isolates were cultured in different $NaNO_3$ concentrations (0.3, 0.15, 0.075 and 0.0 g/l) until stationary phase and the culture was collected to determine the effect of decreasing $NaNO_3$ concentrations on total lipid production and its fatty acids fractions.

Effect of salt stress

The effect of salt stress on algal lipid production were examined by adding NaCl through different concentrations (0.5,1,2.5, and 5 g/L) the algae harvested at stationary phase to study the effect of salt stress on the total lipid production efficiency and its fatty acids profile.

Effect of light source

The selected isolates were cultivated in BG11 medium under continuous illumination (24hrs) with white florescent light at intensity ≈ 2500 Lux. At stationary phase, take equal samples and sub cultured each one twice 15-20 days as adaptation period with continuous illumination under different colors of light emitting diodes (LED), red and warm whit colors with intensity ≈1000Lux and blue color with ≈ 1500 Lux. Then, the isolates were sub cultured again and harvested at stationary phase to determine the effect of light color on the lipid increasing.

Effect of light duration cycle

At stationary phase of selected isolates at controlled conditions, take equal samples and sub cultured each one twice 15-20 days as adaptation period under different colors of LED and white florescent light with illumination cycle 8/16 light/dark. Then the isolates were sub cultured again and harvested at stationary phase to determine the effect of light duration period on the lipid productivity.

Algal biomass harvesting

In order to harvest algal biomass, a suitable harvesting method may involve one or more steps and be achieved in several physical or chemical ways. Filter press operating under pressure was used with *Microcystis aeruginosa* and *Chlamydomonus variabills,* through membrane filter 0.8µm, while *Haematococcus pluvialis* harvested through settling then centrifugation at 2000 rpm for 10 minutes.

Lipid extraction

All species after harvesting were subjected to two methods for oil extraction:

Modified method of Bligh and Dyer and Hexane-Isopropanol Extraction Method according to[15].

RESULTS AND DISCUSSION

Algal growth rate

Chlorophyll a measured as indicator of algal growth revealed that under control condition (BG11 medium, white florescent light with intensity ≈2500 Lux for illumination period 24 hrs) the algal species differed in its biomass and hence lipid production. According to the chlorophyll a, the growth rate of selected microalgae species at stationary phase under control conditions can be arranged in increasing order as following:

Microcystis aeruginosa* > *Haematococcus pluvialis* > *Chlamydomonus variabills

Response of isolated strains to nitrate starvation

Nitrogen limitation is the most frequently reported method of increasing lipid content, as it is cheap, easy to manipulate and has a reliable and strong influence on lipid content in many species[16]. The growth rate of *Chlamydomonus variabills* at different nitrates concentrations continue to increase up to 14 days then started to decline, *Haematococcus pluvialis* continue to grow up to 18 day at 0.3 g/l $NaNO_3$, at concentrations 0.15 and 0.075 g/l it continue to grow up to 14 day. *Microcystis aeruginosa* continue to grow up to 20 days with increasing the chlorophyll content at 0.3, 0.15 and 0.075 g/l, while at zero nitrate *Microcystis aeruginosa* continue to grow up to 12 days then began the stationary phase. The results indicated that the effect of nitrate concentration is species specific and each strain differ in its ability to adapt with nitrate concentration.

The selected isolated strains showed as in Figure 1 that the maximum lipid content was at control condition where $NaNO_3$ concentration equal 1.5 g/l. *Microcystis aeruginosa* and *Chlamydomonus variabills* recorded decreasing in lipid accumulation with nitrate deplation. While, *Haematococcus pluvialis* showed a little decreasing in lipid content by

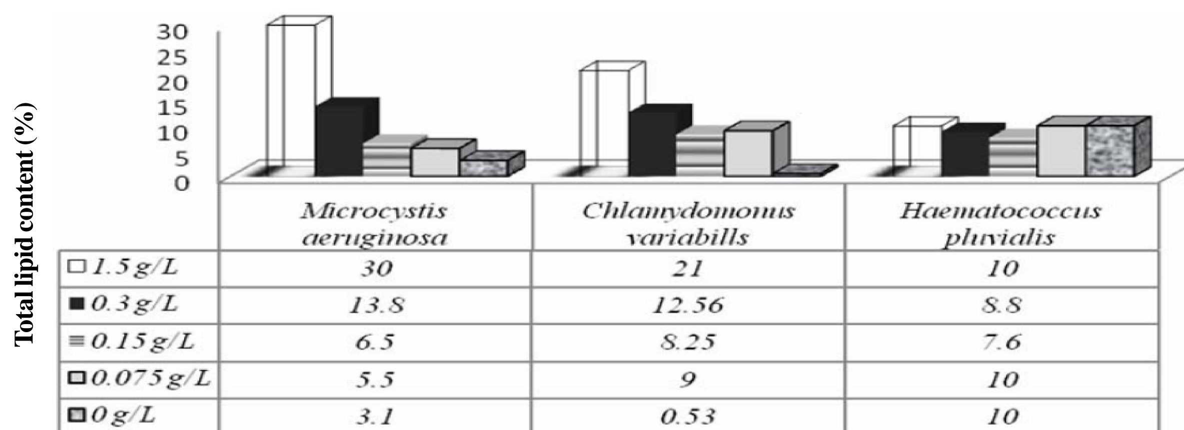

Figure 1 : Effect of nitrate concentration on total lipid content

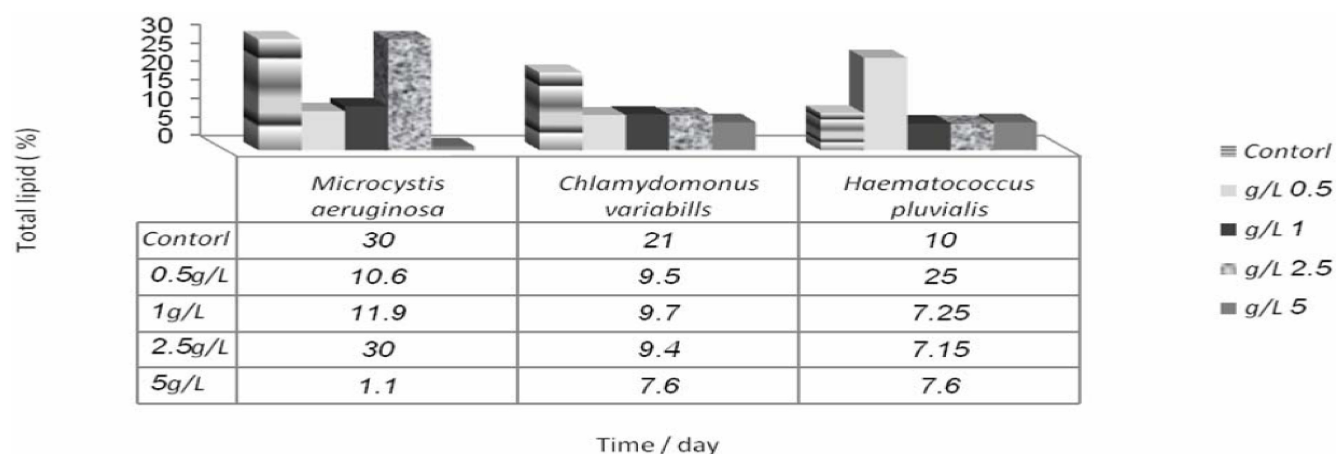

Figure 2 : Effect of sodium chloride concentrations on total lipid content

decreasing nitrate concentration (0.3 and 0.15 g/l), with further decreasing in nitrate (0.075 and 0.0 g/l $NaNO_3$), the lipid accumulation increased again to the maximum value (10%±0.14). This is an economically promising result since this strain (*Haematococcus pluvialis*) can yield the maximum lipid percentage without adding nitrate. It is in agreement with the results of Melinda et al., 2011, where, showed that, the lipid content at 0.15 g/l $NaNO_3$ higher than that at 1.5g/l. Also, the results of Subhasha *et al*, 2011 showed that as decreasing the nitrate concentration in the culture medium, the lipid content increasing. At the present study, the lipid content decreased by decreasing nitrate concentration in case of *Microcystis aeruginosa and Chlamydomonus variabills,* while, lipid accumulation of *Haematococcus pluvialis* recorded the maximum value (10%±0.3) at zero nitrate concentration. Such results prove that, the response to nitrogen limitation is species specific. However, stress conditions that increase lipid content, such as nitrogen limitation, also decrease the growth rate and thus may not improve lipid productivity[17-19].

Response of isolated strains to salinity

The growth rate (as chlorophyll a content) of the selected strains began to grow normally at 0.5 and 1 g/l NaCl concentrations, while at 2.5 and 5 g/l NaCl concentrations, the growth rate began to increase after 6th day till stationary phase from 10th day to 14 days for all strains at all NaCl concentrations.

In Figure 2, *Microcystis* strain recorded the maximum lipid content (30%±0.5) at control medium which was salt free. Increasing NaCl concentrations to 0.5 and 1 g/l decreasing lipid content to11.6%±0.13 and 11.9%±0.4 respectively. At salt concentration 2.5g/l the lipid content reached again to the maximum value. While, increasing NaCl to 5 g/l recorded a depletion in lipid content to 1.1%±0.5. *Chlamydomonus variabills* showed decreasing in lipid accumulation by increasing salt concentrations. while *Haematococcus pluvialis*

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showed increase in total lipids at 0.5 g/l to reach 25%±0.07. The composition of intracellular lipid of microalgae was reported to change in response to environmental salinity.

Increase of NaCl concentration from 0.4M to 4M increased saturated and monounsaturated fatty acids in *Dunaliella* cells isolated from an Antractic hypersaline lake[20], while polyunsaturated fatty acid decreased. The fatty acid composition of polar lipid in *Dunaliella salina* Teodoresco was affected significantly by the change in NaCl concentration[21]. The increase in lipid content at higher NaCl concentration may be due to adaptation under stress conditions which help in accumulation of lipid content and these results are in accordance with the finding of Takagi and his coworkers[9,22].

TABLE 1 showed the G.C. analysis for the selected microalgae strains at control conditions. Also, fatty acids profile at salt concentrations which increased the lipid content in *Microcystis* and the fractions of fatty acids of *Haematococcus* at NaCl concentration and nitrate starvation where the lipid accumulation at these conditions reached to maximum value. The results clear that for *Microcystis* strain, the percentage of PUFAs at saline medium increasing than that at control while monounsaturated fatty acids decreased. The saturated fatty acids which are suitable for biofuel production are present with high percentage at control and saline media. (C16-0, C18-0). For *Haematococcus* F.A. profile showed that C18-0 was not found at control while it appeared at saline medium (0.5g/l NaCl), and at zero nitrate medium with higher percentage. Also, there was an increase in saturated fatty acids and decreasing in unsaturated fatty acids (mono-and poly- unsaturated fatty acids). GC analysis of *Chlamydomonus* at control medium clear that the lipid of this strain containing high percentage of saturated fatty acids which are promising for biofuel production.

TABLE 1 : Fatty acid composition of selected microalgal strains

M.e.:Microcystis aeruginosa, H.p.: Haematococcus pluvialis, Ch.v.: Chlamydomonus variabills

Fatty acids	Common name	*M.e. control*	*M.e. 2.5g/L NaCl*	*H.P. control*	*H.P. 0.5g/L NaCl*	*H.P. 0.0 g/l $NaNO_3$*	*Ch.v. Control*
C:8:0	Caprilic	-	-	-	-	-	4.5
C:10;0	capric acid	1.4	1.7	1.8	3.7	-	-
C:12:0	Lauric	8.1	1.5	2.46	12.6	-	11.7
C:14:0	Myristic	3.1	4.5	10.4	1.6	-	4.3
C:16:0	palmitic	36.4	22.7	2.6	24.3	40.2	24.7
C:17:0	Margaric	3	2.4	14.8	4.5	2.3	3.3
C:18:0	stearic acid	17.7	22.3	-	23.6	32.8	10.7
C:20:0			5.4		3.2	-	-
C:22:0	behenic acid	-	5.9	5.5	-	-	6.7
C:24:0	lignoceric acid	-	2.1	5.5	1.8	-	11.9
C:14:1	myristoleic acid	-	3.3	22.3	-	-	-
C:16:1	palmetolic	5.3	4.4	6.8	6.9	2.9	-
C:18:1	Oleic	17.7	8.4	2.6	5.5	12.4	5.3
C:18:2	Linoleic	5.7	11.4	1.4	10.5	7.6	6.5
C:18:3	Linolenic	1.4	0.8	-	0.9	0.9	5.7
C:20:1	gadoleic acid	-	-	4.4	-	-	-
C:20:2	.	-	-	7.4	-	-	-
C:20:3	.	-	-	5.2	-	-	-
Total fatty acids (%)		99.8	99.3	93.1	99.1	99.1	95.3
Saturated fatty acids (%)		69.7	68.5	43.06	75.3	75.3	77.8
Unsaturatde FA (%)		30.1	30.8	50.1	23.8	23.8	17.5
Monounsaturated FA (%)		23	18.6	36.1	12.4	15.3	5.3
PolyUnsaturated FA (%)		7.1	12.2	14	11.4	8.5	12.2

It is noted that the increase in salinity can increase the lipid content of microalgae, but lowers the growth rate of a species. Salt stress is a major abiotic environmental factor that limits plant growth and productivity. Microalgae differ in their adaptability to salinity and other stress conditions. The ability of cells to survive and flourish in saline environment under the influence of osmotic stress has received considerable attention. Under favorable and unlimited growth conditions microalgae produce primarily polar lipids (e.g. glycolipids and phospholipids), which enrich chloroplast and cellular membranes. However, under unfavorable growth conditions microalgae accumulate neutral lipids in lipid droplets located in the cytoplasm[12].

Effect of light color on total lipid content of isolated strains

The selected isolates were subjected to florescent white light (2500Lux) and three colors of LED light (red, warm white (1000 Lux) and blue (1500Lux)] for 24 hr. Growth rate of each isolate was increased in chlorophyll a with LED light than that at control condition using white florescent light.

As shown in Figure 3 *Microcystis aeruginosa* and *Chlamydomonus variabill* showed the maximum lipid content (30%±0.3 and 21%±0.28) respectively, when subjected to florescent white light for 24 hr. while *Haematococcus pluvialis* showed the maximum lipid content (10%±0.12) when subject for 24 hr. to florescent white light, LED yellow light and LED blue light. Such results prove that, the response to light color and intensity is species specific.

The selected isolates were subjected to florescent white light and three colors of LED light (red, warm white and blue) for 8/16 light/dark cycle. Referring to the growth rate of each isolate, there was an increasing in chlorophyll a with LED light than that at control condition using white florescent light. Figure 4 revealed the result of lipid content with exposure to LED light for 8/16 light dark cycle.

LED warm white light increase the total lipid for, *Chlamydomonus variabills and Haematococcus pluvialis*. The result showed that the light /dark cycle illumination is better than continuous for the selected isolates with LED light instead of their lower intensity than that of florescent white light

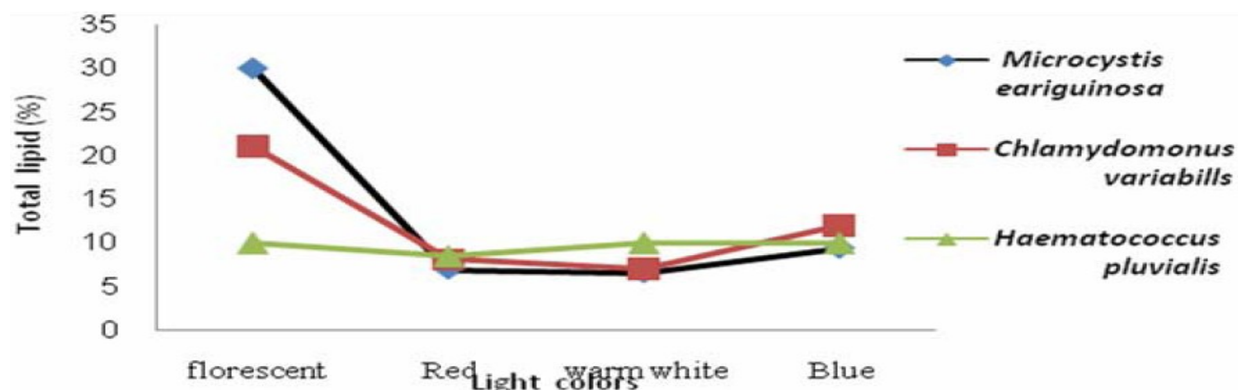

Figure 3 : Effect of light color on lipid content of isolates at illumination period 24hr

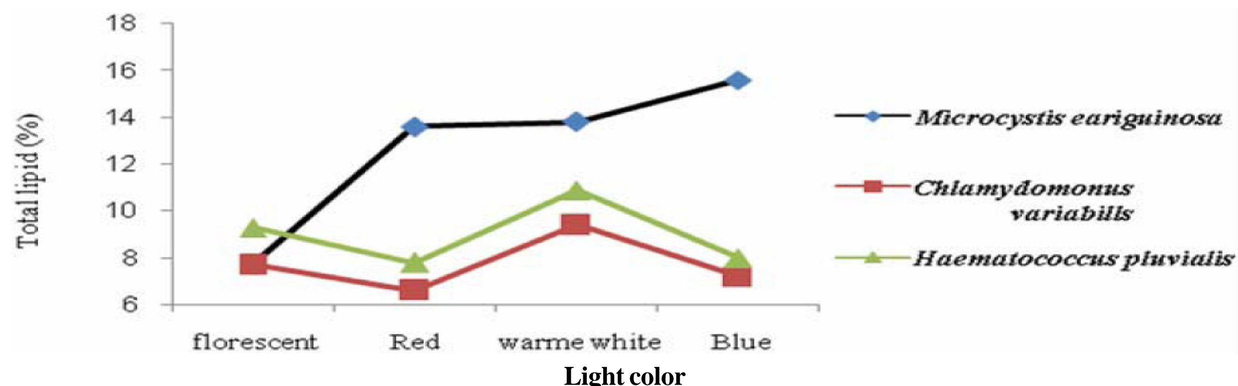

Figure 4 : Effect of light color on lipid content of isolates at illumination period 16/8 light/dark cycle

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While continues illumination period is better with florescent white light. Many studies have investigated the effects of light conditions on the growth of microalgae, including *Isochrysis* galbana, by using continuous light on a dial light/dark cycle and found that it is better for growth[24-28].

CONCLUSION

- The growth rate of selected microalgae species under control conditions can be arranged in order of increasing as following:
  Microcystis aeruginosa ≥ Haematococcus pluvialis ≥ Chlamydomonvariabills
- The effect of nitrate starvation is species specific where microalgae differ in their adaptability to stress conditions.
- Lipid content of *Microcystis aeruginosa* and *Chlamydomon variabills*, decreases with decreasing nitrate concentrations, while, there is no significant effect in total lipid content of *Haematococcus pluvialis* with nitrate deplation.
- *Chlamydomon variabills* showed decreasing in lipid accumulation by increasing salt Concentration.
- *Microcystis aeruginosa and Haematococcus pluvialis can adapt themselves at salt stress 2.5g/l and 0.5g/l to give lipid percentage 30% &25% respectively.*
- Continues illumination period is better with florescent white light while, the light /dark cycle illumination is better than continuous for the selected isolates with LED light instead of their lower intensity than that of florescent white light.
- GC analysis clear that lipid profile of *the Microcystis aeruginosa* and *Chlamydomon variabills* at control medium containing high percentage of saturated fatty acids which are promising for biofuel production.
- Lipid profiles of *Haematococcus pluvialis* at salt stress and nitrate starvation given higher percentage of saturated fatty acids than that at control condition.

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