An attempt to Stimulate lipids for Biodiesel Production from locally Isolated Microalgae in Iraq

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Abstract:

Two locally isolated microalgae (*Chlorella vulgaris* Bejerinck and *Nitzschia palea* (Kützing) W. Smith) were used in the current study to test their ability to production biodiesel through stimulated in different nitrogen concentration treatments (0, 2, 4, 8 g\l), and effect of nitrogen concentration on the quantity of primary product (carbohydrate, protein), also the quantity and quality of lipid. The results revealed that starvation of nitrogen led to high lipid yielding, in *C. vulgaris* and *N. palea* the lipid content increased from 6.6% to 40% and 40% to 60% of dry weight (DW) respectively.Also in *C. vulgaris*, the highest carbohydrate was 23% of DW from zero nitrate medium and the highest protein was 50% of DW in the treatment 8g\l. While in *N. palea* the highest carbohydrate was 25% of DW in the treatment 4g\l, and the highest protein was 15% of DW in 8g\l treatment.

Key words: Microalgae, lipids, Stearic acid, Oleic acid, Biodiesel

Introduction:

Microalgae are photosynthetic organisms that have the ability to fix CO2, so the light energy will transform to chemical energy inside the alga's cell [1]. They may be used in different ways, such as purification of waste water under either autotrophic or mixotrophic conditions [2]extractions of high added value food such as polyunsaturated fatty acids [3] and pigments such as β-carotene and astaxanthin, also pharmaceutical products, in addition to play an important role in the aquaculture business as food for aquaculture and biofuel production which got a great attention in the present century [4,5] Lipid can be produced and extracted from microalgae cells. This lipid can be used in transformation to biofuel especially biodiesel [6] This transformation will be reducing the pollution of petroleum, natural gas, coal, hydro, and nuclear energy [7] which are major source of green house gases emissions (GHG). These emissions are affecting the environment and cause great damages [8] Because of the minifying petroleum advance and increasing environmental worry with the increasing in fossil energy, renewable and cleaner biofuel from microalgae have appeared and got a big attention in recent years [9]The best candidates for fuel production are Microalgae because of: there advantages of higher photosynthetic efficiency, higher biomass production, faster growth compared to other energy crops, they can grow practically anywhere, use far less water than traditional oil seed crops, they have no competition with food crops, and they are the only feed stock that can replace transportation fuels [10]Felizardo *et al*[11] study showed that biodiesel can be made from any oil or lipid source such as vegetable oils and animal fats. Oil contains a glycerol molecule bonded to three fatty acid chains, this structure is called a triglyceride, and it is the major

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component of the oil. Biodiesel fuel has received considerable attention in recent years, as it is a biodegradable, renewable and non-toxic fuel. It contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel [4]. Hu *et al*[12] study showed that there are several ways to make biodiesel, and the most common way is transesterification and biodiesel can be used directly with diesel fuel in diesel engine.Hundreds of microalgae strains capable of producing high content of lipid have been screened and their lipid production metabolisms have been characterized and reported for biodiesel [6,13]This study is the first attempt to isolate locally microalgae from Iraqi aquatic systems and to stimulate the production of biodiesel from these isolates.

Material and Methods:

Two species of algae were isolated from ponds and artificial canal of University of Baghdad in Al-Jadriya campus, also from Tigris River at Al-Rasheed area, Baghdad-Iraq. The studied algae are *Chlorella vulgaris* Bejerinck and *Nitzschia palea* (Kützing) W. Smith. Modified Chu-10 [14] was used for the algal growth (Table 1). Serial dilution method and streaking on plate agar techniques were used for algae isolation and purification in this study. For algae cultivation, 10 ml of isolated culture was added to a flask containing 100 ml of Chu-10 media and incubated for 14 days, then transported to 1000 ml of media and incubated for 14 days; finally the growth was transported to glass pools 5 L for mass culture.The growth curve was determined for the two studied algae. Cell growth was measured by determining the optical density (O.P) daily. Optical density (540 nm) was measured by using spectrophotometer UV-VIS (540 nm). All measurements of the study were triplicates.

The growth rate (K) and doubling time (G) were obtained according to the following equation:

$$K = \frac{(\log OD_t - \log OD_0}{t} \times 3.322 \quad [15]$$

$$G = \frac{0.301}{K} \quad [16]$$

T: time (day)
OD_t: Optical density after (t) day
OD_o: Optical density at beginning of the experiment zero time

Experiment design:

Different concentrations of nitrogen were used in the current study, to stimulate the isolated algae for production lipid that can be used as biodiesel. Nitrate was used as a source of nitrogen in media ($NaNO_3$) by 8g/l and considered as control treatment in the study; also other three concentrations of nitrate (4, 2, zero g/l) were used as treatments. These treatments represent a gradual reduction in concentration of nitrogen (Nitrate) used in the media up to remove completely nitrate from the culture's media.Microalgae had been harvested at the beginning of the stationary phase. Each culture of microalgae was centrifuged in the cooled centrifuge at 3000 rpm for 15 min, supernatant removed but organic precipitate had been washed with distilled water, and then dried at 45 C° for two days. The dry weight was collected for extraction.

Lipid extraction and analysis:

A 1g of dry weight had been put in thimble and carried to specific cylinder in the soxhlet. A 200 ml of solvent (mixture from methanol and hexane

1:1) had been put in the flask after which the process took three-four hours; the solvents color in the cylinder will change from green to colorless. The extracted sample was dried by rotary evaporator at 40 C° for few minutes. The result was poured out to a plate and left in room temperature 25 C° overnight, then transferred to test tube until analysis [17] Samples were analyzed by High Performance Liquid Chromatography (HPLC) system, model SUPELCO. The column is discovery HSC18 dimension 25 cm X 4.6 mm X 5µm injection flow 1 ml/min at UV.

Determination of protein and carbohydrate:

Algae samples were centrifuged by cooling centrifuge model Rotina 380 R for 5000 r/min for 30 min, 4 C°. The supernatant was collected and the protein determined according to Bradford method [18] and the carbohydrate according to phenol sulphuric acid method [19]

Statistical analyses

Complete Randomized Design (C.R.D.) was used as an experimental design. Data were analyzed by using statistical analysis system- SAS [20]to study the effect of different concentration factors on the production of lipid. Least significant difference (LSD) was used to compare the significant difference between means at $P \leq o.o5$.

Results and discussion:

Two isolated algae were obtained successfully, and they were identified according to Prescott [21] and Hustedt [22]

Class: Chlorophyceae
Order: Chlorococcales
Family: Chlorococcaceae
Chlorella vulgaris Bejerinck
Class: Bacillariophyceae
Order: Pennales
Family: Bacillariaceae
Nitzschia palea (Kützing) W. Smith

Different growth was observed for both isolated algae in the treatments, and the harvesting time was also different among the treatments. Figure 1 illustrates the effect of different nitrogen concentrations on *C. vulgaris* biomass growth, figs. 1(a and b) show a noticeable difference between treatment 4g/l and control, while there were different manner in two other treatments (figs.1 c and d), but there were no significant differences among treatments (table 2). The biomass growth of *C. vulgaris* entered a stationary phase in different days among treatments. The stationary phase was identified as day 12, 8, 6 and 4 in treatments 8, 4, 2 and zero g/l nitrogen respectively. The K value increased from 0.1- 0.19 for the treatments 8g/l and zero g/l respectively (fig. 1). The shortest doubling time was 1.5 days in treatment zero g/l while the longest was 2.7 days in control (8g/l), a significant difference was recorded between zero treatment and control, while there were no significant differences among other treatments with control (table 3).The stationary phase was identified as day 10, 8, 6, 5 in treatments 8, 4, 2 and zero g/l (fig. 2). The K value of *N. palea* increased from 0.06 at control treatment to 0.26 at treatment zero g/l. The shortest doubling time (1.1 days) and highest K value occurred at treatment zero g/l, while the longest doubling time (5 days) and lowest K value recorded at control (8 g/l). The harvesting biomasses of both isolated algae were done in stationary phase for lipid,

protein and carbohydrate analysis. Lipid content of algae is an important parameter that determines the economy of biodiesel production from algae [23]The lipid content for *C. vulgaris* ranged from 6.5% at control to 40% at zero treatment, and statistically there are significant differences among treatments except between control and treatment 4g/l where no significant differences (table 3). The same trend was shown for *N. palea.* The lipid contents for *C. vulgaris* and *N. palea* increased from 6.6 % to 40% and 40% to 60% of dry weight respectively, when it was extracted in early stationary phase. The present study revealed that concentration of nitrate is significantly affecting lipid content of both microalgae specially at zero concentration which achieved higher lipid content than control nitrate media and other treatments. Alga lipid content usually increases at nitrogen starvation, due to lipid-synthesizing enzymes are less affected by disorganization than carbohydrate synthesizing enzymes, thus, the major proportion of carbon can be bound in lipids [5,6] These results were also reported in the other literatures [10,24,25,26,27] Shen *et al*[24] revealed that lipid yields of heterotrophic *Chlorella protothecoides* increased from 4 to 5.89% of dry weight, so the studied alga produced more lipids in low-nitrogen-concentration media. Another study [27] showed that nitrogen treatments increased the lipid ratio of alga *Nannochloropsis oculata* from 7% at 0.3 g\l NaNO3 to16% at 0.075 g\l NaNO3.Miao and Wu [10] noticed in their study on alga *C. protothecoides* that the protein level decreased to 10.28% and the lipid level increased to 55.20% during heterotrophic growth. This change in growth parameters was also noticed in the present study that may be the limitation of nitrogen concentration in media growth limited protein biosynthesis thus increasing lipid and carbohydrates were recorded [26,27,28] Oleic acid content (%) showed only significant differences between control and zero treatment, while other treatments have no significant differences with control for *C. vulgaris*. A significant difference in oleic contents for *N. palea* was recorded between treatments (2, zero) and control (table 5). The results of Stearic acid content in both studied microalgae showed the same trend. Higher content was recorded at zero treatment, while lower content at control. There are significant differences among treatments except between control and treatment 4g/l were no significant differences (table 6). Fatty acids (Stearic, Oleic) increased in studied treatments (8, 4, 2and 0) from 0.7% to 26%, 0.05% to 6% and 1.5% to 34.5%, 2% to 15% of total lipid respectively (figures 4 and 6). The present study results were in agreements with those reported by Afify et al. [29]. The Stearic acid (18:0) is considered as the most common fatty acid in biodiesel that is present in this study encourage to use the studied algae to produce biodiesel in addition to increasing of Oleic acid (18:1Δ9). Carbohydrate content of *C. vulgaris* ranged from 16.5% at control to 25% of dry weight at zero treatment. A Significant difference was recorded only between zero treatment and other treatments. While, the carbohydrate content of *N. palea* ranged between 20% at treatments (control and zero) to 25% at treatment 4 g/l, and there was a significant difference between treatment 4g/l and other treatments (table 7).Protein content of both studied microalgae decreased sharply among treatments in contrast with control treatment. The protein content was higher in *C. vulgaris* than in *N. palea*. Significant differences were

recorded among treatments at both studied microalgae (table 8). Carbohydrate and protein concentrations showed differences in their concentrations at studied treatments (figures 3 and 5).

Conclusions:

The different concentrations of nitrogen influenced the lipid, protein and carbohydrate contents of the studied microalgae, and affecting strongly on the lipids productivity of *C. vulgaris* and *N. palea*. Nitrogen also affects the qualitative and quantitative analysis of fatty acids and gives very high values of Stearic acid and Oleic acid that were extracted from the microalgae.

Table (1) The components concentration of modified Chu-10 medium and the concentration of each component

Number of stock solution	Chemical formula of each salt	Concentration g/l
1	$MgSo_4.7H_2O$	10
2	K_2HPO_4	4
3	$NaNO_3$ $CaCl_2$	8 16
4	$FeCl_3$	0.32
5	EDTA-Na	4
6	NaCl	30
7	Na_2CO_3	8
8	$MnCl_2.4H_2O$ $(NH4)\ 6Mo_7O_{24}.4H_2O$ $ZnSO_4.7H_2O$ $CuSO_4.5H_2O$ $COCl_2.6H_2O$ H_3BO_3	0.02 0.028 0.224 0.08 0.004 0.288
9	Na_2Sio_3	5.7

Table2. Effect of different concentrations of nitrogen in the growth medium on growth rate (mean± SE) of studied microalgae.

Concentration (g/l)	Studied microalgae		LSD value
	C. vulgaris	*N. palea*	
Control (8)	0.11 ± 0.04 A	0.06 ± 0.01 B	0.08 NS
(4)	0.14 ± 0.07 A	0.11 ± 0.05 B	0.06 NS
(2)	0.15 ± 0.07 A	0.17 ± 0.07 AB	0.06 NS
Zero	0.19 ± 0.08 A	0.26 ± 0.11 A	0.11 NS
LSD value	0.08 NS	0.122 *	---

NS: non-significant.

The same letters in the column show no statistically different ($P \leq 0.05$)

Table 3 . Doubling time (mean± SE) of studied microalgae at different nitrogen concentrations in the growth medium.

Concentration (g/l)	Studied microalgae		LSD value
	C. vulgaris	*N. palea*	
Control (8)	2.7 ± 0.09 A	5.0 ± 0.32 A	1.47 *
(4)	2.15 ± 0.09 AB	2.7 ± 0.09 B	0.95 NS
(2)	2.0 ± 0.08 AB	1.7 ± 0.08 B	0.69 NS
Zero	1.5 ± 0.08 B	1.0 ± 0.02 B	0.71 NS
LSD value	0.72 *	1.49 *	---

* ($P<0.05$), NS: non-significant.
The same letters in the column show no statistically different ($P\leq 0.05$)

Table4. Effect of different concentrations of nitrogen in the growth medium on total lipid (%) content (mean± SE) of studied microalgae

Concentration (g/l)	Studied microalgae		LSD value
	C. vulgaris	*N. palea*	
Control (8)	6.5 ± 0.52 C	40 ± 2.58 C	6.73
(4)	9.0 ± 0.86 C	45 ± 2.84 C	8.02
(2)	25 ± 1.53 B	50 ± 2.92 B	6.39
Zero	40 ± 2.07 A	60 ± 3.66 A	6.07
LSD value	8.38	6.55	---

The same letters in the column show no statistically different ($P\leq 0.05$)

Table5. Effect of different concentrations of nitrogen in the growth medium on Oleic acid (%) content (mean± SE) of studied microalgae

Concentration (g/l)	Studied microalgae		LSD value
	C. vulgaris	*N. palea*	
Control (8)	0.05 ± 0.01 B	2 ± 0.03 C	0.09
(4)	0.4 ± 0.03 B	5 ± 0.07 BC	1.04
(2)	0.5 ± 0.07 B	8 ± 0.13 B	2.66
Zero	6.0 ± 0.11 A	15 ± 0.74 A	5.82
LSD value	2.17 *	5.38 *	---

The same letters in the column show no statistically different ($P\leq 0.05$)

Table6. Effect of different concentrations of nitrogen in the growth medium on Stearic acid (%) content (mean± SE) of studied microalgae

Concentration (g/l)	Studied microalgae		LSD value
	C. vulgaris	*N. palea*	
Control (8)	0.7 ± 0.09 C	1.5 ± 0.03 C	0.36
(4)	5.7 ± 0.47 C	3.0 ± 0.08 C	0.82
(2)	17 ± 0.94 B	15 ± 0.90 B	1.25
Zero	26 ± 1.53 A	34.5 ± 1.66 A	3.77
LSD value	5.92 *	7.44 *	---

The same letters in the column show no statistically different ($P\leq 0.05$)

Table7. Effect of different concentrations of nitrogen in the growth medium on total carbohydrate contents (mean± SE) of studied microalgae.

Concentration (g/l)	Studied microalgae		LSD value
	C. vulgaris	*N. palea*	
Control (8)	16.5 ± 0.86 B	20 ± 1.04 B	3.76 NS
(4)	19.5 ± 0.94 B	25 ± 1.31 A	3.42
(2)	22 ± 1.23 AB	21 ± 1.16 B	3.50 NS
Zero	25 ± 1.31 A	20 ± 1.04 B	3.75
LSD value	4.74 *	3.92 *	---

NS: non-significant.
The same letters in the column show no statistically different (P≤ 0.05)

Table8. Effect of different concentrations of nitrogen in the growth medium on total Protein contents (mean± SE) of studied microalgae.

Concentration (g/l)	Studied microalgae		LSD value
	C. vulgaris	*N. palea*	
Control (8)	50 ± 2.47 A	15 ± 0.64 A	6.37
(4)	33 ± 1.52 B	10 ± 0.52 B	5.92
(2)	27 ± 1.29 B	7 ± 0.11 BC	5.39
Zero	15 ± 0.64 C	3 ± 0.04 C	4.18
LSD value	8.39 *	4.74 *	---

The same letters in the column show no statistically different (P≤ 0.05)

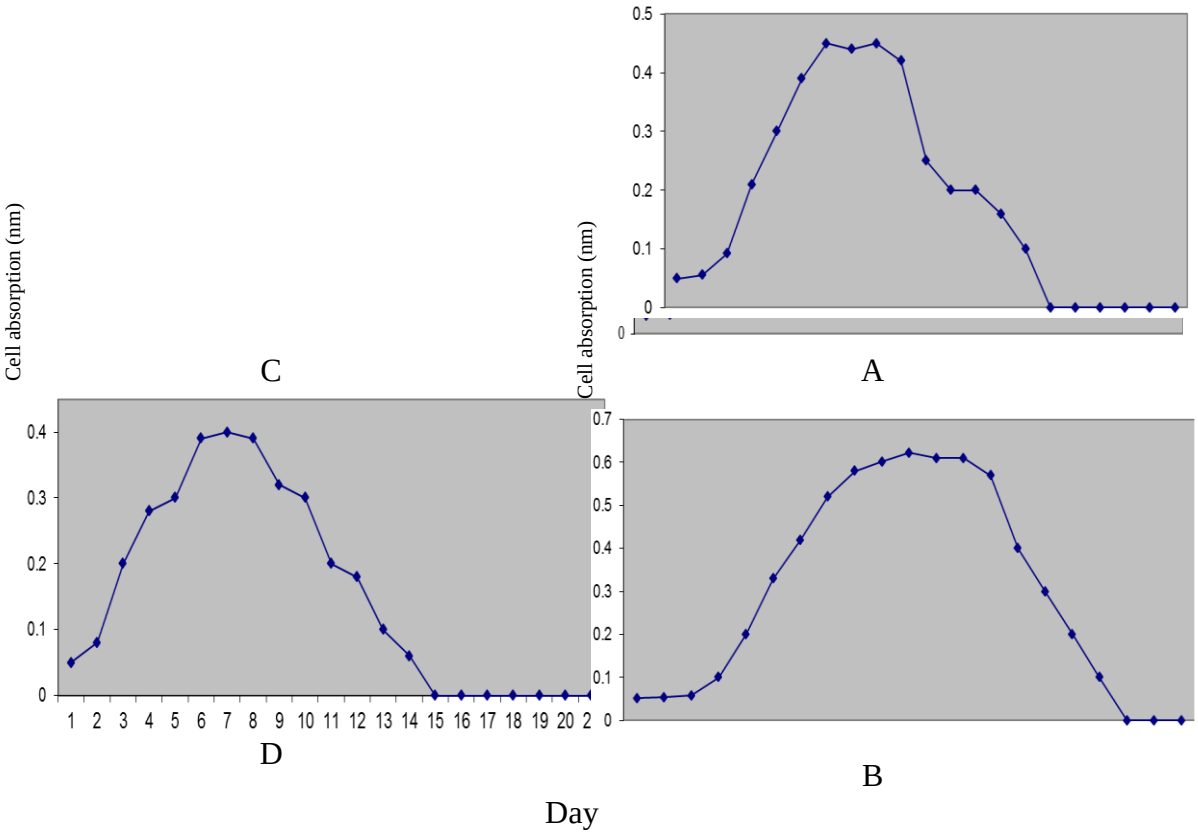

Fig. (1). Growth curve of *C. vulgaris* at different nitrate concentration (mg/l) a=8; b=4; c=2; d=zero

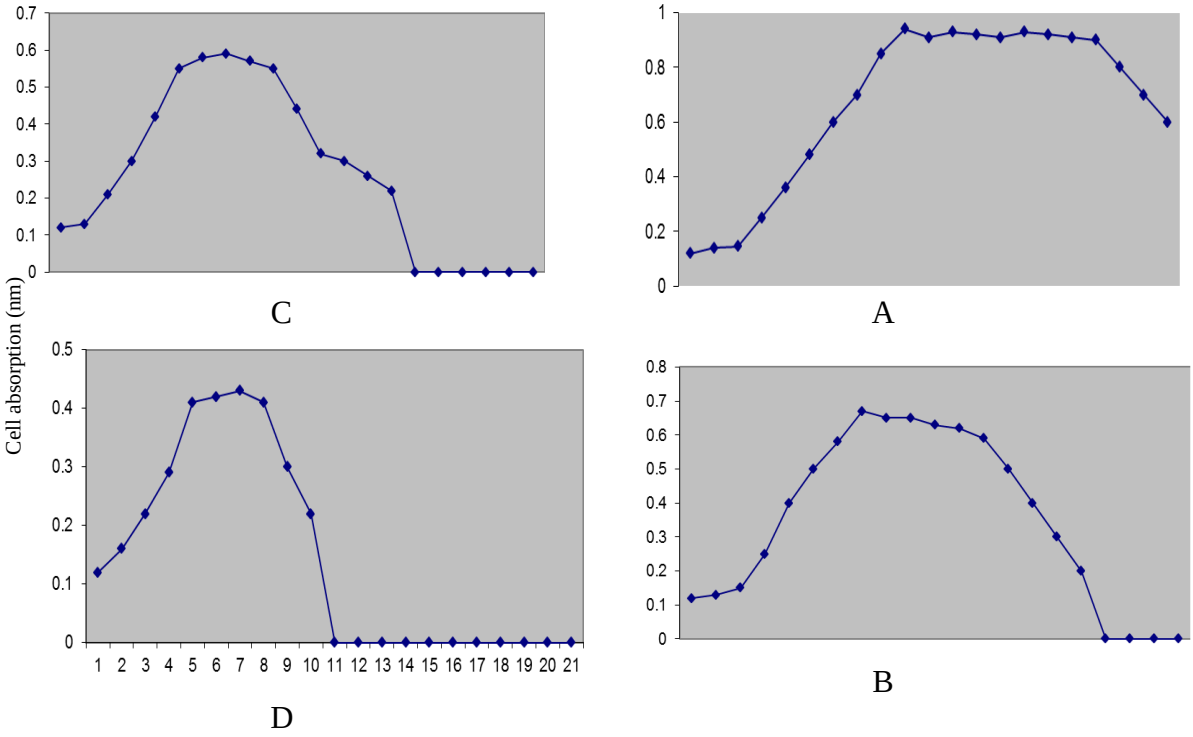

Fig. 2. Growth curve of *Nitizchia palea* at different nitrate concentrations (mg/l). a= 8; b= 4; c= 2; d=zero

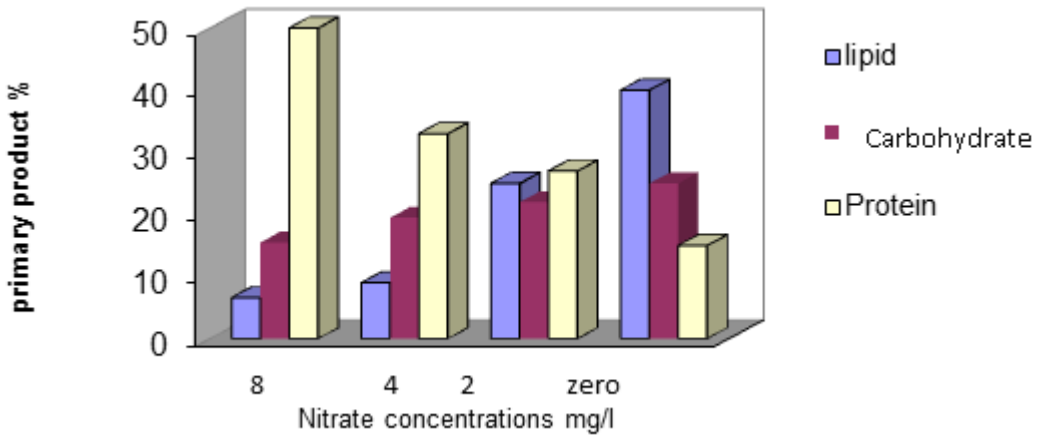

Fig. 3. Total lipid ,carbohydrate and protien of *C.vulgaris* at variouse nitrate concentrations.

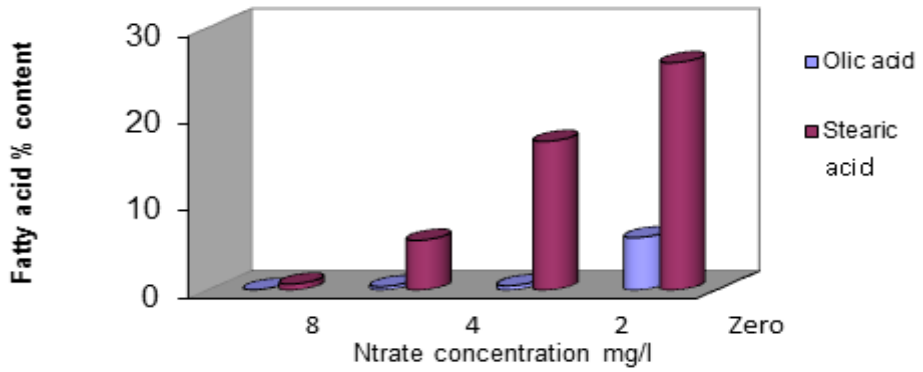

Fig. 4. Fatty acids of *C. vulgaris* at various nitrate concentrations.

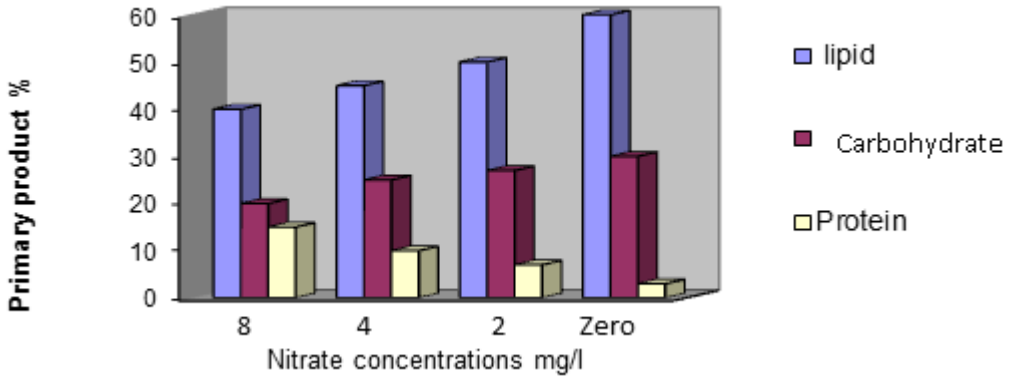

Fig. 5. Total lipid ,carbohydrate and protien of *N.palea* at various nitrate concentrations.

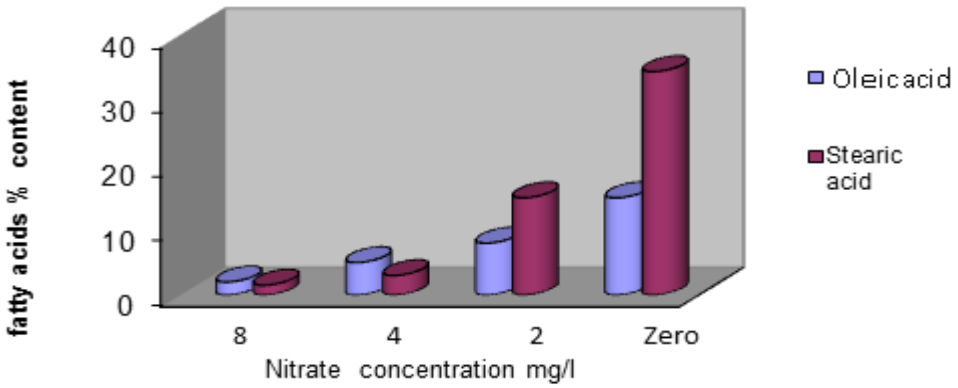

Fig. 6. Fatty acids of *N. palea* at various nitrate concentrations.

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محاولة لتحفيز انتاج الدهون للديزل الحيوي من الطحالب الدقيقة المعزولة محليا في العراق

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الخلاصة:

استخدمت عزلتين محلية من الطحالب الدقيقة (*Chlorella vulgaris* Bejerinck and *Nitzschia palae* (Ktz.) W. Smith) ,لاختبار قدرتها على انتاج الديزل الحيوي من خلال تحفيزها بمعاملات مختلفة من النتروجين (صفر و 2 و 4و 8 ملغم/لتر), وتاثيرها على المنتجات الاساسية (الكاربوهيدرات والبروتين) كذلك على كمية ونوعية الدهون. اظهرت النتائج بان المعاملة ناقصة النتروجين (صفر) حفزت على انتاج عالي من الدهون في كلا من الطحالب الدقيقة *C. vulgaris* و *N. palea* حيث ارتفع محتوى الدهون من 6.6 ٪ الى 40 ٪ ومن 40 ٪ الى 60٪ من الوزن الجاف على التوالي. واظهرت *C. vulgaris* اعلى محتوى للكاربوهيدرات 23٪ من الوزن الجاف في المعاملة صفر نتروجين وسجلت اعلى محتوى للبروتين 50٪ من الوزن الجاف في معاملة 8 ملغم / لتر. في حين *N. palea* كان اعلى محتوى للكاربوهيدرات 25٪ من الوزن الجاف في معاملة نتروجين 4ملغم/لتر واعلى محتوى للبروتين 15٪ من الوزن الجاف في معاملة نتروجين 8 ملغم/لتر.

الكلمات المفتاحية: طحالب دقيقة, دهون, حامض ستيرك , حامض اويلك, الديزل الحيوي