Effect of pHCondition on the Growth and Lipid Content of Microalgae *Chlorella vulgaris&Chroococcus minor*

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Abstract: Two locally isolated microalgae (green algae *Chlorella vulgaris* and blue green algae *Chroococcus minor*) were used in the current study to test their ability to production biodiesel through stimulated in different pH levels treatments (pH 5,pH 9) and effect of pH level on the quantity of protein ,carbohydrate. Showed that the accumulation of lipids in *C.vulgaris* more efficient than *C.minor*, The treatment pH 9 was recorded *C. vulgaris* the highest lipid content from 8% at control to 32% as well as highest carbohydrate content from 18% at control to 25% but showed decreased protein content from 51% to 31%.The treatment pH 9 was recorded in *C. minor* was recorded the highest lipid content from 5% at control to 12% as well as highest carbohydrate content from 15% at control to 18% but showed decreased protein content from 40 % to 30% .The results revealed that Stearic acid and Oleic acid content increased content for both algae at pH 9 levels.

Key words: microalgae, pHlevel, lipid content,biodiesel

1 INTRODUCTION

THE basic sources of energy are fossil fuels, natural gas, and coal, hydro electrical and nuclear. The need of energy is increasing continuously due to the increase in population and industrialization ,These resources of energy are limited [1] and their combustion will lead to generation of the energy-related emissions of greenhouse gases (GHG) such as carbon dioxide, sulfur dioxide, methane and nitrogen oxides [2]. During the last few decades, global atmospheric concentrations of GHG have considerably raised in growth rate of CO_2emissions that main cause of global warming [3]. Biodiesel is one of the better choices among varieties of bioenergy, and microalgae are claimed to be the best cropfor biodiesel production [4, 5]. Renewable and cleaner biofuels from microalgae have attracted widespread attention in recent years [6, 7]. The most common biofuel from microalgae is to produce biodiesel from algal lipids (oil) through transesterificaiton the most important compounds are Triacylglycerides[4, 8].

Microalgae are unicellular photosynthetic organisms that use lightenergy and carbon dioxide, with higher photosynthetic efficiency than plants for the production of biomass[9]. In particular,microalgaecould be used for the production of lipids.Which can be cultured throughout the year, have a simple reproducing system, ability to grow in wastewater/seawater/brackish water, non-interference of food chain, and high-lipid productivity, use water most effectively and do not need rich soil forGrowth [8,10].Most green microalgae produce starch under normal conditions. When exposed to stress under abnormal conditions willstart to synthesize lipids especially, triacylglycerol which is stored in their oil bodies [11]. Several studies have shown that the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions, such as pH variation, temperature and light intensity, nutrient media characteristics, concentration of nitrogen, phosphates and iron [12, 13].

Many reports study fluctuations of the pH in the medium also have been found to alter the lipid composition of microalgae [14,15,16 and17]. The effects of pH on the lipid and FA composition of a *Chlamydomonas* sp. and *Chlamydomonasreinhardtii*In the unidentified *Chlamydomonas* sp., FAs of polar lipids were more saturated than those in C. reinhardtii. grown at pH 1 than that in the cells cultivated at higher pH. The increase in saturation of fatty acids in membrane lipidsof *Chlamydomonas*has been suggested to represent an adaptive reaction at low pH to decrease membrane lipid fluidity [18].

The present study aimed to examine the ability of some isolated local algae to produce lipids in different pH, also to determine the lipids quality that uses in biodiesel.

2 Materials and Methods:

2.1Sampling and Collection of Microalgae

Fresh water samples of algae were collected from the ponds in Al-Mustansiriya University. The samples were collected by sterile container (100 ml) which was marked with date and location of sampling then transported to laboratory immediately to be incubated under suitable condition (268 µE/m²/s, ,16:8 light: dark and 25± 2 C˚) .

2.3 Algae Isolation and Purification

According to [19,20] two methods were used for isolation and purification: streaking on plate agar; Chu-10 media solution solidified by 1.5 % agar-agar and sterilized by autoclave, after sterilization Chu-10 with 45-50 C˚ was poured. Into petri-dishes which left to solidify, sterile loop was used for streaking straight line. Then the plates were keptin a cooled illuminated incubator with light intensity about 268 µE/m²/s, 25± 2 C˚ and 16:8 lights: a dark periodof 10 -14 days. The second method The serial dilution method by using ten test tubes, each one contains 9 ml Chu-10 nutrient solution, 1ml of algal culture was added to the first tube and shake carefully then 1ml from the first tube transported to the second tube and so on then incubated for two weeks . The same way again to the media culture for BG-11

2.4 Preparation and Sterilization of Media

Modified Chu-10 was used for the green algal growth [21],BG-11 culture media for cyanobacteria [22] and described their components in tables(1and 2), respectively, as It was prepared Stock solutions of each salt for macronutrients and stock solutions for micronutrients combined as follows:

TABLE1: Stock Solutions Component of Chu-10

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Al-Mustansiriya University/College of Science Biology department / Botany

A-macronutrient salt	Concentration g/L	B- micronutrient salt	Concentration g/L
Sodium Meta Silicate	5.8	EDTA. Na_2	1.00
$Ca(NO_3)_2.4H_2O$	57.56	H_3BO_3	2.86
K_2HPO_4	10	$MnCl_2.4H_2O$	1.81
$MgSO_4.7H_2O$	25	$ZnSO_4.7H_2O$	0.222
EDTA .Na_2	4.36	$NaMoO_4.5H_2O$	0.390
$FeCl_3.6H_2O$	3.15	$CuSO_4.5H_2O$	0.079
Na_2CO_3	20	$Co(NO_3)_2.6H2O$	0.0494

TABLE 2: stock Solutions Component of BG-11

Macronutrient salt	Concentration g/L	Micronutrient salt	Concentration g/L
$NaNo_3$	150	H_3BO_3	2.86
K_2HPO_4	30	$MnCl_2.4H_2O$	11.8
$MgSO_4.7H_2O$	75	$ZnSO_4.7H_2O$	0.222
$CaCl_2.H_2O$	27.181	$(NH4)Mo_7O_{24}.4H_2O$	0.0124
Citric acid	6	$CuS0_4.5H_2O$	0.072
Ferric ammonium Citrate	6	$CO(NO_3)_2.6H_2O$	0.048
$EDTA.Na_2$	1		
Na_2CO_3	20		

Adjusted pH for media Chu-10 and BG-11 to 6.4 and 7.5, respectively, by adding a few drops of sodium hydroxide or hydrochloric acid (0.01N) then sterilized in autoclave except K2HPO4 at 121Cº, 1.5 j for 15min

2.5 Algae Cultivation forBiomass

A100 ml suitable media for both isolated algae and transfer 10 ml of isolated algae then incubated for 14 days,also transferthis culture growth to 1000 ml of culture media and incubated again for 14 days. Finally, the growth culture transmits to glass pools 4L dimensions (50 cm length, 40 cm width and 30 cm high) for biomass culture [23].

2.6 Determination the Growth Curve

Growth curve was determined for the purpose of identifying growth phases. Then the deposition cultures at the beginning of stationary phase, on the*Chlorella vulgaris* harvested in the twelve day but *Chroococcus minor* in the ten day.Microalga concentration was determined daily by optical density (OD) measurement at 540 nm by UV-Vis spectrophotometer,all measurements of the study were triplicates. The growth rate (K) and doubling time (G) were calculated according to the following equation:

$$K = \frac{(\log OD_t - \log OD_0)}{t} \times 3.322 \quad [24]$$

$$G = \frac{0.301}{K} \quad [25]$$

t: time (day)OD_t: product after (t) dayOD_0: algae beginning of the experiment zero time

The growth curve was determined for the two studied isolated microalgae, in addition to the growth rate (K) and the doubling time (G) were calculated for each treatment of the study experiments (Li *etal*., 2008).

2.7 Stimulation of Algae to Produce Lipid

Many factors affected on the metabolic of algae and in this study pH factors was selected. This is one of the most important factors that encourage the production of lipids in the algae [16].

The pH 6.4 and 7.5 was used for cultivation of green algae and blue green algae respectively , This pH was treated as a control in the study experiments;two levels of pH 5 and pH 9 tested at this study.

2.8 Lipid Extraction:

One gram of dry weight was put in thimble that was transferred to specific cylinder in the soxhlet then 250 ml of solvent (hexane) was put in the flask. After 3-4 hours, the solvent color in the cylinder change from green to colorless, extracted sample were dried by rotary evaporator at $40C^0$ for few minutes. Samples were poured to clean plates and left in room temperature at 25 C^O overnight then samples were transported to test tubes to be analyzed. [26,27].

2.9 Lipid Analysis:

Samples were analyzed by gas chromatography model DANI 2015 in Ministry of Industry and Minerals / IBN SINA state company.

2.10 Determination of Protein and Carbohydrate:

The protein determined according to Bradford, (1976)method [28] and the carbohydrate according to [29].

2.11 Statistical Analyses:

The result express as mean ± sd. Data were analyzed by one way analysis of variance (ANOVA) followed by Fisher's test for multiple comparison, using stat view version 5.0. Different were considers significant when $p < 0.05$.

3 Results and Discussion

The growth density was measured to follow-up phases of growth from zero time (daily) to twenty days to determine the best phase that depending for harvesting, in this study show different harvesting time for both isolated algae in the treatment because observed different growth curve and growth rate.

3.1**The Effect of pH on Growth Curve for *chlorella vulgaris***

In control treatment spent in lag phase one days and then logarithmic phase began until the thirteen day and then entered stationary phase which lasted until the seventeenth day and then observed the beginning of a decline in the number of cells figure (1).

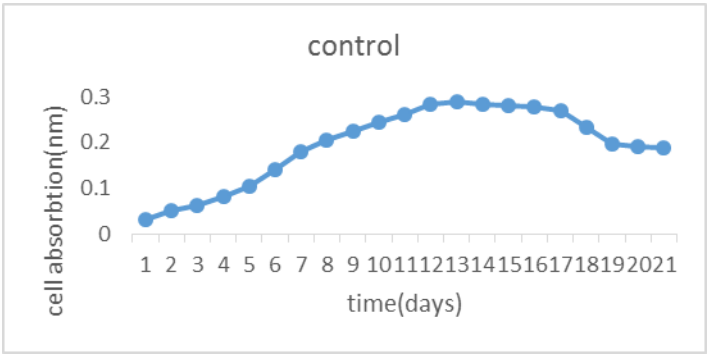

Fig. (1): Growth Curve of *C.vulgaris at* control treatment

In pH 9 treatment spent in lag phase four day and then logarithmic phase began until nine day entered stationary phase which lasted until the fourteenth day and then observed the beginning of a decline in the number of cells and color change from green to yellowish green figure (2)

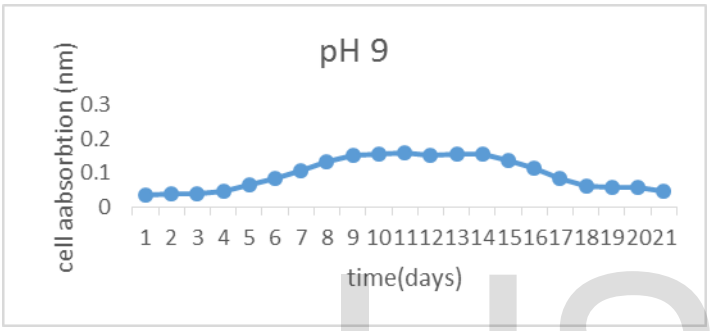

Fig. (2): Growth curve of *C.vulgaris at* pH 9 treatment

Finally, pH 5 treatment spent in lag phase three days and then logarithmic phase began until the nine day and then entered stationary phase until the fourteen day and then observed the beginning of a decline in the number of cells figure (3)

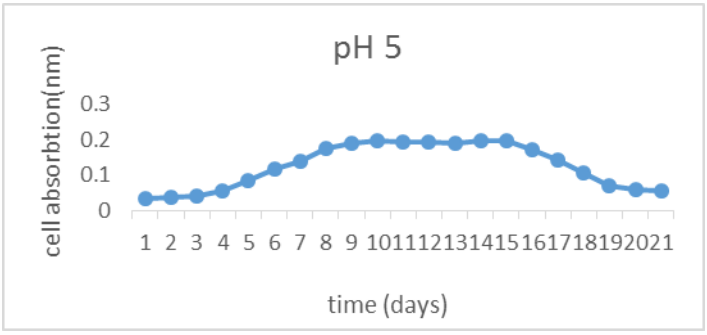

Fig. (3): Growth curve of *C.vulgaris at* pH 5 treatment

The results shown the effect of different pH on growth rate (K) and doubling time (G) for *C.vulgaris* showed a decrease in growth rates but In contrast increased doubling time of all treatment, except control treatment The highest value of growth rat (k) was 0.33 and lowest doubling time 1 day at control treatment while the lowest value of (k) was 0.13 in pH 9 treatment and doubling time is record 3.9 , but at pH 5 treatment the (k) value and doubling time reached 0.20 and 2.2 day respectively. Table (3), Figure (4 and 5)

pH treatment	Growth rate		Doubling time	
	C.vulgaris	*C.minor*	*C.vulgaris*	*C.minor*
Control	0.33±0.021	0.030±0.25	1.00 ±065	1.4 ±0.065
pH 9	0.13±0.026	0.09± 0.026	3.9±0.065	5.3±0.062
pH 5	0.20 ±0.026	0.12±0.026	2.2±0.056	3.6±0.060

TABLE 3 Effect of Different pH Level on Growth Rate (K) and Doubling Time for Both Algae of *C.vulgaris* and *C.minor*

*The mean difference is significant at the P< 0.05 level

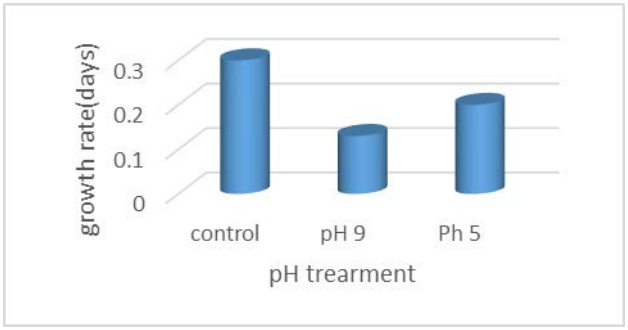

Fig. (4): growth rate of *C.vulgaris* in pH treatment

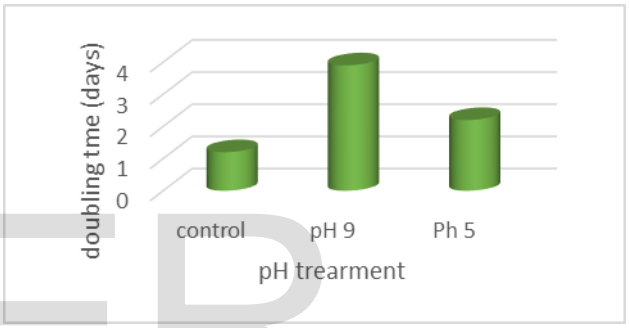

Fig. (5): doubling time of *C.vulgaris* in pH treatment

3.2 The effect of pH on growth curve for *C.minor*

In control treatment spent in lag phase three days and then logarithmic phase began until the ten day and then entered stationary until the seventeen day and then observed the beginning of a decline in the number of cells figure (6)

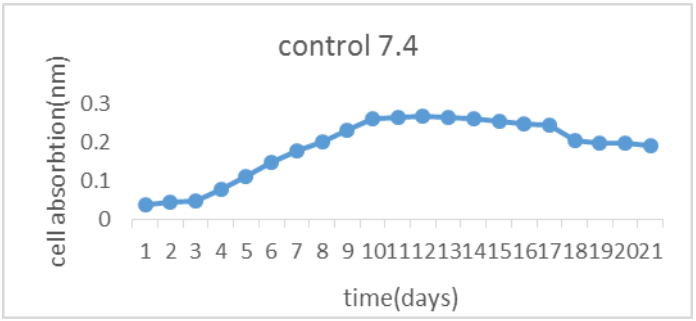

Fig. (6): Growth curve of *C.minor at* control treatment

In pH 9 treatment spent in lag phase four days or then logarithmic phase began until ten day then entered stationary phase until the fourteen day and then observed the beginning of a decline in the number of cells figure (7)

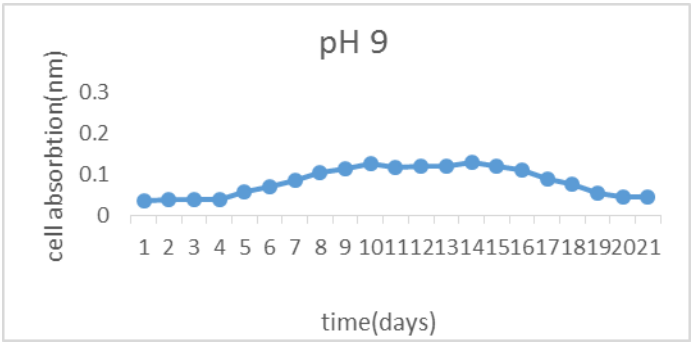

Fig. (7): Growth curve of *C.minor at* pH 9 treatment

Finally, in pH 5 treatment spent in lag phase six days and then logarithmic phase began until eight day then observed the beginning of a decline in the number of cells and change color media in the eighth day figure (8)

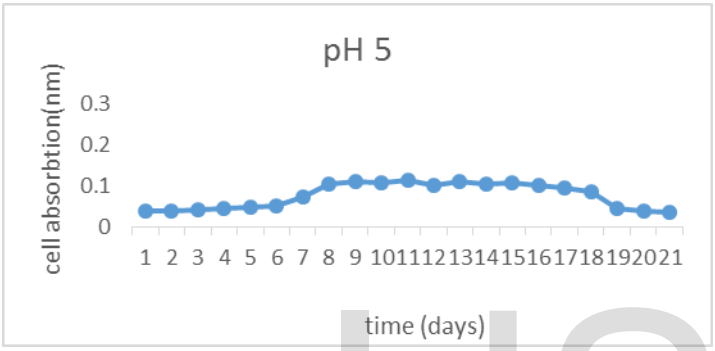

Fig. (8): Growth curve of *C.minor at* pH 5 treatment

And the other hand ,the Effect of different pH on growth rate (K) and doubling time (G) for *C.minor* ,The highest value of growth rat (K) was 0.030 and lowest doubling time 1.4 day at control treatment while the lowest value of (K) was 0.09 in pH 9 treatment and doubling time is record 5.3 , but at pH 5 treatment the (K) value and doubling time reached 0.12 and 3.6 day, respectively table (4) figure (9 and 10)

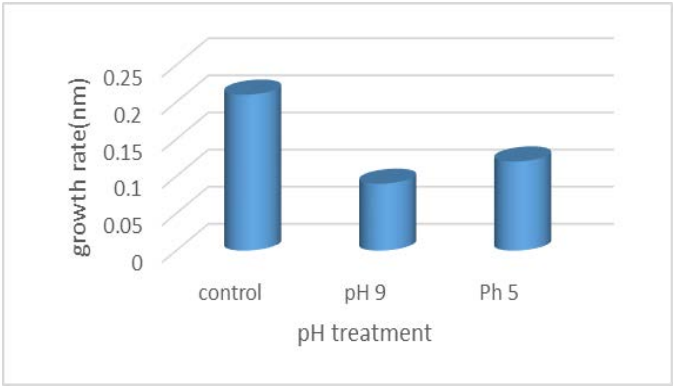

Fig. (9): growth rate of *C.minor* in pH treatment

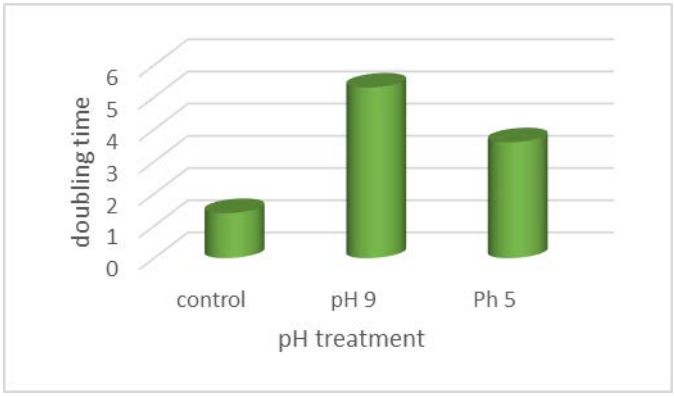

Fig. (10): doubling time of *C.minor* in pH treatment

One of the major factors in the cultivation of algae is pH because it determines the solubility and availability of CO_2 and essential nutrients, and it can have a significant effect on the metabolism of algae [30]. Due to the absorption of inorganic carbon from algae, and can pH significantly higher in cultures of algae [31].

Different growth is observed for each alga isolate in all treatments. The effect of different levels of pH in *C.vulgaris* and *C.minor* biomass growth was significant between (pH5 and pH9). The results showed that increase in pH value to (9) stimulated lipid production by *C.vulgaris* and *C.minor*, in comparison to lower lipid accumulation at 5 pH treatment.

Somchai *"et al."* [32] mentioned that the pH levels less than 7.5 and higher than 9 caused an adverse effect on growth, at pH 6 the filament of blue green alga was broken up into smaller filaments. These results indicated that pH levels at lower and higher than 5 pH could inhibit photosynthesis and affect the morphology of alga. Also, pH values in the range of 8 pH to 9 pH were important for determining the free CO_2 concentrations in the medium. With higher pH values, additional pH effects were observed involving a decrease in the relative high affinity of low CO_2adapted algae to free CO_2[33].

3.3 The effect of pH on lipids accumulation

The extracted lipids content of the early of stationary phase for both algae studies, Table (4) show the result lipids content for C.*vulgaris* was increased from 8% at control treatment to 32% at 9 pH treatment, and its was 21% at treatment5 pH. The same trend was shown for *C. minor* the lipid content increased from 5% at control treatment to 22%, 12% at pH 9, pH 5 treatment respectively, (figure 11 and 12).

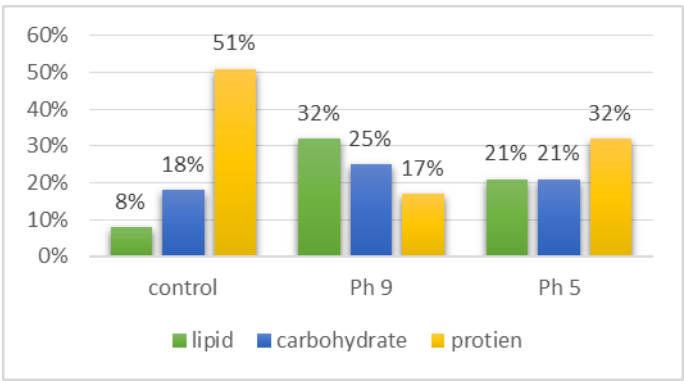

Fig. (11): Total lipid, carbohydrate, and Protein of *Chlorella vulgaris* at different pH

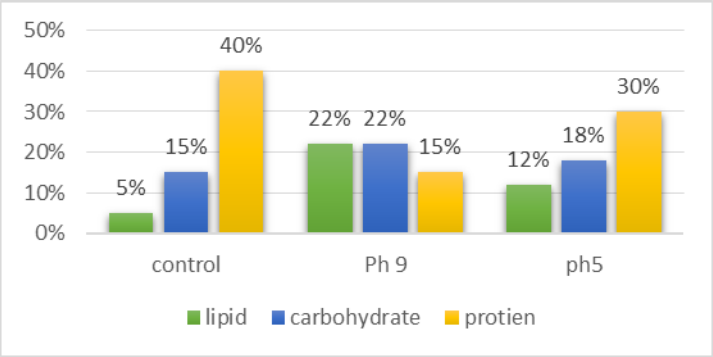

Fig. (12): Total lipid, carbohydrate, and Protein of *chroococcus minor* at different pH

Rai "et al."[34]observed that for *Chlorella* sp. maximum lipid production of 0.1995 g L−1 with lipid accumulation of 23% at pH 8 and24 h photoperiod.

Rodolfi,"et al."[7]noticed better growth of *C.vulgaris* at pH 6.5 and 7.0, and accumulated lipid at pH 7 and 8.5, so optimal for growth and lipid accumulation of *C.vulgaris* was at pH7.0. Alkaline pH increases the flexibility of the cell wall of mother cells, which prevents its rupture and inhibits autospore release, thus increasing the time for cell cycle completion [14].

Moheimani (2013) [35] found pH 7 and 7.5 ideal for accumulation of lipid in *Tetraselmissuecica* and *Chlorella* sp. While we found no significant effect of pH change on lipid accumulation, the treatment with a pH change to 8 exhibited the greatest overall accumulation (averaging 24.75 % by mass). In this study was observed the effects of pH on the biochemical composition of both microalgae *C.vulgaris* and *C.minor*. It found that Increased carbohydrate and decrease in protein content for *C.vulgaris* better than *C.minor* per cell for all treatment.

Carbohydrate content for *C.vulgaris* was increased from 18% at control treatment to 25% at 9 pH treatment, and it was 21% at 5 pH treatment. While Carbohydrate content for *C.minor* was increased from 15 % at control treatment to 22 %at 9 pH treatment, and it was 18 % at 5 pH treatment.

Finally, protein content for *C.vulgaris* decreased for all treatment when compare with 51% control treatment, and it ranged from 17 % to 32% at 9 pH and 5 pH treatment respectively .protein content for *C.minor* also decreased for all treatment , and it ranged from 40% to 15, 30 pH % at 9 and 5 pH treatment respectively(table 4).

TABLE4Effect of Different pH Level on Protein Count (%) of*C. vulgaris* and *C.minor*

pH treatment	***C.vulgaris***		***C.minor***	
	Protein	carbohydrate	Protein	carbohydrate
Control	51	18	40	15
9	17	25	15	22
5	32	21	30	18

Parshikova (2005) [36] said in case of cyanobacteria" the content of chlorophyll a, carotenoides and phycobiline pigments decreases formation the protein and nitrogen value in the cells decreases in connection with their isolation from the cells, Blue green (primarily starch) are another valuable structure of the algal cell, Typical dry weight content of carbohydrates in algae range between 20% to 40% of total the cell mass [37].

Two fatty acids were analyzed using the gas Chromatography (GC), Stearic acid and Oleic acid ,under stress condition of pH (pH9)two fatty acids (oleic, stearic and) appeared, This agreed with study of Thompson, (1996) [38] fatty acids found in Chlorophyceae, C16:0, C18:1, C18:0 were reported as the most common type

TABLE5 Oleic and Acid StearicAcid Count in Both Algae

Treatment	Oleic acid count [mV.s]		Stearic acid count[mV.s]	
	C.vulgaris	*C.minor*	*C.vulgaris*	*C.minor*
stander	14154.342	14154.342	4489.562	4489.562
Control	134.710	271.289	1007.513	617.548
pH 9	527.578	491.708	6876.272	747.024

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