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Changes in the Oil Production and Fatty Acid Composition in *Chlorella vulgaris*

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Abstract

Chlorella vulgaris (single cell microalgae) has recently been paid attention as an alternate source of oil. Effects of several parameters on the yields of oil production and fatty acid (FA) profile of the produced oil by *C. vulgaris* were investigated in this study. Taguchi's experimental approach was used to design the experiments. Yields of oil production were increased when using red and blue lights as sources of photosynthetic energy. Maximum yield of oil production was obtained at 27.0 µM iron at 25±2°C. Increasing the concentrations of NaCl and bicarbonate ion resulted in corresponding decreases in oil production. Maximum level of unsaturated FA was produced when the lowest levels of NaCl and bicarbonate ion were used at 27.0 µm iron levels and at 25±2°C with red light. Ratio of unsaturated to saturated FAs was increased when the oil production was at its optimum level.

Keywords: Microalgae; Oil; Saturated fatty acid; Unsaturated

Introduction

Oil production by algal sources has gained increasing attention in recent years [1-5]. Microalgae grow faster than terrestrial crops [6]. Yield of produced oil by microalgae in one year per hectare of total land area is estimated at ~123 m3 [7], which is higher than that produced by best crop (thirty times more than soybean per unit area) [8]. The oil produced by microalgae has mainly been used for numerous applications such as biodiesels, pharmaceuticals, cosmetics and also for consumption as human food [9]. *Chlorella vulgaris* (green microalgae) is a source of marine oil. If water, sunlight, carbon dioxide and some minerals are available, *C. vulgaris* can produce about 20% oil (w/w, on a dry basis) of high nutritional properties containing linoleic and linolenic acids at 3 and 21% levels, respectively [8,10].

Different methods have been reported to extract oil from microalgae. Use of chemical solvents [11,12] is one approach to extract such oils. Various parameters including silicate level in diatoms [13] as well as nitrogen [12], CO_2 [14], NaCl [9], iron [15] and phosphorus [16] concentrations, temperature [17], and pH [9] impact the yield of oil production and fatty acid (FA) profiles of the produced oil. In the current study, effects of several parameters including temperature and light color and NaCl, $NaHCO_3$ and $FeSO_4.7H_2O$ concentrations on the yield of produced oil and FA composition were investigated using Taguchi's experimental approach.

Materials and Methods

Algal Strain and Culture Conditions

C. vulgaris was obtained from Shahriarriver near the city of Tehran (Iran) and cultivated at different conditions of this study based on Taguchi's experimental approach [18]. Each of the experimental parameters (NaCl, $NaHCO_3$ and $FeSO_4.7H_2O$ concentrations along with the temperature and light color) was applied at four different levels (Table 1). The concentrations of nutrients (in mg/L in distilled water) were $NaNO_3$, 250; KH_2PO_4, 175; $CaCl_2{\cdot}2H_2O$, 25; $MgSO_4{\cdot}7H_2O$, 75; K_2HPO_4, 75; NaCl, 25; EDTA, 50; FeSO4·7H2O, 4.98; H_3BO_3, 11.42; $ZnSO_4{\cdot}7H_2O$, 8.82; $NaMoO_4{\cdot}2H_2O$, 0.72; $CoCl_2{\cdot}6H_2O$, 0.38; $MnCl_2{\cdot}4H_2O$, 1.44; $CuSO_4{\cdot}5H_2O$, 1.57; thiamine, 10; biotin, 0.1 and vitamin B12, 0.01. The pH of the medium was adjusted at 6.0 [8]. Primary cellular density was 4×104 cell/L in the media after the incubation (the cellular densities of cultures were determined by counting the number of cells with light microscopy using Neubauer Hemocytometer lams). Cells were grown in the batch mode using several glass vessels (1L) under constant aeration at controlled temperatures. Continuous light was emitted using four fluorescent lamps in four different colors each with 23-W consumption power. Using air pumps (model U-9900, Royal, Germany) cell suspension was bubbled by atmospheric air at 1 L/min to provide necessary CO_2.

Table 1: Various experimental conditions of this study according to Taguchi's experimental design [17].

Run no.	NaCl (g/L)	$NaHCO_3$ (g/L)	$FeSO_4.7H_2O$ (µm)	Temperature (°C)	Light color
1	0	0	9	20	white
2	20	3	18	20	blue
3	10	6	36	20	red
4	30	9	27	20	yellow
5	0	3	36	25	yellow
6	20	0	27	25	red
7	10	9	9	25	blue
8	30	6	18	25	white
9	0	6	27	30	blue
10	20	9	36	30	white
11	10	0	18	30	yellow
12	30	3	9	30	red
13	0	9	18	35	red
14	20	6	9	35	yellow
15	10	3	27	35	white
16	30	0	36	35	blue

Oil Extraction

After 7 days of incubation, the cultures (cells along with their media) were frozen at -18°C. To extract the oil, 500 mL of thawed algal suspension was thoroughly mixed using glass beads for 30 min according to a procedure reported by Lee, et al. [19]. This procedure was applied to destruct the algal cells. After 10 min of mixing, 30 mL of solvent mixture (methanol: dichloromethane at 1:2 ratio, v/v) was used for the extraction of lipid components and the solvent was separated by using a separatory funnel. According to Wiltshire, et al. [20], this combination of the two solvents has indicated a good performance for the extraction of oil. The extraction was repeated 3 more times and all the organic layers collected throughout the extraction procedure were pooled and the solvent was evaporated using a vacuum oven at 35 °C and the amounts of total lipids were determined gravimetrically according to Abbasi, et al. [21].

Analysis of Fatty Acid Composition

FA methyl ester preparation was the first stage to determine FA compositions of the extracted oils from the microalgae. For this purpose, each sample was mixed vigorously with 1.0 mL alcoholic potassium hydroxide (0.11 g KOH in 1.0 mL pure methanol). The mixture was held for 15 min in water bath at 50°C. Finally, the transparent upper layer of suspension that contained the methyl esters was used for the analysis by gas chromatography (Agilent model 6890N, Santa Clara, CA). An HP-5 capillary column from Agilent (30 m × 0.25 µm i.d. × 320 µm thickness) was used to separate the FA methyl esters. The temperatures for the detector (FID) and the injector were set at 300 and 250°C, respectively. The initial oven temperature was 190°C, which was increased to 220°C at a rate of 1.5°C/min and then was further increased to 240°C at a rate of 3°C/min.

Determination of Mean Values Based on Taguchi's Experimental Approach

Individual effects of NaCl, $NaHCO_3$ and Fe^{+2} ($FeSO_4.7H_2O$) concentrations and also temperature and light color in this study on the oil production and FA composition were determined according to the Taguchi's experimental approach [18]. In the first stage, the mean values of the produced oils and FA compositions for different operational conditions from (Table 1) were determined at each level of a given parameter. Such values for a given parameter (e.g., the temperature) at the levels of that parameter show how the oil production will change when the level of that parameter is changed.

Results and Discussion

Yield of Oil Production

(Table 2) presents the yields of produced oil at the various conditions of this study over the seven days of cultivation. Runs 3, 4, 7, 8, 10, 12, 14 and 16 did not indicate any algal growth during this period and as a consequence no oil was obtained for these experiments.

Table 2: Oil yield along with fatty acid compositions of the produced oils from *C. vulgaris* for the 16 runs of this study (at different conditions) according to Taguchi's experimental approach (Table 1).

		Fatty Acid Concentrations (%, W/W)*										
Run no.	Yield (%, w/w)	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2, C18:3	C20:0	UFA	SFA	UFA/SFA Ratio
1	11.6	24.62	2.74	10.51	9.21	2.95	8.54	27.02	14.41	35.56	64.44	0.552
2	11.9	34.71	10.48	10.24	5.47	0	5.64	24.53	8.98	30.17	69.88	0.432
3	…	…	…	…	…	…	…	…	…	…	…	…
4	…	…	…	…	…	…	…	…	…	…	…	…
5	12.6	11.57	11.32	13.4	1.69	6.94	13.33	31.26	10.51	44.59	55.43	0.804
6	18.9	2.74	13.89	9.31	15.44	5.2	22.52	23.15	7.75	45.67	54.33	0.841
7	…	…	…	…	…	…	…	…	…	…	…	…
8	…	…	…	…	…	…	…	…	…	…	…	…
9	14.7	18.17	19.31	15.45	5.95	1.52	5.5	22.73	11.46	28.23	71.86	0.393
10	…	…	…	…	…	…	…	…	…	…	…	…
11	8.29	6.53	28.98	19.17	5.19	1.56	4.19	22.46	11.91	26.65	73.34	0.363
12	…	…	…	…	…	…	…	…	…	…	…	…
13	5.31	6.35	24.05	13.94	5.72	2.25	7.93	30.21	9.56	38.14	61.87	0.617
14	…	…	…	…	…	…	…	…	…	…	…	…
15	5.33	8.11	24.59	16.19	5.49	2.4	6.27	26.85	10.13	33.12	66.91	0.495
16	…	…	…	…	…	…	…	…	…	…	…	…

- Fatty acids shown above are defined as follows: $C_{10:0}$: capric, $C_{12:0}$: lauric, $C_{14:0}$: myristic, $C_{16\ 0}$: palmitic, $C_{18:0}$: stearic, $C_{18:1}$: oleic, $C_{18:2}$:linoleic, $C_{18:3}$: linoleic and $C_{20:0}$: arachidic acids.
- UFA: Unsaturated fatty acids, SFA: Saturated fatty acids.
- Runs 3, 4, 7, 8, 10, 12, 14 and 16 did not lead to any algal growth.

At concentrations above 6.0 g/L of $NaHCO_3$, no growth was observed for the microalgae if at the same time NaCl were at a concentration greater than 10.0 g/L (runs 3, 7, 10 and 14). However, when $NaHCO_3$ was at a concentration less than 6.0 g/L, the microalgae could grow even at NaCl concentrations of >10.0 g/L (2, 6, 11 and 15). When the concentration of NaCl was above 30.0 g/L (runs 4, 8, 12 and 16), chlorella could not tolerate the excessive salt concentration levels and therefore no algal growth was observed. (Figure 1a) presents the effects of temperature and light color and also those of NaCl, $NaHCO_3$ and $FeSO_4.7H_2O$ concentrations on the yield of oil produced by chlorella. Increasing NaCl concentration (up to 10.0 g/L) resulted in decreases in the yields of produced oils (Figure 1a). However, further increase in the NaCl concentration (from 10.0 to 20.0 g/L) resulted in unfavorable conditions for growing the microalgae but

increased the oil level. Above 30.0 g/L NaCl concentration, oil was not produced since *C. vulgaris* could not grow at this level of NaCl concentration. Borowitzka [22] reported similar effects on the growth of *C. vulgaris* at higher salt concentrations. $NaHCO_3$ indicated a consistent adverse effect on the oil production (Figure 1b). Borowitzka [22] reported that green microalgae could not tolerate bicarbonate ions (i.e., the carbonated condition) at concentrations above 0.2 M. The highest yield of oil in the current study (9.7 g/L) was at 0.0 g/L $NaHCO_3$ indicating that adding bicarbonate at any concentration was not a good choice as a carbon source for the growth of C. vulgaris. Increasing the concentration of Fe^{+2} ($FeSO_4.7H_2O$) to 27.0 µM in the current study enhanced the oil production up to 10.0% (w/w) (Figure 1c).However, concentrations above 27.0 µM resulted in a reduction in the level of produced oil. Slight changes in the concentrations of NaCl and iron (Fe^{+2}) (from 10 g/L NaCl, 18 µM Fe^{+2}) resulted in unfavorable conditions for growing the microalgae but increased the oil production. According to Liu, et al. [15], total lipid content in cultures of *C. vulgaris* supplemented with Fe^{+3} was increased.

The above findings indicate that the reduced form of iron (i.e., Fe^{+2}) did not have positive impact on the growth of *C. vulgaris* at higher concentrations but the oxidized form of iron (i.e., Fe^{+3}) resulted in positive impact at high concentrations. Among the four different temperature levels applied in this study, oil yield was at its highest possible (7.9 g/L) at 25±2°C temperature (Figure 1d).Temperatures above and below that resulted in reductions in the oil yield. Light was the most important parameter for algal growth. In the current study, use of different light colors indicated that the production of oil could be impacted by the type of light color applied (Figure 1e). The highest yield of oil production was obtained using blue and red light colors (among the colors applied in this study). Cervantes [23] reported that blue and red lights enhanced chlorophyll production, which was a prerequisite for oil formation. Among the parameters studied, bicarbonate concentration showed the greatest influence on the oil yield (Table 3) with light color having the least effect. Considering the individual conditions of the current study, the highest yield of oil (18.9%, w/w) was obtained for run no. 6, where NaCl was used at 20 g/L and Fe^{2+} was applied at 27 µM at 25°C with red color selected as light source. Also, Bertoldi, et al. [8] and Rosenberg, et al. [10] reported that if *C. vulgaris* is cultivated in good conditions, it can produce 20% oil with high nutritional value.

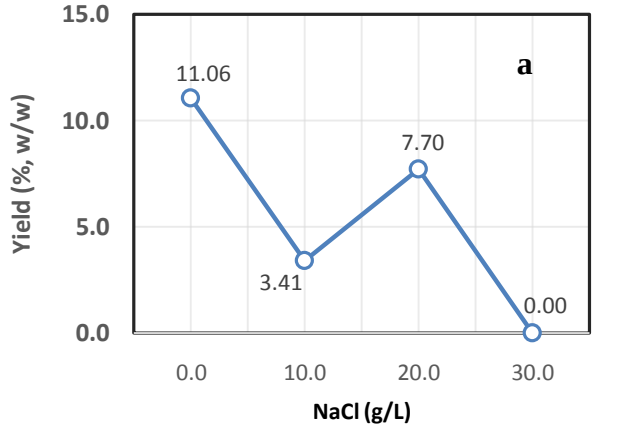


Figure 1: Effects of NaCl.
Figure 1A: $NaHCO_3$.

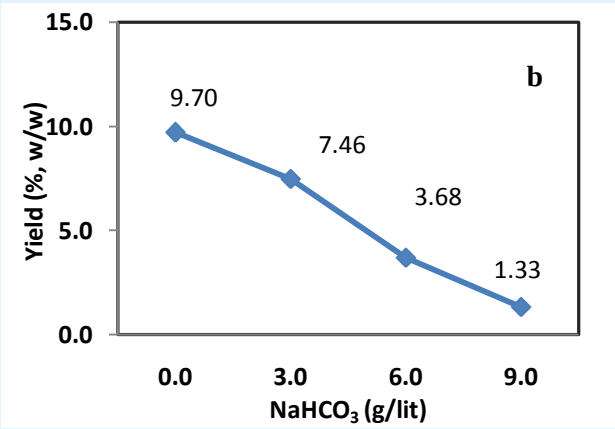


Figure 1B: Andiron.

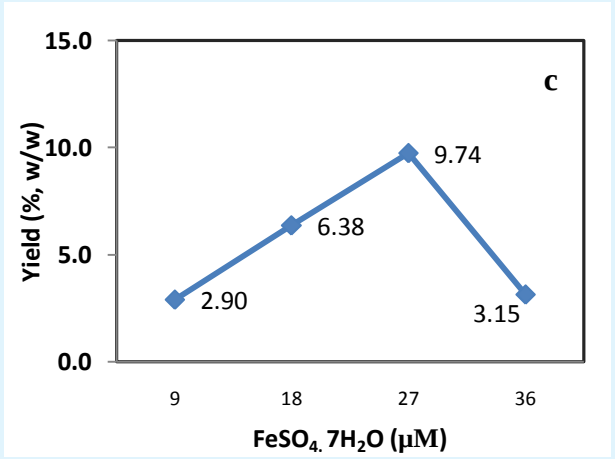
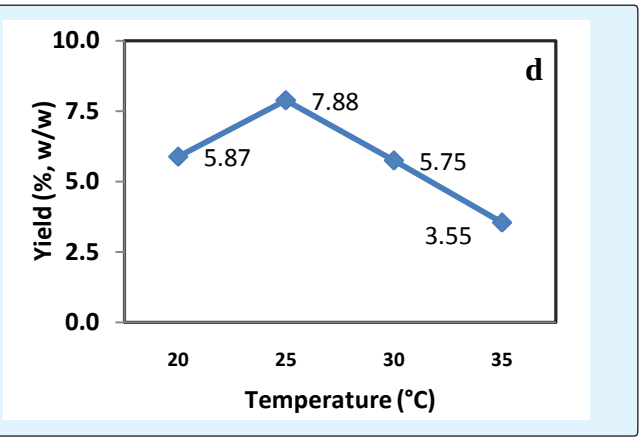


Figure 1C: Concentrations at different temperatures.



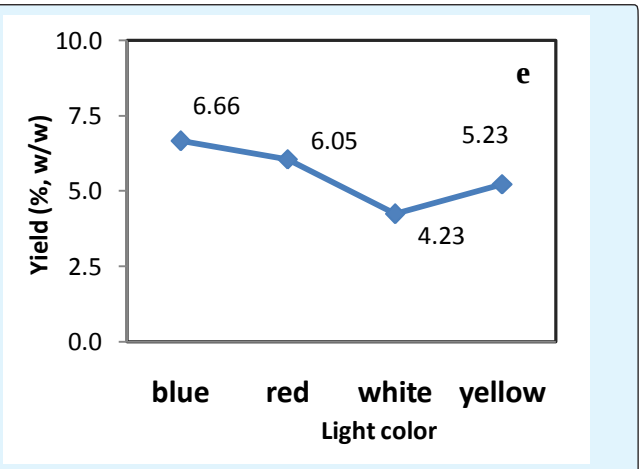


Figure 1: Effects of NaCl (a), NaHCO3 (b) and iron (c) concentrations at different temperatures (d) and light colors (e) on the yield of produced oil (on dry basis) during the seven days of cultivation at the different conditions of this study according to Taguchi's experimental approach (Table 1).

Table 3: Contributions of different parameters in the current study on the yield of oil production during the seven days of *Chlorella vulgaris* cultivation.

Parameter	d*	S*	V*	F *	%p
NaCl	3	0.01	0.004	0.56	16
Bicarbonate	3	0.02	0.006	0.89	26
Fe^{+2}	3	0.01	0.004	0.66	19
Temperature	3	0.01	0.002	0.29	9
Light color	3	0	0		2
Error	3	0.02			
Total	18	0.06			

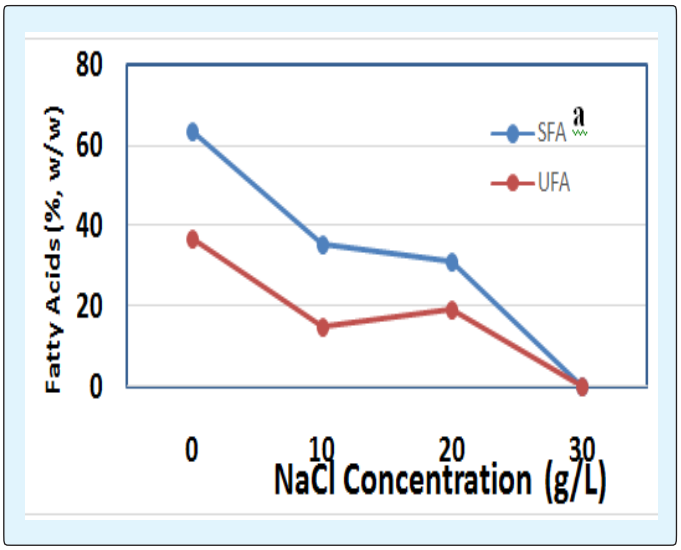
**d*: Degree of freedom, *S*: mean squares, *V*: variance, *F*: V/V_e, V_e: error variance, *P*: S/S_{tot}

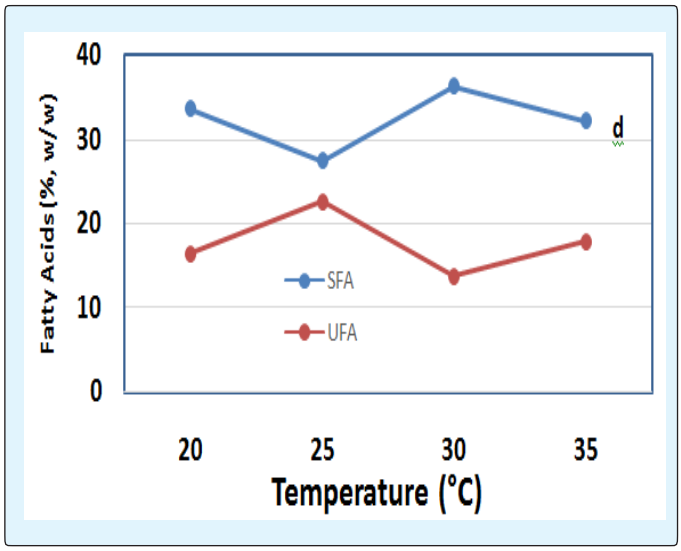
Fatty Acid Composition

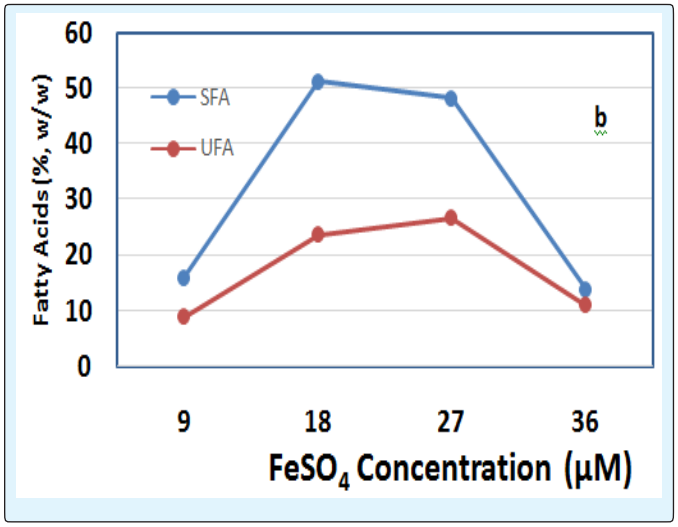
FA compositions of the produced oils in the 16 runs of this study are shown in (Table 2). Nine FAs (capric, lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic and arachidic acids) at different levels were detected in the 8 runs of this study (i.e., where algal growth was observed). Almost all fatty acids were impacted by the different conditions of the study. In all of the specified runs, yields obtained for the saturated fatty acids (SFA) were higher than those for the unsaturated fatty acids (UFA) (Table 2). Concentrations of SFA varied within 54.33-73.34% (w/w).

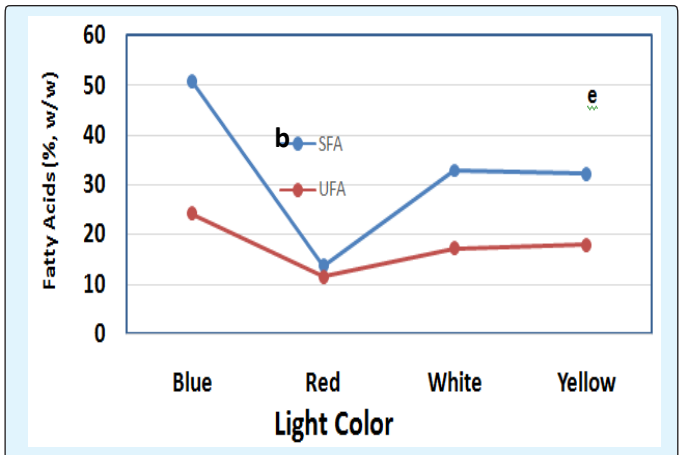
Capric, lauric and myristic acids were the major SFA identified in the oils extracted from the algal samples. Among the runs with biomass production, total UFA were found within 28.23-45.67% and linoleic and linolenic acids were the major UFA (Table 2). Among the UFA, the highest concentration of combined linoleic and linolenic acids was found at 31.26% (run no 5), which is similar to that of rice bran oil (34%) [24]. (Figure 2a-2e) shows the changes in the total SFA and UFA production levels with a change in the concentrations of NaCl, $NaHCO_3$ and $FeSO_4.7H_2O$ at different temperatures and light colors among the different runs of this study. Increasing the concentrations of NaCl and $NaHCO_3$ resulted in a decrease in SFA level. This is due to the fact that C.

vulgaris was sensitive to high concentrations of salt and bicarbonate ions in the culture medium. A slight increase in the level of iron (Fe^{+2}) to 18 µM in the medium resulted in an increase in the production and accumulation of SFA in *C. vulgaris* but additional increase in the concentration of Fe^{+2} to 36 µM resulted in a decrease in these FA to about one fifth of the maximum concentration level. Temperature also indicated certain effects on the FA compositions of the oils produced. Optimum temperature for producing SFA in this study was 30±2°C. Other temperatures resulted in somewhat lower SFA production. At concentration of 27.0 µM iron) II (and at 25±2°C (optimum levels for high yield of oil), the microalgae produced oil with high levels of UFA. Effects of light color on the FA compositions of the produced oils are presented in (Figure 2e). Among the different light colors used in this study, lights with higher energy (blue) increased SFA production in the microalgae.









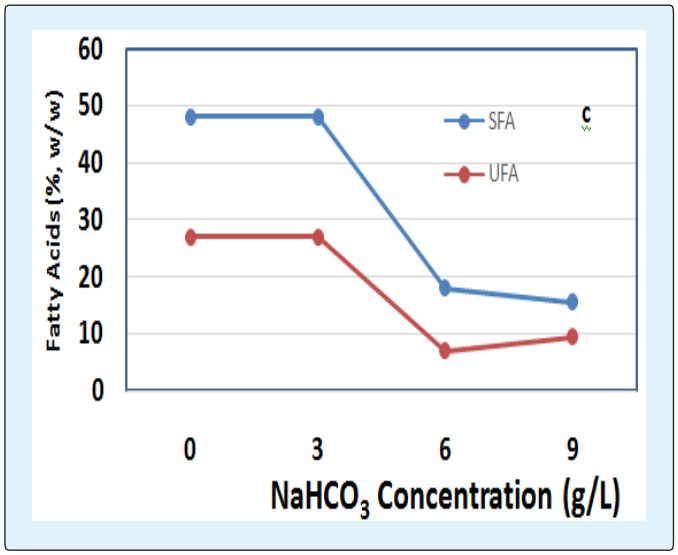


Figure 2: Effects of NaCl (a), NaHCO3 (b) and iron (c) concentrations, cultivation temperature (d) and light color (e) on the yield of saturated and unsaturated fatty acids of produced oil during the seven days of cultivation at the different conditions of this study.

Conclusion

Different parameters applied in the current study indicated major impacts on the oil production and also on the FA compositions of the produced oils. Maximum levels of UFA were produced when the low levels of NaCl and NaHCO3 were used in this study. Increase in the concentration of NaCl to 20.0 g/L resulted in conditions that increased the yield of oil while at the same time the levels of UFA were also increased. At optimum levels for high yield of oil, the microalgae produced oil with high levels of UFA. Slight changes in the concentrations of NaCl and iron resulted in unfavorable conditions for growing the microalgae but increased the oil production. Light color indicated smaller impact on the yield of UFA in this study.

Light at lower energy levels (red) resulted in slight increase in the levels of UFA. Also, when the oil production was at its optimum level (18.9%), ratio of unsaturated to saturated FAs increased (to 0.8406). However, this ratio decreased when the microalgae had the best growth rate (at 30°C, 18 µM Fe^{+2}, yellow light color). Changes in the NaCl concentration resulted in maximal changes in both SFA and UFA in this study (Tables 4 & 5). However, light color had the least effect.

Table 4: Contributions of different parameters of this study on the yield of the saturated fatty acids produced by *Chlorella vulgaris* during the seven days of cultivation in this study.

Parameter	d*	S*	V*	F *	%p
NaCl	3	0.65	0.22	3.82	37.75
Bicarbonate	3	0.39	0.13	2.33	22.98
Fe^{+2}	3	0.49	0.16	2.88	28.43
Temperature	3	0.02	0.01	0.10	0.96
Light color	3	0.01	0.00		0.48
Error	3	0.17			
Total	18	1.72			

**d*: Degree of freedom, *S*: mean squares, *V*: variance, *F: V/V_e*, V_e: error variance, *P: S/S_{tot}*

Table 5: Contributions of different parameters of this study on the yield of the unsaturated fatty acids production during the seven days of *Chlorella vulgaris* cultivation.

Parameter	d*	S*	V*	F *	%p
NaCl	3	0.18	0.06	1.93	34.66
Bicarbonate	3	0.14	0.05	1.47	26.38
Fe+2	3	0.10	0.03	1.00	17.93
Temperature	3	0.02	0.01	0.17	3.08
Light color	3	0.01	0.00		1.53
Error	3	0.10			
Total	18	0.54			

**d*: Degree of freedom, *S*: mean squares, *V*: variance, *F: V/V_e*, V_e: error variance, *P: S/S_{tot}*

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Disclosure

The authors declare that they have no conflict of interest. The manuscript does not contain clinical studies or patient data. The authors alone are responsible for the content and writing of the paper.

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