Investigating the Effects of Several Parameters on the Growth of *Chlorella vulgaris* Using Taguchi's Experimental Approach

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Abstract: Algae are part of photosynthetic organisms that play an important role in the aquatics nutrition. Like plants, algae need water, light and CO_2 to grow. Using Taguchi's experimental approach (5 factors in four levels with 16 runs), effects of several parameters (NaCl, sodium bicarbonate and iron concentrations as well as light and temperature) on the growth of *Chlorella vulgaris* was studied. Increasing the concentrations of NaCl and sodium bicarbonate resulted in corresponding decreases in the growth of *C. vulgaris*. Media with 30.0 g/l NaCl did not indicate any algal growth. Also, with 9.0 g/l sodium bicarbonate, biomass production was decreased. *Chlorella vulgaris* showed different growing behaviors at the various concentrations of iron (Fe^{+2}) and at the different temperatures of this study. Maximum biomass production (approximately 3.56 g dry matter) was obtained at the 0.0 g/l sodium bicarbonate, 10.0 g/l NaCl, 18.0 µmol/l iron and at 30±2 °C. Yellow and blue lights increased the algal growth. Analysis of variance showed that salinity (i.e., the NaCl concentration) had the highest impact on the biomass production.

Keywords: Algae, Biomass, *Chlorella vulgaris*, Taguchi's design.

1. INTRODUCTION

Microalgae can be utilized in the production of nutritional supplements, antioxidants, cosmetics; natural dyes and poly-unsaturated fatty acids [1-3]. Microalgae are supplied in different forms such as tablets, capsules and liquids for human consumption. They can also be used in gum, beverages, candy and snack foods [4]. Under optimal conditions, microalgal population can be doubled within hours, so they are valuable organisms for economic industrial-scale production processes in the 21st century [3]. Spirulina, Chlorella, Dunaliella, Nostoc and Aphanizomenon are among the common algae species [1, 2].

Chlorella is a kind of single-cell green algae living in fresh water that belongs to the phylum Chlorophyta [5]. Chlorella strains consist of *C. protothecoides, C. vulgaris, C. emersonii, C. sorokini* and *C. minuitissima*, which are suitable for biomass production [3, 6].

Chlorella has much higher utilization rate (10-20%) of light energy for the photosynthesis when compared to common plants [7]. Its nutritional value has also great importance for food applications. According to Belasco [8] and Zelitch [5], Chlorella contains 45% protein (w/w, dry basis), 20% fat, 20% carbohydrates, 5% fiber and 10% minerals and vitamins. B-1, 3-glucan is one of the most important substances in Chlorella with a good capacity for scavenging radicals and reducing blood lipids. This compound is also an immunostimulator. Chlorella has also indicated certain health benefits on gastric ulcers and wounds and prevent atherosclerosis, hypercholesterolemia and tumor effects [4]. Production of algal biomass can be worthwhile when considering the high-value metabolites that can be obtained from these sources. Over the 20th century, many studies reported various cultivation technologies for the production of microalgae [9].

Open ponds are one of the primary and effective methods for the large-scale production of microalgae [3, 10]. Photobioreactors are alternate methods for the production of microalgae [3, 11, and 12]. There are several studies reporting on the medium composition for microalgal cultivation. Liu *et al*. [6] enriched sea water with a medium containing $NaNO_3$ 75 mg, $NaH_2PO_4.2H_2O$ 5.65 mg, Na_2EDTA 4.16 mg, $FeCl_3.6H_2O$ 3.15 mg, $CuSO_4.5H_2O$ 0.01 mg, $ZnSO_4.7H_2O$ 0.022 mg, $CoCl_2.6H_2O$ 0.01 mg, $MnCl_2.4H_2O$ 0.18 mg, $Na_2MoO_4.2H_2O$ 0.006 mg, Vitamin B_{12} 0.0005 mg, Vitamin B_1 0.1 mg and Biotin (Vitamin H, B7) 0.0005 mg per liter. Oh *et al*. [13] cultivated Chlorella using a medium containing 40 g glucose, 1 g NH_4NO_3, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot 4H_2O$, 0.05 g NaCl, 0.4 g Ca_2CO_3, 0.1 g yeast extract, 0.1 g Soytone, and 0.1 g Tryptone in 1 l distilled water) with a pH adjusted to 7. The photo bioreactor was stirred at 100 rpm at 25 °C

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and illuminated with continuous fluorescent light and the air was inserted at the bottom of the vessels. Bertoldi *et al*. [14] adjusted the pH to 6.8.

In the current study, effect of several parameters including NaCl, $NaHCO_3$ and $FeSO_4$ concentrations as well as temperature and light color on the growth of *C. vulgaris* was investigated using Taguchi's experimental approach. This design was first reported by Dr. Taguchi and was widely adopted in the 1980_s for quality engineering. Quality engineering at first was used for optimizing the designs (parameter designs) focusing on the functionality systems and then evaluating the system components according to signal, control and error factors with optimization of the systems [15]. Taguchi's orthogonal array design was used for the screening and optimization of medium ingredients in the growth of algae [16].

Daneshvar *et al*. [17] used Taguchi's method for the optimization of biological decolorization of synthetic dye solution from Chlorella. Somnath *et al*. [16] used Taguchi method for lutein production from Microalgae *Auxenochlorella protothecoides* SAG 211-7A.

2. MATERIALS AND METHODS

2.1. Microalgae and Culture Conditions

C. vulgaris was obtained from Shahriar River near the city of Tehran (Iran) and cultivated at different conditions in this study (Table **1**). The medium used in the cultivation of the microalgae especially *C. vulgaris* was Bold's Basal Medium (BBM). This medium stimulate to grow the Chlorella and prevent to grow others so the population of *C. vulgaris* increased by cultivations. The concentrations of nutrients (in mg/l of distilled water) in the culture were from Bertoldi *et al*. [14] (Table **2**). The pH was adjusted to 6.8.

Cells were grown in batch glass vessels under constant aeration at controlled temperatures. Continuous light was emitted from fluorescent lamps in different colors each with 23 W powers. Using air pumps (model U-9900, Royal, Germany) cell suspension was bubbled (sparging method) by atmospheric air at 1 l/min to provide necessary CO_2.

2.2. Harvesting

At the time of harvest, 0.3 g $CaCl_2$ was added as the flocculating agent [13, 18]. After filtering the algal suspensions on pre-weighed Whatman papers (No. 1) [16] and then drying at 100 ºC, the dry weights of algal cells were measured.

2.3. Statistical Analysis and Data Processing

Taguchi's experimental approach (Table **1**) [19] was applied to design the experiments using the five parameters of this study (NaCl, $NaHCO_3$ and $FeSO_4$ concentrations along with temperature and light color) each at four different levels. Individual effects of NaCl, $NaHCO_3$ and Fe^{+2} ($FeSO_4.7H_2O$) concentrations and

Table 1: Various Experimental Conditions of This Study According to Taguchi's Statistical Design Along with Their Equivalent Biomass Yields

Run no.	NaCl (g/l)	$NaHCO_3$ (g/l)	$FeSO4.7H_2O$ (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

Table 2: Nutrients and Concentrations (in mg/l of Distilled Water) of Components Used as the Culture for the Growth of *Chlorella vulgaris* in the Current Study

Nutrients	Concentrations (in mg/l)
$NaNO_3$	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
$FeSO_4{\cdot}7H_2O$	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
Vitamin B_{12}	0.01

also, temperature and light color on the cultivation of Chlorella are also determined according to the Taguchi's approach. At the first stage, the mean values of biomass production were determined at each level of a given parameter (from Table **1**). Such values for a given parameter (e.g., the temperature) show how the biomass production will change when the level of that parameter is changed.

3. RESULTS AND DISCUSSION

Runs 3, 4, 7, 8, 10, 12, 14 and 16 did not indicate any algal growth during this period (Table **1**). The reason for this was the high concentrations of NaCl and $NaHCO_3$ components. Discussions related to each of the parameters studied are as follows.

3.1. Effect of Sodium Chloride Levels (NaCl)

Figure **1a** presents the effect of NaCl on the yield of biomass production at four different levels of salt concentrations (0.0, 10.0, 20.0 and 30.0 g/l). Increasing NaCl concentration resulted in corresponding decrease in the growth of Chlorella. Adding 20.0 g/l NaCl to fresh water reduced the biomass from 1.49 g/l (on a dry basis) to 0.55 g/l. Browitzka [20] reported that the growth of *C. vulgaris* improved in fresh water with no minimal salt concentration. In the current study, when the concentration of NaCl was above 30.0 g/l, Chlorella could not tolerate the excessive salt concentration levels and therefore no algal growth was observed. Table **3** shows the results for the analysis of variance (ANOVA) among the different parameters of this study. NaCl concentration indicated the highest impact on the biomass production of *C. vulgaris* and as a result the biomass was decreased as NaCl concentration increased.

3.2. Effect of Sodium Bicarbonate Levels ($NaHCO_3$)

Figure **1b** presents the effect of added $NaHCO_3$ (0.0, 3.0, 6.0 and 9.0 g/l) on the yield of biomass production. In order to grow, an algae needs to have access to a carbon source [21]. Previously, Lee *et al*. [22] used air enriched with CO_2 as carbon source. Borowitzka [20] reported that green algae could not tolerate bicarbonate ions (i.e., the carbonated conditions) at concentrations above 0.2 M. Therefore, in the current study, $NaHCO_3$ was used as main carbon source at low concentrations. At concentrations above 6.0 g/l $NaHCO_3$, the algae could not grow; NaCl was at a concentration over 10.0 g/l. At lower concentrations of $NaHCO_3$ (<6.0 g/l, runs 2, 6, 11 and 15), the algae could tolerate higher concentrations of NaCl (Table **1**).

Table 3: Analysis of Variance (ANOVA) of the Data Indicating the Contributions of Different Parameters of this Study on the Biomass Production During the Seven Days of *Chlorella vulgaris* Cultivation

Parameter	*d**	*S**	*V**	*F **	*P (%)*
NaCl	3	6.44	2.15	31.04	38.53
Bicarbonate	3	2.23	0.74	10.74	13.33
Fe^{+2}	3	5.88	1.96	28.34	35.17
Temperature	3	1.96	0.65	9.45	11.73
Light color	3	0.83	0.28		4.94
Error	3	0.21			
Total	18	17.54			

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

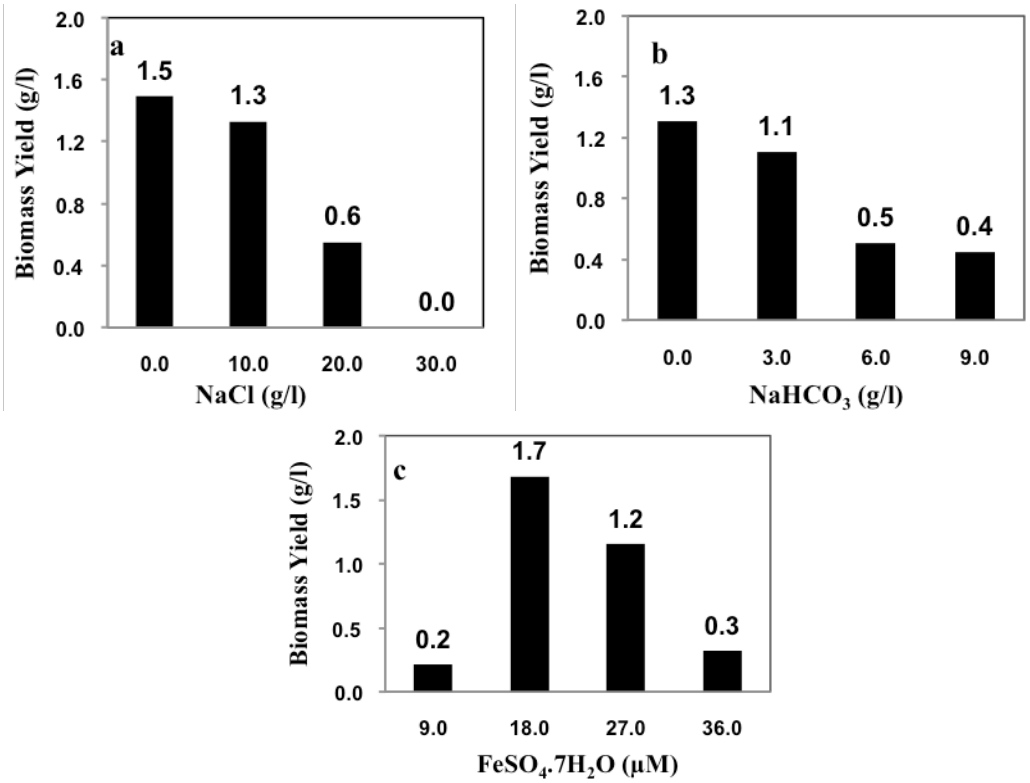

Figure 1: Effects of NaCl (**a**), $NaHCO_3$ (**b**) and $FeSO_4.7H_2O$ (**c**) concentrations on the yield of extracted biomass (on dry basis) during the seven days of cultivation at the different conditions of this study according to Taguchi's experimental design (Table **1**).

Nonetheless, the highest yield (1.31 g/l) was in the absence of $NaHCO_3$, indicating that adding bicarbonate at any concentration was not a good choice as a carbon source for Chlorella growth.

3.3. Effect of Iron

Figure **1c** presents the effect of Fe^{+2} ($FeSO_4.7H_2O$) on the yield of biomass production using four different levels of this ion (9.0, 18.0, 27.0 and 36.0 µM). Results showed that increasing Fe^{+2} levels from 9.0 to 18.0 µM enhanced the biomass production. However, concentrations above 18.0 µM resulted in a reduction. According to Liu *et al*. [6], final cell density was increased by adding chelated Fe^{+3} into the cultures of *C. vulgaris* but adding chelated Fe^{+2} (at levels greater than 18.0 µM) indicated a negative impact on the cell growth. This may be related to the properties of reduced form of iron (Fe^{+2}) that did not have positive impact on the growth of *C. vulgaris* at higher concentrations but the oxidized form of iron (Fe^{+3}) resulted in positive impact at high concentrations.

3.4. Effect of Temperature

Results for the biomass production after seven days of cultivation are shown in Figure **2** for the 16 runs of this study. Run 11 (at 30 °C)indicated the highest biomass yield (3.56 g/l). Kachroo [23] cultivated *Chlorella minutissima* at artificial sea water medium in a batch system (glass house) and obtained only 0.24 g/l algal biomass in 10 days.

This is in agreement with the results of Carlsson *et al*. [1], who reported that optimum temperature for Chlorella growth was 30-35 ºC. The high yields of biomass in the current study were obtained for runs 9, 11, 13 and 15.

Temperature plays an important role in the cell growth and metabolite synthesis [11]. Carlsson *et al*. [1] reported that optimum temperature for Chlorella growth was at 30-35 ºC. Figure **3a** presents the effect of temperature on the algal biomass at four different levels of 20, 25, 30 and 35 ºC. The highest yield in the current study (1.4 g/l) was obtained at 30±2 °C, after which an increase in the temperature (to 35±2 °C) resulted in a drop in the biomass yield (0.89 g/l). Results of this study indicate that *C. vulgaris* colder temperatures reduce the growth rate of this species.

3.5. Effect of Light Color

Light is the most important parameter in growing algae [24]. Light helps photosynthesis in producing

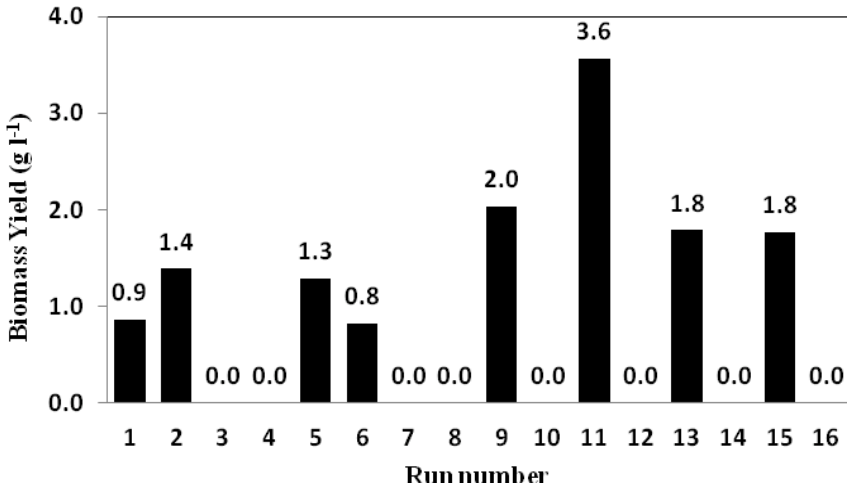

Figure 2: Changes in the biomass production during the seven days of cultivation at the different conditions of this study according to Taguchi's experimental design (Table **1**). Runs 3, 4, 7, 8, 10, 12, 14 and 16 did not lead to any algal growth.

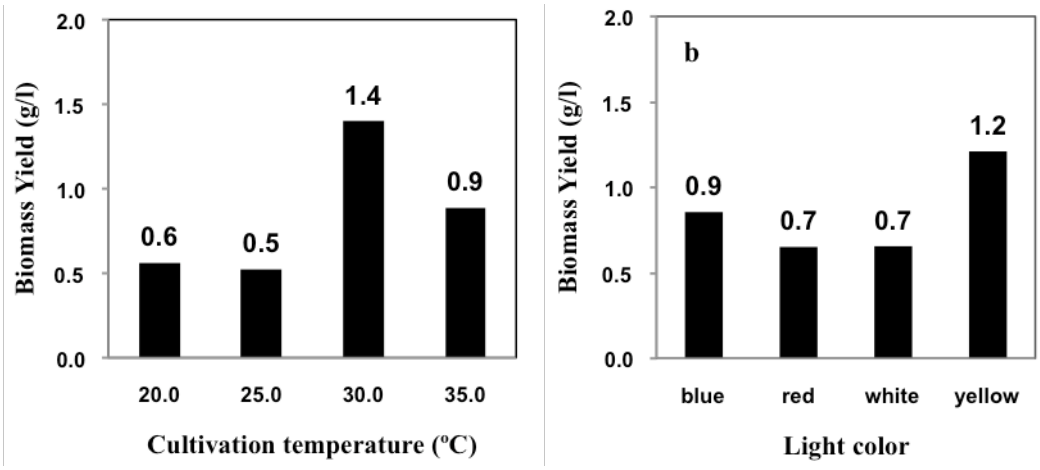

Figure 3: Effects of cultivation temperature (**a**) and light color (**b**) on the yield of extracted biomass (on dry basis) during the seven days of cultivation at the different conditions of this study according to Taguchi's experimental design (Table **1**).

chlorophyll and other metabolites in the algae. In this study, four different colors (white, yellow, blue and red) were applied (Figure **3b**). *C. vulgaris* indicated the best growth rate under yellow light (with low energy) (high yield of photosynthesis). Other colors (blue, white and red) resulted in somewhat less biomass production. According to Cervantes [25], when yellow, red and blue lights were used, the yield of photosynthesis was high. Among the parameters investigated in the current study, light color indicated the least significant effect on the algal production (Table **3**).

4. CONCLUSIONS

Different parameters of this study influenced the growth of *C. vulgaris.* Greatest impact was found in the cultures containing higher concentrations of $NaHCO_3$ and NaCl resulting in no algal growth at such conditions. Fe^{2+}concentration also needed to be at an optimum level (18 µM) for the highest biomass production. Similarly, the cultivation temperature had to be adjusted on an optimum level (30 ºC) for a higher algal growth. Furthermore, among the different light sources used in this study, yellow was the best one resulting in higher biomass production.

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