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Experimental Design of Chlorella Vulgaris Cultivation in Wastewater of Al-rustamiyah South Station

Muna N.AL-Rikabey*

Department of Biochemical Engineering, Al-Khwarizmi College of Engineering, University of Baghdad, Iraq

ABSTRACT

In this research the wastewater used as alternative for culture media of cultivation of Chlorella vulgaris. Different conditions were used to study their effect on growth of Chlorella vulgaris such as temperatures with range (20-35℃), pH (5-11), moreover cultivation time (1-13 day) and dilution ratio (25%-100%). The experimental runs of this work were designed by (Taguchi method). The analyses of Signal to Noise ratio(S/N) and variance (ANOVA) were used to identify the effect and the significance of the process parameters on growth algal. The final results show the significance of all process conditions on growth algae. The order of significance of these conditions is: cultivation time> dilution ratio> temperatures> pH. The moderate temperature at 30℃, dilution rate at 75%, alkaline conditions at pH=9 and 13 day for cultivation are optimum conditions to enhance the growth algal and to obtaining on high optical density with (0.5997).

Keywords: Microalgae; Chlorella vulgaris; Wastewater; Modeling; Algal biomass; Taguchi design

INTRODUCTION

In the last three decades, there are many methods and techniques that use cells or organisms to overcome the depleted resources of our planet. Also, to obtain products that can satisfy certain needs for benefit of a better quality of life [1]. Therefore, the scientists trying to investigate alternative resources, alternative supplements for growth, immunity, reproduction, metabolism, and reduce environmental pollution.

Recently, microalgae biomass is successfully used in the pharmaceutical and cosmetic industry; it is used for the production of food additives, and also for the cultivation of fish. In the long term, it is expected that with an increase in the scale of production, microalgae will be used as raw materials for the production of biofuels and as feed for farm animals. Alternative fuel from microalgae is an object for large investments, primarily from oil and aviation companies [2].

Cultivation of microalgae to obtain high-quality biomass is a promising area in the field of biotechnology. The cultivation of microalgae is relatively easy, which makes them useful in studies of biological, physiological and biochemical laboratories [3]. Microalgae can grow under various cultivation methods either by autotrophic which is based on photosynthesis, without input organic carbon source. Microalgae consume CO_2 for the synthesis of the organic matter via photosynthesis or heterotrophic which is a profitable method of cultivation to cultivate the microalgae to large scale; they are indeed able to use organic carbon as energy source [4] or by mixotrophic which is a mode of culture combining autotrophy and heterotrophy [5].

World algal flora has about 40 thousand species, but the most promising are representatives of families Chlorella, Dunaliella, Scenedesmus, and Spirulina. At the present time, chlorella is used mainly as a high-quality and balanced supplement that provides a complete diet for a person [6]. The attention of scientists was attracted by green microalgae from the genera Chlorella and Scenedesmus, which were mainly cultivated for a variety of applications.

Chlorella vulgaris (*C. vulgaris*) has a size that ranges from 3 to 5 μm, unicellular aquatic plant, microalga, found naturally in fresh water bodies. More recently, chlorella has become an object of commercialization and cultivation on an industrial scale. The biomass of chlorella is predominantly composed of carbohydrates (40 to 70%), proteins (10 to 20%) with all essential amino acids. In addition, chlorella is a rich and balanced source of a variety of vitamins, essential fatty acids, enzymes, chlorophyll, DNA, RNA, antioxidants and a number of other important biological components. Experiments have shown that the use of chlorella has a beneficial effect on the strengthening of the immune system, stimulates the digestive process, cleanses the body of various toxic components[7, 8].

The most promising is the use of algae for sewage treatment of food industry enterprises, fish farms, livestock farms, poultry farms, slaughterhouses. This makes it possible, on the one hand, to purify water, on the other hand, to obtain biomass, which can be used in various fields [9].

Taguchi method based on the orthogonal design is one of the attractive methods used to design the experimental work experiments. Also, it is able to identify the significance of the process parameters on the required response. The signal to noise ratio (S/N) is one of the Taguchi tools that used to identify the effect of each parameter as well as each level of each parameter on the process responses. Furthermore, S/N ratio is used to identify the interaction between the parameters.

Amaral et al, (2015)[10], studied the effect of light intensity, CO2 level, NaNO3 concentration and aeration rate, on the cultivation of the marine Chlorella sp. They use Taguchi L8 orthogonal array at two levels for each factor. The biomass productivity and lipid content were the response variables. The analysis of ANOVA was used to identify the significance of each independent variable on the responses also signal- to-noise ratio was also used to study the effect of each independent variable using larger-the-better response. They found that the highest biomass productivity was 210.9 mg L-1day-1, corresponding to a lipid content of 8.2%. And the aeration rate showed no significant influence on the biomass productivity.

The aim of present study is to evaluate the cultivation of *C. vulgaris* in wastewater obtained from Al- Rustamiyah South Station which is located to the south of Baghdad, Iraq. The cultivation was carried in a photobioreactor. The operating conditions of *C. vulgaris* cultivation are optimized to maximize the yield of biomass. Taguchi method is adopted for this work to design the experiments. The degree of significance of each factor and each level of each factor on the process cell optical density are identified using ANOVA and S/N analysis. The relationship between the cultivation factors and cell optical density is modeled with an empirical equation based regression analysis. \

MATERIALS AND METHODS

Strain Isolation, Purification, and Characterization of Chlorella Vulgaris microalgae

For the realization of this study, it worked with Chlorella Vulgaris species of microalgae which were isolated and purified with the help of experts at Department of Biology/Ibn–Haitham College of Education/University of Baghdad. This species of microalgae was collected from Tigris River then purified as shown in (Figure 1).

Figure 1: Image for *C. vulgaris* after isolation and purification

Inoculum

The inoculum of *C. vulgaris* was prepared by Department of Biology/Ibn–Haitham College of Education/ University of Baghdad. This inoculum is modified BG-11 media in Erlenmeyer flasks closed with a cotton stopper to ensure air exchange. These Erlenmeyer flasks were kept at 25°C, with the air supply containing a CO_2 with 5% by (RS Electrical pump, RS-610, China). The lit provided continuously to the culture with a luminous intensity of (200) μmol m-2 s-1 measured by a light meter ((LI-250A, Biosciences) and stirred at 101 rpm. Each week, the inoculum is refreshed and diluted to 5% with BG-11 media.

Culturing Medias

C .vulgaris is cultured in industrial wastewater which have physical properties as shown in Table 1 was collected from the Rustamiyah wastewater treatment plant which is located in the south of Baghdad Governorate on Tigris River. The secondary and final stages of wastewater station are the quantities which have been taken and subsequently processed several stages:

The first stage: The samples were taken from the secondary stage of wastewater station, and put it inside the biological cabinet (Teslar, Bio Ⅱ A/B, Spain) for two days where exposed to ultraviolet radiation to avoid them of bacteria and unwanted pollutants and remained under the radiation for a full hour.
The second stage: 2 liter were taken from secondary and final wastewater and put in autoclave device (K&K, Guro-GU, seosal, Korea) at 121 ° C for 15 minutes under pressure at 1bars.
The third stage: The wastewater is filtered by the vacuum pump (DV-3E, USA) using 0.45 µm cellulose acetate filter to dispose of particles and suspended that are in the wastewater.
The fourth stage: in the study begin to dilute the wastewater with distilled water because high turbidity of wastewater makes light hard to pass through culture these cause pH decrease, this due to high contamination due to *chlorella vulgaris* to death. To become dilution ratio 25%, 50%,75% and 100% respectively in flask, which volume 500 ml and packed with 400 ml from sterilized wastewater and dilution then injected with 5ml of pure algae of *Chlorella vulgaris* and maintained with temperature of 25̊C, pH=7, and light with (3000) lux measured by (photometer, milkwaukee, china) for light system (16:8) (light: dark) with air supply by(RS Electrical pump, RS-610, China) containing a CO2 concentration of 1.5 1/m. All flasks immersed in water to keep on a certain temperature, that water it is difficult to lose heat and thus maintain it.

Table 1: the properties of wastewater mayoralty of Baghdad sewage board/ Analysis test report Rustamiyah south (Stage 1)

NO	Test/Unit	Values of secondary station
1	BOD(ppm)	20
2	COD(ppm)	51
3	SS(pm)	27
4	pH(-)	7.43
5	Chloride(ppm)	324
6	PO4 (ppm)	2
7	SO4 (ppm)	680
8	NO3(ppm)	1.6

Design of experiment

In this research, Taguchi method was used to optimize cultivation conditions. The effects of four factors such as temperature, pH, cultivation time and dilution ratio on growth of algae were studied. 16 runs were designed by Taguchi experiment L_{16} (4^4) used as orthogonal array for culture and provided with all requirements for cultivation as shown in (Figures 2 and 3).

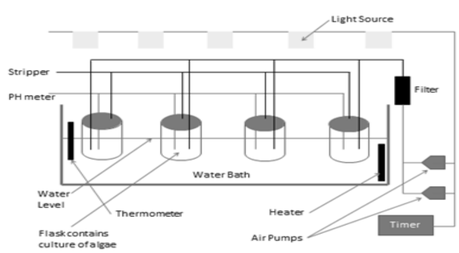

Figure 2: Schematic Diagram for experimental setup of *Chlorella vulgaris* culture

Figure 3: Experimental setup of *Chlorella vulgaris* culture under aeration culture

Determination of cell concentration by optical density

The determination of the concentration and its variation with respect to time was carried out by the direct and indirect method. The first one refers to the direct counting under the microscope by means of a defined method (The hemocytometer is composed of nine equally sized large squares. The central one is divided into 25 small squares with each volume of 4 ×10-6 ml. Chlorella vulgaris were counted in small squares since their sizes are less than 10 μm)[11]. the second a calibration curve is made that relates the optical density with the cellular concentration and that allows them to simply measure the absorbance to know the cellular concentration of future samples without need to count under the microscope, which is a process that demands more time and resources.

The absorbance or optical density was also used as an indirect method to determine the cellular concentration, in the case of the *C. vulgaris*; it was possible to establish linear correlation curves between the cellular concentration and the absorbance. The relationship is obtained from calibration curve between concentration of biomass and absorbance as shown in appendix A.1 and expressed by the following equation:

$X=(Y+0.0446)/0.005$, $R2= 0.9874$ (1)

Where X: Conc. of biomass, Y: is optical density obtained

At $\lambda = 650$ nm [12]

In the case of *C. vulgaris*, due to its size, direct cell counting was difficult under the microscope. Therefore, absorbance-concentration correlation curves obtained from the literature [13].

The absorbance-concentration (cellular) correlations are linear, similar to Beer's Law[14] that correlates the absorbance of a solute linearly with concentration.

RESULT AND DISCUSSION

Taguchi experimental design

The experimental runs of this work were designed by Taguchi method in order to identify the factor influencing the cultivation of *Chlorella vulgaris*. The effects of four factors (temperature, pH, cultivation time and dilution ratio) for wastewater media on the bioprocess responses were simultaneously investigated. In the process, the response represents by optical density for C.V. Data points are mean values of three replicate samples taken throughout the exponential growth period. Table 2 show L16, which represent orthogonal arrays experimental design and the results. The analyses of the responses were performed with the help of MINITAB 18 software package with the using regression model.

Table 2: Taguchi L16 (44) orthogonal arrays with responses for *Chlorella Vulgaris* cultivation in wastewater media

Results of experiments		**Experiments of Taguchi**				
Predicted values of optical density	**Observed values of optical density**	**D, %**	**Cultivation time**	**pH**	**Temp**	**NO.**
0.036	0.035	0.25	1	5	20	1
0.311	0.31	0.5	4	7	20	2
0.48	0.487	0.75	10	9	20	3

0.4385	0.4398	1	13	11	20	4
0.451	0.456	0.75	4	5	25	5
0.355	0.35	1	1	7	25	6
0.416	0.42	0.25	13	9	25	7
0.517	0.521	0.5	10	11	25	8
0.546	0.555	1	10	5	30	9
0.6	0.5997	0.75	13	7	30	10
0.332	0.328	0.5	1	9	30	11
0.283	0.288	0.25	4	11	30	12
0.444	0.44	0.5	13	5	35	13
0.328	0.326	0.25	10	7	35	14
0.399	0.402	1	4	9	35	15
0.309	0.3102	0.75	1	11	35	16

The results indicate the productivity of *Chlorella vulgaris* (i.e. O.D) values ranged from 0.035 to 0.5997 for Wastewater.

Analysis of Signal to noise (S/N) ratio

The values of the signal to noise ratio for optical density for Waste Water media indicated in Table 3

The effect of each control factor on the results is obtained from the response Table 4. These Tables contain the average values of S/N ratio for each level of each factor.

Table 3: Signal to noise ratio(S/N) for the observed values of optical density

S/N	Optical density	Dilution ratio	Cultivation time	pH	Temp	NO.
-28.714	0.035	0.25	1	5	20	1
-10.17	0.31	0.5	4	7	20	2
-6.3318	0.487	0.75	10	9	20	3
-7.1606	0.4398	1	13	11	20	4
-6.9268	0.456	0.75	4	5	25	5
-8.9957	0.35	1	1	7	25	6
-7.6203	0.42	0.25	13	9	25	7
-5.7244	0.521	0.5	10	11	25	8
-5.2686	0.555	1	10	5	30	9
-4.432	0.5997	0.75	13	7	30	10
-9.5696	0.328	0.5	1	9	30	11
-10.971	0.288	0.25	4	11	30	12
-7.0493	0.44	0.5	13	5	35	13
-9.6961	0.326	0.25	10	7	35	14
-7.9805	0.402	1	4	9	35	15
-10.195	0.3102	0.75	1	11	35	16

Table 4: the values of Signal to noise ratio(S/N) for the observed values of optical density for *Chlorella Vulgaris* cultivation in wastewater media

D	Cultivation time	pH	Temp	level	Wastewater media
-14.25	-14.369	-11.99	-13.09	1	(LTB)
-8.128	-9.012	-8.323	-7.317	2	
-6.971	-6.755	-7.876	-7.56	3	
-7.351	-6.566	-8.513	-8.73	4	
7.279	7.803	4.114	5.777	Delta	
2	1	4	3	Rank	

Table 4 indicates that time factor has a large effect on optical density followed by dilution rate, temperature, and pH. Both time and dilution rate factors approximately have the same behavior and have greatest influences on optical density while the pH and temperature have the lower influence on the responses and as illustrated in (Figure 4).The optimum levels of controlling factors depending on the highest value of signal noise ratio as illustrated in Table 1 that temperature with 30℃, pH=7, time=13 day and dilution rate 75%.

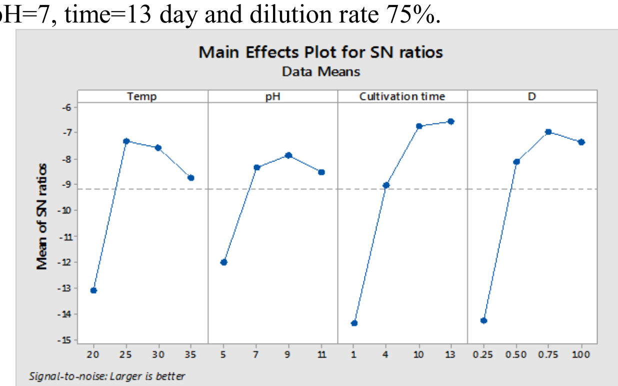

Figure 4: Main effect plots of Signal to Noise ratios for optical density of *Chlorella vulgaris* in wastewater. media

Best fit model section

The highest adjacent R^2 is used for the determination the best fit model. It determines the proportion of the strong model when the value of R^2 for the adjacent is approximate to R^2 predicted, therefore, the data normally distributed. For *Chlorella Vulgaris* cultivation in Wastewater, the values of R^2-adjacent for the proposed models are 99.90% for optical density. It can be concluded that all the proposed models are best to fit the observed data for *C.V*.

Based on the previous criteria, the final regression model equations that describe the responses can be states as follow (for the dependent variable was deduced by ignoring the coefficients by ANOVA that were not significant with p > 0.05):

Regression model for (O.D at 650nm) for Wastewater media of cultivation *Chlorella vulgaris:*

$$\boldsymbol{O.D\ at\ 650nm} = -1.128 + 0.0423\,X1 + 0.06678\,X2 + 1.67\,X4 - 0.000661\,X1^2 - 0.003092\,X2^2 - 0.000644\,X3^2 - 0.7793\,X4^2 + 0.00279\,X2\,X3 - 0.061\,X2\,X4. \quad (2)$$

Effect of physical factors

Temperature, pH, cultivation time all these physical factors are more significant and effect on optical density of cultivation of *Chlorella vulgaris* in wastewater media in the below will widely discussion on all these effects

a) Effect of the temperature on *Chlorella Vulgaris* growth

The temperature one of the important physical factors that influences on the photosynthesis of microalgal species. It strongly influences on the microalgal composition, nutrient uptake rate and carbon dioxide fixation rate. Figure 5 shows the effect of temperature on the *C. vulgaris* growth for wastewater media. It can be observed that the optimum growth temperatures 30℃ had a higher optical density with 0.3997 during 13 day with twenty five percent of dilution ratio of wastewater.

Generally, *C. vulgaris* growth at the wide range of temperature (20-36°C) and depends on *Chlorella* strains and on the environment conditions [6]. This increase in the optical density with increasing temperatures may be linked to enhanced enzymatic kinetics[15]. In fact, an increase of temperature has a positive effect on photosynthesis and cell division through enhancement of the enzymatic activities such as those of the Calvin cycle [16].

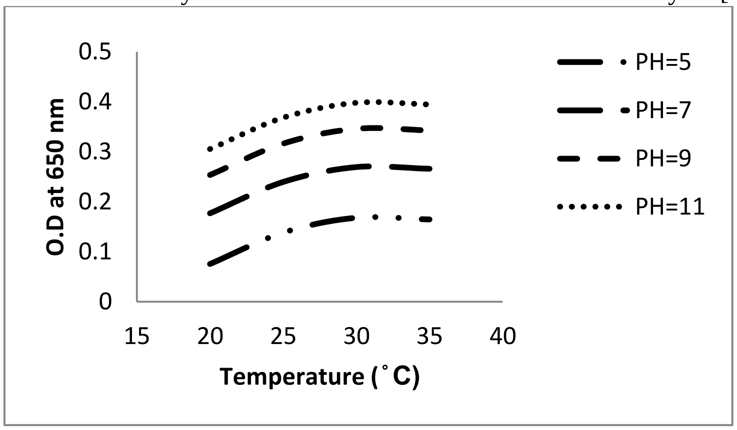

Figure 5: Effect of temperature on (O.D650 nm) for the cultivation of C.V in Wastewater media at different of pH (t=13 day and D=25%)

b)Effect of pH on the optical density of cultivation of *Chlorella Vulgaris*

pH is another physical factor that influence on the algal metabolism. It determines the solubility and availability of CO_2 through photosynthesis consume CO_2 resulting pH increase in algal culture, the complex relation between CO_2 and pH depend on the equilibrium of carbon dioxide in water [17]. In addition pH effect by photoperiod that reaches a maximum with the light period while getting on low with the dark period according to study. That induces to increase biomass for Chlorella vulgaris as shown in (figure.7) which shows the wide range of pH values in five levels from 5 into 13. Exponential phase starts from pH = 5 to 9 until reach maximum of optical density at pH 9 for wastewater. *Chlorella Vulgaris* grow with optimum of CO_2 concentration when beyond this limit the effect on (RUBISCO.) with the high amount of O_2 and CO_2. In pH 11 have lower growth rate than pH 9. This agrees with previous studies [18-26].

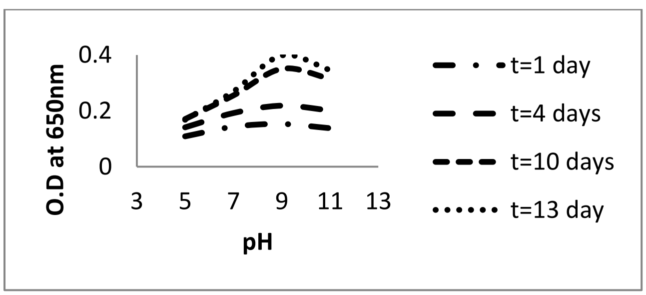

Figure 6: Effect of pH on (O.D650 nm) of cultivation of *Chlorella vulgaris* in wastewater media (T=30̊C and D=25%)

c) Effect of cultivation time of *C .vulgaris* in waste water media on optical density

Through incubated under of photoperiod system light: dark (16:8) as similar to [27]. With the light intensity of 3000lux with cool white fluoresce during 13 days, noticed cultivation time very effective on optical density for wastewater media. In fig.8 indicted for cultivation time significantly with ($p<0.05$), achieved on highest of optical density at the 13th day of cultivation (Figure 6) under 30̊C [28].

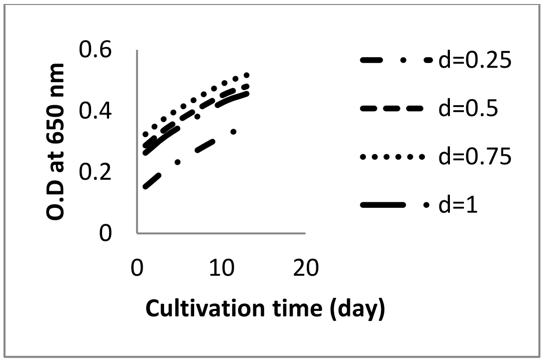

Figure 7: Effect of cultivation time on (O.D650nm) of cultivation of *Chlorella vulgaris* in wastewater media(T=30℃ and pH=9)

d)Effect of dilution rate of cultivation *C.vulgaris* in Wastewater media on optical density

After sterilization of the wastewater and performing some operations on media to be used as a culture medium (figure 7), it was diluted with distilled water with four ratios from (25%,50%,75% and 100%) to determine which is the most suitable ratio for the growth of algae. Optical density data obtained from the culture cultivated in industrial wastewater processed and plotted against dilution rate as shown in fig.9 O.D started with 0.3 at medium containing on 25% of wastewater then increase with increase to reach maximum at 75% of waste then decrease with 100% of it, that the last ratio of medium contains more of disposal material and high level of BOD and COD all these prevent growth of algae due to decrease of optical density. This was the same with protein content reach maximum at 75% of dilution rate and drop with 25%, 50% and 100% of medium agree with previous work [29].

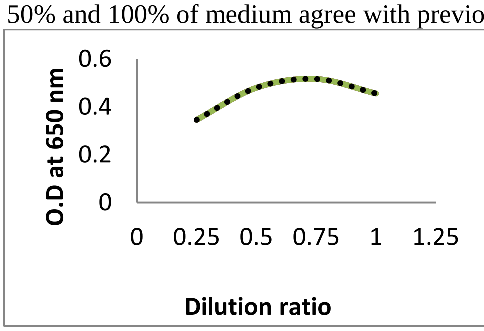

Figure 8: Effect of dilution ratio on the optical density of cultivation of *Chlorella vulgaris* in wastewater media (T=30℃, pH=9 and t=13 day)

CONCLUSION

The results showed that the cultivation time and the dilution ratio of *Chlorella Vulgaris* cultivation in Wastewater media (Figure 8) are more significant on optical density through the range of (1-13 days) and (25%-75%). Both factors of temperature and pH are another keys factors influencing on algal biomass, through ranges of (25-30℃) and (7-9) for temperature and pH respectively, low or high these ranges that due to inhibition of photosynthesis and metabolism inside the cell of algae, thus lead to its death. By employing of Taguchi method indicated that optimum conditions of temperature, pH, time and dilution rate at 30℃, 9, 13 day and 75% respectively. Under these conditions, optical density reached to 0.5597.

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