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The effects of glucose, nitrate, and pH on cultivation of *Chlorella sp.* Microalgae

H. Nouri[1], J. Mohammadi Roushandeh[1], A. Hallajisani[1,*], A. Golzary[2], S. Daliry[1]

[1]*Caspian Faculty of Engineering, College of Engineering, University of Tehran, Rezvanshar, Guilan, Iran*

[2]*School of Environment, College of Engineering, University of Tehran, Tehran, Iran*

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ABSTRACT

BACKGROUND AND OBJECTIVES: Bioenergy is a phenomenon that has attracted humans' attention for about a century. The desirable biological properties of *chlorella sp.* microalgae have turned it to one of the most ideal options for the production of biodiesel. However, the economic issues must be taken into account in its industrial scale production. The present study aims to investigate *chlorella sp.* biomass production and growth conditions by studying the influence of glucose concentration as a carbon source, nitrate concentration as a nitrogen source and pH, as three of the most important factors.

METHODS: For this purpose, design of experiment was done by response surface methodology and each factor was investigated simultaneously under glucose concentration in 2-20 g/L, nitrate concentration in 0-1 g/L and 6<pH<10. During the growing, pH of the culture was measured to identify the correlation between pH and growth rate change. The results were analyzed by response surface methodology as well.

FINDINGS: The results indicated that carbon concentration has maximum effect on growth and biomass production. The best results were obtained in glucose concentration of 2.6-6 g/L, nitrate concentration of 0.2-0.5 g/L and pH values 7-9. Moreover, the maximum biomass production (1.31 g/L), the highest specific growth rate (0.167 1/day), and the highest biomass productivity (0.085 g/L/Day) were obtained in the following conditions: glucose concentration of 2.6 g/L, nitrate concentration of 0.5 g/L, and pH = 8. The optimal C/N ratio was determined and significant correlation was observed between pH and growth rate change.

CONCLUSION: It was concluded that *Chlorella sp.*, if properly adjusted for both chemical and physical parameters could be a valuable source of biomass for biodiesel production in industrial scale.

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*Corresponding Author:
Email: *hallaj@ut.ac.ir*
Phone: +9813 4460 3270
Fax: +9813 44608600

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INTRODUCTION

Fossil fuels have been the most important source of energy for a long time. However, the new challenges concerning the harmfulness of continuous usage of fossil fuels and imminent finishing of their production sources, have made man to look for a reliable alternative for it (Zheng *et al.,* 2017; Gouveia and Oliveira, 2009). The alternative sources must have desirable properties of fossil fuels in terms of widespread use, economical potentials and ease of use, and do not lead to environmental pollution and global warming. Among all available options, biofuel attracted the researchers' attention because of having unique capabilities (Goh *et al.,* 2019; Erazo *et al.,* 2007). Renewability, desirable environmental properties, and economical potentials are some of the characteristics of biofuel as a cheap and clean alternative for fossil fuels (Chen *et al.,* 2018; Huang *et al.,* 2010). Biofuel production sources include three different categories: first-generation fuel sources (food products such as palm oil, sunflower oil, oilseeds, etc.), Second-generation fuel sources (cellulose-containing fuels such as agricultural wastes), and the third generation fuel sources which include the fuel produced from microorganisms like microalgae (Campbell *et al.,* 2011; Schenk *et al.,* 2008). Among these, microalgae seem to be very desirable due to its high growth rate, the capability of cultivation in non-arable land, production throughout the whole year, high photosynthetic efficiency, flexibility in cultivation conditions and corrigibility by biotechnological tools (Campbell, 1988; Chisti, 2007; Chisti, 2008). However, their application in fuel production in an industrial scale is not economically affordable due to the low efficiency of lipid production (Shuba and Kifle, 2018; Benemann, 1997). In recent years, researchers have been trying to economize the fuel production from microalgae. Biomass and cellular lipid production amounts are important parameters in this process. Therefore, examination of the factors influencing biomass and lipid production in microalgae and their optimization are the most important measure (Dickinson *et al.,* 2017; Lv *et al.,* 2010). One of the most important effective factors in microalgae growth is the cultivation regime. Conducted researches in terms of cultivation regime impact show that mixotrophic regime provides the best culture conditions to achieve maximum production of microalgae biomass (Scarsella *et al.,* 2010). Gao *et al.,* (2019) and Kong *et al.,* (2011) examined the impact of triple regimes (autotroph, heterotroph, and mixotroph) on *Chlorella vulgaris* microalgae and introduced mixotroph as the best regime (Gao *et al.,* 2019; Kong *et al.,* 2011). Also, Li *et al.,* (2014) by studying *Chlorella sp.* an equivalent of *C. vulgaris*, reported that the productivity of biomass in mixotroph cultivation is 14 times greater than that of biomass in autotrophic cultivation (Daliry *et al.,* 2017; Li *et al.,* 2014). After the appropriate cultivation regime, the most important parameters influencing microalgae growth are chemical and physical conditions. The chemical conditions consist of type and amount of main nutrients. Among the chemical conditions, type and concentration of carbon and nitrogen sources are more effective. In addition to these two parameters, pH of the cultivation environment (as a physical condition) is of high importance. Chu *et al.,* (2019) and Kong *et al.,* (2011) studied *C. vulgaris* and showed that glucose was the best carbon source and increase of glucose concentration continuously increased biomass production to the extent that the maximum biomass production (2.24 g/L) was achieved at the glucose concentration of 20 g/L (Kong et al. 2011). Scarsella *et al.,* (2010) in their study on *C. vulgaris* also introduced glucose as the best source of carbon, but they measured 6 g/L glucose as the optimal concentration (Pagnanelli *et al.,* 2014). Evaluating the nitrogen source, Feng *et al.,* (2020) and Jiang *et al.,* (2010) found potassium nitrate as the best source of nitrogen for *C. vulgaris*, and reached the maximum biomass concentration (1.2 g/L) when the concentration of potassium nitrate was 0.5 g/L (Lv et al., 2010). Skorupskaite *et al.,* (2015) studied *Chlorella sp.* and reached the maximum biomass concentration and biomass productivity of 1.7 g/L and 0.103 g/L/Day, respectively, when industrial glycerol concentration was 2 g/L and ammonium nitrogen concentration of 0.09 g/L was used (Skorupskaite *et al.,* 2015). Sayadi *et al.,* (2016) studied the ability of *Chlorella vulgaris* to remove nitrate and phosphate from aqueous solutions. After cultivation of *C. vulgaris* in standard BBM medium, they examined the ability of microalgae by adding 0.25-0.45 g/L KNO3, K2HPO4 to municipal water. Finally, they reported that on day 8 the highest nitrate removal was 89.80% in the treatment with 0.25 g/L microalgae and the highest phosphate removal was 88% in the treatment with 0.45g/L microalgae. In investigate the effect of

environment pH, Qiu *et al.,* (2017) and Khalil *et al.,* (2010) studied *C. vulgaris* showed that microalgae could grow under a wide range of pH values (4-10), but pH values of 9 and 10 led to the best cultivation results (Qiu *et al.,* 2017; Khalil *et al.,* 2010). Samiee *et al.,* (2017) investigated the effects of the three parameters on biomass productivity of *Chlorella sp. PTCC 6010*. They examined sodium nitrate (10-200 mg/L) as nitrogen source, dipotassium hydrosulfate (10-70 mg/L) as phosphorus source and light intensity (60-450 µmol photons/m^2/s). They, finally, reported that 200 mg/L sodium nitrate, 70 mg/L dipotassium hydrosulfate and 450 µmol photons/m^2/s^1 resulted in the highest biomass production (0.916 g/L) and biomass productivity (235.8 mg/L/d). (Samiee *et al.,* 2017). The desirable biological properties of *Chlorella sp.,* green single-cell microalgae, such as its high capability in biomass and lipid production, have made it the most ideal option for biodiesel production among other microalgae species (Daliry *et al.,* 2017; Gao *et al.,* 2019; Lv *et al.,* 2010). So far, the impact of carbon source concentration, nitrogen source concentration, and pH on growth and biomass production of *Chlorella sp*. has not been simultaneously examined in a study. Therefore, in this study different glucose and nitrate concentrations, as carbon and nitrogen sources respectively, and different pH values in a specified range were simultaneously investigated by response surface methodology and design of experiments for the first time. This study aims to evaluate the impact of each factor and binary interaction of two of them on growth rate and biomass production, in order to determine the optimal condition for maximum biomass production. It should be noted that protein and chlorophyll content of *Chlorella sp. PTCC 6010* have been also investigated in another study of ours which is under publication. This study has been carried out in Biofuel Laboratory, Caspian Faculty of Engineering, College of Engineering, University of Tehran, Rezvanshahr, Iran during 2017-2019.

MATERIALS AND METHODS

Chlorella sp. PTCC 6010 microalgae was supplied from the Persian Gulf of Iran and used after screening and purifying operations. 50 ml of the obtained microalgae was cultivated during 15-day periods in 4 levels as 0.1, 0.5, 4 and 20 L. The microalgae cultivation environment followed the standard cultivation conditions and was prepared according to Rodik medium with following chemicals in liter (Golzary *et al.,* 2015): $NaNO_3$ (0.3 g), K_2HPO_4 (0.08 g), KH_2PO_4 (0.02 g), NaCl (32.02 g), $CaCl_2$ (0.047 g), $MgSO_4.7H_2O$ (0.01 g), $ZnSO_4$ (0.1 mg), $MnSO_4$ (1.5 mg), $CuSO_4$ (.08 mg), $FeCl_3$ (17 mg), EDTA (7.5 mg) and H_3BO_4 (0.3 mg). All the chemicals were obtained from Merck Company. During the experiments, the cultivation environment was prepared without adding $NaNO_3$ because nitrate concentration was one of the intended parameters. Glucose and nitrate were added to the cultivation environment with different concentrations as carbon and nitrogen sources respectively. Also, KOH and HNO_3 (1 M) were used for initial pH adjustment.

Designing experiments with RSM method and implementation

To achieve the best results through minimum experiment runs and to perform a precise analysis, the experiments design by RSM method based on three parameters including glucose concentration, as carbon source, nitrate concentration, as nitrogen source, and pH of cultivation environment in 5 levels for each parameter. Glucose concentration, nitrate concentration, and pH range of 2-20 g/L, 0-1 g/L and 6-10 were selected according to Central Composite Design (CCD) method. Minitab 17 software was used to design 20 experiments. 250 ml of cultivation environment with the specified pH and glucose and nitrate concentrations was poured into a 500-ml Erlenmeyer and 50 ml of the microalgae was added to it. Temperature of the cultivation environment was set at 30 °C, lighting intensity of 5000 lux was provided using a white fluorescent lamp, and aeration flow rate of 0.05 L/min was provided using a RESUN ACO-004aquarium pump. The pH value of each experiment was measured every day to determine the pH range, and a 5-ml sample of each experiment was taken daily for analysis of concentration specification.

Biomass concentration specification and growth rate

The concentration specification test was conducted on the samples (15 times daily for each experiment) using a Jusco770-Japan spectrophotometer. The biomass concentration was obtained using Eq. 1 as proposed by Golzary *et al.,* (2015), and according to the desired absorption spectra of *Chlorella sp.* biomass (Golzary *et al.,* 2015).

$$W_{(g/L)} = 0.49 \times OD_{550} \quad (1)$$

Where, W is biomass concentration (on dry basis); and OD_{550} is absorption in the desired wavelength, λ=550 nm. The samples of each experiment were put under absorption analysis, and the results were illustrated using the dry biomass concentration-time graphs (growth curve). The pH values-time graphs were also provided with a better understanding of the pH change with the biomass growth.

Data analysis and mathematical model proposal

Results of initial experiments were evaluated by calculating the auxiliary values as a scale, to achieve a better analysis. Specific growth rate and biomass productivity are two vital quantities in the evaluation of growth and production of biomass. Skorupskaite *et al.,* (2015) calculated specific growth rate (μ) and biomass productivity (P) using Eqs. 2 and 3, respectively.

$$\mu = \frac{\ln X_t - \ln X_0}{t_x - t_0} \quad (2)$$

$$P = \frac{X_t - X_0}{t_x - t_0} \quad (3)$$

Where, t_0 and t_x are initial time (the first day) and final time (the 15th day) respectively; X_0 and X_t are biomass concentrations (on dry basis) in t_0 and t_x respectively. Units of μ and P are 1/Day and g/L/Day respectively. Higher specific growth rate and biomass productivity in an experiment show a better growth and higher production of biomass in experiment conditions, respectively.

A more precise analysis was performed using Minitab software, variance analysis (ANOVA) and contour plots. A mathematical model was also proposed based on the experimental results to predict the biomass concentration (W) according to C, N and pH parameters. This model is actually expansion of Eq. 4, wherein x_i values are C, N and pH parameters and β, with different indexes, is a constant factor whose values are calculated in the expanded form of the equation (using computer software). Considering β_0 as equation constant, β_{ij} as binary interaction factor, and Y as response variable or W, its experimental values are predicted by Eq. 4.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad (4)$$

Finally, competence and error percentage of the proposed model are examined in result prediction. Error percentage is calculated using Eq. 5.

$$Error = \frac{W_{exper} - W_{pred}}{W_{exper}} \times 100 \quad (5)$$

Where W_{exper} and W_{pred} are biomass concentration values based on experimental results and model prediction, respectively.

RESULTS AND DISCUSSION

Biomass concentration and growth rate

The final biomass concentration (on dry basis) according to absorption analysis data was calculated using Eq. 1, for all experiments. The obtained results along with the specifications of each experiment are shown in Table 1. Obviously, the best results were obtained when carbon concentration is lower than 11 g/L, nitrogen concentration is in the range of 0.2-0.5 g/L (0.2≤N≤ 0.5) and 7≤pH≤9.

Growth curves were also graphed for each experiment. Analysis of the microalgae growth rate in various experiments implied that in half of the experiments the growth rate was ascending and had a net value, while in other half, it was ascending and descending in a fluctuating behavior, leading to having no net value. Therefore, the results were examined in two groups: the experiments with constant growth rate and the experiments with ascending growth rate. Considering the main objective of this study, it was necessary for the experiments to show the significant net growth rate. Therefore, according to the results, a target group of experiments was performed to analyze the ascending growth. It should be noted that the target group had the highest biomass concentration among the experiments. The two auxiliary variables of specific growth rate (μ) and biomass productivity (P) were calculated for the target group experiments (Table 2).

As shown in Table 2, the highest specific growth rate is 0.167 1/Day, and the highest biomass productivity is 0.085 g/L/day, respectively, in carbon concentration of 2.6 g/L and nitrogen concentration of 0.5 g/L and pH value of 8. Among the target group experiments, five experiments showed the best results in terms of growth rate and amount of the produced biomass. To evaluate the growth rate of the

Table 1: Results of the final biomass concentration (dry basis) according to the experiments design

Runs	C		N		pH		Results
	Code values	Real values (g/L)	Code values	Real values (g/L)	Code values	Real values	W (g/L)
E1	0	11	0	0.5	0	8	0.613
E2	-1.68	2.6	0	0.5	0	8	1.313
E3	0	11	+1.68	1	0	8	0.268
E4	+1	16	+1	0.8	+1	9	0.246
E5	+1	16	-1	0.2	-1	7	0.221
E6	0	11	-1.68	0	0	8	0.217
E7	0	11	0	0.5	0	8	0.251
E8	-1	6	+1	0.8	+1	9	0.926
E9	0	11	0	0.5	-1.68	6.3	0.887
E10	0	11	0	0.5	0	8	0.956
E11	0	11	0	0.5	+1.68	9.7	0.447
E12	+1.68	19.4	0	0.5	0	8	0.221
E13	0	11	0	0.5	0	8	0.246
E14	-1	6	-1	0.2	+1	9	0.931
E15	0	11	0	0.5	0	8	0.300
E16	-1	6	-1	0.2	-1	7	1.289
E17	+1	16	+1	0.8	-1	7	0.314
E18	-1	6	+1	0.8	-1	7	0.59
E19	+1	16	-1	0.2	+1	9	0.419
E20	0	11	0	0.5	0	8	0.793

Table 2: specific growth rate and biomass productivity in the target group experiments

Target group	X_0 (g/L)	X_1 (g/L)	μ (1/day)	P (g/L/day)
E1: 11 , 0.5 , 8	0.128	0.613	0.112	0.035
E2: 2.6 , 0.5 , 8	0.126	1.313	0.167	0.085
E8: 6 , 0.8 , 9	0.109	0.926	0.153	0.058
E9: 11 , 0.5 , 6.3	0.100	0.887	0.156	0.056
E10: 11 , 0.5 , 8	0.157	0.956	0.129	0.057
E11: 11 , 0.5 , 9.7	0.153	0.447	0.077	0.021
E14: 6 , 0.2 , 9	0.153	0.931	0.129	0.056
E16: 6 , 0.2 , 7	0.132	1.289	0.163	0.083
E18: 6 , 0.8 , 7	0.100	0.59	0.127	0.035
E20: 11 , 0.5 , 8	0.148	0.793	0.120	0.046

microalgae under ideal conditions, the growth curves of these experiments were plotted (Fig. 1).

As can be seen in Fig. 1, E2 experiment resulted in the highest produced biomass, while E16 experiment led to the best growth rate. Among the rest of experiments having a biomass concentration of about 1 g/L, experiment E14 shows the best growth rate. The common point in E14 and E16 experiments is equal amount of the used glucose and nitrate. The pH change analysis implied that the pH value in the environment fluctuated within the alkaline range ($9 \leq pH \leq 10$) during the cultivation period. This contradicts with the findings of Kong *et al.,* (2011) who reported that the pH change was in the neutral range (pH=7) during mixotrophic cultivation (Kong *et al.,* 2011). The pH change is important because several studies have shown that alkaline environment can serve as a positive factor in the growth of microalgae (Qiu *et al.,* 2017; Khalil *et al.,* 2010). The range of pH fluctuation during cultivation is also important. This was investigated by plotting the pH-time curves for the five experiments (Fig. 2).

Fig. 2 shows: 1) the increasing trend of pH values in all the experiments, and 2) the tendency of pH change towards pH=10. Studies show the increasing trend of pH in all the target group experiments. The different trend of pH change in experiment E10 indicates a slower pH increase compared to other

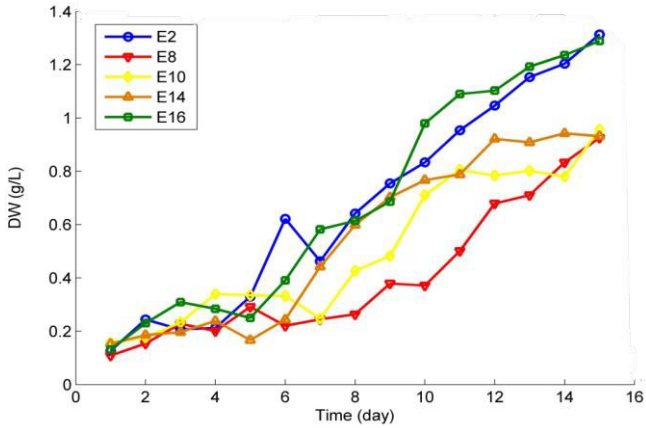

Fig. 1: Growth curves of the five experiments with the best results in terms of growth rate and amount of the produced biomass

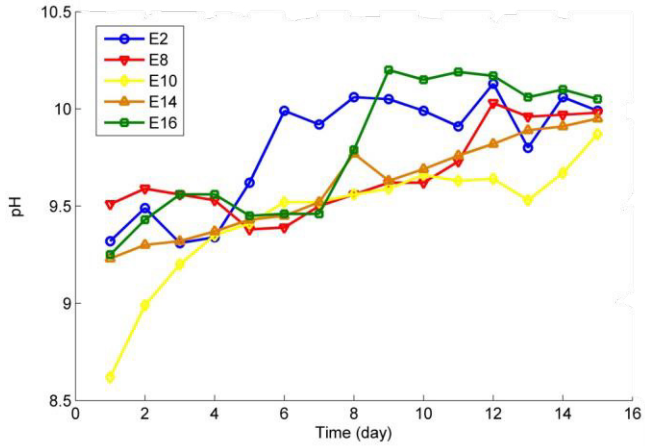

Fig. 2: pH changes for experiments with desired growth

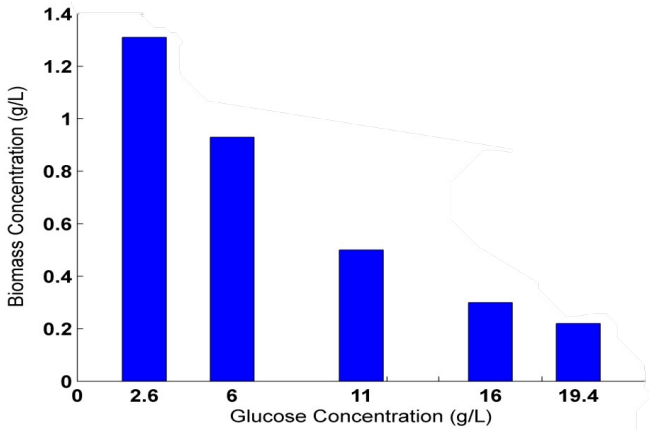

Fig. 3: The average biomass produced in each glucose concentration

experiments. This can be due to higher glucose concentration (11 g/L) in this experiment compared to other experiments.

Effect of glucose concentration on biomass production

The results of biomass production presented in Table 1 and the target group experiments reveal that glucose concentrations of over 11 g/L lead to disruption of biomass growth and thereby significant decrease of biomass production. Moreover, further increase in glucose concentration can have a more inhibitive effect on growth. Fig. 1 showed that glucose concentrations of 6 g/L and 2.6 g/L led to the best growth rate and the highest biomass production, respectively. Moreover, Fig. 2 illustrated that the highest specific growth rate of 0.16 1/Day and the highest biomass productivity of 0.08 g/L/Day were achieved in glucose concentration of 2.6 g/L and 6 g/L, respectively. The average amounts of produced biomass in different concentrations of glucose in all the experiments are illustrated in Fig. 3. It is obvious that the best range of glucose concentration is 2.6-6 g/L, leading to the best growth rate and the highest amount of produced biomass. Moreover, the glucose concentrations of over 6 g/L significantly decrease the biomass production in all the experiments. This is in agreement with the findings of Penno *et al.,* (2019), but contradicts with the results presented by Kong *et al.,* (2011) who declared that increase of glucose concentration to 20 g/L could increase biomass production, specific growth rate and biomass productivity.

Effect of nitrate concentration on biomass production

Fig. 1 shows that the highest amount of biomass (1.31 g/L) is produced in the nitrate concentration of 0.5 g/L. Growth curves illustrated in Fig. 1 indicate a better microalgae growth when nitrate concentration is 0.2 g/L. The highest values for specific growth rate and biomass productivity were also obtained in the nitrate concentration ranging 0.2-0.5 g/L. The average amounts of produced biomass in different concentrations of nitrate in all the experiments are illustrated in Fig. 4. Obviously, absence of nitrate, which means using no nitrogen source in the microalgae cultivation environment, has a significant adverse effect on biomass production. In fact, the least amount of biomass growth and production was observed in the absence of nitrate. On the other hand, the nitrate concentration of over 0.8 g/L significantly decreased the biomass production. Generally, the nitrate concentrations in the range of 0.2-0.5 g/L led to the best results, while the nitrate concentration of 0.8 g/L did not provide a suitable result in terms of biomass growth and production. These results are in agreement with the results obtained by An *et al.,* (2020) and Lv *et al.,* (2010) who reported the nitrate concentration of 0.5 g/L as the optimal concentration leading to the highest amount of chlorella biomass, and introduced the absence of nitrate as a factor leading to a significant decrease in biomass growth and production.

Effect of pH on biomass production

Effect of pH behavior on biomass production and growth rate during the growth was already discussed. However, investigation of the initial pH showed that

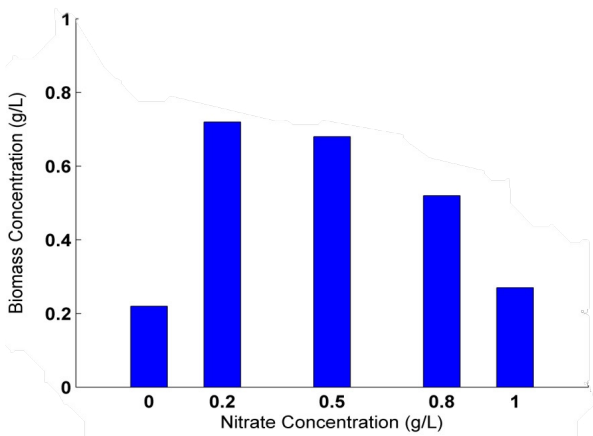

Fig. 4. The average biomass produced in each nitrate concentration

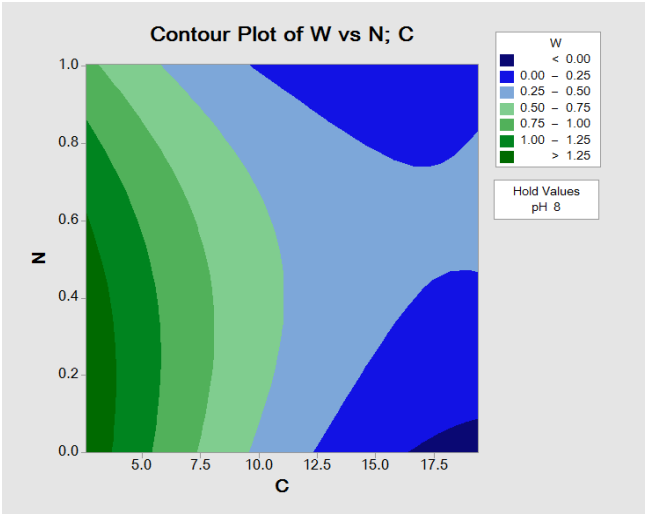

Fig. 5: Contour plot of W vs. C, N

Table 3: The average biomass production (W_{ave}), specific growth rate (μ_{ave}) and biomass productivity (P_{ave}) for different C/N ratios

C/N	5.2	7.5	11	20	22	30	38.8	80	∞
W_{avr}	1.313	0.758	0.268	0.28	0.561	1.11	0.221	0.32	0.217
μ_{avr}	0.167	0.14	0.063	0.052	0.1	0.146	0.046	0.047	0.023
P_{avr}	0.085	0.046	0.011	0.01	0.031	0.07	0.007	0.011	0.004

the microalgae maintained its biomass productivity and growth in the pH range of 6-10. This is in agreement with the findings of Qiu *et al.,* (2017) and Khalil *et al.,* (2010), in terms of growth capability of *Chlorella* in a wide pH range of 4-10. Considering the results of the target group experiments in Table 2, pH>9 decreased the growth and production of biomass and the best results were obtained at 7<pH<9. According to Table 2, the maximum biomass production of 1.31 g/L, specific growth rate of 0.167 1/day, and biomass productivity of 0.085 g/L/Day were achieved in pH=8. This result contradicts with the results obtained by Khalil *et al.,* (2010) who proposed pH of 9-10 as the optimal pH range for cultivation *Chlorella vulgaris,* and Gong *et al.,* (2014) and Gong *et al.,* (2014) who introduced an approximate optimal pH value of 10 for C. *vulgaris* cultivation.

Effects of binary interactions on biomass production

Effects of binary interactions of parameters include the effects of simultaneous change of two parameters on the target variable. The three parameters in this study would form three binary interactions: C-N, C-pH, and N-pH. Contour plots obtained from Minitab software analysis were used to study these interactions. Fig. 5 illustrates a contour plot for C-N interaction. It is obvious that, reduction of glucose concentration (C) to below 10 g/L and nitrate concentration to below 1 g/L at the same time leads to an increase in biomass production (W). Formerly, Pagnanelli *et al.,* (2014) examined the effect of C-N interaction as the effect of C/N ratio on specific growth rate. They suggested the tolerance threshold of microalgae as a specified value for the C/N ratio. According to Gao *et al.,* (2019) and Skorupskaite *et al.,* (2015), there would be a maximum concentration of glucose for each single concentration of nitrate and exceeding this maximum value would result in a significant decrease in specific growth rate and biomass production. They reported this ratio as about 17 for *Chlorella vulgaris*. The effects of different C/N ratios on average biomass production (W_{ave}), average specific growth rate (μ_{ave}) and average biomass productivity (P_{ave}) are presented in Table 3.

Table 3 confirms that there is a specific C/N ratio and exceeding it would lead to a significant decrease in W, μ and P. For *Chlorella sp*., this ratio is 30, but the mentioned decrease can be seen even in lower C/N ratios (C/N=11, 20 and 22). Study of nitrate and glucose concentrations in these ratios revealed that the mentioned decrease occurred as a result of the glucose or nitrate concentrations over the range for appropriate growth. In other words, when both nitrate and glucose concentrations are in the appropriate range, biomass production, specific growth rate and biomass productivity increase in case C/N<30. This point shows the relationship between Fig. 5 and Table 3. Table 3 also shows that the increase of C/N ratio to the values above the tolerance threshold (C/N=30) intensifies the decreasing behavior of specific growth rate and biomass productivity, and according to Fig. 5, biomass production is significantly decreased as well.

The effects of simultaneous change of C-pH and N-pH on biomass production are illustrated in Figs. 6 and 7, respectively.

As it can be seen in Fig. 6, decrease of glucose to less than 10 g/L leads to W increase in a wide range of pH values, and this increase is intensified when glucose concentration is lower than 6 g/L. According to Fig. 7, a simultaneous decrease in nitrate concentration and pH has a positive effect on increase of W. However, this applies to the nitrate concentrations below 0.8 g/L, and pH<7 or pH>9. Comparison of C-pH and N-pH interactions plots indicates that W is more increased by simultaneous decrease of glucose concentration and pH rather than simultaneous decrease of nitrate concentration and pH. This could be due to glucose concentration

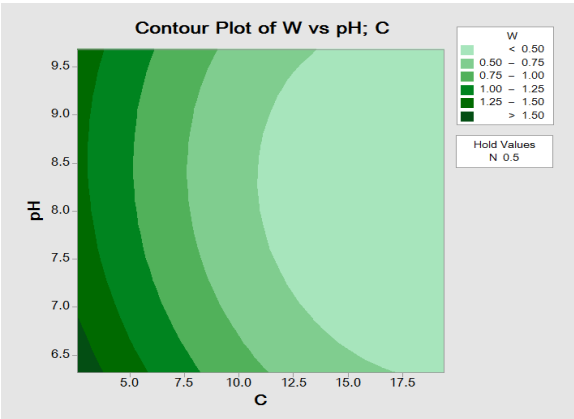

Fig. 6: Contour plot of W vs. C, pH

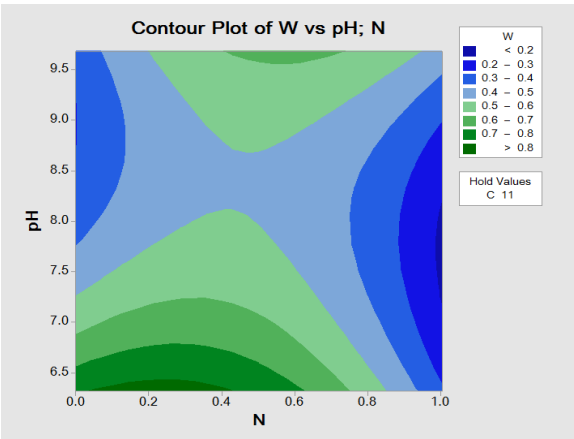

Fig. 7: Contour plot of W vs. N, pH

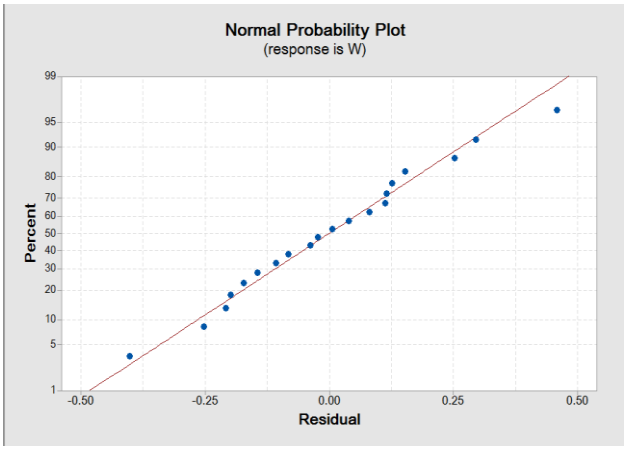

Fig. 8: The normal plot to the investigation of model competency

which has a direct effect on pH changes during the growth. Unlike nitrate, glucose is somewhat alkaline and this could also contribute to stronger interaction of C-pH to N-pH.

Prediction of biomass concentration using the mathematical model

In order to predict the amount of produced biomass (W in g/L) according to the change of glucose concentration (C in g/L), nitrate concentration (N in g/L) and pH, the software proposed a mathematical model as presented in Eq. 6 which is the expanded form of Eq. 4.

$$W=7.78 - 0.213*C - 1.28*N - 1.30*pH + 0.00423*C*C - 0.886*N*N + 0.0703*pH*pH + 0.0520*C*N + 0.0038*C*pH + 0.178*N*pH \quad (6)$$

The three parameters were evaluated to investigate the model competency. The first parameter was the assumption of normality of residuals. In this approach, residual values are marked in normal plot and the fittest line crossing these points is drawn. If all the points are almost covered putting a wide pencil on this line, it is said that the assumption of normality of residuals is true and the model is competent enough. According to the proposed normal plot by Minitab 17 based on the data illustrated in Fig. 8, it can be concluded that the assumption of normality of residuals is true and the proposed model is competent enough.

The second parameter, obtained from analysis of variance, is the P-value parameter. P-value parameter is expressed based on a confidence level of the data, by two parameters of model suitability and lack of fit. Usually, the confidence level of the data is considered as 90-95%. Therefore, the P-value should be less than 0.1 (significant) for model suitability and more than 0.1 (insignificant) for lack of fit. Variance analysis of the model performed by Minitab showed that the P-value was 0.07 (<0.1) for model suitability and 0.82 (>0.1) for lack of fit, confirming the model competency. Moreover, analysis of variance was presented in Table 4 to examine the parameters consisting the P-value quantities and coefficient effects. For P-values the method is the same as above, but for coefficient effects, the sign of coefficients (positive or negative) and their values indicate the type and the amount of their effects on target quantity. As can be seen in Table 4, carbon concentration has the largest coefficient

Table 4: Analysis of variance for biomass production data

Term	P-value	Coefficient
Constant	0.002	0.499
C	0.002	-0.320
N	0.052	-0.051
pH	0.056	-0.046
C*C	0.189	0.106
N*N	0.313	-0.080
pH*pH	0.370	0.070
C*N	0.081	0.078
C*pH	0.095	0.054
N*pH	0.116	0.019

Table 5: Performance evaluation and prediction error percentage of the model

Target group	W_{exper}	W_{pred}	*Error*
E2: 2.6 , 0.5 , 8	1.313	1.351	2.89
E8: 6 , 0.8 , 9	0.926	0.794	14.25
E9: 11 , 0.5 , 6.3	0.887	0.798	10.03
E10 : 11 , 0.5 , 8	0.787	0.519	33.97
E11: 11 , 0.5 , 9.7	0.447	0.647	44.74
E14: 6 , 0.2 , 9	0.931	0.945	1.5
E16: 6 , 0.2 , 7	1.289	1.179	8.53
E18: 6 , 0.8 , 7	0.59	0.814	37.96

effect by negative sign (-0.320). Thus, it can be stated that carbon concentration has the highest reducing effect on biomass production.

The third parameter is R^2 value, which is a measure of model validity and also the extent to which the model covers the data. The nearer is R^2 to 1, the better the model works (in here R^2=0.7). Although it was not a highly desirable value, it was acceptable according to the manner and dispersion of the data. In order to evaluate the performance of the model, the values predicted by the model were compared to the experimental results presented in Table 5. The percentage for each experiment of the target group calculated by Eq. 5 is shown in Table 5 as well.

E_{10} in Table 5 is the central point experiment, and its average value is used due to its 3-time repetition in the target group. The model performance in terms of the experiments with the maximum biomass production (E2, E8, E9, E14, and E16) was acceptable since the error percentage was 10% and proportionate to a confidence level of the data. The minimum predicting error (1.5%) was obtained for experiment E14, and the maximum error (44.74%) was related to experiment E11. In overall, the maximum biomass dry weight was obtained as 1.31 g/L in glucose concentration of 2.6 L/g, nitrate concentration of 0.5 g/L and pH=8. The validation test was done in this optimal condition and the biomass dry weight was obtained as 1.304 g/L. Thus, the optimal biomass dry weight was achieved with only 0.68% error.

CONCLUSION

Due to the desirable biological properties of *Chlorella sp.* microalgae, it is considered as one of the most ideal microalgae species for biodiesel production. Effects of three parameters of glucose concentration, nitrate concentration and pH on growth and production of *Chlorella sp.* biomass were investigated using the response surface methodology. Each factor was examined simultaneously under glucose concentration in 2-20 g/L, nitrate concentration in 0-1 g/L and 6<pH<10. During the growing, pH of the culture was measured to identify the correlation between pH and growth rate change. The results were analyzed by response surface methodology as well. Results showed that glucose concentration was the most effective parameter in biomass growth and production, so that the biomass growth was disrupted and significantly decreased in glucose concentrations of over 10 g/L. Absence of nitrate as a nitrogen source also resulted in disruption of growth and sever decrease in biomass production. It was realized that in case of eligible growth, pH of the cultivation environment increased to pH=10 and was in the range 9-10 during the growth. The best results were achieved when glucose concentration, nitrate concentration and, pH were in the range of 2.6-6 g/L, 0.2-0.5 g/L and 7-9, respectively. Effects of binary interactions of parameters on biomass production were investigated using contour plots. Comparison of C-pH and N-pH interactions plots was indicated that biomass production was more increased by simultaneous decrease of glucose concentration and pH rather than simultaneous decrease of nitrate concentration and pH. This could be due to glucose concentration which has a direct effect on pH changes during the growth. It was demonstrated a significant correlation between C/N ratio and biomass production and optimal C/N ratio of the microalgae was obtained as 30. A model was proposed to predict biomass production. The maximum biomass production, highest specific growth rate and the maximum biomass productivity were obtained as 1.31 g/L, 0.167 1/Day and 0.085 g/L/Day, respectively. It was concluded that *Chlorella sp.*,

if properly adjusted for both chemical and physical parameters, could be a valuable source of biomass for biodiesel production in industrial scale.

AUTHOR CONTRIBUTIONS

H. Nouri and A. Hallajisani performed the literature review and experimental design, analyzed and interpreted the data, prepared the manuscript text, and rendered manuscript edition. S. Dalirinejad helped in the experimental design. A. Golzary and J. Mohammadi Roshande helped in the literature review.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interests regarding the publication of this work. The ethical issues including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy have been completely witnessed by the authors.

ABBREVIATIONS

ANOVA	Analysis of variance
β_0	Constant factor
β_{ij}	Binary interaction factor
C	Carbon source concentration
$CaCl_2$	Calcium chloride
CCD	Central composite design
$CuSO_4$	Copper sulfate
E_i	Experiment number
EDTA	Ethylen diamine tetra acetic acid
Eq.	Equation
$FeCl_3$	Iron(III) chloride
H_3BO_4	Boric acid
HNO_3	Nitric acid
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosfate
KOH	Potassium hydroxide
$Ln\ X_0$	Natural logarithm of initial biomass
$Ln\ X_t$	Natural logarithm of final biomass
$MgSO_4.7H_2O$	Magnesium sulfate 7 hydrate
$MnSO_4$	Manganese (II) sulfate
μ	Specific growth rate
N	Nitrogen source concentration
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
OD	Optical density
λ	Wave length
P	Biomass productivity
pH	Potential of hydrogen
p-value	Probability value
R^2	Coefficient of determination
RSM	Response surface methodology
t_0	Initial time
t_x	Final time
W	Dry biomass weight
W_{exper}	Experimental dry biomass weight
W_{pred}	Predicted dry biomass weight
X_0	Initial biomass concentration
X_t	Final biomass concentration
Y	response variable
$ZnSO_4$	Zinc sulfate

REFERENCES

An, M.; Gao, L.; Zhao, W.; Chen, W.; Li, M., (2020). Effects of nitrogen forms and supply mode on lipid production of microalga Scenedesmus obliquus. Energies, 13(3): 697 **(1 page).**

Benemann, J., (1997). CO2 mitigation with microalgae systems. Energy Convers. Manage., 38: 475-479 (**5 pages**).

Campbell, C., (1988). The coming oil crisis. Multi-science publishing co., Brentwood, England.

Campbell, P.; Beer, T.; Batten, D., (2011). Life cycle assessment of biodiesel production from microalgae in ponds. Bioresour. Technol., **102**(1): 50-56 (**7 pages**).

Chen, J.; Li, J.; Dong, W.; Zhang, X.; Tyagi, R.; Drogui, P.; Surampalli, R.Y., (2018). The potential of microalgae in biodiesel production. Renewable Sustainable Energy Rev., 90: 336-346 (**11 pages**).

Chisti, Y., (2007). Biodiesel from microalgae. Biotechnol. Adv.,

25(3): 294-306 (**13 pages**).

Chisti, Y., (2008). Biodiesel from microalgae beats bioethanol. Trends Biotechnol., 26(3): 126-131 (**6 pages**).

Chu, F.; Cheng, J.; Zhang, X.; Ye, Q.; Chen, S.; Zhou, J.; Cen, K., (2019). Transcriptome and key gene expression related to carbon metabolism and fatty acid synthesis of Chlorella vulgaris under a nitrogen starvation and phosphorus repletion regime. J. Appl. Phycol., 31(5): 2881-2893 (**13 pages**).

Daliry, S.; Hallajisani, A.; Mohammadi, R.J.; Nouri, H.; Golzary, A., (2017). Investigation of optimal condition for Chlorella vulgaris microalgae growth. Global J. Environ. Sci. Manage., 3(2): 217-230 **(14 pages).**

Daliry, S.; Hallajisani, A.; Nouri, H.; Golzary, A., (2017). Effects of Environmental Factors on Chlorella sp. microalgae for Biodiesel Production Purpose: Enhanced Lipid and Biomass Productivity. Recent Innovation in Chem. Eng., 10(2): 119-126 (**8 pages**).

Dickinson, S.; Mientus, M.; Frey, D.; Amini-Hajibashi, A.; Ozturk, S.; Shaikh, F.; Sengupta, D.; El-Halwagi, M., (2017). A review of biodiesel production from microalgae. Clean Technol. Environ. Policy, 19(3): 637-668 (**32 pages**).

Erazo, J.; Sequera, A.; Parthasarathy, R.; Gollahalli, S., (2007). Comparison of spray characteristics of biodiesel and diesel fuel. Proceedings of the 5th International Energy Conversion Engineering Conference and Exhibit, St. Louis, MO, AIAA.

Feng, P.; Xu, Z.; Qin, L.; Alam, M.; Wang, Z.; Zhu, S., (2020). Effects of different nitrogen sources and light paths of flat plate photobioreactors on the growth and lipid accumulation of *Chlorella sp.* GN1 outdoors. Bioresour. Technol., 122762 (**1 pages**).

Gao, F.; Yang, H.; Li, C.; Peng, Y.; Lu, M.; Jin, W.; Bao, J.; Guo, Y., (2019). Effect of organic carbon to nitrogen ratio in wastewater on growth, nutrient uptake and lipid accumulation of a mixotrophic microalgae *Chlorella sp.*. Bioresour. Technol., 282: 118-124 (**7 pages**).

Goh, B.; Ong, H.; Cheah, M.; Chen, W.; Yu, K.; Mahlia, T., (2019). Sustainability of direct biodiesel synthesis from microalgae biomass: A critical review. Renewable Sustainable Energy Rev., 107: 59-74 (**16 pages**).

Golzary, A.; Imanian, S.; Abdoli, M.; Khodadadi, A.; Karbassi, A., (2015). A cost-effective strategy for marine microalgae separation by electro-coagulation–flotation process aimed at bio-crude oil production: Optimization and evaluation study. Sep. Purif. Technol., 147: 156-165 (**10 pages**).

Gong, Q.; Feng, Y.; Kang, L.; Luo, M.; Yang, J., (2014). Effects of Light and pH on Cell Density of *Chlorella vulgaris*. Energy Procedia, 61: 2012-2015 (**4 pages**).

Gouveia, L.; Oliveira, A., (2009). Microalgae as a raw material for biofuels production. J. Ind. Microbiol. Biotechnol., 36(2): 269-274 (5 **pages**).

Huang, G.; Chen, F.; Wei, D.; Zhang, X.; Chen, G., (2010). Biodiesel production by microalgal biotechnology. Appl. Energy, 87(1): 38-46 (**9 pages**).

Jiang, J.; Zhu, Y., (2010). Preliminary and comparative studies on the cultivations of dunaliella salina between outdoors and in the photobioreactor. J. Food Process Eng., 33(1): 104-114 (**11 pages**).

Khalil, Z.; Asker, M.; El-Sayed, S.; Kobbia, I., (2010). Effect of pH on growth and biochemical responses of Dunaliella bardawil and Chlorella ellipsoidea. World J. Microbiol. Biotechnol., 26(7): 1225-1231 (**7 pages**).

Kong, W.; Song, H.; Cao, Y.; Yang, H.; Hua, S.; Xia, C., (2011). The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of *Chlorella vulgaris* under mixotrophic cultivation. Afr. J. Biotechnol., 10(55): 11620-11630 (**11 pages**).

Li, Y.; Tsai, W.; Hsu, Y.; Xie, M.; Chen, J., (2014). Comparison of autotrophic and mixotrophic cultivation of green microalgal for biodiesel production. Energy Procedia, 52: 371-376 (**6 pages**).

Lv, J.; Cheng, L.; Xu, X.; Zhang, L.; Chen, H., (2010). Enhanced lipid production of Chlorella vulgaris by adjustment of cultivation conditions. Bioresour. Technol., 101(17): 6797-6804 (**8 pages**).

Pagnanelli, F.; Altimari, P.; Trabucco, F.; Toro, L., (2014). Mixotrophic growth of Chlorella vulgaris and Nannochloropsis oculata: interaction between glucose and nitrate. J. Chem. Technol. Biotechnol., 89(5): 652-661 (**10 pages**).

Penno, A.; Agar, E.; Divok, J., (2019). Effects of activated carbon on the growth of *Chlorella vulgaris* in an aqueous solution. Stud. Undergraduate Res. Guelph., 11.

Qiu, R.; Gao, S.; Lopez, P.; Ogden, K., (2017). Effects of pH on cell growth, lipid production and CO2 addition of microalgae Chlorella sorokiniana. Algal Res., 28: 192-199 (**8 pages**).

Samiee-Zafarghandi, R.; Karimi-Sabet, J.; Abdoli, M.A.; Karbassi, A., (2018). Increasing microalgal carbohydrate content for hydrothermal gasification purposes. Renewable Energy, 116: 710-719 (**10 pages**).

Sayadi, M. H.; Ahmadpour, N.; Fallahi Capoorchali, M.; Rezaei, M.R., (2016). Removal of nitrate and phosphate from aqueous solutions by microalgae: An experimental study Global J. Environ. Sci. Manage., 2(3): 357-364 (**8 pages**).

Scarsella, M.; Belotti, G.; De Filippis, P.; Bravi, M., (2010). Study on the optimal growing conditions of *Chlorella vulgaris* in bubble column photobioreactors. Chem. Eng., 20: 85-90 (**6 pages**).

Schenk, P.; Thomas-Hall, S.; Stephens, E.; Marx, U.; Mussgnug, J.; Posten, C.; Kruse, O.; Hankamer, B., (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenerg. Res., 1(1): 20-43 (**24 pages**).

Skorupskaite, V.; Makareviciene, V.; Levisauskas, D.; (2015). Optimization of mixotrophic cultivation of microalgae *Chlorella sp.* for biofuel production using response surface methodology. Algal Research, 7: 45-50 (**6 pages**).

Shuba, E.; Kifle, D., (2018). Microalgae to biofuels: Promising'alternative and renewable energy: Review. Renewable Sustainable Energy Rev., 81: 743-755 (**13 pages**).

Zheng, H.; Guo, W.; Li, S.; Chen, Y.; Wu, Q.; Feng, X.; Yin, R.; Ho, S.; Ren, N.; Chang, J., (2017). Adsorption of p-nitrophenols (PNP) on microalgal biochar: analysis of high adsorption capacity and mechanism. Bioresour. Technol., 244 : 1456–1464 (**9 pages**).

AUTHOR (S) BIOSKETCHES

Nouri, H., M.Sc., Caspian Faculty of Engineering, College of Engineering, University of Tehran, Rezvanshar, Guilan, Iran.
Email: *hnourip@gmail.com*

Mohammadi Roushandeh, J., Ph.D., Professor, Caspian Faculty of Engineering, College of Engineering, University of Tehran, Rezvanshar, Guilan, Iran. Email: *roshandeh@ut.ac.ir*

Hallajisani, A., Ph.D., Assistant Professor, Caspian Faculty of Engineering, College of Engineering, University of Tehran, Rezvanshar, Guilan, Iran. Email: *hallaj@ut.ac.ir*

Golzary A., Ph.D., Assistant Professor, Caspian Faculty of Engineering, College of Engineering, University of Tehran, Rezvanshar, Guilan, Iran. Email: *abooaligolzary@ut.ac.ir*

Daliry, S., M.Sc., Caspian Faculty of Engineering, College of Engineering, University of Tehran, Rezvanshar, Guilan, Iran
Email: *saeeddaliry@ut.ac.ir*

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