Increasing microalgal carbohydrate content for hydrothermal gasification purposes

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ABSTRACT

This research examines the growth of Chlorella sp. microalgae under nutrient limitation (10–200 mg NaNO₃ L⁻¹ and 10–70 mg K₂HPO₄ L⁻¹) and different light intensities (60–450 μmol photons m⁻² s⁻¹) for achieving maximum carbohydrate content and biomass productivity using Response Surface Methodology (RSM) technique. According to the results, nutrition limitation had considerable effect on carbohydrate accumulation especially phosphorus concentrations; as in constant light intensities, maximum carbohydrate content was obtained in minimum concentration of K₂HPO₄. Under favorable circumstances; i.e. K₂HPO₄ = 10 mg L⁻¹, NaNO₃ = 105 mg L⁻¹, and light intensity = 255 μmol photons m⁻² s⁻¹ the highest carbohydrate content by 60.9% was achieved. Moreover, Supercritical Water Gasification (SCWG) of carbohydrate enriched microalgal biomass is able to produce much more hydrogen gas in comparison to the basic microalgal biomass. In addition, a 1.85 times increase in amount of produced gas is appeared as a result of a change in biochemical composition of the microalgal biomass.

1. Introduction

Over recent years, renewable energies have become a key strategy in sustainable development realization which can be attributed to disadvantages of fossil fuels, such as having finite resources and supply insecurity, dependence on petroleum exporting countries, contaminations of environment and concerns about the growth of greenhouse gas (GHG) emissions [1]. Biomass is a renewable source of energy that could be converted into biofuels through multiple processes. The first-generation biofuels which are mainly retrieved from edible plants (such as grains and vegetable oils) need extensive lands. The second-generation biofuels are provided mainly from lignocellulose and forestry and agriculture waste sources which do not fall under the category of food crops. However, their sustainability should be examined concerning criteria like minimum lifecycle, GHG reductions, land use change and social standards [2]. Microalgae cultivation for biofuel production is considered as the source of third-generation biofuels. Microalgae are capable of producing high contents of storage compounds (lipid/carbohydrate) within a few days. In comparison to other biomasses, microalgae have attracted noticeable attention as a feedstock in biofuel production due to following causes: higher photosynthetic rate efficiency, requiring less land compared to terrestrial plants, capability of cultivation on non-arable lands with non-potable waters, cultivation in year-round with short harvesting cycles, bio-fixation of CO₂ and high biomass yields [3–5].

The idea of microalgal cultivation for producing renewable fuels was initially presented in 1950 [6], and examined in state research programs of countries such as US, Japan and Australian 1970s [7]. Since 2000, more than $2billion has been assigned to this research field by private institutions [8]. Various studies have been conducted in the field of algae cultivation for producing biofuels such as biodiesel [9], bioethanol [10], biomethane [11], biohydrogen [12], biochar [13], and similar fuels. Among other fuels, hydrogen has the highest amount of energy per mass unit (142 kJ/g) and is the cleanest fuel as it yields only water in combustion [14]. One of hydrogen production methods is utilizing Supercritical Water Gasification (SCWG) technology of biomass. In supercritical water condition (T > 374 °C and P > 221 bar), gaseous products mainly

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CO$_2$, H$_2$, CH$_4$ and CO are produced from decomposition of organic matters. The mechanism of the process is producing the gaseous products from further decomposition of water soluble oligomers which are derived from hydrolysis of feedstock in supercritical water [15]. Hydrogen gas with high efficiency can be produced from wet biomass especially rich-carbohydrate ones [16]. Algal SCWG was introduced in 1990 for the first time [17] and since then numerous studies have been conducted in this field. The most important advantages of this method is requiring no energy to dry the biomass and also reusing the major part of consumed energy for reaching supercritical conditions [17].

Carbohydrates are considered as substantial constituent of microalgal cells which result from photosynthesis and carbon fixation metabolism. Carbohydrates are either accumulated in plastids as storage elements or in cell walls as the main component [18]. Manipulating the carbohydrate content of cells can be accomplished under environmental stress conditions such as nutrient starvation limitation, high light intensities or salinity stress, in which cultivating in optimum situation would be accompanied by alteration of microalgal metabolic pattern and accumulation of storage compounds [19].

Nitrogen is an essential nutrient for growth of microalgae and is known as principal constituent of structural and functional proteins, the synthesis of which depends on the availability of nonorganic nitrogen in a medium [20][21]. Crucial metabolic consequences of nitrogen starvation are disturbance in photosynthesis process, reduction in synthesizing photosystems reaction center proteins and consequent decrease in photosynthetic pigments [22]. In such situation, Pancha et al. [23] suggested that for providing required intracellular nitrogen content for continuing common growth, the nitrogenous compounds in microalgal cells might be degraded. Therefore the flow of the photosynthetically fixed carbon is switched to the lipid or carbohydrate synthesis pathways [24]. Phosphorous which is another essential macronutrient element in algae cells, plays an important role in cellular metabolic processes [25]. Although Phosphorus is required in very small amounts during algal growth cycle, it must be supplied in excess of basic requirement due to bonding phosphates ions with metals ions [26]. Phosphorous starvation in cultivation medium can stimulate carbohydrate or lipid content [24]. For instance, Douskova et al. [27] reported that cultivation of *Chlorella vulgaris* in phosphorus limitation conditions leads to starch accumulation up to 55% dw.

So far, various researches have been conducted on nutrients stresses, including nitrogen, phosphorus or Iron starvation stress and also increase or decrease in light intensity aiming to increase microalgal carbohydrate content. However, the resultant stress from simultaneous effect of two macro nutrients in cultivation media (nitrogen and phosphorus) in the presence of light intensity stress and its effect on microalgal carbohydrate content has not been examined yet. This study aims at assessing the increase in carbohydrate content of *Chlorella* microalgal cells under nutrition stress circumstances (reduction in nitrogen and phosphorus concentrations) and increasing light intensity, in order to produce the suitable feedstock in SCWG process. By designing the experiments, changes in range of variables (10–200 mg NaNO3 L$^{-1}$, 10–70 mg KH2PO4 L$^{-1}$ and light intensities of 60–450 µmol photons m$^{-2}$ s$^{-1}$) on carbohydrate content and biomass productivity will be evaluated. Since nutrient limitation is accompanied by decreasing in growth of the algae, carbohydrate productivity was also examined. The levels of each designing parameter were selected based on pre-experiment. At the end, the SCWG of microalgal biomass rich in carbohydrate content was investigated in comparison to the basic one in batch reactor operating at temperature and pressure of 380 °C and 225 bar respectively and in the reaction time of 30 min.

### 2. Material and methods

#### 2.1. Growth condition

In this study, *Chlorella* sp. (PTCC 6010, Persian Type Culture Collection) was obtained from Iranian Research Organization for Science and Technology (IROST). This microalga is native to the Persian Gulf, resistent and easily adaptable to the environment.

Pre-cultivation of the microorganism was carried out in a 500 ml glass bubble column photobioreactor with 50 µmol photons m$^{-2}$ s$^{-1}$ light intensity in Rodik medium with the following composition (per liter): 0.30 g NaNO$_3$, 0.08 g KH$_2$PO$_4$, 0.02 g KH$_2$PO$_4$, 0.02 g NaCl, 0.047 g CaCl$_2$, 0.02 g MgSO$_4$ 7H$_2$O, 0.1 mg ZnSO$_4$ 7H$_2$O, 1.5 mg MnSO$_4$ 7H$_2$O, 0.08 mg CuSO$_4$ 5H$_2$O, 0.3 mg H$_3$BO$_3$, 0.3 mg (NH$_4$)$_6$Mo$_7$O$_24$ 4H$_2$O, 17 mg FeCl$_3$, 6H$_2$O, 0.2 mg Co(NO$_3$)$_2$ 6H$_2$O, and 7.5 mg EDTA. In order to reach the Persian Gulf sea-water condition, the salinity of cultivation medium was adjusted to 33 g L$^{-1}$.

Prior to the main experiments, the broth at the late exponential phase of the growth curve was centrifuged (5000 rpm) for 12 min and washed with distilled water twice. Then the pre-cultured microalgae were inoculated into 5 L bubble column bioreactor (3 L working volume) to reach an approximate inoculum size of 0.028 g L$^{-1}$. *Chlorella* sp. Microalgal was exposed to different concentrations of NaNO$_3$ (10–200 mg L$^{-1}$) and KH$_2$PO$_4$ (10–70 mg L$^{-1}$) in a new medium.

The temperature was adjusted to 27 ± 1.5 °C and two to eleven 40 W fluorescent lamps mounted on both sides of the bubble column bioreactor with suitable distance to reach the illumination of 60–450 µmol photons m$^{-2}$ s$^{-1}$ which measured by light meter (TES-1330 A, Taiwan). During the cultivation, the bioreactor operation performed with 2.0% v/v CO$_2$ aeration rate of 0.2 vvm continuously. 4 ml liquid sample was collected daily to determine biomass concentration.

#### 2.2. Harvesting

Electro-coagulation was applied as a rapid and cost effective approach to separate microalgae from the cultivation medium. In this process, coagulants form during the electro-oxidation of the sacrificial anode and formation of flocs appears as a result of destabilization and charge neutralization of the suspension [28]. In this research, two parallel aluminum electrodes with effective surface and distance of each other equal to 96 cm$^2$ and 4 cm respectively were placed inside the plexiglas reactor with dimensions of 15 × 15 × 20 cm (useful volume of 2700 cm$^3$). Initial pH of each experiment was adjusted to 6.5 and DC power supply (DAZHENG PS-305D) with a production capacity of 1.4 A was utilized. In order to have mixing during the reaction, the reactor was placed over the magnetic stirrer (Alpha stirrer, made in Iran) with 100 rpm.

At the end of cultivation time, biomass was harvested, washed twice with distilled water and dried in 65 °C oven over the night to analyze the carbohydrate content.

#### 2.3. Determination of biomass concentration

For determining Cell concentration of the culture, optical density was used and the absorbance was measured at wavelength of 550 nm with UV/VIS spectrophotometer (GBC-Model 911). In wavelength of 550 nm, the absorption by chlorophyll and other pigments is the least and negligible [29][30]. These values were converted to *Chlorella* sp. dry weight by using the following equation (equation (1)) which comes from the correlation curve between biomass dried weight per liter (X, mg L$^{-1}$) and the culture absorbance. Biomass productivity (mg L$^{-1}$ d$^{-1}$) was calculated
based on equation (2) where \(X_1\) and \(X_2\) indicate respectively the weight of dried biomass per liter on the first \(t_1\) and final day of experiment \(t_2\).

\[
X = 490 \times \text{OD}_{550} \tag{1}
\]

\[
\text{Biomass productivity} = \frac{X_2 - X_1}{(t_2 - t_1)} \tag{2}
\]

### 2.4. Carbohydrate measurement

Algal biomass carbohydrate determination was assessed by the phenol – sulfuric acid colorimetric method based on the method described by Dobuis et al. [31]. In brief, microalgae biomass was hydrolyzed with 1 M sulfuric acid in autoclave, centrifuged (2000 rpm) for 10 min and then 1 ml of the supernatant was reacted with 1 ml of 5% phenol solution and 5 ml of concentrated sulfuric acid to create a characteristic yellow - orange color. The absorbance was measured at 490 nm with UV/VIS spectrophotometer (GBC-Model 911). Total carbohydrate was quantified based on glucose standard curve. Furthermore carbohydrate productivity (mg L\(^{-1}\)d\(^{-1}\)) was calculated according to equation (3):

\[
\text{Carbohydrate productivity} = \frac{\text{Biomass productivity}}{\text{Carbohydrate content(\%)}} \tag{3}
\]

### 2.5. Lipid content

Bligh and Dyer’s method [32] assisted by ultrasound [33] was applied to estimate total lipid content of each sample. All samples were performed in an ultrasonic bath with working at 50 kHz (POWERSONIC 420 model, HWASHIN TECHNOLOGY, Korea). In brief, lipids were extracted by mixing each sample with chloroform–methanol (1:2 v/v) solvent. During 15 min (three times), the mixture was filtered and the solid phase was removed. Finally an experimental model based on equation (2) was calculated according to the minimum

\[
\text{Variables and their ranges used in RSM design.}
\]

<table>
<thead>
<tr>
<th>Factor</th>
<th>Unit</th>
<th>Low level</th>
<th>High level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light intensity</td>
<td>(\mu)mol photons m(^{-2})s(^{-1})</td>
<td>60</td>
<td>450</td>
</tr>
<tr>
<td>NaN(_3)</td>
<td>mg L(^{-1})</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>K(_2)HPO(_4)</td>
<td>mg L(^{-1})</td>
<td>10</td>
<td>70</td>
</tr>
</tbody>
</table>

### 2.6. Protein determination

The protein content estimation of microalgal biomass was carried out by measuring elemental nitrogen of the biomass measured with a CHNS analyzer (Eager 300 for EA1112). Through multiplying the value by a standard conversion factor of 6.25 [34], the protein content was achieved.

### 2.7. Design of experiments

Among the various methods of designing the experiments, Response Surface Methodology (RSM) is widely used in scientific and industrial studies. RSM applies set of statistical and mathematical techniques to evaluate the effect of independent variables and their interactions on responses through the minimum repetition of experiments and finally an experimental model based on laboratory data will be presented. RSM is also known as an efficient tool to optimize the performance systems and utilizes different experimental designs including Central Composite Design (CCD). CCD is in fact a method to fit the model according to the minimum square technique which defines how variables interact and what their effects on the responses are [35–37].

In this research, CCD was employed to optimize the carbohydrate content of microalgal biomass and biomass productivity. The effect of three parameters namely light intensity, nitrogen and phosphorus sources, and constant values of \(b_0\) come from Analysis of Variance (ANOVA) method.

According to equation (4), fitted second order models were developed to estimate the responses and predict the optimal point [36]. On the following equation:

\[
Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ij} X_i^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} b_{ij} X_i X_j \tag{4}
\]

\(Y\) indicates responses, including carbohydrate content and biomass productivity; \(X_i\) and \(X_j\) are the factors affecting \(Y\), including light intensity, nitrogen and phosphorus sources, and constant values of \(b_0\), \(b_i\), \(b_{ij}\) come from Analysis of Variance (ANOVA) method.

### 2.8. Supercritical water gasification of biomass and calculations

Supercritical water gasification experiments were carried out in a batch stainless steel 316 L reactor with an inner volume of 165 ml (including a vessel and its tubing). The reaction conditions were set under temperature and pressure of 380 °C and 225 bar respectively. In each run, 4.9 wt% suspension of microalgae dried biomass (3.5 g) in deionized water (67 ml) was charged into the reactor vessel and the reactor flange was tightened and sealed through utilizing spiral-wound gaskets. Residual air was removed by pressurizing and venting with pure helium of 5 bar several times. The reactor was heated up by electrical furnace to 380 °C and the temperature maintained with a PID controller. After the reaction time of 30 min the furnace was removed and the reactor was cooled down to ambient temperature by fan. The product gas was collected in a gas sampling bag (SKC, 1 L). The samples were analyzed off-line with an YL-6100 GC gas chromatograph equipped with a proapak Q column and a helium ion detector (HID) to determine \(\text{H}_2\), \(\text{CH}_4\), \(\text{CO}\), and \(\text{CO}_2\) quantification and mole % of each gas in the gaseous product was obtained. Finally the moles of each component were calculated on the basis of general gas equation and the pressure gas produced at the end of the experiment at room temperature.

For Evaluation of SCWG, in addition to measurements of molar fraction (%) of gas product composition samples and amount of gas production, gas yield and gasification efficiency (GE) were calculated by the following equations:

\[
\text{Gas yield} = \frac{\text{Total moles of gas produced}}{\text{Total moles of gas present in feedstock}}
\]

\[
\text{Gasification efficiency} = \frac{\text{Total caloric value of gas produced}}{\text{Total caloric value of feedstock}}
\]
Gas yield (mmol/g) = the mole number of gas product/mass of dry biomass in gr

GE(%) = 100 × mass of gas product/mass of dry biomass

3. Results and discussion

3.1. Growth of microalgae

Fig. 1 shows the microalgal cell growth over time in the main medium at light intensity of 50 μmol photons m⁻² s⁻¹. Cells were grown with air and enriched air with 2% CO₂. Lines between the experimental points show the trend of growth. After inoculation, existence of lag phase during the growth is due to physiological adaptation of the cell metabolism and synthesis of essential enzymes for cell division. Afterwards, substrate is utilized immediately and increasing cell density accelerates during the exponential phase. As seen in Fig. 1, in aeration with 2% CO₂− enriched air, maximum biomass production of 916 mg L⁻¹ achieved through less time duration (7 days) in comparison to cells grown with air (623 mg L⁻¹ in 10 days). It can be concluded that CO₂ as a major carbon source could promote autotrophic growth of microalgal photosynthesis cells. As time progressed, cell division reduced gradually which is mainly related to reduction of nutrient concentrations in the medium and also reduction of the actual light received by each cell due to the shadow of cells caused by cell density increase.

By increasing cultivation time following logarithmic phase, carbohydrate content decreased while cells became enriched in lipid content in long term storage mechanism. Therefore, measuring carbohydrate content of biomass was done in 7th day and was 10.18% for aeration with air and 13.8% in cells cultivation in aeration with 2% CO₂-enriched air. Increasing carbohydrate content in response to increase in CO₂ concentration was reported by Xia et al. [38]. Although increasing in CO₂ content of air did not have significant effect on carbohydrate accumulation, higher light insality and nutrient limitations could stimulate more carbohydrate synthesis in microalgal cells.

3.2. Model presentation

Chlorella sp. microalgae was cultivated for a specific time under different light intensity conditions with different concentrations of nitrogen and phosphorus sources. Carbohydrate experiment was conducted for each sample on dry biomass. As can be seen in Table 2, the maximum biomass productivity was calculated 235.8 mg L⁻¹d⁻¹ in following conditions: NaNO₃ = 200 mg L⁻¹, K₂HPO₄ = 70 mg L⁻¹, and the maximum light intensity of 450 μmol photons m⁻² s⁻¹ and the carbohydrate content calculation equaled to 60.91% under different conditions; i.e. NaNO₃ = 105 mg L⁻¹, K₂HPO₄ = 10 mg L⁻¹, and the light intensity = 255 μmol photons m⁻² s⁻¹. Although achieving the maximum carbohydrate accumulation in microalgal cells was the main aim, carbohydrate productivity is still of immense importance. Hence, it should be mentioned that the maximum carbohydrate productivity of the experimental data was realized under following conditions: NaNO₃ = 200 mg L⁻¹, K₂HPO₄ = 40 mg L⁻¹, and the light intensity = 255 μmol photons m⁻² s⁻¹ which equals 114.28 mg L⁻¹d⁻¹ (according to Table 2).

Table 3 shows the individual and interactive effects of each factor on carbohydrate content and biomass productivity through ANOVA analysis. The significance of a coefficient enhances by p-value reduction and increasing F-value [39]. Therefore, as can be seen on Table 3, light intensity and nitrogen concentration have the highest influence respectively on carbohydrate accumulation and increasing biomass productivity.

P-values less than 0.05 imply the significant relationship between variables and responses and the model fits properly on data. Since the p-values of parameters (light intensity, nitrogen, and phosphorus sources) were less than 0.05, it can be concluded that all parameters have had significant influence on responses. In contrary, interaction of K₂HPO₄ concentration with other parameters (light intensity and NaNO₃ concentration) does not have considerable effect on responses. Moreover, p-values of models’ responses are low enough to prove the suitability of models to be fitted on data and their application in estimating the changes in ranges of each variable and their effects on both responses. Lack of fit F-value of 0.53 and 6.14 respectively for carbohydrate content and biomass productivity implies that lack of fit is not significant due to relative pure error. Hence, the models fit the data properly.

According to the conducted experiments based on RSM and the resultant outputs, suggested models for responses of carbohydrate content and biomass productivity are quadratic and presented as coded variable respectively on Table 4 in equations (7) and (8). These equations help to estimate the responses in assumed levels of
As explained by the model, the coefficient of determination \( (R^2) \) measures the variations around the mean. The more \( R^2 \) approaches 1, it means the model explains the variability in the response successfully. To define a high correlation between the experimental and predicted values, a model should have not only a high \( R^2 \), but also a high adjusted coefficient of determination \( (R^2_{adj}) \). These coefficients were calculated respectively for carbohydrate content model 0.957 and 0.914 and for biomass productivity estimating model 0.965 and 0.930. The subtle difference in \( R^2 \) and \( R^2_{adj} \) proves that regression models are well fitted to laboratory data [40].

### 3.3. Accuracy of the model

Fig. 2(a) and (b) show required charts for evaluation of experimental data which illustrate the normal percent of probability of studentized residues in carbohydrate content and biomass productivity models. As can be seen in both models, residues are approximately along straight lines, following normal distribution and constant variance. As shown in Fig. 2(c) and (d), resultant values from experiments comply with estimated amounts to a high extent that proves the acceptable accuracy of the model in calculating responses. Hence, the model is suitably capable of presenting the effect of variables on responses with acceptable level of accuracy. Fig. 2(e) and (f) depict internally studentized residuals versus experimental runs for both responses. As can be seen, data points are randomly scattered within the limits and do not follow a specific pattern. In addition there is no evidence of unexpected errors that strongly affect the models, as all leverage points show values less than 1 (Fig. 2(g) and (h)).

### Table 2
The matrix of experimental design for carbohydrate accumulation of the microalgae.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>A (µmol photons m(^{-2}) s(^{-1}))</th>
<th>B (mg L(^{-1}))</th>
<th>C (mg L(^{-1}))</th>
<th>Carbohydrate Content (%)</th>
<th>Biomass Productivity (mg L(^{-1}) d(^{-1}))</th>
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</thead>
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<td>Experimental</td>
<td>Model prediction</td>
<td>Experimental</td>
<td>Model prediction</td>
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<td>200</td>
<td>70</td>
<td>46.00</td>
<td>47.52</td>
</tr>
</tbody>
</table>

### Table 3
Analysis of Variance (ANOVA) from CCD design.

<table>
<thead>
<tr>
<th>Source</th>
<th>Carbohydrate Content</th>
<th>Biomass Productivity</th>
</tr>
</thead>
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<tr>
<td>F-value</td>
<td>p-value</td>
<td>F-value</td>
</tr>
<tr>
<td>Model</td>
<td>22.3471</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A-Light</td>
<td>27.38849</td>
<td>0.0008</td>
</tr>
<tr>
<td>B-NaNO(_3)</td>
<td>11.85982</td>
<td>0.0073</td>
</tr>
<tr>
<td>C-K(_2)HPO(_4)</td>
<td>13.69995</td>
<td>0.0049</td>
</tr>
<tr>
<td>AB</td>
<td>39.38015</td>
<td>0.0001</td>
</tr>
<tr>
<td>AC</td>
<td>1.96296</td>
<td>0.1947</td>
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<tr>
<td>BC</td>
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<td>0.2710</td>
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<tr>
<td>A(^2)</td>
<td>62.12664</td>
<td>&lt;0.0001</td>
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<td>B(^2)</td>
<td>1.49855</td>
<td>0.2710</td>
</tr>
<tr>
<td>C(^2)</td>
<td>2.42260</td>
<td>0.154</td>
</tr>
</tbody>
</table>

Lack of Fit 0.52992 | 0.7491 | not significant | 6.13681 | 0.0516 | not significant |

### Table 4
Reduced model for carbohydrate content and biomass productivity.

<table>
<thead>
<tr>
<th>Reduced model</th>
<th>Equation</th>
<th>( R^2 )</th>
<th>( R^2_{adj} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate content (%) = 46.84 + 8.37 A + 5.51B-5.92C + 11.23 A B + 2.51AC + 2.10BC-24.13A(^2)-3.75B(^2)+4.76C(^2)</td>
<td>(7)</td>
<td>0.957</td>
<td>0.914</td>
</tr>
<tr>
<td>Biomass productivity (mg L(^{-1}) d(^{-1})) = 154.55 + 26.52 A + 62.71 B + 18.99C + 22.91 A B + 9.73AC + 10.24BC-25.45A(^2)-26.05B(^2)-13.19C(^2)</td>
<td>(8)</td>
<td>0.965</td>
<td>0.930</td>
</tr>
</tbody>
</table>

Note: A, B and C indicate the coded value of Light intensity, NaNO\(_3\) concentration and K\(_2\)HPO\(_4\) concentration, respectively.
3.4. Influence of individual variables

Perturbation charts show the influence of each parameter on carbohydrate content and biomass productivity when the range of a parameter changes while the other two parameters are at the constant points (Fig. 3).

3.4.1. Influence of light intensity

Among the variables of experimental design, light intensity has the highest influence on bioaccumulation of carbohydrate: raising the light intensity (from 60 to 300 μmol photons m⁻²s⁻¹) increases carbohydrate production (Fig. 3(a)) which is in accordance with the results stated by Guedes et al. [41]. Due to photo inhibition effect,
excessive light intensity has adverse effect on carbohydrate bioaccumulation. It should be considered that light intensity needs to be high enough to stimulate carbohydrate production in microalgae. As stated by Markou et al. [42], although increasing light intensity from 24 to 60 μmol photons m⁻² s⁻¹ raises the biomass production of *Arthrospira platensis*, it does not affect carbohydrate content which is attributed to limited changes in light intensity range. Therefore, in this study the range of light intensity was wide enough to investigate the changes of carbohydrate content and growth of microalgal cells. The experiment has been conducted by Ho et al. [43] through cultivating microalga *Scenedesmus obliquus* under light intensity = 180–540 μmol photons m⁻² s⁻¹ proved that increasing the light intensity up to 400 leads to higher carbohydrate content. Dragon et al. [44] observed that higher light intensity increases polysaccharide production in *Chlorella vulgaris* cells.

Increasing the light intensity from 60 to 360 μmol photons m⁻² s⁻¹ while nitrate and phosphate concentrations are constant at their mid points, leads to acceleration in photosynthesis process and consequently increasing biomass productivity. However, increasing the light intensity from 340 to 450 μmol photons m⁻² s⁻¹, decreases biomass productivity which is due to damaging photosynthetic systems in extreme light intensities. Light is the main supplier of energy for photosynthesis organisms and its intensity affects the growth rate and composition of the biomass [24]. An increase in light intensity leads to a decrease in light harvesting pigments while raises protective agents (such as secondary carotenoids) which protect photosynthetic machinery from harm of light intensity’s excessive energy; besides, they have protective role against oxidative tensions [20,45]. Friedman et al. [46] stated that a fourfold increase in light intensity for two species of marine and fresh water microalgae doubled the growth rate and polysaccharide production increase respectively up to 0.6 and 3 times in them. Through cultivating the microalgae “*Spirulina platensis*” in open spaces, biomass productivity and carbohydrate synthesis in sunny days is considerably higher than in cloudy days [47].

### 3.4.2. Influence of nutrients

Results show that the maximum biomass productivity was accompanied by increasing in nutrient concentration (Fig. 3(b)), but the trend was not the same for carbohydrate content. Maximum value of the latter term was gained at the minimum concentration of phosphate (10 mg L⁻¹ of K₂HPO₄) when the two other variables were constant at the mid points. When the intercellular phosphorus level drops below the threshold, carbohydrates start to accumulate [24]. According to Markou et al. [42], the minimum concentration of phosphorus increases the carbohydrate content in microalgae “*Arthrospira plantensis*” up to 59.64%. Also, Dean et al. [48] reported that decreasing phosphorus concentration of the cultivation medium of *Chlamydomonas reinhardtii* causes considerable rise in carbohydrate content. Moreover, increasing in nitrate concentration to 140 mg L⁻¹, maximizes the carbohydrate accumulation (Fig. 3(a)). Illmaen et al. [49] proved that medium with low nitrogen in different strains of Chlorella leads to different responses in biomass productivity and carbohydrate content as well. However, such a situation lessens the biomass productivity and enhances carbohydrate content up to 55% in *Chlorella vulgaris*. Besides, it was observed that in cultivation mediums with the minimum nitrate concentration, chlorophyll content of the microalgae has decreased drastically; therefore, it is concluded that since chlorophyll is a nitrogenate composition, its pigments could be dependent on nitrate concentration.

![Fig. 4. Response surface plots of interaction effects of variables on carbohydrate content (a, c, e) and biomass productivity (b, d, f).](image-url)
3.5. Influence of interactions

3.5.1. Effect of light intensity and nitrate concentration

The results of ANOVA in Table 3 indicate that light intensity, nitrate concentration and their interaction have significant effects on carbohydrate content and biomass productivity (p-value<0.05). Furthermore, light intensity and nitrogen concentration have the highest influence respectively on carbohydrate accumulation and increasing biomass productivity.

Fig. 4(a) and (b) present how light intensity and NaNO₃ concentration of the medium affect response values. Increasing light intensity up to 290 μmol photons m⁻² s⁻¹ and reduction of nitrate concentration to about 50% of the initial cultivation media, lead to maximizing the bioaccumulation of carbohydrate in microalgal cells (Fig. 4(a)). Carbon partitioning response to light intensity is highly species-specific and there is no consensus on how light intensity influences carbon partitioning under nitrogen constraint [50]. Chen et al. [39] proved increasing production of polysaccharide in Chlorella sp. When nitrogen content and light intensity respectively decreases and increases.

Biomass productivity rises considerably when light intensity and nitrate concentration increase (Fig. 4(b)). According to Sologubchenko et al. [51], increasing light intensity raises biomass productivity. In higher light intensity, the concentration of nitrate content is also determining; i.e., the higher the light intensity, the more concentration of nitrate increases biomass productivity, which is in agreement with results reported by Breuer et al. [52].

3.5.2. Effect of light intensity and phosphate concentrations

It was found that phosphate concentration has a major effect on the responses; in contrary, the interaction of K₂HPO₄ concentration with light intensity does not have considerable effect (Table 3). Fig. 4(c) and (d) illustrate the influence of light intensity and phosphate concentration on carbohydrate content of the microalgae and biomass productivity. Except in runs with limited phosphate, variation in the nutrient did not show significant effect on carbohydrate content in constant light intensity as experienced by Markou et al. [42]. The maximum amount of carbohydrate production occurs in the minimum concentration of phosphorus and light intensity of about 275 μmol photons m⁻² s⁻¹. Higher light intensity and phosphorus concentration cause a decreasing behavior in carbohydrate production. Moreover in low light intensity Biomass productivity increases along with rising light intensity and phosphate concentration which is in accordance with the studies of Markou et al. [42].

3.5.3. Effect of nitrate and phosphate concentrations

Fig. 4(e) and (f) illustrate the influence of two essential macronutrients on carbohydrate content and biomass productivity. Results show that increasing nutrient concentrations leads to high biomass productivity and nitrogen source has the highest influence on increasing biomass productivity. Nutrition starvation limits the microalgae population which is in accordance with the results of Smith [53]. In case of limited nitrogen, proteins cannot be formed. Hence, carbon absorption is assigned to synthesis of other storage elements rather than in cell division and as a consequence, carbohydrate accumulation increases.

As seen in Table 2, the results of this research prove that in minimum light intensity (60 μmol photons m⁻² s⁻¹), the highest amount of carbohydrate production formed in minimum concentration of both NaNO₃ and K₂HPO₄ (10 mg L⁻¹). It is suggested that nutrition limitation in low light intensity leads to increasing carbohydrate accumulation. Also, in steady light intensity, maximum amount of carbohydrate is produced in the minimum concentration of phosphate. However, in lower light intensities, lower concentrations of nitrate stimulated carbohydrate accumulation in microalgae cells.

3.6. Model optimization and validation

The final goal in RSM is optimizing the operating conditions or determining specific ranges in variables space at which operating characteristics are satisfied [36]. In other words, optimization process determines the amount of variable through which the best operating conditions occurs. Depending on requirements, there exist several optimal points in RSM that could be described arbitrarily. In this study, achieving maximum carbohydrate production is the main purpose but it should be noticed that carbohydrate productivity has the highest significance. As shown in Table 5, the optimal amounts of variables for achieving maximum carbohydrate productivity where both carbohydrate content and biomass productivity are designed to be maximized (54.5% and 235.8 mg L⁻¹ d⁻¹ respectively), happens when light intensity = 355 μmol photons m⁻² s⁻¹, NaNO₃ = 198 mg L⁻¹ and K₂HPO₄ = 70 mg L⁻¹. Moreover, the amount of 0.923 of desirability data which approaches to 1 shows that the optimization criteria are well satisfied in such condition.

In order to assess the validity of the experimental model, a confirmation experiment was conducted on assumed optimal condition for carbohydrate productivity. The resultant shows 134.4 mg L⁻¹ d⁻¹ for desirable response which does not have considerable difference from model’s predicted output.

3.7. SCWG of microalgal biomass

On Table 6, chemical compositions of two microalgal biomasses are shown. Stress conditions applied and cultivation was done in presence of 2% CO₂ in air, leading to a sextuple increase in carbohydrate content and also a 25% increase in lipid production, causing a significant decrease in protein bioaccumulation on the other hand.

The results obtained from hydrothermal gasification of the two mentioned biomasses can be seen in Table 7. As tabulated, the major gas product of this process contains 75 and 78% mole fraction of CO₂, which is the consequence of decarboxylation reactions. According to the kinetic model proposed by Guan et al. [54] in SCWG process of microalgal biomass, it is assumed that microalgae turns to two types of intermediate compounds with different conversion rates, and then gas production from produced organic intermediates is done through steam reforming and decomposition paths (equation (9)). Water gas shift (equation (10)) and

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**Table 5**

<table>
<thead>
<tr>
<th>Optimal Operating Conditions</th>
<th>Predicted Response</th>
<th>Carbohydrate (%)</th>
<th>Biomass productivity (mg L⁻¹ d⁻¹)</th>
<th>Carbohydrate productivity (mg L⁻¹ d⁻¹)</th>
<th>Desirability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light intensity (μmol photons m⁻² s⁻¹)</td>
<td>NaNO₃ (mg L⁻¹)</td>
<td>K₂HPO₄ (mg L⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>355</td>
<td>197</td>
<td>70</td>
<td>54.5</td>
<td>235.8</td>
<td>128.5</td>
</tr>
</tbody>
</table>
methanation (equation (11)) reactions may change the composition of produced gases from intermediates. Steam reforming and water gas shift reactions are responsible for hydrogen production, and on the other hand, hydrogen and carbon monoxide are consumed in methanation reaction to produce CH4.

\[
\text{Microalgae} + \text{H}_2\text{O} \rightarrow \text{intermediates} + \text{H}_2\text{O} \rightarrow \text{CO} + \text{CO}_2 + \text{H}_2 \tag{9}
\]

\[
\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2 \tag{10}
\]

\[
\text{CO}_2 + 3\text{H}_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O} \tag{11}
\]

According to the results, gaseous product of carbohydrate enriched microalgae has a greater mole fraction of H2 and a smaller mole fraction of CH4 in comparison with the basic microalgae. This change in composition of the produced gases can be the outcome of the fact that protein hinders hydrogen production. In basic microalgae, which has a greater protein content, parts of H2 and CO gases were consumed in methanation reaction, leading to more CH4 production, which conforms the results proposed by Tiong et al. [55] in SCWG of two types of microalgae.

It can be concluded that the progress in non-catalytic hydrothermal gasification reaction of biomass to the hydrogen or methane production side, is dependent on biochemical composition of the loaded biomass, alongside with operating conditions of reaction. As shown in Table 7, the amount of produced gas in basic microalgae is lesser. Actually, a change in biochemical composition of the biomass has led to a 1.85 and 4.63 times increase in amount of produced gas and hydrogen gas production respectively. In addition GE that indicates the conversion percentage of feedstock to gas phase, shows about 81% increase in rich-carbohydrate biomass. As Kurse et al. [56] proposed, using phyto-mass instead of zoo-mass will definitely lead to increasing gas yield. They reported that proteins can hinder gas production via two ways. Firstly, gasification of proteins needs a longer time compared to carbohydrates; and secondly, proteins or their derivatives can disturb carbohydrate destruction process. In fact, some nitrogen-based compounds are formed in the reaction of protein degradation products and carbohydrate destruction products, which are responsible for free radical scavengers creation. Therefore the rather stable free radicals may have caused disturbance in gasification process through prevention of starting free radical chain reactions [57].

Comparing with water electrolysis, microorganism methanization and pyrolysis, SCWG of biomass, is the most efficient and cost-effective method for retrieving hydrogen [58]. According to the results, high amounts of CO2 were produced during the procedure which can be recycled and used in cultivation medium as carbon source for algal growth that leads to decreasing costs and improving the sustainability of the system. Although in small fractions, methane is also produced during the process which can be separated, collected and transferred for other applications. No need to mention that the details of separating the gases are beyond the framework of this research.

### 4. Conclusion

This research has suggested an appropriate strategy for increasing carbohydrate content and biomass productivity in Chlorella PTCC6010 microalgae, through modifying the cultivation medium and light intensity. It was concluded that higher light intensities has substantial role in accumulation of carbohydrate in microalgal biomass. In this condition, the highest amount of carbohydrate production occurred when the phosphorus concentration was minimized and light intensity and nitrogen concentration were average; while, the highest amount of biomass production was achieved under maximum light intensity and the highest concentrations of nutrients. What distinguishes this research is that unlike existing studies, the stress from two macro nutrients has been examined. Besides, light intensity was used as a variant to increase carbohydrate content. The advantage is that carbohydrate was maximized through simultaneous stress from nitrogen and phosphorus resources in cultivation medium and light intensity. According to the SCWG of microalgal biomass, changing biochemical composition of microalgae cells under nutrients and environmental stress can be considered as a solution to production of appropriate biomass feedstock for using in hydrothermal gasification process.

### References
