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Research Article

Economic medium optimization by response surface methodology for higher biomass productivity and chlorophyll content in *Chlorella vulgaris*

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Abstract

Commercial production of microalgae is often more costly, as it requires significant quantities of various nutrients. This study aimed to utilize response surface methodology (RSM) to identify optimum levels of particular variables in a general economic culture medium, in the presence of sodium bicarbonate as an inorganic carbon source for increasing biomass productivity (BP) and total chlorophyll (CHL) content in *Chlorella vulgaris* microalgae. The different levels of independent variables were based on related references and studies. The predicted amount of biomass productivity and chlorophyll content in RSM was 119.37 mg/L/d and 7.61 mg/L, respectively, at optimized conditions: temperature 25°C, nitrate 716.39 mg/L, phosphate 14 mg/L, and sodium bicarbonate 259 mg/L. The results of the predicted and the actual response differed with R^2: 0.75 and 0.81, and adjusted R^2: 0.51 and 0.64 for BP and CHL, respectively, and were found to be in reasonable agreement with the better reliability model. This method is applied to the optimization of actual BP and CHL experiments and is found to outperform the existing methods. The optimal value found by the proposed method has a high prediction accuracy (less than 10% error), and it can be confirmed that the increased BP and CHL amount is significant (p<0.05). This information is particularly interesting for semi-industrial-scale processes, since the reduction and optimization of medium compounds might represent an improvement in the cost-effectiveness of the process and, eventually, greater profit.

Introduction

Microalgae have numerous industrial applications because they contain high-quality plant proteins, pigments, and polyunsaturated fatty acids. Their feature of not occupying any land makes them a promising alternative to meet the predicted global nutritional requirements (Ejike *et al*., 2017). *Chlorella vulgaris*, an edible microalgae strain with a generally recognized safe status, has been commercially produced for more than half a century (Caporgno *et al.*, 2019). Factors affecting the culture of microalgae include nutrient content, culture method, temperature, light intensity, light period, pH, and CO_2 concentration (Chowdhury *et al.*, 2020). Generally, growth and growth-related metabolism are accelerated with an increase in the supply of carbon, nitrogen, and phosphorus (Kim and Lee, 2014). Nitrate and phosphate uptake in microalgae is dynamic and is influenced by several driving factors, among which carbon, nitrogen, and phosphorus supply are key drivers that can be managed (Chaudhary *et al.*, 2020). Nitrogen (N) and phosphorus (P) are key nutrients for the growth of algae. P and N are categorized as macronutrients that are responsible for cellular integrity (Belotti *et al.*, 2013). Previous studies indicated that biomass productivity was enhanced by providing carbon resources. The cost of organic carbon sources is high compared to all other added nutrients. This high-cost issue can be solved by using an inorganic carbon source. Sodium bicarbonate as an inorganic carbon source is also considered to improve microalgal growth in the cultivating medium. Microalgae can withdraw CO_2 from sodium bicarbonate ($NaHCO_3$) (Wang *et al.*, 2008). There is a study suggesting bicarbonate (1g/L) as a possible source of carbon for biomass and lipid production in *Chlorella vulgaris* (Mokashi *et al.*, 2016). Abedini-Najafabadi *et al* (2014) showed that the highest cell density of *Chlorella vulgaris* was achieved by using sodium bicarbonate in comparison to organic carbon sources in the culture medium. For economic algal biomass production, growth conditions such as temperature, light (intensity and duration), carbon source (for autotrophic metabolism), media type, and nutrient inputs (N and P) should be optimized in algal reactors. Optimization of them is essential for desirable microalgal growth (Suthar *et al.*, 2017). To make microalgae production more economically viable, it is necessary to reduce the production costs associated with the growth medium to be used because various technological and economic constraints limit industrial-scale production. Since this study used a general and economic culture medium, the independent variables investigated were selected based on the constituents of this culture medium and a physical factor (temperature) that affects the growth of chlorella. The simultaneous study of many factors requires many repetitions and treatments, and so high costs, which sometimes makes it impossible. So, in this study, an optimization strategy based on Design of Experiment (DoE) was carried out to simultaneously identify the conditions of four independent variables (nitrate, phosphate, sodium bicarbonate, and temperature) that favored the production of Chlorella biomass by Response Surface Methodology (RSM).

Previously, also this method has been used by some researchers to optimize chlorella cultivation. For example, Kanaga *et al.* (2022) utilized Box-Behnken design (BBD) of the response surface methodology for identifying optimum levels of particular variables for increasing biomass production in *C.vulgaris*. Their results of the predicted and the actual response differed with residuals>2.34% and R^2=98.3 and Adj R^2=90.8 for biomass and were found to be in reasonable agreement with the better reliability model. This work also established the growth of *C.vulgaris* in a general medium base where sodium bicarbonate serves as a source of inorganic carbon in different temperatures by assessing dry biomass productivity and total chlorophyll content. The objectives of this study are maximizing biomass productivity and total chlorophyll content of *C. vulgaris* under optimized culture conditions.

Materials and methods

Preparation and cultivation of C. vulgaris

C. vulgaris stock was obtained from the Caspian Sea Ecological Research Center in Sari, Iran. Algae cultivation was done using BB medium (Stein, 1973) initially to prepare the required stock, followed by the general medium (TMRL) with the presence of sodium bicarbonate in 250 ml containers according to the treatments outlined in Table 2. The initial biomass of algae was 0.10 g/L total dry weights in all containers. The initial culture conditions were as follows: pH=6.80, temperature= 5±1°C, white light with a light intensity of 3000 lux, and a photoperiod of 16:8 light: dark. Each container was continuously aerated.

Cell growth measurement

Algal cell density was assessed by measuring the optical density (OD_{750}) using a UV-Vis spectrophotometer. The result was converted to biomass concentration using a calibration curve relating OD_{750} to biomass concentration. The following formula was used for the conversion:

$$\text{Biomass (g/L)}=0.0703\times OD_{750}+0.0013$$

Biomass productivity (g/L/d) was obtained from the difference in biomass concentration divided by a specific time range (days) based on the formula (Tang *et al.*, 2011):

$$BP\ (g/L/d)=(X_1-X_0)/(T_1-T_0)$$

Where, X_1 and X_0 are the biomass concentration (g/L) on days T_1 and T_0, respectively.

Nitrate, Phosphate and Sodium bicarbonate measurement

Nitrate concentration was measured by the reduction column method using sulfanilamide and N-naphthyl reagents, reading the optical absorption of the colored complex using a spectrophotometer, and finally drawing a standard curve and finding the concentration from the curve. Phosphate concentration was measured with the use of molybdate (Mo) and ascorbic acid reagents, the formation of a colored complex and the reading by a spectrophotometer instrument, and finally drawing a standard curve, bicarbonate measurement was done by titration method using phenolphthalein indicator (APHA, 2017).

Total Chlorophyll (Chlorophyll a and Chlorophyll b) measurement

Fifty ml of the culture were filtered using a Blue Whatman filter (DP 150), and then the sample was weighed. 50 ml of 100% acetone was added to 1 g of the substance to extract the pigment. The sample was homogenized for 1 minute at 1000×g and passed through a Whatman Blue filter, then centrifuged at 2500×g for 10 minutes. The absorbance was measured at 662 and 645, nm. The following formulas were utilized to calculate the pigment concentrations:

$C_a = 11.75\ A_{662} - 2.350A_{645}$

$C_b = 18.61\ A_{645} - 3.960A_{662}$

Where, C_a is the amount of chlorophyll-a and C_b is the amount of chlorophyll-b in $\mu g.g^{-1}$ fresh weight (Lichtenthaler and Wellbum, 1983).

Experiment design and determination of different levels of nitrate, phosphate, and sodium bicarbonate

Statistical design of experiments

To evaluate the effect of four factors of temperature, initial concentration of nitrate, phosphate, and sodium bicarbonate on dry biomass productivity and total chlorophyll content, Response Surface Methodology (RSM) and CCD were used. The different levels of the factors investigated in this study have been selected using various references and studies and of course their achievements and also according to the goals of this research. To simplify the recording of test conditions and data processing, higher levels were indicated by +1, lower levels by -1, and the base level by 0 (Table 1).

Table 1: Independent variables and their values.

Factor	**Range**		
	-1	**0**	**1**
Temperature (°C)	15	22.5	30
Nitrate (mg/L)	225	1237.5	2250
Phosphate (mg/L)	14	78.5	143
Sodium bicarbonate (mg/L)	26	142.5	259

After preparing 30 treatments according to the RSM (CCD) model, the obtained data were analyzed. The presence or absence of a significant relationship between different data was determined by calculating the P-value (in the range of 0.05) and the confidence coefficient of 0.95.

Optimization of independent variables of temperature, initial concentration of nitrate, phosphate, and sodium bicarbonate was done using the RSM statistical model. In this study, the Design of Expert 6.07 software (Stat-Ease Inc., Minneapolis, MN, USA) was used for regression and statistical data analysis (Montgomery, 2001). The experimental data were obtained according to the polynomial model in the second dimension and its regression coefficient. The quadratic polynomial model used in the RSM (CCD) analysis is in the formula:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i.X_i + \sum_{i=1}^{k} \beta_{ii}.X_i^{\ 2} + \sum_{i_{i\le j}}^{k} \sum_{j}^{k} \beta_{ij}.X_i.X_j + e$$

i and j are linear and quadratic coefficients, respectively; β is the regression coefficient and k is the number of factors studied and optimized in the experiment and e is the random error.

Results

The results related to dry biomass productivity (DBP) and total chlorophyll content (CHL) in treatments defined by RSM method are shown in Table 2.

The highest amount of dry biomass productivity (132 mg/L/d) and total chlorophyll content (7.61 mg/L) was in T14, at temperature 30°C, nitrate 2250 mg/L, phosphate 14 mg/L and sodium bicarbonate 259 mg/L. The lowest value of dry biomass productivity (34 mg/L/d) corresponds to T4 (temperature 15°C, nitrate 225 mg/L, phosphate 143 mg/L and sodium bicarbonate 26 mg/L) and the lowest Total chlorophyll content (3.36 mg/L) was related to T18 (temperature 30°C, nitrate 225 mg/L, phosphate 14 mg/L and sodium bicarbonate 26 mg/L.

Table 2: Biomass productivity and chlorophyll content in treatments defined by RSM method.

Treatment	Temperature (°C)	Nitrate (mg/L)	Phosphate (mg/L)	Sodium bicarbonate (mg/L)	Dry biomass productivity (mg/L/d)	Total Chlorophyll (mg/L)
	(15-30)	(225-2250)	(14-143)	(26-259)		
1	22.5	1237.5	78.5	142.5	112	7.1
2	15	225	143	259	54	4.28
3	30	225	14	259	69	6.41
4	15	225	143	26	34	3.53
5	15	1237.5	78.5	142.5	67	4.73
6	15	225	14	259	66	3.81
7	30	225	143	259	54	3.73
8	15	225	14	26	48	3.8
9	15	2250	143	26	71	3.66
10	30	1237.5	78.5	142.5	57	5.4
11	30	225	143	26	64	4.01
12	22.5	1237.5	78.5	142.5	82	5.88
13	30	2250	14	26	44	3.55
14	30	2250	14	259	132	7.61
15	15	2250	14	259	92	3.48
16	22.5	1237.5	78.5	142.5	92	6.25
17	22.5	1237.5	78.5	259	85	6.08
18	30	225	14	26	48	3.36
19	22.5	1237.5	78.5	142.5	105	6.83
20	22.5	1237.5	14	142.5	97	6.05
21	22.5	1237.5	143	142.5	129	6.79
22	30	2250	143	26	49	3.69
23	15	2250	143	259	60	4.21
24	22.5	1237.5	78.5	142.5	86	6.08
25	22.5	225	78.5	142.5	56	3.83
26	22.5	1237.5	78.5	26	90	7.11
27	30	2250	143	259	53	3.88
28	22.5	1237.5	78.5	142.5	107	6.65
29	15	2250	14	26	93	4.15
30	22.5	2250	78.5	142.5	77	4.88

Optimization of C.vulgaris growth medium using surface methodology

The experiments were designed using the CCD model, and around 30 sets of experiments were designed to fit a second-order polynomial equation. The biomass productivity and chlorophyll were analyzed. The factor levels were minimum, central, and maximum, including Temperature (15-30°C), nitrate (225-2250 mg/L), phosphate (14-143 mg/L), and sodium bicarbonate (26-259 mg/L). These factors were used in the CCD for various responses, and their impacts on biomass productivity and chlorophyll of *C.vulgaris* are shown in Table 2. ANOVA was used to evaluate the fitness and adequacy of the predicted models, and the results are tabulated in Table 3. The test statistics for lack of fit is the ratio between the lack-of-fit mean square and the pure error mean square. The results suggest that the models were significant with F-values of 3.22 for biomass productivity and 4.74 for chlorophyll, and probability values of $p<0.0158$ for BP and $p<0.0025$ for CHL. The test of lack of fit showed non-significant results for biomass productivity (2.57) and chlorophyll (4.13), indicating that the models were able to fit the results of the experiment tolerably ($p>0.05$).

Table 3: Analysis of Variance (ANOVA) for biomass productivity and chlorophyll content.

Response Variable	Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value Prob>F
Biomass productivity	Model	14029.49	14	1002.11	3.22	0.0158
	Residual	4665.88	15	311.06	-	-
	Lack Of Fit	3906.55	10	390.65	2.57	0.1543
	Pure Error	759.33	5	151.87	-	-
	Cor Total	18695.37	29	-	-	-
Chlorophyll Content	Model	45.39	14	3.24	4.74	0.0025
	Residual	10.26	15	0.68	-	-
	Lack Of Fit	9.15	10	0.92	4.13	0.0654
	Pure Error	1.11	5	0.22	-	-
	Cor Total	55.65	29	-	-	-

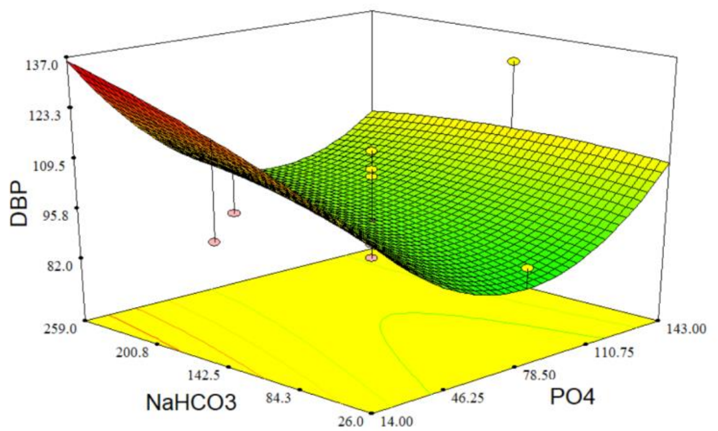
The regression analysis values for response variables R^2=0.75 and 0.81, and Adjusted R^2=0.51 and 0.64 (Measure the drop of magnitude of the estimate of the error variance) for BP and CH, respectively, showed reasonable agreement with the model's reliability. All R^2 values of the experimental data were close to 1.0, indicating the suitability of the model. The signal-to-noise ratio was measured with adequate precision. A signal-to-noise ratio greater than 2.34 is suitable for model validation. A ratio of 6.69 for the biomass productivity model and 6.54 for the chlorophyll model indicated an adequate signal, implying that the models can be used to design three-dimensional graphs. The models of the results based on the analysis of the regression model in the CCD are given in the following equations:

Biomass productivity =93.48-0. 83 temperature+9.89 nitrate-6.72 phosphate+6.89sodium bicarbonate –4.44 temperature × nitrate+0.44 temperature× phosphate+4.81 temperature×sodium bicarbonate – 6.44 nitrate× phosphate + 1,94 nitrate× sodium bicarbonate – 7.69 phosphate× sodium bicarbonate-27.63 temperature^2- 23.13 nitrate^2 + 23.37 phosphate ^{2}- 2.13 sodium bicarbonate^2

Chlorophyll = 6.30+0.33 temperature+0.13 nitrate–0.25 phosphate + 0.37 sodium bicarbonate + 0.071 temperature× nitrate – 0.38 temperature × phosphate +0.40 temperature×sodium bicarbonate – 0.095 nitrate× phosphate + 0.038 nitrate× sodium bicarbonate – 0.33 phosphate × sodium bicarbonate – 1.08 temperature^2 – 1.79 nitrate^2 + 0.28 phosphate^2 + 0.45 sodium bicarbonate^2.

Three-dimensional (3D) plots were used to explore the sensitivity of the responses of two interacting variables by holding the other variables constant at central values. The biomass productivity and chlorophyll content from *C.vulgaris* were predicted to be 119.37 mg/L/d and 7.61 mg/L, respectively, under optimized conditions; these conditions included a temperature of 25°C, nitrate concentration of 716.39 mg/L, phosphate concentration of 14 mg/L, and sodium bicarbonate concentration of 259 mg/L, which were represented in three-dimensional plots. The three-dimensional figures depicting the mutual effects of the effective factors with a higher coefficient on the productivity of biomass and total chlorophyll are shown in Figures 1 and 2.

A



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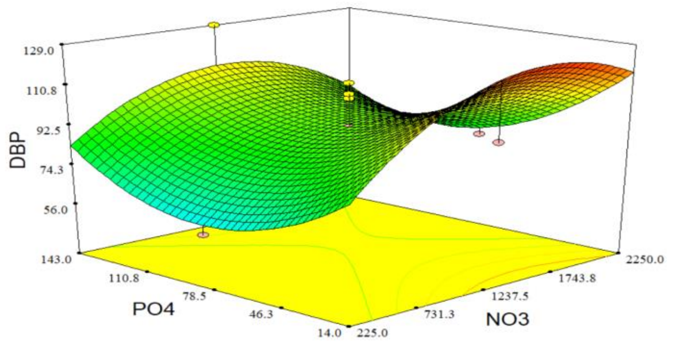

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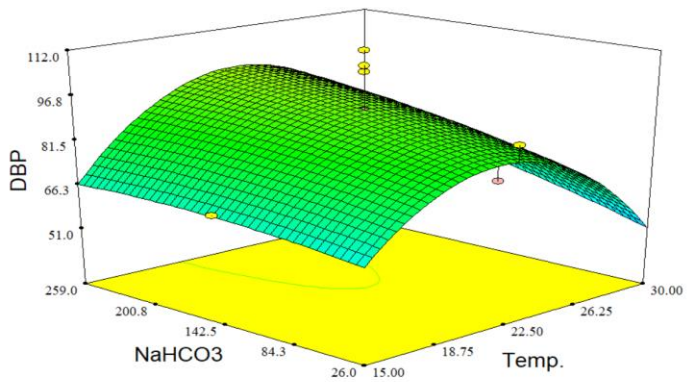

Figure 1: Three dimension plots of response surface method for dry biomass productivity of *C. Vulgaris.*

A

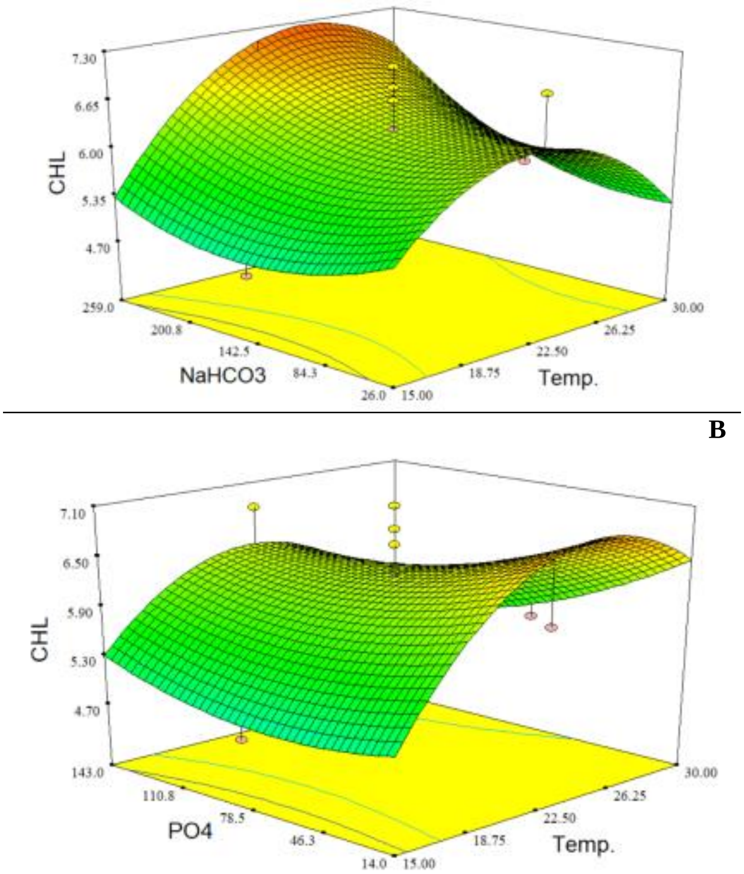

Figure 2: Three dimension plots of response surface method for total chlorophyll of *C. Vulgaris.*

The regression coefficients were found to be significant. The coefficients of the equations indicate that the effect of initial nitrate concentration (+9.89) and sodium bicarbonate concentration (+6.89) on dry biomass productivity was greater compared to the other tested factors. Additionally, the effect of sodium bicarbonate concentration (0.37) and temperature (0.33) on the total chlorophyll content was also higher compared to the other factors. Furthermore, the equation shows that phosphate had an inverse effect on dry biomass productivity (-6.72) and total chlorophyll content (-0.25), while temperature, sodium bicarbonate, and nitrate had a direct effect.

Optimization

The issue of linking between the medium must be addressed, as any increase in the CHL and DBP. On balance, and based on the above discussion, it is better to run an optimization technique to find out the optimal medium condition at which the desirable mechanical properties of the high CHL and DBP joint can be achieved. In fact, once the models have been developed and checked for adequacy, the optimization criteria can be set to find out the optimum medium conditions. In this investigation, one criterion was implemented to maximize both CHL and DBP content. The criterion is to reach maximum CHL and DBP with limitations on either the process parameters or the operating low cost. However, Table 4 summarizes this criterion.

The desirability, which was established from optimum points through numerical optimization, is presented in Figure 3.

Table 4: Optimization criterion used in this study.

Parameter or response	Limit		Importance	Criterion
	Lower	Higher		
Temp.	15	30	3	is equal to 25.00
NO_3	225	2250	3	minimize
PO_4	14	143	3	minimize
$NaHCO_3$	26	259	3	is in range
DBP	34	132	3	maximize
CHL	3.36	7.61	3	maximize

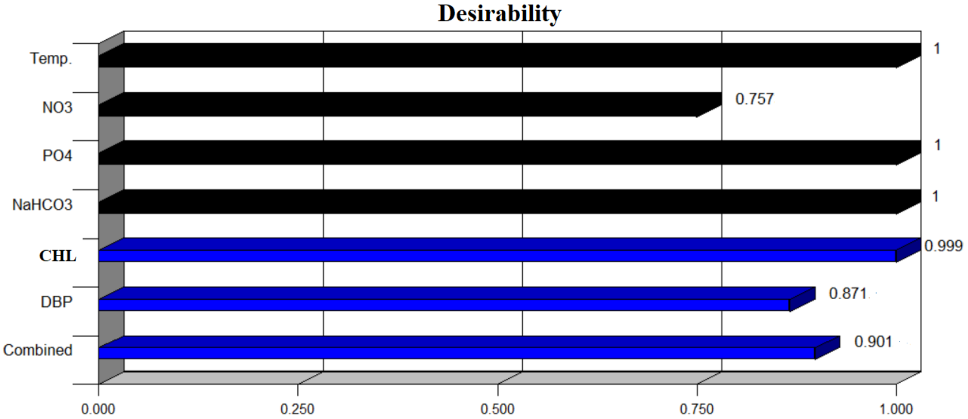

Figure 3: Bar graph of optimization criterion desirability of RSM model.

The optimum points of the model were confirmed by at least three interactions of the experiments. Finally, more than 90% desirability was selected which gives optimized CHL and DBP conditions. The result of the graphical optimization is the overlay plot, which is extremely practical for quick technical use in the workshop to choose the values of the medium parameters that would achieve a certain response value for this type of material. The grey/shaded areas on the overlay plot Figure 4 is the region of optimal CHL and DBP conditions.

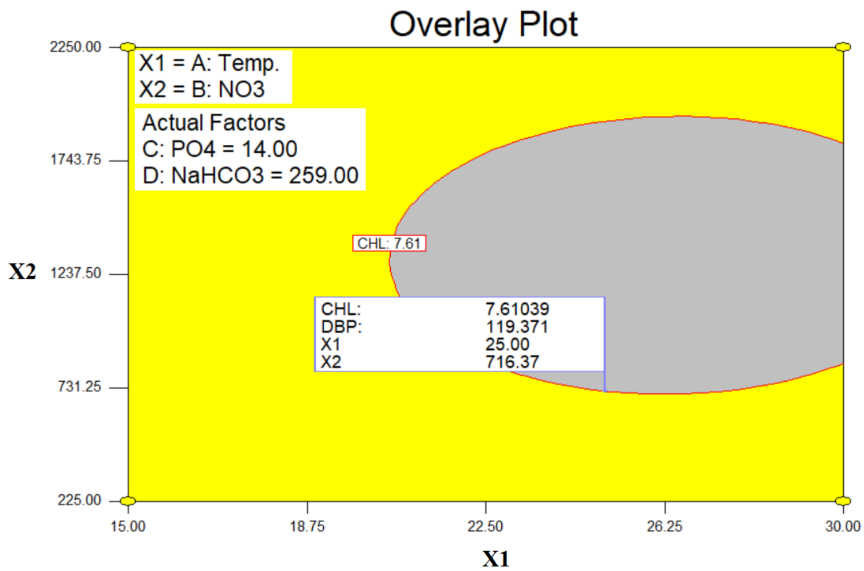

Figure 4: Overlay plot shows the region of optimal CHL and DBP condition.

To validate the developed models, three confirmation experiments were carried out with medium conditions chosen randomly from the optimization results. For the actual responses, the average of three measured results was calculated. The experimental conditions, the average of actual experimental values, the predicted values and the percentages of error are summarized in Table 5. The validation results demonstrated that the models developed are quite accurate.

Table 5: Validation test results.

Experiment No.		CHL	DBP
1	Actual	7.0	111
	Predicted	7.61	119.37
	Error%	8.01%	7.01%
2	Actual	6.90	110
	Predicted	7.61	119.37
	Error%	9.32%	7.84%
3	Actual	6.89	108
	Predicted	7.61	119.37
	Error%	9.46%	9.52%

Discussion

The growth of microalgae is influenced by several abiotic factors, such as nutrients and environmental parameters. Therefore, if optimized in the medium, these parameters can significantly enhance biomass production. Cultivation conditions can maximize algal biomass production rates. In this study, the factors of temperature, nitrate, phosphate, and sodium bicarbonate have been tested and optimized by RSM method, and the significant effect of each of them and their interaction on biomass productivity (BP) and chlorophyll (CHL) have been investigated.

Temperature, as a parameter affecting algal metabolism, definitely governs the biological activity of all metabolic functions taking place inside the cells (Zhao *et al.,* 2020). At higher temperatures, the cells consume the substrate more easily and at a higher rate, and also the activity of enzymes related to the metabolic pathways of nitrogen and phosphorus was improved for the generation of cellular biomass metabolites, leading to enhanced biomass production (Parichehreh *et al.,* 2021). The effect of temperature and other nutrient interactions, especially sodium bicarbonate, was more significant than the effect of temperature itself in this study. The interaction of temperature and sodium bicarbonate had a significant positive effect on both BP and CHL.

Considering the statistical analysis, increasing nitrate concentration in the medium had shown a positive effect on biomass productivity (+9.89). Nitrate is reduced to fulfill nitrogen demands in plants and forms the building block of macromolecules like protein, chlorophyll, RNA, DNA, etc. (Arumugam *et al.,* 2013). The compositional contribution of nitrate in the growth medium is significantly influential. Earlier studies have suggested that dynamics in nitrogen concentration in culture media often result in an increase or decrease in the yield of algal biomass (Lai *et al.,* 2011; Dhup and Dhwan, 2014). Nitrogen is a building block for several metabolites synthesized in plant biomass. Nutrient proliferation leads to catalyzing a series of physiological processes in plants, which in turn accelerates biomass synthesis (Lai *et al.,* 2011). Under nitrogen limitation, algal cells utilize N to synthesize functional proteins (Sakarika and Kornaros, 2016). However, this approach usually comprises a reduction in the maximum biomass concentration (Shrestha *et al.,* 2020). Lai *et al*. (2011) in their study found that growth rates in algae were significantly correlated with N concentration, indicating a strong relationship between algae growth and N concentrations.

Increasing the nutrients available in the medium has shown positive effects on the growth of microalgae; however, if the nutrient concentration reaches a certain value, it can cause inhibitory effects due to nutrient overload. This was also possible to observe in the present study since some of the constituents of the medium (phosphate) had a negative effect on biomass productivity (-6.72) and total chlorophyll (-0.25). The phosphate uptake by algae is a process determined by both environmental conditions and cell state with complex reactions and feedback. Although phosphate is an important metabolite for algal growth, a concentration above the threshold limit results in diminishing algal growth (Chromar and Fallowfield, 1997). For a high amount of phosphate, we observed a decrease in biomass productivity and chlorophyll. High concentrations of P might inhibit growth and hinder the uptake of C and N sources, inflicting severe morphological damage on microalgae cells. The results suggest that high doses of phosphate cause an adverse impact on the metabolic processes of the algae, such as chlorophyll content. A study carried out by Li *et al.* (2018) evaluated the effect of an excessive amount of P on the heterotrophic growth of *Chlorella vulgaris*. Of course, the phosphate concentration in the growth medium directly affects the growth and biomass yield in *C. vulgaris*. The results indicated that algae showed better growth among 10-75 mg/L of phosphate in the media. In the present study, decreasing the phosphate concentration, seemed to enhance biomass productivity. This might be due to phosphorus starvation that can reduce the synthesis and regeneracy of the substrates in the Calvin cycle and also lessen the rate of light used to fix carbon, resulting in a decrease in biomass density and an increase in biomass productivity due to shortening growth time (Parichehreh *et al.*, 2021).

Carbon as one of the most essential components of algal biomass plays a critical role in cellular metabolites biosynthesis and energy generation of microalgae (Kim and Hur, 2013). The results presented in this study revealed that a higher level of dissolved inorganic carbon in the culture media in the form of bicarbonate ($HCO3^-$) was beneficial for the growth of *Chlorella vulgaris* (+6.89). This might have occurred because CO_2 as bicarbonate was more bioavailable than gaseous CO_2 (Hsueh *et al.,* 2007). It was also found that a higher amount of biomass density was achieved when 259 mg/L sodium bicarbonate was supplied in the original medium. The high bicarbonate levels affect several critical enzymes in carbon metabolism, such as carbonic anhydrase and ribulose 1, 5-bisphosphate carboxylase-oxygenase (Rubisco). Therefore, the addition of sodium bicarbonate to the culture medium can stimulate carbonic extracellular anhydrase to assimilate the inorganic carbon (Kamyab *et al.,* 2019). On the other hand, the use of a higher bicarbonate concentration can typically promote the carboxylase activity and suppress the oxygenase activity of Rubisco and increase the photosynthesis rate, hence enhancing biomass production (Gerotto *et al.,* 2020).

In the present study, a significant positive effect on total chlorophyll content (+0.37) with a higher level of sodium bicarbonate, when compared to a lower level, was observed. It was clear that higher photosynthetic efficiency due to an increase in chlorophyll content indicates sodium bicarbonate would promote the growth rate as well as biomass production (Ryu *et al.,* 2009). Furthermore, gaseous CO_2 is costly to store and transport, whereas bicarbonate salts can easily be transported to algal facilities and stored until needed (Gardner *et al.,* 2012). Enhanced inorganic carbon uptake due to the addition of bicarbonate allows cellular material production and thereby achieves maximum productivity (Chi *et al.*, 2013). In this study, a maximum BP of 132 mg/L/d was observed in 259 mg/L (the highest level) sodium bicarbonate addition, and this is in agreement with Yeh *et al.* (2010) who have demonstrated that a high concentration of $NaHCO_3$ was found to be optimum for biomass production in *C.vulgaris*. Bicarbonate supplementation with an optimum concentration of 0.6 g/L in the cultures of *Scenedesmus* sp. resulted in a 23% increase in biomass production (Pancha *et al.,* 2015). So, the supply of bicarbonate to microalgal cultures not only enhances biomass production but also prevents bacterial contamination in outdoor cultivation when the bicarbonate concentration is very high (Tu *et al.,* 2018).

Among all interaction terms studied, the interaction between phosphate and sodium bicarbonate, nitrate and phosphate, and temperature and sodium bicarbonate were found to be the most prominent terms affecting biomass productivity, whereas the other interactions between the independent factors had no noticeable impacts on microalgae growth. The interaction of phosphate and bicarbonate in this study showed that the decrease in phosphate concentration and the increase in sodium bicarbonate concentration, simultaneously, will have a significant effect on biomass production in Chlorella algae per day. The change in biomass productivity is due to the change in the N:P ratio, which is more important than the change in nutrient concentration (Stockenreiter *et al.,* 2016). The increase in biomass productivity at a higher N:P ratio is due to more availability of nitrogen. Other studies such as Vazirzadeh and Moghaddaszadeh (2018), Ward and Rehmann (2019), Parichehreh *et al.* (2021), and Kanaga *et al.* (2022), have also used RSM to optimize some key parameters by the consuming culture medium and also make economic the cultivation of *C.vulgaris* in semi-industrial and industrial scales.

Conclusions

The results of this study can be considered to achieve the optimum BP and CHL of *C.vulgaris* microalgae by using an economic media on a semi-industrial scale. A popular and established technique for simultaneous determination of optimum settings of input variables that can determine optimum performance levels for one or more responses. The experimental findings were in close agreement with the model prediction. Our promising results have shown that *C. vulgaris* has tremendous potential for producing biomass and more cost-effective byproducts, although its commercialization is still challenging.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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