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ORIGINAL ARTICLE

Optimization of biomass production by *Chlorella saccharophila* UTEX 247 employing response surface methodology

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Abstract

Improved productivities of microalgal biomass tend to play a significant role in biorefineries pertaining to multifaceted applications and the inadequate biomass yield in any particular medium is a bottleneck that must be overcome to achieve such sustainability goals. In our present study, we employed new approach to enhance the cell growth of a potential strain *Chlorella saccharophila* (UTEX 247), i.e., media engineering perspective. For better biomass yields, the fundamental constituents are the macronutrients within the growth medium consisting of nitrogen (as $NaNO_3$, sodium nitrate), phosphorus (as K_2HPO_4, dipotassium phosphate) with an additional source of carbon supplementation in the form of $NaHCO_3$, sodium bicarbonate. Our preliminary studies by One Factor at a Time demonstrated no effect on growth with additional carbon supplementation but showed that nitrogen and phosphorus ratios play a significant role in the biomass production. Furthermore, we optimized the biomass yields employing the central composite design associated with the response surface methodology tool to illustrate the combinatorial effects of nitrogen (N) and phosphorous (P). Our results have showed an increase up to 131% dcw in biomass production, i.e., 0.84 g L^{-1} DCW with 26.4 mM and 0.11 mM of $NaNO_3$ and K_2HPO_4 concentrations, respectively, than the control condition ($NaNO_3$: 17.6 mM; K_2HPO_4: 0.23 mM) yielding a biomass content of 0.64 g L^{-1} DCW with a coefficient of variance of 5.12%. In conclusion, the new perspective of media engineering predicts and also evaluates the best condition for the specific strain of interest so that the optimized medium essentially produces higher cell biomass along with other biocommodities of industrial significance.

Keywords Microalgae · Biomass · Macronutrients · Response surface methodology · *Chlorella saccharophila*

Abbreviations

RSM	Response surface methodology
CCD	Central composite design
N	Nitrogen
P	Phosphorus
NaNO3	Sodium nitrate
KH2PO4	Phosphate
NaHCO3	Bicarbonate
CV	Coefficient of variance
EDTA	Fe-Ethylenediaminetetraacetic acid
O.D.	Optical density
SPV	Sulpho-phospho-vanillin
PAM	Pulse amplitude modulation
ANOVA	Analysis of variance
VP	Validation point

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1 Introduction

On a global scale, fossil fuel use has significantly increased demand for renewable fuels due to environmental threats [1–3], such as global warming and melting glaciers [4–6], whereas the development of the first and second generations has been impeded by competition with food production and rising production costs [7–9]. Alternatively, microalgae have

been extensively investigated as a potential feedstock for third-generation biofuels due to their faster growth rates, use of non-arable land resources, carbon sequestration of flue gases, and treatment of wastewater effluents [10]. In recent times, algal biomass has got significant appreciation in the applied research especially for their advancements in the areas of pharmaceuticals, nutraceuticals, and biofuels, and also in the wastewater treatments [11, 12]. Even though the cost of microalgal cultivation seems to be still high, their economic viability and competitiveness in the market can be taken into consideration only with few specialized products [13, 14]. Henceforth, new perspectives are required to enhance the biomass yields in a cost-effective manner using different strategies which will make the overall process sustainable. In the present study, a potential strain of microalgae with industrial relevance namely *Chlorella saccharophila* UTEX 247 having better growth rates with remarkable biocommodities such as lipids, proteins, and other high-value added bioproducts [15–23] has been further optimized for higher biomass yields. A major hurdle in algal biorefineries is production of cell biomass in an optimized medium that can be efficiently scaled up in the industrial photobioreactors.

In such context, our new approach in this study was employing media engineering perspective via response surface methodology (RSM), which is an effective, efficient, statistical tool involved in modeling, analyzing, and predicting experimental datasets wherein the response of interest will be assessed by several factors involved in maximizing their productivities [24]. Henceforth in this study, we have employed a statistical-based experimental design to unveil the interactions between multiple factors simultaneously, thus providing information on their cumulative effects. Furthermore, the number of experiments used in such approaches seems to be reasonable without jeopardizing the accuracy following systematic studies. Thus, the RSM tool seems to be an effective methodology for optimizing numerous variable combinations at the same time for maximizing the output [25–29]. Previously, studies have shown the relevance of using multivariate dose method [30] to optimize the algal growth rates [31–34]. For example, Cheng et al. [35], Mubarak et al. [36], and many others demonstrated the use of the RSM tool for optimization of medium components such as sodium nitrate, phosphate, ethylenediaminetetraacetic acid, $CaCl_2$, and KNO_3 in *Chlorella* sp. for improving biomass yields.

In the present study, the optimization tool such as response surface methodology (RSM) has been employed for enhancing the cell growth of the *C. saccharophila* UTEX 247. The role of essential macronutrients (such as "N" for nitrate, $NaNO_3$; "P" for phosphate, K_2HPO_4; and "C" as bicarbonate, $NaHCO_3$) was studied by varying their concentrations in the medium in context with improving the biomass productivities. Further, the evaluation of growth under optimized conditions has been illustrated via the chlorophyll fluorescence measurements, which demonstrates the enhanced photosynthetic performance in these microalgal cell factories. Overall, our study focused on the deployment of significant macronutrients, either alone or in combination, to obtain optimized biomass yields with other biomolecules such as lipids and carotenoids.

2 Materials and methods

2.1 Strain, culture conditions, and biomass estimation

A freshwater *C. saccharophila* UTEX 247 was procured from The University of Texas at Austin (UTEX) Culture Collection of Algae, Austin, TX, USA, and the strain was cultured in the minimal BG-11 medium in 250-mL Erlenmeyer flasks [37, 38]. The culture was maintained under a light regime of 16:8 h and an illumination of 150 µmol photons m^{-2} s^{-1} photosynthetically active radiation at 24 °C ± 2 °C. The mid-logarithmic phase cells were centrifuged and resuspended in fresh medium with initial concentration of 50 ± 5 mg L^{-1} biomass, and its growth was measured at an interval of every 3 days up to 21 days.

Growth and biomass were evaluated by cell counting method using a hemocytometer [39] and correlated with the optical density (O.D.) measured by a SpectraMax M Series Multimode Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA) at 750 nm [40] using the regression equation:

$$y = 0.0913x + 0.0692 \tag{1}$$

where y corresponds to the cell number and x corresponds to the O.D.

Then the growth rate was determined using the equation below:

$$K = \frac{ln\left(\frac{N_2}{N_1}\right)}{t_2 - t_1} \tag{2}$$

where N_1 and N_2 are the cell count/O.D. at initial (t_1) and final time (t_2), respectively, and similarly doubling time can be estimated using the Eq. 3.

$$\text{Doubling time} = \frac{ln2}{K} \tag{3}$$

2.2 Identification of factors influencing algal biomass

Primarily, the following essential macronutrients namely nitrate ($NaNO_3$) and phosphate (K_2HPO_4) along with addition carbon source as bicarbonate ($NaHCO_3$) were

independently evaluated to predict their effect on the biomass yields. Initially, these three abovementioned variables with different concentrations were carried out employing the strategy of the One Factor at a Time (OFAT) with following experimental setup: $NaNO_3$ ranges between 4.4 (0.25 N), 8.8 (0.5 N), 26.4 (1.5 N), and 35.4 (2 N) mM, respectively, where 17.6 mM (as control [C]); K_2HPO_4 ranges between 0.05 (0.25P), 0.11 (0.5P), 0.34 (1.5P), and 0.46 (2P) mM, respectively, where 0.23 mM (as control [C]); whereas additional carbon was included as follows in the form of $NaHCO_3$: 3.5 (0.5B), 7.0 (B), and 10.5 (1.5B) mM, respectively, where 0 mM (no bicarbonate as control [C]) in BG-11 medium. Summarized tabulation of the OFAT experimental setup with all three variables is clearly illustrated in Table 1. All the OFAT experiments were carried out independently in biological triplicates with all three variables for 21 days to evaluate their effect on the biomass yields in *C. saccharophila*.

2.3 Response surface methodology

An efficient, user-defined decision-making statistical tool known as RSM was employed in this study for optimizing biomass yields following three steps [41]. In the earlier step, essential medium components namely two macronutrients (nitrogen [$NaNO_3$], phosphorous [K_2HPO_4]) and bicarbonate ($NaHCO_3$) supplementation (additional carbon source) were evaluated with three important variables to investigate their influence on the cell's biomass [42]. In the RSM, the specific concentrations of variables which have shown positive effect on enhancement of algal biomass were selected to find the optima using central composite design (CCD) with their corresponding equation. Additionally, a three-dimensional surface plot was reconstructed to assess the interactions of different variables especially nitrate ($NaNO_3$) and phosphate (K_2HPO_4) with reference to their impact on growth. The final step was employed to validate the deduced model using the equation with varying concentrations, thus further confirming the responses between the predicted ($Y_{Pred.}$) and experimental ($Y_{Exp.}$) conditions.

Table 1 Tabulation of OFAT experimental setup with varying concentrations of three different macronutrients considered as the essential factors of growth were evaluated in *C. saccharophila* UTEX 247. Bold represents the varying concentrations of each variable. "C" represents control with $NaNO_3$: 17.6 mM.[1]; K_2HPO_4: 0.23 mM; $NaHCO_3$: 0 mM; and "B" represents the concentration of $NaHCO_3$: 7 mM

S. no	Concentration	$NaNO_3$	K_2HPO_4	$NaHCO_3$
		mM		
1	2 N	35.20	0.23	0.00
2	1.5 N	26.40	0.23	0.00
3	C (control)	17.60	0.23	0.00
4	0.5 N	8.80	0.23	0.00
5	0.25 N	4.40	0.23	0.00
6	2P	17.60	0.46	0.00
7	1.5P	17.60	0.34	0.00
8	C (control)	17.60	0.23	0.00
9	0.5P	17.60	0.11	0.00
10	0.25P	17.60	0.05	0.00
11	1.5B	17.60	0.23	10.50
12	B	17.60	0.23	7.00
13	0.5B	17.60	0.23	3.50
14	C (control)	17.60	0.23	0.00

2.3.1 Step 1: development of model equation using central composite design

The CCD, a second-order experimental setup, was performed for the optimization process [42–44], where the two-level factorials, both the axial and central points, were included in the design to unveil the occurrence and to estimate terms involved in the second-order fitted model equation. The minimum and maximum concentrations were considered at a distance of − 1 and + 1 units, respectively, and the central point of the minimum and maximum concentrations was automatically denoted by the model, which are summarized in Table 2.

A major advantage of setting the experiments with CCD was inclusion of the outliers for each factor at a distance α, thus avoiding any possible error with five replicates at the center point. In this study, the Design-Expert® software, version 13, Stat-Ease, Inc., Minneapolis, MN, USA (www.statease.com), was used to predict the model employing the one-way analysis of variance (ANOVA) analysis. In addition, the model generated the response surface 3D plot with the contour lines, depicting the correlation between the factors and response [45]. The experimental datasets achieve the equation as follows:

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i A_i + \sum_{i=1}^{n} \beta_{ii} A_i^2 + \sum_{i<j}^{n} \beta_{ij} A_i A_j \quad (4)$$

where y is the response; β_0 is the intercept; β_i, β_{ii}, and β_{ij} are the regression coefficients of different variables in linear and

Table 2 Coded levels and their concentrations of medium components for both variables, i.e., nitrogen (N) and phosphorus (P) as denoted by the central composite design (CCD)

Parameters	Label	Coded levels and concentrations		
		− 1	0	+ 1
Nitrogen (mM)	$NaNO_3$	8.80	17.60	26.4
Phosphorous (mM)	K_2HPO_4	0.11	0.17	0.23

quadratic equations; and A_i and A_j are the coded independent variables.

2.3.2 Step 2: validation of the model

The predicted model has been validated with three different concentrations of both factors designated with validation points as VP_1, VP_2, and VP_3 to determine the biomass yields. Overall, the $Y_{Pred.}$ and $Y_{Exp.}$ datasets were compared to analyze the model's accuracy as stated by Eq. 4.

2.4 Quantification of different biocommodities and Chl a fluorescence measurement

2.4.1 Estimation of total pigments using spectrometry

To quantify other biocommodities such as total pigments and lipids obtained in the RSM optimized biomass, the following modified protocols were employed [46]. For the estimation of total pigments, 1 mL of cells was centrifuged, and the pellet was resuspended in 1 mL of methanol. After vortexing, it was incubated at 55 °C for an hour. Later, the absorbance of the supernatant was measured at specific wavelengths of 665, 652, and 470 nm in the SpectraMax M Series Multimode Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA), and the contents of chlorophyll a, chlorophyll b, and total carotenoids [35] were determined using the following equations:

$$\text{Chla} = 16.72A_{665} - 9.16A_{652} \tag{5}$$

$$\text{Chlb} = 34.09A_{652} - 15.28A_{665} \tag{6}$$

$$\text{TotalCarotenoids} = \frac{1000A_{470} - 1.63 * Chla - 104.96 * Chlb}{221} \tag{7}$$

2.4.2 Quantification of total lipids by sulpho-phospho-vanillin assay

The total lipids were estimated by the sulpho-phospho-vanillin method, wherein 2 mL of cells was pelleted, followed by addition of 2 mL of concentrated H_2SO_4 (98%) and incubated at 100 °C for 10 min. After cooling the reaction, 5 mL of freshly prepared phospho-vanillin reagent has been added and incubated at 37 °C for 15 min with continuous shaking at 200 rpm. The absorbance was measured at 530 nm in the SpectraMax M Series Multimode Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA) and the quantification was done using canola oil (MilliporeSigma, USA) as the standard [47].

2.4.3 Chl a fluorescence measurement as photosynthetic efficiency

Non-invasive fluorescence measurements were acquired by using dual-pulse amplitude modulation (PAM) 100 chlorophyll fluorometer (Heinz Walz Gmbh, Effeltrich, Germany) to measure the photosynthetic efficiency of photosystem II (PSII) [48]. Each sample corresponding to at least 20 µg of chlorophyll was incubated in dark for 15 min at 25 °C. For the optimal measurements, the sample was transferred into a quartz glass cuvette (10 × 10 × 40 mm) with a magnetic bead, followed by placing the cuvette into the PAM fluorometer to obtain the induction curve. For minimum fluorescence (F_o) measurement, a measuring light was applied (< 0.1 µmol photons m^{-2} s^{-1}) and for maximum fluorescence measurement (F_m), a saturation pulse light was applied (6000 µmol photons m^{-2} s^{-1}) for 0.8 s in every 10 s). The maximum photosynthesis efficiency of PSII (F_v/F_m) was calculated based on the equation $F_v/F_m = (F_m - F_o)/F_m$, where F_v is the variable fluorescence that elucidates the difference between F_m and F_o [48–50].

2.5 Software and statistical analysis

The mean and standard deviations for all three independent biological triplicates ($n = 3$) were calculated by the ANOVA along with the statistical analysis ($p < 0.05$). The CCD design was performed using the Design-Expert® software, version 13, Stat-Ease, Inc., Minneapolis, MN, USA (www.statease.com). The goodness of fit of these designs was assessed statistically by applying ANOVA to identify the statistically significant terms. The significance of regression coefficients was determined with a confidence level of 95%. Further, the probability plots were drawn between the studentized residual and percent probability of response to confirm data homogeneity.

3 Results

3.1 Optimization of biomass yields using OFAT experiments

The growth profile of *C. saccharophila* UTEX 247 in the minimal BG-11 medium with following macronutrients (17.6 mM $NaNO_3$, 0.23 mM K_2HPO_4, 0 mM $NaHCO_3$—defined as the control [C] in these experiments) demonstrates biomass yields of 640.0 ± 25.0 mg L^{-1} with a specific growth rate (µ) of 0.57 ± 0.02 day^{-1} and doubling time 29.0 ± 2.0 h. The time-course experiments were done at regular intervals as follows: 0, 3, 6, and 9 days. As described earlier in Section 2, Table 1 summarizes the key essential factors with varying concentrations the

components nitrate ($NaNO_3$), phosphate (K_2HPO_4), and bicarbonate ($NaHCO_3$), which were evaluated for their effects on biomass yields individually using the OFAT experimental setup in microalgae *C. saccharophila* (Fig. 1).

Our preliminary data analysis demonstrated that the macronutrients, i.e., nitrogen (as $NaNO_3$, sodium nitrate) and phosphorus (as K_2HPO_4, dipotassium phosphate), showed significant effect on their biomass yields (Fig. 1a, b). The results on the 9th day showed a significant increase in biomass content, i.e., 690 mg L^{-1} with a specific growth rate (μ) of 0.6 ± 0.02 day^{-1} in slightly higher concentration (26.4 mM [1.5 N]) of $NaNO_3$ than the control. In the case of phosphorus (P), the biomass yields ranged between 600 and 680 mg L^{-1} using different K_2HPO_4 concentrations of 0.05–0.46 mM, respectively (Fig. 1a). Also, we have observed a significant enhancement in biomass content, i.e., 680 mg L^{-1} at lower K_2HPO_4 (0.11 mM [0.5 P]) concentration, and the lowest biomass, i.e., 600 mg L^{-1} under the highest K_2HPO_4 (0.46 mM [2.0 P]) concentration on the 9th day (Fig. 1b). Our study demonstrates that higher K_2HPO_4 concentration showed negative impact on biomass yields, whereas the additional carbon supplementation ($NaHCO_3$; 3.5, 7.0, and 10.5 mM) illustrated no impact on their biomass yields in the *C. saccharophila* (Fig. 1c). Henceforth, the $NaHCO_3$ was not included as the essential factor in the further experimentation. In summary, our preliminary study demonstrates that $NaNO_3$ and K_2HPO_4 showed significant effect in enhancing the biomass yields at the concentration of 1.5 N and $NaNO_3$ (26.4 mM) and 0.5 P and K_2HPO_4 (0.11 mM), respectively.

3.2 Response surface methodology

3.2.1 Model designing with two variables for biomass enhancement

Based on the results obtained in the initial step, two variables with different concentrations were further selected in this study as illustrated in Table 2 (Section 2). The reconstruction of the model using response surface methodology was done by employing the CCD module. Also, we performed experiments for demonstrating the interactions between the two essential macronutrients and their impact on the biomass yields with the best-suited model within the selected range of factors. All the combinations used in the CCD model are shown in Table 3, including the 5 replicates around the center point to avoid any possible errors which may occur due to certain artifacts.

Our experimental data analysis of biomass using the parameters predicted by the model ranges between 630.0 and 840.0 mg L^{-1} (Table 3). The range predicted indicates that these two nutrients significantly impacted their growth profiles in *C. saccharophila*. Moreover, the second-order fitted model derived a quadratic equation perfectly suited for the experimentation. In addition, the model also demonstrates non-significant lack of fit (R^2 of 0.87 and adjusted R^2 0.78), which describes the fitness of the data predicted by the model along with the analysis of variance (ANOVA) to evaluate the model's significance [51]. Statistical significance for the response surface quadratic model is given in Table 4. *F*-value (9.09) of the model implies that the model is significant; only 0.57% chance is there that an *F*-value this

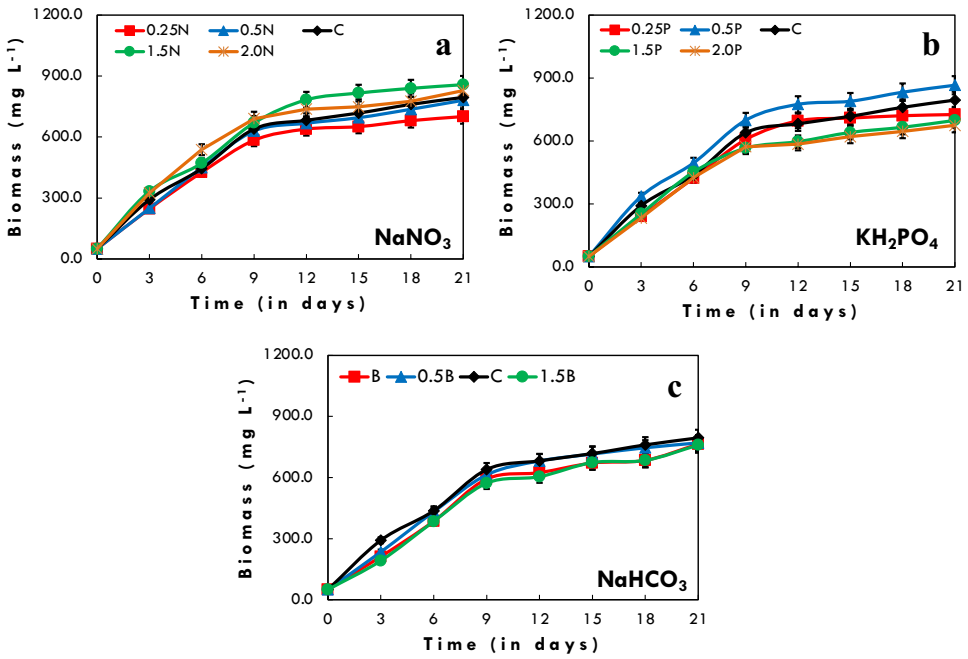

Fig. 1 Growth profiles depicted using three independent variables (**a** $NaNO_3$; **b** K_2HPO_4; **c** $NaHCO_3$) in microalgae *C. saccharophila*. All the samples are represented as the average of three biological replicates ± S.D

Table 3 Summarization of values assigned in the central composite design (CCD) experimental set-up involving two variables with one response (i.e., biomass yield) in the *C. saccharophila* (day 9). All the samples are represented as the average of three biological replicates ± S.D

S. no	Variables		Response (biomass yield)	
	$NaNO_3$ (mM)	K_2HPO_4 (mM)	Y_{Exp} (mg L^{-1})	Y_{Pred} (mg L^{-1})
1	8.80	0.11	680.13 ± 25.2	694.36
2	30.03	0.17	810.15 ± 31.2	801.73
3	17.60	0.17	660.45 ± 24.4	660.00
4	17.60	0.17	660.34 ± 21.6	660.01
5	26.40	0.11	840.72 ± 24.8	832.46
6	8.80	0.23	655.58 ± 25.6	685.04
7	17.60	0.17	660.24 ± 22.8	660.01
8	17.60	0.25	610.65 ± 27.2	577.48
9	17..6	0.17	660.54 ± 28.0	660.01
10	5.04	0.17	720.46 ± 20.8	740.77
11	17.06	0.17	660.72 ± 19.2	660.01
12	17.60	0.09	720.93 ± 21.6	725.02
13	26.40	0.23	600.45 ± 28.5	633.14

large occurs due to noise. *p* values of less than 0.05 indicates that the model terms are significant. In this case, B, AB, and A^2 are significant model terms that affect biomass production. On other hand, values greater than 0.10 indicate that the model terms have no direct significance. Adeq Precision measures the signal-to-noise ratio and a ratio greater than 4 is desirable. Obtained ratio of 10.597 shown in Table 5 indicates an adequate signal so as the model can be used to navigate the design space. All the findings in this study are illustrated in Tables 4 and 5, where the p value < 0.05 indicates the significance of model. For example, K_2HPO_4 (B; $p = 0.004$) and $NaNO_3 \times K_2HPO_4$ (AB; $p = 0.03$) are more significant in terms of the biomass determinants. The quadratic equation has a positive magnitude of A and a negative magnitude of B, indicating their correlation for biomass with the increasing concentration of $NaNO_3$ and the decreasing concentration of the K_2HPO_4. Furthermore, other quadratic terms such as AB and B^2 have a negative magnitude, whereas A^2 has a positive magnitude, which clearly states that the higher concentrations of $NaNO_3$ enhance the growth, i.e., increasing the overall biomass yields, whereas the negative magnitude of term B^2 indicates that it has a negative impact on biomass when it is in very high concentration and the p value indicates that this model term has no direct significance but when it comes to AB and B with p values 0.03 and 0.004, respectively, it is much more significant.

The correlation between the predicted and actual values is illustrated in Fig. 2a with an excellent coefficient of variation (CV) of 5.12. In addition, the 3D surface plot shown in Fig. 2b demonstrates the contour lines with appropriate optimization values and their effect on the biomass yields. It clearly depicted that the higher $NaNO_3$ or lower K_2HPO_4 contributes to better biomass content and any change in their concentration will impact the overall biomass in *C. saccharophila*. With reference to these results, we showed that the highest biomass content obtained was 840 mg L^{-1} with two

Table 4 Analysis of variance (ANOVA) for the regression model for the suggested model

Response	Model term	Coefficient estimate (as a coded factor)	Df	Standard error	Mean square	*F*-value	*p* value
Biomass	Intercept	660.00	1	15.84	11,397.67	9.09	0.0057*
	A ($NaNO_3$)	21.55	1	12.52	3716.36	2.96	0.1289
	B (K_2HPO_4)	−52.16	1	12.52	21,765.24	17.35	0.0042*
	AB	−47.50	1	17.71	9025.00	7.19	0.0314*
	A^2	55.62	1	13.43	21,524.46	17.16	0.0043*
	B^2	−4.38	1	13.43	133.15	0.1061	0.7541

*Significant *p* value

Table 5 Final equation and regression results for the quadratic model

Final equation[a]	R^2	Adjusted R^2	Adequate precision	SD[b]	Mean	CV[c]	PRESS[d]
$Y = 661.29 + 36.15\ A - 53.85\ B - 40.18\ AB + 47.10\ A^2 - 3.45\ B^2$	0.8665	0.7711	10.5969	35.42	691.54	5.12	62,441.94

[a]A: $NaNO_3$ concentration (mM); B: K_2HPO_4 concentration (mM)

[b]Standard deviation

[c]Coefficient of variation

[d]Predicted residual sum of squares

factors, i.e., $NaNO_3$ (26.4 mM) and K_2HPO_4 (0.11 mM). Perhaps, this model accepts some outliers except K_2HPO_4 (0.25 mM) where the experimental ($Y_{Exp.}$) and predicted ($Y_{Pred.}$) response showed significant difference with their standard deviation.

In the present study, we have also estimated the contents of other biocommodities such as total chlorophylls/carotenoids and total lipid content in the optimized biomass to know the changes occurring within the cells subjected to varying macronutrients (Figs. 3a-c). Our results showed biocommodities such as total lipids (120 mg L^{-1}) along with the total chlorophyll and carotenoids, i.e., 10.5 and 6.53 mg L^{-1}, respectively (Figs. 3a-b). In summary, this work also demonstrates the best optimized concentration of two factors, i.e., $NaNO_3$ and K_2HPO_4, for higher biomass production (131%) without compromising any fitness cost on the yield of other biocommodities (122% TC [total chlorophyll], 127% CT (total carotenoids), and 125% total lipids shown in Fig. 3c).

Fig. 3d indicates the F_v/F_m ratios, which are extensively used as a representation of the maximal photochemical efficiency of the PSII reaction centers, and it generally correlates with the photosynthetic performance of the cell. In this study, F_v/F_m (PSII quantum yield) was observed to compare the photosynthetic performance of culture in control and in optimized conditions. In the control, cells attained the highest activity on the 3rd day, i.e., 0.67, whereas in the optimized medium the photosynthetic activity observed was 0.75 on day 6, which was maintained at 0.69 until the 9th day (Fig. 3d). In conclusion, our results demonstrate that the optimized medium showed better photosynthetic efficiency than the control, which indicates the better activity of the photosynthetic machinery thus leading to enhanced cell biomass.

3.2.2 Validation of the model for the accuracy and reliability

Table 6 illustrates the validation of the model for demonstrating their accuracy and reliability in terms of the response on the biomass yield with two optimized variables. The validation points (VP_1, VP_2, VP_3) correspond to biomass yields of 640.25, 600.38, and 760.15 mg L^{-1}, respectively, as shown in Table 6. The difference between the responses as $Y_{Pred.}$ and $Y_{Exp.}$ ranges within the standard deviation thus demonstrating the accuracy of the model. Moreover, the results also affirmed that such model designing approach for the optimization process is quite accurate and reliable.

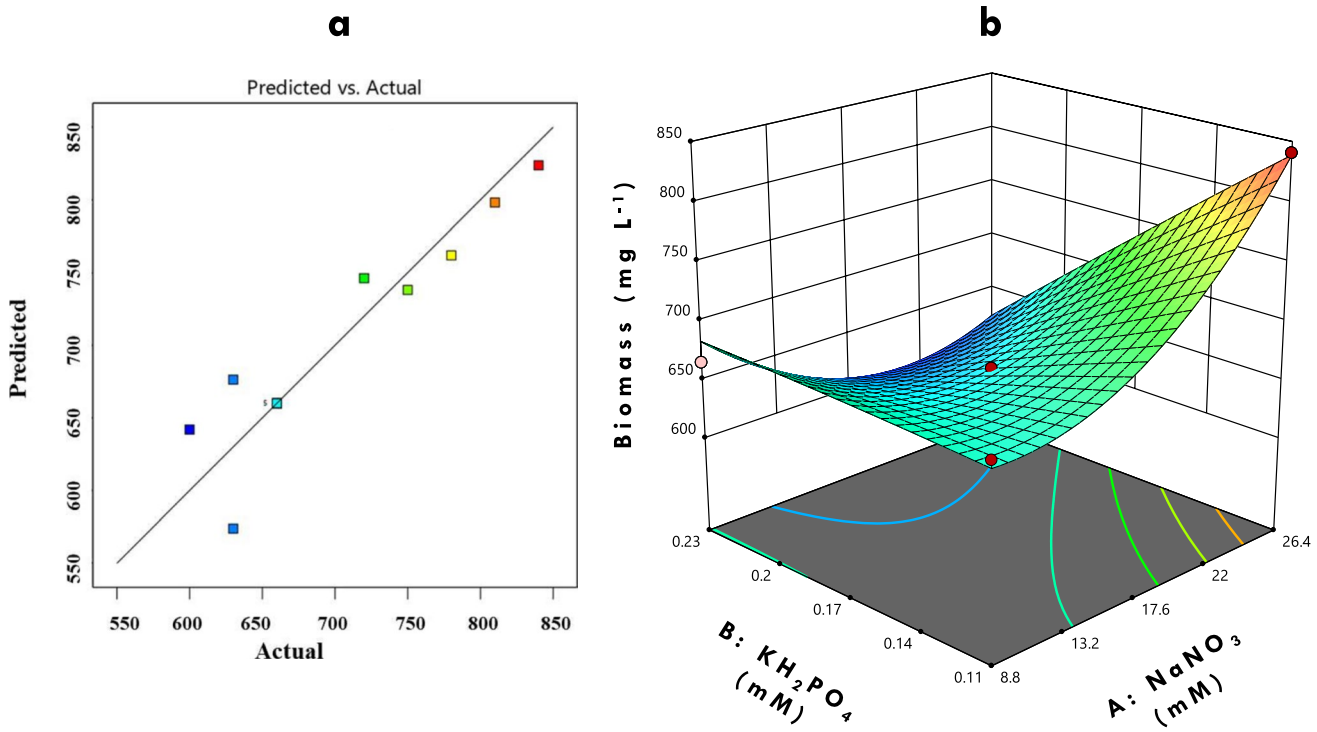

Fig. 2 **a** Parity graphs demonstrating the distribution of actual and predicted values of biomass production in *C. saccharophila*. **b** Response surface and contour lines indicating the impact of $NaNO_3$ and K_2HPO_4 on the biomass yields in *C. saccharophila* with reference to response surface polynomials. Also, the actual data points are shown as red circles

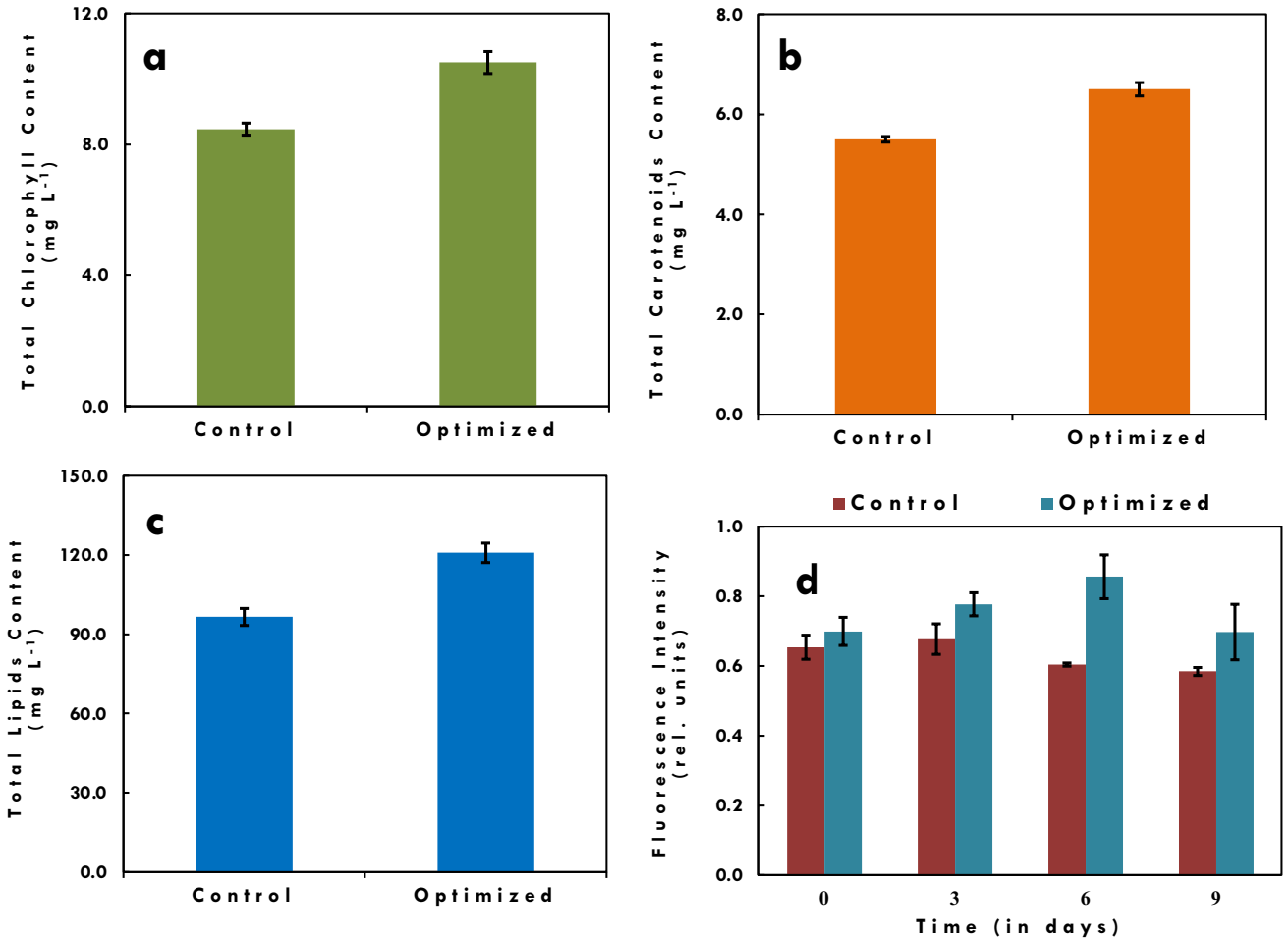

Fig. 3 Biochemical components in *C. saccharophila* at day 9, in two different concentrations of nitrogen ($NaNO_3$) and phosphorus (K_2HPO_4) to compare the total pigments (total chlorophyll [TC] and total carotenoids [Ct.]), total lipid yield (represented as **a**–**c** respectively]. **d** Changes in maximum quantum yield of PSII photochemistry (F_v/F_m), i.e., maximum efficiency at which PSII-absorbed light is utilized to reduce Q_A. All the samples are represented as the average of three biological replicates ± S.D.

Table 6 Validation of the model involving two variables with one response (i.e., biomass yield) in *C. saccharophila*. All the samples are represented as the average of three biological replicates ± S.D

S. no	Label	Variables		Response	
				Biomass yield	
		$NaNO_3$ (mM)	K_2HPO_4 (mM)	Y_{Exp} (mg L^{-1})	Y_{Pred} (mg L^{-1})
1	VP_1	17.60	0.23	640.25 ± 18.0	609.58
2	VP_2	24.17	0.23	600.38 ± 20.8	615.34
3	VP_3	26.4	0.17	760.15 ± 25.6	737.61

4 Discussion

During the past few decades, substantial research has been done with different strains of *Chlorella* sp. for enhancing their biomass yields [20, 52–56]. These reports clearly indicate that the factors influencing the production of biomass include medium composition especially the macronutrients [57–59]. Some of the major nutrients are nitrogen (N), phosphorus (P), and carbon (C); among these, N and P are usually present in the medium and C will be supplemented as additional source. In the present study, we demonstrated the effect of two essential macronutrients including $NaNO_3$ and K_2HPO_4 along with $NaHCO_3$ (additional carbon) independently via the OFAT experiments to illustrate their effect on biomass yields in *C. saccharophila* UTEX 247. Among these, $NaNO_3$ (26.4 mM; 1.5 N) showed a positive impact on its biomass content, thus demonstrating that the nitrogen is the significant growth-enhancing factor in microalgae [60]. Thus, the use of nitrate as N source is the most suitable option for biomass production [61] as it is an essential constituent of the structure of amino acids, proteins, and enzymes.

Moreover, K_2HPO_4 is also important for growth at the lower concentrations. Solovchenko et al. [62] and Lavrinovics et al. [63] demonstrated that there was no effect on the growth when subjected to phosphorus (P) starvation. Moreover, Singh et al. (2021) [64] also reported that less P is giving the better biomass productivity. Other than that, Suthar et al. [65] also reported amount of P for growth is strain specific. In addition, several research groups worked on phosphorous uptake and revealed that microalgae can only absorb additional phosphorus if they develop in a deprived state first [66, 67]. As a result, the subjective reason behind this strategy can be the uptake of inorganic phosphorous and stores it as the poly-P granules. Such poly-P molecules are rich energy source that are able to support the growth of organisms for different metabolic functions within the cells [68]. Furthermore, in the case of higher P, growth was delayed [69]. Henceforth, the P assimilation and/or tolerance is strain-specific. On the other hand, the possibility of P assimilation at higher concentration may lead to irregular N:P ratios, which will significantly impact the biomass yields.

The N:P ratio is known to affect cell proliferation of some micro algae. A research group Zhang et al. (2011) [70] evaluated the effect of N/P ratios on the proliferation and succession of phytoplankton using different nitrogen sources NH_4Cl (N_1) and urea (N_2), and a single source of phosphorous, NaH_2PO_4(P). The optimal N/P ratio that differed among the five species was affected by the source of nitrogen, being as follows (N_1/P, N_2/P in order): *Thalassiosira* sp. (30/1, 20/1), *Heterosigma akashiwo* (30/1, 30/1), *Chroomonas salina* (20/1, 30/1), *Chaetoceros gracilis* (40/1, 60/1), and *Alexandrium* sp. (10/1, 30/1). Thus, the source of nitrogen must be considered when analyzing the N/P ratio. Other than that, Molina et al. 2011 [71] observed the maximum growth rate for N:P between 2.5 and 80. Furthermore, Armitage et al. (2005) [72] reported N:P > 96:1 for Thalassia testudinum at its natural habitat. But the main feature of BG11 medium is that the N:P ratio is deliberately high (~ 80:1) for simple and convenient cultivation of unicellular photosynthetic organisms [73, 74]. However, species-specific medium optimization is necessary for different aspects such as biomass and lipid [73, 75]. In this study, we optimized the medium for better biomass therefore needed more N. As a result, the best biomass obtained in the N:P ration 240:1 which shown as the 5th run in the Table 3. Furthermore, nitrogen is the medium's restraining nutrient and the phosphorus concentration might be even higher after N depletion. Thus, leading to a saturation point where the phosphorus cellular absorption might be restricted [63].

But in the case of $NaHCO_3$, there was no significant improvement in biomass content of *C. saccharophila*. Similar results have been reported earlier [76], where bicarbonate inhibits growth by raising the pH of the medium. Nayak et al. [77] and Richmond et al. [78] investigated the effect of bicarbonate on growth and suggested that the increased pH can be maintained by introducing gaseous CO_2. Henceforth, bicarbonate in some instances cannot be considered a growth-enhancing factor. Chi et al. [79] observed that a few strains can tolerate a higher concentration of different salts including $NaHCO_3$ and/or NaCl. Furthermore, White et al. [76] also worked on bicarbonate and examined the bicarbonate supplementation on two strains and found that it either had no effect on growth or delayed it. However, our present study shows that there is no enhancement in the growth patterns of *C. saccharophila* when subjected with additional carbon supplementation.

Subsequently, two parameters ($NaNO_3$ and K_2HPO_4) were considered to optimize conditions for the better biomass production in *C. saccharophila* employing the CCD module. The CCD is essential for determining the effect of each variable alone or in combination with the total response. Nitrogen (N) is a primary factor which is essential for the synthesis of biomolecules besides growth. Also, it is well known that the nitrogen depletion will lead to decrease in overall protein content which ultimately affects the cell's machinery. In the present study, we demonstrated that the optimal concentration of $NaNO_3$ is required for higher biomass which relates to the work done by Zarrinmehr et al. [80] which stated that increased nitrogen concentration enhances biomass yields. Moreover, our results with the CCD model and the quadratic equation showed that biomass yields in *C. saccharophila* enhance significantly with the specific concentration of higher $NaNO_3$ and lower K_2HPO_4.

Taziki et al. [81] demonstrated use of nitrate as a nitrogen source which has been efficiently utilized by Chlorella sp. to produce higher biomass. Furthermore, Ana−Maria and coworkers [82] enhanced both biomass and lipid yield through the CCD model, and found that nitrate concentration was the growth−enhancing factor. Kim et al. [83] showed that the N and P supplementation in Chlorella sp. further enhanced their growth rate to 0.48 day−1. Recently, Rodrigues−Sousa et al. [84] demonstrated that the nitrogen supplementation enhanced biomass content in Chlorella sp. and stated that a single macronutrient with the specific concentration, including the N:P ratio, will act as an excellent growth−enhancing factor. Similarly, in this study, even NaNO3 and K2HPO4 independently influenced the growth but simultaneously in combination they tend to promote better as the growth−enhancing factors. Such technique demonstrates the importance of the CCD model in the medium optimization process, where a precise concentration and/or their combination of both components generates a promising optimized response for higher yields.

In such context, this is a worthy study of its kind which utilizes RSM tool with CCD model for further enhancing the biomass yields along with other biocommodities as we used

1.5 N and 0.5 P in optimized medium, and achieved 131% biomass, 122% TC (total chlorophyll), 127% CT (total carotenoids), and 125% total lipids as shown in Fig. 3. Kirrolia et al. [30] used similar strategy as a decision−making tool for the medium optimization in Chlorella sp. for enhancing biodiesel production. Increasing the biomass yields of C. saccharophila is an important step in making algal biofuels more economical and sustainable [85, 86]. Therefore, the use of optimized medium for producing better biomass productivities was investigated along with other biocommodities simultaneously. Another salient feature in this study is that the optimized medium showed better photosynthetic performance, which states that the activity of cell's photosystem is functioning at their maximal.

5 Conclusions

In this present study, the microalgae *C. saccharophila* UTEX 247 was subjected to media engineering approach employing response surface methodology for enhancing their biomass productivities. Essential macronutrients in the BG-11 medium, i.e., $NaNO_3$ and K_2HPO_4, influence the biomass yields independently but a better enhancement in the biomass content (840 mg L^{-1}) was achieved by the specific combination of these two factors at the optimized concentrations ($NaNO_3$ (26.4 mM) and K_2HPO_4 (0.11 mM)) as defined by CCD module. The statistical tool used in the current study demonstrated an increase of 131.25% dcw in biomass yields along with other biocommodities such as lipids (125%) and pigments (122% TC [total chlorophyll], 127% CT (total carotenoids) respectively). In conclusion, the optimization of specific growth conditions is essential for each specific strain of industrial relevance for enhancing their growth rates along with other biocommodities, which will lead to sustainable and cost-effective biorefineries.

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Data availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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