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# Combined Effect of Light Intensity, Photoperiod and Phosphorus Levels on Biomass and Chlorophyll Production in *Chlorella vulgaris*

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**Abstract**

Efficient management of economic and sustainable microalgae production necessitates strategically utilizing influential growth factors. A pivotal aspect involves optimizing the utilization of photonic energy in conjunction with environmental parameters by elevating algal efficiency to its maximum potential. This investigation delves into the effect of Light Emitting Diodes (LEDs) on different microalgal species under diverse conditions, specifically exploring the impact of blue light intensity, photoperiod, and phosphorus on biomass and chlorophylls *a* and *b* content in *Chlorella vulgaris*. A Response Surface Methodology (RSM) approach was employed to achieve this objective. The study revealed that the combination of 40 µmol photons. $m^{-2}.s^{-1}$, 12:12 photoperiod (light: dark), and 80 mg/L phosphorus in media yielded a biomass production of $4*10^7$ cells/ml 27.7 mg/L, 9.79 mg/L chlorophyll a and b respectively. Furthermore, response surface analysis identified the optimal condition at 36.58 µmol photons.m-2.s-1, a 12:12 photoperiod, 80 mg/L phosphorus in media, which led to $3.62*10^7$ cells/ml, 27.83 mg/L chlorophylls *a*, and 5.44 mg/L chlorophyll *b*, with a remarkable approval rating of 93 percent. These findings indicate the potential of LED technology to augment biomass production and enhance the content of bioactive compounds in microalgae, thereby endowing them with significant economic value across diverse industries.

## 1. Introduction

Over the last two decades, the large-scale cultivation of microalgae has experienced substantial advancements, primarily in countries such as the United States and Australia. Nonetheless, recent years have witnessed significant growth in microalgae cultivation in various regions worldwide. In Europe, dried algal biomass production reaches approximately 500 tons (Verdelho, 2019). In Asia, Iran presents a promising environment conducive to microalgae cultivation, offering considerable potential for developing the microalgae production industry (Katooli et al., 2021). Despite these opportunities, it is worth noting that this extraordinary capacity remains largely untapped at present.

Chlorella represents a prominent genus of microalgae extensively employed across diverse industries. Its consumption has been scientifically demonstrated to benefit effects on the intestinal microbiota, augment cellular growth, increase leukocyte count and phagocytic activities, and

fortify the human immune system (An et al., 2016; Sani et al., 2021). Moreover, the introduction of specific minerals, such as zinc and selenium, into the culture medium of *Chlorella vulgaris* has been found to result in the production of a nutrient-rich powder, which holds potential for the management and treatment of COVID-19 (Sani et al., 2021).

Given its economic and environmental implications, genetic and metabolic engineering of microalgae has emerged as a promising approach to bolster biomass production. The augmentation of biomass and the accumulation of bioactive compounds in microalgae, pivotal for their commercialization (Hu et al., 2023; Muthukrishnan, 2022), are significantly influenced by various environmental factors (Janjua et al., 2024; Songserm, Nishiyama, & Sanevas, 2024). Nevertheless, given their transgenic characteristics, it is imperative to conscientiously assess the quality of the final products resulting from genetic engineering.

The cultivation of microalgae during upstream processes is subject to various environmental conditions that exert a significant impact on biomass yield and metabolite profiles. This aspect is important since enhancing biomass production is influenced by economic indicators (Ru et al., 2020). Consequently, numerous studies encompassing a wide range of environmental factors have been conducted to augment microalgae yield and ascertain optimal growth conditions (Al-Qasmi et al., 2012; Gani et al., 2019; Songserm et al., 2024). Environmental and nutritional factors have been identified as crucial stimulators of microalgae growth and reproduction (Che et al., 2019; Pelagatti et al., 2023). Notably, photosynthesis and biomass production are profoundly affected by factors such as light, temperature, phosphorus, nitrogen, carbon, and the mixing of the cultivation medium (Gani et al., 2019; Magyar et al., 2024). Additionally, the cell concentration is influenced by light intensity and the doubling time for cell count (Eriksen, 2008). Surface Methodology (RSM) is one promising avenue to yield favorable outcomes, including enhanced and sustainable productivity of microalgae biomass and optimization (Mehra &

Jutur, 2022). Ensuring that microalgae can adapt to physicochemical changes in their natural habitat is paramount for successful biomass production and the synthesis of bioactive compounds (da Silva Ferreira & Sant'Anna, 2017).

Integrating diverse environmental factors at varying levels is essential to attain optimal growth and biomass production of microalgae. Chlorophylls play a pivotal role in facilitating the process of photosynthesis, consequently influencing biomass production (Chen, 2014; Hawrot-Paw & Sąsiadek, 2023; Janjua et al., 2024; Zhang et al., 2023). Nevertheless, recent research has revealed that the accumulation of chlorophylls in microalgae cells may not always directly correlate with cell density or biomass production. Factors of the environmental milieu, such as the composition of the culture medium, can negatively affect cell growth, thereby reducing chlorophyll accumulation within the cells (da Silva Ferreira & Sant'Anna, 2017). Thus, the principal aim of this investigation was to employ RSM to explore the synergistic effects of various levels of blue light intensity, photoperiod, and phosphorus on optimizing cell growth, biomass production, as well as chlorophylls *a* and *b* in *Chlorella vulgaris*

## 2. Materials and Methods

### 2.1. Preparation of microalgae and culture medium

*Chlorella vulgaris* was procured from the Marin Biotechnology division of PTCC Collection at the Iranian Research Organization for Science and Technology. The culture medium employed in this investigation was BG-11, a widely recognized medium extensively utilized for microalgae cultivation (Andersen, 2005). To formulate one liter of the culture medium, all chemical constituents were initially prepared in strict adherence to established protocols in the field. Then, 100 mL of the prepared stock solution was dispensed into 250 mL Erlenmeyer flasks, and the pH was meticulously adjusted using solutions of NaOH and NaCl. Next, the flasks were subjected to autoclaving to sterilize the medium before utilization.

## 2.2. Different levels of light intensity, photoperiod, and phosphorus

The investigation examined the influence of diverse levels of blue light intensity, photoperiod, and phosphorus parameters on microalgae productivity (Abo-State, Shanab, & Ali, 2019; Zhong, Jin, & Cheng, 2018) as summarized in Table 1. Increasing the amount of biomass is through improving the efficiency of photosynthesis. In this process, light and phosphorus indicators are very effective. In addition, the intensity and wavelength of light as the main source of energy for microalgae, influence cell metabolism and biomass.

**Table 1**: Factors and their levels.

| Independent variables | Symbol coded | Coded levels | | |
|---|---|---|---|---|
| | | -L | 0 | L |
| Light intensity (μmol photons . m$^{-2}$ . s$^{-1}$) | A | 36 | 40 | 44 |
| Photoperiod (Light:Dark) | B | 12:12 | 15:9 | 18:6 |
| Phosphorus (mg/L) | C | 40 | 60 | 80 |

## 2.3. Design and experimental conditions

The primary aim of this investigation was to optimize the requisite parameters conducive to achieving the maximum cellular concentration in microalgae. Design-Expert Software (Version 11, Stat-Ease Inc., United States) was employed to construct the design of the experiment for the factors under consideration, which were subsequently implemented in the experimental setup. The specific number of experimental units utilized during this phase is detailed in Table 2. Furthermore, the general conditions governing the phototrophic culture, encompassing the utilization of blue light-emitting diodes (LEDs) with a wavelength range of 460-465 nm, a temperature of 25°C, and an agitation rate of 120 rpm within the photobioreactor, were maintained constant throughout the experimental duration. The light intensity, quantified in lux, was calibrated using a TES 1330A Digital Light meter device.

## 2.3.1. Maximizing Biomass Production through Optimization Strategies

To increase the growth and cell density of *Chlorella vulgaris* in response to different levels of factors investigated in this study, first, the obtained data were analyzed and the variance of each response was reported separately. Then the Box-Cox diagram, which was equal to the estimated value and the actual value obtained, was checked. Data were constructed with real value factor interaction plots. After that, the optimal production amount was determined and the robustness of the model was finally confirmed by three repetitions of design and experimental execution.

Subsequently, the optimum production value was ascertained, and the robustness of the software was validated through three replicates of the experimental design and implementation.

## 2.3.2. Measuring the growth rate

Microalgae proliferation velocity was evaluated through microscopic examination, and successive modifications in cell populace were meticulously recorded on a diurnal cadence (Andersen, 2005). Following the mentioned protocol, the cellular growth rate was calculated utilizing the mathematical formulation denoted as Eq. 1A (Stein-Taylor, 1973).

$$\mu = (3.322 / (t_2 - t_1)) \times \log (N_2 / N_1)$$

Wherein:
3.322 = growth constant, *K* represents the growth rate constant, $t_2 - t_1$ signifies the time interval, log designates the logarithmic function base, $N_2$ indicates the cell count at time $t_2$, $N_1$ corresponds to the cell count at time $t_1$.

## 2.4. Biomass Harvesting and Chlorophylls Extraction

All Erlenmeyer flasks were carefully withdrawn from the incubator during the latter half of the logarithmic phase to obtain biomass samples at an optimal growth stage in the microalgae culture. Then, the collected samples underwent a

systematic procedure for the extraction of chlorophylls *a* and *b*, as follows:

### 2.4.1. Quantification of Biomass and Extraction of Chlorophylls a and b

These crucial steps are instrumental in assessing the photosynthetic pigment composition of the studied samples. The biomass was isolated from the culture medium using high-speed centrifugation. Initially, 500 ml Beckman tubes were weighed prior to the addition of microalgae. Subsequently, the tubes were subjected to centrifugation at 5000 rpm for 10 minutes. Finally, the amount of biomass was obtained from the difference before and after the tubes.

Spectrophotometry was employed to assess the levels of chlorophylls a and b within the microalgae biomass. The concentrations of chlorophylls *a* and *b* were determined using the following Eq. 2A (Lee et al., 2013).

For 100% acetone (mg L$^{-1}$):    (Eq. 2A)

Chlorophyll $a = (12.7 \times A663) - (2.69 \times A645)$

Chlorophyll $b = (22.9 \times A645) - (4.64 \times A663)$

### 2.5. Statistical Analysis

Preliminarily, the normality of the data and the individual impact of each independent variable on the response variable were assessed. Next, a polynomial equation was utilized to decompose the variance of each independent variable into linear, quadratic, and interaction effects. Then, less significant cases were excluded through analysis of the variance table, aiming to enhance the quality of the model under consideration. Response surface plots were then generated to visually illustrate the relationship between the two independent variables and each dependent variable. Dependent variable plots were constructed based on the normality of the data and the model equation derived from the analysis. Analysis of variance was performed for each response variable to determine the significance of the independent variables and their interactions.

Finally, the significance of polynomial effects was evaluated to confirm the appropriateness of the corresponding model (P<0.05).

### 3. Results and Discussion

### 3.1. Impact of Varied Light Intensity, Photoperiod, and Phosphorus Levels on Biomass and Chlorophylls a and b Content in *Chlorella vulgaris*

This study investigates the influence of diverse light intensity, photoperiod, and phosphorus concentrations on biomass productivity and chlorophylls *a* and *b* content of *Chlorella vulgaris*. A comprehensive analysis of the algal growth and pigment synthesis was conducted using controlled experimental conditions. The findings contribute to a deeper understanding of the physiological responses of *Chlorella vulgaris* to distinct environmental factors and provide valuable insights for potential applications in algal biotechnology and ecophysiology.

### 3.1.1. Biomass Production

Results of the analysis of variance in the responses of the biomass, chlorophyll a and b production carried out by RSM are listed in Tables 2-5. The model showed a second-order regression equation for the best description of every three responses production as a function of light-intensity, photoperiod, and phosphorus concentrations Eq. 1-3B:

B1: Biomass = -7340.62000 -101.65417 * Photoperiod + 7.40663 * Light intensity + 29.83237 * Phosphorus + 0.016667 * Photoperiod * Light intensity -1.36458 * Photoperiod * Phosphorus - 5.31250E 003 * Light intensity * Phosphorus + 5.04472 * Photoperiod2 -1.81806E − 003 * Light intensity2 +0.010381 * Phosphorus2

B2: Chlorophyll a = -132.05900 - 1.01896 * Photoperiod + 0.15932 * Light intensity + 0.33038 * Phosphorus + 1.64167E-003 * Photoperiod * Light intensity - 0.063938 * Photoperiod *

Phosphorus + 2.13125E – 004 * Light intensity * Phosphorus + 0.051375 * Photoperiod2 - 5.14094E -005 * Light intensity2 + 2.03719E -003 * Phosphorus2

B3: Chlorophyll b = +110.11400 + 2.06542 * Photoperiod - 0.11846 * Light intensity + 0.031213 * Phosphorus + 7.16667E-004 * Photoperiod * Light intensity +0.014458 * Photoperiod * Phosphorus + 2.50000E -005 * Light intensity * Phosphorus - 0.14242 * Photoperiod2 + 2.58937E – 005 * Light intensity2

Changes in biomass response and chlorophyll a and b as a function of independent variables (light intensity, photoperiod, and phosphorus) fit a quadratic model. The natural logarithm of the residual sum of squares against the confidence interval showed a sudden slope in the region of the best optimal value, as illustrated in Figure 1.

The findings about cell density are graphically depicted in Figure 2. The derived mathematical framework grounded in biomass data is evident through Eq. 1B. Furthermore, the metrics signifying the congruity between the model and empirical data within the context of response surface analysis are meticulously documented in Tables 2 and 5. The outcomes underscore that the utmost proliferation of cellular entities quantified at $4*10^7$ cells/ml, was attained under conditions characterized by an illuminance level of 40 µmol photons.m$^{-2}$.s$^{-1}$, a diurnal light-dark cycle of 12:12, and a phosphorus concentration of 80 mg/L. The present investigation elucidates a notable enhancement of 150% in cellular growth in contrast to outcomes emanating from the synergistic interplay of photoperiod, phosphate,

and nitrate factors impacting *Chlorella vulgaris* (Vazirzadeh & Moghadaszadeh, 2018).

**Figure 1:** Box–Cox plot for Biomass production in *Chlorella vulgaris.*
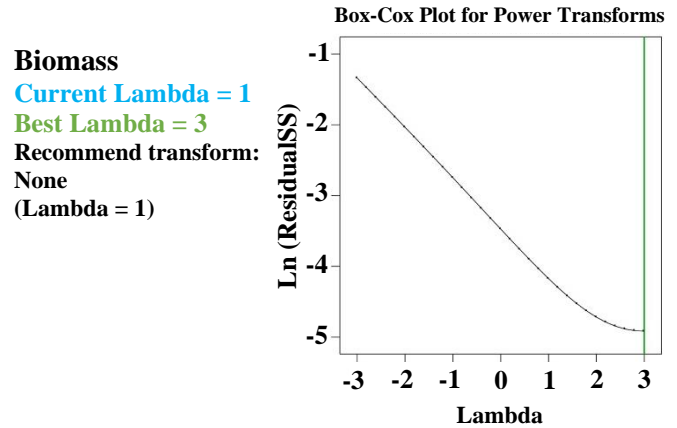


The observed enhancements in cell density and count can be attributed to the utilization of blue LEDs as a light source, as corroborated by previous studies (Baidya et al., 2021; Maltsev et al., 2021; Pelagatti et al., 2023). In line with these results, other investigations have demonstrated that a combination of environmental factors, such as a light intensity of 2500 lux and a photoperiod of 12:12, can significantly augment growth rate and biomass production in *Chlorella vulgaris* (Hariskos, Rubner, & Posten, 2015; Sharma, Singh, & Sharma, 2012). Additionally, higher phosphorus and nitrogen concentrations have been shown to increase cellular biomass in *Chlorella vulgaris* (Abo-State et al., 2019; Magyar et al., 2024).

Comparable findings have been documented in studies involving *Chlorella pyrenoidosa*, indicating a strong correlation between light intensity and cellular biomass production, with blue LEDs yielding favorable outcomes (Guo & Fang, 2020; Yadavalli et al., 2010).

**Table 2.** Results of analysis of variance for the second-degree model and equation for the levels of biomass in the proposed response surface experiment.

| Source | Sum of Squares | Degree of freedom | Mean Square | F-Value | p-value | |
|---|---|---|---|---|---|---|
| **Model (Biomass)** | 374.15 | 9 | 41.57 | 157.04 | < 0.0001 | Significant |
| **A-Photoperiod** | 0.6302 | 1 | 0.6302 | 2.38 | 0.1738 | |
| **B-light intensity** | 13.78 | 1 | 13.78 | 52.06 | 0.0004 | |
| **C-Phosphorus** | 0.0052 | 1 | 0.0052 | 0.0197 | 0.8930 | |
| **AB** | 4.00 | 1 | 4.00 | 15.11 | 0.0081 | |
| **AC** | 134.07 | 1 | 134.07 | 506.45 | < 0.0001 | |
| **BC** | 18.06 | 1 | 18.06 | 68.23 | 0.0002 | |
| **A²** | 68.71 | 1 | 68.71 | 259.56 | < 0.0001 | |
| **B²** | 176.29 | 1 | 176.29 | 665.92 | < 0.0001 | |
| **C²** | 0.5748 | 1 | 0.5748 | 2.17 | 0.1910 | |
| **Residual** | 1.59 | 6 | 0.2647 | | | |
| **Lack of Fit** | 1.17 | 2 | 0.5859 | 5.63 | 0.0688 | Not significant |
| **Pure Error** | 0.4165 | 4 | 0.1041 | | | |
| **Cor Total** | 375.74 | 15 | | | | |

**Figure 1:** A three-dimensional plot displaying the combined effects of varying light intensity, photoperiod, and phosphorus concentrations on biomass efficiency in *Chlorella vulgaris*. a1 represents the outcomes associated with the interaction of factors A and C, a2 illustrates the interaction between factors A and B, and a3 depicts the results of the interaction between factors B and C.
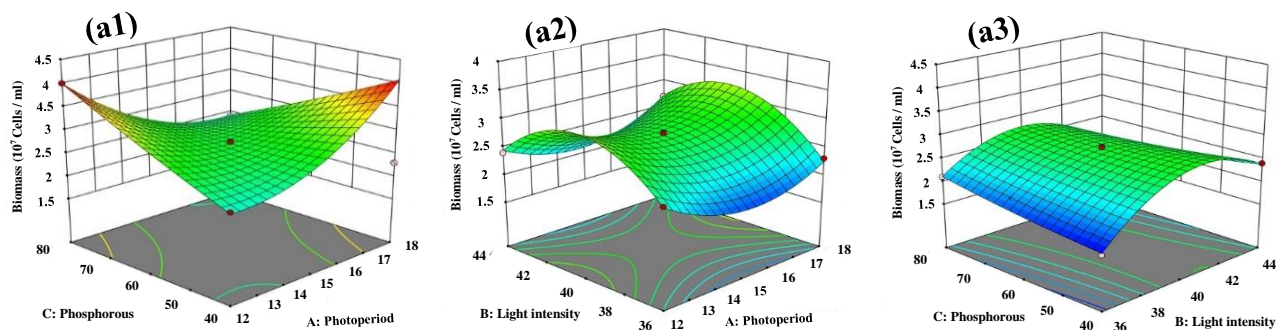


Moreover, investigations on other microalga species such as *Amphidinium carterae*, *Nephroselmis* sp., *Tetraselmis* sp., *Asteromonas gracilis*, and *Dunaliella* sp. have shown that the combined use of salinity and light intensity can significantly enhance biomass production and growth. Specifically, increasing light intensity while reducing salinity in the culture medium led to a notable increase in biomass production (Hotos & Avramidou, 2021).

In a study involving *Dunaliella salina*, the combined effects of salinity, light intensity, and various nitrogen sources on growth and biomass production were assessed. While increased light intensity promoted cell division, it did not have a discernible effect on cellular dry weight, indicating that cellular division alone does not exclusively contribute to augmented biomass production. The observed variations in these outcomes can be attributed to the specific microalga species and the predominant type of pigment it contains. Consequently, the natural growth of microalgae necessitates a specific combination of light wavelength in addition to intensity (Pelagatti et al., 2023). In light of these insights, the cultivation conditions of microalgae must incorporate multiple factors to achieve optimal growth rate stability, consistency, and reduced production costs.

### 3.1.2. Chlorophylls a and b Production

*Chlorella vulgaris* has demonstrated considerable growth potential and the ability to synthesize bioactive compounds under phototrophic, heterotrophic, and mixotrophic cultivation conditions. Results showed, the optimization of chlorophyll *a* yield was achieved by subjecting the specimen to an illuminance of 40 µmol photons.m$^{-2}$.s$^{-1}$ a diurnal rhythm of 12 hours of light succeeded by 12 hours of darkness, and a

phosphorus concentration of 80 mg/L. Conversely, the zenith of chlorophyll *b* production manifested under luminous conditions characterized by an irradiance of 36 µmol photons.m$^{-2}$.s$^{-1}$, a photoperiodic ratio of 15:9, and a phosphorus concentration of 80 mg/liter (refer to Figs 2 and 3), as well as Tables 3 and 4). The empirical thresholds demarcating the upper limits for the generation of chlorophylls *a* and *b* within this experimental milieu were ascertained to be 27.7 mg/L and 9.79 mg/L, respectively.

These findings are in contrast to those observed in a study on *Chlorella ellipsoidea*, where different LED wavelengths resulted in chlorophylls *a* and *b* levels of 7.31 µg/mL and 2.73 µg/mL, respectively (Baidya et al., 2021).

In other study investigating the impact of different light intensities and wavelengths on algae-bacteria growth revealed that the highest concentration of chlorophyll *a* (3.5 mg/L) was synthesized under a light intensity of 100 µmol/m$^2$ in the red spectrum (Katam, Ananthula, Anumala, Sriariyanun, & Bhattacharyya, 2022). The sensitivity of chlorophylls *a* and *b* to varying light wavelengths has been previously reported

(Schulze, Barreira, Pereira, Perales, & Varela, 2014). Our research indicates that the proliferation rate of microalgal cells is not directly correlated to the concentrations of chlorophylls *a* and *b*. In other words, factors contributing to an increase in microalgal cell volume may not significantly affect cell reproduction and the accumulation of chlorophylls *a* and *b* (da Silva Ferreira & Sant'Anna, 2017).

Microalgae division mechanisms involve binary and multiple fissions, and as a consequence, various environmental conditions, particularly fluctuations in light, significantly influence the growth, macromolecule accumulation, cell cycle progression, and division events in microalgal cells (Zachleder, Bišová, & Vítová, 2016). The different stages of cellular differentiation during chromosome pairing have distinct light requirements (Bilcke et al., 2021). Thus, at molecular and physiological levels, cellular cycles and their response to environmental factors play a pivotal role in regulating DNA replication, nuclear and cellular divisions, and ultimately, the biogenesis of microalgal cells (Zachleder et al., 2016).

**Table 3:** Results of analysis of variance for the second-degree model and equation for the levels of chlorophyll a in the proposed response surface experiment.

| Source | Sum of Squares | Degree of freedom | Mean Square | F-Value | p-value | |
|---|---|---|---|---|---|---|
| **Model (Ch a)** | 95.35 | 9 | 10.59 | 8.30 | 0.0090 | Significant |
| **A-Photoperiod** | 0.450 | 1 | 0.450 | 0.0353 | 0.8572 | |
| **B-light intensity** | 25.38 | 1 | 25.38 | 19.88 | 0.0043 | |
| **C-Phosphorus** | 3.77 | 1 | 3.77 | 2.95 | 0.1366 | |
| **AB** | 3.88 | 1 | 3.88 | 3.04 | 0.1319 | |
| **AC** | 29.43 | 1 | 29.43 | 23.05 | 0.0030 | |
| **BC** | 2.91 | 1 | 2.91 | 2.28 | 0.1821 | |
| **A2** | 0.7126 | 1 | 0.7126 | 0.5581 | 0.4833 | |
| **B2** | 14.10 | 1 | 14.10 | 11.04 | 0.0160 | |
| **C2** | 2.21 | 1 | 2.21 | 1.73 | 0.2360 | |
| **Residual** | 7.66 | 6 | 1.28 | | | |
| **Lack of Fit** | 5.16 | 2 | 2.58 | 4.13 | 0.1063 | Not significant |
| **Pure Error** | 2.50 | 4 | 0.6245 | | | |
| **Cor Total** | 103.01 | 15 | | | | |

**Table 4:** Results of analysis of variance for the second-degree model and equation for the levels of chlorophyll b in the proposed response surface experiment.

| Source | Sum of Squares | Degree of freedom | Mean Square | F-Value | p-value | |
|---|---|---|---|---|---|---|
| **Model (Ch b)** | 15.15 | 9 | 1.68 | 36.24 | 0.0002 | Significant |
| **A-Photoperiod** | 0.4219 | 1 | 0.4219 | 9.04 | 0.0236 | |
| **B-light intensity** | 2.23 | 1 | 2.23 | 47.91 | 0.0005 | |
| **C-Phosphorus** | 1.13 | 1 | 1.13 | 24.42 | 0.0026 | |
| **AB** | 0.7396 | 1 | 0.7396 | 15.92 | 0.0072 | |
| **AC** | 1.51 | 1 | 1.51 | 32.39 | 0.0013 | |
| **BC** | 0.0400 | 1 | 0.0400 | 0.8608 | 0.3893 | |
| **$A^2$** | 5.48 | 1 | 5.48 | 117.86 | < 0.0001 | |
| **$B^2$** | 3.58 | 1 | 3.58 | 76.96 | 0.0001 | |
| **$C^2$** | 2.80 | 1 | 2.80 | 60.29 | 0.0002 | |
| **Residual** | 0.2788 | 6 | 0.0465 | | | |
| **Lack of Fit** | 0.1275 | 2 | 0.0637 | 1.68 | 0.2946 | Not significant |
| **Pure Error** | 15.13 | 4 | 0.0378 | | | |
| **Cor Total** | 15.43 | 15 | | | | |

**Figure 3:** Presents a three-dimensional representation depicting the combined effects of varying light intensity levels, photoperiods, and different phosphorus concentrations on the efficiency of chlorophyll a in *Chlorella vulgaris*. In this figure, b1 represents the outcomes associated with the interaction of factors A and C, b2 corresponds to the results of the interaction between factors A and B, and b3 indicates the effects arising from the interaction of factors B and C.
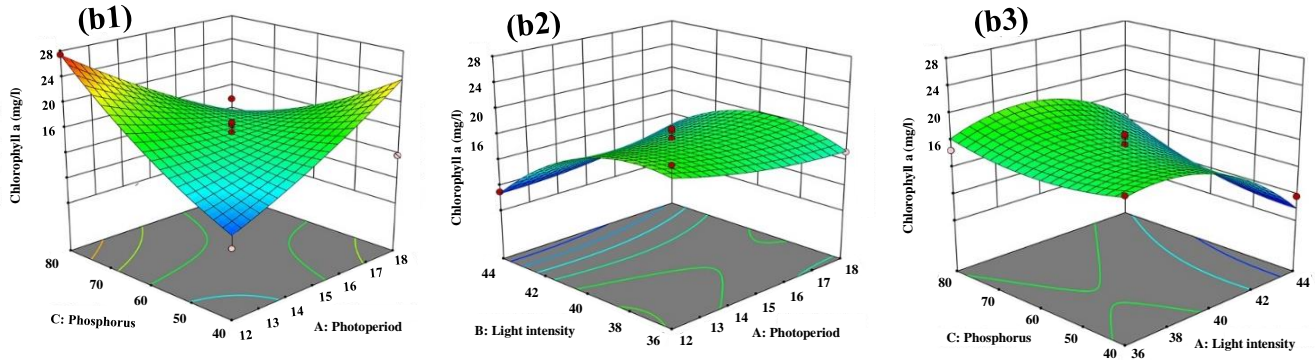


**Figure 4:** Presents a three-dimensional representation depicting the combined effects of varying light intensity levels, photoperiods, and different phosphorus concentrations on the efficiency of chlorophyll b in *Chlorella vulgaris*. In this figure, c1 represents the outcomes associated with the interaction of factors A and C, c2 corresponds to the results of the interaction between factors A and B, and c3 indicates the effects arising from the interaction of factors B and C.
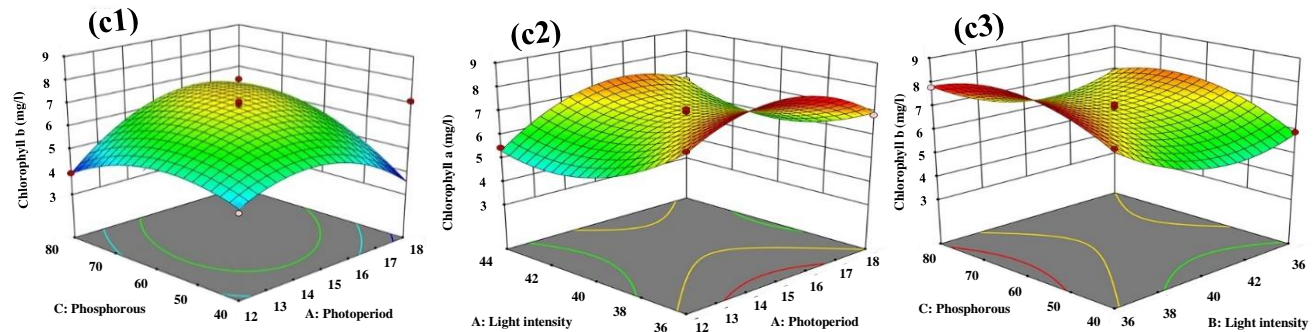
**Table 5:** Quality indices of data fitting in the proposed second-degree equation.

| Calculation of the Standard Deviation for Biomass Response | 0.5145 | R-Squared | 0.9958 |
|---|---|---|---|
| Mean | 25.23 | Adj R-Squared | 0.9894 |
| C.V. % | 2.04 | Pred R-Squared | NA |
| PRESS | NA | Adeq Precision | 56.6212 |
| -2 Log Likelihood | 8.45 | BIC | 36.17 |
| | | AICc | 72.45 |
| Calculation of the Standard Deviation for Ch a Response | 1.13 | R-Squared | 0.9256 |
| Mean | 21.00 | Adj R-Squared | 0.8141 |
| C.V. % | 5.38 | Pred R-Squared | NA |
| PRESS | NA | Adeq Precision | 12.0471 |
| -2 Log Likelihood | 33.62 | BIC | 61.35 |
| | | AICc | 97.62 |
| Calculation of the Standard Deviation for Ch b Response | 0.2156 | R-Squared | 0.9819 |
| Mean | 6.46 | Adj R-Squared | 0.9548 |
| C.V. % | 3.34 | Pred R-Squared | NA |
| PRESS | NA | Adeq Precision | 22.8487 |
| -2 Log Likelihood | -19.39 | BIC | 8.33 |
| | | AICc | 44.61 |

As a result, if the conditions necessary for cell reproduction are not met, checkpoint mechanisms prevent the cell from advancing to the next stages, leading to the formation of larger and bulkier cells (Bišová & Zachleder, 2014; Fang, Reyes, & Umen, 2006). Consequently, an increase in pigment levels is accompanied by an escalation in the number and proliferation of cells. Given the specific objective of enhancing microalgal biomass in our current study, measures were taken to increase the volume of microalgal cells while keeping the conditions for cell proliferation and chlorophylls a and b production constant (da Silva Ferreira & Sant'Anna, 2017). Therefore, we recommend carefully considering the essential factors influencing chlorophyll levels, taking into account both internal and external conditions that affect microalgae. Therefore, we recommend carefully considering the essential factors influencing chlorophyll levels, taking into account both internal and external conditions that affect microalgae.

Overall, this study enhances our understanding of the factors influencing chlorophylls *a* and *b* production and highlights the importance of optimizing cultivation conditions for microalgal growth and pigment synthesis.

## 3.2. Optimization of biomass and chlorophylls a and b production

Based on the findings, optimal conditions for biomass production of Chlorella vulgaris were determined: a light intensity of 36.58 µmol photons.m-2.s-1, a photoperiod of 12 hours of light and 12 hours of darkness, and a phosphorus concentration of 80 mg/L. These conditions were achieved at a cellular density of $3.62*10^7$ cells/ml, with chlorophyll a and b concentrations measured at 27.83 mg/L and 5.44 mg/L, respectively (Figure 5). While all the yellow areas in the overlay plot represent optimal responses, the flagged numerals indicate conditions with more favorable economic implications. The primary objective is to minimize the energy consumption required for biomass production in *Chlorella vulgaris*.

The reciprocal effects of the investigated indices were observed in response to varying environmental conditions. Notably, light intensity emerged as a crucial factor influencing cellular density, either enhancing or reducing it. Additionally, changes in light intensity also affected phosphorus levels and photoperiod, indicating their interdependence and no significant individual effect on biomass production was observed. To optimize the production process, it is recommended to use a combination of various indices, employ a single-wavelength LED source, and maintain controlled conditions in a photobioreactor device.

It is worth noting that research on the synergistic effect of different light intensities, photoperiods, and phosphorus levels on *Chlorella vulgaris* biomass production using RSM is limited. While various studies have explored the impact of different LEDs on microalgae biomass production, conflicting results have been reported (Choi et al., 2015; Mohsenpour, Richards, & Willoughby, 2012; Schulze et al., 2014). These discrepancies may arise from the specific microalgae species under study and their natural habitat, where factors like light absorption, particularly white light compared to blue light, can play a critical role (Baidya et al., 2021; Maltsev et al., 2021).

The process of photosynthesis, being highly reliant on biomass and pigment production, particularly chlorophylls, can be influenced by certain conditions and unknown factors, which may lead to fluctuations in cellular density and total biomass (da Silva Ferreira & Sant'Anna, 2017). To overcome these challenges, future investigations should explore the synergistic effects of abiotic factors alongside genetic manipulation in the target microalgae.

In conclusion, additional research is essential to comprehensively understand the impact of combined abiotic stressors on biomass production and the production of diverse high-value metabolites in *Chlorella vulgaris*. Such insights will be instrumental in optimizing and enhancing the commercial production of microalgae-derived products.

### 3.2.1. Model Validation

The validation of the model is a crucial aspect of this study to ensure the robustness and reliability of the data obtained using the Design Expert software. To address this, a treatment involving specific parameters, namely a light intensity of 36.58 µmol photons.m$^{-2}$.s$^{-1}$, a 12:12 photoperiod,

and a selected concentration of 80 mg/L phosphorus, were applied and rigorously confirmed through three replications. The optimum conditions and verification results are shown in Table 6.

By implementing this carefully validated treatment, the study aims to provide valuable insights applicable at both semi-industrial and industrial scales. The outcomes of this research are expected to constitute a significant advancement toward enhancing biomass cultivation and fostering the production of bioactive compounds in microalgae. As such, this endeavor represents a promising step forward in the field.

**Figure 5:** Response Surface Optimization levels and interactions of Biomass with Chlorophyll a and b. The optimal conditions for light intensity are 36.58 µmol photons.m-2.s-1, a 12:12 photoperiod, and a concentration of 80 mg/L phosphorus for maximizing biomass and chlorophyll production. The values illustrated in the graph confirm the optimal response levels identified in this research study.
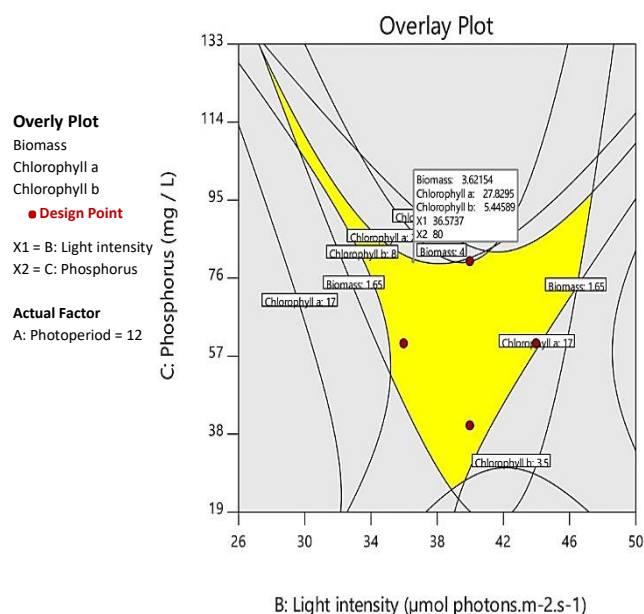
**Table 6:** Results of Optimum Conditions and Validations for Biomass, Chlorophylls a and b.

| Name | Target | Lower limit | Upper limit | Lower weigh | Upper weigh | importance | Desirability |
|---|---|---|---|---|---|---|---|
| A:Photoperiod | minimize | 12 | 18 | 1 | 1 | 5 | |
| B:Light intensity | minimize | 36 | 44 | 1 | 1 | 5 | |
| C:Phosphorus | is in range | 40 | 80 | 1 | 1 | 3 | |
| Biomass | maximize | 16.5 | 40 | 1 | 1 | 5 | 0.93 |
| Chlorophyll a | maximize | 17.36 | 27.7 | 1 | 1 | 4 | 0.93 |
| Chlorophyll b | none | 3.95 | 7.79 | 1 | 1 | 3 | 0.93 |

## 4. Conclusion

The present study investigated the pivotal role of light quality, quantity, and nutrient concentrations in influencing the photosynthesis and growth rate of microalgae. Our findings demonstrated that light intensity exerted a significant impact on biomass and chlorophyll *a* levels, with noteworthy effects observed on factors influencing chlorophyll *b* content. Furthermore, implementing RSM uncovered intricate interactions between all three mentioned factors concerning biomass production. These outcomes suggest the potential for cost reduction in photobioreactor-based cultivation, thereby facilitating a reduction in the overall price of diverse products derived from microalgal biomass. Consequently, optimizing production systems, particularly at industrial scales, is highly recommended for future investigations.

## Conflict of interest

The authors declare that they have no competing interests.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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