

Study of Multi-stress Factors for Biomass and Lipid Enhancement in *Chlorella Pyrenoidosa* and *Scenedesmus Abundans* Using Response Surface Methodology

Chelladurai Chellamboli

c.chilambu@gmail.com

National Institute of Technology Tiruchirappalli <https://orcid.org/0000-0003-1815-0683>

Muthiah Perumalsamy

National Institute of Technology Tiruchirappalli

Mohamed Arshath

St Joseph's College of Engineering

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Additional Declarations:

Tables 1 to 6 are available in the Supplementary Files section.

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**STUDY OF MULTI-STRESS FACTORS FOR BIOMASS AND LIPID ENHANCEMENT IN *CHLORELLA*
PYRENOIDOSA AND *SCENEDESMUS ABUNDANS* USING RESPONSE SURFACE METHODOLOGY**

Chelladurai Chellamboli*¹, Muthiah Perumalsamy², Mohamed Arshath³

¹Research Scholar, Department of Chemical Engineering, National Institute of Technology, Tiruchirappalli, Tamil Nadu, India.

²Associate Professor, Department of Chemical Engineering, National Institute of Technology, Tiruchirappalli, Tamil Nadu, 620015, India.

³Department of Chemical Engineering, St Joseph's College of Engineering, Chennai, Tamil Nadu, 600119, India.

* Corresponding Author: Chelladurai Chellamboli, Department of Chemical Engineering, National Institute of Technology, Tiruchirappalli, Tamil Nadu, India, PO Box 620015, E-mail: c.chilambu@gmail.com

Abstract

This study aimed to identify algal species that sustain biofuel production under stress conditions. *C. pyrenoidosa* and *S. abundans* can be stressed by supplementing or starving multiple nutrients at different light intensities according to response surface methodology. This study showed that algae yield improved during nutrient starvation. The resultant dry biomasses of *C. pyrenoidosa* and *S. abundans* were 98.26 and 110 mg/L, respectively, and the lipid yields were approximately 22.47 and 29.06%, respectively, under optimum conditions. The optimized parameters for *C. pyrenoidosa* were 0.805 g/L NaNO₃, 0.052 g/L K₂HPO₄, 0.099 g/L KH₂PO₄, 17 days of culture and 5168.39 lux of light, whereas *S. abundans* had 1.065 g/L NaNO₃, 0.071 g/L K₂HPO₄, 0.058 g/L KH₂PO₄, 22 days of culture and 2897 lux of light. Studies have confirmed that both species are considered sustainable sources for biodiesel production.

Keywords: Microalgae, Lipid, Biomass, Optimization, Response surface methodology, *Chlorella pyrenoidosa*, *Scenedesmus abundans*

Introduction

To meet the demands of industrial growth, energy is essential for global economic growth. The world has experienced an energy crisis as a result of this unanticipated and excessive level of energy consumption, such as the depletion of the finite supply of fossil fuels. In addition to the depletion of fossil fuel resources, transportation fuels such as gasoline and diesel account for 27% of all energy inputs. However, due to significant environmental risks such as acid rain, global warming, greenhouse gas emissions, and ozone layer depletion, these fuels are widely recognized as nonsustainable sources of energy (IEA 2007 and Ho et al. 2014). It is projected that by 2030, the global use of fossil fuels will increase by 57% (Outlook A.E2010; Nidal et al. 2022). Therefore, it is imperative to find fossil fuel substitutes to minimize air pollution, minimize shortages of gasoline and diesel, and minimize increases in the cost of transportation fuel (Marousek J et al., 2023; Solomon and Susan, 2007). In numerous studies, triacylglycerides (TAGs) have been shown to be promising substitutes for fossil fuels in energy production. In general, an eco-friendly rural economy would benefit from the production of biodiesel from biomass (Nidal et al. 2022; Barnwal et al. 2005). Therefore, the creation of biodiesel from algae was the main focus of this study.

Biodiesel is currently employed as a sustainable energy source for forthcoming energy demands because of its low emission of sulfur and nitrogen and because of the presence of traces of SO_x, NO_x, CO, benzene, and toluene; additionally, a good level of oxygen is liberated into the atmosphere (Liu et al. 2024; Marousek J et al. 2023; Tica et al. 2010). It is generally derived from vegetable oils or animal fats and consists of fatty acid alkyl esters (FAME), which are generated from the transesterification process through the conversion of triacylglycerols (TAGs) into diacylglycerols (DAG), free fatty acids (FFA) and phospholipids (PL) (Sangeetha et al. 2022; Leung et al. 2010; Vyas et al. 2010). Initially, first- and second-generation biofuel sources, such as edible nonedible crops and their residues, were used to produce biodiesel, but this has resulted in less production of biodiesel (Ulgiati 2001; Chisti2013). Therefore, the third generation of microalgae produces fuel oils (Chisti 2007; Hu et al. 2008; Brennan and Owende 2010; Huang et al. 2010). An adequate quantity of algae is an economically inexpensive source for producing biodiesel to replace fossil fuels (Malcata 2011; Pate et al. 2011; Klein-Marcuschamer et al. 2013; Pate 2013).

Many studies have reported that several algal species can secrete oils by limiting nutrient supplementation and stressing cells to produce oil by removing some nutrients (Chisti 2013). Algal crude oil has an energy content of

35,800 kJkg⁻¹, which is equivalent to 80% of the average energy generated from petroleum fuels (Abd Manan et al. 2022; Chisti 2012). Algal growth contributes 40% of the total oxygen available in the atmosphere from 75% of microalgae (Ponnuswamy et al. 2013). Hence, algal crude oil is identified as a potential substitute for transport fuels. This study focused on identifying microalgal strains that produce high lipid yields for the production of biodiesel. Therefore, an optimizing tool was utilized to determine the best combination of operating conditions for culture growth for lipid production intended to synthesize biodiesel.

In conventional optimization practice, the number of experimental combinations might be high, and this process is costly and prolonged. To overcome these issues, response surface methodology (RSM) was used in the present study (Hasan-Beikdashti et al. 2012). RSM is a statistical method for determining a mathematical model to optimize process constraints and is also a powerful tool for evaluating suitable operating conditions for enhancing algal growth and lipid yield by changing multiple factors in a batch culture system. This process is an efficient optimization process used for enhancing desired primary metabolic products in the bioprocessing field (Kanaga et al. 2016).

Algal fuel is recognized as a suitable alternative for transportation fuel (biodiesel) due to its biodegradability, high efficiency, perennial nature, low emission and **short cultivation** time. Hence, many researchers have focused on the production of biodiesel from algae. In the present study, the selected species were *Chlorella pyrenoidosa* and *Scenedesmus abundans*. *Chlorella* is a freshwater green algal species that belongs to the Trebouxiophyceae. Studies have proven that *Chlorella* is an excellent source of biodiesel production because of its rapid growth rate and uncomplicated species for cultivation, and it is also an outstanding algal feedstock for biodiesel production. *C. pyrenoidosa* has an average oil content of 28-32% by dry weight (Chisti 2007). These species have the capacity to hold and simulate triacylglycerols. Most recent studies on this species have assessed the growth of *C. pyrenoidosa* NCIM 2738 using multiple optimization techniques to optimize the factor conditions. Major nutrients, such as nitrate, phosphate and carbon sources, were shown to increase the lipid, biomass and carbohydrate contents by using a central composite design (CCD). Kanaga et al. (2016) showed that the maximum lipid accumulation, biomass production, and glucose consumption can be optimized by 28.9%, 1271.2 mg/L and 89.2%, respectively.

Scenedesmus abundans is also a freshwater green algal species that belongs to the Chlorophyceae family and is suitable for biodiesel production (Chellamboli et al. 2016). The maximum growth rate of *Scenedesmus* was obtained at 0.47 hr^{-1} by changing the environmental conditions such as light, carbon dioxide (CO_2), and temperature (Reddy 2002). Mandotra et al. (2014) investigated *S. abundans* species as potential feedstocks for biodiesel production. *S. abundans* accumulates high amounts of biomass and lipids in modified CHU-13 medium, and the biodiesel obtained from this species is equivalent to the international biodiesel standard. ÁlvaroGonzález-Garcinuno et al. (2014) achieved a maximum lipid yield of 3.55 mg/L/d for *S. abundans* under ammonium nitrate-cultured autotrophic conditions.

The lipids in *Scenedesmus obliquus* were cultured under various conditions. The greatest lipid content (43% of the total dry cell weight (dcw)) was observed under N deficiency, whereas that under the control condition was 12.7%. However, 30% (dcw) of lipids were obtained under P deficiency and thiosulfate conditions. The lipid yield was 61.3% after 8 days of culture using the central composite rotary design (CCRD), which yielded yields of 0.04, 0.03, and 1.0 g/L nitrate, phosphate, and sodium thiosulfate, respectively (Mandalet al. 2009). In 2016, Kanaga et al. optimized multiple parameters, such as nitrate, phosphate and carbohydrate sources, using the central composite design for biomass and lipid production in *Chlorella pyrenoidosa* NCIM 2738. The maximum lipid, biomass and carbon source consumption during the individual response had optimum values of 34.8% (w/w) and 1464.3 mg/L (93.4%), respectively. The maximum lipid, biomass and glucose consumption in multiple responses showed that the optimum values were 28.9% and 1271.2 mg/L 89.2%, respectively. The optimized parameters were 16.8 mM NaNO_3 , 300.9 l M K_2HPO_4 and 2.6% (w/v) glucose.

The growth of *T. suecica* was promoted by the use of different carbon (glucose and sodium acetate) and nitrogen sources (peptone, yeast extract, meat extract, malt extract, urea, sodium nitrate, and ammonium nitrate) in heterotrophic cultivation. The most supportive carbon and nitrogen sources (glucose, peptone, yeast extract, and meat extract) were selected for further optimization. The optimum concentrations of the sources were 5.78 g/L glucose, 9 g/L peptone, 4.48 g/L yeast and 3.01 g/L meat extract. The maximum cell concentration was 28.88 g/L under heterotrophic conditions, which was greater than that in photoautotrophic culture (8.40 g/L). However, the nonoptimized heterotrophic cultivation method yielded 13.81 g/L of biomass. Moreover, glucose consumption was greater in the optimized ($9.31 \text{ g cell/g glucose}$) medium than in the nonoptimized medium ($3.61 \text{ g cell/g glucose}$). In

2013, Chen et al. optimized the lipid content and calorific value of microalgae by changing major variables, such as temperature, light intensity, nitrogen content, and CO₂, using a 24 full factorial design. Among the variables measured, the most important variables for algal growth and lipids were CO₂ and light intensity. This study revealed that algal cultivation results in enough biomass production beginning on the 5th day (Azmaet al. 2011).

Therefore, the present work is focused mainly on improving biomass growth and lipid content for biodiesel production by augmenting experimental conditions, such as the use of various nutrients, to create stress on algae through the accumulation of lipids (Marousek J et al. 2023). The experimental conditions were optimized (using the RSM method) for the effective growth of biomass and maximum lipid content. Based upon these motivating features, the following objectives are framed. The high yield of lipids produced from fast-growing microalgae can be used for commercial production of biodiesel.

Methodology

Preparation of starter culture

C. pyrenoidosa and *S. abundans* were procured from the National Chemical Laboratory, Pune, India. A step-by-step scale-up procedure was performed for the pure strains. The selected algal strains were very well adapted to local environmental conditions. Hence, these strains have a positive impact on cell growth. In this study, seedling cultures were prepared by transferring a pure colony to sterilized media under aseptic conditions, after which the mixture was kept under controlled atmosphere conditions for approximately 30 days. This culture system is constantly supported by artificial illumination light, i.e., 13.514 $\mu\text{moles/m}^2/\text{s}$ (approximately 1000 lux) at a light:dark ratio of 16:8 h (Malebogo 2011).

Experimental Procedure

Inadequate or overnutrition may lead to less algae development. Therefore, this study was designed to supplement or starve targeted multinutrients for lipid production in algae in the presence of different ranges of illumination for better photosynthetic activity. According to this experimental design, the algal species studied were examined under different operating conditions, such as 2 to 30 days of culture, 0 to 1.5 g/L sodium nitrate (NaNO₃), 0 to 0.2 g/L potassium dihydrogen phosphate (KH₂PO₄), 0 to 0.1 g/L dipotassium hydrogen phosphate (K₂HPO₄), and 0 to 10,000 lux of light, as shown in Table 1. Apart from these optimized nutrients, such as NaNO₃, KH₂PO₄, and K₂HPO₄, a known quantity of other nutrients was dissolved in deionized water for the preparation of Bold Basal

Medium. The optimizing nutrients, such as NaNO_3 , KH_2PO_4 , and K_2HPO_4 , were added as per the requirements of the experimental runs and kept for medium sterilization in an autoclave. The sterilized media were kept under atmospheric conditions for 24 h to aid in the incorporation of carbon dioxide. Afterwards, the pure algae mother culture was aseptically grown, and a 10% inoculum concentration was used as a seeding culture for optimization. This research study was fully carried out in laboratory-scale Erlenmeyer flasks kept at ambient temperature under white fluorescent lamps with a light/dark ratio of 16:8 hr. In this investigation, the determination of multiple responses and independent factors affecting the lipid content and biomass of *C. pyrenoidosa* and *S. abundans* was confirmed using CCFD-RSM.

Response Surface Methodology

RSM was used to analyse the influence of the independent variables on a specific dependent variable (response). The independent variables are presumed to be continuous and can be controlled with negligible error. The response (y) is postulated to be a random variable. The individual variables ($x_1, x_2, \dots, x_k$) and the response (y) can be related as given in **Eq. (1)**:

$$y = f(x_1, x_2, \dots, x_k) + e \quad (1)$$

where y is the response of the system,

f is the unknown response function,

$x_1, x_2, \dots, x_k$ are the independent variables,

k is the number of independent variables, and

e is the statistical error.

Postulating a suitable approximation for the true functional relationship between the response (y) and the set of independent variables is the main purpose of response surface methodology (RSM). First, in a region of independent variables, a low-order (first order) polynomial is used. Eq. (2) can be used to express a first-order RSM.

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \varepsilon \quad (2)$$

For maximization problems, experiments are conducted along the path of steepest ascent until no further increase in the response is observed; these paths are known as the optimal region. In most cases, a second-order response surface model is used, which can be given as in **Eq. (3)**:

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k \beta_{ij} x_i x_j + \varepsilon \quad (3)$$

where x_i and x_j are coded independent variables,

$\beta_0, \beta_i, \beta_{ii}, \beta_{ij}$ ($i = 1, 2, \dots, k$),

β_{ij} ($i = 1, 2, \dots, k; j = 1, 2, \dots, k$)

are the regression coefficients for the intercept and linear, quadratic and interaction terms, respectively, and ε is the statistical error. In the present study, the RSM was used to determine the relationships of algal biomass and lipid yield with growth parameters. The uncoded variables are converted to coded variables using **Eq. (4)**:

$$x = \frac{x - [x_{max} + x_{min}]/2}{[x_{max} - x_{min}]/2} \quad (4)$$

where X = the natural variable and x = the coded variable.

The dimensionally coded variables x_1, x_2, x_3 , and x_4 vary between -1 and $+1$, while the variables are designated $-1, 0$, and $+1$, respectively. The mathematical representation of the response Y and the variables is given in **Eqs. (5)** and **(6)**:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 \quad (5)$$

and

$$\beta_{i,j} = 0, 1, 2, 3, 4, \dots, K \quad (6)$$

where β and k are regression coefficients and variables, respectively (Chellamboli et al., 2014).

Quantitative analysis

The most common methods used in this study include the choice of strain, quantification of algal biomass, cell disruption or pretreatment methods and lipid extraction. Furthermore, the analytical and quantification methods used are described in detail. The optimization of the obtained parameters was discussed in detail. Earlier, the literature states that growing algae in open/closed reactors was performed in well-equipped established laboratories or

industries for large-scale cultivation; however, for preliminary studies, Erlenmeyer flasks are sufficient to fix the essential parameters, media, and so on.

Dry weight method

The gleaned cells were harvested from the culture system, and then, centrifugation was performed to separate the algal cells from the solution at 5000 RPM for 10 min. The supernatant portion of the centrifuged culture was discarded, and the precipitated or deposited algal cells were suspended in ammonium solution to wash out the residues. The sample was subsequently dried at 104 °C for 24 hr to quantify the acquired biomass following **Eq. (7)** (Chellamboli et al. 2014):

$$\text{Dry weight of biomass} = \frac{[\text{Final weight of crucible} - \text{Initial weight of crucible}]}{\text{Culture volume}} \text{ g/L} \dots\dots\dots (7)$$

Optical density method

The growth phases of the algal cultures were determined by measuring the absorbance at 660 nm with a UV-2600 Shimadzu, and the corresponding dry weight was calculated from the standard algal growth plot.

Determination of lipids

To determine the total lipid content in algae, several physical disruptions, such as osmotic shock treatment and solvent extraction processes, of intracellular activity were applied to isolate lipids from algae cells. The required amount of algal culture was harvested from the culture system subjected to ultrasonication at 25 kHz for 15 min at 25°C to break the cells. The appropriate concentration of osmotic shock agent was applied to the aliquot culture by dissolving 0.1 g/ml NaCl in the cell culture mixture, followed by mixing n-hexane and methanol at a ratio of 7:3. The prepared sample was continuously shaken and incubated for 24 hr at 100 RPM and 30°C. Furthermore, the sample was transferred to a separating funnel for phase separation. The upper layer was separated and consisted of n-hexane and dissolved lipid cells. The organic phase was removed to a preweighed crucible (W_1) and dried in a vacuum oven at 60°C for 24 hr. The mass of lipids was quantified by calculating the difference between the initial weight (W_1) and the final weight of the dried lipid (W_2) **via Eq. (8)** (GursongYoo et al. 2012):

$$\text{Lipid Yield (\%)} = \frac{\text{Weight of Oil extracted}}{\text{Weight of Biomass}} \times 100 \dots\dots\dots (8)$$

Results and Discussions

This study aimed to identify better growth combination factors for the enhancement of biomass and lipids in *C. pyrenoidosa* and *S. abundans* using RSM. Therefore, supplementation and suppression of several major nutrients were carried out during the growth of algae culture by changing the concentration and varying the illumination range for better photosynthetic activity. Therefore, this exploration addresses the determination of multiple responses and independent factors, as shown in Tables 1 and 2, for lipids and the effect of biomass rate on the growth of *C. pyrenoidosa* and *S. abundans* using RSM. In this study, the independent variables were A- NaNO_3 , B- K_2HPO_4 , C- KH_2PO_4 , D-Time, and E-Light intensity, and the dependent variables were Y_1 -dry biomass and Y_2 -lipid yield percentage for both algae. This multiresponse study can improve the biological activity and characteristics of algae. Even overenrichment may hinder the metabolic improvement of algae. Therefore, this study was executed to determine the exact nutrient levels of the selected algal species.

Optimization of multiple nutrients for determining the biomass concentration of *C. pyrenoidosa* and *S. abundans*

The experimental design included a set of 50 runs with different nutritional concentrations, and light was applied under controlled conditions. The results showed that in the multinutrient optimization study, the dry biomass concentration varied from 1.72 to 99.02 mg/L and from 1 to 222 mg/L for *C. pyrenoidosa* and *S. frugiperda* and *S. abundans*, respectively. The impact of different multinutrient factors on the growth of *C. pyrenoidosa* showed that moderate changes occur at the specified concentration for the production of biomass. When *C. pyrenoidosa* grows, minimum to maximum concentrations are used, and their corresponding response to applied conditions has a modest effect on the growth of algae. Individual factors strongly influence the biomass production of *C. pyrenoidosa*; for example, 99.02 mg/L dry biomass was obtained from the following conditional factors: 0.75 g/L NaNO_3 , 0.05 g/L K_2HPO_4 , 0.1 g/L KH_2PO_4 , 16 days of cultivation and 5000 lux of light. The final quadratic equation for dry biomass obtained for *C. pyrenoidosa* is given below in **Eq. (9)**:

$$Y_1 \text{ biomass of } C. \text{ pyrenoidosa, } \left(\frac{\text{mg}}{\text{L}} \right) = -336.725 + 107.17 A + 5084.65 B + 1599.78 C + 16.402 D + 0.0207 E - 176.87 AB + 378.87 AC - 0.2896 AD - 0.0025 AE - 9505.64 BC - 58.24 BD + 0.024 BE +$$

$$15.099 CD - 0.0013 CE + 0.0002 DE - 80.9128 A^2 A^2 - 31405.4 B^2 - 8199.84 C^2 - 0.4523 D^2 - 2.7 \times 10^{-6} E^2 \dots \dots \dots (9)$$

The experimental results revealed a maximum biomass production of 222 mg/L for *S. abundans* under the combined effect of certain factor conditions, i.e., 1.07 g/L NaNO₃, 0.07 g/L K₂HPO₄, 0.06 g/L KH₂PO₄, 10 days of culture and 2898 lux of light. Hence, the modelling and statistical study revealed accurate conditional factors, which were identified via RSM optimization. The actual quadratic model equation for this dry biomass concentration for *S. abundans* response is shown in **Eq. (10)**. These studies showed optimized dry biomass responses of 98.26 and 110 mg/L for *C. pyrenoidosa* and *S. abundans*, respectively, as shown in Fig. 1(a) and 2(a).

$$Y_2 \text{Biomass of } S. \text{abundans, } \left(\frac{\text{mg}}{\text{L}}\right) = +166.61 + 210.15A + 2437.06 B - 3028 C - 8.143 D - 4.85 \times 10 - 3E + 839.1 AB - 791.96 AC - 4.51AD - 0.0231 AE - 3606.25 BC - 14.65 BD - 0.153 BE + 60.10 CD + 0.2044 CE + 9.041 \times 10^{-4} DE + 8.66A^2 - 12850.58 B^2 + 7837.36 C^2 + 0.03507 D^2 - 1.285 \times 10^{-6} E^2 \dots \dots \dots (10)$$

The individual independent variables A, B, C, D, and E and the interaction of the independent variables AB, AC, AD, AE, BC, BD, BE, CD, CE, and DE had significant effects on the dependent variables. Hence, the model is valid for algae growth.

Effects of multiple stress conditions on the growth of *C. pyrenoidosa* biomass

The maximum growth was obtained at intermediate concentrations because concentrations above 0.75 g/L NaNO₃, 0.05 g/L K₂HPO₄ and 0.1 g/L KH₂PO₄ simultaneously decreased the growth of biomass yield in *C. pyrenoidosa*. Studies have shown that reducing the nitrogen content in algae cultures results in a reduction in biomass, but simultaneously, a reduction in the nitrogen source results in an increase in lipid production. Arumugam et al. (2013) reported that, compared with other nitrogen sources, sodium nitrate improved microalgal growth better. Wu et al. (2021) reported that phosphorus sources play a role in all living organisms during biological processes and growth development. Phosphate sources are used to influence biomass development at relatively low concentrations. An increase in culture time improved the growth of *C. pyrenoidosa*, particularly beginning on the 16th day. Overall, this treatment improved the biomass production of *C. pyrenoidosa*. Light intensity has a great influence on the growth of *C. pyrenoidosa*, but it also has limitations. Even when the light intensity exceeded 5300 lux, light

penetration was greater in *C. pyrenoidosa*. Due to this overillumination, *C. pyrenoidosa* has become intractable, and its range of biomass production has decreased, as shown in Fig. 1(a).

Impact of multiple stress parameters on the biomass production of *S. abundans*

The biomass concentration of *S. abundans* has different impacts on individual factors; for example, an increase in the NaNO_3 concentration has a stable response to the growth of *S. abundans*. An increase in K_2HPO_4 concentration strongly influenced the growth of *S. abundans*, and an increase in KH_2PO_4 concentration above 0.1 g/L had a decreasing effect on the growth of *S. abundans*. Similarly, increasing the culture timing (after the 16th day) slightly decreased the biomass concentration, increased the penetration range under light illumination (>5000 lux), and had a negative impact on the cell growth of *S. abundans*.

Statistical analysis of multiple stress factors involved in *C. pyrenoidosa* and *S. abundans* biomass production

The models for biomass generation were validated through additional regression analysis. The acquired data showed a standard deviation of 10.18, an R^2 of 0.9338, a mean of 43.05, and an adjusted R^2 of 0.8881. The results of the statistical analysis indicate that the biomass production in *C. pyrenoidosa* has an F value of 20.44, indicating the significance of the model. This large F value has a 0.01% probability of being caused by noise. When "Prob > F" values are less than 0.0500, the model terms are considered important. The significant model terms in this instance are D, E, AC, BC, BD, CD, A^2 , B^2 , C^2 , D^2 , and E^2 , which are displayed in Table 3 and Fig. 1(c) for *C. pyrenoidosa*.

Table 4 presents the statistical data on the biomass concentration in response to multinutrient optimization of *S. abundans*. The model is deemed significant based on its F value of 33.73. This large F value has a 0.01% probability of being caused by noise. Prob > F values less than 0.0500 suggest the significance of the model terms. The significant model terms for *S. abundans* in this instance are B, C, D, E, AB, AC, AD, AE, BE, CD, CE, DE, B^2 , C^2 , and E^2 as shown in Fig. 2(c).

Optimization of medium components for enrichment of lipid yield in *C. pyrenoidosa* and *S. abundans*

Lipid yields ranging from 1.12 to 36.3% and 2.5 to 56.98% were obtained for the growth of *C. pyrenoidosa* and *S. abundans*, respectively. The growth of *C. pyrenoidosa* under factorial conditions crossing beyond 0.75 g/L NaNO₃, 0.05 g/L K₂HPO₄, 0.1 g/L KH₂PO₄, a greater 16th day of culture and > 5300 lux of light intensity decreased the response in terms of lipid yield. The corresponding lipid yield quadratic model is shown in **Eq. (11) and (12)**.

$$\begin{aligned} \text{Lipid yield of } C. pyrenoidosa(\%), Y_1 = & +13.08 + 12.58 A + 281.30 B - 368.71 C + 0.6156 D \text{ Time} + \\ & 2.78 \times 10^{-3} E + 163.20 AB + 52.70 AC + 0.4738 AD + 3.77 \times 10^{-3} AE + 86.97 BC + 18.35 BD + \\ & 0.0215 BE + 6.85 CD + 0.0103 CE + 1.23 \times 10^{-4} DE - 31.71 A^2 - 7946.92 B^2 + 715.77 C^2 - \\ & 0.0971 D^2 - 8.92 \times 10^{-7} E^2 \dots\dots\dots (11) \end{aligned}$$

A maximum lipid yield of 56.98% was found for *S. abundans* after combination with 0.43 g/L NaNO₃, 0.03 g/L K₂HPO₄, 0.14 g/L KH₂PO₄, 10 days of culture, 2898 lux of light intensity and the optimized RSM model, as shown in **Eq. (12)**. The optimized response dry biomasses from the experiments were 98.26 and 110 mg/L, with 22.47 and 29.06% lipid yields for *C. pyrenoidosa* and *S. abundans*, respectively, and are shown in Fig. 1 (b) and 2(b).

$$\begin{aligned} \text{Lipid yield of } S. abundans(\%), Y_2 = & -43.26315 - 21.951 A - 79.94 B + 829.42 C + 3.02 D + 6.86 \times 10^{-3} E - \\ & 335.22 AB + 131.12 AC + 0.71 AD + 8.97 \times 10^{-4} AE - 3438.31 BC + 8.063 BD + 0.0538 BE - \\ & 22.75 CD - 0.011 CE - 3.75 \times 10^{-4} DE + 6.433 A^2 + 2441.41 B^2 - 1009.65 C^2 - 2.72 \times 10^{-3} D^2 - \\ & 5.555 \times 10^{-7} E^2 \dots\dots\dots (12) \end{aligned}$$

Influence of different stress parameters on the enhancement of lipid yield in *C. pyrenoidosa* and *S. abundans*

Studies have proven that an increase in nutritional supplements results in a decrease in most nutrients because the growth of microalgae requires only a moderate range of supplements once a certain range of nutrients is present; this range has been noted as nutritional overburden on growing cells. This leads to a decrease in growth development (Geada et al. 2023). This study revealed a low range of phosphates and a moderate range of nitrate sources good lipid yields in *C. pyrenoidosa* and *S. abundans* by using an experimental design, RSM. This study used the CCD model to optimize the nutrient source. The CCD model was also used in nutrient source optimization by controlling the +α and -α values. The combination levels for each numeric factor had a set of five levels, which included +α, -α, -1, 0 and +1. Each experimental run had a different level of combination effect factors to identify the maximum yield of lipid production. Overall, the RSM exploits the individual and interaction effects on operating

parameters and responses. Under these conditions, approximately 50 runs were performed to determine the maximum yield of lipids and biomass in the algae. The combined and individual factors had highly significant effects on the lipid yield.

Statistical analysis of lipid enhancement in *C. pyrenoidosa* and *S. abundans* plants grown under multiple stress conditions

In *C. pyrenoidosa*, a statistical analysis of lipid development yielded a Model F value of 7.59, which implied that the model was significant. There is only a 0.01% chance that an F value above this large value could occur due to noise. A value of "pro > F" less than 0.0500 indicates that the model terms are significant. In this case, A, E, AE, BD, CD, A², B², D², and E² are significant model terms tabulated in Table 5 for the optimization of multiple responses in *C. pyrenoidosa*. The standard deviation was 4.65, the R² value was 0.8396, the mean was 11.06, and the adjusted R² was 0.7291 for the predicted model for *C. pyrenoidosa* Fig. 1(c).

Statistical analysis of *S. abundans* grown under different stress conditions for lipid enhancement showed that the regression model had a standard deviation (SD), R², mean and adjusted R² of 6.47, 0.8286, 24.55, and 0.7103, respectively, which were used to indicate the response of lipid yield in *S. abundans*. A Model F value of 7.01 implies that the model is significant. In this case, C, E, BC, BE, CD, DE, C², and E² are significant model terms and are tabulated in Table 6 and Fig. 2(c).

Conclusion

The production of biodiesel was improved by the application of stressful environmental conditions in the culture system to achieve the highest yield. Supplementation with multiple nutrients at different light intensities was performed for *C. pyrenoidosa* and *S. abundans* grown under stressed conditions, which were investigated using RSM. The optimal conditions were determined using CCFD-RSM, and the results showed that 98.26 and 110 mg/L dry biomass and 22.47 and 29.06% lipid yield were achieved for *C. pyrenoidosa* and *S. abundans*, respectively. The maximum biomass and lipid production were observed for 0.805 g/L NaNO₃, 0.052 g/L K₂HPO₄, 0.099 g/L KH₂PO₄, 17 days of culture and 5168.39 lux for *C. pyrenoidosa*, whereas the maximum *S. abundans* biomass and lipid content were obtained for 1.065 g/L NaNO₃, 0.071 g/L KH₂PO₄, 0.058 g/L KH₂PO₄, 22 days of culture and 2897.76 lux of light. Henceforth, an alga has been treated under different operating conditions, which include starved and enhanced nutrient supplements. Studies have shown that algae can produce more lipids under nitrate and

phosphate starvation conditions. Most algae are universal photosynthetic organisms. Treating algae under nutrient starvation conditions with a different range of illumination (fluorescent light) may improve yield.

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Author details

¹Dr. C. Chellamboli, ²Dr. M. Perumalsamy, ³Mr. Mohamed Arshath, Bioseparation lab, Department of Chemical Engineering, National Institute of Technology, Tiruchirappalli – 620015, Tamil Nadu, India. Tel: 9489799295; Fax: +91-431-2500133; email:c.chilambu@gmail.com. The electronic supplementary information (ESI) is available: [The native format of the figures has also been uploaded].

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Declarations

Ethical Approval

345 This study does not include human or animal objects. Hence, no approval is needed.

Consent to Participate

346 Yes

Consent to Publish

All the authors declare their consent to publish this article.

Author Contribution

347 The data were gathered for publication and additional research investigations by the authors, who also established
348 their research plan and conducted their study experiments in a research laboratory. Then, statistical studies were
349 carried out on the obtained results, which were also included in this manuscript. Dr. C. Chellamboli and Dr. M.
350 Perumalsamy are the primary responsibility for the data representation and final submission of the manuscript; Dr.
351

C. Chellamboli is the corresponding author for this manuscript. Mr. Mohamed Arshath assisted in the analysis of the experiments. All the authors read and approved the final manuscript.

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Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figures

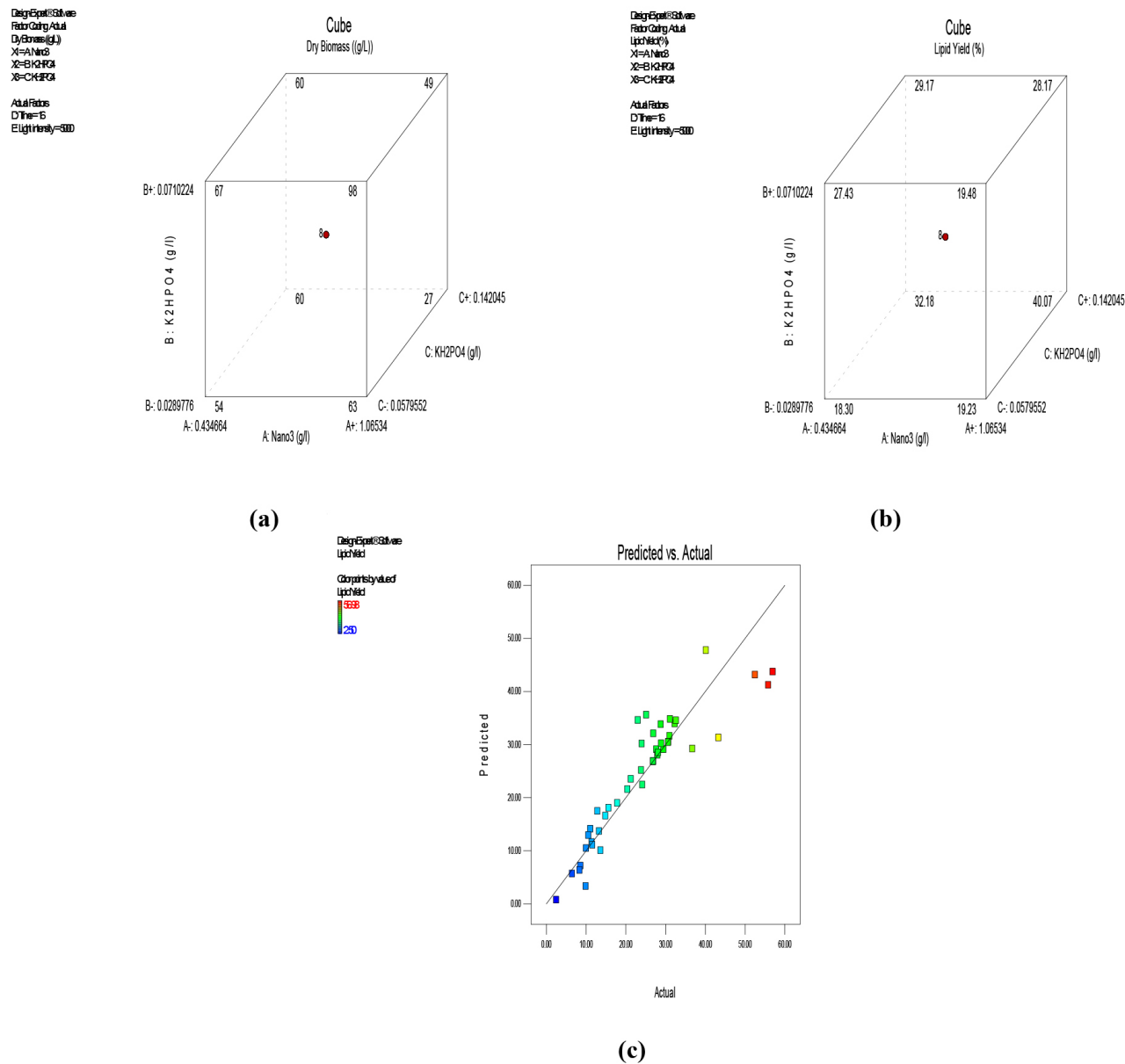
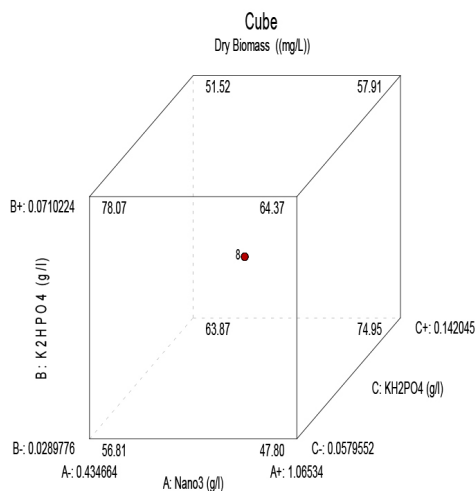


Figure 1: Optimization of medium components for maximization of multiresponse *C. Pyrenoidosa*
(a) Response on dry biomass production in multi factor response of *C. Pyrenoidosa*, (b) Response on lipid yield in multi factor response of *C. Pyrenoidosa*, (c) Depiction of Actual versus Predicted for multiresponse of *C. Pyrenoidosa*

Figure 1

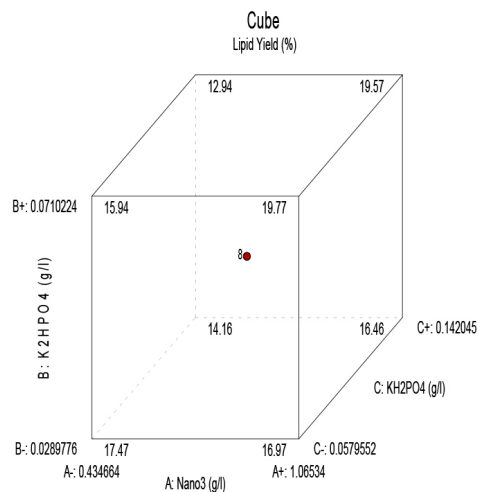
See image above for figure legend.

Design: Split-Block
 Factor: Caring Actual
 Dry Biomass (mg/L)
 X=A: Nano3
 X=B: KH₂PO₄
 X=C: KH₂PO₄
 Actual Factor
 D: Time=16
 E: Light intensity=600



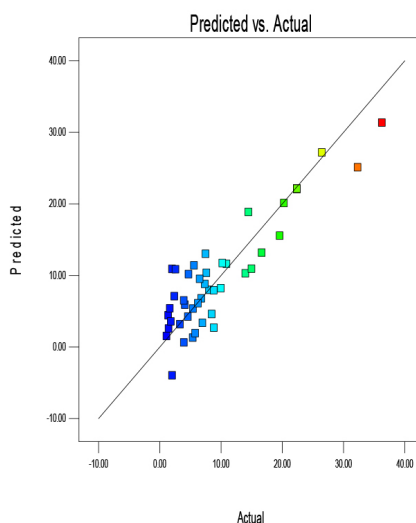
(a)

Design: Split-Block
 Factor: Caring Actual
 Lipid Yield (%)
 X=A: Nano3
 X=B: KH₂PO₄
 X=C: KH₂PO₄
 Actual Factor
 D: Time=16
 E: Light intensity=600



(b)

Design: Split-Block
 Factor: Caring Actual
 Lipid Yield (%)
 X=A: Nano3
 X=B: KH₂PO₄
 X=C: KH₂PO₄
 Actual Factor
 D: Time=16
 E: Light intensity=600



(c)

Figure 2: Optimization of medium components for maximization of multiresponse *S.abundans*

(a) Response on dry biomass production in multi factor response of *S.abundans*, (b) Response on lipid yield in multi factor response of *S.abundans* , (c) Depiction of Actual versus Predicted for multiresponse of *S.abundans*

Figure 2

See image above for figure legend.

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