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Two-stage cultivation of microalga for sustainable biofuel production

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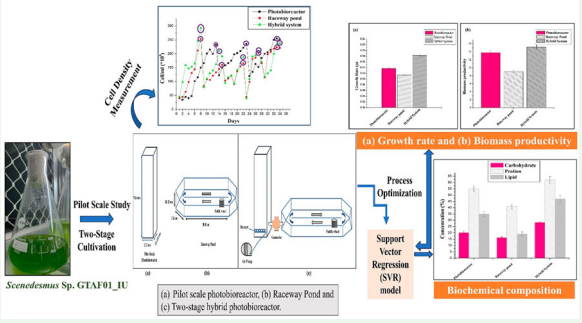
ABSTRACT

This two-stage system comprises an airlift-driven low-tubular photobioreactor for continuous microalgal (strain *Scenedesmus* sp. GTAF_01 IU) growth and an open raceway pond for stress-induced coordinated lipid accumulation due to nutrient deprivation. A support vector regression (SVR) model was used to develop the predictive model to predict the cell biomass on a dry basis at different culture conditions. A maximum biomass (dry weight) of 1.65 g was achieved at a light intensity of 27 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), mixing speed of 150 revolutions per minute, pH 7, and light and dark cycle of 12 h each. The model accuracy was verified by performing experiments at the optimal conditions that resulted in an enhanced biomass (dry weight) of 1.69 g, which was close to the expected one. The results also showed a significant increase in carbohydrate, protein, and lipid content of 27%, 64%, and 47%, respectively. This study provides novel insights into a two-phase cultivation system combining a closed photobioreactor and an open raceway pond for *Scenedesmus* sp. GTAF_01 IU, resulting in enhanced biomass growth and lipid content. This enhancement supports its potential for sustainable biofuel production within a circular economy framework.



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Photobioreactor; raceway pond; lipid; two-stage cultivation; *Scenedesmus* sp; sustainable development; circular economy

1. Introduction

Microalgae are a diverse group of microorganisms having similar characteristics to both prokaryotes and higher plants. They are widely distributed, present in aquatic environments (marine and fresh) and terrestrial ecosystems. Microalgae can exist as single-celled organisms or simple colonial structures, typically displaying rapid growth rates (1). Under optimal conditions, microalgae can convert approximately 9–10% of solar energy into biomass. However, in full sunlight, they often receive more light energy than they can utilize, leading to a photosynthetic efficiency of less than one-third of their theoretical maximum (around 10% of solar light) (2). These microorganisms play a pivotal role in the Earth's ecosystem,

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significantly contributing to the global nitrogen and carbon cycles. Microalgae are known for atmospheric CO_2 fixation by 10–50%, which is notably higher than that of terrestrial plants (3). Through photosynthesis, they harness light energy to produce organic matter, accounting for approximately 50% of the total primary production on our planet. Microalgae possess the remarkable ability to convert atmospheric nitrogen (N_2) into organic nitrogen, thus serving as primary contributors to global biological nitrogen fixation (4).

Currently, around 90% of energy is derived from fossil fuels, whereas only about 10% is derived from renewable energy sources (5). It is clear from the projected increases in energy consumption that economically viable conventional oil sources will disappear after 2050 (6). The excessive use of fossil fuels for energy production has resulted in worldwide environmental pollution, climate change together with biotic health problems and ecological deterioration (7). Consequently, there exists an urgent imperative to advance sustainable, clean, and renewable energy sources and technologies worldwide (8). Furthermore, the mitigation of environmental contamination is a critical global concern. Microalgae can be an outstanding candidate for resolving the environmental issues through the production of biofuel. This has the potential to supplant fossil fuels in an economically viable manner, resulting in carbon dioxide (CO_2) sequestration and reduced greenhouse gas emissions. The emission of CO_2 has increased rapidly in the last few years. As per the report of the Global Carbon Project in 2021, the global industrial CO_2 emission has reached to 36.4 Gt. This exceeds the earth's annual natural carbon sequestration capability of 21 Gt (9). This trend is likely to continue with a projected increase of both global energy consumption and CO_2 emissions by 51% in 2050 as compared to 2005 (10). Intensive carbon capture and storage (CCS) technologies for CO_2 sequestration have been thoroughly evolved to control the arising negative impacts. Microalgae are responsible for fixing carbon in biomass growth through photosynthesis, which is part of the ecosystem. Wet weight of microalgae biomass = 0.3063 × (MJ/kg of CO_2)% because carbon represents about 50% of the dry weight of microalgae biomass, 100 tons of algae biomass ≈183 tons CO_2 (11). Microalgae–CO_2 fixation is the most potential and attractive approach for CCS, due to its natural distribution, high rate of photosynthesis, fast growth rates, and high adaptability towards the environment, as compared to other methods. Various microalgal species exhibit distinct capabilities in carbon dioxide fixation and photosynthetic efficiency, which are critical for biofuel applications and carbon-neutral strategies. Table 1 summarizes recent findings on CO_2 fixation rates and efficiencies of commonly studied microalgae. Microalgae, due to their rich biodiversity, can generate a broad spectrum of value-added substances, including protein, carbohydrate, lipids, pigments, and various other bioactive compounds, which find application in diverse product contexts. Notably, the extensively explored compounds include carotenoids, phycobiliproteins, and polyunsaturated fatty acids (PUFA), such as docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids. Microalgae have versatile applications spanning the food, feed, biofertilizer, and cosmetic industries (11). Biomass derived from microalgae is a sustainable, renewable, and eco-friendly resource that can be utilized for the production of biofuels and bioenergy (19).

Microalgal oil is valuable, particularly as a substitute for omega-3-rich fish oil. Nevertheless, enhancing the economic feasibility of commercial microalgae cultivation for biodiesel, which is a relatively low-value product, requires further refinement. Microalgae can accumulate substantial lipid bodies rich in triacylglycerides under unfavorable conditions, like nutrient restriction (20). During these conditions, microalgae cease division but continue photosynthesis, as well as the accumulation of triacylglycerides, is regarded as a survival strategy to withstand unfavorable circumstances (21). Despite the plausible applications in terms of biofuel production, its commercial viability is limited by several factors, including high biomass production

Table 1. Carbon fixation capacity and photosynthetic efficiency of microalgae.

Microalgal species	CO_2 fixation rate (g CO_2/L/day)	Photosynthetic efficiency (%)	References
Chlorella vulgaris	1.7–2.2	5–6.5	(12)
Nannochloropsis sp.	1.5–2.0	6.0–7.2	(12)
Spirulina platensis	1.0–1.6	3.5–5.5	(13)
Scenedesmus obliquus	5.6	4.5–6.0	(14)
Scenedesmus obliquus CNW-N	1.03	10.5	(15)
Scenedesmus obliquus PF3	0.75	-	(16)
Scenedesmus obliquus SA1	0.25	-	(17)
Scenedesmus obtusiusculus	0.65	-	(18)

rate and low microalgal biofuel yield (22). Different stress methods have been implemented during microalgal growth to enhance microalgal lipid content. These strategies increase the microalgal lipid content, compromising its growth (23). Also, coupling microalgae cultivation with wastewater and CO_2 emanating from power plants can be considered as a promising route for the production of biofuel shortly (24).

In addition to liquid fuels such as biodiesel and bioethanol, microalgal solid biofuels, including biochar, briquettes, and pellets, represent a promising avenue for sustainable energy generation. These solid biofuels are derived from thermochemical processes like pyrolysis and torrefaction, and they offer high energy density, improved transportability, and potential for long-term carbon sequestration. Their application contributes to a more integrated and efficient microalgal biorefinery approach, aligning with circular economy and waste-to-energy principles. Recent reviews have emphasized the significance of both microalgae and macroalgae as viable feedstock for third-generation bioethanol production, owing to their high carbohydrate content and rapid biomass productivity. Furthermore, the challenges and future research prospects in microalgal biofuel production, including scalability, harvesting, and energy input–output ratio, are increasingly being addressed through advancements in process optimization and integrated cultivation systems. These developments underline the critical role of solid biofuels in expanding the scope and viability of microalgae-based energy systems (25,26).

In biodiesel production through microalgae cultivation, the goal is to maximize lipid yields (or lipid productivity), taking into account both lipid contents and growth rates. In batch cultivation systems, microalgae undergo exponential growth to increase their biomass, followed by an induction of lipid synthesis, typically involving nutrient deprivation towards the end of the growth phase. Various lipid induction methods are accessible, and combining them could result in higher lipid concentrations (27). There are two primary ways of microalgae cultivation: open cultivation systems (such as tanks, raceway ponds, and open ponds) and controlled closed cultivation systems using various kinds of bioreactors. The initial attempt to scale up microalgae cultivation was achieved by using open raceway ponds (28). Subsequently, extensive research has been conducted on microalgae cultivation in open systems. Open cultivation systems offer advantages such as minimal capital and operating costs, as well as reduced energy requirements for culture mixing. However, they have drawbacks, including the need for large areas, susceptibility to contamination (e.g. from birds), and vulnerability to adverse weather conditions. High bacterial loads, contamination with other microalgae, and grazers are prevalent in large-scale open pond cultivation systems, with rotifers causing damage to cultures of *Nannochloropsis*, *Scenedesmus*, *Chlorella,* and *Tetraselmis,* and *amoeba* posing a threat to diatoms, often leading to contamination within days of detection (29). Additionally, open pond systems present challenges in controlling growth parameters like evaporation and temperature variation (30).

In contrast, closed cultivation systems, referred to as closed photobioreactors (PBRs), offer improved quality control due to their highly controlled conditions. PBRs can be tailored and optimized for specific strains, occupy less space, enhance light availability, and significantly reduce contamination (31). However, they also have drawbacks such as biofouling, overheating, benthic algae growth, cleaning issues, growth-limiting high dissolved oxygen levels, and notably, high capital costs for design and operation (32). Cultivation system designs and principles vary on the basis of specific requirements (33). Open ponds in wastewater treatment plants can be gravity-driven or circular. Tubular PBR designs have evolved to improve light availability and culture mixing for various product applications. PBRs are highly efficient when operated with continuous cultures (34). Numerous studies have been conducted on closed and open cultivation methods. However, limited attention has been given to two-stage cultivation systems. Two-phase cultivation systems, which separate biomass growth from lipid accumulation, is an advantageous microalgae cultivation system. Recent life cycle analysis suggests that two-stage cultivation may reduce environmental impact as compared to various open and closed cultivation systems (35). A large-scale hybrid cultivation has indicated that PBRs are economically viable for providing a consistent and continuous inoculum for short-term batch open pond cultures. This prevents biological system crashes as compared to longer-term open pond cultures (36).

Among the algal species, a distinctive genus *Scenedesmus* has an immense consideration to turn out biodiesel, as it contains an array of lipid contents. *Scenedesmus* sp., which is part of the ecosystem and is very accessible, is a more environmentally friendly and renewable fuel that can be produced. In terms of oil production, members of the *Scenedesmus* genus have been identified as potential oil-producing species, with both rapid growths, together with relatively high lipid content. *Scenedesmus obliquus* belongs to green microalga that has been extensively researched due to its potential as a source of biodiesel. The use of *Scenedesmus*

obliquus in a recirculatory aquatic system has been investigated for the generation of biodiesel in conjunction with the treatment of waste from fish pond outflow and chicken litter (37). The generated biodiesel's fuel characteristics complied with Indian as well as international standards. *Scenedesmus obliquusis* often found in aquaculture habitats, freshwater, and marine, suggesting its potential as an energy resource. The lipid productivity of *Scenedesmus obliquushas* resulted in an FAME profile suitability. The superabundance of oleate and palmitate in *S. obliquus* biomass makes it an ideal feedstock for biodiesel generation (38). The present investigation aims at the design of a cost-effective airlift-driven low-tubular photobioreactor (PBR) for continuous microalgal culture growth. An open raceway pond was used for producing stress along with lipid accumulation. The productivity variations have also been studied among these systems. The key parameters such as sunlight, nutrients, agitation rate, and pH over time were monitored to understand their significance on microalgae growth and lipid productivity. To find the optimum culture conditions a Support Vector Regression model (SVR) was developed, which was further used as an objective function for genetic algorithm.

Microalgal cultivation and harvesting offer significant potential for advancing circular economy practices and contributing to carbon neutrality, as they enable the recycling of nutrients and wastewater while capturing atmospheric CO_2 through photosynthesis. Integrating microalgae into biorefinery frameworks not only supports resource recovery and waste minimization but also aids in reducing greenhouse gas emissions, making it a sustainable and eco-efficient approach for future bio-based industries (39,40).

2. Materials and methods

2.1. Microalgae cultivation and analysis

Previously, isolated and purified *Scenedesmus* sp. GTAF_01 IU reflected a high lipid concentration under abiotic stresses. The culture was maintained in the cyanobacterial culture room, Department of Bioengineering, Integral University, Lucknow. The temperature was maintained at 27 ± 2°C. BG11 medium with pH 7.0 was used for the growth of the culture. 75 µmol/m^2/s photosynthetic photon flux density (PPFD) was provided by white LED lights (Philips Make, India). 1 µL of acetic acid with 100 µL of culture was used to ensure the loss of its mobility. The growth and doubling time of the *Scenedesmus* sp. was calculated by using Equation (1) (12).

$$\text{Growth rate } (\mu) = \left(\frac{\mathbf{Ln}\left(\frac{\boldsymbol{N2}}{\boldsymbol{N1}}\right)}{\boldsymbol{T2} - \boldsymbol{T1}} \right) \tag{1}$$

where μ = growth rate $N1$ and $N2$ = biomass at time ($t1$) and ($t2$)

$$\text{Doubling time } (t_d) = \frac{\boldsymbol{Ln}(2)}{\boldsymbol{\mu}} \tag{2}$$

2.2. Optimization of culture conditions

One factor at a time approach was used for designing of experiment. A total of 60 experiments were performed by varying four independent parameters, i.e. pH, mixing speed, light intensity, and light–dark time. The dependent variable was biomass on dry weight basis. Out of these, 80% data points were randomly selected for training and 20% data points were used for validation. An SVR model was used to develop the predictive model to predict the cell biomass on dry basis at different culture conditions. SVR can handle nonlinear relationships between the input and output variables, as well as outliers and noise in the data. SVR is based on the idea of kernel methods, which transform the input space into a higher-dimensional feature space, where a linear function can be found more easily (13). SVR has been shown to give better performance than the Artificial Neural Network (ANN) and is less prone to overfitting with the training data (14,15). The trained SVR model was validated using the validation data set. The SVR model was used as an objective function for the Genetic Algorithm (GA) for identifying optimum culture conditions. The optimum values predicted by the GA were validated experimentally.

2.3. Culture scale up and monitoring

A glass bottle filled with fresh autoclaved 1 L BG 11 media was inoculated with 50 mL preculture of pure *Scenedesmus* sp. GTAF_01 IU. Syringe filter was used for supplying the filtered air. This enables uniform mixing of the culture and prevents its stagnation at the bottom of the bottle. After 4 days, 250 mL culture was transferred into 10 L glass bottle filled with 4.75 L autoclaved BG11 media. Millipore syringe filter was used for supplying filtered air. This culture bottle was left undisturbed for 4 days. All subsequent experiment was performed using this culture as the starting culture. 5 mL sample of culture was used daily from closed photobioreactor and raceway ponds for the monitoring of the cell densities. Daily sample collection was done at 5:00 P.M. (IST). During nutrient depletion condition (less than 10%) BG11 media was added in closed PBR and raceway ponds. The nutrition depletion condition was marked when cell density reaches 150×10^4 cells/mL. Once the cell concentration reaches 200×10^4 cells/mL, half of the volume was harvested. In case of two-stage cultivation system, when cell density reaches 200×10^4 cells/mL in closed photobioreactor, half of the culture volume was transferred to open raceway pond for lipid induction phase for around 3–4 days. The transferred volume from closed photobioreactor was again maintained with fresh BG11 media. Parallel to this, the temperature profile was also monitored around 12 AM in the month of January at the time of sampling (Figure 3).

2.4. Determination of biomass composition

The harvested microalgal biomass was lyophilized and resulting microalgae powder (10 mg). The culture was suspended in deionized water (10 mL) and sonicated. 30 µL of phenol (5%) and 150 µL of concentrated sulfuric acid were added to 50 µL of the sample. Finally, optical density (OD) at 490 nm was determined and carbohydrate concentration was measured in equivalent glucose. A 100-mg dried algae suspension and 10 mL of 0.5 M NaOH were prepared to extract protein from microalgae (16). The suspension was vortexed, sonicated, and incubated at 100 °C for 120 minutes. Subsequently, the sample was centrifuged, and 1 mL of supernatant was mixed with 4 mL of Bradford reagent and analyzed at 595 nm (17). 10 mg of dried algae was suspended in 5 mL of methanol and 2.5 mL of chloroform. It was vortexed, sonicated, and centrifuged to collect the supernatant. The same procedure was repeated with half the volume of the solvent. The supernatants were collected, and 4 mL of 1% NaCl, along with 4 mL of chloroform, was added to the test tube. The resulting dark green layer formed after centrifugation, including lipids, was collected carefully in a glass vial, and the lipid content was calculated gravimetrically after evaporation of the chloroform phase (18).

2.5. Determination of pigment content

The determination of chlorophylls (a and b) and carotenoids was done according to the protocol given by Afzal et al. (41). The 10 mL microalgal culture was centrifuged at 500 rpm for 10 minutes. The pellet was separated and mixed with distilled water (1 mL) and acetone (4 mL). This solution was kept in the fridge overnight. Thereafter, it was centrifuged at 500 rpm for 5 minutes, and the OD of the supernatant was taken at 645 nm for chlorophyll (a), 663 nm for chlorophyll (b), and 470 nm for carotenoids. 80% of the acetone was used as a blank. The chlorophyll and carotenoid concentrations were finally estimated by Equations (5)–(7)

$$\text{Chlorophyll(a)} = 15.65A_{663} - 7.34A_{645} \tag{5}$$

$$\text{Chlorophyll(b)} = 27.05A_{645} - 11.21A_{663} \tag{6}$$

$$\text{Carotenoids}(\text{mgL}^{-1}) = (1000A_{470} - 2.86\text{Chla} - 129.2\text{Chlb})/221 \tag{7}$$

The cellular content was expressed in mg L^{-1}.
where Chla is Chlorophyll (a) and Chlb is Chlorophyll (b).

2.6. Microscopic analysis

Following a lipid induction phase, the microalgae cells underwent staining with 2 mg/mL Nile red (Sigma, USA), which was dissolved in acetone for a duration of 15 minutes. Subsequently, the stained cells were captured using a fluorescent microscope (Leica Make, Germany).

2.7. Statistical analysis

All experiments were done with mid-log phase cells and repeated thrice to ascertain the duplicability of the outcomes. Values are denoted as ±Standard error mean ($n = 3$) (SEM). Tests of significance were applied by one-way analysis of variance (ANOVA), followed by Dunnett's test with control to focus on the efficacy of different formulated groups (42) with the help of Graph Pad Prism 5.

3. Results and discussion

3.1. Design and construction of pilot-scale PBR and raceway pond

A closed tubular PBR and an open pond raceway were designed for outdoor algae cultivation. The volume of the PBR was 10 L and that of the raceway pond was 20 L (Figure 1). The PBR and raceway pond had volumes

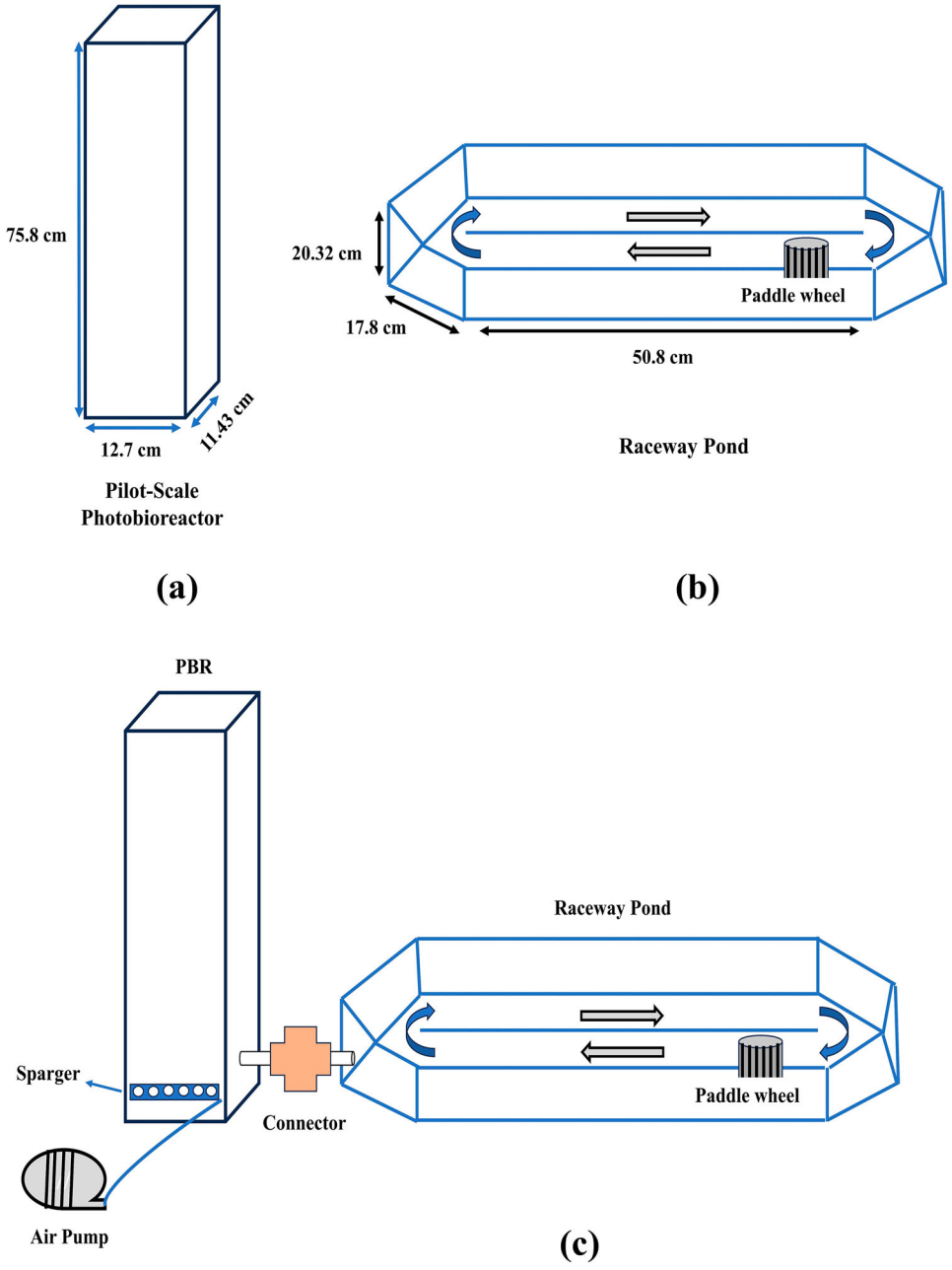

Figure 1. Schematic representation of (a) pilot-scale photobioreactor, (b) raceway pond, and (c) two-stage photobioreactor.

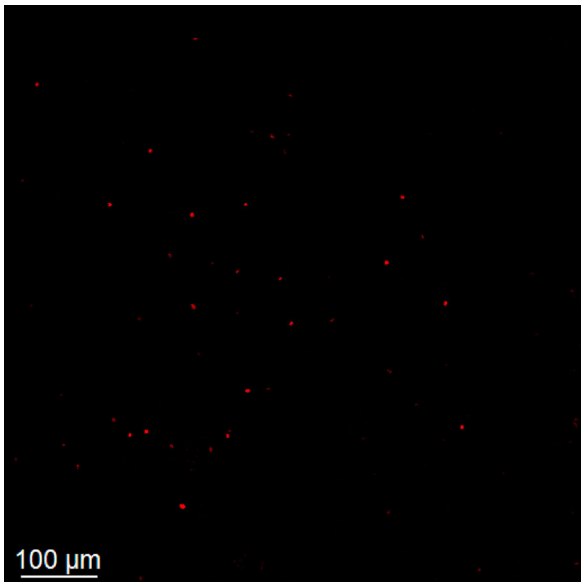

Figure 2. Nile red stained samples of *Scenedesmus* sp. GTAF 01_IU.

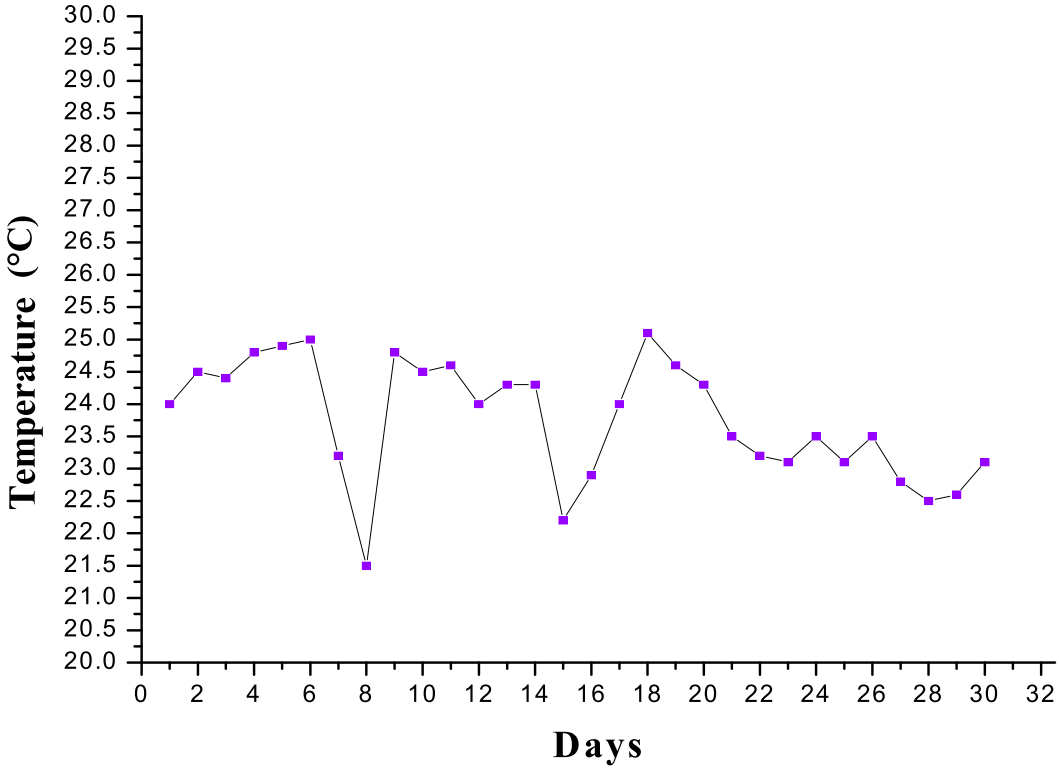

Figure 3. Daily temperature observation at around 12 AM in the month of January at the time of sampling.

of 11 and 32 L, respectively. The airlift mechanism was used for the mixing of culture. The PBR and raceway pond were installed side by side simultaneously for the comparison of these two cultivation systems. These systems promote the algal growth cycle and lipid induction in algal cells. In conditions of nutrient deprivation, lipids synthesized by microalgal cells manifested as vivid yellow globules when stained with Nile red and examined under epifluorescent light (Figure 2). The harvesting was done as soon as the lipid-rich biomass was cultivated. Parallel to this, the temperature profile was also monitored around 12 AM in the month of January at the time of sampling (Figure 3).

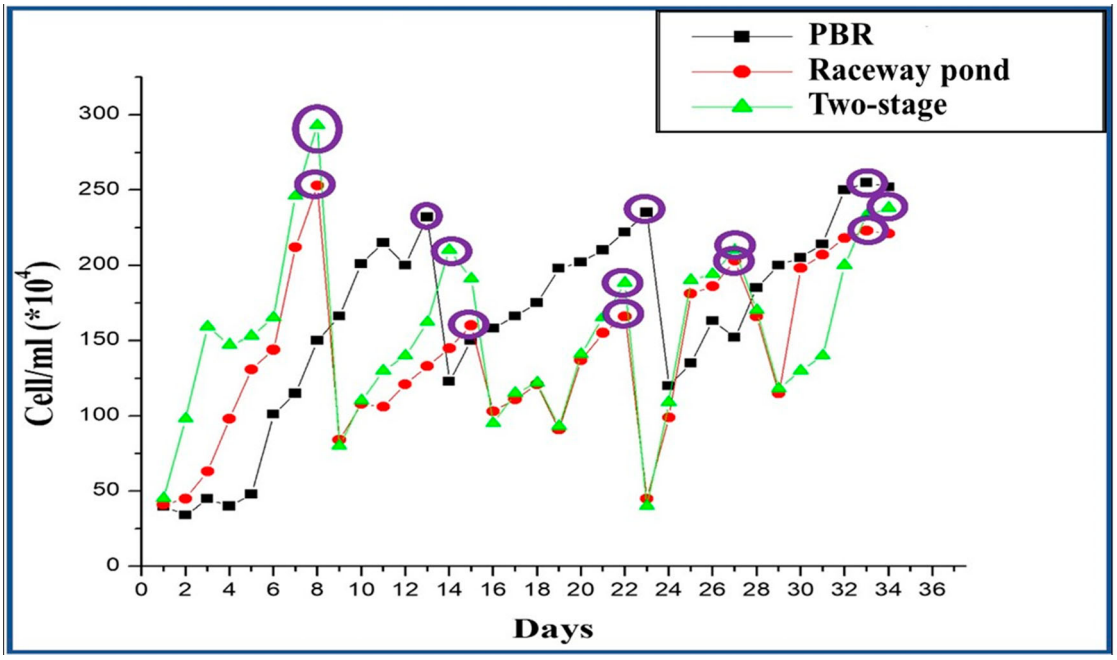

Figure 4. Algal cell density in a PBR, raceway pond, and two-stage system. Three production cycles were evaluated, each of 8 days.

In PBR, the growth of *Scenedesmus* sp. GTAF 01_IU was estimated by cell count (cells/milliliters). The observation was done for 33 days, which included total 3 cycles of growth and harvesting. Only after the confirmation that the biomass is rich in lipid by Nile red staining, the biomass was harvested. In the first cycle, the exponential growth of the algal cell was seen after the 5th day of the inoculation and the nutrient limiting condition was set on 8th day. The monitoring of the lipid induction was again done by Nile red staining. On days 10th and 11th, a low sunlight emission was recorded resulting in the decrease in the cell number and lipid content on 11th and 12th days. On 13th day, half of the culture was harvested and fresh BG 11 media was added on the left half culture in PBR. Similarly in second growth cycle, the nutrient limiting condition was set on day 20th. On 23rd day, the half of the culture was again harvested, and fresh BG 11 media was added on the left half culture. In the third growth cycle, maximum lipid accumulation and cell density was achieved at 32th day. A decrease in the cell numbers was observed on 33rd day, so the complete culture was harvested from PBR.

Similarly, on day 33, a study was done in raceway pond system in which three growth and harvesting cycles were observed. The harvesting of the culture was done on 13th, 23rd, and 33rd days (Figure 4). Similar to the PBR, in raceway pond the first nutrient limiting condition was set at 8th day. On 12th day, a decrease in the cell density was observed. Therefore, half of the culture was harvested and for the rest fresh BG11 media was added in the raceway pond. In the second cycle, the nutrient limiting condition was set on 20th day, where the cell density reached to 220×10^4 cells/mL. At the end of the second cycle on 23rd day half of the culture was again harvested. The left half culture in the raceway pond was added with fresh BG11 media. In the third growth cycle, the maximum cell density of 270×10^4 cells/mL was observed on days 28th and 29th, respectively. Therefore, complete culture was harvested from raceway pond.

3.2. Optimization of culture conditions

The SVR model was developed to predict the biomass of microalgae (on dry weight basis) in different culture conditions. Four independent variables (light intensity, mixing speed, pH and light: dark cycle) were used to develop the model. Different kernels (Gaussian, Linear, and Polynomial) were used to train the SVR model. The best results were observed with the Gaussian kernel. A grid search method was used to optimize the SVR model with the Gaussian kernel. The kernel scale was set to auto. This enables MATLAB to choose a suitable kernel scale based on a heuristic procedure. No kernel offset was used. The model used was a Sequential

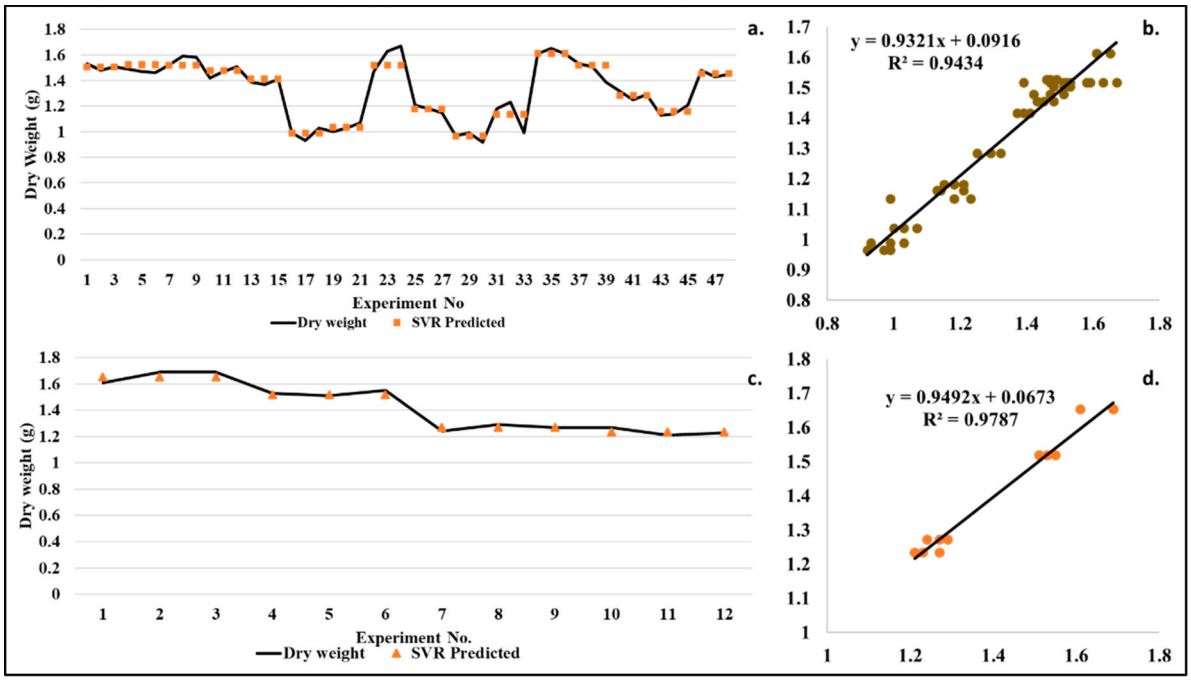

Figure 5. Performance of SVR model in predicting the training (a and b) and validation (c and d) data.

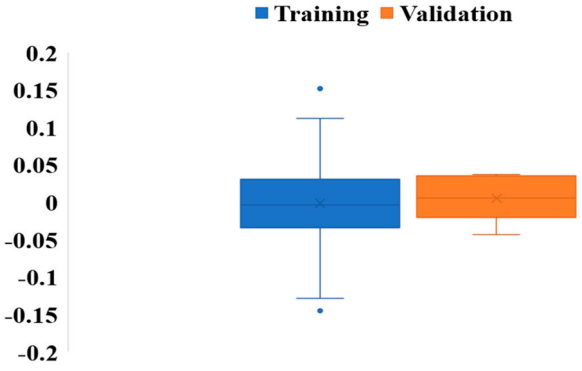

Figure 6. Boxplot showing the residual errors while predicting the dry cell weight in the training and validation data.

Minimal Optimization solver for optimization and the half-width of the kernel sensitive band was set to 0.024. The model converged in 954 iterations and fitted the training data well. The SVR model showed a good fitness of curve with the experimental data with coefficient of determination (R^2) of 0.94 and 0.98 for training and validation data respectively. This suggested that the model can explain about 94% variations in the training data and around 98% variations in the validation data. Mean square error (MSE) and root mean square error (RMSE) were low for both training (0.0028 and 0.053, respectively) and validation data (0.0008 and 0.027, respectively) showing a high goodness of fit (Figures 5). The trained model was then used as objective function for the GA to find the best culture conditions for maximum cell dry weight. MATLAB's optimization toolbox was used to perform the optimization. 50 initial solutions were generated randomly and the 'stochastic uniform' algorithm was applied to select the best ones. The search after 300 iterations was terminated, or when the function value or the constraint violation did not change significantly for 50 iterations, or when they were below 10^{-6} and 10^{-3}, respectively. According to the model, the maximum biomass dry weight of 1.65 g/L was achieved under the following conditions: light intensity of 27 µmol m^{-2} s^{-1}, mixing speed of 150 RPM, pH of 7 and light and dark cycle of 12 h each. The model accuracy was verified by performing experiments at the optimal conditions and obtaining dry cell weight of 1.69 g, which was close to the expected assessment (Figure 6).

3.3. Two-stage cultivation system

In this system, the PBR was connected to raceway pond through a connecter with on–off knob. As soon as the culture in PBR reaches to Log phase, 50% of the culture was transferred to raceway pond from PBR. In the raceway pond, nutrition depleted condition was set. This resulted in lipid biosynthesis and accumulation in the algal cells. In PBR, the cell concentration at the initial of growth was 49×10^4 cells/mL and after the nutrient depletion condition was 290×10^4 cells/mL (Figure 4).

3.4. Comparison of individual open pond, closed PBR and Two-stage cultivation system based on growth rate and biomass productivity of culture

The cultivation of *Scenedesmus* sp. GTAF1 IU in open raceway pond and closed PBR resulted in mainly three growth and harvesting cycles (Figure 4). After the completion of the growth cycle, the biomass rich in lipid content was harvested. On 27th day, an increase in the growth was observed but the resulting biomass showed a low content of lipid therefore it was harvested, and no further cycle was processed with the same biomass (Figure 4). It can be concluded that the raceway pond and closed PBR had only three main growth and harvesting cycles. Parallel to this, in the case of the two-stage system, six growth and harvesting cycles were achieved (Figure 4). Thus the mean growth rate of the two-stage system was higher than that of individual PBR and raceway pond. A possible reason behind this could be the difference in the growth and lipid induction phase. The two-stage system provided a separate area for these two phases that resulted in enhanced biomass and lipid induction. Contrary to this, in case of individual PBR and raceway pond, the *Scenedesmus* sp. GTAF01 IU underwent nutrient limiting (stationary phase) and decline phase that resulted in the inhibition of microalgal growth (Figure 7).

3.5. Biochemical composition of S. GTAF1 IU individual open pond, closed PBR and two-stage cultivation system

The biochemical composition of the closed PBR, open pond, and two-stage cultivation system was evaluated. In closed PBR, the carbohydrate, protein, and lipid were found to be 20.4%, 54.1%, and 34%. Similarly, in raceway pond the carbohydrate, protein, and lipid were 17.7%, 42%, and 19% (Figure 8). A significant decrease

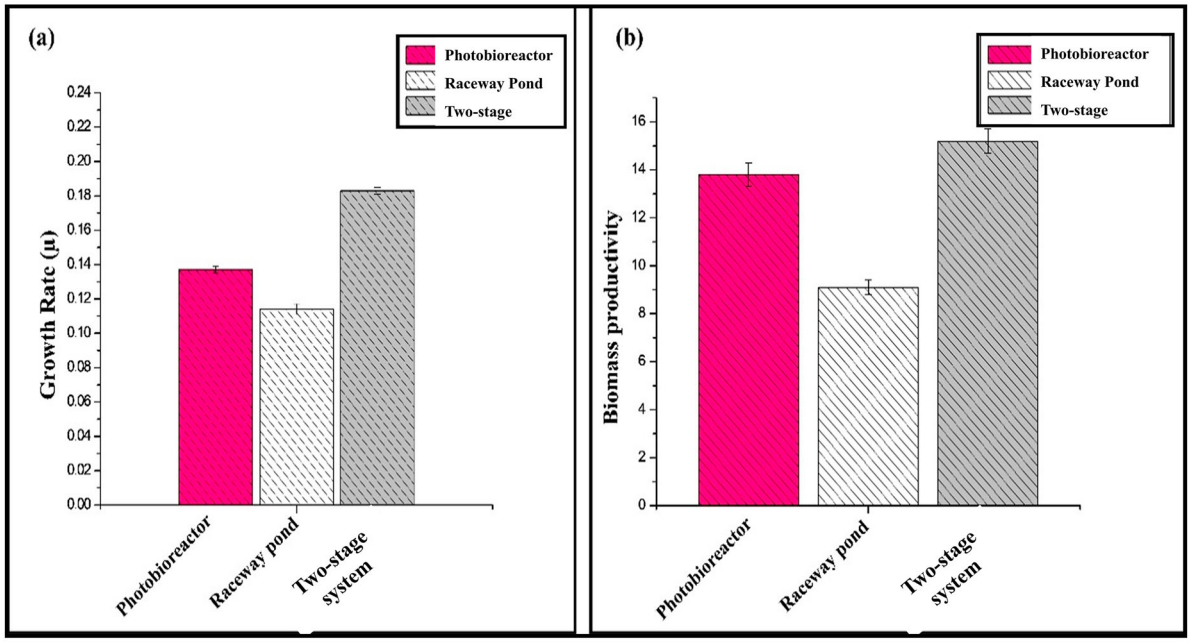

Figure 7. (a) Growth rate and (b) biomass productivity of *Scenedesmus* sp. under photo bioreactor, raceway pond, and two-stage cultivation systems. Values are means ± SE with $n = 3$.

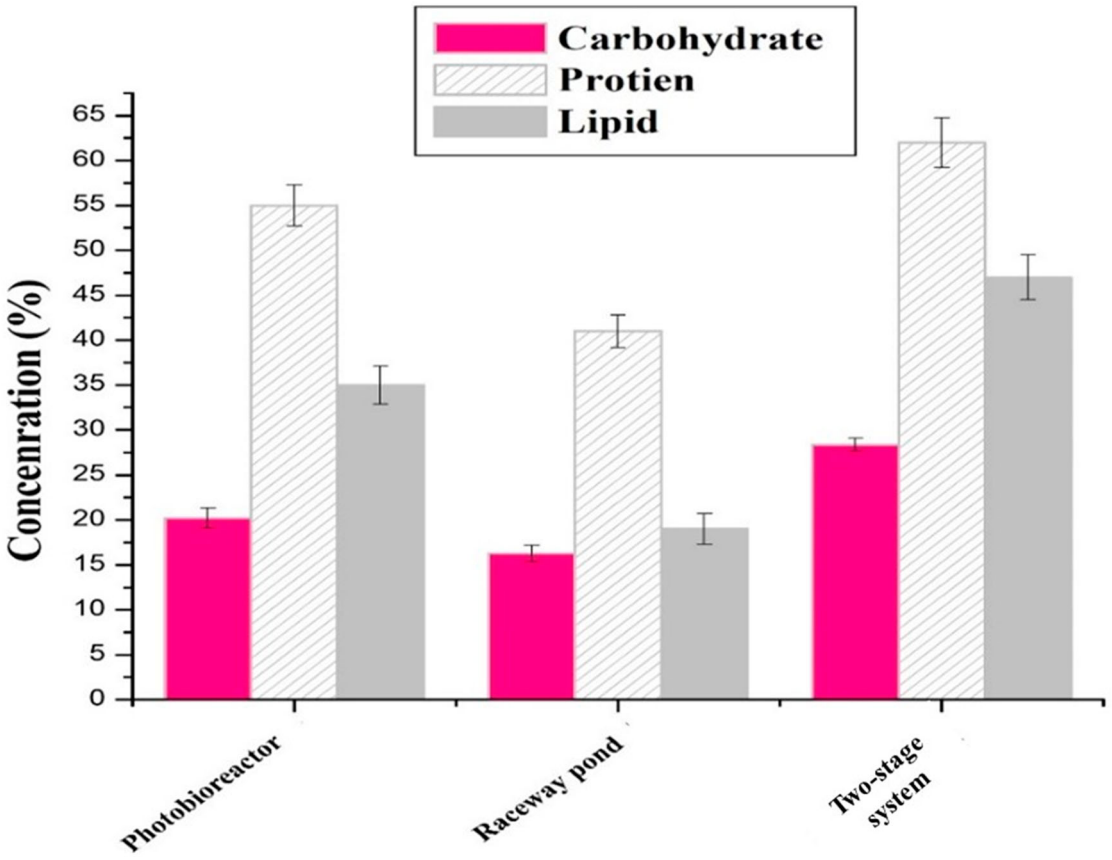

Figure 8. Carbohydrate, protein, and lipid content in photobioreactor, raceway pond, and two-stage cultivation systems. Values are means ± SE with $n = 3$.

in these concentrations was probably due to the uncontrolled growth conditions in the raceway pond. However, in two-stage system, a significant increase in carbohydrate, protein, and lipid of 27%, 64%, and 47% was observed (Figure 8). This was possibly due to the fact that in two-stage cultivation system, the closed PBR provided optimal conditions for the lag to log phase growth of *Scenedesmus* sp. This resulted in a healthy culture with maximum growth rate of 0.186 μ (Figure 7). As soon as the culture approached stationary phase, it was transferred to the attached raceway pond where the increased surface area provided large area for growth and high sunlight absorption. Similar results were obtained by Wu et al. (43), where a significant increase in the carbohydrate was obtained in raceway pond. In another study, an increase in carbohydrate was obtained when *Nostoc calcicola* was grown in raceway pond (44).

Similarly, an increase in the lipid content was obtained at the end of the stationary phase when *Halamphora coffeaeformis* was grown in a raceway pond reactor (45). Iasimone et al. (46) also reported a significant increase in lipid accumulation in *Scenedesmus* sp. and *Chlorella* sp. was observed when grown in a raceway pond. Similarly, when *Chroococcus turgidus* was grown in a raceway pond for 15 days an increase in protein content was observed (47). While total lipid content was assessed, detailed fatty acid profiling (e.g. via GC-FAME analysis) was not conducted, as it was beyond the scope of the current experimental design. This is acknowledged as a limitation, and future studies are recommended to include comprehensive lipid characterization to evaluate the biofuel quality of the produced lipids.

The above results suggested that the hybrid system holds a high potential towards high biomass lipid, carbohydrate, and lipid against individual raceway pond and closed PBR. Additionally, the highest growth rate and biomass productivity were also recorded in the two-stage cultivation system. Considering the improved biomass quantity and biochemical composition the two-stage cultivation system has been proven to be technologically better and should be preferred over raceway pond and PBR. This will ultimately decrease the capital and labor costs compared to raceway pond and PBR. A comparison of raceway pond, photobioreactor, and two-stage cultivation system has been shown in Table 2.

Table 2. Comparison of raceway pond, photobioreactor, and two-stage cultivation system considering and comparing the present and existing literature.

Factors/parameters	Raceway pond	PBR	Hybrid system
Maintenance	Low	High	Moderate
Evaporation loss	High	Low	Moderate
Contamination risk	High	Low	Low
Space required	High	Moderate	High
Biomass quality	Variable	Reproducible	Reproducible
Setup cost	Low	High	Moderate
Operation type	Batch	Batch	Continuous
Maintaining continuous exponential phase	Difficult	Difficult	Easy
Energy input for mixing	Low	High	Moderate

4. Conclusion and future prospective

In the present study, a two-stage photobioreactor has been designed for effective microalgal biomass production. The SVR model was developed to predict the biomass of microalgae (on a dry weight basis) in different culture conditions. The maximum biomass dry weight of 1.65 g was achieved under the following conditions: light intensity of 27 µmol m^{-2} s^{-1}, mixing speed of 150 RPM, pH of 7, and light and dark cycle of 12 hours each. The model accuracy was verified by performing experiments at the optimal conditions and obtaining a dry cell weight of 1.69 g, which was close to the expected value. The results also showed a significant increase in carbohydrate, protein and lipid content. Owing to an enhanced lipid content, by the two-stage cultivation system the microalgal strain can be used in biofuel production.

Thermochemical conversion pathways, such as pyrolysis, gasification, and hydrothermal carbonization, present significant opportunities for valorizing microalgal biomass into solid biofuels like biochar and hydrochar. These processes not only enhance the energy density and stability of the biomass but also contribute to carbon sequestration and soil health when applied as biofertilizers. Future research should focus on optimizing process parameters (e.g. temperature, residence time, catalyst selection) to improve yield and quality of solid biofuels. Moreover, integrating thermochemical conversion with upstream cultivation and downstream biorefinery modules can facilitate energy-positive and economically viable systems. Advanced characterization of biochar properties, lifecycle assessments, and techno-economic evaluations are essential to assess scalability and environmental benefits. Exploring co-processing of microalgae with lignocellulosic residues may also enhance the performance of thermochemical routes. Given the circular bioeconomy goals and global carbon neutrality targets, microalgal solid biofuels warrant deeper investigation as a complementary and sustainable energy solution.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Author Contributions

G.T.: writing – original draft preparation, including figures and tables, conceptualization; P.D., K.O., S.A., I., V.S., S.N.R.: writing – reviewing and editing; A.F., V.M. and E.V.: supervision, writing – reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Data availability statement

Data are contained within the manuscript

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