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Optimization of cultivation conditions for *Microcystis aeruginosa* for biodiesel production using response surface methodology

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Abstract

Background: Biodiesel is expected to play a key role in the development of a sustainable, economical, and environmentally safe source of energy. The third generation of biodiesel is derived from microalgae and cyanobacteria that have sufficient amount of oil. The optimization of biomass and oil content in biodiesel production based on algal cultivation relies upon several factors. The present experimental work aims at optimizing some of the cultivation conditions to obtain maximum oil and biomass yield and create a prediction model that describe the effect of the initial inoculum concentration, and irradiance on the biomass yield and oil concentration were designed using Design Expert 6.0.8.

Results: The results revealed that the optimum surface-to-volume ratio for the airlift bubble column photobioreactor was 0.9, and the most applicable model for describing *Microcystis aeruginosa* growth was the hyperbolic tangent model with a model constant value of 1.294 $mg{\cdot}L^{-1}{\cdot}d^{-1}/\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$. The optimum cultivation conditions were 81 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ irradiance and 67 $mg{\cdot}L^{-1}$ initial inoculum concentration, and these conditions achieved a biomass yield of 163 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ and an oil concentration of 143 $mg{\cdot}L^{-1}$.

Conclusions: This work focused on the cultivation of microalgae in closed systems. Cyanobacteria as *M. aeruginosa* has high lipid content, and high lipid productivity makes it suitable as a lipid feed stock for biodiesel production. The response surface method was the most suitable route to study the simultaneous influence of irradiance and initial inoculum concentration through statistical methods as well as to establish a model for predicting the biomass yield and oil concentration of *M. aeruginosa*.

Keywords: Factorial design, Photobioreactor, Kinetic models, Biomass, Oil content

Background

Microalgae are considered a potential source for biodiesel production. Lipids and fatty acids are the major constituents in algal cells (Lyon and Mock 2014). The major part of non-polar lipids (neutral lipids) of microalgae consists of triglycerides (TAGs) which can be used for biodiesel production (Fahy et al. 2009; Sanchez et al. 2011). Like higher plants, microalgal growth is affected by light capture, the carbon dioxide-to-oxygen ratio, and temperature (Araujo and Garcia 2005; Wang et al. 2012; Zheng et al. 2011), as well as the pH and mixing speed (Hargreaves and Whitton 1976; Janssen et al. 2000). Additionally, because microalgae are cultivated in an aquatic environment, the sterility and cleanliness of the closed cultivation system itself should be considered to avoid contamination (Posten 2009). The synthesis and accumulation of large amounts of TAGs are accompanied by a considerable alteration in the lipid and fatty acid composition in the cell, which occurs when algae are placed under stressful conditions imposed by chemical

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stimuli, such as nutrient depletion, or physical environmental stimuli, such as the light intensity, either individually or in combination (Widjaja 2009).

The effect of light intensity varies according to the algal species and strains (Schulze et al. 2014). With few exceptions, low light intensity favors the formation of polyunsaturated fatty acids (PUFAs), which in turn are incorporated into the membrane structure (Cuellar-Bermudez et al. 2015). However, elevated light intensities alter fatty acid synthesis by producing more saturated and monounsaturated fatty acids, which are the major components of neutral lipids (Ogbonda et al. 2007). Moreover, photoinhibition may occur because of the overproduction of reactive oxygen species synthesized at the elevated light intensity, and these species damage membrane lipids, proteins, and other macromolecules (Zhu et al. 2008).

In this work, the four strains *Scenedesmus obliquus* (green algae), *Nannochloropsis sp.* (genus of algae), *Spirulina platensis* (cyanobacteria), and *Microcystis aeruginosa* (cyanobacteria) were cultivated phototrophically and examined to select the strain most suited for biodiesel production. In addition, the optimum illuminated area-to-culture volume ratio was obtained to define the specifications of the bubble column photobioreactor. A set of experiments were run based on the cultivation of *M. aeruginosa* at irradiances ranging from 20.3 to 176 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to define the best model for describing the relationship between the algal growth rate and irradiance. Finally, two independent variables that affect algal growth were investigated to optimize the cultivation conditions and model the algal growth: irradiance, and initial inoculum concentration. Although most previous studies have indicated that these parameters affect microalgae growth, one of the two parameters was invariably kept constant while the other was varied which eliminated the possibility of detecting potential interactions between the two factors (Wang et al. 2012). Thus, designing experiments using the Response surface methodology (RSM) allows for the study of the simultaneous influence of several parameters using statistical methods. One of the merits of this system is the possibility of determining any nonlinear relationships between independent variables (Montgomery 2003). This step was enhanced by establishing a model to predict the biomass yield and oil concentration of the selected microalgae species.

Material and methods

Inoculum preparation and cultivation system

The microalgae strains were cultivated at a temperature of 20 ± 1 °C, aeration flow rate of 1 *V*/*V* and irradiance of 20.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white cool fluorescent light for 10 days in flasks using BG11 medium. The inoculum concentration was defined for each strain depending on the chlorophyll content, which was determined via methanol extraction (Fogg and Thake 1987). Chlorophyll a is used as an algal biomass indicator, and its constituents are on the average, 1.5% of the dry weight of organic matter of algae (Association APHA 1915). Microalgae were harvested via settling without additives for (30–60 min), and the aeration and irradiance cultivation conditions accelerate the growth rate and increase the pH to 10.5 by the end of the cultivation period, thereby facilitating settling without additives. The sediments were collected and washed twice with water and then centrifuged at 3000 rpm for 10 min. The algal cells were dried at 60 °C overnight. Oil was extracted from dried weighed samples using hexane and isopropanol in a ratio of 3:2 according to the method applied by Halim et al. (2012); 300 mL of the mixture was added to 4 g of microalgae powder; and the mixture of algal powder and solvents was homogenized at 800 rpm for 10 min to rupture the cell wall. The extraction process was applied at 40 °C for 2 h, and the mixture was filtered. The filtrate was washed with water to allow the miscible solution to separate according to polarity.

Strain selection

The four isolated strains of *S. platensis*, *S. obliquus*, *Nannochloropsis sp.*, and *M. aeruginosa* were supplied by the Water Pollution Research Department, Environmental Research Division, National Research Centre in Cairo, where the toxicity test of using microcystin was applied to *M. aeruginosa* because certain species of Microcystis are toxic. The four strains were cultivated using BG11 medium in an airlift bubble column photobioreactor (PBR, as shown in Fig. 1) at a temperature of 20 ± 1 °C, aeration flow rate of 1 *V*/*V* and irradiance of 20.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 10 days. After harvesting, the oil concentration and biomass yield of the four strains were compared to select the most suitable strain for oil production.

Optimization of PBR dimensions and cultivation conditions

The PBR design is based on optimizing the parameters that affect microalgae growth. The main parameter that maximizes algal growth is capturing optimal light, which implies a high illuminated surface area/culture volume ratio (S/V) (Richmond 2004). Thus, four duplicated experiments at various S/V ratios (1.8, 0.9, 0.63, and 0.48) using the previous cultivation conditions were performed to optimize the biomass yield.

Selected models describing the algal growth kinetics

The biomass yield can be expressed as the photosynthetic yield, which can be predicted by growth models. Each species has its own growth characteristics, which differ from that of the others. Thus, the optimal growth

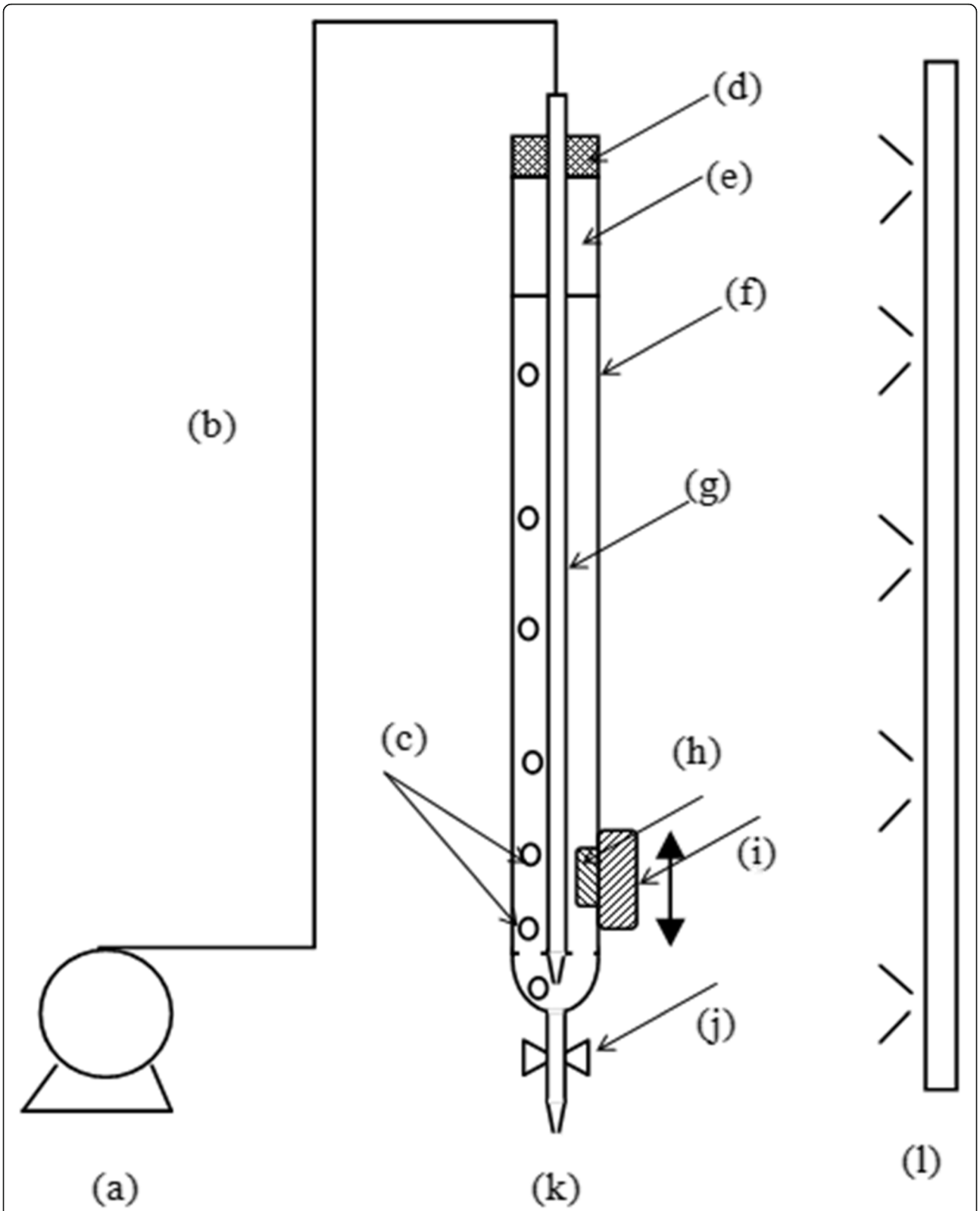

Fig. 1 Simplified sketch of the lab-scale photobioreactor: (a) blower, (b) connection rubber tubes, (c) air bubbles, (d) cotton plug, (e) air gap, (f) glass column, (g) inner glass tube, (h) inner magnet, (i) outer magnet, (j) valve, (k) outlet, and (l) fluorescent lamp

model for describing the selected strain *M. aeruginosa* must be identified. One such model is the hyperbolic tangent model (Kurano and Miyachi 2005):

$$P = P_{\max}.\ \tanh(\alpha I) \quad (1)$$

where

P represents the growth rate ($mg{\cdot}L^{-1}{\cdot}d^{-1}$);

I represents the irradiance ($\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$); and

α is the model constant, which was obtained experimentally ($mg{\cdot}L^{-1}{\cdot}d^{-1}/\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$).

The second model is the Monod model (Jalalizadeh 2012; Kurano and Miyachi 2005; Perez et al. 2008; Sundstrom and Klei 1979; Tamiya 1951), which expresses the growth rate in the following form:

$$\mu = \mu_{\max} I/(I + K_I) \quad (2)$$

where

μ represents the specific growth rate (d^{-1});

I represents the irradiance ($\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$); and

K_I is the model constant, which was obtained experimentally ($\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$).

The third model is a modified Monod model that considers light irradiances as the substrate (Bechet et al. 2013; Jeon et al. 2005):

$$P = P_{\max} I/(I + I_k) \quad (3)$$

where

I_k is the saturated light intensity ($\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$).

The experimental data and constants obtained from eight duplicated runs at irradiance values ranging from 20.3 to 176 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ were used to verify the most applicable model for the selected algal strain. The three models were evaluated by calculating the absolute average deviation (AAD) in the following form (Mejia et al. 2013):

$$\mathrm{AAD} = (1/N) \sum | (\mathrm{Act.} - \mathrm{Pred.})/\mathrm{Act.} | \quad (4)$$

where

AAD means absolute average deviation;

Act. means actual value;

Pred. means predicted value; and

N means number of set values.

Effect of irradiance and initial inoculum concentration on the biomass yield of *M. aeruginosa* and oil concentration

The irradiance and initial inoculum concentration are considered the most significant parameters affecting algal growth that obey phototrophic cultivation (Posten 2009). The range of the examined irradiance was 27–81 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$, and the maximum value of irradiance was determined from the photosynthetic yield–irradiance relation (P–I curve) via the intersection between the initial tangent of the curve and the maximum photosynthetic rate. A dense culture retards light penetration via the PBR wall (Bezerra et al. 2011; Coles and Jones 2000; Jeon et al. 2005). Thus, a range of inoculum concentrations (15.5–67 mg biomass/L) was selected for study. The RSM was chosen as a suitable route for optimizing the interactive effect of irradiance (I) and the initial inoculum concentration (C) on the oil concentration and biomass yield (Montgomery 2003; Silva et al. 2013). The experimental results were statistically analyzed and modeled using the RSM according to Eq. 5 (Montgomery 2003), which was applied using Design Expert-6.0.8 software during a trial period. The extent of the fit of the model was evaluated using the coefficient of determination and analysis of variance (ANOVA).

$$Y = a_0 + a_1 X_1 + a_2 X_2 + a_{11} {X_1}^2 + a_{22} {X_2}^2 + a_{12} X_1 X_2 \quad (5)$$

where

a_0 = the regression constant;

a_0, a_1, a_2, a_{11}, a_{22}, a_{12} = regression coefficients; and

X_1 and X_2 = independent variables investigated here.

Results

The selection of the most suitable strain for biodiesel production was based on the oil content. The results revealed that the oil contents of *Nannochloropsis sp.*, *S. platensis*, *S. obliquus*, and *M. aeruginosa* are 8%, 12%, 16%, and 20%, respectively; the lipid productivities are 2, 4.8, 5.6, and 6 $mg{\cdot}L^{-1}{\cdot}d^{-1}$, respectively; and the biomass productivities are 25, 40, 35, and 30 $mg{\cdot}L^{-1}{\cdot}d^{-1}$, respectively. *M. aeruginosa* has the highest lipid content and the highest lipid productivity which is desired for biodiesel production. Examining the S/V ratios of 1.8, 0.9, 0.63, and 0.48, the results revealed that the biomass yield for *M. aeruginosa* is 23.3, 45, 42.4, and 33.8 $mg{\cdot}L^{-1}{\cdot}d^{-1}$, respectively, and the optimum S/V ratio is 0.9.

The cell productivities of eight duplicated runs in the range of 20.3–176 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ at S/V = 0.9 were plotted versus irradiance using Origin 8.5 program Data Analysis and Graphic Software with an adjusted $R^2 = 0.99$. The intersection between the initial tangent ($\alpha = 1.294$ $mg{\cdot}L^{-1}{\cdot}d^{-1}/\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$) and maximum cell productivity was observed at the saturation irradiance ($I_k = 85$ $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$). A comparison of the specific growth rates versus irradiance showed that the Monod model constant (K_I) determined at $\mu = \mu_{\max}/2$ was 12.5 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ (with adjusted R^2 of 0.99). This result is consistent with the work of Perez et al. (2008), where an adjustment of data was performed via a reciprocal linear regression to obtain a constant of $K_I = 10.2$ $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ and $R^2 = 0.95$.

The three models were compared statistically using the AAD. The hyperbolic tangent model was the optimal model for describing *M. aeruginosa* growth because the AAD was 0.058, while the AADs of the Monod model

Table 1 Evaluation of three kinetic models

I (μmol/m^2·s)	$\mu = \mu_{max}.\ I\ /\ (I + K)$			$P = P_{max}.\ I\ /\ (I + I_k)$			$P = P_{max}.\ \tanh\ (\alpha I)$		
	$\mu_{Act.}$	$\mu_{Pred.}$	μ_{AAD}	$P_{Act.}$	$P_{pred.}$	P_{AAD}	$P_{Act.}$	$P_{pred.}$	P_{AAD}
20.3	0.25	0.223	0.109	25	21.206	0.152	25	25.782	0.031
33.8	0.312	0.263	0.158	45	31.296	0.305	45	41.573	0.076
47.2	0.34	0.285	0.163	59	39.274	0.334	59	55.493	0.059
74.3	0.352	0.308	0.125	82	51.306	0.374	82	77.381	0.056
87.8	0.353	0.315	0.107	92	55.891	0.392	92	85.259	0.073
115	0.359	0.325	0.096	104.7	63.25	0.396	104.7	96.222	0.081
149	0.36	0.332	0.077	110	70.043	0.363	110	103.588	0.058
176	0.36	0.336	0.066	110	74.176	0.326	110	106.556	0.031
AAD			0.113			0.330			0.058

Specific growth rate (μ), cell productivity (P), actual values (Act.), predicted values (Pred.), absolute average deviation (AAD), and irradiance (I)

and Modified Monod model were 0.113 and 0.33, respectively (Table 1). The hyperbolic tangent function provided the best fit, as evaluated by the extended information criterion (EIC).

The effects of irradiance and initial inoculum concentration were studied as two independent variables affecting biomass yield and oil concentration in a total of 13 experiments in accordance with a 22 complete factorial design as shown in Table 2. The data were analyzed using Design Expert 6.0.8 software and by applying the RSM. Table 3 describes the ANOVA for the response surface of a quadratic model for oil concentration response and biomass yield. A statistical analysis of the oil concentration response revealed that the predicted R^2 was 0.99 and the adjusted R^2 was 0.99, whereas an analysis of the yield response revealed that the predicted R^2 and the adjusted R^2 were 0.98 and 0.96, respectively. The coefficients of variation (CVs) of the oil concentration response and yield response were 3.19 and 1.58, respectively. The analysis yielded two linear coefficients (A, B), two quadratic coefficients (A^2, B^2), and one cross-product coefficient for the full model.

Both the adjusted and predicted coefficients of determination (R^2) were approximately 0.99, indicating that the model is highly reliable. Equations 6, 7, 8 and 9 represent the final estimated response model equations of biomass yield and oil concentration in terms of the coded and actual values.

The regression equation for the percent biomass yield in terms of coded variables is as follows:

$$\begin{aligned}\text{Yield} = {} & 96.4 + (34.04 \times A) \\ & + (31.03 \times B) - (2.07 \times A) - \left(4.57 \times C^2\right) \\ & + (7.00 \times A \times B)\end{aligned} \tag{6}$$

The regression equation of the biomass yield response in terms of the actual variables is as follows:

Table 2 Experimental data for five levels of the two-factor response surface analysis

Run	A	B	I (klx)	C (mg/L)	Oil concentration (mg/L)		Biomass yield (mg/L·d)	
					Actual	Predicted	Actual	Predicted
1	0	− α	4.00	4.83	60.00	56.52	45.00	43.37
2	− 1	− 1	2.00	15.50	49.00	52.42	30.00	31.68
3	0	0	4.00	41.25	107.80	105.76	98.00	96.40
4	+ 1	+ 1	6.00	67.00	143.00	140.08	163.00	161.82
5	+ 1	− 1	6.00	15.50	78.00	79.65	85.00	85.77
6	0	+ α	4.00	77.67	110.00	112.98	130.00	131.13
7	0	0	4.00	41.25	106.00	105.76	96.00	96.40
8	0	0	4.00	41.25	105.00	105.76	96.00	96.40
9	0	0	4.00	41.25	103.00	105.76	95.00	96.40
10	− 1	+ 1	2.00	67.00	73.00	71.87	80.00	79.73
11	− α	0	1.17	41.25	55.00	53.50	45.00	44.10
12	0	0	4.00	41.25	107.00	105.76	97.00	96.40
13	+ α	0	6.83	41.25	120.00	121.00	140.00	140.40

Irradiance as the coded variable (A), and initial inoculum as the coded variable (B)

Table 3 ANOVA results for the response surface quadratic model

Source	Biomass yield response		Oil concentration response	
	F-value	Prob > *F*	*F*-value	Prob > *F*
Model	1622.41	< 0.0001	209.87	< 0.0001
A	4340.12	< 0.0001	510.14	< 0.0001
B	3604.77	< 0.0001	356.97	< 0.0001
A^2	14.02	0.0072	66.71	< 0.0001
B^2	68.16	< 0.0001	85.95	< 0.0001
$A \times B$	91.75	< 0.0001	47.05	0.0002
Lack of fit	2.50	0.1984	4.64	0.0861

$$\text{Yield} = -19.00271 + (15.5651 \times I) + (1.23044 \times C) - (0.51875 \times I^2) - (6.8998 \times 10^3 \times C^2) + (0.1352 \times I \times C) \quad (7)$$

The corresponding equation for the percent oil concentration in terms of coded variables is as follows:

$$\text{Oil concentration} = 105.76 + (23.87 \times A) + (19.96 \times B) - (9.25 \times A^2) - (10.50 \times B^2) + (10.25 \times A \times B) \quad (8)$$

The regression equation of the oil concentration response in terms of the actual variables is as follows:

$$\text{Oil concentration} = -5.09018 + (22.23279 \times I) + (1.28624 \times C) - (2.31375 \times I^2) - (0.015843 \times C^2) + (0.19903 \times I \times C) \quad (9)$$

Figures 2 and 3 illustrate a plot of the experimental values versus predicted values for the biomass yield and oil concentration response respectively. The response values predicted from the empirical model are consistent with the actual values over the selected range of operation indicating that the model adequately represents the experimental data. The surface plot and contours of the oil concentration and the biomass yield versus the irradiance and the initial inoculum concentration obtained when individual experimental data were plotted are shown in Figs. 4 and 5. The RSM model equation revealed that biomass yield increases as both the irradiance and initial concentration increase. The results also indicated that the optimum yield and oil concentration can be obtained at 81 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance and 67 $mg \cdot L^{-1}$ initial concentration. The predicted biomass yield and oil concentration were 161.82 $mg \cdot L^{-1} \cdot d^{-1}$ and 140.079 $mg \cdot L^{-1}$, respectively, whereas the actual values were 163 $mg \cdot L^{-1} \cdot d^{-1}$ and 143 $mg \cdot L^{-1}$, respectively.

Discussion

The results for the oil content of *M. aeruginosa* are close to those obtained by Sharathchandra and Rajashekhar (2011) who found that the percentage of extracted oil based on biomass was 28.15 ± 2%, which was also confirmed by the work of El-Ardy et al. (2012). However, the higher percentage obtained by the latter authors could be attributed to the high-temperature range used (20–30 °C). The results achieved by Da Ros et al. (2013) are also close to reported research: the lipid productivity was 3.1 $mg \cdot L^{-1} \cdot d^{-1}$, and biomass productivity was 46.9 $mg \cdot L^{-1} \cdot d^{-1}$. In the present work, the corresponding results are 6 $mg \cdot L^{-1} \cdot d^{-1}$ lipid productivity and 30 $mg \cdot L^{-1} \cdot d^{-1}$biomass productivity. *M. aeruginosa* was selected according to its sufficient oil content and simple cell structure, which can be easily ruptured during oil extraction using chemical methods.

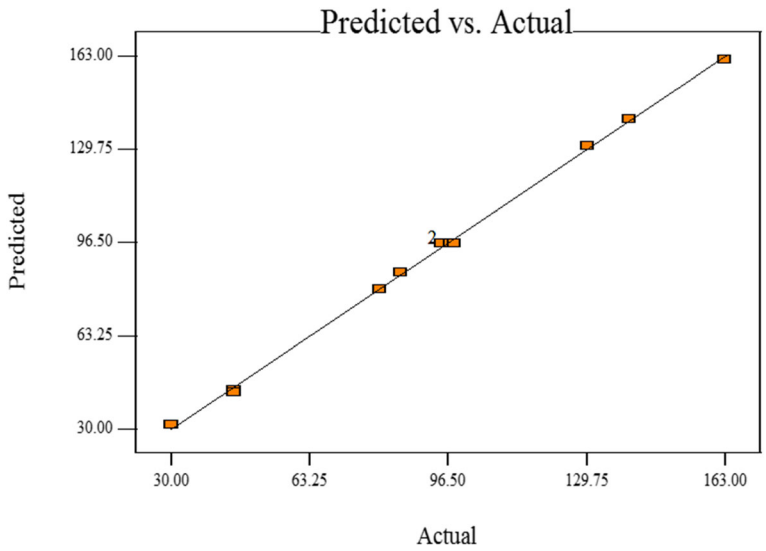

Fig. 2 Actual biomass yield values vs. predicted RSM model

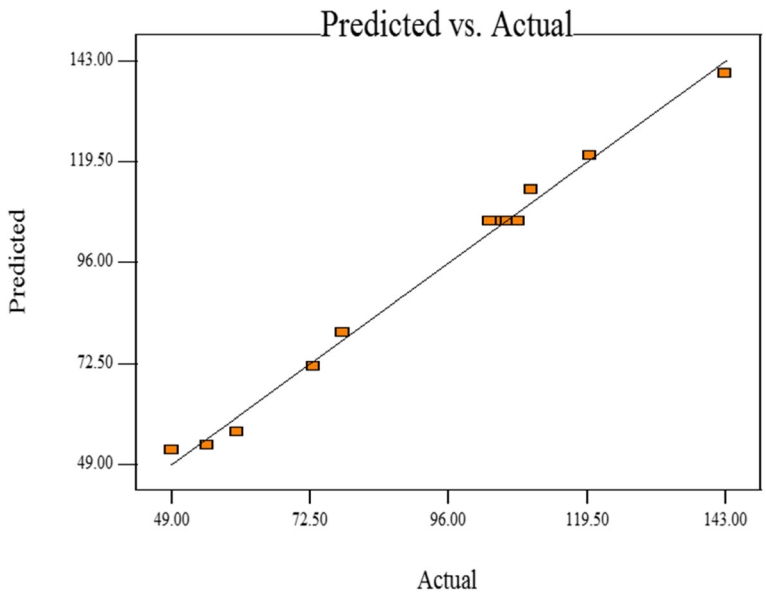

Fig. 3 Actual oil concentration values vs. predicted RSM model

The optimum ratio of illuminated surface area to volume obtained was 0.9. This result is consistent with the work of Richmond (2004), who recommended using a ratio approaching, but not exceeding, unity. The specific nature of algae differs among species according to the nature of the strain thus, the constants of each examined model had to be obtained experimentally and the models of *M. aeruginosa* had to be obtained specifically. The hyperbolic tangent function applied to *M. aeruginosa* provided the best fit, as evaluated by the EIC. This model is necessary to study the relationship between irradiance and growth rate which will enhance further studies on other dependent variables related to irradiance.

Finally, the interactive effect of irradiance and the initial inoculum concentration on the oil concentration and biomass yield was investigated using the RSM. The statistical analysis of each yield response and oil concentration response revealed that the predicted R^2 and adjusted R^2 are similar. In addition, high variance was observed because the CV was greater than one, indicating that the data points distributed at a distance from the mean, and from one another. The *F*-value test was applied to both the oil concentration model and biomass yield model. These models were found to be statistically significant based on an error analysis. The probability test revealed that the models' terms are significant. Model verification was enhanced based on the non-significance of the lack of fit, which indicates non-significant error and suggests that the model can accurately predict the relationships between the reaction factors in the selected range.

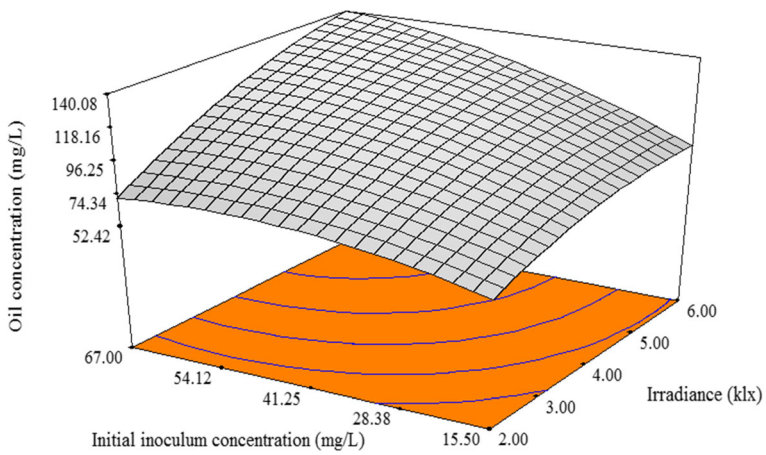

Fig. 4 RSM model graph of the oil concentration response

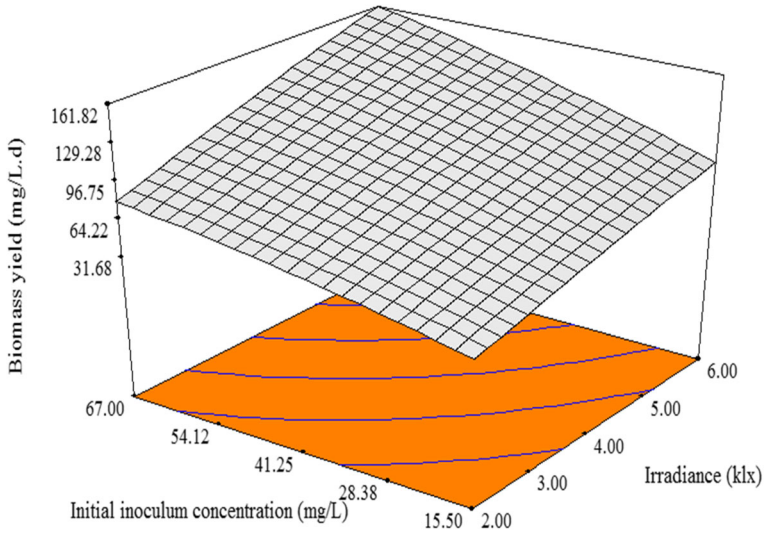

Fig. 5 RSM model graph of the biomass yield response

Conclusions

This work focused on the cultivation of microalgae in closed systems because open ponds are inexpensive but vulnerable to environmental disturbances, such as temperature swings and biological invasions. *M. aeruginosa* has various advantages as a lipid feed stock for biodiesel production, compared with two strains of algae (*S. obliquus*, and *Nannochloropsis sp.*) and a cyanobacterium strain (*S. platensis*), which were locally isolated and cultivated strains. These benefits included a high lipid content and high lipid productivity. In general, the microalgae growth rate or productivity can be predicted using models of algal growth. The hyperbolic tangent model $p = p_{max}\cdot\tanh(\alpha I)$ describes the relationship between irradiance and the growth rate of *M. aeruginosa*. The RSM was used to study the simultaneous influence of two independent variables through statistical methods to optimize these variables, irradiance and initial inoculum concentration, as well as to establish a model for predicting the biomass yield and oil concentration of *M. aeruginosa*. Further research is required to improve the models and simultaneously study more factors using CO_2. The main target of such studies is to convert these batch processes into a continuous cultivation system with minimal cost and effort.

Abbreviations

AAD: Asolute average deviation; C: Inoculum concentration (g/L); CVs: Coefficients of variation; EIC: Information criterion; *I*: Irradiance ($\mu mol\cdot m^{-2}\cdot s^{-1}$); I_k: Saturated light intensity ($\mu mol\cdot m^{-2}\cdot s^{-1}$).; K_i: Model constant ($\mu mol\cdot m^{-2}\cdot s^{-1}$); *P*: Growth rate ($mg\cdot L^{-1}\cdot d^{-1}$); PBR: Photobioreactor; PUFAs: Polyunsaturated fatty acids; RSM: Response surface methodology; S/V: Illuminated surface area/culture volume ratio; TAGs: Triglycerides; *α*: Model constant ($mg\cdot L^{-1}\cdot d^{-1}/\mu mol\cdot m^{-2}\cdot s^{-1}$).; *μ*: Specific growth rate (d^{-1});

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Authors' contributions

SEM and OEA performed the experimental work. NNEI performed the statistical analysis. MA revised the statistical analysis part and was a major contributor in writing the manuscript. NA, AE, and II analyzed and revised the results. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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