Appl Biochem Biotechnol (2015) 175:3322–3335
DOI 10.1007/s12010-015-1503-8

# Improvement of Biomass Production by *Chlorella* sp. MJ 11/11 for Use as a Feedstock for Biodiesel

**Supratim Ghosh · Shantonu Roy · Debabrata Das**

Received: 18 April 2014 / Accepted: 21 January 2015 /
Published online: 19 February 2015


**Abstract** Algal biomass is gaining importance for biofuel production as it is rich in lipids. It becomes more significant when biomass is produced by capturing atmospheric greenhouse gas, $CO_2$. In the present study, the effect of different physicochemical parameters were studied on the biomass and lipid productivity in *Chlorella* sp. MJ 11/11. The different parameters viz. initial pH, nitrate concentration, and phosphate concentration were optimized using single-parameter studies. The interactions between the parameters were determined statistically using the Box-Behnken design of optimization. The optimal values were decided by analyzing them with response surface methodology. The optimum levels of the parameters (pH 6.5, nitrate concentration 0.375 g $L^{-1}$, and phosphate concentration 0.375 mL $L^{-1}$) yielded a maximum biomass concentration of 1.26 g $L^{-1}$ at a constant light intensity of 100 μmol $m^{-2}$ $s^{-1}$ and temperature of 30 °C. The effect of $CO_2$ concentration on the biomass production was also investigated and was found to be a maximum of 4 g $L^{-1}$ at 5 % air-$CO_2$ mixture (*v*/*v*). Maximum lipid content of 24.6 % (*w*/*w*) was observed at 2 % air-$CO_2$ mixture (*v*/*v*). Fatty acid analyses of the obtained algal biomass suggested that they could be a suitable feedstock for biodiesel production.

**Keywords** Biodiesel · Box-Behnken design · *Chlorella* sp. · $CO_2$ concentration · Lipid content

## Introduction

The world is facing a monumental crisis at this period of time. Increases in levels of greenhouse gases along with rapid depletion of fossil fuels have led the world to an eminent energy crisis. This has drawn the attention of different countries to search for alternate avenues of fuels—biofuels [1]. In recent times, studies on biomass-based biofuels have gained importance. Among them, algal biomass is gaining importance for production of biofuels as they are rich in lipids and carbohydrates [2]. Lipid-rich microalgae are suitable for biodiesel production because these organisms have a very minimal nutritional requirement, greatest photosynthetic efficiency, and faster growth rate as compared to plants [3]. Biodiesel yield from microalgae is a factor of biomass concentration as well as oil content of the cells [4, 5]. Lipid productivities

S. Ghosh · S. Roy · D. Das (✉)
Department of Biotechnology, Indian Institute of Technology, Kharagpur 721302, India
e-mail: ddas@hijli.iitkgp.ernet.in

in microalgae vary from about 1–85 % of the dry weight. The productivity values increase to values of 40 % or higher when the cells are subject to stress conditions. But, the commercialization of the biodiesel production process from microalgae is still a distant dream because of the high cost of cultivation incurred. This can be solved by developing an efficient process with optimized parameters for growth and lipid production [6].

Optimization of different physicochemical parameters could improve the biomass productivity as well as the biochemical content of the algal cells [7]. Media compositions have a profound effect on increasing the growth rates and productivities as well as content of other bioactive metabolites in the algal cell [8]. Different physicochemical parameters such as nitrate concentration, phosphate concentration, pH, temperature, light intensity, and $CO_2$ concentration significantly affect the growth of microalgae in autotrophic conditions. Micronutrients required for the growth of microalgae highly influence the biomass productivities as because they are cofactors of various enzymatic reactions [9].

The conventional methods for optimization of physiochemical parameters involve the variation of a single parameter responsible for the process keeping all the other factors constant. This leads to a specific set of levels of the parameters which are then tested for maximization of the product thus making the process laborious and time taking [10]. Different statistical methods for process parameter optimization have been employed by several researchers for maximization of their product yield [11, 12]. The interaction between the dependent variables of different factors is not clearly stated by the traditional single-parameter optimization experiments [13]. The Box-Behnken design of experiment has been widely used for the process evaluation of algal growth and lipid productivity [14–16]. Nitrate concentration plays a very important role in the growth of microorganisms. Nitrogen assimilated in the form of nitrate accounts for 7–10 % of the cell biomass. It is an essential component of all the structural and functional proteins found in algal cells, thereby having a profound effect on the biomass production by microalgae [33]. Phosphate concentrations play a major role in the growth and biomass production of microalgae. Phosphorus is accumulated in the form of orthophosphates and accounts for almost 1 % ca of the dry cell weight of microalgae in nutrient replete conditions [17]. It is an important component of the cell as it is required for different mechanisms of growth and metabolic processes of the cell [18]. Therefore, it is also a determining factor for the final biomass concentration of microalgae.

Algae as well as submersed angiosperms shows a decreased tendency of accumulating inorganic carbon when the $CO_2$ concentrations have been increased from 1 to 5 % [19, 20]. This can be attributed to the fact that there is a decreased tendency of carbonic anhydrase to assimilate inorganic carbon at high $CO_2$ concentrations [21]. Reports on formation of pyrenoids in algal cells [22, 23] and carboxysomes in cyanobacteria [24] were available when algal biomass was grown using different $CO_2$ concentration. Electron microscopic studies reveal an electron dense envelope of chloroplasts in low-$CO_2$ containing cells than high-$CO_2$ containing cells. The reverse has been observed for the thickness of plasma membrane of *Dunaliella tertiolecta* [23]. Thus, it can be said that $CO_2$ concentration has a profound effect on the biochemical composition of the cell.

The present study focuses on the increase in biomass concentration of *Chlorella* sp. MJ 11/11 by optimizing the media parameters. Multiple parameters viz. nitrate concentration, phosphate concentration, and initial pH were optimized by the Box-Behnken model, and surface response plots were generated using the MINITAB software [25, 26]. The optimized parameters were then validated for maximum biomass production. Effect of different $CO_2$ concentrations on the growth and lipid productivities was also studied. The lipid and fatty acid content as well as the variations in fatty acid composition were also investigated by varying the $CO_2$ concentration.

## Materials and Methods

### Microalgae and Culture Medium

The culture of *Chlorella* sp. MJ 11/11 was obtained from NCCUBGA, Indian Agricultural Research Institute, New Delhi. The microalgae was grown in Tris Acetate Phosphate (TAP) medium whose composition is described below [27]. TAP media contained 2.42-g $L^{-1}$ Tris base, 25-mL $L^{-1}$ TAP salt stock solution (15.0 g $L^{-1}$ $NH_4Cl$, 4.0 g $L^{-1}$ $MgSO_4 \cdot 7H_2O$, 2.0 g $L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.375-mL $L^{-1}$ $PO_4$ stock solution (28.8 g per 100 mL $K_2HPO_4$, 14.4 g per 100 mL $KH_2PO_4$), 1-mL $L^{-1}$ Hutner trace metals (21.6 g per 100 mL $H_2O$ EDTA: Titriplex II, 11 g per 50 mL $H_2O$ $ZnSO_4 \cdot 7H_2O$, 5.7 g per 100 mL $H_2O$ $H_3BO_3$, 2.53 g per 25 mL $H_2O$ $MnCl_2 \cdot 4H_2O$, 0.805 g per 25 mL $H_2O$ $CoCl_2 \cdot 6H_2O$, 0.785 g per 25 mL $H_2O$ $CuSO_4 \cdot 5H_2O$, 0.55 g per 25 mL $H_2O$ $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.495 g per 25 mL $H_2O$ $FeSO_4 \cdot 7H_2O$), 1-mL $L^{-1}$ vitamin stock solution (0.5 mg $L^{-1}$ cyanocobalamin (B12), 100 mg $L^{-1}$ thiamine HCl, 0.5 mg $L^{-1}$ biotin), 1 mL $L^{-1}$ glacial acetic acid. Glacial acetic acid was absent in TAP [-acetate] medium. Kumar et al. proposed a change in the composition of TAP [-acetate] medium by replacing $NH_4Cl$ with $NaNO_3$ so as to counteract the pH drop during growth of microalgae in presence of $CO_2$. This was called as modified TAP [-acetate] (mTAP [-acetate]) medium [28]. For the experimental studies, mTAP [-acetate] medium was used. The initial optimization studies were done with TAP medium where acetate is used as a carbon source; therefore, $CO_2$ was not provided in those experiments. Once the optimized initial pH, nitrate, and phosphate concentrations were obtained, further studies were performed where $CO_2$ was the sole carbon source.

### Single-Parameter Optimization

From literature, it was found that biomass production depended on the nitrate concentration, phosphate concentration, and initial pH of the media [29]. Therefore, single-parameter experiments with different initial pH, nitrate, and phosphate concentrations were performed in 250-mL conical flasks with a working volume of 100 mL. The experiments were performed in a refrigerated illuminator shaker (INNOVA, New Brunswick, USA). The temperature and light intensity were adjusted to 30 °C and 100 μmol $m^{-2}$ $s^{-1}$, respectively.

### Multiple Parameter Optimizations

For maximization of biomass production, the cumulative effect of three different factors viz. initial pH, nitrate concentration, and phosphate concentration was studied using multiple parameter optimizations. The variables so chosen were initial pH ($X_1$), nitrate concentration ($X_2$), and phosphate concentration ($X_3$), and the corresponding response variable measured was biomass production (Y). A three-factor Box-Behnken design with 15 unique sets and a triplicate on the center point was used and coded, and actual levels of variables chosen for the statistical design of experiment are given in Table 1. The independent variables were coded according to the following equation:

$$x_i = \frac{X_i - X_{i,mid}}{\Delta X_i} \tag{1}$$

where $x_i$ represents the coded value of the $i^{th}$ independent variable, $X_i$ represents the uncoded value of the $i^{th}$ independent variable, $X_{i,mid}$ represents the uncoded value at the center point for

**Table 1** Coded and actual levels of variables chosen for the statistical design of experiment

| Factors | Levels | Variables |
|---|---|---|
| Initial pH | −1 | 6 |
| | 0 | 7 |
| | 1 | 8 |
| Nitrate concentration | −1 | 2.2 mM |
| | 0 | 4.4 mM |
| | 1 | 6.6 mM |
| Phosphate concentration | −1 | 1.35 mM |
| | 0 | 2.7 mM |
| | 1 | 5.4 mM |

the $i^{th}$ independent variable, and $\Delta X_i$ represents the difference between any two consecutive point of the $i^{th}$ independent variable. Statistical software, MINITAB15, was used for modeling whereby the experimental data were fit into a second degree polynomial equation of the following form:

$$Y = C_1 + C_2X_1 + C_3X_2 + C_4X_3 + C_5X_1^2 + C_6X_2^2 + C_7X_3^2 + C_8X_1X_2 + C_9X_2X_3 + C_{10}X_3X_1 \quad (2)$$

Response surface methodology (RSM) was used to determine the interactions between the variables. The MINITAB software generated 3D and contour plots which gave a graphical insight on the cumulative effect of the concerned variables. The resulting values for interacting were related to the output variable by Eq. (2) [11, 12].

## Effect of $CO_2$ Concentration on Biomass Yield and Lipid Content

The effect of different $CO_2$ concentrations was studied on biomass productivity and lipid yields of *Chlorella* sp. MJ 11/11. The studies were performed in customized airlift photobioreactors which had an $A_d/A_r$ ratio of 4.4 with a constant surface by volume (S/V) ratio of 0.57 $cm^{-1}$. The inner draft tube had a diameter of 3 cm [26]. The working volume for the experiments in the photobioreactors was maintained at 1.4 L. A mixture of air-$CO_2$ (*v*/*v*) was sparged inside the reactor with an air flow of 0.34 vvm. An assembly of rotameters was used for this purpose. The reactor was sparged with 0.03, 1, 2, 3, 5, and 10 % of air-$CO_2$ mixture and was observed for 10 days. During the experiment, the temperature and light intensity were kept constant at 30±2 °C and 100 μmol $m^{-2}$ $s^{-1}$, respectively.

## Analytical Methods

### *Biomass Analysis*

Algal growth in mTAP [-acetate] medium was obtained by measuring the optical density (OD) of sample on a daily basis at 681 nm using Double Beam UV–Visible spectrophotometer (Spectroscan UV 2600, Chemito). Dry cell weight of cells was determined by harvesting a known volume of algal culture by centrifugation at 6500*g* for 10 min, and the pellets were washed twice with deionized water. The cell pellets were then dried at 60 °C for 24 h, and the final concentration was noted when the constant weight was observed [30].

### *Lipid Estimation*

Total lipids were extracted from dried algal biomass using the procedure given by Bligh and Dyer [31]. The fatty acid profiles were analyzed by tranesterifying the obtained lipids with methanolic-HCl mixture at a temperature of 90 °C for 2 h [32]. The sample was analyzed using a gas chromatograph (Clarus 500, PerkinElmer) equipped with Omegawax 250 capillary column (30-m length, 0.25-μm film thickness, and 0.25-mm internal diameter, Sigma) and a flame ionization detector. The oven temperature was programmed from 50 to 240 °C at the rate of 4 °C $min^{-1}$ and at the end held at 240 °C for 15 min. The carrier gas was nitrogen with the flow rate of 1 mL $min^{-1}$. The chromatographic peaks were identified by comparing their retention times and fragmentation patterns with standards of fatty acid methyl ester (FAME) mixture (37mix, Supelco Inc., Bellefonte, PA)

## Results and Discussion

### Optimization of Biomass Production from *Chlorella* sp. MJ 11/11

TAP medium was chosen as the growth medium for *Chlorella* sp. MJ 11/11. Our comparative studies between BBM and TAP-acetate media showed higher biomass yield on using TAP-acetate (data not shown). This observation compelled us to use TAP-acetate media for further studies. The different parameters viz. initial pH, nitrate concentration, and phosphate concentration were optimized by single-parameter optimization in a batch process. The initial pH was varied in a range of 6–9 with an interval of 1 for the determination of optimal pH for maximum biomass. The nitrate and phosphate concentrations were kept constant at 0.375 g $L^{-1}$ and 2.7 mM, respectively. The maximum biomass concentration of 0.8 g $L^{-1}$ was observed at pH 7 with a decrease in biomass concentration at pH higher than 7 (Fig. 1). At lower pH, the biomass concentration of the microalgae decreased which ceased to grow at a pH below 6. Microalgae generally grow at a neutral or slightly alkaline pH. This is due to the fact that most of the enzymes associated with the growth of microalgae become inactive at lower pH [33]. The effect of nitrate concentration on the biomass production was also investigated. The microalgae was grown with different nitrate concentrations in a range of 0–0.75 g $L^{-1}$ while the phosphate concentration and the optimized initial pH were kept constant at 2.7 mM and pH 7, respectively. The maximum biomass concentration of 0.89 g $L^{-1}$ was observed at a nitrate concentration of 0.375 g $L^{-1}$ (Fig. 1). As the nitrate concentration was increased, the biomass concentration also increased. A drop in biomass concentration was observed at lower nitrate levels. The results are in parity with Mallick et al. where a decrease in growth rates was observed with low nitrate concentration [34]. The phosphate concentration was also an important factor in determining the biomass concentration of the microalgae. The initial phosphate concentration was varied within a range of 0–5.4 mM. The maximum biomass concentration was observed at a phosphate concentration of 2.7 mM which corresponded to the value of 0.87 g $L^{-1}$. The biomass concentration was observed to be comparatively lower at lower phosphate concentrations than higher concentrations (Fig. 1). The effect of nitrate and phosphate on growth was supported by Ryan et al., Frink and Machlis, and Sikka and Pramer [35].

### Maximization of Biomass Production by Multiparameter Optimization

The optimized process parameters obtained from single-parameter optimization helped in designing experiment for multiparameter optimization by a three-factor Box-Behnken design.

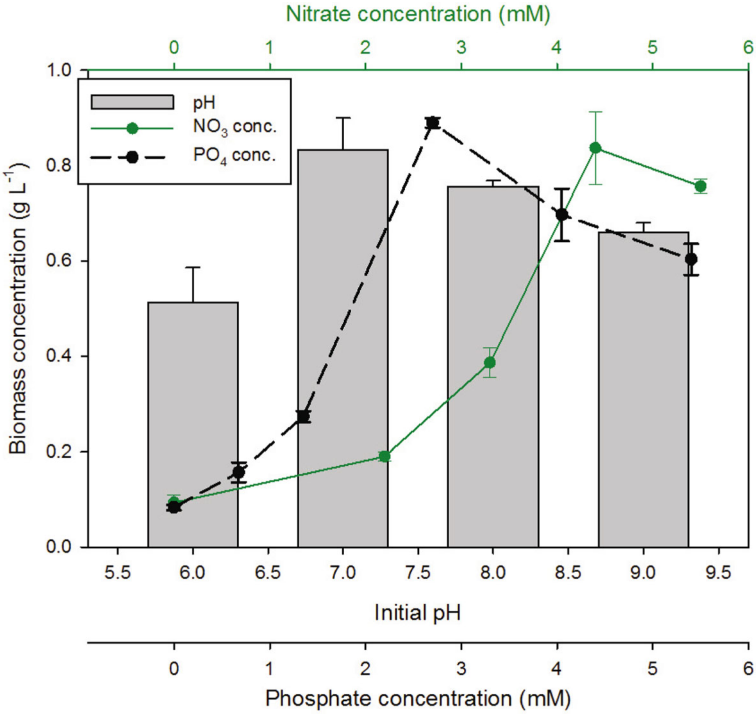


**Fig. 1** Effect of different physicochemical parameters like initial pH, nitrate concentration and phosphate concentration on biomass concentration in a batch system

Effect on biomass production by significant independent variables (initial pH, nitrate concentration, and phosphate concentration) was explored using Box-Behnken design.

Table 2 represents the design of matrix and experimental results. Fifteen unique sets of experiments and a triplicate on the center point were performed. Initial pH was varied from 6 to 8 in steps of 1 pH unit, nitrate concentration was varied from 0.187 to 0.5625 g $L^{-1}$ in steps of 0.187 g $L^{-1}$, and phosphate concentration was varied from 1.35 to 5.4 mM in steps of 2.7 mM and biomass production was observed. The above values were considered using single-parameter optimization.

The theoretical model generated upon training the experimental data is as follows:

$$Y = -10.0190 + 2.5703X_1 + 5.8019X_2 + 0.6802X_3 - 0.1713X_1^2 - 5.9358X_2^2 \\ -0.0565X_3^2 - 0.0746X_1X_2 - 0.0379X_2X_3 - 0.1734X_3X_1 \quad (3)$$

Regression analysis gave an $R^2$ value of 0.94 indicating that the model fails to explain less than 6 % of the variations observed. ANOVA for the response variable revealed 9 degrees of freedom and 5 error degrees of freedom. Fischer variance ratio (F) was found to be 32.50 which is greater than $F_{0.05,\,9.5}$=4.772 (significance level $\alpha$=0.05) which indicates that the data obtained is highly significant.

Using the Minitab software, the response surface curves described by the regression model were constructed (Fig. 2). In these figures, each response surface plot represents the effect of two independent variables on biomass production.

The response curve of nitrate and phosphate concentration on biomass production is shown in Fig. 2a. It shows that there was an increase in biomass production when both nitrate and

**Table 2** Design matrix and results of a three-factor Box-Behnken design

| $V_1$ | $V_1c$ | $V_2$ | $V_2c$ | $V_3$ | $V_3c$ | Y (g $L^{-1}$) |
|---|---|---|---|---|---|---|
| 7 | 0 | 4.4 | 0 | 2.70 | 0 | 1.260 |
| 7 | 0 | 2.2 | −1 | 2.70 | 1 | 0.723 |
| 7 | 0 | 2.2 | −1 | 1.35 | −1 | 0.850 |
| 6 | −1 | 6.6 | 1 | 5.40 | 0 | 0.980 |
| 8 | 1 | 6.6 | 1 | 2.70 | 0 | 0.950 |
| 6 | −1 | 4.4 | 0 | 2.70 | −1 | 0.736 |
| 8 | 1 | 4.4 | 0 | 2.70 | 1 | 0.810 |
| 7 | 0 | 4.4 | 0 | 5.40 | 0 | 1.260 |
| 7 | 0 | 4.4 | 0 | 2.70 | 0 | 1.266 |
| 6 | −1 | 2.2 | −1 | 1.35 | 0 | 0.850 |
| 7 | 0 | 6.6 | 1 | 1.35 | 1 | 0.680 |
| 8 | 1 | 2.2 | −1 | 5.40 | 0 | 0.820 |
| 7 | 0 | 6.6 | 1 | 2.70 | −1 | 1.010 |
| 8 | 1 | 4.4 | 0 | 2.70 | −1 | 0.710 |
| 6 | −1 | 4.4 | 0 | 1.35 | 1 | 0.680 |

*$V_1$* initial pH, *$V_2$* nitrate concentration, *$V_3$* phosphate concentration, *c* coded value, *Y* biomass concentration

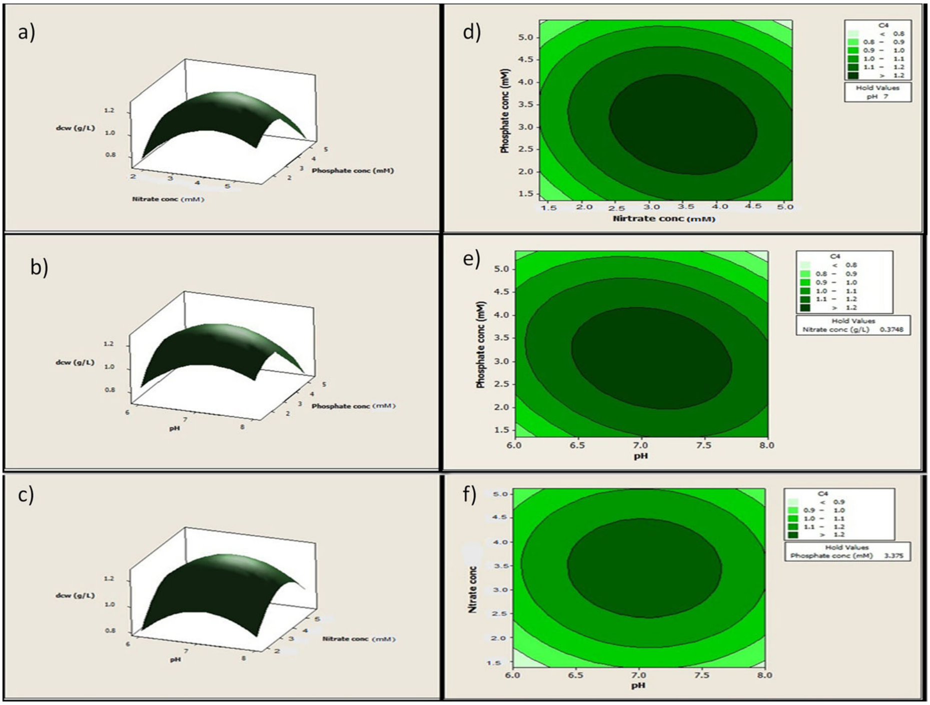


**Fig. 2** Response surface and contour plots on biomass production with respect to the effect of different process parameters and their mutual interactions: (**a**) and (**d**) Nitrate concentration vs phosphate concentration; (**b**) and (**e**) pH and phosphate concentration and (**c**) and (**f**) pH vs nitrate concentration

phosphate concentrations were increased. This can be attributed to the fact that higher nitrate and phosphate concentrations in the medium account for more availability of organic nitrogen and phosphorus. The higher amount of nitrogen and phosphorus leads to increase in biomass production as because the essentials for growth of microalgae are in abundance. The cell machinery is at its peak, and thus, more biomass is produced. This could be an indication that the microalgae could be grown in wastewater containing high amounts of nitrate and phosphate which could in turn lead to bioremediation. But, at very high phosphate concentrations beyond 4 mM, the biomass concentration decreased significantly indicating the negative effect of high phosphate concentrations on biomass production [36]. The contour plot (Fig. 2d) confirmed the synergistic effect of nitrate and phosphate concentrations on the growth of microalgae. A significant interaction was shown by the contour plot suggesting the importance of nitrate and phosphate concentration.

Result obtained in Fig. 2b shows the response curve with respect to phosphate concentration and initial pH on biomass production. A significant increase in biomass production had been achieved by increasing the phosphate concentration from 1 to 4 mM, but a drastic fall in biomass concentration was observed when the phosphate concentrations was increased to higher values. But, an increase in initial pH did increase the biomass concentration which remained almost constant from neutral to alkaline pH. This suggested the stability of different metabolic enzymes of microalgae in neutral or alkaline pH range which may be the cause of higher biomass concentration at neutral pH. Figure 2e shows the interaction between pH and phosphate concentration. Phosphate is assimilated by algae as orthophosphate. This being a strong base, orthophosphate is available to microalgae at neutral and alkaline pH. Thus, the growth is significantly affected at higher pH with higher levels of phosphate.

The response surface plot based on independent variables, i.e., initial pH and nitrate concentration on biomass production, is shown in Fig. 2c. A significant increase in biomass production could be achieved by increasing nitrate concentration from 0.187 to 0.5625 g $L^{-1}$ which remained almost constant at higher nitrate concentrations. This suggested that nitrate concentration is a very important factor for the biomass production in the organism. It also suggested that an increase in the nitrate concentration from the specified end limit of nitrogen concentration could increase the biomass production that provided other parameters that remain constant. This observation could be of use as because the organism could be grown on wastewater containing high amount of dissolved nitrogen and it could easily thrive in those environments. An increase in initial pH from 6 to 7 increased the biomass concentration to a significant level which decreased when the pH increased above 8. This suggests that the maximum rate of photosynthesis at neutral or slightly alkaline pH for microalgae might correspond to higher activity of metabolic enzymes. The parameters showed a nonsignificant interaction as suggested by the contour plot (Fig. 2f). Similar results for biomass concentration under various levels of nitrate and phosphate were observed by Fan et al.. They observed marked increases in the total lipid content under nitrogen deficiency with a reduction of biomass concentration. The different enzymes involved in the de novo fatty acid biosynthesis might be regulating the lipid content in the microalgae. This could be used as a platform for genetic modification of the microalgae for enhanced biofuel production [37].

Thus, optimized value of process parameters, i.e., initial pH, nitrate concentration, and phosphate concentration, came out to be pH 7, 0.375 g $L^{-1}$, and 2.7 mM, respectively, with maximum biomass concentration of 0.89 g $L^{-1}$. The mathematical model given by Eq. (3) gave the maximum point of the model at 1.266 g $L^{-1}$ corresponding to nitrate concentration of 0.399 g $L^{-1}$, phosphate concentration of 3.027 mM, and pH 7.07. Thus, the theoretical value came concurrent with the experimental values. Biomass production increased by 22 % as compared to single-parameter optimization. The cumulative effect of process parameters, i.e., initial pH, nitrate concentration, and phosphate concentration, in multiparameter optimization gave an increased biomass yield as compared to single-parameter optimization.

## Effect of $CO_2$ Concentration on Biomass Production

The effect of $CO_2$ concentrations on the biomass production was observed with the optimal culture conditions. The experiments were performed in a customized airlift reactor. The optimal values of media components were used for the studies. Kumar et al. suggested the change in media composition by changing the nitrate source from $NH_4Cl$ to $NaNO_3$ because he had observed a certain drop in pH when the media was sparged with a mixture of air-$CO_2$ (*v/v*) [28]. Therefore, the media components were varied accordingly, and the initial experiments were performed with $NaNO_3$ as a nitrogen source in place of $NH_4Cl$. Using the optimized values for media components, the organism was grown in presence of air, 1, 2, 3, 5, and 10 % air-$CO_2$ mixtures (*v/v*). As shown in Fig. 3a, the maximum biomass of 4.0 g $L^{-1}$ was obtained at 5 % air-$CO_2$ mixture (*v/v*). Net-specific growth rate in mTAP [-acetate] medium were 0.84, 1.13, 1.45, 1.26, 1.1 $day^{-1}$ at air, 2, 5, 8, 10 % air-$CO_2$ gas mixture (*v/v*), respectively, while the maximum biomass concentration corresponded to 1.8, 2.2, 2.8, 3.3, 4.0, and 2.2 g $L^{-1}$. The microalgae was also grown under high $CO_2$ concentrations, but it yielded lower biomass yields whose data was not shown here. This also suggested that the microorganism could grow at very high $CO_2$ concentrations which in turn could help in biological sequestration of $CO_2$. $CO_2$ mitigation by microalgae can be combined with biofuel production strategies for increasing the yield of the product. Different factors determine the $CO_2$ mitigation by microalgae. One of the major factors includes the photobioreactor configuration and its mixing characteristics. Studies by Fan et al. 2008 reiterated the fact that efficient mixing strategy and bioreactor configuration may be utilized for higher biomass concentration. In addition to this, other factors such as initial pH of the medium and temperature also influence the $CO_2$ sequestration capacity of the microalgae. Process parameters need to be optimized for high rate algal biomass production with efficient biomitigation of $CO_2$ [38].

The pH profile was observed during the growth. It was observed that as soon as the medium is sparged with air, the pH increases from an initial of 6.5 to a pH of around 8.4. A higher $CO_2$ concentration enables the carboxylase activity of ribulose-1, 5 bisphosphate carboxylase/ oxygenase (RuBisCo) thereby enhancing the carbon capturing mechanism of microalgae.

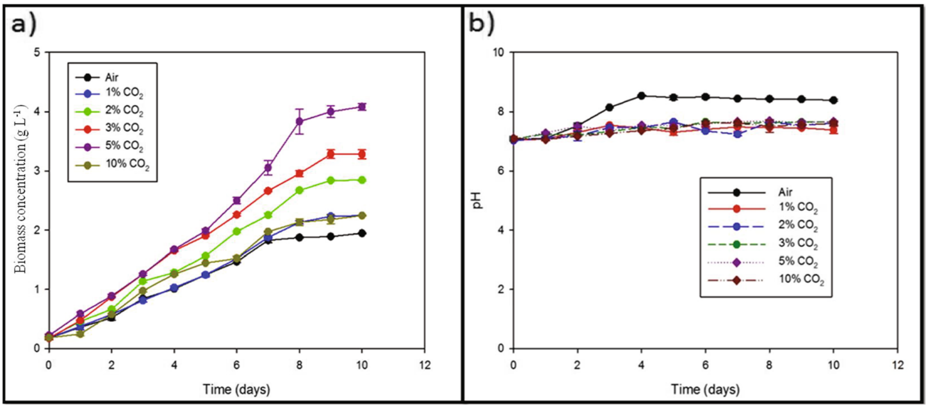


**Fig. 3** (**a**) Cell growth and (**b**) pH profiles of *Chlorella* sp. MJ 11/11 in modified TAP [-acetate] medium using different air–CO2 (*v/v*) gas mixture

But, at lower concentrations of $CO_2$, RuBisCo shifts toward oxygenase activity thereby lowering the carbon capture [39].

## Effect of $CO_2$ Concentration on Lipid Yield and Fatty Acid Composition

The effect of $CO_2$ concentration was studied on the lipid yield and fatty acid composition of *Chlorella* sp. MJ 11/11 (Fig. 4). The biomass obtained at different $CO_2$ concentrations was air-dried and were subjected to lipid extraction. The maximum lipid content was observed with the 2 % air-$CO_2$ mixture (*v*/*v*) sparged sample which corresponded to a value of 24.2 % lipids (*w*/*v*). The lipid content of the cells grown in air, 1, 2, 3, 5, and 10 % air-$CO_2$ mixture (*v*/*v*) was 19.13, 24.6, 10.53, 7.19, 5.6, and 4.8 % (*w*/*v*), respectively. It was observed that with an increase in $CO_2$ concentration, the lipid content of the cells decreased rapidly. The improvement in lipid productivity might be attributed to the fact that the bicarbonates obtained due to externally supplemented $CO_2$ may have played a role in carbon metabolism of the algal cells leading to carbohydrate accumulation. The presence of $CO_2$ for microalgae growth might lead to the storage of lipids under stress microenvironment. Given the stress conditions, the algal cells divert their total lipids into forming neutral lipids rather than forming phospholipids for membrane function and growth [40]. The biomass concentration was higher at higher $CO_2$ concentrations but with lower lipid yields. On the other hand, lower $CO_2$ concentrations corresponded to higher lipid levels in the algal cells lending them a good candidate for biodiesel production. Thus, a compromise is to be reached in order to have a high rate algal biomass production which could be utilized for biodiesel production.

The fatty acid composition was also studied in different $CO_2$ levels (Table 3). Major fatty acids in *Chlorella* sp. MJ 11/11 were palmitic (16:0), palmitoleic (16:1), and oleic (18:1) acids. Myristic (14:0), hexadecadienoic (16:2), stearic (18:0), linoleic (18:2), and arachidic (20:0) acids were also found as minor components (Table 3). An increase in the concentration of unsaturated fatty acids was observed with increased $CO_2$ levels. The results are confirmed by H Zheng et al. [41]. The level of unsaturated fatty acids determines the quality of the biodiesel

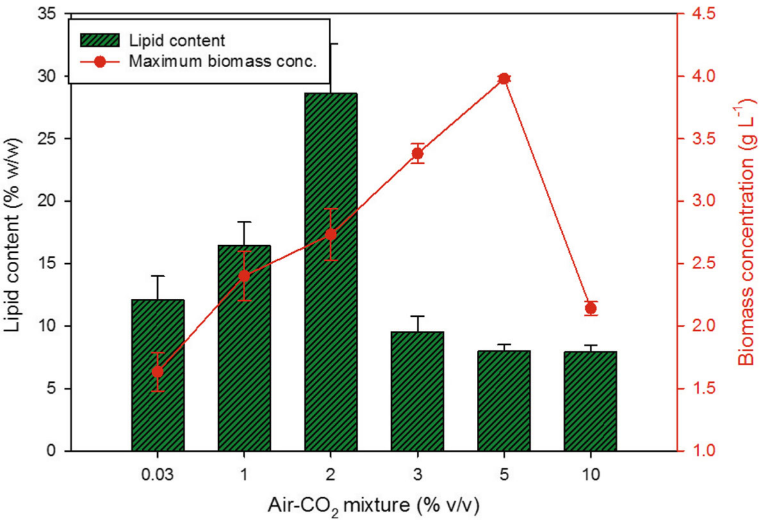


**Fig. 4** Lipid yields and maximum biomass concentrations in different air–CO (*v/v*) gas mixture

**Table 3** Fatty acid compositions of *Chlorella* sp. MJ 11/11 under different $CO_2$ concentrations

| Fatty acids | Air-$CO_2$ concentration (%*v*/*v*) | | | | | |
|---|---|---|---|---|---|---|
| | 0.03 | 1 | 2 | 3 | 5 | 10 |
| C14:0 | 2.32±0.2 | 3.42±0.3 | 2.78±0.2 | 1.61±0.1 | 2.35±0.2 | 0.78±0.1 |
| C16:0 | 19.24±1.0 | 21.63±1.0 | 19.66±1.8 | 18.33±0.7 | 20.52±1.1 | 18.16±0.9 |
| C16:1 | 23.27±0.9 | 24.31±1.1 | 27.34±1.7 | 26.98±0.9 | 24.31±0.8 | 28.05±1.0 |
| C16:2 | 5.55±0.4 | 1.07±0.2 | 1.95±0.2 | 3.54±0.4 | 3.27±0.3 | 4.26±0.5 |
| C16:3 | 0.68±0.5 | 0.72±0.2 | 0.78±0.6 | 0.76±0.2 | 0.78±0.3 | 0.72±0.2 |
| C18:0 | 2.19±0.3 | 1.09±0.1 | 2.64±0.3 | 1.13±0.1 | 1.28±0.1 | 1.62±0.2 |
| C18:1 | 36.58±1.7 | 41.16±0.8 | 36.97±1.4 | 39.72±1.5 | 41.37±1.7 | 38.56±1.8 |
| C18:2 | 3.25±0.3 | 3.34±0.5 | 4.50±0.4 | 5.21±0.6 | 2.73±0.2 | 6.88±0.7 |
| C18:3 | 1.08±0.6 | 1.06±0.3 | 1.06±0.2 | 1.02±0.7 | 1.07±0.3 | 1.06±0.8 |
| C20:0 | 6.60±0.4 | 3.98±0.3 | 4.16±0.4 | 3.48±0.3 | 4.17±0.5 | 1.69±0.2 |
| Unsaturated fatty acids | 69.65±0.7 | 69.88±0.8 | 70.76±0.9 | 75.45±1.0 | 71.68±1.0 | 77.75±1.2 |
| Saturated fatty acids | 30.35±0.6 | 30.12±0.5 | 29.24±0.4 | 24.55±0.7 | 28.32±0.7 | 22.25±0.8 |

produced. Biodiesel with high levels of saturated fatty acids has a very good oxidative stability. The fatty acids obtained had a carbon chain length of C14–C22. This range of carbon chain length has been reported to be suitable for biodiesel production [42]. In Table 4, a comparison regarding various biomass and lipid yields reported in literature using microalgae was done. In the present study, a yield of 4.0-g $L^{-1}$ biomass has been found to be comparable with the earlier study done with *Chlorella vulgaris* under mixotrophic growth conditions. The lipid yield of 34 % (*w*/*w*) was higher as compared to the present study possibly due to the mixotrophic mode of growth [45].

**Table 4** Comparative study on biomass and lipid yield in different microalgae

| Species | $P_{DCW}$ (g $L^{-1}$)/day | Lipids (%*w*/*v*) | TAG (%) | Reference |
|---|---|---|---|---|
| *Nannochloris* sp. UTEX LB1999 | (2.7)/12 | 34.0 | 18.8 | [43] |
| *Chlorella minutissima* | (1.23)/9 | NA | NA | [26] |
| *Chlorella minutissima* UTEX 2341[a] | (12.6)/7 | 16.11 | NA | [16] |
| *Dunaliella* | (0.5)/10 | 67 | NA | [44] |
| *Chlorella vulgaris*[b] | (4.28)/6 | 13.7 | NA | [45] |
| *Chlorella sp*. MJ 11/11 | (4.0)/10 | 24.2 | NA | This study |


| Species | $P_{DCW}$ (g $L^{-1}$)/day | Lipids (%*w*/*v*) | TAG (%) | Reference |
|---|---|---|---|---|
| *Nannochloris* sp. UTEX LB1999 | (2.7)/12 | 34.0 | 18.8 | [43] |
| *Chlorella minutissima* | (1.23)/9 | NA | NA | [26] |
| *Chlorella minutissima* UTEX 2341[a] | (12.6)/7 | 16.11 | NA | [16] |
| *Dunaliella* | (0.5)/10 | 67 | NA | [44] |
| *Chlorella vulgaris*[b] | (4.28)/6 | 13.7 | NA | [45] |
| *Chlorella sp*. MJ 11/11 | (4.0)/10 | 24.2 | NA | This study |


[a] Heterotrophic growth

[b] Mixotrophic growth

## Conclusion

Physicochemical parameters for growth of *Chlorella* sp. MJ 11/11 were optimized. A statistical method was employed for multiparameter optimization for improvement of biomass production. Box-Behnken design was adopted to screen the key process parameters and identify optimal values that contribute maximum biomass production. The results suggested that the statistical experimental design is an effective tool for optimization of process parameters on biomass production. Experimental results show that initial pH, nitrate concentration, and phosphate concentration had significant influence on biomass production. Under optimized conditions, an increase in biomass concentration was observed. This was enhanced when the organism was grown on 5 % air-$CO_2$ mixture (*v*/*v*). The lipid yields at different air-$CO_2$ mixtures (*v*/*v*) were also studied with a maximum lipid yield with 2 % air-$CO_2$ mixture (*v*/*v*). The fatty acid compositions suggested that *Chlorella* sp. MJ 11/11 could be a feedstock for high-quality biodiesel production.

**Acknowledgments** The authors gratefully acknowledge University Grants Commission (UGC), and Council for Scientific and Industrial Research (CSIR) Govt. of India for fellowship; IIT Kharagpur and Department of Biotechnology (DBT), Govt. of India for the financial support.

## References

1. Demirbas, A. (2007). Progress and recent trends in biofuels. *Progress in Energy and Combustion Science, 33*(1), 1–18.
2. Nayak, B. K., Pandit, S., Das. D., Biohydrogen. (2013). In: C., Kennes C., Veiga ría (Eds.), *Air Pollut. Prev. Control* (pp. 345–81). John Wiley & Sons, Ltd.
3. Ngangkham, M., Ratha, S. K., Prasanna, R., Saxena, A. K., Dhar, D. W., Sarika, C., et al. (2012). Biochemical modulation of growth, lipid quality and productivity in mixotrophic cultures of *Chlorella sorokiniana. Springer Plus, 1*, 33.
4. Becker, W. (2004). Microalgae in human and animal nutrition. In A. Richmond (Ed.), *Handbook of microalgal culture* (pp. 312–351). Oxford: Blackwell.
5. Chisti, Y. (2008). Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology, 26*, 126–131.
6. Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances, 25*, 294–306.
7. Behrens, P. W., & Kyle, D. J. (1996). Microalgae as a source of fatty acids. *Journal of Food Lipids, 3*, 259–272.
8. Chaichalerm, S., Pokethitiyook, P., Yuan, W., Meetam, M., Sritong, K., Pugkaew, W., et al. (2012). Culture of microalgal strains isolated from natural habitats in Thailand in various enriched media. *Applied Energy, 89*, 296–302.
9. Barsanti, L., & Gualtieri, P. (2006). *Algae: Anatomy, Biochemistry, and Biotechnology.* Boca Raton: Taylor & Francis.
10. Karemore, A., Pal, R., & Sen, R. (2013). Strategic enhancement of algal biomass and lipid in *Chlorococcum infusionum* as bioenergy feedstock. *Algal Research, 2*(2), 113–121.
11. Roy, S., Ghosh, S., & Das, D. (2012). Improvement of hydrogen production with thermophilic mixed culture from rice spent wash of distillery industry. *International Journal of Hydrogen Energy, 37*, 15867–15874.
12. Roy, S., Vishnuvardhan, M., & Das, D. (2013). Improvement of hydrogen production by newly isolated Thermoanaerobacterium thermosaccharolyticum IIT BT-ST1. *International Journal of Hydrogen Energy.* doi:10.1016/j.ijhydene.2013.06.128.
13. Ferreira, S. L. C., Bruns, R. E., Ferreira, H. S., Matos, G. D., David, J. M., Brandão, G. C., et al. (2007). Box–Behnken design: an alternative for the optimization of analytical methods. *Analytica Chimica Acta, 597*, 179–186.
14. Azma, M., Mohamed, M. S., Mohamad, R., Rahim, R. A., & Ariff, A. B. (2011). Improvement of medium composition for heterotrophic cultivation of green microalgae, *Tetraselmis suecica*, using response surface methodology. *Biochemical Engineering Journal, 53*, 187–195.

15. Patil, P. D., Gude, V. G., Mannarswamy, A., Deng, S., Cooke, P., Munson-McGee, S., et al. (2011). Optimization of direct conversion of wet algae to biodiesel under supercritical methanol conditions. *Bioresource Technology, 102*, 118–122.
16. Li, Z. S., Yuan, H. L., Yang, J. S., & Li, B. Z. (2011). Optimization of the biomass production of oil algae *Chlorella minutissima* UTEX2341. *Bioresource Technology, 102*(19), 9128–9134.
17. Hu, Q. (2004). Environmental effects on cell composition. In A. Richmond (Ed.), *Handbook of microalgal culture* (pp. 312–351). Oxford: Blackwell.
18. Powell, N., Shilton, A., Pratt, S., & Chisti, Y. (2008). Factors Influencing Luxury Uptake of Phosphorus by Microalgae in Waste Stabilization Ponds. *Environmental Science Technology, 42*, 5958–5962.
19. Aizawa, K., & Miyachi, S. (1986). Carbonic anhydrase and $CO_2$ concentrating mechanisms in microalgae and cyanobacteria. *FEMS Microbiology Review, 39*, 215–233.
20. Tsuzuki, M., & Miyachi, S. (1989). The function of carbonic anhydrase in aquatic photosynthesis. *Aquatic Botany, 34*, 85–104.
21. Aizawa, K., & Miyachi, S. (1984). Carbonic anhydrase located on cell surface increases the affinity for inorganic carbon in photosynthesis of *Dunaliella tertiolecta. FEBS Letters, 173*, 41–44.
22. Miyachi, S., Tsuzuki, M., Maruyama, I., Gantar, M., Miyachi, S., & Matsushima, H. (1986). Effects of $CO_2$ concentration during growth on the intracellular structure of *Chlorella* and *Scenedesmus. Journal of Phycology, 22*, 313–319.
23. Tsuzuki, M., Gantar, M., Aizawa, K., & Miyachi, S. (1986). Ultrastructure of *Dunaliella tertiolecta* cells grown under low and high $CO_2$ concentrations. *Plant Cell Physiol, 27*, 737–739.
24. Turpin, D. H., Miller, A. G., & Canvin, D. T. (1984). Carboxysome content of *Synechococcus leopoliensis* (Cyanophyta) in response to inorganic carbon. *Journal of Phycology, 20*, 249–253.
25. Box, G.E.P., Berkum, E.E.M.V. (1960). Some new three level designs for the study of quantitative variables.
26. Mopkar Anand, A. S. D., Sankar, V., & Daniel, D. K. (2013). Optimization of Light intensity, Nitrate concentration and Cultivation time for Biomass production by *Chlorella minutissima* using Response Surface Methodology. *Journal of Applied Sciences Research, 9*(1), 94–99.
27. Skjanes, K., Knutsen, G., Kallqvist, T., & Lindblad, P. (2008). $H_2$ production from marine and freshwater species of green algae during sulfur deprivation and considerations for bioreactor design. *International Journal of Hydrogen Energy, 33*, 511–521.
28. Kumar, K., & Das, D. (2012). Growth characteristics of *Chlorella sorokiniana* in airlift and bubble column photobioreactors. *Bioresource Technology, 116*, 307–313.
29. Yadavalli, R., Rao, S. R., & Rao, C. S. (2013). Response surface methodological approach to optimize process parameters for the biomass production of *Chlorella pyrenoidosa. International Journal of Biotechnology Research, 3*(1), 37–48.
30. Zhang, H., Wang, W., Li, Y., Yang, W., & Shen, G. (2011). Mixotrophic cultivation of *Botryococcus braunii. Biomass and Bioenergy, 35*, 1710–1715.
31. Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*, 911–917.
32. Doan, T. T. Y., Sivaloganathan, B., & Obbard, J. P. (2011). Screening of marine microalgae for biodiesel feedstock. *Biomass Bioenergy, 35*, 2534–2544.
33. Dey, P., Banerjee, J., & Maiti, M. K. (2011). Comparative lipid profiling of two endophytic fungal isolates — *Colletotrichum* sp. and *Alternaria* sp. having potential utilities as biodiesel feedstock. *Bioresource Technology, 102*, 5815–5823.
34. Gimmler, H. (2001). Acidophilic and acidotolerant algae. In L. C. Rai & J. P. Gaur (Eds.), *Algal Adaptation to Environmental Stresses Physiological, Biochemical and Molecular Mechanisms* (pp. 259–290). Heidelberg: Springer Press.
35. Mandal, S., & Mallick, N. (2009). Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. *Applied Microbiology and Biotechnology, 84*(2), 281–291.
36. Fried, S., Mackie, B., & Nothwehr, E. (2003). Nitrate and phosphate levels positively affect the growth of algae species found in Perry Pond. *Tillers, 4*, 21–24.
37. Fan, J., Cui, Y., Wan, M., Wang, W., & Li, Y. (2014). Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors. *Biotechnology for Biofuels, 7*, 17.
38. Fan, L. H., Zhang, Y. T., Zhang, L., & Chen, H. L. (2008). Evaluation of a membrane-sparged helical tubular photobioreactor for carbon dioxide biofixation by *Chlorella vulgaris. Journal of Membrane Science, 325*, 336–345.
39. Jacob-Lopes, E., Scoparo, C. H. G., & Franco, T. T., (2008). Rates of $CO_2$ removal by *Aphanothecemicroscopica* Nageli in tubular photobioreactors. *Chemical Engineering Progress, 47*, 1365–1373.
40. Prathima Devi, M., & Venkata Mohan, S. (2012). $CO_2$ supplementation to domestic wastewater enhances microalgae lipid accumulation under mixotrophic microenvironment: Effect of sparging period and interval. *Bioresource Technology, 112*, 116–123.

41. Zheng, H., Gao, Z., Yin, F., Ji, X., & Huang, H. (2012). Effect of $CO_2$ supply conditions on lipid production of *Chlorella vulgaris* from enzymatic hydrolysates of lipid-extracted microalgal biomass residues. *Bioresource Technology, 126*, 24–30.
42. Demirbas, A. (2010). Biodiesel for future transportation energy needs. *Energy Source Part A, 32*(16), 1490–1508.
43. Yamaberi, K., Takagi, M., & Yoshida, T. (1998). Nitrogen depletion for intracellular triglyceride accumulation to enhance liquefaction yield of marine microalgal cells into a fuel oil. *Journal of Marine Biotechnology, 6*, 44–48.
44. Takagi, M., Karseno, S., & Yoshida, T. (2006). Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. *Journal of Bioscience and Bioengineering, 101*, 223–226.
45. Kong, W. B., Hua, S. F., Cao, H., Mu, Y. W., Yang, H., Song, H., et al. (2012). Optimization of mixotrophic medium components for biomass production and biochemical composition biosynthesis by *Chlorella vulgaris* using response surface methodology. *Journal of the Taiwan Institute of Chemical Engineers, 43*, 360–367.