Enhanced Production of Lipid as Biofuel Feedstock from the Marine Diatom *Nitzschia* sp. by Optimizing Cultural Conditions

Anandapadmanaban Baala Harini,[a] Renganathan Rajkumar,[a,*] and Mohd Sobri Takriff [b,*]

Microalgae were isolated, identified, and cultivated for enhanced biomass production and lipid accumulation. A marine microalgae was isolated from coastal rock pools of Tuticorin, Tamil Nadu, and identified as *Nitzschia* sp. RRSE2 upon microscopic examination and molecular sequence analysis. The experimental results showed that the maximum growth, biomass, and lipid content were obtained at pH 8 using the F/2 medium. These parameters revealed a notable difference when NaCl was added at 3% concentration. Meanwhile, the nutrients $NaNO_3$ (18.75 mgL^{-1}) and NaH_2PO_4 (3.48 mgL^{-1}) were shown to be suitable nitrogen and phosphorus sources, respectively, for the production of lipids. On day 14, the maximum lipid concentration of 77.5 mgL^{-1} was produced using optimized culture conditions. Additionally, the maximum number of 17×10^5 cells mL^{-1} and the biomass concentration of 0.69 gL^{-1} were achieved on this same day. Finally, the fatty acid composition of the algal lipid was analyzed by gas chromatography/mass spectrometry (GC/MS) analysis.

Keywords: Isolation; Identification; Nitzschia; Biomass; Lipid

Contact information: a: Department of Environmental Sciences, Bharathiar University, Coimbatore – 641 046, Tamil Nadu, India; b: Research Centre for Sustainable Process Technology, Faculty of Engineering & Built Environment, University Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia;
** Corresponding authors: micro_rajkumar@yahoo.co.in; sobritakriff@ukm.edu.my*

INTRODUCTION

Microalgae have received much attention as a potential renewable energy source as part of the efforts to mitigate global warming. Microalgae can easily convert solar energy into chemical energy *via* atmospheric carbon dioxide fixation, which is currently under consideration as a promising raw material for biodiesel production (Spolaore *et al.* 2006; Chisti 2007; Mata *et al.* 2010).

In spite of the various advantages microalgae could bring to biodiesel production, their biomass and lipid production cost is relatively very high due to many factors associated with the microalgal cultivation process (Chisti 2013; Singh *et al.* 2015). To improve the economic feasibility of microalgal cultivation, it will be necessary to identify a suitable microalgal strain and explore its response to different cultivation conditions (Chen *et al.* 2015; Negi *et al.* 2015; Singh *et al.* 2016). Recently, many researchers have worked to optimize various factors, such as nitrogen starvation or limitation (Zhila *et al.* 2005; Jiang *et al*. 2012; Pancha *et al*. 2014), silicon deficiency (Lynn *et al.* 2000), phosphate limitation (Mandal and Mallick 2009; Chu *et al.* 2013), salinity (Rao *et al.* 2007; Zhila *et al.* 2011), pH of the medium (Breuer *et al.* 2013), iron content (Liu *et al.* 2008), light intensity (Ruangsomboon 2012), and harvesting time (Zhila *et al.* 2011), to

improve the lipid accumulation by microalgae. When evaluating microalgae under such conditions, Hsieh and Wu (2009) stated that various microalgal strains responded appreciably when the lipid content was increased 30% to 60% of the dry cell weight.

The biomass content of the microalgae can be enhanced when the microalgae are cultivated in photobioreactors. A previous study reported that a high concentration of microalgae cultures was cultivated in photobioreactors that facilitate high light intensity surfaces and high mass transfer rates to improve the biomass productivity (Ugwu *et al.* 2008). It has also been reported that the biomass productivity of *Chlorella vulgaris* was increased 50% in the membrane-sparged helical tubular photobioreactor (MSTR) (Fan *et al.* 2008).

Among these microalgae, diatoms (class Bacillariophyceae) include a large diversity and produce the greatest biomass in the marine environment, though they are not well recognized for biofuel purposes. Diatoms have been identified as promising candidates to produce biodiesel because they give up to 60% of their cellular biomass as triacylglycerols (TAG) that can be converted into biodiesel through the transesterification process (Blanchemain and Grizeau 1996; Yu *et al.* 2009). In general, diatoms have been studied widely for their biochemical constituents that can be used for the larval diet in the aquaculture field. However, only a few reports demonstrate the use of diatoms for biofuel production. For example, *Nitzschia palea* was found to exhibit a high amount of lipid production according to the results given by the Aquatic Species Program (Sheehan *et al.* 1998).

The effect of nutrients and pH on marine diatoms, especially *Nitzschia* sp., for enhancing lipid and biomass production remains an infrequently studied research area. Thus, the present study focused on the isolation of indigenous diatoms from Tuticorin along the Tamil Nadu coast, and studied the effects of cultural conditions on the production of biomass and lipid by the isolate.

EXPERIMENTAL

Materials

Isolation and growth conditions

Seawater samples were collected in sterile plastic vials from coastal rock pools in Tuticorin, Tamil Nadu, India. Pure cultures were isolated and purified by streaking 1.5% agar plates with F/2 medium (Guillard and Ryther 1962). This media was composed of: $NaNO_3$, 75 mgL^{-1}; $NaH_2PO_4.2H_2O$, 5 mgL^{-1}; $Na_2SiO_3.9H_2O$, 30 mgL^{-1}; trace metal stock solution, 1 mL (per 1 L: $FeCl_3.6H_2O$, 3.15 g; $Na_2EDTA.2H_2O$, 4.36 g; 1 mL $CuSO_4.5H_2O$, 9.8 gL^{-1}; 1 mL $Na_2MoO_4.2H_2O$, 6.3 gL^{-1}; 1 mL $ZnSO_4.7H_2O$, 22.0 gL^{-1}; 1 mL $CoCl_2.6H_2O$, 10.0 gL^{-1} and 1 mL $MnCl_2.4H_2O$, 180.0 gL^{-1}), and 0.5 mL of vitamin stock solution (per 1 L: vitamin B1, 200 mg; 1 mL vitamin H, 1.0 gL^{-1} and 1 mL vitamin B12, 1 gL^{-1}). Media chemicals were bought from Himedia, Mumbai, India. The individual colonies were inoculated into the above liquid medium and incubated at 25 ± 1°C in a thermostatically controlled room, with a light intensity of 30 μEm^{-2} s^{-1}, and a photoperiod of 16:8 h (light: dark). The same cultivation conditions were maintained for all the subsequent experiments. Preliminary identification was made for morphological characteristics under a microscope (John *et al.* 2003; Bellinger and Sigee 2010). The purity of the individual culture was confirmed by repeated streaking techniques on the nutrient agar plate. Based on the highest lipid productivity and growth rate, the strain was chosen for further study.

Nile Red Staining

Nile Red (9-(Diethylamino)-5H-benzo[a]phenoxazin5-one) (Himedia, Mumbai, India) staining was performed to observe intracellular lipid droplets according to the method of Greenspan *et al*. (1985). Isolated algal cells were cultured for two weeks and tested with Nile Red staining. The growing culture (0.5 mL) was taken by centrifugation at 1500×g for 10 min and cleaned with a physiological saline solution (0.5 mL). Once the obtained cells were re-suspended in the same saline solution (0.5 mL), the Nile Red solution (0.1 mg mL^{-1} dissolved in acetone) was further added to cell suspensions (1:100 v/v) and kept for 10 min. Subsequently, stained algal cells were observed under fluorescent microscopy.

Molecular Identification

The selected algal strain of approximately 50 mL in the log to stationary phase was used for molecular identification. The isolation of the genomic DNA of the microalgal strain was performed using a plant genomic DNA isolation kit (Sigma-Aldrich, Bangalore, India) following the manufacturer's protocol. The primers SSU-1+ (AAC CTG GTT GAT CCT GCC AGT) and SSU-B (CCT TCT GCA GGT TCA CCT AC) (Sigma-Aldrich, Bangalore, India) were subjected to the polymerase chain reaction (PCR) (Medlin *et al.* 1988; Li *et al.* 2015). The polymerase chain reaction (PCR) amplification was performed in a thermal cycler (Agilent Technologies, Santa Clara, CA, USA) using the following program: initial denaturation for 5 min at 95 °C; 20 cycles for 1 min at 94 °C, 45s at 55 °C, 4 min at 72 °C; and a final extension for 10 min at 72 °C. Electrophoresis was performed with 1% agarose gel (Himedia, Mumbai, India) to separate the PCR products. The amplified product was sequenced at Acme Progen Biotech Pvt. Ltd., Salem, Tamil Nadu, India, and the obtained sequences were analyzed on the National Center for Biotechnology Information (NCBI, Bethesda, MA, USA) server using the Basic Local Alignment Search Tool (BLAST). Further, the nucleotide sequence was submitted to the NCBI to obtain the accession number.

Effect of pH, Salinity, Nutrients, and Cultivation Time on Biomass and Lipid Production

For all experiments, the selected microalgae were grown in an F/2 medium adjusted with different pH ranges (6 to 9). For the analysis of salinity tolerance, the isolated culture was transferred into the same medium added with 0%, 3%, 6%, and 9% of the NaCl concentration. The effect was 0, 18.75, 28.125, 37.5, and 46.875 mgL^{-1} ($NaNO_3$ as nitrogen source). Quantities of 0, 2.32, 3.48, 5, and 7 mgL^{-1} (NaH_2PO_4 as the phosphorus source) were also tested separately. Other than the above cultural parameters, the composition of other elements was the same as the F/2 medium. Experiments were carried out in 500 mL Erlenmeyer flasks containing 200 mL medium. All the flasks were inoculated with the 10% v/v cells of two-week-old culture and incubated in a thermostatically controlled room for 16 days to analyze the growth and lipid productivity every 2 days. The optimum cultural parameter was selected at each experiment based on lipid productivity, and further cultivation was completed in a photobioreactor under the optimized conditions.

Cultivation in Photobioreactor

The chosen microalga was cultivated in a photobioreactor (Lark Innovative Fine Teknowledge, Chennai, India) (2 L capacity) within an optimized F/2 medium to determine the effect of selective factors (Fig. 1).

Fig. 1. Photograph of the photobioreactor for the cultivation of *Nitzschia* sp. RRSE2

The photobioreactor provided a shaft that prevented the culture from settling. Sparger supplied CO_2 with air. Turbines helped to mix the media and culture. A glass vessel was autoclaved and used to grow the *Nitzschia* sp., which was helpful in the overall increase in both biomass and lipid content. For this experiment, 10% of the inoculum was added to the F/2 media (pH 8) with optimized components, such as a salinity of 3%, a nitrate concentration of 18.75 mgL^{-1} ($NaNO_3$), and a concentration of phosphorous of 3.48 mgL^{-1} (NaH_2PO_4). The reactor ran until the decline of the growth phase of the algae, which was up to 14 days. The pH was controlled to remain between 7.9 and 8.1. To maintain a level of 5% CO_2 and to supplement nutrients, the sample was mixed at 150 rpm. The conditions surrounding the reactor were a light: dark maintained as 16:8 h, with 2500 to 3000 lux of light, and a temperature of approximately 25 to 27 °C. A sample of approximately 30 mL was collected every 2 days starting from day 0. The cell concentration and the lipid content were recorded during the cultivation process. On day 14, the grown biomass was collected by filtering with Whatman No.1 filter paper. The collected biomass was oven-dried dried at 65 °C for 1 h, and the crude lipid was extracted. Finally, the extracted lipid was subjected to gas chromatography/mass spectrometry (GC/MS) analysis.

Analytical Methods

Growth and biomass estimation

The cultures were harvested by centrifugation at 10000 rpm for 5 min. Then, the cells were washed twice with distilled water, and the pellet was dried at 70 °C for 1 h. The dry weight of the biomass was determined gravimetrically. The algal growth was expressed in terms of dry cell weight (DCW) per liter (gL^{-1}). Meanwhile, microalgal growth was also monitored by the cell-counting method using a Neubauer hemocytometer (Rohem, India).

Extraction and estimation of lipid

Lipids were extracted from the biomass using the method of Folch *et al.* (1957). Ten mL of grown culture was centrifuged. Then, the pellet was collected and ground with chloroform:methanol (1:2, v/v). Physiological saline was added for phase separation between chloroform and methanol. The above experiment was left overnight. The lower chloroform phase was collected and air-dried. The presence of lipid was treated with the concentrated sulphuric acid and kept at a high temperature for 10 min. Then, vanillin reagent was added to the solution and read at 520 nm. Cholesterol (Himedia, Mumbai, India) with different concentrations was used to prepare for the standard graph to find the unknown weight of the isolated lipid. Simultaneously, transesterification of fatty acids was done by the method of Ichihara *et al.* (1996). The extracted lipid was air dried at room temperature and the obtained lipid was measured gravimetrically. About 10 mg of lipid was dissolved using 2 mL of hexane and 200 µL of 2 M methanolic KOH and vortexed for 2 to 5 min. The FAME containing upper layer (hexane phase) was recovered for further analysis.

Analysis of fatty acid methyl esters

Fatty acid methyl esters were analyzed from the extracted lipid by GC/MS according to the method of Tadashi *et al.* (2009). Gas chromatography/mass spectrometry was performed for the isolated crude lipid and was run in SQ8C GC/MS (Perkin Elmer, Waltham, MA, USA). The crude lipid underwent the basic principle of separation of compounds present in the sample in DB-5ms capillary standard non-polar column (30 m length; 0.32 mm; 0.25 mm; with a flow rate of 0.25 µm). A sample of 1 mL was injected. The oven's initial temperature was 150 °C and progressively raised to 240 °C. A total of 2 µL of sample was injected with flame ionisation detector (FID) temperature of 260 °C. The nitrogen carrier gas was used as a stationary phase that serves as a column with the solvent delay of 3 min. Retention time, a time at which each particular component eluted from the sample, helped in differentiating the elements. Lastly, the GC/MS analysis of this fatty acid composition was performed at Tamil Nadu Agricultural University, Coimbatore, India.

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (IBM Corporation, USA) for one-way ANOVA test. All the experiments were carried out in triplicate. Experimental data were calculated as mean ± SE, and the mean values were determined by using DMRT (Duncan's Multiple Range Test). Significant differences were considered to be at $P < 0.05$.

RESULTS AND DISCUSSION

Isolation and Identification of the Isolate

Nitzschia sp. RRSE2 strain, which was the dominant microalga at the collection site, was isolated from coastal rock pools in Tuticorin by serial dilution, then subjected to plating techniques. The strain revealed the following morphological characteristics under a light microscope: that it was comprised of single cells with bilateral symmetry and isopolar frustules, and in each cell there were two chloroplasts, elongated valves, and a rounded polar end. The cell length was 18.5 µm, and the width was 4.5 µm (Fig. 2a). Nile Red staining of algal cells was also performed to detect intracellular lipid droplets stained

in red using fluorescence microscopy (Fig. 2b). The strain was identified as genus *Nitzschia*, confirmed by 18S rDNA gene sequencing followed by BLAST analysis, demonstrating over 95% similarity with other *Nitzschia* sp. (KT072977.1). Hence, the strain was identified as *Nitzschia* sp. RRSE2 and the sequences were submitted to the NCBI, and an accession number (MK785417) was obtained.

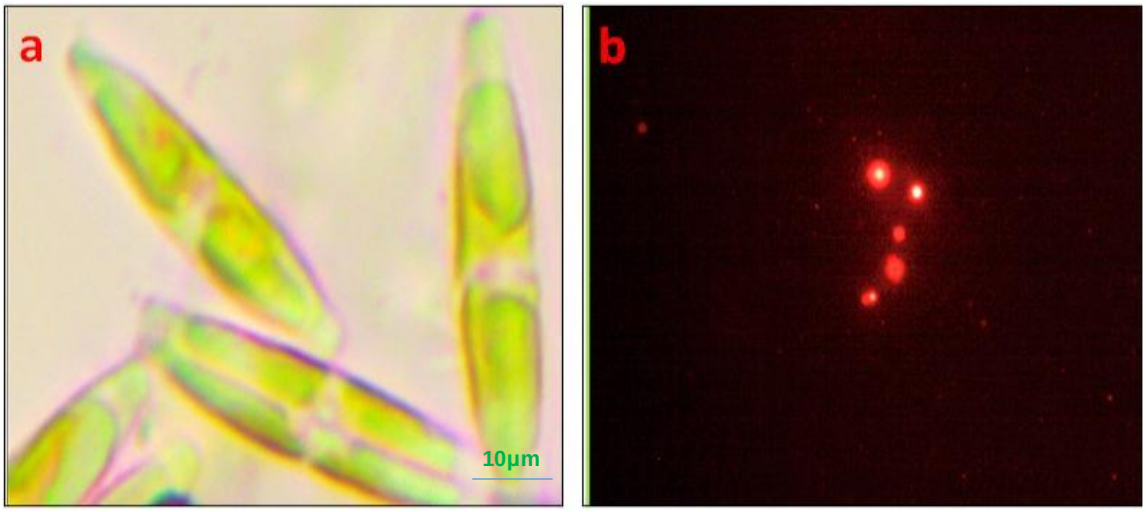


Fig. 2. Microscopy images of the alga, *Nitzschia* sp. RRSE2 grown on F/2 medium: (a) light microscopic (40x) image of the morphology of *Nitzschia* sp. RRSE2; (b) fluorescence images of *Nitzschia* sp. RRSE2 stained with Nile Red

The Effect of pH on Growth and Lipid Production

Diatoms are a highly diverse group of organisms in nature. They can also grow well in a designated medium with a sufficient amount of nutrients and suitable environmental conditions. Wang *et al.* (2010) reported that microalgal growth is controlled by various factors such as pH, nutrient availability, size of the seed culture, *etc*. The F/2 medium supported the growth of different kinds of microalgal species and was used to cultivate the *Nitzschia* in the present study. The growth curves, biomass, and lipid concentrations of *Nitzschia* were evaluated under different pH, and are shown in Fig. 3. The growth of *Nitzschia* demonstrated an optimal growth rate at pH 8 with a maximum cell number of 8.7×10^5 cells mL^{-1} on the tenth day (Fig. 3a). The maximum biomass content 0.36 gL^{-1} with total lipid productivity of 46.5 mgL^{-1} was observed at pH 8 (Fig. 3b and 3c). The biomass and lipid content increased until the twelfth day, and no further increment was noticed subsequently. It was thus concluded that *Nitzschia* was able to tolerate a wide range of pH, an observation that accorded with the reports of Barinova *et al.* (2011) concerning *Fragilaria vaucheriae*, and the reports of Li *et al.* (2017) concerning *Synedra* sp. and *Navicula.* The diatom of the present study, *Nitzschia,* did not grow well in a medium with a lower pH (acidic conditions) to produce frustules and uptake silicic acid (Martin-Jezequel *et al.* 2000).

The Effect of Salinity on Growth and Lipid Production

To study salinity tolerance, *Nitzschia* was subjected to a wide range of salinity gradients. Increasing the salinity of algal cultures can be used to keep cultures free of unwanted algal species (Chaffin *et al.* 2011). As shown in Fig. 4, *Nitzschia* was the fastest growing alga at 6 % NaCl (9.1×10^5 cells mL^{-1}) with a biomass content of 0.32 gL^{-1} on the day 12, followed by 9% and 3% concentrations.

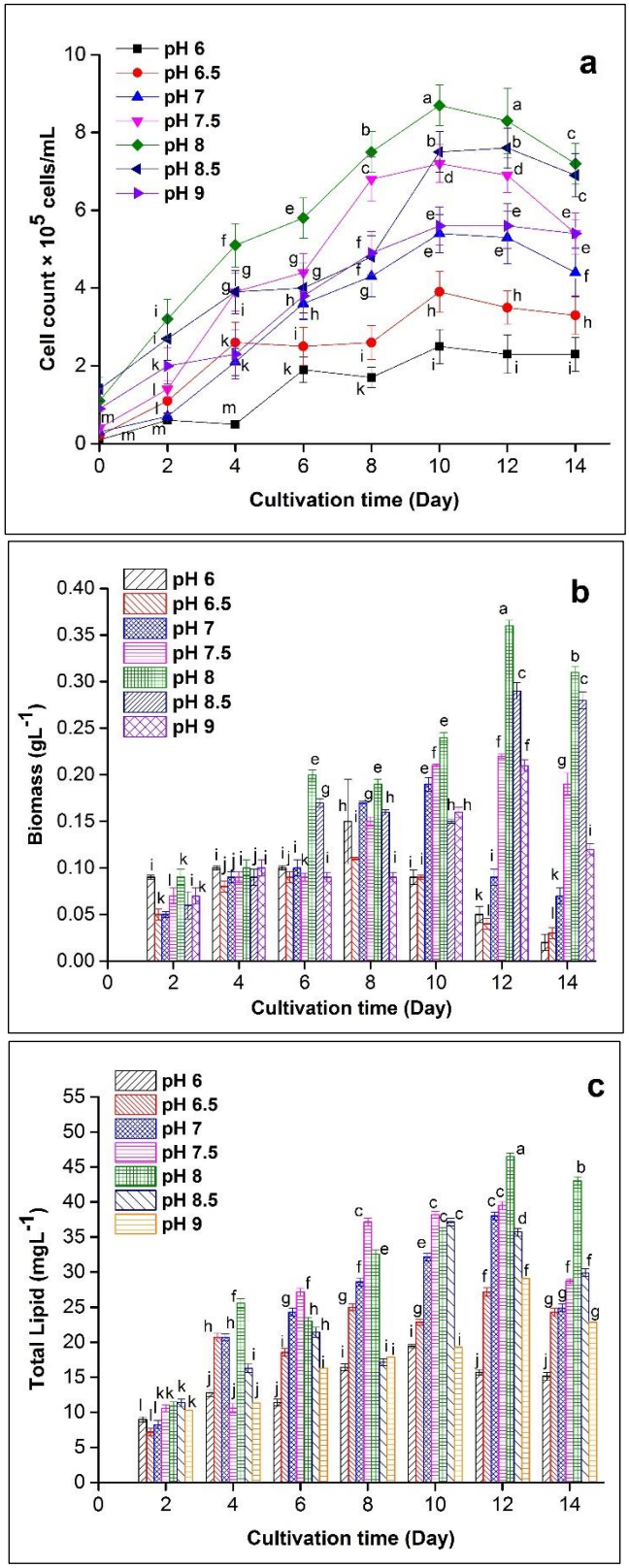


Fig. 3. The effect of pH on (a) cell growth, (b) biomass concentration, and (c) lipid concentration of *Nitzschia* sp. RRSE2 grown in F/2 medium. Lines and bars are represent ± SE (n = 3). Means with different small letters on the lines and bars are significantly different ($p<0.05$).

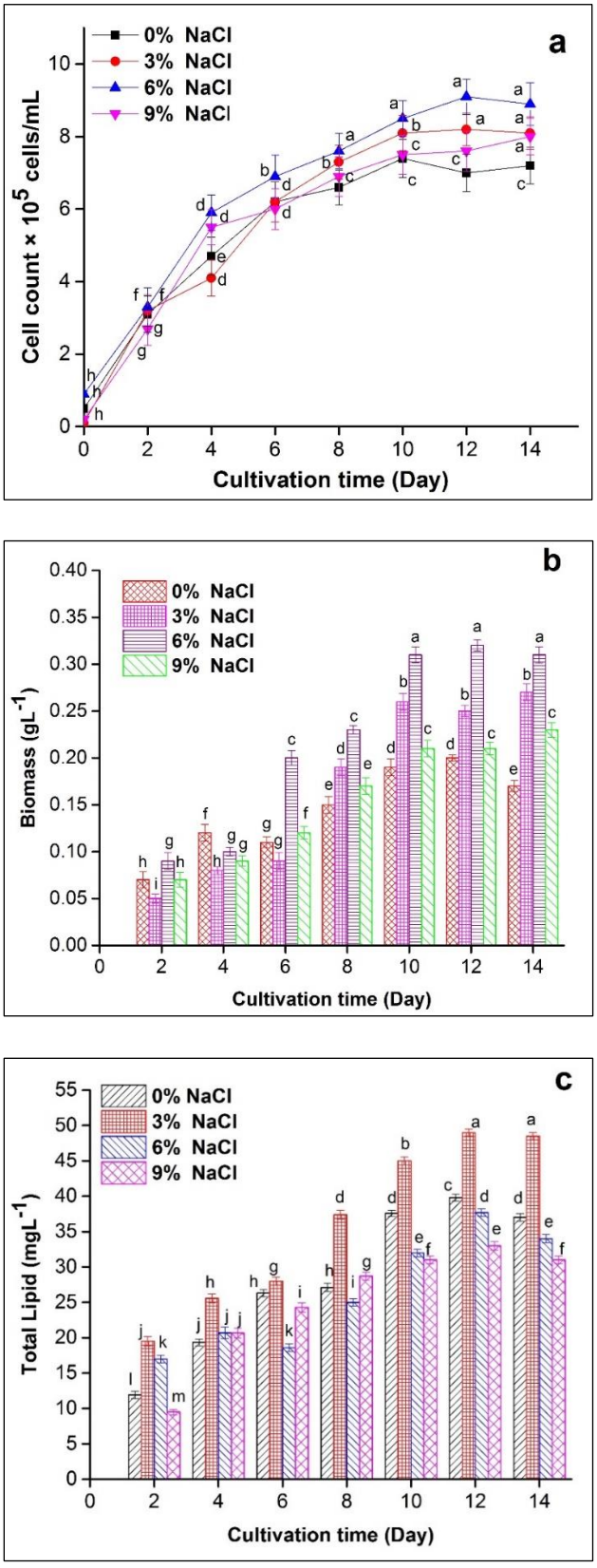
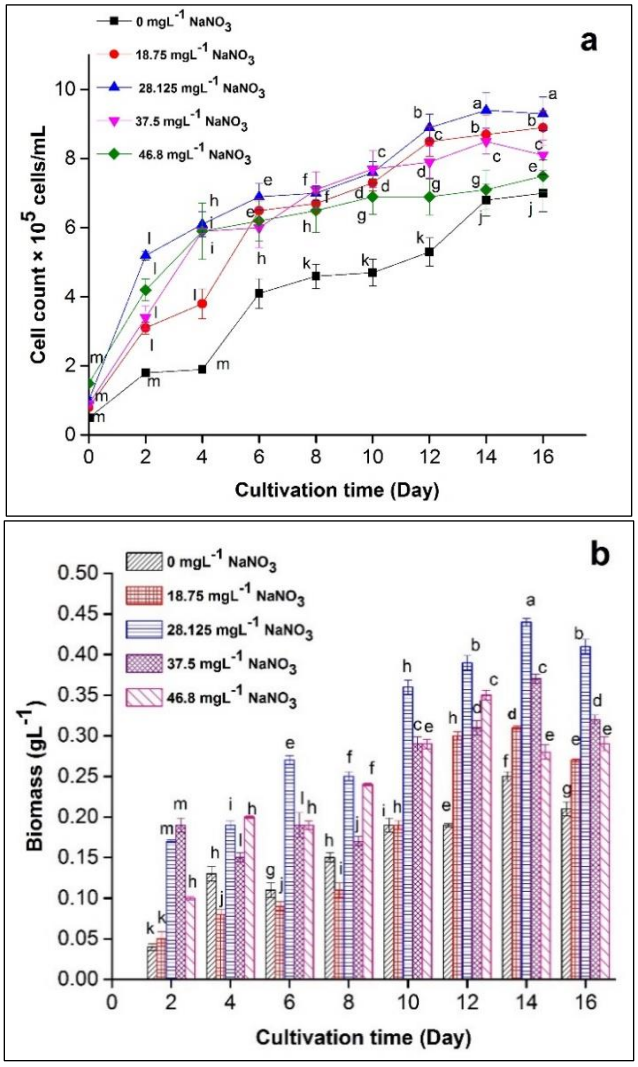


Fig. 4. The effect of NaCl concentration on (a) cell growth, (b) biomass concentration, and (c) lipid concentration of *Nitzschia* sp. RRSE2. Lines and bars are represent ± SE (n = 3). Means with different small letters on the lines and bars are significantly different ($p<0.05$).

Jahnke and White (2003) stated that decreasing marine microalga growth was observed at a low salinity level. However, in the present study, the highest lipid production of 49 mgL^{-1} was observed at low salinity (3% NaCl), followed by 0%, 6%, and 9% NaCl (Fig. 4a through c). Hu (2004) found that an increase in salt concentration may slightly enhance the total lipid content of algae. Ben-Amotz *et al.* (1985) stated that low salinity stress also increases the lipid content in *Botryococcus braunii*.

The Effect of Nitrogen Concentration on Growth and Lipid Production

The cell growth, the concentration of biomass, and lipid were observed, as depicted in Fig. 5. Although the level of lipid concentration increased with decreased $NaNO_3$ concentration in the culture medium, it varied according to the cell concentration and biomass content (Fig. 5a through c). The maximum biomass concentration of 0.44 gL^{-1} was observed after supplying 37.5 mgL^{-1} $NaNO_3$ to the culture medium on day 14, and the maximum lipid productivity of 58.2 mgL^{-1} was seen at a concentration of 18.75 mgL^{-1} $NaNO_3$ on day 12. Similarly, Yu *et al.* (2009) reported that the diatom *Phaeodactylum tricornutum* (FACHB-863) grown in a complete F/2 medium produced biomass equaling 530 mgL^{-1}.



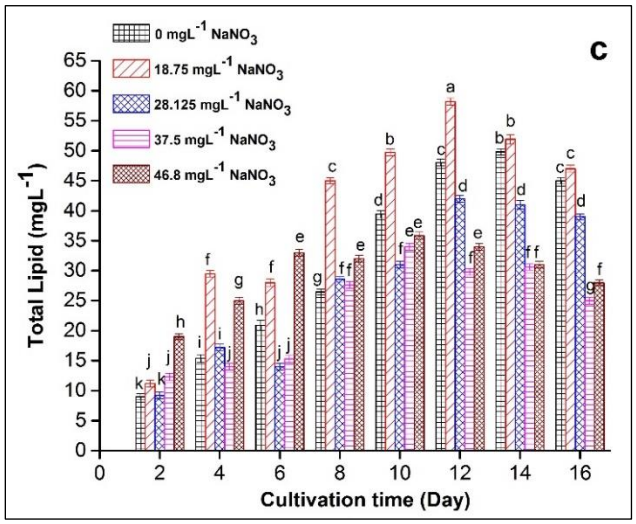


Fig. 5. The effect of $NaNO_3$ concentration on (a) cell growth, (b) biomass concentration, and (c) lipid concentration of *Nitzschia* sp. RRSE2. Lines and bars are represent ± SE (n = 3). Means with different small letters on the lines and bars are significantly different ($p<0.05$).

The results of the previous study accorded with previous reports by Tornabene *et al.* (1983) and Lv *et al.* (2010), which demonstrated that a green alga produces higher lipid content at a lower $NaNO_3$ concentration. Converti *et al.* (2009) reported that a lack of nitrogen limits protein synthesis and hence increases lipid content and, sometimes, the accumulation of carbohydrates in the cells (Hu 2003).

The Effect of Phosphorus Concentration on Growth and Lipid Production

Across microalgae species, phosphorous is among the essential nutrient components of the medium in cellular metabolic processes. The effects of different phosphorus concentrations on cell growth, biomass, and lipid concentration are given in Fig. 6a through c. The highest biomass production and cell number of 0.51 gL^{-1} and 13.4 $\times 10^5$ cells mL^{-1} were obtained at the increased phosphorus concentration of 5 mgL^{-1}. The biomass and cell numbers increased when the phosphorus concentration in the medium increased, but the lipid content decreased. The maximum lipid concentration of 56 mgL^{-1} was recorded with a phosphorus concentration of 3.48 mgL^{-1} in the medium, because phosphate is a vital nutrient for the cell growth and development (excepting lipid yield). Mandal and Mallick (2009) reported that growing *Scenedesmus obliquus* had an increment of lipid only under phosphorus-deficient conditions.

Performance of Microalga Growth and Lipid Production with Photobioreactor Using Optimized F/2 Medium

The preliminary investigation made on *Nitzschia* under different culture conditions revealed that the optimal conditions for enhanced production of total lipids were: (1) pH 8.0, (2) 3% NaCl concentration, (3) 18.75 mgL^{-1} $NaNO_3$, and (4) 3.48 mgL^{-1} NaH_2PO_4 in F/2 medium (other components are fixed amounts). The isolate *Nitzschia* grown under the above conditions for 16 days in 2 L of medium were subjected to cultivation in a photobioreactor, with special attention to the estimation of total lipid and biomass content. Photobioreactors have been used to produce increased algal biomass and even specific molecules, such as lipids (Ugwu *et al.* 2008; Rodolfi *et al.* 2009).

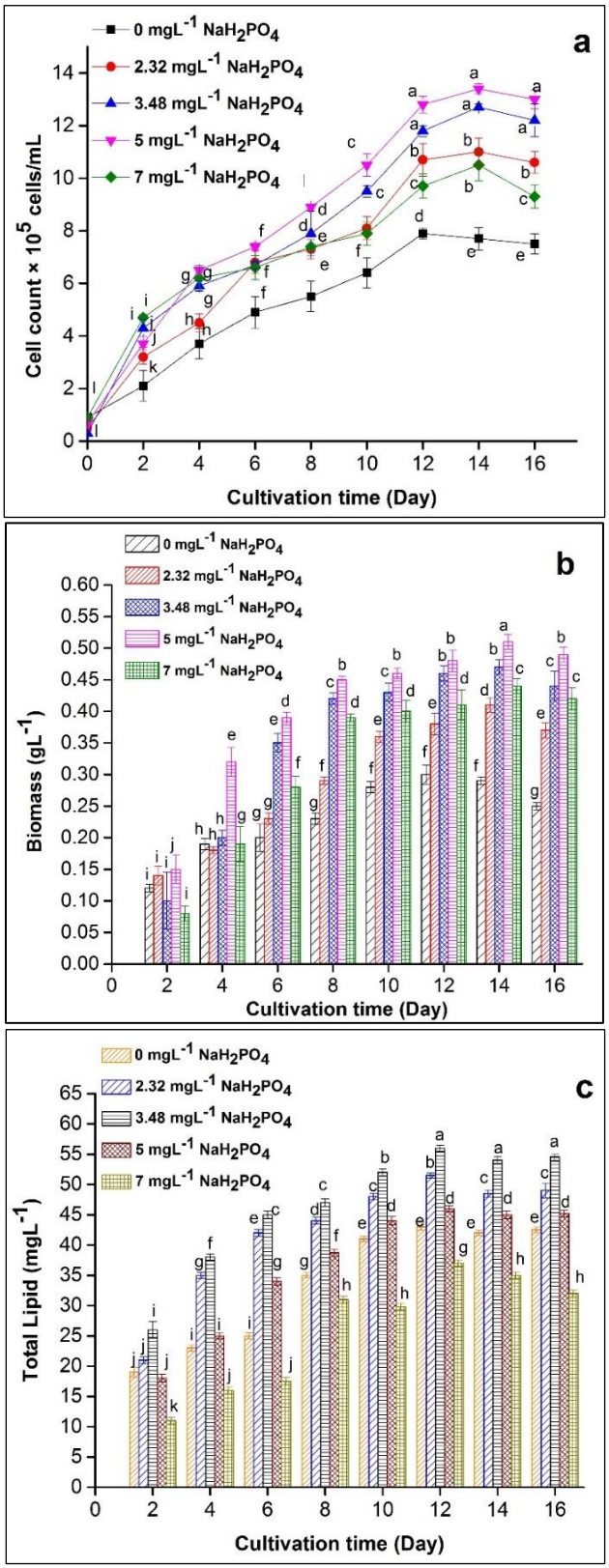


Fig. 6. The effect of NaH_2PO_4 concentration on (a) cell growth, (b) biomass concentration, and (c) lipid concentration of *Nitzschia* sp. RRSE2. Lines and bars are represent ± SE (n = 3). Means with different small letters on the lines and bars are significantly different ($p<0.05$).

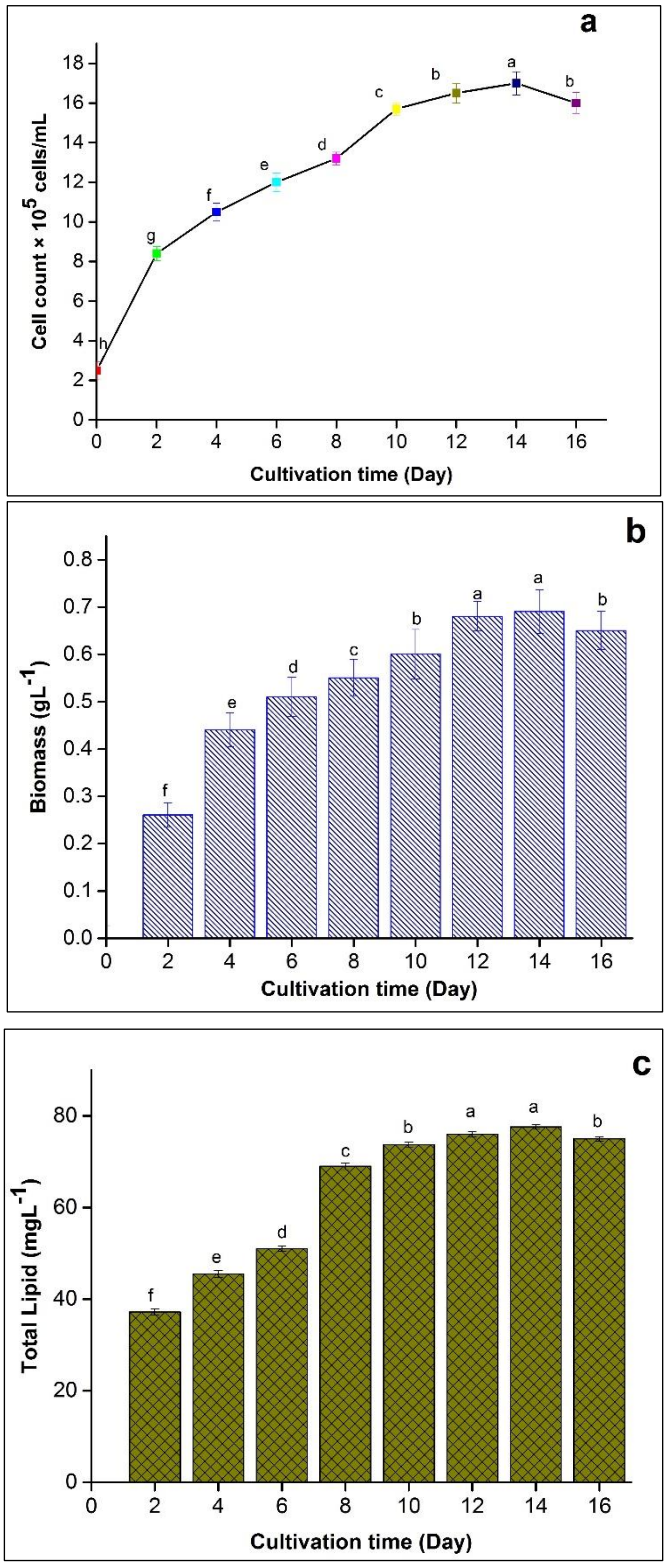


Fig. 7. Cultivation of *Nitzschia* sp. RRSE2 in a 2 L photobioreactor using optimized F/2 medium on the effect of (a) cell growth, (b) biomass concentration, and (c) lipid concentration. Lines and bars are represent ± SE (n = 3). Means with different small letters on the lines and bars are significantly different ($p<0.05$).

It was noteworthy that the results of culturing in the photobioreactor showed the yield of biomass and lipid comparatively higher (0.69 gL^{-1} biomass and 77.5 mgL^{-1} on day 14) than in the shake flask culture (Fig. 7a through c). The maximum number of cells 17 × 10^5 cells mL^{-1} was also recorded on day 14; subsequently, it decreased. The availability of surface area and aeration in the reactor may have been the reason for producing more biomass and lipid.

The present findings are in similarity with those of Arroussi *et al.* (2017). They reported that *Nitzschia* produced biomass up to 0.82 gL^{-1} in the culture medium F/2 supplemented with silicate. Also, in their study, the diatom strains such as *Amphora*, *Chaetoceros* and *Navicula* were also reached biomass level up to 0.5 gL^{-1}. Regarding the lipid content, a nearly 1.7 fold increment was seen in lipid when cultures grown under photobioreactor with optimized medium.

Though the biomass and growth rate is the limiting factor for large scale production of biodiesel in an economic point of view, it has been stated that algal growth can be in inverse proportion to lipid content (Griffiths and Harrison 2009; Rodolfi *et al.* 2009). It may be varied species to species. This study *Nitzschia* had an increasing lipid content across the production stage is considered to be significant indicator for biodiesel production.

The Fatty Acid Profile of *Nitzschia* sp. RRSE2 Cultivated in Optimized F/2 Medium

The diatom was grown in a photobioreactor for the extraction of crude lipid, and it was analyzed by GC/MS to confirm the presence of biodiesel components as presented in Fig. 8. Biodiesel is composed of mainly fatty acid methyl esters, which is derived by the transesterification of produced lipids (Wackett 2008). This author also reported that the quality of biodiesel is significantly affected by the fatty acid components presented in the biodiesel.

In addition to that, differences in the carbon chain length and the unsaturation degree are the important characteristics of biodiesel (Griffiths *et al.* 2009). Table 1 shows that the precursor of biodiesel present in the crude extract *viz.*, decane, octane, nonane, and undecane derivatives, with its following percentages: n-dodecanoic acid (lauric acid (C12:0)) with 10.56%, tetradecanoic acid (myristic acid (C14:0)) with 9.44%, hexadecanoic acid (palmitic acid (C16:0)) with 19.58%, 9-cis-hexadecenoic acid (palmitoleic acid (C16:1)) with 12.9%, octadecanoic acid (stearic acid (C18:0)) with 14.96%, (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid (linolenic acid (C18:3)) with 14.8% and (*Z*)-docos-13-enoic acid (erucic acid (C22:1)) with 5.9%, respectively. The presence of high ratio of octadecanoic acid shows that the compound is presented from the biodiesel transesterified in crude lipid of *Nitzschia* sp. RRSE2. These observations are in agreement with an earlier report, which stated that the presence of 12% linolenic acid is acceptable for the quality biodiesel (Arroussi *et al.* 2017; Purkan *et al.* 2019). It has been reported that the components such as palmitic, oleic, stearic, linoleic and linolenic acid were considered as the most common fatty acids in biodiesel by Knothe (2008).

According to Gao *et al.* (2013), a lipid with a high content of saturated and monounsaturated fatty acids with a low amount of polyunsaturated fatty acids are essential for the production of biodiesel. Therefore, extensive studies are required to determine the fatty acid distribution of *Nitzschia* sp. RRSE2.

Table 1. Compounds Present in Lipid Extracted from *Nitzschia* sp. RRSE2

No.	Retention Time	Area (%)	Components
1.	4.41	1.156	Decane; decane, 2,5,6-trimethyl-; undecane; octane, 3,5-dimethyl-; nonane, 2-methyl-; nonane; octane, 4-ethyl-, decane, 2,6,8-trimethyl-; octane, 4-methyl-, dodecane, 6-methyl-
2.	12.21	1.230	2,4-di-tert-butylphenol; phenol, 2,5-bis(1,1-dimethylethyl)-; phenol, 3,5-bis(1,1-dimethylethyl)-; pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters; phenol, 2,6-bis(1,1-dimethylethyl)-; pentanedioic acid, (2,4-di-t-butylphenyl) mono-ester;phenol,4-(1,1-dimethylethyl)-2-(1,1-dimethylpropyl)-; silane, [4-(1,1-dimethylethyl)phenyl]trimethyl-; 7,9-di-tertbutyl-1-oxaspiro[4,5]deca-6,9-dien-8-one; ethyl 4-t-butylbenzoate
3.	16.20	3.960	Methanone, (1-hydroxycyclohexyl)penyl-; 2-benzoyl-8-octanelactam; cyclohexanol, 1-(2-hexenyl)-; androst-5-en-17one, 2-spiro-2'-1,3-dopxalane-, 2,2'-bioxepane, (2-aminocyclohexyl)-phenyl-methanol; (3,4-dimethoxyphenyl)-hydroxy-phenylacetic acid, 1-methylpiperidin-4-ylester; cyclohexaneethanol, 1-hydroxy-a-nitro-; 1-methyl-f-piperidyl 2-(3,5-dimethoxyphenyl)-2-2hydroxy-2-phenylacetate
4.	20.77	1.459	Diphenylsulfone; 5-phenoxymethyl-furan-2-carboxylic acid; pyracarbolid; 2-propenenitrile, 2-(phenylsulphonyl)-; 1,2-bis(phenylsulfonl)ethane; 2-propenoid acid, 3-(phenylsulfonyl)-, methyl ester; benzene, (1-propenylsulfonyl)-, (E)-; benzene, (ethylsulfonyl)-; [(dibromomethyl)sulfonyl]benzene; bis[phenylsulfonyl]-4-carboxyl phenyl methane
5.	22.01	2.211	5,8,11-heptadecatriyonoic acid, methyl ester; 1,6-jeptadoeme, 2-methyl-6-phenyl-; 10-oxytricyclo[6.4.0.09,12]dodecane-9-carboxylic acid, 11-oxo-12-phenyl-methyl ester; panaxynone; benzene, 1-methyl-3,5-bis(3-methyl-3-butenyl)-; 1-phenylbicyclo(4.1.0), heptane; tricyclo[7.4.0.0(3,8)]tridec-12-en-2-one, 5,6-epoxy-4-methyl-1-(2-propynyl)-; benzene, 1,3,5-tris(3-methyl-3-betenyl)-; 5-methyl-1-phenyl bicyclo[3.2.0]heptane; cyclohexane, 2-(2-nitro-1-phenyl-2-propenyl)-

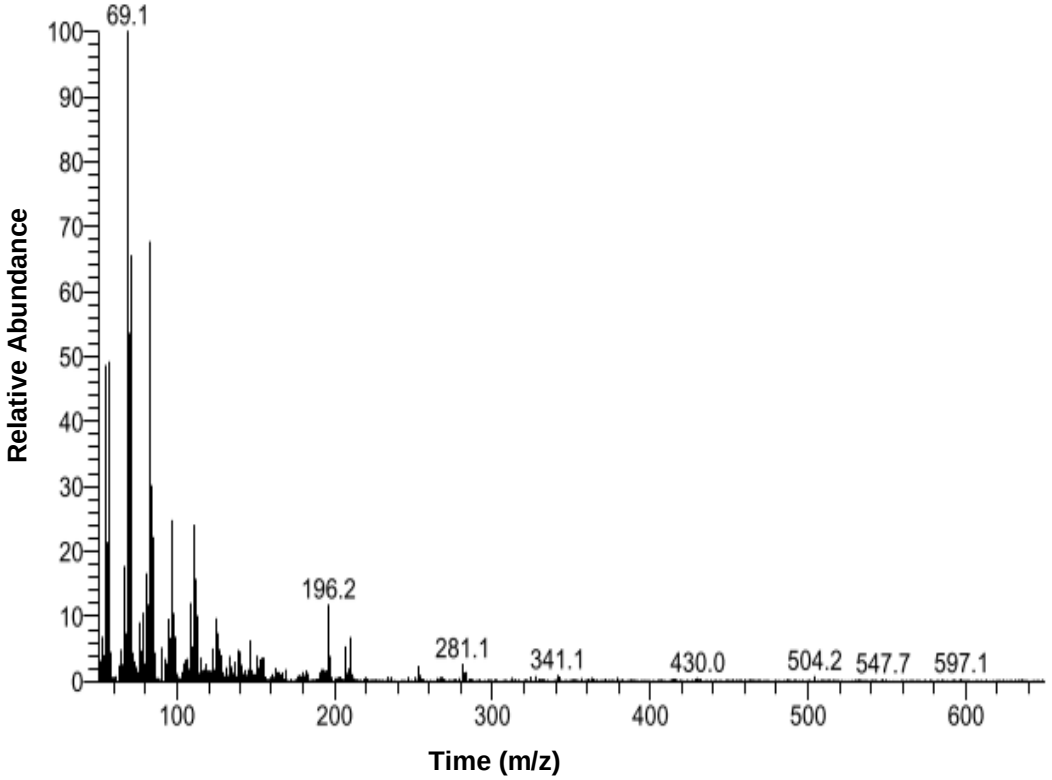


Fig. 8. Gas chromatogram of fatty acid profile extracted from *Nitzschia* sp. RRSE2

CONCLUSIONS

1. A maximum lipid concentration was observed in the conditions of pH 8, 3% NaCl, 18.75 mgL^{-1} nitrogen source, 3.48 mgL^{-1} NaH_2PO_4, and in the F/2 medium.
2. Increased lipid productivity was observed when the alga was cultured and when the nitrogen source was of a low concentration.
3. Gradual increment of lipid (1. 7 fold) was observed in photobioreactor using optimized medium when compared to initial culturing.
4. The GC/MS profile confirmed that the presence of suitable components which are essential for biodiesel production.
5. This study indicated that this indigenous microalga can be cultivated in saltwater medium and it may be adopted to coastal belt for large scale cultivation, thereby avoiding demand for fresh water.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from the Science & Engineering Research Board (SERB), Department of Science and Technology, Government of India (Project number: YSS/2015/001217), and the University Kebangsaan Malaysia (Grant code: FRGS/2018/TK02), Malaysia.

REFERENCES CITED

Arroussi, H. E., Benhima, R., Mernissi, N. E., Bouhfid, R., Tilsaghani, C., Bennis, I., and Wahby, I. (2017). "Screening of marine microalgae strains from Moroccan coasts for biodiesel production," *Renewable Energy* 113, 1515-1522. DOI: 10.1016/j.renene.2017.07.035

Barinova, S., Kukhaleishvili, L., Nevo, E., and Janelidze, Z. (2011). "Diversity and ecology of algae in the Algeti National Park as a part of the Georgian system of protected areas," *Turkish Journal of Botany* 35(6), 729-774. DOI: 10.3906/bot-1009-83

Bellinger, E. G., and Sigee, D. C. (2010). *Freshwater Algae: Identification and Use as Bioindicators*, Wiley-Blackwell, Chichester, UK.

Ben-Amotz, A., Tornabene, T. G., and Thomas, W. H. (1985). "Chemical profile of selected species of microalgae with emphasis on lipids," *Journal of Phycology* 21(1), 72-81. DOI: 10.1111/j.0022-3646.1985.00072.x

Blanchemain, A., and Grizeau, D. (1996). "Eicosapentaenoic acid content of *Skeletonema costatum* as a function of growth and irradiance; relation with chlorophyll *a* content and photosynthetic capacity," *Journal of Experimental Marine Biology and Ecology* 196 (1-2), 177-188. DOI: 10.1016/0022-0981(95)00129-8

Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B., and Wijffels, R. H. (2013). "Effect of light intensity, pH, and temperature on triacylglycerol (TAG) accumulation induced by nitrogen starvation in *Scenedesmus obliquus*," *Bioresource Technology* 143, 1-9. DOI: 10.1016/j.biortech.2013.05.105

Chaffin, J. D., Mishra, S., Kuhaneck, R. M., Heckathorn, S. A., and Bridgeman, T. B. (2011). "Environmental controls on growth and lipid content for the freshwater diatom, *Fragilaria capucina*: A candidate for biofuel production," *Journal of Applied Phycology* 24(5), 1045-1051. DOI: 10.1007/s10811-011-9732-x

Chen, Y., Tang, X., Kapoore, R. V., Xu, C., and Vaidyanathan, S. (2015). "Influence of nutrient status on the accumulation of biomass and lipid in *Nannochloropsis salina* and *Dunaliella salina*," *Energy Conversion and Management* 106, 61-72. DOI: 10.1016/j.enconman.2015.09.025

Chisti, Y. (2007). "Biodiesel from microalgae," *Biotechnology Advances* 25(3), 294-306. DOI: 10.1016/j.biotechadv.2007.02.001

Chisti, Y. (2013). "Constraints to commercialization of algal fuels," *Journal of Biotechnology* 167(3), 201-214. DOI: 10.1016/j.jbiotec.2013.07.020

Chu, F. F., Chu, P. N., Cai, P. J., Li, W. W., Lam, P. K. S., and Zeng, R. J. (2013). "Phosphorus plays an important role in enhancing biodiesel productivity of *Chlorella vulgaris* under nitrogen deficiency," *Bioresource Technology* 134, 341-346. DOI: 10.1016/j.biortech.2013.01.131

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P., and Del Borghi, M. (2009). "Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production," *Chemical Engineering and Processing: Process Intensification* 48(6), 1146-1151. DOI: 10.1016/j.cep.2009.03.006

Fan, L. H., Zhang, Y. T., Zhang, L., and Chen, H. L. (2008). "Evaluation of a membrane-sparged helical tubular photobioreactor for carbon dioxide biofixation by *Chlorella vulgaris*," *Journal of Membrane Science* 325(1), 336-345. DOI: 10.1016/j.memsci.2008.07.044

Folch, J., Lees, M., and Sloane-Stanley, G. H. S. (1957). "A simple method for the isolation and purification of total lipids from animal tissue," *The Journal of Biological Chemistry* 226, 497-509.

Gao, Y., Yang, M., and Wang, C. (2013). "Nutrient deprivation enhances lipid content in marine microalgae," *Bioresource Technology* 147, 484-491. DOI: 10.1016/j.biortech.2013.08.066

Greenspan, P., Mayer, E. P., and Fowler, S. D. (1985). "Nile red: A selective fluorescent stain for intracellular lipid droplets," *Journal of Cell Biology* 100(3), 965-973. DOI: 10.1083/jcb.100.3.965

Griffiths, M. J., and Harrison, S. T. L. (2009). "Lipid productivity as a key characteristic for choosing algal species for biodiesel production," *Journal of Applied Phycology* 21(5), 493-507. DOI: 10.1007/s10811-008-9392-7

Guillard, R. R., and Ryther, J. H. (1962). "Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (cleve) gran," *Canadian Journal of Microbiology* 8(2), 229-239. DOI: 10.1139/m62-029

Hsieh, C. H., and Wu, W. T. (2009). "Cultivation of microalgae for oil production with a cultivation strategy of urea limitation," *Bioresource Technology* 100(17), 3921-3926. DOI: 10.1016/j.biortech.2009.03.019

Hu, Q. (2003). "Environmental effects on cell composition," in: *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*,A. Richmond (ed.),Wiley-Blackwell, Hoboken, NJ, USA, pp. 83-94. DOI: 10.1002/9780470995280.ch5

Ichihara, K., Shibahara, A., Yamamoto, K., and Nakayama, T. (1996). "An improved method for rapid analysis of the fatty acids of glycerolipids," *Lipids* 31, 535-539. DOI: 10.1007/BF02522648

Jahnke, L. S., and White, A. L. (2003). "Long-term hyposaline and hypersaline stresses produce distinct antioxidant responses in the marine alga *Dunaliella tertiolecta*," *Journal of Plant Physiology* 160(10), 1193-1202. DOI: 10.1078/0176-1617-01068

Jiang, Y., Yoshida, T., and Quigg, A. (2012). "Photosynthetic performance, lipid production and biomass composition in response to nitrogen limitation in marine microalgae," *Plant Physiology and Biochemistry* 54, 70-77. DOI: 10.1016/j.plaphy.2012.02.012

John, D. M., Whitton, B. A., and Brook, A. J. (2003). *The Freshwater Algal Flora of the British Isles: An Identification Guide to Freshwater and Terrestrial Algae* Cambridge University Press, Cambridge, UK, pp. 287-389. DOI: 10.5860/choice.40-3394

Knothe, G. (2008). "Designer biodiesel: Optimizing fatty ester composition to improve fuel properties," *Energy Fuels* 22(2), 1358-1364. DOI: 10.1021/ef700639e

Li, C. L., Ashworth, M. P., Witkowski, A., Dazbek, P., Medlin, L. K., Kooistra, W. H. C. F., Sato, S., Zglobicka, I., and Kurzydłowski, K. J. (2015). "New insights into *Plagiogrammaceae* (Bacillariophyta) based on multigene phylogenies and morphological characteristics with the description of a new genus and three new species," *PLoS ONE* 10(10), e0139300. DOI: 10.1371/journal.pone.0139300

Li, X., Marella, T. K., Tao, L., Li, R., Tiwari, A., and Li, G. (2017). "Optimization of growth conditions and fatty acid analysis for three freshwater diatom isolates," *Phycological Research* 65(3), 177-187. DOI: 10.1111/pre.12174

Liu, Z. Y., Wang, G. C., and Zhou, B. C. (2008). "Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*," *Bioresource Technology* 99 (11), 4717-4722. DOI: 10.1016/j.biortech.2007.09.073

Lv, J. M., Cheng, L. H., Xu, X. H., Zhang, L., and Chen, H. L. (2010). "Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions," *Bioresource Technology* 101(17), 6797-6804. DOI: 10.1016/j.biortech.2010.03.120

Lynn, S. G., Kilham, S. S., Kreeger, D. A., and Interlandi, S. J. (2000). "Effect of nutrient availability on the biochemical and elemental stoichiometry in fresh water diatom *Stephanodiscus minutulus* Bacillariophyceae," *Journal of Phycology* 36(3), 510-522. DOI: 10.1046/j.1529-8817.2000.98251.x

Mandal, S., and Mallick, N. (2009). "Microalga *Scenedesmus obliquus* as a potential source for biodiesel production," *Applied Microbiology and Biotechnology* 84(2), 281-291. DOI: 10.1007/s00253-009-1935-6

Martin-Jezequel, V., Hildebrand, M., and Brzezinski, M. A. (2000). "Silicon metabolism in diatoms: Implications for growth," *Journal of Phycology* 36(5), 821-840. DOI: 10.1046/j.1529-8817.2000.00019.x

Mata, T. M., Martins, A. A., and Caetano, N. S. (2010). "Microalgae for biodiesel production and other applications: A review," *Renewable and Sustainable Energy Reviews* 14(1), 217-232. DOI: 10.1016/j.rser.2009.07.020

Medlin, L., Elwood, H. J., Stickel, S., and Sogin, M. L. (1988). "The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions," *Gene* 71, 491-499. DOI: 10.1016/0378-1119(88)90066-2

Negi, S., Barry, A. N., Friedland, N., Sudasinghe, N., Subramanian, S., Pieris, S., Holguin, F. O., Dungan, B., Schaub, T., and Sayre, R. (2015). "Impact of nitrogen limitation on biomass, photosynthesis, and lipid accumulation in *Chlorella sorokiniana*," *Journal of Applied Phycology* 28, 803-812. DOI: 10.1007/s10811-015-0652-z

Pancha, I., Chokshi, K., George, B., Ghosh, T., Paliwal, C., Maurya, R., and Mishra, S. (2014). "Nitrogen stress triggered biochemical and morphological changes in the microalgae *Scenedesmus* sp. CCNM 1077," *Bioresource Technology* 156, 146-154. DOI: 10.1016/j.biortech.2014.01.025

Purkan, P., Nidianti, E., Abdulloh, A., Safa, A., Retnowati, W., Soemarjati, W., Nurlaila, H., and Kim, S. W. (2019). "Biodiesel production by lipids from Indonesian strain of microalgae *Chlorella vulgaris*," *Open Chemistry* 17, 919-926. DOI: 10.1515/chem-2019-0102

Rao, A. R., Dayananda, C., Sarada, R., Shamala, T. R., and Ravishankar, G. A. (2007). "Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents," *Bioresource Technology* 98(3), 560-564. DOI: 10.1016/j.biortech.2006.02.007

Rodolfi, L., Zitelli, G. C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., and Tredici, M. (2009). "Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor," *Biotechnology and Bioengineering* 102(1), 100-112. DOI: 10.1002/bit.22033

Ruangsomboon, S. (2012). "Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, *Botryococcus braunii* KMITL 2," *Bioresource Technology* 109, 261-265. DOI: 10.1016/j.biortech.2011.07.025

Sheehan, J., Dunahay, T., Benemann, J., and Roessler, P. (1998). *A Look Back at the U.S. Department of Energy's Aquatic Species Program – Biodiesel from Algae* (NREL/TP-580-24190), National Renewable Energy Laboratory, Golden, CO, USA.

Singh, P., Guldhe, A., Kumari, S., Rawat, I., and Bux, F. (2015). "Investigation of combined effect of nitrogen, phosphorus and iron on lipid productivity of microalgae

Ankistrodesmus falcatus KJ671624 using response surface methodology," *Biochemical Engineering Journal* 94, 22-29. DOI: 10.1016/j.bej.2014.10.019

Singh, P., Kumari, S., Guldhe, A., Misra, R., Rawat, I., and Bux, F. (2016). "Trends and novel strategies for enhancing lipid accumulation and quality in microalgae," *Renewable and Sustainable Energy Reviews* 55, 1-16. DOI: 10.1016/j.rser.2015.11.001

Spolaore, P., Joannis-Cassan, C., Duran, E., and Isambert, A. (2006). "Commercial applications of microalgae," *Journal of Bioscience and Bioengineering* 101(2), 87-96. DOI: 10.1263/jbb.101.87

Tornabene, T. G., Holzer, G., Lien, S., and Burris, N. (1983). "Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*," *Enzyme and Microbial Technology* 5(6), 435-440. DOI: 10.1016/0141-0229(83)90026-1

Ugwu, C. U., Aoyagi, H., and Uchiyama, H. (2008). "Photobioreactors for mass cultivation of algae," *Bioresource Technology* 99(10), 4021-4028. DOI: 10.1016/j.biortech.2007.01.046

Wackett, L. P. (2008). "Biomass to fuels via microbial transformations," *Current Opinion in Chemical Biology* 12(2), 187-193. DOI: 10.1016/j.cbpa.2008.01.025

Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., and Ruan, R. R. (2010). "Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp.," *Bioresource Technology* 101(8), 2623-2628. DOI: 10.1016/j.biortech.2009.10.062

Yu, E. T., Zendejas, F. J., Lane, P. D., Gaucher, S., Simmons, B. A., and Lane, T. W. (2009). "Triacylglycerol accumulation and profiling in the model diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Bacillariophyceae) during starvation," *Journal of Applied Phycology* 21, 669-681. DOI: 10.1007/s10811-008-9400-y

Zhila, N. O., Kalacheva, G. S., and Volova, T. G. (2005). "Influence of nitrogen deficiency on biochemical composition of the green alga *Botryococcus*," *Journal of Applied Phycology* 17, 309-315. DOI: 10.1007/s10811-005-7212-x

Zhila, N. O., Kalacheva, G. S., and Volova, T. G. (2011). "Effect of salinity on the biochemical composition of the alga *Botryococcus braunii* Kutz IPPAS H-252," *Journal of Applied Phycology* 23(1), 47-52. DOI: 10.1007/s10811-010-9532-8

Article submitted: May 15, 2020; Peer review completed: July 15, 2020; Revised version received: August 5, 2020; Accepted: August 9, 2020; Published: August 13, 2020.
DOI: 10.15376/biores.15.4.7532-7550