Original article

Long-term monitoring of the biomass and production of lipids by *Nitzschia palea* for biodiesel production

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ABSTRACT

Pennate diatom *Nitzschia palea* can be cultured in outdoor vertical-bed photobioreactors to produce biodiesel. To assess the production of biomass and lipids, non-axenic cultures of *Nitzschia palea* were grown outdoors, and the growth of these cultures was measured biweekly. During the annual cycle of algal culture, the culture temperature ranged from 17.3 °C to 33.5 °C, the dry weight biomass ranged from 0.11 g l^{-1} to 0.25 g l^{-1}, light energy] ranged from 1.94 Wm^{-2} to 3.9 Wm^{-2} and intracellular lipid content ranged from 7.1% to 11.4% of biomass weight after drying at 60 °C. Gas chromatography/mass spectroscopy (GC/MS) analysis of n-hexane extracts showed that the intracellular lipids were primarily C14:0 myristic acid (9.01%), C15:0 pentadecyclic acid (8.26%) and two types of C16:0, palmitic acid (41.13%) and palmitoleic acid (29.25%). Gel permeation analysis showed that carboxylic acids comprised 28.9% of lipids, 16.3% of monoglycerides, 27.3% of diglycerides and 24.3% of triglycerides. Alcoholysis of lipids resulted in the conversion of about 93.9% of fatty acids to equivalent fatty acid methyl esters (FAME) or biodiesel, which, on basis of wt%, consisted primarily of C15:0 methyl myristate (8.3%), C16:0 methyl pentadecanoate] (7.2%), C17:1methyl palmitoleate (28.7%) and methyl palimtate](39.8%).
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1. Introduction

Diatoms, members of the class Bacillariophyceae, are unicellular photosynthetic eukaryotes, which differ from other microalgae by having siliceous cell walls. Diatoms constitute more than 25% of global primary producers (Reynolds, 1984; Scala and Bowler, 2001; Sadasivam et al., 1996; Matsumoto et al., 2017). Despite their abundance and diversity, few diatoms are used as live food in aquaculture to provide essential fatty acids, such as eicosapentaenoic acid (EPA) (Robert, 2003; Wen, 2001; Matsumoto et al., 2017). Few biotechnological studies to date have assessed the use of diatom lipids as feedstock for biodiesel (Lebeau and Robert, 2003). Diatoms may be potential candidates for biodiesel production, as certain species of diatoms are rich in glycerides of long chain fatty acids, which are potential feedstock for biodiesel

(Lombardi and Wangersky, 1995). Lipids of some diatoms are exceptionally rich in saturated C16:0 palmitic acid and monounsaturated C16:1 palmitoleic acid, which are ideal fatty acids for high quality biodiesel (Hu et al., 2008; Thomas et al., 1983).

Biodiesel is a biodegradable, renewable and non-lethal fuel, producing lower quantities of vaporous toxins than petroleum diesel (Van Gerpen et al., 2004; Felizardo et al., 2006). Biodiesel consists of a blend of monoalkyl esters of long chain fatty acids, resulting from the esterification of fatty acids and transesterification of glycerides of vegetable oil (Demirbas, 2009). Microalgae may be the only reliable and renewable source of biodiesel and may in future totally supplant fossil diesel as an energy source (Chisti, 2007).

Diatoms may contribute to fossil crude oil (Aoyagi and Neogene, 1992), as evidenced by the wide distribution of the diatom biomarker 24-norcholestane in many crude oil basins worldwide (Holba et al., 1998; Hu et al., 2006). These findings strongly suggest that living diatoms may contribute, at least in part, to the design of a sustainable source of oil (Ramachandra et al., 2009).

The imminent depletion of fossil fuels has emphasized the need to assess whether microalgae, including diatoms, can act as viable alternative resources of renewable biodiesel. Few studies to date, however, have assessed the reliability and feasibility of algal

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biomasses in the production of biodiesel. In the present work is part of a comprehensive research program investigating the production by different species of diatoms of biomass and lipids and determining which, if any, of these species is a feasible source of biodiesel production.

A previous study carried out by authors (Abdel-Hamid et al., 2013) indicated that under favorable laboratory growth conditions, the pinnate diatom *Nitzschia palea* produced relatively higher quantities of biomass and lipids than other tested diatoms. In addition, gas chromatography/mass spectroscopy (GC/MS) analysis indicated the suitability of the structure and composition of *N. palea* derived fatty acids as potential feedstock for biodiesel. Accordingly, *N. palea* was selected as a model test organism for long-term outdoor growth experiments to monitor seasonal variations in biomass and lipid production. Special attention was paid to the suitability of the lipid fraction of this diatom as feedstock for biodiesel based on the physical and chemical characteristics of these lipids. Batch trials were performed to convert diatom lipids to biodiesel through one step acid based esterification. The aim of our long-term monitoring of biomass and lipid production under outdoor conditions was to obtain a realistic assessment of the feasibility of large scale, outdoor diatom-based biodiesel production

2. Materials and methods

2.1. Photobioreactors

Vertical bed photobioreactors were designed and used for outdoor biomass production. Each photobioreactor was composed of a cylindrical clear plastic Perspex® acrylic tube 100 cm in height and 20 cm in diameter (Photo 1). Each tube had exceptional light transmission capacity, allowing transmission of >95% of visible light. Each tube was firmly cast into a transparent Perspex® sheet 3.0 cm thick. A hole 1.5 cm in diameter was made in the center of each basal acrylic sheet, and a tape junction was installed in the hole to sample diatom cultures. The entire photobioreactor unit was fastened with screws to a horizontal plate of 3.0 cm thick white marble, and the entire set of culture-tube vessels (12 tubes) was fixed onto a metallic frame-like table 100 cm above the ground.

Photo 1. The photobioreactor used for a full annual cycle growth of the pinnate diatom *Nitzschia palea*.

2.2. Incubation room and culture conditions

The photobioreactor was placed in a room (100 m long and 50 cm wide) with a roof made of transparent clear Perspex® sheets that allow up to 95% transmission of light. The acrylic sheets were firmly attached to a metal rack installed on the ceiling of the incubation room. The lateral sides of the rack (about 30 cm in height) were covered with stainless steel screens with 0.5 cm sized mesh to allow free exchange of air. The device was specifically designed to allow the upper surface of the transparent plastic roof to be cleaned easily and to handle rain water. The four side walls of the incubation room were cement-brick built without any provision for heat insulation. The room was illuminated during the day only by light from the transparent plastic roof, with direct sunlight maximal around noon time. These incubation conditions largely simulate ambient environmental conditions, especially as no artificial light was provided during the entire incubation period from January 2009 to January 2010.

2.3. Outdoor cultivation of Nitzschia palea

Algae were cultured in standard *Navicula* medium (Starr, 1978), containing 0.1 g/l Ca(NO3)2·.4H2O, 0.14 g/l K2HPO4·.3H2O, 0.025 g/l MgSO4·.7H2O, 0.1 g/l NaSiO3·.9H2O, 0.02 g/; NaCO3, 1.0 ml/l iron solution (i.e. 5.0 g/l FeCl3·.6H2O, 30 g/l Na2. EDTA·2H2O), and 1.0 ml/l trace element solution (i.e. 2.8 g/l H3BO3, 0.9 g/l MnCl2·.4H2O, 0.125 g/l ZnCl2, 0.08 g/l CuSO4·.5H2O, 0.9 g/l Na2MoO4·.2H2O, and 0.014 g/l CoCl2·.6H2O). Nutrient solutions were prepared in dechlorinated tap water filtered with fabric of pore size 5 μm. Twenty liters of liquid nutrient medium were added to each culture vessel (photobioreactor), with four culture vessels used for diatom cultivation. Each vessel was inoculated with 0.01 g dry wt l^{-1} of algae. To ensure vertical mixing of the diatom culture, a continuous air current was introduced into each culture vessel through a transparent flexible plastic tube, with one end firmly connected to an air pump with an output capacity of 160 l min^{-1} and the other end tightly plugged into a perforated silica air stone weighing about 500 g. The flexible plastic tubes were long enough to allow the air stones to settle on the bottom of the culture vessel. Continuous air bubbling through air stones resulted in bottom to top vertical mixing of diatom cells while providing diatom cultures with atmospheric CO2. The mouth (upper end) of the culture tube was covered with nylon gauze (0.05 cm mesh size) to prevent dust and insects from entering into the culture tubes. No bacterial filters were installed on the path of air current and the nutrient solution was not sterilized before use, mimicking outdoor cultivation conditions. Also no artificial light was provided and diatom growth proceeded under natural day and night cycles. The pinnate diatom *Nitzschia palea* was continuously cultivated under these conditions from January 2009 to January 2010.

2.4. Temperature measurements

Culture temperature was measured daily with a Lutron TM-924C two channel thermometer. Measurements were made at around noon. Mean temperatures (°C) were calculated and reported for each sampling period.

2.5. Light measurements

Light intensity was also measured daily around noon time using an LX-107 light meter. The light sensor was horizontally positioned at different levels adjacent to the culture tubes to measure the light intensity of vertically incident day light. Several readings were taken and mean values were calculated. Light intensity was measured in lux and converted to light energy (Wm^{-2}) using an online

energy calculator available at www.metriccalculator.com/index.html. Mean light energy (Wm^{-2}) was calculated for each sampling period.

2.6. Periodic sampling of diatom biomass

Biomass was sampled biweekly during the entire cultivation period (Photo 2). Season was considered throughout sampling. The climate of North Egypt has four distinct seasons: winter (21 December through 21 March), spring (22 March through 21 June), summer (22 June through 21 September) and autumn (22 September through 21 December). Biomass samples were collected biweekly during the early, middle, and late periods of each season.

The sampling schedule ensured accurate representation of seasonal periods involving distinct meteorological factors. Just prior to sampling, each diatom culture was thoroughly mixed with the plastic tube and air stone used for aeration. This resulted in an even distribution of air bubbles and ensured complete and homogeneous vertical mixing of the diatom biomass. While mixing, 10 L of each diatom culture were collected for biomass recovery. The cultures were replenished with 10 L of double strength *Navicula* nutrient medium to ensure continuous diatom growth. Because diatom cells show high autoflocculation, the collected culture samples was allowed to settle for about 2 h until complete sedimentation of diatom cells. The clear spent nutrient solution was carefully decanted, and the dense diatom slurry was transferred to clean, dry pre-weighed glass petri dishes. The slurry was dried for 12 h in an oven at 60 °C and re-weighed to determine diatom biomass, which was expressed as g dry wt l^{-1}.

2.7. Lipid extraction

Dried diatom biomass was wrapped in GF/C filter paperLipids were extracted using the Soxhlet method (Sadasivam et al.., 1996). The lipid fraction was weighed using a sensitive balance (Sartorius Analytical Balance ENTRIS224-1S) and expressed as % (w/w) dry biomass.

2.8. Physical and chemical analyses of lipids

Lipids were subjected to specific physical and chemical analyses to determine their suitability as feedstock for biodiesel. For example, free fatty acids (FFA) and acid value, defined as the mass in milligrams of KOH required to neutralize 1.0 g of lipid, were analyzed as previously described (Cox, 1962). Iodine number, defined as the mass in grams of iodine consumed by 100 g of lipid, and saponification value, defined as the mass in milligrams of KOH required to saponify 1.0 g of lipid, were determined as previously described (Horowitz, 1975).

The density of diatom lipids was determined using density vials with 2.0 ml capacity and of known weight. To one vial was added 1.0 ml of diatom lipids and to a second was added 1.0 ml of distilled water. The vials were plugged and kept overnight at 4 °C, carefully wrapped in dry filter paper to remove any condensed moisture and weighed using a sensitive balance. The specific gravity was calculated as the mass of the lipid sample divided by the mass of an equal volume of distilled water at 4 °C (D, 2638).

The flash point of lipids, defined as the lowest temperature at which vapors passing from lipid samples into air will catch fire spontaneously in the presence of a small flame, was determined according to procedures outline by the American Society for Testing and Materials (ASTM) (D, 1310). Similarly, the fire point, defined as the lowest temperature at which a volatile combustible substance vaporizes rapidly enough to form an air-vapor mixture above its surface that burns continuously when ignited by a small flame, was determined according to ASTM procedures (D, 1310). The intracellular lipids were analyzed by gas chromatography/-mass spectroscopy (GC/MS) and by gel permeation chromatography (GPC) at the laboratories of NesteOil (Keilaranta, Finland).

2.9. Batch biodiesel production by acid-catalyzed esterification of an n-hexane extract of N. palea

Because the n-hexane lipid extract of *N. palea* was found to be rich in FFA (28.9%), it was subjected to one-step acid catalyzed
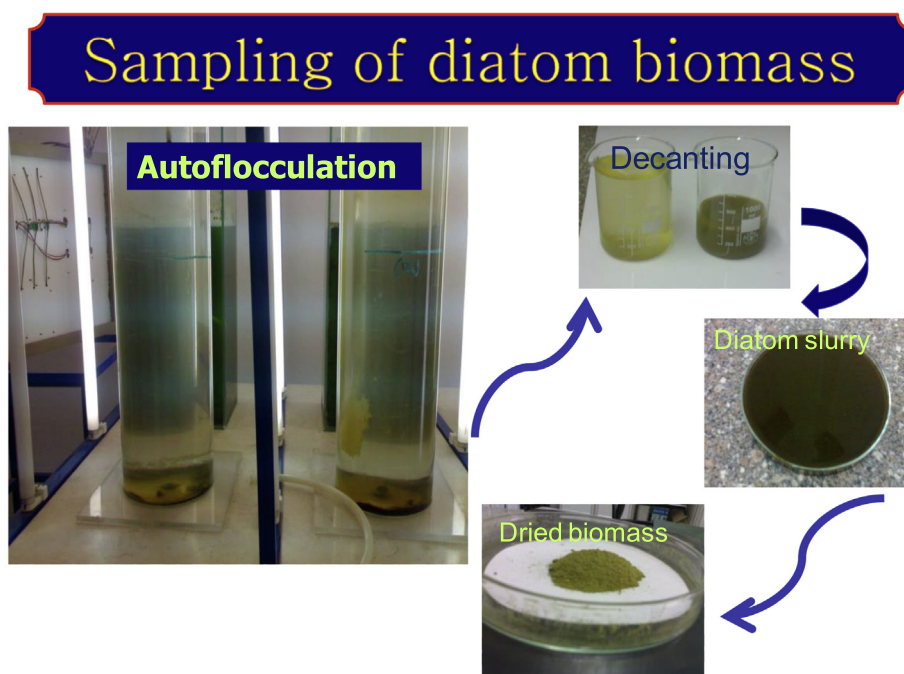

Photo 2. Biomass sampling the pinnate diatom *Nitzschia palea* grown for a full annual cycle in photobioreactors.

alchololysis of lipids (Christie and Christie, 1993) to produce fatty acid methyl esters (biodiesel). During this procedure, four reaction variables were optimized: 1) the molar ratio of oil to alcohol; 2) the wt% or molar ratio of the catalyst (mostly concentrated H2SO4); 3) the reaction temperature; and 4) the reaction time.

The utilized procedures considered the diatom oil to be composed of pure triolein (Van Gerpen et al., 2004; Vicente et al., 2004), a triglyceride in which all three fatty acid chains are oleic acid with a molecular weight of 885.46 Da. The reaction between diatom oil and methyl alcohol in the presence of H2SO4 depends, to a large extent, on their molar ratio (Haas et al., 2009). Pure methanol (HPLC grade) and sulfuric acid (ACS grade, 98% assay) were used. The molar ratio of diatom lipids to methyl alcohol to sulfuric acid was adjusted at 1:8:0.05 (Chongkhong et al., 2007; Goff et al., 2004). The densities (specific gravities) of these reactants were carefully considered when different volumes were mixed in the reaction vessel to exactly yield the adjusted molar ratios.

The esterification reactions were performed in 25 ml screw-cap Pyrex glass tubes. H2SO4 and methanol were mixed first, followed by the addition of diatom lipids (Goff et al., 2004). The reactants were thoroughly mixed until the diatom lipids (semi-solid oily substance) had dissolved completely. To obtain water-free conditions, a small amount (about 0.05 g) of anhydrous sodium sulfate was added to the reaction medium (Molnar-Perl and Pinter-Szakacs, 1986). The mixtures were allowed to stand until sodium

sulfate had precipitated completely. Each supernatant was carefully poured into a clean dry 25 ml screw cap Pyrex glass tube. The cap of each reaction tube was tightly attached, and the tubes were placed in a programmable oven capable of programmed duel temperature control from 30 °C to 300 °C. The oven was maintained at 70 °C (Chongkhong et al., 2007) for 8 h (Goff et al., 2004). The reaction tubes was placed in a desiccator and allowed to cool to room temperature. Each reaction mixture was poured into a separating funnel and left to settle into two phases. The lower FAME layer was separated and purified by repeated washings with distilled water, prior to GC/MS analysis.

3. Results

3.1. Bi-weekly mean variation in temperature of outdoor cultures

Mean variations in the temperatures of diatom cultures are shown in Table 1. Temperatures fluctuated from a minimum of 17.3 °C to a maximum of 33.5 °C with an annual average of 25.7 °C. Biweekly variations in culture temperatures are also illustrated in Fig. 2. Culture temperature exhibited significant ($p \leq 0.05$) but weak positive correlations with biomass dry weight (r = 0.26) and lipid content (r = 0.22), but a strong, significant linear correlation ($p \leq 0.05$, r = 0.71) with light energy (Table 2).

Table 1

Biweekly weekly mean variations in dry weight biomass and intracellular n-hexane lipid content of outdoor cultures of the pinnate diatom *Nitzschia palea* over a full annual cycle (28 January 2009 through 14 January 2010). The corresponding mean variations in culture temperature and ambient light energy are also shown.

Ambient light energy (W/m^2)	Temp. (°C)	wt% Oil content*	D.wt g l^{-1}	Sampling date	Season
2.03	18.9	8.52	0.11	Jan 28,09	Mid winter
2.3	20.1	10.8	0.18	Feb 14,09	Mid winter
2.6	19.2	8.9	0.16	Feb 28,09	Late winter
3.43	22.8	7.7	0.15	Mar 15,09	Late winter
2.7	24.3	8.1	0.2	Mar 29,09	Early spring
2.71	25.5	8.5	0.17	Apr 14,09	Early spring
2.53	26.1	7.4	0.11	Apr 28,09	Mid spring
3.6	26.8	7.2	0.18	May 15,09	Mid spring
3.7	28.5	7.1	0.11	May 30,09	Late spring
3.40	29.2	11.4	0.14	Jun 14,09	Late spring
3.13	32.2	11.12	0.19	Jun 28,09	Early summer
3.54	29.9	9.5	0.14	Jul 13,09	Early summer
3.4	31.1	9.9	0.13	Jul 27,09	Mid summer
3.9	33.5	8.6	0.15	Aug 13,09	Mid summer
3.6	30.8	7.7	0.18	Aug 27,09	Late summer
2.7	27.5	7.2	0.22	Sep 14,09	Late summer
2.83	27.8	7.4	0.25	Sep 29,09	Early autumn
2.34	26	7.3	0.17	Oct 13,09	Early autumn
2.2	25.5	7.9	0.16	Oct 28,09	Mid autumn
2.1	26.5	10.2	0.2	Nov 14,09	Mid autumn
1.98	26.9	9.2	0.15	Nov 29,09	Late autumn
1.95	20.6	8.4	0.14	Dec 14,09	Late autumn
1.94	19	7.5	0. 2	Dec 29,09	Early winter
2.1	17.25	7.4	0.12	Jan 14,010	Early winter
					Basic statistics
2.78	25.7	8.54	0.16	Mean value	
3.9	33.5	11.4	0.25	Max .	
1.94	17.3	7.1	0.11	Min .	

% * wt of oil / wt. biomass dried at 60 °C

Table 2

Linear correlation matrix of various physical and biochemical parameters of lipids in *Nitzschia palea*. Only significant correlations ($P \leq 0.05$) are shown.

Parameter(s)	D. wt biomass g l^{-1}	% Oil content	Temp. (°C)	Ambient light energy (Wm^{-2})
D. wt biomass g l^{-1}	1.00			
% Oil content	0.0.32	1.00		
Temp. (°C)	0.26	0.22	1.00	
Ambient light energy (Wm^{-2})	0.043	0.047	0.71	1.00

3.2. Bi-weekly mean variations in light energy

Throughout a full annual cycle, the light energy ranged from 1.94 Wm^{-2} to 3.9 Wm^{-2} with an annual average of 2.78 Wm^{-2} (Table 1). Light energy manifested considerable biweekly variations (Fig. 1) throughout the outdoor cultivation period.

3.3. Bi-weekly mean variations in biomass dry weight of outdoor cultures

Biweekly biomass samples of *Nitzschia palea* were collected at times representing the early, middle and late periods of the four annual seasons. No substantial differences in dry
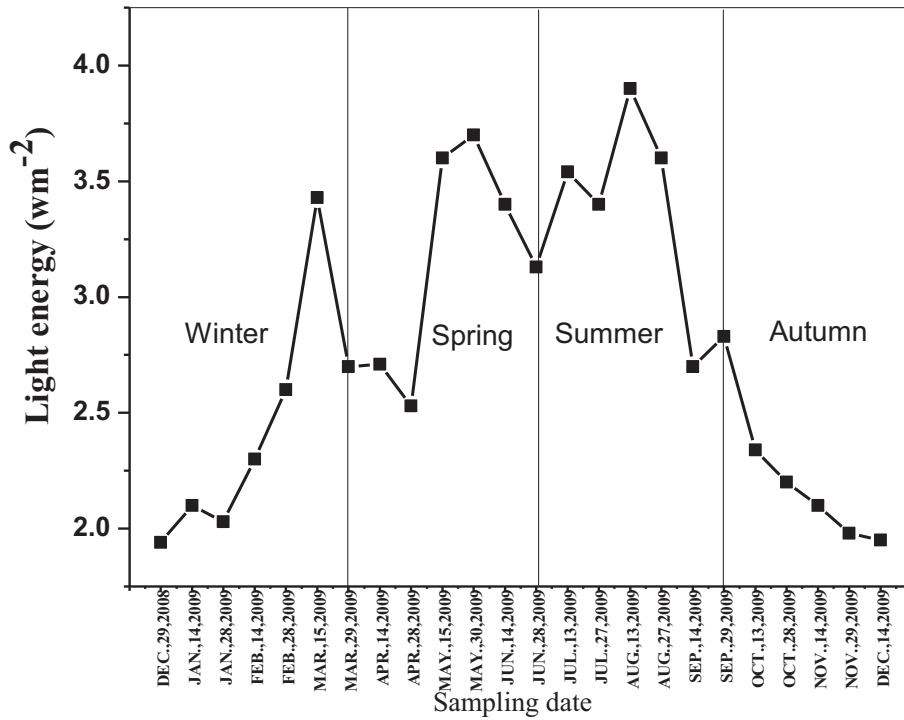

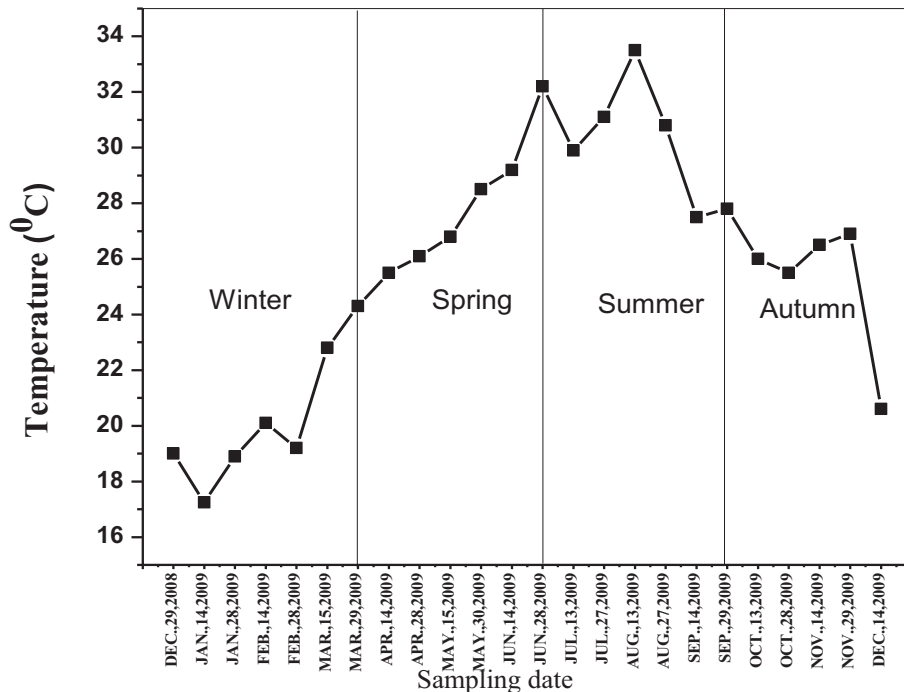**Fig. 1.** Bi-weekly mean variation in light energy.

Fig. 2. Bi-weekly mean variation in temperature of outdoor cultures.

weight biomass were recorded during this study. The dry weight biomass ranged from a minimum of 0.11 g l^{-1} to a maximum of 0.25 g l^{-1} with an annual mean of 0.16 g l^{-1} (Table 1). Seasonal periodic changes in diatom biomass showed no consistent trend (Fig. 3). Although significant (p ≤ 0.05), the dry weight biomass showed a weak positive linear correlation (Table 2) with culture temperature (r = 0.26) and very

week correlations with light energy (r = 0.043) and wt % of intracellular lipids (r = 0.043).

3.4. Biweekly mean variations in wt % of lipids

Considerable and at certain times significant (p ≤ 0.05) variations in wt % of intracellular lipids were recorded during the out-
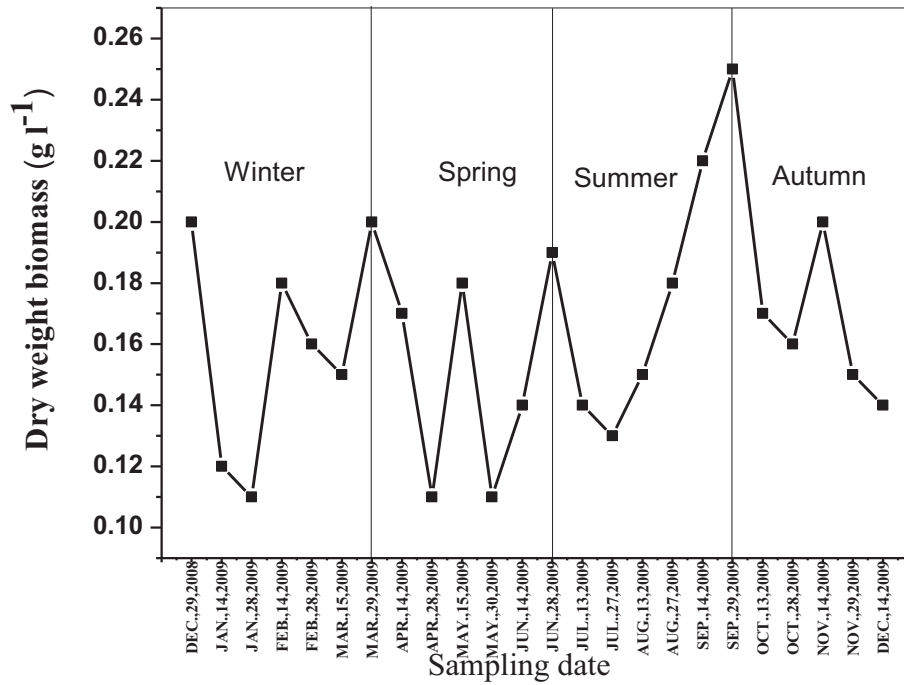

Fig. 3. Bi-weekly mean variation in biomass dry weight of outdoor cultures of *N. palea*.
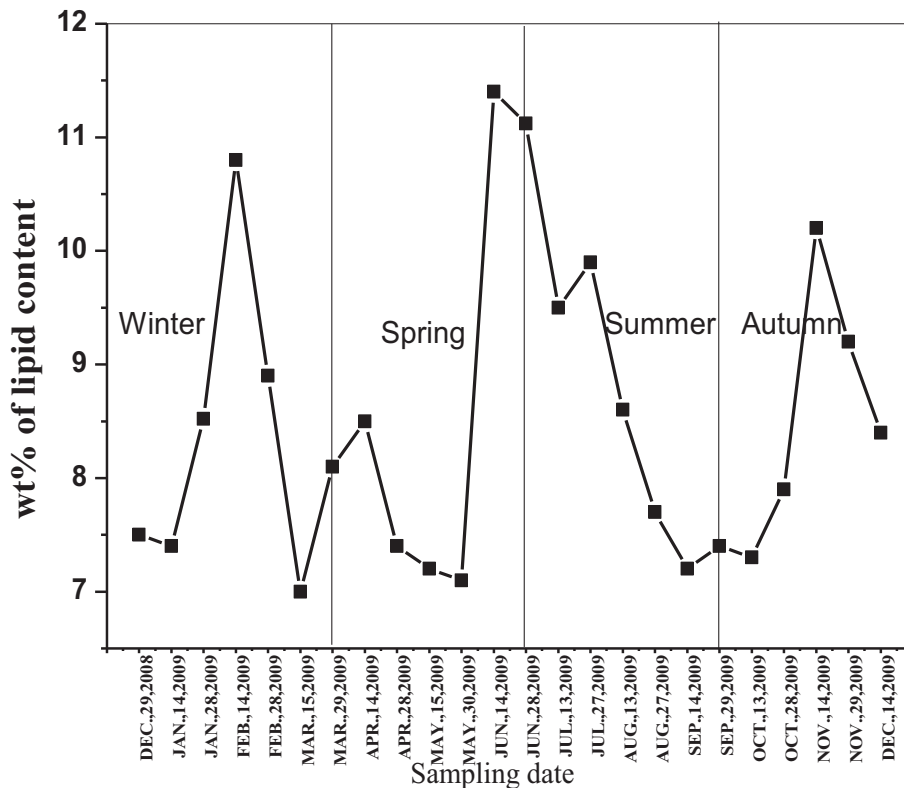

Fig. 4. Bi-weekly mean variation in wt% of lipids of outdoor cultures of *N. palea*.

Table 3
Major free fatty acid (FFA) profile of n-hexane lipid extracts of *N. palea*.

Wt%	Structure	Systemic name	Formula	Fatty acid
9.01	C14:0	Tetradecanoic acid	$C_{14}H_{28}O_2$	Myristic
8.26	C15:0	Pentadecanoic acid	$C_{15}H_{30}O_2$	Pentadecyclic
41.13	C16:0	Hexadecanoic acid	$C_{16}H_{32}O_2$	Palmitic
29.25	C16:1	9-Hexadecenoic acid	$C_{16}H_{30}O_2$	Palmitoleic
2.32	C18:0	Octadecanoic acid	$C_{18}H_{36}O_2$	Stearic
3.22	C18:1	9-Octadecenoic acid	$C_{18}H_{34}O_2$	Oleic
0.48	C18:2	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	Linoleic
1.31	C22:0	Docosapentaenoic acid	$C_{22}H_{44}O_2$	Behenic
1.35	C24:0	Tetracosanoic acid	$C_{24}H_{48}O_2$	Lignoceric

Table 4
Gel permeation chromatography analysis n-hexane lipid extracts of *N. palea*.

%	Component
3.4	Oligomers
24.2	Triglycerides
27.3	Diglycerides
16.3	Monoglycerides
28.9	Carboxylic acids

Table 5
Physical and chemical characteristics of n-hexane lipid extracts of *N. palea*.

Parameter	Unit	Mean values	Specification EDIN 51605
FFA	%	28.9	Max. 1.0%
Acid value	(mg KOH/ g oil)	57.6	Max. 2.0
I_2 value	(mg I_2/ g oil)	39.4	< 120
Specific gravity	(g/cm³)	0.83	0.8 – 0.9
Saponification value	(mg KOH/ g oil)	258	170–179
Flash point	°C	230	Min. 220
Fire point	°C	240	–
Boiling point	°C	260	–

Table 6
GC/MS analysis of biodiesel produced from n-hexane lipids of *Nitzschia palea*.

%wt	Carbon number	Systemic name of FAME	Common name of fatty acid methyl ester (FAME)
8.3	C15:0	Tetradecanoic acid methyl ester	Methyl myristate
7.2	C16:0	Pentadecanoic acid methyl ester	Methyl pentadecyclate
39.8	C17:0	Hexadecanoic acid methyl ester	Methyl palmiteate
28.7	C17:1	9-hexadecenoic acid methyl ester	Methyl palmitoleate
2.0	C19:0	Octadecanoic acid methyl ester	Methyl stearate
3.1	C19:1	9-octadecenoic acid methyl ester	Methyl oleate
0.2	C19:2	9,12-octadecadienoic acid methyl ester	Methyl linoleate
0.5	C23:0	Docosapentaenoic acid methyl ester	Methyl behenate
0.7	C25:0	Tetracosanoic acid methyl ester	Methyl lignocerate

3.5. GC/MS analysis of n-hexane lipid extracts of N. palea

The most frequently detected fatty acids were saturated C16:0 palmitic acid (41.13%) and monounsaturated palmitoleic C16:1 (29.25%) fatty acids (Table 3). Other major fatty acids included myristic C14:0 (9.01%) and pentadecyclic C15:0 (8.26%) fatty acids. Less abundant fatty acids included oleic (3.22%), linoleic (0.48%), behenic (1.31%) and lignoceric (1.35%) acids.

3.6. Gel Permeation Chromatography (GPC) analysis of n-hexane lipid fraction

GPC analysis of the n-hexane lipids of *N. palea* cells showed that the lipid fraction was mainly composed of carboxylic acids (28.9%) followed by diglycerides (27.3%), triglycerides (24.3%) and monoglycerides (16.3%) (Table 4). Oligomers (3.4%), however, were minor constituents.

3.7. Physical and chemical characteristics of the n-hexane lipid extract of N. palea

The results of physical and chemical analyses of the cellular lipids of *N. palea* are shown in Table 5. The physical characteristics included a specific gravity of 0.83 g cm^{-3}, a flash point of 230 °C, a fire point of 240 °C and a boiling point of 260 °C. Chemical characteristics included an FFA content of 28.9%, an acid value of 57.6 mg KOH g^{-1} oil, an iodine number of 39.4 mg I2 g^{-1} oil, and a saponification value of 258 mg KOH g^{-1} oil.

3.8. Composition of biodiesel (FAME) of N. palea

Table 6 lists the common and systematic names of FAMEs resulting from acid-catalyzed esterification of intracellular lipids of *N. palea*. The biodiesel was mainly composed of C17:0 methyl palmitate] (39.8%), C17:1 methyl palmitoleate (28.7%), C15:0 methyl myristate (8.3%) and C16:0 methyl pentadecanoate] (7.2%). Other FAMEs, including methyl stearate, methyl oleate, methyl linoleate methyl behenate and methyl lignocerate, were minor contributors ranging in abundance between 0.2% and 3.1%.

4. Discussion

The present study and its outcomes provide information that may assist in the feasible production of biodiesel from the diatom *Nitzschia palea*. In this study, *N. palea* was grown continuously outdoors for one year to evaluate the seasonal effect on biomass and oil production and to determine mean annual production, which may reliably assess the feasible and sustainable production of biomass-based biodiesel. *N. palea* was cultivated in transparent acrylic tubes that allow 95% penetration of daylight. The culture vessels (photobioreactors) were kept in a room in which no measures were taken to insulate from heat or control the ambient tem-

door growth period. The n-hexane lipid extract was maximum (11.4%) in late spring and minimum (7.1%) in mid-spring with an annual average of 8.54% (Table 1). Inconsistent periodic changes in lipid content (Fig. 4) may indicate that season has a negligible effect on lipid production. Lipid content showed a weak positive correlation (Table 2) with culture temperature (r = 0.22) and a very weak correlation with light energy (r = 0.047).

perature, with free air exchange allowed with the surrounding atmosphere. The transparent acrylic roof also allowed about 95% incidence of light rays. These conditions, in addition to the continuous air bubbling of diatom cultures, ensured thorough mixing of diatom cells and an adequate supply of atmospheric CO2. This simple cultivation system may be applicable to pilot or large-scale cultivation of *N. palea* for biodiesel production.

The selection of *N. palea* as a model test diatom was based on the results of previous studies that assessed biomass and oil production by seven different species of diatoms grown in various nutrient media and under various growth conditions. Compared with other test diatoms, *N. palea* maintained relatively higher production of dry weight biomass and lipid content. GC/MS analysis revealed that the fatty acids of *N. palea* are ideal candidates for biodiesel. All of these characteristics justify the initiation of a long-term outdoor study investigating the seasonal effects on biomass and lipid production by *N. palea*.

The outdoor production of lipids and biomass were monitored for a full annual cycle, involving distinct seasonal variations in ambient temperature and radiant energy, parameters shown to control algal biomass development (Reynolds, 1984) and lipid production (Haas et al., 2009; Hu et al., 2008; Sharma and Singh, 2009; Matsumoto et al., 2017; Pintaka et al., 2017). The dry weight biomass of *N. palea* varied considerably between 0.11 g l^{-1} and 0.25 g l^{-1} with a mean annual value of 0.16 g l^{-1}. Lipid production also varied significantly, ranging between 7.1% dw and 11.4% dw with an annual average of 8.54% dw. Periodic variations in biomass dry weight and lipid production, however, were irregular with no distinct seasonal patterns. Similar results were reported for algal growth under natural environmental conditions (Lebeau and Robert, 2003; Lombardi and Wangersky, 1995; Hu et al., 2008; Thomas et al., 1983; Van Gerpen et al., 2004; Felizardo et al., 2006; Demirbas, 2009; Chisti, 2007; Aoyagi and Neogene, 1992; Holba et al., 1998; Hu et al., 2006; Ramachandra et al., 2009; Abdel-Hamid et al., 2013; Starr, 1978; Cox, 1962; Horowitz, 1975; D, 2638; D, 1310; Christie and Christie, 1993; Vicente et al., 2004; Haas et al., 2009; Chongkhong et al., 2007; Goff et al., 2004; Molnar-Perl and Pinter-Szakacs, 1986; Sharma and Singh, 2009; Griffiths and Harrison, 2009; Sheehan et al., 1998; Basha and Jebaraj, 2009; Knothe, 2005; Sadasivam et al., 1996; Matsumoto et al., 2017)and for lipid production by outdoor cultures of microalgae (Starr, 1978; Cox, 1962; Horowitz, 1975; D, 2638; D, 1310; Christie and Christie, 1993; Vicente et al., 2004; Haas et al., 2009; Chongkhong et al., 2007; Goff et al., 2004; Molnar-Perl and Pinter-Szakacs, 1986; Sharma and Singh, 2009; Griffiths and Harrison, 2009; Sheehan et al., 1998; Basha and Jebaraj, 2009; Knothe, 2005; Sadasivam et al., 1996; Matsumoto et al., 2017).

The results obtained are promising, suggesting the need for pilot studies assessing outdoor diatom growth, with particular focus on the economic feasibility of biomass production for biodiesel. The economically feasible production of microalgae biomass with the least impact on natural resources represents the backbone of sustainable production of algae-based biofuels (Chisti, 2007; Sharma and Singh, 2009; Griffiths and Harrison, 2009; Sheehan et al., 1998; Matsumoto et al., 2017; Wang and Seibert, 2017; Saranya et al., 2018; Abdel-Hamid and Eman, 2019; Almutairi, 2020).

The dry weight biomass of outdoor culture of *N. palea* (x′ = 0.16, n = 24) was significantly (P ≤ 0.05) lower than that under laboratory conditions (x′ = 0.27, n = 8). Moreover, the percentage dry weight biomass (% dw) of the n-hexane eluate of intracellular lipids of this diatom was higher under laboratory (26.2% dw) than under outdoor growth (8.54% dw) conditions, These results are in a perfect agreement with previous findings (Hu et al., 2008; Hu et al.,

2006; Starr, 1978; Sharma and Singh, 2009; Pintaka et al., 2017; Almutairi, 2020).

In agreement with previous studies (Ramachandra et al., 2009; Basha and Jebaraj, 2009; Knothe, 2005; Matsumoto et al., 2017) the fatty acid profiles and physico-chemical characteristics of *N. palea* lipids, as well as the results of GPC analysis, indicated that these lipids may be an excellent feedstock for biodiesel despite the relatively high wt% FFA.

Because the n-hexane extract of *N. palea* was rich in free fatty acids (28.9%), one step acid catalyzed alcoholysis (Christie and Christie, 1993) was sufficient to convert these lipids to biodiesel. Acid-based alcoholysis is regarded as an ideal process to esterify free fatty acids (Goff et al., 2004), in addition to trans-esterification of fat glycerides. The present study found that 93 wt% of the fatty acids of *N. palea* was converted to the corresponding FAMEs (biodiesel).

This result may indicate that acid-based alcoholysis is both useful and technically feasible for the production of biodiesel from fats exceptionally rich in FFAs.

In general the full annual cycle growth experiment of the pinnate diatom *Nitzschia palea* was mainly to assess the adaptability of this diatom to maintain growth development under different seasonal growth conditions. The results obtained are, to some extent, promising and may suggest further pilot and /or large-scale outdoor growth experiments with special focus on feasibility of biomass for economic production of biodiesel.

5. Conclusion

The outdoor production of biomass and lipids by the pinnate diatom *Nitzschia palea* was monitored biweekly for a full annual cycle. The diatom was cultivated outdoors in vertically oriented transparent acrylic tubes.. Marked variations in dry weight biomass and intracellular lipid contents were also observed, with mean annual values of 0.16 g l^{-1} and 8.54 wt%, respectively. Lipid analysis indicated that over 70% of lipids were C16:0 palmitic acid and C16:1 palmitoleic acid, fatty acids considered ideal for the production of biodiesel with exceptionally high technical quality. Except for its relatively higher free FFA level (28.9%), the iodine number, saponification value, specific gravity, flash point, fire point and boiling point of the lipids extracted from *N. palea* indicate that this diatom is chemically suitable as biodiesel feedstock. Due to the high level of FFAs, one step acid catalyzed alcoholysis of lipids was utilized to produce biodiesel. Results indicated that about 94% of fatty acids were converted to the corresponding FAMEs. The results obtained from full annual cycle growth experiment of the pinnate diatom *Nitzschia palea* seem promising and may suggest further pilot and /or large-scale outdoor growth for economic production of biodiesel

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