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Culture Medium and Growth Phase Modulate the Fatty Acid Composition of the Diatom *Nitzschia palea* (Kutzing) W. Smith-Potential Source for Live Feed and Biodiesel

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Abstract

The fresh water diatom *Nitzschia palea* was cultured in four different culture media *viz.,* f/2, Walne's, Chu and WC to determine the quality and production of lipids and fatty acids in this diatom during exponential and stationary phases of growth. The lipid content showed significant variation between culture media i.e. 10.97, 13.98, 25.43 and 21.43% in the exponential phase and 19.09, 21.41, 19.29 and 18.54% in the stationary phase in f/2, Walne's, Chu and WC medium, respectively. The lipid fraction of *N. palea* was mainly composed of myristic acid (11.76 -19.8%), pentadecanoic acid (7.76 - 18.18%), palmitic acid (3.0 - 15.1%), palmitoleic acid (3 - 15.1%), arachidonic acid (5.03 - 21.61%) and eicosapentaenoic acid (2.61 - 19.86%). Saturated fatty acids (SFAs) production was found to increase from exponential to stationary phase in all the culture media studied. Monounsaturated fatty acids (MUFAs) were found to decrease in percentage from exponential to stationary phase in f/2 and Walne's medium whereas in Chu and WC medium MUFAs showed an increase in percentage in the stationary phase. The polyunsaturated fatty acids (PUFAs) showed a decrease in quantity as the culture entered stationary phase. The results revealed that the culture medium and the growth phase affect the quantity of fatty acids considerably and the percentage of fatty acids in each medium and growth phase are significantly different ($p<0.5$). In *N. palea* appreciable amount of saturated and unsaturated fatty acids were found which establishes the importance of this diatom in the field of aquaculture as live feed and biofuel production.

Keywords: *Nitzschia palea,* culture medium, fatty acids, live feed, biofuel

Introduction

Microalgal lipids have been used as a dietary source for metabolic energy and essential components for growth of animals in aquaculture, with the fatty acid content and composition being the central factor in the selection of microalgal species, as this regulates the productivity of the cultured species (Renaud et al., 1999). Thus, microalgae especially diatoms are a critical component of the aquaculture food chain mainly for live feeds for larval culture (Brown et al., 1997). Many of the strains successfully used for bivalves are also used as direct feed for crustaceans (especially shrimp) during the early larval stages, such as *Skeletonema* sp. and *Chaetoceros* sp. Benthic diatoms such as *Navicula* sp. and *Nitzschia* sp. are commonly mass-cultured and then settled onto plates as a diet for grazing juvenile abalone (Brown, 2002). The PUFAs especially EPA derived from diatoms determine the quality, survival, growth and resistance to disease of cultured animal species (Gouveia et al., 2008).

Microalgae are very efficient in metabolizing CO_2, with almost zero level of energy, to highly energetic metabolites including oil which is the feedstock of biodiesel (Spolaore et al., 2006). Approximately 46 tons of oil per hectare per year can be produced from diatoms which can accumulate lipid equivalently or to a greater extent than other algal species such as *Botryococcus braunii, Nannochloropsis* sp., *Neochloris oleoabundans* etc. which produce up to

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50% oil by weight (Demirbas & Demirbas, 2011). Diatoms can rapidly induce triacylglycerol under silicate limitation, avoiding the detrimental effects on photosynthesis, gene expression and protein content associated with N limitation (Hildebrand et al., 2012).

The fuel quality of biodiesel (i.e., cetane number, exhaust emission, heat of combustion, cold flow, oxidative stability, viscosity, and lubricity) depends on the characteristics of the individual fatty acid alkyl esters, which are mainly determined by the structural features of the fatty acids (Knothe, 2005).

The fatty acid profile of diatoms indicates their application in different fields like live feeds in aquaculture and biofuel production. The polyunsaturated fatty acids (PUFA) are essential factors for the growth and survival of larvae and post larvae of fishes, crustaceans and mollusks, or indirectly as food for the live prey (Brown, 2002; Daume et al., 2003 and Gouda et al., 2006).

The lipid and fatty acid profile of microalgae vary in accordance with culture conditions viz. culture medium (Lincymol et al., 2012; Martinez- Cordova et al., 2012; Suman et al., 2012; Abdel- Hamid et al., 2013; Naseera et al., 2013; Krishnan et al., 2016; Menezes et al., 2016; Mitani et al., 2017; Lidiya & Joseph, 2018 and Lidiya et al., 2018), temperature (Rousch et al., 2003; Araujo &Garcia, 2005 and Wah et al., 2015), light intensity (Khoeyi et al., 2012 and Wahidin et al., 2013), photoperiod (Khoeyi et al., 2012; Wahidin et al., 2013 and George et al., 2014), nutrient stress (El- Kassas, 2013; Chagoya et al., 2014; Jiang et al., 2015; Ganga & Joseph, 2016 and Ganga et al., 2016), harvesting technique (Borges et al., 2016) and growth phase (Liang & Mai, 2005; Pratiwi et al., 2009; Costard et al., 2012; Lidiya & Joseph, 2018 and Lidiya et al., 2018). There is paucity in information regarding the impact of different culture media and growth phase on the lipid production and fatty acid profile of *N. palea*. So, the present study was aimed to evaluate the effect of growth media and growth phase on the qualitative and quantitative production of fatty acids in the fresh water diatom *N. palea* with respect to live feed and biodiesel potential.

Materials and Methods

N. palea was collected from freshwater sources like stream and paddy fields. Collection was done by scratching the pebbles and rocks collected from the stream using a brush (Taylor, 2007). Later *N. palea* was isolated from the collected sample by two methods *viz.,* serial dilution and agar plating (Andersen, 2005). The stock culture of *N. palea* was maintained in f/2 medium. The structure, size and morphology of *N. palea* were studied using light microscope. Scanning electron microscope study was carried out for further taxonomic confirmation using the identification key for diatoms (Heurck, 1896).

N. palea was cultured in 1L Erlenmeyer flasks filled with filtered and sterilized fresh water supplemented with desired culture medium. Four media such as f/2 (Guillard, 1975), Walne's (Walne, 1966), Chu (Chu, 1942) and WC (Guillard & Lorenzen, 1972) were selected for the study. The cultures were exposed to 25°C temperature and 1500 lux light on a 12h: 12h light: dark cycle (Andersen, 2005). 10% volume of the total culture medium with a cell density of 10 x 10^4 cells/ ml was used as inoculum (Vicose et al., 2012). Growth kinetics was studied by calculating the cell density on alternate days of culture. The cell density was enumerated following the method described by (Andersen, 2005) using the formula ie.,

Cell density = Total number of cells in 5 columns x 5 x 10^4 cells/ ml

Preliminary studies on growth kinetics revealed that *N. palea* entered into the exponential phase on the 5^{th} day and it lasted to the 15^{th} day with a maximum cell density of 45 x 10^4 cells/ ml (10 days period). The culture entered into the stationary phase on the 16^{th} day and lasted to the 20^{th} day (5 days period). So the culture was harvested during the exponential and stationary phase by centrifugation at 8000 rpm for 20 min in 500 ml centrifuge tubes.

The sample pellet obtained by centrifugation was dried and then lipid extraction was done using chloroform: methanol (1:2) according to the method of Bligh &Dyer (1959). To 1g sample 5ml chloroform: methanol was added and then homogenized thoroughly. Later 5 ml chloroform and 5 ml distilled water were added to the homogenized sample. The sample was shaken well and kept at 4°C overnight. Later the sample was transferred to a separating funnel and the lower chloroform layer containing lipid was collected through Na_2SO_4. Then lipid was concentrated at 45°C in a rotary vacuum evaporator. The fatty acid methyl ester (FAME) was prepared by the method suggested by Metcalfe et al., 1966.

Table 1. Percentage of fatty acids in *Nitzschia palea* cultured in four culture media

Fatty acids	Exponential phase				Stationary phase			
	f/2	Walne's	Chu	WC	f/2	Walne's	Chu	WC
SFA	**40.51±0.15**[a]	**36.09±1.19**[b]	**42.19±1.87**[c]	**35.45±0.49**[d]	**51.57± 0.16**[a]	**52.88±0.58**[b]	**50.4± 0.65**[a]	**46.45±0.24**[b]
C14:0	19.8±0.68	15.07±0.59	16.91±0.84	11.76±0.85	17.29±0.36	19.02±0.83	19.3±0.58	19.74±0.26
C15:0	7.83±0.78	12.98±0.86	7.76±0.75	8.8±0.47	15.16±0.59	18.18±0.62	10.24±0.58	9.96±0.07
C16:0	5.87±0.09	3±0.29	15.1±0.56	12.11±0.42	14.64±0.56	9.61±0.45	13.87±0.89	12.78±0.19
C18:0	1.05±0.26	0.95±0.48	0.32±0.19	0.34±0.14	0.62±0.11	0.8±0.24	1.24±0.19	1.06±0.03
C20:0	2.31±0.33	1.67±0.51	0.79±0.29	0.69±0.09	0.9±0.07	0.62±0.33	2.08±0.22	1.49±0.08
C22:0	1.17±0.17	1.94±0.37	0.72±0.18	0.93±0.31	1.73±0.13	3.41±0.29	2.64±0.22	0.81±0.11
C24:0	2.48±0.58	0.48±0.23	0.58±0.19	0.82±0.08	1.22±0.65	1.24±0.18	1.03±0.46	0.61±0.38
MUFA	**43.5± 1.2**[a]	**22.02±0.68**[b]	**18.33±0.54**[c]	**21.2± 0.46**[a]	**33.49± 1.49**[a]	**19.13±0.11**[b]	**27.98±0.08**[c]	**32.59± 0.12**[b]
C16:1	40.98±0.47	21±0.24	15.72±0.27	19.99±0.86	30.99±0.76	16.06±0.25	26.11±0.29	27.58±0.49
C18:1	1.53±0.32	0.27±0.14	2.07±0.59	0.2±0.09	1.53±0.22	1.17±0.26	0.61±0.4	4.37±0.13
C22:1	0.99±0.42	0.76±0.31	0.54±0.32	1.01±0.32	0.97±0.52	1.9±0.4	1.26±0.18	0.64±0.25
PUFA	**15.99±0.47**[a]	**41.99±0.51**[b]	**39.45±0.69**[c]	**43.88±0.68**[c]	**14.75± 0.44**[a]	**28.02±0.46**[b]	**21.03±1.66**[c]	**20.9± 0.44**[d]
C18:2	3.12±0.11	0.24±0.09	1.33±0.15	3.44±0.25	0.76±0.27	0.98±0.31	0.76±0.27	2.12±0.49
C18:3	4.38±0.29	0.59±0.09	1.17±0.13	1.02±0.29	1.88±0.46	0.56±0.23	0.89±0.73	0.93±0.23
C20:4	5.54±0.49	21.61±0.18	21.37±0.18	19.56±0.43	9.5±0.34	15.07±0.73	5.03±0.37	9.26±0.41
C20:5	2.95±0.78	19.55±0.16	15.58±0.49	19.86±0.4	2.61±0.29	11.4±0.27	14.35±0.29	8.59±0.29

In the row, values (mean±SD) with different letters (a-d) were significantly different at $p< 0.05$

The lipid was refluxed with methanolic NaOH at 80ºC for 25 min under nitrogen atmosphere. Later 5ml BF_3 methanol was added to the sample and refluxed again for 5 min. After cooling the sample, it was transferred to a separating funnel and 5 ml saturated NaCl was added. The solution was shaken well and lower impure layer was removed. Later the sample containing FAME was washed thrice using petroleum ether and distilled water. Then the petroleum ether layer was collected through Na_2SO_4 and concentrated in rotary vacuum evaporator at 40ºC. The FAME thus obtained was then recollected in minimum amount of petroleum ether.

Gas Chromatographic analysis was done on a Perkin-Elmer Clarius 580 Gas chromatograph (HP 5890 Series II, Perkin Elmer, Bridgeport Ave, Shelton, CT, USA) connected with Optima 225 (crossbond 5% diphenyl- 95% dimethyl polsiloxane) capillary column (100 m X 0.25 mm i.d., 0.50 ìm film thickness, Supelco, Bellfonte, PA). The flame ionization detector (FID) equipped with a split injector, was used in the split (1:15) mode. The GLC analyses were accomplished using an oven, injector and detector which were held at a temperature of 110°C, 275°C and 280°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 25 cm/s. Hydrogen was used as the ignition gas at a head pressure of 20 psi. The fatty acid peaks were identified by comparison of retention times with the known standards (SupelcoTM 37 Component FAME Mix, Catalog No. 47885-U).

The experiments were carried out in triplicate and the results were expressed as mean ± standard deviation. SPSS version 22 was used for analysis. The data were compared using one way ANOVA with Duncan's post hoc tests. 'p' values of <0.05 were used as standard for statistical significance.

Results and Discussion

The production of lipids and fatty acids by diatoms in response to nutrients and growth phase is specific. The present study focused on the fatty acid production in *N. palea* when cultured in different culture media and harvested during different growth phases.

N. palea had a lipid content of 10.97, 13.98, 25.43 and 21.43% when cultured in f/2, Walne's, Chu and WC medium, respectively in the exponential phase of growth. In the stationary phase *N. palea* had a lipid content of 19.09, 21.41, 19.29 and 18.54% when cultured in f/2, Walne's, Chu and WC medium, respectively (Krishnan et al., 2016). Statistical analysis revealed that there is significant difference ($P<0.05$) in lipid content in the four culture media during both exponential phase as well as stationary phase.

The major fatty acids found in *N. palea* are shown in Table 1. The results of the present study revealed that the fatty acid composition of *N. palea* can vary significantly($p<0.05$) in response to the culture medium. The result is in accordance with the results published by Opute (1974) who cultured the diatoms *Nitzschia palea, Navicula muralis* and *Navicula inserta* in Chu medium and Abdel-Hamid et al. (2013) in which fatty acid production of *Nitzschia palea* cultured in NAV medium is reported. Extensive studies indicated that diatom lipids are mostly composed of myristic acid, pentadecanoic acid, palmitic acid, palmitoleic acid, arachidonic acid and eicosapentaenoic acid (Opute, 1974; Liang & Mai, 2005; Chen et al., 2007). Other fatty acids namely, stearic acid, arachidic acid, behenic acid, lignoceric acid (SFAs), oleic acid, erucic acid (MUFAs) linoleic acid and linolenic acid exhibited minor quantitative contribution to fatty acid composition.

When *N. palea* was cultured in f/2 medium, MUFAs were found dominating among the total fatty acid content in the exponential phase. Supporting results were reported by Liang & Mai (2005) in *Chaetoceros gracilis, Cylindrotheca fusiformis, Phaeodactylum tricornutum,* and *Nitzschia closterium* and Renaud et al. (1999) in *Nitschia* sp. who showed that MUFA content was higher compared to SFAs and PUFAs. Similarly, Lidiya et al. (2018) reported that *Tetraselmis gracilis* when grown in different culture media viz. f/2, Walne's, Chu and WC, maximum MUFA production was obtained in f/2 medium. As a contrast to these results, Gordon et al. (2006) reported that *Nitzschia laevis, Amphora luciae* and *Navicula lenzii* produce comparatively higher amount of PUFAs than other groups of fatty acids in f/2 medium in the exponential phase. In the present study, in case of stationary phase *N. palea* produced more SFAs compared to MUFAs and PUFAs in f/2 medium. This finding is reminiscent of the results by Knuckey et al. (2002) in the diatom *Atheya septentrionalis.*

In case of Walne's medium, PUFAs were found dominating in the exponential phase. The studies done by Lidiya et al. (2018) in *Tetraselmis gracilis* revealed a similar result with the production of maximum PUFA in Walne's medium. In contrast to this, Umamaheswararao et al. (2015) in *Thalassiosira pseudonana* found out the production of higher concentration of SFAs in the exponential phase. But in case of stationary phase, *N. palea* had a higher concentration of SFAs in Walne's medium. This result is in corroboration with the work of Umamaheswararao et al. (2015).

N. palea when cultured in Chu medium, SFAs were found dominating in the exponential phase. But the studies done by Opute (1974) showed an increased production of MUFAs in this phase. In the present study, when *N. palea* was analyzed for fatty acids in the stationary phase a higher concentration of SFAs was found as in the exponential phase.

A higher percentage of PUFAs and SFAs were found in the exponential phase and stationary phase of *N. palea*, respectively when cultured in WC medium. These results are in contrast to the findings of Calixto et al. (2016) who found out the production of more SFAs in *Chlamydomonas* sp., and MUFAs in *Lagerheimia longiseta* and *Pediastrum tetras* which were cultured in WC medium and harvested in the exponential phase. However, the present observations contradict with the results reported by Menezes et al. (2016) which showed an increased production of MUFAs in *Choricystis minor* in the stationary phase.

As discussed already, *N. palea* when cultured in f/2 medium and Walne's medium, more lipid production was found during exponential phase but when grown in Chu and WC medium more lipid production took place in the stationary phase. Similarly, fatty acid composition also varies with the change in growth phase (Pratiwi et al., 2009; Costard et al., 2012; Lidiya et al. 2018).

During the exponential phase of growth *N. palea* was found to produce maximum saturated fatty acids when cultured in the Chu medium. There was significant difference ($p<0.05$) between the percentage of saturated fatty acids produced in all the culture media. Monounsaturated fatty acid production was highest in f/2 medium and there was significant difference ($p<0.05$) between the percentage of monounsaturated fatty acids produced in all the culture media. Production of polyunsaturated

fatty acids in *N. palea* was more when cultured in WC medium. There was a significant difference between ($p<0.05$) the percentage of polyunsaturated fatty acids produced in f/2 and Walne's medium. But there was no significant difference ($p>0.05$) between the percentage of polyunsaturated fatty acids produced in WC and Chu medium (Fig. 1).

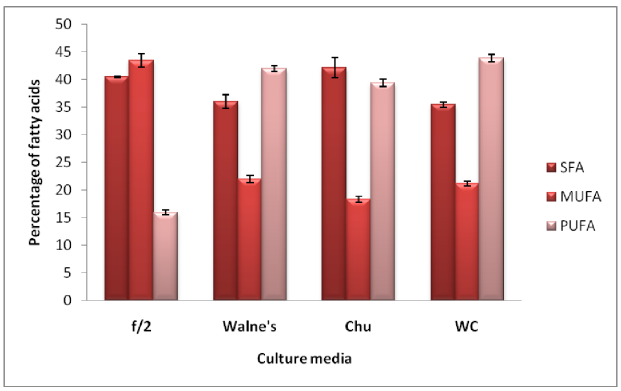

Fig. 1. Fatty acid composition of *Nitzschia palea* during the exponential phase of growth

Highest production of saturated fatty acids in stationary phase was obtained when *N. palea* was cultured in the Walne's medium. There was no significant difference ($p>0.05$) between the percentage of saturated fatty acids produced in Walne's and WC medium. But f/2 and Chu media showed significant difference ($p<0.05$) between them. Among the four culture media an elevation in monounsaturated fatty acid production was found in f/2 medium. MUFA production in all the culture media showed significant difference ($p<0.05$). In case of polyunsaturated fatty acids *N. palea* produced more of them when cultured in Walne's medium. PUFA production in *N. palea* when cultured in all the culture media showed significant difference ($p<0.05$) (Fig. 2).

The growth phase of the diatom also influences the fatty acid production. When *N. palea* was harvested during the exponential and stationary phases of growth and analyzed for fatty acid composition it was found out that saturated fatty acids tend to increase when enter into the stationary phase of growth in all the culture media. In case of monounsaturated fatty acids there found a decrease in production from exponential to stationary phase in f/2 and Walne's medium. But in Chu and WC medium monounsaturated fatty acids showed an increase in the stationary phase of growth. Polyunsaturated fatty acids showed a general tendency to decrease as the culture aged.

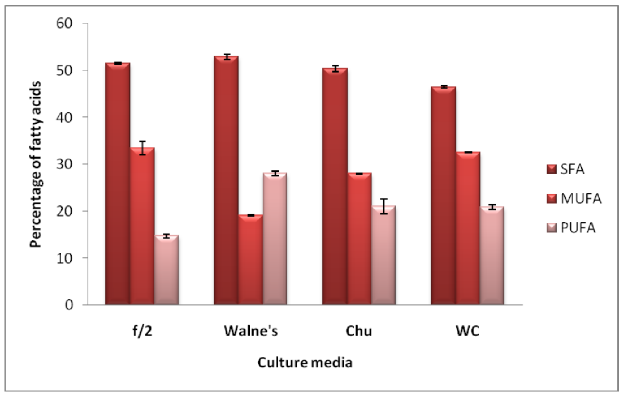

Fig. 2. Fatty acid composition of *Nitzschia palea* during the stationary phase of growth

During *N. palea* harvested in the exponential and stationary phases of growth it was found out that saturated fatty acids tend to increase when enter into the stationary phase of growth in all the culture media. The observation is in accordance with the earlier findings of Pratiwi et al. (2009) in *Chaetoceros gracilis* who found that saturated fatty acids increase in quantity in the stationary phase of culture. In case of monounsaturated fatty acids there found a decrease in production in the stationary phase compared to exponential in f/2 and Walne's medium. This correlates with the results obtained in *Chaetoceros gracilis* by Pratiwi et al. (2009). But in Chu and WC medium monounsaturated fatty acids showed an increase in the stationary phase of growth. Monounsaturated fatty acid percentage was found to increase from exponential to stationary phase in the study done in *Chaetoceros gracilis, Cylindrotheca fusiformis, Phaeodactylum tricornutum,* and *Nitzschia closterium* by Liang & Mai (2005). Polyunsaturated fatty acids showed a general tendency to decrease as the culture aged. *Nannochloropsis oculata* contained more PUFA per cell in logarithmic phase than in stationary phase (Dunstan et al., 1993). Increases in the relative proportions of both saturated and monounsaturated fatty acids and decreases in the proportion of PUFAs were associated with growth phase transition from the exponential to the stationary phase. Liang &Mai (2005) justified this through their study on the effect of growth phase on the fatty acid compositions of diatoms. According to them neutral lipids from microalgae are mainly composed of saturated and monounsaturated fatty acids while polar lipids are

composed of PUFAs. Neutral lipids are considered to be storage lipids for algae while polar lipids are associated with the photosynthetic membranes of algae. Hence, decrease in production of PUFAs in the stationary phase can be in relation with the reduction in photosynthesis in this phase.

The present observations revealed that saturated fatty acids as well as unsaturated fatty acids evenly contribute to the fatty acid composition of *N. palea*. The fatty acid composition of microalgae determines the purpose of their utilization. *N. palea* produced more PUFA in the exponential phase compared to the stationary phase, which indicates that this diatom can be used as a live feed during its exponential phase. It is found to produce a maximum of 19.86% EPA in its exponential phase of growth. Long chain polyunsaturated fatty acids in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are necessary for the growth, development, metamorphosis and reduced mortality of aquatic animals during the larval stage.

Microalgal lipids are also being targeted as a feedstock for the sustainable production of biodiesel. Algae generally produce a lot of polyunsaturates, which may present a stability problem since higher levels of polyunsaturated fatty acids tend to decrease the stability of biodiesel. Basha &Jebaraj (2009) reported that the long chain fatty acids with carbon atoms number between C12 and C24 represent suitable feedstock for biodiesel. Evaluating the results of the present study *N. palea* is found to produce more saturated fatty acids in comparison to polyunsaturated fatty acids and while entering into the stationary phase the percentage of saturated fatty acids tend to increase. According to the studies conducted by Abdel- Hamid et al. (2013), *N. palea* may be suitable feedstock for biodiesel. Hence, *N. palea* can be considered as a good competitor in the field of biodiesel production.

Nitzschia palea is an abundant species of diatom in the natural environment and it can be cultured easily under laboratory conditions. A standard protocol for the culture of *N. palea* under laboratory conditions has been developed. The fatty acid profiling of *N. palea* revealed its significance in the field of aquaculture and biofuel production. Hence, being rich in major fatty acids this diatom can be cultured in mass to satisfy the needs for microalgae as live feeds and a source of biodiesel.

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