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Mass Cultivation of Marine Micro alga *Nannochloropsis gaditana* KF410818 Isolated from Visakhapatnam offshore and Fatty Acid Profile Analysis for Biodiesel Production.

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Abstract

Economically viable algae based oil industry will depend on the high lipid content of microalgal strains. In this paper, native isolate of *Nanochloropsis gaditana* proved that, it is a promising strain for large scale production of biomass and biodiesel. Not all algal oils are adequate for making biodiesel, but suitable oils commonly occur. In this study, the algal growth rate, biomass production, transestrification process, lipid content and productivity of microalgae culture isolated from onshore water was determined. Furthermore, lipid extract from the isolate and its further converted to biodiesel was investigated. Results from the mass culture, 2.67 g L^{-1} biomass were founded on the 10^{th} day under indoor condition with 24 h light intensities in annular column. Maximum FAME yields 20% was observed under direct transestrification process. Fatty acid profile showed that no matter which conditions are provided for growth, but produce profitable biomass under low cost is economical. The approaches present in the study could be employed for the large scale biomass and biodiesel production.

Key words: *Nannochloropsis gaditana*, Growth rate, Fatty acids, Lipid and FAME yield, Biomass productivity, Indoor and outdoor cultures

Introduction

Micro algal biotechnology appears to possess a high potential for biodiesel production. Based on their ability to grow rapidly and to accumulate large amounts of storage lipids, microalgae have been considering for biofuel production. Selection of high lipid producing and FAME yielding algae, suitable outdoor culture locations, efficient cultivation and harvesting methods and oil extraction techniques will be considered for energy and cost efficient production of biodiesel. The effects of light-path length on cell biomass cultivation and EPA output rates (Zou N and Richmond A. J. 1999), as well as the effect of cell density in photoacclimation (Zou N and Richmond A. J. 2000) have been studied. The metabolism of lipids, pigments, chlorophyll in particular, is very dynamic in *Nannochloropsis* sp. There is evidence that rapid changes in pigments (eventually other compounds) contents may be an adaptive response of cells to changes in light environment (Sukenik A et al 1989). The triglycerides are converted by transesterification using methanol and acid catalyst to yield glycerol and the fatty acid alkyl ester or fatty acid methyl ester (FAME). To determine the fatty acid composition of lipid containing tissues FAME preparation was done using a direct transesterification method as an analytical technique.(Park and Goins, 1994; Lepage andRoy, 1984; Rodríguez-Ruiz et al., 1998).The direct derivation methods can result in greater FAME yields than are achieved in the two step extraction followed by transesterificationapproach (Siler-Marinkovic and Tomasevic, 1998; Lewis et al., 2000). A direct approach has been shown to be effective in making biodiesel from both pure (Johnson and Wen, 2009) and mixed cultures of microorganisms (Mondala et al., 2009; Dufreche et al., 2007). In addition to producing FAMEs from TAG and free fatty acids, one study suggests that in-situ transesterification reduced the amount of phospholipids in a sample to a level below detection (Vicente et al., 2009).

Single cell oil has attracted attention during the past decade as a sustainable and biodegradable fuel (Li et al. 2008; Meng et al. 2009). During the biodiesel production process, triacylglycerols are mixed with alcohol and catalysts producing fatty acid esters, with crude glycerol as a primary by-product. Here, we have examined the growth kinetics, culture condition and developed an approach of FAMEs by direct transesterification and two step extraction from high TAG accumulating microalgae such as *Nannochloropsis gaditana*. This study was focused on isolation of high lipid yield native strain from Visakhapatnam coast, find out the productivity of biomass and lipid under indoor and outdoor culture conditions and evaluation of fatty acid profile for cost efficient biodiesel production.

2. Materials and methods

2.1. Isolation and identification of the microalgae

Nannochloropsis gaditana was isolated from the offshore water of East Coast Bay of Bengal, Visakhapatnam, Andhra Pradesh, and Southeast India during August, 2012. Isolation and purification were done using Algal Culturing Techniques (Andersen and Guillard, 2005).

2.2. Stock culture conditions and media

The strains were incubated at 23±2 ^{o}C and constant illumination was used at 60 µE m^{-2} s^{-1} intensity with white fluorescent lamps. The culture was three to five time's manual shaking a day. Walne medium (1970) was used for the stock culture maintenance.

2.3 Light microscopes

Microscopic (Carl Zeiss) examinations of culture were carried out regarding cell size and characteristic morphological feature. Cell images were captured with phase contrast illumination.

2.4. Electron Microscopy Morphology Analysis

To observe the cell morphology and structure, scanning electron microscopy (SEM) studies were carried out. For SEM, 5 ml cultures were harvested and fixed with glutaric dialdehyde for 24 h, separated by centrifugation for 5 min at 1000x *g*, then washed five times with 0.1 M phosphate buffer. Then the cells were embedded using the glutaraldehyde, [Gamliel, H et al., 1983], coated with gold, and examined under the SEM microscope (JEOL- JSM-6610 LV, INCA PENTAFETX3, England). The cells were dehydrated with increasing concentrations of ethanol up to 100% and anhydrous acetone. Photographs were taken under the SEM microscope using JEOL SEM Software.

2.5. Preparation of microalgae inoculums and mass cultures

A stock culture of *N. gaditana* (approximately 1 x 10^5 cells mL^{-1}) was cultured in an Erlenmeyer flask with 800 ml working volume of Conway medium under 23 ± 1^{o}C and 300 µmol m^{-2} s^{-1}. Exponential phase culture was inoculated into 3 L pumpkin flask containing 2000 ml enriched sea water. After the 4th day, 2500 ml was used as a seed culture and inoculated into 17.5 L culture media contains transparent pet jar. Final 20 L of exponential culture was used for mass culture. Indoor mass culture was carried out with 250L capacity of the annular column under 6000 Lux light intensity. Outdoor mass culture was carried out with 250 L capacity of the horizontal FRP tank under natural sunlight. The growth was autotrophic being the inorganic CO_2 from the atmosphere the unique carbon source that reached the culture by bubbling.

2.6. Cell count estimation and Biomass concentrations.

The algal cell density was measured daily by counting using an improved Neubauer haemocytometer and a light microscope. Optical density measurement at 680 nm (Lee et al., 1998) was used to monitor cell growth by UV/Visible spectrophotometer (PerkinElmer Lambda 35). All cultures were initiated with an O.D. of about 0.1. Cells were concentrated by centrifugation, washed with de-ionized water and dried (80°C) to determine dry weight (expressed as g l^{-1}). Based on the calibration curve of O.D. and dry biomass concentration, 1 O.D. corresponds to 2.162 g l^{-1}. So, biomass concentration was calculated by regression equation: y = 2.162 (R2 = 0.98,), where y (g/L) is the dry cell weight, x is the absorbance of suspension at 680 nm.

2.7. Growth kinetics

The calculated cell biomass values were used to calculate the maximum dry weight biomass (Xmax), maximum specific growth rates (µmax, 1/day), doubling time (td, day) and maximum biomass productivity (Pmax, g $l^{-1}d^{-1}$) of each alga. The maximum specific growth rates value was calculated by exponential regression of the logarithmic portion of the growth curve (Bailey and Ollis, 1986) and the doubling time as td = ln2/µmax. Maximum productivity was calculated according to the equation Pmax = (Xt-X0) / (t-t0), where X0 is the initial biomass concentration (g/L) at time t0 and Xt is the biomass concentration in (g/L) at any time t subsequent to t0 (Schmidell, 2001).

2.8. Extraction of oil from algal biomass

The 5 g of dried biomass was ground with a mortar and pestle, and the lipids were extracted with n-hexane (Maxwell *et al*., 1968) followed by extraction with chloroform and methanol (1:2 v/v) (Bligh and Dyer, 1959) for each extraction of the mixture was sonicated at 60Hz with a sonicator (Sonics vibra cell High Intensity Ultrasonic processor with Temperature Controller, Microprocessor controlled-750 WATT Model) at 50 ^{o}C temperature. Each extraction was repeated (total of two times). Phase separation was subsequently carried out following the method of Folch et al., (1957). Here, chloroform and water were added into the mixture in the ratio of mixture: chloroform: water of 10:10:9. After shaking vigorously, the mixture was centrifuged at 3000 RPM for 10 min, resulting in separation into two phases. Methanol, the by-product glycerol and other polar impurities that dissolved in water formed the upper phase while FAMEs, free fatty acids, lipids and other non-polar compounds well dissolved in the chloroform bottom phase. The extracts were dried in a rotary evaporator and weighed. The lipid content was calculated based on total lipid from the first and second extraction steps and then expressed as a dry weight percentage. The lipid yield was calculated as the average value of lipid content multiplied by the average value of biomass concentration.

2.9. Lipid productivity

Daily lipid productivity was calculated using the equation:
Daily lipid production (mg lipid l^{-1} day^{-1}) = DW x (lipid /100/day) x 1,000
Where, DW = algal dry weight (g l^{-1}), lipid = g 100 g−1 DW, and day = growth period.

2.10. FAME yield

The yield of FAME is reported in two different units, defined by Eqs. (1) as follows:

(1) FAME Yield (%by weight of algae) = Weight of FAME obtained after the tranesterified / weight of algae input X 100.

2.11. Two step process of transestrification

Crude lipid extracted by hexane and Chloroform/Methanol was saponified with 25 ml of 0.5 M methanolicNaOH solution at 75 ^{o}C for 20 min, and then submitted to methanolysis with 5% H_2So_4 in methanol at 75 ^{o}C for another 2 h. FAMEs partitioned twice into 2 ml hexane/ether (1:1, v/v). To the combined hexane–ether extracts, 2 ml of 2 % (w/v) NaCl was added for acid neutralization, collecting the top solvent layer. This was evaporated under room temperature and resuspended in hexane for GC analysis. Methyl ester samples were concentrated on rotary evaporator, weighed for FAME yields and analysed by TLC in 90:10-hexane: ethyl acetate (v/v) solvent system for conformation.

2.12. Direct transesterification

5g of algal dry biomass was used to determine the FAME yield under direct transestrification method. Methanol (25 ml) containing sulfuric acid as a catalyst was added to the reaction vessel (150 ml screw cap bottle) containing the algae and a PTFE coated stir bar. The reaction vessel was maintained at 100 ^{o}C with 500 rpm at one hour in heater with magnatic stirrer (Heidolph, MR Hei-Standard, Germany).

The reactions were stopped by the addition of chloroform to the reaction vessel forming a single-phase solution with the methanol. Phase separation was then accomplished by washing the methanol–chloroform solution with water (5 ml) followed by centrifugation. The methanol and sulfuric acid partitioned with the water in the upper phase, while FAME, TAG, and other lipids partitioned with chloroform in the lower, organic phase. The residual biomass formed a layer at the boundary between these two phases. The chloroform phase was removed with a gas tight syringe to a 10 ml volumetric flask. The remaining biomass was washed twice with 2 ml of chloroform to recover residual FAMEs and lipids.

2.13. GCMS/ GC Analysis Protocol

The fatty acid composition of algal oil was analysed qualitatively using GC-MS and quantitatively using GC. The GC/GCMS analyses were carried out using an Agilent 6890N Gas chromatograph connected to an Agilent 5973 mass selective detector at 70 eV (m/z 50-550; sources at 230 ^{o}C and quadruple at 150 ^{o}C) in the electron impact mode with a HP-5 capillary column (30 m x 0.25 mm i.d. X 0.25um film thickness). The oven temperature was programmed for 2 min at 160 °C and raised to 300 °C at 5 °C/min and maintained for 20 min at 300 °C. The carrier gas, helium was used at a flow rate of 1.0 ml/min. The inlet temp was maintained at 300 °C, and the split ratio was 50:1. Structural assignments were based on interpretation of mass spectrometry fragmentation and confirmed by comparison of retention times as well as fragmentation patterns of authentic compounds. GC analysis was performed on HP 6850 Series gas chromatograph equipped with a FID detector and DB- 225 capillary columns (30mm x 0.25 mm i.d. x 0.25 µm film thickness). The injector and detector temperatures were maintained at 300 and 325 °C, respectively. The oven temperature was programmed for 2 min at 160 °C and raised to 300 °C at 5 °C/min and

maintained for 20 min at 300 °C. The carrier gas, nitrogen was used at a flow rate of 1.5 ml/min. the injection volume was 1 µL, with a split ratio of 50:1. The identification of individual fatty acids is based on retention time of authentic fatty acid.

2.14. Statistical analysis.

Triplicates are maintained for the estimation of different parameters. The results are expressed as mean ±SE. Data were analysed statistically using SPSS 17.0 for windows.

3. Results and discussion

The green micro alga N. *gaditana* was isolated from offshore water of the Bay of Bengal, Visakhapatnam, and Andhra Pradesh. The isolate was maintained in both agar (Algal agar, Himedia) and liquid walne medium for maintaining stocks and conducting experiments. The isolate of *N. gaditana* primarily investigated with conical flasks under laboratory condition revealed that, it was produced more than 1.8 g L^{-1} biomass (Data not shown). Keeping this result in mind, mass culture work was carried out in both indoor and outdoor conditions.

Microscopic photos with scanning electron and phase contrast illumination were given in Fig. 1 and Fig. 2 respectively. Calibration curve of *N. gaditana*, cell concentration vs OD and dry weight vs OD were given in Fig. 3 and Fig. 4 respectively. Growth curve of indoor and outdoor cultures were given in Fig 5.

The experiments reported in this work were done to understand the capability of this strain for biomass and lipid production, find out the suitability of fatty acid profile for biodiesel preparation and to confirm this species whether promising strain or not for biodiesel feedstock.

Figure 1: Scanning electron microscopic images (A) group with 5 µm scale bar (B) single with 2 µm bar scale.

Figure 1 (A)

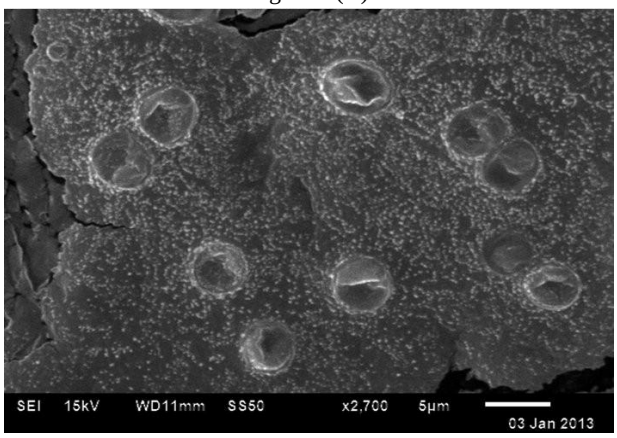

Figure 1 (B)

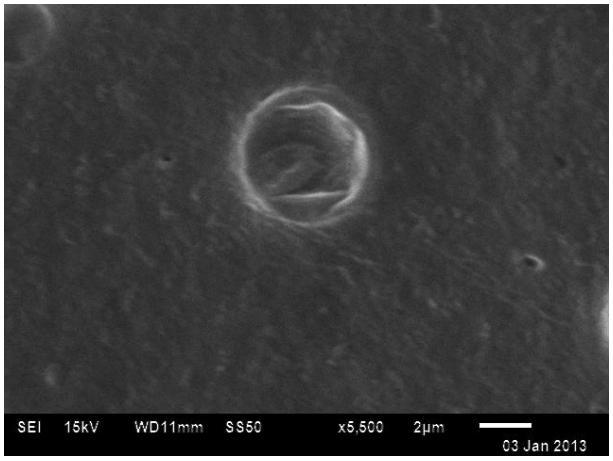

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Figure 2: *N.gaditana* images captured with different illumination using Primo vert microscope. Fig. A scale bar indicating 100 µm scale bar, B and C indicating 50 µm scale bar.

Figure 2 (A)

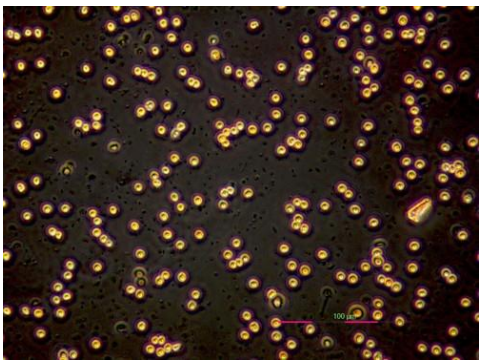

Figure 2 (B)

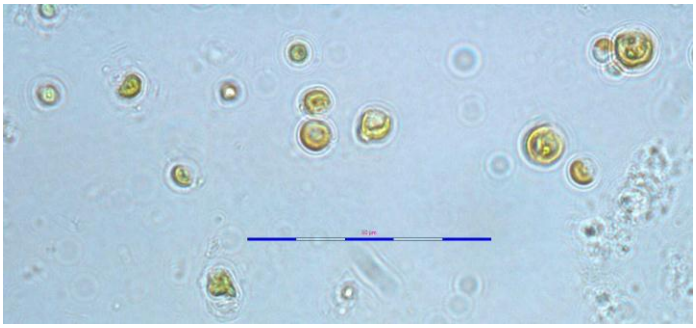

Figure 2 (C)

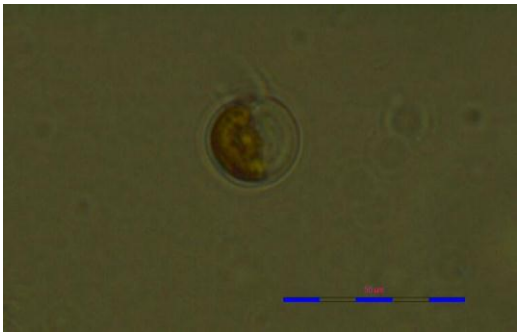

Figure 3. Calibration curves. Cell concentration versus optical density (680 nm).

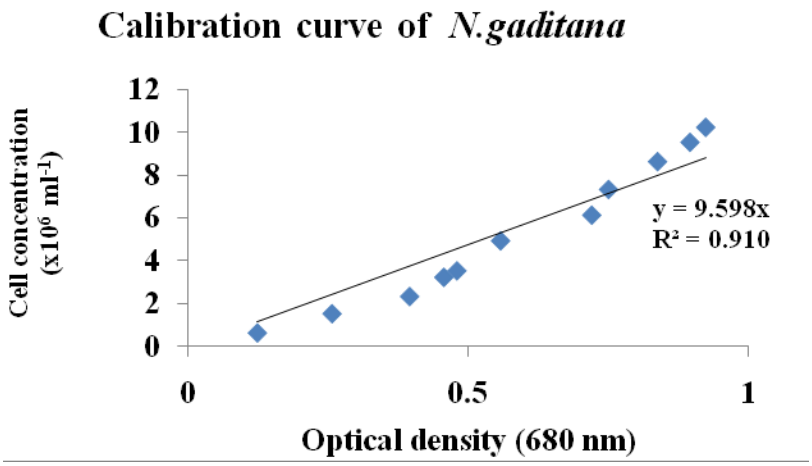

Figure. 4. Calibration curves. Cell dry weight versus optical density (680 nm).

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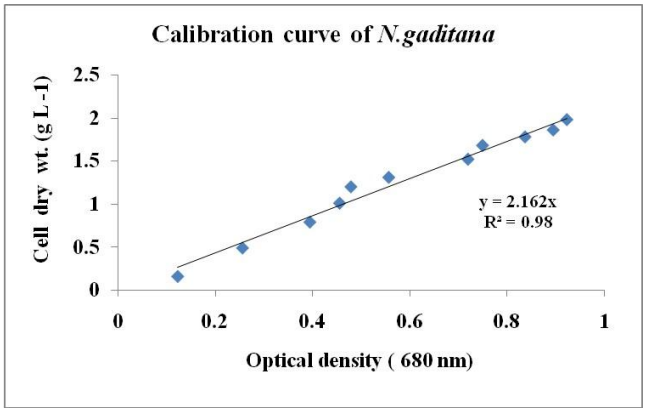


3.1. Biomass content and productivity of mass culture.

Biomass concentration of *N. gaditana* was given in Table 1. The initial concentration of 180 mg l^{-1} biomass was increased in indoor culture and they rose up to 2.67 gl^{-1}. The outdoor culture was started with the initial concentration 150 mg l^{-1} and raised up to 1.84 g l^{-1}. The highest biomass was observed on 10^{th} day of both cultures. Biomass concentration of indoor culture is very similar to the report of J.M.S. Rocha et al 2003 by the same species. The highest per day yield 2.67 and 1.84 g l^{-1} was found under indoor and outdoor cultures respectively. The biomass concentration is not too high because multiplication is highly dependent on environmental condition. B. Cheirsilip and S. Tropee 2012 reported that the growth of *Nannochloropsis* sp. continuously increased up to the maximum level with light intensity up to light intensity of 10,000 Lux. *B. braunii* strains SAG 30.81 and L.B-572 maintained at 16:8 h light and dark cycle with 1.2 and 2.8 g l^{-1}respectively, under laboratory conditions (Dayananda et al, 2007).*Nannochloropsis gaditana* natively exhibits high photoautotrophic biomass and lipid productivities. The average biomass and lipid production (0.65 g l^{-1} d^{-1} and 0.31 g l^{-1} d^{-1}) was reported (Robert E. Jinkerson, 2013).

Table 1. Biomass (dry wt.) content of *N. gaditana* (g L^{-1})

Days	Indoor Culture	Outdoor Culture
0	0.18	0.15
1	0.39	0.33
2	0.47	0.38
3	0.82	0.63
4	1.18	0.76
5	1.13	1.03
6	1.28	1.1
7	1.35	1.18
8	1.48	1.33
9	2.07	1.43
10	2.67	1.84

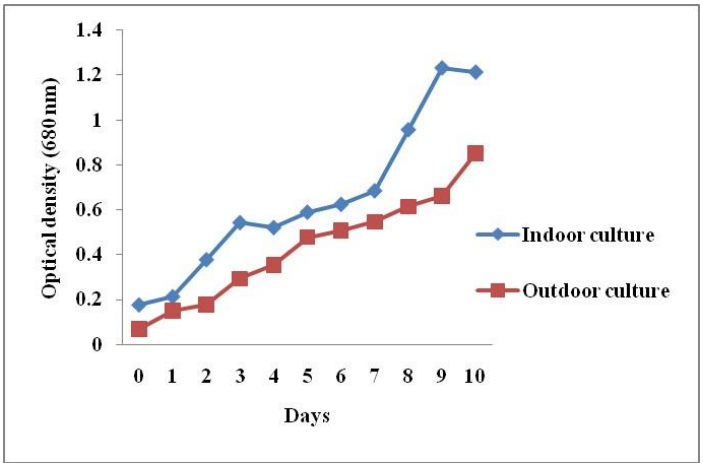
3.2. Growth kinetics of *N. gaditana* indoor and outdoor mass culture

The growth pattern of *N. gaditana* was monitored regularly in both culture systems. Variations in growth kinetics are shown in Table 2. The algal cells were started the exponential phase at first day onwards under both cultures. The indoor culture, stationary phase was started on day 9, while outdoors had on day 10 (Fig 5). Early stationary phase indoor culture biomass concentration was 1.48 g $L^{-1}d^{-1}$ while outdoor culture had 1.43 g L^{-1} d^{-1}. The highest specific growth rate (0.26 µm d^{-1}) was obtained under indoor condition while, specific growth rate 0.22 µm was found in outdoor condition. Outdoor culture gave comparable generation time (T_2) to that under indoor condition. The highest biomass yield (0.25 g L^{-1} d^{-1}) was obtained from indoor culture. The indoor culture, volumetric productivity was five times higher than outdoor cultivation. This suggests their potential to be cultured with 24 h continuous photoperiod. Quinn et al 2012 also obtained the similar volumetric biomass productivity under the outdoor culture of *Nannochloropsis* sp. with photo bioreactor.

Table 2. Growth pattern of *Nannochloropsis gaditana*

Growth kinetics	Indoor Culture	Outdoor Culture
µm(maximum growth rate/day)	0.199	0.3474
K (division per day)	0.2871	0.5049
T_2 (generation times)	3.4829	1.980
P_{max} (g l^{-1} d^{-1})	0.249	0.169
Maximum biomass (g l^{-1})	2.668 (10^{th}day)	1.843 (10^{th} day)
T_i (d)	0 day	0 day
T_f (d)	10	10
X_i (g l^{-1} d^{-1})	0.18	0.15
X_f (g l^{-1} d^{-1})	2.67	1.84

Fig. 5.Growth curves of *Nannocloropsis gaditana.*



3.3. Lipid and FAME yield under indoor and outdoor mass culture

Light is one of the major factors affecting storage product of algae. Scott et al 2010 reported that low light intensity reduce the lipid content. In this study maximum lipid (23 %) content was obtained under indoor condition with 24 ± 1 ^{o}C. A maximum lipid content and yield, 23 ± 1.42 % and 38.3 ± 3.56 mg $L^{-1}d^{-1}$, respectively, were obtained under indoor culture (Table 3). *N. Salina* lipid content was very similar with the results of Jon Van Wagenen et al 2012. Sukenik et al. (1989) reported that de novo lipid synthesis was partially responsible for the increased levels of TAGs in Nannocholoropsis sp. cultures under photoinhibitory irradiances. FAME content 16 % was obtained under indoor condition, this result also similar to Quinn et al 2012 results. Wang ZT 2009 was reported that 0.31 g l-1 d-1 was obtained with *N. gaditana*. This statement strongly supporting for our results that obtained under outdoor conditions. Selection was guided not only by productivity and lipid content of laboratory

cultures, but also by the strain robustness and capacity to dominate for relatively long periods in outdoor culture (data from previous studies).

Table 3. FAME yield, Lipid content and Productivity of *Nannochloropsis gaditana*

Parameters	Indoor Culture	Outdoor Culture
Lipid content (%)	23	19
Lipid productivity (mg l^{-1} d^{-1})	47.3	28.66
FAME yield (%) [a]	20	18
FAME yield (%) [b]	16	14

(a) Direct transestrification, (b) Two step transestrification process

3.4. FAME composition

FAME profiles are shown in Table 4. Fatty acid composition is very important considering factor for biodiesel preparation because it is strongly influenced in the properties of biodiesel. To investigate the suitability of oil for biodiesel production, the fatty acid profile of the species grown under indoor and outdoor conditions was analysed. The primary storage lipid in Nannochloropsis is triacylglycerol (TAG). (Robert E. Jinkerson, 2013). The simple and most abundant chain lengths being the fatty acid composition of N. gaditana is relatively simple, the most abundant chain lengths being 14:0, 16:0, 16:1, 18:1, 20:3(n-3) (ETE) and 20:5(n-3) (EPA), with 16:0 and 16:1 typically dominating. Robert E. Jinkerson, et al 2013 data was supporting with our data. FAME profile at indoor and outdoor culture was very similar, Eicosatrienoic acid (n-3, C20:3) 6.8 % was found in indoor culture while Eicosapentaenoic acid (n-3, C20:5) 5 % at outdoor condition. Many previous studies pointed out that the temperature could affect the FA content, with higher TFA content under lower temperature [Cao CH et al 2006] and low temperature induced the accumulation of PUFAs [Jiang H 2005]. In this study, we found that high temperature was more inductive for accumulation of FAs, with 24% more of TFA than that at low temperature, which is different from previous findings [Cao CH et al 2006].

Table 4: FAME profile of *Nannochloropsis gaditana*

Fatty acids	Indoor Culture	Outdoor Culture
C12:0	0.4	0.7
C14:0	6.5	9.4
C15:0	0.9	1.0
C16:0	58.9	55.6
C16:1	13.0	10.8
C17:0	4.5	3.6
C18:0	4.3	9.2
C18:1	4.4	4.4
C20:0	0.3	0.4
C20:3 (n-3)	6.8	-
C20:5	-	5

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Conclusion

Local microalgae species have a competitive advantage under the local geographical, climatic and ecological conditions. In this study focused on the biomass and lipid productivity as well as fatty acid profile with FAME yield. Locally isolated *N. gaditana* produced much higher biomass and lipid productivity under indoor cultivation compare than outdoor culture. This study confirmed that 24 h light intensity was suitable for the growth of *N. gaditana* and produce high biomass and lipid productivity. *N. gaditana* growth can be improved with a longer light period. This study shows that similar fatty acid profile under both culture conditions. The fatty acid profile is the major key for biodiesel production. Therefore, it is important to standardize the outdoor culture conditions for biomass and lipid production with economical viability. This isolate can be grown in open pond without any major contamination, but the relationship between growth and lipid yield should be understand the specific species before profitable biodiesel production.

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