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Growth and Lipid Production by *Desmodesmus subspicatus* and Potential of Lipids for Biodiesel Production

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ABSTRACT

The objective of this work was to study the growth, lipid production and lipid profile of *Desmodesmus subspicatus,* an isolate from Lahul Spiti, India under optimized culture conditions. Under N limitation, biomass production decreased while amount of lipids increased by 11% over control cultures (29% lipid content). The biomass of the organism increased by 10% and 9% under continuous illumination and in the culture medium with pH 6.4, respectively, while lipid content increased by 9% and 8%. The fatty acid profile of lipids of the organism under optimized conditions also changed. Under nitrogen limitation, saturated fatty acid (SFA) content was 47%, monounsaturated fatty (MUFA) were 30% and polyunsaturated fatty acids (PUFA) were 23%; under continuous illumination SFA were 47%, MUFA were 27% and PUFA were 13% compared to control cultures with 39% SFA, 23% MUFA and 38% PUFA. This indicated that fatty acid profile of lipids under optimized conditions was better from biodiesel production point of view.

1. Introduction

Use of the fossil fuels at large scale has resulted in global warming during the past few decades (Brunekreef and Holgate 2002) which necessitated search for renewable fuels as an alternative to the conventional energy sources (Wargack et al. 2012). First generation biofuels, which had attained economic levels of production, have been mainly obtained from food and oil crops which may create negative impact on global food markets and on food security. According to current knowledge, microalgal biofuels are at the forefront of the next generation of biofuel systems due to their high phototrophic productivity, shorter life cycle, higher nutrient absorption efficiency, fast growth rates (Venkat and Devi 2014), the potential for non-crop land cultivation (Wijffels et al., 2013), ability to trap greenhouse gases (CO_2) and recycle waste water and nutrients (Chen et al., 2015) and significant potential to convert sunlight into reduced carbon molecules, such as carbohydrates and lipids (Quiroz et al., 2015). Microalgae are a diverse group of unicellular or multicellular aquatic organisms with significant potential to produce valuable natural products. A variety of high value biofuels like methane, bio-hydrogen, bio-ethanol and bio-diesel are produced commercially (Harun et al., 2010; John et al., 2011). A number of algae such as *Chlamydomonas reinhardtii, Dunaliella salina, Chlorella* spp., *Botryococcus braunii, Phaeodactylum tricornutum* and *Thalassiosira pseudonana* have been reported as promising biofuel candidates (Scott et al., 2010). Microalgae are seen as a very attractive source of renewable biofuels, especially biodiesel.

In recent years, focus has turned to the production of cellular storage lipids from microalgae (namely triglycerides or TAGs) for the production of biodiesel as sustainable alternative to petroleum fossil fuels (Mata et al., 2010; Ahmad et al., 2011). Algae accumulate lipids such as triglycerides that contain fatty acids important for high value biodiesel production (Li et al., 2014). The lipid accumulation has been reported in several microalgae such as *Nannochloropsis*; Selvakumar and Umadevi, 2014), *Chlorella* (Liu et al., 2010; Herrera-Valencia et al., 2011; Sun et al., 2014), *Scenedesmus quadricauda* (Mohapatra, 2006**),** *Botryococcus* (Deng et al., 2009) *Scenedesmus obliquus* (Becker, 1994), *Chlamydomonas reinhardtii* (Scott et al., 2010), *Scenedesmus subspicatus* (Dean et al., 2012), *Phaeodactylum tricornutum*, *Isochrysis*, *Nannochloris* (Rodolfi et al., 2009).

However, the cost of microalgae production, low lipid yield and water demand still restrict their large scale exploitation. In that sense, the optimization of culture conditions is a key step to minimizing costs and to achieving desirable conditions to exploit the production of microalgae as a biodiesel feedstock (Picardo et al., 2013). Several strategies have been adopted to improve microalgal growth and lipid content. These include optimization of medium compositions e.g. type of carbon source, nitrogen, phosphorus etc. (Mata et al., 2010) and physical parameters e.g. temperature, pH, light etc. Studies on algal biofuels have shown that the

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quantity and quality of lipids within the algal cell biomass can vary as a result of changes in growth conditions (temperature, pH and light intensity) or nutrient media characteristics (concentration of nitrogen, iron and phosphates) (Liu et al., 2008). The accumulation of fatty acids can be controlled by environmental conditions such as temperature, nutrient availability and salinity (Rodolfi et al., 2009; Singh and Dhar, 2011). Screening and selection of microalgae producing high amounts of neutral lipids is very crucial for the commercial success of algae-based biofuel production. Although naturally occurring algal strains have been screened but the criteria for the selection of algal strains as a feedstock for biofuel production have not been well defined (Rodolfi et al., 2009).The present study was focused on optimization of culture conditions not only to increase biomass and lipid productivity but also to get better fatty acids composition from a green microalga *Desmodesmus subspicatus*. Attempt was made to optimize 10 parameters; dry cell weight, net lipid productivity, lipid content (% of Dry Cell Waight), number of fatty acids in lipids, content of C16-C18 fatty acids of total lipid (TLC), content of C=C ≥ 4 fatty acids, content of linolenic acid, content of saturated, monounsaturated and polyunsaturated fatty acids in lipids of test organism.

2. Material and Methods

2.1. Isolation, purification and cultural conditions

The isolate CLS50 was isolated from fresh water source of Koksar village of Lahul Spiti (32º 24' 16.59" N; 77º 15' 49.25" E) of cold desert area of Himachal Pradesh, India. Isolation and purification were performed on BG-11 medium (Rippka et al., 1979) by serial dilution (Stanier 1971). The BG-11 medium contained (g L^{-1}): Na_2CO_3 (0.02), $NaNO_3$ (1.5), EDTA (0.001), Ferric ammonium citrate (0.006), Citric acid (0.006), $CaCl_2.2H_2O$ (0.036), $MgSO_4.7H_2O$ (0.075), K_2HPO_4 (0.04), H_3BO_3 (2.86), $MnCl_2.4H_2O$ (1.81), $ZnSO_4 7H_2O$ (0.22), $CuSO_4.5H_2O$ (0.079), $CoCl_2.6H_2O$ (0.049), $NaMoO_4.2H_2O$ (0.39) Na_2CO_3 (0.02) with pH adjusted to 7.4. The cultures were incubated in a culture room at 28 °C ±2 °C under 44.5 µmol photon flux density m^{-2} s^{-1} at the surface of culture vessels and illuminated for 14 h a day.

2.2. Identification of green algal isolate

The isolate was identified on morphological as well as partial 18S rRNA gene sequence basis. Purified green algal strain was identified based on characteristics given in monographs by Philipose (1967), Parsad and Misra (1992). Identification was done on the basis of colony morphology, number of cells in a colony, cell size, dimensions, chloroplast number, position and shape etc. The cell pellet obtained after centrifugation was suspended in 500 µL of DNA extraction buffer (200 mM Tris HCl, pH 8.5, 250 mM NaCl; 25 mM EDTA, 0.5% SDS) at 60 ºC in a water bath for 90 minutes and genomic DNA was extracted with 350 µL of ice cold phenol: chloroform: isoamyl alcohol (25:24:1 ratio) (Moncalvo et al., 1995). Extracted DNA was precipitated by adding 250 µL of cold isopropanol and centrifuged. The nucleic acid pellet was washed with 70% ethanol, air dried, resuspended in 50 µL of TE buffer (10 mM Tris HCl, pH 8.0 and 1 mM EDTA) and stored at -20 ºC. 18S rRNA gene fragment of 960 bp of the isolate was amplified by using primers: 18SU 467F 52 - ATCCAAGGAAGGCAGCAGGC -32 and 18SU 1310R 52 - CTCCACCAACTAAGAACGGC -32 (Matsunaga et al. 2009). Total PCR reaction mixture was comprised of 2X buffer mixture, 10 µM each of forward and reverse primer, and 50 ng template DNA. Gene amplification was done by an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C and final extension at 72 °C for 8 min. The gel-purified product was obtained using QIA quick PCR purification kit (Qiagen GmBh, Germany). The gene sequencing was done by using AB1310 automatic DNA sequencer (Applied Biosystem, USA). The sequence was analyzed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov/Blast) search algorithm and aligned to the near neighbours. The phylogenetic tree was constructed using MEGA5.05 software package (Tamura et al., 2011).

2.3. Optimization of conditions for growth, lipid production and fatty acid composition

The exponentially growing stock cultures, after two washings with sterilized double distilled water, were inoculated in 100 mL basal medium in 250 mL Erlenmeyer flasks to attain 0.1 absorbance at 720 nm. The cultures were incubated in the culture room and a known volume of culture was withdrawn at the desired time, washed with distilled water and centrifuged at 5,000 g for 15 min and suspended in distilled water to make a known volume. Absorbance at 720 nm, dry cell weight (DCW) and chlorophyll content of the washed cultures were determined. Chlorophyll was extracted with methanol at 60 °C following Jeffrey and Humphrey (1975). Pelleted biomass was oven dried at 70 °C for 24 h to determine its DCW. The effect of nitrogen limitation on growth and lipid content was studied by decreasing concentration of $NaNO_3$ from 17.6 mM to 2 mM through 8 mM and 4 mM. The effect of pH was studied by adjusting pH of medium at 6.4, 7.4, 8.4 and 9.4. The effect of continuous light was studied by incubating the experimental cultures under continuous light without any dark period.

2.4. Lipid Extraction

Total lipids were extracted from the biomass by slightly modifying the method of Bligh and Dyer (1959). The biomass was separated from the medium and washed by centrifugation at 5,000 g for 15 min. The harvested biomass was freeze dried under vaccum at -70 °C. The freeze dried biomass was grounded in a mortar pestle, suspended in methanol and subjected to mild sonication. Then lipids were extracted with a mixture of chloroform:methanol:water (2:1:0.8 v/v). The organic layer was collected, the process was repeated twice and solvent evaporated in a rotary evaporator. The amount of lipids was determined gravimetrically.

2.5. Saponification

A known amount of the lipids (10 mg) was refluxed in a water bath at 70 °C with 50 mL alkaline methanol solution. After cooling the contents, 12 mL distilled water was added. The solution was transferred to a separating funnel and fatty acids were extracted by giving three washings with diethyl ether. Organic layer was collected and solvent was evaporated in a rotatory evaporator.

2.6. Fatty Acids Composition of Lipids

The fatty acid composition of saponified lipids was determined through HPLC (Waters, USA) with refractive index detector using C18W reversed-phase column (4.6 mm×250 mm ID, 5 µm particle size) and pure methanol (Sigma) as mobile phase. The sample was prepared by dissolving fatty acids in methanol to a final concentration of 1 mg mL^{-1}. Twenty microlitre samples were injected into the column and chromatographs obtained. The flow rate of mobile phase was 0.5 mL min^{-1} and the column temperature was maintained at 40 °C. Fatty acids were identified by comparing chromatograms with standards. Fatty acids were quantified by integrating HPLC chromatogram using external standard method.

2.7. Physical properties of lipids

Density of the lipids was measured with a pycnometer. The density of the lipids was calculated according to equation:

$$p = \frac{M_L/V_L}{M_W/V_W}$$

(Mw/M_L = mass or weight of the water/Lipid, Vw/V_L=volume of water/lipid).

Density was expressed in g per cubic meter (g m^{-3}).

The viscosity of the lipids was determined by a U-shaped glass capillary viscometer at 40 °C. The viscosity of the lipids was calculated by following equation:

$\eta_L = d_L t_L / d_w t_w \times \eta_w$

(dL= density of lipids, dw= density of distlled water, tL= flow rate of lipids, tw= flow rate of distilled water ηL= viscosity of lipids, ηw = viscosity of water**)**

2.8. Statistical Analysis

All experiments were performed thrice with three samples in each experiment and the results are expressed as mean value ± SD.

3. Results and Discussion

The isolate is being maintained in our culture collection with PUMCC 3.1.8 code. The cells of the organism are cylindrical and linearly arranged in 4- celled flat coenobium, cell wall is with spines which originate from outer cell wall of lateral sides of the coenobium. Single parietal chloroplast with pyrenoid was present in each cell. Cells are 2-9 µm long and 6-12 µm broad with 6.5-8 µm long spines. The organism belongs to order Chlorococcales and on morphological basis was identified as *Desmodesmus subspicatus.* The morphology based identity of the organism was confirmed and its phylogenetic relationship was determined by 18S rRNA gene sequence analysis. The phylogenetic tree generated by aligning 18S rRNA gene sequence of the test strain with sequences of the microalgal strains obtained from NCBI GenBank showed that strain *D. subspicatus* PUMCC

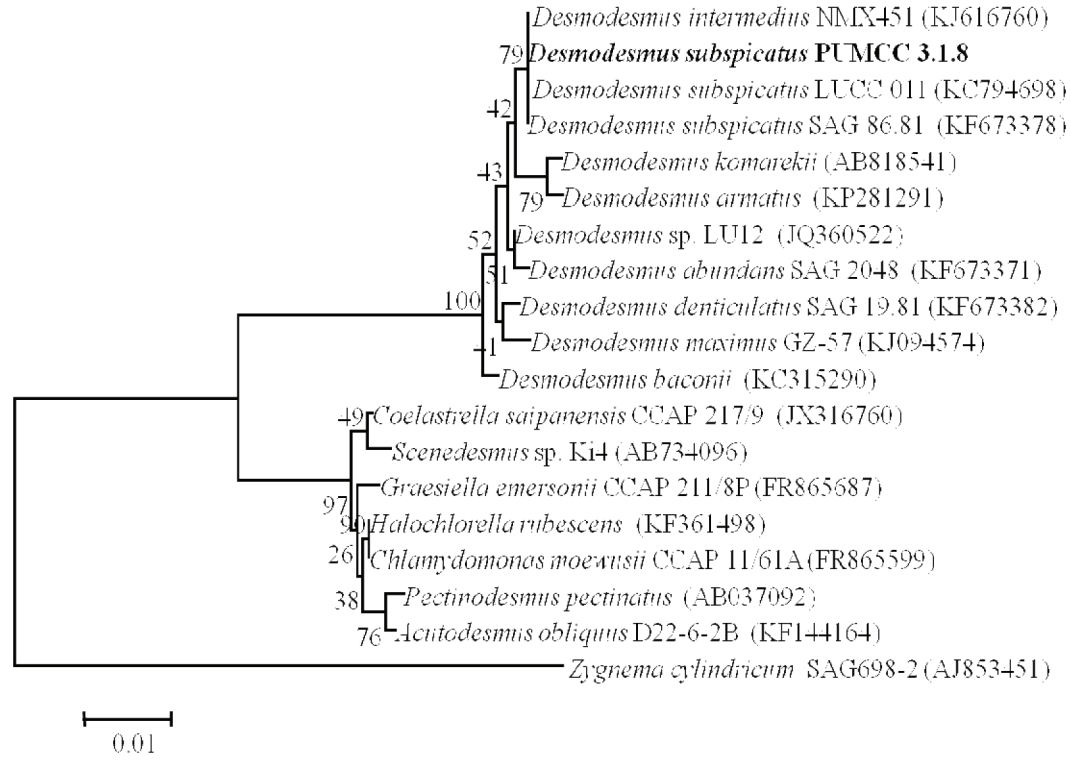

Fig. 1. Phylogenetic tree showing relationship of *D. subspicatus* PUMCC 3.1.8 with closely related taxa based on partial 18S rRNA gene sequence

3.1.8 showed 100% similarity with respective *D. subspicatus* strains studied by other workers (Fig. 1). The nucleotide sequence obtained during the present study has been deposited in NCBI GenBank database with accession number KT151950.

Environmental conditions such as temperature, nutrient availability and salinity regulate growth and accumulation of fatty acids in microalgae (Singh and Dhar, 2011). To investigate the influence of nutritional and physical factors on growth, lipid production as well as on fatty acid composition of lipids, the test strain was subjected to altered nutritional and physical conditions. The selection of algal strains for the purpose of oil production must not be based only on the lipid content but biomass generation capacity should also be considered so as to define the net oil yield or productivity (Provost et al., 2011).

Although biomass productivity of the test strain decreased with decrease in nitrate concentration in the medium but its lipid content increased. When nitrate concentration in medium was decreased to 4 mM, biomass production decreased to 1.8 mg ml^{-1} compared to 2.58 mg ml^{-1} biomass production in control cultures but the amount of lipids increased by 11% over control cultures (29% lipid content) (Fig. 2a, 2b). When net lipid productivity of cultures was taken into account, 4 mM $NaNO_3$ was the optimum nitrate concentration. The relationship between lipid content and day growth rate in microalgae was found to be inversely related in nitrogen deficient condition (Rodolfi et al., 2009; Abomohra et al*.,* 2013). Lipid is one of the biochemical products that microalgae can produce along with starch under nutrient deficient condition (Bartley et al., 2013). This trend of lipid accumulation in response to nitrogen deficiency has been observed in several species or strains of microalgae, including *Botryococcus* sp. (Yeesang and Cheirsilp, 2011), *Chlamydomonas reinhardtii* (Dean et al., 2010), *Chlorella vulgaris*, *Chlorella zofingiensis* (Liu et al., 2010), *Haematococcus pluvialis* (Damiani et al., 2010), *Neochloris oleabundans*, *Parietochloris incisa* (Solovchenko et al., 2010), *Pseudochlorococcum* sp. (Li et al., 2011), *Scenedesmus obliquus* (Mandal and Mallick, 2009), *Scenedesmus rubescens* (Mandal and Mallick, 2009), *Tetraselmis suecica* (Rodolfi et al., 2009), *Chlamydomonas reinhardtii*, *Scenedesmus subspicatus* (Dean et al., 2012), *Nannochloropsis oculata*, *Nannochloropsis* sp. (Rodolfi et al., 2009).

Microalgal growth also depends on pH of the medium as it influenced the nutrient availability, metabolism and biochemical composition of cells (Bajhaiya et al. 2010). pH of the medium also affected the growth and lipid content of the test organism, When pH of the medium was decreased by one unit from 7.4 (control) to 6.4, biomass as well as lipid content of the test organism increased by 9% and 10%, respectively, over control culture (Fig. 3a, 3b). Results further revealed that pH of medium above 8.4 and below 6.4 did not support good growth of the organism.

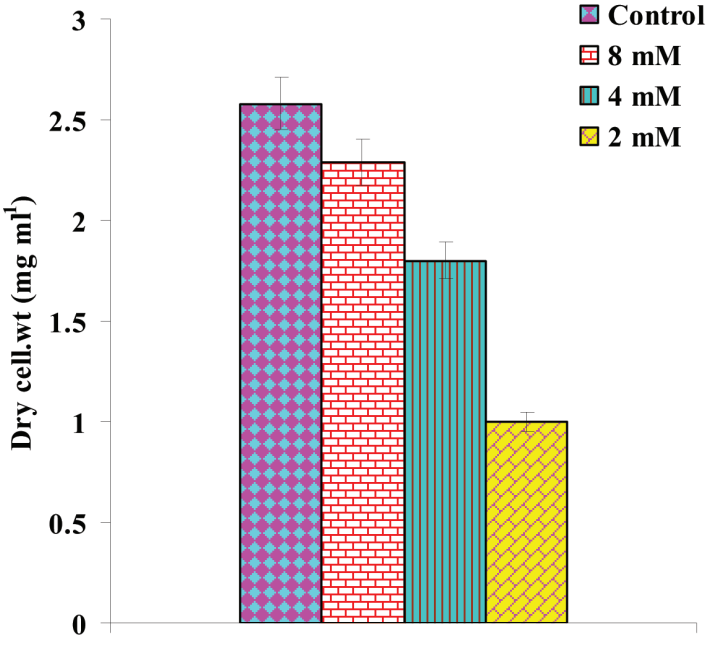

Fig. 2a. Growth of *D. subspicatus* PUMCC 3.1.8 on 24 d in the different concentration of $NaNO_3$ in terms of dry cell weight of cultures

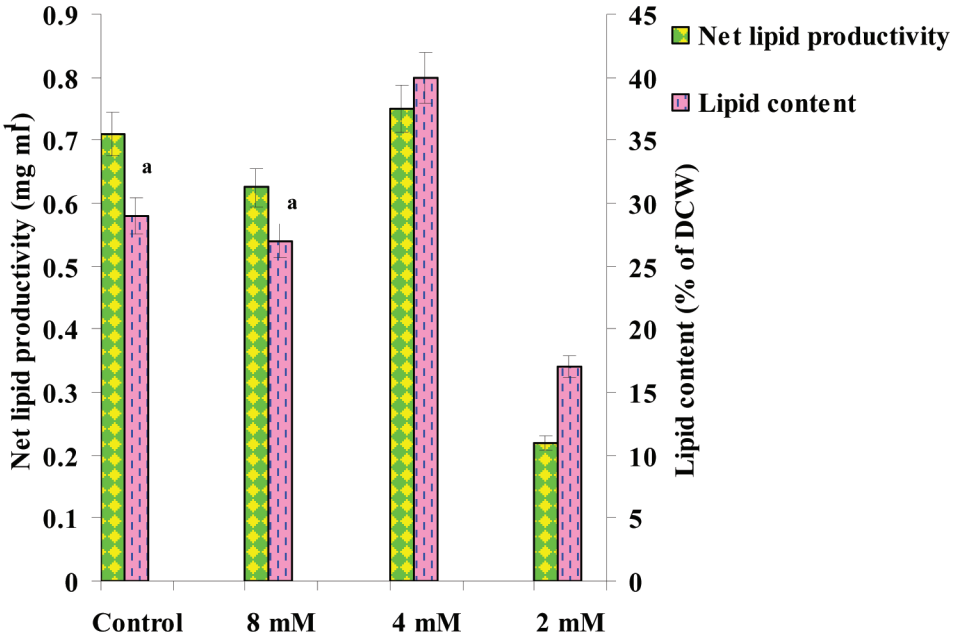

Fig. 2b. Net lipid productivity and lipid content of *D. subspicatus* PUMCC 3.1.8 on 24 d in different concentration of $NaNO_3$
Data on same day with same lower case alphabets are not significantly different from one another at 95% confidence level (P_{cal}<0.025)

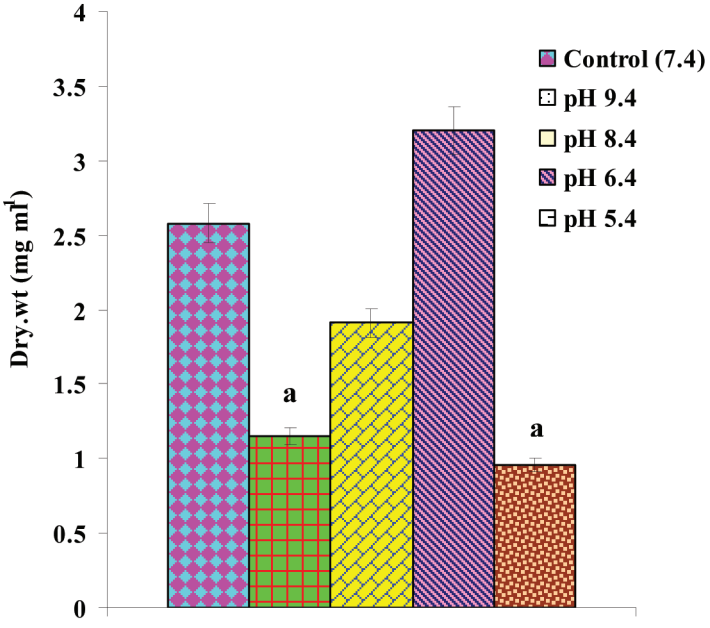

Fig. 3a. Growth of *D. subspicatus* PUMCC 3.1.8 in medium with different pH on 24 d in terms of increase in dry cell weight of cultures
Data with same lower case alphabets are not significantly different from one another at 95% confidence level ($P_{cal<0.025}$)

Medium with pH 6.4 was observed to be the optimum for net productivity of lipids by the cultures of *D. subspicatus.* Higher biomass productivity as well as lipid content in *Chlorella*, *Oedogonium*, *Spirogyra* and *S. obliquus* was observed within pH range of 7.0 to 8.0 (Munir et al., 2015).

Light is an important factor for biochemical composition of microalgae (Fabregas et al., 2002). Different species of microalgae respond differently to irradiance and photoperiod. Under continuous illumination of light, biomass production of the test organism was 3.42 mg ml^{-1} -compared to 2.58 mg biomass ml^{-1} of control cultures. Net lipid productivity of the cultures incubated under continuous light increased to 1.54 mg ml^{-1} from 1.19 mg ml^{-1} of cultures incubated under 14:10 h light:dark cycle while lipid content increased by 11% as compared to control. Thus incubation of cultures under continuous light resulted in more net lipid productivity. Although reports on effect of light intensity on biomass as well as lipid productivity of microalgae are available (Cheirsilip and Tropee, 2012), few studies have reported effect of duration of light on lipid productivity in microalgae. Selvakumar and Umadevi (2014) reported that 24 h illumination with 300 µmol m^{-2} s^{-1} light intensity resulted in high biomass and lipid production in *Nannochloropsis gaditana*. Izabela et al. (2014) reported that biomass productivity in cultures of *Bortyococcus braunii* was more under continuous illumination in comparison to 12L:12D cycle. Culture of *Scenedesmus* sp. AARL G022 produced more biomass but lesser lipid content under mixotrophic condition with a light-dark cycle of 16:8 hr using 0.05 M glucose in comparison to control condition where light-dark cycle was of 12:12 hr and 1.0 M of glucose (Doungpen et al., 2014).

Studies on algal biofuels have shown that the quantity and quality of lipids within the algal biomass varies as a result of changes in growth conditions (temperature, pH and light intensity) or nutrient media characteristics (concentration of nitrogen, iron and phosphorus) (Liu et al., 2008). Along with biomass and lipid productivity, fatty acid composition of lipids of the test organism grown under optimized conditions of nitrate concentration, pH of the medium and continuous light was also studied. Composition of lipids in the test organism grown under particular optimized condition showed variations in composition of all classes of fatty acid compared to control condition. The content of saturated fatty acids and monounsaturated fatty acids in all the optimized conditions increased while content of polyunsaturated fatty acids decreased. The content of saturated and monounsaturated fatty acids in cultures grown in medium with 4 mM NO_3 increased to 47% of total lipid content (TLC) and 30% of TLC, respectively, from 38.9% saturated and 22.6% monounsaturated fatty acids in lipids of control cultures. Most of algal oils possess high amounts of polyunsaturated fatty acids with four or five double bonds which are not desirable from biodiesel point of view (Damini et al., 2010). In the present study, the content of fatty acids with C=C ≥ 4 was very low. Cultures grown in medium with 4 mM NO_3 produced 11% C18:3 fatty acids of TLC and 1% C20:4 fatty acids of TLC which were under the limits of EN1214 standard. Battah et al. (2014) reported

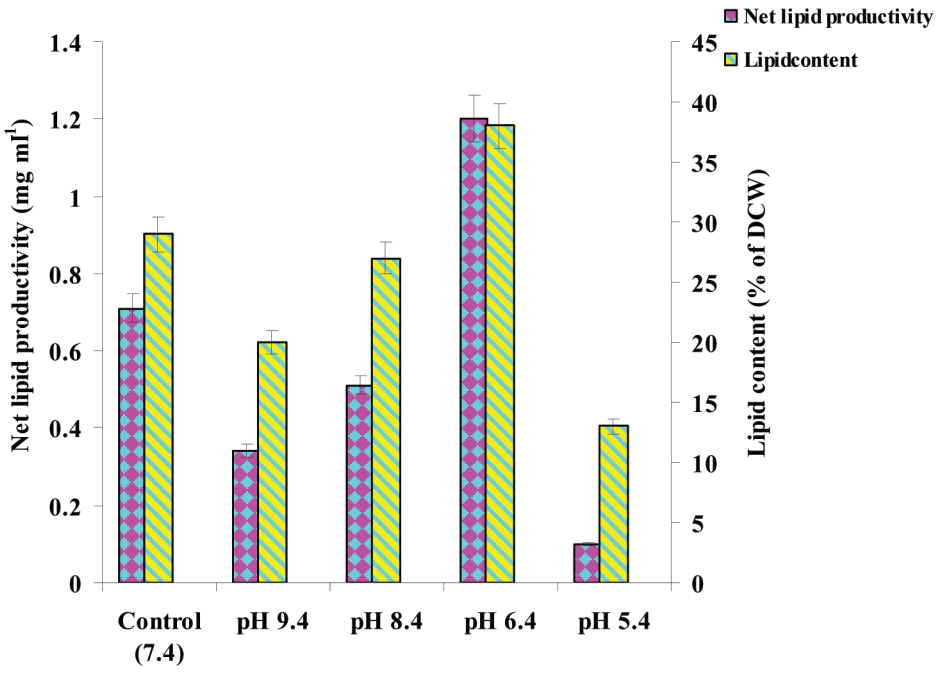

Fig. 3b. Net lipid productivity and lipid content of *D. subspicatus* PUMCC 3.1.8 on 24 d in medium with different pH

increase in content of fatty acids with chain length C14-C18 in *C. vulgaris* under nitrogen limitation of 0.1 mM, high salinity of 0.45 mM and 35.25 µM of ferrous sulphate. In the present study, content of C16-C18 fatty acids in lipids of cultures grown in 4 mM as well as of control culture did not vary significantly (Table 1).

In cultures grown in continuous light, content of saturated fatty acids and monounsaturated fatty acids were increased to 57% of TLC and 29% of TLC from 38.9% and 22.6% respectively of TLC of cultures under 14:10 h light and dark regime. Polyunsaturated fatty acids decreased to 14% of TLC from 38.5% polyunsaturated fatty acids in control culture. Among polyunsaturated fatty acids, the content of C18:3 and C20:4 fatty acids were 12% and 1% of TLC, respectively. The content of fatty acids with chain length C16-C18 was maximum (72%) in cultures incubated in continuous light (Table 1).

Lipids of the cultures grown in medium with pH 6.4 contained 67% C16-C18 fatty acids. Saturated, monounsaturated and polyunsaturated fatty acids in lipids of cultures with pH 6.4 were 50%, 21% and 29% of TLC, respectively, compared to 38.9%, 22.6% and 38.5% of TLC, respectively, in control cultures (Table 1).

Lipids with maximum content of saturated fatty acid and also fatty acids with C16-C18 chain length are highly evaluated for biodiesel production. *D. subspicatus* produced maximum amount of saturated fatty acids (57% of TLC) and fatty acids with C16-C18 (72% of TLC) when incubated under continuous light. Typically, the amount of polyunsaturated fatty acids (structural lipids) increases under low light conditions, whereas high light promotes the accumulation of saturated and mono-unsaturated fatty acids (storage lipids) (Khotimchenko and Yakovleva, 2005). Elumalai et al. (2011) reported that *C. vulgaris* accumulated high level of saturated fatty acids in dark. Combined effect of pH of the medium and intensity of light resulted in increased content of saturated fatty acids in *S. obliquus* .

Density and viscosity of lipids of test organism were also studied. Density and viscosity of lipids of test organism obtained under optimized conditions were within the range prescribed by EN1214 standard for biodiesel (Density= 0.86-0.90 g m^{-3}, viscosity= 3-5 mm^{-2} s^{-1}). Density and viscosity of biodiesel is mainly affected by length of carbon chain and number of double bonds in lipids from which biodiesel is obtained (Mittelbach et al., 2004). Lipids of the test strain had maximum content of fatty acids with medium chain length i.e., C16-C18. The viscosity of oil gets lowered with increase in unsaturated fatty acids. Although the content of unsaturated fatty acids in test organism was slightly decreased under optimized conditions, even then viscosity of lipid was in the range of EN1214 limits. High viscosity of biodiesel causes resistances to the injection pump of engines of vehicles (Mittelbach et al., 2004).

So the best condition for the good quality and high quantity of lipids in *D. subspicatus* was continuous light. Generally, only total lipid content in a particular microalgal species has been considered as an important parameter for the selection of algal strain for biodiesel production, but results obtained from the present study suggested that to determine the suitability of algal biomass as feedstock for lipid based algal biofuel; biomass yield, lipid content, its fatty acid composition should be criteria for selection of a particular microalgal strain.

Table 1. Fatty acid composition of *D. subspicatus* PUMCC 3.1.8 under optimized conditions

Optimized Condition	No. of fatty acid	SFA (% of TLC)	MUFA (% of TLC)	PUFA (% of TLC)	% of C16-C18	Linolenic acid (% of TLC)	% C = C ≥4
Control	9	38.9±1.9	22.6±1.1	38.5±1.9	63±3.1	0	0
4 mM NO_3	9	47±2.3	30±1.5	23±1.1	67±3.3	11±0.6	1±0.09
pH 6.4	9	50±2.3	21±1	29±1.3	67±3.3	8±0.4	1±0.09
Continuous light	9	57±2.3	20±1.4	23±1.3	72±3.4	12±0.6	1±0.09

Conclusions

Ten important parameters were standardized to determine optimized condition for the potential use of *Desmodesmus subspicatus* as algal feedstock for biodiesel production and were successfully used for evaluating the potential of the organism as biofuel feedstock. The present study provided important information regarding the screening strategy as well as fatty acid profile during different optimized conditions so that a particular condition can be selected for better quantity and quality of lipids. *Desmodesmus subspicatus* is a potential candidate for biofuel production at large scale when grown under continuous light where both the quantity as well as quality of lipids was better as compared to other culture conditions. So to obtain maximum net lipid productivity with better fatty acid composition from microalgal cultures, it is recommended that maximum biomass may be obtained under condition optimum for growth and then cultures may be shifted to a condition where high quantity of lipids with good quality are obtained.

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