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# Growth and biochemical response of an indigenous oleaginous microalga *Scenedesmus obtusus* cultivated in outdoor open ponds

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Understanding the response of microalgae to outdoor culture conditions is necessary for the development of large open pond cultivation system for various value added applications. In this context, we evaluated the response of an indigenous oleaginous green microalga *Scenedesmus obtusus* CFR 1-09/FW to outdoor culture conditions. The microalga was cultivated in open ponds at various culture depths under nutrient replete condition. The pond with 3 cm culture depth showed highest biomass productivity (49.05±11.74 mg $L^{-1}$ $day^{-1}$). The high surface solar irradiance (1831 µmol $m^{-2}$ $s^{-1}$) led to a decrease in chlorophyll content (from 12.21 to 4 µg $mg^{-1}$). The long duration exposure to lower temperatures (≤20°C) during night led to an increase in poly unsaturated fatty acids (PUFAs) content (47.21±2.83% w/w mass fraction of FAME). The omega-3 alpha linolenic acid (ALA) content rose significantly reaching 31.01±3.79% (w/w) mass fraction of FAME. The high content of carbohydrate (23.4±0.64% w/w), protein (37.62 ± 2.15% w/w), lipid (21.55±1.43% w/w), palmitic acid (30.97±4.02% w/w mass fraction of FAME) and ALA in outdoor cultures makes this microalga a potential candidate for outdoor cultivation for food and feed applications. The study provides valuable insights for developing outdoor open pond cultivation protocol.

**Keywords:** Alpha linolenic acid (ALA), Biomass productivity, Culture depth, Culture temperature, Surface irradiance

Microalgae have found application as food, feed, an important source of high value products *viz.*, carotenoids, pigments, polyunsaturated fatty acids (PUFA), nutraceuticals, functional foods, etc.[1,2]. Microalgae have also been recognized for their $CO_2$ sequestration potential and the ability to utilize wastewater for growth[3,4]. The microalgae with high lipid or carbohydrate content could be suitable feedstock for biofuel (biodiesel and bioethanol) production[5,6]. The oleaginous microalgae could also be a potential source of omega-3 polyunsaturated fatty acids (n-3 PUFAs). The omega-3 PUFAs are known to impart several health benefits, such as protection against cardiovascular diseases, age-related cognition disorders and inflammation[7]. The fresh water microalgae could be an important source of alpha linolenic acid (ALA), an omega-3 fatty acid[8]. The microalgae derived PUFA rich oil has the potential to be an alternative to fish oil, the main source of PUFAs, and its 'vegetable' nature has been highlighted as a potential marketing differentiator[2,8].

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The systems used for large-scale photoautotrophic cultivation of microalgae range from outdoor open ponds to closed engineered photo bioreactors (PBRs)[9]. Open outdoor cultivation systems are preferred for commercial cultivation of microalgae as these are less expensive to build and operate, and are more durable than PBRs[9]. The outdoor open ponds are widely used for cultivation of *Spirulina*, *Chlorella* and *Dunaliella* sp[2]. It is estimated that approximately 5000-6000 tonnes/annum dry biomass of microalgae is produced worldwide mainly through open pond cultivation[2,10].

The outdoor cultures of microalgae are subjected to the seasonal and diurnal variation of light and temperatures. The availability and distribution of light inside the cultures and culture temperature are considered as the most important factors determining microalgal growth and productivity[9,11]. Light distribution inside microalgal cultures is a function of culture depth where light gets attenuated with increasing culture depth[11]. In open ponds, parameters, such as cell density, turbulence and dilution cycle also affect the efficiency of light utilisation by algal cells[12].

It is important to explore the microalgal biodiversity and select the candidate microalgae for

various desired applications. The indigenous microalgae may be more robust candidate for outdoor cultivation in open ponds as they offer the advantage of adaptation to the natural environment of their habitat[13]. In an attempt towards this objective, we established a culture repository of indigenous freshwater microalgal isolates from various water bodies of India at the CSIR-CFTRI, Mysuru, India. These indigenous microalgal cultures have been characterized for their growth; lipid content, hydrocarbon content and fatty acid profile[8]. The indigenous freshwater isolate, *Scenedesmus obtusus* CFR 1-09/FW, having a high lipid content (32.8±3.69% w/w), a high specific growth rate and short doubling time[8] is a potential candidate for large-scale cultivation for food, feed and fuel related applications and hence was selected for the present study. To be precise, we tried to understand the effect of seasonal and diurnal variations of light and temperature on the growth and metabolite profile (fatty acids, chlorophyll, carotenoids, carbohydrate and protein) of the microalga. The microalga was cultivated in outdoor open ponds maintained at various culture depths, exposed to natural sunlight with intermittent manual mixing.

## Material and Methods

### Organism and culture media

The freshwater microalga *Scenedesmus obtusus* CFR 1-09/FW was used in the present study. The details of the microalga, including ITS-2 gene sequencing (NCBI Sequence No. KJ680145) have been reported earlier[8]. The morphological details of the microalga are provided in Fig 1. The microalgal culture was maintained in Bold's Basal Medium (BBM) having 1.5 g $L^{-1}$ $NaNO_3$. The same medium was used for outdoor cultivation of microalga.

### Outdoor cultivation

The outdoor cultivation studies with microalga were performed in open circular ponds of 40 cm diameter, 30 cm depth with a holding capacity of 33 L. Four ponds were operated at four different culture depths (3, 6, 9 and 12 cm) with working volumes of around 3.83, 7.22, 11 and 14.37 L, respectively. The outdoor cultivation was conducted for one month during April to May, 2015. The initial biomass concentration of the microalga was maintained around 43.1±3.5 mg $L^{-1}$. The cultures were allowed to grow under natural climatic conditions. On every fifth day, a predetermined volume of culture was withdrawn from each pond and fresh media was added to bring the biomass concentration to the initial value and to avoid any nutrient limitation. The requisite volume of water was added every morning to make up for the evaporation losses to maintain the predetermined culture volumes (culture depths). Incidence solar irradiance on culture surface was measured five times a day at 9:30 a.m., 11:30 a.m., 2:00 p.m., 3:30 p.m., and 5:30 p.m. (Indian Standard Time GMT+5:30 hours). Daily culture temperature variations and dissolved oxygen (DO) levels were monitored thrice a day; morning (9:30 am), afternoon (1:30 pm) and evening hours (5:30 pm) using a DO meter (Eutech Instruments, CyberScan PCD 650, Thermo Fisher, Singapore). The culture pH was recorded twice a day, morning (9:30 am) and evening (5:30 pm) with a pH meter (Eutech Instruments, pH Tutor, Thermo Fisher, Singapore). The cultures were mixed manually once every hour during the daylight period. The night temperatures of cultures were recorded every fifth day during the period of the study.

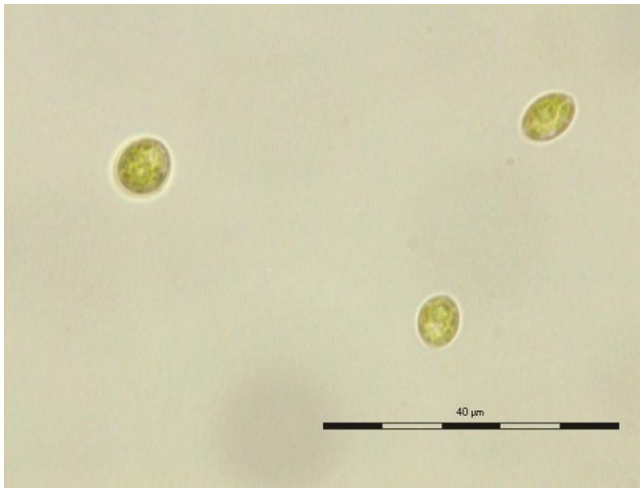


Fig. 1 — Photomicrograph of the microalgal isolate *Scenedesmus obtusus* CFR 1-09/FW

### Measurement of growth, productivity and pigments

The growth of microalga was monitored daily by measuring the optical density (O.D.) of the culture aliquots at 560 nm through a spectrophotometer (UV Spectrophotometer, UV-1800, Shimadzu Corp.)[14]. A relationship between optical density and biomass concentration (g $L^{-1}$) was determined by a calibration curve, and relative biomass concentration was calculated based on the weight of freeze-dried biomass. The following relationship between $O.D._{560}$ and biomass concentration was obtained via calibration curve

($R^2$ = 0.98); Biomass (g $L^{-1}$) = 0.8253 × $O.D._{560}$ – 0.08859

From above correlation, it was found that 1 O.D. was equivalent to 0.737 g $L^{-1}$ of biomass. The specific growth rate (μ) and doubling time (D) of the microalga were calculated[14]. On every fifth day, a predetermined volume of culture was withdrawn from ponds, and centrifuged at 5000 rpm for 5 min (model Sorvall Legend X1R, Thermo Scientific, Osterode, Germany) to obtain the biomass. The obtained biomass was washed with deionised water and freeze dried (Cool safe 55-4 pro, Scanvac, Denmark). The freeze dried biomass was used for further analysis. For analysis of pigments, the culture aliquots were centrifuged, and obtained pellets were extracted with acetone (HPLC grade) for spectrophotometric estimation of chlorophyll and carotenoid using Lichtenthaler's equations[15]. The biomass samples were prepared for lutein extraction[16] and estimation of lutein was done by HPLC using the mobile phase of acetonitrile, dichloromethane and methanol (70:20:10 v/v/v). The pigments were identified by comparing their retention times with respective standard[16].

#### Estimation of total carbohydrates and protein

Total carbohydrate content was determined using phenol-sulphuric acid method[17]. The nitrogen content of biomass was estimated using CHNS elemental analyser (EL-III, Vario, Germany). Protein content was calculated by multiplying the nitrogen content with the conversion factor of 6.25[18].

#### Lipid extraction and fatty acid methyl ester (FAME) analysis

The lipid extraction from freeze dried biomass (100 mg) was carried out with chloroform: methanol (2:1) solvent mixture[14]. Lipids were quantified gravimetrically and lipid content of biomass was expressed as the percentage on dry weight basis. The crude lipid extracts were converted to fatty acid methyl ester (FAME) and were analysed by GC[14]. The FAMEs were identified by comparing their retention times with standard FAME mixture comprising authentic standards (C-8–C-24 FAME mix, Supelco). The fatty acid composition was expressed as relative percentage mass fraction of total FAME.

#### Estimation of moisture, ash and mineral content

The moisture content of biomass was determined by oven drying 1 g of biomass at 110°C to a constant weight. Total ash was determined by calcination at 550°C until constant weight. The ash was used for the estimation of mineral elements (calcium, magnesium, sodium, potassium, iron, zinc, and copper) by AOAC procedure[19] using Atomic absorption spectroscopy (AAS) (iCE 3000AA, ThermoScientific, USA). The concentrations of the elements were determined from their respective standard calibration solutions.

#### Statistical analysis

All the biochemical determinations were performed in triplicates and the results were expressed as mean±SD of five replicates. The difference between the groups were statistically analyzed by using one way ANOVA followed by Tukey-Kramer multiple comparison test at significance level of $P$ <0.05.

## Results

#### Physicochemical parameters, growth, biomass concentration and volumetric productivity of outdoor cultures

During the period of the study, the prevailing weather of Mysuru was characterized by high average rainfall (7.14 mm) and humidity (85.40%)[20]. The maximum and minimum incident solar irradiance on culture surface is presented in Fig. 2. The maximum incident solar irradiance on the culture surfaces were recorded at 11.30 a.m. and values ranged from 226 to 1831 μmol $m^{-2}s^{-1}$. For minimum incidence solar irradiance on culture surfaces, the values recorded at 3.30 p.m. were considered and were in the range of 16 to 227 μmol $m^{-2}s^{-1}$. As the values recorded at 5.30 p. m. were extremely low (between 2 and 15 μmol $m^{-2}s^{-1}$), these values were not considered. The present study suggests that microalgal cultures in open outdoor ponds experience wide diurnal fluctuations in temperatures. The average ambient morning, afternoon and evening temperatures were 25, 30 and 27°C, respectively. The outdoor daytime temperature fluctuations were recorded as shown in Fig. 3A. The night time temperatures were also recorded periodically on every fifth day during the period of the study. The temperature profile of the

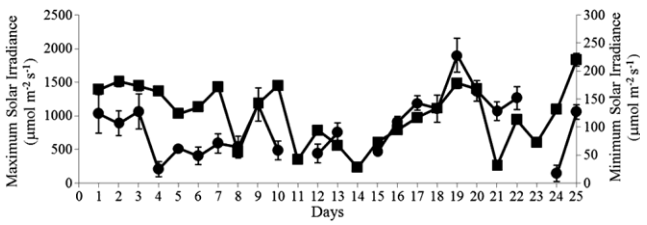


Fig. 2 — Daily solar irradiance measured over 25 day period (■ maximum solar irradiance, ● minimum solar irradiance). [*Minimum solar irradiance for 11th and 23rd day was not recorded due to rainfall]

culture over a period of 24 h showing the diurnal fluctuations is shown in Fig. 3B. The daily diurnal temperatures ranged from an average of 30°C (during afternoon) to 18°C (past midnight) over the period of study.

The dissolved oxygen (DO) concentration in the outdoor cultures ranged between 13.75 and 20.26 mg $L^{-1}$ (Fig. 4). The DO concentrations reached their peak values during afternoon in ponds with 6, 9 and 12 cm culture depths, correlating with the pattern of light. However, during the entire study period, the highest DO levels for ponds with thin layer culture (3 cm culture depth), were recorded during morning hours. The culture pH was observed to rise from initial values of 9.2±0.2 to 10.3±0.3 with growth. The pH was least during morning hours and daily maxima were observed during evening hours (Fig. 5A).

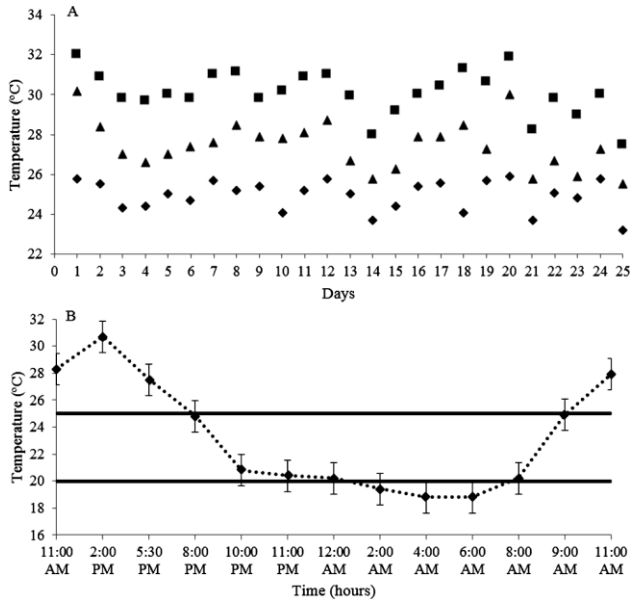


Fig. 3 — (A) Daily temperature (°C) measured over 25 days (◆ daily morning temperature, ■ daily afternoon temperature, ▲ daily evening temperature); (B) Average temperature profile of the culture over a period of 24 hours during the period of study (····· culture temperature)

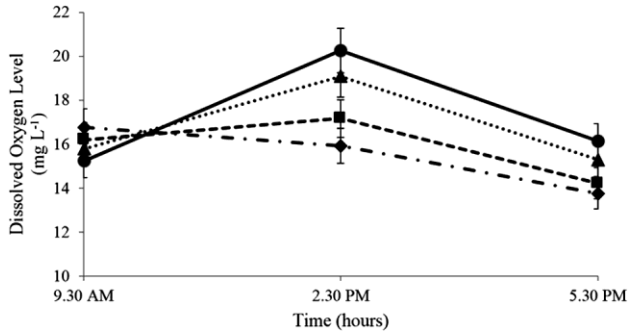


Fig. 4 — Average dissolved oxygen level over 25 days for cultures maintained at different depths (◆3 cm, ■ 6 cm, ▲ 9 cm, ● 12 cm)

The initial biomass concentration of cultures was 0.45 g $L^{-1}$ ($O.D._{560}$ = 0.60). In order to maintain the cultures under nutrient replete conditions, on every fifth day, a predetermined volume of culture was removed and replaced with fresh medium to bring back the biomass concentration to initial levels. The profile of growth, harvest and media replenishment cycle of ponds with 3 cm and 12 cm culture depths is shown in Fig. 5B. It can be seen that the biomass concentration showed a rise reaching a concentration of 0.56 to 0.70 g $L^{-1}$ before a predetermined volume of culture was removed or harvested. In the present study, the biomass concentration, volumetric productivity and specific growth rate were 0.231±0.072 g $L^{-1}$, 49.05±11.74 mg $L^{-1}$ $day^{-1}$ and 0.088±0.024 $day^{-1}$, respectively at 3 cm culture depth (Table 1). These values were significantly higher than the pond maintained at 12 cm culture depth. The specific growth rate at 3 cm culture depth was 1.5 fold higher while biomass concentration and volumetric productivity was almost 2 fold higher than the values obtained at 12 cm culture depth. Increase in culture depth was characterized with a decrease in specific

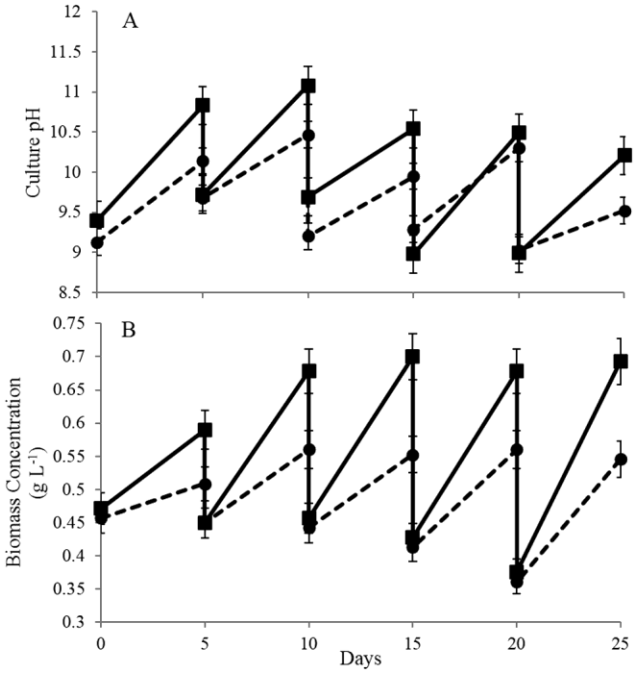


Fig. 5 — (A) Culture pH; and (B) Biomass concentration profile of cultures (■ 3 cm depth, ● 12 cm depth) [The drop in pH and biomass concentration on every fifth day is due to the harvesting of a predetermined culture volume and replenishment with fresh medium]

growth, biomass concentration and volumetric productivity and increase in doubling time (almost 1.7 fold higher in pond with 12 cm culture depth than 3 cm culture depth) (Table 1). It was also observed that the values obtained for specific growth rate and doubling time of the microalga at 6 cm culture depth were similar to those reported for indoor cultures under laboratory conditions[8]. Various studies on the relationship between the volumetric productivities and the culture depths have been reviewed and a first order polynomial equation seems to describe the relationship[21].

In the present study, a first order polynomial relation between the volumetric productivity and culture depth had the $R^2$ value of 0.98;

$y = (2.695)\ x + 56.16$

The $R^2$ value was higher for second order polynomial relation between the two parameters ($R^2 = 0.997$) with the following equation (Fig. 6);

$y = (0.131)\ x^2 - (4.664)\ x + 62.06$

where 'y' represented volumetric biomass productivity and 'x' represented culture depth. Hence, second order polynomial relationship between different culture depths and the resulting volumetric productivity was accepted.

**Proximate composition of biomass**

The proximate composition and mineral content of microalga grown in outdoor open ponds at different depths is presented in Table 2 and Table 3, respectively. The protein content did not vary significantly among different culture depths (37.09±1.83 to 38.07±2.88% w/w). The mineral content of microalgal biomass did not show any correlation with varying culture depths. However, the carbohydrate and lipid content varied among the cultures maintained at different culture depths. The cultures with lowest culture depth i.e., 3 cm had higher carbohydrate content. A decrease in carbohydrate content from 23.4±0.64% (w/w) to 18±1.27% (w/w) was observed when light path length (culture depth) increased from 3 cm to 12 cm.

The lipid content of outdoor cultures was correlated with the growth rate. As the cultures' growth rate decreased, an increase in the lipid content was observed. However, the maximum lipid content (21.55±1.44% w/w) which was observed at a culture

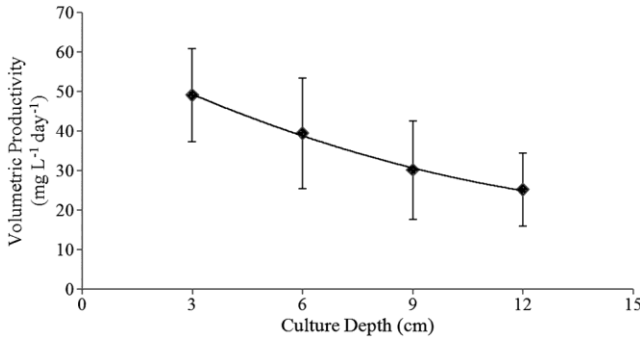


Fig. 6 — Relationship between volumetric productivity and culture depths (*error bars* represent standard deviation)

Table 1 — Specific growth rate, doubling time, biomass concentration and volumetric productivity of *Scenedesmus obtusus* cultivated in outdoor open ponds at different culture depths

| Culture depths (cm) | Specific growth rate μ ($day^{-1}$) | Doubling time (hour) | Biomass concentration (g $L^{-1}$) | Volumetric productivity (mg $L^{-1}day^{-1}$) |
|---|---|---|---|---|
| 3 | 0.088±0.024[a] | 7.91[a] | 0.231±0.072[a] | 49.05±11.74[b] |
| 6 | 0.074±0.026[a] | 9.36[a] | 0.189±0.071[a] | 39.41±13.94[a,b] |
| 9 | 0.059±0.026[a] | 11.71[a] | 0.143±0.064[a] | 30.11±12.39[a,b] |
| 12 | 0.052±0.021[a] | 13.38[a] | 0.121±0.049[a] | 25.20±9.17[a] |

[Data represents mean±SD of five replicates. The superscript (letters a and b) denote significant differences. The mean values in each column sharing a common superscript are statistically not significant at P<0.05 by one way ANOVA]

Table 2 — Proximate composition of *Scenedesmus obtusus* cultivated in outdoor open ponds at different culture depths

| Culture depths (cm) | Lipid content % (w/w) | Carbohydrate content % (w/w) | Protein content % (w/w) | Ash content % (w/w) | Moisture content % (w/w) |
|---|---|---|---|---|---|
| 3 | 16.32±0.82[a] | 23.40±0.64[b] | 38.07±2.88[a] | 16.00±0.80[a] | 8.65±0.43[a] |
| 6 | 16.97±0.75[a] | 20.25±2.12[a,b] | 37.62±2.15[a] | 16.81±0.84[a] | 8.80±0.44[a] |
| 9 | 18.52±1.72[a] | 18.60±2.83[a] | 37.92±1.55[a] | 15.58±0.78[a] | 8.76±0.47[a] |
| 12 | 21.55±1.44[b] | 18.00±1.27[a] | 37.09±1.83[a] | 16.88±0.84[a] | 9.21±0.46[a] |

[Data represents mean±SD of five replicates. The superscript (letters a and b) denote significant differences. The mean values in each column sharing a common superscript are statistically not significant at P<0.05 by one way ANOVA]

Table 3 — Mineral composition of *Scenedesmus obtusus* cultivated in outdoor open ponds at different culture depths

| Culture depths (cm) | Mineral Content (mg $g^{-1}$ DW biomass) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Calcium (Ca) | Potassium (K) | Magnesium (Mg) | Sodium (Na) | Iron (Fe) | Copper (Cu) | Zinc (Zn) |
| 3 | 8.03±0.80 | 5.84±0.66 | 4.52±0.73 | 3.19±0.22 | 3.07±0.19 | 0.03±0.007 | 0.11±0.01 |
| 6 | 7.94±0.79 | 6.40±0.64 | 3.44±0.75 | 3.46±0.24 | 2.82±0.22 | 0.03±0.009 | 0.08±0.02 |
| 9 | 6.87±0.83 | 5.59±0.66 | 3.80±0.71 | 3.54±0.20 | 2.87±0.19 | 0.04±0.008 | 0.11±0.01 |
| 12 | 8.90±0.81 | 4.77±0.67 | 5.03±0.69 | 3.09±0.21 | 3.25±0.20 | 0.05±0.008 | 0.10±0.01 |

[Data represents mean±SD of five replicates]

depth of 12 cm was significantly lower than 32.8±3.69% (w/w) observed for the indoor cultures of the same microalga[8]. The high lipid, protein and carbohydrate content indicated towards the potential application of this microalga as feedstock for aquaculture. The high mineral content, especially the calcium (6.87±0.83 to 8.90±0.81 mg $g^{-1}$ DW biomass), magnesium (3.44±0.75 to 5.03±0.69 mg $g^{-1}$ DW biomass) and iron (2.82±0.22 to 3.25±0.20 mg $g^{-1}$ DW biomass) further implies the nutritional potential of this microalga.

**Pigment profile of microalga**

The total chlorophyll content decreased from 12 µg $mg^{-1}$ upon the transfer of cultures from indoor low light intensities to outdoor high light intensities (Fig. 7). The cultures finally attained a stable total chlorophyll content of 4 µg $mg^{-1}$ irrespective of culture depth. The chlorophyll content of cultures with 9 and 12 cm depths stabilised sooner ($10^{th}$ day, Fig 7C and 7D) than cultures maintained at 3 and 6 cm depths ($15^{th}$ and $18^{th}$ day, respectively, Fig 7A and 7B). The initial carotenoid content of cultures was 4 µg $mg^{-1}$ which decreased and eventually stabilised at 2 µg $mg^{-1}$ when transferred to outdoor ponds. Lutein was identified as the major carotenoid accounting for almost 50% of the total carotenoids. Lutein was present in the range 0.30 to 0.77 µg $mg^{-1}$ in the biomass.

**Fatty acid profile of microalga**

The fatty acid profile of the microalga is shown in the Table 4. The table also shows the fatty acid profile of the indoor culture of the microalga reported earlier[8]. The total saturated fatty acid (SFA) content did not vary much between indoor and outdoor cultures with palmitic acid being the major fatty acid comprising about an average of 30% (w/w) mass fraction of FAME. A significant difference between indoor and outdoor cultures was observed in terms of total mono-unsaturated fatty acid (MUFA) and poly-unsaturated fatty acid (PUFA) content.

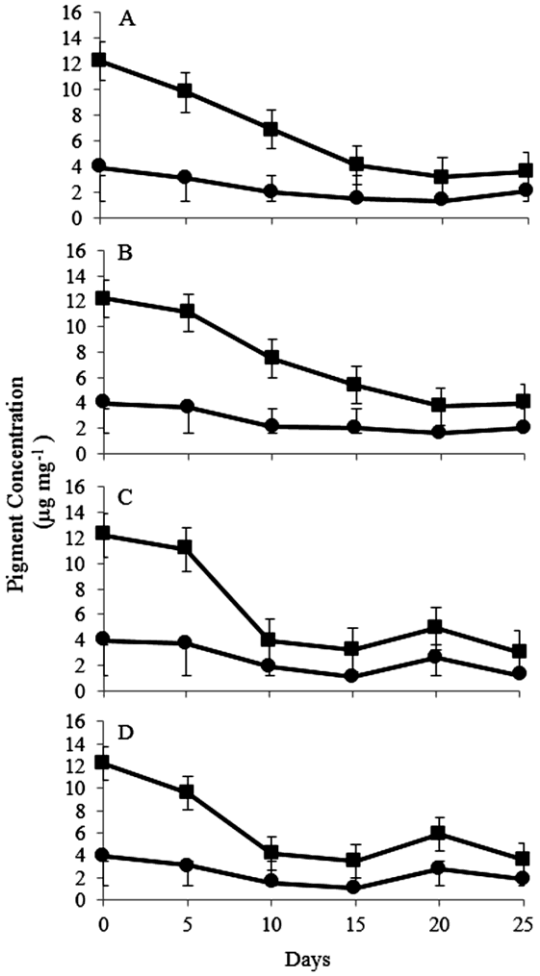


Fig. 7 — Pigment profile of outdoor cultures maintained at different depths; (A) 3 cm (B) 6 cm (C) 9 cm and (D) 12 cm (■ total chlorophyll content, ● total carotenoid content)

Compared to indoor cultures, outdoor showed a two folds decrease in total MUFA content and a two fold increase in total PUFA content. For indoor cultures, omega-6 linoleic acid was the major PUFA while in outdoor cultures; omega-3 alpha linolenic acid (ALA) was the major PUFA comprising about an average of 28 to 31% (w/w) mass fraction of FAME . It was also observed that compared to the fatty acid profile of indoor culture, the outdoor culture showed a 2.5 fold

Table 4 — Fatty acid composition (% w/w mass fraction of FAME) of *Scenedesmus obtusus* cultivated in outdoor open ponds at different culture depths

| | Indoor culture* | Culture depths (cm) | | | |
|---|---|---|---|---|---|
| | | 3 | 6 | 9 | 12 |
| C-16:0 Palmitic acid | 30.59 | 28.92±1.82 | 30.97±4.02 | 30.19±2.89 | 29.83±2.90 |
| C-16:1 n-7 Palmitoleic acid | 3.14 | 2.24±1.15 | 3.09±1.64 | 2.98±1.47 | 2.54±1.42 |
| C-18:0 Stearic acid | 3.08 | 5.04±0.95 | 3.48±1.29 | 3.61±1.09 | 3.65±0.62 |
| C-18:1 n-9 Oleic acid | 36.38 | 14.81±4.57 | 10.7±3.41 | 11.29±2.98 | 10.83±3.40 |
| C-18:2 n-6 Linoleic acid | 14.81 | 12.84±2.30 | 14.9±3.17 | 16.96±4.68 | 18.67±4.15 |
| C-18:3 n-3 α-Linolenic acid | 7.80 | 31.01±3.79 | 30.49±3.8 | 29.84±2.82 | 28.53±4.17 |
| ∑SFA | 37.23 | 35.02±2.07 | 36.65±5.66 | 35.37±3.88 | 34.91±2.72 |
| ∑MUFA | 40.16 | 21.12±3.65 | 17.96±3.79 | 17.83±3.32 | 17.88±3.01 |
| ∑PUFA | 22.61 | 43.86±2.45 | 45.39±4.53 | 46.80±4.76 | 47.21±2.83 |

[Data expressed as mean±SD of five replicates. * Indicates the reported results[8]]

reduction in oleic acid (MUFA) content and four folds increase in ALA content.

## Discussion

The incident surface light intensity varied drastically during the daytime, ranging from ≥1000 µmol $m^{-2}$ $s^{-1}$ to 120 to 150 µmol $m^{-2}$ $s^{-1}$ on most of the days. The short light path length of ponds with lower culture depths helped in better light penetration and distribution leading to enhanced growth profile. Also, since the cultures were never exposed constantly to high light intensity for the longer duration of time, a possibility of light saturation and photo-inhibition was seldom. The increasing light path in ponds with 9 and 12 cm culture depths may have contributed to their comparatively lower growth and productivity profile. Lower culture depths were found to be more effective in improving the biomass productivity[22], reducing energy consumption[23] and water requirements for algal cultivation[24]. Therefore, it is important to discover an optimal culture depth which not only improves biomass productivity but also facilitates energy and water savings. This optimal culture depth may vary from season to season and may also vary for different microalgae.

The ascending trend in afternoon DO levels in ponds having culture depths 6 to 12 cm showed that the build-up of DO in outdoor ponds is also a function of total algal biomass present in the ponds. As the total biomass in ponds increased with increasing culture volumes, a higher build-up of DO was seen. The DO levels observed in the present study were similar to those reported for *Pleurochrysis carterae* in open ponds[22]. The rise in culture pH with growth can be attributed to the alkalization of culture media observed in autotrophic algal cultures. As the algae utilise the dissolved inorganic carbon (DIC) during photosynthesis, the $HCO_3$ converts to $CO_2$ and OH leading to rise in culture pH[25]. The culture regime of removing the culture and replenishing fresh medium on every fifth day helped in maintaining the culture pH in favourable range (Fig. 5A).

The culture regime followed helped in avoiding very high optical densities thereby minimizing the effect of self-shading on culture growth. It was observed that as the culture depths increased, the growth rate slowed down and the cultures maintained at various depths did not show a lag phase. The initial cell density (ICD) has been reported to be an important factor affecting the response of microalgae to the increase in light intensity with low ICD cultures thereby showing a longer lag phase[26]. The absence of a lag phase even in the cultures maintained as thin layer (lower depth, 3 cm) suggested that the ICD used in the present study was optimum and facilitated faster photo-acclimation. The light conditions affect microalgal growth and productivity[27]. Increasing incident light intensities have been shown to enhance the growth of *Scenedesmus* sp.[28,29]. It has also been suggested that, the light saturation constant of microalgae may be lower than that of sunlight e.g., *Dunaliella viridis*, where a light intensity of 700 µmol $m^{-2}$ $s^{-1}$ led to enhanced growth and further enhancement in light intensity to 1500 µmol $m^{-2}$ $s^{-1}$ resulted in a decreased growth[29]. All these studies have been conducted with closed photo bioreactors where the microalgal cultures are constantly exposed to a particular light intensity.

The outdoor cultures differ from these indoor laboratory studies as they are exposed to varying

intensities of sunlight and are never exposed constantly to a particular selected light intensity. The solar irradiance also varies with season and these daily and seasonal variations in solar irradiance pose a major challenge of finding the optimal light path length in outdoor algal cultivation[24]. The short light path length has been reported to promote accelerated growth[30]. The deeper microalgal cultures would not result in higher productivity because in outdoor ponds only the photic zone, i.e., upper 1 to 3 cm of the cell suspension absorbs all the incident light energy while the rest of the culture remains in the dark[31]. The outdoor open pond studies reported high specific growth rate and volumetric productivity of *Pleurochrysis carterae* at least culture depth (13 cm) for autumn season[22]. Another study reported highest biomass concentration of *Scenedesmus acutus* at 7 and 9 cm culture depth for two different seasons in outdoor open raceways[24].

The irradiance and temperature have been reported to influence the proximate composition of *Scenedesmus* sp.[32,33]. A positive correlation between light intensity and carbohydrate content has been reported for *Nannochloropsis* sp. and *Scenedesmus obliquus*[26,28]. In the present study, though all the outdoor cultures received high incident light intensities, the lower depths appeared to have better light penetration and distribution which might have led to the increase in carbohydrate content of these cultures. The increase in microalgal lipid content has been linked to the stress conditions such as depletion of nitrogen or phosphorus (nutritional stress) and increase in salinity[34]. Though nutrient media was replenished periodically, due to the absence of continuous mixing and the length of the culture column, formation of localized nutrient gradients leading to nutritionally starved regions cannot be ruled out[11]. These phenomena might be collectively responsible for comparatively higher lipid content at 12 cm culture depth as compared to other depths.

The decrease in chlorophyll content on transfer of the indoor cultures to outdoor open ponds could be attributed to the phenomenon of photo-acclimation. As an adaptive response, the indoor cultures on their transfer to outdoor conditions, characterised by high light intensities, undergo rapid changes in their pigment content, especially the chlorophyll[26]. The changes in pigment content helps in optimizing the ability of cells to harvest available light and thereby compensates for changes in light intensity[35]. It was observed that decline in chlorophyll content of *Nannochloropsis* sp. cultures in photobioreactor with 1 cm light path was significantly higher than the cultures in photobioreactors with 3 cm light path length when light intensity was increased to 3500 µmol $m^{-2}$ $s^{-1}$ [26]. A decrease in chlorophyll content of *Scenedesmus* sp. cultures on exposure to high light intensities have also been reported[29]. In the present study, *Scenedesmus obtusus* culture was grown indoors at low light intensity[14] and subsequently transferred to outdoor ponds. The profile of decline and stabilisation of the total chlorophyll and carotenoid content was similar in cultures irrespective of culture depths. The thin layer culture maintained at 3 cm showed a similar response as shown by cultures maintained at 6,9,12 cm depths. The profile of total chlorophyll and carotenoid content, and; absence of lag phase in thin layer cultures observed in the present study suggests that the indigenous *Scenedesmus obtusus* isolate has the ability to adapt to varying and high light intensities experienced in outdoor conditions and therefore could be a potential candidate for outdoor cultivation.

The culture temperatures have been reported to influence the fatty acid composition of microalgae with low temperature enhancing the PUFA content[32,33,36-38]. These temperature induced changes in fatty acid composition are considered to be an adaptive response of microalgae to maintain membrane fluidity[39]. Unlike the studies where microalgae are maintained indoor at constant temperatures, the outdoor open pond system is susceptible to a wide range of temperature fluctuations. The minimum night time temperatures have been suggested to be a key factor influencing the fatty acid composition[40]. The temperature profiles of the outdoor cultures in the present study shown in Fig. 3A and 3B clearly shows that cultures experienced wide diurnal fluctuations in temperature. During the present study, the cultures spent nearly 10 h at temperatures ≤20°C per day, with 6 h at temperatures between 26 and 30°C and remaining 8 h between 20 to 25°C. A long duration (10 hours) exposure of culture to lower temperatures i.e., ≤20°C might have resulted in enhanced accumulation of PUFA, especially omega-3 ALA. The enhancing effect of high temperature on SFA was not seen in the present study as the residence time of culture at temperatures ≥30°C was significantly short.

Temperatures below the cell membrane fluidity threshold would induce the accumulation of structural lipids to facilitate the membrane fluidity[36] and these threshold temperatures may vary from species to species[39]. Therefore, the lower temperature range which induced the PUFA accumulation in *Scenedesmus obtuse* in the present study might be specific for this strain. Also, the results of this study may be specific to the particular season in which the study was carried out.

Therefore, the indigenous microalga *Scenedesmus obtusus* CFR 1-09/FW could advantageously adapt to outdoor culture conditions, showed higher growth rate, lipid, carbohydrate and PUFA content, making it a potential candidate for large scale outdoor cultivation. The microalga can be employed for production of biomass for food and feed applications owing to its high carbohydrate, protein and omega-3 ALA content. The study suggests that seasonal variations in biochemical composition of the microalga could offer the algal biomass targeting specific applications depending upon the season of cultivation.

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## Conflict of Interest

The authors declare no conflict of interest and mutually agree for submission of the manuscript to Indian Journal of Experimental Biology (IJEB).

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