Biomass production of multipopulation microalgae in open air pond for biofuel potential

P Selvakumar* & K Umadevi

Department of Marine Living Resources, Andhra University, Visakhapatnam-530 003, Andhra Pradesh, India

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Biodiesel gains attention as it is made from renewable resources and has considerable environmental benefits. The present investigation has focused on large scale cultivation of multipopulation microalgae in open air pond using natural sea water without any additional nutritive supplements for low cost biomass production as a possible source of biofuel in large scale. Open air algal pond attained average chlorophyll concentration of 11.01 µg /L with the maximum of 43.65 µg /L as well as a higher lipid concentration of 18 % (w/w) with lipid content 9.3 mg/L on the 10th day of the culture; and maximum biomass of 0.36 g/L on the 7th day of the culture. Composition analysis of fatty acid methyl ester (FAME) was performed by gas chromatography and mass spectrometry (GCMS). Multipopulation of algal biomass had 18% of total lipid content with 55% of total saturated fatty acids (SFA), 35.3% of monounsaturated fatty acids (MUFA) and 9.7% of polyunsaturated fatty acids (PUFA), revealing a potential source of biofuel production at low cost.

Keywords: Biodiesel, FAME, Mass culture, Renewable energy

Biofuels, as an alternative of fossil fuel, are in demand from various sectors, transport (blending mandate), power (renewable energy for electricity), and domestic1,2. The Medium-Term Renewable Energy Market Report (MTRMR) 2015 has estimated biofuels growth by 2020 at 4% of road transport demand despite the current lower crude oil price environment while USDA has predicted global ethanol production to grow up to 40% by 202223. As most biofuels are produced from crops such as corn, sugar cane, sugar beet, sorghum, palm, rapeseed, soy, etc., supply to industrial/domestic use as indicated above has impact on food production and prices, carbon stores, land use, etc.2,4. Moreover, existing policies restrict the expansion of grain-and oilseed-based biofuel production5. Such issues have urged use of ‘non-feed’ feed stocks viz., agricultural residues, food wastes and waste oils and algae as potential source of bioenergy2. Recently, Bhatia & John5 have demonstrated the potential of pineapple peel for low-cost production of bioethanol using yeast under optimized process conditions in anaerobic batch fermentation.

In this context, algal biomass production with target algal strains produced in considerable quantities for industrial application as biofuel is a profitable business. The economic feasibility of algal mass culture for biofuel production depends on its ability to increase micro algal biomass productivity6. Mass cultivation is essentially focused on enhancing growth rates while minimizing the nutrient inputs. Certain culture conditions could result in higher quantities of storage lipids, such as triglycerides7. Diatoms, in particular, are useful neutral lipid sources, both as liquid fuel precursor and as food for zooplanktons8. Manipulation of micro algal or diatom lipid quantity and quality is necessary for effective use of this renewable resource as fuel. Microalgae can grow autotrophically, with a wide range of tolerance to temperature, salinity, pH and nutrient availabilities9.

Building an algal bioreactor is expensive. Comparatively, establishing and maintaining large scale cultures of multipopulation micro algal species in the open air pond for biomass production is economical and easy to operate. The micro-algal cells in open ponds utilize the sunlight and CO2 in the atmosphere. However, the quality and quantity of natural sunlight are affected by daily and seasonal fluctuation10,11. Here, we explored whether multi population micro algal in open air pond can achieve growth and lipid yields necessary to serve as a potential feedstock for production of biofuels.

*Correspondence:
E-mail: bioselvas@gmail.com
Materials and Methods

Geographic location and weather condition of site
The study area was Bheemunipatnam, Visakhapatnam, Andhra Pradesh, India (Location of open air pond 17° 54′N, 83° 27′E). A monsoon fed river, Gosthani opens into the Bay of Bengal at this place. Satellite image (Fig 1) shows the pond site and the creek with onshore of Bay of Bengal. The site selected for the study was based on availability of adequate sunlight, optimum air temperature, abundant rainfall and proximity to coastal area. Ramasamy et al. (2012) has measured the average day temperature of this area to be >30°C during summer and >22°C in winter, and the average year irradiance, 4.08 kWh/m²/day. The hill ranges of the Eastern Ghats surround the place on the land side and at two points they butt into the sea. The coast is rocky interspersed with sandy patches. The bulk of the rainfall is brought by the southwest monsoon which commences late in June and lasts till early October. The northeast monsoon closely follows the southwest monsoon and extends till December. While the average annual rainfall of the place is about 38", the period under review had a rainfall of 37-22".

Open air pond construction
A square shaped experimental open air pond (14×14 m) measuring 144 m² in surface area and 0.6 m depth was constructed with four side walls with 0.4 m thickness using simple clay dike and lined with 2.0 mm thick high density polyethylene (HDPE) sheets (The floors and walls were completely covered. Although the water was only 0.3 m deep, the boundary walls were of 0.7 m high for reasons of stability). Culture pond was designed to promote algal growth—shallow in order to allow maximum light penetration, and was operated at short hydraulic retention time (HRT) in the range of 5 to 30 days depending on climatic conditions, reducing the required surface area. During high tide, the sea water reached the stagnation point through the estuary where the pump house was located.

Open air pond mass culture
The pond was filled with sea water filtered through 20 micron plankton net in order to remove zooplankton and larger organisms and cultivation was carried for two months, Nov.-Dec. 2012. Dimensions of the culture maintained area of the pond was 14×14×0.31 m. The algal culture was mixed manually during day time throughout the culture period to prevent settling as well as to enhance the dissolved CO₂ concentration.

Hydrograpy and hydrochemical analysis
Measurements of atmospheric and surface water temperatures, pH and salinity were made in situ. Atmospheric and surface seawater temperatures were measured using standard thermometer; salinity with the refractometer (Atago, S/Mill – E, Japan), and pH using a portable pH meter (Perkin Elmer, accuracy, + 0.01). The pH meter was calibrated with standard buffers just before use.

Identification and characterization of multipopulation of microalgae
No attempt was made to inoculate a particular microalgae species into the pond initially. Considerable number of diatoms, blue green algae and dinoflagellates were observed in the initial day of culture.

Chlorophyll concentration was monitored regularly to ensure continuous growth of microalgae, and nutrient concentrations were monitored every alternate day in order to confirm the nutrient uptake by microalgae. The composition of this mixed microalgae algal culture was maintained for 10 days and characterized roughly under the microscope (Carl Ziess, Primovert) regularly. Identification of algal taxa was carried out using taxonomic keys following Desikachary and Kandari et al.

Biomass estimation (as chlorophyll a) and nutrient analysis
The nutrients of the culture were analyzed by using Strickland and Parsons method. Chlorophyll a, b and c concentrations were estimated by spectrophotometry. Briefly, samples were collected at four corners and the middle section of the pond using clean amber colour
plastic bottles from the surface culture. Small pinch of magnesium carbonate was added into the culture before filtering (0.45 µm, 47 mm) with vacuum pump (max. 5 mm Hg). The filter paper was placed in an acid free test tube containing 15 mL of 90% acetone and kept in a refrigerator for 24 h. The acetone soaked filter paper was ground using a mortar and centrifuged for 5 min at 4000 rpm. The resultant extract was measured using spectrophotometer (PerkinElmer Lambda 35, UV visible spectrophotometer) against 90 % acetone as blank at wavelengths of 750, 665, 645 and 630 nm, i.e., the maximum absorption wavelengths of the pigments. Cultures were sampled from all four corners and middle portion of the pond to estimate the mean value of chlorophyll concentration (µg/L).

Growth kinetics
Algal culture specific growth rate \( \mu \) (d\(^{-1}\)) was calculated using following Equation.

\[
\mu (\text{day}^{-1}) = \frac{\ln(C_1/C_0)}{t_1-t_0}
\]

where \( C_1 \) and \( C_0 \) were the biomass concentration (as chlorophyll a) (µg L\(^{-1}\)) on days \( t_1 \) and \( t_0 \), respectively. In this work, the chlorophyll a concentration was used to quantify the cell density values.

Algal biomass harvesting
The algal cell settled at the bottom in the open air pond was harvested on 11th day. After auto flocculation or gravity sedimentation, the supernatant was changed to another pond and the settled biomass was transferred into 20 L capacity Polyethylene terephthalate (PET) jar for total separation of biomass from water and allowed to further settle for 6-8 h. Concentrated biomass was washed with tap water and cells were allowed to settle. This process was repeated thrice in order to remove the salt in the algal biomass. The washed algal cells were spread on aluminum tray with a white plastic sheet and dried under sunlight for 8 h followed by hot air oven (Thermo scientific precision) drying at the 80°C for 24 h.

Lipid extraction from algal biomass
The 10 g of dried biomass was ground with a mortar and pestle, and the lipids were extracted with n-hexane\(^18\) followed by extraction with chloroform and methanol\(^19\). Each extraction of the mixture was sonicated at 60Hz with a sonicator (Sonic High Intensity Ultrasonic processor with Temperature Controller, Microprocessor controlled-750 WATT Model) at 50°C. Phase separation was subsequently carried out following Folch et al.\(^20\). Here, chloroform and water were added into the mixture in the ratio of 10:10:9 (Mixture: chloroform: water). After vigorous shaking, the mixture was centrifuged (Eppendrof, cooling centrifuge 5810 R, Germany) at 5000 rpm for 10 min, resulting in separation into two phases. The extracts were dried in a rotary evaporator (Heidolph Hei-VAP precision with glassware set G3) and weighed using high precision electronic weighing balance (Shimadzu ≤0.01 g). The lipid content was calculated based on total lipid from the first and second extraction steps and then expressed as a dry wt. percentage.

Lipid productivity
Daily lipid productivity was calculated by the following equation:

\[
\text{Daily lipid production (mg lipid L}^{-1}\text{day}^{-1}) = \text{DW} \times (\text{lipid/100/day}) \times 1000
\]

where DW = algal dry wt. (g L\(^{-1}\)), lipid = g 100 g\(^{-1}\) DW, and day = growth period.

Transesterification process
Solvent based derivatization
Total lipid extracted by chloroform/methanol was saponified with 25 mL of 0.5 M methanolic NaOH solution at the 75°C for 20 min, and then submitted to methanalysis with 5% H\(_2\)SO\(_4\) in methanol at 75°C for another 2 h. Two mL of hexane/ether (1:1, v/v) was added twice to collect FAME content and 2 mL of 2% (w/v) NaCl was added for acid neutralization before collecting the top solvent layer. This was evaporated under room temperature (33°C) and dried with the vacuum and FAME yield was calculated.

Direct transesterification
The direct derivatization process was done according to the acidic method described by Lepage and Roy\(^21\) with the modification introduced by Cohen et al.\(^22\). Dried FAME was re-suspended in hexane for GC analysis. Methyl ester samples were concentrated on rotary evaporator, weighed for FAME content and analyzed by TLC using hexane: ethyl acetate (v/v) (90:10) solvent system for conformation. FAME yield was measured according Griffiths et al.\(^23\).
### FAME concentration

The yield of FAME calculated using the equation:

\[
\text{FAME yield (\% by weight of algae) = } \frac{\text{Weight of FAME obtained after transesterified}}{\text{Weight of algae input} \times 100.}
\]

### GCMS/GC analysis protocol

The fatty acid composition of algal oil was analyzed qualitatively using GC-MS and quantitatively using GC. The GC-MS 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°C and quadrupole at 150°C) in the electron impact mode with a HP-5 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). The oven temperature was programmed for 2 min at 160°C and raised to 300°C at 5°C min⁻¹ and maintained for 20 min at 300°C. The carrier gas helium was used at a flow rate of 1.0 mL min⁻¹. The inlet temperature was maintained at 300°C, and the split ratio was 50:1. Structural assignments were based on interpretation of mass spectrometric 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 (30 m × 0.25 mm i.d. × 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 the 5°C min⁻¹ and maintained for 20 min at 300°C. The carrier gas, nitrogen was used at a flow rate of 1.5 mL min⁻¹. The injection volume was 1 µL, with a split ratio of 50:1. The identification of individual fatty acids was based on standard retention time of authentic fatty acids.

### Results

Figure 2 shows the open air algal pond; auto flocculation; collection of auto settled biomass; and concentrated algal biomass while live culture.

#### Physico-chemical parameters and microalgae population of open air pond

The physico-chemical parameters of the open air pond during the culture period were as follows: air temperature 34-37°C, culture temperature 32-37°C, salinity 32-37 ppt, pH 8.8 to 11.6, photoperiod 12:12 h (light:dark) (Table 1).

The microalgae population of the pond were mainly composed of 15% blue green algae, 20% dinoflagellates, and 65% diatoms, as shown in Fig. 3. The complete composition of the mixed algal population at the initial day is given in Table 2. Diatoms were found to be dominant than dinoflagellates and blue green algae. Among diatoms, genera *Nitzschia* and *Pleurosigma* contributed were most common and *Skeletoneema* sp. (>300 numbers per mL) (data not shown) was more abundant from the initial day to end of the culture period. A considerable number (200-300 per mL) (data not shown) of *Gyrosigma* sp. and *Pleurosigma* sp. were observed in the middle of the culture period which continued till the end. *Nitzschia* sp. gradually increased from day one and reached up to a concentration of 3 million per mL on day 8 (Data not shown).

<table>
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<tr>
<th>Days</th>
<th>Salinity (ppt)</th>
<th>pH</th>
<th>Air Temp. (°C)</th>
<th>Water Temp. (°C)</th>
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<tr>
<td>10</td>
<td>37</td>
<td>11.6</td>
<td>37</td>
<td>31</td>
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</table>

![Fig. 2](image-url) — (a) A view of open air algal pond; (b) Auto flocculation occurred pond; (c) Collection of auto settled biomass; and (d) Concentrated algal biomass while live culture.
shown). Dinoflagellates disappeared on 3\textsuperscript{rd} day and blue green algae \textit{Oscillatoria} sp. and \textit{Synechococcus} sp. were found till the end. A shift in phytoplankton community in the experimental open pond was observed on day 5, possibly due to increased pH.

### Chlorophyll and nutrient concentrations

The chlorophyll concentrations of multipopulation micro algal cultures are presented in Table 3. The initial concentration of chlorophyll ‘a’ 0.573 µg L\textsuperscript{-1} (day 1) had increased to 43.64 µg L\textsuperscript{-1} on the 9\textsuperscript{th} day;
chlorophyll ‘b’ increased from 0.285 µg L⁻¹ (day 1) to 31.09 µg L⁻¹ on the 8th day; and chlorophyll ‘c’ from 2.66 µg L⁻¹ to 39.99 µg L⁻¹. To monitor the biomass concentration, regular chlorophyll concentration was measured as well as microscopic observation was done to calculate the maximum percentage of phytoplankton among the multipopulation without fertilizing natural sea water.

The nutrients concentration of multipopulation micro algal culture are presented in Table 4. On the first day of the culture, nutrient concentration was as follows: nitrite 4.35 µm/L, nitrate 28.85 µm/L, phosphate 5.65 µm/L and silicate 29.25 µm/L, and it gradually reduced day by day.

Micro algal growth rate

The growth rate (µ, 1 d⁻¹) was calculated by exponential regression of the logarithmic portion of the growth curve. In the present study, the chlorophyll ‘a’ concentration values were used for growth rate calculation. A high growth rate 0.34 day⁻¹ was recorded for multi population microalgae that were cultured under open air algal pond. Division per day (K) and generation time (T₆₇) was 0.5 and 2.0, respectively (Table 5).

Concentrations of biomass, lipid and FAME

Maximum biomass concentration 0.36 g L⁻¹ was obtained on the 7th day. The lipid content was 18%, lipid yield 65 mg L⁻¹ and lipid productivity was 9.26 mg L⁻¹ d⁻¹. FAME yield was 7.8 ± 1.61% through solvent based transesterification while through the solvent based transesterification method it was 6.2 ± 1.92%.

Comparison of relative FAME yield between the solvent based transesterification and direct transesterification was >1.5%.

FAME profile

Relative fatty acid composition data (% dry wt.) obtained using the direct derivatization are depicted for multipopulation of microalgae (Table 6). The

<p>| Table 3—Chlorophyll concentration of mixed population microalgae under open air algal pond |</p>
<table>
<thead>
<tr>
<th>Days</th>
<th>Chl’a’ (µg L⁻¹)</th>
<th>Chl’b’ (µg L⁻¹)</th>
<th>Chl’c’ (µg L⁻¹)</th>
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<tr>
<td>1</td>
<td>0.573</td>
<td>0.285</td>
<td>2.667</td>
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<tr>
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<td>1.356</td>
<td>1.146</td>
<td>3.939</td>
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<tr>
<td>3</td>
<td>2.766</td>
<td>1.977</td>
<td>1.446</td>
</tr>
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<td>4</td>
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</tr>
<tr>
<td>10</td>
<td>43.647</td>
<td>23.586</td>
<td>19.704</td>
</tr>
</tbody>
</table>

<p>| Table 4—Nutrient concentrations in culture pond |</p>
<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Days</th>
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<th>3</th>
<th>5</th>
<th>7</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite µm L⁻¹</td>
<td>4.35 ± 0.545</td>
<td>2.65 ± 0.25</td>
<td>2.69 ± 0.78</td>
<td>1.52 ± 0.54</td>
<td>0.65 ± 0.18</td>
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<tr>
<td>Nitrate µm L⁻¹</td>
<td>28.85 ± 6.54</td>
<td>23.60 ± 5.65</td>
<td>20.80 ± 7.28</td>
<td>12.32 ± 1.35</td>
<td>1.25 ± 0.65</td>
<td></td>
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<tr>
<td>Phosphate µm L⁻¹</td>
<td>5.65 ± 1.025</td>
<td>4.75 ± 1.64</td>
<td>4.64 ± 1.67</td>
<td>1.587 ± 0.96</td>
<td>0.55 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>Silicate µm L⁻¹</td>
<td>29.25 ± 6.52</td>
<td>26.24 ± 0.65</td>
<td>20.38 ± 6.37</td>
<td>13.25 ± 3.25</td>
<td>6.35 ± 1.64</td>
<td></td>
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</table>

(Mean Value (n=5) ± Standard deviation)
major acids in the multipopulation microalgae were identified as C16:0, C16:1, C18:1; PUFA comprising C18:2 (n-6) C18:3 (n-3), C20:3 n-3, C20:4 and C20:5. C16:0 constituted 34 % of the total 55.5% saturated fatty acids while C16:0 and C18:1 accounted for 11.8 and 23.5% of total mono-unsaturated fatty acid (35.3%). Total poly-unsaturated fatty acid (9.7%) had 2.1% ω3 and 7.6% ω6 fatty acids.

Discussion

This study focused on growing mixed algal species in outdoor shallow pond systems with natural marine water supply without any prior treatment and assessed the biomass potential and lipid productivity of such culture. The mass culture of marine microalgae depends up on natural seawater, both in open tanks and in closed controlled systems. Near-shore seawater is generally used directly in mass culture without addition of nutrients, trace elements and vitamins. However, use of artificial medium is also not uncommon.

In multipopulation microalgal cultures, as we observed here, not all the species survive throughout the study period. It could be due to the differential nutrient requirements of phytoplanktons and other species. However, diatoms such as Nitzschia sp., Pleurosigma sp., Gyrosigma sp. and Skeletonema sp.; blue green algae (Trichodesmium sp. Oscillatoria sp., Lyngbya sp.); and dinoflagellates Prorocentrum sp. and Gymnodinium sp. survived till the end of culture period. While most of the microalgal species disappeared after 6th day only a few genera as mentioned above survived of which Nitzschia dominated. Environmental factors, water mixing and nutrients are known to affect the composition of phytoplankton community. Further, lower N:P ratio and increased Si:N ratio provided an ideal nutrient distribution for the dominance of diatoms.

In our experiment, we observed low nitrate+nitrite: silicate ratio from day 1, and it explains to some extent, the dominance of diatoms till the end of culture period. The observed low values could be attributed to the uptake of nutrients by microalgae for their biological activity. The sudden fall in the nitrate, nitrate and phosphate concentrations during the culture period could be due to the increased density of particular microalgae. Johansen et al. reported that the lipid content in nitrogen-stressed cultures to be more than 2 folds than that of the unstressed cultures.

For a low-cost, high-volume product, such as lipid for biodiesel, gravity sedimentation followed by flocculation seems promising methods. Sedimentation is a suitable method for harvesting of multipopulation microalgae which are naturally having high sedimentation rates. After auto flocculation the biomass that gets formed floats like a mat on the surface of the pond. The floating biomass then gather in a corner of the pond and settles by gravity sedimentation. Gravity sedimentation is preferred as it requires only few inputs apart from transferring to a settling tank and gives sufficient time for the cells to settle.

An important characteristic for any biodiesel feedstock is the suitability of the fatty acid profile. Few studies have investigated the quality of microalgal biodiesel. The distributions are typical of most green algae, with C16 and C18 PUFA being most abundant. Algal fatty acid composition is influenced by growth conditions such as temperature and nutrient availability. The different carbon chain lengths and number and position of unsaturated bonds found in fatty acids influence the cetane number (CN), iodine value, oxidative stability, cold flow properties and viscosity of the fuel.

Conclusion

By this work, we have demonstrated that multipopulation of marine microalgae can be economically cultured on large scale in natural seawater. Based on considerable tolerance to a broad range of temperature, and large quantity of intracellular lipid and the biomass growth, multipopulation of microalgae may have potential for exploitation as a renewable precursor to liquid fuels or as a lipid source. The feasibility of using microalgal lipids as a fuel precursor on large scale is based not only on productive lipid-producing algae but also on the environment conducive for multipopulation of microalgal culture. Culturing mixed unscreened natural marine algal species viz., green algae, BGA and diatoms in open air ponds could be a feasible option of feedstock for biodiesel production.

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