

Article

Lipid enhancement in microalgae by temporal phase separation: Use of indigenous sources of nutrients

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ABSTRACT

Microalgae have been recommended as superior candidate for fuel production because of their advantages of higher photosynthetic efficiency, biomass & lipid productivity, and faster growth rate as compared to other energy crops. To meet up all these criteria, we have developed a continuous outdoor micro-algal raceway pond reactor (RPR) and a lab scale indoor tubular photo bioreactor (PBR) for biofuel production. An attempt to utilise indigenous sources of nutrients to improve the economics also revealed that micro-algal culturing can also be used as a mode of nutrient removal and water treatment. The photosynthetic rate and lipid production were enhanced by arresting daytime cell division and promoting night-time cell division. A 50% lipid improvement was observed for the particular algal consortia. Microscopic studies revealed that temporal phase separation could be achieved by adjusting nutrient distribution pattern. To monitor temporal phase separation, it is required to know DNA multiplication model. Quantification of gDNA in RPR confirmed that cell division happens during the night which positively affects the photosynthetic efficiency and lipid productivity of microalgae.

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1. Introduction

The need for clean, biodegradable and carbon neutral alternative energy has led the R&D focus on microalgae as a biofuel source [1,2]. Microalgae are considered a future source of biofuel, because it does not compete with agricultural land and fresh water resources, and has the potential to be cultivated in quantities required for substituting mineral oil. However, at productivities achieved so far, algal oil production costs are several times that of mineral oil. Mass culturing methods of algae have to be optimised along with the cost optimisation of culturing methods so that the algal biofuels can compete with the ever-depleting and polluting, fossil fuels [3]. A proper optimisation of all the steps involved in the production process is vital for improving the economics of the whole process [4]. Raceway ponds are shallow artificial ponds used in the cultivation of microalgae which is the most economical method for algal culture [5,6]. The shortcomings of raceway ponds are that they are highly susceptible to environmental factors [7]. A closed photobioreactor could be a better companion while studying the growth parameters of algal culture [8]. The simultaneous studies of parameters of growth in a closed system along with a larger open system could reveal valuable insights into maximisation of lipid production and cost optimisation.

The preference of microalgae is due to its less complex structure and generation time also they are filled with protein, lipid and various other high value products such as nutraceuticals and fatty acids [9]. Similar to

any photoautotrophs microalgae also derive its energy from solar irradiance and process the energy through photosynthetic pathways to stored energy [10]. Contemporary research efforts with microalgae are concentrated on finding high yielding species and have yielded lipid up to around 20% with a biomass yield of around $1000 \text{ mg} \cdot \text{L}^{-1}$. The selection of an algal species is a significant step in the commercialization of bio-oil as a source of fuel [11]. There are many methods for selection of algae [12]. The R&D effort on microalgae has followed two approaches: a) collecting, culturing and cultivating natural algal strains that have high lipid content or b) genetic engineering of algae to improve lipid content. Natural selection engineering is a new approach to the problem. In our novel method, a phototrophic consortium rather than specific strains is cultivated. The consortium is modified through natural selection by control of process conditions so that it has desired characteristics. To improve the photosynthetic rate and lipid production of microalgae, daytime cell division should be arrested and instead, night time division should occur since the lipid accumulation in microalgae cells happens during daytime [13]. Daytime cell division can be arrested by facilitating a nutrient exhausted condition [14]. The energy harnessed by photosynthesis during the day should be bypassed to storage instead of cell division. Microscopic studies revealed that it is possible by adjusting nutrient distribution pattern.

Besides CO_2 and light, algae require nutrient to grow, nitrogen (N) and phosphorous (P) being the most important ones [15]. According to Redfield ratio, the nutrient availability should be equal to or higher than 16:1 (N: P) [16,17]. The usage of indigenous, alternative sources of nutrients like sewage or seawater [18], apart from the vast

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diversity and availability, can significantly reduce the running cost of nutrient additions or water cost [19,20]. The amount and kind of nutrients needed for algal growth depend very heavily on the species, but as an indication for the most important nutrients, about 5% (typically 7%–8%) of algal dry matter consists of nitrogen (N) and 1% of phosphorous (P) [13]. Ammonium chloride (NH_4Cl), Potassium dihydrogen orthophosphate (KH_2PO_4) and trace metal solution are the nutrients used. In this work, our aim is to get a consortium that has high lipid productivity; accordingly the nutrient concentration is changed. Ammonium chloride acts as the nitrogen source, and potassium dihydrogen orthophosphate acts as the phosphorous source.

2. Methodology

2.1. Experimental setup

2.1.1. Photobioreactor

A tubular photobioreactor (PBR) of volumetric capacity 10 L, made of transparent polycarbonate tubes was made to culture the algal

consortium (Fig. 1(a)). The irradiant area was 0.5 m^2 and was illuminated by two 40 W fluorescent tubes providing lighting equivalent to $418.92 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (31000 lx) on the tube surface. The temperature of the system was uncontrolled and closely monitored, $(28 \pm 3)^\circ\text{C}$. The circulation of the media was carried out using a peristaltic (Watson-Marlow) pump. Continuous monitoring of dissolved oxygen and pH was carried out using (Ponsel) probes. The aeration tank was provided with a constant mechanical stirring, at $20 \text{ r} \cdot \text{min}^{-1}$, just to prevent the settling of microalgae inside the aeration tank and not causing any shear damage to the cells. The CO_2 required was continuously supplied by compressed air sparged into the aeration/degassing tank.

2.1.2. Raceway pond reactor

An algal raceway pond reactor (RPR) made of opaque fibreglass was used to cultivate the microalgae consortium (Fig. 1(b)). It had an irradiant area of 1 m^2 ; liquid depth is 0.30 m and a volumetric capacity of 300 L. Agitated by a two bladed paddle system controlled by a three-phase regulator. The reactor was fed with only fresh sea water, and the

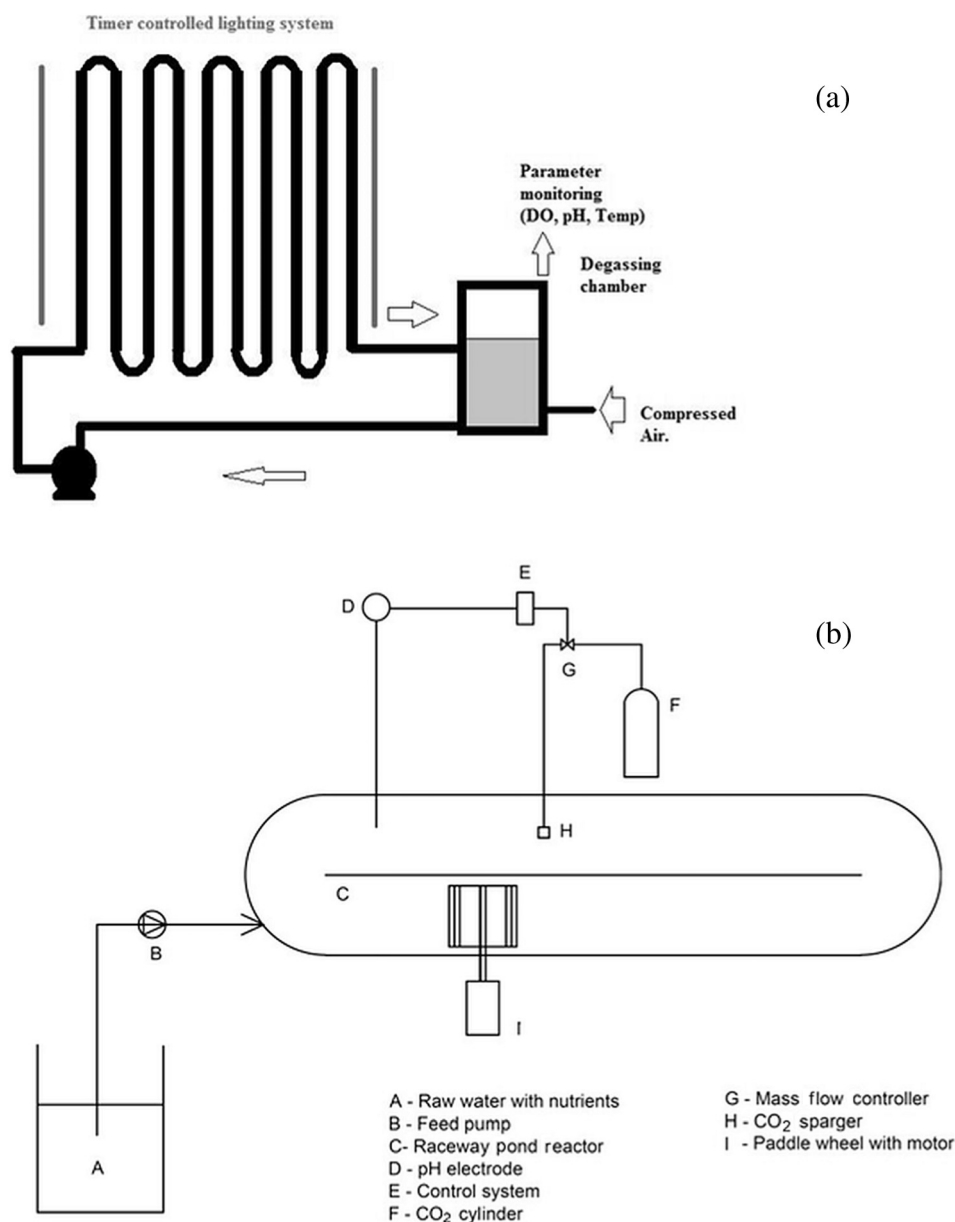


Fig. 1. (a) Schematic diagram of tubular photobioreactor. (b) Schematic diagram of raceway pond reactor.

composed nutrients are given overnight through a peristaltic (Watson-Marlow) pump. Harvesting is done on a daily basis of 50% to give a two-day cell retention time. The incident radiation was monitored using a pyranometer. A photosynthetically active radiation sensor (PAR) was provided outside and at the floor of the RPR. Continuous monitoring of dissolved oxygen and pH was carried out using outdoor probes (Mettler Toledo, InPro Series). Carbon dioxide was supplied to the RPR as the primary carbon source. The CO₂ addition was feedback controlled by pH at 8.0. The daily solar irradiance, on an average, ranged from 0.1 to 1.5 CCM (5000 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) over 830–1730 h. The temperature fluctuated diurnally, based on the solar irradiance and was controlled only by natural evaporation. The media temperature during the experiment was ranging from 24 °C to 29 °C.

2.2. Source of water and microorganism

The use of natural sources of nutrient would aid us in having a positive effect on the ergonomics of the developed system. The sources of water used in this work are (1) sewage sludge, collected from Sewage Treatment Plant, Sewage Farm, Valiyathura, Thiruvananthapuram, Kerala, India (8° 27' 34.2966" N, 76° 56' 12.8076" E) and (2) sea water, collected from Vizhinjam Wharf, Thiruvananthapuram, Kerala, India (8° 22' 24" N, 76° 59' 23" E). The naturally occurring algal consortium from each water source was enhanced and modified to yield lipid with nutrient stress and temporal phase separation protocol. The use of indigenous, naturally occurring consortia, consisting of *Arthrospira* sp. along with *diatoms*, *Scenedesmus* sp., *Chlorella* sp. and *Enteromorpha* sp., improves the chances of survival and will have the highest biomass yield as the organisms are well acclimatized with the nutrient conditions [8,21].

2.3. Nutrients and its composition

Besides CO₂ and light, algae require nutrient to grow. The following formulation was used in RPR daily and was based on the water analysis. NH₄Cl – 2.8 g, KH₂PO₄ – 0.8 g, trace metal solution (16.7 ml) from a stock containing per litre: FeCl₃ – 2000 mg, MnCl₂·2H₂O – 500 mg, ZnCl₂ – 50 mg, NH₄Mo₇O₂₄·4H₂O – 50 mg, CoCl₂·6H₂O – 50 mg, CuCl₂·2H₂O – 50 mg, EDTA – 500 mg, NaSeO₃ – 100 mg, H₃BO₃ – 50 mg, AlCl₃ – 50 mg, HCl – 1 ml. The nutrients were made up to 1 L and pumped at a flow rate of 100 ml·h⁻¹. In order to shift the cell division pattern, nutrient addition to the reactor was done during night time. In PBR sewage was used as primary nutrient.

2.4. Microscopic observation

Regular monitoring of samples with an epifluorescence microscope (Leica DM2500) gives valuable responses [22]. Photoactive algae in consortia were observed by autofluorescence of the consortia under UV illumination. Accumulations of lipid in cells in the consortia were observed by staining with Nile Red dye, and fluorescence with Y3 filter [23]. The gross morphology of the algal consortia was observed with a Nikon stereo microscope. Underwater attached growth in the raceway pond was observed with a borescope.

2.5. Total biomass quantification

Total suspended solids (TSS) are solid materials including organic and inorganic that are suspended in the water i.e. neither dissolved nor settled [24]. A known amount of sample is filtered carefully using the filtration unit employing a pre-weighed Whatman No.1 filter paper pre-dried at 105 °C. The residue collected along with the filter paper is then transferred to a pre-dried and weighed silica crucible and heated at 105 °C for 1 h. After cooling, the crucible and its contents

are weighed again, and TSS is calculated from the difference in mass. The formula used:

$$\begin{aligned} \text{Total suspended solids (mg} \cdot \text{ml}^{-1}) \\ = (\text{Mass of crucible with dried biomass} \\ - \text{Mass of crucible without biomass}) / \text{Sample volume} \end{aligned}$$

2.6. Quantitative lipid content estimation: (chloroform–methanol 1:1)

Standardisation of extraction procedure has been carried out with various ratios of chloroform–methanol mixture. Maximum lipid extracted with 1:1 ratio of chloroform–methanol solvent. 100 mg of lyophilized sample is weighed accurately to which 4 ml methanol, 4 ml chloroform, 2 ml water and mix well. Centrifuge at 2000 r·min⁻¹ for 10 min, at 5 °C to 25 °C. Remove the upper layer (methanol/water) using a Pasteur pipette. Transfer the lower layer into a transparent tube with a Pasteur pipette. Leave the solids behind, if present. Re-extract the solids left at the bottom using 2 ml of 1:1 (v/v) chloroform/methanol. Pass the combined layers through anhydrous sodium sulphate using Whatman No. 1 filter paper in a funnel, into a pre-weighed container suitable for a rotary evaporator. Remove the solvents using a rotary evaporator under reduced pressure, at 40 °C. Calculate the mass of the lipid:

$$\text{mass of lipid} = (\text{mass of container} + \text{extracted lipid}) - (\text{mass of container})$$

Calculate the lipid content by mass difference, as given below

$$\begin{aligned} \text{Lipid content} = \text{amount of lipid extracts} / \text{mass of original sample} \\ \times 100\% \end{aligned}$$

2.7. Physico-chemical analysis

The pH and dissolved oxygen were determined by portable probes (Ponsel). Nitrate and phosphate were measured using a UV-visible spectrophotometer [25]. EDTA titrimetry was followed for calcium and magnesium estimation [25]. Estimation of silicate was done by molybdosilicate method [25]. BOD and COD were estimated by standard methods [24].

2.8. Statistical analysis

For batch studies as well as pilot plant studies, duplicates were carried out to confirm the findings. For PBR studies, all the nutrient loads were carried out twice to confirm the results. For DNA studies in RPR, the results were confirmed using replicates. The data acquired were subjected to statistical analysis for variability check and standard errors. The level of replication was subjected to T-Test for assuring the acceptance of the results.

2.9. Total genomic DNA estimation

Optimised DNA extraction procedure was used on RPR samples collected at various time intervals in a day. Samples were collected at every 10 am and 5 pm to investigate the cell division pattern. Collected samples were immediately subjected to further DNA extraction procedure.

1 L of the microalgal sample was collected, settled, concentrated and fed into a mechanical attritor for cell disruption. The cell suspension was collected and stored in a clean 2 ml Eppendorf tube. Organic solvents such as phenol and chloroform interact with hydrophobic components of protein and lipoprotein, causing denaturation. The precipitate of denatured cellular material remains within the organic phase or collect at the interface between the two phases which is separated by

centrifugation. Lipids will partition effectively into the organic layer. Chloroform was used to separate aqueous and organic phase and also help phenol to remove proteins. Isoamyl alcohol was used as an antifoaming agent during extraction. The ratio of phenol:chloroform:isoamyl alcohol was 25:24:1.

The DNA from cell lysates and the suspension was recovered by the use of isopropanol. With the aid of isopropanol, reversible denaturation (i.e., the helical structure is extensively destroyed) and subsequent precipitation of DNA occur. Ethanol (70%) was used for washing the DNA because alcohol soluble salts are removed in this step and making final DNA clearer. Absolute alcohol could not be used due to its high evaporation rate. The DNA pellets were resuspended in TE buffer or Milli-Q water (if stored). Suspension was analysed in a Biophotometer.

2.10. Growth modelling

The growth dynamics of microalgae in consortia is significantly different than that in a pure culture. But the growth rate of consortia is still a function of irradiance and follows equation $\mu = f(I_{avg})$ where μ is the specific growth rate (d^{-1}) and I_{avg} is the average irradiance received by the culture ($\mu mol \cdot m^{-2} \cdot s^{-1}$). The biomass concentration was determined daily by measuring absorbance at 750 nm with a spectrophotometer (UV/Vis Spectrophotometer, Thermo Fisher Scientific Inc., USA). Incident irradiance was measured by a PAR sensor. Assuming monod kinetics, specific growth rate, μ follows as:

$$\mu = \frac{\mu_{max} I_{avg}}{I_k + I_{avg}}$$

Here μ is the specific growth rate (d^{-1}), μ_{max} is maximum specific growth rate (d^{-1}), I_{avg} is the average irradiance ($\mu mol \cdot m^{-2} \cdot s^{-1}$), and I_k is irradiance saturation constant ($\mu mol \cdot m^{-2} \cdot s^{-1}$). The average irradiance was calculated from the expression:

$$I_{avg} = \frac{I_0}{\varphi_{eq} K_a X} \left(1 - \exp(-\varphi_{eq} K_a X) \right)$$

Here, K_a is the extinction coefficient of the biomass, I_0 is the incident irradiance on the culture surface, and φ_{eq} is the length of the light path from the surface to any point in the bioreactor. For RPR system, ideally, $\varphi_{eq} = d$, the depth of RPR. If $\varphi_{eq} < d$, then light does not reach the bottom of the RPR. For tubular photobioreactor systems, φ_{eq} is a function of tube diameter φ and the angle of declination (θ) of the light source from the vertical. In our case, θ is 0° . $\varphi_{eq} = \varphi / \cos \theta$.

For industrially viable large scale production of microalgae, volumetric productivity (P_v) and areal productivity (P_a) of lipid rich biomass must be maximised. The volumetric productivity, P_v is represented as $P_v = \mu X$. Here, P_v is the volumetric biomass productivity ($mg \cdot L^{-1} \cdot d^{-1}$), μ is the specific growth rate (d^{-1}) and X is the biomass concentration of culture ($mg \cdot L^{-1}$), which was measured regularly. Consecutively, areal productivity (P_a) needs to be calculated. The volumetric productivity calculation is common for both open pond system and tubular PBR system, but areal productivity varies as the incident area is different. For tubular PBR, areal productivity is calculated as:

$$P_a = P_v \left(\frac{\pi \varphi^2}{4N\varphi} \right)$$

Here, P_a is areal biomass productivity ($mg \cdot L^{-1} \cdot d^{-1}$), P_v is the volumetric biomass productivity ($mg \cdot L^{-1} \cdot d^{-1}$), φ is the tube diameter (m) and N is the number tubes in the array.

Similarly, in an open pond system, areal productivity depends on the area of the pond exposed.

$$P_a = P_v \frac{A}{d}$$

Here, A is the exposed area (m^2) and d being the depth of the pond (m).

3. Results

3.1. Chemical analysis of nutrient sources

Table 1 depicts the physicochemical estimation of the seawater collected for the use as culture media. The supplementary nutrients were decided based on this evaluation. Table 2 illustrates the physicochemical values of the sewage sludge collected from the sewage farm. A 50% diluted sludge has the nutrient composition as represented in Table 3.

Table 1
Physico-chemical parameters of collected seawater

Parameters	Values
Initial phosphate (PO_4^{3-}) in fresh seawater/ $mg \cdot L^{-1}$	0.149
Initial silica (SiO_2) in fresh seawater/ $mg \cdot L^{-1}$	0.169
Salinity	33×10^{-12}
pH	7.9
DO/ $mg \cdot L^{-1}$	8.1
Total suspended solids/ $mg \cdot L^{-1}$	10

Table 2
Physico-chemical parameters of collected sludge

Parameter	Values
Biological oxygen demand (BOD)/ $mg \cdot L^{-1}$	8
Chemical oxygen demand (COD)/ $mg \cdot L^{-1}$	83
Total suspended solids (TSS)/ $mg \cdot L^{-1}$	15
Coliform presence	<4 MPN
pH	7.19

Table 3
Chemical estimation of 1:1 diluted sludge (used as media)

Parameter	Values
Silica (Si)	2.40×10^{-7}
Nitrate (NO_3^-)	2.5×10^{-8}
Calcium (Ca)	2.004×10^{-5}
Magnesium (Mg)	6.141×10^{-5}
Phosphate (PO_4^{3-})	1.63×10^{-7}

3.2. Cell disruption

The samples were subjected to cell disruption by different methods like physical grinding and attrition with zirconium beads. The time duration of beating, weight of zirconium beads and speed of rotor rotation were optimised. The attritor was calibrated at $1500 \text{ r} \cdot \text{min}^{-1}$ for 20 min with 150 g of zirconium beads. With this calibration, bead beating seems to be very effective for the better quantity, but purity was not much improved. Total gDNA extraction via this method results in the improved quantity of total genomic DNA than extraction but purity obtained was reduced. Another method adopted for cell disintegration was a cell beater that has the same principle as that of an attritor the difference being the beads used and the speed of operation. The beads used

were glass beads of 0.1 mm to 1 mm, and the speed of operation was $5000 \text{ r} \cdot \text{min}^{-1}$. This method gave sufficient purity and greater quantity. The results are as depicted in Table 4.

Table 4
Optimisation of cell disruption

Cell disruption methods	Biophotometer reading	A_{260}/A_{280}	A_{260}/A_{230}	Quantity of DNA/ $\mu\text{g} \cdot \text{ml}^{-1}$
Physical grinding	21.4	1.65	2.17	0.5715
Physical grinding with lysozyme	84.8	1.54	1.43	2.15
Attritor	9.3	1.62	2.51	4.45
Cell beater	6.2	1.30	2.21	24.6

3.3. Biomass and lipid profiles

Fig. 2(a) displays the biomass profile of culture in PBR with sewage under various lighting cycles. Fig. 2(b) depicts the Biomass, Dissolved oxygen and pH profile of microalgal culture in a PBR at different nutrient loadings. The light exposure was 16 h of light and 8 h dark. The nutrient load provided was $\text{NH}_4\text{Cl} - 52.22 \text{ mg} \cdot \text{L}^{-1}$; $\text{KH}_2\text{PO}_4 - 3.75 \text{ mg} \cdot \text{L}^{-1}$; Trace nutrients $- 1.2 \text{ mg} \cdot \text{L}^{-1}$ and a one-time addition of 1 g NaSiO_2 for enhancing the growth. The nutrient levels were set in accordance

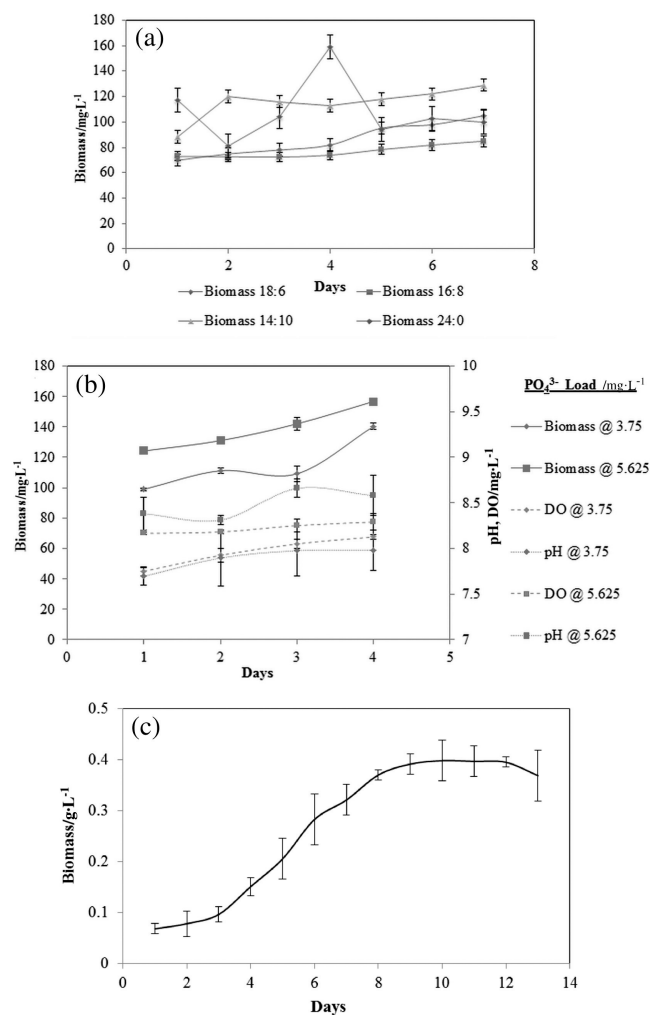


Fig. 2. (a) Biomass profile of culture in PBR with sewage under various lighting cycles (light hours:dark hours). (b) Biomass, pH and DO profile of seawater culture at various nutrient concentrations in PBR. (c) Batch growth curve of consortia with sewage.

with the Redfield ratio, an atomic ratio of nutrients found in phytoplankton, N:P = 16:1 [8].

The continuous analysis of the microalgal sample collected from the raceway pond reactor, under seawater as nutrient source, twice daily over a period of 10 days was carried out in triplicate and the data obtained is as shown in Fig. 3. The biomass profiles of the system during the period of study are presented in Fig. 3. The quantification of Total gDNA of the sample is done by the optimised CTAB protocol for microalgal species. The expected DNA quantity plotted gives an idea of how the genetic matter is increasing overnight. The Total gDNA is an indication of the microalgal cell division [26]. The process of daily 50% harvesting halves the genetic matter of the whole system, which is readjusted by the overnight cell division.

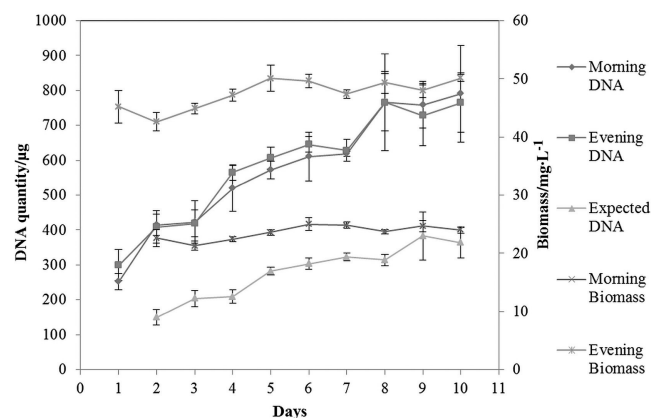


Fig. 3. Biomass profile and Total gDNA quantity over the days and the Expected morning values.

A plot of the Total genomic DNA values over the days along with the calculated Biomass values during the morning will give an overview of how the biomass growth responds. The difference of the TgDNA values between morning and the evening samples describes the cell division during daytime and the difference of the morning value to the previous day evening value represents the overnight multiplication of genetic matter. The comparison of lipid profile and the DNA difference values over the period of study are given in Fig. 4. In Fig. 4, Day difference (E-M) denotes the difference between Total gDNA quantities of evening samples and morning samples of the same day (cell division during

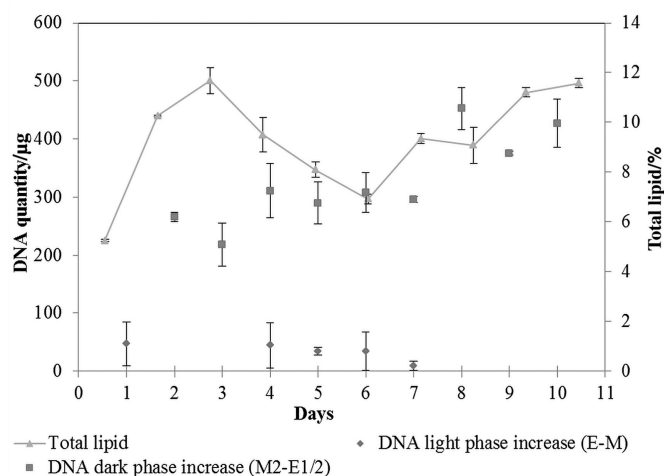


Fig. 4. Total lipid profile versus day time and night time DNA division.

day), Night difference (M2–E1) denotes the difference between Total gDNA quantities of next day morning samples and evening samples of the previous day (cell division during night).

A statistical analysis of the results shows ample replicability, and it was also observed that the replication was in accordance and supports the hypothesis.

Fig. 5 depicts the microscopic observations of lipid yielding consortia present.

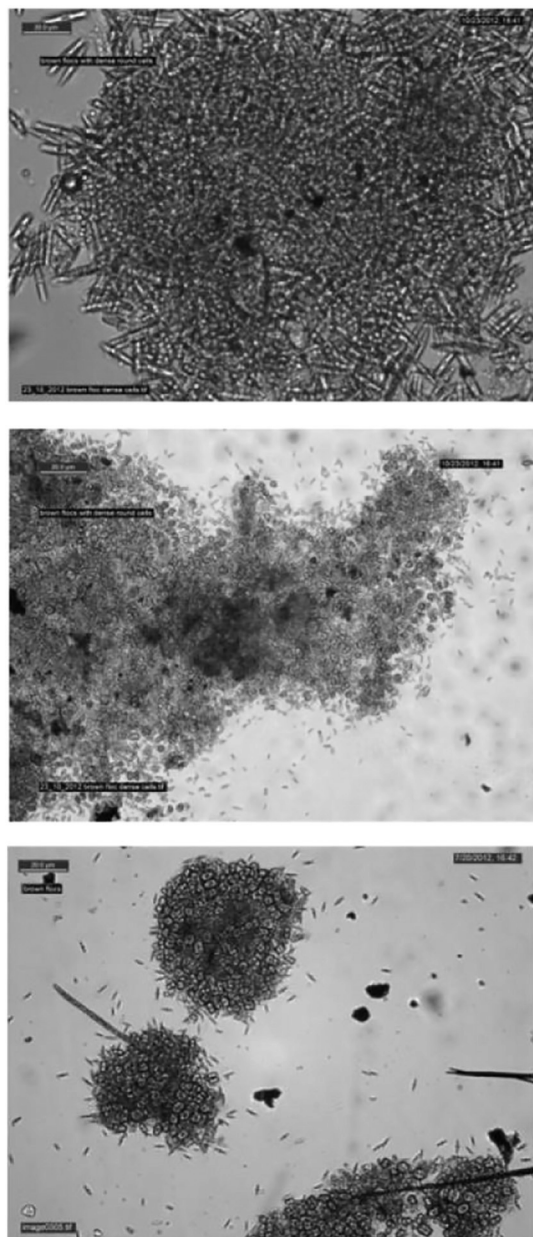


Fig. 5. Self-settling flocs of lipid producing microalgae consortium.

3.4. Effect of pH

At pH 6.5 condition biomass production was $140\text{--}150\text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$. Whereas at pH 8.1 it was $90\text{--}100\text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ (Fig. 6). At pH 6.5, total lipid production of biomass was up to 14%. But at pH 8.1, total lipid production of biomass improved up to 20%. Under N/L 2 condition with pH 6.5 total lipid production of biomass was up to 14% while with pH 8.1 it was 20% (Fig. 6). A 30% higher total lipid production was obtained under pH 8.1 and a higher biomass was obtained at a pH of 6.5.

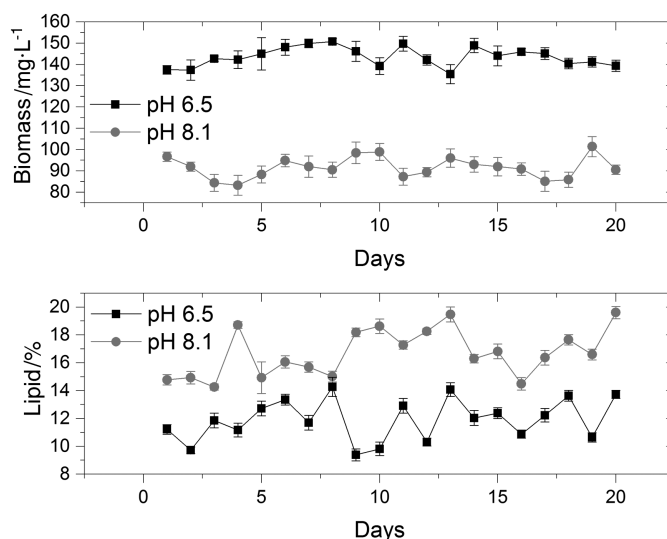


Fig. 6. Biomass and lipid profile at different pH.

4. Discussions

The objective of this study was to check the hypothesis of improving the lipid yield of microalgal consortium by providing temporal phase separation of cell division. By nature, cell division is an energy-intensive primary process of any cell [27,28]. The energy harnessed by a cell from photosynthesis will be primarily diverted to cell division, under nutrient-rich conditions, followed by biomass production and the leftover energy will be converted to storage food, protein or lipid depending on the ambient conditions [29]. To use algae as a lipid producer, it is required to bypass this energy consumption pattern, and to harvest the lipid, as and when it is produced, before it is lost to cellular respiration. To achieve this, temporal phase separation of energy harvesting phase and energy expending phase, nutrient loading pattern was altered. To arrest cell division (energy expending phase) during daytime (energy harvesting phase), a nutrient deplete condition was provided. By providing nutrient deplete condition during photosynthesis period has a two-fold effect, the consortia will produce maximum storage food as well as the production cycle will be leaning towards lipid production rather than protein. On the other hand during night time, to facilitate cell division, nutrient loading was performed. Biomass harvest was aptly placed between these two periods, to extract maximum amount of lipid.

The use of indigenous sources of water other than fresh water reduces the load on the overall economics of the system [30]. In order to accomplish this mission, non-potable and potentially nutrient rich sources like municipal sewage and seawater were considered. The physicochemical analysis of the sources was carried out in order to fix an appropriate supplementary nutrient. Tables 1, 2 and 3 depict the analysis results. The nutrient concentration should be adjusted such that the availability of phosphate and nitrogen follows the Redfield ratio [31]. The phosphates are essential for proper cell division whereas nitrogen is one of the major building blocks in cellular mass development.

In order to make algal biofuel economically viable at practical biomass yields, the phototrophic biomass should have the following characteristics:

- should be stable and perpetual
- should have inexpensive and complete separability from water either by coarse straining or rapid gravity settling for harvesting
- should be neutrally buoyant during daytime for resuspension in raceway ponds
- should have high lipid content

- e) there should be only limited grazing activity
f) should be capable of cultivation in clay lined open raceway ponds.

By employing the natural selection engineering protocol in conjunction with temporal phase separation we produced biomass which are automatically stable and perpetual, provided the process conditions are held stable. Inherent variability of seasonal temperatures, irradiances and the length of daylight, in case of RPR, cannot be controlled. Therefore, there will be variation in biomass. The day to day changes in irradiance leads to changes in yield rather than in diversity. When the culture is perpetual, there is no need to raise seed cultures to inoculate the cultivation ponds. Hydrodynamic selection pressures are applied to selected algae that are easily harvested. Even though different water sources were used the consortia was dominated by *Arthrospira* sp. along with *diatoms*, *Scenedesmus* sp., *Chlorella* sp. and *Enteromorpha* sp. The *Arthrospira* is a species which can survive in seawater, brackish as well as nutrient rich conditions [32].

For seawater culture with 16:8 light–dark cycles, the biomass, pH and DO profile at different nutrient concentrations in PBR are as depicted in Fig. 2(b). A higher nutrient load would generally result in higher biomass, but there is a higher limit to the biomass increase based on light availability and pH. The consecutive batch experiments revealed that the biomass tends to fall at higher pH. There is a need to adjust the pH at around 8.8 so as to get maximum biomass [33]. The batch studies reveal the effect of pH over biomass growth and lipid production (Fig. 6). It was evident that the levels of pH need to be adjusted to optimise the growth in the system. Generally lower pH favours an increase in biomass but lipid accumulation is favoured at higher pH. The pH can be adjusted by a feedback control over the CO₂ pumped into the system. From the batch experiment, it was also observed that the culture was saturated at 5.625 mg·L^{−1} of phosphate concentration and did not respond to further increase. The culture may be considered to be light limited inside the PBR.

A semi-continuous process on RPR was devised to facilitate maximum lipid accumulation in algae. In order to promote maximum lipid recovery, biomass harvest was introduced before the cells are allowed to utilise the energy for cellular respiration. Also, the cell division was restricted to night time by a time controlled addition of nutrients since the lipid accumulation in microalgae cells happens during daytime through photosynthesis under stress conditions. In order to shift the cell division pattern, nutrient addition to the reactor was done during night time and nutrient consumption pattern in the reactor shows added nutrients were depleted within 12 h of nutrient addition. Figs. 3 and 4 shows the effect of night time nutrient loading. In spite the 50% harvest of biomass, the cell number was auto adjusted at par with the pre-harvest consortia. However, the biomass of the consortia is not drastically improved as biomass increase requires energy from photosynthesis (Fig. 3).

The nutrient rich night conditions favour cell division and the nutrient deplete daytime arrests the cell division. This condition combined with evening time biomass harvest ensures minimum loss of lipid to cellular respiration. Fig. 4 gives the effect on lipid. It can be observed that whenever the cell division is less during the day, the harvested lipid levels are high, which confirms our hypothesis.

Table 5

Statistics of Biomass profile of seawater culture at various nutrient concentrations in PBR, corresponding to Fig. 2(b)

	PO ₄ ^{3−} load/mg·L ^{−1}	
	5.625	3.75
Variability based on sample	1.414	1.414
Average standard error	1.503	1.741
Degree of freedom	6	6
α	0.025	0.025
T-value	2.447	2.447
T-Stat	−0.422	0.310

Table 6

Statistics of Biomass profile and Total gDNA quantity over the days in RPR, corresponding to Fig. 3

	Biomass	DNA
Variability based on sample	0.919	9.192
Average standard error	0.631	2.907
Degree of freedom	23	23
α	0.025	0.025
T-value	2.069	2.069
T-Stat	0.220	1.643

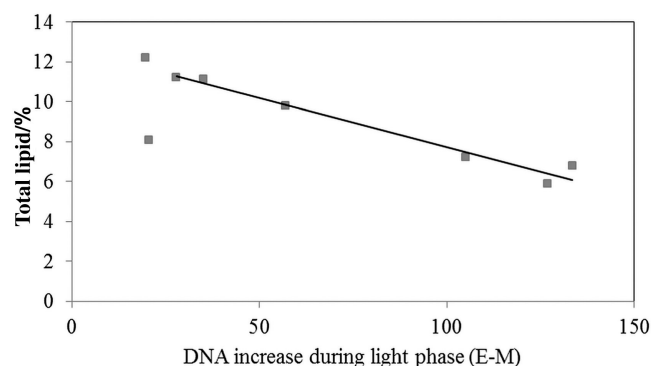
Statistical analysis (Tables 5–7) reveals that the replication of experiment supports the conclusion as well proves the replicability of the experiments under similar conditions.

Table 7

Statistics of total lipid profile versus night time DNA division in RPR, corresponding to Fig. 4

	DNA night increase	Lipid/%
Variability based on sample	4.243	0.0530
Average standard error	1.414	0.615
Degree of freedom	21	23
α	0.025	0.025
T-value	2.08	2.069
T-Stat	1.355	0.954

An inverse association can be formed with cell division during day time and the lipid produced. Fig. 7 depicts the relationship between lipid production and daytime cell division.

**Fig. 7.** Relation between lipid and cell division.

5. Conclusions

In the quest for sustainable alternate energy, microalgal biodiesel is the most promising outcome which could connect the old technologies of internal combustion engines to new age pollution free technologies. A novel approach of natural selection of consortia for lipid production was established in this work. The use of consortia would bring down the production cost of biodiesel as they do not require sterile conditions. The lipid maximisation of the consortia was successfully carried out with novel temporal phase separation protocol in combination with pH control. The consortia yielded up to 22% lipid with biomass of around 800 mg·L^{−1} in PBR. In outdoor open pond the yield was around 12%–15% lipid and around 600 mg·L^{−1} biomass. From the work it can be confirmed that the temporal phase separation has a positive lipid accumulation capability. Moreover approximately 40% increase in lipid levels could be achieved, in both modes of cultivation, without much loss in biomass.

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