

Fluorescence Transients as a Selection Tool for Marine Microalgal Consortia in a Raceway Pond Reactor for Biofuel Production

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In the search for renewable fuels, microalgae are seen as a potential source of oil that can be cultivated at large scale without competing with terrestrial food agriculture. Among the unsolved problems in the large scale production of microalgae for biofuel, is a rapid selection process based on lipid productivity. The fluorescence transients of consortia can be used as a measure of its lipid yield, thereby providing faster selection criteria. In order to test this hypothesis, a continuous (part harvested daily) raceway pond reactor with perpetual flocs containing microalgae consortia was developed. It is possible to select the dominant microalgal strains in the consortium by changing the process conditions. In this approach, process conditions that lead to higher lipid yield are desired. In this paper the photosynthetic activity of components of the algal consortia is measured by fluorescence transients and compared with lipid yield. It was found that, in our culture, brown microalga diatom dominant association had higher photosynthetic efficiency than a green filamentous microalgae association, and had higher lipid content.

Keywords: microalgae, biodiesel, fluorescence, photosynthetic efficiency, selection criteria

Introduction

Algae have received attention as a new biomass source for the production of biodiesel. Some of the main characteristics which set algae apart from other biomass sources are that it can have a high biomass and lipid yield per unit of light and area, do not require agricultural land or fresh water and cultivation is possible by wastewater and CO₂ from combustion gases¹. Marine microalgae can potentially be employed in an economically effective¹ and environmentally sustainable manner in a raceway pond reactor. Marine species are capable of generating as much as 40 times crude oil than other plants per acre of land area². An important criterion for selection of algae species for biofuel production is lipid yield per unit area^{3,4,5}. A phototrophic consortium has advantages over single species, if these consortia are stable and perpetual. In nature, the species that form the consortia are selected by its environmental conditions. In a reactor, these conditions can be engineered. Therefore, in order to select a suitable

consortium for lipid production, we do not select the species that make up the consortia, but we select the process conditions that lead to highest lipid yield. Photosynthetic efficiency is a measure of phytoplankton's ability to convert light energy to free energy of biomass. Photosynthetic efficiencies can be defined and measured by fluorescence, carbon dioxide fixation rate measurement and O₂ production rate measurement^{6,7,8,9}. The transient fluorescence method (OJIP) has the advantage of a very short measurement period⁹ and can be performed in the field. Fluorescence transients provide information on the efficiency of conversion of absorbed photons to electron transport in photosynthetic membrane under various physiological conditions. Under certain assumptions, it also allows the estimation of CO₂ fixation rates^{10,11,12,13,14}. In this paper we report OJIP fluorescence transients in phototrophic consortium developed under defined conditions in a raceway pond reactor (RPR) for biofuel production and its relation to lipid content. Higher efficiencies of energy utilization are expected to high rate of storage of energy in the form of lipid or carbohydrates.

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Materials and Methods

Experimental Setup

An open raceway pond reactor (RPR) made of opaque fibreglass was used to cultivate the microalgae consortium. It had an irradiant area of 1 m^2 , liquid depth of 0.30 m, and volumetric capacity of 300 L. The RPR was placed outdoor in an elevated platform to get sunlight with no shadows. The RPR was circulated with a two bladed paddle system and velocity was approximately 25 cm/s.

The medium was sea water collected from Vizhinjam Wharf, Kerala, India ($8^\circ 22' 24'' \text{ N}$, $76^\circ 59' 23'' \text{ E}$). Nutrients were supplied through a peristaltic pump. 50% of the culture was harvested daily, giving a 2 day hydraulic retention time and mean cell age. A pyranometer measured the radiation incident on pond surface. Photosynthetically Active Radiation Sensor (PAR) was provided outside and at the floor of the RPR. The irradiance was completely seasonal. For this experiment the illumination was 6-8 hours daily. A dissolved oxygen and pH probe were provided for continuous monitoring. Carbon dioxide was supplied to the RPR from bottles. CO_2 addition was feedback controlled by pH at 8.0. The temperature fluctuated based on the solar irradiance and was controlled only by natural evaporation. The following nutrients were supplied to the RPR daily: NH_4Cl – 2.8 g, KH_2PO_4 – 0.8 g, trace metal solution (16.7 ml) from a stock containing per litre: FeCl_3 -2000 mg, $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ -500 mg, ZnCl_2 -50 mg, $\text{NH}_4\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ -50 mg, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ -50 mg, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ -50 mg, EDTA-500 mg, NaSeO_3 -100 mg, H_3BO_3 -50 mg, AlCl_3 -50 mg, HCl -1 ml. The nutrients were made up to 1 litre and pumped at a flow rate of 100 ml/hr. This formulation was used based on the water analysis. 50% of the culture was harvested daily. The RPR was refilled with seawater and remaining biomass. Lipid content in harvested biomass was measured regularly. Samples were collected for study after the culture has been stabilised after several cycles of cell growth. The cell acclimatisation and culture stabilisation was given around 14 - 21 days. Also the experiment was conducted after several cycles of trial runs.

Microscopic observation

Samples from raceway pond were observed immediately with epifluorescence microscope (Leica DM 2500). Photoactive algae in consortia were observed by autofluorescence of the consortia under UV illumination. Cyanobacteria in the consortia

could be distinguished by autofluorescence under green light illumination, by the use of Y3 filter. Accumulations of lipid in cells in the consortia were observed by staining with Nile Red dye, and fluorescence with Y3 filter¹⁵. There is a clearly distinguishable difference in tint between fluorescence of cyanobacteria (greenish yellow) and lipid stained with Nile Red (reddish yellow). The gross morphology of the filaments was observed with a Nikon stereo microscope. Underwater attached growth in the raceway pond was observed with a borescope.

Photosynthetic efficiency measurement

The consortia were separated into green filaments and brown flocs by picking out the green filaments manually, leaving the brown. Fluorescence transients by pulse modulation¹⁶ measurement was carried out with AquaPen-C AP-C 100, PSI, Czech Republic, a portable, ultra-high sensitive (10 ng Chl/l) device. The OJIP responses are measured under twice on a sample. The samples that are placed in a cuvette immediately on removal from raceway pond and rapid fluorescence transient – OJIP protocol were recorded. The samples were left in the cuvette inside the instrument for 20 minutes for dark adaptation and the transients of dark adapted culture was recorded. Fluorescence transients are measured at excitation wavelengths of 450 and 620 nm. The fluorescence transient with 450nm excitation gives the microalgae photosynthetic activity and the transient with 620nm excitation, the activity of cyanobacteria. The OJIP protocol includes the following extracted and technical parameters. These parameters are experimentally derived from the fluorescence transients OJIP curve with logarithmic time axes. Standard points are identified on the OJIP curve with logarithmic time axis for comparison - F_0 (at 0 s), F_J (at 2-3 ms), F_i (at 60 ms) and F_p (Maximal fluorescence). Using these

Table 1—Extracted and Technical parameters

Formula	Formula Explanation
F_0	$F_0 = F_{50\mu s}$, fluorescence intensity at 50 μs
F_J	F_J = fluorescence intensity at J-step (at 2 ms)
F_i	F_i = fluorescence intensity at i-step (at 60 ms)
F_M	F_M = maximal fluorescence intensity
F_V	$F_V = F_M - F_0$ (maximal variable fluorescence)
V_J	$V_J = (F_J - F_0) / (F_M - F_0)$
Φ_{P_0}	$\Phi_{P_0} = 1 - (F_0 / F_M) = (F_V / F_M)$
Ψ_0	$\Psi_0 = 1 - V_J$
Φ_{E_0}	$\Phi_{E_0} = (1 - (F_0 / F_M)) \cdot \Psi_0$

points we calculate the the following measures of efficiency – Φ_{P_0} , Ψ_0 , and Φ_{E0} gives the photosystem II efficiency, Electron Transport Chain (ETC) efficiency and overall photosynthetic efficiency respectively. Φ_{P_0} effectively gives the light taken up by the PS II from the available photo synthetically active radiation. Ψ_0 signifies the conversion of captured photons to electrons and transferred into the ETC. Φ_{E0} is the product of Φ_{P_0} and Ψ_0 , and signifies the total efficiency of photosynthesis under the given condition¹⁷.

Total Biomass Quantification

Biomass is estimated as total suspended solids (TSS)¹⁸. 1 L of mixed sample from raceway pond was filtered in a pre-dried, pre-weighed GF/C filter. The retentate with filter was collected and transferred to a pre-dried and weighed silica crucible and dried at 105 °C. After 1 hour the crucible with filter cake was allowed to cool for 30 minutes, in a desiccator. The crucible along with filter cake was weighed and TSS was calculated.

$$\text{Total suspended solids (mg/l)} = \frac{[(A-B) \times 10^6]}{\text{sample volume, ml}}$$

where:

A = weight of filter paper + dried residue + weight of crucible in g, and

B = weight of filter paper + crucible in g.

Quantitative total lipid estimation: (chloroform-methanol 1:1)

Lipid was extracted from algal biomass using solvent extraction techniques by chloroform-methanol mixture¹⁹. For lipid estimation, the total algal biomass was collected from the reactor and dried. 100 mg of dry biomass was weighed, to which 4 ml methanol, 4 ml chloroform, 2 ml water is added and mixed well. The mixture was centrifuged at 2000 rpm for 10 min at 25 °C. The upper layer of methanol/water was removed using a pasteur pipette. The lower layer was transferred into a clear tube. The solids, if present are re-extracted using 2 ml of 1:1 chloroform/methanol. The lower layers were passed through anhydrous sodium sulphate using Whatman no.1 filter paper in a funnel, into a pre-weighed container suitable for rotary evaporator. The solvents were removed using a rotary evaporator under reduced pressure, at 40 °C. The weight of the lipid was calculated:

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

$$\text{Lipid content (\%)} =$$

$$\frac{\text{amount of lipid extracts (gram)}}{\text{weight of original sample (gram)}} \times 100$$

Results

Microscopic observations

The culture developed in RPR has two distinct phytoplankton colonies, one that grew as long green filaments, and the other as brown flocs. The green consortia had mainly *Enteromorpha sp.* The brown flocs had an association of *diatoms* and *cyanobacteria*. The green filamentous colonies were of two types: Free floating colonies dominated by *Enteromorpha sp.* in flower like formation, emerging from a central dark floc. The dark floc had *cyanobacteria*. The other types of growth were long filaments of *Enteromorpha sp.* rooted in a dark floc having association of cyanobacteria. These colonies had apparently been detached from walls. The conditions of the culture were modified such that the organisms are having a very limited chance of getting attached to the surfaces. Once *Enteromorpha* are attached to any surface by a disc like holdfast, they tend to outgrow every other organism. Figure 1 shows the observations of a selected brown floc under different filters for its activity and the presence of neutral lipids. The larger green filaments were manually removed to select the brown culture for microscopic observations.

Fluorescence transients of brown flocs and green filaments

The OJIP curves plots the absorbance readings with respect to time. The OJIP curve with logarithmic time axis gives a detailed view of the initial phase of OJIP curve where the transients give greater information. The OJIP curve with standard time axis gives an overview of the total duration of OJIP. The PSII efficiency for brown algae is consistently greater than that of green filaments. Both types of colonies are not light saturated as dark adapted fluorescence is marginally less than the light adapted fluorescence. The ETC efficiency is higher for brown flocs on nearly all days. The overall photosynthetic efficiency is higher for brown flocs as compared with green filaments. The 620 nm Φ_{E0} is marginally higher for brown flocs as compared with green filaments.

Total lipid and biomass

The total lipid content in biomass is shown in Figure 3a. The total biomass was also quantified and plotted against photosynthetic efficiency (Figure 3b).

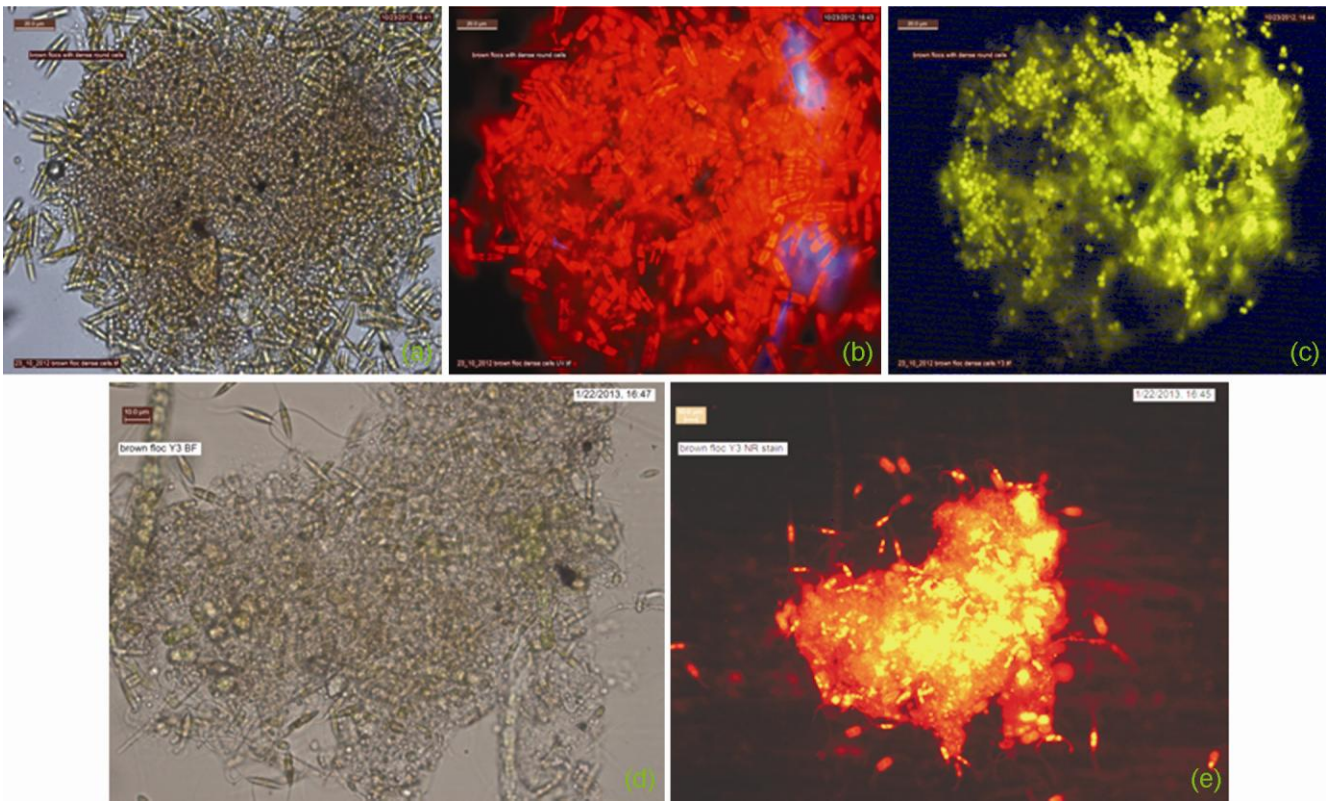


Fig. 1—(a) Brown floc comprising diatoms (in bright field); (b) same field under UV shows autofluorescence from diatoms; (c) same field under Y3 filter autofluorescence showing association of cyanobacteria inside the brown flocs; (d) Bright field view of a brown floc and (e) Nile red stained floc showing presence of lipids in diatoms

Table 2—Fluorescence transient (OJIP) parameters derived from OJIP curve

	GREEN RL450	GREEN RD450	BROWN RL450	BROWN RD450	GREEN RL620	GREEN RD620	BROWN RL620	BROWN RD620
F_o	290	307	25576	37387	110	130	14467	20503
F_i	413	395	45472	60351	142	152	21826	30126
F_i	443	449	46057	56850	149	166	21208	27049
F_m	484	473	65141	81168	156	159	26096	36173
F_v	194	166	39565	43781	46	29	11629	15670
V_i	0.634	0.53	0.503	0.525	0.696	0.759	0.633	0.614
F_m/F_o	1.669	1.541	2.547	2.171	1.418	1.223	1.804	1.764
F_v/F_o	0.669	0.541	1.547	1.171	0.418	0.223	0.804	0.764
Φ_{P_o}	0.401	0.351	0.607	0.539	0.295	0.182	0.446	0.433
Ψ_o	0.366	0.47	0.497	0.475	0.304	0.241	0.367	0.386
Φ_{E_o}	0.147	0.165	0.302	0.256	0.09	0.044	0.164	0.167

Notation: - Green: Green filament; RL immediate measurement of sample taken from pond in sunlight. 450: - excitation wavelength 450 nm. RD: - Measurement after keeping sample 20 minute dark condition

Discussion

Microscopic observations show that lipids are accumulating mainly in the diatoms. (Figure 1 d & e). The green filaments show no neutral lipid accumulation, except in certain filaments where pigmentation is lost and the filaments are starting to produce spores. Cyanobacteria are seen associated

with the brown flocs. We expect these to be nitrogen fixing cyanobacteria in a symbiotic association with lipid accumulating algae under nitrogen limiting steady growth conditions in the pond. For biofuel production, cyanobacterial association with algal consortia is undesirable, as there is an exchange of fixed carbon (secreted as lipid) for fixed nitrogen

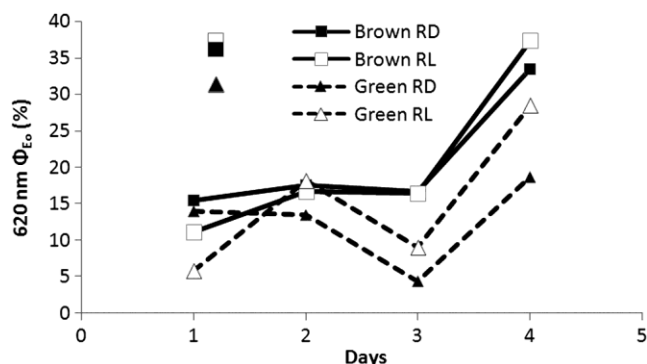


Fig. 2—Overall photosynthetic efficiency Φ_{E0} , with 620 nm excitation, in brown flocs and green filaments. It indicates the activity of cyanobacteria

between the alga and the cyanobacteria, resulting in overall decrease in lipid and increase in protein in the biomass. Lipid content in the total biomass can be fairly correlated to Φ_{P0} , Ψ_o , Φ_{E0} . The total biomass in the system did not always increase with Φ_{P0} , Ψ_o , Φ_{E0} . The consortia of brown flocs showed higher photosynthetic efficiencies Φ_{P0} , Ψ_o , Φ_{E0} than green filaments on all days. As the lipid accumulated mainly in brown diatoms, we find a relation between the overall photosynthetic efficiency and lipid. The figure 3c depicts the correlation between Φ_{E0} and lipid content. The results can be explained by noting that the fluorescence transients indicate the efficiency of energy transduction, higher efficiencies signifying greater PSII efficiency in conversion of absorbed light energy into free energy of ATP and NADPH. The ATP and NADPH generated may be used for carbon fixation to the reduction level of sugar (G3P), or it may be used to further reduce the fixed carbon to the reduction level of lipid. Higher Φ_{P0} , Ψ_o , Φ_{E0} with lower biomass yield indicates greater accumulation of lipid. Carbon fixation to the level of sugar increases mass. The total energy fixed remains constant whether the product is sugar or lipid.

More insight is gained by reading 620- Φ_{E0} (signifying cyanobacterial activity) along with 450- Φ_{E0} read with Figure. 3b). Symbiotic cyanobacterial activity is expected to be greater when algae accumulates and secretes lipid, adversely impacting further lipid accumulation. The increase in 620- Φ_{E0} on Day 4, is therefore interpreted not as a cause but as a consequence of increased lipid content. The net effect of cyanobacterial activity is increase protein biomass, through N-fixation, under N-limiting conditions. Cyanobacteria are undesirable in the consortium approach to algal biofuel. The lower

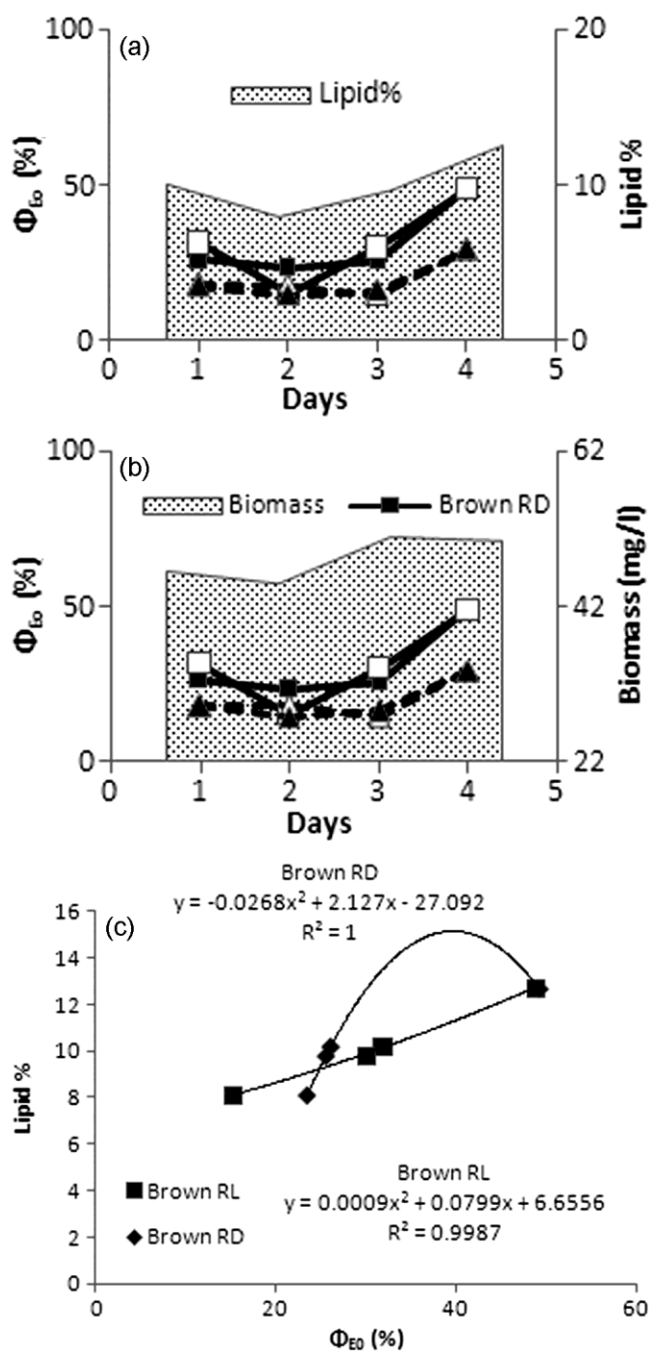


Fig. 3—(a) Lipid content in total biomass and overall photosynthetic efficiency (Φ_{E0}); (b) Total biomass and the photosynthetic efficiency (Φ_{E0}) and (c) Correlation between Φ_{E0} (%) and Lipid %

photosynthetic efficiency of the green filaments (*Enteromorpha sp.*) implies slower growth rate as compared with brown flocs. The question arises as to how does it survive in the system with the brown flocs? This is because of its ability to grow as wall attached growth, which is not removed or harvested

from the system efficiently. Hence, despite slower growth rates, it is able to establish a slow domination, because of its greater retention time in the system. In addition, the ability to periodically produce large number of zoospores, enables the wall growth filaments to outcompete suspended growth species over the medium term. Interestingly, lipid accumulated in sporulating filaments. Wall growth is undesirable in the consortium approach to algal biofuel.

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