Print: ISSN 2394-4315
Online: ISSN 2582-2276

Original Research Article

Isolation, Growth and Characterization of Lipids from *Pandorina morum* for Biofuel Production

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Received: December 2, 2019; revised: April 10, 2020; accepted: April 27, 2020

DOI: 10.17605/OSF.IO/XYT2B

Abstract: To meet the growing global energy demand, nations worldwide have considered renewable sources of fuel to offset the dependence on non-renewable sources like fossil fuels. Microalgal lipids can be exploited to produce oil, and to meet energy demands. The indigenous species can be studied for their efficiency in biomass and lipid production. This could minimize the growth and harvesting costs and thereby increasing the overall efficiency of biofuel production. In the present study, a locally well growing freshwater green alga *Pandorina morum* was isolated and studied its biomass and lipid yielding potential. Preliminary screening for the presence of lipids was carried out by staining the algal cells with Nile red dye. Presence of yellow globular structures indicated the presence of intercellular lipid droplets. FTIR analysis of the extract confirmed the presence of lipids. Growth studies in different media such as Chu- 10, Bold's Basal Medium and BG-11 were conducted. BG11 was found to be best medium to obtain maximum biomass. Total lipid was estimated using Bligh and Dyer extraction method. Extracted lipids were transesterified and qualitatively analyzed by GC-MS. The highest biomass production was observed in BG-11 medium with 2.0 × nitrate concentration (0.1884±0.09 g $L^{-1}day^{-1}$). The highest yield of lipid was noted in BG-11 medium with 0.5 × nitrate concentration (15.6888±0.21% of lipids by dry mass basis). The GCMS analysis revealed high abundance of hexadecenoic acid methyl ester. Nitrogen starvation increased lipid productivity with compromised biomass production. Growth conditions were studied to maximize biomass and lipid production. Aeration and polymer substrate were found to be helpful to increase the yield. *P.morum* showed promising growth and lipid yielding characteristics in optimized growth conditions, which could be further investigated to produce commercial quality-biofuel in accordance to biofuel standards.

Key words: Biofuel, lipids, microalgae, nutrient stress, *Pandorina*,

Introduction

Depleting stocks of fossil fuels and increment of anthropogenic carbon dioxide in the atmosphere have necessitated the research on sustainable environment-friendly energy resources. Biofuels are superior to fossil fuels in terms of decreasing carbon emission and reduction in overall climate foot prints (Wright *et al.*, 2011). One of the promising sources of biomass for alternative fuel production is rapidly growing microalgae. This third generation algal-based biofuel production technology can reduce the food-fuel competition, which can arise owing to the use of food crops for conventional biofuel production (Prieler *et al.*, 2013; A. Singh *et al.*, 2011b). Spent biomass after the fuel extraction can be effectively used in biochar production, which acts as an effective carbon sink (Bird *et al.*, 2011; Sayre, 2010). Nutraceutical applications of the spent biomass is also a topic of interest to produce livestock feed, proteins and extractable bioactives (Ravishankar *et al.*, 2012).

The capability of algae to grow in industrial, municipal and agricultural wastewaters and seawater can be utilized for biofuel production (McGinn *et al*., 2011). Some algal strains synthesize and accumulate a large amounts of neutral lipids (20–50% of dry weight) mainly in the form of triacylglycerol (TAG), which is stored in the cytosol. Algal biofuel production is more efficient compared to conventional crop-based fuels in terms of harvesting time and lipid productivity per unit area (Bux, 2013). It has been demonstrated that the growth conditions such as temperature, nutrient profile and other competing organisms like bacteria, fungi and protozoa affect the growth patterns and lipid and biomass productivity (Higgins & VanderGheynst, 2014). Hence screening of local, robust, well-established algal species for higher lipid productivity is the key factor in optimization of the overall efficiency of algal biofuel production. Biomass and lipid production of an algal species depend on various factors. pH and Nutrient optimization is one of the key feature to increase the yield. Nitrate, Phosphate and sulphate concentrations in the media were known to alter the lipid compositions. Hence, in this study, levels of those ions were studied for their effect on biomass and lipid productivity. Higher lipid accumulation can be achieved by nutrient deprivation. In few algal species such as *Chaetoceros gracilis* and *Chlorella vulgaris* nutrient deprivation can increase the lipid content ranging from 20 to 30% (Araujo GS *et al*., 2011).

Lipid extraction procedures seemed to be a key factor in the overall efficiency of the biofuel production technology. The effectiveness of cell disruption methods is crucial in efficient lipid extraction. Chemical methods such as solvent extraction is widely used. Single solvent systems especially n-hexane, chloroform, petroleum ether are used, however, these methods fail to lyse the cell membrane and extraction of lipids are not satisfactorily achieved. Combination of hydrophilic and hydrophobic solvents like methanol-chloroform is used to extract polar lipids such as phospholipids and glycolipids along with non-polar lipids. Several physical lipid extraction procedures could also be employed (Ranjith Kumar *et al*., 2015). However, cost involvement in physical extraction procedure is high, so that such extractions are limited (Borowitzka 1999; Singh &Gu 2010; Singh *et al*. 2011b).

The efficient lipid producing algal species should contain more than 30% of their cell weight as lipids (Mondal *et al*., 2017). The best performing microalgae can be obtained by screening of a wide range of naturally available isolates. However, every algal species will have its own lipid profile and it is, therefore, important to utilize species that have a suitable lipid profile for biofuel production. Therefore, this work is an attempt to screen the suitability of one such algal species, for biofuel production. To the best of our knowledge, no study has been carried out in the characterization of lipids from common freshwater green alga *Pandorina morum*. Accordingly, the objective of the study is to characterize the lipids from *P.morum*, which was isolated from a freshwater pond located at Mangalore, India. This study also tries to understand the growth rate, biomass production, and effects of different media on growth and lipid accumulation.

Materials and methods

Isolation and culture

Pandorina morum was isolated from the local freshwater pond, near Mangalore, India, and identified referring to the ICAR monographs on algae (Chlorococcales) (Philipose M T, 1967). About 100 ml of the samples from local pounds were collected in pre-autoclaved glass bottles. The sample was aseptically centrifuged, the pellet was inoculated into BG11 (Stein, 1973) medium. Isolation was performed according to (Andersen, 2005). Pure cultures were obtained by following standard culturing techniques on BG-11 medium. Batch cultures were grown in BG-11 medium in 100 ml borosilicate culture flasks and were maintained at 25 ± 2°C under a 16:8 light–dark cycle and an irradiance of ~30 µmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent lamps. Colonies from the exponential growth phase were used as inoculum in the subsequent experiments.

Monitoring lipids by Nile red staining

The algal cells were stained with a lipophilic stain Nile red (9-diethylamino-5-benzo á-phenoxazinone) at a concentration of

0.25mg ml^{-1} in acetone (Cooksey *et al*., 1987), observed under a fluorescence microscope(Olympus BX51). 436 nm excitation filter and 580 nm emission filter were used to capture the photographs. Nile red binds to intra cellular lipids to produce yellow colour at an excitation wavelength of 436 nm. Lipids stored as intracellular droplets appear as yellow globular structures.

Monitoring the cell growth

Cell growth was monitored by gravimetry (Hempel *et al*., 2012). Each determination made in triplicates every 5 days. The conical flask containing algal culture was centrifuged at 3000 g for 10 min. Pellet was washed twice with deionised water to remove residue salt content. The pellet was dried in a hot air oven at 60°C for 48 h. Dry biomass was determined using an analytical weighing balance.

Growth and lipid profile

The speciûc growth rate, doubling time, generation time, lipid content, lipid productivity and biomass productivity was calculated according to (Hempel *et al*., 2012; Moheimani *et al.,* 2013)

The speciûc growth rate was determined:

Specific growth rate (day^{-1}), $\mu = \frac{\ln(x_1) - \ln(x_0)}{t_1 - t_0}$ —— (1)

(where, x_1 and x_0 are the dry biomass weight at the end and the beginning of the exponential phase

t_1-t_0 is the duration of the exponential phase).

The time required for the cells to double their cell number was calculated:

Doubling time (days) = $\frac{\ln 2}{\mu}$ ———————— (2)

Generation of cells produced per day was calculated:

Generation time (day^{-1}) = $\frac{\mu}{\ln 2}$ ———————— (3)

Biomass productivity was calculated by the following equation

Biomass productivity (g $L^{-1}day^{-1}$) = $\frac{x_2 - x_1}{t_2 - t_1}$ ———————— (4)

[where X_1 and X_2 are the dry biomass per liter of the medium (g L^{-1}) on days t_1 (start point of cultivation) and t_2 (end point of cultivation) respectively, represented as an average productivity].

Lipid content was calculated:

Lipid content = $\frac{WL}{WA} \times 100$ ———————— (5)

WL and *WA* are weights of extracted lipids and weight of dry algal biomass

Lipid productivity was calculated:

Lipid productivity (mg $L^{-1}day^{-1}$) = Biomass productivity (BP) × lipid content (LC) ——— (6)

Efficiencies of the lipid extraction

One gram of the dry biomass, harvested at its logarithmic phase was used to study the lipid extraction methods. Chloroform, hexane (Ranjith Kumar *et al*., 2015), Bligh and Dyer (Bligh and Dyer, 1959) and Folch (Folch *et al*., 1957) methods were tested with a slight modification by incorporating 10 min of bath sonication. Sonication was given after the addition of $CHCl_3$: MeOH solution.

FTIR (Fourier-transform infrared spectroscopy) analysis

Functional groups of the crude lipid extract were analysed using ZnSe crystal attenuated total reflectance (ATR) FTIR spectroscopy (Thermoscientific Nicolet™). Scanning range was set to 200-4000 cm^{-1} and spatial resolution was set to 1 cm^{-1}. The solvent-free crude extract was compared with commercial olive oil (FIGARO, Lot no: 6111207) Functional groups were identified by comparing wave numbers to the previously reported literature (Vlachos *et al*., 2006).

Optimisation of growth

Inoculum preparation

Pure cultures were grown in BG-11 liquid medium till cultures attain exponential phase. Cultures were then centrifuged at 112 g in sterile centrifuge tubes. The pellet was washed and resuspended in respective medium to remove salts of the previous medium. The inoculum was then transferred to the respective medium

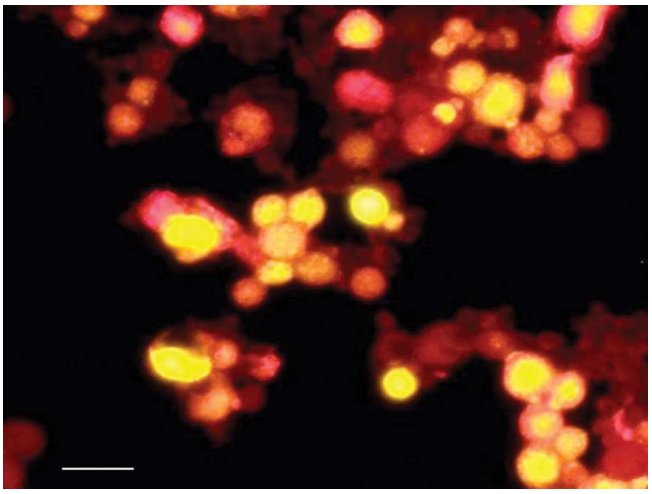

Fig. 1. Fluorescence microscopic image of algal cells stained with Nile red. Excitation filter was at 436 nm and emission filter was at 580 nm. Yellow-gold fluorescent droplets indicate the presence of intracellular lipids in the algal cells.

Culture conditions

For cell growth and lipid accumulation monitoring experiments, cells were grown in 250 ml of the liquid medium in Erlenmeyer flasks. Batch cultures were maintained at pH 6.5 (except in pH variation studies), temperature of 25 ± 2°C under 16/8 light/dark cycle. An irradiance of ~30 µmol photons $m^{-2}s^{-1}$ was provided by the cool white fluorescent lamps. Cultures were shaken daily at 100 rpm for 1 hr using an orbital shaker to reduce clumping and settling down of the algal cells. Weight of the dry biomass and extracted lipids were recorded to assess the biomass and lipid accumulation profile.

Effect of media on growth and lipid accrual

Effect of different media on growth and lipid content was assessed in BG -11, Chu-10,(Chu, 1942) and Bolds basal medium (BBM) (Bischoff and Bold, 1963). Biomass of whole flask was harvested at intervals of 5 days to analyze dry weight and lipid content. Lipids were extracted using sonication assisted Bligh and Dyer method. The experiments were carried out in triplicates.

Effect of pH on growth

Effect of pH on algal growth was studied in the BG-11 medium. Initial pH was set to 1-12 at an interval of 0.5 pH difference.

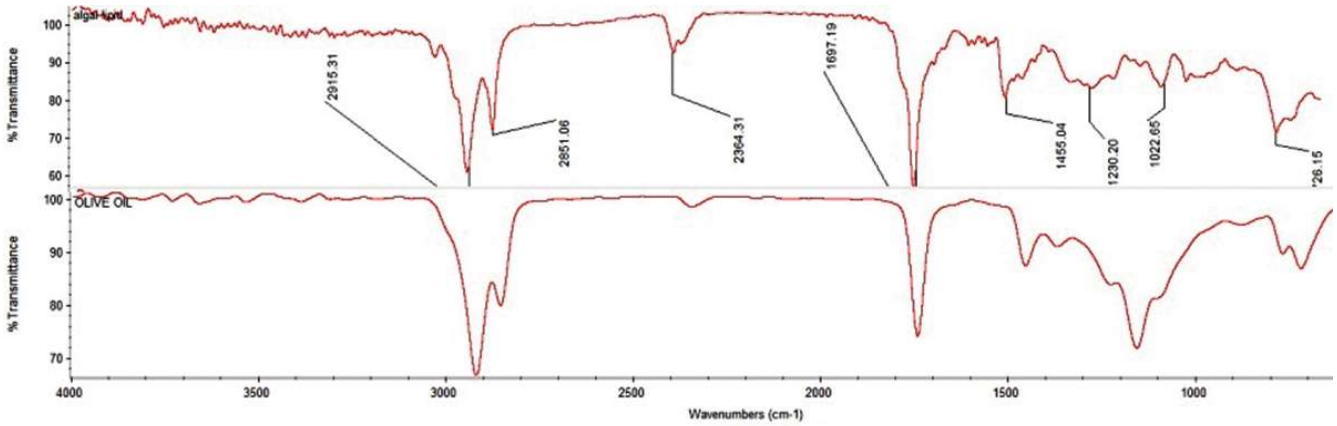

Fig. 2. Fourier transform infrared spectrogram of a) Algal lipids b) olive oil. Close resemblance of IR spectrum of the extracted lipid to that of commercial olive oil indicates the presence of the triglyceride. Bands at 2915 cm^{-1}, 2851 cm^{-1} (stretching and vibration of the aliphatic CH_2), 1697 cm^{-1} (C=O), 1455 cm^{-1} (bending of C-H), 1230 and 1022 cm^{-1} (C-O) confirms the presence of lipids

Constant pH was maintained by correcting the pH using 0.1M HCl or 0.1M NaOH at an interval of 10 days. Biomass productivity was assessed to select the best pH value for further experiments.

Effect of nitrates, sulphates and phosphates growth and lipid accrual

Major nutrients like nitrate, phosphate and sulphates of the BG-11 medium were selected for the study. 0x, 0.5x, 1.0x, 1.5x, 2.0x concentrations of nitrates(NO_3^-), sulphates(SO_4^{-2}), and phosphates(PO_4^{-3}) were prepared which corresponds to 0, 8.8, 17.6, 26.4, 35.2 mM of nitrates(NO_3^-), 0, 0.115, 0.23, 0.345, 0.46 mM of sulphates(SO_4^{-2}), 0, 0.25, 0.5, 0.75, 1.0 mM of phosphates (PO_4^{-3}), respectively in the final concentration. Variation in the concentrations was tested individually. The pH of all the media were set to 6.5 and cell growth and lipid accumulation were monitored.

Optimization of growth conditions for the upscale

The study was carried out in 1 l borosilicate Erlenmeyer flasks and 3.5 l capacity semi-hollow spherical glass bowls. Algae was cultured in three different set up: i) liquid medium; ii) liquid medium with aeration (filtered through 0.2 µm, PTFE); iii) liquid medium with aeration and 15 cm ×15 cm polypropylene strips as supports. The experiment was carried out for a duration of 50 days in BG-11 medium with 1.0X NO^{3-} and 0.5X NO^{3-} concentration.

Transesterification and identification of lipids

Extracted lipids were transesterified according to (Cancela *et al.*, 2012), briefly, the algal crude lipid extract was suspended in methanol containing 1% w/v NaOH, at 1:6 ratio. The solution was subjected to microwave radiation for 1 min. This solution was heated to 65°C for 15 min and cooled to room temperature. The FAMEs (Fatty acid methyl ester) were extracted thrice with 5 volumes of hexane; hexane is volatilized at room temperature to obtain the transesterified lipids. Qualitative analysis was done using a GC-MS (Gas chromatography–mass spectrometry) (Agilent7890 A, MS 5975) J&W 5Ms: 30m x 0.25mm ID x 0.25 film thickness column was used to separate the analyte. Column flow was 1.0 ml per min at constant helium flow.

Results

Preliminary detection of presence of lipids

Yellow-gold fluorescent droplets were observed in the algal cells under a fluorescence microscope (Fig. 1), indicating the presence of intracellular lipids in the algal cells. Presence of lipids is indicated by grease spot test. The FTIR bands at 2915 cm^{-1} and 2851 cm^{-1} indicated the presence of the symmetric and asymmetric stretching and vibration of the aliphatic CH_2 group. A band at 1697 cm^{-1} indicated the presence of C=O group, 1455 cm^{-1} indicated bending band of C-H group. Bands

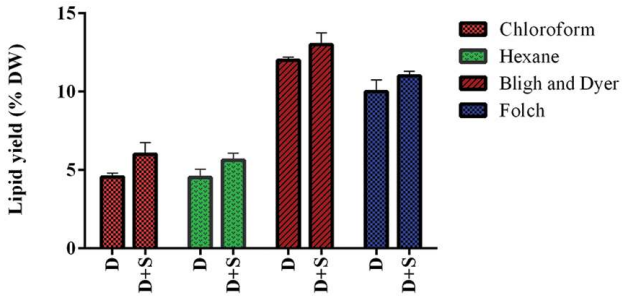

Fig. 3. Lipid extraction protocols with and without sonication treatment (D = Direct extraction; D+S = Direct extraction with sonication) Bligh and Dyer method showed the highest yield (13.0± 1.0%) whereas hexane is the least (4.53±0.53 %) (n =3)

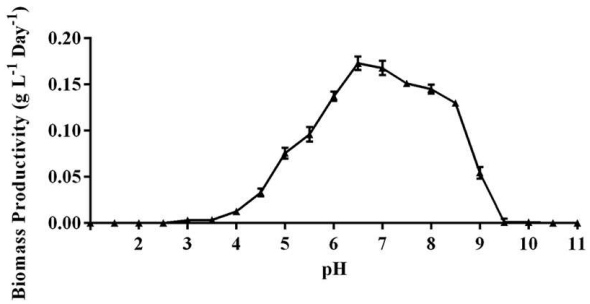

Fig. 4. Effect of pH on algal growth. pH at 6.5 is optimum for highest biomass production i.e. 0.1729± 0.007 g $L^{-1}Day^{-1}$ (n =3)

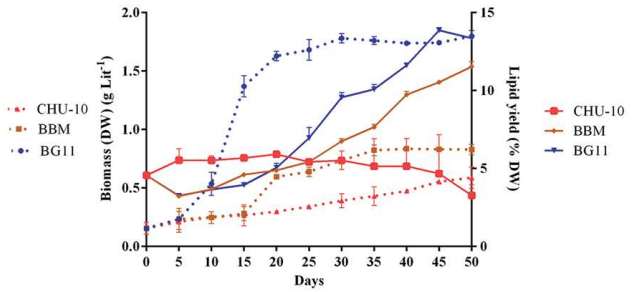

Fig. 5. Growth characteristics and lipid accumulation in *Pandorina morum* in different media. Highest growth rate and biomass production is observed in BG-11 medium. Specific growth rate is 0.081±0.002 and biomass production is 0.1645±0.02g $L^{-1}day^{-1}$ (n =3)

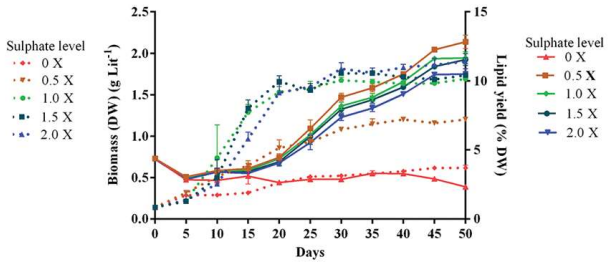

Fig. 8. Growth characteristics and lipid accumulation in *Pandorina morum* in BG11 medium with different Sulphates (SO_4^{-2}) levels. Highest biomass production (41.0171±12.45 $gL^{-1}day^{-1}$) is achieved in BG-11 medium containing 2.0 x SO_4^{-2} concentration. Lipid yield is highest (12.8278±0.47 %) in 0.5 x SO_4^{-2} concentration. (n =3)

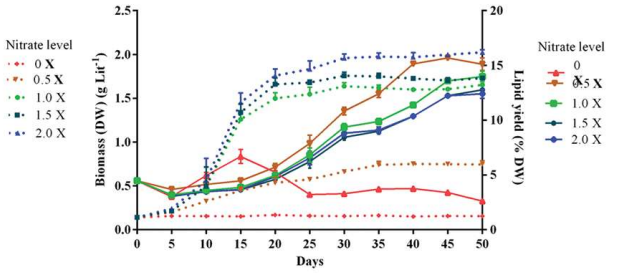

Fig. 6. Growth characteristics and lipid accumulation in *Pandorina morum* in BG11 medium with different nitrate (NO_3^-) levels. The highest biomass production is (0.1884±0.09 g $L^{-1}day^{-1}$) observed in BG-11 medium containing 2.0 x NO_3^- concentration. The highest lipid yield is observed (15.6888±0.21%) in 0.5 x NO_3^- concentration (n =3)

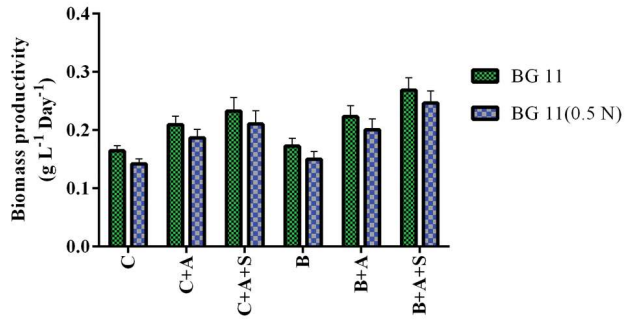

Fig. 9. Effect of different growth conditions on lipid productivity. (C = Erlenmeyer flask; B = Semi hollow spherical bowl; A = Sparging by aeration; S = Supported by polypropylene sheets) Aeration in combination with supporting sheets of polypropylene is found to be helpful to increase the lipid productivity (n =3)

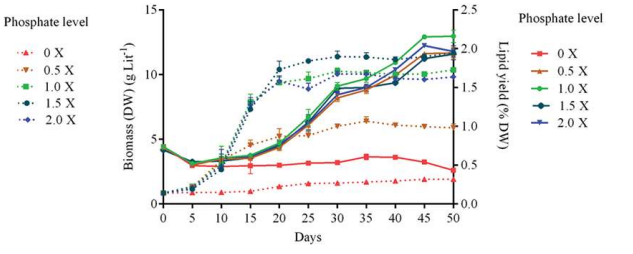

Fig. 7. Growth characteristics and lipid accumulation in *Pandorina morum* in BG11 medium with different Phosphates (PO_4^{-3}) levels. Highest biomass productivity is (41.0171±12.45 $gL^{-1}day^{-1}$) observed in BG-11 medium containing 1.5 x PO_4^{-3} concentration. Highest lipid yield is (12.9574±0.48%) in 1.0 PO_4^{-3} concentration. (n =3)

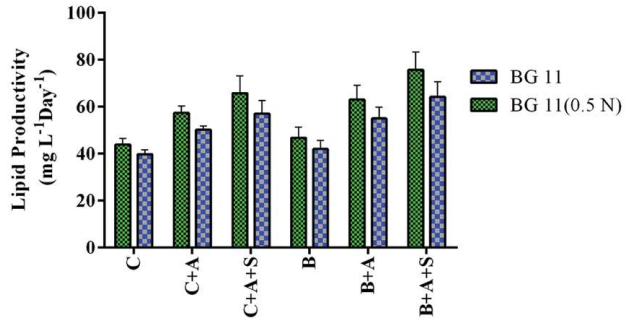

Figure 10. Effect of different growth conditions on biomass productivity. (C = Erlenmayer flask; B = Semi hollow spherical bowl; A = Sparging by aeration; S = Supported by polypropylene sheets) Aeration in combination with supporting sheets of polypropylene is found to be helpful to increase the Biomass productivity (n =3)

at 1230 and 1022 cm^{-1} indicated the presence of C-O group. (Vlachos *et al.*, 2006) (Fig. 2). Close resemblance of IR spectrum of the extracted lipid to that of commercial olive oil confirms the presence of the triglycerides.

Lipid extraction protocol

Among all the tested protocols, sonication assisted Bligh and Dyer method showed the highest yield of 13.0± 1.0% of the lipids per gram of the dry biomass (Fig. 3), whereas the least amount of lipids were extracted in hexane (4.53±0.53 %). There was a significant increase ($p<0.001$; t-test) in the efficiency of lipid extraction when sonication was carried out during the extraction procedure. Hence, further experiments were carried by sonication assisted Bligh and Dyer method.

Effect of pH on growth

Growth of *Pandorina morum* was assessed for the pH of 2–11 (Fig. 4). A pH of 6.5 was found to be the optimum for highest biomass production i.e. 0.1729± 0.007 g $L^{-1}Day^{-1}$. There was a significant decrease in the biomass production even when pH altered by 0.5 from the optimum. Biomass production at pH 6.0 and 7.0 were found to be 0.1372±0.004 and 0.1678±0.007 g L^{-1} Day^{-1}, respectively.

Effects of media on growth and lipid accrual

Among three media, Chu-10 (Stein 1973), Bold Basal Medium (Bischoff and Bold, 1963) and BG-11 (Stanier *et al.* 1971) showed the highest growth rate and biomass production with BG-11 medium (Fig. 5). Specific growth rate of 0.081±0.002 and a biomass production of 0.1645±0.02g $L^{-1}day^{-1}$ was observed in BG 11medium (Table 1). The ANOVA showed a highly significant change in the growth rate and biomass productivity in three different media. Further, the Bonferroni test indicated higher growth rate and biomass productivity in BG-11 medium ($p < 0.05$) compared to Chu-10 and Bold basal media. Lipid yield was highest in Chu-10 medium till the 20th day. But maximum lipid yield was observed in the BG-11 medium on the 45th day. Table 1 shows the results of specific growth rate, doubling time, generation time, biomass productivity and lipid production.

Effects of nitrates, sulphates and phosphates on growth and lipid accrual

In all the nutrient variation experiments, there was a significant change ($p< 0.001$) in the biomass productivity and lipid yield when nutrient levels were changed. The higher biomass production up to 0.1884±0.09 g $L^{-1}day^{-1}$ was seen in BG-11 medium with 2.0 NO_3^- concentration. The highest lipid yield up to 15.6888±0.21% per dry weight was seen in 0.5 x NO_3^- concentration on 45th day (Fig 6) There is a significant increase in the lipid yield when nitrates and sulphates are reduced by half of the original concentration. In phosphates (PO_4^{-3}) variation experiments, highest biomass productivity is achieved in 1.5 PO_4^{-3} (41.0171±12.45 $gL^{-1}day^{-1}$). Increase in the PO_4^{-3} level to 2.0 × results in a decreased biomass production (28.8279±2.84 $gL^{-1}day^{-1}$). There is a highest lipid yield in 1.0 ′ PO_4^{-3} (12.9574±0.48% lipids by dry biomass at 50th day). There is a significant decrease in the lipid yield in other concentrations of phosphate (Fig. 7). In sulphate (SO_4^{-2}) variation experiments, highest biomass production was achieved in 2.0 ′ SO_4^{-2} (41.0171±12.45 $gL^{-1}day^{-1}$). And a decrease in SO_4^{-2} level led to decreased biomass productivity (Fig. 8). Lipid yield was highest in 0.5 ′ SO_4^{-2} (12.8278±0.47 % of lipids by dry biomass at 50th day) and found to be decreasing in other concentrations of sulphate. The Table 1 shows the results of specific growth rate, doubling time, generation time, biomass productivity and lipid productivity.

Optimizing the growth conditions for upscale

Highest lipid productionty (Fig. 9) (75.6692±7.71 mg L^{-1} Day^{-1} in BG-11 with 0.5 × NO_3^-) and biomass productivity (Fig. 10) (0.2689±0.021 g L^{-1} Day^{-1}in BG-11with 1.0 × NO_3^-) was noted in the cultures of aerated semi hollow spherical bowl with immersed polypropylene strips support, whereas the least lipid productivity (39.7576±1.91 mg L^{-1} Day^{-1} in BG-11 with 1.0 × NO_3^-) and biomass productivity (0.1420±0.008 g L^{-1} Day^{-1}in BG-11 with 1.0 × NO_3^-) was observed in cultures in Erlenmeyer flasks. The ANOVA showed a significant increase ($p< 0.001$) in biomass and lipid productivity in cultures with aeration and

Table 1. Growth analysis of *Pandorina morum* in different media values are expressed as mean ± SD. Specific growth rate is maximum in BG 11 medium containing 1.5× $PO4^{-3}$ concentration (μ=0.0919±0.01 day^{-1}), Doubling time is least in BG 11 medium containing 1.5 × PO_4^{-3} concentration (7.5446±2.95 days), Generation time (Generation of cells produced per day) is maximum(0.1326±0.01 day^{-1}) in BG 11 medium containing 1.5× $PO4^{-3}$ concentration, Biomass productivity is highest (0.1884±0.09 $gL^{-1}day^{-1}$) in BG 11 medium containing 2.0 x NO_3^- concentration and Lipid productivity is maximum in in BG 11 medium containing 2.0X $NO_3^{\%}$concentration (42.4757±2.48 $mgL^{-1}day^{-1}$) is plotted against various medium and respective nitrate, phosphate and sulphate concentrations. (n=3)

Medium	Specific growth rate μ (day^{-1})	Doubling time (days)	Generation time (day^{-1})	Biomass productivity ($gL^{-1}day^{-1}$)	Lipid productivity ($mgL^{-1}day^{-1}$)
BG 11	0.081±0.002	8.5613±0.22	0.1168±0.01	0.1645±0.02	39.5144±3.01
BBM	0.054±0.005	12.9891±1.15	0.0775±0.01	0.0672±0.01	6.4288±0.06
CHU-10	ND	ND	ND	ND	0.8339±0.01
BG 11 (0.0X NO_3^-)	ND	ND	ND	ND	0.0071±0.00
BG 11 (0.5X NO_3^-)	0.0474±0.04	14.6168±0.57	0.0685±0.01	0.0605±0.00	6.8723±1.02
BG 11 (1.0X NO_3^-)	0.0840±0.03	8.3210±0.25	0.1028±0.04	0.1513±0.01	32.7723±6.48
BG 11 (1.5X NO_3^-)	0.0843±0.01	8.2264±0.21	0.1216±0.02	0.1590±0.08	32.4015±0.88
BG 11 (2.0X NO_3^-)	0.0836±0.01	8.2917±0.214	0.1206±0.01	0.1884±0.09	42.4757±2.48
BG 11 (0.0X SO_4^{-2})	0.0242±0.00	28.6293±0.64	0.0349±0.01	0.0476±0.00	0.6886±0.09
BG 11 (0.5X SO_4^{-2})	0.0491±0.01	14.1095±0.53	0.0709±0.02	0.1060±0.06	13.3610±1.23
BG 11 (1.0X SO_4^{-2})	0.0810±0.03	8.5613±0.22	0.1168±0.07	0.1549±0.09	33.5981±1.03
BG 11 (1.5X SO_4^{-2})	0.0843±0.02	8.2264±0.21	0.1216±0.01	0.1594±0.03	31.9153±9.42
BG 11 (2.0X SO_4^{-2})	0.0830±0.02	8.3597±0.92	0.1197±0.04	0.1758±0.04	38.9076±6.96
BG 11 (0.0X PO_4^{-3})	0.0242±0.00	28.6293±3.46	0.0349±0.01	0.0177±0.02	0.1471±0.02
BG 11 (0.5X PO_4^{-3})	0.0616±0.00	11.2484±0.10	0.0889±0.02	0.0839±0.02	9.6620±1.40
BG 11 (1.0X PO_4^{-3})	0.0810±0.00	8.5613±0.22	0.1168±0.02	0.1585±0.03	35.4638±6.32
BG 11 (1.5X PO_4^{-3})	0.0919±0.01	7.5446±2.95	0.1326±0.01	0.1806±0.12	41.0171±12.45
BG 11 (2.0X PO_4^{-3})	0.0827±0.02	8.3811±0.75	0.1193±0.03	0.1495±0.05	28.8279±2.84

polypropylene strips supports compared to liquid cultures with aeration.

Identification of lipids

Saturated fatty acids like dodecanoic acid methyl ester (C12:0), methyl tetradecanoate (C14:0), tetradecanoic acid (C14:0), pentadecanoic acid methyl ester (C15:0), hexadecanoic acid methyl ester (C16:0), octadecanoic acid (C18:0), eicosanoic acid (C20:0) and hexadecanoic acid (C16:0) were observed. Hexadecanoic acid methyl ester (C16:0) is found to be most abundant.

The following unsaturated fatty acids were observed during analysis. Linolenic acid methyl ester (C19:3) an ω-3 fatty acid with double bonds at Δ9, Δ12, Δ15 positions, 9-Hexadecenoic acid methyl ester (C16:1) a Δ9 mono

Table 2. Transesterification and qualitative identification of algal lipids and fatty acid methyl esters (FAME) by GC-MS. Hexadecanoic acid methyl ester is found to be relatively most abundant (22.94%).

No	RT	Name	Molecular weight	Relative abundance (%)
1	16.32	(E)-5-Octadecene	252.4784	0.36
2	18.244	Dodecanoic acid methyl ester	214.3443	0.87
3	19.141	3-Octadecene	252.4784	0.52
4	19.235	Hexadecane	226.4412	0.99
5	20.714	Methyl tetradecanoate	242.3975	1.83
6	20.116	Tetradecanoic acid	228.3709	0.66
7	21.484	Hexadecyl trichloroacetate	387.812	0.43
8	21.817	Pentadecanoic acid, methyl ester	256.4241	0.57
9	22.518	Linolenic acid methyl ester	292.4562	0.86
10	22.655	9-Hexadecenoic acid, methyl ester	268.4348	11.64
11	22.869	Hexadecanoic acid, methyl ester	270.4507	22.94
12	23.057	cis-9-Hexadecenoic acid	254.4082	3.63
13	23.27	Hexadecanoic acid	256.4241	12.22
14	24.023	10-heptadecen-8-ynoate methyl ester	278.43	0.54
15	24.433	[E]-5-Eicosene	280.5316	13.52
16	24.561	Linolenolinic acid methyl ester	292.4562	1.03
17	24.655	Phytol	296.531	1.64
18	24.801	Heptadecanoic acid, 16-methyl-, methyl ester	298.5038	0.56
19	25.143	Octadecanoic acid	284.4772	4.74
20	25.339	Eicosanoic acid	312.5304	0.73
21	25.98	5,8,11,14-Eicosatetraenoic acid, methyl ester	318.4935	0.87
22	26.032	5,8,11,14,17-Eicosapentaenoic acid, methyl ester	316.4776	3.11
23	26.869	(Z)-9-Octadecenamide	281.4766	1.23
24	27.066	Di(2-ethylhexyl) adipate	370.5665	0.61

unsaturated fatty acid, cis-9-Hexadecenoic acid(C16:1) a $\Delta 9$ mono unsaturated fatty acid, 10-heptadecen-8-ynoate methyl ester (C19:1) a $\Delta 10$ unsaturated fatty acid with triple bound at $\Delta 8$ position, linolenolinic acid methyl ester (C18:2) a $\Delta 9$ $\Delta 12$ di unsaturated fatty acid, 5,8,11,14-eicosatetraenoic acid, methyl ester (C20:4) $\Delta 5$ $\Delta 8$ $\Delta 11$ $\Delta 14$ tetra unsaturated fatty acid, 5,8,11,14,17-eicosapentaenoic acid methyl ester (C21:5) $\Delta 5$ $\Delta 8$ $\Delta 11$ $\Delta 14$ $\Delta 17$ penta unsaturated fatty acid. Among these 5,8,11,14,17-eicosapentaenoic acid, methyl ester was found to be most abundant.

Long chain alkenes such as (E)-5-octadecene, 3-octadecene, hexadecane, 5-eicosene and modified fatty acids such as hexadecyl trichloroacetate (C16:0), heptadecanoic acid 16-methyl methyl ester (C18:0) with methylation at $\Delta 16$; (Z)-9-octadecenamide(C18:1) with $\Delta 9$ unsaturation; phytol, a acyclic diterpene alcohol; and Di(2-ethylhexyl) adipate were recorded.

Among all the recorded molecules, C16 species contributes relative abundance of 52.85% in which hexadecenoic acid and its methyl ester is most abundant.

Discussion

Obtaining well growing pure cultures of algae depends on several factors including physical and chemical parameters of their naturally growing conditions such as pH, salinity, temperature, major and micro nutrients and so on(Andersen, 2005). The FTIR could be used to assess the quality of extracted lipids. Contaminants in crude lipid extracts can also be observed in FTIR spectrum. According to (Stehfest *et al.*, 2005), 3000-2800 cm^{-1} indicates the symmetric stretching of C-H group, ~1650 cm^{-1}indicates C=O of amides and proteins, N-H Symmetric deformation is observed at ~1540 cm^{-1}, ~1455 cm^{-1} indicates asymmetric deformation of CH_3 and CH_2 groups of proteins, ~1398 cm^{-1} indicates symmetric deformation of CH_3 and CH_2 groups of proteins, it also suggests symmetric stretch of C-O and COO^- groups. ~1250-1230 cm^{-1} shows asymmetric stretch of P=O groups of nucleic acids, ~1200-900 cm^{-1} indicates C-O-C symmetric starch of polysaccharides, ~1075 cm^{-1} is symmetric starch of silicates (Si-O). In our sample bands at 1455.04 cm^{-1} and 1230.20 cm^{-1} are probably due to contamination of proteins and nucleic acids respectively.

There are several quantitative lipid extraction methods such as electroporation, enzyme assisted extraction, microwave assisted extraction, hydrothermal liquefaction etc. However for commercial biodiesel production extraction process need to be energy efficient. Efficient extraction of total lipids cannot be achieved by a single solvent. Hydrophobic solvents such as hexane, petroleum ether, chloroform etc. fail to penetrate into the cells when used with fresh algal biomass. Our observation showed single solvents such as chloroform or hexane are less efficient when compared with multi solvent extraction techniques. In addition, we also observed that the sonication during extraction yields highest lipid content. Although mechanical force such as grinding or sonication is required to break cell walls to facilitate maximum lipid extraction, the energy required for the mechanical cell disruption is considerably high and contributes to the overall fuel production cost (Ranjith Kumar *et al.*, 2015; Wang *et al.*, 2015).

Nutrient stress reduces biomass productivity. Stress-induced lipid accumulation is correlated with cessation of cell division and decrease in the rate of synthesis of proteins and carbohydrate. This in turn increases the lipid content. Nitrogen is essential in overall protein synthesis; hence nitrogen deprived environment decreases biomass productivity. In the present study, increment of nitrate concentration in media increased the biomass productivity, while the lipid productivity was lowered. This may be the indication of the activity of those enzymes which are responsible for lipid metabolism. Nitrogen deprivation will lead to lipid accumulation (Boyle *et al.*, 2012; Mujtaba *et al.*, 2012). Nitrogen deprivation leads upregulation of several genes including those, responsible for the transportation of NO_3^-, NO_2^-, NH_4^+ and organic N sources. *GLN3* gene which encodes for the assimilation of NH_4^+ by the Gln synthetase-Glu synthase cycle was up-regulated. Hence nitrogen deprivation seems to be triggering those pathways which recruits new nitrogen sources, whereas biosynthetic pathways that utilize the assimilated nitrogen remain relatively unaffected (Miller *et al.*, 2010). RNA-Seq analysis suggests the correlations between DGAT1, DGTT1, and PDAT1 genes which encode for the pathways involved in accumulation of triacylglycerol (TAG) in *Chlamydomonas.* Nitrogen starvation effects membrane composition and protein biosynthetic pathways. Re-introduction of nitrogen leads to the reappearance of those proteins those involve in stress recovery. This suggests that cells implement normal cellular activities and vegetative growth after the re-introduction of nitrogen sources.

Nitrogen deprivation led to increased lipid productivity, whereas, when phosphate in the media lowered, lipid productivity was reduced. Increase in sulphate concentration did not result in higher biomass productivity, however when concentration is lowered in the media, biomass productivity is decreased whereas in lipid productivity increased.

Presence of unsaturated FAME and other unsaturated alkanes are prone to autooxidation process, rate of this process depends on position and degree of unsaturation. The bis-allylic positions

are more susceptible than allylic double bounds. Relative oxidation rates are 1 for methyl or ethyl esters of oleates (C18:1, Δ 9), 41 for linoleates (C18:2, Δ 9, Δ 12) 98 for linoleates (C18:3, Δ 9 Δ 12, Δ 15). Since most of the biodiesel sources contain higher amounts of esters of oleic, linoleic or linolenic acids, quality of the biodiesel will depend on relative amounts of these fatty acids. European B100 biodiesel standard (EN 14214), a limit of 12 percentage by total weight is set for the mono, di and tri unsaturated fatty acids whereas 1% if the unsaturation is more than four double bounds. ASTM D6751-02 do not have restriction on unsaturation levels (Barabás and Todoruþ, 2011). Higher chain number, high branching, and increased saturation in fatty acids result in poor ignition quality and cold flow properties. It also negatively effects the cetane number of the fuel. Whereas unsaturated short chain fatty acids yield better cetane number and good cold flow properties (Islam *et al*., 2013; Pinzi *et al*., 2013). In our experiment saturated C16 species are observed in higher abundance. Although the relative concentration of saturated fatty acids is relatively high, there are significant amount of unsaturated fatty acids (Stein, 1973; Chen and Durbin, 1994; Sheehan *et al*., 1998; Knothe, 2005; Guschina and Harwood, 2006; Boyle *et al*., 2012; Slade and Bauen, 2013; Valledor *et al*., 2014; Pavia *et al*., 2014; Ranjith Kumar *et al*., 2015; Monisha Miriam *et al*., 2017). Total percentage of unsaturated fatty acid molecules do not exceed more than 12 percentage, which compliance with the European B100 biodiesel standard (EN 14214).

Conclusion

In the last few decades various researchers have worked on the use of oil seeds for the production of biofuels. Production of second-generation fuels such as bioethanol and biodiesel from the oil-seeds of land plants has resulted in food-fuel controversy. Unlike other oil crops, microalgae grow very rapidly and many are exceedingly rich in oil. Therefore, production of biodiesel using microalgae is a viable alternative. In the present study, the preliminary investigations on nutrient stress studies and Fatty acid methyl ester quality assessment show that *Pandorina morum* is a promising candidate for the production of biodiesel. Nitrogen starvation leads increased lipid accumulation compromising with the biomass production. There is a need of selection and strain improvement in order to increase biomass production and lipid production to cope with fuel demand and cost of fuel production. Therefore, information of detailed cellular pathways and proteomic expression profiles are necessary to understand the lipid accumulation process in order to increase the efficiency of the process of algal biofuel production.

Acknowledgments

Sachin SP acknowledges the logistical support of the Star College scheme of the Department of Biotechnology, Ministry of Science and Technology, Govt. of India. The Authors would like to thank, Mangalore Jesuit Educational Society (MJES) for additional financial support. Authors express gratitude to Rev Dr Leo D'Souza S J for the review of the manuscript.

References

Singh A, Nigam PS, Murphy JD. 2011 . Mechanism and challenges in commercialisation of algal biofuels. Bioresource technology. 102(1): 26-34.

Andersen RA. 2005. Algal Culturing Techniques. Academic Press.pp:260-276

Araujo GS, Matos LJ, Gonçalves LR, Fernandes FA, Farias WR. 2011. Bioprospecting for oil producing microalgal strains: evaluation of oil and biomass production for ten microalgal strains. Bioresource technology. Apr 1;102(8):5248-50.

Barabás, I. and Todoruþ, I.A. 2011. Biodiesel quality, standards and properties. Biodiesel-quality, emissions and by-products. Intechopen. pp.3-28.

Bird MI, Wurster CM, de Paula Silva PH, Bass AM, De Nys R. 2011. Algal biochar–production and properties. Bioresource technology. 102(2):1886-91.

Bischoff HW, Bold HC. 1963. Some soil algae from Enchanted Rock and related algal species. University of Texas, Austin, Tex.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Canadian J. Biochem. and Physiol. 37(8): 911–917.

Borowitzka MA. 1999. Commercial production of microalgae: ponds, tanks, and fermenters. InProgress in industrial microbiology. Elsevier. Vol. 35, pp. 313-321.

Boyle NR, Page MD, Liu B, Blaby IK, Casero D *et al.* 2012. Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in Chlamydomonas. The Journal of Biological Chemistry. 287(19): 15811–15825.

Bux F. 2013. Biotechnological applications of microalgae: biodiesel and value-added products. CRC Press. Pp 86-91

Cancela A, Maceiras R, Urrejola S, Sanchez A. 2012 . Microwave-assisted transesterification of macroalgae. Energies. 5(4): 862–871.

Chen CY, Durbin EG. 1994. Effects of pH on the growth and carbon uptake of marine phytoplankton. Marine Ecology Progress Series. 109(1): 83–94.

Chu SP. 1942. The influence of the mineral composition of the medium on the growth of planktonic algae: Part I. Methods and Culture Media. J. Ecol. 30(2): 284–325.

Cooksey KE, Guckert JB, Williams SA, Callis PR. 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. J. Microbiol. Methods. 6(6): 333–345.

Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. The Journal of Biological Chemistry. 226(1): 497–509.

Guschina IA, Harwood JL. 2006. Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research. 45(2): 160–186.

Hempel N, Petrick I, Behrendt F. 2012. Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. J. Applied Phycol. 24(6): 1407–1418.

Higgins BT, VanderGheynst JS. 2014. Effects of *Escherichia coli* on mixotrophic growth of *Chlorella minutissima* and production of biofuel precursors. PLoS One. 9(5).

Islam MA, Ayoko GA, Brown R, Stuart D, Heimann K. 2013. Influence of fatty acid structure on fuel properties of algae derived biodiesel. Procedia Engineering. 56(1): 591–596.

Knothe G. 2005 . Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Processing Technology. 86(10): 1059–1070.

McGinn PJ, Dickinson KE, Bhatti S, Frigon JC, Guiot SR, O'Leary SJ. 2011. Integration of microalgae cultivation with industrial waste remediation for biofuel and bioenergy production: opportunities and limitations. Photosynthesis research. 109(1-3): 231-47.

Miller R, Wu G, Deshpande RR, Vieler A, Gärtner K *et al.* 2010. Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen deprivation predict diversion of metabolism. Plant Physiol. 154(4): 1737–1752.

Moheimani NR, Borowitzka MA, Isdepsky A, Sing SF. 2013. Standard methods for measuring growth of algae and their composition. In Algae for biofuels and energy. Springer, Dordrecht. pp. 265-284.

Mondal M, Goswami S, Ghosh A, Oinam G, Tiwari ON, Das P, Gayen K, Mandal MK, Halder GN. 2017 . Production of biodiesel from microalgae through biological carbon capture: a review. 3 Biotech. 7(2): 99.

Monisha Miriam LR, Edwin Raj R, Kings AJ, Adhi Visvanathan M. 2017 . Identification and characterization of a novel biodiesel producing halophilic *Aphanothece halophytica* and its growth and lipid optimization in various media. Energy Conversion and Management. 141(1): 93–100.

Mujtaba G, Choi W, Lee C-G, Lee K. 2012. Lipid production by *Chlorella vulgaris* after a shift from nutrient-rich to nitrogen starvation conditions. Bioresource Technology. 123(1): 279–283.

Pavia DL, Lampman GM, Kriz GS, Vyvyan JA. 2014 . Introduction to spectroscopy. Cengage Learning. pp:24-50

Philipose M T. 1967. I.C.A.R. monographs on algae (Chlorococcales). Indian Council of Agricultural Research. Vol 8. pp 150-200

Pinzi S, Rounce P, Herreros JM, Tsolakis A, Pilar Dorado M. 2013. The effect of biodiesel fatty acid composition on combustion and diesel engine exhaust emissions. Fuel. 104(1): 170–182.

Prieler S, Fischer G, van Velthuizen H. 2013. Land and the food–fuel competition: insights from modeling. Wiley Interdisciplinary Reviews: Energy and Environment. 2(2):199-217.

Ranjith Kumar, Ramanathan, Polur Hanumantha Rao, and Muthu Arumugam. 2015. Lipid extraction methods from microalgae: a comprehensive review. Frontiers in Energy Research. 2(1): 61.

Ravishankar GA, Sarada R, Vidyashankar S, VenuGopal KS, Kumudha A. 2012. Cultivation of micro-algae for lipids and hydrocarbons, and utilization of spent biomass for livestock feed and for bio-active constituents. Biofuel Co-products As Livestock Feed: 423.

Sheehan J, Dunahay T, Benemann J, Roessler P. 1998. Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae; Close-Out Report. No. NREL/TP-580-24190. National Renewable Energy Lab. Golden. pp:260-275

Slade R, Bauen A. 2013. Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. Biomass and Bioenergy. 53(1): 29–38.

Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriological reviews. Jun;35(2):171.

Stehfest K, Toepel J, Wilhelm C. 2005 . The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. Plant physiology and biochemistry. 43(7): 717–726.

Stein JR. 1973. Handbook of phycological methods: physiological and biochemical methods. Cambridge University Press. pp:121-160

Valledor, Luis, Takeshi Furuhashi, Luis Recuenco-Muñoz, Stefanie Wienkoop, and Wolfram Weckwerth. 2014. System-level network analysis of nitrogen starvation and recovery in *Chlamydomonas reinhardtii* reveals potential new targets for increased lipid accumulation." Biotechnology for biofuels. 7(1): 171.

Vlachos N, Skopelitis Y, Psaroudaki M, Konstantinidou V, Chatzilazarou A *et al.* 2006. Applications of Fourier transform-infrared spectroscopy to edible oils. Analytica Chimica Acta. 573(1): 459–465.

Wang D, Li Y, Hu X, Su W, Zhong M. 2015. Combined Enzymatic and mechanical cell disruption and lipid extraction of green alga *Neochloris oleoabundans.* International J. Mol. Sci. 16(4): 7707–7722.

Wright LA, Kemp S, Williams I. 2011. Carbon footprinting: towards a universally accepted definition. Carbon management. 2(1):61-72.