Lipid composition in microalgal community under laboratory and outdoor conditions

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Abstract

Microalgae are the most sought after sources for biofuel production due to their capacity to utilize carbon and synthesize it into high density liquid. Current energy crisis have put microalgae under scanner for economical production of biodiesel. Modifications like physiological stress and genetic variation is done to increase the lipid yield of the microalgae. A study was conducted using a microalgal consortium for a period of 15 days to evaluate the feasibility of algal biomass from laboratory as well as outdoor culture conditions. Native algal strains were isolated from a tropical freshwater lake. Preliminary growth studies indicated the relationship between the nitrates and phosphates to the community structure through the days. The lipid profile done using Gas chromatography - Mass spectrometry, revealed the profile of the algal community. Resource competition led to isolation of algae, aided in the lipid profile of a single alga. However, further studies on the application of the mixed population are required to make this consortium approach economically viable for producing algae biofuels.

Introduction

Microalgae are found in both marine and freshwater ecosystems. They contribute to more than half of the total primary production at the base of the food chain (Gushina & Harwod, 2009). The ability of microalgae to survive or proliferate over a wide range of environmental conditions results in the production of an array of many secondary metabolites, which are of considerable value in biotechnology fields including aquaculture, health and food industries (Andersen, 1996). Lipids act as a secondary metabolite in microalgae, maintaining specific membrane functions and cell signaling pathways while responding to the environment changes.

Aquatic Species Program [National Renewable Energy Laboratory (NERL), Department of Energy (DOE), USA)], provided impetus to the global biofuel research. Important advances in microalgal strain are isolation and characterization, physiology and biochemistry, genetic engineering process development and microalgal culture piloting apart from establishing a collection of over 3000 algae gathered from various sites and they were screened for their tolerance to environmental conditions and their ability to produce neutral lipids (Sheehan *et al.,* 1998). Major crop based feedstock for biodiesel are soybeans, canola oil, animal fat, palm oil, corn oil, waste cooking oil and jatropha oil (Demirbas, 2009). These feedstock's have limitations such as low biomass productivity, requirements of large land area, non renewability and dissatisfaction towards meeting the existing demand for fuel (Christi, 2007). In recent years, microalgae based biofuels are considered as viable alternative, in the context of food security and also requirement of large stocks to meet the growing fuel demand.

Triacylglycerol producing capabilities of microalgal cells are genetically controlled and the quantity and quality of oils produced by algal cells are directly proportional to the stimulus received from the surroundings. The major environmental factors include the nutrient deficit, light intensity, pH and temperature (Borowitzka, 1999). The optimization of these factors can maximize the oil production irrespective of the cultivation system. Production of algal oil need to be economically competitive and sufficient stock is required to meet the demand. Therefore to reduce the cost, most of the cultivation systems use freely available sunlight and sea water, despite daily and seasonal variations in light levels. The growth for each algal species will depend on environment conditions- temperature 20-30°C, essential medium of nitrogen, phosphorus, iron which are generally inexpensive. The convenient commercial scale production of microalgal biomass can be achieved by two methods, conventional open pond system and closed photo bioreactors. Open pond system: Algae cultivation in open pond production system has been used since1950's (Borowitzka, 1999). Large scale cultivation of microalgae in open pond systems relies on natural light for illumination. The most common strains used for the open pond systems are *Anabaena* sp., *Chlorella* sp., *Dunaliella* sp., *Haematococcus* sp. and Nostoc sp. (Chisti, 2006). The open ponds have a variety of size and shapes depending upon the location of cultivation. The algal biomass productivity achieved in open pond systems range between 10 - 50 gm^{-2}d^{-1}(Verma *et al.*, 2010), but they are economically more favorable due to lower establishment cost.

Closed photobioreactor: These are preferred over open ponds as they can be established and maintained either indoor or outdoor (Pulz, 2001). These bioreactors allow the cultivation of single microalgal species for prolonged duration under controlled conditions (Carvalho *et al.*, 2005) with the enhanced productivity while

operating cost serves as a major drawback. Open pond systems have certain advantages like low cost, low energy input requirement and large scale production but it has less efficiency, inefficient mixing and temperature fluctuation in the growth medium and less light availability compared to photobiorector.

Over the past few decades, several thousand algae and cyanobacterial species have been screened for high lipid content of which many species have been isolated and characterized under in situ and or outdoor conditions (Hu *et al.*, 2008). Algae provide natural material in the form of a lipid rich feed stock and scope for manipulation for production of biofuel. However, understanding of lipid content and metabolism to enable the manipulation of the process physiologically and genetically is still in infant stage. Biofuel through algae provide sustainable gasoline while addressing the current energy crisis (Ramachandra *et al.*, 2009). Beyond the level of lipid metabolism, the fundamental understanding of the regulatory mechanisms of lipid production and how it is related to the environmental control is required, which have been addressed in this work. The objectives of the study were: 1) to check the role of environmental parameters on the growth of the consortium in outdoor as well as indoor culture conditions; 2) lipid characterization of the consortium to understand the efficiency for biofuel production.

Materials and methods

Study area

Hesarghatta lake, the chosen sampling site for microalgae, is situated at a distance of 18 kms to the North West of Bengaluru city, Karnataka in India This sampling site was chosen so as to get maximum microalgae diversity and to enable characterization of lipid.

Collection of samples

Water quality: 500 ml of water sample was collected and bought to Aquatic ecology lab for the water quality analysis. The physical parameters such as pH, temperature (0C), salinity (mg L-1), total dissolved solids (mg L-1) and electrical conductivity (µScm-1) was measured on the site using Extech pH/conductivity EC500. The nitrate and phosphate concentration of the sample was measured in the laboratory using standard protocols as given in APHA (APHA, 1998).

Microalgal biomass: The epiphytic (*Hydrilla* sp.) microalgal sample was collected and stored in non-reactive plastic bottles following the procedure given by Kelly *et al.*, 2008.

Analysis of samples

Water quality analysis: The water quality parameters such as pH, salinity, total dissolved solids, electrical conductivity, nitrate concentration, phosphate concentration of triplicate samples were determined according to the standard protocols. The parameters were measured once in 2 days for the indoor and outdoor culture samples.

Community structure analysis: In order to study the variation in microalgal community for the cultured samples, the sample was analyzed two days once by observing 1 ml of the samples under microscope (40X magnification). The community structure of natural sample on 1st, 3rd, 5th, 7th, 9th, 11th, 13th and 15th day samples were analyzed.

In situ culturing

The culturing of the microalgae was done using Chu's freshwater medium (Bold & Wynne, 1978), since the sample showed presence of large number of diatom species. WC vitamin solution (Guillard & Lorenzen, 1972) was added to this medium to minimize the growth of bacteria in the inoculums (Debenest *et al.*, 2009).

Experimental setup

The experimental setup of outdoor and indoor culture conditions was maintained for comparative analysis of lipid quality with diverse community in each of the individual setup.

Outdoor culturing: Outdoor culturing refers to culturing of microalgal species in natural conditions. The outdoor culturing was maintained for 15 days with 2 replicates per day. 100mL media was taken in sterilized cotton plugged culture flask and to each 15 mL of inoculum (microalgal sample) was added.

Indoor culturing: The term indoor culturing refers to the culturing of microalgae in fixed laboratory conditions. Alike outdoor conditions, the culture was maintained for 15 days 2 replicates per day. 100mL media was taken in sterilized cotton plugged culture flask and to each flask 15 mL of inoculum (microalgal sample) was added. The light intensity of 1320 lx was provided using Compact fluorescent lamps (Philips Genie, Made in India) with 16:8 hours light and dark phase respectively.

Fig. 1. Process for the extraction and purification of lipids from microalgae
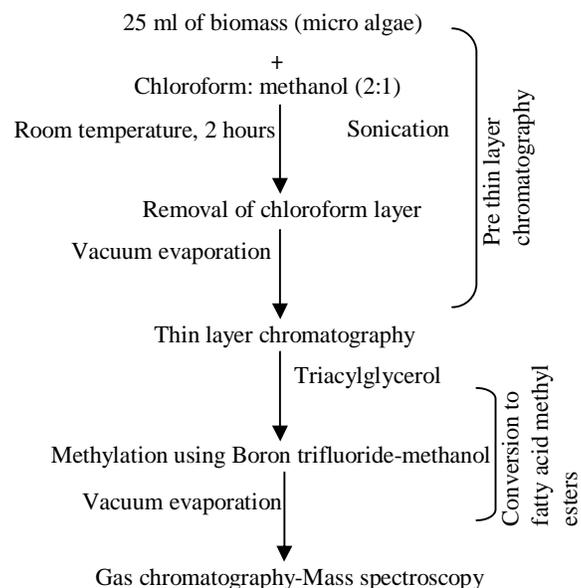
Analysis

The growth rate of microalgal community was analyzed. The percentage relative abundance for each alga every odd day was calculated using the following formula:

$$\text{Relative abundance} = \frac{\text{Abundance of individual species}}{\text{Total abundance of the community}} \times 100$$

Lipid analysis (Fig 1)

Pre-thin layer chromatography: 25 ml of the microalgal sample was sonicated (Pernet &Tremblay, 2003) in water bath for 2 hours at room temperature in order to disrupt the cell membranes, chloroform: methanol (2:1) was added as the extraction solvent. The chloroform layer was evaporated using rotary evaporator (Eppendorf Vacuum Concentrator 5301) to obtain lipids. This step was important since lipids are highly sensitive to hydrolysis and oxidation processes during storage (Sasaki & Capuzzo, 1984).

Thin layer chromatography: All samples were reconstituted in chloroform to make stock solutions. The stock solutions were spotted in bands onto silica gel TLC plates (Merck KGaA). The mobile phase consisted of a solvent system of hexane/diethyl ether/acetic acid (70:30:1 by volume) [Maloney, 1996]. The plates were developed by exposing the vapors of iodine crystals to stain the plates for visualizing neutral lipids. The samples were extracted and stored in -20 ^{0}C until further analysis (Mansour *et al.*, 2005).

Gas chromatography-mass spectrometry analysis: After the initial thin layer chromatography (TLC) lipid screening, the extracts were converted into fatty acid methyl esters (FAME) using Boron trifluoride-methanol and was heated in water bath at a temperature of 60^0C for 1 hour. The methylated sample was then purified further for GC-MS. The main focus of using GC-MS was purely for lipid identification rather than quantification. The injector and detector temperatures were set at 250^0C while the initial column temperature was set at 40^0C for 1 min. A 1 µL sample volume was injected into the column and ran using a 50:1 split ratio. After 1 min, the oven temperature was raised to 150^0C at a ramp rate of 10 ^{0}C min^{-1}. The oven temperature was then raised to 230^0C at a ramp rate of 30C min^{-1}, and finally the oven temperature was increased to 300^0C at a ramp rate of 10^0C min^{-1} and maintained at this temperature for 2 min. The total run time was programmed for 47.667 min. The mass spectra were acquired and processed using Agilent Chem Station (5975 C; Agilent, USA).

Table 1. Physico-chemical variables of natural and media composition

Water quality variables	Sample	
	Natural	Media composition
pH	9.42	5.26
Electric conductivity (µS)	150.7	152.30
Nitrates (mgL^{-1})	0.22	1.20
Phosphates (mgL^{-1})	0.17	0.40

Results and discussion

Physico-chemical parameter

The analysis of water quality variables such as pH, electric conductivity, nitrates and phosphates under natural condition and of media measured on alternative days is listed in Table.1

Table 2. Physico-chemical variables for 15 days in indoor culture conditions

Days	pH	Electrical conductivity (µScm^{-1})	Nitrates (mgL^{-1})	Phosphates (mgL^{-1})
1	7.56	264.50	1.57	0.13
3	7.45	270.50	0.81	0.15
5	7.47	264.00	0.89	0.12
7	7.25	254.00	0.49	0.17
9	7.78	280.00	0.68	0.11
11	8.02	244.50	0.85	0.26
13	7.66	312.50	1.03	0.19
15	8.09	269.00	1.37	0.31

Indoor culture conditions: pH in *in situ* culture conditions did not vary much (range 7-8, Table 2). This is due to the controlled light and dark conditions (16:8 hrs of light intensity 1320 lx). The electrical conductivity was also similar across days [Table 2] due to the restricted conditions. On the 1st day, the nitrate level increased due to the change in the micro algal community (mention

Fig. 2. Concentration of nitrates (mg L^{-1}) in indoor culture conditions

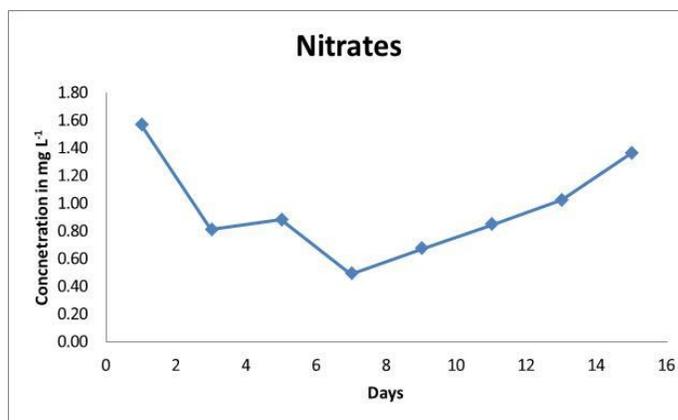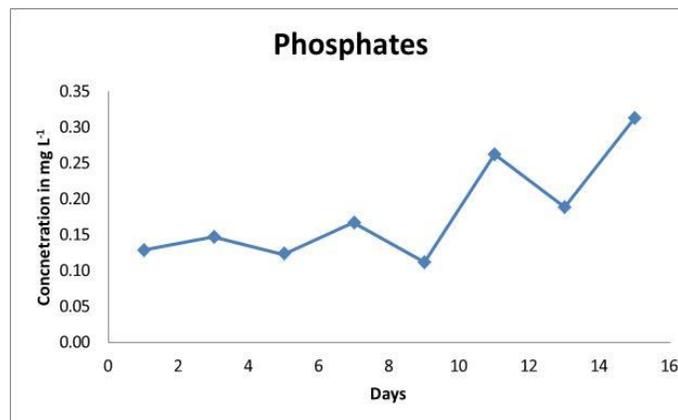Fig.3. Phosphate concentration (mg L^{-1}) in indoor culture conditions

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Table 3. Physico-chemical variables for 15 days in outdoor culture conditions

Days	pH	Electric conductivity (μScm^{-1})	Nitates (mgL^{-1})	Phospahates (mgL^{-1})
1	7.25	252.50	1.04	0.18
3	7.67	265.00	0.67	0.17
5	8.95	245.00	0.54	0.11
7	9.45	215.30	0.51	0.14
9	8.24	248.50	0.24	0.15
11	8.71	246.50	0.17	0.07
13	9.05	205.65	0.17	0.11
15	8.79	232.00	0.14	0.14

reason e.g., uptake of reproduction etc).

The nitrate thereafter showed steep decrease on 7th day (0.49 mg L^{-1}) with thereafter only increase in the nitrate level on subsequent days (Fig.2). Whereas, the phosphate level showed a decrease throughout the 15 days (Fig.3) with only slight increase on the 7th, 11th and 15th day (Table 2).

Outdoor culture conditions: In this investigation, water quality in the *ex situ* was alkaline pH, low conductivity and phosphates values. The pH ranged from acidic i.e., 5.26 (0th day) to alkaline i.e., 9.45 (7th day). The acidic pH, elevated nitrates and phosphates on 0th day are mainly

Fig. 4. Concentration of nitrates (mg L^{-1}) in outdoor culture conditions

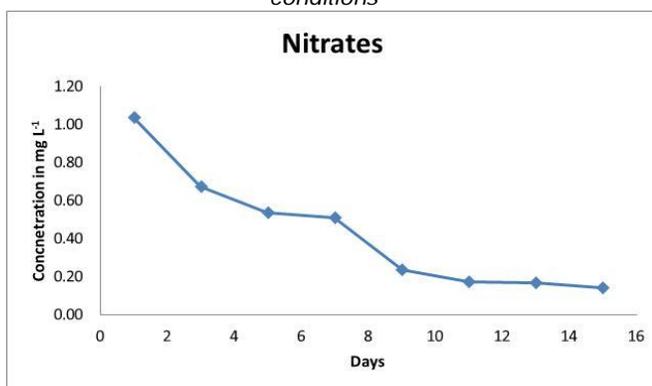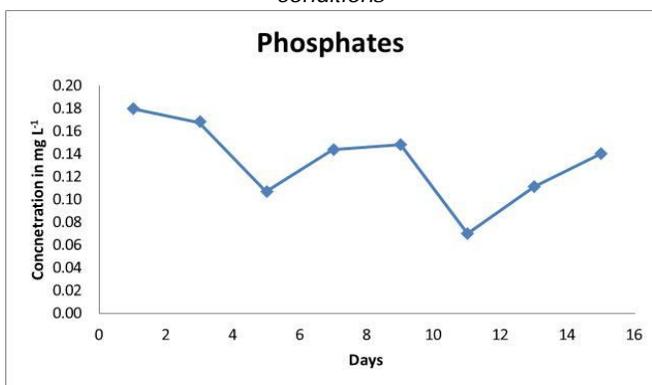*Fig. 5.Phosphate concentration (mg L^{-1}) in outdoor culture conditions*

Table 4. Community structure showing mixed population of days 1 and 9 in indoor culture conditions

Microalgae	Relative abundance (%)	
	Day 1	Day 9
Anabena sp.	10.69	11.34
Chlorella sp.	17.56	< 5%
Unidentified filamentous (2)	9.16	11.34
Unidentified filamentous (1)	25.95	25.77
Gomphonema sp.	5.34	16.49
Nitzschia sp.	< 5%	7.22

Table 5. Community structure (> 5% abundance) in indoor culture conditions

Microalgae	Days	Relative abundance (%)
Chlorella sp.	3rd , 15th	24.12%, 39.53%
Gomphonema	5th , 7th , 11th	21.88%, 45.45%
Nitzschia sp.	5th, 13th	37.50%, 41.67%
Anabena sp.	7th	17.78%
Unidentified filamentous (1)	9th	25.77%

Table 6. Community structure showing mixed population in outdoor culture conditions (Day 1)

Microalgae	Relative abundance (%)
Anabena sp.	6.60
Chlorella sp.	8.49
Unidentified filamentous (2)	7.55
Gomphonema sp.	16.04
Nitzschia sp.	12.26
Scenedusmus sp.	16.04

Table 7. Community structure (> 5% abundance) in outdoor culture condition

Microalgae	Days	Relative abundance (%)
Anabena sp.	13th	28.32
Chlorella sp.	15th	39.81
Unidentified filamentous (2)	5th	85
Unidentified filamentous (1)	3rd , 7th	48.57, 92.59
Scenedusmus sp.	9th, 11th	20.73, 27.20

due to the addition of required salts and acid supplied as nutrients to the algal community. Neutral pH on 1st and 3rd day was elevated to alkaline conditions on 5th while conductivity accounted to be high on 3rd day and slowly showed a decline in the values (Table 3). The nitrates vary from 1.04 mg L^{-1} on 1st day to 0.14 mgL^{-1} on 15th day. The outdoor conditions which varied according to the changes in the surrounding environment could be the reason for the nitrate peak on 7th day (0.51 mgL^{-1}) and thereby a decrease in the following days (Fig.4).

The phosphate levels were found decreasing throughout the 15 days (Fig. 5). The community structure (Table 6) is evident of the change in the nutrient and phosphate levels. The change in the N: P ratio could be

Table 8. Fatty acid methyl esters of microalgae consortium on each day

Fatty acid methyl esters	Days															
	I1	D1	I3	D3	I5	D5	I7	D7	I9	D9	I11	D11	I13	D13	I15	D15
	Microalgal consortium															
	C+F1	G+S	C	F1	G+N	F2	A+G	F1	F1+G	S+C	G+N	S+N+C	N+C	A+C+N	C+N	C
: 9-Hexadecenoic acid, methyl ester																+
: 9-Octadecenoic acid, methyl ester															+	
11-Octadecenoic acid, methyl ester														+		
Decanoic acid, methyl ester						+	+	+						+	+	
Docosanoic acid, methyl ester								+								
Dodecanoic acid, methyl ester	+		+	+	+	+	+	+	+	+	+		+	+	+	+
Eicosanoic acid, methyl ester			+	+				+	+	+	+	+		+	+	+
Heptadecanoic acid, methyl ester			+	+					+	+	+	+				
Hexadecanoic acid, 14-methyl-,	+	+				+	+				+	+	+			+
Hexadecanoic acid, methyl ester	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Hexanoic acid, methyl ester														+	+	
Methyl 13-methyltetradecanoate	+												+			
Methyl tetradecanoate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Octadecanoic acid, methyl ester	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Octanoic acid, methyl ester						+	+	+			+	+		+	+	+

attributed to the diverse community of algae (Table 5; Table 7) in the media.

Competition for space and nutrients may occur between and among different species due to the variation in the resources (Tilman, 1982). Since the population is of mixed type consisting of 6 genus (*Anabena* sp., *Chlorella* sp., *Scenedusmus* sp., Unidentified Filamentous algae, *Nitzschia* sp. & *Gomphonema* sp.) competition for the resources occurs, which is evident in the community structure (Table 5; Table 7). The N: P ratio does not explain the community structure completely due to mixed population.

Community analysis

Cells showing more than 5% of relative abundance were considered for the analysis. The natural community showed the presence of *Gomphonema* sp., *Navicula* sp., *Rhopholodia* sp., *Chlorella* sp., *Closterium* sp., *Pandorina* sp. and *Anabena* sp. with dominant being *Navicula* sp. and *Anabena* sp.

Indoor culture conditions: On Day 1 and 9 the community had a mixed population structure with no clear dominance of any alga (Table 4). The different alga dominated on various days is given in Table 6.

Outdoor culture conditions: On Day 1 the community had a mixed population structure with no clear dominance of any alga (Table 6). The different alga dominated on respective days is given in Table 6. The change in the N: P values can be attributed to this diverse abundance observed in all the days. Low nitrogen and Low phosphorus conditions often favor the growth of Cyanobacteria in fresh and saltwater systems (Sellner, 1997). In the same way when nitrate and phosphate levels are sufficiently high, it may offer a competitive advantage for diatoms thereby resulting in the reduction of other algae (Pinckney *et al.*, 1995). An increase of nitrogen and phosphorus nutrients may increase the primary production (Cadée, 1986), but the change in the nutrient composition may also affect the phytoplankton composition (Officer & Rhyther, 1980). The dominance of filamentous algae was found due to the consumption of the excessive phosphates (Machnicka, 2006).

Lipid analysis

The fatty acid methyl esters of the microalgae are listed in Table 8. The pattern of fatty acids varies according to the internal and external factors working on the algal cell (Cohen, 1988; Thompson *et al.*, 1990) which concludes that growth rate and the mixed population which competes for the resources, influences on fatty acid composition. Although there are many micro organisms which have the ability to accumulate oils under some special cultivation they have different prospects for biodiesel production in terms of oil yield lipid coefficient and lipid volumetric productivity (Li et al., 2008; Doan *et al.*, 2011). Berglund *et al.*, (2001) reported that both the quantity and quality of lipids produced will vary with the identity of the algal species. Microalgae are efficient biological factories capable of taking zero-energy form of carbon and synthesizing it into a high density liquid form of energy (natural oil) and are capable of storing carbon in the form of natural oils or as a polymer of carbohydrates (Benemann & Oswald, 1996). Under natural growth conditions phototrophic algae absorb sunlight, and assimilate carbon dioxide from the air and nutrients from the aquatic habitats. Therefore, as far as possible, artificial production should attempt to replicate and enhance the optimum natural growth conditions (Hu

et al., 2008). Growth of many species of algae is limited by the availability of nitrogen and phosphate (Lapointeand Connell, 1988; Larned, 1998; Russ & Mc Cook, 1999) for eg. Nutrient-enhanced (mixed N and P) cyanobacterial growth was observed by Miller *et al.*, (1999). A number of factors could have contributed to this effect, including temperature, competition and that the algae were in different phases of growth (eg. reproduction, vegetative, senescent) (Kuffnerand Paul, 2001). The resources will influence competitive interactions between algal and cyanobacterial species, the outcomes of which will vary along gradients in environmental variables (Carpenter *et al.,* 1990). Phytoplankton has often to rely on nitrate as nitrogen source (Berman *et al.*, 1984). Since the population was of mixed type, the other algae could have contributed to the lipid profile. The lipid content, lipid class composition and the proportions of the various fatty acids in a microalgae vary according to the environmental or culturing variables such as light intensity, growth phase photoperiod, temperature, salinity, CO_2 concentration, nitrogen and phosphorous concentration (Dunstan *et al.*, 1993; Zhu *et al.*, 1997; Wu *et al.*, 2011).

Conclusions

Indoor and outdoor culture conditions could support good growth of specific strains of fresh water algae evident from the community structure. The lipid characterization of the consortium of algae provided insights into applying mixed population for enhanced lipid productivity and hence biofuel. This study established the proof-of-concept for production of biodiesel from a consortium of algae from both indoor as well as outdoor culture conditions. The changes in neutral lipid emphasize the importance of knowing how nutrient levels play an important role in each of the microalgae for an enhanced accumulation of neutral lipids. Modifications using genetic engineering can be used to convert the autotrophic microalgae to heterotrophic microalgae which can accumulate maximum oils. For further application of this technique, role of each keystone microalgae species in the contribution towards lipid production in a consortium with its ecological preference has to be studied. This can be achieved by not limiting the consortium to just one alga, but for other classes as well. Biodiesel produced from the mass cultivation of microalgae potentially offers a high attractive and ecologically friendly biodiesel but after almost half a century of research the full promise of microalgae as a feed stock for biofuel production has remained largely unfulfilled.

Acknowledgements

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