

# POTENTIAL FOR BIOFUEL PRODUCTION FROM SEWAGE EFFLUENT USING INDIGENOUS MICROALGAE

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**Abstract:** Proliferous growth of green microalgae in sewage effluent can provide a cost-effective method for biofuel production. The aim of the present study was to investigate the efficiency of sewage effluent on the growth, biochemical changes and lipid accumulation capability of indigenous microalgae such as *Monoraphidium contortum*, *Psuedomuriella sp.* and *Chlamydomonas sp.* isolated from water bodies of Madurai, South India. Compared to other two selected algae, *Chlamydomonas sp.* exhibited greater growth rate ( $0.457 \text{ day}^{-1}$ ) with high biomass density (0.74 mg/ml) during the exponential phase of growth (20<sup>th</sup> day). Profuse growth of *Monoraphidium contortum* and *Psuedomuriella sp.* were also observed in untreated wastewaters with a mean growth rate ( $\mu$ ) of 0.439 and  $0.277 \text{ day}^{-1}$  respectively. Carbohydrates, proteins, lipids and fatty acid contents were also affected significantly by the effluent composition and growth stages of microalgae. *Chlamydomonas sp.* achieved a maximum lipid content of 27.7% (w/w) followed by *Monoraphidium contortum* with 26% (w/w). Fourier transform infrared spectra showed clear transitions in biochemical composition with an increased lipid/protein ratio at the end of the culture. Gas chromatography indicated the presence of high content of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) adding to the biodiesel quality. Efficient nutrient uptake, profuse biomass and high lipid content (comprised of saturated fatty acids) make the *Chlamydomonas sp.* as the best microalgae and viable source for biofuel production in sewage effluent. The results also emphasized that the sewage effluent can serve as a cost effective medium for biofuel production and thereby preventing eutrophication.

**Index Terms-** Biofuel production, fatty acids, lipids, microalgal biomass, sewage effluent.

## I. INTRODUCTION

Disposal of domestic sewage has become a serious issue in many parts of the globe including India. Sewage contains enormous nutrients, which if not treated properly may lead to eutrophication. Hence, instead of directly discharging the effluents into water bodies, it has been used for irrigation or fodder cultivation. The economic value of sewage can be assessed based on its nutritional value. The nutritional value of sewage in terms of nitrogen 30 mg/L, phosphate 7.5 mg/L, and potassium 25 mg/L is provided by CPCB [1997]. The total value of nutrients in sewage assuming @ Rs. 4220/- per tone of nutrient (as per 1996 cost), works out to be Rs. 1018 million, i.e., Rs. 890.6 million towards nutrients plus Rs. 127.4 million toward the cost of water. Among other Indian cities, the city of Madurai generates the least with 84.46 MLD [Rajendran and Sekaran, 2015]. Large portion of pollutants are removed by treatment plants and the remaining pollutants are allowed to undergo natural purification process. But, currently available technologies are considered inappropriate in India due to the economic barrier apart from the complexity in the operation and management. This imposes a need for simple, economically viable and sustainable wastewater treatment system at decentralised levels. Algae-based wastewater treatment has been adopted for a long time for cost-effective treatment as well as for nutrient recovery (Pittman *et al.*, 2011). Microalgal species have been employed for municipal wastewater treatment due to their abilities to uptake nutrients and grow profusely in wastewater with varied carbon (C), nitrogen (N) and phosphorus (P) concentrations (Mahapatra *et al.*, 2011). Earlier studies (Bhatnagar *et al.*, 2010; Ruiz-Marin *et al.*, 2010; Wang *et al.*, 2010) have reported high biomass productivity and complete removal of nutrients by *Chlorella* and *Scenedesmus sp.* Previously, algal growth and nutrient removal have also been reported in artificial wastewaters (Feng *et al.*, 2011; Yujie *et al.*, 2011).

Energy generation from algal biomass was promoted in 1960s by Oswald and Golueke, which gained interest in recent years, owing to the exhaustion of fossil-based reserves and hike in fuel prices (Ramachandra *et al.*, 2009). Among the many species of algae that grow in wastewaters, green microalgae species act as a possible feedstock for biodiesel production (Chisti, 2007). Simple growth requirements, high photosynthetic ability as compared to other terrestrial crops and very short regeneration time makes microalgae a good alternative for biofuel feed stock. Various synthetic media are used for growing microalgae, but for economic viability of lipid production, feed stock for algal cultivation must be inexpensive. Waste water is a cheap source of nutrients that could

be utilized for sustainable microalgal cultivation thereby reducing the cost of nutrients and water resources (Ravindran *et al.*, 2016). But the type of lipids (saturates, unsaturates, polyunsaturates or TAGS) and the quantity of lipids produced depend on the species and its growth conditions (Chisti, 2007; Griffiths and Harrison, 2009). The role of microalgae like *Chlorella* and *Scenedesmus* has been widely reported in the wastewater treatment and lipid production (Wang *et al.*, 2010; Li *et al.*, 2010). However, the indigenous microalgae that are isolated from wastewater can adapt and grow better than the other commercially available algal strains (Li *et al.*, 2010). The current study investigates the role of indigenous microalgae isolated from wastewater in assimilation of nutrients from sewage water collected from wastewater treatment plant at Velakkal, and lipid productivity.

## II. METHODS

### 2.1 Microalgal strains and culture conditions

Microalgae such as *Monoraphidium contortum*, *Pseudomuriella sp.* and *Chlamydomonas sp.* used in this study were isolated from the water bodies of Madurai city, South India. The pure cultures were maintained in CHU-10 media at a light intensity of 60 photons ( $\mu\text{moles}/\text{m}^2/\text{s}$ ) with 12 hours light/ 12 hours dark cycle at  $25\pm 2^\circ\text{C}$  for 25 days. During incubation, the monospecificity of the cultures were verified periodically by microscopic observation.

### 2.2 Sewage water characteristics

To investigate the efficiency of wastewater as a feedstock for biodiesel production from microalgae, the waste water from a sewage treatment plant at Vellakal, Madurai was collected and analyzed for its physico-chemical characteristics. For uniformity in methodology and to enable comparison with standards, approved procedures in standard manual (APHA, 2005) was adopted for analyzing parameters such as acidity, alkalinity, total solids, total dissolved solids, total suspended solids, chloride, dissolved oxygen, chemical oxygen demand, biological oxygen demand, inorganic phosphorus and nitrate. Physical parameters like pH and temperature were carried out at the field site itself. Analytical grade chemicals and double distilled water were used for preparing solutions for analysis.

### 2.3 Batch experiments

The selected green algal strains were cultivated in sterilized waste water and subjected to a series of physiological and biochemical characterization such as growth rate, dry weight, pigment composition, carbohydrate and protein content, lipid weight and fatty acid analysis at an interval of ten days. All the experiments were carried out in triplicates and the average values were recorded.

#### 2.3.1 Algal growth and dry weight

Growth rate of the microalgae were recorded continuously for 30 days at an interval of ten days by measuring the absorbance at 665nm in a HITACHI U-2001 spectrophotometer (Huang *et al.*, 2002). Proliferous rate of algal strains were calculated by using the following formula

$$K = \frac{[\log OD_t - \log OD_0]}{T} \times 3.332$$

Where  $OD_t$  = Terminal OD,  $OD_0$  = Initial OD, T = Number of days

The dry weight of the freeze dried algal biomass was determined gravimetrically (Dayananda *et al.*, 2010) and the growth was expressed in terms of dry weight grams per liter.

#### 2.3.2 Microalgal pigments and biochemical composition

Pigments such as chlorophyll *a* and carotenoids were extracted from microalgae by using 80% acetone and the absorbance was read after incubation in dark for 10 minutes, at 663 and 450nm respectively, as described by Subramanian (1993). Chlorophyll *a* and carotenoid content were calculated by using the following formula

$$\text{Chlorophyll } a \text{ (mg/ml)} = A_{663} \times 12.63 \times \text{Volume of acetone} / \text{Volume of sample}$$

where, 12.63 is the correction factor.

$$\text{Carotenoids (mg/ml)} = \frac{A_{450} \times \text{Volume of sample} \times 10}{20500}$$

where, 20500 is the extinction co-efficient

Carbohydrate and protein content in the selected microalgal strains were estimated by Anthrone method (Hedge and Hofreiter, 1962) and Lowry method (1951) respectively. Total lipids were extracted from microalgal biomass using a modified method of Bligh and Dyer (1959). The lipid dry weight was calculated by using the following formula

$$\text{Lipid content (\% dry weight)} = \frac{\text{Lipid weight}}{\text{Cell dry weight}} \times 100$$

### 2.3.3 Fatty acid composition

Fatty acid composition analysis was determined by two consecutive steps including preparation of fatty acid methyl ester (FAME) and GC analysis. FAME was prepared from the extracted lipids by a one-step extraction–transesterification method described by (Lewis *et al.*, 2000). GC analysis was performed at ten days intervals upto 30 days, for identifying the fatty acid composition. Fatty acid analysis was done by Gas Chromatograph 2010 Plus (Shimadzu, Japan) using Flame Ionization Detector (FID). Injector and Detector temperature was set at 225<sup>o</sup>C and 250<sup>o</sup>C respectively. One microlitre of the sample was injected in a split mode (35:1) at a flow rate of 184.9 ml/min with Nitrogen as the carrier gas onto a FAMES-RTX-2330 column (length 105.0m, Film thickness 0.20 µm, total run time 40min). Peak areas were integrated using the GC solution software. The fatty acid methyl esters were identified using fatty acid standards (Sigma, Supleco, 37 FAMES).

### 2.3.4 FTIR Spectroscopy

Harvested biomass of *M.contortum*, *Pseudomuriella* sp., and *Chlamydomonas* sp. on the 10<sup>th</sup>, 20<sup>th</sup> and 30<sup>th</sup> day of the algal growth was freeze dried and analyzed for their macromolecular composition using FTIR spectrometer. The spectra were recorded using Thermo Scientific Nicolet 380 FTIR spectrometer (Thermo Electron Corporation, USA) in the Transmission E.S.P mode with 32 scans for each spectrum in the wave number range of 4000 - 500 cm<sup>-1</sup> at 4 cm<sup>-1</sup> resolution. The spectral data were then exported using Omnic 7 software and were baseline corrected using the automatic baseline correction algorithm and were analyzed using Origin Pro8 software.

## III. RESULTS AND DISCUSSION

The dependence of algal growth and succession in wastewater, on the availability of nutrients and prevailing ecological conditions, it is indispensable to study the physico-chemical profile of the sewage effluent. Although phytoplankton species composition and diversity changes with environmental conditions such as nutrient levels, temperature, light, predator pressure etc, the relative importance of these factors varies considerably among different taxa. For the algae grown on wastewater, selection of fast-growing and high-lipid producing strains is essential for the commercial production of wastewater-based green biofuel. Thus the biomass, growth rate, biochemical contents including total lipid and fatty acid profile of selected microalgal strains were systematically examined in this study.

### 3.1 Physico-chemical characteristics of sewage wastewater

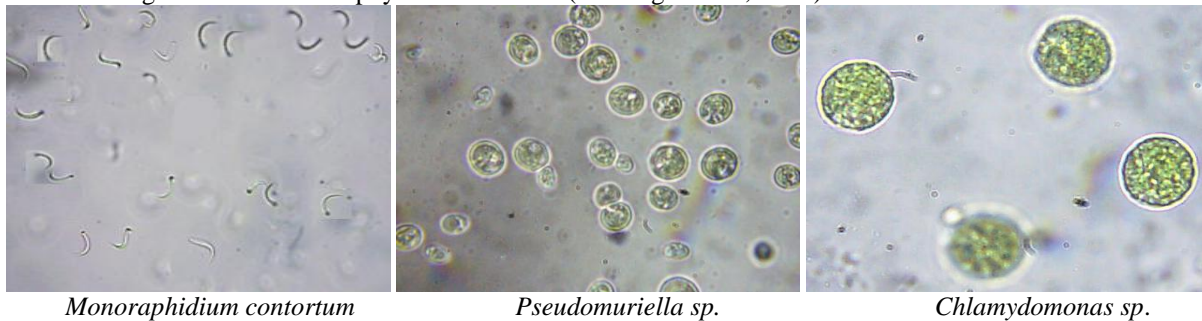
The characteristics of the wastewater differed among the Indian cities depending on the standard of living of the people and other industrial activities in the city. Wastewater from sewage treatment plant at Velakkal, Madurai mainly comprised of water (99.9%) together with relatively small concentrations of suspended solids (0.062 mg/ml) and dissolved solids (0.063 mg/ml). pH value ranged about 7.3 which was within the permissible limits (pH- 6.9 to 9.2) of WHO (1993). Alkalinity of 38 mg/L indicated the ability of water to resist changes in pH (buffering capacity). Surface water temperature may vary during different seasons and it was about 31<sup>o</sup>C during the sampling period. Chloride content of 103.2 mg/ml was recorded. WHO's guidelines for water quality (WHO, 1993) pointed out that the permissible range of chloride is 250 mg/L. BOD and COD are the most important parameters for the design and operation of sewage treatment plants. BOD levels of municipal sewage will be several times higher that of domestic sewage. In this study, the amount of dissolved oxygen and BOD in the sewage effluent measured about 6.4 mg/L and 38.42 mg/L respectively. COD measurements in natural waters are affected by domestic or industrial wastes. The COD value of 196.3 mg/L was noticed during the sampling period. Nitrogen and phosphorus being an essential nutrient, its optimum concentration is important for proper functioning of biological treatment methods. The concentration of nitrogen and phosphorus in domestic sewage is generally adequate to support aerobic biological wastewater treatment. 51.3 mg/L of nitrate and 5.9 mg/L of phosphate were detected in the Velakkal sewage effluent exhibiting the N/P ratio of 8.69. The optimal inorganic N/P ratio for freshwater algae growth was suggested to be in the range of 6.8–10 [Wang *et al.*, 2009].

### 3.2 Batch cultivation of selected microalgae

CHU-10 medium, which was commonly used for autotrophic cultivation of green microalgae (McKinley and Wetzel, 1979), was used for the enrichment of isolated microalgal species in this study. These selected strains (**Fig. 1**) were further investigated for their stability in small flasks with autoclaved sewage effluent for 30 days. The results confirmed that the selected 3 microalgal strains adapted well in the sewage effluent which coincides with other reports (Chinnasamy *et al.*, 2010). It can be hypothesized that the species that is naturally isolated from wastewater can adapt and grow better in wastewater than other commercially available algal strains (Li *et al.*, 2010).

Among various green microalgae, very few species such as *Scenedesmus*, *Chlorella*, etc. have the capability to survive in the diverse environment (Maity *et al.*, 2014). Organic carbon in wastewaters can also be utilised by microalgae such as *Euglena* sp. (Mahapatra *et al.*, 2013), *Chlorella* sp. (Liang *et al.*, 2009) and *Scenedesmus* sp. (Li *et al.*, 2010). Marchetti *et al.*, (2013) reported that the growth and biochemical composition of microalgal species may vary depending upon the cultural conditions such as light, pH, temperature, nutrients, aeration etc. In this study, the sewage effluent without any pre-treatment favoured algal growth, as compared

to previous studies on highly concentrated centrate water (sludge-concentrated water) and dairy wastewater (Wang *et al.*, 2010). Nutrient enrichment in sewage effluent collected from Velakkal facilitates the biomass abundance. All the selected microalgae were able to grow in the sewage effluent and it was evident from the biochemical data that the tremendous increase in biomass was due to the rapid nutrient uptake by the microalgae. Observations also exhibited high tolerance and adaptability of the selected three green microalgae to raw sewage like other Chlorophyceae members (Bhatnagar *et al.*, 2010).

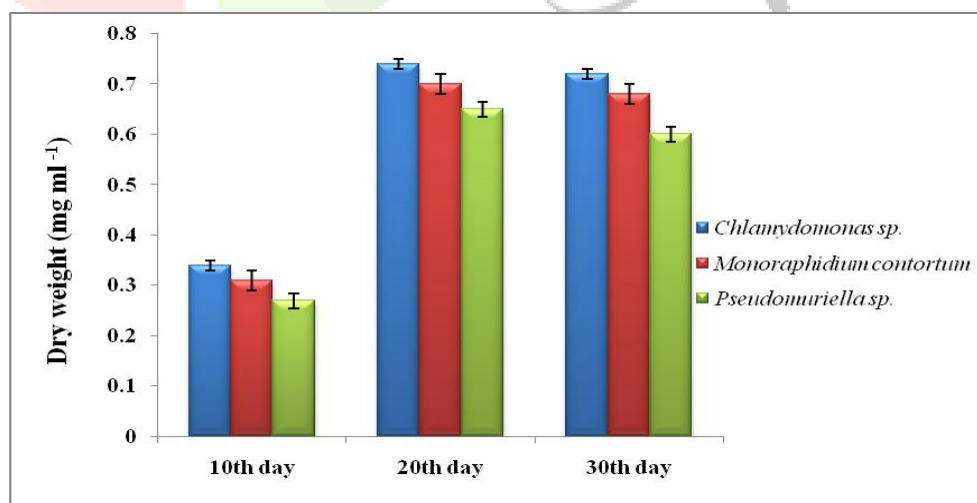


**Fig. 1** Microscopic appearance of selected green microalgae (100X)

### 3.3 Comparison of growth rate and biomass productivity of the selected microalgae

One of the best parameters to monitor microalgal cultivation is the estimation of growth, generally expressed in biomass, algal dry weight and pigment content over a certain period of time (Becker, 1994). Growth rate of a microalgae is a measure of an exponential increase in biomass over time. Growth rate expresses the relative ecological success of a species or a strain in adapting to its natural environment. After an initial lag period, there was a gradual increase in the optical density of all algal strains and reached maximum value on the 20<sup>th</sup> day. This might be due to the enhanced algal growth with the availability of enormous nutrients in wastewater as mentioned in previous studies (Bhatnagar *et al.*, 2010). The microalgal strains entered the exponential phase after 10<sup>th</sup> day and it extended upto the 20<sup>th</sup> day. Based on the optical density measurement ( $OD_{665nm}$ ), *Chlamydomonas* sp and *Monoraphidium contortum* showed higher growth rate ( $0.457$  and  $0.439 \text{ day}^{-1}$  respectively) on the 20<sup>th</sup> day of growth. These growth rates were comparable with those microalgae grown in municipal primary settled sewage ( $0.277 \text{ day}^{-1}$ ) and in municipal settled sewage ( $0.35\text{--}0.5 \text{ day}^{-1}$ ) wastewaters (Tam and Wong, 1990; Lau *et al.*, 1995). Growth rate was very slow in *Pseudomuriella* sp. ( $0.287 \text{ day}^{-1}$ ) in comparison to other green algae. During stationary phase, biomass is often very high and exhaustion of nutrients, limiting  $\text{CO}_2$  and light limitations are the primary causes of declining growth. Large population of cells may become stressed, photoinhibited and the culture enters the death phase.

Under suitable environmental conditions and sufficient nutrients, microalgae can grow profusely. Algae growth was measured in terms of an increase in biomass dry weight ( $\text{mg ml}^{-1}$ ) and is represented in **Fig. 2**. Biomass increased over the cultivation time in all experiments until the 20<sup>th</sup> day. The biomass was significantly higher for *Chlamydomonas* sp ( $0.74 \text{ mg/ml}$ ) followed by *Monoraphidium contortum* ( $0.70 \text{ mg/ml}$ ) and *Pseudomuriella* sp ( $0.65 \text{ mg/ml}$ ) cultivated in wastewater. Higher biomass was obtained in wastewater medium than in CHU-10 medium and similar results were reported by (Cho *et al.*, 2013).



**Fig. 2** Growth of microalgal strains in wastewater.

Error bar shows mean  $\pm$  standard deviation ( $n=3$ )



### 3.4 Pigment composition of green algal isolates

Algal biomass is usually analyzed on the basis of pigment concentration (Geider *et al.*, 1993). The pigment composition in green microalgae includes both carotenoids and chlorophylls. Chlorophyll *a*, the main photosynthetic pigment accounted for the highest concentration and the other chlorophyll pigments are accessories which may or may not be in combination with chlorophyll *a* (Richardson *et al.*, 1983). Chlorophyll *a* content varied during various phases of growth of all green algal species. Chlorophyll *a* content increased gradually from the lag phase to the exponential phase. When the dry weight of green algae increased during the exponential phase, a two fold increase in chlorophyll *a* and one fold increase in carotenoid was observed. The present study showed that a maximum amount of chlorophyll *a* was noted during the exponential phase of growth of algae (20<sup>th</sup> day) and high pigment values were probably due to the greater cell density. Chlorophyll *a* and carotenoid content varied significantly with respect to the algal taxa (Dere *et al.*, 2003). Chlorophyll *a* content was high in *Monoraphidium sp.* (8.12±0.09 µg/ml) followed by *Chlamydomonas sp.* (7.17 ±0.07 µg/ml) on the 20<sup>th</sup> day of growth. Exponential culture of *Pseudomuriella sp.* contained about 6.59 ±0.08 µg/ml of chlorophyll *a*. The results indicated that as chlorophyll is a nitrogenous compound, its content and composition are greatly influenced by nutrient concentrations in the growth medium. Chlorophyll *a* per cell was highest in the exponential phase which was also confirmed by the study of Eker-Develi *et al.*, (2008).

Highest value of Carotenoids was observed during the decline phase (30<sup>th</sup> day). *Monoraphidium sp.* (5.89 ±0.05 µg/ml) and *Chlamydomonas sp.* (5.31 ±0.04 µg/ml) produced higher amount of carotenoids followed by *Pseudomuriella sp.* (3.06±0.02 µg/ml) during 30<sup>th</sup> day. Decline in the production of chlorophyll pigments was greater than the carotenoids, when the cells became nutrient limited (Lagorio *et al.*, 2015) as carotenoids act as photoprotective pigments and prevent photooxidation (Mulders *et al.*, 2015).

### 3.5 Biochemical constituents of selected algal strains

The typical major metabolites of algal cells such as proteins, carbohydrates, lipids and fatty acid profile of the selected microalgal strains were analyzed and compared at an interval of 10 days until the 30<sup>th</sup> day of growth.

Several microalgal species possess high carbohydrate content, such as *Spirogyra sp.* (33%–64%), *Porphyridium cruentum* (40%–57%), *Chlorella emersonii* (37.9%), *Chlorogloeopsis fritschii* (37.8%) (Ravindran *et al.*, 2016). However, the carbohydrate percentage of the cells depends on the microalgal species, cultivation, and environmental conditions. The carbohydrate content in the medium in terms of total sugars increased with the growth of the algae. Carbohydrate content of *Monoraphidium contortum* (0.39 g/L) peaked during the 20<sup>th</sup> day of growth indicating an increased polysaccharide content in the biomass due to rapid assimilation of carbon followed by *Chlamydomonas sp.* and *Pseudomuriella sp.* (0.21 and 0.22 g/L respectively). When nutrients deprivation is imposed upon a culture, photosynthesis continues at a reduced rate and the flow of fixed carbon is diverted from protein to either lipid or carbohydrate synthesis (Shifrin and Chisholm, 1981). These energy-rich compounds are hydrophobic in nature and are efficiently packed in small compartment of cells for being utilized during adverse conditions for cell survival and proliferation (Courchesne *et al.*, 2009). Such high carbohydrate content can be converted into biofuels by various biochemical or thermochemical processes such as anaerobic digestion, anaerobic fermentation, and biological biohydrogen production (Markaou *et al.*, 2013).

Microalgae have the ability to synthesize all essential amino acids within their cell, leading to high levels of proteins. Cellular proteins are the major constituent of the photosynthetic apparatus, cell growth machinery and CO<sub>2</sub> fixation pathways. Ravindran *et al.*, (2016) reported relative protein content of certain microalgae species such as *Spirulina maxima* (60%–71%), *Synechococcus sp.* (63%), *Anabaena cylindrica* (43%–56%), and *Chlorella vulgaris* (41%–58%). The protein content in the cells increased relative to an increase in biomass density of the selected microalgal strains and was declined significantly with the depletion of nutrients in wastewater. Protein content was higher in *Pseudomuriella sp.* (0.096 g/L), followed by *Monoraphidium contortum* (0.082 g/L) and *Chlamydomonas sp.* (0.037 g/L) during the exponential phase which declined gradually. These findings are in agreement with other studies that the protein content decreased with the age of the culture (Pancha *et al.*, 2014). However, the high protein content implies high nitrogen content, which is undesirable for biofuel production (Bi and He, 2013).

### 3.6 Lipid content and fatty acid profile of selected microalgae

The algal lipids can be categorized as polar lipids and non-polar lipids. Polar lipids are also known as structural lipids containing maximum content of polyunsaturated fatty acids (PUFAs). Sterols and polar lipids are the key structural components of cell membranes, providing the matrix for different metabolic processes. It also serves as key intermediates in cell signaling pathways. Nonpolar lipids are also called as storage lipids or neutral lipids. These storage lipids mostly include triacylglycerols (TAGs), which contains predominantly saturated fatty acids along with a few unsaturated fatty acids that can be converted to biodiesel by transesterification (Sharma *et al.*, 2011).

Many microalgal species have the ability to accumulate oil in the form of non-polar glycerolipids such as triacylglycerol (TAG) (Hu *et al.*, 2008). Microalgal lipids and the fatty acid composition of algal cells depend upon the genetic and phenotypic factors, including environmental and culture conditions. Profiling of lipids in biomass feed stocks is critical for the production of quality biodiesel as well as other algal biofuels. Lipids are accumulated in microalgae under specific nutritional stress conditions, such as phosphate or nitrogen limitation [Bellou and Aggelis, 2012]. In the present study, screening for lipids during the early exponential

phase showed trace amount of lipid accumulation. A similar type of trend in lipid accumulation was also observed in *Chlorella minutissima* (Ordog *et al.*, 2012). As the age of the culture increased, there was a considerable amount of increase in lipid in all the selected green algae. The lipid accumulation increased during the decline phase (30<sup>th</sup> day) when the nutrients were depleted from the medium (Fig. 3). This result was in concordance with the findings of Hu *et al.*, (2008). He reported that when the age of the culture increased, the cells undergo stressful conditions and more amount of lipids are accumulated in algal strains because of the shift in lipid metabolism from structural lipid biosynthesis to the storage of neutral lipids. In waste water medium, 30<sup>th</sup> day culture of *Chlamydomonas sp.* (27.7%) showed higher lipid content followed by *M.contortum* (26%) and *Pseudomuriella sp.* (25.1%). This is comparable to lipid content (8–20 mg/ L/day) in other algae such as *Nannochloropsis oculata* and *Chlorella vulgaris* (Converti *et al.*, 2009). Previously, *Chlamydomonas sp.* TAI-2 and *Chlorella sp.* were reported to accumulate the lipid up to 18.4% while cultivating in waste water (Wu *et al.*, 2012). Lipid production of 24 mg/ L/day was obtained in a semi-continuous mode growing polyculture of *Chlorella sp.*, *Micractinium sp.* and *Actinastrum sp.* (Woertz *et al.*, 2009). From the results, it was clear that the availability of enormous nutrients in waste water favoured the growth of algal strains and down regulated the lipid productivity during early stages of growth and the mechanism reversed during later growth stages.

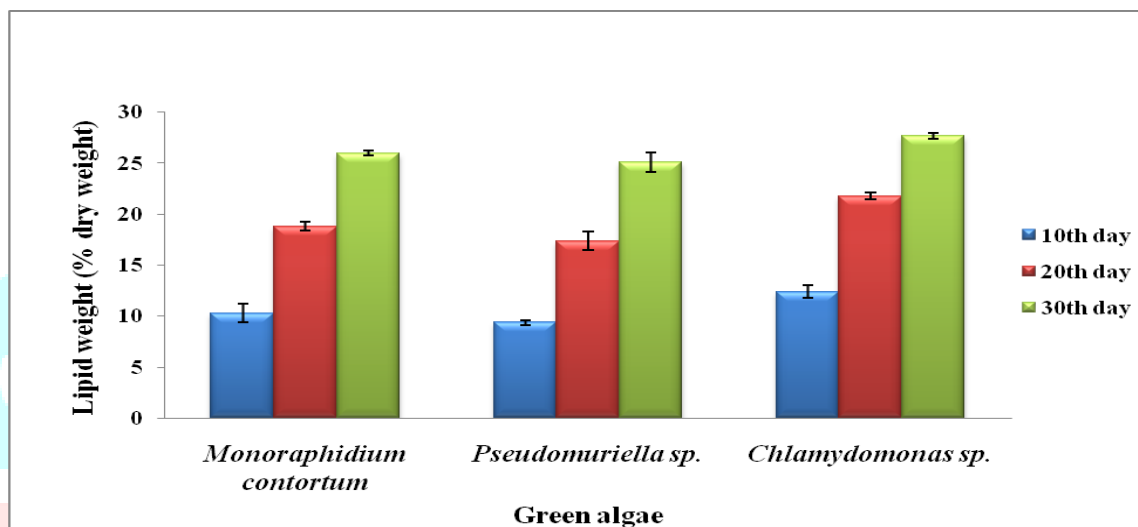
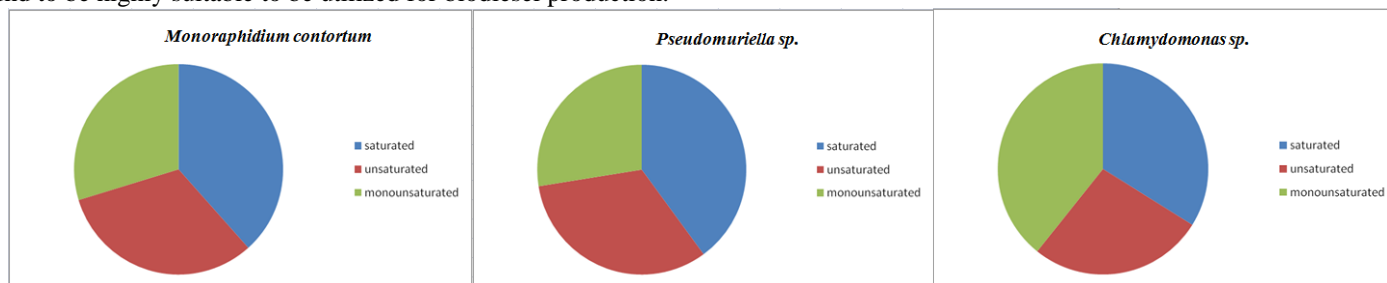


Fig.3 Lipid dry weight of selected green microalgae over growth period

Strain selection was essential in order to drive down the economic cost and optimize the biodiesel properties (Day *et al.*, 2012). Fatty acid composition was used to predict the quality of fatty acid methyl esters of lipids for use as biodiesel (Knothe, 2009). The fatty acids vary in their carbon chain length and in their number of unsaturated bonds. The major fatty acids present in all the green algae ranged between C12:0 to C24:0, but the composition of fatty acids differed between the microalgae. The fatty acids such as stearic acid (C18:0), palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) are commonly found in green microalgae (Durrett *et al.*, 2008; Singh and Singh, 2010). As previously indicated, the fatty acid composition can widely vary both quantitatively and qualitatively based on the microalgae's physiological status and the growth conditions such as medium composition, aeration rate, temperature, the ratio of light/dark cycle, and illumination intensity (Halim *et al.*, 2012).

GC analysis indicated a higher percentage of saturated fatty acids in all the three algal strains examined (Fig. 4). In waste water medium, higher percentage of saturated fatty acids were present in *Pseudomuriella sp.* (38.7%) followed by *M.contortum* (37.6%). *Chlamydomonas sp.* contained 33.4% of saturated fatty acids. The major saturated fatty acids present in algal strains were butyric acid, capric acid, decanoic acid, lauric acid and arachidic acid. The composition of fatty acids varied from 10<sup>th</sup> day to 30<sup>th</sup> day of growth. High percentage of lauric (10.9%), myristic (20.7%) and linolenic (38.2%) acid was observed during the log phase of growth. More amount of polyunsaturated (58%) and less amount of saturated (26%) fatty acids were observed on the 10<sup>th</sup> day of growth. The percentage of palmitic, stearic and oleic acid gradually increased from 10<sup>th</sup> to 30<sup>th</sup> day of growth. These results confirmed the accumulation of monounsaturated fatty acids, when the nitrogen concentration in the growth medium was decreased (He *et al.*, 2013). GC analysis indicated a higher percentage of saturated and monounsaturated fatty acids and less percentage of polyunsaturated fatty acids at the end of 30<sup>th</sup> day of growth in green algae. Piorreck *et al.*, (1984) also reported that during early stages of growth, green algae produced relatively large amount of polar lipids and polyunsaturated C16:0 and C18:0 fatty acids. The percentage of saturated fatty acids increased with the age of the culture (Pratoomyot *et al.*, 2005). The lipid class important from biofuel prospects indicates that C16–C18 are essential fatty acids with the desirable biofuel properties such as palmitic, stearic, oleic and linolenic acids (Knothe 2008). Palmitic acid (C16:0) was the major fatty acid in the selected green algae and highest percentage of palmitic acid was present in *Pseudomuriella sp.* (41.7%). Oleic acid (C18:1) content ranged between 3.4% to 41.3% in the selected microalgal strains. For biodiesel, oleic acid was a strong candidate for improving fuel properties. High amount of oleic acid (C18:1) was observed in the *Chlamydomonas sp.* (37.9%). Higher oleic acid content increases the oxidative stability of fuel, decreases the cold filter plugging point

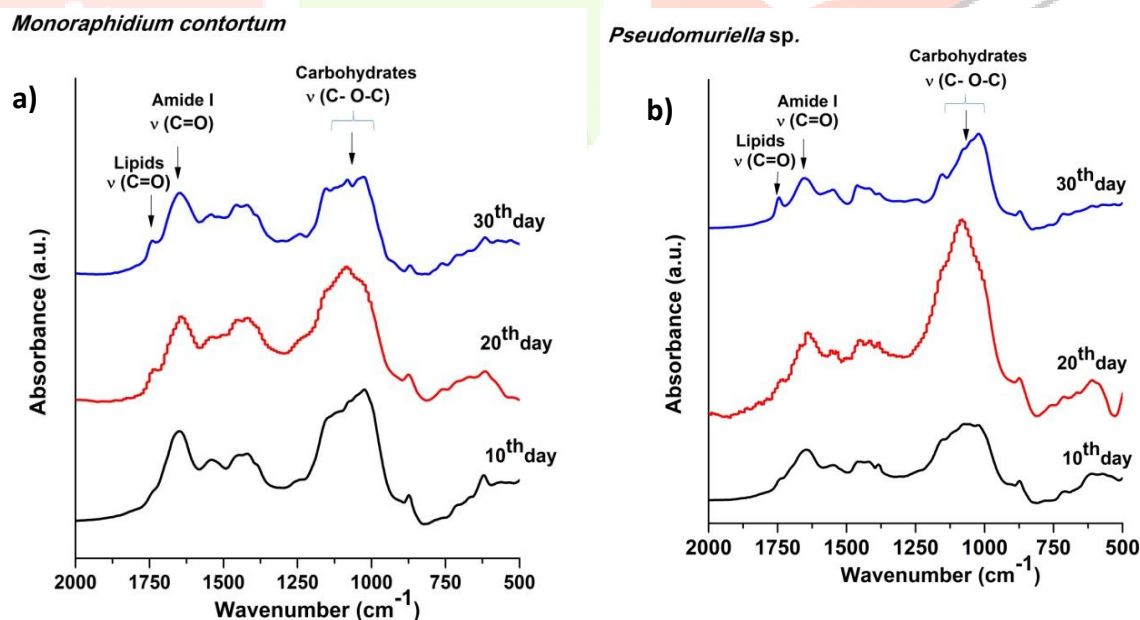
of the fuel and allows it to be used in cold regions (Knothe, 2008). The composition of fatty acids in the selected microalgae was found to be highly suitable to be utilized for biodiesel production.

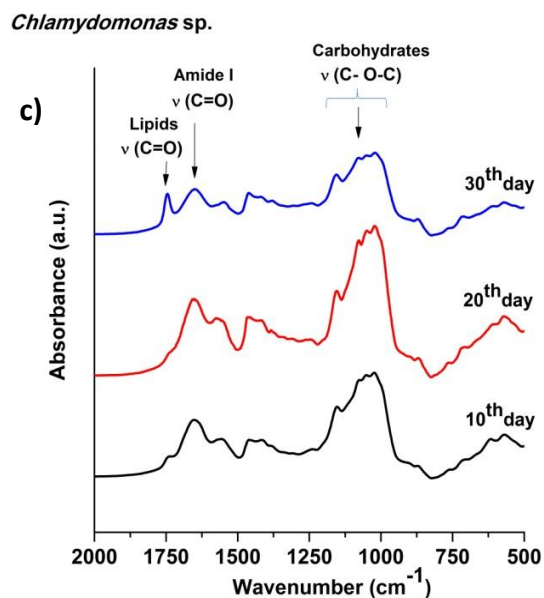


**Fig. 4 Fatty acid distribution of selected microalgal strains**

### 3.7 Biochemical transitions and Relative lipid content through FTIR analysis:

The spectra of the three algal species showed distinct absorption bands corresponding to the macromolecules in the wave numbers ranging from 2000 - 500  $\text{cm}^{-1}$  (Fig. 5). These bands were assigned to their specific functional groups using published studies (Stehfest *et al.*, 2005, Murdock and Wetzel, 2009). The band assignments are provided in Table 1. Out of all the bands listed, three bands were of particular interest, the band at  $\sim 1740 \text{ cm}^{-1}$  which is associated with  $\nu(\text{C}=\text{O})$  of ester groups, from lipids and fatty acids, the amide I band from proteins at  $\sim 1652 \text{ cm}^{-1}$  and the region from 1200 - 1000  $\text{cm}^{-1}$  corresponding to  $\nu(\text{C}-\text{O}-\text{C})$  stretching of polysaccharides from carbohydrates. The FTIR spectra shown in Figure 5, also illustrates the biochemical transitions that took place in the microalgal cells during the different phases of growth. These transitions or the compositional changes were traced by analysing the intensities of the peaks corresponding to lipids, proteins and carbohydrates. Relative content of lipids and carbohydrates were determined by calculating the ratios of the peak intensities of lipid to the amide I band and carbohydrates to the amide I (Dean *et al.*, 2010, Meng *et al.*, 2014). All the three algal species exhibited an increase in the lipid/amide I ratio (L/P) (Fig.6). Among the three species, *Chlamydomonas sp.*, showed a two fold increase in the L/P ratio from an initial value of 0.37 on the 10<sup>th</sup> day to 0.9 on the 30<sup>th</sup> day. Lipid/amide I ratio in *Monoraphidium contortum* and *Pseudomuriella sp.*, increased from an initial value of 0.3 and 0.38 to 0.42 and 0.56 on the 30<sup>th</sup> day of growth. An increase in the carbohydrate/amide I ratio (C/P) from the 10<sup>th</sup> to the 20<sup>th</sup> day was observed in all the three species. But on the 30<sup>th</sup> day, their ratios had decreased, due to the biochemical transitions within the macromolecules during different phases of growth. Greater accumulation of lipids and low content of protein and carbohydrates was noticed on the 30<sup>th</sup> day of growth as the microalgae entered the stationary phase (Ami *et al.*, 2014).



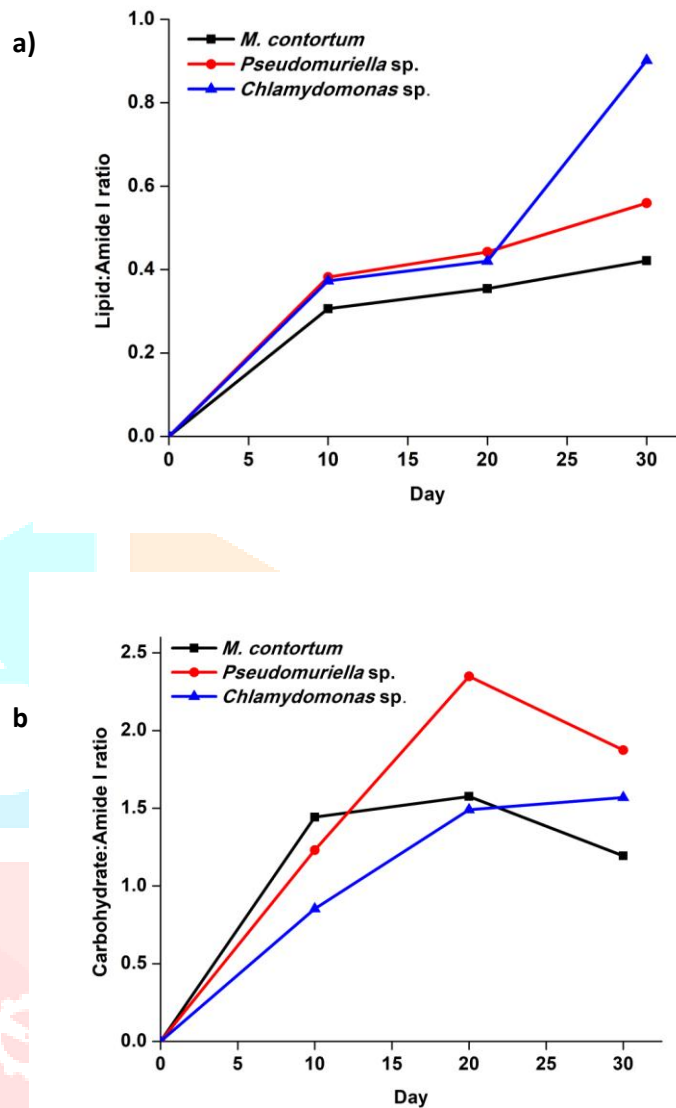


**Fig.5** FTIR absorption spectra of the microalgal biomass: a) *M.contortum* b) *Pseudomuriella* sp., and c) *Chlamydomonas* sp. The major band assignments are illustrated as functional groups.

**Table 1:** Band assignments for the FTIR spectra of the selected green microalgae

Wavenumber ( $\text{cm}^{-1}$ )	Band Assignments
~ 1740	$\nu$ (C=O) of esters from lipids and fatty acids
~ 1652	$\nu$ (C=O), $\nu$ (C-N) stretching and $\delta$ (N-H) bending of Amide I from proteins
~ 1546	$\delta$ (N-H) bending and $\nu$ (C-N) stretching of Amide II from proteins
~ 1460	$\delta_{\text{as}}$ ( $\text{CH}_2$ ) and $\delta_{\text{as}}$ ( $\text{CH}_3$ ) bending of methyl groups from proteins
~ 1380	$\delta_{\text{s}}$ ( $\text{CH}_3$ ) bending of methyl and $\nu_{\text{s}}$ (C-O) stretching of $\text{COO}^-$ groups
~ 1240	$\nu_{\text{as}}$ (>P=O) stretching of phosphorus compounds from nucleic acids
~1200 - 1000	$\nu$ (C-O-C) stretching of polysaccharides from carbohydrates





**Fig. 6 Determination of Relative content: a) Changes in the Lipid: Amide I ratio  
b) Changes in the Carbohydrate: Amide I ratio**

#### IV. CONCLUSIONS

The results clearly emphasized that the sewage effluent is a cost effective growth medium for higher biomass and lipid production in microalgae. As the developing nations are in a need to develop economically feasible and eco-friendly technologies to treat wastewater, algae-based wastewater treatment coupled with biodiesel production would serve as an effective strategy. This would also facilitate mitigation of green house gases through assimilation of carbon (as algae are photosynthetic organisms). Based on the ability to grow in sewage effluent and lipid productivity, *Chlamydomonas sp.* was considered as promising strains chosen for further research to improve the technologies for sustainable algal-biofuel production and effective wastewater treatment. The fatty acid profiles of the selected microalgae also authenticated them as a good candidate for energy generation. But, the major bottlenecks in the production of algal biofuels are the cost involved in its harvesting and extraction of metabolites. Manipulation of genes for increasing the energy efficiency and generation of resistant variety for avoiding field contamination could serve as an alternative. Further upscaling to industrial level could lead to sustainable biodiesel production from waste water resources.

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