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Euglena sp. as a suitable source of lipids for potential use as biofuel and sustainable wastewater treatment

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Abstract Prolific algal growth in sewage ponds with high organic loads in the tropical regions can provide cost-effective and efficient wastewater treatment and biofuel production. This work examines the ability of *Euglena* sp. growing in wastewater ponds for biofuel production and treatment of wastewater. The algae were isolated from the sewage treatment plants and were tested for their nutrient removal capability. Compared to other algae, *Euglena* sp. showed faster growth rates with high biomass density at elevated concentrations of ammonium nitrogen (NH_4-N) and organic carbon (C). Profuse growth of these species was observed in untreated wastewaters with a mean specific growth rate (μ) of 0.28 day^{-1} and biomass productivities of 132 mg L^{-1} day^{-1}. The algae cultured within a short period of 8 days resulted in the 98 % removal of NH_4-N, 93 % of total nitrogen 85 % of *ortho*-phosphate, 66 % of total phosphate and 92 % total organic carbon. Euglenoids achieved a maximum lipid content of 24.6 % (*w*/*w*) with a biomass density of 1.24 g L^{-1} (dry wt.). Fourier transform infrared spectra showed clear transitions in biochemical compositions with increased lipid/protein ratio at the end of the culture. Gas chromatography and mass spectrometry indicated the presence of high contents of palmitic, linolenic and linoleic acids (46, 23 and 22 %, respectively), adding to the biodiesel quality. Good lipid content (comprised quality fatty acids), efficient nutrient uptake and profuse biomass productivity make the *Euglena* sp. as a viable source for biofuel production in wastewaters.

Keywords Algal nutrient uptake · Biomass productivity · *Euglena* · FAME · Lipid · Wastewater

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Introduction

The treatment and disposal of domestic sewage has become a serious issue in many parts of the globe including India. Among the various technologies used, waste stabilisation ponds or lagoons are adopted due to its low operation and maintenance (OM) costs and adequate land availability. These treatment systems work with an initial anaerobic stabilisation followed by the facultative and aerobic oxidation ponds (Mahapatra et al. 2011a, b, c) or high-rate oxidation ponds (Park et al. 2011). In India >40 billion L day^{-1} of wastewaters are generated mostly from the urban areas (CPCB 2011), and these untreated or partially treated wastewaters find their way into receiving water bodies enriching the aquatic systems with nutrients (Mahapatra et al. 2011a, b, c). The suspended and attached growth bacterial systems are not popular in India due to the economic barrier apart from the complexities involved in the operation and management. This necessitates a simple, economic and sustainable wastewater treatment system at decentralised levels.

Algae-based domestic wastewater treatment has been practised for a long time for cost-effective treatment and nutrient recovery (Oswald et al. 1957; Pittman et al. 2011). These systems would offset OM costs through biomass use as bio-

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fertilisers, bio-chemicals as proteins, pigments etc., (de la Noue et al. 1992; de Bashan and Bashan 2004). Energy generation from algal biomass was advocated in 1960s (Oswald and Golueke 1960) and gained momentum in recent times owing to the exhaustion of fossil-based reserves resulting in increased fuel prices and also due to increased green house gas (GHG) emissions (Ramachandra et al. 2009; Wang et al. 2010a, b; Pittman et al. 2011). Among the many species of algae that grow in wastewaters, oleaginous algae species act as a possible feedstock for biodiesel production as a viable alternative for fossil fuels (Chisti 2007). Oleaginous algal species have been used 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. 2011a, b, c; Noue et al. 1992; Pittman et al. 2011). However, there are constraints like light absorption due to dense algal growth, longer retention time, etc. (Savage 2011).

Earlier studies (Bhatnagar et al. 2010; Ruiz-Marin et al. 2010; Wang et al. 2010a) have reported high biomass productivity and complete removal of nutrients by *Chlorella* and *Scenedesmus* sp. Removal experiments include either batch or semi-continuous laboratory or pilot-scale cultures (Ruiz-Marin et al. 2010; Wang et al. 2010b). *Chlorella minutissima* grown in wastewaters have higher growth rates of 380 mg L^{-1} under heterotrophic conditions compared to 73 mg L^{-1} under phototrophic conditions in raw sewage (Bhatnagar et al. 2010). This suggests that chlorophycean algae such as *Chlorella* are better assimilators of nutrients. In addition to these, algal growth and nutrient removal have also been reported from artificial wastewaters (Voltolina et al. 1999; Li et al. 2010; Feng et al. 2011; Yujie et al. 2011).

Wastewaters provide a sustainable growth medium for algal biofuel feedstock (Chanakya et al. 2012a, b), and the nature, type of lipids (saturates, unsaturates, polyunsaturates and glyco/phospholipids or TAGS) and the quantity of lipids produced depend on the species and its growth conditions (Borowitzka 1992, 1999; Chisti 2007; Griffiths and Harrison 2009; Ramachandra et al. 2009). Reports reveal high concentrations of lipids in algal cells (Griffiths and Harrison 2009) and low algal biomass productivities (Dean et al. 2010), due to stress induction (N and P limitation). However, unlimited nutrient supply in wastewaters often results in the enhanced algal cell densities with larger amounts of lipid (Wang et al. 2010a). Nutrient deprivation studies have reported an increased lipid/protein (L/P) ratio and carbohydrate/amides (C/P) ratio (Stehfest et al. 2005; Dean et al. 2010, 2012). Laboratory studies carried out in batch and semi-continuous cultures in bioreactors and ponds have reported lipid accumulation in the wastewater-grown algae, yielding low (<15 %), moderate (25–30 %) lipid content and in many cases a very high lipid yield. Rapid ways of quantification for algal lipid accumulation and tracking lipids over the culture period include Fourier transform infrared (FTIR) spectroscopy (Stehfest et al. 2005; Dean et al. 2010, 2012; Sigee et al. 2002), which helps in the study of C transformations and allocation in algal cells (Dean et al. 2010, 2012). The role of mixotrophic algae like *Chlorella* and *Scenedesmus* has been widely reported in the wastewater treatment and lipid production (Wang et al. 2010a; Li et al. 2010). However, there are no studies on euglenoids (*Euglena* spp.) for their abilities of nutrient removal and biofuel production. Euglenoids grow profusely under anoxic conditions in sewage with high organic loads. The current study investigates the role of mixotrophic indigenous alga *Euglena* sp. in the removal of nutrients from wastewater and lipid production. The objectives of the present study were as follows:

1. To evaluate the utility of *Euglena* sp. in wastewater treatment and assess the nutrient (C, N and P) removal capability
2. To assess the cell compositional changes in the algae with the culture time
3. To determine the biomass and lipid productivities together with fatty acid composition for assessing the suitability of wastewater-grown *Euglena* sp. as a candidate for sustainable biofuel production

Materials and methods

Experiments were carried out using wastewater generated in Indian Institute of Science (IISc) Campus, Bangalore, India. The characteristics and features of the raw wastewater are summarized in Table 1.

Municipal wastewater used for *Euglena* sp. (cultivation)

The values of ammonium nitrogen (NH_4-N), total nitrogen (TN), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), *ortho*-phosphates (OP), total phosphorus (TP), total organic carbon (TOC) in domestic wastewater culture broth were determined following the HACH protocols. Physico-chemical parameters were analysed as per standard protocol (APHA et al. 2005). C and N analyses were performed by CHN elemental analyser (LECO, True Spec CHNS). Domestic wastewater (sewage) used as the medium for culturing microalgae was first allowed to settle for several hours. Then the supernatant was sterilised, filtered and then used as the culture medium (as per Zhou et al. 2012). All experiments were carried out in triplicate and mean with standard deviation computed to understand variability.

Algae species culturing and growth conditions

Euglena sp. was isolated from the sewage collected from treatment firms (between 12.273681°–12.270031°N and

Table 1 Physico-chemical characteristics of domestic wastewaters used for the experiment

Physico-chemical parameter	Mean ± SD
Total nitrogen (mg L^{-1})	32 ± 1.72
Ammonium nitrogen (mg L^{-1})	25 ± 1.44
Nitrate nitrogen (mg L^{-1})	0.1 ± 0.021
Nitrite nitrogen (mg L^{-1})	0.8 ± 0.066
Total phosphorus (mg L^{-1})	18 ± 1.22
Ortho-phosphates (mg L^{-1})	16 ± 1.45
Total organic carbon (mg L^{-1})	250 ± 21
Chemical oxygen demand (mg L^{-1})	660 ± 64
Total solids (mg L^{-1})	1,400 ± 130
Total suspended solids (mg L^{-1})	540 ± 84
Total volatile solids (mg L^{-1})	344 ± 66
pH	6.8 ± 0.1
Redox potential (mV)	−120 ± 34

76.650737°–76.655947°E) at Mysore, Karnataka and was maintained in the Bolds Basal (BB) medium (Grobbelaar 2004). Inocula of the algae were prepared and added to the reactors (10 L) (~10^6 cells mL^{-1}, inoculum volume 20 mL). Reactors were kept on the rooftop under sunlight (12:12 h light/dark period), and cultures were mixed periodically. Algal cultures were maintained for 9 days at ambient temperatures, and the growth was monitored. Every day, 100 mL of broth was collected and centrifuged, and replaced with deionised water. The supernatant was used for analysis of nutrients, and the algae pellet was washed repeatedly and then used for dry weight estimation, total lipid content and spectroscopic and elemental analysis. Algae broth of 100 mL was centrifuged, and pellets formed were then dried and weighed to assess the algae biomass (cell dry weight in the culturing medium, g L^{-1}). The specific growth rate (μ) was calculated by Eq. 1, considering dry weight of cells during 9 days of culture:

$$\mu\left(\text{day}^{-1}\right) = (\ln N/N_0)/T_2 - T_1 \quad (1)$$

where $T_2 - T_1$ (day) is the time between the two measurements, and N and N_0 (g L^{-1}) are the concentrations of biomass at day T_2 (next day for 8 days) and T_1 (first day).

The biomass productivity at the time of culturing is given by Eq. 2:

$$\text{Biomass productivity}\left(\text{g L}^{-1}\text{day}^{-1}\right) = (\text{N} - \text{N}_0)/\text{T} \quad (2)$$

where N (g L^{-1}) is the concentration of biomass at the end of the cultivation, N_0 (g L^{-1}) is the concentration of biomass at the beginning, and T is the duration of cultivation (9 days).

Biochemical transitions through FTIR analysis

Macromolecular biochemical compositions of algal cells were monitored using a FTIR spectroscope (Alpha Bruker). Algae dry biomass after freeze drying was analysed with attenuated total reflectance (ATR) using an ATR–FTIR spectroscope in the absorbance mode (range 1,800–800 cm^{-1} wave numbers) with 128 scans at a spatial resolution of 2 cm^{-1}. IR absorption spectra were collected for the daily monitoring. The data analysis was carried out with Origin Pro software with an initial base line correction and scaled up to Amide I max (Stehfest et al. 2005). L/P and C/P required for biochemical composition assessment were derived from the area under the curve (Giordano et al. 2001; Murdock and Wetzel 2009).

Lipid measurements

The total lipids were determined according to Bligh and Dyer (1959). Cell suspensions from cultures were centrifuged at 1,162×g for 10 min. Then, the algal pellet was stored at −20 °C until use. The calculation of the total lipid content is given by Eq. 3:

$$\text{Lipid content algae}\left(\text{g g}^{-1}\right) = \text{wt. (lipids)} \,/\, \text{wt. (dry biomass)} \quad (3)$$

The lipid productivity is given by Eq. 4:

$$\text{Lipid productivity}\left(\text{g L}^{-1}\text{day}^{-1}\right) = (\text{L}_\text{f}\text{N} - \text{L}_0\text{N}_0) \,/\, \text{T} \quad (4)$$

where L_0 (g g^{-1}) is the lipid content at the beginning, L_f (gg^{-1}) is the lipid content at the end of the batch culture, N_0 (g L^{-1}) is the biomass concentration at the beginning, and N (g L^{-1}) is the biomass concentration at the end of the batch culture (8 days)

Fatty acid composition analysis (FAME)

Disruption of cells was by sonication using an ultrasonic bath (frequency 35 kHz) for 30 min. Lipids were extracted with chloroform/methanol (2:1, *v*/*v*) and separated into chloroform and aqueous methanol layers by the addition of methanol and water to give a final solvent ratio of chloroform/methanol/-water 2:1.1:0.9. Twenty millilitres of 5 % NaCl solution was used for washing the chloroform layer and then evaporated using a rotary vacuum evaporator with a water bath temperature of 60 °C. Methylation of lipids was by using boron trifluoride-methanol (BF_3-MeOH) to convert all fatty acids to their corresponding methyl esters. The methylated sample was loaded onto silica column with helium gas as carrier in splitless mode. The total run time was calculated to be

47.667 min. Fatty acids were identified by comparing the retention time to that of known standards. The composition of the fatty acid methyl ester (FAME) was assessed by gas chromatography mass spectrometry (Agilent Technologies 7890C, GC System; Agilent Technologies 5975C insert MSD with Triple-Axis Detector). Both the initial column temperature and the injection port temperature were maintained at 250 °C. Detector temperature was 280 °C and was increased to 300 °C at a temperature gradient of 10 °C min^{-1}. The oven temperature was then raised to 230 °C at a ramp rate of 3 °C min^{-1}, and finally, it was raised to 300 °C at a ramp rate of 10 °C min^{-1}. This temperature was maintained for 2 min. The components were identified based on their retention time, abundance and fragmentation patterns by comparing with a known standard. These experiments were carried out in triplicate, and average values are reported. Results were analysed using PAST 1.86b. Correlation tests, ANOVA and Tukey's post hoc analysis were used to determine the significance of difference wherever applicable.

Results

Algae growth was measured in terms of increase in biomass concentration (mg L^{-1}) and is shown in Fig. 1. There was an initial lag phase observed during the growth of *Euglena*. However, after day 2, they grew very fast in these wastewater cultures. However, the growth curve was not consistent as there was another growth stress observed at the end of day 4. With the starting density of 0.18 ± 0.06 g L^{-1}, the dry biomass cell weight of the algae cells went up to 1.24 ± 0.74 g L^{-1} (dense culture) (Fig. 1). There was sudden increase up to five times in the density from the 2nd to the 4th day, following a small lag phase further reaching a maximum on the 8th day. The CN elemental analysis of the dry biomass showed a significant increase in the N content from 3.3 ± 0.76 to 4.4 ± 0.24 % ($p < 0.05$) at the end of day 4 and thereby a significant decrease in the C value from 33.63 ± 1.34 to 18.01 ± 3.23 ($p < 0.01$) at the end of day 5 (Fig. 2). The C/N ratio significantly decreased from 10.09 ± 2.12 to 7.72 ± 1.34 ($p < 0.05$) at the end of day 4.

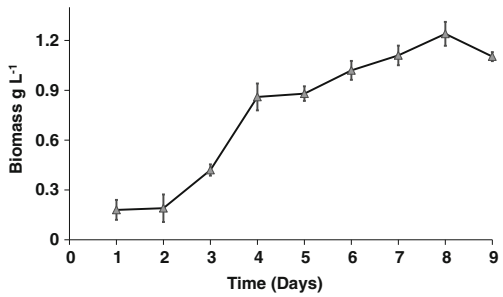

Fig. 1 Growth of *Euglena* sp. in the wastewater culture. *Error bar* shows mean + SD ($n = 3$)

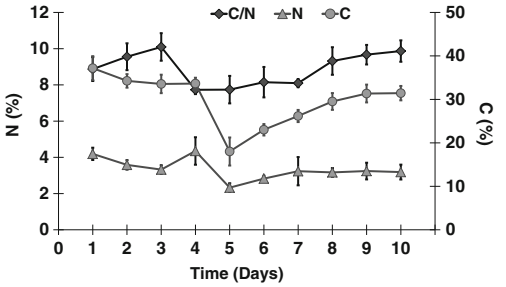

Fig. 2 C and N contents of *Euglena* sp. during growth and C/N ratio. *Error bar* shows mean + SD ($n = 3$)

pH values showed a consistent increase from pH6.1 ± 0.1 to 9.16 ± 0.57 ($p < 0.01$) (Fig. 3). The specific growth rates (μ) peaked in the initial period and reached maximum on day 4 ($\mu = 0.79$ day^{-1}), after which it decreased significantly. TOC (90 %) was removed rapidly within a period of 6 days from 256 ± 21 to 20.75 ± 12.5 mg L^{-1} as shown in Fig. 4. Although there was a lag phase in the initial growth stages, this did not affect the TOC removal process which started from the day of initiation of the culture experiment. TOC was the lowest on day 6, after which it slowly increased to an insignificant extent ($p < 0.05$) (Fig. 4), and majority of the organic carbon was uptaken during the first 3 days of the culture experiment.

Total nitrogen removal of 93 % was observed in just 8 days (Fig. 5). From an initial concentration of 32.5 ± 0.13 mg L^{-1}, the values dropped to 2.28 ± 0.06 mg L^{-1}. Most of the N removal happened from day 2 to day 7. Similarly, NH_4-N was almost totally removed, i.e. more than 98 % at the end of day 8 (Fig. 5). The major NH_4-N removal also occurred during the peak growth period until day 6 when almost 90 % of NH_4-N was removed in just 4 days. However, there was no significant nitrification ($p < 0.01$) taking place in spite of mixing and prevalence of oxidising conditions. The NO_3-N values increased to 0.9 mg L^{-1} from 0.1 mg L^{-1} on day 6 and then decreased (Fig. 5). The proportion of NO_3-N compared to the NH_4-N was 1:25. Similarly, NO_2-N levels were insignificant and increased to a certain extent during the growth from 0.89 to 0.03 mg L^{-1} (Fig. 5). As compared to the C and N removal, the efficiency of phosphorus removal was observed to be low. Around 66 % (16.25 ± 1.22 to 5.49 ± 1.32 mg L^{-1}) of TP were

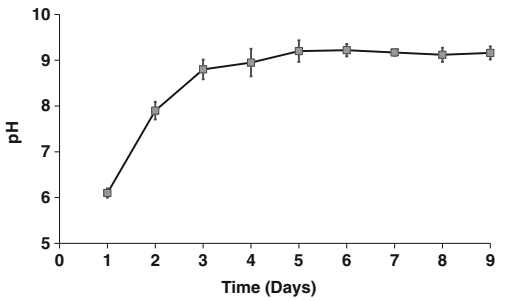

Fig. 3 pH of culture. *Error bar* shows mean + SD ($n = 3$)

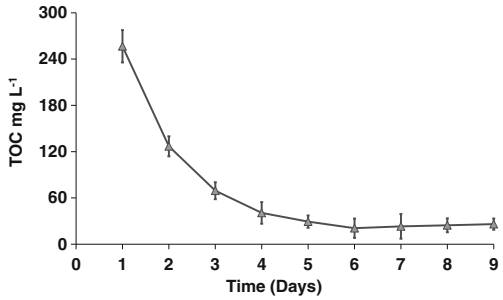

Fig. 4 TOC removal in algal culture. *Error bar* shows mean + SD (n = 3)

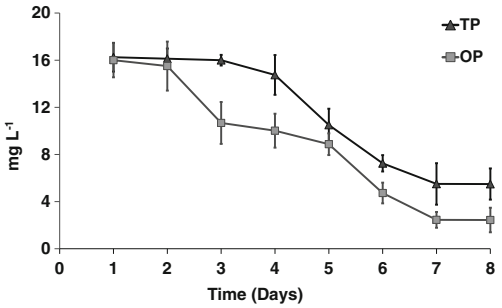

Fig. 6 TP and *ortho*-P removal. *Error bar* shows mean + SD (n = 3)

removed, and the removal started from the end of day 3 up to the end of day 7, after which it was very slow (Fig. 6). The *ortho*-phosphate was reduced from 16 ± 1.22 to 2.44 ± 1.32 mg L^{-1} giving a removal efficiency of 85 %.

ATR–FTIR spectra of the *Euglena* cells showed seven distinct absorption bands with the wave numbers ranging from 1,800–800 cm^{-1} (Fig. 7). These bands were assigned to specific functional groups following biochemical standards and published matter (Stehfest et al. 2005). Assignments of bands corresponding to the functional groups are provided in Table 2. Out of all these bands, three bands were most important for the study, i.e. ~1,740 cm^{-1} for ester/fatty acid, ~1,655 cm^{-1} for proteins and ~1,150–950 cm^{-1} for carbohydrates. The characteristic lipid peak can be very distinctively visualised in the FTIR spectra at ~1,700 cm^{-1} during the final phases of the culture (Fig. 7). Figure 7 also illustrates the changes in the FTIR spectra with the culture time from day 1 to day 9. The compositional changes were traced by the carbohydrate/amide (C/P) and lipid/amide (L/P) changes of *Euglena* sp. Cultures experienced an increase in the L/P band ratio from an initial value of 0.025 to 0.26. There was a significant increase in the ratio during day 2 and day 3 ($p < 0.05$). C/P ratio increased significantly from 0.94 to 2.4 ($p < 0.01$) on day 6 (discussed later in Fig. 10).

The lipid content ranged from 9 ± 0.77 to 24.62 ± 0.98 % dry weight of algae biomass. The daily measurement of lipid content of the centrifuged and dried algae biomass showed an increase in the lipid content on day 2 of the culture (18 ± 1.23 %), after which there was a steep decrease. Lipid

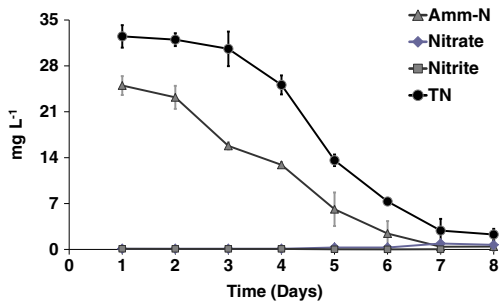

Fig. 5 TN, NH_4-N, NO_3-N and NO_2-N removal. *Error bar* shows mean + SD (n = 3)

increased from day 6 and was maximum (25.6 %) on day 9 (Fig. 8). FAME composition of lipids through GC–MS analysis showed 13 different types of fatty acid methyl esters, wherein almost 51 % were unsaturates. Among the fatty acid methyl esters, palmitic acid (C16:0) was dominant with 46 % followed by α linolenic acid (~23 %) then followed by linoleic acid (22 %) and stearic acid (3 %) (Fig. 9). Unsaturated fatty acids were comparatively higher than saturates, and polyunsaturated fatty acids (PUFA) were 46.6 %.

Discussion

Euglena sp. isolated from urban wastewaters was able to grow in domestic wastewater utilising the dissolved nutrients and minerals in culture solutions. Observations showed high tolerance of *Euglena* sp. to raw sewage and the ability to grow mixotrophically as other Chlorophyceae do (Bhatnagar et al.

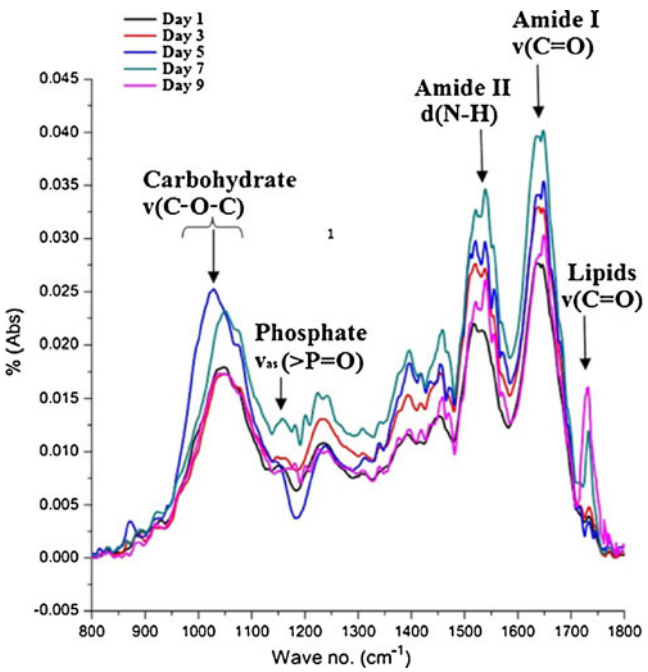

Fig. 7 FTIR spectra of the algal biomass during the culture period. The major band assignments are illustrated as functional groups

Table 2 Band assignments for the various functional groups/substrates in algal cells

Band assignment (cm^{-1})	Functional groups
~1,740	ν(C=O) stretching of ester groups, primarily from lipids and fatty acids
~1,655	ν(C=O) stretching of amides from proteins (amide I)
~1,545	δ(N–H) bending of amides from proteins (amide II)
~1,455	$\delta_{as}(CH_2)$ and $\delta_{as}(CH_3)$ bending of methyl from proteins
~1,380	$\delta_s(CH_2)$ and $\delta_s(CH_3)$ bending of methyl and ν_s(C–O) stretching of COO–
~1,240	ν_{as}(>P=O) stretching, associated with phosphorus compounds
~1,000	ν(C–O–C) stretching from polysaccharides

2010). *Euglena* sp. is a potential indicator of the presence of high quantity of organic carbon in wastewaters (Mahapatra et al. 2011a) and hence its eco-chemical characteristics are presently being used for wastewater decontamination (Ahmed and Hader 2011). Heterotrophy is advantageous as it reduces the dependency on bacteria to decompose complex organic matter in sewage and hence treat the water faster. TOC, TN and TP concentrations of the feed were intermediate compared to the earlier reports (Wang et al. 2010a; Zhou et al. 2102) and does not require pre-treatment through anaerobic digestion (Park et al. 2011). The domestic wastewater without any pre-treatment favours algae growth (as in current experiments) compared to highly concentrated wastewater samples as in centrate water (sludge-concentrated water) and dairy wastewaters (Wang et al. 2010a, b). Organic carbon in wastewaters can manifest into acids like acetate, butyrate or alcohols in the absence of pre-treatment (Zhou et al. 2012) and can be easily utilised by mixotrophic algae such as *Euglena* (current study), *Chlorella* (Liang et al. 2009) and *Scenedesmus* (Li et al. 2010). Voluminous nutrient content in such domestic wastewater helps in the profuse biomass growth and rapid nutrient uptake.

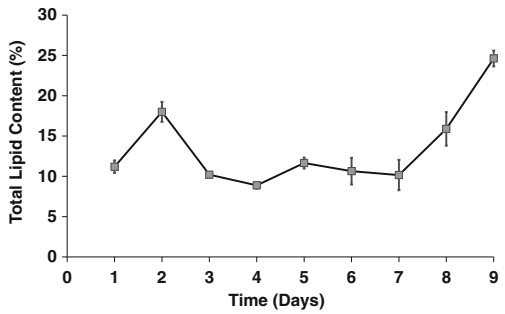

Fig. 8 Changes in lipid content of *Euglena* sp. *Error bar* shows mean + SD ($n = 3$)

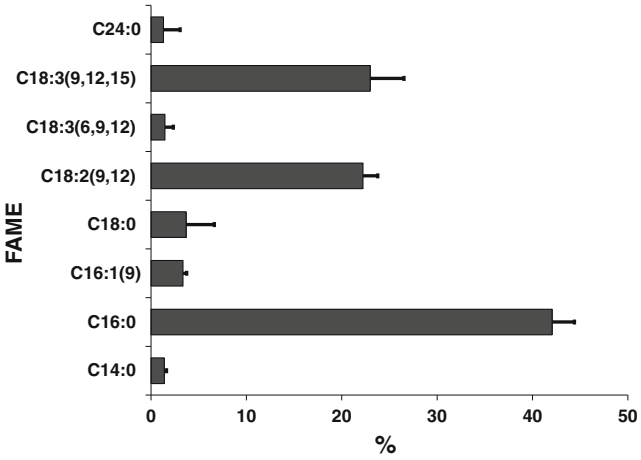

Fig. 9 Major FAME obtained during the analysis. *Error bar* (*whisker*) shows mean + SD ($n = 3$)

After the initial lag period, there was a rapid increase in the average specific growth rate (μ) from 0.05 day^{-1} (day 2) to 0.72 day^{-1} (day 4) during culturing (Table 3); this was due to heterotrophic algal growth with the availability of abundant soluble organic matter as mentioned in earlier studies (Bhatnagar et al. 2010). TOC is correlated significantly with the specific growth rate μ ($R^2 = 0.77$). Decline of TOC with the increase in μ suggests rapid organic C consumption during the initial stages of the culture, similar to the earlier reports (Ogbonna et al. 1998). This highlights the importance of soluble C for good growth of cells and indicates healthy heterotrophic growth of *Euglena* sp. The absence of visible suspended particles in the culture solution initially aided light penetration and algal growth as algal cells adapted to the wastewater environment.

Euglena sp. entered the exponential phase after 2 days of lag period indicating the stress due to changes in the environment (as it was previously grown in BB media) and slowed down between the 4th and the 6th day (Table 3). Elemental analysis of carbon and nitrogen in the algae biomass indicated the transition through increased protein synthesis due to the rapid N uptake and turnover during day 4. This is also evident from the decline of C/N ratio from day 4 onwards indicating the slowing down of the growth (Fig. 2). TOC/TN ratio of the culture broth also showed a lower value during days 4 and 5 of the culture period indicating rapid C fixation into algae biomass compared to N fixation. These observations help in understanding C and N dynamics including C partitioning in the algae biomass; however, it requires further investigation.

Removal of nutrients with a lower N/P ratio of 2:1 is observed compared to the earlier experiments with N/P ratio of 20–11 (Ho et al. 2003; Boelee et al. 2011). N removal assessment shows faster NH_4-N removal by rapid consumption, favoured by organic C availability in the medium and subsequent decline in algae growth due to exhaustion of NH_4-N (Fig. 5). Higher N removal efficiencies were observed

Table 3 Variations in the growth rate, biomass productivities and lipid productivities with specific growth rates (μ) with the culturing time (T, in days)

Time (day)	Biomass productivity (g $L^{-1}day^{-1}$)				Lipid productivity (%)		
	Productivity (mg $L^{-1}day^{-1}$)	Growth rate	Increase (%)	μ (day^{-1})	Lipid content (%)	Daily accumulation rate	Increase (%)
1	0.18				11.20		
2	0.19	5.26	85.48	0.05	18.00	60.71	54.51
3	0.42	54.76	84.68	0.79	10.20	−43.33	26.89
4	0.86	51.16	66.13	0.72	8.88	−12.94	58.57
5	0.88	2.27	30.65	0.02	11.65	31.19	63.93
6	1.02	13.73	29.03	0.15	10.64	−8.67	52.68
7	1.11	8.11	17.74	0.08	10.16	−4.51	56.78
8	1.24	10.48	10.48	0.11	15.89	56.40	58.73
9	1.10	−12.42	0.00	−0.12	24.62	54.94	35.46

compared to other studies conducted in the batch mode (Woertz et al. 2009; Wang et al. 2010a, b). The highest N removal was observed during the 5th and the 6th day possibly owing to very high growth rates. NH_4-N removal of ~98 % in 8 days was observed, contrary to TN removal of only 31 % with *Scenedesmus* sp. (Li et al. 2010) with a decrease in biomass density at NH_4-N loads of 15 mg L^{-1}. *Scenedesmus acutus* removed ~90 % of NH_4-N in 8 days of a batch culture in ammonia-rich (NH_4-N ~32 mg L^{-1}) wastewater (Doria et al. 2012).

High NH_4-N removal is observed during the 5th to the 7th day (~60 %, Fig. 5). In certain cases, NH_4-N removal takes place by NH_3 volatilization at high temperature under alkaline conditions (Tam and Wong 1990), due to high urea content in wastewaters (Matusiak et al. 1976). In the present study, NH_4-N removal can be attributed to algal uptake rather than volatilization as temperature was always below 26 °C and urea was not a major constituent of the wastewater. Small fraction of NH_4-N is also absorbed through the cell walls of microorganisms (Volensky 2001; de Philippis et al. 2007). Nitrate (NO_3-N) and nitrite (NO_2-N) values reveal low nitrification during the culture growth. There was an increase in NO_3-N due to excess of NH_4-N in the system initially, up to day 6 after which it decreased. Though algae assimilate NH_4-N (at a higher rate), still some NH_4-N was available for nitrification up to day 6. After day 6, N started to be limiting thus ceasing subsequent nitrification (Fig. 5). Decrease in the nitrate content after day 6 is due to the uptake of nitrates and nitrite along with NH_4-N by the algae as they were in the exponential phase comparable to the earlier experiment (Stehfest et al. 2005). NO_2-N values consistently decreased showing low nitrification. *Chlorella* sp. also showed a decrease in nitrite content accompanied by a decrease in nitrate content at an initial N concentration of 17 mg L^{-1} (Wang et al. 2010b). Low nitrification can be attributed to the unavailability of the N sources, i.e. NH_4-N at the time of nitrification, lack of substrates for the attachment and growth of the nitrifiers and low culture time as nitrifiers are slow growers (Wiesmann 1994).

TP was reduced by 66 % from 16.5 to 5.5 mg L^{-1}, and most of the P was removed on days 5 and 6. P removal efficiency was lower compared to the 72 % removal in digested dairy manure (Wang et al. 2010a), 86 % removal from concentrated wastewater (Wang et al. 2010b), 90 % removal from artificial wastewater (Yujie et al. 2011) and ~85 % removal from secondary treated wastewater. Lower TP removal of 66 % highlights the availability of large quantity of unutilised P and with a small supplemental C and N to the broth would aid in the algal growth and hence the complete removal of nutrients. There are chances of P precipitation under high pH conditions (Song et al. 2002). As the culture experienced a rise in pH up to pH9, the total phosphate removal can be attributed to both algal uptake and P precipitation. *Ortho*-P was reduced by 85 % through uptake during the exponential stages of the algal growth. Approximately 15 % of the easily accessible P was still unutilised in the medium due to reduced algae growth with C and N limitation. Similar findings were also reported by Zhou et al. (2012). Earlier studies (Aslan and Kapdan 2006) attribute the underutilisation of P to light limitation due to dense biomass in the culture. The N/P ratio of the algal biomass normally ranges between 6.8 and 10 (Wang 2010b). N/P ratio of 2 was observed during the initial phase of algal culture which declined to 0.42, indicating excess P concentration all along the growth phase. This alteration however did not affect the algae growth and N consumption as in other studies (Wang et al. 2010a; Park et al. 2011; Wang and Lan 2011) as long as there was sufficient C supply. In such conditions, algae store excess of P (Wang et al. 2010a, b) as polyphosphates through luxury P uptake (Aitchison and Butt 1973; Powell et al. 2008).

The FTIR assignments of bands to the spectra reveal high carbohydrate and protein peaks during the initial phase of the culture. Enhanced lipid/amide (L/P) ratio at the stationary phase illustrates an increase in lipid content with a

decrease in protein content, which correlates with the total lipid content during day 2 of the culture (Fig. 8). Decline of L/P ratio after day 3 correlates with the increase in the carbohydrate/amide (C/P) ratio from days 4 to 5. In the present experiment, the L/P ratio increased from 0.02 to 0.26 (12-fold increase on day 9), which is relatively higher compared to the earlier studies (Stehfest et al. 2005; Dean et al. 2010). Investigations of *Phaeodactylum tricornitum* under nutrient sufficient conditions and N deplete conditions showed an L/P ratio of 0.23 (1.27-fold increase on day 14) and 0.3 (1.2-fold increase on day 26), respectively, (Stehfest et al. 2005). In contrast, *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* under lower N regimes showed an L/P ratio of 1.4 (6.75-fold increase on day 28) and 1.5 (7.1-fold increase on day 15) (Dean et al. 2012). *Pediastrum duplex* under natural conditions showed an L/P ratio of 0.2 (Dean et al. 2012).

Carbohydrate/amide ratio peaked on day 5 of the culture (Fig. 10), indicating increased polysaccharide content in the biomass due to rapid TOC assimilation (from days 2 to 6 in the culture). Lower C/P ratio initially is attributed to an increase in the protein content in the cells relative to carbohydrates with the increase in biomass densities. Enhanced C/P ratio shows potential N limitation that would have triggered the lipid synthesis (Dean et al. 2012). In the present experiment, there was an increase in C/P ratio from 0.94 to 2.41 (2.56-fold increase on day 5). However, studies on *P. tricornitum* under nutrient-sufficient and N-deplete conditions showed a C/P ratio of 0.8 (1.14-fold increase on day 14) and 0.3 (2.42-fold increase on day 26), respectively, and a 6.7-fold increase in *Microcystis auregonosa* and 3.5-fold increase in *Chroococcus minutus* (Stehfest et al. 2005). *C. reinhardtii* and *S. subspicatus* under lower N regimes showed a C/P ratio of 2.4 (9.6-fold increase on day 10) and 1.5 (3.75-fold increase on day 10), respectively, (Dean et al. 2012). *P. duplex* under natural conditions showed a C/P ratio of 0.6 (Dean et al. 2012).

Lipid content of *Euglena* sp. was 24.6 %, and there are few studies on lipid quantification of *Euglena*. During 1960s, studies were focussed on physiology, photosynthetic ability and lipid biosynthesis of *Euglena gracilis* (Rosenberg 1963). It has been reported that due to their heterotrophic nature,

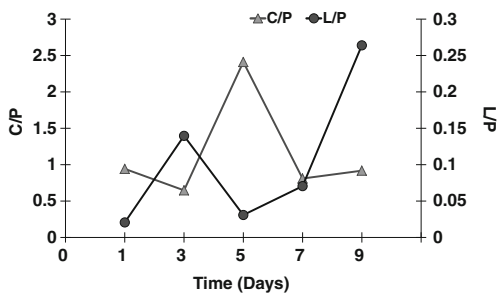

Fig. 10 Changes in the carbohydrate/amide ratio and lipid/amide ratio of *Euglena* sp.

Euglena can assimilate more soluble C from solutions and can accumulate lipids faster than other algae in the dark (Coleman et al. 1988). Regnault et al. (1995) reported lipid accumulation of *E. gracilis* to be independent of NH_4-N limitation and C sensitive, which increased in the presence of lactate. In the present study, the lipid content of *Euglena* sp. suddenly increased on day 2 of the culture owing to C fixation into lipids under heterotrophic/mixotrophic mode due to availability of organic carbon. A possible reason can be alteration in C metabolism due to adaptation to new wastewater conditions (Pittman et al. 2011). Average lipid production in *Euglena* sp. was 16.15 mg $L^{-1}day^{-1}$, and the biomass productivity was 155 mg $L^{-1}day^{-1}$. This is comparable to 8–20 mg $L^{-1}day^{-1}$ in other algae such as *Nannochloropsis oculata* and *Chlorella vulgaris* (Converti et al. 2009). A very high lipid productivity of 505 mg $L^{-1}day^{-1}$ was reported from *C. reinhardtii* (bio-coil) in concentrated wastewater with the biomass productivity of 2 g $L^{-1}day^{-1}$ (Kong et al. 2010). Lipid production of 24 mg $L^{-1}day^{-1}$ was obtained in a semi-continuos mode growing polyculture of *Chlorella*, *Micracitinium* and *Actinastrum* (Woertz et al. 2009). *Euglena* sp. grown in continuous systems would help in treating domestic wastewater while providing biofuel and ensuring the sustenance of energy at decentralised levels.

Culture condition does play a vital role in determining the quality and quantity of lipids in algae (Solovchenko et al. 2008). The quality of biofuel depends on the composition of methyl esters in lipids in addition to the structural characteristics like chain length and degree of saturation (Griffiths and Harrison 2009). There were thirteen different fatty acids identified in the FAME mixture extracted from *Euglena* sp. grown in domestic wastewaters, among which palmitic acid (C16:0), linolenic acid (C18:3) and linoleic acid (C18:2) are the abundant fatty acids. The percentage composition of each fatty acid with their time of retention is shown in Table 4. It has been observed that vegetative oil that has good biofuel properties as linseed, rape seed, etc. mostly contain C18 fatty acids comprising of stearic, oleic, linoleic and linolenic acids (Lang et al. 2001). The lipid profile shows a dominance of C16 (42 %) and C18 (50 %) methyl esters, emphasising that the lipids produced from *Euglena* sp. are similar to vegetative oil and have good biofuel properties. Lipids with the larger share of palmitate and oleate contents have biofuel properties in terms of ignition, better oxidative stability and lubrication (Knothe 2005). PUFA content of 46 % observed in the present study could lead to susceptibility to oxidation (instability) during storage, requiring a partial catalytic hydrogenation for stability and to be used as biodiesel (Dijkstra 2006). *E. gracilis* cells grown heterotrophically showed FAME comprised of C18:3 (31 %) > C16:0 (25.2 %) > C16:4 (14.8 %) > C18:0 (7 %) with a total lipid content of 27.2 % (Constantopoulos and Bloch 1967). Similar work on municipal wastewater algae showed FAME consisting of C18:3 > C16:1 > C16:0 (Sturm et al.

Table 4 Percentage composition of FAME with the retention time in columns

C N:U	FAME composition	Retention time	Corresponding % max	% FAME
C12:0	Dodecanoic acid methyl ester	19.023	0.1	0.043
C14:0	Methyl tetradecanoate	25.274	3.33	1.399
C15:0	Pentadecanoic acid methyl ester	28.529	0.27	0.113
C16:0	Hexadecanoic acid methyl ester	31.83	100	42.051
C16:1(9)	9-Hexadecenoic acid methyl ester	31.038	7.98	3.357
C17:0	Heptadecanoic acid methyl ester	34.84	0.25	0.106
C18:0	Octadecanoic acid methyl ester	37.859	8.76	3.682
C18:1(11)	11-Octadecenoic acid methyl ester	37.238	2.6	1.093
C18:2(9,12)	9,12-Octadecadienoic acid methyl ester	36.917	52.84	22.22
C18:3(6,9,12)	6,9,12-Octadecatrienoic acid methyl ester	36.352	3.48	1.465
C18:3(9,12,15)	9,12,15-Octadecatrienoic acid methyl ester	37.131	54.46	22.979
C20:0	Docosanoic acid methyl ester	48.817	0.43	0.179
C24:0	Tetracosanoic acid methyl ester	51.964	3.12	1.31
				100
Saturated fatty acids (saturates)				48.886
Monoenoic fatty acids (mono-unsaturated fatty acids)				4.45
Polyenoic fatty acids (poly-unsaturated fatty acids)				46.664
Total unsaturated fatty acids				51.114
C16–C18 (fatty acids important from bio-diesel perspective)				96.953
Max total lipid content				24.6

2012) and *Chlorella pyrenoidesa* with FAME C18:3 (40 %) > C16:0 (16 %) > C18:2 (18 %) > C16:2 (8.5 %) had 21 % lipid (D'Oca et al. 2011). The environmental variables like light play a key role in deciding the composition of fatty acids (Su et al. 2011) that is evident from a relatively higher saturated fatty acids (SFA) compared to poly-unsaturated fatty acids (PUFA) and mono-unsaturated fatty acids when exposed to high intensities of sunlight and longer dark periods. This is contrary to the reduced PUFA when exposed to high light intensities (Seyfabadi et al. 2011). *Isochysis galbana* showed high SFA accumulation during the day and PUFA accumulation in the night (Zhu et al. 1997). The percentage of FAME within C16–C18 was ~97 % in the present study. 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 2005, 2008). It has been reported that the presence of high amount of saturates imparts relatively poor cold flow properties to the fuel (Chiu et al. 2004). In the present FAME study, the percentage of saturated fatty acids was relatively high compared to PUFA with the major share of essential fatty acids.

In conclusion, the present study highlights the possibility of treatment of domestic wastewater with biofuel production using *Euglena* sp. (indigenous algae). The results indicate that culturing algae in mixotrophic mode offers an efficient removal of TOC, N and P from domestic wastewater without any pre-treatment. Developing nations in tropical region need to safeguard and sustain existing freshwater resources through technically feasible, economically viable and environmentally sound technologies to treat wastewater. Algal-based wastewater treatment would be effective in tropical countries in reducing eutrophication. *Euglena* sp. with high biomass productivities accumulate substantial lipid in the cells while removing the nutrients from wastewater. The lipid profiles of the extracted algal oil were similar to the vegetative feedstock oils, indicating a good quality fuel for energy generation. The recycling of nutrients by converting the sewage to algal biomass and then to biofuel is a major environmental benefit to the system. This would also help in mitigating GHGs through assimilation of carbon (as algae are photosynthetic organisms). Algae-based pond systems deployed in the rapidly growing cities would aid in the sustainable management of wastewater through low-cost treatment with the prospects of biofuel in addressing the regional energy demand.

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