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Leachate for Producing 3rd Generation Microalgal Oils

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Abstract: The ability of microalgae to grow well under certain wastewater conditions has indicated the potential of these resources as environmentally sustainable growth medium for producing 3rd generation biodiesel feedstock. Ultra-membrane treated landfill leachate was used as a nutrient medium for growing native microalgal cultures to produce oil for future biodiesel energy conversion. A total of three lab scale experimental sets with different dilutions of leachate were tested with altering pH and phosphate addition. At the end of batch experiment, priliminary results showed highest microalgal dry biomass (2.5 g L^{-1}) in 50% leachate with minimum total cell lipid content. Microalgae grown in 10% leachate (~ 50 mg L^{-1} $N\text{-}NH_4^+$) produced maximum total cell lipid content (114.64 mg g^{-1} dry biomass). Phosphate addition in leachate enhanced the microalgal dry biomass production in higher leachate dilutions (10-50% TL) but total lipid content did not show any significant increase. Leachate stress can be screened further for inducing microalgal lipid production.

Key words: microalgal lipids, landfill leachate, biodiesel feed stock

1. Introduction

1.1 Microalgal Biomass — A Sustainable Alternative Feedstock for Biodiesel

The investigation on microalgae as a sustainable alternative energy source for transportation fuel is not new but the prevailing oil crisis in the oil producing regions, fast depleting fossil oil reserves and environmental pollution concerns (release of green house gases GHG etc.) have made it imperative for organizations and countries to invest more time and efforts into research on sustainable, renewable, environmental friendly-carbon neutral feedstock for biodiesel such as microalgae [1-6].

The renewed interest in microalgae for producing oils is due in part to the high lipid content of some species — 10-30% of dry weight. Lipids and fatty acids form a major part of a microalgal cell as membrane components, metabolites and storage products [7]. Microalgae contain storage lipids (triglycerides TAG) suitable for transesterification for biodiesel conversion [2, 8]. In fact paleobotanical evidence has also suggested that microalgae are responsible for major sources of hydrocarbon (fossil fuels) in a variety of oil-rich deposits dating from the Ordovician period to the present [1, 9, 10].

Eukaryotic algae contain a diverse composition of acyl lipids and their fatty acids. Even within divisions, individual algae contain a bewildering array of lipid compositions (such as saturated fatty acids, polyunsaturated fatty acids, glycolipids or triacylglycerols). Their lipid content differ from strain to strain and can also be adjusted through altering nutrient (C, N, P ratios) and growth conditions (temp, light intensity or pH etc.) [2, 11-13].

1.2 Utilizing Wastewater Resources for Growing Microalgae

The statement made by Chisti [14] in his research article that microalgae produce 15-300 times more oil for biodiesel production than traditional crops on an area basis, further intensified the interest in research

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and development of 3rd generation microalgal biofuels [1, 3, 4, 6]. Some researchers suggested that the claim made by Chisti [14] was not realistic with the then present technology and available strains, and the only way for microalgae to compete with terrestrial crops in the race of 2nd generation biofuels was to use wastewater [2,15,16].

Microalgae are known to grow more abundantly in nutrient rich (high N and P) eutrophic waters leading frequently to algal blooms [1]. If this nutrient requirement for large scale microalgal cultivation is provided by chemical fertilizers then it will provide an immense upstream burden to the life cycle analysis LCA of microalgae biodiesel production system [17-19]. The processing stages of microalgae biofuels need to be simplified with virtually zero energy input for long-term sustainability and environmental benefits [20]. Zhou et al. [21] evaluated that using waste organic biosolids from only three major sources (municipal wastewater, livestock manure and food waste) could potentially support enough bio-crude oil production to completely replace the US demand for petroleum imports.

Dual-use microalgae cultivation for wastewater treatment coupled with biofuel generation has been an attractive option in terms of reducing the energy cost, GHG emissions, and the nutrient (fertiliser) and freshwater resource costs of biofuel generation from microalgal large scale cultivation [6, 11, 22].

1.3 Sanitary Landfill Leachate

A sanitary landfill is a biological reactor to treat and allow final disposal of municipal solid wastes around the world. Leachate is a strongly contaminated liquid that accumulates beneath the landfill site as a result of natural infiltration processes, bio-chemical reactions from both natural degradation as well as compression of the waste and rain water. Landfill leachates LFL often consist of increasing concentrations of toxic xenobiotic organic compounds, Ammoniacal nitrogen ($N\text{-}NH_4^+$), salinity (chloride ions Cl^-) and heavy metals etc. [23-25].

The major potential environmental impact of LFL is pollution of soil, surface water and groundwater. The toxics in LFL can over load the ecosystem, disrupting the natural recycling processes such as photosynthesis, respiration, nitrogen fixation, precipitation and evaporation [11]. To use leachate as a source of fertilizer and water for microalgal oil production, bring an added value which otherwise would be considered as waste needing treatment.

1.4 Leachate to Produce Microalgal Biomass-to-Bioenergy Generation

Since landfills continue to produce leachates throughout its lifespan even after closure, it could be used as a continuous nutrient source for large scale microalgal biomass production for biodiesel generation [26]. Depending on constantly varying characteristics of leachate, researchers around the world have investigated leachate in particular as a growth medium for microalgae for the treatment of toxic heavy metals, ammonia and organics etc. [25-30]. But literature on leachate treatment coupled with microalgal lipid production is scarce [31]. The objective of the present study was to evaluate the potential of ultra-membrane treated landfill leachate TL, taken from Istanbul municipal landfill (Odayeri Istaç-Istanbul Buyuk Şehir Belediyesi) to sustainably grow native microalgal cultures and to screen lipids in microalgal cells for supplying raw material for future biodiesel conversion.

2. Material and Methods

2.1 Culture Conditions

Mixed culture of fresh water microalgal species of Chlorella v*ulgaris* and Chlamydomonas *reinhardii* were obtained from Bioenergy department, Ege University. Ultra-membrane treated leachate TL was kindly provided by Odayeri management and stored at 4°C in 20 L air tight plastic containers in dark until use. Physico-chemical characteristics of autoclaved TL is presented in (Table 1).

Table 1 Characteristics of ultra-membrane treated leachate TL.

Parameter	(mg L^{-1})
Total organic carbon TOC	135.6
Ammonium NH_4	485
Ortho-phosphate PO4	5
Total dissolved solids TDS	18.74
Conductivity mS/cm	26.7
Salinity ppt	23.9
Chloride ion Cl^{-1}	9060
pH	7.5

Exponentially growing inoculum at a volumetric ratio of 20% (v/v) measured at an absorbance of 680 nm (using spectrophotometer, model U-2001, HITACHI, Japan) was used to start and monitor growth in the 3 experimental sets. BG11 UTEX medium used in the experiments consisted of the following nutrients: $NaNO_3$, $MgSO_4$, KCl, $CaCl_2$. $2H_2O$, $NaHCO_3$, $NaNO_3$, KH_2PO_4, K_2HPO_4, Fe Ammonium citrate, Citric acid, and trace elemental solution. Trace elemental solution includes $CuSO_4$. $5H_2O$, $ZnSO_4$. $7H_2O$, $MnCl_2$. $4H_2O$, $CoCl_2$. $6H_2O$, $FeSO_4$. $7H_2O$, EDTA, H_3BO_3, $NaMO_4$. $2H_2O$.

Cultures were put in 1L glass bottles with 500 ml working volume, continuous air bubbling was supplied at a rate of 3 L/min (flow rate in each bottle was around 0.31 ml min^{-1}), continuous artificial irradience of 60 µmol $m^{-2}s^{-1}$ was provided through white fluorescent lamps (measured by a digital light meter - linkoln USA) and maintained at room temperature of 25±1°C. Cultivation was carried out under batch mode in dublicate for 27 days. The data was statistically analyzed by Student's t test comparing the control at $p \leq 0.05$. Values presented in the results section are averages of dublicate cultures.

2.2 Lab Experimental Setup

A set of 3 experiments were conducted with same cultivation conditions as mentioned in section 2.1. Different dilutions of autoclaved (20 min at 1 atm, 121°C) treated leachate TL (i.e., 10%, 30%, 50%, 70%, 90%, 100%) with distilled water (dw) as –ive control and regular BG11 media as +ive control, were formulated to evaluate microalgal growth and lipid content. 0.5 N $NaOH/H_2SO_4$ was added manually on alternate days for pH control. The only difference in the experiments was as follows:

- In experimental set 1, pH was maintained within a range of 6.5-8.5.
- In experimental set 2, pH was maintained within a range of 6.5-7.5 and
- In experimental set 3, pH was maintained at a range of 6.5-7.5 with phosphate addition (same as BG11 media with N/P ratio 40:1).

2.3 Microalgal Biomass Dry Weight and Lipid Extraction and Staining

Biomass dry weight was measured by filtering the microalgal samples through whatman filter papers (0.7 pore size, GF/F) at the start and end of each batch cultures. Filters were oven dried at 60°C until constant weight (~ 3 days) and then weighted on a measuring balance.

Dried algal biomass was homogenized in mortar and pestle and 500 mg from each experimental set was subjected to lipid extraction following folch method [32] with sonication. 10 ml mixture of chloroform and methanol (2:1) was added to dried biomass in a glass tube and undergone sonication (50 Hz) for 20 min. The extract was equilibrated with 1/4th its volume of a saline solution and vortexed. The tubes were centrifuged at 2000g for 10 min for the separation of two layers. Lower chloroform layer containing lipids was carefully transferred to pre-weighted glass vials and dried under fume hood at 80°C. The dried lipids were then gravimetrically measured.

Nile red (9-diethylamino-5H-benzo [α] phenoxazine-5-one, $C_{20}H_{18}N_2O_2$) staining of microalgal intact cells containing neutral lipids was carried out using fluorescence microscopy (OLYMPUS BX50 with attached camera) [33]. Oven dried microalgal biomass was homogenized in pestal and mortar. Wasted with phosphate buffer and

centrifuged twice and then 10 µm was spread on glass slide with 10 µm nile red stain and 30% ethanol solution. Slides were visualized under florescence microscopy.

3. Results and Discussion

3.1 Microalgal Biomass Growth in the 3 Experimental Sets

Microalgae usually accumulate lipids as part of their grown up cells, that's why growth curve was first carefully monitored to check if TL was supporting microagal biomass growth. The high nitrogen content of TL makes it attractive for microalgae cultivation as green algae demand more nitrogen and phosphorus than do many other plant species but the same nitrogen source $N\text{-}NH_4^+$ can become toxic in higher concentrations. Excessive $N\text{-}NH_4^+$ can damage photosynthesis organs (chloroplast) and decrease photochemical efficiency [34].

In the present study both stimulatory and inhibitory effects of TL was observed on miroalgal growth in the 3 experimental sets. Irrespective of an adaptation time (~month) given to microalgae before the start of experiments, cultures still showed a lag phase of around 3-5 days in all the formulations (10%-100% TL) due primarily to high total dissolved solids (susceptable for fresh water species) and inconsistency of nutrient compositions in leachate or inhibition by some possible hidden element in leachate medium (Figs. 1-3; Table 1). In experimental sets 1 and 2 microalgal growth in all the dilutions (10%-100% TL) was significantly lower than regular nutrient media BG11 as shown by growth curves (Figs. 1, 2). Experimental sets 1 and 2 were only different in pH ranges, which was established to check its effect on biomass growth. pH above 8.5 can start formation of free ammonia gas, which can leave the liquid medium and could no longer be available for microalgae to assimilate as a nitrogen source for its growth. But the growth curves did not show any significant effect of varying pH ranges on the growth curves of microalgae, which could imply that nitrogen was not lost from the medium in experimental set 2 in the form of ammonia gas (Figs. 1, 2).

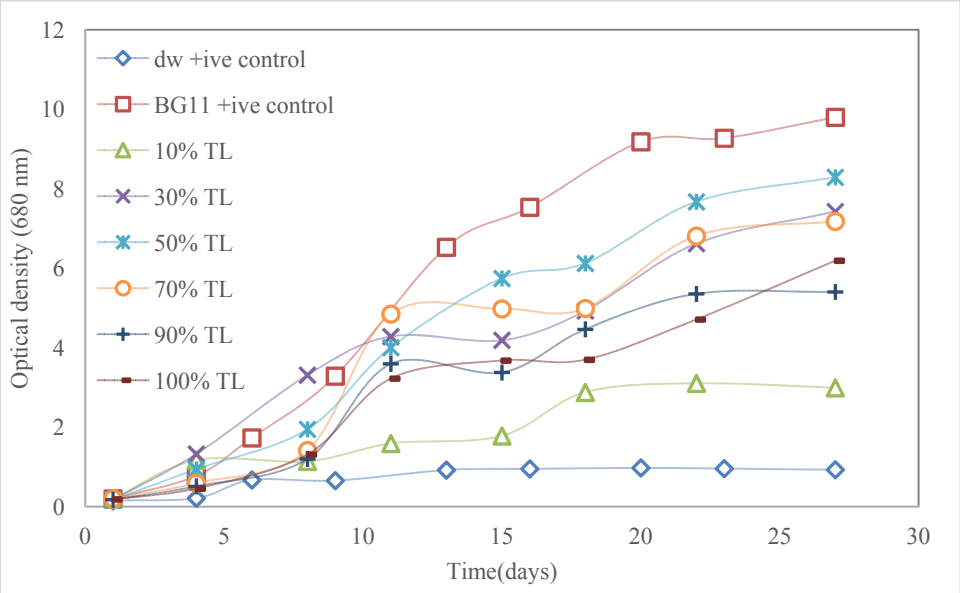


Fig. 1 Experimental set 1 — Biomass growth curve of microalgae in different dilutions of TL.

In general all the dilutions supported microalgal growth in a bell shaped curve, with 50% TL supporting the highest biomass growth in all the 3 experimental sets and the rest of dilutions showing decrease in successive order (Fig. 4). In 10% TL, nutrients seemed insufficient due to dilution and the growth curve stayed

closer to negative control (dw) and very low dry biomass was observed (Figs. 1, 2, 4). In higher formulations (70%-100% TL) growth was slow and steady in all the 3 experimental sets, with growth curves not showing onset of proper stationary phase, but after day 20th there was an upward increase in growth curve albeit at a very slow pace.

TL had sufficient nitrogen source in the form of $N\text{-}NH_4^+$ to support microalgal growth but was limited in phosphorus P, which is an essential nutrient for growth [35-37]. Experimental set 3 was supplemented with phosphate $P\text{-}PO_4^-$. Since at pH 8 or more phosphate can precipitate [38], in experimental set 3, pH was kept within 6.5-7.5 range to make sure any phosphate elimination from the medium was from metabolic uptake by microalgae. All the TL dilutions after $P\text{-}PO_4^-$ addition showed better growth curves and dry biomass than previous experimental sets 1 and 2 (Figs. 3, 4). After $P\text{-}PO_4^-$ addition, 50% TL showed the highest microalgal growth almost equal to BG11 media with 2.50 g L^{-1} dry biomass (Figs. 3, 4, 6b).

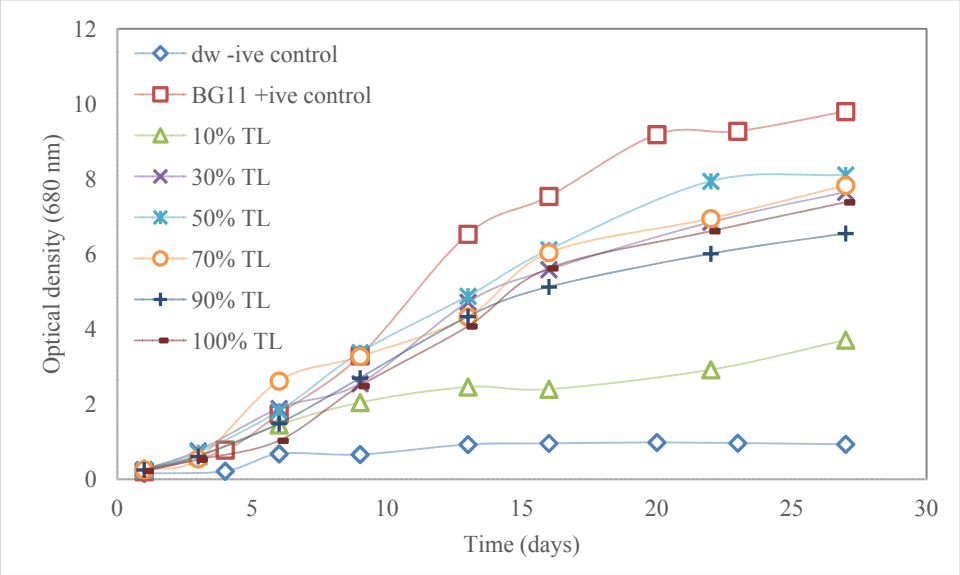


Fig. 2 Experimental set 2 — Biomass growth curve of microalgae in different dilutions of TL.

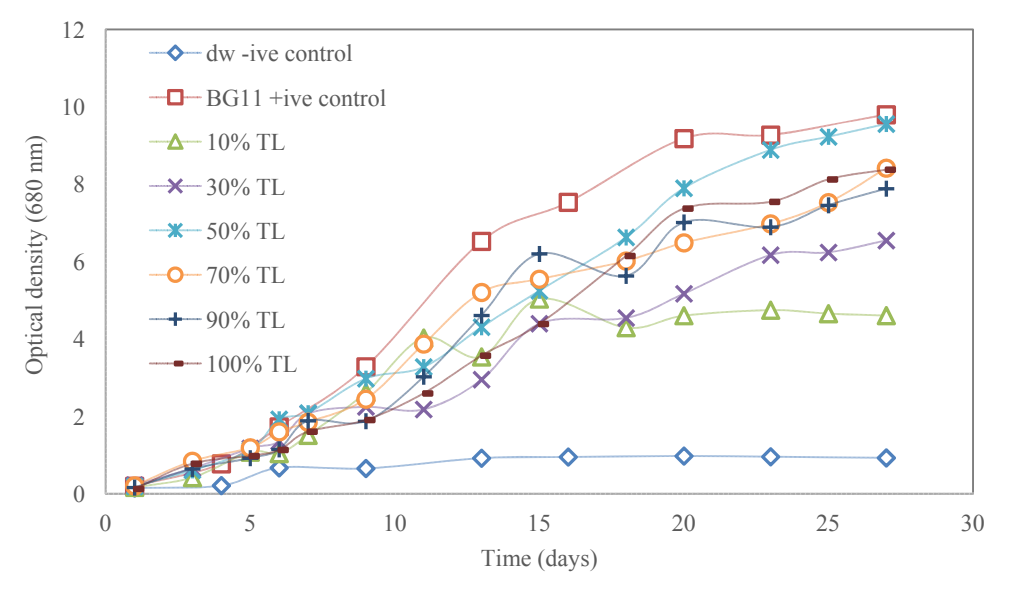


Fig. 3 Experimental set 3 — Biomass growth curve of microalgae in different dilutions of TL.

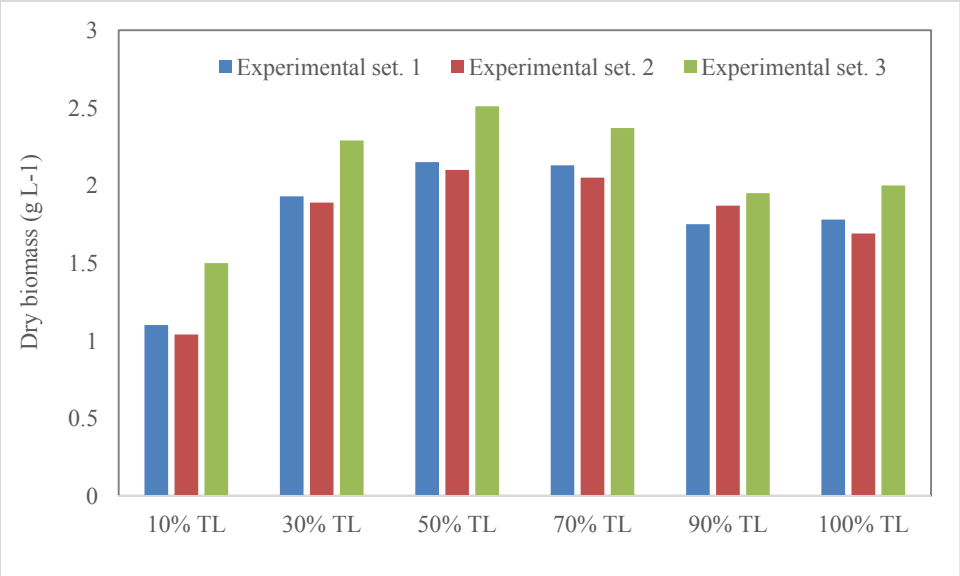


Fig. 4 Microalgal dry biomass (g L^{-1}) in different dilutions of TL in the 3 experimental sets.

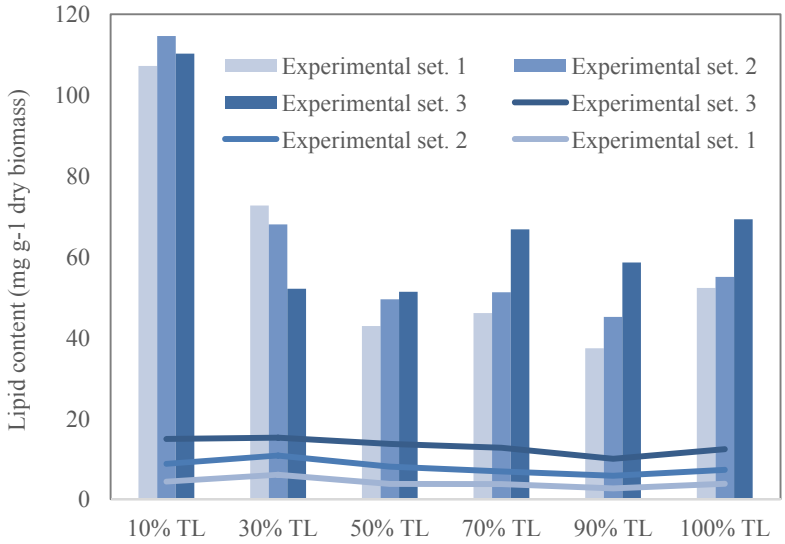


Fig. 5 Microalgal total cell lipid content (bar graph) and lipid productivity (stacked line) in different dilutions of TL for the 3 experimental sets.

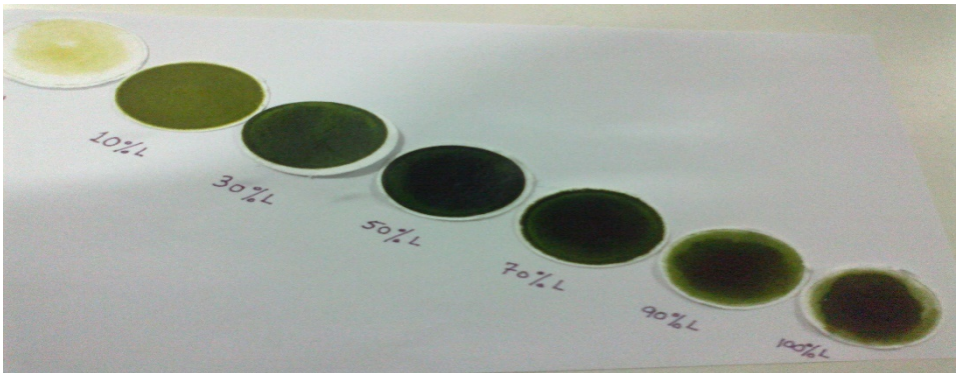
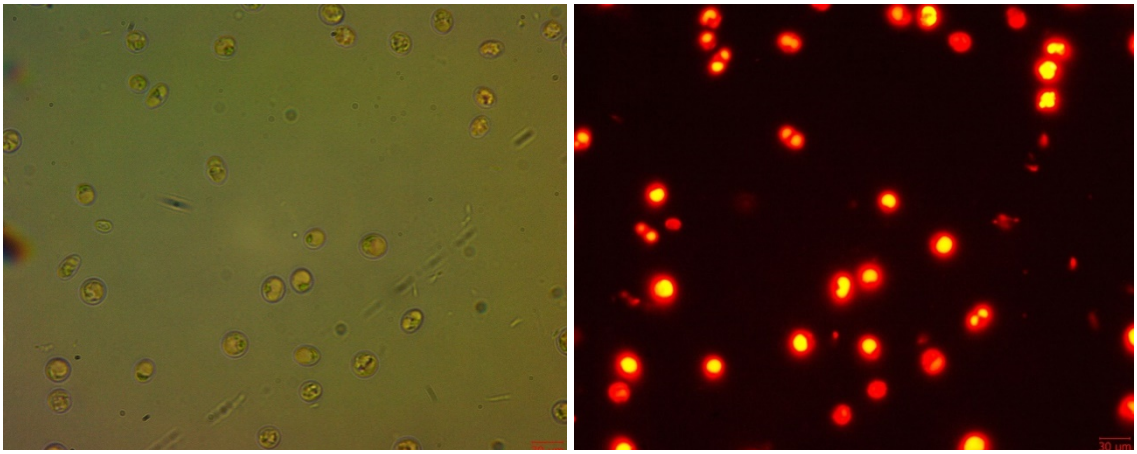


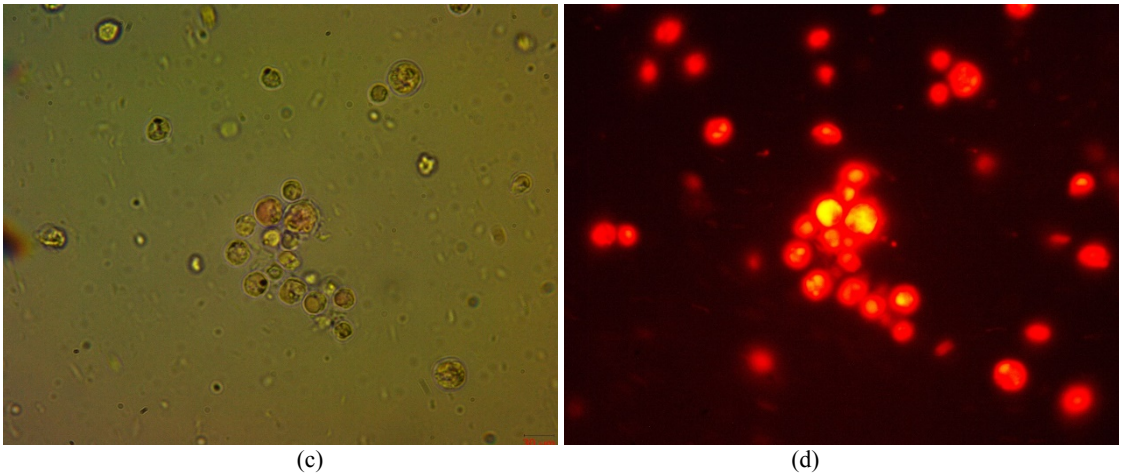
Fig. 6a Oven dried filtered dry weights of microalgal cells grown in different dilutions of TL.

Fig. 6b Cultures in different dilutions of TL at the end of batch cultures.



(a) (b)

Fig. 7 Nile red staining of dried microalgal cells grown in 10% TL: (a) Dried cells under 100x magnification using normal microscope; (b) Same cells with their stored lipids (yellow region) stained with nile red as observed under florescence microscope (green excitation filter at 604 nm).



(c) (d)

Fig. 7 Nile red staining of dried microalgal cells grown in 50% TL: (c) Dried cells under 100x magnification using normal microscope; (d) Same cells with their stored lipids (yellow region) stained with nile red as observed under florescence microscope (green excitation filter at 604 nm).

3.2 Microalgal Lipid Content in the 3 Experimental Sets

In any process aimed at oil production by photosynthesis, the key objective is a high photosynthetic efficiency of lipid production. According to literature survey environmental stress conditions particularly decreased nitrogen in the medium, induces oil production in microalgal cells. Rodolfi et al. [2] evaluated that when nitrogen deprivation is imposed upon a culture exposed to suitable irradiences, photosynthesis continues, albeit at a slow rate and the fixed carbon flow is diverted from protein to either lipid or carbohydrate synthesis. The major limitation of this approach is that despite the fraction of lipids may increase, biomass productivity of the microalgal cells is often very low and so overall lipid productivity will not be high. Growth conditions that focus on providing high biomass productivity instead may ultimately be more economical and may be a more efficient means of increasing total lipid productivity [2, 11, 39]. Similar trend was observed in the present study where 10% TL (~ 50 mg L^{-1} $N\text{-}NH_4^+$) had the lowest biomas yield, but it produced highest lipid content (107.24-114.64 mg g^{-1} dry biomass) (Fig. 4, 5). 50% TL (~ 248 mg L^{-1} $N\text{-}NH_4^+$) showed highest microalgal growth interms of dry biomass in all the 3 experimental sets but lipid content was almost half when compared with 10% TL (Figs. 5, 7bd). Microalgae growing in 50% TL and 100% TL produced almost same lipid content, which implied that TL can be used without dilution in further studies to optimize lipid yield.

3.3 Effect of Phosphate on Lipid Content

To enhance oil yield of algae cultures the cell lipid content should be increased over the basal value without significant losses of productivity. The high biomass productivities (in some cases high lipid productivities) of the wastewater-grown microalgae suggests that there is real potential in the utilisation of these high nutrient resources for cost-effective biofuel generation and production of sustainable and renewable energy [39]. Although culture composition and growth conditions may be less managable in municiple wastewater and most microalgae have relatively low total lipid content per cell under wastewater conditions, ranging from low (< 10% dry biomass) to moderate (25-30% dry biomass) lipid content, the high biomass productivity potentially can translate to significant total lipid productivity [6, 11].

In the present study Phosphate $P\text{-}PO_4^-$ was added in experimental set 3 to enhance the growth of microalgae which was hypothesized to further increase the cells lipid content and productivity. Phosphate $P\text{-}PO_4^-$ addition significantly enhanced the biomass growth curves and dry biomass of microalgae in higher dilutions (10%-50% TL) but the lipid content did not show any significant difference when compared with experimental sets 1 and 2 (Figs. 3-5). Phosphate addition increased lipid content and productivity in lower dilutions (70%-100% TL) (Figs. 5, 6a). Chu et al. [40] pointed out that phosphorus plays a significant role in lipid production under nitrogen deficiency. In their study excess phosphate (35 mgL^{-1}) in nitrogen starvation conditions achieved highest lipid productivity (58.39 $mgL^{-1}day^{-1}$) but lipid content remained the same after 14 days of cultivation. While in the present study, it seemed that phosphate $P\text{-}PO_4^-$ was mainly metabolically uptaken for growth purposes and was not sufficient enough to induce lipids production in microalgal cells in higher dilutions (10%-50% TL) but in lower dilutions (70%-100% TL) $P\text{-}PO_4^-$ induced lipid production with no enhanced growth (Figs. 4, 5).

Study by Xin et al. [36] suggested high lipid content (53%) and productivity (0.075 gL^{-1}) under phosphorus limitation. However under nitrogen limitation lipid content was enhanced (30%) but productivity per unit volume of the culture medium was rather low because the algal biomass was also very low (0.05 g L^{-1}). In the present study, cultures in

10% TL produced the highest lipid content irrrespective of phosphate limitation (sets 1 and 2) or addition (set 3) (Fig. 5). The cultures in 10% TL turned pale green after around 10 days and remained so until the end of each batch cultures (Fig. 6b).

Nile red staining of the pale green cultures of 10% TL further confirmed the increased lipid content when compared with 50% TL cultures (Figs. 6a, b; 7a, b, c, d). Almost all cells surviving in 10% TL had lipid storage in them, which absorbed nile red staining and readily responded to florescence microscopy. Since phosphorus addition and limitation had no significant effect on lipid content for higher dilutions (10-50% TL) in the 3 experimental sets, the only reason for increased lipid content in 10% TL can be attributed to nitrogen limitation (Figs. 4, 5). Under N-limited conditions microalgal cells degrade the intracellular abundant proteins to recycle amino acids into proteins more suited for survival. Another quick nitrogen source utilized under stress conditions is chlorophyll and any changes in chlorophyll content are directly reflected in the nitrogen content of microalgal biomass [41]. In the present study the pale green cultures in 10% TL can be attributed to intracellular degradation of chlorophyll for survival of stressed microalgal cells.

In the present study dry biomass and lipid productivity was opposite to what Zhao et al. [31] had observed in their study. They grew Chlorella *pyrenoidosa* in a mixture of leachate and municipal wastewater with no external phosphate addition and their evaluated microalgal biomass was 1.58 g L^{-1} and lipid production was 24.1 mg L^{-1} d^{-1} in 12 days. While in the present study lipid productivity in 10% TL (with highest total lipid content per cell) was very low (~ 4.42 mg L^{-1} day^{-1}) but dry biomass was higher 2.5 gL^{-1} (50% TL). The lipid productivity observed in the present study was in the lowest range according to literature [15, 20]. Leachate is a complex mixture of constantly varying compounds and it might have some possible inhibition or toxic effect on lipid production. Also the possible positive impact of waste addition (stressed condition) on lipid induction may sometimes not proceed as expected. As observed by Kim et al. [42] where microalgal growth following the addition of fermented swine urine was increased by nearly 3-fold (197 mg L^{-1} dry biomass in treated cells over 76.5 mg L^{-1} in control cells) over a 31 day growth period, but the total fatty acid content was significantly reduced (9 mg g^{-1} dry biomass in treated cells compared to 46 mg g^{-1} in control cells).

4. Conclusion

Optimized biomass production is central to economic biodiesel production and this in turn requires careful optimzation of microalgal cultivation systems. In the present study priliminary results suggested that irrespective of high N-NH_4^+ and other stresses in leachate media, fresh water microalgae were able to grow biomass in all the experimental sets and produced lipids. Altering pH of the medium had no significant effect on biomass and lipid content in the 3 experimental sets but addition of phosphate significantly increased the biomass in higher dilutions (10-50% TL) and lipid content in lower dilutions (70-100% TL) of microalgal cultures. Total lipid content of dry biomass was in the lowest range and further screening is required to optimize the microalgae-to-bioenergy system using leachate to produce more lipid for future biodiesel production.

Acknowledgements

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