Phycoremediation of municipal wastewater by microalgae to produce biofuel

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Abstract

Municipal wastewater (WW), if not properly remediated, poses a threat to the environment and human health by carrying significant loads of nutrients and pathogens. These contaminants pollute rivers, lakes and natural reservoirs where they cause eutrophication and pathogen-mediated diseases. However, the high nutrient content of WW makes it an ideal environment for remediation with microalgae that require high nutrient concentrations for growth and are not susceptible to toxins and pathogens. Given that an appropriate algal strain is used for remediation, the incurred biomass can be refined for the production of biofuel. Four microalgal species (*Chlamydomonas reinhardtii*, *Chlorella* sp., *Parachlorella kessleri*-I and *Nannochloropsis gaditana)* were screened for efficient phycoremediation of municipal WW and potential use for biodiesel production. Among the four strains tested, *P. kessleri*-I showed the highest growth rate and biomass production in 100% WW. It efficiently removed all major nutrients with a removal rate of up to 98% for phosphate after ten days of growth in 100%

municipal WW collected from Delhi. The growth of *P. kessleri*-I in WW resulted in a 50% increase of biomass and a 115% increase of lipid content in comparison to growth in control media. The FAME and fuel properties of lipids isolated from cells grown in WW complied with international standards. The present study provides evidence that the green alga *P. kessleri*-I effectively remediates municipal WW and can be used to produce biodiesel.

Keywords

microalgae, wastewater management, biodiesel, nutrient removal efficiency, bioremediation of polluted water.

Abbreviations

WW Wastewater

TIC - Total inorganic carbon

TOC - Total organic carbon

COD - Chemical oxygen demand

BOD - Biochemical oxygen demand

DW - Distilled water

TC - Total carbon

ASW - Artificial Sea water

1. Introduction

The global freshwater reservoir is about 3% of the total water present on earth but only 0.5% of it is available in liquid form.[1] Wastewater (WW) generally is classified into different categories like municipal, agricultural and industrial WW. Every year, there is plenty of municipal WW generated. It is estimated that human societies produce about 3 billion tons of domestic WW every year[2] and ~4400 million cubic meters of it only in Delhi[3] because of the growing population and modernized lifestyle in this fast expanding city. Moreover, a leakage of WW in pristine natural water resources such as lakes, rivers and groundwater decreases the availability of drinking water.[4] An increasing global human population especially in developing countries such as India demands innovative and affordable solutions to tackle the ever increasing threat of water pollution. Using microalgae for WW remediation might be an effective and affordable approach especially for developing countries as physical and chemical remediation approaches are costly, and if toxic substances are being used for remediation, this expose an additional threat to the environment.[5,6]

The major composition of municipal WW is sewage and it comprises majorly pathogens (e.g. bacteria, viruses, and parasitic worms) and non-pathogenic bacteria and a mixture of natural organic and inorganic materials as well as diverse man-made compounds including toxins.[7] Carbohydrates, fats, proteins, amino acids, and volatile acids can make up to three-quarters of organic carbon in sewage.[8] Phycoremediation with microalgae represents a promising approach as it can be efficient if appropriate algal strains are being used. For many microalgae, WW contains essential nutrients for their growth such as nitrogen, phosphorus and organic compounds (e.g. carbohydrate, amino acids and vitamins).[9] . In contrast to other organism,

microalgae are unique, having ability to grow mixotrophically and utilizing organic/inorganic carbon substrate to produce biomass and an efficient low-cost bioremediation treatment for wastewater along with biomass production. Algal utilization on secondary and tertiary treatment processes might provide unique and elegant solution on the removing of substances originated from various sources and microalgal mitigation of nutrients originated from municipal wastewater has shown great applicability towards biomass production that can be used as a biofuel feedstock. However, not all microalgae are able to tolerate WW environment which toxic due to multiple factors that depends on the source of waste and the type of wastewater.[10] Thus, screening for suitable algal strains is a prerequisite for algal based phycoremediaton of WW. The selection of strains most likely will be dependent on the chemical and biological composition of the WW.

The use of WW to produce algal biofuel at a commercial scale was proposed by Oswald and Golueke[11]. However, so far, only a few microalgae strains were tested for WW treatment and subsequent biofuel production. The most common strains were *Chlorella vulgaris,*[12] *Chlorella pyrenoidosa,*[13-15] *Chlamydomonas polypyrenoideum,*[16] *Scendesmus obliquous*[17] and *Botryococcus braunii.*[18] Seawater microalgae species have also been used to treat WW despite their salinity requirements.[19] Growth of microalgae in WW has been studied under various physiochemical conditions such as light, temperature, pH, CO_2 concentration, and nutrients, but the use of WW for the production of algal-based biofuel has not been explored extensively yet. [20, 21] Thus, the scope of this study was to identify an algal strain that efficiently remediates municipal WW and can be used for biofuel production. [22,10,15]

WW for this study was obtained from sewage draining into the Neela Hauz Lake, New Delhi. A civil litigation was filed at the Delhi High Court to remediate this sewage based on a public initiative complaining about the pollution of Neela Hauz Lake. Despite promises in Court, there seems little hope for the restoration of Delhi's water body. This lake is the main source of drinking water in the area and one of the largest natural water bodies of South Delhi. We think that phycoremediation of sewage polluting Neela Hauz Lake is an efficient and cost effective approach to improve its water quality. Therefore, in present study, a comparative evaluation of four microalgae was undertaken in term of growth, and identified the green alga *P. kessleri*-I as being most appropriate for efficient nutrient removal, biomass production and synthesis of FAMEs that comply with international standards for biodiesel production.

2. Materials and Methods

2.1 Microalgae strain and growth condition

Axenic cultures of four microalgal strains (*Chlamydomonas renhadtii, Chlorella* sp. *Parachlorella kessleri*-I, *Nanochloropsis gaditana*) were obtained from *Chlamydomonas* Genetics Centre, Duke University (USA), Indian Agricultural Research Institute (India), Indian Institute of Technology Madras (India) and National Centre For Marine Algae (USA), respectively. These strains were maintained on respective medium in 250 ml Erlenmeyer flasks (100 mL of culture) at 25 ± 1°C at a ligh-dark cycle of 16/8 hours under white LED light with an irradiance of 5000 lux. The cultures were continuously shaken on an orbital shaker at 150 rpm. The Tris/Acetate/Phosphate (TAP) and ASW medium were used for fresh water and marine water microalgal strains, respectively.[23,24] Microalgal strains were grown at varying

concentrations of WW, adjusted by the addition of distilled water (DW), i.e. (25%WW+75%DW), (50%WW+50%DW), (75%WW+25%DW) and (100%WW). Each microalga was grown in their respective medium (TAP/ASW) as a control. For testing their ability to accumulate biomass and for the analysis of FAMEs, the selected strains were grown in 1L flasks under conditions mentioned above.

2.2 Estimation of specific growth rate and doubling time

The specific growth rate (μ) and cell doubling time were estimated based on the optical density at 750nm[25] using the following equation,

$\mu = \ln(N_1/N_2)/ t_1-t_2$

where, $N_1$and N_2 stand for the optical density at 750 nm of the culture suspension at the beginning (t_1) and end (t_2) of the selected time intervals.

Doubling time = $\ln (2)/\mu$ (See Table S1).

2.3 Wastewater collection, filtration and storage

Wastewater was collected in bulk from the Neela Hauze Lake situated between 28.528950° N latitude and 77.170910° E longitude, New Delhi. Sedimentation and filtration through 0.2μm filters (Corning® bottle-top vacuum filters) removed solid particles. After filtration, wastewater was stored at 4°C in the dark until needed for the experiments.

2.4 Nutrient removal analysis of wastewater

The nutrient removal capacity were analysed on beginning (0 day) and end (10 day) of the experiment by measuring physico-chemical parameters such as total nitrogen, total phosphate, COD, BOD, TOC, TIC, iron magnesium, alkalinity and hardness. These parameters were studied following standard methods reported by the American Public Health Association (APHA).[26] On

beginning of the experiment, wastewater was filtered using 0.2µm filters (Corning® bottle-top vacuum filters) and filtrates were analysed for physic-chemical parameter. On end of the experiment (10^{th}), algal biomass was harvested by centrifugation at 3000 rpm for 15 min at 4^{o}C as reported,[27] and remaining wastewater (supernatant) was filtered using 0.2µm filters. Then, filtrates were subjected to physico-chemical analysis.

The percentage removal of nutrients was calculated using the following equation:

% nutrient removal efficiency = $[(C_o - C_F)/ C_o] \times 100$

where, C_o and C_F stand for initial concentration on beginning of the experiment (0 day) and final concentration on end of experiment (10 day), respectively.

2.5 Measurment of photosynthetic quantum yield of PSII (Fv/Fm)

Chlorophyll (Chl) fluorescence measurements were conducted to determine the combined effect of nutrients present in wastewater on the photosynthetic quantum yield of *P. kesseleri*-I using a Dual-PAM 100 Chlorophyll Fluorometer (Heinz Walz, Germany). For Chl fluorescence induction analyses, cell suspensions of *P. kesseleri*-I were adjusted to a yield of 2.5 µgChl mL^{-1}. After 15 min incubation in the dark to completely oxidise PSII, an actinic flash of 100µs was used to induce maximum Chl fluorescence. Fv/Fm (maximum quantum yield of photosynthesis) was calculated according to the following equation:

Fv/Fm=(Fm-Fo)/Fm

Where, Fm is maximum fluorescence, and Fo is minimum fluorescence resulting in the variable fluorescence Fv.

2.6 Biomass harvesting and dry weight measurement

Microalgae cultures were harvest with a Sorvall RC6 Plus centrifuge (Thermofisher Scientific, Waltham, MA) at 4500 rpm for 10min and the harvested biomass was dried at 60°C until it reached to constant weight according to Rai et al.[28]

2.7 Lipid analysis

2.7.1 Visualization of cellular lipids by Nile Red

Intracellular lipid bodies were visualized via Nile Red (9-diethylamino-5H-benzo[a]-phenoxazine-5-one) staining.[29,30] Briefly, 1 ml of the the algal culture was centrifuged at 12,000 rpm for 10 min. The pellet was re-suspended in 1 ml of 20% DMSO and vortexed for 1min at room temperature. Cells were centrifuged at 12,000 rpm for 5 min. The pellet was suspended in 1 ml of water and vortexed before adding Nile Red (5 µl of 1 $mgml^{-1}$ in DMSO) and incubated for 5 min in the dark at room temperature. Stained cells were visualized under a fluorescent microscope (Nikon TE2000-U) using UV light with excitation and emission at 485 nm and 552 nm, respectively.

2.7.2 Gravimetric analysis of total lipids

Extraction of lipids was done following protocol by Bligh and Dyer[31] with some modifications. To a 10 ml glass vial containing a known amount of algal biomass, 2 mL methanol, 0.9mL water and 1 mL chloroform were added and kept for 24 h at room temperature. The mixture was vortexed for 2 min. and 1 mL of chloroform was added. This mixture was then shaken vigorously for 1 min; 0.9 mL of distilled water was added, and the mixture was agitated in a vortex again for 2 min. The different layers were separated by centrifugation for 10 min at 2000 rpm. The lower layer was filtered through Whatman No. 1 filter paper into a previously weighed clean vial (W1). Evaporation was carried out in a water bath, and the residue was dried at 80°C

for 30 min. The weight of the vial was recorded (W2). The lipid content was calculated by subtracting W1 from W2.

2.8 FTIR spectrometry

Fourier Transform Infra-Red (FTIR) Spectrometer of PerkinElmer was used to analyze the extracted algal oil samples. The FTIR spectrophotometer was equipped with a Universal Attenuated total reflectance (UATR) single reflection diamond accessory to clear differences in regions of the spectrum corresponding to alkene functional groups. The FTIR spectra were recorded over a range of wave number from 4000 to 750 cm^{-1}. Each sample was analysed in triplicate by using instrument specific software.

2.9 Fatty acid analysis of transesterified lipids

Fatty acids were analyzed using the method of Ichihara et al.[32] Briefly, 10 mg of lipid was dissolved in 2 ml of hexane and 200 µl of 2 M methanolic KOH (used as a catalyst). The mixture was vortexed for 5 min followed by brief centrifugation. The upper hexane layer was collected for FAME analysis. Quantification of FAME was carried out using gas chromatography (Agilent GC) equipped with Omega Wax 250 column (30 m×0.25 mm×0.25 µm) and flame ionization detector (FID). The operating conditions were as follows: split ratio 1:10, injection volume 1 µL, nitrogen carrier gas with constant linear velocity 33.9 cm/s, H2at 40 ml/min, air at 400 ml/min, makeup gas (nitrogen) at 30 ml/min; injector temperature of 270°C, detector temperature of 280°C, oven temperature started at 140°C for 5 min and increased at the rate of 4°C/min to 240°C, and hold time of 20 min at 240°C. Methylheptadecanoate was used as the internal standard.

3.0 Estimation of biodiesel fuel properties

Predictive equations based on fatty acid composition were used for the calculation of critical biodiesel properties. Saponification and iodine values were determined according to the method.[33] Higher heating values of biodiesels were calculated according to Ayhan Demirbas model.[34] Cetane number, kinematic viscosity and density of biodiesel were calculated from the FAMEs composition according to the protocol.[35]

3.1 Statistical analysis

Data were analyzed using statistical analysis software (OriginPro). All data represent the mean (± standard deviation, SD) of three independent experiments and each experiment was performed in triplicate. Student's *t* tests were performed to distinguish significantly different results ($P < 0.05$).

4. Results

4.1 Growth analysis of microalgae in WW

Among the four microalgae strains tested, *P. kessleri*-1 showed the highest growth rate in 100% WW ($\mu = 0.49$/day and a doubling time of 1.42 days (Figure 1 and Table S1). The growth of *P. kessleri*-1 was lowest in the 25%WW+75%DW with a specific growth rate (μ) of 0.38/day and a doubling time of 1.83 days (Figure 1 and Table S1). *P. kessleri*-I showed higher growth rates in 100% WW compared to control ASW (Figure 1 and Table S1). Growth rates and the biomass yield of the other three strains (*Chlamydomonas reinhardtii*, *Chlorella* sp., *Nannochloropsis gaditana*) were significantly lower (Figure 1). Consequently, *P. kessleri*-1 was selected for all subsequent physiological studies conducted in 100% WW to test its suitability for phycoremediation and the production of biodiesel.

4.2 Removal of nutrients from WW

Nutrient composition of WW is shown in Table 1. The nutrient removal efficiency (Figure 2), was calculated at the end of the growth experiments (10^{th} day).Total nitrogen, phosphate, ammonical nitrogen, magnesium, and iron concentration were reduced by 81% and 98%,89%, 84% and 63% respectively. The COD, BOD, TOC, and TIC were reduced by 69%, 68%, 48% and 69% respectively. The alkalinity and hardness of WW were reduced by 68% and 47% respectively (Figure 2).The initial pH of WW on day 0 was 7.2, which, however, increased to 9.8 at day 10 due to photosynthetic activity of the microalgae.

4.3 Comparison of photosynthetic quantum yield (Fv/Fm) in WW and ASW

The photosynthetic quantum yield of *P. kessleri*-I in WW was measured in the mid-exponential growth phase (day 5) as well as at the end of the growth experiments (day 10). The maximum quantum yield of PSII was measured in control (ASW) and WW. *P. kessleri*-I had Fv/Fm values of 0.66 in ASW (control) and 0.79 in WW in the mid-exponential growth phase (Figure 3). However, Fv/Fm slightly decreased in the stationary growth phase in both, ASW (0.63) and WW (0.74) (Figure 3).

4.4 Biomass and lipid yield

The total biomass and lipid content was determined by dry weight (DCW) after harvesting one-liter cultures of *P. kessleri*-I using 100% WW and control (ASW) medium as shown in Table 2. The dry weight was higher (309 mgL^{-1}) in WW compared to ASW medium (205 mgL^{-1}), which represents an increase of ca. 50% in WW. Similarly, the total lipid yield was higher (96.4 mgL^{-1}) in WW compared to control medium (44.9 mgL^{-1}), reflecting a significant increase of lipid production (115%) in WW. Specifically, the neutral lipid content seems to have increased in cells grown in WW according to Nile Red staining (Figure 4).

4.5 Lipid analysis by FTIR

An FTIR spectrum of algal oils was obtained using a PerkinElmer Spectrum 400 FTIR/FT-NIR spectrometer. FTIR spectroscopy can be used to obtain physiological fingerprints to study the structure and chemical bonding of the algal oil, primarily though to identify functional groups. The transmittance (%T) spectra of *P. kessleri*-I oil from WW and ASW grown biomass was compared with soyabean oil as standard.[36] The FTIR transmittance spectra in the range 3000-1000 reveal six prominent peaks. These peaks were present in the following ranges: 2900- 2800 cm^{-1}, 1700-1400 $cm^{-1}$and 1200 cm^{-1}-900 cm^{-1} (Figure S1).

4.6 Analysis of FAME and fuel properties for biodiesel production

After transesterification of the algal oil, FAME composition was studied using GC-MS (Table 3). *P. kessleri*-1 grown in WW showed a higher content of C18:1, oleic acid (28%) in comparison to cells grown in ASW medium (6%) (Table 3), whereas the opposite was the case for linolenic acid content (WW grown cells: 11%; ASW grown cells: 29%). The properties of biodiesel such as density, saponification value, iodine value, cetane number, higher heating value and viscosity were determined and compared with international standards (EN, ASTM) as summarized in Table 4. The density, viscosity, and iodine values were found being within the range specified by biodiesel standards for FAMEs derived from algal cells grown in WW and ASW medium. However, the cetane number of biodiesel obtained from cells grown in ASW was marginally lower than the EN and ASTM standards from cells grown in WW (Table 4). Thus, cells grown in WW did not only produce higher biomass but were also producing FAMEs compliant with international biodiesel standards.

5. Discussion

The concept of phycoremediation is not new, but the major obstacle lies in finding a suitable algal species that can produces higher biomass for subsequent applications such as the production of biodiesel. Previous studies have shown that both freshwater and marine algae are suitable for phycoremediation of different types of WW such as municipal, industrial and agricultural.[37] In present study, therefore, four species of oleaginous microalgae were investigated in term of growth using different concentrations of WW from Neela Hauz Lake near New Delhi. Our comparative analysis revealed that *P. kessleri*-I was most capable of growing in 100% WW. This is in agreement with Osundeko et al.[38] who observed that *P. kessleri* has high tolerance to the wastewater environment. Further, the sewage water that drains in the lake is rich in inorganic and organic nutrients, which according to our data, are effectively utilised by *P. kessleri*-I (Table 1). Regarding the efficiency of nutrient removal, *P. kessleri*-I showed a remarkable absorption capacity for removing total nitrogen and phosphorus by 81% and 98% respectively (Figure 2). Similarly, Renuka et al.[39] studied four microalgal strains (*Calothrix* sp., *Lyngbya* sp., *Ulothrix* sp and *Chlorella* sp.) for phytoremediation of sewage and found that *Chlorella* sp. has maximum nutrient removal efficiency of 78% and 91% for NO_3-N and PO_4-P, respectively. These nutrients are needed for the synthesis of biomolecule such as amino, nucleic acids and ATP.[40] Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) are important indicators to estimate organic pollutants in WW. In our experiment, both BOD and COD were reduced by more than 60% (Figure 2). Previously, *Chlorella* sp. was reported to efficiently reduce BOD and COD by about 50%.[41] According to Silambarasan et al.,[42] *Pithophora* sp. seems also relatively efficient in removing nutrients from WW, and COD and BOD was decreased by 61.65% and 64.67%, respectively. However, *Pithophora* sp. grew in

dairy effluent and not municipal WW. Other abundant constituents in many different types of WW are TOC (sugar, alcohol, and petroleum products) and TIC (dissolved carbon dioxide, bicarbonate, and carbonate). *P. kessleri*-I was able to reduce TIC and TOC by 68% and 48%, respectively (Figure 2). Similar reduction rates for total carbon (56.9%) and COD (57.7%) were observed for *Scenedesmus obliquus,* but based on growth in brewery effluent.[43] Thus, *P. kessleri*-I seems to be potential candidate to treat municipal WW and the reason for that might been high algal growth rate and efficient photosynthetic activity (Fv/Fm) as compared to control.

The higher concentrations of these nutrients in WW most likely caused a significantly increased biomass yield in stationary phase at the end of the experiment (day 10). However, an overall decrease in Fv/Fm in the stationary growth phase seems to have been caused by nutrient limitation even in the WW cultures. This potentially has caused the increase in lipid production. However, the lipid content of cell from WW in stationary phase was still higher than in cells from control growth medium even though nutrient stress was more severe in the latter cultures according to lower Fv/Fm. Potentially, the avaibility of organics and/or nutrients in WW was the reason for higher lipid production in these cultures. Along with the rise of total lipids, especially neutral lipids were more accumulated in WW cultures based on Nile Red staining.

The FTIR spectra of the lipids revealed the existence of important functional groups such as those involved in stretching, bending and double bond absorption. Overall though, there were no significant differences between absorption peaks from WW and ASW lipid samples. However, there were slight variations using soyabean oil as a standard. The asymmetric and symmetric vibrational modes of methylene groups showed peaks at 2929 and 2850 cm-1, respectively. The

peak was attributed to t(C=O) stretching of ester at 1735 $cm^{-1}$and 1300 $cm^{-1}$from lipids and fatty acids. The stretching vibrations produce a sharp peak in the 1200–900 cm^{-1} region associated with t(C–O–C) stretching of polysaccharides. The bands were assigned to distinct molecular groups by biochemical standards and published studies as described by Stehfest et al.[44] and Laurens et al.[45]

The FAME analysis of *P. kessleri*-I grown in WW and control media revealed that palmitic acid methyl ester (26%, C16:0) and Oleic acid methyl ester (28%, C18:1) were produced in higher concentrations in algal cells from WW (Table 3). According to Knothe,[46] fatty acids with chain lengths ranging from C16 to C18 should be high in potential feedstock for suitable biodiesel production. In order to compromise between cold flow and oxidative stability, Hu et al.[47] suggested that FAME with high percentage of monounsaturation (C16-C18) is most desirable. Thus, WW induced the production of significant more oleic acid methyl ester (C18:1) biodiesel. Similar observation was reported in *Desmodesmus* sp. S1 by Mar et al.[48] Besides, FAMEs with higher concentrations of palmitate and oleate indicate good biodiesel properties such as quality ignition, higher oxidative stability and lubricity.[49,50,47]

Physical properties important to assess the suitability for biodiesel production were analyzed by FAME composition as shown in Table 4. Properties like density, viscosity, high heating value and saponification values were not significantly different between ASW and WW grown algae and complied to international standards (EN and ASTM standard). Furthermore, it was found that iodine concentrations were lower in *P. kessleri*-I when grown in WW (104g I_2/100g) as compared to cells grown in ASW medium (134 g I_2/100 g) due to a decrease in unsaturation.[51] According to the EN standard, the maximum limit of 120 g Iodine/100 g is acceptable for

commercial fuel quality. The Cetane Number (CN) is an essential parameter to determine the ignition quality of fuel. Generally, CN value should be high for better ignition of the fuel and vice versa.[52,51] Also CN was higher in *P. kessleri*-I (51) from WW compared to cells grown in control medium (44). According to international standards, CN numbers should be as follows: ASTM (≥47) and EN 14214 (≥51). Consequently, biodiesel produced from *P. kessleri*-I grown in WW has the appropriate combination of saturated and monounsaturated fatty acids.

6. Conclusion

With this study, we have shown that *P. kessleri*-I is a suitable algal species to remediate municpal WW as it grows quickly in polluted waters without dilution and produces a significant amount of biomass. Due to its ability to synthesise and accumulate a lot of lipids in WW, it holds great potential not only for being an efficient alga for phycoremediation but also for the production of biodiesel. Thus, our approach would reduce the production costs of algal-based biofuel to improve it's competitiveness and at the same time help to remediate municipal WW.

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Table 1: Chemical composition of WW before growing the algal strain.

Parameter	Concentration (mgL^{-1})
Total Nitrogen	7.4 ± 0.042
Total Phosphorous	3.7 ± 0.033
Iron	0.3 ± 0.004
Magnesium	18 ± 0.235
Ammonical Nitrogen	5 ± 0.186
Chemical Oxygen Demand (COD)	84 ± 2.309
Biological Oxygen Demand (BOD)	21 ± 1.155
Total Organic Carbon (TOC)	64 ± 1.201
Total Inorganic Carbon (TIC)	54 ± 0.843
Alkalinity	190 ± 1.121
Hardness	243 ± 2.032

Data is an average value of three experiments ± S.D (n=3).

Table 2: Comparative biomass and total lipid yield in *Parachlorella kessleri*-I grown in WW vs ASW medium on the 10^{th} day.

Growth Medium	Biomass yield (mg L^{-1})	Lipid yield (mg L^{-1})
WW	308.5 ± 9.1*	96.4 ± 6.2*
ASW	205.0 ± 9.8	44.9 ± 7.0

Data is an average values of three experiments ± S.D (n=3). Differences between WW and ASW were analyzed by Student's *t* test. Asterisks indicate statistically significant differences compared the WW with ASW at $*P < 0.05$.

Table 3: Major changes in the FAME of *Parachlorella kessleri*-I, grown in WW vs ASW medium on the 10th day of culture.

FAME (Carbon chain length)	Relative FAME composition (%)	
	ASW	**WW**
Palmitic acid methyl ester (C16:0)	26 ± 1.46	26 ± 2.69
Oleic acid methyl ester (C18:1)	6 ± 0.31	28 ± 0.49*
Linoleic acid methyl ester (C18:2)	11 ± 0.80	14 ± 2.17
Linolenic acid methyl ester (C18:3)	29 ± 1.12	11 ± 0.46*

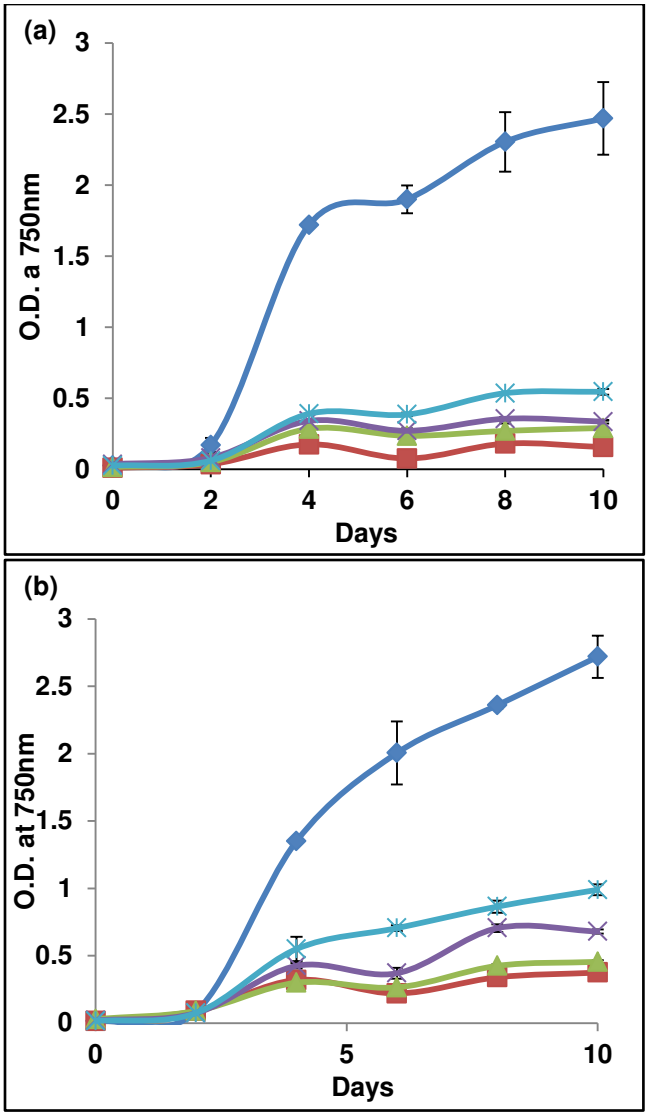
Data is an average values of three experiments ± SD (n=3). Differences between WW and ASW were analyzed by Student's *t* test. Asterisks indicate statistically significant differences compared the WW with ASW at

*$P < 0.05$.

Table 4: Comparison of FAME (biodiesel) properties of *Parachlorella kessleri*-I, grown in WW vs ASW medium using international standards.

Physical properties	ASW	WW	EN 14214:2008	ASTM D6751
Density ($g.cm^{-3}$)	0.881	0.877	0.860-0.900	0.875-0.900
Saponification value (mg $KOH.g^{-1}$)	196	196	–	–
Iodine value(g I_2.100 g^{-1})	134	104	<120	–
Cetane number	44	51	≥51	≥47
Higher Heating value($MJ.Kg^{-1}$)	39	40	–	>35
Viscosity ($mm^2.s^{-1}$)	3.8	4.1	3.5-5.0	1.9-6.0

Data is an average value of three experiments.



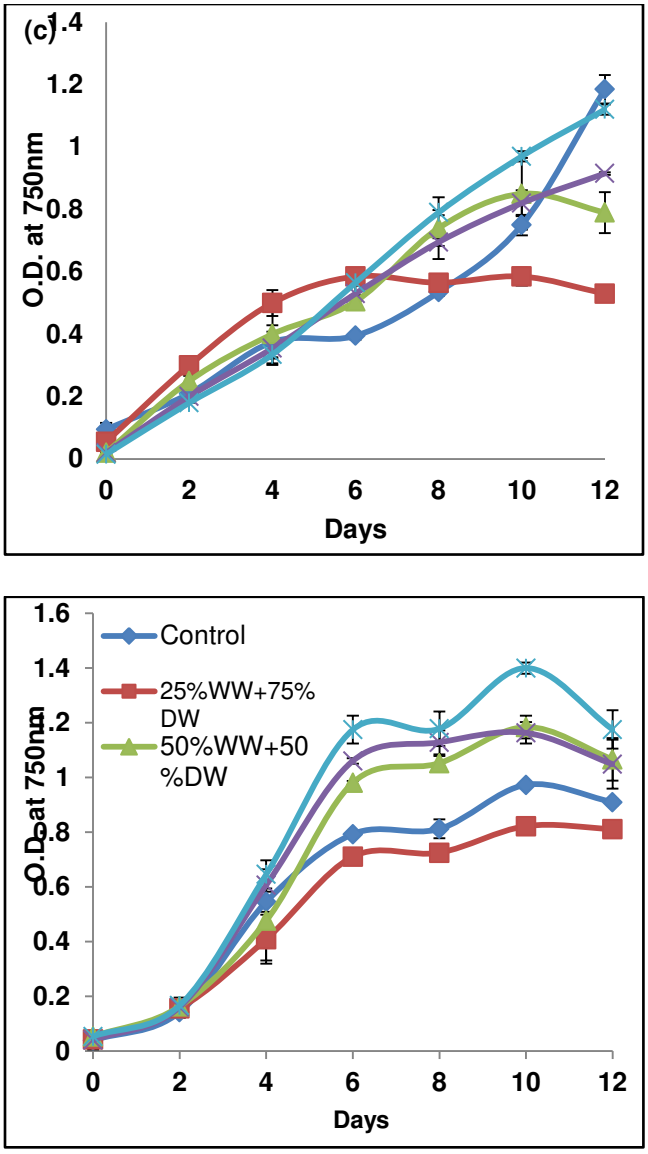


Figure 1 Growth study of microalgae in WW. (a) *Chlamydomonas reinhardtii*, (b) *Chlorella* sp. (c) *Nannochloropsis gaditana* and (d) *Parachlorella kessleri*-I. Data is an average value of three experiments ± S.D.

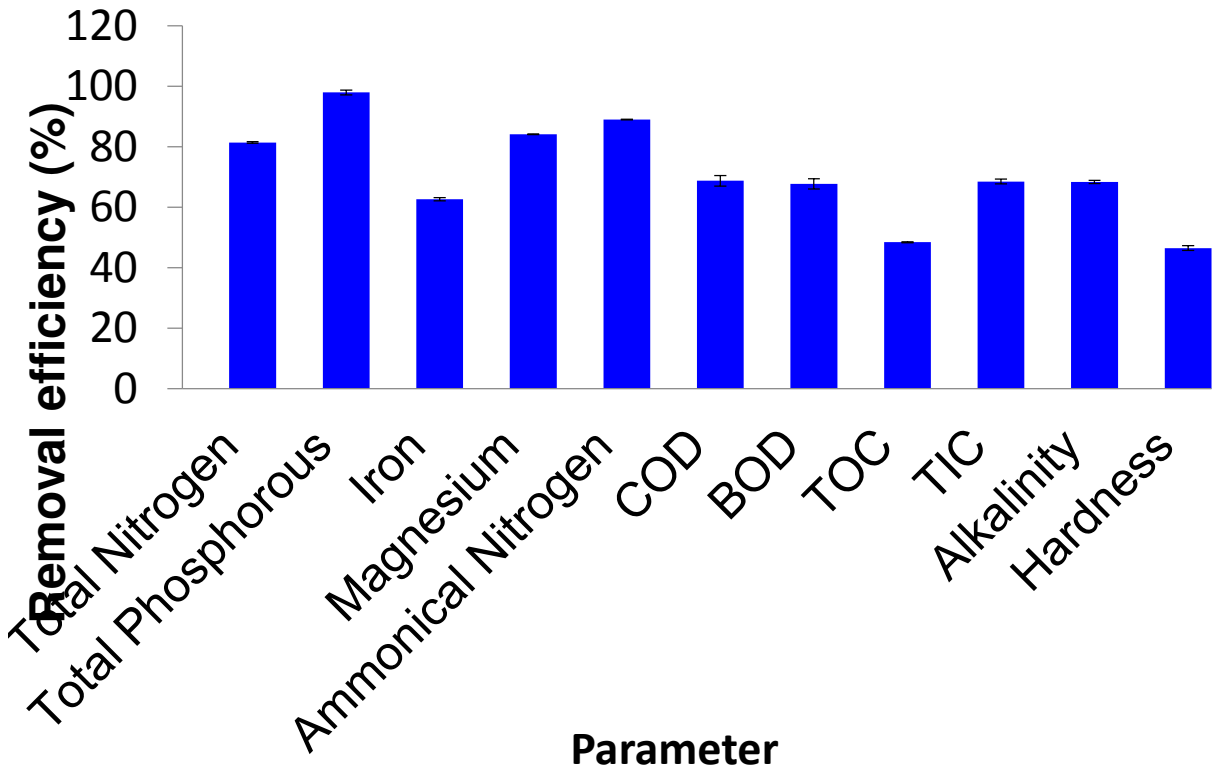


Figure 2. Percentage removal of nutrients from the WW by an oleaginous marine alga, *Parachlorella kessleri*-I.

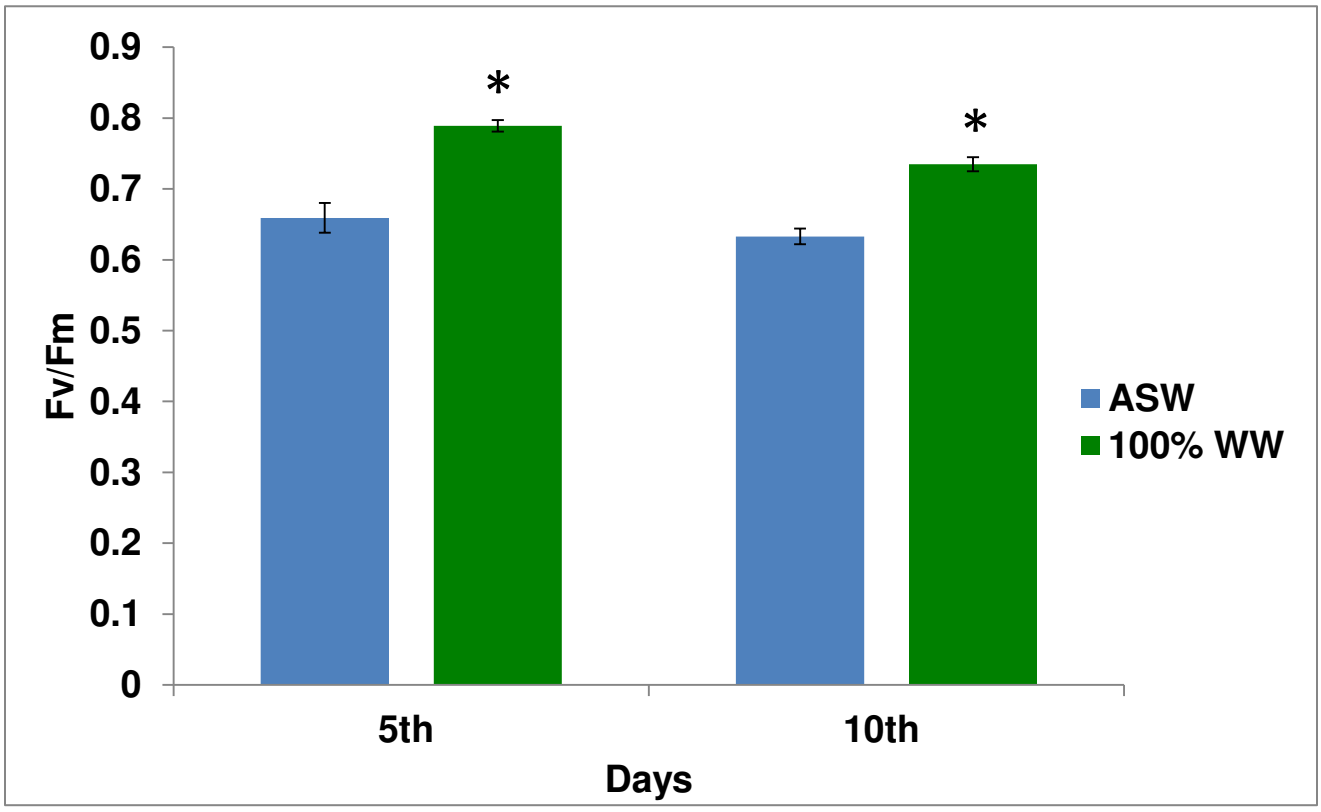


Figure 3. The maximum quantum yield of PSII (Fv/Fm) of *Parachlorella kessleri*-I under ASW and WW media. Differences between WW and ASW were analyzed by Student's *t* test. Asterisks indicate statistically significant differences compared the WW with ASW at $*P < 0.05$.

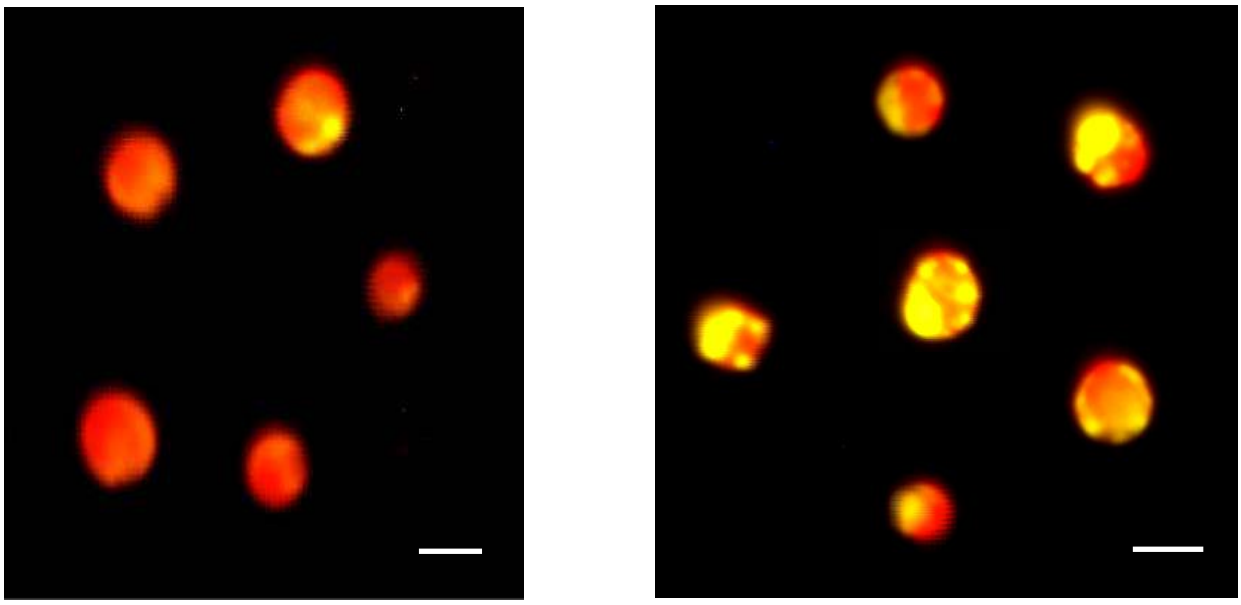


Figure 4. Nile red fluorescence study of *Parachlorella kessleri*-I cells grown in WW and ASW (control medium) for 10 days. Algal cells with lipid droplets in golden yellow colour in ASM medium (a) vs WW (b). Cultures were viewed at 600×using a fluorescence microscope at 485 nm excitation and 552 nm emission filters showing the lipid globules. Scale bars = 10 µm.