

Research Article

Efficacy of EDTA and Phosphorous on Biomass Yield and Total Lipid Accumulation in Two Green Microalgae with Special Emphasis on Neutral Lipid Detection by Flow Cytometry

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Chlorella ellipsoidea and *Chlorococcum infusionum*, promising microalgae for biodiesel feedstock production, were treated with ethylenediaminetetraacetate (EDTA) and phosphorous to induce stress which was then followed by flow cytometry to study the enhanced intracellular neutral lipid content. Treatment resulted in up to a threefold increase in total lipid content of *Chlorella* ($41.8 \pm 1.9\%$ at 16 days of incubation period) and more than twofold increases in *Chlorococcum* ($31.3 \pm 1.0\%$ at 18 days of incubation period) under phosphorous starvation in the culture. It was observed that maximum biomass yields in *Chlorella* and *Chlorococcum* were 1.56 ± 0.06 and 2.17 ± 0.12 g/L at 1.5 g/L of phosphorous after 20 and 18 days of incubation periods, respectively. The qualitative analyses of neutral lipid bodies under stress conditions were performed by confocal microscopy and revealed bright golden-yellow lipid droplets in stress exposed cells. Significant increase of monounsaturated fatty acids under the nutrient limited conditions was suitable to produce biodiesel. The maximum biomass (g/L) and lipid content (% dry cell weight) at different stresses showed significant results ($p < 0.05$) by single-factor Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT).

1. Introduction

Continued exploitation of the world's fossil fuel reserves is unsustainable due to their depleted status and simultaneous steady accumulation of greenhouse gases in the atmosphere. Development of sustainable energy sources is therefore necessary to overcome this problem. The production of biodiesel from photosynthetic microorganisms is considered as an effective strategy to produce renewable energy. One of the most promising candidates for production of alternative energy is microalgae because of their higher growth rate and oil content than those of the conventional energy crops such as jatropha, soybean, palm, canola, and corn [1–3]. Additionally, some conditions of cultivation should be strategically changed to enhance microalgal lipid. Most recent studies have found that the lipid accumulation in microalgae could be increased through altering parameters such as temperature, light, CO₂, salinity, and nutrient concentration [4–10].

Nutrient is the most important regulator of microalgal growth and lipid accumulation. Many microalgal species

can synthesize large quantities of lipid and carbohydrates along with other bioactive molecules under varied nutrient supplement conditions. These newly synthesized lipids and carbohydrates can be directly converted to biodiesel and bioethanol, respectively [1, 11, 12]. Under different nutrient starvation, microalgae can accumulate large quantities of neutral lipid in the form of triacylglycerol (TAG) within the cell cytoplasm [11]. A few reports were available on the effect of ethylenediaminetetraacetate (EDTA) on growth and lipid productivity in microalgae. In *Nannochloropsis oculata* the dry weight of the biomass and lipid gradually increased with increased concentration of EDTA [10]. The marine diatom *Amphiprora paludosa* synthesized 65.64% lipid under 0.026 mM of Fe-EDTA [13].

Simultaneous effect of phosphorous (P) influences the growth and lipid synthesis in microalgae [9, 14–17]. Effect of P deficiency in *Chlorella vulgaris* was studied by Mutlu et al. [9]. The lipid accumulation and growth characteristics of *Chlorella kessleri* under different concentration of P were

investigated by El-Sheek and Rady [17]. In other microalgae it was observed that P starvation limited the growth but triggers the lipid productivity. Microalgae *Skeletonema costatum* and *Scenedesmus obliquus* were found as good candidates for biodiesel production due to their high lipid productivity and fatty acid content [2, 3]. Different experimental conditions using P starvation were applied to improve lipid productivity in *S. obliquus*, *Chlorella kessleri*, and *Monodus subterraneus* [3, 17, 18]. The lipid productivity in *S. obliquus* was increased up to 54% under P starvation or limitation but growth ceased due to insufficient nutrient [3]. The high lipid and triacylglycerol (TAG) content was studied in different microalgae under P and EDTA limitation [8, 13, 19].

Flow cytometry is considered to be a most promising and powerful technique which enables the analysis of the physiological states of living cells at the single cell level [20]. Fluorescence activated cell sorting (FACS) also can be employed to screen the microalgae from a mixture of cell populations due to the autofluorescence properties of algal native pigments like chlorophylls, carotenoids, and phycobilins [21–24]. Flow cytometry in combination with a lipophilic fluorescent probe was applied to screen high lipid containing microalgae for several times [25–27]. The application of flow cytometry to detect the neutral lipid in stress induced microalgal cells was studied by a few group of researchers. Temperature is one of the most important physical factors which enhanced neutral lipid accumulation in microalgae other than the nutrients [28]. Lipid metabolism and production of high neutral and polar lipid in the snow alga *Chlamydomonas nivalis* were analyzed by flow cytometry under NaCl stress [29]. Various mechanical (freezing, ice cooling, and centrifugation) and chemical (saponin and H₂O₂) stresses were applied to study the lipid metabolism in a toxic dinoflagellate *Alexandrium minutum* by flow cytometry [23]. The reports available on neutral lipid study by flow cytometry were mainly based on light, temperature, CO₂, and NaCl but no reports were available for the nutrient based synthesis of neutral lipid.

This investigation examined the effects of EDTA and phosphorous on biomass yield and lipid accumulation with special emphasis on neutral lipid study by flow cytometry and confocal microscopy. In this study, two green microalgae, *Chlorella ellipsoidea* and *Chlorococcum infusionum*, isolated from Sundarbans Biosphere Reserve were applied to demonstrate the rapid accumulation of lipid under P and EDTA stress conditions.

2. Materials and Methods

2.1. Microalgal Strain and Culture Conditions. The microalgal genera were collected from Sundarbans mangrove forest and isolated in the laboratory in unialgal form. The microalgae were identified by handbook of Smith [30] and Prescott [31]. *Chlorella ellipsoidea* (CUH/AI/MW-189) was isolated in Zarrouk's media [32] and *Chlorococcum infusionum* (CUH/AI/MW-190) was isolated in Bold basal medium [33]. Both cultures were maintained in the culture room at 22°C under 16 : 8 light-dark cycles. The axenic cultures were mixed well with bubbling air containing 2% CO₂. The cultures were

grown in batch culture mode and were shaken regularly at 120 rpm for 2-3 h per day in Eyla horizontal shaker incubator.

2.2. Preparation of Experimental Media. The microalgae *Chlorella ellipsoidea* and *Chlorococcum infusionum* were subjected to different concentrations of EDTA (Na₂-EDTA) and P (K₂HPO₄) for growth and lipid estimation. To study the effects of EDTA and P on growth and lipid accumulation, microalgal cultures were grown in different media with varied concentrations of EDTA and P. The conditions were achieved by addition and depletion of EDTA and P salts with respect to the control condition. The various modified concentrations for both the culture media (BBM and Zarrouk's) were 0 g/L, 0.075 g/L, 0.15 g/L, 0.5 g/L, 1.5 g/L, 2.5 g/L, and 5 g/L for P salt and 0 g/L, 0.01 g/L, 0.05 g/L, 0.08 g/L, 0.1 g/L, 0.5 g/L, and 1 g/L for EDTA salt. Three replicates of each set were incubated for 30 days and biomass and lipids were quantified as described later.

2.3. Measurement of Dry Cell Weight (dcw). The biomass was harvested at two-day interval from the date of inoculation. Three sets of culture were harvested for both the algal genera. The cultures were centrifuged at 8000 g for 5 min and washed 2-3 times with double distilled water to remove excess nutrients. After washing, the biomass was transferred to preweighed vials for oven drying at 70°C to get a constant dry weight. Biomass yield was calculated by the gravimetric method and expressed as g/L [27, 34].

2.4. Lipid Extraction, Transesterification, and Analysis of Fatty Acid Methyl Esters. Lipid extraction was done in the same vials after estimation of dcw. The lipids were extracted using chloroform and methanol in the ratio of 2:1 (v/v) following the method of Bligh and Dyer [35]. The fatty acid methyl esters (FAME) were prepared using acid catalyzed transesterification reaction. The test was performed at 60°C using lipid:methanol:HCl in the molar ratio of 1:80:4 and incubated for 6.4 h. The upper organic phase, which contained oil, was pipetted out and taken for GC-MS analysis using Agilent 6890N Gas Chromatograph connected to an Agilent 5973 Mass Selective Detector at 70 eV. The instrument was equipped with a HP-5 MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The identification of individual fatty acids was performed on the basis of retention time.

2.5. Staining of Neutral Lipid. Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one), a lipid soluble dye, was purchased from Sigma (CAS number: 7385-67-3; Catalogue number: N 3013) and used in this experiment. The cells containing high lipid were selected for staining. The cells were centrifuged at 8000 g for 5 min at 20°C and pellets were washed 2-3 times with phosphate buffer saline (PBS) to remove salts and cell debris. The cells were then stained with standardized protocol of Satpati and Pal [27].

2.6. Neutral Lipid Analysis by Flow Cytometry. The maximal accumulation of enhanced neutral lipid was quantified by

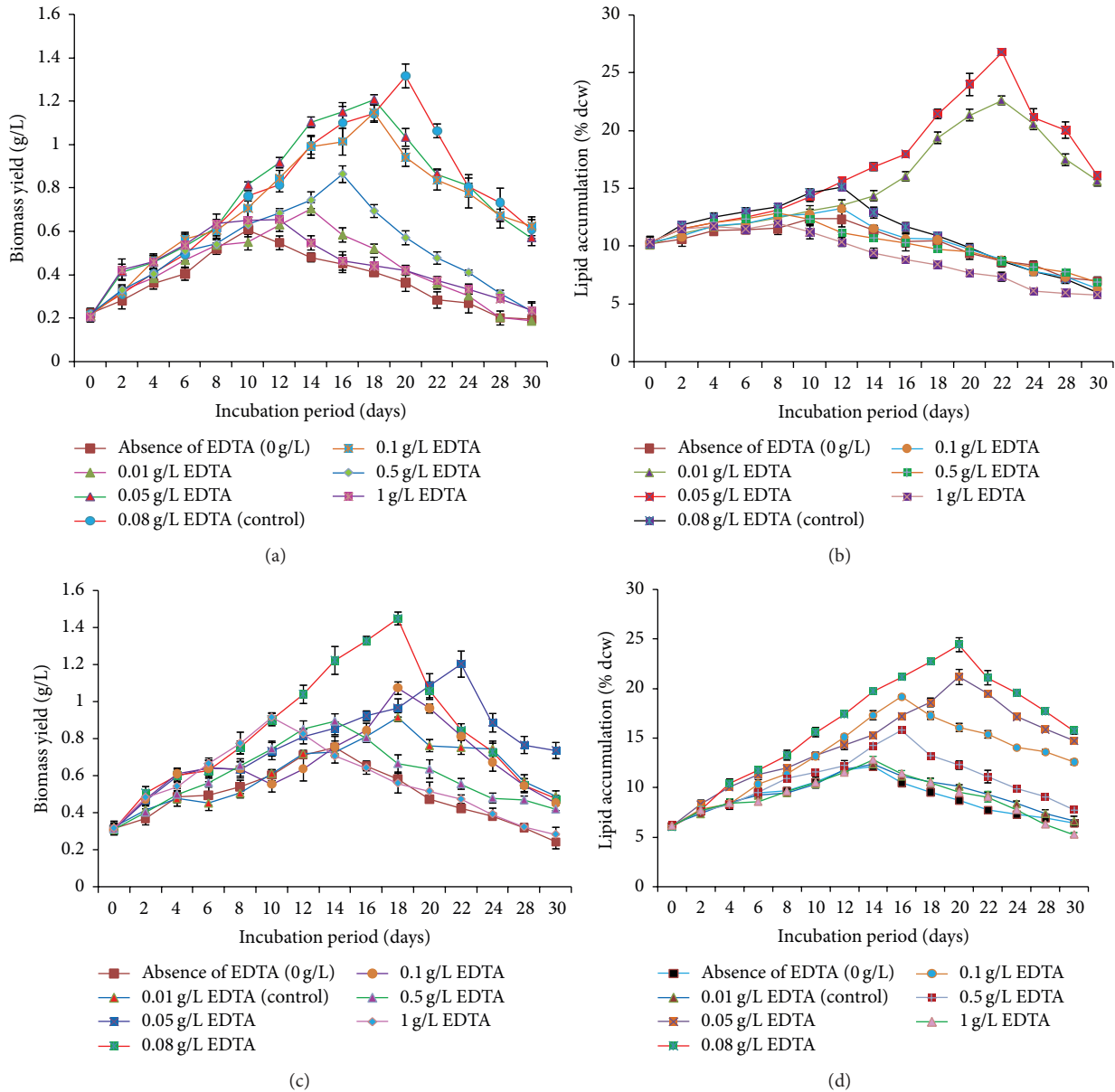


FIGURE 1: Biomass yield (g/L) and lipid accumulation (% dcw) in (a)-(b) *Chlorella ellipsoidea* and (c)-(d) *Chlorococcum infusionum* under different EDTA concentrations.

FACSVerse flow cytometer equipped with 488 nm solid state laser. The emission signal was recorded at 586/42 nm (PE-A) for detecting neutral lipid. The cellular morphology of size and granularity were studied by forward scatter (FSC) and side scatter (SSC) dot plots. The fluorescence intensity in Nile red stained cells was detected by flow cytograms. The accumulation of neutral lipid in P and EDTA starved cells was observed by confocal microscopy (Olympus IX 81) equipped with the Flow view FV 1000 software.

2.7. Statistical Analysis. The maximum values of mean biomass (g/L) and lipid (% dcw) concentrations obtained for different levels of a given treatment (EDTA and P) were used to perform single-factor Analysis of Variance (ANOVA) at

$p < 0.05$ in Microsoft Excel, followed by Duncan's Multiple Range Test (DMRT) using MSTATC software package. The median and coefficient of variation (CV) were analyzed to quantify neutral lipid in EDTA and P starved cells by FACSVerse software.

3. Results

3.1. Effect of EDTA on Biomass Yield and Total Lipid Accumulation. In control growth media the EDTA acts as a chelator at concentrations of 0.08 g/L for *Chlorella* and 0.01 g/L for *Chlorococcum*. Increasing or decreasing concentrations of EDTA in *Chlorella* culture did not show any positive effect

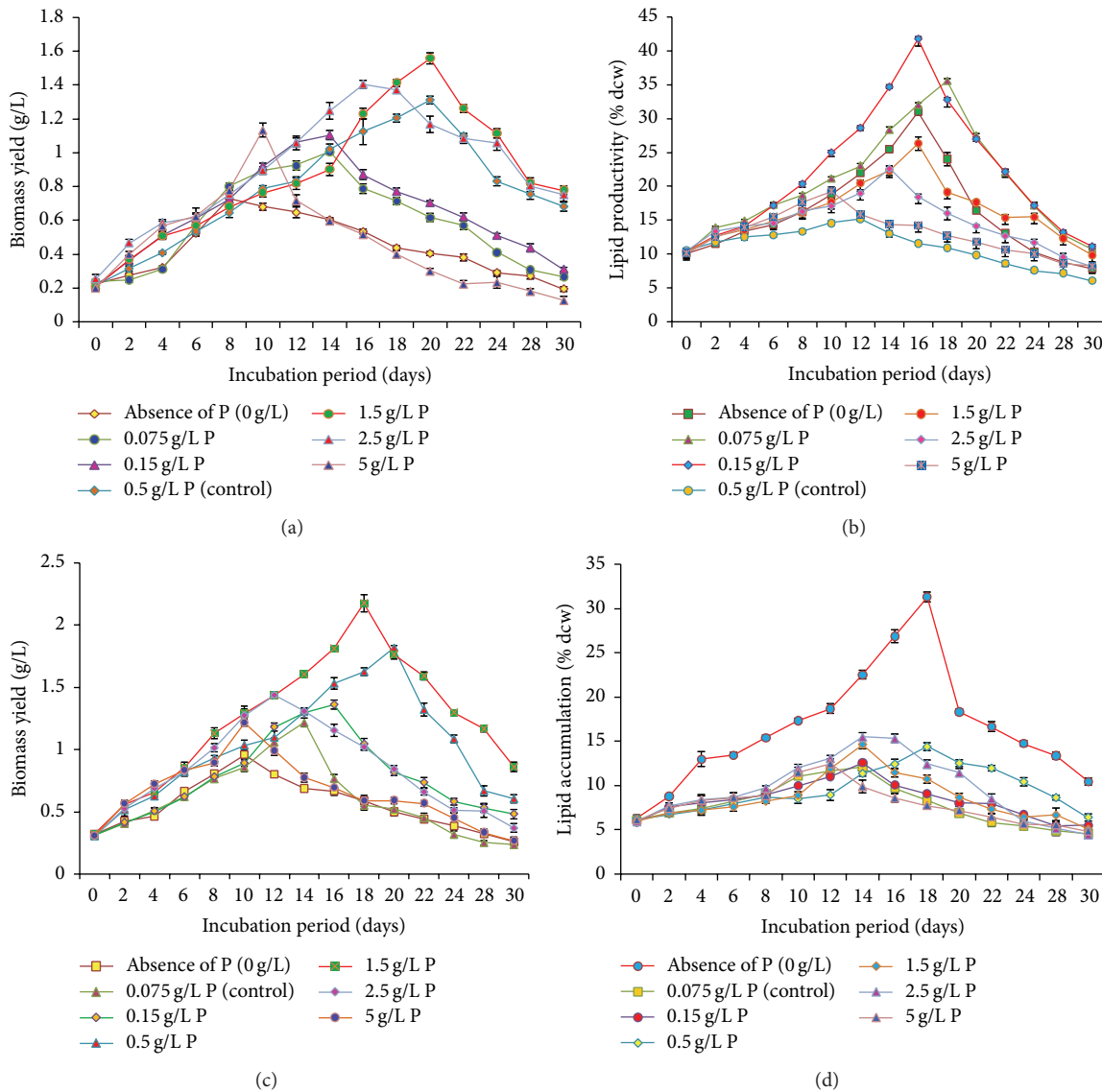


FIGURE 2: Biomass yield (g/L) and lipid accumulation (% dcw) in (a)-(b) *Chlorella ellipsoidea* and (c)-(d) *Chlorococcum infusionionum* under different P concentrations.

on biomass yield. But in *Chlorococcum* the increased concentration (0.08 g/L) showed a positive effect on growth. In *Chlorella* the maximal biomass obtained was 1.32 ± 0.1 g/L after 20 days of incubation at control cultured condition (Figure 1(a)). Culture of *Chlorococcum* showed biomass yield of 1.45 ± 0.06 g/L after 18 days of incubation when exposed to 0.08 g/L EDTA (Figure 1(c)). In both *Chlorella* and *Chlorococcum* significant growth ($p < 0.05$) was observed in different concentrations of EDTA (Figures 6(a)-6(b)).

The lipid accumulation in tested microalgae was studied under various concentrations of EDTA. In control medium the lipid accumulation was $15.1 \pm 0.4\%$ for *Chlorella* and $12.4 \pm 0.5\%$ for *Chlorococcum* after 12 and 14 days of incubation (Figures 1(b) and 1(d)). The maximum lipid accumulation was obtained as $26.8 \pm 0.4\%$ dcw (1.7-fold) for *Chlorella* (22 days of incubation) and $24.4 \pm 1.2\%$ dcw (1.9-fold) for

Chlorococcum (20 days of incubation) at 0.05 g/L and 0.08 g/L EDTA, respectively (Figures 1(b) and 1(d)). In *Chlorella* the low concentrations of EDTA showed enhanced lipid accumulation whereas in *Chlorococcum* the lipid accumulation was maximum for a higher concentration of EDTA than in the control (Figures 1(b) and 1(d)). A significant amount ($p < 0.05$) of lipid was accumulated in both *Chlorella* and *Chlorococcum* at different concentrations of EDTA (Figures 6(a)-6(b)).

3.2. Effect of P on Biomass Yield and Total Lipid Accumulation.

In the experimental media the normal concentrations of P salt (K_2HPO_4) were 0.5 g/L for *Chlorella* and 0.075 g/L for *Chlorococcum*. When the concentrations were altered, the algae exhibited a rapid change in growth and in lipid accumulation (Figures 2(a)-2(d)). At normal culture conditions

TABLE 1: FAME compositions (%) of *Chlorella ellipsoidea* and *Chlorococcum infusionum* under P and EDTA stress.

Fatty acids	Fatty acid compositions (%) [*]					
	<i>Chlorella ellipsoidea</i>			<i>Chlorococcum infusionum</i>		
	Control	0.15 g/L P	0.05 g/L EDTA	Control	0.00 g/L P	0.08 g/L EDTA
Lauric (12:0)	1.93 ^b ± 0.06	—	0.46 ^c ± 0.04	2.68 ^a ± 0.26	0.40 ^c ± 0.08	—
Myristic (14:0)	5.78 ^b ± 0.23	5.29 ^b ± 0.30	7.74 ^a ± 0.39	9.23 ^{ab} ± 0.32	8.63 ^b ± 0.40	1.97 ^c ± 0.30
Palmitic (16:0)	8.92 ^a ± 0.44	4.26 ^c ± 0.31	4.86 ^b ± 0.27	8.63 ^b ± 0.36	10.84 ^a ± 0.72	10.59 ^a ± 0.36
Palmitoleic (16:1)	10.12 ^c ± 0.19	17.21 ^a ± 1.13	16.24 ^a ± 0.79	13.99 ^c ± 1.19	21.49 ^a ± 1.06	14.53 ^c ± 1.11
Stearic (18:0)	2.17 ^c ± 0.21	9.26 ^a ± 0.78	2.12 ^c ± 0.22	3.87 ^d ± 0.22	10.44 ^c ± 0.99	17.98 ^a ± 0.98
Oleic (18:1)	13.73 ^d ± 0.77	21.62 ^c ± 0.94	30.05 ^b ± 2.16	9.52 ^c ± 0.31	30.32 ^a ± 2.68	29.80 ^a ± 1.43
Linoleic (18:2)	19.52 ^a ± 0.74	14.12 ^b ± 0.86	10.17 ^c ± 0.99	22.92 ^a ± 1.10	13.05 ^c ± 1.19	15.02 ^d ± 0.75
α-Linoleic (18:3)	10.84 ^a ± 0.73	10.88 ^a ± 0.36	11.08 ^a ± 0.13	22.02 ^a ± 0.75	1.81 ^d ± 0.29	7.88 ^c ± 0.50
Arachidic (20:0)	19.04 ^a ± 0.77	9.71 ^c ± 0.49	11.38 ^b ± 1.13	0.60 ^b ± 0.13	—	1.23 ^a ± 0.11
Arachidonic (20:4)	0.24 ^c ± 0.06	6.62 ^a ± 0.37	5.92 ^b ± 0.56	2.08 ^a ± 0.22	—	0.25 ^b ± 0.15
Eicosapentaenoic (20:5)	7.71 ^a ± 0.14	—	—	1.19 ^b ± 0.38	1.61 ^a ± 0.07	—
Behenic (22:0)	—	1.03 ^a ± 0.23	—	2.68 ^a ± 0.54	—	—
Lignoceric (24:0)	—	—	—	0.60 ^b ± 0.06	1.41 ^a ± 0.11	0.74 ^b ± 0.11
SFA	37.84 ± 1.71	29.55 ± 2.11	26.56 ± 2.05	28.29 ± 1.89	31.72 ± 2.19	32.51 ± 1.86
MUFA	23.85 ± 0.96	38.83 ± 2.07	46.29 ± 2.95	23.51 ± 1.50	51.81 ± 3.74	44.33 ± 2.54
PUFA	38.31 ± 1.67	31.62 ± 1.59	27.17 ± 1.68	48.21 ± 2.45	16.47 ± 1.55	23.15 ± 1.40

^{*} Values are mean ± SD, $n = 3$. Values in each column superscripted by different alphabets (a–d) are 559 significantly different from each other ($p < 0.05$, DMRT).

the biomass yield in *Chlorella* was 1.31 ± 0.04 g/L (20 days of incubation) and in *Chlorococcum* it was 1.22 ± 0.06 g/L (14 days of incubation). Maximum biomass yield was achieved with 1.5 g/L P salt for both *Chlorella* and *Chlorococcum*, being, respectively, 1.56 ± 0.06 g/L (Figure 2(a)) and 2.17 ± 0.12 g/L (Figure 2(c)). The biomass yield was significantly increased ($p < 0.05$) with different concentrations of P salt in *Chlorella* and *Chlorococcum* culture (Figures 6(c)–6(d)).

Figures 2(b) and 2(d) show lipid accumulation up to $15.2 \pm 0.4\%$ dcw for *Chlorella* and $12.1 \pm 0.2\%$ in *Chlorococcum* after 12 days and 14 days of incubation under control growth media. The lipid obtained by gravimetric method was $41.8 \pm 1.9\%$ dcw (2.7-fold) in *Chlorella* (after 16 days of incubation) and $31.3 \pm 1.0\%$ dcw (after 18 days of incubation) in *Chlorococcum* (2.5-fold) when the cultures were supplemented with 0.15 g/L and 0 g/L of P, respectively (Figures 6(c)–6(d)). Increasing and decreasing concentrations of P diminished and enhanced the lipid accumulation, respectively, in the medium. The decreasing concentration of P was significantly induced by ($p < 0.05$) lipid accumulation in the culture of *Chlorella* and *Chlorococcum* (Figures 6(c)–6(d)).

3.3. Analysis of FAME under EDTA and P Stress. The GC-MS analysis of FAME revealed elevated levels of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) in *Chlorella ellipsoidea* and *Chlorococcum infusionum* under EDTA and P stress (Table 1). The major SFA in *Chlorella* biodiesel were lauric, myristic, palmitic, stearic, and arachidic acids, whereas in *Chlorococcum* behenic and lignoceric acids were additionally present. The FAME profile of

Chlorella and *Chlorococcum* biodiesel confirmed the presence of major MUFA like palmitoleic and oleic acid methyl esters. Both microalgal oils were found to contain major PUFA like linoleic, α-linoleic acid esters with large proportions under the control conditions except for arachidonic acid in *Chlorella* and eicosapentaenoic acid in *Chlorococcum*. Under EDTA and P stresses, the biodiesel was predominated with both SFA and MUFA methyl esters. The palmitoleic, stearic, oleic, and linoleic acids were increased under EDTA and P stresses for both the microalgae. In *Chlorella*, eicosapentaenoic and lignoceric acid methyl esters completely disappeared whereas arachidic, arachidonic, and behenic acid esters were absent in *Chlorococcum* under P limited cultures. Under EDTA starved condition, eicosapentaenoic and behenic acid esters were completely removed in both microalgae.

3.4. Effects of EDTA and P in Neutral Lipid Accumulation. The accumulation of neutral lipid in Nile red stained cells was detected and quantified by flow cytometry. In *Chlorella* and *Chlorococcum* the maximal lipid containing cells (determined by gravimetric method) were taken for flow cytometric analysis to quantify the neutral lipid. In Figures 4–5, FSC versus SSC scattered plot, flow cytograms, and confocal images showed the accumulation of high neutral lipid in microalgal cells under EDTA and P stress with respect to the control (Figure 3). The FSC versus SSC two-dimensional dot plots showed uniformly scattered cells in *Chlorella* and highly scattered cells in *Chlorococcum*. The flow cytograms of Nile red stained cells emit fluorescence of neutral lipid at 586/42 (PE-A) signal. The confocal images confirmed the synthesis of

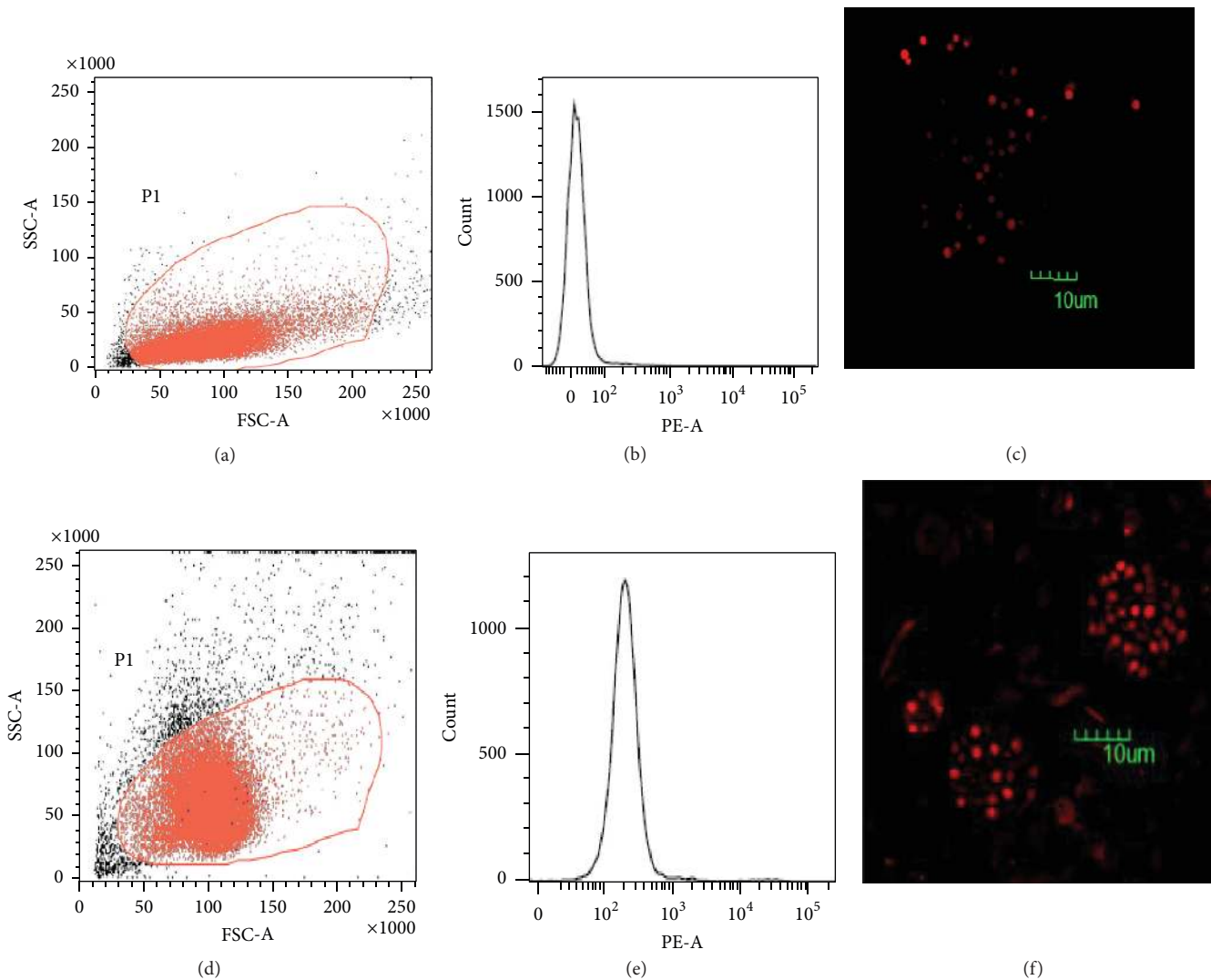


FIGURE 3: Flow cytometric analysis of unstained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum* under control condition. (a), (d) FSC versus SSC two-dimensional dot plots of scattered cells. (b), (e) Flow cytograms of unstained cells showing fluorescence intensity. (c), (f) Unstained cells showing chlorophyll autofluorescence.

neutral lipid in cell cytoplasm. High median and CV values were found in EDTA and P stressed cells when compared to the control culture suggesting the positive staining of neutral lipid containing parent cells (Table 2). The EDTA stressed cultures showed median value as 4,152 for *Chlorella* and 3,426 for *Chlorococcum* whereas the CV values were found as 140.81 for *Chlorella* and 106.39 for *Chlorococcum*, respectively. In P stressed cells of *Chlorella* the median value was obtained as 6,006 and for *Chlorococcum* it was found to be 4,171. The CV values were 141.57 for *Chlorella* and 117.57 for *Chlorococcum* under P stress. In EDTA and P stressed cells the % grandparents (% of positive stained parent cell population) were obtained as 30.41% and 51.26% for *Chlorella* whereas for *Chlorococcum* they were 28.31% and 30.54%, respectively (Table 2).

4. Discussion

For economical production of biodiesel from microalgae, biomass and lipid content play an important role. Different approaches have been used to enhance the biomass yield and lipid accumulation in microalgae for the last few years. All of them have concluded that improved culture strategies are needed to obtain high quality of oil in microalgal cells [11, 36, 37]. Previous studies have demonstrated that lipid accumulation in microalgae could be modified by various growth conditions other than the EDTA and P. The biomass yield and lipid accumulation in microalgal cells were also enhanced by nitrogen [5, 8, 9, 38], sulphur limitation [39, 40], silicon deficiency [12], high salinity [4, 29, 34], calcium [34, 41], magnesium [34, 42], and heavy metal such as cadmium

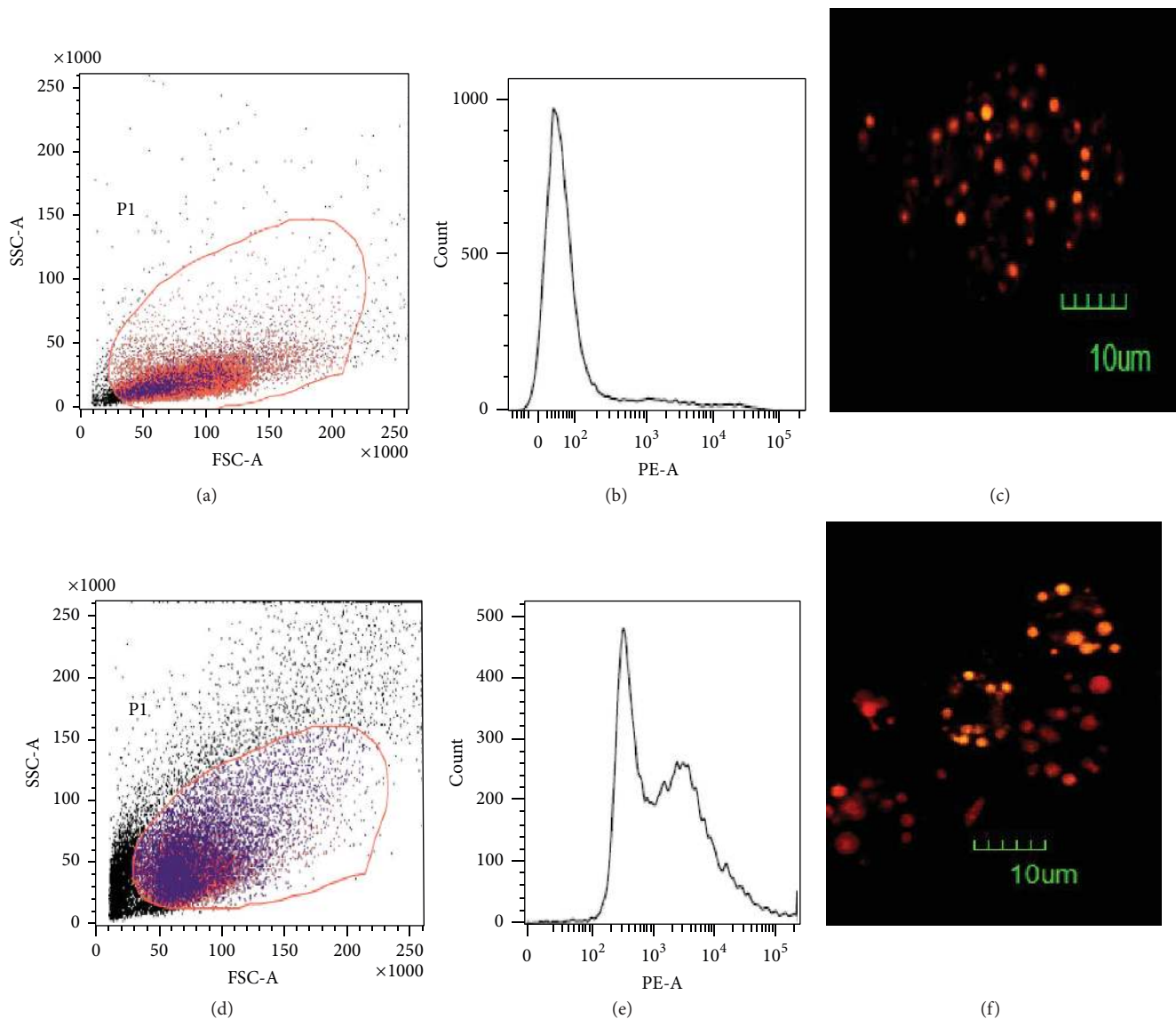


FIGURE 4: Flow cytometric analysis of Nile red stained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum* under EDTA starvation. (a), (d) FSC versus SSC two-dimensional dot plots. (b), (e) Flow cytograms of Nile red stained cells showing fluorescence intensity. (c), (f) Neutral lipid bodies showing yellow-gold fluorescence (the colour red indicating the cells with chlorophyll autofluorescence and violet indicating cells with neutral lipids in the scattered plots).

[15, 38] and iron [43]. It has also been found that some microelement and superoxide radical, namely, manganese, cobalt, and hydrogen peroxide, can induced lipid biosynthesis in microalgae [6].

Very few reports were available on biomass and lipid accumulation in *Chlorella ellipsoidea* and *Chlorococcum infusionum*. The biomass and lipid content in *Chlorella ellipsoidea* YJ1 were 425 mg/L and 43% (w/w), respectively, when isolated in domestic secondary effluent [44]. Twofold increase of unsaturated fatty acids was obtained in *Chlorella ellipsoidea* at low temperature stress [38]. High amount of lipid accumulation ($40 \pm 2\%$ dcw) in *Chlorococcum infusionum* at 1.75 mM of sodium nitrate was reported by Karemire et al. [45].

In the present study we applied different concentrations of EDTA and P to test the effect on biomass yield and lipid

accumulation in green microalgae. The lipid classes were characterized by GC-MS. In this study we demonstrated flow cytometry for neutral lipid accumulation under P and EDTA stress. Gas chromatography-mass spectrometry revealed the information about different levels of saturated, monounsaturated, and polyunsaturated fatty acids. Neutral lipid is the most essential parameter for biodiesel production. In microalgae, nonpolar or neutral lipid was accumulated in the form of triacylglycerol (TAG). The synthesis of intracellular TAG in microalgae was analyzed by flow cytometry use of a fluorescent probe in terms of fluorescence intensity. Simultaneously, confocal microscopy revealed the qualitative analysis of neutral lipid in microalgae in terms of lipid droplets in cytoplasm. Both qualitative and quantitative analyses of neutral lipid were required for biodiesel production. Thus in

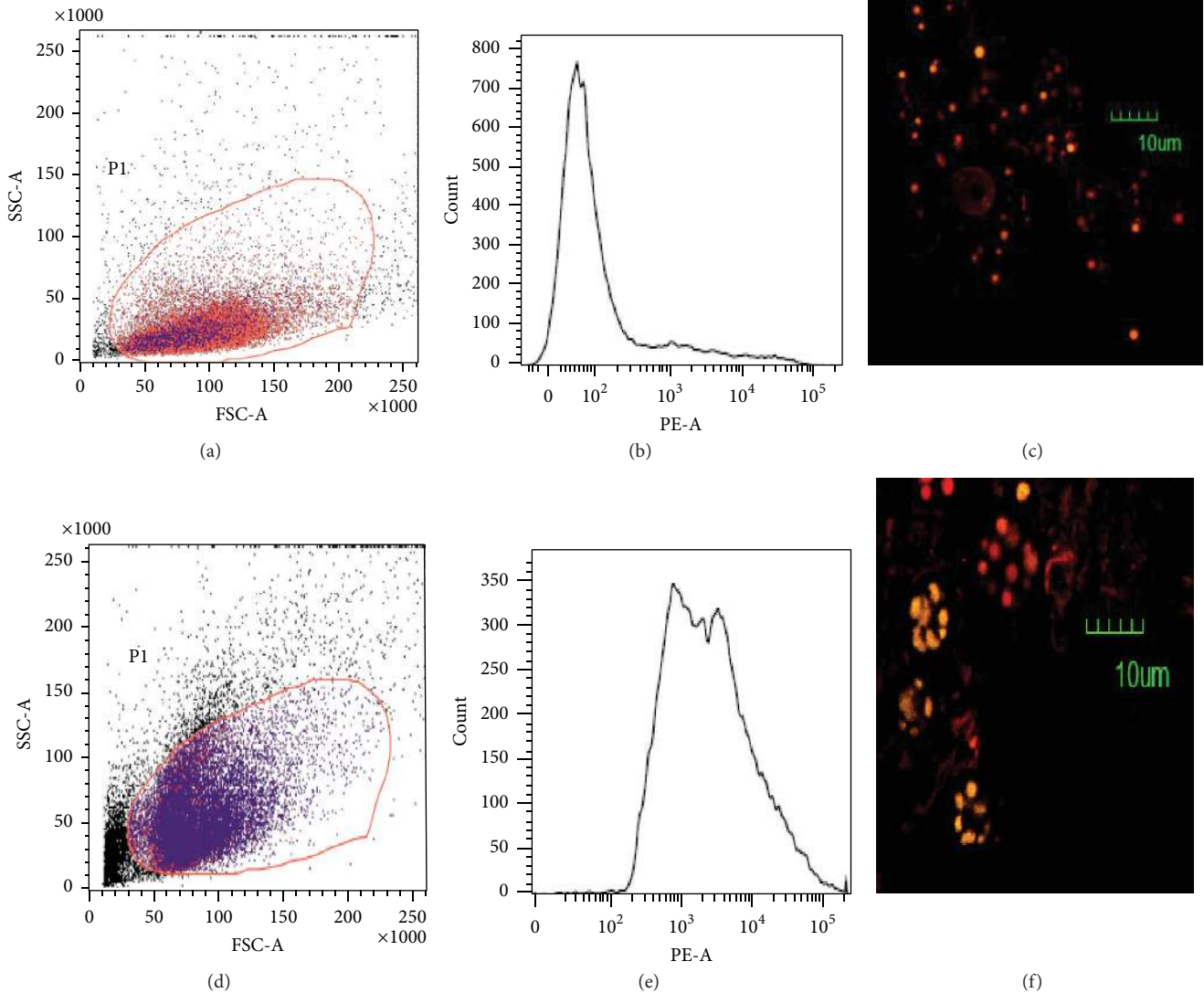


FIGURE 5: Flow cytometric analysis of Nile red stained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum* under phosphorous starvation. (a), (d) FSC versus SSC two-dimensional dot plots. (b), (e) Flow cytograms of Nile red stained cells showing fluorescence intensity. (c), (f) Neutral lipid bodies showing yellow-gold fluorescence (the colour red indicating the cells with chlorophyll autofluorescence and violet indicating cells with neutral lipids in the scattered plots).

TABLE 2: Statistical parameters of Nile red stained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum* under different concentrations of P and EDTA (CV: coefficient of variation).

Stress factors	<i>Chlorella ellipsoidea</i>			<i>Chlorococcum infusionum</i>			
	% grandparent	Median	CV	Stress factors	% grandparent	Median	CV
Control	9.89	1,217	30.45	Control	7.67	1,112	26.74
0.15 g/L P	51.26	6,006	141.57	0.00 g/L P	30.54	4,171	117.57
0.05 g/L EDTA	30.41	4,152	140.81	0.08 g/L EDTA	28.31	3,426	106.39

this study we applied GC-MS, confocal microscopy, and flow cytometry for detailed lipid analysis in microalgae.

In this study we reported highest biomass yield of 1.32 ± 0.1 g/L in *Chlorella* after 20 days of incubation whereas in *Chlorococcum* it was found to be 1.45 ± 0.06 g/L at 18

days of incubation. In both the microalgae the maximum biomass yields 0.08 g/L EDTA. In *Chlorella* and *Chlorococcum* maximum lipid accumulation was $26.8 \pm 0.4\%$ (dcw) and $24.4 \pm 1.2\%$ (dcw) at 22 and 20 days of incubation, respectively. In *Nannochloropsis oculata* the biomass and lipid productivity

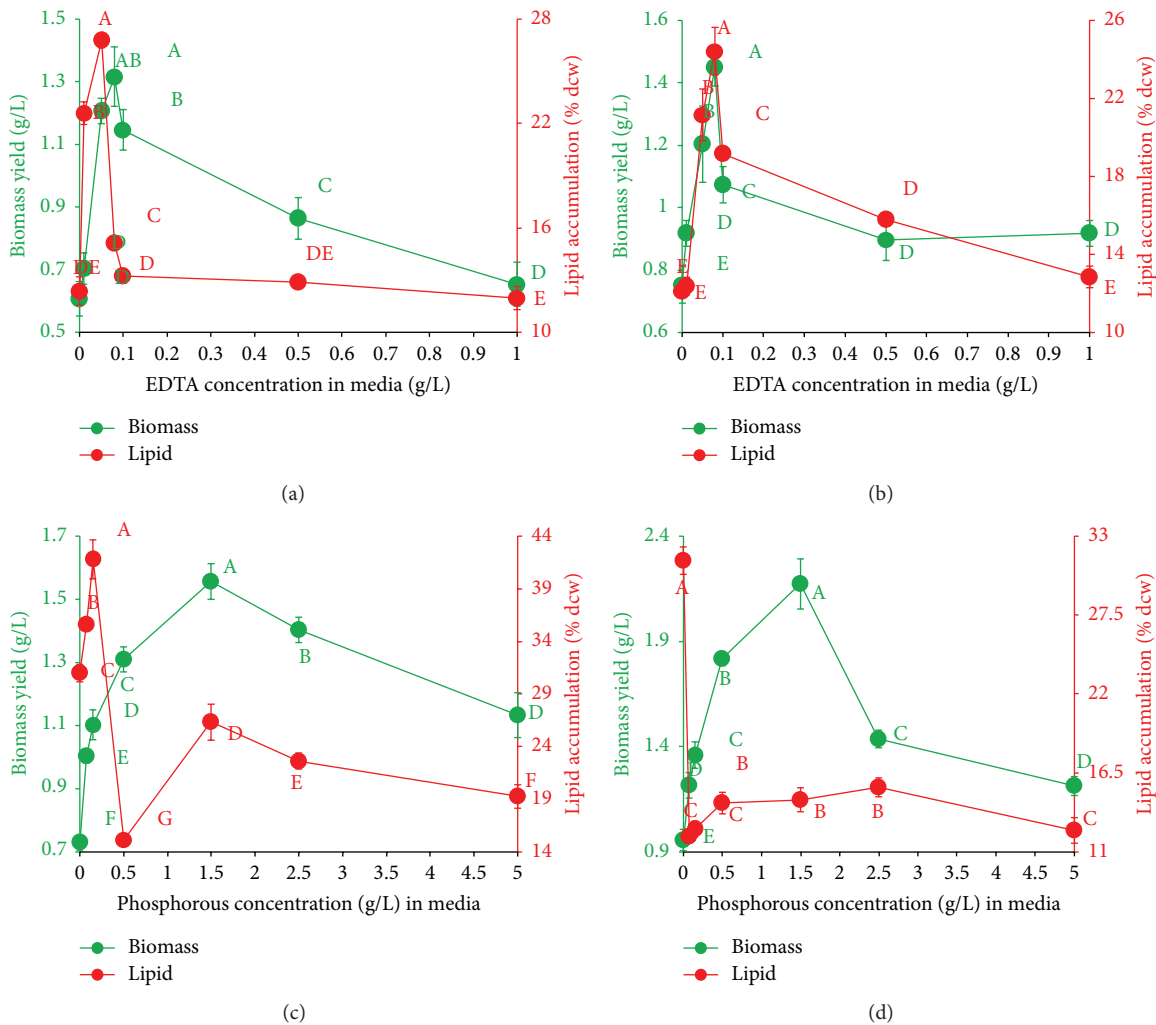


FIGURE 6: Maximum values of biomass and lipid concentrations obtained from different levels of (a)-(b) EDTA and (c)-(d) P treatment on *Chlorella ellipsoidea* and *Chlorococcum infusionum*. Values are mean \pm SD, $n = 3$. Values in each point superscripted by different alphabets (A–G) are significantly different from each other ($p < 0.05$, DMRT).

were increased with the increasing concentrations of EDTA [10]. The lipid accumulation in *Amphiprora paludosa* was 65.64% at 0.026 mM EDTA [13]. EDTA can increase the availability of other metal nutrients for growth and lipid accumulation. Maximal lipid accumulation in microalgae can reach up to 40–50% under EDTA stress. In both the microalgae MUFA increased and subsequently PUFA decreased under P and EDTA stress. It has been reported that the concentration of MUFA increased simultaneously with lowered phosphate concentration [15–18]. In *Chlorella ellipsoidea*, PUFA significantly decreased with decreasing EDTA concentration whereas opposite result was found for *Chlorococcum infusionum*. Similar results were obtained with decreasing P concentration.

The biomass and lipid content in microalgae can be increased by limitation of P in the culture medium [14–18]. In the present investigation we employed various concentrations of P in the culture medium for *Chlorella* and *Chlorococcum*. The maximum biomass yield in *Chlorella* was 1.56 ± 0.06 g/L

and in *Chlorococcum* was 2.17 ± 0.12 g/L at 1.5 g/L P. The maximum lipid accumulation in *Chlorella* and *Chlorococcum* was $41.8 \pm 1.9\%$ and $31.3 \pm 1.0\%$ (dcw) under various concentrations of P. Similar studies were obtained in *Phaeodactylum tricornutum*, *Chaetoceros* sp., *Isochrysis galbana*, and *Pavlova lutheri* [14]. They showed that palmitic and oleic acid methyl esters were also increased under P deprivation. The overall triacylglycerol (TAG) production was increased from 6.5% to 39.3% under P limitation [17]. The cellular total lipid content in terms of TAG accumulation was also reported in *Monodus subterraneus* under P limitation [18].

Flow cytometry coupled with a fluorescent probe is an efficient tool for determination of neutral lipid content in microalgae. In our study we applied lipid soluble fluorescent probe Nile red in combination with flow cytometry to evaluate the neutral lipid in test microalgae. Various studies were performed on accumulation of neutral lipid in different algal groups. Guzmán and coworkers used four species of microalgae, namely, *Isochrysis galbana*, *Tetraselmis suecica*,

Dunaliella salina, and *Chlorella pyrenoidosa* and found low CV values in FL2 and FL3 signals [25]. The fluorescence signal for neutral and polar lipid in *Cryptocodinium cohnii*, obtained through flow cytometry, was linearly correlated with the lipid obtained from gravimetric method [26]. In the present communication high CV and median values were obtained in *Chlorella* and *Chlorococcum* suggesting the lipid containing cells were positively stained with Nile red. The maximum fluorescent intensity (% grandparent) of positive stained cell populations was 51.26% in *Chlorella* and 30.54% in *Chlorococcum* under 0.15 g/L of P. The neutral lipid accumulation in conditional mutants of two microalgae *Chlamydomonas reinhardtii* and *Chlorella vulgaris* was studied at elevated temperatures [28]. The enhanced cellular lipid containing mutants of *Nannochloropsis* sp. was isolated by flow cytometric cell sorting [22]. They obtained fourfold increase in total fatty acid content followed by 30% increase in palmitic acid and 45% decrease of eicosapentaenoic acid relative to that of the wild type. The intracellular lipid bodies in *Chlamydomonas reinhardtii* by flow cytometry was studied using two different lipophilic fluorescent dyes, Nile red and BODIPY^{505/515} (4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a-Diaza-s-Indacene) [46]. The results they revealed showed that BODIPY^{505/515} is more effective for intracellular neutral lipid accumulation and cell sorting. In our study we found the positive staining of Nile red and detection of intracellular neutral lipid bodies under P and EDTA stress. The *Chlorella ellipsoidea* and *Chlorococcum infusionum* resulted in high lipid accumulation and biomass yield under various stress conditions and therefore can be considered as good candidates for biodiesel production. It has been proved that EDTA improves staining efficiency as well as neutral lipid accumulation in microalgae. In combination with a fluorescent probe, EDTA increases the porosity of the cell wall and cell membrane to stain intracellular neutral lipid [47]. Simultaneously it has been found that intracellular lipid accumulation and cell quality were checked by confocal or fluorescent microscopy. EDTA enhances cell permeability to penetrate the dye easily to stain intracellular neutral lipid [10, 47]. Flow cytometry and confocal microscopy confirm the accumulation of intracellular neutral lipid bodies in microalgae which is commercially important for biodiesel production.

5. Conclusion

In concluding remarks, it can be stated that the flow cytometry is the most modern and useful technique to screen potential microalgae suitable for biodiesel production. It is more effective than other conventional techniques of lipid study. Enhanced lipid containing cells determined by gravimetric method were screened through flow cytometry to confirm the accumulation of intracellular lipid droplets in *Chlorella ellipsoidea* and *Chlorococcum infusionum*. Confocal images also confirmed the accumulation of neutral lipid bodies under EDTA and P starvation. Enhanced production of monounsaturated fatty acids also supported that these

microalgae can be used as potential feedstock for production of biodiesel.

Conflict of Interests

The authors do not have any conflict of interests.

Acknowledgments

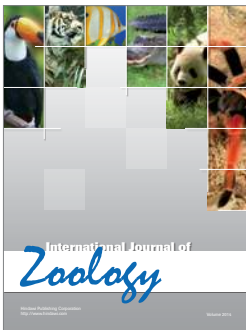
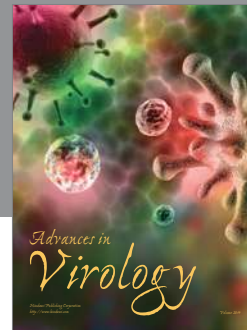
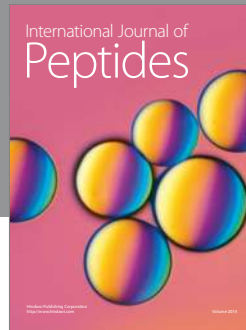
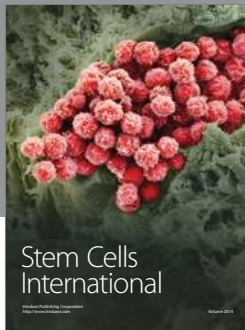
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