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ORIGINAL ARTICLE

Rapid detection of neutral lipid in green microalgae by flow cytometry in combination with Nile red staining—an improved technique

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Abstract A staining protocol for rapid in situ detection of neutral lipid using flow cytometry in combination with Nile red staining was optimized. Staining efficiency was tested in terms of fluorescence intensity (% grandparent) in varied concentrations of Nile red and dimethyl sulfoxide (DMSO), with variable incubation period, temperature and pH level. The improved method was tested using two microalgae: *Chlorella ellipsoidea* and *Chlorococcum infusionum*. Maximum staining efficiency was recorded with a concentration of 5 μg mL^{-1} Nile red and 40 % DMSO in a 15-min incubation at 40 °C for both taxa (pH 6.5). The forward (FSC) and side scatter (SSC) two-dimensional dot plot showed highly scattered cells containing neutral lipid. The coefficient of variation, standard deviation, mean and median values were determined for quantification of neutral lipid. We also applied this modified method to detect the elevated level of neutral lipid in nitrate ($NaNO_3$)-depleted cells; the efficiency of this technique was justified indicating a prominent 3- to 4-fold increase in neutral lipid in treated cells. Confocal images of stained cells also revealed accumulation of high levels of neutral lipid in treated microalgal cells.

Keywords Flow cytometry · Nile red · Nitrate depletion · Novel staining method · Physico-chemical parameters

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Introduction

Microalgal lipids, like those of higher plants, are composed of both polar and neutral lipids (Li et al. 2010). In favorable environmental conditions, microalgal cells can accumulate large amount of polar lipids viz. phospholipid and glycolipid, in the cell membrane. Microalgae are considered as promising candidates for an alternative fuel source due to their high growth rate and accumulative power of large amount of neutral lipid also in the cytosol in the form of triacylglycerol (TAG) (Day et al. 1999; Hu et al. 2008; Chen et al. 2009). TAG is an energy-rich compound that can be converted to biodiesels and jet fuels (Radakovits et al. 2010; Goodson et al. 2011; Velmurugan et al. 2013). Microalgae accumulate large quantities of neutral lipid suitable for biodiesel production, especially in exponential phase or when environmental conditions are stressful for cellular growth (Illman et al. 2000). It has been reported previously that depletion of nitrate in the culture medium enhances lipid productivity (Feng et al. 2012; Sun et al. 2014). Nitrogen starvation is thus one of the key factors used widely to trigger lipid synthesis in microalgae (Breuer et al. 2012; Santos et al. 2013; Mandotra et al. 2014; Chu et al. 2014). Besides this, various physical and chemical stresses reportedly enhanced microalgal lipid production (Renaud et al. 2002; Li et al. 2008; Converti et al. 2009; Bellou and Aggelis 2012). Further thorough investigation is needed for screening of the most suitable microalgal species with high neutral lipid content and to move from laboratory to commercial production of biodiesel in a cost effective way.

Most conventional methods for lipid extraction, such as gravimetric determination, solvent extraction and supercritical fluid extraction (Bligh and Dyer 1959; Halim et al. 2012), are time- and energy-dependent processes and cannot screen large numbers of microalgal species. The quantification of neutral

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lipid further requires solid–liquid phase separation, thin layer chromatography (TLC) and gas chromatography (GC). Alternative in situ methods to measure lipid content are most effective for screening of microalgae for biodiesel production (Izard and Limberger 2003; Chen et al. 2009).

Nile red (9-diethylamino-5H-benzo [α] phenoxazine-5-one) is a lipid-soluble fluorescent dye showing golden-yellow fluorescence for neutral lipid, red for chlorophyll auto fluorescence and polar lipids. It is frequently employed for determination of lipid content in different groups of microorganisms (Chen et al. 2009). In microalgae it can penetrate easily through the cell wall and membrane to combine with nonpolar lipids within the cytoplasm giving yellow-gold fluorescence (Elsey et al. 2007; Huang et al. 2009; Doan and Obbard 2011a, 2011b; Sun et al. 2014; Pancha et al. 2014). On the other hand, non-polar lipid shows green fluorescence when combined with BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a-diaza-s-indacene). The latter is also more effective and is used frequently for sorting microalgal cells according to their fluorescence properties (Brennan et al. 2012; Xu et al. 2013).

Flow cytometry or fluorescence-activated cell sorting is the most powerful technique currently available for the analysis of different features or physiological parameters such as cell size and granularity in a variety of microorganisms, viz., bacteria, fungi, yeast, mammalian cells and microalgae (Velmurugan et al. 2013; Hyka et al. 2013). Flow cytometry can screen different microalgae by autofluorescence emission of photosynthetic pigments such as chlorophylls, carotenoids and phycobilins (Hyka et al. 2013). The combination of flow cytometry and a suitable fluorescent dye is a highly effective way to rapidly isolate high-lipid-containing microalgae for biodiesel production (Cooper et al. 2010; Yao et al. 2012; Doan and Obbard 2012; Velmurugan et al. 2013).

In this study, we combined flow cytometry with the lipid soluble dye Nile red to determine the staining efficiency of accumulated intracellular lipid under variable conditions. The staining method was modified by manipulating incubation time, temperature, pH, solvent and dye concentration. These treatments successfully enhanced the effectiveness and efficiency of Nile red to stain the intracellular lipid bodies of two microalgae, *Chlorella ellipsoidea* and *Chlorococcum infusionum*. These two taxa were tested in the present investigation, as green micro algae have already been reported as potential strains for biodiesel production (Liu et al. 2011; Yang et al. 2011; Karemore et al. 2013). The improved method of Nile red staining in combination with flow cytometry can be used as a high throughput screening of microalgae for biodiesel production. In the present study, we applied this improved staining method to in situ elevated neutral lipid detection in nitrate-depleted cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum*.

Materials and methods

Collection and isolation of microalgae

The microalga *Chlorella ellipsoidea* was collected from coastal mangrove forests (N 22°12.109', E 088°42.856') and *Chlorococcum infusionum* was collected from a brackish water pond (N 22°01.355', E 088°41.168') in the Sunderbans. The samples were collected with the help of a phytoplankton net of 20 μm mesh size. The microalgal species were isolated in axenic culture under sterile conditions in the laboratory. After complete establishment of axenic culture, voucher specimens were prepared and named CUH/Al/MW- 189 for *Chlorella ellipsoidea* and CUH/Al/MW-190 for *Chlorococcum infusionum* and deposited with the Calcutta University Herbarium (CUH).

Microalgae culturing

The microalgae *Chlorella ellipsoidea* and *Chlorococcum infusionum* were isolated and maintained under laboratory conditions. *Chlorella ellipsoidea* was cultured in modified Zarrouk's growth medium (Zarrouk 1966) and *Chlorococcum infusionum* was cultured in Bold Basal Medium (Bold 1949). Both microalgal species were maintained at 22 °C in 16:8 light:dark cycles. The cultures were mixed well with bubbling air containing 2 % CO_2 (v/v) and vigorous shaking at 120 rpm in an Eyla horizontal shaker-incubator for 2–3 h per day.

Organic solvent and reagents

The universal organic solvent dimethyl sulfoxide (DMSO) was used for dissolving the dye. Nile red was purchased from Sigma-Aldrich (St. Louis, MO, CAS number: 7385-67-3; catalogue number: N3013) and used to stain the algal cells.

Determination of growth by measurement of dry cell weight

For measuring microalgal growth, cell suspensions were harvested on day 0, 3, 5, 7, 14, 21 and 28 of growth. An aliquot of 50 mL of each algal culture was collected and centrifuged at 10,000 rpm for 5 min and transferred to pre-weighed vials for oven drying at 70 °C to attain constant weight. Oven-drying is the most commonly used method and is preferred over other drying methods as it is convenient, efficient and cost effective. The biomass was estimated

by the gravimetric method in terms of g L^{-1}. Cells of log-exponential phase (21-day-old culture) were used for optimization of the staining method.

Physical and chemical parameters for optimizing Nile red staining

DMSO concentration

An aliquot of 500 μL of each algal culture from the log exponential growth phase (21-day-old culture) was transferred to a 2-mL Eppendorf tube and 5 μL 0.1 mg mL^{-1} Nile red solution was added. Then, 295 μL DMSO aqueous solution at concentrations ranging from 1 % to 70 % (v/v) was added. The tubes were vortexed (110 rpm) to mix the dye with the algal cells and solvent and then incubated in the dark at room temperature for 10 mins. After staining, the algal cells were washed 2–3 times with phosphate-buffered saline (PBS) solution and observed under a confocal microscope. The unstained culture and stained cells at different concentrations of DMSO were run through flow cytometry. Three replicates of each treatment were analyzed.

Nile red dye concentration

Different concentrations of Nile red dye ranging from 0.1 μg mL^{-1} to 100 μg mL^{-1} were used to optimize the concentration of dye for staining of intracellular lipid of microalgal cells. In this study, 40 % DMSO aqueous solution was applied as stain carrier as standardized previously.

Variation in staining temperature

A range of staining temperatures from 20 °C to 90 °C was tested to obtain the maximum efficiency of staining using 5 μg mL^{-1} Nile red and 40 % DMSO aqueous solution as standardized previously.

Incubation time

Algal suspensions of log exponential phase were stained with 5 μg mL^{-1} Nile red in 40 % DMSO aqueous solutions and incubated at 40 °C elevated temperature. Efficiency of staining was optimized using different values of incubation time of 5 min to 60 min.

Variation in pH

An aliquot of 5 μg mL^{-1} 40 % aqueous DMSO solution was used as staining solvent. Different pH values of the staining solution, viz. 3, 5, 5.5, 6, 6.5, 7.5, 8.5, 9.5, 10 and 12 were employed to optimize the staining procedure.

Flow cytometric analysis of intracellular lipid

The accumulation of intracellular lipid was detected by high speed FACS Verse flow cytometer (Becton Dickinson Instruments, Franklin Lakes, NJ). The fluorescence signals of both the control and stained cells were obtained using a solid state laser with an excitation of 488 nm and an emission of 586/42 nm for neutral lipid. The unstained cells were used as an auto-fluorescence control. Cell morphology including cell size and granularity were correlated with two scattering signals by flow cytometry, namely forward scatter (FSC) and side scatter (SSC) signals. The voltage was set for different wavelengths of light to study the subpopulations of cells in detail. Data were expressed as fluorescence arbitrary units and percentage (%) grandparent of stained cell population. The mean, median, standard deviation (SD) and coefficient of variation (CV) of unstained (auto-fluorescence) and stained cells (neutral lipid) were analyzed using FACS Verse statistical software (Becton Dickinson).

Confocal microscopy

The distribution of intracellular neutral lipid bodies in microalgal stained cells was studied by confocal microscopy. After staining with Nile red, the slides (Blue star; http://www.bluestarglass.net/) were prepared using 10 % glycerin (v/v) and observed under confocal microscope (Olympus IX 81, Olympus, Tokyo, Japan) equipped with Flow view FV 1000 software (Olympus). The excitation wavelength was set to 470 nm and the emission wavelength to the 530–630 nm range for both neutral and polar lipid.

Preparation of experimental media

The $NaNO_3$ concentration varied in experimental media from 0 (absence of nitrate) to 10 g $L^{-1}$for both the culture medium BBM and Zarrouk's medium, and pre-cultured microalgal cells of log-exponential phase (14 days for *Chlorella* and 21 days for *Chlorococcum*) were inoculated into the stressed media.

Extraction of total lipid

Microalgal biomass under different concentrations of $NaNO_3$ was harvested at different time intervals and dried in a pre-weighed vial in a hot air oven at

70 °C. Lipid was extracted from dried biomass from the same vials using the protocol of Bligh and Dyer (1959). The percentage of total lipid was calculated by the gravimetric method.

Results

Biomass yield

Growth in terms of biomass yield (g L^{-1}) with time was determined for both microalgal species. From Fig. 1a, it was clear that maximum biomass yield was 1.71 g L^{-1} (dry cell weight) after 14 days of culture for *Chlorella* and 3.07 g L^{-1} after 21 days for *Chlorococcum*. Biomass from the exponential growth phase was used for neutral lipid staining.

Optimization of staining

Effect of DMSO concentration

With increasing concentration of DMSO, the staining intensity of the algal cells increased, showing a maximum level at 71.68 % grandparent in *Chlorococcum* and 17.89 % in *Chlorella* at 40 % DMSO (Fig. 1b). Figure 2 show two-dimensional (2D) dot plots and cytograms for both unstained and 40 % DMSO-assisted stained cells. The unstained cells of both *Chlorella* and *Chlorococcum* exhibit reddish color auto fluorescence of endogenous chlorophyll, carotenoid, phycoerythrin and phycocyanin molecules (Fig. 2a-1 and b-1). In both cases, highly scattered FSC and SSC signals were observed. A uniform cellular neutral lipid (violet color) was observed from the dot plots of FSC vs SSC signals (Fig. 2c-1 and d-1). The fluorescence intensity for neutral lipid was shown by the flow cytograms (Fig. 2c-2 and d-2) showing the emitted fluorescence at stationary phase. The statistical data in terms of mean, median, SD and CV were analyzed for both unstained and stained cells (Table 1). In 40 % DMSO-assisted staining solution, all the values of neutral lipids increased rapidly with respect to that of the unstained sample (table 1). In *Chlorella* the final fluorescence mean, median and SD intensities were found to be greater than those of *Chlorococcum* except the CV value, suggesting variation in lipid accumulation within the cells. Confocal images of stained cells show yellow-gold droplets of neutral lipid (Fig. 2c-3 and d-3), while unstained cells showed reddish color corresponding to auto fluorescence of pigments and polar lipids (Fig. 2a-3 and b-3).

Effect of Nile red concentrations

We recorded maximum fluorescence intensity of neutral lipid after staining with Nile red at 5 μg mL^{-1} showing 68.62 % grandparent in *Chlorococcum* and 40.21 % in *Chlorella* (Fig. 1c). The 2D dot plots showed highly scattered signals in FSC and SSC for both the algal species (Fig. 3a-1 and b-1). The fluorescence intensity were almost equal in terms of cell count in both *Chlorella* and *Chlorococcum* (Fig. 3a-2 and b-2). Flow cytograms revealed that the cells of the exponential phase were stained maximally with Nile red in *Chlorella* whereas in *Chlorococcum* maximal staining was in stationary phase. The confocal images showed yellow-gold fluorescence for neutral lipids (Fig. 3a-3 and b-3). The CV for *Chlorococcum* was found to be 165.4 suggesting maximum fluorescence intensity, compared to the medium fluorescence intensity of *Chlorella* (97.84) (Table 1).

Effect of temperature

Figure 1d shows that the percentage grandparent stained cell population reached maxima of 53.9 % in *Chlorella* and 67 % in *Chlorococcum* at 40 °C temperature. The results further revealed that 30–40 °C was the optimum temperature for Nile red staining for *Chlorococcum infusionum*, while for *Chlorella ellipsoidea* the optimum temperature was 40–50 °C. Temperatures above 70 °C significantly decreased the fluorescence intensity of Nile red stained cells. At 40 °C, a uniform scattered fluorescence signal of both polar and non-polar lipid was observed in *Chlorella* whereas *Chlorococcum* showed highly scattered SSC and FSC for fluorescence of polar and neutral lipid (Fig. 3c-1 and d-1). The fluorescence intensity was measured by flow cytogram of cell count vs. 586/42 emission signal (PE-A) for neutral lipid (Fig. 3c-2 and d-2). High intracellular lipid bodies accumulated at the optimum temperature for both algal species (Fig. 3c-3 and d-3). The high CV, SD and mean values also suggested high fluorescence intensity at 40 °C (Table 1).

Effect of incubation time

The changes in staining efficiency of both *Chlorella* and *Chlorococcum* with increasing time are shown in Fig. 1e. The optimum incubation period was found to be 15 min, revealing 40.88 % grandparent for *Chlorella* and 68.41 % for *Chlorococcum*. Beyond the incubation period of 15 min a sharp decline in staining efficiency in terms of percentage grandparent was observed. The confocal images showed neutral lipid accumulation in the cytosol of both algal species (Fig. 4a-3 and b-3). Uniform scattered cells of *Chlorella* were found in the SSC region of the 2D dot plots (Fig. 4a-1). The other green alga *Chlorococcum* showed highly scattered cells in the SSC and FSC region (Fig. 4b-1). The flow cytograms

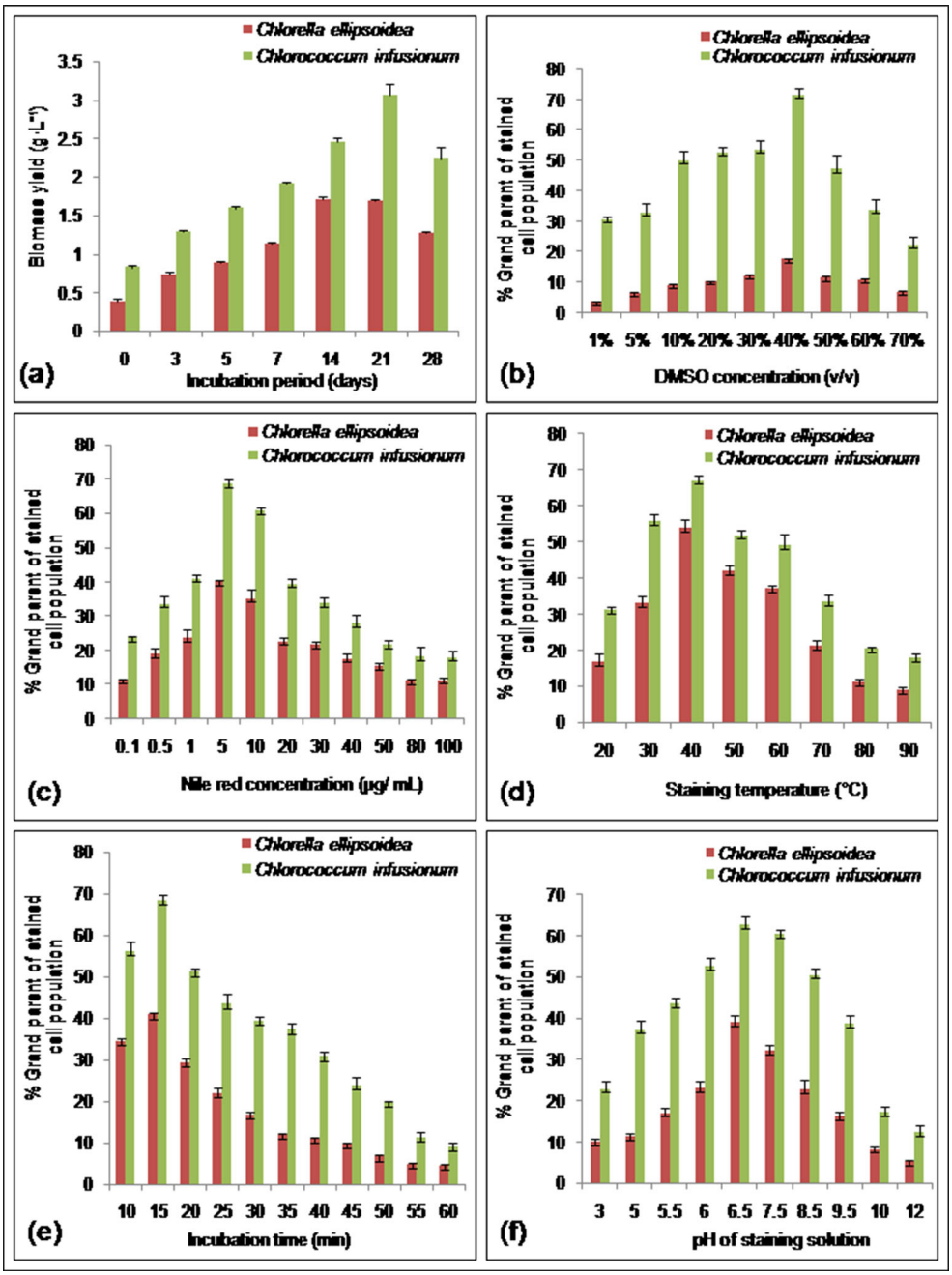

Fig. 1 Biomass yield (g L^{-1}) and the effect of different physical and chemical parameters on fluorescence intensity (% grandparent) of Nile red stained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum*. Data represented as mean±standard deviation (SD) of triplicates

showed high fluorescence intensity at 15 min incubation time (Fig. 4a-2 and b-2). The CV and SD value of *Chlorococcum* was higher than that of *Chlorella* indicating high non polar lipid accumulation in the cell cytoplasm (Table 1).

Effect of pH

From Fig. 1f it is clear that, at pH 6.5, the cells of both microalgal species showed maximum fluorescence intensity in terms of percentage grandparent (39.07 in *Chlorella* and 62.97 in *Chlorococcum*). A sharp decline in fluorescence intensity was recorded with any further increase in pH. In *Chlorella* the FSC vs SSC scattered plot showed uniformly scattered cells (Fig. 4c-1) while highly scattered FSC and SSC signals were observed in *Chlorococcum*. The flow cytograms of *Chlorella* indicated high fluorescence intensity in early growth phase (Fig. 4c-2) while high intensity was observed in mid-growth phase for *Chlorococcum* (Fig. 4d-2). Confocal microscopic images also showed the yellow-gold fluorescence of neutral lipid bodies at pH 6.5 (Fig. 4c-3 and d-3). Maximum CV, SD, mean and median values were also recorded at pH 6.5 (Table 1).

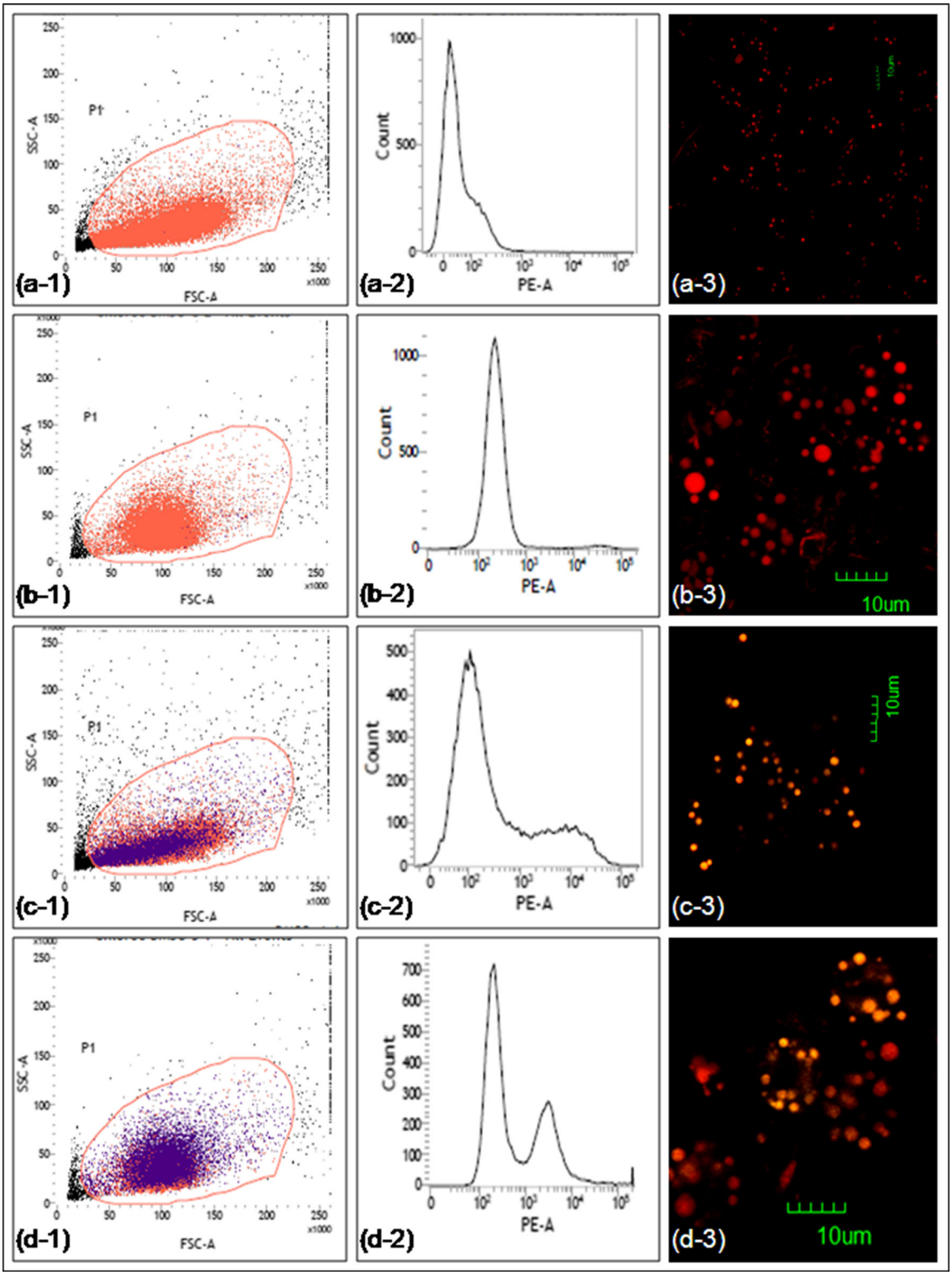

Fig. 2a–d Flow cytometric analysis of unstained and Nile red stained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum* at 40 % DMSO concentration. **a**-1, **b**-1 Forward scatter (FSC) vs side scatter (SSC) two-dimensional (2D) dot plots of unstained cell population. **c**-1, **d**-1 FSC vs SSC 2D dot plots of stained cell population. **a**-2, **b**-2 Flow cytograms of unstained cell showing cell count vs fluorescent signal (586/42 or PE-A). **c**-2, **d**-2 Flow cytograms of stained cell showing cell count vs fluorescent signal (586/42 or PE-A). **a**-3, **b**-3 Chlorophyll auto fluorescence of unstained cell populations. **c**-3, **d**-3 Neutral lipid bodies of Nile red stained cells. In scattered plot *red* indicates indigenous chlorophyll and polar lipids, and *violet* indicates neutral lipid. **a**, **c** *Chlorella ellipsoidea*; **b**, **d** *Chlorococcum infusionum*

Table 1 Statistical analysis of unstained control and Nile red stained cells in terms of mean, median, standard deviation and coefficient of variation by flow cytometry. *SD* Standard deviation, *CV* coefficient of variation

Physical and chemical parameters	*Chlorella ellipsoidea*				*Chlorococcum infusionum*			
	Mean	Median	SD	CV	Mean	Median	SD	CV
Unstained control algal culture	3,125	2,425	1,816.77	58.14	2,044	1,360	1,642.36	80.35
DMSO concentration 40 %	11,263	6,967	12,437.28	110.42	5,075	3,188	8,786.67	173.15
Nile red concentration (5 μg mL^{-1})	16,855	11,610	16,491.56	97.84	4,874	2,998	8,061.84	165.4
Staining temperature (40 °C)	12,957	8,545	13,199.91	101.87	7,908	4,021	12,422.21	157.08
Staining time (15 min)	11,307	4,686	11,367.11	98.35	6,535	3,426	11,501.12	145.05
pH (6.5)	4,525	3,218	9,877	89	4,285	2,231	8,351.05	134
Unstained nitrate depleted cells	2,516	1,725	4,315	78	3,105	2,834	5,351	101
Nitrate depleted cells (14 days)	6,116	5,756	8,978.32	129.52	5,695	6,754	9,445.23	132.14

Accumulation and detection of enhanced neutral lipid under nitrogen ($NaNO_3$) stress

Maximum lipid (% dcw) accumulation was detected in nitrate-depleted conditions for both microalgal species when exposed to 0–10 g L^{-1} nitrate salts (Fig. 5). Lipid accumulation increased up to 51.3 % (3–4 fold) in *Chlorella* and 40.43 % (3–4 fold) in *Chlorococcum* in the absence of nitrate (Fig. 5). An increase in $NaNO_3$concentration in the growth medium was found to decrease lipid accumulation in microalgal cells.

The flow cytometric analysis of maximum lipid yielding cells (51.3 % and 40.43 % dcw) revealed uniformly scattered cells in *Chlorella* and highly scattered cells in *Chlorococcum* (Fig. 6c-1 and d-1). The FSC vs SSC area plot showed scattered cells containing neutral lipid after Nile red staining (Fig. 6c-1 and d-1). The flow cytogram of *Chlorella* showed high fluorescence intensity in stained cells of log exponential phase (Fig. 6c-2) while in *Chlorococcum* high intensity was found in mid stationary phase (Fig. 6d-2). Confocal microscopic images of both algal species showed yellow-gold fluorescence of neutral lipid bodies in the cell cytoplasm (Fig. 6c-3 and d-3). Statistical analysis also supported the results (Table 1).

Discussion

Green microalgae have received significant attention due to their inherent high lipid content and potential for biodiesel production; their high lipid yielding properties make them of great interest for biodiesel applications. The total lipid content of green microalgae has been reported to vary from 15 % to 75 %, as revealed by previous studies, and is considered as a lipid feed stock for biodiesel research (Chisti 2007; Rajvanshi and Sharma 2012). Lipid extraction and determination of the percentage of neutral lipid are two significant steps for biodiesel production in addition to screening for better strains. Therefore, rapid detection of in situ neutral lipid production is a current challenge in biofuel research.

Most common methods of lipid extraction from microalgae use cell disruption methods (Lee et al. 2010). The dry cell biomass is disrupted using various methods such as microwave exposure (Cravotto et al. 2008; Virot et al. 2008; Mahesar et al. 2008; Lee et al. 2010), sonication (Engler 1985; Lee et al. 1998), autoclaving together with osmotic shock using 10 % NaCl solution (Lee et al. 2010) or the bead-beating method (Lee et al. 1998; Geciova et al. 2002). Lee et al. (2010) applied these methods to cells of *Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp. and found that highest lipid productivity in *Botryococcus* sp. (5.7 mg L^{-1} day^{-1}) was achieved by a microwave-based cell disruption method. However, this process was time consuming and required input of energy therefore it would not be effective for screening purposes. In this study we applied an easy, rapid screening procedure to screen microalgal species of high lipid content for biodiesel production.

We optimized the staining efficiency of Nile red in combination with flow cytometry. Several research groups have used Nile red to stain neutral lipid bodies in microalgae in combination with different organic solvents such as acetone, glycerol and DMSO (Elsey et al. 2007; Chen et al. 2009; Doan and Obbard 2011a; Velmurugan et al. 2013). Chen et al. (2009) reported the optimum concentration as 0.5 μg mL^{-1} for Nile red staining, with an optimum exposure time of 10 min. In the present investigation, we found that a concentration of Nile red of 5 μg mL^{-1} gave best results for both microalgal species with an incubation time of 15 min. The maximum fluorescence intensity of Nile red stained cells was found at a DMSO concentration of 25 % (v/v), as recorded in *Chlorella vulgaris* (Chen et al. 2009). In our study, we found 40 % DMSO concentration (v/v) to be optimum for high fluorescence intensity. Several authors have reported a significant correlation between the fluorescence intensity of Nile red stained cells and lipid content in different algal groups such as

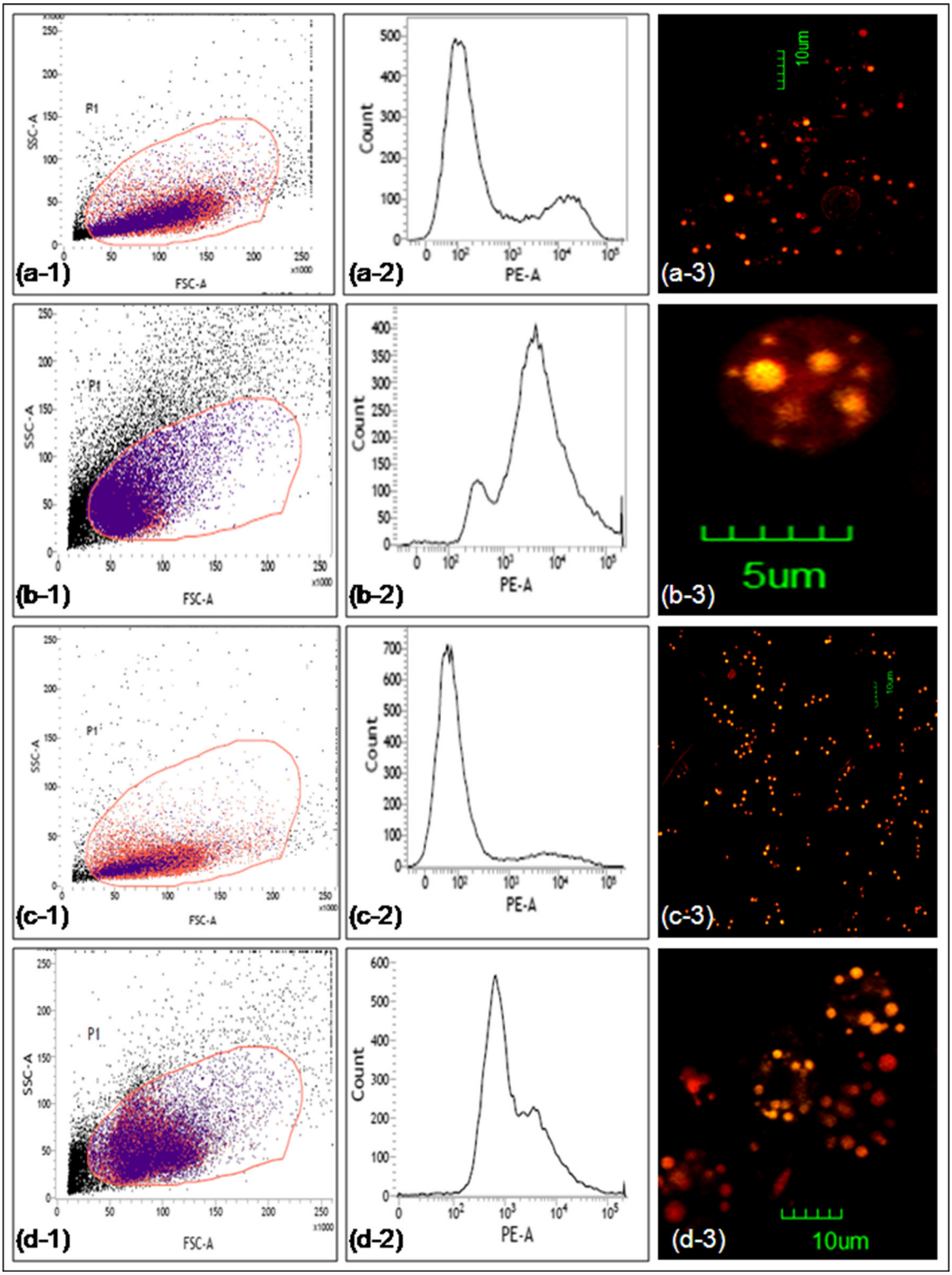

Fig. 3a–d Flow cytometric analysis of 5 μg mL^{-1} Nile red stained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum* at an incubation temperature of 40 °C. **a**-1, **b**-1, **c**-1, **d**-1 FSC vs SSC 2D dot plots of stained cells. **a**-2, **b**-2, **c**-2, **d**-2 Flow cytograms of cell count vs fluorescent signal (586/42 or PE-A). **a**-3, **b**-3, **c**-3, **d**-3 Neutral lipid bodies of Nile red stained cells. In scattered plot *red* color indicates indigenous chlorophyll and polar lipids and *violet* indicates neutral lipid. **a**, **c** *Chlorella ellipsoidea*; **b**, **d** *Chlorococcum infusionum*

dinoflagellates (de la Jara et al. 2003), green algae (Chen et al. 2009; Velmurugan et al. 2013) and other microalgae (Cooksey et al. 1987; Lee et al. 1998; Elsey et al. 2007). In this study, we demonstrated flow cytometric determination of intracellular lipid accumulation in factorial combinations of different physical and chemical parameters to optimize the staining

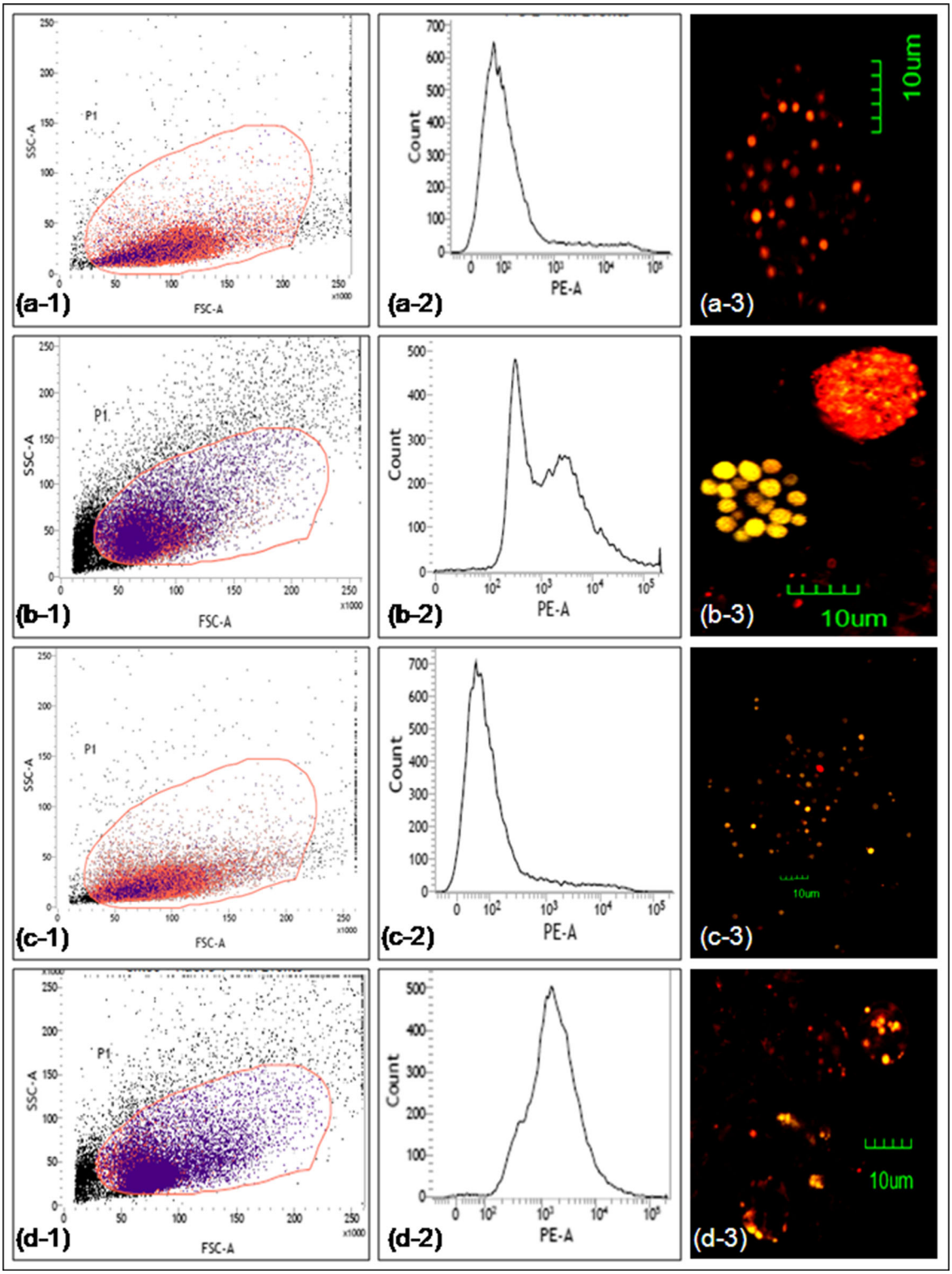

Fig. 4a–d Flow cytometric analysis of Nile red stained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum* at 15 min incubation periods and pH 6.5. **a**-1, **b**-1, **c**-1, **d**-1 FSC vs SSC 2D dot plots of stained cells. **a**-2, **b**-2, **c**-2, **d**-2 Flow cytograms of cell count vs fluorescent signal (586/42 or PE-A). **a**-3, **b**-3, **c**-3, **d**-3 Neutral lipid bodies of Nile red stained cells. In the scattered plot *red* indicates indigenous chlorophyll and polar lipids and *violet* indicates neutral lipid. **a**, **c** *Chlorella ellipsoidea*; **b**, **d** *Chlorococcum infusionum*

effectiveness of Nile red. Fluorescence activated cell sorting (FACS) is the most effective method for sorting cells according to their fluorescence properties. The 2D scatter plots distinguish polar and non-polar lipids according to their emission wavelengths. The FSC vs SSC plot demonstrated uniform fluorescence signal in *Chlorella* and highly scattered

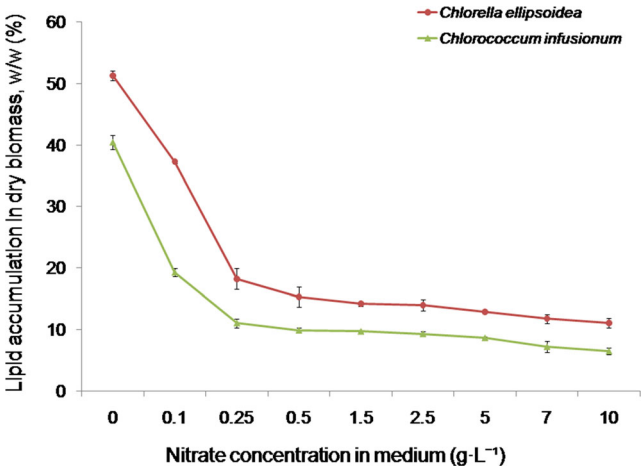

Fig. 5 Lipid accumulation in dry biomass, w/w (%) under different concentrations of $NaNO_3$ (g L^{-1}) in *Chlorella ellipsoidea* and *Chlorococcum infusionum*. Values are presented as mean±SD of triplicates

stained cells in *Chlorococcum*. Two dimensional dot plots of *Chlamydomonas reinhardtii* cells with different fluorescence signals were investigated by Velmurugan et al. (2013), who evaluated intracellular lipid bodies by flow cytometry and compared the two fluorescent probes Nile red and $BODIPY^{505/515}$. Doan and Obbard (2012) used *Nannochloropsis* sp. and induced random mutagenesis to enhance intracellular lipid by flow cytometric cell sorting. In our study, unstained cells of *Chlorella* and *Chlorococcum* showed auto fluorescence of chlorophyll and other photosynthetic pigments, viz. carotenoids, phycocyanin, etc. The enhanced rate of intracellular lipid accumulation in terms of high CV, SD, median and mean values was found to be significant for both microalgal species. In previous studies, the mean, SD and CV values in FL2 and FL3 channel by flow cytometry were investigated in four microalgal species and the interspecific variation in relative fatty acid composition was analyzed (Guzman et al. 2011). Flow cytometric determination of lipid content in a marine dinoflagellate, *Crypthecodinium cohnii* was studied by de la Jara et al. (2003). They used Nile red as a lipid-specific fluorescent probe and employed in vivo lipid quantification. The variation in dye concentration in our study revealed a high percentage of grandparent in the stained cell population. Spectro-fluorometric quantification of Nile red stained cells have been studied extensively (Chen et al. 2009). Different concentrations of DMSO also enhanced the fluorescence intensity in terms of percentage grandparent of positively stained cells. In our study, we found the optimum DMSO concentration for Nile red was 40 %. A similar study by Chen et al. (2009) found the optimum concentration of DMSO to be 25 %. The fluorescence intensity in terms of CV, SD, mean and median values was found to be higher in both *Chlorella* and *Chlorococcum*. The effect of staining temperature and time was found to be optimized at 40 °C and 15 min. Chen et al. (2009) performed a similar study in *Chlorella vulgaris* where the staining efficiency of Nile red was determined at longer than 10 min incubation time at a temperature of 40 °C. Our present study incorporated a new effort to optimize the pH affecting staining efficiency of Nile red. A pH of 6.5 was found to be optimal for high fluorescence intensity of Nile red. Flow cytometry in combination with Nile red can thus be used to screen green microalgae for biodiesel production. In association with green algae, this modified staining method is more useful for complex cell structures than conventional methods of lipid extraction.

A few strains of *Chlorella* have already been investigated for biodiesel production. Yang et al. (2011) reported 43 % (w/w) lipid content in another strain of *Chlorella ellipsoidea*, YJ1, isolated from domestic secondary effluent treated with activated sludge technology. A two-fold increase in unsaturated fatty acid was obtained in *Chlorella ellipsoidea* at low temperatures (Sharma et al. 2012). Another group of researchers reported the total lipid content of *Chlorella ellipsoidea* as 45.35±2.31 % when cultured in laboratory bioreactors (Liu et al. 2011). In *Chlorococcum infusionum*, the accumulation of lipid was 30–35 % (dcw) under nitrogen-starved conditions compared to control values of 12–15 % dcw (Karemore et al. 2013).

Nutrient limitation is one of the most important key regulators for triggering lipid synthesis in microalgal cells. Nitrogen limitation in microalgal culture is used to reduce cell growth and simultaneously induce lipid synthesis (Chu et al. 2014). The highest lipid productivity (87.1 mg L^{-1} day^{-1}) in nitrogen deficient culture of *Chlorella zofingiensis* was reported by Feng et al. (2012). Other studies have shown a significant rise in total lipid production and TAG accumulation under nitrogen starvation in microalgae, cyanobacteria and diatoms (Hu 2006; Hsieh and Wu 2009; Yeh and Chang 2011; Praveenkumar et al. 2012; Sun et al. 2014). Other than nitrogen, different physical and nutritional stress has been shown to alter lipid accumulation in microalgae. Previous studies have demonstrated that lipid accumulation in microalgae could be modified by various growth conditions other than nitrogen, such as phosphorous limitation (Reitan et al. 1994; Bellou and Aggelis 2012; Chu et al. 2014), sulphur limitation (Sato et al. 2000; Matthew et al. 2009), silicon deficiency (Griffiths and Harrison 2009), high salinity (Takagi and Yoshida 2006; Zhu et al. 2007; Chen et al. 2008) and the presence of heavy metals such as cadmium (Guschina and Harwood 2006). Li et al. (2008) reported enhanced lipid production (3- to 7-fold) upon iron supplementation. In several microalgae, the total lipid content and lipid composition are reported to be changed in altered physical conditions such as light and temperature. Light-induced changes in lipid accumulation of microalgae have been reported by many authors (Khotimchenko and Yakovleva 2005; Carvalho and Malcata 2005; Bellou and Aggelis 2012). Ultra violet radiation (UV-A and UV-B)-mediated lipid

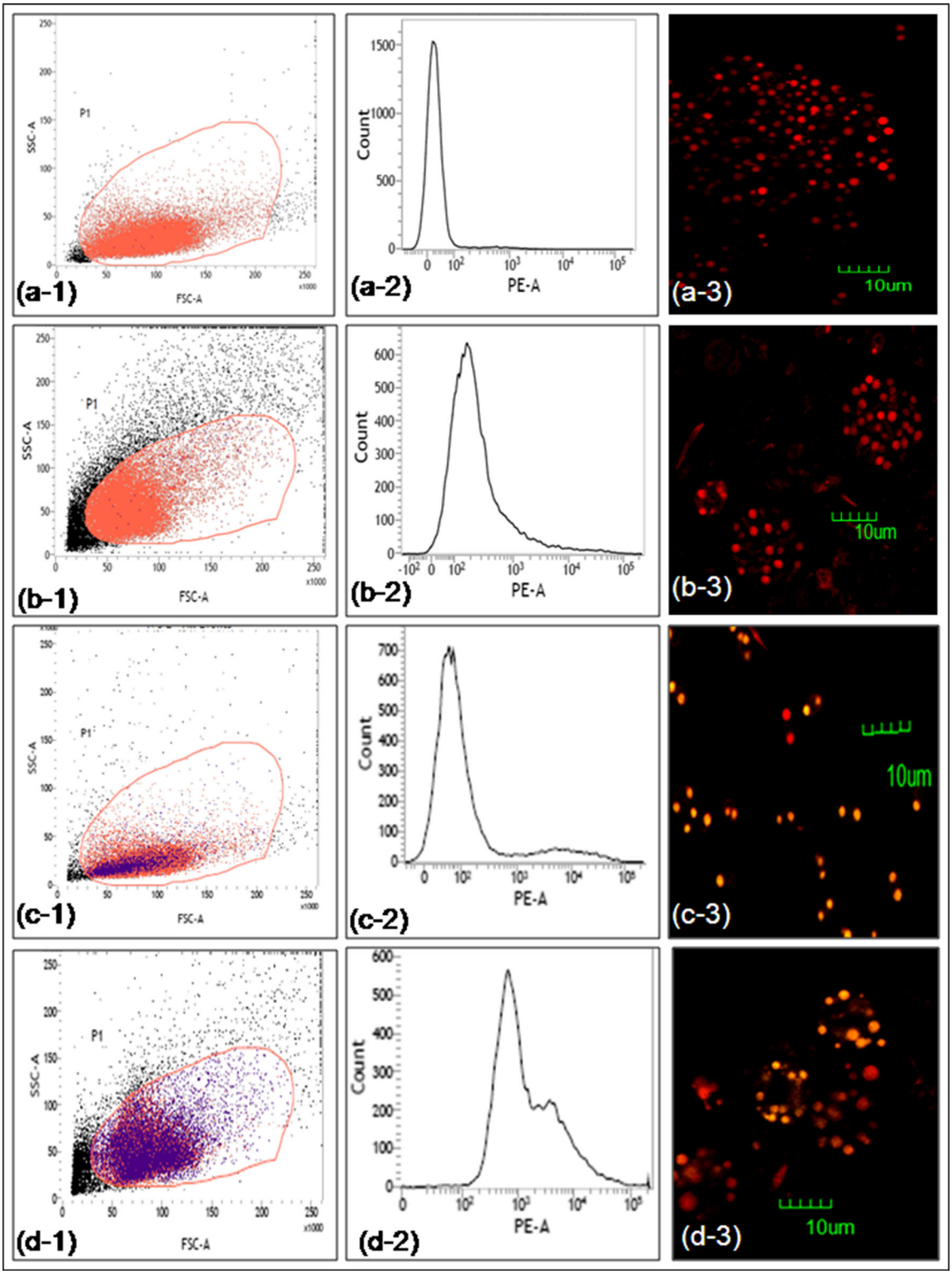

Fig. 6a–d Flow cytometric analysis of Nile red stained cells of *Chlorella ellipsoidea* and *Chlorococcum infusionum* under nitrate depleted conditions using the optimized method. **a**-1, **b**-1, **c**-1, **d**-1 FSC vs SSC 2D dot plots of stained cells. **a**-2, **b**-2, **c**-2, **d**-2 Flow cytograms of cell count vs fluorescent signal (586/42 or PE-A). **a**-3, **b**-3, **c**-3, **d**-3 Neutral lipid bodies of Nile red stained cells. In the scattered plot *red* indicates indigenous chlorophyll and polar lipids and *violet* indicates neutral lipid. **a**, **c** *Chlorella ellipsoidea*; **b**, **d** *Chlorococcum infusionum*

accumulation has been studied in many microalgae by different research groups (Liang et al. 2006; Forjan et al. 2011). However, other studies report high and low temperature-induced changes in lipid accumulation (Renaud et al. 2002;

Converti et al. 2009). Our present study has demonstrated a flow cytometric approach with modified staining strategy to detect micro-algal lipid synthesis in the form of neutral lipid (TAG) only, as required in biodiesel production.

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