Growth, lipid productivity and cellular mechanism of lipid accumulation in microalgae *Monoraphidium* sp. following different phosphorous concentrations for biofuel production

Saumya Dhup[1,*], Dheeban Chakravarthi Kannan[2] and Vibha Dhawan[2]

[1]Bioresources and Biotechnology, TERI University, 10 Institutional Area, Vasant Kunj, New Delhi 110 070, India
[2]Biotechnology and Management of Bioresources Division, TERI, India Habitat Centre, Lodhi Road, New Delhi 110 003, India

Stress to an algal species can be caused by several factors ranging from temperature extremes to varying light intensities under which they accumulate lipids. Stress caused by nutrient limitation is known to induce maximum lipid accumulation in microalgae. In order to obtain an appropriate phosphate concentration exhibiting both high biomass and high lipid contents, four different concentrations of phosphate were implemented. Mechanism of lipid accumulation was also studied. Of the tested concentrations, no significant difference between growth rates was observed. Cultures with phosphate concentration 2.7 mg/l exhibited maximum increase in biomass productivity compared to control. But phosphate concentration 0.5 mg/l demonstrated the highest lipid productivity (8.75 mg/l/day), in accordance with lipid content (21.8%). Further, representation of TEM and fluorescent microscopic images depicted differences in lipid accumulation and sub-cellular ultrastructure at different concentrations of phosphate. Disappearance of phosphate and nitrate from the medium was also evaluated to study the efficient nutrient concentration and to better understand mechanism of lipid enhancement.

Keywords: Lipid accumulation, *Monoraphidium* sp., phosphate concentration, nutrient removal efficiency, transmission electron microscopy.

INTEREST in using microalgae as a feedstock is not new, but, in recent times, it has become an emerging option to generate biodiesel. Their potential as biofuel feedstock is well known and documented in the literature, and the selection is largely based on higher yields per hectare, short duration, lower production cost, capability of utilizing nutrients from a variety of agricultural, industrial and municipal waste and the ability to grow on degraded lands[1].

*For correspondence. (e-mail: saumya.dhup.18@gmail.com)

Oil productivity, mass of oil produced per unit volume of microalgal broth per day, is dependent on growth rate and oil content of algal biomass[2]. Therefore, the prime objective currently lies in identifying a locally adapted strain which can withstand varying environmental conditions to provide higher oil productivities on a larger scale to meet future requirements[3]. It is well documented in the literature that lipid production can be increased under stress conditions which can be artificially created by changing the temperature, pH and nutrient saturation. However, most of these treatments also result in reduction in multiplication[4]. It was earlier reported that under nutrient limitation, considerable changes in biochemical composition of the algal strain are seen, provided there is enough light and CO_2 available for photosynthesis. The amount of changes depends on the type of nutrient limited and also the degree to which it has been limited. Nitrate and phosphorus being the two important nutrient sources majorly alter growth and metabolism of a species depending on its concentration in the medium. When these concentrations are limited, it leads to a shift in the metabolic pathway of cells from membrane lipids to synthesis of neutral lipids in the form of triacylglycerides (TAG)[5]. Nitrogen is a fundamental constituent for formation of all structural and functional proteins in algal cells. Stress caused by nitrogen depletion has long been studied for increasing lipid content. Phosphorus, on the other hand, is another nutrient known to induce lipid accumulation in the cells. It has a key role in transportation of metabolic energy and is a vital structural element of phospholipid molecules and nucleotides in living cells[5]. Studies have shown that lipid content under nutrient limitation is increased by blocking starch production[6–8]. However, such stresses lower the growth rate, lipid quality and productivity which is a major bottleneck for using oleaginous microalgae for biofuel production on a commercial scale. Thus, for optimal algal growth and lipid quantity, requirement of phosphorus differs from species to species[9].

In the present study, four different concentrations of phosphorus in the medium with BG11 were used for targeting standardization of the appropriate phosphorus concentration for higher overall lipid production per unit volume of *Monoraphidium* sp. T4X. The study also deals with analysing the progression of lipid content and changes in cell components throughout the production process over time with different phosphorus concentrations. Microscopy and chromatography were used to detect the increase in lipid bodies and fatty acid profile of algal cells throughout a research scale production pathway. This study is in continuation of the previous study wherein, nitrate concentration was optimized for higher lipid productivity. It was observed that with a lower nitrate concentration there was a higher uptake of phosphorus concentration. Therefore, to determine the role of phosphorus in growth metabolism, four different concentrations were used.

Materials and methods

Algal strain and growth conditions

Algal strain, *Monoraphidium* sp. T4X, was collected and isolated from a freshwater pond at the India Habitat Centre, New Delhi, India (28.5897°N, 77.2249°E). The strain was maintained in liquid BG11 medium consisting of (g/l): K_2HPO_4 (0.04), $MgSO_4 \cdot 7H_2O$ (0.075), $CaCl_2 \cdot 2H_2O$ (0.036), citric acid (0.006), ferric ammonium citrate (0.006), $NaHCO_3$ (0.02), Na_2EDTA (0.001) and $NaNO_3$ (1.5). The pH of the medium was adjusted to 7.5. The medium was sterilized at 121°C for 15 min. *Monoraphidium* sp. cultured in BG11 medium[10] was maintained in a 250 ml glass jar as stock, incubated at 25 ± 2°C.

Experimental procedure

The experiment was designed with three biological replicates for four batches. The factors differentiating the batches were four different concentrations of di-potassium phosphate (g/l): 0.001, 0.005, 0.04 and 0.1 used in the medium BG11. The batch with concentration 0.04 g/l is already reported as a typical concentration of di-potassium phosphate in BG11 medium and therefore it was considered as control. A concentration of 0.5 g/l of sodium nitrate was used in the medium. This concentration was optimized in a previous study[11]. A pre-incubation step was carried out to negate the carry-over effect of the strain on the experiment. These cultures with phosphorus concentration (mg/l) corresponding to 0.5, 2.7, 21.8 and 54.5 were then used as inoculum and added to 1 litre Erlenmeyer flask, the working volume being kept as 800 ml. Initial inoculum of the strain was added at a proportion of 10% (v/v) into the solution. As seen in Figure 1, the experiment was conducted for six passages. The results of the sixth passage were recorded for analysis. The inoculum used for the last phase was 50% (v/v) of the previous passage. Therefore the culture for final phase was inoculated at a proportion of 50% (v/v). The concentration of the culture for every passage was maintained to initial dosage by replenishing the exhausted nutrients (nutrient disappearance from the medium was calculated by using spectrophotometric methods listed in the *American Journal of Public Health* as APHA standards for nutrient analysis). Culture flasks were operated under 16 : 8, light and dark conditions at an rpm of 180. The data obtained was analysed statistically using one way ANOVA.

Growth measurements

The growth was observed by measuring optical density at 680 nm using a Shimadzu spectrophotometer and samples (3 ml) were taken every 4 days for growth kinetics and in the late exponential phase for lipid and FAME analyses. The dry cell weight was determined by filtering 3 ml of the cell culture through a 0.22 mm pore-size glass fibre filter (milipore). The filters with biomass were washed with water and dried in a hot air oven at 100°C. The increased weight of the dry filter was measured.

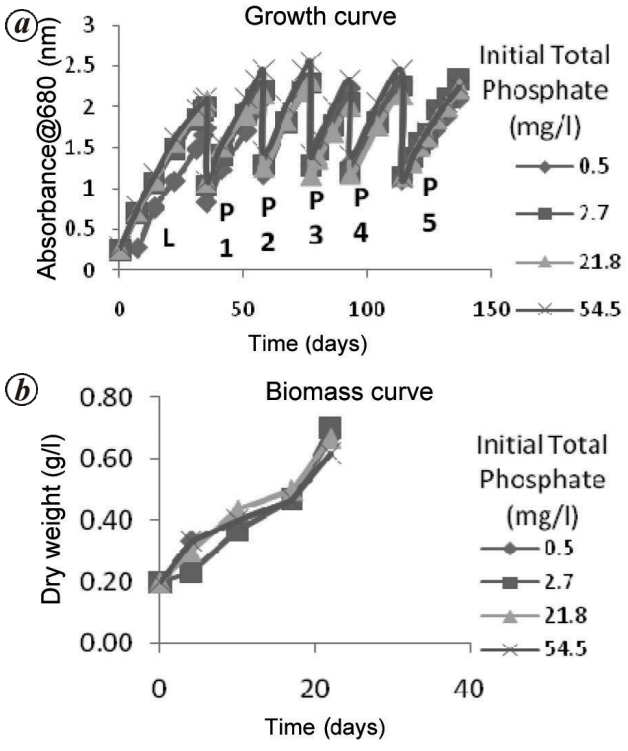

Figure 1. Phosphate optimization with four different concentrations. ***a***, Periodic fluctuation of growth for *Monoraphidium* sp. in semi-continuous culture. L represents pre-culture period. P1 to P5 show nutrient recharge and biomass removal intervals. ***b***, The biomass growth of *Monoraphidium* sp.

Nutrient analysis

Culture samples (2 ml) for nutrient measurements were taken at an interval of 4 days until it was harvested. Collected samples were filtered through glass fibre filters for analysis. Nutrients were analysed photometrically using standard methods from APHA standards. Total disappearance rate (TDR) was calculated by summing the disappearance rates of all nitrogen forms in a particular medium. Nutrient disappearance rates (NDR, mg-nutrient/l/day) was calculated as

$$\text{NDR} = (C_0 - C_t)/t,$$

where C_0 and C_t are the nutrient concentrations (mg/l) at the beginning and end of the experiment respectively, and t is the time interval (days).

Nitrogen estimation (chromotropic method)

In this method, two moles of nitrate nitrogen react with one mole of chromotropic acid to form a yellow reaction product having maximum absorbance at 410 nm. A nitrate standard in the range of 0.1 to 5.0 mg/l was prepared by diluting 0, 1.0, 5.0, 10.0, 25.0, 40 and 50 ml of standard nitrate solution to 100 ml with water. The suspended matter present was filtered suitably. Around 2 ml of the standard nitrate solution, sample (filtered supernatant of algal culture) and water blank was pipetted into dry 10 ml volumetric flask. One drop of sulphite urea reagent was added to each flask. These flasks were placed in a tray of cold water (10–20°C) and 2 ml of antimony reagent was added. The flasks were swirled following an addition of each of the reagent. After about 4 min in the water bath, 1 ml of chromotropic acid reagent was added, swirled and allowed to stand in cooling bath for 3 min. Sulphuric acid was added to bring the volumes near 10 ml mark. The samples were allowed to stand for 45 min at room temperature and the volume was adjusted to 10 ml with concentrated sulphuric acid. Final mixing was performed gently to avoid bubbling in the sample. Absorbance was measured at 410 nm between 15 min and 24 h after the volume adjustment.

$$\text{Nitrate nitrogen (mg/ml)} = \frac{\mu\text{g of nitrate nitrogen in 10 ml of final volume}}{\text{volume in ml of sample taken for test}}.$$

Phosphate estimation (stannous chloride method)

In this method, the molybdate–phosphoric acid formed is reduced to an intensely coloured complex molybdate blue by stannous chloride. About 100 ml of clear, colourless filtered sample was taken to which 4 ml of molybdate reagent and 0.5 ml of stannous chloride were added through continuous mixing. After 10 min of incubation, absorbance was measured at 690 nm.

$$\text{Phosphorus (mg/ml)} = \frac{\text{mg of phosphorus corresponding to control standard}}{\text{volume in ml of sample}} \times 100.$$

Qualitative analysis of lipids by Nile red

Nile red is a lipid soluble phenoxazone dye used to evaluate lipid content of micro-organisms by partitioning into the cytoplasmic oil bodies, after which the cells become fluorescent. In this study, validity of the method was done to visualize oil bodies in *Monoraphidium* sp. Lipid droplets of the strains were visualized under fluorescent microscope using Nile red. Algal suspension (5 ml) having an absorbance of 0.8 at 680 nm was taken and 600 µl of Nile red 601 (2.5 mg/10 ml of acetone) was added to it in dark conditions. The mixture was thoroughly shaken by rapid inversion and incubated in dark for 10 min at room temperature. The stained cell cultures were then applied to a glass slide covered with a cover slip and observed under a fluorescent microscope (ZIESS), with 450–490 nm excitation wavelength and 515 nm emission wavelength. Pictures were acquired randomly at least 25 cells per sample. Typical images are presented here.

Ultrastuctural analysis by transmission electron microscopy

A suspension of algae was fixed using 1.2% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M cacodylate buffer for an hour. Glutaraldehyde was washed out with same buffer (3 × 10 min) and the cells were post fixed using a mixture of 0.1 M cacodylate buffer and 4% (w/v) osmium tetraoxide solution for 1 h. The fixative was then washed out (3 × 15 min) and the cells were dehydrated in ascending sequences of acetone (10%, 30%, 50%, 70%, 90%, 95% and 100%), each for 10 min. Samples were then immersed into sequences of the mixture consisting of spur's resin and 100% acetone (2 : 1 and 1 : 1 for 1 h, 1 : 2 for 1.5 h) and then immersed in 100% spur's resin and placed overnight in a rotator. Finally they were again embedded in 100% spur's resin with activator and polymerized at 60°C for 24 h. Resin blocks with sample were then cut using a diatome diamond knife on a Powertome–PC (RMC products) and ultra thin sections were attached to grids. Sections were examined in Fei Tecnai G2 T20 Twin transmission

electron microscope (TEM). Images were recorded with a Gatan Orius camera and processed using Adobe Photoshop 7.0.

Lipid extraction, esterification and fatty acid analysis

Two batches of samples were harvested by continuous centrifugation (8000 rpm for 5 min) after completion of their respective time. Concentrated algal samples were frozen overnight at –80°C and freeze-dried under vacuum. Algal mass was accurately weighed and lipid extraction carried out according to Folch *et al.*[12].

Lipid content (%) and lipid productivity (g/l/day) were calculated as

$$\text{Lipid content } (C_{\text{lipid}}) = (\text{wt of lipid/wt of sample}) \times 100,$$

$$\text{Lipid productivity} = (C_{\text{lipid}} \times \text{DCW})/t,$$

where C_{lipid} is the lipid content (%), DCW the dry cell weight (g/l) and t is the time interval (days).

Lipid extracts were converted to methyl esters with methanolic HCl and hexane. Fatty acid methyl esters were prepared by adding 1 ml of concentrated HCl with 5 ml methanol. The mixture was heated at 80°C to 90°C in a water bath for 30 min. Hexane (1 ml) was added to the vial after methylation. Top hexane layer containing methyl esters was placed into GC vials for GC analysis (Agilent 6890N (USA)) equipped with a DB-5 column (0.2 mm ID, 30 m, 0.25 mm film by Agilent). The temperature programme was started at 2°C and increased by 50°C min^{-1} to 250°C. Peaks were integrated with Chemstation and identified by comparison of retention times with pure standard (Sigma). System performance was checked with blanks and standard samples prior to analysis. Concentration was expressed in mg/ml which was converted to percentage. All tests were performed in triplicates.

Results and discussion

Effect of phosphorus concentrations on growth kinetics

The present study was carried out with four different phosphorus concentrations to study its impact on biomass production and lipid content. Growth curve of *Monoraphidium* sp. T4X grown for six semi-continuous cycles is shown in Figure 1 *a*. The initial cycles were carried out to negate the carryover effect on the experiment. The results for lipid and biomass were recorded for the final cycle. Figure 1 suggests that concentration of 0.005 g/l (2.7 mg/l of phosphate) of di-potassium phosphate exhibited enhanced growth compared to other concentrations with a higher exponential increase in growth curve. The effect of various phosphorus concentrations on growth, biomass and lipid productivity was analysed statistically and presented in Table 1. It has been commonly reported that limitation or starvation of essential nutrients growth affects algal cell growth. El Kassas[13] reported that the growth rate of *Pichochlorum* sp. retarded with decreasing phosphorus concentration. Unlike the above observations, our study showed that the growth rate of this species was not significantly affected by varying phosphorus concentrations ($P > 0.05$).

Ferriols *et al.*[14] also reported no marked difference with change in phosphorus concentration. In the present study, it was observed that with concentrations (g/l) from 0.001–0.04, there was no significant difference between the growth rate, but the biomass productivity decreased with increasing concentrations. At concentration 0.1 g/l, an evident decline in growth rate and dry weight concentration of cells was observed (Table 1 and Figure 1 *b*). From this, it can be inferred that increasing concentration of the nutrient beyond a limit results in toxicity for the cells, leading to decrease in growth and metabolism[11]. The medium with 0.005 g/l phosphorus concentration showed an increase in biomass productivity by 66% in 23 days compared to control and medium with concentration of di-potassium phosphate –0.001 g/l (0.5 mg/l of phosphate) showed an increase of 33%. Maximum growth rate and biomass productivity in this study were exhibited by phosphate concentration 2.7 mg/l (0.005 g/l of dipotassium phosphate). Thus, in the present study, it was concluded that lower concentrations of phosphorus (0.001 g/l and 0.005 g/l) are better for overall growth.

Effect of phosphorus concentrations on nutrient removal by Monoraphidium sp.

In a previous study[11], it was reported that lower nitrogen concentration led to an increased removal of phosphorus from the medium, suggesting that nitrogen concentration has a significant effect on phosphorus usage. This observation was further investigated to obtain more information on whether phosphorus removal increased as a result of replacement of nitrogen or is it an equally important nutrient needed for growth and metabolism of the cells. Thus, the effects of varying phosphorus concentration on nitrogen and phosphorus removal were studied.

The corresponding removal efficiencies, after 23 days of cultivation, are shown in Table 2. Phosphorus removal efficiencies were significantly dependent on phosphorus concentration ($P < 0.05$). Phosphorus removal efficiency decreased when the phosphate concentration was higher than 2.7 mg/l. It was also observed that with a phosphorus concentration 0.5 mg/l, phosphorus removal efficiency

Table 1. Growth parameters and productivity of *Monoraphidium* sp.

Di-potassium phosphate (g/l)	Biomass productivity (g/l/day)	Lipid content (%)	Lipid productivity (mg/l/day)	Specific growth rate (day^{-1})
0.001	0.04	22	8.75	0.0213
0.005	0.05	16	8.08	0.0212
0.04	0.03	15	4.39	0.0211
0.1	0.03	12	3.65	0.018

Table 2. Nutrient disappearance and removal efficiency for *Monoraphidium* sp. with four different phosphate concentrations

Phosphate concentration added (mg/l)	N concentration		Nutrient removal efficiency (%)	P concentration		Nutrient removal efficiency (%)
	Initial	Final		Initial	Final	
0.5	39.22	21.62	44.88	0.119	0.067	43.70
2.7	42.04	21.62	48.58	0.24	0.083	65.42
21.8	41.69	21.27	48.98	1.6	0.674	57.88
54.5	40.98	20.21	50.68	2.15	1.244	42.14

decreased, but, was still higher at phosphate concentration of 21.8 and 54.5 mg/l. As mentioned earlier, higher phosphorus concentration becomes toxic to the culture effecting its growth leading to inefficient usage of resources. The removal efficiency of a nutrient decreases because the cells make an attempt to avoid uptake of the nutrient after a certain level. Lower nutrient concentrations also lead to a decline in algal growth, with limited photosynthesis when optimum amount of light is given. In order to continue photosynthesis at lower nutrient concentration, cells replenish their pool of electron carriers by shifting their metabolism from growth to fatty acid production for energy storage[15]. With this, increased uptake of nutrients by the cells also takes place for growth before shifting their metabolism to lipid production. Concurrently, Juneja *et al.*[5] stated that the immediate effect of phosphorus limitation is on inhibition of photosynthesis. In the present study, an increase in phosphorus removal efficiencies was observed with decrease in phosphorus concentration.

The table also shows that nitrogen removal efficiency significantly decreased with decreasing phosphorus concentration ($P < 0.05$) but a slight difference was found between the disappearance of nitrogen for each concentration. Xin *et al.*[4] reported a decrease in nitrogen removal with decreasing phosphorus concentration. Ferriols[14] observed that although the difference between the removal efficiencies of nitrogen with varying phosphorus was not significant, there was a slight increase in nitrogen uptake rate with elevated levels of phosphorus. Therefore, from these and the previous study[11], it can be inferred that in both cases (varying phosphorus and nitrogen concentration), nitrogen is the major source of nutrient which is taken up first when present in optimum amounts. In the absence of nitrogen, phosphorus, considered the next best optimum source is taken up; therefore, it acts as a replacement nutrient to negate the nutrient stress caused to the cell. Also, when phosphorus concentration is reduced, the rate of nitrogen uptake is reduced. This suggests that phosphorus also acts as an inducer nutrient for nitrogen uptake. Therefore, it can be stated that the combination of an appropriate lower concentration of phosphorus and nitrogen can be used efficiently in a medium at large scale, thereby increasing biomass productivity with improved nutrient usage. Thus, it can be stated that, phosphorus limitation may be the cause for a decrease in nitrogen uptake, suggesting that phosphorus induces nitrogen removal. Increase in lipid content was also observed with an increase in phosphorous removal efficiency (Tables 1 and 2). Similarly, Estevez-Landazabal *et al.*[16] reported an increase in lipid percentage upon higher phosphorous consumption in *Chlorella vulgaris* UTEX 1803. Previous studies also reported that nitrogen and phosphorus co-starvation made it possible for an increase in total lipid productivity with a visible shift in lipid composition from polar to non polar lipids. This was observed due to depletion of nutrients over time causing stress to the cells[17,18].

Effect of phosphorus concentration and cultivation time on subcellular ultrastucture of Monoraphidium sp. T4X

Most algal cells store neutral lipids within their cytoplasm. These neutral lipids are stored in the form of round bodies of oil called lipid bodies. Previous studies so far have demonstrated the effect of nitrogen deprivation on lipid accumulation, formation and mobilization of lipid bodies in various algal species and diatoms such as *Chlorella vulgaris* and *Phaeodactylum tricornutum* respectively[19,20]. But to the best of our knowledge, the

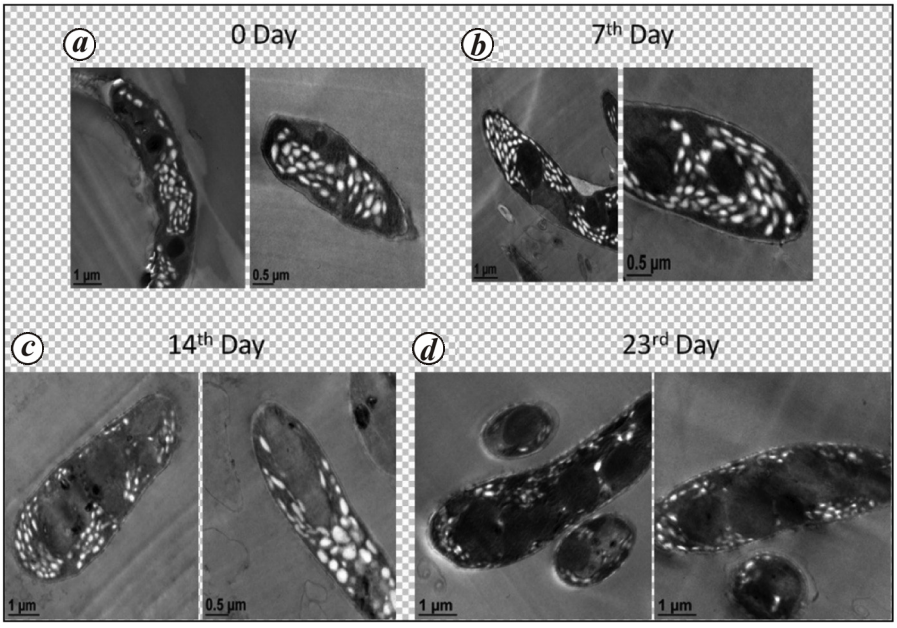

Figure 2. Progression of storage lipids with time in *Monoraphidium* sp. at a lower concentration (0.5 mg/l). ***a***, Cells at the beginning of the cultivation cycle with minute lipid droplets. ***b***, Enlargement of lipid bodies after 7 days of cultivation. ***c***, Starch and lipid bodies become equivalent as storage compounds after 14 days. ***d***, Cells after 23 days; huge dark lipid bodies oppresses the light starch bodies by filling almost the entire cell volume.

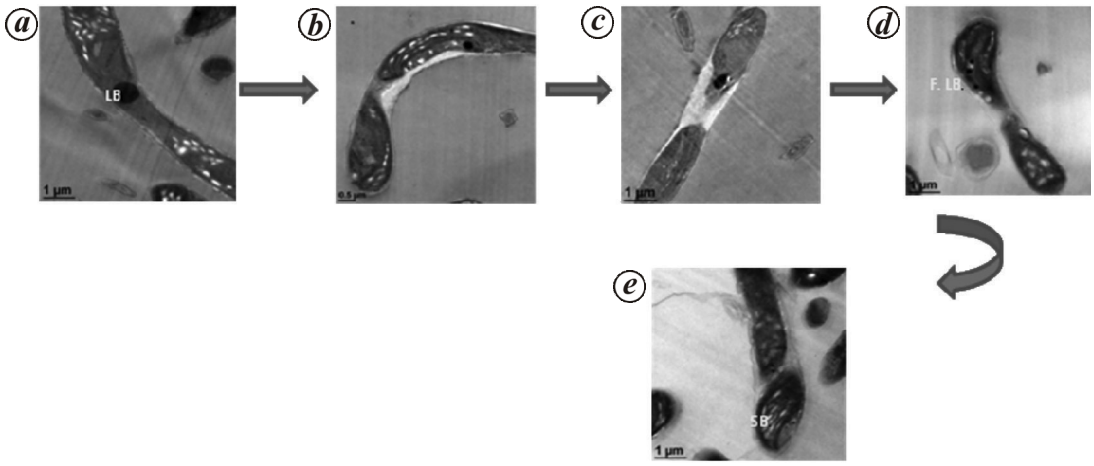

Figure 3. Biogenesis of lipid bodies during cell division. ***a***, Cell just before division with a large lipid body. ***b***, ***c***, Beginning of division of a cell; lipid body progresses to one of the daughter cell. ***d***, Fragmentation of lipid body into minute droplets within the cell. ***e***, Prevalence of starch bodies after division. LB = Lipid bodies; F. LB, Fragmented lipid bodies; SB, Starch bodies. Scale bars for ***a***, ***c***, ***d***, ***e*** – 1 µm, for ***b*** – 0.5 µm.

effect of different phosphorus concentration on lipid accumulation by studying the progression of lipid bodies and changes in cell structure by TEM in *Monoraphidium* sp. has not been studied till date.

Formation and progression of lipid bodies over time in Monoraphidium sp. T4X

Algal species usually utilize both oil and starch as storage compounds, but differently in accordance with growth phase and conditions. Progression of lipid bodies during the course of biofuel production in this study was shown with the lowest concentration of phosphorus (0.5 mg/l), since this concentration led to storage of maximum lipid bodies as protective mechanism under stress. It was observed that at the start of the cycle, where intensively dividing cells formed a part of the inoculum, large prominent starch globules localized in the chloroplast with minute lipid bodies (0.1–0.3 µm in diameter) found in the chloroplast and cytosol (Figure 2 *a*). After 7 days of cultivation, enlargement of lipid bodies was seen with starch granules still being the major storage compound

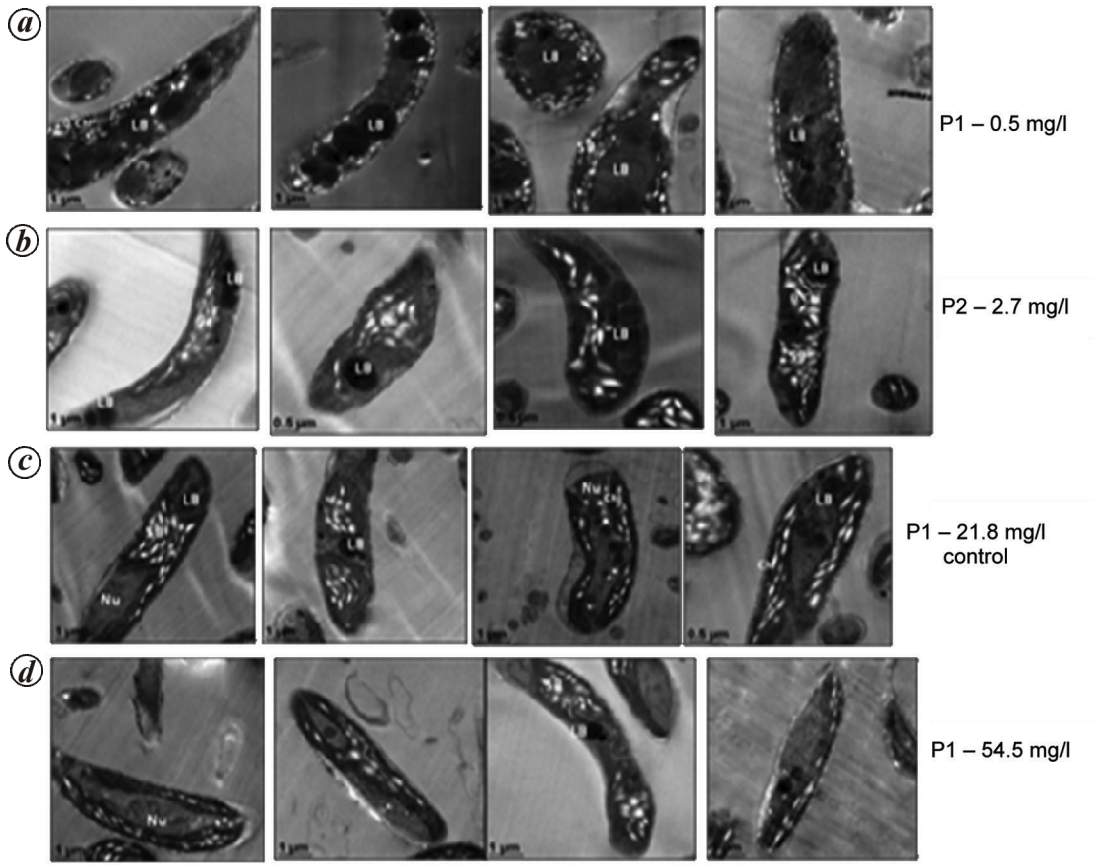

Figure 4. Effects of four different phosphate concentrations on *Monoraphidium* sp. ultrastucture. Transmission electron micrographs showing difference in subcellular ultrastructure and number of lipid bodies at different phosphate concentrations. LB, Lipid bodies; Chl, Chloroplast; SB, Starch bodies; D. Chl, Deformed chloroplast; Nu, Nucleus.

(Figure 2 *b*). Intensive enlargement of lipid bodies by fusion of smaller bodies into larger ones was observed after 14 days of cultivation (Figure 2 *c*). During this period, the cell apparently seemed to contain approximately equivalent amounts of lipids and starch as storage compounds. Enlargement in lipid bodies is observed over time as a result of stress built up in the cells due to depletion of nutrients, phosphorus and nitrogen from the medium. During such conditions, cells block the production of starch and shift their metabolism towards the production of lipid bodies, hence leading to enlargement of lipid bodies[6]. After 23 days of cultivation, the oil bodies prevailed over starch bodies, where a huge lipid body filled up almost the entire cell. Few diminishing starch granules were also seen (Figure 2 *d*).

Since these results recorded were for the sixth passage of a semi-continuous cycle, the inoculum used for this phase was 50% (v/v) of the previous passage. Therefore the culture for final phase was inoculated at a proportion of 50% (v/v). Apparently, this inoculum is subjected to contain cells from the exponential phase of a previous passage, therefore, when a new medium replenishing the exhausted nutrients is added, the cells start dividing intensively. Large lipid bodies were observed to be present in these cells just before division, since they were still in there exponential phase (Figure 3 *a*). During division in the presence of replenished nutrients, these lipid bodies start fragmenting into a large number of smaller droplets or one medium-sized globule (Figure 3 *b–d*). Starch bodies in these nascent daughter cells again become prevalent as a major storage compound for the cell (Figure 3 *e*).

Difference in subcellular ultrastructure of Monoraphidium sp. at different phosphorus concentration

Thin sections of the cells from exponential phase were observed by transmission electron microscopy to assess the impact of four different phosphorus concentrations on their ultrastructure. Numbers of lipid bodies with varying sizes were present at these concentrations. However, the total volume of lipid bodies was substantially higher at lower phosphorus concentrations. Lower concentration usually contained fusion of enlarged lipid bodies filling up most of the cell volume. The lipid bodies at lower concentrations were compact, globular and mostly

1.0–2.0 μm in diameter. In the case of concentrations above 2.7 mg/l (21.8 mg/l – control and 54.5 mg/l), small (0.1–0.7 μm in diameter) dispersed droplets of lipids were observed (Figure 4 *c*, *d*).

The number of starch granules in control and higher phosphorus concentration was more unlike at lower concentration, where diminishing or no starch granules were observed (Figure 4 *a*). In addition to significant changes being observed for storage compounds with different concentrations, changes to sub-cellular structure were also observed. At a concentration range of 0.5–2.7 mg/l, cell structure deformation and wrinkling of the membrane were evidently seen. A less defined nucleus and a poorly organized chloroplast structure were also observed with decreasing phosphorus concentration (Figure 4 *a*, *b*). In contrast, cellular structure in control and higher concentrations exhibited a well-defined nucleus, and a properly organized thylakoid membrane system housing large granules of prominently visible starch globules in them.

Effect of phosphorus concentration on lipid productivity and accumulation

Quantitative differences were observed in lipid productivities of four different phosphorus concentrations. Figure 5 shows the lipid content (%), algal biomass (g) and lipid productivity obtained with different phosphorus concentrations. It has been long studied that environmental stress plays a vital role in determining the quality and quantity of lipids[20]. Nutrient limitation is the most widely used lipid accumulation technique in microalgae. Despite reduced growth rates, a significant increase in lipid content due to nutrient stress makes that particular concentration an efficient dosage concentration for large scale cultivation. Phosphorus deprivation significantly influencing the lipid content of various microalgae has been shown in many studies. It was observed in the present study that with increasing concentration of phosphorus, there was reduction in lipid content of the cells (Figure 5).

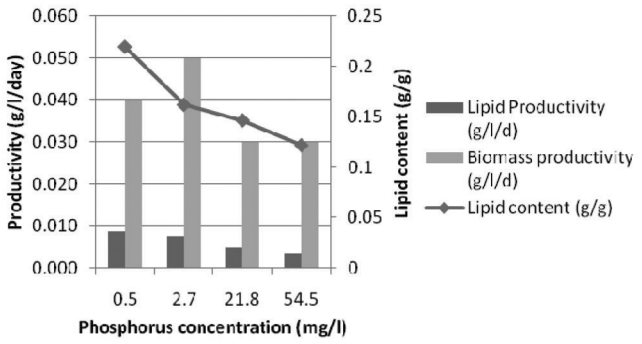

Figure 5. Biomass productivity (g/l/day), lipid content (%) and lipid productivity (g/l/day) of *Monoraphidium* sp. after 23 days of cultivation for different phosphate concentrations.

When *Monoraphidium* sp. was at its lowest phosphorus concentration (0.5 mg/l), lipid accumulation was enhanced and lipid content obtained was as high as 21.88% (Table 1). Lipid content at this concentration was significantly higher (33.2%) than that of control (0.4 g/l of di-potassium phosphate). Studies have also reported an increase in total lipid content by three-fold (41.8 ± 1.9%) and two-folds (31.3 ± 1.0%) in *Chlorella* sp. and *Chlorococcum* sp. respectively, under phosphorus starvation[21]. Phosphorus at 2.7 mg/l gave higher biomass and removal efficiencies, showed a slight (9.5%) increase in lipid content when compared to control. El Sheek *et al.*[22] also reported an increase in lipid productivity of *Scenedesmus obliquus* up to 54% upon phosphorus starvation. But they also reported that the growth rate ceased due to insufficient nutrients. Further, due to enhanced lipid accumulation, despite lower growth and biomass productivity, 0.5 mg/l concentration exhibited higher lipid productivity compared to other tested concentrations (Table 1). Similar results were demonstrated in a study by Xin *et al.*[4], wherein, lipid productivity at total phosphorus of 0.5 mg/l was the highest. Highest lipid content under phosphorus starvation was also reported by Dahmen *et al.*[23], on *Picochlorum* sp. Accumulation of lipids under nitrogen limitation occurs because, under high energy input conditions, the non-dividing cells are forced to channel excess energy obtained through light and carbon into intracellular high energy compounds–lipid[20]. Due to phosphorus limitation, replacement of membrane phospholipids by non-phosphorus glycolipids takes place, resulting in significant enhancement in lipid accumulation in *Monoraphidium* sp. as a reflection of lower nitrogen uptake by the cells at reduced phosphorus levels. A combination of these two creates an appropriate environment within cells which shifts the metabolic pathway from synthesis of membrane lipids to neutral lipids. Therefore, despite lower growth rates, cells exhibit higher lipid productivity.

Lipid content and accumulation of cultures were also assessed using Nile red and fluorescence microscopy. Nile red staining exhibited bright green fluorescence indicating intracellular lipid content in the cells. The size and number of oil bodies in sampled cells were observed after an interval of seven days during a culture cycle of 23 days. Substantially more oil bodies were observed in lower phosphorus concentrations than in control and higher phosphorus concentration. The size of oil bodies in lower concentrations was slightly bigger than that of control (Figure 6). Also, they usually contained large fused oil bodies filling most of the cell volume. Higher concentrations contained more of smaller oil bodies. The progression of oil bodies with time is also shown in Figure 6. The number and volume of oil bodies progressed with time. Also, the total volume of oil bodies was clearly higher in culture with lower phosphorus concentration than control, which was basically in line with the lipid

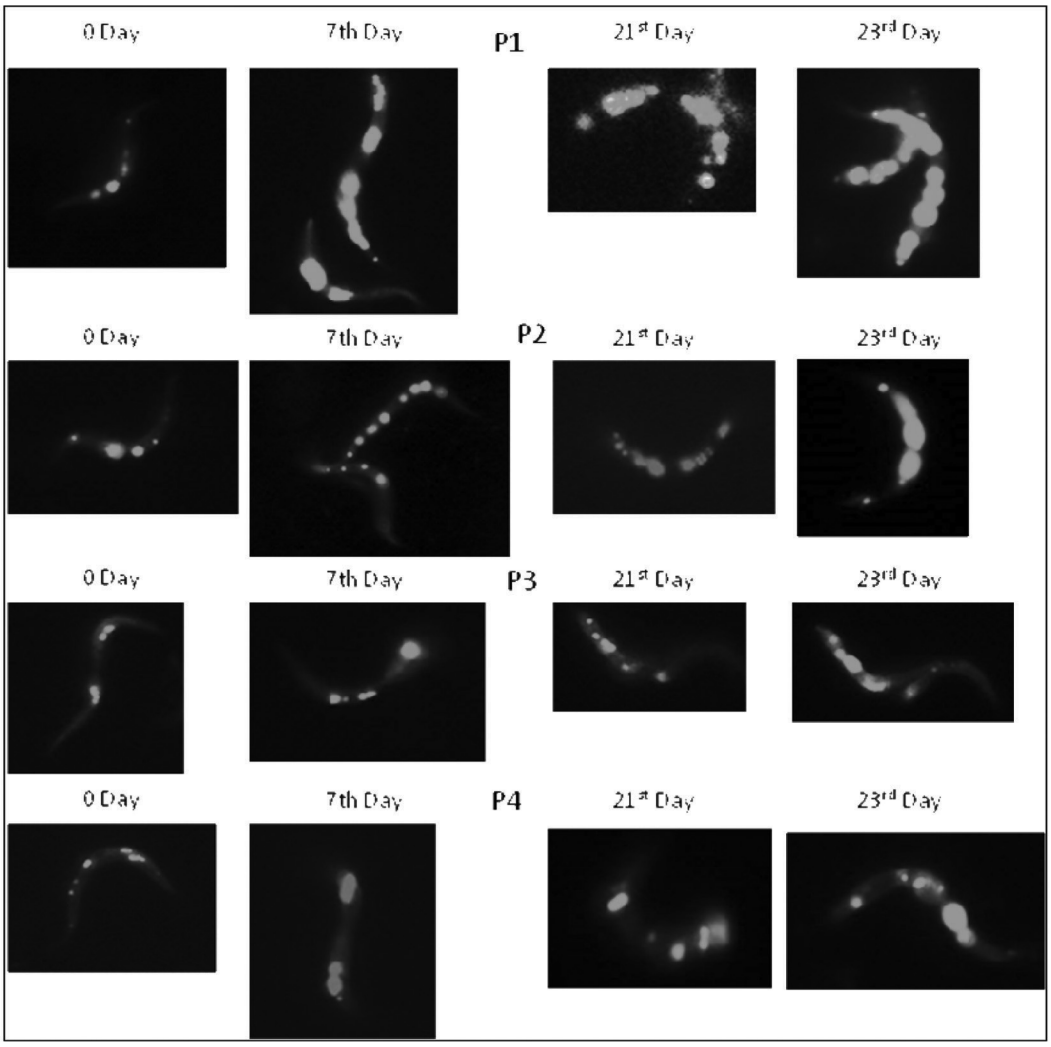

Figure 6. Representative fluorescent microscope images of *Monoraphidium* sp. showing oil bodies after Nile red staining. P1 – 0.5 mg/l, P2 – 2.7 mg/l, P3 – 21.8 mg/l (control) and P4 – 54.5 mg/l depict difference in accumulation of lipid bodies for different phosphorus concentrations along with progression of lipid bodies over time for each concentration.

Table 3. Fatty acid composition of *Monoraphidium* sp. at different phosphorus concentration

Phosphate concentration (mg/l)	0.5	2.7	21.8	54.5
Saturated fatty acid	36.28	18.49	17.32	13.61
Monounsaturated fatty acid	55.33	76.65	75.86	80.33
Polyunsaturated fatty acid	8.39	4.86	6.82	6.07
Degree of unsaturation	72.10	86.37	89.50	92.46

content of lowest concentration (21.88%) and control (14.62%) (Table 1). These results confirmed that lower phosphorus concentrations induced substantially higher lipid accumulation in *Monoraphidium* sp., making it a promising biofuel.

Fatty acid composition

Fatty acid composition analysis for *Monoraohidium* sp. at different phosphorus concentrations was done. It was observed (Table 3) that saturated fatty acids were highest at the lowest phosphorus concentration, which was almost double that of higher phosphorus concentration. This was observed due to an increased percentage of C18 : 0 at phosphorus concentration 0.5 mg/l. For monounsaturated fatty acids, an increasing trend was observed, where it was higher for higher phosphorus concentrations, but individually, no significant difference for the values of oleic acid was seen. Polyunsaturated fatty acids were observed to be lower at all phosphorus concentrations with no evident trend with difference in concentration. Also, the degree of unsaturation, associated

with biofuel property and cetane number, is seen to increase with increasing phosphorus concentration. To obtain a higher cetane number such that ignition delay is avoided, a species is required to have a lower degree of unsaturation. Therefore, phosphate concentration 0.5 mg/l, which has the lowest degree of unsaturation (Table 3) along with suitable fatty acid composition, can be further used for efficient growth and lipid productivity.

Conclusion

An optimum phosphate concentration providing appropriate growth and lipid productivity, of *Monoraphidium* sp. was studied. Four different concentrations were also tested for formation and accumulation of lipid bodies. Results suggest that though phosphorus is an important element for structural and metabolic processes, its presence in the medium even at minimal levels can lead to increased biomass and lipid productivity despite reduced growth rate, thereby leading to an effective nutrient usage at large scale. Our findings also shed light on the mechanism of sub-cellular accumulation of lipid bodies at exponential growth phase, division of cells upon medium replenishment and fragmentation of lipid bodies on division. Thus, from our results, it can be stated, that a lower concentration of phosphorus in combination with an optimum lower concentration of nitrogen can be used efficiently in a medium at large scale, thereby increasing biomass productivity with improved nutrient usage. The results of this study can serve as useful reference for future research on lipid accumulation of various other species.

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