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ORIGINAL ARTICLE

Role of C/N ratio on microalgae growth in mixotrophy and incorporation of titanium nanoparticles for cell flocculation and lipid enhancement in economical biodiesel application

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Abstract
Present study aimed to evaluate the influence of carbon/nitrogen ratio (C/N) on mixotrophic growth of microalgae and role of nanomaterial in cell recovery and lipid improvement. In this study, three microalgae species were isolated, screened from local freshwater body for lipid assimilation. The microalgae were identified as *Chlorococcum sp., Scenedesmus sp.,* and *Euglena sp.* Mixotrophic cultivation of each microalgae strain using various organic carbon sources was preferred in contrast with photoautotrophic mode. Sucrose represented as the preeminent source for enhancing the microalgae biomass of 3.5 g/L and lipid content of 58.35%, which was a significant improvement as compared to control. Later, response surface methodology–central composite design (RSM–CCD), tool was employed to optimize the C/N ratio and demonstrated the maximum biomass production of 5.02 g/L along with the increased lipid content of 60.34%. Ti nanoparticles (Ti nps) were added to the culture for lipid enhancement in the stationary phase and biomass removal was performed by nanoparticle (np)-mediated flocculation technique. Optimized concentration of 15 ppm Ti nps determined the cell harvesting efficacy of 82.46% during 45 min of sedimentation time and 1.23-fold lipid enhancement was reported. Extracted lipid was converted to fatty acid methyl esters (FAME) by the process of transesterification and analyzed by gas chromatography–mass spectrometry (GC–MS). Characterization of FAME revealed the presence of 56.31% of saturated fatty acid (SFA) and 29.06% unsaturated fatty acids (UFA) that could be processed towards sustainable biodiesel production. Hence, our results suggested that integration of mixotrophic cultivation and Ti nps emerged as a new cost-effective approach for biomass and lipid enhancement in microalgae *Chlorococcum sp.*

Keywords Microalgae · Mixotrophic cultivation · Nanoparticles · Flocculation · Lipid · FAME

Introduction

The detrimental effect of global warming and energy crisis due to fossil fuel depletion has paved the substitutive way for the development of green energy production (Ansari et al. 2017). Among the biological resources, microalgae are considered as the most potential and renewable feedstock for feasible biodiesel. Microalgae possess photosynthetic efficiency by harvesting solar radiation and able to distribute the photonic energy towards cell division, allowing for rapid growth rate, results in high biomass production and large amount of oil content that has raised sustainable attention of researchers towards microalgae for biodiesel production (Hossain and Mahila 2019; Zhu et al. 2019; Khanra et al. 2018; Mondal et al. 2017). They offer high amount of carbohydrate and protein with zero lignin content that helps to improve the quality of biodiesel. Furthermore, microalgae

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carry high biomass characteristic index, energy density, and fuel value index that make them a potential source of biofuel feedstock for heat and electricity (Hossain et al. 2018). The calculated price of 10,000 tonnes of microalgae with 30% lipids is about $2.80/L that is comparatively higher than the petroleum fuel price ($1.10/L) (Chia et al. 2018). However, with the continuous escalating price of petroleum diesel, biodiesel produced from algae appears extremely attractive source as crude oil, even if the assumed capital and operating costs herein prove to be optimistic (Gallagher 2011). Hence, there is a need of emerging cost-effective methods of biofuel production from microalgae, mainly focusing on availability of suitable feedstock, easy biomass recovery, and high lipid yield.

The selection of indigenous and prospective oleaginous microalgal strain for producing lipids remains one of the crucial hurdles for commercial biodiesel production (Sehgal et al. 2019). Therefore, isolation and profiling of potential microalgae for their lipid productivity and the fatty acid composition became essential. Presently, the screening of lipid-rich microalgae and their mode of cultivation plays a vital role in achieving competitive biodiesel yield. It is well known that India has an adequate source of natural lakes with ample nutrients as well as enough solar radiation that provides a persuasive atmosphere for the abundant proliferation of microalgae. In this respect, the selection of algae from local water bodies is preferred. Naturally proliferating microalgae are usually accredited by several metabolic pathways for promoting cell growth and lipid assimilation. However, low biomass and lipid productivity in photoautotrophic mode are evolved as one of the major hurdles in the entire microalgae cultivation system. Therefore, mixotrophic conditions by utilizing organic carbon sources are benefitted over photoautotrophy for improved biomass and lipid yield (Sun et al. 2014).

In the mixotrophic regime, CO_2 and organic carbon are assimilated, where the photosynthesis and respiratory metabolism occur simultaneously. Wang et al. (2016) stated that the high C/N ratio improved the lipid accumulation in microalga. So, the effect of C/N ratio on microalgal photosynthetic system is highly influential, where the balanced ratio is required to achieve higher biomass. Statistical optimization of C/N under RSM can be a suitable technique for media optimization and to understand the interaction among variables to obtain maximum biomass and lipid production in microalgae. The RSM model allows the researchers to investigate the optimum conditions for several variables in single experiment. Further, CCD under RSM helps to optimize the estimation of a second-order model, allowing for reduced costs and time required during experimentation (Bartley et al. 2015).

Another major bottleneck associated with microalgae biodiesel production is the high cost of biomass harvesting and the lack of a suitable method for lipid enhancement. Hence, the blooming of a feasible integrated process is desired, which concomitantly attribute to the higher lipid productivity with subsequent cell removal. A number of conventional harvesting techniques have opted these days; however, most of them are either expensive or non-renewable and thereby, they are not favored for large-scale applications. To circumvent the problems, the concept of nanotechnology appeared to be the spotlight for improve biofuel production. Various nps such as ZrO_2, TiO_2, Al_2O_3, and SiO_2 have been explored for biofuel yield enhancement (Hossain et al. 2019). In a study of Garcia et al. (2018), iron oxide nps achieved the harvesting efficiency of > 95%, whereas TiO_2 in conjugation with amino clay (3 g/L) observed the biomass recovery of 85% at very high concentration for *Chlorella sp*. in the findings of Lee et al. (2014). Generally, such engineered nps have high surface to volume ratio, and they have positively charged cations. Hence, the interaction between positive charge metals and negative charge microalgae surface occurs strongly and, therefore, microalgae get flocculated. A few studies reported the enhancement in lipid accumulation by incorporating nps in the culture medium. Hua et al. (2016), observed a significant increase in lipid content of 23.4 ± 0.7 g-lipid g-algae^{-1} in microalgae *Scenedesmus dimorphus* by adding Ti_4O_7 nps. In another study, the lower dose of carbon nanotubes (CNTs), MgO, Fe_2O_3 nps improved the lipid productivity with 8.9, 39.6 and 18.5%, respectively, in *Scenedesmus obliquus*. (He et al. 2017). NiO nps found significant for the high lipid content (91.08%) in *Chlorella vulgaris* (Huang and Kim 2016).

Hence, the present study is contextualized about the key aspect of mixotrophic cultivation integrated with Ti nps to functionalize the lipid synthesis and cell recovery economically. Therefore, the major objectives of this study include (i) the optimization of C/N using CCD under RSM for enhanced biomass and lipid synthesis (ii) incorporation of Ti nps for sequential harvesting and improved lipid production. The fatty acid composition of the extracted lipids has been identified via GC–MS characterization for the possible application in biodiesel.

Methods and materials

Screening of microalgae and culture

The samples containing mixed algal communities were collected from a natural lake, located at New Delhi, India. Algal species were isolated via serial dilution technique and incubated in a growth chamber (28 °C, 16:8 h light: dark cycle, 100 µmol photons m^{-2} s^{-1} light intensity). Subsequently, the axenic cultures were obtained by culture media

supplementation by 1.5% agar provided with ampicillin and kanamycin. The detailed isolation and screening procedure were followed by Rai et al. (2018). The algal strains were cultured and cultivated in 250 ml Erlenmeyer flask, having 150 ml autoclaved Foggs, BG-11 and Hutner's medium for their adaptability in photoautotrophic mode (Rai and Gupta 2017; Khanra et al. 2017).

Morphological determination

The screened microalgal cells were determined morphologically using light microscopy (A1 Nikon Confocal). Additionally, scanning electron microscopy (SEM- Carl Zeiss microscope, SUPRA 40, Germany) was also used for morphological characterization. Neutral lipid droplets present in microalgae were determined via nile red staining, where the cells were stained during stationary phase of the cell growth and observed using confocal microscopy via laser light with excitation and an emission wavelength of 552 and 636 nm, respectively, (Jeyakumar et al. 2020).

Microalgae cultivation in photoautotrophic mode

Growth of the identified microalgae was determined by taking the dry cell weight (DCW) in their respective stationary phase. 2 ml of cultures were centrifuged at 10,000 × g for 8 min (Eppendorf 5810R), the sediment was dried in a hot air oven at 60 °C and gravimetrically weighed until the constant weight of the biomass was obtained. Biomass concentration, biomass productivity, and specific growth rate were calculated using following formulae (Khanra et al. 2017):

$$\text{Biomass concentration } (g/L) = W(g)/V(L), \quad (1)$$

$$\text{Biomass productivity}(g/L/d) = \text{Biomass concentration}\left(\frac{g}{L}\right)\Big/T(d), \quad (2)$$

$$\text{Specific growth rate}(\mu) = lnX_2 - lnX_1/T_2 - T_1, \quad (3)$$

where, W, V, and T were expressed as weight of dried biomass, sample volume, and time; X_2 and X_1 showed final and initial biomass concentration; T_2 and T_1 represent final and initial time, respectively.

The lipid extraction was carried out by Bligh and Dyer method (Bligh and dyer, 1959) using the chloroform and methanol in the ratio of 2:1. The estimation of lipids was calculated using the following formulae (Khanra et al. 2017):

$$\text{Lipid production } (g/L) = \text{mass of lipid}(g)/\text{Volume}(L), \quad (4)$$

$$\text{Lipid productivity } (g/L/d) = \text{mass of lipid}(g)/\text{Volume}(L) \times \text{cultivation time}(d), \quad (5)$$

$$\text{Lipid content}(\%) = \text{mass of lipid } (g)/\text{mass of culture}(g) \times 100. \quad (6)$$

Optimization of various nitrogen and carbon sources for biomass and lipid production

Various nitrogen sources like urea nitrogen, NO_3 nitrogen, NO_2 nitrogen and NH_4 nitrogen, and carbon sources including glucose, fructose, sucrose, cellobiose, sodium acetate, starch, glycogen, and glycerol were applied to the culture medium for the estimation of biomass as well as lipid biomolecules. The best nitrogen and carbon source selected from the experimental analysis were optimized for its most suitable concentration.

Experimental design using response surface methodology–central composite design (RSM–CCD)

The best nitrogen and carbon sources were selected for the optimization using RSM–CCD linear model design expert-11 (RSM–CCD) tool. The experiment was designed with nine runs under pH 7.0. The selected range for one factor was 0–5.0 g/L for sucrose concentration and 0–3.0 g/L for NO_3-N concentration as second factor. The variation in C/N ratio was optimized by RSM–CCD and responses were recorded as biomass production, lipid production, and lipid content.

Flocculation by adding Ti nps to the culture medium

Ti nps were applied at various concentrations (15, 30, 45, 60, 75, 90, and 105 mg/L) at the start of the stationary phase of the culture medium for observing the cell flocculation efficiency (Koley et al. 2017). The harvesting efficiency was observed at several sedimentation time after adding Ti nps to the microalgae culture. The microalgae cells could settle down along with nanoparticle and absorbance of the supernatant was taken at 540 nm. Lipid productivity was also measured after addition of Ti nps by Bligh and Dyer method prescribed earlier. Imaging of lipid globules was observed under fluorescence microscopy.

Flocculation efficiency was determined using the following formulae (Koley et al. 2017):

$$\text{Flocculation efficiency}(\%) = \left(1 - \frac{A}{B}\right) \times 100 \quad (7)$$

where A = final optical density and B = Initial optical density.

Tranesterification of oil to FAME and its characterization analysis

The extracted lipids were esterified to FAME by incorporating methanol and acid catalyst using standard transesterification protocol (Rai et al. 2013). The FAME extract was recovered using 1 ml of hexane and characterized by GC–MS (Shimadzu QP-2010 Plus with Thermal Desorption System TD 20) equipped with flame ion detector (FID). Supelco-37 FAME mix was used as an internal standard. The operation was set by supplying nitrogen as carrier gas with flow rate of 1.21 ml/min and injection temperature of 260 °C. The FAME peaks were identified, and the retention time was compared with authentic standard GC further quantified by normalization.

Results and discussion

Screening and morphological analysis of microalgae strains

Initially, three distinct pure microalgal strains were screened from freshwater site. All the strains were morphologically categorized by confocal and SEM to corroborate the topographical analysis. According to morphometric evaluation, the identified microalgae were *Chlorococcum sp*., *Scenedesmus sp*., and *Euglena sp.* (Fig. 1). All the aforesaid algal strains are chlorophycean in nature, except *Euglena sp.* which belongs to eugleneaceae family. The morphological characteristics of each microalga were described below:

Chlorococcum sp.

Morphological characterization of this particular microalgal strain demonstrated that the monoalga belongs to the genera chlorococcum and order chlorococcaceae. The microscopic (confocal) image of this particular strain exhibited the spherical shape having cell size as 3–6 µm (Fig. 1; row 1). This microalga had a parietal chloroplast with a single pyrenoid, surrounded at the opposite of the sphere. This structure is particularly designated as chlorococcoid (Fritsch 1948). Each coccoid bears a thin and inconspicuous mucilage layer. The SEM study also explored the presence of warts to vary the size of the cell wall.

Scenedesmus sp.

These microalgal cells were green, ellipsoidal, and crescent in shape belonging to order Sphaeropleales and family Scenedesmacea. The morphological characteristics of this particular candidate include the presence of ribs on the cell surface. The most remarkable feature of its cell-wall

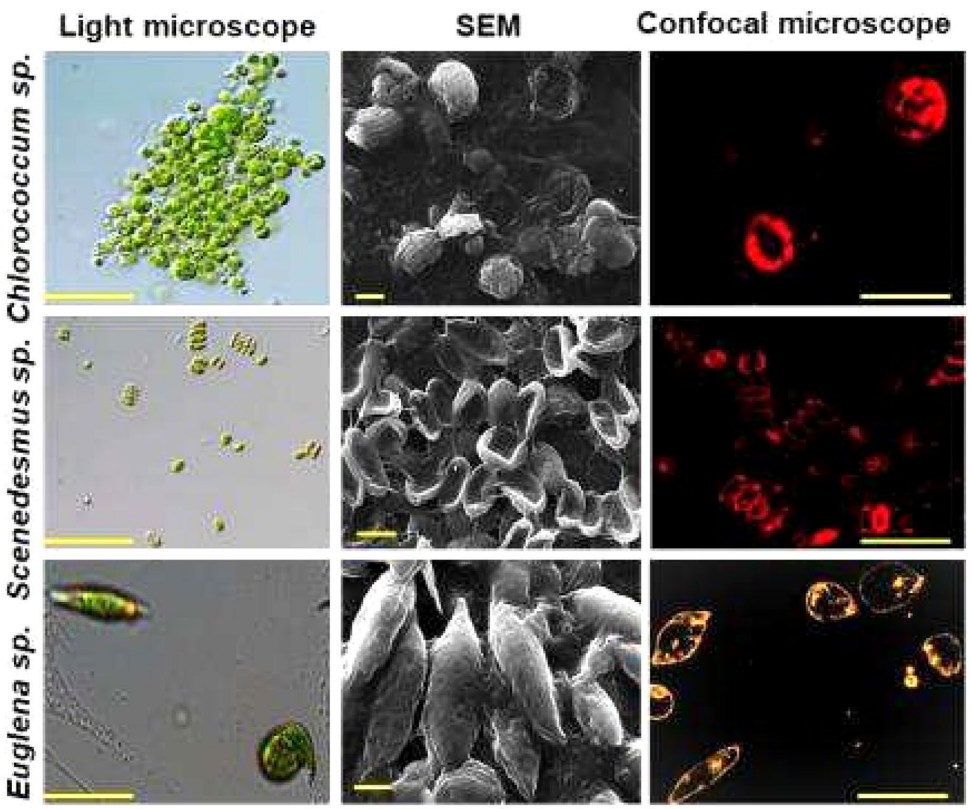

Fig.1 Light microscopic, SEM, and confocal microscopic via laser light images for the three microalgae strains; In row 1, row 2 and row 3, light microscopic (scale bar 10 µm), SEM (scale bar 2 µm) and confocal microscopic images (scale bar 10 µm) of *Chlorococcum sp., Scenedesmus sp.,* and *Euglena sp.* respectively

decoration is the continuous pattern of ribs and the absence of large warts which lie with the physical appearance of *Scenedesmus* as revealed by microscopic and SEM analysis (Fig. 1; row 2). The cells were heavily granulated and mostly organized in the form of tetrads, but single- and double-celled forms were also observed; such type of structural topography is known as coenobia (Fritsch 1948). The conical forms of this particular strain possess some spiny projections.

Euglena sp.

Morphometric arrangements of this monoalga exhibit that they are composed of an elastic axial thread, surrounded by a contractile envelope of alveolar cytoplasm from where the threads have emerged (Fig. 1, row 3). *Euglena sp.* belongs to the class euglenoideae, order euglenales and family euglenaceae. Literature depicted that a large number of these particular cells lack chromatophores, but in the case of holophyticeugleneace, there are existed mostly several or even numerous chloroplasts (Fritsch 1948). The paramylon grans present in each cell, originate in the cytoplasm. Many of them can store fat globules as food reserves. The flagella emerge from the canal and appear normal to the posterior end of the reservoir. The nucleus also existed in such strain is usually large and prominent and generated a central caryosome with a surrounding chromatin reticulum. The chromatin sometimes demonstrates a radial arrangement during the early prophase of division. SEM image of this particular strain revealed the average size of 2–3 µm.

Studies of cell proliferation and lipid analysis under photoautotrophy

The biomass yield and lipid synthesis of three isolated microalgal strains were explored primarily in Fogg's and Hutner's medium. *Chlorococcum sp*. and *Scenedesmus sp.* were cultivated in Fogg's medium while *Euglena sp.* grown in Hutner's medium, maintaining the culture conditions under photoautotrophic mode. The growth dynamic pattern of each microalga was demonstrated in Fig. 2a. According to this figure, *Chlorococcum sp*. attained highest cell density of 2.9 g/L, followed by 0.924 g/L in *Scenedesmus sp*, wherein, *Euglena sp*. had the lowest biomass production of 0.165 g/L on 16th day of culture. The algal growth was also studied by measuring the specific growth rate (µ) d^{-1} of three microalgae. It is defined as the increase in number of cells per unit time. The µ was found to be 0.286, 0.214, and 0.192 d^{-1} in *Chlorococcum sp*. *Scenedesmus sp.* and *Euglena sp*, respectively. The microalgal strains were also investigated for their lipid synthesis under similar kind of environmental conditions as shown in Fig. 2b. The maximum lipid production obtained by *Chlorococcum sp*. was found to be 1.02 g/L along with lipid content of 35.23%. On the other hand, *Scenedesmus sp.* achieved the lipid production and lipid content of 0.27 g/L and 29.38%, respectively. Wherein, *Euglena sp.* showed the maximum lipid production (0.035 g/L) and lipid content (21.23%) during stationary phase. Earlier reports suggested that lipid production in microalgae can be stimulated by several factors like composition of the growth medium, pH, temperature, light

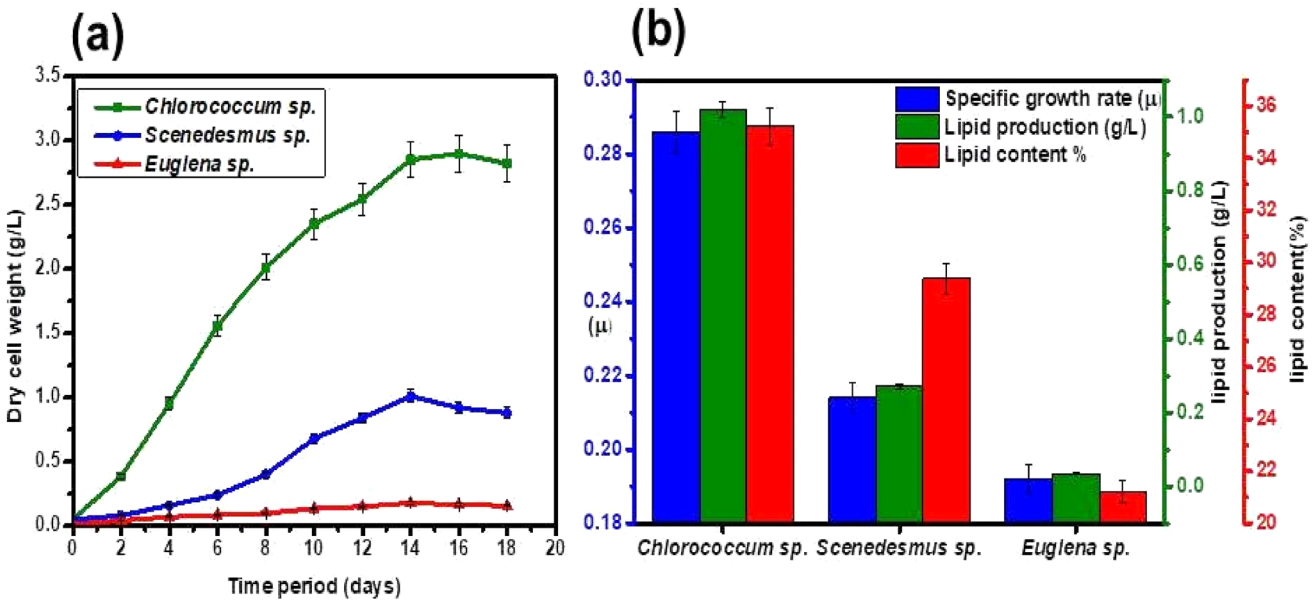

Fig. 2 Comparative growth and lipid assimilation analysis of *Chlorococcum sp., Scenedesmus sp.,* and *Euglena sp.*; **a** growth curve in photoautotrophic mode; **b** specific growth rate (µ), lipid production and lipid content

illumination, and the age of culture. It has also been notified that the lipid composition and production appeared to species oriented (Lin and Wu 2015).

The average lipid content determined for microalgal strains is in the range between 11–30% of the dry mass fraction. In the present study, *Chlorococcum sp., Scenedesmus sp.*, and *Euglena sp.* showed lipid assimilation > 20% at the stationary phase, hence they were regarded as high lipids producing strains.

Effect of different nitrogen sources on *Chlorococcum sp.* for biomass density and lipid production

To explore the biomass and lipid accumulation, *Chlorococcum sp.* has been selected owing to its maximum growth capacity and lipid production. Hence, the effect of different nitrogen sources was visualized on this particular strain. Nitrogen is recognized to be one of the most efficient nutrients for cell growth and lipid metabolism in microalgae. To elucidate the response of nitrogen sources on biomass density and lipid production, four different nitrogen sources were employed to the selected microalga strain under a photoautotrophic mode of culture condition.

According to Fig. 3, NO_3-N provided to the culture medium, demonstrated as favorable substrate for higher biomass production (2.8 g/L) than the cells grown with other nitrogen sources, whereas, the culture had lowest biomass productivity of 0.59 g/L/d in nitrogen free medium. Under ammonium and nitrite supplementation, the microalgae cells displayed a moderate cell density of 2.46 and 2.39 g/L, respectively. The possible explanation may be due to the high concentration of ammonium and nitrite nitrogen, which were a little bit toxic to the cell. The extreme transport of $NH4^+$ and $NO2^-$ ions to the microalga could hamper the ATP formation in chloroplast owing to the down

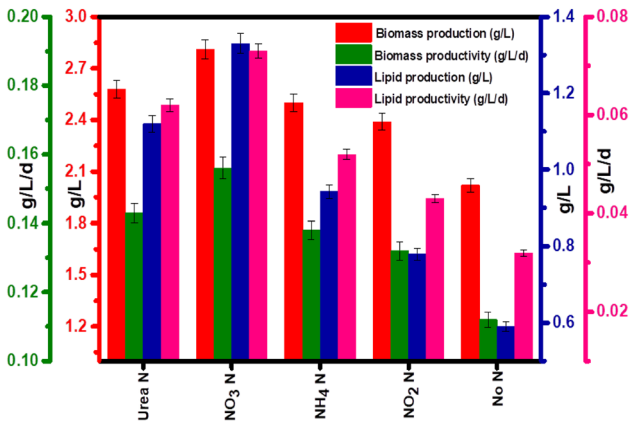

Fig. 3 Effect of various inorganic nitrogen sources on biomass production, biomass productivity, lipid production, and lipid productivity of microalgae *Chlorococcum sp*

regulation of photosynthesis while NO_3 transport is well controlled by the cell causing up-regulation of photosynthesis to generate maximum NO_3 influx (Feng et al. 2020).

Effects of the above-mentioned nitrogen sources on lipid accumulation were also estimated. Through a comprehensive analysis of lipid biomolecules, nitrate nitrogen treatment displayed the maximum lipid production of 1.33 g/L along with the lipid productivity of 0.094 g/L/d as compared with other sources. Lin and Lin (2011) also proved that NO_3-N was the best nitrogen source for enhanced lipid content up to 35% in *S. rubescens.* This is probably because of more metabolic flux generated by NO_3-N which was assimilated during photosynthesis, leading to high lipid accumulation in comparison with other N sources. On the other hand, the total lipid yield of urea-N was found lower than NO_3-N, higher than NO_2-N, NH_4-N and N deficient medium, respectively. This is likely since urea-N produces urease enzyme which helps in the formation of ammonium and CO_2 followed by urea amino hydrolase pathway. This additional CO_2 (except atmosphere CO_2) is segregated from HCO_3- or CO^{2-} ions to produce more carbon flux towards lipid synthesis and generate more growth (Chen et al. 2020). However, in the current study, N-deficient medium had the lowest lipid production, which was plausibly due to the inhibitory effect of cell growth causing inefficiency for high lipid yield in contrast with N- added medium. In a study of Feng et al. 2020, the lipid productivity and lipid concentrations of *Chlorella sp*. GN1 under nitrogen restriction conditions were found significantly lower than those obtained under nitrogen sufficient conditions. It might be anticipated that the algal cell growth was limited under nitrogen deficient condition in contrast to nitrogen sufficient medium. This phenomenon leads to limited biomass production, resulting in low lipid productivity in microalgal cells. Many previous studies had such conclusions (Hu et al. 2019; Nayak et al. 2019). Tan et al. (2016) has explained in their work that microalgae *D. tertiolecta* have ability to survive in hostile environmental changes, as the energy deposits can easily be organized when growth conditions are re-established. Lipid accumulation in N-starved cells strictly depends on carbon augmentation. It was stated that under N depletion condition central carbon metabolism (CCM) play major role. Hence, genes in the (CCM) pathways, particularly those of the tricarboxylic acid cycle (TCA), were simultaneously up regulated, indicating a robust interchange of carbon skeletons for metabolic processes and so prefers starch synthesis. In contrast, fatty acid and triacylglycerol synthesis genes were down regulated, suggesting that lipids are not a preferred form of storage in these cells during N-starved conditions.

Adaptation of isolated microalgae in mixotrophic growth condition

Microalga strains were cultivated in mixotrophic mode by utilizing glucose as primary carbon source offers higher growth and lipid production in contrast to photoautotrophic mode (Yu et al. 2020). Maximum biomass productivity of 0.395 g/L/d was achieved in *Chlorococcum sp.* as compared to *Scenedesmus sp.* (0.18 g/L/d) and *Euglena sp* (0.1 g/L/d) (Fig. 4). In addition, 1.2-fold and 1.53-fold enhancement of lipid content was obtained in *Chlorococcum sp.* when compared with *Scenedesmus sp*. and *Euglena sp*, respectively. Under mixotrophic condition, monosaccharide glucose is first converted to glucose 6 phosphate and successively to pyruvate through glycolysis, afterwards enters TCA cycle followed by mitochondrial oxidative phosphorylation for ATP production. Indirectly, glucose acts as a substrate for triacylglycerol (TAG) synthesis and simultaneously increases the lipid production. In addition, the incorporation of carbon source significantly increases the acetyl CoA/malonyl CoA pool, which symbolizes central carbon donor for fatty acid synthesis, possibly it promotes the lipid synthesis (Yu et al. 2020; Sharma et al. 2016).

Response of various organic carbon sources on cell growth and lipid production

In mixotrophic mode of cultivation, different varieties of organic carbon sources including monosaccharides, disaccharides, and polysaccharides were induced for growth and lipid assessment on selected algae. According to Fig. 5, the growth rate of *Chlorococcum sp.* was promisingly higher in presence of glucose-, fructose-, and sucrose-mediated culture in 8 days. However, sucrose-mediated cultivation demonstrated the maximum biomass production of 3.5 g/L among others. In addition, the lipid synthesis in

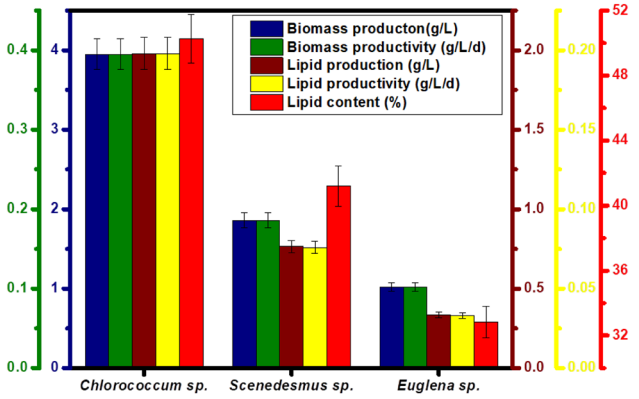

Fig. 4 Biomass composition and lipid analysis of *Chlorococcum sp, Scenesdesmus sp and Euglena sp* grown under mixotrophic mode using glucose

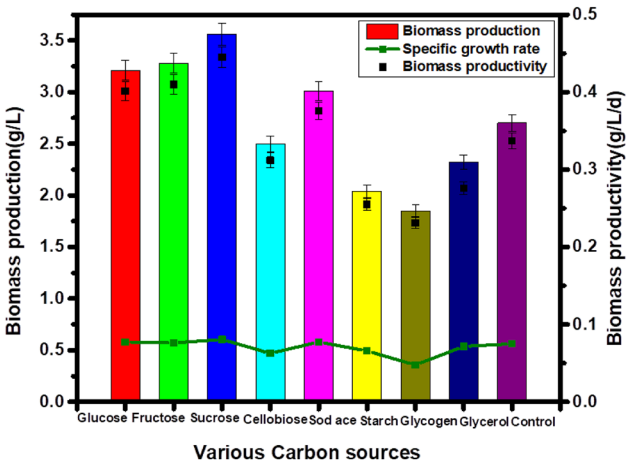

Fig. 5 Effect of various organic carbon sources on biomass production, biomass productivity, and specific growth rate of *Chlorococcum sp.* cultured under mixotrophic mode

Chlorococcum sp. using the different organic carbon sources was represented in Table 1. According to Table 1, sucrose induced culture medium attributed maximum lipid production of 2.02 g/L along with lipid content of 58.35%. The possible reason could be explained by the fact that sucrose can be easily decomposed to yield a glucose molecule and a fructose molecule and, hence, microalgae have the capacity for absorbing directly sucrose by modulating their intracellular metabolism (Jiang et al. 2015).

Statistical optimization of C/N for yield enhancement

The optimization of carbon source sucrose and nitrogen source NO_3-N was evaluated for the increase in biomass and lipid analysis with the help of RSM-CCD quadratic model. In view of this model, the derived equations are as follows:

Table 1 Lipid production and lipid content of *Chlorococcum sp.* in mixotrophic mode using various organic carbon sources

Organic carbon sources	Lipid production (g/L)	Lipid content (%)
Glucose	1.81	54.67
Fructose	1.44	49.29
Sucrose	2.02	58.35
Cellobiose	1.19	47.60
Sodium acetate	1.53	51.78
Starch	1.13	45.59
Glycogen	1.09	45.04
Glycerol	1.16	45.69
Control	1.07	41.14

$$\text{Biomass}\,(g/L) = 5.02 + (0.264 \times A) + (0.5835 \times B) + (0.3125 \times AB) - (1.03 \times A^2) - (1.04 \times B^2), \quad (8)$$

$$\text{Lipid}\,(g/L) = 3.18 + (0.239 \times A) + (0.5308 \times B) + (0.2395 \times AB) - (0.9738 \times A^2) - (0.8977 \times B^2), \quad (9)$$

$$\text{Lipid content}\,(\%) = 63.34 + (3.58 * A) + (9.28 \times B) + (0.1150 \times AB) - \left(11.66 \times A^2\right) - \left(9.73 \times B^2\right), \quad (10)$$

where, A, B are the concentration of sucrose and nitrate, respectively, the positive symbol indicates the synergistic effects and showing more interaction towards response, whereas negative symbol designates the antagonistic effects and thus less interaction towards the response was observed (Anahas and Muralitharan 2019).

According to Table 2, 2.5 g/L sucrose and 1.5 g/L nitrate demonstrated the maximum biomass of 5.02 g/L along with the lipid content of 60.34%. On the other hand, an excess amount of sucrose may compromise the cell growth due to the perturbation of whole enzymatic system.

The graphical representations in the form of 3D response surface plots are constructed by plotting the relative effect of two experimental factors on the response i.e. sucrose and

Table 2 Biomass production, lipid production, and lipid content of *Chlorococcum sp.* using different concentrations of sucrose and NO3-N

Run order	Factor 1 Sucrose (g/L)	Factor 2 NO_3-N (g/L)	Biomass production (g/L)	Lipid production (g/L)	Lipid content (%)
1	4.27	0.43	2.51	0.97	38.09
2	4.27	2.56	4.79	2.62	54.86
3	2.5	0	2.33	0.71	30.56
4	0	1.50	2.90	1.02	35.23
5	2.5	3.00	3.29	1.77	54.01
6	5	1.50	2.76	1.14	41.59
7	2.50	1.50	5.02	3.02	60.34
8	0.73	2.50	3.01	1.46	48.79
9	0.73	0.43	1.98	0.56	28.48

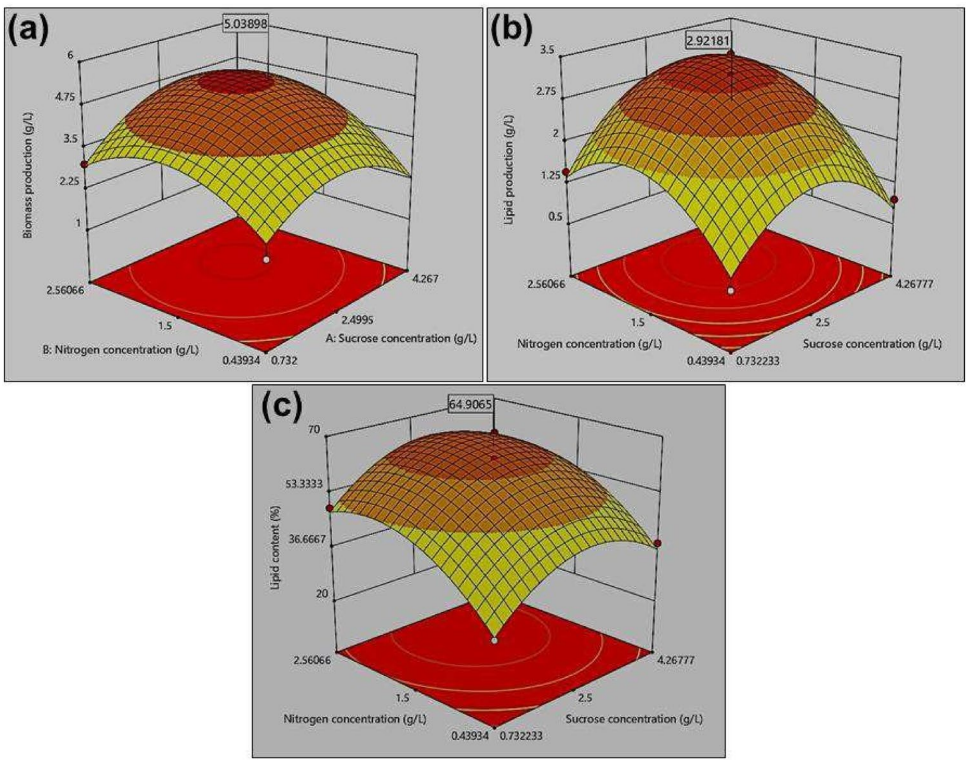

Fig. 6 Three-dimensional surface (3D) plots showing interaction between nitrogen and sucrose and its effect on **a** biomass production (g/L) **b** lipid production (g/L) **c** lipid content (%) using RSM–CCD model

nitrate (Fig. 6). Figure 6a shows the moderate concentration of sucrose (2.5 g/L) and nitrate (1.5 g/L) increased the biomass production as 5.02 g/L experimental value and 5.03 g/L as predicted value indicating major portion of carbon has paved the path towards cell density. The interactive effect of sucrose and nitrate on lipid production was observed in Fig. 6b, indicating the experimental value and predicted value as 3.02 and 2.92 g/L, respectively, those are very close figures. Furthermore, Fig. 6c represents lipid content having experimental value of 60.34%, whereas the predicted value was observed 64.90%. It was noticed that the model predicted values from the RSM–CCD satisfactorily matched with experimental values for biomass production, lipid production and lipid content.

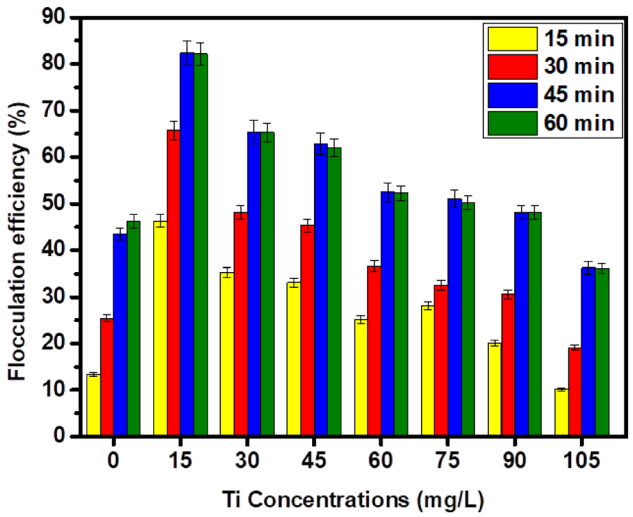

Fig. 7 Effect of various dosage of Ti nps on flocculation efficiency of microalgae *Chlorococcum sp* at different sedimentation time

Microalgae cell harvesting and lipid extraction efficacy using Ti nps

The role of advanced engineering-based nps spurred the researchers towards microalgae harvesting and lipid extraction. The present study (Fig. 7) revealed that the harvesting efficiency of 15 ppm concentrations of Ti nps were achieved at 82.46%, whereas, the control (without nanoparticle) cells were demonstrated as 46.22% flocculation efficacy during 45 min of sedimentation time. This phenomenon could be explained by the strong electrostatic attraction of positively charged Ti nps with negatively charged microalgal cell surface, addressing towards adsorption. It was observed that lower dose of Ti nps expressed better flocculation efficiency compared to higher doses. At higher concentration, nanoparticles get coagulated formed aggregates and hence availability of nanoparticles to the cells decreased. It is well known that particles above their critical coagulation concentration (CCC) get attracted by each other following DLVO theory and so agglomeration of the particles occur. CCC depends on the size, shape, electrostatic charge, and outer environment of the particle (Hsu and Liu 1998).

Figure 8a indicated the morphological distribution of Ti nps, where it was clearly shown about the uniformity of Ti nps. In addition, Fig. 8b represented that the nps were adsorbed onto the cell surface. To express the lipid determination, Ti np-mediated cell culture indicated about 1.23-fold enhancement in contrast with control, as shown in Table 3.

Table 3 Comparative analysis of lipid content in *Chlorococcum sp* treated with Ti nps and control (untreated cells)

Name of the condition	Lipid content (%)
Ti mediated algae culture	74.29
Control	60.34

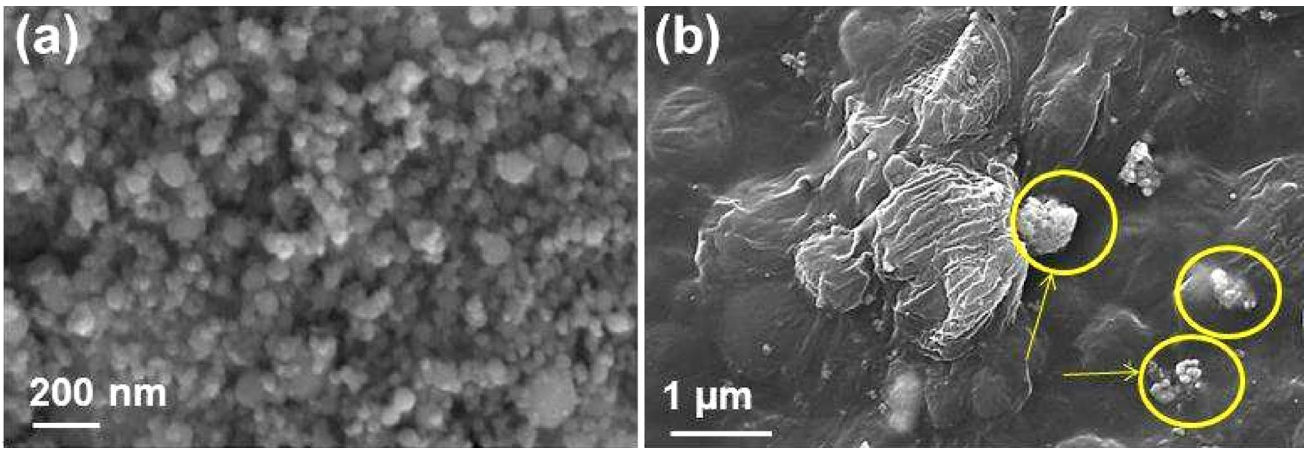

Fig. 8 Morphological characterization: **a** SEM image of Ti nps; **b** SEM image showing attachment of Ti nps onto the algae cell surface

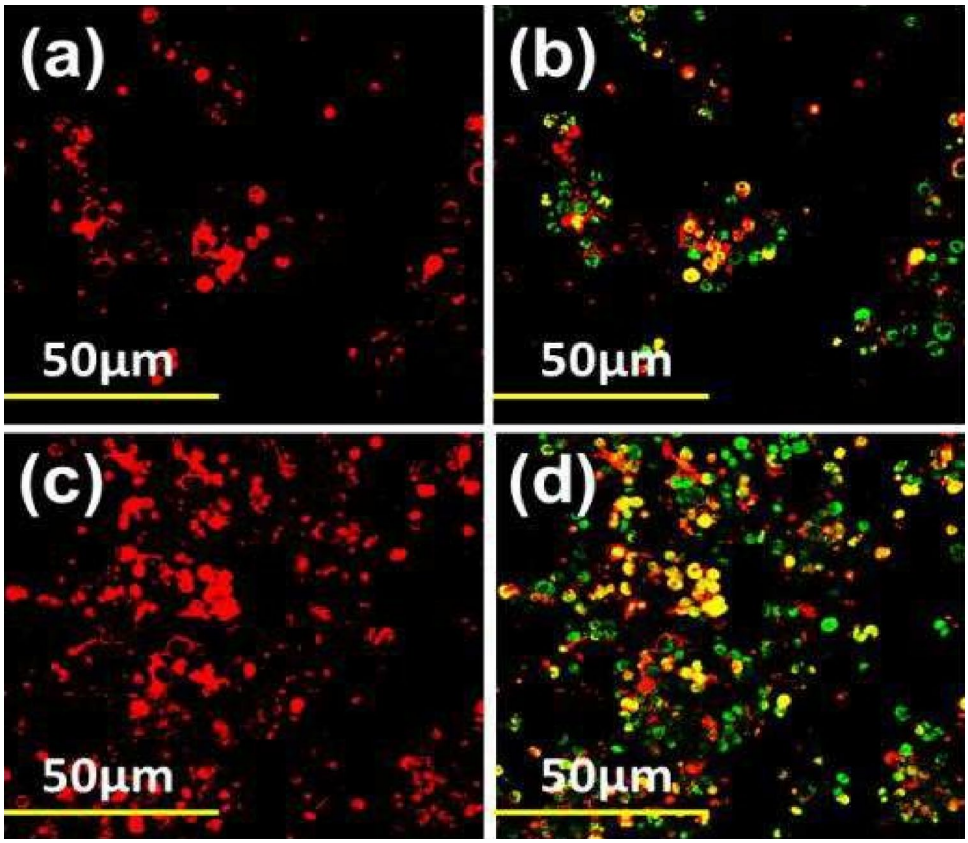

Fig. 9 Intracellular lipid globules analysis in *Chlorococcum sp*. by confocal microscope, **a** and **b** nile red stained image of control cells, **c** and **d** cells treated with Ti nps. Note: different filters were used, showing lipid droplets as red in color, whereas yellow droplets representing the merging of green and red

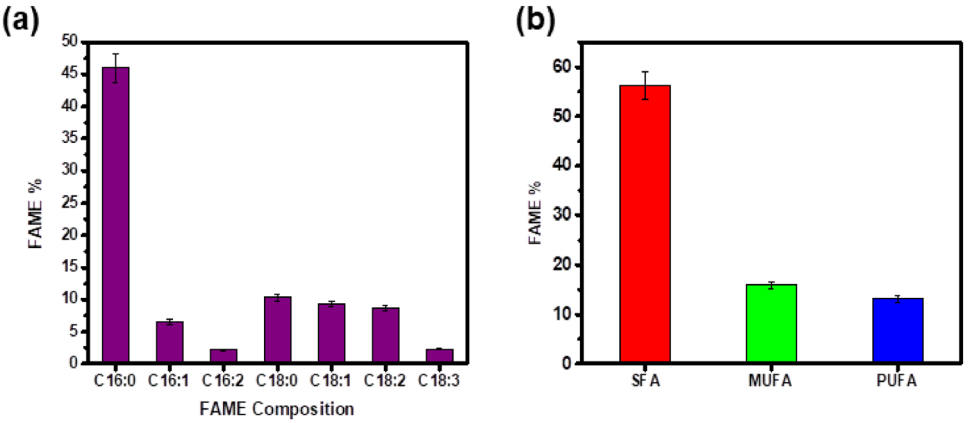

Fig. 10 FAME composition analysis in *Chlorococcum sp*; **a** Percentage of FAME components present in *Chlorococcum sp*. **b** cumulative amount of SFA, MUFA, and PUFA present in FAME extract of *Chlorococcum sp*;

This comparison was clearly visualized with the fluorescence images of microalgae, treated with and without nps, as Fig. 9. Hence, we believe that Ti nps have a great catalytic activity which leads for better cell lysis.

FAME production and characterization

The increased lipid content that was obtained from *Chlorococcum sp*. under sucrose-mediated mixotrophic culture condition, assessed for FAME production. In view of FAME profile, represented in Fig. 10a, palmitic acid (C16:0) existed at the maximum amount of 46.01% of total FAME components. In addition, this microalga had a maximum amount of total saturated fatty acid (SFA) of 56.31%, followed by 15.90% and 13.16% of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), respectively (Fig. 10b). The balancing ratio of this SFA, MUFA, and PUFA ensures the microalgae derived oil applicable to biodiesel application (Arora et al. 2017). It was suggested from our current findings that the obtained FAME profile was promisingly suitable for requisite biodiesel production.

Therefore, the above discussed results provide insight into the application of nanomaterials as they offer high efficiency and yield in microalgae technology. Nanomaterials in microalgae cultivation not only augment the biomass but also accumulate carbon-based compounds making the process economically viable (Nguyen et al. 2019). Hence, it is strongly believed that applying nanomaterials at various stage of microalgae culture to the final product, has a strong possibility to improve the economic feasibility.

Conclusion

The present report emphasized the effect of C/N ratio using organic carbon sucrose and NO_3-N on microalgae *Chlorococcum sp.* biomass and lipid production. A remarkable increment of 1.73-fold in biomass production and 1.71-fold in lipid content was achieved in the presence of RSM–CCD optimized concentrations of sucrose (2.5 g/L) and sodium nitrate (1.5 g/L) treated medium compared to photoautotrophy. Ti nps were incorporated in the medium and found an effective flocculant as they have given maximum cell harvesting efficiency of 82.46% in the presence of 15 ppm concentrations and sedimentation time of 45 min. Further, the increased lipid productivity was obtained in the presence of Ti nps and fluorescence images also supported significant improvement in lipid. Hence, these findings could make our preferred microalga, capable to use the optimized C/N ratio for biomass production and Ti nps for subsequent lipid synthesis, making it viable feedstock for biodiesel application. Industrial waste effluent enriched in sucrose could serve as an economical nutrient source for large-scale production of biodiesel. Furthermore, the energy consumption, production cost, and recycle of nanomaterial in regard to microalgae biorefinery process need to be addressed in future.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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