Phase-feeding strategy for *Chlorella vulgaris* to enhance biomass and lipid productivity

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Abstract: A phase-feeding strategy of nutrients based on requirements of *Chlorella vulgaris* in different physiological phases was examined to maximize the biomass and lipid productivity. This strategy includes reduction of duration in adaption phase (stage-I), enhancement of biomass in growth phase (stage-II) and improvement of lipid productivity in stationary phase (stage-III). The duration of microalgae in adaption phase was reduced from 52 h to 34 h at nitrogen and phosphorus feeding rates of 5.11 mg/(L·d) and 0.54 mg/(L·d), whereas the maximum biomass concentration during growth phase was improved to (4.03±0.25) g/L at nitrogen and phosphorus feeding rates of 20.04 mg/(L·d) and 4.21 mg/(L·d). In stationary and decline phases, a maximum lipid productivity of 132.30 mg/(L·d) was achieved when nutrients supply was stopped at 128 h, which was 28.86 mg/(L·d) higher than that when nutrients supply was stopped at 104 h. This multi-phase cultivation could be a promising strategy for simultaneous enhancement of microalgae biomass and lipid productivity.

Keywords: cultivation strategy, lipid productivity, microalgae, nutrients regulation

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1 Introduction

With respect to the rapid depletion of fossil fuels and ever-increasing atmospheric CO_2 concentration, microalgae has been extensively studied in recent years, as they can capture CO_2 whilst producing biofuels, simultaneous alleviating energy and environmental pressures[1,2]. In addition, microalgae can be cultivated on non-arable cropland at a growth rate of ten or more times higher than conventional oil crops, such as soybean, sunflower and palm[3]. Among all microalgae-based energy conversion approaches (such as biodiesel, biohydrogen and bioethanol), the most common one is biodiesel production[4]. However, the productivity of biodiesel from microalgae is still too low for commercialization because high lipid contents and robust algae growth are usually mutually exclusive[5]. During past decades, poor lipid contents were observed when microalgae were cultivated in nutrition-rich medium. For example, *Scenedesmaceae* sp. and *Chlorella* sp. contained less than a half of oil when cultivated in nutrition-rich conditions compared with nutrition-poor conditions[6,7]. On the other hand, it was found that many high lipid-accumulating microalgae cultures are

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associated with slow growth rates under nutrients (mostly nitrogen and phosphorus) limited conditions, causing little improvement or even a decrease in overall lipid productivity[8,9].

It is known that microalgae cultivation mainly contains four physiological phases: adaption, growth, stationary and decline phase, and each with a different nutrient requirement[10]. More specifically, adaption phase is a period for microalgae cells to adapt to a new environment. A suitable inoculating environment with low nutrients concentration is beneficial for microalgae cells to shorten the duration of adaption phase, which is essential to improve the productivity of microalgae biodiesel[11]. Growth phase is a period for algae cells to accumulate biomass, in which sufficient nutrients is necessary to maximize biomass accumulation[12]. In stationary and decline phases, biomass concentration has reached a peak point thus needs little nutrients. Limitations of some essential nutrients (e.g., nitrogen and phosphorus) in these phases are beneficial for microalgae cells to induce cellular carbon flux from protein synthesis to lipid synthesis, which can significantly improve the overall lipid productivity[1]. Therefore, a stepwise nutrients feeding strategy considering the nutrients requirements by microalgae in different physiological phases was expected to simultaneously enhance microalgae biomass accumulation and lipid synthesis.

Recently, it was demonstrated that a two-stage cultivation strategy can simultaneously tune algae growth and lipid accumulation[5,13-15]. In the two-stage cultivation strategy, microalgae are first cultivated in nutrients sufficient conditions to produce high biomass concentration. Once certain biomass concentration reached, the microalgae cells were harvested by centrifuging and re-suspended into nitrogen-deficient medium for lipid accumulation. Mujtaba et al.[13] reported that the lipid productivity of *Chlorella vulgaris* increased from 31.5 mg/(L·d) to 77.1 mg/(L·d) using the two-stage cultivation strategy. However, the nutrients supply amount in the two-stage cultivation strategy did not follow nutrients requirements of microalgae in different phases and the adaption phase not taken into consideration in the previous studies. Moreover, the methods of transferring algae cells from nutrition-rich to nutrition-depletion medium in previous studies (mainly by centrifuging) are energy-intensive, operation-discommodious and possibly companied by microbial contamination[5].

To further enhance the economical feasibility of microalgae biodiesel, a continuous stepwise nutrients feeding strategy using an novel anion-exchange-membrane photobioreactor (AEM-PBR) was proposed. In the AEM-PBR, nutrients continuously permeated from nutrients feeding medium to microalgae cultivating medium through an anion-exchange-membrnae. The strategy considered the adaption phase of microalgae growth and comprehensively followed the nutrients demands by microalgae in different phases. In addition, the energy cost and possibility of microbial contamination by transferring algae cells from nutrition-rich to nutrition-starvation medium is altered by using the AEM-PBR. In the three-stage cultivation strategy, a small quantity of nutrients (nitrogen and phosphorus) were first continuously supplied in adaption phase (stage-I) to expedite to the adaption to new environment. Subsequently, the feeding amounts of nutrients increased in growth phase (stage-II) to enhance biomass accumulation. Finally, the nutrients feeding was stopped in stationary and decline phases to create a nutrients-starvation environment for lipid synthesis. Results showed that biomass accumulation and lipid synthesis were synergistically improved by the introduction of the multi-phase cultivation strategy.

2 Materials and methods

2.1 Microorganisms and culture conditions

The microalgae *C. vulgaris* (FACHB-31) was purchased from the Institute of Hydrobiology, Chinese Academy of Science (Wuhan, China). All the experiments were performed at a constant temperature of (25±0.5)°C. Continuous illumination was provided with fluorescent lamps at 7000 lx from single side of the PBRs. CO_2 enriched gas (5% CO_2) was bubbled into cultures through two stainless steel tubes (with orifice of 1 mm) at a gas flow rate of 300 mL/min. Mass flow meters (FMA-2606A, Omega, Switzerland) were used in this work to control the flow rates of CO_2 and air. The initial inoculum density of culture was 0.022 g/L.

2.2 Anion exchange membrane photobioreactor

Figure 1 shows the schematic of AEM-PBR, which has been described in our previous study[16]. The cultivating chamber was sandwiched by two nutrient feeding chambers, which were separated by anion exchange membranes (AMI-7001, AMFOR INC. USA). The effective working volumes of cultivating chamber and feeding chamber were 1600 mL and 500 mL, respectively. In the AEM-PBR, the nutrients (primarily N and P) permeated into the cultivating chambers from feeding chambers under the concentration difference of nutrients.

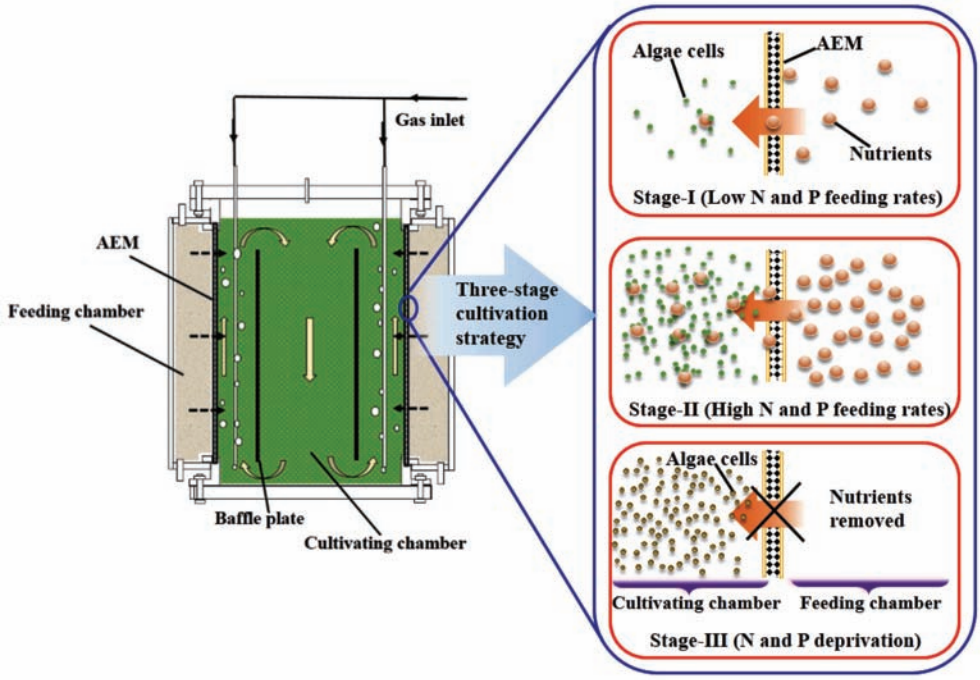

Figure 1 Schematic of anion exchange membrane photobioreactor and the process of three-stage cultivation strategy[16]

2.3 Culture medium composition in cultivating chamber

For all experiments, Brostol's solution (also known as SE medium)[17] devoid of nitrate and phosphate was adopted as culture medium in cultivating chamber. The modified Brostol's solution contains 0.075 g $MgSO_4 \cdot 7H_2O$, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.025 g NaCl, 0.005 g $FeCl_3 \cdot 6H_2O$, 1 mL EDTA-Fe, 1 mL A5 solution and 40 mL of soil extract water in 1 L deionized water. Nitrogen (N, in the form of nitrate, NO_3^-) and phosphorus (P, in the form of orthophosphate, PO_4^{3-}) continuously permeate into culture medium through AEM to satisfy the growth of microalgae. The feeding rates of N and P were regulated based on the growth requirements of microalgae cells in different physiological phases.

2.4 Optimization of N and P feeding rates for adaption phase, growth phase, stationary and decline phase

Considering the inhibiting effects of excessive nutrients (N and P, etc.) on low inoculum density of microalgae in adaption phase (stage-I), suitable nutrients concentrations are important to reduce duration of this period. Therefore, feeding rates of N and P with relatively low levels were set in stage-I to obtain optimum conditions. Pre-experiments were conducted to estimate the permeating rates of N and P from feeding chambers (with medium containing known N and P concentration) to cultivating chamber (with SE medium devoid of N and P). Then six experimental groups with different N feeding rates (2.06 mg N/(L·d), 5.11 mg N/(L·d) and 10.31 mg N/(L·d)) and P feeding rates (0.27 mg P/(L·d) and 0.54 mg P/(L·d)) in all combinations were conducted (as shown in Table 1) for stage-I. They were denoted as N-2.06&P-0.27, N-5.11&P-0.27, N-10.31&P-0.27, N-2.06&P-0.54, N-5.11&P-0.54 and N-10.31&P-0.54 for convenience.

According to the study by Mattos et al.[10], when microalgae biomass concentration increased to eight times of the initial inoculum concentration, it is regarded that the culture had entered growth phase (stage-II). To

optimize N and P feeding rates for stage-II, newly inoculated cultures (same initial inoculum density with stage-I) were first cultivated in the aforementioned optimal N and P feeding rates for stage-I. Once these cultures reached stage-II, the feeding mediums were replaced by new feeding mediums containing higher concentration of N and P. Thus, the feeding rates of N and P were improved to higher levels (with 10.31 mg N/(L·d), 20.04 mg N/(L·d), 30.12 mg N/(L·d) for N and 2.15 mg P/(L·d), 4.21 mg P/(L·d) for P, respectively) in all combinations (as shown in Table 2). For convenience, these six groups were denoted as N-10.31&P-2.15, N-20.04&P-2.15, N-30.12&P-2.15, N-10.31&P-4.21, N-20.04&P-4.21 and N-30.12&P-4.21, respectively, in latter discussion. To avoid the decline of N and P permeating rates caused by N and P concentration decrease in feeding medium during cultivation process, the feeding medium was refreshed once N and P concentration decrement in feeding medium reached 10% of total N and P concentration.

Table 1 N and P concentration in feeding mediums and the corresponding feeding rates in stage-I

Experimental group*	N concentration in feeding medium /mg·N·L^{-1}	N permeating rate /mg·N·L^{-1}·d^{-1}	P concentration in feeding medium /mg·P·L^{-1}	P permeating rate /mg·P·L^{-1}·d^{-1}
N-2.06&P-0.27	54	2.06	30	0.27
N-2.06&P-0.54	54	2.06	56	0.54
N-5.11&P-0.27	120	5.11	30	0.27
N-5.11&P-0.54	120	5.11	56	0.54
N-10.31&P-0.27	220	10.31	30	0.27
N-10.31&P-0.54	220	10.31	56	0.54

Note: * N-2.06&P-0.27, N-2.06&P-0.54, N-5.11&P-0.27, N-5.11&P-0.54, N-10.31&P-0.27 and N-10.31&P-0.54 represent six possible combinations of nitrogen feeding rates (2.06 mg N/(L·d), 5.11 mg N/(L·d) and 10.31 mg N/(L·d)) and phosphorus feeding rates (0.27 mg P/(L·d)and 0.54 mg P/(L·d)).

Table 2 N and P concentration in feeding mediums and the corresponding feeding rates in stage-II

Experimental group*	N concentration in feeding medium /mg·N·L^{-1}	N permeating rate /mg·N·L^{-1}·d^{-1}	P concentration in feeding medium /mg·P·L^{-1}	P permeating rate /mg·P·L^{-1}·d^{-1}
N-10.31&P-2.15	220	10.31	212	2.15
N-10.31&P-4.21	220	10.31	410	4.21
N-20.04&P-2.15	415	20.04	212	2.15
N-20.04&P-4.21	415	20.04	410	4.21
N-30.12&P-2.15	607	30.12	212	2.15
N-30.12&P-4.21	607	30.12	410	4.21

Note: * N-10.31&P-2.15, N-10.31&P-4.21, N-20.04&P-2.15, N-20.04&P-4.21, N-30.12&P-2.15 and N-30.12&P-4.21 represent six possible combinations of nitrogen feeding rates (10.31 mg N/(L·d), 20.04 mg N/(L·d) and 30.12 mg N/(L·d)) and phosphorus feeding rates (2.15 mg P/(L·d) and 4.21 mg P/(L·d)).

For enhancement of lipid synthesis in stationary and decline phases (stage-III), N and P feedings were stopped to create a nutrients-starvation environment and the different ceasing time of nutrients supply were investigated to explore the optimal nutrients feeding strategy for enhancement of lipid productivity.

2.5 Analytical methods

2.5.1 Determination of biomass concentration

Biomass concentration of microalgae *C. vulgaris* FACHB-31 was determined using gravimetric method. A 10 mL of culture medium was centrifuged (GL-21M, Xiangyi Instrument, Hunan, China) at 8000 r/min for 8 min. The obtained microalgae pellet was washed twice with deionized water and dried at 105°C until constant weight was obtained.

The maximum biomass concentration and the maximum specific growth rate of *C. vulgaris* were obtained by fitting the evolution of microalgae biomass concentration with the Verhulst logistic kinetic model[18,19], as shown in Equation (1):

$$\frac{\mathrm{d}X}{\mathrm{d}t}=\mu_{\max}\left(1-\frac{X}{X_{\max}}\right)X \tag{1}$$

where, dX/dt represents the growth rate of microalgae, g/(L·d); $\mu_{\max}$ represents the maximum specific growth rate, d^{-1}; $X_{\max}$ and X denote the maximum biomass concentration and real biomass concentration at time t, g/L, respectively.

Biomass productivity of *C. vulgaris* was calculated with Equation (2) according to Xia et al.[5]:

$$P=\frac{X-X_0}{t} \tag{2}$$

where, P is the biomass productivity of microalgae, g/(L·d); t is the cultivation time, d; X and X_0 are the biomass concentration at time t and at the beginning of the experiment, g/L, respectively.

2.5.2 Determination of nutrients concentration

To estimate nutrients concentration, 5 mL of microalgae culture was centrifuged at 8000 r/min for 10 min with centrifuge (GL-21M, XiangYi Instrument, Hunan, China). Then the supernatant was collected and injected into ion chromatograph (ICS-5000, ThermoFisher, USA) to detect ion concentration. Anion analytical column (4×250 mm, AS11-HC, ThermoFisher, USA) and self-regenerating suppressor (4 mm, ASRS 300,

ThermoFisher, USA) were used for ion concentration determination.

Nutrient consumption rate for each cultivation phase was calculated with Equation (3):

$$\gamma = \frac{S_{tot} - S_{res}}{t} \quad (3)$$

where, γ denotes the consumption rate of the nutrient, mg/(L·d); S_{tot} denotes the total feeding amount of the nutrient from feeding medium to cultivating medium during the cultivation process, mg/L; S_{res} denotes the residual amount of the nutrient in culture medium at the end of cultivation process, mg/L; t denotes the lasting time of the cultivation process, d.

2.5.3 Determination of lipid content and fatty acid methyl esters (FAMEs) composition

Total lipids were gravimetrically quantified using the method proposed by Bligh and Dyer[20] with some modifications. Briefly, 100 mg of dry algae powder was mixed with 7.6 mL of chloroform/methanol/water (1:2:0.8, v/v/v), ultrasonicated for 30 min at 100 W with ultrasonic cell crusher (JY92-2D, Xinzhi Instrument Ltd., NingBo, China), and then vibrated for 1 h at ambient temperature of 35°C in water bath. Then the mixer was centrifuged at 6500 r/min for 5 min to collect the supernatant, and the residual biomass was extracted twice more. Chloroform (6 mL) and water (6 mL) were added to the combined supernatant to ensure a final volume ratio of 1:1:0.9 (chloroform/methanol/water). The chloroform phase was carefully transferred to a new tube and evaporated over 24 h in a drying oven at 60°C. Finally, total lipid was gravimetrically measured. The lipid composition was analyzed as FAMEs by acidic transesterification of the lipids extracted previously[21]. The FAMEs were dissolved in hexane with nonadecanoic acid (C19:0) as an internal standard. After then, the mixture was incubated at 85°C for 2.5 h, and 1 μL of the mixture after incubation was injected into gas chromatograph (GC-2000, Shimazu, Japan) for determination of individual FAMEs.

3 Results and discussion

3.1 Nutrients-moderate condition to reduce duration of adaption phase in stage-I

Adaptation phase (stage-I) is desirable to be as short as possible. To reduce the duration of stage-I, effects of nutrients feeding rates on the duration of stage-I were investigated, as shown in Figure 2. Given that the initial inoculum density was 0.022 g/L, it regarded that microalgae reached stage-II when microalgae biomass concentration reached 0.172 g/L, eight times higher than the initial inoculum density.

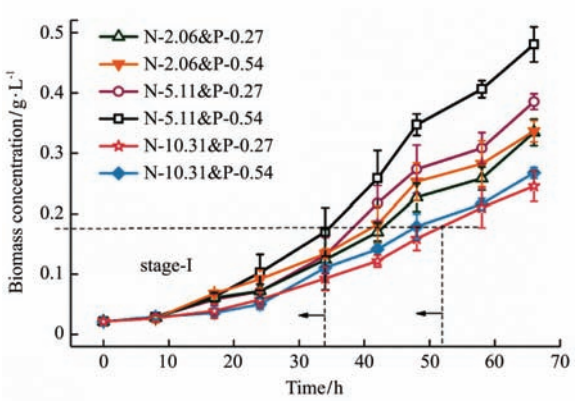

Figure 2 Effects of N and P feeding rates on microalgae growth in adaption phase (stage-I)

At low N and P feeding rates (i.e. N-2.06&P-0.27), the duration of stage-I was around 43 h, 9 h longer than that under N-5.11&P-0.54. One possible reason for the prolonged duration was that the N and P were not enough for microalgae growth and resulted in insufficient synthesis of necessary intracellular macromolecules, like nucleic acids and proteins[22]. Table 3 shows the N and P concentrations in cultivating medium at 34 h and 48 h under different N and P feeding rates. It shows that N and P concentrations in cultivating medium under N-2.06&P-0.27 stayed at 0 mg/L during the cultivation process, indicating that the N and P sources were insufficient for microalgae growth under N-2.06&P-0.27. Compared to the cultivating medium under N-2.06&P-0.27, the residual P concentration under N-2.06&P-0.54 at 34 h and 48 h were (0.07±0.04) mg P/L and (0.11±0.06) mg P/L, indicating that P was sufficient for microalgae growth under N-2.06&P-0.54. However, the duration of stage-I under N-2.06&P-0.54 was almost the same with that under N-2.06&P-0.27, with a value of 42 h, demonstrating that N-deficiency was the major limiting factor for microalgae growth under N-2.06&P-0.27 and N-2.06&P-0.54. By increasing the N feeding rate to 5.11 mg N/(L·d), the duration of stage-I was reduced to 34 h and 38 h under N-5.11&P-0.54 and

N-5.11&P-0.27, respectively, suggesting that sufficient N supply can enhance the acclimation of microalgae to new environment. However, when the N feeding rate was further improved to 10.31 mg N/(L·d), the duration of stage-I was prolonged to 48 h and 52 h under N-10.31&P-0.54 and N-10.31&P-0.27. This was probably because that the microalgae cell density was low at initial, excess N supplied in culture medium under N-10.31&P-0.54 and N-10.31&P-0.27 caused detrimental effects on microalgae growth. This is in accordance with reported results by researchers[12,23].

Among all the experimental groups in this study, the shortest duration of stage-I for *C. vulgaris* was 34 h under N-5.11&P-0.54, as shown in Figure 2. Considering the shortest duration of stage-I under N-5.11&P-0.54, the feeding rates of 5.11 mg/(L·d) for N and 0.54 mg/(L·d) for P are regarded as the optimal N and P feeding rates for microalgae growth in stage-I in this study. In addition, based on Equation (3), N and P consumption rates of *C. vulgaris* in stage-I under N-5.11&P-0.54 were calculated as 3.61 mg N/(L·d) and 0.49 mg P/(L·d), respectively.

Table 3 N and P concentration in cultivation medium in stage-I (means ± SD)

	Time/h	N-2.06&P-0.27	N-2.06&P-0.54	N-5.11&P-0.27	N-5.11&P-0.54	N-10.31&P-0.27	N-10.31&P-0.54
N concentration /mg·N·L^{-1}	34	0	0	3.11±0.15	2.12±0.20	5.81±0.31	6.34±0.40
	48	0	0	3.18±0.43	0.71±0.12	13.28±0.28	12.85±0.32
P concentration /mg·P·L^{-1}	34	0	0.22±0.04	0	0.07±0.04	0	0.17±0.08
	48	0	0.31±0.06	0	0.11±0.06	0	0.26±0.09

3.2 Nutrients-repletion condition to enhance biomass production in stage-II

For microalgae in growth phase (stage-II), sufficient nutrients (N and P, et al.) are prerequisites for improvement of biomass concentration. However, excessive nutrients may cause toxic effects on microalgae cells, resulting in little increase or even decrease of biomass[2,23]. To explore the optimal N and P feeding rates for biomass accumulation in stage-II, all experimental groups were first cultivated in identical conditions of N-5.11&P-0.54, which was the optimal N and P feeding rates obtained in stage-I, until the cultures entered stage-II (i.e., the cell density reached eight times of the initial inoculum density). Then the feeding rates of N and P were enhanced in stage-II to supply more nutrients for microalgae. Table 4 shows the kinetic parameters of *C. vulgaris* (i.e., the maximum biomass concentration, X_{max}; the maximum biomass productivity, P_{max}; and the maximum specific growth rate, μ_{max}) according to Equations (1) and (2). The highest values of X_{max}, P_{max} and μ_{max} were obtained under N-20.04&P-4.21, with values of (4.03±0.25) g/L, (0.64±0.02) g/(L·d) and (1.19±0.06) d^{-1}, respectively. In addition, the growth profiles of microalgae in stage-II under different N and P feeding rates are shown in Figure 3a. Microalgae cultivated under N-20.04&P-4.21 cost less time (176 h) to reach the maximal biomass concentration than that under other N and P feeding rates. These results demonstrated that N-20.04&P-4.21 was a superior N and P feeding rates for biomass accumulation in stage-II.

Table 4 The maximum biomass concentration (X_{max}), maximum biomass productivity (P_{max}) and maximum specific growth rate (μ_{max}) of *Chlorella vulgaris* under different N and P feeding rates (means ± SD)

	N-10.31&P-2.15	N-10.31&P-4.21	N-20.04&P-2.15	N-20.04&P-4.21	N-30.12&P-2.15	N-30.12&P-4.21
X_{max}/g·L^{-1}	3.47 ± 0.14	3.45 ± 0.17	3.99 ± 0.11	4.03 ± 0.25	3.75 ± 0.18	3.64 ± 0.13
P_{max}/g·(L·d)$^{-1}$	0.47 ± 0.04	0.50 ± 0.04	0.56 ± 0.06	0.64 ± 0.02	0.50 ± 0.02	0.49 ± 0.03
μ_{max}/d^{-1}	0.88 ± 0.04	1.03 ± 0.02	0.95 ± 0.01	1.19 ± 0.06	0.90 ± 0.04	0.83 ± 0.01

When N feeding rates was the same while P feeding rates increased from 2.15 mg P/(L·d) to 4.21 mg P/(L·d), no significant difference of X_{max} was observed, but the μ_{max} was obviously improved. For example, as shown in Table 4, the X_{max} under N-10.31&P-2.15 and N-10.31&P-4.21 were almost same ((3.47±0.14) g/L and (3.45±0.17) g/L, respectively), while the μ_{max} increased from (0.88±0.04) d^{-1} under N-10.31&P-2.15 to

(1.03±0.02) d^{-1} under N-10.31&P-4.21. It demonstrated that the increase of P feeding rate has more remarkable influence on specific growth rate than the maximum microalgae biomass concentration. The possible reason is that cellular energy transduction is closely associated with inorganic P[24]. The increase of P feeding rates can enhance the production and transduction of metabolic energy, accelerating the growth rate of microalgae[9,25]. However, the μ_{max} under N-30.12&P-4.21 ((0.83±0.01) d^{-1}) was not improved than that under N-30.12&P-2.15 ((0.90±0.04) d^{-1}), which might be because that the detrimental effect caused by excessive N (as shown in Figure 3b) in cultivating medium was the major influencing factor for microalgae growth. As shown in Figure 3c, there were about 10 mg/L of P remaindered in cultivating mediums at the end under P feeding rate of 4.21 mg P/(L·d), indicating that P feeding rate of 4.21 mg P/(L·d) has already supplied enough P for microalgae growth.

When N feeding rate increased from 10.31 mg N/(L·d) to 30.12 mg N/(L·d), the X_{max} first increased from about 3.5 g/L to about 4.0 g/L, but then decreased to about 3.7 g/L (as seen in Table 4). Under N feeding rate of 10.31 mg N/(L·d), the growth of microalgae was mainly limited by the N-limitation condition (with N concentration of 0 mg N/L in cultivating medium through the cultivating process, as seen in Figure 3b), which triggered the degradation of nitrogenous compounds in algae cells and decreased the X_{max}[2]. By increasing the N feeding rate to 20.04 mg N/(L·d), there were about 10 mg N/L of N remaindered in cultivating mediums (Figure 3b), demonstrating that N feeding rate of 20.04 mg N/(L·d) had provided enough N for microalgae growth in stage-II. At this time, the sufficient but not excessive N concentration in cultivating medium contributed to the increase of X_{max} to about 4.0 g/L. In contrast, when N feeding rate was further improved to 30.12 mg N/(L·d), the N concentration in cultivating medium was about 120 mg N/L at 248 h (Figure 3b). Excessive N in cultivating medium caused over-accumulation of intracellular ammonium and nitrite, both of which are toxic to algae cells[1,23,26], leading to the decrease of X_{max} to ca. 3.7 g/L.

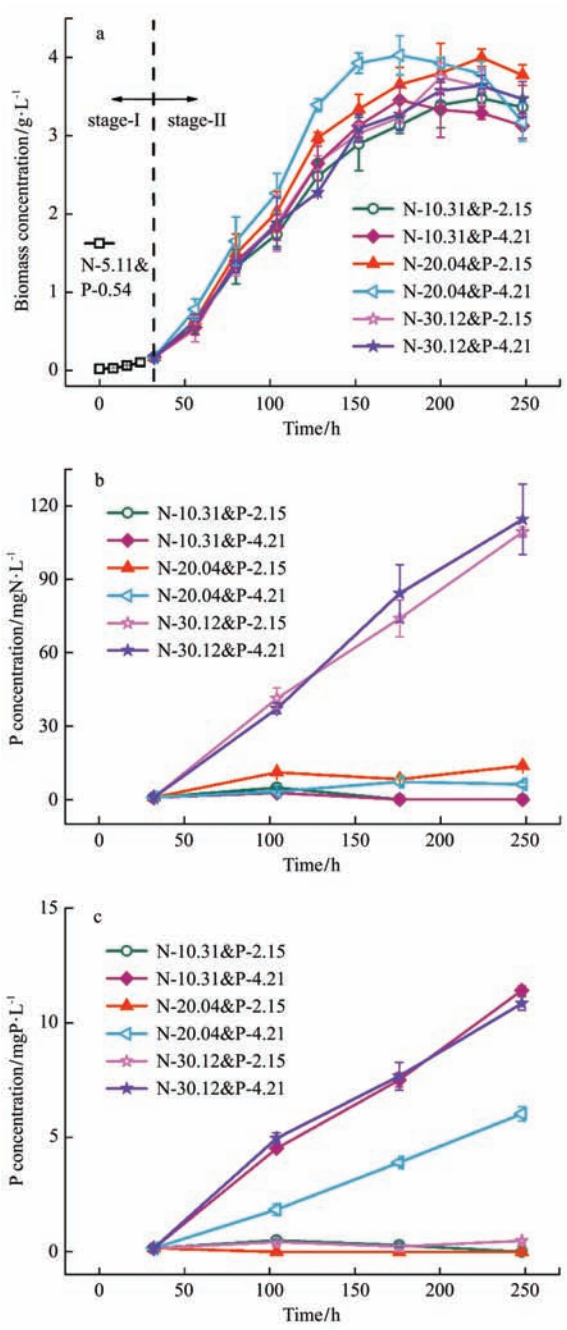

Figure 3 Variations of (a) microalgae biomass concentration, (b) N concentration in cultivating medium and (c) P concentration in cultivating medium in response to different N and P feeding rates in growth phase (stage-II)

3.3 Nutrients-deprivation condition to enhance overall lipid productivity in stage-III

To enhance the lipid productivity of microalgae, newly inoculated microalgae cultures (with the same inoculum cell density as before) were first provided with the optimal N and P feeding rates obtained in stage-I (i.e., N-5.11&P-0.54) and stage-II (i.e., N-20.04&P-4.21). Then, nutrients supplied to microalgae cultures were stopped at different time to create nutrients deprivation environments by removing nutrients feeding mediums.

Based on the obtained X_{max} of (4.03±0.25) g/L under N-20.04&P-4.21 in Section 3.2, the time when the biomass concentration reached half of the X_{max}, three-quarter of X_{max} and X_{max} were chosen as three nutrients supply ceasing time, which were denoted as S_{t1} (104 h), S_{t2} (128 h) and S_{t3} (176 h) for convenience. Figure 4 shows the effects of nutrients supply schedule on biomass concentration, lipid content, lipid productivity and lipid yield. In the Figure, lipid productivity means the obtained lipid amount from microalgae for each day, and the lipid yield means the cumulative lipid productivity. It shows that the X_{max} of microalgae cultures continuously increased from (3.02±0.3) g/L to (4.12±0.21) g/L when nutrients supply ceasing time extended from S_{t1} (104 h) to S_{t3} (176 h) (Figure 4a). On the other hand, the highest lipid content of microalgae biomass monotonically decreased from 35.8% to 26.1% with the delay of nutrient supply ceasing time (Figure 4b).

Variations of N and P concentrations in cultivating medium at different nutrients supply ceasing time are shown in supplementary materials. When nutrients supply was stopped at S_{t1} (104 h), both N and P in cultivating medium were used up before 176 h, and thereafter the cultivating medium became N and P starving. At this time, the microalgae had the largest lipid content (35.8%), much higher than that under nutrients supply ceasing time of S_{t2} (34.1%) and S_{t3} (26.1%). This was mainly because that under N and P supply ceasing time of S_{t1} (104 h), the N and P starvation condition increased the intracellular content of fatty acid acyl-CoA and activated diacylglycerol acyltransferase, which could enhance the conversion of fatty acid acyl-CoA to triglyceride hydrolysis, leading to a higher lipid content of microalgae cells[27]. However, since the biomass concentration under N and P ceasing time of S_{t1} was low ((3.02±0.30) g/L) attributing to the deficiency of N and P, the maximal lipid productivity and lipid yield were only 103.44 mg/(L·d) and 845.94 mg/L, respectively. In contrast, when N and P feeding were stopped at S_{t3} (176 h), the N and P sources were abundant for microalgae growth throughout the cultivating process. The biomass concentration was obviously enhanced to (4.12±0.21) g/L because of the abundant N and P supplements. On the other hand, the plentiful N and P in cultivating medium adversely affected the lipid content in algae cells, resulting in a low lipid content (26.1%) at the end of cultivation (Figure 4b). The maximal lipid productivity and lipid yield under nutrients supply ceasing time of S_{t3} (176 h) were 119.06 mg/(L·d) and 931.89 mg/L, respectively. Compared to the cases of S_{t1} and S_{t3}, the microalgae biomass accumulation and lipid synthesis were coordinated when N and P were stopped at S_{t2} (128 h). The maximal biomass concentration and lipid content were (3.64±0.26) g/L and 34.1%, respectively. As a result, the lipid productivity and lipid yield obtained under N and P supply ceasing time of S_{t2} were largest among all three nutrients supply ceasing time of S_{t1}, S_{t2} and S_{t3}, with values of 132.30 mg/(L·d) and 1022.91 mg/L, respectively.

Table 5 shows the comparison of lipid productivity of microalgae with different cultivation strategies. The lipid productivity obtained in this study with phase-feeding strategy was 132.30 mg/(L·d), much higher than that obtained in conventional and two-stage cultivation strategies. For example, the lipid productivity of *C. vulgaris* cultivated under conventional method was only 32.6 mg/(L·d)[7]. Mujtaba et al.[13] adopted a two-stage cultivation strategy to enhance lipid productivity, containing improvement of biomass in N-repletion medium in stage-I and enhancement of lipid accumulation in N-starvation medium in stage-II. Compared to the conventional cultivation method, the lipid productivity was improved to 77.80 mg/(L·d) in the work by Mujtaba et al.[13]. However, the lipid productivity in the two-stage cultivation strategy was still limited by the poor biomass productivity since nutrients supply did not follow nutrients demand by microalgae in different phases. Moreover, the adaption phase of microalgae growth, which is essential to improve the overall biomass productivity of microalgae, was not taken into consideration. The three-stage cultivation strategy proposed in this study comprehensively considered the nutrients demand by *C. vulgaris*, including the adaption phase of microalgae growth, and thus the lipid productivity was effectively improved to 132.30 mg/(L·d).

In addition, compared to the previous reported two-stage cultivation strategies[5,13], the three-stage cultivation strategy is cost-effective by introduction of the AEM-PBR because the energy cost on algae cells transferring from nutrient-rich to nutrient-starvation medium (mainly by centrifuging) is avoided.

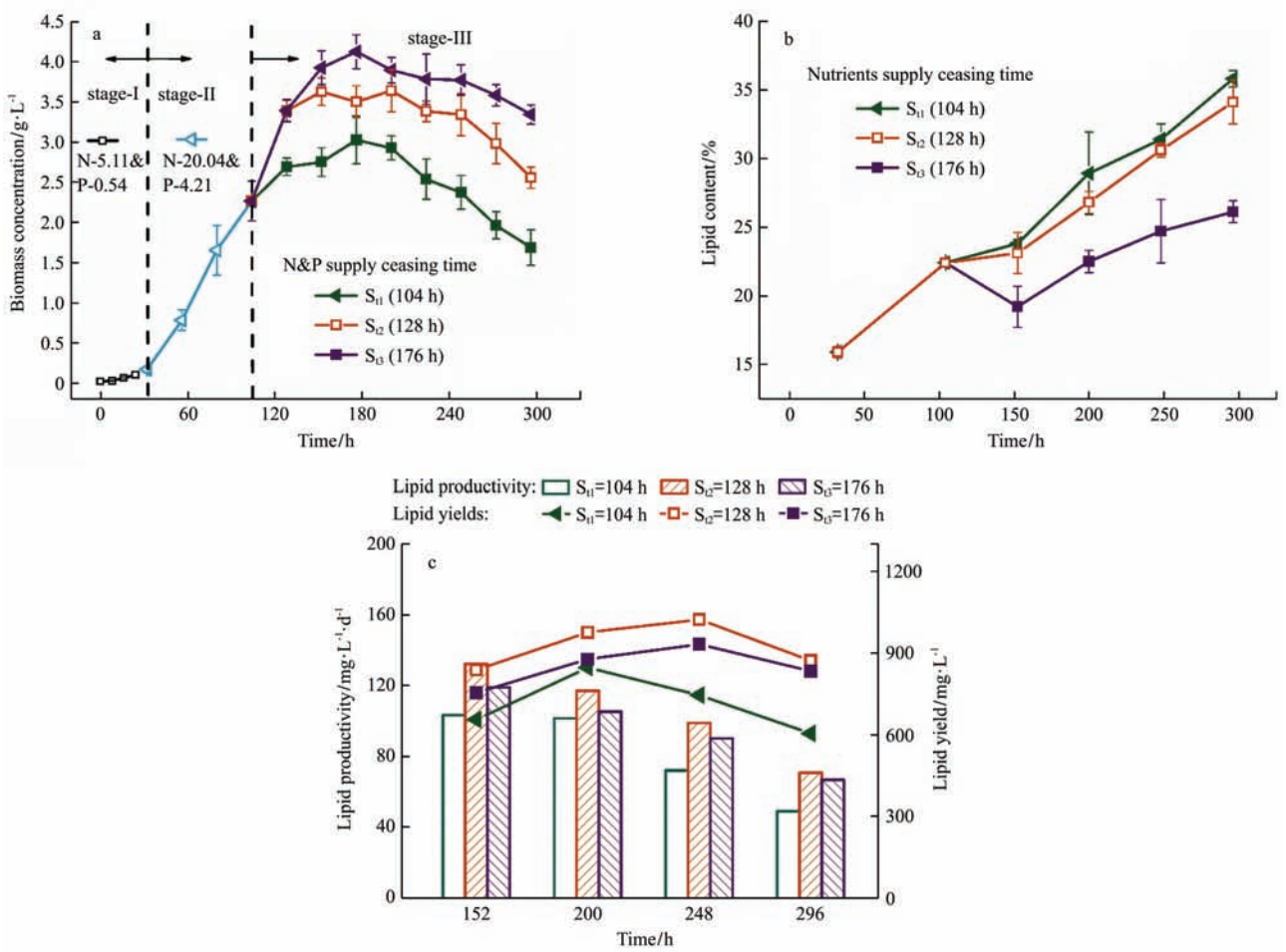

Figure 4 Variations of (a) microalgae biomass concentration, (b) lipid content and (c) lipid productivity and lipid yield in response to different N and P supply ceasing time in stationary and decline phases (stage-III) (Lipid productivity means the obtained lipid amount from microalgae for each day, and the lipid yield means the cumulative lipid productivity)

Table 5 Comparisons of biomass concentration (BC)/biomass productivity (BP), lipid content (LC) and lipid productivity (LP) of this study with representative literatures

Lipid-induction strategy	Microalgae strain	Implementation method	BC/g·L^{-1} or BP/g·L^{-1}·d^{-1}	LC/%	LP/mg·L^{-1}·d^{-1}	Reference
Conventional strategy						
Without any strategy	*C. vulgaris*	--	0.17 g·(L·d)$^{-1}$	19.2	32.6	[7]
N deficiency	*Chlorella* sp.	0.025 g/L of urea	0.46 g·L^{-1}	66.1	51	[30]
P deficiency	*Chlorella* sp.	32 μM of P	1.8 g·L^{-1}	23.6	15.7	[31]
Two-stage strategy						
N-repletion (stage-I) to N-starvation (stage-II)	*C. vulgaris*	Centrifugation	1.8 g·L^{-1}	43.0	77.8	[13]
High salinity (stage-II)	*Scenedesmus obtusus*	20 g/L of NaCl addition	2.6 g·L^{-1}	47.7	60.7	[5]
Three-stage strategy						
Low N&P (stage-I) to high N&P (stage-II) to N&P starvation (stage-III)	*C. vulgaris*	Anion exchange membrane PBR	3.64 g·L^{-1}	34.1	132.3	the present study

Besides the lipid productivity, fatty acid compositions of microalgae is another important characteristic since it ultimately affects the quality of biodiesel product. Table 6 shows the fatty acid compositions of *C. vulgaris* FACHB-31 grown under different N and P supply ceasing time in stage-III. Overall, the compositions are very similar, with 31.53%-34.55% saturated fatty acids, 21.72%-25.40% mono-unsaturated fatty acids and

40.05%-45.75% poly-unsaturated fatty acids. The predominant fatty acids in *C. vulgaris* FACHB-31 were unsaturated fatty acids, occupying 65.45%-68.47% of the total fatty acids. It demonstrated that the lipids have good cold flow characteristics[28,32]. Since high linolenic acid (C18:3) content in microalgae lipid can cause degradation of the lipid and then reduce the quality of biodiesel product, a linolenic acid content lower than 12% was suggested in previous studies[29]. In this study, the linolenic acid content are 8.99%-10.86%, demonstrating that the lipids obtained using the three-stage cultivation strategy can well satisfy the quality requirements.

Table 6 Fatty acid profiles (%, w/w total fatty acid) of *Chlorella vulgaris* grown under different N and P supply ceasing time in stage-III

FAME composition	FAME content/%		
	S_{t1} (104 h)	S_{t2} (128 h)	S_{t3} (176 h)
C16:0	29.81	29.53	27.62
C16:1	2.75	1.86	3.46
C16:2	2.99	2.33	4.61
C16:3	6.48	5.54	6.66
C18:0	3.83	5.02	3.91
C18:1	21.72	23.54	18.26
C18:2	22.15	21.31	26.50
C18:3	10.29	10.86	8.99
Total saturated	33.64	34.55	31.53
Total mono-unsaturated	24.47	25.40	21.72
Total poly-unsaturated	41.89	40.05	45.75

4 Conclusions

This study examined a three-stage microalgae cultivation strategy based on nutrients demand of *C. vulgaris*, which can realize simultaneous enhancement of microalgae biomass and lipid productivity. This strategy based on an AEM-PBR, thereby lowering the energy cost on microalgae cells transfer and avoiding microbial contamination. The duration of adaption phase of microalgae cultivation was reduced from 52 h to 34 h, whereas the lipid productivity was improved from 103.44 mg/(L·d) to 132.30 mg/(L·d). This cultivation strategy can effectively improve the biodiesel productivity from microalgae.

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[References]

[1] Chen M, Tang H, Ma H, Holland T C, Ng K Y, Salley S O. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresour. Technol., 2011; 102: 1649–1655.

[2] Pancha I, Chokshi K, Maurya F R, Trivedi K, Patidar S K, Ghosh A, et al. Salinity induced oxidative stress enhanced biofuel production potential of microalgae *Scenedesmus* sp. CCNM 1077. Bioresour. Technol., 2015; 189: 341–348.

[3] Chisti Y. Biodiesel from microalgae. Biotechnol. Adv., 2007; 25(3): 294–306.

[4] Tan C H, Show P L, Chang J-S, Ling T C, Lan J C-W. Novel approaches of producing bioenergies from microalgae: A recent review. Biotechnol. Adv., 2015; 33(6): 1219–1227.

[5] Xia L, Ge H, Zhou X, Zhang D, Hu C. Photoautotrophic outdoor two-stage cultivation for oleaginous microalgae *Scenedesmus obtusus* XJ-15. Bioresour. Technol., 2013; 144: 261–267.

[6] Griffiths M J, Harrison S T L. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J. Appl. Phycol., 2009; 21(5): 493–507.

[7] Rodolfi L, Chini Zittelli G, Bassi N, Padovani G, Biondi N, Bonini G, et al. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng., 2009; 102(1): 100–112.

[8] Chang H X, Fu Q, Huang Y, Xia A, Liao Q, Zhu X, et al. An annular photobioreactor with ion-exchange-membrane for non-touch microalgae cultivation with wastewater. Bioresour. Technol., 2016; 219: 668–676.

[9] Chu F F, Chu P N, Shen X F, Lam P K, Zeng R J. Effect of phosphorus on biodiesel production from *Scenedesmus obliquus* under nitrogen-deficiency stress. Bioresour. Technol., 2014; 152: 241–246.

[10] Mattos E R, Singh M, Cabrera M L, Das K C. Effects of inoculum physiological stage on the growth characteristics of *Chlorella sorokiniana* cultivated under different CO_2 concentrations. Appl. Biochem. Biotechnol., 2012; 168(3):

519–530.

[11] Roleda M Y, Slocombe S P, Leakey R J G, Day J G, Bell E M, Stanley M S. Effects of temperature and nutrient regimes on biomass and lipid production by six oleaginous microalgae in batch culture employing a two-phase cultivation strategy. Bioresour. Technol., 2013; 129: 439–449.

[12] Arumugam M, Agarwal A, Arya M C, Ahmed Z. Influence of nitrogen sources on biomass productivity of microalgae *Scenedesmus bijugatus*. Bioresour. Technol., 2013; 131: 246–249.

[13] Mujtaba G, Choi W, Lee C G, Lee K. Lipid production by *Chlorella vulgaris* after a shift from nutrient-rich to nitrogen starvation conditions. Bioresour. Technol., 2012; 123: 279–283.

[14] Ra C H, Kang C-H, Kim N K, Lee C-G, Kim S-K. Cultivation of four microalgae for biomass and oil production using a two-stage culture strategy with salt stress. Renew. Energ., 2015; 80: 117–122.

[15] Su C H, Chien L J, Gomes J, Lin Y S, Yu Y K, Liou J S, et al. Factors affecting lipid accumulation by *Nannochloropsis oculata* in a two-stage cultivation process. J. Appl. Phycol., 2010; 23(5): 903–908.

[16] Fu Q, Chang H-X, Huang Y, Liao Q, Zhu X, Xia A, et al. A novel self-adaptive microalgae photobioreactor using anion exchange membranes for continuous supply of nutrients. Bioresourc. Technol., 2016; 214: 629–636.

[17] Cheng J, Huang Y, Feng J, Sun J, Zhou J, Cen K. Improving CO_2 fixation efficiency by optimizing *Chlorella* PY-ZU1 culture conditions in squential bioreactors. Bioresour. Technol., 2013; 144: 321–327.

[18] Vogels M, Zoeckler R, Stasiw D M, Cerny L C. Verhulst's "notice sur la loi que la populations suit dans son accroissement" from correspondence mathematique et physique. Ghent, vol. X, 1838. J. Biol. Phys., 1975; 3(4): 183–192.

[19] Chang H X, Huang Y, Fu Q, Liao Q, Zhu X. Kinetic characteristics and modeling of microalgae *Chlorella vulgaris* growth and CO_2 biofixation considering the coupled effects of light intensity and dissolved inorganic carbon. Bioresour. Technol., 2016; 206: 231–238.

[20] Bligh E G, Dyer W J. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 1959; 37(8): 911–917.

[21] Miao X L,Wu Q Y. Biodiesel production from heterotrophic microalgae oil. Bioresour. Technol., 2006; 97(6): 841–846.

[22] Guo F, Wang H, Wang J, Zhou W, Gao L, Chen L, et al. Special biochemical responses to nitrogen deprivation of filamentous oleaginous microalgae *Tribonema* sp. Bioresour. Technol., 2014; 158: 19–4.

[23] Chen W, Zhang Q, Dai S. Effects of nitrate on intracellular nitrite and growth of *Microcystis aeruginosa*. J. Appl. Phycol., 2009; 21(21): 701–706.

[24] Beardall J, Roberts S, Raven J A. Regulation of inorganic carbon acquisition by phosphorus limitation in the green alha *Chlorella emersonii*. Can. J. Bot.-Rev. Canadienne De Botanique, 2005; 83(7): 859–864.

[25] Wu H, Miao X. Biodiesel quality and biochemical changes of microalgae *Chlorella pyrenoidosa* and *Scenedesmus obliquus* in response to nitrate levels. Bioresour. Technol., 2014; 170: 421–427.

[26] Feng D, Chen Z, Xue S, Zhang W. Increased lipid production of the marine oleaginous microalgae Isochrysis Zhangjiangensis (*Chrysophyta*) by nitrogen supplement. Bioresour. Technol., 2011; 102: 6710–6716.

[27] Takagi M, Watanabe K, Yamaberi K, Yoshida T. Limited feeding of potassium nitrate for intercellular lipid and triglyceride accumulation of *Nannochloris* sp UTEX LB1999. Appl. Microbiol. Biotechnol., 2000; 54(1): 112–117.

[28] Ho S H, Chen W M, Chang J S. *Scenedesmus obliquus* CNW-N as a potential candidate for CO_2 mitigation and biodiesel production. Bioresour. Technol., 2010; 101(22): 8725–8730.

[29] Singh B, Guldhe A, Rawat I, Bux F. Towards a sustainable approach for development of biodiesel from plant and microalgae. Renew. Sustain. Energ. Rev., 2014; 29(7): 216–245.

[30] Hsieh C H, Wu W T. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresour. Technol., 2009; 100: 3921–3926.

[31] Liang K, Zhang Q, Gu M, Cong W. Effects of phosphorus on lipid accumulation in freshwater microalgae *Chlorella* sp. J. Appl. Phycol., 2012; 25(1): 311–318.

[32] Zheng Y P, Huang Y, Liao Q, Zhu X, Fu Q, Xia A. Effect of wettability on the growth of *Scenedesmus obliquu*s biofilm attached on glass surface coated with polytetrafluoroethylene emulsion. Int. J. Hydrogen Energ., 2016; 41(46): 21728–21735.