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Effect of phosphorus and sodium acetate on lipid accumulation from *Ankistrodesmus* sp. IFRPD 1061 in an open pond

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ABSTRACT

Ankistrodesmus sp, has been comprehensively studied for their potential in the production of biodiesel due to their biomass productivity and high lipid content. This study examined the biomass productivity, and concentration, lipid productivity, and concentration, and lipid contents of *Ankistrodesmus* sp. IFRPD 1061 under several phosphorus concentrations. The optimum conditions were attained at 0.12 g/L KH_2PO_4. The highest lipid content reached to 35.950 ± 4.253% (w/w) in 22 days cultivation. An open pond cultivation system was used with the addition of 10 mM sodium acetate on every fourth day (0, 4, 8 and 12) of cultivation and KH_2PO_4 on twelfth day of cultivation. The obtained biomass productivity and concentration, lipid productivity and concentration and lipid content were 0.709 ± 0.027 g/L, 48.304 ± 1.894 mg/L/day, 0.214 ± 0.004 g/L 14.550 ± 0.215 mg/L/day and 30.154 ± 1.627% (w/w) in 14 days of cultivation, respectively. The results exhibited that addition of 10 mM sodium acetate and KH_2PO_4 may enhance lipid accumulation within algae cells in an open pond cultivation system.

1. Introduction

The intensive natural fuel (fossil fuel) utilization at the expense of extensive energy consumption has increased the risk of energy depletion round the globe. Presently, 90% of the energy requirement of the world is fulfilled by the fossil fuels, and it is estimated that reserves of these natural resources may be exhausted by the year 2050 [1]. In addition, the burning of fossil fuels release harmful greenhouse gases into the atmosphere, endangering both human health and the ecosystem [2]. The use of photosynthetic microorganisms such as microalgae is an alternate method of CO_2 removal [3]. Soluble carbonates, a carbon form, can be used for cell development, either directly or by converting carbonate (via carbonhydrase activity) to free CO_2 [4,5]. Microalgae with quick life cycle development, have the ability to gather solar energy, and adapt to a variety of environmental situations. When microalgae are used for biodiesel production, they not only eliminate CO_2 but also convert it into biomass [6]. Microalgae are a promising alternative fuel

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source since they are one of the most efficient biological producers of oil and a flexible biomass source [7]. Additionally, there are many algal species that can be stimulated to increase fatty acid concentration for biodiesel production under environmental stress, such as nutrient deficiency and salt stress [8]. Microalgae, particularly green microalgae species, propagate quicker in contrast with numerous crops of oil and can be cultivated in non-arable places. Microalgae based biodiesel is considered to be one of the best sources of renewable energy, which may substitute the natural fuels by fulfilling global transportation energy demand in the future [9]. Phosphorus is a macronutrient that is essential for growth, metabolism, and other crucial functions like adenosine triphosphate (ATP) generation, photosynthesis, signal transduction, and respiration. Additionally, in microalgae growth acceptable concentration of phosphorous regulates its metabolic activities which significantly influence lipids and fatty acid yield [10]. Cell division might be hindered because of phosphate deficiency in growth medium [11]. Photosynthesis, on the other hand, is less likely to be disrupted by this circumstance, which resultantly slows down the cell division and enlargement of cells: however, carbon accumulation in cell is in the form of lipids, a high energy yielding substance [2].

Some microalgae exhibit heterotrophic behavior, utilizing organic sources of carbon, whilst others may exhibit both autotrophic and heterotrophic features, concurrently [12]. It is thought that mixotrophic cultivation combines the pluses of both types of cultivation i.e., phototrophic and cultivation. The chemical energy through microalgae in mixotrophic cultivation is collectively generated from organic (acetate, glucose, glycerol, fructose, maltose, and sucrose) and inorganic carbon (photosynthesis) sources [13].

Open cultivation systems, for instance: tanks, raceway ponds, and open ponds, are common means of large-scale microalgae cultivation. Open pond is the oldest and most basic technique for producing microalgae. Studies have just begun to assess the economics of open pond production method as the most viable solution for large-scale commercial production [14]. There are several benefits of an open cultivation system such as cost-effectiveness, requirement of less energy for mixing the culture, but it also suffers different challenges i.e., contamination, temperature control, intensity of light, evaporation, CO_2 utilization, and maintainability [15]. The depth of open pond (usually, 20 cm–30 cm) is critical as shallower ponds have higher depth and cost less operational expenses by lowering the overall volumetric flow rate that the paddle wheels must maintain. Moreover, shallow ponds are believed to produce abundant biomass density with lower harvesting cost [16]. Nevertheless, an open system needs larger area, and it is vulnerable to contamination and adverse weather conditions. Furthermore, the growth parameters including temperature of medium, and evaporation are hard to be controlled in an open pond system [17].

The current study was aimed to investigate the influence of varying phosphorus concentrations during the stationary growth phase on the lipid accumulation of the green microalgae *Ankistrodesmus* sp. IFRPD 1061. The effects of phosphate and acetate on lipid accumulation and the quality of fatty acid profile in an open pond culture were also analyzed.

2. Results and discussion

Microalgae growth and nutrient assimilation under different potassium dihydrogen phosphate (KH_2PO_4) concentration.

The growth of *Ankistrodesmus* sp. 1061 in modified NS III medium (control) are shown in Fig. 3. During 32 days experimental span, the highest values (on day 32 of cultivation) for biomass and lipid concentrations found were 7.260 ± 0.113 g/L, and 2.453 ± 0.018 g/L, respectively. Highest biomass and lipid productivity obtained (after 2 days of cultivation) were 386.250 ± 15.910 mg/L/day, and 124.089 ± 19.409 mg/L/day, respectively. Maximum lipid content achieved (on day 6 of cultivation) was 46.057 ± 4.121% (w/w). The nitrate concentration was dropped rapidly after day 12 of cultivation: whereas, in control condition (32 days of cultivation), the nitrate concentration decreased from 0.696 ± 0.005 g/L to 0.000 ± 0.000 g/L. On the other hand, the concentration of phosphorus decreased from 0.050 ± 0.002 g/L to 0.013 ± 0.000 g/L after 32 days cultivation in control condition.

The modified NS III medium for the growth of *Ankistrodesmus* sp. 1061 contained 0.24 g/L of KH_2PO_4. Fig. 4 exhibits that 0.12 g/L KH_2PO_4 was added to the medium during stationary phase (day 20 of cultivation). The peak values for biomass and lipid concentrations were observed on day 32 i.e., 7.360 ± 0.566 g/L and 2.948 ± 0.124 g/L, respectively. Highest biomass, lipid productivity, and lipid content observed were 317.500 ± 17.678 mg/L/day (day 4 of cultivation), 95.725 ± 0.269 mg/L/day (day 28 of cultivation), and 40.101 ± 1.401% (w/w) (day 32 of cultivation), respectively. The nitrate concentration dropped rapidly after 16 days cultivation,

Figure: 1. Chamber for culture *Ankistrodesmus* sp. 1061.

Figure: 2. Open microalgae pond for culture *Ankistrodesmus* sp. 1061.

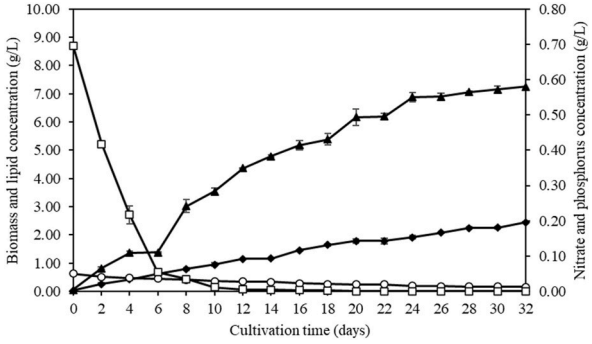

Figure: 3. Change in biomass (▲) nitrate (□) phosphorus (◯) and lipid (◆), concentration of the microalgae cultivated in modified NS III medium (control).

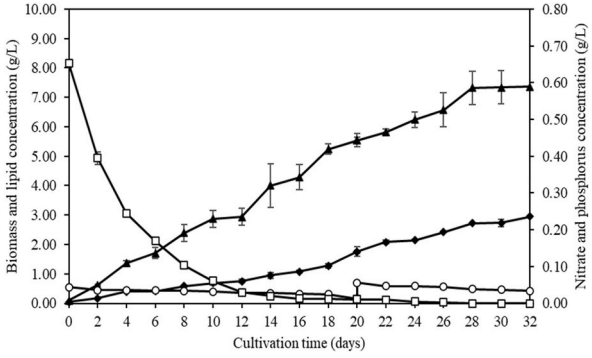

Figure: 4. Change in biomass (▲) nitrate (□) phosphorus (◯) and lipid (◆), concentration of the microalgae cultivated in modified NS III medium with addition 0.12 g/L KH_2PO_4 concentration at stationary growth phase.

decreased from 0.652 ± 0.013 g/L to 0.000 ± 0.000 g/L on day 32 of cultivation in control condition. The concentration of phosphorus decreased from 0.043 ± 0.000 g/L to 0.034 ± 0.002 g/L on day 32 days of cultivation in control condition. On day 20, addition of 0.12 g/L KH_2PO_4 increased the phosphorus concentration to 0.056 ± 0.001 g/L from the original concentration of 0.013 ± 0.002 g/L.

The growth of *Ankistrodesmus* sp. 1061 in modified NS III medium was supplemented with 0.24 g/L KH_2PO_4 on day 20 (stationary phase) (Fig. 5). Peak values obtained for biomass, lipid concentration, biomass productivity, lipid productivity, and lipid content were: 8.020 ± 0.113 g/L (day 32 of cultivation), 3.015 ± 0.050 g/L (day 30 of cultivation), 351.875 ± 0.884 mg/L/day (day 8 of cultivation), 99.383 ± 1.522 mg/L/day (day 30 of cultivation), and 57.775 ± 14.717% (w/w) (day 4 of cultivation), respectively. The nitrate concentration was dropped rapidly after 12 days cultivation with 0.000 ± 0.000 g/L on day 32 of cultivation in controlled condition: whereas phosphorus decreased from 0.047 ± 0.000 g/L to 0.068 ± 0.004 g/L (day 32 of cultivation), in control condition. Phosphorous concentration increased by addition of KH_2PO_4 on day 20 using 0.24 g/L KH_2PO_4 to 0.080 ± 0.001 g/L from its original value of 0.017 ± 0.000 g/L.

The growth of *Ankistrodesmus* sp. 1061 in modified NS III medium containing 0.24 g/L KH_2PO_4 and addition of 0.36 g/L KH_2PO_4 in culture medium (on day 20 during stationary phase) is shown in Fig. 6, exhibiting 7.567 ± 0.566 g/L biomass (day 32 of cultivation),

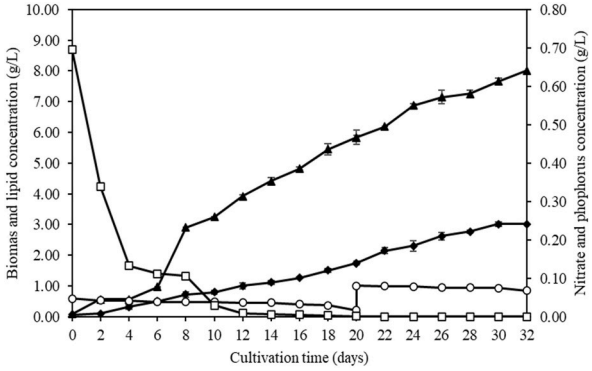

Figure: 5. Change in biomass (▲) nitrate (□) phosphorus (◯) and lipid (◆), concentration of the microalgae cultivated in modified NS III medium with addition 0.24 g/L KH 2PO4 concentration at stationary growth phase.

2.348 ± 0.230 g/L lipid (day 32 of cultivation), 423.750 ± 5.303 mg/L/day biomass productivity (day 4 of cultivation), 116.397 ± 17.749 mg/L/day lipid productivity (day 4 of cultivation), and 34.221 ± 6.275% (w/w) lipid content (day 8 of cultivation) as maximum attained values. In control conditions, the nitrate and phosphorous concentration were dropped from 0.750 ± 0.076 g/L to 0.000 ± 0.000 g/L and from 0.050 ± 0.002 g/L to 0.071 ± 0.004 g/L, respectively during time span of 32 days. Addition of KH_2PO_4 during stationary phase (day 20) using 0.36 g/L KH_2PO_4 enhanced the phosphorous concentration from 0.024 ± 0.003 g/L to 0.093 ± 0.007 g/L. Mechanism and growth techniques for microalgal cultivation need improvement for optimized biodiesel production. The selection of highest lipid productivity and less cultivation time are imperious step that affect the cost of biodiesel production. Table 1 illustrates kinetic parameters for *Ankistrodesmus* sp. IFRPD 1061 under different phosphorus concentrations in stationary growth phase in modified NS III medium.

The addition of KH_2PO_4 (0.24 g/L) at day 20 of cultivation enhanced the highest lipid productivity at day 22 of cultivation i.e., 96.048 ± 0.666 mg/L/day. Conversely, the addition of KH_2PO_4 of 0.36 g/L at day 20 of cultivation resulted in decreasing lipid productivity. In terms of the control condition, the result displayed a gradual decrease in lipid productivity after 16 days cultivation. Highest biomass concentration of 6.550 ± 0.118 g/L was achieved using 0.36 g/L KH_2PO_4 concentration. Although, the lowest biomass concentrations of 5.180 ± 0.170 g/L followed by 5.820 ± 0.226 g/L, and 6.190 ± 0.071 g/L were found in the culture supplemented with 0, 0.12 and 0.24 g/L of KH_2PO_4. Increase phosphorus concentration in stationary growth phase, the biomass concentration of the microalgae increases. Highest lipid concentration of 2.143 ± 0.018 g/L was gained using 0.24 g/L of KH_2PO_4. The results of varying KH_2PO_4 on lipid concentration indicated that no significant difference was observed on 22 days of cultivation. Highest value for biomass productivity in control condition was 320.469 ± 10.386 mg/L/day. The lowest biomass productivity of 260.000 ± 10.285 mg/L/day followed by 277.500 ± 3.546 mg/L/day, and 291.591 ± 5.678 mg/L/day was found in the culture supplemented with 0.12, 0.24, 0.36 g/L KH_2PO_4, respectively. Highest lipid content of 35.950 ± 4.253% (w/w) was achieved using 0.12 of g/L KH_2PO_4. Nonetheless, lipid content using rest of phosphorus concentrations gave nearly the same results. The lipid content in the absence of phosphorus (28.192 ± 0.432%) was relatively lower than the presence of various phosphorus concentrations. The results of lipid content were non-significant at day 22 of cultivation. On the other hand, the culture with lower phosphate (control) concentration recorded the lowest lipid percentage of 28.192 ± 0.432%. Moreover, the obtained lipid content percentage was found significantly higher when compared with the results of Radha et al. [25] (33.00 ± 4.253%) using similar specie of *Ankistrodesmus*.

There is a well-known hypothesis that if phosphate and nitrate concentrations in the culture medium fall, so does the biomass of microalgae [26,27]. The lipid buildup of microalgae is increased in nitrogen-limited or deficient conditions. Nitrogen depletion,

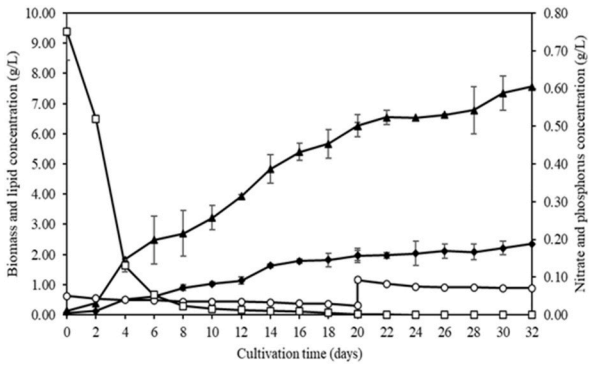

Fig. 6. Change in biomass (▲) nitrate (□) phosphorus (◯) and lipid (◆),concentration of the microalgae cultivated in modified NS III medium with addition 0.36 g/L KH $_2PO_4$ concentration at stationary growth phase.

Table 1
Kinetic parameters: biomass concentration (C_x), lipid content percentage (LC), lipid concentration (C_p), lipids productivity (Q_p), and biomass productivity (Q_x) of *Ankistrodesmus* sp. IFRPD 1061 cultured in modified NS III medium at stationary growth phase under different phosphorus concentrations.

KH_2PO_4 concentration (g/L) [1]	C_x (g/L)	C_p (g/L)	Q_x (mg/L/d)	Q_p (mg/L/d)	LC (% w/w)
0 g/L[2]	5.180 ± 0.170[c]	1.460 ± 0.026[b]	320.469 ± 10.386[a]	90.325 ± 1.543[a]	28.192 ± 0.432[a]
0.12 g/L[3]	5.820 ± 0.226 [bc]	2.088 ± 0.166[a]	260.000 ± 10.285[b]	93.252 ± 7.360[a]	35.950 ± 4.253[a]
0.24 g/L[4]	6.190 ± 0.071 [ab]	2.143 ± 0.018[a]	277.500 ± 3.546[b]	96.048 ± 0.666[a]	34.616 ± 0.681[a]
0.36 g/L[5]	6.550 ± 0.118[a]	1.975 ± 0.170[a]	291.591 ± 5.678 [ab]	87.927 ± 7.685[a]	30.134 ± 2.049[a]

1 = KH_2PO_4 concentration were added at stationary growth phase on day 20 of cultivation; 2 = Calculated kinetic parameters from day 16 of cultivation; 3, 4, 5 = Calculated kinetic parameters from day 22 of cultivation.

together with the availability of a carbon supply, shifts the cellular carbon flux from protein synthesis to lipid synthesis. Aside from that, neutral lipids in the form of triacylglycerols become the dominating lipid components in nitrogen-depleted cells [28,29]. However, increased lipid accumulation is associated with poorer biomass productivity and, as a result, lowers the overall lipid. To achieve a high growth rate as well as a high fat content, a two-stage technique consisting of nutrition sufficient cultivation followed by nitrogen starvation cultivation could be used [30,31].

In contrast, in nitrogen deprivation, lipid percentage of microalgae increases with the decrease in phosphate concentration. Muthuraj et al. [32] discovered that under phosphorus restricting conditions, the carbohydrate content of the alga decreased up to 2.5 times with an increase in neutral lipid content. Acetyl-CoA carboxylase (ACCase) enzyme catalyses the conversion of acetyl-CoA to malonyl-CoA, which is followed by the creation of fatty acids [33,34]. During phosphate limitation, most of the intracellular carbon reserve generated by carbohydrate catabolism is diverted to the Krebs cycle to boost fatty acid synthesis; phosphate constraint increases the expression of ACCasa [2].

Effect of phosphorus and sodium acetate concentration on biomass and lipid productivity in an open pond.

The growth and lipid accumulation, nitrate, and phosphorus concentration during nutrient sufficient cultivation of *Ankistrodesmus* sp. 1061 in NS III medium and added 10 mM sodium acetate on day 0, 4, 8 and 12 of cultivation, using air containing 2% CO_2 under mixotrophic cultivation in an open pond is presented in Fig. 7. The highest values for various parameters attained were: 0.734 ± 0.016 g/L biomass (day 16 of cultivation), 0.215 ± 0.001 g/L lipid concentrations (16 days of cultivation), 88.750 ± 1.768 mg/L/day biomass productivity (2 days of cultivation), 14.550 ± 0.215 mg/L/day lipid productivity (6 days of cultivation), and 33.152 ± 0.000 lipid content (20 days of cultivation), respectively. A rapid drop in nitrate concentration was observed after 10 days cultivation. The concentration of nitrate decreased from 0.498 ± 0.002 g/L to 0.177 ± 0.000 g/L on 20 days cultivation: while phosphorus concentration declined slowly from 0.044 ± 0.000 g/L to 0.038 ± 0.000 g/L in 20 days. Under the mixotrophic condition, carbon, nitrogen and phosphate sources can play significant role in the growth of microalgae and lipid biosynthesis [35]. Additionally, different carbon sources and their concentrations are very important to be optimized for enhancing the growth and lipid accumulation in mixotrophic conditions [36]. Among the organic carbon sources, glucose and sodium acetate are the most effective carbon sources for the growth of mixotrophic microalgae [37]. Sodium acetate is also one of the most used organic carbons in mixotrophic cultivation, that could promote both growth and lipid content in many microalgae [38]. Additionally, there are few major limitations for mixotrophic cultures in open pond such as limitation in controlling contaminations, less control of process parameters, poor mixing, and light distribution.

3. Conclusions

This study reveals the effects of various phosphorus concentrations on growth and lipid productivity of *Ankistrodesmus* sp. IFRPD 1061 under stationary phase using KH_2PO_4. It was observed that 0.12 g/L concentration of KH_2PO_4 enhanced the lipid concentration, lipids productivity and lipid content percentage during phototrophic cultivation. The mixotrophic cultivation using 10 mM sodium

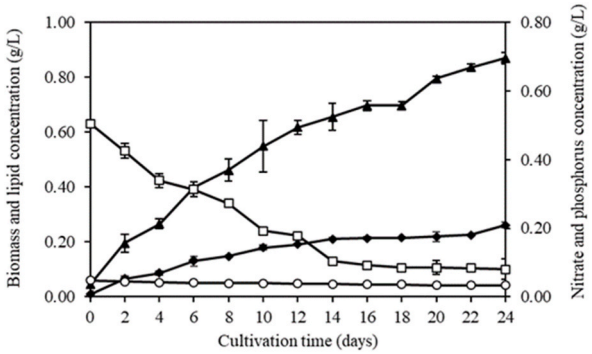

Fig. 7. Change in biomass (▲)concentration, nitrate (□) phosphorus (◯) and lipid (◆),concentration of the microalgae cultivated in modified NS III medium with addition KH $_2PO_4$ concentration at stationary growth phase.

acetate as a carbon source (initially and each fourth day of cultivation) and phosphorus gave high lipid productivity in an open pond.

4. Materials and methods

4.1. Microalgae strain and cultivation

Green microalgal strains were obtained from the Institute of Food Research and Product Development (IFRPD), Kasetsart University, Bangkok Thailand. *Ankistrodesmus* sp. IFRPD 1061 were cultivated in a 50 mL NS III medium (NS III), which was composed of (per liter): 0.015 g $CaCl_2 \bullet 2H_2O$; 0.11 g NaCl; 0.12 g $MgSO_4 \bullet 7H_2O$; 0.28 g $K_2HPO_4 \bullet 3H_2O$; 1.01 g KNO_3; Solution A (0.021 g LiCl; 0.077 g H_3BO_3; 0.415 g KI; 0.592 g KBr; HCl; 2 mL); Solution B (0.05 g $MnCl_2 \bullet 4H_2O$; HCl; 2 mL); Solution C (0.75 g EDTA, 0.81 g Fe $(NO_3) \bullet 9H_2O$; 2 mL) [18,19] in a 200 mL microalgae tube under a thermostat chamber (Fig. 1).

Microalgae cultivation for phosphorus concentration tests in modified NS III medium consisted of 0.24 g/L of KH_2PO_4, with a further addition of KH_2PO_4 at 0, 0.12, 0.24 and 0.36 g/L concentrations during stationary phase (day 20 of cultivation). These microalgae were cultivated and maintained in the light: dark ratio at 16:8 h (10–12 klux of light intensity) at 30 °C, and aeration supplemented with 2% CO_2 for longer time. Later, in an open pond, microalgae were cultured in 100 L modified NS III medium [19].

4.2. Open pond cultivation

Microalgae inoculum was photo-trophically cultivated in a 20 L medium present in an open, transparent, and large round plastic water tank (40 L). The outdoor cultivation using an open transparent, and large round plastic water tank 40 L with 1.0 m wide, 1.2 m long and 0.2 m deep, facilitated with a water turbine for stirring the media at rotation speed 18 rpm shown in Fig. 2. The liquid culture medium was propagated in a 100 L modified NS III medium, adding 10 mM sodium acetate on day 0, 4, 8 and 12 of cultivation, and 0.12 g/L KH_2PO_4 in stationary phase on day 12 of cultivation. An air stream, augmented with 2% (v/v) CO_2 was used to aerate the cultivation with the help of a flow meter at 0.036 vvm. The microalgae were cultivated under natural light, while light intensity, temperature were measured at 9:00am, 12:00pm and 16:00pm. The optical density of initial microalgal medium measured was about 0.2, at OD_{680}. The kinetics of biomass and lipid production were investigated on cultivating the microalgae for a longer period.

4.3. Biomass productivity and concentration determination

Dry cell weight (g) was estimated by filtering 10 mL microalgae culture through Whatman GF/C microfiber filter (diameter 4.7 milli-meter (mm); twice rinsed with distilled water and dried at 105 °C for 24 h. The biomass concentration (C_x, g/L) and the productivity (Qx, g/L/day) was calculated using equations given below:

$$C_x = \frac{C_2 - C_1}{\mathrm{V} \times 10^{-3}}$$

where, C_X: biomass concentration (g/L), C_1: dry blank microfiber filter weight (g), C_2: dry weight of microfiber filter and microalgae cell (g), V: volume of sample for dry cell weight (mL) [21].

$$Q_X = \frac{(X_t - X_0)}{(t_t - t_0)}$$

where, X_t and X_o are dry biomass concentration (g/L) at time t_t and t_0, respectively [22].

4.4. Lipid production measurement

Bligh and Dyer [20] technique (modified) was considered for total lipids extraction. Microalgal cell mass was extracted by centrifugation of suspended sample (50 mL) for 15 min at 6000 rpm; re-suspended in 1 mL sterile water and added with chloroform, methanol, and water mixture at a ratio of 1/2/1, v/v/v (4 mL), sonicated at 100 W and 20 kHz for 15 min (VCX 130, Sonics & Materials Inc., CT, USA). The mixture was subjected to vortex for 30 s; addition of chloroform (1 mL) and water (1 mL); vortexed for 30 s and centrifuged for 15 min at 6000 rpm. The upper layer (methanol/cell) was extracted; whereas the bottom phase (chloroform/water) was transferred to another test tube and evaporated for 24 h in a drying oven at an 80 °C. Lipid content (LC) was quantified as a percentage of dry cell weight, and lipid productivity was computed.

$$\mathrm{LC} = \frac{W_L}{W_A} \times 100\%$$

where, W_L (g) is weight of the extracted lipids and W_A (g) is the dry cell microalgae. The lipid productivity was calculated by:

$$Q_P = \frac{Q_X \times \mathrm{LC}}{100}$$

where Qp is productivity of lipids, Qx is productivity of biomass; and LC is the content of lipids and were given as percent dry weight

[22].

4.5. Fatty acid profiles

Harvested microalgal cells were used for free fatty acids' extraction which were further treated modified method of Bligh and Dyer [20]. Fatty acid methyl ester (FAME) was operated by using the modified method of Rios et al. [23] through Agilent J&W GC columns, (DB-WAX, length, 30 m; diameter, 0.25 mm; film, 0.25 μm; temperature limits: 20–250 °C); chromatographic conditions: 1 μL injection volume, 50:1 split ratio. The FAMEs were identified using standard FAME mixes C8:0–C22:0 (Sigma, Supelco CRM18920). The initial oven temperature i.e., 140 °C for 5 min, was raised with flow rate at 4 °C/1 min until 230 °C and held at 250 °C for 5 min; with a detector temperature of 250 °C. Helium was used as a carrier gas at 1 mL/1 min flow rate.

4.6. Nitrate determination

Determination of nitrate in the growth medium was performed by American Public Health Association techniques [24]. Briefly, the filtration of microalgal cells was carried out through a 0.22 μm pore size filter. A 5 mL sample after proper dilution with water was added to 0.1 mL HCl solution, which was used for analysis of absorbance at 220 nm to acquire a NO_3–N reading and at 275 nm to detect interference owing to dissolved organic matter using a spectrophotometer (Shimadzu, UV-1201, Japan). Potassium nitrate was used for calibration between nitrogen concentration and absorbance.

4.6.1. Phosphorus determination

A well explained method of American Public Health Association [24] was used for the measurement of phosphorus concentration in medium by filtering the microalgal cells through a 0.22 μm pore size filter.

A 1 mL diluted sample, and 1 mL of Vanadomolybdophosphoric acid (A mixture of reagent A i.e. 25 g Ammonium molybdate, $((NH_4)_6Mo_7O_{24}{\cdot}4H_2O)$ in 300 mL distilled water, and reagent B i.e. 1.25 g Ammonium meta-vanadate, (NH_4VO_3) in 300 mL boiled distilled water) were mixed up, and made the final volume up to 1 L by adding 300 mL concentrated HCl and water. The samples were analyzed for absorbance at 450 nm by using a spectrophotometer (Shimadzu, UV-1201, Japan) after proper dilution. Potassium dihydrogen phosphate standard was used for calibration between the absorbance and phosphorus concentration.

4.7. Statistical analysis

All the experiments (determination of dry weight, growth rates, and biochemical assays) were performed in triplicate for data accuracy. SPSS software (Version 22) was used for statistical analysis. The differences between treatments were assessed using one-way analysis of variance (ANOVA). In case of significant ANOVA effects, post hoc least significant differences were used to compare the different means (LSD). The standard deviation and the error bars are shown in the figures; the probability values were <0.05.

Authors contribution

Conceived and designed the experiments: Paninee Jarungkeerativimol, Pramuk Parakulsuksatid, Afrasiab Khan Tareen.
Performed the experiments: Paninee Jarungkeerativimol, Imrana Niaz Sultan, Afrasiab Khan Tareen.
Analyzed and interpreted the data: Muhammad Waseem Khan.
Contributed reagents, materials, analysis tools or data: Pramuk Parakulsuksatid,
Wrote the paper: Paninee Jarungkeerativimol, Muhammad Waseem Khan, Imrana Niaz Sultan.

Ethical approval

Not applicable.

Consent to participate

All authors contributed equally.

Funding

Not applicable.

Consent to publish

The authors gave their approval to publish this work.

Availability of data and materials

All the data has been declared.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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