Requirement of Cyanobacteria *Synechococcus elongatus* for the biomass and lipid production

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**Abstract.** *Synechococcus elongatus* is unicellular Cyanobacteria having fast growth and can conduct photosynthesis process in its cells. This research purposed to evaluate the potential of *Synechococcus elongatus* as an candidate of biodiesel feedstocks by evaluating its biomass and lipid content in appropriate nutrients. The study was conducted at Asian Institute of Technology, Thailand. The Factorial Completely Randomized Design was used as experimental design in this study. The species was grown in BG II medium which was added by different doses of NaNO3 and KHPO4. Cultivation was conducted for seven days for several parameters, namely appropriate dose of nutrients, specific growth rate, biomass, and lipid content. This study resulted the optimal dose of nitrate from NaNO3 and phosphate from KHPO4 for good growth of *Synechococcus elongatus* which were 289.11 mg/L of NO3- and 22.26 mg/L of HPO4. This species grew well in BG-II medium added optimal nutrients at specific growth rate of 0.34µg/day. The optimal lipid productivity of *Synechococcus elongatus* was achieved at day 6th of cultivation which its dried biomass was 0.21±0.03 g/L and total lipid was 1.89±0.28%. Based on the results, *Synechococcus elongatus* cultivated in BG-II medium was potential tobe biodiesel source with its fast growth and lipid content in its cell.

1. Introduction

Global energy demand has encouraged to develop biofuel as an alternative energy source that has more advantages than energy source based on fossil fuel. Biodiesel which belongs to biofuel contains monoalkyl esters which is derived from organic oils (animal or plant oil) through the transesterification process [1]. Nowadays, utilization of Cyanobacteria as biodiesel feedstock becomes a new research topic that is interested to be explored. Some studies exhibit that the different
cyanobacteria with their various morphological from different habitats could have potential to produce lipids as a precursor of biofuel feedstock. The saturated and unsaturated fatty acids which influence the properties of biofuel, are also found in cyanobacteria [2].

Cyanobacteria belongs to microorganisms that have capability to synthesize primary and secondary metabolites, included hydrocarbons. The photosynthetic process in Cyanobacteria can identify the electrons appearing from the main processes and convert them immediately into the H₂ formation. The calvin cycle of Cyanobacteria organizes the process to produce the proteins, carbohydrates, fatty acids, and lipids. Furthermore, these lipids content can be derived to be biodiesel through complex transformation [3]. This characteristics of Cyanobacteria indicate that it is almost similar to microalgae characteristics.

There are several advantages of microalgae as biodiesel feedstock. Microalgae is microorganism group that can grow rapidly to produce huge biomass in short time and numerous species have 20-50% of oil content in their dried biomass. Microalgae becomes perennial simple plants so its oil production surpasses the oil production of best plants. The necessary of water in microalgae cultivation is less than the necessary of terrestrial plants thus the usage of water amount during cultivation can be decreased. Microalgae also can support in bio-fixation of waste CO₂, so it is ecosystem friendly. Finally, microalgae have ability to conduct the photobiological production of bio-hydrogen and produce valuable co-products [4].

The potential Cyanobacteria that is explored as a biodiesel feedstock is *Synechococcus elongatus*. This statement is based on the composition of fatty acid in *Synechococcus elongatus* that can be a precursor of biodiesel source. The study [5] showed that *Synechococcus elongatus* contained saturated and unsaturated fatty acids and the pathway of saturated fatty acid synthesis in *Synechococcus elongatus* might be modified to improve unsaturated fatty acid production such as omega-3 fatty.

This species is one of unicellular Cyanobacteria that has rapid growth and belongs to a group of photosynthetic prokaryotes that are attractive hosts for biotechnological applications [6]. The Cyanobacterium *Synechococcus elongates* PCC7942 was treated to produce the free fatty acids (FFA) in recent experiment. For upgrading the lipid formation, over expression of acetyl-CoA carboxylase (ACCase) has been tried onto *Synechococcus elongatus* [7]. Finally, The characterizations of lipid composition in *Synechococcus elongatus* consists of monounsaturated fatty acid and short-chain saturated in huge proportion, and lack of sterols and PUFAs [8]. The existence of lipid in the cells of *Synechococcus elongatus* becomes attractive point for resulting high lipid yield as biodiesel precursor. While, the high lipid yield is also influenced by lipid productivity of this species.

For supporting the lipid productivity of *Synechococcus elongatus*, it needs to produce high biomass of this species through supporting its growth by providing several appropriate nutrients for its growth. Based on this description, the research of Nutrient Requirement of Cyanobacteria *Synechococcus elongatus* for the Biomass and Lipid Production is interested to be explored. This research aimed to evaluate the nutrient requirement of Cyanobacteria *Synechococcus elongatus* for the biomass and lipid production as an effort to develop biodiesel based on Cyanobacteria feedstock.

2. Materials and Method

The study was conducted at Asian Institute of Technology, Thailand with the materials of this research were *Synechococcus elongatus* TISTR 8500 gained from Thailand Institute of Scientific and Technological Research (TISTR) and some chemicals used during the experiment. Those chemicals such as BG-II media consisted of K₂HPO₄·H₂O, NaNO₃, CaCl₂·H₂O, MgSO₄·H₂O, Na₂CO₃, Fe₃(SO₄)₂·H₂O, citric acid, EDTA, and trace elements and petroleum ether as a solvent in lipid extraction.

*Cyanobacteria stock culture*

Biomass cultivation was started by conducting stock culture of *Synechococcus elongatus*. The standard medium used was BG-II medium. The initial inoculum of cyanobacteria at 5 mL was grown
in 100 mL BG-II medium under photoautotrophic growth condition with continuous illumination at 2000 lux. The temperature of incubation was at 28 °C with placement of stock culture in a continuous aeration. The stock culture was renewed by aseptically culturing the inoculums 10% into new BG-II medium every day 7th of cultivation day. During the experiment, the BG-II medium and used equipments were sterilized by using autoclave at temperature 121°C for 15 minutes. The composition of BG-II medium is shown in Table 1.

**Required of nitrogen-phosphate level of cyanobacteria and lipid yield**

Determination of nitrogen-phosphate requirements of *Synechococcus elongatus* was conducted through calculating the concentration of NO₃ and HPO₄ (the molarity of nitrate and phosphate) contained in BG-II medium which was composited of 10 mL of NaNO₃ and 1 mL of K₂HPO₄. The concentrations of NO₃ and HPO₄ which were determined in this experiment were categorized into several levels. Determination of nitrate and phosphate levels were based on basic NaNO₃ and K2HPO4 amount added into stock solution of BG-II medium for *Synechococcus elongatus*. Then, those basic nutrients amounts were varied into several levels.

The levels of NO₃ were:
- a. 6 gram/200 mL of NaNO₃ stock solution containing 128.94 mg/L NO₃
- b. 12 gram/200 mL of NaNO₃ stock solution containing 289.11 mg/L NO₃
- c. 18 gram/200 mL of NaNO₃ stock solution containing 386.81 mg/L NO₃
- d. 24 gram/200 mL of NaNO₃ stock solution containing 515.74 mg/L NO₃
- e. 30 gram/200 mL of NaNO₃ stock solution containing 644.68 mg/L NO₃

The levels of HPO₄ were:
- a. 1.6 gram/200 mL of K₂HPO₄ stock solution containing 19.41 mg/L HPO₄
- b. 3.2 gram/200 mL of K₂HPO₄ stock solution containing 20.82 mg/L HPO₄
- c. 4.8 gram/200 mL of K₂HPO₄ stock solution containing 22.27 mg/L HPO₄
- d. 6.4 gram/200 mL of K₂HPO₄ stock solution containing 23.63 mg/L HPO₄
- e. 8 gram/200 mL of K₂HPO₄ stock solution containing 25.16 mg/L HPO₄

Daily lipid was observed per day through analyzing lipid content using soxhlet method. Lipid content of *Synechococcus elongatus* was observed for 7 days during cultivation. Lipid content was extracted by using petroleum ether as the lipid solvent.

**Table 1. BG-II Composition for *Synechococcus elongatus* Cultivation.**

| Chemical           | Stock solution (g/200 ml) | Culture solution ADD: ml/L |
|--------------------|---------------------------|----------------------------|
| NaNO₃              | 12                        | 10                         |
| K₂HPO₄·H₂O         | 4.8                       | 1                          |
| MgSO₄·H₂O          | 15                        | 1                          |
| CaCl₂·H₂O          | 7.2                       | 1                          |
| Na₂CO₃             | 4                         | 1                          |
| Citric acid        | 1.2                       | 1                          |
| Fe₂SO₄·H₂O         | 1.2                       | 1                          |
| EDTA               | 0.2                       | 1                          |
| Trace elements     | *                         | 1                          |
| - H₂BO₃            | 2.68                      | 1                          |
| - MnCl₂·H₂O        | 1.81                      | 1                          |
| -ZnSO₄·H₂O         | 0.22                      | 1                          |
| -Na₂MoO₄·H₂O       | 0.39                      | 1                          |
| - CuSO₄·H₂O        | 0.079                     | 1                          |
| - Co(NO₃)₂·H₂O     | 0.049                     | 1                          |
Biomass harvesting

\textit{Synechococcus elongatus} biomass was collected when the accumulation of lipid was high in its dried cells. The harvest process was conducted by centrifugation (in small volume) and flocculation (in huge volume). The centrifugation method was used in the speed of 4000 rpm for 10 minutes. The effective flocculation was achieved by adjusting pH ranging 8.6-10.6 with 10% alum \([Al_2(SO_4)_3]\) in 1mL/50mL of culture. \(CaCO_3\) was added into harvested stock culture to optimize the pH of biomass through neutralizing the alum contained in harvested stock culture.

Microalgae oil extraction

Extraction of lipid content of \textit{Synechococcus elongatus} was done by using soxhlet method. The lipid extraction used petroleum ether as the solvent. The lipid content in sample was calculated by using this formula below:

\[
\text{Lipid content of sample (\%)} = \frac{(W_3 - W_2)}{W_1} \times 100\% \quad \text{.....................................}[9]
\]

Where, 
- \(W_1\) : Weight of sample (gram of dry weight)
- \(W_2\) : Weight of cup
- \(W_3\) : Weight of cup + lipid

Data Analysis

The experimental design was Factorial Completely Randomized Design consisted of two factors, which were different levels of nitrate and different levels of phosphate. The observed data were analyzed by using two ways anova with replication at confidence level 95% and processed by SPSS software. The summary of data was shown through graphic and table.

3. Result and Discussion

\textit{Synechococcus elongatus} had ability to produce huge biomass by generating new cells through binary fission in short time. This species has specific green color with its cylindrical cells form. The existence of chlorophyll-a was proven by its green color and it is implied that it is photosynthetic microorganism. The cell morphology of \textit{Synechococcus elongatus} is shown at Figure 1 below. According to the result of this study, this species needed low nitrate and phosphate concentration for its growth.

![Figure 1. Synechococcus elongatus culture and its cells (100 x Magnification).](image)

The specific growth rate (SGR) of \textit{Synechococcus elongatus} was significantly affected by different levels of nitrate concentration (P-value 0.005 < 0.05). The best growth performance was shown by 289.11 mg/L of nitrate concentration (NO\(_3\)), whereas the value of the specific growth rate was 0.34 \(\mu\)/day. The nitrate concentration at 289.11 mg/L was significantly different from 128.94 mg/L and 386.81 mg/L. While, the concentrations of NO\(_3\) at 515.74 mg/L and 644.68 mg/L showed the similar effect to 289.11 mg/L of NO\(_3\) concentration on specific growth rate parameter. The performance of specific growth rate (SGR) of \textit{Synechococcus elongatus} at various nitrate-phosphate levels are shown in Figure 2. The small amount of nitrate consumed by \textit{Synechococcus elongatus}
correlate to salts mobility occurred on membranes from nitrate-adapted cell that had interaction or NO₃⁻ binding. [10] Consumption of nitrate on other cyanobacteria, namely *Microcystis viridis* was also low. The uptake and assimilation of nitrate in the cell of that cyanobacteria was lower than ammonium as medium nutrient.

![Figure 2. The specific growth rate of *Synechococcus elongatus* at various nitrate-phosphate levels added in BG-II medium.](image)

The adapted cells cultured in low nitrate medium suffered low mobility of plasmalemma vesicle. The vesicles from adapted cells in low nitrate medium became the more positively charged and less mobile by adsorption of Na⁺ or K⁺ ions. In which, a high ‘nitrate protein’ content was correlated with a low mobility and conversely [11]. Therefore, nitrate consumption of *Synechococcus elongatus* was low in BG-II medium. This result was in same line with the statement revealed by [12] that *Synechococcus* peaked at 10⁴ cell/mL when nitrogen concentrations were lowest inshore.

Different from *Synechococcus elongatus*, [13] the study of *Nostoc muscorum* showed that it needed high concentration of nitrate and phosphate for its life to perform high biomass. The multicellular cyanobacteria species *Nostoc muscorum* required 644.6795 mg/L of NO₃⁻ and 25.1566 mg/L of HPO₄²⁻. This difference may be caused by their morphology and the mechanisms of producing new cells. The multicellular species will need higher energy and nutrients to produce new cells than unicellular species.

The performance of specific growth rate of *Synechococcus elongatus* was also significantly affected by various phosphate levels (P-value 0.00< 0.05). The best specific growth rate was shown by 22.26 mg/L of phosphate concentration, with the value of SGR was 0.34 µ/day. Based on statistical analysis, the concentration at 22.26 mg/L of phosphate was significantly different from 19.41 mg/L and 20.82 mg/L of phosphate concentration. However, concentration 23.63 mg/L of phosphate gave similar effect to 22.26 mg/L of phosphate concentration on growth parameter. The appropriate nitrate and phosphate nutrients resulted high specific growth rate on *Synechococcus elongatus*. The specific growth rate of *Synechococcus elongatus* in various levels of phosphate can be seen at Figure 3.
Figure 3. The specific growth rate of *Synechococcus elongatus* at various levels of nitrate-phosphate added in BG-II medium.

Phosphorus is involved as the important trace-elements for supporting life of organisms and microorganisms. Cyanobacteria as one of autotrophic organisms can assimilate the form of phosphorus, namely Orthophosphate. In this study, *Synechococcus elongatus*, utilized the phosphate nutrient for metabolism and production of DNA and proteins through forming acid soluble phosphate (ASP) inside the cells in their growth [14]. In addition, the accumulation of acid insoluble phosphate as polyphosphate was also stored inside the cells of Cyanobacteria. In where, the maximum phosphate that could be utilized by *Synechococcus elongatus* was 22.26 mg/L of phosphate (>15 mg/L of phosphate concentration).

The absorption of phosphorus in microalgae was influenced by phosphate concentration, light intensity, pH, energy, and temperature [14][15]. According to the reference, continuous light intensity during cultivation of *Synechococcus elongatus* also influence the absorption of phosphate. The synthesis of ASP could encourage the metabolism and production of DNA and proteins for Cyanobacteria’s growth.

During the study, cultivation of *Synechococcus elongatus* was conducted in phototrophic condition with energy source from continuous light intensity at 2000 lux and certain carbon source from inorganic carbon (Na$_2$CO$_3$). The lipid productivity and lipid content parameter related inversely each other. It meant that high biomass for lipid productivity would be achieved at low lipid content of biomass. While, the high lipid content might be caused by deprivation of nutrients and other stressed conditions [16]. The general principle of lipid formation related to nitrogen extinction is that inadequate nitrogen for producing protein required for growth causes the excess metabolite of carbon from photosynthesis is channelled into triacylglycerols or starch as storage molecules.

The total lipid of *Synechococcus elongatus* decreased during cultivation for 7 days. This decreasing total lipid of *Synechococcus elongatus* related to enrichment BG-II medium with the optimal nitrogen concentration supporting the growth of species. The growth of *Synechococcus elongatus* was rapid which could reach 0.26 g/L of dried biomass for 7 days culture (Table 2). High nitrate content encouraged the increment of cell size to be larger or longer than previous cells which was initiated by protein synthesis and utilization of carbon as energy in the cell. Expansion of the cells naturally resulted cell division through binary fission therefore, reproduction of *Synechococcus elongatus* became rapid in good nutrient condition. Evaluation of biomass and total lipid of *Synechococcus elongatus* cultured in BG-II medium is shown in Table 2.
Table 2. Evaluation of biomass and total lipid of *Synechococcus elongatus* cultured in BG-II medium.

| Day of culture | Biomass (g/L) | Total lipid (%) | Lipid productivity (mg/g) |
|---------------|--------------|----------------|----------------------------|
| 0             | 0.05±0.01    | 12.85±2.66     | 128.48±26.64               |
| 1             | 0.06±0.00    | 4.49±0.25      | 44.94±2.47                 |
| 2             | 0.07±0.00    | 4.17±0.24      | 41.67±2.37                 |
| 3             | 0.12±0.00    | 1.70±0.19      | 16.96±1.94                 |
| 4             | 0.14±0.01    | 1.97±0.03      | 19.66±0.34                 |
| 5             | 0.17±0.00    | 0.65±0.07      | 6.53±0.73                  |
| 6             | **0.21±0.03**| **1.89±0.28**  | **18.87±2.79**             |
| 7             | 0.26±0.01    | 0.57±0.00      | 5.67±0.03                  |

Comparative study of biomass and total lipid of *Synechococcus elongatus* cultured in BG-II medium in Figure 4 showed the contrast proportion in which biomass of *Synechococcus elongatus* had tendency to increase during culture for 7 days observation while the total lipid tended to decrease dramatically. Determination of the optimal lipid productivity was decided through determining high biomass with the optimal lipid content during cultivation. Therefore, high lipid productivity at day 6th was determined by considering the high biomass production with the optimal lipid content at day 6th. Biomass at 0.21 gram/L and lipid productivity at 18.87 mg/g could be expected to give best choice for harvesting and extracting the lipid from *Synechococcus elongatus*. Therefore, the best time of harvesting *Synechococcus elongatus* was on day 6th.

![Figure 4. Comparative study of biomass and total productivity of Synechococcus elongatus cultured in BG-II.](image_url)

Limitation of nutrient is an efficient trigger in increasing the lipid content per algal biomass, and it has been reported by many other researchers. For example, [17] the experiment was conducted in 110-L panel photobioreactor, alteration of culture condition from “nitrogen sufficient” to “nitrogen deficient”, the lipid content of *Nannochloropsis* increased from 32% to 60%. [17] Under the condition of phosphorus limitation causing the dramatic increase in TAG levels from 6.5% up to 39.3% of total lipids, the total cellular lipid content of starved cells also tended to increase.
4. Conclusion

*Synechococcus elongatus* could grow in BG-II medium added optimal nutrients at specific growth rate of 0.34µg/day. The optimal lipid productivity of *Synechococcus elongatus* was achieved at day 6th of cultivation which its dried biomass was 0.21±0.03 g/L and total lipid was 1.89±0.28%. *Synechococcus elongatus* required 289.11 mg/L of nitrate (NO$_3^-$) and 22.26 mg/L of phosphate (HPO$_4^{2-}$) which showed the highest specific growth rate.

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