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THE EFFECT OF NITROGEN STRESS IN MEDIUMFOR INCREASING CARBOHYDRATE AS A BIOETHANOL SOURCE AND CAROTENOID AS AN ANTIOXIDANT FROM CHLORELLA ZOFINGIENSIS CULTURE

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ABSTRACT

Chlorella zofingiensis is a prospective microalgae because it is mainly ascarotenoid producer, such as astaxanthin. However, its carbohydrates could be also as promising source ofbioethanol. Furthermore, Nitrogen stress treatment is reported used for increasing both carbohydrate and carotenoid of some microalgae. Therefore, this study aimed to increase carbohydrate and carotenoid of microalgae *C. zofingiensis* by using low and high nitrogen excessin the growth medium. The mediums were consisted of local compound fertilizer (farmpion), urea and ZA with a ratio of 0.25:0.5:1(low nitrogen excess medium) and 0.5: 1: 2(high nitrogen excess medium). Its cells density, carotenoid, and carbohydrate were measured every day for 7 days. The cell density was calculated using haemocytometer under light microscope. The carotenoid was measured using spectrophotometer with absorbance at a wavelength of 470, 645 and 662 nm. The carbohydrate was measured using sulfuric acid method. The results showed that the nitrogen stress treatment was able to increase carbohydrates and carotenoids approximately twice in *C. zofingiensis* as culture as source of bioethanol and antioxidant.

Keywords: chlorella zofingiensi, nitrogen, carotenoid, carbohydrate.

INTRODUCTION

Chlorella zofingiens is freshwater green algal that classified into class and family Chlorophyceae and ordo Chlorococcales [1] and indicates having high carbohydrate content as bioethanol source [2]. That product has been proposed as good alternative to non-renewable fossil fuels [3]. Microalgal is as a potential new generation of feedstock for biofuel production because of its high growthh rates and high photosynthetic efficiencies when grown on specific environments [4].

When the microalgae is cultivated and grown in extreme environmental conditions and under stress circumstances, it will synthesize various secondary metabolites to increase the possibility for surviving under those stress conditions [5, 6]. One of the secondary metabolites produced by the microalgae is carotenoid asantioxidant property [7]. Microalgae *C. zofingiensis* is reportedable accumulate the carotenod, especially astaxhanthin as a king of antioxidant [8], so that that the microalgae is potentially as astaxanthin producer [9].

Aprospective method for enhancing its valuable contents is by modifying Nitrogen concentration in the growth medium. Nitrogen is an essential nutrient for increasing *C. Zofingiens* is carbohydrate and carotenoid [10]. Nitrogen is used for producing chlorophyll which is an important compound to accelerate the photosynthesis rate.

Therefore, the aim of this research is to increase carbohydrate and carotenoid of microalgae *C. zofingiensis* by nitrogen excess treatments in the medium. The research will be an alternative method for producing bioethanol from high carbohydrate microalgae as a future renewable energy as well as producing carotenoid as a valuable antioxidant.

MATERIALS AND METHODS

Materials

This research used medium agricultural fertilizers: urea: ZA with a ratio of 0.5: 1: 2 (high excess nitrogen medium) and 0.25: 0, 5: 1(low excessnitrogen medium).

Method

The study was conducted in Wukir Sari, Cangkringan, Pakem, Sleman, Daerah Istimewa Yogyakarta (DIY). *C. zofingiensis* was cultured on a mass scale in the 3600 litre pool. Environmental parameters measured were temperature, pH, and density. The mediums were local agricultural fertilizer (farmpion), urea and ZA with ratio of 0.25: 0, 5: 1 (low excess nitrogen medium) and 0, 5: 1: 2 (high excess nitrogen medium). As a control, *C. zofingiensis* was cultivated in medium with local agricultural fertilizer (farmpion) without the addition of urea and ZA. Nitrogen content in fertilizers and ZA 21%, while the nitrogen contentinUrea46%. Samples were taken every day for 7 days. The parameters measured were cells density, carotenoid, and charbohydrate.

The Calculation of the Cells Density

Cell counting using Haemacytometer, samples were taken 800µL inserted into the tube 2mL was added 200µL 70% alcohol, and wait about 20 minutes before counted under a microscope. Calculations done by counting cells with two fields of view on Haemacytometer then calculated using the following formula:

$$cellsdensity\frac{cell}{mL} = \frac{numberofcellscounted}{5} xdilutionfactorx\ 25\ x10^4$$

Calculation of Carotenoids

Samples were taken 10 mL inserted into the tube, then centrifuge at a speed of 3300 rpm for 15 minutes. The supernatant was discharged; andthe sample was added by 2 mL of acetone, then centrifuged again with a rate of1800 rpm for 10 minutes. The sample was transferred into a glass cuvette spectrophotometer and calculated by using absorbance at a wavelength of 470, 645 and 662 nm. The carotenoids could be calculated by using the following equation [11].

$$Carotenoid\left(\frac{mg}{L}\right) = \frac{(A470nm - A662nm)x\ 25\ x\ 1000}{200\ xsamplevolume\ (mL)}$$

Calculation of Carbohydrates

Standard curve made used standard glucose, with concentration of 0,025 g/l, 0,05 g/l, 0,1 g/l, 0.25 g/l and 0.5 g/l was observed by using a spectrophotometer with a wavelength 0f 492 nm. The samples were taken and added 30 mL phenol and 150 mL sulfuric acid, then let stand for 30 minutes and the absorbance was measured using a spectrophotometer with a wavelength of 490 nm.

RESULTS AND DISCUSSIONS

As a photosynthetic organism, *C. zofingiensis* has pigments used for harvesting light energy. Chlorophyll was the main pigment of photosynthesis. Chlorophyll was composed of tetraphyrrole ring containing atoms of magnesium, nitrogen and long chain terpenoids [12]. So that the provision of nitrogen could increase the formation of chlorophyll. Increased formation of chlorophyll could increase the rate of photosynthesis and the more energy was produced. Nitrogen was an important element in the growth of microalgae. Nitrogen was an important nutrient after the formation of carbon in biomass [13]. Nitrogen was generally available in the form of nitrate (NO_3^-) and ammonia (NH_4^+). Nitrogen sources in this experiment were urea and ZA. Urea was a nitrogen source that could provide CO_2 for photosynthesis [14]. A high nitrogen content that was 2-3 times higher than normal concentration in the medium increased the synthesis of chlorophyll pigments in cells [15].

When the chlorophyll content increased, the rate of photosynthesis would also increase. If the process of photosynthesis increased, the carbohydrates produced from photosynthesis process would also increase. Carbohydrates formed through the photosynthesis process, most carbohydrates were used as energy through respiration reaction [16]. While others were stored in carbohydrate glandular or in the form of cellulose of cell walls, to result in increased cell biomass [17]. Carbohydrates were used for energy sources. Energy produced through photosynthesis was used to multiply the number of cells and stored in the form of carbohydrates, especially starch and cellulose [13].

In this study, increasing of carbohydrate production in photosynthesis was as source of energy. The carbohydrate could be used for bioethanol production to conduct an alternative energy. Microalgae had the ability to alter their biomass composition under stress conditions to accumulate more carbohydrates. The carbohydrates were broken into their sugars. These sugars could be fermented to produce bioethanol [2].

As could be seen in Figure-1, the carbohydrate per cells in both low and high excess nitrogen mediums tend to increase. In all treatments, the carbohydrate per cell decreased significantly at the day 5 as the number of cells was at the pick. However, the trend increased dramatically at the day 6 to 7. It was predicted that the cell growth slowly, but the amount of carbohydrate still increased at those days.This indicated that the nitrogen concentration affected on the formation of carbohydrates indirectly. The nitrogen as an element that forming the light-harvesting chlorophyll accelerated photosynthesis products accumulated in the formation of carbon biomass [13].

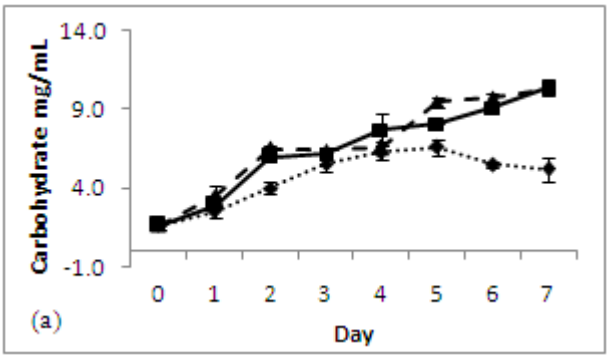

Figure-1. (a) Carbohydrate total in *C. zofingiensis* (b) Carbohydrate per cell content in *C. zofingiensis* Control Low nitrogen excess High nitrogen excess.

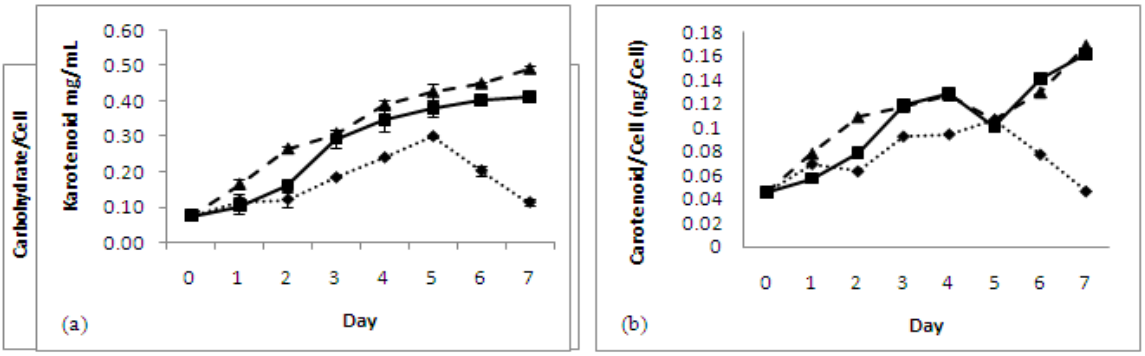

Figure-2. (a) Total carotenoid in *C. zofingiensis* (b) Carotenoid per cell content in *C. Zofingiensis* Control Low nitrogen excess High nitrogen excess.

Some microalgal species cultured under stress conditions could accumulate specific secondary metabolites, such as pigments, vitamins or lipids [18]. As shown in Figure-2, the carotenoid per cell tend to increase, but the ratio of carbohydrates to carotenoids tend to decline (Figure-3) in both low and high nitrogen excess mediums. It was assumed that both treatments tend to form more carotenoids than carbohydrate. Carotenoids were pigments that were involved in the light harvesting reaction and protection of algal cellorganelle against single

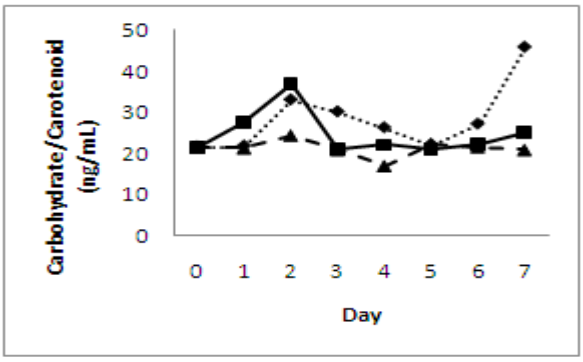

Figure-3. Carbohydrate per carotenoidtotal in *C. zofingiensis* Control Low nitrogen excess High nitrogen excess.

Oxygen damage [19]. Carotenoids in green algal were produced in two different pathways, i.e. acetic-mevalonate pathway and phosphegly ceraldehyde-pyruvate pathway [20, 21]. More carotenoids produced due to environmental stress or adverse culture conditions such nutrient excess and starvation [22]. The nitrogen stress stimulated the formation of carotenoids, especially astaxan thin [23].One of the studies that have been done on *Haematococcuspluvial is* also showed that heantioxidant pigment astaxan thin produced in a variety of stress conditions such as high light, salinity, nutrient stress, the concentration of carbon or high nitrogen [24, 25, 26]. It could be clearly stated that nitrogen was an important nutrient for biomass production [13] and carotenoid production [27].

Synthesis of astaxanthin required nitrogen, and most likely reflected the need for continuous synthesis of protein in order to support the massive accumulation of pigments [28]. Nitrogen was essential element for de novo synthesis of enzymes responsible for carotenoid formation [27]. The nitrogen stress could also increase the carbohydrate content in the cells. The carbohydrate could be broken to be sugars and fermented to produce bioethanol [2] and the carotenoid could be used as antioxidant. It could be concluded that both low and high nitrogen increased carbohydrate and carotenoid content in *C. Zofingiensis.*

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