The Effect of Copper Nanoparticle to Astaxanthin Content in Microalgae

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ABSTRACT *Haematococcus pluvialis* is kind of microalgae which produces high yield of astaxanthin under stress, such as when exposed to high temperature, pH, salinity, light condition, and nitrogen deficiency. The aim of this study is to determine the effect of Cu nanoparticle to the growth of *H. pluvialis* and the production of astaxanthin in the mcroalgae. The microalgae were cultured and exposed to 10 mg/L, 100 mg/L, and 200 mg/L of Cu nanoparticle respectively, with growth cycle of 20 days. The astaxanthin were extracted and quantified at 474 nm using a spectrophotometer in every 4 to 5 days. The results showed the presence of Cu nanoparticle diminished the cell growth and reduced the content of astaxanthin in *H. pluvialis*. Cu nanoparticle is not a good stress agent for astaxanthin production in *H. pluvialis*. However, the microalgae are proven sensitive to the presence of Cu nanoparticle. The potential of astaxanthin in *H. pluvialis* as bioindicator of Cu nanoparticle can be further explored.

KEYWORDS: Astaxanthin; Cu nanoparticle; Cell toxicity; Microalgae; Photosynthetic pigment

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INTRODUCTION

Astaxanthin is a type of colour pigment which can be widely found in many marine animals, e.g. shrimps, salmons, crayfish and lobster (Chen *et al.*, 2015). Besides, the pigment can be found in yeast and microalgae. Astaxanthin is produced in plants and photosynthetic microbes, sharing same biosynthesis pathways with several types of carotenoids, e.g. lutein, lycopene and carotenoids (Borowitzka, 2013; Wang *et al.*, 2014).

Astaxanthin is a member of carotenoid family. Due to the natural characteristic of providing vivid colouring and strong antioxidant property (Naguib, 2000; Seabra & Pedrosa, 2010), the pigment has been widely used as health supplements, skin care and cosmetic products, and colouring pigment for aquaculture industry.

Haematococcus pluvialis is a green microalga that is found to produce a large amount of astaxanthin (Guerin *et al.*, 2003). The accumulation of astaxanthin occurs during the conversion of the green vegetative cells to the aplanospore phase (Borowitzka, 2013). The green algae have been widely used as a source for naturally produced astaxanthin. The productivity of astaxanthin has been found related to cellular stress or unfamiliar conditions like high temperature, high salinity, extreme light condition, and nitrogen deficiency (Guerin *et al.*, 2003).

Although extensive attempts had been conducted to induce stress to *H. pluvialis*, the stress from a kind of relatively new environmental toxicant- metal nanoparticles has not been explored. Nanoparticles are generally referred to particles with one or more dimensions of the order or 100 nm or less. Many of these particles are artificially produced. The toxicity of nanoparticle to higher plants (Lee *et al.*, 2008) and microalgae (Suman *et al.*, 2015) has been reported.

The exposure of metal nanoparticles to microalgae would cause changes in cellular antioxidants, suggested that the presence of metal nanoparticles would increase oxidation stress in cells. Reports confirmed the presence of Cu nanoparticles caused inhibition of growth of microalgae

Pseudokirchneriella subcapitata (Aruoja *et al.*, 2009) *Chlamydomonas reinhardtii* (Perreault *et al.*, 2012). However, the effect of Cu nanoparticle to *H. pluvialis* has not been reported before. Thus in this study, the effect of Cu nanoparticle to the growth and the content of astaxanthin in *H. pluvialis* was studied.

METHODOLOGY

Preparation of Cell Culture

Microalga *H. pluvialis* was obtained from the University of Texas at Austin, Texas, United States of America. Pure copper (II) oxide nanoparticles in powder form (diameter > 100 nm), Bold's Basal (BB) stock medium (50x), and HPLC grade acetone were purchased from Sigma-Aldrich, Kuala Lumpur, Malaysia. For culturing work, 100 mL of BB culture medium was prepared by adding two mL of BB stock medium to 98 mL of distilled water. A volume of 3 – 5 mL of microalgae was transferred into a conical flask with 100 mL of BB medium. The microalgae was then grown at room temperature, with dark and light conditions maintained at 8 hours and 16 hours. The light was provided by fluorescence light. Aeration was carried out manually on a daily basic. All the culturing work was conducted in sterile condition. The cell growth was monitored through cell count using light microscope (Y-103 RaxVision) and hemocytometer (Neubauer, Marienfeld).

Copper Nanoparticles Exposure

Three different concentrations of Cu nanoparticle (10 mg/L, 100 mg/L, and 200 mg/L) were prepared by adding the nanoparticle to the culture (w/v). The cultures were then incubated for 20 days. The content of carotenoids were then determined every 4 to 5 days. The culture without the exposure to Cu nanoparticle was used as negative control. All the exposure tests were conducted in triplicates.

Astaxanthin Content Determination

Astaxanthin was extracted by following the method developed by Sarada *et al.* (2006). A volume of 5 mL of the culture was extracted and mixed with 1 mL of HCl for 5 minutes at 70 °C, followed by centrifugation 5000 rpm for 6 min at 4 °C. The pellets were gently washed with distilled water. The pellet was then resuspended with 1 mL of acetone (HPLC grade). The content of astaxanthin was determined with spectrophotometer at 474 nm (GeneQuant 1300, GE), using Equation 1 (Kelley & Harmon, 1972).

$$\text{Astaxanthin estimation } (\mu g/g) = (A \times D) / (100 \times G \times d \times E_{1cm}^{1\%}) \quad (1)$$

Where,
A is absorbance at 474 nm
D is the volume of extract in acetone, in this study = 3.5 mL
G is the weight of sample in g, in this study = 0.0045 g
d is the width of cuvette, in this study = 1 cm
E is extinction coefficient, in this study = 2100

RESULT AND DISCUSSION

The cell count indicated *H. pluvialis* for negative control (without Cu nanoparticle) grew fast on the first 5 days of culture. The number of cell reached maximum on day 10, and maintained at the plateau until day 20 (Figure 1). The cell growth profile did not exhibit a clear log and exponential phases. However, after day 10, the cell growth entered stationary phase where the number of viable cells remained the same. The rate of cell growth was equal to the rate of cells death, due to the

exhaustion of nutrients in the media (Navarro Llorens *et al.*, 2010). The cell growth in the culture with Cu nanoparticle showed lower growth rate compared to negative control.

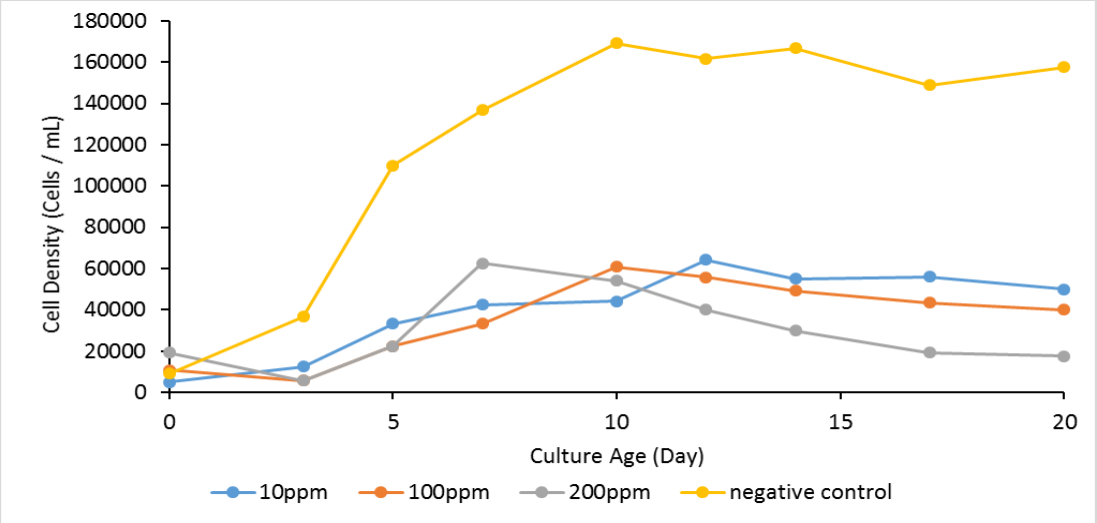

Figure 1. The growth of *H. pluvialis* in 20 days of culture.

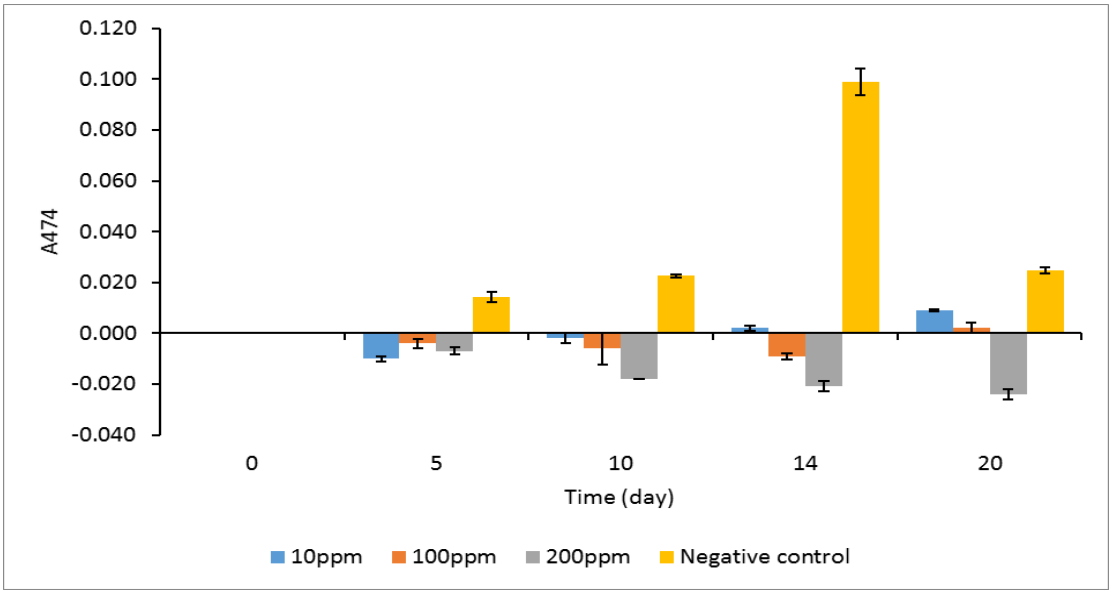

Figure 2. The change in astaxanthin content with exposure to different concentration of Cu nanoparticle. The content of astaxanthin at day-0 is used as baseline.

The cells for negative control reached maximum growth on day-10, while the maximum production of astaxanthin recorded on day-14 (Figure 2). The content of astaxanthin was lowered with the presence of Cu nanoparticle. Therefore the presence of Cu nanoparticle had interrupted the metabolisms of the cells. The presence of nanoparticle might affect the cells viability by inhibiting photosynthesis process, damaging cell membrane, entering the cells and destroying thylakoid, and causing reduction of the photosynthetic pigments (Djearamane *et al.*, 2018; Oukarroum *et al.*, 2012). Long term exposure to nanoparticle might cause cell membrane rupture and cell death. According to the report by Aruoja *et al.* (2009), growth inhibition of microalgae *P. subcapitata* was confirmed with the exposure to CuO, ZnO, and Ti_2O nanoparticles. Another study done by Perreault *et al.* (2012) confirmed the presence of CuO nanoparticle reduced the number of microalgae *C. reinhardtii*.

The quantity of astaxanthin decreased as the concentration of the Cu nanoparticle increased (Table 1). The presence of 200 mg/L of Cu nanoparticle in the medium caused highest reduction in

astaxanthin content. Although aggregation of the nanoparticle in water with the presence of organic matters might alleviate the toxicity effect of the nanoparticle (Chekli *et al.*, 2015; Prathna *et al.*, 2011), the effect could only be reflected on the *H. pluvialis* exposed to Cu nanoparticle concentration of 10 mg/L (day 14). The reduction of astaxanthin might cause by the deterioration of biosynthesis pathway of the pigment, or by the reduction of viable cells.

Microscopic examination on different stages of *H pluvialis* showed the cells were disrupted by the presence of Cu nanoparticle. Green coccoid cells (palmelloid) was observed at log phase (Figure 3), intermediate cells and cysts (aplanospore) were available at the end of exponential phase, and when the cells entered stationary phase, the colour of the cells faded, membrane deteriorated, and organelles deformed. The results were in line with the study on the effects of ZnO nanoparticles to *Spirulina platensis* done by Djearamane *et al.* (2018).

Table 1. The content of astaxanthin in *H. pluvialis* exposed to different concentrations of Cu nanoparticles

Time (day)	Average content of astaxanthin (μg / g), n = 3			
	10 mg/L	100 mg/L	200 mg/L	Negative control
0	0.006	0.010	0.019	0.005
5	0.003	0.008	0.016	0.010
10	0.006	0.007	0.012	0.014
14	0.007	0.006	0.011	0.042
20	0.010	0.010	0.010	0.014

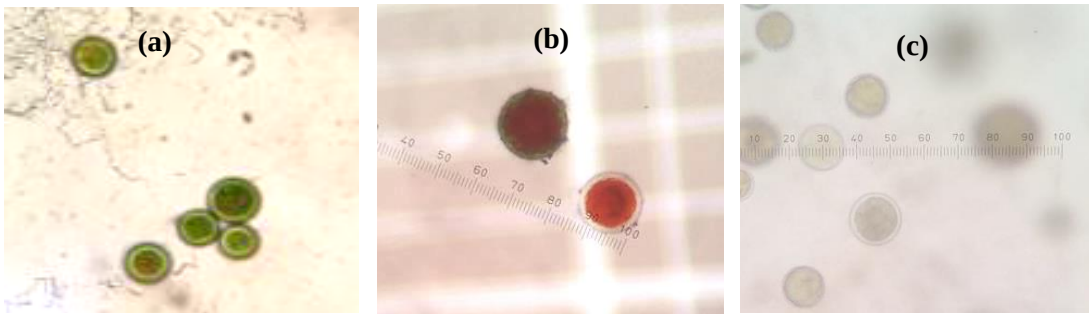

Figure 3. *H. pluvialis* treated with 200 mg/L of Cu nanoparticle on (a) day 0 showing green coccoid cells, (b) day 7 with intermediate cells and cysts, and (c) day 14 with dead cells.

CONCLUSIONS

The content of astaxanthin in *H. pluvialis* was affected by the presence of different concentration of Cu nanoparticle. As the content was lowered by the presence of 10 mg/L, 100 mg/L, and 200 mg/L of Cu nanoparticle, the nanoparticle was proven to cause inhibition of astaxanthin production in *H. pluvialis*. The long term exposure to Cu nanoparticle caused the decrease in cell viability and affected the growth of the microalgae. The sensitivity of *H. pluvialis* to Cu nanoparticle can be further exploited to develop as bioindicator for the nanoparticle in environment.

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