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THE EFFECT OF ARGENTUM AND CADMIUM TOWARDS ASTAXANTHIN CONTENT IN GREEN ALGAE, *HAEMATOCOCCUS PLUVIALIS*

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Abstract - *Haematococcus pluvialis* is a kind of microalgae with high potential in the production of astaxanthin- a secondary carotenoid with high commercial values. However, the mass production of astaxanthin is still not possible due to the low yield of astaxanthin obtained from this alga. The present study is aimed to determine the potential of Ag and Cd to enhance the production of astaxanthin in *H. pluvialis* at optimal pH and salinity. The alga was cultured in a series of different concentrations of Ag and Cd nanoparticles for 30 days, with pH 7 and salinity of 0.25%. The cell density and astaxanthin content were determined at day 15 and day 30. Highest accumulation of astaxanthin in the algae were found in the exposed concentration 10 ppm for both Ag(2.83 ± 0.70 mg/L) and Cd (4.98 ± 0.09 mg/L) nanoparticles at day 30. The amount of cell density and astaxanthin content in the algae decreased with the exposure of higher concentration of metal nanoparticles. Argentum nanoparticles were more toxic to the algae as both cell density and astaxanthin production for day 15 and 30 were lower than control. It can be concluded that Cd is a good inducer of astaxanthin production in *H. pluvialis*.

INTRODUCTION

Astaxanthin is recognized as one of the most important natural by occurring pigments in living organisms (Li *et al.*, 2011). This red lipophilic pigment belongs to the xanthophyll group and is known to be the main source of colour pigmentation in many organisms such as salmon, shrimps, krill, trout, microalgae and yeasts (Okada *et al*., 2009; Brambilla *et al.*, 2009; Ambati *et al*., 2014; Wu *et al*., 2015). The pigment has high antioxidant activities, even more potent than vitamin E and ß-carotene (Sarada *et al.*, 2006; Dong *et al*., 2014).

Due to its antioxidant property, astaxanthin has been widely applied in various industries in the market. In pharmaceutical and nutraceutical industries, astaxanthin has been used as nutritional supplements due to their anti-inflammatory, anti-cancer and anti-diabetic properties (Ambati *et al*., 2014). Animal feeds enhanced with astaxanthin to obtain the favoured colour pigmentations in fishes or crustaceans were also practiced in the aquacultural industry (Brambilla *et al*., 2009). Therefore, it is of particular interest for these industries to research on the a large-scale production of this substance.

Currently, the main commercialized astaxanthin found in the market are synthetically synthesized from petrochemical origin, and has been subjected solely to aquacultural applications (Li *et al*., 2011). Therefore, synthetic astaxanthin has not been approved for human consumption due to its safety concern (Guerin *et al.*, 2003). On the other hand, production of natural astaxanthin has so far been modest due to the difficulties and cost to maintain the production rate of natural astaxanthin (Olaizola, 2000; Liu *et al*., 2014). Green algae *H. pluvialis* is a popular source of natural astaxanthin, which has been reported to have the ability to accumulate astaxanthin up to 9.2 mg/g cell, many

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times higher compared to bacteria, yeast and plants (Choi *et al*., 2002; Guerin *et al*., 2003; Dong *et al*., 2014; Zhang *et al*., 2016). Nevertheless, the extraction of astaxanthin from *H. pluvialis* has been proven to be safe and approved by US FDA as dietary supplement in 1999 (Wu *et al*., 2015).

The fact that *H. pluvialis* is able to enhance its production of natural astaxanthin under stressed environment has opened up a pathway to large scale production and commercialization of natural astaxanthin (Shah *et al*., 2016). Much effort has been done in order to maximize the production of astaxanthin in this species by inflicting different environmental stresses upon the green algae (Kobayashi *et al*., 1991; Chaumont and Thépenier, 1995; Nagaraj *et al*., 2012). One of the major problem of producing astaxanthin by this microalgae was the slow cell growth that hinder the mass production of commercially available astaxanthin (Shah *et al*., 2016). An interesting approach by using heavy metal stressor, Fe, has given promising results on astaxanthin accumulation in the green algae in which the maximum amount of astaxanthin obtained was 30.70 mg/g biomass cell (Cai *et al*., 2009). This result indirectly signified the potential of metals as the inducer of astaxanthin production. To date, no reported studies on metals nanoparticle induction of astaxanthin accumulation in the green algae were found in the literature. Therefore, the focus of this study is to investigate the potential of using Ag and Cd nanoparticles in combination of different environmental stresses, such as salinity and pH, to induce the production of astaxanthin in *H. pluvialis*.

METHODOLOGY

Optimizations of algae strain culture

Haematococcus pluvialis was obtained from Unitex Enterprise Sdn. Bhd., Malaysia and maintained in Bold Basal Medium (BBM) (Sigma Aldrich, U.S.). Optimization was done to determine the ideal pH and salinity for the growth of the algae in laboratory condition to yield optimal astaxanthin production. About 20 mL of the mother culture was used as the standard inoculum (Sarada *et al*., 2002). The cultures were grown under room temperature 25±2 °C with the light and dark condition maintained at 16 hours to 8 hours. Two sets of cultures were prepared separately in Erlenmeyer flasks in a series of different: (i) pH (4.0, 5.0, 6.0, 7.0, and 8.0) and (ii) salinity (0.1, 0.25, 0.5, 1.0 and 2.0% w/v). Twenty millimeter of the mother culture was inoculated in each culturefor both sets of different pH and salinity and left to grow for 30 days. At day 15 and day 30, the cultures were harvested and analyzed for astaxanthin content. The pH (7) and salinity (0.25 % w/v) that showed the highest production of astaxanthin was used in the subsequent step in which they were treated with different concentration of Cd nanoparticles (Sigma Aldrich, U.S.).

Cd and Ag nanoparticles exposure and extraction of astaxanthin from algae strain

Different concentrations (10, 50, and 100 mg/L) of Ag and Cd nanoparticle was added into new sets of cultures grown in the optimized salinity (0.25% w/v) and pH(7) for 30days. All the cultures were carried out in triplicates.

Extraction and analysis of astaxanthin were done on day 15 and 30. As recommended by Sarada *et al.,* (2006), the extraction analysis were done by taking 5 mL of cell suspension from the metal exposed cultures and centrifuged at 5000 rpm at 4 ºC for 5 minutes. The supernatant was discarded later on and the cell pellet was treated with 3 mL of 4 M HCl in a microcentrifuge tube at 70 °C in a water bath for 2 min. The samples were then cooled and centrifuged at 5000 rpm for 5 min at 4 ºC. The HCl-treated samples were washed twice with distilled water and resuspended in 3 mL acetone for 1 hour before the estimation of astaxanthin content via UV-spectrophotometer (Biochrom, U.K.) were carried out. The absorbance of the extracts measured at 480 nm by using a UV-spectrophotometer. The concentrations of the astaxanthin (mg/L) were calculated by using the following formula (Gao *et al*., 2013):

Astaxanthin (mg/L) = OD480 x 4.5mg/L x Va/Vb,

where Va and Vb represent volumes of acetone and microalgae sample, respectively.

CELL COUNT

Cell count was done at day 15 and day 30 for all the cultures by using Neubauer chamber (Marienfeld, Germany) and observed under light microscope (Y103, RaxVision). The cell count was expressed in cell density by using followed formula:

Cell Density (cells/mL) = Average Cells per Large Square×10^4 ×Dilution Factor

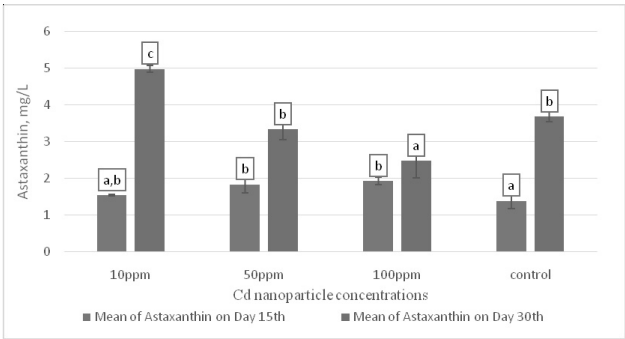
Statistical analysis

Data obtained were analyzed with One-way ANOVA analysis by using IBM SPSS software (Version 20).

RESULTS AND DISCUSSION

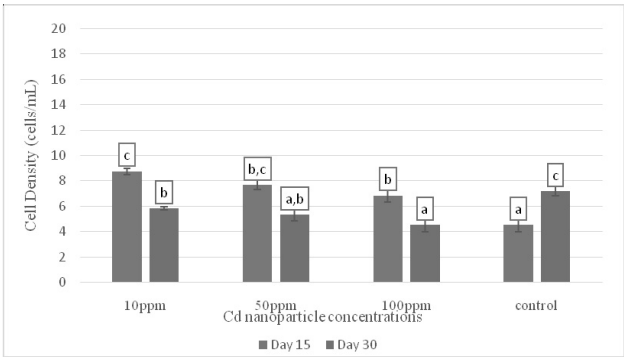
Effects of Cd nanoparticles to the production of astaxanthin

Figure 1 and 2 show the effects of Cd towards the astaxanthin production and cell density of *H. pluvialis* for 30 days, respectively.The accumu- lation of astaxanthin (4.98 ± 0.09 mg/L) by the algae was the highest ($p<0.05$) at 10 ppm exposure on the 30th day and decreased significant ($p<0.05$) in the subsequent higher Cd nanoparticles concentrations (Figure 1). However, no significant ($p>0.05$) variations were found in the production of astaxanthin for all Cd nanoparticle concentrations at day 15. Nanoparticles were able to generate oxidative stress, via the formation of Reactive Oxygen Species (ROS) in *H. pluvialis* and induce the production of astaxanthin as a protective mechanism against the detrimental effects of ROS (Qin and Hu, 2008). Kobayashi *et al*., (1993) also found similar trend of astaxanthin production in *H. pluvialis* when exposed to ferrous ion (Fe2+) as a surviving strategy of algae under stressed condition. This could explain the higher production of astaxanthin at 10 ppm Cd nanoparticles concentration as compared to control.



*The different letters represent significant differences at $p<0.05$.

Fig. 1 Effect of different concentration of Cd nanoparticle on astaxanthin content of *H. pluvialis* at day 15 and 30. Standard error bar was based on Standard Deviation (n=3).



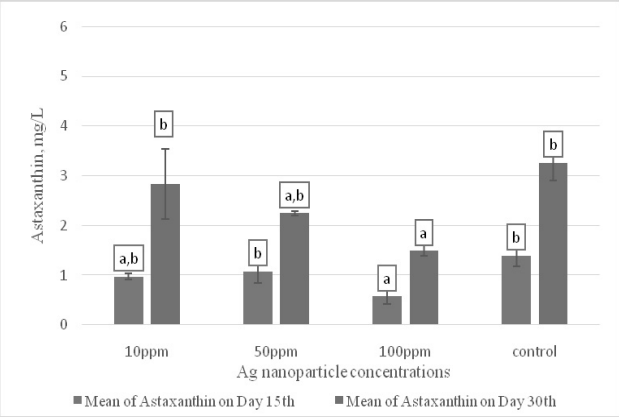
*The different letters represent significant differences at $p<0.05$

Fig. 2 The effect of different concentration of Cd nanoparticle on cell density of *H. pluvialis* at day15 and 30. Standard error bar was based on Standard Deviation (n=3).

The decreasing astaxanthin and cell production showed signs of the inability of the algae's adaptation towards toxic effects of high concentration of Cd nanoparticle (Miazek *et al*., 2015). This is supported by the decreasing density of algae in the order of 10 > 50 > 100 ppm concentrations of Cd nanoparticles for both 15 and 30 day (Figure 2). Despite the higher yield of astaxanthin at day 30 for all Cd concentrations, the cell density were visibly lower ascompared to day 15. A possible explanation of the low cell growth of the algae at higher levels of Cd nanoparticles could be due to the inhibition of photosynthesis activity of the algae when exposed to nanoparticle by blocking the electron transfer chain (Li *et al*., 2015). Therefore, most of the energy obtained in the algae was used for the production of astaxanthin to adapt to the environ- mental stress instead of growth. Another possible reason could be the disorder uptake of essential elements such as calcium and magnesium caused by the presence of Cd, which in turn suppressed the growth of the algae (Shariati & Yahyaabadi, 2006).

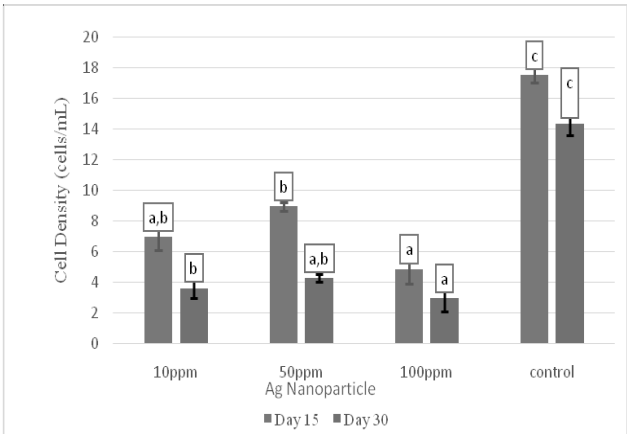
Effects of Ag nanoparticles to the production of astaxanthin

The results of Ag exposure show in similar trend of astaxanthin production (Figure 3) and cell density (Figure 4) as compared to those in Cd nanoparticle exposure for all concentrations. The production of astaxanthin by the algae in all concentrations of Ag nanoparticle exposure were mostly lower ($p<0.05$) than control for both days 15 (1.39 ± 0.22 mg/L) and 30 (3.26 ± 0.36 mg/L). Among the 3 different concentration of Ag nanoparticle for both days, the algae in 10ppm were found to produce the highest



*The different letters represent significant differences at p<0.05

Fig. 3 Effect of different concentration of Ag nanoparticle on astaxanthin content of *H. pluvialis* at day 15 and 30. Standard error bar was based on Standard Deviation (n=3).



*The different letters represent significant differences at p<0.05.

Fig. 4 The effect of different concentration of Ag nanoparticle on cell density of *H. pluvialis* at day 15and 30. Standard error bar was based on Standard Deviation (n=3).

amount of astaxanthin (2.83 ± 0.70 mg/L) on day 30. Argentum nanoparticles, similar to any other metals, stressed the algae through formation of ROS and induced the production of astaxanthin (Higuera-Ciapara *et al*., 2006; Oukarroum *et al*., 2012).

Although similar in trend, as compared to Cd nanoparticles exposure, the cell density of Ag were significantly lower (p<0.05) than those in control for both day 15 and 30. This phenomenon could be attributed to the significant toxic effect of Ag nanoparticle towards the growth of photosynthetic algae by disrupting the biological and morphological mechanisms of the cells leading to cell rupture and degradation (Dash *et al*., 2012). Inhibitory effect of nanoparticle has led to serious chemical or physical alteration of *H. pluvialis* even before they could react or produce more astaxanthin to cope with the surrounding (Oukarroum *et al*., 2012). This could explain the lower range of astaxanthin production in Ag as compared to Cd (0.58 - 2.83 mg/L) nanoparticles exposure.

This study had proven that Cd nanoparticle is a good inducer for astaxanthin production in *H. pluvialis* where the highest yield is obtained at 10 ppm exposure. On the other hand, Ag nanoparticle is not a good in inducer as it the exposure inhibits the normal growth of the algae rather than promoting astaxanthin production. However, further studies need to be conducted confirm the potential of nanoparticle as a promoting agent for astaxanthin production in *H. pluvialis*.

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