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Effects of Silver Nanoparticles on the Carotenoid Production from *Haematococcus pluvialis*

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Abstract

Carotenoids are known to be potent antioxidants with high commercial value. They have been widely utilized as supplements or animal feed in the industries. *Haematococcus pluvialis* is a microalgae that is able to produce high levels of carotenoid under environmental stresses such as temperature, salinity and polutants. Hence, this study was conducted to investigate the production of the carotenoid by exposing *H. pluvialis* to different concentrations (10 ppm, 100 ppm and 200 ppm) of silver (Ag) nanoparticles. The concentrations of carotenoid production by the microalgae due to metal exposure were conducted by using a GeneQuant 1300 Spectrophotometer at 470 nm. Based on the results, the maximum carotenoid production (0.458 ± 0.002 mg/L) was obtained from 10 ppm of Ag nanoparticles exposures to the microalgae at day-14. The growth of the microalgae was minimal for the first 7 days when exposed to the various metals and increased from day 8 of exposure onwards. The interruption of growth at the beginning was expected, as Ag is known to be toxic to the microalgae while the increment of growth in the following days of exposure could be due to adaptation of the microalgae to the stressed environment. This was proven by the bright red appearance of the *H. pluvialis*, which is a sign of carotenoids accumulation in the cells as a mode of protection against unfavourable conditions. This study will serve as a fundamental research on the induction of potential carotenoids production by exposing *H. pluvialis* to different concentrations of Ag nanoparticles.

Keywords: *Haematococcus pluvialis;* Carotenoid; Ag nanoparticles; Microalgae

1. Introduction

Carotenoids are naturally occurring pigments in all living organisms that undergo photosynthesis (Fiedor and Burda, 2014). To date, many different kinds of carotenoids have been studied and they are highly regarded as potent antioxidants (Johnson, 2002). Carotenoids act as part of the defence mechanism that shield living cells against oxidative damage by stabilizing the cell membranes and reduce free radical activities in our body (Guerin *et al.*, 2003).

In addition, several studies have reported that carotenoids possess antidiabetic and anticancer properties, as well as protection against cardiovascular diseases (Yuan *et al.*, 1997; Guerin *et al.*, 2003; Lorenz & Cysewski, 2000). This has led to the increased interests in researchers in finding ways to maximise the production of various potent carotenoids in order to meet the market's need.

Haematococcus pluvialis is a freshwater microalga that is commonly used in the production of carotenoid due to its rich content of carotenoids as compared to other natural sources (Biswal, 2014). Numerous studies have been conducted to stimulate the production of carotenoids in *H. pulvalis* by increasing their exposure to environmental stresses (Boussiba & Vonshak, 1991).

These include metals such as silver (Ag) which is known to stimulate high production of carotenoids in microalgae cultures (Miazek *et al*., 2015). However, silver could, on the other hand, inhibit the growth of microalgae by generating free radicals, which targets the membrane lipids of living cells, leading to the disruption of their normal biological functions (Kim *et al*., 2007). In this study, Ag nanoparticles were used due to its minute size as compared to normal Ag. The smaller sized Ag nanoparticles display larger surface area, which quickens the ions dissolution and toxicity interaction with the cell (Sinouvassane *et al*., 2016). However, limited reported studies were found to have used Ag nanoparticles to stimulate the production and accumulation of carotenoids in *H. pluvialis.*

Therefore, the aim of this study was to determine and compare the growth rates and carotenoid production of *H. pulvialis* with different Ag nanoparticles concentrations.

2. Materials and Methods

2.1 Preparation of Haematococcus pluvialis culture

One hundred fifty ml of fresh microalgae, *H. pluvalis* (UTEX, United States of America) were transferred into a 250 ml conical flask with 1X Bold Basal Medium (BBM, Sigma-Aldrich, Malaysia) and served as the mother culture of this study. The culture was incubated under fluorescent light for 16 hours and followed by dark conditions for 8 hours daily. The cultures were shaken daily to avoid clumping of the microalgae cells.

2.2 Exposure of Ag nanoparticles.

A series of different concentrations of Ag nanoparticles were prepared: from 0 ppm (negative control), 10 ppm, 100 ppm and 200 ppm. Triplicates of culture were prepared for each concentration of Ag nanoparticles where 3 ml of *H. pluvialis* were transferred from the homogenized mother culture into each set of concentrations. All cultures were optimized at pH 7 and contained 0.25 % NaCl solution, prior to incubation for 20 days.

2.3 Determination of cell density and growth pattern of Haematococcus pluvialis.

The cell density was determined using a haemocytometer. Briefly, 0.5 mL of the microalgae culture, was pipetted into a haemocytometer (Neubauer, Marienfeld) and observed under the light microscope (E200, Nikon) to determine the cell count. This procedure was repeated on every 2 to 3 day interval for 20 days to obtain the microalgae cell growth curve (Smith-Baedorf, 2012). Cell density was calculated using Equation 1 below (Ghoshal and Srivastava, 1991):

Cell density (cells/mL) = Average cells per large square × 10^4× dilution factor (Eq. 1)

2.4 Extraction and analysis of carotenoid.

Carotenoid extraction was carried out using the hydrochloric acid-acetone (HCl-ACE) extraction method once every 4 to 5 days (Sarada *et al*., 2002). The HCl-ACE extraction method involved treating the carotenoid with HCl followed by acetone-ethanol solution. Briefly, the extraction method began with the treatment of 5 mL of suspended cells with 1 mL of 4 M HCl. The mixture was heated at 70 ºC for 2 min. Then, the mixture was centrifuged at 5000 rpm (4 ºC) for 5 min. The resulting pellet was washed twice with 2 mL of distilled water and resuspended in 1 mL of HPLC-acetone followed by 1 hour of incubation at room temperature (28 ºC). This was followed by centrifugation at 5000 rpm (4 ºC) for 6 min and the supernatant was used for determination of carotenoid content by using the GeneQuant 1300 Spectrophotometer at 470 nm. The calculation of carotenoid concentrations was based on Equation 2 (Gao *et al*, 2013) below:

$$\text{Concentration (mg/L)} = (4.5 \times \text{OD470} \times \text{Va})/\text{Vb} = (4.5 \times \text{OD470} \times 0.001\ \text{L})/0.005\ \text{L} \quad \text{(Eq. 2)}$$

Where, Va represents the volume of extract; Vb represents the volume of the sample.

2.5 Statistical analysis.

The mean value, standard deviation and One-way ANOVA were determined by using the statistical software, IBM Statistical Package for Social Sciences (SPSS Version 20) based on the data collected. ANOVA tests were conducted separately based on different sets of exposure days (Days 0, 5, 10, 14, 20).

3. Results and Discussions

The growth of cultures exposed to 10 ppm and 100 ppm of Ag nanoparticles showed a lag phase from day-0 to day-7 and exponential phase from day-7 until day-20. The culture in 10 ppm of Ag nanoparticles showed the highest growth rate of *H. pluvialis* at day-20 (Figure 1). As for the growth pattern of *H. pluvialis* exposed to 200 ppm of Ag nanoparticles, lag phase was observed from day-0 to day-7 followed by a rapid increase in the cell number from day-7 to day-14, before entering the stationary phase from day-14 to day-20.

In comparison with negative control, the microalgae culture showed similar growth pattern for the first 12 days with both 10 ppm and 100 ppm of Ag nanoparticles. As for the culture with 200 ppm of Ag nanoparticles, the growth showed a slight decline for the first 12 days as compared to negative control. Silver is a non-essential metal, which carries no biological functions in the growth of the *H. pluvialis* and therefore, it will not induce the growth of the species at the beginning of the exposure (Ivanova, *et al*., 2008). The decline in growth could be attributed to the high concentration of Ag nanoparticles. According to AshaRani *et al*. (2008), silver nanoparticles interact with protein and disrupt ATP synthesis in the cell, which then damages the DNA. The blockage of cell division from high concentrations of Ag metal could also affect the metabolism of the microalgae causing cell death (Monteiro *et al*., 2012). In this study, the cell growth of *H. pluvialis* from day-12 onwards continued to increase by 2 to 3 folds.

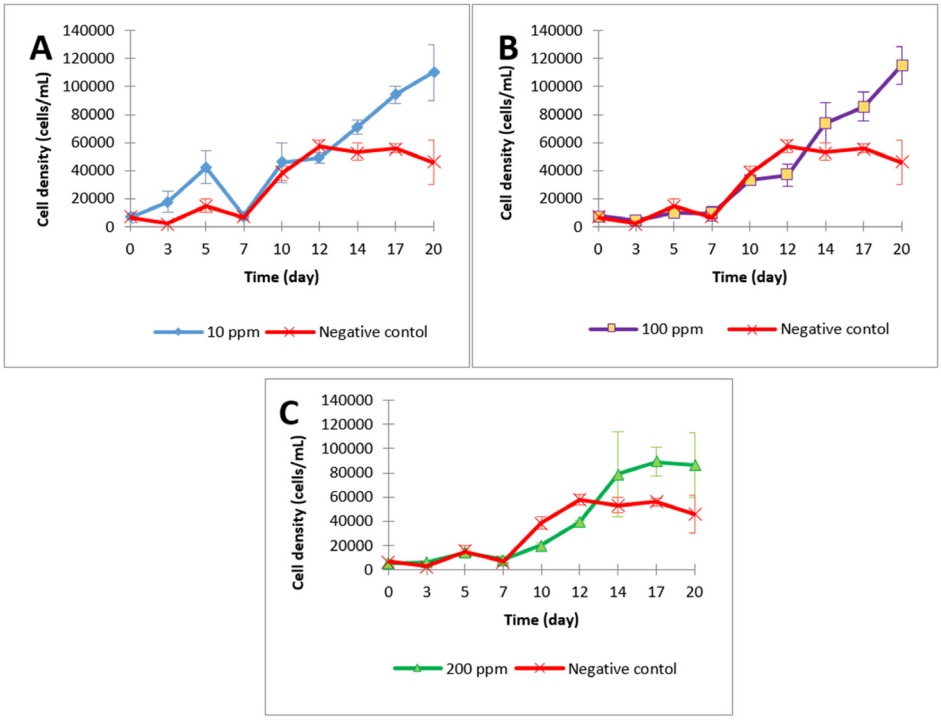

Figure 1: Growth curve of *H. pluvialis* over 20 days of cultivation: (A) 10 ppm of Ag nanoparticles with negative control, (B) 100 ppm of Ag nanoparticles with negative control and (C) 200 ppm of Ag nanoparticles with negative control. Standard error bar was based on standard deviation (n = 3)

This could be due to the adaptation of the species to the presence of the protective carotenoid, which was able to protect the cells from Ag nanoparticle stress (Shah *et al*., 2016). This was supported by the observation under the microscope of the microalgae during the exposure period to Ag nanoparticles. Figure 2 shows that at Day-0, the *H. pluvialis* was in an ideal form, denoted by their green vegetative stage appearance, at the early stage of metal exposure (Trifonov & Kolev, 2016). The microalgae were in the encystment stage at Day-20 (Figure 2), where they were vigorously producing the red carotenoid, astaxanthin, as protection against the toxic metal exposure (Shah *et al*., 2016). This study is in agreement with several other reported studies on astaxanthin, where they had stated that the highest level of carotenoid accumulation in the *H. pluvialis* was at the encystment stage which occured during the stationary phase (Borowitzka *et al*., 1991; Kobayashi *et al*., 1997; Nagaraj *et al*., 2012).

The presence of Ag nanoparticles, regardless of the concentration, enhanced the production of carotenoid, with Day-14 showing higher production compared to other days as compared to the negative control (Figure 3). Maximum carotenoid production (0.458 ± 0.002 mg/L) by *H. pluvialis* was found with 10 ppm of Ag, which resulted in an increment of 3.5 folds of carotenoid as compared to the negative control at day-14. The carotenoid production in the microalgae of 100 ppm and 200 ppm Ag nanoparticle exposure was lower than those of 10 ppm exposure, which could be the a sign of adaptation (100 ppm) or death (200 ppm) of microalgae.

Several studies demonstrated that the exposure of algae to Ag metals was able to induce the synthesis of carotenoids (Oukarroum *et al*., 2012; Miazek *et al*., 2015; Cheng *et al*., 2018). *Haematococcus pluvialis* produces antioxidants, which are mainly carotenoids as protection by stopping the free radical process in the cell, which explains the highest production of carotenoid at Day-14 for all metal concentrations as compared to other days of metal exposure. Silver is known to be toxic to microalgae, whereby it disrupts the normal functions of the microalgae by releasing free radicals that bind to the membrane lipids of cells (Kim *et al*., 2007). Nanoparticles of smaller size, as compared its parental form (Ag), pose higher toxicity towards cells (Sinouvassane *et al*., 2016). Therefore, it is plausible that the microalgae were adapting to the stressed environment with a reduction in growth while producing more carotenoid for protection at Day-14. The increase in growth rate and reduced amount of carotenoid production at day-20 could be a sign of adaptation of the microalgae where less carotenoid is required as protection. Thus, this study showed that exposure of *H. pluvialis* to Ag nanoparticles was able to induce synthesis of carotenoid.

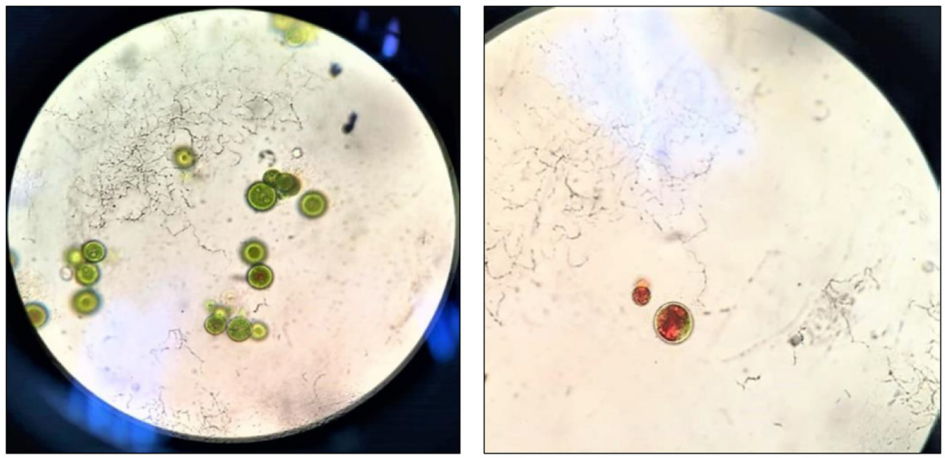

Figure 2 *Haematococcus pluvialis* during the exposure period of Ag nanoparticles at Day-0 and Day-20 under light microscope (10× magnifications)

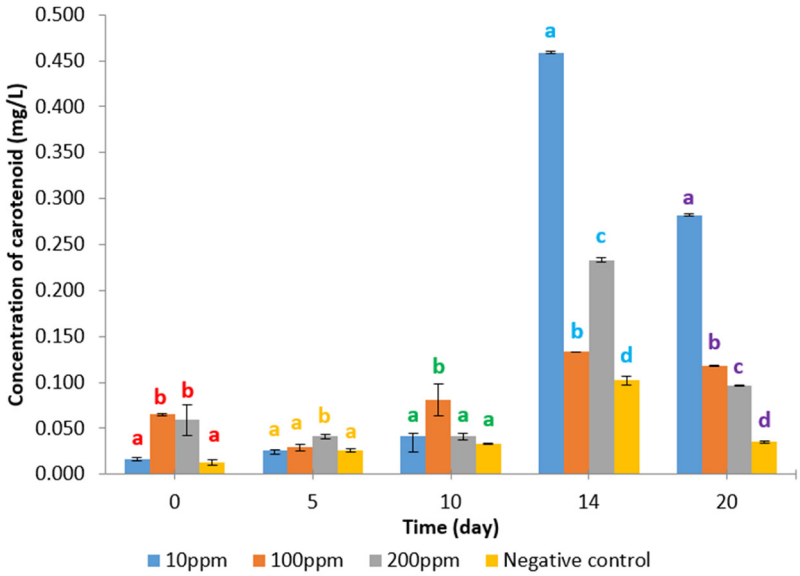

Figure 3 Carotenoid content from *H. pluvialis* under different concentrations of Ag nanoparticles over 20 days (n = 3). (The different alphabets represent significant differences at $p < 0.05$; ANOVA analysis were conducted separately for each exposure day (Day 0, 5, 10, 14, 20) indicated with different colour.)

4. Conclusion

This study served as a preliminary study of the potential of Ag nanoparticles in inducing the production of natural carotenoid in *H. pluvialis*. The results of this study suggested that 10 ppm of Ag nanoparticles was able to induce the highest production of carotenoid in *H. pluvialis* as compared to the other concentrations. Therefore, future studies, such as the determination and extraction of specific carotenoid, are necessary to generate better understanding on the effect of Ag nanoparticles induction on specific carotenoid production in *H. pluvialis*.

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