THE PRODUCTION OF ASTAXANTHIN IN FRESHWATER ALGAE, *Haematococcus Pluvialis* WITH LEAD NANOPARTICLE EXPOSURE

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ABSTRACT

Haematococcus pluvialis is a freshwater micro algae that produces up to 4 - 5 % dry weight of astaxanthin making it the most effective organisms for astaxanthin production. Astaxanthin is a ketocarotenoid with high antioxidant properties. It is used in feed, nutraceutical, food, pharmaceutical and various other industries owing to its high antioxidant level. Astaxanthin is accumulated under stress in *H. pluvialis* cells. Therefore, the cells need to be stressed to produce astaxanthin. This study investigated the growth of *H. pluvialis* and the effect of lead (Pb) nanoparticle exposure on the production of astaxanthin in *H. pluvialis*. In this experiment, *H. pluvialis* was cultured in Bold Basal Medium (BBM) and exposed to three concentrations (10 mg/L, 100 mg/L and 200 mg/L) of lead nanoparticles in the form of PbO nanoparticles. Cell quantification was done in every 2 - 3 days using cell counting chamber and light microscope. Astaxanthin was then extracted and quantified at 470 nm using a spectrophotometer in every 4 - 5 days. Negative control without PbO nanoparticles was also cultured in order to compare astaxanthin production in stressed and unstressed cells. The data were statistically analyzed using simple tools in Microsoft Excel 2016. Growth curve of *H. pluvialis* cells showed that the cells experienced three phases of cell growth and showed the highest cell density of 16.92×10^4 cells/mL. The results showed that the cells exposed to PbO nanoparticles did not yield a higher percentage of astaxanthin compared with unstressed cells due to the decreased cell number. The maximum amount of astaxanthin (0.235 mg/L) was accumulated in negative control cells. Therefore, *H. pluvialis* underwent lag phase, log phase and stationary phase. Exposure of lead nanoparticles did not yield a high amount of astaxanthin compared with negative control due to the less incubation period and PbO causing a negative effect on H. pluvialis cell density.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
%	percentage
w/v	weight per volume
µmol photons m^{-2} s^{-1}	micromole photons per metre squared per second
µm	micrometre
mg	milligram
µL	microlitre
mL	millilitre
g/mol	gram per mole
nm	nanometre
BBM	Bold Basal Medium
cells/mL	cells per millilitre
mg/L	milligram per litre
min	minutes
hr	hours
rpm	revolutions per minute

CHAPTER 1

INTRODUCTION

Astaxanthin is a ketocarotenoid belonging to the xanthophyll family with a bright red color. Chemical formula of astaxanthin is 3,3'-dihydroxy-β-carotene-4,4'-dione which is synthesized *de novo* by some plants, bacteria, yeast, and microalgae. Metabolic reactions and processes occurring in the body cause the production of free radicals such as singlet oxygen, and peroxyl radicals among others. These free radicals damage DNA and oxidize lipids and proteins. Antioxidants such as astaxanthin quench these free radicals and render them inactive (Olaizola & Huntley, 2003). Astaxanthin has proven to have anticancer activity, effective against *Helicobacter pylori* infections, acts as a modulator and a booster of the immune system, prevents cardiovascular diseases, et cetera (Ciapara, Valenzuela & Goycoolea, 2006).

Astaxanthin is a super antioxidant as it is stronger than β-carotene by 54 times, 10 times more potent than some carotenoids, more powerful than vitamin C by 65 times and 100 times more effective compared to α-tocopherol making it extremely useful in the nutraceutical, medical, aquaculture, food and cosmetic industry (Yuan, Peng, Yin & Wang, 2011). Nevertheless, synthetic astaxanthin dominates over natural astaxanthin due to the practicality of mass producing it. Despite this, natural astaxanthin is preferable and is at a higher demand in the market (ranging from US$ 2500 - 7000 /kg) compared to synthetic astaxanthin as synthetic astaxanthin raises concerns regarding the safety of direct human consumption (Panis & Carreon, 2016). *Haematococcus pluvialis* is a microalgae capable of producing up to 4 - 5 % dry weight of astaxanthin.

Microalgae produce vitamins, fatty acids, dyes, polysaccharides, minerals, proteins and various secondary carotenoids that are of great interest to the nutraceutical, pharmaceutical and various other industries (Galvão, Santana, Fontes & Sales, 2013). *Haematococcus pluvialis* belongs to the family Chlorophyceae. It is a unicellular green microalgae found in many habitats worldwide. This mobile microalgae is capable of photosynthesizing, undergoing morphogenesis and accumulating the secondary carotenoid, astaxanthin in response to environmental stress (Minggang, Zhe & Anxiang, 2009). Although several micro algae strains have proven to produce astaxanthin, accumulation of astaxanthin

when exposed to stress in *H. pluvialis* exceeds any other micro algae, making it the most favorable producer of natural astaxanthin in nature (Panis et al., 2016). *H. pluvialis* exists in two forms; green vegetative cells and red aplanospores or red hematocysts (Minggang et al., 2009).

Both the red cells and the green cells require different culture conditions. Vegetative cells pre-dominate when the cells are exposed to favorable conditions. Under unfavorable conditions, including high temperature, salinity, pH, light and low nutrient content level, they transform into resting vegetative, non-motile palmella cells (Wang, Han, Sommerfeld, Lu & Hu, 2013). The continuous subjection of the cells to environmental stress ceases cell division and palmella transforms into asexual aplanospores which accumulate astaxanthin. Aplanospores contains an acetolysis resistant secondary cell wall which protects the cell from harsh environmental stress. In mature aplanospores, the cells appear bright red due to the high amount of astaxanthin accumulation in the cytoplasm (Han, Li & Hu, 2013).

Physical conditions such as pH, salinity, temperature, and nutrition affect the astaxanthin accumulation greatly in *H. pluvialis*. Astaxanthin accumulates best at temperatures of 20 ℃ and 28 ℃. Salinity ranging from 0.25 - 0.50 % w/v NaCl and pH 7.00 - 7.85 have proven to be the optimum range with which highest concentration of astaxanthin accumulates. A light intensity of 70, 80 or up to 177 µmol photons m^{-2} s^{-1} and low nutrient content (limitation of nitrogen, the presence of micronutrients such as chromium and selenium) results in increased astaxanthin production (Shah, Liang, Cheng & Daroch 2016).

The aim of this research was to culture and measure the growth of *H. pluvialis* and to determine the effect of lead (Pb) nanoparticles to the production of astaxanthin in *H. pluvialis*.

CHAPTER 2

LITERATURE REVIEW

2.1 ASTAXANTHIN

2.1.1 Biochemistry Of Astaxanthin

Astaxanthin is a xanthophyll which is used in aquaculture as a pigmentation source (Kobayashi, Kakizono, Nishio, Nagai, Kurimura & Tsuji, 1997). Since synthetic astaxanthin raises concerns about food safety, direct human consumption is not approved. It is only used as a fish feed additive for pigmentation purposes (Dong, Huang, Zhang, Wang & Liu, 2014). The long carbon chain of astaxanthin is held together with double bonds which is also known as chromophore. A hydroxyl and a keto group on each of the ionone rings at both the ends of the chromophore allows the molecule to be esterified and alters its optical characteristics. Esterification gives the molecule a polar configuration in addition to its anti-oxidant activity (Ambati, Phang, Ravi & Aswathanarayana, 2014).

The conjugated double bonds which acts as strong antioxidants in the astaxanthin molecule gives it the distinguishing red color Antioxidants are molecules which can prevent oxidation. Oxidation initiated by reactive oxygen species (ROS) react with proteins, lipids and, DNA in a chain reaction causing damage to DNA and oxidation of lipids and proteins. Antioxidants render the free radicals inactive by donating the electrons which reacts with the free radicals, quenching them and making them more stable and terminating the chain reactions. (Guerin, Huntley & Olaizola, 2003) Astaxanthin is considered as a better carotenoid compared with other carotenoids due to its ability to be present both inside and outside the cell membrane providing the cells a better protection (Ambati et al., 2014).

As seen in Figure 2.2, carbon atoms with the chiral centers at the 3 and 3' allow the molecule to exhibit three forms of isomers; 3S, 3'S : 3R, 3'S : 3R, 3'R, with 1:2:1 ratio (Moretti et al., 2006). Among these, 3S, 3'S is the most common isomer found in nature and is the main isomer which exists in *H. pluvialis* (Olaizola et al., 2003). Due to its stability, *trans* isomers of astaxanthin are the most abundant in nature. Due to its sensitivity towards

oxidation, astaxanthin is often found conjugated to proteins or esterified with the fatty acid(s), making them soluble (Olaizola, 2007).

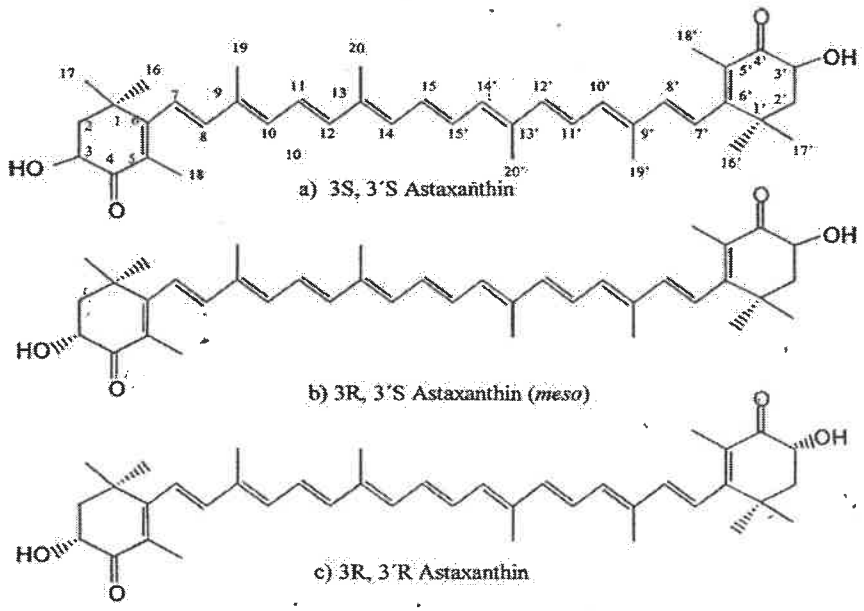

Figure 2.1 Three isomers of astaxanthin. Among the three isomers, 3S, 3'S is the most common in nature (Ciapara et al., 2006).

2.1.2 Health Benefits Of Astaxanthin

The human body produces various antioxidants such as catalase, dismutase, peroxidase and other molecules in response to oxidative stress (Ciapara et al., 2006). Although, at times, these antioxidants are not enough to neutralize the oxidative stress. Therefore, dietary intake of antioxidants is necessary. The inability for mammals to synthesize astaxanthin results in them acquiring it via diet. The high antioxidant activity of astaxanthin enables the molecule to provide various health benefits. Esterification of astaxanthin provides the molecule it's health benefits as it affects the absorbability of the molecule in the organism along with the transport of the molecule inside the body. Esterification causes change in polarity of the carotenoid making it more polar when it is as free astaxanthin and decrease polarity from monoester to diester (Yuan et al., 2011).

Astaxanthin quenches singlet oxygen molecules along with ROS in order to act as antioxidants. The energy of the singlet oxygen or the reactive species is absorbed onto the

molecule's chain, causing degradation of the astaxanthin molecule. During this process, other molecules and tissues are prevented from any damage (Yuan et al., 2011). Free radicals in a chain reaction are produced as a result of degradation of polyunsaturated fatty acids which causes degradation of lipid membranes at a faster rate. Astaxanthin can prevent the formation of these chain reaction of free radicals, protecting the lipid membranes (Rao, Sarada, Baskaran & Ravishankar, 2009).

Apart from antioxidant activity, astaxanthin also shows anti-inflammatory effects. ROS may cause aggravation of inflammation which leads to other health complications such as asthma, exercise-induced muscle damage, ulcers due to *Helicobacter pylori* and inflammation of gastric tissues (Olaizola, 2007). Plasma membranes of immune cells contain high concentration of polyunsaturated fatty acids, causing more production of oxidative products and are thus, sensitive to oxidative stress. According to a study, astaxanthin could increase the immune response and decrease inflammation and DNA oxidative damage biomarker (Yuan et al., 2011).

In diabetic patients, oxidative stress results in tissue damage, dysfunction of pancreatic β-cells and various other problems. Astaxanthin decreases the oxidative stress on pancreatic β-cells significantly along with decreasing blood glucose levels, increasing serum insulin levels and improving glucose tolerance in diabetic patients (Bhattacharjee, 2014). Other than above mentioned health benefits, astaxanthin has shown to be effective against gastro related diseases, cardiovascular diseases, neurological diseases, skin diseases, ocular diseases, anti-cancer effects and many other health benefits (Guerin et al., 2013).

2.1.3 Other Benefits Of Astaxanthin

Apart from the clinical applications, astaxanthin in its natural form, as well as synthetic form, is used to improve coloration of salmon and trouts, in the poultry industry for the improved color of egg yolk sack and in ornamental fish breeding (Göksan & Ak, 2006). Fishes do not have the ability to synthesize carotenoids *de novo*, which requires carotenoids to be administered to the fishes via fish additives or by ingesting other aquatic food (Kaur & Shah, 2017). Providing the fishes with the necessary carotenoids such as astaxanthin for skin pigmentation will not only increase its ornamental value but also it will increase the quality of the fishes. Astaxanthin containing diet provided to chickens has proven to produce high breast

mass, increased efficiency of feed and overall faster weight gain in comparison with chickens provided with regular meals (Lorenz & Cysewski, 2000). Moreover, egg yolk color is also increased which is preferable by consumers in some parts of the world.

2.1.4 Synthesis Of Astaxanthin

Astaxanthin and various other carotenoids are produced in microalgae as a defense mechanism to stress. When the microalgae are exposed to metal stress, it synthesizes various compounds such as peptides, pigments, lipids, carotenoids and other compounds which protects and helps in withstanding the harsh environmental stress (Miazek, Iwanek, Remacle, Richel & Goffin, 2015). The high light intensity of 1000 $\mu mol.photons.m^{-2}.s^{-1}$ was noted to produce a significant amount of carotenoids in microalgae *Gracilaria tenuistipitata* (Pinto et al., 2011). Environmental stresses induce oxidative stress which causes the production of free radicals which damages the DNA, proteins, and lipids. The microalgae produce the carotenoids which act as antioxidants and protect the damages caused by oxidative stress.

2.2 *Haematococcus pluvialis*

2.2.1 Life Cycle Of *Haematococcus pluvialis*

The first morphology exhibited by *H. pluvialis* in the green vegetative phase is called macrozooids (zoospores) which appears pear-shaped, spherical or ellipsoidal, consisting equal length of two flagella on the anterior end of the algae. Zoospores are 8 - 20 μm long with dispersed pyrenoids and cup-shaped chloroplasts. These zoospores under favorable conditions undergoes mitosis to produce 2 - 8 daughter cells and predominate during the early stages of vegetative phase. When the cells are exposed to unfavorable culture or environmental conditions, they transform into palmella in green vegetative phase. During this process, macrozooids lose their flagella, becoming non-motile and expand in cell size. This process is called encystment where palmella becomes resting vegetative cells (Han et al., 2013).

Persistent of the environmental or culture stress such as nutrient deprivation, high salinity, high temperature and high light intensity ceases cell division and palmella transforms into asexual non-motile aplanospores (Eonseon, Lee & Polle, 2006). Aplanospores have thick, rigid cell wall consisting of acetolysis-resistant material, making it resistant to harsh culture or