ASTAXANTHIN PRODUCTION BY *HAEMATOCOCCUS PLUVIALIS* UNDER DIFFERENT LIGHT EMITTING DIODES (LEDs) ILLUMINATION

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ABSTRACT

The freshwater phototrophic green microalga *Haematococcus pluvialis* is a well-known microorganism which is rich with powerful antioxidant astaxanthin and beneficial in pharmaceutical and cosmetic industries. The aim of this study is to investigate the effect of different LEDs illumination on the astaxanthin accumulation in the microalgae cell. Experiments were carried out by growing the microalgae using fluorescent light until it reached stationary phase. The growth was calculated by measuring the optical density (OD) spectrophotometrically at 750nm, cell counting and cell dry weight (CDW). The culture began its exponential phase on day-2 and reached stationary phase on day-13 with cell density, CDW, specific growth rate, μ and division time of 3.0 x 10^7 cells/mL, 6.58 g/L, 0.272 day^{-1} and 0.392 respectively. The *Haematococcus pluvialis* culture were then subjected to astaxanthin induction process by exposing the culture to (i) red, (ii) blue and (iii) red-blue LEDs at light intensity of 200 μmol m^{-2} s^{-1}. The astaxanthin accumulation were observed for 14 days and results found that mix red-blue LED exhibited the best result in terms of astaxanthin accumulation at 3.04mg/L compared to other red and blue LED, at 2.17mg/L and 2.49mg/L respectively. The usage of LED in astaxanthin production is proven effective and more study needed to determine the optimal induction setting for maximum astaxanthin production from *Haematococcus pluvialis*.

Keywords: *Haematococcus pluvialis*; astaxanthin; LEDs, cultivation; extraction.

1. INTRODUCTION

The freshwater green microalgae *Haematococcus pluvialis* is the best microbial source of astaxanthin [1]. Astaxanthin (3,3'-dihydroxy-diketo-β, β'-carotene-4,4'-dione) is one of the most powerful antioxidants among carotenoids with many applications in nutraceuticals and in the food and feed industries because of its strong anti-aging, anti-inflammatory, sun proofing, and immune system boosting effects on organisms. *Haematococcus pluvialis* is believed to demonstrate the highest capacity to accumulate natural astaxanthin under environmental stress conditions [2] which protects itself by secreting the astaxanthin when faced to the undesirable conditions as a response to adverse conditions that result in the cessation of growth while photosynthesis is still active [3] [4]. *Haematococcus pluvialis* species is considered as the most promising producer of astaxanthin, as it accumulates the (3*S*, 3'*S*)-isomer of astaxanthin, mostly in its mono- and di-ester forms in cytoplasmic lipid bodies as a secondary carotenoid [5]. There has been an emerging development in facilitating the commercial mass production of *Haematococcus pluvialis* due to its ability to secrete and produce powerful antioxidants for pharmaceutical and nutraceutical industry. This carotenoid also principally consumed in aquaculture industry specifically for salmon and lobster colorant. Hence, more commercial cultivation farm being constructed and research began to venture on alternative light source for astaxanthin production to replace sunlight. Conventional artificial light source was reported to produce higher biomass due to large illumination area, high stability of light source and low construction cost [6]. LEDs can serve as an ideal light source for algal growth due to its advantages: (i) narrow spectral output, which can overlap with the absorption spectra of microalgae; (ii) high electric-to-light conversion efficiency, which generates less heat; (iii) no emission outside of photosynthetic active radiation (PAR), such as ultraviolet and infrared regions, which makes the light delivery system simpler; (iv) small weight and volume characteristics, which makes LEDs to be incorporated into virtually all types of PBRs for both internal and external light sources; (v) many other advantages, such as long life expectancy, solid state, safe (powered by low DC voltage), extremely short rise and fall time [7].

In the present study, the potential of LED light to induce astaxanthin accumulation in *Haematococcus pluvialis* is studied. The different wavelength of LEDs (i) red, (ii) blue and (iii) red-blue LED illumination bars were chosen as alternative artificial light source throughout the astaxanthin induction process to test their effect on astaxanthin accumulation in *Haematococcus pluvialis* culture.

2. MATERIALS AND METHODS

2.1. Collection of Microalgae Sample and Culture Maintenance

The strain of *Haematococcus pluvialis* was obtained from the Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah (UMS), Malaysia and maintained both in liquid culture and agar plates. Agar transfer was performed every month and single colony was picked from agar plates which then transferred into Erlenmeyer flasks containing 50mL culture media and grown at 21 ºC under continuous fluorescent light with intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 160 rpm as stock cultures.

2.2. Inoculation and Cultivation of *Haematococcus Pluvialis*

Modified Bold's Basal Medium (BBM) is the medium used for *Haematococcus pluvialis* cultivation. The freshwater microalgae species which was maintained in dormant condition (on agar plate) and liquid form (stock culture) was inoculated in modified BBM. The modified BBM formulation (per L) is as follows: 25 g $NaNO_3$, 2.5 g $CaCl_2$, 7.5 g$MgSO_4.7H_2O$, 7.5 gK_2HPO_4, 17.5 g KH_2PO_4, 2.5 g NaCl, EDTA Solution (50 g EDTA + 31 g KOH), trace element solution (8.82 g $ZnSO_4.7H_2O$, 1.14 g $MnCl_2.4H_2O$, 0.88 g $(NH_4)_6Mo_7O_{20}.4H_2O$, 1.57 g $CuSO_4.5H_2O$, 0.49 g $Co(NO_3)_2.6H_2O$), 11.42 g H_3BO_3 and 4.98 g $FeSO_4.7H_2O$.

The *Haematococcus pluvialis* was inoculated in 200mL Erlenmeyer shake flasks which contain 10 mL seed culture and 90 mL medium. The cultivation was performed at 21ºC pH6.8$\pm$0.2 and under a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 24:0 light:dark cycle for 5 days on the shaker. On the 5th day, 40 mL of culture was transferred into sterilized 500 mL Erlenmeyer flasks which contain 360 mL medium for microalgae growth observation for 14 days with continuous aeration. Each experiment was performed in duplicates to ensure reproducibility of result. After the growth profile was obtained and the stationary phase was identified, another set of experiment was performed to induce the astaxanthin production. A seed culture was prepared and inoculated into 6 500 mL Erlenmeyer flasks which contain 30 mL microalgae culture and 270 mL growth medium. All 6 flasks were made to grow under fluorescent light for 11 days before transferred to the induction rack to induce astaxanthin production under different LEDs illumination wavelength (red, blue and red-blue) at light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 24:0 light:dark cycle.

2.4 Growth Analyses and Quantification of Astaxanthin

Haematococcus pluvialis cultures OD readings were taken daily and observed using Shimadzu UVmini-1240 UV-vis spectrophotometer at 750 nm. Meanwhile the cell counting was determined by direct counting with haemacytometer using a 0.1 mm-depep Neubauer chamber (BOECO, Hamburg, Germany) under a light microscope (Olympus CX31, Japan). Lastly, 10 mL of the culture were used for determining the CDW by filtering the sample through the Glass Microfiber Filters, GF/C (Whatmann) and then dried at 100 ºC to a constant weight in an oven (Model: UNB 500, Memmert GmbH, Schwabach, Germany). All CDW samples were cooled in desiccator before weigh.

Astaxanthin from samples were quantified to confirm astaxanthin accumulation in the cell. The cultures were sampled every two days (48 hours). 5 ml of sample was taken for every flask. Then, centrifuge for 5 min at 2500 rpm. The supernatant was removed. Resuspended the pellet in 5 ml solution of 5% KOH in 30% (v/v) methanol in water bath at 70 °C for 5 min. Mixture was centrifuged again at 2500 rpm for 5 min. Then, pellet was extracted with mixture 5 ml DMSO and 5 drops of acetic acid in water bath at 70 °C for 5 min. Mixture was centrifuged at 2500 rpm for 5 min and supernatant was collected to

measure the absorbance at 490nm.The absorbance of the combined extracts was determined at 490 nm, and the per unit volume astaxanthin concentration (c) was calculated as:

$$c(mg/L) = 4.5 \times A_{490} \times V_a / V_b$$

where V_a (L) was the volume of extracts, $V_b(L)$ is the volume of the culture sample, and A_{490} is the absorbance of extract at 490 nm [8].

3. RESULTS AND DISCUSSION

The growth profile of *Haematococcus pluvialis* in terms of optical density (OD) at 750 nm and cell count is depicted in Figure 1. The optical density for the biomass exhibits an exponential curve where a lag phase was observed for the first 2 days, followed by the growth phase after the 3rd day and finally reached the stationary phase after day 13. The maximum cell density and CDW were 3.0 x 10^7 cells/mL and 6.58 g/L respectively.

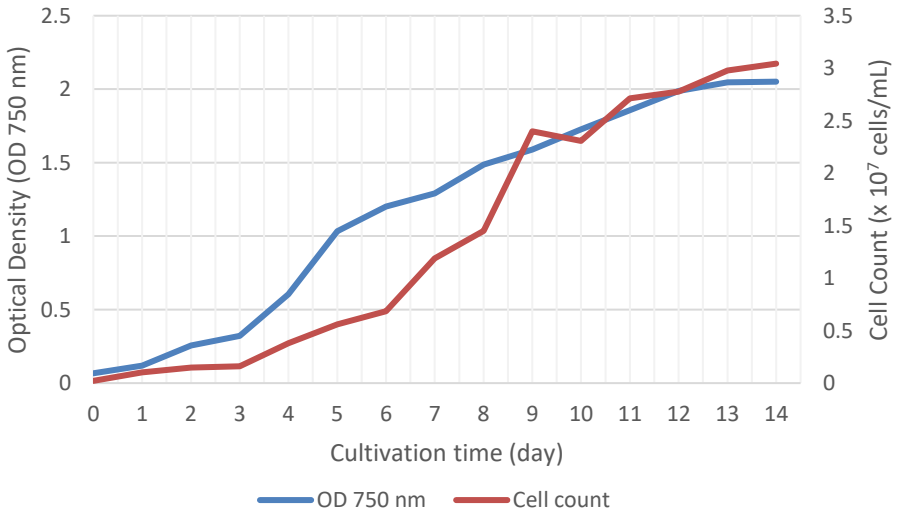

Figure 1. Optical density (OD) reading and cell count of Haematococcus pluvialis culture during 14 days of cultivation.

Specific growth rate is defined as the increase in cell mass per unit times. The growth rate of population is a measure by the increase in biomass over time and it is determined from the exponential phase. The specific growth rate and division time of *Haematococcus pluvialis* grown were, 0.272 day^{-1} and 0.392 respectively.

Table 1. Astaxanthin Accumulation (mg/L) Under Different LEDs Illumination

Day	Astaxanthin (mg/L)		
	Red LED	Blue LED	Red + Blue LED
2	1.222	0.5643	1.1219
4	1.2182	1.0382	1.4648
6	1.4459	1.5863	1.6839
8	1.6988	1.9143	2.2932
10	1.8077	1.8531	2.1987
12	2.1717	2.4633	2.3436
14	2.1744	2.4917	3.0407

The second part of the experiment is the induction phase which was carried out to investigate the effect of LED illumination light (red, blue and red-blue) on the astaxanthin accumulation within the cell structure when exposed to 24:0 hours light:dark cycle at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 14 days. After 14 days of induction, the flasks showed color changes demonstrating that the cell began to accumulate astaxanthin when subjected to stress, in this case the high light intensity. Table 1 showed that the culture flasks induced by red-blue LED light showed highest astaxanthin accumulation after 14 days of induction with the value of 3.0407 mg/L followed by red and blue LED, which revealed astaxanthin concentration of 2.4917 mg/L and 2.1744 mg/L respectively.

Previous work [5] reported that incorporating blue and red LEDs illumination as external light coupled with fluorescent internal illumination during induction process showed promising results in enhancing the astaxanthin accumulation in *Haematococcus pluvialis* culture compared to fluorescent illumination alone (both internal and external illumination) at 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. It is believed that LED illumination have effect on increased transcript levels of carotenoid biosynthesis genes under both blue and red-light conditions [9]. Based on current finding, the mixture of red-blue LEDs showed greater astaxanthin accumulation than monochromatic LED illumination.

4. CONCLUSION

Based on the results obtained, high intensity LED illumination is proven capable to create stress environment to the *Haematococcus pluvialis* culture to induce astaxanthin accumulation in the cell structure acting as self-defense mechanism to protect itself from the extreme environment changes. However, much study is needed as to further investigate the optimal and economical LED illumination strategy for faster and maximum astaxanthin accumulation in *Haematococcus pluvialis* cell structure.

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