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Research Article

Astaxanthin Production from Green Microalga *Haematococcus pluvialis* under Various Bean Sprout Media Concentrations and Duration of UV Radiations

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ABSTRACT
Astaxanthin (AX) is known as a very strong antioxidant and has been utilised in many kinds of products such as foods, pharmaceutical, cosmetics, aquaculture, etc. One of the natural resources of AX is *Haematococcus pluvialis* which has been investigated by some researchers in order to enhance the AX production. However, the production of AX from the microalgae is still costly, hence, this present research is proposing low-cost methods namely bean sprout media (BSM) as an alternative growth media and UV radiation. The variations of BSM concentrations (2, 4, and 6 %) and times of UV radiation (1.5 and 3 hrs) were treated to *H. pluvialis* in laboratory conditions. BSM 4 % treatment showed an optimum growth of the microalga at 427 x 10^4 cell/ml (day 8) which also exhibited macrozooid, palmella, and aplanosore phases. UV radiation for 3 hr revealed that the concentration of AX production was as much as 17.37±0.04 mg/l. The research results were potential to be developed further in order to discover better and cheaper methods for scaling up AX production.

INTRODUCTION
Astaxanthin (AX) is a natural carotenoid which shows strong antioxidant, that is 500 times and 38 times higher than vitamin E and β-carotene, respectively (Han et al. 2013; Nurdianti et al. 2017; Han et al. 2019). It has a potency in health care products for skin, eyes, heart, liver, detoxification, photo protectant, immune response, and anticancer (Iwamoto 2000; Guerin et al. 2003). In addition, AX has also been utilized as natural pigment in food and aquaculture feed industries (Higuera-Ciapara et al. 2006). Therefore, demand for AX in some fields and industries are increasing worldwide and its market is estimated to gain 800 million USD in 2022 (Khazi et al. 2021).

AX belongs to a xanthophyll group, that dissolves in lipid and can be synthesised by some microalgae and marine animals (Ambati et al. 2014; Park et al. 2014; Hernayanti & Simanjuntak 2019). One of the microalgae that is rich in AX is *Haematococcus pluvialis* that can produce more than 30 g/kg dry weight (Butler et al. 2018). In order to fulfil the rising demand, researchers are trying to find best and low-cost methods

to increase AX production from *H. pluvialis* (Tocquin et al. 2011; Liu 2018).

There are some factors affecting AX production in microalgae such as light intensity, nutrition, salt content, pH, CO_2 concentration, and oxidative stress (Kavitha et al. 2015; Kwak et al. 2015; Huber & Blaha-Robinson 2016; dos Santos & Lombardi 2017). Nutrient contents in the growth media enhanced AX production in *H. pluvialis*, since nutrition compositions and nutrient concentrations determine biomass and metabolite productions (Prihantini et al. 2007; Putra et al. 2015; Trikuti et al. 2016). Natural medium such as bean sprout extract was common to be utilised for growing microalgae due to the nutrition compositions that compose of both macro and micro nutrients, such as protein, carbohydrate, lipid, P, K, Ca, Fe, Na, Cu, Zn, thiamine, riboflavin, nisin, and vitamin C (Ministry of Health of The Republic of Indonesia 2018).

Different light intensities and light qualities have been utilised in order to induce AX production (Kobayashi et al. 1992; Imamoglu et al. 2009; Saha et al. 2013). There were also some researches combining both light and nutrient factors to enhance AX production in *H. pluvialis* (Su et al. 2014; Ghosh et al. 2017; Zhang et al. 2018; Mehariya et al. 2020). Ultraviolet (UV) radiation and variation of growth media were reported to increase AX production (Muzaki 2008; Kavitha et al. 2015). However, there is no research report on the treatment of natural growth media (bean sprout media) and times of UV radiation to AX production of *H. pluvialis*.

Therefore, the objectives of this present research are to determine the optimum concentration of bean sprout medium (BSM) on the growth of *H. pluvialis* and to determine the time of UV radiation in order to enhance AX production.

MATERIALS AND METHODS

Materials

Stock culture of *H. pluvialis* was obtained from Estuarine Fishery Research Centre, Jepara, Central of Java. Acclimatization of *H. pluvialis* with initial culture at 100,000 cell/ml was done by providing Walne medium added with vitamin B12 and incubated at 25 °C with LED light 3200 lux illumination for 8 days (Putri & Alaa 2019; Khazi et al. 2021).

Methods

Bean sprout medium (BSM) preparation

As much as 750 g of bean sprouts were washed with tap water to cleanse the dirt. Clean bean sprouts were put into a bowl with 2000 ml distilled water and were boiled for 45 min. After getting warm, the bean sprouts were separated from the water by filtration. The filtered water was put into a flask and was utilized as BSM stock solution. Growth medium treatment for *H. pluvialis* were varied as follows BSM 2, 4, and 6 % respectively, with Walne as positive control medium and distilled water as negative control medium. All the growth media were sterilized at 121 °C for 20 min (Prihantini et al. 2007; Panis & Carreon 2016).

Inoculation of *H. pluvialis* in the growth media

Each medium was taken as much as 150 ml and put into 500 ml Erlenmeyer flasks and 150 ml of culture of *H. pluvialis* with density 100,000 cell/ml was added into every flask. Additional of 0.15 ml vitamin B12 was added into all the flasks. The cultures were incubated at 25 °C with aeration and were given LED 3200 lux light intensity with 10:14 dark:light cycle (Muzaki 2008; Kavitha et al. 2015).

Cell count and morphology of *H. pluvialis*

Cell count and morphology of the microalgae conducted before and during the treatments. Cell count was done utilising haemocytometer (Madigan et al. 2015; Liu 2018). The observation of cell morphology was done every two days throughout the research.

UV radiation treatment

As much as 150 ml media (BSM optimum treatment) and 150 ml *H. pluvialis* initial culture were mixed in a flask. Every flask was treated with UV lamp radiation for 1.5 and 3 hr. After irradiation, all the flasks were incubated for 18 days in order to achieve aplanospore phase (red phase) (Muzaki 2008; Han et al. 2013; Shang et al. 2016; Butler et al. 2018).

Astaxanthin (AX) extraction and analysis

Extraction of AX was done by taken as much as 5 ml *H. pluvialis* culture and was subsequently centrifuged for 15 min at 5900 rpm. KOH 5 % and methanol 30 % (v/v) solutions were prepared and then heated at 70 °C. The pellet was rinse three times with distilled water to discard alkali compound. Furthermore, the pellet was added with 5 ml DMSO (Wang et al. 2018). Extraction treatment was repeated until cell debris became colourless and the final volume reached 15 ml (Molino et al. 2018). The sample extract was diluted into 15 ml Dimethylsulfoxide (DMSO) and then was put into cuvettes. Every cuvette was placed into spectrophotometer and the absorbance was measured at 490 nm (Li et al. 2012). The concentration of AX (C_{AX}) was calculated utilizing the following formula (Liu 2018).

$$C_{AX}\ (mg/l) = 4.5 \times A_{490} \times (V_a/V_b)$$

where:
A_{490} = wavelength at 490 nm
V_a = extraction volume
V_b = sample volume

Data analysis

All treatments conducted in quintuplicates. Cell numbers of *H. pluvialis* data from different media treatments were analysed utilising Excel™ program. Anova with 95 % degree of confidence was applied for data of UV radiation treatments. Duncan Multiple Range Test (DMRT) was carried out whenever there were significant differences between the treatments.

RESULTS AND DISCUSSION

Cell morphology of *H. pluvialis*

Cell morphology of *H. pluvialis* was observed utilising trinocular microscope with 10 x 45 magnification (Figure 1). This present research found three different phases of cell morphology of *H. pluvialis*, namely macrozooid, palmella, and aplanospore. The round phase macrozooid was green in colour, but no flagellum visible.

According to Shah et al. (2016) macrozooid cells were round to ellipsoid or pear shape with 8-12 µm diameter. The disappearance of flagellum was due to the stress condition and cells were expanded in size and subsequently loss of their flagella (Figure 1A). Palmella phase was marked with reddish colour surrounding the nucleus which determines the accumulation of AX (Figure 1B). Wayama et al. (2013) reported that cell of *H. pluvialis* under stress condition will grow bigger with thicker

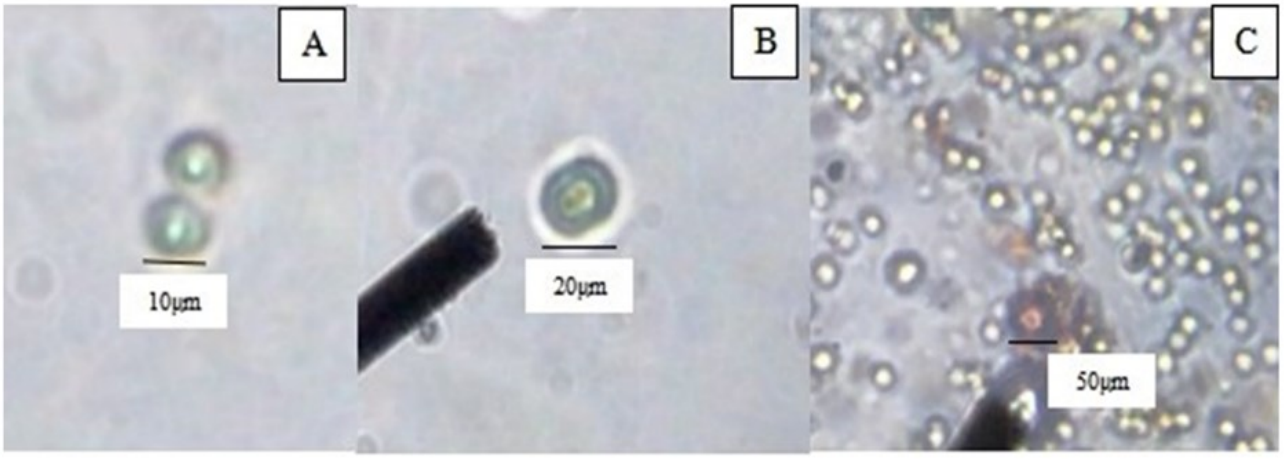

Figure 1. *Haematococcus pluvialis* cell's morphology. (A) round cell phase macrozooid, (B) early palmella phase, and (C) red phase aplanospore.

cell walls and reddish colour around the nucleus. If the stress condition continues the cell will transform into aplanospore phase with round shape, thick cell wall, and red colour in the cytoplasm (Figure 1C). Similar cell's morphological appearances in *H. pluvialis* were reported by Levin and Fleuerence (2018) and He et al. (2020).

Growth of *Haematococcus pluvialis* on various of media

The growth of *H. pluvialis* on various of media treatment was observed for 12 days. The growth was ended with death phase and followed by the decreasing of cell numbers in the media (Table 1, Figure 2).

In general, growth curve of *H. pluvialis* in different media reached stationary phase at day 8 and subsequently went into death phase at day 10. Negative control treatment showed no growth due to no additional nutrients for the cell. The highest cell number was obtained at BSM 4 % and considered as optimum concentration (Figure 2). Kavitha et al. (2015) observed *H. pluvialis* growth in Walne medium showed more turbid appearance in the flask. The media became more turbid and dark-green in colour due to the highest growth of the microalga at day 8 (Figure 3).

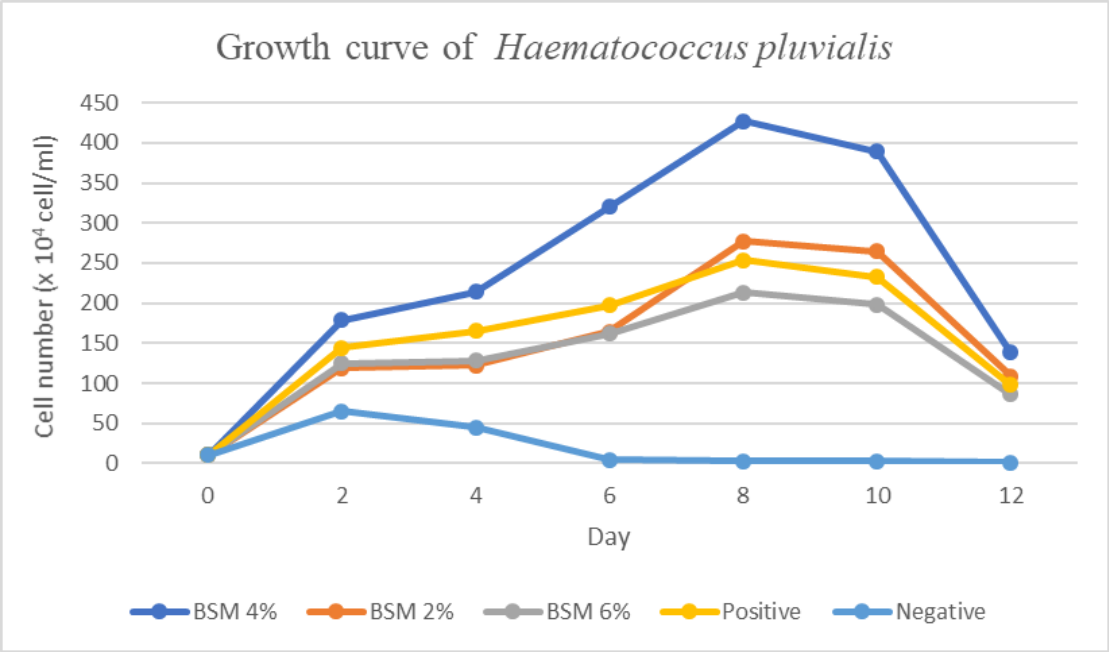

Figure 2. Growth curve of *H. pluvialis* in various BSM concentrations treated.

Table 1. Cell numbers of *H. pluvialis* (x 10^4 cell/ml) in various growth media.

Medium	Days of observation						
	0	2	4	6	8	10	12
BSM 2 %	10	119	122	164	277	265	109
BSM 4 %	10	179	214	320	427	389	138
BSM 6 %	10	125	128	162	213	198	8.6
Control +	10	144	165	197	254	233	9.8
Control -	10	65	45	40	30	30	1

Notes:
Control +: control positive (Walne medium)
Control -: control negative (distilled water)

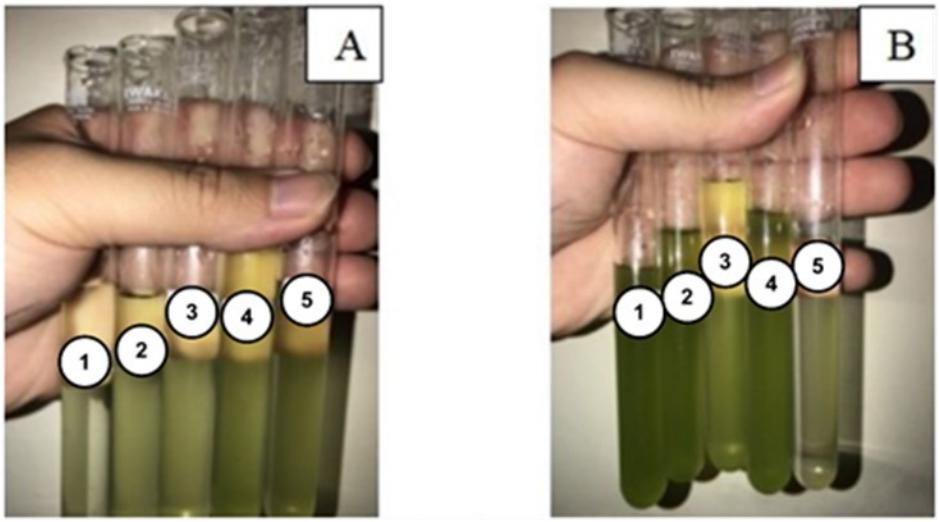

Figure 3. Visual observation of *H. pluvialis* growth in various media treated. (A) Day 0 and (B) Day 8. Notes: 1. BSM 4 %, 2. BSM 2 %, 3. BSM 6 %, 4. Control +, 5. Control –.

Astaxanthin (AX) production from *H. pluvialis*

The optimization of media variation revealed that BSM 4 % treatment showed the highest cell count of *H. pluvialis* and therefore BSM 4 % treatment was utilised in the subsequent step. *H. pluvialis* culture in the BSM 4 % medium was radiated with UV in order to put *H. pluvialis* in the stress condition and subsequently the cell will produce AX (Butler et al. 2018). UV radiations conducted within time variations 1.5 and 3 hr (Shang et al. 2016). The results were presented in Table 2.

No significant differences between treatments were shown from the growth of *H. pluvialis* cell cultures after UV radiation. The growth of the microalga cells was not affected by UV radiation variations for 1.5 and 3 hrs, although the cell numbers at UV radiation for 3 hr were higher than 1.5 hr treatment (Table 2). Previous researches reported that both light intensities and wave lengths affected the growth of *H. pluvialis* cultures (Evens et al. 2008). Logaritmic phase was ended at day 8 and the accumulation of AX required at least 10 days which were divided into three phases namely macrozooid phase (4 days), palmelloid phase (3 days) and aplanospore phase (3 days) (Butler et al. 2018).

Stress stimulation on the *H. pluvialis* cell cultures utilizing UV radiation can be observed in Figure 4. The figure revealed that *H. pluvialis* produces red colour in the cells which were expected coming from the AX. Therefore, the cell cultures were extracted utilising maceration method with DMSO as the solvent to reveal the AX contents in the cells (Wang et al. 2018). AX concentrations extracted from UV-stressed cell *H. pluvialis* were shown in Table 3.

Table 2. Growth of *H. pluvialis* cells (x 10^4 cell/ml) under UV radiation treatments.

Time of radiation (hr)	Medium	Day of observation									
		0	2	4	6	8	10	12	14	16	18
1.5	BSM 4%	10	144	183	217	348	204	128	111	99	91
	Ctrl +	10	82	165	175	266	158	103	68	42	35
	Ctrl -	10	37	12	5	3	0	0	0	0	0
3	BSM 4%	10	132	213	235	394	220	139	119	104	94
	Ctrl +	10	86	179	193	249	165	111	69	45	37
	Ctrl -	10	31	10	4	2	0	0	0	0	0

Notes:
Ctrl +: control positive (Walne medium)
Ctrl -: control negative (distilled water)

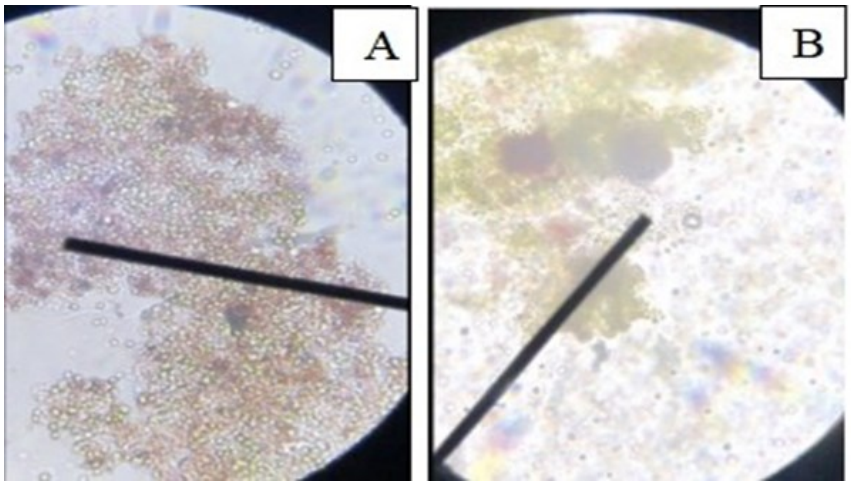

Figure 4. Red colour appearances were shown from *H. pluvialis* cell growth under UV radiation. (A) 3 hr radiation and (B) 1.5 hr radiation.

Table 3. Astaxanthin (AX) concentrations (mg/L) from *H. pluvialis* under UV radiation.

Medium	UV radiation (hr)	
	1.5	3
BSM 4 %	11.41 ± 0.03^a	17.37 ± 0.04^a
Control +	8.72 ± 0.02^a	12.36 ± 0.04^a
Control -	0.22 ± 0.01^b	0.24 ± 0.01^b

Notes: numbers with the same alphabet in the same row were no significant difference at $P<0.05$. Numbers were mean $\pm$ standard deviations (SD) from quintuplicates.

There was no significant difference between UV radiation variation treatments for 1.5 and 3 hrs, however AX concentrations at UV radiation for 3 hr were higher compare to 1.5 hr treatment (Table 3). This result was supported by the appearances of red cells after UV radiation for 3 hr (Figure 4). The length of radiation time was expected to stimulate the reactive oxygen species (ROS) and furthermore induced the AX production in the cell of *H. pluvialis* (Kavitha et al. 2015). This present research showed potential results and need to be explored further in order to discover better method with specific regard to scaling up the AX production. Therefore, some researchers have been proposing combination of growth media, radiation, and transgenic factors to enhance AX production from *H. pluvialis* (Gómez et al. 2013; Gao et al. 2015; Le-Feuvre et al. 2020).

CONCLUSION

Haematococcus pluvialis was able to grow optimally in bean sprout medium (BSM) 4 % with the highest cell number (427 x 10^4 cell/mL) achieved at day 8. Ultraviolet radiation for 3 hr enhanced astaxanthin production by the green microalga as much as 17.37±0.04 mg/L.

AUTHORS CONTRIBUTION

B.R.R.H. designed and conducted the research, B.R.S. supervised and wrote the manuscript, I.S.A. collected and analysed the data.

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CONFLICT OF INTEREST

None of the authors declare conflict of interests in publishing the research data as well as the research funding.

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