ICNTC-2019 Special Issue

J. Indian Chem. Soc.,
Vol. 96, September 2019, pp. 1143-1148

The acuumulation of astaxanthin from *Haematococcus pluvialis* in different stress factors

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Manuscript received online 26 April 2019, revised and accepted 20 July 2019

Astaxanthin is a high antioxidant which is red carotenoid pigment obtained from many microalgae species. It is an important preservative of the immune system and it is used in the treatment of cancer, skin and heart diseases. The most efficient source of astaxanthin is *Haematococcus pluvialis* microalgae. In this study, the optimum cultivation conditions of *H. pluvialis* and the accumulation of astaxanthin were investigated. Bold Basal media (BBM) and Bristol media (BM) were used for growth media. Different stress conditions were applied and the astaxanthin accumulation was examined. The stress conditions were the application of different illumination power, the addition of tap water and iron, nitrogen deficiency in both BBM and BM. The amount of astaxanthin, dry cell weight and cell count were measured. BBM was found to be more efficient than BM on growth of microalgae. The nitrogen-free BM was more efficient for production of astaxanthin than BBM. Using tap water has been also found advantageous in terms of cost. The effect of illumination power did not make any differences on astaxanthin accumulation.

Keywords: Microalgae, *Haematococcus pluvialis*, astaxanthin production, stress, medium.

Introduction

Astaxanthin (3,3′-dihydroxy-α,β-carotene-4,4′-dione) is a red ketocarotenoid which has highly strong biological activity in the human bodylike anti-inflammatory and antitumoral activity, thus presenting important applications in the nutraceutical and pharmaceutical industries[1]. Synthetic astaxanthin is much more cost-effective rather than natural astaxanthin which exhibits an increased capacity for free radical elimination, as well as increased bioavailability and safety for human health[2]. Therefore, natural astaxanthin is more desirable and great efforts have been made to identify feasible sources. Natural astaxanthin can be extracted from carapace, plants, bacteria and fungi. However, most of these sources have very low contents of astaxanthin, which made these extract processes high costs and inefficiency[3]. Natural astaxanthin is predominantly present in a broad range of seafood, such as shrimp, lobster, salmon, red seabream, trout and fish eggs. However, animals naturally containing astaxanthin do not have the ability to biosynthesize astaxanthin themselves, therefore, they must obtain astaxanthin from environmental sources, such as some microalgae species[4]. Due to the high content of astaxanthin (1–5% of dry cell weight), *Haematococcus pluvialis* is regarded as the best biological source of natural astaxanthin and the most promising microbial producer for commercial production[4]. *H. pluvialis*, unicellular freshwater microalgae, belongs to the class Chlorophyceae, order Volvocales, family Haematococcaceae. *H. pluvialis* has a distinctive lifecycle in which it undergoes a morphological change from motile bi-flagellated green vegetative cells during the growth phase to a red non-motile cell phase for astaxanthin accumulation[5]. Screening and isolation of an excellent strain of *H. pluvialis* and optimization of culture conditions are very important for obtaining high biomass and astaxanthin concentrations[4]. Therefore, the accumulation of astaxanthin in *H. pluvialis* is induced by various stress conditions. Factors inducing astaxanthin production include a wide variety of unfavorable environmental stimuli such as high light intensity, pH, salin-

ity, high temperature, nitrogen limitation, and phosphate deficiency[6,7].

In the present study, we investigated to improve astaxanthin productivity from microalgae *H. pluvialis* with optimum cultivation conditions. The appropriate microalgae cultivation conditions were determined for obtaining higher astaxanthin accumulation. Stress conditions were different content of media, differences of ventilation, illumination, temperature, nitrogen deficiency, using tap water and iron. Those effects were investigated for the productivity of microalgae and the accumulation of the astaxanthin.

Experimental

Materials and methods:

Microalgae culture *Haematococcus pluvialis* CCAP 34/8 was brought from culture collection of algae and protozoa (CCAP, Droop, Tvarminne, Finland).

Bold Basal media (BBM) and Bristol media (BM) were prepared. Components of BBM (for 400 mL) were $NaNO_3$ 10.0 g, $MgSO_4.7H_2O$ 3.0 g, NaCl 1.0 g, K_2HPO_4 3.0 g, KH_2PO_4 7.0 g, $CaCl_2.2H_2O$ 1.0 g. Trace elements stock solutions (1 mL) were prepared with using $ZnSO_4.7H_2O$ 8.82 mg, $MnCl_2.4H_2O$ 1.44 mg, MoO_3 0.71 mg, $CuSO_4.5H_2O$ 1.57 mg, $Co(NO_2)_3.6H_2O$ 0.49 mg, H_3BO_3 11.42 mg, EDTA 50.0 mg, KOH 31.0 mg, $FeSO_4.7H_2O$ 4.98 mg, H_2SO_4 1.0 mL. Components of BM (for 400 mL) were $NaNO_3$ 10.0 g, $MgSO_4.7H_2O$ 3.0 g, NaCl 1.0 g, K_2HPO_4 3.0 g, KH_2PO_4 7.0 g, $CaCl_2.2H_2O$ 1.0 g, 1.0% $FeCl_3$0.05 μL. Stock solutions of all chemical components of BBM and BM were prepared at pH 7.5–8.0 and sterilized in autoclave at 121°C for 20 min, separately. *H. pluvialis* was activated on BBM and BM contains agar. Then microalgae were cultured in BBM and BM for activation the microalgae. The temperature was set to 27±2°C. Illumination was performed with daylight (36 W) for 24 h.

Cultivation conditions of H. pluvialis microalgae:

H. pluvialis was cultivated under stress and stress-free conditions with using solid and liquid BBM and BM. The effects of two different solid media, temperature and illumination differences on the growth of microalgae were investigated. Firstly, the production of microalgae on solid media was performed by incubating 12 h daylight (36 W) for 12 h in dark environment at 25±2°C. After, the microalgae were incubated for 24 h of light at 27±2°C in the same media. Differences in the growth rates of the microalgae were qualitatively investigated in these conditions.

H. pluvialis were incubated in test tubes containing BBM and BM (10 mL/tube) for 24 h in daylight and at 27±2°C. Manual rinsing was performed 2–3 times in certain intervals. The optical densities (OD) were measured at 680 nm using UV/Vis spectrophotometer (Scinco S-3100, Seoul, Korea)[8] and cell numbers were calculated by Thoma counting method using an Olympos CX40 microscope every day for 15 days. The microalgae was inoculated into flasks containing 250 mL both liquid media. It was incubated at 27±2°C for 24 h in daylight (36 W). Manual rinsing was performed, OD were measured, and cell counts were performed during 15 days. Thereafter the culture was then inoculated into flasks containing 500 mL both liquid media. The three of cultures in the BBM and BM (500 mL) was stirred by manual rinsing for 2–3 times daily. The ventilation was applied to the another three of algae cultures. Daily OD measurements and cell counts were performed for 15 days. After this period, different stress conditions were applied to algae cultures. The cultures were harvested by centrifugation at 3500 rpm for 5 min at the end of incubation in liquid medium studies. Afterwards, three different stress conditions were studied.

Firstly 500 mL sterile tap water was added to the harvests of the cultures in BBM and BM. Cultures were incubated for 24 h in daylight (36 W) and at 27±2°C. Secondly, 500 mL of sterile nitrogen-free media were added to the harvest of the cultures. Incubated for 24 h in daylight (36 W) and at 27±2°C. Finally, 500 mL of sterile nitrogen-free media were added to the harvest of the cultures and incubated for 24 h at 72 W and 27±2°C. OD measurements and cell counts were performed daily for all stress conditions. Dry cell weight and astaxanthin analysis were performed every three days.

Determination of dry cell weight:

Whatman GF/C filter paper was used for dry cell weight determination. The filter paper was dried at 105°C for 1 h in the oven, cooled in the desiccator, then weighed on the analytical balance and tared. The well-mixed 5 mL sample was filtered through the filter paper. The residue in the filter paper was dried for 2 h at 105°C in an oven and cooled in a desiccator. Analytical balance was measured and the result was calculated[9].

Determination of astaxanthin content:

The sample was centrifuged at 3000 rpm for 5 min

(Hettich, Germany) and the supernatant was poured. Thus, the stress environment was removed. It was dried at 105°C for 1 h in the oven (Binder, Germany) and after cooling at the desiccator, 5 mg of sample was taken and placed in the tube. 0.5 mL water was applied by humidifying. The KOH-methanol solution (5% (v/v) methanol solution – 5% (w/v) KOH solution in water) was used to remove the chlorophyll pigment. The sample was centrifuged at 3000 rpm for 5 min and the supernatant removed. 2.5 mL acetic acid were added dropwise and 5 mL of DMSO was added. After standing for 10 min in a water bath at 70°C, centrifuge at 3000 rpm for 5 min and this time the supernatant was removed. This process was continued until white cells were obtained. The collected supernatant was measured at 490 nm and 750 nm by spectrophotometer. Calculation was carried out as follows[9,10].

$$\% \text{ Astaxanthin} = \frac{(A_{490} - A_{750}) \times 5.6 \times \text{Volume}}{\text{Sample weight}} \quad (1)$$

Results and discussion

In the study, *H. pluvialis* production was performed with using BBM and BM. The effect of different media on the growth efficiency of the microalgae was investigated. The effects of stress conditions on astaxanthin production efficiency were determined.

Cultivation conditions of H. pluvialis microalgae:

The effects of two different solid media, temperature and illumination differences on the growth of microalgae were compared. Using different illumination period and temperature on the microalgae growing was effective in solid BBM after 15 days (Table 1). In the first 15 days after the cultivation, any microalgae growing on solid BM was not observed in the first cultivation condition. In the second cultivation condition, the microalgae showed significant growth on solid BM.

Table 1. Differences of cultivation conditions on *H. pluvialis* microalgae production in solid BBM

Cultivation conditions	Illumination period and power	Temperature	Results
1	12-h daylight (36 W)	25±2°C	No growing after 15 days
2	24-h daylight (36 W)	27±2°C	Growing after 15 days

Astaxanthin accumulation from H. pluvialis:

After 12 weeks period, increasing the accumulation of astaxanthin was observed due to the spontaneous stress application on the microalgae. It is clearly that effective photoinduction is the primary factor affecting biomass growth and astaxanthin accumulation of *H. pluvialis*, due to its specific growth characteristics[9]. Despite many previous involving optimized the light conditions, such as light intensity, spectral distribution (light quality), and light period research in this arena has seldom taken the combined effects of the light path of photobioreactors and the illumination mode into consideration[4]. In the cultivation of microalgae with using both media (Table 2), the amount of reproduction in the cases where ventilation was applied and non-applied were compared by OD measurement (Fig. 1) and cell count (Fig. 2). Incubation temperature and illumination period were 27±2°C, 24 h daylight (36 W), respectively. The OD values shows that BBM has more effective on microalgae growth than BM and ventilation application had low effect for using both media. The cell number results of the microalgae in ventilated and non-ventilated BBM and BM were 118×10^5 cfu/mL, 104×10^5 cfu/mL, 101×10^5 cfu/mL and 86×10^5 cfu/mL, respec-

Table 2. Cultivation conditions on *H. pluvialis* microalgae

Cultivation conditions	Media	Ventilation
1	BBM	Applied
2	BBM	Non-applied
3	BM	Applied
4	BM	Non-applied

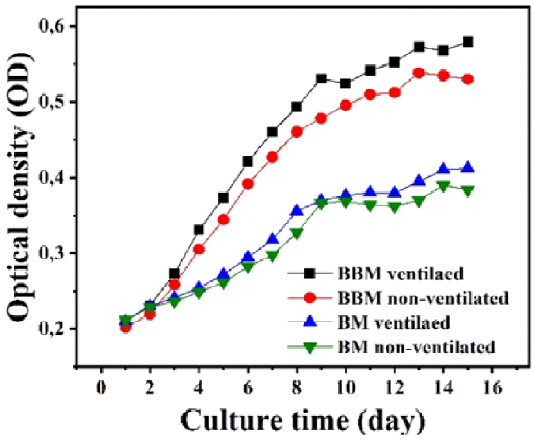

Fig. 1. OD results of ventilation effects on BM and BBM.

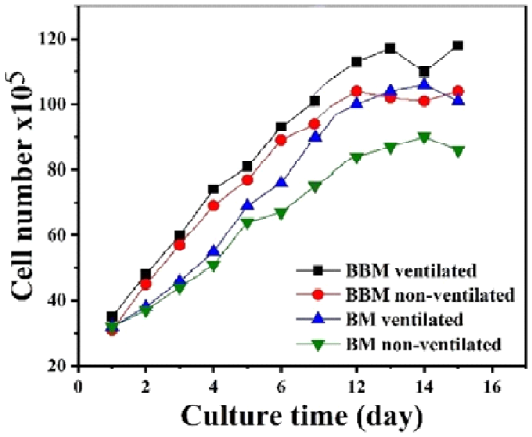

Fig. 2. Cell number results of ventilation effects on BM and BBM.

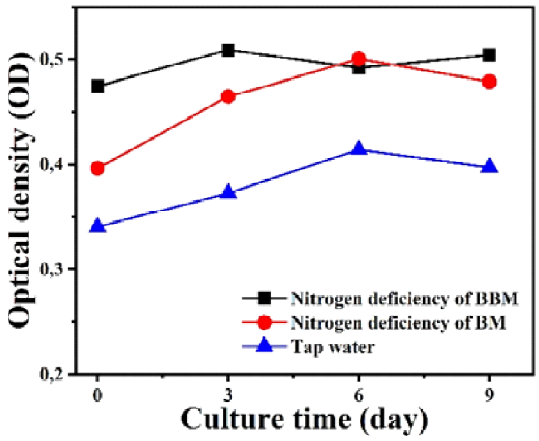

Fig. 3. OD results of *H. pluvialis* at 1, 2 and 3 stress conditions.

tively. The cell number results coincided with OD measurements. We can say that the BBM is more efficient for the culture of *H. pluvialis* than the BM.

The effect of stress conditions on the cell growing efficiency was investigated with using different media, illumination power and incubation time. Incubation temperature was 27±2°C (Table 3). The OD results were given in Fig. 3 under stress conditions for 1–3 samples in Table 3. In the study,

Table 3. Stressed cultivation conditions on *H. pluvialis* microalgae

Stressed cultivation conditions	Media	Illumination period and power	Incubation time (days)
1	Nitrogen-free BBM	24-h daylight (36 W)	0–9
2	Nitrogen-free BM	24-h daylight (36 W)	0–9
3	Tap Water	24-h daylight (36 W)	0–9
4	Nitrogen-free BM	24-h daylight (36 W)	6–12
5	Nitrogen-free BM + Fe	24-h daylight (36 W)	6–12
6	Nitrogen-free BM	24-h daylight (72 W)	6–12

conducted in tap water and nitrogen-free BM, OD values increased until the 6th day and then decreased until the 9th day. At the nitrogen-free BBM, OD values increased at the 3rd day. Fig. 4 displays the dry cell weight analysis results of three stress conditions. The linear increase in dry cell weight was occur. The cell counts decreased between 4th and 6th day using nitrogen-free BBM, BM and tap water but after the 6th day cell number increased for all stress conditions (Fig. 5). Astaxanthin accumulation in *H. pluvialis* at 1, 2 and 3

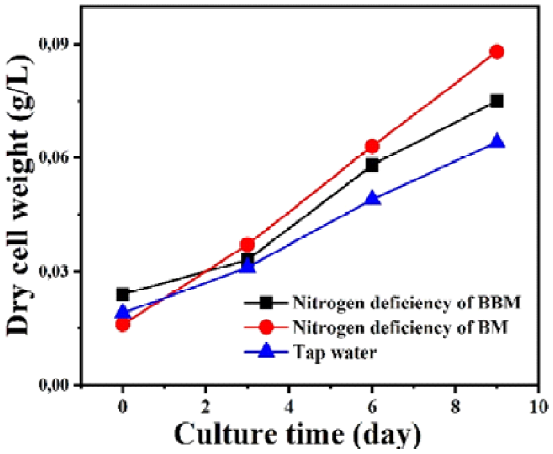

Fig. 4. Dry cell weight *H. pluvialis* at 1, 2 and 3 stress conditions.

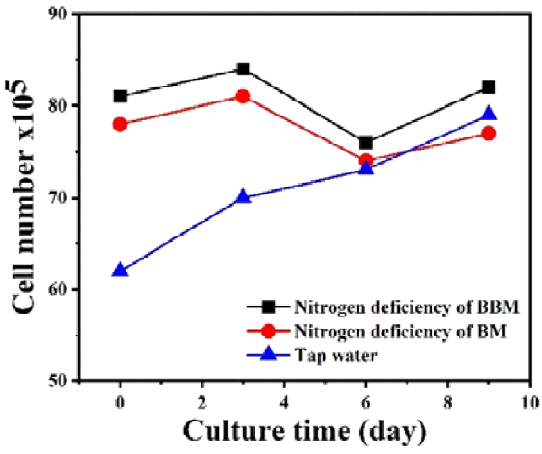

Fig. 5. Cell count of *H. pluvialis* at 1, 2 and 3 stress conditions.

stress conditions were analyzed, and results shown in Fig. 6. Astaxanthin accumulation reached the maximum levels at the 6th days but astaxanthin levels began to decrease at the end of the 6th day. While the accumulation of astaxanthin in the tap water and the nitrogen-free BBM was in the range of 1.1–1.2%, the yield in the study on the nitrogen-free BM reached 1.83%. Nitrogen-free BM was found to be efficient

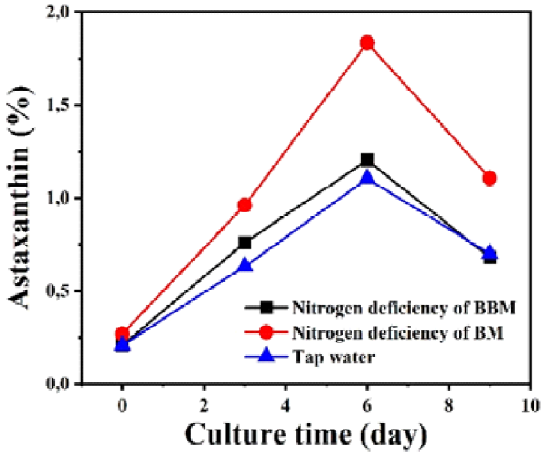

Fig. 6. Astaxanthin (%) accumulation in *H. pluvialis* at 1, 2 and 3 stress conditions.

in terms of astaxanthin accumulation in stress conditions. Fig. 7 shows the OD results of *H. pluvialis* at 4, 5 and 6 stress conditions. The OD values decreased slightly for these stress conditions. On the other hand, dry cell weight of *H. pluvialis* increased at the 4, 5 and 6 stress conditions (Fig. 8). The cell number of *H. pluvialis* increased up to the 9th

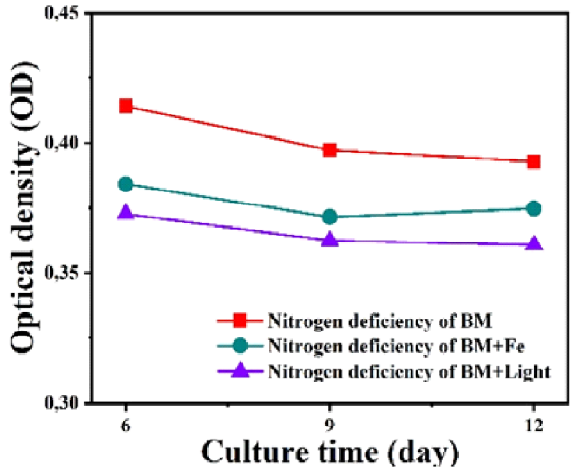

Fig. 7. OD results of *H. pluvialis* at 4, 5 and 6 stress conditions.

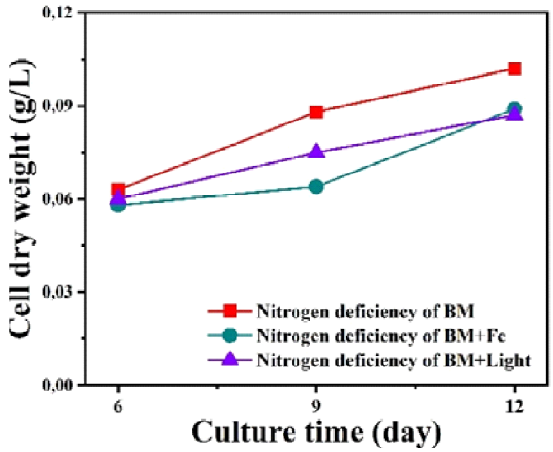

Fig. 8. Dry cell weight *H. pluvialis* at 4, 5 and 6 stress conditions.

day in the BM. But after the 9th day decreasing in cell numbers was observed for all stress conditions (Fig. 9). The cell count results demonstrated that nitrogen-free BM stress condition is much more effective than other stress conditions on *H. pluvialis* microalgae. Astaxanthin accumulation in *H. pluvialis* at 4, 5 and 6 stress conditions were analyzed, and results shown in Fig. 10. Astaxanthin accumulation decreased for all stress conditions. It was observed that the accumulation of astaxanthin decreased from 1.6% to 0.9% at the end of 12th day.

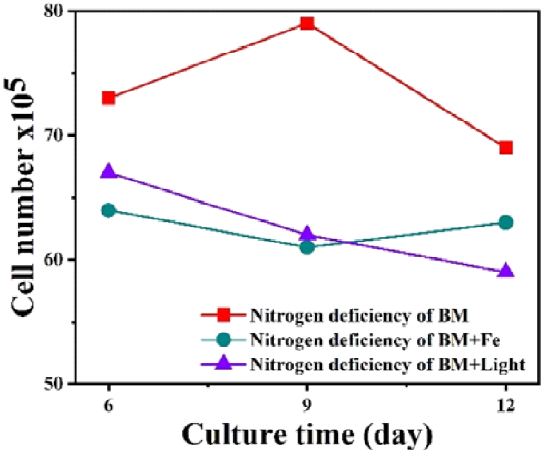

Fig. 9. Cell count of *H. pluvialis* at 4, 5 and 6 stress conditions.

The aim of this study was to investigate the high astaxanthin productivity at low cost. According to the results, the productivity of *H. pluvialis* culture shows the highest results in BBM while the productivity of astaxanthin accumula-

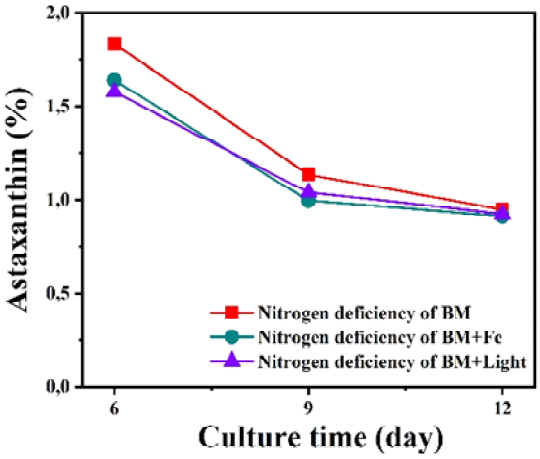

Fig. 10. Astaxanthin (%) accumulation in *H. pluvialis* at 4, 5 and 6 stress conditions.

tion represent the highest results in nitrogen-free BM. Using tap water in stress media showed no significant effect to production astaxanthin but it is recommended for economic astaxanthin productions.

Conclusions

The optimum cultivation conditions of *Haematococcus pluvialis* microalgae and the accumulation of astaxanthin from *H. pluvialis* were investigated in this study. The production of microalgae in the BBM with ventilation applied provides better results. On the other hand, astaxanthin production is more advantageous in the stress environment by using nitrogen-free BM. When tap water was compared to the BBM, astaxanthin deposition rates was close to each other. But the using of tap water in culture conditions is recommended in terms of cost for the feasibility studies. The approximate costs of the experiments conducted in the BBM and BM were found to be almost the same. However, since the production of astaxanthin is more efficient in the nitrogen-free BM, the use of this environment is recommended to produce astaxanthin.

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