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ASTAXANTHIN PRODUCTION BY TROPICAL MICROALGAE STRAINS ISOLATED FROM ENVIRONMENT IN MALAYSIA

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Abstract – Astaxanthin is one of the most important secondary metabolites with superior antioxidant property and is widely applied in nutraceuticals and pharmaceuticals industry. Recently, the production of astaxanthin from natural source has been mainly focused on the green microalgae, *Haematococcus pluvialis*, as it can accumulate large amounts of astaxanthin and its esters. However, the slow growth with low biomass yield and easy contamination by other fast-growing organisms are the main problems faced by *H. pluvialis*. In this study, the preliminary study on growth characteristic and amount of astaxanthin by tropical microalgae strains isolated from environment in Malaysia was reported. Two newly isolated microalgae including *Acutodesmus obliquus* and *Coelastrum* sp. were compared under various stress inductive conditions to identify the microalgae culture that can accumulate high amount of astaxanthin. The objective of this study is to compare and characterise astaxanthin accumulation using different species of green microalgae as mentioned above under different stress conditions. Findings of the preliminary studied indicated that the exposure of microalgae culture to high light intensity and nitrogen starvation in mixotrophic culture is a potential inducer of high amount of astaxanthin production. Among the two microalgae studied, *Coelastrum* sp., exhibited the highest tolerance to stress conditions. Outcomes of this work have shown that, astaxanthin produced in *Coelastrum* sp. is the most comparable to *H. pluvialis* and can be the potential alternative to current astaxanthin production.

INTRODUCTION

Astaxanthin (3,32 -dihydroxy–β, β2–carotene-4,42-dione) from carotenoid pigment is known as one of the most valuable compounds with a great potential in the market. Recently, the production of astaxanthin from natural sources has become one of the most successful activities in biotechnology that has been widely applied in the nutraceuticals, cosmetics, food, feed additive and pharmaceutical industries (Shah *et al*., 2016). It has been now proven that astaxanthin can help the human body to maintain a healthy state as it has strong pigmentation function, powerful antioxidative activity and is more effective in scavenging free radicals (Dragos *et al*., 2016).

Currently, only a minor portion of the market is contributed by natural astaxanthin while synthetic astaxanthin monopolises the market. However, the production of astaxanthin from natural sources has received a great demand in the recent market since the production of natural astaxanthin has significantly greater antioxidant capacity than the synthetic astaxanthin (Shah *et al*., 2016).

Even though there are various organisms capable of producing astaxanthin, only a small number of

these organisms are commercially cultivated (Ranga *et al*. 2014). Microalgae constitute very promising bio-catalysts to be implemented in the increasing field of biotechnology (Wijffels *et al*., 2013). It is represented with the most potential for natural astaxanthin production and considered as the best natural source of astaxanthin (Ranga *et al*., 2014). Due to that, the current study on production of astaxanthin by green microalgae is very encouraging.

Among the commercially potential microalgae, *Haematococcus pluvialis* is known to be one of the richest sources of natural astaxanthin as it can accumulate large quantities of astaxanthin compared to other organisms (Dragos *et al*., 2016). Although *H. pluvialis* can accumulate a high amount of astaxanthin, it grows relatively slow with a low biomass yield, easy to be contaminated by other fast-growing organisms and is sensitive to high hydrodynamic stress (Lorenz and Cysewski, 2000; Ravi *et al*., 2014). Consequently, this hindered the commercial production of astaxanthin as it is not cost effective and unable to compete with synthetic astaxanthin (Milledge, 2011).

Therefore, the search for other strains to overcome these problems is an important area to be explored in the production of natural astaxanthin (Ranga *et al*., 2014). The objective of the present study is to extensively compare the astaxanthin profile using tropical microalgae strains isolated from environment in Malaysia with *H. pluvialis*. These microalgae were screened and characterised with respect to their cell growth and astaxanthin accumulation under different stress inductive conditions. The contribution of this study is obvious as the resulting outcomes can be the potential alternative in producing huge amount of astaxanthin from microalgae to enhance the bio based economy worldwide.

MATERIALS AND METHODS

Generally, it has been known that there are two stages for commercial application in producing astaxanthin from microalgae; the first stage (green stage) and the second stage (red stage) (Spolaore *et al*., 2006). The first stage is carried out under optimum growth conditions to achieve high growth rate and high cellular densities. Once the desired cellular density is achieved, the microalgae were then exposed to stress conditions in the second stage to induce and increase the accumulation of astaxanthin (Herrero *et al*., 2012).

Microalgae strains and culture conditions

Two tropical microalgae strains isolated from environment in Malaysia were selected for experimental purpose. The microalgae strains of *Acutodesmus obliquus* and *Coelastrum* sp. were isolated from a sampling site at Hulu Langat river, Kuala Selangor, Malaysia by Algal Biomass team members from Malaysia-Japan International Institute of Technology (MJIIT) and identified using 18S rDNA analysis. *Haematococcus pluvialis* (NIES-144) obtained from National Institute for Environmental Studies (NIES), Japan was used as a control in this study. The strains were cultured in Af-6 medium comprising $NaNO_3$, NH_4NO_3, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, Fe-citrate, Citric acid, KH_2PO_4, K_2HPO_4, Trace metal solution ($FeCl_3.6H_2O$, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, $Na_2EDTA.2H_2O$) and mix of vitamin (Biotin, Pyridoxine, Thiamine) according to media recipe available in the Microbial Culture Collection National Institute for Environmental Studies (NIES-collection), Japan.

These microalgae underwent two stages of production namely first stage (green stage) for optimum growth production and second stage (red stage) which was used to increase astaxanthin accumulation. During the first stage, the strains were all grown under normal condition in culture room at 25±1 °C with continuous aeration and illuminated at 12 h: 12 h (Light: Dark) cycle with florescence light at normal photon flux densities (PFD) of 70 µmol photons $m^{-2} s^{-1}$. The inoculum was cultured in 250 mL Erlenmeyer flasks contained 100 mL culture. The method of Boussiba and Vonshak (1991) was used to determine the biomass weight and expressed as gL^{-1}.

To determine the effects of different stress inductive conditions on growth and induction of astaxanthin synthesis, the green cells of all the microalgae cultures including control in the late exponential growth phase during the first stage were subsequently exposed to continuous illumination of high photon flux densities (PFDs) of 250 µmol photon $m^{-2} s^{-1}$ to induce the accumulation of astaxanthin. Another set of cultures were harvested by centrifugation at 2000 xg for 10 min at 4 °C and re-suspended in new culture medium without nitrogen source. Meanwhile, last set of cultures were further subject to combined stresses, namely high light, nitrogen starvation and cultured

in mixotrophic medium supplemented with 100 mM sodium acetate. The cells were then subjected to extraction of astaxanthin and all the experiments were carried out in triplicates.

Extraction of astaxanthin

Extraction of astaxanthin was carried out according to the method by Sarada *et al*., (2006). To measure the astaxanthin content, 50 mL of microalgae cultures were centrifuged at 2000 xg for 5 min at 4°C. The samples then were freeze dried for 24 h (Eyela, FDU-1200, Tokyo Rikakikai Co., Ltd). The dried biomass was treated with 5% KOH in 30% (v/v) methanol at 70 °C for 5 min and washed by resuspending it with distilled water. This step was used to remove the chlorophyll when pure astaxanthin is required. The cells were then centrifuged at 2000 xg for 10 min at 4 °C and the supernatant was discarded. Following that, the pre-treatment of astaxanthin was done to cleave the vital bond in the cell wall of a cell to facilitate the astaxanthin extraction by adding 4N hydrochloric acid (HCl) to the cell and heated at 70 °C for 2 min, cooled and washed with distilled water. The astaxanthin extraction was followed by solvent extraction with acetone and left to react for 1 hour. The supernatant was analysed by HPLC. All steps were carried out under dim light.

Determination of astaxanthin

The extracted astaxanthin from microalgae was identified and quantified using HPLC (Agilent Technologies 1220 Infinity LC) with a reverse phase C_{18} column (250 x 4.6 mm, 5 µm) equipped with photodiode array detector (Brinda *et al*., 2004). The mobile phase used was (A) acetone (B) methanol: water (9:1 v/v) at a flow rate of 0.8 mL/min with column temperature of 25°C. The injection volume was 10 µL. The absorption spectra of the pigment were shown between 250 and 700 nm. All the peaks of carotenoid were integrated at a wavelength of 476 nm to quantify astaxanthin. The separated carotenoids were identified by comparing retention times and against astaxanthin standard (Sigma-Aldrich, St. Louis, USA). The amount of astaxanthin accumulated in all microalgae studied was compared before (green stage) and after exposure to stress conditions in red stage.

RESULTS AND DISCUSSION

Growth curve and total biomass production

The performance of microalgae production can be monitored by measuring the biomass of microalgae in the culture (Orosa *et al*., 2000). During the green stage which is before the microalgae cultures were transferred to different stress inductive medium, they were first grown under identical conditions to compare the growth profile of different species of microalgae studied. Results as shown in Fig. 1 shows the growth profile of two tropical microalgae strains of *Acutodesmus obliquus* and *Coelastrum* sp. with *H. pluvialis* used as a control. The growth profile of *Acutodesmus obliquus* was found to be higher than that of *Coelastrum* sp. with highest total biomass yield during green stage and was shown in Fig. 2. Meanwhile, as expected *H. pluvialis* showed the slowest growth with lowest total biomass yield during green and red stage.

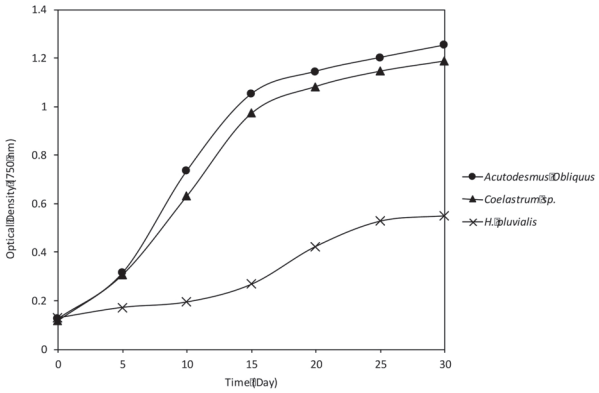

Fig. 1. The growth profile of *Acutodesmus obliquus*, *Coelastrum sp.* and *H. pluvialis*

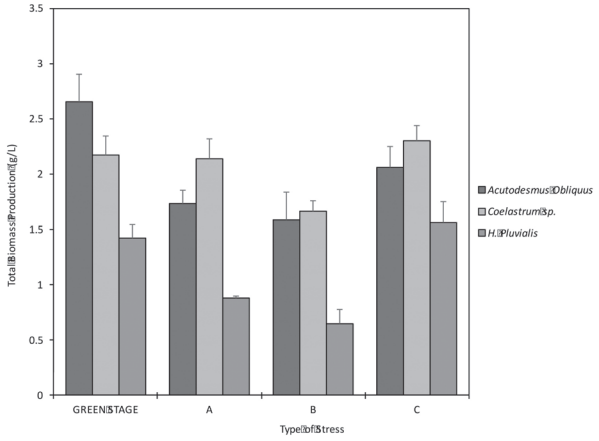

Fig. 2. Total biomass production of microalgae during green stage and under different stress inductive conditions (A) high light; (B) high light and nitrogen starvation; (C) high light, nitrogen starvation and mixotrophy medium

Astaxanthin production at green stage

Under normal condition before induction of stress

(green stage), the extract of the cell from *Acutodesmus obliquus*, *Coelastrum* sp. and *H. pluvialis* showed a peak at the same retention time as the astaxanthin standard, which confirmed the presence of astaxanthin in the cell. The concentration of standard astaxanthin was used as a reference to calculate the concentration of astaxanthin in a sample according to HPLC peak area. Fig. 3 displays concentration of astaxanthin was highest in *Coelastrum* sp. with 1.68 mg/L followed by *H. pluvialis* (0.99 mg/L) and *Acutodesmus obliquus* (0.96 mg/L).

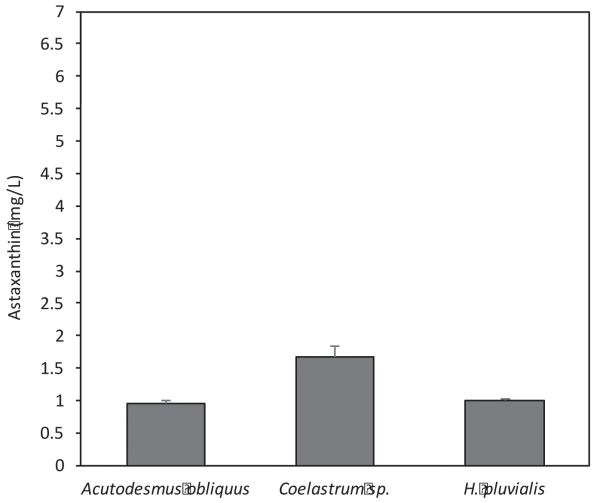

Fig. 3. Production of astaxanthin before induction of stress during Green stage

During green stage, which was before induction of stress, all the microalgae studied showed the presence of astaxanthin in the exponential growth phase. The presence of astaxanthin in logarithmic growth phase could suggest some nutritional deprivation during this growth phase. Intriguingly, *Coelastrum* sp. showed higher and early production of astaxanthin which was 1.7-fold higher than *H. pluvialis* during the green stage production.

Astaxanthin production at red stage

In the mid to late exponential growth phase, all the microalgae studied were harvested and transferred to different inductive stress media to compare the amount of astaxanthin accumulation under (A) high light intensity, (B) high light intensity and nitrogen starvation and (C) high light intensity, nitrogen starvation and addition of carbon source. At this stage, these microalgae cultures were slowly turned to yellow-orange, which may depict the accumulation of secondary carotenoids.

The results of HPLC analysis towards astaxanthin accumulation under different stress conditions as depicted in Fig. 4 suggested that the addition of sodium acetate as carbon source can lead to the production of highest astaxanthin accumulation with 4.51 mg/L, 3.77 mg/L and 4.8 mg/L in *Coelastrum* sp., *Acutodesmus obliquus* and *H. pluvialis* respectively. On the other hand, nitrogen deprivation and high light intensity led to a smaller production of astaxanthin compared to that by adding carbon source.

Surprisingly, *Coelastrum* sp. showed a special ability of synthesising large concentration of astaxanthin under high light intensity. Astaxanthin production was able to reach 4.38 mg/L, which was almost four-fold higher than in culture of *Acutodesmus obliquus* (1.14 mg/L) under high light intensity. These findings were somewhat surprising as the production of astaxanthin in *Coelastrum* sp. was two-fold higher than *H. pluvialis* with the production of astaxanthin 2.19 mg/L as shown in Fig. 4. This could be due to the lowest biomass yield and slowest growth rate of *H. pluvialis* that may affect the low production of astaxanthin.

Nitrogen starvation and high light intensity are the primary factors in accumulating secondary carotenoids especially astaxanthin (Orosa *et al*., 2001). However, these conditions can inhibit the growth of microalgae and thereby lead to cell death during stress inductive conditions. If the cell of microalgae died, it will not be able to synthesise astaxanthin from carotenoids, thereby decreasing the amount of astaxanthin. Therefore, the combined effect of carbon and nitrogen is an important factor to allow the synthesis of a large amount of astaxanthin. Thus, the ratio of high carbon concentration with low nitrogen concentration can trigger the accumulation of high amount of astaxanthin (Liu *et al*., 2013).

By adding the carbon source during the inductive stress condition, the growth of microalgae was able to be sustained since less number of nitrogen and high light induction were applied during this stage. The yield of biomass of microalgae under stress inductive conditions can influence the amount of astaxanthin production. According to the result shown in Fig 2., the addition of sodium acetate as carbon source in stress inductive medium led to the highest biomass in *Coelastrum* sp. (2.3 g/L), *Acutodesmus obliquus* (2.06 g/L) and *H. pluvialis* (1.56 g/L).

Carbon is the most important element for

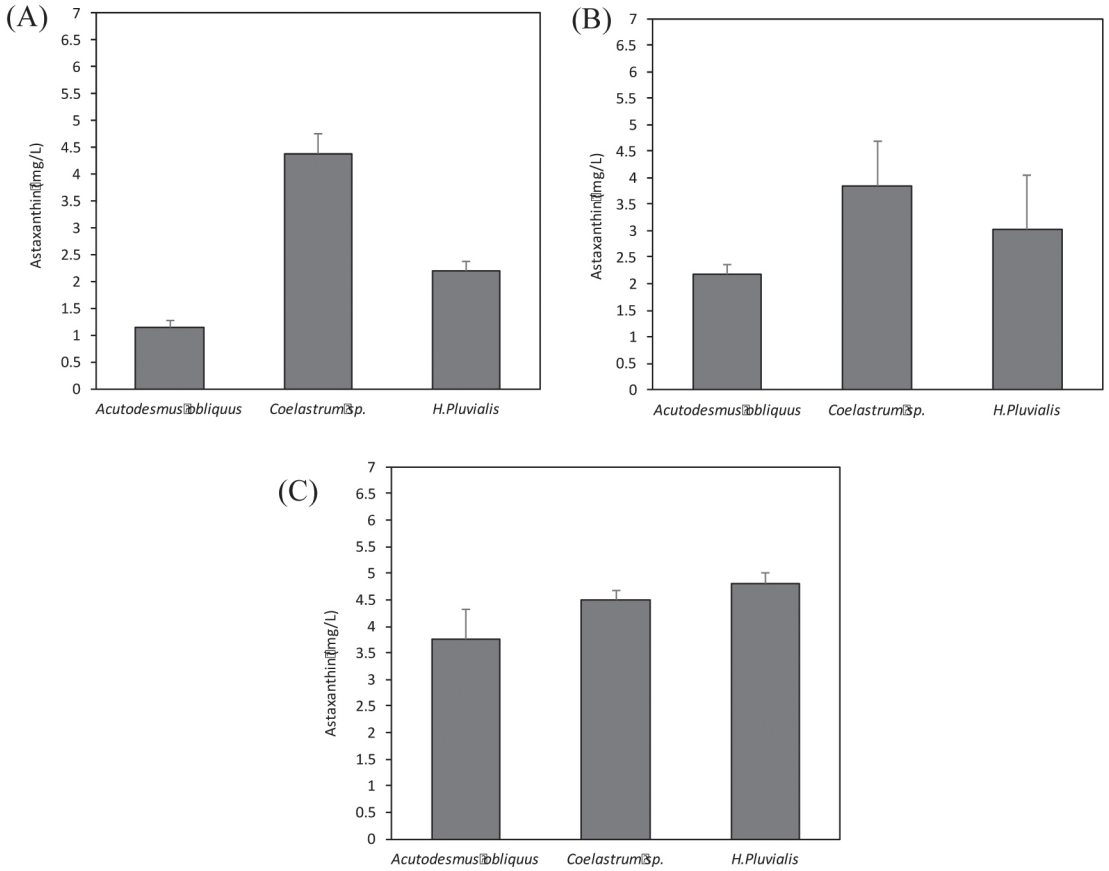

Fig. 4. Effect of different stress inductive conditions towards astaxanthin production (A) high light; (B) high light and nitrogen starvation; (C) high light, nitrogen starvation and mixotrophy medium

microalgae cultures as it is made up of 50% of microalgae biomass (Liu *et al*., 2014). Besides, acetate has been commonly used as the major carbon source of growth and astaxanthin production by *H. pluvialis* (Borowitzka *et al*., 1991). In this study, it was found that the biomass yield and the production of astaxanthin improved from the addition of 100 mM of sodium acetate under mixotrophic condition. This condition led to a higher production of astaxanthin in all microalgae studied.

Study by Choi *et al*., (2002) also reported that the addition of acetate as the carbon source with the combination of nitrogen starvation has increased the accumulation of astaxanthin in *H. pluvialis* (Choi *et al*., 2002). This indicated that the balance ratio between initial carbon and nitrogen in culture medium can give a great impact on carotenogenesis.

The outcomes of this study indicated that among two different species of newly isolated tropical microalgae strains, the amount of astaxanthin accumulated by *Coelastrum* sp. was the highest even if compared to *H. pluvialis*, under high light and nitrogen starvation conditions. Besides, *Coelastrum* sp. which was the newly isolated strain from Hulu Langat, Kuala Selangor, Malaysia has an important feature with relatively early astaxanthin accumulation and higher amount of astaxanthin compared to *H. pluvialis* with almost two-fold higher than control during the green stage. Apart from that, *Coelastrum* sp. showed high capability of biomass yield in a short time compared to *H. pluvialis*. These characteristics could make *Coelastrum* sp. as the potential candidate for astaxanthin production thus overcoming the slow growth associated with *Haematococcus*.

CONCLUSION

The main goal of the current study was to determine the alternative of potential microalgae strain in producing natural astaxanthin. The productivity of astaxanthin of microalgae can be greatly influenced by a variety of nutrient and environmental factors. The result of this study has identified that,

Coelastrum sp., a newly isolated strain from Malaysia to be a potential strain for producing astaxanthin from natural source as it was able to accumulate higher amount of astaxanthin than *H. pluvialis* under high light and nitrogen starvation conditions. Besides, these findings able to provide the following insights for future research since this strain was locally isolated from Malaysia' environment. Hence, ongoing research can be continued under existing climate for cultivation of *Coelastrum* sp. for astaxanthin production.

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