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ASTAXANTHIN-OVERPRODUCTION FROM MICROALGAE *HAEMATOCOCCUS PLUVIALIS* UNDER PHYSICAL AND CHEMICAL INDUCTION OF STRESS

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ABSTRACT

H. pluvialis is a microalgae that can accumulate astaxanthin up to 5% dry weight of cells under stress conditions. The aim of study was to increase production of astaxanthin through physical, chemical induction of stress and the combination of physical and chemical induction of stress. *H. pluvialis* was cultivated using Walne's medium. On day 7, *H. pluvialis* was added with iron (II) heptahydrate with serial concentrations 450, 550, 650 µM as chemical stress induction. Physical stress induction is carried out by irradiating UV-B with exposure time 30, 60, and 90 minutes. Physical and chemical stress induction by irradiating UV-B with 90 minutes exposure time and the addition of Fe^{2+} 550 µM. Culture *H. pluvialis* was cultivated for 16 days to induce astaxanthin accumulation. *H. pluvialis* was extracted by maceration method using DMSO solvent. The results of quantitative analysis using a spectrophotometer, showed that UV-B irradiation for 90 minutes, the addition of Fe^{2+} with a concentration of 550 µM, and the combination of both can increase astaxanthin content 0.745 m /L, 0.926 mg/L and 0.771 mg/L respectively, than the control 0.342 mg/L. The results of the qualitative analysis using TLC silica gel F_{254} showed that the extract assumed contain astaxanthin.

Keywords: Astaxanthin, *H. pluvialis*, UV-B, Fe^{2+}

INTRODUCTION

Astaxanthin (3,3'-dihydroxy-β-carotene-4,4'dione) is a secondary metabolite that belongs to the carotenoid group (1). Astaxanthin is a high-value secondary

metabolite product in the pharmaceutical, nutritional and cosmetic because astaxanthin is very strong antioxidant with an IC_{50} 39,1 ± 1,14 ppm (2). The antioxidant activity of astaxanthin is 65 times higher than vitamin C, 54 times stronger than β-carotene, 14 times higher than vitamin E and 20 times stronger than synthetic astaxanthin (3). Astaxanthin also can be used in supporting therapies for several degenerative diseases such as type 2 diabetes mellitus, hypertension, even cancer (4).

The natural sources of astaxanthin are found in marine animals such as red snapper, salmon, and lobster (5, 6). But for the production of astaxanthin on a large scale, the source of marine animals is less efficient because of their long growth. So that the natural astaxanthin sources are selected from several microorganisms such as the group basidiomycota *Phaffia rhodozyma* (5), mikroalgae *Haematococcus pluvialis* (6), *Chlorella zofingiensis* (7), gram negative bacteria *Agrobacterium aurantiacum* (8) and *Paracoccus carotinifaciens* (9) whose growth is relatively fast. But of these microorganisms, *Haematococcus pluvialis* has the highest astaxanthin content up to 5% of the dry weight cells to accumulate astaxanthin under stressful conditions due to unfavorable environments (10).

The market value of astaxanthin varies usually from $2500–7000/kg, while its global market potential was estimated at 280 tons and was valued at $447 million in 2014. Of this market, more than 95% refers to synthetically derived astaxanthin, since it involves lower production costs (around $1000/kg) than the algal alternative (11). Natural astaxanthin approximately 20 times stronger in free radical elimination than synthetic astaxanthin (3). Synthetic astaxanthin is produced from petrochemical sources, which raises the issues of food safety (because are different stereochemistry between natural astaxanthin and synthetic astaxanthin, also potential toxicity in the final product). In fact, to date, synthetic astaxanthin can only be used as an additive to fish feed for pigmentation purposes and has not been approved for direct human consumption in food or supplements (12).

The microalgae *Haematococcus pluvialis* is capable of accumulating natural astaxanthin under stress condition such as high light (13), UV-B (14), Fe^{2+} (15), nitrogen and phosphorus deprivation (16), salinity (17) and using various chemicals to trigger or enhance accumulation of astaxanthin (18).

According to Zahra (2017), one stressor can only induce the synthesis and accumulation of astaxanthin at a low level. Therefore,

further research is needed on the combination of stress induction to increase astaxanthin production. The aim of study was to increase production of astaxanthin from *Haematococcus pluvialis* using UV-B radiation, Fe^{2+} and a combination of both as a stressor. The results of this study could help to be an alternative method for increasing the production of astaxanthin from *Haematococcus pluvialis.*

MATERIALS AND METHODS

The materials used in this study are glass sampling bottle 1 L, aerator, cuvette quartz, *microtube* 1,5 ml (Biologix®), vial 10 ml, micropipette 100-1000 µl (*Fisher brand*™), tip micropipette, light microscope (Yazumi), *autoclave*, TL lamp (Philips®), UV-B lamp, UV ʎ 254 nm and ʎ 365 nm lamp, *haemocytometer* (Neubauer®), luxmeter, thermometer, spectrofotometer UV-Vis (Shimadzu UV1800®), *microsentrifuga* (MPW-55®), sonicator (Elma S 40 H®).

Haematococcus pluvialis was obtained from Balai Besar Perikanan Budidaya Air Payau (BBPBAP) Jepara Jawa Tengah, mineral water AQUA®, Walne medium, iron (II) heptahydrate ($FeSO_4.7H_2O$), Neurobion® tablet, aquades, DMSO (*Dimethyl Sulfoxide*), solution of 5% (w/v) KOH in 30% (v/v) methanol, TLC *silica gel* F_{254} (Meck®), acetone, formic acid, n-hexane.

Sterilization Method

Sterilization using autoclave at 121° C (249° F) for around 15–20 minutes and the pressure is 15 pounds/sq inch (1 atmosphere).

Acclimatization

Acclimatization *H. pluvialis* for adaptation to a new natural enviroments will normally occur within 5–7 days at room temperature (19).

Medium Selection

The enriched medium used in the present study was Walne's medium with vitamin B_{12} sourced from Neurobion tablet and Walne's medium that already contained vitamin B_{12} (obtained from BBPBAP Jepara). The total volume of culture is 900 mL, inoculum cell 10% (v/v) 90 mL microalgaee liquid culture was added with 810 mL sterile mineral water, were added 500 µL Walne's medium, aeration 24 hours, at room temperature (25 ± 1 ° C), photoperiod 12:12 (dark:light) cycles with a light intensity of 3000 lux (20, 21).

Activation of Microalgae Cells

Activation microalgae cells is carried out under the same conditions as in the media selection procedure. The media used were selected media (Walne's medium with vitamin B_{12} obtained from BBPBAP Jepara).

Cultivation

H. pluvialis cultivation was carried out under the same conditions as activation microalgae

cells conditions, the initial algal cell concentration (inoculum cells) was about 1 x 10^6 cell/mL, calculated using a haemacytometer under a light microscope with a 40x objective lens (magnification of 400x).

Microalgae Growth Curve Analysis (OD)

5 mL algal cells were harvested, and optical density (OD) values of the alga were measured under 680 nm absorbance using spectrophotometer (20).

Physical and Chemical Induction

H. pluvialis was treated with thephysical and chemical induction at the exponential phase or end of logaritmic phase (for negative control, the culture was not given any treatment). Physical induction is carried out by exposing UV-B with different time of exposure (30, 60 and 90 minutes) (22). Chemical induction is carried out with different concentrations of Fe^{2+} (450, 550, 650 µM) (15). Combination ofmultiple stress induction treated with UV-B radiation (exposure time of 90 minutes) and Fe^{2+} (550 µM).

Extraction

To estimate the astaxanthin content of the cells, 10 mL *H. pluvialis* during induction period (day 12, 13, 14, 15, 16) were collected by centrifugation. The cell pellet was washed twice with distilled water and then treated with a solution of 5% (w/v) KOH in 30% (v/v) methanol at 65°C for 15 min to destroy the chlorophyll. The pellet was centrifuged for 5 min at 5000×g and washed three times with distilled water. The treated pellet was ultrasonicated with 5 mL dimethylsulfoxide (DMSO) for 10 min and extracted in a 45°C water bath for 20 min. The extraction procedure was repeated as necessary until the pellet became colourless (23, 24).

Supernatant Analysis

The supernatants (for step remove chlorophyll) were analyzed by Thin-Layer Chromatography (TLC) silica gel F_{254} plate, the mobile phase n-hexane-acetone-formic acid (4: 2: 10 drops). TLC plates were visualized under UV at ʎ 254 nm and UV at ʎ 366 nm.

Astaxanthin Accumulation Determination

Total astaxanthin was spectrophotometrically determined by measuring absorbance at 490 nm. All experiments were conducted in triplicate. The astaxanthin content (mg/L) was calculated according to the following equation:

$$C\ (mg/L) = 4{,}5 \times A490 \times \frac{Va}{Vb}$$

where Va (mL) and Vb (mL) are the volume of DMSO and the algal samples, respectively, and A490 is the absorbance of the extracts at 490 nm (25, 23, 24, 26).

Identification of Astaxanthin by TLC

Astaxathin was analysed by TLC silica gel F_{254} plate, for this experiment ± 20 microliter of *H. pluvialis* algal extracts were spotted on the silica gel F_{254} plate along with standard astaxanthin. Spot development was done by using mobile phase; n-hexana-acetone (3:1). The separated bands were identified using standard astaxanthin and internationally accepted R_f values for astaxanthin by Lorenz Todd (27).

RESULTS AND DISCUSSION

Selection of culture medium for growth of *H. pluvialis*

The enriched media used in the present study was Walne's medium with vitamin B_{12} sourced from Neurobion tablet and Walne's medium that already contained vitamin B_{12} (obtained from Jepara BBPBAP). Walne's medium with vitamin B_{12} sourced from Neurobion tablet show that the microalgae are not able to grow as shown in **Figure 1**. This occurs assumed because there are other ingredients in Neurobion tablet that are not suitable for *H. pluvialis* microalgae growth.

Walne's medium which already contains vitamin B_{12}show that the microalgae was growth as shown in **Figure 2**. Based on the results of media selection, the best medium for growth *H. pluvialis* is Walne's medium which already contains vitamin B_{12}.

***H. pluvialis* Growth Curve**

H. pluvialis growth curveas shown in **Figure 3**.

The logarithmic growth phase (log phase) occurs since the early age of culture. At the beginning of the age of culture, it is seen macroscopically the culture is still clear in color as shown in **Figure 4**. The logarithmic phase is the phase where cells divide rapidly so that an increase in the number of cells occurs. At the end of the logarithmic phase, which is on the 7th day, macroscopically the culture is green as show in **Figure 5**, and on that day stress induction is carried out to increase the number of microalgae cells that accumulate astaxanthin. According in the study of Shang *et al* (2016), stress induction at end of the logarithmic phase produces higher astaxanthin levels compared to stress induction carried out at other phases.

The decline in growth rate occurs at the age of culture on the 8th day, the phase where there is still an increase in the number of cells, but the growth rate has decreased because there is high competition in the culture media, where the available food substances are not proportional to the number of cells, so that only some cells get enough nutritions and can carry on their lives.

The stationary phase occurs at the age of culture 8 to 10 days, which is the phase

where the growth is stopped. In this phase, the number of cells is constant. This is due to the depletion of nutrients in the media or due to the accumulation of toxic metabolic products, resulting in stalled microalgae growth.

Extraction

The extraction process is carried out by the maceration method using organic solvent Dimethyl Sulfoxide (DMSO). DMSO is an aprotic polar solvent that can dissolve polar or nonpolar compounds. Extraction using the DMSO solvent is also called the "sucking process" extraction method. This is because DMSO can increase the permeability of thick *H. pluvialis* microalgae cell walls, and can also penetrate well without damaging the cell walls (29). Also, according to the literature comparing types of organic solvents for astaxanthin extraction, DMSO is a solvent that can produce extracts with a fairly high astaxanthin content, which is 66.64 ± 0.61% (30).

Extraction procedures based on the literature of (23) in Zhao *et al* (2018) (24). First, several stages of sample preparation are carried out before the extraction process, including the following:

Microalgae were harvested using centrifugation method. The cell pellet was washed twice with distilled water to remove residual medium and then treated with a solution of 5% (w/v) KOH in 30% (v/v) methanol at 65°C for 15 min to destroy the chlorophyll (lipohilic). Chlorophyll can be saponified in the presence of sodium hydroxide, leading to the production of water-soluble chlorophyllin and phytol (31). Based on the saponification reaction, chlorophyll can be easily separated from other liposoluble biochemical compounds and the supernatant will look green and the pellets look yellow to orange as in **Figure 6**. Accordingly, this saponification treatment is often used in the determination of carotenoids content. Due to the similar absorption wavelength of chlorophyll and carotenoids at 440–490 nm, chlorophyll in biomass greatly interferes the determination of carotenoids. After the chlorophyll is removed, DMSO is added to the pellet and then sonicated. Sonication can damage the cell walls of microalgae, so as to streamline the extraction process (30).

Supernatant Analysis

The supernatant that is thought to contain chlorophyll were analyzed by thin-layer chromatography (TLC) silica gel F_{254} plate. The chromatogram as shown in **Figure 7**.

Based on the results as shown in **Figure 7**, show a bright fluorescence red spot under UV at 366 nm. According to Francis (2000),

all chlorophyll derivatives show a bright red fluorescence under UV ultraviolet light. This shows that in the supernatant there is chlorophyll.

Astaxanthin Accumulation Determination

In this study, determining the estimated profile of astaxanthin was measured only from the first pellet extraction. However, absorbance measurements were carried out on the extracts from the extraction results 1, 2 and 3 as shown in **Figure 8**. The measurement results showed absorbance values that are much different at ʎ 490 nm between the results of the first pellet extraction, 2 and 3 as shown in **Table 1**.

The estimated profile of astaxanthin levels was measured during induction period (day 12, 13, 14, 15, 16) for determine the age of harvesting *H. pluvialis* with the maximum content of astaxanthin.

Profile Estimation of Astaxanthin Content treated with UV-B

Ultraviolet (UV) radiation, especially UV-A (320–400 nm) and UV-B (280–315 nm), can induce reactive oxygen species (ROS) and has significant effects on the growth and productivity of microalgae and plants. Microalgae and plant have developed two major classes of antioxidants to mitigate the damage from UV-induced ROS: enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and non-enzymatic antioxidant such as ascorbate, flavonoids, and carotenoids (14).

Based on the results of the study, an estimated profile of astaxanthin levels with induction stress of UV-B radiation as shown in **Figure 9**.

Based on **Figure 9**, showed that an increase in astaxanthin levels with UV-B induction radiation, compared with negative controls. This is following research (22), that the induction stress of UV-B can increase astaxanthin levels.

The highest estimated astaxanthin levels is generally on day 14 after stress induction. However, in the treatment of UV-B stress induction with 90 minutes exposure time, the highest astaxanthin level increase occurs early (on day 12 after stress induction). This occurs assumed because of UV-B radiation with the exposure time 90 minutes, causing the large production of free radicals in microalgae.

The peak value of estimated astaxanthin content (0,745 mg/L) occurred in the UV-B with exposure time 90 minutes on day 12 after induction stress. This value is two times higher when compared to negative controls on day 12 (0.342 mg/L).

In the UV-B radiation treatment with exposure time 30 minutes and 60 minutes showed an estimate of astaxanthin levels lower than the 90 minutes exposure treatment. It is allegedly that the time of UV-B exposure at that time is not enough to cause stress conditions in *H. pluvialis* microalgae.

Profile Estimation of Astaxanthin Content treated with Fe^{2+}

The growth of microalgae is affected by light, temperature, nutrients (mainly N, P) and some trace elements. Among trace elements, iron is one of the most essential elements required by microalgae. Iron deficiency has been demonstrated to limit the growth of microalgaee even in high nutrient environments (32). Furthermore, ferrous ion addition has been suggested promoting astaxanthin formation (33). The ferrous form of Fe in particular is known to give rise to free radical formation (especially hydroxyl radicals, HO•) via Fenton chemistryand it has been suggested that free radicals may play a role in the accumulation of astaxanthin (33).

Based on the results of the study, an estimated profile of astaxanthin levels treated with Fe^{2+} as shown in **Figure 10**.

Based on **Figure 10**, showed that an increase in astaxanthin levels treated with Fe^{2+} stress induction, compared with negative controls. The highest estimated astaxanthin levels is generally found on day 15 after stress induction. However, in the addition of Fe^{2+} stress induction with a concentration 550 µM,the highest astaxanthin level increase occurs early (on day 12 after stress induction).

The peak value of estimated astaxanthin content (0,926 mg/L) occurred in the 550 µM Fe^{2+} treatment on day 12 after induction stress. This value is three times higher when compared to negative controls on day 12 (0.342 mg/L).

However, astaxanthin accumulation under 450 µM Fe^{2+} treatment was strongly decreased. According to Zahra (2017), the addition of Fe^{2+} in the range of 0-450 µM is thought to be too low to cause stress conditions in *H. pluvialis*. Then, astaxanthin accumulation under 650 µM Fe^{2+} treatment also decreased. Allegedly the concentration is too high and causes too much accumulation of free radicals that inhibits the survival of *H. pluvialis*.

Profile Estimation of Astaxanthin Content treated with Combination of Multiple Stress Induction

Based on the results of the study, an estimated profile of astaxanthin levels treated with Fe^{2+} as shown in **Figure 11**. Based on **Figure 11**, showed that an increase in

astaxanthin levels treated with combination of multiple stress Induction (Fe^{2+} 550µM and 90 minutes exposure to UV-B radiation) compared with negative controls. The highest estimated astaxanthin levels was on day 14th day after stress induction (0.771 mg/L).

According to Zahra (2017), astaxanthin levels can be increased by using a combination of multiple stress factors. In this study a combination of stress factor (UV-B and Fe^{2+}) did not increase astaxanthin levels higher than a single stress induction.

IDENTIFICATION OF ASTAXANTHIN BY TLC

Based on the resultsdetermination of astaxanthin by the spectrophotometric method, the highest concentration of astaxanthin was obtained by the treatment of the addition Fe^{2+} 550 µM. Furthermore, the extract with the highest astaxanthin concentration was selected for TLC analysis. The results of a qualitative analysis of astaxanthin using TLC silica gel showed as **Figure 12**.

Based on the results as shown in **Figure 12**, show a spot (or band) under UV at 366 nm with R_f value 0, 33. According to Lorenz (1998), the R_f values obtained for free astaxanthin is 0, 30 and this occurs assumed that *H. pluvialis* algal extract contain astaxanthin.

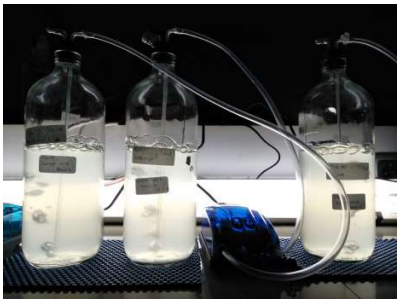


Figure 1: Effect of Walne's medium with vitamin B_{12} sourced from Neurobion tablet on the growth of *H. pluvialis*

Figure 2: Effect of Walne's medium wich already contain vitamin B_{12} on the growth of *H. pluvialis*

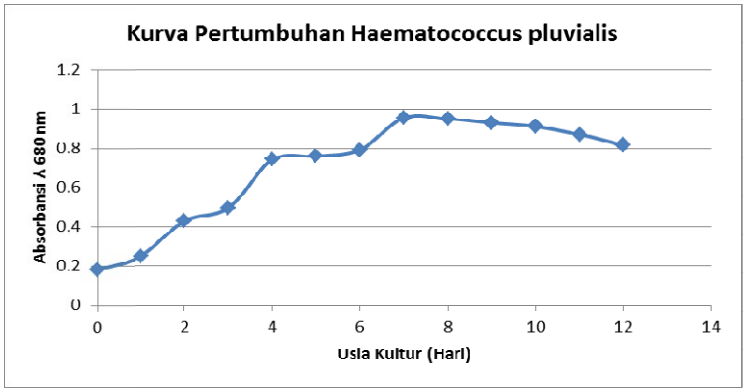


Figure 3: *H. pluvialis* growth curve in Walne's Medium

Figure 4: *H. pluvialis* culture on day 0

Figure 5: *H. pluvialis* culture on day 7

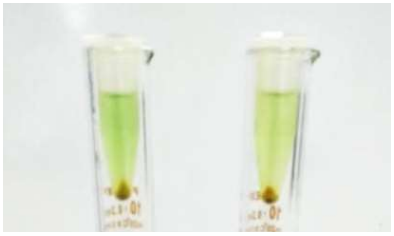


Figure 6: The supernatant contains the chlorophyll

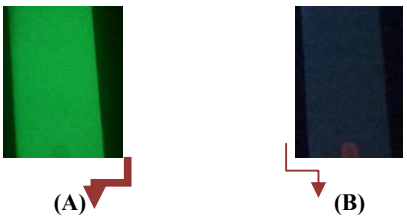


Figure 7: Thin Layer Chromatography of supernatant contain chlorophyll, stationary phase silica gel F_{254}; mobile phase is n-hexane-acetone-formic acid (4: 2: 10 drops) (A) visualized under UV at ʎ 254 nm and (B) UV at ʎ 366 nm.

Figure 8: *H. pluvialis* algal extract

Table 1: Extract Absorption at ʎ 490 nm

Extract	Absorbance
1	0,274
2	0,070
3	0,080

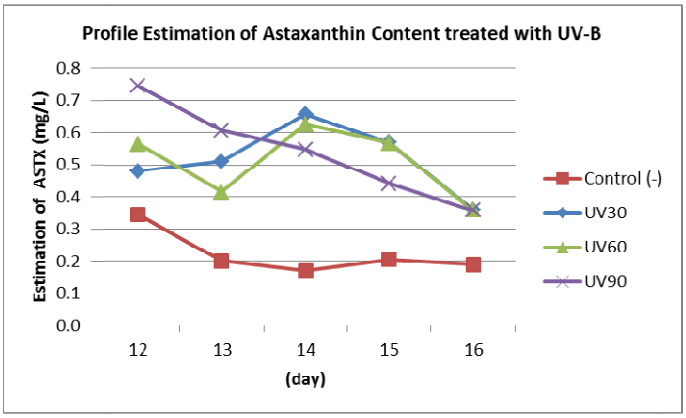


Figure 9: Profile Estimation of Astaxanthin Content treated with UV-B

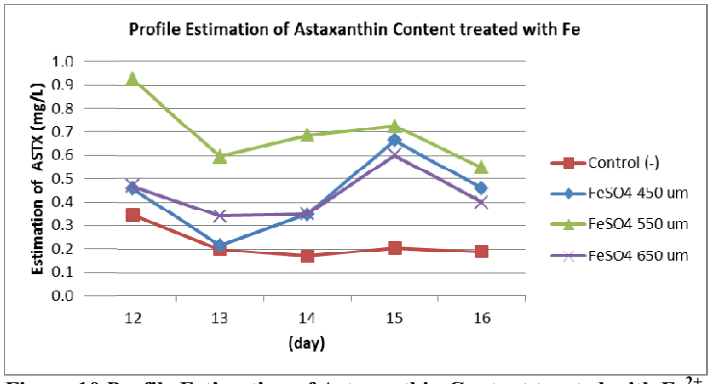


Figure 10.Profile Estimation of Astaxanthin Content treated with Fe^{2+}

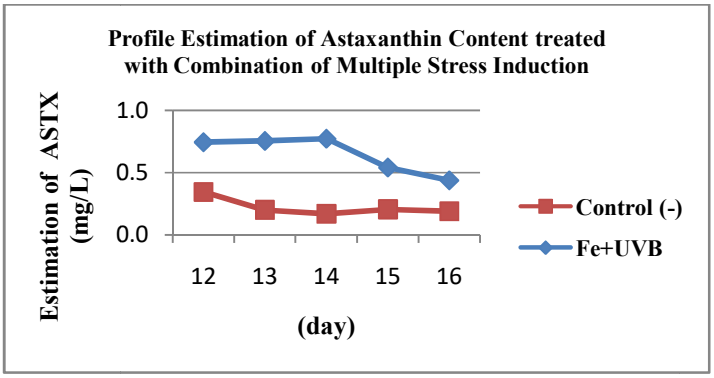
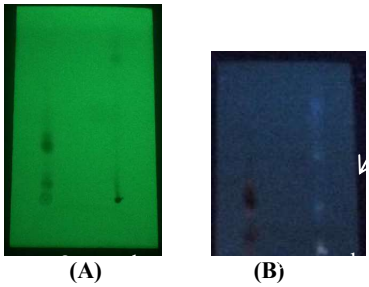


Figure 11: Profile Estimation of Astaxanthin Content treated with Combination of Multiple Stress Induction



(A) **(B)**

Figure 12: Thin Layer Chromatography of (a) standar astaxanthin (b) *H. pluvialis* algal extract; stationary phase silica gel F_{254}; mobile phasen-hexana-aceton (3:1) (A)visualized under UV at ƛ 254 nm and (B) UV at ƛ 366 nm

CONCLUSIONS

The results of this study show that UV-B radiation, Fe^{2+} treatment and also combination of multiple stress induction physical and chemical stress induction (UV-B and Fe^{2+}) can increase the production of astaxanthin in *Haematococcus pluvialis*.

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