Manipulation of Environmental Stress Towards Lutein Production in *Chlorella fusca* Cell Culture

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Abstract: All carotenoids originate from a single, common precursor, phytoene. The colour of carotenoids is determined by desaturation, isomerization, cyclization, hydroxylation and epoxidation of the 40-carbon phytoene. The conjugated double-bond structure and nature of end ring groups confer on the carotenoids properties such as colour and antioxidant activity. Algae may become major sources of carotenoids but the extent of environmental stress and genetic influences on algae carotenoid biosynthesis are poorly understood. Carotenoid biosynthesis can be influenced by many aspects and is liable to geometric isomerization with the existence of oxygen, light and heat which affect the colour degradation and oxidation. Therefore, in this study carotenoid biogenesis is investigated in cell culture of *Chlorella fusca* as a potential model system for rapid initiation, and extraction of carotenoids by providing stringent control of genetic, developmental and environmental factors. The value of this experimental system for investigating key factors controlling the carotenoid accumulation is then tested by assessing the effects of environmental variables, such as drought stress, light intensity, nutrient strength and media formulation on carotenoid accumulation. Our findings revealed that the conversion of violaxanthin to lutein is due to irradiance stress condition, nutrient strength as well as drought stress. As a result, manipulation of environmental variables will up-regulate lutein concentration. This reaction will restrict the supply of precursors for ABA biosynthesis and the algae cell culture responds by increasing carotenogenic metabolic flux to compensate for this restriction. In conclusion, selecting the appropriate algae species for the appropriate environmental conditions is not only important for yield production, but also for nutritional value quality of carotenoid.

Keywords: Carotenoid, phytoene, carotenogenesis, *Chlorella fusca*, violaxanthin, lutein.

INTRODUCTION

Carotenoids biosynthesis can be found in all photosynthetic organisms (bacteria, algae and plants) and also appear in some of the non-photosynthetic fungi and bacteria. Carotenoid produced by microalgae is a high value-added product that benefits especially to human nutrition and health. For the past few decades, there have been a drive of interest in microalgal biotechnology for producing valuable molecules which include pigments, sterols, vitamins, therapeutic protein and biofuels, and polyunsaturated fatty acids. Algal pigments are categorised into chlorophylls a, b, and c, phycobilin pigments and carotenoid. Beta-carotene, lutein, and astaxanthin have a potential possibility of high market value [1]. However, it still requires significant studies to improve the method to be highly economic and competitive in markets [2]. Generally, the carotenoids steady-state levels are depending by the rate of biosynthesis, the storage capacity of the cell and the rate of catabolism and degradation. The mass spectrum has identified the carotenoid profiles which indicate lutein, violaxanthin, zeaxanthin, astaxanthin and beta-carotene. The carotenoid produced from algae is different for different species and it depends on the type of culture condition of the algae. Higher plants synthesised xanthophylls, for example; violaxanthin, antheraxanthin, zeaxanthin, neoxanthin and lutein that may also be synthesised by microalgae with some additional xanthophylls which are loroxanthin, astaxanthin and canthaxanthin [3]. Primary carotenoid is the structural and functional components of the cellular photosynthetic apparatus, and they are aiding in the survival of the microalgae [4]. The secondary carotenoid will be produced only after some exposure to specific environmental stimuli. Due to its hydrophobic properties, xanthophylls may associate with a membrane or covalently binding with protein and are usually located in the thylakoid membrane [4]. On the other hand, a secondary carotenoid may be found in lipid vesicles either in the plastid or stroma [4]. In microalgae, carotenoids play several roles besides harvesting light where it involves

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in stabilising the structure, dissipating excess energy, and scavenging reactive oxygen species [5]. As for the environmental stress, it is caused by the external conditions that can affect growth, productivity, and development. According to [6], carotenoids can play a symbolic role to protect photosynthetic organisms from the excessive light, and it can be shown *in vitro* of photosystem II complexes [7]. One of the factors that can cause photo-inhibition and photo-oxidation in plants is when light that has been stressed to the plants and caused the increase in production. In the carotenoid biosynthesis, light plays its role and make it one of the factors for the regulation of biosynthesis [8]. In the production of several carotenoids and metabolism of microalgae, quality of light was affected and being one of the most serious limiting factors [9].

MATERIALS AND METHODS

Mass Production of *Chlorella fusca* Cell Culture

Chlorella fusca was selected in this study, Polyethylene glycol (PEG) (1g/L, 3g/L, 5g/L), sodium chloride (NaCl) (10g/L, 20g/L, 60g/L) and salicylic acid (SA) (25µg/L, 50µg/L, 75µg/L) were added to the media as environmental stress agent. Microalgae cell culture will be incubated in a growth room at 24ºC temperature for day and night, with a 16-h photoperiod under a cool white fluorescent light. Bold's Basal Medium (BBM) was sterilised by autoclaving (15 min, 121ºC) and 4-liter aliquots poured into pre-sterilised 5 liter Schott bottle. 10% of inoculum with absorbance reading of 0.6AU from the stock culture was transferred into the sterilizing media.

Effect of Environmental Factors on Carotenoid Biosynthesis in *C. fusca* Cell Culture

In five independent experiments the influence of light on media formulation, water-stress, salt-stress, drought-stress and nutrient availability towards carotenoid biosynthesis were tested in *C. fusca* cell culture under the following conditions:

1. Incubation of *C. fusca* cell culture developing in BBM, BBM modified with vitamin B and Bristol media formulation under cool-white, fluorescent lamps (80-85 µmol m-2 s-1; 16 h photoperiod) with a dark condition imposed by carefully wrapping the culture vessels in aluminium foil.

2. Incubation of *C. fusca* cell culture developing in BBM, BBM with vitamin B and Bristol media formulation in response to 0.5x, 5.0x and 10.0x $NaNO_3$ salt stress with light and dark condition.

3. Incubation of *C. fusca* cell culture developing in BBM in response to 1.0, 3.0 and 5.0 g/L PEG stress with light and dark condition.

4. Incubation of *C. fusca* cell culture developing in BBM in response to 25, 50 and 75 mg/L salicylic acid stress with light and dark condition.

5. Incubation of *C. fusca* cell culture developing in BBM in response to 10.0, 40.0 and 60.0 g/L NaCl salt stress with light and dark condition.

Cell Culture Extraction and Analysis of Carotenoids

The extraction procedure followed from by the methods described by [10]. For each of the sample, powdered freeze-dried materials were rehydrated by adding 1 mL of distilled water, followed by 5 mL of acetone and methanol mixture (7:3) that has been premixed with calcium carbonate ($CaCO_3$). The crude extracted was then centrifuged for 5 min at 10 000 g and stored at 4ºC in the dark prior to analysis. To extract carotenoids an equal volume of hexane and distilled water was added to the combined supernatants. The solution was then allowed to separate and the upper layer containing the carotenoids was collected. The combined upper phase was then dried to completion under a gentle stream of oxygen-free nitrogen.

Saponification

Samples were saponified with a mixture of acetonitrile and water (9:1) and methanolic potassium hydroxide solution (10% w/v). Base carotenoids were then extracted by addition of 2 ml hexane with 0.1% butylated hydroxytoluene (BHT), followed by addition of 10% NaCl until phase separation was achieved. The extracts were washed with distilled water, dried under a gentle stream of oxygen-free nitrogen and re-suspended in ethyl acetate for spectrophotometry and HPLC analysis [10].

HPLC Analysis of Carotenoids

The HPLC analysis of carotenoids was performed on an Agilent model 1200 series comprised of a quaternary pump with autosampler injector, micro-degassers, column compartment equipped with a thermostat and a diode array detector. The column used was a ZORBAX Eclipse XDB-C_{18} end-capped 5 µm, 4.6x150 mm reverse phase column (Agilent Technologies, USA). The eluents used were (A) acetonitrile: water (9:1 v/v) and (B) ethyl acetate. The column separation was allowed via a series of gradient

such as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 mL min^{-1}. The column would be allowed to re-equilibrate in 100% A for 10 min prior to the next injection. The temperature of the column was maintained at 20°C. The injection volume is 10 µL each. Detection of individual carotenoids was made at the wavelengths of maximum absorption of the carotenoids in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), zeaxanthin (452 nm), β-carotene (454 nm), β-cryptoxanthin (450 nm) and α-carotene (456 nm). Compounds were identified by co-chromatography with standards and by elucidation of their spectral characteristics using a photo-diode array detector. Detection for carotenoid peaks was in the range of 350 to 550 nm. Individual carotenoid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas, to total carotenoid content determined by spectrophotometry. The total and individual carotenoid concentration would be expressed in terms of a milligram per 1.0 g dry weight of freeze-dried matter (µg/g DW).

Statistical Analysis

All measurements were calculated in triplicates (n=3) and the values were averaged with standard deviation (±SD). Statistical analysis was completed by using Statistical Analysis Software (SAS). The differences between the means were analysed by ANOVA test followed by Tukey's post-hoc test. A significant difference was considered at the level of $P< 0.0001$.

RESULTS AND DISCUSSION

Analysis of variance on the effect of light on media formulation, water-stress, salt-stress, disease-stress and nutrient availability towards carotenoid biosynthesis in *Chlorella fusca* cell culture established highly significant differences in all their interaction for all the carotenoid content and composition.

Effect of Light and Media Formulation on the Carotenoid Accumulation in *C. fusca* Cell Culture

C. fusca cell culture accumulated two individual carotenoids compounds (violaxanthin and lutein) when developing in both dark and light at three different media. The BBM and BBM + vitamin B were observed accumulated only trace of violaxanthin whereas C. fusca cell culture developing in Bristol accumulated violaxanthin and lutein in either dark or light treatments (Figure **1**). However, development of *C. fusca* cell culture in light resulted in an approximate doubling of the total lutein content (44.19 µg/g DW) compared *C. fusca* cell culture developing in darkness (16.08 µg/g DW) (Figure **1**). The amount of violaxanthin remained below 5.0 µg/g DW upon development in light and dark, for all types of medium.

Effect of Nutrient Stress on the Carotenoid Accumulation in *C. fusca* Cell Culture

As shown in Figure **2**, only lutein was found in all types of medium grown in response to light at varying $NaNO_3$ salt strengths, but upon development in darkness none was detected. After development in light, when $NaNO_3$ salt strength increased from 0.5x to 10.0x, total lutein content increased, compared to the first treatment as shown in Figure **1**. In BBM + vitamin B medium, when $NaNO_3$ salt strength increased from 0.1x to 10.0x, lutein content increased from 157.97 to 244.98 µg/g DW. In contrast, upon further increases in $NaNO_3$ salt strength, 0.5x to 10.0x in BBM and Bristol media, lutein content slightly decreased from 162.07 to 158.98 µg/g DW and 176.27 to 161.28 µg/g DW respectively. No individual carotenoid was observed in response to darkness in all types of medium when $NaNO_3$ salt strength increased from 0.1x to 10.0x for the development of *C. fusca* cell culture.

Effect of PEG on the Carotenoid Accumulation in *C. fusca* Cell Culture

C. fusca cell culture developing in the BBM medium in the presence of light and PEG (Figure **3**) exhibited an increased lutein content from 1.0 g/L (142.72 µg/g DW) to 3.0 g/L (160.01 µg/g DW). The lutein content was slightly decreased in 5.0 g/L (88.90 µg/g DW) as compared to the 1.0 g/L and 3.0 g/L. No individual carotenoid was observed in response to darkness in BBM medium when PEG concentration increased from 1.0 to 5.0 g/L for the development of *C. fusca* cell culture.

Effect of Salicylic Acid (SA) on the Carotenoid Accumulation in *C. fusca* Cell Culture

As shown in Figure **4**, when SA concentration increased from 25.0 to 50.0 mg/L in *C. fusca* cell culture developing in the BBM medium in the presence of light, total lutein content increased from 135.0 to 156.51 µg/g DW. However, upon further increase to 75.0 mg/L, lutein slightly decreased to 131.33 µg/g DW. Again, no individual carotenoid was detected in

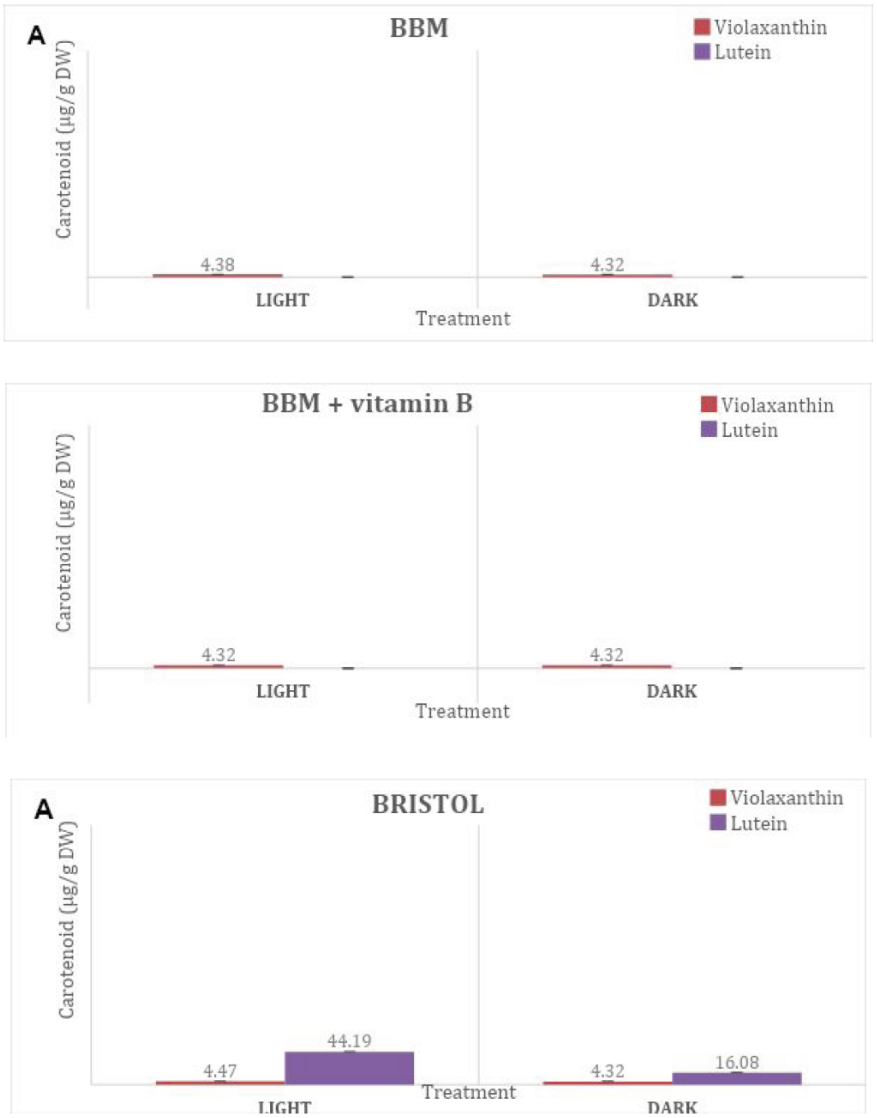

Figure 1: Analysis of carotenoid content (µg/g DW) of *Chlorella fusca* cell culture developing in BBM, BBM with vitamin B and Bristol media formulation in response to light and dark condition. Error bars represent ± SE.

response to darkness in BBM medium when SA concentration increased from 25.0 to 75.0 mg/L for the development of *C. fusca* cell culture.

Effect of NaCl on the Carotenoid Accumulation in *C. fusca* Cell Culture

Analysis of individual carotenoid content for *C. fusca* cell culture developing in BBM medium in the presence of light and NaCl salt stress (Figure **5**), only lutein was detected accumulated at NaCl concentration of 40.0 g/L (138.45 µg/g DW). None was detected in NaCl at 10.0 g/L and 60.0 g/L as well as in response to darkness in NaCl concentration increased from 10.0 to 60.0 g/L for the development of *C. fusca* cell culture.

The development of *C. fusca* cell culture model system has proved to be an effective experimental system for investigating the environmental factors involved in regulating carotenoid biosynthesis. This potential model system has been used because of several advantages:

i. rapid initiation of cell culture within four weeks;

ii. the environmental conditions are easy to control because of the small volume of the cell culture;

iii. cell culture was easily exposed to different types of environmental treatments effect

iv. the variation between cell culture was minimised; and

v. extraction and analysis of carotenoids can be done by using cell culture.

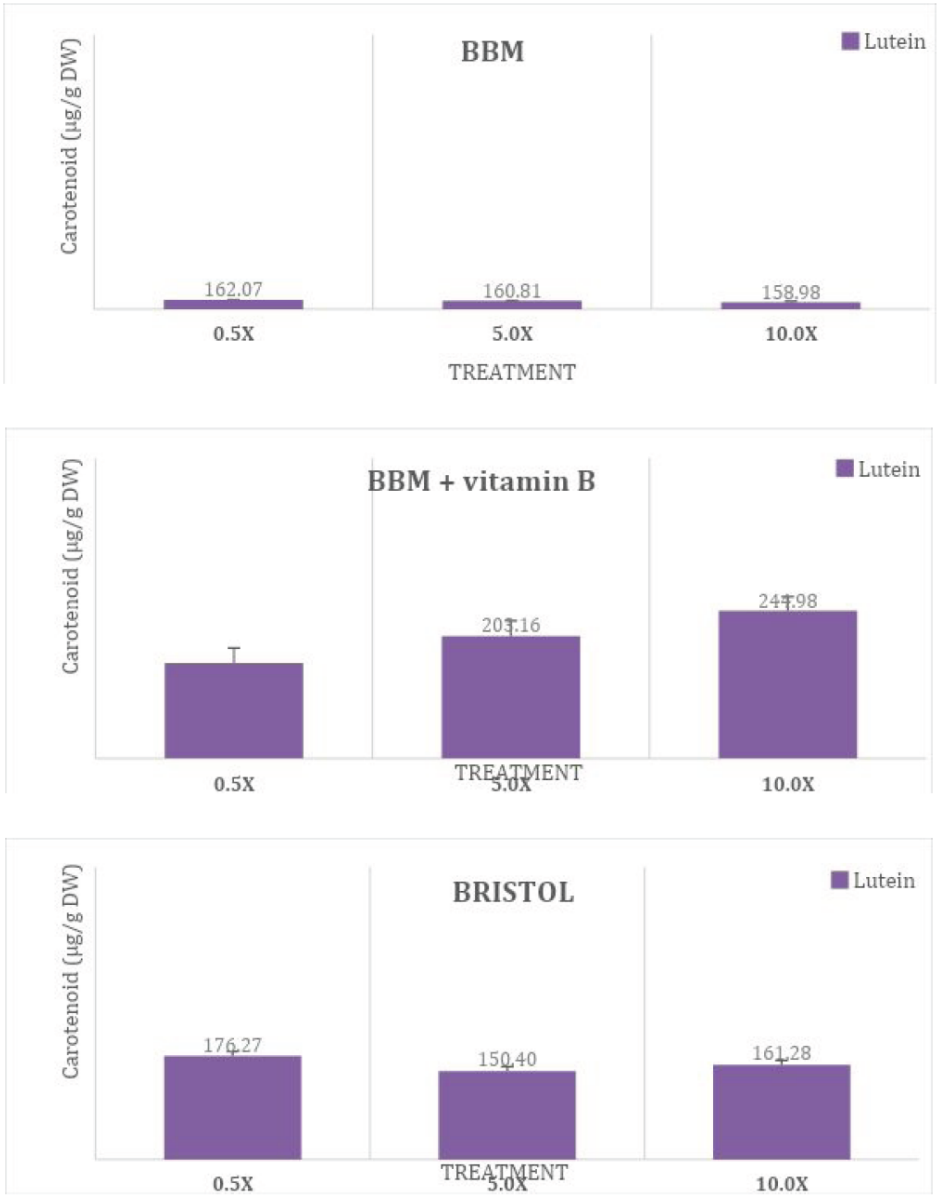

Figure 2: Analysis of carotenoid content (µg/g DW) of *Chlorella fusca* cell culture developing in BBM, BBM with vitamin B and Bristol media formulation in response to 0.5x, 5.0x and 10.0x $NaNO_3$ salt stress with light condition. Error bars represent ± SE.

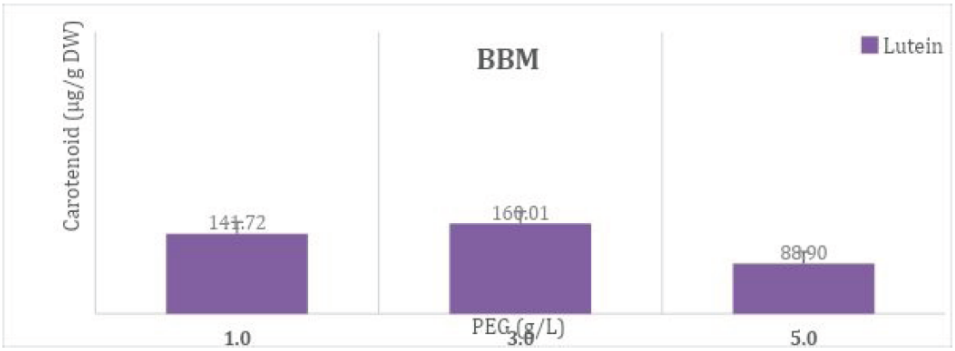

Figure 3: Analysis of carotenoid content (µg/g DW) of *Chlorella fusca* cell culture developing in BBM in response to 1.0, 3.0 and 5.0 g/L PEG stress with light condition. Error bars represent ± SE.

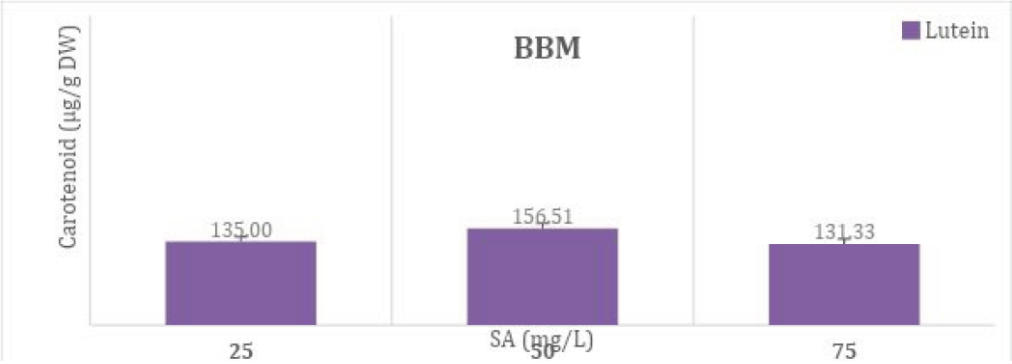

Figure 4: Analysis of carotenoid content (µg/g DW) of *Chlorella fusca* cell culture developing in BBM in response to 25.0, 50.0 and 75.0 mg/L salicylic acid stress with light condition. Error bars represent ± SE.

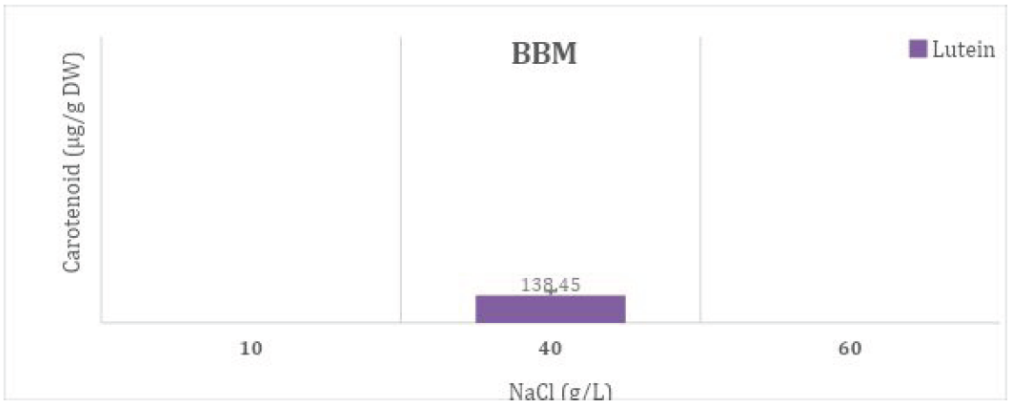

Figure 5: Analysis of carotenoid content (µg/g DW) of *Chlorella fusca* cell culture developing in BBM in response to 10.0, 40.0 and 60.0 g/L NaCl salt stress with light condition. Error bars represent ± SE.

[10] defined environmental stress as external conditions that adversely affect growth, development, or productivity. Light can be one of the important parts of changing the chemical composition of microalgae. In these studies, *C. fusca* was claimed to divide faster in the presence of light and related factor such as carbon dioxide. Furthermore, light intensity and photoperiod provide an effect on the growth, biomass, and other metabolites [11]. In the culture of Bolds Basal Medium (BBM), there were two types of carotenoid being produced from *C. fusca*, which were; lutein and violaxanthin. In addition to that, when the BBM media was modified with vitamin B, the production of lutein was the highest. In Bristol media, *C. fusca* cell culture produced lutein and trace of violaxanthin. It was reported that, *C. fusca* contained high lutein content [12]. Nitrate also play an important role in influencing the growth and lipid accumulation in algae [13]. The rate of cell metabolism can be influenced by different forms of nitrogen [14]. In these studies, the elicitors (PEG, SA, NaCl) were observed to stimulate the production of lutein in *C. fusca* cell culture. Different elicitors have differently influenced in the production of total lutein content. SA and PEG were proven to increase the production of lutein in *C. fusca* cell culture. On the other hand, NaCl and dark restricted the production of lutein. Therefore, in this study elicitors can be used to exploit lutein production in *C. fusca* cell culture. Different stress conditions such as water, drought, cold, light, and temperature result in increased amounts of phytohormone abscisic acid (ABA). Stress recognition may have activated signal transduction pathways that transmit information within the individual cell and throughout the plant. In order to manipulate the biochemical composition of microalgae, it can be done by changing the environmental stresses and the growth conditions [15]. This may induce changes in gene expression that modify growth and development and even influence the carotenoid biosynthesis. Stress will have triggered and altered cellular metabolism, and as a result, lutein and violaxanthin had been produced in this study. Light is one of the crucial regulators for photosynthesis and its related metabolism including the carotenogenesis. Different media are behaving uniquely towards a light to regulate carotenoids. Cultures with different carbon sources concentration

are having the varying capability to synthesis, sequester and store carotenoids. The operation is more related to the biotechnological process, in order to stimulate the synthesis for the desired product [16]. Lutein and violaxanthin are major carotenoids in *C. fusca*, and in carotenogenesis biosynthesis both carotenoids were derived from the activity of zeaxanthin epoxidase and β-Carotene hydroxylase. To sum up, the type of elicitors and concentration of elicitors can be manipulated to up-regulate or down-regulate β-Carotene hydroxylase activity towards the production of lutein and violaxanthin.

CONCLUSION

Microalgae will respond to stress if the gene expression was transformed, trigger cellular metabolism, and variations in growth rates and yields. The categories of stress will be concluded as two types, which were:

i) Biotic- organisms was influenced in this condition

ii) Abiotic- the reaction from physical and chemical environment inadequacy

From the abiotic reaction, the physical and chemical environment plays an important role as it can trigger stress and regulate carotenoid biosynthesis. Due to this; light, water stress, disease and nutrient stress were among the important factors which can affect the *C. fusca* cell culture. From this scenario, the factors that can contribute to the production of lutein from *C. fusca* cell culture, are; light, medium formulation, nutrient, drought and disease stress.

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