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Screening of lutein content in several fresh-water microalgae

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Abstract

Lutein is the most abundant xanthophylls in the photosynthetic organisms and its dietary intake is thought to protect against the development of age-related macular degeneration. Microalgae are predicted to be the major contributors of lutein in the future and to date, various species of microalgae has been analyzed for its lutein content. This study examined the lutein content of six chlorophycean microalgae species grown in the laboratory culture under the comparable environmental condition in which the population doubling time (t_d) as well as the maximum growth rate (μ_{max}) for each species was evaluated. Using batch culture operation grown under the heteroautotrophic condition, *Scenedesmus dimorphus* was observed to accumulate the highest lutein content (60.11 mg/g) with maximal biomass of 0.349 g/L culture. Based on the observations available, it appears that strains of unicellular microalgae of smaller size have appreciably higher intrinsic growth rates than algae of the larger size (*Chlorella* sp. B > *Chlorella* sp. TLL > *Scenedesmus dimorphus* > *Scenedesmus obliquus* TLL > *Desmodesmus* sp. TH > *Ankistrodesmus* sp. TH).

Introduction

Lutein ((3R,3'R,6'R)-β,ε-Carotene-3,3'-diol) is yellow plant pigment and this xanthophyll (oxygenated carotenoids) is present abundantly in higher plants and green algae (Ceron *et al*., 2008; Dall'Osto *et al*., 2006). It exists in ubiquitous and abundant in nature on account of its pivotal role in the photosynthetic process as primary light-harvesting pigments. Lutein acts as a very efficient filter to the visible blue light in the photosynthetic organisms, and when ingested by a human, it serves as a filter to the macula of the retina, hence protecting retina against age-related macular degeneration (AMD) (Landrum *et al*., 1997). AMD is a disease commonly affecting the Western population. Notwithstanding, Asian population is also at risk as they are exposed to the blue wave emitted from devices and many advanced technologies.

Carotenoid's accumulation and composition differ at various stages of development and maturation. Like other naturally occurring carotenoids, lutein differs in concentrations at different levels of development. Lutein can be seen accumulated in a tremendous amount at a particular stage of life and decreased gradually after the onset of maturation. This is true for many divisions of plants and its parts such as seeds, fruits, peels, and leaves. Lutein at higher concentration can be found in the seeds of early developing yellow-typed soybean Enrei 25 DAF (Monma *et al*., 2014), in the fruits of ripening Chenin grapes (Marais *et al.*, 1991), in the peel of immature gac fruit *Momordica cochinchinesis* Spreng (Kubola and Siriamompum, 2011) and also in the leaves of young tea *Camellia sinensis* Var. *Assamica* near the terminal shoot (Venkatakrishna *et al.*, 1977).

Among higher plants, petals of Aztec marigold *Tagetes erecta* (Compositae) have drawn considerable attention for its capability of significant accumulation of lutein (up to 0.2% of cell dry weight) (Sievel *et al.*, 2014). This feature renders marigold petals as the most important source of biotechnologically produced natural lutein. Despite the ample occurrence of lutein in marigold, lutein consists mainly as esters (Breithaupt *et al*., 2002). Free lutein is more appreciated and desired as it is readily absorbed by the human body compared to the ester form (Kemin Foods L.C., 2004). While searching for free lutein from natural sources, it is observed that in microalgae, free lutein appears as a major form and the accumulated lutein far outweighs the marigold. A strain of microalga can store up from

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0.5% to 1.2% dry weight of lutein content (Molina *et al.*, 2005; Ceron *et al*., 2008). In addition, the growth of microalgae is more rapid than marigold. For example, microalgae are ready to reproduce within hours while marigolds are seasonal plants that bloom bright during the summer months of October.

This study, therefore, focuses on the lutein content in six chlorophycean microalgae obtained from local indigenous and commercial microalgae collection. The study of growth characteristic for each strain was also investigated. Variability and bias of the growth rate and biomass of microalgae for six different cultures were minimized by growing the cultures under the comparable environmental condition and restricted from other sources that could contribute to the variance (such as light, temperature, air composition and nutrient variation).

Materials and Methods

Algal species

Six algal species were studied, three of which were isolated from the local environments with two identified only to the genus level and one identified to the species level. Two other strains were purchased from Universiti Sains Malaysia (USM) and one was bought from University of Texas (UTEX) Culture Collection of Algae. They are *Chlorella* sp. B (isolated from freshwater in Bentong, Pahang, Malaysia), *Chlorella* sp. TLL and *Scenedesmus obliquus* TLL (isolated from Layang-layang lake, Kepong, Selangor, Malaysia); *Ankistrodesmus* sp. TH and *Scenedesmus* sp. TH (culture collection from USM) and *Scenedesmus dimorphus* (culture collection from UTEX).

Microalgae isolated from the environment were liberated from bacteria and fungi using the method of Mustapa *et al.* (2016) through a cocktail of ampicillin, cefotaxime and carbendazim at the concentrations of 700 µg/mL, 200 µg/mL and 1 µg/mL, respectively. Microalgae were streaked twice onto Tris-acetate-phosphate, TAP agar containing the aforementioned cocktail formulation, each incubated for one week. Clean microalgae were subsequently streaked onto non-treated TAP agar and further proceeds with mass cultivation.

Culture conditions

The cultures were grown in a medium of the following composition (in grams per liter): Tris base, 2.42; NH_4Cl, 0.4; $MgSO_4.7H_2O$, 0.1; $CaCl_2.2H_2O$, 0.05; K_2HPO_4, 0.108; KH_2PO_4, 0.054; $ZnSO_4.7H_2O$, 0.022; H_3BO_3, 0.0114; $MnCl_2.4H_2O$, 0.00506; $CoCl_2.6H_2O$, 0.00161; $CuSO_4.5H_2O$, 0.00157; $(NH_4)6Mo_7O_{24}.4H_2O$, 0.0011; $FeSO_4.7H_2O$, 0.00499. After the cells reach their mid-logarithmic phases (determined spectrophotometrically and by cell counting), 80 mL culture from the parent cells containing 1 x 10^6 cells per mL were inoculated into a 1L Schott bottle containing 720 mL culture volume (10% (v/v) inoculation ratio). Cultures were aerated with compressed air at 0.3L/min, temperature at 24°C ± 1 and illuminated under the 12:12 hr light/dark cycle with white fluorescent lamp.

Analytical methods

Cell density

Daily samples of 3 mL culture volume were taken for measuring the cell growth. Cell growth was monitored by measuring the optical density at 680nm with a UV-Vis spectrophotometer.

Cell count

Daily samples taken from the cultures and living microalgal cells were counted under the microscope with a Neubauer haemocytometer using trypan blue.

Biomass

Cultures were harvested at their respective end of exponential growth. Dry biomass was determined via freeze-dried cultures for 3 days prior to weighing.

Carotenoid extraction

Carotenoid extraction was conducted based on Othman (1991) method with slight modifications. For pigment (or carotenoid; or lutein) analysis, the 800mL culture of cell suspension was centrifuged at 9000 x g, 10°C for 20 minutes, after which the pellet was freeze-dried. The freeze-dried microalgae were rehydrated by adding 1 mL of distilled water, followed by 5 mL of an acetone and methanol mixture (7:3) which has been mixed earlier with calcium carbonate to allow efficient solvent penetration. The solution was allowed to stand overnight in the darkness at the room temperature. On the following day the solution was vortexed and centrifuged at 9000 g for 5-8 minutes and the supernatant was transferred into a fresh 50 mL graduated polypropylene centrifuge tube. This procedure was repeated by adding 5 mL of acetone and methanol (7:3) without additional calcium carbonate to the microalgae pellet until the collected supernatant was colourless. Then, the collected supernatant was re-extracted with an equal volume of hexane and distilled water. The mixed supernatant was later allowed to separate and the upper layer containing the carotenoids was collected. This procedure was repeated with hexane alone and

Table 1. Culture profile for six species of microalgae.

Chlorophycean microalgae	*Density of cells (x 10^6cells/mL)	*Optical density (at 680 nm)	End of exponential growth (day)	Maximum growth rate, μ_{max} ($hours^{-1}$)	Doubling time, t_d ($hours^{-1}$)	*Biomass (g/L)	*Total carotenoid (mg/g)	*Lutein (mg/g)
Scenedesmus dimorphus	10.1	1.377	9	0.028	35.71	0.349	100.21	60.11
Chlorella sp. B (self-isolated)	68.4	1.891	4	0.155	6.45	0.409	70.11	33.35
Ankistrodesmus sp. TH	7.7	0.797	14	0.014	71.42	0.258	70.72	30.70
Chlorella sp. TLL (self-isolated)	77.7	1.931	4	0.113	8.85	0.399	56.25	28.41
Scenedesmus obliquus TLL (self-isolated)	9.0	1.400	8	0.027	37.04	0.295	43.27	27.13
Desmodesmus sp. TH	1.7	0.912	5	0.023	43.47	0.427	22.76	10.25

*at the end of exponential growth

the combined upper layer fraction was dried under the stream of liquid nitrogen. To remove chlorophyll and lipid impurities from the dried extract, saponification was performed as followed. The dried extract was wetted with 10-40 µL of ethyl acetate followed by addition of 390-360 µL of acetonitrile and water (9:1). The mixture was further diluted with 400 µL of methanolic potassium hydroxide solution (10% w/v) to remove triglyceride from the lipid-rich sample and allowed to stand overnight in darkness at room temperature. Carotenoids were extracted by addition of 4mL hexane and 0.1% butylated hydroxytoluene (BHT) followed by 2 mL 10% NaCl. BHT is used as a stabilizer to inhibit the auto-polymerization of organic peroxides and to scavenge metal impurities which can initiate decomposition. The solution was then vortexed and centrifuged at 9000 g for 5-8 minutes. The upper layer of the solution was collected into a fresh 50 mL graduated polypropylene tube while the bottom layer was re-extracted with hexane and 0.1% BHT until the upper layer became colourless. Few mL of distilled water were added into the collected upper layer and centrifuged for washing debris and another particulate. The upper layer was sent for drying under the stream of liquid nitrogen.

Analysis of lutein using HPLC

The extracted lutein was quantified using HPLC system (Agilent) fitted with a reverse phase column C_{18} consisting 5 µm particle size, length of 250 mm and inner diameter of 4.6 mm (Sommerburg, 1998). The method employed binary gradient with mixture of acetonitrile and water (9:1) as Solvent A and ethyl acetate as Solvent B. The gradient profile for the system was developed as followed: 0-40% solvent B (0-20 mins), 40-60% solvent B (20-25 mins), 60-100% solvent B (25-25.1 mins), 100% solvent B (25.1-35 mins) and 100-0% solvent B (35-35.1 mins) at a flow rate of 1.0mL min^{-1}. Lutein was measured at 447 nm using diode-array detector (DAD). Quantitative analysis was performed by plotting a standard graph of lutein in the range of 0.2-0.8 µg/µL.

Results and Discussion

Analysis of microalgae growth profile

A detailed description of culture profiles i.e. cell density, optical density, and biomass; growth rate and doubling time in all six species are provided in Table 1. It is apparent that maximal biomass varied greatly among species and the biomass showed a positive correlation with algal growth rate. For instance, *Chlorella* had the most rapid growth rate ($0.155h^{-1}$) and the highest biomass ($0.409gL^{-1}$) while *Ankistrodesmus* had the slowest growth rate ($0.014h^{-1}$) and the lowest biomass ($0.258gL^{-1}$). This positive correlation between biomass and growth rate however did not seemed to occur in *S. dimorphus* and *Desmodesmus*. As expected, among all six strains, both *Chlorella* species have rapid growth rate and shorter doubling time. Only 4 days was required for both *Chlorella* under the stated culture conditions to reach the late exponential phase. On the other hand, *Ankistrodesmus* sp. TH takes 14 days to achieve the same phase, the longest time among the six strains. It is observed that there is no exceptional increase in the biomass of *Ankistrodesmus* sp. in spite of the longer incubation time due to slow doubling time of the population.

A decline in the maximal density along with an increase in cell size under the uniform condition is generally reported in the study of various taxonomic groups of freshwater and marine algae (Agusti *et al*.,

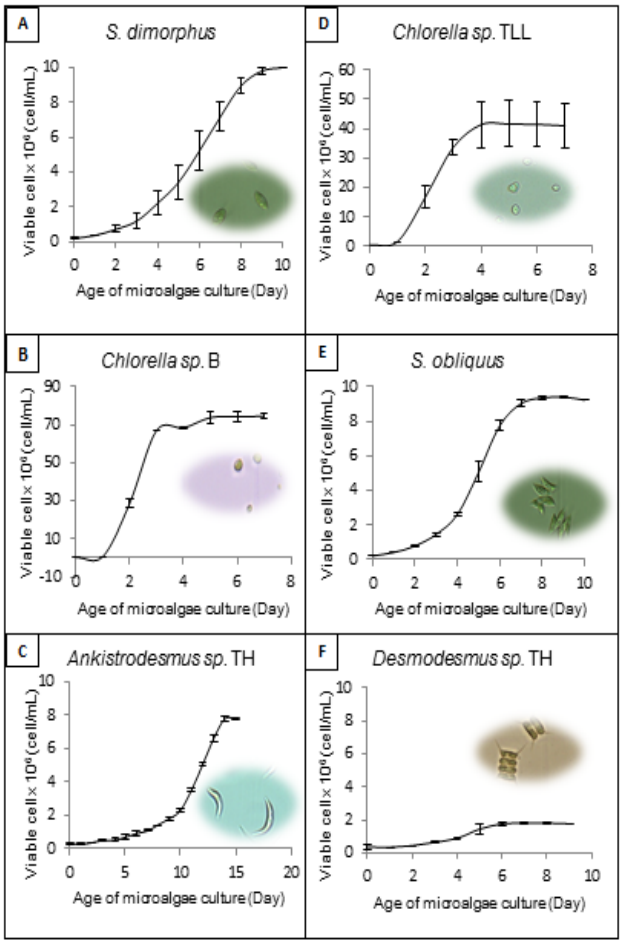

Figure 1. Growth profiles of six species microalgae in relation to viable cell number

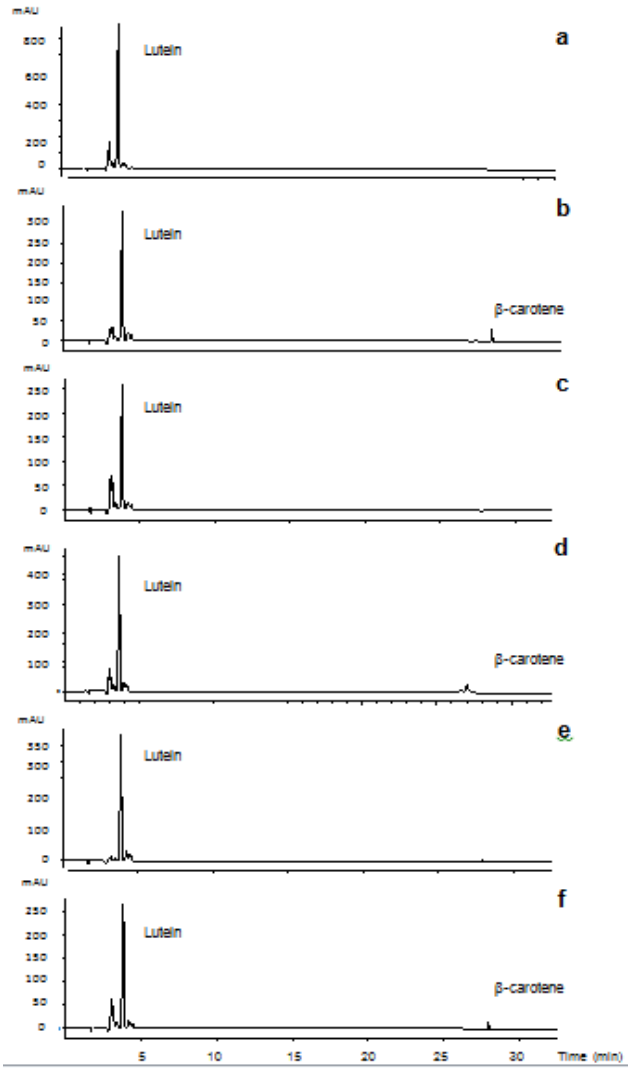

Figure 2. Carotenoid analyses by reverse-phase HPLC from top (highest lutein content) to bottom (lowest lutein content) of the chromatogram. a. *Scenedesmus dimorphus* b. *Chlorella* sp. B c. *Ankistrodesmus* sp. TH d. *Chlorella* sp. TLL e. *Scenedesmus obliquus* TLL f. *Desmodesmus* sp. TH

1987). For microalgae strains which have a smaller size such as *Chlorella,* their growth can be seen materially faster than any other microalgae strain especially from the strain that has spines, which add up to a larger size such as *Desmodesmus* sp. TH and their abundant number of cells can also be seen occupying the space. Several explanations have been proposed with respect to the discovery and Munk and coworker suggested that smaller size cells may have a shorter distance to transport nutrients from their outer membrane, thus reproducing offspring in a shorter generation time as compared to the cells of larger size (Munk and Riley, 1952). The recent studies supported the theory and elaborated that a relationship between photosynthetic efficiency, growth rate, and cell volume are the crucial factors (Straskraba, 1976; Nielsen, 2006; Lopez-Sandoval *et al.*, 2014).

Figure 1 depicts the evolution with time of growth for six microalgae species. Cell numbers can be seen increased at an exponential rate following the onset of inoculum and undergo a plateau for a long period. No lag phase was detected in all six species as the inoculums were taken from the exponentially growing parent cells. When it comes to producing a product at a cost-effective level, shorter running time of the product cultivation is often being the subject of interest in the production line. This method of culture has been the practice of the commercially available product as it reduces the time for harvesting yield and simultaneously reduces the cost of operating.

Carotenoid analysis on microalgae strain

Carotenoid contents of six chlorophycean microalgae measured in the late exponential phase of algal growth presented lutein as the major component (Figure 2). Of all six strains, freshwater microalga *Scenedesmus dimorphus* was found to be an excellent source of lutein as it showed substantial accumulation of lutein when grown under the comparable environmental condition. The lutein content was

6-fold higher in the cells of *Scenedesmus dimorphus* than alga of the same genus, *Desmodesmus* sp. TH. As seen in the chromatograms, secondary carotenoids accumulation was found negligible in most of the strains except in *Chlorella* sp. B, *Chlorella* sp. TLL and *Desmodesmus* sp. TH whereby the substance took 26-27 mins to be eluted in the system. The substance was identified as β-carotene, and its proportion to lutein is less than 10%. A delay in harvesting the biomass is expected to be the most probable cause which aids the conversion of precursor phytoene to β-carotene, hence reducing the accumulation of lutein pigment.

Regulated by age, it is inferred that lutein is synthesized by the cells in the exponential phase of growth, but the lutein content is greatly enhanced between the late exponential and early stationary phase. This statement is supported by the observation reported by Del Campo *et al*. (2004) that the yield for lutein in batch culture of *Chlorella zofingiensis* was maximum on the fifth day to day eight (late exponential phase) while another study found that the maximum yield for lutein of the same *Chlorella* species on a ninth day to day ten (deceleration phase) (Cordero *et al*., 2011). In different studies, the content of lutein in other strains of green algae such as *Chlorella vulgaris, Ankistrodesmus gracilis, Chlorella protothecoides, Chlorella zofingiensis, Scenedesmus obliquus* was also lower during stationary phase than in cells of exponentially growing state (Del Campo *et al.,* 2004; Chue *et al.*, 2012; Chan *et al*., 2013). The collection of this data, together with the late exponential of lutein accumulation in the present study provides conclusive facts that the manipulation of the cultivation conditions did not affect the accumulation of lutein and/or other carotenoids at a specific growth phase. It is also apparent that in the culture of a similar strain, the duration of exponential phase may vary depending on the size of inoculum as well as the cultivation medium used for the support of algal growth. In terms of lutein content and duration of cultivation, the present study shows better lutein productivity and shorter cultivation time as compared to previous reports.

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