PRODUCTION OF VALUE ADDED SUBSTANCES BY TROPICAL MICROALGAE

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ABSTRACT

Microalgae recently appeared to be a new source of renewable biofuel that is capable of meeting the global demand for transport fuel due to its ability to accumulate high amount of lipid in their intracellular body. In addition, massive accumulation of ketocarotenoid astaxanthin, which is one of the secondary metabolites produced by microalgae have also gained much attention for its potential applications in pharmaceuticals, nutraceuticals as well as cosmetics products. Four green microalgae morphologically identified as Staurastrum sp., Scenedesmus sp., Desmodesmus sp., and Ankistrodesmus sp. were isolated from Kuala Selangor Nature Park and Hulu Langat River, Selangor. The intracellular lipid bodies of the microalgae were stained with BODIPY 493/503 for the screening of the potential microalgae for biodiesel production. From the staining, more lipid bodies can be seen from Scenedesmus sp. compared to the other three isolated microalgae species. In astaxanthin complex quantification analysis, Ankistrodesmus sp. showed the highest accumulation of astaxanthin complex and therefore has the potential to be utilized for natural supplement applications.

Keywords— Astaxanthin, biodiesel, isolation, lipid, microalgae

1. INTRODUCTION

Microalgae, the sunlight-driven microorganisms are currently gaining much attention from the researchers due to its ability to produce a wide range of metabolites includes, proteins, lipids, carbohydrates, carotenoids and vitamins [1]. Nowadays, special attention is paid to the coupling of the carotenoid and lipid biosynthesis as well as the deposition of astaxanthin in the microalgal cells.

Recent volatility in crude oil prices attributed to increase demand and limited resources, tied with the urge to reduce pollutant emissions and greenhouse gases, have created a major focus in the production of sustainable biofuel. At present, microalgae had become a promising alternative and being considered as the potential feedstock for the production of third generation of biofuels [2]. This is due to the reasons that biofuel derived from microalgae gives some merits than those other alternative feedstocks. Microalgae have the potential of giving twenty times more productivity in terms of oil than oilseed crops [3]. Microalgae also grow faster and this high growth rate results in high biomass yield in a short period of time [4]. Besides, the cultivation of microalgae can use the non-arable land and they give a high per-acre yield [5]. Lipids in microalgae are accumulated specifically under the conditions of excess carbon and limited of other nutrients, especially nitrogen [6]. The accumulated lipid is in the form of discrete oil droplets and in some cases it can occupy up to 85% of the cell volume [7]. Microalgal total lipid is mainly composed of neutral lipids (NLs), glycolipids (GLs) and phospholipids (PLs) [8]. The NLs are mainly composed of triacylglycerols (TAGs) or triglycerides which is the main component for the production of microalgal biodiesel. Different species of microalgae accumulated different amount of lipid in their cell bodies (Table 1) and commonly, the lipid content is in the range of 20-50% by weight of their dry biomass [3]. However, the compositions and structure of fatty acid esters of the microalgal lipid including the unsaturation degree and carbon chain length need to be scrutinized to determine its feasibility for producing high quality of biodiesel. This is because, fatty acid methyl esters (FAMEs) oxidizes easily and forms products that shown to have unfavourable effects on vehicle fuel systems, especially those that contained high level of unsaturation [9]. High level of unsaturation indicates low oxidation stability and this is unfavourable for the production of biodiesel.

Table 1 Lipid content of some microalgae [3]

Microalgae	Lipid content (% dry weight)
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	>20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp.	50-77
Tetraselmis sueica	15-23

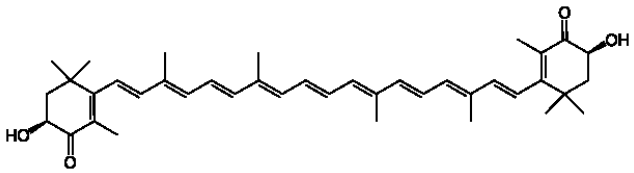


Fig. 1 Skeletal structure of astaxanthin [11].

Other than lipid, the high accumulation of carotenoids in the microalgal cells, especially astaxanthin has increased the attention towards microalgae to be one of the major sources for the production of natural supplement [10]. In the recent years, there is considerable interest among the researchers in extracting bioactive compounds from natural resources due to its efficacy towards treatment of human diseases. This is due to the fact that synthetic compound is always classified as compound that might give side effect to the consumer. Synthetic astaxanthin is usually produced from petrochemical and the production is not preferred in some cases because it contains a mixture of stereoisomer (3R, 3S') and is potentially unsafe. Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'dione) is a group of xanthophyll carotenoids that possess a red colour pigment that can be found in many microorganisms and marine organisms [11]. Astaxanthin has the antioxidant properties which are very beneficial in blocking the activity of free radical chemicals that are highly reactive and have potential to cause cell damage that may lead to cancer [11]. Scientific literatures have revealed the significant evidence that astaxanthin surpasses the antioxidant benefits of beta-carotene, zeaxanthin, canthaxanthin, vitamin C and vitamin E [12].

Generally, the massive accumulation of secondary metabolites of lipid and ketocarotenoid astaxanthin in the microalgal cells are dominant under the adverse growth conditions (mostly nutrient starvation) that limits the cell growth due to the restrictions of nitrogen content that inhibits the cell to perform cell divisions [10, 13]. The accumulation of astaxanthin is strongly related with the biosynthesis of fatty acid such as oleic acid associated predominantly with triacylglycerols [14]. However, the inhibition of the lipid biosynthesis abolished the accumulation of astaxanthin, but blocking the biosynthesis of astaxanthin did not prevent the accumulation of neutral lipids and formation of lipid bodies [15]. Eukaryotic marine microalgae, *Haematococcus pluvalis* was found to boost high amount of astaxanthin accumulation [16]. The bulk of astaxanthin in *Haematococcus pluvalis* is in the form of mono- and diesters of palmitic acid (16:0), oleic acid (18:1) or linoleic acid (18:2) [10]. There were attempts to estimate the suitability of conversion of the *Haematococcus pluvalis* biomass to biodiesel, however, nowadays, the application of *Haematococcus pluvalis* biomass for the extraction of astaxanthin is much more economically viable than production of biodiesel [10]. Nevertheless, the knowledge on the coupling of the secondary metabolites biosynthesis of lipid and astaxanthin of freshwater microalgae is currently much unknown.

(a) (b)

Fig. 2 Sampling sites. (a) Brackish water in Kuala Selangor Nature Park, (b) Freshwater in Hulu Langat River Selangor.

The objectives of this study were focused on the characterization and comparison of the isolated tropical freshwater microalgae on their feasibility for biodiesel production as well as the assessment of the potential applications of secondary ketocarotenoid astaxanthin as the natural supplements.

2. MATERIAL AND METHOD

2.1 Isolation and inoculation

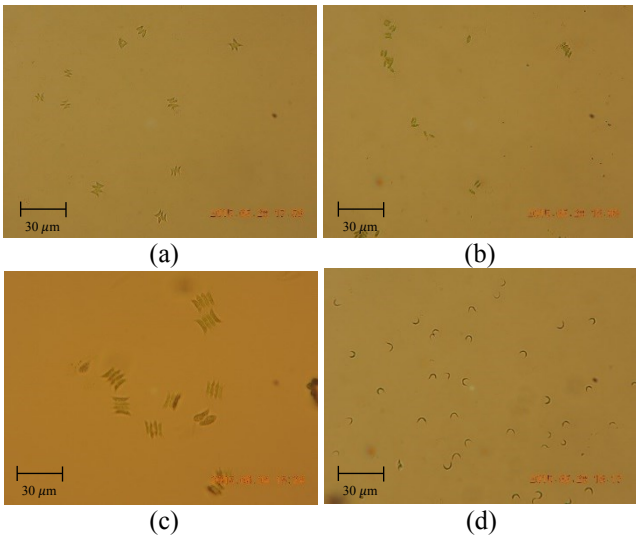
Microalgae samples were collected from brackish water in Kuala Selangor Nature Park and freshwater in Hulu Langat River with a plankton net (25µm open mesh). Four individual cells of microalgae were isolated from the water samples using sterile micropipette washing method. The isolated microalgae were subjected to purification by serial dilution followed by inoculation in 96 well plates containing artificial freshwater (AF-6) medium at ambient temperature under continuous illumination of 45 µmol photons $m^{-2}s^{-1}$ light intensity. The purity of the isolated microalgal culture was ensured by repeated plating and by regular observations under the microscope. The isolated microalgae were morphologically identified as *Staurastrum* sp., *Scenedesmus* sp., *Desmodesmus* sp., and *Ankistrodesmus* sp. by referring to Encyclopaedia of Microbes in Freshwater: Visual Guidebook of Protozoa as shown in Fig. 3.

2.2 Algal cultures

The isolates were cultured in 100 mL Erlenmeyer flasks containing 75 mL of AF-6 medium at ambient temperature. The isolates were allowed to grow under continuous illumination of 45 µmol photons $m^{-2}s^{-1}$ light intensity. The optical densities (OD) of each sample were determined using UV-VIS spectrophotometer (Hach DR6000) at 750nm by withdrawing aliquots of the microalgae cultures at interval of every 24 hours. All experiments were carried out at least in duplicate.

2.3 Fluorescent staining

A type of the available lipophilic dyes which is BODIPY 493/503 (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) was prepared in dimethyl sulfoxide (DMSO) to give a stock solution of 1 mg/mL and stored in dark tube protected from light.



(a) (b)

(c) (d)

Fig. 3 Visualization of isolated cultures of microalgae at 40X magnification. Isolates were morphologically identified as (a) *Staurastrum* sp. (b) *Scenedesmus* sp. (c) *Desmodesmus* sp. (d) *Ankistrodesmus* sp. by referring to Encyclopaedia of Microbes in Freshwater: Visual Guidebook of Protozoa.

1 mL of microalgae cultures were stained with 1 µL of BODIPY 493/503 to stain the fatty acid contained in the microalgae followed by incubation in darkness for 10 minutes before being observed under the fluorescence microscope [17].

2.4 Quantification of astaxanthin complex

Astaxanthin content in the isolated microalgae cultures were analysed spectrophotometrically by extraction using acetone as the solvent. 5 mL of each microalgae culture were harvested by centrifugation at 4000 g for 5 minutes. The supernatant was then removed before adding 3.5 mL of solution mixture of 5% potassium hydroxide (KOH) in 3% methanol (CH_3OH). The mixture was then incubated at 70°C for 5 minutes. After the incubation, it was then centrifuged again at 4000 g for 5 minutes. The supernatant was removed and 3 to 5 drops of acetic acid were added into the sample tubes. Next, 5 mL of acetone was added into each tubes followed by incubation at 70°C for 1 night. The sample cultures were then analysed by UV-VIS spectrophotometer (Hach DR6000) at 470 nm wavelength. All experiments were carried out at least in duplicate. The astaxanthin concentration was simply calculated by dividing the measured absorbance by the extinction coefficient for pure astaxanthin at the specific wavelength and corresponding for dilution of the product sample as shown in the equation below [18].

Concentration of astaxanthin, μg/mL

$$= \text{Absorbance x } \frac{10000}{2100}$$

3. RESULTS AND DISCUSSIONS

3.1 Growth Analysis

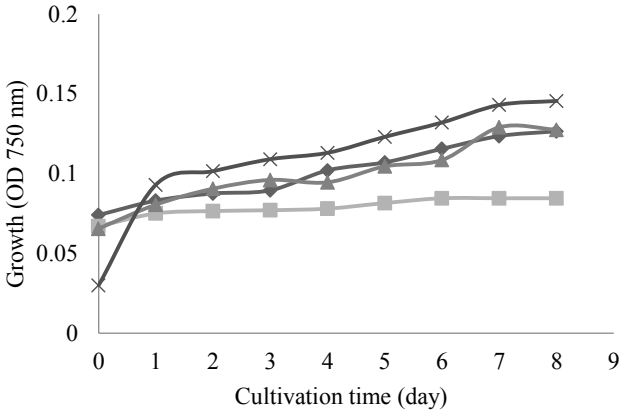
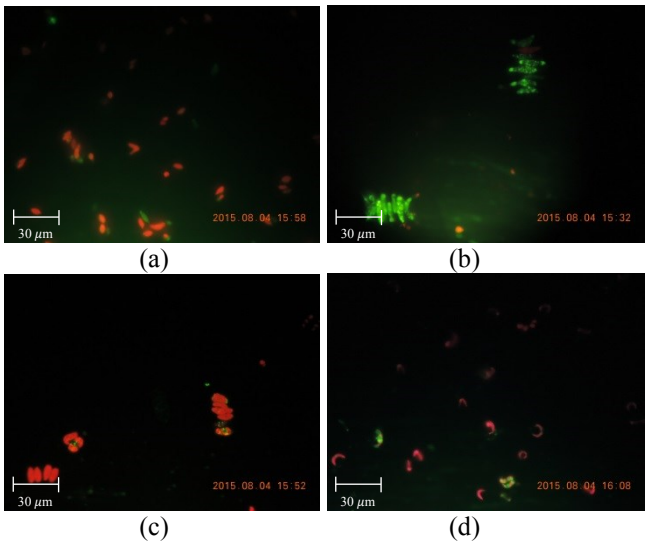


Fig. 4 Growth curve of four isolated microalgae *Staurastrum* sp. (solid diamond), *Scenedesmus* sp. (solid square), *Desmodesmus* sp. (solid triangle) and *Ankistrodesmus* sp (cross).

Fig. 4 shows the comparison of growth curve among four isolated microalgae based on the optical density analysis. Based on Fig. 4, *Ankistrodesmus* sp. showed the fastest growth followed by *Desmodesmus* sp., *Staurastrum* sp. and the slowest one was the *Scenedesmus* sp. After 8 days cultivation, the microalgal growth was still at the exponential phase. This means that there were still enough nutrients in the medium for the growth of the microalgae. Upon reaching the stationary phase where most of the essential nutrients were deprived, the microalgae stop their cell division and start to accumulate lipid and astaxanthin as the secondary metabolites in their cell bodies [2]. Accordingly, different species of microalgae have different ability to maintain their photosynthetic rates under conditions which are unfavourable for growth [19].



(a) (b)

(c) (d)

Fig. 5 Visualization of intracellular lipid bodies of four isolated microalgae cells under fluorescent light at 40X magnification when staining with BODIPY 493/503. (a) *Staurastrum* sp. (b) *Scenedesmus* sp. (c) *Desmodesmus* sp. (d) *Ankistrodesmus* sp.

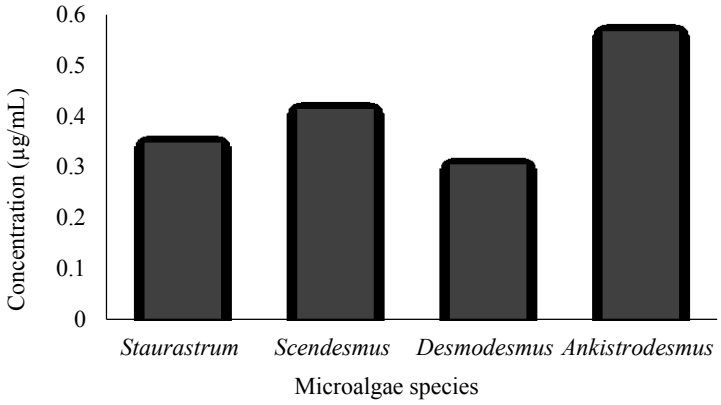


Fig. 6 Astaxanthin concentrations in four different isolated microalgae.

3.2 Fluorescent staining

The isolated cultures were stained with BODIPY 493/503 and being observed under fluorescent microscope. This was done because staining the microalgae with BODIPY 493/503 aids in the detection of intracellular lipid bodies by fluorescence microscopy [17]. This is due to the fact that fluorescence intensities could be correlated to the lipid content within the microalgal cells. The BODIPY 493/503 stained lipid bodies showed bright green fluorescence in the microalgal cells as shown in Fig. 5. Meanwhile, the strong red autofluorescence was from the chlorophyll of the microalgae.

From Fig. 5, it can be deduced that *Scenedesmus* sp. contained more lipid bodies compared to the other isolated microalgae. Strong green fluorescence separated from the red fluorescence stained by the BODIPY 493/503 can be seen clearly in *Scenedesmus* sp. cells. Not much lipid bodies contained in *Staurastrum* sp., *Desmodesmus* sp., and *Ankistrodesmus* sp. cells as only some green lipid droplets can be seen. More lipid bodies were expected to be seen when staining the microalgae cells after the cultures reached the stationary phase of growth as the lipid accumulation is increased under the nutrient starvation condition, especially in nitrogen limitation. Accordingly, the degree to which photosynthetic capacity is decreased under nutrient limitation is species-specific and can strongly influence the amount of energy that is available for triacylglycerol (neutral lipid) synthesis [19]. However, gravimetric analysis involving transesterification process and gas chromatography are needed to study and characterize the composition of the lipid content of the isolated microalgae for the determination of their potential in producing high quality biodiesel.

3.3 Astaxanthin complex quantification

Astaxanthin was determined as the most important carotenoid in pigmentation. In this study, the concentration of the astaxanthin complex was quantified by spectrophotometric assay method. The light absorbance of the extract solvent containing the astaxanthin is measured at a wavelength that corresponds to the maximum absorbance for astaxanthin (usually between 470 and 480 nm). From Fig. 6, it shows that *Ankistrodesmus* sp. contained the highest concentration of astaxanthin with 0.567 μg/mL. It was followed by *Scenedesmus* sp. with 0.414 μg/mL and *Staurastrum* sp. with 0.348 μg/mL. *Desmodesmus* sp. shows the least accumulation of astaxanthin concentration among the isolates with 0.305 μg/mL. However, the astaxanthin concentrations difference among *Scenedesmus* sp., *Staurastrum* sp. and *Desmodesmus* sp. were not really significant compare to *Ankistrodesmus* sp. In accordance to lipid synthesis, the accumulation of secondary ketocarotenoid astaxanthin in microalgal cells is also more preferable under adverse conditions (under excessive irradiance, nutrient deficiency, extreme temperatures, high salinity, and their combinations) which slowing down the cell division and photosynthesis reaction [20]. Thus, it was also expected that higher accumulation of astaxanthin complex will be obtained when performing the extraction under these conditions.

4.0 CONCLUSIONS

Recent insights had unveiled the amazing ability of microalgae in the accumulation of lipid and ketocarotenoids astaxanthin as the secondary metabolites. These highly valued substances have the potential for the production of biodiesel as well as the natural supplements. In this study, the tropical microalgae from brackish water and freshwater in Selangor, Malaysia were isolated and their lipid and astaxanthin content were analysed and compared. Among 4 isolated microalgae, it was found that *Scenedesmus* sp. contained more lipid bodies based on the fluorescent staining by BODIPY 493/503. Meanwhile, *Ankistrodesmus* sp. showed the highest accumulation of astaxanthin concentration compared to *Staurastrum* sp., *Scenedesmus* sp. and *Desmodesmus* sp. This study has revealed that these two species of microalgae have a great potential for the production of biodiesel as well as in other various applications of pharmaceutical, nutraceuticals and cosmetics products.

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