Introduction

Natural antioxidants are becoming more important as alternatives to synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) because the synthetic ones are known to be carcinogenic and toxic to humans. Moreover, natural antioxidants contain higher therapeutic values as compared to synthetic antioxidants (Li et al., 2007; Hajji et al., 2010; Kong et al., 2012). Phenolic or polyphenolic compounds found in many plants have received attention due to their physiological function as scavengers of singlet oxygen and free radicals. Phenolic hydroxyl groups from this compound donate an electron or hydrogen atom to form a stable end product that does not initiate or propagate lipid oxidation (Craft et al., 2012). Previous studies have reported that plants, fruits and macroalgae are well known for their high content of phenolic compounds and antioxidant properties with diverse chemical structures and biological activities (Lester et al., 2012). However, they are associated with many disadvantages such as land limitation and slow growth. Furthermore, plant and macroalgae are important food sources in developing countries and thus, their application in biotechnology will create a shortage in food supply (Balasubramanian et al., 2011).

Microalgae have received increasing attention as an alternative antioxidant source because they are the fastest-growing plants in the world and hence, they have higher...
productivities compared to land plants and macroalgae (Demirbas, 2010). Li et al. (2008) reported that microalgae produce biomass at a rate of 50 times higher than the fastest growing terrestrial plant. Microalgae are known to produce many kinds of pigments including chlorophylls, β-carotene, xanthophylls, astaxanthin and phycobiliproteins, (Begum et al., 2015) and have high potential to be a sustainable source of feedstock for biofuel (Medipally et al., 2015). In recent years, microalgae biomass market produces about 5000 t of dry matter per year and generates a turnover of approximately US$ 1.25×10^7/year (Spolaore et al., 2006). Other advantages of microalgae include less space and water requirement if compared to land plants because they can be cultivated in open ponds, closed systems and photobioreactors (tubular, flat plat or other designs) for their production. Some microalgae can use saltwater, brackish water or wastewater to grow and thus, they do not compete with conventional agriculture for water and land resources (Krohn et al., 2011). For these reasons, it is possible for microalgae to satisfy the massive demands as an alternative antioxidant source using limited land resources, is cost effective compared to conventional farming and contributes to the reduction of greenhouse gases that are responsible for global warming as microalgae directly use carbon dioxide (Balasubramanian et al., 2011). Hence, several researchers had tried to extract antioxidant compounds from various algae biomass for nutritional, aquaculture and cosmeceutical purposes (Khatoon et al., 2009; Hajimahmoodi et al., 2010; Custódio et al., 2012).

The biochemical composition of microalgae is influenced by different types of environmental factors such as the culture medium (Vega et al., 2010), temperature (Renaud et al., 2002), pH (Scherholz and Curtis, 2013), light intensity (Saavedra and Voltolina, 1994) and time of harvesting (Chu, 1942). Nevertheless, the light condition is totally different in large-scale cultures because penetration and light requirement for different microalgae cell may vary (Sánchez Mirón et al., 1999). Most of the previous experiments on microalgae in the laboratory were done in small-scale batch experiments. However, for large-scale bioreactors, a continuous process is used in the commercial algae production instead of batch or semi-continuous processes. Therefore, for the effective production of antioxidants from microalgae biomass, identification of the stage of the cell cycle and the culture conditions to attain high quality and concentration of biochemical compounds should be evaluated in a large-scale reactor operated by continuous process.

The objective of the present study was to evaluate the productivity of antioxidant compounds in the continuous culture of microalgae, T. tetrathele for 56 days by using 1201 photobioreactor. A comparative study of antioxidant compound productivity in different culture media (f/2 and Conway) was also evaluated.

**Materials and Methods**

**Culture conditions**: *Tetraselmis tetrathele* (West) Butcher (isolate UPM-A007) was obtained from the Laboratory of Marine Biotechnology, Institute of Bioscience Universiti Putra Malaysia. Two different culture media; f/2 and Conway media were used to grow *T. tetrathele* in four 120 l annular photobioreactors (two treatments with duplicates). Before growing the *T. tetrathele* in photobioreactors, at least 10% of the microalga culture volume was prepared as inoculum. Subcultivation and inoculation for the experiment were prepared from logarithmic phase culture. Microalgae cultures in 120 l photobioreactors were grown and maintained under a constant light of 120 μmolphotonm⁻²sec⁻¹, photoperiod of 12 hrs light : 12 hrs dark at 25±2°C. The annular photobioreactors (series F & M – T1 50/40) used in this study was a vertical reactor, comprised of two Plexiglas cylinder fixed on a base. The photobioreactor was designed as an air-bubbled column with a high surface to volume ratio. The photobioreactor ring was 2.05 m in height and 50 cm in diameter, which allowed a maximum volume capacity of 120 l. The reactor had a thickness of 4.5 cm between the cylinder rings for the cultivation of microalgae (F & M Ltd, Florence, Italy). Aeration was supplied by an air pump to a pipe placed at the bottom between the cylinders, where the pipe was drilled with 2 mm wide holes. Pure carbon dioxide was supplied to the cultures through an aeration tube with an air stone submerged in the microalga cultures.

**Growth assessment**: Treatments were performed in duplicate using continuous culture for high biomass production. The culture was harvested partially by as much as 40% and equal amount of fresh medium was replaced for continuous cycle so as to maintain a dry weight of about 0.8 g l⁻¹ to 1.2 g l⁻¹. This procedure was repeated for six consecutive runs and samples were collected during days 32, 36, 40, 46, 50 and 56. The growth rates were monitored every 24 hrs by determining dry biomass, optical density (spectrophotometer) and cell count (Neubauer haemocytometer). Based on cell density and dry biomass, cell size was calculated by using the following formula:

$$ F \ (g \ cell^{-1}) = \frac{C \ (g l^{-1})}{N \ (cells l^{-1})} \quad (1)$$

where, F is the cell size of microalgae, C is the dry biomass and N is the cell number.
The microalgae harvested at logarithmic phase cultures were centrifuged at 10,000 rpm for 30 min and washed with 0.5 mol⁻¹ ammonium formate in order to remove soluble salts. The paste was then dried in a vacuum at -47°C under 17.5 Pa and stored at 4°C for 4 hrs followed by keeping in -20°C until further chemical analysis.

Chemical analyses: The freeze-dried biomass was ground in a mortar and extracted with 100% (v/v) methanol in 1 : 20 ratio and stirred at 80 rpm in an orbital shaker (LabTech, Daihan LabTech Co., Ltd) for four days at room temperature. Whatman No. 1 filter paper was used for separating the extract from the residue. The remaining residue was re-extracted four times and then the obtained extracts were pooled. The methanolic extract solvent was removed under reduced pressure at 6666.1 Pa pressure and 40°C using a rotary evaporator. Again, the methanolic extract was produced in triplicates. The same extraction procedure was carried out for all the samples for determination of antioxidant and total phenolic compounds. Methanolic crude extracts were dried in a vacuum to remove excess methanol solvent and purged to dry using nitrogen and kept at 80°C until further use.

Determination of total phenolic compounds: Total phenolic content was determined by the modified protocol of Folin-Ciocalteu method (Li et al., 2007). In this method 25 μl of diluted sample was mixed with 125 μl of 1 : 10 diluted Folin-Ciocalteu reagent in 96-well microtiter plates and allowed to stand at room temperature for 4 min. After that 100 μl of sodium carbonate solution (75 g l⁻¹) was added to the mixture followed by incubation for 2 hrs at room temperature. The absorbance was measured at 765 nm and gallic acid was used as standard for calibration curve. The results were expressed as gallic acid equivalent (GAE) d. wt. of microalgae. All the measurements were taken in triplicate and the mean value ± standard deviation (SD) were reported.

DPPH radical scavenging assay: Diphenylpicrylhydrazyl (DPPH) assay determines the scavenging of stable radical species of DPPH by antioxidant compounds that can be quantified using a spectrophotometer. Radical scavenging activity was evaluated by a modified DPPH method (Natrah et al., 2007). Briefly, a 50 μl aliquot of test sample (in methanol) was added to 195 μl of 0.1 mmoll⁻¹ DPPH methanolic solution. Different concentrations of microalgae extracts were prepared in 96-well microtiter plates; the mixture was swirled for one minute and then left to stand at room temperature for 60 min in dark. The absorbance was measured at 517 nm, and the radical scavenging activity was calculated as percentage inhibition relative to blank. Blank, containing 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox, 1 mg ml⁻¹), was used as a positive control. The ability to scavenge DPPH radical was calculated using the following equation:

\[
\text{DPPH radical scavenging activity (\%) } = \left( \frac{A_0 - A_i}{A_0} \right) \times 100 \tag{2}
\]

Where, \(A_0\) is the absorbance of control (DPPH solution without sample), and \(A_i\) is the absorbance of test sample (DPPH solution plus test sample).

ABTS radical scavenging assay: Radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) is also known as equivalent antioxidant capacity (TEAC) assay, based on spectrophotometry on the capacity of an antioxidant species to scavenge free radical cation ABTS by donating electron or hydrogen atom (Craft et al., 2012). ABTS radical scavenging activity on the microalgae extracts was conducted according to the modified method as explained by Li et al. (2007). In this test, 7.0 m mol l⁻¹ of ABTS was incubated with 2.45 m mol l⁻¹ of potassium persulfate for 16 hrs in dark at ambient temperature. The ABTS stock solution was prepared by mixing with distilled water until the initial absorbance reading reached 0.700 ± 0.050 at 734 nm. Trolox and each crude extract of microalgae were dissolved in methanol to the required concentration. The diluted sample (0.1 ml) in triplicate, was mixed with 0.9 ml of ABTS stock solution. After 2 min, the absorbance was quickly measured at 734 nm. The ABTS scavenging activity was expressed in milligram Trolox Equivalent (TE) per gram of dry weight. Samples were assayed in four replicates and results are given as averages ± SD. The capacity to scavenge ABTS radical was calculated by the following equation:

\[
\text{ABTS radical scavenging activity (\%) } = \left( \frac{A_0 - A_i}{A_0} \right) \times 100 \tag{3}
\]

Where, \(A_0\) is absorbance of the control (ABTS solution without sample), and \(A_i\) is the absorbance of test sample (ABTS solution plus test sample).

Ferric reducing antioxidant power (FRAP) assay: FRAP measures the reducing potential of an antioxidant reacting with ferric tripyridyltriazine Fe³⁺-TPTZ (iron[III] – 2,4,6 – tripyridyl–s–triazine) complex and producing a blue colored ferrous tripyridyltriazine (Fe²⁺-TPTZ) at low pH. The FRAP assay was carried out according to the method of Goh et al. (2010) with modified concentrations. The FRAP reagent was...
freshly prepared by mixing 10 mmol l\(^{-1}\) 2,4,6-tripyridyl-s-triazine (TPTZ) with 40 mmol l\(^{-1}\) HCl, 20 mmol l\(^{-1}\) of aqueous FeCl\(_3\), and 0.3 mol l\(^{-1}\) acetate buffer (pH 3.6) in the ratio of 1 : 1 : 10 and warmed to 37°C. After that, 180 μl of FRAP reagent was mixed with 20 μl of sample solution and the absorbance at 593 nm was measured every 30 min before and after the incubation periods. Reducing activity of microalgae's crude extracts was expressed as milligrams Trolox Equivalent (TE) per gram dry weight of microalgae.

Statistical analysis: All the results were calculated using SPSS (SPSS 20, Inc., USA) software. T-test was applied to test significant difference on total phenolic content and antioxidant activity between two media and two cell sizes (p<0.05). Meanwhile, one way ANOVA and Duncan post-hoc tests were applied to test significant differences in phenolic contents and antioxidant activity amongst different algal species at p<0.05. The correlation between antioxidant capacity, and the amount of phenolic compounds was analyzed using Pearson correlation test.

Results and Discussion

Growth, phenolic content and antioxidant activities: Cell densities reached a steady state after 25 days under constant culture conditions of light intensity, temperature, pH and nutrients (Fig. 1). The assessment of *T. tetrathele* crude extract showed that there were no significant differences (p>0.05) in total phenolic content or antioxidant activities between microalgae cultured in the f/2 or Conway media (Table 1). Evaluation of homogeneity of algal biomass in culture media revealed that high correlation coefficients were observed between optical density and dry biomass with R\(^2\) = 0.554 and R\(^2\) = 0.609, respectively (p<0.05) (Fig. 2a and 2b), but the correlation between cell density and dry biomass was low (Fig. 2c and 2d). This suggests that the difference in cell size affected the correlation coefficient (R\(^2\)) of cell number and biomass. Cell size and their distribution throughout the experiment is shown in Fig. 3. One major group of cell size of about 3.0-6.5x10\(^{-11}\) g cell\(^{-1}\) was observed in the Conway medium (Fig. 3a). Meanwhile, two types of cell sizes, such as small sized-cells (3.0-5.0x10\(^{-11}\) g cell\(^{-1}\)) and big sized-cells (5.5-8.0x10\(^{-11}\) g cell\(^{-1}\)), were identified in the f/2 medium (Fig. 3b).

Effect of cell size on total phenolic content and antioxidant activities: Evaluation on the production of phenolic compounds between different cell sizes in the f/2 medium showed that the amount of total phenolics in small sized-cells was significantly higher (p<0.05) than in big

### Table 1: Total phenolic compounds and antioxidants assays of *Tetraselmis tetrathele* crude extract cultured in two different media; f/2 and Conway media

<table>
<thead>
<tr>
<th>Assays</th>
<th>f/2</th>
<th>Conway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolic content (mg GAE/g)</td>
<td>2.47±0.71</td>
<td>2.04±0.67</td>
</tr>
<tr>
<td>DPPH free radical scavenging power (%)</td>
<td>18.20±1.76</td>
<td>17.20±1.81</td>
</tr>
<tr>
<td>ABTS' radical scavenging activity (mg Trolox/g)</td>
<td>2.54±0.33</td>
<td>2.50±0.43</td>
</tr>
<tr>
<td>Ferric reducing antioxidant power (mg Trolox/g)</td>
<td>3.44±1.44</td>
<td>2.49±0.66</td>
</tr>
</tbody>
</table>

Different letters in the same row indicate significant differences by t-test at p<0.05

### Table 2: Total phenolic contents and antioxidant activities of different cell size of *Tetraselmis tetrathele* in f2 media

<table>
<thead>
<tr>
<th>Assays</th>
<th>Small cell</th>
<th>Big cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolic content (mg GAE/g)</td>
<td>2.99±0.14*</td>
<td>1.85±0.48</td>
</tr>
<tr>
<td>DPPH free radical scavenging power (%)</td>
<td>10.89±3.16</td>
<td>25.26±3.69*</td>
</tr>
<tr>
<td>ABTS' radical scavenging activity (mg Trolox/g)</td>
<td>2.41±0.20</td>
<td>2.71±0.37</td>
</tr>
<tr>
<td>Ferric reducing antioxidant power (mg Trolox/g)</td>
<td>4.22±1.25</td>
<td>3.55±0.68</td>
</tr>
</tbody>
</table>

**"*** indicates statistically different at confidence level of 95% (p<0.05).

### Table 3: The p values and correlation coefficients (R\(^2\)) between the content of phenolic compounds and antioxidant activities in different cell size (obtained from f2 media culture)

<table>
<thead>
<tr>
<th>In vitro antioxidant assays</th>
<th>Small cell</th>
<th>Big cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R(^2)</td>
<td>p value</td>
</tr>
<tr>
<td>DPPH free radical scavenging power (%)</td>
<td>0.824*</td>
<td>0.012</td>
</tr>
<tr>
<td>ABTS' radical scavenging activity (mg Trolox/g)</td>
<td>0.809*</td>
<td>0.015</td>
</tr>
<tr>
<td>Ferric reducing antioxidant power (mg Trolox/g)</td>
<td>0.416</td>
<td>0.166</td>
</tr>
</tbody>
</table>

**"*** indicates that correlation is significant at p<0.05.
sized-cells with 2.99 ± 0.14 mg GAE g⁻¹ and 1.85 ± 0.48 mg GAE g⁻¹, respectively (Table 2), which means that small sized-cells contain 1.6 times higher phenolics than the big sized-cells. Big sized-cells showed higher activities of DPPH free radical scavenging activity power (p<0.05) and ABTS⁺ radical scavenging activity which were 25.26 ± 3.69% and 2.71 ± 0.37 mg Trolox g⁻¹, respectively. However, from the FRAP assay, the antioxidant activity in small sized-cells was slightly higher as compared to big sized-cells, which was 4.22 ± 1.25 mg Trolox g⁻¹ but this was not significantly different (p>0.05).

The relationship between antioxidant activities and the amount of total phenolic compounds were determined by correlation coefficient (R²) as shown in Table 3 and contributed to explaining the variation in the antioxidant activity of different cell sizes. Strong correlation coefficients (R²) were illustrated among small and big sized-cells for DPPH, which were R² = 0.824 and R² = 0.967, respectively. Meanwhile, weak correlations were observed between big sized-cells for ABTS (R² = 0.604) and small sized-cells for FRAP (R² = 0.416). Few studies have investigated the role of phenolic-antioxidant activities in microalgae since they have been suggested as a potential rich source of natural antioxidants (Maisuthisakul et al., 2007). Phenolic compounds and their derivatives, which contain aromatic rings and hydroxyl groups such as simple phenols, flavanoids, phenylproponoids, lignins and tannins, have the ability to scavenge free radicals. Phlorotannins from brown microalgae, pheophytin from green microalgae and phlorotannins from brown microalgae are examples of phenolic compounds that are reported as antioxidant compounds (Goh et al., 2010). A previous study reported that phenolic compounds have redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. However, not all phenolic compounds are reducing agent substances and contribute to the antioxidant activity (Zhang et al., 2007). There are some other compounds such as carotenoids, polyunsaturated fatty acids (PUFA) and polysaccharides that act as antioxidants, even though the phenolic contents in cells are low (Valentão et al., 2010). In fact, microalgae can produce a wide range of antioxidant compounds such as carotenoids, polysaccharides and polyunsaturated fatty acids (PUFA). A wide range of antioxidant compounds produced in microalgae cells, which are not phenolic compounds, might contribute to variation in correlation coefficients between phenolic compounds and each antioxidant activity. There was a general trend of a significant decrease to total phenolic contents after the first harvest (Fig. 4a), whereby the DPPH assay showed a significant increase (Fig. 4b). The ABTS assay showed constant growth (Fig. 4c) while growth in the FRAP assay varied and fluctuated (Fig. 4d). Interestingly, antioxidant levels were not constant in the extract, even though growth and concentration were stable under constant conditions.

![Graph](image_url)

**Fig. 1:** Daily changes in biomass for 56 days culture in *T. tetratele*. Triangle and circle show f/2 and Conway media treatment respectively. Line indicates the volume of media in the photobioreactor. Harvest time for analyses are indicated by arrows of H1, H2, H3, H4, H5 and H6 during day 32, 36, 40, 46, 50 and day 56 respectively.
culture conditions. The main reason for instability of phenolic compound production and antioxidant activities of *T. tetrathele* might be due to the effect of continuous culture conditions on the physiology of cell itself.

Generally, the algal yield for biochemical compound production is divided into two steps (Lorenz and Cysewski, 2000). The first step is green culture process to produce and increase algal biomass, which emphasizes on high growth and nutrient uptake rate. The second step is accumulation process for increasing the antioxidant content in the cells. Normally, the growth rate in the second step is much lower than first step, since the antioxidant content in algal biomass increase under stressful conditions, such as combined effect of both high light and nitrogen starvation (Rise *et al.*, 1994), low nutrient loading (Ip *et al.*, 2004) and salt stress (Pelah *et al.*, 2004). Most of the previous studies on the production of antioxidant compounds were focused on the second step for the estimation of the antioxidant content and high antioxidant activities (Hajimahmoodi *et al.*, 2010). Goh *et al.* (2010) reported that the total phenolic content in *Chaetoceros* sp. extract was 98.67 mg g⁻¹ when cultured in a strong light condition and Alberto Viera

![Graphs showing correlation between optical density, cell density, and dry biomass](image)

**Fig. 2:** Correlation between optical density (a, b), cell density (c, d), with dry biomass. Triangle and circle show f/2 and Conway media, respectively.
Costa et al., (2004) reported that the biomass of *Spirulina platensis* production increased 2.67 times by supplementing with 1.125 mg l\(^{-1}\) of urea for the microalgal growth. However, the total production rate of antioxidant and phenolic compounds decreased under stressful conditions, despite antioxidant content being high. In the present study, the concentrations of phenolic compounds were relatively low as compared to previous studies because the present study was conducted in stressful conditions where cells were in green process with high production rate (Ip et al., 2004; Pelah et al., 2004).

Fig. 3 : Cell size distribution in Conway (a) and f/2 (b) media

Fig. 4 : Total phenolic content and antioxidant activity in each series of harvesting time for f/2 and Conway (a, b, c, d) media's culture
This study illustrated that only f/2 medium gave two groups of cell size even though that there were no significant differences between the total phenolic and antioxidant activities between the f/2 and Conway media. There were significant differences to the contribution of total phenolic content and DPPH free radical scavenging power between small sized-cells and big sized-cells. Therefore, the results of the present study showed that the microalgae cell size significantly influenced the antioxidant activities and total phenolic contents. This result suggests that effective antioxidant and phenolic compound production can be achieved by controlling cell size only in single culture condition without stress.

Acknowledgments

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