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## Relationship Between Total Phenolics Content and Antioxidant Activities of Microalgae Under Autotrophic, Heterotrophic and Mixotrophic Growth

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### ABSTRACT

A study was carried out to explore the relationship between growth conditions and antioxidant properties of microalgae. Further, correlation between phenolics and antioxidant activities were studied to determine whether antioxidant activity depends on microalgal phenolics content under varying culture conditions. Total phenolics and antioxidant properties of *Chlorella vulgaris* and *Scenedesmus obliquus* grown under autotrophic, heterotrophic and mixotrophic conditions were evaluated. Domestic water, Bold's medium and sewage water were used to cultivate the microalgae and the extracts were prepared in methanol and analyzed for biochemical (total phenolics) and antioxidant properties (DPPH assay, super oxide scavenging assay and antioxidant potential). The experiments were done in triplicates and significant correlation coefficients between antioxidant properties against phenolic content and growth conditions were interrelated. The amount of total phenolics content varied in growth conditions and ranged from 0.11-0.55 mg GAE g<sup>-1</sup>. Significant correlation co-efficient between phenolics and antioxidant properties of microalgae determined by DPPH, superoxide anion scavenging and total antioxidant activities were found in the study. The strongest positive correlation was found to be between total phenolics and DPPH activity in *C. vulgaris* ( $r = 0.997$ ). In *S. obliquus*, the strongest positive correlation was between total phenolics and antioxidant potential ( $r = 0.091$ ) at  $p < 0.01$  followed by superoxide scavenging ( $p < 0.05$ ). The findings indicated that phenolic compounds were the major contributors to the antioxidant properties of microalgae. The results demonstrated that strongest positive correlation was observed in mixotrophic conditions followed by autotrophic conditions in *Chlorella* whereas the correlation was significant under heterotrophic conditions in *Scenedesmus* followed by mixotrophic conditions.

**Key words:** Microalgae, phenolics, antioxidant, mixotrophic, sewage water

### INTRODUCTION

Oxidative stress due to the production of Reactive Oxygen Species (ROS) contribute to the pathogenesis of cardiovascular diseases, atherosclerosis and cancer (Dhalla *et al.*, 2000; Finkel and Holbrook, 2000; Madhavi *et al.*, 1996). Synthetic antioxidants such as Butylated Hydroxyl Toluene (BHT), Butylated Hydroxyl Anisole (BHA),  $\alpha$ -tocopherol and propyl gallate have been used to reduce oxidative damages in the human body (Gulcin *et al.*, 2002) but need to be replaced with natural antioxidants, as they were found to be toxic and carcinogenic in animal models (Ito *et al.*, 1986; Safer and Nughamish, 1999).

Microalgae are being investigated for properties beneficial to the nutraceuticals and health foods industries and are promising alternative source of antioxidants (Li *et al.*, 2007;

Natrah *et al.*, 2007; Rodriguez-Garcia and Guil-Guerrero, 2008; Chacon-Lee and Gonzalez-Marino, 2010; Lee *et al.*, 2010). Microalgae exhibit adaptative responses to oxidative and radical stresses by producing potential chemicals via stimulation of their antioxidant defence system (Srivastava *et al.*, 2005; Tsao and Deng, 2004). Microalgal antioxidant products are recognized as safe (Abe *et al.*, 1999; El-Baz *et al.*, 2002). Although many microalgal species have been reported widely for their antioxidant activity (Li *et al.*, 2007; Rao *et al.*, 2006; Duan *et al.*, 2006; Wu *et al.*, 2005; Herrero *et al.*, 2006, 2005; Murthy *et al.*, 2005; Tannin-Spitz *et al.*, 2005; Kuda *et al.*, 2005; Guzman *et al.*, 2001; Mirada *et al.*, 1998) there has been limited information on antioxidant levels of microalgae under varying culture conditions. Few studies reported that phenolic compounds had a high antioxidant capacity (Jaime *et al.*, 2005; Geetha *et al.*, 2010; Custodio *et al.*, 2012), while another study found the opposite (Goh *et al.*, 2010) hence it is not clear whether phenolic substances are important antioxidants in microalgae.

This research was attempted to explore the relationship between growth conditions and antioxidant properties of microalgae and also the correlation between phenolics and antioxidant activities. The experimental work encompassed screening of freshwater microalgae for antioxidant behaviour grown under autotrophic, heterotrophic and mixotrophic conditions. Phenolic content was determined and correlation coefficient was used to estimate and compare the contribution of phenolic compounds to the measured antioxidant activities.

## **MATERIALS AND METHODS**

**Sample collection and identification:** Waste water was collected from Bangalore Water Sewerage and Supply Board (BWSSB), Bengaluru (13°04'N, 77°58'E), India and poured into a closed 250 mL bottle and exposed in sunlight for 3 weeks. The upper layer of the water was inoculated in BG11 medium enriched agar plates containing 200 µg mL<sup>-1</sup> ampicillin. The plates were incubated at 25±2°C under cool white fluorescent light (40 µ mol photons m<sup>-2</sup> sec<sup>-1</sup>; 15 h light/9 h dark) until algal growth was detected. Single green colour colonies were inoculated into BG11 medium and identified as *Chlorella vulgaris* and *Scenedesmus obliquus* according to Anderson (2005) and Round (1973).

**Culture conditions:** Microalgal cultivations were performed in 500 mL conical flasks in different growth medium namely normal water (autotrophic), Bold's media (heterotrophic) and sewage water (mixotrophic).

**Extract preparation:** Microalgal extracts were prepared by centrifuging 10 mL of a 30 day-old cultures of *Chlorella* and *Scenedesmus* at 2500 rpm for 10 min. The pellet was then resuspended and homogenized in methanol (1:1 w/v) and then subjected to centrifugation at 4000 rpm for 5 min. The supernatant was collected and used for biochemical and antioxidant assays.

**Determination of polyphenols:** Total phenolics in the extracts were determined with Folin-Ciocalteu method (Javanmardi *et al.*, 2003) using gallic acid as a standard phenolic compound (2-20 mg mL<sup>-1</sup>). Aliquots (200 µL) of each extract were added with 1.0 mL of Folin-Ciocalteu reagent and 800 µL of 7.5% sodium carbonate. The mixture was allowed to stand for 30 min in dark and the absorbance was measured at 765 nm. The total phenolic content was expressed as gallic acid equivalents (GAE g<sup>-1</sup>) dry weight of microalgae and calculated as mean value±SD.

### **Antioxidant evaluation assays**

**DPPH assay:** Free radical scavenging activity of the samples was determined according to the modified methodology of Brand-Williams *et al.* (1995). Algal extracts (200  $\mu\text{L}$ ) were mixed with 1.8 mL of the methanolic DPPH solution (0.5 mM). The absorbance was measured at 517 nm immediately after mixing and after standing at room temperature for 30 min. The percent of scavenging has been calculated as the ratio of the absorption of the sample relative to the control DPPH solution without extract. The radical scavenging activity was calculated as the percentage of DPPH discoloration using the equation:

$$\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where,  $A_{\text{sample}}$  is the absorbance of the solution when the sample solution has been added at a particular level and  $A_{\text{control}}$  is the absorbance of the DPPH solution.

**Super oxide free radical scavenging activity:** Measurement of superoxide anion scavenging activity of the samples was done based on the methodology of Nishikimi *et al.* (1972). Two hundred microliter aliquots of the extracts and ascorbic acid (2-20  $\text{mg mL}^{-1}$ ) were added with 100  $\mu\text{L}$  of Riboflavin solution (20  $\mu\text{g}$ ), 200  $\mu\text{L}$  EDTA solution (12 mM), 200  $\mu\text{L}$  methanol and 100  $\mu\text{L}$  NBT (Nitro-blue tetrazolium) solution (0.1 mg). The absorbance of solution was measured at 590 nm using phosphate buffer as blank after illumination for 5 min.

**Antioxidant potential assay:** Antioxidant potential of the extracts has been assessed with the phosphomolybdenum reduction assay according to Prieto *et al.* (1999). The reagent solution contained ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM) mixed with the extracts. The samples were incubated for 90 min at 90°C and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. Ascorbic acid standard solutions (2-20  $\text{mg L}^{-1}$ ) were used to plot the calibration curve and the reducing capacity of the extracts has been expressed as the ascorbic Acid Equivalent Antioxidant Content (AEAC).

**Statistical analysis:** The assays were carried out in triplicate and the results were expressed as mean values and the Standard Deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) ( $p < 0.05$ ). Correlations were established using Pearson's correlation coefficient ( $r$ ) in bivariate linear correlations ( $p < 0.05$  and  $p < 0.01$ ). These were carried out using Microsoft office Excel 2007 and SPSS version 16.0 program.

## **RESULTS AND DISCUSSION**

**Total phenolics content:** The phenolic content of algal extracts were ranged from 0.11-0.55  $\text{mg GAE g}^{-1}$  and extracts from autotrophic conditions showed low contents of phenolic compounds ( $< 0.2 \text{ mg GAE g}^{-1}$ ). *Scenedesmus* from mixotrophic had the highest phenolic content (0.55  $\text{mg GAE g}^{-1}$ ) followed by *Chlorella* (0.39  $\text{mg GAE g}^{-1}$ ). Phenolic contents under heterotrophic growth varied from 0.20 to 0.37  $\text{mg GAE g}^{-1}$ . In general, *Scenedesmus* has exhibited higher phenolics content than *Chlorella*.

**Antioxidant activities of microalgae:** As shown in Table 1, *Chlorella* exhibited highest DPPH free radical scavenging activity (52.3%) when grown under mixotrophic conditions whereas under

Table 1: Total levels of phenolics, DPPH, superoxide anion scavenging and antioxidant potential of microalgae under varying growth conditions

Parameters	Autotrophic		Heterotrophic		Mixotrophic	
	<i>Chlorella</i>	<i>Scenedesmus</i>	<i>Chlorella</i>	<i>Scenedesmus</i>	<i>Chlorella</i>	<i>Scenedesmus</i>
Polyphenols (mg GAE g <sup>-1</sup> )	0.191±2.7 <sup>e</sup>	0.111±3.1 <sup>f</sup>	0.209±3.4 <sup>d</sup>	0.375±0.3 <sup>c</sup>	0.397±1.1 <sup>b</sup>	0.550±2.6 <sup>a</sup>
DPPH (% inhibition at mg mL <sup>-1</sup> )	37.04 <sup>e</sup>	43.5 <sup>b</sup>	39.1 <sup>c</sup>	15.64 <sup>f</sup>	52.3 <sup>a</sup>	37.9 <sup>d</sup>
Super oxide (% inhibition at mg mL <sup>-1</sup> )	27.31 <sup>c</sup>	29.89 <sup>a</sup>	14.91 <sup>f</sup>	18.42 <sup>e</sup>	29 <sup>b</sup>	25 <sup>d</sup>
Antioxidant potential (mg AEAE g <sup>-1</sup> )	1836.48±0.3 <sup>c</sup>	1815.60±0.4 <sup>d</sup>	1520.48±0.6 <sup>f</sup>	1542.96±0.2 <sup>e</sup>	4322.58±0.5 <sup>b</sup>	5096.76±0.5 <sup>a</sup>

Data are expressed as mean±SD. In each column different letters indicate significant differences (p<0.05), DPPH: 2, 2-diphenyl-1-picrylhydrazyl

Table 2: Correlation showing the interrelation among antioxidant activity in *Chlorella vulgaris*

Parameters	Phenolics	DPPH	Superoxide	Antioxidant potential
Phenolics	1.000	0.997**	0.277*	0.967**
DPPH	0.997**	1.000	0.237*	0.949**
Superoxide	0.277*	0.237*	1.000	0.451*
Antioxidant potential	0.967**	0.949**	0.451*	1.000

\*\*Correlation is significant at the 0.01 level (2-tailed), \*Correlation is significant at the 0.05 level (2-tailed), DPPH: 2, 2-diphenyl-1-picrylhydrazyl

Table 3: Correlation showing the interrelation among antioxidant activity in *Scenedesmus obliquus*

Parameters	Phenolics	DPPH	Superoxide	Antioxidant potential
Phenolics	1.000	0.091*	0.277*	0.576*
DPPH	0.091*	1.000	0.997**	0.152*
Superoxide	0.277*	0.997**	1.000	0.023*
Antioxidant potential	0.576*	0.152*	0.023*	1.000

\*\*Correlation is significant at the 0.01 level (2-tailed), \*Correlation is significant at the 0.05 level (2-tailed), DPPH: 2, 2-diphenyl-1-picrylhydrazyl

autotrophic conditions, *Scenedesmus* had highest free radical scavenging activity (43.5%). Lowest activity was observed under heterotrophic conditions in both algal extracts.

Among the growth conditions, mixotrophic and autotrophic growth exhibited considerable superoxide anion scavenging activity. Heterotrophic growth showed lower activity. Both *Chlorella* and *Scenedesmus* indicated variations in activities with 27.31 and 29.89% in autotrophic and 29 and 25% under mixotrophic conditions.

The total antioxidant activity of algal extracts was evaluated using phosphomolybdate method which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. Mixotrophic growth has significantly influenced the total antioxidant activity of both *Chlorella* and *Scenedesmus* (4322.5 and 5096.7 mg AEAE g<sup>-1</sup>).

**Correlation between phenolic content and antioxidant properties:** The correlation coefficient between the phenolic content and antioxidant activities of *Chlorella* and *Scenedesmus* grown under varying cultivating conditions were determined (Table 2 and 3). From the results, phenolic compounds have contributed to the antioxidant properties of microalgae irrespective of cultivating conditions. The strongest positive correlation was found to be between total phenolics and DPPH activity in *C. vulgaris* (r = 0.997). A strong positive correlation also exists between phenolics and antioxidant potential (r = 0.967). A significant correlation was obtained between phenolics and super oxide scavenging activity (p<0.05). In *S. obliquus*, the strongest positive correlation was between total phenolics and antioxidant potential (r = 0.091) at p<0.01 followed by superoxide (p<0.05). However, the interrelation among DPPH and superoxide was higher in *Scenedesmus* (r = 0.997).

Table 4: Correlation showing the interrelation among antioxidant activity in *Chlorella vulgaris*

Growth conditions	<i>Chlorella</i>	<i>Scenedesmus</i>
Autotrophic	0.700*	0.676
Heterotrophic	0.595	0.765**
Mixotrophic	0.704*	0.729**

\*\* correlation is significant at the 0.01 level (2-tailed), \* Correlation is significant at the 0.05 level (2-tailed)

**Correlation between growth conditions and antioxidant properties:** The correlation between growth conditions and antioxidant properties were determined to find the influence of autotrophic, heterotrophic and mixotrophic conditions. Strongest positive correlation observed in mixotrophic conditions followed by autotrophic conditions in *Chlorella* (Table 4). However, the correlation was significant under heterotrophic conditions in *Scenedesmus* followed by mixotrophic conditions.

Reactive oxygen and free radicals are produced during oxygenic photosynthesis by microalgae. Antioxidant compounds are produced by microalgae as their defence to avoid oxidative damage (Lu and Foo, 1995) and are potent chemical blockers of UV radiation (Mata *et al.*, 2010). Biochemical composition of microalgae can be fine tuned by cultural operations (Fabregas *et al.*, 2001; Otero and Fabregas, 1997; Gigova and Ivanova, 2015) and determination of optimum culture conditions towards biomass growth and antioxidant synthesis in microalgae have been reported (Celekli and Yavuzatmaca, 2009). Influence of growth medium on antioxidant properties of cyanobacteria is reported by Tarko *et al.* (2012). Studies also show that antioxidant activities are influenced by illuminance while culturing (Madhyastha *et al.*, 2009). The effect of pH and temperature on antioxidant productivity in *Scenedesmus* were assessed by Guedes *et al.* (2011) and found that pH plays an important role in antioxidant content. The effect of antioxidants on DPPH free radical scavenging was considered to be due to their hydrogen donating ability. In this study, extracts of both algae showed notable activities indicating the higher efficacy for scavenging of free radicals. *Chlorella* and *Scenedesmus* have exhibited higher free radical scavenging activity when cultivated under autotrophic and mixotrophic conditions respectively. A superoxide anion radical generally forms first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Liu and Ng, 2000). In this study, significant superoxide scavenging activities of microalgae grown under autotrophic mixotrophic conditions were observed. Phenolic compounds donate a hydrogen atom or an electron in order to form stable radical intermediates thereby serves as important antioxidants. A number of phenolic compounds are present in microalgae (Klejdus *et al.*, 2010; Kovacic *et al.*, 2010) and were reported to responsible for microalgal antioxidant properties. But the contribution of phenolics to the antioxidant activity of microalgae needs to be understood in a better way. The amount of total phenolics by Folin-Ciocalteu method was varied in growth conditions. Correlation analyses indicated significant contribution of phenolics to antioxidant activity as measured by the DPPH, superoxide and total antioxidant assays. It was found that phenolic compounds are important contributors to antioxidant activities in *Chlorella* and *Scenedesmus* and the results are in accordance with previous studies (Hajimahmoodi *et al.*, 2010; Goiris *et al.*, 2012). In general, heterotrophic cultivation is having many advantages than autotrophic cultivation (Perez-Garcia *et al.*, 2011) but in this study mixotrophic cultivation using municipal sewage water has increased antioxidant properties of microalgae. The use of wastewater for microalgal cultivation for higher biomass and lipid production has been reported widely (Clarens *et al.*, 2010; Pittman *et al.*, 2011) but to our knowledge, influence of municipal sewage water on antioxidant properties of microalgae is reported for the first time through this study.

## CONCLUSION

Relationship between phenolics and antioxidant properties of microalgae grown under autotrophic, heterotrophic and mixotrophic conditions were studied. The results demonstrated that growth conditions play an important role in contributing antioxidant properties of microalgae. In general, mixotrophic culture using sewage water exhibited higher antioxidant activities followed by autotrophic culture. Correlation co-efficient studies revealed that antioxidant activity is depending on the phenolic content of microalgae which is varying under growth conditions.

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