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Variations among Antioxidant Profiles in Lipid and Phenolic Extracts of Microalgae from Different Growth Medium

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ABSTRACT

Up-scaling the production of value added products from microalgae requires reliable techniques. This study has been carried out in order to determine whether microalgae have an inherent tendency to contain similar proportions of antioxidant properties in its lipid and phenolic extracts under varying growth medium. *Chlorella vulgaris* and *Acutodesmus obliquus* (*Scenedesmus obliquus*) were cultivated in normal water, Bold's medium and sewage water followed by extraction of lipids and phenolics. The extracts were subjected to in vitro antioxidant assays performed in triplicates in which higher scavenging activity of DPPH and super oxide radical was observed in phenolic extracts. However, there was a significantly higher antioxidant potential found in lipid extracts suggests that next to the well studied phenolic compounds, microalgal lipids should also be considered when using microalgae as a source of natural antioxidants. Further, antioxidant profile of lipid and phenolic extracts from same species varied with growth medium.

Key words: Microalgae, lipid extract, phenolic extract, antioxidant capacity, *Chlorella*, *Acutodesmus*

INTRODUCTION

Reactive oxygen and free radicals would attack key biological molecules such as DNA, protein and lipid leading to degenerative diseases. Removal of free radicals and Reactive Oxygen Species (ROS) is one of the most effective defenses of a living body against various diseases by enzyme mediated and non-enzymatic factors. Synthetic oxidants have been used to retard the oxidation process however use of synthetic antioxidants is under strict regulation due to their potential health hazards (Branen, 1975; Park *et al*., 2001) and natural antioxidants are of great interest as alternatives. Food industries prefer natural antioxidants as they prevent rancidity of fats and oils in foods.

Microalgae are a group of heterogeneous microorganisms having natural source of biologically active compounds. Microalgae use light energy and inorganic nutrients to develop and synthesize bio-compounds having therapeutic and nutritional values. Many studies have reported that microalgae can produce different chemical compounds with different biological activities (Li *et al*., 2007; Markou and Nerantzis, 2013; Costa and Morais, 2013; Plaza *et al*., 2009; Spolaore *et al*., 2006). Microalgae respond with physiological changes to the environmental conditions where they

grow (Valenzuela-Espinoza *et al*., 2002; Scragg *et al*., 2002). Microalgae exhibit adaptative responses to oxidative stresses, via stimulation of their antioxidant defence system (Hong *et al*., 2008; Srivastava *et al*., 2005).

Up-scaling the production of value added products from microalgae requires reliable techniques. Changes in the external environment cause microalgae to change their intracellular environment and manipulation of the culture conditions by presence or absence of nutrients stimulates the biosynthesis of specific compounds. The research effort described in this paper attempts to expand the uses of microalgae by specifically taking advantage of their antioxidant features by using varying growth medium and extracts. The experimental work encompassed screening of extracts derived from *Chlorella vulgaris* and *Acutodesmus obliquus* (*Scenedesmus obliquus*) grown under three different medium for antioxidant potential. Both lipid and phenolic extracts were considered, so as to comprehensively characterize the microalgae in terms of total phenolics content, DPPH, super oxide radical scavenging and total antioxidant capacity.

MATERIALS AND METHODS

Microorganisms: *Chlorella vulgaris* and *Acutodesmus obliquus* were isolated from waste water treatment plant, Bengaluru (13°04' N, 77°58' E), India and identified according to Anderson (2005) and Round (1973).

Culture conditions and growth medium: In order to determine variations in antioxidant properties of microalgal extracts, three different growth medium were used. The cultures were inoculated into 500 mL conical flasks and cultivated in growth room provided with cool white fluorescent light (40 μmol photons m^{-2} sec^{-1}, 15 h light 9 h dark) at 25±2°C. Growth media used in the study were normal water, Bold's basal medium and municipal sewage water.

Intracellular extraction

Lipid extract preparation: Lipid extraction was done by centrifuging the algal cells followed by addition of 10 mL of ice cold 0.2 N $HClO_4$. After 15 min at 4°C, the sample was centrifuged and 10 mL of chloroform-methanol (2:1 v/v) solution was added. The mixture was allowed to stand for 5 min at 4°C and centrifuged. To the supernatant, 0.2 volumes of distilled water were added and the solutions were shaken for 5 min before centrifugation for 15 min at 2000 rpm to separate the phases. The lower organic phase was collected and the chloroform-methanol solution was evaporated under a steam of nitrogen (Folch *et al*., 1957).

Phenolic extract preparation: Phenolic compounds were extracted by homogenizing the microalgae with 20 mL of methanol in an orbital shaker at 25°C for 60 min at 200 rpm (De Souza *et al*., 2009). The filtrate was added with equal volume of hexane and the mixture was dried in a rotary evaporator at 50°C under reduced conditions. Dried extract was dissolved in 25 mL of distilled water and clarified with 5 mL each of barium hydroxide (0.1 M) and of zinc sulphate (5%).

Determination of polyphenols: Total phenolics in the extracts were determined by Folin-Ciocalteau (FC) method (Javanmardi *et al*., 2003) using gallic acid as standard (2-20 mg mL^{-1}). Aliquots (200 μL) of microalgal extracts were added with 1.0 mL of FC reagent and 800 μL of sodium carbonate (7.5%). 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) dry weight of microalgae and calculated as mean value ±SD.

In vitro free radical scavenging and antioxidant assays

DPPH radical scavenging assay: Antioxidant capacity of the extracts was confirmed by the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay according to Brand-Williams *et al*. (1995) with slight modifications. Algal extracts (200 μL) were mixed with 1.8 mL of the methanolic DPPH solution (0.5 mM). The absorbance has been 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:

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

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

Super oxide radical scavenging assay.

Super oxide radical scavenging assay: Measurement of superoxide radical scavenging activity of the samples was done by the reduction of NBT according to earlier method (Nishikimi *et al*., 1972). Two hundred micro liters aliquots of the extracts and ascorbic acid (2-20 mg mL^{-1}) were added with 100 μL of Riboflavin solution (20 μg), 200 μL EDTA solution (12 mM), 200 μL methanol and 100 μL 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 was 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 mins at 90°C and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. Ascorbic acid standard solutions (2-20 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 Standard Deviation (SD) using Microsoft excel.

RESULTS

Estimation of phenolic and antioxidant content through assays in lipid and phenolic extracts shows different levels irrespective of growth medium. Total polyphenols from lipid extract were in the range of 0.012-0.122 mg GAE g^{-1} (Fig. 1) and sewage water grown microalgae recorded highest polyphenols content (0.122 mg GAE g^{-1}) followed by Bold's basal medium. Lower levels of polyphenols were present in phenolic extract (0.014-0.063 mg GAE g^{-1}) in which Bold's medium produced maximum polyphenols. Total phenolics were higher in lipid extracts of *A. obliquus* whereas, it was higher in phenolic extract of *C. vulgaris*.

As depicted in Fig. 2, phenolic extracts exhibited highest DPPH radical scavenging activity than lipid extracts. From the data obtained, *Chlorella* contains potential free radical scavenging activity (65.41%) when grown in sewage water where as Bold's media produced higher DPPH scavenging

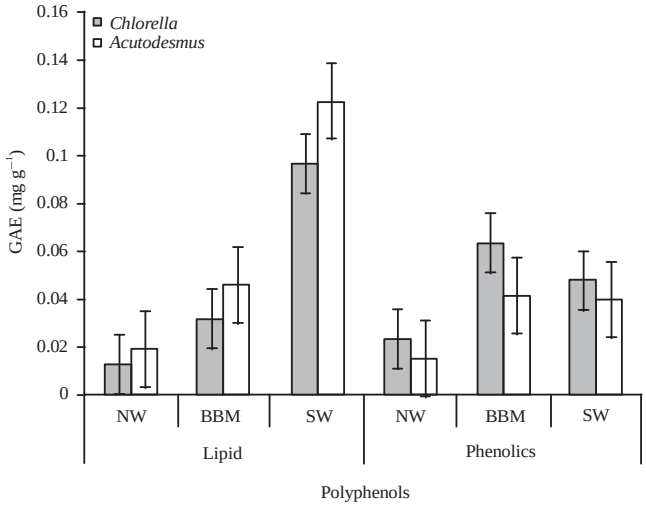

Fig. 1: Polyphenols content of lipid and phenolic extracts from *Chlorella vulgaris* and *Acutodesmus obliquus*, NW: Normal water, BBM: Bold's basal medium, SW: Sewage water

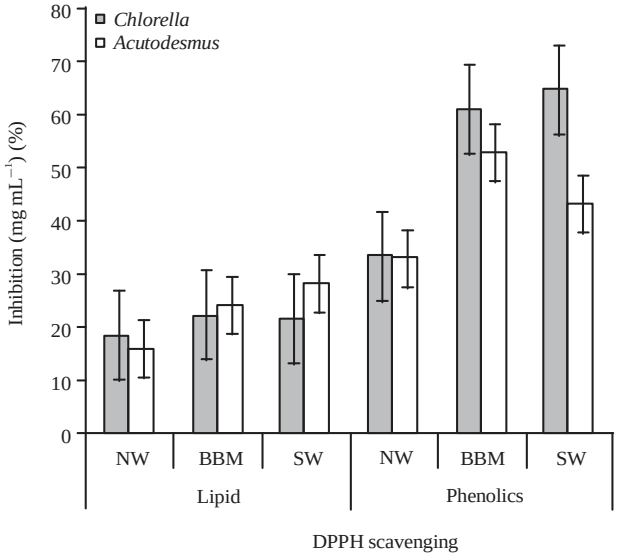

Fig. 2: DPPH radical scavenging activity of lipid and phenolic extracts from *Chlorella vulgaris* and *Acutodesmus obliquus*, NW: Normal water, BBM: Bold's basal medium, SW: Sewage water

activity in *Acutodesmus* (53%). Lipid extracts exhibited lower levels of free radical scavenging (15.69-24.29%) and *Acutodesmus* recorded higher activity than *Chlorella*. Significant superoxide anion scavenging activity (Fig. 3) was found in phenolic extracts of *Chlorella* and *Acutodesmus* (28.86-55.9%) in which highest activity was from microalgae grown in Bold's media (55.9%). The activity was relatively low in lipid extracts and sewage water produced maximum scavenging activity in *Acutodesmus* (24.42%). Antioxidant potential of microalgae revealed that lipid extracts from sewage water grown microalgae having three fold higher levels of activity (1511.71 mg AEAE g^{-1}) than normal water and Bold's medium (Fig. 4). Phenolic extracts of both microalgal species exhibited lower activities and Bold's medium has positively influenced the antioxidant potential when compared to sewage water.

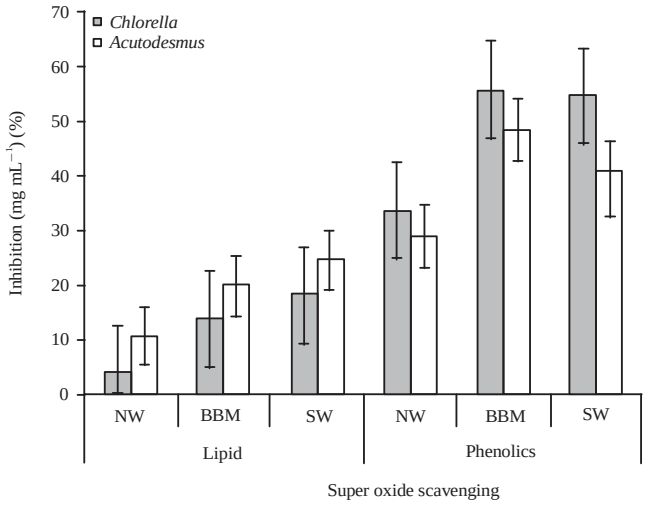

Fig. 3: Superoxide radical scavenging activity of lipid and phenolic extracts from *Chlorella vulgaris* and *Acutodesmus obliquus*, NW: Normal water, BBM: Bold's basal medium, SW: Sewage water

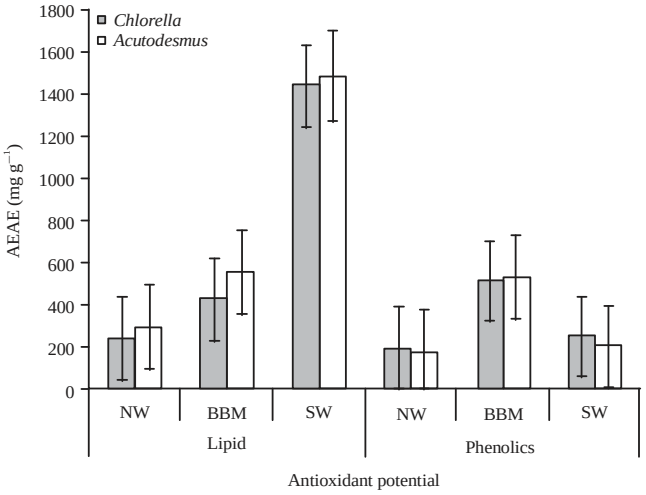

Fig. 4: Total antioxidant potential of lipid and phenolic extracts from *Chlorella vulgaris* and *Acutodesmus obliquus*, NW: Normal water, BBM: Bold's basal medium, SW: Sewage water

DISCUSSION

Formation of reactive oxygen species have been linked in pathogenesis of several human diseases and investigations on natural antioxidants is increasing. Algae live in extreme environmental conditions and to survive, varieties of biologically active compounds are produced in which antioxidants have attracted major interest. The conditions for microalgal cultivation are important that influence the metabolism, thus directing the synthesis of specific compounds of interest. Efforts to increase the productivity of microalgal cultures in terms of biomass and lipid production have been focused, but little attention has been paid to identify the type of extracts and nutritional requirements of microalgae in order to improve antioxidant capacity.

Culture operation has been demonstrated to be a key factor in biochemical composition of microalgae biomass (Fabregas *et al*., 2001; Otero and Fabregas, 1997). Variations in biochemical composition of *C. vulgaris* grown under different media were reported earlier (Chia *et al*., 2013). Influence of growth medium on antioxidant properties of Cyanobacteria was determined by Tarko *et al*. (2012). Temperature and pH also has relevant effects on antioxidant production in microalgae (Guedes *et al*., 2011). Substantial differences in antioxidant enzyme activities of microalgae in response to exogenous nitrogen levels are found recently (Gigova and Ivanova, 2015). Hence, three different media were used in this study and growth medium triggered variations in antioxidant profiles of lipid and phenolic extracts were determined. The results revealed significant changes in antioxidant activity influenced by change in growth medium in which sewage water produced sound results followed by Bold's medium.

Phenolic compounds can act as antioxidants by chelating metal ions, preventing radical formation, improving the antioxidant endogenous system and combat free radicals (Al-Azzawie and Alhamdani, 2005; Estrada *et al*., 2001). Higher amount of phenolics were found in lipid extracts of *Acutodesmus* and significant antioxidant levels was observed thus confirming the role of phenolic compounds in antioxidant properties. Similar results from *Scenedesmus* were obtained by previous studies (Guedes *et al*., 2013; Aboul-Enein *et al*., 2003) with higher antioxidants levels and activity. DPPH radical is widely used to test the free radical-scavenging ability of various samples. Free radical scavenging and lipid peroxidation reducing compounds were identified from *Chlorella* (Spolaore *et al*., 2006). In this study, phenolic extracts of *C. vulgaris* and *A. obliquus* grown in sewage water and Bold's medium has possessed relatively higher scavenging activity for DPPH. At the same time, lower activity was seen with lipid extracts and there was no significant influence by the growth medium. Superoxide radicals could initiate lipid peroxidation due to reduction of transition metals, releasing protein-bound metals and formation of perhydroxyl radicals (Aikens and Dix, 1991; Elias *et al*., 2008). A significant scavenging activity of phenolic extract from both microalgae against super oxide radical was observed in the study which suggests the lipid peroxidation inhibition activity of *Chlorella* and *Acutodesmus*. Inhibition of lipid peroxidation by other microalgae was reported earlier (Natrah *et al*., 2007). An interesting finding of the study was potential free radical scavenging activity was found in phenolic extracts but total antioxidant potential values were doubled in lipid extracts of both microalgae grown in sewage water.

Apart from biofuel feed stock, algal lipids have been studied for beneficial food additives and high value products (Schenk *et al*., 2008; Adarme-Vega *et al*., 2012). Several microalgal genera contain potent antioxidants, both from lipophilic and hydrophilic nature. Antioxidant activity of lipid extracts of marine microalgae was found by Abd El Baky *et al*. (2014). Earlier findings revealed microalgal fractions that were rich in phenolic compounds had a high antioxidant capacity (Jaime *et al*., 2005; Geetha *et al*., 2010; Custodio *et al*., 2012) whereas, Li *et al*. (2007) found no relation between phenolic content and antioxidant capacity. Non-enzymatic factors such as carotenoids and fatty acids are able to protect microalgae from oxidative damage (Goiris *et al*., 2012; Herrero *et al*., 2006; Sies and Stahl, 1995). This study has compared the antioxidant properties of both lipid and phenolic extracts and the results clearly indicated that next to the well-studied phenolic compounds, lipids also contribute significantly to the antioxidant capacity of microalgae. Further, antioxidant profile of the lipid and phenolic extracts from same species varied with growth medium.

CONCLUSION

This study investigated the differences among antioxidant properties of lipid and phenolic extracts from microalgae grown in three different media. It was found that both lipid and phenolic

extracts of *Chlorella* and *Acutodesmus* exhibited variations in antioxidant properties. The fact that the antioxidant properties differed between lipid and phenolic extracts of the same species suggests the presence of multiple bioactive compounds influence the antioxidant capacity of microalgae. Further, the biochemical properties in microalgae may be optimized by selecting the appropriate growth medium.

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