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Tocopherols, Phycocyanin and Superoxide Dismutase from Microalgae: as Potential Food Antioxidants

Ivonne Sandra Santiago-Morales[1], Lourdes Trujillo-Valle[2], Facundo Joaquín Márquez-Rocha[3], Jenny Fabiola López Hernández[4]*

1- Industries Institute, University of Sea, Puerto Ángel, Oaxaca, CP 70902.

2- Aquaculture Department, Center for Scientific Research and Higher Education of Ensenada, B.C., CP 22860.

3- Regional Cleaner Production Center, National Polytecnic Institute, Cunduacán, Tabasco, CP 86691.

4- National Technology Institute of Mexico at, Balancán, CP 86930, México.

Abstract

Background and Objective: Microalgae are photosynthetic organisms that are in contact with several reactive oxygen species, and under these conditions microalgae produce a wide variety of antioxidant compounds to protect from highly oxidant growth conditions, these facts can be used to optimize antioxidant production, however, firstly studies of antioxidant production by microalgae should be done. This work was focused to establish differences of antioxidants formation among microalgae species and elucidate some antioxidant properties of phycocyanin.

Material and Methods: Superoxide dismutase activity was performed by using a commercial kit and determined by spectrophotometry. Phycocyanin and carotenoids were quantified by spectrophotometry and tocopherols were analysed by high pressure liquid chromatography with a fluorescence detector. Trolox equivalent antioxidant activity was determined by using 2,2´-azinobis (3-ethylbenzothiazoline-6-sulphonic) reagent, scavenging and synergic anti-oxidant activities were also ascertained.

Results and Conclusion: The microalgae used in this study produces different amounts of superoxide dismutase, carotenoids, α, δ and γ-tocopherols, and phycocyanin. Trolox equivalent antioxidant activity varied according with the amount of antioxidants produced. Phycocyanin scavenge superoxide and hydrogen peroxide radicals work well to produce synergy with α-tocopherol, increasing protection of biomolecules against oxidation. One microalgae specie may be selected to produce one of the antioxidants mentioned above, but more detailed studies on growth phases are required to improve the antioxidant production. Microalgae has the potential to be considered as a natural antioxidants source.

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***Corresponding author:**

Facundo J. Márquez-Rocha,

Centro Regional para la Producción más Limpia, Instituto Politécnico Nacional, Cunduacán, Tabasco, CP 86691, México.

Tel: +52-57296000
Fax: +52-914-1041023
E-mail: fmarquez@ipn.mx

1. Introduction

The importance of natural antioxidants are their use as effective compounds to delay or inhibit the biological process of molecular oxidation and to avoid the use of synthetic antioxidants on the assumption that these produce carcinogenesis. In fact, there has been an increased interest on the search for new sources of natural compounds which display free radicals scavenging properties, mainly for the use in biomedicine, food formulation and fish nutrition [1]. Among natural antioxidants, phycocyanin has shown a great potential for several applications [2].

Recent studies have shown that marine algal extracts possess important antioxidant properties [3]. The marine algae are photosynthetic organisms that developed special mechanisms against oxidative damage [4]. The algae have antioxidant enzymes and several small metabolites, such as tocopherols, carotenoids, phenolic molecules and ascorbic acid, which may play an important role in the oxidative defence mechanisms [4].

The principal reactive oxygen species (ROS), the oxygen singlet ($^1O_2$), hydrogen peroxide (H_2O_2), the superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) radical, represent the most common examples of nucleophilic attacks to biomolecules. Oxidation of food components caused by ROS, can be prevented by addition of antioxidants [1], being antioxidants from microalgae an alternative.

Most of the studies about natural phytochemicals have been carried out on terrestrial plants and some information has been generated in algae and microalgae [4-6], but more

information of which microalgae produce one or more antioxidants, extraction methods, and better production is needed. Also the specific mechanism of antioxidant activity needs to be determined. The molecular diversity and feasibility of microalgae industrial cultivation make the idea to consider them as good candidates for natural antioxidant source. Microalgae species due to its genetic variability may produce different amount of antioxidants such as superoxide dismutase (SOD), α, δ, γ-tocopherols, β-carotene and phyc-ocyanin and there are few reports on the antioxidant mech-anism of phycocyanin.

This work studied the different antioxidants content among microalgae species and elucidated some of the antioxidant properties of phycocyanin.

2. Materials and Methods

2.1 Chemicals

All reagents were of analytical grade from SIGMA-ALDRICH and were as follows: thiobarbituric acid, trichlo-roacetic acid, ethylenediaminetetraacetic acid, 2,2´-azinobis (3-ethylbenzothiazoline-6-sulphonic) (ABTS), dithiotrei-tol, phenazine methasulphate (PMS), nico-tinamide adenine dinucleotide reduced (NADH), nitroblue tetrazolium (NBT), 2-tert-butyl-4-hydroxyanisole (BHA), 3,5-di-tert-4 butylhydroxytoluene (BHT), α, β, γ-tocopherols and bovine liver CuZn-SOD from Oxis (Portland, OR).

2.2 Organisms and Culture

The microalgae strains used in this work were *Tetraselmis* (*T.*) *suecica*, *Nannochloropsis* sp., *Chaetoceros* (*C.*) *muelleri*, *Spirulina* (*S.*) *maxima* (purchased from Instituto Politécnico Nacional microalgae culture colletion, Mexico), *Synechococcus* sp., *Porphyridium* (*P.*) *cruentum* (purchased from Centro de Investigación Cientifica y Estudios Superiores de Ensenada microalgae culture colletion, Mexico). All strains were grown on 'f medium', prepared with filtered seawater, trace minerals and vitamins as described by Guillard and Ryther [7], except Spirulina which grew on standard medium SOT, bicarbonate as sole source of carbon [8]. The culture conditions were: salinity 33-34%, temperature 24±2°C, continuous light supply of an intensity of 100 μmol quanta$\cdot s^{-1}m^{-2}$ throughout fluorescent lamps with manual mixing twice a day.

2.3 Extracts Preparation

2.3.1 Preparation of ESOD (extracts for the determination of SOD) [EC: 1.15.1.1] activity

Approximately 0.05 g of microalgal biomass was sonic-ated (Ultrasonic Processor Mod. GE100) for 2 min on 1 ml of potassium phosphate buffer (0.2 M, pH 8.0, 5 mM EDTA, 1 mM dithiotreitol), then centrifuged (Heraeus Mod. D-37520) at 12,000 × g, 4°C, 30 min. The supernatants were stored at -70°C until use.

2.3.2 Preparation of EPC extracts for phycocyanin determination

Lyophilized biomass (0.04 g) was re-suspended in 5.5 ml of K-phosphate buffer 0.01 M, pH 6.5, 0.15M NaCl. The suspension was frozen at −20°C, thawed and sonicated, then left at 5°C overnight. The suspension was centrifuged at 12,000×g, 15 min, 4°C supernatant contained phycocyanin extract.

2.3.3 Preparation of lipophilic extracts (LE), for tocopherols and carotenoids determination.

Approximately 0.07 g of microalgal biomass were sonicated (Aquasonic Mod. 150D) for 10 min in 2 ml of a mix containing ethanol 60%, pyrogallol (6%) and 0.87 ml of KOH (60%) under nitrogen gas atmosphere. The mixture was incubated 1 h at 70ºC while protected against light, then the mixture was chilled on ice; 3.13 ml of NaCl (2%) were added and an extraction with n-hexane was carried out (1:1 vv^{-1}). The extract was concentrated under N_2, and re-suspen-ded in 0.7 ml of methanol and filtered through a 0.45 μm membrane. The extract was stored at -70°C until use.

2.4 Phycocyanin purification and quantification

The soluble blue extracts (EPC) obtained in phosphate buffer 0.01 M, pH 6.5 were used to determine the phyco-cyanin content. The absorbance was measured at 615 and 652 nm and the amount of phycocyanin was calculated by PC (mg ml^{-1}) = $[(A_{615}–A_{652})/5.34)$. The crude extract cont-aining phycocyanin was precipitated in 50% $(NH_4)_2SO_4$ and recovered by centrifugation at 10,000 × g for 10 min. The colourless, clear supernatant was discarded and the blue precipitate was dissolved in a small volume of 2.5 mM Na-phosphate buffer (pH 7.0) and dialyzed against the same buffer. The dialyzed mixture was then placed in a 2.5×30 cm column packed with hydroxylapatite resin. Following a stepwise elution with a phosphate buffer of increasing ionic strength at pH 7.0 (2.5-100 mM), the absorbance at 615 and 652 nm were measured through the eluted fractions. Two main fractions (c-phycocyanin and allophycocyanin) were collected and the fraction exhibiting the larger absorbance at 615 nm contained purified c-phycocyanin.

2.5 Determination of SOD Activity

The SOD activity was measured by the SOD assay kit, Bioxytech SOD-525 (Percipio Bioscience, Inc., Portland, OR). The reaction was measured at 525 nm at 30°C for one min. The extracts ESOD obtained for each microalgae (30 to 100 μg of protein ml^{-1} of reaction mixture) were used for this assay. Commercial SOD was used as is standard. Protein content was measured by using BioRad regent at 595 nm in a spectrophotometer (DR6000, Hach, US).

2.6 Tocopherols and β-Carotene Quantification

The lipophilic extracts were used to determine tocopherols content by HPLC (Algilent 1100A, Germany). A Zorbax XDB-C8 (4.6 mm x 150 mm, 5 μ) column was used. Ten to twenty microliters were eluted with a isocratic solution of methanol:water (95:05) by a flux of 1.0 ml min^{-1}. Tocopherols content was measured with a fluorescence detector (Algilent G1321A) at an excitation λ of 292 nm, an emission wavelength of 340 nm and UV detector (Algilent G1314A) at a wavelength of 295 nm.

Commercial α, δ and γ-tocopherols served as the standard for quantification and detection. Total carotenoids were measured with the LE by dilution with methanol at 450 nm of absorbance in a spectrophotometer UV-Vis (DR6000, Hach), an absorption coefficient of ε=221.l $g^{-1}cm^{-1}$ was used to determined carotenoids concentration [9].

2.7 Determination of the Antioxidant Equivalent Activity (Trolox Equivalent: Teq)

To compare the antioxidant effect of a particular compound or a combination of two, the reduction of the absorbance of $ABTS^{\cdot+}$ radical at 734 nm was measured. The cation $ABTS^{\cdot+}$ was prepared in an aqueous solution with 7 mM of ABTS and 2.45 mM potassium persulfate. The solution was fixed, by dilution with ethanol, to an absorbance of 0.714 at 734 nm and kept in the dark until used. One ml of the $ABTS^{\cdot+}$ solution was mixed with 70 µl of each microalgae extract. Reaction mixtures were incubated for 5 min at 30°C and the absorbance was determined at 734 nm [10]. The trolox equivalent activity was quantified by the means of a reference curve at 734 nm.

2.8 Superoxide Anion Scavenging Activity (SASA)

Superoxide radicals are generated in PMS -NADH-NBT system, by the oxidation of NADH and followed by the reduction of NBT. The decrease of absorbance at 560 nm with the extracts indicates consumption of $O_2^{\bullet-}$ in the reaction mixture. The reaction mixture (1 ml) contained phosphate buffer 16 mM, pH 8.0, NBT 16.6 µM, NADH 26 µM, 3.3 µM PMS and 70 µl of Synechococcus -ESOD, Spirulina-EPC, Tetraselmis-LE extracts and 10 µl of 1:10 dilution of SOD commercial. The reaction started by the addition of PMS. The mixture was incubated at 25°C for 5 min and measured at 560 nm. L-ascorbic acid (5 mM) was used as the positive control. The percentage inhibition of the superoxide anion ($O_2^{\bullet-}$) generation was calculated by the equation: Eq. 1

$$\%\ \text{of inhibition of } O_2^{-} reactivity = \left[\frac{A_0 - A_1}{A_0}\right] x100$$

Where A_0 is the absorbance of the blank and A_1 is the absorbance of the extracts or the positive control.

2.9 Hydrogen Peroxide Scavenging Activity (HPSA)

To prepare 1 ml of mixture reaction in phosphate buffer 0.1 M, pH 7.4, the following reagents were added one at a time; 70 µl of each extract (Synechococcus-ESOD, Spirulina-EPC, Tetraselmis-LE), 2.0 µg α-tocopherol, ascorbic acid 5 mM and 10 µl of 1:10 dilution of commercial SOD. Then the reaction start with the addition of H_2O_2 to a final concentration of 43 mM. The mixture reaction was incubated at room temperature for 30 min. The absorbance was measured at 230 nm. The percentage of the hydrogen peroxide scavenging activity was calculated as: Eq. 2

$$\%\ inhibition\ of\ [H_2O_2]\ reactivity = \left[\frac{B_0 - B_1}{B_0}\right] x\ 100$$

Where B_0 is the absorbance of the blank (H_2O) and B_1 is the absorbance in the presence of extract [11].

2.10 Hydroxyl Radical Scavenging Activity (HAS)

To prepare 1 ml of a mixture reaction, 200 µl of each of the following solutions were added: deoxyribose 16.8 mM, $FeCl_3$: ethylene diamine tetraacetic acid(1:1, 300 mM:1.2 mM), H_2O_2 16.8 mM, buffer KH_2PO_4/KOH 10 mM, pH 7.4. Then one at a time the following solutions were added: 70 µl of each microalgal extract (Synechococcus-ESOD, Spirulina-EPC, Tetraselmis-LE), 2.0 µg α-tocopherol, ascorbic acid 5 mM, and 10 µl of 1:10 dilution of commercial SOD. Then 100 µL of ascorbic acid at 1.2 mM were added to start the reaction. The reaction was incubated at 37°C for 1 h, then it was stopped by the addition of 1 ml of thiobarbituric acid 1% (w v^{-1}) in 50 mM NaOH and 1 ml of trichloroacetic acid 2.8%. The mixture was incubated at 80°C for 20 min and chilled to room temperature; then the absorbance was measured at 532 nm. The hydroxyl scavenging activity was calculated by Eq. 3:

Eq. 3

$$Inhibition\ of\ [OH^{\cdot}]\ reactivity\ (\%) = \left[1 - \frac{C_1 - C_2}{C_3}\right] \times 100$$

Where C_1 is the absorbance of the extract in the assay mixture; C_2 is the absorbance of the extract in 1 ml of water and thiobarbituric acid and trichloroacetic acid solutions; C_3 is the absorbance of the distilled water of the assay mixture.

2.11 Synergic effect of phycocyanin

The antioxidant activity of Spirulina-EPC was evaluated according to the thiocyanate method described by Gülçin et al. [12]. Linoleic acid emulsion (50 ml base mixture) was prepared with 175 µl Tween-20, 155 µl linoleic acid in potassium phosphate buffer 0.04 M, at pH 7.0. For 1 ml of the mixture reaction, 900 µl of base mixture and one at a time of the following reagents were added: control 100 µl potassium phosphate buffer, BHT 0.05%, 2.0 µg α-toco-pherol, ascorbic acid 5 mM, 70 µl of Spirulina-EPC, BHA 0.05 %, 35 µl of Spirulina-EPC + 1.0 µg of α-tocopherol, and 2.5 mM of ascorbic acid + 35 µl of Spirulina-EPC. The reaction was followed during 9 h at 500 nm, room tempera-ture. Reaction was stopped by addition of 1 ml of an ethanol solution (70%) of thiocyanate (30%), $FeCl_2$ (3.5%) incubated at 30°C for 60 min. The percentage of oxidant inhibition was calculated from the same reaction without any antioxidant in the reaction mixture as 0% inhibition.

2.12 Statistical Analysis

Multiple *t*-test was applied to compare ROS scavenging, statistical significance determined using the Holm-Sidak method ($p \leq 0.05$). One sample *t*-test was used to compare amounts of each compound with $p \leq 0.05$. All statistical analyses were performed using the software GraphPad PRISM version 7.03 (Inc. La Jolla, CA 92037, USA). All experiments were done in triplicate.

3. Results and Discussion

3.1 Microalgae growth characteristics

Microalgae growth characteristics data are summarized in Table 1. The day of harvest varies between 5 (*T. suecica*) to 12 (*P. cruentum*). The growth rates were between 0.13 (*P. cruentum*) to 0.76 (*T. suecica*) d^{-1} and biomass amounts between 0.14 (*Chaetoceros* sp.) to 0.29 (*T. suecica*) g l^{-1} at the harvest day, Sigaud et al, [13] obtained a growth rate of 0.87 d^{-1} for *T. gracilis*, close to the growth rate reached in this paper for *T. suecica* 0.76 d^{-1}.

Table 1. Some growth characteristics of the microalgae used in this study

Microalgae	Day of harvest	Growth rate (d^{-1})	Biomass (dw g l^{-1})
Nannochloropsis sp	8	0.31	0.16
Spirulina maxima	10	0.21	0.18
Tetraselmis suecica	5	0.76	0.29
Chaetoceros	6	0.22	0.14
Synechoccocus	7	0.57	0.21
Porphiridium cruentum	12	0.13	0.18

3.2 SOD activity measurement and phycocyanin, tocopherols and β-carotene content analyses.

The values of SOD activity in the microalgae strains are showed in Table 2. The activity in Synechococcus extract was 157 UA mg^{-1} protein; this value shows a significant difference compared with Nannochloropsis and Cheato-ceros extracts, with values of 127 and 73 UA mg^{-1} protein, respectively. SOD activity in Spirulina and Tetraselmis were 30 and 20.4 UA mg^{-1} protein, respectively. Sigaud et al, measured SOD in *T. gracilis* obtaining 170 UA mg^{-1} protein at beginning of growth curve, which means 8 times the SOD activity reported in the present work for *T. suecica*. However SOD activity decreased during the growth of *T. gracilis*, at 5 days of growth, the harvested SOD activity fell close to 50 UA mg^{-1} protein [13]. Similar behaviour of SOD activity evolution was found for the diatom *Minutocellus polymorphus* [13]. These facts are perhaps due to the harvest time of the growth curve.

The phycocyanin content in *S. maxima* was 31 mg PC per biomass, in Porphyridium and Synechococcus phyco-cyanin contents were 5.5, and 1.34 mg PC g^{-1} biomass, respectively (Table 2). Phycocyanin in *Nannochloropsis Sp.*, *T. suecica.*, and *Chaetoceros Sp.* was not detected. Marquez et al, measured 165 mg g^{-1} biomass of phycocyanin content in *S. platensis,* 5 times high the amount obtained for *S. maxima* in present contribution, phycocyanin content increases during growth in autotrophic and mixotrophic conditions [14].

Contents of tocopherols are summarized in Table 2. α-Tocopherol was present in all microalgae studied, in *S. maxima* an amount of 2.5 mg g^{-1} biomass was found, being the largest value found in all microalgae, followed by *Nannochloropsis* sp and *T. suecica* with 1.7 mg of α-tocopherol g^{-1} biomass, The values for this tocopherol are noticeable low compare with α-tocopherol reported in *C. calcitrans* 72 mg g^{-1} biomass [15], but considerably high compared with 0.013 mg g^{-1} reported for commercial Spirulina biomass [16]. Durmaz reported 2.3 mg of α-tocopherol g^{-1} biomass in *N. oculata* which is a comparable value obtained in this study for *Nannochloropsis* sp, 1.7 mg of α-tocopherol g^{-1}[17]. δ-Tocopherol was not detected in Nannochloropsis, Spirulina, Synechococcus and Porphirid-ium, whereas δ-Tocopherol was present in Tetraselmis and Chaetoceros, 1.1 and 0.83 mg of δ-tocopherol g^{-1} biomass, respectively. γ-Tocopherol was present in all strains studied with the larger amounts than α-tocopherol, Tetraselmis with 17 mg g^{-1} biomass of γ-tocopherol another Tetraselmis strains have similar amount (data not shown) of γ-tocopherol and Chaetoceros with 10.5 mg g^{-1} biomass, there is not much information about the presence of this tocopherol in microalgae, in *Porphyridium cruentum* amounts of 0.055 and 0.051 mg g^{-1} of α and γ-tocopherols respectively have been reported [18] low amounts of γ-tocopherol was found in lipophilic extracts of *T. suecica* [19]. The content of β-carotene vary from one specie to another (Table 2). *T. suecica*, *S. maxima* and *P. cruentum* with similar amount between 3.93-4.85 mg of β-carotene g^{-1} biomass, without significant differences, *C. mulleri* 3.1 mg of β-carotene g^{-1} biomass with significant differences with respect to *T. suecica*, *S. maxima* and *P. cruentum*, whereas in Synechococcus 1.1 and Nannochloropsis 0.3 mg β−caro-tene g^{-1} biomass were found. The values for β-carotene in this study are lower compared with *N. oculata* 21-28 mg g^{-1} biomass and 15-17 mg g^{-1} biomass obtained in *C. calcitrans* [15] but slightly higher compared to *T. gracilis* [13]. Differences of pigments and tocopherols contents may be due to harvesting time (not mentioned), culture medium may provide stress condition by using seawater and carotenoid content was determined by using a calibration curve [15] instead of using extinction coefficient in the present data.

Table 2. SOD □activity and phycocyanin, α, δ, γ-tocopherols, and β-carotene contents in microalgae

Compound	Microalgae strain					
	Nannochoropsis	Spirulina	Tetraselmis	Chaetoceros	Synechoccocus	Porphiridium
SOD	127 ± 21	30 ± 0.6	20.4 ± 2.7	73 ± 5	157 ± 23	58 ± 1.7
Phycocyanin	nd[§]	31	nd	nd	1.34	5.5
α-tocopherol	1.7	2.5 ± 0.4	1.7 ± 0.2	0.89 ± 0.1	1.4	1.3 ± 0.2
δ-tocopherol	nd	nd	1.1 ± 0.4	0.83 ± 0.1	nd	nd
γ-tocopherol	0.4 ± 0.1	1.4 ± 0.3	17.0 ± 2.0	10.5 ± 0.1	0.85 ± 0.2	3.42 ± 0.5
β-carotene	0.3 ± 0.1	3.9 ± 0.5	4.6 ± 0.3	3.09 ± 0.3	1.1 ± 0.1	4.5 ± 0.9

□ SOD (UA mg^{-1} protein). Phycocyanin, tocopherols and β-carotene (mg g^{-1} biomass)
[§] Not-determined

Many factors affect the amount of pigments, SOD and tocopherols, such as growth conditions, microalgae specie, phase of the growth, extraction method and etc. Optimization data have not been reported, it is necessary to have a full characterisation of each factor to define the best conditions to produce the maximum amount of each compound.

3.3 Determination of the Antioxidant Equivalent Activity (Trolox Equivalent: Tq)

The Figure 1 correlate the activity of ESOD extracts found in the microalgae (Figure 1A) with the trolox antioxidant activity of these extracts (Figure 1B). SOD activity (Figure 1A) correlate well with the antioxidant activity expressed as trolox equivalents (Figure 1B). Trolox activity in *Nannochloropsis* sp. and *Synechococcus* sp. were 25.6 and 26.2 μmol trolox equivalent g^{-1}, respectively, no significant differences were found between them, followed by *C. mulleri* and *P. cruentum* with 20.5 and 21.62 μmol trolox equivalent g^{-1}, respectively, with no significant differences, but with significant differences with respect to *T. suecica* and *S. maxima*. SOD activity and Tq in Nannochloropsis and Synechococcus correlate well, the two activities were the largest obtained in this study.

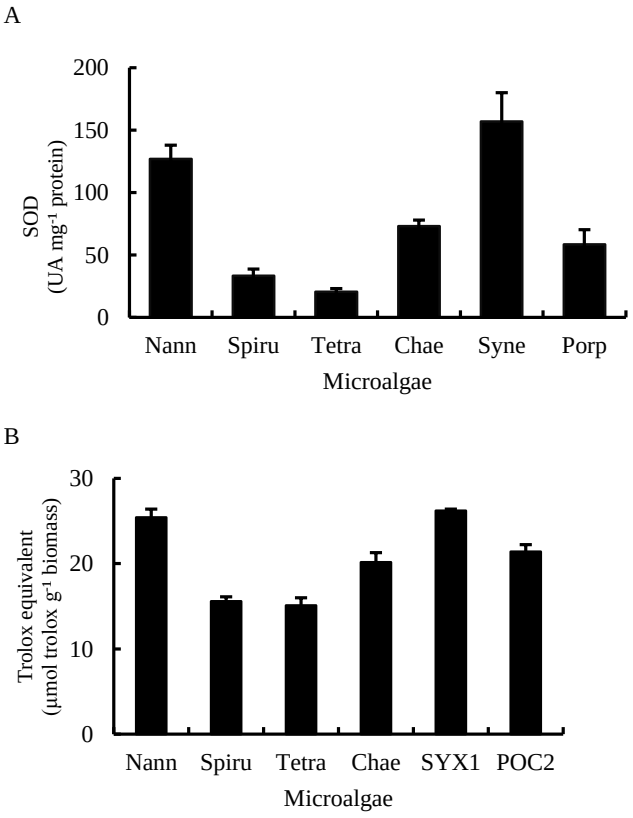

Figure 1. Correlation between SOD and Trolox activity. A) SOD activity in microalgae extracts. B) Trolox equivalent activity of ESOD microalgae extracts

Figure 2 shows the correlation between phycocyanin content (Figure. 2A) and trolox activity (Figure. 2B) for EPC extracts. A value of 148 μmol trolox equivalent g^{-1} for *S. maxima* was obtained with a value of 30 mg phycocyanin g^{-1} biomass, the other two EPC extracts have showed important trolox activity, Synechococcus and *P. cruentum*, 22 and 63 μmol trolox equivalent g^{-1}sample Figure 2B, but at least 6.5 and 2 times lower than *S. maxima*, respectively. Phycocyanin content correlate well with respect to Tq activity. These data clearly demonstrate that phycocyanin content strongly enhances the antioxidant activity. Gantar et al found antioxidant activity on phycocyanin from Limno-thrix and two commercial samples of dry powder of Spiru-lina, a commercial sample has lower antioxidant activity than Limmnothris, the content of phycocyanin obtained in two commercial samples of Spirulina biomass were different. Source of phycocyanin, purification pro-cedure and store condition are important for final product content and properties[20]. In this paper, three strains have produced very clean phycocyanin with antioxidant activity with only one extraction step.

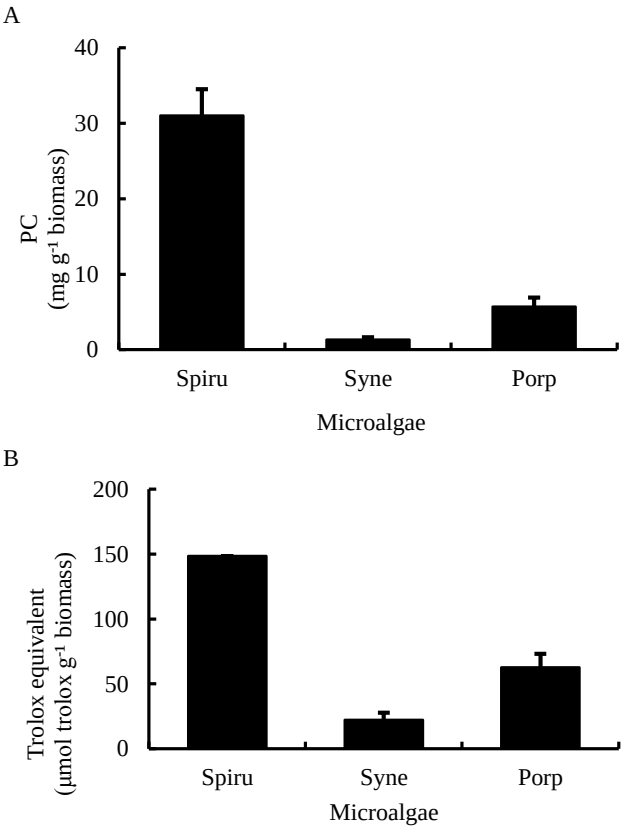

Figure 2. Correlation between Phycocyanin content and Trolox activity. A) Phycocyanin content of three EPC microalgae extracts. B) Trolox equivalent activity of EPC microalgae extracts

Furthermore, the Tq measured in lipophilic extracts have shown an important antioxidant activity, Tq activity depends on the amount of antioxidants present in each extract. The sum of α, δ and γ tocopherols (Σ = α, δ, γ-toco-pherols) in each microalgae in Figure 3A correlated well with the values of Tq obtained in Figure 3B. In *T. suecica* and *C. mulleri* tocopherols were 19.8 and 12.2 mg g^{-1} biomass, whereas trolox activities were 71.7 and 38.8 μmol Tq g^{-1} biomas, respectively (Figure 3A, B). In this Figure, tocopherols seems to be the major components responsible of Tq.

All strain have been found with a different arrangement of antioxidant compounds, hydrophilic components such as SOD and phycocyanin or lipophilic components such as tocopherols and β-carotene.

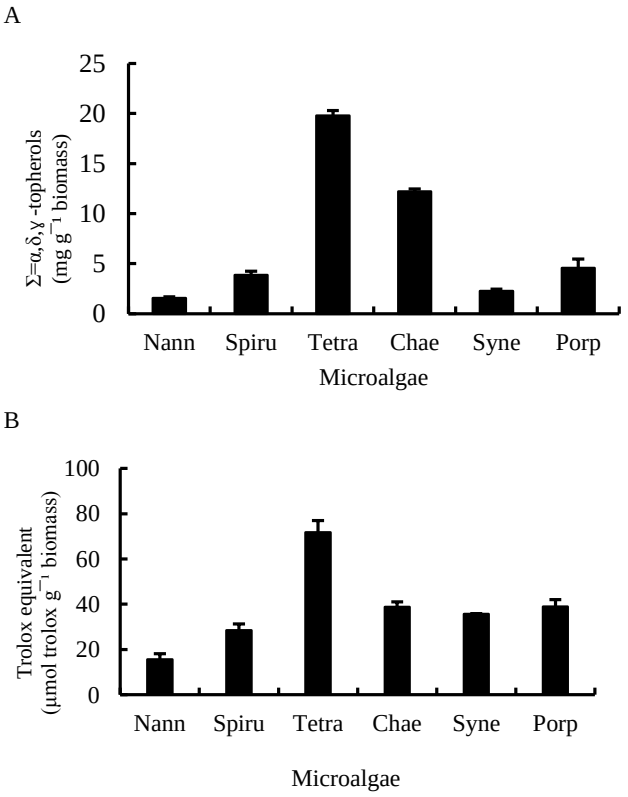

Figure 3. Correlation between tocopherols content and Trolox activity. A) Sum of α, δ, γ- tocopherols in each LE microalgae extracts. B) Trolox equivalent activity of LE microalgae extracts

3.4 Scavenging Activity

For scavenging activity Synechococcus ESOD, Spirulina EPC, and Tetraselmis LE extracts were selected to determine superoxide anion (SASA), hydrogen peroxide (HPSA), and hydroxyl (HAS) radicals scavenging activities comparing them to commercial preparations of CuZn-SOD (0.83 ng ml^{-1}), ascorbic acid (5 mM), and α-tocopherol (2 μg ml^{-1}).

The SASA was carried out by the inhibition of the NBT reduction drive by the PMS-NADH coupling reaction. Synechococcus ESOD and Spirulina EPC extracts showed an SASA inhibition activity of 66% and 67% (Figure. 4A), respectively, slightly lower than those found with CuZn-SOD (78%) and ascorbic acid (79%). The scavenging activity of $O2^{\bullet -}$ (Figure. 4A) observed in ESOD extract of Synechococcus might be supported by its high SOD activity (Figure. 1A), although the presence of other metabolites cannot be excluded due to the polar character of the extract. The high O2•- scavenging activity of phycocyanin extract from Spirulina (Figure. 4A), seems to depends on the presence of phycocyanin (Figure 2A). Potential applications of phycocyanin as natural dye, fluorophore in diagnostics immunoassays and nutraceutical have been mentioned by Kuddus et al [2], but no specific antioxidant activity have been reported until the present study.

The Spirulina EPC produced a 94% of scavenging for HPSA compared with 10% for the commercial α-tocopherol. The HPSA of Spirulina was significantly different to the values obtained for the extracts of Synecho-coccus ESOD (18.19%) and Tetraselmis LE (14.63 %), Figure 4B. Cyclic double bond molecules such as bioflavonoids and DNA are attacked by H_2O_2 [20], phycocyanin can be used as a similar H_2O_2 scavenging mechanism like these compounds due its molecular structure.

The HSA activities observed in Tetraselmis LE (89.54%) was significantly different to Synechococcus ESOD (24.89%), and Spirulina EPC (23.71%) HSA actitvities showed in Figure 4C. The HSA activity observed in the Tetraselmis LE was significantly different compared to those obtained for α-tocopherol (63.72%), Synecho-coccus ESOD (24.89%) and Spirulina EPC (23.71%) extracts, no significant difference between Synechococcus ESOD and Spirulina EPC can be observed. The scavenging activity mediated by tocopherols present in *T. suecica* lipofilic extract is due by the presence of γ-tocopherol which has been found as the main tocopherol component of lipophilic extract.

3.5 Synergic Activity of Phycocyanin

Partially purified phycocyanin (PC) from *Spirulina maxima* was assayed for synergic activity combined with commercial α-tocopherol and ascorbic acid by the thiocyanate method, Table 3. Synergic activity was referred to the half time (min) required to reach the maximum oxidation in the reaction mix. The data reports significant differences between the blank test and the others as follows: blank (55.4 min) ≤ BHT (56.4) ≤ α-tocopherol (80.3) ≤ ascorbic acid (113.0 min) ≤ PC (286.3 min) ≤ BHA (395.3 min) ≤ α-tocopherol + PC (537.4 min) ≤ ascorbic acid + PC (slope was very low to define half time, after 550 min).

Table 3. Synergic effect of purified phycocyanin

Antioxidant	Antioxidant Effect		
	% inhibition	Half time (min)	% synergic effect
Control	0	55	-
BHT	0	56	-
α-tocopherol	31	80	-
ASA	51	113	-
EPC	80	286	-
BHA	86	395	-
α–tocopherol + Phycocyanin	88	537	55
ASA + Phycocyanin	88	>537	55

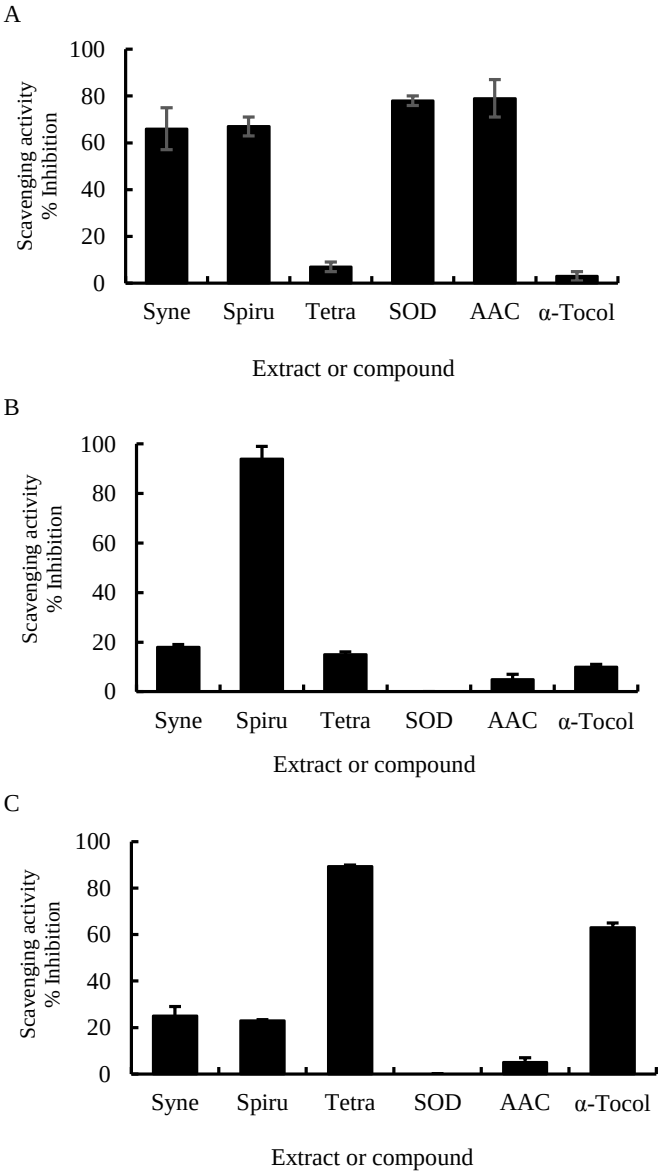

Figure 4. Scavenging activity of microalgae extracts. Synechoccocus-ESOD, Spirulina-EPC and Tetraselmis-LE extracts and commercial SOD, ascorbic acid (AA), and α-tocopherol were used to measuring scavenging activity.

A) Scavenging activity for superoxide anion radical ($O_2^{\bullet-}$).

B) Scavenging activity for hydrogen peroxide (H_2O_2).

C) Scavenging activity for hydroxyl radical ($OH^{\bullet}$)

Several studies have shown synergism between α-tocopherol and ascorbic acid, glutathione, phospholipids, polyphenols, β-carotene, chlorophyll-a and phycocyanin [2-5]. In the present study, the extract from *S. maxima*, which contains phycocyanin with one purification step, has shown important antioxidant and synergic activity (Table 3) in the presence of commercial α-tocopherol and ascorbic acid. Gantar et al. [21], used purified phycocyanin from *Limno-thrix* and *Spirulina* sp commercial powder to evaluate antioxidant activity, a concentration of 0.08 $mgml^{-1}$ of phycocyanin have EC_{50} of antioxidant activity, while in the present study a concentration of 0.015 mg ml^{-1} in the antioxidant reaction, a comparable activity was found with five times less amount of phycocyanin. In the other hand, Gantar et al did not detect any antioxidant activity in the commercial sample of Spirulina, these results often happen because downstream processes may damage phycocyanin content of dry biomass, so data is not shown [21].

4. Conclusion

Microalgae used in this work produced different amounts of SOD, α, δ and γ tocopherols, carotenoids, and phycocyanin under same culture conditions. However, several factors may have influence the antioxidant production, such as growth phase, nutrient availability, light supply and intensity as well as oxygen concentration and stress conditions, but also the recovery of these compounds are affected by the processes of extraction and purification. To identify the factors that have major influence on antioxidant production, experimental procedures should be performed.

Phycocyanin is an excellent antioxidant, scavenge superoxide (O2•-) and hydrogen peroxide H2O2 radicals efficiently works well to produce synergy with α-tocopherol, increasing protection against oxidation. Purified phycocyanin and phycocyanin in one step extraction from Spirulina can be used with good results as antioxidant.

Microalgae has the potential to be considered as a natural antioxidants source, but studies to improve production and extraction are need.

5. Acknowledgements

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6. Conflict of Interest

The authors declare that there is no conflict of interest

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توکوفرول، فیکوسیانین و سوپراکسید دیسموتاز به‌دست آمده از ریزجلبک: به عنوان آنتی‌اکسیدان‌های غذایی

ایونه ساندرا سانتیاگو-مورالس[۱]، لوردس تروجیلو-وال[۲]، فاکاندو جوآکویین مارکز-روشا*[۳]، حنی فابیولا لوپز هرناندز[۴]

۱- انستیتو صنایع، دانشگاه سی، پورتو آنجل، اواکساکا، کد پستی ۷۰۹۰۲

۲- گروه آبزی پروری، مرکز دوم تحقیقات علمی و آموزش عالی انسنادا، کد پستی ۲۲۸۶۰

۳- مرکز تولید تمیز منطقه ای، انستیتو ملی پلی تکنیک، کانداکان، تاباسکو، کد پستی ۸۶۶۹۱

۴- انستیتو فناوری های پیشرفته ریورز، بالکان، کد پستی ۸۶۹۳۰، مکزیک

چکیده

سابقه و هدف: ریزجلبک‌ها به عنوان ریزاندامگان فتوسنتز کننده هستند که در تماس با بسیاری از انواع اکسیژن واکنش دهنده، طیف گسترده‌ای از پادأکسنده‌ها یا آنتی‌اکسیدان‌ها را تولید می‌کنند، تا از شرایط بسیار أکسندگی رشد محافظت کنند. این امر می‌تواند برای بهینه‌سازی تولید پادأکسنده مورد استفاده قرار گیرد. مطالعه حاضر بر تعیین تفاوت پادأکسنده‌های تولید شده در انواع ریزجلبک و توضیح برخی خواص پادأکسندگی فیتوسیانین متمرکز شده است.

مواد و روش‌ها: فعالیت سوپراکسیداز دیسموتاز با استفاده از کیت تجارتی و روش طیف سنجی تعیین شد. فیتوسیانین و کاروتنوییدها به روش طیف‌سنجی و توگوفرول با کروماتوکرافی مایع فشار بالا مجهز به آشکارساز فلورسنس تعیین مقدار شدند. فعالیت هم افزایی پادأکسندگی و حذف با تعیین فعالیت پادأکسندگی معادل تورولوکس با استفاده از معرف ۲، '۲ آزینوبیس [۳-اتیل بنزوتیازولین-۶- سولفونیک]، تعیین شد.

یافته‌ها و نتیجه‌گیری: ریزجلبک مورد استفاده در این مطالعه مقادیر متفاوتی از سوپراکسیددیسموتاز، کاروتنوییدها، α، β و γ توکوفرول و فیکوسیانین تولید می‌کند. فعالیت پادأکسندگی معادل تورولوکس بر اساس میزان ترکیب پادأکسنده تولید شده متفاوت بود. فیکوسیانین سوپراکسید و رادیکال‌های هیدروژن پراکسید را حذف کرد، هم‌افزایی خوبی با α توکوفرول داشت و مولکول‌های زیستی را در برابر أکسندگی محافظت کرد. گونه-ای از ریزجلبک می‌تواند برای تولید پادأکسنده‌های مورد اشاره در بالا مورد استفاده قرار گیرد، اما برای بهبود تولید پادأکسنده لازم است مطالعات دقیق‌تری بر روی مراحل رشد انجام گیرد. ریزجلبک می‌تواند به عنوان منبع بالقوه پادأکسنده طبیعی در نظر گرفته شود.

تعارض منافع: نویسندگان اعلام می کنند که هیچ تعارض منافعی وجود ندارد.

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واژگان کلیدی

- ریزجلبک
- فیکوسیانین
- سوپراکسید دیسموتازف توکوفرول‌ها
- فعالیت هم افزایی

***نویسنده مسئول**

فاکاندو جوآکویین مارکز-روشا،
مرکز تولید تمیز منطقه ای، انستیتو ملی پلی تکنیک، کانداکان، تاباسکو، کد پستی ۸۶۶۹۱

تلفن: ۵۷۲۹۶۰۰۰-۵۲+
دورنگار: ۱۰۴۱۰۲۳-۹۱۴-۵۲+
پست الکترونیک:
fmarquez@ipn.mx