

Molecular characterization and antioxidant potential of Andean Chlorophytes from Ecuador

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ABSTRACT

Microalgae are an important bio resource for the productive matrix within the energy, agricultural and food sectors. In Ecuador, there are several studies on the use of native microalgae for bioremediation, food production and as alternative fuels. However, it is not common to identify this macrophyte at the species level, as well as the determination of nutritional and pharmaceutical properties. Classical identification of microalgae are based on their morphology and, in general, needs experienced taxonomists to achieve specific typifications. Molecular methods allow us to perform species-level identifications based on the amplification of multiple loci, where the sequences obtained can be compared to available public databases. Amplified DNA fragments show the presence of endemic species of nutraceutical interest and, together with chemical extraction processes, could provide reliable information for the use in the agricultural industry. Under this condition, the present research was carried out with endemic species of the genus *Chlorella* sp., belonging to the Cayambe Coca ecological reserve with antioxidant potential and for the use of environmental remediation as functional feed for livestock farms.

INTRODUCTION

The Ecuadorian Andean regions preserve a high diversity of cyanobacteria and microalgae with potential use for medicine, food, bioremediation, biofuels, and other applications (Joao and Rivero, 2012). Ecuadorian irradiation maintains a daily average of 4.72 to 5.46 kWh/m² during the year, where the angle of incidence of sunlight is perpendicular to the earth's surface and it is increasing in height areas (Peralta *et al.*, 2013). This environmental condition determines the specific cellular structures of cyanobacteria and microalgae for the survival and it is increasing the concentrations of antioxidant molecules which act in the active center of molecular complexes (photosystems),

oxygenate photosynthesis and protect the cellular structures against photo-oxidation and photo inhibition (Costa *et al.*, 2013). This condition generates nutraceutical properties which needs a detailed study so that this could be useful for food and pharmaceutical industry.

In Ecuador, there are more than 1500 species of algae identified in different aquatic ecosystems, where 106 are present in lacustrine areas over 3500 meters on sea level (Maldonado *et al.*, 2011, Steinitz-Kannan, 1997). Pachacama *et al.*, (2016) have collected Chlorophyta algae from the Cayambe-Coca reserve in the Andean mountain in Ecuador and tested for their bioremediation capabilities and as a functional food for productive systems (Villafuerte *et al.*, 2016). According to their isolation and morphological characterization, these species were initially identified as *Chlorella* sp. However, since morphology is often incompatible with phylogenetic relationships in coconut algae (Luo *et al.*, 2010), these procedures are inadequate or may generate an error in the specific identification of new isolates.

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With this background, the present research was taken up using biotechnological tools to find the molecular characteristics with precision at the level of genus and species, as well as to generate an approximation of its antioxidant properties for industrial use.

MATERIALS AND METHODS

Samples collection

Green algae strains CN001 and CL001 were collected and purified from water samples from the Cayambe Coca Reserve, specifically from the Loreto lagoon (17M, 818079, 9964895, 3748 m) and De Antojos lagoon (17M, 818039, 9968023, 4093 msnm) respectively. These strains were isolated by conventional methods (Lavenset *et al.*, 1996) and maintained in complete BG 11 culture medium (NaNO₃ (1.5 g/L), MgSO₄ · 7H₂O (7 g/L), CaCl₂ · 2H₂O (3.6 g/L), K₂HPO₄ · 3H₂O (4 g/L), EDTA (0.1 g/L), Na₂CO₃ (2 g/L), H₃BO₃ (2.86 g/L), MnCl₂ · 4H₂O (0.8 g/L), Na₂SO₄ · 4H₂O (0.39 g/L), CuSO₄ · 5H₂O (0.8 g/L), Co(NO₃)₂ · 6H₂O (0.25 g/L), in volumes of 200 mL, constant air flow of 0.79 L/min, temperature 20 °C, light intensity of 78.8 μmol quanta/m² s.

DNA isolation and amplification

Total genomic DNA was extracted from CN001 and CL001 strains using cetyltrimethylammonium bromide (CTAB) described by Doyle (1991), with modifications. The algal samples were filled in 2 ml tubes from 19 days of algal culture, centrifuged at 4000 rpm for 3 minutes, forming a pellet at the tube bottom. After discarding the supernatant, this procedure was repeated once again in the same tube. Four sterile glass beads 2 mm in diameter were added together with 500 μL of extraction buffer [100 mM Tris/HCl, pH 8.0, 20 mM EDTA, 2.5% CTAB, 1.4 M NaCl, 2% 2-mercaptoethanol]. The tubes were shaken at 3150 rpm vortex (VM-300, Gemmy Corp., Taiwan) for 10 min and incubated in a Thermo block (ProvoCell, Esco Technologies, USA) for 30 min at 60 °C and 600 rpm. Two extractions of chloroform are made from the mixture. For each extraction, 500 μL of chloroform were added to the tubes, samples were homogenized by shaking for 1 min and centrifuged for 5 minutes at 14500 rpm. The supernatant from each extraction was recovered in different tubes, an equal volume of 2-propanol was added to each tube, along with 150 μL of 3 M sodium acetate and 300 μL of 70% ethanol. Samples were brought to -20 °C for 2 h, then centrifuged for 10 min at 14500 rpm. The supernatant was discarded and the tubes containing DNA pellets were allowed to dry inside a laminar flow hood for 30 min. The pellets were resuspended in 100 μL of nuclease-free water and 1.5 units of RNase (RNase ONE, Promega, USA) were added. Samples were incubated for 20 min at 37 °C and stored at -20 °C for further use.

The 18S regions and internal transcriptional spacer region (ITS), including ITS1, 5.8S, and ITS2, from ribosomal DNA, were amplified using primer pairs 1F-1528R (Medlin *et al.*, 1988) and ITS1-ITS4 (White *et al.*, 1990). PCR reactions contained 1X GoTaq green Master Mix (Promega, USA), 0.2 μM

of each primer, 1 ng/μL DNA sample, 0.4 μg/μL bovine serum albumin (BSA), and nuclease-free water in a final volume of 25 μL. Cycles for 18S amplification were: 94 °C for 5 minutes, thirty-five cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 90 s, followed by a final extension at 72 °C for 7 min. The cycles for the ITS region were 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 90 s, followed by a final extension at 72 °C for 7 min. Amplification was confirmed by electrophoresis through a GelStar 1% agarose gel (Lonza BioScience, USA). PCR products were sequenced by the dideoxy chain termination method (Macrogen Inc., South Korea).

Sequence analysis

The PCR products were sequenced, and compared to the NCBI nucleotide database, using mega BLAST (Zhang *et al.*, 2000). These sequences, along with sequences from other species of the class Trebouxiophyceae, were used to elaborate a multigene phylogenetic tree of maximum likelihood. In order to determine to which species the Ecuadorian isolates belonged, a multiple alignment was made based on the secondary structure of the ITS2, and the number of compensatory base changes (CBCs) between the most conserved regions of the ITS2 of the study isolates and several Isolated from closely related species.

Antioxidant potential in relation to its phenolic content

Phenolic content extraction was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA), methanol, chloroform, Folin-Ciocalteu, sodium carbonate (E. Merck, Darmstadt, Germany).

Samples Preparation

The strains CN001 and CL001 of chlorophytes were treated in a single exhaustive extraction. For each sample, four solvents of different polarities were used: methanol, 40% methanol, chloroform/methanol (1:1, v/v) and chloroform. 1g of dry sample was placed in test tubes with 2 ml of solvent for 48 h at room temperature (24 °C), homogenized on an orbital shaker for 1 h. Subsequently centrifuged at 4500 rpm for 15 min and the supernatant were recovered for the respective chemical tests.

Total phenol content

The total phenolic content was determined by spectrophotometry using the Folin-Ciocalteu method (Singleton *et al.*, 1999). Gallic acid was used as standard (10-200 mg/mL), 200 μl of a sample where 1.5 mL of Folin-Ciocalteu reagent diluted 1:10 was added. After 4 minutes, 7.5% sodium carbonate solution was added. It was allowed to stand for 30 minutes at room temperature. The formation of a blue color was measured at a wavelength of 765 nm in a spectrophotometer (Genesys 10S). The total mass fraction of phenolic compounds was calculated and expressed as gallic acid GAE/fresh equivalent weight (mg/100g). Four replicates were used for each biomass sample.

described based on morphological characteristics that have been recognized as the product of an independent evolution of different taxonomic groups (Friedl 1997). The multigene phylogenetic tree revealed that the Ecuadorian strains formed a discrete, monophyletic clade with both *Micractinium reisseri* and *Chlorella vulgaris*, forming part of the true *Chlorella* that includes the genus *Micractinium*, *Didymogenes*, *Actinastrum*, *Meyerella* and *Hegewaldia* (Luo *et al.*, 2010). According to the analysis of BCCs the species closest to the Ecuadorian strains, with a CBC of difference, is *Mycorrhaphium pusillum*. This difference implies a high probability that the Ecuadorian strains do not belong to any of the microalgae species currently described (Müller *et al.*, 2007). The phylogenetic tree generated on the basis of the CBCs locates the Ecuadorian strains within true *Chlorella*, clade *Micractinium*.

Antioxidant capacity of Andean microalgae

The total phenolic contents in the two strains analyzed had concentrations of 65.89 to 258.83 mg GAE/g extract. The higher concentration is evident with the solvent methanol. The phenolic contents are directly related to the light intensity and UV incidence in the photosynthetic processes, which produces in the algae, a state of stress and the generation of bioproducts for cellular protection (Copia *et al.*, 2012). On the other hand, the phenolic contents of Andean microalgae at 3500 m above sea level are substantially higher than those detected in other microalgae species, ranging from 3.59 to 60.35 mg GAE/g extract (Li *et al.*, 2007, Gomez *et al.*, 2016).

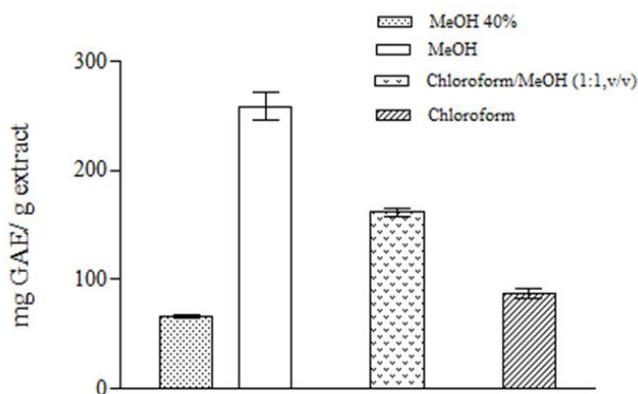


Fig. 2: Total phenolic compounds expressed as gallic acid equivalent (GAE) mg/g fresh algae extract CN001.

The capture of free radicals (AA %) varied from 17.8 to 72.5%. The highest free radical scavenging activity was detected with the 40% methanol solvent represented with a 72.5%. The percentage of antioxidant activity of ascorbic acid used as control was represented by $35.7 \pm 0.05\%$.

Comparative studies with *Chlorella pyrenoidosa* showed a DPPH activity of 39.71% in 2mg/mL where the best solvent was 40% methanol (Chen *et al.*, 2016), on the other hand, *Chlorella vulgaris* and *Spirulina platensis* maintained a DPPH equal to or less than the results obtained in the present study (Wu *et al.*, 2005). The antioxidant activity of the extracts indicates the

presence of compounds that have the ability to interact with free radicals and act as electron donors. Some studies correlate the antioxidant activity of plant extracts with the presence of phenolic compounds (Li *et al.*, 2008). However, other studies show little correlation (Stagos *et al.*, 2012).

The present study clearly indicates the presence of correlation between antioxidant activity and phenolic content in the strain CL and CN001. It should be noted that the results of DPPH indicate the presence of other compounds than phenolics, which contribute significantly to the antioxidant potency of high altitude microalgae.

Table 1: Capture of free radicals (AA%) under different extraction solvents.

Extraction solvent	Percentage of free radical capture (AA%)
MeOH	43.2 ± 0.05
MeOH 40%	72.5 ± 0.07
Chloroform/MeOH(1:1, v/v)	24.7 ± 0.02
Chloroform	17.8 ± 0.19

All data present the mean ± SD for each sample prepared CN001.

CONCLUSIONS

The present research shows the microalga is an endemic species of Ecuadorian Andean, with show a unique identity in height ecotypes. The cellular structure of these species, generate important concentrations of secondary metabolites, many of them could be used by the nutritional and pharmaceutical industry. It is evident that the phenolic content as well as the antioxidant potency making it viable that many of these species could be used as functional food in several industries.

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Conflict of Interests: The authors' declare no conflict of interest.

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