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Simultaneous production of antioxidants and starch from the microalga *Chlorella sorokiniana*

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ABSTRACT

In recent years, microalgae have gained considerable importance as potential source of biofuels and bioplastics. However, these markets are still developing, as the high costs of cultivation ask for exploiting microalgae into new areas and with a biorefinery approach towards a multicomponent cascade extraction process. Here, a sequential processing strategy was used to extract starch with high yield from *Chlorella sorokiniana* under biocompatible conditions. The extract residue was then tested as a potential source of antioxidants. We found a strong protective activity of the extract residue towards oxidative stress *in vitro* on human colon cancer cells and *in vivo* on *Caenorhabditis elegans* nematodes, by inhibiting ROS production and activating DAF-16/FOXO transcription factor pathway. A pool of molecules from three different classes (fatty acids, photosynthetic pigments and carotenoids) was identified as responsible for the antioxidant activity. To our knowledge, this is the first report on the obtainment, from a "waste" fraction, of high value products endowed with antioxidant activity tested in cell-based models and *in vivo*.

1. Introduction

The circular bioeconomy, based on the biorefinery approach, seems to be a good and long-term solution to harmonise the use of natural resources to sustain economic growth, ensure human wellness, reduce the increase of CO_2 concentration in the atmosphere and reduce waste production. Microalgae have been often regarded as a potential candidate for biorefinery as they are an incredible reservoir of compounds endowed with biological activities that could be used in different fields [1].

Microalgal biomass is renewable, as CO_2 and sun light energy are both required for their growth. As no competition exists with food culture for the use of arable land, many recent studies have been focused on the exploitation of various microalgal components in order to obtain the maximum economic benefit, as the conversion of biomass into different products produces minimal waste to the environment. Currently, the research is mainly focused on the use of microalgae as sources of lipids for biodiesel production [2], carbohydrates for methane production *via* anaerobic fermentation [3], proteins for animal nutrition [4] and food additives. Several process alternatives have been proposed to exploit microalgal components in a multi-product biorefinery approach [5]. However, most of these fractions have been tested only as raw fractions, not characterized in their single components to validate them.

In our experimental system, we found that *C. sorokiniana*, requires, for starch accumulation, nitrogen depletion [6]. This stressful condition, in turn, can be exploited to obtain pigments endowed with antioxidant activity. In principle, the cultivation conditions (light intensity and nutrient supply strategy) must be selected to optimize the accumulation of all the main products to be extracted within the biorefinery.

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It is known that microalgae, when exposed to high oxygen and radical stress in their natural environment, develop several efficient protective systems against reactive oxygen species (ROS) and free radicals [7], by producing pigments, such as chlorophylls, carotenoids, xanthophylls and phycobiliproteins [8]. In particular, astaxanthin, β-carotene, zeaxanthin and lutein possess antioxidant activity exceeding that of α-tocopherol [9] and are required from food, cosmetic, nutraceutical and pharmaceutical industries. Thus, the obtainment of an active pigment fraction can significantly influence the economic balance of the whole process (cultivation plus biorefinery).

The main objective of this contribution was to obtain a second high-value class of molecules, such as antioxidants, starting from the discarded fraction after starch extraction [6]. Moreover, we identified the antioxidants obtained and validated their activity in a real applicative environment, *i.e.* both in cell-based models and *in vivo* on a *C. elegans* model, which is widely used in this context [10]. This contribution provides a clear validation of the feasibility of a multiproduct biorefinery applied to *C. sorokiniana* at lab-scale.

2. Materials and methods

2.1. Microalgal strain and culture conditions

Chlorella sorokiniana Shihiraet Krauss strain ACUF 318 (http://www.acuf.net) is a fresh water terrestrial alga. The culture medium used was Bold's Basal Medium (BBM) containing $NaNO_3$ as nitrogen source at concentration of 0.25 g L^{-1}. The growth was carried out in inclined square bubble column photobioreactors, as reported by Olivieri et al. [11]. The culture was continuously irradiated with white fluorescent lamps on the front side of the reactors with a light intensity of 300 μmol m^{-2} s^{-1}. Aeration and mixing was provided by feeding air supplemented with 2% of CO_2. Gas flow rate was set at 0.2 vvm. The temperature was maintained at 25 °C by air-conditioning system. Nitrogen depletion was reached after the regular uptake of $NaNO_3$ for biomass growth and the biomass was harvested in the second day of nitrogen depletion, optimal condition for starch accumulation and productivity [12].

2.2. Pigment extraction

A conventional solvent extraction method was carried out to obtain starch and antioxidants from *C. sorokiniana*. Pure ethanol (99.8%, Sigma-Aldrich) was used as solvent. The extraction protocol follows that reported by Aremu et al. [13] with some modifications. Briefly, the microalgal biomass was dried and ground to 0.150 mm powder. 200 mg of microalgal powder were suspended in 2 mL of ethanol and disrupted in bead beater (2000 g for 3 cycles of 1 min space out with 2 min breaks in ice) equipped with 0.5 mm glass beads. The disrupted biomass was moved to dark flasks and the beads were washed twice with ethanol and the final volume was led to 20 mL. The mixture was shaken for 24 h at 250 rpm in the dark on a magnetic stirrer. Then the mixture was centrifuged at 12000*g* for 10 min. The supernatant was dried by using N_2 stream, and represents the ethanol extract (EE), which was solubilized in 1 mL of DMSO for further analysis on a cell-based model and *in vivo*. The recovered pellet (EP) was dried to recover starch.

2.3. Biochemical composition

Initial microalgal biomass, EE and EP were characterized in terms of lipids, proteins, total sugars, starch and pigments. Lipids were extracted according to the protocol proposed by Breuer et al. [14] followed by gravimetric quantification of the chloroform extract, previously dried at 100 °C for 1 h.

Proteins were assayed by BCA Protein Assay Kit (Thermo Scientific). 10 mg of sample (biomass or dried pellet) was dissolved in 1 mL of lysis buffer (60 mM Tris, 2% SDS) and disrupted in a bead beater (700 g for 3 cycles of 4 min space out with 1 min breaks in ice) equipped with 0.5 mm glass beads. The samples were incubated at 100 °C for 30 min for the extraction of membrane proteins and then centrifuged at 2500*g* for 10 min. Supernatants were analyzed by BCA Kit. Total sugars concentration was measured by spectrophotometric method of Anthrone modified with respect to that described by Chen and Vaidyanathan [15]. In particular, the disruption of the biomass and of the pellet was carried out according to the procedure described for proteins. Starch was measured using Total Starch kit by Megazyme (Wicklow, Ireland) and according the manufacture's protocol with some modifications. The mass of sample was set at 10 mg and the cells disruption was carried out in the bead beater, as previously reported [16]. Pigments were directly measured by spectrophotometric measurements at 663, 646 and 470 nm. chlorophyll *a*, b and carotenoids concentration were calculated according to Wellburn [17].

Analyses were performed in triplicates. Simple sugars are calculated as difference between total sugars concentration and starch concentration. The recovery yields of the biomolecules were calculated according to mass balances. The recovery yield (Y, %) was defined as reported in the following formula:

$$Y = \frac{m_{x\ EP}}{m_{x\ initial}} \cdot 100$$

where $m_{x\ EP}$ is the mass (mg) of the compound x (proteins, lipids, total sugars, starch) in EP and $m_{x\ initial}$ is the mass (mg) of the compound x in the initial biomass.

2.4. Cell culture and cell survival assay

Human epithelial colorectal adenocarcinoma cells (LoVo) and human immortalized keratinocytes (HaCaT) were obtained from ATCC and cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich), supplemented with 10% foetal bovine serum (HyClone), 2 mM L-glutamine and antibiotics, all from Sigma-Aldrich, in a 5% CO_2 humidified atmosphere at 37 °C. Every 3–4 days the culture medium was removed and cells were rinsed with PBS, detached with trypsinEDTA and diluted in fresh complete growth medium for sub-culturing.

For dose-dependent survival assays cells were seeded in 96-well plates (100 μL/well) at a density of 4×10^4 cells *per* cm^2. 24 h after seeding, increasing amount of microalgae extract (from 20 μg mL^{-1} to 2 mg mL^{-1}) were added to the cells for 24–48 h. At the end of treatment, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent and the cells were incubated for 4 h at 37 °C in a humidified 5% CO_2 incubator. At the end of the incubation, medium was removed and the converted dye was solubilized with isopropanol containing 0.01 mol L^{-1} HCl (100 μL per well). Absorbance was measured at a wavelength of 570 nm using an automatic plate reader (Microbeta Wallac 1420, PerkinElmer, Waltham, MA, USA). Cell survival was expressed as percentage of viable cells in the presence of the microalgae extract under test compared with control cells grown in the absence of the extract. The assay was carried out in triplicates for at least 3 times. Control experiments were performed either by growing cells in the absence of the extract or by adding to the cell cultures identical volumes of DMSO. The method used avoids any possibility of a DMSO effect on the final results.

2.5. Oxidative stress

To analyze if EE was able to protect cells from oxidative stress, LoVo cells were plated at a density of 4×10^4 cells *per* cm^2. 24 h after seeding, cells were incubated for different length of time (24 or 48 h) in the presence or absence of EE (200 μg mL^{-1}), and then incubated in the presence of 300 μM SA for 45 min at 37 °C. At the end of treatment, cell viability was assessed by the MTT assay as reported above.

To detect the minimal pre-treatment time, cells were plated as reported above and then incubated for different length of time