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Cultivation of Microalgae *Chlorella* Using Wine Industry by-Products †

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Abstract: An approach of new and sustainable uses for by-products generated in the wine production industry, one of the agro-food sectors of importance, has been studied. Wine lees, a sediment obtained in different processes of decantation of wine, have been used to produce biomass of microalgae enriched in carotenoids as high added value biomolecules. Experiments to incorporate chemical components of wine lees into microalgae biomass to understand the effect of these residues on the growth and biosynthesis of carotenoids into commercial microalgae *Chlorella sorokiniana* have been done. Algal culture system has been optimized and preparation of culture media have been obtained by extracting in water the soluble nutrients contained in the lees at different concentrations between 5% and 50% *w*/*v*. Optimal growth was obtained using extraction of wine residues at 5% and 10% *w*/*v*. At 10% oxidative stress, measured as carotenoids production (specially lutein) and antioxidant activity (DPPH method), was more intense than the obtained using residues at 5%. Our results show that growth in culture media prepared with wine lees extracts stimulated the antioxidant activity and the production of carotenoids in *C. sorokiniana* cells. Preliminary information, not only to produce sustainable growth media for biomass of microalgae enriched in high value molecules, but also to reuse nutrients contained in wine industry by-products what is of particular interest in the context of a circular economy is provided.

Keywords: *C. sorokiniana*; wine industry by-products; microalgae biomass; carotenoids

1. Introduction

Valorization of agroindustrial by-products in biotechnology research is one of the most important challenges of circular economy. In the case of wine production industry, it has become important to find new uses for the large amounts of residues generated each year which represents an environmental issue [1]. As by-products, wine lees are considered a source of bioactive compounds with antioxidant activity due to phenolic compounds and could be valorized and recycled to avoid environmental pollution [2].

Microalgae are able to assimilate organic compounds for biomass production and previous studies have described supplementation of algae culture media with agricultural waste such as dairy manure, wastewater from fruit, vegetable or cultivated plant processing [3]. However, the incorporation of wine lees, a sediment obtained in different processes of decantation of wine, has been scarcely explored to obtain microalgae biomass. The aim of the present study was focused on

the development of new strategies to use agro-food industry by-products from wine production, in order to produce biomass of microalgae *C. sorokiniana* enriched in bioactive molecules, as carotenoids.

2. Materials and Methods

2.1. Microorganism and Culture Conditions

Microalgae *C. sorokiniana* was obtained from the UTEX culture collection. It was maintained in modified M-8 medium [4] that was prepared as follows: KH_2PO_4, 0.74 g L^{-1}; $Na_2HPO_4 \times 2H_2O$, 0.26 g L^{-1}; $MgSO_4 \times 7H_2O$, 0.4 g L^{-1}; $CaCl_2 \times 2H_2O$, 0.013 g L^{-1}; KNO_3, 3 g L^{-1}; EDTA ferric sodium salt, 0.116 g L^{-1}; $Na_2EDTA \times 2H_2O$, 0.0372 g L^{-1}; H_3BO_3, 6.18×10^{-5} g L^{-1}; $MnCl_2 \times 4H_2O$, 1.3×10^{-2} g L^{-1}; $ZnSO_4 \times 7H_2O$, 3.20×10^{-3} g L^{-1}; $CuSO_4 \times 5H_2O$, 3.2×10^{-3} g L^{-1}. The pH was adjusted to 6.7–6.8. Cultures were grown at 25 °C, bubbled with air containing 2.5% (*v*/*v*) CO_2, as unique carbon source, and continuously illuminated with fluorescent lamps with 100 μmol of photons/m^2/s of white light at the surface of the flask. The irradiance was measured with a photoradiometer Delta OHM (mod. HD9021).

2.2. Analytical Determinations

Pigments were measured spectrophotometrically using aliquots (1 mL) of the cultures, whose cells were spun down after 5 min at 4400 rpm at 4 °C. To the obtained pellet, 4 mL of pure methanol was added and the resulted suspension incubated first at 60 °C for 30 min and after at 0 °C for 10 min, and centrifuged for 5 min at 4400 rpm. Chlorophyll and total carotenoid concentration were determined in the supernatant, using the equations proposed by Wellburn [5].

2.3. Pigment and Antioxidant Activity Analysis

Pigments were extracted with methanol and measured spectrophotometrically as described in [6]. Specific carotenoids—lutein and β-carotene—were separated by HPLC equipped with a diode-array detector (L-7420, TermoQuest, San Jose, CA, USA) and a RP18 column (LichroCart RP18, Merck, Kenilworth, NJ, USA), 5 μm, size 250 × 4 mm. In the mobile phase, solvent A was ethyl acetate and solvent B was acetonitrile and water (9:1, *v*/*v*) (mobile phase flow rate was 1 mL per minute). Carotenoids detection was at 450 nm and the carotenoids were quantified using lutein and β-carotene standards supplied by DHI-Water and Environment (Hørsholm). Antioxidant activity measured as DPPH was determined as described by [7].

2.4. Dry Weight Measurements

To measure the dry weight, 5 mL of each culture was taken and the liquid medium was removed by means of a vacuum pump using a cellulose acetate filter (weighed before using it) with 0.45 μm pore size from Sartorius (Goettingen) to separate the cells from the medium. The cells were then washed with demineralized water before drying the filters. The filters with the cells were dried and stoved at 100 °C. The filters with the dried cells were weighed after 24 h.

2.5. Maximum Photosystem II Quantum Yield

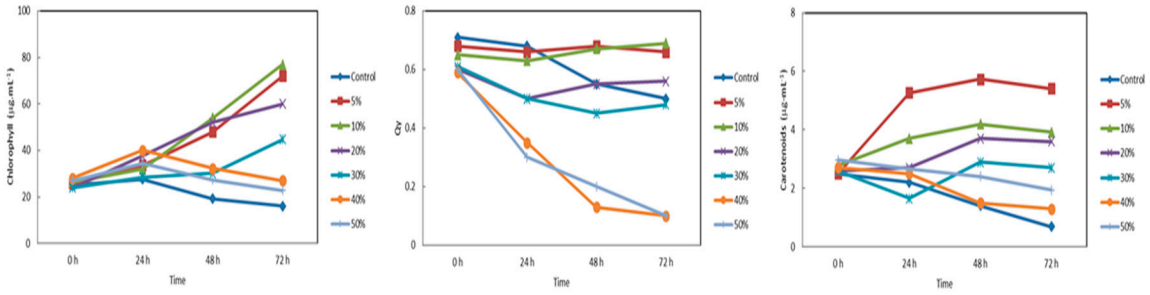
The photosynthetic efficiency was evaluated by measuring the chlorophyll fluorescence in dark-acclimated cells, considered as the maximum photosynthetic efficiency of photosystem II. This parameter was determined using a portable pulse amplitude modulated fluorometer (AquaPen-C AP-C 110, Photo Systems Instruments, Drasov, Czech Republic), according to the method described by [8].

3. Results and Discussion

Cultivation procedure of the microalgae *C. sorokiniana* using by-products from the wine industry.

First step to obtain stable growth of the microalgae *C. sorokiniana* was to optimize culture media prepared with soluble nutrients (MSN) extracted from lees as wine residues. For this, lees were extracted with distilled water at room temperature, using them at different concentrations between 5 and 50% *w*/*v*.

Cell cultures of the microalgae were incubated with media obtained by extraction of wine residues at 0% (control culture), 5, 10, 20, 30, 40 and 50% *w*/*v*. The cultures were grown at 25 °C, bubbled with air containing 5% (*v*/*v*) CO_2 and continuously illuminated with fluorescent lamps. Throughout 4 days (96 h) of experiment, samples of the different cultures were taken to determine the evolution of specific growth parameters as chlorophyll, total carotenoid content and quantum yield (QY) as an indicator of cell viability and status of the photosystem II. Results are shown in Figure 1A–C. As can be seen in Figure 1A, the highest values in chlorophyll concentration were obtained after 48 h with MSN cultures prepared with 5, 10 and 20% *w*/*v* of lees. Maximum values of QY were determined with 5 and 10% MSN indicating an optimal growth of cells in both concentrations. A loss of cell viability was observed in cultures of 40 and 50% *w*/*v* indicating a toxic effect of wine by-products in *C. sorokiniana* cells. Total carotenoid profile (Figure 1C) shows a higher production, and a stronger antioxidant response, with both 5 and 10% MSN cultures.



(**A**) (**B**) (**C**)

Figure 1. Evolution of chlorophyll, quantum yield and total carotenoid content in *C. sorokiniana* cultures prepared with MSN at different concentrations. Cell cultures of the microalgae were incubated with media obtained by extraction of wine residues (MSN) at 5, 10, 20, 30, 40 and 50% *w*/*v*. Both cultures were inoculated with 25 µg Chl/mL, with 100 µmol of photons/m^2/s of white light, incubated at a temperature of 25 °C and were fluidized with air enriched in CO_2 (5% *v*/*v*). Chlorophyll content (µg mL^{-1}) (**A**); quantum yield (QY) (**B**) and total carotenoid content (µg mL^{-1}) (**C**) were calculated throughout the experiment as indicated in Materials and Methods section.

To evaluate the antioxidant response of cultures due to residues from the wine industry, specific carotenoids were identified and quantified from cultures under standard conditions. First, a typical chromatogram obtained from methanol extracts of *C. sorokiniana* is shown in Figure 2. HPLC analysis of the accumulated carotenoids showed that lutein plays a significant role in the antioxidant response of the microalgae as it appears as the main carotenoid in terms of quantity. Other carotenoids as β-carotene, neoxanthin, violaxanthin and zeaxanthin are involved in the antioxidant response by scavenging reactive oxygen species and light excess dissipation [9]. Peak identification was done using commercial standards.

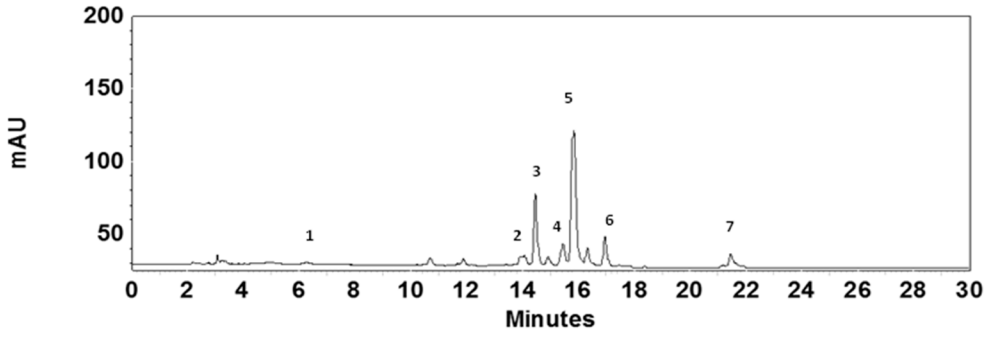
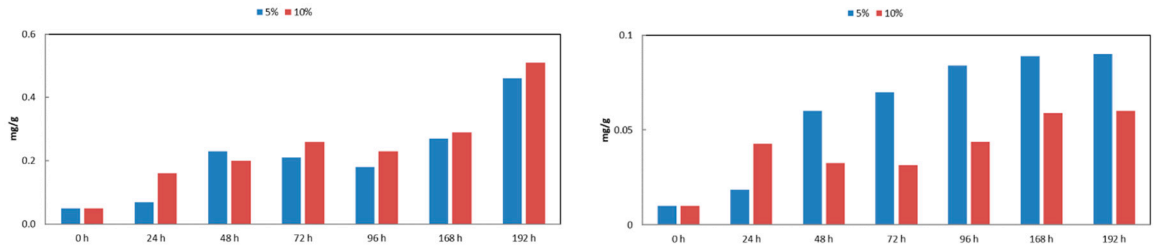


Figure 2. HPLC analysis of carotenoids from *C. sorokiniana* extracts. Peak assignment is as follows: (1) Neoxanthin, (2) Violaxanthin, (3) Lutein, (4) Zeaxanthin, (5) Chlorophyll b, (6) Chlorophyll a, (7) β-carotene. The separation and identification procedure is described in Materials and Methods section.

To evaluate if the antioxidant response of *C. sorokiniana* cells increased using MSN media, a growth experiment was performed with cultures prepared at 5 and 10% *w/v* where maximum values of chlorophyll, QY and total carotenoid were determined (Figure 1). Throughout 8 days (192 h) of experiment, samples to determine carotenoids lutein and β-carotene by HPLC were taken and content [mg/g dw] in cultures prepared with MSN at 5 and 10% are shown in Figure 3.



(**A**) (**B**)

Figure 3. Carotenoid content of *C. sorokiniana* cultures prepared with MSN at different concentrations. Microalgae cultures were incubated with media obtained by extraction of wine residues (MSN) at 5% and 10%. Both cultures were inoculated with 25 µg Chl/mL, with 100 µmol of photons/m²/s of white light, incubated at a temperature of 25 °C and were fluidized with air enriched in CO_2 (5% *v/v*). Content of lutein (**A**) and β-carotene (**B**), expressed in mg/g, were calculated by HPLC throughout the experiment (192 h) as indicated in Materials and Methods section.

In media obtained by extraction of wine residues (MSN) at 5% and 10% *w/v*, the synthesis of all carotenoids of *C. sorokiniana* was stimulated, except for zeaxanthin in which after the first 48 h, there was a decrease in both cultures at 5 and 10% (data not shown). In the case of lutein, a higher production was observed for cells grown in MSN cultures at 10% *w/v*, although this microalga is not one of the major producers of this carotenoid of commercial interest. For neoxanthin and violaxanthin (data not shown), an increase in their synthesis was also observed at the end of the experiment using MSN cultures of 10% *w/v*. For β-carotene the highest increase appeared in media prepared at 5% *w/v*.

The antioxidant activity of *C. sorokiniana* grown in 5 and 10% MSN cultures was also determined, using 1,1-diphenyl-2-picrylhydrazyl (DPPH) that measures the ability of antioxidants to scavenge a radical [7]. Results at the end of the experiment (192 h) are shown in Figure 4. As can be seen, highest antioxidant activity was reached using MSN cultures at 10% *w/v* what it could be the cause of the increase in carotenoid content (specially in lutein) observed in Figure 3A [10].

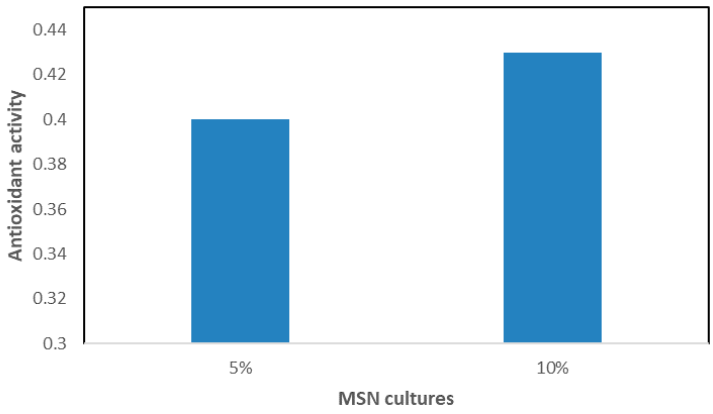


Figure 4. Antioxidant activity (DPPH) of *C. sorokiniana* cultures prepared with MSN at different concentrations. Microalgae cultures were incubated with media obtained by extraction of wine residues (MSN) at 5 and 10% *w/v*. Both cultures were inoculated with 25 µg Chl/mL, with 100 µmol of photons/m²/s of white light, incubated at a temperature of 25 °C and were fluidized with air enriched in CO_2 (5% *v/v*). Antioxidant activity expressed as (µmol DPPH min^{-1} mL^{-1} 10^2), was calculated at the end of the experiment (192 h) as indicated in Materials and Methods section.

4. Conclusions

1. Culture media to grow microalgae *C. sorokiniana* using by-products from the wine industry has been optimized, extracting with distilled water at room temperature the soluble nutrients contained in wine lees using them at different concentrations *w/v*.
2. Stable cultures of the microalgae *C. sorokiniana* have been obtained using culture media prepared with soluble nutrients (MSN) extracted from lees as wine residues at concentrations from 5 to 30% *w/v*. Optimal growth was reached with MSN cultures prepared at both 5 and 10% *w/v*.
3. At 10% oxidative stress, measured as carotenoids production (specially lutein) and antioxidant activity (DPPH method), was more intense than the obtained using residues at 5%. Our results show that growth in culture media prepared with wine lees extracts stimulated the antioxidant activity and the production of carotenoids in *C. sorokiniana* cells.
4. A first approach in the search for new and sustainable uses of wine industry by-products in the context of a circular economy is presented as these residues could be used to obtain carotenoid-enriched microalgae biomass.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

MSN	Medium prepared with Soluble Nutrients of wine by-products
w/v	weight/volume
DPPH	1,1-diphenyl-2-picrylhydrazyl

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