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Pressurized liquid extraction of *Neochloris oleoabundans* for the recovery of bioactive carotenoids with anti-proliferative activity against human colon cancer cells

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ABSTRACT

In recent years, the green microalgae *Neochloris oleoabundans* have demonstrated to be an interesting natural source of carotenoids that could be used as potential food additive. In this work, different *N. oleoabundans* extracts obtained by pressurized liquid extraction (PLE) have been analyzed in depth to evaluate the influence of different culture conditions (effect of nitrogen, light intensity or carbon supplied) not only on the total carotenoid content but also on the carotenoid composition produced by these microalgae. Regardless of the cultivation conditions, lutein and carotenoid monoesters were the most abundant carotenoids representing more than 60% of the total content in all extracts. Afterwards, the effect of the different *N. oleoabundans* extracts and the dose-effect of the most potent algae extracts (namely, N9, PS and CO_2 (−)) on the proliferation of human colon cancer cells lines (HT-29 and SW480) and a cell line established from a primary colon cancer cell culture (HGUE-C-1) were evaluated by an MTT assay whereas a stepwise multiple regression analysis was performed to get additional evidences on the relationship between carotenoid content and the antiproliferative activity. Results revealed that, as a general trend, those extracts with high total carotenoid content showed comparably antiproliferative activity being possible to establish a high correlation between the cell proliferation values and the carotenoid constituents. Monoesters showed the highest contribution to cell proliferation inhibition whereas lutein and violaxanthin showed negative correlation and diesters and zeaxanthin showed a positive significant contribution to cell proliferation.

1. Introduction

Nowadays, cancer constitutes one of the leading causes of death. Cancer research has been mainly focused on the search for curative treatments, and few studies have aimed to develop preventive strategies that can be useful in the long period of carcinogenesis of the tumors. This period is suitable for employing chemopreventive strategies based on the use of natural, synthetic or biological substances to reduce the risk of developing a cancer (Talero et al., 2015). In this regard, the exploration of compounds from natural sources with health effects with the aim of developing a new functional food or nutraceutical is an intense field of research.

Marine environment, which contains a vast array of organisms (bacteria, cyanobacteria, fungi, algae, microalgae or small invertebrates with unique biological properties), represents a huge and underutilized natural source to isolate bioactive compounds to be used in the food and pharmaceutical industries. Among all the marine sources, microalgae have raised an enormous interest. These microorganisms may be used as natural bioreactors since depending on the cultivation conditions they are able to stimulate the synthesis of compounds with health effects, including carotenoids (Herrero, Mendiola, Castro-Puyana, & Ibáñez, 2012; Ibáñez & Cifuentes, 2013). In fact, contents up to 10% carotenoids (w/w) have been described in some microalgae species and may be of high interest as source of pure carotenoids (Guedes, Amaro, & Malcata, 2011). The beneficial effects of carotenoids as antioxidant

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compounds are well documented. Moreover, they have been also associated with relevant bioactivities such as lower risk of coronary heart diseases and prevention of cancer. Several studies have demonstrated the antiproliferative activity of carotenoids, such as violaxanthin, zeaxanthin, lutein or fucoxanthin, isolated from microalgae against different cancer cells (Cha, Koo, & Lee, 2008; Christaki, Bonos, Giannenas, & Florou-Paneri, 2013; Guedes et al., 2011; Moghadamtousi et al., 2014; Pasquet et al., 2011). A key point to carry out these analyses is obviously the extraction of carotenoids from the microalgae. From the viewpoint of the extraction processes, the new challenges involve the development of green and sustainable processes which enable a fast, selective and efficient extraction and isolation of bioactive compounds from natural matrices (Barba, Grimi, & Vorobiev, 2015; Herrero & Ibáñez, 2015; Roselló-Soto et al., 2015). In this regard, advanced extraction techniques based on the use of compressed fluids (Supercritical Fluid Extraction (SFE), pressurized liquid extraction (PLE)) (Herrero, Castro-Puyana, Mendiola, & Ibañez, 2013) and the employment of new strategies based on ultrasound-assisted extraction (Parniakov, Apicella, et al., 2015) and pulsed electric field assisted extraction (Parniakov, Barba, et al., 2015a; Parniakov, Barba, et al., 2015b) are among the most promising techniques. These techniques are considered alternative processes to extract nutritionally valuable compounds from microalgae while complying with green chemistry principles and sustainability. Among them, PLE (technique based on the extraction at high temperatures (usually above the boiling point of the solvent) and pressures that maintain the solvent in the liquid state during the extraction) has demonstrated being an interesting alternative to extract carotenoids from different microalgae such as *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella vulgaris*, *Spirulina platensis*, etc. (Cha et al., 2010; Denery, Dragull, Tang, & Li, 2004; Herrero, Jaime, Martín-Álvarez, Cifuentes, & Ibáñez, 2006; Jaime et al., 2005; Jaime et al., 2010; Plaza et al., 2012). Recently, PLE has also been successfully applied to the extraction of carotenoids from *Neochloris oleoabundans* (Castro-Puyana et al., 2013). Besides the production of lipids, *N. oleoabundans* has recently demonstrated its potential to accumulate relevant amounts of carotenoids when it is grown under certain conditions so that it can be considered as a novel source of natural carotenoids (Castro-Puyana et al., 2013; Chue et al., 2012; Goiris et al., 2012; Urreta et al., 2014).

Considering that microalgae structure has a marked influence in cell disruption and thus in the degree of extraction of intracellular valuable compounds, extraction conditions should be optimized targeting the compounds of interest. In this sense, it is important to focus not only on the family of compounds of interest, for instance carotenoids, but also on the specific compounds responsible for a described bioactivity. Therefore, it is crucial also to perform an in depth chemical characterization, using potent analytical techniques, of the compounds present in the bioactive extracts and to correlate them with the associated bioactivity.

Therefore, the aim of this work was to study the composition and content of carotenoids in different *N. oleoabundans* extracts obtained by PLE in order to evaluate the differences in the type and amount of carotenoids produced when different culture conditions (effect of nitrogen, light intensity or carbon supplied) are employed. Furthermore, the activity of the different *N. oleoabundans* extracts against two colon cancer cell lines and one primary colon cancer cells was also investigated to establish correlations between not only the carotenoid content but also carotenoid constituents and their antiproliferative activity.

2. Material and methods

2.1. Chemicals and reagents

Ethanol was supplied by Panreac Quimica (Barcelona, Spain). Methanol, methyl *tert*-butyl ether (MTBE), hexane, and acetone were obtained from LabScan (Gliwice, Poland). Sea sand was from VWR (Leuven, Belgium). Standard samples of β-carotene, lutein, chlorophyll *a* (from *Anacystis nidulans* algae), and chlorophyll *b* (from spinach) were obtained from Sigma-Aldrich (Madrid, Spain), whereas astaxanthin monopalmitate and astaxanthin dipalmitate were purchased from CaroteNature GmbH (Lupsingen, Switzerland).

2.2. Samples

Neochloris oleoabundans (UTEX#1185) was obtained from the culture collection of algae at the University of Texas (Austin, TX, USA). Modified Bold's Basal Medium (BBM) was used as medium for cultivation (Andersen, Berges, Harrison, & Watanabe, 2005). Cultures were established in BBM containing 9 mM KNO_3 until cell reached optical density (OD 660 nm) of 5.0. Aliquots of 200 mL were centrifuged (520 × g during 5 min), washed with distilled water once and used as inoculum in three consecutive experiments aimed to study the effect of nitrogen, light and CO_2 in the carotenoid and chlorophyll cellular content.

All the experiments were carried out in a culture chamber at 24 ± 2 °C, under 16-h photoperiod. Cultures were grown in batch mode, using 9-cm wide glass reactors containing a working volume of 1 L and subjected to continuous stirring by bubbling air at a constant flow rate.

To evaluate the effect of nitrogen, cultures were grown in BBM supplemented with KNO_3 (used as nitrogen source) at concentrations of 3, 6 and 9 mM (samples N3, N6 and N9, respectively). Pure CO_2 was supplied automatically to the bubbling air for 30 s every 10 min to maintain the pH below 8 and ensure carbon sufficiency. The incident light intensity on the reactor surface was 400 μmol photons m^{-2} s^{-1} (Philips TLD 58 W).

To determine the effect of light intensity, cultures were grown in BBM supplemented with 3 mM KNO_3 and subjected to two different incident light intensities on the reactor surface: 240 μmol photons m^{-2} s^{-1} 1 (sample LL) and of 400 μmol photons m^{-2} s^{-1} (sample HL). Pure CO_2 was automatically supplied as is described above.

The effect of carbon supply was investigated in cultures grown in BBM supplemented with 3 mM KNO_3 and under an incident light intensity of 400 μmol photons m^{-2} s^{-1} which were cultivated suppressing the CO_2 addition during cultivation (sample CO_2 (−)) or supplementing CO_2 automatically as is described above (sample CO_2 (+)).

In all of the above experiments, cells were harvested by centrifugation (520 × g, 5 min) 6 days after reaching the stationary phase of growth, which was induced by the depletion of the initially supplemented nitrogen, as described in Urreta et al. (Urreta et al., 2014). The obtained algal biomass was pre-frozen at −20 °C, lyophilized and stored under dry and dark conditions until further use.

In addition to those samples described above, other two samples were included in this study. The first of them was obtained from cultures grown in MBB medium at 3 mM KNO_3, using an incident light intensity of 400 μmol photons m^{-2} s^{-1} and with automatic injection of CO_2 (therefore under similar culture conditions than those used for samples N3, HL and CO_2 (+)). In this case, cells were harvested 10 days after reaching the stationary phase of growth instead of after 6 days (sample D10).

The last sample (sample PS) was collected from a culture operated at pilot plant scale which was used to reproduce the culture conditions corresponding to sample N3, therefore grown with 3 mM KNO_3, 400 μmol photons m^{-2} s^{-1} of light intensity and automatic supply of CO_2, until 6-days after reaching the stationary phase of growth. This culture was grown in a 30-cm wide tubular photo bioreactor with a working volume of 40 L.

2.3. Pressurized liquid extraction (PLE)

Before PLE, between 1.2 and 2.5 g of lyophilized algae samples were treated by 3 cycles of cryogenic grinding using a Mixer Mill CryoMill (Retsch, Haan, Germany) to break down the cell wall to obtain the highest extraction yield. Three steps were carried out in each cycle: