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Natural Beta-Carotene: a Microalgae Derivate for Nutraceutical Applications

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Nutraceuticals have gained growing attention during the last year, becoming one of the most relevant areas of investigation. Among the existing natural sources of high value bioactive compounds, microalgae have assumed paramount importance. Indeed, they are a natural source of carotenoids, essential omega-3 fatty acids, proteins, carbohydrates, pigments, vitamins and minerals. Among these components, beta-carotene, a potent antioxidant pigment, is particularly attractive to the nutraceutical and cosmetic sector, being an ingredient in many supplements, functional, cosmetic and pharmaceutical products. Moreover, beta-carotene is one of the forms (precursor) of Vitamin A, which may be used in the manufacture of food supplements according to Directive 2002/46/EC. Even though some cyanobacteria produce beta-carotene (80% of total carotenoids) as the major components followed by zeaxanthin, the commercial biological source of beta-carotene is *Dunaliella salina*. This microalga lives in very saline environment and has the capacity to produce beta-carotene up to 10% of the dry weight. In this work, beta-carotenewas extracted by using supercritical CO_2 under optimized operational conditions, resulting in increased amount of carotenoids (from 3.46% to 18.62%), proteins (from 10.03% to 43.08%), and lipids (from 3.49% to 7.93%). Then, the investigation on the main operational parameters affecting extraction from *Dunaliella salina* and a cost-effectiveness evaluation of the process are presented; the results evidence as 194.3-366.6 kWh/day reflecting a cost of 26.89-52.60euros/day are required for natural based beta-carotene recovery(59.3%) from *Dunaliella salina*.
Keywords: nutraceutical, microalgae, beta-carotene, *Dunaliella salina*, supercritical extraction.

1. Introduction

The term "nutraceutical" was coined in 1989 by Stephen De Felice, founder and chairman of the Foundation for Innovation in Medicine, an American organization located in Cranford, New Jersey and is formed by coupling two words, i.e. "nutrient" (a nourishing food component) and "pharmaceutical" (a medical drug). A nutraceutical is defined as "any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease". At global level, the nutraceutical market amounted to ~170 billion euros in 2016, with a growth forecast that will allow it to reach ~300 billion euros by 2022 with a compound annual growth rate (CAGR) of ~7% from 2016 to 2022 (Globenewswire Report, 2019). The greatest market share is represented by the Asian-Pacific region which constitutes 47% (Inkwoodresearch report, 2019) and the second major player is North America (31%). Europe plays an important role for the nutraceutical market, accounting for 15% globally. Even though the European market is less pronounced than in other countries mainly due to the presence of rigorous legislation, the placing on the market of functional foods and drinks, and food supplements is finely regulated offering a high protection for human health. Interestingly, among the nutraceutical products, carotenoids, mainly referring to

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beta-carotene, astaxanthin, lutein, lycopene, canthaxanthin and others (zeaxanthin, capsanthin, etc.) haveremarkably increased (Molino et al., 2019a; Molino et al., 2019b). Indeed, international carotenoid market gained 1.3 billion euros in 2017 and it is expected to increase up to 1.8 billion euros by 2022, with a CAGR of 5.7% in the next 5 years (Bccresearch report, 2019).Commercially available carotenoids are synthetic or natural-based; the chemically-synthesized forms coverthe majority of the total market (90%);however, the demand is considerably increasing. Beta-carotene (Figure 1) is one of the most required and effective pro-vitamin A, whose use has been allowed according to Directive 2002/46/EC as food supplement (Directive 2002/46/EC) with a market of about 180 million euros in 2010 and expected to become 480 million euros by 2020 (Food and Agriculture Organization of the United Nations, 2010).
Due to its numerous biological functions and the inability for humans to synthesize carotenoids, beta-carotene dietary supply is necessary.

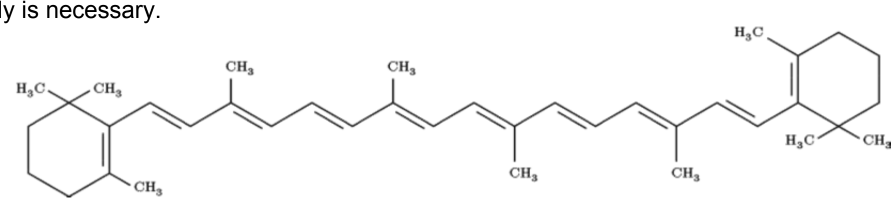

Figure 1. Chemical structure of beta-carotene

Currently, 97-98% of beta-carotene market is covered by the synthetic form, and only the remaining part, equal to 2-3%, is produced from bio-resources, such as fruits and vegetable, algae, fungi, and bacteria.The fruits and vegetables are generally considered as important source of carotenoids, whose type and availability in these vegetal matrix can be predicted by fruit or vegetable color. The yellow-orange vegetables and fruits are generally rich in α- and β-carotene, while lycopene pigment, responsible for bright red color, is the major constituents of tomatoes and tomato products. However, the lutein and β-carotene are the predominant forms of carotenoids in green leafy vegetables, and significant high contents of α-carotene can be found in dark green vegetables, such as green beans, spinach and broccoli (Saini et al., 2015).
Microalgae represent a valuable source for the extraction of carotenoids suitable for the replacement of synthetic ones and to meet the growing demand of such bioactive compounds. In addition, considering that the presence of carotenoids in fish oil finds its origin in the microalgae, the cultivation of these microorganism could be even more favored.
In particular, the microalgae genus *Dunaliella* can accumulate up to 10% beta-carotene of the dry weight (Lv et al., 2016), typically composed of all-trans and 9-cis stereoisomers.
Dunaliella sp. includes eukaryotic photosynthetic organisms, exhibiting the capability to reproduce and growth into a large variety of salty environments, such as oceans and brine lakes. *Dunaliella salina* (Figure 2), in addition to beta-carotene, also produces polyunsaturated fatty acids, such as oleic, palmitic and linolenic acid.

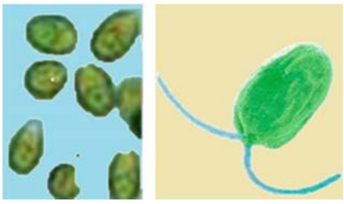

Figure 2. Dunaliella salina microalgae

Beta-carotene is used as an ingredient for multivitamins supplements, as additive in cosmetic formulations, as colouring agent in animal feeds and above all as antioxidant in foods. There are many oncological and epidemiological investigations that evidence as a nutritional regimen rich in carotenoids can reduce the incidence of several degenerative and cancer diseases. The placing on the market of beta-carotene in the European Countries has been already approved as food ingredient by the European Food Safety Authority the responsible agency which assesses the safety of any new food and feed compounds before they are authorized for production and commercialization. European multinationals such as BASF and DSM have recently acquired some of the main global producers of beta-carotene located in the United States and Australia. Natural beta-carotene extracted from *Dunaliella salina* is predominantly commercialized by BASF as Betatene® (40–50 tons per year), NBT in Israel (2–3 tons per year), and the Indian E.I.D Parry (1–3 tons per year) (Hu, 2019;Boussiba, 2012). For beta-carotene production, *Dunaliella* is generally cultivated by means of

a two-stage process.First, microalgaegrow ina culture medium rich in nitrates, phosphates and other minerals to obtain the optimal biomass production. Second, the cells are moved in a larger production pond characterized by high nutrient deficiency. Tafreshi et al. (2006) reported that, in addition to the medium poor of essential components, also a high concentration (2.5 M) of sodium chloride as stress condition, promoted a further increase of beta-carotene in the algal biomass.
In this work, carried out within VALUEMAG project (Horizon 2020-Grant Agreement No 745695) a biorefinery mainly composed of a microalgae cultivation stage, followed by biomass pretreatment and beta-carotene extraction through supercritical CO_2, was investigated and a preliminary economic assessment for the entire process was performed.

2. Materials and Methods

VALUEMAG project is based on the use of a specific reactor (named SOMAC) developed for the algal growth andwhich works continuously. SOMAC reactor, located in Cyprus and essentially consisting of a steel conical structure having 6 m base diameter and 6 m height, was designed, produced and tested by National Technical University of Athens (NTUA) and NOMASICO LTD Company, coordinator and partner of VALUEMAG project, respectively. Specific preliminary transformation led to the introduction of super paramagnetic nanoparticles in the microalgae strain, thus converting the cells into MAGnetic Modified microAlgae (MAGMA). After transformation, a mechanical pump forces water with MAGMA to reach the peak of the reactor and the magnetic areas of SOMAC surface resulted in trapping MAGMAs. The overall system involveda dewatering membrane-based unit which operated for 30 minutes for a day with the aim to increase the microalgae concentration up to 100 g/L for all the volume of microalgae produced in 24hoursinthe cultivation reactor starting from an initial microalgae concentration of 3-5 g/L.More precisely, the dewatering section was composed of a cartridge (39.4 cm length with an external diameter of 10.4 cm) containing microfiltration polysulfone membrane (0.2 μm pore size) which, under the action of an hydraulic pressure generated by a pump activation, performed the separation of water from the algal biomass. For the preliminary economic evaluation of process feasibility, also drying and pretreatments operations were included in the process. Each test was carried out for three consecutive times and the average was calculated and reported in the Result and Discussion section (the relative standard error was less than 5% in all cases). Thus, by supposing that after the growth nutrient content in the recycled water was negligible, the required amount of nutritional compounds for each day of growth was estimated and reported in Table 1.

Table 1: Culture medium composition for the growth of Dunaliella salina microalgae in the VALUEMAG plant

Nutrient	Content (g/day)
NaCl	200-500
KNO_3	150-225
$NaH_2PO_4*H_2O$	1.5-2.25
$MgSO_4*7H_2O$	120-180
KCl	7.50-11.25
$CaCl_2*2H_2O$	3.30-4.95
$NaHCO_3$	84-126
H_3BO_3	0.10-0.150
Na_2*EDTA	10-15(x10^{-3})
$FeCl_3*6H_2O$	10-15(x10^{-3})
$CuSO_4*5H_2O$	5.0-7.5(x10^{-3})
$ZnSO_4*7H_2O$	22-33(x10^{-3})
$MnCl_2*4H_2O$	0.18-0.27
$MnSO_4*7H_2O$	0.25-0.37
$Na_2MoO_4*2H_2O$	5.0-7.5(x10^{-3})
TOTAL NUTRIENTS	**567**÷ 1,050

The supercritical carbon dioxide equipment for the beta-carotene extraction is composed by an extractor, a separator, a condenser sub-cooling device, a recirculation pump and a heating exchanger (the set-up has been described in more detail in the recent work of Molino et al., 2019a).
The recovery of beta-carotene is expressed as Eq(1):

$$\text{Recovery (\%)} = (W_e/W_t)*100 \qquad (1)$$

where $W_e$is the weight of the extracted compound (mg); W_t is the beta-carotene content (mg) in the sample processed by supercritical CO_2 extraction.
On the basis of a recent published work (Molino et al., 2019a), the adjusted conditions for supercritical CO_2 used for the extraction of beta-carotene from *Dunaliella salina* correspond to a 350 bar pressure and 65 °C temperature and a flow rate of 0.31 Kg/min for a complete extraction time of 240 minutes (Table 2).

Table 2: Optimum supercritical CO_2 extraction conditions for Dunaliella salina and characterization of the resulting extract

Supercritical CO_2 extraction: operational conditions		
Dunaliella salina loaded biomass	G	48.3
Bulk density	g/L	86.9
Pressure	Bar	350
Temperature	°C	65
CO_2 flow rate	Kg/min	0.31
Extraction time	Min	240

Costs related to the entire VALUEMAG proposed biorefinery have been calculated by examining the contributes given by the nutrients, biomass harvesting, biomass pretreatment and CO_2 extraction stage, and starting from the energetic demand of each section, assessing the cost of installed power (kW), compressed air (L/s), and energy consumption (kWh/day).
For the growth phase, energy assessment took into account the use of ball valves, pneumatic control valves, a blower and led lamps necessary for the microalgae cultivation; for the pretreatment, the use of a lyophilizer and a ball mill, that assure an optimized biomass disruption and homogenization prior to carry out the beta-carotene extraction, have been included. The use of supercritical CO_2 as extraction solvent needed to consider the energetic and economic contributes of a compression pump, a condenser/sub-cooling system, a heat exchanger, auxiliaries and a potential gas loss.

3. Results and Discussion

The extraction of beta-carotene from *Dunaliella salina* microalgae is an integral part of the VALUEMAG project biorefinery (Figure 3).

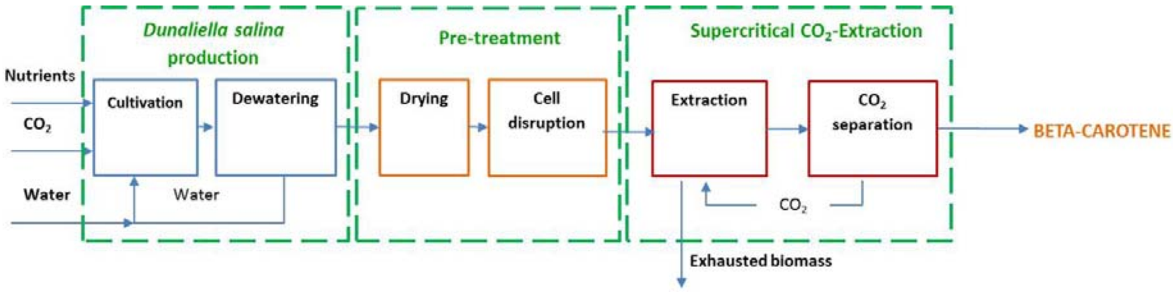

Figure 3. Beta-carotene production through VALUEMAG project approach: Dunaliella salina production followed by biomass pre-treatment and supercritical CO_2 extraction

As illustrated in Figure 3, *Dunaliella salina* is first cultivated in saline water by supplying the correct amount of nutrients, CO_2 and light irradiation. The microalgae growth is followed by a pre-treatment, mainly based on drying and planetary ball mill cell disruption (Molino et al., 2019a) and finally the resulting dried biomass undergoes to supercritical CO_2 treatment with the aim to extract beta-carotene.
The entire process implies a dewatering step after the microalgae cell growth in order to reduce water consumption, improving at the same time the energy consumption and the environmental impact (Marino et al., 2019). Moreover, the CO_2 employed in the extraction stage is recovered and re-used for subsequent extraction steps.
Table 3 reports the comparison between the composition of the initial microalgae biomass and the extract after using supercritical carbon dioxide.

Table 3: Dunaliella salina characterization and extract quality

	Biomass	**Extract**
Ash	48.74%	19.86%
Protein	10.03%	43.08%
Carbohydrates	25.31%	1.66%
Total dietary fibers (TDFs)	8.97%	8.85%
Carotenoids	3.46%	18.62%
Lipids	3.49%	7.93%
of which FAMEs:	90.44%	88.84%
Carotenoids composition:		
β-carotene	98.55%	100%
Lutein	1.42%	<Ldl*
FAMEs Composition:		
SFAs	37.03%	72.77%
MUFAs	68.88%	12.57%
PUFAs	33.46%	14.66%

**low the detection limit.*
FAMEs=fatty acids methyl ester; SFAs=Saturated fatty acids;
MUFAs=Monounsaturated fatty acids; PUFAs=Polyunsaturated fatty acids

The extract obtained in the tests at pilot scale corresponded to 10.46% with a protein content that increased from 10.03% to 43.08% in the extract. At the same time, Table 3 shows that carotenoids content increased up to 18.62%, composed of β-carotene while carbohydrates decrease till 1.66%. The extraction method resulted in a good yield of fatty acid methyl esters (FAMEs), or commonly known as biodiesel, that are renewable and environmental-friendly energy sources, superior to exhaustible fossil fuels in terms of biodegradability,carbon emission, and sulfur content. However, regarding the fatty acids content, the Saturated (SFAs), Monounsaturated (MUFAs),and Polyunsaturated (PUFAs) fatty acids were analyzed: SFAs content increased up to 72.77%, while MUFAs and PUFAs decrease till 12.57% and 14.66%, respectively.*Dunliellasalina* is a microalgae that grow with an high NaCl concentration that produce an ashes content of about 48% while after the extraction process its content achieve value of about 20%.On the basis of the results obtained in Table 2 and Table 3, a preliminary evaluation on the energetic and economic impact on the overall process was performed, as reported in Table 4, showing for each process phases/steps the corresponding energy consumption and average cost. Subtotal for the growth stage is 16.3-21.7 kWh/day and 2.45-3.25 euros/day (Table 4).Examining the contribute deriving from the growth phase, the LED lights account for almost half of the energy consumption, equal to 10-12 kWh/day resulting in 1.50-1.80 euros/day cost.The apportion of the pretreatment step is higher than that of the growth and the nutrient supply phases.

Table 4: Energetic and economic preliminary evaluation for the growth phase of Dunaliellasalina microalgaeand beta-carotene extraction by means supercritical CO_2

Contribute	**Energy consumption range kWh/day**	**Average cost range euros/day euros/kg$_{dry}$**
I.Growth phase	16.3-21.7	2.45-3.25
II. Supplied nutrients	-	0.19-0.85
III. Pretreatment step	31.0-62.0	4.65-9.30
Subtotal I+II+III	47.3-83.7	7.29-13.40
IV. Supercritical CO_2 extraction	130.6-261.3	19.60-39.20
TOTAL I+II+III+IV	**194.3-366.6**	**26.89-52.60**

As highlighted in Table 4, the extraction stage suffered the highest energy consumption and average estimated costs, which exceeded those of the previous subtotal steps (growth phase + supplied nutrients + pretreatment step).Nevertheless, the beneficial effects correlated with the use of supercritical fluids for the extraction of thermolabile microalgal pigments such as beta-carotene promoted its use, since it allowed to preserve the bioactive compound properties without any further additional purification steps at the end of the extraction.

Conclusions

In this work, a preliminary economic assessment on the beta-carotene production from *Dunaliella salina* microalgae as natural source was presented, even though cultivation parameters have been simulated. The investigated process consisted in an algal biorefinery in which, after an optimized cultivation step, a dewatering step for water recovery and recycle was performed. The microalgae growth was followed by biomass drying and cell disruption by means of a planetary ball mill pre-treatment for guaranteeing a better supercritical CO_2 penetration though the *Dunaliella* cells. Beta-carotene (59.3% recovery) was extracted by using supercritical CO_2 under the previous optimized operational conditions (350 bar, 65 °C, 0.31 Kg/min).
The characterization of the extract obtained after using supercritical carbon dioxide revealed as the carotenoids amount increased from the initial 3.46% to 18.62% and similarly also the proteins content passed from 10.03% to 43.08%, while lipids increased from 3.49% to 7.93%.
Energetic and economic evaluation allowed to observe as, among the different steps, the extraction played the major contribute, in both terms of energy consumption and average cost (130.6-261.3 kWh/day and 19.60-39.20 euros/day, respectively). Despite this, the use of supercritical fluids assured the integrity and a high purity of the obtained compounds, thus promoting its use when compared to conventional extraction techniques.

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