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Extraction and milking of astaxanthin from *Haematococcus pluvialis* cultures†

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The microalga *Haematococcus pluvialis* is capable of accumulating natural astaxanthin when subjected to external stress and shifted towards a red-cyst phase, characterized by a robust and multilayered cell wall. In the present study, still-unexplored solvents were applied for extracting astaxanthin from *H. pluvialis*, directly from algae culture and without any pre-treatment of the cells. Among the tested solvents, some of them (*e.g.* ethyl acetate and 2-methyltehydrofuran) gave excellent astaxanthin recovery (>80%) in a short time (30 min) and others (*e.g.* isoamyl acetate, well known as a human-compatible solvent and already in use as a food additive) gave an astaxanthin recovery close to 90% in 1 hour and thus they are exploitable in the natural astaxanthin market. Almond oil is proved to be able to extract astaxanthin and keep *H. pluvialis* alive, without affecting the algal photosynthetic activity, providing the possibility to milk and regeneratively cultivate *H. pluvialis* and avoid an uneconomical loss of biomass.

Introduction

Microalgae are important photosynthetic producers of many bioactive compounds, especially of those that cannot be obtained by non-algal sources.[1] Carotenoids extracted from algae (*e.g.* β-carotene, lutein, astaxanthin, and cantaxanthin) are among the most promising examples in terms of actual or potential industrial applications.[2] The case of astaxanthin is paradigmatic. Astaxanthin is a ketocarotenoid belonging to the class of xanthophylls, produced by a few organisms (*e.g.* microalgae, yeasts) *via* carotenogenesis. Synthetic astaxanthin covers more than 95% of the market (with production costs around 1000 \$ kg^{-1}) but since it has not been approved for direct human consumption in food or supplements, it can be used only as an additive to fish feed for pigmentation purposes.[3,4] Natural astaxanthin, despite today's niche use,[5] has a huge market value (2500–7000 \$ kg^{-1}) and has recently been approved by the U.S. Food and Drug Administration as Generally Recognized As Safe (GRAS) food ingredients.[6] Additionally, natural astaxanthin is obtained as a single stereoisomer (3*S*,3′*S*) and mainly in a mono- or di-esterified form (linked usually to 16 : 0, 18 : 1 and 18 : 2 fatty acids), while the current synthetic pathway provides a mixture of three stereoisomers (3*R*,3′*R*), (3*R*,3′*S*) and (3*S*,3′*S*), in the free form.[7]

The main producer organism of natural astaxanthin is the green microalga *Haematococcus pluvialis*, able to accumulate the highest concentration among all the living organisms (1.5–5% by dry weight). Astaxanthin production in *H. pluvialis* involves two growth stages: (i) a green vegetative phase, cultivated under near-optimal growth conditions of pH, light, temperature and nutrient levels, and in which the carotenoid pattern is composed of lutein (75–80%) and β-carotene (10–20%); (ii) a red-cyst phase induced by specific stress stimuli like light, pH, and nutrient starvation, in which astaxanthin accounts for ~80% of the carotenoid fraction contained in the mature aplanospore.[4] Red-cysts have a robust cell wall composed of a highly resistant outer trilaminar sheath made of algaenans and a very thick secondary wall mainly constituted of mannan polymers that can limit the availability of astaxanthin.[8] Thus, energy-intensive cell disruption approaches like expeller pressing under high pressure and bead milling are required to enhance the recovery of astaxanthin from the cell, but such harsh operations can affect both the quality and quantity of the extracted astaxanthin.[9,10] Various combinations of pre-treatments and sustainable solvent-assisted extractions have been investigated so far to find a good compromise between the astaxanthin extraction efficiency, environmental compatibility, economics, quality and safety of the recovered astaxanthin.[9,11–25] All of these protocols are based on cell wall disruption/permeabilization and

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H. pluvialis cells become unavoidably broken and no more productive at the end of the process. Since most of microalgae are known to have a low productivity, both in terms of raw biomass (slower cell growth rates than the theoretical ones) and product formation (secondary metabolites like astaxanthin are produced only when algae are under stress conditions and the growth is limited), the "algal biomass loss" is one of the major bottlenecks in algae exploitation for obtaining bioactive compounds.[26] This could be an even more relevant economical issue for *H. pluvialis* biomass that has a market value of 30 \$ kg^{-1} and a laborious cultivation.[1] As to environmental aspects, previous studies reported that harvesting and drying of microalgae can play an important role in the overall environmental footprint of the production of functional extracts, due to the consumption of high amounts of energy.[27] Apart from the impact associated with dewatering algal biomass, the extraction of secondary metabolites from algae is further affected by solvent use and removal that can make the overall process impactful: comparative life cycle analysis (LCA) aimed to evaluate the astaxanthin extraction process sustainability is highlighted as solvent-extraction of astaxanthin affects mainly the eutrophication, marine aquatic ecotoxicity and global warming, while abiotic depletion impact can be minimized through the recycling of solvents.[28]

"Milking" secondary metabolites from microalgae is an appealing option for keeping algal cells alive and reusable for a continuous production of high-value compounds, analogous to the milking of cows for a continuous production of milk:[26,29] the use of a suitable hydrophobic "algae-compatible" organic solvent allows a selective and continuous extraction of metabolites from live cells, preserving their integrity and productivity, by-passing the harvesting and dewatering steps (that have a large impact on the overall energy consumption and economics of the process) and biomass loss. Furthermore, due to the short contact time between algal culture and solvent, the milked cells are potentially able to grow again, without the necessity to re-establish a new culture. The milking concept has been successfully applied for the extraction of β-carotene from *Dunaliella salina* with dodecane,[30] bio-hydrocarbons from *Botryococcus braunii* with hexane/heptane,[31,32] and lipophilic substances from *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Acutodesmus obliquus* and *Scenedesmus obliquus* with non-ionic surfactants.[33–35] Milking secondary metabolites from microalgae (*e.g.* hydrocarbons from *B. braunii*) has been demonstrated to have a clear advantage over single extraction regarding the use of resources, above all in terms of nitrogen consumption (10-times reduction under milking conditions) and land foot print (3-times reduction). This holds especially true for algae with a very low growth like *B. braunii*., for which a repetitive extraction could reduce the dependence of process economy on the biomass productivity of slow growing algae and improve biomass utilization by recycling the algae themselves.[36]

To the best of our knowledge, there is only one study about the application of an advanced single crystalline gold nanoscalpel (Au-NS) to incise *H. pluvialis* cells and regeneratively extract astaxanthin in a milking approach.[37] Potentially biocompatible alkanes (from C8 to C16) have been also investigated as solvents for extracting astaxanthin from *H. pluvialis*, but the reuse of red cyst cells for continuous astaxanthin production was not achieved.[38] Being a monoesterified diketo-dihydroxy-carotenoid, natural astaxanthin is a more oxygenated molecule than β-carotene and bio-hydrocarbons,[4] thus more affine to solvents with a medium lipophilicity than to alkanes. This feature, combined with the structural resistance of the red-cyst cell wall makes the extraction of astaxanthin in a milking-mode quite challenging.

The aim of the present study is to investigate the potential of still unexplored solvents for the extraction and/or milking of astaxanthin directly from *H. pluvialis* culture, avoiding any algal cell breaking or pre-treatment, and bypassing dewatering and harvesting steps. The selected candidates are representative of various solvent categories: alcohols (butanol), alkanes (cyclohexane), alkyl carbonates (dimethyl carbonate and diethyl carbonate), esters (ethyl acetate, 3-methylbutyl acetate, and almond oil), ethers (2-methyltetrahydrofuran) and ketones (methyl isobutyl ketone). Each candidate solvent has been evaluated by taking into consideration its extraction efficiency, and its eventual algae compatibility. This last point is crucial for the development of an extraction process in the milking-approach aimed at a regenerative production of algal biomass and astaxanthin; therefore, the viability of *H. pluvialis* cells after each solvent treatment was evaluated in terms of photosynthetic efficiency, to determine the potential application of the selected solvents in a novel milking process.

Experimental section

Chemicals

All the chemicals and standards were purchased from Sigma Aldrich. The tested solvents were butanol (BuOH), cyclohexane, dimethyl carbonate (DMC), diethyl carbonate (DEC), ethyl acetate (EtOAc), 3-methylbutyl acetate (isoamyl acetate), 2-methyltetrahydrofuran (2-MeTHF) and methyl isobutyl ketone (MIBK). Almond oil was cosmetic grade (I Provenzali, Italy).

Algal cultivation

Haematococcus pluvialis (strain HP5) was isolated in July 2014 in a freshwater sample collected in Ravenna (Italy). Algal cultures were set up in triplicate in a 1 L air-insufflated bottle using a modified BBM medium,[39] and grown in the green vegetative phase at a temperature of 21 ± 1 °C, a light intensity of 90–100 μmol of photons per m^2 per s and a 16 h light:8 h dark cycle. After reaching a dry weight of 0.7 g L^{-1} at day 20, cultures were stressed under a high light intensity (450–500 μmol of photons per m^2 per s) and nutrient starvation by 3-times dilution of the algal culture. After 15 days, mature aplanospores (red-cysts) were obtained and cultures were used for the milking experiments.

Astaxanthin analysis

100 mL of red-cyst culture was collected and centrifuged at 2550*g* for 10 min at 4 °C. The supernatant was removed and the algal pellet was freeze dried. The pellet was then extracted with a mixture of cyclohexane/ethanol/acetone (2/1/1, 100 mL) for 24 h at room temperature. The solution was filtered, and the solvent phase was evaporated under vacuum. The carotenoid-enriched extract obtained (46 ± 2 wt%) was suspended in DMSO (5 mL), and an aliquot (0.1 mL) was withdrawn and diluted in DMSO (1 mL), and then measured spectrophotometrically (UV/VIS/NIR, JASCO V-650, Tokyo, Japan) at 530 nm. This wavelength is reported to be one of the most suitable for astaxanthin spectrophotometric determination in *H. pluvialis* crude extract.[40]

The astaxanthin content in the carotenoid extract was determined using a calibration curve prepared with standard astaxanthin in the free form (0.85–21.25 μg mL^{-1}) and found to be 9.7 mmol in 100 g of carotenoid extract (corresponding to 5.8 ± 0.4 wt% of astaxanthin). The astaxanthin content in the algal cells was determined considering the yield of the carotenoid extract (46 ± 2 wt%) and the astaxanthin content in the carotenoid extract (5.8 ± 0.4 wt%) and it resulted to be 4.5 mmol in 100 g of dried *H. pluvialis* biomass (corresponding to 2.7 ± 0.3 wt% of astaxanthin).

Astaxanthin extraction

Red-cyst culture with an algal density of 2.7 g L^{-1} and a measured astaxanthin content of 2.7 ± 0.3 wt% was put in contact with each solvent phase and gently stirred with a magnetic bar at 50–100 rpm for 5, 10 and 30 min. Three algal culture-to-solvent volume ratios were preliminary tested: 3 : 1, 1 : 1, 1 : 3. Isoamyl acetate and cyclohexane were also tested in a wider timeframe (up to 60 min), whereas the performance of almond oil was additionally investigated on a larger scale (30 mL of culture, 10 mL of oil) and for longer times (24 and 48 h).

The extracted astaxanthin amount through each solvent was determined after 5, 10 and 30 min by withdrawing an aliquot of the solvent phase (100 μL), diluting in DMSO (1 mL) and analysing spectrophotometrically at 530 nm, as previously described. Astaxanthin recovery (%) was determined by dividing each extracted astaxanthin amount by the astaxanthin content in the algal cells (2.7 ± 0.3 wt%). Extract amounts after solvent evaporation (apart from almond oil) have been reported in the ESI (Table 1S†).

Photosynthetic activity

H. pluvialis vitality before and after the extraction experiments was evaluated through PAM fluorometry measurements. The kinetics and parameters of Photosystem II (PSII) were measured by means of pulse-amplitude modulated fluorometry. The model used was 101-PAM (H. Walz, Effeltrich, Germany) connected to a PDA-100 data acquisition system, high power LED Lamp Control unit HPL-C and LED-Array-Cone HPL-L470 to supply saturated pulses, US-L655 and 102-FR to provide far red light and measuring light, respectively. Before and after the tests, algal cultures (3 mL) were analysed in cuvettes (10 × 10 mm) mounted on an optical unit ED-101US/M. Measurement of the photosynthetic efficiency was derived from the maximum quantum yield of PSII (Φ_{PSII}),[41] calculated as:

$$\Phi_{PSII} = \frac{F_m \quad F_0}{F_m} = \frac{F_V}{F_m}$$

The minimal fluorescence (F_0) was measured on dark adapted cultures for 20 min, by using modulated light of low intensity (2 μmol m^{-2} s^{-1}). Then, a short saturating pulse of 3000 μmol m^{-2} s^{-1} for 0.8 s induced the maximal fluorescence yield (F_m). Photosynthetic activity (%) was calculated by dividing the maximum quantum yield of PSII (Φ_{PSII}) after the extraction by the maximum quantum yield of PSII (Φ_{PSII}) of the culture before the extraction.

Milking experiments with almond oil and cyclohexane

Milking experiments were performed by using a red-cyst culture with a cell density of 3.1 g L^{-1} and an astaxanthin content of 1.1 ± 0.08 wt% (corresponding to 1.8 mmol in 100 g of dried *H. pluvialis* biomass).

After a first 48 h extraction with almond oil or 1 h with cyclohexane, each red-cyst culture (culture A) was divided into two aliquots:

(1) an aliquot was 3 times diluted with sterilized water and exposed to high light intensity (450–500 μmol of photons per m^2 per s) to keep the red phase. After 26 days from the beginning of the test, cells were concentrated to have a cell density of 3.1 ± 0.1 g L^{-1} (culture B)

(2) an aliquot was 10 times diluted with fresh growth medium and exposed to a low light intensity of 90–100 μmol per photons per m^2 per s^1 to induce the vegetative phase. After 15 days, when algal cells reached the green phase, the culture was 3 times diluted with water and stress under high irradiance (light intensity 450–500 μmol of photons per m^2 per s) to re-induce the red-cyst phase (reached after 11 days). After 26 days from the beginning of the test, cells were concentrated to have a cell density of 3.1 ± 0.1 g L^{-1} (culture C).

Both red-cyst aliquots were subjected to a second milking extraction experiment (almond oil for 48 h and cyclohexane for 1 h).

The recovery of astaxanthin and cell viability after the second milking were determined for almond oil and cyclohexane as described before.

Results and discussion

Solvent selection

The solvents for performing astaxanthin extraction experiments were selected by considering their greenness, bio-based nature and/or safety for humans, to explore potentially better alternatives to hydrocarbons (*e.g.* cyclohexane), known to be "problematic/hazardous" compounds.[42]

• Butanol (BuOH) and ethyl acetate (EtOAc) are granted GRAS by the Flavour and Extract Manufacturers Association (FEMA), classified as "recommended" solvents, and synthesizable from renewable resources (potentially bio-based).[42]

• Dimethyl carbonate, DMC, and diethyl carbonate, DEC, are CO_2-derived solvents available in large amounts and at low prices, with low (eco)toxicity and complete biodegradability. Due to their low vapor pressure, they are considered as promising alternatives to replace VOCs.[43]

• Methyl isobutyl ketone (MIBK), widely used in many industrial applications (like reactions in biphasic systems *e.g.* production of furans from renewable resources), is generally considered as a "recommended" solvent.[42] MIBK is also naturally present in fruits and meats, and it has been approved for use as a component of synthetic flavoring substances (granted GRAS by FEMA) and as a denaturant in alcohol and rum (maximum concentration 4%).[44,45]

• 2-Methyltetrahydrofuran (2-MeTHF) has a potential low environmental footprint because of its renewability (synthesis from furfural or levulinic acid) and low persistence in the environment, but it can also form hazardous peroxides under ambient conditions.[42] Thus, even if it was claimed to be a promising replacement for cyclic-based solvents such as THF because of its better chemical–physical properties (*e.g.* low water miscibility),[46] it is actually considered a "problematic" solvent.[42]

• 3-Methylbutyl acetate (isoamyl acetate) is commercially available as both a natural and synthetic product and used as a solvent, flavoring additive, perfume and fragrance for various consumer products (*e.g.* soaps and detergents, creams, lotions, perfumes, non-alcoholic beverages, candies and chewing gums). Granted GRAS by FEMA.[47]

• Almond oil is widely used in cosmetic formulations as a moisturizer and emollient and for its nutritional benefits because it is rich in mono- and polyunsaturated fatty acids (60 and 30%, respectively, mainly oleic acid) and the antioxidant α-tocopherol (0.02–0.05 wt%). In this case the solvent cannot be separated from the extracted astaxanthin, but since almond oil is considered as one of the best carrier oils in cosmetic preparations,[48] this peculiarity could open the way to its use as an astaxanthin-enriched formulation.

Astaxanthin extraction

All tested solvents were "hydrophobic" enough to form a multiphase system with water; therefore, a direct extraction of astaxanthin from algal culture, without applying any kind of pretreatment (thus using unbroken algal cells) and bypassing dewatering and harvesting steps, was investigated. This extraction mode could suffer from the fact that astaxanthin is included within the (highly resistant) algal cells which are, in turn, surrounded by water; however, it is at the same time highly attractive in terms of the overall economics and energy-demand of the process. Specifically, the extraction performance of the selected solvents (1 mL) has been evaluated as astaxanthin recovery from red phase *H. pluvialis* culture (3 mL) with an algal biomass of 2.7 g L^{-1} and an astaxanthin content

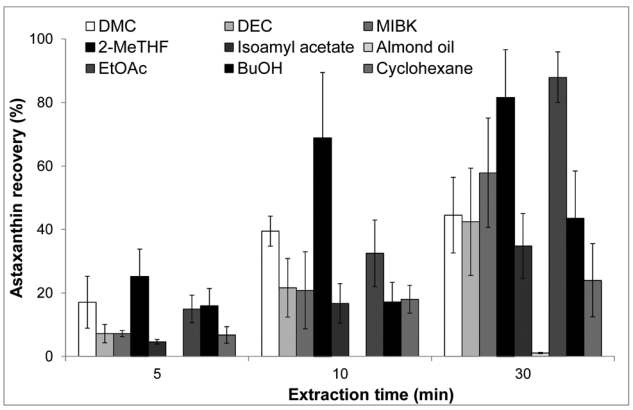

Fig. 1 Astaxanthin recovery (expressed on the basis of the percentage of the astaxanthin content in *H. pluvialis* cells) with the selected sol vents. Data are expressed as mean ± standard deviation of two indepen dent experiments on different algal cultures.

of 2.7 ± 0.3 wt% (Fig. 1). After 5 min of contact between the solvent and algal culture, none of the tested solvents achieved 50% of astaxanthin recovery. In addition, by testing other algal culture-to-solvent volume ratios (1 : 1 and 1 : 3) even lower astaxanthin recoveries were obtained.

A more efficient astaxanthin extraction was achieved for most of the tested solvents prolonging the contact time to 30 min:

• EtOAc and 2-MeTHF were the best solvents, reaching the highest astaxanthin recovery (>80%) after 30 min, with a recovery rate close to 3% min^{-1};

• the performances of DMC, DEC, MIBK and butanol were similar, with the final astaxanthin recoveries ranging between 40–60% after 30 min. The efficiency increased from 2 (for DMC and BuOH) to 8 fold (MIBK) by increasing the extraction time from 5 to 30 min, with recovery rates of 1.4–1.9% min^{-1};

• isoamyl acetate and cyclohexane behaved similarly (astaxanthin recovery of 35 and 24%, respectively), whereas almond oil provided the worst performance (recovery <2% after 30 min). The same results (data not shown) were achieved with corn, soybean, olive and karate oils. Therefore, a deeper investigation by prolonging the extraction timeframe was performed for maximizing the performances of isoamyl acetate, cyclohexane and almond oil (Table 1).

The astaxanthin recovery with isoamyl acetate was improved by almost 2.5-fold increasing the time from 30 to 60 min, reaching a final value close to 90%. This result was particularly relevant because it demonstrates for the first time the potential of this GRAS and green solvent in the extraction of natural compounds.[47]

The recovery achieved with almond oil after 30 min was improved by 8-fold increasing the time to 48 h, reaching a recovery close to 10%. This result is almost 10-times lower than the one reported in the literature by Kang and Sim (recovery of 87% after 48 h with various vegetable oils),[25] reasonably due to the vigorous stirring applied in their case to disrupt the red-cysts and due to a higher oil-to-culture ratio (1 : 1) than the one applied in the present study (1 : 3).

Table 1 Astaxanthin recovery (expressed on the basis of the percentage of the astaxanthin content in *H. pluvialis* red cysts) with isoamyl acetate, almond oil and cyclohexane in a longer timeframe. Data are expressed as mean ± standard deviation of two independent extraction experiments on different algal cultures

	Astaxanthin recovery (%)		
Extraction time (min)	Isoamyl acetate	Almond oil	Cyclohexane
1	0.6 ± 0.07	nd	nd
2	1.4 ± 0.4	nd	nd
3	3.2 ± 1.5	nd	nd
5	4.6 ± 0.7		6.8 ± 2.6
10	16.7 ± 6.2		18.1 ± 4.4
30	34.8 ± 10.2	1.1 ± 0.2	24.2 ± 11.5
60	88.2 ± 2.3	nd	20.6 ± 1.0
24 (h)	nd	5.5 ± 0.4	nd
48 (h)	nd	9.4 ± 2.8	nd

The result achieved with cyclohexane clearly demonstrated that the performance of this solvent did not improve by increasing the time, the astaxanthin recovery being almost constant (close to 20%) after 10, 30 and 60 min.

On comparing all the results achieved here by extracting directly the algal culture with various solvents and those obtained with other conventional/unconventional solvents reported in the literature without any cell pre-treatment, the solvents tested here resulted to be highly promising and more fit for the purpose: recoveries <1% are described for conventional solvents like acetone, ethanol or dodecane.[9,15,20,38] On the other hand, innovative solvents like switchable hydrophilicity solvents (SHS) recover about 40% of astaxanthin from *H. pluvialis* paste in 1 h,[11] but this is due to a drastic break of the cell wall that causes cell lysis.[11,49]

All of the carotenoid-enriched extracts were mainly characterized by bound fatty acids (BFA), as esters of glycerol or esters of astaxanthin (see Fig. 1S in ESI†); no free fatty acids or sterols, typically present in algae samples, were detected by ^{1}H NMR or GC-MS, even upon silylation (see Table 1S in ESI†). The composition of BFA in each extract was similar, with acid oleic c18:1 (35–37%), linoleic acid c18:2 (24–25%) and palmitic acid c16:0 (21–25%) as the main compounds (see Fig. 2S in the ESI†). This corresponds to the main fatty acids found as the monoesters of astaxanthin in *H. pluvialis* reported in the literature.[50]

All the chromatograms obtained by HPLC UV-Vis at 470 nm were similar and predominantly characterized by the peaks of astaxanthin monoesters (with astaxanthin typical maxima at 470 nm but higher retention times); minor signals ascribable to astaxanthin in the free form and canthaxanthin were detected, whereas no β-carotene was found in all the extracts (see Fig. 3S in the ESI†).

Haematococcus pluvialis viability

The evaluation of *H. pluvialis* cell viability after each solvent treatment, crucial to develop a milking-mode astaxanthin extraction, was evaluated as measure of the photosynthetic efficiency (maximum quantum yield of algal photosystem II, Y(II), measured after dark adaptation), after astaxanthin extraction with all tested solvents. The algal culture had an initial maximum quantum yield Y(II) of 0.57. Even if algal cells seemed intact and with different astaxanthin contents (according to the extraction efficiency of each solvent) under a light-microscope (see the ESI†), only MIBK, isoamyl acetate, cyclohexane and almond oil were able to maintain to a certain extent the algal photosynthetic efficiency after contact between the algal culture and the solvent itself (Table 2):

Table 2 Residual photosynthetic activity of *H. pluvialis* cells after each solvent treatment (expressed on the basis of the percentage of the photosynthetic activity of the culture before the extraction)

Solvent	Extraction time (min)	Residual photosynthetic activity (%)
MIBK	5	13
	10	9
	30	
Isoamyl acetate	1	44
	2	41
	3	37
	5	26
	10	4
	30	4
	60	
Almond oil	24 (h)	98
	48 (h)	85
Cyclohexane	5	100
	10	100
	30	74
	60	50

- the photosynthetic activity recorded after 5 and 10 min of extraction with MIBK was close to 10%, and algae was completely inhibited after longer times;
- isoamyl acetate halved the photosynthetic activity in the first 3 min of extraction, reaching a complete inhibition after 10 min, following the general trend "higher algae compatibility-shorter contact time";
- cyclohexane and almond oil were completely algae-compatible, the photosynthetic efficiency (partially or entirely) being maintained even at a prolonged time. This holds especially true for almond oil: after 48 h of contact there was a very limited effect on the vitality of algal cells.

These more "algae-compatible" solvents are actually the ones with the highest log *P*(o/w) values and the lowest water solubility, in line with what was reported in the literature (solvents with log *P*(o/w) > 6 have been found to be completely "algae-compatible").[25] Here, even solvents with log *P*(o/w) between 2–4 (*e.g.* 3.44 for cyclohexane and 2.25 for isoamyl acetate) were partially compatible, probably due to the fact that *H. pluvialis* red-cysts are forms of resistance against environmental stress and therefore quite resistant also to a solvent attack.

Milking experiments with almond oil and cyclohexane

The possibility of re-accumulating astaxanthin after an initial extraction, and therefore developing a real regenerative cultivation of *H. pluvialis* and milking-mode extraction was investigated. The red-cyst cultures after a first extraction with almond

oil and cyclohexane (named cultures A) were divided into two aliquots, B and C:

• culture B remained red under prolonged stress conditions (high irradiance);

• culture C was supplied with fresh nutrients and incubated in an optimal mode until the green vegetative phase was achieved again; then the green cultures were stressed under high irradiance to re-get the red-cyst phase.

After 26 days, a second extraction experiment (1 h for cyclohexane and 48 h for almond oil) was performed on both cultures B and C (Fig. 2).

The results achieved with the two solvent systems are the following (Table 3):

• almond oil: cultures A and B had the same astaxanthin content, meaning that a prolonged stress condition (high irradiance for 26 days) did not increase the astaxanthin productivity. Also culture C had the same astaxanthin content, meaning that *H. pluvialis* did not increase astaxanthin production upon regenerative cultivation. This finding was opposite to what was recently demonstrated by a study in which the *H. pluvialis* cell wall was incised with a gold nano-scalpel, causing a 2-fold over-production of astaxanthin as a response to the stress experienced by the cells.[37] However, despite the same content, a large difference in the recovery of astaxanthin after milking was observed: the recovery from culture C was 2–3 times higher than the recovery from culture B. This result could be tentatively justified as a response to the stress (first extraction) experienced by the cells that could have induced some changes in the mechanical resistance of the cells re-grown under optimal conditions until the vegetative stage (culture C), maybe altering the organization of the layers in the cell wall. The photosynthetic efficiency of both cultures B and C after the second milking was close to 90%.

• cyclohexane: cultures A, B and C had almost the same astaxanthin content but a great difference was observed in terms of astaxanthin recovery and residual photosynthetic activity after milking. The recovery of astaxanthin from culture B was about 20% and the residual photosynthetic efficiency was close to 50%. The recovery of astaxanthin from culture C was lower (13.5 ± 3.4%), as well as its photosynthetic efficiency (24%). An incapacity of algal cells to completely recover their photosynthetic activity after treatment with cyclohexane can be assumed, worsened when *H. pluvialis* cells are switched to the green phase and then re-stressed again to red-cysts (culture C).

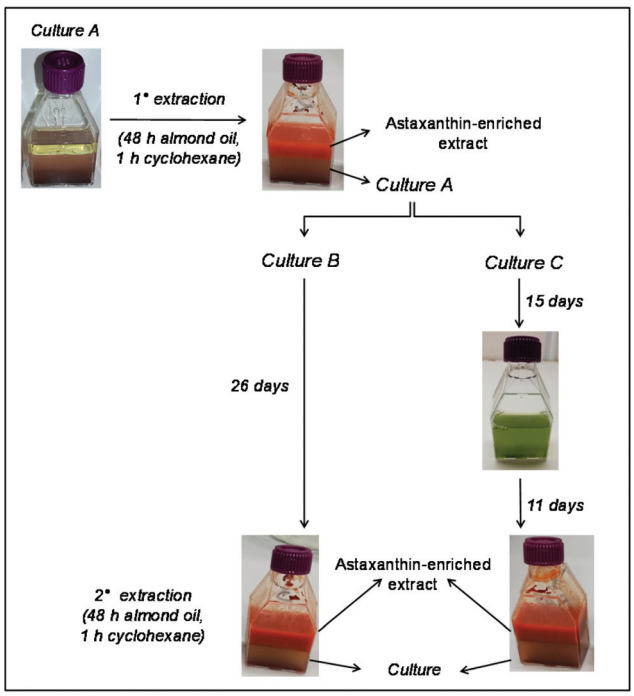

Fig. 2 Regenerative cultivation of *H. pluvialis* and astaxanthin extraction through two extraction processes based on cyclohexane and almond oil in a milking mode.

Table 3 Milking experiments with almond oil and cyclohexane

Solvent	Culture	Astaxanthin recovery (%)	Residual photosynthetic activity (%)
Almond oil	A	11 ± 0.4^{a}	85^{a}
	B	8.4 ± 1.8^{b}	87^{b}
	C	28.7 ± 5.0^{b}	84^{b}
Cyclohexane	A	20.6 ± 1.0^{a}	50^{a}
	B	17.9 ± 2.3^{b}	47^{b}
	C	13.5 ± 3.4^{b}	24^{b}

[a] After the first extraction (48 h for almond oil and 1 h in for cyclo hexane). [b] After the second extraction (48 h for almond oil and 1 h in for cyclohexane).

Conclusions

The solvents here proposed have never been used before for the extraction of astaxanthin from *H. pluvialis*, nor in the "conventional" approach nor in a milking-mode approach. Here, all of them have been applied directly on algal culture, without any thermal, mechanical or chemical pre-treatment, by-passing dewatering and harvesting steps, known to be energy intensive and largely impacting on the overall economics of algal-based process/productions, as demonstrated by a few LCA studies on the entire algal metabolite production process.[27,28] The possibility of applying human-compatible (granted GRAS) and efficient solvents like EtOAc, MIBK, isoamyl acetate and BuOH can provide new opportunities in the natural astaxanthin market, especially for what concerns isoamyl acetate, already in use as a food additive for human consumption.

The results obtained with a milking approach showed that almond oil can be exploited for extracting astaxanthin by keeping *H. pluvialis* alive, without affecting the algal photosynthetic activity. The possibility of increasing the recovery of astaxanthin by both milking and cultivating regeneratively *H. pluvialis* (switching the red-cysts to green cells and then switching-back green cells again to red-cysts) is also feasible with almond oil, avoiding an uneconomical loss of biomass. Cyclohexane can be applied following the same approach, even if its algae-compatibility is lower than that of almond oil. The

use of almond oil in this context is highly promising since it is fully human biocompatible, has proven nutritional benefits (rich in mono- and polyunsaturated fatty acids) and is suitable for applications in the food, nutraceutical and cosmetic industries. Actually, almond oil can be seen as an "atypical" solvent because it is not separable from the extracted compounds, but this can be usefully exploited for the formulation of novel functional foods or "super" cosmetics with an improved oxidation stability and an extended shelf life,[51] avoiding potential hazards from mishandling and simplifying the entire productive process. The replacement of organic solvents with food grade alternatives (vegetable or essential oil) could also lead to a slight reduction of the overall impact even though the yield is lower.[28]

Conflicts of interest

There are no conflicts to declare.

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