

Effect of Lipids and Carbohydrates Extraction on Astaxanthin Stability in *Scenedesmus sp.*

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Elevated costs of biomass downstream processing represent a severe limit to the industrial development of microalgal production systems. Biorefinery solutions allowing simultaneously deriving biofuels and extracting high value compounds must be explored to enhance economic feasibility. In this work, the possibility to extract carbohydrates, lipids and astaxanthin from a strain of *Scenedesmus sp.* is investigated. The analysis is mainly focused on analyzing the effect of consolidated procedures of extraction of carbohydrates and lipids on the degradation and recovery of astaxanthin.

Microalgae were cultivated till achieving stationary phase and maintained in this phase to enhance lipids and astaxanthin accumulation. The fractions of total lipids, carbohydrates and astaxanthin of the produced biomass were 17 %, 33 % and 0.02 % respectively. No statistically significant difference in the astaxanthin content determined following Soxhlet extraction and a more gentle extraction method (Yuan et al. 2002) was found. The effect of transesterification conditions was also evaluated revealing a scarce degradation of astaxanthin in response to the achievement of elevated temperature, NaOH and dissolved oxygen concentrations. Reductions in astaxanthin content were in contrast obtained in response to the addition of H₂SO₄. These reductions were proportional to acid sample concentration. However a regeneration of astaxanthin was obtained by NaOH addition indicating reversibility of the degradation process. In accordance with these results, the possibility to perform biomass saccharification for carbohydrate extraction at progressively lower acid concentrations was investigated.

1. Introduction

Microalgae offer a promising alternative to the production of biofuels and high value chemicals. They can grow and produce new biomass by utilizing cheap and renewable resource as solar light, water, CO₂ and mineral salts (Pagnanelli et al. 2014). However, microalgae based processes aimed at the production of biofuels have not reached economic feasibility yet (Slade and Bauen, 2013). A practicable way to enhance economic feasibility is the development of biorefinery solutions where several products can simultaneously be produced from the cultivated biomass. Microalgae can be employed to produce different biofuels as biodiesel, bioethanol, biogas and biohydrogen (Altimari et al. 2014), and several chemicals as polymers, proteins (for feed and food), carotenoids, essential polyunsaturated fatty acids (DHA, EPA), omega 3, etc. (Mata et al. 2010).

Experimental studies of microalgal downstream processing have been focused on the analysis of processes allowing for the recovery of a prescribed microalgal fraction. Extraction techniques implemented to separate a prescribed fraction can however degrade and thus rule out the possibility to successively recover additional compounds of interest. It is therefore fundamental to analyse the impact of any implemented separation stage on the degradation of other compounds of interest (Vanthoor-Koopmans et al. 2013). In this work, the possibility to extract carbohydrates, lipids and astaxanthin from a strain of *Scenedesmus sp.* is investigated. This strain can achieve elevated content of lipids and carbohydrates under different stressed conditions (as, for example, nitrogen starvation) and store, in the same conditions, interesting astaxanthin content (Qin et al. 2008). Astaxanthin is largely used in aquaculture as pigment for salmon feed. Moreover, it exhibits bioactivity

properties as antioxidant, anticancer and can enhance immune response, and can therefore be employed as supplement for human diet. The high market price between 2000-7000 \$/kg makes the extraction of astaxanthin an important factor to achieve economic sustainability in production of microalgae (Markou and Nerantzis, 2013). Generally not all astaxanthin is in free form but a variable amount is linked to one or two fatty acids by ester bonds. A hydrolytic process is therefore necessary to extract all astaxanthin. However esterified astaxanthin has greater stability and greater bioactivity than non-esterified astaxanthin (Rao et al. 2013). Astaxanthin is a hydrophobic molecule which can be extracted with the same techniques employed for other lipids. However, severe conditions as, for example, high temperature can be achieved in these procedures leading to the degradation of astaxanthin. As a consequence, astaxanthin is generally extracted by specific procedures to minimize its degradation while the effect of procedures employed to extract other compounds as triglycerides or carbohydrates on its stability are generally not evaluated.

In this study, the effect on astaxanthin stability of typical operating conditions imposed in triglyceride and carbohydrate extraction processes for the production of biodiesel and bioethanol is investigated. Extraction methods generally adopted for lipids extraction typically rely on the application of organic solvents because their high efficiency in lipid recovery. The increase of the temperature is a key factor to raise lipid extraction efficiency but astaxanthin stability can be compromised. To evaluate the effect of the exposition to high temperature for long time, the recovery of astaxanthin with a conventional method for lipid extraction (extraction by Soxhlet) was compared with that attained with a gentle method, specific for carotenoids, developed by Yuan et al. (2002). After extraction, transesterification is the consecutive step to produce biodiesel from lipids. The reaction conditions of the transesterification can increase astaxanthin recovery by hydrolysing ester bonds of astaxanthin esters. Transesterification reaction for biodiesel production can be acid, base or enzyme catalysed. The most consolidated transesterification procedure is based on the use of base catalysts because they are cheap and ensure high rate of reaction at low temperature and pressure. However as demonstrated by Yuan and Chen (1999), astaxanthin can be degraded when hydrolysis is carried out at excessive NaOH concentration for long time. In order to evaluate stability of astaxanthin during transesterification in biodiesel production, a method with high temperature, NaOH concentration and exposition to oxygen was compared with a more gentle method described by Yuan and Chen (1999).

To produce bioethanol from microalgae biomass, polysaccharides must be breakdown to easily fermentable sugars (mainly glucose) through a saccharification process. This process is generally catalysed by acid and can be carried out after lipid extraction. However in some wet processes developed in latest years to avoid drying step (very energy-intensive phase) these processes are simultaneous. In order to evaluate the influence of these simultaneous extractions on astaxanthin recovery, its stability after different addition of H₂SO₄ has been also evaluated. Finally the possibility to extract carbohydrates through a saccharification process characterized by lower acid concentration and thus attenuating degradation of other compounds has been investigated.

2. Materials and methods

2.1 Microalgae cultivation

A strain of microalga *Scenedesmus sp.* was selected in Siracusa (Sicily, Italy) and maintained in Petri dishes in BG11 solid medium: 15 g·L⁻¹ agar, 1,000 mg·L⁻¹ NaNO₃, 30.5 mg·L⁻¹ K₂HPO₄, 75 mg·L⁻¹ MgSO₄·7H₂O, 36 mg·L⁻¹ CaCl₂·2H₂O, 20 mg·L⁻¹ CaCO₃, 6 mg·L⁻¹ ammonium Fe(III) citrate, 1 mg·L⁻¹ EDTA, 0.287 mg·L⁻¹ ZnSO₄·2H₂O, 0.161 mg·L⁻¹ MnSO₄·2H₂O, 0.0125 mg·L⁻¹ (NH₄)₆Mo₇O₂₄·2H₂O, 0.061 mg·L⁻¹ H₃BO₃, 0.0025 mg·L⁻¹ CuSO₄·2H₂O.

Microalgae were firstly transferred from the Petri dishes to 1,000 mL roux bottles in BG11 liquid medium and then, they were re-inoculated until to reach 6,000 mL final volume in column bioreactors (d = 10 cm) through a 1 to 10 dilution. In this latter dilution the medium was prepared adding nutrient (equal concentration to BG11) to tap water. The columns were supplied with 1.7 L·min⁻¹ of air and maintained under magnetic agitation and constant illumination (80 ± 10 µE m⁻² s⁻¹) at room temperature (298-303 K). The experiment was carried out in duplicate.

2.2 Determination of biomass concentration

Biomass concentration was determined filtering 10 mL of solution through an acetate cellulose membrane filter of 0.45 µm pore size. After drying filter at 378 K biomass concentration was gravimetrically determined. Determination were carried out in duplicate.

2.3 Total lipids extraction (TLE) and biodiesel production

At the end of the cultivation microalgae were centrifuged at 3,000 rpm and washed twice with distilled water. Then they were put in a mortar and pre-treated by hand milling in order to obtain a homogeneous granulometry. Soxhlet extractor was employed to determine total lipid content in microalgae. Extractions were

carried out by putting 200 mg of biomass in the chamber of a 100 mL Soxhlet extractor loaded with 90 mL of chloroform and 40 mL of methanol. Extractions were carried on for 7 hours and then total lipids were gravimetrically determined after solvent evaporation through rotary evaporator (IKA RV 10 Digital). The triglycerides were then transesterificated to methyl-esters (biodiesel) by adding 50 mL of 1.5 % NaOH solution in methanol for two hours at boiling temperature under magnetic agitation. This latter treatment lead to the hydrolysis of astaxanthin esters and from now on it will be denoted as HTL. After hydrolysis 100 μ L of H₂SO₄ (96%) were added to 5 ml of the sample (AHTL) and then neutralized by addition of 20 μ L NaOH 50% w/w (AHTL + base).

2.4 Carbohydrates extraction

For carbohydrates extractions two different procedures denominated A and B were followed. In procedure A, biomass was treated by a two steps process according to M1 method reported by Moxley and Zhang (2007) for ligno-cellulosic biomasses. In the first step 100 mg of dried biomass was treated with 1 mL of H₂SO₄ solution (72 % w·w⁻¹) at 303 K for 1 h and in the second step 28 mL of distilled water was added until reaching a 4 % solution of H₂SO₄ and the suspension was kept at 393 K for 1 h. In procedure B, only the second step of procedure A was followed. For both the procedures at the end of the extraction the suspension was centrifuged at 8,000 rpm and total carbohydrates dissolved in supernatant were spectrophotometrically determined by Dubois method (Dubois et al. 1951). To avoid interference, determination was done with and without phenol addition according procedures recommended by Chow and Landhäusser (2004).

2.5 Specific astaxanthin extraction (SAE) and hydrolysis (SAH)

A modified method specific for carotenoids extraction reported by Yuan et al. (2002) was followed. Dried biomass was put in a mortar and pre-treated by hand milling with a pestle in order to obtain a homogeneous granulometry. Milled biomass (100 mg) was then put in a glass vial and 2 mL of solvent mixture (25 % dichloromethane and 75 % methanol) were added. Through a pestle, the biomass was shaken to allow carotenoids extraction. Vessel was centrifuged at 3,000 rpm, supernatant was preserved and the biomass was rinsed with fresh solvent mixture. The procedure was repeated until a colourless solvent mixture was maintained. Astaxanthin hydrolysis was carried out by adding 0.4 mL of a methanol solution with NaOH equal to 0.02 M to 2 mL of solvent mixture obtained from extraction. The reaction mixture was maintained in dark, under constant agitation and nitrogen atmosphere for 2 hours according to the procedure developed by Yuan and Chen (1999). After hydrolysis, an aliquot of 20 μ L of H₂SO₄ (96 %) was firstly added and then a further second addition of 20 μ L was carried out.

2.6 Astaxanthin analysis

Analysis of astaxanthin was carried out by using a reverse-phase high performance liquid chromatography (HPLC). HPLC was composed by a P4000 pump, a C₁₈ Hypersil GOLD column (particle size: 5 μ m, dim. 250 x 4.6 mm) and a UV-Vis detector (SpectraSystem UV1000). After filtration in a 0.2 μ m pore size filter, an aliquot (20 μ L) of sample was injected in the column at room temperature. The mobile phase consisted of a solvent mixture of dichloromethane: methanol: acetonitrile; water, in the ratio of 5.0:85.0:5.5:4.5 (v·v⁻¹). The flow rate was maintained at 1 mL·min⁻¹ and the absorbance was detected at 480 nm. Quantification was carried out by a standard curve obtained through astaxanthin standard purchased from Sigma Aldrich.

2.7 Elemental composition determination

Elemental composition analysis was carried out through a CHN elemental analyzer. Protein content has been determined by multiplying nitrogen content by 6.25.

3. Results and discussion

3.1 Biomass production

During cultivation microalgae were growing until reaching a stationary phase and maintained in this phase for some days (Figure 1). This phase is generally characterized by different changes in metabolism as, for example, storage of energy and carbon as carbohydrates and/or lipids.

Table 1: elemental composition of biomass at two different phase of cultivation

Cultivation Day	C (%)	H (%)	N (%)	S (%)	C/N
20th	39.2	5.7	6.0	1.1	6.5
31th	40.5	5.9	5.1	1.1	7.9

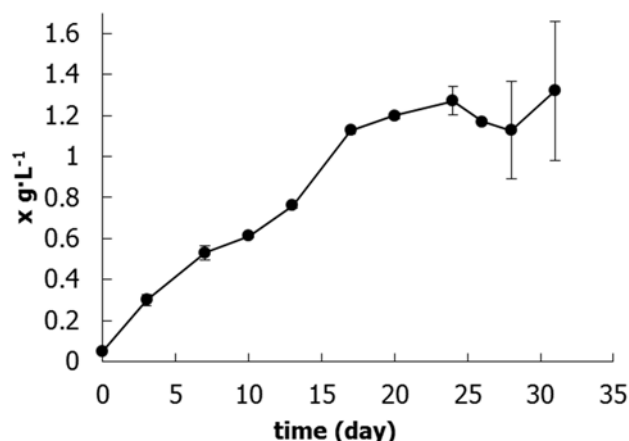


Figure 1: evolution in the time of biomass concentration of *Scenedesmus sp.*

Moreover in some species an astaxanthin accumulation also occurs during stationary phase (Markou and Nerantzis, 2013). As reported in Table 1, during the stationary phase there was an increment in carbon content on dry weight and a decrement in nitrogen content with a global increment in C/N ratio. As reported in Table 2, the mass balance on microalgae accounting for all main microalgal fraction could not be closed. This is due to the influence on whole mass of minor components and, probably, to the inefficiency in lipid extraction. It should be considered that after Soxhlet extraction biomass was not completely white but a light green colour persisted.

3.2 Astaxanthin extraction through SAE and SAH

In order to minimize astaxanthin degradation caused by high temperature, light, and exposition to oxygen, astaxanthin content has been evaluated by modified specific method employed by Yuan et al. (2002) for carotenoid determination. For this purpose, also hydrolysis was carried out by utilizing condition optimized by Yuan and Chen (1999) to minimize degradation caused by temperature and OH⁻. By this procedure, a recovery of $0.18 \pm 0.01 \text{ mg}\cdot\text{g}^{-1}$ was attained after extraction and of $0.15 \pm 0.01 \text{ mg}\cdot\text{g}^{-1}$ after hydrolysis. This difference is not statistically significant indicating that there was no relevant concentration of astaxanthin esters and not relevant degradation by NaOH occurred. Astaxanthin esters have generally retention time higher than astaxanthin and can be identified as a series of peaks which appear after peak of astaxanthin (Yuan and Chen, 1999). In samples from *Scenedesmus sp.* employed in this study these peaks were absent (data not shown). The astaxanthin content found is very lower than the content achieved by *Haematococcus pluvialis* (4 % w·w⁻¹). However *Scenedesmus sp.* can be a good candidate for astaxanthin production owing to the fast growth rate and good resistance in stressed condition (e.g. growth in wastewater). To simulate conditions experienced in acid saccharification processes, degradation of astaxanthin was evaluated by consecutive H₂SO₄ additions. As is shown in Figure 2, acid additions led to decrement in astaxanthin content. When acid concentration was increased after SAH, by two further addition of H₂SO₄, astaxanthin concentration also decreased from about $0.15 \text{ mg}\cdot\text{g}^{-1}$ to $0.04 \text{ mg}\cdot\text{g}^{-1}$. This latter result indicates high sensitivity of astaxanthin to acid condition. A similar trend was found for other peaks, relatives to other carotenoids (unidentified).

Table 2: percentage content of proteins, lipids and carbohydrates in biomass at different cultivation times

Cultivation day	Proteins (%)	Lipids (%)	Carbohydrates (%)	Total (%)
20th	37 ± 1	24 ± 9	34 ± 6	90 ± 20
31th	32 ± 1	17 ± 4	32.7 ± 0.7	82 ± 6

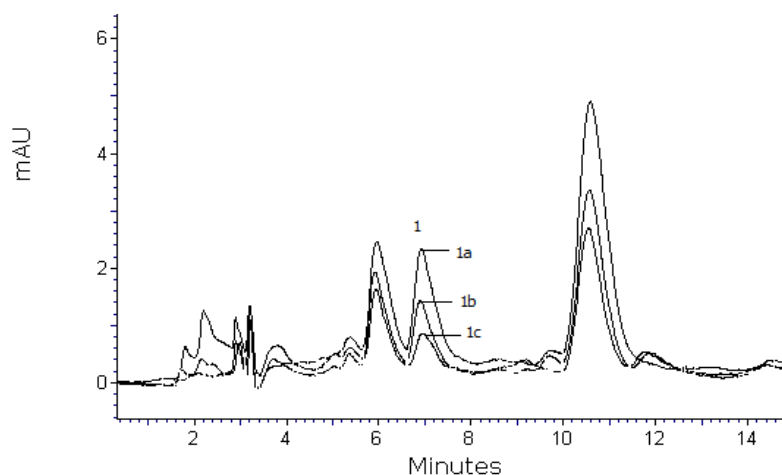


Figure 2: chromatograms of carotenoid extract from *Scenedesmus* sp. 1): astaxanthin peak, 1a) specific astaxanthin hydrolysis after specific extraction (SAH), 1b) first H_2SO_4 addition, 1c) second H_2SO_4 addition

3.3 Effect of total lipid extraction and biodiesel production on astaxanthin recovery

To evaluate the influence of typical conditions of biodiesel production processes on astaxanthin stability, astaxanthin recovery was evaluated after lipid extraction by Soxhlet and subsequent transesterification process. As shown in Figure 3, there are not significant variations in astaxanthin recovery comparing TLE and SAE. This indicates a good stability of astaxanthin to exposition at temperature of about 330-340 K for several hours. HTL treatment, similarly to SAH, did not influence astaxanthin recovery suggesting feasibility of simultaneous astaxanthin hydrolysis and transesterification of triglycerides in biodiesel production. Acidification after hydrolysis confirms the sensitivity of astaxanthin to elevated acid concentration, but, as shown in Figure 3, when pH was again raised, by addition of NaOH, initial astaxanthin concentration was partially restored. This latter result suggests that a reversible process is responsible of astaxanthin disappearance in acid condition. Further studies are required for a better understanding of involved mechanism.

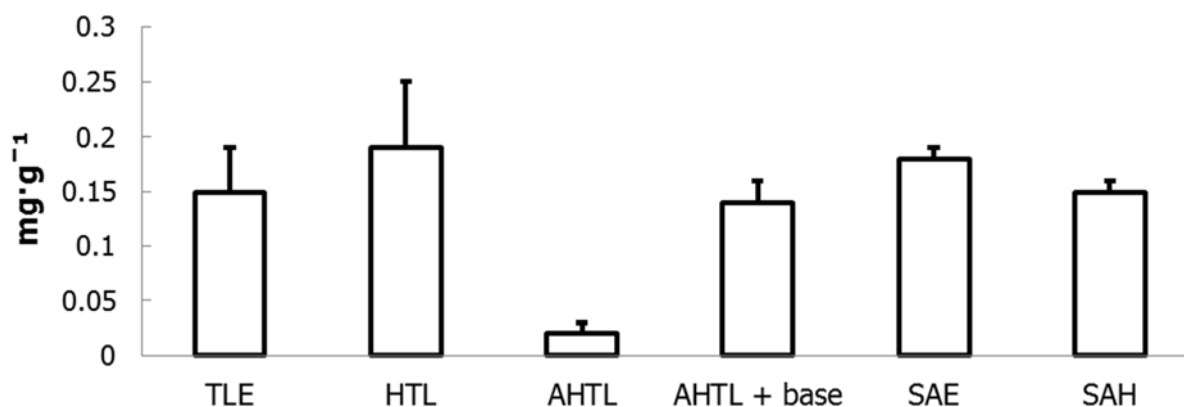


Figure 3: astaxanthin content after different extraction procedures is reported. TLE: total lipid extraction, HTL: hydrolysis after total lipid extraction; AHTL: acidification after HTL; AHTL + base: basification after AHTL; SAE: specific method for astaxanthin extraction; SAH: specific astaxanthin hydrolysis after SAE. Values are expressed as mg of astaxanthin on g of microalgal dry biomass

3.4 Effect of different saccharification procedures on carbohydrates recovery

In last years, the possibility to employ wet processes for contemporary lipid and carbohydrate extraction has been investigated by various studies (Sathish and Sims, 2012). This is a promising solution to reduce energy consumption in extraction processes because two extractions are integrated in one phase and biomass drying is avoided. Since the results on astaxanthin recovery evidence astaxanthin sensitivity to acid condition, the influence of a saccharification modified method on carbohydrates recovery was evaluated. Elimination of the first step, with the higher acid concentration (72 %), did not lead to a significant reduction in carbohydrate

recovery with values that changed from 32.7 ± 0.7 % to 32 ± 2 %. This indicates the possibility to use more gentle acid exposition in carbohydrate extraction process from microalgae compared to conditions developed for ligno-cellulosic biomasses.

4. Conclusions

Results reported in this study indicate feasibility of the extraction of astaxanthin following biodiesel production. Astaxanthin produced by *Scenedesmus sp.* is present mainly in not-esterified form, allowing an equal recovery after and before transesterification process. Our results proved astaxanthin instability at acid pH. However the mechanism involved seems to be reversible because astaxanthin can be partially restored by lowering again H^+ concentration by adding NaOH. This process should be further investigated for better understand involved mechanism. In order to reduce exposition to acid, carbohydrate extraction with different acid concentration values were compared. This analysis revealed that more gentle extraction processes can be carried out for microalgae without decreasing carbohydrate recovery.

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