Advantageous enzyme selective extraction process of essential spirulina oil

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**ARTICLE INFO**

**Keywords:**
Arthrospira sp., Spirulina, Alcalase®, Extraction, Biofactory, Fatty acid

**ABSTRACT**

*Spirulina* is an important source of metabolites and nutrients. It grows rapidly with low land requirements, and it is an ideal feedstock for implementation of a biofactory of bioactive products. A Part of intracellular bio-components are not extracted with conventional methods, but they can be using green and selective enzyme-assisted extractions. Different selective degradations of cyanobacterial murein layer with peptidoglycans and lipopolysaccharide polymers were studied for extraction of spirulina oil. The effects of most important parameters of biomass degradation with two proteases and two glucanases were studied in the range of pH (5-9), temperature (30–50 °C), enzyme loading (0.5%–2% v/w) and time (0–24 h).

Each procedure yields different products and oil recovery yields. Vinoflow® gave the highest oil extraction effectiveness (8.1% w/w), resulting 74% higher and being 1.8 times richer in unsaturated fatty acids (64.92 mol %) than with the simple solvent extraction; the more abundant acids in this oil extract were: palmitic (32.7%), palmitoleic (19.6%), linolenic (18.8%) and linoleic (18.9%). However, Alcalase® gives rise to the most important destruction of cell integrity by transmission electronic microscopy and the highest extraction yield of hydrophilic biocomponents recovery (36.50% w/w).

1. Introduction

*Arthrospira platensis* and *Arthrospira maxima* (commonly named *Spirulina platensis* and *Spirulina maxima*) is very abundant cyanobacteria in oceans. It mainly grows in Africa, Asia and South America [1] and plays a relevant nutritional role [2–5]. It was already consumed by the Aztecs two-three thousands of years ago, and currently it is being used in health recovery programs for populations with nutrition deficiencies. In modern society, spirulina biomass is widely used in foods, due to its nutritional characteristics of interest for different types of healthy diets. In 2011, spirulina was classified as a safe ingredient in class A diet supplements, being the single blue microalgae (cyanobacteria) with GRAS status [6,7]. Spirulina is very rich in protein, it has all the essential amino acids, and it is also a good source of vitamins, carbohydrates, fatty acids, sterols, macro- and trace minerals, and other nutrients. Percent composition by dry weight of spirulina is 64–73 % protein, 12–17 % carbohydrate, 5–7 % lipids, 0.9 % P and 10.3–11.6 % N [8]. A typical fatty acid composition of spirulina is 48.2% of saturated, 13.6% of monounsaturated, 14.5% of 18:2 n-6 (linoleic acid) and 21.1% of 18:3 n-3 (linolenic acid) [9]. Blue pigments (phycocyanins) of spirulina contribute to increase the protein and iron availability [10,11]. The positive effects of consuming spirulina in the immune system have been related with the presence of anti-oxidants (like β-carotene) and the essential α-linolenic acid [12]. Moreover, spirulina has important therapeutic effects (anticancer, antiviral, antiinflammatory, antimicrobial) [13].

Spirulina is a very appropriate feedstock for obtaining food additives or supplements for the food, cosmetics and pharmaceutical applications. Most attention has been paid to the extraction of polar spirulina bioactive peptides. But only a few hydrophilic peptides [14,15] and antioxidants [16] have been isolated using specific extraction processes. But also, given the high omega-3 and omega-6 fatty acid contents of spirulina oil, essential lipids, essential phospholipids and other apolar biocomponents, exhibiting interesting bioactivities (antiviral and anticancer properties, etc) could be obtained from spirulina [12,13].

Many bioactive products obtained are formed as a result of a specific biomass degradation carried out before the proper recovery of biocomponents. Consequently, the chemical composition and functional properties of the obtained bioactive extracts depend on the whole extraction procedure (especially on prior biomass degradation) and its operation conditions [14,15]. Hence, in order to increase the knowledge of spirulina potential as a source of great variety of bioactive products, it is of great interest to investigate different extraction processes, particularly those involving different selective biomass degradations. Enzyme technology constitutes an alternative to traditional
physical or chemical methods for cellular degradation prior to recovery of biomass constituents [17]. Selective enzymatic degradation of biomass could permit not only selective formation of specific products, but also more efficient extraction of the biomass components. Enzyme degradation processes of vegetable biomass is a “white biotechnology” for sustainable production of food supplements and metabolites [13]. In fact, the use of industrial biotechnology, based on the employ of biomaterials and naturally occurring production processes, is a promising alternative for manufacture of food products and derivatives [18].

The cell membrane makes difficult the extraction of intracellular biocomponents. Hence, biomass degradation facilitates metabolites recovery from the cytoplasm and organelles [19]. In the case of lipids, they mainly accumulate in subcellular lipid droplets (oleosomes) [20]. Oleosomes have a nucleus of neutral lipids surrounded by a monolayer of lipoproteins [21]. Since most lipid reserves are inside the cell [22], complete lipid extraction can only be achieved after destruction of the cellular and subcellular structures [19,23].

The cyanobacteria cell membrane is structurally similar to that of Gram-negative bacteria [24], containing peptidoglycan: a heteropolymer resistant to lysozyme treatment [12,25]. The cell membrane of Gram-negative bacteria is constituted by two lipid membrane layers (cellular and cytoplasmatic), separated by the layer of murein. Murein is integrated by complex polymers peptidoglycans and lipopolysaccharides. Peptidoglycans are formed by disaccharides and tetrapetides covalently linked forming a rigidmacromolecular structure. They are placed between cellular and cytoplasmatic membranes, and linked to the external membrane layer by lipoproteins. Lipopolysaccharides are formed by a lipid and a complex polysaccharide chain. The cellular membrane has proteins non-covalently linked to lipids; while the cytoplasmatic membrane is formed by lipoproteins (proteins covalently linked to lipids) [12].

Different types of enzymes are used to degrade the cellular membrane of cyanobacteria and microalgae. It is an advantageous technology for obtaining glucose via degradation with cellulases, and fatty acids using lipases to hydrolyze membrane phospholipids [26]. Enzyme degradation of spirulina with lysozyme has been widely studied, mainly in the area of molecular biology [27]. Lysozyme treatment of spirulina for 24 h destroys the bacterial cells [27].

Among the commercial enzymes used in this study, Alcalase® is a serine endopeptidase (EC. 3.4.21.62) from Bacillus licheniformis (mainly substilisine A; 27,300 Da mole weight). It hydrolyzes amino acids including heterocyclic amino esters. Flavourzyme® is a peptidease from Aspergillus oryzae, widely used for protein hydrolysis. Eight enzymes in the range of 19–85 kDa have been identified in Flavourzyme®: two aminopeptidases, two dipeptidyl peptidases, three endopeptidases, and one α-amylase [28]. Ultrafloro® L contains 5–10% (w/w) β-glucanase (endo-1,3(4)-) and 1–5% (w/w) xylanase (endo-1,4-) as the main activities (EC 3.2.1.6 and EC 3.2.1.8) produced by a strain of Humicola insolens. It is used to hydrolyze polysaccharide gums in the brewing industry, and beta-glucan and xylans in cereals [29]. These two types of enzymatic activities in Ultrafloro® are cellulases and catalyze the hydrolysis of complex sugars in the amorphous regions of the cellular membrane. It is used primarily with well-modified malt, and marketed within the European Union as feed-additive “Pentopan/Biofeed Plus.” The more abundant enzyme has around 60,000 Da of mole weight, and the second activity declared is due to two similar enzymes of 6000 Da. and 21,000 Da mole weight, respectively. Viniflow® Max A is a β-glucanase (exo-1,3-) preparation with 31,500 u. It is used to speed up the aging process, accelerating clarification of the wine [30]. All these enzyme preparations (Alcalase®, Flavourzyme®, Ultrafloro® and Viniflow®) are Gras type hydrolyses declared by the American Center for Food Safety and Applied Nutrition Food And Drug Administration [31].

Different types of degradation products of spirulina biomass can be obtained using different types of enzymes and operation conditions. The enzyme assisted extraction procedure may affect to the composition and properties of the extract (for example, different degree of peptidoglycans, pipopolysaccharide polymers or lipopeptide degradations). Hence, to go further on the study of the spirulina potential as source of bioactive products, the implementation and study of operation parameters of different selective enzyme assisted methods is required.

In this work, the great potential of spirulina and the limited number of extraction studies reported, different enzyme assisted extraction processes of the apolar spirulina biocomponents were studied, optimized and compared. The biomass has been enzymatically degraded using four different commercial enzyme preparations. Particularly, two different enzyme treatments based on the degradation of membrane proteins, (lipoproteins and peptidoglycan) by two proteases (Alcalase® and Flavourzyme®), and two other treatments using the endo- and exo-glucanases Ultrafloro® and Viniflow® to attack the sugar polymer structure, were comparatively studied. Spirulina biocomponents were extracted using a mixture of solvents of low toxicity. The effects of the most important parameters of the enzymatic pre-treatment for biomass degradation were determined. The processes were also carried out in larger scale at their respective best conditions, and the obtained extracts were compared with those of the control extract (obtained without any enzyme assistance). Fatty acid compositions of the different oil extracts were compared. Changes occurred at cellular level after the distinct extraction processes were also comparatively analyzed by transmission electronic microscopy (TEM).

2. Materials & methods

2.1. Materials

Spirulina platensis dry biomass (lyophilized dry powder for nutrition use) was purchased to ASN Leader S.L. (Murcia, Spain). According to the manufacturer, the composition was 50–65 % proteins, 6–7.5 % lipids, 18–22 % carbohydrates, 15 % minerals, 0.2 % fiber and 390 cal/100 g. Chloroform, methanol and isopropanol were HPLC grade. n-Hexadecane was used as internal standard and sodium sulfate as desiccant. All of them were from Sigma-Aldrich (Madrid, Spain). Buffer solutions used were CH3COONa/CH3COOH (pH 5–6), Na2HPO4/NaH2PO4 (pH 6.5–8) and NaCO3/NaHCO3 (pH 8.5–9). Alcalase® 2.4 L FG, Flavourzyme®, Ultrafloro® L and Viniflow® Max A were liquid commercial enzyme preparations kindly donated by Novozymes A/S (Denmark). Alcalase® has a declared activity of 2.4 AU–A/g using the Anson haemoglobin method, where denatured haemoglobin was degraded during the incubation of enzyme and substrate under standard conditions (0.02–0.39 AU/litre enzyme in 0.0067 M phosphate pH 7.5 was incubated with the substrate for 10 min. at 25 °C). Undigested haemoglobin was precipitated using trichloroacetic acid (TCA). The amount of TCA-soluble haemoglobin fraction was determined using Folin & Ciocalteu’s phenol assay at pH 11.4–11.6 for 6–10 min. and color was determined at 750 nm wavelength according with the analytical standard method of Novozymes® No.: EB-5M-0349.02/01. Flavourzyme® has at least 1000 LAPU/g (leucine aminopeptidase units/g determined by hydrolysis of leucine aminopeptidase (exopeptidase, LAPU) activity. In this assay hydrolysis of 1.53-mm l-leucine-nitroanilide in Tris buffer 0.1 M, at pH 8.0 and 40 °C was measured for 10 min in a spectrophotometer at 405 nm (Novozymes® standard method No.: EB-5M-0298.02/02). But the manufacturer indicates that it is not the single activity type in this preparation. Ultrafloro® L main activity is β-glucanase (45 fungal β-glucanase (FBG) per g. determined by Novozymes® standard method: 0.5% β-glucan hydrolyzed by 1.5–4.6 % β-glucanase in 28 mM phosphate buffer pH 5.5 at 50 °C for 20 min. and color reaction at 405 nm wavelength with 42 mM PAHBH in 56 mM tartrate with 4.5 mM Bi3+, 146 mM NaOH at 50 °C for 20 min. 1 FBG is the amount of enzyme that produces reducing carbohydrate equivalent to 1 μmol of glucose per min. In addition, it has approximately 470 arab xylanase units (FXU) per g. Activity was determined by Novozymes®

standard method No.:EB-SM-0364.02/01, based on hydrolysis of 0.45% w/v remazol-stained wheat arabinoxylan substrate at pH 6.0 and 50 °C for 30 min., and color determination at 590 nm. Viniflow® Max A has declared Botryis GlucanEx Units of activity of 46 BGXU/mL, determined by Novozymes® standard method No. EB-SM-0202.02. This is based on hydrolysis of β-1,6 bonds in Botryis glucan forming reducing sugars including gentiobiose and glucose. The number of bonds hydrolyzed in the reaction is determined colorimetrically by reaction with alkaline 2-hydroxy-3,5-dinitrobenzoic acid (DNBA-R) and subsequent heating to 100 °C. The resulting yellowish brownish color is measured spectrophotometrically at 540 nm.

2.2. Enzyme assisted extraction of spirulina biocomponents

The extraction technology was investigated using four different commercial enzyme preparations, namely Alcalase® 2.4L FG, Flavourzyme®, Ultraflo® L and Viniflow® Max A. The extraction methods studied only differ in the step of cellular degradation (protein or sugar hydrolysis). The enzymatic treatment was followed by a step of solvent extraction of biomass. The same solvent extraction procedure carried out without any prior enzyme assistance (control experiment) was also compared with them.

The effects of the more important parameters of the enzymatic step were studied: pH 5–9, temperature 25–50 °C and enzyme loading 0.5–2 (v/w, volume of enzyme preparation per weight of biomass suspension) except for Ultraflo® (0–4 % v/w). Optimal operation conditions of the four enzyme assisted extraction processes were determined for the recovery of oil bio-components.

A suspension of spirulina biomass (0.2 g/mL aqueous buffer) containing the corresponding loading of enzyme preparation (or an equivalent volume of milli-Q water in the case of the control experiment) was kept under magnetic agitation (500 rpm) for the indicated time at controlled temperature. The study was carried out at short and long reaction times (duration of enzyme pretreatment: 4 and 24 h).

The effect of pH was studied at 40 °C and 1 % (v/v) enzyme solution. Temperature effect was studied with 1 % (v/v) enzyme solution at the optimal pH value determined previously for each biocatalyst. Effect of the enzyme loading was investigated at the optimal values of pH and temperature previously determined for each biocatalyst. The process extraction was followed at 1, 2, 4, 6, 8 and 24 h.

2.2.1. Solvent extraction step

Each aliquot (0.5 mL) of the enzyme-biomass suspension was dissolved in 1 mL hexane-isopropanol mixture (3:2, v/v), and the resultant solution was centrifuged for 15 min at 10,000 rpm to obtain two separated phases. After separation of the oil phase, the oil phase extraction protocol was repeated twice, using 0.25 mL hexane-isopropanol mixture. Finally, the three extracts obtained were mixed. A 0.5 mL aliquot of the total mixture of oil extract was dried under nitrogen and dissolved in 1.25 mL of a mixture of chlorofrom. To this solution, sodium sulphate was added as desiccant, and 1 mL n-hexadecane (1 mL) was added to serve as internal standard of HPLC analyses.

2.2.2. Scaled up extraction

In order to obtain greater amounts of aqueous and oil extracts as well as residual biomass, all the extraction processes (enzyme assisted and control extractions) were carried out at a scale factor of 2.5 at their respective optimal conditions for 24 h enzyme pretreatment under magnetic agitation (500 rpm). Scaled up extraction processes were carried out as follows: pH 6.5, 30 °C and 1 % v/w Alcalase®; pH 6.0, 30 °C and 1 % v/w Flavourzyme®; pH 7.0, 30 °C and 1 % v/w Ultraflo®; pH 6.5, 40 °C and 2 % v/v Viniflow®. The extraction without enzyme digestion (control) was obtained using milli-Q water instead of the enzymatic preparation in buffer at 30 °C and 24 h. After the indicated time, the enzyme-biomass mixture was centrifuged for 30 min at 14,000 rpm and 10 °C. All the corresponding oil and aqueous extracts were dried and weighted in a four digits digital balance (Metler-Toledo, Spain). Aqueous extracted phases were lyophilized for 4 days, and extracted oil phases were dried in a rotoevaporator at 35 °C to eliminate most of the solvent, and they were ultimately dried for 4 h at 20 °C under nitrogen until complete elimination of solvent traces was reached. All dry weight values of the aqueous extracts were corrected by subtraction of the corresponding weight of the buffer enzyme solution (lower than 1% w/w). Residual biomasses obtained were dried under nitrogen. This study permitted to obtain sufficient amount of residual biomasses and extracts to determine their corresponding values of dry weights. It also allowed us to have the necessary amount of samples to run their compositional analyses of fatty acids via gas chromatography. All the experiments were carried out by triplicate.

2.3. Analysis and characterization of oil extracts

A High-Performance Liquid Chromatography apparatus coupled to an evaporative light scattering detector (HPLC-ELSD) was used for optimization of the enzymatic extraction process. All different oil extracts were analyzed to determine their fatty acid composition by Gas Chromatography coupled to a Flame Ionization Detector (GC-FID). Fatty acids identification was performed with a GC coupled to a Mass Spectrometry detector (GC-MS).

2.3.1. HPLC-ELSd analyses

These analyses permitted to determine concentration changes of bio-components in the oil extracts obtained at different times of enzyme treatment. Analyses were carried out with a Hitachi D-7000F apparatus (Germany) with a silica column from Kromasil (5 μ, 250 x 4.6 mm) connected to a Sedex 55 ELSD detector (Sedere, France). A chloroform (99 %) HPLC quality) solution of the samples (20 μL) was injected and analyzed at 30 °C for 30 min with a gradient mobile phase at 1.5 mL/min. The phase A was n-hexane/formic acid (100:0.1 v/v) and the phase B was hexane/isopropanol/ethyl acetate/formic acid (80:10:10:0.1 v/v). The composition of the phases (A:B) varied as follows: from 99:1 to 98:1 in the first 20 min, the composition was maintained for the next 3 min (up to min 23), then the mobile phase returned to the first composition (99:1) in 1 min, and it remained constant for the rest of the analysis. Each sample was analyzed by triplicate.

2.3.2. GC analyses

GC-FID analyses of all the oil extracts were performed to determine their fatty acid composition. To analyze total (free and esterified) fatty acids in the oil extracts, a method previously described was followed to achieve complete derivatization of the samples [32]. Briefly, 200 μL of a chloroform solution of the oil extract (ca. 20 mg/mL) was methylated by addition of 1 mL of 0.2 N methanolic HCl. This mixture was heated to 60 °C for 4 h, and then 200 μL of distilled water were added. The resulting solution was extracted twice with 1 mL of n-hexane and dried with sodium sulfate for at least 2 h prior to injection to the GC. Analyses of fatty acid methyl esters (FAME) were conducted by GC according with the method reported [32]. Two μL of sample were injected into an Agilent (Palo Alto, CA) gas chromatograph (model 6890 N) fitted with a Zebron, ZB-WAX column (30 μ x 0.25 mm x 0.25 μm film thickness) purchased from Supelco (Bellefonte, PA) and a flame ionization detector (FID). Injector and detector temperatures were 250 and 300 °C, respectively. The temperature program was as follows: starting at 50 °C for 2 min and then heating to 220 °C at 30 °C/min, holding at 220 °C for 20 min, followed by heating from 220 to 255 °C at 5 °C/min. Finally, the temperature was held at 255 °C for 10 min. Identification of the various fatty acids was made by comparing their retention times with those of a Supelco 37 Component FAME Mix. Each sample was analyzed by
triplicate. Fatty acid identification was confirmed by GC–MS analyses under the same conditions.

2.3.3. Transmission electronic microscopy analyses, TEM

Spirulina biomasses (no digested/no extracted and residual/extracted) were visualized in a transmission electronic microscope. Thus, morphological changes on the residual biomass after the enzyme assisted extraction process were compared. A Jeol Jem 1010 apparatus (100Kv, Yokyo Japan), coupled to digital camera Orius SC200 (Gatan Inc., Pleasanton, California) and Digital Micrograph v 3.4 software for images acquisition, were used. Prior to analysis, all the samples were treated as follows: fixation with aldehyde (2.5% w/v) for 2 h 40 min., washed wit cacodylate buffer 0.1 M (pH 7.3), fixation with osmium tetroxide (1% w/v) for 1 h 40 min., dehydration with absolute alcohol and acetone, inclusion in a durecupan resin via polymerization at 60 °C during 48 h. Samples were cut in ultrafine layers (60 nm) with a Leica ultracut S. Finally the samples slices were dyed with uranyl and lead acetates. For each sample, at least 5–6 different regions of the corresponding slice were visualized. The most representative micrographs are reported.

3. Results & discussion

In this work, a commercial dry biomass of the cyanobacteria was used in the extraction experiments. According with the manufacturer, it can provide elevated amounts of protein (50–65 %) and all types of amino acids, some of them are essential. It also provides 6–7.5 % w/w lipids containing important amounts of omega-3 and omega-6 fatty acids (linoleic and γ-linolenic (GLA) acids), which are essential for a healthy life, as well as antioxidants and bioactive products. The bulk of the weight of dry biomass spirulina ASN corresponds to proteins, being lipids minor components. However, this lipid fraction is rich in unsaturated and polyunsaturated fatty acids.

3.1. Enzyme assisted extraction study

The effect of the most important parameters of the four different enzyme assisted degradation processes of spirulina biomass was first studied, determining the optimal values of pH, temperature, time and enzyme loading. With this aim, changes in the peaks area of the HPLC-ELSD chromatograms of the oil phase were first determined. All significant peaks of the HPLC chromatogram exhibited the same dependence of the studied parameters, that is, the same variation (increase or decrease) of their area values with the studied parameter value, and the same optimal value. Thus for each parameter study, values of total area of all significant peaks of the chromatograms could comparatively be analyzed. Hence, for the sake of clarity, the influences of pH, time, temperature and enzyme loading are depicted with respect to the total area values of all significant peaks

3.1.1. Influence of pH

This study was carried out at moderate temperature (40 °C) and 1% (v/w) of the corresponding commercial enzyme solution (units in volume per weight of total reaction sample; namely, the enzyme + biomass suspension). Oil extracts were obtained after 4 h of biomass degradation via enzyme pretreatments at different pH values. Fig. 1 depicts the changes of the total peaks area of HPLC chromatograms of samples obtained with Alcalase®, Flavourzyme®, Ultraflow® (Fig. 1A) and Vinoflow® (Fig. 1D).

For the oil extraction with the two proteases assayed (Alcalase® and Flavourzyme®), pH values of 6.5 and 6.0 were the best ones determined, respectively (Figs. 1A and B, respectively). The dependence of the extraction yield with pH is a consequence of its effect on the process of biomass degradation by the enzyme. An identical study of the influence of pH on the total area of all significant peaks appearing in the HPLC chromatogram of aqueous extracts, indicated that the same pH value permits to obtain maximal weight yields of both, aqueous and oil extracts with each studied enzyme (not shown).

A different pH value were used by Lu et al for the extraction of an antihypertensive peptide from spirulina using Alcalase® (pH 8.5 at 50 °C for 10 h) after three cycles of freeze-thawing the biomass [15]. Also, Kim et al. used different operation conditions for obtaining the iron-chelating peptide with a multienzymatic biomass degradation (Alcalase® pH 8.0 at 50 °C for 1 h + Flavourzyme® pH 7.0 at 50 °C for 8 h) [14]. The resultant products of their peptide degradation were different from those of this study (work in progress). Similarly, In et al. used different proteases of those herein studied to obtain different spirulina oils, determining an optimal pH range of 7.5–10 for the best biocatalyst [33]. Ismaiel et al. found a group of enzymes with high activity and other group with low activities at basic pH values in the extraction of spirulina antioxidants [16]. Zhang & Zhang found a wide range of optimal pH values (pH 2–8.5) for different enzymes of the ones herein studied, including papain with an optimal pH of 6.5, when studying the extraction of anticancer biocomponents from spirulina [34]. All these studies suggest the great potential of different selective enzyme degradation methods for obtaining great variety of high value spirulina biocomponents.

In this study, the best pH values determined were pH 6 for oil extraction with Flavourzyme®, pH 6.5 for the extraction with Alcalase® and Vinoflow® and pH 7.0 for the one with Ultraflow®. Best values of temperature and biocatalyst loading where next determined at their respective best pH values of these four extraction processes.

3.1.2. Effect of temperature

The effect of this parameter was studied after 4 h and 24 h biomass treatment. Fig. 2 depicts the total area of peaks of the HPLC-ELSD chromatograms of the corresponding oil phases obtained with Alcalase® (Fig. 2A), Flavourzyme® (Fig. 2B), Ultraflow® (Fig. 2C) and Vinoflow® (Fig. 2D).

The best temperature was 30 °C for the extractions with the two proteases and Ultraflow®, corresponding to the lower temperature at which the maximum value of peaks area was achieved after 24 h pretreatment. At this low temperature, products susceptible of oxidation (polyunsaturated fatty acids, vitamins, antioxidants, etc) are better preserved. In the case of Vinoflow®, the maximum recovery yield was achieved at temperature of 40 °C or greater (Fig. 2D). Use of higher temperature favors the process kinetics, reducing the necessary time for complete extraction of biocomponents, but it increases the energy expenses and the products lability, and reduces the operational stability of biocatalysts. In fact, the area decrease observed when the enzyme pretreatment of the biomass increased from 4 h to 24 h at the higher temperature processes with some of these enzymes (Fig. 2), can be due to a decay of the enzyme stability. Hence, the temperature value selected for further experiments was 30 °C in all the cases, except for Vinoflow® (40 °C). An identical study of the influence of temperature on the aqueous extract indicated that the same temperature value permits to obtain maximal yields of both, aqueous and oil extracts with each studied enzyme (not shown).

Zhang & Zhang found that trypsin, pepsine and papaine work optimally at 42 °C, 37 °C and 55 °C, respectively, and that temperature affects more than the pH to the enzyme assisted extraction of spirulina [34]. The best temperature values determined in this study are 10–20 °C lower than that used by Lu et al. and Kim et al. to obtain their anti-hypertensive and iron-chelating peptides [14,15]. Minimal temperature values typically employed for degradation of cyanobacteria and microalgae with cellulases is 37 °C [23] but also 50 °C or greater the temperatures are frequently used [20]. The processes herein described works optimally at temperatures of 30–40 °C, fact that permits energy savings and facilitates the enzyme activity preservation.

These extractions operate at moderated temperatures compared with alternative extraction methods, such as Soxhlet (70–80 °C) [35,36], pervaporation, subcritical water (324 °C), etc. [37]. Moreover,
alternative methods using supercritical fluids work at much higher pressures (> 7.38 MPa) depending on the product to be extracted [37], than the extractive method at atmospheric pressure described here.

3.1.3. Effect of the enzyme loading

Different studies revealed the important effect of this parameter, affecting to the speed of biomass biodegradation prior to solvent extraction and it determines the extraction yield [38]. Use of high enzyme loadings reduces the time to complete the desired biomass degradation, but it increases the expenses in biocatalyst.

In general, the total peaks area continuously increased when the enzyme pretreatment progressed during 24 h with all the enzyme loadings studied (Fig. 3). But use of 4% v/w Ultraflo®, resulted an excessive enzyme concentration. In the case of the two proteolytic enzymes and with Ultraflo®, the oil extraction yield also decreased using > 1% v/w enzyme. This effect suggests that an excessive enzyme concentration causes a more intensive biodegradation of the biomass. As a result of that, further biodegradation of the initially extracted products might occur, decreasing their concentration in the obtained oil extract. Liang et al., also found an important decay in the extraction recovery with enzyme loadings greater than 4% (w/w) [23].

Notably, the four enzyme assisted extractions studied (24 h enzyme treatment in their respective optimal operation conditions) yield important increases in the oil recovery, compared with the extraction process without any enzyme pretreatment of the biomass (control, Fig. 3). These results probe the favorable contribution of the biomass degradation by the studied enzymes for the oil extraction.

The same enzyme charge value permits to obtain maximal weight yields of both, aqueous and oil extracts after 24 h enzyme treatment with each studied enzyme (not shown). The best value of enzyme loading determined was 1% v/w for all the biocatalysts, except for Vinoflow® (2% v/w). Considering the density values of these commercial enzyme solutions, best loadings were determined (1–2% v/w corresponded to around 1.2–2.4% w/w).

3.2. Extraction yields of the processes at higher scale

Once best operation conditions were determined for all the cases, the four different enzymes assisted extractions were carried out at higher scale. The scale up factor was 2.5, using 10 g of spirulina biomass for each experiment. All the extracts were prepared in triplicate in their respective best conditions (pH 6.5, 1% v/w and 30 °C for Alcalase®; pH 6.0, 1% v/w and 30 °C for Flavourzyme®; pH 7.0, 1% v/w and 30 °C for Ultraflo®; pH 6.5, 2% v/w and 40 °C for Vinoflow®). The control extract without any enzyme assistance was obtained in this scale using milli-Q water instead of the enzyme solution, at 30 °C and 24 h.

The recovery yields obtained for oil and aqueous extracts, as well as for the residual biomasses (expressed in dry weight percent with respect to the starting spirulina biomass) are summarized in Table 1. The total yield values corresponding to the three phases (oil, aqueous and residual biomass) were 86.0–94.2%, due to inevitable loses of material during the process, especially in the filters (90.8% and 94.0% in the cases of Alcalase® and control, respectively).

All enzyme assisted extractions studied permitted to obtain greater yields of both, oil and aqueous extracts than the control extraction. Among them, the highest yield of oil extract was obtained with Vinoflow® (1.7 and 1.2 times higher than with the control and Alcalase methods, respectively). The higher yields of aqueous extracts (rich in amino acids and peptides) were obtained with proteolytic biocatalysts (Alcalase® and Flavourzyme®). Hence, the highest total extraction yield (oil + aqueous extracts) was found with Alcalase®. This biocatalyst

![Fig. 1. Effect of pH on the enzyme assisted extraction of the apolar spirulina biocomponents. Conditions: 1% (v/w) enzyme loading and 40 °C. A) Alcalase®, B) Flavourzyme®, C) Ultraflo® and D) Vinoflow®. A.U., arbitrary units. Data shown as mean +/− SD, n = 3.](image-url)
permitted to increase 1.4 and 1.9 times the weight of oil and aqueous extracts, respectively, with respect to the ones obtained in the control extraction process without any enzyme assistance (Table 1).

In the process with Alcalase®, the yield of oil extract obtained (6.65 ± 0.06% (w/w) is in the value range of lipids content declared by ASN Leader manufacturer of spirulina biomass (6.0–7.5 % w/w). Consequently, 89–100 % of total lipids recovery was extracted with this biocatalyst. In the cases of Flavourzyme® and Ultraflow®, the recoveries of oil obtained were 86–100 % and 78–98 % of total declared lipids, respectively. Remarkably, Vinoflow® permits to obtain the highest oil weight recovery. Alcalase® and Vinoflow® methods permit to extract comparatively higher levels of intracellular lipids than the other studied methods.

Different extraction yields of oil from spirulina platensis biomass have been reported when different solvent media were employed [39,40]. Amounts of oil extracts depended on the lipid content of the starting biomass. Chaiklahan et al. obtained very high recovery yield of lipids of 94% (w/w), although process requires the use of a seven steps extraction with ethanol [41]. In this work, the results of the extraction with a 3:2 (v/v) hexane/isopropanol (control) were compared with the one involving an enzyme pretreatment of biomass (Table 1). The manufacturer declares oil content of 6–7.5 % (w/w) of ASN spirulina biomass. The results of Table 1 indicate that the four enzyme assisted extraction methods are advantageous, especially the one using Vinoflow® (8.10% oil w/w oil with respect to the starting biomass) and Alcalase® (6.65% w/w oil with respect to the starting biomass).

These results demonstrate the superiority of the Vinoflow® for recovery of spirulina oil, and the superiority of Alcalase® for recovery of hydrophilic spirulina biocomponents. Further studies are being carried out to identify the different biocomponents of extracts obtained with the distinct selective degrading enzymes. These results will facilitate the investigation of the spirulina potential as a source of high value products through different selective enzyme degradation of cyanobacteria biomass. The process is of interest for a modern and advanced integrated biofactory-biorefinery, where high added-value spirulina biocomponents are obtained for application in food, cosmetic and medicine, besides to a residual biomass susceptible of its further conversion on renewable biogas (CH₄ y CO₂) via anaerobic digestion.

3.3. Compositional fatty acid analysis of the oil extract

The fatty acid compositions of the oil extract obtained with the best enzyme assisted extraction method was determined via gas chromatography (GC) analyses. Table 2 summarizes the different fatty acids composition by weight of oil extracts obtained with Vinoflow® and control processes.

The most interesting fatty acids from Spirulina platensis for food, cosmetic and medicine industries are those mono- and polyunsaturated species, being 64.92% w/w of the fatty acids in the oil extract obtained. Among unsaturated fatty acids, the most abundant are palmitoleic (19.5%), γ-linolenic (18.3%) and linoleic (18.9%) ones. The most abundant fatty acid in this extract is saturated palmitic acid (32.7%). Linoleic acid is an essential compound that facilitates cholesterol regulation and reduces the body fat. Chaiklahan et al reported a multistep extraction with ethanol at the 30 °C obtaining an extract with 21% w/w linoleic acid and 18% w/w γ-linolenic acid [41].

The oil extracts of spirulina obtained in this work are relatively rich in essential fatty acids (Table 2). Importantly, extraction with Vinoflow® reduced the saturated fatty acids content of the extract from 63% to 36%, while it increased the respective MUFA and PUFA contents (44% higher total content). Polyunsaturated fatty acids (PUFAs) are of great interest because of their nutraceutical properties, such as...
anticarcinogenic, antibiotic, antifungal, and antiviral [3,5]. They participate in processes of oxygen and electron transport, they facilitate membrane fluidity and heat adaptation [42,43]. Preliminary analyses of the different oil extracts indicate that, the different fatty acids determined are combined in different chemical structures, many of them having relevant biological functions (work in progress). For example, different lipopeptides of cyanobacteria (e.g., spirulina) with immune effects (cytotoxic, antitumor, antiviral, antibiotics, antimalarial, antymycotics, multi-drug resistance reversers, antifeedant, herbicides and immunosuppressive agents) [3]. Hence, considering the potential bioactivity of the extracts obtained in this study, further studies are being carried out to identify and characterize the different biocomponents of the extracts obtained in this work.

3.4. Biomass analyses by TEM

It is known that a short time (4 h) lysozyme treatment of spirulina leads to the advent of permeaplasts displaying a normal internal structure, but a more prolonged (24 h) lysozyme treatment destroys the bacterial cells. Moreover, some cells are more resistant than others to enzyme degradation [27]. The effect of the four studied enzyme treatments in the cyanobacteria biomass was investigated by TEM analyses. Residual biomasses of the four different enzyme assisted extractions, residual biomass of the control extraction and non-extracted dry commercial biomass, were compared.

Fig. 4A is a micrograph of commercial dry spirulina biomass not treated with enzymes neither extracted with any solvent, where a longitudinal section through a trichome, as well as several transversally cuts of other trichomes are visualized. The longitudinal cut of trichome corresponds to a sequence of ten cyanobacteria cells in the same plane (Fig. 4A). In Figures B and C, a detail of the cellular membrane and intracellular components can be observed. In all these micrographs the complete integrity of the cellular membrane is evident. The black spots correspond to regions of lipids or lipids inclusions [9]. In the non-treated/extracted biomass, the cytoplasm and thylakoidal system is compressed against the internal cell membrane. In Figure C the cell membrane formed by four layers is visible [9]. In Fig. 5, the micrographs of both a non-extracted biomass (Fig. 5A) and a biomass

Table 1

<table>
<thead>
<tr>
<th>Phase</th>
<th>Alcalase*</th>
<th>Flavourzyme*</th>
<th>Ultrafl®</th>
<th>Vinoflow®</th>
<th>Control**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>6.65 ± 0.06</td>
<td>6.43 ± 0.08</td>
<td>5.86 ± 0.06</td>
<td>8.10 ± 0.20</td>
<td>4.65 ± 0.15</td>
</tr>
<tr>
<td>Aqueous</td>
<td>36.50 ± 0.10</td>
<td>31.80 ± 0.10</td>
<td>19.70 ± 0.10</td>
<td>26.30 ± 0.10</td>
<td>19.20 ± 0.20</td>
</tr>
<tr>
<td>Residual Biomass</td>
<td>47.65 ± 0.35</td>
<td>47.75 ± 0.95</td>
<td>61.42 ± 1.22</td>
<td>57.10 ± 0.30</td>
<td>70.13 ± 0.37</td>
</tr>
</tbody>
</table>

* In weight percent with respect to the starting spirulina biomass.
** No enzyme pretreatment.
Table 2
Fatty acid composition of oil extract of spirulina from ASN Leader SL. obtained with Vinoflow® assistance in optimal conditions (extraction after 24 h of 2% v/w Vinoflow® pretreatment at pH 6.5 and 40 °C) and without enzyme (Control extraction).

<table>
<thead>
<tr>
<th>FATTY ACID</th>
<th>Retention Time (min)</th>
<th>Control</th>
<th>Vinoflow®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miristic Acid, C14:0</td>
<td>7.71</td>
<td>1.50 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Palmitic Acid, C16:0</td>
<td>8.17</td>
<td>32.71 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Estearic Acid, C18:0</td>
<td>8.95</td>
<td>1.34 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>TOTAL SATURATED</td>
<td>63.07 ± 0.19</td>
<td>35.55 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Palmitoleic Acid,</td>
<td>8.22</td>
<td>0.51 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>C16:1n7</td>
<td></td>
<td>19.54 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Oleic Acid, C18:1n9</td>
<td>9.03</td>
<td>2.60 ± 0.05</td>
<td>5.83 ± 0.01</td>
</tr>
<tr>
<td>Eicosenic Acid, C20:1n9</td>
<td>9.92</td>
<td>1.45 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>TOTAL MUFAs</td>
<td>4.64 ± 0.10</td>
<td>26.82 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Linoleic Acid, C18:2n6</td>
<td>9.27</td>
<td>18.10 ± 0.03</td>
<td>18.85 ± 0.05</td>
</tr>
<tr>
<td>γ-Linolenic Acid,</td>
<td>9.41</td>
<td>14.18 ± 0.04</td>
<td>18.33 ± 0.08</td>
</tr>
<tr>
<td>C18:3n6</td>
<td></td>
<td>0.44 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>α-Linolenic Acid, C18:3n3</td>
<td>9.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL PUFAs</td>
<td>32.28 ± 0.07</td>
<td>38.10 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

extracted after Alcalase® treatment (Fig. 5B) are compared at the same scale. Original trichomes observed in the first case are completely disappeared in the second one, being an evidence of the cellular degradation by this protease. In Fig. 5B, disordered rests of cellular material obtained after biomass treatment with Alcalase® are visualized. This fact is the result of an important destruction of the cellular integrity. After extraction with Alcalase® assistance, the residual cellular material is considerably low compared with the starting material (Fig. 5 A, B). In Fig. 6, the micrographs of residual biomasses obtained after the simple solvent extraction process, namely control (Fig. 6A), or after the different enzyme assisted extractions, namely the one with Alcalase® (Fig. 6B), Ultraflow® (Fig. 6C) and Vinoflow® (Fig. 6D), are compared. Unlike the non-treated/extracted cyanobacteria biomass (Fig. 5), cells submitted to control (simple solvent) extraction exhibited an electronically less dense cytoplasm and their thylakoidal system is not any more compressed against the internal cell membrane (Fig. 6A). This phenomenon was early observed in lysozyme degraded spirulina [44]. Cells obtained in the control extraction exhibited an inflation phenomenon, but not all are detached from the trichome. Dwain et al. reported changes in shape to more spherical cells and their inflation, which make cells more detachable from the trichoma after Lysozyme treatment of spirulina and other cyanobacteria [45]. In our case, the enzyme treatments for 24 h clearly gave rise to more efficient cell degradation and, in all the cases very few cells were not detached from thylachoidal system (Fig. 6B–D).

After the simple solvent extraction (control), there are visible parts of the trichome formed by several cells organized in linear sequence, where the integrity of most of the internal cellular material remains mostly intact. By contrast, all the enzyme assisted extractions produces severe destruction of the cellular integrity. After extracting with Ultraflow® and Vinoflow® (Fig. 6C & D), less cellular material remained in the residual biomasses compared with the control experiment (lower

Fig. 4. Micrographs of dry commercial spirulina biomass not treated with any enzyme neither extracted by any solvent; A) a longitudinal cut trichome and several transversally cut trichomes; B) two contiguous cyanobacteria of a trichome separated by their cell walls; C) detail of the cell wall.
electronic density is observed). Amounts of cellular material in all these three residual biomasses are greater than in the one extracted with Alcalase® (Fig. 6B). These findings are in good agreement with the values of extraction yields obtained for both the aqueous and oil phases (Table 1), where Alcalase® extraction process results the most effective one for biocomponents recovery of spirulina (especially the hydrophilic ones).

Lindsey et al. suggested that elder cells are more refractory to a proteolytic (lysozyme) treatment [44]. The micrographs of this study show that some cells were better preserved than others in the extracted biomasses (Fig. 6). But in general after 24 h of enzyme treatment, the number of degraded trichomes and spheroplasts were considerably
high, especially in the case of Alcalase®. Earlier TEM studies on * Spirulina platensis* cyanobacterial spheroplasts by Vladimirescu [27], also revealed the existence of differences in enzymatic sensitivity of cells.

The comparative inspection of micrographs (Fig. 6) suggests that the higher biocomponents recovery by the pretreatment of the biomass with Alcalase® corresponded to a greater cellular degradation. The biomass extracted after Alcalase® treatment appeared with very low electronic density, as a result of the greater extraction of their biocomponents (Fig. 6B-D).

Enzymatic processes have a wide range of applications in different industrial areas [46]. The extraction with the enzymes herein studied leads to increased yield and quality of extracts from spirulina. The enzyme technology implemented uses food grade enzymes and hexane accepted by regulatory agencies for food and drugs safety. This enzyme technology looks promising for a more efficient, safe and environmentally clean industrial production of cyanobacteria and microalgae extracts with high value in nutrition, cosmetic and pharmaceutical industries.

4. Conclusions

Different selective biodegradations of spirulina biomass prior to extraction of its biocomponents were studied. Biomass degradation with Vinoflow® gave the highest weight yield of the oil extract, and nearly duplicates the content in unsaturated fatty acid species (MUFA). C.M. Verdasco-Martín, et al. [31] gave the highest weight yield of the oil extract, and nearly duplicates the content in unsaturated fatty acid species (MUFA and PUFA) compared with the non-enzyme-assisted oil extract. Among nearly duplicates the content in unsaturated fatty acid species (MUFA). C.M. Verdasco-Martín, et al. [31] gave the highest weight yield of the oil extract, and nearly duplicates the content in unsaturated fatty acid species (MUFA and PUFA) compared with the non-enzyme-assisted oil extract. Among nearly duplicates the content in unsaturated fatty acid species (MUFA and PUFA) compared with the non-enzyme-assisted oil extract. Among nearly duplicates the content in unsaturated fatty acid species (MUFA and PUFA) compared with the non-enzyme-assisted oil extract.


