Biostimulants obtained after pilot-scale high-pressure homogenization of *Scenedesmus* sp. grown in pig manure

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A B S T R A C T

Microalgae are becoming an important source of high-value products such as biostimulants, biopesticides, and other low-value products such as biofertilizers or aquafeed (fatty acids and carotenoids), among others. However, extracting the biomolecules contained in the microalgae cells is difficult due to the structure and composition of the microalgal cell wall. To overcome this difficulty, mechanical cell disruption methods, such as high-pressure homogenization (HPH), can be applied to facilitate the extraction of the compounds of interest.

This work focuses on optimizing the extraction of biostimulants from microalgae biomass and obtaining amino acid concentrates that can be used in several applications. The research aims to study the effect of HPH pre-treatment on the production of these different bioproducts using *Scenedesmus* sp. biomass grown in pig sludge as the raw material.

For each product, the HPH pretreatment of the wet biomass must be optimized to select the optimal conditions and the number of passes. Soft treatment of the wet biomass at 200 bar is sufficient to observe a 10% increase in the germination index (GI) of watercress seeds, while greater treatment intensities reduce the GI with respect to the untreated biomass, possibly because this increases the concentration of the molecules responsible for the bioactivity, thus causing a toxic effect on the seeds. In the case of the amino acid concentrates, better results were obtained at 600 bar, achieving a hydrolysis level of 72.9%. Lastly, the concentrations of the phenolic compounds and the salicylic acid, related with enhanced biostimulant activity, were determined using spectrophotometric methods and High-Performance Liquid Chromatography- diode array (HPLC), respectively.

1. Introduction

The potential of microalgae as a source of various important agricultural or aquaculture products has attracted considerable interest over recent decades. Nowadays, agriculture has to contend not only with the growing global population, and the consequent resource depletion, but also the effect of climate change on crops [3,8]. Therefore, it is a priority to develop commercial products from environmentally friendly resources, such as microalgae, that enhance agricultural yields.

Using microalgae as the raw material to obtain high-value products has several advantages. For instance, (i) microalgae have a very short harvesting life cycle and so allow multiple and continuous collection; ii) they can be grown in a wide range of environments, including on non-arable land and in brackish or salt water, thus minimizing the environmental impact; and iii) they produce a carbon-neutral fuel from the photosynthetic fixation of atmospheric carbon dioxide, helping to

reduce the levels of this gas. However, microalgal production at the large scale must overcome certain drawbacks, such as requiring a considerable input of growth nutrients, with the associated high costs. To solve this problem, using pig manure as part of a nutrient recovery system in microalgae production seems a good alternative for reducing the cost associated with the nutrient requirements, recycling the excess nitrogen and phosphorus from the pig manure and, moreover, helping to prevent the environmental damage caused by piggery wastewater [2,4,22]. In addition to the nutrients introduced from the pig manure, one needs to develop a production process that allows the integral use of the harvested microalgal biomass, obtaining commercial high-value products such as biostimulants as well as other low-value products such as biofertilizers, which together make microalgae a profitable raw material [32].

Furthermore, including microalgae cell breakage as a prior step to solvent extraction has been found to considerably improve the

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extraction yield of the compounds of interest, especially in those species that possess a rigid cell wall such as *Scenedesmus*. Cell disruption methods can be classified into two major groups: mechanical, which includes high-pressure, bead mill or ultrasound homogenization, and non-mechanical methods (physical, chemical and/or biological) [14]. In general, it would seem that mechanical treatments are more advantageous than the other cell disruption techniques as they prevent the cells from becoming contaminated with chemicals; also, their effectiveness is less dependent on the microalgae species [13]. With regard to the mechanical methods possible, high-pressure homogenization has attracted a lot of interest over the last few years as it can be scaled up easily to process large volumes of microalgae; it is also effective in aqueous environments, thus allowing one to reduce energy consumption by avoiding a drying step [26,27,31].

Many authors have researched the positive effects of algal extracts on seed germination and growth promotion, for example, Hernández-Herrera et al. (2014), who studied the effect of different seaweed extracts on tomato seedling growth, or Garcia-Gonzalez and Sommerfeld [8], who found that applying aqueous extracts from the microalga *Acutodesmus dimorphus* enhanced the potential yield of tomato plants. It is widely reported that this positive effect observed on crops is related to the presence of phytohormones and other phenolic compounds, which play an important role in plant development [7,8,20].

The objective of this study, therefore, is to optimize the biomass pretreatment of *Scenedesmus* sp. grown in pig manure by means of high-pressure homogenization, modifying not only the treatment pressure but also the number of passes through the homogenizer. Subsequently, we will evaluate the influence of this pretreatment on obtaining amino acid concentrates by enzymatic hydrolysis, and the efficacy of the aqueous extracts as plant biostimulants. We will also determine the concentrations of phenolic compounds and salicylic acid present using spectrophotometric methods, and an HPLC-diode array to contrast the relationship between them.

2. Material and methods

2.1. Microalgae, enzymes and chemicals

Wet paste biomass from *Scenedesmus* sp. was supplied by "*Las Palmerillas, Cajamar*" research centre (El Ejido, Almería, Spain) and used as the raw material to obtain the bioproducts. The cells were grown from September to November 2017 in an open, thin-layer cascade reactor, of 32 m^2 (1.2 m^3), used for pig manure remediation. The reactor is equipped with a 1 m^3 sump in which the pH is fixed at 8 by the on-demand injection of pure CO_2 at 5 L min^{-1}; alternatively, air is supplied at 50 L min^{-1} to remove oxygen. In the thin-layer reactor, the culture is circulated at 0.2 m s^{-1} using a pump that drives the culture up from the sump to the first layer, 0.5 m above the sump culture level [15]. In our study, the reactor was operated in semi-continuous mode at a dilution rate of 0.3 day^{-1}. It was initially filled with the pig manure diluted in water (10% pig manure: 90% water) and inoculated with 10% total volume of *Scenedesmus almeriensis*. The biomass was harvested daily by centrifugation (RINA continuous centrifuge, Riera Nadeu SA, Spain) and used within the two days following reception. The biochemical composition of the biomass was determined and shown to include: 14.66% total lipids (6.21% saponifiable lipids (fatty acids)), 30.09% proteins, 7.47% ash and 47.8% carbohydrates. The analytical procedures used for their determination are explained in Section 2.6.

The enzymes used in the enzymatic hydrolysis to produce the amino acid concentrates were Alcalase 2.5 L (2.5 AU-A/g) and Flavourizyme 1000 L (1000 AU-A/g), both supplied by Novozymes A/S (Bagsvaerd, Denmark). These are commercial enzymes from yeast and other substrates used in the production of amino acid hydrolysate, which are finally commercialized as fertilizers. The chemicals used for the analytical methods were sodium tetraborate decahydrate, DL-dithiothreitol, phthaldialdehyde, dodecyl sulfate and DL-serine, all of

which were obtained from Sigma-Aldrich (St Louis, MO, USA). Glacial acetic acid from Panreac S.A (Barcelona, Spain) and methanol (99.9% purity, Carlo Erba Reagents, Rodano, Italy) were also used. All the reagents utilised in the analytical determination were of analytical grade. Standards were purchased from Sigma-Aldrich (St Louis, MO, USA) and used without further purification.

2.2. Biomass pretreatment by high-pressure homogenization

Biomass from *Scenedesmus* sp. grown in pig manure was pretreated by high-pressure homogenization (HPH) modifying not only the pressure but also the number of passes through the Panda plus 2000 homogenizer (model S.N. 8983) purchased from Gea Niro Soavi S.p.A (Parma, Italy) in order to determine the influence of these parameters on extracting the compounds of interest for agriculture, taking into account the energy consumption in each case. Cell disruption assays were carried out by passing wet *Scenedesmus* sp. biomass (with a concentration of 40 g L^{-1}) through the homogenizer at pressures from 200 to 800 bar and repeating this process up to 5 times. Under these conditions, flow rates of around 9.9 and 11.1 L h^{-1} of wet biomass were obtained at 200 and 800 bar, respectively. To observe the effect of the treatment intensity on the cell disruption, samples were centrifuged at 10835 g for 4 min (Micro-centrifuge Minispin®plus, Eppendorf, with an F-45-12-11 rotor) and the absorbance of the supernatants was measured in 96-well microplates (Greiner Bio-One) using a monochromator-based microplate reader (Synergy™ Mx, BioTek) at wavelengths from 200 to 800 nm.

2.3. Determination of the biostimulant activity

To determine the biostimulant activity of the microalgal extracts, 50 mL of the homogenized biomass solution, with an initial concentration of 40 g L^{-1} dry biomass (obtained by applying each HPH condition), were transferred to 100 mL Erlenmeyer flasks and incubated at 30 °C for 2 h with magnetic stirring at 300 rpm on a magnetic heating-plate stirrer (Multimix Heat D, Ovan, Spain). The extracts were centrifuged at 10835 g for 5 min (Micro-centrifuge Minispin®plus, Eppendorf, with an F-45-12-11 rotor) and the supernatants were collected and diluted to 1, 0.5 and 0.1 g L^{-1}; these were used to perform the bioassays for quantifying the germination index of the seeds in contact with each extract.

In each experiment, 100 tested watercress seeds (*Lepidium sativum L.*, Sonnentor) were placed on Whatman No 5 filter papers in four sterilized 90 mm Petri dishes and then treated with 8 mL of either distilled water (the control) or the extracts resulting from each treatment condition (a pressure of between 200 and 800 bar and from 1 to 5 passes). The seeds were allowed to grow for 3 days at 24 °C in the dark. Then, free *Image J* software was used to measure the seed length in each case. All measurements were duplicated. Finally, the germination index of each sample was determined using the following equation:

$$GI\ (\%) = \frac{G*L}{Gw*Lw}*100 \tag{1}$$

where G and L are the number of germinated seeds and their length, in the case of the microalgal extracts, and Gw and Lw are the same parameters but for the control (distilled water).

The data shown for the biostimulant activity experiments are, therefore, the results from measuring 200 seeds for each treatment condition.

2.4. Enzymatic hydrolysis method

The enzymatic hydrolysis method applied in this work has been described by Romero García et al., (2012). The hydrolysis was performed in a 100 mL Erlenmeyer flask, which was heated and stirred (300 rpm) on a magnetic heating-plate stirrer (Multimix Heat D, Ovan,

Spain) at a temperature of 50 °C and with the pH controlled. The experiments were performed on the wet *Scenedesmus* sp. biomass described in Section 2.1. The hydrolysis was carried out in two steps, each under the optimal conditions recommended by the enzyme's suppliers. In the first step, 50 mL of the microalgal biomass, with a concentration of 40 g L^{-1}, is transferred into an Erlenmeyer flask and heated to 50 °C. The pH is adjusted to 8 by adding NaOH 1 M. Then, 0.08 g of Alcalase 2.5 L (4% *w*/w with respect to the dry biomass) are added into the reactor and the reaction begins. Free amino acids are released as the reaction proceeds, this acidifies the medium leading to a decrease in the pH, which is compensated for by adding NaOH 1 M to maintain the pH at 8. Two hours after starting the reaction, the pH is lowered to 7 by adding H$_2$SO$_4$ 1 M and then 0.1 g of Flavouryzime 1000 L (5% *w*/w with respect to the dry biomass) are added to the reaction medium. After three hours, the reaction is considered finished, and the solution is heated to 75 °C for 15 min to deactivate the enzymes. The final concentrate, rich in amino acids, is separated from the biomass by centrifugation at 7943 *g* (Centrifuge Brand Supelco 4–15, Sartorius, Germany). The enzymatic hydrolysis was performed twice for each HPH condition.

2.5. Degree of hydrolysis

The degree of hydrolysis is defined as the ratio between the number of free amino acids in each sample and the total number of amino acids available for hydrolysis. The degree of hydrolysis was determined using the OPA (o-phthaldialdehyde) method with serine as the standard, and assuming the following parameter values for the model $\alpha = 1$, $\beta = 0.4$ and yht$_{ot} = 8$ [18].

2.6. Biochemical composition determination

The protein content was determined following the method described by González López et al. [10], which is a modification of the Lowry method, with BSA (Bovine serum albumin) as the standard. The saponifiable lipids content (SLs, fatty acids) from *Scenedesmus* sp. was determined by direct transesterification of the microalgal biomass with methanol: acetyl chloride (10:0.5 *v*/v) to transform all the SLs into fatty acid methyl esters (FAMEs). The FAMEs were then analysed by gas chromatography using an Agilent Technologies 6890 gas chromatograph (Avondale, PA, USA). More details regarding the method have been described by Navarro López et al. [17]. The total lipids were determined using the Kochert (1978) method obtaining the extract with chloroform: methanol (2:1 *v*/v). The total ash content was determined by incineration of a representative 0.5 g sample in an oven at 500 °C for 24 h while the carbohydrate content (%) was determined as the difference between 100 and the sum of the percentages of the other fractions (ash, protein and lipids) [5].

2.7. Extraction and determination of the salicylic acid and total phenol content by HPLC and spectrophotometric methods

Both the total phenols and the salicylic acid were measured after the incubation of the pretreated biomass (for 2 h at 30 °C and 300 rpm). Then, the samples were centrifuged at 12000 rpm for 5 min (Microcentrifuge Minispin®plus, Eppendorf, with an F-45-12-11 rotor), and the supernatants were collected for characterization. The total polyphenols were determined using the Folin-Ciocalteu method (Waterman and Mole, 1994). Aliquots (0.1 mL) of the supernatants were taken and transferred into the test tubes and their volumes made up to 6 mL with distilled water. After adding 0.5 mL of Folin reagent, 1.5 mL of 20% sodium carbonate solution was added between 1 and 8 min, and finally the volumes were adjusted to 10 mL by adding 1.9 mL of distilled water. The tubes were agitated, and the absorbance of the blue-coloured samples were measured after 2 h at 760 nm against a blank containing 0.1 mL of extraction solvent and the reagents. The total polyphenol content was calculated as gallic acid equivalents from the calibration

curve of gallic acid (covering a concentration range of between 0 and 0.6 mg mL^{-1}, and expressed as mg gallic acid g of dry biomass^{-1}). All measurements were carried out twice.

For the salicylic acid quantification, a previous step of sample purification is needed following the method described by Ghanem et al. [9] with several modifications. In this case, the supernatants were collected and allowed to pass through Sep-Pack plus C-18 cartridges (Sep-Pack plus, Waters, USA) to remove interfering lipids and some of the plant pigments. The cartridges were conditioned with 2 mL of water and 2 mL of methanol. Subsequently, 2 mL of these purified supernatants were lyophilized to eliminate the water; then the dry samples were resuspended in 0.5 mL of methanol-water 80:20 *v*/v and filtered through Millex Nylon membrane filters with a 13 mm diameter and a 0.22 μm pore size (Millipore, Bedford, MA, USA), and injected into a Shimadzu SPDM10AV High Liquid Performance Chromatograph equipped with a photodiode array detector and a LiChrospher© 100 RP-18 (5-μm) column (4.6 × 150 mm), following the method described by Nour et al., (2013) with several modifications. The injection volume of each sample was 20 μL. Chromatograms were acquired at 300 nm according to the maximum absorption of salicylic acid. This was identified in each sample by the retention time and by comparison with ultraviolet (UV) spectra of the standard at 300 nm. The salicylic acid (Sigma-Aldrich, St Louis, MO, USA) concentration was based on a calibration curve taken at different standard concentrations.

2.8. Statistical analyses

Statistical data analyses were performed using the Statgraphics Centurion XVII software package. Data, in percentage, were arcsin(x1/2) transformed. The normality and homogeneity analyses were performed using the Kolmogorov–Smirnov and Levene tests, respectively. Multifactor ANOVA tests were used to study the effect of the factors (pressure, number of passes through the homogenizer, and extract concentration) and their interactions (pressure-concentration and number of passes-concentration) at a 95% confidence level for the germination index, the aim being to decide the most influential factor in the germination index of the watercress seeds.

3. Results and discussion

3.1. Influence of high-pressure homogenization on the extraction of several compounds of interest

To perform the mechanical cell disruption pretreatment, the wet *Scenedesmus* sp. paste (with an initial concentration of 0.21 g dry biomass g wet biomass^{-1}) was diluted to 40 g L^{-1}. Then, the samples for obtaining each product were prepared as explained before. Although cell counting is the most reliable method to quantify the cell disruption, measuring the supernatant absorbances of the pretreated samples also provides information about the increase (or not) in the quantity of water-soluble components, and thus on the degree of cell disruption achieved in each case [24]. Fig. 1 shows the comparison of absorbances determined at wavelengths between 600 and 800 nm as a measure of the cell disruption achieved applying each treatment intensity. As can be observed, any HPH condition applied enhanced the cell disruption compared to the control (*Scenedesmus* sp. at 40 g L^{-1} without any pretreatment). The same occurred when increasing the number of passes, which, in turn, led to an increase in absorbance (data not shown). Therefore, it can be concluded that the greater the pretreatment intensity of the biomass (both by increasing the pressure and the number of passes), the greater the cell disruption observed. Fig. 1 shows the normalized absorbances, calculated as the relationship between the absorbance of each sample and the absorbance of the control (untreated biomass).

Another important parameter that should be considered is the energy consumption associated with this treatment. Table 1 shows the
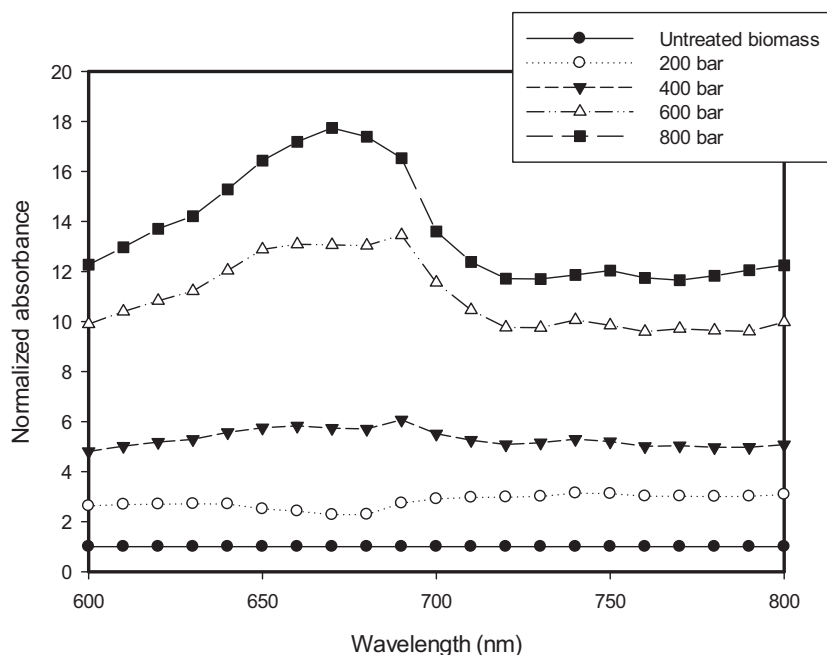
Fig. 1. Measurement of absorbances between 600 and 800 nm for each high-pressure homogenization (HPH) treatment pressure applied.

Table 1
Energy consumptions (MJ Kg dry biomass^{-1}) for a biomass concentration of $40\,g\,L^{-1}$ as a function of the high-pressure homogenization conditions, the pressure, and the number of passes.

Pressure (bar)	Energy consumption (MJ Kg dry biomass^{-1})				
	Number of passes				
	1	2	3	4	5
200	0.45	0.97	1.61	2.45	3.88
400	0.84	1.93	3.45	4.95	6.20
600	1.13	2.45	3,74	5.82	6.92
800	1.83	4.21	6.92	8.16	11.43

homogenizer energy consumption expressed as MJ kg-1 of dry biomass for each pretreatment condition considered in this study.

Obviously, the energy consumption increases along with the treatment intensity, that is, with the increase in pressure and the number of passes through the homogenizer. Note that, for several of the compounds of interest, it is not necessary to reach the maximum pressure in order to obtain a high extraction yield; therefore, in each case, the treatment pressure which leads to the best results needs to be studied. As is shown in Table 1, the maximum energy consumptions were obtained with a pressure of 800 bar; these ranged from 1.8 to 11.4 MJ Kg dry biomass^{-1} depending on the number of passes. Other authors, such as Jiménez Callejón et al. [11], used HPH as a cell disruption method in the processing of *Nannochloropsis gaditana* biomass containing a 14% solid content, describing an energy consumption of 4.5–5.3 MJ Kg dry biomass^{-1} at 500 and 1500 bar, respectively, and with only one pass through the homogenizer. This energy consumption is greater than that described in our work (0.45 and 1.83 MJ Kg dry biomass^{-1} at 200 and 800 bar, respectively) because of the lower solid content (0.4%) of the *Scenedesmus* sp. biomass; in other words, the greater the solid content of the biomass, the higher the pretreatment energy consumption.

3.1.1. Biostimulants.

3.1.1.1. Effect of the HPH treatment on the germination index of watercress seeds.. To observe the influence of high-pressure homogenization on the

germination index (GI) of watercress seeds, calculated using Eq. (1), the wet *Scenedesmus* sp. biomass was homogenized at different pressures between 0 and 800 bar, changing the number of passes through the homogenizer (from 1 to 5 passes) for each biomass batch. The samples were prepared as described in Section 2.3 and diluted to 1, 0.5 and $0.1\,g\,L^{-1}$ in order to carry out the bioassays. A GI of 100% is assumed when seeds are in contact with distilled water, so only those extracts that result in a GI higher than 100% are considered as promoting biostimulant activity. Fig. 2 shows the effect of pressure, extract concentration, and the number of passes on the germination index of the seeds in contact with these extracts (i.e. an increase or decrease in the GI with respect to the control), whereas Table 2 provides a statistical analysis of the above-mentioned factors.

As is shown in Fig. 2, the lowest germination index (GI) for all the HPH conditions tested was obtained when the extracts were diluted to $1\,g\,L^{-1}$, and this GI decreased as the pretreatment pressure increased. As can be observed in Fig. 1, cell disruption increased along with the treatment intensity, reaching maximum disruption at 800 bar. Therefore, it is possible that the lower GI obtained at such high pressures could be due to the higher concentration of the particular stimulant compounds, which exert a toxic effect on the seeds, thus affecting their growth. Authors such as Charles and Hemingway (1965) reported that this higher GI obtained at the lowest concentrations might be due to the presence of some plant-growth regulators, micro and macro nutrients that cause an inhibitory effect on seed growth when these compounds exceed a certain concentration. On the other hand, when extracts were diluted to $0.1\,g\,L^{-1}$, higher GIs were obtained, except when the biomass pretreatment was performed at 800 bar, for which a maximum GI of 98% was achieved. Apparently, in this latter case, it was necessary to further dilute the extracts to reduce the concentration of the biostimulant molecules. This effect has been reported by other authors, such as Kumar and Sahoo [12], who also observed growth inhibition in *Triticum aestivum* var. Pusa Gold along with an increase in the seaweed liquid extract concentration. Some authors, such as Niemann and Dörffling [19], have described the presence of an inhibitory complex that deters plant growth in the bioassays. It was supposed by these authors that certain bioactive compounds, such as abscisic acid (ABA), produced shoot growth suppression when acting on their own, or formed an inhibitory complex with other biomolecules such as lunularic acids,
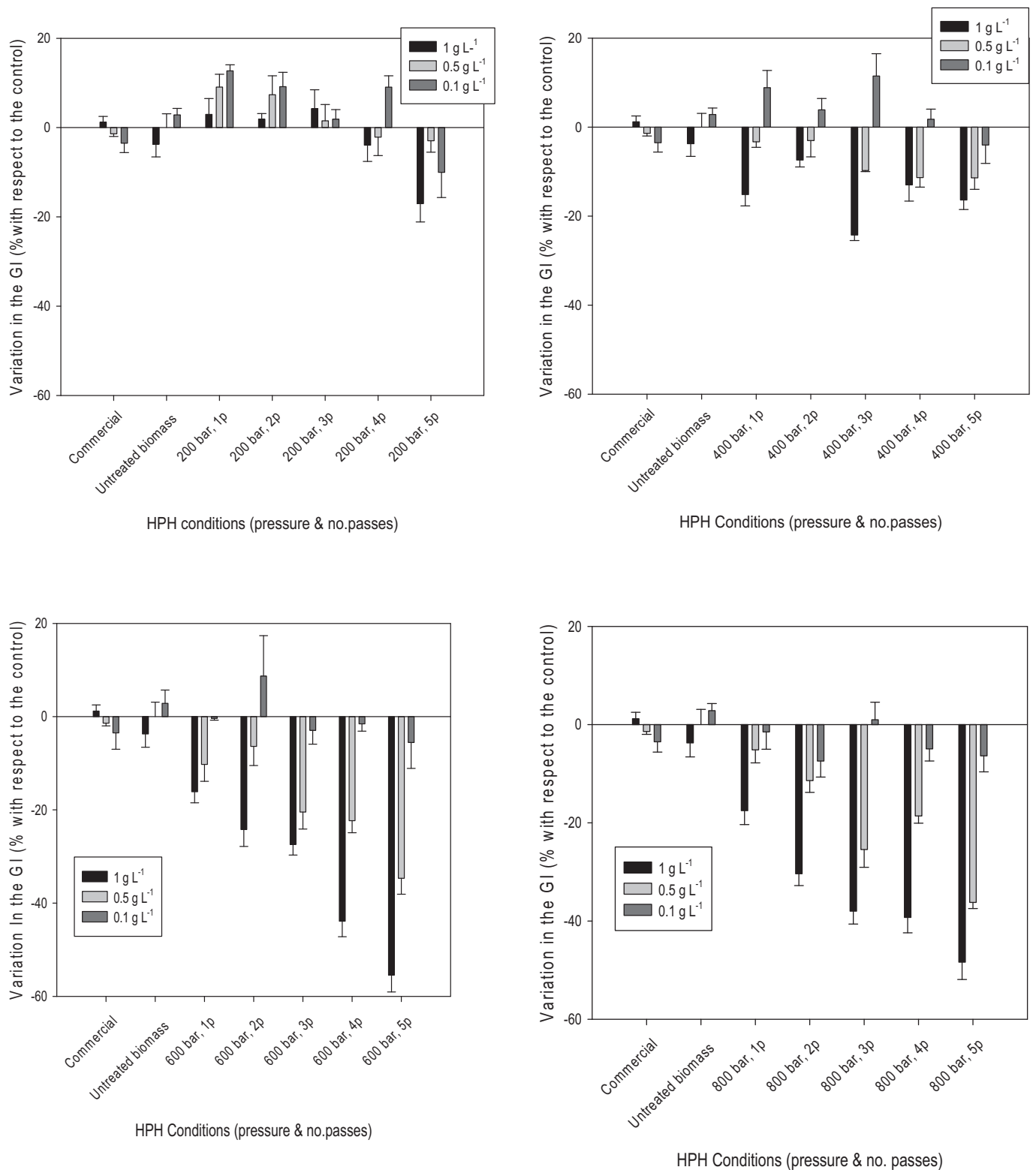
Fig. 2. Influence of the high-pressure homogenization (HPH) conditions (pressure & no. of passes) on the germination index of the watercress seeds, diluting the microalgal extracts to 1, 0.5 and 0.1 g L^{-1}. Results are shown as mean ± SD ($n = 2$).

salicylic acid and other unidentified compounds. This might be the reason for the results obtained in this work, showing an inhibitory effect on the bioassays applied when the pretreated *Scenedesmus* sp. microalgal biomass are diluted at the highest concentration, 1 g L^{-1}; given that the biomolecules responsible for growth inhibition are also at their highest concentration. Authors such as Plaza et al. [23] analysed the hormone

content in hydrolysed *Scenedesmus* sp. and *Arthrospira* sp., and determined an ABA content of 3718.3 and 1.03 ng g^{-1} for each microalgae, respectively, along with other phytohormones such as gibberellins or salicylic acid.

In any case, when *Scenedesmus* sp. microalgal biomass (without pretreatment) was used to perform the bioassays, the GI of the seeds was

Table 2
Multifactor ANOVA testing the effect of pressure (P), the number of passes through the homogenizer (N) and the extract concentration (C) on the germination index of the watercress seeds. The data variability is attributable to the main effect of each factor separately (P, N and C) and the interaction found on two levels (P—C and N—C), as indicated by the *p*-value. The contribution of each factor was expressed as the percentage variation of the response (the F ratio of each factor relative to the sum of all F ratios).

Response	Statics	P	N	C	P-C	N-C
Germination index (%)	Variation (%)	36.18	15.23	42.75	4.88	0.95
	p-Value	0.0000	0.0000	0.0000	0.0000	0.0000

96.28, 100 and 102.85% for sample dilutions of 1, 0.5 and $0.1\,g\,L^{-1}$, respectively. These results obtained with the untreated biomass are very similar to those obtained with the commercial biofertilizer product, which leads to GIs of 101.2, 98.6 and 96.5% at 1, 0.5 and $0.1\,g\,L^{-1}$, respectively. According to the supplier's instructions, this product should be used at $2\,g\,L^{-1}$, but in order to compare with the microalgae extracts, the commercial product was diluted to the same concentrations as tested in this work. These results could be improved with biomass pretreatment by HPH at 200 bar and only one pass through the homogenizer, which slightly improved the cell disruption with respect to the control (Fig. 1), obtaining in this case 102.97, 109.09 and 112.72%, respectively, corresponding to an improvement of almost 10% in the germination index. Other authors, such as Garcia-Gonzalez and Sommerfeld [8], also reported a higher germination velocity and a significant improvement in the appearance of tomato seeds in contact with cell extracts from the microalgae *Acutodesmus dimorphus*.

A multifactor ANOVA was carried out to analyse the effect of the factors and their interactions studied herein (pressure, number of passes and extract concentration) on the germination index observed in the watercress seeds, although the only interactions observed with the extract concentration were between the pressure and the number of passes; these results are shown in Table 2. All the factors, including the two interactions analysed, had a statistically significant effect on the studied variables at the 95% confident level (*p*-value<0.05). Regarding Table 2, the extract concentration at which it is necessary to dilute samples to carry out the bioassays is the most important factor affecting the germination index. The number of passes through the homogenizer for each biomass batch is not as important as other factors such as the pressure or the extract concentration. Therefore, given that energy consumption increases with each pass (Table 1), and that this parameter does not have much influence on obtaining the biostimulants (as will be seen later in extracting other bioproducts), it is preferable that the biomass only passes through the homogenizer once.

Stirk et al. [28] analysed the biological activity of different extracts from *Chlorella* sp., *Chlorella vulgaris* and *Scenedesmus acuutus* after pretreatment with different cell disruption methods, and observed a decrease in the activity of the sonicated and milled extracts from *Scenedesmus acuutus*, this negative response being greater the higher the extract concentration. These authors point out that the decrease in biostimulant activity may be due to several reasons such as the possibility that the bioactive metabolites were heat sensitive, and were thus degraded by the highest treatment intensities reached in these cell disruption methods, or the possibility that, in the case of the disrupted samples, the active compounds could be oxidised or degraded faster during the storage of the extracts prior to carrying out the bioassays.

In the case of the HPH, an increase in the treatment intensity led to an increase in the pretreated biomass temperature (from 18 °C in the initial biomass to 40 °C after treatment at 800 bar and 5 passes). Consequently, to determine if the decrease in biostimulant activity resulting from the treatment intensity might be due to the molecular degradation caused by the higher temperatures associated with this pretreatment, rather than due to greater cell disruption (and the resulting higher concentration of certain compounds), the microalgal biomass samples were kept at temperatures ranging from 20 to 90 °C for 30 min, after which time the biostimulant activity of the supernatants was determined by bioassays to calculate the GI under these conditions.

As Fig. 3 shows, the temperature does not seem to have an influence on the biostimulant activity in the microalgal extracts since only a slight decrease was observed in the GI when the biomass was maintained at 90 °C for 30 min - a temperature far higher than any reached in our experiments.

3.1.1.2. Quantification of salicylic acid and total phenols. Previous studies have demonstrated that certain microalgal extracts enhance the growth of agricultural crops; this has been attributed to the presence of plant-growth regulators in the microalgal biomass such as gibberellins, cytokinins and auxins, as well as micro and macro nutrients, which have been widely reported as playing an important role in plant development ([30]; Shaaban et al., 2001). However, several authors have reported an inhibitory effect on seed growth when certain biomolecules are present, or when they are present in the extracts at certain concentrations. This is the case with Niemann and Dörffling [19] and Charles and Hemingway (1965), who established that the cause of this growth inhibition may be because certain biomolecules, such as abscisic acid, form inhibitory complexes with lunularic or salicylic acid, among others. Likewise, Davies (2006) and Tarakhovskaya et al. [30] reported that phytohormones are bioactive compounds that influence physiological processes in plants at low concentrations and can inhibit at higher concentrations.

In this study, the total phenols and salicylic acid were quantified for each HPH condition applied in the pretreatment; this was done in order to discount treatment intensity as the cause of these biomolecules' degradation, and to try to relate the seed germination inhibition to increasing concentration. Samples were prepared as explained in Section 2.7, and determined by spectrophotometric methods and HPLC-DAD, respectively.

As Fig. 4 shows, both the total phenols and salicylic acid concentrations in the treated biomass increased with pressure and the number of passes. This is because with higher treatment intensity comes greater cell disruption (Fig. 1) and thus a higher concentration of the compounds of interest was obtained. In both cases, the maximum concentrations of total phenols and salicylic acid in the extracts diluted to $0.5\,g\,L^{-1}$ were obtained at the highest treatment intensity, namely 800 bar and 5 passes ($0.128\,mg\,GAE\,g\,DW^{-1}$ and $4.5\cdot10^{-3}\,\mu g\,SA\,g\,DW^{-1}$, respectively).

However, the samples with the higher total phenols and SA concentrations resulted in lower GIs for the three extract concentrations tested (1, 0.5 and $0.1\,g\,L^{-1}$), which may be due to the excess concentration of certain compounds that turn out to have a toxic rather than biostimulant effect on the seeds. At each treatment pressure, the highest contents of salicylic acid and total phenols were obtained when the biomass passed 5 times through the homogenizer, supposedly when the cell disruption (at each pressure tested) is maximum (salicylic acid content: $6.2\cdot10^{-4}$, $1.37\cdot10^{-3}$, $2.35\cdot10^{-3}$ and $4.55\cdot10^{-3}\,\mu g\,SA\,g\,DW^{-1}$ and total phenols content: 0.055, 0.0693, 0.0863, 0,1283 mg GAE g DW^{-1} for pressures of 200, 400, 600 and 800 bar, respectively). These maximum concentrations obtained of salicylic acid and total phenols coincide with the minimum germination index observed when seeds were in contact with these microalgae extracts diluted to $0.5\,g\,L^{-1}$ (97.05, 88.61, 65.33 and 63.78%, respectively). Therefore, there is a toxic effect on the watercress seeds in contact with these microalgae extracts when the concentration of the biostimulant molecules analysed in this work increases.

Many authors have characterized a variety of extracts to determine their phenol and phytohormone contents, given their direct relationship to plant development. Pereira et al. [21] analysed different microalgae extracts from species such as *Picohlorum* sp. and *Nannochloropsis* sp. observing that, in both, microalgal salicylic acid was the main phenolic
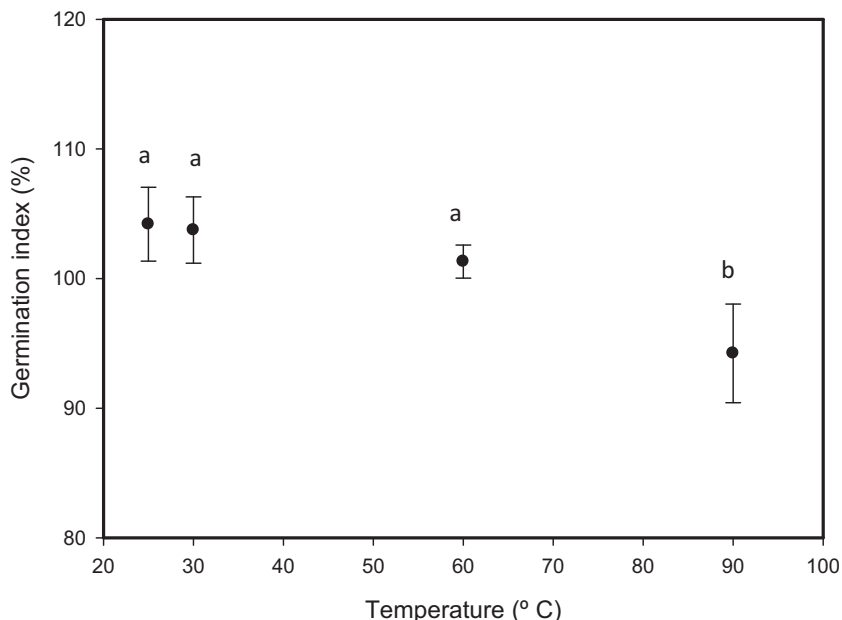
Fig. 3. Stability of the molecules related to the biostimulant activity with the increased incubation temperature of the microalgal extracts. Results are shown as mean $\pm$ SD ($n = 2$). Values with different cover letters are significantly different, *p*-value<0.05.
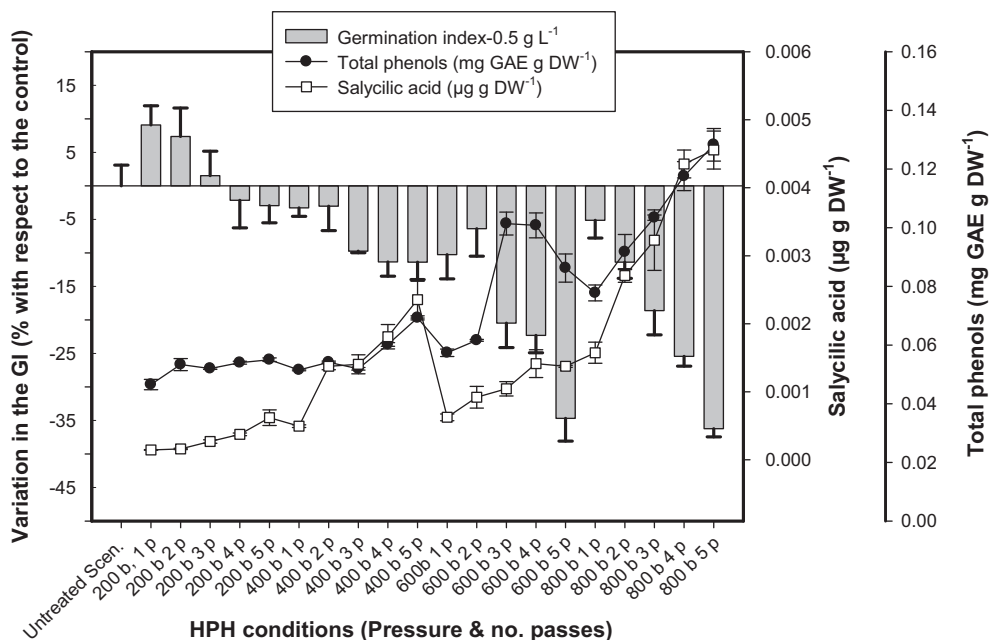

Fig. 4. Total phenols and salicylic acid contents compared to the variation in the germination index obtained for each high-pressure homogenization (HPH) condition (pressure & no. of passes) for an extract concentration of 0.5 g L^{-1}. Results are shown as mean $\pm$ SD ($n = 2$ for the germination index and $n = 3$ for the total phenols and salicylic acid contents).

compound with concentrations of 0.64 mg g extract^{-1}, DW and 0.14 mg g extract^{-1}, DW, respectively, which are higher than those observed in *Scenedesmus*. Other authors, such as Mouget [16], analysed the antioxidant activity of three microalgae species and determined a total phenol content of 17.8, 16.9 and 4.7 mg Gallic acid g extracts^{-1} for *I. galbana*, *Tetraselmis Chuii* and *Dunaliella salina,* respectively.

3.1.2. Effect of HPH on the degree of hydrolysis
Carrying out enzymatic hydrolysis, with the goal of obtaining free amino acid concentrates from the microalgal biomass, requires three

steps. The first consists of protein solubilization, the second, hydroxylation, and the last involves the separation of the products from the waste biomass [25]. To perform the first protein solubilization step on the *Scenedesmus* sp. biomass, it is necessary to consider which cell disruption method to use for breaking down the cell walls and for making the reaction and extraction of products easier. In this case, only mechanical cell disruption by HPH was considered. Therefore, as explained before, the experiments were carried out by applying different treatment intensities and the resulting treated biomass was subjected to enzymatic hydrolysis. The results are shown in Fig. 5.
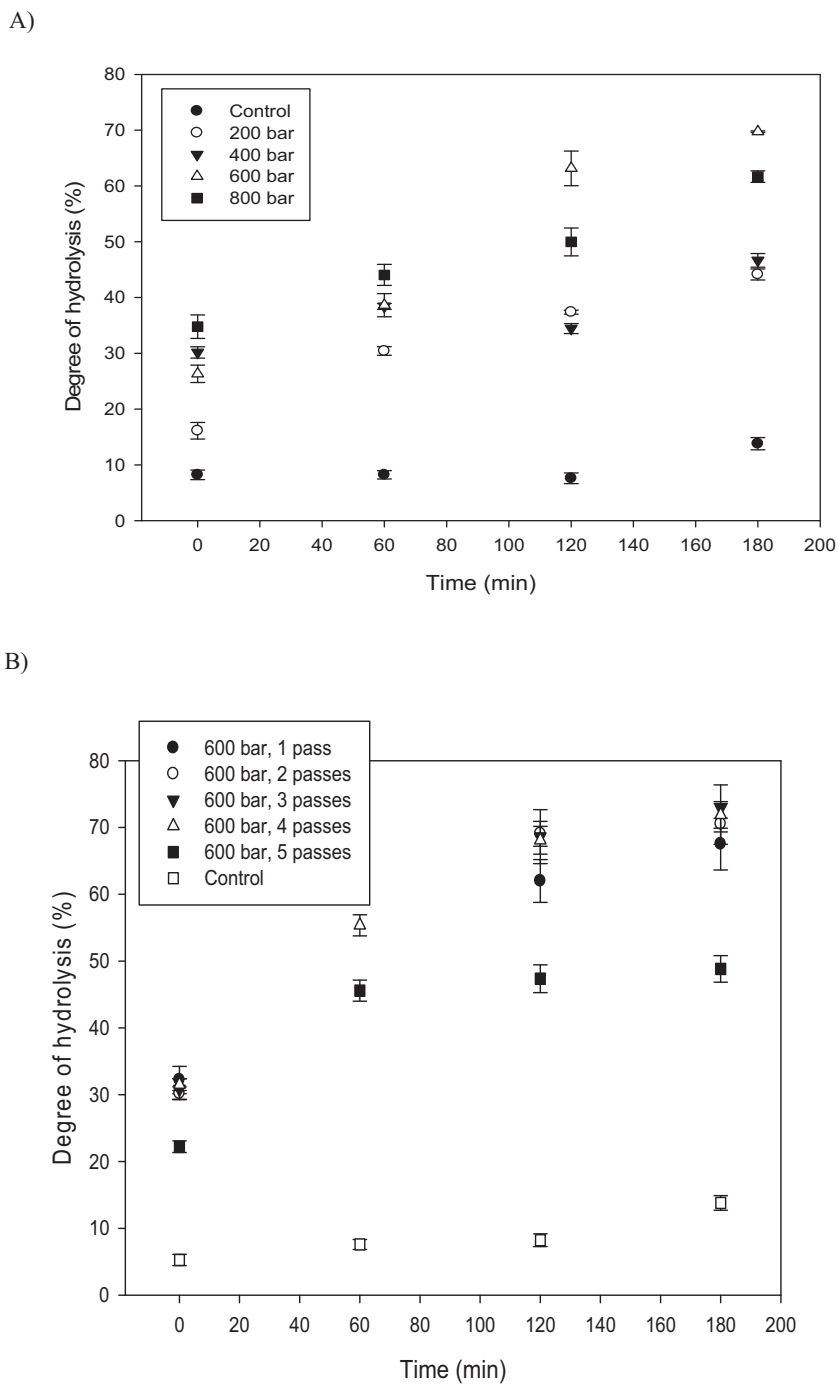
A)

B)

Fig. 5. Influence of the high-pressure homogenization (HPH) conditions on the enzymatic hydrolysis of *Scenedesmus* sp. biomass. A) Homogenization pressure (0–800 bar). B) Number of passes through the homogenizer (1–5 passes) at a treatment pressure of 600 bar. The enzymatic hydrolysis was performed twice and, at each time, the samples were analysed in triplicate; therefore, data points are averages and vertical bars are standard deviations (SD).

Fig. 5A shows the degree of hydrolysis achieved for each treatment pressure. One can observe that the degree of hydrolysis increases in line with the pressure at which the pretreatment is carried out, both at the initial stage when the reaction has not yet begun and after the enzymatic treatment. The results show that it is not necessary to apply the maximum treatment intensity to achieve a high degree of hydrolysis. In this case, the highest degree of hydrolysis, 65.7%, was achieved when treating the biomass at 600 bar; whereas applying 800 bar led to a degree of hydrolysis of only 61.7%. In all instances, applying any of the homogenization pressures tested in this work resulted in higher degrees of hydrolysis compared to the untreated biomass (in which a degree of hydrolysis of only 13% was reached after 180 min) - thus justifying the use of this mechanical cell disruption method to obtain the amino acid concentrates that can be used as a biofertilizer product. Once the optimal treatment pressure was selected, the next step was to optimize the number of passes that the biomass made through the homogenizer at 600 bar (Fig. 5B). The results are shown in Fig. 5B; they reveal that the lowest degree of hydrolysis was reached when the biomass passed through the homogenizer 5 times, maybe because the biomass was degraded at this higher treatment intensity, leading to destabilization of the weak protein bonds and thus to changes in certain functional properties of the proteins such as water binding capacity, rheological

properties, solubility and enzyme activity, all negatively affecting the degree of hydrolysis [24]. Therefore, the number of passes increased the degree of hydrolysis from 65% (when the biomass passed through the homogenizer only once) to 72.9% (when the same batch passed through three times). In any case, the higher the treatment intensity, the greater the energy consumption of the process, so lower treatment intensities are desirable to reduce the cost associated with biofertilizer production. Other authors, such as Romero García et al. [25], optimized the enzymatic hydrolysis of the microalgae *Scenedesmus almeriensis* obtaining yields of around 60% under the optimally established conditions; the results were very similar to those obtained in this work.

One of the main applications of these amino acid concentrates is as biofertilizers used to increase agricultural crop yield. Authors such as Plaza et al. [23] studied the foliar application of *Artrosphira platensis* and *Scenedesmus* sp. hydrolysates and observed higher rates of plant growth and earlier flowering. Other microalgae bioproducts are sold mainly as a source of amino acids and are used in various applications; these include as antioxidants [1], human and animal feed [6,29] or biofuels [25].

4. Conclusions

From the work carried out in this study, it can be concluded that *Scenedesmus* sp. grown in pig manure can be used as a microalgal raw material for producing biostimulants and amino acid concentrates, which have several applications. For each product, the pretreatment of the wet biomass using high-pressure homogenization must be optimized to select the most favourable conditions. We found that a soft treatment of *Scenedesmus* sp. biomass at 200 bar was enough to improve the GI by almost 10%. In addition, a toxic effect was observed on the seed growth as the pretreatment intensity increased, probably because of the higher concentrations of certain biomolecules (salicylic acid and phenolic compounds among them), which begin to inhibit seed growth. The same inhibitory effect was observed when microalgae extracts with a concentration of $1 \, g \, L^{-1}$ were directly applied to the seeds. In the case of the amino acid concentrates, the best results were obtained at 600 bar, achieving a 65.7% degree of hydrolysis when passing the biomass through the homogenizer only once; this was markedly higher than that obtained for the untreated biomass, which reached only 14% after 180 min of reaction.

CRediT authorship contribution statement

Elvira Navarro-López: Methodology, Data curation, Writing - original draft. **María del Carmen Cerón-García:** Visualization, Supervision, Writing - review & editing. **Mercedes López-Rodríguez:** Methodology, Data curation, Formal analysis. **Francisco Gabriel Acién-Fernández:** Supervision, Writing - review & editing, Project administration. **Emilio Molina-Grima:** Supervision, Writing - review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

All the authors have contributed to the manuscript. All contributed to the research design, data analyses, revision, editing and approval of the final manuscript.

References

[1] A.E.M.M.R. Afify, G.S. El Baroty, F.K. El Baz, H.H. Abd El Baky, S.A. Murad, Scenedesmus obliquus: antioxidant and antiviral activity of proteins hydrolyzed by three enzymes, J. Genet. Eng. Biotechnol. (2018), https://doi.org/10.1016/j.jgeb.2018.01.002.

[2] A. Bai, L. Stündl, P. Bársony, M. Fehér, P. Jobbágy, Z. Herpergel, G. Vaszkó, Algae production on pig sludge, Agron. Sustain. Dev. 32 (2012) 611–618, https://doi.org/10.1007/s13593-011-0077-2.

[3] M.A. Borowitzka, High-value products from microalgae—their development and commercialisation, J. Appl. Phycol. 25 (2013) 743–756, https://doi.org/10.1007/s10811-013-9983-9.

[4] L. Brennan, P. Owende, Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products, Renew. Sust. Energ. Rev. 14 (2010) 557–577, https://doi.org/10.1016/J.RSER.2009.10.009.

[5] J. Camacho-Rodríguez, A.M. González-Céspedes, M.C. Cerón-García, J.M. Fernández-Sevilla, F.G. Acién-Fernández, E. Molina-Grima, A quantitative study of eicosapentaenoic acid (EPA) production by Nannochloropsis gaditana for aquaculture as a function of dilution rate, temperature and average irradiance, Appl. Microbiol. Biotechnol. 98 (2014) 2429–2440, https://doi.org/10.1007/s00253-013-5413-9.

[6] A. Clemente, Enzymatic protein hydrolysates in human nutrition, Trends Food Sci. Technol. 11 (2000) 254–262, https://doi.org/10.1016/S0924-2244(01)00007-3.

[7] F. Du, G. Ruan, H. Liu, Analytical methods for tracing plant hormones, Anal. Bioanal. Chem. 403 (2012) 55–74, https://doi.org/10.1007/s00216-011-5623-x.

[8] J. Garcia-Gonzalez, M. Sommerfeld, Biofertilizer and biostimulant properties of the microalga Acutodesmus dimorphus, J. Appl. Phycol. 28 (2016) 1051–1061, https://doi.org/10.1007/s10811-015-0625-2.

[9] M.E. Ghanem, A. Albacete, C. Martínez-Andújar, M. Acosta, R. Romero-Aranda, I.C. Dodd, S. Lutts, F. Pérez-Alfocea, Hormonal changes during salinity-induced leaf senescence in tomato (Solanum lycopersicum L.), J. Exp. Bot. 59 (2008) 3039–3050.

[10] C.V. González López, M. del C.C. García, F.G.A. Fernández, C.S. Bustos, Y. Chisti, J.M.F. Sevilla, Protein measurements of microalgal and cyanobacterial biomass, Bioresour. Technol. 101 (2010) 7587–7591, https://doi.org/10.1016/J.BIORTECH.2010.04.077.

[11] M.J. Jiménez Callejón, A. Robles Medina, M.D. Macías Sánchez, E. Hita Peña, L. Esteban Cerdán, P.A. González Moreno, E. Molina Grima, Extraction of saponifiable lipids from wet microalgal biomass for biodiesel production, Bioresour. Technol. 169 (2014) 198–205, https://doi.org/10.1016/J.BIORTECH.2014.06.106.

[12] G. Kumar, D. Sahoo, Effect of seaweed liquid extract on growth and yield of Triticum aestivum var, Pusa Gold. J. Appl. Phycol. 23 (2011) 251–255, https://doi.org/10.1007/s10811-011-9660-9.

[13] A.K. Lee, D.M. Lewis, P.J. Ashman, Disruption of microalgal cells for the extraction of lipids for biofuels: processes and specific energy requirements, Biomass Bioenergy 46 (2012) 89–101, https://doi.org/10.1016/J.BIOMBIOE.2012.06.034.

[14] A.P. Middelberg, Process-scale disruption of microorganisms, Biotechnol. Adv. 13 (1995) 441–501.

[15] del M. Morales-Amaral, C. Gómez-Serrano, F.G. Acién, J.M. Fernández-Sevilla, E. Molina-Grima, Outdoor production of Scenedesmus sp. in thin-layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source, Algal Res. 12 (2015) 99–108, https://doi.org/10.1016/J.ALGAL.2015.08.020.

[16] I.W. Mouget, Z. M., P.K. H., S. R., H. Y., L. V., B. N., L. J., Antioxidant activity of three microalgae Dunaliella salina, Tetraselmis chuii and Isochrysis galbana clone Tahiti, IOP Conf. Ser. Earth Environ. Sci. 55 (2017) 12067.

[17] E. Navarro López, A. Robles Medina, P.A. González Moreno, M.J. Jiménez Callejón, L. Esteban Cerdán, L. Martín Valverde, B. Castillo López, E. Molina Grima, Enzymatic production of biodiesel from Nannochloropsis gaditana lipids: influence of operational variables and polar lipid content, Bioresour. Technol. 187 (2015) 346–353, https://doi.org/10.1016/J.BIORTECH.2015.03.126.

[18] P.M. Nielsen, D. Petersen, C. Dambmann, Improved method for determining food protein degree of hydrolysis, J. Food Sci. 66 (2006) 642–646, https://doi.org/10.1111/j.1365-2621.2001.tb04614.x.

[19] D.I. Niemann, K. Dörffling, Growth-inhibitors and growth-promoters in enteromorpha compress A (chlorophyta)1, J. Phycol. 16 (1980) 383–389, https://doi.org/10.1111/j.1529-8817.1980.tb03049.x.

[20] V. Nour, I. Trandafir, S. Cosmulescu, HPLC determination of phenolic acids, flavonoids and Juglone in walnut leaves, J. Chromatogr. Sci. 51 (2013) 883–890.

[21] H. Pereira, L. Custódio, J.M. Rodrigues, B.C. de Sousa, M. Oliveira, L. Barreira, D.N. Neng, M.J. Nogueira, A.S. Alrokayan, F. Mouffouk, M.K. Abu-Salah, R. Ben-Hamadou, J. Varela, Biological activities and chemical composition of Methanolic extracts of selected autochthonous microalgae strains from the Red Sea, Mar. Drugs. (2015), https://doi.org/10.3390/md13063531.

[22] J.K. Pittman, A.P. Dean, O. Osundeko, The potential of sustainable algal biofuel production using wastewater resources, Bioresour. Technol. 102 (2011) 17–25, https://doi.org/10.1016/J.BIORTECH.2010.06.035.

[23] B.M. Plaza, C. Gómez-Serrano, F.G. Acién-Fernández, S. Jimenez-Becker, Effect of microalgae hydrolysate foliar application (Arthrospira platensis and Scenedesmus sp.) on Petunia x hybrida growth, J. Appl. Phycol. 30 (2018) 2359–2365, https://doi.org/10.1007/s10811-018-1427-0.

[24] B.C. Porto, A.A.L. Tribst, M. Cristianini, Dynamic high pressure effects on bioplymers: polysacharides and proteins, Bioplymers for food design (2018) 313–350.

[25] J.M. Romero García, F.G. Acién Fernández, J.M. Fernández Sevilla, Development of a process for the production of l-amino-acids concentrates from microalgae by enzymatic hydrolysis, Bioresour. Technol. 112 (2012) 164–170, https://doi.org/10.1016/J.BIORTECH.2012.02.094.

[26] N. Samarasinghe, S. Fernando, R. Lacey, W.B. Faulkner, Algal cell rupture using high pressure homogenization as a prelude to oil extraction, Renew. Energy 48 (2012) 300–308, https://doi.org/10.1016/J.RENENE.2012.04.039.

[27] E.M. Spiden, B.H.J. Yap, D.R.A. Hill, S.E. Kentish, P.J. Scales, G.J.O. Martin, Quantitative evaluation of the ease of rupture of industrially promising microalgae by high pressure homogenization, Bioresour. Technol. 140 (2013) 165–171, https://doi.org/10.1016/J.BIORTECH.2013.04.074.

[28] W.A. Stirk, P. Bálint, M. Vambe, C. Lovász, Z. Molnár, J. van Steden, V. ÖrdÖg, Effect of cell disruption methods on the extraction of bioactive metabolites from microalgal biomass, J. Biotech. 307 (2020) 35–43, https://doi.org/10.1016/j.jbiotec.2019.10.012.

[29] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae, J. Biosci. Bioeng. 101 (2006) 87–96, https://doi.org/10.1263/JBB.101.87.

[30] E.R. Tarakhovskaya, Y.I. Maslov, M.F. Shishova, Phytohormones in algae, Russ. J. Plant Physiol. 54 (2007) 163–170, https://doi.org/10.1134/S1021443707020021.

[31] B.H.J. Yap, G.J. Dumsday, P.J. Scales, G.J.O. Martin, Energy evaluation of algal cell disruption by high pressure homogenisation, Bioresour. Technol. 184 (2015) 280–285, https://doi.org/10.1016/J.BIORTECH.2014.11.049.

[32] H.-W. Yen, I.-C. Hu, C.-Y. Chen, S.-H. Ho, D.-J. Lee, Microalgae-based biorefinery – from biofuels to natural products, Bioresour. Technol. 135 (2013) 166–174, https://doi.org/10.1016/J.BIORTECH.2012.10.099.