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

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Abstract

Microalgae are becoming an important source of high-value products such as biostimulants, biopesticides, and other low-value products like 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 drawback, mechanical cell disruption methods such as high-pressure homogenization (HPH), can be applied to facilitate extracting the compounds of interest.

This work focuses on optimizing the extraction of two major groups of products from microalgae biomass - biostimulants and biofertilizers. The aim of this research is to study the effect of the HPH pre-treatment on the production of these different bioproducts starting with *Scenedesmus* sp. biomass grown in pig sludge as the raw material.

For each product the pre-treatment of the wet biomass by HPH must to be optimized to select the optimal conditions for the pressure and the number of passes. A soft treatment of the wet biomass at 200 bar is enough 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 applying greater treatment intensities increases the concentration of the molecules responsible for the bioactivity thus causing a toxic effect on the seeds. In the case of biofertilizers, better results have been obtained at 600 bar, achieving a hydrolysis level of 72.9 %. Finally, the determination of the concentrations of phenolic compounds and salicylic acid, related with enhanced biostimulant activity, was carried out using spectrophotometric methods and High-Performance Liquid Chromatography- diode array (HPLC), respectively.

Keywords: Bioproducts, high-pressure homogenization, microalgae, salicylic acid, phenols

1. Introduction

The potential of microalgae as a source of various important agricultural or aquaculture products has attracted considerable interest over recent decades. Currently, agriculture has to face not only the growing global population with the consequent resource depletion but also the effect of climate change on crops (Borowitzka, 2013; Garcia-Gonzalez and Sommerfeld, 2016). Therefore, it is a priority to develop commercial products from environmentally friendly resources, such as microalgae, which 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 due to the photosynthetic fixation of atmospheric carbon dioxide, facilitating a reduction in the levels of this gas. However, microalgal production at the large scale must overcome several drawbacks such as requiring a considerable input of growth nutrients, with the associated high costs. To solve this problem, using pig manure in microalgae production as part of a nutrient recovery system seems to be a good alternative for reducing the cost associated with the nutrient requirements, recycling the excess nitrogen and phosphorous from the pig manure and, moreover, helping to prevent the environmental damage caused by piggery wastewater (Bai et al., 2012; Brennan and Owende, 2010; Pittman et al., 2011). In addition to the nutrients introduced from the pig manure, it is necessary 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 (Yen et al., 2013).

Moreover, including cell breakage of the microalgae as a prior step to solvent extraction has been found to considerably improve the extraction yield of the compounds of interest, especially in those species which 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) (Middelberg, 1995). In general, it seems that mechanical treatments are more advantageous than the other cell-disruption techniques as they prevent cells from becoming contaminated with chemicals and their effectiveness is less dependent on the microalgae species (Lee et al., 2012). With regard to the possible mechanical methods, high-pressure homogenization has attracted much 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 (Samarasinghe et al., 2012; Spiden et al., 2013; Yap et al., 2015).

Many authors have researched the positive effects of algae extracts on seed germination and growth promotion such as Hernández-Herrera et al. (2014), who studied the effect of different seaweed extracts on tomato seedling growth or García-Gonzalez et al. (2016), 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 with the presence of phytohormones and other phenolic compounds which play an important role on the plant development (Du et al., 2012; Garcia-Gonzalez and Sommerfeld, 2016, Nour et al., 2013)

The objective of this study, therefore, is to optimize the biomass pre-treatment 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 pre-treatment in obtaining biofertilizers 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 for obtaining the bioproducts. The cells were grown from September-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 culture level in the sump (Morales-Amaral et al., 2015). 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 with 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 biofertilizers were Alcalase 2.5L (2.5 AU-A/g) and Flavourizyme 1000L (1000 AU-A/g), both supplied by Novozymes A/S (Bagsvaerd, Denmark). These are commercial enzymes used in the production of amino acid hydrolysate from yeast and other substrates, 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). Acetic acid glacial 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 pre-treatment by high-pressure homogenization

Biomass from *Scenedesmus* sp. grown in pig manure was pre-treated 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 in 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) with 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} of dry biomass (obtained by applying each HPH condition), were transferred to 100 mL Erlenmeyer flasks and incubated at 30 ºC for 2 hours with magnetic stirring at 300 rpm in a heating magnetic stirrer (Multimix Heat D, Ovan, Spain). The extracts were centrifugated 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} to carry out 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 distilled water (the control) or the extracts resulting from each treatment condition (a pressure of between 200-800 bar and from 1-5 passes). Seeds were allowed to grow for 3 days at 24ºC in darkness and 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 by the following equation:

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

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

The data shown in the biostimulant activity experiments are, therefore, the results from measuring 200 seeds for each 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) with a heating magnetic stirrer (Multimix Heat D, Ovan, Spain) at a temperature of 50 ºC 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 50ºC. The pH is adjusted to 8 by adding NaOH 1M. Then, 0.08 g of alcalase 2.5L (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 1M to maintain the pH at 8. Two hours after starting the reaction, the pH is lowered to 7 by adding H_2SO_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 75ºC for 15 min to deactivate the enzymes. The final concentrate, rich in amino acids, is separated from the biomass by centrifugation at 7000 rpm (Centrifuge Brand Supelco 4-15, Sartorius, Germany).

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 by the OPA (o-phthaldialdehyde) method using serine as the standard, and assuming the following parameter values for the model α= 1, β=0.4 and yht_{ot}=8 (Nielsen et al., 2006).

2.6. Biochemical composition determination

The protein content was determined following the method described by González López et al., (2010), 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., (2015). 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 ºC for 24 h while the carbohydrate content (%) was determined as the difference between 100 and the sum of the percentages of the other fractions (ashes, protein and lipids) (Camacho-Rodríguez et al., 2014).

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 pre-treated biomass (for 2 hours at 30 ºC and 300 rpm). Then, the samples were centrifuged at 12000 rpm for 5 min (Micro-centrifuge Minispin®plus, Eppendorf, with an F-45-12-11 rotor), and the supernatants were collected for characterization. The total polyphenols were determined by the Folin-Ciocalteu method (Waterman and Mole, 1994). Aliquots (0.1 mL) of 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 minutes, 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 hours 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 duplicated.

For the salicylic acid quantification, a previous step of sample purification is needed following the method described by Ghanem et al., (2008) with several modifications. In this case, supernatants were collected and allowed to pass through Sep-Pack plus C-18 cartridge (Sep-Pack plus, Waters, USA) to remove interfering lipids and some of the plant pigments. Cartridges were conditioned with 2 mL of water and 2 mL of methanol. Subsequently, 2 mL of these purified supernatants were lyophilized to eliminate water and 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 its 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 pre-treatment, 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 absorbances of supernatants of the pre-treated samples also gives information about the increase or not of the quantity of water-soluble components and thus, of the degree of cell disruption achieved in each case (Porto et al., 2018). Figure 1 shows the comparison of absorbances determined at wavelengths between 600-800 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 if compared to the control (*Scenedesmus* sp. at 40 g L^{-1} without any pre-treatment). The same occurred increasing the number of passes, with in turn led to an increase in absorbance (data not shown). Therefore, it can be concluded that the greater the pre-treatment intensity of the biomass (both with an increase in the pressure and the number of passes), the greater the cell disruption observed. Figure 1 shows the normalized absorbances, calculated as the relationship between the absorbance of each sample and the absorbance of the control (untreated biomass).

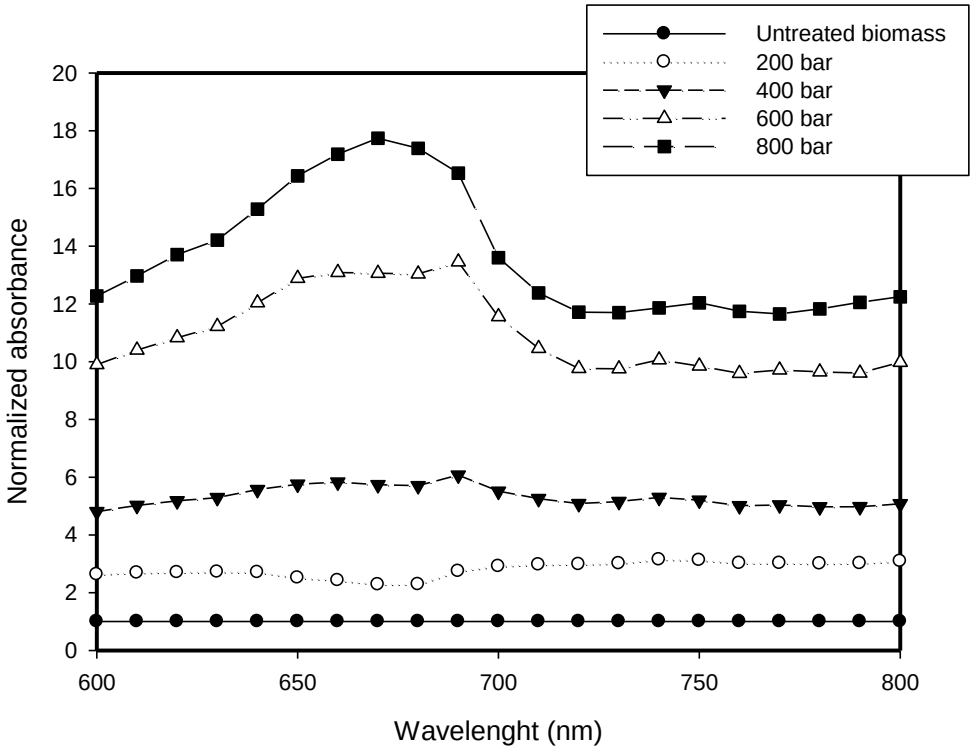


Figure 1. Measure of absorbances between 600-800 nm for each HPH treatment pressure applied.

Another important parameter that should be considered is the energy consumption associated with this treatment. Table 1 shows the homogenizer energy consumption expressed as MJ kg-1 of dry biomass for each pre-treatment condition considered in this study.

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.

	Energy consumption (MJ Kg dry biomass^{-1})				
Pressure (bar)	**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

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 it is not necessary to reach the maximum pressure in order to obtain a high extraction yield for several of the compounds of interest, so 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 1.8-11.4 MJ Kg dry biomass^{-1} depending on the number of passes. Other authors as Jiménez Callejón et al., (2014) used the HPH as cell disruption method on the processing of *Nannochloropsis gaditana* biomass with a 14% of solid content and described an energy consumption of 4.5-5.3 MJ Kg dry biomass^{-1} applying pressures of 500 and 1500 bar, respectively, and only one pass through the homogenizer. This energy consumption is greater to that this described in this 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, so the greater solid content of the biomass, the higher energy consumption of the pre-treatment.

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 following the equation 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 supposed when seeds are in contact with distilled water, so only extracts that result in a GI higher than 100 % are considered as promoting biostimulant activity. Figure 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 (increase or decrease of the GI with respect to the control), whereas Table 2 provides a statistical analysis of the above-mentioned factors.

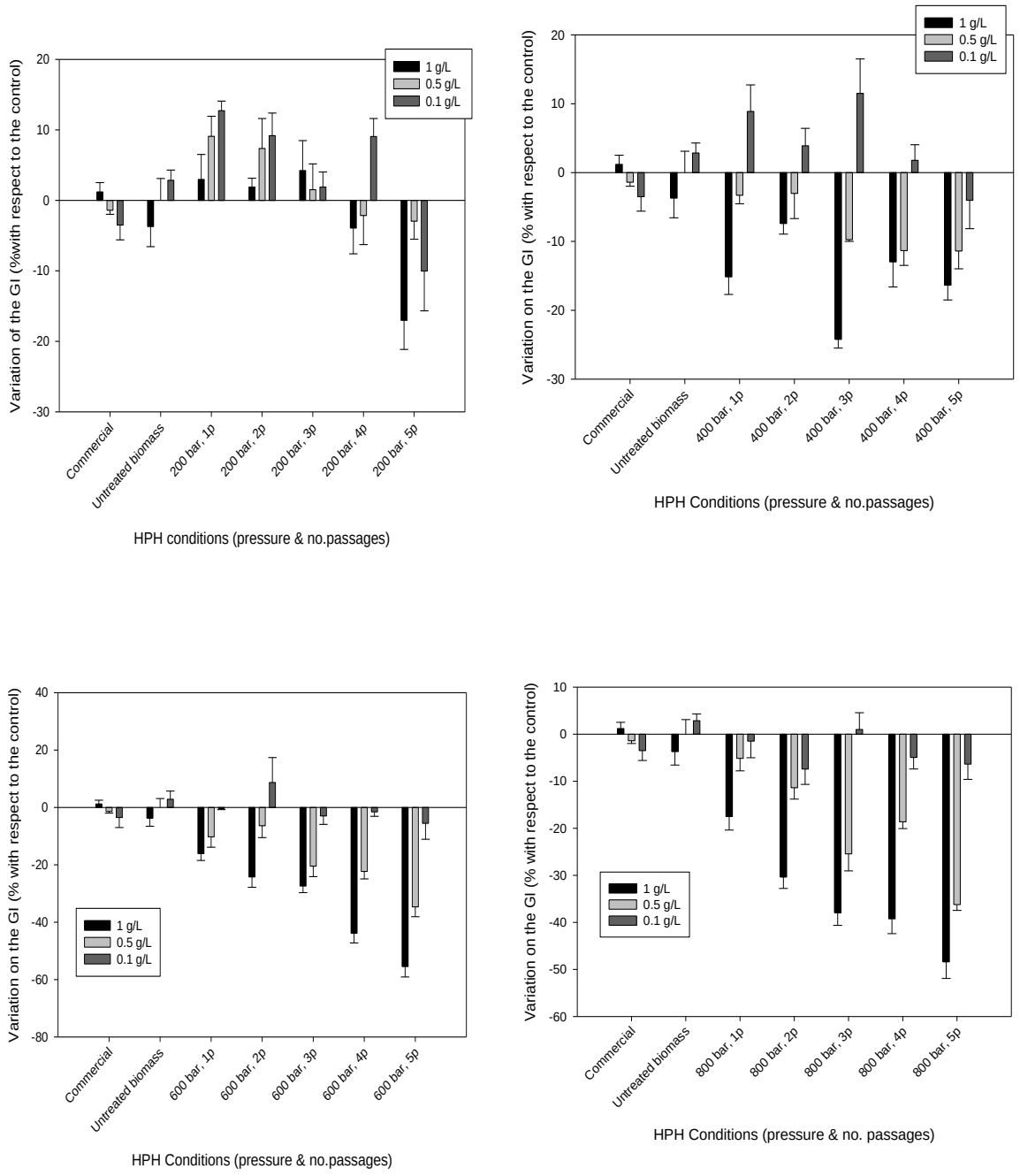


Figure 2 Influence of the HPH conditions (pressure & no.passages) on the germination index of the watercress seeds diluting the microalgal extracts to 1, 0.5 and 0.1 g/L.

As it is shown in Figure 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 pre-treatment pressure increased. As can be observed in Figure 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 determined stimulant compounds, which exert a toxic effect on the seeds 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 the 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 pre-treatment was performed at 800 bar, where 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 (2011), who also observed growth inhibition in *Triticum aestivum* var. Pusa Gold along with an increase in the seaweed liquid extract concentration. Some authors as Niemann and Dörffling, (1980) have described the presence of inhibitory complex that avoid the plant growth in the bioassays. It is supposed by these authors that some bioactive compounds as abscisic acid (ABA) produced shoot growth suppression when they act on their own or forming inhibitory complex with other biomolecules such as lunularic acids, salicylic acid and other unidentified compounds. This fact could be the reason of obtaining results which show inhibitory effect on the bioassays applied in this work when the pre-treated microalgal biomass of *Scenedesmus sp.* and moreover when the microalgal extracts are diluted to the highest concentration, 1 g L^{-1}, because of the highest concentration of these biomolecules responsible of the growth inhibition. Authors as Plaza et al., (2018) analysed the hormone content in the hydrolysed of *Scenedesmus* sp. and *Arthrospira*

sp. and determined an ABA content of 3718.3 and 1.03 ng g^{-1} in both microalgae respectively, among others phytohormones such as gibberellins or salicylic acid.

In any case, when *Scenedesmus* sp. microalgal biomass (without pre-treatment) was used to perform the bioassays, the GI of the seeds were 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 GI 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 tested in this work. These results could be improved with biomass pre-treatment by HPH at 200 bar and only one pass through the homogenizer, which slightly improved the cell disruption with respect to the control (Figure 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, (2016) 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 in order 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.

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

On the other hand, an increase in the treatment intensity leads to an increase in the pre-treated biomass temperature. Consequently, to determine if the decrease observed in the GI that occurred as a result of increased treatment intensity might be due to molecular degradation at the higher temperatures associated to this pre-treatment rather than due to the greater cell disruption (and the resulting higher concentration of certain compounds), the microalgal biomass samples were kept at temperatures ranging from 20-90 ºC for 30 min, after which the biostimulant activity of the supernatants was determined by bioassays to calculate the GI under these conditions.

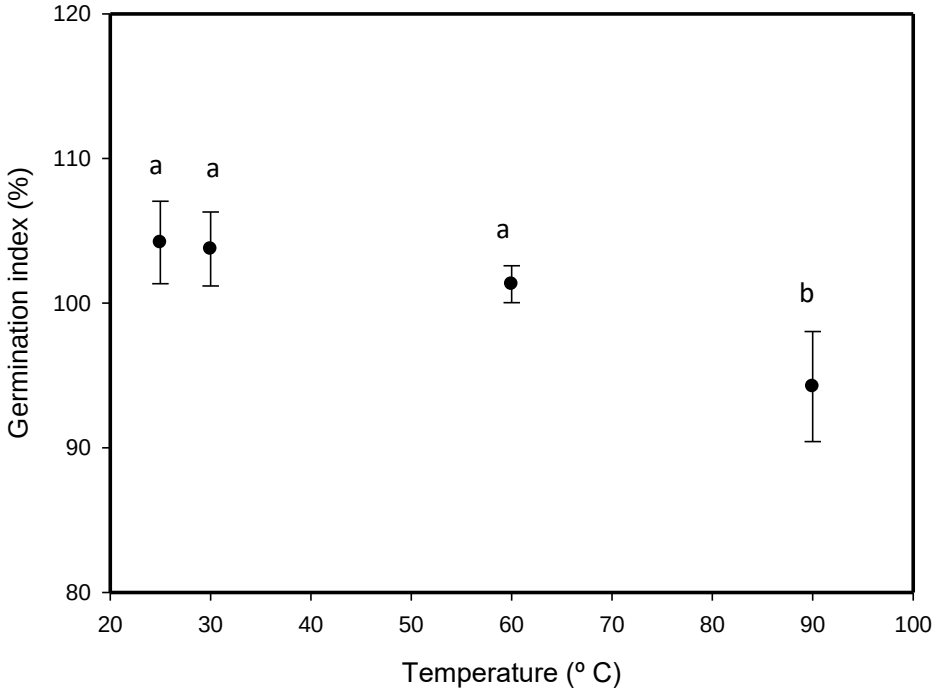


Figure 3 Stability of the molecules related to the biostimulant activity with the increased incubation temperature of the microalgal extracts. Values with different cover letters are significantly different, p-value<0.05.

As is shown in Figure 3, it seems that the temperature had no 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 90ºC 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 microalgae extracts enhance the growth of agricultural crops, which 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 (Tarakhovskaya et al., 2007; Shaaban et a., 2001). In this study, the total phenols and salicylic acid were quantified for each HPH condition applied

in the pre-treatment. Samples were prepared as explained in Section 2.7 and determined by spectrophotometric methods and HPLC-DAD, respectively.

As Figure 4 shows, both the total phenols and salicylic acid concentrations in the treated biomass increase with pressure and the number of passes. This is because with higher treatment intensity comes greater cell disruption (Figure 1) and, thus, higher concentration of the compounds of interest were 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 $4.5 \cdot 10^{-3}$ µg SA g DW^{-1}, respectively).

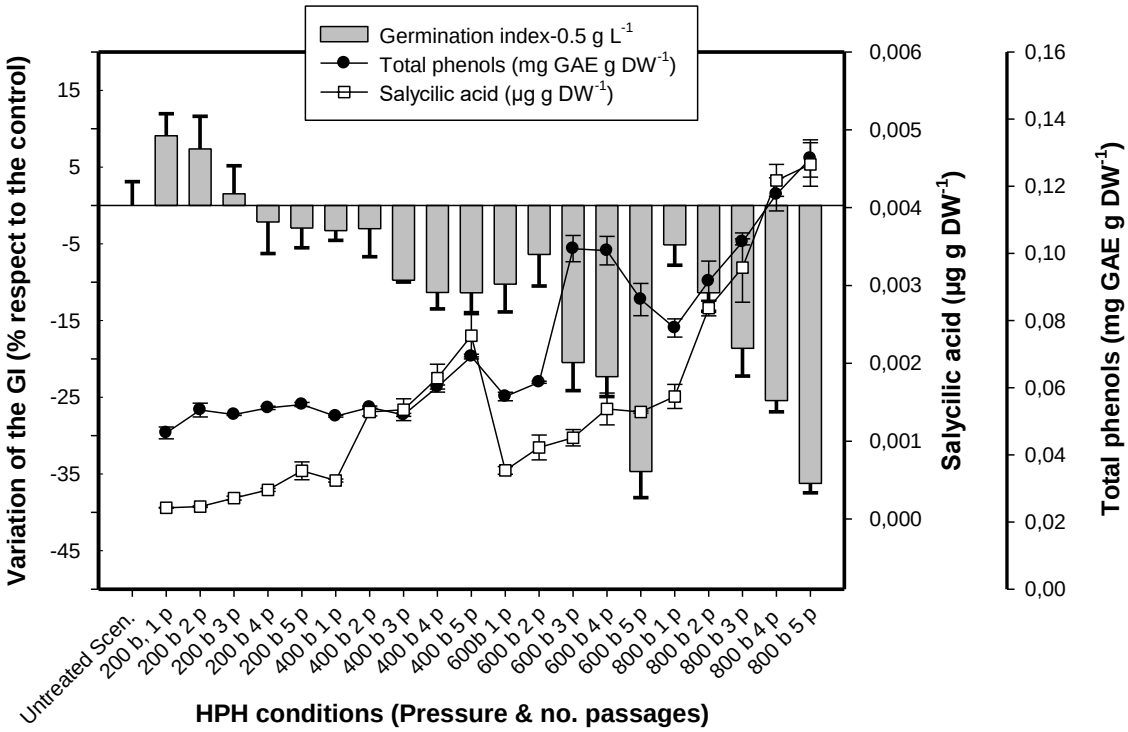


Figure 4 Total phenol and salicylic acid contents compared to the variation of the germination index obtained in each case for an extract concentration of 0.5 g/L.

However, these 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, when the cell disruption (at each pressure tested) is supposedly the maximum (salicylic acid content: $6.2 \cdot 10^{-4}$, $1.37 \cdot 10^{-3}$, $2.35 \cdot 10^{-3}$ and $4.55 \cdot 10^{-3}$ µg 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 of salicylic acid and total phenols obtained 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.

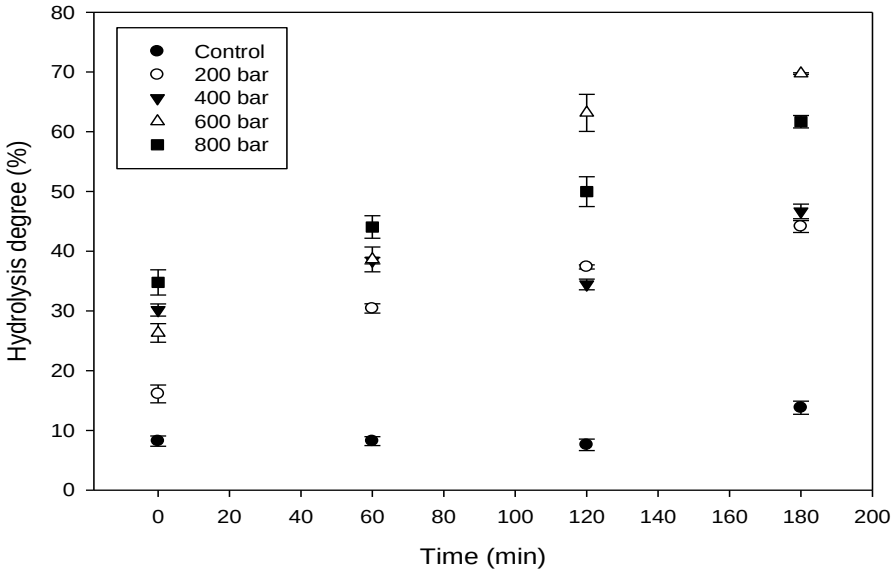
Many authors have characterized a variety of extracts to determine their phenol and phytohormone contents, given their direct relationship to plant development. Pereira et al. (2015) analysed different microalgae extracts from species such as *Picohlorum* sp. and *Nannochloropsis* sp. observing that, in both, microalgal salicylic acid was the main phenolic 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 (2017) 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. Biofertilizers: 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 (Romero García et al., 2012). To perform the first protein solubilization step from *Scenedesmus* sp*.* biomass, it is necessary to consider which cell disruption method to use for breaking down cell walls and to make 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 Figure 5.

A)



B)

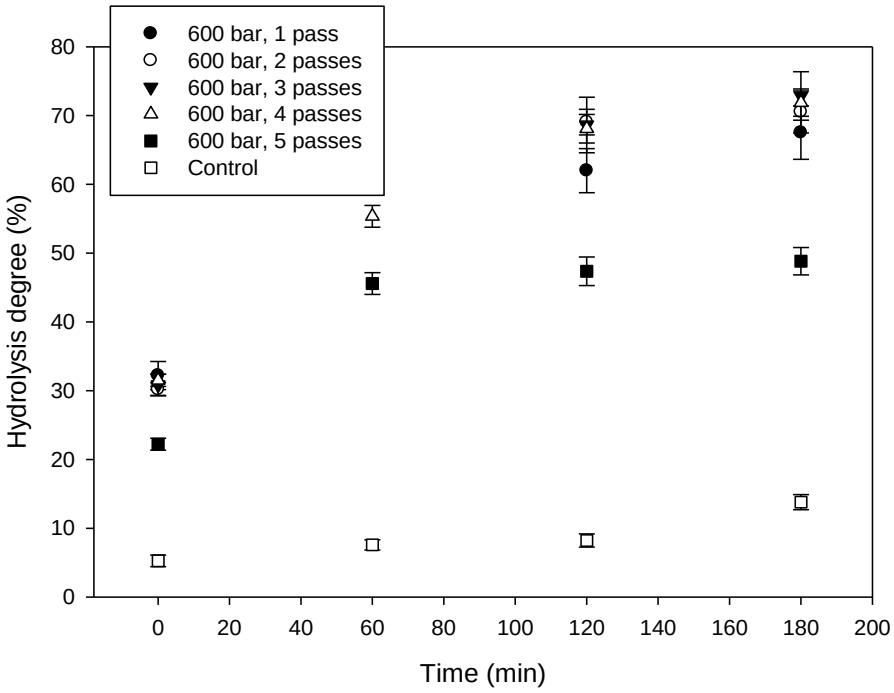


Figure 5 Influence of the HPH conditions on the enzymatic hydrolysis of Scenedesmus sp. biomass. A) Homogenization pressure (0-800 bar). B) Number of passes through the homogenizer (1-5 passes) at a treatment pressure of 600 bar.

Figure 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 pre-treatment is carried out, both in 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 minutes) - thus justifying the use of this mechanical cell disruption method for obtaining biofertilizer products. Once the optimal treatment pressure for obtaining the biofertilizers was selected, the next step was to optimize the number of passes that the biomass made through the homogenizer at 600

bar (Figure 5B). The results are shown in Figure 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 which leads to destabilization of the weak protein bonds and therefore to changes in certain functional properties of proteins such as water binding capacity, rheological properties, solubility and enzyme activity, affecting negatively the hydrolysis degree (Porto et al., 2018). 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. (2012) optimised 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.

Biofertilizers are currently used with many agricultural crops. Most of these products come from microalgae and are mainly sold as a source of amino acids for various applications, not just as biofertilizers; these include as antioxidants (Afify et al., 2018), as human and animal feed (Clemente, 2000; Spolaore et al., 2006) or as biofuels (Romero García et al., 2012).

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 the microalgal raw material for the production of biostimulants and biofertilizers. For each product, the pre-treatment of the wet biomass by 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 pre-treatment 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 biofertilizers, the best results were obtained at 600 bar, achieving a 65.7 % degree of hydrolysis while passing the biomass through the homogenizer only once; this was markedly greater than that obtained for the untreated biomass, which reached only 14 % after 180 min of reaction.

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Declarations

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Author contributions

All authors have contributed to the manuscript. All authors contributed to the research design, data analyses, and all authors have revised, edited and approved the final manuscript.

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