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Bioprocess to produce biostimulants/biofertilizers based on microalgae grown using piggery wastewater as nutrient source

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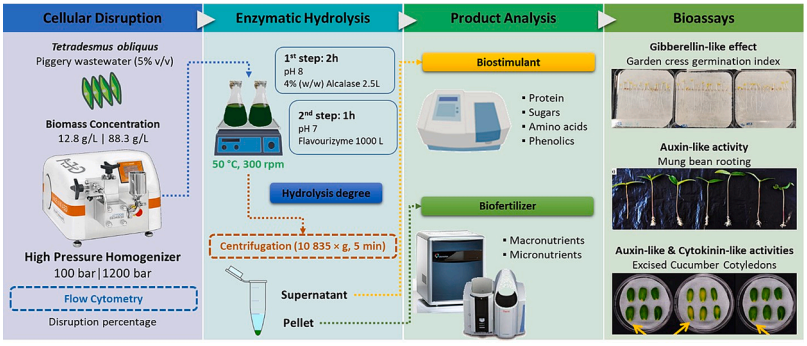
HIGHLIGHTS

- Extracts rich in amino acids and phenols were achieved.
- The *T. obliquus* culture (without treatment) achieved the highest germination index.
- The supernatant and culture of HPH-1200 showed the highest auxin-like effect.
- Only the initial *T. obliquus* culture showed cytokinin-like effect.
- Both biomasses had similar NPK contents compared to commercial organic fertilizer.

GRAPHICAL ABSTRACT



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ABSTRACT

In the present work, two downstream processes – high-pressure homogenization at 100 (HPH-100) and 1200 bar (HPH-1200), and enzymatic hydrolysis (EH) – were tested to produce biostimulant extracts from *Tetradesmus obliquus* grown in piggery wastewater at two concentrations (12.8 and 88.3 g/L). Extracts before and after centrifugation (C) were evaluated in four bioassays using garden cress (germination), mung bean (auxin-like activity), and cucumber (auxin- and cytokinin-like activity) relative to distilled water. The initial microalgal culture, without any treatment, had the best germination results (162 % at 0.2 g/L) and the only one that showed cytokinin-like activity (141 % at 0.5 g/L). In both auxin-like bioassays, the HPH-1200 + C and EH + C originated high values (186 and 155 % for cucumber, 290 and 285 % for mung bean, respectively). For mung bean, the HPH-1200 achieved the highest auxin-like effect (378 %). Finally, the extracted biomass contained essential nutrients for biofertilization, complementing the biostimulant extracts for sustainable agriculture application.

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1. Introduction

Nowadays, agriculture not only has to fulfil the food demands of the growing population but must cope with resource depletion and climate change impacts. It is a priority to promote more sustainable food production using environmentally friendly resources like microalgae. The potential of microalgae to obtain high-value products for agriculture results from their permanent exposure to abiotic and biotic stress, which prompted them to develop robust protection mechanisms, including the synthesis of growth-stimulating compounds, such as phytohormones, polysaccharides, amino acids, polyamines, and fatty acids (Ronga et al., 2019).

Large scale microalgae production still faces major hurdles, including high nutrient requirements and associated costs. The use of wastewater as a low-cost source of water and nutrients can benefit the economic feasibility of microalgae production, while recovering nutrients, which could otherwise contaminate water bodies and cause eutrophication problems (Mariyappan et al., 2024).

Microalgae biomass grown in nitrogen rich wastewater is mostly composed of proteins. Proteins could be an important source of peptides and amino acids, which are useful as bio-stimulants in agriculture (Mógor et al., 2018). However, they are usually intracellular compounds, requiring the disruption of the microalgal cell walls.

High-pressure homogenization (HPH) has attracted considerable interest related to its high rupturing efficiency, easiness to scale up, and capacity to process large volumes of wet microalgae biomass (avoiding drying) (Spiden et al., 2013). Protein hydrolysates can be obtained by different methods, but enzymatic hydrolysis (EH) has more advantages, including lower environmental impact and energy requirements, since it is carried out under mild conditions (pH and temperature) and the protein hydrolysate is rich in essential amino acids and free of toxic degradation products (Rojo et al., 2021; Romero García et al., 2012).

This paper focused on the development of a process, including high-pressure homogenization and enzymatic hydrolysis, to produce extracts with biostimulant activity derived from *Tetradesmus obliquus* biomass, grown using piggery wastewater (PWW) as a low-cost medium. Extracts before and after centrifugation (C) were evaluated in different bioassays: i) gibberellin-like effect (garden cress); ii) auxin-like activity (mung bean and cucumber); and iii) cytokinin-like effect (cucumber). The spent biomass after centrifugation was evaluated as a biofertilizers through the quantification of macro and micronutrients. The integral and fractional valorisation of microalgal biomass in a biorefinery concept, obtaining high-value products such as biostimulants and low-value products such as biofertilizers, will help minimize waste streams, while improving biomass profitability and promoting a circular bioeconomy.

2. Materials and methods

2.1. Culture conditions

The microalga *Tetradesmus obliquus* (ACOI 204/07, ACOI Culture Collection, Coimbra University, Portugal) was cultivated in diluted piggery wastewater (5 % v/v). The PWW had the following composition: pH 7.72, 1855 ± 0.3 mg TKN/L (total Kjeldahl nitrogen), 1257 ± 14 mg NH_4^+/L (ammoniacal nitrogen), 198 ± 46 mg PO_4^{3-}/L, and 8305 ± 169 mg O_2/L (chemical oxygen demand). These cultures were produced in 300 L outdoor open raceway ponds (2 m^2 exposed area) at the LNEG's Lumiar Campus in the city of Lisbon, located on the west coast of Portugal (38°420 N, 9°110 W) during the month of August 2023. Microalgal cultivation was carried out for 15 days under natural light cycles with 14 h light (average radiation of 508.7 ± 22.6 W/m^2) and 10 h dark and average ambient temperature of 24.3 ± 2.9 °C. Microalgal cultures were agitated by paddlewheels at approximately 5 m/min. The cultures were inoculated with an initial concentration of 0.2 g/L and reached a final concentration of 1.2 g/L. Biomass was concentrated by gravimetric sedimentation to achieve ten times concentration slurry with a biomass concentration of 12.8 g/L, and then further left to settle until reaching a maximum of 88.3 g/L.

2.2. Protein and amino acid profile

Protein content was estimated through the Lowry method with BSA (Bovine serum albumin) as standard (Lowry et al., 1951), after extraction with NaOH 1.0 N at 100 °C for 60 min. Amino acid identification and quantification was conducted in the analytical platform at GreenCoLab – Associação Oceano Verde (Algarve, Portugal). Microalgal samples were first hydrolysed with HCl 6 N at 121 °C for 72 h. The extracts were then concentrated to dryness in a speed vacuum system (Concentrator plus, Eppendorf), and resuspended in HCL 0.02 N, followed by derivatization according to Waters AccQ-Tag™ for hydrolysate amino acids procedure for High-Performance Liquid Chromatography (HPLC) (Wilson and Plumb, 2024). Amino acids determination was performed by HPLC (Chromaster, Hitachi, VWR) with Fluorescence detector (5440 FL detector, Hitachi, VWR). Chromatographic conditions were set according to the certified Waters AccQ-Tag™ for hydrolysate Amino acids (Wilson and Plumb, 2024).

2.3. Downstream processing of microalgal biomass

The influence of different downstream processing strategies into the bioactivity of the microalgal biomass was studied. A complete factorial experimental design was performed considering three main steps: cell disruption by high-pressure homogenization, enzymatic hydrolysis at previously optimized conditions, and centrifugation (Fig. 1). The wet microalgal biomass from *Tetradesmus obliquus* at 12.8 (LC) and 88.3 g/L (HC) was first subjected cell disruption by HPH. After that, both the disrupted and the undisrupted biomass were submitted to EH. Finally, biomass and supernatant were separated by centrifugation. Treatments are numbered from T0 which is the initial biomass without any pretreatment, until T6 which corresponds to the biomass on which the three treatments have been applied (HPH + EH + C) and R1 and R6 are the residues of biomass after separation from supernatant.

2.3.1. High-pressure homogenization

Cell permeabilization/disruption was carried out by HPH using in a PandaPLUS 2000 homogenizer (GEA, Düsseldorf, Germany). The wet microalgal biomass (100 mL), at two concentrations 88.3 and 12.8 g/L (ash-free dry weight), was passed through the homogenizer for 1 cycle at two different pressures: 100 bar to induce partial disruption with membrane permeabilization and 1200 bar to assure complete disruption, according to preliminary tests (to be published) Samples were collected and immediately analysed through flow cytometry (FC) to evaluate cell integrity (intact/permeabilized/disrupted cells).

The microalga samples were analysed using a CytoFLEX flow cytometer (Beckman Coulter Life Sciences, USA), equipped with a blue argon laser (488 nm). Light scatter properties from cells were measured in the Forward (FSC) and Side Scatter (SSC) detectors to distinguish different cell sizes and internal complexities, respectively. Fluorescence channel Phycoerythrin Cyanin 7 (PC7) at 575 nm was used for detection of chlorophyll to select the microalgal cells' population. For cell membrane integrity, samples were stained with Sytox-green, and the fluorescence was measured by the Fluorescein isothiocyanate (FITC) detector at 525 nm. Staining was first optimized (see supplementary material), and the following conditions were selected: Sytox-green 0.12 μM and 25 min in the dark. Heat-treated *T. obliquus* cells (incubated in a water bath at 100 °C for 60 min) were used as the positive control, while untreated cells were used as the negative control. Microalgae samples were diluted with phosphate saline buffer (PBS) to adjust cell concentration to 100–150 events per second in a final volume of 500 μL. Samples were pre-treated in an ultrasound bath for 10 s to destroy possible cell aggregates. The cell permeabilization and disruption

percentage after HPH treatments at 100 and 1200 bar were calculated in relation to the untreated microalgal cells (negative control) according to equations (1), and 2. The plot FTIC vs. FSC was used to distinguish intact (unstained) from permeabilized (stained) microalgal cells, while the plot SSC vs. FSC was used to determine the disrupted microalgal cells.

$$Permeabilization(\%) = \frac{Untreatedmicroalgalcells - Stainedmicroalgalcells}{Untreatedmicroalgalcells} \times 100 \quad (1)$$

$$Disruption(\%) = \frac{Untreatedmicroagalcells - Treatedmicroalgalcells}{Untreatedmicroagalcells} \times 100 \quad (2)$$

2.3.2. Enzymatic hydrolysis

The enzymatic hydrolysis was conducted in 100 mL Erlenmeyer flasks stirred (300 rpm) in two steps, according to Romero García et al. (2012). Each step was done at the optimal conditions recommended by the enzyme's suppliers (Novozymes, Bagsværd, Denmark). In the first step, 50 mL of the untreated or previously disrupted microalgal biomass were transferred into Erlenmeyer flasks and heated to 50 °C. The pH was initially adjusted and maintained at 8 with NaOH 1 M. Then, 4 % (w/w) of Alcalase 2.5 L (from *Bacillus licheniformis*) was added into the reactor and the reaction began. After two hours, pH was lowered and maintained at 7 by adding H_2SO_4 1 M. The second enzyme, Flavourizyme 1000 L (from *Aspergillus oryzae*), was added to the reaction medium. After three hours, it was considered that the reaction was finished, and the solution was heated to 75 °C for 15 min to deactivate the enzymes.

2.3.3. Centrifugation

The resulting microalgal slurries from the downstream processes were centrifuged at 7000 × g for 5 min and 4 °C (6–16 KS, Sigma) to obtain two separate fractions (microalga extract and residue).

2.4. Analysis of extracts

2.4.1. Extraction yield

The extraction yields were quantified gravimetrically by drying the extract at 105 °C for 3 h.

2.4.2. Protein and carbohydrates

Protein content was estimated through the Lowry method based on the absorbance at 750 nm. BSA (Bovine serum albumin) was used as the protein standard (Lowry et al., 1951). Carbohydrates content was determined through the phenol sulphuric method measuring the absorbance at 490 nm. Glucose was used as the sugar standard (DuBois et al., 1956).

2.4.3. Amino acid content and hydrolysis degree

The amino acid content was quantified before and after the enzymatic reaction to assess the hydrolysis degree (HD). The HD is defined as the ratio between the number of free amino acids of each sample and the total number of amino acids-protein available for hydrolysis. The hydrolysis degree was determined by equation (4) and (5) using the OPA (o-phthaldialdehyde) method using serine as standard assuming the following value of the parameters of the model $\alpha = 1$, $\beta = 0.4$ and $h_{tot} = 8$ (Nielsen et al., 2001).

$$HD(\%) = \frac{Serine_{NH_2} - \beta}{\alpha \times h_{total}} \times 100 \quad (4)$$

$$Serine_{NH_2} = \frac{Abs_{sample}}{Abs_{standard}} \times 0.9516 \times V \times \frac{100}{X \times P} \quad (5)$$

Where V, X, and P are the volume, mass, and protein content of the sample, respectively.

2.4.4. Phenolic content

Total phenol content in the extracts was determined using the Folin–Ciocalteu procedure (Singleton and Rossi, 1965). The absorbance of samples was measured at 750 nm using a microplate reader (Epoch2, Agilent Biotek, California, USA) and gallic acid was used as standard. The content of phenolic compounds was expressed as mg gallic acid equivalents (GAE) mL^{-1} extract. All experiments were performed in triplicate.

2.5. Analysis of residues

Elemental analysis and metals were determined at the Laboratory of Biofuels and Biomass (LBB), an accredited laboratory according to NP EN ISO/IEC 17025: 2018, at LNEG (Lisbon, Portugal). The amounts of elemental carbon, nitrogen, hydrogen, and sulphur were simultaneously measured in freeze-dried samples, with the Elementar Vario Macro Cube CARBO analyser, following the guidelines of ISO 16948 and the manufacture instructions. For metals and phosphorus analysis, samples were first submitted to acid digestion in closed vessels according to an in-house procedure adapted from EPA 3050A. All digestions were performed in a microwave heating temperature-controlled (Milestone Ethos Plus). Samples of 0.5000 ± 0.0100 g were measured into a high pressure TFM vessel of 100 mL and adding, in a first round, HNO_3/H_2O_2 acid

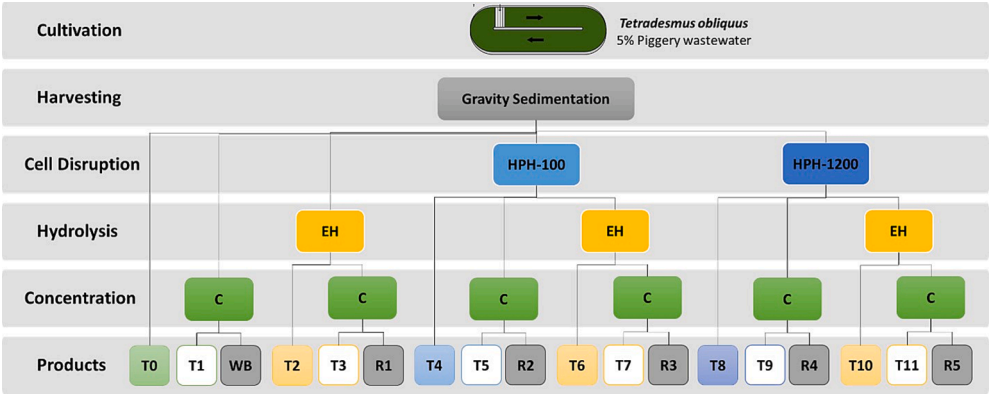

Fig. 1. Downstream processing of the wet microalgal biomass: high-pressure homogenization at 100 bar (HPH-100) and 1200 bar (HPH-1200), enzymatic hydrolysis (EH), and centrifugation (C). A total of 11 treatments (extracts and supernatants) and 5 residues (spent biomass) were tested. Treatments: T0 – Initial microalgal culture (without any treatment); T1 – C; T2 – EH; T3 – EH + C; T4 – HPH-100; T5 – HPH-100 + C; T6 – HPH100 + EH; T7 – HPH100 + EH + C; T8 – HPH-1200; T9 – HPH-1200 + C; T10 – HPH-1200 + EH; T11 – HPH-1200 + EH + C. Residues: WB: Whole biomass; R1 – EH + C; R2 – HPH-100 + C; R3 – HPH-100 + EH + C; R4 – HPH-1200 + C; R5 – HPH-1200 + EH + C.

mixture (5:2), under a heating program using a gradual ramp to 190 °C over 11 min (approximately 28 °C/min heating rate until 90 °C, followed by 130 °C and 190 °C at a 8 °C/min and 17 °C/min respectively) and a mantained at 190 °C, during 10 min. After cooling down to room temperature, a second round was performed adding HNO_3/HCl acid mixture (5:1) to the digested solutions from the previous round, under the same heating program conditions. Metals were then determined by flame atomic absorption spectrometry (THERMO SCIENTIFIC iCE3000 Series) and phosphorous by molecular absorption spectrometry (UNICAM UV300).

2.6. Bioassays for biostimulant potential of extracts

2.6.1. Germination index

The germination tests were performed in 120 mm square Petri dishes using garden cress (*Lepidium sativum* L.) seeds. Each Petri dish was covered with 2 filter papers and 15 seeds were placed. A volume of 5 mL of each extract was added to the seeds in triplicate. Distilled water and gibberellic acid (GA, >90 %, CAS-No: 77-06-5, Thermo Scientific) at 2.5 μM were used as the negative and positive controls, respectively. Seeds were incubated at 23 °C in the dark for 3 days in a growing chamber (FITOCLIMA S600 PL). The number of germinated seeds in each Petri dish was counted, and the root and shoot lengths were measured using ImageJ software. The germination index (GI) was determined according to Zucconi et al. (1981) using equation (6):

$$GI(\%) = \frac{G \times L}{G_w \times L_w} \times 100 \quad (6)$$

Where G, Gw correspond to the total number of germinated seeds and L, Lw to the root length for the tested conditions and the negative control (distilled water), respectively.

2.6.2. Mung bean rooting bioassay

Commercial mung bean *Vigna radiata* (L.) seeds were planted according to Zhao et al. (1992). Seeds are sown at 1 cm depth in moistened perlite in plastic trials maintained at 23 °C and illuminated with fluorescent lamps in light cycles of 12 h in a grown chamber (FITOCLIMA S600 PL). After 10 days, five seedlings were then cut 3 cm under the cotyledon, placed in vials containing the microalgal extract and incubated in the same conditions for 5 days. A stock solution of Indol-3-butyric acid (IBA, I5386, BioReagent, Sigma-Aldrich) was prepared at 100 mg/L using distilled water and adding 2 mL/L of ethanol (≥99.9 %, Carlo Ebra). Dilutions were made to prepare the standard curve of IBA (0.2–100 mg/L) for comparison. Each condition was tested in triplicate (15 samples). At the end, the number of roots (longer than 1 mm) were counted on each plantlet. The number is directly proportional to the auxin concentration within the assay range. The mean number of roots, derived from each vial, was compared to the negative control (distilled water).

2.6.3. Cucumber cotyledon expansion and rooting bioassays

Commercial seeds of cucumber *var.* Marketeer (*Cucumis sativus* L.) were used to evaluate the stimulant effect on germination, according to the method described by Zhao et al. (1992). The seeds were placed in plastic trays with 0.7 % agar medium and then transferred to an incubator maintained at 23 °C for 5 days in the dark. Cotyledons were excised from hypocotyl (1–2 mm at the base) and transferred to Petri dishes of 60 mm diameter (10 cotyledons per Petri dish in triplicate) containing filter paper. The microalgal extracts were prepared at 0.5 g/L. A stock solution of 6-Benzylaminopurine (BAP, 99 %, 226410010, Acros Organics) was prepared at 100 mg/L in distilled water, adding a few drops of NaOH 1 N. Dilutions were made to prepare the standard curve of BAP (0.2–100 mg/L) for comparison.

The filter papers were moistened with 6 ml distilled water (negative control), the microalgal extracts, or BAP solutions. For the cotyledon expansion, the excised cotyledons were initially weighted before transferring to the Petri dishes and after 3 days of incubation in the dark. The weight increase was evaluated against the negative control (distilled water).

For the rooting, the Petri dishes were maintained for 6 days and the mean number of roots, from each treatment, was compared to the negative control and evaluated using a standard curve of IBA (0.2–100 mg/L) for comparison.

2.7. Statistical analyses

Statistical data analyses were performed using the Jamovi software version 2.5.3.0. Data were log10 transformed when necessary. One-way Welch's ANOVA was used to describe the effects of the different conditions on the various bioassays. Games-Howell's post hoc test was used for comparisons among treatments and controls. The normality and homogeneity assumptions were verified through the Shapiro-Wilk and Levene tests, respectively. Multifactor ANOVA tests were used to study the effect of the factors (growth medium, biomass loading, extraction temperature, and microalga concentration) and their interactions at a 95 % confidence level. The p-values resulting from the sum of square analyses were used to describe the impact of the factors. For all tests a significance level of $\alpha = 0.05$ was considered.

3. Results and discussion

3.1. Biomass composition

The protein content of the microalgal biomass was determined and the amino acid profile was analysed (see supplementary material). *T. obliquus* cultivated in PWW (5 % v/v) was composed of 25.6 % protein, which is lower than the previous reported one (34.5 %) (Ferreira et al., 2021). This can be explained by the differences in PWW composition and cultivation conditions, as the present work was done outdoors with natural daylight cycles and variable meteorological conditions.

The amino acid profile presented a suitable essential-to-total amino acid ratio (35.7 %), which was in the same range as the ones determined by Lorenzo-Hernando et al. (2019) and Rojo et al. (2021) for *Scenedesmus almeriensis* grown in piggery wastewater (29.3–32.7 %). The major amino acids (AAs) were glutamic acid (6.69 %), leucine (5.45 %), and aspartic acid (4.42 %). Moreover, Lorenzo-Hernando et al. (2019) and Rojo et al. (2021) also obtained glutamic acid and aspartic acid at similar percentages than the present work.

A study by Abdelkader et al. (2023) showed an improvement in plant development by applying different AAs such as methionine, proline, tryptophan, and glutamine. These AAs were present in *T. obliquus* biomass, hinting on its biostimulant potential for germination and root development.

3.2. High-pressure homogenization

Flow cytometry was used to evaluate the impact of HPH on the cell membrane integrity (Fig. 2). The gates for intact, permeabilized, and disrupted cells were established based on the controls (untreated and heat-treated biomass). Fig. 2(a–f) shows an example of cytograms for the concentration of 88.3 g/L, which were equivalent to the ones for the one at 12.8 g/L.

The gate for the microalgal cells was established based on their chlorophyll content in the plot PC7 vs. SSC in the initial culture (T0, Fig. 2a). Events located in the upper quadrant of the FSC vs. SSC plot with high PC7 fluorescence were considered the microalga cells, which maintained their size and internal content as well as high chlorophyll (Fig. 2a,b).

After sample staining, intact and permeabilized cells were identified as those located at the left lower (Sytox- cells, Fig. 2c) and left upper (Sytox + cells, Fig. 2f) quadrants of the FITC vs. SSC plot, respectively.

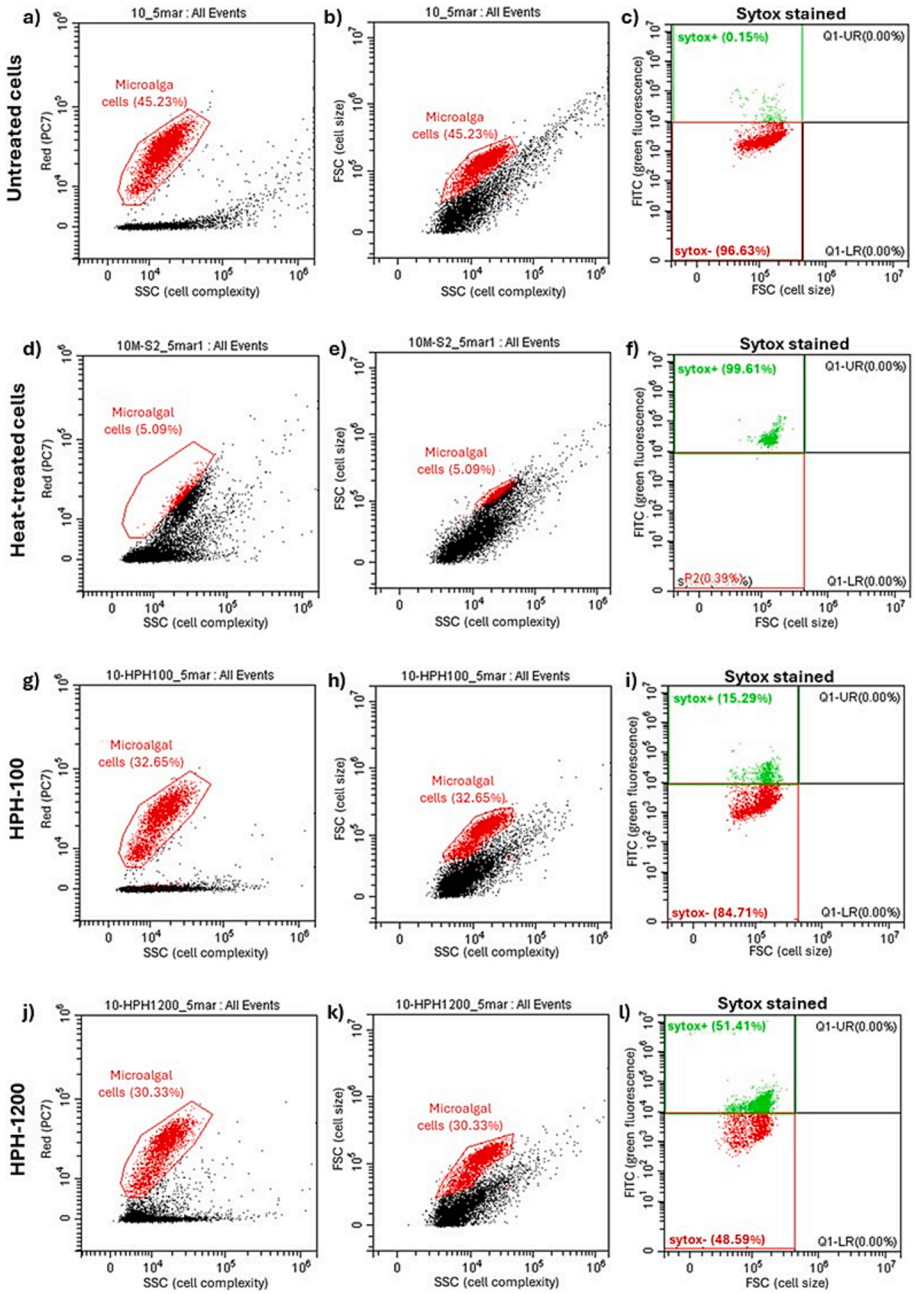

Fig. 2. Flow cytometry analysis. Disruption controls: a) FSC vs. SSC and b) PC7 vs. SSC plots of unstained untreated cells (negative control); c) FTIC vs. FSC of stained untreated cells (negative control). d) FSC vs. SSC and e) PC7 vs. SSC plots of unstained heat-treated cells (positive control); f) FITC vs. FSC of stained heat-treated cells (positive control). Microalgal cells are highlighted in green. Percentages of intact, permeabilised and disrupted *Tetradesmus obliquus* cells on the initial (untreated) culture or after high-pressure homogenization at 100 bar (HPH-100) and 1200 bar (HPH-1200) at g) 12.8 g/L and h) 88.3 g/L, determined by flow cytometry. Data are shown as mean ± standard deviation ($n = 2$).

The quadrants were established by analysing the cytograms of both untreated and heat-treated cultures. The disrupted cells were detected at lower FSC and SSC, meaning they no longer maintained their structure and formed debris after HPH treatments. The disrupted cells were calculated by applying the same gate related to microalgal cells (Fig. 2a) to all the analysed samples and subtracting the decreasing number of events within the gate.

The initial culture (T0) at both concentrations contained already a reduced number of intact cells (37.6–40.1 % of total number of captured events). This means that the microalgal was already at a fragile state related to the fact that they were stored at −4 °C until the HPH treatment. Cytometry analysis showed that the process of freezing and thawing already provoked the disruption of microalgal cells prior to HPH treatments, which can be estimated to be less than 10 % by comparing with the cytograms for the PWW medium without microalgal cells. Loureiro et al. (2023) also obtained some percentage of disruption by applying several cycles of freezing-thawing, although much lower (23.5 %) than other harder disruption methods like sonication or bead milling (>90 %).

As expected, the percentage of intact cells decreased with increasing pressure, while the permeabilized and disrupted cells increased (Fig. 2g–l). At 100 bars, the permeabilization and disruption percentages were 46.9–48.7 % and 26.9–37.8 %, respectively (Table 1). This means that 100 bar was already capable of disrupting the biomass, contrary to the observed by Ferreira et al. (2022), which required 3 cycles at 100 bar to obtain full disruption of the fresh cells. This could be related to the

initial more fragile state of the microalga cells as previously mentioned. At 1200 bar, the permeabilization and disruption percentages were 79.6–87.8 % and 39.6–39.7 %, respectively. The disruption percentage is still low after 1 cycle at 1200 bar (<40 %), meaning that higher pressures or number of cycles might be required to fully disrupt *T. obliquus* cells. This species has a complex extracellular matrix with an inner layer mainly made up of polysaccharides and a tri-laminar outer layer of algaenan with an estimated thickness of 110 nm (do Carmo Cesário et al., 2022). Algaenan is a non-hydrolysable biopolymer that protects microalgae from degradation which makes them extremely difficult to break (Alhattab et al., 2019). Ferreira et al. (2022) observed that additional HPH cycles had a cell-weakening effect on *T. obliquus*, suggesting that compromised cell walls are more prone to cell disruption in subsequent applied cycles. Thus, more HPH cycles might be necessary to achieve higher disruption levels.

At lower concentration (12.8 g/L), the permeabilization and disruption percentages are always slightly lower than at 88.3 g/L. This suggests that higher cell concentration could benefit disruption.

At both pressures, the loss of chlorophyll was lower than 20 % (Fig. 2g,j). From 100 to 1200 bar, only a slight increase of 2–3 % was verified. However, when comparing with the positive control (Fig. 2d), where heat was applied, the loss of chlorophyll was higher (34.9–42.3 %). Considering that HPH is a purely mechanical on–off disruption process, and the microalgal cells were only submitted to one pass, the rise in temperature was negligible and no thermal degradation occurred. Furthermore, although higher pressures are preferred for promoting cell disruption, repeated cycles at lower pressures like 100 bar might be beneficial for avoiding sample overheating and compound degradation (Bernaerts et al., 2019).

3.3. Enzymatic hydrolysis

As previously stated, the cell wall of *T. obliquus* is mainly formed by algaenan, proteins, and polysaccharides (Alhattab et al., 2019), so the most used enzymes for cell disruption are proteases and cellulases. Proteases include a wide-ranging group of enzymes that catalyze peptide-bond cleavage in proteins to convert them into shorter proteins, peptides, or amino acids (Alavijeh et al., 2020). The two enzymes used in the present work were Alcalase 2.5L, which is an endoprotease, to break down the proteins into peptides, and Flavourzyme 1000L, an exoprotease to cleave the peptide bonds, producing single AAs.

The production of free AAs from microalgae biomass generally entails the release of intracellular proteins. The effect of both HPH pressures on the enzymatic reaction was studied and compared with EH alone by calculating the hydrolysis degree (Table 1).

The composition of the cell wall of *T. obliquus,* as previously mentioned protects it from degradation from chemical or enzymatic hydrolysis, which justifies the low hydrolysis degree (<20 %). The degree of hydrolysis is improved when HPH is previously applied. Increasing the pressure from 100 to 1200 bar, approximately doubles the hydrolysis degree. This means that increased cell disruption improves the enzymatic hydrolysis by more than 60 % in the case of the highest pressure (1200 bar).

The use of fresh biomass has been reported to provide higher hydrolysis degrees compared to dried biomass, which brings the additional benefit of avoiding a costly and energy-intensive drying step (Romero García et al., 2012). Moreover, higher biomass concentration led to a slightly higher hydrolysis degree, which produced extracts much richer in amino acids (Table 2).

3.4. Extraction yield and extract composition

The extraction yield of each step, as well as the composition of extracts in terms of protein, sugars, phenolics, and AAs is presented in Table 2.

The extraction yield shows that by applying more downstream processes, more compounds were extracted. Increasing the HPH pressure from 100 to 1200 bar increases the extraction yield of all compounds, related to the higher disruption (Table 2) and release of compounds (Alavijeh et al., 2020).

During HPH, intercellular compounds such as lipids, proteins, nucleic acids, and polysaccharides are released into the medium, while cell wall and membrane debris are produced. EH greatly increases the extraction yield compared to cell disruption, which could be explain by the fact that the latter only releases intracellular content, while EH breaks the compounds into smaller molecules that become soluble and are extracted into the liquid phase. Nonetheless, HPH + EH has a positive effect on the extraction yield related to a reduction in cell size. A smaller biomass particle size increases the contact areas between the enzymes and the interparticle bonds during the hydrolysis process (Alavijeh et al., 2020). In addition, the permeabilization of microalgal cells gives access to intracellular proteins, which are not available without cell disruption. In this case, when EH is directly applied to biomass without any pre-treatment, most extracted proteins might be a part of cell wall rather than internal compounds (Rojo et al., 2021).

In contrast to what was expected, the extraction yield of HPH-100 + EH was slightly higher than HPH-1200 + EH. This could relate to the fact that the latter releases more compounds from the microalgal cells into the medium (e.g. polysaccharides), which builds the medium viscosity (Magpusao et al., 2021), which could have a negative effect on the EH.

3.5. Germination index

The germination index (GI) is calculated in relation to the negative control (distilled water), which means that only microalgal extracts that lead to values higher than 100 % are considered to have biostimulant activity (Fig. 3).

Microalgal suspensions at 0.2 g/L from treatments T0, T2, T5, T6, T7, and T8 had a significant positive effect on the GI (>128.8 %). At the same concentration, T1 had a significant negative effect (61.7 %), while

Table 1
Disruption parameters (cell permeabilization and disruption percentages, loss of chlorophyll) and hydrolysis degree of *Tetradesmus obliquus* biomass grown in piggery wastewater submitted to high-pressure homogenization at 100 bar (HPH-100) and 1200 bar (HPH-1200) and enzymatic hydrolysis for biomass concentrations of 12.8 and 88.3 g/L. Boiling for 60 min was used as the positive control for high-pressure homogenization, while enzymatic hydrolysis without prior disruption was used to compare the effects of disruption. Data is shown as mean ± mean deviation ($n = 2$).

Treatment	Biomass concentration (g/L)	Disruption parameters			Hydrolysis degree
		Permeabilization (%)	Disruption (%)	Loss of chlorophyll (%)	
EH	12.5	–	–	–	13.4 ± 0.9
	88.3				17.9 ± 1.8
HPH-100 + EH	12.5	46.9 ± 2.2	27.4 ± 2.2	11.0 ± 0.3	26.6 ± 0.5
	88.3	48.7 ± 0.3	37.8 ± 0.3	8.2 ± 1.0	26.5 ± 2.7
HPH-1200 + EH	12.5	79.6 ± 2.7	40.0 ± 2.3	14.2 ± 0.3	45.7 ± 0.1
	88.3	87.8 ± 0.1	39.7 ± 1.4	10.3 ± 0.9	48.4 ± 5.1
Boiling	12.5	100.0 ± 0.0	93.1 ± 0.0	42.3 ± 2.3	–
60 min	88.3	100.0 ± 0.0	84.1 ± 0.6	34.9 ± 1.3	

Table 2
Extraction yield and composition of extracts (protein, sugars, total phenolics, and amino acids) of each downstream process of *Tetradesmus obliquus* biomass at 12.8 (LC: Low Concentration) and 88.3 g/L (HC: High Concentration): not treated (NT); high-pressure homogenization at 100 bars (HPH-100); high-pressure homogenization at 1200 bar (HPH-1200), enzymatic hydrolysis (EH) and combination among them. Data is presented as mean ± mean deviation ($n = 2$).

Downstream Process	Extraction yield (%)	Content (mg/L)			
		Protein	Sugars	Phenolics	Amino acids
LC: 12.8 g/L					
NT	–	23.01 ± 4.36	54.8 ± 2.5	31.2 ± 0.0	252.9 ± 16.9
EH	35.2 ± 3.8	383.5 ± 3.2	856.0 ± 42.5	66.9 ± 0.4	696.5 ± 32.0
HPH-100	13.9 ± 1.8	53.94 ± 0.40	551.7 ± 13.3	27.5 ± 0.2	213.4 ± 5.5
HPH-100 + EH	**43.6 ± 9.3**	**1093 ± 13**	596.8 ± 36.2	82.8 ± 1.8	1772 ± 8
HPH-1200	20.0 ± 4.8	150.7 ± 5.9	364.8 ± 4.8	22.2 ± 0.5	153.9 ± 39.4
HPH-1200 + EH	35.2 ± 3.8	719.0 ± 0.0	**772.1 ± 20.2**	87.8 ± 3.0	**2059 ± 174**
HC: 88.3 g/L					
NT	–	104.7 ± 0.4	293.8 ± 3.6	77.4 ± 0.2	263.0 ± 5.3
EH	43.3 ± 7.1	3413 ± 169	7466 ± 149	385.8 ± 14.2	5549 ± 71
HPH-100	18.9 ± 5.5	340.3 ± 1.6	1614 ± 69	76.2 ± 0.3	1079 ± 36
HPH-100 + EH	**53.7 ± 1.1**	**6680 ± 514**	2713 ± 177	209.7 ± 3.1	7371 ± 59
HPH-1200	23.1 ± 1.7	2546 ± 52	2756 ± 154	182.5 ± 0.0	2682 ± 93
HPH-1200 + EH	47.9 ± 10.8	5555 ± 388	**9813 ± 404**	**576.2 ± 9.8**	**8676 ± 529**

the rest was not statistically different from the control ($p > 0.05$). On the other hand, all the microalgal suspensions at 0.5 g/L either had no significant effect compared to distilled water (T0, T3, T6, T8, T9, T10) or had a negative effect (T1, T4, T5, T7, T10). In fact, only the microalgal suspension EH (T2) at 0.5 g/L had a positive significant effect (128.8 %). The best GI result was obtained for T0 at 0.2 g/L (162.2 %), which is the microalga culture without any treatment. The microalgal suspensions prepared from less downstream processes and at lower concentration (0.2 g/L) have more significant effect on GI (T6: 154.9 %; T7: 131.9 %; T8: 134.9 %; T2: 128.8 %; T5: 128.8 %), result that has also been previously highlighted by Navarro-López et al. (2020b). These results are statistically higher than the negative control and, in some cases, the positive control (gibberellic acid).

For the same condition, the GI values were always higher for the microalga cultures rather than the supernatants. This could mean that the biomass might adsorb some metabolites around the cells, which are not released into the medium. Another explanation might be the lower amount of metabolites on the suspensions prepared from the microalga cultures. Because they are prepared from concentrated biomass, they require lower volumes to prepare diluted suspensions, while in the case of supernatants only the dissolved metabolites are considered and, thus, more volume of supernatants is added in these suspensions. Furthermore, at 0.2 g/L significantly higher GI values were obtained than at 0.5 g/L for the same treatment. This means that lower concentrations might be required to avoid potential inhibition (Navarro-López et al., 2020b).

3.6. Auxin-like activity

3.6.1. Cucumber rooting

The presence of auxin-like activity was evaluated in excised cucumber cotyledons and the results obtained are presented in Fig. 4a.

There was an increase in root induction from 0.2 to 1 mg/L of synthetic IBA, while higher concentrations become inhibiting for rooting. This is because phytohormones are usually beneficial at micromolar concentrations and the balance between the concentration of various phytohormones plays a significant role on the stimulation (Sosnowski et al., 2023). Regarding the microalgal suspensions, the supernatants seemed to offer a higher positive effect than the biomass. The only exception was HPH-100 + EH, whose culture (T6) produced a positive effect (125.9 %) while the respective supernatant (T7) was slightly inhibiting (83.2 %). Both culture (T10) and supernatant (T11) of HPH-1200 + EH were inhibiting for rooting.

The supernatant from HPH-1200 (T9) produced the highest rooting percentage (186.2 %) equivalent to 1 mg/L of synthetic IBA (199.5 %). Also, the supernatants from EH (T3) and HPH-100 (T5) generated high percentages (154.9 and 141.3 %, respectively) equivalent to IBA 0.5 mg/L (131.8 %). For suspensions prepared from the initial culture (T0), EH (T2), and HPH-100 (T4), a high inhibition was verified compared to the negative control (<68 %). The HPH-1200 + EH supernatant (T11) had the worst negative effect on the rooting (33.9 %).

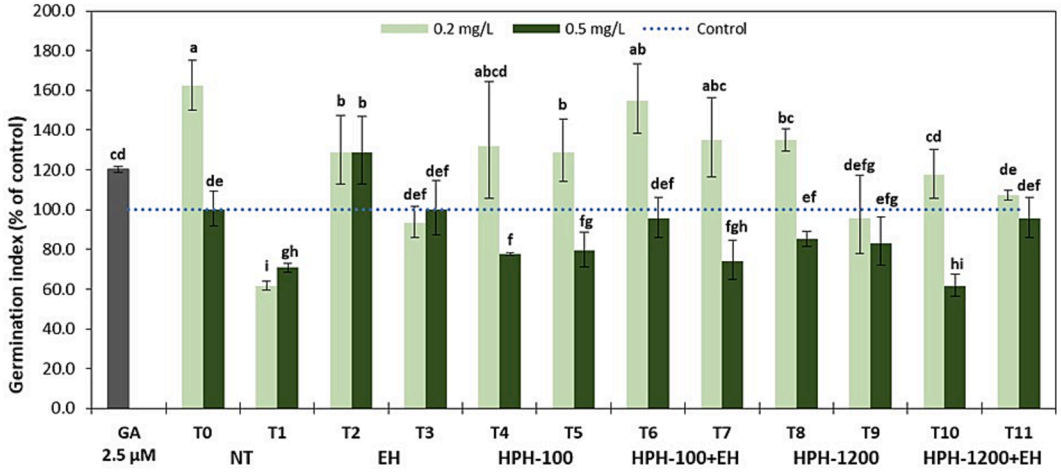

Fig. 3. Germination index of garden cress seeds with application of the *Tetradesmus obliquus* suspensions (biomass and supernatant after centrifugation): non-treated culture (NT: T0, T1) or after enzymatic hydrolysis (EH: T2, T3), high-pressure homogenization at 100 bar (HPH-100: T4, T5) and 1200 bar (HPH-1200: T8, T9), or a combination of both (HPH-100 + EH: T6, T7 and HPH-1200: T10, T11). Distilled water and gibberellic acid (GA) at 2.5 µM were used as the negative and positive controls, respectively. The columns and error-bars represent mean ± standard deviation ($n = 3$). Different letters indicate significant difference ($p < 0.05$) among treatments according to Games-Howell's two-sided post-hoc test.

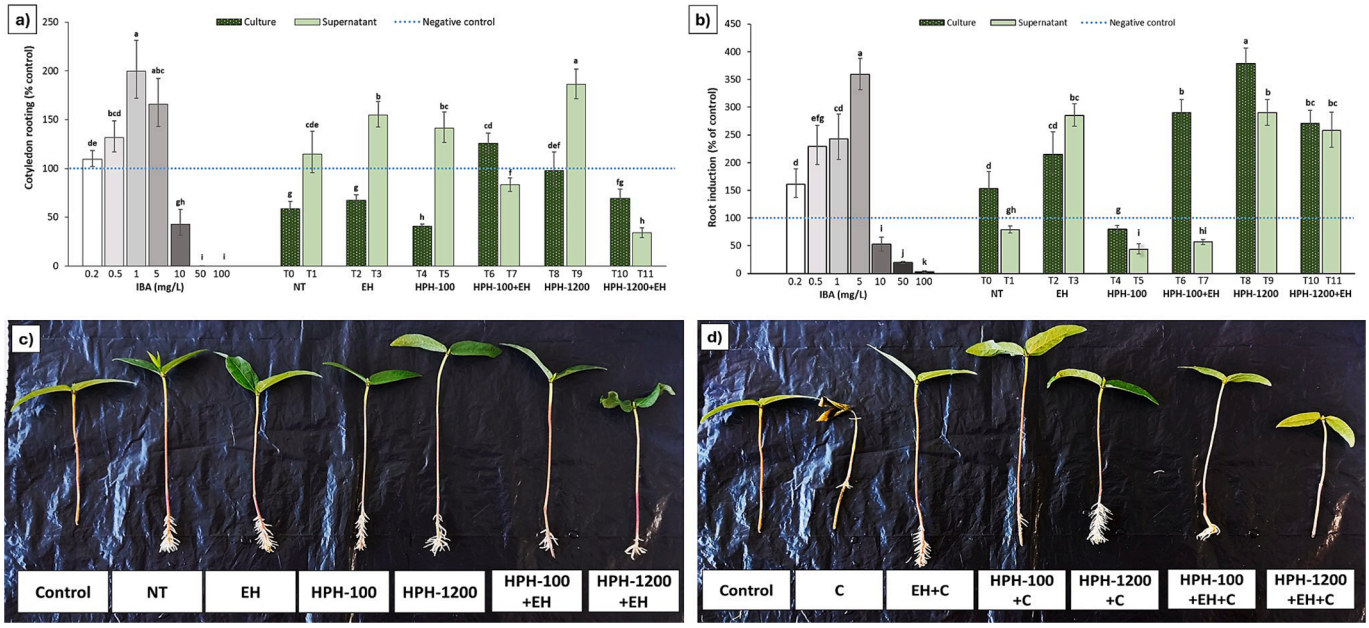

Fig. 4. Root development of plants treated with *Tetradesmus obliquus* suspensions at 0.5 g/L (culture and supernatant after centrifugation (C)) of non-treated (NT: T0, T1) biomass or after enzymatic hydrolysis (EH: T2, T3), cell disruption by high-pressure homogenization at 100 (HPH-100: T4, T5) and 1200 bar (HPH-1200: T8, T9), or a combination of both (HPH-100 + EH: T6, T7 and HPH-1200: T10, T11). Distilled water was used as the negative control (dotted blue line) and a standard curve of Indole-3-acetic Acid (IBA) was used as positive control (0.2–100 mg/L). Root induction of a) excised cucumber cotyledons, and b) mung bean plants. The columns and error-bars represent mean ± standard deviation ($n = 3$). Different letters indicate significant difference ($p < 0.05$) among treatments according to Games-Howell's two-sided post-hoc test. c) Photograph of mung bean plants treated with biomass suspensions. d) Photograph of mung bean plants treated with supernatant suspensions.

The treatments T1 and T8 are not significantly different ($p > 0.05$) from distilled water and, thus, are not considered to have auxin-like effect. While these suspensions might require higher concentrations of application to offer a beneficial significant effect, the inhibiting ones need to be diluted to decrease the excessive amounts of metabolites. On the other hand, some harmful compounds might be present in the PWW, which negatively impact the root development. A previous study by Navarro-López et al. (2020b) evidenced the auxin-like effect of *T. obliquus* biomass grown in brewery wastewater, submitted to ultrasonication and EH. The produced microalgal suspensions also had rooting percentages higher than 140 %, similar to supernatants from EH, HPH-100, and HPH-1200. Furthermore, Navarro-López et al. (2020a) obtained much higher rooting percentage than the present results in cucumber cotyledons treated with *Scenedesmus* sp. submitted to different downstream processes, reaching 274–275 % with HPH at 200 bar, followed or not by EH. Unlike the present work, both studies did no evidenced any inhibition at both concentrations tested (0.5 and 2 g/L).

3.6.2. Mung bean rooting

By cutting the roots from the mung bean stem there was no production of endogenous auxins by roots, meaning that all induction effects will come from the extracts where plants were submerged. The results obtained are presented in Fig. 4(b–d).

Most microalgal suspensions had a positive effect on rooting of mung bean (Fig. 4b), except for treatments T1, T4, T5 and T7, which had a negative effect compared to the control (<100 %). The last three were all related to the condition HPH-100, meaning the permeabilization induced by this condition might not be adequate for auxin-like activity. However, the culture of HPH-100 + EH (T6) induced a significant high rooting percentage (289.7 %), equivalent to 1 mg/L synthetic IBA.

The best results were achieved by the culture of HPH-1200 (T8, 378.4 %), which was similar to synthetic control IBA 5 mg/L (358.9 %), followed by the respective supernatant (T9, 289.7 %), comparable to 1 mg/L IBA (243.2 %). The culture of EH (T2, 215.3 %) had an equivalent effect than 0.5 mg/L IBA, while the respective supernatant (T3, 285.1 %) had a higher effect, statistically like 1 mg/L IBA. Both culture (T10, 271.0 %) and supernatant (T11, 257.6 %) of HPH-1200 + EH had effects equivalent to 1 mg/L IBA.

For both culture and supernatants, EH increased the mung bean root formation results of the initial culture (T0, T1) and the culture submitted to HPH at 100 bar (T6), but decreased for the one after HPH at 1200 bar (T10). This could be related to the amount of AAs, which is higher than the optimum after EH, producing a inhibiting effect. The same was verified by Navarro-López et al. (2020a), which obtained lower rooting percentages when EH was applied.

This bioassay showed a more pronounced auxin-like effect of microalgal suspensions compared to the cucumber bioassay (Fig. 4a). Furthermore, a general higher effect is obtained from suspensions prepared from microalga culture (Fig. 4c) compared to supernatants (Fig. 4d), which contradicts the results of the cucumber bioassay (Fig. 4a). However, both evidence that HPH-1200 generates the best auxin-like activity.

3.7. Cytokinin-like activity

The cucumber cotyledon root expansion test was performed to determine the cytokinin-like activity of the microalgal extracts obtained from the different treatments (Fig. 5).

Almost all treatment solutions at 0.5 g/L showed no significant cytokinin-like effect or differences among them ($p < 0.05$). Only the initial culture (T0), without any treatment, showed positive significant effect (141.3 %) comparable to BAP 0.2–0.5 mg/L. On the other hand, both culture and supernatant of HPH-100 + EH showed a significant negative effect (61.7 and 81.3 %, respectively). A previous study by Navarro-López et al. (2020b) tested *T. obliquus* biomass grown in brewery wastewater. Likewise, most treatments showed no significant cytokinin-like effect, including the initial microalga culture, differently to the present study. On the same study, two microalgal extracts obtained after EH (culture and supernatant) showed a significant positive effect at 2 g/L but not at 0.5 g/L, suggesting that *T. obliquus* might

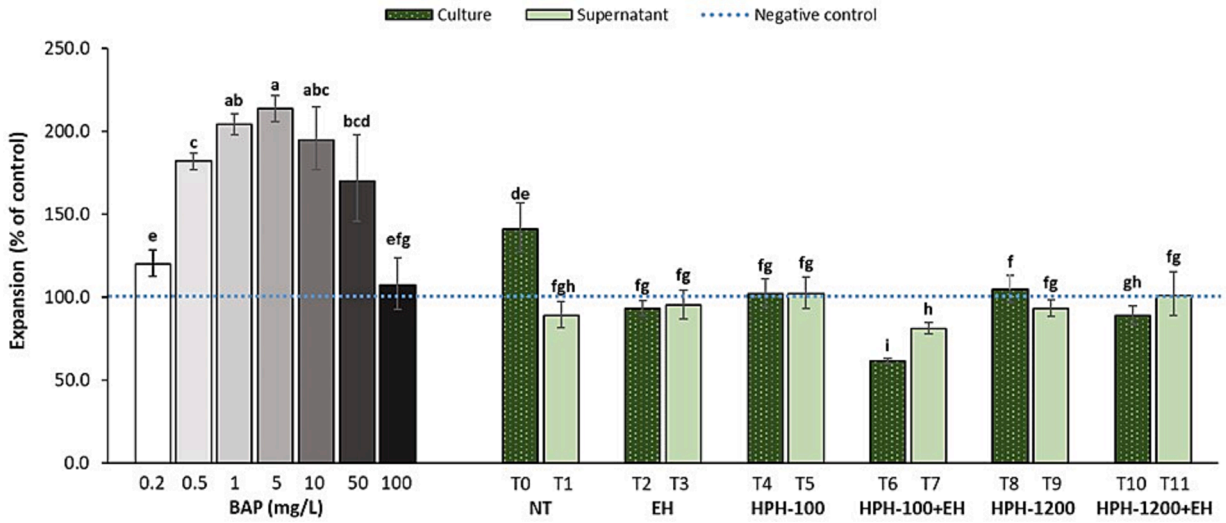

Fig. 5. Expansion of excised cucumber cotyledons treated with application of the *Tetradesmus obliquus* suspensions at 0.5 g/L (culture and supernatant after centrifugation) of non-treated (NT) biomass or after enzymatic hydrolysis (EH), cell disruption by high-pressure homogenization at 100 (HPH-100) and 1200 bar (HPH-1200), or a combination of both (HPH + EH). Distilled water was used as the negative control (dotted blue line) and a standard curve of 6-benzylaminopurine (BAP) was used as positive control (0.2–100 mg/L). The columns and error-bars represent mean ± standard deviation ($n = 3$). Different letters indicate significant difference ($p < 0.05$) among treatments according to Games-Howell's two-sided post-hoc test.

possess a low amount of cytokinin, requiring a higher concentration to detect the cytokinin-like activity.

3.8. Effect of downstream processes in bioassay results

Multifactor ANOVA was used to quantify the effects of cell disruption by HPH, EH, C and microalgal extract concentration in the various bioassays performed (see supplementary material).

For GI, HPH, C, and EC were all significant factors, except for EH (0.1772 %, $p > 0.05$). Both C and EC had a high effect compared to the other ones (>20 %). This means that, for germination of garden cress seeds, the concentration of the microalgal suspensions closely followed by centrifugation highly influenced the results. Lower concentrations generated positive results, while the use of the supernatants (C) had a negative effect. Both observations could be related to the amount of metabolites present in the microalgal suspensions, which might be excessive in higher concentrations and supernatants, since a higher volume of original suspensions are used.

Elevated HPH pressures prompted more cell disruption and, consequently the release of bioactive metabolites in amounts beyond the optimum. The initial biomass, without any treatment, at 0.2 g/L, produced the best GI, meaning that this should be the chosen condition.

For the cucumber cotyledon bioassay for testing auxin-like activity, EH and C were statistically significant factors, while HPH did not significantly influence rooting. However, the interaction HPH × EH induced the highest variation (21.97 %), followed by the interaction between all three factors (17.66 %). This means that the combination of the various factors was more significant than their individual effect.

For the auxin-like bioassay with mung bean, all individual factors played a significant role in rooting, being HPH the most significant one (26.24 %), closely followed by C (24.53 %). Regarding the interactions between factors, the HPH × C and the HPH × EH × C interactions had a significant effect, probably related to the strong effect of their individual factors HPH and C. Both auxin-like bioassays, with cucumber and mung bean, confirmed the significant effect of centrifugation.

For cytokinin-like activity, all parameters were statistically significant, including the interactions between factors. The EH imposed the highest variation (23.45 %), followed by HPH (13.95 %), and C (1.790 %). It is interesting to notice that the interaction between C with the other factors induced a higher variation in the cotyledon expansion compared to its effect alone.

3.9. Residues for biofertilizers

The whole biomass and residues (spent biomass) obtained after the downstream processes were analysed for their content in macro and micronutrients for plants (Table 3). Both biomass and residues had double the C content than the commercial organic fertilizer but exhibited significantly lower nutrients levels compared to mineral fertilizer. Specifically, NPK levels in the biomass and residue were notably lower than in mineral fertilizers, though N and P contents were closer to the ones of the organic fertilizer, but K was substantially lower than both types of commercial fertilizers. The lower P and K levels are related to the PWW composition, where *T. obliquus* was cultivated. When grown in wastewater, microalgae tend to adapt their composition to match the nutrient profile of the wastewater (Choi and Lee, 2015), which can lead to variations from expected composition based on standard growth medium. Low P and high Ca concentrations were also previously obtained by Ferreira et al. (2021), when cultivating *T. obliquus* in PWW. However, the K levels were higher, and the Mg was even lower than the limit of detection. This can be explained not only by PWW variability among batches, but also from different growth conditions. In Ferreira et al. (2021), microalgae cultivation was done in controlled indoor conditions with continuous light, while the present one was done in non-controllable outdoor conditions, with variable temperature and light.

Comparing the residues with the whole biomass, C and N contents decreased as more downstream process steps were applied, but P becomes more concentrated. The EH had a more significant impact in these changes due to protein hydrolysis, where C and N are released into the extracts, but P seemed to remain in the biomass (Miladinovic et al., 2021). K content was maintained throughout the downstream processing, while S, Ca, and Mg became more concentrated in the spent biomass. The Mg contents were equivalent to the one present in the mineral fertilizer (12 g/kg), while the S contents were up to 5 times lower. The microalgal residues possessed Cu, Fe and Mn, which were not mentioned in the composition of the commercial fertilizers. The micronutrients became slightly more concentrated in residues with increasing downstream processing. All residues could be an alternative source of organic fertilizers, especially in terms of N, P, and micronutrients, while contributing for reducing the dependence on mineral

Table 3
Contents of essential macro- and trace elements of residues (spent biomass) of *Tetradesmus obliquus* grown in piggery wastewater after cell disruption (by high-pressure homogenization (HPH) at 100 and 1200 bar), enzymatic hydrolysis (EH) or combined (HPH + EH). Whole biomass (without any treatment), and commercial mineral and organic fertilizers from SIRO® were used for comparison. NPK is given as percentage of N, P (as P_2O_5), and K (as K_2O).

Nutrients	Whole Biomass	EH	HPH-100	HPH-100 + EH	HPH-1200	HPH-1200 + EH	Mineral Fertilizer SIRO®	Organic Fertilizer SIRO®
Essential macro-elements (g/kg)								
C	423	373	406	379	420	369	–	276
N	58.7	40.7	56.5	38.4	55.9	33.6	120	60
P	6.88	7.19	6.71	7.68	6.93	10.7	13.1	8
K	6.92	6.06	6.22	4.98	6.41	5.62	70.5	33
S	3.70	4.30	4.10	4.00	5.00	5.10	24	–
Ca	71.9	80.7	70.8	93.6	77.6	97.4	–	–
Mg	12.2	14.3	11.4	14.0	11.4	14.8	12	–
Ratio C/N	7	9	7	10	8	11	–	4.6
NPK	6-6-2	4-7-2	6-6-2	4-7-1	6-6-2	4-10-1	12-12-17	6-7-8
Essential trace elements (mg/kg)								
Zn	652	660	640	656	694	813	100	–
Cu	93.4	80.0	89.9	79.4	99.1	82.5	–	–
Fe	2010	2120	2000	2470	2180	2660	–	–
Mn	428	483	425	543	453	582	–	–

fertilizers. The carbon content could improve the organic matter of soil, improving its structure and fertility, while the presence of Fe and Zn could help overcome soil deficiencies in these essential elements.

4. Conclusions

The produced biomass of *Tetradesmus obliquus* effectively served as a biostimulant for garden cress, mung bean, and cucumber. The initial microalga culture at 0.2 g/L displayed favourable results across all bioassays. The minimal downstream processing offers advantages of reduced energy, chemical, and time inputs, enhancing product profitability. Multifactor ANOVA analysis showed a preference for using biomass instead of supernatant for all bioassays. In germination trials, concentration played an important role, justifying its exploration to the other bioassays. The biomass residues contained essential macro and micronutrients for plants, comparable with commercially available organic fertilizers.

CRediT authorship contribution statement

Alice Ferreira: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Diego O. Corrêa:** Writing – review & editing, Methodology, Investigation. **Belina Ribeiro:** Methodology, Investigation. **Teresa Lopes da Silva:** Writing – review & editing, Methodology. **Cláudia Marques-dos-Santos:** Writing – review & editing, Supervision. **F. Gabriel Acién:** Writing – review & editing, Supervision. **Luisa Gouveia:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131619.

Data availability

Data will be made available on request.

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