Environmental Technology & Innovation 36 (2024) 103797

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

Innovative approach in sustainable agriculture: Harnessing microalgae potential via subcritical water extraction

Alice Ferreira [a,b,*], Jelena Vladić [c], Diego de Oliveira Corrêa [a,d], Valéria Louzada Leal Butzke [e], Pedro L. Martins [a,b], Belina Ribeiro [a], Cláudia Marques-dos-Santos [b], F. Gabriel Acién [f], Luisa Gouveia [a,g]

[a] *LNEG, National Laboratory of Energy and Geology I.P., Bioenergy and Biorefineries Unit, Estrada do Paço do Lumiar 22, Lisbon 1649-038, Portugal*

[b] *Forest Research Centre, Associate Laboratory TERRA, School of Agriculture, University of Lisbon, Tapada da Ajuda, Lisboa 1349-017, Portugal*

[c] *NOVA School of Science and Technology, NOVA University Lisbon, Caparica 2829-516, Portugal*

[d] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná (UFPR), PO Box 19046, Curitiba, Paraná CEP 81531-980, Brazil*

[e] *Postgraduate Program in Environmental Technology, University of Santa Cruz do Sul (UNISC), Santa Cruz do Sul, Rio Grande do Sul, Brazil*

[f] *Chemical Engineering Department, University of Almeria, Ctra. Sacramento, s/n, Almería 04120, Spain*

[g] *GreenCoLab, Green Ocean Technologies and Products Collaborative Laboratory, University of Algarve, Campus de Gambelas, Faro 8005-139, Portugal*

ARTICLE INFO

Keywords:
Wastewater treatment
Tetradesmus obliquus
Subcritical water extraction
Biostimulants
Biofertilizers
Amino acids

ABSTRACT

Microalgae can contribute to sustainable agriculture and wastewater treatment. This study investigated *Tetradesmus obliquus*, grown in piggery wastewater (To-PWW), as a biostimulant/biofertilizer compared to biomass grown in synthetic medium (To-B). Subcritical water extraction was tested for disruption/hydrolysis of wet biomass, at three temperatures (120, 170, and 220 °C) and two biomass loads (1:10 and 1:80 (g dry biomass/mL water)). Extracts were evaluated for germination, and root formation/expansion. Residues were quantified for nutrient composition to assess their biofertilizer potential and tested for their affinity to oil compounds for bioremediation. The best germination was achieved by To-B extracts at 170 °C (1:10: 148 % at 0.2 g/L, 1:80: 145 % at 0.5 g/L). Only To-PWW extracts at 0.2 g/L had a significant germination effect (120 °C: 120–123 % for both loads; 170 °C: 115 % for 1:80). To-PWW extract at 120 °C and 1:10 significantly affected cucumber and mung bean root formation (224 and 268 %, respectively). Most extracts significantly enhanced root expansion, with all To-B extracts at 1:10 showing the best results (139–181 %). The residues contained essential nutrients (NPK), indicating their biofertilizer potential, helping decrease synthetic fertilizers demands. To-B residues had high affinity to toluene and diesel but lower to used cooking and car oils. To-PWW showed very low affinity to all oil compounds. Finally, all residues were only able to form stable emulsions with the used car oil. This study fully exploits the use of microalgal biomass in sustainable agriculture, producing biostimulant extracts, and residues for biofertilizer and bioremediation, from a low-cost wastewater source.

* Corresponding author at: LNEG, National Laboratory of Energy and Geology I.P., Bioenergy and Biorefineries Unit, Estrada do Paço do Lumiar 22, Lisbon 1649-038, Portugal.

E-mail addresses: alice.ferreira@lneg.pt (A. Ferreira), jelenavladic@fct.unl.pt (J. Vladić), diego.biodoc@gmail.com (D. de Oliveira Corrêa), valeria.louzadaleal@gmail.com (V.L.L. Butzke), pblouromartins@gmail.com (P.L. Martins), belina.ribeiro@lneg.pt (B. Ribeiro), cms@isa.ulisboa.pt (C. Marques-dos-Santos), facien@ual.es (F.G. Acién), luisa.gouveia@lneg.pt (L. Gouveia).

https://doi.org/10.1016/j.eti.2024.103797
Received 14 June 2024; Received in revised form 28 July 2024; Accepted 17 August 2024
Available online 24 August 2024

1. Introduction

Enhancing global food security requires prioritizing sustainable approaches in the agricultural and livestock sectors. In the current scenario, agriculture must not only meet the increasing food demands of a growing population but also cope with resource depletion, water scarcity, soil degradation, and climate change. A key focus for advancing sustainable food production is utilizing environmentally friendly resources such as microalgae.

These microorganisms possess significant potential for yielding high-value agricultural products due to their constant exposure to abiotic and biotic stress. This exposure prompts the development of robust protection mechanisms, leading to the synthesis of growth-stimulating compounds like phytohormones, polysaccharides, amino acids, polyamines, and fatty acids (Ferreira et al., 2023). However, large-scale microalgae production faces major challenges, such as high nutrient costs and water consumption. The utilization of wastewater as a cost-effective source of water and nutrients can enhance the economic viability of microalgae production. This approach not only aids in the recovery of nitrogen (N) and phosphorus (P) but also prevents these nutrients from reaching water bodies, mitigating eutrophication issues. Consequently, microalgae offer a dual benefit by contributing to both wastewater treatment and addressing agricultural fertilization concerns (Acién et al., 2016).

The integral utilization of microalgal biomass is crucial to fully exploit all valuable fractions within a biorefinery concept. This comprehensive approach yields high-value products such as biostimulants and lower-value ones like biofertilizers, that collectively enhance the profitability of the biomass for sustainable agriculture. However, most bioactive metabolites in microalgae are produced and confined within cells, requiring efficient extraction technologies for release without compromising their bioactivity.

Subcritical water extraction (SWE) uses water as both the extraction solvent and catalyst within the temperature range from 100 °C (boiling point) to 374 °C (critical point) and high pressure to maintain water in liquid state. Under these conditions, water exhibits unique properties, such as higher diffusion coefficient, lower viscosity, surface tension and dielectric constant, allowing the extraction of compounds with different polarities. The possibility of modifying the properties of water by varying operational conditions such as temperature and pressure makes it a promising reaction medium for high rates of conversion at short extraction times (Thiruvenkadam et al., 2015; Zakaria and Kamal, 2016). For example, subcritical water can be a cheaper alternative to enzymatic hydrolysis to produce peptides and amino acids (Zainan et al., 2022). Likewise, the higher ionic strength of subcritical water can enhance depolymerization of polysaccharides to oligomers and to monomeric units (Álvarez-Viñas et al., 2020).

SWE is a technically efficient and environmentally friendly technology (Cvjetko Bubalo et al., 2018). Its overall sustainability, coupled with the growing research interest in microalgal high-value products, has incited research on the application of SWE for optimal microalgae extraction. However, still few studies exist, and most were done with dried biomass from pure microalga cultures grown in synthetic medium (Awaluddin et al., 2016; Rodríguez-Meizoso et al., 2010; Zainan et al., 2022; Zakaria et al., 2017). The application of SWE on microalgal biomass, particularly from wastewater-grown microalgae, is an unexplored topic. Despite successful applications for extracting high-value compounds from microalgal biomass in general, the application of SWE to biomass obtained from wastewater treatment is not widely spread due to safety concerns for human and animal uses. The stringent legislation associated to human consumption and health applications, such as pharmaceutical, cosmetics, and food, blocks the use of wastewater-grown biomass. Recent studies by Juárez et al. (2020), Ferreira et al. (2022), and Vladić et al. (2023) showed promising results with microalgae grown in pig manure, brewery, and poultry wastewater by producing extracts cleaned from the various pathogens that were initially present in the biomass. These findings mark a significant stride toward expanding the use of wastewater-grown microalgae biomass for high-value applications. However, more research is needed for the application of subcritical water for extraction of microalgae biomass grown in different types of wastewaters, as some industrial effluents, for example, might contain harmful chemical or toxic heavy metals which are assimilated by microalgae. Still, subcritical water has already been reported as an important environmental remediation tool for different emerging contaminants (Aminzai et al., 2024). The use of wet algal biomass could also avoid the energy-intensive and costly drying step, but even fewer studies exist, and they are solely focused on lipid extraction for biofuel production (Reddy et al., 2014; Zullaikah et al., 2019).

To the best of the authors' knowledge, the use of SWE for recovering bioactive compounds from microalgae biomass focusing on the biostimulant potential of extracts has not been reported to date. In fact, most studies focus on high-value products since the associated profits can offset cultivation and extraction costs. However, this significantly limits the studies to microalgal biomass produced with synthetic fertilizers, which account for greater cultivation costs. The biostimulant activity of extracts from microalgae can be evidenced by carrying out different bioassays to evaluate their effect on improving plant germination and growth (Stirk et al., 2002). Although this approach is more practical than performing the complete chemical analysis of elemental composition, amino acids and phytohormone profiles, various bioassays need to be performed to assess the different biostimulant effects. Furthermore, the spent biomass after extraction was shown to contain essential macro and micronutrients for plant development suggesting their use as biofertilizer (Vladić et al., 2023; Ferreira et al., 2022; Juárez et al., 2020; Vladić et al., 2023), and even characteristics that could lead them to new applications, such as biosurfactants.

Biosurfactants are natural amphiphilic compounds produced as secondary metabolites of microbial sources such as bacteria, yeasts, algae, and fungi (Silva et al., 2024). These compounds feature both hydrophobic and hydrophilic components within their molecular structure, enabling them to modify surfaces or interfaces by reducing the interfacial tension between immiscible substances (Silva et al., 2024). This property makes biosurfactants highly valuable across various industries, including pharmaceuticals, cosmetics, and food production, as well as in environmental applications such as soil and water treatment, enhanced oil recovery, and as adjuvants in agro-bioproducts (Jamal et al., 2023; Silva et al., 2024; Zahed et al., 2022). Compared to their synthetic counterparts, they have lower toxicity levels, improved biodegradability, and favourable physicochemical properties (Miao et al., 2024; Silva et al., 2024; Zahed

et al., 2022). However, while current research is predominantly directed towards health, cosmetic, and food-related fields, the exploration of biosurfactant applications in agriculture, particularly concerning soil and crop health and productivity, remains notably limited (Silva et al., 2024). The authors particularly noted this research gap in the context of biosurfactants derived from microalgal biomass.

Soil is a very slowly renewable natural resource, and it is important that its use does not lead to its decline to future generations. Microalgae, with their unique cellular and metabolic characteristics, can play a crucial role in soil remediation. They have been shown to help prevent erosion, recover damaged soils, or remove toxic pollutants, such as heavy metals, trace elements, hydrocarbons (Acea et al., 2001; Decesaro et al., 2017; Hu et al., 2002; Kheirfam et al., 2017; Malam Issa et al., 2007; Nisha et al., 2007; Priya et al., 2014; Saadatnia and Riahi, 2009; Tripathi et al., 2008). Microalgae can degrade the pollutants enzymatically or adsorb them onto their surfaces due to the high metal binding capacity of polysaccharides, proteins, or lipids present on their cell walls (Saavedra et al., 2018). Crops can experience increased productivity as biosurfactants' molecules assist in efficiently distributing micronutrients and metals in the soil, stimulating plant immunity, and enhancing soil hydrophilization, ensuring optimal moisture levels and uniform fertilizer distribution (Kumar et al., 2021b; Miao et al., 2024). Overall, utilizing microorganisms for bioremediation and as sources of biosurfactant molecules presents opportunities for sustainable agriculture practices. This approach represents a green and sustainable remediation option based on natural processes that are less aggressive and more suited to the ecological balance. Additionally, it represents a lower cost option when compared with alternative conventional remediation technologies (da Silva et al., 2020; Michael-Igolima et al., 2022).

The objective of the present study was to evaluate the subcritical water extraction for producing microalgal extracts with biostimulant activity from *Tetradesmus obliquus* grown in piggery wastewater (PWW) through four bioassay methodologies: i) gibberellin-like effect through germination index of garden cress seeds; ii) auxin-like activity through rooting of mung bean and excised cucumber cotyledons; and iii) cytokinin-like effect in excised cucumber cotyledons. The resulting residue (spent biomass) was also evaluated as a potential biofertilizer and as an agent for bioremediation and bioemulsification of oily compounds. This approach presents a step-forward from previous works since it directly uses wet biomass, avoiding the need for energy intensive drying step and using the treated wastewater, avoiding the water requirements of this extraction technique. Finally, this study offers new insights on the applicability of this green technology to fully exploit all microalgae biomass potential for producing safe biostimulant extracts, biofertilizers and bioremediation agents for application in sustainable agriculture and soil bioremediation, from a low-cost source (wastewater), which has not been previously assessed in literature.

2. Materials and Methods

2.1. Culture conditions

The microalga *Tetradesmus obliquus* (ACOI 204/07, ACOI Culture Collection, Coimbra University, Portugal) was cultivated in synthetic medium (Bristol) and diluted piggery wastewater (5 % v/v) (Table 1). These cultures were produced in a 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). Microalgal growth was conducted for 15 days during the month of August 2023 under natural light cycles (approximately 14 h light/10 h dark) with an average of 508.7 ± 22.6 W/m^2 and average ambient temperature of 24.3 ± 2.9 °C. The cultures were inoculated with an initial concentration of 0.2 g/L. Biomass and water were separated after gravimetric sedimentation to concentrate the biomass for following studies.

2.2. Analysis of microalgal biomass

T. obliquus biomass samples were centrifuged (6–16 KS, Sigma) at 4500 × g for 5 min and freeze-dried (Heto Power Dry LL3000, Thermo Scientific) for biochemical analysis as follows.

2.2.1. 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). 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.

Table 1
Piggery wastewater composition: pH, conductivity (k), total Kjeldahl nitrogen (TKN), ammonia (NH_4^+), phosphate (PO_4^{3-}), and chemical oxygen demand (COD).

pH	k (mS/cm)	TKN (mg N/L)	NH_4^+ (mg/L)	PO_4^{3-} (mg/L)	COD (mg O_2/L)
7.72	22.2 ± 0.3	1855 ± 0.3	1257 ± 14	198 ± 46	8305 ± 169

2.2.2. Carbohydrates

Microalgal biomass was submitted to quantitative acid hydrolysis using 72 % (w/w) H_2SO_4 (30 °C, 60 min) followed by dilution to 4 % (w/w) H_2SO_4 and autoclave hydrolysis (121 °C, 60 min) (Wychen and Laurens, 2023). The acid-insoluble organic residue was quantified by filtration through 1.22 μm glass fiber filters (VWR, USA), after correction for ash and protein.

Monosaccharides (glucose, mannose, xylose, and galactose), and furans (furfural and 5-hydroxymethylfurfural) were analyzed in an HPLC system (Agilent 1100 Series, Waldbronn, Germany), equipped with a refractive index (RI) detector and a diode array detector (DAD). Monosaccharides were analyzed using an Aminex HPX-87 P column (Bio-Rad, Hercules, USA) in combination with a cation Pb^{2+}-guard column (Bio-Rad). Elution took place at 80 °C with water as eluent at a flow rate of 0.6 mL/min (Branco et al., 2015). Samples were neutralized when needed, using barium hydroxide. Furans (furfural and 5-hydroxymethylfurfural) were analyzed using an HPX-87 H column (Bio-Rad, Hercules, USA) and a Micro-Guard Cation-H Refill Cartridge from Bio-Rad (Bio-Rad, Hercules, USA), with sulfuric acid 5 mM as mobile phase, column temperate of 50 °C and flow rate of 0.6 mL/min. Detection was carried out using a refractive index detector and a diode array detector set at 280 nm. All samples were filtered through 0.22 μm nylon membrane filters (VWR, USA) before HPLC analysis.

The percentage of the polymeric sugars was calculated from the concentration of the corresponding monomeric sugars (S), according to Eq. 1. An anhydro correction (A) of 0.90 (162/180) for C-6 sugars (glucose, galactose, and mannose) and 0.88 (132/150) for C-5 sugars (xylose) was used. Moreover, a correction factor for sugar degradation during post-hydrolysis (F) was also considered, corresponding to 1.04 for hexoses and 1.09 for pentoses.

$$Polymeric\ sugar(\%) = \mathrm{A} \times \mathrm{F} \times \frac{100}{\rho} \times \frac{S \times W_{sol}}{DW} \quad (1)$$

Where W_{sol} and DW are the weight of the solution and the biomass sample dry weight, respectively, and ρ is the volumetric mass density of the solution (g/L).

2.3. Subcritical water extraction (SWE) of microalga biomass

SWE extraction was performed in a 2 dm^3 batch-type extractor (Parr 4520, Moline, IL, USA). The extractions were conducted at 120, 170, and 220 °C, according to previous studies with the same microalga (Ferreira et al., 2022; Gouveia et al., 2021), while pressure was held constant at 3 MPa (provided by injecting nitrogen). Two different biomass loadings were tested: 1:10 and 1:80 (g dry biomass/mL water). The first, more concentrated one was used to compare with previous works using dried biomass (Gouveia et al., 2021), while the latter corresponds to a concentration that can be obtained after sedimentation. The extraction was carried out for 10 min after reaching the desired temperature. Heating times were different depending on the final temperature (19, 24, and 32 min for 120, 170, and 220 °C, respectively). After the extraction, the extractor was immediately cooled to reach room temperature, and nitrogen was discharged from the extractor. Extracts were separated from residues (spent biomass) by centrifugation at 6000 rpm for 10 min. Extracts were then filtered through 0.45 μm PTFE filter and stored at 4 °C in the dark until analysis. Solid residues were freeze-dried (Heto Power Dry LL3000, Thermo Scientific) and stored until the analysis. A total of 12 extracts and 12 residues were produced.

2.4. Analysis of extracts

The extraction yield of each downstream step was quantified gravimetrically after drying the extract at 105 °C for 3 h. Protein content was estimated by absorbance measures at 750 nm following the Lowry method with BSA (Bovine serum albumin) as standard (25–500 mg/L) (Lowry et al., 1951). The amino acid content was quantified by measuring the samples' absorbance at 750 nm after the OPA (o-phthaldialdehyde) method, using a standard curve made with serine (10–250 mg/L) (Nielsen et al., 2001). These determinations were done in duplicate. Carbohydrates content was determined through the phenol sulphuric method by measuring the absorbance at 490 nm and using glucose as standard (10–100 mg/L) (DuBois et al., 1956). Total phenol content in extracts was determined using the Folin–Ciocalteu procedure (Bobo-García et al., 2015). 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 (0–250 mg/L). The content of phenolic compounds was expressed as mg gallic acid equivalents (GAE)/mL extract. These determinations 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. Metals were determined according to an in-house method, by flame atomic absorption spectrometry (THERMO SCIENTIFIC iCE3000 Series), after sample microwave acid digestion with HNO_3/H_2O_2 followed by HNO_3/HCl acid mixture. The acid digested samples were also used to determine the phosphorous content 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 (2.5 μM) were used as the negative control and positive control, 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 lengths were measured using ImageJ software. The germination index (GI) was determined according to Zucconi et al. (1981) using Eq. 2:

$$GI(\%) = \frac{G \times L}{G_w \times L_w} \times 100 \quad (2)$$

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 cycles of 12 hours of light in a grown chamber. After 7 days of incubation, five seedlings were cut, placed in vials containing the microalgal extract and incubated in the same conditions for 5 days. Each condition was tested in triplicate so three vials (15 samples) were needed per treatment. At the end, the number of roots (longer than 1 mm) were counted on each hypocotyl. 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), and a standard curve made by a specific auxin (Indol-3-butyric acid, IBA) at 0.2–100 mg/L.

2.6.3. Cucumber cotyledon expansion and rooting bioassays

Commercial seeds of cucumber *var.* Marketeer (*Cucumis sativus* L.) 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 with small (1–2 mm) hypocotyl section were excised from seeds and transferred to Petri dishes of 60 mm diameter (10 cotyledons per Petri dish in triplicate) containing filter paper moistened with 6 mL distilled water (negative control) or the SW extracts at 0.5 g/L. 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 of the control and the treatments was measured and evaluated using a standard curve for comparison made by a standard curve of a specific cytokinin (6-Benzylaminopurine, BAP). For the rooting, the Petri dishes were maintained for 6 days and the mean number of roots, from each treatment, was compared to the control and evaluated using a standard curve for comparison made by a specific auxin (Indol-3-butyric acid, IBA).

2.6.4. Statistical analyses

Statistical data analyses were performed using the Jamovi software version 2.5.3.0. Data were log10 transformed when necessary. Datasets were verified for normality and homogeneity assumptions through the Shapiro-Wilk and Levene tests, respectively. One-way Welch's ANOVA was used to describe the individual effects of the different microalgal extracts in the various agricultural bioassays, due to its robustness against the assumption of homogenous variances, which was not complied by the datasets. Mean comparisons among treatments were done using the Games-Howell's two-side post-hoc test. 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.

2.7. Hydrophobicity of residues

The residues were evaluated for their potential for remediation of hydrocarbons. For this, the Microbial Adhesion To Hydrocarbons (MATH) method was used (Rosenberg et al., 1991). The residues were resuspended in PUM (Phosphate-Urea-$MgSO_4$) buffer to standardize the initial absorbance at 750 nm to approximately 0.5 (~0.5 g/L), using the PUM buffer as blank. Prior to resuspension, a spectral scan of the sample was performed to determine the optimal wavelength for measurement. The standardized suspension was transferred to a test tube and toluene, diesel oil, used cooking oil, and used car oil were added in the same proportion (1:1). The mixture was incubated in a water bath at 30 °C for 10 min. After, the mixture was agitated on a vortex mixer for 2 min and allowed to settle for 15 min at room temperature. Finally, approximately 1 mL of the aqueous phase (bottom layer) was collected for measuring the residual absorbance at 750 nm. The relative hydrophobicity (RH) was calculated according to Eq. 3 (Zoueki et al., 2010).

$$RH(\%) = \frac{Abs_0 - Abs_f}{Abs_0} \times 100 \quad (3)$$

The partition coefficient (*Kp*) is a measure of the differential solubility of a compound in two immiscible solvents. In simple terms, the division coefficient measures how much of a solute dissolve in the aqueous and organic phase. Furthermore, $log_{10}(K_p)$ is also widely

used to assess the hydrophobicity of an organic compound. A negative value means the compound has greater affinity for the aqueous phase (hydrophilic), while a positive value denotes a greater affinity for the organic phase (hydrophobic). When equal to zero, the compound is equally divided between both phases. This value is calculated according to Eq. 4 (Ravera et al., 1997).

$$\log_{10}(K_p) = \log_{10}\left(\frac{\mathrm{Abs}_0 - \mathrm{Abs}_f}{\mathrm{Abs}_f}\right) \quad (4)$$

For both Eqs. 3 and 4, Abs_0 is the initial absorbance at 750 nm of standardized residues suspensions and Abs_f is the final absorbance at the same wavelength of the aqueous phase (bottom layer), after phase separation.

2.8. Emulsification activity of residues

The emulsification activity of residues was evaluated by mixing the residue suspensions at 0.5 g/L with an equal volume of toluene, diesel oil, used cooking oil, and used car oil. Distilled water and Sodium dodecyl sulfate (SDS) at 0.5 g/L were also mixed with those compounds serving as negative and positive controls, respectively. After being vortexed for 2 min, the mixtures were left settling at room temperature. After 5 min, the formation of emulsification layers was observed, and the bottom and top layers were measured with a ruler. The mixtures were then left for settling for 24 hours, and again measured the bottom and top layers (Hong et al., 2017). The emulsification index (EI) was calculated according to Eq. 5. The experiments were performed in duplicate.

$$EI_t \quad (\%) \quad = \frac{L_E}{L_T} \quad \times 100 \quad (5)$$

Where L_E and L_T are the length of the emulsion layer and total length, respectively.

3. Results and discussion

3.1. Biomass composition

The biomass composition as well as the amino acid and sugar profiles of *Tetradesmus obliquus* biomass were analysed and the results are presented in Table 2. *T. obliquus* was composed of 36.7 % protein when cultivated in synthetic Bristol medium, and 25.6 % in PWW. The protein content of To-B was similar to the one reported by Khatoon et al. (2019), but higher than reported by Ferreira et al. (2018), 38.9 and 20.4 %, respectively. For To-PWW, the content was slightly lower than the previous reported one (34.5 %) (Ferreira et al., 2021). The latter could be related to the fact that indoor cultivation 24-hour light was performed in that previous study, while the present one was done outdoors with natural daylight cycles.

Both To-B and To-PWW have similar amino acid (AA) contents of approximately 51 %, being richer in non-essential AAs. Commonly, 17 AAs, except for tryptophan, are reported in *T. obliquus* cells (Liu et al., 2019). Most AAs were present in contents closer

Table 2
Composition of Tetradesmus obliquus (g/100 g biomass) grown in synthetic Bristol medium and piggery wastewater (PWW, 5 % v/v).

Compound (g/ 100 g biomass)		Bristol	PWW
Protein		36.7 ± 0.8	25.6 ± 0.3
Amino acids (AA)		50.93	50.77
	Essential AA	18.4	18.1
	Histidine (His)	1.04	1.08
	Isoleucine (Ile)	2.00	1.95
	Leucine (Leu)	5.67	5.45
	Lysine (Lys)	2.75	2.69
	Methionine (Met)	0.930	1.00
	Phenylalanine (Phe)	3.17	3.21
	Threonine (Thr)	2.80	2.75
	Non-essential AA	32.6	32.6
	Valine (Val)	3.00	29.6
	Arginine (Arg)	3.98	39.4
	Cysteine (Cys)	0.500	0.460
	Tyrosine (Tyr)	1.81	1.78
	Aspartic acid + Asparagine (Asx)	4.49	4.42
	Glutamic acid + Glutamine (Glx)	6.47	6.69
	Alanine (Ala)	0.420	0.411
	Glycine (Gly)	0.330	3.42
	Proline (Pro)	2.85	2.90
	Serine (Ser)	1.97	1.96
Carbohydrates		8.16 ± 0.33	15.0 ± 0.2
	Glucan	5.45 ± 0.29	9.67 ± 0.11
	Mannan	2.04 ± 0.07	4.06 ± 0.28
	Galactan	0.37 ± 0.02	0.84 ± 0.09
	Xylan	0.30 ± 0.02	0.46 ± 0.09

to the lower range reported by Oliveira et al. (2021), while leucine (Leu), phenylalanine (Phe), arginine (Arg), cysteine (Cys), glutamic acid (Glx), and glycine (Gly) where within the range. Both biomasses had suitable essential-to-total amino acid ratios (35.7–36.0 %), slightly higher than the ones determined for *Scenedesmus almeriensis* grown in piggery wastewater (29.3–32.7 %) (Lorenzo-Hernando et al., 2019; Rojo et al., 2023, 2021). The major AAs were glutamic acid (6.47–6.69 %), leucine (5.45–7.67 %), which is an essential AA, and aspartic acid (4.42–4.49 %). Glutamic and aspartic acids are usually reported as the main AAs present in algae (Trigueros et al., 2021). Lorenzo-Hernando et al. (2019) and Rojo et al. (2021, 2023) obtained glutamic acid and aspartic acid as the major AAs, at similar percentages than the present work.

The exogenous application of amino acids in agriculture has been shown to improve germination, photosynthesis rates, chlorophyll biosynthesis, stomata, and gene expression within the plant (Khan et al., 2019). They are also used as chelators of metal ions for accelerating their absorption and transportation within the plant (e.g. cysteine, glutamine, glycine, histidine, and lysine). Furthermore, they are involved in plant tolerance mechanisms to abiotic stresses (Sowmya et al., 2023). A study by Abdelkader et al. (2023) showed an improvement in germination rates, vigour index, and root development by applying different amino acids such as methionine, proline, and glutamine. The presence of these AAs in both To-B and To-PWW hints on their biostimulant potential for germination and root development.

Polysaccharides are polymeric carbohydrate macromolecules with complex structures in terms of monosaccharide composition. Xylose, galactose, glucose, rhamnose, and mannose are usual constituent monomers in microalgal polysaccharides (Chanda et al., 2019). They are mainly found as structural polymers (forming part of the cell wall) or energy storage polymers for various metabolic processes. In addition, some microalgae can produce exopolysaccharides (EPS) which are released into the medium (Moreira et al., 2022). In the biomass of *T. obliquus*, glucose, xylose, galactose, and mannose were identified (Table 2). Glucose, expressed as glucan was the dominant sugar, accounting for 64–67 % of total sugars, followed by mannose (25–27 %) and galactan and xylan with less than 10 %. The biomass grown in PWW has almost double the number of sugars than the one from synthetic medium, 15.0 and 8.16 %, respectively. The carbohydrate content was similar to the one reported by Martins et al. (2022) for *T. obliquus* grown in brewery wastewater (16.3 %) but lower than the previously reported for the same biomass in Bristol (27.7–30.7 %) (Ferreira et al., 2018; Khatoon et al., 2019), brewery wastewater (30.2 %) (Ferreira et al., 2018) and PWW (25.5 %) (Ferreira et al., 2021).

3.2. Extraction Yield

The extraction yields of biomass grown in synthetic medium (To-B) are generally lower compared to biomass grown in effluent (To-PWW), regardless of the biomass load. However, at 220 °C, the extraction yields for To-B are higher than To-PWW when the biomass load is 1:10. This discrepancy in yields could be attributed to differences in the composition of compounds within or surrounding the biomass from each growth medium (Fig. 1).

The extraction yield of To-B increases with temperature, whereas for To-PWW, this trend is observed only between 120 and 170 °C. At 170 °C, the biomass load does not significantly affect the extraction yield for both growth media. However, at 220 ºC, To-B exhibits different behaviours: at 1:10, the extraction yield increases, whereas at 1:80, it remains constant. This variation might be due to the different amounts of biomass, and consequently, the quantity of intracellular compounds. At 170 °C, for a biomass load of 1:80, the maximum extraction yield is achieved at 170 °C, which implies that most of the intracellular compounds were extracted, while for 1:10, some compounds remain inside the cells and are extracted at higher temperatures (220 °C). Comparing these results using wet biomass with previous ones with dried biomass (Gouveia et al., 2021), at the same temperature range and biomass load of 1:10, the extraction yield at 120 ºC is almost double with dried biomass (21.6 %) than with wet one (12.3 %). For higher temperatures, the wet biomass results in greater yields, especially at the highest temperature (24.9 and 37.7 %, respectively for dried and wet biomass at 220

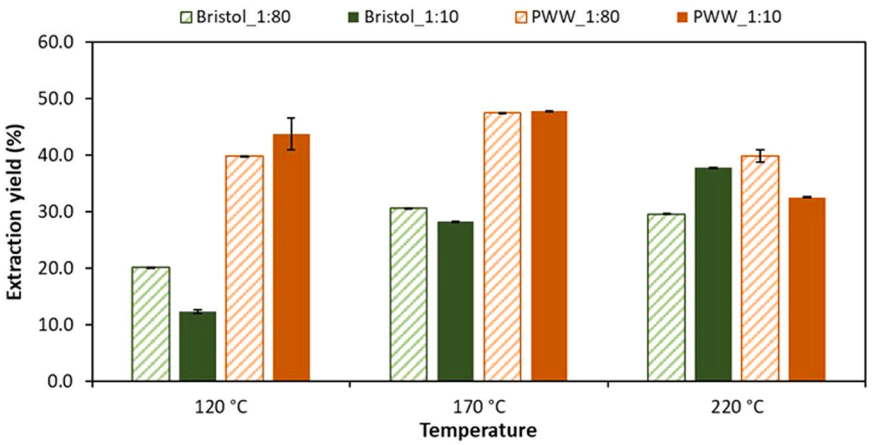

Fig. 1. Extraction yield (%) of microalga *Tetradesmus obliquus*, grown in synthetic Bristol medium or piggery wastewater (PWW), submitted to subcritical water extraction at different temperatures (120, 170, and 220 °C) and biomass loadings (1:10 and 1:80 (g dry biomass/mL water)). The columns and error-bars represent mean ± mean deviation ($n=2$).

°C). This could be explained by the fact that by not being submitted to drying processes, the wet biomass presents lowers risks of compound degradation. Furthermore, by drying the biomass, the formation of solid particles leads to the closing of the surface, forming a crust that makes the entrance of solvents more difficult (Oliveira et al., 2021). A previous study by Sarkar et al. (2020) has also shown a better extraction of pigments from wet microalgal biomass compared to the dried one. In the case of lipids, although dried biomass generates higher extraction yields, various studies have been studying the use of wet biomass to avoid the energy intensive drying step that hinders the economic feasibility of large-scale production of microalgae (Chen et al., 2012; Derwenskus et al., 2019; Reddy et al., 2014; Wang et al., 2021; Wetterwald et al., 2023).

For To-PWW, there is a decrease in extraction yield at the highest temperature (220 °C), possibly due to compound degradation, which is more expressive at a higher biomass load (1:10). This suggests that the optimal extraction temperature falls within the range of 170 and 220 °C for both types of biomasses. Additionally, a higher biomass load may be more advantageous as it allows for the extraction of more compounds without compromising extraction yield, thereby improving the economic feasibility of the extraction process. In fact, preliminary energy measurements taken throughout the experiments (data not shown) evidenced that higher biomass load could lead to savings as the same energy requirements are the same, independently of the biomass load.

3.3. Extract composition

The generated extracts were analysed in terms of protein, sugars, phenolics, and amino acids (Table 3), and extraction yields were calculated for each compound (Fig. 2).

3.3.1. Effect of Temperature

The extraction yields of proteins (Fig. 2a, b) and phenolics (Fig. 2e, f) show a general increasing trend with temperature across both biomass loads and growth media. For instance, at 120 °C, both protein and phenolic contents in To-B extract are significantly lower compared to that at 220 °C (Table 3), indicating that higher temperatures enhance the extraction efficiency of these compounds. This trend is consistent for both To-B and To-PWW at both biomass loadings, and it was also previously evidenced for *T. obliquus* grown in other effluents (Ferreira et al., 2022; Gouveia et al., 2021). For *Nannochloropsis* sp., increasingly higher protein contents were obtained from 120 to 240 °C, decreasing for higher temperatures related to potential thermal degradation or hydrolysis of the extracted proteins to amino acids (Zainan et al., 2022). For *Chlorella vulgaris*, maximum protein extraction was obtained at 277 °C (Awaluddin et al., 2016). Lower protein yields were achieved in the present work compared to those studies since a lower temperature range was tested. Unlike proteins and phenolics, the extraction of sugars displays a nuanced response to temperature (Fig. 2c, d). While increasing the temperature from 120 to 170 °C enhances sugar extraction, further raising it to 220 °C results in a drastic reduction in extraction yield, especially evident in To-B. This decline at higher temperatures may be attributed to the rapid decomposition of sugars (e.g. caramelization and Maillard reaction) when exposed to higher temperatures, as indicated by the burnt smell and caramel-like texture observed in the extracts (Awaluddin et al., 2016; Listyaningrum et al., 2021). Only trace amounts of furfural and 5-hydroxymethylfurfural were detected in some conditions. At a biomass load of 1:10, these compounds were only found in trace amounts in 220 °C extracts, with furfural being present at 0.2 g/L. As such, the severity of this treatment indicates some effect on pentose degradation and this temperature should be used carefully to preserve sugar integrity. These compounds were not detected at 1:80. However, the sugar degradation effect at the highest temperatures (220 °C) should not be discarded, as the use of 1:80 resulted in very diluted solutions that could leave the presence of these compounds below the detection and quantification threshold. Careful consideration should be given to avoid excessive temperatures that may lead to degradation and a decline in extraction yield, especially for sugars. Based on the observed trends, the optimal extraction temperature falls within the range of 170 and 220 °C for proteins, phenolics, and sugars. At 1:10, there is an increase of AAs extraction with temperature. However, at 1:80, it increases between 120 and 170 °C, decreasing after.

Table 3
Composition of extracts (protein, sugars, total phenolics, and amino acids) of *Tetradesmus obliquus* grown in different media (synthetic Bristol medium and piggery wastewater) submitted to subcritical water extraction at different temperatures (120, 170, and 220 °C) and biomass loadings (1:10 and 1:80).

Content (μg/mL extract)	Bristol				PWW			
	Protein	Sugars	Total Phenolics	Amino acids	Protein	Sugars	Total Phenolics	Amino acids
1 g/10 mL								
120 °C	3847 (± 48)	979.2 (± 4.2)	102.8 (± 6.7)	1258 (± 6)	5703 (± 16)	25807 (± 414)	359.4 (± 16.4)	1741 (± 10)
170 °C	11207 (± 0)	2085 (± 76)	663.7 (± 0.0)	2827 (± 82)	10129 (± 127)	17204 (± 976)	1577 (± 44.1)	1537 (± 9)
220 °C	12318 (± 64)	857.0 (± 24.4)	1312 (± 21.9)	3251 (± 6)	14666 (± 95)	4602 (± 214)	2599 (± 76.6)	1766 (± 3)
1 g/80 mL								
120 °C	357.3 (± 1.6)	560.0 (± 22.0)	34.8 (± 1.6)	318.3 (± 0.9)	430.3 (± 4.8)	2283 (± 48)	48.1 (± 1.8)	258.3 (± 6.2)
170 °C	920.1 (± 21.8)	752.9 (± 37.2)	87.3 (± 1.4)	358.3 (± 3.8)	1097 (± 28)	3642 (± 181)	126.3 (± 0.7)	261.9 (± 0.3)
220 °C	1107 (± 2)	424.0 (± 20.1)	193.5 (± 7.1)	281.8 (± 9.1)	2037 (± 40)	1056 (± 95)	367.5 (± 2.6)	318.9 (± 0.0)

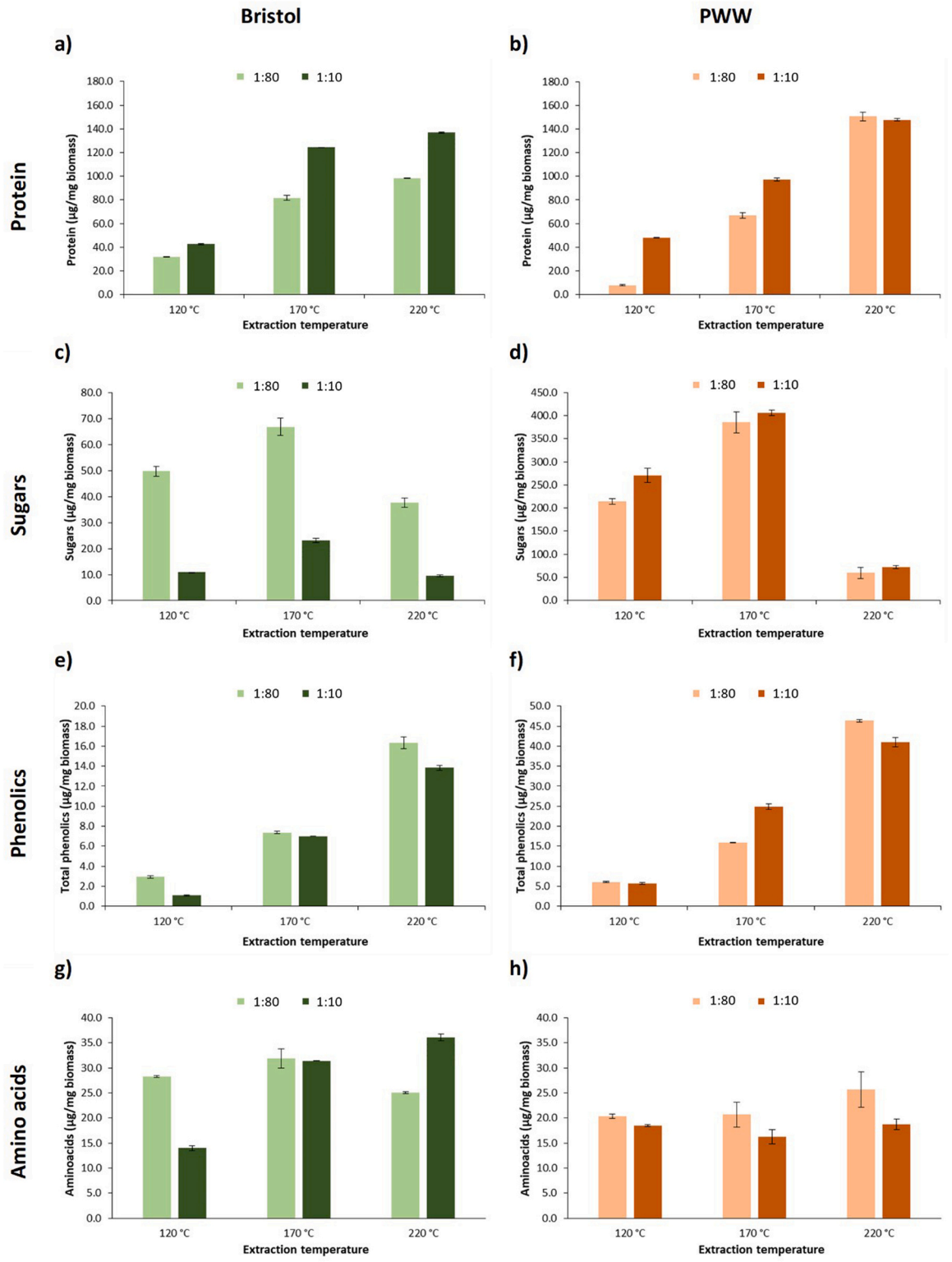

Fig. 2. Extraction yield of extracted compounds from *Tetradesmus obliquus*, grown in synthetic Bristol medium or piggery wastewater (PWW), submitted to subcritical water extraction at different temperatures (120, 170, and 220 °C) and biomass loadings (1:10 and 1:80 (g dry biomass/mL water)): a) Protein; b) Sugars; c) Phenolics; and d) Amino acids. The columns and error-bars represent mean ± mean deviation (n=2).

3.3.2. Effect of Biomass Load

Awaluddin et al. (2016) previously identified the biomass loading as the most significant parameter in protein extraction of *C. vulgaris*. In the present case, higher biomass loads generally led to higher extraction yields of proteins and phenolics. This is evident from the comparison between 1:10 and 1:80, where the extraction yields were consistently higher at the former load across all temperatures and growth media. The same trend in protein content was obtained by Zainan et al. (2022) when increasing the biomass load from 1 to 5 % w/v, but slightly decrease after that. An excess of an insoluble substrate, in this case algae, could prevent the effective contact of protein with water and interfere with the cleavage of peptide bonds (Zainan et al., 2022). The same can be said for AAs extraction, which is always higher at lower biomass load. In contrast, for sugars, a lower biomass load seems to facilitate higher extraction yields, as observed in both To-B and To-PWW. The same trend was observed for *Nannochloropsis sp.* carbohydrates (Listyaningrum et al., 2021) and Mohd Thani et al. (2019) also obtained a negative effect on the sugar yield with increasing solid loads of bakery leftovers. The authors suggested that the increase in raw materials and low availability of water could lead to saturation problems, reducing the reaction efficiency (Awaluddin et al., 2016; Gong et al., 2015). In terms of extract composition (Table 3), high biomass loads will give more concentrated extracts in all compounds (protein, sugars, phenolics, and amino acids) as expected.

3.3.3. Effect of Growth Medium

While no significant differences are observed in protein extraction yields between biomass grown in Bristol and PWW, there is a slight reduction in amino acid extraction yield for To-PWW (Fig. 2g,h). This discrepancy could be attributed to differences in nutrient composition between both growth media, influencing the synthesis and accumulation of amino acids in microalgae biomass (Table 2). Higher AAs yields were generally achieved in To-B (1.40–3.61 g AA/100 g biomass) and To-PWW (1.62–2.57 g AA/100 g biomass) compared to the maximum reported for *Nannochloropsis* sp. (1.959 g AA/100 g biomass) (Zainan et al., 2022). Notably, To-PWW exhibits higher extraction yields of sugars and phenolics compared to To-B across various temperatures and biomass loads. This difference may be attributed to the presence of pre-existing metabolites in the effluent, which could contribute to the higher content of sugars and phenolics in To-PWW. Thus, the nutrient medium within the biomass grows greatly influences the composition of extracts at least in terms of sugars and phenolics. Except for amino acids, protein, sugars, and phenolics contents are higher in To-PWW extracts that the ones from To-B for all conditions (Table 3). This means that although more protein exists in To-PWW than in To-B, there are more free amino acids, in the latter one. The influence of the nutrient medium is further evidence in previous studies, which studied subcritical extracts from *T. obliquus* grown in brewery and poultry wastewater (Ferreira et al., 2022; Vladić et al., 2023; Ferreira et al., 2022). For example, the extracts from *T. obliquus* in brewery effluent had similar phenolics contents than the ones from To-B across the same range of temperatures, while the ones from poultry wastewater-wastewater grown biomass, were even higher than the ones from To-PWW at 170 °C.

3.4. Germination index

The germination index (GI) is calculated in relation to the negative control (distilled water), which means that only microalgal

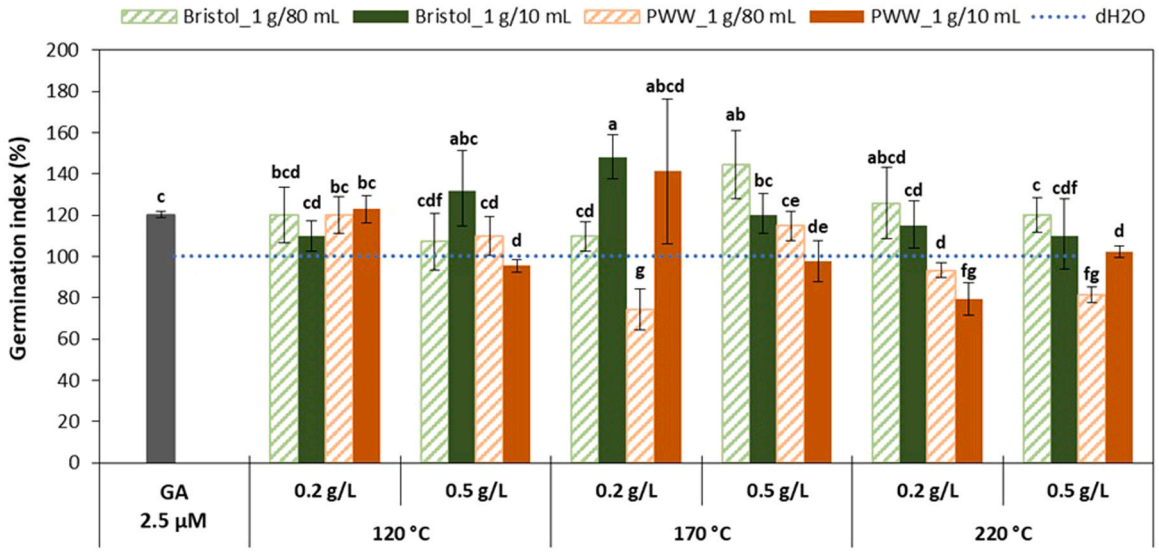

Fig. 3. Germination index of garden cress seeds treated with extracts of *Tetradesmus obliquus*, grown in Bristol medium or piggery wastewater (PWW), after subcritical water extraction at different temperatures (120, 170, and 220 °C) and biomass loadings (1:10 and 1:80 (g dry biomass/mL water)). Distilled water (dotted blue line) and gibberellic acid (GA) at 2.5 µM were used as 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.

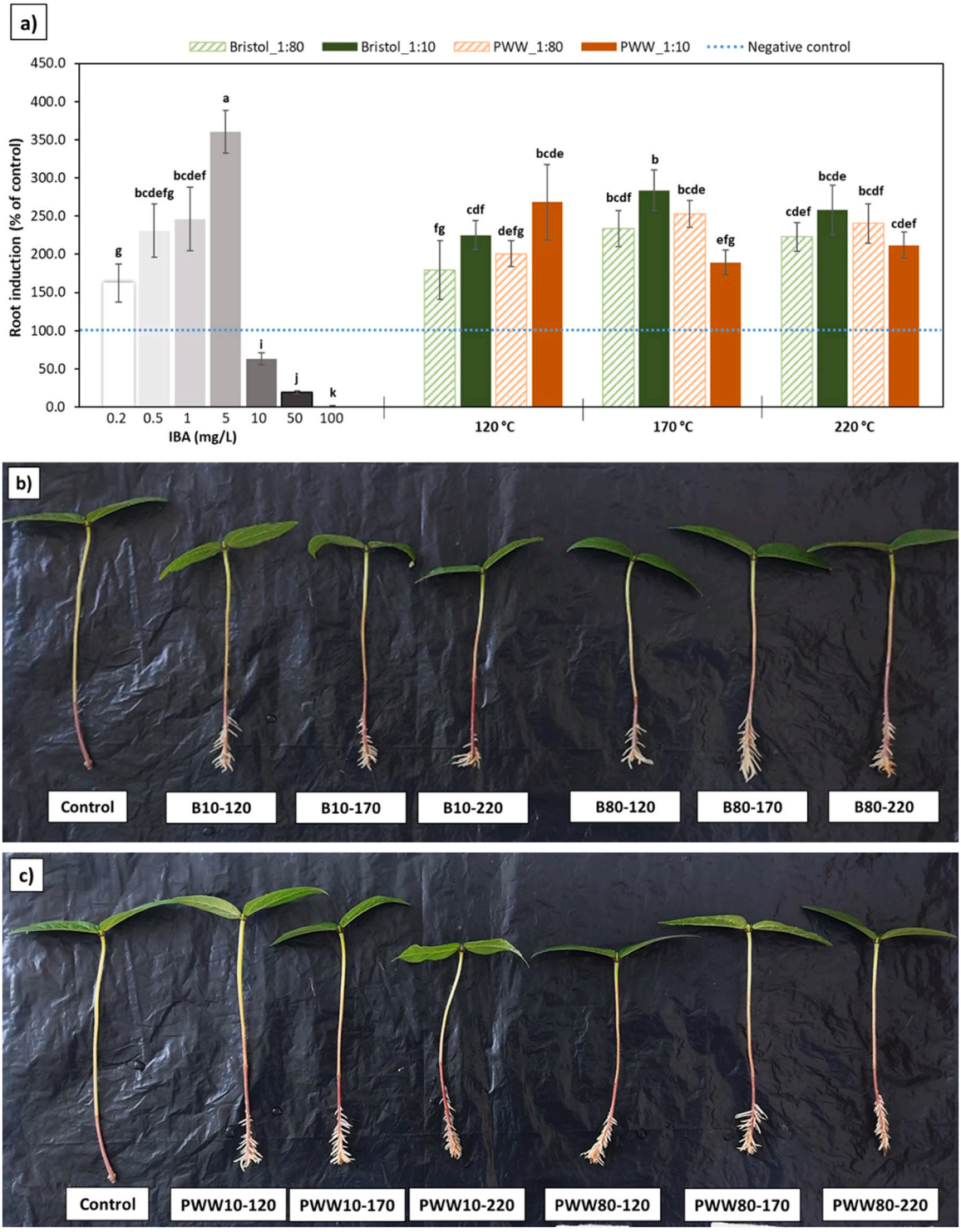

Fig. 4. Root development of mung bean treated with extracts (at 0.5 g/L) of *Tetradesmus obliquus*, grown in Bristol medium or piggery wastewater (PWW), after subcritical water extraction at different temperatures (120, 170, and 220 °C) and biomass loading (1:10 and 1:80 (g dry biomass/mL water)): a) Root induction (%). 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). 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; b) Photograph of mung bean plants treated with extracts from biomass grown in Bristol (B) medium; c) Photograph of mung bean plants treated with extracts from biomass grown in piggery wastewater (PWW).

extracts that lead to values higher than 100 % are considered to have biostimulant activity. The results obtained are presented in Fig. 3.

To-B generates extracts with higher biostimulant effect compared to To-PWW. The To-B extracts obtained at 170 °C gave the best GI results (148 % at 0.2 g/L for 1:10 and 145 % at 0.5 g/L for 1:80). These results were even higher than the positive synthetic control (120 %). For the biomass loading of 1:10, the extract at 120 °C showed no significant effect compared to the negative control but when the concentration was more than doubled (0.5 g/L), it produced a significant effect (132 %). For the same conditions, the opposite occurred with the extract at 170 °C (122 % at 0.5 g/L). This could mean that 170 °C allowed a higher extraction of compounds beneficial for germination compared with 120 °C, observation that is also supported by the higher extraction yield (Fig. 1). Thus, the extract at 120 °C allows a similar effect than the one at 170 °C by increasing the concentration, while the latter decreases its positive effect, probably due to concentrations of metabolites higher than the optimum. This is the case of phytohormones, which influence physiological process in plants at low concentrations and can inhibit at higher concentration (Davies, 2004; Tarakhovskaya et al., 2007). For both concentrations, the extract at 220 °C did not present any significant effect for the biomass loading of 1:10. However, for 1:80, the extracts at 220 °C showed an equal significant effect to synthetic GA (126 % at 0.2 g/L and 120 % at 0.5 g/L). The other To-B extracts did not generate significantly higher GI compared to distilled water control.

For To-PWW, only 3 extracts showed significant positive effect compared to the negative control. They were the extracts at 120 °C for both biomass loadings at 0.2 g/L (123 % for 1:10 and 120 % for 1:80) and the extract at 170 °C for a biomass loading of 1:80 at 0.5 g/L (115 %). Their effect was also equivalent to synthetic GA (120 %). All the other extracts produced no significant effect compared to the control, and 3 produced significant negative outcomes, including two obtained at the highest temperature (220 °C). This detrimental effect could be related to contents of metabolites higher than the optimum or the formation of harmful compounds related to the higher temperature of extraction.

These results demonstrate the presence of molecules with biological activity for inducing germination, mainly gibberellins. Other molecules with germination inducing ability are amino acids, especially aspartic acid, lysine, methionine, phenylalanine, and threonine (Sowmya et al., 2023). These amino acids were present in both biomasses (To-B and To-PWW).

3.5. Auxin-like activity

3.5.1. Mung bean rooting

This bioassay using mung bean was used to detect the presence of auxins by looking into their capacity to induce rooting. By cutting the roots from the mung bean stem we make sure that there is no production of endogenous auxins by roots, which means that all induction effect is coming from the extracts were plants are submerged. The results are presented in Fig. 4.

As it can be seen in Fig. 4, all microalgal extracts from biomass grown in both Bristol and PWW media highly induce the formation of roots, indicating that they possess auxin-like activity. They all have a significantly ($p<0.05$) higher effect (>179 %) than the negative control meaning that the induction of rooting is coming from the microalgal extracts. Furthermore, they all have statistically similar auxin-effect than the synthetic IBA in the range of 0.5–1 mg/L (Fig. 4a). It is very clear from Fig. 4b and Fig. 4c that these extracts stimulate the formation of roots comparable to the control. Auxins play an important role in the initiation of root formation and induction of elongation growth (Tarakhovskaya et al., 2007). Thus, the present results suggest the presence of this phytohormones in the extracts applied to mung bean plants. The presence of auxins in *Tetradesmus* (formerly known as *Scenedesmus*) species has been previously evidenced by Stirk et al. (2002), Navarro-López et al. (2020) (the same *T. obliquus* used in the present work), and Navarro-López et al. (2023), supporting the findings of this work.

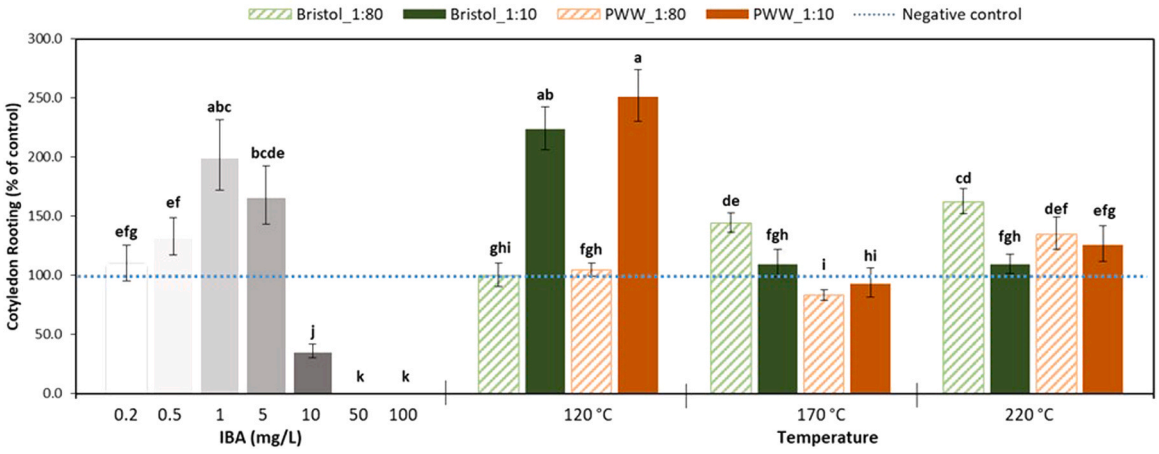

Fig. 5. Rooting of excised cucumber cotyledons treated with extracts (at 0.5 g/L) of *Tetradesmus obliquus*, grown in Bristol medium or piggery wastewater (PWW), after subcritical water extraction at different temperatures (120, 170, and 220 °C) and biomass loading (1:10 and 1:80 80 (g dry biomass/mL water)). Distilled water (dotted blue line) and a standard curve of Indole-3-acetic Acid (IBA, 0.2 – 100 mg/L) were used as 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.

3.5.2. Cucumber rooting

According to the results presented in Fig. 5, there is an increase in root induction from 0.2 to 1 mg/L of synthetic IBA. For higher concentrations, an inhibition is observed conducting to lower induction values. Regarding the extracts, 5 showed a positive significant effect compared to distilled water. The best results were achieved by the extract from To-PWW at 1:10 and 120 °C (252 %), closely followed by the one from To-B at the same conditions (224 %). Both are comparable to the best positive control (IBA 1 mg/L, 200 %). The extracts from To-B at 1:80 and extraction temperature of 170 and 220 °C are also statistically higher than the control together with the one from To-PWW at the same biomass loading (1:80) at the highest temperature (220 °C). Their effects are comparable to IBA between 0.5 and 1 mg/L. The only extract that produces a significantly lower effect than the negative control is the one from To-PWW at 1:80 and 170 °C (83 %). All the other extracts are not statistically different from distilled water, suggesting no auxin-like activity. These results contrast slightly with the one from the mung bean assay, which suggest a strong auxin-like activity from all extracts. This could be because mung bean might be more enduring of higher auxin concentration, showing no inhibition at the tested concentration. Furthermore, it could be more resistant to toxic compounds eventually present in the microalgal extracts.

3.6. 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 SWE conditions. Cytokinins are a group of phytohormones that are responsible for cell division, bud development or development of the leaf blade (Tarakhovskaya et al., 2007). Fig. 6 depicts the results of the different downstream processing and compared with the standard curve of the cytokinin 6-benzylaminopurine (BAP).

The microalgal extracts from To-B at 1:10 and temperatures 120 and 170 °C led to the highest percentages of cotyledon expansion (172 and 181 %, respectively), which is comparable to the synthetic BAP at 0.5 mg/L (180 %). Also, the microalgal extracts of To-B at 1:80 resulted in cotyledon expansion values of 135 and 134 %, respectively, at 170 and 220 °C, while the one at 120 °C did not have a significant effect compared to the control (116 %). The extracts of To-PWW were all statistically higher than the negative control, except for the one at 1:10 and 220 °C, which slightly inhibited the expansion of the cotyledon (95 %). Among then, there was no significant difference. This means that, at the tested conditions, the most extracts of both To-B and To-PWW present cytokinin-like activity. An older study by Stirk et al. (2002) applied the same bioassay to evaluate the presence of cytokinins in ten different strains of microalgae and obtained a significant effect in all species, including *Scenedesmus quadricauda,* which belongs to the same family as *T. obliquus.* A more recent study by Navarro-López et al. (2020) using the same *T. obliquus* grown in brewery wastewater also demonstrated significant cytokinin-like effects of the microalgal extracts at 0.5 and 2 g/L.

3.7. Effect of extraction parameters in bioassay results

Multifactor ANOVA was used to quantify the effects of the growth media (GM), and extraction parameters - biomass loading (BL) and temperature (T) - in the various bioassays performed. For germination index, an additional factor was considered, the extract concentration (C), making a total of 4 factors. The results of the 4-way ANOVA for the GI are shown in Table 4. For auxin- and cytokinin-like activity bioassays, only 3 factors were considered (GM, BL, and T) and the results of the 3-way ANOVA are presented in

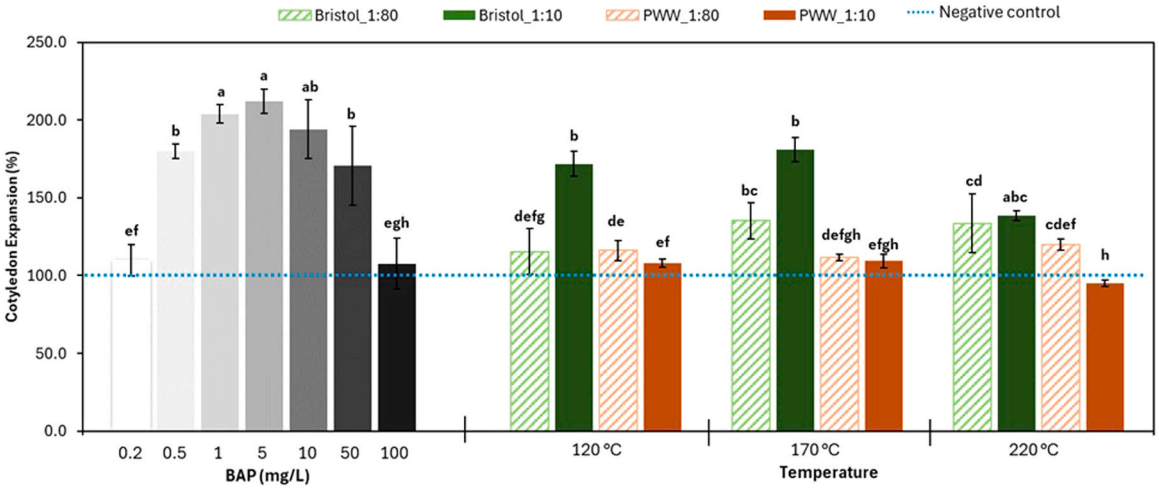

Fig. 6. Expansion of excised cucumber cotyledons treated with extracts of *Tetradesmus obliquus*, grown in Bristol medium or piggery wastewater (PWW), after subcritical water extraction at different temperatures (120, 170, and 220 °C) and biomass loading (1:10 and 1:80 (g dry biomass/mL water)), compared to distilled water (negative control – dotted blue line). 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.

Table 5.

All factors have a significant effect on the germination of garden cress seeds, except for the extract concentration (Table 4). GM has the highest influence in the germination index, with extracts from biomass grown in synthetic Bristol medium having a more pronounced significant effect than the ones from wastewater-grown biomass. Temperature also has a significant effect, with the extracts obtained at 170 °C offering the best results, which can be justified by the extraction yields (Fig. 1) and beneficial extract composition (Fig. 2 and Table 3). The biomass loading has a slightly less significant effect. However, the extracts have different behaviours at the two extract concentrations. For 0.2 g/L, extracts from 1:10 have a higher GI than the ones at 1:80, while at 0.5 g/L, the opposite occurs. This is shown by the interaction between BL and C, which has a significant effect on GI ($p<0.001$). Moreover, except for the interaction GM × BL, all the other ones have a significant effect.

Likewise, growth media has a significant effect for the cucumber bioassays, with extracts from biomass grown in synthetic medium producing more beneficial results to cotyledon root formation and expansion compared to PWW. In the case of mung bean, however, the effect of GM is negligible. Biomass loading was shown to have a significant effect for all bioassays, generating similar a similar variation percentage (Tables 5, 9.4–9.5%). Once again, temperature was a significant factor for both cucumber bioassays, especially in auxin-like effect (24.68 %), while for mung bean it had no significant effect. The interactions between factors have a general significant effect on all bioassays, except for GM × BL × T in cotyledon rooting (auxin-like effect).

Overall, GM has the most significant effect in germination index of garden cress and cytokinin-like effect in cucumber. Biomass grown in Bristol medium generates extracts with higher GI and cytokinin-like activity. BL has the most significant effect in mung bean bioassay (auxin-like effect), while temperature is the most influential factor in cucumber auxin-like bioassay. Still, all tested parameters (GM, BL, and T) significantly influence the biostimulant potential of extracts. These results suggest that the subcritical extracts from the same microalga biomass grown in different growth media have different effects, and thus should be investigated separately, especially when dealing with wet biomass, where wastewater composition should be considered. Moreover, more extraction runs should be done within the range of 170 and 220 °C to ensure the optimum extraction yields and biostimulant potential. In general, extracts obtained from a higher biomass loading (1:10) have a higher biostimulant potential in all tested bioassays. This is not only beneficial from the agricultural standpoint but also from an economic view since the processing of higher amounts of biomass will decrease the energy costs and produce richer extracts. Concentrations higher than 0.5 g/L should be tested to further understand the effect of extract concentration on biostimulant activity.

3.8. Residue composition for biofertilizer

After SWE of *T. obliquus* biomass, two fractions are generated, a liquid extract and a solid residue. This residue can still possess essential nutrients for plant development, which are shown in Table 6.

The residues of To-PWW are richer in C than the ones from To-B, which is a result of the organic matter present in the effluent, where the microalga was grown. It is also interesting to notice that the C content of To-B residues does not change with temperature, while it increases in To-PWW residues. This difference could be explained by the presence of organic matter in the pig effluent, which is either contained on the biomass or within the microalgae culture used. Either way, all residues are 1.5–2 times richer in C content than the commercial control. All residues have less than half the content of N compared to the mineral fertilizer, but they are similar to the commercial organic fertilizer, having a higher C/N ratio (>6 %). The residues from To-B are richer in P compared to the ones from To-

Table 4
Multifactor ANOVA testing the effect of the different factors – Growth media (GM), biomass loading (BL), temperature (T), and microalgal extract concentration (C) - on the germination index of garden cress seeds. The data variability is attributable to the main effect of each factor and the interaction found, as indicated by the p-value. The contribution of each factor was expressed as the percentage variation of the response (F ratio of each factor relative to the sum of all F- ratios).

	Variation (%)	*p-value*
GM	18.30	<0.001 (***)
BL	1.135	0.004 (**)
T	9.833	<0.001 (***)
C	0.2431	0.177 (ns)
2-factors interaction		
GM × BL	0.4424	0.069 (ns)
GM × T	5.183	<0.001 (***)
GM × C	3.810	<0.001 (***)
BL × T	0.7374	0.019 (*)
BL × C	3.083	<0.001 (***)
T × C	0.8960	0.036 (*)
3-factors interaction		
GM × BL × T	2.505	<0.001 (***)
GM × BL × C	0.6947	0.023 (*)
GM × T × C	2.631	<0.001 (***)
BL × T × C	22.55	<0.001 (***)
4-factors interaction		
GM × BL × T × C	3.827	<0.001 (***)

Table 5
Multifactor ANOVA testing the effect of the different factors (GM: growth medium, BL: biomass loading, and T: temperature) on the the presence of auxins (root development with mung bean and cotyledon root development with cucumber) and cytokinins (cotyledon root expansion with cucumber). The data variability is attributable to the main effect of each factor and the interaction found, as indicated by the p-value. The contribution of each factor was expressed as the percentage variation of the response (F ratio of each factor relative to the sum of all F- ratios).

Response	Statistics	GM	BL	T	Interaction GM × BL	Interaction GM × T	Interaction BL × T	Interaction GM × BL × T
Mung bean Rooting	Variation (%)	0.02026	9.462	1.054	8.145	15.78	15.24	20.00
	p-value	0.804 (ns)	<0.001 (***)	0.204 (ns)	<0.001 (***)	<0.001 (***)	<0.001 (***)	<0.001 (***)
Cotyledon Rooting	Variation (%)	0.7238	9.360	24.68	3.424	5.119	51.17	0.1881
	p-value	0.002 (**)	<0.001 (***)	<0.001 (***)	<0.001 (***)	<0.001 (***)	<0.001 (***)	0.2778 (ns)
Cotyledon expansion	Variation (%)	14.89	1.687	1.299	6.456	0.8556	2.856	0.5830
	p-value	<0.001 (***)	<0.001 (***)	<0.001 (***)	<0.001 (***)	<0.001 (***)	<0.001 (***)	<0.001 (***)

Table 6
Macro and micronutrient contents of residues (spent biomass) of *Tetradesmus obliquus* (grown in Bristol medium and piggery wastewater, PWW) after subcritical water extraction at different temperatures (120, 170, and 220 °C) and biomass loading (1:10 and 1:80). Commercial mineral and organic fertilizers from SIRO® were used for comparison.

Nutrients	Bristol				PWW				Mineral Fertilizer SIRO®	Organic Fertilizer SIRO®
	WB	120 °C	170 °C	220 °C	WB	120 °C	170 °C	220 °C		
Macronutrients (mg/kg)										
C	37.2	41.6	42.8	41.2	42.3	53.1	57.5	64.7	-	27.6
N	6.88	6.89	5.82	4.01	5.87	4.79	4.59	4.49	12	6
P	4.25	4.52	5.34	6.63	0.688	0.517	0.795	0.956	12	5
K	0.321	0.160	0.132	0.141	0.692	0.499	0.300	0.284	17	12
S	0.540	0.950	0.640	0.470	0.370	0.470	0.430	0.400	2.4	-
Ca	4.99	5.27	6.67	8.39	7.19	1.32	1.54	1.74	-	-
Mg	0.746	0.730	0.815	1.10	1.22	0.151	0.152	0.156	1.2	-
Ratio C/N	5	6	7	10	7	11	13	14	-	4.6
NPK	5–39–0.8	7–41–0.4	6–49–0.3	4–61–0.3	7–6–2	5–5–1	5–7–1	5–9–1	12–12–17	6–7–8
Micronutrients (mg/kg)										
Zn	149	214	262	323	652	548	698	834	100	-
Cu	69.8	78.7	100	124	93.4	124	162	203	-	-
Fe	21200	21400	25800	34300	2010	731	734	1010	-	-
Mn	1470	1430	1740	2290	428	158	183	192	-	-

PWW, probably because Bristol is much richer in P than the pig effluent, which is more assimilated to *T. obliquus* cells. Furthermore, the P becomes more concentrated in the residue with increasing temperature. The Bristol-grown biomass and respective residues have a much higher content of P compared to the commercial mineral fertilizer, indicating their potential as a P fertilizer. In terms of K, all residues have much lower contents than both commercial controls. Like in C, temperature does not change the K content in residues, but it decreases in To-PWW. In To-B, S becomes more concentrated in the residue obtained at 120 °C, but then its content decreases with temperature. The same is observed for To-PWW but not significant changes happen at higher temperatures. The S contents are 10x lower than the ones present at the commercial mineral fertilizer. In terms of Mg, the To-B residue at 220 °C and the initial biomass of To-PWW both have similar contents than the commercial mineral fertilizer, while the other residues are lower. Ca increases with temperature in To-B residues, while in To-PWW, it follows the same trend as S, being 10x higher in the initial biomass than in the residues. Despite this, all residues can be alternative sources of organic fertilizers especially in terms of N and P, while contributing for reducing the dependence on mineral fertilizers.

In terms of micronutrients, their quantities in the residues increase along with temperature. The microalgal residues have much higher contents of Zn compared to the mineral fertilizer (2x and 5x, respectively, for To-B and To-PWW). They also possess Cu, Fe and Mn, which are not mentioned in the composition of the commercial fertilizers. Between residues of To-B and To-PWW, the latter has almost double the amount of Cu and triple the amount of Zn. On the other hand, To-B has much higher quantities of Fe and Mn (about 30 and 10x, respectively) than To-PWW. Micronutrients like Fe and Zn play very important roles in the physiological processes of crop plants in very little amounts. Fe is required for chlorophyll synthesis and maintenance of chloroplast structure and functions. High quantities of Fe are present in soil, but with limited bioavailability. Hence, Fe-deficiency is a common nutritional disorder in many crops. Likewise, Zn is required for optimum plant growth, as it influences cell proliferation, carbohydrate metabolism, P-Zn interactions, and enzyme synthesis. Fe and Fe deficiencies in plants lead to reduced plant growth, lower yields, and poor nutritional quality of the produce. However, a high concentration of both is toxic for plants (Kumar et al., 2021a).

3.9. Hydrophobicity of residues

Aside from their fertilizer potential, the affinity of microalgal residues for hydrocarbons was assessed using the Microbial Adhesion To Hydrocarbons (MATH) assay, which could indicate their capacity to produce biosurfactants molecules and capability to remediate oil residues from contaminated soils (Table 7).

The whole biomass (WB) from both To-B and To-PWW, before extraction, was also evaluated. Comparing the initial WB from both tested mediums, To-B has a higher affinity for all tested compounds. Both show the highest affinity to toluene (84.4 and 71.7 %, respectively for To-B and To-PWW), followed by diesel oil (72.5 and 52.0 %, respectively for To-B and To-PWW), and used car oil (65.3 and 53.3 %, respectively for To-B and To-PWW). Both WB differ the most for used cooking oil, where the RH of To-B is more than double the one of To-PWW.

After extraction, the residues show different behaviours towards the different oily compounds, depending on growth media and extraction temperatures. Compared to its initial WB, To-B residue at 120 °C had a lower RH for toluene (72.0 % *vs.* 84.4 %) and the used cooking oil (50.4 % *vs.* 56.8 %), but higher for diesel oil (77.9 % *vs.* 72.5 %) and used car oil (79.4 % *vs.* 65.3 %). The residue at 170 °C presented lower RH values, except for the used car oil, which was slightly higher (68.4 %) than the WB (65.3 %). The residue at 220 °C had a higher RH for diesel oil (80.4 %), similar RH for toluene (84.8 %), and more significantly lower RH values for the used oils (<40 %) compared to the WB. Overall, among the residues of To-B, the RH values decreased with temperature for the used oils, while for toluene it increased.

For To-PWW, the residues present a lower RH for toluene and diesel but a higher affinity for the used cooking oil compared to their respective initial WB. In the case of the used car oil, it was only possible to determine the RH for the residue at 170 °C since no separation of phases was obtained at the other temperatures. Nonetheless, the RH of the residue is similar to the WB. In the case of used cooking oil, the residues showed a higher RH than the initial WB. No trend was observed regarding temperature.

Based on $\log_{10}(K_p)$ values, the To-B residues were generally hydrophobic ($\log_{10}(K_p) > 0$), except for the used cooking oil, where it became more hydrophilic with increasing temperature ($\log_{10}(K_p) < 0$). In fact, the initial biomass had a low hydrophobic character, while at 120 ºC, the residue was equally partitioned between the organic and aqueous phase ($\log_{10}(K_p) = 0$). For higher temperature, it becomes hydrophilic. In contrast, most To-PWW had a hydrophilic to low hydrophobic nature. For example, for diesel, the initial biomass and the residue at 220 °C were equally partitioned between both phases, while the others were hydrophilic. The opposite trend was observed for To-PWW for the used cooking oil, where the initial biomass started being hydrophilic and the residues became increasingly more hydrophobic with temperature. Nonetheless, the results suggest that the residues of *T. obliquus* biomass grown in synthetic medium have affinity to pure oil compounds like toluene and diesel oil, but the oil residues are more difficult to adsorb.

3.10. Emulsification activity of residues

To further address the potential of microalgae to produce biosurfactant molecules and their extraction residues to remediate contaminated soil, emulsification trials were conducted using the same hydrocarbons and oily compounds as those used in the hydrophobicity test. The emulsification assay is used to screen for biosurfactants, which will be able to emulsify hydrocarbons. Measurements were taken 5 min after mixing the suspensions and the oil compounds and after 24 hours to establish the stability of the formed emulsion. The results are presented in Fig. 7 and Table 8.

The results show that although the tested suspensions seem to form small emulsion layers between both phases after 5 min for all tested compounds, these are highly unstable being mostly gone after 24 hours, as shown by the low EI_{24}/EI_5 ratios (Table 8). Both the initial WB and residue at 120 °C from To-PWW form higher emulsion layers after 5 min for diesel oil (Fig. 7c) and used cooking oil (Fig. 7e). However, after 24 hours it is completely unformed (Fig. 7d, f). In the case of toluene, there is also the formation of a more pronounced emulsion layer to To-PWW biomass compared to the To-B after 5 min (Fig. 7a) which is still visible after 24 hours

Table 7
Relative Hydrophobicity (RH) and logarithmic base 10 of the coefficient partition ($\log_{10}(K_p)$) of initial whole biomass (WB) and spent biomass (residues) of *Tetradesmus obliquus* (grown in Bristol medium and piggery wastewater, PWW) after subcritical water extraction at different temperatures (120, 170, and 220 °C). Data is presented as mean ± mean deviation (*n=2*).

Oil compound	Parameter	Bristol				PWW			
		WB	120 °C	170 °C	220 °C	WB	120 °C	170 °C	220 °C
Toluene	RH (%)	84.4 (± 1.4)	72.0 (± 2.1)	82.4 (± 0.0)	84.8 (± 1.8)	71.7 (± 1.0)	63.7 (± 2.3)	66.6 (± 0.1)	61.9 (± 1.6)
	$\log_{10}$ (*Kp*)	0.7	0.4	0.7	0.7	0.4	0.2	0.3	0.2
Diesel oil	RH (%)	72.5 (± 0.8)	77.9 (± 1.2)	68.6 (± 0.1)	80.4 (± 0.1)	52.0 (± 0.4)	45.4 (± 2.6)	19.9 (± 1.0)	50.6 (± 0.8)
	$\log_{10}$ (*Kp*)	0.4	0.5	0.3	0.6	0.0	−0.1	−0.6	0.0
Used cooking oil	RH (%)	56.8 (± 1.0)	50.4 (± 1.5)	23.8 (±)	30.0 (± 2.3)	24.7 (± 1.5)	59.7 (± 0.0)	49.7 (± 3.2)	61.2 (± 0.0)
	$\log_{10}$ (*Kp*)	0.1	0.0	−0.5	−0.4	−0.5	−0.3	0.0	0.2
Used car oil	RH (%)	65.3 (± 3.2)	79.4 (± 0.0)	68.4 (± 0.0)	37.6 (± 0.0)	53.3 (± 3.3)	-	57.6 (± 0.0)	-
	$\log_{10}$ (*Kp*)	0.3	0.6	0.3	−0.2	0.1	-	0.1	-

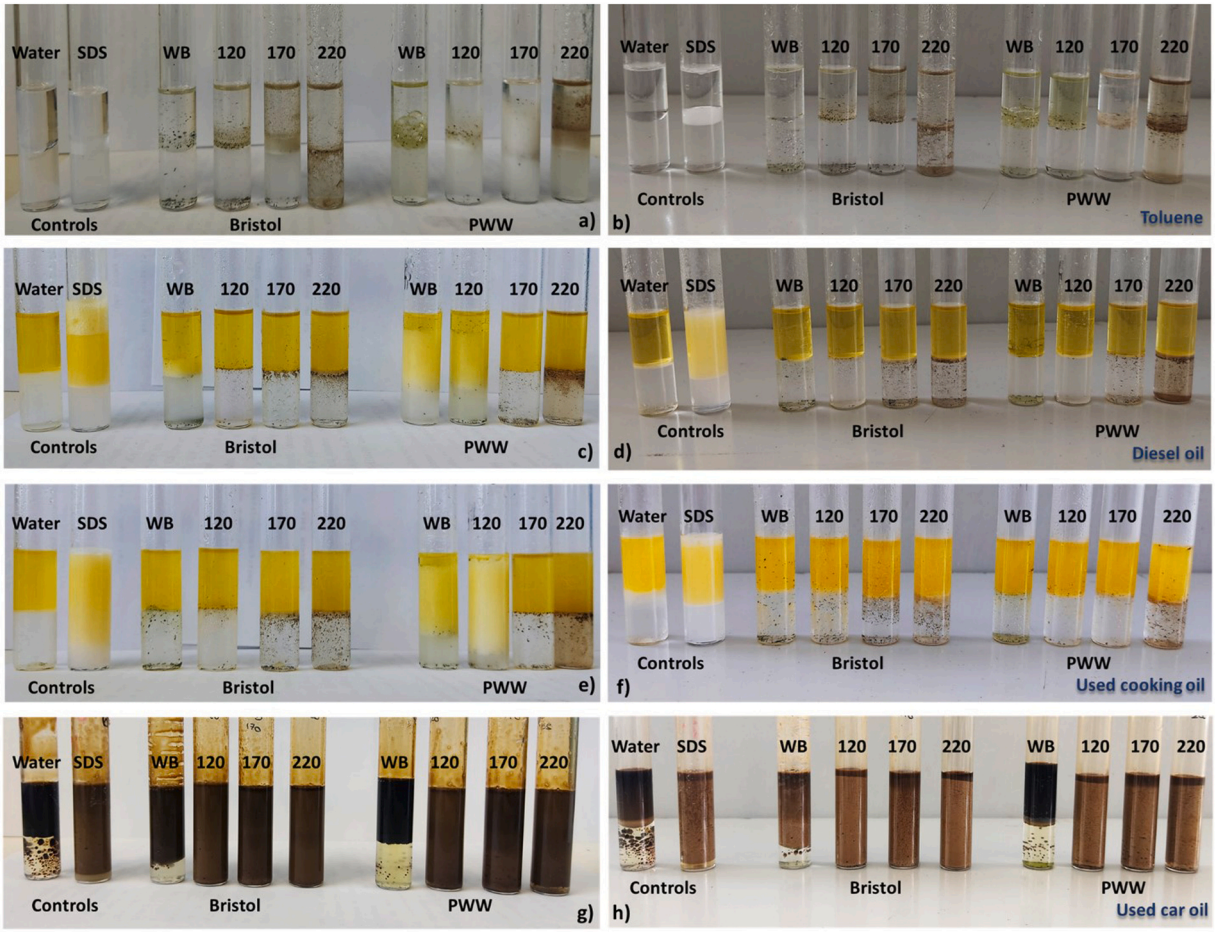

Fig. 7. Emulsification activity of initial whole biomass (WB) and spent biomass (residues) of *Tetradesmus obliquus*, grown in Bristol medium or piggery wastewater (PWW), after subcritical water extraction at different temperatures (120, 170, and 220 °C) for toluene (a, b), diesel oil (c, d), used cooking oil (e, f) and used car oil (g, h). The figures represent the mixtures after 5 min (a, c, e, g) and after 24 hours (b, d, f, h). Distilled water and Sodium Dodecyl Sulfate (SDS) at 0.5 g/L were used as the negative and positive control, respectively.

(Fig. 7b), although much smaller than the initial one. For the used car oil, all residues form stronger emulsion layers which are maintained even after 24 hours (Fig. 7h). In the case of the initial WB in Bristol medium, there is the formation of an emulsion layer which is maintained after 24 hours, but not in the case of the biomass from PWW.

An emulsion can be a heterogeneous system, comprising one immiscible liquid dispersed in another in the form of droplets. There are two types of emulsions: oil-in-water (o/w) or water-in-oil (w/o) emulsions. Both are not stable thus requiring biosurfactants to stabilize them (Sharma et al., 2022). In the case of car oil, the bio emulsifier compounds present in the microalgal suspensions produce o/w emulsions, since oil droplets are observed in the lower aqueous phase (Fig. 7g, h), while for the other compounds, w/o emulsions are formed in the upper layer (Fig. 7a, c, e). This could explain the lower stability of these w/o emulsions since water droplets are highly mobile, which leads their sedimentation, flocculation, or coalescence (Colucci et al., 2020).

The assays for evaluating hydrocarbon affinity and the emulsifying potential of extraction residues were conducted in an exploratory manner, aiming for the full utilization of *T. obliquus* biomass. These activities are mostly studied using extracts from plants and other microorganisms like bacteria and yeast, while there is a lack of studies related to microalgae. Although no studies were found directly testing the biosurfactant and bioemulsifying potential of microalgae biomass, it is known that they can produce an array of biomolecules that can act as emulsifiers or surfactants. These molecules can be attached to their cell walls, like extracellular polymeric substances, which include polysaccharides, proteins, nucleic acids, lipids, polysaccharides, and phospholipids, or they can be intracellular molecules (e.g. cytosolic proteins, membrane lipids, lipoproteins, glycolipids, and other) (Law et al., 2018).

The results of the present study suggest that biosurfactant and bioemulsifying fractions predominantly remain in the extracts rather than in the residues, which would explain the observed indices. However, the observation of some degree of emulsion formation from the residues suggests the occurrence of a Pickering emulsion, where disrupted cell debris adhered to the hydrophobic surface of the solvent droplets, stabilizing the immiscible fluids with solid particles (Law et al., 2018).

These findings highlight the potential for the biotechnological exploitation of biological activities that have been relatively

Table 8
Emulsification index after 5 min (EI_5) and after 24 hours (EI_{24}) and stability ratio (EI_{24}/EI_5) of initial whole biomass (WB) and spent biomass (residues) of *Tetradesmus obliquus* (grown in Bristol medium and piggery wastewater, PWW) after subcritical water extraction at different temperatures (120, 170, and 220 °C). Distilled water and Sodium Dodecyl Sulfate (SDS) at 0.5 g/L were used as the negative and positive control, respectively. Data is presented as mean ± standard deviation (*n=3*). NE means no emulsion layer formed.

Oil compound	Parameter	Controls		Bristol				PWW			
		Water	SDS	WB	120 °C	170 °C	220 °C	WB	120 °C	170 °C	220 °C
Toluene	EI_5 (%)	0.0 (± 0.0)	16.3 (± 2.2)	0.0 (± 0.0)	4.7 (± 0.1)	4.7 (± 0.1)	4.7 (± 0.1)	9.6 (± 2.6)	9.6 (± 0.1)	16.4 (± 13.3)	7.2 (± 0.3)
	EI_{24} (%)	0.0 (± 0.0)	14.8 (± 0.9)	0.0 (± 0.0)	0.0 (± 0.0)	4.7 (± 0.0)	0.0 (± 0.0)	2.3 (± 0.0)	12.4 (± 0.2)	9.1 (± 2.3)	4.7 (± 0.1)
	EI_{24}/EI_5	NE	0.9	NE	0.0	0.0	0.0	0.3	1.3	0.6	0.7
Diesel oil	EI_5 (%)	0.0 (± 0.0)	62.5 (± 4.3)	12.1 (± 2.8)	8.3 (± 2.8)	5.2 (± 0.4)	4.7 (± 0.2)	12.5 (± 1.1)	10.4 (± 1.3)	0.8 (± 1.1)	0.0 (± 0.0)
	EI_{24} (%)	0.0 (± 0.0)	61.4 (± 3.7)	2.3 (± 0.0)	0.0 (± 0.0)	2.4 (± 0.1)	4.7 (± 0.2)	4.7 (± 0.1)	4.4 (± 0.1)	0.0 (± 0.0)	0.0 (± 0.0)
	EI_{24}/EI_5	NE	1.0	0.2	0.0	0.5	1.0	0.3	0.1	0.0	NE
Used cooking oil	EI_5 (%)	0.0 (± 0.0)	70.5 (± 1.5)	55.5 (± 2.2)	53.1 (± 3.6)	68.0 (± 4.1)	53.2 (± 1.0)	53.2 (± 2.1)	56.7 (± 5.5)	53.3 (± 2.3)	71.2 (± 1.4)
	EI_{24} (%)	0.0 (± 0.0)	64.8 (± 4.5)	0.0 (± 0.0)	0.0 (± 0.0)	2.4 (± 0.1)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	2.3 (± 0.1)	3.9 (± 1.1)
	EI_{24}/EI_5	NE	0.9	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1
Used car oil	EI_5 (%)	0.0 (± 0.0)	90.0 (± 7.6)	76.5 (± 8.5)	93.1 (± 1.9)	96.1 (± 1.1)	96.9 (± 1.1)	0.0 (± 0.0)	84.7 (± 1.2)	88.9 (± 4.6)	91.1 (± 1.3)
	EI_{24} (%)	0.0 (± 0.0)	83.9 (± 4.3)	68.8 (± 4.4)	89.2 (± 2.2)	86.2 (± 3.5)	86.7 (± 3.8)	3.7 (± 1.0)	87.4 (± 4.6)	87.3 (± 4.0)	89.0 (± 2.3)
	EI_{24}/EI_5	NE	0.9	0.9	1.0	0.9	0.9	NE	1.0	1.0	1.0

underexplored for microalgal biomass. For example, in bioremediation, biosurfactants can be used to solubilize pollutants and enhance their biodegradability, thereby improving hydrocarbon removal through biodegradation, solubilization, mobilization, or emulsification (Silva et al., 2024). Previous studies have evidenced the ability of microalgae to perform the remediation of soils by removing hydrocarbons, heavy metals, trace elements, among others (Ferreira et al., 2023). For example, extracts of *Arthrospira platensis* containing phycocyanin have been shown to remove diesel from contaminated soils (63.9 %), while the whole biomass was more effective in biodiesel removal (88.8 %) (Decesaro et al., 2017). Other studies evidenced the use of microalgae to bioremediate aquatic environments contaminated by crude oil. *T. obliquus* (formerly *Scenedesmus obliquus*) and *Chlorella vulgaris* were shown to effectively grow and degrade crude oil under heterotrophic conditions (El-Sheekh et al., 2013). More recent studies by Xaaldi Kalhor et al. (2017), Das and Deka (2019) and Kuttiyathil et al., (2021) further supported the high ability of *C. vulgaris* to degrade crude oil hydrocarbons.

In the agricultural sector, biosurfactant molecules can act as adjuvants for seed germination and plant growth, facilitate beneficial microbe interactions, and enhance the absorption of herbicides and foliar nutrients. Bioemulsifiers used in irrigation systems can promote better delivery and stabilization of other bioproducts, such as biopesticides and biofertilizers (Silva et al., 2024).

3.11. Future perspective for research and large-scale application

Future research on SWE of microalgal biomass should focus on systematically studying the effects of varying process conditions like temperature, pressure, biomass loadin on the yield and quality of extracted bioactive compounds for specific applications. Given its high flexibility and versatility, SWE can be a promising choice for processing different microalgae biomass, as process parameters can be tailored to their unique composition and target application. This technology can be especially relevant for wastewater-derived biomass since the harsh temperature and pressure conditions have been shown to eliminate pathogens, producing safe extracts and residues (Ferreira et al., 2022; Vladić et al., 2023). As growth media was shown to influence extract composition, different microalgae-wastewater combinations should always be previously optimized. Exploring different ranges of temperature is also important not only for selective extraction of polar, moderately polar, and nonpolar bioactive compounds from natural sources, but also to avoid thermal degradation of compounds, as optimal operating conditions differ for protein, carbohydrate, and lipids (Zainan et al., 2020). Different biomass loads should also be tested, aiming for higher biomass loads to decrease energy consumption per processed biomass, but without compromising extraction yields and compound quality, and whenever possible avoiding energy-intensive dewatering steps, as this will aggravate downstream costs (Fasaei et al., 2018). Preliminary energy measurements taken during extraction runs (data not shown), showed no relevant differences between 1:10 and 1:80. Heating and cooling were only affected by temperature, with higher temperatures requiring longer heating and cooling times. This means that higher biomass load was more cost-effective since energy requirements per processed biomass were much lower. The use of co-solvents could also be explored to improve extraction efficiency (Zullaikah et al., 2019). Wet biomass instead of dried biomass should be considered as it will avoid energy-intensive drying steps, and reduce downstream costs (Derwenskus et al., 2019; Reddy et al., 2014; Zullaikah et al., 2019). In fact, extraction yield obtained with wet biomass of *T. obliquus* (present work) was higher than previously reported for dried biomass in Bristol medium (Gouveia et al., 2021).

As previously stated, few studies exist on SWE of microalgae biomass, and all exploratory studies are at laboratory scale. No pilot-

scale have been found using specifically microalgae biomass, but a recent study by Trigueros et al. (2023) evidenced the feasibility of industrial SWE of a red alga residue after agar extraction by performing a scaling-up from the lab to a pilot system. Lower but reproducible results were obtained at 130 °C in both scales, while the pilot system was able to achieve better yields using higher loadings. However, they pointed out that the heating systems need to be upgraded to ensure that temperature is maintained throughout the whole process. This means that scaling up SWE for microalgae biomass is promising but at the current bench-scale, it is difficult to assess the real costs and perform a thorough techno-economic evaluation to compare with already established large-scale processes. In fact, Sganzerla et al. (2021) performed a techno-economic analysis on subcritical water hydrolysis of brewer's spent for sugar production, showing that the scale-up process from pilot to industrial scale decreased the manufacturing costs by 80 %.

The implementation of the proposed approach at large-scale requires specialized equipment. High-pressure reactors and continuous flow systems are commercially available and can be easily adapted for large-scale SWE operations, ensuring consistent and high-throughput extraction processes. Full-scale applications of SWE have been described, mainly for resource recovery from waste, flavour, fragrance, health and cosmetic ingredients, meaning this technology falls into the TRL range of 7–9 (Essien et al., 2021). This specialized equipment leads to initial high investment costs, and the high temperature and pressure conditions entail high energy expenditure. In literature, recent studies show the potential to decrease manufacturing costs to compete with traditional solvent extraction. In a study by Todd and Baroutian (2017), the manufacturing cost of SWE of grape marc was very close to that of ethanol extraction, 52.38 and 47.75 USD/kg product. More recently, Essien et al. (2021) obtained a lower manufacturing cost for SWE compared to ethanol extraction of kanuka leaves, 1.27 and 3.32 USD/kg product, mainly due to the differences in cost between water and ethanol as raw material, the additional energy to remove ethanol from the product, and the shorter extraction times, which allow to run higher number of batches. Although the total capital investment was substantial, the Net Present Value was positive and 20 % higher than ethanol extraction, meaning that investment will return bigger profits.

In addition to the economical viewpoint, the assessment of the environmental impacts of these technologies is extremely important. Life cycle analysis (LCA) is a very powerful tool to examine the environmental impacts and energy requirements of a specific process or product throughout the course of their life cycles with specified inputs and outputs within pre-determined boundaries (Guler et al., 2024). LCA plays an important role in the identification of critical parts of operations that might require optimization or more sustainable alternatives. Few LCA studies exist on SWE for different raw materials, but they all conclude that SWE is environmentally superior to traditional solvent extraction, owing to concerns with solvent volatility in the latter (Essien et al., 2021; Todd and Baroutian, 2017). The use of water as the solvent is inherently safer and more environmentally friendly compared to organic solvents that require subsequent removal and disposal, not to mention much cheaper.

LCA studies focusing on SWE of microalgae biomass are even scarcer. Only Ponnusamy et al., (2014) conducted an LCA study focused on the SWE of lipids from *Nanochloropsis salina*. They reported that SWE required 3–5 times less energy than traditional solvent (ethanol) extraction; and the contributions to CO_2 emissions were considerably lower (0.6 *vs.* 11.4 kg CO_2/kg biodiesel). In addition, SWE can extract wet biomass directly, significantly reducing the energy needed for drying and the environmental impact.

Finally, it is important to point out conventional extraction with organic solvents or, in the case of microalgal biostimulants, where enzymatic hydrolysis is usually employed, a mechanical or chemical disruption step might be necessary to break the microalgal cells for the solvents or enzymes to capture the desired compounds (Romero-García et al., 2022). When adding wastewater to the mix, it complicates the process because it requires an additional step of sterilization of the biomass, which of course further aggravates costs. On this context, SWE could be a more cost-effective alternative by performing hydrolysis, extraction and sterilization in a single step using water instead of costly enzymes or toxic organic solvents (Ferreira et al., 2022; Vladić et al., 2023). In this case, LCA will be fundamental to fully recognize SWE as a better economic and environmentally friendly alternative in microalgal products.

4. Conclusions

Subcritical water extraction can be successfully used as an alternative to traditional microalgae processing, especially considering sustainability since it uses water as the solvent and requires short extraction times. Furthermore, due to its flexibility, SWE can be seen as the perfect candidate to process microalgae biomass, given their diversity and unique characteristics, as process parameters can be tailored to the biomass and target applications.

Subcritical water extraction of wet microalgal biomass, whether grown in synthetic medium or piggery wastewater, showed promising potential in obtaining extracts for biostimulants. The observed positive results, particularly in terms of germination enhancement and root development, are likely attributed to the presence of amino acids, phenolics, and phytohormones. Additionally, the solid residues from extraction can serve as alternative sources of organic fertilizers, as they have shown the presence of essential nutrients for plant development, especially in terms of nitrogen and phosphorus, in concentrations comparable to commercially available organic fertilizers. Moreover, the residues also demonstrated potential as biosurfactants and bioemulsifiers due to their affinity for hydrocarbons and oily compounds, which suggest potential applications in soil bioremediation and as adjuvants for agricultural products.

Multifactor ANOVA statistical analysis revealed that all factors were relevant for different bioassays. Growth media played a major role in results, suggesting the need for further testing and optimization on various combinations of microalgae-wastewater. Optimal extraction temperatures between 120 and 170 °C prevented thermal degradation of bioactive metabolites, ensuring better preservation of desired compounds, which translates into more significant biostimulant effect. A higher biomass loading not only had a positive effect on the bioassays, but the use of more concentrated biomass could improve the economic feasibility because less energy per processed biomass would be required, and richer extracts would be generated. The use of wet biomass instead of dried one potentially eliminates energy-intensive drying steps, improving economic efficiency. Future research should focus on systematically studying the

effects of varying process conditions on the yield and quality of extracted bioactive compounds. Fine-tuning these parameters could enhance extraction efficiency and reduce energy costs. The addition of different modifier of extraction can also be explored.

Overall, the use of SWE on wastewater-derived microalgal biomass offers a promising path for sustainable agriculture by (i) recycling nutrients from wastewater, avoiding their excessive release and contamination of surrounding soils and water bodies; (ii) capturing carbon dioxide during photosynthesis, which can help offset greenhouse gas emissions of conventional agriculture and livestock practices, and (ii) offering effective natural biostimulants, biofertilizers and biosurfactants, with proven beneficial effects of crop productivity, which will help reduce the reliance on chemical fertilizers.

Funding

This work was supported by the project Bilateral Portugal-India DRI/India/0609/2020 - Project WCAlgaeKIT+- Combination of Vertical Wetlands, Microalgae Photobioreactor and Microbial Fuel Cell (KIT) for wastewater treatment in small pig production farms; Biomass and Bioenergy Research Infrastructure (BBRI)- LISBOA-01–0145-FEDER-022059, supported by Operational Programme for Competitiveness and Internationalization (PORTUGAL2020), by Lisbon Portugal Regional Operational Programme (Lisboa 2020) and by North Portugal Regional Operational Programme (Norte 2020) under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (ERDF); Red CYTED P320RT0025 - RENUWAL - Red Iberoamericana para el Tratamiento de Efluentes con Microalgas; FCT/MCTES (LA/P/0008/2020 DOI 10.54499/LA/P/0008/2020, UIDP/50006/2020 DOI 10.54499/UIDP/50006/2020, and UIDB/50006/2020 DOI 10.54499/UIDB/50006/2020). A. Ferreira and P. Martins are pleased to acknowledge their PhD grants awarded by Fundação para a Ciência e Tecnologia (SFRH/BD/144122/2019 (DOI: 10.54499/SFRH/BD/144122/2019) and SFRH/BD/121704/2016, respectively). D. Corrêa and V. L.L Butzke also acknowledge their PDE and PhD grants 200258/2022–2 and 142081/2020–5, respectively, awarded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) - finance code 001. Jelena Vladic would like to acknowledge her CEEC grant CEECIND/04909/2022 awarded by Fundação para a Ciência e Tecnologia. Forest Research Centre (CEF), a research unit funded by Fundação para a Ciência e a Tecnologia (FCT), Portugal, grant number UIDB/00239/2020 and Laboratory for Sustainable Land Use and Ecosystem Services – TERRA (LA/P/0092/2020).

CRediT authorship contribution statement

Pedro L. Martins: Writing – review & editing, Investigation, Data curation. **Belina Ribeiro:** Methodology, Investigation. **Cláudia Marques dos-Santos:** Writing – review & editing, Supervision. **F. Gabriel Acién:** Writing – review & editing, Supervision. **Alice Ferreira:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Jelena Vladić:** Writing – review & editing, Methodology, Investigation. **Diego de Oliveira Corrêa:** Writing – review & editing, Methodology, Investigation, Data curation. **Valéria Louzada Leal Butzke:** Writing – review & editing, Methodology, Investigation. **Luisa Gouveia:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Valorgado for supplying the piggery wastewater. The authors also acknowledge Graça Gomes and Natércia Sousa (LNEG) for laboratorial assistance and maintenance of the microalgae cultures, and Cristina Oliveira, Rita Sousa, Sandra Calisto, Jorgiana Branco and Amélia Caldeira for their assistance in the physicochemical analysis at LBB.

References

Abdelkader, M., Voronina, L., Puchkov, M., Shcherbakova, N., Pakina, E., Zargar, M., Lyashko, M., 2023. Seed priming with exogenous amino acids improves germination rates and enhances photosynthetic pigments of onion seedlings (Allium cepa L.), 2023, Vol. 9, Page 80 Horticulturae 9, 80. https://doi.org/10.3390/HORTICULTURAE9010080.

Acea, M.J., Diz, N., Prieto-Fernández, A., 2001. Microbial populations in heated soils inoculated with cyanobacteria. Biol. Fertil. Soils 33, 118–125. https://doi.org/10.1007/s003740000298.

Acién, F.G., Gómez-Serrano, C., Morales-Amaral, M.M., Fernández-Sevilla, J.M., Molina-Grima, E., 2016. Wastewater treatment using microalgae: how realistic a contribution might it be to significant urban wastewater treatment. Appl. Microbiol Biotechnol. 100, 9013–9022. https://doi.org/10.1007/s00253-016-7835-7.

Álvarez-Viñas, M., Rodríguez-Seoane, P., Flórez-Fernández, N., Torres, M.D., Díaz-Reinoso, B., Moure, A., Domínguez, H., 2020. Subcritical water for the extraction and hydrolysis of protein and other fractions in biorefineries from agro-food wastes and algae: a review, 2020 14 Food Bioprocess Technol. 3 (14), 373–387. https://doi.org/10.1007/S11947-020-02536-4.

Aminzai, M.T., Yabalak, E., Kalderis, D., Gizir, A.M., 2024. Environmental remediation of emerging contaminants using subcritical water: a review. J. Environ. Manag. 366, 121800 https://doi.org/10.1016/J.JENVMAN.2024.121800.
Awaluddin, S.A., Thiruvenkadam, S., Izhar, S., Hiroyuki, Y., Danquah, M.K., Harun, R., 2016. Subcritical water technology for enhanced extraction of biochemical compounds from *Chlorella vulgaris*. Biomed. Res Int 2016, 1–10. https://doi.org/10.1155/2016/5816974.
Bobo-García, G., Davidov-Pardo, G., Arroqui, C., Vírseda, P., Marín-Arroyo, M.R., Navarro, M., 2015. Intra-laboratory validation of microplate methods for total phenolic content and antioxidant activity on polyphenolic extracts, and comparison with conventional spectrophotometric methods. J. Sci. Food Agric. 95, 204–209. https://doi.org/10.1002/JSFA.6706.
Branco, P.C., Dionísio, A.M., Torrado, I., Carvalheiro, F., Castilho, P.C., Duarte, L.C., 2015. Autohydrolysis of *Annona cherimola* Mill. seeds: Optimization, modeling and products characterization. Biochem Eng. J. 104, 2–9. https://doi.org/10.1016/J.BEJ.2015.06.006.
Chanda, M. joan, Merghoub, N., EL Arroussi, H., 2019. Microalgae polysaccharides: the new sustainable bioactive products for the development of plant bio-stimulants? World J. Microbiol Biotechnol. https://doi.org/10.1007/s11274-019-2745-3.
Chen, M., Liu, T., Chen, X., Chen, L., Zhang, W., Wang, J., Gao, L., Chen, Y., Peng, X., 2012. Subcritical co-solvents extraction of lipid from wet microalgae pastes of Nannochloropsis sp. Eur. J. Lipid Sci. Technol. 114, 205–212. https://doi.org/10.1002/EJLT.201100120.
Colucci, G., Santamaria-Echart, A., Silva, S.C., Fernandes, I.P.M., Sipoli, C.C., Barreiro, M.F., 2020. Development of water-in-oil emulsions as delivery vehicles and testing with a natural antimicrobial extract. Molecules 25. https://doi.org/10.3390/MOLECULES25092105.
Cvjetko Bubalo, M., Vidović, S., Radojčić Redovniković, I., Jokić, S., 2018. New perspective in extraction of plant biologically active compounds by green solvents. Food Bioprod. Process. 109, 52–73. https://doi.org/10.1016/J.FBP.2018.03.001.
Das, B., Deka, S., 2019. A cost-effective and environmentally sustainable process for phycoremediation of oil field formation water for its safe disposal and reuse, 2019 9:1 Sci. Rep. 9, 1–15. https://doi.org/10.1038/s41598-019-51806-5.
Davies, P.J., 2004. Plant Hormones.Biosynthesis, Signal Transduction, Action! Springer International Publishing.
Decesaro, A., Rampel, A., Machado, T.S., Thomé, A., Reddy, K., Margarites, A.C., Colla, L.M., 2017. Bioremediation of soil contaminated with diesel and biodiesel fuel using biostimulation with microalgae biomass. J. Environ. Eng. 143, 04016091 https://doi.org/10.1061/(asce)ee.1943-7870.0001165.
Derwenskus, F., Metz, F., Gille, A., Schmid-Staiger, U., Briviba, K., Schließmann, U., Hirth, T., 2019. Pressurized extraction of unsaturated fatty acids and carotenoids from wet Chlorella vulgaris and Phaeodactylum tricornutum biomass using subcritical liquids. GCB Bioenergy 11, 335–344. https://doi.org/10.1111/GCBB.12563.
DuBois, M., Gilles, K., Hamilton, J., Rebers, P., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem.
El-Sheekh, M.M., Hamouda, R.A., Nizam, A.A., 2013. Biodegradation of crude oil by *Scenedesmus obliquus* and *Chlorella vulgaris* growing under heterotrophic conditions. Int Biodeterior. Biodegrad. 82, 67–72. https://doi.org/10.1016/J.IBIOD.2012.12.015.
Essien, S.O., Udugama, I., Young, B., Baroutian, S., 2021. Recovery of bioactives from kānuka leaves using subcritical water extraction: Techno-economic analysis, environmental impact assessment and technology readiness level. J. Supercrit. Fluids 169, 105119. https://doi.org/10.1016/J.SUPFLU.2020.105119.
Fasaei, F., Bitter, J.H., Slegers, P.M., van Boxtel, A.J.B., 2018. Techno-economic evaluation of microalgae harvesting and dewatering systems. Algal Res 31, 347–362. https://doi.org/10.1016/j.algal.2017.11.038.
Ferreira, A., Bastos, C.R.V., Marques-dos-Santos, C., Acién-Fernandez, F.G., Gouveia, L., 2023. Algaeculture for agriculture: from past to future. Front. Agron. 5, 1064041. https://doi.org/10.3389/FAGRO.2023.1064041/BIBTEX.
Ferreira, A., Marques, P., Ribeiro, B., Assemany, P., de Mendonça, H.V., Barata, A., Oliveira, A.C., Reis, A., Pinheiro, H.M., Gouveia, L., 2018. Combining biotechnology with circular bioeconomy: from poultry, swine, cattle, brewery, dairy and urban wastewaters to biohydrogen. Environ. Res 164. https://doi.org/10.1016/j.envres.2018.02.007.
Ferreira, A., Melkonyan, L., Carapinha, S., Ribeiro, B., Figueiredo, D., Avetisova, G., Gouveia, L., 2021. Biostimulant and biopesticide potential of microalgae growing in piggery wastewater. Environ. Adv. 4, 100062 https://doi.org/10.1016/j.envadv.2021.100062.
Ferreira, A., Molnar Jazić, J., Gouveia, L., Maletić, S., Tomić, M., Agbaba, J., Vladić, J., 2022. Valorisation of microalga Tetradesmus obliquus grown in brewery wastewater using subcritical water extraction towards zero waste. Chem. Eng. J. 437, 135324 https://doi.org/10.1016/J.CEJ.2022.135324.
Gong, Y., Zhang, X., He, L., Yan, Q., Yuan, F., Gao, Y., 2015. Optimization of subcritical water extraction parameters of antioxidant polyphenols from sea buckthorn (Hippophaë rhamnoides L.) seed residue. J. Food Sci. Technol. 52, 1534–1542. https://doi.org/10.1007/S13197-013-1115-7/FIGURES/2.
Gouveia, L., Molnar Jazić, J., Ferreira, A., Maletić, S., Cvetković, D., Vidović, S., Vladić, J., 2021. Green approach for the valorization of microalgae Tetradesmus obliquus. Sustain Chem. Pharm. 24, 100556 https://doi.org/10.1016/J.SCP.2021.100556.
Guler, B.A., Tepe, U., Imamoglu, E., 2024. Sustainable point of view: life cycle analysis for green extraction technologies. ChemBioEng Rev. 11, 348–362. https://doi.org/10.1002/CBEN.202300056.
Hong, Y.H., Ye, C.C., Zhou, Q.Z., Wu, X.Y., Yuan, J.P., Peng, J., Deng, H., Wang, J.H., 2017. Genome sequencing reveals the potential of Achromobacter sp. HZ01 for bioremediation. Front Microbiol 8, 1507. https://doi.org/10.3389/FMICB.2017.01507/FULL.
Hu, C., Liu, Y., Song, L., Zhang, D., 2002. Effect of desert soil algae on the stabilization of fine sands. J. Appl. Phycol. 14, 281–292. https://doi.org/10.1023/A:1021128530086/METRICS.
Jamal, A., Ali, M.I., Badshah, M., Masood, A., Bin, 2023. Role of biosurfactants in agriculture management. Adv. Biosurfactants Res. 277–308. https://doi.org/10.1007/978-3-031-21682-4_15.
Juárez, J.M., Vladic, J., Rodríguez, S.B., Vidovic, S., 2020. Sequential valorisation of microalgae biomass grown in pig manure treatment photobioreactors. Algal Res 50, 101972. https://doi.org/10.1016/J.ALGAL.2020.101972.
Khan, S., Yu, H., Li, Q., Gao, Y., Sallam, B.N., Wang, H., Liu, P., Jiang, W., 2019. Exogenous application of amino acids improves the growth and yield of lettuce by enhancing photosynthetic assimilation and nutrient availability, 2019, Vol. 9, Page 266 Agronomy 9, 266. https://doi.org/10.3390/AGRONOMY9050266.
Khatoon, H., Rahman, N.A., Suleiman, S.S., Banerjee, S., Abol-Munafi, A.B., 2019. Growth and proximate composition of scenedesmus obliquus and selenastrum bibraianum cultured in different media and condition. proceedings of the national academy of sciences India section. B - Biol. Sci. 89, 251–257. ⟨https://doi.org/10.1007/S40011-017-0938-9/FIGURES/5⟩.
Kheirfam, H., Sadeghi, S.H., Homaee, M., Zarei Darki, B., 2017. Quality improvement of an erosion-prone soil through microbial enrichment. Soil Tillage Res 165, 230–238. https://doi.org/10.1016/j.still.2016.08.021.
Kumar, S., Kumar, Santosh, Mohapatra, T., 2021. Interaction between macro- and micro-nutrients in plants. Front Plant Sci. 12, 665583 https://doi.org/10.3389/FPLS.2021.665583.
Kumar, A., Singh, S.K., Kant, C., Verma, H., Kumar, D., Singh, P.P., Modi, A., Droby, S., Kesawat, M.S., Alavilli, H., Bhatia, S.K., Saratale, G.D., Saratale, R.G., Chung, S.M., Kumar, M., 2021b. Microbial biosurfactant: a new frontier for sustainable agriculture and pharmaceutical industries. Antioxidants 10, 1472. https://doi.org/10.3390/ANTIOX10091472.
Kuttiyathil, M.S., Mohamed, M.M., Al-Zuhair, S., 2021. Using microalgae for remediation of crude petroleum oil–water emulsions. Biotechnol. Prog. 37, e3098 https://doi.org/10.1002/BTPR.3098.
Law, S.Q.K., Mettu, S., Ashokkumar, M., Scales, P.J., Martin, G.J.O., 2018. Emulsifying properties of ruptured microalgae cells: barriers to lipid extraction or promising biosurfactants? Colloids Surf. B Biointerfaces 170, 438–446. https://doi.org/10.1016/J.COLSURFB.2018.06.047.
Listyaningrum, N.B., Azis, M.M., Sarto, Rosdi, A.N., Harun, M.R., 2021. Kinetic study of subcritical water extraction of carbohydrate from microalgae nannochloropsis sp. ASEAN J. Chem. Eng. 21, 11–18. https://doi.org/10.22146/AJCHE.60015.
Liu, X., Wang, K., Zhang, J., Wang, J., Wu, J., Peng, F., 2019. Ammonium removal potential and its conversion pathways by free and immobilized Scenedesmus obliquus from wastewater. Bioresour. Technol. 283, 184–190. https://doi.org/10.1016/J.BIORTECH.2019.03.038.
Lorenzo-Hernando, A., Ruiz-Vegas, J., Vega-Alegre, M., Bolado-Rodríguez, S., 2019. Recovery of proteins from biomass grown in pig manure microalgae-based treatment plants by alkaline hydrolysis and acidic precipitation. Bioresour. Technol. 273, 599–607. https://doi.org/10.1016/J.BIORTECH.2018.11.068.
Lowry, O., Rosebrough, N., Farr, A., Randall, R., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem.

Malam Issa, O., Défarge, C., Le Bissonnais, Y., Marin, B., Duval, O., Bruand, A., D'Acqui, L.P., Nordenberg, S., Annerman, M., 2007. Effects of the inoculation of cyanobacteria on the microstructure and the structural stability of a tropical soil. Plant Soil 290, 209–219. ⟨https://doi.org/10.1007/S11104-006-9153-9/FIGURES/6⟩.
Martins, P.L., Reis, A., Duarte, L.C., Carvalheiro, F., 2022. Effective fractionation of microalgae biomass as an initial step for its utilization as a bioenergy feedstock. Energy Convers. Manag.: X 16, 100317. https://doi.org/10.1016/J.ECMX.2022.100317.
Miao, Y., To, M.H., Siddiqui, M.A., Wang, H., Lodens, S., Chopra, S.S., Kaur, G., Roelants, S.L.K.W., Lin, C.S.K., 2024. Sustainable biosurfactant production from secondary feedstock—recent advances, process optimization and perspectives. Front Chem. 12, 1327113. https://doi.org/10.3389/FCHEM.2024.1327113/BIBTEX.
Michael-Igolima, U., Abbey, S.J., Ifelebuegu, A.O., 2022. A systematic review on the effectiveness of remediation methods for oil contaminated soils. Environ. Adv. 9, 100319 https://doi.org/10.1016/J.ENVADV.2022.100319.
Mohd Thani, N., Mustapa Kamal, S.M., Taip, F.S., Sulaiman, A., Omar, R., 2019. Effect of sub-critical water hydrolysis on sugar recovery from bakery leftovers. Food Bioprod. Process. 117, 105–112. https://doi.org/10.1016/J.FBP.2019.07.002.
Moreira, J.B., Vaz, B. da S., Cardias, B.B., Cruz, C.G., Almeida, A.C.A. de, Costa, J.A.V., Morais, M.G. de, 2022. Microalgae polysaccharides: an alternative source for food production and sustainable agriculture, 2022, Vol. 3, Pages 441-457 Polysaccharides 3, 441–457. https://doi.org/10.3390/POLYSACCHARIDES3020027.
Navarro-López, E., Ruiz-Nieto, A., Gallardo-Rodríguez, J.J., Cerón-García, M.C., González-López, C.V., Acién-Fernández, F.G., 2023. Downstream processing of Scenedesmus sp. to obtain biostimulants. J. Appl. Phycol. 35, 2193–2203. https://doi.org/10.1007/S10811-023-03039-3/FIGURES/6.
Navarro-López, E., Ruíz-Nieto, A., Ferreira, A., Gabriel Acién, F., Gouveia, L., 2020. Biostimulant potential of *Scenedesmus obliquus* grown in brewery wastewater. Molecules 25, 1–16. https://doi.org/10.3390/molecules25030664.
Nielsen, P.M., Petersen, D., Dambmann, C., 2001. Improved method for determining food protein degree of hydrolysis. J. Food Sci. 66, 642–646.
Nisha, R., Kaushik, A., Kaushik, C.P., 2007. Effect of indigenous cyanobacterial application on structural stability and productivity of an organically poor semi-arid soil. Geoderma 138, 49–56. https://doi.org/10.1016/j.geoderma.2006.10.007.
Oliveira, C.Y.B., Oliveira, C.D.L., Prasad, R., Ong, H.C., Araujo, E.S., Shabnam, N., Gálvez, A.O., 2021. A multidisciplinary review of Tetradesmus obliquus: a microalga suitable for large-scale biomass production and emerging environmental applications. Rev. Aquac. 13, 1594–1618. https://doi.org/10.1111/RAQ.12536.
Ponnusamy, S., Reddy, H.K., Muppaneni, T., Downes, C.M., Deng, S., 2014. Life cycle assessment of biodiesel production from algal bio-crude oils extracted under subcritical water conditions. Bioresour. Technol. 170, 454–461. https://doi.org/10.1016/J.BIORTECH.2014.07.072.
Priya, M., Gurung, N., Mukherjee, K., Bose, S., 2014. Microalgae in removal of heavy metal and organic pollutants from soil. Microb. Biodegrad. Bioremediat. 519–537. https://doi.org/10.1016/B978-0-12-800021-2.00023-6.
Ravera, F., Ferrari, M., Liggieri, L., Miller, R., Passerone, A., 1997. Measurement of the Partition Coefficient of Surfactants in Water/Oil Systems. ⟨https://doi.org/10.1021/LA962096⟩.
Reddy, H.K., Muppaneni, T., Sun, Y., Li, Y., Ponnusamy, S., Patil, P.D., Dailey, P., Schaub, T., Holguin, F.O., Dungan, B., Cooke, P., Lammers, P., Voorhies, W., Lu, X., Deng, S., 2014. Subcritical water extraction of lipids from wet algae for biodiesel production, 133, 73–81. https://doi.org/10.1016/j.fuel.2014.04.081.
Rodríguez-Meizoso, I., Jaime, L., Santoyo, S., Señoráns, F.J., Cifuentes, A., Ibáñez, E., 2010. Subcritical water extraction and characterization of bioactive compounds from *Haematococcus pluvialis* microalga. J. Pharm. Biomed. Anal. 51, 456–463. https://doi.org/10.1016/J.JPBA.2009.03.014.
Rojo, E.M., Filipigh, A.A., Bolado, S., 2023. Assisted-enzymatic hydrolysis vs chemical hydrolysis for fractional valorization of microalgae biomass. Process Saf. Environ. Prot. 174, 276–285. https://doi.org/10.1016/J.PSEP.2023.03.067.
Rojo, E.M., Piedra, I., González, A.M., Vega, M., Bolado, S., 2021. Effect of process parameters on the valorization of components from microalgal and microalgal-bacteria biomass by enzymatic hydrolysis. Bioresour. Technol. 335, 125256 https://doi.org/10.1016/J.BIORTECH.2021.125256.
Romero-García, J.M., González-López, C.V., Brindley, C., Fernández-Sevilla, J.M., Acién-Fernández, F.G., 2022. Simulation and techno-economical evaluation of a microalgal biofertilizer production process. Biol. (Basel) 11. https://doi.org/10.3390/BIOLOGY11091359.
Rosenberg, M., Barki, M., Bar-Ness, R., Goldberg, S., Doyle, R.J., 1991. Microbial adhesion to hydrocarbons (math). Biofouling 4, 121–128. https://doi.org/10.1080/08927019109378202.
Saadatnia, H., Riahi, H., 2009. Cyanobacteria from paddy fields in Iran as a biofertilizer in rice plants. Plant Soil Environ. 55, 207–212. https://doi.org/10.17221/384-pse.
Saavedra, R., Muñoz, R., Taboada, M.E., Vega, M., Bolado, S., 2018. Comparative uptake study of arsenic, boron, copper, manganese and zinc from water by different green microalgae. Bioresour. Technol. 263, 49–57. https://doi.org/10.1016/J.BIORTECH.2018.04.101.
Sarkar, S., Manna, M.S., Bhowmick, T.K., Gayen, K., 2020. Extraction of chlorophylls and carotenoids from dry and wet biomass of isolated Chlorella Thermophila: Optimization of process parameters and modelling by artificial neural network. Process Biochem. 96, 58–72. https://doi.org/10.1016/J.PROCBIO.2020.05.025.
Sganzerla, W.G., Zabot, G.L., Torres-Mayanga, C.ésar, Buller, P., Mussatto, L.S., Forster-Carneiro, S.I., 2021. Techno-economic assessment of subcritical water hydrolysis process for sugars production from brewer's spent grains. T. https://doi.org/10.1016/j.indcrop.2021.113836.
Sharma, N., Lavania, M., Lal, B., 2022. Biosurfactant: a next-generation tool for sustainable remediation of organic pollutants. Front Microbiol 12, 821531. https://doi.org/10.3389/FMICB.2021.821531/BIBTEX.
da Silva, I.G.S., de Almeida, F.C.G., da Rocha e Silva, N.M.P., Casazza, A.A., Converti, A., Sarubbo, L.A., 2020. Soil bioremediation: overview of technologies and trends, 2020, Vol. 13, Page 4664 Energies 13, 4664. https://doi.org/10.3390/EN13184664.
Silva, M. da G.C., Medeiros, A.O., Converti, A., Almeida, F.C.G., Sarubbo, L.A., 2024. Biosurfactants: promising biomolecules for agricultural applications, 2024, Vol. 16, Page 449 Sustainability 16, 449. https://doi.org/10.3390/SU16010449.
Sowmya, R.S., Warke, V.G., Mahajan, G.B., Annapure, U.S., 2023. Effect of amino acids on growth, elemental content, functional groups, and essential oils composition on hydroponically cultivated coriander under different conditions. Ind. Crops Prod. 197, 116577 https://doi.org/10.1016/J.INDCROP.2023.116577.
Stirk, W.A., Ördög, V., Van Staden, J., Jäger, K., 2002. Cytokinin- and auxin-like activity in Cyanophyta and microalgae. J. Appl. Phycol. 14, 215–221. https://doi.org/10.1023/A:1019928425569.
Tarakhovskaya, E.R., Maslov, Y.I., Shishova, M.F., 2007. Phytohormones in algae. Russ. J. Plant Physiol. https://doi.org/10.1134/S1021443707020021.
Thiruvenkadam, S., Izhar, S., Yoshida, H., Danquah, M.K., Harun, R., 2015. Process application of subcritical water extraction (SWE) for algal bio-products and biofuels production. Appl. Energy. https://doi.org/10.1016/j.apenergy.2015.05.076.
Todd, R., Baroutian, S., 2017. A techno-economic comparison of subcritical water, supercritical CO2 and organic solvent extraction of bioactives from grape marc. J. Clean. Prod. 158, 349–358. https://doi.org/10.1016/J.JCLEPRO.2017.05.043.
Trigueros, E., Ramos, C., Alonso-Riaño, P., Beltrán, S., Sanz, M.T., 2023. Subcritical water treatment for valorization of the red algae residue after agar extraction: scale-up from laboratory to pilot plant. Ind. Eng. Chem. Res 62, 3503–3514. https://doi.org/10.1021/ACS.IECR.2C04132/SUPPL_FILE/IE2C04132_SI_001.PDF.
Trigueros, E., Sanz, M.T., Filipigh, A., Beltrán, S., Riaño, P., 2021. Enzymatic hydrolysis of the industrial solid residue of red seaweed after agar extraction: extracts characterization and modelling. Food Bioprod. Process. 126, 356–366. https://doi.org/10.1016/J.FBP.2021.01.014.
Tripathi, R.D., Dwivedi, S., Shukla, M.K., Mishra, S., Srivastava, S., Singh, R., Rai, U.N., Gupta, D.K., 2008. Role of blue green algae biofertilizer in ameliorating the nitrogen demand and fly-ash stress to the growth and yield of rice (Oryza sativa L.) plants. Chemosphere 70, 1919–1929. https://doi.org/10.1016/j.chemosphere.2007.07.038.
Vladić, J., Jazić, J.M., Ferreira, A., Maletić, S., Cvetković, D., Agbaba, J., Vidović, S., Gouveia, L., 2023. Application of green technology to extract clean and safe bioactive compounds from tetradesmus obliquus biomass grown in poultry wastewater. Molecules 28, 2397. https://doi.org/10.3390/MOLECULES28052397/S1.
Wang, Q., Oshita, K., Takaoka, M., 2021. Effective lipid extraction from undewatered microalgae liquid using subcritical dimethyl ether. Biotechnol. Biofuels 14, 1–13. https://doi.org/10.1186/S13068-020-01871-0/TABLES/2.
Wetterwald, L., Leybros, A., Fleury, G., Delrue, F., Dimitriades-Lemaire, A., Chambonniere, P., Hertz, A., 2023. Supercritical CO2 extraction of neutral lipids from dry and wet Chlorella vulgaris NIES 227 microalgae for biodiesel production. J. Environ. Chem. Eng. 11, 110628 https://doi.org/10.1016/J.JECE.2023.110628.
Wychen, S.Van, Laurens, L., 2023. Determination of total carbohydrates in algal biomass: laboratory analytical procedure (LAP).

Xaaldi Kalhor, A., Movafeghi, A., Mohammadi-Nassab, A.D., Abedi, E., Bahrami, A., 2017. Potential of the green alga Chlorella vulgaris for biodegradation of crude oil hydrocarbons. Mar. Pollut. Bull. 123, 286–290. https://doi.org/10.1016/J.MARPOLBUL.2017.08.045.

Zahed, M.A., Mohammad, Matinvafa, A., Azari, Aryandokht, Mohajeri, L., 2022. Biosurfactant, a green and effective solution for bioremediation of petroleum hydrocarbons in the aquatic environment, 2022 2:1 Discov. Water 2, 1–20. https://doi.org/10.1007/S43832-022-00013-X.

Zainan, N.H., Sapardi, M.A.M., Ho, B.C.H., Siajam, S.I., Kamal, S.M.M., Danquah, M.K., Harun, R., 2022. Kinetic and thermodynamic characterization of amino acids generation via subcritical water reaction of microalgae Nannochloropsis sp. biomass. Biomass-.-. Convers. Biorefin 12, 2001–2014. https://doi.org/10.1007/S13399-019-00538-7/TABLES/6.

Zainan, N.H., Thiruvenkadam, S., Danquah, M.K., Harun, R., 2020. Biochemical analysis and potential applications of aqueous and solid products generated from subcritical water extraction of microalgae Chlorella pyrenoidosa biomass. J. Appl. Phycol. 32, 111–126. https://doi.org/10.1007/S10811-019-01960-0/TABLES/5.

Zakaria, S.M., Kamal, S.M.M., Harun, M.R., Omar, R., Siajam, S.I., Zakaria, S.M., Kamal, S.M.M., Harun, M.R., Omar, R., Siajam, S.I., 2017. Subcritical water technology for extraction of phenolic compounds from *Chlorella* sp. microalgae and assessment on its antioxidant activity. Molecules 22, 1105. https://doi.org/10.3390/molecules22071105.

Zakaria, S.M., Kamal, S.M.M., 2016. Subcritical water extraction of bioactive compounds from plants and algae: applications in pharmaceutical and food ingredients. Food Eng. Rev. 8, 23–34. https://doi.org/10.1007/s12393-015-9119-x.

Zhao, Z.R., Wu, Z.L., Huang, G.Q., Li, G.R., 1992. An improved disk bioassay for determining activities of plant growth regulators. J. Plant Growth Regul. 11, 209. https://doi.org/10.1007/BF02115479.

Zoueki, C.W., Tufenkji, N., Ghoshal, S., 2010. A modified microbial adhesion to hydrocarbons assay to account for the presence of hydrocarbon droplets. J. Colloid Interface Sci. 344, 492–496. https://doi.org/10.1016/J.JCIS.2009.12.043.

Zucconi, F., Forte, M., Monaco, A., De Bertoldi, M., 1981. Biol. Eval. Compost Matur. Biocycle 22, 27–29.

Zullaikah, S., Jessinia, M.C.P., Rinaldi, Yasmin, M., Rachimoellah, M., Wu, D.W., 2019. Lipids extraction from wet and unbroken microalgae chlorella vulgaris using subcritical water. Mater. Sci. Forum 964, 103–108. https://doi.org/10.4028/www.scientific.net/MSF.964.103.