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Research Article

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Posted Date: November 28th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11557275/v1

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Bioplastics production from high-rate algal pond biomass

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Acknowledgements: The authors would like to thank Dr. Maria Lúcia Calijuri for providing access to the high-rate algal ponds used to produce the biomass for this study.

Abstract

Purpose
Growing interest in sustainability has intensified the search for alternatives to conventional plastics while promoting resource recovery from wastewater. This study aimed to produce bioplastics from microalgae–bacteria biomass cultivated in high-rate algal ponds (HRAPs) treating raw domestic sewage (septic tank effluent).

Methods
Protein extraction was optimized by response surface methodology using a Doehlert design, and bioplastic films were prepared by casting whole biomass or biomass protein solution (BPS) with gelatin and glycerol.

Results
Within nine days, HRAPs achieved complete ammonium ($N\text{-}NH_4^+$) removal, an 84% reduction in organic nitrogen, eliminated estrogenic activity from the solid fraction, and produced protein-rich biomass (43.3% dry weight). Optimal extraction conditions were pH 12, 93 °C, and 47 min, with a predicted protein yield of 11.3% from biomass at 4 g L^{-1}. The films exhibited homogeneous, crack-free surfaces. Although initial formulations showed moderate phytotoxicity toward Eruca sativa seeds (Germination Index 62–78.8), the combined addition of magnesium oxide and corn starch to BPS-based films raised the Germination Index to 106, indicating non-phytotoxicity and growth promotion.

Conclusion
These findings demonstrate an integrated strategy linking wastewater treatment, protein recovery, and sustainable bioplastic production within a circular bioeconomy framework.

Keywords: Bioremediation. Microalgae. Phytotoxicity. Proteins. Sewage. Sustainability

1 Introduction

As the demand for sustainable solutions to mitigate environmental degradation and dependence on fossil resources grows, microalgae represent a promising path toward a circular bioeconomy. These photosynthetically efficient and highly adaptable organisms offer a triple benefit: they sequester atmospheric CO_2, treat wastewater, and yield valuable biomass products [1, 2].

A prominent application of microalgal biomass is bioplastic production, as an alternative to conventional petroleum-based plastics. Unlike first-generation bioplastics derived from terrestrial crops, which impact food security and demand intensive land and chemical use, microalgae can be grown in wastewater or marine environments without occupying arable land [3]. Furthermore, their rapid growth and metabolic diversity enable various bioplastic production strategies, either directly using whole microalgal cells or indirectly by extracting cellular biopolymers such as polyhydroxyalkanoates (PHA), starch, and proteins [4–6].

Bioplastics produced from microalgal biomass can be used to manufacture biodegradable grow bags [7]. In contrast to conventional plastic containers, which generate waste and require manual removal before transplanting, these bioplastic containers can be planted directly into the soil, where they biodegrade and may release nutrients [8].

However, before agricultural application, it is necessary to verify that these bioplastics do not affect seed germination or seedling development. Compounds released from the material during degradation may have phytotoxic effects. Therefore, phytotoxicity should be evaluated using standardized assays, such as the Germination Index (GI) [8, 9].

Furthermore, the technical performance of microalgae-based bioplastics depends strongly on the characteristics of the raw material. Using whole microalgal cells for bioplastic production simplifies processing and reduces costs by minimizing or eliminating energy-intensive extraction steps [10, 11]. Despite these advantages, this approach often produces materials with high heterogeneity and low water resistance due to the presence of non-polymeric cellular components [11, 12].

The protein, carbohydrate, and lipid contents of microalgae biomass directly determine the properties of the resulting bioplastic [10, 11, 13]. Proteins can form stable bioplastic films through intermolecular interactions between protein chains that generate cohesive three-dimensional polymeric networks [14]. Protein-rich microalgae, such as *Chlorella*

(51–58%) and *Spirulina* (46–63%), are suitable for bioplastic fabrication because their macromolecular chains exhibit functional properties comparable to petrochemical polymers [15]. The three-dimensional protein networks provide higher mechanical resistance than polysaccharide or lipid-based bioplastics [16]. Glycerol plasticizes the protein matrix by increasing polymer chain mobility, thereby reducing film brittleness and improving flexibility and elasticity [17, 18].

Various processing methods yield microalgae-based bioplastics, including hot compression molding, solvent casting, injection molding, and extrusion [5]. The method chosen directly determines the final properties of the bioplastic [19]. For instance, additive-free hot pressing of *Spirulina* spp. biomass yields resistant bioplastics with soil biodegradability and mechanical properties comparable to polystyrene [20].

Although microalgae have a high protein content, cell walls restrict protein accessibility [21]. Researchers have used mechanical, chemical or enzymatic methods to release intracellular components. However, protein extraction from microalgal consortia remains challenging due to strain diversity, cell wall variability, and differences in protein solubility [21–23].

While several studies explore microalgal biomass for bioplastic production, most research focuses on pure cultures grown in controlled synthetic media [13, 20, 24, 25]. Few investigations address microalgae consortia cultivated in real wastewater, an approach that aligns with the principles of sustainability resource recovery [11].

Therefore, this study aimed to cultivate microalgal biomass in wastewater, optimize protein extraction from the biomass and produce bioplastics from both the biomass and the extracted protein solution. This approach integrates wastewater treatment and bioplastic production within a circular bioeconomy framework.

2 Materials and Methods

2.1 Wastewater treatment and biomass production

The domestic sewage was collected at the exit of a septic tank at a wastewater treatment plant (WWTP) located in the Romão dos Reis neighborhood, in the city of Viçosa, Minas Gerais. The effluent was transported to the Laboratory of Sanitary and Environmental Engineering (LESA) at the Federal University of Viçosa (UFV) and stored in five-thousand-liter tanks until fed to the high rate algal ponds (HRAPs).

Wastewater treatment was carried out in batch mode for nine days during May and June 2024 at the LESA experimental area. Minimum and maximum temperatures during the treatment period were 10.4 °C and 27.7 °C; the average relative humidity was 83%

and global radiation was 1,148 KJ m^{-2}. There was no rainfall during the experimental days. Three fiberglass HRAPs equipped with paddles composed of six stainless steel blades, rotating at a speed of approximately 0.13 m s^{-1} were used. The HRAPs were 1.28 m wide and 2.86 m long with a working depth of 0.26 m and a working volume of 860 liters.

Each HRAP was inoculated with 10^6 cells L^{-1} of microalgae, corresponding to 20% (v/v) of inoculum. At the end of the treatment, the paddles were turned off for sedimentation of the produced biomass. The clarified treated wastewater was discarded and the collected biomass was centrifuged (Hitachi CR 21GIII) at 2,683 *g* for 15 minutes, the resulting pellets were frozen and lyophilized (Solab, SL-404) at -40 °C and -700 mmHg for six days. The lyophilized biomass was macerated with a mortar and pestle and passed through a 0.3 mm sieve (48 mesh).

2.2 Wastewater and biomass characterization

Wastewater characteristics were measured using the following standard methods (APHA 2023). Treatment efficiency was evaluated by measuring initial and final organic (DOC) and inorganic (DIC) dissolved carbon (5310 B), soluble phosphorus (P_{sol}) (4500-P D) and ammonium-nitrogen ($N\text{-}NH_4^+$) (4500-NH_3 B and C). Nitrate-nitrogen ($N\text{-}NO_3^-$) was quantified using a NitraVer reagent kit (Hach Company). Before DOC, DIC, P_{sol}, and $N\text{-}NO_3^-$ quantification, samples were filtered through 0.45µm cellulose ester membranes.

Dissolved oxygen (DO) (4500-O G) and pH (4500-H^+ B) were also monitored during the treatment process. Samples for total (TSS) and volatile (VSS) suspended solids (2540 D and E) were filtered using 1.2 µm porosity glass fiber filters (FilterPro) and 0.7 µm glass fiber filters (Whatman GF/F) for chlorophyll-a (10200 H) analysis. Microalgal growth was monitored throughout the treatment by measuring chlorophyll *a* content, and the development of the microalgae-bacteria consortium by measuring VSS.

Sewage estrogenicity was evaluated via the Yeast Estrogen Screen (YES) following the method proposed by Routledge and Sumpter [26], with modifications. For the liquid sewage samples, 100-mL aliquots were filtered through 0.45 µm membranes and then concentrated using solid phase extraction (SPE) cartridges (Strata-X, 500 mg/6 mL, Phenomenex, USA). Estrogenic activity was also evaluated in solids from three distinct sewage fractions separated by sequential filtration: large solids (> 0.7 µm), intermediate solids (0.45 - 0.7 µm) and dissolved solids (< 0.45 µm). After filtration, the membranes containing the respective solid fractions were placed in 20 mL of methanol and the solids

were recovered by ultrasonic extraction in three 10-min cycles. The extracted fractions were diluted to 200 mL with ultrapure water and processed via SPE [27]. The material retained in the SPE cartridges was eluted with acetone, evaporated to dryness, and reconstituted in 1 mL of HPLC-grade ethanol for use in the YES test. A standard curve was prepared from a stock solution of 17β-estradiol (54.48 μg L^{-1}) and results were expressed as 17β-estradiol equivalents per liter (E2-eq L^{-1}).

The carbohydrate content of the biomass was determined by quantitative acid hydrolysis [28] followed by the phenol-sulfuric acid method [29]. Absorbances were measured at 490 nm (spectrophotometer Hach, DR 3800) and results obtained by interpolation of a glucose standard curve. Protein content was quantified according to method 4500-NorgC [30], applying a nitrogen-to-protein conversion factor of 6.25 [31]. Total organic carbon (TOC) was measured using an SSM-5000A analyzer coupled to a TOC-VCSH system (Shimadzu).

Moisture, ash, volatile matter, and fixed carbon contents were determined according to the American Society for Testing and Materials [32]. Initially, 1 g of biomass was dried at 105 ± 2 °C for 1 h. Moisture content was determined by the mass difference before and after drying. Subsequently, the sample was heated at 900 ± 5 °C for 7 min to determine volatile matter. Ash content was determined at 700 ± 5 °C for 4 h. Finally, the fixed carbon content was then calculated by difference as: fixed carbon (%) = 100 − [moisture (%) + volatile matter (%) + ash (%)].

Finally, microorganisms present in the freeze-dried biomass were identified by Neoprospecta Microbiome Technologies (Florianópolis, Brazil) via 16S rRNA amplicon sequencing for bacteria and 18S rRNA sequencing for microalgae

2.3 Protein extraction

To optimize protein extraction from the microalgae-bacteria consortium, response surface methodology with a Doehlert experimental design was used. The previously sieved biomass was dissolved in purified water at a ratio of 1:250 w/v (4 g L^{-1}) and homogenized for 30 minutes on a magnetic stirrer.

After pH adjustment, the mixture was kept in a water bath under conditions predefined by the experimental design. At the end of the reaction time, samples were centrifuged at 2,500 *g* for 3 minutes to remove microalgae cells. The response variable was the concentration of solubilized protein in the supernatant (biomass protein solution - BPS).

Three independent variables (operational factors) were evaluated: pH, time (min), and temperature (°C). The lower and upper levels for each factor, pH (2–12), time (10–50

min), and temperature (35–95 °C), were predefined based on preliminary experiments. The design comprised 19 experimental runs, including the center point (pH 7, 65 °C, 30 min), which was performed in septuplicate for Doehlert matrix analysis [33]. All experiments were conducted in random order. Statistically significant factors were then optimized through response surface methodology. Data were analyzed using Minitab software (version 22.1.0).

Experiments were conducted to validate the model at three pH values (4, 8, and 12), three temperatures (40, 49, and 60 °C), and three times (15, 25, and 50 min), in addition to the optimal point found by the model (pH 12, 93 °C, and 47 min). The validation points were defined to test the model's robustness in different regions of the experimental space, including central, extreme and optimal conditions.

2.4 Bioplastic synthesis

The experimental steps for bioplastic production are schematized in Figure S1 (Supplementary Material). The bioplastic films were synthesized via the casting method, using biomass and BPS, both with commercial gelatin (Dr. Oetker, Brazil) as a film-forming agent and glycerol (Êxodo Científica, Brazil) as a plasticizer. Corn starch (Maizena, Unilever, Brazil) and magnesium oxide (Vetec Química, Brazil) were also added to some bioplastics to evaluate their effects on film water resistance.

A Taguchi L9 (3^4) orthogonal design was employed to evaluate the effects of biomass-to-gelatin ratio (25%, 50%, and 75%), glycerol (0.4, 0.6, and 0.8 g), temperature (60, 70, and 80 °C), and reaction time (10, 20, and 30 min) on film formation. This design was applied to both the raw biomass and the BPS, as outlined in Table 1.

Table 1 Experimental design for selecting time, temperature, and proportions of biomass, gelatin, and glycerol in bioplastic production from microalgae biomass.

ID	Biomass - gelatin	Glycerol (g)	Temperature (°C)	Time (min)
1	25% - 75%	0.4	60	10
2	25% - 75%	0.6	70	20
3	25% - 75%	0.8	80	30
4	50% - 50%	0.4	70	30
5	50% - 50%	0.6	80	10
6	50% - 50%	0.8	60	20
7	75% - 25%	0.4	80	20
8	75% - 25%	0.6	60	30
9	75% - 25%	0.8	70	10

Initially, for film synthesis, the biomass was dissolved in ultrapure water and the solution was adjusted to pH 4. This pH was selected based on preliminary tests evaluating

plastic formation at pH values ranging from 2 to 12. In a second set of experiments, BPS (obtained after extraction under optimal conditions of 93 °C, 47 min, and pH 12) replaced the biomass used to synthesize bioplastic. The total solids ratio (biomass + gelatin) to water volume was kept constant at 1:17 w/v (60 g L^{-1}) for all films. Preparation involved hydrating the gelatin in the respective biomass solution or protein extract.

After adding glycerol, the mixture was kept in a water bath under mechanical stirring, following the times and temperatures defined in Table 1. At the end of the process, the liquid was poured into plastic molds (8.5 cm diameter) and dried in an incubator at 45 ± 1 °C for 48 h. After drying, the films were demolded, and the swelling index of each was evaluated. Formulations resulting in lower swelling were selected for the subsequent stage. Figure 1 illustrates the bioplastic preparation stages.

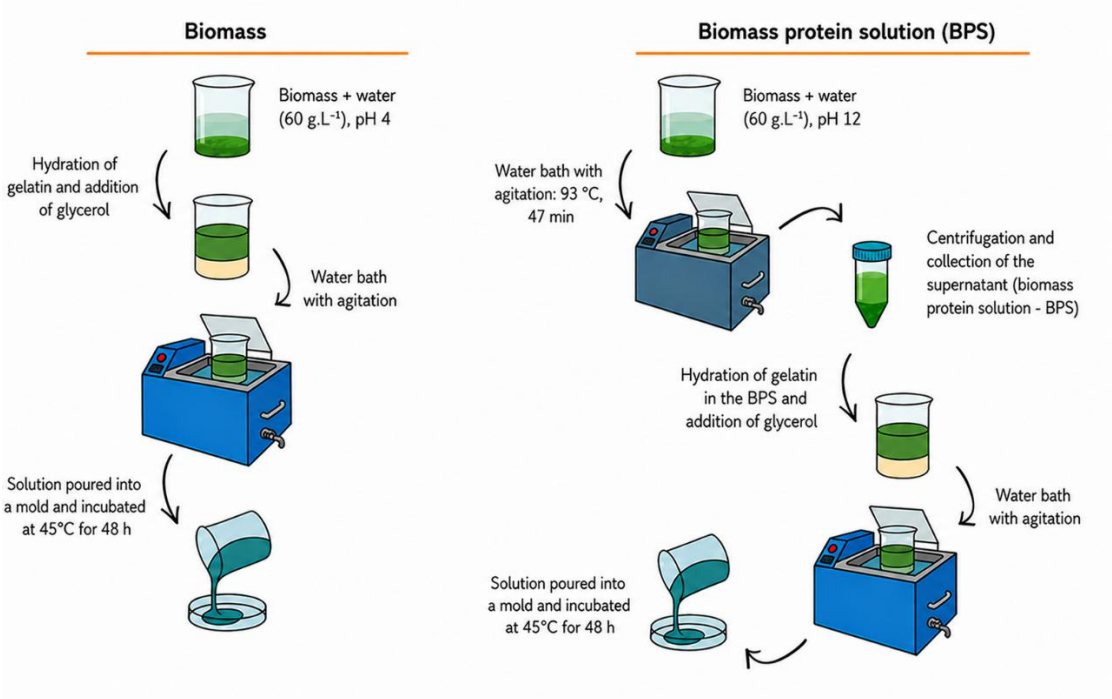


Fig. 1 Graphical representation of bioplastic production processes using biomass and biomass protein solution

In a third stage, the influence of magnesium oxide and corn starch additives was investigated in the previously selected formulations, as detailed in Table 2. Finally, the best-performing bioplastics were visualized by scanning electron microscopy (SEM) and their toxicity evaluated in seed germination assays.

Table 2 Experimental design to verify the influence of magnesium oxide and corn starch additives on bioplastics with the lowest swelling index.

ID	Treatment	MgO (% m/m)	Corn starch (% m/m)
C	Control	0	0
Mg	MgO	1	0
CS	Corn starch	0	5
MCS	MgO + corn starch	1	5

2.5 Water uptake capacity (Swelling)

Swelling was determined based on Jiménez-Rosado et al. [34], with modifications. Pieces of plastics (2.5 x 2.5 cm) were kept in a desiccator containing silica gel and subjected to repeated water immersions for periods of 2, 15, and 45 min. At the end of the determined times, excess water from the plastics was removed by blotting with filter paper. Swelling was calculated using Equation 1:

$$\text{Swelling (\%)} = \frac{w2 - w1}{w1} \times 100 \quad (1)$$

where w_1 is the weight (g) of the dry plastic before water immersion and w_2 is the weight (g) of the plastic after immersion.

2.6 Scanning electron microscopy

Surface images of the produced films were obtained using a JSM-6010LA scanning electron microscope (JEOL) housed in the Department of Physics, UFV. Images were obtained with an acceleration voltage of 10 kV. Samples were fixed on aluminum stubs covered with carbon tape and sputter-coated with a thin layer of gold using a Quorum Q150R S coater from the Microscopy and Microanalysis Center at UFV.

2.7 Germination assay

Bioassays consisted of incubating arugula (*Eruca sativa*) seeds (Isla, Brazil) in contact with a bioplastic solution at 25 ºC for 4 days, according to Young et al. [35], with modifications. For solution preparation, 20 mg of bioplastic were added to 30 mL of ultrapure water and the mixture was stirred for 60 min. Subsequently, the solution was left to rest for 15 min, and 4 mL of the supernatant was added to Petri dishes, each containing 10 *E. sativa* seeds.

Three replicates were prepared for each solution and ultrapure water was used as a control. After the incubation period, germinated seeds were counted and root lengths were measured using a 150 mm digital caliper with 0.001 cm precision (MTK-5100). The

absolute germination, in percentage (% AG), relative radicle growth (RRG) and the germination index (GI) were obtained using Equations 2 - 4 to determine the degree of phytotoxicity of the bioplastic [35, 36].

$$\% \text{ AG} = \frac{\text{Mean number of seeds germinated in the treatment}}{\text{Mean number os seeds germinated in the neutral control}} \times 100 \quad (2)$$

$$\text{RRG} = \frac{\text{Mean radicle length in the treatment}}{\text{Mean radicle length in the neutral control}} \quad (3)$$

$$\text{GI} = \frac{(\%\text{GA}) \times (\%\text{RRG})}{100} \quad (4)$$

Toxicity was classified based on the RRG and GI results. RRG values lower than 0.8 indicated root growth inhibition, values from 0.8 to 1.2 indicated no significant effect, and values above 1.2 indicated stimulation of root growth [35]. GI results below 30 were classified as highly phytotoxic, from 30 to 60 as phytotoxic, from 60 to 80 as moderately phytotoxic, from 80 to 100 as non-phytotoxic, and above 100 as indicative of germination stimulation [36].

2.8 Statistical analysis

To verify significant statistical differences in phytotoxicity, Jamovi Project software (version 2.5) was used. First, the Shapiro-Wilk test was performed to evaluate the normal distribution of measurements. Subsequently, Levene's test was used to test the homogeneity of variance. After verifying the assumptions of normality and homogeneity, analysis of variance (ANOVA) was performed, followed by Tukey's post hoc tests, at a 5% level of significance ($\alpha = 0.05$).

3 Results and Discussion

3.1 Wastewater treatment in HRAPs

The dominant microorganisms in the biomass produced in the HRAPs were the bacteria *Romboutsia timonensis* (12.1% of reads), *Xanthomonadaceae bacterium* (10.54% of reads) and *Rhodanobacteraceae bacterium* (9.76% of reads). The dominant microalgae were *Scenedesmus abundans* (54.4% of reads) and *Acutodesmus obliquus* (31.4% of reads).

The $N\text{-}NH_4^+$ concentration in the septic tank effluent was 62 mg L^{-1}. At the end of the

HRAP treatment, ammonium nitrogen was no longer detected. There was an 84% reduction in organic nitrogen, combined with nitrate formation, which was 625% higher than the initial level. The initial P_{sol} concentration was 8.0 mg L^{-1} and the average removal efficiency across the three HRAPs was 50% (Table 3).

Table 3 Nutrient levels in septic tank and HRAP effluents after 9-day batch treatment

Parameter	Septic tank (mg L^{-1})	HRAP (mg L^{-1})
Norg	83 ± 0.11	13.5 ± 0.17
$N\text{-}NH_4^+$	62 ± 3.4	< LoD
$N\text{-}NO_3^-$	20 ± 1.5	145 ± 11
P_{sol}	8.0 ± 0.3	4.0 ± 1.2

HRAPs: high-rate algal ponds. P_{sol}: soluble phosphorus. LoD: Limit of Detection.

Chlorophyll *a* production by microalgae increased during the first seven days, dropped on the eighth day, and increased slightly at the end of the last day of treatment. Similar behavior was observed for biomass, quantified as VSS (Fig. 2). The final average chlorophyll *a* production was 1.4 mg L^{-1}. Similar production (1.1 mg L^{-1}) was obtained in HRAPs with an 8-day HRT using UASB reactor effluent [37]. The final average VSS level was 225 mg L^{-1}, slightly higher than found in UASB-HRAP systems in other studies [38, 39].

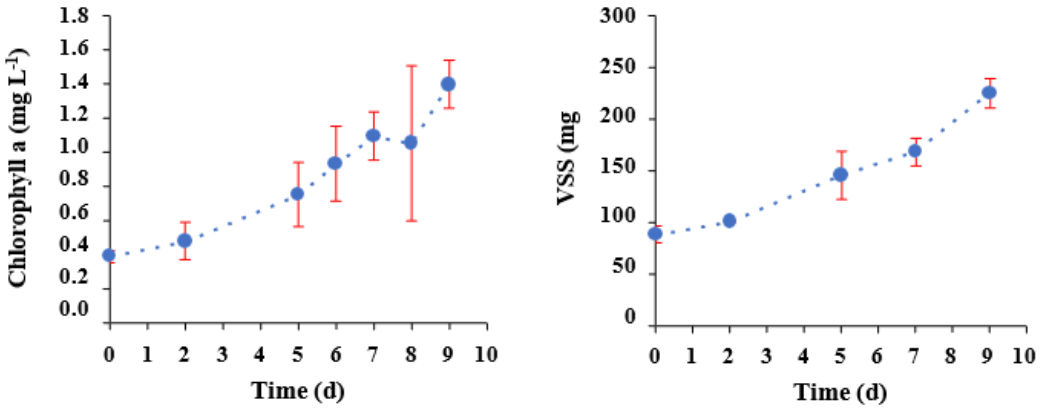


Fig. 2 Microalgal (chlorophyll *a*) and biomass (VSS) growth over 9 days of batch treatment of primary sewage in HRAPs (mean and standard deviation of three HRAPs)

Photosynthetic reactions decreased the initial inorganic carbon concentration from 84 mg L^{-1} to 5 mg L^{-1} and increased dissolved O_2 concentration in the HRAPs (Fig. 3).

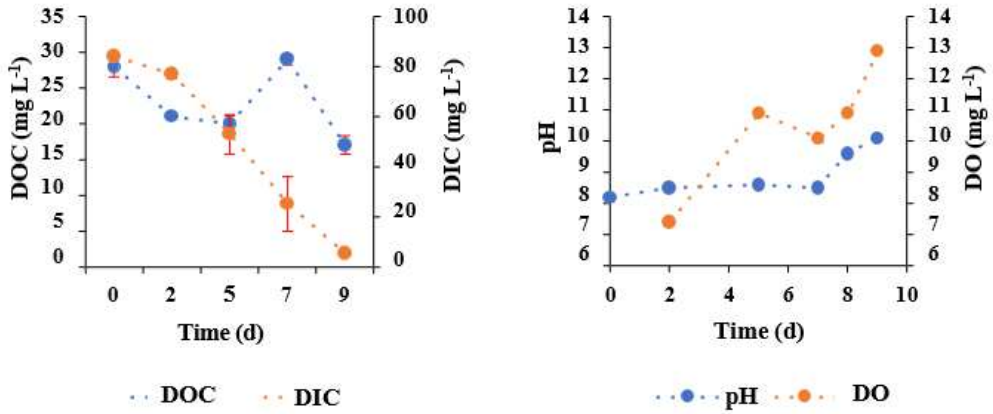


Fig. 3 Dissolved organic (DOC) and inorganic (DIC) carbon concentrations, dissolved oxygen (DO) and pH values over 9 days of batch treatment of primary sewage in HRAPs (mean and standard deviation of three HRAPs)

The data in Figures 2 and 3, coupled with the identification of dominant microorganisms, elucidate the nitrogen removal mechanisms in the system. The reduction in organic nitrogen (84%) observed after nine days of HRAP treatment, along with the complete removal of $N\text{-}NH_4^+$ and the 625% increase in $N\text{-}NO_3^-$ evidences the synergistic action of multiple biological processes within the microbial community identified in the system. These processes include N_{org} mineralization, nitrification, microalgae assimilation, and possibly NH_3 volatilization. Organic nitrogen mineralization, or ammonification, initiates the conversion of this nutrient into assimilable forms for microalgae. The heterotrophic bacterium *Romboutsia timonensis*, dominant in the biomass, degrades complex nitrogenous compounds such as amino acids and peptides, generating $N\text{-}NH_4^+$ [40]. This conversion makes nitrogen available for subsequent stages of the cycle.

Next, nitrification emerges as a central process. The total absence of $N\text{-}NH_4^+$ in the treated effluent, initially present at 62 mg L^{-1}, associated with the increase in $N\text{-}NO_3^-$ from 20 to 145 mg L^{-1}, indicates nitrifying activity. The presence of *Xanthomonadaceae bacterium* has the potential to participate in ammonia and nitrite oxidation through pathways not yet characterized, reinforces this hypothesis [41]. Nitrification was favored by system conditions, including N_{org} mineralization, high dissolved oxygen concentration, and high pH, the latter two resulting from intense photosynthetic activity.

Simultaneously, nitrogen assimilation by microalgae was an important component of $N\text{-}NH_4^+$ removal. The dominant species, *Scenedesmus abundans* and *Acutodesmus obliquus*, are widely recognized for their efficiency in absorbing $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$

from wastewaters [42, 43]. Microalgal growth was evidenced by the increase in chlorophyll *a* and VSS. Active nitrogen absorption by microalgae was driven by high metabolic demand for nutrients for protein and nucleic acid synthesis.

Furthermore, the high final pH (10.9) reflects CO_2 consumption in photosynthetic reactions that led to a drop in inorganic carbon from 84 mg L^{-1} to 5 mg L^{-1}. The alkaline environment may have promoted ammonia (NH_3) volatilization, especially during the peak of photosynthetic activity, further contributing to N-NH_4^+ removal.

Therefore, the reduction in N_{org} may be due to its biological transformation and remobilization in the reactive HRAP ecosystem. The microalgae-bacteria consortium demonstrated a high bioremediation capacity by converting complex organic nitrogen into assimilable inorganic forms and immobilizing it within the microbial biomass.

Finally, the removal of estrogenic activity by HRAP treatment was evaluated. Estrogenicity was not detected in the dissolved solids (< 0.45 μm) and large solids (> 0.7 μm) fractions of the primary sewage but was found in the intermediate solids (0.45 - 0.7 μm) with an average estrogenic activity of 19.1 ng L^{-1} Eq-E2. After HRAP treatment, estrogenic activity was not detected in the final effluent or biomass in any of the HRAPs (Table 4).

Table 4 Estrogenic activity in soluble and solid fractions after septic tank effluent treatment in HRAPs

Size fraction (μm)	**[a]Eq-E2 (ng L^{-1})**	
	Initial	**Final**
< 0.45	< LoD	< LoD
0.45 – 0.7	19.1 ± 5.9	< LoD
> 0.7	< LoD	< LoD

[a]Eq-E2:17β-estradiol equivalents. Limit of detection (LoD): 1.4 ng L^{-1}

Vassalle et al. [39] evaluated the removal capacity of micropollutants (including estrogens) by a UASB system followed by HRAPs. The authors observed high efficiency in removing most compounds, concluding that the combination of anaerobic treatment followed by aerobic treatment with a microalgae-bacteria consortium proved effective in removing various pollutants, corroborated by the present study, in which the consortium proved effective in biodegradation of compounds conferring estrogenic activity to the wastewater, as well as their possible estrogenic by-products.

3.2 Biomass production

At the end of the treatment, the total biomass production in the three HRAPs reached 657 g TSS. Carbohydrates corresponded to 17.2 ± 0.9% and proteins 43.3 ± 0.18% of the dry weight of the biomass (lyophilized and sieved). Other biomass characteristics are presented in Table 5. The high protein content reinforces the consortium's efficiency in assimilating nitrogen from the effluent, previously observed by the reduction of $N\text{-}NH_4^+$ and N_{org} and the increase in chlorophyll *a*.

The biomass had a low moisture content (7.5 ± 0.5%) and high volatile matter content (76.7 ± 1.2%). The ash content (14.6 ± 1.4%) was within the expected range for biomass grown in wastewater, which may indicate salt or mineral accumulation during the growth process [44, 45].

Table 5 Characterization of the microalgae biomass produced in this study

Parameter	(%)
Ash	14.6 ± 1.4
Moisture	7.5 ± 0.5
Volatile matter	76.7 ± 1.2
Fixed carbon	1.2 ± 1.1
Phosphorus	1.7 ± 0.4
Total organic carbon	40.8 ± 0.6

The total organic carbon content (40.8 ± 0.6%) reinforces the predominance of organic compounds in the biomass and, together with the low fixed carbon (1.2 ± 1.1%), points to a predominantly volatile organic fraction. Phosphorus (1.7 ± 0.4%) was also present at a relevant concentration, which may reflect its absorption from the medium and structural integration into cellular components such as phospholipids and nucleic acids [46].

3.3 Protein extraction

The Doehlert matrix experimental design explored the effects of time, pH, and temperature on the amount of extracted protein (Table 6).

Table 6 Experimental design for protein extraction from microalgal biomass and protein extraction results.

Run	Factors			Response
	pH	Time (min)	Temperature (°C)	Protein (%)
1	7.0	50	65	6.80
2	12.0	40	65	10.61
3	8.7	40	95	7.28
4	7.0	10	65	6.02

5	2.0	20	65	6.68
6	5.3	20	35	5.33
7	2.0	40	65	7.18
8	5.3	40	35	5.34
9	12.0	20	65	10.62
10	10.3	30	35	6.62
11	8.7	20	95	7.12
12	3.7	30	95	5.30
13	7.0	30	65	7.07
14	7.0	30	65	6.46
15	7.0	30	65	6.51
16	7.0	30	65	6.49
17	7.0	30	65	6.42
18	7.0	30	65	6.13
19	7.0	30	65	6.51

Response surface regression analysis revealed that the model was significant, with an R^2 of 98.42%, indicating a good fit to the data (Supplementary material Tables S1 – S4). The factors pH and temperature, along with their quadratic and interactive effects, had significant influences on protein extraction, as shown in the Pareto chart in Fig. 4.

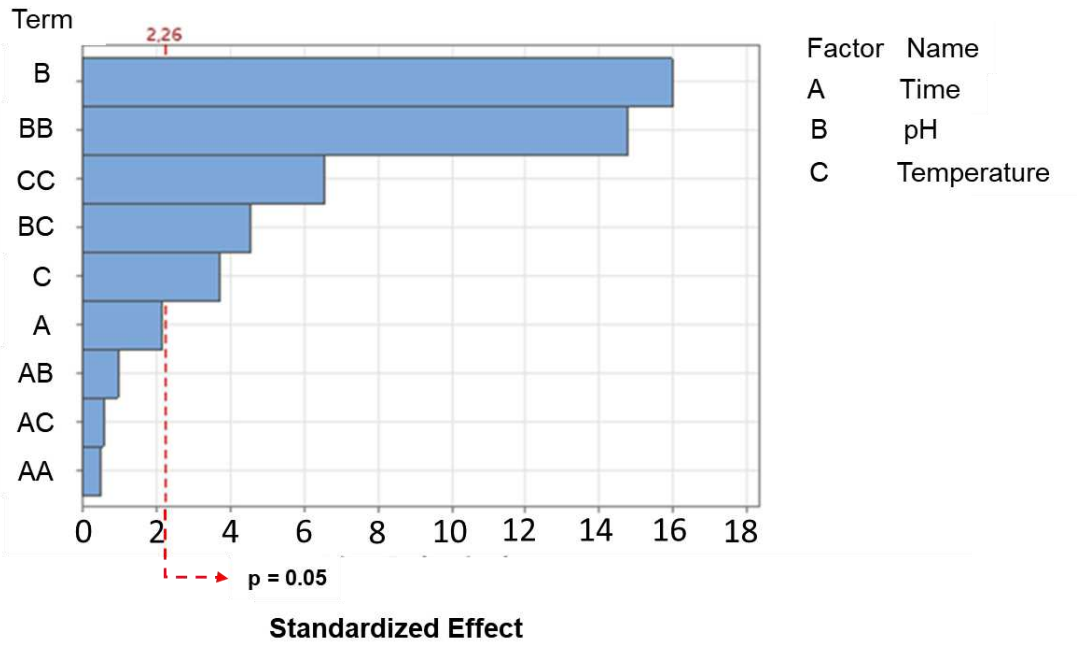


Fig. 4 Pareto plot elaborated with the protein extraction (%) results

The model response surface indicated that maximum protein extraction would be obtained at a time of 47.17 minutes, pH of 12, and temperature of 93.18 °C. Under these conditions, the model predicted 11.3% extracted protein. Figure 5 presents the contour plot of protein extracted as a function of pH and temperature, at a time of 30 min (center

point of the experimental design). The study demonstrated that pH and temperature were critical parameters in protein extraction from the biomass and that the combination of high values of these factors can lead to greater efficiency in the extraction process. These are the optimal conditions within the range of values tested, and true optimum values might fall outside the ranges tested.

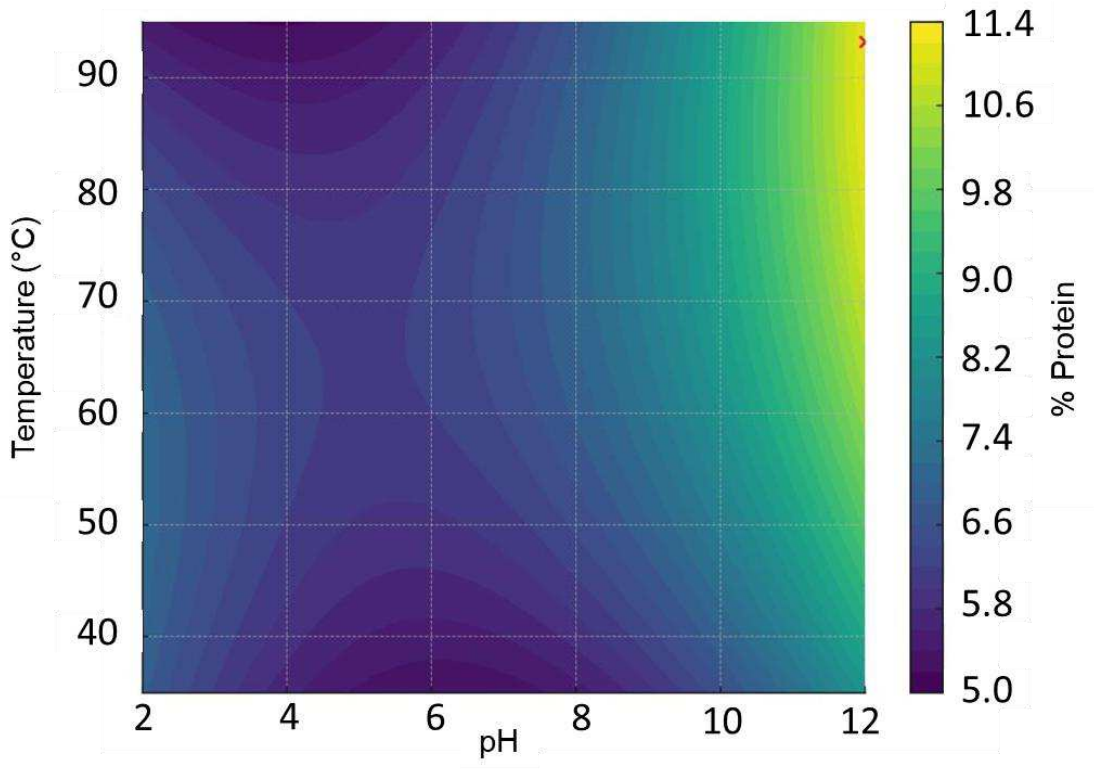


Fig. 5 Contour plot of protein extracted as a function of pH and temperature at 30 min (center point of the experimental design)

Predicted and experimental protein values obtained in the model validation step are presented in Table 7). For validation run 1 (50 min, pH 12, 49 °C), the model underestimated the experimental results. Observed values (11.3 ± 0.26) were consistently higher than predicted and were outside the 95% prediction interval (8.22 - 10.57%). For validation runs 2 (15 min, pH 8, 40 °C) and 3 (25 min, pH 4, 60 °C), the predicted values were in good agreement with the experimental results, with the mean of observed values within the prediction interval. Under optimal conditions (run 4), the model underestimated the protein extraction observed experimentally (13.07 ± 0.26%), with a predicted value of 11.23% (10.15 - 12.41%).

The analysis suggests that although the response surface regression model had a high R^2 (98.42%), indicating a good fit to the experimental data, its predictive capacity varied

in different regions of the experimental space. In particular, it was less precise under high pH and temperature conditions and underestimated the protein extraction potential under optimized conditions. This may indicate that the model, while well-fitted to initial data, may not fully capture the complex relationship between variables in the upper part of the experimental space.

Table 7 Results of protein extraction model validation experiments

Test	Experimental condition			Protein (%)		
	Time (min)	pH	Temperature (°C)	Observed	Predicted	*95% PI (Lower – Upper)
1	50	12	49	11.1		
1	50	12	49	11.4	9.398	8.22 – 10.57
1	50	12	49	11.6		
2	15	8	40	5.5		
2	15	8	40	5.2	5.741	4.91 - 6.57
2	15	8	40	5.2		
3	25	4	60	5.4		
3	25	4	60	5.4	6.179	5.54 – 6.82
3	25	4	60	6.1		
4	47	12	93	13.01		
4	47	12	93	13.3	11.278	10.15 – 12.41
4	47	12	93	12.78		
4	47	12	93	13.2		

*95% prediction interval (PI)

This underestimation suggests that the true optimal condition for maximizing protein extraction may be slightly different from that predicted, and the maximum achievable protein yield may be higher than the model's 11.3% prediction. The model's inaccuracy at extreme conditions of the experimental space indicates that precautions should be taken when extrapolating conclusions outside the initial data range or when implementing the process on an industrial scale, where variations may occur.

Despite the discrepancies found in the model, the optimal time of 47 minutes can be compared with other studies. Wang et al. [22] observed that extraction efficiency increased with time and stabilized after 60 minutes. Ng et al. [21] found an increase in efficiency up to 60 minutes, with a plateau thereafter. Safi et al. [23] observed an increase in protein and pigment concentration with time for different extraction methods.

Alkaline extraction is effective for solubilizing proteins, as demonstrated in other studies [21, 23, 47]. In the study by Safi et al. [23], chemical hydrolysis at 40 °C with an alkaline solution (NaOH) slowly weakened the cell wall of *C. vulgaris*, allowing the recovery of proteins (26 ± 0.8%) bound to the cell wall and some smaller cytoplasmic

proteins. However, this technique failed to penetrate the phospholipid bilayer of the chloroplast, where pigments are located, resulting in the absence of pigments in the aqueous phase. Protein diffusion was initially rapid but became very slow over time, suggesting hindered diffusion within pores and confirming that complete cell disruption did not occur. Microscopic observations showed intact cells after this treatment.

Ng et al. [21] investigated an integrated approach of dry bead milling and alkaline solubilization to increase protein recovery from the microalga *C. vulgaris*. Without the bead milling step, protein extraction yield at 37 °C was 5.0% with water and 16.8% with 0.1 mol L^{-1} NaOH, from a biomass concentration of 20 g L^{-1}. The integrated approach significantly improved results, reaching a maximum protein extraction yield of about 47.3% at a biomass concentration of 100 g L^{-1}. The significance of bead milling lies in its ability to disrupt the rigid cell wall of the microalga, improving protein release into the extraction medium. Alkaline treatment further assists in cell breakdown and promotes protein solubilization.

Variation in cell wall structure, protein content, and protein solubility among different species can make extraction more challenging. The aforementioned studies focused on a single species, which may facilitate process optimization. It is important to consider that direct comparison with other studies is limited by differences in microalgae species, extraction methods, and experimental conditions. However, comparative analysis allows for the identification of common trends and challenges in microalgae protein extraction, contributing to the development of more efficient and sustainable methods.

3.4 Bioplastic

3.4.1 Biomass

In the experiment on bioplastic formation with whole biomass, only the bioplastic made with 50% biomass/50% gelatin and 0.4 g glycerol, at 70 °C for 30 min did not form a regular-looking film, as it became brittle (Table 8). Through visual observation, it was possible to perceive that the lower the proportion of gelatin, the more malleable the formed plastics were.

Table 8 Visual and swelling results after 2 and 45 min of bioplastics produced with total HRAP biomass (*refer to Table 1 for plastic composition)

ID*	Visual observation	Swelling (%) 2 min	Swelling (%) 45 min
1	Visually homogeneous	75 ± 20	-
2	Visually homogeneous	78 ± 11	-

3	Visually homogeneous	70 ± 7	333 ± 16
4	No film formation	-	-
5	Visually homogeneous	56 ± 5	-
6	Visually homogeneous	-	-
7	Visually homogeneous	61 ± 0.06	-
8	Visually homogeneous	44 ± 2	-
9	Visually homogeneous	38 ± 9	-

– Not determined due to absence of film formation or loss of film integrity during the swelling test

Most biomass combinations tested did not result in plastics water-resistant enough to complete the swelling test and non-response occurrences limited the availability of valid data. Consequently, no statistical comparisons could be made.

Bioplastic 6 (50% biomass/50% gelatin, 0.8 g glycerol, 60 °C, 20 min) did not resist the first immersion and could not therefore be weighed at the start of the analysis. Bioplastics 1, 2, 5, 7 and 9 fell apart after the first weighing (2 min), and it was not possible to proceed with the swelling analysis. Only plastic 3 (25% biomass/75% gelatin, 0.8 g glycerol, 80 °C, 30 min) formed a regular-looking plastic that allowed measuring water absorption for up to 45 minutes of immersion.

The high solubilization rate of the films indicates that material integrity is a crucial challenge for the development of functional bioplastics from this biomass. The performance observed in bioplastic ID 3, however, offers a promising starting point, suggesting that certain combinations of factors may favor the formation of a more stable polymer matrix that, although having a high swelling index, maintains its integrity.

This behavior is closely related to the raw microalgal biomass, which constitutes a highly heterogeneous matrix of proteins, carbohydrates, lipids, pigments, cell fragments, and mineral compounds [4, 10]. Such chemical complexity likely resulted in low cohesiveness and weak formation of a continuous polymer matrix, necessary for film integrity. On the other hand, the high proportion of gelatin and prolonged heating possibly favored more stable structural interactions [15], as observed in the only film that remained intact at the end of the test.

To improve the water immersion resistance of films produced from microalgal biomass and expand their range of applications, further experiments employing new approaches are recommended. Extraction of fractions with higher film-forming activity, such as proteins would reduce the heterogeneity of the raw material and concentrate the components most suitable for forming stable polymeric networks. Incorporation of crosslinking additives to promote interactions between the polymer chains of the biomass

and gelatin, which might enhance both mechanical strength and water resistance [16]. Application of alternative processing methods, such as hot pressing or injection molding, which generally involve higher shear and pressure conditions, might favor homogenization and the formation of denser and more interconnected polymeric networks [20]. Modification of the medium pH to influence protein structural conformation and expose functional sites might favor formation of stronger intermolecular interactions (such as ionic and hydrogen bonds) and promote crosslinking between polymer chains [4, 15]. Finally, a more comprehensive experimental design to evaluate additional combinations of the selected factors might uncover more favorable conditions for plastic formation.

3.4.2 Biomass protein solution (BPS)

In the swelling test with bioplastics produced with BPS (Table 9), plastics 6 (50% BPS/50% gelatin, 0.8 g glycerol, 60 °C, 20 min) and 8 (75% BPS/25% gelatin, 0.6 g glycerol, 60 °C, 30 min) did not form homogeneous films, demonstrating that these specific proportions and conditions were not suitable. Plastics 2 and 4 did not resist the first immersion, and plastic 1 resisted only the first weighing (2 min). The other plastics (3, 5, 7 and 9) remained intact after 45 min of testing. Through visual observation, it was also possible to perceive that the lower the proportion of gelatin, the more malleable the formed plastics were. Non-response occurrences also limited the availability of valid data for the experiment, and consequently, no statistical analysis was possible.

Table 9 Visual and swelling results of bioplastics produced from HRAP biomass protein solution (BPS) at varying gelatin/BPS ratios, temperatures and times (*refer to Table 1 for plastic composition and formation conditions)

ID*	Visual observation	Swelling (%) 2 min	Swelling (%) 45 min
1	Visually homogeneous	66 ± 16	-
2	Visually homogeneous	-	-
3	Visually homogeneous	73 ± 10	646 ± 174
4	Visually homogeneous	-	-
5	Visually homogeneous	130 ± 15	659 ± 40
6	No film formation	-	-
7	Visually homogeneous	198 ± 44	502 ± 83
8	No film formation	-	-
9	Visually homogeneous	181 ± 32	464 ± 9

– Not determined because of the absence of film formation or loss of film integrity during the swelling test

When BPS was used instead of total biomass, the number of formulations that successfully resisted the swelling test for 45 minutes increased. Plastics with greater water resistance were obtained when processed at higher temperatures, which favored cross-linking and the increase of the protein network, as found in other studies [15, 48]. Different degrees of swelling were observed for the plastics that remained intact. Since plastic 9 (75% BPS/25% gelatin, 0.8 g glycerol, 70 °C, 10 min) contained a higher proportion of BPS compared to plastics 3 (25% BPS) and 5 (50% BPS), and lower preparation temperature and time relative to plastic 7 (80 °C, 20 min), it was selected for further testing.

3.4.3 Addition of composites to the bioplastic

The plastics with the highest water immersion resistance and lowest degree of swelling were selected for the addition of MgO (1% w/w), corn starch (5% w/w), and MgO + corn starch (1 and 5% w/w, respectively), as detailed in Table 2. The unamended plastics were produced from total biomass (Table 8, 3 = 25% biomass/75% gelatin, 0.8 g glycerol, 80 °C, 30 min) and BPS (Table 9, 9 = 75% biomass/25% gelatin, 0.8 g glycerol, 70 °C, 10 min). All eight compositions formed regular-looking plastics (Figure 6a and 6b). Plastics produced with biomass had an average thickness of 0.55 ± 0.029 mm while plastics produced with BPS had an average thickness of 0.37 ± 0.032 mm.

The only bioplastic produced with whole biomass that remained intact throughout the 45-min swelling test was the unamended control, which exhibited a swelling index of 227 ± 1.4% (Figure 6c). Among the BPS-based films, both the unamended control (Figure 6d) and the formulation containing magnesium oxide and corn starch (Figure 6e) remained intact after 45 min, with swelling indices of 467 ± 8.5% and 551 ± 43%, respectively. The individual addition of MgO or corn starch did not improve the performance of either bioplastic during water immersion. Although the combined addition of MgO and corn starch allowed the BPS-based film to maintain its integrity throughout the test, it did not reduce water uptake, as indicated by the higher swelling index compared with the unamended control.

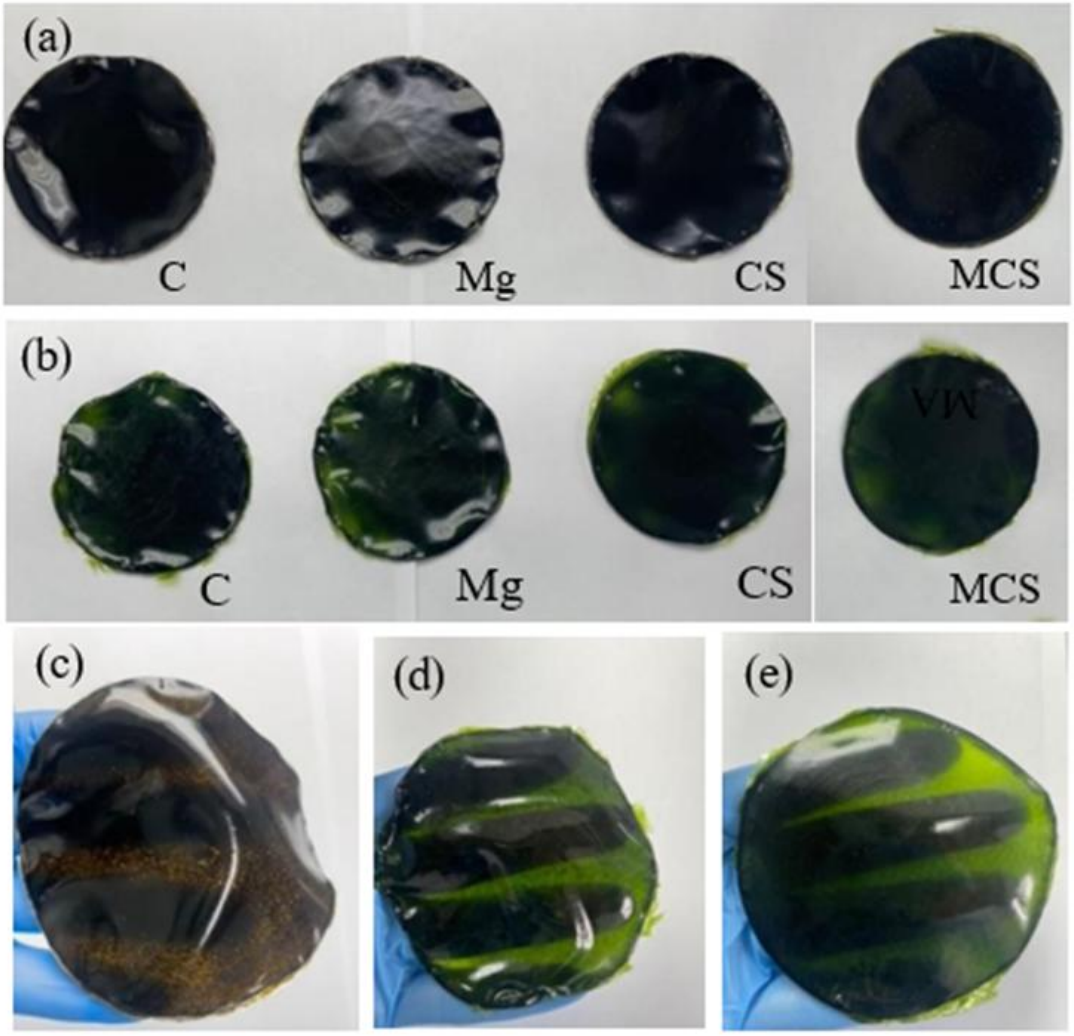


Fig. 6 Plastics produced with the addition of magnesium oxide and corn starch. (a) Plastics produced with biomass. (b) Plastics produced with biomass protein solution (BPS). (c) Control plastic (biomass without additives). (d) Control plastic (BPS without additives). (e) MCS – BPS plastic with the addition of MgO + corn starch.
C: control plastics. Mg: plastics with MgO addition (1% w/w). CS: plastics with corn starch addition (5% w/w). MCS: plastics with joint addition of MgO (1% w/w) and corn starch (5% w/w)

3.4.4 Scanning electron microscopy

Micrographs of the bioplastic samples are presented in Figure 7. The bioplastic synthesized with biomass exhibited a more homogeneous surface, without cracks, compared to plastics synthesized with BPS. The latter showed failure points and particle agglomeration, indicating poor dispersion, aggregation, or less integration of the composites added to the plastic formulation.

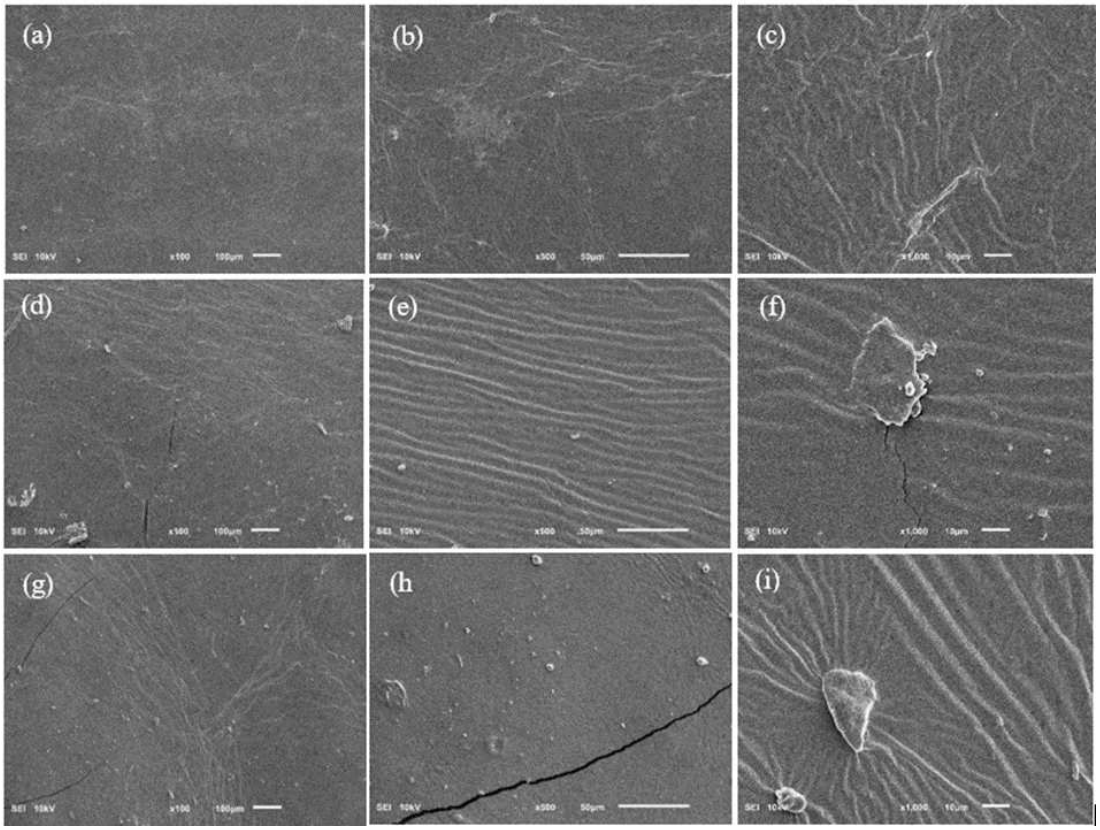


Fig. 7 SEM images of microalgae-based bioplastics. (a), (b), and (c) control plastic produced with biomass, 100x, 500x, and 1000x. (d), (e), and (f) control plastic produced with BPS, 100x, 500x, and 1000x. (g), (h), and (i) plastic produced with BPS with joint addition of MgO and corn starch, 100x, 500x, and 1000x

3.4.5 Germination assays with arugula seeds

Table 10 presents the results obtained in the germination assay with bioplastics. No significant differences were found in radicle length between the control (ultrapure water) and bioplastic treatments. The biomass treatment resulted in 100% germination, but the relative radicle growth (RRG) of 0.79 indicated root growth inhibition and the germination index of 78.8 classified the bioplastic as moderately phytotoxic.

The BPS bioplastic treatment resulted in 88.9% AG and an 0.70 RRG of, which also indicated root growth inhibition. The GI of 62 classified this treatment as moderately phytotoxic. The BPS bioplastic treatment with joint addition of MgO and corn starch resulted in 100% AG and an RRG of 1.06, indicating no significant effect on root growth. The GI of 106 suggests that this treatment enhanced germination. Corn starch and magnesium appear to have had a beneficial effect on radicle development.

Table 10 Radicle length, absolute germination index (AG), relative radicle growth (RRG), and germination index (GI) of arugula seeds exposed to produced bioplastic extracts. (Mean values and standard deviation, n = 3)

Treatment	Length (mm)	AG (%)	RRG	GI
Control	27.8 ± 7.95	-	-	-
Biomass bioplastic	21.9 ± 11.44	100	0.79	78.8
BPS bioplastic	19.4 ± 9.43	88.9	0.70	62
BPS bioplastic with MgO + corn starch	29.6 ± 13.21	100	1.06	106

BPS: Biomass protein solution.

Although the results did not reveal statistically significant differences at the 5% level in radicle length between the control and bioplastic treatments, the trends observed in group means (lower growth with total biomass and solubilized protein, and higher growth with MgO + corn starch) are consistent with the toxicity classifications (inhibition and enhancement) obtained based on RRG and GI parameters.

4. Conclusion

This study demonstrated the feasibility of microalgal biomass production in primary domestic sewage, achieving removal of estrogenic activity in the solid phase and producing a protein-rich biomass (43.3%) suitable for bioplastic production. The response surface analysis revealed that pH, temperature and their interaction had significant influences on biomass protein extraction yield, whereas extraction time was not significant. Under the conditions evaluated, the model predicted an extraction yield of 11.3% protein from an initial biomass concentration of 4 g L^{-1}. Ultimately, the optimization analysis identified the optimal extraction conditions as 93 °C, pH 12, and 47 min. These results can be used as a starting point to improve the process by optimizing the applied biomass load or by combining the optimal conditions with a cell disruption method, such as bead milling

The synthesis of visually homogeneous bioplastics from HRAP total biomass and BPS was successful. The nine combinations of time, temperature, biomass/gelatin ratio, and glycerol amount tested resulted in plastics with low water immersion resistance, with a large part solubilizing in just 15 minutes of immersion. Of the nine bioplastics produced with total biomass, only one resisted to the end of the 45-min swelling test, with an index of 333 ± 16%. Of the nine plastics produced with BPS, four reached the end of the swelling test, with indices between 464 and 659%.

The combined addition of MgO and corn starch produced a BPS-based film that remained intact after 45 min of water immersion, despite its high swelling index (551 ± 43%), which exceeded that of the control (467 ± 8.5%). The formulation achieved a germination index of 106, indicating no phytotoxicity according to the study's criteria. However, no significant differences in radicle length were observed. Further improvements are needed to reduce water uptake and enhance film properties.

This work advances the development of a sustainable and technically simple approach for bioplastic production from biomass produced in HRAP treating wastewaters. The results obtained provide important information for optimizing protein extraction processes and bioplastic synthesis and pave the way for future research focused on improving bioplastic properties and evaluating their economic and environmental viability on a large scale.

Supplementary Information: The online version contains supplementary material available as Online Resource 1.

References

1. Rawat, I., Ranjith Kumar, R., Mutanda, T., Bux, F.: Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Appl. Energy. 88, 3411–3424 (2011). https://doi.org/10.1016/j.apenergy.2010.11.025
2. Calijuri, M.L., Silva, T.A., Magalhães, I.B., Pereira, A.S.A. de P., Marangon, B.B., Assis, L.R. de, Lorentz, J.F.: Bioproducts from microalgae biomass: Technology, sustainability, challenges and opportunities. Chemosphere. 305, 135508 (2022). https://doi.org/10.1016/j.chemosphere.2022.135508
3. Mogany, T., Bhola, V., Bux, F.: Algal-based bioplastics: global trends in applied research, technologies, and commercialization. Environ. Sci. Pollut. Res. 31, 38022–38044 (2024). https://doi.org/10.1007/s11356-024-33644-9
4. Lutzu, G.A., Ciurli, A., Chiellini, C., Di Caprio, F., Concas, A., Dunford, N.T.: Latest developments in wastewater treatment and biopolymer production by microalgae. J. Environ. Chem. Eng. 9, 104926 (2021). https://doi.org/10.1016/j.jece.2020.104926
5. Rajpoot, A.S., Choudhary, T., Chelladurai, H., Nath Verma, T., Shende, V.: A comprehensive review on bioplastic production from microalgae. Mater. Today Proc. 56, 171–178 (2022). https://doi.org/10.1016/j.matpr.2022.01.060
6. Tennakoon, P., Chandika, P., Yi, M., Jung, W.-K.: Marine-derived biopolymers as potential bioplastics, an eco-friendly alternative. iScience. 26, 106404 (2023). https://doi.org/10.1016/j.isci.2023.106404
7. Iqbal, K., Mishra, A., Sreedharan, S.M.: The quintessence of algal biomass in bioplastic production: insightful advancement and sustainable use. Bioresour. Bioprocess. 12, 91 (2025). https://doi.org/10.1186/s40643-025-00908-2
8. Liwarska-Bizukojc, E.: Phytotoxicity assessment of biodegradable and non-biodegradable plastics using seed germination and early growth tests. Chemosphere. 289, 133132 (2022). https://doi.org/10.1016/j.chemosphere.2021.133132
9. Ranucci, E., Treccani, S., Ferruti, P., Alongi, J.: The Seed Germination Test as a Valuable Tool for the Short-Term Phytotoxicity Screening of Water-Soluble Polyamidoamines. Polymers (Basel). 16, 1744 (2024). https://doi.org/10.3390/polym16121744

10. Roy Chong, J.W., Tan, X., Khoo, K.S., Ng, H.S., Jonglertjunya, W., Yew, G.Y., Show, P.L.: Microalgae-based bioplastics: Future solution towards mitigation of plastic wastes. Environ. Res. 206, 112620 (2022). https://doi.org/10.1016/j.envres.2021.112620
11. López Rocha, C.J., Álvarez-Castillo, E., Estrada Yáñez, M.R., Bengoechea, C., Guerrero, A., Orta Ledesma, M.T.: Development of bioplastics from a microalgae consortium from wastewater. J. Environ. Manage. 263, 110353 (2020). https://doi.org/10.1016/j.jenvman.2020.110353
12. Arora, Y., Sharma, S., Sharma, V.: Microalgae in Bioplastic Production: A Comprehensive Review. Arab. J. Sci. Eng. 48, 7225–7241 (2023). https://doi.org/10.1007/s13369-023-07871-0
13. Yang, J.H., Hong, J.S., Lee, J.S., Sim, S.J., Ahn, K.H.: Dispersion of unfractionated CO2-derived protein-rich microalgae (Chlorella sp. HS2) for ecofriendly polymer composite fabrication. Mater. Today Commun. 31, 103769 (2022). https://doi.org/10.1016/j.mtcomm.2022.103769
14. Yiasmin, M.N., Al Azad, S., Easdani, M., Islam, M.S., Hussain, M., Cao, W., Chen, N., Uriho, A., Asaduzzaman, M., Liu, C., Hua, X.: The State-of-the-Art on Exploring Polysaccharide-Protein Interactions and Its Mechanisms, Stability, and Their Role in Food Systems. Food Rev. Int. 42, 315–350 (2025). https://doi.org/10.1080/87559129.2025.2482150
15. González-Balderas, R.M., Felix, M., Bengoechea, C., Guerrero, A., Orta Ledesma, M.T.: Influence of mold temperature on the properties of wastewater-grown microalgae-based plastics processed by injection molding. Algal Res. 51, 102055 (2020). https://doi.org/10.1016/j.algal.2020.102055
16. Mubarak Aldawsari, H., Kotta, S., Asfour, H.Z., Vattamkandathil, S., Abdelkhalek Elfaky, M., Ashri, L.Y., Badr-Eldin, S.M.: Development and evaluation of quercetin enriched bentonite-reinforced starch-gelatin based bioplastic with antimicrobial property. Saudi Pharm. J. 31, 101861 (2023). https://doi.org/10.1016/j.jsps.2023.101861
17. Leite, I. de sá, Pinto Jr, W.R., SILVA, L.D.A.: Biofilme a partir de gelatina e glicerol com a adição de íons de prata como agente antimicrobiano. Lat. Am. J. Energy Res. 6, 1–11 (2020). https://doi.org/10.21712/lajer.2019.v6.n2.p1-11
18. Ben, Z.Y., Samsudin, H., Yhaya, M.F.: Glycerol: Its properties, polymer synthesis, and applications in starch based films. Eur. Polym. J. 175, 111377 (2022).

https://doi.org/10.1016/j.eurpolymj.2022.111377

19. Adamu Ugya, Y., Chen, H., Sheng, Y., Ajibade, F.O., Wang, Q.: A review of microalgae biofilm as an eco-friendly approach to bioplastics, promoting environmental sustainability. Environ. Res. 236, 116833 (2023). https://doi.org/10.1016/j.envres.2023.116833
20. Iyer, H., Grandgeorge, P., Jimenez, A.M., Campbell, I.R., Parker, M., Holden, M., Venkatesh, M., Nelsen, M., Nguyen, B., Roumeli, E.: Fabricating Strong and Stiff Bioplastics from Whole Spirulina Cells. Adv. Funct. Mater. 33:2302067 (2023). https://doi.org/10.1002/adfm.202302067
21. Ng, J.W., Teh, T.M., Melanie, W., Talukder, M.M.R.: Integrating bead milling and alkaline solubilization for enhanced protein recovery from microalgae: A comprehensive approach. Futur. Foods. 9, 100385 (2024). https://doi.org/10.1016/j.fufo.2024.100385
22. Wang, Z., Ye, W., Wu, Y., Lin, X., Luan, C., Xie, X., Peng, Y., Sun, X., Shi, C., Lv, Y., Shen, J., Astatkie, T., He, Q. (Sophia), Li, Z., Yang, J.: Protein extraction from chlorella pyrenoidosa microalgae: Green methodologies, functional assessment, and waste stream valorization for bioenergy production. Bioresour. Technol. 397, 130508 (2024). https://doi.org/10.1016/j.biortech.2024.130508
23. Safi, C., Frances, C., Ursu, A.V., Laroche, C., Pouzet, C., Vaca-Garcia, C., Pontalier, P.-Y.: Understanding the effect of cell disruption methods on the diffusion of Chlorella vulgaris proteins and pigments in the aqueous phase. Algal Res. 8, 61–68 (2015). https://doi.org/10.1016/j.algal.2015.01.002
24. Moreira, A.S.P., Gonçalves, J., Sousa, F., Maia, I., Pereira, H., Silva, J., Coimbra, M.A., Ferreira, P., Nunes, C.: Potential of Coccolithophore Microalgae as Fillers in Starch-Based Films for Active and Sustainable Food Packaging. Foods. 12, 513 (2023). https://doi.org/10.3390/foods12030513
25. Ciapponi, R., Turri, S., Levi, M.: Mechanical Reinforcement by Microalgal Biofiller in Novel Thermoplastic Biocompounds from Plasticized Gluten. Materials (Basel). 12, 1476 (2019). https://doi.org/10.3390/ma12091476
26. Routledge, E.J., Sumpter, J.P.: Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Toxicol. Chem. 15, 241–248 (1996). https://doi.org/10.1002/etc.5620150303
27. Argolo, A. dos S., Gomes, G., Bila, D.M.: (Anti)estrogenic activity impacted by complex environmental matrices: A DOM and multiphase distribution approach.

Chemosphere. 310, 136917 (2023). https://doi.org/10.1016/j.chemosphere.2022.136917

28. Hoebler, C., Barry, J.L., David, A., Delort-Laval, J.: Rapid acid hydrolysis of plant cell wall polysaccharides and simplified quantitative determination of their neutral monosaccharides by gas-liquid chromatography. J. Agric. Food Chem. 37, 360–367 (1989). https://doi.org/10.1021/jf00086a020
29. DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F.: Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28, 350–356 (1956). https://doi.org/10.1021/ac60111a017
30. APHA, A.P.H.A.: Standard Methods for the Examination of Water and Wastewater. , Washington, DC (2023)
31. Zhong, W., Zhang, Z., Luo, Y., Qiao, W., Xiao, M., Zhang, M.: Biogas productivity by co-digesting Taihu blue algae with corn straw as an external carbon source. Bioresour. Technol. 114, 281–286 (2012). https://doi.org/10.1016/j.biortech.2012.02.111
32. ASTM, A.I.E.-82(2012): Standard Test Method for Volatile Matter in the Analysis of Particulate Wood Fuels. ASTM International, West Conshohocken, PA, USA (2012)
33. Teófilo, R.F., Ferreira, M.M.C.: Quimiometria II: planilhas eletrônicas para cálculos de planejamentos experimentais, um tutorial. Quim. Nova. 29, 338–350 (2006). https://doi.org/10.1590/S0100-40422006000200026
34. Jiménez-Rosado, M., Rubio-Valle, J.F., Perez-Puyana, V., Guerrero, A., Romero, A.: Use of heat treatment for the development of protein-based bioplastics. Sustain. Chem. Pharm. 18, 100341 (2020). https://doi.org/10.1016/j.scp.2020.100341
35. Young, B.J., Riera, N.I., Beily, M.E., Bres, P.A., Crespo, D.C., Ronco, A.E.: Toxicity of the effluent from an anaerobic bioreactor treating cereal residues on Lactuca sativa. Ecotoxicol. Environ. Saf. 76, 182–186 (2012). https://doi.org/10.1016/j.ecoenv.2011.09.019
36. Peduto, T.A.G., Jesus, T.A. de, Kohatsu, M.Y.: Sensibilidade de diferentes sementes em ensaio de fitotoxicidade. Rev. Bras. Ciência, Tecnol. e Inovação. 4, 200 (2019). https://doi.org/10.18554/rbcti.v4i2.3698
37. Couto, E., Calijuri, M.L., Assemany, P., Cecon, P.R.: Evaluation of high rate ponds operational and design strategies for algal biomass production and domestic wastewater treatment. Sci. Total Environ. 791, 148362 (2021).

https://doi.org/10.1016/j.scitotenv.2021.148362

38. Santiago, A.F., Calijuri, M.L., Assemany, P.P., Calijuri, M. do C., Reis, A.J.D. dos: Algal biomass production and wastewater treatment in high rate algal ponds receiving disinfected effluent. Environ. Technol. 34, 1877–1885 (2013). https://doi.org/10.1080/09593330.2013.812670
39. Vassalle, L., García-Galán, M.J., Aquino, S.F., Afonso, R.J. de C.F., Ferrer, I., Passos, F., R Mota, C.: Can high rate algal ponds be used as post-treatment of UASB reactors to remove micropollutants? Chemosphere. 248, 125969 (2020). https://doi.org/10.1016/j.chemosphere.2020.125969
40. Gerritsen, J., Hornung, B., Ritari, J., Paulin, L., Rijkers, G.T., Schaap, P.J., de Vos, W.M., Smidt, H.: A comparative and functional genomics analysis of the genus Romboutsia provides insight into adaptation to an intestinal lifestyle, http://biorxiv.org/lookup/doi/10.1101/845511, (2019)
41. Baskaran, V., Patil, P.K., Antony, M.L., Avunje, S., Nagaraju, V.T., Ghate, S.D., Nathamuni, S., Dineshkumar, N., Alavandi, S. V., Vijayan, K.K.: Microbial community profiling of ammonia and nitrite oxidizing bacterial enrichments from brackishwater ecosystems for mitigating nitrogen species. Sci. Rep. 10, 5201 (2020). https://doi.org/10.1038/s41598-020-62183-9
42. González-Garcinuño, Á., Tabernero, A., Sánchez-Álvarez, J.M., Martin del Valle, E.M., Galán, M.A.: Effect of nitrogen source on growth and lipid accumulation in Scenedesmus abundans and Chlorella ellipsoidea. Bioresour. Technol. 173, 334–341 (2014). https://doi.org/10.1016/j.biortech.2014.09.038
43. Liu, X., Wang, K., Wang, J., Zuo, J., Peng, F., Wu, J., San, E.: Carbon dioxide fixation coupled with ammonium uptake by immobilized Scenedesmus obliquus and its potential for protein production. Bioresour. Technol. 289, 121685 (2019). https://doi.org/10.1016/j.biortech.2019.121685
44. de Assis, L.R., Calijuri, M.L., do Couto, E. de A., Assemany, P.P.: Microalgal biomass production and nutrients removal from domestic sewage in a hybrid high-rate pond with biofilm reactor. Ecol. Eng. 106, 191–199 (2017). https://doi.org/10.1016/j.ecoleng.2017.05.040
45. de Souza, R.A.S., Saldanha-Corrêa, F.M.P., Gallego, A.G., Neto, A.M.P.: Semi-quantitative determination of ash element content for freeze-dried, defatted, sulfated and pyrolysed biomass of Scenedesmus sp. Biotechnol. Biofuels. 13, 63 (2020). https://doi.org/10.1186/s13068-020-01699-8

46. Gao, B., Liu, J., Zhang, C., Van de Waal, D.B.: Biological stoichiometry of oleaginous microalgal lipid synthesis: The role of N:P supply ratios and growth rate on microalgal elemental and biochemical composition. Algal Res. 32, 353–361 (2018). https://doi.org/10.1016/j.algal.2018.04.019
47. Hildebrand, G., Poojary, M.M., O'Donnell, C., Lund, M.N., Garcia-Vaquero, M., Tiwari, B.K.: Ultrasound-assisted processing of Chlorella vulgaris for enhanced protein extraction. J. Appl. Phycol. 32, 1709–1718 (2020). https://doi.org/10.1007/s10811-020-02105-4
48. Fernández-Espada, L., Bengoechea, C., Cordobés, F., Guerrero, A.: Thermomechanical properties and water uptake capacity of soy protein-based bioplastics processed by injection molding. J. Appl. Polym. Sci. 133, (2016). https://doi.org/10.1002/app.43524

Statements and Declarations

Author contributions: Priscila Romana da Silva: Conceptualization, methodology, investigation, formal analysis, writing – original draft. **Ann H. Mounteer:** Conceptualization, resources, writing – review and editing, project administration, funding acquisition. **Eder Carlos Lopes Coimbra** – Methodology, writing – review and editing.

Funding: This work was supported by the National Council for Scientific and Technological Development (CNPq) [grant number 408971/2023-1]

Competing interests: The authors declare no competing interests.

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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