Algal Research 69 (2023) 102941

Contents lists available at ScienceDirect

Algal Research

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

Microalgal cultivation on grass juice as a novel process for a green biorefinery

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ARTICLE INFO

Keywords:
Novel protein
Feed
Circular economy
Green juice
Bioeconomy
Microalgae

ABSTRACT

Green biorefineries aim to sustainably produce chemicals, materials, proteins and energy by processing green biomass, such as grass, into a solid fraction (fibers) and a liquid fraction (green juice) for further refining. Here, we propose to incorporate microalgae cultivation in the green biorefinery concept to obtain a higher protein production from green juice obtained from grass. A mixed culture of *Chlorella sorokiniana* and *Acutodesmus obliquus* was cultivated on multiple dilutions and after different pre-treatments of green juice from agricultural grass. After 19 days, 1.01 ± 0.06 g/L of algal biomass was reached in a 10 % dilution pre-treated by sedimentation and pH adjustment to 8. Further treatments to reduce the microbial load in the grass juice did not increase algal productivity. The produced biomass had a 41 % protein content, and its heavy metal content and microbial load complied with safety norms for feed, except for yeast and Enterobacteriaceae. Overall, these findings offer new perspectives for protein production in a green biorefinery.

1. Introduction

Microalgae are photosynthetic microorganisms that have high productivity, do not require arable land, can be harvested throughout the year and have a biomass composition (proteins, lipids, carbohydrates) that is attractive for several applications [1–4]. However, despite their enormous potential, the commercialization of microalgae still lags behind expectations. This is mainly due to high production costs, difficulties extrapolating laboratory data to industrial-scale cultivation, limited use of algae strains and legislative hurdles [5–9]. In addition, the cultivation of microalgae requires large amounts of nutrients and (fresh) water which augments its environmental footprint [10–12].

One promising approach to lower the costs and the environmental footprint of microalgae cultivation is to use organic streams as a source of nutrients [13–15]. A more efficient and economically viable process might also be obtained if microalgae are incorporated as a step in a larger process instead of as a stand-alone process. In doing so, costs and facilities could be shared and a larger scale of operation can be obtained. For this, green biorefineries are an interesting option, as they produce a nutrient-rich liquid stream that could be used for microalgae cultivation.

Green biorefineries have been proposed for over a decade for the production of renewable chemicals, materials, food, feed and energy from green biomass such as cultivated grass, alfalfa and clover [16]. This process entails a step in which the fiber and liquid fractions are separated to increase the refining possibilities of the used biomass, besides significantly reducing the cost of drying the fibers for further applications such as insulation or feed [17–19]. The liquid fraction, i.e. green juice, is often used for the production of proteins and lactic acid [16,20]. The juice is not only rich in protein (29–40 %) but also contains amino acids, organic acids and dyes [21]. It can be used as an ingredient for animal feed, or as feedstock for biogas or bioethanol production [21,22]. Furthermore, it is rich in nitrogen, phosphorus and multiple trace elements crucial for algal cultivation [18,23,24].

The economic viability of the current process of obtaining proteins in a green biorefinery mainly depends on the nitrogen content of the green juice [25,26], making juices that are low on nitrogen less economically attractive. In an attempt to also valorize low-nitrogen green juices, microalgae could help to produce and concentrate proteins in their biomass [27]. Microalgae cultivation requires a rather diluted medium and previous studies showed that green juices obtained from cattail

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https://doi.org/10.1016/j.algal.2022.102941
Received 10 July 2022; Received in revised form 25 November 2022; Accepted 7 December 2022
Available online 12 December 2022

(*Typha latifolia*) and miscanthus (*Miscanthus* × *giganteus*) diluted 10× were able to provide sufficient nutrients for the growth of *Chlorella* sp. when compared to a mineral growing medium [28,29]. However, mostly only algal growth was assessed in these studies while the chemical composition of the produced biomass was not characterized [28,29].

In addition, the mentioned previous research on this topic used green juices produced on a lab scale and frozen before use, which might affect the physical characteristics, nutrient content and microbial load of the produced juices [28,29]. Therefore, the use of representative green juices and the characterization of the produced biomass are necessary if we are to assesss the real potential of integrating microalgae cultivation in a green biorefinery.

To assess the feasibility of using green juice from grass (hereafter called grass juice) as a growing medium for microalgae, a mixed culture of *Acutodesmus obliquus* and *Chlorella sorokiniana* was grown on grass juice after several treatments to improve light penetration and reduce the initial microbial load. A mixed culture was chosen to lower the risk of a culture collapse [30,31], and *A. obliquus* and *C. sorokiniana* were picked for their known potential to grow on complex streams [32,33]. The grass juice was produced by processing fresh cultivated grass in a pilot-scale screw press and was used within a few days-weeks of its production, being either kept at room temperature or refrigerated, but never frozen. Next to microalgal biomass productivity, the nutritional quality and safety of the biomass were determined to assess the potential for feed applications. These proof-of-concept assessments aim to contribute to an alternative process for producing proteins in a green biorefinery when using green juices with low nitrogen content. They are instrumental in the development of a more sustainable bioeconomy, in particular through microalgae technology and its applications for animal feed production.

2. Materials and methods

2.1. Microalgae strains and culture conditions

Acutodesmus obliquus (SAG 276-3d) and *Chlorella sorokiniana* (SAG 211-31) were purchased from SAG (Department of Experimental Phycology and Culture Collection of Algae, University of Goettingen, Germany). They were selected based on their robustness to grow on different types of organic streams [15,34,35] and their commercial potential [36–38]. Stock cultures of these strains were maintained in 250 mL conical flasks on an orbital shaker at 90 rpm with 15 μmol m^{-2} s^{-1} light exposure (cool-white fluorescent) in an incubator at 22 °C under a 16/8 h light/dark cycle. For upscaling and the experiment itself, 70 μmol m^{-2} s^{-1} light exposure was used. The used freshwater medium was autoclaved at 121 °C for 20 min and had the following composition (based on the SAG basal medium version 10.2008): 252 mg/L HNO_3, 22 mg/L H_3PO_4, 247 mg/L KOH, 6.3 mg/L Fe-DTPA, 42 pg/L $CuSO_4.5H_2O$, 2.8 μg/L $ZnSO_4$, 7.2 μg/L $MnSO_4$, 4.3 μg/L Na_2MoO_4, 40 μg/L $Na_2B_4O_7$, 0.2 g/L $NaHCO_3$ and 23 mg/L $MgSO_4.7H_2O$. To produce inoculum for the experiments described below, a mixed *A. obliquus* and *C. sorokiniana* stock culture (containing approximately 20 % *A. obliquus* and 80 % *C. sorokiniana*; this ratio was reached naturally after several cycles of co-cultivation) was cultivated in aerated (ambient air) 1 L bottles till an OD_{720} of approximately 1.

2.2. Preparation of grass juice

The used grass clippings were a mixture of *Festulolium* and perennial ryegrass mowed from a farmland near Ghent, Belgium in June 2021. The liquid fraction from the grass (grass juice) was obtained by screw pressing fresh clippings on the same day of grass harvesting with a yield of 50 % (200 kg of grass resulted in 100 L of grass juice). The used press, EYS SP400, was a proprietary screw press from Releaf (Drongen, Belgium). The temperature within the screw press was uncontrolled, but the temperature of the produced grass juice was monitored and did not exceed 40 °C, with a usual temperature range of 15–20 °C depending on the ambient temperature. The grass juice was produced on the same day of harvesting and was allowed to sediment overnight under two conditions: (i) grass juice was diluted at 5 %, 10 % and 15 % and then left to sediment in covered 50 mL plastic volumetric tubes in a refrigerator at 4 °C and (ii) undiluted grass juice was left to sediment at room temperature (20 ± 2 °C) in a 100 L conical glass tank to mimic a more commercial, less controlled condition. After sedimentation, the supernatant from (i) was recovered and stored for 2 weeks at 4 °C before further use, while the supernatant from (ii) was filtered through a 50 μm filter (Sentinel, Filtermat Belgium) before being stored, undiluted, in air-tight recipients with a pressure release valve under dark conditions at 19 ± 2 °C before further use.

2.3. Microalgal cultivation on grass juice

2.3.1. Influence of dilution and filtration of grass juice on microalgae growth

To assess microalgal growth on different dilutions and the influence of filter sterilization, we conducted a first experiment in which microalgae were grown for 11 days in 5 %, 10 % and 15 % supernatant from (i) as described in Section 2.2. The diluted supernatants were either used unfiltered or after filtration with a 0.2 μm sterile filtration unit (Nalgene, Thermo Scientific, USA). All supernatants had their pH values adjusted from pH 4 to 7 by the addition of NaOH (1 N) before algal inoculation. A control with the freshwater medium described in Section 2.1 was used. 250 mL flasks with 100 mL of each medium were inoculated with approximately 2 × 10^6 cells/mL from healthy cultures in their exponential growth phase, and cultivation was carried out on an orbital shaker as described in Section 2.1. Flasks were capped with hydrophobic cotton stoppers to allow for gas exchange but hinder the entering of contaminants. Samples (1 mL) were taken on days 4 and 11 for cell counting as described in Section 2.4.1. Each condition was tested in triplicate.

2.3.2. Influence of alternative grass juice pretreatments on microalgae growth

To assess microalgal growth after different pretreatments of the supernatant to reduce microbial load, a second experiment was run in aerated 1 L bottles, provided with filters (0.2 μm) on both the aeration in-and outlet, using supernatant from (ii), as described in Section 2.2, after 6 weeks of storage. The supernatant was used at a 10 % concentration (v/v) achieved with the addition of unsterilized tap water and the start-pH was adjusted from 4.35 to 8 by the addition of NaOH (1 N). Three pretreatments were tested: (i) only pH adjustment (P), (ii) filtration (5 μm) in combination with pH adjustment (PF), and (iii) a heat treatment for 15 min at 100 °C in combination with pH adjustment and filtration (5 μm) (PFH). The pH was adjusted to 8 to lower the number of unwanted microorganisms, the 5 μm filtration was aimed at removing larger fungal cells, clumps of cells and organic matter, and heating was performed to semi-sterilize the medium.

Each condition was tested in triplicate and growth was monitored for 19 days. Sterile freshwater mineral medium (see Section 2.1) was used as a control medium. The used inoculum consisted of 85 mL of a microalgal culture with an OD_{720} of 1, and was added to a total volume of 850 mL to reach a start OD_{720} of 0.1 (corresponding to approximately 1.6 × 10^6 cells/mL). Cell counts were done at the start, three times each week (Monday, Wednesday and Friday) and at the final day of the experiment as described in Section 2.4.1. To generate enough biomass for a qualitative and quantitative analysis of the microalgal biomass (e.g., presence of macro-nutrients, heavy metals, protein- and mineral content), microalgae were also grown for 12 days in 10 % grass juice (filtered through 50 μm) at a start pH of 8 in aerated 2 L bottles.

2.4. Analytical methods

2.4.1. Growth determination

To monitor microalgal growth during the experiments, a Bürker counting chamber and microscope (DM50, Leica) were used for microscopic observation and to assess total cell count (*C. sorokiniana* plus *A. obliquus*). In the 1 L bottle experiments, also dry weight (DW) measurements were performed at the start and the end of the experiment. For this, a 5 mL sample was filtered on a pre-weighted glass microfiber membrane (0.45 μm, washed in deionized water). Samples were dried at 70 °C for 24 h and transferred to a desiccator before weighing.

2.4.2. Nutrient content of grass juice and uptake during growth

The grass juice supernatants (obtained after sedimentation) were analyzed for their elemental composition. Hot-plate digestion at the boiling point of a mixture containing 2.5 mL grass juice supernatant, 2.0 mL HNO_3 and 1 mL H_2O_2 was carried out until all color and sediments disappeared. The digested supernatants were then analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES; Varian Vista MPX, USA). CN content was determined with a CN analyzer (Leco, USA) directly in the undigested grass juice supernatant. Nutrient uptake during algal growth was evaluated by measuring total organic-N, PO_4^{3-}, NO_3^- and NH_4^+-N concentrations at the start and end of the growth experiments in 1 L bottles. 50 mL samples were centrifuged for 10 min at 1550 ×*g* at 20 °C (Sorvall lynx 4000, Thermo Fisher Scientific) before nutrient analysis. Total organic nitrogen content was determined according to the Kjeldahl method (Gerhardt, Kjeldatherm, Vapodest 20 s). PO_4^{3-} and NO_3^- contents were determined by ion chromatography (Metrohm Eco IC using a Metrosep A Supp 17 – 250/4.0 column) using a 6 mM Na_2CO_3 buffer after filtering through 0.45 μm and 0.20 μm disposable PET-filters (NBN EN ISO 10304-1). NH_4^+-N content was determined by steam distillation (Vapodest 200, Gerhardt). Each sample was diluted in a phosphate buffer and ammonia was expulsed from this weak alkaline solution by distillation (4 min, 100 % steam power). Ammonia was subsequently collected in a 2 % boric acid solution and volumetrically determined by titration with 0.01 M HCl (CMA/2/I/E.3 ISO 5664:1984). To measure the pH of the cultures, a pH meter (Edge meter, HI11310 probe, Hanna Instruments) was used.

2.4.3. Quality and safety of the cultivated algal biomass

The biomass cultivated in 2 L bottles (see Section 2.3.2) was collected by centrifugation at 1550×*g* for 10 min. The nutritional quality of the microalgal biomass was assessed through a Weende analysis. Specifically, moisture content (ISO 1442), raw fat content (ISO 1443), inorganic substance (mineral fraction, ISO 936), crude protein level (N-containing substance × 6.25 following ISO 1871), crude fiber content (SM00121), starch content (Ewers method SM00120) and total carbohydrates (SM00093, calculated) of the microalgal biomass were determined.

To evaluate the microbial safety of the microalgal biomass for feed applications, the presence of microbial pathogenic indicator organisms was also assessed. For this, 8 to 9 pathogen groups were selected based on the EU directive 183/2005/EC, Regulation (EC) 142/2011 and the Feed Chain Alliance Standard (OVOCOM) regarding safety norms for feed production: coliforms (ISO 4832), enterococci (NEN 6817), *Salmonella* sp. (AFNOR BRD-7/11-12/05, only measured at the start), *Campylobacter* sp. (Microval MV2008LR12), yeasts (ISO 21527), fungi (ISO 21527), sulfite-reducing anaerobes (ISO 15213), coagulase-positive staphylococci (ISO 6888-1) and Enterobacteriaceae (AFNOR BRD-7/24-11/13). The presence of these microbial pathogens was determined at the beginning and end of the microalgal growth in 1 L bottles and at the end of the growth in 2 L bottles (see Section 2.3.2). Pathogen presence was tested using standard methods for determining colony-forming units (CFU). Microbial and Weende Analyses were done by LOVAP NV (Geel, Belgium) following accredited methods.

To determine the heavy metal content, the collected biomass was frozen at −20 °C and freeze-dried (L200, Büchi) until a constant mass was reached. Then, 0.1 g freeze-dried samples were pre-digested with 10 mL HNO_3 for 30 min, followed by 30 min in an ultrasonic water bath. The samples were then subjected to microwave digestion (UltraWAVE, Milestone, Italy) and the total metal concentrations for Cd and Pb in the digested samples were analyzed by ICP-OES.

2.5. Statistical analysis

Statistical analyses were performed in R version 4.0.2 (R Core Team, 2022) at a significance level of $\alpha = 0.05$. Model assumptions, including distributional fit and homogeneity of variance, were verified graphically for all analyses. Microalgal growth in both the 250 mL flasks (see Section 2.3.1) and 1 L bottles (see Section 2.3.2) was analyzed using mixed modelling with Gaussian error distribution (lme4 package; [39]) with cell count (per mL) as the dependent variable. Condition (i.e., pre-treatment type as explained in Sections 2.3.1 and 2.3.2) and time, including their interaction, were added as fixed factors to the model. Culture identity was added as a random effect to account for between- and within-culture variation over time. The significance of the fixed factors in the model was tested with type III Wald chi-square tests. Post-hoc differences were assessed using Tukey-corrected pairwise comparisons (lsmeans package; [40]).

3. Results and discussion

3.1. Microalgal growth on diluted grass supernatant with and without filtration

Freshly pressed grass juice had a green, opaque color due to suspended chlorophyll-containing particles (Fig. S1). Even after a 20× dilution, the color was too intense for proper light penetration necessary for the growth of photoautotrophic microalgae suggesting the need for gravitational sedimentation. After 1 h of sedimentation, a clearer supernatant was recovered (Fig. S1) with high nutrient content, indicating that this simple pretreatment was effective for recovering the nutrients in grass juice while improving its light penetration properties.

The composition of the treated grass juice (undiluted as well as the 5, 10 and 15 % conditions) and the mineral medium is shown in Table 1. This characterization indicates that a concentration between 10 and 15 % grass juice is needed to match the N content of the mineral medium. Moreover, while all N in the mineral medium is found as nitrate, grass

Table 1
Elemental composition of grass juice after sedimentation and storage, and of the mineral medium used as control in microalgae growth experiments.

(mg/L)	Grass juice				Mineral medium
	Undiluted	5 %	10 %	15 %	
C_{total}	6717 ± 110	36	672	1008	–
N_{total}	520 ± 54	19[a]	38[a]	60[a]	56
$N\text{-}NH_4^+$	97 ± 15	3[a]	7[a]	9[a]	–
$N\text{-}NO_3^-$	11 ± 3	4[a]	8[a]	15[a]	56
P	192 ± 2	10	19	29	7
K	2215 ± 45	111	222	332	172
S	109 ± 2	5	11	16	3
Ca	487 ± 6	24	48	73	–
Fe	0.43 ± 0.06	0.02	0.04	0.06	0.75
Cu	BDL				$1e^{-8}$
Zn	17.17 ± 0.17	0.9	1.7	2.5	0.001
Mn	4.85 ± 0.06	0.2	0.5	0.7	0.003
Na	104 ± 3	5	10	16	0.06
Mg	168 ± 3	8	17	25	2.24
Al	2.16 ± 0.04	0.1	0.2	0.3	–

BDL – below detection level.
Cd, Co, Cr, Ni and Pb were all below detection level for the grass juice sample.
[a] Determined analytically; all other elements were calculated by multiplying the undiluted content by the dilution factor.

juice has also some ammonia and about 50 % of its N is present in organic form as amino acids, peptides or proteins. Microalgae are known to be able to uptake amino acids, but it is still not entirely clear if peptides and proteins are also used as organic N sources [41].

The N/P ratio of grass juice was appropriate, and K and S contents were also in line with the mineral medium for all the dilutions tested. The Fe content of grass juice was significantly lower than that of the mineral medium, but it is known that such medium recipes are not optimized and tend to have much higher micronutrient concentrations than those required by the microalgae. Finally, Zn, Mn and Mg were all in much higher concentrations in the diluted grass juices than those of the mineral medium; nevertheless, these elements do not have any expected toxicity at the present concentrations. As only the liquid fraction was used, it is expected that all these elements are in their mineral form and not solid-bound, and are therefore available for uptake by the microalgae.

Overall, the grass juice after dilution showed a balanced composition for microalgal growth suggesting that any nutrient supplementation was unnecessary. In contrast, significant amounts of bacteria, yeast and filamentous fungi were observed in the fresh grass juice which had an acidic pH of 4. Neither of these conditions are ideal for the growth of the selected microalgae [42,43], and suggested that filtration to reduce the initial microbial load and pH adjustment could be necessary. The results of these treatments are presented in Fig. 1 and Table S1.

On day 4, corresponding to the middle of the exponential phase, both filtered and unfiltered conditions of all tested grass juice concentrations yielded similar cell counts as the control cultures grown in the mineral medium (Fig. 1, $P > 0.05$). On day 11, when the cells were at the beginning of the stationary growth phase, both filtered and unfiltered conditions at 5 and 10 % grass juice concentrations resulted in significantly higher cell counts when compared to the control (Fig. 1; Table S1, $P < 0.05$). Microalgal density on day 11 did not differ between the 15 % grass juice conditions (filtered and unfiltered) and the control (Fig. 1).

Overall, cultures grown on filtered grass juice only had bacterial contamination. In the unfiltered samples, not only bacteria, but also a high number of yeast cells were observed; nevertheless, their presence declined during microalgal cultivation. A filamentous fungus was only observed once in an unfiltered sample, even though abundant fungal growth was found in the fresh grass juice. This reduced presence of filamentous fungi and yeast can be related to the higher pH of the algal culture, which went up to 10 by the end of the experiment, while fungi have been reported to thrive in more acidic environments [44]. A concomitant increase in pH with algal biomass production is typically an indicator for dissolved carbon uptake during photosynthesis, mediated by the bicarbonate buffer system, and nutrient uptake, e.g. assimilation of nitrate [45–48]. Finally, the grass juices at 15 % were the most microbially-contaminated media, which can explain the lower microalgae cell count in the unfiltered medium compared to the other conditions (Fig. 1, day 11). Even in the filtered medium (15 %), the concentration of bacteria was higher than in the other two, possibly due to its higher sugar content.

From this first experiment, we conclude that both 5 and 10 % grass juice could provide a good nutrient source for algal growth if proper treatment is done to reduce the initial microbial load. Since the 10 % treated condition had more nutrients to sustain higher algal growth than the 5 % condition, this was selected for further tests in a higher volume.

Interestingly, the best juice concentration found in the previous studies using cattail and miscanthus juices for microalgae cultivation was also 10 %, even though their nutrient content was overall much higher than the grass juice used in this study [28,29]. Nevertheless, it is advisable to further optimize the best concentration of grass supernatant to use in future studies, e.g., in a more continuous setup in which nutrients are provided in regular intervals to reach higher microalgae yields while maintaining sufficient light penetration and keeping the growth of undesired microorganisms to a minimum.

3.2. Microalgal growth on grass supernatant after different pretreatments

To assess microalgal growth on grass juice in different settings, a sedimentation step without temperature control, as refrigerated storage of the green juice may not necessarily reflect a potential commercial setting, was conducted on a larger scale (Fig. S2). This grass juice supernatant was subsequently used for microalgal cultivation.

In the previous experiment, an abundant microbial load (mainly bacterial and large yeast cells >5 μm) was observed. These microorganisms can compete for resources with the microalgae and reduce the growth rate and final yields of microalgae biomass, suggesting that pretreatments to reduce the initial load of unwanted microorganisms

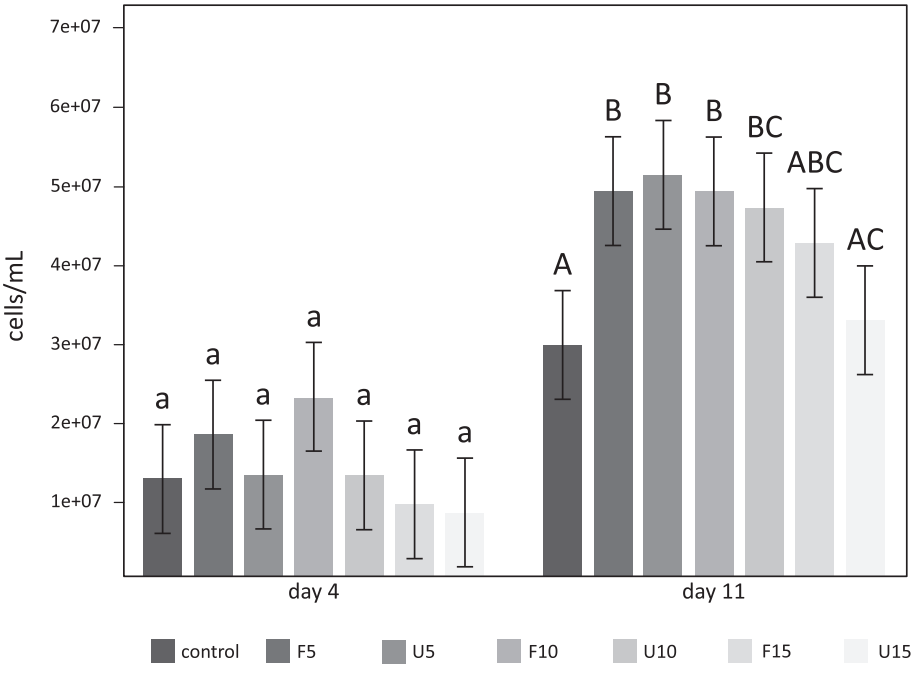

Fig. 1. Microalgal cell concentration (*C. sorokiniana* + *A. obliquus*) after 4 and 11 days of growth in grass juice supernatant obtained after dilution (0 %, 5 %, 10 % and 15 % final concentrations) followed by sedimentation and pH adjustment to 7. The supernatants were either used unfiltered (U) or after a filtration step with a 0.2 μm filter (F). Freshwater mineral medium was used as a control. The average cell concentration of 3 independent replicates is given, and whiskers represent the standard error. Different letters indicate a significant difference between the values obtained either on day 4 (lowercase) or day 11 (uppercase) based on Tukey-corrected pairwise comparison ($P < 0.05$).

could be necessary. Therefore, simple and scalable pretreatment techniques were tested in a second experiment. The results are shown in Fig. 2 and microscopic observations at the start and end are shown in Fig. S3.

Average cell count over time differed depending on the pretreatment of the medium (Table S1), with higher growth being observed in the condition with only pH adjustment compared to when the medium was also microfiltered and/or heated (Fig. S4). The pH rapidly increased from 8.0 to 8.9 after 2 to 4 days and slowly increased until reaching a final pH between 9.3 and 9.6 for the remainder of the experiment. A similar final DW between 0.9 and 1.0 g/L was reached for all the tested conditions, indicating that the additional filtration and heat treatments were not needed and that the pH adjustment was the main factor influencing cell growth. Although difficult to compare due to differences in experimental setup, this final DW is comparable with other studies evaluating the growth of *Acutodesmus* and *Chlorella* species in wastewater [15,49–51]. However, the DW achieved in this study was lower compared to *Acutodesmus* sp. cultivated in energy grass digestate effluent [52,53].

The nutrient content (nitrogen and phosphorous) in each medium is shown in Table 2. For the 3 conditions tested, the nutrient composition was similar at the start of cultivation, albeit with slightly higher nitrogen content in the medium that was only pH-adjusted. All nitrogen and almost all phosphorous present in the medium were effectively assimilated by the microalgae and removed from the medium by the end of the experiment (Table 2), confirming the bioavailability of the nutrients present in the grass juice for algae cultivation.

The results of the influence of the tested treatments on the presence of indicator pathogenic organisms in the used grass juice are shown in Table 3. As expected, the pre-treatment with pH adjustment in combination with extra filtration and heating of the supernatant resulted in the lowest CFU counts at the start of the experiment. However, the CFU count for coliforms and Enterobacteriaceae at the end of the experiment was higher in this condition (PFH) compared to when the medium was only pH-adjusted (P). The addition of a filtration step (PF) caused an increase in CFU counts for Enterococci and yeast compared to the non-filtered sample (P) at the start condition. The reason for this increase at the start in the filtered-treated media is unclear, but might be due to additional manipulation of the sample.

Combining pH adjustment, filtration, and heating of the supernatant was most effective at reducing the presence of indicator organisms in the medium, but it did not result in the best microalgal growth (Fig. 2). While medium sterilization by autoclaving is often used to reduce the microbial load, it can also alter the water chemistry and result in a less suitable medium for microalgal growth [54–56]. A similar explanation can be given for the filtration, which might remove potential nutrients for the microalgae. Solely adjusting the pH resulted in higher CFU counts for enterococci, fungi and yeast at the end of the cultivation period. Nevertheless, at the end of the growth experiment, most of the tested pathogens in the algal cultures were within the acceptable limits as described for animal feed according to the EU directives 183/2005/EC, Regulation (EC) 142/2011 and Feed Chain Alliance Standard (OVOCOM). Therefore, solely adjusting the pH might be sufficient for growing algae in grass juice, as there was no indication that the presence of competing microorganisms inhibited microalgal growth in this condition.

3.3. Quality and safety of the algal biomass cultivated in 10 % pH-adjusted supernatant

The nutritional quality of the biomass grown on grass juice (Weende analysis, Table 4) was comparable with reported compositions for *Chlorella* and *Acutodesmus* species [34,57–59]. Specifically for the protein content, the biomass (dry product) had a protein content of 41 %, similar to values reported in the literature for *Acutodesmus* or *Chlorella* species [60–62] and higher compared to *Acutodesmus* sp. cultivated in energy grass digestate effluent (16 %) [52]. Furthermore, this protein content is similar to – or even better than – traditional crops such as safflower (23–43 %), linseed (35 %), soy (44 %) and sesame (40–45 %) [63]. While this study applied the commonly used conversion factor of

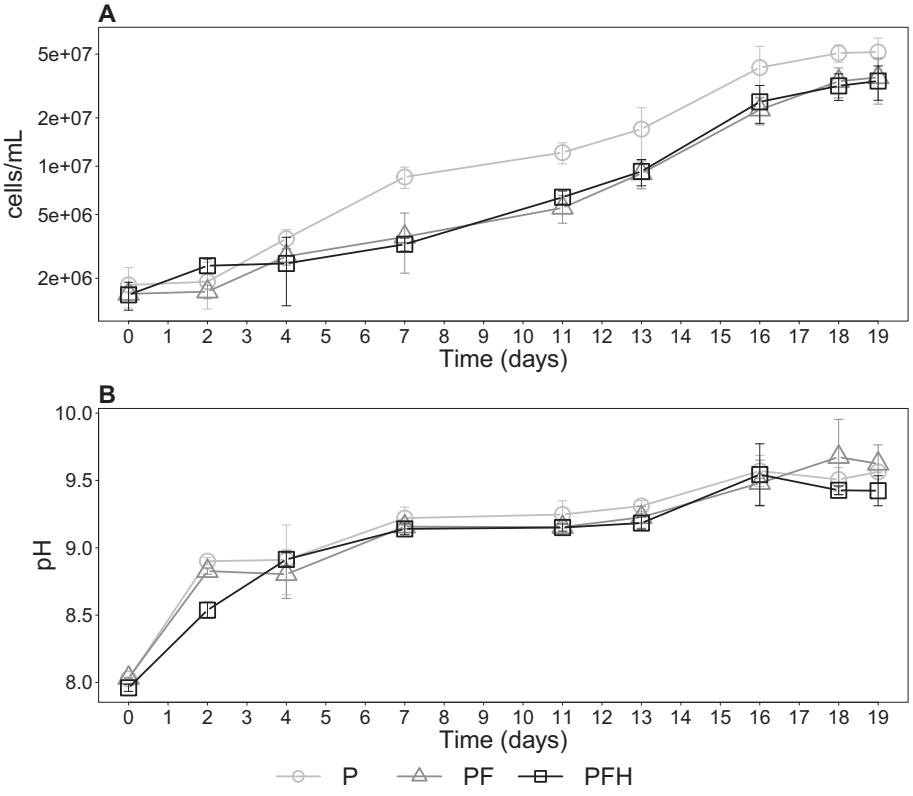

Fig. 2. (A) Microalgal cell concentration (*C. sorokiniana* + *A. obliquus*) profile during growth in grass juice supernatant obtained after sedimentation, dilution to a final concentration of 10 % (v/v) and treatment by (i) only pH adjustment to 8 (P), (ii) filtration (5 μm) followed by pH adjustment to 8 (PF), and (iii) filtration (5 μm) followed by heat treatment for 15 min at 100 °C followed by pH adjustment to 8 (PFH). (B) pH profile of the cultures described above during microalgal growth. Markers show the average of 3 independent replicates and error bars represent the standard deviation of the replicates.

Table 2
Nutrient concentrations in the different growth media at the start and end of the cultivation period. Mean concentration (mg/L), standard deviation (SD, n = 3) and average nutrient consumption (%) are given for NH_4^+-N, NO_3^-, PO_4^{3-} and Total organic N. (P) pH adjustment to 8; (PF) filtration (5 μm) followed by pH adjustment to 8; (PHF) filtration (5 μm) followed by heat treatment for 15 min at 100 °C followed by pH adjustment to 8.

		NO_3^-		NH_4^+		PO_4^{3-}		Total N	
		Mean	%	Mean	%	Mean	%	Mean	%
P	Start	11 ± 1	100	13.2 ± 0.2	100	39.01 ± 3.9	88	58 ± 3.5	100
	End	0		0		<5[a]		0	
PF	Start	8.3 ± 0.6	100	11.6 ± 1.0	100	31.14 ± 3.3	84	48 ± 0	100
	End	0		0		<5[a]		0	
PFH	Start	9.6 ± 2.1	100	15.0 ± 0.1	100	38.7 ± 5.5	88	52 ± 7	100
	End	0		0		<5[a]		0	

[a] Due to a technical problem only 1 sample could be measured.

Table 3
Presence of indicator pathogenic organisms per tested condition. Results are shown in CFU per g sample. Samples were prepared by filtering the stored supernatant through a 50 μm filter bag before pH adjustment to 8 (P), extra filtration at 5 μm (PF) and additional heating of the supernatant for 15 min at 100 °C (PFH). Pathogen abundance was measured at the onset (start) and at the end of the 19-day cultivation period.

Condition	Start (CFU/g)			End (CFU/g)		
	P	PF	PFH	P	PF	PFH
Coliforms	<10	<10	<10	140	2400	1100
Enterococci	1500	8100	<10	5300	502	250
Sulfite-reducing anaerobes	10	<10	<10	<10	<10	<10
Fungi	<100	<100	<10	500	<100	<100
Yeasts	500	1400	<10	2000	<100	<100
Enterobacteriaceae	<10	<10	<10	260	3100	2100
Coagulase-positive staphylococci	<100	<100	<10	<100	<100	<100
Campylobacter sp.	<100	<100	<10	<100	<100	<100

Table 4
Macronutrient composition and microbial load of algae paste cultivated for 12 days on 10 % unsterilized grass juice after sedimentation and pH adjustment to 8. Dry mass was calculated based on the macronutrient composition of fresh algae paste considering a residual 17 % moisture level (measured). Yeast and Enterobacteriaceae (bold numbers) exceeded the thresholds. Cd and Pb were below the quantification levels.

Weende analysis (g/100 g)	Algae paste	Dry product
Moisture	82.8	17
Inorganic substance	2.91	16.9
Total protein	7.1	41.2
Starch	1	5.8
Crude fiber	0.5	2.9
Total lipids	2.0	11.6
Total carbohydrates	4.7	27.3
Indicator organism (CFU/g)		
Coliforms	<10	
Enterococci	<10	
Sulfite-reducing anaerobes	<10	
Fungi	2500	
Yeast	**180,000**	
Enterobacteriaceae	**330,000**	
Coagulase-positive staphylococci	<100	
Campylobacter sp.	<100	

6.25 to estimate dry mass protein content from Kjeldahl N, this can be an overestimation [64]. Follow-up studies should directly measure the amino acid content of the biomass to facilitate more accurate and in-depth assessments.

The heavy metal content of the produced biomass was also assessed to verify its safety for feed application. Only As, Hg, Cd, and Pb in feedstuffs are regulated by Directive 2002/32/EC of the European Parliament and may not exceed the respective limits of 2, 0.1, 1, and 10 mg/kg feedstuff. Cd and Pb were analyzed in the present study and were found to be below quantification levels (0.01 and 0.1 mg/kg, respectively) and, therefore, were within the regulatory limits.

Besides nutritional value and the presence of contaminants such as heavy metals, also microbial safety of the algal biomass determines its applicability for animal feed production. Six of the eight tested microbial pathogens stayed well below the thresholds described for animal feed according to the EU directive 183/2005/EC, Regulation (EC) 142/2011 and Feed Chain Alliance Standard (OVOCOM) (Table 4). In contrast, the total amount of yeast and Enterobacteriaceae exceeded the thresholds, suggesting that extra treatment steps may be needed to reduce microbial pathogens in the algal biomass before use as an animal feed ingredient. Possibly, a longer microalgal cultivation period would further reduce the pathogen load as suggested by the lower pathogen load in the 19-day (Table 3) compared to the 12-day (Table 4) growth test. Also, post-harvest treatment steps could help in reducing the number of pathogens in the biomass. While we performed the microbial analysis on fresh algae paste, algal biomass is usually processed into a dry formulation before use, which could lead to a further reduction of the pathogen load [65]. Future research is recommended to study the effect of downstream processing on the microbial safety of the produced algal biomass.

3.4. Integrating microalgae cultivation in a green biorefinery

In this proof-of-concept study, we assessed the potential of using grass juice as growth medium to cultivate microalgae for the production of protein within a green biorefinery. In a classic green biorefinery process (i.e. without microalgae), protein production from grass juice is highly dependent on its N content, and a techno-economic assessment showed that a 10 % decrease in N content would significantly decrease the viability of the process [26]. In the current study, the used grass juice had a N content of only 0.5 g/L, while usual green juices used for protein precipitation have a N content between 1.6 and 4.6 g/L. Therefore, this low N juice would most likely not result in an economically-attractive protein source in a green biorefinery.

By using grass juice as a culture medium for microalgae, the protein content can be concentrated in the algal biomass. In the current study, the use of a grass juice diluted at 10 %, with a N concentration of about 0.05 g/L, resulted in the production of 1 g/L of algae with a N content of about 6 %. Therefore, all N present in the juice was converted into algal biomass, which can be harvested and could be used as animal feed, providing not only proteins but also other nutritional factors such as antioxidants, polyunsaturated fatty acids, minerals and vitamins [66].

A techno-economic assessment of the process was conducted based on the laboratory results found in the current study and the use of grass from farm edges as a way to diversify the business models of farms and to increase their sustainability. The integrated use of grass juice for microalgae cultivation and fibers for either anaerobic digestion or production of biocomposites showed positive Net Present Values for *Chlorella* market prices between 30 and 40 euros/kg and payback periods of 3–5 years (unpublished results). Therefore, the proposed process

could potentially be feasible and attractive to be installed at a farm scale considering a 10,000 L algae reactor.

With the use of grass juice as a growing medium, a more sustainable approach to microalgae cultivation could be achieved. However, only a limited set of parameters influencing the microalgal growth, biomass quality and safety was assessed in this study. Yet, other parameters should be investigated to optimize the growth and quality of algae cultivated in grass juice such as the species of algae, inoculation concentration and ratio of different strains in mixed cultures, N/P ratio in the growth medium, pH, light intensity and growth temperature. In addition, different types of grass, including waste clippings from roadside verges, could be used. Grasslands represent a substantial part of the agricultural area and often periodic mechanical cutting is vital [18]. Currently, grass clippings are often left to rot, used to make compost, mulch or used to produce bioenergy [67–69]. Besides the types of grass, also the storage of the grass juice and potential seasonal effects should be taken into account. Furthermore, pilot-scale studies will be needed to validate the use of grass juice as a growing medium for microalgae and to better refine the existing techno-economic assessment with more relevant data.

4. Conclusion

This study shows that the juice from cultivated grass clippings may be used as a nutrient source in the production of protein-rich microalgae biomass. However, the acidity of the juice may warrant the need to artificially increase the pH of the culture medium for optimal growth of the microalgae and reduce the microbial load during cultivation. While the resulting biomass had high nutritional quality and heavy metal content was below safety norms for use as an animal feed ingredient, downstream processing may be needed to further reduce the pathogen content. Moreover, future (pilot-scale) studies are needed to further explore the potential of using grass juice as a nutrient source for microalgal growth. Nevertheless, the present findings pave the way to a more flexible green biorefinery approach for using low N feedstocks.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Funding

This work was funded by operationele groep 2020, Grass2Algae, Departement Landbouw en Visserij, Vlaamse Regering.

CRediT authorship contribution statement

Floris Schoeters: Conceptualization, Methodology, Formal analysis and interpretation, Investigation, Writing - original draft, Writing - review & editing; **Eli S.J. Thoré**: Conceptualization, Methodology, Formal analysis and interpretation, Investigation, Writing - original draft, Writing - review & editing; **Audrey De Cuyper**: Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing; **Isabelle Noyens**: Investigation, Writing - review & editing; **Sarah Goossens**: Investigation, Writing - review & editing; **Sander Lybaert**: Resources, Writing - review & editing; **Erik Meers**: Obtaining of funding, Conceptualization, Writing - review & editing; **Sabine Van Miert**: Conceptualization, Writing - review & editing; **Marcella Fernandes de Souza**: Obtaining of funding, Conceptualization, Project administration, Formal analysis and interpretation, Investigation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

We thank Kris Heirbaut for helping with sampling for the acquisition of data, and Ward Ceunen and Marie-Chantal Heerteler for helping with the chemical analyses. We also thank Emily Van Cantfort for helping with the graphical abstract.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2022.102941.

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