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Impacts of harvesting methods and medium recycling on rheology and composition of recycled medium and cell concentrates of *Limnospira platensis* semi-continuous cultures

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ABSTRACT

Recycling microalgae growth media is essential to reduce the water and nutrient demands, and increase the viability of biomass production. However, it is not clear whether the harvesting method affects the outcomes of medium recycling on culture performance. In this study, *Limnospira platensis* was grown in a semi-continuous mode with daily harvest and dilution (0.25 d^{-1}) for 50 days with medium refresh (control), or recycle after 0.22 μm or 10 μm filtration, or centrifugation. The concentration of extracellular polymeric substances (EPS) in the medium was 4- to 6-fold higher during semi-continuous growth when compared to batch mode and the highest after centrifugation. Medium recycling reduced growth rates by 30 %, but productivity remained unaltered during the experiments (0.24 ± 0.02 g L^{-1} d^{-1}). EPS extracted from recycled media had similar compositions (mainly proteins and hydrocarbons) and no influence in the viscosity of solutions. The biochemical composition and rheology of cell concentrates varied widely, especially after centrifugation. The harvesting method affected the partitioning between free EPS in the medium and EPS adhered to the cell wall. If carefully applied to the microalgae industry, these findings can lead to more circular and sustainable biomass production.

1. Introduction

Producing microalgal biomass, extracts, and/or bioproducts involves dewatering a liquid culture, which results in a cell-free growth medium and a cell concentrate that can be further processed depending on the desired end product. It is essential to maximize the recycling of medium to reduce the water footprint and increase the viability of industrial microalgae production [1]. From an economic perspective, medium recycling ensures optimal usage of water and nutrients, whereas, from an environmental point of view, it reduces the amount of freshwater resources that the ecosystem is deprived of due to the cultivation system [2]. However, the outcomes of prolonged medium recycling on the physiology of cells and, hence, on the performance of cultures, are still unclear. Existing literature reports highly heterogeneous results ranging from the improvement of productivity in *Tetraselmis* sp. [3] to the complete collapse of *Nannochloropsis* sp. [4] and *Limnospira platensis* [5] cultures. Results seem to be case-specific, and the same species can respond differently to recycled media, as in the case of *L. platensis* [5–7]. Some strategies to improve medium recyclability have been investigated, such as physicochemical treatments of the growth medium before recycling [8,9] and spectrophotometric analysis to evaluate if the medium is adequate for recycling [10]. Still, given the current relevance of microalgal biotechnology, it is clear that this topic must be better understood.

Even though the mechanistic effects are still unclear, it is assumed that the accumulation of organic molecules released from the cells to the environment is responsible for the effects of medium recycling in cell physiology [11]. Such molecules – generally called extracellular

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polymeric substances (EPS) – can be complex conjugates, and their function and structure in microalgae are still obscure [12]. Indeed, while EPS research is extensive for bacterial systems, there is limited knowledge about microalgae EPS and their impact on microalgae processes [13]. Other than being in solution, EPS can also be adhered to the cell wall, affecting the microalgal cell concentrates. Positive growth results in co-cultures suggest that EPS may have an allelopathic effect on microalgae [14,15]. It has been suggested that the accumulation of EPS can alter the rheological behavior of cell concentrates, increasing up to 60 % of the energy consumption for harvesting and downstream processing in microalgal production plants [16]. EPS can also be valorized as a co-product within a biorefinery framework because they are commercially attractive to industries due to their gelling, adhesive, rheology-modifying, and flocculant capacities [12,17,18]. Recently, cyanobacterial EPS have received attention due to their biological activities, such as being antiviral, antioxidant, anti-inflammatory, and more [19,20]. De Jesus et al. [17] reported that the synthesis of EPS by *L. platensis* LEB-18 was approximately 10 times higher than the biomass productivity after 30 days of cultivation, highlighting the biotechnological potential of using post-harvest supernatant to obtain high-value bioproducts. Therefore, the exploitation of cell-free medium as a valuable side stream of microalgal production has been strongly considered [21,22]. From a physiological perspective, relatively high concentrations of EPS in microalgae cultivation may have positive effects on non-gaseous mass transfer, extracellular electron transfer, and resistance to osmotic and oxidative stress. However, gas transfer and light utilization are negatively affected [13].

This work assessed the effects of prolonged medium recycling in lab-scale *L. platensis* cultures as a function of the harvesting method used (10 and 0.22 μm filtration, and centrifugation). This species was selected because it is one of the most widely produced and commercialized microalga in the world, known commercially as Spirulina platensis. It has been hypothesized that the harvesting method could affect the outcomes of recycling because it is known that high-molecular-weight EPS can be retained by filtration. The biochemical composition and rheological behavior of recycled medium and cell concentrates were analyzed. To the best of these authors' knowledge, medium recycling has traditionally been assessed using batch mode. However, the semi-continuous mode, which involves daily harvesting with a fixed dilution rate, is more commonly used by commercial microalgae producers to maintain a stable culture environment [23]. This study aims to bridge this research gap by evaluating the outcomes of culture medium recycling in semi-continuous *L. platensis* cultures. Even though the applicability of lab-scale results on commercial-scale production is not straightforward, this study can guide microalgae producers to improve growth medium recyclability, recycling the harvested medium without compromising the fitness of the culture. This action can save resources and decrease the water footprint of microalgal biomass production, in alignment with circular economy principles and the UN's Sustainable Development Goals.

2. Material and methods

2.1. Cultivation conditions

Limnospira platensis (BEA005B) cells were cultivated in 1 L spherical flasks containing 500 mL of modified Zarrouk medium. The final composition of the growth medium (pH 9.8) contained the following per liter: 8.4 g $NaHCO_3$, 0.90 g $NaNO_3$, 0.18 g $MgSO_4$, 0.14 g KH_2PO_4, and 0.03 g Karentol® Mix Super(Kenogard, Spain). Cultures were gassed with a 1.0 ± 0.1 L min^{-1} air flow of filtered (0.22 μm) atmospheric air, exposed to continuous illumination of 300 μmol photons m^{-2} s^{-1} (measured in the surface of flasks) provided by fluorescent lamps, and maintained at 22 ± 2°C. The volume of the culture medium was monitored and, when necessary, adjusted by adding sterile distilled water to minimize evaporation effects.

Experiments were operated in batch mode until cells reached the stationary phase, followed by 50 days of semi-continuous cultivation mode with a dilution rate of 0.25 d^{-1}. Cell growth was followed on a dry weight basis. Aliquots of the cell suspension were filtered through pre-weighted paper filters (retention 0.5 μm; Macherey-Nagel) and washed twice with the same volume of distilled water for dry weight determination. Filters were reweighted after drying at 80°C until constant weight was reached. Biomass productivity was determined as the product of the biomass concentration and the dilution rate. Specific growth rates were estimated from the linear coefficients of the equations describing the exponential portion of the logarithmic growth curves. Daily growth rates during semi-continuous harvesting were estimated in the same way but considering the interval between harvests. The maximum quantum yield of photosystem II (F_v/F_m) was measured using an AquaPen-C PAM fluorometer (Photon Systems Instruments, Czech Republic) in samples dark adapted for 15 min.

2.2. Harvesting methods and medium recycling

Four harvesting methods and medium recycling treatments were carried out during the semi-continuous cultivation (Fig. 1), as follows:

- Control - the harvested volume was completely replaced by fresh growth medium. Biomass for analyses was separated through dead-end filtration (pore size 8–10 μm; Filterlab) coupled to a vacuum pump (2 mbar; Vacuubrand).
- Filtration 0.22 μm - cells were separated through dead-end filtration using a cellulose microfilter (pore size 0.22 μm; Millipore) coupled to a vacuum pump (2 mbar; Vacuubrand) and the permeate was reinserted in the culture.
- Filtration 10 μm - cells were separated through dead-end filtration using a paper filter (pore size 8–10 μm; Filterlab) coupled to a vacuum pump (2 mbar; Vacuubrand) and the permeate was reinserted in the culture.
- Centrifugation - cells were separated via centrifugation (10 min at 9119 x g; Sigma 4–16S) and the supernatant was reinserted in the culture.

To avoid nutritional limitations, nutrients were replenished daily in all treatments based on the amount of harvested biomass, as described by Depraetere et al. [5]. To ensure comparable nutrient concentrations between treatments, nitrogen, phosphate, and bicarbonate levels were periodically measured in the recycled growth media. Cell concentrates were collected manually as a wet paste (i.e., filtration cakes or centrifugation pellets). All culture and medium handling was carried out in sterile conditions. When necessary, the harvested biomasses and cell-free media immediately were stored at −20°C in the darkness until further analysis.

2.3. Crude EPS concentration and characterization using nuclear magnetic resonance (NMR)

Crude EPS from the recycled medium were precipitated by the addition of 95 % ethanol in a ratio of 3:1 (v/v) and stored at 4 °C for 24 h. The precipitated material was centrifuged at 10,000 x g at 4°C for 10 min and the remaining solvent was evaporated in an oven at 35°C. The concentration of crude EPS was determined gravimetrically. Then, 60 mg of dried EPS samples were first prepared in 0.6 mL of deuterated methanol (CH_3OH-d_4) with 0.01 % (w/v) tetramethylsilane (TMS). These samples were sonicated for 15 min, centrifuged for 5 min (12,300 g) and transferred into oven-dried 5 mm NMR tubes. The pellets were completely redissolved in 0.6 mL of a mixture of CH_3OH-d_4 and heavy water (D_2O) (1:1) containing the sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP 0.01 %, w/v) after being submitted to the same extraction procedure. Samples were then transferred into oven-dried 5 mm NMR tubes and measured. The measurements of CH_3OH-d_4

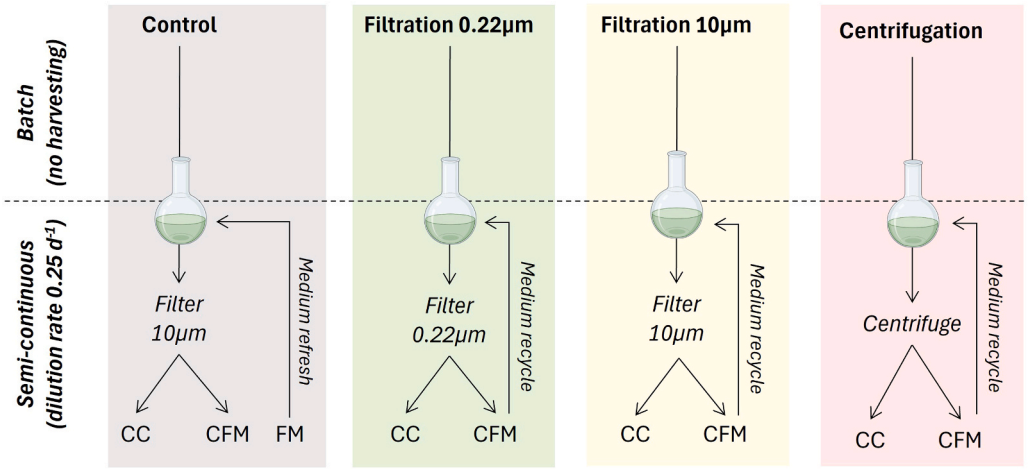

Fig. 1. Schematic representation of the cultivation process used in this study. Abbreviations: CC = cell concentrate, CFM = cell-free medium; FM = fresh medium.

and CH_3OH-d_4-D_2O extracts were taken at 300 ± 0.1 K on a Bruker Avance III 600 spectrometer (USA) operating at a proton frequency of 600.13 MHz using a 5 mm QCI quadruple resonance pulse field gradient cryoprobe. The spectrometer transmitter was locked to the CH_3OH-d_4 frequency. The pre-saturation pulse sequence (Bruker 1D noesygppr1d) was used. Acquisition and processing of spectra were carried out with TOPSPIN software (version 3.7.0). Spectra were calibrated to TMS or TSP peaks at 0.0 ppm. Acquisition parameters were the following: 32 scans, 4 dummy scans, 65536 data points, 20.0 ppm of spectral width, 2.73 s of acquisition time, 5.0 s of relaxation delay, 0.36 Hz of FID resolution and 10 ms of mixing time.

The subsequential solubilization of the pellets with CH_3OH-d_4-D_2O (1:1) generated similar spectra with the same metabolic profile (data not shown), meaning that the insolubilized material from the first extraction was not due to solubilization reduction but probably to a saturation of the solution. Therefore, only CH_3OH-d_4 spectra followed subsequent analysis. NMR CH_3OH-d_4 spectra were processed by identifying individual peaks within the δ_H 0.2–10.0 ppm range using AMIX 3.9.15 software (Bruker BioSpin GmbH, Rheinstetten, Germany). Subsequently, spectral areas corresponding to these peaks were integrated to generate data buckets. Peaks from residual H_2O suppression and methanol were excluded from the bucketing. Before conducting any statistical analyses, the intensity of individual peaks was scaled to the intensity of the TMS peak for all spectra.

2.4. Rheological analyses

The rheological characterization of recycled medium (crude EPS solutions) and cell concentrates was carried out using a Brookfield DV-II+ Pro viscometer (Brookfield, USA). According to the viscosity and volume of samples, the viscometer was equipped with one out of four cylindrical spindles (LV1, LV2, LV3, or LV4), a small sample adapter, and an ultralow viscosity adapter. The selection criteria of the available devices were described elsewhere [24]. Rheological tests were performed at a constant temperature of 25°C, controlled using a TECTRON 200 thermostatic bath (P Selecta, Spain).

The rheological data were analyzed using the Power Law model. This model describes the non-linear variation of shear stress as a function of shear rate (Eq. 1), where τ is the shear stress (Pa), γ is the shear rate (s^{-1}), K is the consistency index (Pa·s^n), and n is the behavior index of the fluid. When $n < 1$, the fluid is termed pseudoplastic and when $n > 1$, it is termed dilatant. If $n = 1$, it is a Newtonian fluid. The viscosity is defined as the ratio between the shear stress and the shear rate (Eq. 2), which is termed the apparent viscosity of the fluid, μ_a (Pa·s).

$$\tau = K \bullet \gamma^n \quad (1)$$

$$\mu_a = \frac{\tau}{\gamma} = K \bullet \gamma^{n-1} \quad (2)$$

2.5. Biomass composition

The contents of proteins and carbohydrates, the main macromolecular components of EPS, were determined in the resulting cell concentrates from different harvesting methods. Protein content was determined based on the interaction of cupric ions from the side chains of some amino acids with the Folin-Ciocalteau reagent. The reaction produced a blue-green color that was detected at 750 nm and whose intensity is proportional to the amount of proteins in the sample [25]. A calibration curve was made using bovine serum albumin as standard. Carbohydrate content was also determined spectrophotometrically, using the phenol-sulfuric method as described by Dubois et al. [26]. Briefly, carbohydrates were digested and dehydrated in sulfuric acid. The resulting compounds then reacted with phenol to produce a yellow-gold color that could be detected at 490 nm. A calibration curve was made using glucose as standard.

2.6. Data treatment and analytics

Four measurements (two biological replicates x two instrumental replicates) were performed for chemical analysis. When necessary (i.e., NMR), biomasses from each biological replicate were mixed in equal parts before analysis. Data in this study are reported as mean ± standard deviation (SD). The effects of categorical factors on results were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honest significant difference test to determine whether there were significant differences between each treatment. All statistical analyses used a confidence level of 95 % ($\alpha = 0.05$).

In NMR analysis, the processed dataset was subjected to multivariate data analysis using MetaboAnalyst 6.0. Hierarchical clustering analysis (HCA) was generated with Euclidean distance measure and Ward clustering algorithm using SIMCA-P software (v. 18.0, Umetrics, Sweden) to understand similarities between NMR profiles of crude EPS samples. A heatmap containing all the integrated NMR regions was also generated with MetaboAnalyst 6.0 to visually compare the constitutions of different samples.

3. Results and discussion

3.1. Cell growth and EPS accumulation

All growth curves presented a similar overall behavior. Batch growth

lasted 10 days to reach the stationary phase, reaching essentially the same maximal biomass concentration. After the daily semi-continuous harvesting started, the biomass concentration decreased to acclimate to the imposed dilution rate before stabilizing (Fig. 2). During batch cultivation, when physiological and operational conditions were the same for all treatments, all cultures presented a specific growth rate of 0.53 ± 0.02 d^{-1} and an average final biomass concentration of 2.33 ± 0.15 g L^{-1} (Table 1). Likewise, during the 50 days of semi-continuous cultivation mode, the biomass productivity remained unaltered among all treatments (on average, 0.24 ± 0.02 g L^{-1} d^{-1}). However, the harvesting method affected the final biomass concentration of the semi-continuous cultivation, being significantly lower ($p < 0.05$) when cultures were harvested using centrifugation. Given that the same dilution rate (0.25 d^{-1}) was employed in all treatments, different biomass concentrations ultimately indicate growth impairment. Additionally, at the end of the experiments, the photosynthetic quantum yield (F_v/F_m) was significantly lower in the centrifugation treatment ($p < 0.05$; Fig. 2). The quantum yield represents a robust indicator of whether the culture is subjected to stress conditions [27]. Given that all other parameters were the same in this study, it indicates that medium recycling through centrifugation led to physiological stress for *L. platensis*.

It has been shown that *L. platensis* growth can be severely damaged when subject to medium recycling. Other studies reported up to 90 % decrease in biomass concentration after recycling two to four times the corresponding culture volume [5,28]. However, it is noteworthy that the cultivation modes, harvesting frequencies, and dilution rates used differed from those in the present study. For example, Depraetere et al. [5] harvested 80 % of their culture every 10 days, whereas Han et al. [28] harvested 80–90 % of their culture considering a 9-day cycle. In continuous cultivation mode, Pattaro et al. [29] showed that recycling 90 % of the medium at a biomass residence time of 1.2 day did not inhibit *Limnospira maxima* growth, whereas higher recycling ratios did. By employing a higher harvesting frequency (daily) and lower dilution rate (0.25 d^{-1}), it was possible to recycle the medium more than 10 times the culture volume with milder outcomes. It is higher than generally reported in other studies using lab-scale controlled conditions in the literature. Furthermore, Han et al. [28] improved the recyclability of the growth medium by harvesting cells using ultrafiltration (UF; 5–10 kDa), keeping the algal organic matter in the retentate, which significantly altered the composition of cell concentrates. Likewise, the results showed that the thinner filtration resulted in higher biomass concentration. Still, even thinner membranes were not considered in this study because they were considered too cost- and energy-intensive for large-scale *L. platensis*. production plants [30]. Another key factor that may influence the results of medium recycling is nutrient management, which varies greatly in the literature. It is essential to replenish nutrients in a way that prevents limitation or accumulation of nutrients in the medium [15]. For example, in semi-continuous cultivation, cultures are often refreshed with a fully enriched medium, which can lead to the overaccumulation of nutrients that are not incorporated into biomass [15,28]. In this study, nutrient replenishment was carried out on demand based on the amount of harvested biomass, as suggested by Depraetere et al. [5]. Since the biomass productivity was essentially the same in all treatments, the amount of nutrients reintroduced to the growth media was also very similar. Therefore, it is highly unlikely that nutritional factors influenced the results presented here.

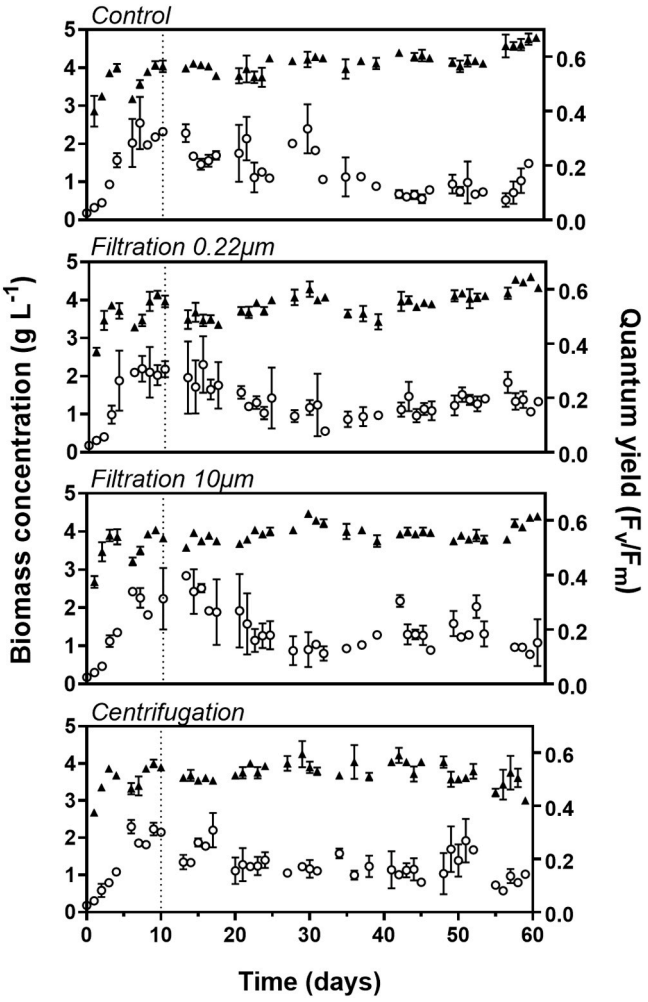

Fig. 2. Photoautotrophic growth (white circles) and maximum quantum yield of photosystem II (black triangles) of *Limnospira platensis* subject to daily semi-continuous harvesting followed by either complete medium refreshment (control), growth medium recycling using filtration (0.22 and 10 µm), or centrifugation. The data represent the means of two biologically independent replicates. Error bars show standard deviation.

It is well known that, during growth, microalgae produce and secrete into the environment a wide range of dissolved organic compounds or polymeric metabolites, usually called EPS [18]. Their composition, function, and bioactivity are still unclear, in contrast to the comprehensively studied EPS from heterotrophic bacteria [13]. The available information on microalgae EPS indicates that they can improve culture performance under certain conditions [14,15]. However, their overaccumulation is often considered the cause of physiological stress during prolonged medium recycling [5,29]. Herein, EPS were mainly accumulated in the recycled medium during the semi-continuous growth, and crude EPS of all treatments were at least four-fold higher than those observed at the end of the batch (Fig. 3). At the end of the semi-continuous cultivation mode, the accumulation of crude EPS in the recycled medium was significantly affected by the harvesting method. The 10-µm filtration and centrifugation treatments presented the highest crude EPS values, 50 % higher than that of the control group. On average, the 0.22 µm filtration treatment had intermediate crude EPS concentrations between the control and the other two treatments. However, this trend was not statistically substantiated ($p > 0.05$). Interestingly, the treatment with the highest accumulation of crude EPS in the supernatant (i.e., centrifugation) had a significant negative impact on the growth rate and biomass yield during semi-continuous cultivation (Table 1).

These findings are in line with the hypothesis that growth impairment is caused by EPS accumulation. However, it should be noted that no experiments were conducted in this study to directly test this hypothesis. This causality has been elegantly demonstrated previously in *L. platensis* cultures by inoculating fresh cells in recycled medium and vice versa [5]. Here, we focused on the effect of different harvesting methods on growth impairment potentially caused by EPS. Zhou et al. [13] suggested two key factors contributing to this impairment: (i) EPS accumulation in microalgae hinders efficient CO_2 and O_2 mass transfer

Table 1
Growth parameters of *Limnospira platensis* during batch cultivation and during daily semi-continuous harvesting followed by either complete medium refreshment (control), growth medium recycling after filtration (0.22 and 10 μm), or centrifugation.

Treatments	Batch			Semi-continuous		
	Specific growth rate (d^{-1})	Biomass productivity (g L^{-1} d^{-1})	Max. biomass concentration (g L^{-1})	Specific growth rate (d^{-1})	Biomass productivity (g L^{-1} d^{-1})	Final biomass concentration (g L^{-1})
Control	0.55 ± 0.03	0.25 ± 0.10	2.54 ± 0.69	0.55 ± 0.28^{a}	0.25 ± 0.13	1.24 ± 0.76^{a,b}
Filtration 0.22 μm	0.51 ± 0.05	0.22 ± 0.08	2.20 ± 0.34	0.41 ± 0.16^{a,b}	0.22 ± 0.12	1.39 ± 0.28^{a}
Filtration 10 μm	0.52 ± 0.01	0.23 ± 0.04	2.26 ± 0.26	0.43 ± 0.21^{a,b}	0.25 ± 0.13	1.15 ± 0.43^{a,b}
Centrifuge	0.52 ± 0.01	0.23 ± 0.01	2.30 ± 0.18	0.37 ± 0.16^{b}	0.23 ± 0.13	0.82 ± 0.18^{b}

Data represent means and standard deviation (± SD) of two biological replicates. Different superscript letters on the same column represent statistically significant differences (one-way ANOVA, $p < 0.05$).

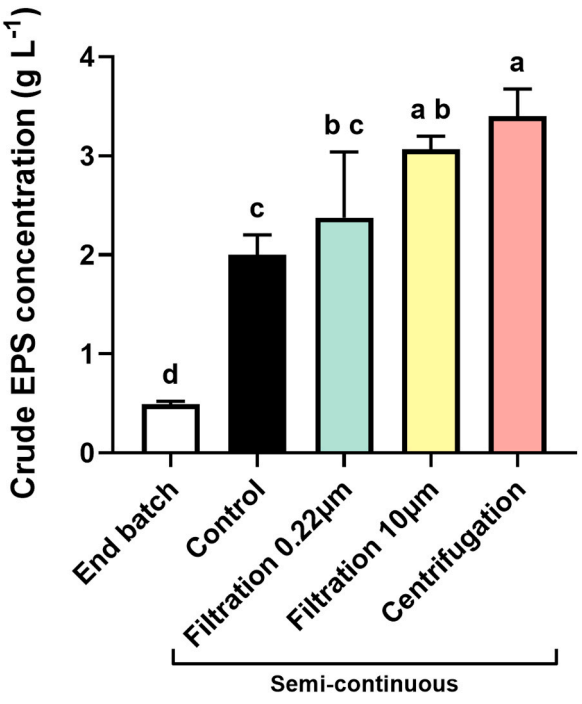

Fig. 3. Crude EPS concentration on the cell-free growth media of harvested *Limnospira platensis* cultures at the end of batch growth (white bar), or at the end of semi-continuous growth where harvest was carried out either by complete medium refreshment (control; black bar), growth medium recycling using 0.22 μm filtration (green bar), 10 μm filtration (yellow bar), or centrifugation (red bar). The data represent the means of two biologically independent replicates. Error bars show the standard deviation.

due to the lower diffusion coefficient of these gases in EPS compared to free water; and (ii) a thick EPS layer on the microalgae cell surface might reduce light transmission, resulting in lower intracellular light intensity, which restricts photosynthesis and biomass production. Unfortunately, the information available in the literature is too quantitatively and methodologically heterogeneous to allow a comprehensive assessment. For example, while de Jesus et al. [17] isolated around 10 g L^{-1} of EPS in *L. platensis* batch cultures using ethanol precipitation followed by dialysis, Depraetere et al. [5] measured 0.25 g L^{-1} of total sugars in semi-batch *L. platensis* cultures using the phenol-sulfuric method. Interestingly, only the latter reported microalgal growth impairment. Unlike what was reported by de Jesus et al. [17], ethanol precipitation was not followed by dialysis to remove ions and salt in this study. However, the lower values compared to those reported by the aforementioned authors, as well as the alignment with values found in the literature, suggest that the salts had minimal interference with the gravimetric results. Such interference should be even less significant in the NMR spectra, as they can reliably distinguish between organic and inorganic components. Nevertheless, it is important to consider the presence of salts and the potential for different carryover between different harvesting methods when interpreting our data. A wide array of factors may affect the production and effects of EPS from microalgae, such as the physicochemical cultivation conditions, the harvesting frequency and method, the presence of contaminants, and the growth phase [24]. Therefore, the available information is somewhat confusing.

3.2. *Effects of harvesting methods and medium recycling on the rheology and biochemical composition of cell concentrates*

In an industrial *L. platensis* production facility, the biochemical composition of cell concentrates must be monitored because they are generally the products of interest. Ideally, the composition of the main macromolecular pools (i.e., proteins, carbohydrates, and lipids) should be maintained in an optimal range for the biomass application [10]. An influence of the harvesting method on the biochemical composition of cell concentrates was observed. Depending on the harvesting method, the protein and carbohydrate concentrations in *L. platensis* biomass varied from 32.7 to 52.2 % and 15.7 – 29.0 %, respectively. These results are consistent with those reported in the literature for different *L. platensis* strains and cultivation conditions [23]. The highest concentrations of both macromolecules were observed in cell concentrates harvested by 0.22 μm filtration, whereas the lowest concentrations of proteins and carbohydrates were found in cells harvested by centrifugation and 10 μm filtration, respectively (Table 2). Since carbohydrates are presumably the major components of EPS, the carbohydrate content of the different treatments indicates that EPS is preferentially retained in the 0.22 μm filter cake and not in the 10 μm filter cake or in the centrifuge pellet. Interestingly, the carbohydrate contents of cells harvested by 10 μm filtration and centrifugation were similar to that of the control group. This highlights the complex effects that shear rates and cake formation of different harvesting methods have on the harvested biomass. The heterogeneity of EPS is also important in the interpretation of these results. For example, Han et al. [28] showed that the removal of algal organic matter from recycled media was not directly proportional to the filter pore size.

Table 2
Biochemical composition of *Limnospira platensis* biomass grown semi-continuously and harvested with complete medium refreshment (control), medium recycling using filtration (0.22 and 10 μm), or centrifugation.

Treatments	Proteins (%)	Carbohydrates (%)
Control	45.7 ± 0.50^{b}	17.7 ± 0.34^{b}
Filtration 0.22 μm	52.2 ± 2.23^{a}	29.0 ± 0.22^{a}
Filtration 10 μm	47.3 ± 0.50^{b}	15.7 ± 0.20^{c}
Centrifugation	32.7 ± 1.70^{c}	16.9 ± 0.47^{b}

Data represent means and standard deviation (± SD) of two biological replicates. Different superscript letters on the same column represent statistically significant differences (one-way ANOVA, $p < 0.05$).

The rheological behavior of cell concentrates was also affected by the harvesting methods used. The rheological data showed a pseudoplastic behavior ($n < 1$) for cell concentrates from all methods (Fig. 4; Table 3). Comparison between harvesting methods revealed a substantially higher apparent viscosity on cell concentrates obtained using centrifugation, whereas those obtained from 0.22 μm filtration showed the lowest. Likewise, n was higher in the centrifugation. All rheological parameters analyzed (μ, K, and n) were higher in cell concentrates obtained using centrifugation, whereas the lowest values were found in the control or 0.22 μm filtration as depicted by the distribution of values in Fig. 4 (Table 3). The differences found in the rheological parameters of cell concentrates were statistically significant ($p < 0.05$). Notably, the cell concentrate obtained from centrifugation showed the most notable differences compared to all other groups, which aligns with its much higher viscosity values.

Notably, centrifugation separates cells from liquid based on their density under the influence of increased gravitational force, allowing solubilized compounds (e.g., EPS) to remain in solution. In contrast, filtration separates cells based on size by exerting pressure toward a filter of known pore size, favoring the accumulation of EPS in the resulting cake [31]. Given the results obtained here, the diverse separation methods used to harvest microalgae seem to allocate EPS differently in the cell concentrates and cell-free growth medium, leading to different outcomes. For example, EPS preferentially left in recycled medium from centrifugation led to growth impairment when recycled, whereas EPS accumulated in cell concentrates from 0.22 μm filtration led to more drastic changes in biomass composition. Similarly, Belachqer El-Attar et al. [24] observed that the presence of heterotrophic bacteria also affected the partitioning between free- and adhered-EPS. Those findings are extremely relevant for algal biotechnology since the energy consumption of downstream processing can increase by up to 60 % due to the rheological behavior of cell concentrates [16].

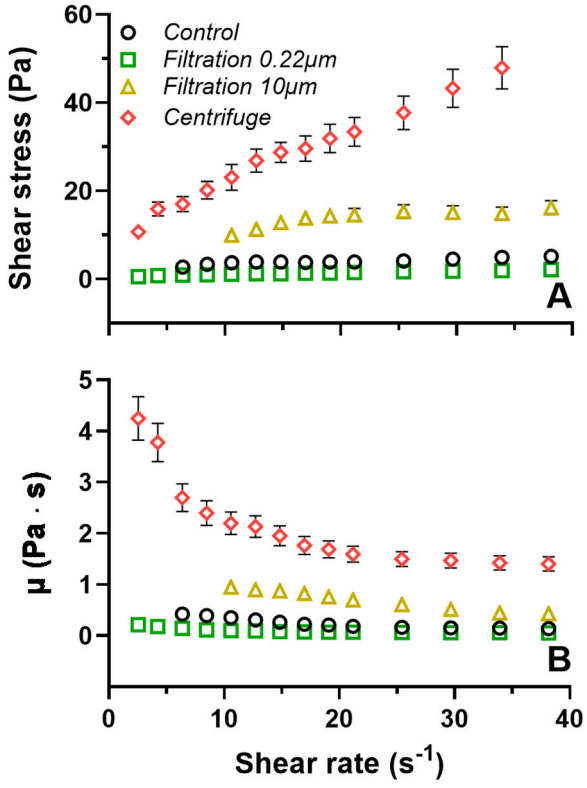

Fig. 4. (A) Shear stress and (B) apparent viscosities as a function of sheer rate for cell concentrates of *Limnospira platensis* grown semi-continuously and harvested with complete medium refreshment (control; black circles), complete medium recycling using 0.22 μm filtration (green squares), 10 μm filtration (yellow triangles), or centrifugation (red diamonds). The data represent the means of two biologically independent replicates.

Table 3
Apparent viscosities (μ), consistency index (K), and behavior index (n) from cell-free growth media and cell concentrates of *Limnospira platensis* grown semi-continuously and harvested with complete medium refreshment, complete medium recycling using 0.22 or 10 μm filtration, or centrifugation.

	Treatments	Apparent viscosity, μ (Pa·s)*	Power Law	
			K (Pa·s^n)	n
Cell-free growth medium	Control	1.35 ± 0.13	(1.34 ± 0.04)·10^{-4}	1.50 ± 0.01
	Filtration 0.22 μm	1.16 ± 0.03	(0.85 ± 1.96)·10^{-4}	1.59 ± 0.05
	Filtration 10 μm	1.41 ± 0.14	(1.70 ± 0.04)·10^{-4}	1.47 ± 0.01
	Centrifuge	1.20 ± 0.08	(0.96 ± 0.13)·10^{-4}	1.57 ± 0.02
Cell concentrate	Control	306 ± 0.03^c	1.55 ± 0.22^c	0.31 ± 0.04^c
	Filtration 0.22 μm	90.5 ± 0.10^d	0.36 ± 0.04^d	0.46 ± 0.05^b
	Filtration 10 μm	900 ± 0.89^b	5.17 ± 0.33^b	0.32 ± 0.03^c
	Centrifuge	2130 ± 0.20^a	6.50 ± 0.00^a	0.55 ± 0.01^a

Data represent means and standard deviation (±SD) of two biological replicates. Significant effect of harvesting method over the parameter was observed only on cell concentrates, where different superscript letters indicate significant differences (one-way ANOVA, $p < 0.05$).

* Apparent viscosity is presented as the value achieved using 60 rpm in rheological analysis.

3.3. *Effects of harvesting methods and medium recycling on the composition and rheology of recycled medium (EPS solutions)*

From a biotechnological perspective, the presence of EPS on the recycled medium of *L. platensis* makes them interesting sources of alternative products, like bioflocculants or biolubricants depending on their characteristics [13]. However, in contrast to the cell concentrates, the EPS solutions analyzed showed a Newtonian behaviour, with no relevant differences observed as a function of the harvesting method or medium recycling (Fig. 5; Table 3). Likewise, mild variations in composition were found in crude EPS extracted from the recycled media.

A typical NMR spectra obtained from the CH_3OH-d_4 extract of a control EPS sample is presented in the Supplementary material. The other samples, obtained after 0.22 and 10 μm filtration or centrifuge, were similar except for some metabolic differences. The analysis of the NMR spectra showed the following very intense broad signals that may derive from proteins and also hydrocarbons: at δ_H 0.88 ppm, there was a triplet from terminal CH_3 from hydrocarbons; at δ_H 1.20 – 1.50 ppm, there was a high intense peak from CH_2 and CH_3 from proteins and hydrocarbons [32]; at δ_H 1.88 ppm, there was acetyl moieties; and at the aromatic region at δ_H 7.55 ppm, there was a broad peak probably generated from phenolic moieties of proteins. Other metabolites include trimethylamine (δ_H 3.01 ppm), glycerol (δ_H 3.51 and 3.58 ppm), fatty acids chains (δ_H 0.88, 1.27, 1.60, 2.14, and 2.31 ppm), acetic acid (δ_H 1.88 ppm), formic acid (δ_H 8.55 ppm) and 3-hydroxybutyrate (δ_H 1.20, 2.33, 2.54 and 4.06 ppm). The latter was only found in the control EPS solution (see supplementary material).

The complexity of these macromolecules hindered their adequate analysis. Metabolomics techniques were applied to compare the spectra obtained from crude EPS obtained from the four treatments and the

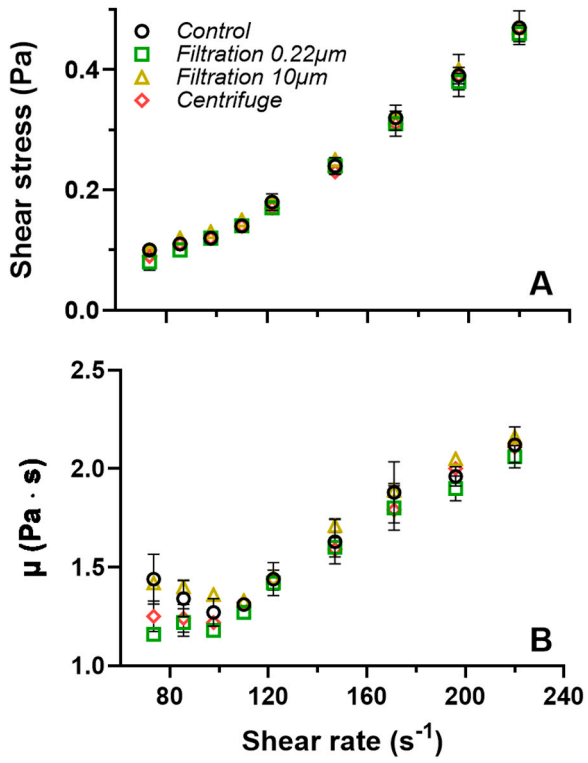

Fig. 5. (A) Shear stress and (B) apparent viscosities as a function of shear rate for crude EPS from cell-free growth media of *Limnospira platensis* grown semi-continuously and harvested with complete medium refreshment (control; black circles), complete medium recycling using 0.22 μm filtration (green squares), 10 μm filtration (yellow triangles), or centrifugation (red diamonds). The data represent the means of two biologically independent replicates.

intensity and appearance of new signals and correlate these results with biological parameters previously discussed. Firstly, an HCA of ^{1}H NMR data was generated with the EPS extracted from supernatants of all treatments (Fig. 6A). The results allow to conclude that 0.22 μm filtration and centrifugation treatments produced the most similar EPS metabolic profiles and the one generated from 10 μm filtration was the most different. The EPS from the control had some unique signals, such as 3-hydroxybutyrate and other non-assigned signals (see supplementary material). Further analysis of the heatmap (Fig. 6B) indicates that the broad signals from macromolecules were more intense for the EPS from 0.22 μm filtration, suggesting a higher content, and less intense for the EPS from 10 μm filtration. The low-intense anomeric signals from sugars suggest a decrease in the number of these compounds in EPS extracted from the centrifugation supernatant. Yuan et al. [33] found that medium recycling promoted bacterial contamination in mass cultures (700 m^2 raceway ponds). This indicates that the differences found here could be from bacterial consumption, which could be inhibited by their retention in 0.22 μm filters. However, in contrast to those authors, the lab-scale operations in this study were carefully carried out under sterile conditions and frequently monitored for bacterial contamination. Therefore, the results reported here are likely a consequence of the physical and structural properties of EPS in the recycled medium. This is one example of the necessary careful interpretation of lab-scale results when compared to industrial-scale scenarios.

From a chemical point of view, EPS are mainly composed of polysaccharides, proteoglycans, glycoproteins, or other types of biopolymers [34]. The chemical composition, the type, and the amount of the EPS produced by a given cyanobacterial strain depend on the species and the cultivation conditions. The variability of monosaccharides in EPS, the carbohydrates/proteins ratio, molecular weight variability, and diversity of monosaccharide linkage types lead to a wide range of possible structures and architecture of these complex macromolecules [35]. Surprisingly, in the spectra obtained in this study, only limited signals arising from carbohydrates were found. These were mostly characterized by their anomeric H1 signals at δ_H 5.34 and 4.14 ppm. Usually, carbohydrates are a very important fraction of EPS. Indeed, EPS chemical composition produced by 25 cyanobacterial strains was characterized by Halaj et al. [36] to have a range of carbohydrate and protein contents of 9–67 % and 0–37.8 %, respectively. The authors identified most of these macromolecules as glycoconjugates (i.e., glycoproteins or proteoglycans).

3.4. Implications and challenges to the microalgae biotechnology sector

The rapid ascent of microalgal biotechnology as a clean and sustainable alternative for numerous industrial sectors underlined that some aspects of microalgal biomass production still need to be better understood. For example, the optimal medium recycling potential is still unclear, as the available information is too variable [37]. Also, little is known about the production and secretion of microalgal EPS and their physiological and operational outcomes, especially when compared to heterotrophic bacteria [13]. Many authors have been focusing on pre-treatments such as foam fractioning, ultrafiltration, and UV treatment to increase medium recyclability. Still, it involves adding another step to the downstream processing, and the question about the influence of the harvesting method remains. In this study, a holistic investigation regarding (i) microalga growth and physiology, (ii) rheology and composition of cell concentrates, and (iii) rheology and composition of EPS from recycled media was carried out. Ultimately, the results indicated that, in the cultivation conditions described, long-term harvesting using centrifugation negatively impacts growth and increases the viscosity of cell concentrates faster than the other methods, potentially hindering downstream processing. Still, it cannot be ruled out that even longer recycling would also lead to growth impairment in the 10 and 0.22 μm filtration treatments. Pelletization of centrifuged samples visibly deteriorated over time during the experiment (not objectively measured). Such a negative effect of prolonged medium recycling on harvesting efficiency over time has been recently reported in a commercial *L. platensis* plant [23]. Although several factors must be considered by a *L. platensis* producer when choosing a harvesting method (e.g., cost, energy consumption, etc. [30]), the results reported in this study highlight the importance of considering the effects on growth and biomass.

The biotechnological exploitation of microalgal EPS from recycled medium and/or cell concentrates remains a promising alternative. However, extracting EPS adhered to the biomass would require adding another unit operation to the process, which would eventually increase costs. Additionally, the potential market price of EPS as a co-product remains unknown. The extraction methods for cyanobacterial EPS and their potential as future co-products to enhance the biorefinery framework are still research challenges. It is noteworthy that EPS are secondary metabolites predominantly produced under stress conditions, which are more evident in larger-scale outdoor facilities. This study used laboratory conditions to replicate the conditions of commercial production of *L. platensis*. Therefore, the results should not be directly applied to large-scale production systems, especially from a technoeconomic perspective. For instance, the capital and energy requirements for microalgae production decrease as the scale of production increases because industrial harvesting and downstream machinery are more energy- and cost-efficient than laboratory instruments [38]. Therefore, it is suggested that future studies should perform similar analyses under larger-scale outdoor conditions.

4. Conclusion

The harvesting methods investigated affected virtually all analyzed parameters and, to a milder extent, the composition of EPS isolated from

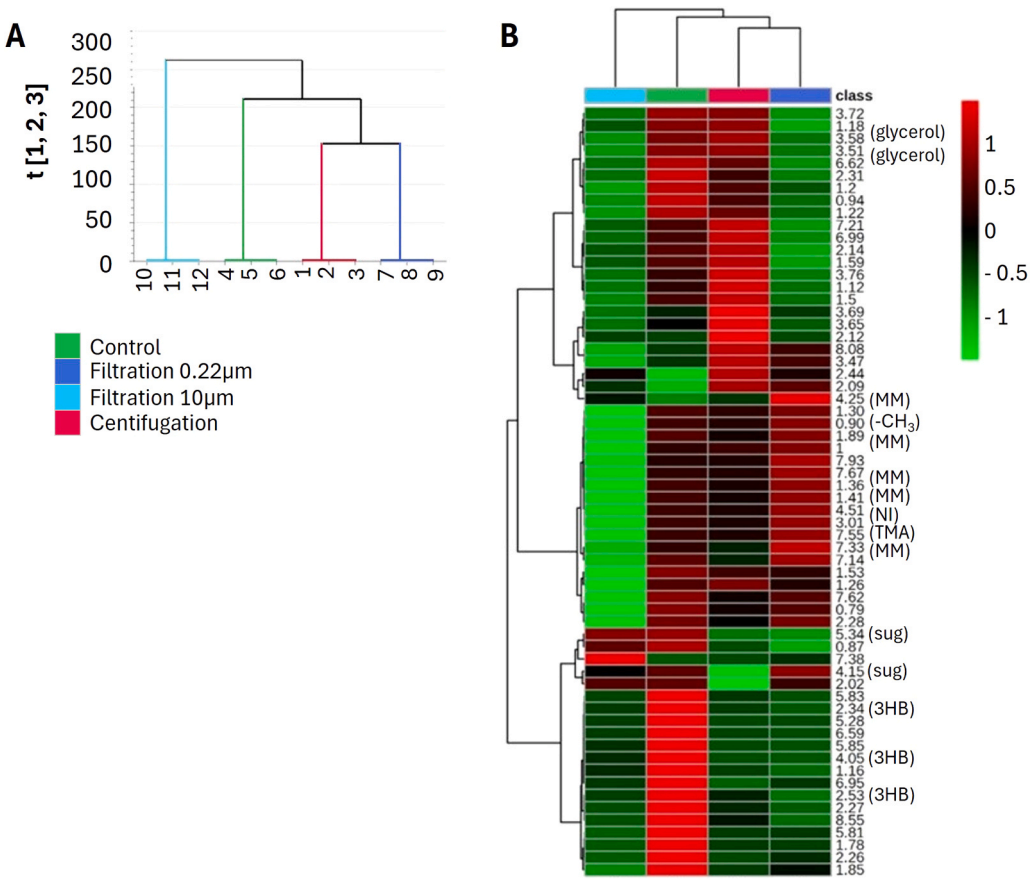

Fig. 6. (A) HCA built from ^{1}H NMR bucketed data of the four EPS types. Euclidean Distance Measure and Ward Clustering Algorithm. (B) Heatmap built with ^{1}H NMR data of EPS samples. Abbreviations: MM = macromolecule; NI = non-identified; TMA = trimethylamine; sug = sugars; 3HB = 3-hydroxybutyrate.

recycled media. Microalgal EPS composition determined using NMR showed the most intense peaks arising from macromolecules such as proteins and hydrocarbons and a minor content of carbohydrates. Harvesting with centrifugation led to the most negative outcomes on culture performance and apparent viscosity of cell concentrates, which result in negative economic impacts in an *L. platensis* production plant. The results suggest that *L. platensis* production must consider strategies to improve medium recyclability and achieve long-term operation stability.

CRediT authorship contribution statement

Andrea Schievano: Writing – review & editing, Resources, Funding acquisition. **Solaima Belachqer-El Attar:** Writing – review & editing, Methodology, Formal analysis. **Luiggi Pessôa:** Writing – review & editing, Methodology, Formal analysis. **Martina Ciardi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Daniel Kurpan:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Ignacio Fernández:** Writing – review & editing, Methodology, Formal analysis. **Ana Cristina Abreu:** Writing – review & editing, Methodology, Formal analysis. **Gabriel Acién:** Supervision, Resources, Funding acquisition. **Antonio Idà:** Writing – review & editing, Supervision, Resources, Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.119570.

Data availability

Data will be made available on request.

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