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Impact of harvesting via flocculation on protein yield from *Chlorella vulgaris* following pulsed electric field and high-pressure homogenization treatments

Viviane de Carvalho Arabidian [a], Alexander S.K. Müller [b], Luiz Antonio de Almeida Pinto [a], Débora Pez Jaeschke [a,*], Tito Roberto Sant'Anna Cadaval Jr. [a], Wolfgang Frey [b], Christian Gusbeth [b]

[a] School of Chemistry and Food, Federal University of Rio Grande – FURG, Italia Avenue 8 km, 96203–900, Rio Grande, RS, Brazil
[b] Karlsruhe Institute of Technology, Institute for Pulse Power and Microwave Technology (IHM), Karlsruhe, Germany

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ABSTRACT

The growing interest in sustainable biomass sources has encouraged research on microalgae, particularly focusing on improving the efficiency of cell separation and extraction processes. Currently, the harvesting step is primarily performed by centrifugation, which requires high energy consumption, especially for large-scale cultivation volumes. Therefore, this study aimed to explore coagulation/flocculation as an alternative harvesting method for *Chlorella vulgaris*, using $FeCl_2$, $FeCl_3$, and chitosan. Additionally, this study aimed to evaluate the effect of the different flocculants on subsequent protein extraction, comparing extraction yields obtained by pulsed electric field (PEF) and high-pressure homogenization (HPH) treatment for the first time. The results indicated that $FeCl_3$ had the highest flocculation efficiency, reaching over 99% with a lower dosage than the other flocculants (200 mg L^{-1}). Although $FeCl_3$ achieved the highest flocculation efficiency (over 99% at a low dosage of 200 mg·L^{-1}), the subsequent protein extraction yields were lower compared to centrifuged biomass. Protein recovery from $FeCl_3$-flocculated biomass reached only 9.22% of cell dry weight (CDW) with HPH and 0.5% of CDW with PEF, whereas centrifugation resulted in higher yields of 49.4% and 19.5% of CDW, respectively. These results indicate that while floc formation is effective for biomass harvesting, it may hinder protein extraction and reduce biomolecule recovery. Additionally, the protein profile analyzed by SDS-PAGE confirmed the negative impact of using flocculants, showing a disappearance or reduction of bands.

1. Introduction

As the population grows and the demand for food and energy rises, seeking renewable and alternative food and energy sources becomes essential [1]. In this context, microalgae can be utilized in producing food, biofuels, cosmetics, and pharmaceuticals, as these microorganisms synthesize various compounds, including lipids, proteins, carbohydrates, and bioactive compounds [2,3]. Moreover, compared to terrestrial plants, these microorganisms require less land and water for cultivation, and their growth rate is significantly higher than that of terrestrial plants [4,5].

However, the industrial commercialization of microalgae products remains limited due to high production costs. Harvesting is one of the bottleneck operations because of low biomass concentration at the end of cultivation (typically up to 1 g L^{-1}) and due to the cell characteristics, such as small size and charged surface [6]. The commonly used methods for microalgae separation include centrifugation, coagulation/flocculation, flotation, and filtration. The choice of method depends on the characteristics of the microalgae cells and the intended use of the biomass or other final products. Centrifugation is known for its high separation efficiency, which is achieved in short periods, allowing the separation of small cells (<30 μm). However, the main limitation is the high energy consumption and the challenges of scaling up. Filtration can be easily scaled up but faces the issues of low flux and membrane fouling, which require extended operational time [7,8].

Coagulation/flocculation is an alternative method that has lower

* Corresponding author.
E-mail addresses: viarabidian@hotmail.com (V.C. Arabidian), alexander.s.mueller@web.de (A.S.K. Müller), dqmpinto@furg.br (L.A.A. Pinto), deborapj@furg.br (D.P. Jaeschke), titoeq@gmail.com (T.R.S. Cadaval), wolfgang.frey@kit.edu (W. Frey), christian.gusbeth@kit.edu (C. Gusbeth).

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operational costs and allows scalability. Coagulation facilitates the destabilization of particles in suspension or solution, while flocculation occurs when these particles aggregate to form flocs [9,10]. However, the main issue with this method is the addition of chemical compounds that remain in the biomass after separation from the cultivation medium, and the presence of the flocculant can contaminate the biomass, affect its color and degrade its quality. Therefore, it is important to develop materials and processes that achieve high separation efficiency while maintaining biomass quality for the industrial application of microalgae-based products [8].

In this context, various materials have been utilized for microalgae flocculation, including inorganic compounds (metal salts, such as $Fe_2(SO_4)_3$, $FeCl_3$, $Al_2(SO_4)_3$) and inorganic and organic polymers (polyaluminum chloride, chitosan, and cellulose) [11–13]. Chitosan, for instance, is a natural biopolymer derived from chitin that has been widely explored for wastewater treatment from several sources such as dairy industry [14], textile industry [15,16], fishery industry [17], oil refineries [18,19], removal of microplastics [20,21], and, more recently, began to be investigated as a flocculant for microalgae harvesting [22,23] due to its abundant positively charged amino groups, which facilitate charge neutralization and bridging interactions with negatively charged algal cell surfaces, promoting floc formation. In addition, chitosan is biodegradable, non-toxic, and derived from renewable waste streams, making it especially suitable for applications where downstream product quality is critical [12,24,25].

More recently, inorganic metal salts such as $FeCl_3$ and $FeCl_2$ have been applied to microalgal biomass harvesting [23,26], achieving flocculation efficiencies often exceeding 90%. These coagulants are already well recognized and widely established for drinking water treatment in several countries [27,28]. They primarily neutralize algal surface charge through cation interactions, enabling efficient recovery across diverse microalgal species. Ferric salts have demonstrated robust flocculation under a wide range of operational conditions and can significantly reduce harvesting costs compared with non-flocculated solid–liquid separation methods [9,22]. Moreover, compared with chitosan, these flocculants are considerably cheaper. Industrial-grade chitosan typically costs 20–40 USD per kg, whereas ferric chloride costs approximately 2.4 USD per liter [29,30].

Although many flocculants result in high separation efficiency, they may compromise cell structure and modify the cell composition. However, few studies address this aspect, as most concentrate on separation efficiency. Furthermore, even less is understood about the impact of flocculant presence on downstream processing and the extraction of target compounds [7,31]. For example, Imbimbo et al. [32] evaluated different flocculants across multiple microalgae strains and cyanobacterium, demonstrating that flocculation can be an effective alternative to centrifugation, but the downstream assessment focused mainly on carotenoid extraction, concluding that flocculation did not negatively affect carotenoid yields when wet biomass.

In contrast, Labeeuw et al. [31] investigated the performance of synthetic polyacrylamides and chitosan across multiple microalgae species, focusing on flocculation efficiency and biomass integrity. Their study demonstrated that certain flocculants may compromise cell integrity, potentially leading to the release of intracellular compounds into the surrounding medium. However, the analysis was restricted to harvesting performance and medium recyclability, without extending the assessment to downstream processing. In a study conducted by Hidayah et al. [33], *C. vulgaris* was harvested using chitosan dissolved in 1% acetic acid. Compared to centrifugation, which resulted in a lipid content of approximately 50%, flocculation yielded a significantly lower amount, around 22%. Unlike the present study, the focus was not on protein extraction, and cell permeabilization techniques using HPH and PEF were not applied.

For the extraction of biocompounds, high-pressure homogenization (HPH) is widely used as a benchmark method for cell rupture of microalgae, employing pressures ranging from 20 to 120 MPa. This process promotes turbulence and shear stresses, resulting in the rupture of the cell wall and the release of intracellular compounds [34,35]. Besides HPH, different methods have been studied to disrupt cell membranes, including ultrasound [36], and electrical technologies, such as moderate electric field and pulsed electric fields (PEF) treatments [37]. PEF enables the formation of pores on the cell membrane, allowing the extraction of intracellular compounds, such as proteins [38–40], lipids [41], and pigments [34]. Owing to its low thermal impact and potential for selective permeabilization, PEF has been widely recognized as a promising technology for microalgae processing. Its industrial application, however, remains limited due to challenges related to process integration. In particular, PEF efficiency is strongly influenced by medium conductivity, often requiring low-conductivity environments to avoid excessive energy dissipation, as well as by biomass characteristics that can lead to variable extraction yields and incomplete permeabilization, especially for species with robust cell walls or aggregated structures. In addition, large-scale implementation requires optimized high-voltage systems and treatment chambers capable of handling dense microalgal suspensions [42–44]. Addressing these operational and integration challenges is therefore essential to fully exploit the potential of PEF in microalgal biorefineries.

To date, most studies have concentrated on cell disruption and the efficiency of extraction methods such as HPH and PEF, with limited investigation into the effects of flocculation on the composition and recovery of extracted biocompounds. Furthermore, no studies to date have applied PEF to coagulated microalgae. Therefore, this study aims to evaluate the flocculation efficiency of $FeCl_2$, $FeCl_3$, and chitosan for harvesting *Chlorella vulgaris*, as well as to assess the impact of these flocculants on protein extraction, using HPH as a reference method for comparison with PEF.

2. Materials and methods

2.1. Cultivation of microalgae

Chlorella vulgaris strain 211–12 was obtained from SAG (Sammlung von Algenkulturen Göttingen, Germany). It was cultivated in a modified Tris-Acetate-Phosphate medium [45], which contained: 1× TAP salts, Tris Base (0.02 M), phosphate buffer (0.001 M), 1× Hutner's trace elements, acetic acid (0.001%). The pH was adjusted to 7 ± 0.2 with chlorhydric acid. The cultures were inoculated for 7 d in Erlenmeyer flasks (400 mL of medium and ≅ 22 mL of a previous inoculum) under constant agitation (150 rpm) in a thermostatic cabinet under constant exposure to light (23 ± 1 °C, 90 μmol m^2 s^{-1}). The initial optical density (OD) at 750 nm of the cultures was 0.1 ± 0.05. When the cells had reached the stationary phase, with a cell dry weight ≅ 0.8 g L^{-1}, the flocculation experiments were performed.

2.2. Flocculants

Iron (II) chloride (Merck, Germany), iron (III) chloride (Merck, Germany), and chitosan (Roth, Germany) were used as flocculants. The iron salts were dissolved in distilled water and chitosan was dissolved in acetic acid 5% (*v*/v) to produce a stock solution with a concentration of 10 g L^{-1}.

2.3. Chlorella vulgaris harvest: Flocculation and centrifugation experiments

The initial experiments were carried out to determine the best flocculant dosage. For this purpose, 400 mL of microalgae cultivated for 7 d were used, at an initial pH of 8.5 ± 0.1, which remained stable throughout cultivation. This ensured that any effects on later protein extraction were due solely to the flocculants, without interference from pH changes.

The flocculants were added at different dosages (from 0.1 to 0.5 g

L^{-1}) and the suspensions remained under agitation in an overhead stirrer (IKA Eurostar 20 Digital). The agitation was carried out in two distinct stages: the first consisted of rapid agitation at 200 rpm for 1 min, followed by slow agitation at 50 rpm for 15 min. After agitation, the mixture of microalgae and flocculated microalgae was left to rest in a graduated cylinder at ambient room conditions (23 ± 2 °C). At each time interval (30, 60, 90 min, and 24 h), aliquots of the supernatant were removed to evaluate the optical density, and to determine the flocculation efficiency. The flocculant dosage corresponding to the highest efficiency at the optimal time interval was selected for subsequent protein extraction experiments. The pH of these samples was measured prior to protein extraction. No pH adjustment was performed at any stage of the flocculation or extraction experiments, as the objective was to evaluate the direct and impact of flocculant addition on downstream protein extraction. Centrifugation (10,000 ×*g*, 10 min, 23 ± 1 °C) of microalgae suspension was used as the control for the flocculation process.

2.3.1. Flocculation efficiency

The flocculation efficiency (η) was determined based on optical density measurements of the supernatant, taken before (OD_i) and after (OD_f) the flocculation experiments, using a UV–Vis spectrophotometer (Thermo Scientific - Genesys 10 UV) at 750 nm, according to Eq. 1.

$$\eta = \frac{OD_i - OD_f}{OD_i} \times 100 \quad (1)$$

2.3.2. Determination of the cell dry weight

The dry weight of the cells was measured after flocculation and centrifugation, as this value was required to determine the protein content on a dry biomass basis. Approximately 2.5 mL of each sample was transferred to aluminum caps and dried in an oven at 80 °C for 24 h. The CDW was calculated based on the mass difference before and after drying, using an analytical balance. CDW values were expressed in g L^{-1} of suspension. For flocculated samples, CDW values were corrected using Eq. 2, which accounted for the gravimetrically determined CDW (CDW_0), the initial suspension volume prior to flocculation (V_0), and the volume of the flocculated fraction (V_f).

$$CDW = CDW_0 \times \frac{V_0}{V_f} \quad (2)$$

2.4. Protein extraction

2.4.1. High pressure homogenization

Fig. 1 illustrates the experimental workflow, schematically depicting the sequence of PEF and HPH applications to the different biomasses. For protein extraction by HPH, 25 mL of the flocculated and centrifuged biomasses underwent 5 passes using a high-pressure homogenizer (EmulsiFlex-C3 Avestin, Canada) at pressures ranging from 1000 to 1500 bar. Upon completion of the 5th pass, the sample was collected from the device and centrifuged at 12,000 *g* for 10 min. The supernatant, containing the solubilized proteins, was carefully separated and stored under refrigeration at −20 °C for use in subsequent experiments to determine protein content, as illustrated in Fig. 1.

2.4.2. Pulsed electric field treatment

The continuous flow PEF treatment of algal suspension was performed with a home-made transmission line pulse generator which delivered rectangular pulses of 1 μs duration and pulse amplitudes of 8 kV. The suspensions were treated in a polycarbonate treatment chamber with plane-parallel stainless-steel electrodes, with a gap of 2 mm between electrodes, as previously described by Krust et al. [46]. The suspensions were pumped through the PEF treatment system using a peristaltic pump (ISMATEC ISM832C, Cole-Parmer GmbH, Germany). For PEF treatment, 120 mL of each flocculated and centrifuged (as control) samples were used. The parameters used are detailed in Table 1. The resulting specific treatment energy was 10.7 J mL^{-1} for PEF#1 and 107.5 J mL^{-1} for PEF#2.

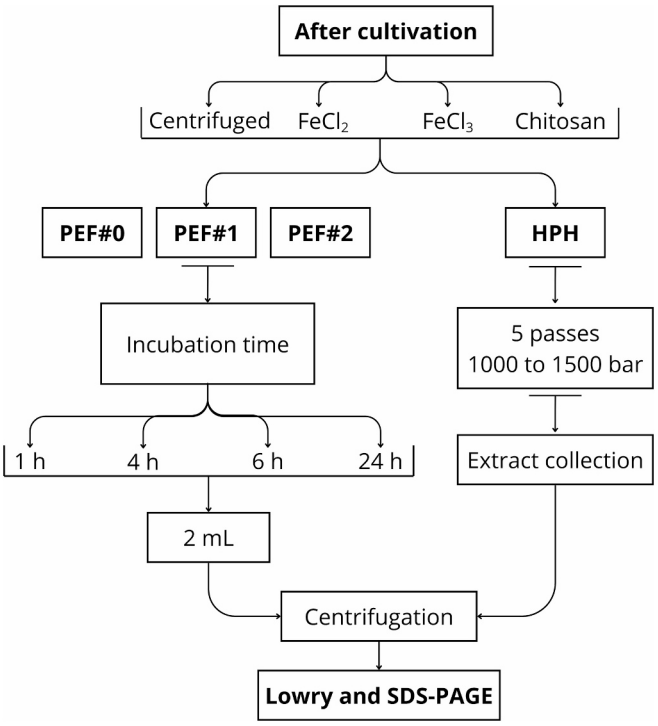

Fig. 1. Experimental flowchart illustrating the application of PEF and HPH treatment to different biomass types: centrifuged, and flocculated using $FeCl_2$, $FeCl_3$, or chitosan.

Following PEF treatments, the samples were placed in falcon tubes and allowed to rest while awaiting the incubation period, which ranged from 1 to 24 h. This incubation time was chosen based on previous studies indicating that a minimum of 2 h post-treatment enhances the diffusion of proteins, making them more accessible for subsequent quantification [39,40]. Aliquots of 2 mL were collected at incubation intervals of 1, 4, 6, and 24 h and centrifuged at 12,000 *g* for 10 min. After centrifugation, the supernatant was refrigerated and stored at −20 °C for later analysis, as illustrated in Fig. 1.

2.5. Analysis of the extracts

2.5.1. Protein content

Subsequent to the HPH and PEF treatments, the protein content in the collected extracts was assessed using the Lowry protocol. This methodology utilizes the reduction of the Follin reagent (comprising molybdate, tungstate, and phosphoric acid) when it interacts with proteins in the presence of the Cu^{2+} catalyst (DC Protein Assay, Bio-Rad Laboratories, US), producing a compound with a maximum absorption

Table 1
Parameters used for the PEF treatment of the suspension, performed using a transmission line pulse generator.

Parameters	Symbol	Unit	PEF#1	PEF#2
Electric field intensity	E	$V{\cdot}cm^{-1}$	40,000	40,000
Pulse duration	t	10^{-6} s	1	1
Suspension conductivity	σ	$S{\cdot}cm^{-1}$	0,0016	0,0016
Repetition rate	f	s^{-1}	0.40	4.00
Chamber volume	V_O	cm^3	0.524	0.524
Liquid flow	Φ	$mL{\cdot}s^{-1}$	0.050	0.050
Number of pulses	N	–	4	42
Specific energy	ΔW	$J{\cdot}mL^{-1}$	10.7	107.5

at 750 nm [47].

The analysis was performed combining 5 μL of the protein-containing supernatant from each sample with 25 μL of alkaline copper tartrate and 200 μL of the Follin reagent. The mixture was stirred for 5 s at 420 rpm, followed by a 15-min pause, after which it was stirred for an additional 5 s. Finally, the absorbance of each sample was measured at 750 nm using a spectrophotometer (Thermo Scientific - Genesys 10 UV), and the protein content was calculated based on the absorbance values in relation to a previously established standard curve with bovine serum albumin (DC™ Protein Assay, BioRad).

2.5.2. SDS-PAGE

To verify the integrity and composition of the extracted proteins, the SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) technique was employed, which was conducted using a vertical electrophoresis cell (Mini-PROTEAN Tetra, Bio-Rad Laboratories, US). This method relies on the denaturation of proteins by sodium dodecyl sulfate (SDS), which confers a consistent negative charge to the proteins. As a result, separation occurs solely based on molecular size when an electric field is applied to polyacrylamide gel. The gels were prepared following the Tris-Glycine Laemmli system [48] using a 10% polyacrylamide concentration. Before electrophoresis, the samples were treated with Tris buffer at 98 °C for 15 min. Electrophoresis proceeded in two phases: the stacking phase was carried out at a voltage of 50–60 V for 30 min, followed by the separation phase at 100–150 V for 60 min. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, US) and the protein bands were identified using a molecular marker (Precision Plus Protein Standard Dual Color, Bio-Rad Laboratories, US).

2.6. Statistical analysis

All experiments were conducted in replicate, and the results were expressed as mean ± standard deviation. Statistical analysis was performed using ANOVA to assess significant differences among treatments, followed by Tukey's test at a 95% confidence level ($p < 0.05$) using the software Statistica® (12.0, StatSoft Inc., Tulsa, USA).

3. Results and discussion

3.1. Flocculation efficiency for Chlorella vulgaris harvesting

The optimal flocculant dosage was determined to establish standardized conditions for subsequent experiments evaluating the influence of flocculants on protein extraction. The flocculation efficiencies over time for the various flocculant dosages are presented in Fig. 2. The results showed that the optimal dosages for $FeCl_2$ and chitosan were both 400 mg L^{-1}, while $FeCl_3$ achieved its highest performance at a lower dosage of 200 mg L^{-1}. Among the flocculants tested, $FeCl_3$ demonstrated the highest flocculation efficiency (99.8 ± 0.08%), compared to chitosan (76.4 ± 1.2%) and $FeCl_2$ (56.7 ± 0.7%). These findings indicate that $FeCl_3$ is the most effective flocculant, providing superior efficiency at a reduced dosage.

Fig. 3 illustrates the temporal progression of the flocculation process using sequential images captured at different time intervals. Each flocculant was applied at its optimal dosage, resulting in final pH values of 8.3, 7, and 4.6 for $FeCl_2$, $FeCl_3$, and chitosan, respectively. The pH influences flocculation efficiency by modulating the surface charge of microalgal cells and the resulting electrostatic interactions with flocculating agents [49,50]. For inorganic flocculants, optimal performance is generally observed within a pH range of 6–9 [51,52]. This interval matches the pH of the cultivation medium in the present study, both prior to flocculation and after the addition of $FeCl_2$ and $FeCl_3$. Under neutral conditions, Fe^{2+} and Fe^{3+} undergo controlled hydrolysis, forming positively charged amorphous hydroxides that destabilize negatively charged microalgal cells via charge neutralization [52,53]. As pH increases, the formation of insoluble metal hydroxides is enhanced, promoting sweep flocculation, in which suspended particles become enmeshed within precipitated hydroxide matrices [53].

In contrast, studies using chitosan report high flocculation efficiencies under mildly acidic conditions (pH 5–6) [23,54], as well as at neutral to slightly alkaline pH (7–8) [55,56], and even in alkaline environments up to 10 [57]. This variability is attributed to factors such as cultivation medium composition, microalgal species, and the physicochemical properties of chitosan, including source, degree of deacetylation, and molecular weight, which influence solubility, protonation state, and interaction with negatively charged cell surfaces.

According to optical density measurements of the supernatant, $FeCl_3$ achieved maximum flocculation efficiency within just 20 min (Fig. 3). In contrast, $FeCl_2$ and chitosan required over 4 h to reach their peak efficiencies, which remained lower than that of $FeCl_3$. These results highlight the superior performance of $FeCl_3$ in the flocculation process. The reduced effectiveness of $FeCl_2$ and chitosan may be attributed to differences in their flocculation mechanisms and the chemical interactions these compounds have with microalgae. In water, $FeCl_3$ dissociates into Fe^{3+} and Cl^-, while $FeCl_2$ yields Fe^{2+}. The presence of Fe^{3+} plays a more

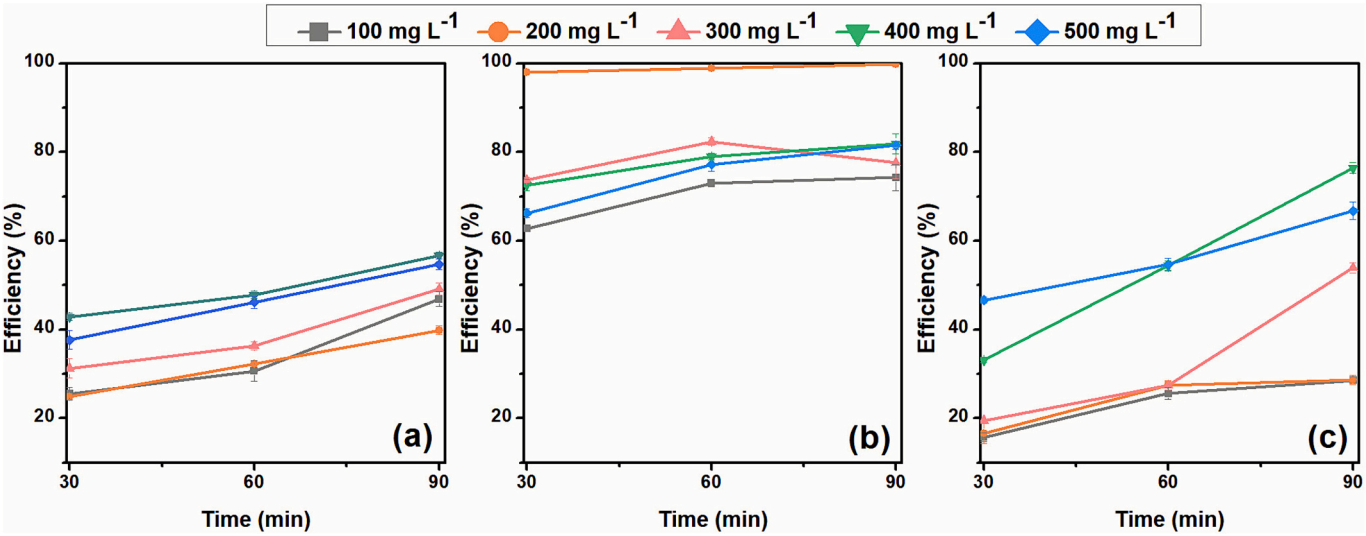

Fig. 2. Flocculation efficiency over time at varying flocculant dosages using: (a) $FeCl_2$, (b) $FeCl_3$, and (c) chitosan.

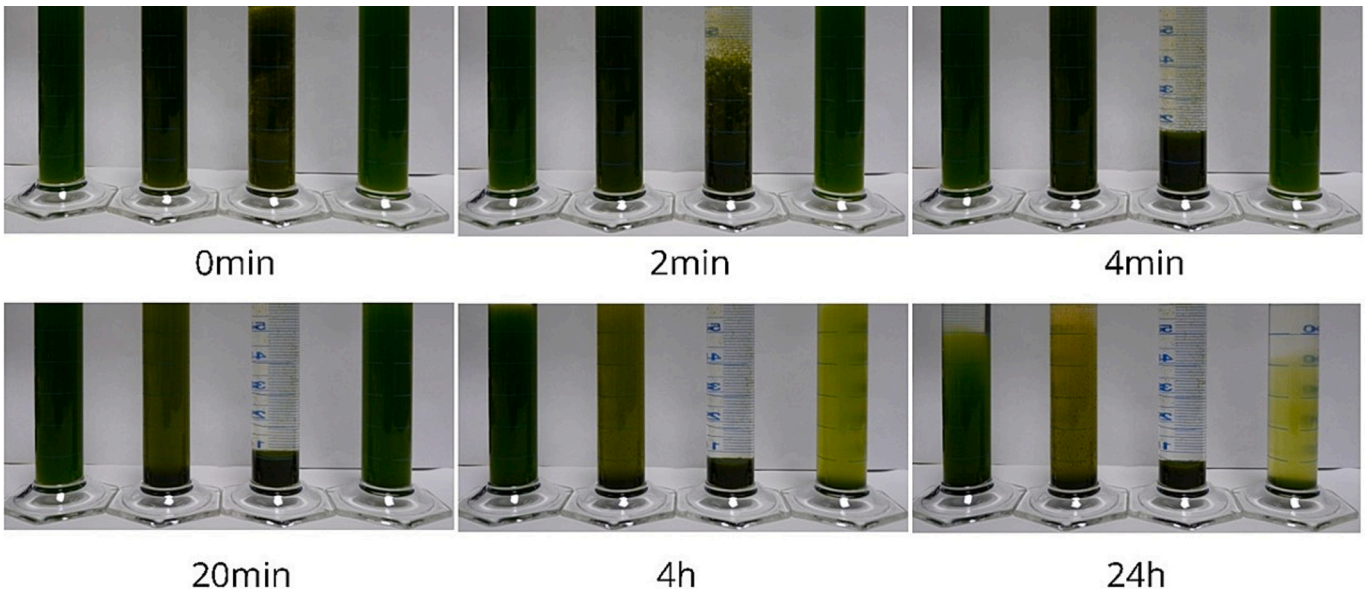

Fig. 3. Flocculation overtime from left to right: no flocculant, $FeCl_2$, $FeCl_3$, and chitosan.

significant role in neutralizing the negative charges of microalgae compared to Fe^{2+}, as the former has a greater positive charge, which in turn enhances cell aggregation. Similar results were reported by Seo et al. [58], which also employed $FeCl_3$ as a flocculant to separate *Chlorella* sp. These researchers reported approximately 90% of flocculation efficiency using the same dosage as the present study (200 mg L^{-1}) after 20 min. However, their medium was more acidic (pH 3) and had a higher cell concentration (1.7 g L^{-1}), more than twice that used in the current work. According to the authors, flocculation efficiency exceeded 90% only at a lower pH value, as $FeCl_3$ becomes more protonated under acidic conditions, enhancing its ability to neutralize the negatively charged surfaces of the microalgae.

In the study performed by Machado et al. [23], on the other hand, lower flocculant efficiencies were obtained for $FeCl_3$. The researchers studied organic and inorganic flocculants, among them $FeCl_3$, to harvest *C. vulgaris* at a dry biomass concentration of approximately 343 mg L^{-1}. Iron chloride showed the poorest performance compared to the organic flocculants, with a flocculation efficiency of approximately 9.4% at a concentration of 100 mg L^{-1}, which is about ten times lower than that observed in the present study.

Other researchers reported higher separation efficiencies for chitosan. Zhu et al. [59] applied chitosan dissolved in 1% acetic acid as a flocculating agent for the harvest of *C. vulgaris*, under initial conditions of biomass concentration of 1.2 g L^{-1} and pH 9.5. The maximum harvest efficiency, corresponding to 91.9%, was obtained with a dosage of 0.25 g L^{-1} of flocculant, a concentration 1.6 times lower than the present study. These differences can be explained due to the different culture media, as well as the microalgae strain. In the study conducted by Machado et al. [60], chitosan was also employed for harvesting *Chlorella vulgaris*, achieving a flocculation efficiency of 99.3% at pH 5, a significantly more acidic condition than the typically slightly alkaline culture medium. The flocculant dosage used was 10 mg L^{-1}, substantially lower than that applied in the present study. However, the initial dry biomass concentration was approximately 335 mg L^{-1}, considerably lower than the 800 mg L^{-1} used in this study. This lower biomass concentration, along with the acidic pH, which enhances chitosan protonation and thus increases its positive charge, may account for the higher flocculation efficiency observed [61].

3.2. Protein extraction by HPH

3.2.1. Protein yield after HPH treatment

Fig. 4 illustrates the protein content obtained after the application of HPH on flocculated and centrifuged biomass. The centrifuged sample exhibited the highest protein content (49.4 ± 0.5% of CDW), which corresponds to the typical values observed for this microalgal species [39,46]. The second highest protein content was achieved using $FeCl_2$, yielding approximately 31 ± 1% of CDW, followed by chitosan with 23.7 ± 2.7%, while $FeCl_3$ resulted in the lowest protein content at around 9.22 ± 0.03%.

These results demonstrate that flocculation and the type of flocculant significantly influence protein extraction. Although $FeCl_3$ demonstrated greater efficiency in harvesting microalgae, its use resulted in a significant reduction in protein content (by approximately 84%), if compared to the centrifuged sample. Similarly, $FeCl_2$ and chitosan also resulted in lower protein yields relative to the centrifuged biomass. Therefore, protein decline may be attributed to two factors: i) the interaction between the flocculants and the proteins released into the medium,

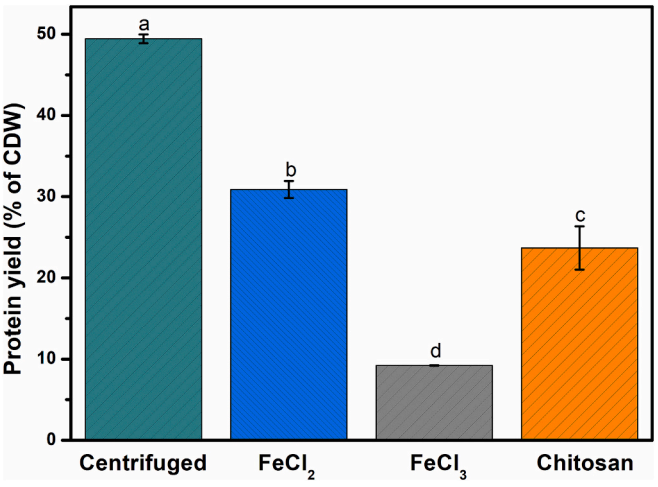

Fig. 4. Protein recovery from *Chlorella vulgaris* after HPH treatment when algae were harvested through centrifugation and flocculation with $FeCl_2$, $FeCl_3$ and chitosan, respectively. Means followed by different lowercase letters differ significantly ($p > 0.05$).

possibly influenced by the pH conditions established after flocculant addition, which may reduce protein solubility or impede their detection; ii) the formation of flocs that could obstruct the cell disintegration process by HPH, potentially necessitating higher energy inputs in terms of pressure to achieve efficient extraction of proteins from within the cells.

These hypotheses are based on previous studies showing that metal ions interact with negatively charged amino acid side chains, leading to the formation of protein–metal complexes that promote aggregation or precipitation and consequently reduce protein solubility [62]. For chitosan-based flocculation, the results can be attributed to electrostatic interactions between the cationic polymer and proteins, which are known to form stable protein–chitosan complexes and reduce effective protein solubilization [63]. In addition to direct flocculant–protein interactions, the pH conditions resulting from flocculant addition may have contributed to the observed differences. Chitosan and $FeCl_3$ lowered the suspension pH to approximately 4.6 and 7, respectively, from an initial pH of around 8.5. This acidification likely influenced protein solubility, as many proteins tend to precipitate near pH 4.5 and may also partially precipitate at neutral pH. Consequently, this precipitation reduces the amount of protein remaining in solution, complicating accurate quantification [36,64,65].

Residual flocculants remaining associated with harvested microalgal biomass may further affect downstream processing. Tang et al. [66] demonstrated that a substantial fraction of flocculants remained associated with *Auxenochlorella pyrenoidosa* biomass after harvesting, including 58% of chitosan, 67% of γ-polyglutamic acid (γ-PGA), and 74% of aluminum from $Al_2(SO_4)_3$ treatments. Likewise, Tran et al. [67] showed that residual polyaluminum chloride inhibited the transesterification activity of immobilized lipase and SrO/SiO_2 catalysts during biodiesel production from *Chlorella vulgaris*. In contrast, biomass harvested with chitosan showed performances comparable to centrifuged samples, indicating that residual chitosan did not impair biocatalytic activity. These findings highlight that the chemical nature of residual flocculants is critical for downstream compatibility, with metal-based coagulants being more prone to interfere with catalytic reactions than biopolymer-based flocculants. These results support the discussion that residual flocculants may differentially affect protein recovery and purity.

Beyond chemical interactions, physical limitations associated with floc structure may also play an important role. Although HPH is widely recognized in the literature as an effective method for disrupting microalgae cells, the formation of dense flocs can impose physical mass-transfer limitations that hinder effective cell permeabilization and protein release. The diffusion of proteins from the floc interior to the bulk medium is governed by intrafloc diffusion. This phenomenon, previously reported for activated sludge flocs, may delay net protein release after cell disruption or limit the penetration of extraction buffers and mechanical energy into interior cells, ultimately reducing protein extraction efficiency [68,69].

Limited studies have focused on the application of HPH for the extraction of biocompounds from microalgae harvested through flocculation, with even fewer research efforts evaluating the implications of this harvesting method on protein extraction [70–72]. The majority of the existing literature concentrates on cell disruption and the efficiency of HPH without directly addressing the impact of flocculation on the composition and recovery of extracted biocompounds. One study explored the use of HPH for lipid extraction from flocculated biomass; however, it was limited to analyzing changes in microalgal particle size post-HPH treatment and did not assess the impact of flocculation on lipid quality [72]. The study conducted by Magpusao et al. [70] explored the effects of HPH on the microstructural and rheological properties of the microalgae species *Arthrospira platensis*, *Isochrysis*, *Nannochloropsis*, and *Tetraselmis*. The results confirmed that HPH induced significant cell rupture, although *Nannochloropsis* exhibited lower disruption due to the enhanced resistance of its cell wall. Notably, none of the microalgal species studied were harvested through flocculation.

In the study of Moreira et al. [71], HPH was employed for protein extraction from *Chlorella vulgaris*; however, the biomass was obtained through filtration. This process was executed in a single cycle at a pressure of approximately 1000 bar, and protein quantification, conducted via the Kjeldahl method, revealed a protein content of approximately 26.6% on a dry biomass basis. In comparison, this value is significantly lower than that obtained in the present study, where protein concentration reached approximately 50% in the centrifuged sample. The observed discrepancies can be ascribed to several factors, including variations among the strains utilized, differences in cultivation conditions and biomass composition, and principally the application of higher pressures and an increased number of cycles in HPH, which enhanced cell rupture and facilitated protein release.

3.2.2. SDS-PAGE analysis after HPH treatment

Polyacrylamide gel electrophoresis was used to evaluate protein extraction under different experimental conditions. Fig. 5 shows the protein profile of samples treated with different flocculants, compared to the centrifuged sample (C). The molecular weight marker (M) allowed the estimation of the size of the proteins present, with visible bands in the ranges of 250 to 15 kDa.

The SDS-PAGE profiles provided qualitative findings into how different flocculants influence total protein recovery and the distribution of extracted proteins across molecular weight ranges. The reduced band intensity observed in flocculated samples compared to the non-flocculated control suggests that protein recovery is not uniformly affected across all fractions. The attenuation or disappearance of specific bands is consistent with selective under-extraction of certain protein classes. Similar trends have been reported in microalgal systems, where upstream processing steps altered protein accessibility or solubilization, leading to the under-representation of specific molecular weight bands in SDS-PAGE profiles despite comparable total protein contents [73,74].

Metal-based coagulants, particularly ferric salts, are known to form coordination complexes with negatively charged amino acid residues (e.g., carboxyl and phosphoryl groups), promoting cross-linking and the formation of insoluble aggregates. Such aggregates may remain in the pellet during sample preparation or may not be fully dissociated under SDS-denaturing conditions, resulting in attenuated or absent bands in the electrophoretic profile [75]. Similar mechanisms have been described for algogenic organic matter (AOM), which contains significant protein fractions, where metal ions promote complexation and reduced solubility [76]. In this context, some studies report direct evidence of the formation of complexes between proteins and coagulants, although not all are conducted specifically with microalgae. For example, Takaara et al. [77] demonstrated that polyaluminum chloride can form chelate complexes with extracellular and cellular organic matter, including proteins. Additionally, Zhang et al. [78] reported increased precipitation of bovine serum albumin in the presence of Fe^{2+} ions, reinforcing the role of metal–protein interactions in aggregation phenomena.

Moreover, the absence of pronounced low-molecular-weight smearing or extensive fragmentation across treatments suggests that protein degradation was not the dominant mechanism. Instead, the SDS-PAGE data support the conclusion that flocculation primarily affects protein extractability and solubility through flocculant–protein interactions and extraction efficiency, thereby influencing the qualitative protein profile recovered after downstream processing.

In general, the results obtained by SDS-PAGE analysis confirmed the findings obtained using the Lowry method. It was possible to observe that the centrifuged sample (C) had the highest protein bands intensity. This is consistent with the protein yield measured by Lowry and confirms that the highest efficiency of HPH is achieved with the algal suspension harvested by centrifugation. The sample treated with $FeCl_2$ displayed a profile similar to that of the centrifuged, indicating that this

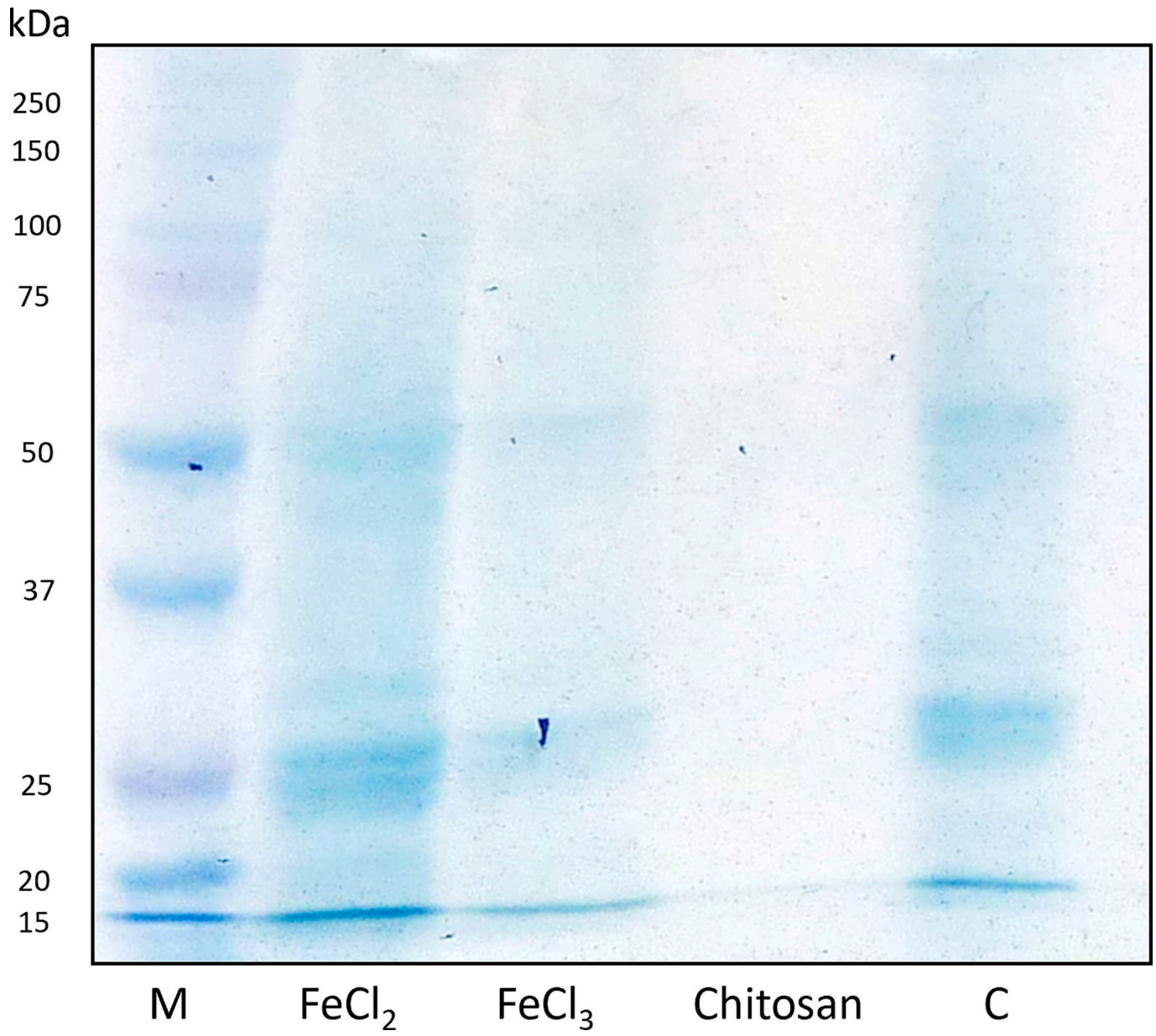

Fig. 5. Protein profiles obtained by SDS-PAGE after HPH treatment of biomass harvested via centrifugation and flocculation using $FeCl_2$, $FeCl_3$, or chitosan (M: marker; C: centrifuged biomass).

flocculant facilitated protein release, although in smaller amounts. In contrast, the use of $FeCl_3$ and chitosan as flocculants resulted in a decrease in protein detection after HPH treatment. Notably, the sample treated with chitosan yielded the least favorable results, showing nearly absent bands except for a single band at 15 kDa.

3.3. Protein extraction by PEF treatment

3.3.1. Protein yield after PEF treatment

Fig. 6 presents the protein content in each treatment (PEF#0 (control), PEF#1, and PEF#2), at the respective incubation times (1, 4, 6, and 24 h), for the different flocculants tested ($FeCl_2$, $FeCl_3$ and chitosan), as well as for the centrifuged sample. As expected for the centrifuged sample, the absence of a PEF treatment (PEF#0) resulted in negligible protein release. However, the application of PEF led to a progressive increase in protein extraction over the incubation period, with the most pronounced effect observed at 24 h. This shows that an extended incubation time enhances the diffusion and release of proteins due to the cell autolysis process triggered by cell death, as demonstrated in previous studies [39,46].

For PEF#0, $FeCl_2$, and $FeCl_3$ samples exhibited similar protein content across all incubation times, with no significant protein release detected. In contrast, the use of chitosan induced protein release from the earliest incubation time of 1 h through to 24 h, although no significant differences were observed among the different incubation periods. When chitosan was used as flocculant, a comparable trend was observed for both PEF treatments (PEF#1 and PEF#2), suggesting that neither incubation time nor increased electric field energy significantly enhanced protein extraction. For $FeCl_3$-flocculated samples, protein release was absent, as no increases in protein content were detected under any incubation time or PEF energy condition.

Application of a lower energy electric field (PEF#1) resulted in a significant increase in protein release from the centrifuged samples only after 1 h of incubation, with no significant differences observed among 4, 6, and 24 h. This behavior was similarly observed under the higher energy treatment (PEF#2), indicating that a 1 h incubation time does

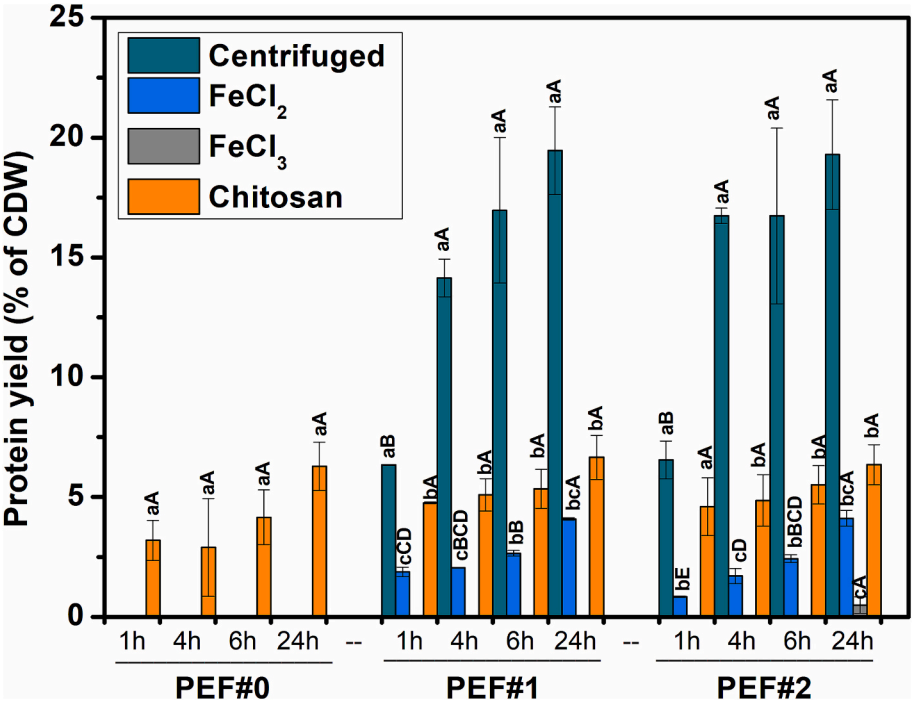

Fig. 6. Protein yield after PEF treatment of biomass obtained by centrifugation and flocculation with $FeCl_2$, $FeCl_3$ and chitosan. Lowercase letters indicate comparisons among means obtained at the same time and under the same treatment (e.g., PEF#0–1 h). Uppercase letters indicate comparisons among means corresponding to the same flocculant (centrifuged, $FeCl_2$, $FeCl_3$, and chitosan). Means followed by the same letter do not differ significantly ($p > 0.05$).

not sufficiently promote protein extraction. Moreover, increasing the electric field strength by a factor of 10 did not enhance protein release, suggesting that high energy input is unnecessary for effective PEF-assisted extraction. For $FeCl_2$-flocculated samples, a significant increase in protein content was observed at 24 h compared to earlier incubation times. For PEF#2 treatment, protein content of $FeCl_2$-flocculated samples were not significantly higher than those obtained with PEF#1. These results demonstrate that, similar to the centrifuged samples, applying higher specific treatment energy does not necessarily improve protein release efficiency.

In comparison to the centrifuged suspension, all flocculants reduced the efficiency of protein extraction, with $FeCl_3$ exhibiting the least favorable performance. One possible explanation for the observed results is the formation of denser and more agglomerated flocs induced by the flocculants, which may have hindered the effectiveness of PEF treatment and subsequent the protein extraction. As demonstrated by Akaberi et al. [40] and Guittet et al. [79], the mutual electric shielding effect among cells can reduce the amplitude of the induced transmembrane voltage, thereby diminishing protein release following PEF treatment. Nevertheless, the kinetics of protein release from *C. vulgaris* post-PEF appear to be independent of the specific energy applied [39]. In this context, protein release is primarily driven by enzymatic activity. However, in suspensions obtained through flocculation, protein extraction remained limited even after HPH, suggesting that diffusion processes may be hindered, likely due to the formation of dense cells or floc aggregates that restrict mass transfer. Given that diffusion plays a crucial role in enzymatic extraction, such structural constraints likely contributed to the observed reduction in overall efficiency. Moreover, as previously discussed, the pH conditions established after flocculant addition may also have contributed to the observed protein levels, potentially affecting protein solubility and, consequently, quantification.

Interestingly, chitosan showed a distinct behavior. Protein release occurred even in the absence of PEF treatment, indicating that the flocculant itself may have facilitated cell membrane permeabilization or improved protein solubilization. Among all the flocculants tested, chitosan led to the highest protein yield. However, its concentration remained nearly constant throughout the incubation period and still did not reach the levels obtained in the centrifuged control sample without any flocculant.

As with HPH, there are few studies investigating the application of PEF treatment to microalgae harvested by flocculation and their impact on protein extraction. Most studies with PEF focus on the processing of biomass obtained by centrifugation, as in the study by Carullo et al. [80]. These authors evaluated cell membrane permeabilization, morphological characteristics, and the extraction of intracellular compounds from *Chlorella vulgaris* suspensions subjected to PEF at various electric field intensities (10 to 30 kV cm^{-1}), comparing these results with those obtained by HPH. Their findings showed that PEF induced cell membrane permeabilization in an intensity-dependent manner without generating cellular debris. In contrast, HPH, which causes complete cell disruption, resulted in the release of small cell fragments along with the intracellular compounds. Regarding extraction efficiency, PEF selectively released carbohydrates (36% *w*/w) and low molecular weight proteins (5.2% w/w), likely because no incubation period was provided to facilitate protein release. Conversely, HPH, achieved yields 1.1 times higher for carbohydrates and 10.3 times higher for proteins compared to PEF. The results obtained in the present study show differences when compared to those of Carullo et al. [80]. In the present study, the maximum protein content extracted by PEF from the centrifuged sample was approximately 20% of the cell dry weight (CDW). Furthermore, this value aligns well with literature results, which report protein contents reaching up to 25% of the CDW [39].

Fig. 7 summarizes the protein content results obtained previously, highlighting the differences observed between the treatments applied. When comparing the maximum protein contents obtained in each sample by HPH with those of PEF, the greater efficiency of the first methodology becomes evident. This greater efficiency of HPH is justified because the method is based on the complete rupture of the cell envelope, which provides greater release of the compounds present inside the cells. In contrast, PEF consists of electroporation, a much less invasive mechanism, which consequently generates lower protein content values.

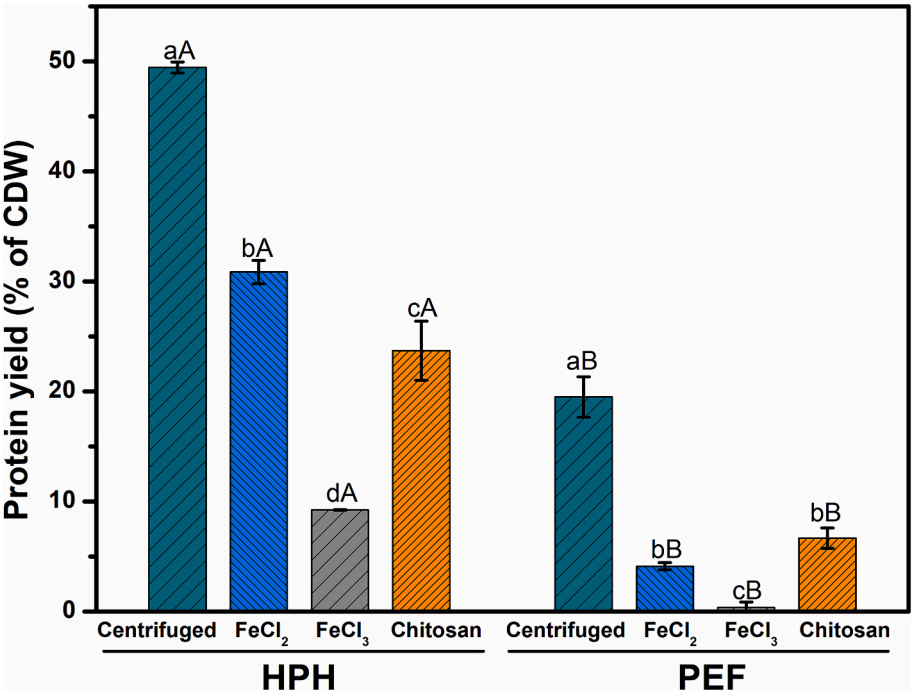

Fig. 7. Maximum protein content obtained from centrifuged and flocculated biomass following HPH and PEF treatments. Means followed by the same lowercase letter within a treatment (HPH or PEF) or by the same uppercase letter between treatments (HPH or PEF) do not differ significantly ($p > 0.05$).

The comparisons presented in Fig. 7 also provided insights into the flocculation impact of protein extraction: lower protein contents were observed in all flocculated biomasses, in comparison to centrifugated biomass. As previously discussed, two main hypotheses may explain these results: (i) interactions between the flocculants and the proteins released into the medium, potentially altering protein structure or reducing their detectability, as certain flocculants, such as chitosan or $FeCl_2$, may adsorb or aggregate released proteins, leading to their entrapment in the flocs and reduced detection in the supernatant; and (ii) the formation of flocs that could hinder the cell permeabilization process. It is likely that both effects contributed to the outcomes observed. The formation of flocs may be the main effect, accounting for the lower protein yields following PEF treatment. However, structural alterations or degradation of proteins could explain the reduced efficiency observed after HPH.

3.3.2. *SDS-PAGE analysis after PEF treatment*

Fig. 8 shows the protein profile of samples treated with different flocculants, as well as the centrifuged sample, under PEF#0, PEF#1, and PEF#2 treatment conditions after 24 h of incubation. Supporting the results obtained by the Lowry method in determining protein content, the sample centrifuged without PEF application (PEF#0) did not show detectable protein bands. On the other hand, in the PEF#1 and PEF#2 treatments, it is possible to have a clear visualization of several bands.

In the samples flocculated with $FeCl_2$ and without PEF application, as well as distributed to the centrifuged sample, there was no protein release. However, in the PEF#1 and PEF#2 treatments, protein bands appeared, with the intensity being greater in the PEF#1 treatment. The samples flocculated with chitosan showed similar profiles in the three PEF conditions evaluated. Discrete bands associated with proteins of approximately 150 kDa were observed, and more intense bands around 15 kDa, confirming the protein content results that indicated release even in the absence of PEF application.

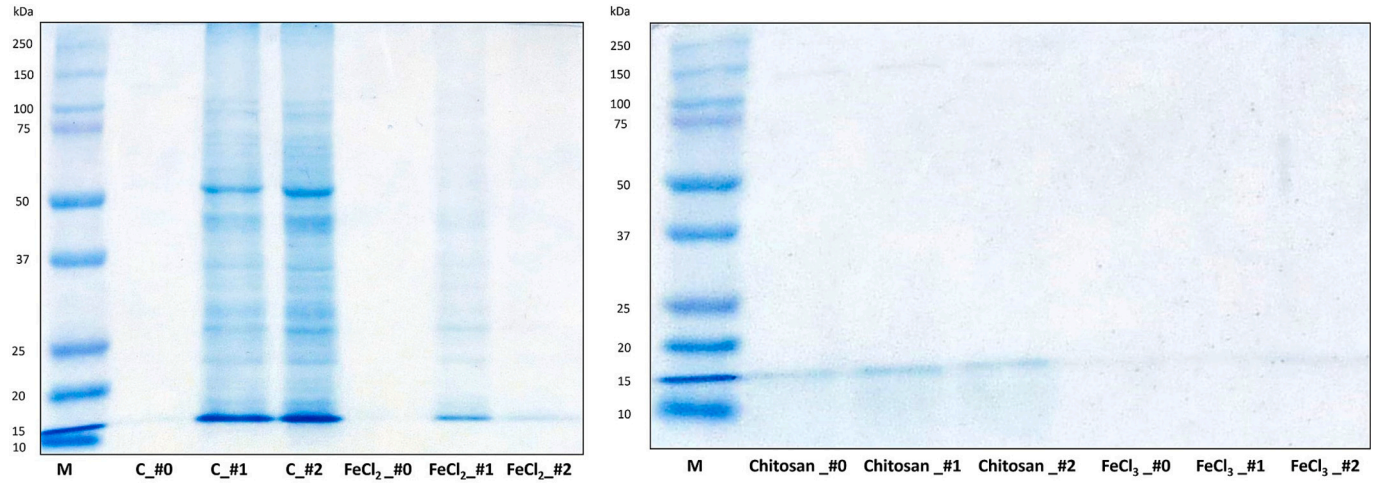

Fig. 8. Protein profile obtained by SDS-PAGE after PEF treatment. (M:marker; C: centrifuged; #0: PEF#0; #1: PEF#1 and #2: PEF#2).

Consistent with the protein content analysis, in the samples flocculated with $FeCl_3$ the majority of protein bands diminished compared to the centrifuged samples, with only one band remaining around 15 kDa. The impact of flocculants on protein extraction was evident, as the protein profiles showed either a reduction or complete absence of bands in the flocculated samples compared to the centrifuged sample. As explained earlier, the presence of metal-based or polymeric flocculants may promote the formation of protein–flocculant complexes that limit mass transfer and reduce effective protein solubilization. Consequently, proteins released upon electroporation may remain entrapped within the floc structure or undergo aggregation, leading to reduced detection in SDS-PAGE profiles.

Overall, the SDS-PAGE results confirm that both flocculant type and PEF application influence the qualitative protein profiles recovered. Rather than indicating protein degradation, the data support the conclusion that flocculation primarily affects protein extractability and solubility through flocculant–protein interactions and extraction efficiency, thereby shaping the protein profile obtained after downstream processing.

While both HPH and PEF treatments effectively disrupt microalgal cells in non-flocculated suspensions, their efficiency was significantly reduced when applied to biomass flocculated with $FeCl_3$, $FeCl_2$ and chitosan. These results highlight a key limitation of conventional cell disruption technologies when downstream protein extraction must be integrated with chemical harvesting strategies. Flocculants cannot be considered inert once harvesting is completed, as substantial fractions may remain associated with the microalgal biomass and cause pronounced effects on downstream processing. These findings reinforce the importance of explicitly considering flocculant residues when designing integrated microalgal biorefineries, as the choice of harvesting strategy can directly influence downstream extraction efficiency, product purity, and overall process viability. Future development should focus on optimizing the compatibility between flocculation and cell permeabilization methods. Potential strategies include tailoring HPH and PEF parameters, such as number of passes, field strength and pulse duration, to enhance penetration into flocculated biomass, or exploring alternative biopolymer-based flocculants that exert minimal interference with protein recovery.

4. Conclusions

This study provided novel insights into the influence of flocculation on protein extraction from *Chlorella vulgaris*, addressing a gap in the literature where most previous research has focused solely on flocculation or cell disruption techniques. This work evaluated the flocculation efficiency of $FeCl_2$, $FeCl_3$, and chitosan as harvesting agents and their impact on subsequent protein extraction using HPH and PEF applied to flocculated microalgal biomass. Although $FeCl_3$ achieved the highest flocculation efficiency (around 99%), all flocculants significantly reduced subsequent protein extraction yields compared to centrifuged biomass, revealing a critical trade-off between harvesting efficiency and extractability. This limitation is particularly relevant for industrial biorefineries aiming at protein-rich fractions.

The reduced protein yields may result from combined physical and chemical effects, including floc-induced mass-transfer limitations and flocculant–protein interactions that may alter solubility and extractability. The superior performance of HPH relative to PEF confirms that complete cell disruption partially overcomes these constraints; however, even HPH could not fully compensate for the limitations introduced during flocculation. These findings indicate that the selection of harvesting strategies must be guided not only by biomass recovery efficiency and operational cost, but also by their compatibility with subsequent processing steps. Flocculation and cell disruption should therefore be strategically co-designed rather than sequentially optimized.

Future research should focus on quantifying mass-transfer resistance in flocculated biomass, investigating protein structural modifications induced by metal salts and biopolymer flocculants, and optimizing flocculation conditions, such as pH. The development of flocculants with minimal downstream interference also represents a key research direction. Overall, this work underscores the need for integrated process design in microalgal biorefineries, where harvesting and extraction strategies are co-optimized to ensure both economic viability and product quality.

CRediT authorship contribution statement

Viviane de Carvalho Arabidian: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Alexander S.K. Müller:** Visualization, Methodology, Investigation. **Luiz Antonio de Almeida Pinto:** Funding acquisition, Formal analysis. **Débora Pez Jaeschke:** Writing – review & editing, Visualization, Validation, Conceptualization. **Tito Roberto Sant'Anna Cadaval:** Supervision, Funding acquisition, Formal analysis. **Wolfgang Frey:** Resources, Funding acquisition, Formal analysis, Conceptualization. **Christian Gusbeth:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Conceptualization.

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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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Data availability

Data will be made available on request.

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