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Pulsed electric field treatment enhances lipid bioaccessibility while preserving oxidative stability in *Chlorella vulgaris*

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**ABSTRACT**

There is growing demand for gentle technologies to improve the lipid bioaccessibility (BA) of *Chlorella vulgaris* biomass while preserving cell integrity and therefore oxidative stability. Pulsed electric field treatment (PEF, 5 μs at 20 kV cm\(^{-1}\), 31.8 kJ kg\(^{-1}\) \(\text{wet}\)) led to an enhancement in lipid BA from 4–7.8% (untreated) to 18.7–20.9%. To reach such a level of BA, incubation in buffer after the treatment (12 h at 25/37 °C, 48 h at 4 °C) was required. As hypothesized, PEF preserved cell integrity, as shown by particle size and scanning electron microscopy analyses, as well as oxidative stability of the biomass over 3 months at 40 °C. Proteome analysis identified four proteins that may be involved in cell wall lytic activity during incubation after PEF. Future work should focus on further understanding the mechanism behind incubation after PEF and studying the potential effect played by endogenous cell wall-degrading enzymes.

1. Introduction

Microalgae are unicellular organisms that represent a source of several valuable nutrients, such as polyunsaturated fatty acids (PUFAs), protein and minerals. Currently, *Chlorella* sp., together with *Arthospira* sp. (‘Spirulina’), account for the largest percentages of commercially produced microalgae and are among the few species that can be consumed as food according to the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) (Enzing et al., 2014; García et al., 2017). Generally, *Chlorella* whole cells are mostly used as food supplements because of their complete nutritional profile instead of extracting specific compounds (Rego et al., 2015). Therefore, from an industrial point of view, the bioaccessibility of a component in the whole biomass is a more meaningful parameter than the extraction yield with organic solvents. Our previous study showed that the in vitro protein bioaccessibility of *Chlorella* was approximately 40–70%, whereas the lipid bioaccessibility was below 20% because it was limited by the indigestibility of the cell wall (Canelli, Murciano Martínez, Maude Hauser, et al., 2021; Canelli, Tarnutzer, et al., 2020). These results indicate a strong need for research aimed at improving the bioaccessibility of lipids from *C. vulgaris*. Complete disruption of *C. vulgaris* cells by high-pressure homogenization (HPH, 100 MPa) showed a significant increase in lipid bioaccessibility of up to 60%. However, harsh disintegration of the cell simultaneously led to a loss in oxidative stability due to the exposure of PUFAs to oxidation initiation factors (e.g., oxygen, light) (Canelli, Murciano Martínez, Maude Hauser, et al., 2021). In addition, cavitation during HPH can produce free radicals that trigger lipid oxidation (Günerken et al., 2015). Lipid oxidation is one of the main causes of quality loss in a food product due to the production of off-flavours (Shahidi & Zhong, 2010). Therefore, to preserve biomass quality, research should focus on finding a disruption process that increases the bioaccessibility of the lipid fraction while avoiding lipid oxidation.

The literature on techniques to improve lipid bioaccessibility is scarce. However, we can refer to methods used to enhance lipid extractability by organic solvents. In addition to mechanical treatments such as HPH mentioned earlier, several mild techniques can be used. Among those, the use of cell wall-degrading enzymes was previously...
investigated (Canelli, Murciano Martínez, Maude Hauser, et al., 2021). The combination of galactanase, chitinase, and rhamnohydrolase was able to enhance the protein bioaccessibility to 59%; however, the lipid bioaccessibility remained unchanged compared to the untreated biomass.

Pulsed electric field (PEF) is a physical technology that consists of applying an electric field that it is pulsed (duration from nanoseconds to microseconds). PEF treatment can be used to apply harsher or milder disruptions, depending on the operating parameters. The specific energy input (\(W_s, J kg^{-1}\)) that is applied depends on several factors as described in Eq. (1): electric field strength (\(E, kV cm^{-1}\)), pulse width (\(\tau_p, \mu s\)), pulse shape, medium conductivity (\(\sigma, mS cm^{-1}\)), and pulse number (\(n, -\)).

\[ W_s = E^2 \cdot \tau_p \cdot n \cdot \sigma \]  

(1)

time when the applied field strength is sufficiently high to cause a transmembrane potential (TMP) larger than the critical TMP, electroporation occurs. The critical TMP is approximately 0.1–1 V (Teissie & Tsong, 1981; Zimmermann et al., 1974). Moreover, depending on the process intensity, reversible or irreversible electroporation is possible. Destabilization of the cell membrane leads to higher membrane conductivity and permeability (electroporation) while facilitating the release of intracellular matter (Carullo et al., 2018; Jaeschke et al., 2019; Silve, Kian, et al., 2018). The effect of PEF on the cell membrane is widely known; however, there is no clear evidence that the cell wall surrounding the cell membrane is affected by PEF. T Lam et al. (2017) showed that the cell wall acted as an obstacle for the release of protein from Chlamydomonas reinhardtii after PEF, deducing that the cell wall was unaffected by PEF. Hydrophilic proteins were completely released after PEF in cell wall-free mutants, while wild-type cell wall-containing cells showed three times lower protein yields. Although several studies report that PEF does not have a direct impact on the cell wall (Bensalem et al., 2018; Papachristou et al., 2020; T Lam et al., 2017), an increase in the extractability of microalgae intracellular compounds (e.g., protein, lipid, pigments, carbohydrates) by organic solvents or buffer systems after PEF treatment has been described (Aguilar-Machado et al., 2020; Buchmann et al., 2019; Kemptes et al., 2015; Silve, Kian, et al., 2018). There are different theories to explain how these compounds could migrate out of the cell wall. Among them, one focuses on the role played by autolytic enzymes that are naturally produced by C. vulgaris during the reproductive cycle or in the case of programmed cell death under environmental stress conditions. These enzymes (autolysins) were described as a mixture of proteases, phosphatases, esterases and glycosidases capable of degrading cell walls, e.g., releasing daughter cells (Burczyk & Loos, 1995; Demuez et al., 2015). Jaeschke et al. (2019) suggested that PEF induces a programmed cell death mechanism that promotes apoptosis by the action of endogenous enzymes. According to this theory, autolytic enzymes might be:

a. **Expressed**: PEF treatment may induce the expression of autolytic enzymes, as PEF was reported to be able to regulate gene expression (Bai et al., 2020).

b. **Liberated**: Autolytic enzymes might be released from secretory vesicles, move through electroporated disorganized membranous systems and come in contact with the cell wall (Aguilar-Machado et al., 2020; Martínez et al., 2020).

It is important to consider the incubation of a microalgae suspension after PEF treatment, regardless of the mechanism explaining the PEF effects on the cell wall. The studies that showed no impact of PEF on protein extractability (T Lam et al., 2017) or algae mechanical stability (Papachristou et al., 2020) had short or even absent, respectively, incubation of the algae suspension after PEF. In contrast, Jaeschke et al. (2019) showed how a minimum incubation step, although short (15 min), was necessary to extract protein from A. platensis after PEF treatment (40 kV cm\(^{-1}\), 1 \(\mu s\), 28–112 J mL\(^{-1}\)sup). With longer incubation times, the protein extractability was further improved. Similarly, an incubation time of 24 h at room temperature clearly improved the protein release into the supernatant from Nannochloropsis salina by 400% (Coustets et al., 2013). In yeasts, autolysis can be triggered after PEF treatment, and the consequent release of endogenous enzymes such as proteases and β-glucanases leads to cell self-degradation (Martínez & Cebrián, 2016). This leads to loss of membrane function, hydrolysis of intracellular polymers and the consequent release of hydrolytic products in the extracellular space.

The aim of this study was to define an interval of PEF parameters (electric field strength and pulse width) targeting an enhancement of the lipid extractability of C. vulgaris. Additionally, it was examined whether PEF treatment could improve the lipid bioaccessibility of C. vulgaris, with a close understanding of the effect of the incubation time and temperature after PEF. Finally, the combination of exogenous cell wall-degrading enzyme treatment and PEF was assessed in terms of lipid bioaccessibility, cell integrity and oxidative stability.

### 2. Material and methods

#### 2.1. Microalgae cultivation

C. vulgaris (CCALA 256) was cultivated in biological triplicate of independent cultures (duplicate for the lipid extractability experiment) in bold basal medium complemented with 20 g L\(^{-1}\) glucose in 500-mL Erlenmeyer flasks (working volume 250 mL) in the dark in a shaking incubator (25 °C, 150 rpm, Multitron Pro, Infors AG, Bottmingen, Switzerland). The cells were harvested in stationary phase after four days of cultivation and centrifuged (10,000 x g, 10 min, 4 °C). The supernatant was discarded, and the pellet (fresh biomass) was utilised for further experiments.

#### 2.2. Disintegration techniques

##### 2.2.1. Pulsed electric field

Prior to cell electroporation, the conductivity and concentration of the microalgae suspensions were standardized. For all PEF experiments, the microalgae concentration was set at 70 g L\(^{-1}\). The fresh biomass pellet was resuspended in potassium phosphate buffer (pH 6) and adjusted to a conductivity (\(\kappa\)) of 2 mS cm\(^{-1}\), resulting in a final conductivity of 1.7 mS cm\(^{-1}\) in the microalgae suspensions. After vortexing, the microalgae suspensions (1 mL) were treated into plate-plate electroporation cuvettes (VWR International, Leuven, Belgium) for PEF treatment. The samples were treated at a 4-mm electrode distance, with a cuvette holder connected to an RUP6-15CL pulse generator (GBS-Elektronik, Radeberg, Germany). Pulses were measured with a voltage probe (P6015A, Tektronix, Beaverton OR, USA) coupled with an oscilloscope (Wave Surfer 10, Teledyne LeCroy, Heidelberg, Germany). The applied voltage was varied between 8 kV and 12 kV, resulting in electric field strengths of 20 kV cm\(^{-1}\) and 30 kV cm\(^{-1}\), respectively. Pulse widths of 5 \(\mu s\) up to 25 \(\mu s\) and a pulse number of 10 were applied. Different combinations were tested systematically to investigate their impact on lipid extractability. Following the PEF treatment, biomass aliquots from several cuvettes were combined and incubated at different temperatures (4, 25, 37 °C) for different times (0, 1, 6, 12, 18, 24, 48, 72 h) at 300 rpm. After incubation, the biomass were immediately snap-frozen with liquid nitrogen and stored at −20 °C until further analysis. Untreated samples (control) were concentrated and standardized to the same conductivity, incubated and then snap-frozen.

##### 2.2.2. Enzymatic treatment

Fresh microalgae biomass was suspended in potassium phosphate buffer (50 mM, pH 6) at 20 g L\(^{-1}\). A solution of chitinase (700 μL, 1 mg ml\(^{-1}\) in microalgae suspension, Sigma Aldrich, Switzerland), rhamnogalacturonan rhamnomehydrolase (35 μL, NZYTech, Portugal), and endo-1,4-β-galactanase (1.75 μL, 750 U ml\(^{-1}\) in potassium phosphate buffer,
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pH 6, Megazyme, Ireland) was added to the microalgae suspension. After mixing, the samples were incubated at 37 °C at 300 rpm for 24 h on a stirring plate. After incubation, the biomass was immediately snap-frozen with liquid nitrogen and stored at −20 °C until further analysis. For the control, potassium phosphate buffer (pH 6, 50 mM) was used instead of the enzyme solution. All samples were incubated for 24 h at 37 °C on a stirring plate at 300 rpm, and, after incubation, immediately cooled in crushed ice.

2.2.3. PEF combined with enzymatic treatment

Microalgal biomass was treated with a pulse width of 5 μs, an electric field strength of 20 kV cm−1, and 10 pulses. The biomass coming from several electroporation cuvettes was combined, and the following enzymes were added before diluting the suspension to 20 g L−1 with potassium phosphate buffer (50 mM, pH 6): chitinase (700 μL, 1 mg mL−1 in potassium phosphate buffer, pH 6), hirnoglucanexorotanase (35 μL, NYTech, Portugal), and endo-1,4-β-galactanase (1.75 μL, 750 U mL−1 in potassium phosphate buffer, pH 6, Megazyme, Ireland). After mixing, the samples were incubated at 37 °C for 24 h at 300 rpm on a stirring plate. After incubation, the biomass was immediately snap-frozen with liquid nitrogen and stored at −20 °C until further analysis.

2.2.4. High-pressure homogenisation

Microalgal cell suspension (200 mL, 40 g L−1) was disintegrated by high-pressure homogenisation (HPH) in a microfluidizer (M110EH, Microfluidics, Newton, MA, USA) at 100 MPa, applying four passes. The outflow temperature did not surpass 40 °C. Part of the sample was directly cooled on ice, while another aliquot was incubated for 24 h at 37 °C and 300 rpm. The biomass was then snap-frozen with liquid nitrogen and stored at −20 °C until further analysis.

2.3. Lipid extractability

Lipid extractability was assessed by measuring the hexane:isopropanol (HI) extraction efficiency as reported by Bernaerts et al. (2019), with slight modifications. HI extraction efficiency is expressed as the ratio of the free lipids extracted by hexane:isopropanol (3:2 v−1) to the total lipids determined by direct transesterification (Eq. (2)). HI difficulty penetrates the intact microalgal cell wall, producing a lower extraction yield.

\[
HI\ extraction\ efficiency\ (\%) = \frac{Extracted\ lipids}{Total\ lipids} \times 100
\]  

Hexane:isopropanol (3:2 v−1, 1.5 mL) was added to 10 mg of freeze-dried PEF-treated microalgal biomass, and the mixtures were vortexed for 30 s. After centrifugation (10 min, 750 x g, 25 °C), the solvent containing the extracted lipids was recovered. Overall, the extraction was repeated four times. After pooling all solvent layers, the solvent was evaporated under a nitrogen stream. The extracted and total lipids were measured by gas chromatography (GC) upon transesterification to fatty acid methyl esters (FAMEs) as previously reported (Canelli, Neutsch, et al., 2020). Lipid bioaccessibility was defined as the amount of lipid contained in the micellar phase divided by the amount of lipid in the full digesta, expressed as a percentage (%)(Eq. (3)).

\[
Lipid\ bioaccessibility\ (%) = \frac{Lipid_{micellar\ phase}}{Lipid_{full\ digesta}} \times 100
\]

2.5. Proteome analysis

A comparative quantitative protein analysis between untreated microalgae cells and cells treated with PEF (5 μs, 20 kV cm−1, 31.8 kJ kg−1 sus) with and without a subsequent incubation (24 h, 37 °C) was conducted to identify enzymes potentially involved in cell wall degragation after PEF treatment. The full algae suspension was centrifuged (4 °C, 5 min, 10,000 x g), and the supernatant containing the released protein was evaluated.

2.5.1. Protein digestion

For each sample, proteins were precipitated with trichloroacetic acid (5%) and washed two times with cold acetone. The dry pellets were dissolved in 45 μL of buffer (10 mM Tris +2 mM CaCl2, pH 8.2). Reduction and alkylation of the proteins was performed by adding 2 mM Tris(2-carboxyethyl)phosphine-hydrochloride and 15 mM iodoacetamide. After 30 min at 60 °C, the samples were cooled to room temperature, and digested in a microwave instrument (Discover System, CEM, Kamp-Lintfort, Germany) for 30 min at 5 W and 60 °C after adding 500 ng of Sequencing Grade Trypsin (Promega, Dübendorf, Switzerland). The samples were dried to completeness and re-solubilized in 10 μL of 0.1% formic acid in water for LC-MS/MS analysis. The peptide concentration was determined using a Lunatic (Unchained Labs, Pleasanton, USA) instrument.

2.5.2. Liquid chromatography (LC)-mass spectrometry (MS) analysis

The analysis was carried out on an Orbitrap Fusion Lumos (Thermo Scientific, San Jose, USA) equipped with a Digital PicoView source (New Objective, Littleton, USA) and coupled with an M-Class UPLC (Waters GmbH, Wilmslow, UK). Column A had 0.1% formic acid and channel B had 0.1% formic acid and 99.9% acetonitrile. For each sample, peptides corresponding to 0.56 absorbance were loaded on a commercial MZ Symphony C18 Trap Column followed by a nanoEase MZ C18 HSS T3 Column. The peptides were eluted at a flow rate of 300 nL min−1. After an initial 3 min hold at 5% B, the peptides were eluted by a gradient from 5 to 22% B in 80 min, 32% B in 10 min and 95% B for 10 min. Data-dependent mode was used for the mass spectrometer, acquiring full-scan MS spectra (300–1500 m/z) at a resolution of 120,000 at 200 m/z after accumulation to a target value of 400,000. All relevant data were deposited to the ProteomeXchange Consortium via the PRIDE (http://www.ebi.ac.uk/pride) partner repository with the dataset identifier PXD027770.

2.5.3. Protein identification and label-free protein quantification

The raw MS data were processed by MaxQuant (version 1.6.2.3) and proteins were identified using the integrated Andromeda search engine.
2.6. Particle size

Protein along with the blastp evaluation. As a result of this process, each lyzed in triplicate with a LS 13320 laser diffraction particle size analyser significantly different concentrations among the tested conditions. These monumental material). Proteins that showed a log2fold change |log2(FC)|

protein entry had an annotation consisting of the top hit pfam protein reference proteome of Cox & Mann, 2008). Spectra were searched against a Six Chlorophyta database (downloaded from http://chlorella.genomprojectsolutions-databases.com/, version from 2020 to 08-31), concatenated to its reversed decoyed fasta database and common protein contaminants. The oxidation of methionine and the acetylation of N-terminal protein were set as variables, while the carbamidomethylation of cysteine was set as a fixed modification. Enzyme specificity was set to trypsin/P, permitting a minimal peptide length of seven amino acids and a maximum of two missed cleavages. MaxQuant Orbitrap default search settings were employed. The maximum false discovery rate (FDR) was set to 0.01 for peptides and 0.05 for proteins. Label-free quantification was allowed, and a 2-min window for matching between runs was applied. A group of functions executed in the R package SRMService (Wolski et al., 2018) was used to filter for proteins with two or more peptides, permitting for a maximum of four missing values, to normalize the data with a modified robust z-score transformation and to compute p values using the t-test with pooled variance. If all measurements of a protein were missing in one of the conditions, a pseudofold change was computed replacing the missing group average by the mean of the 10% smallest protein intensities in that condition.

2.5.4. Selection of proteins of interest based on proteome analysis

Annotion was performed by searching the protein database (the Six chlorophyta database) against the pfam protein family database (https://pfam.xfam.org/) using the Hmmer program (http://hmmer.org/) and the default parameter settings (Supplementary material). Additionally, the protein sequences were blasted (blastp) against the UniProt reference proteome of C. reinhardtii, and the description lines were extended with the best matching blast orthologue with the C. reinhardtii protein along with the blastp evaluation. As a result of this process, each protein entry had an annotation consisting of the top hit pfam protein family and the accession number of the UniProt orthologue (Supplementary material). Proteins that showed a log2fold change |log2(FC)| ≥ 1 and an adjusted p value <0.05 were defined as occurring at significantly different concentrations among the tested conditions. These proteins were screened for their putative function in the C. reinhardtii database from the UniProt consortium (24.08.2021), and proteins associated with hydrolytic, proteolytic or carbohydrate catabolic activity were selected as potential candidates of interest.

2.6. Particle size

The particle size of untreated and treated microalgae cells was analysed in triplicate with a LS 13320 laser diffraction particle size analyser (Beckman Coulter, Brea, Canada), as explained in Canelli, Murciano Martínez, Maude Hauser, et al. (2021), to determine the cell integrity. The results were reported as the mean diameter of the volume-based droplet size distribution (d43).

2.7. Scanning electron microscopy (SEM)

The cell suspension was gently spun down, and the resulting pellet was transferred to cellulose capillary tubes. The small tubes were cut to approximately 5 mm and closed at the same time by a scalpel in a drop of PBS. Sample processing for chemical fixation was performed in a PELO BioWave Pro+ microwave system (Ted Pella, USA), following a microwave-assisted fixation and dehydration procedure. Fixation was performed using 0.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS buffer (pH 7). After washing, postfixation was performed in 1% aqueous osmium tetroxide (OsO4) in 0.1 M PBS (pH 7), followed by washing in dd-H2O three times. Dehydration was performed in a gradient series of ethanol (50%, 75%, 90%, 98% and 3 times 100%) on ice. The samples were finally critical point dried (CPD 931, Tousimis, USA). The cellulose capillary tubes were cut open with a blade and glued by double-adhesive conductive carbon tape on aluminium stubs. Before imaging, samples were sputter-coated with 7.5 nm platinum/palladium using a CCU10 sputter coater (Safematic, Switzerland). Imaging was performed at 2 kV by collecting secondary electrons using an Everhart-Thornley Detector (SE2) on a Zeiss Merlin FE Scanning Electron Microscope (Zeiss, Germany).

2.8. Lipid oxidative stability

Lipid oxidation was evaluated according to the method of Rohfritsch et al. (2021) with certain adjustments, as reported by Canelli, Murciano Martínez, Maude Hauser, et al. (2021). Secondary lipid oxidation products were measured in triplicate in freeze-dried biomass subjected to a storage at 40 °C for up to 12 weeks. Additionally, the stored samples were randomized and assessed by sniffing on odour ranking and flavour notes by a four people-panel.

3. Results and discussion

3.1. Parameter screening: Effects of electrical field strength, pulse width, and specific energy input on lipid extractability

The optimization of a PEF treatment requires the setting/tuning of several parameters, such as pulse width and electric field strength, which results in a specific energy input W. The impact of the pulse width was evaluated for values between 5 μs and 25 μs, while the electric field strength was kept constant at 20 kV cm−1 (Fig. 1A). In a second experiment (Fig. 1B), the effect of the field strength was assessed (20–30 kV cm−1) at a constant pulse width of 5 μs. In both experiments, the biomass suspension was incubated for 1 h at 25 °C after PEF treatment. The measured electric field strength must be distinguished by the theoretical strength. The actual energy inputs calculated based on the measured electric field strengths are reported in Fig. 1. The measured electric field strengths and the theoretical energy inputs are reported in the Supplementary material.

As shown in Fig. 1A, lipid extractability was enhanced by PEF treatment at 5 μs compared to the untreated control. Treatments at higher pulse widths, and therefore higher energy inputs, did not cause an improvement in lipid extractability. Thus, 5 μs was selected for further experiments. Lipid extractability was assessed at various electric field strengths and at a constant pulse width of 5 μs, and the results are shown in Fig. 1B. When the biomass was treated at 20 kV cm−1, the lipid extractability was 15.8% higher than that of the control, but the highest increase was reached when the biomass was treated at 25 kV cm−1 (+26.9%). At higher electric field strengths (30 kV cm−1) and therefore

![Fig. 1. Lipid extractability (%) of C. vulgaris biomasses treated at 20 kV cm−1 and different pulse widths (5–25 μs) (A), and biomasses treated at 5 μs and different electric field strengths (20–30 kV cm−1) (B). The results are shown for biomass incubated for 1 h at 25 °C after the PEF treatment. The actual energy input (kJ kg−1sus) is indicated for each treatment. Error bars show the absolute deviation between biological duplicates (n = 2).](image-url)
greater energy inputs, the lipid extractability was reduced. Notably, when treating at 25 kV cm$^{-1}$ or higher, system instabilities occurred. Therefore, 20 kV cm$^{-1}$ was selected for further experiments, as it caused a clear increase in lipid extractability while avoiding side effects that were present at higher field strengths in our settings. PEF conditions that will be further investigated regarding their impact on lipid bioaccessibility were pulses of 5 μs at 20 kV cm$^{-1}$. This represents a μsPEF treatment with an energy input of 31.8 kJ kg$^{-1}$ sus.

Previous research indicated that the specific energy input might be the predominant operating parameter, impacting nutrient release (Eing et al., 2013; Lam et al., 2017). (Goettel, Eing, Gusbeth, Strassner, & Frey, 2013) showed that variation in electric field strength barely impacted cell disintegration, whereas a greater energy input led to greater membrane permeabilization and therefore a higher release of intracellular material. In the range investigated by our study, there was no clear trend suggesting the most impactful processing parameter.

Several studies reported increased lipid yields when extracting after μsPEF treatment of microalgae (Kempkes et al., 2015; Silve, Kian, et al., 2018). With an energy input of 150 kJ kg$^{-1}$ sus and 20 h of incubation, Silve, Kian, et al. (2018) achieved a lipid yield of 68.8% of total lipids in Auxenochlorella protothecoides. Overall, in this study, PEF treatments improved the lipid extractability from C. vulgaris biomass compared to the untreated control. μsPEF treatment is known to induce membrane permeabilization, but there is no evidence that it can directly impair the cell wall structure (Bensalem et al., 2018; Papachristou et al., 2020; Lam et al., 2017). Membrane permeabilization might have facilitated the penetration of solvents (Silve, Papachristou, et al., 2018). However, it remains unclear how lipid extractability could be enhanced if the intact cell wall still acts as a barrier. Degradation of the cell wall by endogenous autolytic enzymes of the microalgae after PEF treatment could further facilitate solvent interaction (Silve, Kian, et al., 2018). Such an autolysis-like process might be accelerated due to permeabilization of the cell membrane after PEF treatment (Silve, Kian, et al., 2018). Aguilar-Machado et al., 2020 suggested that disorganized molecular transport through the electroporated cell membrane could facilitate the entry of water and therefore causing a reduction of the cell osmotic pressure, producing the lysis of lysosomes and other organelles. This would cause the release of hydrolytic enzymes such as carbohydrates and proteases, acting on the cell wall (Aguilar-Machado et al., 2020). Accordingly, Bensalem et al. (2020) showed for the first time by transmission electron microscopy that PEF caused losses in structure and density in the C. reinhardtii cell wall. It is expected that if PEF treatment increases lipid extractability by indirectly triggering autolytic degradation of the cells, then the incubation time should play a significant role. When looking at other studies, it seems important that incubation after PEF treatment is known to induce membrane permeabilization, but there is no evidence that it can directly impair the cell wall. In this work, Leonhardt et al. (2020) observed no improvement in lipid extractability.

### 3.2. Effect of Incubation after PEF treatment on lipid bioaccessibility

Since Chlorella biomass is mostly used as whole cells, the bioaccessibility of lipids in the whole biomass was investigated. To further investigate the role played by incubation after PEF treatment, this study analysed lipid bioaccessibility by an in vitro digestion model of C. vulgaris biomass that was PEF-treated (5 μs at 20 kV cm$^{-1}$), followed by incubation conditions at different temperatures (4 °C, 25 °C, 37 °C) and for up to 72 h (Fig. 2). After incubation, the algae suspension was immediately snap-frozen in liquid nitrogen and freeze-dried for subsequent in vitro digestion. This combination of PEF parameters was selected among the feasible parameters previously tested in this study as yielding the highest lipid extractability. The lipid bioaccessibility better reflects the release of lipids in the gastro-intestinal conditions (enzymes, pH) and their inclusion in micelles by bile salts to be available for further absorption.

PEF treatment without incubation did not improve lipid bioaccessibility compared to the untreated control. Incubation (4 °C, 72 h) without PEF treatment led to a lipid bioaccessibility of 10.4 ± 1.7% which is lower than the values obtained for PEF-treated samples followed by incubation for at least 6 h at 25 °C or 37 °C and incubation for at least 24 h at 4 °C. A longer incubation time after PEF treatment caused an increase in lipid bioaccessibility for any incubation temperature. Fig. 2 shows a plateau of maximum lipid bioaccessibility at 18–19%. This value is reached after 12 h of incubation for the biomass kept at either 25 °C or 37 °C. A longer incubation time (48 h) was necessary at 4 °C to yield a lipid bioaccessibility of 18.7 ± 1.6%. The reason why no further increase was reached could be that the incubation combined with PEF led to the coalescence of the lipid droplets in a large unique body, as previously shown, which was harder to access by digestive enzymes (Aguilar-Machado et al., 2020; Bensalem et al., 2018; Bodénes et al., 2016).

To our knowledge, the bioaccessibility of lipids upon PEF treatment has not been investigated thus far. Rego et al. (2015) studied the bioaccessibility of chlorophyll a and b in Chlorella sp. after a PEF treatment (15 kV cm$^{-1}$, 5 μs) without a subsequent incubation and did not observe any increase in bioaccessibility in the treated biomass compared to the control.

An increase in lipid bioaccessibility with longer incubation times would agree with the theory of PEF triggering the release of autolytic enzymes that are subsequently degrading the cell wall (Martínez et al., 2018), favouring an increase in the bioaccessibility of lipids. Moreover, the kinetic behaviour of lipid bioaccessibility was proportional to temperature, which further suggests that the role played by endogenous enzymes in such a mechanism is enzymatic activity temperature-dependent. Such an enzymatic-driven process may indeed be slowed down at nonphysiological temperatures, i.e., 4 °C (Scherer et al., 2019). Accordingly, Silve, Kian, et al. (2018) observed that lipid extractability after PEF was less efficient at lower temperatures (on ice compared to 20 °C) but still beneficial when the subsequent incubation was extended to 20 h.

Accordingly, Martínez and Cebrián (2016) observed that μsPEF treatment can activate yeast autolysis, which leads to self-degradation of the yeast cell constituents by their own endogenous enzymes (proteases, glucanases). As the membranous system becomes disorganized during electroporation, endogenous enzymes are able to come into contact with the cell wall. μsPEF has the capability to induce and speed up this process by membrane permeabilization. To be effective, aqueous incubation for at least 6 h was recommended to improve carotenoids extraction.
Selection of secreted proteins tentatively involved in the hydrolysis of cell wall-related structures. A positive log2(FC) indicates that a certain protein was more abundant in PEF + incubation samples compared to the untreated/PEF sample. The analysis was performed with four biological replicates (n = 4). The effect of incubation was evaluated by comparing a sample that was treated by PEF only to one treated by PEF + incubation. Additionally, also a comparison between PEF + incubation and untreated samples was performed.

Table 1

<table>
<thead>
<tr>
<th>UniProt orthologue</th>
<th>Annotations</th>
<th>PEF + Incubation vs. Untreated</th>
<th>PEF + Incubation vs. PEF</th>
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<tbody>
<tr>
<td>A8J3T4</td>
<td>Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds</td>
<td>Log2(FC): 5.9, Adj. p value: 0.0006</td>
<td>Log2(FC): 5.2, Adj. p value: 0.0098</td>
</tr>
<tr>
<td>A8IDP6</td>
<td>Calcium-mediated signalling</td>
<td>Only present in PEF + incubation</td>
<td>Only present in PEF + incubation</td>
</tr>
<tr>
<td>A0A2K3D822</td>
<td>Modification-dependent protein catabolic process</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0A2K3D796</td>
<td>Hydrolysis</td>
<td>3.7</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

OS = Organism; GN = Gene; pfmd = Protein Family Domain; desc = pfmd description.

3.3. Proteomic analysis of secreted proteins

Label-free protein quantification was conducted on the proteins secreted by untreated cells and cells treated with PEF (5 μs, 20 kV cm⁻¹, 31.8 kJ kg⁻¹ su) with and without a subsequent incubation (24 h, 37 °C) to identify enzymes potentially involved in cell wall degradation after PEF treatment. A total of 201–290 proteins were quantified with more than 2 peptides, with less than 5 missing values and a false discovery rate (FDR) of 0.5–0.7% (Supplementary material).

Protein levels in the three tested conditions were compared by performing two-group analyses. Protein levels were considered significantly different with an adjusted p value < 0.05 and a |log2(FC)| ≥ 1. A positive log2(FC) indicated that a protein was present in higher amounts in PEF + incubation samples compared to the untreated/PEF sample. No significantly different proteins were detected when comparing the untreated sample to the PEF-treated sample. In contrast, 73 proteins were significantly different when comparing untreated vs. PEF + incubation, while 61 proteins significantly differed between PEF and PEF + incubation. All significantly different proteins were screened for their putative function in the C. reinhardtii database from the UniProt consortium. Table 1 reports proteins that were selected as potentially involved in the hydrolysis of the cell wall after PEF + incubation treatment because of significantly differently regulated in PEF + incubation vs. PEF/untreated samples and reportedly associated with protein or carbohydrate degradation. It is important to highlight that, given the lack of annotated genome and proteome sequence data for microalgae, there may be other proteins associated with cell wall lytic activity that were not identified because they have not yet been discovered and/or deposited in UniProt.

Among the analysed proteins, A8J3T4 was selected for its putative cell wall lytic activity. This protein was present in higher amounts in the cells treated with PEF followed by incubation, agreeing with previously shown data (Fig. 2) for which an incubation step is necessary to cause an effect on the cell wall and therefore an improvement in lipid bioaccessibility. Indeed, it is during an incubation time that enzymes can explicit their activity according to their kinetics. A8J3T4 has hydrolase activity, and it is reported to act on carbon-nitrogen (but not peptide) bond. This protein is also related to poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase activity, which can be associated with the deacetylation of N-glucosamine present in the C. vulgaris cell wall (Canelli, Mericano Martínez, Austin, et al., 2021).

Similarly, A0A2K3D822 was included in Table 1 because of its involvement in the protein catabolic process. Information on this protein is still very scarce; therefore, we can only hypothesize that it may be involved in the hydrolysis of protein bound to the cell wall, which is estimated to be approximately 20% of the total protein present in C. vulgaris (Berliner, 1986).

Additionally, A8IDP6 was selected because it was significantly more abundant in PEF + incubation samples than in both untreated cells (log2(FC) = 5.6) and PEF cells (log2(FC) = 5.3). A8IDP6 has calcium ion binding activity, which is relevant for the mechanisms triggered in the cell after PEF treatment. Indeed, the literature has shown that transient cytosolic calcium hubs are involved in the cellular response to stress induced by PEF (Haberkorn et al., 2021).

In addition to the direct proteome analysis, a measurement of the autolytic products in the medium could bring further evidence; however, these data would not represent any direct proof. Moreover, previous research described the monosaccharides as the main products of in vivo lysis of algae cell wall and these would be quickly assimilated by the young developing cells without leaving any evidence (Loos & Meindl, 1984). Our results go beyond what was described by Loos and Meindl (1984), who could not detect cell wall lytic activity in the medium of Chlorella fusca and suggested, therefore, that the wall-degrading activity is either inactivated or remains bound to the remnants of the old cell wall. However, to obtain a more complete overview of the enzymes
involved in cell wall degradation during incubation after PEF, further work should also assess the proteins present in the full cell suspension. Moreover, it is of utmost importance to have a more comprehensive characterization of the proteome of *C. vulgaris* to obtain a complete and well-described database.

### 3.4. PEF and cell wall-degrading enzymes

#### 3.4.1. Lipid bioaccessibility

Treatment with cell wall-degrading enzymes is another mild and targeted process that showed an increase in protein bioaccessibility in previous research (Canelli, Murciano Martínez, Maude Hauser, et al., 2021). Even though enzymatic treatment alone did not improve the lipid bioaccessibility of *C. vulgaris* cells, in this work, we included the addition of enzymes (chitinase, rhamnogalacturonase, and galactanase) in combination with PEF treatment. Cell wall-degrading enzymes may be more effective when combined with PEF because of the looser cell wall structure induced after PEF and incubation (Akaberi et al., 2019; Kalum & Hendriksen, 2010), which may facilitate the access of exogenous enzymes to their substrate.

In Fig. 3, we compared the lipid bioaccessibility of biomass that was PEF treated (5 μs at 20 kV cm⁻¹, 31.8 kJ kg⁻¹, sus), enzymatically treated (chitinase, rhamnogalacturonase, and galactanase), treated with PEF in combination with enzymes, and mechanically treated with HPH at 100 MPa. After the respective treatment on the fresh biomass, samples were incubated in potassium phosphate buffer (pH 6, 50 mM) at 37 °C for 24 h. Untreated biomass, referred to as the control 24 h, was incubated in the exact same manner.

HPH led to the highest lipid bioaccessibility, confirming what was described by Canelli, Murciano Martínez, Maude Hauser, et al. (2021). Interestingly, incubation after this treatment did not have any effect on lipid bioaccessibility, with values of 55.4 ± 2.4% for HPH without incubation and 56.5 ± 2.2% for HPH with 24 h incubation. Accordingly, Buchmann et al. (2019) did not observe any additional benefit in protein extraction with an incubation period after HPH. The mechanical disruption by HPH is severe and fast and leads to a sudden release of intracellular compounds into the surrounding medium (Carullo et al., 2018). The lipid fraction was already made accessible without the need for additional incubation.

PEF treatment showed an increase in lipid bioaccessibility by 17%, whereas enzymatic treatment did not have any relevant effect on lipid bioaccessibility (4.3% ± 3.6%) compared to the control (4.0% ± 2.1%). When the enzymes were added to the biomass in combination with a PEF treatment, no clear effect was shown (24.4% ± 2.8%) compared to PEF-treated biomass (20.9% ± 3.2%). In this experiment, the enzymes were added after the PEF treatment for practical reasons and because no difference was reported when enzymes were added before the PEF treatment (data not shown). Our hypothesis that the combination of enzymatic treatment and PEF could lead to a synergistic effect improving bioaccessibility could not be confirmed.

#### 3.4.2. Particle size

The effects of different disintegration treatments on cell integrity were also assessed by measuring the mean diameter of the volume-based droplet size distribution (d₄₃). The volume density distribution (q₃) is reported in the Supplementary material. Fig. 4 displays the mean particle diameters of untreated (control), PEF-treated, enzymatically treated (galactanase, chitinase, and rhamnogalacturonase), PEF + enzymatically treated, and treated with HPH at 100 MPa. Error bars represent the standard deviation of triplicates (n = 3).

![Fig. 4. Mean diameter of the volume-based droplet size distribution (d₄₃) of *C. vulgaris* cells that were untreated (control), PEF-treated, enzymatically treated (chitinase + rhamnogalacturonase + galactanase), PEF + enzymatically treated, and treated with HPH at 100 MPa. Error bars represent the standard deviation of triplicates (n = 3).](image)

#### 3.4.3. Scanning electron microscopy (SEM)

The effects of the different treatment conditions on *C. vulgaris* cells were further evaluated by SEM (Fig. 5). HPH at 100 MPa clearly disrupted the cells yielding to cell debris, corroborating the particle size measurements (Fig. 4). No visible effects of PEF or PEF followed by enzymatic treatment were observed, with cells maintaining their original shape and structure. Similarly, Papachristou et al. (2020) did not find any major external modification on *C. vulgaris* and *A. protothecoides* after PEF, as did T’Lam et al. (2017) in *C. reinhardtii*. Additionally, no major morphological changes in *A. protothecoides* after PEF were described by Silve, Papachristou, et al. (2018), who only observed slight shrinkage.

#### 3.4.4. Oxidative stability

The oxidative stability of the biomasses was assessed by measuring the formation of secondary oxidation products after storage for 12 weeks at 40 °C (Fig. 6). The volatiles were monitored in the untreated biomass.
Fig. 5. Representative scanning micrographs of C. vulgaris cells that were untreated (A, control), PEF treated (B), enzymatically treated (C, chitinase + rhamnolipidase + galactanase), PEF + enzymatically treated (D), and HPH treated (E).

Fig. 6. Evolution of secondary oxidation products - A) (Z)-3-hexenal and B) hexanal - during 12 weeks of storage at 40 °C for C. vulgaris biomass samples that were untreated (●, control), PEF treated (●, 5 μs, 20 kV cm⁻¹, 31.8 kJ kg⁻¹ sus), enzymatically treated (●, ET, chitinase + rhamnolipidase + galactanase), PEF + enzymatically treated (△), and treated with HPH at 100 MPa (▲). The signal is expressed as area of the targeted compound divided by the area of the internal standard (ISTD) hexanal-d₁₅. Error bars represent the standard deviation of triplicates (n = 3).

The signals of (Z)-3-hexenal and hexanal in untreated, PEF-treated, ET-treated and PEF + ET-treated biomasses did not increase during storage, showing remarkable oxidative stability. These results indicate that both PEF and ET are mild processes that preserve the quality of the lipid fraction. The signal intensities in HPH-treated biomass of hexanal and (Z)-3-hexenal were, respectively, 4.1- and 7.3-fold higher than that of the untreated biomass after 12 weeks, showing the severe effect that HPH had on lipid oxidation. Both hexane and (Z)-3-hexenal signals decreased from week 0 to week 4, which may be an indication of major oxidation occurring already throughout the disintegration treatment prior to storage. Some of the produced aldehydes could also further degrade into organic acids or form co-oxidation products with proteins, which were not detected with the current analysis. The outcomes of the sensory are in alignment with the analytical data. HPH-treated biomass presented a rancid flavour already at time 0, whereas the untreated and enzymatic- and PEF-treated biomasses showed a subtle smell over the entire storage period.

This study highlights the relevant potential of PEF treatment: by employing this technique, we could improve lipid bioaccessibility in C. vulgaris biomass while preserving its oxidative stability.

4. Conclusion

PEF successfully enhanced the lipid bioaccessibility of C. vulgaris biomass while preserving the oxidative stability. The addition of exogenous enzymes to the PEF treatment did not lead to any clear increase; as a result, our hypothesis was rejected. Moreover, it was shown how incubating the biomass suspension after PEF treatment was necessary to increase lipid bioaccessibility. Proteome analysis identified four proteins that may be involved in cell wall lytic activity during incubation after PEF. Future work should focus on further understanding the mechanism behind incubation after PEF, understanding the dynamics of pore formation, and studying the potential effects mediated by endogenous cell wall-degrading enzymes. Moreover, cell viability could be an interesting indicator to study as it can relate to the cell lysis induced by endogenous cell wall-degrading enzymes after PEF and incubation.

Declaration of Competing Interest

The authors declare that Nestlé Research supported the research conducted for this manuscript. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifsset.2021.102897.

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