Effect of Pulsed Electric Fields and High Pressure Homogenization on the aqueous extraction of intracellular compounds from the microalgae *Chlorella vulgaris*

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Abstract

Pulsed Electric Fields (PEF) and High Pressure Homogenization (HPH) are promising and scalable cell disruption technologies of microalgae cells. In this work, the permeabilization degree, morphological properties, and extractability of intracellular compounds from microalgae *Chlorella vulgaris* suspensions (1.2%, w/w) were investigated as a function of PEF treatment at different electric field strengths (10–30 kV/cm) and total specific energy input (20–100 kJ/kg), in comparison with the more disruptive HPH treatment (150 MPa) at different number of passes (n_P=1-10). The conductivity and the particle size analyses, as well as the SEM images, clearly showed that PEF induces the permeabilization of the cell membranes in an intensity-dependent manner, without producing any cell debris, whereas HPH treatment causes the total disruption of the algae cells into small fragments. Coherently with the lower permeabilization capability, PEF promoted the selective extraction of carbohydrates (36 %, w/w, of total carbohydrates), and low molecular weight proteins (5.2 %, w/w, of total proteins) with a relatively low energy input (2.9 kWh/kg_{DW}). On the other hand, at the biomass concentration tested in this work, HPH required a significantly higher energy (20.0 kWh/kg_{DW}) to induce the undifferentiated release of all the intracellular content, resulting in a 1.1 and 10.3 fold higher yields than PEF, respectively of carbohydrates and protein. These results suggest that, in a multi-stage biorefinery, PEF could represent a suitable cell disruption method for the selective recovery of small-sized cytoplasmic compounds, while HPH should be placed at the end the cascade of operations allowing the recovery of high molecular weight intracellular components.

Keywords— Microalgae; Pulsed electric fields; High pressure homogenization; Cell disruption; Proteins; Carbohydrates.

1. Introduction

Chlorella vulgaris is a freshwater eukaryotic microalga with a mean diameter ranging from 2.5 to 5 μm [1] belonging to the division of Chlorophyta. It has drawn large attention over the last decades because of its capability to accumulate large amounts of valuable components, especially proteins (51 – 58 %), but also polyunsaturated fatty acids (14 – 22 %), carbohydrates (12 – 17 %), nucleic acids (4 – 5 %), vitamins and minerals [2, 3]. Moreover, it accumulates also chlorophyll (1-2%) that imparts the characteristic green color, masking the other less concentrated pigments, such as lutein and other carotenoids [4]. The extraction of all these intracellular compounds, which can be used as natural additives or active ingredients for food, cosmetic, pharmaceutical and animal feed products, as well as in the production of biofuels [5, 6], is crucial for achieving an economically feasible microalgae biorefinery [7].

However, these compounds are located in different parts of the cells, protected by the rigid cell wall and membranes surrounding the cytoplasm and the internal organelles (e.g., chloroplast), which greatly limit their rate of mass transfer during extraction. Conventional extraction processes of these intracellular compounds are often conducted from dry biomass with organic or aqueous solvents, depending on the polarity of the target compounds [8, 9]. However, these methods suffer from several limitations, namely the long extraction times and the use of relatively large amounts of solvent, and may lead to the co-extraction of undesirable components, with increased downstream processing costs [7, 10]. In addition, the drying of microalgal biomass is reported to be one of the major energy-consuming steps within the overall process and is responsible for significant losses of valuable compounds [5, 7].

For these reasons, the application of conventional or innovative cell disruption methods to wet biomass may considerably promote the implementation of the biorefinery concept on microalgae, enabling a faster and more efficient release of intracellular compounds at low temperature. This also contributes to limit the degradation of the extracts and promotes the reduction of energy costs, of solvent consumption, as well as of the extraction time [7, 10].

Among the cell disruption methods, the Pulsed Electric Fields (PEF) and the High Pressure Homogenization (HPH) treatments have emerged as promising methods for the mild and complete disruption of biological cells, respectively [9 – 14]. Moreover, both PEF and HPH can be easily scaled up to process large volumes of wet biomass in a wide range of solids concentration, thus avoiding the need for energy-intensive drying and possibly allowing to reduce the energy demand per unit biomass [5, 15 – 18].

In PEF processing, the biomaterial is placed between two electrodes of a treatment chamber and exposed to high intensity electric fields (10-50 kV/cm), applied in the form of repetitive pulses of very short duration (from several nanoseconds to few milliseconds), which induce the permeabilization of cell membranes by electroporation, facilitating the subsequent release of intracellular matter [19]. Several studies highlighted the effectiveness of PEF to enhance the selective recovery of intracellular compounds from wet microalgal biomass, including lipids [20, 21], pigments [8, 10, 14, 22 – 23], carbohydrates, and water-soluble proteins of small molecular weight [6, 9, 14, 18, 23].

However, the extraction of molecules of higher molecular weight, or more bounded to the intracellular structure (e.g., proteins), requires the application of more effective cell disruption techniques, such as HPH [10].

HPH is a purely mechanical process, during which a liquid dispersion of plant material or a cell biosuspension is forced by high pressure (50-300 MPa) through a micrometric disruption chamber, where the velocity increases rapidly and the pressure decreases to atmospheric conditions as the suspension exit the unit [15]. As a result, the biological cell suspension is subjected to extremely intense fluid-mechanical stresses (shear, elongation, turbulence, and cavitation), which cause the physical disruption of the cell wall and membranes [16, 24, 25].

Due to its high cell disruption efficiency [7], HPH is reported to markedly increase the extraction yield of several intracellular compounds from microalgae [7, 14, 26 – 28]. However, the HPH treatment causes the non-selective release of intracellular compounds, with the concurrent

dispersion of cell debris, complicating the downstream separation processes [14]. Moreover, because of the intense interfacial shear stresses and inherent heating occurring in the homogenization valve, which might induce the degradation of compounds, such as proteins [29 - 30], HPH treatments always require an efficient heat dissipation at the homogenization valve.

Although several studies have already highlighted the potential of PEF and HPH pre-treatments in the microalgae biorefinery, to date, only the study of Safi et al. [28] has addressed the comparison of their efficiency in terms of cell disintegration, energy input and release of soluble proteins from microalgae *Nannochloropsis gaditana.* However, suspensions of this microalgae were prepared from a frozen paste and at different biomass concentration for PEF (15-60 g_{DW}/L) and HPH (100 g_{DW}/L) treatments.

Moreover, a deeper knowledge regarding the impact of these novel technologies at micro and macro scale is required, which is thoroughly necessary in view of their use in a cascade biorefinery approach of microalgae, where the control of the degree of cell breakage could be exploited to enable the fine tuning of the recovery process of intracellular components [6, 7, 31].

Therefore, the aim of this study is to investigate comparatively the effects of the main process parameters of both PEF and HPH treatments on the cell disintegration degree, the energy consumption, and the release of intracellular compounds (ionic substances, proteins, and carbohydrates) from fresh *C. vulgaris*, in order to select, for each investigated technology, the best treatment conditions in the perspective of their implementation in a biorefinery scheme.

2. Materials and Methods

2.1. Microalgae and cultivation

The microalgal strain used in this study was *Chlorella vulgaris* (CCAP 211), purchased from the Culture Collection of Algae and Protozoa (Argyll, UK). It was cultivated in modified Bold's basal medium [32] at pH 7.0 ± 0.5, in a 5 L horizontal tubular photobioreactor illuminated by four 40 W fluorescent lamps from one side [33]. The composition (per liter of distilled water) of the modified

118 medium was as follows: 1.5 g $NaNO_3$, 0.45 g $MgSO_4 \cdot 7H_2O$, 0.15 g NaCl, 0.45 g $K_2HPO_4 \cdot 3H_2O$,
119 1.05 g KH_2PO_4, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.003 g vitamin B_1, 7.5 10^{-6} g vitamin B_8, 7.5 10^{-6} g vitamin
120 B_{12} and 6 mL of P-IV solution (Sigma Aldrich, Milan, Italy). The culture was aerated at a rate of
121 1000 cm^3/min with an air flow containing 2 % (v/v) carbon dioxide. Growth conditions were
122 monitored by optical density (OD) measurements at 625 nm using a UV–Vis spectrophotometer
123 (Lambda 25 model, Perkin Elmer, Milan, Italy). The pH of the culture medium was monitored
124 during the experiments using a pH meter (pH211, HANNA Instruments, Woonsocket, RI).
125 Microalgae were harvested during the end of the exponential phase at a biomass concentration of
126 about 3 g_{DW}/L of suspension and then concentrated by centrifugation (centrifuge model 42426,
127 ALC, Milan, Italy) at 4000×g for 10 min at 20 °C up to a final concentration of 12 g_{DW}/L. The
128 concentrated biomass was pre-packed in high-density polyethylene bottles (Nalgene) cooled at 4
129 °C, and sent to the laboratories of ProdAl Scarl (University of Salerno, Italy). Samples were
130 transported in an EPS box under refrigerated conditions and delivered within 24 hours. PEF and
131 HPH treatments were performed on the delivery day. The initial electrical conductivity of algae
132 suspension was about 1.78±0.03 mS/cm at 25 °C (Conductivity meter HI 9033, Hanna Instrument,
133 Milan, Italy).
134

135 **2.2. PEF Treatment**

136 PEF treatments were conducted in a bench-scale continuous flow PEF unit, described in detail in a
137 previous work [6]. Briefly, the unit consisted of a peristaltic pump to control the flow rate of the
138 algae suspension through the system. The inlet temperature of the algae suspension was controlled
139 using a stainless steel coil immersed in a water heating bath. The PEF treatment zone consisted of
140 two modules, each made of two co-linear cylindrical treatment chambers, hydraulically connected
141 in series, with an inner radius of 1.5 mm and a gap distance of 4 mm. The treatment chambers were
142 connected to the output of a high voltage pulsed power (20 kV-100 A) generator (Diversified
143 Technology Inc., Bedford, WA, USA) able to deliver monopolar square pulses (1-10 μs, 1-1000

Hz). The maximum electric field intensity (E, in kV/cm) and total specific energy input (W_T, in kJ/kg_{susp}) were measured and calculated as reported in Postma et al. [6]. T-thermocouples were used to measure the product temperature at the inlet and outlet of each module of the PEF chamber.

During PEF treatment, the algae suspension (12 g_{DW}/L) was pumped, from a feeding tank under stirring, through the treatment chamber at a constant flow rate of 33.3 mL/min. The pulse length was fixed at 5 μs, while the electric field strength (E) of 10, 20 and 30 kV/cm and total specific energy input (W_T) of 20, 60, and 100 kJ/kg_{susp} were set by varying the applied voltage and the pulse repetition frequency, respectively. All the experiments were carried out at an inlet temperature of each module of PEF chamber of 25 °C, while the maximum temperature increase at the exit of each module due to Joule effect never exceeded 10 °C.

At the exit of the treatment chamber, treated and untreated (without applying PEF treatment) algae suspensions were collected in plastic tubes and placed in an ice water bath to be rapidly cooled up to a final temperature of 25 °C before undergoing the aqueous extraction process.

2.3. HPH treatment

HPH treatments were carried out by using an in-house developed laboratory scale high-pressure homogenizer [34]. The *C. vulgaris* suspensions, at the same concentration as for PEF treatment tests (12 g_{DW}/L), were forced to pass through a 100 μm diameter orifice valve (model WS1973, Maximator JET GmbH, Schweinfurt, Germany) upon pressurization by means of an air driven Haskel pump (model DXHF-683, EGAR S.r.l., Milan, Italy). The pressure drop across the orifice and the volumetric flow rate of the suspension were 150 MPa and 155 mL/min, respectively. In this work, the algae suspensions were treated with a different number of passes (n_P = 1 – 10). In order to prevent excessive heating, after each pass, the suspensions were cooled at 25°C by passing through a tube-in-tube heat exchanger, located downstream of the orifice valve.

2.4. Water extraction

After processing, untreated and treated (PEF, HPH) samples were allowed to stand for 1 h at 25 °C under shaking at 160 rpm to allow intracellular components to diffuse out of the cells. After this resting time, the cell suspensions were centrifuged (10 min, 5700×g) (PK121R model, ALC International, Cologno Monzese, IT) and the supernatants were transferred to fresh tubes and stored at -20 °C until further analysis.

2.4.1. Electrical conductivity measurement

Changing of the electrical conductivity (σ) of untreated and treated (PEF, HPH) algae suspensions was monitored periodically (Conductivity meter HI 9033, Hanna Instrument, Milan, Italy) over time for up to 24 h by maintaining the samples in a water bath set at a constant temperature of 25 °C.

The collected data were used also to evaluate (Eq. 1) the cell disintegration index (Z_p), which has been successfully used as a reliable macroscopic indicator of the degree of cell membrane permeabilization induced by PEF [35-36]:

$$Z_P = \frac{\sigma_{PEF,t} - \sigma_0}{\sigma_{MAX} - \sigma_0} \quad (1)$$

where $\sigma_{PEF,t}$ is the electrical conductivity of PEF treated biosuspensions measured at time t, σ_0 is the conductivity of untreated algae suspension at time 0, and σ_{MAX} is the conductivity of biosuspension with completely disrupted algae cells (HPH treatment: P = 150 MPa, n_P = 5). The Eq. (1) gives Z_p=0 for intact algae cells and Z_p=1 for fully disrupted cells.

2.4.2. Particle size distribution (PSD) analysis

PSD of untreated and treated (PEF or HPH) algae suspensions were analyzed by laser diffraction at 25 °C, using a MasterSizer 2000 particle size analyzer (Malvern, United Kingdom). Using the Fraunhofer approximation, which does not require the knowledge of the optical properties of the sample, the size distribution of the algal suspension was determined, from which the mean particle

size expressed as volume moment mean diameter ($D_{4,3}$) was evaluated for each processing condition. The parameters used in the determination of the PSD were the properties of water at 25 °C (refraction index = 1.33), which was used as dispersant medium.

2.4.3. Scanning Electron Microscopy (SEM) analysis

The morphological features and cellular details of algae cells were analyzed by using a Scanning Electron Microscopy (SEM). Pellets derived from the centrifugation of untreated and treated (PEF or HPH) algae suspensions were prepared as described by Kunrunmi et al. [37] with some modifications. At first, samples were fixed by immersion in a 2 % (v/v) glutaraldehyde phosphate buffer solution. The buffer was then removed and the pellets were osmotically dehydrated with ethanol solutions of increasing concentration (25%, 50%, 75%, and 100% (v/v)). Afterwards, ethanol was removed from the pellet with supercritical CO_2 in a Quorum K850 critical point dryer (Quorum Technologies Ltd, London, UK) and the latter was then metallized by means of the Agar Auto Sputter Coater 103A (Agar Scientific Ltd, Stansted, UK), before being analysed in a high-resolution ZEISS HD15 Scanning Electron Microscope (Zeiss, Oberkochen, Germany).

2.4.4. Dry Matter (DM) content analysis

Approximately 40 mL of the supernatants collected from the centrifugation of untreated and treated (PEF or HPH) algae suspension were placed in aluminum cups and dried in an oven (Heraeus, Germany) at 80 °C until constant mass was achieved. DM was gravimetrically determined by weighing the samples before and after drying on an analytical balance (Gibertini, Italy). The dry mass content was expressed as g of dry matter/kg of supernatant ($g_{DW}/kg_{SUP.}$).

2.4.5. Proteins Analysis

The water-soluble protein concentration in the supernatants was evaluated using the Lowry method [38], with some modifications. The Folin-Ciocalteau reactive [39], purchased from Sigma Aldrich

(Milan, Italy), was initially diluted in two volumes of ultra-pure water (1:2, v/v); then 0.5 mL of the diluted reactive were added to 1 mL of supernatant, previously mixed with 5 mL of the reactive "C" [50 volumes of reactive "A" [(2% (w/v) Na_2CO_3 + 0,1 N NaOH) + 1 volume of reactive "B" (1/2 volume of 0.5% (w/v) $CuSO_4 \cdot 5H_2O$ + 1/2 volume of 1% $KNaC_4H_4O_6 \cdot 4H_2O$)] (Sigma Aldrich, Milan, Italy). Absorbance was measured at 750 nm against a blank (5 mL reactive "C" + 1 mL deionized water + 0.5 mL Folin-Ciocalteau reactants) 35 min after the start of the chemical reaction by using a V-650 Spectrophotometer (Jasco Inc. Easton, MD, USA). Bovine serum albumin (BSA) (A7030, Sigma Aldrich, Milan, Italy) was used as standard and the results were expressed as mg equivalent of BSA per g of dry biomass.

2.4.6. Carbohydrates Analysis

The total carbohydrates concentrations of the supernatants were analyzed according to the method of DuBois et al. [40]. 0.2 mL of 5% (w/w) phenol and 1 mL of concentrated sulfuric acid (Sigma Aldrich, St. Louis, USA) was added to 0.2 mL of diluted supernatant (Dilution Factor = 5). Samples were incubated at 35 °C for 30 min before reading the absorbance at 490 nm against a blank of 0.2 mL 5% (w/w) phenol, 1 mL concentrated sulfuric acid and 0.2 mL of deionized water. D-Glucose (G8270, Sigma-Aldrich, Milan, Italy) was used as a standard and the results were expressed as equivalent mg of D-glucose per g of dry biomass.

2.5. Statistical Analysis

All treatments and analyses were performed in triplicate and the results were reported as mean values with their respective standard deviations. Statistically significant differences ($p \leq 0.05$) between the means were evaluated using one-way analysis of variance (ANOVA), performed with SPSS 20 (SPSS Inc., Chicago, USA) statistical package, and the Tukey's test.

3. Results and Discussion

3.1. Impact of PEF and HPH treatments on the release of ionic intracellular components

The results of the measurements of the electrical conductivity of microalgae suspension have been successfully used as a valuable indicator to assess and quantify the amount of ionic intracellular components released from algae upon the application of the different cell disruption methods [9, 14, 18, 41].

Figure 1 shows the effect of PEF treatment intensity (E, W_T), as well as the number of HPH passes (n_P) on the conductivity profiles of *C. vulgaris* suspensions over time at 25 °C.

For the sake of comparison, in the same graphs, also the time-conductivity profile of the untreated algae suspension is shown. Results demonstrate that the initial conductivity (1.78 mS/cm) of untreated suspension increased only slightly with the incubation time, likely due to a spontaneous release of a small fraction of intracellular ionic compounds, reaching a saturation value (1.82 mS/cm) already after 30 min of incubation.

The electroporation effect induced by the application of PEF treatment at different field strength (10-30 kV/cm) and energy input (20-100 kJ/kg) promoted a rapid release of the ionic intracellular compounds, which resulted in a substantial increase in the electrical conductivity, with respect to the untreated suspension (Figs. 1a-c). After PEF treatment, the saturation value, reached after 1 h of incubation, increased with the increase of the field strength and energy input, due to a faster diffusion of the ionic intracellular substances into the aqueous phase, which is in agreement with the electroporation theory. A further increase of the incubation time did not cause any significant increase in the conductivity value, which leveled off to a final value in the range between 2.08- and 2.21 mS/cm, depending on the PEF treatment intensity applied.

A progressive increase of the content of ionic compounds in the extracellular medium when increasing the intensity of the PEF treatment was also observed by Goettel et al. [18], which also reported that 79% of the total released ions from *Auxenochlorella protothecoides* already occurred in the first hour after treatment. Similarly, Postma et al. [6] and Pataro et al. [9] reported that

increasingly intense PEF treatments promoted the progressive permeabilization of the *C. vulgaris* cells, and that an incubation time of 1 h was sufficient to allow small ions to diffuse out of the cells, in agreement with the results reported in Figs. 1a-c.

The data of Figs. 1a-c suggest the achievement of an irreversible electroporation after PEF treatment [18], by markedly improving the mass transfer rate of ionic compounds through the cell structure, which is partially damaged by the electrical treatment.

Coherently with this assumption, when compared to PEF treatments, the HPH treatments resulted in a significant increase in the conductivity of *C. vulgaris* suspension, whose extent was greater when increasing the number of HPH passes, as shown in Fig. 1d. More specifically, the mechanical disruption of the algae cells appeared to be extremely fast, leading to an almost instantaneous diffusion of the intracellular compounds into the aqueous phase, as observed also by Safi et al. [26]. Considering that HPH is a purely mechanical on-off disruption process, it is likely that after each pass a certain fraction of algae cells are completely broken, while the residual cells remain intact, in agreement with the observation of the significant extraction yield of ionic compounds after the multi-pass HPH treatment, as reported in Fig. 1d.

Coherently, the results of Fig. 1d also show that above 5 passes, the conductivity did not change significantly, and tended to an asymptotic value of 2.3 mS/cm, because the residual fraction of intact cells has become extremely small. However, such asymptotic value was significantly higher than that measured after the application of the most intense PEF treatment, confirming that the release of ionic compounds by PEF is incomplete.

Thus, setting the conditions of Z_P=1 in correspondence of 5 HPH passes, the cell disintegration efficiency of PEF varied in a range dependent on the treatment intensity applied: the lowest value of Z_P (0.47) was observed for a PEF treatment intensity of 10 kV/cm and 20 kJ/kg, whereas, increasing the electric field strength and energy input, a maximum Z_P value of 0.85 was recorded.

3.2. Effect of PEF and HPH treatment on *C. vulgaris* cell structure

298 In this work, particle size distribution (PSD) analyses and SEM observations were carried out in
299 order to gain insight on the impact of PEF and HPH treatments on the size and structure of *C.*
300 *vulgaris* cells.
301 Fig. 2 depicts the mean particle size $D_{4,3}$ for untreated (control), PEF treated at variable field
302 strength and energy inputs, and HPH (n_P = 5) treated microalgae suspensions.
303 The PSD curves of untreated algae suspension revealed the presence of a single peak between 1 and
304 10 μm (data not shown), which was characterized by a mean cell size of 3.03±0.03 μm (Fig. 2).
305 The size distribution curve of PEF-treated algae suspension was very similar to that of the untreated
306 sample (data not shown), showing only a slight decrease of the mean cell size with increasing the
307 treatment intensity (E and W_T). In fact, the value of the mean cell size significantly ($p \leq 0.05$)
308 decreased by about 6% only upon the application of the most intense PEF treatment conditions
309 (E=30 kV/cm, $W_T \geq 60$ kJ/kg) (Fig. 2). These results seem to confirm that PEF is a relatively mild
310 cell disruption method, preserving the initial structure of the algae cells.
311 The application of 5 HPH passes, instead, led to a significant change in the PSD curves of the
312 microalgae suspension, highlighting a bimodal distribution, in which a second peak between 0.1 and
313 1 μm appeared (data not shown). As a result, a strong reduction in the mean cell size down to a
314 value of 2.22±0.04 μm was observed (Fig. 2), which is likely due to the complete cell disruption
315 and the consequent formation of cell debris.
316 Partially in contrast with these results, Spiden et al. [42] found that the effect of an HPH treatment
317 on *Chlorella* microalgae at different pressures (P = 30 – 107 MPa) only led to a slight decrease in
318 the mean cell size, which was in agreement with the only partial fragmentation achieved.
319 Eventually, in our case, the application of a higher pressure (P = 150 MPa) was capable of inducing
320 the complete disruption of the cells, which is in agreement with the previous findings of Safi et al.
321 [28]. Similarly, Shene et al. [27] and Samarasinghe et al. [17], studying the effect of HPH

processing (P = 70 – 310 MPa, n_P = 1 – 6) on *Nannochloropsis oceanica* microalgae, reported that the cells were fully disrupted in fragments, with a corresponding decrease in mean particle size.

In order to better interpret the results of Figs. 1 and 2, also SEM analyses were carried out on untreated, PEF-treated (E = 20 kV/cm; W_T = 20 – 100 kJ/kg), and HPH-treated (n_P=5) microalgae, as shown in Fig. 3.

Untreated *C. vulgaris* cells exhibited their characteristic near-spherical shape and a diameter ranging from 1.5 and 4.5 μm, which relate to the findings reported in the current literature [43].

The SEM images of Fig. 3 clearly show the different impact of PEF and HPH treatments on the microalgal cell structure. Interestingly, the results clearly show, for the first time, the occurrence of a shrinkage phenomenon in PEF-treated algae cells, which, gradually lose their initial near-spherical shape with increasing the applied energy input but were never disintegrated into cell debris. The observed shrinkage could be associated with the partial release of the intracellular compounds through the electroporated cell membranes (Fig. 1b), which led in some cases to cell collapse (Fig. 3). Similar results were observed at different electric field strengths (data not shown).

In contrast, a complete disruption of the cells and the formation of small fragments was observed after 5 passes HPH treatment, which was consistent with the results of Figs. 1 and 2.

Similarly, the formation of cell fragments was observed by other authors upon the application of HPH treatments to *Chlorella* [26, 44] and *Neochloris abundans* [45] microalgae, highlighting the strong efficacy of HPH treatment as a method of complete cell disruption.

3.3. Influence of PEF and HPH treatments on the release of intracellular compounds

The cell disruption efficiency of PEF and HPH treatments were also compared by monitoring the extractability of intracellular compounds by dry matter analyses and by measuring the amount of water-soluble compounds (proteins and carbohydrates) released into the supernatants obtained from untreated and treated (PEF, HPH) algae suspension.

3.3.1. Dry Matter of supernatants

348 The total amount of released intracellular compounds was evaluated by measuring the dry matter
349 content in the supernatant of untreated, PEF-treated at different field strength and energy inputs, and
350 HPH-treated (n_p=5) microalgae suspensions.
351 The results showed in Fig. 4 are in agreement with the conductivity measurements of Fig. 1. The
352 application of PEF treatment markedly increased the dry matter content of supernatants, when
353 compared with the untreated sample. A higher field strength and energy inputs resulted in a higher
354 degree of membrane permeabilization, leading to a significantly ($p \leq 0.05$) higher release of
355 intracellular compounds into the aqueous phase. The maximum value of dry matter content was
356 detected at the most intense PEF treatment conditions (E = 30 kV/cm; W_T = 100 kJ/kg), which was
357 2.4 times higher than that detected in the supernatant of the untreated microalgae suspension.
358 However, among PEF treated samples, statistically significant differences ($p < 0.05$) were observed
359 only between samples treated at 10 kV/cm and 20 kJ/kg with those treated either at 20 kV/cm and
360 100 kJ/kg or at 30 kV/cm for an energy input greater than 20 kJ/kg. Remarkably, the results of Fig.
361 4 are in agreement with the previous findings of Goettel et al. [18]. The authors observed a
362 continuous increase of cell components in the medium surrounding *Auxenochlorella protothecoides*
363 when the energy input was increased up to 200 kJ/kg at a constant field strength (34 kV/cm).
364 Moreover, in our case, the release of intracellular soluble compounds by PEF varied in the range 13
365 – 18 % of total cell dry weight, which is also in agreement with the results obtained by Goettel et al.
366 [18], who found that a PEF treatment at 30.5 kV/cm and 155 kJ/kg caused the spontaneous release
367 of intracellular matter up to 15% of the initial biomass dry weight (109 g/kg_{DW}). Pataro et al. [9]
368 also observed a slightly higher leakage of intracellular matter from *C. vulgaris* cells with increasing
369 the field strength (from 27 to 35 kV/cm) and energy input (from 50 to 150 kJ/kg).
370 The stronger cell disintegration effect, achieved after 5 passes HPH treatment (Figs. 1-3), led to a
371 highly efficient extraction of intracellular matter (Fig. 4), whose extent reached up to 64% of the
372 total cell dry weight.

373 The results of Fig. 4 were also confirmed by visual observation of the supernatants. In fact, while
374 the supernatants obtained from centrifugation of fresh and PEF treated microalgal suspensions
375 appeared colorless, those obtained from HPH treated samples were characterized by a green color
376 (data not shown). This was likely due to the presence of cell debris (Fig. 3) containing green
377 pigments, which, being extremely reduced in size, did not precipitate in the pellet after
378 centrifugation [26]. With this assumption, it can be stated that part of the supernatant dry matter
379 content from the HPH treated cells could be due to the presence of submicrometric residues, which
380 remained suspended in the aqueous phase, making the downstream separation processes more
381 difficult.
382
383 **3.3.2. Extractability of carbohydrates and proteins**
384 Fig. 5 shows the concentration (on DW basis) of carbohydrates (Fig. 5a) and proteins (Fig. 5b)
385 detected in the aqueous supernatant obtained 1 h after PEF treatment of *C. vulgaris* suspensions at
386 different field strength and energy input.
387 When no PEF treatment was applied, only very low amounts of carbohydrates (7.06 mg/g_{DW}) and
388 proteins (1.65 mg/g_{DW}) were released in the aqueous phase, which may be ascribed to either a
389 concentration gradient across the intact cell membranes or to a spontaneous cell lysis.
390 The permeabilization effect of the cell membranes induced by the application of PEF treatment,
391 instead, improved the mass transfer of intracellular compounds, leading to a significantly ($p \leq 0.05$)
392 higher content of both carbohydrates and proteins, as compared to the untreated samples, being the
393 extraction efficiency increased up to 20-fold for proteins and 8-fold for carbohydrates.
394 Among the PEF treated samples, the effect of the field strength applied (Fig. 5) appeared less
395 important than that of the energy input within the investigated range, especially for the protein
396 extraction, which is in agreement with previous findings [9, 41]. In particular, a significant ($p \leq$
397 0.05) increase in the content of both intracellular compounds was detected only when the field
398 strength was increased from 10 to 20 kV/cm and for a fixed energy input of 100 kJ/kg for proteins,

399 and 20 kJ/kg for carbohydrates, respectively. In contrast, while significant differences ($p \leq 0.05$) in
400 the protein content were detected when PEF treatments were carried out at different energy inputs
401 (Fig. 5a), regardless of the field strength applied, only a slighter effect of the energy input was
402 observed for the extraction of carbohydrates, which was significant ($p \leq 0.05$) only when the energy
403 input was increased from 20 to 60 kJ/kg at 10 kV/cm and between 20 and 100 kJ/kg at 30 kV/cm
404 (Fig. 5b).
405 A slightly increasing trend when increasing the energy input from 50 to 150 kJ/kg was previously
406 observed by both Goettel et al. [18] with the microalgae *A. protothecoides* at a fixed field strength
407 applied of 34 kV/cm, and Pataro et al. [9] with the microalgae *C. vulgaris* at a fixed field strength
408 applied of 27 kV/cm. Postma et al. [6], instead, did not find any significant difference in the release
409 of carbohydrates from *C. vulgaris* treated by PEF at 50 and 100 kJ/kg at 17.1 kV/cm.
410 From the results of Fig. 5 it can be concluded that a field strength of 20 kV/cm and an energy input
411 of 100 kJ/kg could be sufficient to achieve efficient protein and carbohydrates extraction by PEF.
412 In particular, assuming a carbohydrates and proteins content of 16% and 61 % on DW, respectively
413 [6], the amount of these compounds released after PEF treatment (20 kV/cm, 100 kJ/kg) was 35.8%
414 (w/w) of total carbohydrates (approximately 5.7% DW biomass) and 5.2% (w/w) of total proteins
415 (approximately 3.2% DW biomass). These values are in the same range of values reported by other
416 authors [6, 12, 13, 22, 28]. In the study of Postma et al. [6], for example, it was observed that the
417 application of a PEF treatment at room temperature resulted in an extraction yield of 22-24% for
418 carbohydrates, and 3.2-3.6% for proteins, when the energy input was increased between 50 and 100
419 kJ/kg at a field strength applied of 17.1 kV/cm. Moreover, no further improvement of the diffusion
420 kinetics of intracellular compounds was detected when PEF effect was combined with the thermal
421 treatments at a higher temperature [6] or elevated pH [23].
422 These results suggest that PEF was successful in opening pores on membranes of *C. vulgaris* cells
423 (Figs. 1, 3), allowing the selective release of carbohydrates and small-sized cytoplasmic proteins,
424 while hindered simultaneously the diffusion of most proteins, which are likely larger and more

425 bonded to the cell structure. This hypothesis is supported by some literature evidence. In fact, it has
426 been reported that the proteins of *C. vulgaris* species have molecular weights ranging from 12 to
427 120 kDa [26], and that PEF was able to selectively enhance only the extraction of small protein
428 materials, with molecular weight lower than 20 kDa, while larger molecules remained entrapped
429 inside the cells, being unable to cross the permeabilized cell membrane [6]. In contrast, as suggested
430 by the SEM images (Fig. 3), PEF merely electroporated the algae cells without altering the
431 extremely resistant rigid cell wall of *C. vulgaris*, which represents a further barrier against the
432 extraction of proteins [46]. Moreover, it is estimated that 20% of *C. vulgaris* proteins are bonded to
433 the cell wall [47], and therefore they likely remained entrapped in the pellet along with the water-
434 insoluble fraction of proteins. This would contribute to further explain the relatively low amount of
435 proteins released after PEF (Fig. 5b).
436 Therefore, the disruption of the rigid cell wall of *Chlorella vulgaris* appears to be a crucial step to
437 enhance the protein release [48], hence requiring a more effective cell disruption techniques, such
438 as high pressure homogenization [10].
439 Fig. 6 reports the amount of carbohydrates and proteins released upon the application of HPH
440 treatment (150 MPa) as a function of the number of passes. In agreement with the results of Fig. 1d,
441 a significant fraction *C. vulgaris* cells was already disrupted after 1 pass and water gained the
442 access to the cytoplasmatic content, allowing the release of a certain amount of carbohydrates and
443 proteins.
444 The subsequent HPH passes led to the further release of carbohydrates and proteins, whose amount
445 gradually increased up to reaching a saturation value after 5 passes, which was, with respect to the
446 control sample, 9-fold higher for carbohydrates and 200-fold higher for proteins.
447 An asymptotic behavior in the extraction yield of intracellular compounds, such as chlorophyll and
448 carotenoids, as a result of the increased degree of cell disruption with increasing the number of
449 passes has previously been shown by Xie et al. [49]. These authors reported that the release of these
450 pigments from HPH-processed *Desmodesmus* microalgae could be enhanced by increasing the

number of passes up to a saturation value above which no additional leakage of interest compounds could be achieved.

From the results of Fig. 6, using the same assumption for the composition of *C. vulgaris* cells used for PEF [6], the amount of carbohydrates and proteins released after 5 HPH passes was 41.9% (w/w) of total carbohydrates (approximately 6.7% DW biomass) and 54.1% (w/w) of total proteins (approximately 33.0% DW biomass).

Similarly, Safi et al. [26, 48] found that, among the different cell disruption techniques, including the chemical treatments, ultrasonication, and manual grinding, HPH was the most efficient one, and that after an HPH treatment (P=270 MPa, n_p=2) water gained rapid access to the cytoplasmic proteins and infiltrated the chloroplast to recover 50-66% of proteins from the total protein content of *C. vulgaris* cells. However, even from these results it appears that, despite the high cell disruption efficiency of the HPH treatment, the complete release of all the proteins contained in the algae could not be reached, because of the rigidity of the cell wall [50], as well as the insoluble nature of some proteins that remained in the pellet [51]. In this frame, it has been demonstrated that the combination of higher HPH pressure than that used in our work with chemical cell lysis could further improve the extractability of protein from algae cells. In particular, Ursu et al. [52] observed that 2 HPH passes at 270 MPa allowed the recovery of 98% of total protein content of the microalgae *C. vulgaris* when the pH of the suspension was maintained at 12.

The comparison between the results of Figs. 5 and 6 highlights the capacity of PEF to efficiently release low molecular weight molecules, such as carbohydrates, to an extent comparable to the one obtained from HPH treatment for a sufficiently high number of passes (85.4%). This selectivity of PEF towards the carbohydrates could be advantageously exploited for specific applications [41]. In contrast, despite the huge increase in protein extraction caused by PEF processing with respect to untreated microalgae suspension, the protein yields are still relatively low being 10 fold lower than that detected in HPH treated samples.

476 However, next to the extraction yield of valuable intracellular compounds, the feasibility of a cell
477 disintegration technique should also take into account the total energy consumed. In this work, to
478 enable the comparison between PEF and HPH, on the basis of the work of Günerken et al. [7], the
479 total energy consumed (in kWh/kg_{DW}) was calculated as the energy to disrupt 1 kg of dry
480 microalgae biomass (= consumed energy / (treated biomass · cell disruption yield)), considering a
481 cell disruption yield of, respectively, 100% for 5 passages HPH treatment (Z_P=1), and 81%
482 (Z_P=0.81) for PEF treatment (20 kV/cm, 100 kJ/kg). For HPH, an overall efficiency of the pumping
483 system of 87% was considered.
484 The results showed that HPH is always an extremely energy intensive cell disintegration technique,
485 with a total consumed energy 20.0 kWh/kg_{DW}, whereas PEF, despite the lower yields is
486 characterized by a total consumed energy of 2.9 kWh/kg_{DW}. These results are in contrast with the
487 findings of Safi et al. [28], who demonstrated that PEF was energetically less efficient (10.42
488 kWh/kg_{DW}) than HPH (0.32 kWh/kg_{DW}) after only one passage at 100 MPa when applied for the
489 recovery of proteins from suspensions of *Nannochloropsis gaditana* microalgae with a cell
490 concentration of, respectively, 60 g/L and 100 g/L. Probably, this difference can be somehow
491 explained in terms of the peculiarity of the tested microalga, the different biomass concentrations as
492 well as on the different PEF and HPH systems. For example, in agreement with previous findings
493 [53], it is likely that the energy efficiency of the continuous flow PEF system used in the present
494 work is higher than that of the batch chamber (electroporation cuvette with a maximum capacity of
495 400 μL) used in the work of Safi et al. [28]. On the other hand, it has been reported that processing
496 biomass with higher solid concentrations than the diluted suspension used in our work, could
497 positively affect the energy efficiency of both HPH and PEF treatment.
498 To this regard, for example, when Yap et al. [15] processed suspensions of *Nannochloropsis sp.* by
499 HPH at different concentrations, they found the same extent of cell rupture, but the energy demand
500 of HPH was about 28 kWh/kg_{dw} at 0.25 % w/w solids and 0.28 kWh/kg_{dw} at 25 % w/w solids.
501 Moreover, they also demonstrated that large scale HPH equipment is considerable more energy

efficient than lab-scale apparatus. Thus, from these results it appears that processing of concentrated algae biomass using large scale HPH equipment could require up to 10 fold less energy than that required in our experiments where diluted suspensions were processed in a lab-scale PEF unit.

On the other hand, it has been also reported that the energy demand of PEF could be reduced by increasing the biomass content of the suspension. For example, Goettel et al. [18] using a lab-scale PEF unit found that for an algae suspension containing 100 g_{dw}/kg_{sus} algae the energy demand was 0.44 kWh/kg_{dw}, while for a suspension containing 167 g_{dw}/kg_{sus} algae, the energy demand of PEF was reduced up to 0.25 kWh/kgdw. Similarly, Safi et al. [28] found that increasing the biomass concentration from 45 to 60 g/L resulted in an almost double amount of released proteins (from about 5% w/w to 10 % w/w).

Thus, as previously observed for HPH [15], it cannot be excluded that also for PEF the processing of high biomass concentration could positively affect the extraction yield of intracellular compounds and reduce the energy requirements per unit biomass. Further research is, therefore, needed in order to achieve for both PEF and HPH optimal conditions in terms of extraction yield and energy consumption as well as to achieve a more general conclusion about the energy efficiency of PEF and HPH.

Moreover, for the first time, the comparison between PEF and HPH has also been carried out in terms of the energy consumed to extract 1 kg of carbohydrates or proteins, which were, respectively, 40.5 kWh/kg of glucose equivalent and 72.3 kWh/kg of BSA equivalent for PEF, and 311.8 kWh/kg of glucose equivalent and 60.4 kWh/kg of BSA equivalent for HPH. These estimated energy consumptions clearly show that, at least for the biomass concentration tested in this work, the carbohydrates can be efficiently recovered through PEF at comparable yields with HPH, but with higher purity and lower energy consumption . This is a remarkable result, because a selective release of carbohydrates may result in a less intensive fractionation in the later biorefinery stages.

In the case of proteins, instead, HPH is slightly more energetically efficient that PEF, because of significantly higher yields. However, PEF represents a viable option when considering the lower

528 purity of HPH extracts and the need of more complex downstream purification process. In addition,
529 PEF and HPH significantly differ also in the composition of the protein extracts, and therefore
530 future research should address in deeper detailing the effect of microalgae pretreatment on the
531 molecular composition of the protein extract.

4. Conclusions

The present study provides additional insights into the impact of PEF and HPH treatments on the disintegration efficiency of *C. vulgaris* cells and into the subsequent recovery of intracellular compounds, namely carbohydrates and proteins.

PEF resulted in being a relatively mild cell disruption method, which merely electroporates the algae cells without the formation of any cell debris, allowing to selectively enhance the extraction yield of small ionic substances and carbohydrates to an extent comparable to that achieved by HPH. The extraction efficiency of proteins, instead, was relatively low and did not exceed 5.2% of the total.

HPH, instead, was able to disrupt completely the microalgae cells, favoring an instantaneous and efficient release of all the intracellular material, including a large amount of proteins, whose release was 10.3 fold higher than by PEF. However, despite the higher extraction efficiency, the formation of large amounts of finely sized cell debris by HPH significantly complicates any downstream separation process.

Moreover, the HPH treatment resulted in being significantly more energy-intensive than PEF to achieve a comparable release of carbohydrates, while shown a slightly higher energy efficiency when used for the extraction of proteins.

In the ongoing work, the optimal cell disruption conditions identified in this work for individual PEF (E = 20 kV/cm; W_T = 100 kJ/kg_{SUSP}) and HPH (n_P = 5) treatment, are tested in a cascade biorefinery, in order to maximize in a selective and sustainable way the extraction yield of target compounds, by reducing the overall processing costs, which nowadays represent the main bottleneck to the full exploitation of microalgal biomass.

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725 **Figure captions**

726 **Figure 1.** Effect of incubation time after PEF and HPH treatment on electrical conductivity at 25 °C
727 of (a–c) PEF (E=10–30 kV/cm; W_T=20–100 kJ/kg) and (d) HPH (150 MPa; n_p=1–10) treated *C.*
728 *vulgaris* suspension at a different number of passes. Control means untreated suspension. Data
729 shown is the mean ± SD, n=9.

730 **Figure 2.** Mean particle size of untreated (control), PEF treated (E=10–30 kV/cm; W_T=20–100
731 kJ/kg) and HPH treated (P=150 MPa; n_P=5) *C. vulgaris* suspension. Different letters above the bars
732 indicate significant differences among the mean values of the samples ($p \leqslant 0.05$). Data shown is the
733 mean ± SD, n=9.

734 **Figure 3.** Scanning electron microscopy (SEM) of *C. vulgaris* cells before (Control) and after PEF
735 (20 kV/cm) at total specific energy input of 20 kJ/kg (PEF1), 60 kJ/kg (PEF2), 100 kJ/kg (PEF3),
736 and HPH (P=150 MPa; n_p=5) treatment of the microalgal suspension.

737 **Figure 4.** Dry matter content in the supernatant of untreated (Control) and treated *C. vulgaris*
738 suspension 1 h after PEF (E=10–30 kV/cm; W_T=20–100 kJ/kg) or after HPH (P=150 MPa; n_P=5)
739 treatment. Different letters above the bars indicate significant differences among the mean values of
740 the samples ($p \leqslant 0.05$). Data shown is the mean ± SD, n=9.

741 **Figure 5.** Concentration of carbohydrates (a) and proteins (b) in the supernatant of untreated (0
742 kV/cm) and treated *C. vulgaris* suspension 1 h after PEF treatment as a function of the field strength
743 and for different energy input. Different letters above the bars indicate significant differences
744 among the mean values of the samples ($p \leqslant 0.05$). Data shown is the mean ± SD, n=9.

745 **Figure 6.** Concentration of proteins and carbohydrates in the surpenatant of untreated (n_P=0) and
746 HPH (P=150 MPa) treated *C. vulgaris* suspension as a function of the number of passages. Data
747 shown is the mean ± SD, n=9.

748

749

Figure 1

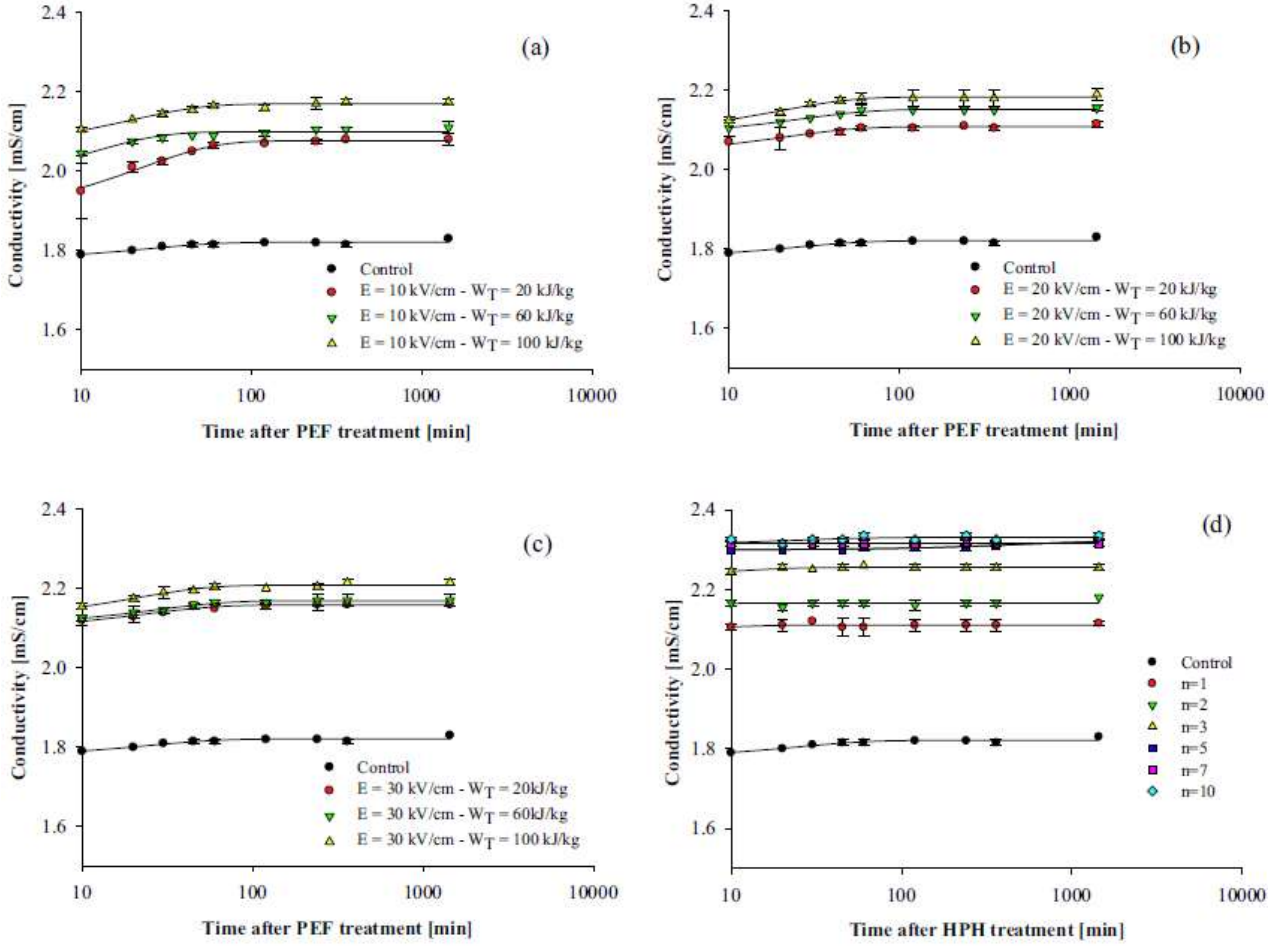

(a)
Conductivity [mS/cm]
Time after PEF treatment [min]
Control
E = 10 kV/cm - W_T = 20 kJ/kg
E = 10 kV/cm - W_T = 60 kJ/kg
E = 10 kV/cm - W_T = 100 kJ/kg
(b)
Conductivity [mS/cm]
Time after PEF treatment [min]
Control
E = 20 kV/cm - W_T = 20 kJ/kg
E = 20 kV/cm - W_T = 60 kJ/kg
E = 20 kV/cm - W_T = 100 kJ/kg
(c)
Conductivity [mS/cm]
Time after PEF treatment [min]
Control
E = 30 kV/cm - W_T = 20kJ/kg
E = 30 kV/cm - W_T = 60kJ/kg
E = 30 kV/cm - W_T = 100 kJ/kg
(d)
Conductivity [mS/cm]
Time after HPH treatment [min]
Control
n=1
n=2
n=3
n=5
n=7
n=10

Figure 2

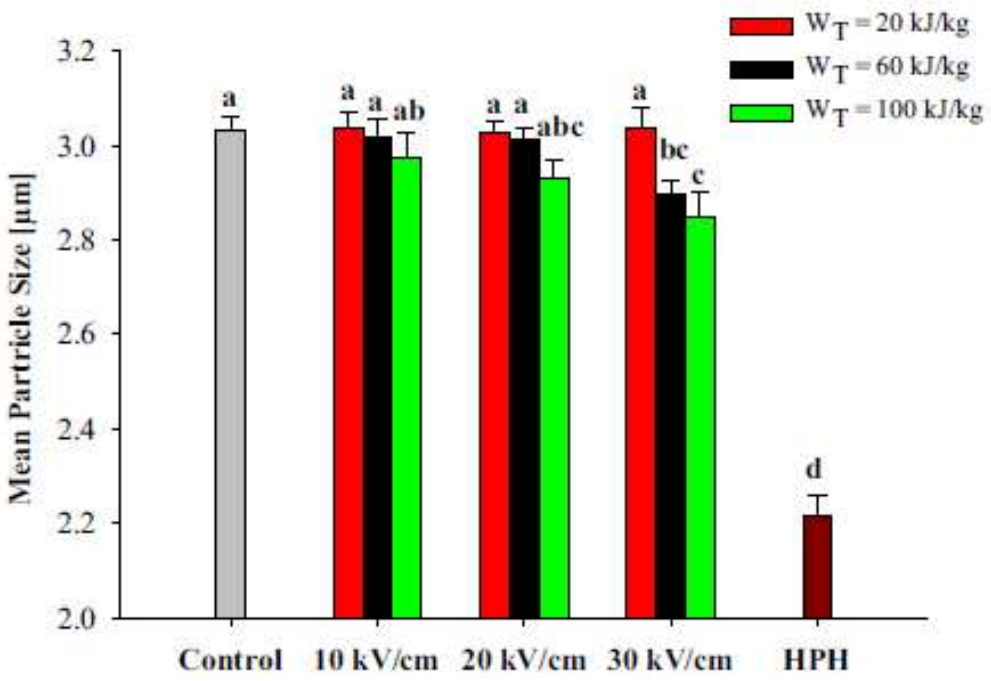

W_T = 20 kJ/kg
W_T = 60 kJ/kg
W_T = 100 kJ/kg
Mean Partricle Size [µm]
3.2
3.0
2.8
2.6
2.4
2.2
2.0
a
a a ab
a a abc
a bc c
d
Control
10 kV/cm
20 kV/cm
30 kV/cm
HPH

Figure 3

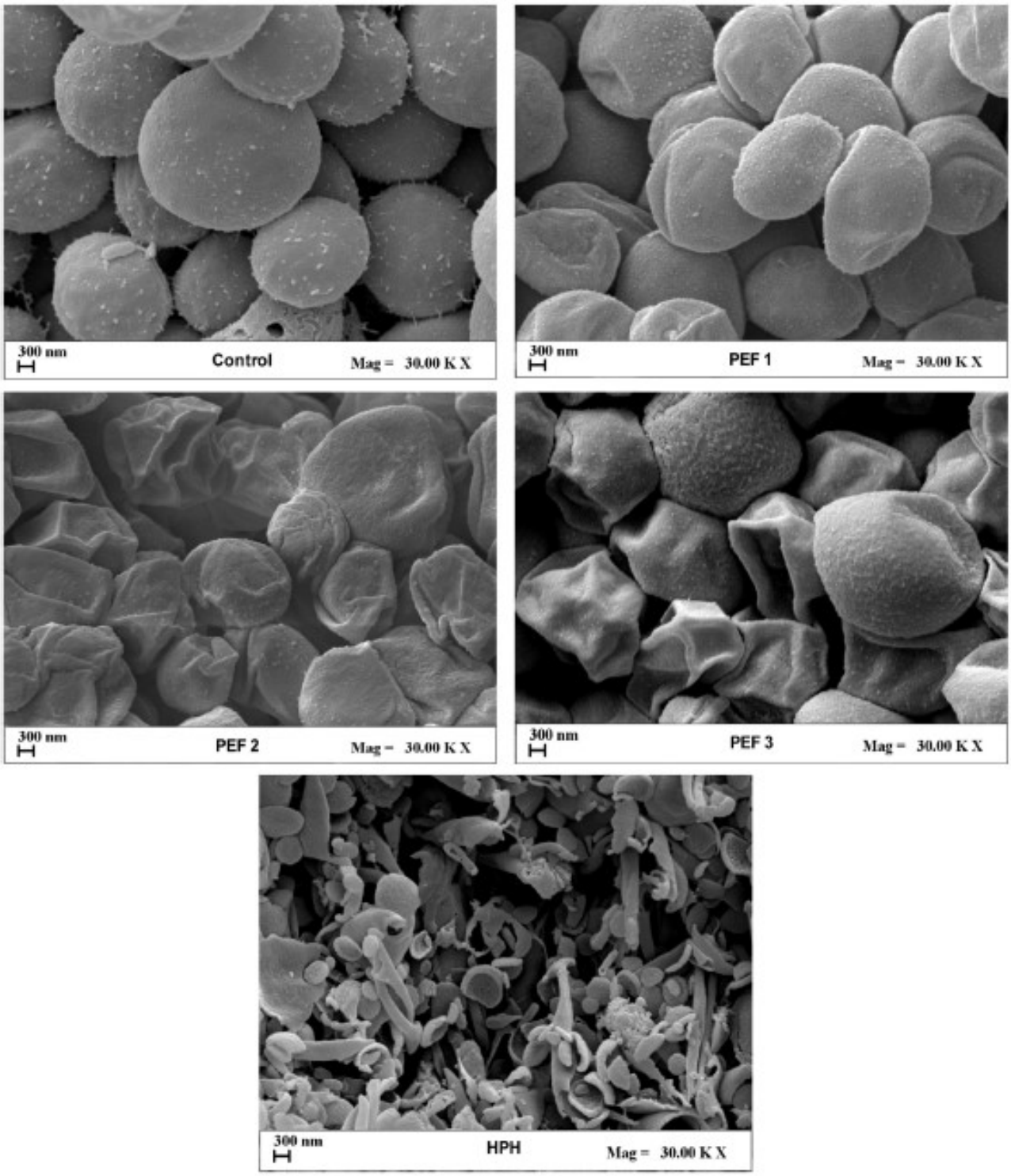

300 nm
Control
Mag = 30.00 K X
300 nm
PEF 1
Mag = 30.00 K X
300 nm
PEF 2
Mag = 30.00 K X
300 nm
PEF 3
Mag = 30.00 K X
300 nm
HPH
Mag = 30.00 K X

Figure 4

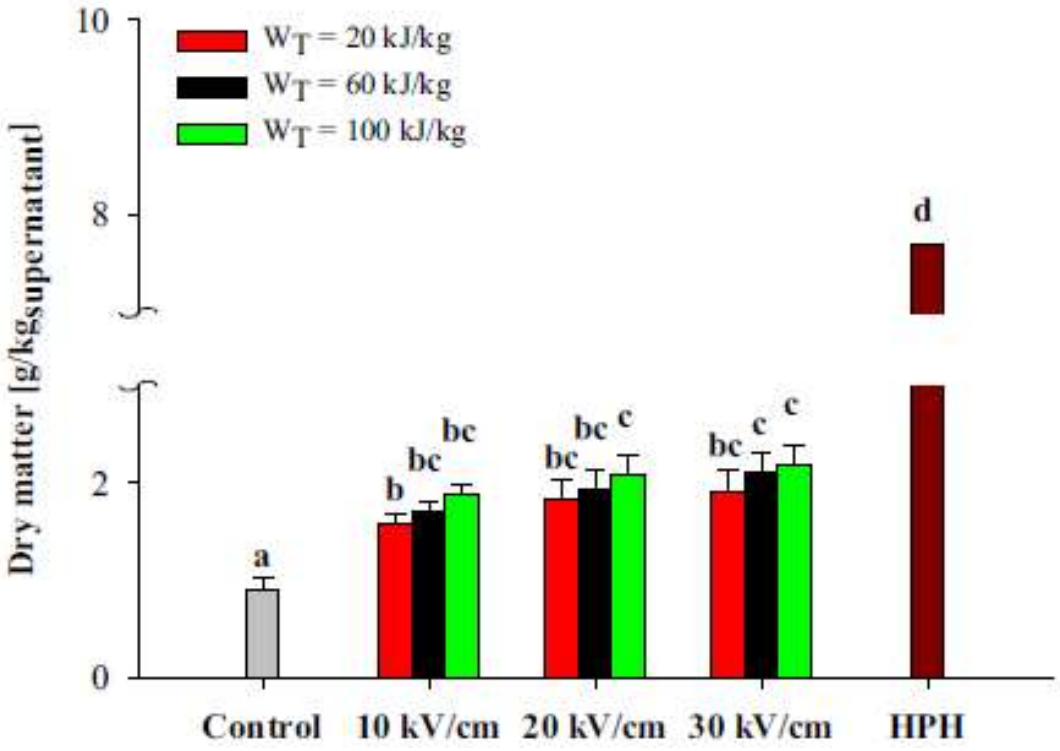

10
8
2
0
W_T = 20 kJ/kg
W_T = 60 kJ/kg
W_T = 100 kJ/kg
Dry matter [g/kgsupernatant]
a
b
bc
bc
bc
bc
c
bc
c
c
d
Control
10 kV/cm
20 kV/cm
30 kV/cm
HPH

Figure 5

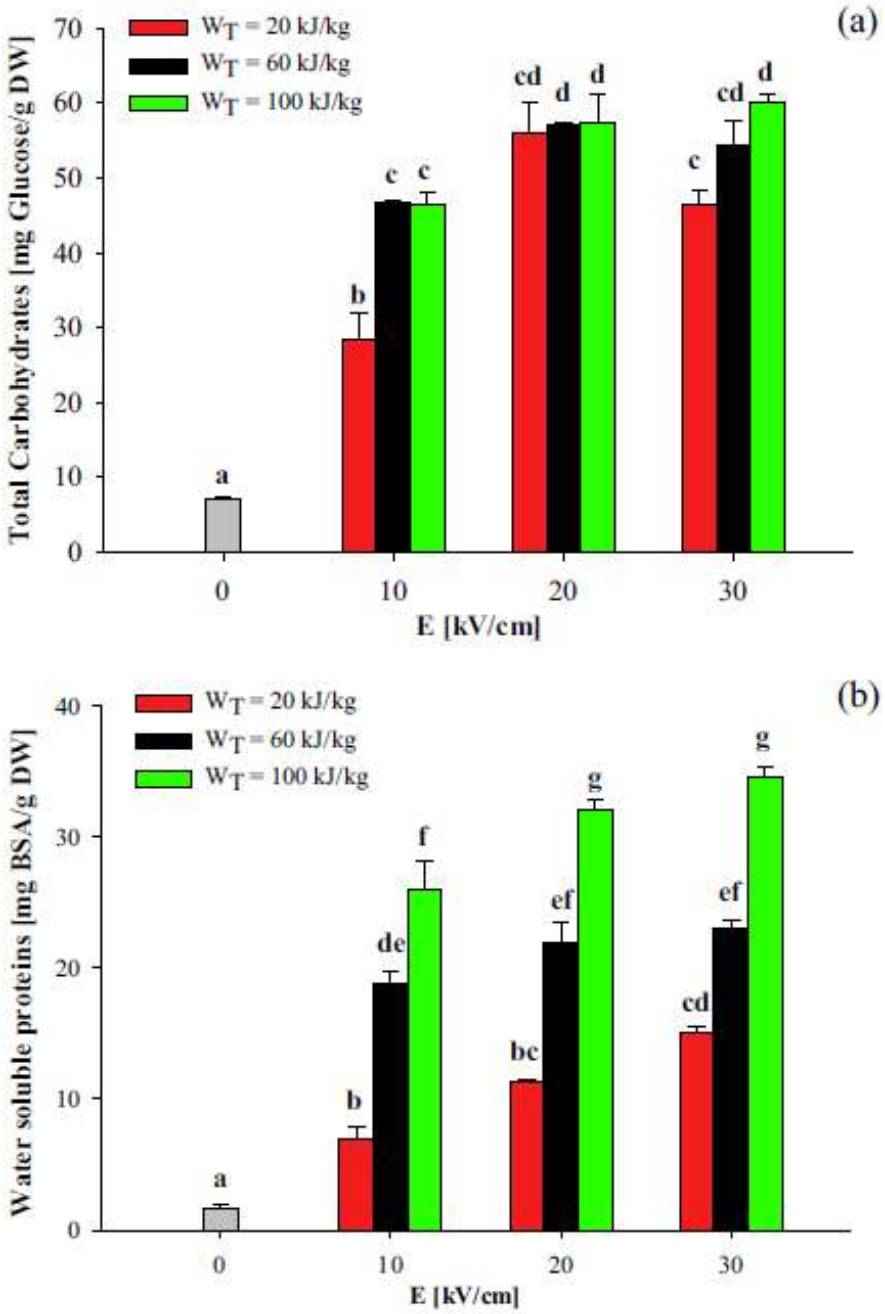
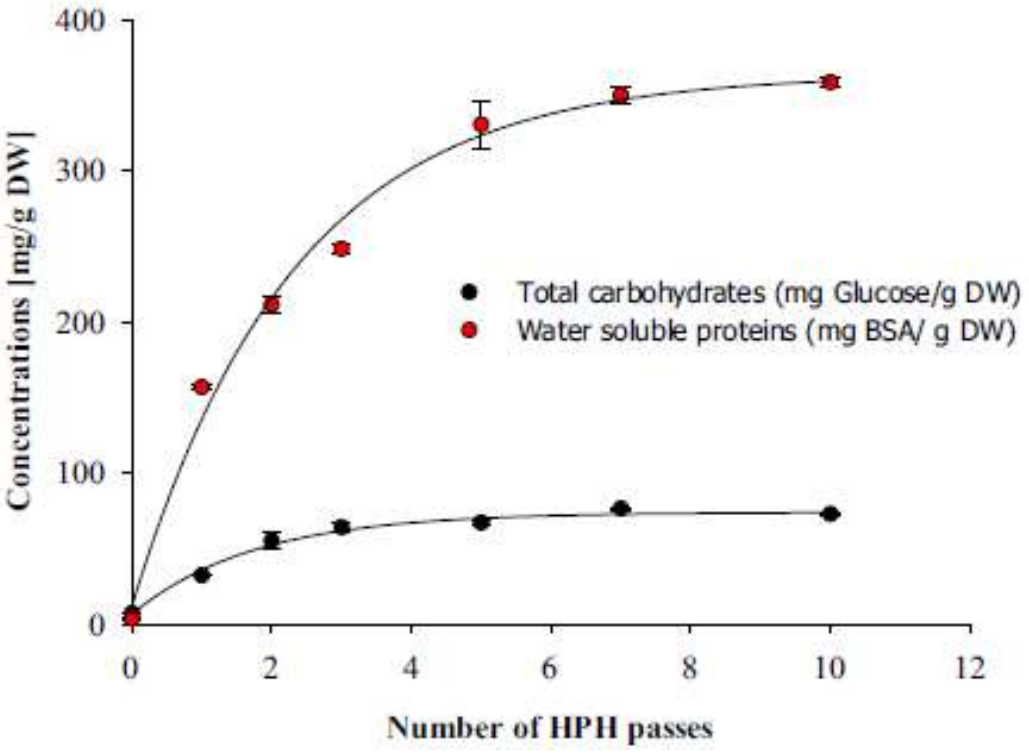

(a)
W_T = 20 kJ/kg
W_T = 60 kJ/kg
W_T = 100 kJ/kg
Total Carbohydrates [mg Glucose/g DW]
E [kV/cm]
(b)
Water soluble proteins [mg BSA/g DW]
E [kV/cm]

Figure 6


400
300
200
100
0
Concentrations [mg/g DW]
0
2
4
6
8
10
12
Number of HPH passes
Total carbohydrates (mg Glucose/g DW)
Water soluble proteins (mg BSA/ g DW)