Selective extraction of intracellular components from the microalga *Chlorella vulgaris* by combined Pulsed Electric Field-Temperature treatment

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Abstract

The synergistic effect of temperature (25-65 °C) and total specific energy input (0.55-1.11 kWh kg_{DW}^{-1}) by pulsed electric field (PEF) on the release of intracellular components from the microalgae *Chlorella vulgaris* was studied. The combination of PEF with temperatures from 25-55 °C resulted in a conductivity increase of 75% as a result of cell membrane permeabilization. In this range of temperatures, 25-39% carbohydrates and 3-5% proteins release occurred and only for carbohydrate release a synergistic effect was observed at 55 °C. Above 55 °C spontaneous cell lysis occurred without PEF. Combined PEF-temperature treatment does not sufficiently disintegrate the algal cells to release both carbohydrates and proteins at yields comparable to the benchmark bead milling (40-45% protein, 48-58% carbohydrates).

Keywords

Microalgae; pulsed electric field; temperature; selectivity; mild cascade biorefinery

1 Introduction

Microalgae are promising for the production of multiple components like proteins, carbohydrates, lipids and pigments to be used as functional additives for cosmetic, nutraceutical, chemical, food and feed products as well as for the production of biofuels (Batista et al., 2013; Vanthoor-Koopmans et al., 2013). In order to have an economically feasible production process all intracellular components have to be used in a biorefinery approach (Günerken et al., 2015; Wijffels et al., 2010).

Biorefinery comprises the downstream processing (i.e. recovery, fractionation and purification) of added value ingredients from biomass. Most interesting products from microalgae are commonly stored either in the cytoplasm or in internal organelles. The complex cell structure of microalgae, comprises several organelles such as the chloroplast, mitochondria, Golgi apparatus, nucleus etc., and all these organelles have a different composition and structure (Eppink et al., 2012). The first step in the biorefinery is the use of a cell disintegration technique able to break the cell wall and cell membranes facilitating the release of these high value added components (Günerken et al., 2015; Vanthoor-Koopmans et al., 2013). Cell disintegration should be done avoiding the use of severe processing conditions that could negatively affect the quality of the extracts, diminishing the product value. Therefore, effort should be put in finding mild cell disintegration methods, effective enough to facilitate the release of the target compounds from the inner parts of the cells.

In the past 5 years, pulsed electric field (PEF) has been claimed to be a promising mild technique able to induce the permeabilization of the microalgal cell

membrane by electroporation and to enhance the spontaneous release of intracellular components at specific energy consumptions between 0.06 –239 kWh kg_{DW}^{-1} (Coustets et al., 2014; Diversified Technologies, 2010; Eing et al., 2013; Flisar et al., 2014; Goettel et al., 2013; Grimi et al., 2014; Lai et al., 2014; Luengo et al., 2015; Parniakov et al., 2015; Zbinden et al., 2013). Originally PEF was widely investigated and applied in the medical field (e.g. electrochemotherapy, gene transfer) as well as in processes of the food industry which are based either on microbial inactivation (cold-pasteurization) or on mass transfer of liquids and valuable compounds from the inner parts of plant cells (extraction, drying) (Donsì et al., 2010; Kotnik et al., 2015; Pataro et al., 2011). Moreover, Ganeva et al. (2003) showed that PEF can be a mild technique to disintegrate yeast, where 70-90% of the total enzyme activity was maintained and large proteins up to 250 kDa could be released with a yield up to 50%. To our knowledge, these yields of large proteins have so far not been obtained for microalgae.

Several parameters influence the PEF efficacy, which mainly include electric field strength and total specific energy input (Pataro et al., 2014). In general, depending on the settings of these parameters reversible or irreversible pores are formed (Kotnik et al., 2012, 2015). However, in some cases, it is necessary to apply intense process conditions (high field strengths and energy inputs) to obtain a sufficient high permeabilization degree of the cell membrane (Pataro et al., 2011). Therefore, in order to obtain the required permeabilization effect with less severe processing conditions, or to achieve higher efficacy at the same treatment conditions, PEF has also been applied in a hurdle approach. For example, additive or synergistic

effects have been observed by combining PEF with moderate heating above 35 °C for microbial inactivation (Timmermans et al., 2014). As per the literature survey, only Luengo et al. (2015) described the effect of temperature (10-40 °C) on the release of the pigment lutein during PEF treatment of microalga *Chlorella vulgaris.* They found that, at 25 kV cm^{-1}, increasing the temperature of the biomass from 10 °C to 20 °C increased the extraction yield by 35%, but an increase lower than 10% was observed by raising the treatment temperature from 30 to 40 °C. Therefore, further studies are necessary to better elucidate the interactions between electric field and temperature and the dependence of the extraction yield of the target components on the process parameters of the combined treatment.

The objective of this work was to investigate the effect of the processing temperature during PEF treatment of the microalga *Chlorella vulgaris* on the release of intracellular components. Release of ions (conductivity), carbohydrates and proteins was followed. Additionally, the effect of this treatment on the product quality was measured with gel electrophoresis and a Rubisco activity assay.

2 Materials and Methods

2.1 Microalgae, cultivation and logistics

C. vulgaris (SAG 211-11b) was obtained from the Culture Collection of Algae at Göttingen University and was cultivated in M-8a medium as described by Kliphuis et al. (2010) using a 12L stirred tank photobioreactor (Postma et al., 2015). After harvesting, the biomass was concentrated by means of centrifugation (4000 x g, 15min and 4 °C) up to a final concentration (C_x) of 25 g_{DW} kg^{-1} with an initial conductivity (σ) of about 0.6 mS cm^{-1} at 25 °C (Conductivity meter HI 9033, Hanna Instrument, Milan, Italy).

The concentrated biomass was pre-packed in high-density polyethylene bottles (Nalgene) and cooled to 4 °C. The transport of the biomass to ProdAl Scarl (University of Salerno, Fisciano (SA), Italy) was conducted by courier within 24 hours in an EPS box in which the refrigerated temperature was maintained using gel-packs. PEF treatments were performed on the delivery day.

2.2 PEF experimental set-up

PEF experiments were conducted in a bench-scale continuous flow PEF system of which a schematic overview is shown in Figure 1. The PEF unit design was based on the unit described by Pataro et al. (2014), but adjusted in order to include two PEF treatment zones. In short, a peristaltic pump (Pump Drive PD5201, Heidolph Instruments GmbH, Germany) provided a continuous flow of 33 mL min^{-1} of the algae biomass suspension. Prior to entering each of the PEF treatment zones, the algae

suspension flowed through a stainless steel coil immersed in a water heating bath to control the temperature between 25 – 65 °C. Each PEF treatment zone consisted of a module made of 2 co-linear treatment chambers, hydraulically connected in series, with an inner diameter of 3.1 mm and a gap distance of 4 mm. Monopolar square wave electric pules were supplied by a high voltage pulse generator (Diversified Technology Inc., Bedford, WA, USA). The voltage and current signals at the treatment chamber were measured by a high voltage probe (P6015A,Tektronix, Wilsonville, OR, USA) and a Rogowsky coil (2-0.1W,Stangenes, Inc., USA), respectively. The measurements were recorded and displayed using a 300 MHz digital oscilloscope (TDS 3034B,Tektronix, Wilsonville, OR, USA) connected to a PC. The maximum electric field intensity (*E*, $kVcm^{-1}$) was evaluated as the peak voltage divided by the inter-electrode gap. The total specific energy input (W_{PEF}, $kWh\ kg_{DW}^{-1}$) was calculated with the following equation:

$$W_{PEF} = \frac{n}{3600 \cdot \rho \cdot v \cdot C_x} \int_0^{\infty} U(t) \cdot I(t) dt \quad (1)$$

where *n* is the total number of pulses applied in the four treatment chambers, ρ is the density of the biomass, *v* is the volume of the treatment zone in each chamber and C_x is the biomass concentration in $kg_{DW}\ kg_{susp}^{-1}$. *U(t)* and *I(t)* represent the voltage across the electrodes and the current intensity through the product at time *t*, respectively.

During the treatment a single voltage value of 8 kV was set resulting in an electric field strength (*E*) of 20 kV cm^{-1}. The pulse length was fixed to 5 µs and the pulse frequency was adjusted between 50 – 200 Hz to provide a specific energy input

(W_{PEF}) of 0.55 and 1.11 kWh kg^{-1} dry weight (kWh kg_{DW}^{-1}). Four thermocouples were used to measure the product temperature at the inlet and outlet of the PEF chamber module.

An overview of the conducted experiments and process conditions is shown in Table 1. At the exit of the unit the treated algae suspension was collected in plastic tubes and placed in an ice water bath. After cooling, the samples were allowed to stand for 1 hour at 25 °C under shaking at 140 RPM to allow intracellular components to diffuse out of the cells. After this resting time, the cell suspensions were centrifuged (10 min, 5300 x g) and the supernatant was transferred to fresh tubes and stored at -20 °C until further analysis.

2.3 Synergy of PEF and temperature

Energy was provided via two ways in the current study: (1) heating (increased processing temperature using water heating baths) and (2) via electrical pulses, abbreviated as W_T and W_{PEF}, respectively. Assuming a specific heat capacity of 1.14 10^{-3} kWh $(kg\ K)^{-1}$ for the algae suspension, it can be observed that up to 1.83 kWh kg_{DW}^{-1} additional energy was consumed when the processing temperature was increased from 25 up to 65 °C (Table 2). Where W_{PEF} was 0.55 or 1.11 kWh kg_{DW}^{-1}, on the laboratory scale plant used in this work, this had a substantial effect (i.e. $W_T \approx W_{PEF}$) on the total amount of consumed energy for a single operating condition. Nevertheless, on an industrial scale, heat exchangers could be used to recover the energy from the outlet stream. Besides, the energy input of the PEF (W_{PEF}) also increased the temperature of the suspension (5-10 °C). This increase can be sufficient to exchange

enough energy between the outlet and inlet, reducing the amount additional W_T to a minimum. Therefore, only W_{PEF} was considered in comparison to other studies.

2.4 Analytical methods

2.4.1 Protein analysis

The total protein content on dry weight (DW) and the water soluble protein content of supernatants were determined according to Postma et al. (2015). In short, for total protein content on DW, 6 mg of freeze dried algae were bead beaten in 1.0 mL lysis buffer I (60 mM Tris, 2% SDS, pH 9.0) in a lysing matrix E tube (6914-500, MP Biomedicals Europe, France). The tubes were beaten using a bead beater (Precellys 24, Bertin Technologies, France) for 3 cycles of 60 s at 6500RPM with 120 s breaks between cycles.

For analysis of the water soluble protein content, supernatant obtained from PEF-treated samples was diluted 2 times using lysis buffer II (120 mM Tris, 4% SDS, pH 9.0).

Subsequently, samples for both total protein content on DW and water soluble protein content from supernatant were incubated at 100 °C for 30 min before quantification using a commercial kit (DC^{TM} Protein assay, Bio-Rad, USA) similar to the Lowry assay (Lowry et al., 1951). Bovine serum albumin (A7030, Sigma-Aldrich, USA) was used as protein standard. The absorbance was measured at 750 nm. The protein yield (Y_p) was expressed as:

$$Y_p = \frac{C_{p,sup}}{C_{p,biomass}} \quad (2)$$

where $C_{p,sup}$ is the protein content in the supernatant (%$_{DW}$) and $C_{p,biomass}$ is the total protein content on DW (%$_{DW}$).

2.4.2 Carbohydrate analysis

In order to analyze the carbohydrate content on DW, ~1 mg of algae DW was hydrolyzed in 1 mL 2.5 M HCl in a heat block at 100 °C for 3 hours. Samples were neutralized using 1 mL of 2.5 M NaOH.

Both, hydrolyzed samples and supernatant of PEF treated samples were analyzed according to DuBois et al. (1956). 0.2 mL of 5% w/w phenol and 1 mL of concentrated sulfuric acid were added to 0.2 mL of (diluted) sample (hydrolyzed sample or supernatant obtained from PEF). The samples were incubated at 35 °C for 30 minutes before reading of the absorbance at 485 nm against a blank of 0.2 mL 5% w/w phenol, 1 mL concentrated sulfuric acid and 0.2 mL of deionized water. Glucose was used as a standard. The carbohydrate yield (Y_c) was expressed as:

$$Y_c = \frac{C_{c,sup}}{C_{c,biomass}} \quad (3)$$

in which $C_{c,sup}$ is the carbohydrate content in the supernatant (%$_{DW}$) and $C_{c,biomass}$ is the total carbohydrate content on DW (%$_{DW}$).

2.4.3 Polyacrylamide gel electrophoresis

Supernatant samples from PEF experiments were thawed and 6x concentrated using Amicon Ultra-0.5 3K (Merck Millipore, USA) centrifugal tubes. Concentrated samples were kept on ice until further use.

Native PAGE was conducted using a 4-20% Criterion TGX gel (#567-1094, Biorad). 50 µl of Native sample buffer (#161-0738, Biorad) and 125 µg of protein was mixed and made up to 100 µL using Milli-Q®. 25 µg of protein was loaded per lane. NativeMark™ (LC0725, life technologies) was used as marker for size estimation. Tris/Glycine (#161-0734, Biorad) was used as running buffer at 200 V constant for 35 min.

Bio-Safe Coomassie stain (#161-0787, Biorad) was used to stain the Native PAGE gel for 120 min followed by overnight rinsing with de-ionized water to increase background contrast before scanning.

2.4.4 Rubisco activity assay

The Rubisco activity was analyzed spectrophotometrically according to Desai et al. (2014). NADH oxidation was measured at 340 nm using quartz cuvettes over a period of 6 minutes. The Rubisco activity was calculated using a molar extinction coefficient of 6.22 mM^{-1}. The final reaction mixture (3 mL) contains 259 mM Tris, 5 mM magnesium chloride, 67 mM potassium bicarbonate, 0.2 mM β-nicotinamide adenine dinucleotide (reduced form), 5 mM adenosine 5’ -triphosphate, 5 mM glutathione (reduced form), 0.5 mM D-ribulose 1,5-di-phosphate, 5 units alpha-glycerophosphate

dehydrogenase trios phosphate isomerase, and 5 units glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglyceric phosphokinase. Either, PEF treated supernatant or bead mill supernatant was added just before the measurement, a reaction mixture without Rubisco was used as blank.

3 Results and Discussion

The cell composition of *C. vulgaris* used in this study was quantified as follows: 61.1 $\%_{DW}$ protein and 16.2 $\%_{DW}$ carbohydrates. The results of this study will be presented in terms of extraction yields expressed with respect to this composition. Cell membrane permeabilization was monitored by measurement of electrical conductivity (Donsì et al., 2010). The release of relatively small and large molecules was monitored by the release of carbohydrates and proteins, respectively. Furthermore, a detailed analysis on the size distribution and activity of the released protein is presented in comparison to our benchmark bead milling. Subsequently, the role of PEF in a biorefinery approach is discussed.

3.1 Effect of combined PEF-temperature treatment on the release of macromolecules

3.1.1 Permeabilization of the algal cells

In order to quantify the maximal increase in conductivity after combined PEF-temperature treatment, a sample of the microalgal suspension was subjected to a beat beater step followed by measurement of the conductivity. The microalgal suspension

had a maximal absolute increase of 1.0 mS cm^{-1} with respect to the fresh sample (0.6 mS cm^{-1}). This maximal conductivity increase was further used as a benchmark value to calculate the relative increase of conductivity for PEF treated samples 1 h after PEF treatment. From the results shown in Figure 2A it can be observed that there was a strong increase in conductivity after PEF treatment under all conditions applied. This is a clear indication that PEF caused permeabilization of the algal cell resulting in leakage of small ions as also observed by Goettel et al. (2013) and Grimi et al. (2014). Besides, it can be observed that processing temperatures between 25 and 55 °C did not result in additional spontaneous release of ions without PEF treatment. However, when the algae were heated up to 65 °C without PEF treatment, a strong increase of the conductivity up to 62.5% of the maximal possible was observed. As a result of the PEF treatment at 25 – 55 °C, the conductivity increased up to 60-75% of the maximal conductivity value. Applying PEF at an energy input of 0.55 or 1.11 kWh kg_{DW}^{-1} at 65 °C only resulted in a further increase of the conductivity up to 65.5% or 67.5% respectively. The maximal relative conductivity increase was 75% achieved after a PEF treatment at 1.11 kWh kg_{DW}^{-1} at 45 °C. From these results, which are in agreement with the findings of Grimi et al. (2014), it can be concluded that, under the processing conditions investigated, no complete extraction of ionic components was achieved.

3.1.2 Release of carbohydrates

The analysis of the carbohydrates in the supernatant (Figure 2B) shows that only a small fraction (< 5%) of the total carbohydrates content in the microalgae was released at processing temperatures between 25 and 55 °C without any PEF treatment. However, when the temperature was further increased up to 65 °C, a substantial increase in the release of carbohydrates was observed. This was likely due to the thermal disintegration of the cell membranes, resulting in an extraction yield of more than 35% of the total carbohydrate content. Figure 2B also shows the carbohydrate release yields achieved after the combined PEF-temperature treatments. When PEF was applied at processing temperatures between 25 and 45 °C, carbohydrate yields between 22 and 25% were obtained. Nevertheless, no positive interaction between PEF and temperature can be noted in this range of temperatures. Further increase of the processing temperature up to 55°C, instead, showed a clear synergistic effect of the combined treatment leading to an increase of the carbohydrate yield up to 39%. However, no difference could be detected at any temperature when the total specific energy input was increased from 0.55 to 1.11 kWh kg_{DW}^{-1}. This synergistic effect was most likely caused by a less stable cell membrane due to the increased temperature, making the lipid bilayer of the cell membrane more sensitive for electric pulses (Timmermans et al., 2014) allowing a larger amount of carbohydrates to be released. This effect could even be further enhanced because at increased temperature values, diffusivity and solubility of carbohydrates tend to be higher. In contrast, no further release of carbohydrates was observed at a processing temperature of 65 °C when PEF was applied. This is likely due to the fact that, at this

high temperature value, the thermal effect was enough to break the cell membranes masking the PEF effect. Goettel et al. (2013) reported a carbohydrate release of about 8 g L^{-1} using a specific energy input of 0.40 kWh kg_{DW}^{-1} from a suspension of 109 g_{DW} kg^{-1} with the microalgae Auxenochlorella *prototheco*ides. Assuming a carbohydrate content of 33% on DW (Bohutskyi et al., 2015), a carbohydrate yield of 22% was achieved which is in the same range as the current study.

3.1.3 Release of water soluble protein

Figure 2C shows the protein yield achieved after either heating or combined PEF-temperature treatment. The results show that no or hardly any protein could be detected in the supernatant of the suspension when no PEF treatment was applied at a processing temperature between 25 and 55 °C. This is either because no protein was released or the protein content was below the limit of quantitation (0.05 mg mL^{-1}). However, similarly to the results on conductivity (Figure 2A) and release of carbohydrates (Figure 2B), a substantial increase in the released protein up to an extraction yield of 3.7% was found at a processing temperature of 65 °C without any PEF treatment. The application of a PEF treatment at room temperature resulted in an extraction yield of 3.2% at 0.55 kWh kg_{DW}^{-1} and 3.6% at 1.11 kWh kg_{DW}^{-1}. A slight synergistic effect was observed when the electrical treatment was combined with heating of the biomass. However, the absolute maximum yields are still a tenfold lower than the yields obtained with the bead mill (40-45% at 25 g_{DW} kg^{-1}) (Postma et al., 2015). Nevertheless, it appeared that above a certain critical temperature, no further improvement could be observed. For the PEF treatment at 0.55 and 1.11 kWh

kg_{DW}^{-1}, the maximum protein extraction was obtained at 55 °C (Y_p: 4%) and 45 °C (Y_p: 4.4%), respectively.

Although for mild treatments generally temperatures below 35 °C are used to prevent any damage to the protein structure, this is only a prerequisite if long treatment times (i.e. order of minutes to hours) are applied. In the current work, the residence time of the biomass inside the treatment chambers was only 0.22 s, while the total residence time at each processing temperature was lower than 25 s. Moreover, the treated algae suspension collected at the exit of the PEF unit was immediately cooled in a water-ice bath. Studies on the denaturation kinetics of whey proteins showed that it takes over 200 seconds at 65 °C to denature 1% of β-lacto-globulins or α-lactalbumin (Dannenberg and Kessler, 1988).

In recent PEF studies, similar protein yields and corresponding specific energy inputs (W'_{PEF}) to the results presented in this study were reported. Grimi et al. (2014) found a relative yield of 3.6% with and energy consumption of 1.50 kWh kg_{DW}^{-1} using *Nannochloropsis sp*. Parniakov et al. (2015) even obtained a protein yield between 5-10% although this required 4.00 kWh kg_{DW}^{-1}. In addition, these authors used frozen/thawed *Nannochloropsis sp.* algae prior to the application of PEF, which most likely weakened/damaged the cell structure. On the other hand, Goettel et al. (2013) were able to enhance the spontaneous release of protein slightly (from 8 µg L^{-1} before PEF to 10.5 µg L^{-1} after PEF) from fresh cells of *A. protothecoides* at a specific energy input of 0.40 kWh kg_{DW}^{-1}.

3.2 Protein size distribution and activity

Since low protein yields (< 5 %) were obtained in comparison to the yields obtained with the bead mill (40 - 45% protein yield at a C_x of 25 g kg^{-1}) (Postma et al., 2015), and proteins are generally very water soluble, it is expected that only small proteins were released. Therefore, the size distribution of the released proteins was determined via gel electrophoresis. This gives better understanding on the release behavior and location of the proteins.

3.2.1 Native PAGE

Native PAGE provides understanding whether the released protein is negatively affected in size (i.e. degradation or aggregation). Figure 3 shows the Native PAGE gel in which bead mill samples (lanes 14-16) are compared to the PEF treated samples at an energy input of 1.11 kWh kg_{DW}^{-1} at 25- 65 °C (lane 2-10, 12, 13). From lanes 2-13 it can be observed that a wide range of proteins of different molecular sizes was released by combined PEF-temperature treatment. From 45 °C onwards, a more intense group of proteins is visible between 20-66 kDa, and at 65°C even additional bands occur. This indicates that elevated processing temperatures do negatively affect the native state of the released protein, irreversibly damaging the protein structures, despite the short processing time as described in section 3.1.3. Conclusively, only processing temperatures up to 35 °C should be applied if native proteins are desired. Comparing the overall profile of the proteins released by bead milling and PEF, it can be observed that the bead mill samples reveal a strong band at ~540 kDa (i.e. the size of native Rubisco) next to a large range of proteins in different sizes, both larger and smaller.

The samples subjected to PEF also reveal a band at ~540 kDa although less distinct. Based on the soluble protein (Lowry) assay, equal amounts of protein were loaded on the gel per lane. However, it can be observed that the higher molecular weight proteins are more distinct in the bead mill samples than in the PEF samples. Instead, below 20 kDa an intense band of low molecular weight protein material can be observed which is more pronounced for the PEF samples. To summarize, according to the results, it appears that PEF releases more small proteins rather than large proteins.

Similar to the observations in this work, Azencott et al. (2007) found that Bovine Serum Albumin (BSA) was able to move across the cell wall and cell membrane of a wild-type *Chlamydomonas reinhardtii* when subjected to electrical pulses. Although, virtually all cells were able to take up the fluorescent dye calcein (~0.6 kDa), only a fraction of the much larger BSA (~66 kDa) was taken up.

3.2.2 Rubisco activity

To confirm whether the released multimeric Rubisco was still biologically active a Rubisco activity assay was performed. Figure 4 shows the specific Rubisco activity of the protein released in the supernatant after PEF treatment (processing temperature: 35 °C, W_{PEF}: 1.11 kWh kg_{DW}^{-1}) in comparison to a supernatant sample obtained from bead milling (agitator speed: 9 m s^{-1}). It can be observed that the specific activity of the bead mill sample was about 10 times higher than the PEF sample, indicating that the purity (i.e. amount of Rubisco per amount of total protein) of the PEF sample was lower. This confirms the observations from the Native PAGE gel leading to the suggestion that during PEF treatment less intracellular organelles were disintegrated

than during bead milling. Bead milling completely disintegrates the algal cells including larger and smaller internal organelles. Therefore, also Rubisco stored in the chloroplast (free in stroma and/or inside pyrenoids) would be released. Nevertheless, since active Rubisco was observed after combined PEF-temperature treatment, it is likely that part of the chloroplast was disintegrated causing free Rubisco from the stroma to diffuse out of the cell. Though, this hypothesis cannot be confirmed based on the current results.

3.3 The role of PEF in a cascade biorefinery

A multi-stage biorefinery should exploit the full potential of the cell in terms of compartmentalization and products (Eppink et al., 2012). Mahnič-Kalamiza et al. (2014) envisioned the application of PEF as a first disintegration step in such a multi-stage biorefinery followed by two extraction stages. In the first stage, water soluble components should be extracted and in a second stage an environmentally friendly solvent could be applied to extract pigments or other hydrophobic components. Our findings indicate that maximum 39% of the carbohydrates and less than 5% of the proteins could be released, but only at a combined PEF-temperature treatment at 55 °C and 0.55 or 1.11 kWh kg_{DW}^{-1}. On the other hand, the results of the native PAGE showed that from 45 °C onwards the proteins seem to be negatively affected. Albeit a lower processing temperature of 35 °C yields active Rubisco, a carbohydrate and protein yield of only 25% and 3.8% were obtained, respectively.

In the ideal case, PEF would allow selective release of small soluble molecules (e.g. carbohydrates) resulting in relative pure fractions without negatively harming the

other components. In this way extensive fractionation of complex protein and carbohydrate mixtures (e.g. by membrane filtration) could be omitted. Consequently, effort should be made to release the high-value water soluble proteins and likewise to release the remaining carbohydrates in a subsequent stage before any solvent is applied (Figure 5). Bead milling has shown to be a promising candidate to release up to 45% of the water soluble protein (Postma et al., 2015) and 48-58% of carbohydrates (data not shown). To conclude, further studies are necessary in order to optimize the processing conditions (e.g. electric field strength) of the combined PEF-temperature treatment as well as its integration in a cascade biorefinery in order to maximize the selective recovery of the high value components with minimal energy consumption.

4 Conclusion

PEF showed to be an effective technique to release small ionic solutes and carbohydrates up to 75% and 39%, respectively. Nevertheless, permeabilization was not effective enough to release high quantities of large molecules such as protein (< 5%). Though, the released protein fraction contained the biologically active multimeric Rubisco showing that PEF is a mild technique at 35 °C. In conclusion, under the processing conditions investigated in this work, the combined PEF-temperature treatment seems not able to sufficiently disintegrate the algal cells to release both carbohydrates and proteins at yields comparably to the benchmark bead milling (40-45% protein, 48-58% carbohydrates).

Acknowledgements

This project is financed by the IPOP Biorefinery of Wageningen University and Research Centre. Part of this work was conducted in the framework of a COST TD1104 action (www.electroporation.net) at ProdAl Scarl (University of Salerno, Fisciano, Italy).

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Figure captions

Figure 1 Schematic overview of continuous flow PEF system. O: oscilloscope, UB: untreated biomass, ST: magnetic stirrer, HVPG: high voltage pulse generator, P: peristaltic pump, WB: water bath, HV+: high voltage, T: thermocouple, TC: treatment chamber, TB: treated biomass, WIB: water ice bath

Figure 2 Relative increase of electrical conductivity of the biomass suspension evaluated with respect to the conductivity of the bead beaten sample (1.6 mS cm^{-1}) (A), Carbohydrate yield (B) and Protein yield (C) as a function of the total specific energy input WPEF (E=20 kV cm^{-1}) and for different processing temperatures. The conductivity increase, carbohydrate release and protein release were measured 1h after PEF treatment.

Figure 3 Native PAGE gel for PEF samples treated at WPEF 1.11 kWh $kgDW^{-1}$. Values in kDa. M: marker; 2: 25 °C; 3: 25 °C; 4: 35 °C; 5: 35 °C; 6: 45 °C; 7: 45 °C; 8: 45 °C; 9: 55 °C; 10: 55 °C; 11: 65 °C (no PEF); 12: 65 °C; 13: 65 °C; 14: bead mill 6 m s^{-1}; 15: bead mill 9 m s^{-1}; bead mill 12 m s^{-1}

Figure 4 Specific Rubisco activity (Units/mg protein) of Rubisco standard (ST), supernatant obtained from PEF (20 kV cm^{-1}, 1.11 kWh kg_{DW}^{-1} and 35 °C), and from bead milling (BM) (9 m s^{-1}).

Figure 5 A schematic representation of a single disintegration approach (top) compared to a multi-stage or cascade biorefinery approach (bottom).

Figure 1
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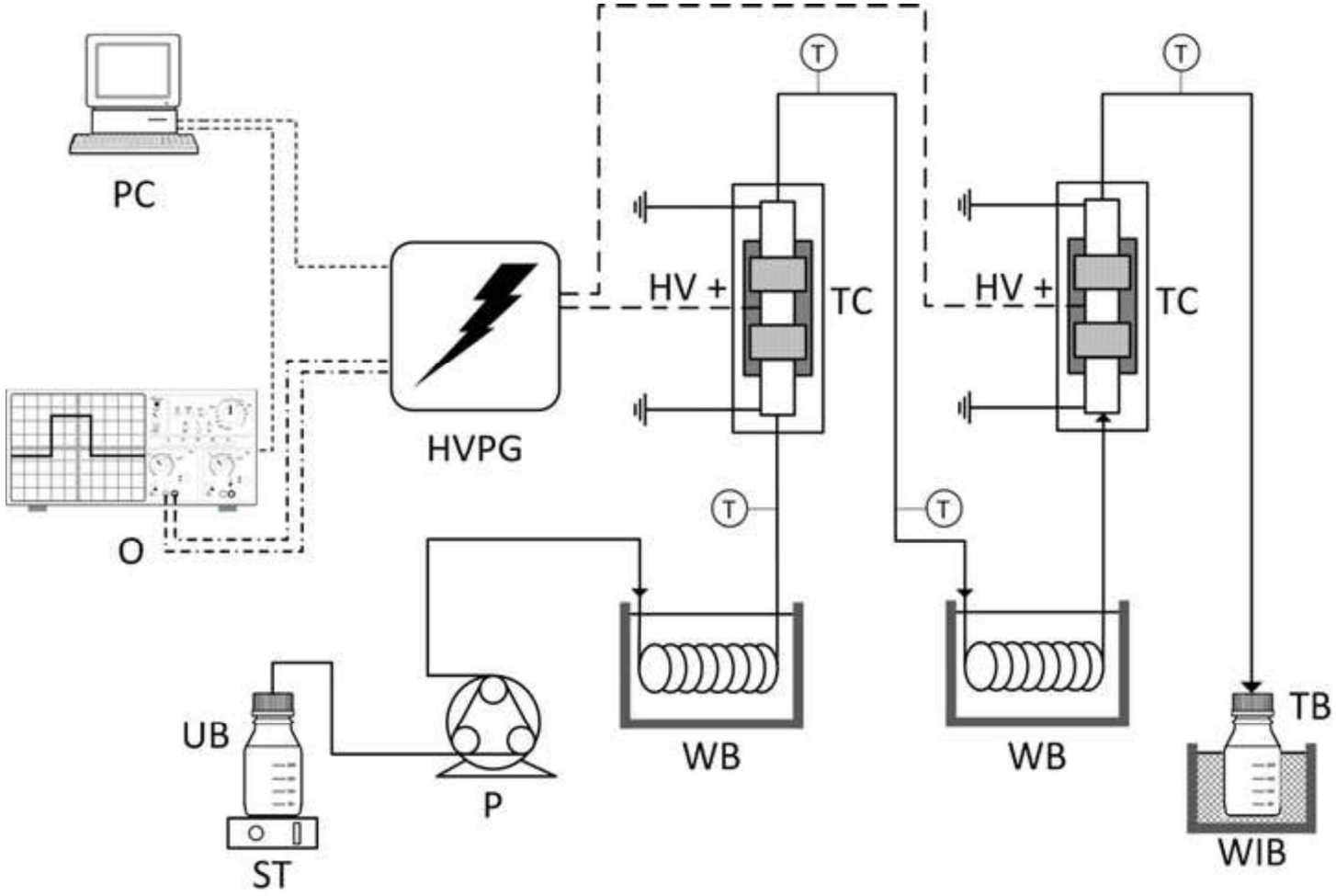

Figure 2

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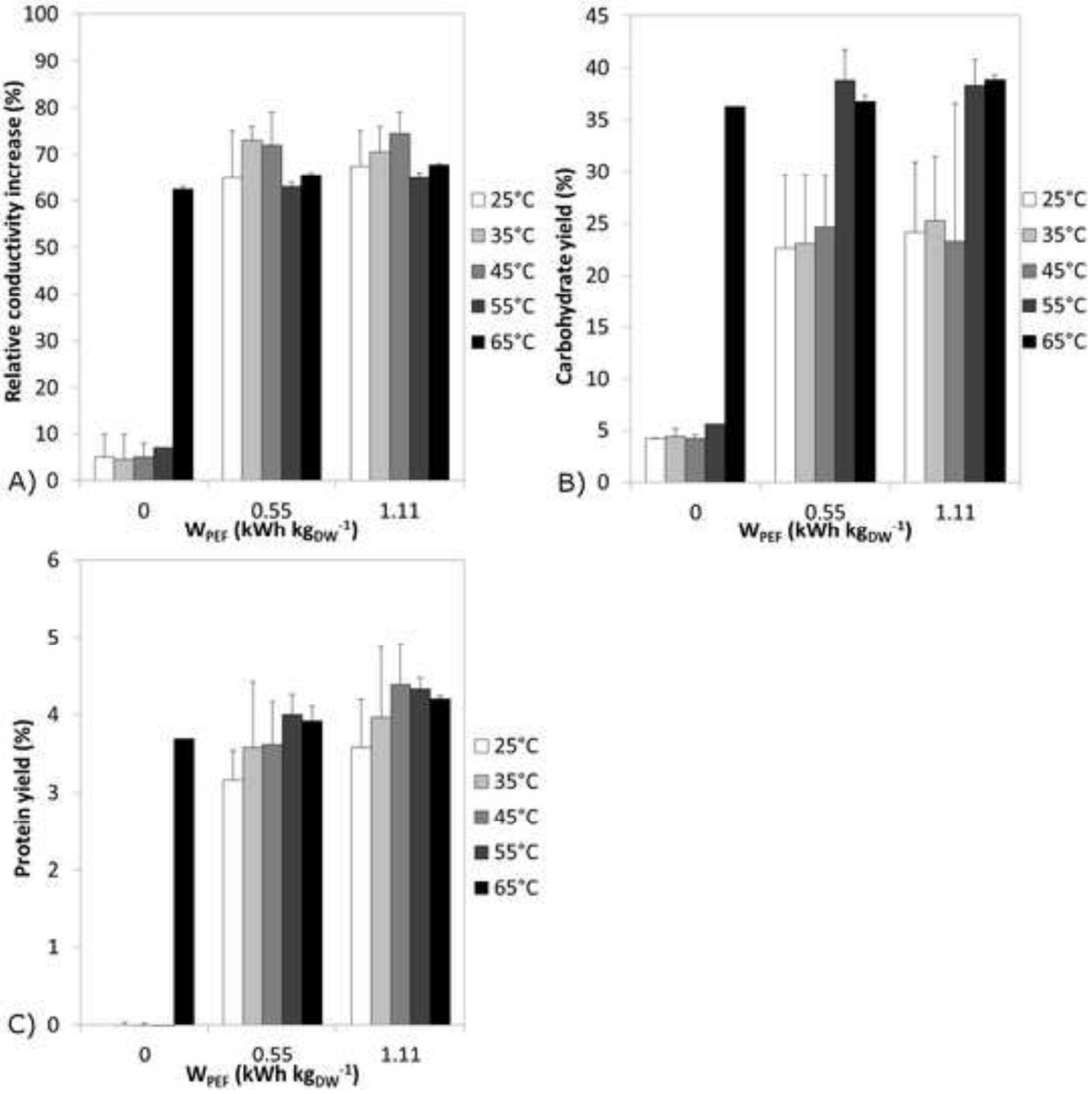

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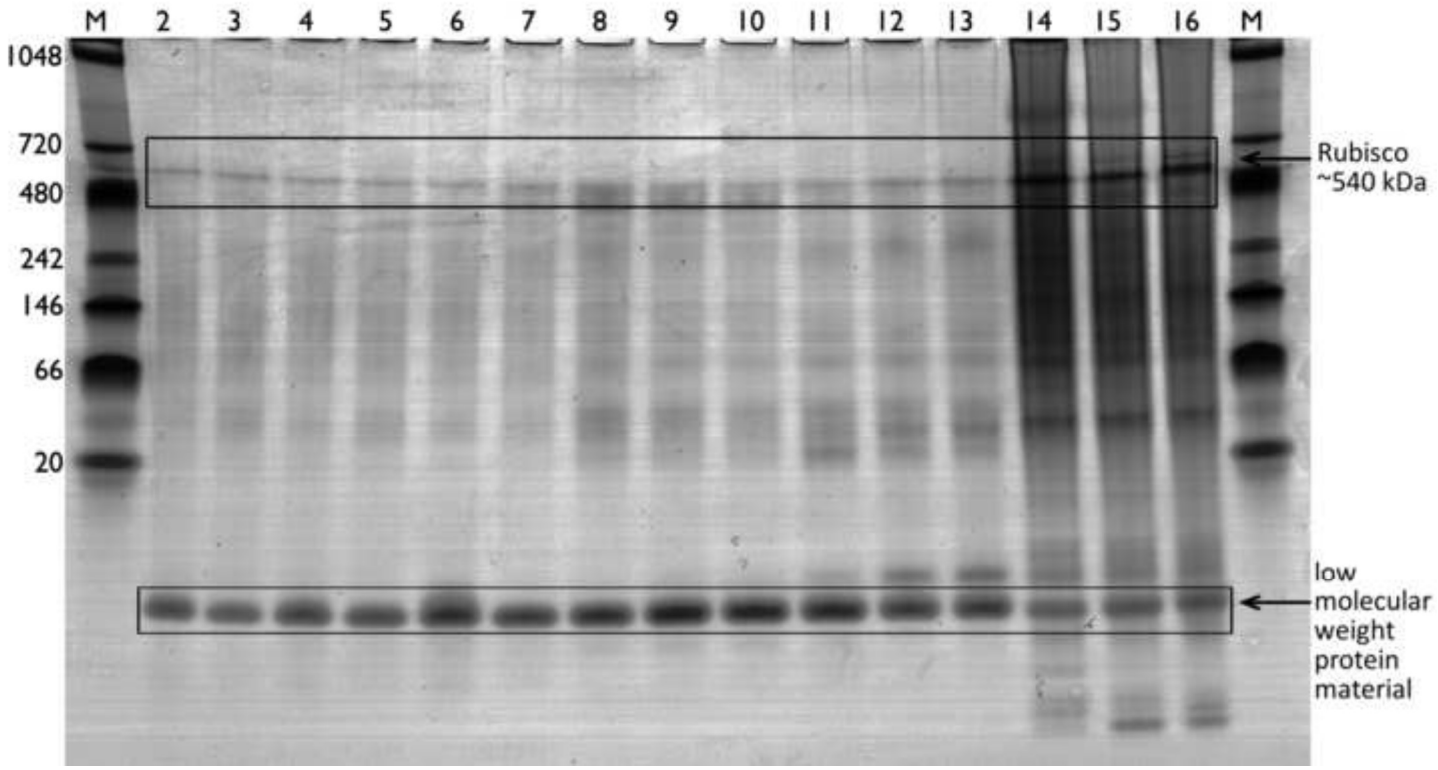

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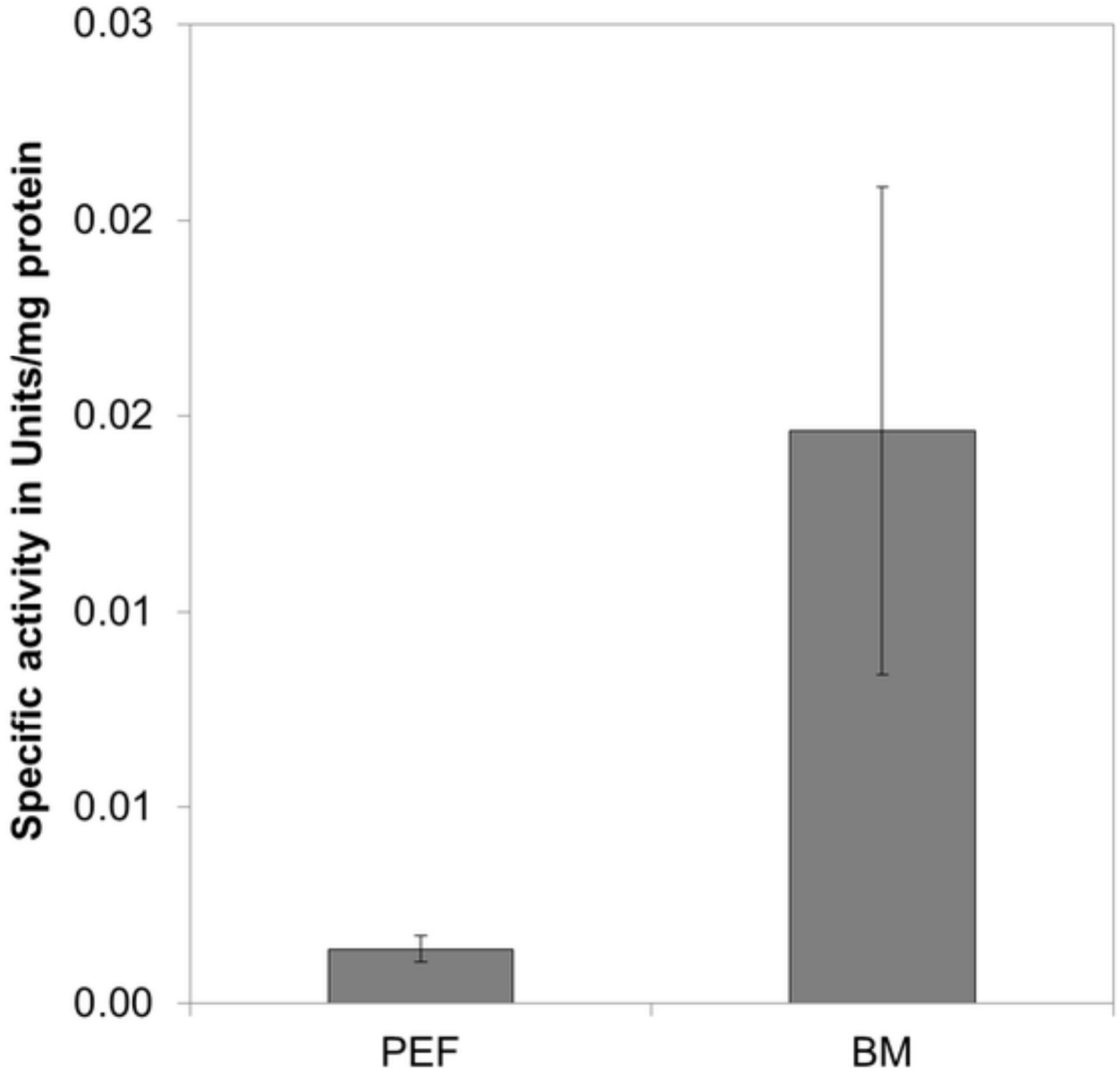

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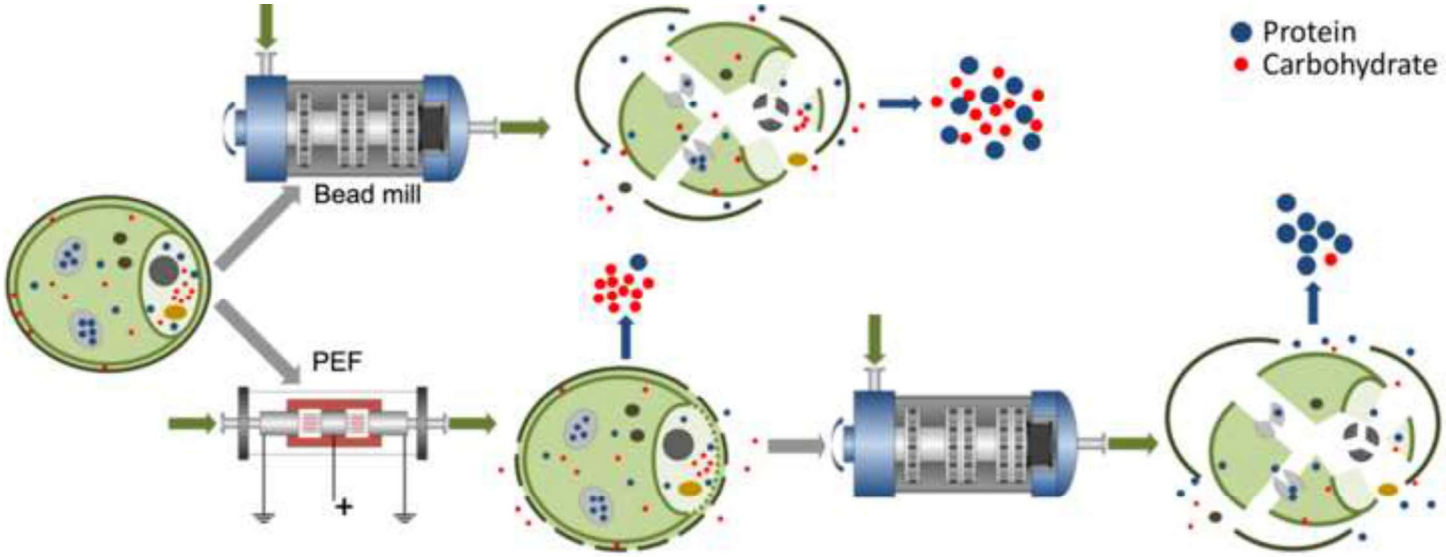

Tables

Table 1 Overview of conducted PEF experiments and process conditions

Sample	E (kV cm^{-1})	W_{PEF} (kWh kg_{DW}^{-1})	Temperature (°C)
C-25	0	0	25
P1-25	20	0.55	25
P2-25	20	1.11	25
C-35	0	0	35
P1-35	20	0.55	35
P2-35	20	1.11	35
C-45	0	0	45
P1-45	20	0.55	45
P2-45	20	1.11	45
C-55	0	0	55
P1-55	20	0.55	55
P2-55	20	1.11	55
C-65	0	0	65
P1-65	20	0.55	65
P2-65	20	1.11	65

Table 2 Specific energy input from heating (WT). A heat capacity of 1.14 10^{-3} kWh $(kg\ K)^{-1}$ is used for a 25 gDW kg^{-1} algae suspension based on an assumed biomass composition of 60% protein, 16% carbohydrate, 10% lipids and 14% ash.

Temperature (°C)	W_T (kWh kg_{DW}^{-1})
25	0
35	0.60
45	0.92
55	1.38
65	1.83