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Effect of PEF Treatment on Extraction of Valuable Compounds from Microalgae *C. vulgaris*

Gianpiero Pataro[a*], Martina Goettel[b], Ralf Straessner[b], Christian Gusbeth[b], Giovanna Ferrari[a,c], Wolfgang Frey[b]

[a] Department of Industrial Engineering, University of Salerno, via Giovanni Paolo II 132, 84084 Fisciano (SA), Italy
[b] Karlsruhe Institute of Technology (KIT), Institute for Pulsed Power and Microwave Technology (IHM), 76344 Eggenstein-Leopoldshafen, Germany
[c] ProdAlScarl – University of Salerno, via Ponte don Melillo, 84084 Fisciano (SA), Italy
gpataro@unisa.it

Recovery of intracellular components from microalgae requires a cell disruption step. In recent years, pulsed electric field (PEF) treatment has been discussed to be an efficient alternative to conventional cell disruption techniques, i.e. homogenization or bead milling.
In this work the effect of the main processing parameters of the PEF treatment on the extraction of valuable compounds from microalgae *Chlorella Vulgaris* was investigated.
Culture of *C. vulgaris* strain inoculated in TAP-medium, were cultivated in batch 26 L photo-bioreactor. The algae were harvested after 24 days and concentrated up to a final biomass concentration of 40.8 g_{dw}/kg of suspension. PEF experiments at different field strength (E=27-35 kV/cm) and energy input (W_T=50-100-150 kJ/kg) were carried out in a laboratory scale continuous flow unit. Determinations of time-conductivity profile as well as quantification of dry matter, carbohydrates, protein content, and total phenolics of the supernatant collected after 1 h extraction, were performed.
Results showed a higher increase of the electrical conductivity of PEF treated suspension, when compared to the untreated samples, as a result of the irreversible electroporation induced by pulse treatment. Moreover, the PEF treatment increased the dry matter content as well as the amount of carbohydrates, proteins and phenolic compounds released into the supernatant from inside the algae cells.
The results obtained from this study demonstrate the potential of PEF for improving extraction yield of valuable compounds from microalgae.

1. Introduction

In recent years the cultivation and exploitation of microalgae biomass has stimulated intensive research due to both its high productivity, when compared to agriculturally grown biomass, and the large content of valuable intracellular components, like lipids, proteins, polysaccharides, antioxidants, vitamins and pigments, which can be used as natural additives or active ingredients for food, cosmetic, pharmaceutical and feed products, as well as for the production of biofuels (Goettel et al., 2013; Golberg et al., 2016; Postma et al., 2016).
However, these valuable compounds are confined in internal organelles or in the cytoplasm of the algae cells protected by the rigid cell wall and especially by an intact cytoplasmic membrane. The latter acts as semipermeable barrier that greatly influences extraction of these compounds (Golberg et al., 2016; Poojary et al., 2016)
Traditionally, extraction of algae intracellular products is conducted from dry biomass with organic or aqueous solvents, depending on the polarity of the compound to be extracted (Luengo et al., 2015). However, these methods are time consuming, and often require the usage of relatively large amounts of solvents, which is expensive and not environmental friendly (Poojary et al., 2016). In addition, drying of microalgal biomass requires a significant amount of energy and may cause loss of valuable food compounds through oxidation (Golberg et al., 2016).

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For these reasons, over the last years, several research groups have investigated the use of innovative non-conventional technologies for processing of wet biomass in order to enhance the rate of mass transfer of high-added value compounds from the intracellular space, while preventing their degradation, reducing the energy costs, the solvent consumption and shortening the treatment time (Poojary et al., 2016).
Among these technologies, pulsed electric field (PEF) has gained increased interest as a non–thermal, green extraction technique for targeting intracellular compounds from plant or biosuspensions. PEF processing involves the application of repetitive short duration pulses (from several nanoseconds to several milliseconds) of high intensity electric fields to a biological material placed between two electrodes in either batch or continuous flow treatment chamber (Raso et al., 2016).
As a result, PEF induces an increase in the permeability of the cell membranes by electroporation that facilitates the release of intracellular components. Several parameters influence the PEF efficacy, which mainly include electric field strength (E) and total specific energy input (W_T) (Postma et al., 2016). In general, depending on the settings of these parameters reversible or irreversible pores are formed (Kotnik et al., 2015). Specifically, the application of PEF for improving the extraction of intracellular compounds from algae cells, requires irreversible electroporation in order to avoid that pores created by the electric field are able to reseal (reversible electroporation) after the treatment (Luengo et al., 2014).
Recently, several studies have demonstrated the potential of PEF to enhance the extraction yield of a variety of intracellular compounds from wet microalgal biomass such as lipids (Lai et al., 2014), pigments (Grimi et al. 2014; Luengo et al., 2015; Parniakov et al., 2015; Poojary et al., 2016), carbohydrates and proteins (Goettel et al., 2013; Grimi et al. 2014; Postma et al., 2016).
However, more detailed research is necessary to gain insight on the influence of the main PEF processing parameters on the cell membrane permeabilization of algae cells and subsequent release of intracellular compounds. Moreover, most data have been obtained applying PEF treatment in batch chambers of small capacity, and only few papers deal with the processing of microalgal biomass in a continuous flow unit.
This experimental study aims at investigating the extraction of valuable intracellular components from microalga *C. vulgaris* pre-treated in a continuous flow PEF unit. Particularly, the effect of the two main PEF processing parameters affecting the extent of electroporation process (E, W_T) on the release of ions (conductivity), as well as on the content of dry matter, carbohydrates, proteins, and total phenolics of the extracts, was evaluated.

2. Materials and methods

2.1 Microalgae and cultivation

The microalgae strain used throughout this work was *C. vulgaris* obtained from the Culture Collection of Algae at Göttingen University. Algae cells from a preculture were inoculated in sterile TAP-medium. The batch cultivations were carried out in a 26 L annular bubble column at 25 °C. The photo-bioreactor (Goettel et al., 2013) was illuminated continuously at 600 µE/s/m^2 by 8 fluorescence lamps (Osram fluora, 36 W). The culture was aerated at a rate of 1000 cm^3/min with an air flow containing 2.5% (v/v) carbon dioxide. Growth conditions were monitored by optical density (OD) measurements at 750 nm. The algae cells were harvested after 24 days at a biomass concentration of about 3.5 g_{dw}/ kg of suspension (kg_{sus}). The algal suspensions from the photo-bioreactor were concentrated 15 times by centrifugation at 3000 g for 7 min at 20 °C. In this way, algae suspensions with biomass concentrations of 40.8 g_{dw}/kg_{sus} were obtained. The electrical conductivity of the concentrated suspensions was not adjusted and was 1.3 mS/cm at 25°C. The concentrated suspensions were stored at ambient temperature and PEF treated within 1 h after the concentration step.

2.2 PEF Treatments

PEF experiments were conducted in a bench-scale continuous flow PEF system manufactured at the Institute of Pulsed Power and Microwave Technology (Karlsruhe Institute of Technology, Germany) and described in detail in a previous work (Goettel et al., 2013).
Briefly, it consisted of a treatment chamber made of two stainless steel electrodes with a diameter of 60 mm paired in parallel and separated to a gap distance of 4 mm by a transparent polycarbonate housing. The treatment chamber was connected to the output of a transmission line pulse generator that delivered square pulses with a voltage amplitude between 8 kV and 20 kV. 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 (2–0.1 W, Stangenes, Inc., USA), respectively, and used to evaluate the field strength and the energy input applied to the processed algae suspension.
During PEF treatment, the algae suspension, placed in a feed vessel and kept under stirring at ambient temperature, was pumped through the chamber with a peristaltic pump (ISMATEC ISM 834C, Switzerland) at a constant flow-rate of 24 mL/min. The pulse length was fixed to 5 µs, while electric field strengths (E) of 27

and 35 kV/cm and total specific energy input (W_T) of 50, 100, and 150 kJ/kg_{sus} were set by varying the applied voltage (10.8 and 14 kV) and the pulse repetition frequency (1-6 Hz), respectively. All the experiments were carried out at an inlet temperature of 20 °C, while the maximum temperature increase due to Joule effect was of 25°C.
At the exit of the treatment chamber, treated and untreated algae suspension were collected in plastic tubes and placed in an ice water bath to be rapidly cooled up to a final temperature of 25°C. After cooling, the samples were allowed to stand for 1 h 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, 5300g) and the supernatant was transferred to fresh tubes and stored at -20 °C until further analysis.
In each processing conditions, the experiments were performed in duplicate and each collected sample was analysed in duplicate. The mean values and standard deviations of the experimental data were calculated.

2.3 Analyses

Changing of the electrical conductivity (σ) of untreated and treated algae suspension with time after PEF treatment, was monitored at constant temperature of 25°C for 24h.
Dry matter was assed using the method described by Goettel et al. (2013). The content of carbohydrates and proteins was determined according to the method reported by Postma et al. (2016). Total polyphenol content (Folin-Ciocalteu method) was quantified using the method described by Singleton et al. (1999).

3. Results

The evolution with time of electrical conductivity of the algae suspensions subjected to PEF treatment, has been successfully used by various authors as reliable indicator for quantifying the efficiency of the treatment parameters for the release of intracellular ionic substances, as a result of the increased cell membrane permeabilization (Goettel et al., 2013; Grimi et al., 2014; Luengo et al., 2014). Figure 1 shows a typical time-conductivity profile at 25°C of untreated and PEF treated algae suspension at different intensities (E and W_T). Results show that the conductivity of untreated samples increased only slightly with incubation time. The initial conductivity value was 1.3 mS/cm and reached the saturation value of 1.4 mS/cm after 3 h of incubation at 25°C.
On the other hand, PEF treatment yielded a faster increase of the conductivity up to the value of 2.2 mS/cm (on average), already after 1 h of incubation. Within this interval of time, an increase of the field strength and energy input led to a notably higher rate of release of ionic substances.

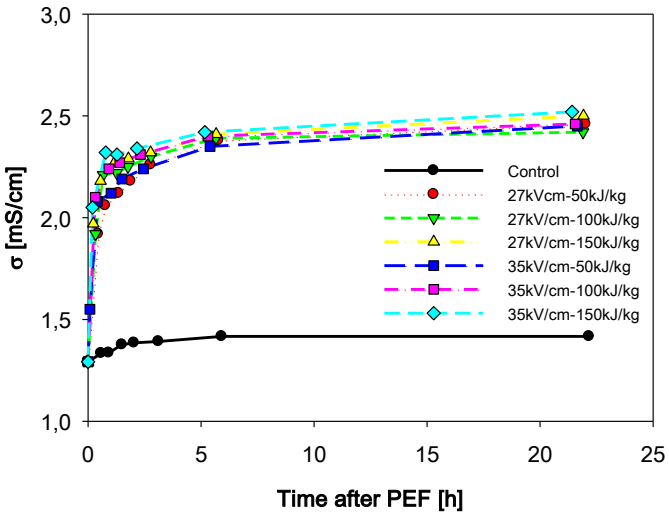

Figure 1: Effect of incubation time after PEF treatment on electrical conductivity at 25 °C of untreated (control) and PEF treated algae suspension.

These results are a clear indication that PEF caused permeabilization of the algal cell resulting in leakage of small ions as previously observed (Goettel et al., 2013; Grimi et al.; 2014; Postma 2016). Moreover, in agreement with previous findings (Goettel et al., 2013; Luengo et al. 2014), it is likely that under the processing conditions used in this work (E>20kV/cm), most of the population of *C. vulgaris* exhibited a high

degree of membrane permeabilization. Interestingly, results of this work clearly highlight the key role played by the energy input in inducing a conductivity increase during the first minutes after treatment, irrespective of the value of field strength applied (Figure 1). Further increasing of the incubation time, did not cause significant increase in conductivity value, which tended to level off to a final value of 2.5 mS/cm after 24 h of incubation, regardless of the PEF treatment intensity. According to these findings, an incubation time of at least 1 h after the PEF treatment should be integrated into algae processing for an efficient extraction of intracellular components from *C. vulgaris*. Therefore, for further investigations, supernatant obtained by centrifugation of algae suspensions 1 h after PEF treatment, were subjected to determination of dry matter, carbohydrates, proteins content, total phenolics.

The dry matter content in the supernatant of untreated and PEF treated algae suspension as a function of the energy input and for different field strength applied, is shown in Figure 2. As expected, these results are in agreement with those reported in Figure 1. The application of PEF treatment markedly increased the dry matter content, when compared with the untreated sample. In particular, after a PEF treatment at a field strength of either 27 or 35 kV/cm at 50 kJ/kg, the dry matter increased 3.6 times. However, for energy input greater than 50 kJ/kg, higher values of electric field intensity and energy input led only to a slightly higher release of intracellular matter content.

This is in contrast to the results found by Goettel et al. (2013), who observed a continuous increase of cell components in the medium surrounding *Auxenochlorella protothecoides* up to 200 kJ/kg. Here, at a treatment energy of 50 kJ/kg, the dry weight of released solids is already close to the maximum value. The yield of extracted cell components from *Chlorella vulgaris* starts to saturate earlier at 50-100 kJ/kg.

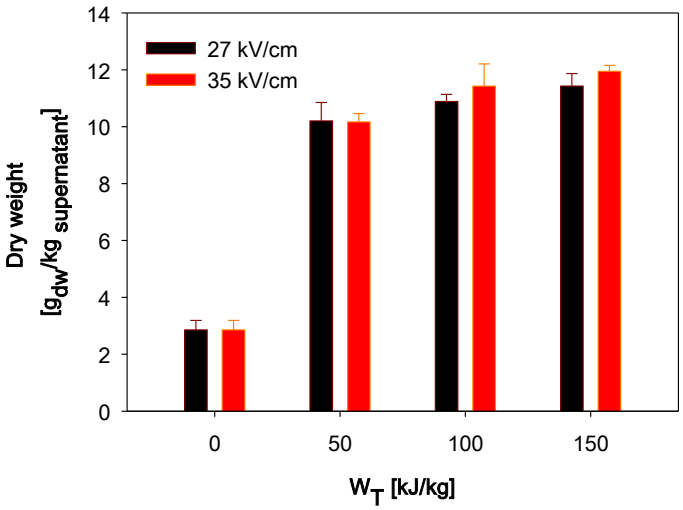

Figure 2: Dry mass content in the supernatant of untreated (0 kJ/kg) and treated algae suspension 1h after PEF treatment of different intensities.

Carbohydrates, proteins and phenolics compounds extracted from algae cells might be used as high-added value ingredients in food and pharmaceutical industry.

Figure 3 shows the content of carbohydrates, proteins and total phenolics of the supernatant obtained after PEF treatment of algae suspension at fixed energy input of 100 kJ/kg by varying the field strength applied (Figure 3a), and at a fixed field strength of 27 kV/cm and for different energy input (Figure 3b).

The content of carbohydrates released in water without any PEF treatment and after 1 h of extraction was 2.7 g/L of supernatant. When the PEF treatment was applied, a substantial increase in the release of carbohydrates was observed. However, the effect of the field strength applied (Figure 3a) appeared less important than that of the energy input (Figure 3b), at least in the range of values investigated. Specifically, as compared with the control sample, when PEF treatment at 27 and 35 kV/cm and at fixed energy input were applied (Figure 3a), the carbohydrates content in the supernatant increased 2.7 and 2.6 times, respectively. On the other hand, the application of 50, 100 and 150 kJ/kg at 27 kV/cm, increased the extraction of carbohydrates 2.2, 2.7, and 2.8 times, respectively (Figure 3b). A similar increasing trend when increasing the energy input from 50 to 150 kJ/kg at a fixed field strength applied of 35 kV/cm was observed by Goettel et al. (2013) with the microalgae *A. protothecoides*. In contrast, Postma et al. (2016), did not find any significant difference in the release of carbohydrates from *C. vulgaris* treated by PEF at 50 and 100 kJ/kg at room temperature. However, at the field strength applied by these authors (<20 kV/cm), it is likely that the degree of

membrane permeabilization was substantially lower (Luengo et al., 2014). Thus, based on the presented results it can be concluded that the field strength for efficient carbohydrate extraction has to be higher than 20 kV/cm.

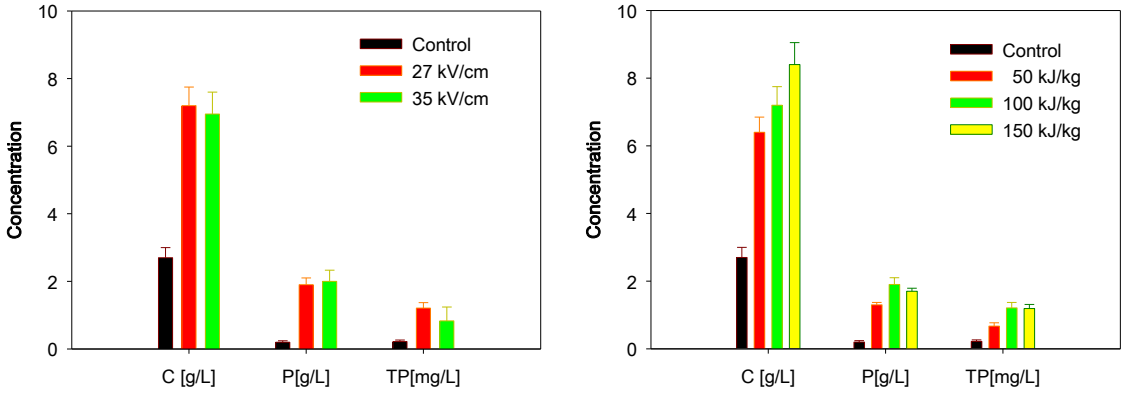

Figure 3: Carbohydrate (C), Proteins (P), and Total phenolics (TP), measured in the surpenatant of untreated (Control) and treated algae suspension 1 h after PEF treatment at a) fixed energy input (W_T=100 kJ/kg) and for different field strength applied, and b) at fixed field strength (E=27 kV/cm) and for different energy input.

The analyses of the proteins in the supernatant, showed that very low amount of proteins (0.2 mg/mL of supernatant) could be extracted when no PEF treatment was applied. However, similarly to the results on the release of carbohydrates, significantly higher contents of proteins were extracted from the PEF treated algae suspension, as compared to the control sample. Moreover, no appreciable difference in the protein content was detected when PEF treatments were carried out at different field strength (Figure 3a), while significant increase was observed in the samples collected after treatment at different energy input ((Figure 3b), but only when the energy was increased from 50 to 100 kJ/kg. Further increase of the energy input up to 150 kJ/kg, led to a slight reduction in the amount of proteins as compared with treatment at 100 kJ/kg. Overall, the maximum content of proteins was 2 mg/mL, which was detected in the samples treated at 35 kV/cm and 100 kJ/kg, corresponding to an extraction yield of about 8%. This value is on the same order as achieved by Grimi et al. (2014) and Postma et al. (2016), who concluded that a substantial amount of water-soluble algal proteins remains inside the algae cell. Moreover, results of this work show that protein release was not limited by an insufficient high field strength value (Figure 3). Thus, it can be hypothesized that PEF preferentially releases water-soluble proteins of small molecular weight, while most proteins, which are likely larger and more bounded to intracellular structure, would require the application of more effective cell disruption techniques than PEF like high voltage electrical discharges, or powerful mechanical disintegration methods such as bead milling or high pressure homogenization (Poojary et al., 2016)

Finally, regarding the extraction of phenolic compounds, the results reported in Figure 3 shows that the algae cells were able to spontaneously release only a little amount of phenolic compounds (0.22 mg/L of supernatant). After PEF treatment, a substantial increase in the release of total phenolics up to a maximum concentration of 1.21 mg/L was detected in the sample treated at 27 kV/cm and 100 kJ/kg. Moreover, similarly to the results on the release of proteins, only a positive effect of the energy input was detected between 50 and 100 kJ/kg, while the effect of field strength was negligible in the investigated range. To date, only few works focused on PEF-assisted extraction of phenolic compounds from algae cells. In particular, Parniakov et al., (2015) found that PEF pre-treatment increased the total phenolic content in a manner dependent on the concentration of the aqueous organic solvent.

4. Conclusions

The results obtained in this study allow to conclude that PEF treatment carried out under processing conditions (E>20 kV/cm) able to induce irreversible permeabilization of algae cells, is an effective technique to release substantial amount of small ionic solutes, carbohydrates and phenolic compounds. Moreover, in these conditions it appears that an increase in the energy input can be more effective in promoting the leakage of intracellular compounds than a further increment in the field strength applied. Nevertheless, the degree of cell membrane permeabilization was not effective enough to release high quantities of large molecules such as protein (an extraction yield <8%).

Thus, more research should be carried out in order foresee the application of PEF as a first selective disintegration stage in a multi-stage biorefinery, which should include two consecutive extraction stages for the recovery of both water soluble components (e.g. carbohydrates, small soluble protein molecules) and hydrophobic components (pigments), followed by the application of a more effective cell disruption technique for the recovery of high molecular weight components (e.g. large protein molecule).

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