

Post-incubation pH Impacts the Lipid Extraction Assisted by Pulsed Electric Fields from Wet Biomass of *Auxenochlorella protothecoides*

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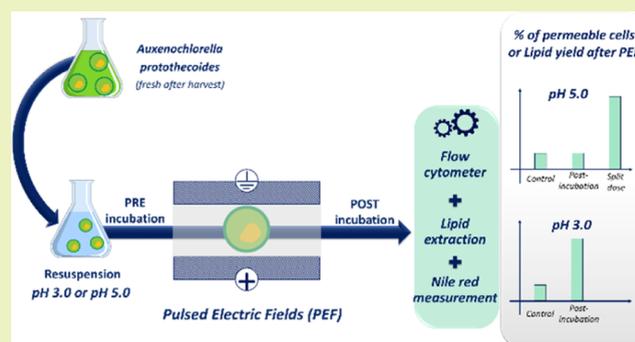
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ABSTRACT: Pulsed electric field (PEF) treatment is a promising technology for efficient lipid extraction from microalgae. This study focusses on under-investigated processing parameters, such as media pH, pulse application, and incubation protocols. The lipid yield and electroporation level of PEF-treated *Auxenochlorella protothecoides* were determined at a medium pH of 3.0 and 5.0 under variation of the pre- or post-PEF incubation time and for split-dose treatments. Low energetic PEF treatments with 40 kV/cm and 1 μ s pulses at 9.6 and 19.2 kJ/L were performed either in batch mode or in continuous flow. Post-PEF incubation significantly increased the shared electroporated cells (>60%) in medium pH 3.0, while no change was observed at pH 5.0. Split-dose PEF treatments at pH 5.0 caused significantly higher electroporation levels and lipid extraction yields than equivalent single-dose treatments. Results have shown that medium pH is critical in the final electroporation and lipid extraction yields of *A. protothecoides* and therefore should be considered in further studies.

KEYWORDS: microalgae, lipid extraction, pulsed electric fields, electroporation, media pH, incubation



1. INTRODUCTION

Microalgae are a promising sustainable source of many biocompounds, thanks to their capacity to use inorganic carbon as a nutrient by means of photosynthesis and to the possibility to cultivate them on non-agricultural land.¹ Lipids from oleaginous microalgae are one of the most investigated compounds because of their versatile applications in the food, feed, pharmaceutical, and chemical industry or for production of biofuels. Their final industrial application is directly related to the fatty acid composition and largely to the degree of fatty acid saturation.² While most microalgae accumulate saturated and monosaturated fatty acids excellently suited for biofuel production, some species produce more polyunsaturated fatty acids with high value for human nutrition or animal feed.²

However, the extraction of these lipids of interest is made difficult due to the particularities of microalgae. First, they are very small and possess a very robust cell wall that sometimes makes difficult the use of traditional extraction techniques such as French press. Furthermore, although a drying pre-step has been shown to facilitate lipid extraction by organic solvents from microalgae, the enormous drying energy costs make it non-competitive for current marketing. Therefore, the actual challenge of research is to develop an efficient and affordable method for lipid extraction from wet microalgae biomass. This

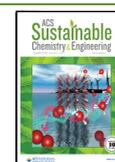
approach is essential to obtain competitive microalgae lipids for large-volume production at an industrial scale. From an economic point of view, the right choice of the cell disruption method is also important. Physical disruption methods usually investigated include bead milling, high-pressure homogenization, ultrasound, along with other less conventional approaches such as microwaves, freezing–thawing, osmotic shocks, or pulsed electric fields (PEFs).³ An appropriate pre-method for lipid extraction from microalgae should not only be a guarantee of high performance in terms of lipid yield but also avoid heat damages, allow continuous treatment, applicability at large-scale, and further downstream processing with minimum operating costs (energy consumption, further purification steps).

Among the current non-thermal emerging technologies, PEF treatment is one of the most promising for intracellular lipid extraction due to the fact that it is a mild disruption technique,

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thanks to its very low energetic requirements (typically reported: 1.5–2 MJ/kg_{dw} in maximum), and it does not generate debris, which favors product residue separation.^{3,4} PEF technology increases the permeability of the cytoplasmic membrane of biological cells facilitating the mass transfer of water-soluble outside or inside the cell. This well-known phenomenon called electroporation or electroporability is the consequence of an external electric field. In practice, pulses of high electric field intensity and short duration (μ s–ms) are delivered to a biological matrix located between two electrodes. PEF treatment was successfully used to enhance the extraction of different valuable compounds in a large range of microalgae strains, eventually with solvent assistance. One can mention macromolecules such as proteins, carbohydrates, and lipids or more specific ones such as carotenoids, pigments, or enzymes.^{3,5}

Although plenty of studies have proven the benefit of PEF as a microalgae pre-treatment, some of the underlying mechanisms are still unknown. While some small water-soluble compounds, such as ions, are immediately liberated by a simple diffusion process, larger molecule extraction has shown to require in many cases long incubation durations after PEF treatment. Regarding protein extraction, the benefit of incubation after PEF in the recovery yields has been demonstrated in *Haematococcus pluvialis*, *Chlorella vulgaris*, *Porphyridium cruentum*, and *Arthrospira platensis*.^{6–10} This phenomenon has been related not only to the kinetics of diffusion of proteins outside of the cells but also to a protein release process that might be mediated by enzymes. Scherer et al.¹¹ demonstrated that adding a protease inhibitor in *C. vulgaris* suspensions after the PEF decreased the final protein yields, suggesting the enzymatic key role in this incubation process.

In the case of lipid extraction from microalgae, PEF has been proved to be an efficient pre-treatment enabling the accessibility of solvents to the inner lipids.^{12,13} However, Goettel et al.¹² found that while soluble compounds are released spontaneously after PEF, lipid droplets remain inside microalgae cells. In the same way, it was reported that the disruption of *C. vulgaris* by PEF resulted in a deficient carotenoid extraction even when 90% of cells were electroporated and resuspended immediately in ethanol.¹⁴ Deeper studies have demonstrated that larger incubation times (20–24 h) after PEF treatments (40 kV/cm; 1 μ s, 1.5 MJ/kg_{dw}) enabled almost the total lipid extraction from *Auxenochlorella protothecoides* and *Scenedesmus almeriensis*.^{15,16} Furthermore, this incubation phenomenon was further investigated in *A. protothecoides*, and it was shown that it allows us to significantly reduce the required energy of PEF treatment (from 1.5 to 0.25 MJ/kg_{dw}), while still recovering 97% of the total lipids.¹⁷ This enhancing effect of after-PEF incubation has been suggested to be related to an autolysis process triggered by PEF, as already reported for yeasts.^{18,19} In the case of microalgae, an analogous approach was performed for the carotenoid extraction from *H. pluvialis* by PEF treatment combined with a 6 h post-PEF aqueous incubation.²⁰ The authors observed an enormous release of the esterase enzyme in the supernatant of PEF-treated suspensions in the first hours after PEF, in comparison to the untreated ones. The bioaccessibility of microalgae lipids has also been suggested to be improved by the incubation after PEF.^{4,21} More recently, PEF has been shown to induce programmed cell death in the microalgae *C. vulgaris*.²² Sublethal PEF treatments would induce the production by

microalgae of a cell death factor that is released and increases population mortality with incubation after PEF. However, the mechanism of the incubation after PEF in microalgae is under-investigated, and the actual biological responses behind are still barely identified.

On the other hand, the effect of the repetition rate of pulses and the application of pulses in split-dose has been scarcely investigated in microbial cells. Most of the time, the repetition rate (frequency) has been considered not to influence the final microbial electroporation/inactivation outcome by PEF for energy-equivalent treatments.²³ However, the range of the repetition rate used in major studies is extremely broad and spreads from 0.5 to 500 Hz. When lower repetition rates were investigated in potato tissue (long delay between pulses), results showed that very low frequencies (0.1–0.02 Hz) resulted in more effective electroporation.²⁴ Regarding microbial cells, the applications of split-train of pulses revealed higher inactivation levels than a single equivalent pulse train in *Salmonella typhimurium*.²⁵ These findings were attributed to the *electro-desensitization* theory, which suggests that reversibly electroporated cells during PEF application might be “resistant” to subsequent pulses due to the loss of the cytoplasmic membrane integrity needed to generate a transmembrane voltage. On the contrary, *electro-sensitization* theory which is based on observations in mammalian cells attributes the effectivity of split PEF to an increment in the sensitivity of cells due to the treatment itself.^{26,27} However, none of the (de)sensitization processes proposed has been fully demonstrated yet. The phenomena of *electro-sensitization* and *electro-desensitization* have been barely investigated, although they could help to understand some of the biological behaviors of cells subjected to PEF and facilitate the industrial application of this technology. Furthermore, it represents a potential strategy for reducing energetic requirements in lipid extraction from microalgae using PEF processing.

Finally, although the medium pH has been reported to be a key factor in the final inactivation of bacteria cells, it has never been evaluated in microalgae electroporation and subsequent lipid extraction. Generally, the effect of pH has been found to be different between Gram-positive and Gram-negative bacteria, attributing the differences to the capacity or difficulty to recover the sublethal damages produced in their cytoplasmic membranes.²⁸

In general, there is plenty of research regarding microalgae as a source for lipids and the potential of PEF as a pre-treatment, prior to extraction. However, most of the research has been focused on a very practical application approach, forgetting sometimes the fundamental knowledge about the effect of PEF and the biological responses of microalgae. Therefore, this study aimed to evaluate different protocols of pre-PEF and post-PEF incubation and the application of split-dose treatments in media of different pH. The main objective was to obtain substantial knowledge about microalgae behavior against PEF and to propose adequate strategies to optimize PEF treatments for industrial requirements.

2. MATERIALS AND METHODS

2.1. Microalgae and Cultivation Conditions. **2.1.1. Microalgae Strain and Mixotrophic Cultivation.** The microalgae strain *A. protothecoides* (211-7a) was purchased from SAG, Culture Collection of Algae, Göttingen, Germany. Axenic cultures were maintained in cultivation flasks in a modified Wu medium, as detailed by Silve et al.¹⁶ Erlenmeyer flasks with 400 mL of Wu medium properly

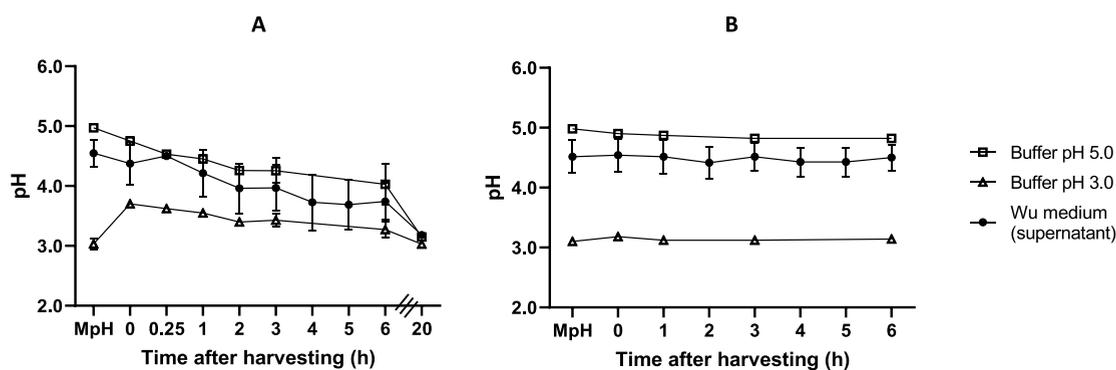


Figure 1. pH evolution along time after resuspending (A) concentrated (100 g_{dw}/L) and (B) non-concentrated (10 g_{dw}/L) microalgae biomass in its own medium and McIlvaine buffers at pH 3.0 and 5.0. MpH: Original pH of the supernatant and buffer media before resuspending microalgae cells.

autoclaved were inoculated to obtain an optical density (OD at 750 nm) of 0.1. Inoculated flasks were cultivated at 25 °C on the ORBIT 5000 analogue shaker (VWR International, Bruchsal, Germany) at 100 rpm. Illumination was provided by LED (LUMINUX COMBI LED-N, 980 lm, 3000 K, 10 W, OSRAM) at 60 μmol/m² s. Microalgae growth was monitored by OD, cell dry weight (CDW), and the number of cells measured. After 10 days when microalgae suspension achieved around 10 g_{dw}/L and 3 × 10⁸ cells/mL, microalgae were harvested for further PEF experiments.

2.1.2. Optical Density. OD at 750 nm was measured by a spectrophotometer (Genesys 10S UV-vis, Thermo Scientific) using a disposable cuvette (ref 634-0676, VWR).

2.1.3. Cell Dry Weight. Between 5 and 10 mL of the whole microalgae suspension and supernatant were placed in pre-weighed aluminum caps in a precision balance. Both cups were dried at 90 °C overnight in a drying oven (Universalschrank Model U, Memmert, Germany) and weighted again. The CDW [g_{dw}/L] was calculated by subtracting the dry weight of the supernatant medium from the dry weight of the microalgae suspension.

2.1.4. Microalgae Concentration and pH-Buffer Resuspension. Once the microalgae suspension was harvested (10 g_{dw}/L), it was subjected to a centrifugation process at 3000g using a Sigma 8k centrifuge with a swinging-bucket rotor (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The microalgae pellet was resuspended in the appropriate amount of cultivation media or pH-buffers to concentrate the biomass to reach a CDW of 100 g_{dw}/L. Buffers used were citrate-phosphate McIlvaine of pH 3.0 and 5.0, adjusted to 1 mS/cm. When the biomass concentration was not required, the pellet was resuspended in the same volume of the supernatant drawn after centrifugation, in order to maintain the same CDW (10 g_{dw}/L). The exact final concentration was always measured. The pH and its evolution in the different cell suspensions were measured by a pH-meter (Multi 3510 IDS, Xylem Analytics Germany GmbH, Weilheim, Germany).

2.2. PEF Processing. Microalgae suspensions of 10 or 100 g_{dw}/L resuspended in the cultivation medium (Wu medium) or in McIlvaine buffers of pH 3.0 and 5.0, with a conductivity adjusted to 1 mS/cm, were subjected to different PEF treatments in batch and continuous flow conditions.

PEF treatments were accomplished using a custom-made transmission-line generator that delivers square waveform pulses. The voltage was monitored by a high voltage probe (P6015, Tektronix, Beaverton, OR, USA) connected to an oscilloscope (TDS640, Tektronix). The current was measured sporadically with a current transformer (model 411, Pearson Electronics, Palo Alto, CA, USA).

The electric field strength was fixed at 40 kV/cm and pulse duration at 1 μs width. In the case of batch experiments, 2 to 60 pulses were applied at 1 Hz, corresponding to a range of 4.8–144 kJ/L of the total specific energy. Pulses were applied in an electroporation cuvette (VWR, Radnor, Pennsylvania, USA) with a parallel electrode configuration distant of 0.4 cm. In order to

accomplish equivalent PEF treatments in continuous flow, samples were pumped at 0.53 mL/s using a peristaltic pump (Masterflex L/S, Cole Parmer Instrument Company, Vernon Hills, Illinois, USA), and frequencies were modified to deliver continuous flow equivalent specific energies as in the batch treatment. Treatments were applied at room temperature, and the maximum temperature achieved was lower than 28 °C.

After harvesting, pre- and post-PEF treatment incubations of different durations (0, 3, or 6 h) at room temperature were applied, in order to evaluate their effect on the subsequent electroporation rates and on the efficiency of lipid extraction.

2.2.1. Split-Dose PEF-Treatments. The effect of the application of split-dose treatments was evaluated by comparing a single dose of 8 pulses with the outcome of the equivalent treatment-applied split as 4 + 4 pulses, delayed by 3 and 6 h in between. The incubation between the two PEF doses was carried out at room temperature in the treatment medium. The effect of split-dose treatment was assessed by the electroporation rates and the lipid extraction efficiency.

2.3. Electroporation Measurements by a Flow Cytometer. Microalgae electroporation was measured by detecting the uptake of the fluorescent dye Yo-Pro [YO-PRO-1 Iodide (491/509) Invitrogen, Thermo Fisher Scientific]. The initial microalgae samples were diluted to 1.0 g_{dw}/L in their own treatment medium (previously filtered) and mixed with an adequate amount of Yo-Pro to obtain 0.1 μM of the final concentration. Samples were incubated for 10 min in the dark at room temperature and then diluted 1:5 before input in the flow cytometer. The flow cytometer used was the Attune NxT (Thermo Fisher Scientific) with a 488 nm laser as the excitation source. The emission fluorescence signal was detected using the green filter of the device (530/30). For each sample, 5000 cells were analyzed. The number of electroporated cells was calculated by comparison with the negative control and positive control (70 °C, 10 min) obtained using the same staining protocol.

2.4. Lipid Extraction Protocol. The lipid extraction protocol used in this study was similar to the one described by Silve et al.,¹⁶ with slight modifications. 30 mL of the microalgae suspension (10 g_{dw}/L) was centrifuged, and the wet pellet was resuspended in a mixture of hexane/ethanol to achieve a final extraction system of water/ethanol/hexane 1:18:7.3 v/v/v (water was not added but is the residual water in the microalgae pellet). The extraction system was left overnight under agitation in the dark. Afterward, tubes were centrifuged (10 000g/10 min), and 6 mL of the supernatant was mixed with 18 mL of hexane and 3 mL of water to accomplish phase separation by means of vigorous agitation. The upper hexane phase was recovered in a pre-weighed glass flask and evaporated in a rotary evaporator (Hei-VAP Expert, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). The amount of lipids was determined gravimetrically in a precision balance.

2.5. Nile Red Measurements. Nile red dye (Invitrogen) was used for the detection of the remaining intracellular lipid inside microalgae after the lipid extraction procedure. Nile red staining was

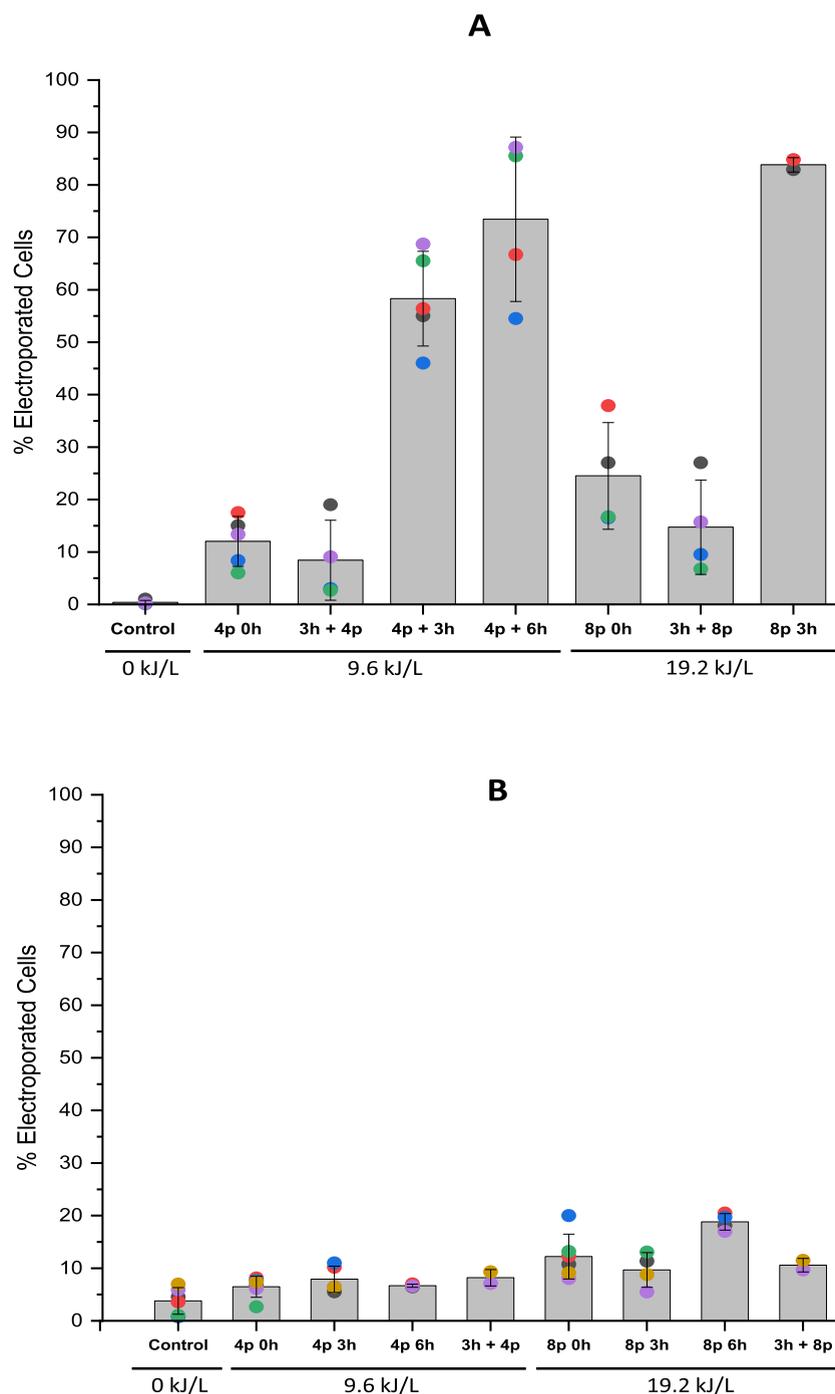


Figure 2. Influence of incubation after and before PEF on the electroporation levels (%), obtained by PEF treatments (40 kV/cm) of 4 pulses (9.6 kJ/L) and 8 pulses (19.2 kJ/L) at pH 3.0 (A) and 5.0 (B).

performed on the biomass kept at $-20\text{ }^{\circ}\text{C}$ after the lipid extraction. The microalgae biomass was resuspended in PBS until an OD (750 nm) of 0.05 was reached. From this suspension, 1 mL was centrifuged at 5000g for 5 min, the supernatant was removed and replaced with 800 μL of PBS plus 200 μL of a 30 $\mu\text{L}/\text{mL}$ Nile red solution in DMSO. The sample was left for 15 min at $40\text{ }^{\circ}\text{C}$ in the dark for staining and then washed twice by centrifugation. The samples were then kept on ice before further analysis on the flow-cytometer using the 488 nm excitation laser and the 530/30 emission filter.

2.6. Statistical Analysis. For each measurement, at least three samples from independent cultivation were analyzed. Data are expressed as the mean \pm the standard deviation. GraphPad Prism (Graph-Pad Software, San Diego, California, United States) was used

for statistical analyses to evaluate the significance of differences among the mean values by one-way analysis of variance and the Tukey test. Differences were considered significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of *A. protothecoides* Biomass Concentration on the pH of the Microalgae Suspension.

Working at high concentration, typically, 100 $\text{g}_{\text{dw}}/\text{L}$ is usual in microalgae downstream processing since increasing concentration enables to reduce operating costs.² In order to achieve the usual working biomass concentration, the microalgae suspension was concentrated from 10 to 100 $\text{g}_{\text{dw}}/\text{L}$ after

harvesting either in their own medium of growth of pH 4.6 (value at the end of cultivation) or in 2 McIlvaine buffers at pH 3.0 and 5.0. The pH of the three microalgae suspensions was measured for 20 h after the concentration step, and results are shown in Figure 1A.

Suspensions immediately after harvesting (0 h) slightly decrease their pH in the case of microalgae resuspended in buffer pH 5.0 and in the supernatant but rise up to 3.7 in buffer pH 3.0. During the hours following the concentration/resuspension, the pH of the three suspensions falls down, reaching around 3.0 for all suspensions after 20 h. Even after just 6 h, the pH of the suspension resuspended in supernatant media decreased from 4.6 to 3.7. On the contrary, when microalgae were resuspended without concentrating the biomass (concentration 10 g_{dw}/L), the pH of all suspensions remained constant during the 6 h after harvesting (Figure 1B). These results suggest that the concentration process itself triggers some acidification processes, which decreases the pH of the suspension. This observation might be only detected when the number of microalgae is sufficient (high concentrations) to change the pH. The observed decrease of pH with time implied non-constant conditions along the experimental time that might involve methodological variabilities in the results. Therefore, pH being one of the target parameters to investigate in this study, the following experiments were carried out without concentrating the biomass in order to accomplish reproducible results. Furthermore, pH monitoring was also performed after the application of a PEF treatment (40 kV/cm; 25 kJ/L) in non-concentrated microalgae biomass in their own growing medium. The results obtained showed non-significant variations of pH after PEF, pH being constant even after 6 h (data not shown). Measurements during the following experiments performed in buffers did not show any pH variations after PEF treatments either.

3.2. Influence of Incubation of Microalgae at Different pH before and after PEF Treatment on Electroporation Levels and on the Lipid Extraction Efficiency.

A. protothecoides was subjected to PEF treatment, and the microalgae suspension was incubated before or after PEF. Experiments were performed in buffers at either pH 3.0 or 5.0, and the microalgae concentration was always set at 10 g_{dw}/L. Figure 2 shows the percentage of electroporated microalgae cells (Yo-Pro positive cells) at pH 3.0 (A) and 5.0 (B), when incubations before or after PEF of 3 and 6 h were accomplished. The application of 4 pulses and 8 pulses of 40 kV/cm and 1 μs at pH 3.0 with no incubation electroporated 12 and 24% of microalgae cells, respectively. While pre-incubation at pH 3.0 for 3 h had no significant effect on these percentages, incubation post-PEF greatly increased the number of permeabilized cells. After 3 h of post-PEF incubation, the levels of electroporation increased from 12 to 58% in the case of 4 pulses and from 24 to 83% for 8 pulses. On the contrary, at pH 5.0, neither the pre- nor post-PEF incubation significantly affected the number of microalgae cells electroporated, obtained by 4 and 8 pulses. Suspensions with untreated microalgae did not exhibit any significant variation in the electroporation levels achieved after pre- or post-PEF incubation at both studied pH values. The percentage of permeabilized cells was always below 5% (data not shown).

According to these results, the pH of the treatment media seems to play an important role in the final electroporation outcome of *A. protothecoides* when incubation time after PEF is applied. It is known that the damages created in the

cytoplasmic membrane of cells, as a consequence of an external electric field, can be reversible (sublethally injured cells). Once the treatment is finished, cells recover their membrane integrity and survive after PEF treatment, which is generally attributed to resealing of pores. The capacity of microbial cells for resealing has been reported to be dependent on the recovery conditions, where the pH value is a key parameter.^{29,30} However, the resealing process after PEF has been poorly studied, and several other possible cellular outcomes have been proposed to explain the various microbial behaviors observed.²³ The fact that cells become permeabilized, even though they repair their membranes and survive, could indicate stress processes due to the loss of homeostasis. Permeabilized cells could recover their membrane integrity immediately after PEF, but the damage could be sufficient to cause membrane permeabilization and death under less than optimal recovery conditions.

In this study, when *A. protothecoides* was treated and incubated at pH 3.0, a strong increase in permeability for Yo-Pro was observed over time. In buffer pH 5.0, closer to the physiological conditions (growth media pH 4.6), the number of electroporated cells remained constant along time after PEF treatment. This fact points out that under adverse conditions (acidic pH), *A. protothecoides* cells subjected to an external electric field, and thus external stress would not be able to maintain their membrane integrity during the post-PEF incubation. This phenomenon could be explained by the fact that the repairing mechanisms of *A. protothecoides* and its capacity to overcome alterations do not work correctly at acidic pH. García et al.³¹ observed an increase of approximately 3.0 Log₁₀ cycles in the inactivation of *Escherichia coli* treated and incubated in apple juice (pH 3.8), 6 h after PEF. The authors evidenced that the final inactivation obtained after the incubation corresponded to the sublethally injured population. On the contrary, rapid pore resealing (<3 min) was reported after treating *Chlamydomonas reinhardtii* by very moderate PEF treatment (4.5 kV/cm and 2.1 kJ/L).³² Therefore, it can be presumed that the PEF treatment applied in this study consisted of a sublethal treatment that affected 70% of the population. While at pH 5.0, most of the affected cells were able to repair their damage, under acidic conditions of incubation, they ended up being permeabilized. According to our data, to achieve similar electroporation levels (65%) without incubation, the PEF treatment energy should be at least 48 kJ/L, that is, approximately 20 pulses. This means that by applying PEF treatment in a medium with an acidic pH value (non-optimal), combined with a post-incubation of 6 h, the specific energy used can be reduced by a factor of 5, while maintaining the same efficiency. These results contribute to the suggested theory that the electroporation level is not dependent on the characteristics of the media but that its composition is crucial for the post-PEF recovery or not the native state of microbial cells.²⁵ In other words, the same PEF treatment would affect (electroporate) the same percentage of cells, regardless of media, while the final outcome of the treatment is highly dependent on the capacity of cells to repair their damages, which in turn depends on the media properties.

In parallel to the electroporation measurements, lipid extraction experiments were carried out in order to evaluate the effect of the pH on the final lipid extraction yields. Figure 3 shows the lipid extraction yields after PEF treatments for different pH and different post-PEF-treatment incubation durations. When samples were not incubated after PEF-

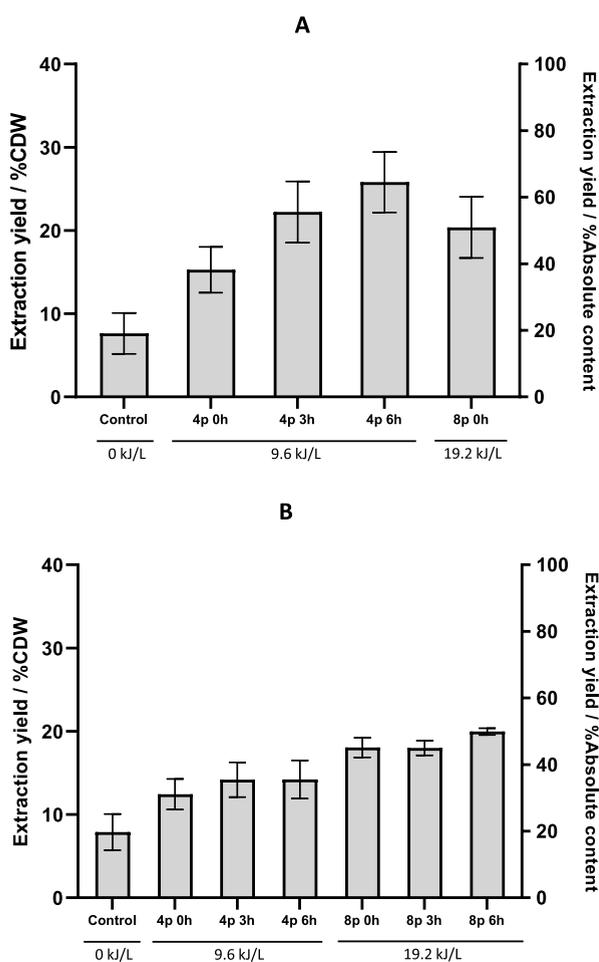


Figure 3. Influence of incubation after PEF on the lipid extraction yield obtained by PEF treatments (40 kV/cm) of 4 pulses (9.6 kJ/L) and 8 pulses (19.2 kJ/L) at pH 3.0 (A) and 5.0 (B).

treatment, the lipid extraction yields after 4 and 8 pulses at 40 kV/cm were similar for both pH 3.0 and pH 5.0. However, as was observed in the electroporation levels, the incubation of cells post-PEF enhanced the extraction yields only in the suspension at pH 3.0. For example, the application of 4 pulses allowed the extraction of 15.3% of lipids reported for CDW, that is, 38.2% of the absolute lipid content, while with an additional 6 h of incubation after PEF treatment, the lipid yield increased up to 25.8% of the CDW, that is, 64.5% of the total lipids. In contrast, in medium pH 5.0, no increase of the lipid extraction yield was observed by any post-PEF incubation, in accordance with Yo-Pro uptake results. For example, the application of 4 pulses provoked the extraction of 12.5% of CDW, that is, 31% of the total lipids, and the yield obtained by a post-PEF incubation of 6 h were just 14.2 and 35%, respectively. The positive effect of incubation after PEF-treatment on the extraction of intracellular molecules has already been reported, although mainly for aqueous compounds.⁸ Furthermore, in the case of lipid extraction, Silve et al.¹⁷ demonstrated that this effect was not a consequence of the diffusion process. These authors obtained 70% of the total lipid content using very low energetic PEF treatment (15 kJ/L) and 20 h of incubation. In the present study, similar yields (64.5%) were found at pH 3.0, when applying just 9.6 kJ/L and 6 h of incubation. Silve et al.¹⁷ suggested that endogenous enzymes could mediate this process during incubation, as attributed to

the autolysis reported for yeast. This phenomenon has also been attributed to enzyme activity in microalgae processing, enhancing the extraction of phycoerythrin from *P. cruentum* and carotenoids from *H. pluvialis*.^{6,20} According to the latter, the extraction of carotenoids would be facilitated by an enzyme driven process that is triggered by PEF and needs incubation time. These enzymes would dissociate the intracellular structures promoting subsequent solvent extraction.

In parallel, a supporting effect was observed by post-PEF incubation at pH 3.0 with respect to the number of electroporated cells and the lipid extraction yield, but the great rise in the permeabilized cells was not fully reflected in the lipid extraction yield. While drastic increases in permeabilized cells were observed 6 h after PEF (>60%), the amount of extracted lipids barely increased by 20% of the total content. Hence, there was no perfect linear correlation between the number of electroporated cells and the lipid extraction yield at pH 3.0. These discrepancies could be a consequence of the irregular distribution of lipids among the different microalgae cells. On the other hand, this might indicate that not all the lipids are immediately extracted after the electroporation of microalgae cells at pH 3.0, and although Yo-Pro can enter into the cells, some lipids cannot be extracted. This unextractability could be related to their size. It is known that the damages provoked by an external electric field can greatly differ and hence an electroporated cell can be permeable to small ions (K or Na) but not to bigger molecules such as sucrose.³⁰ Since most of the inner lipids in microalgae are triglycerides, which are large molecules, the evolution of the integrity of the membrane with incubation after PEF might be very relevant to their extraction. This could effect that even though cells are electroporated some lipids of big size will not be able to cross the pores created. However, the unextractability could also be a consequence of the lipid bond to other structures that solvents cannot break. According to some authors,^{18,20} enzymes could be involved in the unbinding process of lipids in cells treated by PEF facilitating their solvent-extractability. It is widely known that enzyme activity is very dependent on the environmental conditions, and in this case, pH 3.0 could be a non-optimal one for their action.

To our knowledge, this is the first time that the great impact of the pH medium has been evidenced for the lipid extraction effectiveness from microalgae pretreated by PEF. However, the pH relevance had already been reported in protein extraction from microalgae *Nannochloropsis* and *A. platensis*.^{7,33} Similarly, the impact of the pH in the PEF-triggered yeast autolysis was shown to be significant, and acidic pH decelerated the extraction kinetics observed at pH 7.0.³⁴ These authors suggested that it could be a consequence of the influence of pH in the enzyme activity that might mediate this process, although this still needs to be fully elucidated. Our results pointed out that pH should be taken into account for future studies in which lipid extraction from microalgae is under investigation since it is a variable to be optimized.

3.3. Influence of Split-Dose PEF Treatments in the Electroporation and the Lipid Extraction at pH 5.0.

Application of PEF in split doses has shown greater results than equivalent single doses both in permeabilization and inactivation studies on mammalian, vegetative, and microbial cells.^{25,26,35,36} This previously neglected strategy to enhance PEF effectiveness could help to reduce energetic requirements for lipid extraction from microalgae. With the objective of exploring this new approach, PEF treatment of 8 pulses in

buffer pH 5.0 were split into 4 plus 4 pulses with 3 and 6 h of delay in between. Electroporation levels and lipid extraction yields obtained following a PEF split-dose protocol are shown in Figure 4.

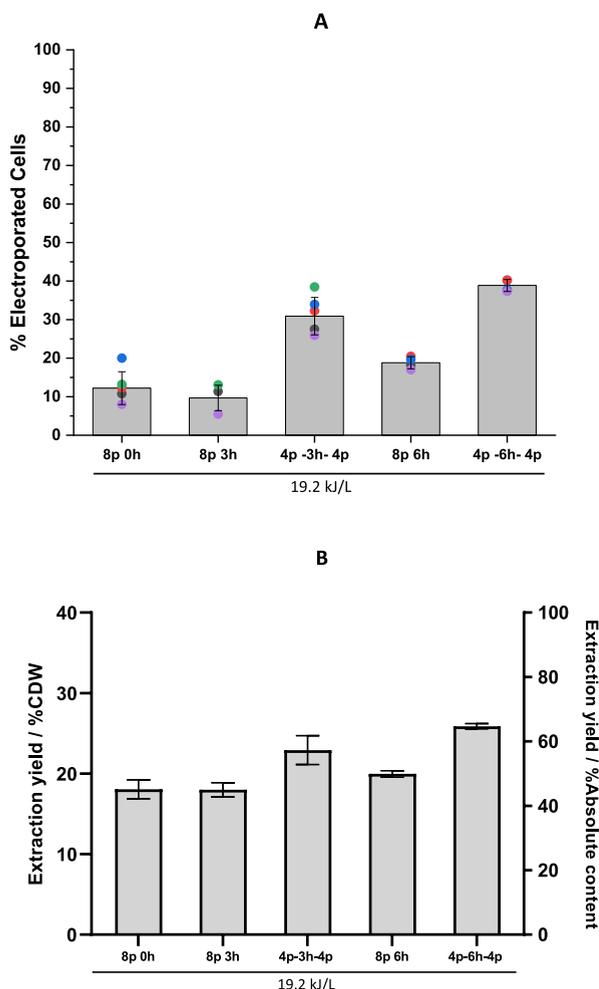


Figure 4. Influence of split-dose PEF treatment at pH 5.0 on the percentage of electroporation levels (A) and lipid extraction yields (B) obtained by PEF treatments (40 kV/cm) of 8 pulses (19.2 kJ/L) applied as a single dose of 8 pulses or split into two trains as 4 + 4 pulses with 3 or 6 h between them. For comparison purposes, the results of 8 pulses in a single dose with 3 or 6 h of incubation post-PEF are shown.

Regarding electroporation levels obtained, it can be noticed that the treatments applied as two split doses of four pulses were more efficient than a single dose of eight pulses. While 8 pulses just achieved electroporation levels ranging from 10 to 20% with and without incubation post-PEF, the application of the same number of pulses, split into two trains separated by a time delay, achieved up to 40%. Thus, by letting microalgae suspensions, treated by 4 pulses, for 6 h in the same medium and by subsequent application of another 4 pulses, the electroporation levels could be doubled. These results show for the first time the enhancement effect of the split-dose protocol in microalgae. This observed effect could be a consequence of the *electrosensitization* process occurring in a percentage of damaged but non-electroporated cells. According to this theory, some cells that get permeabilized during PEF could reseal their membrane but remain damaged. The damage

would make them more sensitive to the second train of pulses, and hence, the obtained electroporation outcome could be higher.²⁷ Indeed, Jensen et al.³⁷ demonstrated the sensitization process in mammalian cells by split-dose treatments being enhanced at higher incubation temperatures and with larger wait times between trains.

On the other hand, the *electrosensitization* process could also be suggested to explain the observed results. This hypothesis pointed out that once a cell gets electroporated, it loses the capability of accumulating an induced transmembrane voltage when the next pulses are delivered, which is necessary for electroporation.^{24,25}

Therefore, in a treatment of 8 pulses, only the first ones could really have an efficient impact. Cebrián et al.³⁸ demonstrate that *Staphylococcus aureus* cells became impermeable to propidium iodide rapidly after PEF treatment but not to NaCl. Therefore, if some small pores or defects (not detected by Yo-Pro) remain in the cytoplasmic membrane of cells of *A. protothecoides* after PEF, the cells would be “insensitive” to subsequent pulses. By means of time after PEF in which cells could completely repair these damages, they would be again susceptible to be electroporated again by the next train of pulses.

Once observed the effect of the split-dose PEF-treatments, the corresponding lipid extraction was performed. As expected, the variations found in the electroporation levels were similarly detected for the lipid yields. The split PEF treatment of 4 + 4 pulses with 6 h of delay in between increased the extraction of the total lipids by 15% in comparison with the yield obtained after 8 pulses in a single dose. Therefore, there seems to exist a linear correlation between the percentage of the electroporated cells detected by Yo-Pro and the lipid yields achieved at pH 5.0. Nevertheless, this correlation is not observed at pH 3.0 since the percentage of electroporated cells does not directly reflect the percentage of extracted lipids (cf. Section 3.2). A simple explanation could be that the lipid content is not uniform among the cells and therefore that some cells containing no or low amounts of lipids could contribute to the fraction of electroporated cells and not to the lipid yield and vice versa. In order to test this point, the cells extracted were stained with Nile red and analyzed with a flow cytometer.

3.4. Nile Red Staining Correlation with the Lipid Extraction at pH 3.0 and 5.0. Nile red is a fluorescent dye staining the lipids so that cells which are positive to Nile red staining have lipids remaining inside, while negative cells are expected to be fully extracted. Figure 5 shows the different fractions of Nile red negative cells (%) as a function of lipid yields (data are a collection of all conditions studied above, as shown in Figures 3 and 4). There is a linear correlation ($R^2 = 0.88$ and $R^2 = 0.93$) between lipid yield and the fraction of Nile red negative cells. This correlation means that in general terms, the lipid distribution among microalgae cells is uniform, and each extracted cell contributes the same amount of lipids. However, according to the data, for the same amount of extracted lipids, a higher number of cells are Nile red negative at pH 3.0 than at pH 5. For example, an extraction of 50% of lipids corresponds to 60% of cells that are Nile red negative at pH 5.0, while at pH 3.0, it is 80%. These discrepancies could be a consequence of the properties of the Nile red molecule which has an excitation and an emission spectrum strongly influenced by the polarity of its environment and therefore by the pH. Additionally, some unspecific staining of other structures such as the cell wall can interfere with the results.³⁹

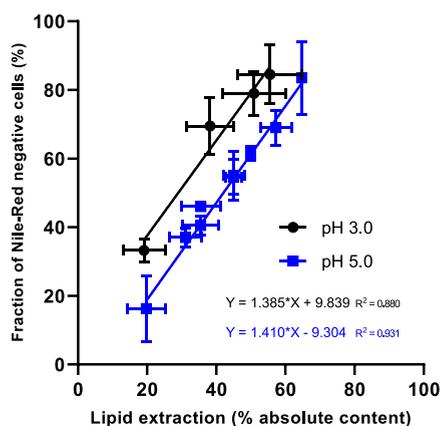


Figure 5. Correlation between the fraction of Nile red negative cells (%) and the lipid extraction (% absolute content), depending on the pH medium.

Further experiments should be necessary to elucidate whether some cell biological reasons regarding the pH effect could explain these differences.

4. CONCLUSIONS

This study has highlighted some under-investigated parameters and conditions within PEF processing of microalgae that can be crucial for improving lipid extraction processing. The concentration of the microalgae biomass might be responsible for unintended pH changes in the medium and thus for uncontrolled cell responses. Indeed, the pH of the medium during post-PEF incubation was shown to have a great impact on the electroporation outcome of *A. protothecoides* and on subsequent lipid extraction. However, the pH effect proved much more evident in the electroporation enhancement rather than in the lipid extraction. This strengthens the idea that microbial lipid extraction by PEF is a complex process that depends on the level of the electroporated cells. Finally, the efficacy of split-dose treatments of microalgae has been shown for electroporation and lipid extraction efficiency for the first time. This study has evidenced that there are still many unknown mechanisms in microalgae PEF processing that need further investigation to optimize downstream-processing conditions.

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C.D. and A.S.: conception and design of the study. C.D., N.N., and R.W.: acquisition of data. N.N. and R.W.: methodology. C.D. and A.S.: analysis and interpretation of the data. C.D. and A.S.: drafting of the article. I.Á., J.R., and W.F.: critical review of the article and funding acquisition.

Notes

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