Assessment of a Pulsed Electric Field based Treatment for Marine Microalgae Cell Disruption

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Abstract The rampant exploitation of fossil based resources and its negative environmental impact is triggering the demand for renewable feedstock alternatives, such as microalgae. However, cultivating microalgae only to obtain (bio)fuel precursors does not offset the demanded investment and operational costs. One way of circumventing this problem is by extending the extraction process to other microalgae added-value intracellular compounds. To recover them, a mild and energy-efficient cell disruption method is required. In this thesis, Pulsed Electric Field (PEF) is assessed as such method, aiming at intracellular water soluble proteins and pigments from two never tested marine strains, A and B. A Proof-of-Concept is performed along with an in-depth study of the effect of process and electric parameters towards PEF efficiency improvement. Furthermore, a novel Pre-treatment protocol is developed to enable the application of PEF to both marine strains. Finally, a comparative analysis between PEF and Bead milling is performed for the first time based on protein yields and respective energy consumption. Unlike A, B could not withstand the Pre-treatment, so it did not qualify for PEF. The maximum protein yield achieved with PEF for A was 13% but no pigment release was observed, providing evidence that PEF was not able to disrupt this species. Nonetheless, it was possible to improve PEF efficiency by reducing the theoretical energy input by twenty-fold while maintaining the same maximum protein yield. Compared to Bead milling, PEF revealed to be not yet competitive, requiring a twenty-fold higher energy input range for a four-fold lower protein yield. Follow-up studies and recommendations are presented to enhance PEF process for marine microalgae intracellular content recovery.

Keywords— Pulsed Electric Field, Bead milling, Microalgae cell disruption, Marine microalgae, A, B.

I. INTRODUCTION

Nowadays, PEF is a widely acknowledged alternative to the traditional thermal pasteurization processing of liquid foods. When using PEF, microbial inactivation is achieved by applying high intensity, periodic and short duration electric pulses (micro to milliseconds) to the processed stream, instead of applying heat \[^1\]. These pulses cause the formation of irreversible pores at the cell membrane of contaminant microorganisms (electroporation), deactivating them but also conserving the organoleptic properties of food \[^2\-4\].

This same principle could be extended for microalgae cell disruption. Microalgae host a large diversity of intracellular compounds such as proteins, vitamins, sugars, pigments, antioxidants, and lipids that are valuable for food, feed, pharmaceutical and biofuel industries. However, to efficiently recover those compounds while conserving their functional properties, an appropriate cell disruption method is required. Conventional cell disruption methods based on non-mechanical and mechanical treatment usually require harsh conditions such as high temperatures and towering pressures that only allow the selective extraction of a specific component while damaging the remaining \[^5\-7\]. The use of such severe conditions also impacts the energy input, resulting in higher operating costs \[^6\, 7\]. Thus, novel microalgae cell disruption technologies that are milder and less energy intensive need to be studied.

One potential candidate that might feature the above requirements is PEF \[^8\-13\]. The underlying mechanism of PEF over microalgae cell membrane-wall systems has not been fully elucidated up to now. However, a general consensus supports the theory that in the presence of an external electric field, the cell membrane potential shifts, resulting in the rearrangement of the phospholipid bilayer and consequent increase in membrane permeability. This increase in permeability not only is strain-specific but it also depends on the fine tuning of particular process parameters

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like biomass concentration, sample conductivity, applied electric field strength, number of pulses, pulse shape and duration. According to the available literature, PEF has been regarded as a promising microalgae cell disruption technique for the recovery of intracellular valuables, such as proteins and lipids. However, issues concerning its application, like species variety, extraction yields and energy consumption, need to be tackled before placing PEF among other well-known cell disruption methods. The majority of the studies published so far on the use of PEF for microalgae cell disruption, only feature freshwater species. The use of freshwater to grow microalgae is known to increase the water and nutrient footprint. So from a process point of view for large scale implementation, microalgae grown on a marine environment are preferred. Although marine microalgae seem to be a good alternative, they entail an additional hurdle when applying PEF. One of the parameters that can influence PEF performance is the sample conductivity. Microalgae grown on a marine environment present a higher conductivity (5 Sm⁻¹) than freshwater’s (5-50 mSm⁻¹). The higher it is, the greater is the energy consumption rendering the process less efficient. So in order to apply PEF, marine microalgae have to be washed first with a low conductivity solvent prior to treatment. Besides the marine aspect, the lack of data in the literature regarding yields of extraction and respective energy consumption makes it hard to place PEF in perspective against benchmark technologies. Before a qualitative conclusion on the feasibility of PEF can be drawn, these gaps in the existing literature should be cleared up. This study seeks to fill these gaps aiming for:

1. A Proof-of-Concept study to assess the ability of PEF to successfully disrupt marine Neochloris oleoabundans and Phaeodactylum tricornutum.

2. A process optimization towards PEF efficiency improvement.

3. A comparative analysis between PEF and Bead milling set as benchmark technology, based on protein extraction yields and respective energy consumption for optimized process parameters.

To attain the first two objectives, process (sample conductivity, species cell wall, biomass concentration) and electric (electric field, number of pulses and pulse length) parameters are explored to understand their role. Furthermore, a novel Pre-treatment protocol is developed to enable the application of PEF to both marine strains. The detailed equations and calculations that support the findings and conclusions summarized in the next sections can be found in the author’s reference.

II. MATERIALS AND METHODS

A. Study Design

The experimental part of this study was mainly structured in two parts: (i) Pre-treatment step and the (ii) PEF treatment. The Pre-treatment step comprised two stages: a washing treatment to reduce both A and B samples conductivity so that PEF could be applied and a concentration treatment to set the desired working concentrations. Milli-Q water was the solvent selected to feature this pre-treatment, not only due to its reduced conductivity but also because it can be easily translated into a large scale scenario. The resilience of both species to this pre-treatment was also assessed. Species losing a great percentage of its intracellular content in the pre-treatment make PEF useless for them. PEF is used only in species able to withstand the washing treatment.

PEF treatment was applied using a batch lab-scale electroporator. This brings some limitations discussed ahead but does not detract a meaningful assessment of PEF to disrupt the tested species and to further set process optimization strategies. Two sets of experiments were conducted to evaluate the role of process and electric conditions (Figure 1). In the first set, electric parameters (electric field, number of pulses and pulse length) were fixed to study the effect of biomass concentration while in the second one, biomass concentration was fixed to study the effect of the above mentioned electric parameters.

![Figure 1 – Study Design schematic representation.](image-url)
conceive a preliminary assessment of its feasibility in a larger scale.
Because process conditions and parameters reported in the literature for these techniques are so scattered and most of them do not present yields and respective energy consumption or lack data to compute these, this comparison had to be narrowed to the available data. Postma et al. [25] published a complete study on the release of intracellular products from *Chlorella vulgaris* using Bead milling and performed an elaborate analysis on the respective energy consumption. Bead milling was thus set as the benchmark technology against which PEF was compared grounded on Postma et al. study.

B. Strains and Culture Conditions

A was obtained from the culture Collection of Algae (UTEX1185, University of Texas, Austin) and B was obtained from the SAG culture Collection (SAG1090-1-B). A pre-culture was used to inoculate a 3 L stirred tank reactor (PhotoBioSTR3, Applikon Biotechnology, Netherlands) operated continuously in a chemostat mode. The working volume was 2.2 L. Temperature and pH set points were 25 °C and 7.5, respectively, the latter being controlled using CO2 sparging. Mixing was provided by a central located stirrer operating at 150 rpm. The reactor was roundly and continuously illuminated (40 Watt panel) with an average incident light intensity of 200 μmol·m⁻²·s⁻¹. The outgoing stream of 24h was collected in an autoclaved 2 L harvesting bottle placed in a Styrofoam box completely filled with ice. The dilution rate was set to 500 mL/day. On the other hand, B pre-culture was used to inoculate a 1.9 L flat panel reactor (Labfors 5 Lux, LED Flat Panel Option, Infors HT, Switzerland) operated continuously in a turbidostat mode. The working volume of 1.7 L. Temperature and pH set points were set at 20 °C and 7.2, respectively, the latter being controlled with acid/base supply. The average incident light intensity on the reactor was of 266 μmol·m⁻²·s⁻¹. The outgoing stream of 24h was collected in an autoclaved 1 L harvesting bottle placed in a Styrofoam box completely filled with ice. When both reactors reached steady state the daily harvest of A and B was used for the Pre-treatment step and PEF treatment.

C. Pre-treatment Step

1) Washing treatment

Milli-Q water was obtained from a Milli-Q Integral Water Purification System (Milli-Q® Advantage A10 Water Purification System, Merk Millipore, USA). 500 mL of A and B harvested suspensions were distributed over 10x50 mL Falcon tubes. These were centrifuged at 4500 x g for 10 min (Allegra X-30R, Beckman Coulter, USA). The supernatant was discarded and 50 mL of Milli-Q water was added. The new suspension was then vortexed for 10 s resulting in what was named the “After Washing 1x” sample. The same procedure was repeated resulting in the “After Washing 2x” sample. Figure 2 depicts the schematic representation of this procedure.

![Figure 2 – Washing treatment schematic representation.](image)

To assess the impact of the washing treatment, “After Washing 1x” and “After Washing 2x” samples were centrifuged at 4500 x g for 10 min and the supernatant was used for conductivity and water soluble protein analysis, performed according to the analytical methods described ahead.

2) Concentration treatment

A and B were centrifuged after one washing cycle at 4500 x g for 10 min (Allegra X-30R, Beckman Coulter, USA). The supernatant was discarded and Milli-Q water was added until the desired OD₇₅₀ and belonging working concentration was reached. The new suspension was vortexed for 10 s. After vortexing, Milli-Q water was added, resulting in what was named the “Before PEF” sample (Figure 3).

![Figure 3 – Concentration treatment schematic representation](image)

To assess the impact of the concentration treatment, “Before PEF” samples were centrifuged at 4500 x g for 10 min and the supernatant was used for conductivity, water soluble protein and pigment analysis, performed according to the analytical methods described ahead.

D. PEF treatment

1) PEF experimental set-up
“Before PEF” samples were PEF treated using a lab-scale electroporator (Gene Pulser Xcell™, BIO-RAD, USA). This equipment consists of a pulse forming network capable of producing monopolar exponential pulses or square pulses (truncated exponential) for cell electroporation with adjustable voltage ($V_0$), electrode gap ($l$), pulse length ($PL$), pulse interval ($PI$) and number of pulses ($NP$) for a low (LV) and high (HV) circuit voltage.

### 2) Experimental conditions

“Before PEF” samples were subjected to two sets of experiments: (i) one varying biomass concentration ($C_b$), while fixing electric parameters (electric field, $EF$, number of pulses, $NP$, and pulse length, $PL$) compatible with electroporator available options and (ii) the other varying electric parameters while fixing biomass concentration. Both sets were performed using the high circuit voltage (HV circuit) with $V_0 = 3000$ V and using monopolar square-wave pulses. The pulse interval ($PI$) between two consecutive pulses was set to 5 s in the electroporator and the time between each two pulses was defined to 10 s, the latter being controlled with a timer.

An appropriate cell model was used to calculate the cell response to the applied $EF$ and determine the required value to rupture the cell membrane. This was evaluated as $EF > 3.3 \text{ e}^{[34]}$. The same model also predicts the condition $PL > 1.76 \mu$s. Tables 1 and 2 showcase the experimental conditions for both sets of experiments based on the equipment available options.

Table 1 - Experimental conditions used to study the effect of different biomass concentrations on PEF performance.

<table>
<thead>
<tr>
<th>$EF$ (e)</th>
<th>$NP$</th>
<th>$PL$ (μs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>10</td>
<td>0.005</td>
</tr>
</tbody>
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Table 2 - Experimental conditions used to study the effect of different electric parameters on PEF performance.

<table>
<thead>
<tr>
<th>Fixed Biomass Concentration ($C_b$)</th>
<th>$EF$ (e)</th>
<th>$NP$</th>
<th>$PL$ (μs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.5</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>18</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2</td>
<td>0.5</td>
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<td></td>
<td>15</td>
<td>18</td>
<td>0.5</td>
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</tbody>
</table>

### E. PEF versus Bead milling experiment

To compare PEF against Bead milling in terms of water soluble protein yields and energy consumption, a Dyno-Mill Research Lab (RL) (Willy A. Bachofen AG Maschinenfabrick, Switzerland) was used for the Bead milling experiment with A according to $[25]$. $\Omega = 1$ mm ZrO$_2$ beads (Tosoh YTZ, Japan) were used at a milling chamber filling percentage of 65% (v/v). A biomass concentration of 4 c$_b$ was set, following the same concentration treatment used for PEF. Samples were harvested after 0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0, 45.0 and 60.0 min. These samples were then centrifuged at 20000 x $g$ for 20 min and the supernatant was stored at -80 °C until further use.

### F. Analytical methods

1) Conductivity

“Initial”, “After Washing 1x”, “After Washing 2x”, “Before PEF” and “After PEF” (more specifically, after 3h of post-pulse-incubation time) samples were centrifuged at 10000 $g$ for 10 minutes and the conductivity was measured (S230 Seven compact™, Mettler Toledo, USA) in the supernatant, at room temperature.

2) Protein analysis

a) Water soluble protein content

Water soluble protein content was determined using the commercial kit Modified Lowry Protein Assay (Thermo Scientific, BIO-RAD, USA). “Before PEF” and “After PEF” (more specifically, after 3h of post-pulse-incubation time) samples were centrifuged at 10000 $xg$ for 10 min. To determine the water soluble protein concentrations before and after PEF, 200 μL of the supernatant was collected from
the respective samples for the Modified Lowry Protein Assay.

b) **Total protein content**

Total protein content was determined so that protein yields could be computed. This was ascertained using the same commercial kit presented in the previous subsection for water soluble proteins. A total protein content % (DW) was estimated in 27.65±0.56% (DW) while B in 40±0.01% (DW).

10 mg of freeze-dried A from the harvested suspensions was resuspended in 1 ml lysis buffer I (60 mM Tris, 2% SDS, pH 9.0) in lysing matrix E tubes (6914–500, MP Biomedicals Europe). The tubes were bead beaten for 3 cycles of 60 s at 6500 rpm with breaks of 120 s between cycles using a bead beater (Precellys 24, Bertin Technologies, France). Subsequently, the samples for total protein content were incubated at 100°C. After 30 min at this temperature, samples were centrifuged at 3500 x g and the supernatant was recovered for a new eppendorf being then 2x diluted with lysis buffer. 200 μL of the supernatant was collected from the sample for the Modified Lowry Protein Assay.

3) **Pigment analysis**

Before and after applying PEF (more specifically, after 3h of post-pulse-incubation time), A samples were centrifuged at 10000 x g for 10 min. 200 μL of the supernatant was mixed with 1300 μL methanol and then incubated in the dark for 1 h at 45°C. The samples were then centrifuged at 10000 x g for 10 min at 20°C. The organic phase containing the pigments was then recovered and analyzed.

### III. RESULTS AND DISCUSSION

A. **Pre-treatment Step**

1) **Washing treatment**

After reaching steady state, both marine A and B samples were taken from the reactor and washed twice with Milli-Q water. Figure 4 shows the efficiency of the washing treatment step for sample conductivity reduction. For A, the sample conductivity decreased approximately by two orders of magnitude after the first washing cycle and one order of magnitude after the second washing cycle. On the other hand, for B, the sample conductivity decreased approximately by one order of magnitude between washing cycles (Figure 4). This confirms that for both species, the used washing protocol was able to reduce significantly sample conductivity.

When washing, the ion gradient between the inside and outside of the cells is suddenly changed due to cell transfer from a higher conductivity solvent to a substantial lower one (Milli-Q water conductivity is two-three orders of magnitude lower than the original sample conductivity).

This results in an osmotic shock that can ultimately lead to the release of intracellular compounds like proteins. The impact of the washing treatment step in the release of these compounds was thus assessed for both species and it is showcased in Figure 5.

These results suggest that both A and B have been affected by this pre-treatment, but at different extents. B revealed to be much more vulnerable, with a sharper protein release in the supernatant, after two washing cycles and for a lower osmotic gradient (from brackish to fresh conditions) to which the cells were exposed during the washing steps. One possible reason might be the fact that this species was cultivated in a naked silica medium. Diatoms cell wall is mainly composed of silica.

### Figure 4 - Conductivity measured in the supernatant initially, after one and two washing cycles with Milli-Q water in logarithmic scale, for A (black bars) and B (brown bars) cells suspensions. All measurements were performed in duplicate (technical) and error bars represent standard deviations. The concentration in the reactor at steady-state was 1.15 c, for A and 1.73 c, for B.

Although B is one of the few that is able to grow and survive in silica naked environments, its cell wall becomes weaker in the absence of this component and is less resistant to sudden environmental changes such as salinity shock. When diatoms are subjected to a change in salinity their silica based constituents (valves and girdle bands) are
triggered to re-shape so the osmolytes flux balance is again restored and cells are able to adapt to the new condition. If no silica is present, such cannot happen [30].

In total, this washing pre-treatment step has cost B a 78% loss of its total protein content (Figure 5B). Applying PEF subsequently on this species would be useless given the small remaining protein fraction to be recovered. A, on the other hand, was relatively able to withstand the washing treatment step, losing in total 22% of its total protein content (Figure 5B) after two washing cycles despite the larger osmotic gradient (from seawater to fresh conditions). A is a species that can be found in extreme environments like deserts (it was first isolated there), where sudden up and down salinity shocks are permanently occurring due to water scarcity. This may explain why this species was able to better resist to a sudden down salinity shock.

Based on the above results, one washing cycle was considered enough to reduce sample conductivity to acceptable values (see author’s reference [34]). Furthermore, additional protein loss due to the second washing cycle would also be prevented. For these reasons, only one washing cycle was carried out on the samples before and after PEF treatment.

2) Concentration treatment

After one washing cycle and prior to PEF application, A and B samples concentration had to be set. As previously described, this concentration step involves a centrifugation and multiple vortex steps, until the measured OD matches the desired concentration. Based on the protein release observed only for the washing pre-treatment, and considering that the procedure for the concentration pre-treatment is harsher, it was hypothesized that the latter would also damage the cells. Thus, to assess its impact, both cells of A and B were concentrated up to the highest working concentration – 26 c – in order to consider the worst case scenario, where the number of centrifugation and vortex steps would be maximum.

Although the conductivity measured for both species did not differ (Figure 6A), the same was not noticed for protein and pigment release (Figure 6B and Figure 7).

Regarding pigment release, this was one-two orders of magnitude higher for B. Because pigments are stored within the chloroplast, which has a robust structure hard to puncture, these results indicate that the biomass was already partially disrupted or even destroyed during the single-washing cycle and concentration steps.

In this concentration treatment, B lost 10% of its total protein content whereas A lost 5%, as depicted in Figure 7.

![Figure 6](image_url) – A. Conductivity and B. Pigments measured in the supernatant, after the concentration step up to 26 c.

The results obtained for the washing and concentration pre-treatment steps revealed that B is more sensitive to the whole pre-treatment itself, losing a great fraction of the intracellular content that PEF was supposed to recover. Since PEF requires the described pre-treatment to reduce sample conductivity and to set the desired concentration, it was concluded that B is not a good choice to assess PEF. Following the study design of Figure 1, B was discarded and A, that seemed to be relatively able to resist both washing and concentration treatments, was used thereon.
B. PEF treatment

1) Effect of biomass concentration

Figure 8 exhibits the conductivity measured in the supernatant before and after applying PEF as a function of the biomass concentration. As expected, the release of ions, after PEF treatment, increased with the increasing biomass concentration, since the number of cells per unit volume is also larger. The increase in conductivity after PEF treatment within each concentration suggests that pores are being formed, although nothing can be said about their reversibility.

Regarding protein release, this was also observed for all biomass concentrations (Figure 9), but at different extents. Like for conductivity, one would expect increasing release of proteins with increasing concentration after PEF treatment, since there are more cells per unit volume. However, when computing protein yields for each biomass concentration, it was observed that, even though the difference between them was not so evident, a maximum occurred at 14 c×.

A possible explanation for the protein yield reduction at the highest working concentration, 26 c×, might rely on a decreased release of intracellular content in the supernatant resulting in the fast increase of the sample conductivity beyond the permitted value for the electroporator. The consequence is that the electroporator cannot maintain a constant applied EF beyond the permitted value for the electroporator. The consequence is that the electroporator cannot maintain a constant applied EF at the electrodes decaying very fast during the PL, modifying the test conditions [34].

Another possible explanation consists in the presence of more divalent ions like Mg²⁺ or Ca²⁺ (that were present in the medium) outside the cells before PEF treatment for a biomass concentration of 26 c× (see Figure 8), contributing to the enhanced stabilization of the cell membranes thus hampering pore formation and so protein recovery [31].

2) Effect of the electric parameters

The conductivity measured in the supernatant before and after applying PEF is presented in Figure 10A for the experimental conditions from Table 2. An increase in the release of ions for all of them was observed, suggesting that electroporation was occurring. The influence of the EF and PL seemed not to produce a notable effect on the conductivity, based on the similar release of ions for different values of EF and PL. On the other hand, ions release increased with increasing NP, regardless the EF or the PL applied.

The same trend was observed for protein yields as depicted in Figure 10B. For all experimental conditions, there was protein release that mainly increased with the NP, but once again independently from the EF and PL.

However, despite the clear threshold, one would expect within each NP, an increase in conductivity and protein yields according to the applied electric field and PL, i.e. that lower values of conductivity and protein yields would match lower values of applied EF and PL and vice versa. For instance, the conductivity values for 15 e and 0.005 t should be higher than the ones observed for 7.5 e and 0.005 t for all tested NP. Likewise, the water soluble protein yields for 7.5 e and 0.0005 t should be lower than the other tested experimental conditions for 2 and 10 pulses, which oddly did not happen.
For the studied conditions, $NP$ seemed to be the most relevant electric parameter for the recovery of intracellular compounds, unlike one would expect. In Figure 10, it is also possible to identify a $NP$ threshold (within each experimental condition) – 10 pulses, from which the release of ions and proteins stopped varying.

![Figure 10](image_url)

**Figure 10** - A. Conductivity and B. Water soluble protein yields after applying PEF, as a function of the number of pulses for $A$ after applying 7.5 e, 0.0005 t (black solid line), 7.5 e, 0.005 t (grey solid line), 15 e, 0.0005 t (brown solid line), 15 e, 0.005 t (green solid line). Note: $\sigma_{\text{Beadmill}} = 548$ J (for all experiments).

Since the protein yields obtained for the studied range, seem to be more dependent on $NP$ rather than on $EF$ and $PL$, there was room for optimization of the specific energy input, $ET$. Thus, reducing $EF$ and the $PL$ for a fixed $C_x$ and $NP$, enables to lower $ET$ rendering the process more energy efficient and so more economically feasible. Table 3 showcases the impact of the $EF$ and $PL$ reduction on the specific energy input for the tested experimental conditions. By maintaining $NP$ at 10 and reducing $EF$ and $PL$ to 7.5 e and 0.0005 t respectively, the specific energy input can be decreased by forty-fold with respect to the harsher test conditions while maintaining the same maximum water soluble protein yield.

After analyzing the role of the different parameters in PEF performance, it is important to notice though, that the source of the proteins released from $A$ was not ascertained in this work, so it is not clear whether they come from the cell wall, cell membrane or from the inside of the cell. In addition, for all these experiments varying biomass concentration or the electric parameters, there was never pigment release (data not shown).

These results entail two possibilities: the experimental conditions were not harsh enough to disrupt the internal and smaller organelles like the chloroplast, or else (in case of successful chloroplast disruption), the environment after PEF treatment was too hydrophilic for the pigments, which are hydrophobic, to diffuse. The main reason why PEF protein yields reported herein are so low for microalgae can be attributed to its rigid cell wall. The results of this work show that PEF alone is not able to disrupt $A$, i.e., to completely break its system cell membrane-wall. To circumvent this problem, cultivation conditions could be played to weaken the cell wall or PEF could be coupled with another cell disruption technology, like enzymatic treatment, that would first break the cell wall for subsequent application of PEF.

A. PEF versus Bead milling

Bead milling was the mechanical cell disruption technology selected as a benchmark, because only one study was found that contained enough information for a complete evaluation of the process efficiency. Postma *et al.* (2014) where an elaborate analysis of the water soluble protein yields versus energy consumption.

These two cell disruption methods were compared based on their water soluble protein yields, energy consumption and temperature increase. Postma *et al.* showed that an optimum in water soluble protein yields versus energy input could be found for *C. vulgaris*, as a function of the treatment time, i.e., further increasing the energy input did not result in an enhanced protein yield.

Based on this knowledge, Bead milling was optimized by reducing the energy input while maintaining a maximum protein yield of 42% with *C. vulgaris*.

A similar experiment according to Postma *et al.* was performed for $A$ in order to quantify its maximum protein yield using beadmilling. This was determined to be 53%. At the highest energy input with PEF however, the protein yield was not higher than 13%. This illustrates that although the harshest possible conditions were applied, a similar degree of cell disruption and protein yield was not observed. This can be related with the underlying disruption mechanism of both techniques. PEF simply open pores not being harsh enough to release proteins that might be entrapped within other structures in the cell. Bead milling, on the other hand, completely destroys the biomass and so those structures that prevent proteins to be released end up being destroyed as well.

In addition, according to this study of Postma *et al.*, the energy consumption with Bead milling was determined in
Table 3 - Difference in the initial energy input by reducing the value of the electric field, $EF$, and pulse length, $PL$, for a fixed biomass concentration, $C_s$. $NP$ was chosen based on the lowest value required to assure the higher water soluble protein yield.

<table>
<thead>
<tr>
<th>Harshest Conditions</th>
<th>$C_s$</th>
<th>$EF$</th>
<th>$NP$</th>
<th>$PL$</th>
<th>Specific Energy Input ($J$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best Conditions</td>
<td>14</td>
<td>15</td>
<td>10</td>
<td>0.005</td>
<td>122.43</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7.5</td>
<td>10</td>
<td>0.0005</td>
<td>3.06</td>
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</table>

4 et protein released. With PEF, however, the energy input was determined to be 79 et protein released.

Both lower and upper energy consumption limits for PEF are one order of magnitude higher than the ones Postma et al. observed for Bead milling. Thus, even with an approximately twenty fold higher energy input than the one reported for Bead milling, a similar protein yield was not observed. Although in terms of protein yield and energy input, PEF is not competitive with our set benchmark, both technologies did prove to be mild. In both treatments, independent of the degree of severeness, the maximum final temperature did not exceeded 40 °C. This is an important criterion to maintain the integrity of vulnerable compounds such as antioxidants and proteins.

Summarizing, the performance of PEF is not competitive with Bead milling, at least relying on this preliminary analysis carried out with an electroporator. With a twenty fold higher energy input, a fourfold lower yield was obtained. However, PEF is proven to be controllable in terms of temperature increase. This comparison shows that further and considerable improvements should be taken to enhance the feasibility of PEF as a cell disruption technology.

IV. CONCLUSIONS

PEF is a well established technology for non-thermal pasteurization processing of liquid foods. Its extension for permanent rupturing of cell membrane-wall systems to recover its intracellular contents, namely for microalgae, is still in its infancy. The drive of this thesis was to contribute to this study trend, specially focused on marine microalgae, which pose specific challenges for PEF and where there are scarce reported studies so far.

This thesis had a threefold goal: (i) a proof-of-concept study to ascertain the ability of PEF to successfully disrupt A and B cultivated in a marine environment, (ii) a process optimization towards PEF efficiency improvement and (iii) a comparative analysis with a benchmark technology to qualitatively assess the feasibility of PEF when translated to a larger scale.

The proof-of-concept study entailed two questions:

- Is it possible to use PEF to disrupt marine microalgae?
- What is the effect of using different marine microalgae strains?

The main challenge behind the first question is brought by salinity of the marine microalgae that impacts on PEF. To address this challenge, A and B had to be subjected to a pre-treatment step to reduce their high conductivity so that PEF could be applied. Unlike A, B was not able to resist to this pre-treatment, losing most of its intracellular content. Thus, the Pre-treatment revealed to be strain-specific, restricting the application of PEF just to marine species that are able to withstand it. Given these results, PEF could only be applied to A.

The increase in conductivity after PEF treatment for all experiments performed with A suggested that pores might have been formed. However, the source of the water soluble proteins released was not determined, so it was not clear whether they come from the cell wall, cell membrane or from the inside of the cells. In addition, there was never pigment release which indicates that the conditions used were not severe enough to open intracellular organelles. If any pores were formed, this data set does not allow one to confirm if these were reversible or irreversible. This is an important issue since when applying PEF one is aiming for irreversible pores to maximize the product extracted. However, the low protein yields obtained when compared with the set benchmark technology and the non release of pigments, strongly suggest that there was no cell disruption. The rigidity of the system cell membrane-wall might be one of the main reasons of such result. Process conditions could be played or PEF could be coupled with another cell disruption technology that would first disrupt the cell wall for subsequent application of PEF, freeing it of an extra barrier (See recommendations in author’s reference [34]). Since B did not qualify for PEF treatment, because it lost most of its intracellular content for the pre-treatment step, a comparison between both strains concerning PEF performance itself was not possible. However, since B appeared to be much more vulnerable than A to the pre-treatment, one could hypothesize that this species would be more prawn to cell disruption by PEF treatment using even milder conditions. Considering the pre-treatment and PEF as a whole, one can say based on this study that marine strains that are more robust suit PEF better.

The second objective raised a different question:

- Is it possible to maintain the same optimal yields with a lower energy input?

The role of biomass concentration and electric parameters on PEF performance was studied. An optimal concentration for which the protein yield was maximum was found for the tested range, suggesting that when setting this parameter, a compromise between protein yields and energy consumption has to be reached. Although the higher the concentration, the lower is the energy input, water soluble protein yields start decreasing from a specific concentration, and so the low energy input does not offset the amount of product recovered.

Regarding the study of the electrical parameters, it was found that the protein yields are independent of the electric...
field and pulse length for the tested range. Also, a number of pulses threshold was found for which the water soluble protein yields do not vary anymore, regardless the electric field and pulse length. These discoveries allowed the optimization of the process by reducing the specific energy input by fortyfold when compared to the harshest conditions used.

Finally, in the third objective one tried to understand where PEF stands when compared to a competitive benchmark technology, this being set as Bead milling. Based on the established assumptions, PEF performance is not yet competitive with Bead milling with a four-fold lower protein yield requiring twenty-fold higher energy input. However, PEF is proven to be controllable in terms of temperature increase (according to the procedure used to measure temperature), which evidences its potential as a mild cell disruption technique. Based on this preliminary assessment of PEF, it is still not feasible to apply it on a larger scale. Yields have to be increased requiring a substantial lower amount of energy.

It is noteworthy though, that these conclusions are based on the results obtained using an eletroporator. The electroporator has two shortcomings that may have conditioned the results in some of the test cases: i) The voltage that creates the applied electric field is supplied by a capacitor in a \( R \)\( -\)\( C \) circuit, which has intrinsically an exponential decay with time during the pulse length. This decay becomes sharper as the sample conductivity increases (sample resistance decreases), In the limit of sample resistance much lower than 600 \( \Omega \), the applied \( EF \) may decay below the rupture value. (There are PEF dedicated machines that can maintain better the field level during the pulse.) ii) The second shortcoming is the very small volume of the shocking chamber (400 \( \mu L \)). The smaller the volume fraction under test, the larger the expected spread of results associated with measurement uncertainty.

Overall, this thesis helped contributing to fill the gaps in the literature concerning PEF, namely the use of marine microalgae and the determination of intracellular recovery yields (in this case, water soluble proteins). For the first time, the impact of the pre-treatment step required to apply PEF on marine microalgae was quantified, a never tested marine microalgal species was used - A and a comparison based on yields and energy consumption was made with a benchmark technology.

Furthermore, this thesis has evidenced the physical mechanisms involved in the PEF process and how they relate to the obtained results. This enabled to set the optimum protocols and electrical parameters values for PEF treatment of A. These are now being used (by the Biorefinery Unit of Bioprocess Engineering Research Group at Wageningen University) to set the parameters for a continuous process PEF machine using this strain.