Evaluation of Pulsed Electric Fields effect on the microalgae cell mechanical stability through high pressure homogenization

I. Papachristou[a], A. Silve[a], A. Jianu[a], N. Nazarova[a], R. Wüstner[a], G. Müller[a], W. Frey[a]

[a] Karlsruhe Institute of Technology, Institute for Pulsed Power and Microwave Technology (IHM), Eggenstein-Leopoldshafen, Germany

Abstract

Pulsed Electric Fields (PEF) is a known technique for the permeabilization of cell membranes, which can considerably foster intracellular component extraction from microalgae. During this phenomenon, the cells are subjected to short electrical pulses leading to the deconstruction of the cell membrane. However, it is currently uncertain in what way, if any, the microalgae cell wall is affected during pulsing. In this study, freshly harvested *Auxenochlorella protothecoides* (AP) and *Chlorella vulgaris* (CV) were subjected to PEF treatment with an energy input of 1.5MJ per kilogram of dry matter and then fed into a High Pressure Homogenizer (HPH) for 5 passes at 1500 bar. The percentage of intact cells after each pass was determined and compared with Control biomass that underwent the same homogenization. AP and CV autotrophic had almost 40% intact cells at the end of homogenizing whereas AP mixotrophic 20%. In all cases, no major difference on the disruption degree of pulsed and control samples was observed, indicating that the resistance to mechanical stress of the cell, a function of the cell wall, is not affected by PEF. Scanning Electron Microscopy (SEM) observation of the cells also showed no superficial or structural cell alteration after pulsation.

Keywords:

- Microalgae
- PEF treatment
- Electroporation
- Cell wall
- Auxenochlorella protothecoides

1. Introduction

Microalgae have attracted considerable research attention due to their fast growth rate and flexible outputs. A wide array of products, ranging from proteins to lipids and various other compounds can be sourced from them [1]. Microalgal lipids, more specifically, were initially considered as an excellent substitute for biodiesel with the focus of microalgae utilization nowadays slowly shifting to other applications such as aqua-feed and cosmetics industries [2]. A considerable bottleneck to any large scale commercial exploitation of microalgae, however, is the high cost of the extraction of intracellular components which in the case of biodiesel can represent 30 to 50% of the overall process [3]. The main obstacles that are usually cited as necessary to be overcome prior to successful extraction are the large amount of water present in the system and the cell wall surrounding the cell [4].

The cell wall is an integral part of the microalgae since it encloses all the intracellular components and provides protection against external threats. It is usually composed of

cellulose, protein, glycoprotein and polysaccharide [5]. However, the composition and thickness can vary greatly between different microalgae species or even depending on the growth stage. It has been observed, for example, that the cell wall of the green microalga *Chlorella vulgaris* (CV) has an initial thickness of 2 nm for a newly formed cell while it reaches 17-21 nm upon maturity [6]. The most common theory in the field of lipid extraction is, that the cell wall acts as a barrier preventing the interaction between the lipids or any other targeted analytes and the solvent [7]. It has also been speculated that the higher the thickness of the cell wall (that is, higher cellulose composition) the more the diffusion of lipid particles into the solvent is hampered [8].

To counter this, a disruption or pre-treatment method is usually required in order to modify or fracture the cell structure offering thus better solvent accessibility to the targeted compounds. This pre-treatment process can be physical (mechanical, thermal, electrical, etc), chemical, biological or a combination of the above [9]. An ideal disruption technique should be effective on wet algae, energy efficient and scalable [10]. If a cascade process with multiple outputs is designed (according to the biorefinery scheme [11]) then it is additionally crucial that the applied disruption method will not contaminate or destroy any of the desired compounds and that it enables further separation and fractionation of the biomass after each extraction step.

Pulsed Electric Field (PEF) treatment is one such pre-treatment method. During PEF treatment, an external intense electric field is applied across the microalgae suspension for a short period ranging from nanoseconds to milliseconds. It is theorized and

experimentally demonstrated that these short electrical pulses cause an increase of the transmembrane potential of the cell membrane. This leads to an increase of the cytoplasmic membrane permeability enhancing thus interchange between intracellular and extracellular space, an effect known as electroporation. Being a mild and low energy consuming method, PEF, has already been utilized to facilitate microalgae lipid extraction by different research teams [12]. Our group has also demonstrated in recent studies, that almost total lipid extraction using monophasic solvent system, could be achieved from wet, freshly harvested *Auxenochlorella protothecoides* (AP) after PEF-treatment, in stark contrast to untreated microalgae [13,14].

The phenomenon of electroporation or electropermeabilization has quite a history of applications in the medical and biological sector [15], however the exact mechanisms involved are still not fully explained [16]. Different theories have been discussed, usually focusing on the ways the cell membrane is modified [17]. This seems natural when mammalian cells are examined but raises the question in which way, if at all, the cell wall is affected when plant cells are processed. ‘t Lam *et al* showed that the presence of a rigid cell wall acted as a barrier for the spontaneous release of intracellular components (such as proteins) after PEF treatment of the microalgae *C. reinhardtii* concluding that the cell wall was unaffected [18], a conclusion which was previously shared by Azencott *et al* [19]. Observations on other microorganisms than microalgae however, suggest that the effect of PEF might not be limited to the cell membrane. Pillet *et al* working on bacterial inactivation, observed cell debris and cell wall degradation [20]. Cell debris and cell fractionation were also reported by Sheng *et al* when they treated the cyanobacteria

Synechocystis PCC 6803 with electric fields, visualized with Scanning Electron Microscope (SEM) [21]. Working with yeast and rectangular pulses, Ganeva *et al* reported an increased cell wall porosity as determined with lyticase uptake after incubating pulsed biomass at 30^0C for 1hr hour [22].According to the authors, PEF-treatment did not cause any direct cell lysis but allowed higher enzyme uptake (and therefore cell lysis), an effect which intriguingly increased with time. It should be noted, however, that literature research on this topic can be challenging, since often any positive effect of pulsing will be attributed on increased cell wall permeability either ignoring the cell membrane or combining it with the cell wall, causing thus some confusion regarding the actual effect of PEF.[5,10,12,23]

The goal of this work was to study whether PEF treatment has a degrading effect on the microalgae cell wall's mechanical stability. In order to achieve that, microalgae suspension that had been prior pulsed, was fed into a high pressure homogenizer (HPH) and the degree of cell disruption was compared to the one obtained from non-pulsed cells that underwent the same homogenization. The hypothesis was that if PEF causes some alteration or degradation on the cell wall, then the disruption degree of pulsed cells after HPH should be higher compared to untreated cells, since they were prior weakened from PEF. HPH is a proven disruption technique on its own, during which the cells are being destroyed due to high shear stress when forced to flow through a small orifice under high pressures. This approach of evaluating the cell structural weakening through HPH, which is functioning essentially as a diagnostic method after another pre-treatment method, has been used before by Halim et al [24]. This allows, however, for a qualitative assessment

only, instead of a measurement of the microalgae mechanical properties that would require more complex methods [25]. Cell rupture was quantified with cell counting in a counting chamber, a fast and simple method, able to deliver reliable results [26]. The microalgae used, were *Auxenochlorella protothecoides* (AP) and *Chlorella vulgaris* (CV), two strains that have been recognized to have a rigid and strong cell wall [6,27] and therefore are suitable candidates for this study. Experiments were performed on mixotrophic AP and on autotrophic AP and CV. In addition, SEM pictures were taken for a visual inspection of the cells after PEF treatment and for detection of potential direct external modifications.

2. **Materials and Methods**

The microalgae cultivation, harvest and pulse treatment protocol followed in this work is very similar to the conditions that have been described in detail before [13]. Therefore, only a brief description for each step shall be given here.

2.1. Microalgae cultivation

AP strain number 211-7a and CV strain 211-12 were obtained from SAG, Culture Collection of algae, Göttingen, Germany.

AP was cultivated autotrophically and mixotrophically. As the names imply, in the first case, CO_2 was the only carbon source supplied to the microalgae while in the second case the microalgae were cultivated with glucose to achieve faster growth rates. AP

mixotroph was cultivated in a modified Wu medium, similar with [13] in 1L conical polycarbonate cultivation flasks (VWR International, Bruchsal, Germany). The pH of the medium was fixed at 6.8 ± 0.1. The freshly prepared medium was then autoclaved. New cultivations started after inoculation from previously existing ones with a targeted initial optical density at 750 nm (OD_{750}) of ~0.1. Experiments were performed with 10-day old culture, which corresponds to the beginning of the stationary phase after the exhaustion of the glucose in the medium.

Autotrophic AP was cultivated in 25L photobioreactors (PBR) under sterile conditions. The starter-culture was cultivated mixotrophically as described above for 5 days and then used as inoculum for the PBR. The cultivation medium of the PBR was tris- phosphate (TP) medium as described in [28] however without the addition of any acetate and with supplementation of 40 µg/L Thyamine. For illumination, LED lamps were used (WU-M-500-840, 4000 K, Panasonic) with a light intensity of 200 µmol $m^{-2}s^{-1}$ for the first 24hr and afterwards increased to 600 µmol $m^{-2}s^{-1}$. The temperature and pH of cultivation were constantly monitored and a CO_2 flow of 3% volume in sterile air 60 L/h was supplied. Microalgae were harvested after 3 weeks, in the late lipid accumulation phase.

Autotrophic CV was cultivated in the same PBR in TP medium, with identical illumination and aeration conditions. Likewise, microalgae were then harvested in the stationary phase after 10-15 days.

2.2. Microalgae harvest

The microalgae were concentrated using a Sigma 8k centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) operating at 3000g. Once the majority of the medium was removed, the separated biomass was resuspended in an adequate amount of supernatant in order to achieve the desired final concentration. The targeted final concentration in each experiment was 100 g microalgae dry weight per liter of suspension (100 g_{DW}/L) with the exact value verified gravimetrically by drying at 90^0C overnight in a drying oven (Universalschrank Model U, Memmert, Germany).

2.3. Pulsed Electric Field (PEF) treatment

PEF treatment of the concentrated biomass took place in a continuous-flow, uniform-field treatment chamber consisting of two parallel circular stainless steel electrodes separated by a polycarbonate housing. The electrode distance was d = 4 mm and the treatment volume amounted to 2.05 ml. A full detail of the experimental setup can be found in a previous work [13]. The applied rectangular pulses had a duration of 1 μs and a field magnitude of 40 kV/cm. They were applied with a repetition rate of 3 Hz on the suspension flowing at 0.1 mL/s, corresponding to an input energy of 150 kJ per liter of suspension i.e. 1.5 MJ/kg_{DW}. Full details on the energy calculation can be found on [14]. Conductivity and temperature of the microalgae suspension were measured immediately after pulsing using a conductivity meter (Endress + Hauser, CLM, 381) in order to validate the efficiency of the PEF treatment. Control suspension refers to microalgae suspension pumped through the system but without applying the electric pulses. After PEF treatment,

the samples were stored on ice and were immediately fed to the homogenizer, first the pulsed biomass and then the Control.

2.4. High Pressure Homogenizer (HPH)

HPH treatment took place in a EmulsiFlex-C3 homogenizer (Avestin Europe GmbH, Germany). The pressure was manually adjusted to 1500 bar. During operation, occasional overshoots up to 2000 bar occurred. Working volume per condition was 40 mL of suspension. Once the entirety of the microalgae suspension was pumped through, designated as ‘one pass’, 1mL sample was removed for cell counting and the rest was fed again in the homogenizer. In total, five passes were done with all removed samples stored on ice until cell counting was performed.

2.5. Cell counting

Cell counting took place after proper dilution in the range of 5000 dilution factor, in a cell counting chamber (‘Glasstic Slide 10 with Grids, Kova International Inc., USA.) under a straight microscope (Axioplan 2, Zeiss, Jena, Germany) using a x63 magnifying objective (×63 LD Plan-Neofluar, Zeiss, Jena, Germany). The number of intact cells after each pass $\mathbf{n_p}$ divided by the initial untreated cells $\mathbf{n_i}$ gives the percentage of overall intact cells (equation 1)

$$\%cells\ intact = \frac{n_p}{n_i} x100 \qquad (1)$$

2.6. Preparation of samples for Scanning Electron Microscopy

Immediately after PEF treatment, cells were fixed by incubating them for 1 hour in a Phosphate-Buffered-Saline (PBS) solution diluted to have the same osmolarity as the cultivation medium and supplemented with 2.5% Glutaraldehyde. 200 µL of the cell suspension were deposited on a coverslip previously coated with 0.1 % Polyethyleneimine and left for 1 hour to adhere. The coverslip was rinsed three times with PBS followed by submersion in an increasing ethanol concentration bath (10 %, 30 %, 50 %, 70 %, 90 %, 100 %) each time for ten minutes and finally dried in a supercritical CO2 dryer.

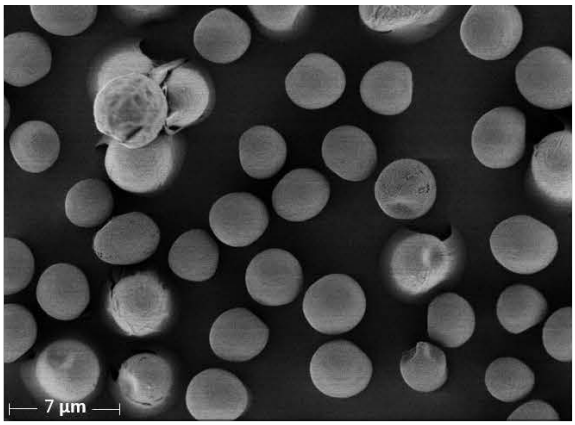
2.7. Scanning Electron Microscopy (SEM)

The morphology of the cells was imaged using SEM (Hitachi S-4800 FE-SEM) operating at 0.5 or 1kV for AP samples and 20kV for CV under deceleration mode. Freeze-dried microalgae samples mounted on the coverslips were coated with a thin conductive layer of gold before observation.

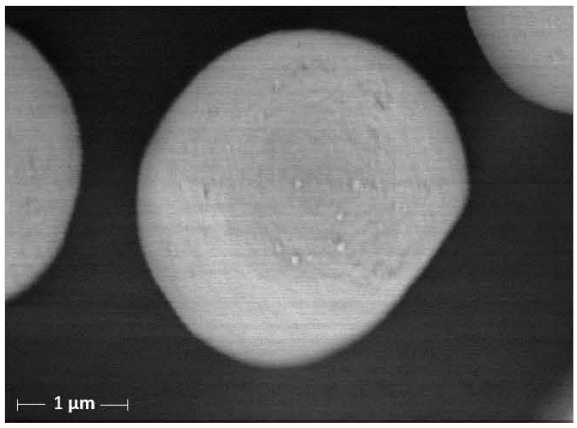
3. Results

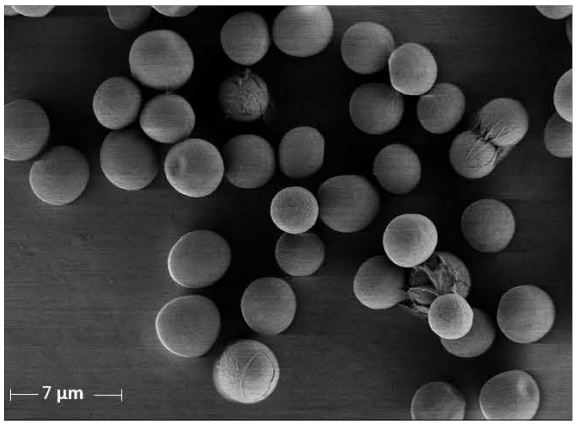
3.1. SEM imaging

Both untreated and PEF_treated microalgae cells of $AP_{autotrophic}$ and CV were examined using scanning electron microscopy (SEM). Representative images are displayed in Figure 1.

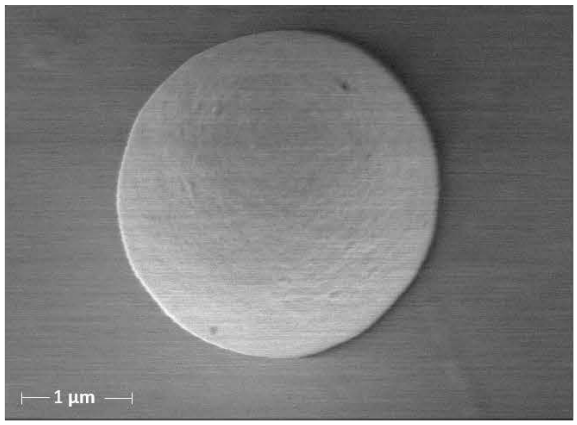


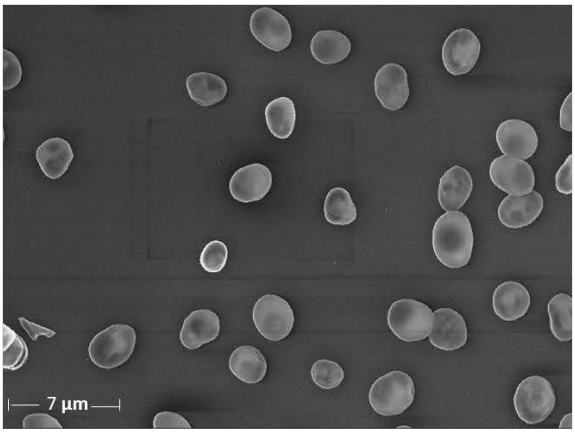
A



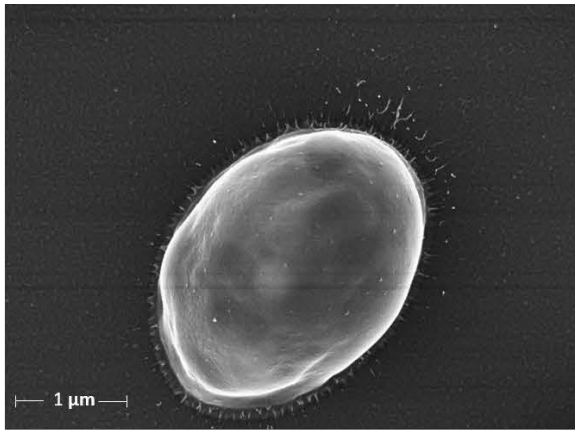


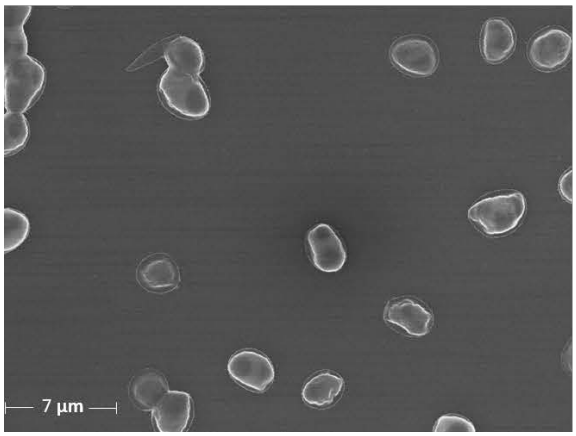
B





C





D

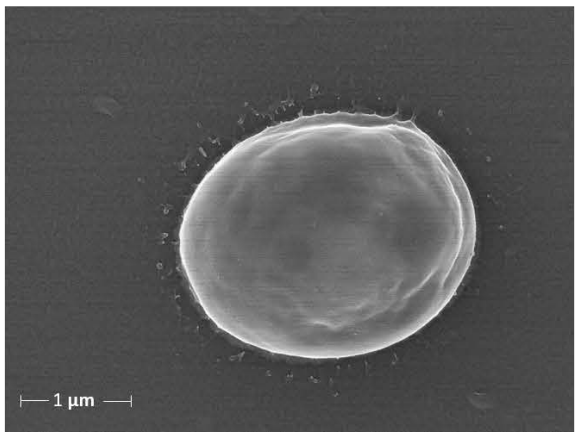


Figure 1 *SEM imaging of microalgae cells with or without PEF. A refers to AP autotrophic control at x3500 (left) and x20000 (right) magnification, B to AP autotrophic after PEF at x3500 (left) and x20000 (right) magnification, C to CV Control at x3500 (left) and x20000 (right) magnification and D to CV PEF at x3500 (left) and x20000 (right) magnification. CV images were conducted at 20kV compared to 1kV for AP.*

The size of the cells varied between 2-3 μm with a typical eukaryotic shape. No major external modification of microalgae could be observed after PEF treatment with both AP and CV cells retaining their original structure and shape. This confirms that PEF is affecting the cells in more mild ways without any obvious external deformation or destruction of the external part of the cell- wall.

3.2. Impact of PEF-treatment on cell-wall mechanical stability assessed by HPH disruption

The goal of this experiment was to evaluate if PEF treatment had an effect on the mechanical stability of the cell wall of the microalgae. In order to evaluate the mechanical stability, control and pulsed cells were submitted to five passes of HPH immediately after PEF-treatment and the number of intact cells after each pass was determined by cell counting. The microscopy pictures in Figure 2 are representative for untreated AP cells, prior to any HPH treatment (left), after one pass (middle) and after five passes (right). The pictures illustrate the increase of the number of disrupted cells with increasing number of passes and highlights the effectiveness of HPH.

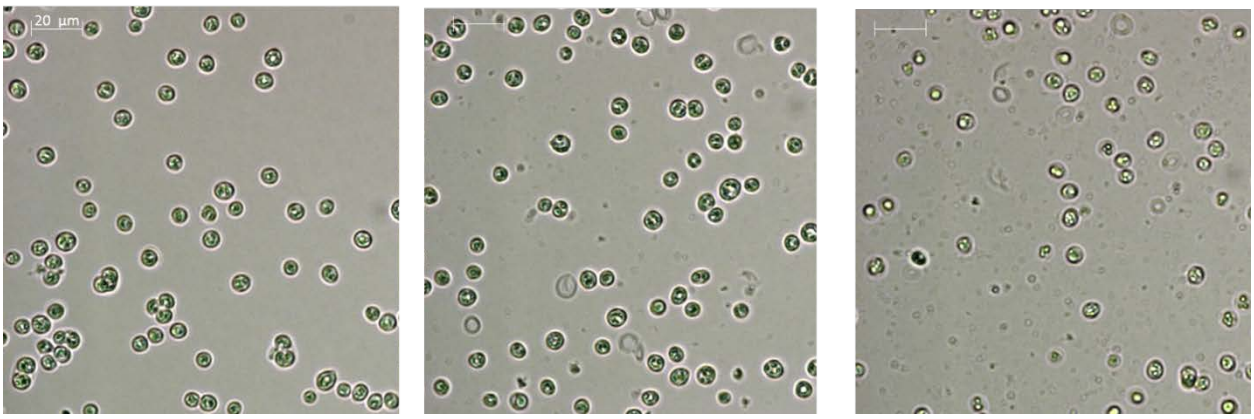


Figure 2: Microalgae suspension after HPH treatment as seen in the microscope. From left to right, the cell suspension untreated, after one pass of HPH and after five passes.

The quantitative results obtained by cell counting are presented on Figure 3. The three graphs correspond to AP cultivated autotrophically (top), AP cultivated mixotrophically (middle) and CV cultivated autotrophically (bottom).

Regarding $AP_{mixotrophic}$ without any pulsing after the first pass through HPH, 32% of cells were destroyed. A second pass through HPH, diminishes again significantly the number of intact cells, however, after the 3rd pass the rate of disruption is decreasing. At the 5th and final pass, 21% of the initial cells are remaining. $AP_{autotrophic}$ displayed a similar pattern although after the first pass, a higher number of cells was intact (78%) and the percentage of disruption seemed to stabilize at 43% at the fifth and final pass. For both AP cultivation modes, the results were identical for the microalgae, which had been previously subjected to PEF-treatment.

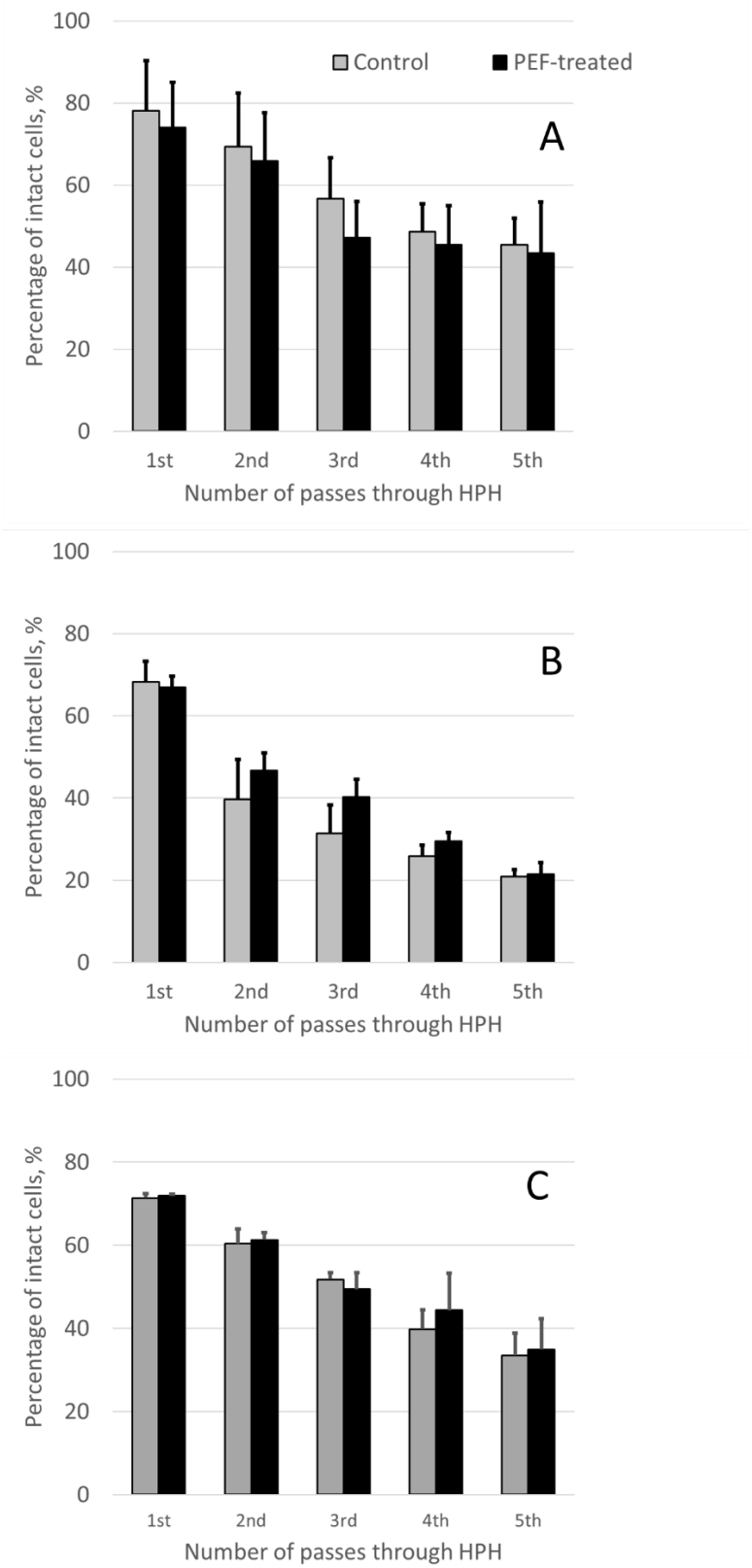

100
80
60
40
20
0
Percentage of intact cells, %
Control
PEF-treated
A
1st
2nd
3rd
4th
5th
Number of passes through HPH
B
C

Figure 3 Percentage of intact cells after each pass through HPH for unpulsed and pulsed microalgae. From top to bottom, AP autotrophic (A), AP mixotrophic (B) and CV (C). Results are the average + std of three independent experiments, two for CV.

Control CV retain approximately 71% intact cells after the first pass through HPH with 33% of cells remaining after the fifth and final pass. As with AP, Control and Pulsed microalgae had the same survival rate of 33% and 35% respectively.

4. Discussion

As shown in a previous study [13], AP without any prior pre-treatment is quite resistant to lipid extraction using an ethanol/hexane solvent blend, with CV exhibiting a similar pattern in unpublished experiments. The fact that ethanol (and other short chain alcohols) has a destabilizing effect on cell membranes [29] but is still unable to penetrate the cell in order to access the lipids, seems to imply that indeed the cell wall is the main obstacle that needs to be overcome. PEF, however, as discussed earlier, is mainly known as a cell membrane affecting process. On the same time, though, PEF has been proven as an effective microalgae pretreatment method indicating thus that it has a potential effect on the cell wall as well, an important parameter that needs to be examined. Cell wall, in plant cells is often referred to as the ‘skeleton’ of the plant [30] and it is quite possible it exhibits the same function in the microalgae as well. It is within reason then, to assume that should PEF-treatment has a degrading effect on the cell wall, this would be reflected in the disruption rate after HPH.

Based on the above results, no effect of PEF on the mechanical stability of the cells through homogenizing can be observed. After each pass for each microalgae type studied, PEF and Control had similar percentages of intact cells. It can be thus concluded that immediately after PEF there is no direct change in the mechanical stability of the cells

as determined with this experimental approach. SEM images further verified that PEF causes no obvious external modifications of the cells.

It can also be ascertained that AP autotrophic are the most resistant against high pressure homogenization. CV are less resilient and AP mixo can be disrupted by HPH the most efficient. This allows remarking that mixotrophic cultivation conditions produce cell walls, which are more susceptible to HPH disruption. This difference clearly shows that cultivation conditions have a major influence on disruptability by HPH. Compared to the differences in all disruption by different pretreatments, cultivation conditions have a dominant impact on disruptability in this study.

While special care was paid to the reproducibility of the microalgae photobioreactors, there were some slight differences during their cultivation, such as the cultivation duration. The fluctuation observed in some measurements, especially for AP autotrophic could be attributed to that fact. The results from AP mixotrophic, which present much less uncertainty, seem to verify this, since their cultivation was in all cases identical. Another explanation could be the occasional overshoots of the HPH to higher pressures, or indeed undershoots, which could also had an impact on the final count.

It must be stated though that the applied methodology in this study, does not provide information about inner morphologic or molecular changes of the cell wall constituents. Further study is required on this complex phenomenon. The isolation of the cell wall and determination of its composition could help in this direction. This would allow the observation of any possible degradation of polysaccharide constituents of the cell wall and provide a link with PEF treatment. Considering that with SEM imaging no external

PEF effect on the cell structure, it would be also interesting if Transmission Electron Microscopy (TEM) could reveal any modifications from the inside of the cell.

5. Conclusions

Elucidating the PEF-treatment effect on microalgae cell would allow for a more efficient optimization of any intracellular extraction process. In this study, the effect of PEF, a well-known membrane affecting method, on the cell wall is studied, Microalgae that were prior PEF-treated at 150kJ/L underwent high pressure homogenization and the degree of disruption was compared to untreated biomass in order to test whether PEF affects the cell mechanical stability. Results for both conditions were similar after homogenization at 1500bars, 5 passes. SEM imaging allowed for an external examination of the cells without detecting any modification after PEF-treatment.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Statement of informed consent

No conflicts, informed consent, human or animal rights applicable.

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Declaration of contributions

Conception and design of the study: IP, AS, WF. Experiments: IP, NZ. Analysis and interpretation of the data: IP and AS. Microalgae cultivation: IP, RW, NZ. Drafting of the article IP. Critical revision of the article AS, GM, WF.

IP takes responsibility for the integrity of the work as a whole from inception to finished article (ioannis.papachristou@kit.edu).

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