Supplementary information Part A, belonging to:

Mild and selective protein release of cell wall deficient microalgae with Pulsed Electric Field.

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Appendix A – PEF on cell wall deficient microalgae at low energy input

To confirm that PEF yields in a high protein release at low energy input, additional experiments were performed. The energy input in these experiments ranged between 0.01 and 0.5 kWh/kg which is lower than the threshold of 0.68 kWh/kg_{DW}, provided by Coons et al.[1]. Interestingly, the used energy input was also substantial lower as the consumptions that are reported by other (mechanical disruption technologies[2]. Safi et al.[3] reported for example an energy consumption of 7.5 kWh/kg_{DW} for the disruption of *C. vulgaris* using high pressure homogenisation. In addition, although the energy consumption of bead milling is already strongly reduced, the energy consumption is still between ~ 0.5 - 1 kWh/kg_{DW})[4].

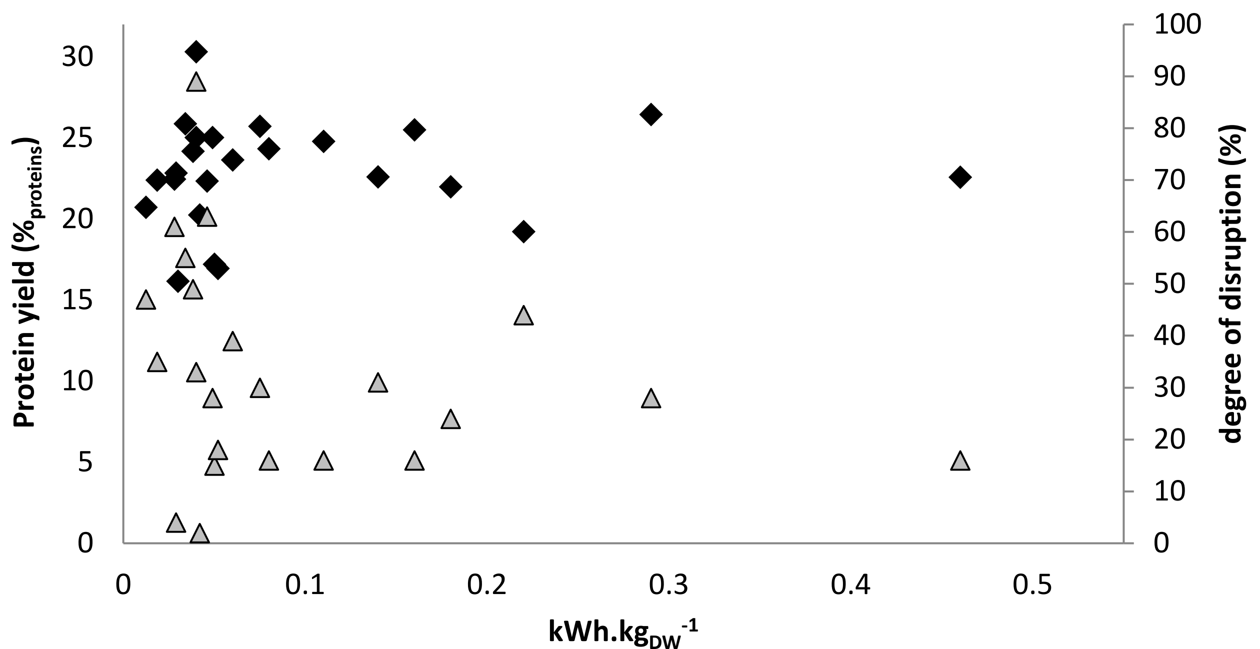

Figure S1: Protein yield and degree of disruption as a function of the applied energy. ♦ = protein yields after PEF. ▲= degree of disruption. Operating conditions were 7.5 kV/cm, 0.05-0.2 ms pulse length and 1-15 of pulses. A pulse interval of 10 seconds was applied and samples were washed with demi-water prior to PEF-treatment. DW ranged between 2-5 g/L.

The protein yields obtained after PEF treatment are on average 23%$_{proteins}$ ± 3.3 with a maximum of 30%$_{proteins}$ at 0.04 kWh/kg$_{DW}$. Bead beating resulted in an average protein yield of 34%$_{proteins}$ ± 4.2 (n=3). Thus the average protein yield using PEF was 68% of the protein yield obtained after bead beating.

Supplementary information Part B, belonging to:

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Appendix B – Native PAGE analysis of the released proteins after PEF

To investigate if the released proteins after PEF-treatment are still native, additional native-PAGE analysis was performed on the biological replicates of the experiments performed at 7.5 kV/cm, 0.1 ms pulse length, 10 seconds pulse interval and either 3 or 5 pulses. The biomass concentration in these experiments was 2.5 g/L. As a fairly low biomass concentration was present during PEF treatment, a silver staining (Thermo Fischer scientific) was used according to the manufactures protocol.

Figure S2 shows the size distribution of the proteins present in both the marker and the samples. For both experimental conditions, three samples have been analysed.

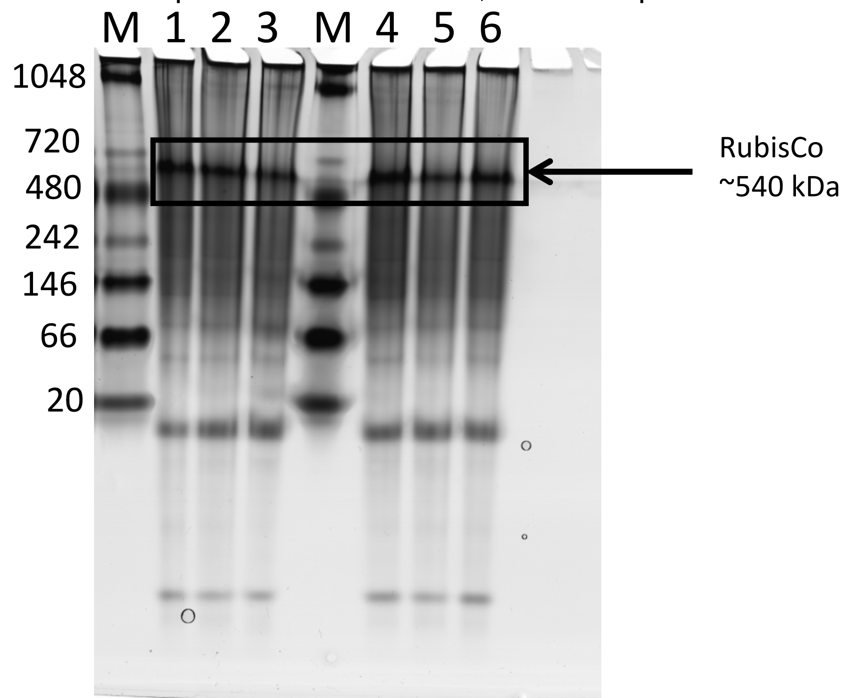

Figure S2: Native-PAGE using Silver staining (Thermo-Scientific). ***M*** = marker; ***1,2 and 3*** = biological replicates of 3p, 7.5 kV/cm, 0.1 ms pulses, 10 sec interval. ***4, 5 and 6*** = biological replicates of: 5 p, 7.5 kV/cm, 0.1 ms pulses and 10 seconds pulse interval.

In this analysis, RubisCo was used as a biomarker to investigate if the proteins remain intact. RubisCo is a protein that is present in large amounts in microalgae, and is active in the photosynthetic machinery of microalgae. RubisCo has a size of approximately 540 kDa and consists of eight small (approx. 13 kDa) subunits and eight large (approx. 56 kDa) subunit. The subunits are non-covalently bound to each other to form native RubisCo, dissociation of the subunits would destroy the protein and influence the mildness of the treatment.

According to Figure S2, in all 6 experiments there was a clear band visible between 400 and 700 kDa, illustrating that intact RubisCo was released in its native form during PEF. Next to the presence of RubisCo after PEF, also other large proteins (between 66 and 480 kDa) are present in all samples. It is therefore reasonable that PEF is able to release large proteins without destroying them to a large extend.

Similar observations were already made in the study of Postma et al[5]. However, in that study, the cell wall containing microalgae *Chlorella vulgaris* was subjected to PEF. Due to the presence of a cell wall, and despite some release of native RubisCo, the absolute protein yields remained in all experiments substantial lower as the observed yields during bead milling as reference.

Supplementary information Part C, belonging to:

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Appendix C – Enzymatic incubation as potential pre-treatment

To investigate the potential of an enzymatic pre-treatment, we subjected the wild type *C. reinhardtii* cc-124 to an enzymatic pre-treatment for 3 hours at neutral pH and 40 °C. Both strains were cultivated in shake flasks according to the first part of this work (described in material and methods).

The applied enzyme dosages ranged between 4 and 7$\%_{w/w}$ which is in accordance with applied enzyme dosages of other studies[6,7]. After incubation, the biomass was washed to remove the enzymes and samples were taken. After sampling, the biomass was PEF-treated, and again samples were taken for further analysis

Figure S3 shows the protein release after enzymatic pre-treatment followed by a PEF-treatment. An experiment with PEF treatment as well as a beat beating treatment without enzymes were performed as reference. On the vertical axis the protein release is presented in '$\%_{proteins}$'.

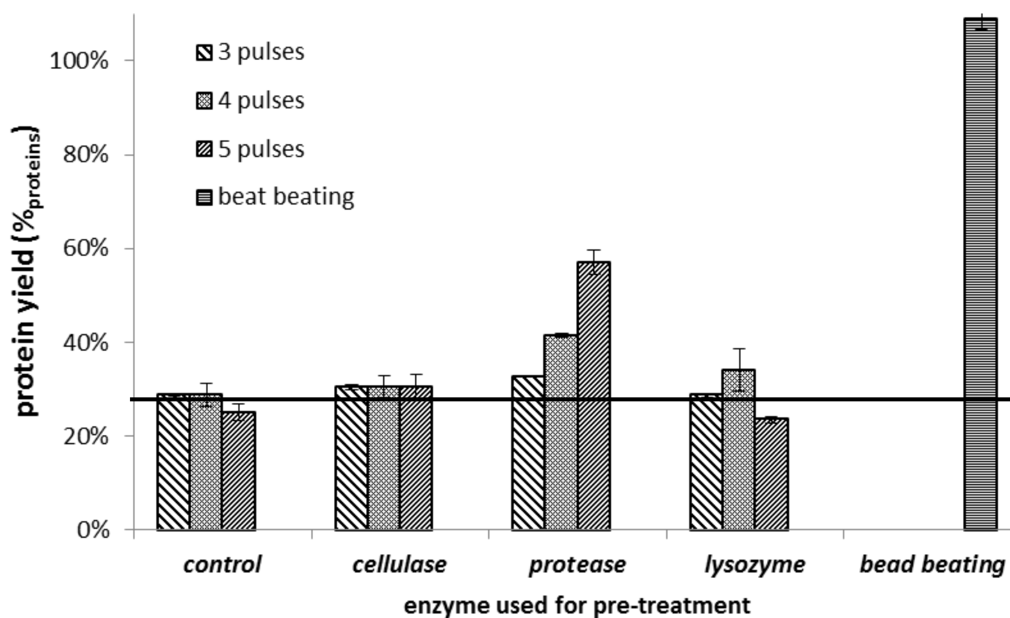

Figure S3: Protein yield after E-PEF treatment using different enzymes. Treatment conditions were: C_x of 1.45 g/L 7 $\%_{w/w}$ enzymes, 7.5 kV/cm, 0.05 ms pulse length, and 5 pulses. Error bars represent technical duplicates. Beat beating was included as positive control whereas the sample that was incubated without enzymes acted as a negative control.

Cellulase incubation combined with PEF treatment did not result in substantial higher yields, compared to the control experiment. Although it has been reported that glycoproteins are present in the cell walls of *C. reinharditii,* they appear not to be vulnerable for cellulose treatment[8].

Similar results were obtained after lysozyme incubation and PEF-treatment. Although lysozyme has been reported to be a very promising enzyme for successful weakening of the microalga *C. vulgaris*[9], no increased protein yield was observed after lysozyme incubation and PEF treatment. The difference in effect with lysozyme between the study of Gerken et al.[9] and our results suggest that the effect of an enzymatic pre-treatment is very strain specific. This selectivity of enzymes was also mentioned as a major advantage for specific breakage of cell wall linkages for mild disruption in the review of Demuez et al.[7]. The consequence of this high selectivity is the necessity to optimize the enzymatic processing for each specific algal strain[7].

Protease incubation resulted in a substantial increase in protein release compared to the control experiments. The protein yield was even more than twice as high as the control experiment and compared to the other enzyme treatments (cellulase, lysozyme). These results suggest that the cell wall of *C. reinhardtii* includes protein (like) structures that are vulnerable for a protease incubation. Similar results were reported using proteases for the degradation of *C. reinhardtii* to enhance methane production from the biomass[10]. In addition, Wang et al.[6] reported successful application of enzymes prior to other cell disruption techniques. In their study, various enzymes were successfully applied as pre-treatment before high pressure homogenization (HPH) and sonication as cell disruption technologies. The conditions that were used during incubation (up to 50˚C) as well as cell disruption (e.g. high

pressures) may have impact on the protein integrity[6]. In all the enzyme assisted experiments in this study, the temperature of the samples after PEF-treatment was always below 35 ˚C. This mild temperature increase, argues in favour of E-PEF as a mild cell disruption technology.

Only incubation at elevated temperatures, without enzymes resulted in a fourfold increase in protein yield for both the control PEF experiment and beat beating (Figure S3), compared to the yields that were obtained at room temperature. Thus only incubation of *C. reinhardtii* at an increased temperature results in an increase in protein yields. Similar results have been reported in the study of Postma et al.[5] as well as in the review published by Sari et al.[11].

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