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Pulsed Electric Field for protein release of the microalgae *Chlorella vulgaris* and *Neochloris oleoabundans*

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ABSTRACT

Pulsed Electric Field (PEF) is currently discussed as promising technology for mild and scalable cell disintegration of microalgae. In this study *Chlorella vulgaris* and *Neochloris oleoabundans* have been subjected to batch and continuous PEF treatments under a wide range of operating conditions (1–40 pulses, 0.05–5 ms pulses, 7.5–30 kV cm^{-1}, 0.05–150 kWh kg_{DW}^{-1}). In many cases after treatment, both algal species show release of ions, which indicates that PEF treatment resulted in permeabilization of the algal cell. However, the electroporation effect was not sufficient to substantially release intracellular proteins. Even at severe energy input (10 to 100 times higher than bead milling) only up to 13% of proteins released from the cells in comparison to 45–50% after bead milling.

1. Introduction

Microalgae are a promising feedstock for the production of bulk commodities because of their interesting composition [1–3]. It has been proposed in literature to increase the potential value of the biomass by adopting a biorefinery approach instead of a single-product downstream process [2,4–7]. By applying biorefinery, all the components, such as proteins, pigments and carbohydrates, can be valorised [6]. Though, the biorefinery should be mild to maintain the integrity of the components.

The majority of these components are present in the cytoplasm or in internal organelles (e.g. chloroplast) and they are difficult to access due to the rigid algae cell walls [8]. However, harsh cell disintegration technologies are not preferred if especially proteins are foreseen to be extracted in their native form [6].

PEF has already been mentioned as a promising technology for mild cell disintegration in literature [9–11]. By applying short electrical pulses (in the order of magnitude of ms or even μs), the cell membrane can be charged sufficiently to cause a rearrangement of the membrane, resulting in pore formation [10]. Due to the short electrical pulses applied, this technology requires a low energy input (even lower than 1 kWh kg_{DW}^{-1}, see Table 1). In addition, the method is mild for the molecules that should be released because they are subjected to a limited temperature increase and limited shear forces during the treatment.

An overview of studies on the application of PEF for disintegration of microalgae and cyanobacteria biomass for the release of proteins and lipids is presented in Table 1. From this overview, it can be deduced that not only various experimental approaches, but also various results have been obtained. When looking to the protein yields, it can be seen that over a wide range of specific energy inputs (0.02–239 kWh kg_{DW}^{-1}) very low to low protein yields have been obtained.

These low protein yields are in contradiction with the current consensus in literature on the general feasibility of PEF [10]. It is therefore difficult to create a consensus about the performance of PEF for the disintegration of microalgae or cyanobacteria. In addition, even though PEF is regarded as a promising technology for releasing hydrophilic proteins, an elaborate study that evaluates PEF over a similar range of processing conditions in direct comparison to benchmark disintegration technologies is not presented yet. Further, some studies applied marine cultivated microalgae, although the effect of desalination prior to the PEF treatment has not been addressed yet [12,14–16].

This work therefore presents a systematic screening of the operating conditions required to spontaneously release ions and proteins from the

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Table 1
Literature overview of previous performed PEF studies.

Microorganism	Product of interest	Conditions	Outcome	Reference
Nannochloropsis salina	Protein	15.4–30.9 kWh/kg, 37 °C outflow temperature, 0.0545–0.109% DCW	4 fold more extraction with water than methanol extraction of untreated cells	Coustets et al. [12]
Chlorella vulgaris	Protein	2.3 kWh/kg, 37 °C outflow temperature, 0.73% DCW	2 fold more extraction with water than methanol extraction of untreated cells	Coustets et al. [12]
Auxenochlorella prothecoides	Lipid	0.15–0.6 kWh/kg, 10% DCW	Over 3 fold more extraction with ethanol	Eing et al. [13]
Auxenochlorella prothecoides	Protein	0.15–0.6 kWh/kg, 14–22 °C temperature increase, 3.6–16.7%DCW	2 μg/L of protein release in the supernatant	Goettel et al. [14]
Nannochloropsis salina	Protein	0.4–1.5 kWh/kg, 1.0% DCW	3.6% protein release after PEF treatment	Grimi et al. [15]
Nannochloropsis salina	Protein	0.02–14 kWh/kg, 5.74–34.45 °C temperature increase, 1%DCW	Protein release in the supernatant of 10%	Parniakov et al. [16]
Chlorella vulgaris	Protein/Carbohydrate	0.6–1.1 kWh/kg, 2.5% DCW, continuous flow (33 mL min^{-1})	4.9%protein release after PEF treatment	Postma et al. [17]
Synechocystis PCC 6803	Lipid	59.7–239 kWh/kg, 0.03% DCW	25–75% increased lipid recovery	Sheng et al. [18]
Scenedesmus spp.	Lipid	6.9 kWh/kg, 0.44% DCW	3.1 fold increase in lipid recovery	Lai et al. [19]

fresh water species *Chlorella vulgaris* and the marine water cultivated species *Neochloris oleobundans* using two different PEF devices in a wide range of operating conditions. The results obtained with PEF are compared with those found for bead milling as a mechanical benchmark [21]. By doing so, a quantitative insight on the current state-of-development of PEF compared to a benchmark technology for both freshwater and marine cultivated microalgae is obtained.

2. Material and methods

2.1. Study design

This study is divided in three different parts: biomass pre-treatment, batch PEF operation and continuous PEF operation: The biomass pre-treatment describes the effect of washing and concentrating on the integrity of both microalgal strains. After the pre-treatment, various experiments were performed using a batch mode PEF to determine the effect of operating conditions and the energy input on the release of ions and proteins. Finally, to eliminate an effect of the equipment design, additional experiments using a continuous mode PEF were performed.

2.2. Biomass supply and preparation

C. vulgaris (SAG 211-11b, EPSAG Göttingen, Germany) was cultivated according to Postma et al. [21] using repeated batch cultivation in a fully controlled 12 L stirred tank reactor. The light intensity was increased during the cultivation from 400 up to 1100 μmol · m^{-2} · s^{-1}. The temperature was kept constant at 25 °C and *C. vulgaris* was cultivated in M8a medium at pH 7.0 according to Kliphuis et al. [22]. The microalgae were harvested each time at late linear growth phase at an OD_{750nm} of ~15.

N. oleoabundans (UTEX 1185, Austin, USA) was cultivated in a continuous mode operated 3 L stirred tank reactor. During cultivation the incident light intensity was kept constant at 200 μmol · m^{-2} · s^{-1}. Temperature and pH were kept constant at 25 °C and 7.5 respectively. *N. oleoabundans* was cultivated in artificial sea-water according to Breuer et al. [23]. After harvesting, the biomass of both species was stored in a cooled (4 °C) and dark environment for maximum 72 h.

Samples were centrifuged at 4000 × *g* for 15 min and the pellet was washed with Milli-Q water (*N. oleoabundans*) or with a 0.04% NaCl solution (*C. vulgaris*) to adjust the conductivity of the samples to an electrical conductivity of maximum 1.5 mS cm^{-1} prior to PEF treatment. After washing the biomass, the concentration was adjusted to the desired concentration. The effect of a possible osmotic shock after washing the algal biomass was determined by analysis of protein release before and after washing.

2.3. Batch mode PEF treatment

Batch mode screening of PEF conditions was performed in a lab-scale electroporator (Gene-Pulser Xcell™ Bio-Rad, USA), also commonly used for electrotransformation of algae cells [24–26], using cuvettes with gap distances of 1, 2 and 4 mm (Bio-Rad, Hercules, CA, USA). By altering the voltage between 1.6 and 3.0 kV the electric field strength could be varied between 7.5 and 30 kV cm^{-1}. Further, 1–40 square wave pulses with various lengths (0.05–5 ms) were applied each 5 s. With *N. oleoabundans*, after filling the cuvettes they were cooled to a temperature of 4 °C before PEF treatment. Electroporation of *C. vulgaris* was always conducted at room temperature. After treatment, the temperature was measured and it never exceeded 40 °C for all experiments of both algae.

The treated samples were gently mixed for 1 h to allow intracellular components to diffuse out of the biomass. After mixing, the suspension was centrifuged (20,000 × *g*, 10 min) and the release of intracellular components was measured in the supernatant.

2.4. Continuous flow PEF treatment

Continuous PEF experiments were performed on a previously described lab-scale PEF system [27] as a downscaled copy of a pilot-scale PEF apparatus [28]. Special attention was paid to downscale criteria to guarantee electric field homogeneity. In short, the algae suspension was pumped at room temperature (20 °C) with a flowrate of 13 mL min^{-1} through two co-linear treatment zones placed in series with a diameter of 1 mm and a gap distance of 2 mm, resulting in a total residence time of 13.5 ms in the treatment chambers. Directly after leaving the treatment chambers, the suspension was cooled down by pumping through a coil placed in ice-water, to a temperature below 20 °C. PEF processing was applied using square wave monopolar pulses at an electric field strength of 20 kV cm^{-1} with a pulse duration of 2 μs. The pulse waveform, voltage and intensity were monitored with a digital oscilloscope (Rigol DS1102, Beaverton, USA). By varying the pulse frequency, the total number of applied pulsed was changed leading to different maximum temperatures (Table 2).

Temperature increase for each condition was calculated, based on Eq. (1):

$$dT = \frac{E^2 \cdot \sigma \cdot \tau}{\rho \cdot c_p} \quad (1)$$

where E is electric field strength (V m^{-1}), σ is electrical conductivity (S m^{-1}), τ is pulse duration (s), ρ is density of the algae suspension, c_p is the specific heat (kJ(kg K)$^{-1}$), being 4.12 kJ(kg K)$^{-1}$. The used biomass concentration in this experiment was 25 g kg^{-1} for both algae,

Table 2
Process conditions used for PEF treatment of algae suspensions on continuous flow system.

Suspension	Frequency (Hz)	Number of pulses	Electrical field strength (kV cm^{-1})	T_{in} (°C)	T_{out} (°C)	dT (°C)
C. vulgaris	964	14.0	20.6	21.7	30.4	8.7
	390	5.7	20.4	21.8	25.7	3.9
	120	1.7	20.1	21.8	23.2	1.4
	0	0.0	0	21.9	21.9	0
N. oleoabundans	964	14.0	19.7	20.8	31.4	10.6
	390	5.7	20.3	21.0	25.3	4.3
	120	1.7	20.7	21.2	22.7	1.5
	0	0.0	0.0	21.3	21.3	0

resulting in a specific energy input of 0, 0.05, 0.165 and 0.41 kWh kg_{DW}^{-1} (respectively 0, 180, 594 and 1476 kJ kg_{DW}^{-1}).

2.5. Bead mill experiments

The protein release after bead milling reported for *C. vulgaris* by Postma et al. [21] was used to evaluate the performance of using PEF for this species. For *N. oleoabundans*, additional bead mill experiments were performed similar to Postma et al. [21]. A Dyno®-Mill ECM-AP 05 bead mill was operated using zirconia beads with bead sizes of 0.3 and 0.5 mm. The treatment chamber was filled for 70% and the applied tip speed was 8 m s^{-1}. Biomass concentrations ranging between 50 and 100 g_{DW} kg^{-1} were treated in different modes of operation: single pass, double pass and with a batch recirculation. In all experiments the liquid throughput was 10 kg h^{-1}. After treatment, the protein release in the supernatant was measured. To determine the increase in conductivity, lab scale experiments using beat beating were performed.

2.6. Electrical conductivity measurement

Before and after every treatment, the electrical conductivity of the supernatant was measured at room temperature using a Mettler Toledo® SevenCompact™ probe without temperature compensation. All samples were analysed at the same temperature (room temperature). As a positive control, bead-beated biomass was measured and results were used for further calculations.

2.7. Protein analysis

The total protein content on biomass dry weight (DW) was determined according to de Winter et al. [29]. In short, the biomass was freeze dried and then beat beated in a cell lysis buffer to solubilize all proteins. After bead beating the samples were incubated for 30 min at 100 °C.

Modified Lowry protein assay kits (Thermo Scientific and Bio-rad) were used to measure the total protein content and the soluble protein release before and after PEF treatment. The absorbance was measured at 750 nm. Bovine serum albumin was used as a proteins standard.

2.8. Determination of the specific energy input

The volumetric specific energy input (W_V), previously described as the treatment intensity (*TI*) by Salerno et al. [30] and Sheng et al. [18], was calculated based on the operating conditions (electrical field strength, pulse number) and the conductivity before PEF as:

$$W_V\ (\mathrm{kWh\ m^{-3}}) = \frac{E^2 \cdot t_p \cdot N \cdot \sigma}{3600000} \quad (2)$$

in which E is the electrical field strength in V m^{-1}, t_p is the pulse length (s), N are the number of pulses and σ is the initial electrical conductivity (S m^{-1}) at room temperature.

The mass specific energy input (W_M) was subsequently calculated as:

$$W_M\ \left(\mathrm{kWh\ kg_{DW}^{-1}}\right) = \frac{W_V}{C_x} \quad (3)$$

in which C_x is the biomass concentration (kg_{DW} m^{-3}).

2.9. Determination of the relative ion yield and protein yields

The permeabilization of the cell membrane was monitored by measurement of the electrical conductivity [31].

Similar to other studies, the relative ion yield (σ_R) was expressed as the specific increase in electrical conductivity with PEF over the specific electrical conductivity increase after bead beating. The increase in electrical conductivity was defined as the difference in electrical conductivity before and after treatment. In the reference beat beating experiments, the biomass concentrations were 25 g kg^{-1} (*C. vulgaris*) and 26 g kg^{-1} (*N. oleoabundans*). The electrical conductivity increase after bead beating was measured to be 0.98 mS cm^{-1} for *C. vulgaris* and 1.06 mS cm^{-1} for *N. oleoabundans*.

$$\sigma_R(\%) = \frac{\left(\sigma_{after\ PEF} - \sigma_{before\ PEF}\right)}{\left(\sigma_{after\ bead\ beating} - \sigma_{before\ beat\ beating}\right)} \quad (4)$$

Finally, the amount of released proteins was expressed as the increase in released proteins in the aqueous phases divided over the total amount of proteins present in the biomass:

$$\text{Protein yield}\ (\%) = \frac{PR_{sup}\ (\%_{dw})}{\text{total protein content}\ (\%_{dw})} \quad (5)$$

in which the 'proteins released in supernatant (PR_{sup})' are expressed as:

$$PR_{sup}\ (\%_{dw}) = PR_{sup\ after\ PEF}\ (\%_{dw}) - PR_{sup\ before\ PEF}\ (\%_{dw}) \quad (6)$$

by using '$PR_{sup\ before\ PEF}$', and not the initial amount of proteins present in the supernatant, the effect of the osmotic shock can be distinguished from the effect of the PEF treatment.

2.10. Statistical analysis

To ensure reliability of the experimental data, all analytical procedures have been performed in at least technical duplicates. During the batch-electroporator campaign of experiments, additional tests at extreme conditions ($E > 90$ kWh/kg_{DW}) were performed. An independent samples *t*-test with a significance level of $p = 0.05$ (assuming equal variances) was used for statistical analysis.

To exclude possible effects of the equipment design and to confirm the obtained results in the batch mode PEF, additional experiments were performed under continuous mode PEF. During continuous mode experiments, next to performing all analysis in technical replicates, drifts in the pulse delivery were eliminated by ensuring steady state operation prior to sampling.

3. Results and discussion

In this section, first the results obtained using the batch mode PEF are presented followed by the results of the continuous flow PEF. Finally, the current state-of-development is discussed.

3.1. Batch mode PEF

3.1.1. Effect of pulse parameters on PEF

Pre-treatment of *C. vulgaris* by resuspending in 0.04% NaCl did not result in release of any protein, even if an osmotic shock occurred. Fig. 1 presents the specific ion release and the protein yield for *C. vulgaris*

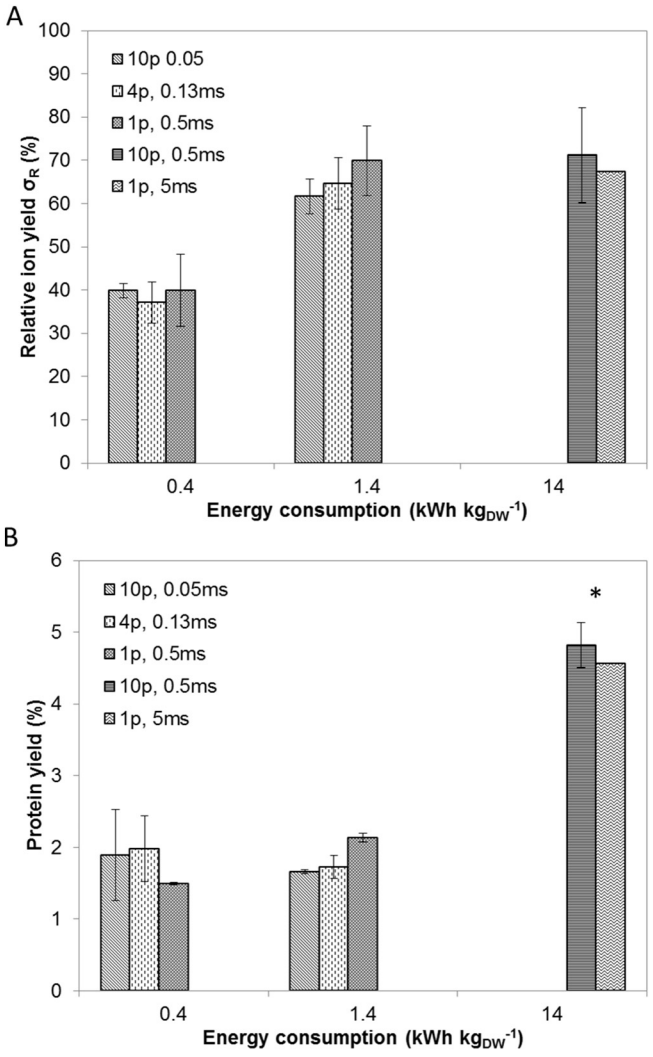

Fig. 1. Relative ion yield (σ_R) after PEF treatment as a function of pulse parameters for *C. vulgaris* (A). Protein yield measured 1 h after PEF as a function of pulse parameters for *C. vulgaris* (B). The electric field strengths were 8, 15 and 15 kV cm^{-1} for 0.4, 1.4 and 14 kWh kg$_{DW}^{-1}$ respectively. *14 kWh kg$_{DW}^{-1}$ significant different from 0.4 and 1.4 kWh kg$_{DW}^{-1}$. Errors bars show standard deviation ($n = 2$).

after applying a PEF treatment at three different energy consumptions for a fixed biomass concentration of 25 g kg^{-1}. At each energy input, the pulse length and number of pulses were changed to determine the effect of these individual parameters. The used energy inputs were; 0.4, 1.4 and 14 kWh kg$_{DW}^{-1}$. The field strength in these experiments was either 8 or 15 kV cm^{-1}. With an increasing pulse length, the number of pulses was decreased proportionally at a given specific energy input (Fig. 1).

The results in Fig. 1 show that with a specific energy input similar to the ones reported for bead milling [21], a substantial increase in electrical conductivity was obtained. These results imply that small components such as ions can be successfully released using PEF-treatments. Even though high amounts of ions were released, the protein yields were at best 6–8 fold lower in comparison to the mechanical benchmark bead milling [21]. Noteworthy are the results by Sheng et al. [18] and Ganeva et al. [32], who treated the cyanobacteria *Synechocystis* PCC68003 and the yeast *Saccharomyces cerevisiae*, respectively. A volumetric specific energy input (i.e. treatment intensity) W_V of ~30 kWh m^{-3} appeared in their study sufficient to successfully disintegrate the cyanobacteria and yeast cells. Yet, this work showed that in the case of eukaryotic microalgae, a W_V of 35 kWh m^{-3} (1.4 kWh kg$_{DW}^{-1}$) or even 350 kWh m^{-3} (14 kWh kg$_{DW}^{-1}$) was merely enough to release small ionic substances.

Next to the release of proteins, Fig. 1B also illustrates that individually varying the pulse length or number of pulses did not affect the protein yield. Instead, it appears that only the energy input affects the performance of PEF, as being illustrated the increase in release from about 1.8% at 0.4 kWh kg$_{DW}^{-1}$ up to 4.8% at 14 kWh kg$_{DW}^{-1}$ ($p < 0.05$). No difference could be observed between 0.4 and 1.4 kWh kg$_{DW}^{-1}$ ($p = 0.82$). This suggests that the electrical field strength is that high, that the specific energy input is the most important parameter affecting the operation. Similar results have been reported by Coustets et al. [20]. In their study 30 pulses of 1 ms and 15 pulses of 2 ms resulted in the same protein release at a fixed field strength of 4.5 kV cm^{-1}.

3.1.2. Release of intracellular components

The results of Fig. 1 showed that only the specific energy input affects the overall performance of PEF (given the same biomass concentration). Since a high release of ions was observed in all experiments, it is most likely that a sufficiently high field strength was applied to evoke a successful electroporation of the cells. Under these conditions, apparently the specific energy input is the pre-dominant operating parameter. Therefore, additional experiments were performed in which the ion release and the protein yield were investigated as a function of the energy input (electrical field strength ranged between 7.5 and 30 kV cm^{-1}). The goal of these experiments was to identify operating conditions at which both a high release of ions and a high release of proteins could be obtained. This was done by extending the energy input range from 0.03 up to 150 kWh kg$_{DW}^{-1}$. In these experiments, both *C. vulgaris* and the seawater cultivated *N. oleoabundans* were subjected to a PEF treatment.

Prior to PEF-treatment, also *N. oleoabundans* was washed similar to the washing applied on *C. vulgaris* (see Section 3.1.1). The washing resulted in a decrease of medium electrical conductivity from 45 mS cm^{-1} to <0.5 mS cm^{-1}. The protein release caused by this pretreatment was at maximum 4.8%$_{DW}$ after washing (3.4%$_{DW}$) and concentrating (1.4%$_{DW}$).

In Fig. 2, the ion-yield for both microalgae is presented as a function of the mass specific energy input W_M. The results show that due to the PEF treatment, a relative increase up to 79% with *C. vulgaris* and up to 76% with *N. oleoabundans* compared to beat beating (100%) as positive control was obtained. These results suggest that only small pores were formed in the cell membrane and cell wall allowing ions to be released.

Similar results were reported by Goettel et al. [14], after PEF treatment and 6 h of resting time, an increase in conductivity of 1 mS cm^{-1} was observed using biomass concentrations ranging between 36 and 167 g kg$_{DW}^{-1}$. Also in the study of Eing et al. [13], a conductivity increase of 1 mS cm^{-1} at a biomass concentration of 100 g kg$_{DW}^{-1}$ was obtained. Although a relative increase (σ_R) was not calculated in those studies, the absolute increase in electrical conductivity after PEF

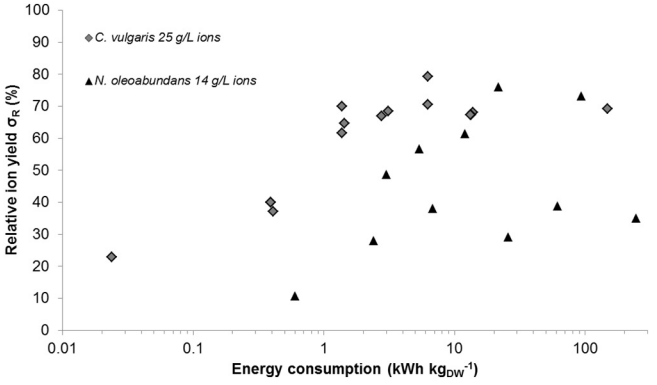

Fig. 2. Relative ion yield (σ_R) for *C. vulgaris*, and *N. oleoabundans* after PEF treatment. Part of *C. vulgaris* originates from Fig. 1

treatment was in the same order of magnitude as the increase obtained in this study.

Next to achieving a reasonable high ion-yield, part of the aim was to further enhance the protein release. Fig. 3 shows the protein yield as a function of the specific energy input W_M.

From Fig. 3 it can be observed that for both strains treated with PEF, the protein release did not exceed 13%. With bead milling however, the protein release ranged between 30 and 50% for both *C. vulgaris* and *N. oleoabundans*. Moreover, in the study of Safi et al. [33], a protein release of 51.7% was observed after high pressure homogenization of *C. vulgaris*. These results are in agreement with the protein release presented in Fig. 3 Even at energy inputs higher than applied during bead milling, no protein release close to the one by mechanical disintegration was observed ($p < 0.05$).

The results obtained with PEF as shown in Fig. 3 are in agreement with results reported in other studies as well [34] reported with *Nannochloropsis salina* a protein yield of maximal 10%. In addition, Goettel et al. [14] reported a protein yield of <1% with *Auxenochlorella protothecoides* (assuming a total protein content of 50% on DW). Also in the study of Postma et al. [17], which investigated the effect of processing temperature during PEF-treatment, for *C. vulgaris*, similar protein yields to the ones reported in this study were obtained. Furthermore, Grimi et al. [15] obtained a protein yield of 3.6% with *N. salina*. Coustets et al. [12] measured proteins after PEF-treatment as well. Although it was not possible to calculate a yield, the protein concentrations in the supernatant were equal, or lower than the protein concentrations measured in this study. In addition as already illustrated by Table 1, the degree of protein release or disintegration was not provided in all studies. Instead only absolute concentrations of components such as carbohydrates, pigments or 'total organic components' were provided [11,13]. It is therefore difficult to compare our results elaborately with other work.

Overall, the results presented in Fig. 1, Fig. 2 and Fig. 3 suggest that small pores were formed allowing ions to be liberated through the cell wall and membrane. The performance of PEF with respect to protein release was not as efficient as with bead milling limited by the pore formation and/or disintegration.

3.2. Continuous flow PEF

To quantify the impact of the PEF apparatus design on the observed yields, a continuous flow PEF unit was used and compared to the batch PEF unit. Based on the results presented in Fig. 1, only the specific energy input was varied in this experiment. By varying the pulse frequency the specific energy input was varied, while keeping the field strength and biomass concentration constant at 20 kV cm^{-1} and 25 g kg_{DW}^{-1}, respectively. The used field strength of 20 kV cm^{-1} is in agreement with the range used during batch-electroporation (7.5–30 kV cm^{-1}).

Fig. 4 shows that a protein yield between 2.5 and 3.2% was obtained for *C. vulgaris* and between 1.9 and 2.5% for *N. oleoabundans*. These yields are in the same order of magnitude as the ones presented in Fig. 3, and remained substantially lower than the yields obtained after bead milling. With a similar specific energy consumption of 0.4 and 0.6 kWh kg_{DW}^{-1} for *C. vulgaris* and *N. oleoabundans* during batch mode PEF yields up to 2.3% and 10.5% were obtained, respectively. So, for *N. oleobundans* even lower protein yields were obtained as with the batch mode PEF. The results of Figs. 3 and 4 imply that regardless of the energy input and the pulse length (2 μs for continuous PEF and 0.05–5 ms for batch PEF) similar results were obtained.

Both strains were cultivated in fresh water for the experiments shown in Fig. 4 instead of using artificial seawater medium for *N. oleoabundans*. As more biomass was required for these experiments, *N. oleoabundans* was cultivated in a fully controlled air-lift photobioreactor according to Postma et al. [35]. No proteins were released before treatment as can be observed in Fig. 4 at 0 kWh kg_{DW}^{-1}, whereas washing of marine cultivated *N. oleoabundans* did release protein and thus caused an osmotic shock (see paragraph 3.1.2). In any case, the results of Fig. 4 confirm the general trend that proteins remained entrapped intracellular.

3.3. General discussion

In this study, the highest yield of proteins of 13% was obtained with *N. oleoabundans* cultivated in seawater medium in a batch mode PEF. Despite the effect of an osmotic shock that *N. oleoabundans* suffered during the washing treatment, no yields similar to bead milling were obtained. Also in other studies, similar protein yields after PEF were observed [14–16].

This study showed, that regardless of the high amount of released ions, PEF was not feasible yet for either a complete disintegration, or for selectively releasing proteins. Although only low protein yields were observed after PEF, several other studies already reported that increased lipid yields could be obtained using extraction after PEF-treatment for both microalgae and also cyanobacteria [13,18,36]. It may be that the electroporation performed in this study is sufficient to allow enhanced lipid extraction, making PEF an interesting technology for lipid-scenarios. However, the native state of the soluble proteins is most likely negatively affected diminishing the total biomass value. Therefore, we believe that for a successful biorefinery strategy, first native proteins should be released.

It should be considered that the mode of PEF operation is different from bead milling. Where bead milling causes a complete cell

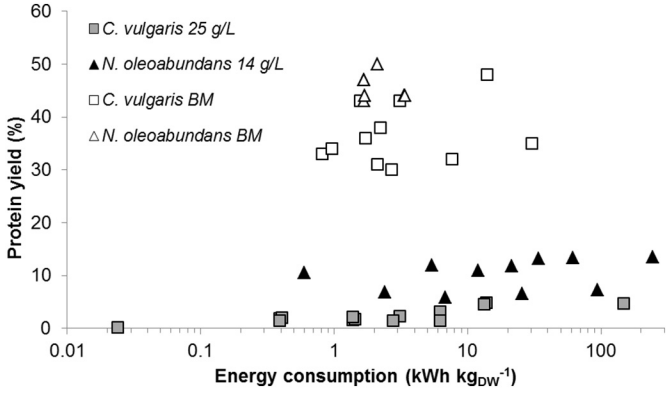

Fig. 3. Protein yield as function of the specific energy input. Protein yield measured 1 h after application of PEF. Specific energy consumption calculated based on initial conductivity at 25 °C. Benchmark by bead milling BM for *C. vulgaris* [21] and *N. oleoabundans* (this study).

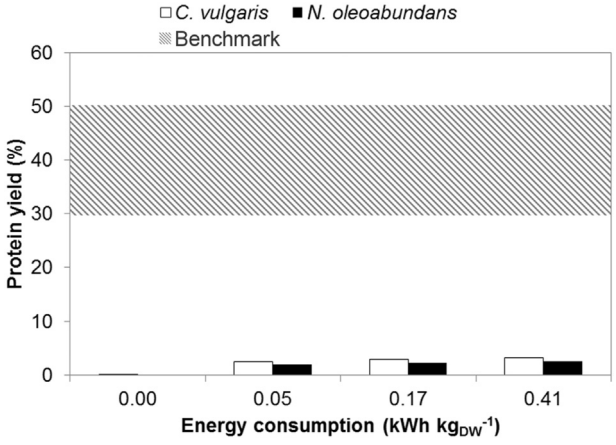

Fig. 4. Protein release yield measured after 1 h versus specific energy consumption for continuous flow PEF. Marked area represents benchmark yields range.

disintegration [21] PEF merely electroporates the cell. The kinetics of PEF may therefore require a longer incubation time after PEF compared to bead milling. In the experiments presented in this study, an incubation time of 1 h was used. Goettel et al. [14] presented in their work the effect of the diffusion kinetics after PEF. They reported that already 79% of the total released ions were released in the first hour after PEF treatment, which is in agreement with the results obtained in Figs. 1 and 2. In addition, Parniakov et al. [16], showed in their work the release kinetics of proteins after PEF treatment. According to their results, >80% of the total released proteins, were released in the first hour of resting time. It is therefore likely that an incubation time of 1 h was sufficiently long to observe at least a substantial release of intracellular components. In addition, other work reported the combined temperature-PEF effect, or combined pH-PEF effect [16,17]. Neither an elevated pH, nor higher temperatures contributed to the diffusion kinetics. The study of Postma et al. [17] did show however, that carbohydrates could be released selectively whereas the proteins remained entrapped. Although the carbohydrate yield was not as high as with benchmark bead milling, this selectivity may be advantageous for specific applications.

Besides the reported enhanced lipid extraction from microalgae and cyanobacteria, and the potential selectivity of the technology, other work showed that PEF was successful in opening cell membranes to inactivate/disintegrate microorganisms lacking a cell wall [27,37]. However, microalgae often have an additional rigid cell wall. Recently, Scholz et al. [38] proposed for example that the *Eustigmatophyceae Nannochloropsis gaditana* has a bilayered cell wall composed of a thick layer of cellulose and algaenans. It may be that other microalgae such as the species used in this study have similar properties, limiting the performance of PEF. This observation was also done by Azencott et al. [39] who found that the cell wall of *Chlamydomonas reinhardtii* was limiting the uptake of relatively large (66 kDa) protein molecules.

Next to the protein yield, the energy consumption is influencing the feasibility of PEF. By assuming a total energy content of 6.82 kWh kg_{DW}^{-1} in combination with an energy input <10%, the resulting energy consumption should be equal or lower than 0.682 kWh kg_{DW}^{-1} [40]. According to this criterion, next to low protein yields, the belonging energy input with PEF was substantially higher than 0.682 kWh kg_{DW}^{-1}.

4. Conclusion

The high release of ions illustrated that the application of PEF for the disintegration of fresh and marine cultivated microalgae, resulted in a weakening of the cell membrane suggesting the formation of pores. Nevertheless, with respect to the mechanical benchmark, no sufficient amounts of protein were liberated by the application of PEF. Moreover, the required energy input for PEF was higher than the mechanical benchmark.

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