Impact of incubation conditions on protein and C-Phycocyanin recovery from *Arthrospira platensis* post-pulsed electric field treatment

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Abstract

Pulsed electric field (PEF) was conducted for the extraction of proteins/C-Phycocyanins from *Arthrospira platensis*. The cyanobacterial suspension was treated with 1 µs long pulses at an electric field strength of 40 $kV \cdot cm^{-1}$ and a treatment energy of 114 $kJ \cdot kg_{sus}^{-1}$ and 56 $kJ \cdot kg_{sus}^{-1}$. For benchmarking, additional biomass was processed by high pressure homogenization. Homogeneity of the suspension prior to PEF-treatment influenced the protein/C-phycocyanin extraction efficiency. Stability of C-Phycocyanin during post-PEF incubation time was affected by incubation temperature and pH of the external medium. Biomass concentration severely affect proteins/C-Phycocyanins extraction yield via PEF-treatment. The optimum conditions for extraction of proteins/C-Phycocyanin was obtained at 23 °C while incubating in pH 8-buffer. The energy demand for PEF-treatment amounts to 0.56 $MJ \cdot kg_{dw}^{-1}$ when processing biomass at 100 $g_{dw} \cdot kg_{sus}^{-1}$. PEF treatment enhances the protein/C-Phycocyanin extraction yield, thus, it can be suggested as preferential downstream processing method for the production of C-Phycocyanin from *A. platensis* biomass.

Keywords: pulsed electric field treatment; *Arthrospira platensis* biomass; C-Phycocyanin; protein; extraction efficiency

1. Introduction

Arthrospira platensis, more commonly known as *Spirulina*, is a cyanobacteria that has oftentimes been mistaken as microalgae. This cyanobacteria has been receiving enormous attention during the last decades due to its high content of proteins (up to 70% of dry weight) that can be used for food or pharmaceutical applications (Ali and Saleh, 2012). Among various proteins, phycobiliproteins like C-Phycocyanins are supplementary light-absorbing complexes that are present in high concentration in *A. platensis* (up to 20% of dry weight) (Safaei et al., 2019). Not only can phycobiliproteins like C-Phycocyanin be used as pharmaceuticals, they can also be utilized as natural blue colorants for food applications (Chaiklahan et al., 2012).

So far, extraction of C-Phycocyanin from *A. platensis* has been conducted using diverse cell disruption methods, such as sonication, bead milling, high pressure homogenization (HPH), or freeze-thawing (Patel et al., 2005; Duangsee et al., 2009). In addition to the energy demand of these cell disruption methods, purification of C-Phycocyanin from a mixture of cell debris is also counted as a major drawback. Furthermore, stability of C-Phycocyanin during extraction processes is also an issue, since these molecules are found to be sensitive to light, temperature, pH and protein concentration, which is all affecting their aggregation state (Berns and MacColl, 1989; Jespersen et al., 2005; Chaiklahan et al., 2012). In fact, C-Phycocyanin are oligomeric proteins that are composed of α and β subunits (monomers) (Berns and MacColl, 1989). In general, they can be found as trimers $(\alpha\beta)_3$ or hexamers $(\alpha\beta)_6$ (Safaei et al., 2019).

Pulsed electric field (PEF) treatment has already been introduced as a pre-treatment method for the extraction of proteins from microalgae (Goettel et al., 2013; ’t Lam et al., 2017; Carullo et al., 2018). Exposing cells to an external electric field induces additional transmembrane voltage across the cell membrane, which leads to

the formation of pores across the cell membrane. This phenomena, also known as permeabilization, enables transfer of molecules across the cell membrane (Golberg et al., 2016). In fact, cell viability can also be influenced by the intensity of the PEF treatment parameters. At high intensities membrane permeabilization cannot be recovered, also known as irreversible permeabilisation, which leads to cell death and intensive release of intracellular compounds (Golberg et al., 2016).

Since C-Phycocyanin are water-soluble proteins located on the outer membrane of thylakoids (Safaei et al., 2019), PEF treatment can greatly enhance their release into the suspension. Indeed, Scherer et al., (2019) have also shown that the extraction of membrane-bound proteins by PEF treatment are often limited, whereas water-soluble proteins can be extracted very efficiently. In the current study, irreversible PEF treatment has been conducted as a pre-treatment method for the extraction of proteins and C-Phycocyanin from *A. platensis*. PEF treatment has already been tested for the extraction of C-Phycocyanin from of *A. platensis* (Martínez et al., 2017). They have shown that the maximum amount of C-Phycocyanin were released using 150 µs pulses at electric field strengths of 25 $kV \cdot cm^{-1}$ and incubation time of at least 300 min post-PEF treatment. In contrast to them, Jaeschke et al., (2019) have reported the highest amount of C-Phycocyanin that were extracted within 30 min after PEF treatment using 1 µs pulses at electric field strengths of 40 $kV \cdot cm^{-1}$. Apparently, the electric field intensity impacts the post-PEF incubation time that is required for extracting C-Phycocyanin. Nevertheless, these studies have focused on C-Phycocyanin extraction with only one post-treatment condition while using relatively diluted biomass (1 $g \cdot l^{-1}$). Studies are limited regarding the impact of different post-PEF incubation conditions on extraction yield and C-Phycocyanin stability.

Although PEF as a treatment method has the potential for protein or C-Phycocyanin recovery for industrial applications, upscaling of the process has to be

achievable. The influence of the biomass concentration on protein extraction efficiency after PEF treatment has been shown on *Chlorella vulgaris* (Scherer et al., 2019). They have shown maximum protein recovery from PEF treated *C. vulgaris* using diluted biomass (2.5 $mg \cdot ml^{-1}$), whereas increasing the biomass concentration from 2.5 to 12.5 $mg \cdot ml^{-1}$ reduced the protein extraction efficiency from 50% to 30% of total protein content. Presumably the diffusion inefficiency can influence the protein extraction yield after PEF treatment, i.e. the concentration difference of proteins from intracellular to extracellular medium impact the yield of PEF-extraction (Scherer et al., 2019). Thus, processing of highly concentrated biomass through PEF treatment for industrial scale needs to be investigated.

This study aims to investigate how post-PEF incubation parameters can impact the recovery of proteins and C-Phycocyanin from *A. platensis*. In the first step of the study, the impact of high biomass concentration on protein and C-Phycocyanin recovery after PEF treatment was investigated. In addition, the stability of C-Phycocyanin during incubation time after PEF-treatment in relation to temperature, pH of the external medium, and homogeneity of the suspension prior to PEF-treatment were also studied.

2. Materials and Methods

2.1. Cyanobacteria biomass

The cyanobacteria strain *A. platensis* (SAG strain 21.99) was used throughout this work. Cultivation of this cyanobacteria was carried out in Erlenmeyer flasks using Full Zarouk medium (Aiba and Ogawa, 1977), and the initial pH of 9.2 at 23 °C. The flasks were shaken at 150 rpm to prevent cell sedimentation, and illuminated 24 h at 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The biomass was harvested after 7 d, when the cells were in the stationary phase of growth (data not shown). Cells were harvested by centrifugation

at 10000 ×g, 12 min at 10 °C. Since the high initial conductivity of the suspension at 22 $mS \cdot cm^{-1}$ can increase the energy requirement of PEF treatment (Silve et al., 2018), cells were washed using sodium-phosphate buffer pH 7.2 at 0.1 $mS \cdot cm^{-1}$, in order to reduce the conductivity of the cyanobacterial suspension. The conductivity of the suspension was adjusted to 1.7 $mS \cdot cm^{-1}$ using sodium-phosphate buffer and the medium.

2.2. Homogeneity of the suspension prior to treatment

While concentrating biomass via centrifugation it has been observed that the biomass tend to form aggregates. The most probable reason for the aggregate formation can be attributed to the filamentous structure of the *A. platensis.* These relatively long spiral cells clump together and form large aggregates of cells. However, for subsequent processing steps, especially for PEF treatment, cell suspension has to be homogeneous to deliver PEF treatment energy equally to all cells and avoid any mutual shielding effect (Pavlin et al., 2002; Pucihar et al., 2007) (Guittet et al., 2017). Homogeneity of the suspension was provided by pipetting and magnetic homogenisation for at least 5 min (until no visible clusters of cells could be detected) prior to treatment. Homogeneous suspensions were then introduced to pre-treatment. Cell dry weight (CDW) was determined according to (Akaberi et al., 2019).

2.3. Treatments

PEF treatment was applied using a continuous flow treatment chamber and a transmission line based pulse generator developed at the Institute of Pulsed Power and Microwave Technology (Karlsruhe Institute of Technology, Germany), for details see (Goettel et al., 2013). Part of the biomass was processed via HPH as a positive control i.e. a mechanical cell disruption method that ensures maximal release of proteins and C-Phycocyanins. For PEF treatment, the pulse parameters from

Jaeschke et al., (2019) were chosen since they were shown to be efficient for permeabilization of *A. platensis*.

PEF treatment was applied using square pulses of 1 µs duration, an electric field strength of 40 $kV \cdot cm^{-1}$ and a treatment energy of either 114 $kJ \cdot kg_{sus}^{-1}$ or 56 $kJ \cdot kg_{sus}^{-1}$. The energy input of PEF treatment was selected by adjusting the pulse repetition frequency at 4 Hz for 114 $kJ \cdot kg_{sus}^{-1}$ or at 2 Hz for 56 $kJ \cdot kg_{sus}^{-1}$ at a constant suspension mass flow of 3 $ml \cdot min^{-1}$ through the treatment chamber that was separated by 2 mm gap (for details concerning the treatment chambers see (Frey et al., 2013; Jaeschke et al., 2019).

HPH treatment was conducted by using an EmulsiFlex-C3 homogenizer (Avestin, Canada). HPH samples were processed at 2000 bar and 5 passes.

After either treatment (PEF or HPH), in order to monitor the rate of protein and C-Phycocyanin release, samples were taken by pipetting 1.5 ml of cell suspension followed by centrifugation at 12300 ×g, 20 min at 10 °C. For the following experiments agitation during incubation was conducted on orbital shaker (80 rpm).

2.4. Incubation condition after PEF-treatment

For optimization of the extraction efficiency after PEF treatment, effect of the agitation (using orbital shaker or rotator) was examined. Agitation via orbital shaker or rotator has not influenced the protein and C-Phycocyanin extraction efficiency (data not shown). Nevertheless, agitation on orbital shaker (80 rpm) was conducted during incubation.

2.4.1. Effect of temperature on protein/ C-Phycocyanin release

To study the effect of temperature on protein/C-Phycocyanin release, the concentration of fresh *A. platensis* biomass was adjusted to 2.6 ± 0.5 $g \cdot kg_{sus}^{-1}$.

Untreated and PEF-treated samples were incubated in initial buffer under agitation at 4, 23, and 40 °C in dark. Samples were taken every hour till 4 h post-treatment, as described before in section 2.3. The supernatant was then analyzed for the protein and C-Phycocyanin content.

2.4.2. Post-PEF incubation in pH-buffers

Untreated and PEF-treated samples were split in different batches. All samples were centrifuged at 10000 ×g, 12 min at 10 °C. After centrifugation, the supernatant was exchanged by a desired pH-buffer (100 mM sodium-phosphate buffer at pH 6 or pH 8), resuspended and kept under agitation at 23 °C in dark. To allow a thorough comparison of the impact of the incubation-pH on protein/C-Phycocyanin release, resuspension and incubation in initial buffer was also followed. Samples were taken every hour till 5 h post-treatment and after 24 h, as described before in section 2.3. It has to be considered that the pH-buffers have the conductivity of 7 and 11.9 $mS \cdot cm^{-1}$ for pH 6- and pH 8-buffers, respectively.

2.5. Protein and C-Phycocyanin determination

The obtained supernatant was processed for protein determination using a modified Lowry method (DC™ Protein Assay, BioRad), and bovine serum albumin (DC™ Protein Assay, BioRad) as standard (Lowry 1951).

For C-Phycocyanin determination, the obtained supernatant was measured concerning its absorption spectra using a UV-spectrophotometer (Thermo Scientific, Genesys 10S, Germany). For spectrum analysis, the extract was also measured within a wavelength range of 350 to 750 nm. The C-Phycocyanin concentration was calculated according to Eq. 1 (Bennett and Bogobad, 1973; Yoshikawa and Belay,

2008) and Eq. 2. First the supernatant was measured spectrophotometrically at 620 nm (A_{620nm}, Eq. 1), and at 652 nm (A_{652nm}, Eq. 1).

Eq 1. $$[C-PC]\ (g\cdot l^{-1}) = \frac{A_{620nm} - 0.474\cdot A_{652nm}}{5.34}$$

Eq 2. $$[C-PC]\ (\%CDW) = \frac{[C-PC](g\cdot l^{-1})}{[CDW](g\cdot l^{-1})}\cdot 100$$

2.6. Absorption spectra and purity of crude extract

Effect of either pre-treatment on the purity of the C-Phycocyanin extract was investigated. Absorption spectra of crude extracts of PEF-treated samples after 24 h incubation either in initial buffer or in pH 8-buffer, as well as HPH treated samples were evaluated by measuring the absorption spectrum from 250 to 750 nm.

Furthermore, the purity of crude extracts were also monitored by measuring the purity ratio of A_{620}/A_{280} according to (Patel et al., 2005).

2.7. Statistical data analysis

All experiments were performed at least in duplicate. The graphs show average values of 2-4 independent experiments with their standard error.

3. Results and Discussion

In order to investigate the behaviour of the protein and C-Phycocyanin extraction using PEF treatment, different parameters (incubation temperature, pH of the external medium, incubation time, biomass concentration) were examined.

3.1. Effect of incubation temperature on protein and C-Phycocyanin release following PEF treatment

To investigate the effect of incubation temperature on protein and C-Phycocyanin release. PEF treated *A. platensis* biomass (2.6 ± 0.5 $g\cdot kg_{sus}^{-1}$) was incubated at 4, 23, and 40 °C. The kinetics of released proteins and C-Phycocyanin

into the external medium were represented after PEF treatment (Figure 1). The yield of protein released into the suspension was strongly influenced by incubation temperature (Figure 1a). Within 1 h of incubation after PEF treatment a high amount of proteins (45% of CDW) was released at 40 °C. It has to be mentioned that HPH treatment has obtained maximum amount of proteins (60% of CDW). However, after 2 h of incubation following PEF treatment the saturation value (~58% of CDW) was reached in biomass incubated either at 23 or at 40 °C. In contrast, the biomass incubated at 4 °C showed drastically lower protein recovery that has not been compensated even after 4 h of incubation after PEF treatment. Moreover, regarding these temperatures, at least 2 h of incubation after PEF treatment is required to obtain the comparable amount of proteins as has been obtained via HPH treatment.

It is known from the literatures that various factors can affect the PEF extraction efficiency. Indeed, PEF-treatment enables protein release via diffusion, which is strongly time-dependent. In other words, to attain efficient extraction yield an incubation of the PEF-treated biomass is obligatory to enable diffusion. Goettel et al., (2013) have also recommended an incubation time of at least 2 h post-PEF treatment to ensure sufficient diffusion of *Auxenochlorella protothecoides*. Influence of temperature on the release of proteins during post-PEF incubation have been investigated in *C. vulgaris* (Scherer et al., 2019). Consistently, their findings also revealed high protein release at 20-40 °C. They have proposed the incubation of the PEF-treated biomass within this temperature range to ensure autolysis, which additionally contributed to protein release after PEF-induced cell death.

C-Phycocyanin release after PEF treatment was also severely affected by incubation temperature (Figure 1b). The amount of C-Phycocyanin released after 1 h of incubation at 23 and 40 °C was identical (5.5 and 4.9% of CDW), whereas it was

significantly lower at 4 °C (0.5% of CDW). However, HPH treatment obtained 9.9% C-Phycocyanin with regard to CDW. While incubating the biomass at 40 °C, the C-Phycocyanin content was gradually reduced over time (2.7% after 4 h post-PEF treatment). The sever reduction of the C-Phycocyanin content could be an indication for molecular degradation. Incubation at 23 °C showed an initial increase of C-Phycocyanin till 2 h of incubation, followed by the subsequent decrease that happened with 1 h delay, in comparison to the incubation temperature of 40 °C. Contrary to incubation at 23 and 40 °C, at 4 °C C-Phycocyanin was released steadily over time and showed hardly any decrease. However, the amount of maximum release was not comparable to incubation at 23 and 40 °C.

Chaiklahan et al., (2012) have investigated the stability of dried C-Phycocyanin that was dissolved in citrate phosphate buffer. In order to enhance C-Phycocyanin stability at high temperatures Chaiklahan et al., (2012) have suggested to reduce the pH of the citrate phosphate buffer, since temperature and pH reversely affected the C-Phycocyanin stability. Coherently Sarada et al., (1999) have also indicated the C-Phycocyanin degradation at temperatures above 45 °C. Furthermore, regarding C-Phycocyanin stability at 25 °C the authors have suggested to maintain the pH range at 5-7.5. Based on our findings, we propose a mechanism for releasing the C-Phycocyanin during post-PEF incubation. In general, we have two reaction kinetics that are happening simultaneously. On the one hand, C-Phycocyanins are released over time, whereas, on the other hand, the released molecules that undergo degradation due to their unfavourable surrounding medium (unfavourable temperature or pH). Therefore, the reported value shown in the graphs can be inferred as the absolute C-Phycocyanin concentration (Eq.3).

Eq 3. $[C-PC](t)_{absolute} = [C-PC](t)_{released} - [C-PC](t)_{degraded}$

The result from the incubation temperatures convinced us to choose 23 °C as the optimal post-PEF incubation temperature. To get insight into the effect of the pH of the external medium on protein and C-Phycocyanin liberation and stability during post-PEF incubation, the effect of incubation in different pH-buffers (pH 6- and pH 8-buffers) in addition to the incubation in initial buffer was investigated.

3.2. Effect of the post-PEF incubation pH on protein and C-Phycocyanin release

This experiment was conducted using biomass concentration of 3.6 $g \cdot kg_{sus}^{-1}$. Incubating cells in pH 6- and pH 8-buffers at this biomass concentration actually enhances the protein extraction efficiency (Figure 2a). Incubation in pH 6-buffer showed a gradual increase in the amount of released proteins till 5 h post-PEF treatment, which was slightly reduced within 24 h (40.5% of CDW). In contrast, incubation in pH 8-buffer showed higher recovery of proteins over time which lasted even till 24 h post-PEF treatment (47.6% of CDW). The maximum amount of proteins (56.7% of CDW) were released via HPH treatment. Although incubation in initial buffer showed an initial increase of the protein release, it is reduced gradually over time and reached the lowest yield after 24 h of incubation (37.3% of CDW). Incubating untreated biomass in initial buffer or pH-buffers showed negligible amount of protein release during incubation. Thus, pH-buffers have not influenced the cells integrity.

During post-PEF incubation, it was observed that the pH of the PEF treated sample incubated in initial buffer is considerably falling over time, while the pH of incubation-buffers have enough capacity to maintain the desired-pH. At this biomass concentration untreated biomass that was incubated in initial buffer has a pH of 10.5 before treatment. Though, after PEF treatment the pH of the initial buffer decreased

over time and reached a value of 7.6 after 24 h. Thus, a drastic shift in pH of the initial buffer was observed.

The efficiency of PEF treatment in combination with pH-medium for protein extraction from *Nanochloropsis spp.* have been investigated (Parniakov et al., 2015). The aforementioned study showed that PEF-treated biomass at 20 $kV \cdot cm^{-1}$ and incubated in normal pH-medium of 8.5 gained higher protein extraction yield than the PEF treated biomass incubated at basic medium (pH of 11.0), which is in agreement with the high protein yield obtained in the current study using pH 8- buffer.

Regarding C-Phycocyanin extraction yield (Figure 2b), incubation at pH 8-buffer obtains the relatively high amount within 3 h post-PEF (10.5% of CDW), whereas incubation in initial buffer showed a strong decrease of C-Phycocyanin content (5.7% of CDW) within 3 h post-PEF. Decrease in C-Phycocyanin content in initial buffer continued drastically, whereby the lowest C-Phycocyanin concentration (1.3 % of CDW) was determined after 24 h post-PEF treatment. In other words, drastic degradation of C-Phycocyanin was observed over time as the blue colour was faded. Although, other studies have not reported any C-Phycocyanin degradation during post-PEF incubation of *A. platensis* (Martínez et al., 2017; Jaeschke et al., 2019). Nonetheless, incubation in pH 6-buffer has yielded higher C-Phycocyanin (11.2% of CDW) in comparison to pH 8-buffer. C-Phycocyanin concentration of the PEF treated biomass incubated in pH 6-buffer increased steadily over time and showed rarely any drop. Although C-Phycocyanin concentration of the PEF treated biomass incubated in pH 8-buffer showed an initial increase, decrease was also revealed over longer incubation. Untreated biomass did not show any liberation of C-Phycocyanin. Thus, incubation in pH-buffers without PEF treatment have not influenced the cells even after 24 h of incubation.

The observed reduction in C-Phycocyanin concentration in the initial buffer most probably caused by degradation could be attributed to the significant pH fall. One possible reason for pH drop could be attributed to the externalization of free amino acids by PEF treatment, which can acidify the medium (Akaberi et al., 2019). Generally severe pH alterations result in protein denaturation as the pH affects the charges of the ionisable groups of the proteins and eventually the protein stability The integrity of C-Phycocyanin with an isoelectric point of about 4.5 to 5.0 (Safaei et al., 2019) can be altered in a pH far from its isoelectric point.

It has to be mentioned that the ionic strength of the suspension was reduced through the obligatory washing step prior to PEF treatment for conductivity adjustment. Indeed, the initial sodium phosphate buffer used for washing the biomass has a conductivity of 0.1 $mS \cdot cm^{-1}$. Thus, the initial buffer has a significantly lower ionic strength than the pH-buffers used in this study. Furthermore, it has to be considered that the ionic strength of the initial buffer is considerably lower than the commonly used phosphate buffers at pH 6 for C-Phycocyanin extraction in other studies. At the pH ranges of 5.5-7.0 C-Phycocyanin can mostly be found in their stable trimeric or hexameric structures (Kao et al., 1975; Adams et al., 1979). It is known that ionic strength influences the C-Phycocyanin aggregation state (Berns and MacColl, 1989), though various salts can affect the aggregation state of C-Phycocyanin in a different manner. For instance, MacColl et al., (1971) have shown that increasing the ionic strength of solvents by addition of phosphate salts or sodium chloride enhanced the C-Phycocyanin aggregation. Thus, in the current study the relatively reduced ionic strength in initial buffer might also influence C-Phycocyanin stability. It can be concluded that the incubation in pH-buffers with considerably higher conductivity values (i.e., increased ionic strength) not only impede the pH fall, but

also compensate the low ionic strength. Thus, it enhanced the overall stability of C-Phycocyanins.

3.3. Effect of the post-PEF incubation pH on protein and C-Phycocyanin release at higher biomass concentration

For further up-scaling processes effect of the post-PEF incubation pH was also examined using higher biomass concentration (9.7 $g \cdot kg_{sus}^{-1}$). As can be seen from Figure 3a, the maximum protein extraction yield over time is comparable to the results obtained previously at 3.6 $g \cdot kg_{sus}^{-1}$ (Figure 2a). However, the kinetics of protein release are different. The PEF-treated sample incubated in pH 8-buffer did not show the initial faster kinetics. Using 3.6 $g \cdot kg_{sus}^{-1}$, PEF treated biomass in pH 8-buffer obtained the maximum amount of protein release within 3 h post-treatment, whereas using high biomass concentration the amount of protein release increased steadily over time and did not show the saturation phase as has been observed previously. However it seems that at high biomass concentration using pH-buffers still enhances protein extraction yield following PEF treatment.

With respect to C-Phycocyanin yield at high biomass concentration, the drastic reduction observed in PEF-treated and incubated in initial buffer using 3.6 $g \cdot kg_{sus}^{-1}$ was not appeared i.e., only a mild reduction in C-Phycocyanin yield was observed (Figure 3b). While at 3.6 $g \cdot kg_{sus}^{-1}$ (Figure 2b), the maximum C-Phycocyanin yield was obtained within 1 h post-PEF incubation in initial buffer, at high biomass concentration (Figure 3b), the maximum C-Phycocyanin yield was released after 5 h of incubation. Consequently, the C-Phycocyanin kinetics at high biomass concentration is significantly slower in comparison to the kinetics obtained at 3.6 $g \cdot kg_{sus}^{-1}$.

The first possible reason for the observed different in release kinetics can be due to the slower diffusion rate in concentrated biomass. In other words, at

3.6 g·kg_{sus}^{-1} the efficient diffusion leads to faster release of C-Phycocyanin, whereas at high biomass concentration the inefficient diffusion causes gentler C-Phycocyanin liberation. From another point of view, the diffusion rate may affect the amount of liberated ions and charged molecules which can reduce the pH and consequently the C-Phycocyanin stability, also in accordance with our suggested mechanism (Eq. 3). Another possible reason for an improvement in C-Phycocyanin stability at high biomass concentration can be attributed to the amount of released proteins. Consistently to our observations, Houghton JD, (1996) have shown that at high protein concentrations, C-Phycocyanin are found as trimers or hexamers (i.e. stable structures), whereas at low protein concentrations they dissociate to form monomers (i.e. unstable structures).

One possibility to improve the diffusion rate could be to dilute the biomass after PEF treatment. However, this can also increase the downstream costs at industrial scale. Nonetheless, the results of the current study clearly reveal the dependency of PEF performance on the biomass concentration. Other studies showed that more than 80% of the total proteins were extracted within 1 h post-PEF treatment (Goettel et al., 2013; Parniakov et al., 2015), which are in agreement with our results obtained using biomass concentration at 3.6 g·kg_{sus}^{-1} (Figure 2), since at high biomass concentration longer post-PEF incubation is mandatory. The results of our study are in good agreement with the findings of Chaiklahan et al., (2012) for the effect of pH on C-Phycocyanin stability.

3.4. PEF treatment at lower energy input of 56 kJ·kg_{sus}^{-1}

Further reduction of PEF energy demand was applied by introducing the fresh biomass (at 9.12 g·kg_{sus}^{-1}) to the treatment energy of 56 kJ·kg_{sus}^{-1}. Effect of PEF treatment at 56 kJ·kg_{sus}^{-1} and post-PEF incubation in pH-buffers on release of protein and C-Phycocyanin is illustrated in Figure 4. The kinetics of the protein/C-

Phycocyanin release were significantly different from the ones obtained at PEF treatment of 114 $kJ \cdot kg_{sus}^{-1}$. PEF treatment at 56 $kJ \cdot kg_{sus}^{-1}$ did not show the initial faster increase as has been observed previously using PEF treatment of 114 $kJ \cdot kg_{sus}^{-1}$ (Figure 3). To recover the maximal protein yield at lower energy treatment a longer incubation is mandatory. At this biomass concentration and the treatment energy of 56 $kJ \cdot kg_{sus}^{-1}$ hardly any advantage regarding incubation in pH-buffers was observed.

The pH changes of the PEF treated biomass incubated in initial buffer was followed over time (Table 1). Though the initial pH of 10.2 before PEF treatment, the pH of the initial buffer falls rapidly to 7.6 directly after PEF treatment due to the tremendous release of intracellular compounds into the medium. During post-PEF incubation the pH of the initial buffer falls gradually and reached the value of 6.7 after 24 h. Regarding pH alteration, it was observed that PEF treatment at this specific energy (i.e. 56 $kJ \cdot kg_{sus}^{-1}$) has slightly lower pH than the final pH of 7.3 that has been obtained after PEF treatment at 114 $kJ \cdot kg_{sus}^{-1}$. On the other hand, using lower biomass concentration (3.6 $g \cdot kg_{sus}^{-1}$), the pH variation during post-PEF incubation is considerably different in comparison to high biomass concentration. The pH of the PEF treated biomass at 3.6 $g \cdot kg_{sus}^{-1}$ remained more alkaline over a short post-PEF incubation. This observation can be attributed to the lower amount of biomass and subsequently lower amount of released molecules that can acidify the external medium.

The extraction efficiency induced by PEF treatment at lower energy input (56 $kJ \cdot kg_{sus}^{-1}$) promoted a slow release of the intracellular proteins, as only 10.8% of proteins were released after 4 h post-PEF incubation (Figure 4). Whereas PEF treatment at high energy input (114 $kJ \cdot kg_{sus}^{-1}$) obtained 40.7% proteins after 4 h post-PEF treatment (Figure 3), also shown in Table 2, which resulted in a faster release of

proteins. Irrespective of the impact of the external medium, PEF treatment at higher energy (114 $kJ \cdot kg_{sus}^{-1}$) reached the saturation value over a shorter incubation after PEF treatment. Regardless of the protein/C-Phycocyanin extraction kinetics, the maximum yield were obtained using either PEF treatment energies. However, to obtain the maximum protein/C-Phycocyanin yield at PEF treatment energy of 56 $kJ \cdot kg_{sus}^{-1}$ longer post-PEF incubation is necessary.

In the current study, the PEF treatment energies are calculated for a biomass concentration of 100 $g \cdot kg_{sus}^{-1}$, which corresponds to 1.14 and 0.56 $MJ \cdot kg_{dw}^{-1}$ for the energy input of 114 and 56 $kJ \cdot kg_{sus}^{-1}$, respectively. By reducing the PEF energy input at 56 $kJ \cdot kg_{sus}^{-1}$, the saturation value reached after 24 h of incubation. This can probably be seen as a result of the ineffective membrane permeabilization and the subsequent small release of intracellular valuables. Goettel et al., (2013) have shown the considerable release of various components into the external medium using PEF treatment *A. protothecoides* at high energy inputs (150 $kJ \cdot kg_{sus}^{-1}$). They have determined a higher degree of membrane permeabilization at higher PEF treatment energies that are in agreement with the findings of the current study. Thus, efficient permeabilisation at the energy input of 114 $kJ \cdot kg_{sus}^{-1}$ enables faster release of intracellular valuables into medium, which results in efficient protein/C-Phycocyanin extraction yield over a shorter post-PEF incubation. In contrast, the PEF lower energy input of 56 $kJ \cdot kg_{sus}^{-1}$ provided comparable protein/C-Phycocyanin extraction rates only after longer post-PEF incubation, which corresponds to inefficient permeabilization. Principally post-PEF incubation is indispensable in order to enhance the diffusion process for obtaining satisfactory release of intracellular valuables (Goettel et al., 2013; Coustets et al., 2015; Silve et al., 2018) also exhibited the necessity of the post-PEF incubation time for gaining comparable lipid extraction yield of *A. protothecoides* while reducing the PEF treatment energy. Besides

diffusion, Silve et al., (2018) have proposed enzyme-dependent processes that happened during post-PEF incubation i.e. PEF treatment can induces autolysis-like process, which subsequently can improve lipid-extraction yield even at lowest energy treatment. Though, PEF-induced autolysis have been indicated extensively in yeast (Alexandre and Guilloux-Benatier, 2006; Martínez et al., 2016). More recently Scherer et al., (2019) have shown that proteolytic enzymes that are released as a result of cell death during post-PEF incubation might assist in protein recovery of *C. vulgaris*. They have argued that autolysis-like processes, similar to yeast can also occur in algae following PEF treatment. It is known from bacterial decontamination studies that efficiency of PEF treatment scales with intensity of the specific treatment energy. In fact, Gusbeth et al., (2009) have shown that the specific treatment energy affected the bacterial inactivation rate. This is consistent with the results of the current study that at high PEF treatment energy (114 $kJ \cdot kg_{sus}^{-1}$) more cells can be affected by the electric field thus leads to the quicker release of intracellular compounds. The lower PEF treatment energy (56 $kJ \cdot kg_{sus}^{-1}$) impacts cells gently, albeit irreversibly, however longer post-PEF incubation is necessary. Interestingly the results of our study were not in agreement with the findings of previous works in terms of the kinetics of protein/C-Phycocyanin release after PEF-treatment (Martínez et al., 2017; Jaeschke et al., 2019). For instance, Martínez et al., (2017) demonstrated a gap phase of about 150 min post-PEF treatment for detecting C-Phycocyanin probably due to the lower electric field intensity and lower biomass concentration. Since at lower biomass concentration, the amount of released compounds over a short incubation time were probably far from the range of being detected. Though we have used the same PEF parameters as Jaeschke et al., (2019), the maximum yield of C-Phycocyanin was not obtained within 30 min at either treatment energies. Our results indicated that the release of protein/C-Phycocyanin directly after PEF treatment is a

matter of biomass concentration. Nevertheless, the conclusion of the latter study is in agreement with our findings obtained after PEF treatment of biomass at 3.6 $g \cdot kg_{sus}^{-1}$ and high treatment energy (114 $kJ \cdot kg_{sus}^{-1}$) (Figure 2a).

3.5. Influence of cell aggregation on PEF extraction efficiency

Long filamentous structure of *A. platensis* clump together and form large aggregates of cells during harvest. However, for subsequent PEF treatment this aggregates might affect the permeabilization and thus the subsequent extraction efficiency. Hence, an experiment was designed to get insight into the effect of cell aggregation in the suspension prior to PEF treatment on the amount of protein/C-Phycocyanin release. Effect of homogeneity of the suspension (without visible clusters of cells) on the protein/C-Phycocyanin extraction yield was examined by testing a heterogeneous cell suspension where the cells are clustering in addition to a homogeneous suspension prior to PEF treatment. The kinetics of protein/C-Phycocyanin release were considerably influenced by the homogeneity of the suspension prior to PEF treatment (Figure 5). PEF treatment of a homogeneous suspension obtained fast kinetics of protein/C-Phycocyanin release, whereas PEF treatment of a heterogeneous suspension showed slow kinetics. PEF treatment of a homogeneous suspension obtained higher amount of proteins (53.3% of CDW) than PEF-treatment of a heterogeneous suspension (49.8% of CDW) after 24 h incubation. Though, HPH-treatment obtained the maximum amount of proteins (59.9% of CDW).

Regarding C-Phycocyanins, PEF treatment of a homogeneous suspension also gained higher amount (12.7% of CDW) than PEF-treatment of a heterogeneous suspension (10.9% of CDW) 24 h post-incubation. Whereas HPH-treatment attained 12% C-Phycocyanin.

The slower release kinetics and the reduced yield of the heterogeneous suspension containing visible cell aggregates (clusters of cells), can be attributed to

the mutual electric shielding of cells, which causes a decrease in the amplitude of induced transmembrane voltage (Guittet et al., 2017). It is known, that the amplitude of transmembrane voltage induced on spherical cells progressively decreases when cells are brought closer together (Pavlin et al., 2002). These authors show that volume fractions and cell arrangement both influence the induced transmembrane potential. Other numerical computations of the induced transmembrane voltage on suspended cells of various volume fractions, are supporting this conclusion (Pucihar et al., 2007). These authors also concluded, that higher pulse amplitudes were required to achieve the same fraction of cell permeabilization in dense cell suspensions. In addition, heterogeneous cells clusters with irregularities on the edge, such as in case of *A. platensis* - due to their long spirals - lead to a further reduction of induced transmembrane potential for the cells in these areas. The C-Phycocyanin release kinetics we measured for the suspension with visible clusters of cells are consistent with those findings. Furthermore, the time course of C-Phycocyanin release from suspensions containing visible clusters of cells (Figure 5) is similar to that obtained with less specific treatment energy (56 $kJ \cdot kg_{sus}^{-1}$, Figure 4b). This supports the assumption of mutual electric shielding of cells packed in dens clusters. Therefore, a gentle homogenisation of cell suspension is necessary to dissolve cell aggregation in order to maintain a high extraction efficiency.

3.6. Purity of crude extract: PEF- vs. HPH-treatment

Effect of either pre-treatment (PEF *vs.* HPH) on the purity of the C-Phycocyanin extract was investigated. Absorption spectra of crude extracts of PEF-treated samples after 24 h incubation either in initial buffer or in pH 8 buffer, as well as HPH treated samples were evaluated. Absorption spectra of crude extract obtained after HPH-treatment showed chlorophyll contamination, whereas the crude extract of PEF-treated samples barley showed chlorophyll contamination.

The purity of crude extracts was also examined by measuring the purity ratio of A_{620}/A_{280}. Crude extract obtained 24 h post-PEF treatment and incubated in initial buffer has a purity ratio of 0.36, whereas crude extract of PEF treated sample incubated in pH 8 buffer has a purity ratio of 0.51. This result clearly indicated the enhancement of the C-Phycocyanin purity using PEF-treatment assisted by incubation in pH 8-buffer. In contrast, crude extract gained after HPH treatment has a purity ratio of 0.32. This result clearly indicated the enhancement of C-Phycocyanin purity using PEF-treatment. Martínez et al., (2017) have also reported the purity ratio (A_{615}/A_{280}) of 0.51 after PEF treatment of *A. platensis* using distilled water as a solvent rather than pH-buffer. PEF-treatment can therefore be employed as a pre-treatment method, which enables C-Phycocyanin extraction by avoiding chlorophyll contaminations as well as large amount of cell debris. Comparison of PEF treatment at optimal incubation condition and HPH treatment on protein/C-Phycocyanin extraction efficiency is presented in Table 3.

4. Conclusion

Herein this study, we have extracted proteins and C-Phycocyanins from fresh *A. platensis* biomass using PEF treatment. Our results showed that cell aggregation in suspension prior to PEF treatment influenced the kinetics of protein/C-Phycocyanin release due to the mutual electric shielding which causes a decrease in the amplitude of induced transmembrane voltage. Biomass concentration severely affects proteins and C-Phycocyanins extraction yield using PEF-treatment. Optimum condition for extraction of proteins and C-Phycocyanin was obtained at 23 °C and incubation in pH 8-buffer. PEF treatment followed by subsequent incubation in pH 8-buffer enhanced the purity ratio of C-Phycocyanin.

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Figures

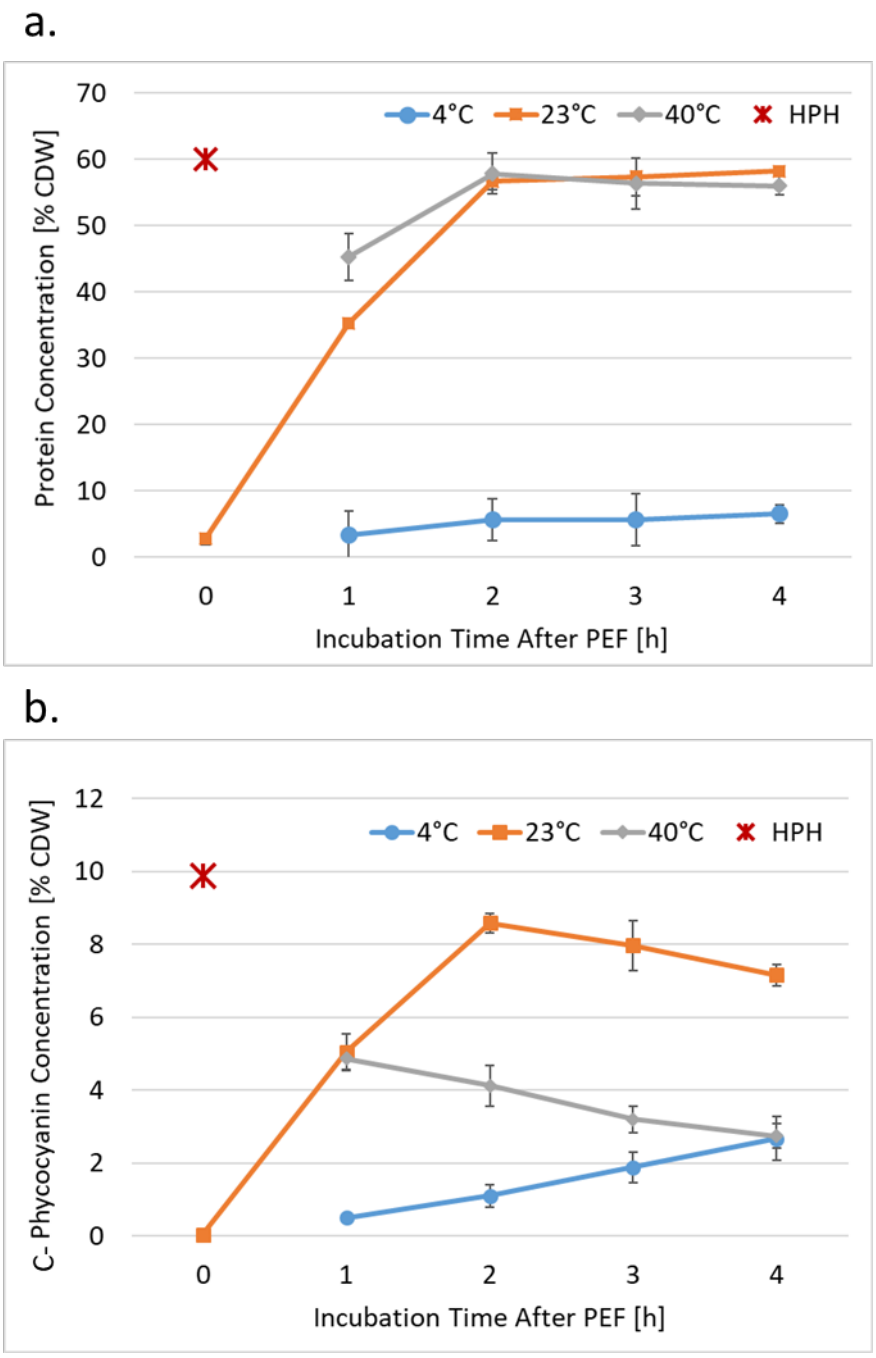

Figure 1 Effect of temperature on protein and C-Phycocyanin release of *A. platensis* following PEF treatment. **a.** Protein concentration **b.** C-Phycocyanin concentration (%CDW) of *A. platensis* fresh biomass at 2.6 ± 0.5 $g \cdot kg_{sus}^{-1}$ after PEF treatement at 114 $kJ \cdot kg_{sus}^{-1}$. The experiment was performed in triplicate. Error bars represent standard errors.

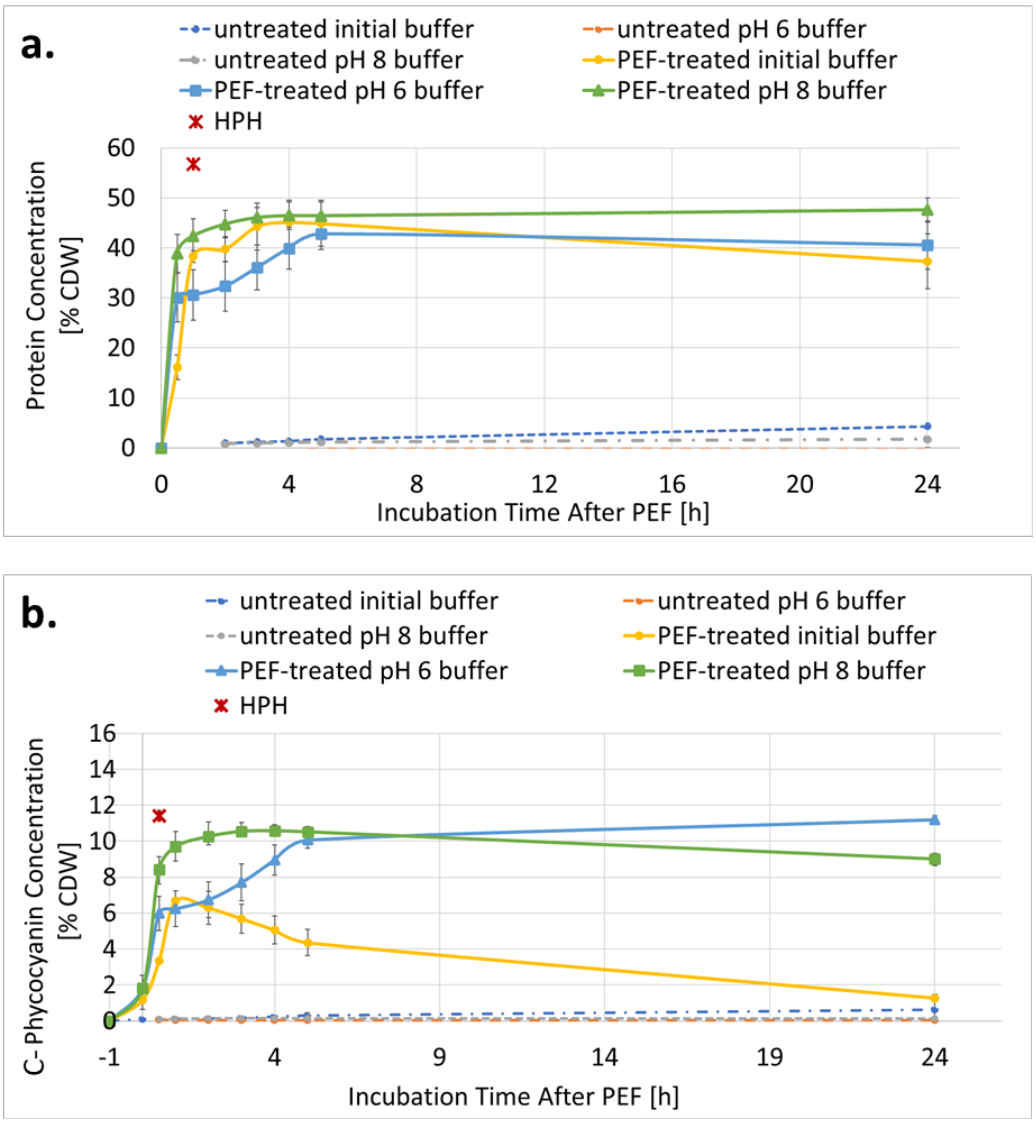

Figure 2 Effect of the pH of the external medium on protein and C-Phycocyanin release of *A. platensis* following PEF treatment. **a.** Protein concentration **b.** C-Phycocyanin concentration (%CDW) of *A. platensis* fresh biomass at 3.6 $g \cdot kg_{sus}^{-1}$ after PEF treatment at 114 $kJ \cdot kg_{sus}^{-1}$. The experiment was repeated for four times. Error bars represent standard errors.

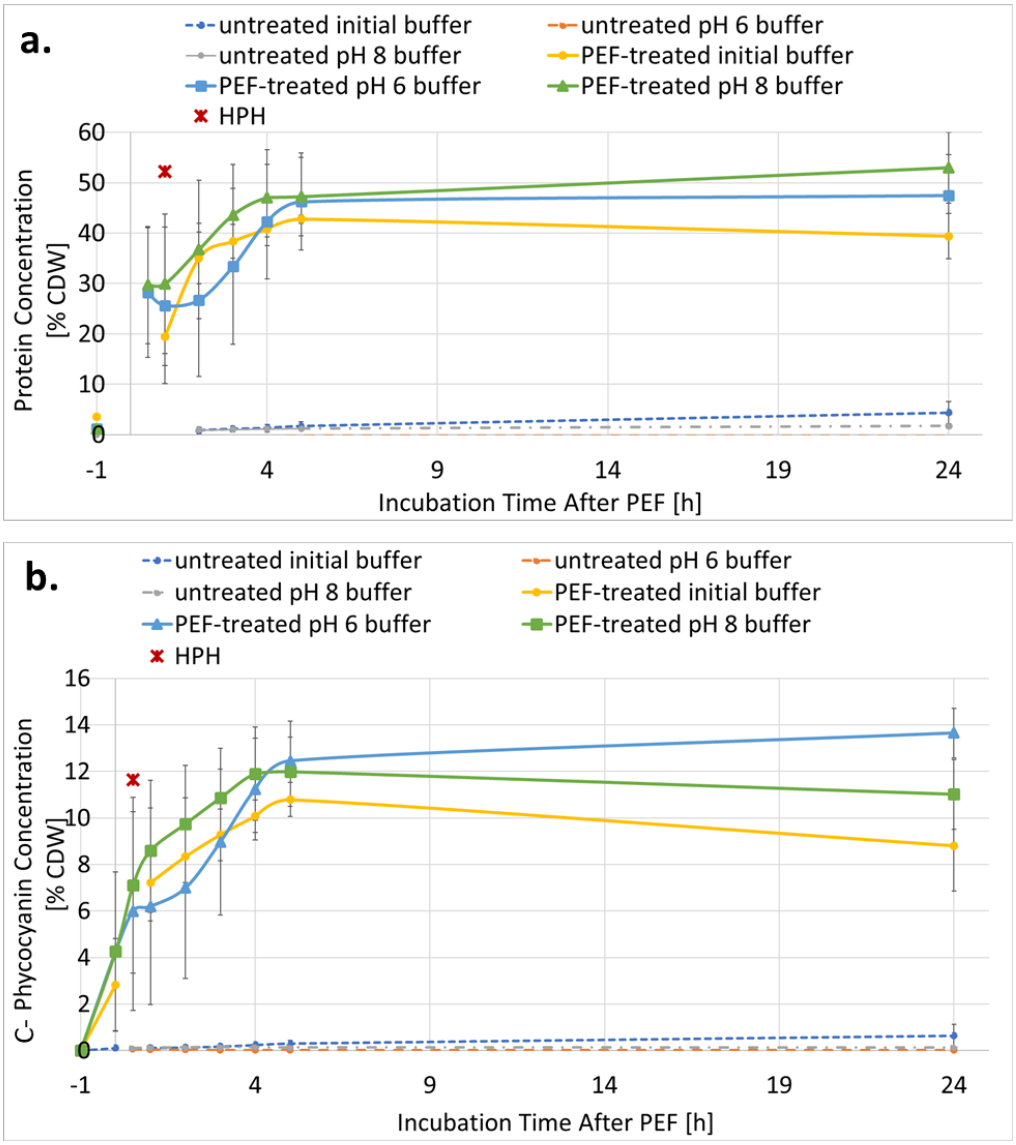

Figure 3 Effect of the pH of the external medium on protein and C-Phycocyanin release of *A. platensis* following PEF treatment. **a.** Protein concentration **b.** C-Phycocyanin concentration (%CDW) of *A. platensis* fresh biomass at 9.7 $g \cdot kg_{sus}^{-1}$ after PEF treatment at 114 $kJ \cdot kg_{sus}^{-1}$. The experiment was repeated for three times. Error bars represent standard errors.

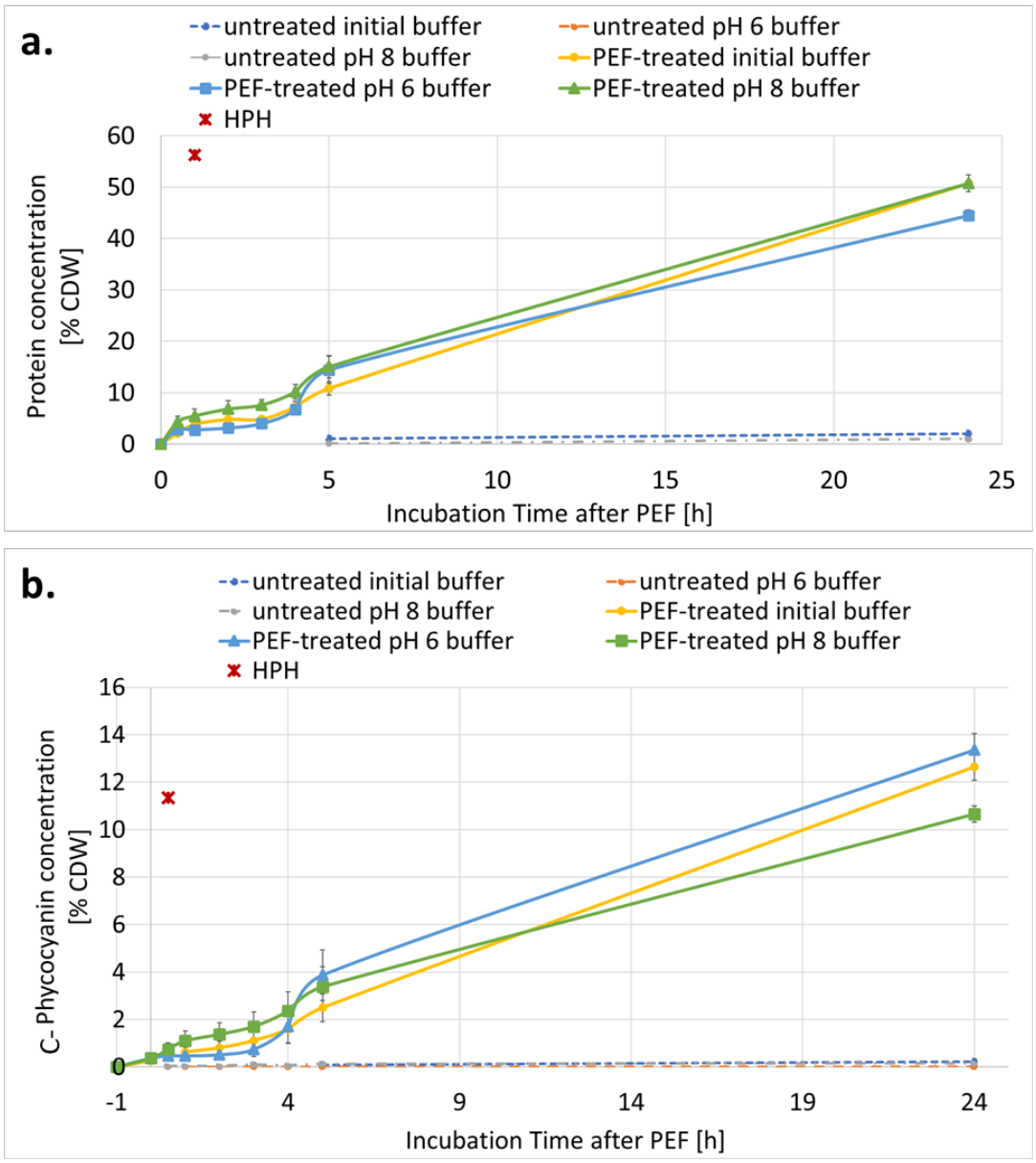

Figure 4 Effect of PEF-treatment at 56 kJ·kg$_{sus}^{-1}$ and the pH of the external medium on protein and C-Phycocyanin release of *A. platensis* **a.** Protein concentration **b.** C-Phycocyanin concentration (%CDW) of *A. platensis* fresh biomass at 9.12 g·kg$_{sus}^{-1}$ after PEF treatment at 56 kJ·kg$_{sus}^{-1}$. The experiment was repeated for three times. Error bars represent standard errors.

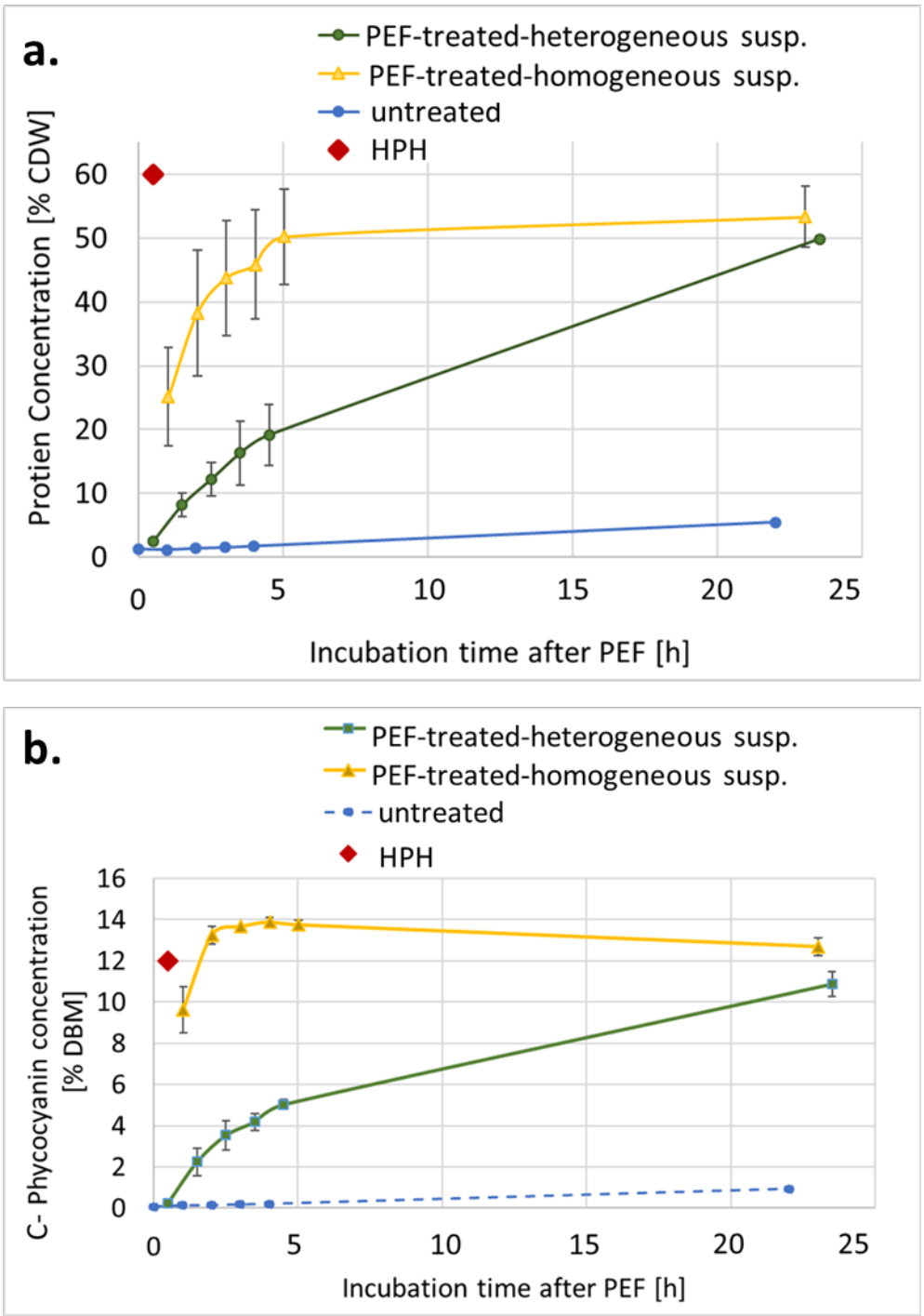

Figure 5 Influence of cell aggregation on PEF extraction efficiency of *A. platensis.* **a.** Protein concentration **b.** C-Phycocyanin concentration (%CDW) of *A. platensis* fresh biomass at 11.3 $g \cdot kg_{sus}^{-1}$ after PEF treatment at 114 $kJ \cdot kg_{sus}^{-1}$. The experiment was repeated in duplicates. Error bars represent standard error.

Tables

Table 1 Effect of PEF treatment at 114 and 56 kJ·kg_{sus}^{-1} on pH post-PEF incubation. Changes of the pH of *A. platensis* fresh biomass at 3.6, 9.7 and 9.12 g·kg_{sus}^{-1} during post-PEF incubation in initial buffer is followed till 24 h.

Incubation time in h	pH after PEF treatment at 114 kJ·kg_{sus}^{-1} (3.6 g·kg_{sus}^{-1})	pH after PEF treatment at 114 kJ·kg_{sus}^{-1} (9.7 g·kg_{sus}^{-1})	pH after PEF treatment at 56 kJ·kg_{sus}^{-1} (9.1 g·kg_{sus}^{-1})
0 h	**9.8**	**8.5** ± 0.2	**7.6** ± 0.1
1 h	9.6	7.9 ± 0.2	7.2 ± 0.0
2 h	9.5	7.8 ± 0.2	7.2 ± 0.0
3 h	9.3	7.9 ± 0.1	7.2 ± 0.0
4 h	9.2	7.9 ± 0.1	7.4 ± 0.0
24 h	**7.6**	**7.3** ± 0.3	**6.7** ± 0.1

* Data points represent mean values ± standard error (n = 3).

Table 2 Effect of PEF treatment at 114 and 56 kJ·kg_{sus}^{-1} on protein release (%CDW) of *A. platensis* fresh biomass at 9.7 and 9.12 g·kg_{sus}^{-1} incubated in initial buffer.

Incubation time in h	Protein %CDW PEF treatment at 114 kJ·kg_{sus}^{-1}	Protein %CDW PEF treatment at 56 kJ·kg_{sus}^{-1}
0 h	-	-
1 h	19.5 ± 5.8	3.8 ± 0.6
2 h	35.0 ± 5.1	4.8 ± 1.0
3 h	38.0 ± 3.3	7.2 ± 0.3
4 h	**40.7** ± 1.5	**10.8** ± 1.1
24 h	**39.4** ± 4.4	**50.7** ± 1.6

* Data points represent mean values ± standard error (n = 3).

Table 3 Comparison of PEF treatment at 56 kJ·kg_{sus}^{-1} incubated in pH-8 buffer for 24 h and HPH treatment on protein/C-Phycocyanin extraction yield, purity ratio of crude C-Phycocyanin extract and energy input of *A. platensis* fresh biomass at 9.12 g·kg_{sus}^{-1}.

Cell disruption method	Protein %CDW	C-Phycocyanin % CDW	Purity ratio of crude C-Phycocyanin	Energy input

PEF treatment at 56 kJ·kg_{sus}^{-1} (24h post-treatment in pH-8)	50.7 ± 1.6	12.65 ± 0.6	0.51	less energy
HPH treatment	56.2 ± 3.8	11.35 ± 0.7	0.32	energy intensive

* Data points represent mean values ± standard error (n = 3).