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Pulsed electric field assisted extraction of intracellular valuables from microalgae

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ABSTRACT

This paper discusses the application of pulse electric field (PEF) treatment for the cell disintegration of the fresh water microalgae *Auxenochlorella protothecoides*. The algae were cultivated under controlled conditions in a closed photo-bioreactor. After algae harvest the algae suspensions were concentrated and PEF treated with square pulses with a duration of 1 μs. We investigated the influence of specific treatment energy (52–211 kJ/kg suspension), electric field strength (23–43 kV/cm) and biomass concentration (36–167 g dry weight per kg suspension) on cell disintegration. For all pulse parameters applied, the PEF induced cell disintegration resulted in the release of soluble intracellular matter into the suspension. The disintegration efficiency increased with increasing specific treatment energy, whereas the field strength hardly had any influence. For suspensions with a biomass content of 100 g dry weight per kg suspension the electrical energy input necessary for considerable cell rupture was in the range of 1 MJ/kg dried algae. This is equivalent to 4.8% of the upper heating value of the algae. Although the treated algae contained lipids, PEF treatment only led to the spontaneous release of soluble components. The selectiveness of the process might offer the opportunity to use PEF treatment in a biorefinery concept, where soluble algae ingredients are extracted before solvent extraction of lipids is performed.

1. Introduction

In the past years microalgae gained a lot of attention because they are considered to be a promising, renewable feedstock for food, feed, fine chemicals and biofuel production. For example algae can produce lipids, pigments, unsaturated fatty acids or polysaccharides at comparably high production rates [1]. However, up to now they are mainly used for the production of high value low volume products. Among the reasons for this are the costly scale-up of algae cultivation and the rather difficult downstream processing [2].

Many valuables produced by microalgae are stored intracellular and the extraction of these products involves a cell disintegration step. Algae cells have strong cell walls [3], making cell disintegration and extraction rather difficult and energy intensive. For example, in the case of biodiesel production from microalgae, 30–50% of the production cost is due to the extraction step which includes also cell disruption [2]. Hence, the search for efficient, appropriate cell disintegration methods is vital to increase competitiveness of algae for the production of high volume, low cost substances like biofuels.

An appropriate cell disintegration process has to maximize the yield and the value of the compounds extracted [4]. In other words it disintegrates all the cells precisely without chemical contamination or degradation of the target compounds. For large scale production it is also important that the disintegration process can be scaled up and that it is rapid. In addition the integration of the cell disruption into the downstream processing has to be easy and it should not have a negative impact on subsequent processing steps, e.g. by hampering separation. All these properties influence the overall efficiency of the disintegration process and therefore its overall energy consumption, which is the crucial issue in biofuel production.

Different procedures of algae cell disruption were investigated, e.g. chemical, mechanical, thermal, or enzymatic methods. In most cases solvent extraction alone was less efficient than its combination with cell disruption methods [5–7]. Lee et al. and Prabakaran and Ravindran compared the effectiveness of autoclaving, bead-beating, sonication, osmotic shock and microwaves for the disintegration of different microalgae species [5,6]. Both groups found that the performance of the solvent extraction did not only depend on the disintegration method but also on the algae species. Teixeira who performed the lysis of different microalgae with hydrophilic ionic liquids reported a complete deconstruction of different microalgae species at temperatures between 100 and 140 °C [2]. Another approach is the cell lysis with alkali or acids at ambient temperatures, which has been found to be counterproductive in the case of astaxanthin recovery from

Abbreviations: TOC, Total Organic Carbon; HOC, Hydrophobic Organic Carbon; UHV, Upper Heating Value; CH, Carbohydrates; dw, dry weight; sus, suspension; R, Release-factor; 0, control untreated suspension; P, PEF treated suspension; σ_0, conductivity of untreated suspension; σ_{PEF}, conductivity of PEF treated suspension; c_x, biomass concentration; MO, Microorganism.

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Haematococcus pluvialis due to product degradation [8]. Seibert and Halim et al. performed cell disruption of microalgae using high-pressure homogenization and ball milling. According these studies, high-pressure homogenization was the more effective disintegration method for microalgae [9,10]. In most of the studies carried out, the scale up, the integration in the overall process and the energy consumption were not discussed. The studies revealed that further research is needed, in order to find other cell disruption methods that fulfill the above mentioned criteria.

Pulsed electric field (PEF) treatment may be a promising alternative to conventional cell disintegration methods. The exposure of biological cells to high intensity electric field pulses can alter the structure of the cell membrane. The external field provokes a charging of the membrane. At a sufficiently high transmembrane voltage (~0.5–1 V) the arrangement of the phospholipid molecules changes [11,12]. As a result the membrane looses its barrier function and becomes permeable, a phenomenon often referred to as "electroporation" or "electropermeabilization" [11,13]. According to literature, the major electrical parameters that influence the electropermeabilization efficiency are field strength of the external field, pulse shape, pulse duration, number of pulses applied, and specific treatment energy [11,14].

Depending on the electrical conditions, this membrane permeabilization can be reversible or irreversible. For an effective extraction of intracellular valuables, an irreversible cell membrane permeabilization is necessary. This membrane rupture promotes the release of intracellular matter and facilitates solvent access into the cell. PEF is a comparably gentle cell disintegration process, since it is usually performed at ambient temperatures and does not introduce additional impurities into the process. Hence, it helps to prevent undesirable changes in the target material [15]. PEF can be performed in batch and continuous mode and it is scalable. For example Sack et al. realized an industrial scale PEF device for the continuous treatment of sugar beets that can process 10 t beets per hour [12]. PEF has not only been used to foster the extraction of soluble compounds from plant tissue, it has also been employed to accelerate the extraction of vegetal oils, such as olive oil and maize oil [16].

Although PEF has been used for the enhancement of the extraction of lipids and other cell ingredients from terrestrial plants its application to algae is relatively new [17]. This work aimed to investigate the efficiency of the PEF treatment for the disintegration of microalgae cells. The fresh water microalgae *Auxenochlorella protothecoides* were PEF treated in a continuous process at different pulse parameters. The influence of electric field strength, specific energy input, algae concentration and diffusion time on the extraction efficiency is discussed. In addition the study addressed issues of the energy consumption and the integration of the PEF treatment in the downstream processing.

2. Materials and methods

2.1. Microalgae and cultivation

The microalgae strain used throughout this work was *A. protothecoides* obtained from EPSAG Göttingen, SAG strain number: 211-7a (Fig. 1). The algae were grown mixotrophically and axenically in TAP-medium [18], exhibiting an initial conductivity of 1.4 mS/cm at 25 °C. The batch-cultivations were carried out in a 26 l annular bubble column at 25 °C. The photo-bioreactor was illuminated continuously at 600 μE/s/m^2 by 8 fluorescence lamps (Osram fluora, 36 W). The culture was aerated at a rate of 1000 cm^3/min with an air carbohydrate mixture containing 2.5% carbon dioxide. For maximizing the amount of biomass available for PEF parameter studies, the algae were harvested after 29–35 days during the stationary growth-phase at a biomass concentration of 4.5 ± 0.5 g_{dw}/kg_{sus}. The algal suspensions from the photo-bioreactor were concentrated 10–35 times using a centrifuge (swinging-bucket rotor, 3000 *g*, 20 °C). In this way, algae suspensions with biomass concentrations of 36 to 167 g_{dw}/kg_{sus} were obtained. The conductivity of the concentrated suspensions was not adjusted and ranged between 0.9 and 1.0 mS/cm. The concentrated suspensions were stored at ambient temperature. The PEF treatment was performed within 3 h after the concentration step.

2.2. Pulsed electric field treatment

The algae suspensions were treated in the treatment chamber, shown in Fig. 2. The chamber was constructed for continuous flow treatment of biomass suspensions and manufactured at the Institute of Pulsed Power and Microwave Technology (Karlsruhe Institute of Technology, Germany). It consists of two stainless steel electrodes with a diameter of 60 mm paired in parallel and separated to a gap distance of d = 4 mm by a transparent polycarbonate housing. The treatment volume, length = 47 mm and width = 11 mm, was perfused from bottom to top allowing gas bubbles to exit the chamber. The field distribution in the treatment volume is uniform. The treatment chamber was connected to the output line of a transmission line pulse generator that delivered square pulses with a voltage amplitude between 8 kV and 20 kV, corresponding to field strengths of 20 kV/cm to 50 kV/cm. Fig. 3 shows a typical waveform of the output pulse measured across the treatment chamber by a 40 kV high voltage probe (P6015, Tektronix) during continuous treatment of pre-concentrated microalgae suspension. A pulse amplitude of 14 kV represents an electric field strength of 35 kV/cm in the treatment volume. The pulse rise time is less than 20 ns. The details on the transmission line pulse generator and the acquisition system were reported in our previous work [19].

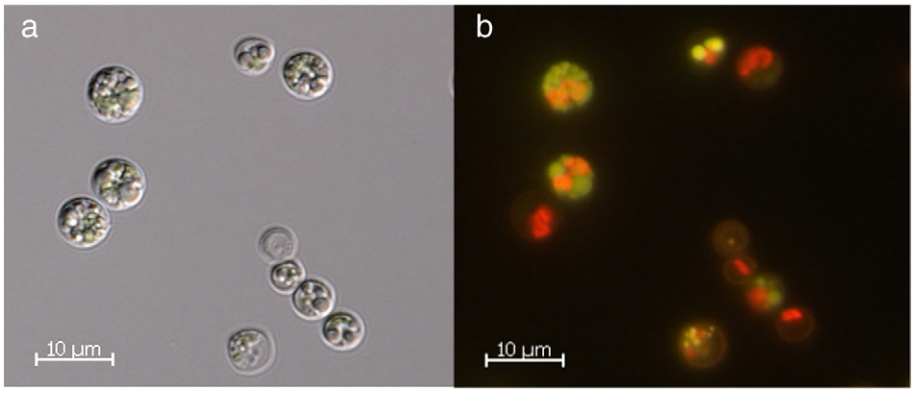

Fig. 1. *Auxenochlorella protothecoides*; 630 times magnified: (a) light-microscope; (b) lipid droplets dyed with Nile-Red (yellow fluorescence).

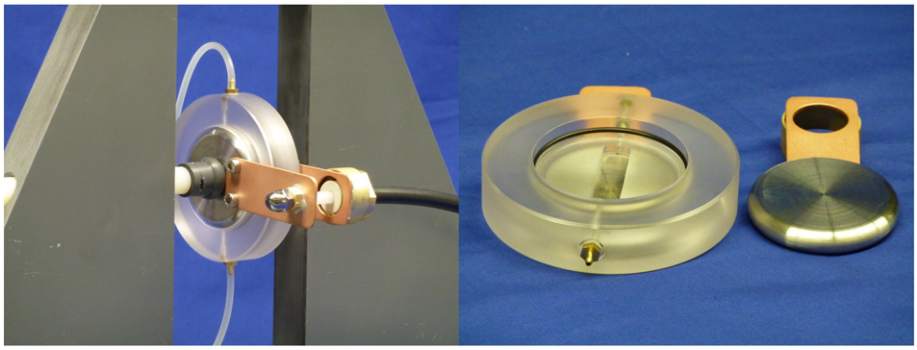

Fig. 2. Flow-cell for PEF treatment of algae suspensions; volume 2 ml; gap between electrodes 4 mm.

The suspensions were fed to the chamber with a peristaltic pump (ISMATEC ISM 834C, Switzerland) at a constant flow-rate of 6 ml/min. The suspensions in the feed vessel were stirred and kept at ambient temperature. In order to detect the temperature change due to the PEF treatment the temperature in the feed vessel and at the treatment chamber outlet was measured using a digital thermometer (Qtemp 500, VWR International, Germany). Samples from the treated suspension were collected after the temperature at the outlet reached steady state.

The influence of the electric field strength, the specific energy input, and the algal biomass concentration on the cell disintegration was investigated. The pulse duration was 1 μs throughout the experiments. For a constant specific energy delivery of 160 ± 2 kJ/kg_{sus} at different electric field strengths (23–43 kV/cm) the frequency of the pulses was varied from 1.0 to 5.5 Hz. In the same way a variation of the specific energy input (52–211 kJ/kg_{sus}) at a nearly constant electric field strength (33.5 ± 4.5 kV/cm) was accomplished.

For all algae suspensions control treatment cycles were performed identical to the PEF treatment procedure. The suspensions were pumped through the cell but without applying electric field pulses.

The PEF treated and the control suspensions were stored in the dark at 25 °C. The storage time was 40 min unless specified otherwise. After storage the conductivity and the pH of the suspensions were measured with a handheld conductivity meter (CLM 381, Endress + Hauser, Germany) and a pH meter (WTW pH 330i, Germany). Subsequently the suspensions were centrifuged at 10,000 *g* and 25 °C. The supernatant was taken for further analysis. The supernatant was either analyzed directly or stored in the freezer at −18 °C.

2.3. Analytical methods

2.3.1. Determination of biomass concentration

For the determination of the biomass concentration approximately 20 ml of untreated algae suspension was weighted and then washed three times. The washing was effected by centrifuging the suspension at 10,000 *g* and replacing the supernatant by deionized water. After the third centrifugation step the pellet was dried in a circulating air drying oven (Memmert, Germany) at 80 °C and weighted (balance AE163, Mettler Germany) when constant mass was achieved. For each suspension the biomass concentration was determined three times. Biomass concentrations are expressed in g dried biomass per kg algae suspension (g_{dw}/kg_{sus}).

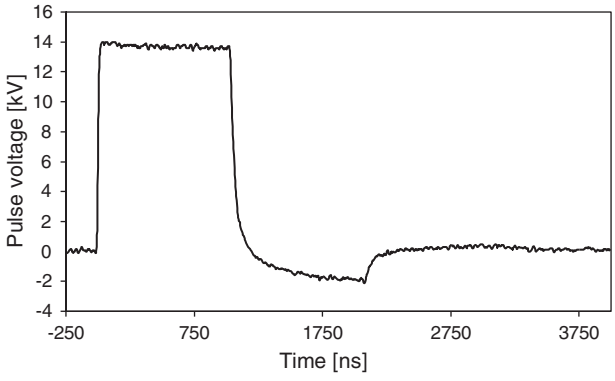

Fig. 3. Pulse voltage waveform across the treatment chamber during continuous PEF treatment of microalgae suspension, c_x = 98 g_{dw}/kg_{sus}, at a pulse repetition frequency of 2 Hz.

2.3.2. Dry matter content analysis

Approximately 20 ml of the fresh supernatant was dried in a drying oven at 80 °C until constant mass was achieved. The weight was determined before and after the drying. The dry mass content in the supernatant of untreated and treated algae suspensions is expressed in dry matter per kg of supernatant (g_{dw}/kg).

2.3.3. Organic carbon analysis

For the determination of the total organic carbon content (TOC), frozen samples of supernatant were unfrozen and diluted 100 times with carbonless water. A sample of 8000 μl was acidified with 30 μl of 2 mol HCl. Then the TOC was analyzed according to EN 1484 with a TOC-analyzer (multi N/C 2000, analytikjena, Germany). Each measurement was repeated at least three times.

The hydrophobic organic carbon content HOC of the supernatant was determined by an outside laboratory (DOC-LABOR Dr. Huber, Germany) according to the method described by Huber et al. [20]. The supernatant samples were taken immediately after centrifugation and stored on ice. The analysis was started within 4 h after centrifugation.

2.3.4. Carbohydrate analysis

Determination of carbohydrate concentration was performed with the organic reagent anthrone as described by [21]. The anthrone-reagent consisted of 0.2% w/v anthrone in sulfuric acid H_2SO_4 (95%). Glucose at different concentrations was used as a standard. Defrosted samples of supernatant were diluted 20 times. 0.2 ml of the diluted supernatant and 2 ml anthrone-reagent were mixed, incubated at 110 °C and mixed again. After 10 min incubation at room temperature the absorbance at 625 nm was measured in a spectrophotometer (Genesys 10S UV–VIS, Thermo Scientific, USA).

2.3.5. Protein analysis

Protein quantitation assay was performed with a commercial kit (Roti-Nanoquant K880, Roth, Germany) using bovine serum albumin as a standard.

2.3.6. Visualization of lipid content

For the visualization of lipids inside the algae the cells were dyed with Nile Red (5H-Benzo[α]phenoxazin-5-one, 9-(diethylamino)-, Invitrogen, Germany) (Fig. 1b) [22,23]. The algae cells were diluted to an optical density of 1.0. 1.6 ml of the algae suspension was mixed with 400 μl of Nile Red stocking solution (30 μg/ml in dimethylsulfoxide

(DMSO)). Staining was performed within 30 min in the dark at room temperature. Afterwards the cells were washed twice and centrifuged for 5 min at 500 rpm. The supernatant was discarded and the cells were resuspended in 1.8 ml of deionized water.

The Nile Red stained algae cells were analyzed by light- and fluorescence microscopy (Axioplan 2 imaging, Zeiss, Germany). Nile Red emission was observed with 460 ± 10 excitation and 515 long pass emission filter (Zeiss filter set 09) and with 546 ± 12 excitation and 575–640 band pass emission filter (Zeiss filter set 20).

2.3.7. Determination of the upper heating value

The upper heating value of freeze dried algae samples were determined with a calorimeter (Kalorimeter C 5000, IKA, Germany) according to DIN 51900, ISO 1928.

3. Results and discussion

3.1. Overall effect

With the pulse parameters chosen for the experiments, considerable disintegration of *A. protothecoides* was achieved. All the PEF treatment experiments performed resulted in an increase in conductivity and a drop of pH of the treated suspensions. In addition to ionic substances, organic substances such as carbohydrates and proteins drained out of the cells (Fig. 4). Compared to the control samples, the total organic carbon content (TOC) of the supernatant was up to 6 times higher after PEF treatment. Dry weight measurements revealed that 11–15% of the initial biomass remained in the supernatant. Although the algae contained up to 20% w/w lipids, hydrophobic organic carbon HOC was not detectable, neither in the supernatant of the control nor in the supernatant of the treated suspensions. These findings evidence that PEF treatment only fostered the spontaneous release of soluble cell ingredients from *A. protothecoides* cells. The lipid droplets remained inside the cells. However, solvent extraction experiments performed in our laboratory with untreated and PEF treated *A. protothecoides* cells revealed that PEF treatment led to higher lipid-yields [24].

3.2. Diffusion kinetics

Fig. 5a shows the typical time course of the conductivity of a microalgae suspension inside the treatment chamber during start-up of a PEF treatment experiment. In order to completely fill the entire volume of the treatment chamber by liquid medium and to control for removal of unwanted gas bubbles, initially tap water (0.6 mS/cm) was pumped through the chamber, indicated by the low conductivity value of 0.6 mS/cm at the beginning of the waveform displayed in Fig. 5a. Subsequently, as shown 25 s later in the diagram, the algae suspension entered the chamber and the conductivity increased to 1 mS/cm, which represents the conductivity of the untreated suspension. Since the PEF treatment of the algae cells caused the release of intracellular ionic substances, the conductivity increased further. After about 60 s, steady-state was reached inside the treatment chamber and the conductivity saturated at a value of 1.5 mS/cm right after pulsing.

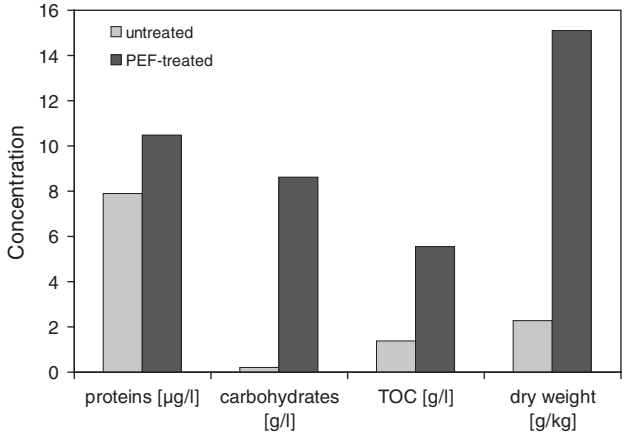

Fig. 4. Release of intracellular matter 40 min after PEF treatment $c_x = 109\ g_{dw}/kg_{sus}$; specific treatment energy 155 kJ/kg; field strength 30.5 kV/cm; pulse duration 1 µs.

After PEF treatment, additional ionic matter continued to leak out of the algae cells, indicated by a further increase in conductivity (Fig. 5b) exceeding the value of 1.5 mS/cm detected right after pulsing. Two hours after PEF treatment the conductivity started to saturate at a value between 2.6 and 3 mS/cm. During that time the conductivity of the untreated suspension remained almost constant at 1.1 mS/cm. The fact that the leakage of intracellular matter persisted for hours after the treatment shows that the electrical conditions were sufficient to achieve irreversible membrane permeabilization.

The release of ionic substances through a permeabilized membrane is a diffusion driven process. Therefore, waiting time is required to allow for sufficient diffusion after PEF treatment. In literature, diffusion times of 4 h for yeast [13], 2 h for skin tissue of grapes [12] and 1.5 h for chicory roots [25] are recommended. According to our findings a waiting time of at least 2 h after the PEF treatment should be integrated into algae processing for an efficient extraction of intracellular valuables from *A. protothecoides*.

3.3. Influence of pulse parameters

In order to find the optimal pulse conditions for electropermeabilization of *A. protothecoides*, the influence of specific treatment energy input and electric field strength was investigated. The pulse duration and the pulse shape were not varied. In our previous work we investigated the inactivation of the bacterium *Pseudomonas pudita* at different field strengths (40–120 kV/cm) and at constant treatment energy input (120 kJ/kg) [11]. For this microorganism the electropermeabilization efficiency was independent of pulse shape and pulse duration (100 ns–10 µs).

A variation of the specific treatment energy was performed by increasing the pulse repetition frequency from 1 Hz (50 kJ/kg_{sus}) to 4 Hz (200 kJ/kg_{sus}) while keeping other processing parameters (flow rate, field strength, pulse duration) constant. A higher energy input resulted in a higher degree of membrane permeabilization and therefore a stronger release of intracellular matter into the extracellular medium (Fig. 6). All concentrations measured (dry weight, total organic carbon and carbohydrates) showed the same trend as the conductivity. Hence, they also can be used to detect the extent of membrane permeabilization.

A strong augmentation of intracellular matter in the extracellular medium was already achieved at 50 kJ/kg_{sus}. Above 100 kJ/kg_{sus}, the concentrations and the conductivity started to saturate. The fact that the specific energy input influences the degree of membrane permeabilization has been reported by several authors who studied the electropermeabilization of biological cells [11,26,27].

The temperature was not controlled during the PEF treatment. Depending on the energy input the ohmic temperature elevation was in the range of 7 to 33°. Since the temperature rise was proportional to the specific energy input, the highest energy 206 kJ/kg_{sus} provoked the largest increase in temperature ($\Delta T = 33$ °C). In this case, the temperature at the outlet of the treatment chamber was 54 °C. It cannot be ruled out that a minor part of the enhancement of the extraction efficiency at high treatment energies is due to thermal effects. Supposed that component release would predominantly be caused by thermal effects, an above-average increase of component release should have been measured instead of the saturation behavior obtained for PEF treatment energy values higher than 100 kJ/kg_{sus}.

The temperature at the outlet of the treatment cell was 38 °C ($\Delta T =$ 15.5 °C) at an energy input of 100 kJ/kg_{sus}. It is very unlikely that this

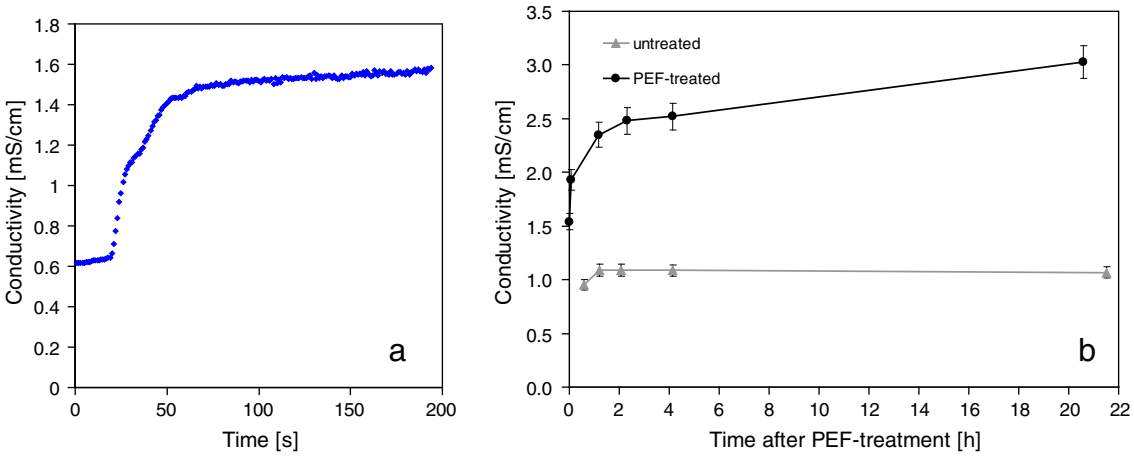

Fig. 5. Conductivity of algae suspension; (a) inside the treatment chamber during PEF treatment start-up; (b) after PEF treatment.

temperature rise will lead to a degradation of target products. Other authors used mechanical cell disintegration methods like ultrasonication, high-pressure homogenization or bead beating [9,10,28]. They report that they cooled the suspensions during the disintegration process, in order to provide for overheating and product degradation. Hence, the temperature elevation seems to have been considerable. Unfortunately, no values for temperature rise or cooling power are given and a comparison with our process is not possible.

The data displayed throughout in the diagrams were acquired at the same day from the same starting suspension prepared from one cultivation batch except the data shown in Fig. 7. Here, the variation of the TOC in the supernatant over treatment energy input is depicted for different cultivations. Hence, each experiment represents a different batch of microalgae. This comparison of the results allows estimating the influence of biomass condition. For all experiments performed the results shown in Fig. 7 exhibit the same trend on treatment energy variation as described above. Although the conductivities of the starting suspensions were not adjusted they were all in the same range (0.9–1.1 mS/cm at 25 °C). Also the TOC concentrations of the control samples did not vary much (1.3 g/l ± 10%) either. However, after PEF treatment variation was much more significant. The largest relative deviation from the average was obtained at a treatment energy of 100 J/kg and amounts to 31%. The relative error caused by the TOC-analyzer was determined to be 2%. The systematic measurement error increased but was less than 5% when additionally dilution errors were taken into account.

One source for the observed variation of the measured values might be differences in biomass concentration of the starting suspensions. Due to material adhesions inside the pipette tip and resulting pipetting errors, this concentration was difficult to adjust by optical density measurements and it varied in the range of 89 to 111 g_{dw}/kg_{sus}, which represents a relative deviation of 13%. This deviation is considerably lower than the observed variation in TOC results. The scatter of the TOC values most probably is due to variations of the biomass condition. Although the algae were cultivated under controlled conditions and harvested during the stationary phase, batch to batch differences in algae composition like cell wall porosity or thickness are unavoidable. Based on this analysis it should be mentioned, that the error bars displayed in the diagrams refer to the error of the diagnostic method and not to variations of the biomass. Absolute values of released compounds given in this study might be afflicted by an additional error on the order of 30%. Nevertheless, the validity of the general trend of the results on treatment energy variation could be confirmed repeatedly.

The required treatment field strength for membrane permeabilization in general linearly scales with the diameter of the targeted cells [29]. Because of the cell size of *A. protothecoides* (5–8 μm) the field strengths were chosen in accordance to research on the inactivation of heterotrophic microorganisms such as *E. coli* (2–6 μm, length) and *Saccharomyces cerevisiae* (5–10 μm). We assumed that both, efficient inactivation of microorganisms and efficient extraction of intracellular matter, required the formation of irreversible pores. Typical field strengths applied for inactivation of microorganisms (MO) lie in the range of 10–60 kV/cm [30–32]. Hence, our PEF experiments were performed at electric field strengths between 23 and 43 kV/cm. For field variation experiments the energy was kept at a

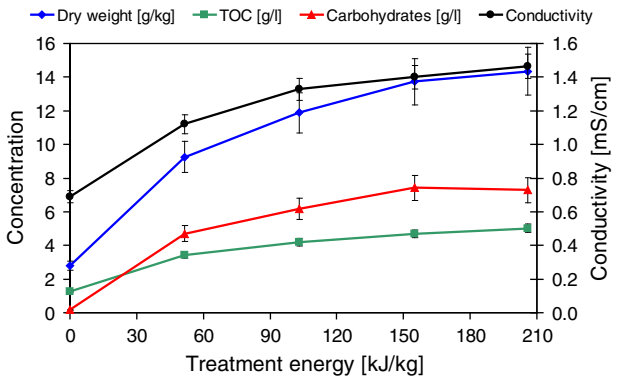

Fig. 6. Influence of treatment energy input on conductivity of algae suspensions and concentration of different bulk parameters in the supernatant; $c_x = 98\ g_{dw}/kg_{sus}$; field strength 34 ± 4 kV/cm; pulse frequencies 0, 1, 2, 3 and 4 Hz; pulse duration 1 μs, centrifugation 40 min after PEF treatment.

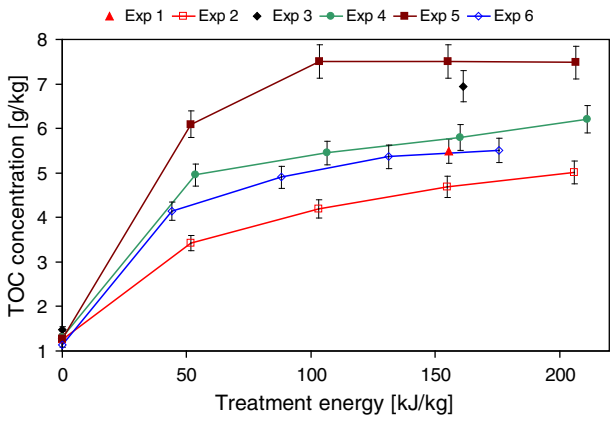

Fig. 7. Influence of treatment energy input on TOC concentration of the supernatant, experiment to experiment variation E = 32 ± 6 kV/cm; pulse duration 1 μs, centrifugation 40 min after PEF treatment. Exp 1: $c_x = 109\ g_{dw}/kg_{sus}$; exp 2: 98 g_{dw}/kg_{sus}; exp 3: 107 g_{dw}/kg_{sus}; exp 4: 102 g_{dw}/kg_{sus}; exp 5: 111 g_{dw}/kg_{sus}; exp 6: 89 g_{dw}/kg_{sus}.

constant value of 160 kJ/kg_{sus}. Using these comparably strong electric fields, a variation of field strengths did not have a great influence on the amount of intracellular compounds released (Fig. 8). The lowest field strength applied (23 kV/cm), was already high enough to rapidly achieve the membrane voltage (~0.5–1 V) that is necessary for the growth of pores [11,12] and to provide whole cell surface membrane permeabilization. At the same time the energy input was sufficient to achieve irreversible permeabilization.

Contrary to our findings, it is reported that an increasing field strength yielded in a better PEF treatment efficiency, i.e. a decrease of the survival fraction of MO treated [30]. However, in some cases experimental conditions are described incompletely and it is not clear whether this is due to the effect of a stronger electrical field and/or a higher energy input. The discrepancy to our findings might be also due to the fact that MO inactivation is not proportional to extraction efficiency. In addition optimal PEF treatment parameters do not only depend on the cell diameter but also on other properties of the target organism, e.g. the electrophysical properties of cell membranes and fluids and the tendency to form cell aggregates [15,27].

3.4. Influence of biomass concentration

The influence of the biomass concentration of the conductivity of PEF treated and untreated suspensions is depicted in Fig. 9. The higher the biomass concentration c_x of the suspension, the lower was the conductivity of the untreated suspension σ_0. For treated suspensions the converse observation could be made. Initially the more concentrated suspensions contained less extracellular ions since a larger part of the volume was occupied by algae cells. After PEF treatment more ions were released into the medium, simply because more disintegrated cells were in the higher concentrated suspensions, which resulted in the higher conductivity of the treated suspensions σ_{PEF}. Our data suggest a linear relationship between σ_{PEF} and c_x at a field strength of ~32 kV/cm. El Zakhem et al. [27] who treated yeast cells at moderate fields (<7.5 kV/cm) also report a nearly linear relation between σ_{PEF} and c_x up to a threshold value of about 16 wt.%. Above this limit yeast concentration, the conductivity σ_{PEF} decreased again. The authors supposed that in very highly concentrated yeast suspensions a part of the yeast cells remain unaffected, because they were hidden in low electrical field sites. Hence, in high concentrated yeast suspensions PEF treatment was less effective. They call this the percolation threshold. In our experiments the highest c_x investigated was about 160 g_{dw}/kg_{sus} and the described deterioration of PEF efficiency was not observed. This was according to our expectations since our c_x did not exceed the reported percolation threshold and the electrical field was remarkably stronger.

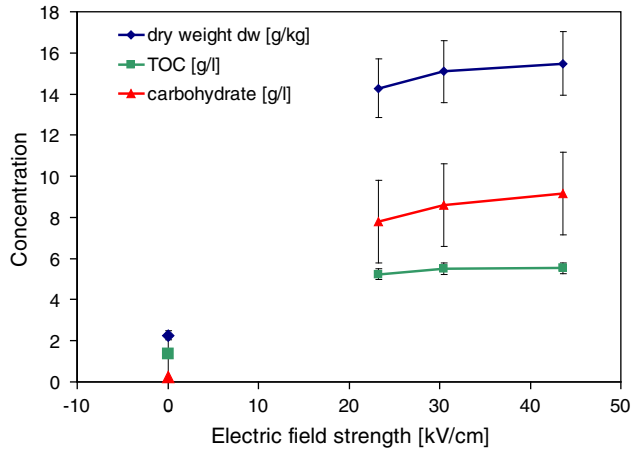

Fig. 8. Influence of electric field on the concentrations of intracellular matter in the supernatant. c_x = 109 g_{dw}/kg_{sus}; specific treatment energy 155 kJ/kg; pulse frequency 0, 5.5, 3 and 1.5 Hz; pulse duration 1 μs, centrifugation 40 min after PEF treatment.

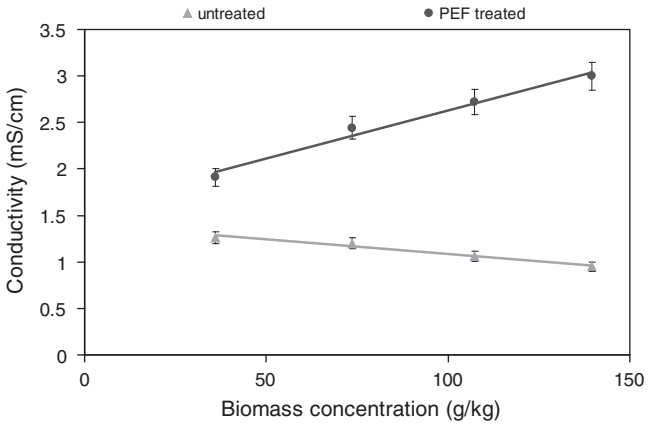

Fig. 9. Influence of biomass concentration c_x on the conductivity of treated and untreated algae suspensions. Specific treatment energy 162 kJ/kg; field strength 32 kV/cm; pulse duration 1 μs, centrifugation 40 min after PEF treatment.

An increasing σ_{PEF} only indicates a higher PEF treatment efficiency in the case of a constant biomass concentration in the suspension to be treated. The same is valid for the concentration of intracellular substances in the supernatant of the suspensions. If the biomass concentration varies, it has to be taken into account that at higher c_x theoretically more intracellular substances can be released. In order to compare the PEF treatment efficiency at different c_x a release-factor R for dry weight dw, carbon total organic TOC, and carbohydrates CH was calculated as follows:

$$R_{dw} = (c_{dw\text{-}P} - c_{dw\text{-}0})/c_x \quad (1)$$

$$R_{TOC} = (c_{TOC\text{-}P} - c_{TOC\text{-}0})/c_x \quad (2)$$

$$R_{CH} = (c_{CH\text{-}P} - c_{CH\text{-}0})/c_x \quad (3)$$

where $c_{dw\text{-}0}$ is the dry weight concentration in the supernatant of the untreated suspension and $c_{dw\text{-}P}$ is the dry weight concentration in the supernatant of the PEF treated suspension. The release factors for TOC and carbohydrates (CH) were calculated accordingly. Up to c_x of 160 g_{dw}/kg_{sus} the release factors increased slightly (Fig. 10). In conclusion, a higher algae concentration did not have negative effects on the extraction efficiency. It even seems to be favorable. In mechanical processes such as ultrasonication, high-pressure homogenization, or bead beating an adverse behavior was observed [9,10].

3.5. PEF energy demand

For biofuel production from microalgae, the energy demand of the algae processing is crucial. Microalgae processing efficacy usually is assessed by the required treatment energy per kilogram of dry microalgae biomass. In the biomass variation experiments, the treatment energy applied to the algae cells was approximately 160 kJ/kg_{sus}. This specific treatment energy is calculated per kg of treated suspension. Hence, for an algae suspension containing 100 g_{dw}/kg_{sus} algae the energy demand per kg_{dw} treated algae is 1.6 MJ/kg_{dw}, which is about 7% of the upper heating value (UHV) of unwashed dried *A. protothecoides* (UAV = 21 MJ/kg_{dw}). The lowest energy demand (0.9 MJ/kg_{dw}) was achieved for a suspension containing 167 g_{dw}/kg_{sus} algae.

According to our findings at high field strengths (~30 kV/cm) and a c_x of 100 g_{dw}/kg_{sus}, a specific treatment energy input of 100 kJ/kg_{sus} is necessary for sufficient extraction efficiency. Hence, the energy demand for the PEF treatment is 1 MJ/kg_{dw}. The energy input for the cell rupture of *Phaeodactylum tricornutum* cells (biomass content 5% w/v) with a ball mill and a high-pressure homogenizator was found to be 3.7 MJ/kg_{dw} and 3.0 MJ/kg_{dw}, respectively [9]. These values from literature are

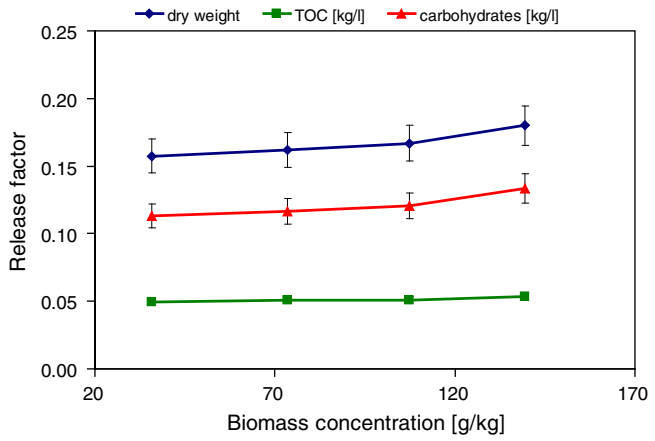

Fig. 10. Influence of biomass concentration on the extraction efficiency of intracellular matter after PEF treatment. Specific treatment energy 162 kJ/kg; field strength 32 kV/cm; pulse duration 1 μs, centrifugation 40 min after PEF treatment.

higher than the energy input in our PEF treatment experiments but in the same order of magnitude. However, a direct comparison of these values is difficult. In both studies, the degree of cell disintegration was measured with different parameters and the experiments were performed with completely different microalgae species.

Although an energy demand of 1 MJ/kg_{dw} for the PEF treatment might be comparably low, future studies have to aim to develop optimized pulse protocols that allow reducing the specific treatment energy necessary for PEF treatment. Another way to bring down the energy demand for PEF treatment is to increase the biomass content of the treated suspensions. The maximum c_x is reached in a dewatered algae paste containing nearly no extracellular water (~30 wt.%) [33]. PEF treatment of such slurries at the conditions that have been applied in this study would lead to a theoretical minimum energy demand of 0.33 MJ/kg_{dw} which is 1.5% of the UHV. However, it has to be kept in mind that high mass–density slurries are not pumpable anymore and cannot be treated by continuous-flow PEF processing. The process would have to be modified from a continuous to a batch process. It is also necessary to identify the limits that might be due to high c_x, like the percolation threshold or the loss of pumpability. In addition for an overall assessment the influence of c_x on the pumping energy and the energy necessary for the separation of algae has to be taken into account.

3.6. PEF process integration

For an economic and sustainable production of biofuels from algae biorefinery concepts are often proposed [34]. The extraction and commercialization of high value products such as pigments, enzymes, proteins or polysaccharides from the algae biomass are supposed to be necessary for an economic viability of an energetic use of microalgae. Cell disintegration by PEF treatment offers the opportunity to develop selective extraction processes that can be integrated in an algae biorefinery concept. PEF induced cell disintegration did not cause a spontaneous release of lipids. However, it has been found to increase the lipid yield from *A. protothecoides* in a one stage solvent extraction [24]. The authors observed a three fold increase in extraction efficiency on average. Therefore, after PEF treatment, in a two stage process water soluble cell ingredients can be extracted prior to solvent extraction of lipids.

In the first stage soluble organic and inorganic substances would be removed from the biomass. Consequently the relative amount of lipids in the remaining cell suspension would be higher and it may be possible to reduce the amount of solvent necessary for lipid extraction. Another advantage of cell disintegration by PEF treatment is the fact that hardly any cell debris is produced. This can be favorable in an extraction procedure involving several solid–liquid separation steps.

4. Conclusions

The effect of PEF treatment on the extraction of intracellular compounds from the fresh water microalgae *A. protothecoides* was studied. The results clearly evidence that a considerable cell disintegration of this microalgae was achieved. In the range investigated the variation of electric field strength hardly changed cell disintegration, whereas the variation of the specific energy input had a large influence. For an algae suspension containing 100 g_{dw}/kg_{sus} algae, the energy demand for sufficient cell disintegration by PEF treatment was 1 MJ/kg_{dw} algae. Experiments with algae suspensions of different algae concentrations (36–167 g_{dw}/kg_{sus}) revealed that the suspension's biomass content did not have a negative influence on the efficiency of cell disintegration. Hence, it is possible to further reduce the energy demand of PEF treatment by increasing the biomass content of the suspensions.

Although the algae used in this study contained up to 20% of lipids, only soluble substances leaked out of the cells. PEF treatment did not cause spontaneous release of lipids but enhances solvent extraction of lipids. Hence, PEF treatment is proposed to be used for cell disintegration and selective two-step extraction in biorefinery concepts. In a first step, PEF treatment allows to separate water soluble intracellular substances. In a subsequent step, lipids can be extracted by solvents at high efficiency.

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References

[1] C. Posten, R. Rosello-Sastre, Microalgae reactors, Ullmann's Encyclopedia of Industrial Chemistry, Wiley-VCH Verlag GmbH & Co. KGaA, 2011, http://dx.doi.org/10.1007/s00232-013-9547-6.
[2] R.E. Teixeira, Energy-efficient extraction of fuel and chemical feedstocks from algae, Green Chemistry 14 (2) (2012) 419–427.
[3] U.W. Goodenough, J.E. Heuser, The chlamydomonas cell-wall and its constituent glycoproteins analyzed by the quick-freeze, deep-etch technique, The Journal of Cell Biology 101 (4) (1985) 1550–1568.
[4] H.C. Greenwell, et al., Placing microalgae on the biofuels priority list: a review of the technological challenges, Journal of the Royal Society, Interface 7 (46) (2010) 703–726.
[5] J.Y. Lee, et al., Comparison of several methods for effective lipid extraction from microalgae, Bioresource Technology 101 (Suppl. 1) (2010) S75–S77.
[6] P. Prabakaran, A.D. Ravindran, A comparative study on effective cell disruption methods for lipid extraction from microalgae, Letters in Applied Microbiology 53 (2) (2011) 150–154.
[7] A. Ranjan, C. Patil, V.S. Moholkar, Mechanistic assessment of microalgal lipid extraction, Industrial and Engineering Chemistry Research 49 (6) (2010) 2979–2985.
[8] M.M. Mendes-Pinto, et al., Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-availability, Journal of Applied Phycology 13 (1) (2001) 19–24.
[9] A. Seibert, Entwicklung eines Verfahrens zur Gewinnung von EPA-Ethylestern aus Phaeodactylum tricornutum mit überkritischen Fluiden, Stuttgart, Fraunhofer Verlag, 2012.
[10] R. Halim, et al., Microalgal cell disruption for biofuel development, Applied Energy 91 (1) (2012) 116–121.
[11] W. Frey, C. Gusbeth, T. Schwartz, Inactivation of *Pseudomonas putida* by pulsed electric field treatment: a study on the correlation of treatment parameters and inactivation efficiency in the short-pulse range, Journal of Membrane Biology (2013), http://dx.doi.org/10.1007/s00232-013-9547-6.
[12] M. Sack, et al., Research on industrial-scale electroporation devices fostering the extraction of substances from biological tissue, Food Engineering Reviews 2 (2) (2010) 147–156.
[13] V. Ganeva, B. Galutzov, J. Teissié, High yield electroextraction of proteins from yeast by a flow process, Analytical Biochemistry 315 (1) (2003) 77–84.
[14] G. Saulis, Electroporation of cell membranes: the fundamental effects of pulsed electric fields in food processing, Food Engineering Reviews 2 (2) (2010) 52–73.
[15] J. Ben Ammar, et al., Impact of a pulsed electric field on damage of plant tissues: effects of cell size and tissue electrical conductivity, Journal of Food Science 76 (1) (2011) E90–E97.
[16] M. Guderjan, et al., Impact of pulsed electric field treatment on the recovery and quality of plant oils, Journal of Food Engineering 67 (3) (2005) 281–287.

[17] P. Mercer, R.E. Armenta, Developments in oil extraction from microalgae, European Journal of Lipid Science and Technology 113 (5) (2011) 539–547.
[18] D.S. Gorman, R.P. Levine, Cytochrome F and plastocyanin — their sequence in photosynthetic electron transport chain of *Chlamydomonas reinhardi*, Proceedings of the National Academy of Sciences of the United States of America 54 (6) (1965) 1665.
[19] C.J. Eing, et al., Effects of nanosecond pulsed electric field exposure on *Arabidopsis thaliana*, IEEE Transactions on Dielectrics and Electrical Insulation 16 (5) (2009) 1322–1328.
[20] S.A. Huber, et al., Characterisation of aquatic humic and non-humic matter with size-exclusion chromatography–organic carbon detection–organic nitrogen detection (LC–OCD–OND), Water Research 45 (2) (2011) 879–885.
[21] W.E. Trevelyan, R.S. Forrest, J.S. Harrison, Determination of yeast carbohydrates with the anthrone reagent, Nature 170 (4328) (1952) 626–627.
[22] W. Chen, et al., A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae, Journal of Microbiological Methods 77 (1) (2009) 41–47.
[23] G.H. Huang, G. Chen, F. Chen, Rapid screening method for lipid production in alga based on Nile red fluorescence, Biomass and Bioenergy 33 (10) (2009) 1386–1392.
[24] C.J. Eing, et al., Pulsed electric field treatment of microalgae — benefits for microalgae biomass processing, IEEE Transactions on Plasma Science (2013), (accepted for publication).
[25] K.V. Loginova, et al., Acceleration of soluble matter extraction from chicory with pulsed electric fields, Journal of Food Engineering 96 (3) (2010) 374–379.
[26] M. Bazhal, E. Vorobiev, Electrical treatment of apple cossettes for intensifying juice pressing, Journal of the Science of Food and Agriculture 80 (11) (2000) 1668–1674.
[27] H. El Zakhem, et al., Behavior of yeast cells in aqueous suspension affected by pulsed electric field, Journal of Colloid and Interface Science 300 (2) (2006) 553–563.
[28] G. Cravotto, et al., Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves, Ultrasonics Sonochemistry 15 (5) (2008) 898–902.
[29] J.C. Weaver, Y.A. Chizmadzhev, Theory of electroporation: a review, Bioelectrochemistry and Bioenergetics 41 (2) (1996) 135–160.
[30] Q.H. Zhang, et al., Inactivation of microorganisms in a semisolid model food using high-voltage pulsed electric-fields, LWT - Food Science and Technology 27 (6) (1994) 538–543.
[31] G.A. Evrendilek, et al., Processing of fruits and fruit juices by novel electrotechnologies, Food Engineering Reviews 4 (1) (2012) 68–87.
[32] R.E. Bruhn, et al., Heat conduction in microbes exposed to pulsed electric fields, IEEE Transactions on Dielectrics and Electrical Insulation 5 (6) (1998) 878–885.
[33] Evedos, Totally Dewatering Algae, http://www.evodos.eu/market-specific-solutions/totally-dewatering-algae.html, (cited 2012 12.12.2012).
[34] C. Griehl, S. Beiler, C. Posten, Konzentrierte grüne Energie, in: T. Bührke, R. Wengenmayer (Eds.), Erneuerbare Energie, Wiley-VCH Verlag GmbH & Co. KGaA, 2012, pp. 79–82.