Extraction of lipids from wet microalgae *Auxenochlorella protothecoides* using pulsed electric field treatment and ethanol-hexane blends

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**Abstract**

Pulsed Electric Field (PEF) treatment was used as pre-treatment on the microalgae strain *Auxenochlorella protothecoides* (A.p.) prior to organic solvent extraction of lipids. Experiments were performed on fresh biomass from mixotrophic or autotrophic culture which both had an evaluated lipid content of 30–35% of cell dry weight. Lipid yield was determined gravimetrically and compared to the reference lipid content assessed by bead-milling and subsequent Soxhlet extraction. The biomass was concentrated at 10% w/w solids prior to PEF-treatment and further dewatered afterwards to approximately 25% w/w before extraction. PEF-treatment with an energy input of 1.5 MJ per kilogram of dry matter induced electropermeabilisation of the microalgae cells detected by the increase of the conductivity of the microalgae supernatant. This greatly increased the lipid yield upon subsequent monophasic solvent extraction. A mixture of Water/Ethanol/Hexane 1:18:7.3 vol/vol/vol enabled to recover 92%, and 72%, of the evaluated lipid content of mixotrophically, and autotrophically respectively, grown A.p., after 2 h of extraction. Recovery increased to 97%, and 90% respectively, after 20 h of extraction. The same extraction system on untreated biomass yielded maximum 10% of lipid content. The highest yields were obtained with 80 mL of solvent for 1 g dry biomass but solvent volume could be reduced by a factor two in case of mixotrophically grown microalgae. However, the solvent:biomass ratio still remains high, and includes a water-miscible solvent, ethanol. Total lipid extraction was confirmed by nile red staining of residual biomass combined with fluorescence microscopy imaging and flow cytometry. Gas chromatography analyses of extracted lipids after transesterification revealed that PEF-treatment did not alter their fatty acid composition. Overall PEF-treatment shows promising features for upscaling especially in a biorefinery concept since it avoids potentially harmful temperature increase and small debris problematic for further processing.

**1. Introduction**

Microalgae are among the highest organisms with the highest yield per cultivated unit area \cite{1} and the highest proportion of intracellular valuable components. In the past decades, they gained a lot of attention because they are considered to be a promising, renewable feedstock for food, feed, fine chemicals and biofuel production. However, although a lot of research efforts have been expended, up to now microalgae have been mainly marketed for the production of high-value low-volume products and not for commodities like feed additives or biofuels. One of the limiting factors is that microalgae are small, i.e. in the micrometer range and have very robust cell walls preventing application of simple extraction techniques such as oil presses to recover intracellular lipids. In order to make microalgal lipids competitive for commodity markets, there is a compulsory need for an efficient extraction process with low-energy requirements \cite{2}. The main consensus that has been reached is that biomass processing should not include a drying step which represents prohibitive costs in particular for energetic utilization \cite{3,4}. Research is however still ongoing to find the most efficient and appropriate cell disintegration and extraction method in order to increase competitiveness of microalgae for the production of high-volume, low-cost substances like lipids for commodities.

Various methods have been proposed as pre-treatment for lipid extraction that in principal could work on wet-biomass. The most
studied ones include high-pressure-homogenization [5,6], bead-milling [7,8] and ultrasound but other less conventional techniques such as pulsed-electric fields, microwaves or osmotic shocks are also being tested [9–11]. The success of a method should be evaluated based on the success of further lipid extraction after pre-treatment, on the overall costs i.e. investment and operating costs, and on the ease of implementation at large scale [11].

Pulsed electric field (PEF) treatment consists of delivering pulsed electric field on biological cells or tissues in order to modify their membrane's properties. During the application of the electric field, membranes get charged, which results in an increase of the transmembrane voltage which in turns modifies the properties of cells external membrane [12–14]. The most commonly observed consequences are the increase of membrane's conductivity and of its permeability to normally non-permeant water soluble molecules. PEF treatment is therefore often referred to as electroporation or electropermeabilisation. These effects have been reported and documented not only on biological cells and tissues but also on artificial membranes which suggests that one of the main targets of PEF treatment is the lipid double layer which is the main constituent of the cells' plasma membrane. However, no precise description of the molecular changes at the level of the membrane is available. Some theories predict the formation of pores in the nanometer range [15–19] and recently, molecular dynamic simulations have confirmed the possibility for the existence of such pores [20–25]. However, the experimentally observed long-lasting permeabilisation state contradicts a very rapid pore closure predicted by theories and by molecular dynamic simulations. Therefore most scientists in this field agree that simple pores are not sufficient to describe electropermeabilisation [26,27]. Despite the lack of precise understanding of the permeabilisation phenomenon, PEF treatment was already successfully implemented in various applied fields in both the medical area [28] and in the food industry [29–32]. It can be used to deliver non-permeant cytotoxic molecules for anti-cancerous treatment [33–36] or nucleic acids for simple transfection [37–39], for gene therapy and gene vaccines [39,40]. PEF treatment is also successful for extraction of compounds from various multicellular organisms such as grapes, sugar beets or other higher plants [29,32,41].

PEF treatment appears to be a powerful potential tool for exploiting the intracellular content of microalgae. It shares numerous advantages with HPH which is one of the most promising available technologies, such as the possibility to operate on wet biomass, the feasibility of scale-up as well as the easy operation in continuous mode, and it is therefore conceivable to bring PEF-treatment to industrial scale. Previous studies have already shown that it is possible to extract small-sized water-soluble components with an energy input not exceeding 1.5 MJ per kilogram of dry biomass [42]. Moreover, although PEF alone does not lead to the spontaneous release of the coveted lipids, preliminary studies in our laboratory have shown that pretreatment of wet microalgae with PEF could increase the efficiency of a solvent extraction [43]. This paper targets on precise quantification of the impact of PEF pre-treatment on subsequent solvent extraction of intracellular lipids and on advantageous processing conditions for achieving highest lipid recovery rates. All experiments were performed on Auxenochlorella protothecoides (A.p.) which is known for accumulating large lipid quantities [44]. Despite the fact that only autotrophic growth can be reasonably foreseen for large scale biofuel production, all studies were performed both on mixotrophic and autotrophic culture in order to investigate how the mode of cultivation might affect the efficiency of extraction.

2. Materials and methods

2.1. Microalgae strain

All experiments were performed with Auxenochlorella protothecoides (A.p.), strain number 211¬7a obtained from SAG, Culture Collection of algae, Göttingen, Germany.

2.2. Mixotrophic cultivation

Axenic mixotrophic cultivation was performed in 1 L conical NALGENE polycarbonate cultivation flasks (VWR International, Bruchsal, Germany) in a modified Wu medium [170 mM glucose, 5 mM KH₂PO₄, 1.7 mM K₂HPO₄, 1.2 mM MgSO₄, 10 μM FeSO₄, 1 mM glycin and 4 g/L of yeast extract]. The pH was adjusted at 6.8 ± 0.1 with KOH 1 M solution and medium was autoclaved. Erlenmeyer flasks, each containing 400 mL of medium, were inoculated at an optical density (OD) of 0.1 (750 nm) and cultivated at 25 °C on an ORBIT 5000 analogue shaker (VWR International, Bruchsal, Germany). Agitation was fixed at 100 rpm. Illumination was provided by LED (LUMINUX COMBI LED¬N, 980 lm, 3000 K, 10 W, OSRAM) and fixed at 60 μmol m⁻² s⁻¹. Growth was monitored with OD measurement. The cells were harvested after 10 days for further PEF experiment. The typical cell dry weight (CDW) always assessed one day before harvesting was 10 g/kgₕₚₑₜₚₑₜ.

2.3. Autotrophic cultivation

Autotrophic axenic cultivation was started using A.p. starter-cultures cultivated mixotrophically in modified Wu medium for 5 days as previously described. The starter culture was subsequently used as inoculant of a photobioreactor (PBR) of 25 L filled with Tris-acetate–phosphate (TAP) medium (recipe adapted from [45,46]; detailed composition given in [47]) supplemented with 40 μg/L Thiamine. Preparation of the PBR consisted in autoclaving the reactor filled with water and sterile addition of the medium stock solution and of the inoculum. The amount of inoculum was calculated in order to start the cultivation with an OD of 0.1 (750 nm). The PBR was illuminated by LED (WU-M-500-840, 4000 K, Panasonic) with a light intensity at the surface of the reactor of 200 μmol m⁻² s⁻¹ for the first 24 h and 600 μmol m⁻² s⁻¹ afterwards. The temperature was regulated at 25 °C and the CO₂ flow (3% volume in sterile air 60 L h⁻¹) was kept constant. The cultivation was daily monitored with OD measurement and microalgae were harvested after approximately 16 days. Typical CDW one day before harvesting was between 4.5 and 5 g/kgₕₚₑₜₚₑₜ.

2.4. Optical density (OD) measurement

OD was measured at 750 nm with a spectrophotometer (Genesys 10S UV–Vis, Thermo Scientific) using disposable cuvette (ref 634-0676, VWR).

2.5. Cell dry weight (CDW)

About 5 mL of microalgae suspension, respectively supernatant, were deposited in pre-weighted aluminum caps. The exact masses of microalgae suspension and supernatant medium were measured with a precision balance. Both cups were then left to dry overnight at 90 °C in a drying oven (universalshrank model U, Memmert, Germany) and precision balance. Both cups were then left to dry overnight at 90 °C in a drying oven (universalshrank model U, Memmert, Germany) and measured again. CDW [g/kgₕₚₑₜₚₑₜ] was obtained by subtracting the dry weight of the medium from the dry weight of the microalgae suspension.

2.6. Concentration of microalgae suspension

For both type of cultivation, the harvested microalgae suspension was concentrated by centrifugation at 3000g using a Sigma 8 k centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), with a swinging-bucket rotor. The microalgae pellet was resuspended in the appropriate amount of cultivation medium in order to obtain the desired CDW of 100 g/kgₕₚₑₜₚₑₜ. The exact final CDW was always measured as described above. The average delay, induced by the step of
concentration of the microalgae, from the beginning of the harvest to the beginning of the PEF experiment, was typically 2 h.

2.7. Soxhlet extraction

Total lipid extraction was performed with a commercial Soxhlet apparatus (behrettest® Kompakt-Apparatur KEX 30 from Behr Labor-Technik). Approximately 0.5 g of dried bead-milled (Mixer mill, MM400, Retsh, Haan, Germany) microalgae biomass was precisely weighted and placed inside a permeable paper thimble (“Extraction Thimbles Cellulose”, 90022080, Albet LabScience, Dassel, Germany) and deposited inside the Soxhlet chamber. Approximately 50 mL of hexane was used with a heating temperature of 170–200 °C. The extraction was run for at least 3 h which corresponded to at least 20 extraction cycles. At the end of the extraction, the solvent was siphoned out of the apparatus and the boiling traction cycles. At the end of the extraction, the solvent was siphoned along with extracted lipids was removed, let to cool down under Nitrogen atmosphere, and the lipid yield was determined gravimetrically.

2.8. Nile red (NR) staining

Nile red (5H-Benzol[α]phenoxazin-5-one, 9-(diethylamino)-) was purchased from Invitrogen. Optical density of the fresh probes was adjusted to 0.05 (750 nm) by dilution with distilled water. Probes were then directly stained. For staining, 1 mL of each probe was centrifuged at 6000 g for 5 min and 200 μL of the supernatant was removed and replaced with 200 μL of NR solution at 30 μg mL⁻¹ in dimethylsulfoxide (DMSO) or 200 μL of pure DMSO for blank staining. The samples were vortexed vigorously and left for 10 min in the dark at 40 °C in a thermostatic cabinet. Afterwards, the probes were centrifuged at 6000 g for 5 min at 4 ºC, supernatant was removed and cells were resuspended in distilled water. Then samples were kept on ice before microscopy imaging or flow cytometer analysis.

2.9. Microscopy imaging

Microscope imaging was performed with a straight microscope (Axioplan 2, Zeiss, Jena, Germany) and a magnifying objective (× 63 LD Plan-NeoFluar, Zeiss, Jena, Germany). Images were acquired with an Axiocam HRc (Axioplan 2, Zeiss, Jena, Germany) and a magnifying objective (×63 fluar set 09 from Zeiss (Excitation BP 450-490, Beam Splitter FT 510, Emission LP 515).

2.10. Flow cytometer analysis

Flow cytometer measurements were conducted on a Millipore Guava with a 488 nm laser as excitation source. Emission fluorescence signal was collected with the green filter of the device (525/30). For each samples, 5000 cells were analyzed.

2.11. Pulsed electric field (PEF) treatment

The concentrated microalgae suspension was treated in a continuous flow chamber consisting of two parallel circular stainless steel electrodes separated by a polycarbonate housing. Distance between the electrode was 4 mm. The setup insured a uniform electric field distribution in the whole volume of the treatment chamber (Vchamber = 48 × 11 × 4 mm³) which had no sharp angles. Photos of the treatment chamber and of the electrodes can be found in [42]. PEF treatment was performed with a custom-made transmission-line generator. Pulse duration, was fixed at Δt = 1 μs, electric field intensity at 4 MV/m and repetition rate at 3 Hz. During PEF treatment, the voltage across the electrodes was systematically controlled and acquired with a 40 kV high voltage probe (P6015, Tektronix, Beaverton, OR, USA) and an oscilloscope (TD640, Tektronix, Beaverton, OR, USA). Current measurements were performed on a Millipore Guava with a 488 nm laser as excitation source. Emission fluorescence signal was collected with the green filter set 09 from Zeiss (Excitation BP 450-490, Beam Splitter FT 510, Emission LP 515).

Fig. 1. Typical voltage pulse applied on the electrode of the treatment chamber (left) and current flowing through the electrodes (right) during PEF treatment of the microalgae suspension.

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E_{fl,avg} = \left( \int_0^{\Delta t} u(t) i(t) \, dt \right) \frac{I_{rep}}{Q} \tag{1}
\]

Temperature measurements were performed just at the output of the treatment chamber using a digital thermometer (Qtemp 500, VWR International, Germany) in order to evaluate the temperature increase due to PEF treatment.

2.12. Conductivity measurement

Conductivity measurements were performed with a conductivity meter (Endress + Hauser, CLM 381). No temperature compensation was used and conductivity measurements are therefore reported as
measured at room temperature i.e. between 20 °C and 24 °C.

2.13. Lipid extraction protocol

The extraction protocol was inspired from Molina et al. [49] and adapted to wet microalgae. In the standard extraction protocol, 5 mL of microalgae suspension which corresponds to approximately 0.5 g of dry weight microalgae were placed in a 50 mL falcon. The exact amount \( W_{\text{algae susp}} [g] \) was determined on a precision balance. Samples were centrifuged at 10000 g for 10 min. The separated supernatant (approximately 3 mL) was collected, its conductivity measured and then discarded. The microalgae pellet was then resuspended in 27 mL water, ethanol and 11 mL n-hexane. Based on estimation of the remaining water left in the pellet, the extraction system therefore consisted in Water Ethanol Hexane Total

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\begin{array}{c|ccc|c}
\text{Composition of the different extraction systems. Values are given in mL normalized to 1 g of dry biomass. Percentage values in brackets are the mass fraction of the respective solvent.} \\
\hline \\
\text{Extraction system} & \text{Water} & \text{Ethanol} & \text{Hexane} & \text{Total} \\
\hline \\
\text{E} & 3 (5.0\%) & 54 (71.0\%) & 22 (24.0\%) & 79.0 \\
\text{E}' & 3 (9.5\%) & 27 (67.6\%) & 11 (22.9\%) & 41.0 \\
\text{E}^* & 3 (13.6\%) & 18 (64.5\%) & 7.3 (21.8\%) & 28.3 \\
\text{E}'' & 3 (13.6\%) & 18 (64.5\%) & 7.3 (21.8\%) & 28.3 \\
\hline
\end{array}
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For the chosen duration of the extraction, the samples were centrifuged again at 10000 g for 5 min. The upper phase was collected into another tube where 8 mL of distilled water were added as a washing step. The samples were vortexed very well for 1 min and centrifuged at 6000 g for 5 min. The upper phase containing the hexane and the product of the transesterification was removed into a glass vial. The vials were flushed with \( N_2 \) and stored at \(-20 \, ^\circ C\) for gas chromatography analysis.

2.15. Gas chromatography

Gas chromatography (GC) with a flame ionization detector (FID) was used for the analyses. The device was the model 7890A with autosampler 7693 both from Agilent. The column was Otima WAX 30 m, 0.25 mm, 0.25 \( \mu \)m from Macherey & Nagel and helium served as carrier gas. The evaluation of the results was done with Chemstation Software from Agilent over calibration with FAME-Mix standard (FAME Mix C4-C24, 18,919-1AMP Supelco).

3. Results

3.1. Reference extraction for determination of lipid content

In order to determine the lipid content of the microalgae, freeze-dried microalgae were bead-milled and then extracted with a Soxhlet device, using n-Hexane as extraction solvent. The top picture on Fig. 2 displays freeze-dried autotrophic A.p. and suggests that freeze-drying did not affect the general shape and structure of the microalgae although it had a major impact on the size of the cells which appeared much smaller than when they were observed fresh in their cultivation medium (see Fig. 4 top image for comparison). On the bottom picture which was acquired after bead-milling, only cell debris can be observed and no intact cells. Similar results were observed for mixotrophically grown A.p.

Extraction yields after Soxhlet extraction are displayed in Fig. 3. For both, mixotrophically and autotrophically grown microalgae, yields are very low, around 2–3%, when no bead-milling is performed. With bead-milling, yields are considerably increased, reaching values of about 34–35% for both types of cultivation. Alternative methods for lipid content determination were also screened for applicability in this study. Bligh and Dyer extraction performed on freeze-dried bead-milled material. Slightly higher values could be obtained when applying the MTBE-method after [51]. In this case, lipid recovery from freeze-dried material was 28% on average, but individual values exhibited wide scattering. Thus, in the following, the lipid yield obtained with Soxhlet, performed on freeze-dried and bead-milled microalgae, is considered as the reference lipid content. This method is systematically used in all further experiments.

3.2. Effect of PEF on the microalgae

All PEF-treatment experiments were performed on freshly harvested and concentrated biomass using a continuous-flow treatment chamber. The parameters for the PEF treatment were not optimized in this study but were chosen based on previous work of our group on the same microalgae [42]. In this study PEF treatment always consists of pulses of 1 \( \mu \)s duration, with a field magnitude of 4 MV/m applied with a repetition rate of 3 Hz on the microalgae suspension flowing at 0.1 mL/s (details can be found in the Materials and methods section). Control
sample were also pumped through the treatment chamber but not submitted to the pulsed electric field. After treatment, microalgae were observed under the microscope. The observation did not reveal any major change of morphology of the microalgae. The general shape and size were maintained even though slight shrinkage of the cells could be observed after PEF-treatment. PEF-treatment in any case did not lead to disruptions of cells and no cells debris were to be observed (Fig. 4).

In order to validate the efficiency of PEF treatment, the microalgae suspension was centrifuged approximately 20 min after the treatment and the conductivity of the supernatant was measured. Results for control and treated samples, are displayed in Fig. 5 for both, mixotrophically and autotrophically grown microalgae. The supernatant of control samples had a conductivity at room temperature of $1.67 \pm 0.08$ mS/cm, and $1.32 \pm 0.08$ mS/cm, for mixotrophically and autotrophically grown A.p. respectively. The supernatant of treated samples showed a decrease in conductivity, which was more pronounced for mixotrophically grown A.p. (Fig. 5).
and autotrophically grown A.p., respectively. The conductivity of the PEF treated samples was approximately twice higher (Fig. 5) due to the release of the microalga's intracellular ionic content [42] and it could therefore be concluded that the PEF-treatment induced electro-permeabilisation.

PEF-treatment also induced a temperature elevation due to Joule effect [52]. Temperature measured just at the output of the treatment chamber was between 45 °C and 47 °C which corresponds to a temperature increase of about 26 °C.

3.3. Lipid extraction

After PEF treatment, the microalgae suspension was centrifuged, supernatant was discarded and the microalgae pellet was resuspended in a mixture of ethanol and n-hexane for lipid extraction. The respective amounts of solvents were adapted from Grima et al. who had proposed a similar solvent composition also including water but applied for extraction of lipids from dry biomass [49]. The exact composition of extraction system E is given in Table 1. Note that no water has been added and that the water present in the extraction system is the remaining water left after centrifuging the microalgae and discarding the supernatant. After a given extraction time, the mixture was centrifuged to separate solvent from the residual biomass, solvent was collected and additional hexane and water were added in order to obtain a biphasic system S for separation of the lipids. The upper hexane phase was collected and evaporated and lipid yield was determined gravimetrically. The compositions of the extraction system E and the phase separation system S are displayed in the hexane/ethanol/water phase diagram in Fig. 6.

Lipid yields obtained after 1 h, 2 h or 20 h of extraction duration are displayed in Fig. 7. For both mixotrophically and autotrophically grown microalgae, extraction procedure was inefficient for untreated microalgae and lipid yields always ranged between 1 and 4%. PEF treatment had an outstanding effect on the extraction. In the case of mixotrophically grown microalgae, the lipid yield after only 1 h of extraction was on average 31% of CDW and therefore very close to the reference lipid content of 37%. The yield was further increased after 20 h of extraction and reached 36% of CDW. A similar pattern can be observed for autotrophically grown microalgae. Here, the yields after 1 h and 2 h of extraction are slightly lower, 23% and 21%, i.e. about two thirds of the reference lipid content. Increasing the extraction duration to 20 h, resulted in lipid yield values of 26% of CDW i.e. 90% of the reference lipid content (Fig. 7).

Fig. 6. Hexane/Ethanol/Water phase diagram (% w/w/w). Data for phase separation limit were taken from [53]. Point E indicates the solvent fractions during the extraction. Point S indicates solvent fractions during the phase separation step.

Fig. 7. Influence of PEF treatment on lipid yields obtained with solvent extraction for different extraction durations. Experiments were performed with extraction system E and separation system S. Bars show the average ± standard deviation of at least three independent experiments. Markers show individual data points. The straight lines indicate the average ± std of Soxhlet extractions. Results were obtained from mixotrophically grown microalgae, upper diagram, and from autotrophically grown microalgae, lower diagram.

3.4. Reduction of extraction solvent

Attempts were made to reduce the amount of solvent required for the lipid extraction. Amounts of ethanol and n-hexane were therefore reduced by a factor 2 (extraction system E′) or 3 (extraction system E″). The water amount could however not be reduced since water was present in the wet microalgae and could not be removed with a simple cost-efficient procedure. This implies that not only the biomass to solvent ratio is modified but also the overall composition of the extraction mixture. Table 1 summarizes the different amounts of solvents used in different extraction conditions normalized to 1 g of microalgae CDW.

Extraction yields after 2 h of extraction are presented in Fig. 8. The results show that for both types of cultivation, the reduction of the solvent volume lowered the overall extraction yield when extraction was performed for only 2 h. With extraction system E′, which corresponds to a reduction of the total solvent volume by a factor two, the recovered lipids were only about 81% of what could be recovered with extraction system E in case of mixotrophic cultivation and 57% in case of autotrophic cultivation.

The extraction yield for the different extraction systems after 20 h of extraction are presented in Fig. 9. For the mixotrophically grown algae, (Fig. 9, top), it appears that extraction system E′, i.e. reduction of solvent volume by a factor 2, enables to recover the same lipid amount as system E. However, a further reduction of the solvent volume (extraction system E″) dramatically decreased the extraction yield. In case of
autotrophically grown A.p., already the reduction of solvent by a factor two resulted in a decrease of the extraction yield (Fig. 9, bottom).

3.5. Control of lipid content by nile red staining

NR staining of the microalgae was performed in order to visually confirm the extent of lipid extraction. Staining of mixotrophically grown microalgae was performed on untreated samples (Absolute Control), on treated samples (Sham or PEF) before and after lipid extraction. The acquired images are displayed in Fig. 10. Before extraction, the majority of the control microalgae display no fluorescence signal just like the absolute control, while PEF treated microalgae are all intensively stained. The difference of staining can be directly explained by the effect of PEF treatment which increases the permeability of the membrane to the normally impermeant NR molecules. After 20 h of extraction (extraction system E), the control microalgae display bright NR fluorescence suggesting that lipids were not successfully extracted but that the extraction process modified the membrane properties which became permeable to NR. The PEF-treated and extracted microalgae display a very weak fluorescence signal which might be due to traces of non-extracted lipids or to some non-specific binding of NR.

These qualitative observations were quantified using flow-cytometry. Typical histograms of the green fluorescence signal (FITC 525/30) are displayed in Fig. 11. Unstained control cells before extraction are displayed in the top histogram. Similar distributions could be observed with all unstained cells, with and without PEF treatment and before or after extraction (histograms not shown). For untreated cells, NR staining was efficient only on average in 23% of the cell population while the majority of the cells displayed the same signal as unstained cells, (Fig. 11, diagrams in the middle, sham). On the contrary, after PEF treatment, more than 90% of the cells are highly stained with an average fluorescence increase of more than one order of magnitude, confirming that PEF-treatment facilitated the penetration of NR into the cells which subsequently stained lipid bodies. After 20 h of extraction, on average 84% of control cells display an intense NR signal while more than 97% of the PEF-treated-extracted cells have the same fluorescence intensity as unstained cells. Note that for those cells, a residual fluorescence signal could be detected with the flow cytometer when using a detection filter at higher wavelength (583/26) confirming the observation of the microscopy imaging and suggesting that the residual fluorescence signal results from unspecific binding of NR.[54]

3.6. Fatty acid determination

Fatty acid determination was performed with gas chromatography FID. Methylation was done by transesterification of the extracted lipid using methanol and sulfuric acid as a catalyst. Fig. 12 displays the main identified FAME obtained from mixotrophically and autotrophically grown A.p. after extraction with Soxhlet or after PEF-treatment and solvent extraction. For both type of cultivation, the main FAME identified were the same although the proportion of the different FAME were slightly different. For the mixotrophic growth, C18:1 represented about three quarters of all identified FAME while for the autotrophic growth, there was a more equal distribution between C18:1 and C18:2
which represented together more than 80% of the total identified FAME. For both type of growth, very similar FAME profile can be observed in the lipids obtained with Soxhlet extraction and in the lipids obtained after PEF treatment combined with solvent extraction. These results therefore suggest that PEF treatment did not affect the fatty-acid composition.

4. Discussion

The results of this study show that PEF treatment can be used as an effective pre-treatment technique to improve the efficiency of solvent based extraction of lipids from the microalgae Auxenochlorella protothecoides. Soxhlet after bead-milling was used as a reference method to assess lipid content of A.p. Up to 97% of the lipid evaluated content could be recovered after PEF treatment and ethanol/hexane extraction for mixotrophically grown A.p and 90% for autotrophically grown A.p. Moreover, completeness of extraction was confirmed by NR staining and subsequent microscopy imaging and flow cytometer analysis. Furthermore, NR experiments impressively demonstrated the permeabilizing effect of PEF treatment on A.p. cells.

PEF-assisted lipid extraction was performed on wet biomass and did not require any energy-expensive drying step. A concentrating step was however needed it order to process the biomass at 100 g/kg but partial dewatering is common to any microalgae treatment process and is not specific to PEF-treatment. The energy expense of PEF is directly linked to the volume of suspension which is treated and is independent on the biomass concentration. This was verified in our laboratory for concentration up to 150 g/kg [42]. Concentrating the biomass before the treatment therefore enables to considerably reduce the energy input per kg of dry biomass like it is also the case with HPH [6]. The treatment concentration chosen in this study was 100 g/kg and therefore, further dewatering to approximately 250 g/kg was required before solvent extraction. An alternative to this procedure would be to treat directly the biomass at 250 g/kg. This could in principle further reduce the PEF...
According to Yap and colleagues, HPH can achieve energy consumption values \[8\]. However, it is still higher than the lowest reported values for HPH. By measuring the conductivity increase of the extracellular medium and cell disruption, such short pulses moreover prevent or at least reduce side effects of PEF treatment such as microalgae precipitation on electrodes \[42\]. Depending on the targeted application, both options should therefore be considered and evaluated in terms of total energy consumption.

Microscopy images have shown that PEF treatment preserved the cellular integrity which guarantees an easy further processing of the biomass after extraction, in particular for biorefinery processes where subsequent mechanical separation steps are required. Moreover, the process can easily be run in continuous mode and PEF treatment has already been proven to be successful on large scale in other applications of lipid extraction\[32\].

In this study, pulses parameters were fixed at 1 μs for the pulse duration, 4 MV/m for electric field intensity and 3 Hz for the repetition rate. These parameters translate into an energy input of 150 kJ/kg_DW for a flow rate of 0.1 mL/s, i.e. 1.5 MJ/kg_DW for a biomass concentration of 100 g/kg_DW. The release of intracellular ions was indirectly evaluated by measuring the conductivity increase of the extracellular medium and suggested that those pulse parameters provided efficient cell permeabilisation. Such short pulses moreover prevent or at least reduce side effects of PEF treatment such as microalgae precipitation on electrodes \[47\] and are therefore well suited for a large-scale process implementations. However, the degree of efficiency of permeabilisation was not studied in detail and other pulse parameters might enable comparable lipid extraction rates but with even lower energy input. Optimization of PEF parameters should therefore be investigated in order to really estimate the minimum required PEF treatment energy for lipid extraction.

Comparison of the required energy with other treatment method is not straightforward since, for any type of pre-treatment, the energy consumption depends largely on the criteria chosen to evaluate the success of the treatment, on the microalgae specie and to some extend at which point of the cultivation the microalgae were harvested. It can however be noted that required energy is lower than the lowest reported value for bead-milling i.e. 1.8 MJ/kg_DW in optimized conditions \[8\]. However, it is still higher than the lowest reported values for HPH. According to Yap and colleagues, HPH can achieve efficient cell disruption of some microalgae species such as Tetraselmis suecica with energy as low as 0.16 MJ/kg_DW. In case of strong microalgae species such as Nannochloropsis sp., energy requirement is however much higher and reaches 3.4 MJ/kg_DW although it can be reduced to 0.4 MJ/kg_DW in case the microalgae are previously weakened by a 15 h incubation at 37 °C \[6,55,56\]. In this study the energy consumed for PEF-pretreatment was 1.5 MJ/kg_DW although the PEF parameters were not optimized. PEF-treatment could therefore compete with HPH in case it is efficient on very resistant strains.

Unfortunately, based on the literature, it is not clear whether Auxenochlorella protothecoides possesses a very strong cell wall. Evaluation of the stiffness of this microalgae and tests of PEF-treatment on very resistant known species are therefore necessary to evaluate the full potential of this approach.

Some differences in extraction yield were observed between mixotrophically and autotrophically grown microalgae. In particular, the volume of solvent required to achieve total lipid extraction was different. In case of mixotrophically grown microalgae, after 20 h of extraction, extraction system E enabled to recover 98% of the total lipid content while only 40% could be recovered in case of autotrophically grown microalgae, although the reference lipid content was 30–35% of the dry weight for both types of cultivation. The lipid composition, especially the type of fatty-acid, which depends on the type of cultivation might partially explain the observed differences. Most probably, other constituent of the microalgae such as protein or starch might be very different depending on the cultivation mode and might also cause significant differences in extraction performances by interacting with lipids or with the solvent itself.

Analysis of the FAME with GC-FID, revealed the same qualitative composition of the fatty-acids extracted after PEF treatment and extraction according to our reference protocol with Soxhlet. Based on this analysis, PEF treatment does not affect the quality of the recovered lipid fraction. However, a thorough quantitative analysis of the extracted lipids should be performed in future in order to precisely determine its detailed composition. Concluded from the color of the extract, it already can be ascertained that it contains pigments such as chlorophylls. In particular future work should focus on lipid component selectivity of PEF-assisted lipid extraction and its influence on the quality of the recovered lipid fraction.

In the current state and based on up-scaling considerations starting from lab-scale experimental results, in case extraction system E is used, the proposed method requires at least 76 L of solvent per kg of microalgae dry weight just for the extraction step, i.e. without the lipid separation step. Assuming a lipid content of 40%, this translates into 190 L of solvent per kg of extracted lipids distributed into 135 L of ethanol and 55 L of hexane. While Hexane is established as well recyclable solvent in industrial processes and is already used for large scale extraction of lipids for food \[57\], ethanol might not be appropriate for energy-efficient large scale processing considering the high amount of energy required for recycling due to its high vaporization energy and the difficulty to separate it from water \[58\]. The next step of this work should therefore include a techno-economic analysis of the whole extraction process. Improvement of the extraction solvent and in particular removal of ethanol should be tested since extraction with hexane only was already shown to be feasible for lipid extraction from wet biomass of Nannochloropsis sp. \[56\]. Restricting the solvent to Hexane only might additionally enable selective extraction of non-polar lipids and therefore could foster fractionation of lipids which might facilitate development of biofuel applications \[59\].

Finally, it is crucial to study and understand the mechanisms by which PEF treatment enhances lipid extraction. Most of the pre-treatment methods that have been tested in combination with solvent extraction, lead to the destruction of cells integrity and very often the performance of a pre-treatment is assessed by cell counting to quantify the number of intact cells that overcome the pre-treatment \[11\]. Indeed, a pre-treatment method that can achieve cell disintegration guarantees opening of cell wall and therefore full accessibility of extraction solvents to the lipids. However, PEF treatment was shown to be
extremely efficient without impacting the over cell structure and with probably negligible effect on cell-wall at all [60]. Further experiments and in particular electron microscopy imaging should be performed in order to better evaluate if cell wall is affected by PEF-treatment but in any case, it is clear that PEF-treatment did not efficiently disrupt the cell wall since no cell debris were observed after treatment. This raises the question of the mechanisms which make PEF-treatment efficient as a pre-treatment method. Spontaneous release of lipid droplets has never been observed after solely PEF-treatment and would be very surprising considering the size of the lipid droplets. One possible mechanism would be that permeabilisation induced by PEF-treatment facilitates penetration of solvents. This is relatively unlikely in the case of ethanol since it is known to permeate membranes [61,62]. However, an impact on the membrane that might facilitate uptake of n-Hexane cannot be excluded. Additionally, PEF treatment might facilitate solvent extraction simply by inducing release of small intracellular compounds which stabilize lipid droplets or the electric field might have a direct destabilising effect on the lipid droplets and especially on the phospholipid surfactants at the surface of the droplets. Accumulating profound understanding about these mechanisms will not only help to optimize and in particular electron microscopy imaging should be performed in extremely

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