DOWNSTREAM PROCESSING OF MICROALGAE UTILIZING PULSED ELECTRIC FIELDS (PEF)

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ΠΕΡΙΛΗΨΗ

Fast growing, diverse and with flexible outputs, microalgae have attracted considerable research interest. Recognized as prime candidates for 3rd generation biofuels, mainly due to their ability to be cultivated on non-arable land and their high lipid content (up to 50% of dry weight for some strains), they have also been considered for a number of bio-technological applications such as aqua-feed industry and high value dietary supplements.

The main challenges for lipid extraction from microalgae are the selection of the appropriate set of chemical solvents and the strong, rigid cell walls that surrounded them. Microalgal lipids can be either polar or neutral and lipids generally dissolve in a solvent of similar polarity, meaning a mixture of co-solvents for total lipid extraction is usually required. As for the presence of cell walls, a pre-treatment process is necessary. Said processes, either physical, chemical or biological in nature, should be mild, economic and harmless to the targeted product. Pulsed Electric Fields (PEF) have been successfully demonstrated as one such microalgae pre-treatment method. During PEF, the cells are subjected to an external intense electric field for very short periods of time, leading to an increase of the transmembrane potential of the cell membrane. As a result, the permeability of the cell membrane is increased and the exchange between intracellular components and an external solvent is greatly enhanced.

In this study the complete permeabilization of the lipid-rich microalgae strain Auxenochlorella Protothecoides (AP) after PEF-treatment along the spontaneous release of various intracellular carbohydrates and other water-soluble components is demonstrated. The microalgae biomass is then further mixed with Ethanol:Hexane (1:0.41 vol/vol), two common industrial solvents, and almost total lipid extraction is achieved (90% of total lipids as determined with other reference methods). The above results can be further enhanced and the PEF treatment energy greatly reduced (from 150kJ/kg_{susp} down to 25kJ/kg_{susp}) with the introduction of an incubation step of the pulsed biomass, ranging between 2-24hrs at inert conditions, showing that even in low energies PEF initiates a number of complex biological processes that influence the extraction.

Gas chromatography analysis of Fatty Acid Methyl Ester (FAMEs) produced from transesterified lipids indicate that AP has a fatty acid profile mainly composed of C16-C18 fatty acids, including large amounts of mono-unsaturared C18:1 , making it thus a good candidate for biodiesel production. PEF has no effect either on the FAME composition compared to reference methods, or on the overall mechanical structure of the cell as determined by microscopy. Future works are focused on further illuminating the PEF mechanism and the more selective extraction of different microalgal components such as carotenoids.

ΕΙΣΑΓΩΓΗ

Microalgae, eukaryotic photosynthetic microorganisms, are able to grow in a variety of, sometimes extreme, environmental conditions. This has led to the development of wildly different strains with very different properties, biochemical composition, and therefore highly diverse product outputs. Depending on the cultivation conditions and stage, microalgae can accumulate significant amounts of lipids, proteins and other valuable compounds such as carotenoids[1]. This output diversity, coupled with fast growing rates and high productivities, has attracted considerable research interest on microalgae cultivation and extraction of their intracellular components (commonly referred as

'upstream' and 'downstream' processes, respectively).

Lipids produced from microalgae can be broadly classified into two categories. One is polar lipids (phospholipids and glycolipids) which serve structural functions and are encountered in the cell membrane. The other is neutral lipids, used for energy storage, located in the cytoplasm[2]. Neutral lipids consist mainly of triacylglycerols (TAGs) and are considered an excellent base material for biodiesel production. Microalgae have been proven to accumulate significant amounts of TAGs (up to 50-60% of dry weight for certain strains) when they are subjected into stress conditions such as nitrogen starvation[3]. It has been estimated that the oil production per hectare from microalgae is much higher compared to other conventional land plants[4]. This, combined with the fact that no arable land is required for cultivation, make microalgae an excellent candidate for biodiesel production.

However, a number of challenges that must be resolved remain for microalgae downstream processing. A significant one is the fact that as a rule, microalgae cells are quite resistant to extraction[5]. The cell wall, a complex structure enclosing the intracellular components is cited as the main barrier for successful extraction[6]. To overcome this, a pre-treatment method is usually applied prior to extraction to disrupt the cells. Said techniques, should be scalable, inexpensive, not harmful to the desired product, and effective on wet basis. The latter is of the utmost importance if biofuels is the final usage, in order to avoid any energy intensive drying steps.

Pulsed Electric Fields (PEF) is one appropriate such pre-treatment method. The basis of this technique is the phenomenon known as electroporation or electropermeabilization during which the cell membrane is made permeable with the application of short electric field pulses[7]. Electroporation has been intensively studied and applied in other scientific fields; however, the exact mechanisms remain yet not fully understood[7]. The most accepted theory is that an increase of the charge of the cell membrane results in a high transmembrane voltage and modification of its properties with the eventual formation of pores[8]. While an increase of membrane's conductivity and permeability can be detected and measured, experimental confirmation of such pores is still missing and debated[8]. What is also unknown is in what ways, if any, PEF affects the microalgae cell wall. In literature, little attention is paid on this part with often no distinction being made between the two cell components[9]. Despite the above ambiguities though, PEF has already seen a number of research and industrial applications.

Pulsation of microalgal biomass is first accompanied by a spontaneous release of various intracellular components such as proteins and other water solubles in the surrounding aquatic medium[10]. For the extraction of lipids, though, the introduction of an organic solvent is necessary. A co-solvent system is often required given the aforementioned diverse polarity of the lipids produced from the microalgae. The most common lipid extraction method used historically, the chloroform:methanol process (Folch or Bligh and Dyer method) is not appropriate at industrial scale, given the toxicity of the chemicals involved[11]. Multiple other solvents and combinations thereof have been examined as potential replacements, with the ternary system ethanol/hexane/water a popular one. A description of the extraction mechanism can be found in Cooney et al[11]. In short, a monophasic system is formed with the blending of the solvents and wet biomass, during which the polar solvent disrupt the hydrogen bonds between polar lipids and membrane associated proteins and the TAGs dissolve in the neutral solvent. Once extraction is complete, water (and potentially additional neutral solvent) is introduced το facilitate the creation of two separate phases. The polar solvent with similar polarity to water will partition to the aquatic phase, whereas the highly hydrophobic lipids will remain with the neutral solvent. The extracted lipids can then be further purified and upgraded to Fatty Acid Methyl Esters.

In this study, PEF is evaluated as a pre-treatment method of microalgae. Lipids were extracted from the lipid rich strain *Auxenochlorella Protothecoides* (AP) and were further converted to FAMEs prior to their gas chromatography analysis. Additionally, the fact that PEF is a dynamic effect offered the

opportunity to explore the potential for the reduction of the required treatment energy for total lipid extraction, with the introduction of an incubation period. By monitoring the release of carbohydrates and the extraction of lipids at different time points with different energies, some further insights of the PEF-assisted extraction mechanism are offered.

ΠΕΙΡΑΜΑΤΙΚΟ ΜΕΡΟΣ

The experimental procedure can be found in previous publications[8,10], therefore only a brief description will be given below.

Microalgae cultivation and harvest

AP strain number 211-7a and CV strain 211-12 were obtained from SAG, Culture Collection of algae, Göttingen, Germany.

Autotrophic AP was cultivated in 25L photobioreactors (PBR) in sterile conditions. The cultivation medium of the PBR was tris- phosphate (TP) medium as described elsewhere[8] where the full composition can be seen. LED lamps (WU-M-500-840, 4000 K, Panasonic) provided light with an intensity of 200 μmol m^{-2} s^{-1} for the first 24hr and 600 μmol m^{-2} s^{-1} afterwards. The temperature and pH of cultivation were constantly monitored and a CO2 flow of 3% volume in sterile air 60 L/h was supplied. Microalgae were harvested after 3 weeks, in the late lipid accumulation phase. The microalgae were harvested by centrifugation with a Sigma 8k centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), at 3000g until ~100g/L concentrations were reached.

Pulse Electric Field Treatment

PEF treatment was performed in a continuous-flow, uniform-field treatment chamber consisting of two parallel circular stainless-steel electrodes with a 4mm distance. The applied rectangular pulses were fixed at 1 μs and a field magnitude of 40 kV/cm. The repetition rate was adjusted between 0.1 and 3 Hz corresponding to a wide energy input range between 15kJ and 150kJ per liter of suspension, respectively. A full description of the PEF setup and energy calculations can be found in Slive et al[10]. After pulsation, the biomass was processed either immediately or was incubated after some determined period. The samples were flashed with N_2 and incubated in the dark at 25^0C.

Water soluble extraction

Prior to extraction, the samples were centrifuged at 10.000g for 10 minutes. The supernatant was removed and its conductivity σ [μS/cm] measured with a conductivity meter (Endress+Hauser, CLM 381). No automatic temperature compensation was used, but temperature T[°C] was recorded in parallel with conductivity. The equivalent conductivity at 25 °C was then calculated[10]

The carbohydrate content of the supernatant was determined using the Anthrone Sulfuric Acid assay[10]. In brief, the samples were diluted with distilled water to concentrations ranging between 0.1 and 0.4g/L. As standards for calibration, fresh starch aqueous solutions were prepared from starch powder (Merck 1.01257). 400μL of diluted sample were mixed with 800μL anthrone reagent, 0.1% w/w anthrone in 95% sulfuric acid in 1.5ml Epperdorf Safe Lock tube. After 5 min incubation in ice, the solution was transferred into a thermo-incubator at 95^0C for 16 minutes at 300rpm. Then, the samples were cooled in ice for 5 minutes and the optical density was measured at 625nm. The carbohydrate concentration was then calculated based on the standard curve of starch.

Lipid extraction

After the removal of the supernatant, the biomass slurry was blended with the ethanol/hexane co-solvent in ratios EtOH/Hex/Water 1:0.41:0.05 vol/vol/vol. Extraction took place in Teflon tubes, in the dark and constant agitation for 24hrs. Once extraction was complete, the samples were

centrifuged at 10.000g for 10minutes, in order to remove the solvent (along the extracted lipids) from the spent biomass. Additional water and hexane were added (final system composition EtOH/Hex/Water, 1:4.85:0.80 vol/vol/vol) to the collected supernatant to facilitate the formation of two phases. The upper hexane phase was collected and evaporated under N_2. The amount of lipids removed was then determined gravimetrically.

Total lipid extraction

Total lipid extraction was performed with a commercial Soxhlet apparatus (behrotest® Kompakt-Apparatur KEX 30 from Behr Labor-Technik). Approximately 0.5 g of freeze-dried biomass was bead milled (bead mill, Mixer mill MM400, Retsch, Haan, Germany). Consequently, it was transferred 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 as extraction solvent 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 flask, along with extracted lipids, was let to cool down under N_2 atmosphere, followed by gravimetric determination of the lipid yields.

Transesterification and gas chromatography analysis

Approximately 30mg of extracted lipids were dissolved in 6mL methanol along with 0.3mL sulfuric acid as catalyst in sealed pyrex tubes. The transesterification reaction took place at 70^0C for 3hrs on a heat block with vigorous shaking every 30 minutes. The mixture was then transferred in fresh tubes along with 12mL distilled water and 12 mL hexane. After 15minutes, the samples were centrifuged at 6000g for 5minutes. The upper phase was collected and further washed with additional 8 mL water and a second centrifugation. The upper phase along the final product was collected and the hexane was evaporated under N_2. The samples were then stored until gas chromatography analysis. The device was the model 7890A with autosampaler7693, both from Agilent. The column was Otima WAX 30 m, 0.25 mm, 0.25 μm 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).

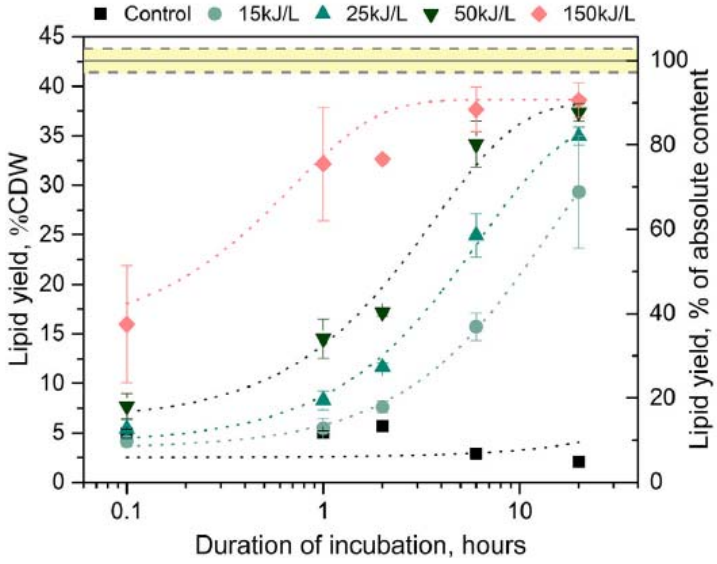
ΑΠΟΤΕΛΕΣΜΑΤΑ ΚΑΙ ΣΥΖΗΤΗΣΗ

In this study, PEF was proven as an effective pre-treatment method for lipid extraction from wet AP. In most cases, immediately after pulsation almost total lipid extraction was achieved as verified with the reference method. As can be seen in figure 1, untreated AP would yield negligible yields, whereas after pulsing with 150kJ/kg, significantly higher amounts of lipids are extracted. A waiting period of only 2hrs would lead to almost total lipid extraction as verified with Soxhlet method (~38% vs ~42% on dry weight).

On the same figure, the impact of the energy treatment can be seen along with its evolution over time. The more the energy treatment is reduced, the less efficient the PEF treatment gets, with the lipid yields decreasing. At 15kJ or 25kJ, the lipid yields immediately after PEF are barely higher than the untreated ones. However, by pulsing and then incubating the biomass, the lipid yields increase steadily. After 24hr incubation, total lipid extraction is achieved for almost all the treatment conditions studied here.

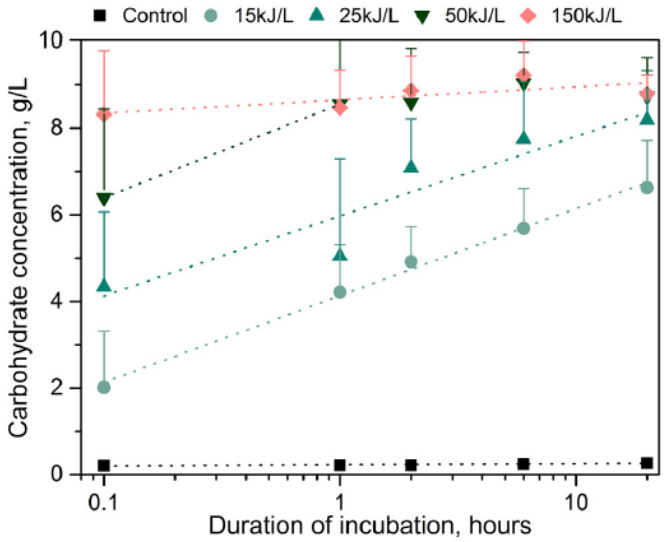
Interestingly enough, the same pattern is repeated with the water-soluble carbohydrates. As can be seen in graph 2, untreated AP releases no carbohydrate to the surrounding medium even after 24hrs. After pulsing with 150kJ/kg, approx. 8 g/L of carbohydrates are detected in the supernatant immediately after PEF. That amount, which would correspond to 10% of dry biomass would be stable even after 24hrs incubation. Regarding the other treatment conditions, it is observed that

immediately after PEF, modest to little amount of carbohydrates are released. Incubating though, leads again to the same intracellular release as 150 kJ. It must be noted, though, that the dynamic of this release is quite different compared to the lipid yields. For carbohydrates, it is more linear, whereas for the lipids it is more complex.



Graph 1. *Lipid yields as a function of incubation duration at different treatment energies. Results are the average of 3 independent experiments along with the std. Yellow line corresponds to the total lipid content.*

Analysis of the FAMEs produced from the lipids also allows for an evaluation of the appropriateness of AP to serve as biodiesel and whether PEF has a negative impact on the quality of the final product. FAMEs after Soxhlet with hexane and PEF with ethanol:hexane are remarkably similar, the only difference being slightly higher amounts of linoleic acid (C18:2) in the former. It can thus be claimed that PEF does not affect the FAME composition. Compared to rapeseed oil, it can be seen that AP has a similar FAME output with higher amounts of poluunsatured FAMEs. All the above results are summarized on table 1.



Graph 2. *Carbohydrate release in the supernatant as a function of duration of incubation for different treatment energies. Results are the average of 3 independent experiments along with the std.*

Table 1. *FAMEs produced by AP after lipid extraction with Soxhlet with hexane and PEF with ethanol:hexane. Results are from three different experiments*

FAME	Soxhlet (hexane)	PEF (ethanol: hexane)	Rapeseed oil
C16	10.4	12.3	4.7
C18:1	48.6	41.8	66.2
C18:2	36	39.2	19.8
C18:3	5.2	6.8	9.3

ΣΥΜΠΕΡΑΣΜΑΤΑ

In this study, the effectiveness of PEF as pretreatment method for microalgae downstream processing was demonstrated. By pulsing at 150 kJ/kg, significant lipid yields were observed after extraction with ethanol:hexane with total extraction achieved after introducing an incubation step. Said incubation step, also offered the possibility to greatly reduce the energy treatment up to 25kJ/kg. Untreated biomass consistently had negligible yields. Analysis of water-soluble carbohydrates painted the same picture, with releases at various conditions gradually increasing and reaching a plateau after 24hr incubation. No clear correlation can be established yet between the release of water solubles and efficiency of lipid extraction, but any further insight in the biomass evolution during this incubation step will enable further optimization of this process.

ΕΥΧΑΡΙΣΤΙΕΣ

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