Excess of Water Enables Efficient Lipid Extraction from Wet Pulsed-Electric Field-Treated *A. protothecoides* Microalgae Using Immiscible Solvents

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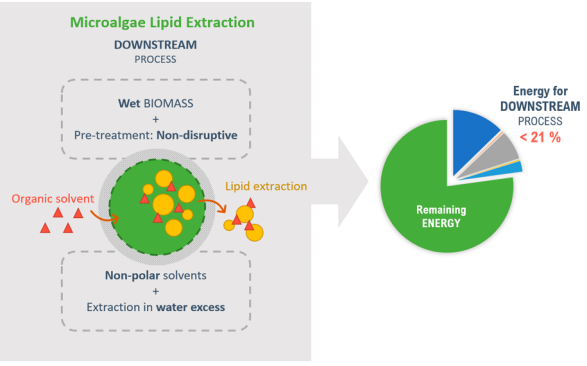
ABSTRACT: This article presents a new processing approach to perform lipid extraction on the wet microalgae *A. protothecoides* using nonpolar solvents or solvents of low polarity. It was demonstrated that solvents such as hexane, heptane, or 2-butanol exhibit a negligible extraction efficiency (<4% of total lipid content) when added to the pulsed-electric field (PEF)-treated and dewatered biomass slurry. In contrast, extraction efficiency after PEF treatment could substantially be increased by adding water and solvent to the biomass slurry. For this case, additional water admixture during extraction enabled to recover 67.3, 62, or 82.3% of the total lipid content, respectively. When utilizing methyl terbutyl ether (MTBE), ethylacetate, and 2-methyltetrahydrofuran (2-MeTHF) as extracting solvents, the yield increase by additional water admixture is far less pronounced. In all cases, the extraction process was performed after a PEF treatment using 1.50 MJ/kg$_{DW}$ plus 24-h incubation and 20 h of mixing time with the solvents and the solvent/water mixtures. Estimations on the energy required for the whole downstream process showed that this process variant allows an energy consumption of 31.48 MJ/kg$_{biodiesel}$ at maximum and 7.98 MJ/kg$_{biodiesel}$ at minimum. The low energy requirements (only <22% of the energy content of the lipids) open pathways to energetical applications of microalgae lipids.



KEYWORDS: *microalgae, lipids, pulsed electric field, energy, solvent*

1. INTRODUCTION

Microalgae are an important potential source of energy, especially for biofuel production, thanks to the large amount of lipids they can accumulate. Their advantages have long been claimed, in particular, their productivity, which exceeds that of any other agricultural crop, and the fact that they do not require the use of arable land. However, after decades of research efforts, microalgae are still not fulfilling their promises in the energy sector, despite the growing pressure to find alternatives to fossil fuels in order to limit the impacts of climate change.[1−4] The production of quantities of microalgae biomass compatible with the development of energy applications implies facing considerable technological scaling-up challenges and finding strategies to reduce the still-too-high production costs.[5] The downstream processing of the microalgae biomass is also not yet mature, and among various challenges, the extraction of the lipids from microalgae represents a major challenge to achieve costs or, in particular, net energy gains compatible with biofuel commercialization.

Many solutions proposed in the literature to extract lipids from microalgae or other microbial cells use organic solvents on dry biomass since organic solvents work very well in the absence of water.[2] In particular, this approach is almost systematically used in the laboratory for all analytical methods aimed at the quantification and characterization of lipids. However, those approaches are not realistic for industrial applications due to the extremely high costs of drying,[6−9] estimated to be 3.3−3.9 MJ per kg of water, which assumes a microalgae pellet containing 30% dry weight (DW) after mechanical dewatering, implies final drying energy requirements of 7.7−9.1 MJ/kg$_{DW}$. Considering a lipid content of 40%$_{DW}$, the energy demand for drying per kg of lipids already closely approaches the chemical energy content of lipids of around 30 MJ/kg. For that reason, permanent efforts are made to develop extraction methods that operate directly on wet biomass, i.e., an extraction system that works in the presence of water. Most of those methods can be separated into two main categories:

> The so-called "monophasic" approach in which the used solvents are perfectly miscible with water (or at least in the chosen proportions) so that the whole extraction mixture

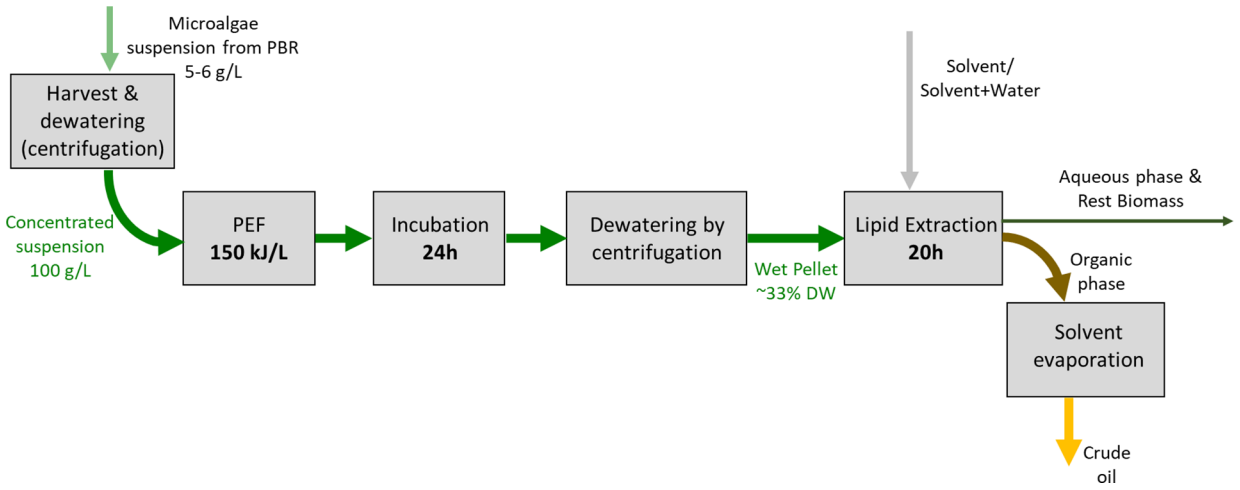


Figure 1. Schematic diagram of a lipid extraction experiment. The biomass is processed directly after harvesting and dewatering. The different parameters such as PEF treatment energy, incubation duration, and lipid extraction duration were deliberately chosen not to be limiting.

forms a single phase. These approaches systematically use a polar solvent which then is difficult to separate from the remaining water and is generally problematic to recycle.[2] In the worst case, the solvent forms an azeotrope with the water, e.g., in the case of ethanol, which considerably increases energy requirements for solvent separation.

The "biphasic" approach in which the wet biomass is in contact with a nonmiscible solvent, i.e., a nonpolar solvent or a solvent of very low polarity. Examples of solvents that are tested for those approaches are hexane or heptane but not only. A consensus is that such a biphasic extraction needs a preliminary cell disruption step which liberates the lipids by destroying the cell wall and allows the solvent to contact them.[10−12] "Cell disruption" is intended for any approach that destroys the mechanical integrity of the cell and in particular the integrity of the cell wall. Commonly used technologies are high-pressure homogenization and bead milling. Using those techniques, several authors have demonstrated a perfect correlation between lipid extraction yield and disruption rate.[11,13] However, those approaches are typically limited by the high energy demand associated with the disruption step, i.e., on average 3.5 MJ/kg_{DW}[14] or 8.75 MJ/kg_{lipids} (lipid content: 40%$_{DW}$), although some promising innovations such as autolytic incubation enable to reduce the specific energy needed.[15]

Another problem associated with such strategies is that the disruption of biomass implies the generation of extremely small debris, which is hard to separate and can form extremely stable emulsions.[16−18] The ultimate consequence is an increase in the complexity and energy demand of the following downstream steps.

Pulsed electric field (PEF) treatment has been widely studied for its ability to extract valuable intracellular compounds from microorganisms.[19] Its mechanism of electroporation increases cytoplasmic membrane permeability, which allows mass transfer processes through it, without cell disruption. This results in no cell debris being created, making PEF an attractive pretreatment method due to its low energy demands and ease of application. Previous work of our group has demonstrated that PEF treatment, despite exhibiting a direct effect primarily on the cell's membrane only and not on the cell wall,[20,21] can facilitate lipid extraction from microalgae[14,22−24] and oleaginous yeast[25] at an energy demand in the range of 0.25 to 1.5 MJ/kg_{DW} (0.625 to 3.75 MJ/kg_{lipid}). The lower value was achieved by including a post-PEF incubation step.[14] These demonstrations were performed using ethanol/hexane blends, following the above-described monophasic approach and using large quantities of solvents associated with high-solvent recycling costs in terms of processing energy, prohibitive for the production of low-added-value products such as biodiesel. In more recent work, still using ethanol/hexane blends but this time in a hybrid monophasic/biphasic approach, our group has demonstrated the possibility to reduce considerably the volumes of solvents required while still recovering 27%$_{DW}$ of lipids, i.e., 67.5% of total lipids.[23] Nevertheless, recycling of ethanol was evaluated to represent a minimum energy expense of 30 MJ per liter of lipids (energy consumption ratio (ECR) > 1), i.e., an energetical expenditure that is still too high to consider lipids for biofuel application.[23] Based on these findings, it was decided to concentrate our research efforts on extraction systems using only solvents that can be recycled at reasonable energy expenditures, i.e., excluding *de facto* solvents such as ethanol, creating nonfavorable azeotrope mixture with water.

Ideally, the solvent used should be nonmiscible with water in order to facilitate the separation between the organic phase and the water phase after extraction.[13,26,27] Nonpolar solvents such as hexane, heptane, or cyclohexane belong to the preferred candidate and according to the well-known rule "like dissolves like",[28,29] they are particularly suitable when neutral lipids are the main target. Specifically for biodiesel production, most of the saponifiable lipids that can be used are in the form of triglycerides, which are neutral molecules, and therefore, the possibility to extract specifically the neutral lipids can reduce efforts in the further refining steps.[10] In the case that polar lipids are also interesting for the pursued application, slightly polar solvents will be preferred. The solvent chosen should also have a low boiling point and a low heat of vaporization to facilitate recycling. Finally, the volume of solvent used should be kept as low as possible in order to keep solvent recycling efforts to a minimum.[27]

Finally, the solvents used should also be compatible with health, environmental, and safety regulations.[13,30] They should be manageable in a controlled industrial environment and in order to limit environmental hazards and impact on biodiversity. The process should target zero losses of solvents.

The present study was thus designed based on all these considerations, with the goal to develop a sustainable and economically viable organic lipid extraction process from microalgae for biofuel purposes. To achieve this, we conducted a comprehensive investigation of different solvents based on their effectiveness and, more importantly, their energy sustainability for extracting lipids from wet microalgae.

2. MATERIALS AND METHODS

2.1. Microalgae Strain and Cultivation Conditions. Experiments were performed with *Auxenochlorella protothecoides*, strain no. 211−7a obtained from SAG (Culture Collection of algae, Göttingen, Germany). *A. protothecoides* was cultivated autotrophically in a 25L photobioreactor (PBR) under sterile conditions.

The starter culture was cultivated mixotrophically for 3 days in a modified Wu medium[31] as described in previous publications[24] and then used as inoculum for the PBR. The cultivation medium was tris-phosphate (TP) medium[31] with the supplementation of 40 μg/L thiamine. For illumination, LED lamps were used (WU-M-500−840, 4000 K, Panasonic) with a light intensity of 200 μmol m^{-2} s^{-1} for the first 24 h, afterward increased to 600 μmol m^{-2} s^{-1}. The temperature was regulated at 21 °C, and the cultivation was aerated with sterile air at 60 L/h (0.04 vvm) supplemented with 3%vol CO_2.

A. protothecoides was harvested after 14 days in the late lipid accumulation phase and concentrated into a dense microalgae slurry using a disc stack centrifuge (STC 3−06−107, GEA Westfalia Separator Group GmbH). The obtained biomass slurry was then diluted in some of the supernatants to adjust a cell concentration of 0.1 kg_{DW}/L. During dilution to 0.1 kg_{DW}/L, the suspension was homogenized using a magnetic agitator. Subsequently, it was processed as displayed in Figure 1 and described in the following chapters below.

Furthermore, microalgae samples were collected at different time points during the extraction experiments and observed under a microscope (Axioplan 2, Zeiss, Jena, Germany) and a magnifying objective (×63). Images were acquired with an Axiocam HRc instrument (Zeiss, Jena, Germany).

For all experiments, a fraction of the final suspension (0.1 kg_{DW}/L) was freeze-dried in a laboratory freeze-drier (Alpha 1−4 LDplus, Christ) and stored in vacuum-sealed bags at −20 °C for further analysis of the biomass, in particular for total lipid content determination, described inSection 2.5.

2.2. Pulsed Electric Field Treatment. The concentrated microalgae suspension (0.1 kg_{DW}/L; 1.2 mS/cm ± 0.1) was treated in continuous mode with a constant flow rate of Q = 0.1 mL/s. The treatment chamber consisted of two parallel circular stainless-steel electrodes separated by a polycarbonate housing. The distance between the electrodes was 4 mm. The setup ensured a uniform electric field distribution in the whole volume of the treatment chamber ($V_{chamber}$ = 48 × 11 × 4 mm^3), which had no sharp angles. Photos of the treatment chamber and of the electrodes have been previously published.[32] PEF treatment was performed with a custom-made transmission-line generator. Pulse duration was fixed at Δt = 1 μs, and electric field intensity was at E = 4 MV/m. The pulse repetition rate f_{rep} was fixed at 3 Hz, in order to deliver a specific treatment energy of 150 kJ/L. More details about the instrumentation and the energy calculation were given in previous publications.[14,24]

After PEF treatment, control and PEF-treated samples were incubated for 24 h by depositing the biomass suspension in glass vessels. The air above the suspension was removed by flushing with nitrogen gas. The vessels were closed and placed in the dark at room temperature (23 °C in our laboratory) without any agitation.

2.3. Lipid Extraction. After 24 h of incubation, the microalgae suspension was deposited in Teflon tubes (Nalgene Oak Ridge Centrifuge Tubes, Teflon FEP, 50 mL Thermo Scientific) or borosilicate glass tubes (KIMBLE, Centrifuge tubes, round-bottom, H/S 45600−30, 30 mL) and centrifuged for 5 min at 7000g. The supernatant was removed, and solvent or solvent plus deionized water was added onto the wet pellet, which exhibits a biomass concentration of about 33%$_{DW}$. The pellet was resuspended using a vortex, and the extraction tubes were then placed on an orbital shaker (shaker DOS-10L, neoLab) at 350 rpm for 20 h. Different biomass-to-solvent ratios ranging from 15 to 300 g_{DW} per liter of solvent were evaluated in this step.

After the extraction time, the tubes were then centrifuged for 2 min at 7000g, and 1−5 mL of the upper organic phase was collected and evaporated under nitrogen gas in a preweighted glass tube. The gravimetric crude yield was then calculated based on the evaluated volume of the organic phase, which can be calculated using the miscibility properties of the solvents.[13,30,33,34]

2.4. Crude Extract Washing. In order to quantify the amount of lipid extracted, we washed the crude extracts obtained after extraction. It was performed by resuspending the crude extracts in 7 mL of chloroform−methanol (2:1, v:v) and completing with 3 mL of a HCl solution (0.1 M) and 0.3 mL of MgCl solution (0.5%w). The mixture was then vortexed and centrifuged at 3500g for 4 min. The lower phase was carefully removed with a Pasteur pipet in preweighted glass tubes. The remaining water phase was washed with an additional 5 mL of chloroform/methanol (2:1, v:v), and again, the lower organic phase was pipetted and added to the first one. The solvent was then evaporated under nitrogen flow, and a gravimetric yield of pure lipids was obtained.

2.5. Total Lipid Content. The total lipid extraction was performed with a commercial Soxhlet apparatus (Behr R-254-S from Behr Labor-Technik). Approximately 0.5 g of freeze-dried biomass was bead-milled (Mixer mill, MM400, Retsch, Haan, Germany) recovered in a thimble (Extraction Thimbles Cellulose, 90022080, Albet LabScience, Dassel, Germany) and deposited inside the Soxhlet chamber. Approximately 50 mL of hexane was used. The heating temperature was 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, the boiling flask along with extracted lipids was removed and allowed to cool under a nitrogen atmosphere, and the lipid yield was determined gravimetrically.

2.6. Energy Evaluation. In order to roughly assess the feasibility of biodiesel production based on the methodology previously exposed, evaluations of the energy balance were performed. Those evaluations are not intended to replace a more detailed techno-economic analysis but serve as an initial indication of the potential of our approach. Only the downstream part was analyzed, assuming that the starting point is a microalgae biomass concentrated at C_{algae} = 0.1 kg_{DW}/L, as typically available after a harvest. The main downstream steps to be considered are the ones displayed in Figure 1, namely, the PEF treatment, the dewatering by centrifugation, the lipid extraction (especially the agitation/mixing), the separation of the organic phase (centrifugation step), and finally the evaporation of the organic solvent. The used equations are described in the following sections, and the values for each parameter of the equations are displayed in Table 1. Furthermore, the evaluation was performed assuming two scenarios, conservative and optimistic for the parameters of the fraction of saponifiable lipids (η_{sap}) and conversion efficiency (η_{conv}). In the case of lipid content (x_{lipid}) and

Table 1. Parameters Considered in the Energy Evaluation[a]

parameters		values	
biomass concentration ($C_{microalgae}$, kg_{DW}/L)		0.1	
PEF treatment energy (E_{PEF}, MJ/L)		0.15	
centrifugation energy (E_{cent}, MJ/m^3)		2.5	
mixing energy (E_{cent}, MJ/m^3)		3.6	
		conservative	**optimistic**
lipid content (x_{lipid}, wt %)*		35%	45%
lipid extraction yield (η_{ext}, %)*	MTBE	63.75%	81.25%
	Hexane:Water	48.63%	65.0%
fraction of saponifiable lipids (η_{sap}, %)		70%	90%
conversion efficiency (η_{conv}, %)		80%	95%
energetical content of biodiesel (MJ/$kg_{biodiesel}$)		37.8	

[a]Energy calculated assuming an energetical content of biodiesel of 37.8 MJ/$kg_{Biodiesel}$.[37]

Table 2. Properties of the Solvent[a]

solvent	hexane	heptane	MTBE	ethylacetate	MeTHF	2-butanol
molecular formula	C_6H_{14}	C_7H_{16}	$C_5H_{12}O$	$C_4H_8O_2$	$C_5H_{10}O$	$C_4H_{10}O$
density (kg/L)	0.66	0.68	0.74	0.90	0.85	0.81
boiling T (°C)	69	98	55	77	80	100
enthalpy of vaporization ΔH (kJ/mol)	31.6	36.6	29.3	35.6	32.3	49.7
heat of vaporization ΔH (kJ/kg)	366.2	365.0	332.4	404.0	375	613.6
relative static permittivity	1.89	1.92	4.5	6.02	6.97	16.6
octanol−water partition coef. Log P	3.9	4.66	1.06	0.73	n.a.	0.61
solubility (g/L)	<0.001	<0.001	40	80	140	210

[a]All data are given at 25 °C unless stated otherwise. Data for MeTHF from Sicaire et al.[34] and others from PubChem.

lipid extraction yield (η_{ext}), the values for the energy estimation were fixed according to the experimental data obtained within this study (conservative: average std; optimistic: average + std).

The analysis does not include the production and initial concentration of the biomass and also not the energy required for the transesterification itself as well as for the refining of the extract. Additionally, in the downstream process itself, the energy of pumping, e.g., is also not included. The impact of the loss of solvent or of the treatment of the remaining water and rest biomass on the energetical balance is also not investigated.

2.6.1. PEF Treatment. Calculations were made by selecting a PEF treatment energy of E_{PEF} = 0.15 MJ per liter of suspension and assuming an efficiency of the generator of η_{pef} = 0.7.[35] The energy required ξ_{PEF} [kJ/kg$_{Biodiesel}$] can be expressed as

$$\xi_{PEF} = \frac{E_{PEF}}{\eta_{pef} C_{algae} x_{lipid} \eta_{ext} \eta_{sap} \eta_{conv}}$$

2.6.2. Centrifugation for Dewatering. The calculation of the energy required by centrifugation is based on technical data from the company GEA Westfalia Separator Group, which declares requiring an energy of 0.4−0.8 kWh/m^3 when operating at large industrial scales,[36] i.e., 1.44−2.88 MJ/m^3. A value of E_{cent} = 2.5 MJ/m^3 was taken for the calculation, and therefore, the energy needed for the dewatering ξ_{cent1} [kJ/kg$_{Biodiesel}$] is given by

$$\xi_{cent1} = \frac{E_{cent}}{C_{algae} x_{lipid} \eta_{ext} \eta_{sap} \eta_{conv}}$$

2.6.3. Energy of Mixing during Extraction. The total volume to mix V_{tot} is the sum of the volume of the initially dewatered pellet plus the volume of the solvent and eventually the volume of the solvent plus extra water. In first approximation, the volume of the dewatered pellet is considered to be 3 L/kg$_{DW}$, i.e., assuming extraction will be done at C_{extr} = 300 g$_{DW}$/L$_{solvent}$, it implies V_{tot} = 6.3 or 13.0 L/kg$_{DW}$ depending if a pure solvent is used or a solvent:water mixture (1:2, v:v). The energy of mixing is assumed to be 1 kWh/m^3 as given in the literature,[27] i.e., E_{mix} = 3.6 MJ/m^3, and the mixing step lasts for t_{mix} = 20 h. Therefore, the specific energy needed for the mixing ξ_{mix} [kJ/kg$_{Biodiesel}$] is given by

$$\xi_{mix} = \frac{E_{mix} t_{mix} V_{tot}}{x_{lipid} \eta_{ext} \eta_{sap} \eta_{conv}}$$

2.6.4. Separation of Organic Phase by Centrifugation. The energy required is assumed to be E_{cent} = 2.5 MJ/m^3, as in the previous centrifugation step and the volume to centrifuge is V_{tot} previously calculated in the mixing section, which implies an energy for this second centrifugation step ξ_{cent2} [kJ/kg$_{Biodiesel}$] as follows:

$$\xi_{cent2} = \frac{E_{cent} V_{tot}}{x_{lipid} \eta_{ext} \eta_{sap} \eta_{conv}}$$

2.6.5. Evaporation of Solvent. The energy expense of evaporation $\xi_{evaporation}$ [kJ/kg$_{Biodiesel}$] is calculated using the latent heat of evaporation of the solvent ΔH [MJ/kg$_{solvent}$] (see Table 2) and assuming an efficiency of heat recovery of η_{heat} = 0.7

$$\xi_{evaporation} = \frac{\Delta H(1 - \eta_{heat})}{C_{extr} x_{lipid} \eta_{ext} \eta_{sap} \eta_{conv}}$$

2.6.6. Energy Balance. The energy balance or the ECR of the whole downstream process was calculated as the sum of energies of each individual step referred to 1 kg of biodiesel divided by the energy content of the biodiesel (37.8 MJ/kg$_{biodiesel}$) as reported by Lardon et al.[37]

$$ECR = \frac{\xi_{PEF} + \xi_{cent1} + \xi_{mix} + \xi_{cent2} + \xi_{evaporation}}{\text{Energy content of } \mathbf{1} \text{ kg of biodiesel}}$$

2.7. Statistical Analysis. Results were obtained from two or three independent experiments with internal duplicates in each experiment. Data are expressed as the mean ± the standard deviation (std). OriginPro 2023 (OriginLab Corporation, Northampton, MA, United States) was used for statistical analyses to evaluate the significance of differences among the mean values by one-way analysis of variance and the Tukey test. Differences were considered significant at $p \leq 0.05$.

3. RESULTS

3.1. Lipid Extraction Performance of Different Solvents on Wet, PEF-Treated *A. protothecoides* Biomass. The capacity of six selected solvents (hexane, heptane, MTBE, ethyl acetate, MeTHF, and 2-butanol) to extract lipids from PEF-treated wet biomass of *A. protothecoides* was evaluated. Figure 2A shows the crude yields obtained when pure solvents were used in untreated and PEF-treated biomass in a ratio of 15 g$_{DW}$/L$_{solvent}$. Figure 2B shows the yield results when these solvents were added with extra water content (solvent:water, 1:2 v/v) with a biomass solvent ratio of 30 g$_{DW}$/L$_{solvent}$. The yellow line and filled area are the average ± SD of the evaluated total lipid content. Data are presented as crude yield, but further analyses have demonstrated a lipid purity in all the extracts above 97% except for lipids obtained with MeTHF and 2-butanol from PEF-treated and control samples, respectively. Crude yields and washed yield values are given in Table S1 of supplementary data.

In the case of pure solvent extraction (Figure 2A), the higher lipid yields were obtained from PEF-treated biomass samples extracted by MTBE and ethylacetate with 20 and 22.4%$_{DW}$, respectively. When extraction was performed on microalgae that did not receive any pretreatment (control), the crude yields were <1%$_{DW}$ for hexane, heptane, and MTBE <5%$_{DW}$ for ethylacetate and MeTHF and reached a maximum of 8.8%$_{DW}$ for 2-butanol, although a purification of this extract revealed that only 2%$_{DW}$ correspond to lipids, the rest can be assigned to impurities (see Table S1).

On the other hand, when the extraction was performed with the same solvents but in excess of water (Figure 2B), the lipid yields significantly increased for all the solvents evaluated in PEF-treated biomass. The maximum value was obtained by

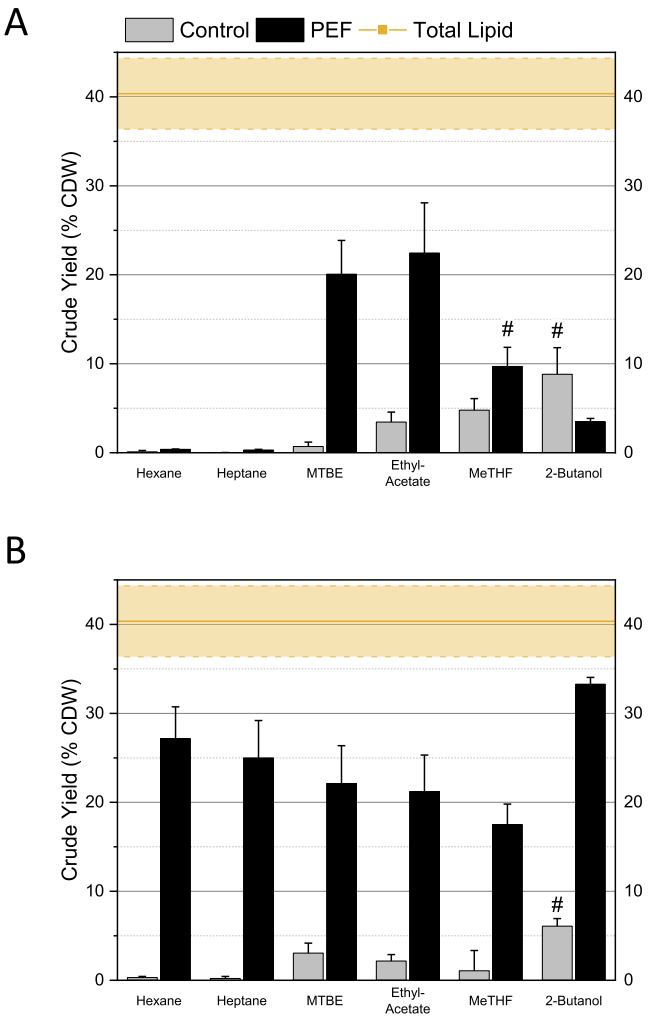


Figure 2. Crude lipid yields ($\%_{DW}$) obtained after extraction with pure solvent (A) or with solvent and an excess of water (B) (solvent:water 1:2/v:v). The experiments were performed on fresh *A. protothecoides* with 15 $g_{DW}/L_{solvent}$ (A) or 30 $g_{DW}/L_{solvent}$ (B). Solvent extraction was applied either directly after harvesting (Control) or after PEF treatment and incubation. Total lipid content ($\%_{DW}$) is represented in yellow. Results are the average ± SD of three independent experiments with internal duplicates.

means of 2-butanol:water with 33.3% $_{DW}$ of crude yield, meaning an extraction of approximately 82% of the total lipid content. For the rest of the solvents evaluated, crude yields from 27%$_{DW}$ for hexane:water to 17.5%$_{DW}$ for MeTHF:water were found. The lipid yields from untreated biomass remained neglectable for all the solvents.

Pictures of microalgae samples collected at different time points during the extraction experiments and observed under the microscope are displayed in Figure 3. Immediately after the PEF treatment, the microalgae cells have a similar aspect as the control cells, and no debris is observed. Even after 24 h of incubation, cells appear slightly shrunk but have kept their overall shape. It can be noted that some contaminants started to develop as expected since sterility was broken at the moment of the harvest. Some cells were also observed after extraction with MTBE pure (Figure 3D) or with hexane:water (1:2 v:v) (Figure 3E). In the first case, some shed cell walls are observed, and most cells appear as shrunk so that the cell wall seems to be detached from the cell. In the second case, cells have a more standard appearance and only a few debris can be noted floating around the cells. In both cases, it is evident that the whole extraction process did not lead to disruption of the microalgae cell.

3.2. Influence of the Biomass to Solvent Ratio on the Lipid Yield. In order to evaluate the influence of a higher biomass-to-solvent ratio, increasing concentrations of biomass during the solvent extraction step were studied for the systems that resulted in the highest yields of pure ethylacetate and MTBE or hexane:water and 2-butanol:water (1:2, v/v). Figure 4 shows the crude yields referred to the DW for extraction performed in PEF-treated biomass by means of the mentioned solvent/solvent systems when increasing the concentration of biomass to the amount of solvent from 15 and 30 $g_{DW}/L_{solvent}$ up to 300 $g_{DW}/L_{solvent.}$ For comparison purposes, the yields of samples not processed by PEF (control) for the lowest biomass-to-solvent ratio were evaluated.

The lipid yields for both pure solvent extraction, ethylacetate, and MTBE, when increasing the biomass concentration, remained very consistent. Even when the highest biomass:solvent ratio (300 $g_{DW}/L_{solvent}$) was tested, values of 25.7 and 29.1%$_{DW}$ for ethylacetate and MTBE were found, respectively, with no statistical differences to the yields at the lowest biomass:solvent ratio (15 $g_{DW}/L_{solvent}$). For the two other extraction systems, i.e., hexane:water and 2-butanol:water, the crude extraction yields tended to decrease for biomass concentrations above 60$g_{DW}/L_{solvent}$. Crude yields were reduced from 29.8 to 22.7%$_{DW}$ in the case of hexane:water when increasing from 30 to 300$g_{DW}/L_{solvent}$, and from 35.5 to 28.8%$_{DW}$ in 2-butanol:water in the same conditions. Apart from the reduction of yield in the two extraction systems previously mentioned, the increase of the biomass:solvent ratio did not pose any technical problems. The agitation, in particular, remained efficient. Some pictures that illustrate the aspect of the

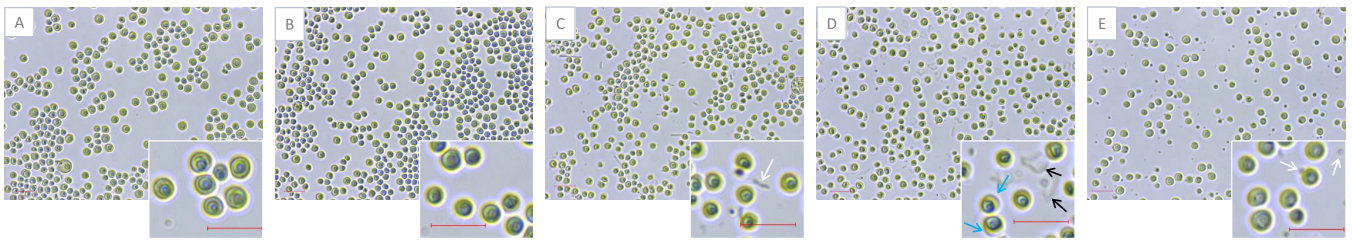


Figure 3. Microscope observation (x63 magnifying objective) of microalgae cells *A. protothecoides* during the extraction process. Images were done on cells, frozen during the experiment. (A) Control cells immediately after harvest and concentrating step, (B) cells immediately after PEF treatment with 150 kJ/L, (C) after an additional 24-h long incubation step, (D) after extraction with MTBE, and (E) after extraction with hexane:water mixture (1:2 v:v). Some shed cell walls are visible (black arrows), and on the majority of cells, the cellwall appears as detached from the cells (blue arrow). Few debris are visible (white arrows). The red bar scale represents 20 μm.

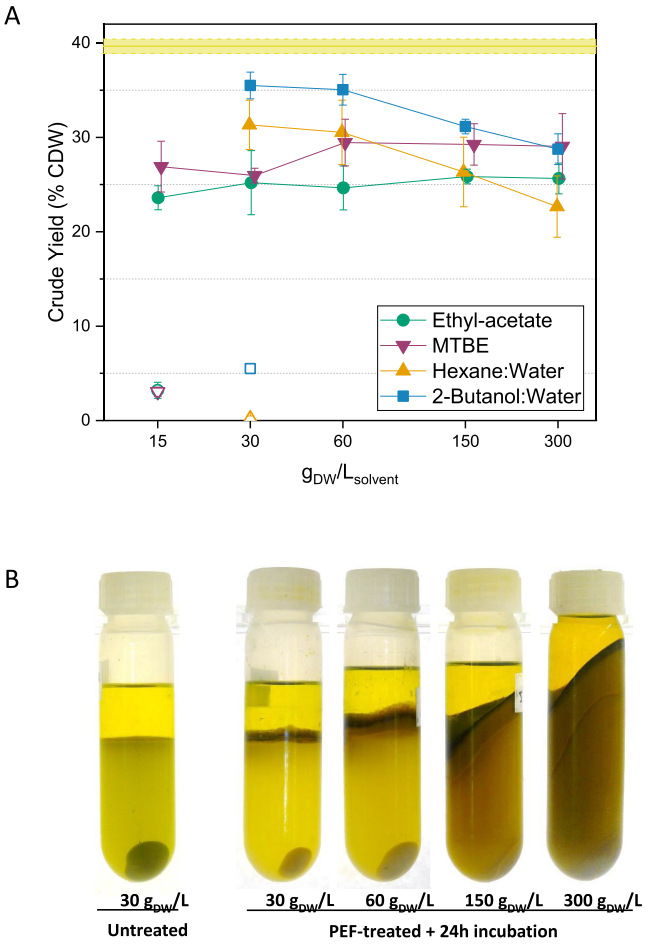


Figure 4. Influence of biomass:solvent ratio. (A) Crude lipid yield obtained by extraction on wet pellet using pure ethylacetate, pure MTBE, hexane:water (1:2, v:v), or 2-butanol:water (1:2, v:v). Experiments were performed on fresh *A. protothecoides* either unprocessed (empty symbols) or after PEF treatment followed by incubation for 24 h (full symbols). Results are the average + SD of 2 independent experiments with internal duplicates. Note that data were slightly shifted along the *x*-axis for a better visualization of all error bars. The yellow line and filled area are the avg ± std of evaluated total lipid content. (B) Aspect of the extraction tubes when performing extraction with hexane:water (1:2 v:v) with different biomass to solvent ratio. The photos were taken after centrifugation at the end of the 20 h of extraction on orbital shaker.

extraction tubes with the solvent hexane:water and for different biomass concentrations are displayed in Figure 3B. They were taken at the end of the extraction, after the centrifugation step preceding the recovery of the organic phase.

3.3. Energy Assessment of the Downstream Process for Biodiesel Production from Wet PEF-Treated *A. protothecoides* Using Pure MTBE and Hexane:Water (1:2) as Selected Solvent Systems. Calculations were made for two different extraction strategies: MTBE pure and hexane:water (1:2 v:v) and for the two scenarios, conservative and optimistic, presented in Table 1. In the case of lipid content and extraction yields, values used for calculation were obtained from the maximum and minimum of our experimental data (average ± SD). Therefore, 35 and 45%$_{DW}$ of total lipid content were used for conservative and optimistic scenarios, respectively, while extraction yields for MTBE were fixed at 67.75 and 81.25% and for hexane:water at 48.63 and 65% (Figure 4). The results of the energy demand estimations for obtaining 1 kg of biodiesel are detailed for the different processing steps (PEF treatment, dewatering, mixing, separation, and evaporation of solvent) in Figure 5. Furthermore, the remaining energy (designated as "Rest", Figure 5) and the ECR are also detailed for each solvent system and the conservative or optimistic approach.

The total required energies calculated for MTBE extraction for the two scenarios sum up to 19.96 and 7.98 MJ/kg$_{biodiesel}$ while slightly higher values of 31.48 and 12.0 MJ/kg$_{biodiesel}$ were obtained for hexane:water. These estimated energy requirements for all evaluated scenarios showed an ECR below 1.0, namely, 0.53 to 0.21 for pure MTBE or 0.83 to 0.32 for hexane:water.

For all cases, the PEF processing step exhibits the highest energy demand. It amounts to 35.3 and 46.3% of the total available energy in the conservative scenarios or 14.1 and 17.6% in the optimistic ones, each for MTBE and hexane:water, Figure 5. Mixing represents the second largest share of processing energy demand. It ranges from 3.9%, MTBE, optimistic case, up to 26%, hexane:water, conservative case. Solvent evaporation varies between 2.8 and 9.4% of the total energy content for the same cases. Dewatering and separation processes resulted in the lowest energetic consumption, all of which were below 1%.

4. DISCUSSION

The present study investigated several organic solvents in order to perform lipid extraction on wet biomass from *A. protothecoides*, a microalgae well-known for its high productivity and its high neutral lipid content ideal for biofuel production.[38−40] A recent GC analysis of the FAME profile, carried out in our laboratory, confirmed the low content of linolenic acid methyl ester (<7%), 13% is accounted for by saturated fatty acids, and C18:1 and C18:2 have a share of 80%,[41] which exceeds the FAME composition requirements for biodiesel according to EN14214.

The positive impact of PEF treatment on subsequent lipid extraction was already demonstrated on this strain using ethanol/hexane blends.[24]

In later studies, the beneficial effect of an incubation step after PEF processing and before lipid extraction was also demonstrated.[14,42] Incubation improved the efficiency of the extraction process, allowing for a reduction in extraction time or a reduction in specific PEF energy demand while maintaining the same lipid yield.

During our solvent extraction processing, the parameters employed were: PEF treatment energy of 150 kJ/L$_{sus}$, 24 h incubation time after PEF and 20 h of solvent mixing time. Figure 1 represents upper limit values from previous studies on solvent extraction from *A. protothecoides*. These conditions were intentionally selected to guarantee the most optimal and nonlimiting conditions for lipid recovery.

The screening was performed with six solvents that were either nonpolar (hexane and heptane) or had relatively low polarity (MTBE, ethylacetate, MeTHF, and 2-butanol) (Table 2) in order to facilitate the separation of the organic phase from the residual biomass suspension after extraction. The selection was performed based on three big criteria: "extraction capability", "solvent recycling", and "health and safety".[13] Hexane and heptane were chosen since they are nonpolar and therefore nonmiscible with water which are ideal conditions for recycling. The other four solvents have a relatively low polarity: MTBE, ethylacetate, MeTHF, and 2-butanol are only partially miscible with water. 2-butanol shows the highest miscibility of

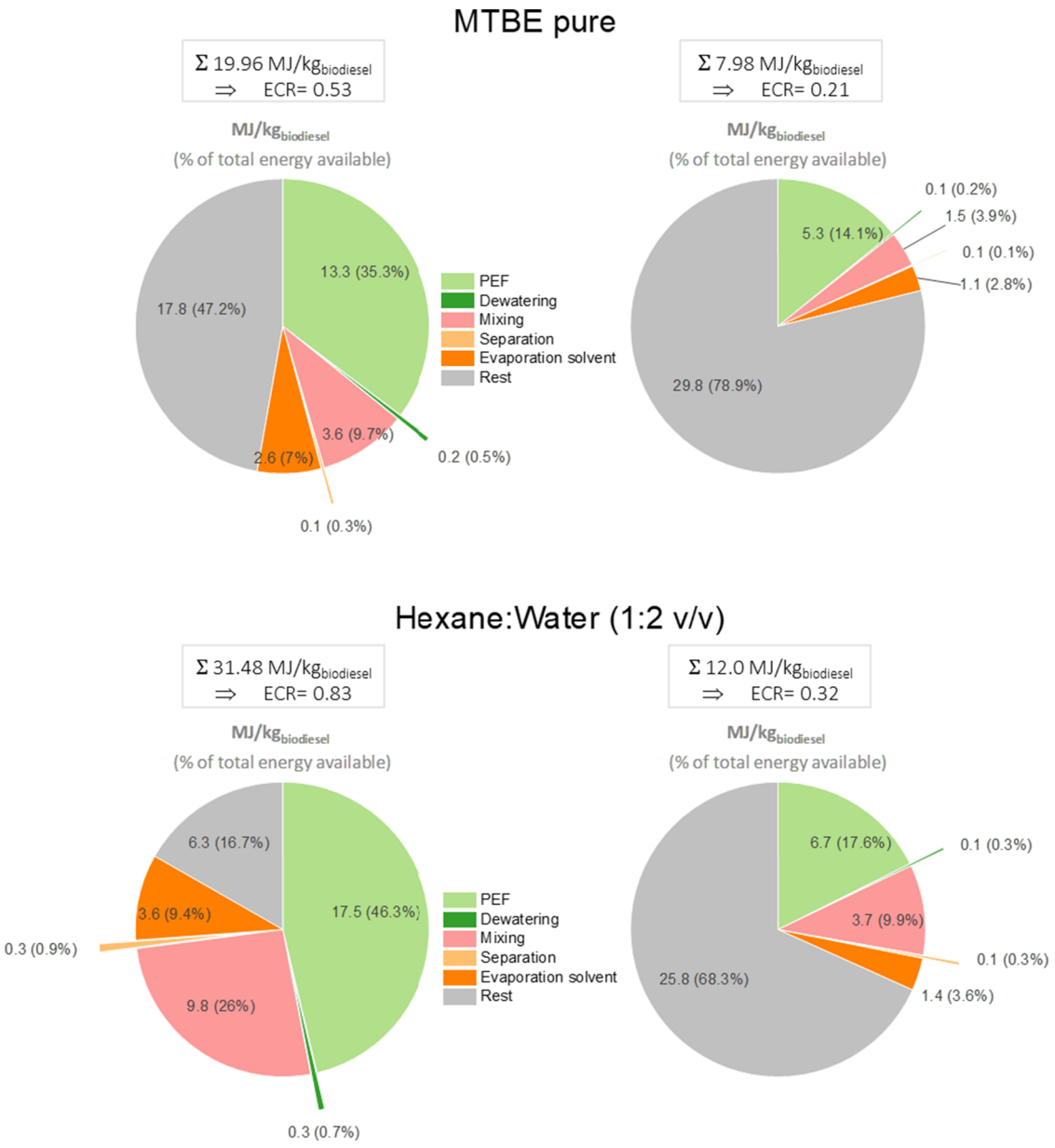


Figure 5. Evaluations of the energy demand of the main steps of the downstream processing of *A. protothecoides*. Calculations were done for extraction with pure MTBE or with hexane:water (1:2 v:v). The left graphs correspond to the conservative scenario, and the ones on the right correspond to the optimistic scenario. The numbers on the charts are the energies in $MJ/kg_{Biodiesel}$ expressed in brackets in percentage of the total recoverable energy.

210 g/L. Furthermore, ethyl acetate and MeTHF are considered green solvents due to their renewable origin, ease of recycling, harmlessness, and biodegradability.[34,43−45] Both solvents are recommended alternatives for use in sustainable industries. All solvents have a low heat of vaporization (<410 kJ/kg at 25 °C) except for 2-butanol, which requires 613.3 kJ/kg to evaporate, which makes it less advantageous in terms of higher energy demand for recycling. Nevertheless, it was included in the screening study since lipid extraction capabilities from wet microalgae were attested to be excellent in the literature.[30]

The first set of experiments consisted of adding pure solvent to the wet pellet (i.e., only the water remaining in the pellet is present). In a second round of experiments, not only solvent was added to the microalgae pellet but also water in a solvent:water proportion of 1:2 (v:v). All solvents were more efficient when applied in combination with an excess of water, despite the fact that they are not miscible with water. This was especially true for hexane and heptane, two fully nonmiscible solvents, totally inefficient in extracting lipids when applied purely on wet biomass pellets but enabling to achieve high yields when applied in combination with water, i.e., 27.2 and 25.0%$_{DW}$ after PEF treatment for hexane and heptane, respectively. These results are very significant as they contradict the general assumption that the presence of water hinders the action of nonpolar solvents. In fact, our results show that the additional water not only has no negative impact but also improves lipid extraction. Furthermore, it has to be remarked that this extra water effect has also been observed when another microalgae strain was investigated (data not shown). The neglected lipid extraction in untreated samples (control) excludes the possibility of hypoosmotic shock caused by the addition of water. On the other hand, this phenomenon can be associated with the capacity of the presence of extra water to enhance the contact between hexane or heptane and PEF-treated microalgae cells and their intracellular lipids. In the

absence of extra water, the concentrated microalgae biomass (aqueous phase) remained in a single clump, not dispersed in hexane or heptane (organic phase). The extra water admixture resulted in better dispersion of the biomass through the organic phase when mixing. Here, water also might facilitate the formation of solvent droplets that enhance hexane diffusion into the aqueous phase (microalgae + water) by reducing the interface tension, as it has been reported for higher and faster lipid extraction from diatom cells.[46]

A consensus in the literature is that when extraction is performed with a solvent that is nonmiscible with water, i.e., a nonpolar solvent such as hexane or solvent of low polarity, a preliminary cell disruption step is required to disrupt the cell wall and therefore give solvents access to the lipids.[11−13] The results presented in this study demonstrate the invalidity of this dogma since high lipid extraction yields (>20%$_{DW}$) were achieved with hexane and heptane, without any obvious fracturing of the microalgae cell wall as shown in Figure 3. This strongly supports the hypothesis that lipid transport happens *through* the cell barrier.

The use of hexane or heptane which are nonpolar is of particular interest since they have a good selectivity to neutral lipids which can therefore minimize downstream fractionation/purification costs. Additionally, they belong to extremely volatile solvents, which limits the energy needed for distillation and solvent recycling. The extremely low miscibility of those solvents with water also implies fewer traces of solvent in the final water fraction and consequently simplifies further processing and causes fewer solvent losses in general.

Based on the yields obtained during the solvent screening, four different extraction systems were selected and further investigated: pure MTBE, pure ethylacetate, hexane:water (1:2, v:v), and 2-butanol:water (1:2, v:v). The screening evaluation was performed with our standard ratio of biomass to solvent of 15 $g_{DW}/L_{solvent}$ (pure solvent extraction) and 30 $g_{DW}/L_{solvent}$ (in excess of water solvent extraction). Therefore, the influence of an increase in the biomass:solvent ratio was investigated due to its direct impact on the energy consumption derived from the downstream process.

For the conditions studied, lipid yields remained efficient even when increasing 10 times this biomass:solvent ratio, although some yield reductions (≤7%) were observed in the solvent systems (hexane:water and 2-butanol:water). This observation might reinforce the idea that water improves solvent dispersibility in the aqueous phase, facilitating contact with microalgae cells and thus increasing lipid extraction. Consequently, by increasing the biomass:solvent ratio, the biomass-to-water ratio also increases, which might reduce the interphase contact between solvent (dispersed in water) and microalgae cells. Nevertheless, these results evidence the robustness of the solvent extraction procedure for being efficient under more realistic conditions to be applied on a large scale, enabling a reduction of energy costs. Furthermore, according to the energy balance (Figure 5), the mixing step within the whole downstream process represents the second more energy-demanding procedure. However, further optimization on this step might be evaluated by increasing biomass to solvent ratio, reducing mixing times, or looking for more suitable mixing methods. It should be noted that under biphasic conditions, the separation step may be more energetically demanding due to the interaction of solvent and biomass, which may make it difficult to separate composites if the previous mixing was too vigorous. Similarly, when partially water−miscible solvents are used, a percentage of the organic phase dissolves in the aqueous phase. The ease of subsequent water−solvent separation here becomes of high relevance for the final energy balance and should be considered. These points need further investigation in future studies.

The energy calculations have demonstrated that all the approaches proposed in the current work enable the extraction of lipids with a reasonable amount of processing energy. The required energy for the downstream processing of *A. protothecoides* for biodiesel applications represented up to 83% (ECR = 0.83) of recoverable energy in conservative scenarios and 21−32% (ECR = 0.21−0.32) for the optimistic scenarios. These results turn out to be of high importance since the first of our studies on this matter found ECRs between 1 and 12,[14,24,42] i.e., that the energy necessary for the downstream process was higher than the one available in the recovered lipids. Studies should certainly focus on this point, considering the importance of the type of solvent and its properties, which allow a reasonable energy demand and therefore a reduction of the ECR, so that microalgae lipids become a real alternative for the industrial production of biodiesel.

It should be noted that the objective of this study was not to fully optimize the extraction process and that several parameters were not investigated, which would allow to reduce the overall energy demand of the process. In particular, it should be investigated whether it is possible to

- Reduce the PEF treatment energy for pretreatment of *A. protothecoides* either by simply reducing the delivered volumetric energy or by increasing the concentration of the biomass since this microalga can easily be concentrated up to 200 $g_{biomass}/L_{solvent}$, and the cell suspension remains liquid and well pumpable.
- Reduce extraction duration and thus the associated mixing energy.
- Reduce the amount of water when performing extraction with a solvent:water approach to reduce energy related to mixing since water was here in clear excess.
- Reuse solvent for several microalgae batches before performing evaporation since solvents are currently far from being saturated with lipids.

Note that the energy analysis currently omits certain requirements such as pumping, transesterification, purification, and treatment of the rest water/biomass etc. However, the energy that can be recovered from the rest biomass using, i.e., anaerobic digestion[47] is also not considered.

5. CONCLUSIONS

The use of PEF to assist in the extraction of lipids from the wet biomass of *A. protothecoides* has shown potential as a downstream process for industrial-scale biodiesel production. The proposed methods successfully demonstrate lipid extraction, using nonpolar solvents in a reduced volume and without any mechanical cell disruption technique. This approach avoids the high costs associated with biomass drying, solvent/water separation, solvent recycling, and further purification steps. Up to 80% of the total lipid content was extracted from *A. protothecoides* biomass using either pure MTBE or hexane:water in a high biomass to solvent ratio (300 $g_{DW}/L_{solvent}$) with PEF as pretreatment. The energy balance calculations indicate that lipids can be extracted from microbial biomass with reasonable energy input (7.89−31.48 MJ/$kg_{biodiesel}$), using in some cases less than 25% of the recoverable energy with the perspective of

even further reducing this value by simple optimization of the process.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.3c06966.

Crude Yields and Washed Yields obtained after lipid extraction during solvent screening experiment on *A. protothecoides* (PDF)

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Notes

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