Direct transesterification of microalgae after Pulsed Electric Field (PEF) treatment

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Abstract

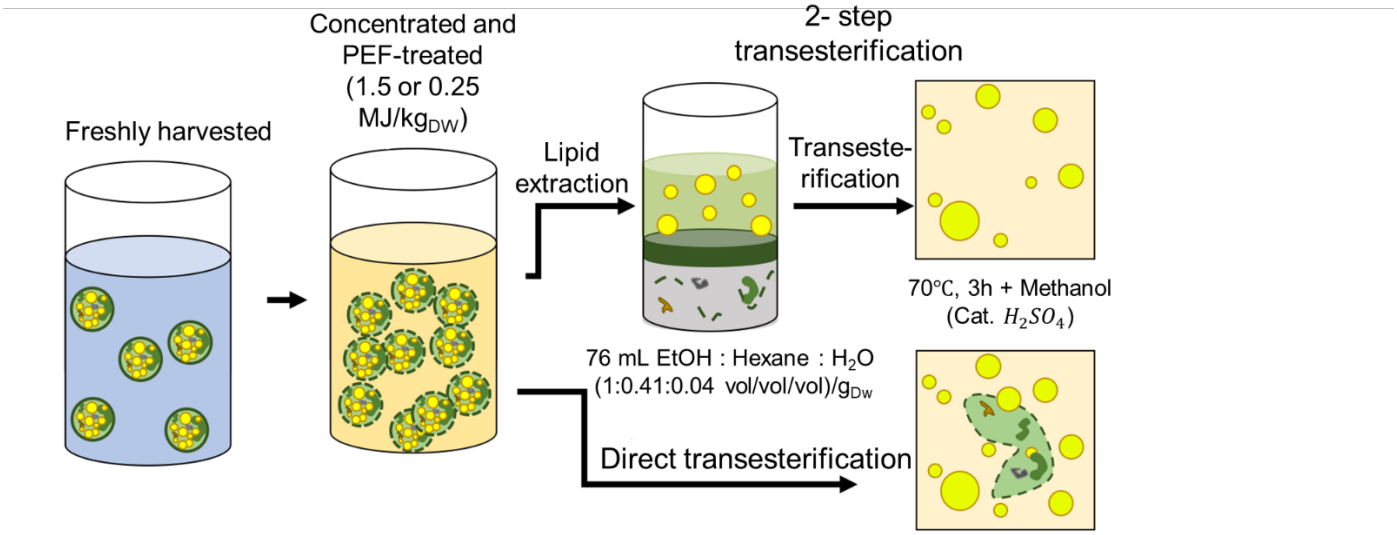
Background: Lipid extraction is a major bottleneck for the commercialization of microalgae due to energy costs involved during solvent recycling. Direct transesterification offers the possibility to bypass the extraction step by immediately converting the lipids to fatty acids methyl esters (FAMEs). In this study, the efficiency of direct transesterification after pulsed electric field (PEF) was evaluated. Freshly harvested *Auxenochlorella protothecoides* (*A. protothecoides*), cultivated either autotrophically or mixotrophically,

was subjected to PEF. Two treatment energies were tested, 0.25 MJ/kg_{dw} and 1.5 MJ/kg_{dw} and results were compared with conventional two-step transesterification.

Results: For autotrophically grown *A. protothecoides*, the percentage of the total FAMEs recovered from untreated biomass and microalgae treated with 0.25 MJ/kg_{dw} was 30% for both cases while for 1.5 MJ/kg_{dw} it was 65%. A 24-h incubation step between PEF-treatment and direct transesterification significantly improved the results. Untreated biomass remained stable with 30% of FAMEs, while with both treatment energies a 97% FAME recovery was achieved. However, for mixotrophic *A. protothecoides* the process was not as effective. Approximately 30% of FAMEs were recovered for all three conditions immediately after PEF with only a marginal increase after incubation. The reason for this different behavior of the two cultivation modes is unknown and under investigation.

Conclusions: Overall, the synergy between PEF and direct transesterification was proven to have potential, in particular for autotrophic microalgae. Its implementation and further optimization in a biorefinery therefore merits further attention.

Graphical abstract



Keywords: PEF, oleaginous microorganisms, biodiesel, two-step transesterification

Abbreviations: PEF: Pulsed Electric Field, DW: dry weight, GC: gas chromatography, FAMEs: Fatty acid methyl esters.

1. Introduction

Biodiesel, also known as FAME, is a mixture of fatty acid methyl esters derived by vegetable oils and used as transportation fuel [1]. Microalgae are aquatic microorganisms that have been extensively studied for biofuel production in general and in particular for biodiesel [2]. Under nitrogen starvation, certain microalgae strains are capable of high lipid accumulation, reaching up to 50% of the cell dry weight (DW) [3]. Other advantages offered by microalgae include cultivation on non-arable land, fast growth rates [4] and coproduction of other useful compounds such as proteins, carbohydrates and pigments that if exploited can improve the economics of a biorefinery [5].

In conditions favoring lipid accumulation, the main constituent of microalgae lipids is usually triglycerides (TAGs), molecules composed of three long chain fatty acids attached to glycerol [6]. TAGs are converted to

FAMEs through a reaction known as transesterification during which they react with methanol to form a mixture of esters with glycerin as a byproduct [7]. Overall, transesterification is a well-established technology with plenty of commercial applications to convert vegetable oils from sources such as rapeseed and soybean seed [8]. Important parameters that affect the reaction yields include the alcohol amount, type of catalyst, temperature and reaction time.

Methanol is typically selected for this reaction although other alcohols such as ethanol may be used [9]. In equilibrium, one mole of triglyceride reacts with three moles of methanol to produce three moles of FAMEs. In practice, an excess of solvent is applied in order to drive the reaction towards the products. This leads however, to the additional challenge of recovering the unused solvent [10].

The reaction can be catalyzed by either acids or bases. Usage of the latter offers faster reaction rates [4] but in presence of water and high free fatty acid contents the use of a base induces a risk of saponification and therefore reduction of the yields [11]. Acid catalysts do not share these problems but exhibit slower conversion kinetics and may lead to equipment oxidation. A potential strategy involves a combination of the two, with an initial acid transesterification to convert the free fatty acids followed by an alkaline one as demonstrated by Dong et al [12]. A different strategy is also the utilization of enzymes as catalysts such as lipase [13] or heterogeneous catalysts [14] although their industrial application is still limited. The selection of the catalyst has an effect on the reaction temperature as well. In principle, alkaline catalysts require lower temperatures than acidic [15].

For commercial production of FAMEs from microalgae, significant challenges need to be considered. The conventional pathway would first require lipid extraction with organic solvents from the biomass and their subsequent conversion to FAMEs. This ‘two-step transesterification’ faces the bottleneck of demand of large solvent volumes for lipid extraction and the associated energy costs for their recycling [16]. A different approach, would be to bypass the extraction step and to convert the lipids to FAMEs by applying the transesterification directly on the entire microalgae biomass. This ‘Direct Transesterification’, also

encountered as '*in situ* transesterification', was previously employed as an analytical technique for the determination of the total FAME content [17,18]. Recently however, its usage as a downstream processing method is coming under evaluation. The elimination of the extraction step is expected to offer a number of benefits including solvent reduction and higher FAME yields [19].

An additional obstacle to consider in processes aiming at the production of FAME, is the natural resistance against extraction exhibited by microalgae cells. This is typically attributed to their rigid cell wall, a microfibrillar layer that surrounds the cell and usually is composed of cellulose, hemicellulose and other polysaccharides [20]. In general a pretreatment method has to be applied in order to overcome this barrier and enhance the accessibility to the targeted molecules [21]. The nature of this pretreatment can be physical (mechanical, thermal), chemical, biological or a combination of the above [22]. An optimal disruption technique should be effective on wet biomass, energy efficient and suitable for large scale industrial applications [23]. Common pretreatment methods include high pressure homogenization, microwave treatment and ultrasonication.

Pulsed Electric Field (PEF) treatment is one such pretreatment technique. During PEF-treatment, the microalgae are subjected to an external electric field. The ion flow induced by the electric field causes an increase of the transmembrane voltage of the cell membrane [24] leading to a phenomenon known as 'electroporation', during which a rearrangement of the membrane's structure and its eventual collapse [25] occurs. PEF is considered a mild technology and with proven industrial scalability in certain applications, particularly in the Food industry [26]. After treatment, some intracellular components such as hydrophilic proteins and carbohydrates are spontaneously released in the surrounding aqueous medium. Lipids can subsequently be extracted by the addition of appropriate organic solvents. Unlike conventional physical techniques, PEF generates no debris since the overall cell structure remains intact after treatment. This in turn, allows for easier cascade extraction of different components[27,28]. PEF is also a non-thermal method which should prevent any damage to heat sensitive components such as pigments [29]. Low energy input

with PEF-treatment is another significant advantage with only 1.5 MJ/kg_{dw} being sufficient to pretreat wet *Auxenochlorella protothecoides* as shown in a previous study from group where total lipid extraction was achieved in these conditions[30].

PEF has been applied for extraction of lipids [30–32], proteins [33–35], carbohydrates [36–38] and pigments [31,38–40] from microalgae. In literature, direct transesterification is usually coupled with microwave technology [41–43] or ultrasounds [41,44,45] (often dubbed as 'assisted' if treatment is taking place during the reaction itself [46]). To the best of our knowledge however, no work has been reported on performing direct transesterification on microalgae after PEF-treatment.

The goal of this study was therefore to evaluate the direct transesterification of microalgae using PEF as a pretreatment method. *Auxenochlorella protothecoides* (*A. protothecoides*) was used as model microalgae due to its high lipid content and ability to grow autotrophically and mixotrophically [30]. Two PEF-treatment energies, proven effective for lipid extraction in a previous work [37], were tested; 0.25 MJ/kg_{dw} and 1.5 MJ/kg_{dw}. As a rule, the effectiveness of microalgae pretreatment is proportional to the treatment energy input. In a previous study with conventional lipid extraction from our group however, it was demonstrated that incubation of the biomass between PEF-treatment and extraction, compensated for the reduction of the treatment energy while retaining similar yields [37]. Incubation's potential effect on direct transesterification was therefore included in this study as well.

2. Materials and methods

Experiments were conducted on wet biomass, either fresh (processed within 15 minutes after PEF-treatment) or incubated at inert conditions (flushed with N_2 and stored for 24 h at 25 °C, in the dark without agitation). All chemicals used were of analytical grade. Results from two independent cultivations are shown, each with internal duplicates.

2.1. Microalgae cultivation and harvest

A. protothecoides strain number 211-7a was obtained from SAG culture collection of algae, Göttingen, Germany. The cultivation of the biomass took place in sterile conditions either autotrophically or mixotrophically. In brief, in autotrophic mode, the microalgae were cultivated in 25 L annular photobioreactors in tris-phosphate (TP) medium for approximately 19 days. In the mixotrophic cultivation, the microalgae were supplied with glucose in 1 L conical polycarbonate cultivation flasks (VWR International, Bruchsal, Germany) in modified Wu medium [30] and grown for 10 days. The duration of the cultivation for the two microalgae was selected based on experimental data on the time required for the stationary phase to be reached. Detailed description of the cultivation modes were given in [47].

The microalgae were harvested through centrifugation. In the case of autotrophic mode, a separator was used (STC 3–06-170, GEA Westphalia, Germany). For the mixotrophic cultivation, a Sigma 8k centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) was used at 3,000 x g. In both cases, the concentrated microalgae were re-suspended in a portion of the removed supernatant. The final cell dry weight was 100 g/L, a concentration where the microalgae suspension is still pumpable through the PEF-apparatus yet dense enough for the reduction of the input energy. The concentration was verified by overnight drying of known amounts of the final suspension and supernatant in a drying oven (Universalschrank model U, Memmert, Germany) at 90 ˚C. From each harvest, a portion of the harvested biomass was lyophilized (Alpha 1–4 LDplus, Christ) and stored in vacuum-sealed bags at -20 °C.

2.2. Pulsed Electric Field (PEF) treatment and incubation

The equipment used is described in detail in [28,47]. A custom-made treatment chamber was utilized, capable of delivering uniform-field treatment. It consisted of two parallel circular stainless-steel electrodes 4 mm apart, separated by a polycarbonate housing. Treatment took place in continuous mode, with a flow rate of 0.1 mL/s. Rectangular pulses were applied with a duration of 1 μs and a field magnitude of 40 kV/cm.

149 The repetition rate of the pulses was either 0.5 or 3 Hz, resulting in an input energy of 0.25 MJ/kg_{dw} and
150 1.5 MJ/kg_{dw}, respectively (corresponding energy calculations are explained in [37]).

151 For incubation, biomass was kept in polypropylene falcons with screw cap (CELLSTAR® 50mL PP tubes,
152 Greiner Bio-One, Frickenhausen, Germany). After flushing with N_2, the samples were sealed and stored in
153 the dark, without agitation, at 25 °C for 24 h. The further processing of the incubated biomass was identical
154 to the fresh one.

155 2.3. Direct transesterification of *A. protothecoides*

156 The protocol from Breuer et al. [48] was adapted for the transesterification reaction. With a precision
157 balance, the weight of 1 mL of microalgae suspension was measured (containing approx. 100 mg dry
158 biomass) in borosilicate glass tubes with screw caps ('culture tubes', 16/36/26 MP, Pyrex, England). The
159 samples were then centrifuged at 1,800 x g for 5 minutes. The supernatant was removed (corresponding
160 to 0.6 mL of medium) and 6 mL of methanol, 0.3 mL sulfuric acid (96% wt. purity) were added along with
161 1 mL of hexane (methanol:hexane 1:0.17 vol:vol), given the reported increased efficiency of the
162 transesterification reaction with a co-solvent [49]. The final microalgae concentration at the beginning of the
163 reaction was thus 250 g/L. The tubes were then vortexed and placed into a heating block.

164 The reaction took place at 70 °C with vortexing every 30 minutes for 1 or 3 h in total. At the end of the
165 reaction, the mixture was transferred into new polypropylene falcons where 12 mL of distilled water and
166 12 mL of hexane were added. The samples were vortexed and left to agitate for 15 minutes, followed by
167 centrifugation at 10,000 x g for 5 minutes. 10.5 mL were removed from the upper phase into a new falcon
168 where 8 mL of distilled water were added as a washing step. After vortexing for 1 minute and
169 centrifugation at 10,000 x g for 5 minutes, 9 mL of upper phase were removed into pre-weighted glass
170 tubes and the hexane was evaporated under N_2. Afterwards, the glass tubes along the FAMEs were flushed
171 with N_2, sealed with paraffin and stored at -20 °C for gas chromatography analysis.

2.4. Two-step transesterification

The conventional two-step transesterification is divided into two processes, lipid extraction and transesterification. For lipid extraction, a protocol from a previous work was adapted [30], the main difference being the scaling down of the microalgae and solvents volumes. In brief, 3 mL concentrated microalgae suspension were measured in Teflon tubes (Nalgene® Oak Ridge Centrifuge Tubes, Teflon® FEP, 50 mL Thermo Scientific) using a precision balance. The samples were then centrifuged (Heraeus™; Megafuge™ 8R, ThermoFischer Scientific, Germany) at 10,000 x g for 10 minutes and the supernatant was removed (2-2.1 mL). The biomass pellet was then resuspended by the addition of 16.1 mL ethanol and 6.6 mL hexane which were combined with the remaining water from the previous dewatering step (approx. 0.6 mL) and resulted in a monophasic co-solvent ethanol:hexane:water, 1:0.41:0.04 (vol/vol/vol). Lipid extraction then commenced for 24 h in the dark and under constant agitation on an orbital shaker.

For the separation of the solvent and dissolved lipids from the residual biomass, the samples were subjected to centrifugation at 10,000 x g for 10 minutes. From the supernatant, 6.1 mL were removed in a separate falcon, where 18.2 mL hexane and 2.9 mL water were added. From the resulting two-phase system, 15 mL were transferred from the upper hexane lipid-rich phase into pre-weighted glass tubes and evaporated under N_2. The lipids were then measured using a precision balance and yields were calculated gravimetrically.

At the end of the extraction, lipids (typically, around 30 mg per sample, depending on the condition) were dissolved in 4 mL hexane and transferred to glass tubes with screw caps. The hexane was evaporated under N_2. The transesterification protocol as described in section 2.3 was then followed.

2.5. Evaluation of total fatty acid methyl esters (FAMEs) content

The total fatty acid methyl esters (FAME) content was evaluated with direct transesterification of freeze-dried biomass. Lyophilized *A. protothecoides* was bead-milled at 30 Hz, 5 times for 15 s (Mixer mill,

MM400, Retsch, Haan, Germany) and approximately 100 mg measured with a precision balance were transferred to glass tubes with screw caps. Transesterification was then performed according to the protocol described in section 2.3.

A similar transesterification protocol was then followed as described in section 2.3.

2.6. Evaluation of total lipid content (Kochert method)

A chloroform:methanol extraction, based on Kochert method [50], was performed on freeze-dried *A. protothecoides* after every harvest in order to determine the total lipid content. Freeze-dried biomass was bead-milled at 30 Hz, 5 times for 15 sec (Mixer mill, MM400, Retsch, Haan, Germany). Approximately 100 mg were recovered and the exact weight was measured with a precision balance. 2 mL of chloroform:methanol (2:1 vol/vol) were mixed with the biomass, vortexed and immediately centrifuged at 1,800 ×g for 4 min. After the centrifugation, the supernatant was removed and collected into a separate glass tube. 2mL of fresh solvent were added to the biomass and the above process was repeated. In total, 7 mL of solvent were used, in four separate extraction steps (3x2 mL and 1x1 mL for the last step). Into the glass tube with the collected solvent, 3 mL of HCl 0.1 N and 0.3 mL $MgCl_2$ 0.5% were added to facilitate phase separation. The lower phase along the lipids was removed with a Pasteur pipette into pre-weighted glass tubes and evaporated under N_2. The lipid yield was determined gravimetrically. All samples were performed in duplicates.

2.7. Gas chromatography (GC) analysis of fatty acid methyl esters (FAMEs)

The stored FAMEs were initially re-suspended in 4 mL hexane and subsequently filtered (Chromafil Ca-20/25, 0.20 µm, filter 25 mm, Macherey-Nagel, Dueren, Germany). The samples were then diluted 1:4 with hexane. For samples with high FAME concentration an additional 1:20 dilution was performed. Gas chromatography (GC) with a flame ionization detector (FID) was used. The device was the model 7890B with autosampler 7693 from Agilent. The column was Stabilwax 30 m, 0.25 mm, 0.25 µm from Restek with

helium as carrier gas. The evaluation of the results was done with Chemstation Software from Agilent over calibration with FAME-mix standard (Food Industry FAME Mix (37 components), Restek).

2.8. Reproducibility of results

Experiments were performed on two independent microalgae harvests with internal duplicates. The average and standard deviation was calculated.

3. Results

3.1. Two step transesterification

Freshly harvested biomass, cultivated either autotrophically or mixotrophically, was subjected to PEF-treatment either with 0.25 MJ/kg_{dw} or 1.5 MJ/$kg_{dw.}$ Lipid extraction then commenced either immediately after PEF-treatment or after a 24h incubation period at inert conditions. The extracted lipids were then transesterified as described in section 2.3. The gravimetric measurement of the extracted lipids along with the GC analysis of the FAME after transesterification are shown in Figure 1 for both types of cultivation.

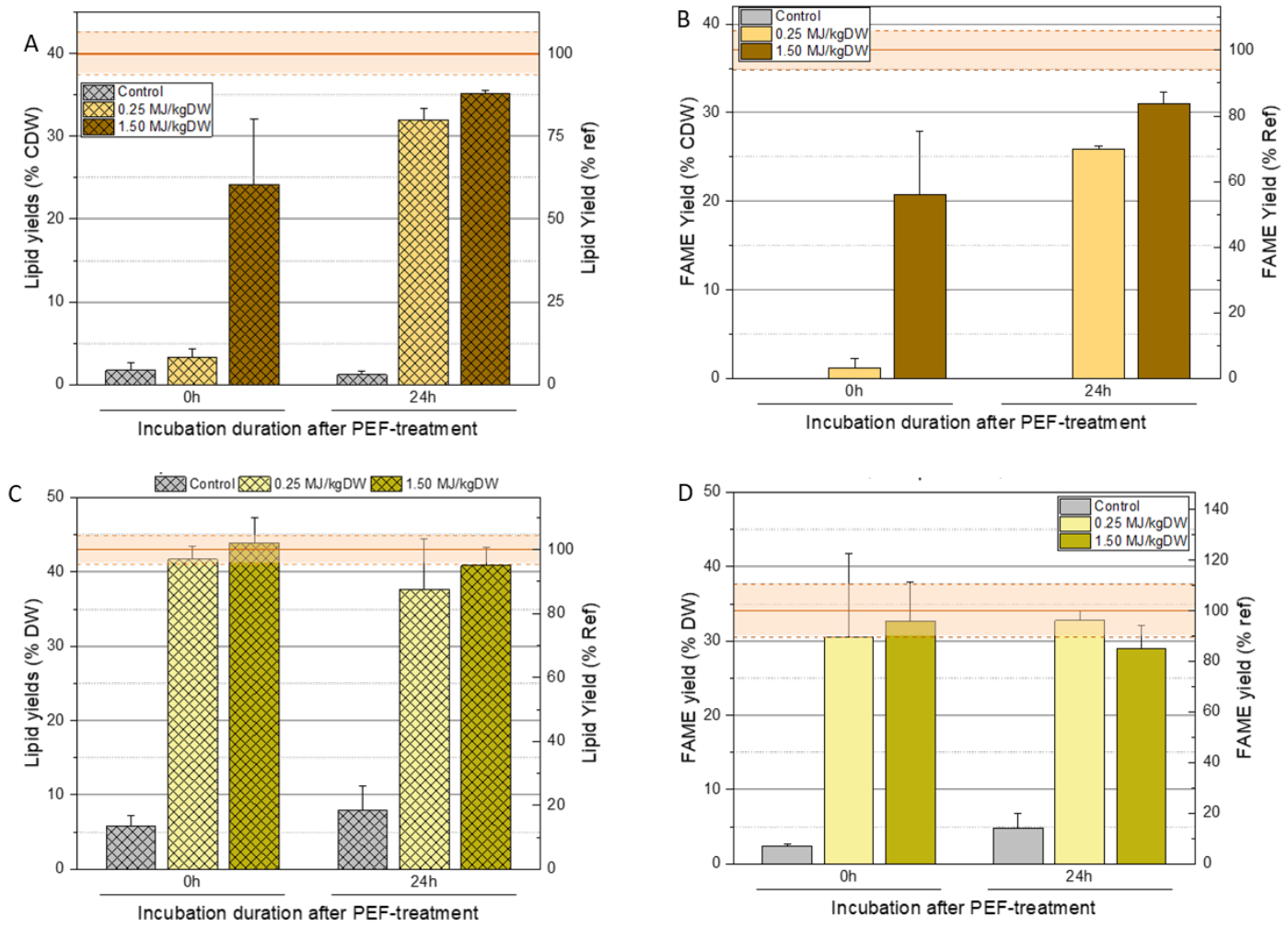


Figure 1: Two step transesterification of *Auxenochlorella protothecoides* cultivated in either autotrophic or mixotrophic mode. The gravimetric measurement of the lipids after solvent extraction and the FAME conversion after transesterification with gas chromatography analysis are shown in A and B for autotrophic cultivation. The respective results for the mixotrophic cultivation are shown in C and D. Two PEF-treatment energies were tested, 0.25 MJ/kg_{DW} and 1.5 MJ/kg_{DW}, while Control refers to untreated biomass. On the left y-axis the yields are displayed as percentage per dry weight while on the right y-axis as percentage of the total estimated content. In straight red line, the total lipid and FAME content of the biomass is represented. Control was not analyzed with GC due to the very low yields after lipid extraction. Lipid extraction took place with a monophasic co-solvent ethanol:hexane:water, 1:0.41:0.04 (vol/vol/vol) on freshly harvested, wet biomass after PEF-treatment with 0.25 MJ/kg_{dw} or 1.5 MJ/kg_{dw} either immediately

after treatment or after a 24-h incubation step at inert conditions. For transesterification a methanol: hexane, 1:0.17 vol/vol mixture was used. The Kochert protocol was used as reference method for the evaluation of the total lipid content while for FAME content, the transesterification protocol was applied on lyophilized, bead-milled biomass. Results are the average and standard deviation of two independent experiments with internal duplicates.

Lipid extraction was ineffective on untreated biomass for both cultivations without any effect from a 24-h incubation, with 1.7% dry weight yields from fresh biomass and 1.2% from incubated. For autotrophic *A. protothecoides* (Figure 1A), PEF-treatment with 0.25 MJ/kg_{dw} displayed minimal yields immediately after PEF, 3.3% dry weight. Lipid yields were significantly increased when an incubation step was interjected between treatment and lipid extraction, rising up to 32% dry weight. PEF-treatment with 1.5 MJ/kg_{dw} without incubation resulted in moderate lipid yields, 24.1% dry weight. Incubating after treatment was effective in improving the yields in this case as well, leading to an increase up to 35.1% dry weight. Lipid extraction from mixotrophic biomass displayed more immediate high yields as seen in Figure 1C. For both treatment energies, lipid yields were in similar range, equal to 42% and 44% dry weight for 0.25 MJ/ kg_{DW} and 1.5 MJ/kg_{dw} respectively. Incubation of biomass after PEF-treatment led to no further increase.

In Figure 1B and 1D for the autotrophic and mixotrophic cultivations respectively, the transesterification conversion of the extraction lipids is displayed. Compared to the total evaluated FAME content (37.1%± 1.6 dry weight for the autotrophic mode and 34.1% ± 2.5 for the mixotrophic respectively), incubating after PEF-treatment with 1.5 MJ/kg_{dw} offered the best FAME yields for the autotrophic cultivation, reaching 83% of total FAME conversion while 0.25 MJ/kg_{dw} achieved 70%. High FAMEs conversion was observed from the mixotrophic cultivation as well. Without any incubation, 90% and 96% of total FAMEs were recovered from biomass treated with 0.25 MJ/kg_{dw} and 1.5 MJ/kg_{dw} respectively. After incubation, 96% of total FAMEs were converted from 0.25 MJ/kg_{dw} although for 1.5 MJ/kg_{dw}, the conversion was reduced to 85%.

The above results verify the effectiveness of PEF utilization in the two-step transesterification along with the importance of incubating the biomass after treatment. The results were in agreement with observations which are discussed in extension in previous publications [30,37] and served as benchmark for direct transesterification.

3.2. Conversion of lipids through direct transesterification

Freshly harvested biomass, cultivated either autotrophically or mixotrophically, was subjected to PEF-treatment either with 0.25 MJ/kg_{dw} or 1.5 MJ/kg_{dw} and direct transesterification took place for 3h either immediately after PEF-treatment or after a 24h incubation period at inert conditions. The end product of the reaction was analyzed with GC. The results are displayed in Figure 2.

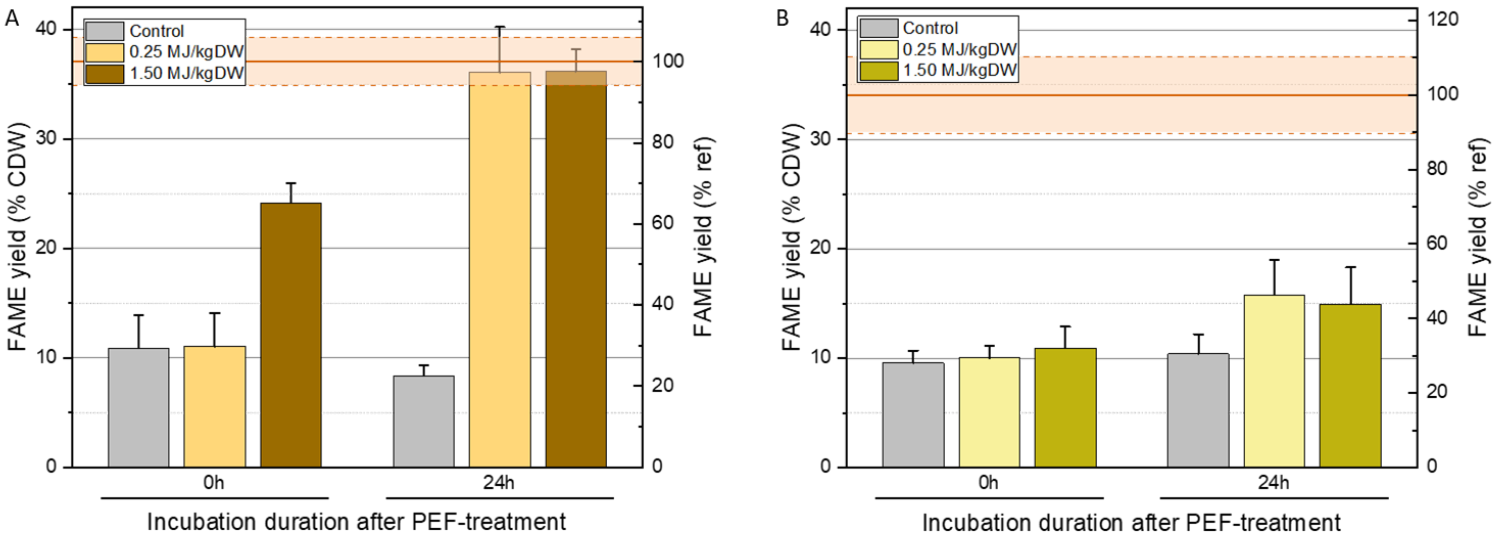


Figure 2: FAME recovery after direct transesterification of *A. protothecoides* cultivated in either autotrophic or mixotrophic mode. GC analysis of the reaction product is shown in A and B for autotrophic and for mixotrophic cultivation respectively. Two PEF-treatment energies were tested, 0.25 MJ/kg_{DW} and 1.5 MJ/kg_{DW}, while Control refers to untreated biomass. On the left y-axis the yields are displayed as percentage per dry weight while on the right y-axis, as percentage of the total estimated FAME content. In straight red line, the total FAME content of the biomass is represented. Transesterification took place

with a methanol: hexane, 1:0.17 vol/vol mixture for 3 h at 70°C. As reference method for the evaluation of the total FAME content, the same transesterification protocol was applied for 3h on lyophilized, bead-milled biomass. Results are the average and standard deviation of two independent experiments with internal duplicates.

The FAME recovery from autotrophic *A. protothecoides* without any incubation after PEF-treatment, were 11% dry weight or 27% of the total evaluated FAMEs for samples treated with 0.25 MJ/kg_{dw} and untreated ones. Samples treated with 1.5 MJ/kg_{dw} displayed better results with 24% dry weight i.e. 65% of total evaluated FAMEs. Incubation after PEF-treatment significantly improved the results. While untreated biomass was unaffected, an almost total FAME recovery was achieved for the two PEF-treatment energies (36% dry weight i.e. 97% of total evaluated FAMEs for both cases).

When the same method was applied on mixotrophic *A. protothecoides*, the results were not as successful. Without any incubation, the FAME recovery was relatively low, even for 1.5 MJ/kg_{dw} (10% dry weight i.e. 32% of total evaluated FAMEs for all three conditions). Unlike autotrophic biomass however, incubation after PEF had no significant effect, although there was a marginal increase of FAME recovery for the PEF-treated samples up to 15% dry weight i.e. 44% of the total evaluated FAMEs.

The FAME composition of the end product both of the two-step and direct transesterification was analyzed with GC and presented in Table 1 and Table 2 for the autotrophic and mixotrophic cultivation respectively. In the same tables, the total FAME content of the microalgae as evaluated from direct transesterification of freeze-dried and bead-milled biomass ("reference method") can also be found.

As it can be seen in Table 1, the FAMES produced by the autotrophically cultivated *A. protothecoides* were mainly oleic acid (C18:1), linoleic acid (C18:2) and in lesser amounts palmitic acid (C16:0) and the polyunsaturated γ-linolenic acid. More specifically, the FAMEs were composed by 9.8% C16:0, 39.5% C18:1, 41.6% C18:2 and 6.1% C18:3n6. The FAMEs of the PEF-treated samples, either after a two-step or a direct transesterification were very similar to the reference method. The direct transesterification approach thus did not have an effect on the FAME composition. Equally important is the result that incubating the microalgae after PEF-treatment for 24-h did not affect negatively the end product, especially the unsaturated FAMEs.

The mixotrophic *A. protothecoides*, shown Table 2, had a slightly different FAME composition from the autotrophic cultivation. The main fatty acid was C18:1 i.e. 67.3%, followed by 21.8% C18:2, 9.1% C16:0 and 8.3% C18:3n6. The FAME composition at the end of the two-step transesterification was similar to the above for various experimental conditions, although loss of the PUFA C18:3n6 was noted. The FAME composition at the end of the direct transesterification displayed reduced C18:1 compared to the reference method although given the reduced overall yields of the reaction, no concrete conclusion can be made for this case.

From the above, it can be summarized that direct transesterification is most efficient when coupled with PEF-treatment and incubation for the autotrophically cultivated *A. protothecoides*. In fact, under these conditions the FAME recovery was even higher compared with the conventional two-step transesterification. Neither direct transesterification nor incubation had an effect on the FAME composition of the end product. It is currently unknown however, why the yields from the mixotrophic biomass were repeatably low.

3.3. Further examination of the direct transesterification-incubation after PEF synergy

As seen previously, incubation after PEF-treatment had a considerable effect on the FAME yields, when processing autotrophically cultivated biomass. The exact mechanism of incubation is still largely

unexplored, however. On parallel with the previous experiments, samples were stopped after only 1h direct transesterification reaction in order to gain further insights on the involved mechanisms and whether the reaction time could be reduced. The results are displayed in Figure 3.

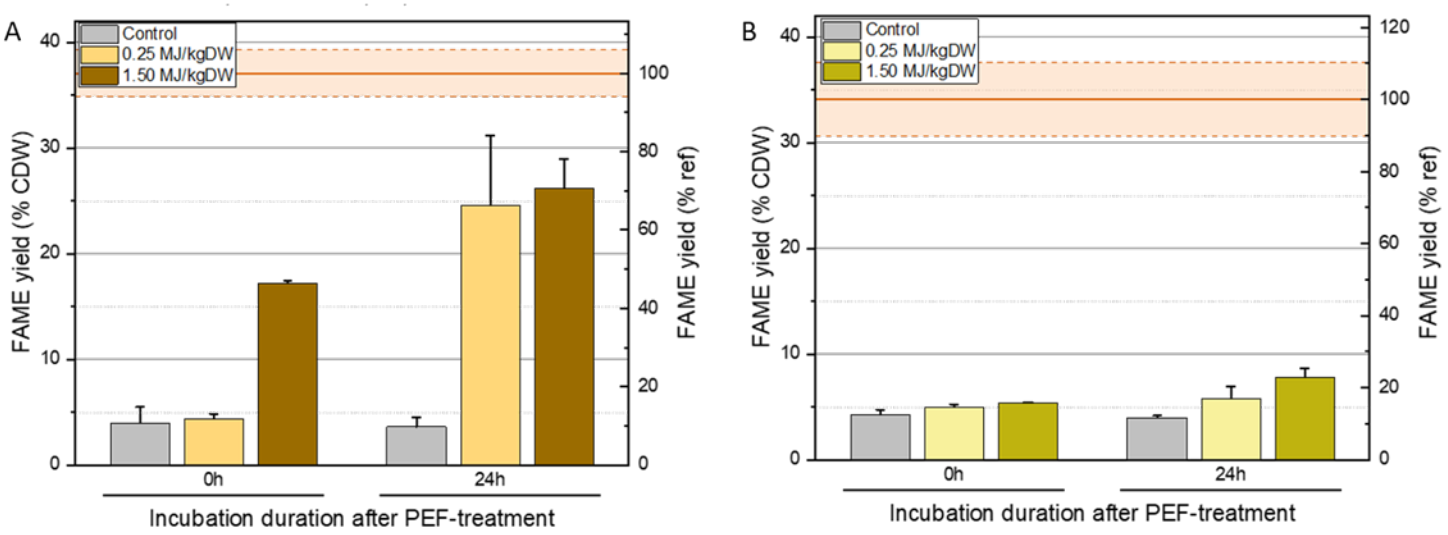


Figure 3: FAME recovery after direct transesterification with 1h reaction time of *Auxenochlorella protothecoides* cultivated in either autotrophic or mixotrophic mode. GC analysis of the reaction product is shown in A and B for autotrophic and for mixotrophic cultivation respectively. Two PEF-treatment energies were tested, 0.25 MJ/kg_{DW} and 1.5 MJ/kg_{DW}, while Control refers to untreated biomass. On the left y-axis the yields are displayed as percentage per dry weight while on the right y-axis, as percentage of the total estimated FAME content. In straight red line, the total FAME content of the biomass is represented. Transesterification took place with a methanol: hexane, 1:0.17 vol/vol mixture. As reference method for the evaluation of the total FAME content, the same transesterification protocol was applied for 3h on lyophilized, bead-milled biomass. Results are the average and standard deviation of two independent experiments with internal duplicates.

Examining the FAME yields after 1h reaction from Figure 3 and comparing them with the respective 3h ones from Figure 2, it is evident that there is a decrease in product output. More specifically as seen in

Figure 3A for the autotrophic cultivation, untreated and PEF-treated with 0.25 MJ/kg_{dw} biomass the FAME recovery was reduced by half, down to 4% dry weight. Yields from microalgae treated with 1.5 MJ/kg_{dw} were also reduced although not as strongly: 17% dry weight or 46% of total FAMEs. However, incubating the biomass after PEF-treatment improved the results. Untreated biomass remained unaffected, while both 0.25 MJ/kg_{dw} and 1.5 MJ/kg_{dw} displayed 26% dry weight FAMEs i.e. 70% of total evaluated FAMEs. The mixotrophically cultivated microalgae displayed reduced FAME yields with only a marginal increase after incubation.

3.4. Discussion

As seen from the presented results, incubating the microalgae after PEF-treatment had a positive effect not only on the FAME yields but also on the reaction time. While a total recovery was not achieved for 1h reaction time, the FAME yields nonetheless increased on that time point when compared with the one without incubation (70% of total FAMEs were recovered for 1.5 MJ/kgdw with incubation against 46% without). To the best of our knowledge, there is no reason to assume that PEF-treatment has an effect on the kinetics of the reaction itself. This increase then, is most likely due to a modification of the microalgae structure, caused by PEF-treatment which subsequently triggers biological functions during incubation. One possibility, as proposed by Martínez et al, could be the release of hydrolytic enzymes from the interior of the microalgae after PEF which then proceed to degrade the cell wall during incubation[51]. This would result to enhanced solvent penetration to the microalgae cell and accelerated removal of FAMEs. To prove this theory however, more study is required and in particular identification of these enzymes along with their mechanism.[51]

It is challenging to explain the ineffectiveness of direct transesterification on the mixotrophic *A. protothecoides*. Apart from a lesser amount of C18:2 compared to autotrophic microalgae, the final lipid content and FAME profile of the two cultivation modes were relatively similar. Moreover, the lipid extraction was very efficient on the mixotrophic cultivation, even more than on the autotrophic, which

suggests that the cells are more vulnerable to solvents. One assumption for this behavior was that leftovers from the cultivation medium might prevent the transesterification to take place since the mixotrophic cultivation took place with the addition of glucose. To test this hypothesis, the biomass pellet was washed once with distilled water before proceeding with the direct transesterification without any improvement of the results, however (data not shown). More in-depth examination is thus required, including evaluating the reaction conditions themselves.

3.5. Benefits and challenges of implementing direct transesterification

The previous results validate the efficiency of direct transesterification for the autotrophically cultivated microalgae. Significantly, the process is effective on wet microalgae, avoiding thus any drying expenses, a critical parameter for biofuel production. The fact that incubation after PEF-treatment is required in order to achieve total FAME recovery is not necessarily a drawback. Apart from increased FAME yields and reduction of the treatment energy, incubating the biomass after PEF can bring an additional benefit if the process is examined in the context of a biorefinery. A spontaneous release of water soluble microalgae components has been reported after PEF-treatment [52,53]. This has been verified in the case of *A. protothecoides* in a previous work [37] where extrusion of carbohydrates was reported over a 24-h incubation after PEF-treatment followed by nearly complete lipid extraction. Direct transesterification can act in a similar manner as a second step in a cascade process for the exploitation of the spent biomass after the removal of the water-soluble components. The two-step transesterification as shown in Figure 1 was effective enough but at the demand of large solvent amounts. For the processing of just 1 L of lipids, more than 140 L of ethanol and 680 L of hexane would be required. Bypassing the lipid extraction step by immediately converting them to FAMEs would remove not only equipment costs but also significant energy expenses for the solvent recycling.

Direct transesterification is not without challenges, though. Moisture content is reported to decrease the reaction's efficiency [54]. Even though in this study total conversion was achieved from wet biomass at

concentrations of 250 g/L, the water content might have a negative effect on further optimization of the reaction conditions or in case an alkaline catalyst is used for the reaction. A final point of discussion is the amount of methanol used in this direct transesterification protocol. The stoichiometric molar ratio of triglycerides to alcohol is 1:3 although in practice higher ratios are applied. In literature, for conventional transesterification processes an excess of 1:6 is often reported [55] although these typically use alkaline catalysts. In one comprehensive review, for acid catalyzed transesterification, like this study, various methanol molar excesses are reported, from 1:6 up to 1:56, depending on the feedstock and the reaction conditions [9]. Based on the total fatty acid composition from Table 1 and Table 2, the average molecular weight of lipids produced from autotrophic *A. protothecoides* could be calculated as equal to 0.883 kg/mol. For the conversion of 1 L of saponifiable lipids i.e. 1.16 moles, based on our experimental conditions, 14.8 L of methanol would be required which would correspond to 364 moles. That would mean, that the molar ratio of lipid to alcohol is currently equal to 1:311, considerably higher than the ones mentioned previously. The reduction of methanol in this methodology (a goal beyond the scope of this work) could be part of future works.

4. Conclusions

The goal of this study was to examine the application of direct transesterification on microalgae after PEF-treatment. The process was very successful on autotrophically cultivated *A. protothecoides* with a total FAME recovery achieved with treatment energy as low as 0.25 MJ/kg_{dw}, if a 24 h incubation step is implemented after PEF. On the contrary, very low yields were observed for mixotrophically cultivated *A. protothecoides* for still unknown reasons. This question along with the further optimization of the overall direct transesterification should be further explored in future experiments.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to publish this article.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no competing interest.

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Author's contributions

Ioannis Papachristou: conceptualization, methodology, investigation, writing-original draft preparation. Shiqi Zhang: investigation, Visualization. Olga Gorte: investigation, resources. Katrin Ochsenreither: validation, Rüdiger Wüstner: Investigation. Natalja Nazarova: investigation, resources. Wolfgang Frey: writing - review & editing, project administration. Aude Silve: conceptualization, supervision, methodology, writing - review & editing.

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574 Table 1: Gas chromatography analysis of the FAME content of autotrophic *Auxenochlorella protothecoides* after two-step transesterification or
575 direct transesterification. The biomass was processed either immediately or after a 24-h incubation after PEF-treatment. As reference method,
576 freeze-dried biomass was bead-milled and subjected to direct transesterification with a methanol: hexane, 1:0.17 vol/vol co-solvent. The average of
577 duplicates from two independent cultivations is presented along with standard deviation.

	% of Total detected Fatty Acid					
	Autotrophic cultivation, no incubation after PEF-treatment					
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3n6
Total FAME from reference method	1.0 ± 0.1	9.8 ± 0.3	2.1 ± 0.1	39.5 ± 0.6	41.6 ± 0.4	6.1± 0.1
Two-step transesterification 0.25 MJ/kg_{DW}	0.0 ± 0.0	7.3 ± 2.9	0.0 ± 0.0	40.7 ± 1.0	44.7 ± 3.4	7.2 ± 0.4
Two-step transesterification 1.5 MJ/kg_{DW}	1.1 ± 0.0	10.3 ± 0.1	2.3 ± 0.0	39.5 ± 1.3	42.6 ± 1.5	4.2 ± 2.8
Direct transesterification Control	0.9 ± 0.1	10.3 ± 0.1	1.5 ± 0.2	31.7 ± 1.9	47.3 ± 2.1	8.3 ± 0.3
Direct transesterification	1.0 ± 0.1	10.3 ± 0.0	1.6 ± 0.0	31.5 ± 2.1	46.7 ± 1.0	8.8 ± 1.1

0.25 MJ/kg_{DW}						
Direct transesterification 1.5 MJ/kg_{DW}	1.1 ± 0.1	10.1 ± 0.2	2.1 ± 0.2	36.8 ± 0.1	42.1 ± 0.7	7.6 ± 0.6
Autotrophic cultivation, 24 h incubation after PEF-treatment						
Two-step transesterification 0.25 MJ/kg_{DW}	1.0 ± 0.1	9.8 ± 0.1	2.5 ± 0.1	39.2 ± 0.3	41.0 ± 0.6	6.5 ± 0.2
Two-step transesterification 1.5 MJ/kg_{DW}	1.1 ± 0.0	9.7 ± 0.1	2.7 ± 0.1	39.2 ± 0.5	40.4 ± 0.4	6.9 ± 0.0
Direct transesterification Control	1.0 ± 0.1	10.1 ± 0.3	1.9 ± 0.3	32.1 ± 2.6	46.0 ± 2.1	8.9 ± 0.4
Direct transesterification 0.25 MJ/kg_{DW}	0.9 ± 0.1	9.8 ± 0.1	2.3 ± 0.1	38.9 ± 0.9	41.5 ± 0.1	6.5 ± 0.5
Direct transesterification 1.5 MJ/kg_{DW}	1.0 ± 0.2	9.7 ± 0.2	2.4 ± 0.1	38.9 ± 0.7	41.3 ± 0.1	6.7 ± 0.3

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581 Table 2: Gas chromatography analysis of the FAME content of mixotrophic *Auxenochlorella protothecoides* after two-step transesterification or
582 direct transesterification. The biomass was processed either immediately or after a 24-h incubation after PEF-treatment. As reference method,
583 freeze-dried biomass was bead-milled and subjected to direct transesterification with a methanol: hexane, 1:0.17 vol/vol co-solvent. The average of
584 duplicates from two independent cultivations is presented along with standard deviation.

	% of Total detected Fatty Acid					
	mixotrophic cultivation, no incubation after PEF-treatment					
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3n6
Total FAME from reference method	0.5 ± 0.0	9.1 ± 0.0	1.2 ± 0.0	67.3 ± 0.3	21.8 ± 0.4	8.3± 0.2
Two-step transesterification 0.25 MJ/kg_{DW}	0.9 ± 0.4	8.6 ± 0.3	4.3 ± 2.8	65.1 ± 2.6	19.7 ± 0.0	3.1 ± 1.4
Two-step transesterification 1.5 MJ/kg_{DW}	0.5 ± 0.0	9.6 ± 0.1	2.2 ± 0.6	66.4 ± 0.8	20.1 ± 0.6	1.8 ± 0.0
Direct transesterification Control	0.7 ± 0.0	11.3 ± 0.0	0.5 ± 0.3	53.0 ± 0.9	30.4 ± 0.7	3.7 ± 0.3
Direct transesterification	0.7 ± 0.1	10.9 ± 0.9	0.5 ± 0.3	53.7 ± 0.6	30.3 ± 0.8	3.6 ± 0.4

0.25 MJ/kg_{DW}						
Direct transesterification 1.5 MJ/kg_{DW}	0.7 ± 0.0	11.0 ± 0.4	0.5 ± 0.3	53.9 ± 1.2	29.9 ± 0.4	3.6 ± 0.3
mixotrophic cultivation, 24 h incubation after PEF-treatment						
Two-step transesterification 0.25 MJ/kg_{DW}	0.6 ± 0.0	9.2 ± 0.4	1.9 ± 0.3	65.5 ± 0.2	21.2 ± 0.7	1.9 ± 0.0
Two-step transesterification 1.5 MJ/kg_{DW}	0.6 ± 0.0	9.6 ± 0.2	1.8 ± 0.4	64.6 ± 0.4	21.8 ± 0.1	2.1 ± 0.0
Direct transesterification Control	0.6 ± 0.1	10.2 ± 0.6	0.5 ± 0.3	54.0 ± 0.9	31.0 ± 0.3	3.3 ± 0.4
Direct transesterification 0.25 MJ/kg_{DW}	0.7 ± 0.0	11.3 ± 0.3	0.5 ± 0.4	53.7 ± 3.2	30.0 ± 2.6	3.6 ± 0.4
Direct transesterification 1.5 MJ/kg_{DW}	0.7 ± 0.0	11.0 ± 0.5	0.6 ± 0.3	57.1 ± 1.0	27.2 ± 0.4	3.1 ± 0.1

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