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Alkaline direct transesterification of different species of *Stichococcus* for bio-oil production

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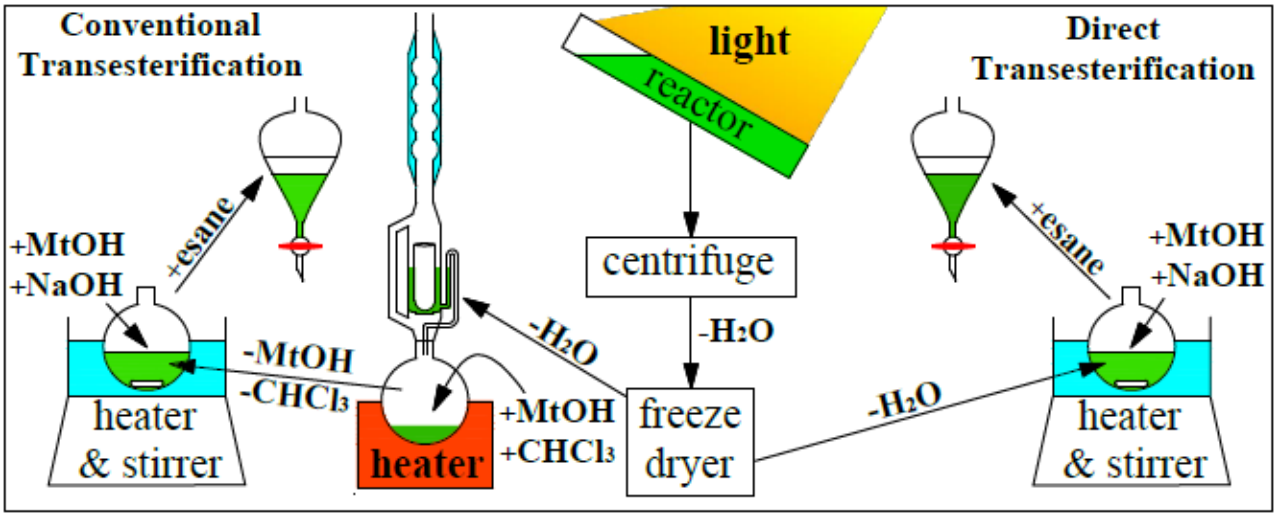
Graphical abstract



Highlights

- Direct transesterification of *Stichococcus* genus without any extraction
- The maximum bio-oil yield was close to 17 % under alkaline conditions
- Influence of the catalyst, methanol/biomass weight ratio, time and temperature
- The pre-contact time did not affect bio-oil yield
- The increase of biomass water content significantly reduced the bio-oil yield

Abstract

The cost of bio-oil refining from microalgal biomass can be significantly reduced by combining extraction and transesterification. The characterisation and optimisation of the combined steps have been carried out on strains of *Stichococcus bacillaris*, focusing on catalyst type and concentration, reaction time and temperature, methanol/biomass ratio, pre-mixing time and water content in the biomass. The bio-oil yield has been referenced as production of fatty acid methyl esters (FAMEs). The maximum yield (~17%) was achieved using dried biomass with

alkaline catalyst at 60°C and methanol/biomass weight ratio of 79:1. Alkaline catalyst conditions gave faster reaction rates and higher bio-oil yields than acid catalyst. Yield was also strongly affected by water content in the biomass. A mechanistic interpretation has been proposed to elucidate the effect of the different operating conditions. However, the structural characteristics of the Chlorophyta cell wall can be very different, leading to different bio-oil yields when the same protocol is applied. Therefore, the optimised protocol of direct transesterification for *Stichococcus bacillaris* strains was tested on other *Stichococcus* strains and several other Chlorophyta species characterised by a different cell wall structure. It was clearly demonstrated that different results for bio-oil yield were obtained within the same microalgal species and much more within different microalgal genera.

Keywords: Bio-oil; microalgae; biorefinery; transesterification; *Stichococcus*.

Introduction

Microalgae, as feedstock for food/feed, chemicals, fuels and "specialties" in cosmetics and healthcare, have received strong interest in regard to three issues [1-6] namely: i) microalgae production does not compete for use of arable land with food/feed plant cultivation; ii) microalgae production may be considered an effective carbon capture and storage process contributing to the reduction of carbon dioxide concentration in the atmosphere [7-9]; iii) microalgae are capable of accumulating significant quantities of lipids, polar-lipids, wax and sterols [10,11]. In particular, microalgae, like higher plants, produce storage lipids in the form of triacyglycerols (TAGs). Although TAGs can be used to produce a wide variety of chemicals,

the majority can be employed to produce fatty acid methyl esters (FAMEs), as a substitute for fossil-derived diesel [12]. This bio-oil, known as biodiesel, can be synthesised from TAGs through a simple transesterification reaction in the presence of alcohol under acidic or basic conditions [2,5,7]. Recent cost assessments concerning bio-oil from microalgae have highlighted that not only the upstream (cultivation and harvesting) but also the downstream (oil-extraction and refining) processes can be bottlenecks in terms of capital and operating costs [12-14]. Oil extraction from algal biomass and its transesterification accounts for approximately 40% of the process energy requirement [15,16]. In particular, the crude microalgae are characterised by a biodiesel energy content ranging from 20 to 38 MJ/kgDW: only 0.37 MJ/MJbiodiesel of energy are required in microalgal oil extraction for wet extraction and cultivation under low-nutrient conditions, while the energy required for transesterification is 0.0024 MJ/MJbiodiesel [17]. Consequently bio-oil refining does need significant improvements to impact on the process economy [18,19]. The transesterification of TAGs in a one-pot process, suppressing the pre-extraction step, has been proposed as a significant simplification of the bio-oil production [20-22]. This approach - known as *in situ* or *direct* transesterification - was adopted in the past as an analytical technique in the preparation of FAMEs in connection with the determination of fatty acid composition in lipid incorporating tissues [20]. Attempts to use the process as an alternative approach for transesterification of oil palm pulp and other materials have been previously reported [21,22].

Recently, direct transesterification of some microalgal species under acidic conditions has been reported [23]. The authors found that the amount of total FAMEs produced by direct transesterification was significantly higher compared with that obtained by extraction followed by transesterification. It was suggested [24] that direct transesterification is capable of producing more FAMEs than expected from the TAG content alone, suggesting also the capture of fatty acids from membrane phospholipids. However, it was pointed out [25] that the process

yield is affected by many process variables. The authors investigated the effects of reaction time, temperature, type and concentration of catalyst and methanol/biomass ratio on direct transesterification of *Chlorella vulgaris*. The preliminary results indicated that alkaline conditions may ensure higher conversion of TAG in a shorter time with respect to that assessed under acidic conditions. Salam et al., [26], investigated the kinetics of direct alkali-catalysed transesterification of *Chlorella vulgaris* to produce bio-oil. They showed that the maximum bio-oil yield can be achieved in 10 minutes before saponification occurs. Moreover, it was demonstrated that other reactions, such as FAMEs and triglycerides saponification and free fatty acids neutralisation, occur with the desired bio-oil diesel synthesis in a direct NaOH-catalysed transesterification [26].

Others have pointed out that direct transesterification of oil seeds was quite tolerant to the biomass water content with respect to conventional extraction and transesterification processes [27]. More recently, acidic *in situ* transesterification was successfully applied directly on wet microalgae with a process yield higher than 90% [28]. Reaction temperature, wet cell weight, reaction time, and catalyst volume all affect the conversion yield. Moreover, direct transesterification results were more tolerant of the level of water in biomass compared to the two-steps transesterification [26].

FAME production from *Stichococcus bacillaris* cultures has been previously evaluated. This microalgal strain can produce up to 0.256 g/(Ld) of biomass when grown under autotrophic conditions at a pH ranging from 3.0 to 8.5 [3]. Moreover, higher biomass concentration can be established in thin flat photobioreactors (up to 4.7 g/L) without TAG concentration modification [1]. *Stichococcus* is a green microalgae characterised by a homogeneous cell wall structure in which cellulose or cellulose derivatives are the main skeletal building-blocks.

This work therefore was designed to characterise and optimise the production of bio-oil by direct transesterification of *Stichococcus bacillaris* under alkaline conditions. Moreover, it aimed to understand if a single optimised protocol can be used with similar results on different microalgae belonging to the same species and to different genera. Tests were carried out with six different *Stichococcus* species (*S. bacillaris, S. chodatii, S. cylindricus, S. fragilis, S. deasonii and S. jenerensis*) and four different genera (*Chlorella vulgaris, Chlamydomonas reinhardtii, Scenedesmus vacuolatus,* and *Maesotaenium caldariorum).*

The bio-oil yield was affected by different operating conditions, namely: mixing time, catalyst concentration, methanol/dried-biomass weight ratio, temperature, reaction time and water content in the biomass. The results were compared with data obtained using a conventional method of extraction-transesterification [22,29]. A comparison of the results with those obtained through the adoption of literature protocols under acidic conditions was also performed.

Materials and Method

2.1. Algal culture and biomass preparation

The following species from the ACUF collection were investigated: *Stichococcus bacillaris* ACUF-155, *Stichococcus chodatii* ACUF-110, *Stichococcus cylindricus* ACUF-103, *Stichococcus fragilis* ACUF-108, *Stichococcus deasonii* ACUF-579 and *Stichococcus jenerensis* ACUF-610, *Chlorella vulgaris* ACUF-059, *Chlamydomonas reinhardtii* ACUF-027, *Scenedesmus vacuolatus* ACUF-053, and *Maesotaenium caldariorum* ACUF-611 [31]. The experimental procedures for algal culture were performed as follows. The microalgal cells were

grown under autotrophic conditions in a sterilised Bold Basal Medium (BBM) containing essential inorganic ions and a minimal quantity of organic compounds, such as vitamins [30]. Algae were grown at pH 6.8 in 2 L inclined square bubble column photobioreactors thermostated at 23±1 °C [1].

The lamps (light irradiance from fluorescent tubes: 250 μE/(m^2s), M2M engineering), fixed to the ceiling of the climate chamber, continuously illuminated the upper side of the photobioreactors. For the aeration and mixing, air was sparged (0.5 vvm) at the bottom of the photobioreactor by means of a plastic tube with numerous holes at a distance of about 1 cm. The gas-off line was connected to a gas analyser (Solaris biotechnology) to determine CO_2 concentration in the effluent air streams. Microalgal biomass was harvested by centrifugation (Eppendorf Centrifuge 5804 R) for 20 minutes at 5000 rpm and 5°C to separate the microalgal biomass from the medium. The harvested biomass was stored at 20°C. Depending on experimental designs, the cell pellets were either freeze-dried or used as wet biomass. The biomass dry weight has only a residual water content in the freeze dried samples of 2%.

2.2. Production of bio-oil from algal biomass

Bio-oil was produced from microalgal biomass using two methods: (i) lipid extraction from with Soxhlet according to the Bligh-Dyer method followed by transesterification, and (ii) direct transesterification. Freeze-dried biomass or wet biomass was used as feedstock during direct transesterification tests.

Each test run was carried out in triplicate and the standard deviations (SD) are reported.

2.2.1. Conventional transesterification

In laboratory practice [17], both non-polar and polar organic solvents were added to the microalgal cells to ensure the complete extraction of TAG and polar lipids from algae [32]. Thus, the lipid fraction was extracted from the algal cells according to a method previously described [33-35]. A pre-weighed spherical flask (empty flask) (0.25 L) was loaded with (i) a cellulose extraction thimble with about 0.4 g of freeze-dried algal biomass placed into the main chamber of the Soxhlet extractor, and (ii) the extraction solvents comprising chloroform/methanol in a 2:1 ratio. The flask was immersed in an oil bath and heated until a solvent reflux was observed from the condenser. At the end of the extraction procedure, solvents were evaporated at 60°C under vacuum. The lipids, contained in the spherical flask, were weighed after solvent evaporation (full flask). The total lipid content was calculated as:

$$Lipid\ yield(\%) = \frac{Lipid\ mass}{Dry\ algal\ mass} \times 100$$

The extracted samples were stored at 4°C. A literature procedure was adopted for the conventional alkaline transesterification reaction [22]. A given amount of extracted lipids was mixed with an appropriate volume of an alkaline solution of methanol in a flask equipped with a condenser and thermostated at 65°C in a water bath for 3 min with magnetic stirring. The mixture was then filtered and the liquid phase stored at 4°C prior to gas chromatograph (GC) analysis. Acid-catalysed conventional transesterification was carried out according to two protocols [37].

2.2.2. Direct transesterification in alkaline-catalysed conditions

A sample of microalgal biomass (0.1g dry matter) was suspended in a closed glass test tube containing alkaline methanol solution. Tests were carried out under a wide range of operating conditions obtained by changing the methanol/freeze-dried biomass weight ratio (r_{mdb}), the concentrations of NaOH in methanol (C_{NaOH}), the pre-mixing time (t_p) and the temperature and

reaction time (T and t_r, respectively). The biomass water content (defined as $w_{H2O}/w_{biomass}$%) and the methanol/wet biomass weight ratio (r_{mwb}) was also investigated. The concentrations of NaOH in methanol were increased up to 2.0% (w/w). The reaction temperature was set in the interval 20-80°C while the reaction time investigated was varied from 1 to 20 minutes. The pre-mixing time – mixture stirring period before being heated up to reaction temperature – was increased up to 6 h.

For the experiments on biomass with different water content, samples of wet microalgal biomass recovered from the bioreactor underwent a freeze drying process for different operation times. The residual water content inside dried biomass was estimated to be around 1%. Samples with 9, 10, 12, 19 and 21% of biomass water content were thus obtained and used in the direct transesterification experiments under the operating conditions previously optimised on freeze-dried biomass. With a fixed biomass water content, the methanol/wet biomass weight ratio was varied from 24:1 to 790:1.

The reaction was stopped by cooling the samples to room temperature. After transesterification, bio-oil was extracted 3 times using 3 mL of hexane each [34]. The organic layers containing bio-oil were collected and transferred to a separate tube in which they were combined. The hexane phase was stored at 4°C, prior to further GC analysis.

A linear optimisation procedure was adopted to find the best values for the operating conditions.

2.2.3. Direct transesterification in acidic-catalysed conditions

Acidic-catalysed direct transesterification was carried out according to two previously reported protocols [35]. Samples of freeze-dried algal biomass (0.2 g) were placed in different closed glass test tubes and mixed with a methanol (1 mL) and sulfuric acid (0.2 mL) mixture. Depending on the experimental design, 1 mL of chloroform (or methanol) was added to the

tube. The transesterification process was carried out at 90 °C for 40 minutes with magnetic stirring and controlled reflux. After reaction, samples were cooled to room temperature and mixed with 3 mL of double-distilled water and 3 mL of hexane. The samples were centrifuged at 5000 rpm for 10 min at 5°C; the organic phase, containing bio-oil, was collected and washed with 2 mL of double-distilled water. The samples were again centrifuged at 5000 rpm for 10 min at 5 °C and the hexane phase was then filtered and stored at 4 °C before GC analysis.

2.3. Bio-oil analysis

The qualitative and quantitative analysis of bio-oil in terms of FAMEs were carried out using an Agilent 7820A GC equipped with a Flame-ionisation detector (FID) and Agilent DB-WAXTER column (30m x 0.320mm x 0.50 film thickness). The temperature was increased from 100 °C to 230 °C at 10 °C min^{-1}. Helium (1mL/min in constant flow) was used as carrier gas and the detector temperature was 300 °C.

Peak areas were used to quantify each FAME relative to the internal standards. The bio-oil yield was defined as:

$$Bio-oil\ yield(\%) = \frac{Total\ FAME\ mass}{Dry\ algal\ mass} x\ 100$$

After conventional transesterification, it was also possible to calculate the transesterified lipids yield, expressed as:

$$Transesterified\ lipid\ yield(\%) = \frac{Total\ FAME\ mass}{Lipid\ mass} x\ 100$$

The yield of each methyl ester was expressed as:

$$Methyl\ ester\ yield(\%) = \frac{Methyl\ ester\ mass}{Dry\ algal\ mass} x\ 100$$

Each of the above procedures for transesterification is based on different sequences of several laboratory steps. For each single procedure, a sample of dried microalgae was divided into three aliquots and the complete sequence of steps repeated **3** times.

Results and Discussion

3.1. Conventional alkaline-catalysed transesterification with *Stichococcus* species

An amount of 0.4 g of dried biomass was used to determine the lipid content within the biomass and the bio-oil yield after conventional alkaline transesterification (1.5% NaOH w/w, 3 min of reaction, methanol/biomass weight ratio 79:1, 60°C) with *S. bacillaris, S. cylindricus, S. fragilis, S. jenerensis*, *S. deasonii* and *S. chodatii* (Figure 1).

To verify completeness of extraction, residual biomass was subjected to direct alkaline catalysed transesterification. The same transesterification conditions (1.5% NaOH w/w, 3 min of reaction, methanol/biomass weight ratio 79:1, 60°C) were employed for the residual biomass. The results in terms of bio-oil yield were close to zero, thus indicating that, after extraction, no further esterifiable lipids remained in the biomass residue.

Lipid yield ranged from 8.6% (*S. fragilis*) to 35.5% (*S. bacillaris*) and was very similar for *S. cylindricus* and *S. deasonii* (~24%), and *S. jenerensis* and *S. chodatii* (~18%) strains. Bio-oil yield ranged from 1.4% for *S. jenerensis* to 9.5% for *S. deasonii*. Calculating the FAME percentage with respect to lipid content (esterified lipid yield), the best value was obtained with *S. deasonii* (~46%), while the lowest was reached by *S. bacillaris* (~14%) (Figure 1). Based on these observations, it can be concluded that significant variations may be found in both extracted lipids and transesterified lipid yield even within same genus.

It is however important to note that *S. bacillaris* gave the highest lipid yield (35.5%), but the lowest transesterified lipid yield (13.7%). Because of its high lipid content among the

investigated microalgal species, *S. bacillaris* was selected to significantly improve the bio-oil yield by means of direct alkaline transesterification.

The results of two preliminary tests on *S. bacillaris* for both direct and conventional alkaline transesterification are reported in Figure 2. Under the adopted conditions (1.5% NaOH, 60 ºC, 3 min of reaction and 79:1 methanol/biomass weight ratio), a higher bio-oil yield (17.2%) was obtained by applying direct transesterification compared to the conventional one (6.7%). The results indicated that the predominant methyl esters were palmitate (C16:0), stearate (C18:0), oleate/elaidate (C18:1), linoleate (C18:2), linolenate (C18:3) and arachidate (C20:0). The methyl ester yield for the direct transesterification was in all cases twice that obtained using the conventional protocol.

These results encouraged us to improve the direct transesterification protocol for *Stichococcus*. Attention was focused on *S. bacillaris* and the optimised protocol has since been tested on other selected *Stichococcus* species and then on other microalgal **genera.**

3.2. Direct transesterification under alkaline conditions

Effect of catalyst concentration

Figure 3a shows the results of direct alkaline catalysed transesterification as a function of catalyst concentration. Tests were carried out with freeze-dried microalgal biomass in alkaline-methanol solutions characterised by a wide interval of concentrations: NaOH catalyst in methanol was increased up to 2.0% by weight. Fixed operating conditions were reaction temperature (T=60 °C), reaction time (t_r=3 min) and methanol/freeze-dried biomass weight ratio (r_{mb}=79:1). The results indicated that bio-oil was produced only when NaOH was present. The yield was appreciably affected by catalyst concentration, with the maximum obtained with

1.5% NaOH. With a further increase of NaOH concentration, the saponification reaction led to a decreased yield.

These results were consistent with those reported by others [36,37]. In particular, Dorado et al. [36] investigated the effect of KOH concentration in the range 0 to 2.3% (weight of KOH/weight of oil) and Encinar et al. [37] studied the influence of NaOH concentration in the range 0 to 1.0% (w_{NaOH}/w_{oil}). Literature results have shown that the ester conversion was zero without catalyst and that marked increases of alkaline catalyst concentration gave rise to a small decrease in the bio-oil production yield.

Thus, a catalyst concentration of 1.5% NaOH was chosen for the following investigations.

Effect of reaction temperature

Bio-oil yield in tests at temperatures ranging between 20°C and 80°C are reported in Figure 3b. Operating conditions of the tests were: freeze-dried biomass, methanol to biomass weight ratio 79:1, NaOH concentration in methanol 1.5% (by weight) and transesterification reaction time at 3 min. The results indicated that bio-oil yield increased significantly with temperature and approached the highest values (16.25%) at 60°C. For the highest temperatures (80°C), there was a marked decrease in yield of up to 7.7%. This can again be attributed to the increased relevance of the saponification process by the alkaline catalyst compared to methanolysis [38].

A reaction temperature of 60 °C was thus chosen for the following investigations.

Effect of reaction time

The results of the performance of direct alkaline transesterification of freeze-dried microalgal biomass with a reaction time set in the range 1-20 min are reported in Figure 3c. Operating conditions employed for the tests were: reaction temperature 60 °C, methanol to biomass weight

ratio 79:1 and catalyst concentration of 1.5%NaOH (w/w). Bio-oil yield increased with a reaction time between 1 and 3 min and was quite constant at about 17%, with a reaction time within the interval 3 -12 min and decreased for more prolonged reaction times.

These results are partially in agreement with those reported elsewhere. Freedman et al. [39] pointed out that the conversion of seed-oils (soybean, peanut, cotton seed, and sunflower) to FAMEs did not alter significantly (93-98%) for reaction times ranging between 1 and 60 minutes, whereas other [40] observed high FAMEs yield from *Spirulina platensis* only after 10 min of reaction. Similar results were reported regarding the transesterification of beef tallow with methanol [41] where it was found that the reaction was slow during the first minute due to the mixing of methanol and beef tallow, proceeding faster in the following 4 min. The amounts of mono and diglycerides increased at the beginning and decreased after 15 min of reaction time. Others, instead, showed that the maximum bio-oil yield from microalgae can be achieved in 10 min before saponification occurs [26].

It is reasonable that prolonged reaction times (12-20 min, under the conditions adopted) may lead to higher losses of FAMEs due to thermal degradation processes or different chemical reactions, such as alkaline hydrolysis to fatty acids (FA) [42], "Claisen-like" condensation reactions [43], and alcoholysis with phytol and other alcohols derived from the lysis of algal material.

As indicated in Figure 4, a parallel-series reaction scheme can be hypothesised to summarise the observed effects of methanol, catalyst and reaction temperature and time.

Effect of methanol/biomass weight ratio

Direct transesterification experiments were carried out with methanol to freeze-dried biomass weight ratios (r_{mb}) set at 24:1, 39:1 and 79:1 (data not shown). The operating conditions were:

alkaline catalyst concentration 1.5%% (w/w), reaction temperature 60°C and reaction time of 3 min. The bio-oil yield did not change with the methanol to biomass weight ratio within the range 39:1 - 79:1. These results were in accord with those previously reported [24] where it was also noticed that the FAMEs yield did not change with the methanol/freeze-dried biomass weight ratio even when as high as 118:1. These results seem to indicate the absence of methanol limitation under the investigated operating conditions and therefore to exclude any problem of methanol diffusion inside the biomass. If methanol diffusion was a limiting step, a first order dependence on methanol concentration would have been clearly observed.

Thus, the subsequent direct transesterification experiments were carried out with the same methanol/ biomass weight ratio used in the previous experiment (r_{mb} = 79:1).

Effect of pre-mixing

The effect of pre-mixing time was also investigated in order to validate the previous consideration about a possible methanol diffusion-limiting process. Tests were carried out with freeze-dried algal biomass in 1.5% NaOH w/w alkaline-methanol solutions. Fixed operating conditions were: reaction temperature (T 60 °C), reaction time (t_r 3 min) and methanol/freeze-dried biomass weight ratio (r_{mb} 79:1).

On increasing the pre-mixing time from 0 to 6 hours, results indicated that the bio-oil yield did not change significantly and ranged from 16 to 18% (data not shown). As a consequence of the finding that pre-mixing did not affect the reaction rate, the effect of diffusion-limited rate can be further excluded.

Effect of biomass water content

The tests on biomass water content were carried out under the following operating conditions: methanol/biomass weight ratio 79:1, catalyst concentration 1.5% NaOH (w/w), reaction

temperature 60 °C, reaction time 3 min and pre-mixing time 0 h. Figure 3d reports data referring to the bio-oil yield as a function of biomass water content. The results showed that the water content did not affect bio-oil yield (14-16%) if biomass/water content ratio was less than 10%, while water content higher than 10% reduced the bio-oil yield to 0.2%. This drastic reduction of bio-oil yield with increasing water content in the biomass is in agreement with results reported by others [24]. The authors pointed out that the FAMEs yield decreases to 50% of the expected FAMEs when the water content of biomass increases from 0 to 400 % (w/w % of biomass).

The explanation of this process is twofold. During direct transesterification, at temperatures higher than ambient, a competition between lipolysis, promoted by lipases present in the cells [43], and methoxy ions, present in the alkaline solution, takes place. It should be considered that although methoxy ions are more nucleophilic than water molecules and consequently capable of a faster attack on the lipids, these ions need first to enter the cells before reacting. Therefore, the transesterification yield is not only the result of a simple attack of methoxy ions on the lipid molecules, but it is also influenced by their ability to diffuse from the external solution into the cell.

A second explanation for the observed behaviour derives from some studies reporting that the lyophilisation process may break up the membrane protein matrix [44, 47], or be sufficient to weaken the cell membrane [48], thus creating an easy access for the alkaline catalyst and promoting direct transesterification. Through this mechanism, the lower the residual water content, the higher will be the degree of cell membrane damage. From this point of view it could be assumed that, for a residual water content lower than 10%, a high degree of cell damage is obtained which enables methoxy ions to easily attack lipid molecules.

A possible solution is to counteract the detrimental effect of biomass water content on FAMEs yield by increasing the methanol volume. Figure 5 reports data obtained using biomass with a water content of 18% under the following operating conditions: catalyst concentration 1.5% NaOH (w/w), reaction temperature 60 °C, reaction time 3 min and pre-mixing time 0 h. The analysis of the data showed that the bio-oil yield increased with the methanol/wet-biomass ratio in the range from 79:1 to 790:1. Notably, the yield increased from 0 to 7%, under the operating conditions investigated.

In addition, the positive effect on bio-oil yield of increasing the methanol volume could be ascribed to the fact that methanol, which is cell disruptive [49], may additionally favour the disruption of the microalgal wet agglomerates.

3.3. Conventional *vs* direct alkaline and acid transesterification

For *Stichococcus* species, the results for both direct and conventional alkaline transesterification are reported in Figure 6. Under the conditions adopted, methanol/biomass weight ratio 79:1, catalyst concentration 1.5% NaOH (w/w), reaction temperature 60°C, reaction time 3 min and pre-mixing time 0 h, for all *Stichococcus* species, with the sole exception of *S. fragilis*, a higher bio-oil yield was gained by applying direct transesterification compared with the conventional one. The higher performance of direct *vs.* conventional transesterification is in agreement with previous investigations [34]. The direct transesterification of *Schizochytrium limacinum* was characterised by 10%–20% higher FAMEs yield compared to the conventional transesterification method [34]. These results may be related to a partial thermal degradation of lipids resulting from elevated temperatures and prolonged times during the Soxhlet extraction step [29] and/or to the release of fatty acids from the alkaline hydrolysis of cellular membranes and disruption of cell walls [50].

To compare alkaline and acidic transesterification process, experiments were also performed under acidic conditions for all investigated *Stichococcus* species (Figure 6). Bio-oil yield, for direct transesterification, carried out under alkaline conditions, was higher than that measured for both direct and conventional processes under the adopted acidic conditions (90 °C, 40 min). Some acidic transesterification tests (data not reported), performed at the same temperature and reaction time for the alkaline process (60 °C, 3 min), showed lower bio-oil yield than that reached adopting the conditions reported by Johnson and Wen [35]. In previous studies [25] it was reported that, in terms of time, the alkaline catalyst (NaOH) outperformed the acid catalyst (H_2SO_4) obtaining higher conversions at lower reaction times.

Moreover, there were no significant differences between results obtained for tests carried out according to the two acidic protocols. These results indicated that the presence of the solvent in acidic-catalysed transesterification, under the adopted experimental conditions, is not essential. In contrast, Johnson and Wen reported that in a solvent free system, FAMEs yield obtained from *Schizochytrium limacinum* by direct transesterification, under acidic conditions, was very low, indicating that the solvent was essential for the reaction [35].

3.4. Conventional vs direct alkaline and acid transesterification on different microalgal genera

The diversity of microalgae within the species and much more within the genera is very large, and the structural characteristics and rigidity of the cell wall can be different from one to the others. These differences may lead to different bio-oil yields when the same protocol is applied. Therefore, the optimised protocol of direct transesterification for *Stichococcus bacillaris* was also tested on four microalgae belonging to different genera of Chlorophyta used for bio-oil production and characterised by a different cell wall. Direct and conventional transesterification under acid and alkaline condition were carried out on *Chlorella vulgaris*, *Chlamydomonas*

reinhardtii, Scenedesmus vacuolatus, and *Maesotaenium caldariorum*. The selected microalgae were characterised by the following differences in cell wall composition. *Chlorella vulgaris* (also known as *Chlorella fusca*) cell wall is complex and resistant. Its composition was studied by Atkinson et al. [51]. Provided that cytokinesis has produced naked autospores within the mother cell wall, cell wall formation starts outside the autospore plasma membrane with the formation of small trilaminar plaques. These expand while the granular material mass inter-autospore decreases, and they eventually fuse to produce a complete trilaminar sheath around each autospore. A microfibrillar, cellulase digestible, layer is deposited between the trilaminar component and the plasma membrane. Meanwhile the corresponding microfibrillar component of the mother cell wall is digested leaving only its resistant trilaminar component. This includes a substance considered to be the polymerised carotenoid, sporopollenin, responsible for the resistance to extreme extraction procedures including acetolysis, and its infrared absorption spectrum [51].

Chlamydomonas reinhardtii cell wall of the biflagellate microalgae is a multilayered, extracellular matrix composed of carbohydrates and 20-25 polypeptides [52]. This cellulose-deficient cell wall is composed of two separate domains, one of approximately 20 proteins held together by non-covalent interactions, and a second domain comprising a few proteins, which are the framework of the wall. *Scenedesmus vacuolatus* cell wall contains ketocarotenoids and sporopollenin [53]. Canthaxanthin, astaxanthin and unidentified ketocarotenoid and lutein were found as integral cell wall components. They are bound to the outer (trilaminar) layer of the complete cell wall which also contains sporopollenin. *Maesotaenium caldariorum* cell wall is very thin. There appear to be no reported studies regarding its chemical composition.

Operating conditions used for direct transesterification were: dried biomass, t_p 0 h, t_r 3 min, T 60 °C, r_{mb} 79:1, C_{NaOH} 1.5% (w/w). Two protocols were adopted for direct acidic transesterification [35]. Table 1 reports the results of the transesterification process for the

investigated strains. Bio-oil yield is reported as % of FAMEs per gram of dried biomass. With the sole exception of *Scenedesmus vacuolatus*, the yield assessed for conventional and direct transesterification under alkaline conditions was higher than that measured for direct and conventional processes under acidic conditions. For all selected genera, the yield obtained by direct transesterification was higher or similar than to that of the conventional process. Moreover, the process under alkaline conditions was more efficient in terms of bio-oil yield than the acidic ones.

Conclusion

The results of alkaline direct transesterification experiments carried out on *Stichococcus bacillaris* indicated that triglycerides were not converted without an alkaline catalyst and approached a maximum value with a catalyst concentration of 1.5% NaOH (w/w). Under alkaline conditions this led to the following: (i) the pre-mixing time did not affect bio-oil yield (~17%); (ii) the yield increased with temperature and approached a maximum at close to 65°C; (iii) this did not change significantly with the methanol to biomass weight ratio within the range 39:1 - 79:1; iv) the yield gradually increased within the first minutes of reaction, approached a constant value within a 3-12 min interval and decreased for times over 12 min. Biomass drying was observed to play an important role in direct transesterification: the bio-oil yield reduced with an increase in biomass water content. A higher yield was obtained increasing the methanol/wet biomass ratio. Under alkaline catalysis conditions the direct transesterification process was more efficient than the acidic ones producing higher yield.

The comparison between the results of tests carried out according to the conventional transesterification method and those according to the direct transesterification method demonstrated that the latter method ensures the highest bio-oil yield for all the investigated *Stichococcus* species and microalgal genera with the sole exception of *Scenedesmus vacuolatus*.

However, not all *Stichococcus* strains and other Chlorophyta species used achieved the expected results in terms of bio-oil yield when the same protocol was applied. This suggests that it is not possible optimise a protocol of direct transesterification for a single microalgal species and use it for other species or genera. It was clearly demonstrated that different results for bio-oil yield were obtained within the same microalgal species and were much more variable within the different microalgal genera.

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References

[1] Olivieri G, Gargano I, Andreozzi R, Marotta R, Marzocchella A, Pinto G, Pollio A. Effects of photobioreactors design and operating conditions on *Stichococcus bacillaris* biomass and biodiesel production. Biochem Eng J 2013;74: 8-14.

[2] Mallik N, Mandal S, Sing AK, Bishai M, Dash A. Green microalga *Chorella vulgaris* as a potential feedstock for biodiesel. J Chem Technol Biotechnol 2012; 87:137-145.

[3] Olivieri G, Marzocchella A, Andreozzi R, Pinto G, Pollio A. Biodiesel production from *Stichococcus* strain at laboratory scale. J Chem Technol Biotechnol 2012; 86:776-783.

[4] Tang D, Han W, Li P, Miao X, Yhong J. CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. Bioresour Technol 2011; 102:3071-3076.

[5] Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Danzins A. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 2008; 54: 621-639.

[6] Sawayama S, Inoue S, Dote Y, Yokoyama SY. CO_2 fixation and oil production through microalga. Energ Convers Manage 1995; 36:729-731.

[7] Zeng X, Danquah M, Chen XD, Lu Y. Microalgae bioengineering: From CO_2 fixation to biofuel production. Renew Sust Energ Rev 2011; 15:3252-3260.

[8] Wang B, Li Y, Wu N, Lan CQ. CO_2 bio-mitigation using microalgae. Appl Microbiol Biot 2008; 79:707-718.

[9] Benemann JR. CO_2 mitigation with microalgae systems. Energ Conver Manage 1997; 38:475-479.

[10] Spolaore P, Joannis-Cassan C, Duran E,. Isambert A. Commercial applications of microalgae. J Biosci Bioeng 2006; 101:87-96.

[11] Metting FB. Biodiversity and application of microalgae. J Ind Microbiol 1996; 17:477-489.

[12] Chisti Y. Biodiesel from microalgae. Biotechnol Adv 2007; 25:294–306.

[13] Stephens E, Ross IL, King Z, Mussgnug JH, Kruse O, Posten C, et al. An economic and technical evaluation of microalgal biofuels. Nat Biotechnol 2010; 28:126-128.

[14] Norsker NH, Barbosa M, Vermuë MJ, Wijffels RH. Microalgal production – A close look at the economics. Biotechnol Adv 2011; 29:24-27.

[15] Ma F, Hanna MA. Biodiesel production: a review. Bioresour Technol 1999; 70:1-15.

[16] Munir N, Sharif N, Naz S, Saleem F, Manzoor F. Harvesting and processing of microalgae biomass fractions for biodiesel production (A review). Sci Tech Dev 2013; 32:235-243.

[17] Zhu L. Biorefinery as a promising approach to promote microalgae industry: An innovative framework. Renew Sustain Energy Rev 2015; 41:1376-1384.

[18] Lardon L, Hèlias A, Sialve B, Steyer J, Bernard O. Life-cycle assessment of biodiesel production from microalgae. Environ. Sci. Technol. 2009; 43:6475-6481.

[19] Krawczyk T. Biodiesel – Alternative fuel makes inroads but hurdles remain. INFORM 1996; 7:801-829.

[20] Lepagè G, Roy CC. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res 1984; 25:1391-1396.

[21] Rashid U, Anwar F. Production of biodiesel through base-catalysed transesterification of safflower oil using an optimised protocol. Energ Fuel 2008; 22:1306-1312.

[22] Rashid U, Anwar F. Production of biodiesel through optimised alkaline-catalysed transesterification of rapeseed oil. Fuel 2008; 87:265-273.

[23] Lewis T, Nichols PD, McMeekin TA. Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. J Microbiol Method 2000; 43:107–116.

[24] Wahlen DB, Willis RM, Seefeldt LC. Biodiesel production by simultaneous extraction and conversion of total lipids from microalgae, cyanobacteria, and wild mixed-cultures. Bioresour Technol 2011; 102:2724–2730.

[25] Velasquez-Orta SB, Lee JGM, Harvey A. Alkaline in situ transesterification of *Chlorella vulgaris*. Fuel 2012; 94:544-550.

[26] Salam KA, Velasquez-Orta SB, Harvey AP. Kinetics of fast alkali reactive extraction/in situ transesterification of *Chlorella vulgaris* that identifies process conditions for a significant enhanced rate and water tolerance. Fuel Proces Technol 2016; 144:212-219.

[27] Haevey A, Lee J, Zakaria R. Reactive extraction of biodiesel from rapeseed. In: Engineers AIoC, editor. AIChE Annual Meeting. Philadelphia, USA; 2008.

[28] Im HJ, Lee HS, Park MS, Yang JW, Lee JW. Concurrent extraction and reaction for the production of biodiesel from wet microalgae. Bioresour Technol 2014; 152:534–537.

[29] McNichol J, MacDougall KM, Melanson JE, McGinn PJ. Suitability of Soxhlet extraction to quantify microalgal fatty acids as determined by comparison with in situ transesterification. Lipids 2012; 47:195-207.

[30] Richmond A, Becker EW. Technological aspect of mass cultivation – A general outine. In: Richmond A, editor. Handbook of microalgal mass culture. CRC Press, Boca Raton, Florida; 1986, p. 245-264.

[31] ACUF, http://www.biologiavegetale.unina.it/acuf.html

[32] Halim R, Danquah MK, Webley PA. Extraction of oil from microalgae for biodiesel production: A review. Biotechnol Adv 2012; 30:709-732.

[33] De Swaaf ME, De Rijk TC, Van Der Meer P, Eggink G, Sijtsma L. High-Cell-Density Fed-Batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*. Biotechnol Bioeng 2003 103:21-29.

[34] Wang G, Wang T. Characterisation of lipid components in two microalgae for biofuel application. J Am Oil Chem Soc 2012; 89:135-143.

[35] Johnson MB, Wen Z. Production of biodiesel fuel from the microalga *Schizochytrium limacium* by direct transesterification of algal biomass. Energ Fuel 2009; 23:5179-5183.

[36] Dorado MP, Ballesteros E, Lopez FJ, Mittelbach M. Optimisation of alkali-catalysed transesterification of *Brassica Carinata* oil for biodiesel production. Energ Fuel 2004; 18:77-83.

[37] Encinar JM, Gonzalez JF, Sabio E, Ramiro MJ. Preparation and properties of biodiesel from *Cynara cardunculus* L. oil. Ind Eng Chem Res 1999; 38:2927-2931.

[38] Ramadhas AS, Jayaraj S, Muraleedharan C. Biodiesel production from high FFA rubber seed oil. Fuel 2005; 84:335-340.

[39] Freedman B, Pryde EH, Mounts TL. Variables affecting the yields of fatty esters from transesterified vegetable oils, J Am Oil Chem Soc 1984 61:1638-1643.

[40] Rodrigues da Silva Baumgartner T, Medes Burak JA, Baumgartner D, Zanin GM, Arroyol PA. Biomass production and ester synthesis by in situ Transesterification/Esterification using the microalga *Spirulina platensis*. International Journal of Chemical Engineering 2013; Article ID 425604, http://dx.doi.org/10.1155/2013/425604.

[41] Ma F, Clements LD, Hanna MA. The effects of catalyst, free fatty acids and water on transesterification of beef tallow. A Soc Agric Eng 1998; 41:1261-1264.

[42] Mazo PC, Rios LA. Esterification and transesterification assisted by microwaves of crude palm oil. Homogeneous catalysis. Latin Am Appl Res 2010; 40:337-342.

[43] Ryckebosch E, Muylaert K, Eeckhout M, Ruyssen T, Foubert I. Influence of drying and storage on lipid and carotenoid stability of the microalga *Phaeodactylum tricornutum*. J Agric Food Chem 2011; 59:11063-11069.

[44] Mata TM, Martins AA, Caetano NS. Microalgae for biodiesel production and other applications: A review. Renew Sust Energ Rev 2010; 14:217–232.

[45] Lee SJ, Yoon BD, Oh HM. Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. Biotechnol Techno1 1998; 12:553–556.

[46] Van Leeuwe MA, Villerius LA, Roggeveld J, Visser RJW, Stefels J. An optimised method for automated analysis of algal pigments by HPLC. Mar Chem 2006; 102:267–275.

[47] Molina Grima E, Acien Fernandez FJ, Robles Medina A. Biotechnology and applied phycology. In: Richmond A, editor. Handbook of Microalgal Culture. Blackwell Publishing, Oxford, UK; 2004.

[48] Pasquet V, Chérouvrier JR, Farhat F, Thiéry V, Piot JM, Berard JB, Kaas R, Serive B, Patrice T, Cadoret JP, Picot L. Study on the microalgal pigments extraction process: performance of microwave assisted extraction. Process Biochem 2011; 46:59–67.

[49] Kusdiana D, Saka S. Two-Step preparation for catalyst-free biodiesel fuel production. Appl Biochem Biotech 2004; 113-116:781-791.

[50] Kim J, Yoo G, Lee H, Lim J, Kim K, Kim CW, Park MS, Yang JW. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnol Adv 2013; 31:862-876.

[51] Atkinson Jr AW, Gunning BES, John PCL. Sporopollenin in the cell wall of *Chlorella* and other algae: ultrastructure, chemistry, and incorporation of 14C-acetate, studied in synchronous cultures. Planta 1972; 107(1):1-32.

[52] Imam SH, Snell WJ. The *Chlamydomonas* cell wall: characterisation of the wall framework. J Cell Biol 1985; 101(4):1599–1607.

[53] Burczyk J, Szkawran H, Zontek I, Czygan FC. Carotenoids in the outed cell-wall layer of *Scenedesmus* (Chlorophyceae). Planta 1981; 151: 247-250.

Legends of Figures and Table

Figure. 1 – Lipid and bio-oil yield under **the** conventional method of extraction and transesterification.

Figure 2 – Methyl esters yield and composition of bio-oil produced with conventional and direct transesterification

Figure. 3 - Direct alkaline catalysed transesterification. a) Effect of catalyst concentration on bio-oil yield; operating condition: r_{mb} 79:1, T 60 °C, t_r 3 min. b) Effect of reaction temperature on bio-oil yield; operating condition: C_{NaOH} 1.5% (w/w), r_{mb} 79:1, t_r 3 min. c) Effect of the reaction time on the bio-oil yield; operating condition: C_{NaOH} 1.5% (w/w), r_{mb}=79:1, T=60 °C. d) Effect of biomass water content; operating conditions: t_p=0 h, t_r=3min, r_{mb}=79:1, T=60 °C, C_{NaOH}=1.5% (w/w) in methanol.

Figure 4 – A parallel-series reaction scheme to describe the observed effects of methanol, catalyst and reaction temperature and time.

Figure 5 - Direct alkaline catalysed transesterification: effect of methanol/wet biomass weight ratio. Operating conditions: t_p 0 h, t_r 3min, r_{mb} 79:1, T 60 °C, C_{NaOH} 1.5% (w/w) in methanol, biomass water content = 18% (w/w). Curve fitting: 2nd order polynomial.

Figure 6 – Bio-oil production under conventional method of extraction and transesterification and direct transesterification. Operating conditions adopted for direct alkaline transesterification were: t_p 0 h, t_r 3 min, T 60 °C, r_{mb} 79:1, C_{NaOH} 1.5% (w/w). For direct acidic transesterification, two protocols were adopted [35].

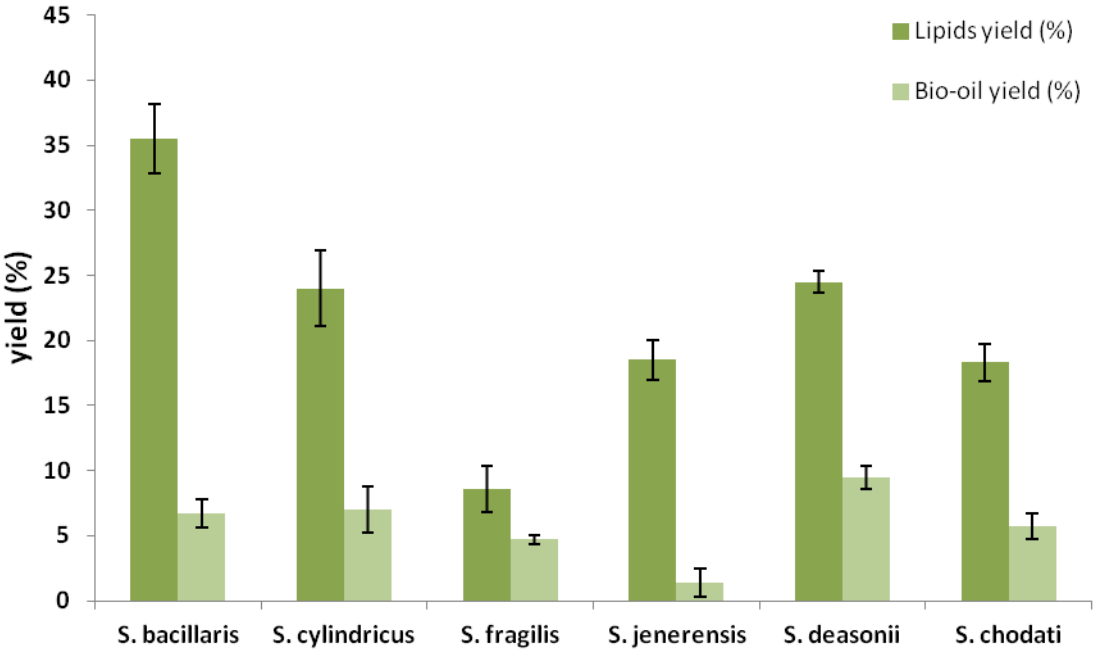

Figure 1

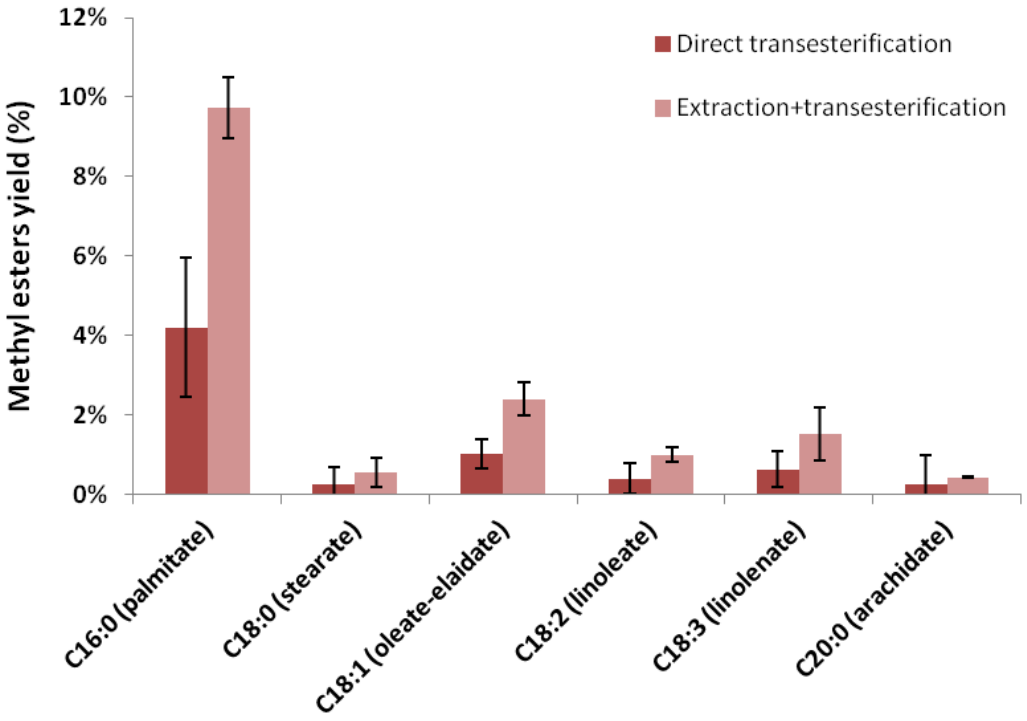

Figure 2

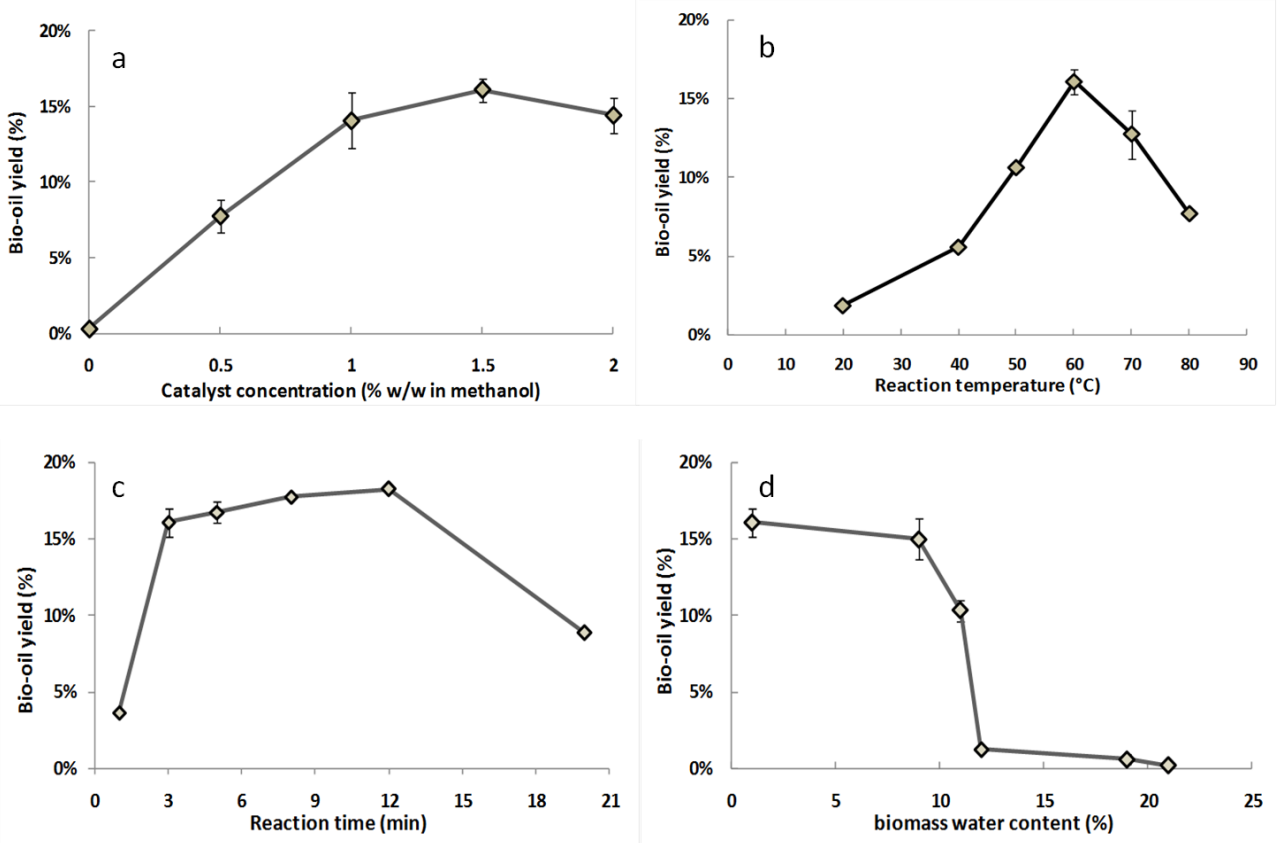

Figure 3

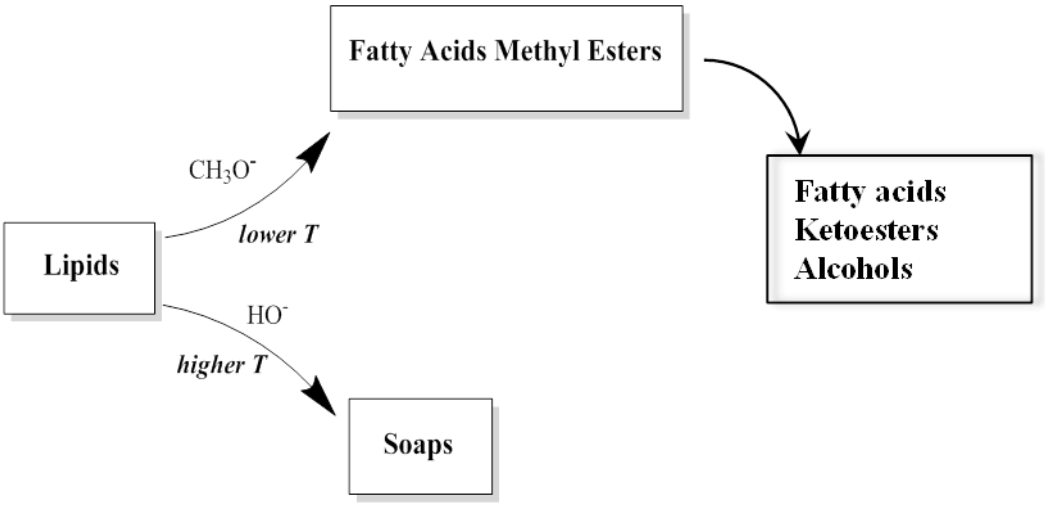

Figure 4

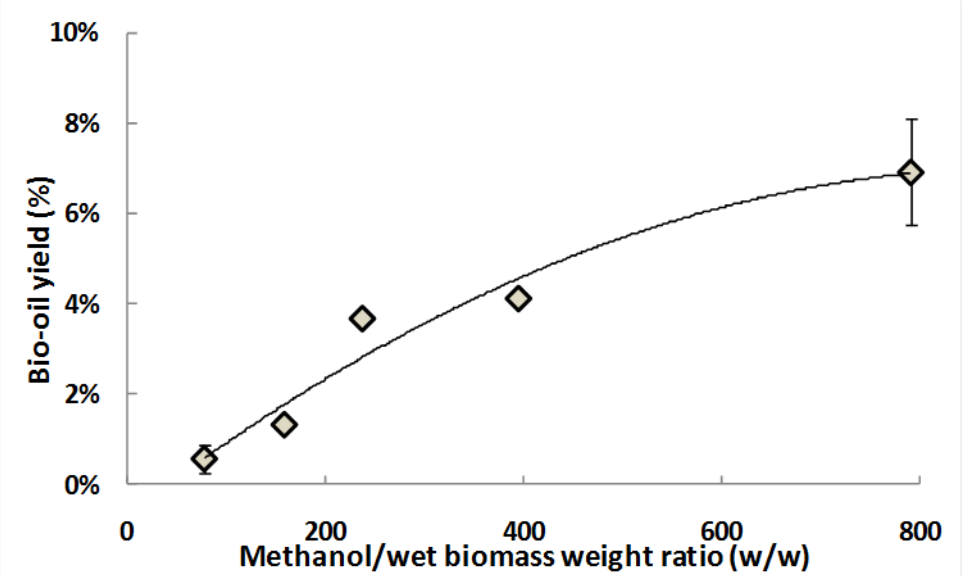

Figure 5

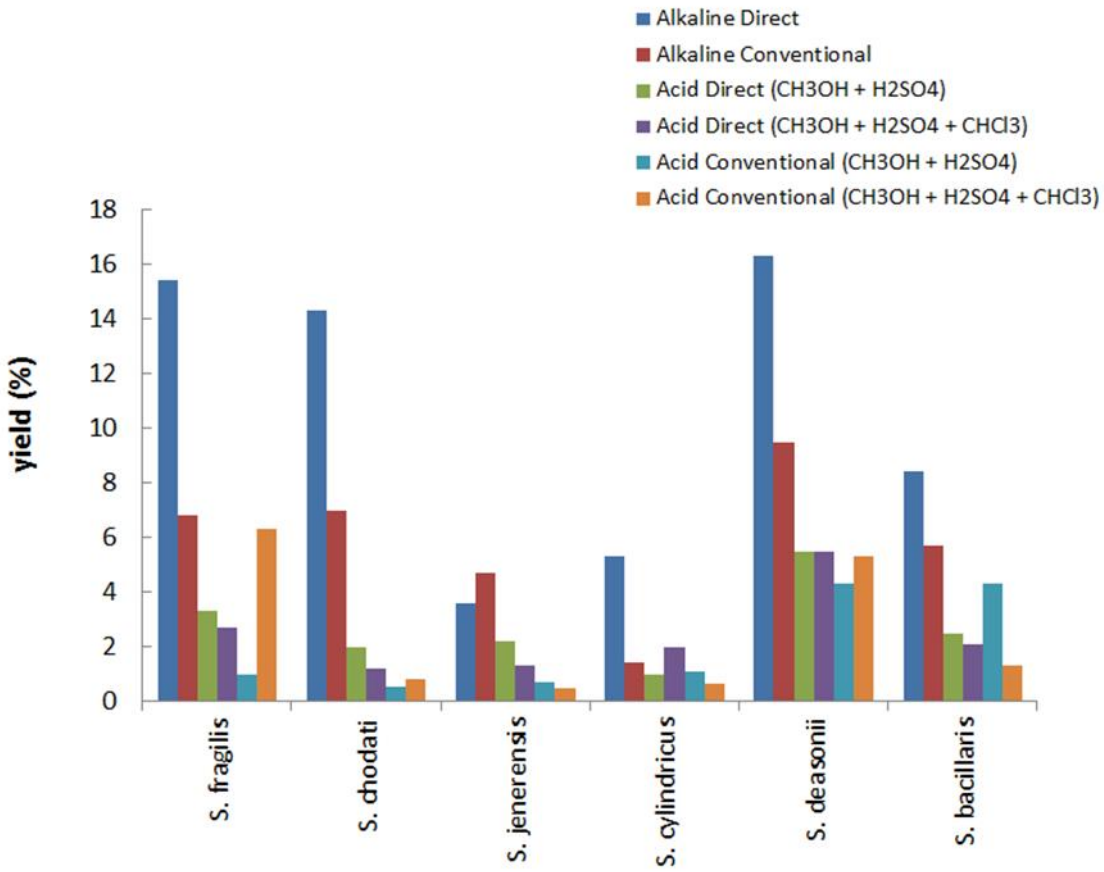

Figure 6

Table 1: Results of the transesterification process for *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Scenedesmus vacuolatus*, and *Maesotaenium caldariorum*. Bio-oil yield is reported as % of identified FAMEs per gram of dried biomass.

	Scenedesmus vacuolatus	***Maesotaenium caldariorum***	***Chlorella vulgaris***	***Chlamydomonas reinhrdtii***
Alkaline transesterification				
Direct	**2.9**	**5.6**	**6.5**	**7.6**
Conventional	**6.3**	**5.8**	**1.7**	**1.7**
Acidic transesterification				
Direct ($CH_3OH+H_2SO_4+CHCl_3$)	**2.3**	**2.6**	**5.8**	**2.0**
Conventional ($CH_3OH+H_2SO_4+CHCl_3$)	**1.0**	**0.4**	**2.4**	**2.4**