Journal of Sustainable Bioenergy Systems, 2017, 7, 149-164
http://www.scirp.org/journal/jsbs
ISSN Online: 2165-4018
ISSN Print: 2165-400X

Effect of Soaking Pre-Treatment on Reactive Extraction/*in situ* Transesterification of *Nannochloropsis occulata* for Biodiesel Production

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How to cite this paper: Salam, K.A., Velasquez-Orta, S.B. and Harvey, A.P. (2017) Effect of Soaking Pre-Treatment on Reactive Extraction/*in situ* Transesterification of *Nannochloropsis occulata* for Biodiesel Production. *Journal of Sustainable Bioenergy Systems*, **7**, 149-164.
https://doi.org/10.4236/jsbs.2017.74011

Received: September 19, 2017
Accepted: October 20, 2017
Published: October 23, 2017

Abstract

Microalgal phospholipid bilayer contributes to the molar excesses of methanol and high acid concentration required in reactive extraction to achieve high fatty acid methyl ester (FAME) yield. This study reports an investigation into the effects of pre-soaking *Nannochloropsis occulata* in methanol at 600:1 and 1000:1 methanol to oil molar ratios prior to acid-catalyzed *in situ* transesterification at 8.5:1 and 15:1 H_2SO_4 to oil molar ratios on the FAME yield. The results showed that the pre-soaked *Nannochloropsis occulata* produced a higher FAME yield at the two tested methanol to oil molar ratios and acid concentrations than the un-soaked, resulting in a reduction in methanol volume and acid concentration. A maximum FAME yield of 98.4% ± 1.3% was obtained for the pre-soaked *Nannochloropsis occulata* at 1000:1 methanol to oil molar ratio and 15:1 H_2SO_4 to oil molar ratio. Both the phosphorus mass balance and conversion of the isolated phospholipids into FAME revealed that pre-soaking solubilizes the phospholipid bilayer to some degree, and contributes to an increased FAME yield.

Keywords

Pre-Soaking, Microalgae, Cell Disruption, Glycerol, Transesterification, *in situ* Transesterification (Reactive Extraction)

1. Introduction

The depletion of fossil fuel and the associated negative climatic changes have

generated significant global interest in biodiesel. Biodiesel is a renewable transport fuel that can be produced from microalgae through reactive extraction ("*in situ* transesterification") or a two-step transesterification. In a two-step transesterification, pre-extracted oil from microalgae can be converted into fatty acid methyl ester (FAME) ("biodiesel") with alkalized or acidified methanol. Production of algal biodiesel via a two-step transesterification usually includes dewatering, conventional drying, solvent extraction, oil degumming, trans/esterification, neutralization and product purification.

Alternatively, algal FAME can be produced via i*n situ* transesterification by contacting the algal biomass directly with an alcohol containing a catalyst [1] [2]. It is potentially a cost-effective alternative way of producing algal FAME due to its elimination of the solvent extraction step and its higher water tolerance [1] [2]. The solvent extraction and drying steps account for ~90% of the process energy in a two-step transesterification of algal oil to FAME [3].

Additionally, micro-algal cell walls consist of phospholipid bilayer membranes (about 7 - 8 nm thick), which are embedded with integral and peripheral proteins [4]. A carbohydrate coat in the form of glycolipids, glycoproteins and polysaccharides encloses the membrane [4]. This configuration provides structural rigidity for the cells to adapt to their environments. However, the cell wall's resistance adversely affects the efficiency of algal bioprocesses such as genetic transformation, fermentation, anaerobic digestion, oil extraction and biodiesel production. Indeed, it leads to significant solvent requirements and energy load during the extraction processes [5]. For instance, direct FAME production requires a significant methanol to oil molar ratio, which can be as high as 1570:1 [6]. Dhar and Kirtania [7] reported that, for a transesterification process operating at 6:1 and 40:1 methanol to oil molar ratios, 662 and 6450 kW respectively were required for a 95% methanol recovery. By extrapolation, 16,658 - 171,898 kW will be required for the same methanol recovery by a reactive extraction process operating at 100:1 - 1000:1 methanol to oil molar ratio. This means the downstream distillation heat load required to recycle unreacted methanol (>94% of it) would significantly increase the operating cost. In addition, FAME yield was reported to increase with rising acid concentration during *in situ* transesterification [1] [2] [8], but the need to neutralize the unreacted acid in the product streams will increase the operating cost to some extent.

This work studied the effect of pre-soaking microalgae in methanol, prior to H_2SO_4 catalyzed *in situ* transesterification of *Nannochloropsis occulata* on FAME yield. The purpose is to determine whether methanol pre-soaking can enhance the solubilization of the phospholipid bilayers, thereby compromising the cell wall integrity. Additionally, evaluation of the isolated micro-algal phospholipids for FAME production was investigated. This was to determine whether microalgal phospholipids are converted into FAME during the *in situ* transesterification of microalgae.

2. Methods

2.1. Materials

Concentrated *Nannochloropsis occulata* was purchased from Varicon Aqua Solution (London, UK). Methanol (anhydrous, 99.8%), chloroform (99%), iodine (99.8% solid), HNO_3 (70%), hexane and potassium chloride were supplied by Sigma-Aldrich, UK. Glacial acetic acid (99%), propanol (99.5%), H_2O_2 (>30% w/v) and NaOH (98%) were supplied by Fisher Scientific, UK. Di-ether (99%) was supplied by VWR, UK.

2.2. Total Lipid Content

Nannochloropsis occulata was freeze dried at −40˚C for ~24 h in a Thermo Modulyo D-230 Freeze Dryer (Thermo Electron Corporation, UK) and then homogenised. The microalgae was further dried with a MB 45 Moisture Analyzer (Ohaus, USA) at 60˚C until a constant mass. Drying was performed at 60˚C to preserve the biochemical compositions of the samples [9]. The moisture content of the resulting microalgae was taken as reference point, 0% (w/w) dry algae. The total lipids from the microalgae was extracted overnight (~12 h) using chloroform: methanol (2:1, v/v) solvent mixture, based on the procedure of Folch *et al*. [10]. The biomass was then filtered out under vacuum using Whatman glass microfiber filter paper, GF/A (70 mm diameter). An aqueous solution (0.88%) of KCl at 25% of the volume of the extracting solvents was added to the filtrate in a separating funnel and thoroughly mixed. It was then allowed to form a biphasic layers. The lower chloroform layer was carefully removed into a pre-weighed conical flask and weighed. Chloroform was allowed to dry off in a fume cabinet until a constant mass of the lipids.

2.2.1. Fractionation of the Total Lipids and Identification of Each Fraction

The micro-algal total lipids were fractionated using solid phase extraction's method of Kaluzny *et al.* [11]. This involves dissolving about 10 mg of total lipid mixture in chloroform (Sigma Aldrich, UK). The solution is then fed to an amino propyl column (Bond Elut NH_2; 500 mg, 12 ml, Agilent Technology, UK) under vacuum. The columns were pre-conditioned using hexane (Fischer scientific, UK). The chloroform in the mixture eluted, leaving the lipid classes adsorbed onto the column. Then, the lipid classes were eluted using solvent mixtures of varying polarities into pre-weighed tubes. All neutral lipids were eluted with chloroform-2-propanol (2:1); free fatty acids were eluted with 2% acetic acid (Fischer scientific, UK) in diethyl ether (VWR, UK) while methanol (Sigma Aldrich, UK) was used to isolate the phospholipids. The solvent in the lipid fractions was completely evaporated under inert using Nitrogen gas and their dry mass recorded. The solid phase extracted (SPE) lipid fractions were evaluated by a Reversed Phase Hydrocarbon Impregnated Silica Gel Thin layer Chromatography (TLC) with dimension 5 × 20 cm, 250 microns (Analtech, UK). The de-

veloping solvent for the TLC was a hexane/diethyl ether/acetic acid mixture (80:20:1, v/v/v) [12]. Spots were visualized using iodine vapor [13].

2.2.2. Transesterification of Phospholipids

A 5 mg of the isolated phospholipids was transesterified in 15 ml glass tubes. A high 450-rpm agitation was used to prevent mass transfer limitation. The tubes were loaded in an IKA KS 4000 "icontrol" incubator shaker (IKA, Germany) maintained at a constant temperature of 60˚C and a stirring rate of 450 rpm. A 0.138 ml of methanol containing concentrated H_2SO_4 at 1.8% v/(v methanol) was used for the reaction. The reaction was run for 20 h. The reaction was quenched using calcium oxide (CaO) to neutralize the acid catalyst. The mixture of methanol, FAME and by-products was stored in a pre-weighed tube and the mass of the mixture was recorded. The FAME concentration in the mixture was then measured by gas chromatography, as explained in Section 2.5.

2.3. Effect of Soaking Pre-Treatment

Soaking is a pre-treatment of microalgae for cell disruption achieved by allowing a solvent such as methanol to percolate through the microalgae biomass in order to solubilize the phospholipid bilayer. A full factorial design on Minitab® 16 statistical software (Minitab, UK) was used with each factor at two levels. The microalgae was either pre-soaked or un-soaked. All *in situ* transesterification were conducted in 15 ml glass tubes containing 100 mg of microalgae. The tubes were loaded in an IKA KS 4000 icontrol incubator shaker (IKA, Germany) and kept at a constant temperature of 60˚C. A 100% H_2SO_4 (w/w oil) was used according to what reported to be optimum by Ehimen *et al.* [14]. In this studies, 100% H_2SO_4 (w/w oil) equals to 8.5:1 H_2SO_4 to oil molar *i.e.* 0.087 µl H_2SO_4/(mg algae). A 15:1 H_2SO_4 to oil molar ratio equals to 0.154 µl H_2SO_4/(mg algae) was compared with 8.5:1 H_2SO_4 to oil molar to check the effect of acid concentration on the FAME yield. The molar ratio of methanol to oil was 600:1 or 1000:1 methanol oil molar ratio, which equals to 0.0047 or 0.0078 ml methanol/(mg algae), respectively.

In a reactive extraction, algal biomass absorbs methanol. Therefore, it is necessary, that some methanol remain in the liquid phase for the transesterification to proceed, and for the fatty acid methyl ester ("biodiesel") formed to remain in the solvent (liquid) phase. In order to achieve this in the current studies, a 600:1 methanol to oil molar ratio was required. A 1000:1 methanol to oil molar was compared with 600:1 methanol to oil molar to check the effect of the increase in methanol volume on the FAME yield. A 100 mg of microalgae was used in all experiments and 880 g/(mol.) was the molecular mass of oil used to calculate the entire ratios. The microalgae were pre-soaked by placing 100 mg of the biomass in methanol inside 15 ml glass tubes. The tubes were loaded in an IKA KS 4000 icontrol incubator shaker (IKA, Germany) which was agitated at 300 rpm, kept at 17˚C and run for 14.5 h. Then, the *in situ* transesterification commenced by introducing the catalyst into the mixture and the reaction was run for 24 h at 60˚C; 450 rpm. A temperature of 60˚C was used for all the experiments as most

previous reports on *in situ* transesterification of microalgae were optimized at 60˚C [2] [6] [13]. The un-soaked micro-algae were run at the same process conditions. The acid catalyst in each sample taken at each specified *in situ* transesterification was neutralized with calcium oxide (CaO) to quench the reaction. The biomass was separated from the liquid by centrifugation. The biodiesel filtrate (a mixture of methanol, FAME and by-products) was stored in pre-weighed tubes and weighed. The FAME concentration in the mixture was measured by gas chromatography, as explained in Section 2.5.

2.4. Determination of Maximum FAME Content

The maximum FAME (mg) produced in each algal species was quantified using method of Garces and Mancha [15]. A methylating mixture of methanol, toluene, 2, 2-dimethoxypropane, and sulphuric acid at a volumetric ratio of 39:20:5:2 was prepared. The mixture was thoroughly mixed using a vortex mixer. A homogeneous mixture containing 3.3 ml of the methylating mixture and 1.7 ml of heptane was added to 0.2 g of each microalgae sample and vortexed well. After this, the mixture was loaded in an IKA KS 4000 icontrol incubator shaker at 60˚C; agitated at 450 rpm for 12 h. The reaction was quenched using calcium oxide (CaO) and further kept in a freezer. The resulting upper FAME layer was carefully pipetted into a pre-weighed centrifuge tube and weighed. The FAME concentration of the upper layer was measured by gas chromatography as explained in Section 2.5. The maximum FAME content in the sample was calculated by multiplying the FAME concentration obtained by the mass of the upper FAME layer.

2.5. Analytical Techniques

The European standard procedure [16] was used to determine the FAME concentration after the *in situ* transesterification. The biodiesel filtrate was mixed with 0.1 ml of an internal standard solution: methyl heptadecanoate (Sigma Aldrich, UK, 10 mg/(ml methanol) in 2 ml vials. 1 µl of the homogeneous mixture was injected into the GC and data was collected using Data Apex Clarity software, UK. The gas chromatography, GC was set to the following conditions: carrier gas: helium, 4.8×10^4 Pa; air pressure, 2.2×10^5 Pa; hydrogen pressure, 1.5×10^5 Pa; a capillary column head pressure was adjusted to 3.1×10^4 Pa. The oven temperature was maintained at 230˚C for 25 minutes. Heat rate was 15˚C/min; initial temperature was set at 150˚C and held for 2 min; final temperature was set at 210˚C and held for 20 min; injection temperature was 250˚C while detector temperature was 260˚C. The carrier gas flow rate was 2 ml/min. The column used was CP WAX 52 CB 30 m × 0.32 mm (0.25 µm) (Agilent, Netherlands). The mass of FAME obtained in the biodiesel-rich phase from the experiments was calculated by multiplying the mass of the final biodiesel mixture obtained and the FAME concentration measured by the GC. The FAME yield was calculated by dividing the mass of FAME obtained by the maximum FAME available in the algae.

$$\text{FAME Concentration}(C) = \frac{(\sum A) - A_{Ei}}{A_{Ei}} \times \frac{C_{Ei}V_{Ei}}{m} \times 100\% \quad (1)$$

where:

$\sum A$ are the total peak areas from C8 to C20:1. This includes all FAME between the range.

A_{Ei} is the peak area of the methyl heptadecanoate.

V_{Ei} is the volume in ml of the methyl heptadecanoate used.

C_{Ei} is the concentration in mg/(ml of the methyl heptadecanoate solution), and

m is the mass of the sample in mg.

The mass of the methyl ester in the sample was calculated by multiplying the FAME concentration (C) with the mass of the biodiesel reach filtrate from the *in situ* transesterification.

$$\text{Mass of the methyl ester}(\text{mg}) = C(\%) \times w(\text{mg}) \quad (2)$$

where w is the mass of the biodiesel filtrate.

Yield (% w/w) was the determined by comparing the mass of methyl ester obtained with the maximum FAME in the sample.

$$\text{Yield}(\%\,\text{w/w}) = \frac{\text{Mass of methyl ester from the experiments}(\text{mg})}{\text{Mass of the maximum FAME in the sample}(\text{mg})} \times 100\% \quad (3)$$

FAME Profile of *Nannochloropsis occulata*

In order to determine the algal FAME profiles, a standard grain FAME mix (Sigma Aldrich, UK, 10 mg/ml) and pure FAME compounds including C16:0, C17:0 and C18:2 (Sigma Aldrich, UK) were injected into the GC at the same conditions as the maximum FAME. Every FAME peak on the standard chromatogram that has the same retention time as the sample FAME peak was assigned the same fatty acid.

2.6. Phosphorus Quantification

A phosphorus mass balance on the extracts and the residue for pre-soaked; *in situ* transesterification or pre-soaked plus *in situ* transesterification was conducted. All the experimental conditions were the same as stated in Section 2.3. The methanol in the extracts and the residues was evaporated at 60˚C until a constant mass. A 10 mg of microalgae, extract or residue was digested in a mixture of 600 µl hydrogen peroxide (30% w/v)and 1200 µl HNO_3 (70%) at 140˚C for 4 h [17]. The resulting liquid mixture was made up to 10 ml with 1% HNO_3 aqueous solution. The phosphorus content in this mixture was then determined using ICP-AES. A known amount of standard aqueous solution of phosphorus was used as the reference.

3. Results and Discussion

3.1. Total Lipids/Fraction Analysis

The total lipids in the *Nanmochloropsis occulata* were determined as 17% ±

0.8% (w/w dry algae). The neutral lipids were determined as 22.5% ± 2.5% (w/w total lipids). The polar lipids were determined as 50% ± 0% (w/w total lipids) while the free fatty acids were determined as 18.3% ± 2.4% (w/w total lipids). The *Nannocloropsis occulata* contained higher quantity of polar lipids (phospholipids and glycolipids) than the neutral lipids and free fatty acids. This agrees with the findings of Scragg and Leathers [18] that phospholipids represent a large proportion of the algal total lipids. The isolated lipid fractions of the microalgae were further identified using thin layer chromatography, as shown in Figure 1. This was done by comparing the spot height of the elutes (1, 2 and 3) obtained from the isolated algal neutral lipid (NL) with standard monoglyceride (MG (elute 1)), standard diglyceride (DG (elute 2)) and standard triglyceride (TG (elute 3)). Based on this analysis, it can be seen clearly that the algal neutral lipids contained mono-, di- and triglycerides. The same procedure was used to confirm the isolated algal phospholipids (PL) with the standard phospholipid (STD PL), which also confirmed that the isolated sample was actually phospholipids.

Cobelas and Lechado [19] reported that the major phospholipids (cell wall lipids) in *Nannochloropsis sp.* contain two fatty acid moieties bonded to a glycerol backbone, and a phosphorus-containing moiety. In contrast, triglycerides contain three fatty acids bonded to a glycerol backbone but no phosphorus containing moiety, as shown in Figure 2 by Wood [20].

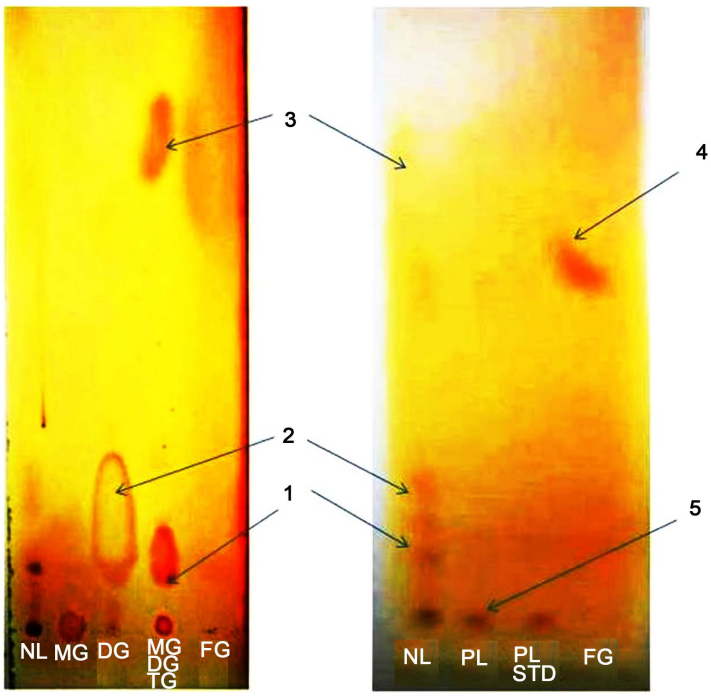


Figure 1. Thin layer chromatogram showing neutral and phospholipids fractions of total lipids: NL: Neutral lipids fraction of the sample; PL: Phospholipids fraction of the sample; MG: Standard monoglyceride; DG: Standard diglyceride; TG: Standard triglycerides; FG: Standard FAME grain mixtures. 1: Monoglycerides; 2: Diglycerides; 3: Triglycerides; 4: FAME grain mix; 5: Phospholipids.

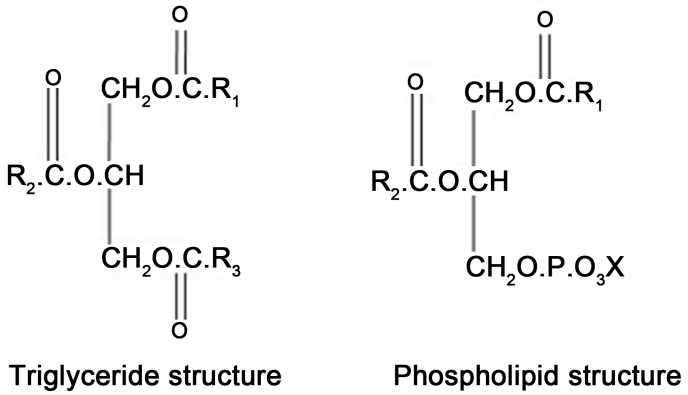


Figure 2. Comparison between the structure of triglyceride and that of phospholipids. The symbol "X" in phospholipids can be choline, ethanolamine, serine, water, glycerol and phosphatidyglycerol while R_1, R_2 and R_3 denote carbon chain of fatty acids. Source: Wood [20].

3.2. Evaluation of Phospholipids for FAME Production

The isolated micro-algal phospholipids were evaluated for FAME production by transesterifying them under the same conditions as the *in situ* transesterification. The results show that 9.6% ± 1% of the phospholipids in *Nannochloropsis occulata* converted into FAME. This shows that microalgae cell wall lipids (membrane lipids) convert into FAME during acid-catalyzed *in situ* transesterification. This result empirically explained why Wahlen *et al.* [1] obtained significantly more biodiesel than would be expected from the conversion of triglycerides alone during acid-catalyzed *in situ* transesterification of microalgae, cyanobacteria and wild mixed-cultures. It also explained why a greater FAME yield was obtained from *in situ* transesterification than from the two-step transesterification of pre-extracted oil [21] [22] [23].

3.3. FAME Profiles for the *Nannochloropsis occulata*

The FAME profiles determined for *Nannochloropsis occulata* are shown in **Table 1**.

Each fatty acid represents the percentage of the maximum total FAME obtained from *Nannochloropsis occulata* through reactive extraction ("*in situ* transesterification"). The maximum FAME was quantified as per the explanation in the section outlining the materials and method. It is known that the properties of biodiesel are strongly affected by its fatty acid methyl ester profile [24]. As shown in **Table 1**, ~87% of the total fatty acid methyl ester produced from the microalgae is saturated and mono-unsaturated which is good, as it improves the quality of the biodiesel obtained.

The maximum number of unsaturated (double bonds) FAME detected in the algae was 2, which is found in linoleic acid (C18:2n6c) methyl ester. This poly-unsaturated fraction accounts for 13% of the fatty acids as shown in **Table 1**. The FAME contain no poly unsaturated fatty acids such as eicosapentaenoic (C20:5) and docosahexaenoic acid (C22:6) acids. Absence of such poly unsatu-

Table 1. Fatty acids profile for *Nannochloropsis occulata.*

FAME type	FAME produced (%)
Caprylic (C8:0)	0.1 ± 0.005
Capric (C10:0)	0.4 ± 0.024
Decanoic (C10:1)	0.7 ± 0.039
Lauric (C12:0)	0.4 ± 0.028
Lauroleic (C12:1)	1.1 ± 0.066
Myristic (C14:0)	5.4 ± 0.27
Myristoleic (C14:1)	2.7 ± 0.154
Palmitic (C16:0)	26.7 ± 1.07
Palmitoleic (C16:1n9c)	31.1 ± 0.933
Stearic (C18:0)	1.2 ± 0.048
Elaidic (C18:1n9c)	7.3 ± 0.365
Linoleic (C18:2n6c)	12.8 ± 0.512
Arachidic (C20:0)	2.8 ± 0.123
Eicosenoic (C20:1)	7.1 ± 0.284
Total	
Saturated	37
Mono-unsaturated	50
Poly-unsaturated	13

rated methyl esters has been shown to improve oxidation stability [25]. According to standard EN 14214, polyunsaturated fatty acid methyl ester (≥4 double bonds) should be ≤1%, while linolenic acid (an 18 carbon chain fatty acid methyl ester with 3-double bonds) should be ≤12%.

3.4. Effect of Soaking Pre-Treatment on Methanol Molar Excess and Acid Concentration

Figure 3 shows the effect of pre-soaking *Nannochloropsis occulata* in methanol prior to acid catalyzed *in situ* transesterification on the FAME yield at 600:1 and 1000:1 methanol to oil molar ratios and 8.5:1 and 15:1 catalyst to oil molar ratios.

The figure shows that soaking pre-treatment enhances the FAME yield for the two tested methanol to oil molar ratios and acid concentrations. It is notable that there is no significant difference ($p > 0.05$) between a 67.2% ± 0.9% FAME yield obtained for the pre-soaked micro-algae at a 600:1 methanol to oil molar ratio, 8.5:1 acid to oil molar ratio and 54.6% ± 4% FAME yield obtained for the un-soaked microalgae at 1000:1 methanol to oil molar ratio, 8.5:1 acid to oil molar ratio. However, pre-soaking at that process condition resulted in a 42% reduction in the methanol to oil molar ratio. Similarly, 89% ± 2.5% FAME yield was obtained for pre-soaked microalgae at 8.5:1 H_2SO_4 to oil molar ratio, and

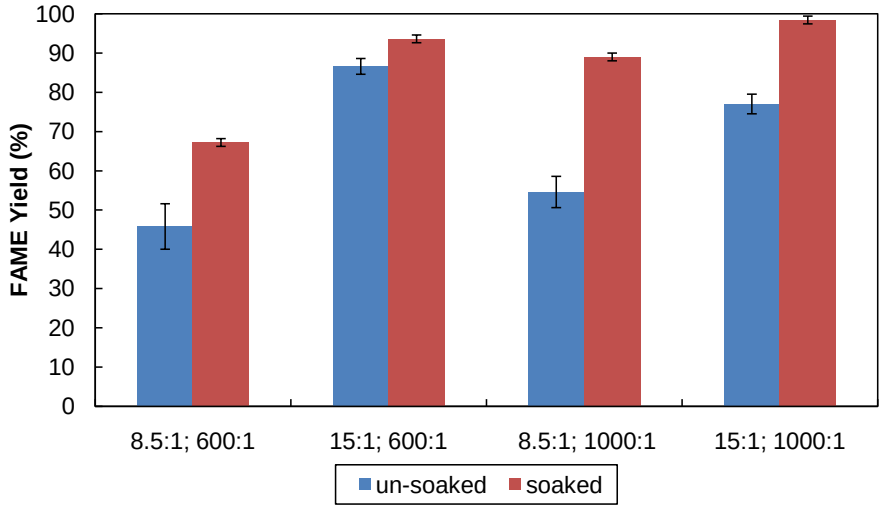


Figure 3. Effect of pre-soaking on reactively extracted FAME yield from *Nannochloropsis occulata.* Process conditions: Pre-soaking time: 14.5 h, agitation rate: 450 rpm, temperature: 60˚C, reaction time: 24 h, mass of *Nannochloropsis occulata*: 100 mg. Methanol to oil molar ratio: 600:1; 1000:1. H_2SO_4 to oil molar ratio: 8.5:1; 15:1.

1000:1 methanol to oil molar ratio which was significantly greater than ($p < 0.05$) the 77% ± 2.5% FAME yield obtained at the same methanol to oil molar ratio, and at a 15 :1 H_2SO_4 to oil molar ratio. This resulted in a ~40% reduction in the concentration of the acid catalyst. Methanol pre-soaking is simple and can be easily scaled up.

Figure 4 shows that all the factors (acid concentration, methanol to oil molar ratio, and pre-soaking time of *Nannochloropsis occulata* in methanol) increased the FAME yield. However, the methanol to oil molar ratio has lesser effect than the acid concentration and pre-soaking time as shown by the slope of the main effect plot.

Figure 5 shows that all the two way interactions: the acid concentration and methanol to oil ratio; acid concentration and pre-soaking time; methanol to oil molar ratio and pre-soaking time, significantly affected the FAME yield ($p < 0.05$), but not the three way interaction ($p = 0.915$).

This is the first investigation on soaking pre-treatment prior to acid catalyzed reactive extraction, therefore the FAME enhancement was compared with the existing disruption process *i.e.* ultrasound. The results of the current studies agree with increase in FAME yield achieved by Koberg *et al.* [26] in a reactive extraction of Nannochloropsis cells due to algal pre-treatment by microwave and ultrasound irradiation. The authors obtained 99% FAME conversion when the algal cells were treated with microwave irradiation while 92% FAME conversion was obtained using ultrasound treatment. Their control experiments involving neither treatments nor stirring produced only 10.7% FAME conversion. Although their techniques are different from soaking pre-treatment reported here, nevertheless they all resulted in increase in FAME yield than the un-treated samples (the control experiment).

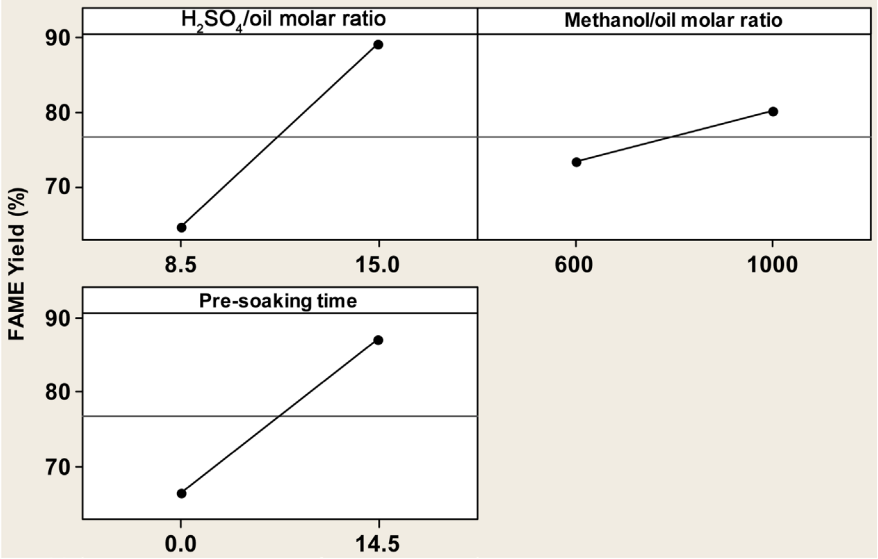


Figure 4. Main effect plot of FAME yield vs. pre-soaking time; methanol to oil molar ratio; acid concentration. Process conditions: pre-soaking time: 14.5 h; agitation rate: 450 rpm; temperature: 60˚C; reaction time: 24 h; mass of *Nannochloropsis occulata*: 100 mg. Data shown are mean values of duplicate experiments.

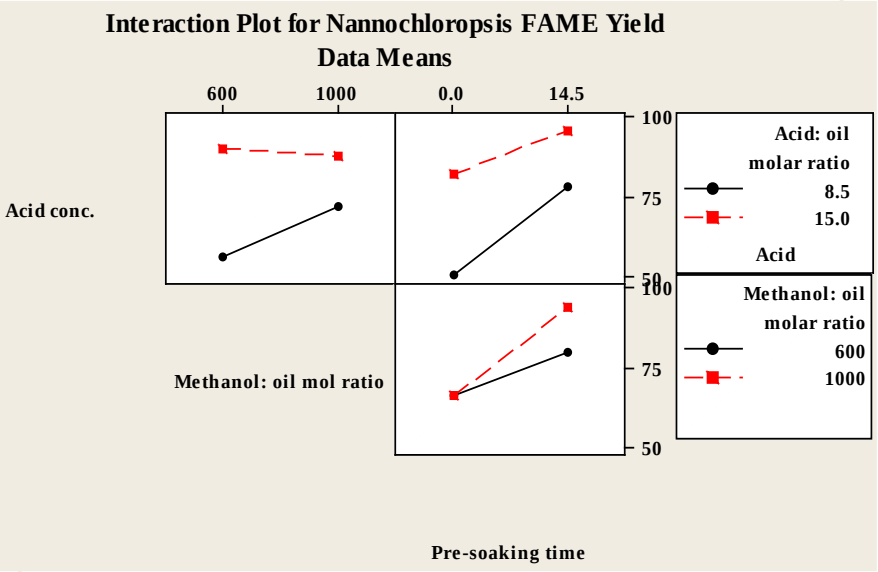


Figure 5. Interaction effect plot of pre-soaking time, acid concentration, methanol to oil molar ratio on FAME yield. Process conditions: Pre-soaking time: 14.5 h; agitation rate: 450 rpm; temperature: 60˚C; reaction time: 24 h; mass of *Nannochloropsis occulata*: 100 mg. Data shown are mean values of duplicate of experiments.

3.5. Maximum FAME Yield Obtained from *Nannochloropsis occulata*

A maximum FAME yield of 98.4% ± 1.3% was obtained for the pre-soaked *Nannochloropsis occulata* at 1000:1 methanol to oil molar ratio and 15:1 H_2SO_4 to oil molar ratio. The maximum FAME yield obtained in this species was not significantly different from a maximum FAME of 98% obtained by Zhao and Liu

[27] during H_2SO_4-catalysed reactive extraction of *Rhodosporidium toruloides* with 868:1 methanol to oil molar ratio. The maximum FAME obtained in the current studies is greater than 77% FAME yield obtained by Wahlen *et al.* [1] during H_2SO_4-catalysed reactive extraction of *Chlorella sorokiniana* with 1831:1 methanol to oil molar ratio.

The volume of methanol used in this study is significantly less than what were reported in the literatures [1] [27]. In our experiment a 600:1 methanol to oil molar ratio equals to 0.0047 ml methanol/(mg algae) while 1000:1 methanol to oil molar ratio equals to 0.0078 ml methanol/(mg algae). These volumes of methanol are significantly less than 1831:1 methanol to oil molar ratio which equals to 0.02 ml methanol/(mg algae) used by Wahlen *et al.* [1] and 868:1 methanol to oil molar ratio which equals to 0.02 ml methanol/(mg algae) used by Zhao and Liu [27].

The concentration of catalyst used was according to the optimum reported by Ehimen *et al.* [14]. The authors used 100% H_2SO_4 concentration (w/w algal oil). In our experiment this amount equals to 8.5:1 H_2SO_4 to oil molar ratio which equals to 0.087 µl H_2SO_4/(mg algae) while 15:1 H_2SO_4 to oil molar ratio equals to 0.154 µl H_2SO_4/(mg algae). These values are in the range of what other investigators reported for acid-catalyzed reactive extraction of microalgae. For instance, El-shimi *et al.* [8] obtained a maximum FAME yield of 84.7% when 100% H_2SO_4 (w/w oil of algae) was used during acid catalyzed reactive extraction of *Spirulina-platensis* for FAME production. The amount of H_2SO_4 used by these authors equals to 0.19 µl H_2SO_4/(mg algae).

3.6. Phosphorus Mass Balance

The increase in FAME yield achieved by soaking pre-treatment was also explained by solubilization of phospholipids in methanol as shown in **Figure 6**. Phosphorous content was positively correlated with the phospholipids of each sample. **Figure 6** shows the differences in phospholipid content between the different phases.

As can be seen in the Figure, a substantial portion of the phosphorous remained in the residue after each step. However, the small amount which was solubilized in methanol was significant enough to be related to the phospholipids contained in the cell wall (% phospholipids in the cell wall). The phosphorous solubilization was attributed to pre-soaking in methanol, as subjecting the biomass to subsequent *in situ* transesterification led to no further reduction. This shows that the equilibrium dissolution of the phosphorus in the algae biomass has been attained. It is possible that the dissolution of phospholipids in methanol increased its FAME conversion rate as they were no longer bonded to the cell wall. The removal of the phospholipids from the cell wall looses the triglycerides from the cellular matrix, which results in an increased FAME conversion rate. This could explain why pre-soaking caused FAME enhancement in this species when using a 1000:1 methanol to oil molar ratio, as seen in **Figure 3**.

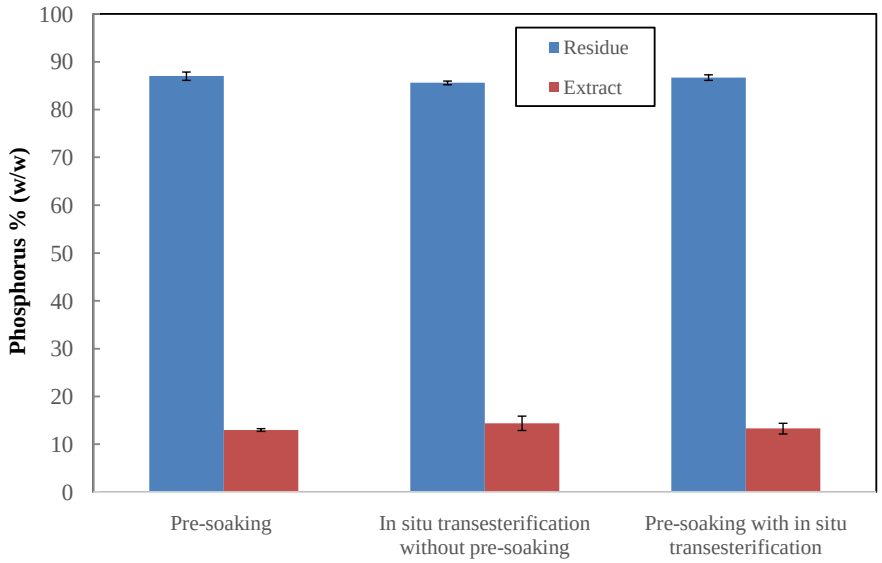


Figure 6. Phosphorus content of algal residue and extract compared with initial value. Pre-soaking time: 14.5 h; pre-soaking agitation rate: 300 rpm; reaction agitation: 450 rpm; reaction temperature: 60˚C; reaction time: 24 h; methanol to oil molar ratio: 600:1; acid to oil molar ratio: 8.5:1; mass of microalgae: 100 mg.

3.7. Minimum Pre-Soaking Time Required

The time used for pre-soaking in the experiments was 14.5 h to ensure a long mass transfer completion time. In order to compare the pre-treatment time with other similar processes found in the literature, an optimization was conducted by trying to see what would be the minimum residence time needed for pre-soaking. **Figure 7** shows that after 1 h, the same amount of phosphorous was obtained from the cells as after 14.5 h. That means that the increased reaction time needed is short and could be conducted in the same reactor vessel as for the transesterification by first adding the methanol and then, after 1 h, the catalyst.

3.8. Proposed Mechanism for Pre-Soaked Micro-Algae Undergoing Reactive Extraction

Pre-soaking microalgae in methanol prior to acid catalyzed *in situ* transesterification solubilizes the phospholipid bilayer to some degree. This can be seen clearly from the phosphorus mass balance (**Figure 6**) and phosphorus-time profile (**Figure 7**). Though a significant amount of phosphorus remained in the residue, the phosphorus removed from the microalgae was largely due to pre-soaking. The removal of the phosphorus (phospholipids) from the micro-algal cell wall could certainly compromise its integrity. This looses the triglyceride from the cellular matrix which enhances its conversion into FAME. The solubilized phospholipids easily converted into FAME because they were no longer bound up in the cell wall. This is clearly shown by conversion of the isolated phospholipids into FAME. The scheme is shown in **Figure 8**.

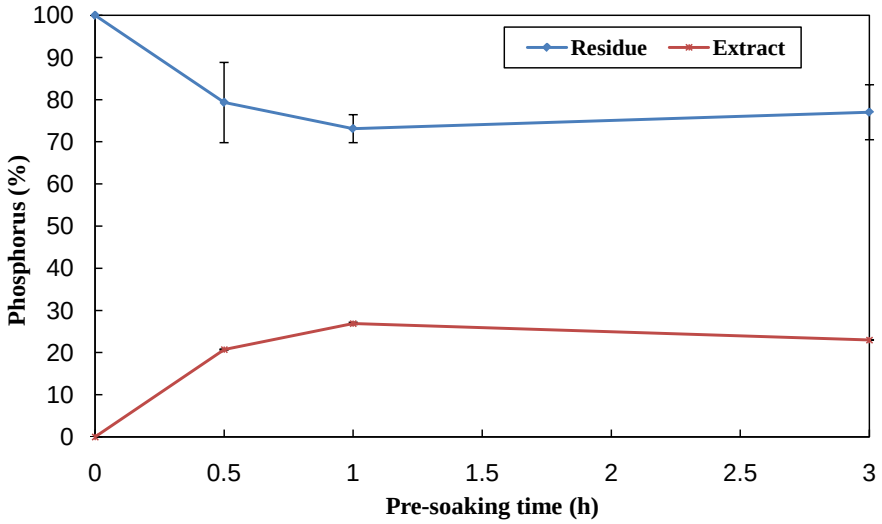
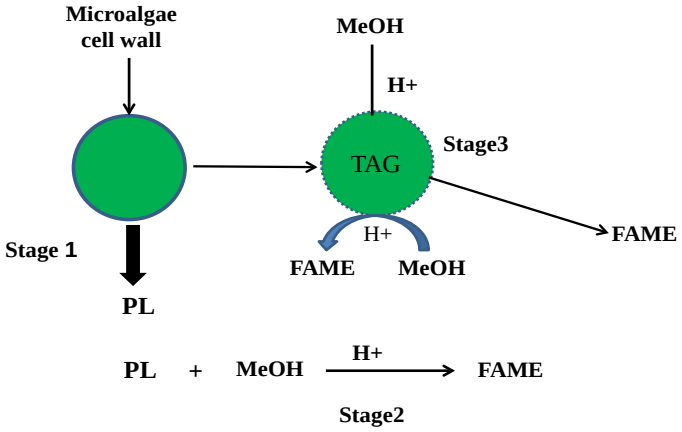


Figure 7. The phosphorus time profile for the soaking pre-treatment.



- Stage1 (Pre-soaking) : **PL** solubilised into methanol
- Stage2: **PL** in the cell wall and bulk fluid convert into FAME
- Stage3: TAG converts into FAME

Figure 8. Proposed scheme for pre-soaked microalgae prior to acid catalyzed *in situ* transesterification. FAME: Fatty acid methyl ester; PL: Phospholipids; TAG: Triglyceride and MeOH: Methanol.

4. Conclusion

Pre-soaking pre-treatment solubilizes phospholipids bilayer to some degree, and results into greater enhancement in reactively extracted FAME yield in *Nannochloropsis occulata*. It causes reduction in methanol to oil molar ratio and H_2SO_4 to oil molar ratio required to catalyze reactive extraction ("*in situ* transesterification") of *Nannochloropsis occulata* for biodiesel production. It is empirically shown that acid-catalyzed reactive extraction involves phospholipids conversion into biodiesel, and contributes to higher biodiesel yield observed in reactive extraction than two-step transesterification of pre-extracted oil.

Acknowledgements

Dr. Velasquez Orta acknowledges the Newton Fund Institutional Links Mexico (ID: 275897070) for the funds provided to be able to communicate this research while Dr. Salam acknowledges Petroleum Technology Development Fund (PTDF) Nigeria for sponsoring the Research Project.

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