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Original Research Paper

Surfactant-assisted direct biodiesel production from wet *Nannochloropsis occulata* by *in situ* transesterification/reactive extraction

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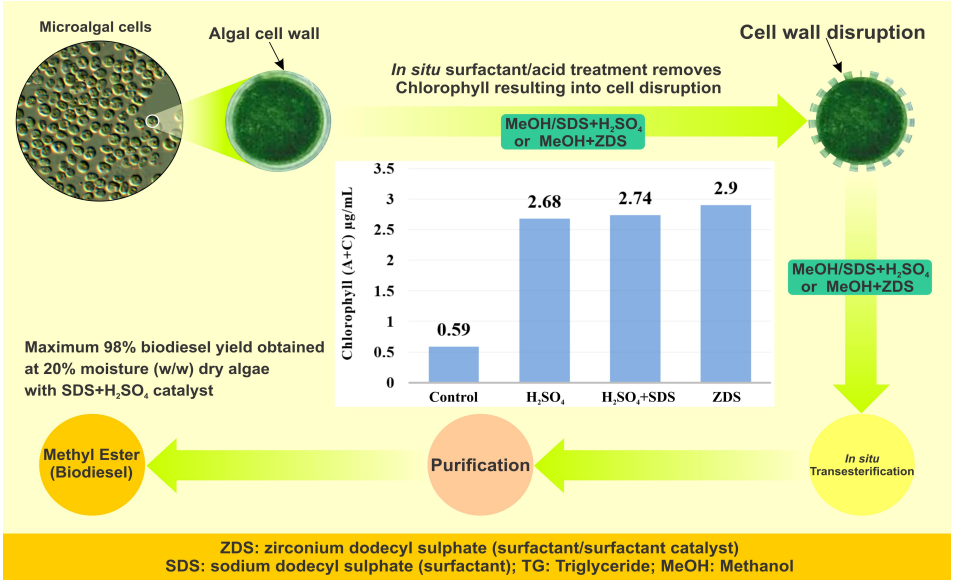
HIGHLIGHTS

- Surfactant assisted *in situ* transesterification of wet algae was studied.
- A surfactant catalyst ("ZDS") produced high yields in *Nannochloropsis occulata*.
- Inclusion of SDS in H_2SO_4 increased FAME production in the wet algae.
- The process was not adversely affected by water in the algae up to 20%.

GRAPHICAL ABSTRACT



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ABSTRACT

This article reports an *in situ* transesterification/reactive extraction of *Nannochloropsis occulata* for fatty acid methyl ester (FAME) production using H_2SO_4, sodium dodecyl sulphate (SDS) plus H_2SO_4 and zirconium dodecyl sulphate (ZDS). A maximum 67 % FAME yield was produced by ZDS. Effect of inclusion of sodium dodecyl sulphate (SDS) in H_2SO_4 for FAME enhancement and water tolerance was also studied by hydrating the algae with 10 % - 30 % distilled water (w/w) dry algae. Treatment with SDS in H_2SO_4 increases the FAME production rate and water tolerance of the process. Inclusion of SDS in H_2SO_4 produced a maximum 98.3 % FAME yield at 20 % moisture in the algae. The FAME concentration began to diminish only at 30 % moisture in the algae. Furthermore, the presence of a small amount of water in the biomass or methanol increased the lipid extraction efficiency, improving the FAME yield, rather than inhibiting the reaction.

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1. Introduction

The need to produce alternative renewable transport fuels has generated considerable global interest in biodiesel (Meng et al., 2009). Consequently, different biodiesel feedstocks have been explored, including food oil crops (Zeng et al., 2008), non-food oil crops such as *Jatropha curcas* (Kasim and Harvey, 2011), and microalgae (Wahlen et al., 2011; Velasquez-Orta et al., 2012). Food oil crops are not sustainable, as freshwater and considerable hectares of arable land are required for their cultivation (Chisti, 2007). On the other hand, non-food oil crops and waste oil can only supply limited quantities of biofuels, so cannot meet world transport fuels requirements.

There are still a number of challenges for algae to be used as fuel feedstocks including limited supply of concentrated CO_2, full utilisation of nitrogen or phosphorous nutrients, adverse effect of small quantity of fresh water even if marine algae is used, and efficient utilisation of algal residues after oil extraction (Chisti, 2013). Additionally, economic construction of large algae photobioreactors, and reducing the drying costs, perhaps by increasing the water tolerance of the reaction step need to be done for micro algal biodiesel to become a commercial reality. Regardless of these challenges, microalgae could still serve as alternative biodiesel feedstock as it has short growing time, high lipid productivity while it is capable of capturing concentrated CO_2 and can potentially be used in waste water remediation.

Biodiesel can be made either by reactive extraction ("*in situ* transesterification") (Wahlen et al., 2011; Velasquez-orta et al., 2012) or by two step transesterification of pre-extracted oil (Eze et al., 2014). A major advantage of *in situ* transesterification over the two step transesterification is that it reduces the number of process steps (by eliminating the solvent extraction steps) by contacting the biomass directly with the reactants. This could reduce the cost of biodiesel production. However, the major drawback of *in situ* transesterification is that it requires a high molar ratio of methanol to oil. The need to recycle the unreacted methanol (over 94 % of it) increases the process costs. Additionally, extraction of intracellular lipids from microalgae requires a significant excess of solvent because of the chemical resistance and structural toughness of algal cell walls (Gerken et al., 2012).

The relatively low permeability of polar solvents such as methanol and ethanol, as well as non-polar solvents such as hexane through the walls of dried oil-bearing cells can significantly reduce lipid extraction effectiveness, but it can be improved by addition of a small amount of water (Cohen et al., 2012). The water swells the cellular structure of polysaccharide-containing biomass, which increases the solvent permeability through the cell walls (Cohen et al., 2012). Similarly, the inclusion of water in alcohol such as methanol or ethanol was effective for extraction of polar lipids such as phospholipids or glycolipids (Zhukov and Vereshchagrin, 1981). Polar lipids are the major components of algal cell walls. Their removal from micro algal cell walls compromises their integrity, which can improve fatty methyl ester (FAME) recovery during *in situ* transesterification.

The most common homogeneous catalysts for *in situ* transesterification of microalgae are NaOH and H_2SO_4. NaOH is seldom used in microalgae if its lipids contain high free fatty acid (FFA) perhaps due to long term storage (Chen et al., 2012) to prevent soap formation (Canakci and Gerpen, 1999; Ma and Hanna, 1999).When H_2SO_4 is used, a high concentration of the catalyst is always required to achieve high yields (Wahlen et al., 2011; Velasquez-Orta et al., 2013). However, the need to neutralise the unreacted acid in the product streams will increase operating costs.

A surfactant catalyst (cerium (III) trisdodecyl trihydrate) has been evaluated for a two-step FAME production from soybean oil and oleic acid (Ghesti et al., 2009). The authors concluded that the surfactant catalyst efficiently promoted the transesterification of triglycerides and the esterification of free fatty acids. Similarly, use of cetyltrimethylammonium bromide (CTAB) (a cationic surfactant) with an alkali catalyst resulted in an increased FAME yield and reduction in catalyst concentration during *in situ* transesterification of *Jatropha curcas* by acting as a phase transfer catalyst (Hailegiorgis et al., 2011).

Park et al. (2014) reported that inclusion of sodium dodecyl benzene sulfonate (SDBS) in H_2SO_4-catalysed hot water enhanced extraction of FFA and lipids from *Chlorella vulgaris*. They reported that the inclusion of SDBS in H_2SO_4 significantly reduced the amount of H_2SO_4 required to convert the pre-extracted algal oil into FAME using a two-step transesterification. Inclusion of sodium dodecyl sulphate (SDS) in water has also been reported to increase oil extraction from canola seeds (Tuntiwiwattanapun et al., 2015). In a different study, SDS has been used for lysing cells to recover intracellular components (Brown and Audet, 2008).

Effect of inclusion of SDS in H_2SO_4 for a direct FAME production from wet microalgae has not been investigated. Similarly, an *in situ* transesterification of microalgae by a surfactant catalyst has not been reported in the literature. Therefore, this paper reports on the usage of zirconium dodecyl sulphate ("ZDS") (a surfactant catalyst) to catalyse *in situ* transesterification of *Nannochloropsis occulata*. Cell wall disruption by ZDS was explored for FAME enhancement.

In addition, the inclusion of SDS in H_2SO_4 was used in this report for improving water tolerance of the *in situ* transesterification of *N. occulata*. This is entirely a new approach to produce biodiesel from wet microalgae through *in situ* transesterification. It is worth quoting that even small amounts of water have been reported to significantly decrease conversion during a two-step transesterification of vegetable oil (Canakci and Gerpen, 1999). On the other hand, complete drying of algae is energy intensive, which significantly increases the cost of algae pre-treatment. Hence, the findings of the present study are important, as the significant amounts of energy required to dry microalgal biomass or microalgal oil to the levels required in a two-step biodiesel production render the process uneconomic, and is currently one of the major technical challenges to micro algal biodiesel production.

2. Materials and methods

2.1. Microalgae culture and their major biochemical compositions

Concentrated wet *N. occulata* was purchased from Varicon Aqua Solutions (London, UK). Guldhe et al. (2014) has shown that there was no significant differences in the lipid extraction yield of *Scenedesmus* sp. dried by three techniques: freeze-drying, oven- drying, and sun-drying. Therefore, a frozen sample was freeze-dried at -40°C for ~24 h in a Thermo Modulyo D Freeze Dryer as this method is faster than the other drying techniques. A moisture analyser was used to further dry the algae at 60°C to preserve its biochemical compositions (Widjaja et al., 2009) until their moisture remained constant. The moisture content of the resulting dry microalgae was taken as 0 % (w/w dry algae). The total lipids content were measured using the method described by Folch et al. (1956). The FFA and cell wall lipids (phospholipids and glycolipids) of the species were measured using the solid phase extraction method of Kaluzny et al. (1985).

2.2. Determination of maximum FAME content

The maximum FAME concentration was quantified using the procedure described by Garces and Mancha (1993). 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 microalgae and vortexed well. After this, the mixture was transesterified in an IKA incubator at 60°C; 450 rpm for 12 h. Subsequently, the acid catalyst was neutralised with calcium oxide (CaO) to quench the reaction. The resulting upper FAME layer was carefully pipetted into a pre-weighed centrifuge tube and weighed. After that, it was prepared for FAME analysis and its concentration was measured by gas chromatography. The maximum FAME content in the sample was calculated by multiplying the FAME concentration obtained by the mass of the upper FAME layer.

2.3. Catalyst synthesis

Zirconium (IV) dodecyl sulphate (Zr $[OSO_3C_{12}H_{25}]_4$) was synthesised using the modified method presented by Zolfigol et al. (2007) as follows by inclusion of 4 % KCl (w/w zirconium dodecyl sulphate solution):

(i) 2.86 g (8.9 mmol) of zirconium oxychloride octahydrate (Sigma Aldrich, UK) was dissolved in 100 ml of distilled water at room temperature;

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(ii) 12.13 g (42 mmol) of sodium dodecyl sulphate (VWR, UK) was put in a three-neck 500 ml round bottom flask. Then, 300 ml of distilled water was added to this at room temperature;
(iii) A zirconium oxychloride octahydrate solution was added to the sodium dodecyl sulphate solution whilst mixing at 500 rpm and stirred for 30 min;
(iv) 4 % KCl (w/w zirconium dodecyl sulphate solution) was added to increase catalyst recovery;
(v) The precipitate was centrifuged and washed repeatedly with 150 mL distilled water;
(vi) The resulting white solid was further calcined at 80°C for 4 h and was then dried in a desiccator (Duran vacuum desiccator).

2.4. Quantification of cell disruption after in situ transesterification

The amount of chlorophyll extracted from the microalgae has been correlated with cell wall disruption by Gerde et al. (2012). The total chlorophyll A and C obtained after the *in situ* transesterification by the different catalysts was measured using a modified version of the method previously described by Gerde et al. (2012). To study the extent of cell disruption in *N. occulata*, 0.47 mL of methanol was added to a 100 mg of dried microalgae in a 2.5 mL tube followed by the addition of 100 % H_2SO_4 (w/w oil). To another tube containing the same amount of microalgae, methanol, H_2SO_4, and 9 mg SDS was added to study the effect on cell disruption by including SDS in H_2SO_4. A third test tube was used with 100 % ZDS (w/w lipids), 100 mg of microalgae, and 0.47 mL of methanol. The reactions were allowed to progress for 24 h, at 32°C to avoid degradation of the chlorophyll at a stirring rate of 450 rpm using IKA KS 4000 icontrol incubator shaker (IKA, Germany). At the end of the reaction, the samples were centrifuged at 17,000 ×g for 10 min using an Accu Spin Micro 17 centrifuge (Fisher Scientific, UK). Methanol was used as blank. The absorbance of the supernatant obtained was measured at 664, 647, and 630 nm and the chlorophyll concentrations in µg /mL were calculated using the formulae presented by Jeffrey and Humphrey (1975) (**Eqs. 1 and 2**):

$$Chla = 11.93\ A_{664} - 1.93 A_{647} \quad \text{Eq. 1}$$

$$Chlc = -3.73\ A_{664} + 24.36\ A_{630} \quad \text{Eq. 2}$$

Where $Chla$ is chlorophyll a and $Chlc$ is chlorophyll c.

2.5. Experimental designs

An 8.5 mol. H_2SO_4/(mol. lipids) which equals to 100 % (w/w lipids) was used. ZDS was fixed as 100 % ZDS (w/w lipids). These amounts of catalysts used in this study were based on the optimum of 100 % H_2SO_4 (w/w oil) reported by Ehimen et al. (2010).

A 9 mg of SDS was added to H_2SO_4 to study the effect of combination of a surfactant and homogeneous H_2SO_4 catalyst on FAME yield. This amount of SDS was significantly greater than 2 mol. SDS/(mol. oil) reported to be enough to solubilise the phospholipid bilayer (Tan et al., 2002). The molar ratio of methanol to lipid was 600:1, which equals to 0.0047 mL/(mg algae cells). A temperature of 60°C was used for all the experiments as most previous reports on *in situ* transesterification of microalgae were optimised at 60°C (Haas and Wagner, 2011; Li et al., 2011; Velasquez-Orta et al., 2013). An 880 g/(mol.) was the average molecular mass of the oil used to calculate the entire molar ratios. Rehydrated samples of *N. occulata* were prepared by adding 10 %, 20 %, and 30 % of distilled water (w/w dry algae), then allowing the samples to equilibrate for 1 h. The resulting wet biomass was then transesterified using H_2SO_4, with or without SDS, to isolate the water tolerance effect.

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 high stirring rate of 450 rpm was used to prevent mass transfer limitations. The acid catalyst in each sample taken at each specified *in situ* transesterification was neutralised with 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 biodiesel filtrate was measured by gas chromatography, as explained in the Section 2.6.

2.6. Analytical techniques

The Standard UNE-EN 14103 (2003) 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. Then, 1µL of the homogeneous mixture was injected into the GC and data was collected using the Data Apex Clarity software (UK). The gas chromatograph was operated at the following conditions: carrier gas: helium, 7 psi; air pressure, 32 psi; hydrogen pressure, 22 psi, and capillary column head pressure, 4.5 psi. The carrier gas flow rate was 2 mL/min. The oven temperature was maintained at 230°C for 25 min. 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 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 (**Eq. 3**).

$$\text{FAME Concentration (C)} = \frac{(\sum A) - A_{Ei}}{A_{Ei}} \times \frac{C_{Ei} V_{Ei}}{m} \times 100\ \% \quad \text{Eq. 3}$$

Where $\sum A$ is the total peak areas from C12 - C20:1, A_{Ei} is the peak area of the methyl heptadecanoate, V_{Ei} stands for 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) by the mass of the biodiesel filtrate from the *in situ* transesterification (**Eq. 4**).

$$\text{Mass of the methyl ester (mg)} = \text{C (\%)} \times \text{w (mg)} \quad \text{Eq. 4}$$

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 as follows (**Eq. 5**):

$$\text{Yield (\% w/w)} = \frac{Mass\ of\ methyl\ ester\ from\ the\ experiments\ (mg)}{Mass\ of\ the\ maximum\ FAME\ in\ the\ sample\ (mg)} \times 100\ \% \quad \text{Eq.5}$$

3. Results and discussion

3.1. In situ transesterification using H_2SO_4

The amount of total lipids was determined as 17±0.8 % (w/w dry algae) while the FFAs were determined as 18.3±2.4 % (w/w total lipids).This level of FFA necessitates the use of acid rather than base catalysts. Lotero et al. (2005) reported an upper limit of 0.5 % FFA content to prevent saponification for two-step alkali-catalysed transesterification. **Figure 1** shows that the FAME yield increased with increasing the reaction time as expected. The maximum FAME yield was 53.8±8 % occurring at 24 h.

Increasing the acid concentration to 0.15 µL/(mg algae) resulted in increased FAME yield from 53 to 87 %, in 24 h. El-shimi et al. (2013) observed a 53% increase in FAME yield during H_2SO_4-catalysed *in situ* transesterification of *Spirulina platensis* by increasing acid volume from 0.0016 to 0.19 µL/(mg algae). Other researchers also reported increases in the yield of biodiesel with an increase in acid concentration during acid-catalysed *in situ* transesterification of microalgae (Wahlen et al., 2011; Velasquez-Orta et al., 2013). One reason for this is that acids can be involved in other reactions, such as hydrolysis of carbohydrates during acid-catalysed *in situ* transesterification as well. Consequently, higher acid concentrations may be required to achieve high FAME yields.

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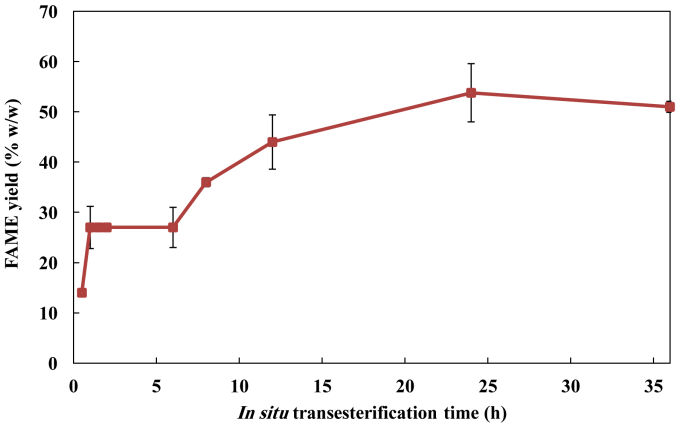

Fig.1. Reactively extracted FAME profile of *Nannochloropsis occulata* with H_2SO_4 catalyst. Process conditions: 600 mol methanol/(mol lipids) = 0.47 mL methanol/(mg algae), agitation rate = 450 rpm, temperature = 60°C, mass of microalgae = 100 mg, 8.5 mol H_2SO_4/ (mol lipids) = 0.087 µL/ (mg biomass).

3.2. *In situ transesterification using SDS/H_2SO_4*

The total amount of phospholipids and glycolipids in the *N. occulata* was determined as 50±0 % (w/w total lipids). A 3.2 mol SDS/(mol lipids) was added to H_2SO_4 to study its effect on FAME enhancement. As mentioned earlier, this amount of SDS in H_2SO_4 was significantly greater than 2 mol SDS/(mol phospholipids) required to effectively solubilise the phospholipids bilayers as reported by Tan et al. (2002). The effect of the inclusion of SDS in H_2SO_4 on FAME yields for *N. occulata* is shown in **Figure 2**.

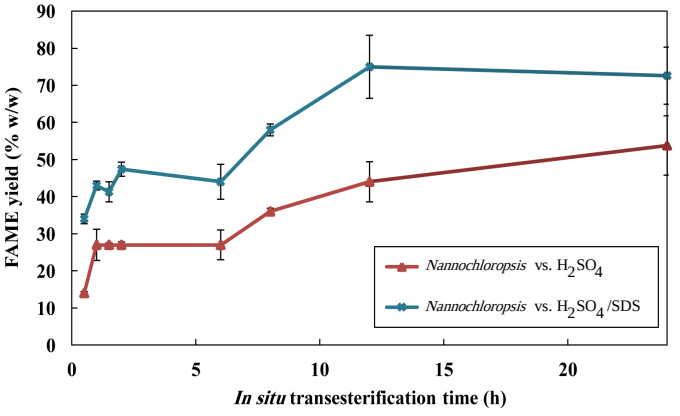

Fig.2. Reactively extracted FAME profile of *Nannochloropsis occulata* with H_2SO_4 *vs.* SDS plus H_2SO_4. Process conditions: 600 mol methanol/(mol lipids) = 0.47 mL methanol/(mg algae), 8.5 mol H2SO4/ (mol lipids) = 0.087 µL/ (mg algae), agitation rate = 450 rpm, temperature = 60°C, mass of microalgae = 100 mg.

It can be seen clearly in the figure that the inclusion of SDS in H_2SO_4 caused higher FAME yields compared with the H_2SO_4 alone at each data point. At 24 h, a 72.6 ± 7.7 % maximum FAME yield was obtained using H_2SO_4/SDS while a 53.8 ± 8 % FAME yield was obtained in this species at the same duration with H_2SO_4 alone. This FAME yield represents 35 % increase. This is significantly higher than the 11 % increase obtained by the inclusion of cetyltrimethylammonium bromide (CTAB) (a surfactant) in NaOH for *in situ* ethanolysis of *Jatropha curcas* L (Hailegiorgis et al., 2011), it is difficult to attribute this to the effect of surfactant though, given the different catalysts used.

3.3. *In situ transesterification with surfactant catalyst ("ZDS") vs. H_2SO_4*

The performance of the synthesized "surfactant catalyst" (zirconium dodecyl sulphate, or "ZDS") for FAME production from *N. occulata* was compared with the FAME yield obtained using H_2SO_4 alone as shown in **Figure 3**.

As can be seen in the figure, the FAME yield produced by both catalysts increased with increases in time as expected. FAME production rate by the ZDS was greater than that produced by H_2SO_4 between 12-36 h. This result shows that *in situ* transesterification of *N. occulata* could be catalysed by ZDS and that ZDS performed more efficiently than the conventional homogeneous H_2SO_4 catalyst.

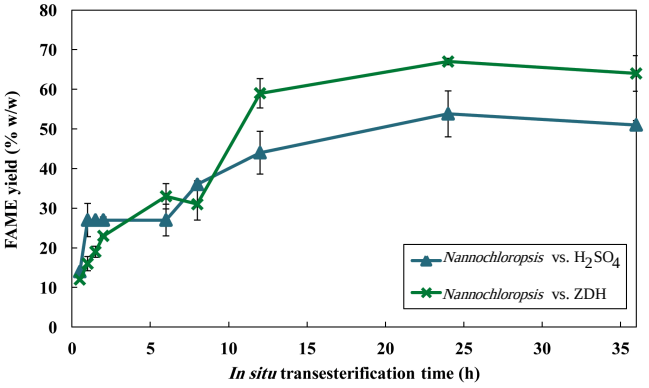

Fig.3. Reactively extracted FAME profile of *Nannochloropsis occulata* with H_2SO_4 vs ZDS. Process conditions: 600 mol methanol/ (mol lipids) = 0.47 mL methanol/(mg algae), 8.5 mol H_2SO_4/ (mol lipids) = 0.087 µL/ (mg algae), 100 % ZDS (w/w lipids), mass of microalgae = 100 mg, agitation rate = 450 rpm, temperature = 60°C.

3.4. *Mechanisms of enhancement of FAME yield by the ZDS catalyst*

The differences in the FAME production by the different catalysts used could be explained in terms of the chlorophyll extracts after the *in situ* transesterification, as shown in **Table 1**.

Table 1.
Chlorophyll content as a measure of cell disruption in *Nannochloropsis occulata.*

Catalyst	Total chlorophyll (µg/mL)	Statistical analysis (P, *t* tests)
Control experiment	0.59±0.02	-
Acid	2.68±0.12	0.01
Acid+SDS	2.74±0.19	0.03
ZDS	2.90±0.29	0.03

Total chlorophyll, i.e., chlorophyll A+C. Process conditions: 600 mol methanol/(mol lipids) = 0.47 mL methanol, 8.5 mol H_2SO_4 / (mol lipids) = 0.087 µL H_2SO_4/(mg algae), agitation = 450 rpm, temperature = 32°C, mass of microalgae = 100 mg, mass of SDS = 9 mg, 100 % ZDS/(w/w lipids), reaction time = 24 h. The control experiment contained no catalyst but other conditions were the same.

Chlorophyll concentration has been positively correlated with cell wall disruption (Gerde et al., 2012). Based on this measurement, H_2SO_4, H_2SO_4+SDS, and ZDS significantly disrupted the cells (i.e. $p<0.05$) than the control experiment but there was no significant differences in cell wall disruption between H_2SO_4 and H_2SO_4/SDS even though there was a significant difference between the FAME yields as presented in **Table 2**. However, the highest chlorophyll extract was produced when using ZDS. Clearly, ZDS disrupted *N. occulata's* cell wall more effectively than H_2SO_4 which explains why it produced greater FAME yield than H_2SO_4 alone.

H_2SO_4 concentrations of 8.5 and 15 mol/(mol lipids) were equivalent to 0.326 and 0.578 mmol H^+, respectively. Increase in H_2SO_4 concentration from 8.5 to 15 mol/(mol lipids) resulted in increases in FAME production rate. The maximum FAME yield produced at 15 mol/(mol lipids) was greater than that produced by ZDS. However, 100 % ZDS (w/w lipids) used was equivalent to 0.0624 mmol H^+ indicating that ZDS was more efficient on a mass for mass basis than H_2SO_4 catalyst. The highest FAME yield (98%) was obtained using SDS+H_2SO_4 at 20% moisture content in

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the microalgae indicating that moisture did not adversely affect this process at this level when catalyst/surfactant was used.

Table 2.
Maximum FAME yields from *Nannochloropsis occulata*.

Catalyst	FAME yield % (w/w)	Reaction time (h)
[a]H_2SO_4	54±8	24
[b]H_2SO_4	87±2	24
SDS + [a]H_2SO_4	73±7.7	24
SDS + [b]H2SO4	98 ± 6.7	24
ZDS	67±1	24

[a]H2SO4 = 8.5 mol/(mol lipids); [b]H_2SO_4 = 15 mol/(mol lipids); SDS + [a]H_2SO_4 for dry algae; SDS + [b]H_2SO_4 for wet algae at 20 % moisture (w/w dry algae). Process conditions: 600 mol methanol/(mol lipids), agitation rate = 450 rpm, temperature = 60°C, mass of microalgae = 100 mg, mass of SDS = 9 mg, 100 % ZDS (w/w lipids).

3.5. *Effect of inclusion of SDS in H_2SO_4 on water tolerance*

It has been shown that acid-catalysed direct transesterification exhibits higher water tolerance to microalgae-bound water (Velasquez-Orta et al., 2013) and free water (Wahlen et al., 2011). In order to investigate the level of water tolerance of H_2SO_4, with and without SDS, samples with 10, 20, and 30 % distilled water (w/w dry algae) were prepared and allowed to equilibrate for 1 h. Surprisingly, there was an increase in the FAME rate for H_2SO_4, with or without SDS, with increase in moisture content in the algae as shown in **Figure 4**.

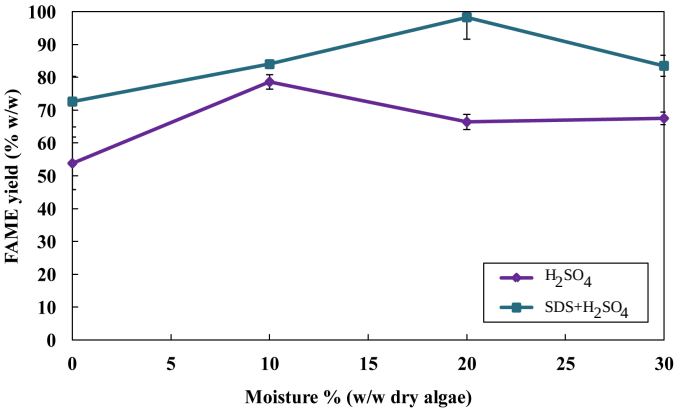

Fig.4. Reactively Extracted FAME produced from re-hydrated *Nannochloropsis occulata* with H_2SO_4 or H_2SO_4 + SDS. Process conditions: 600 mol methanol/(mol lipids) = 0.47 mL methanol, 8.5 mol H_2SO_4/(mol lipids) = 0.087 µL/ (mg biomass), agitation = 450 rpm, temperature = 60°C, mass of SDS = 9 mg, mass of microalgae = 100 mg.

The FAME production rates begin to decrease at 30 % moisture content. Cell wall lipids, such as phospholipids and glycolipids may be disrupted by polar organic solvents such as methanol, ethanol, other alcohols, and water (Cohen et al., 2012). However, the poor permeability of these solvents into the cells of completely dry oil-bearing biomass can significantly reduce their lipid extraction efficiency (Cohen et al., 2012). This can be counteracted to some extent by addition of a small quantity of water, as it swells the cell wall. The inclusion of water in extracting solvents including methanol or ethanol has been reported to increase extraction of phospholipids (Zhukov and Vereshchagrin, 1981). Removal of the cell wall lipids (phospholipids and glycolipids) from the algal cell walls compromises their integrity, i.e., it disrupts the cell wall to some degree thereby increasing accessibility of the solvent (methanol) to the internal body lipids (triglycerides). In addition, the interaction of water and methanol with cell wall proteins could compromise their integrity. The enhancement observed in the present study could be some combination of these two effects and the swelling effect. Therefore, the observed water tolerance in the re-hydrated microalgae was probably due to increased lipid extraction by moist methanol. This could be a key method of increasing the FAME yield in *in situ* transesterification of wet microalgae.

However, beyond 20 % moisture content, a drop in the FAME yield was observed, which showed that the water tolerance was exceeded for both catalysts. The amount of water tolerance achieved herein was greater than 10 % (w/w dry mass) obtained by Velasquez-Orta et al. (2013), perhaps because their moisture content was based on bound, rather than the free water used in this current investigation. However, the water tolerance achieved herein was lower than the 50 % (w/w dry mass) of free water during acid-catalysed *in situ* esterification of *C. gracilius* reported by Wahlen et al. (2011). It was also lower than the 80 % (w/w dry mass) of free water during acid-catalysed *in situ* transesterification of *N. gaditana* reported by Kim et al. (2015). It should be noted that Wahlen et al. (2011) used 0.04 mL methanol/(mg algae) while Kim et al. (2015) used 0.01 mL methanol/(mg algae). These methanol volumes/(mg algae) were significantly higher than the 0.0047 mL/(mg algae) used in this study. Therefore, their corresponding higher water tolerance than what observed herein is expected. *In situ* esterification of microalgae using H_2SO_4 as catalyst exhibited the same water tolerance, with or without SDS. However, the inclusion of SDS in H_2SO_4 produced greater FAME yields than H_2SO_4 alone at each moisture content as shown in **Figure 4**.

Park et al. (2014) has shown that the inclusion of sodium dodecyl benzene sulfonate (SDBS) in H_2SO_4 enhanced the extraction of FFAs and lipids from *Chlorella*. They also reported that SDS did not produce the same corresponding enhancement as SDBS (Park et al., 2014). It should be noted that their experiments were fundamentally different from what is reported herein. They investigated the effect of the inclusion of SDBS or SDS in H_2SO_4-catalysed hot water on the extraction of FFAs and lipids from *C. vulgaris*. They conducted additional experiments on the effect of including SDBS in H_2SO_4 for FAME production from the pre-extracted algal oil through a two-step transesterification. In better words, the approach used by Park et al. (2014) involved making biodiesel from pre-extracted algal oil which is fundamentally different from the single step transesterification ("*in situ* transesterification") reported in this study.

4. Conclusions

In situ transesterification has been shown to be technically feasible for FAME production from *N. occulata* using H_2SO_4, H_2SO_4/SDS (a surfactant), or ZDS (surfactant catalyst). ZDS produced a maximum 67±1 % FAME yield. SDS addition to H_2SO_4 enhanced the FAME yield and caused some levels of water tolerance. Addition of SDS in H_2SO_4 at 20 % moisture content produced a maximum FAME yield of 98.3±6.7 %. Finally, not only the process was more tolerant to water than transesterification-based routes, but the presence of a small quantity of external water increased the FAME yields in *in situ* transesterification, rather than inhibiting the reaction. This effect was apparent for all conditions up to 20-30 % water (w/w dry algae) which was significantly greater than the maximum of 0.5 % water (w/w oil) required in a two-step transesterification.

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