Reactive Extraction of Microalgae for Biodiesel Production

By

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Abstract

Fatty acid methyl ester (FAME “biodiesel”) is a renewable transport fuel that can be produced from waste/refined oil, pre-extracted oil from oilseeds or microalgae. The most common method converts extracted oil from biomass to FAME through transesterification using acidified or alkalised methanol. Alternatively, FAME can be made by contacting the oil-bearing biomass directly with an alcohol containing a catalyst. This approach is potentially a cost-effective alternative way of making algal FAME due to its elimination of the solvent extraction step and its higher water tolerance. This study reports reactive extraction of *Nannochloropsis occulata* and *Chlorella vulgaris* for FAME production using NaOH, H_2SO_4, zirconium dodecyl sulphate (“ZDS”) or H_2SO_4/SDS (a surfactant) as catalysts. It is possible to produce FAME using all of them. A relationship was found between FAME yield, catalyst concentration, methanol to oil molar ratio, moisture content or algal cell wall chemistry. NaOH is the most effective catalyst, producing high FAME yields (96 %) in relatively short reaction times (10 min), at 925:1 methanol to oil molar ratio and 0.5N NaOH. This was achieved despite high levels of free fatty acid (6 % lipid) in *Chlorella vulgaris*. A numerical model derived by Eze *et al*. (2014) fitted with experimental data from this study shows that other side reactions including FAME and triglyceride saponification, free fatty acid neutralisation occur alongside the desired FAME synthesis in a NaOH-catalysed reactive extraction. Regardless of the catalysts used, methanol to oil molar ratios in the range 600:1-1277:1 caused 5-30 wt %/(wt dry algae) moisture tolerance: significantly greater than the 0.5 wt % oil moisture required in conventional transesterifications. Both the phosphorus mass balance and conversion of the isolated algal phospholipids into FAME revealed that pre-soaking pre-treatment solubilises the phospholipid bilayer to some degree, and

[Abstract continued]

contributes to an increased FAME yield in *Nannochloropsis occulata* (98.4 %) and *Chlorella vulgaris* (93.4 %). Residual protein loss in *Chlorella vulgaris* and *Nannochloropsis occulata* were respectively 6.5 and 10 %. The carbohydrate content was significantly reduced by 71 % in *Chlorella vulgaris* and 65 % in *Nannochloropsis occulata*.

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ABBREVIATIONS

BS	British standard
CHN	Carbon-hydrogen-nitrogen analysis
COD	Chemical Oxygen Demand
CTAB	Cetyl trimethyl ammonium bromide
DD	Diphosphatidyl glycerol
DG	Diglyceride
DHA	Docosahexanoic
EPA	Eicosapentaenoic
FFA	Free fatty acid
FT IR	Fourier transform infrared
GC FID	Gas chromatography flame ionization detector
ICP AES	Inductively coupled plasma atomic emission spectroscopy
MG	Monoglyceride
NL	Neutral lipid
PA	Phosphatidic acid
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PG	Phosphatidyl glycerol

PL	Phospholipid
PS	Phosphatidyl serine
RSM	Response surface methodology
SDS	sodium dodecyl sulphate
SPE	Solid phase extraction
STD	standard
TBHQ	Tert-butyl hydroquinone
TG	Triglyceride
TLC	Thin layer chromatography
TSS	Total solid suspension
UV-Vis	Ultraviolet-visible
ZDS	zirconium dodecyl sulphate

Chapter 1. Introduction

1.1 Research background

World energy consumption continues to increase due to population and economic growth. British Petroleum (2014) reported that there was 2.3 % increase in global primary energy consumption in 2013 over its value in 2012. Fossil fuel continues to dominate worldwide energy usage. As shown in figure 1.1, in 2013, 87 % of primary energy consumption was based on fossil fuel (oil: 33 %, coal: 30 %, gas: 24 %), whereas hydroelectricity accounted for only 7 %, nuclear, 4 % renewable energy contributed only to 2 %.

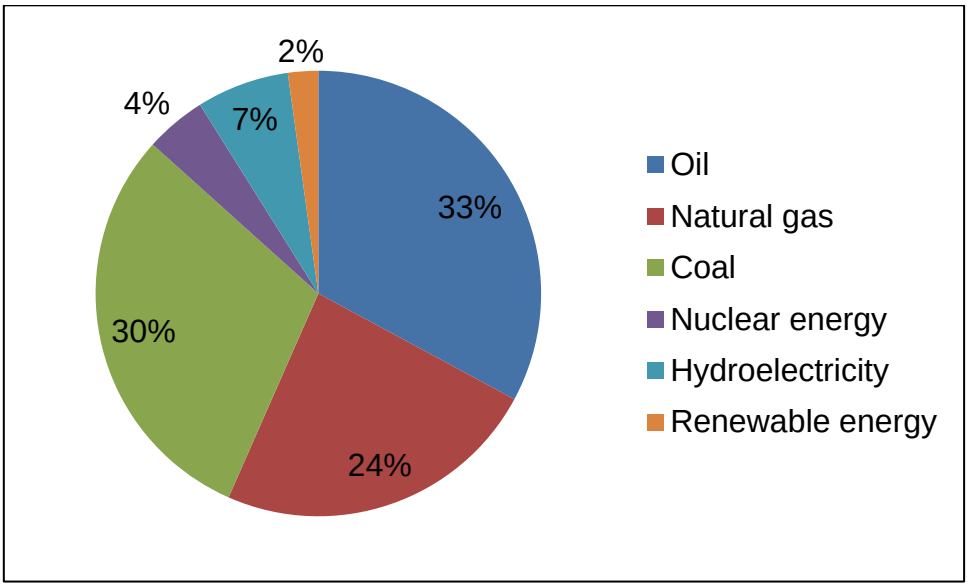


Figure 1.1: Global primary energy consumption in 2013 (BP, 2014)

Combustion of fossil fuel causes the CO_2 concentration in the atmosphere to increase. The Intergovernmental Panel on Climate Change, IPCC (2014) reported that total anthropogenic greenhouse gas emissions rose to 49±4.5 Gt CO_2 eq / yr, in 2010. Fossil fuel combustion and industrial processes accounted for about 78 % of

the total emissions in 2010. This has led to a significant increase in the average atmospheric temperature. Global warming has already adversely affected man, plants, animals and the environment causing prolonged heat waves, higher sea levels, glacial and polar ice cap recession.

However, the impact of CO_2 emissions on the Earth is more than global warming. In the last 200 years, approximately 50 % of anthropogenic CO_2 has been absorbed by the ocean (The Royal Society, 2005). This has led to a change in its carbonate chemistry which manifests as a reduction in its pH and carbonate ion concentrations (The Royal Society, 2005; Orr *et al*., 2005). It was reported that the pH of surface seawater decreased from 8.18-8.07 (0.1unit), which translates into a 30 % increase in the concentration of hydrogen ion (The Royal society, 2005). It has been anticipated that the pH could fall by 0.5 in the 22 nd century, if the current rate of CO_2 emission remains unchanged. Increased ocean acidity has significant impacts on ecosystems, as it prevents coral from building skeletons and shellfish from building their shells. Coral plays a key role in water ecosystems by providing shelters for other marine organisms.

A further problem, particularly for transport fuel and petrochemical supplies, is that the global oil reserve is not evenly distributed. As can be seen in figure 1.2, in 2013 47.9 % of oil reserves were located in the Middle East, 19.5 % in South and Central America, 13.6 % in North America, 8.8 % in Europe and Eurasia, 7.7 % in Africa; and 2.5 % shared by the Asia-pacific region. Similarly, the distribution of global gas reserves in the same year was: 43.2 % in the Middle East, 30.5 % in Europe and Eurasia, 8.2 % in Asia, 7.6 % in Africa, 6.3 % in North America, and 4.1 % of the gas reserves were shared by South and Central America.

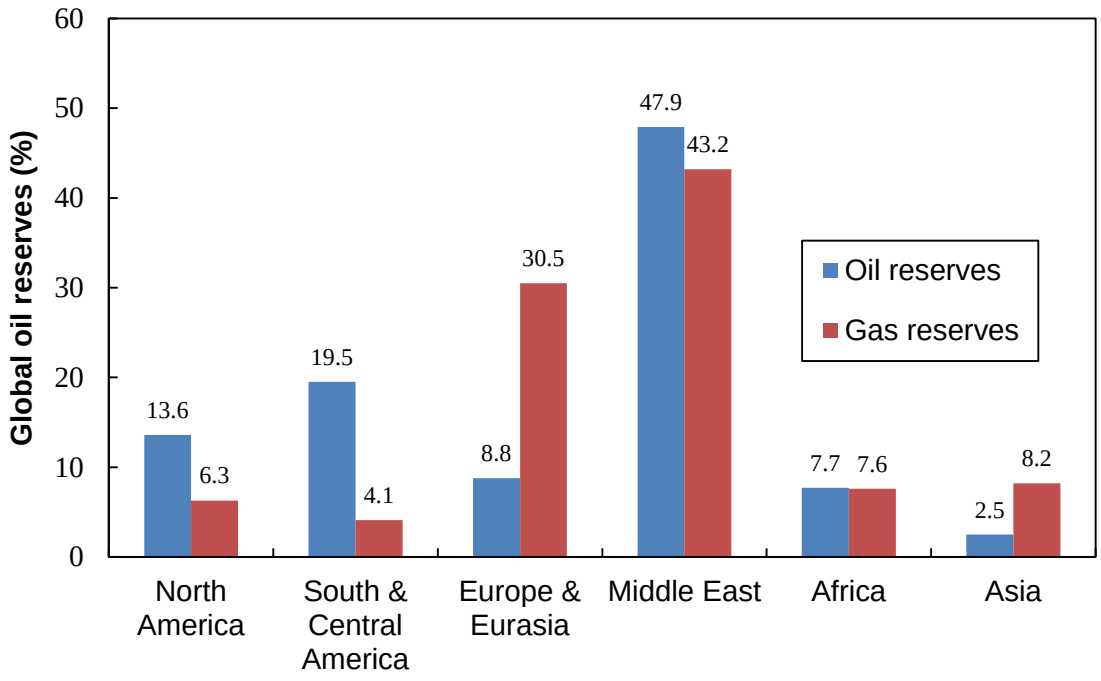


Figure 1.2: Gobal oil and gas reserves in 2013 (BP, 2014).

Since the largest percentage of the global oil and gas reserve is in the Middle East, instability in this region could lead to a global oil shortage or an over-supply as now. Regardless, it is anticipated that the global oil and gas reserves can only serve the world for about 53.3 and 55.1 years, respectively if current energy consumption is unchanged (British Petroleum, 2014).

1.2 Alternative fuels

Irreversible depletion of fossil fuel and the climatic changes due to its combustion, has led to a significant global interest in biofuel production. Between 2003 and 2013, there has been a continued increase in global production of biofuel such as biodiesel and bioethanol, as shown in figure 1.3.

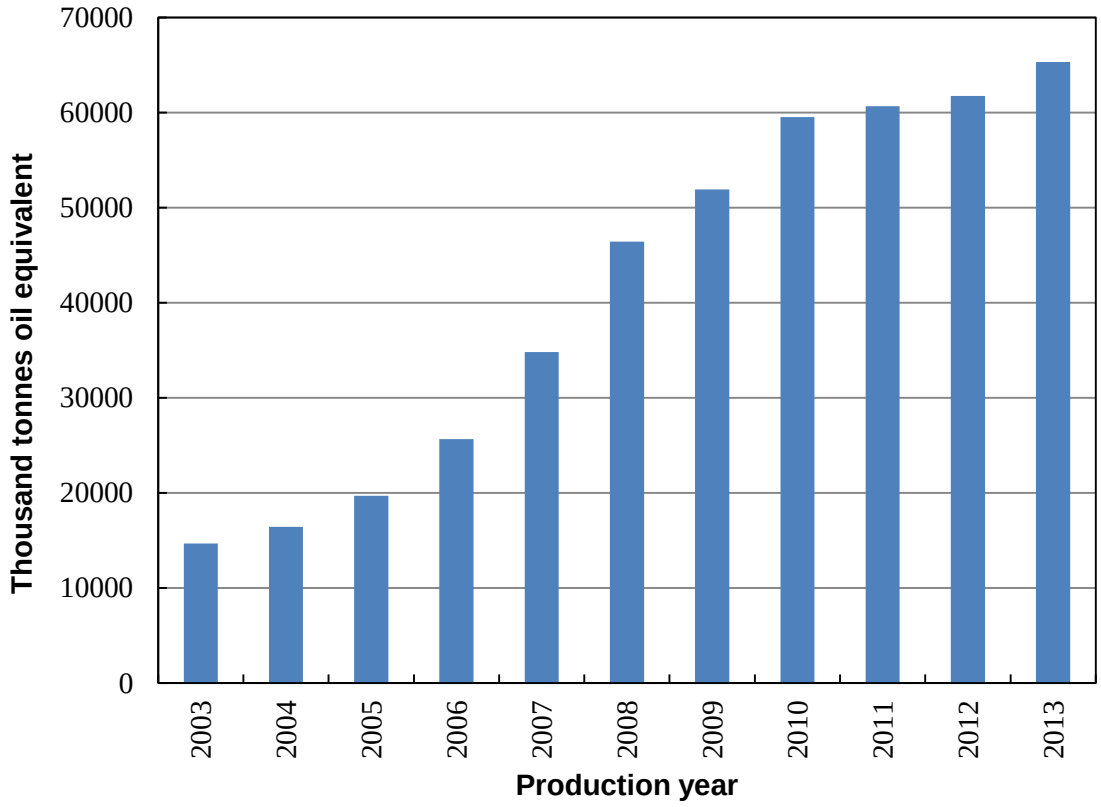


Figure 1.3: Global biofuels production from 2003 to 2013 (BP, 2014).

Global biofuel production in 2013 shows that 45.1 % was accounted for by North America, 28.9 % by South and Central America, 16.8 % by Europe and Eurasia, and 9.8 % by Asia. The Middle East and Africa made an insignificant contribution in 2013 (British Petroleum, 2014).

1.3 Food vs. fuels

Biodiesel is commonly produced from refined vegetable oil made from soybean, rapeseed, sunflower and palm oil. In Europe and America, it is largely produced from rapeseed and soybean, respectively, due to their availability and the cold flow properties of resulting biodiesel (Gui *et al.*, 2008). Edible oil crops account for ~95 % of worldwide biodiesel feedstocks (Gui *et al*., 2008). However, using these crops for biodiesel would require significant amounts of freshwater and arable lands (Chisti,

2007). This can create competition for use of these resources for agriculture and domestic purposes. In Europe and America, the increased cost of edible oil crops has been strongly influenced by their use as energy crops (Mitchell, 2008). High prices may be beneficial to farmers, but this might lead to food shortages in many developing countries, particularly where almost 50 % of their earnings are used for food (Mitchell, 2008). It could also lead to global food insecurity.

Competition for land between food and fuel can also have a negative environmental implication. Recently, vast areas of rainforests particularly in Malaysia, Indonesia and Thailand have been exploited for cultivation of palm oil as it is in high demand for food and fuel (Butler, 2006). This has caused deforestation and negatively affects the forest ecosystems. In order to provide raw materials for large-scale production of biodiesel, non-edible feedstocks that require marginal land and insignificant freshwater are preferable. Such feedstocks include waste oils, non-edible oil crops and microalgae. Non-edible oil crops and waste oils can only supply limited quantities of biofuels, so cannot meet world transport fuels requirements. Usage of microalgae as biofuel feedstock has a number of compelling advantages including: short growing time, high lipid productivity and capturing concentrated CO_2. Since microalgae can be cultivated on non-arable land using wastewater; and are adaptable to harsh environments, it is an alternative renewable energy crop. Using microalgae can improve global energy security.

1.4 Problem statement

Biodiesel can be produced from microalgae through conventional transesterification or reactive extraction ("*in situ* transesterification"). In a conventional two stage transesterification, microalgal oil is pre-extracted usually with hexane and transesterified with alcohol containing a catalyst. A major advantage of reactive

extraction over the conventional process is that it reduces the number of process steps by contacting the biomass directly with the reactants and eliminating the solvent extraction steps. Substantial process energy is required to extract lipids from microalgae. Indeed ~ 90 % of the process energy is accounted for lipid extraction from *Chlorella vulgaris* (Lardon *et al*., 2009).

Producing biodiesel via reactive extraction is potentially cost-effective. However, one major drawback of reactive extraction is that it requires high molar ratio of methanol to oil which is usually in the range of 100:1 to 1000:1. This is significantly greater than the 6:1 methanol to oil molar ratio required by conventional transesterification. The need to recycle unreacted methanol (over 94 % of it) increases the process costs as substantial distillation heat load will be needed for the methanol recovery.

Further problem particularly with microalgae is their small size (3-30 µm) and low concentration, commonly less than 500 mgL^{-1} (Grima *et al*., 2004; Rodolfi *et al*., 2009; Chinnasamy *et al*., 2010). However, biomass productivity of *Chlorella protothecoides* reached 7300 mg/L when cultivated heterotrophically (Xiong *et al*., 2010). Water from such microalgae needs to be quickly removed to avoid spoilage particularly in a hot climate (Grima *et al*., 2004). Dewatering of microalgae usually results in 5-35 % TSS of the algae concentrate (Grima *et al*., 2004; Show *et al*., 2013) which commonly accounted for 20-30 % of the cost of microalgal biomass recovery (Gudin and Therpenier, 1986). Soeder and Pabst (1975) showed that 15,700 kcal of heat were required to evaporate 18.2 kg water per unit kg algae, to an endpoint of 4 % moisture. It was concluded that maintaining the biomass at 10 % moisture content instead of 4 to 5 % would significantly reduce the biomass processing cost. Drying microalgae to the level required by conventional transesterification (<0.5 % wt moisture/ (wt lipid)) is significantly energy intensive and

currently contributing to a significant cost of microalgal pre-treatment. Reduction of drying cost could be achieved by increasing the water tolerance of the reaction step.

A surfactant catalyst (cerium (III) trisdodecyl trihydrate) has been evaluated for conventional FAME production from soybean oil and oleic acid (Ghesti *et al.*, 2009). They concluded that the surfactant catalyst efficiently promoted transesterification of triglycerides and 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 ethanolysis of *Jatropha curcas* (Hailegiorgis *et al.*, 2011). Inclusion of sodium dodecyl sulphate (SDS) in water has been reported to increase oil extraction from canola seeds (Tuntiwiwattanapun *et al*., 2013). SDS has been used for lysing cells to recover intracellular components (Brown and Audet, 2008). These properties of surfactants have not been exploited in reactive extraction of microalgae.

Acid catalysts are commonly used for reactive extraction of microalgae. High acid concentrations are required for high FAME yields. The need to neutralise the catalyst from the product streams, coupled with the longer reaction times required for acid catalysis increase the operating cost. Alkali, surfactant catalysts and combination of surfactant with acid catalyst in reactive extraction of microalgae have not been fully explored. In addition, unlike conventional transesterification the reaction scheme and kinetics of alkali-catalysed reactive extraction have not been well investigated. Such a model would reduce the number of experiments required to find the process optimum.

In combination with the lipids (substrate for biodiesel production), microalgae contain carbohydrate and protein (Sheehan *et al.,* 1998; Becker, 2007) that could add value

to the process economy. Thus, the residual protein and carbohydrate of both *Nannochloropsis occulata* and *Chlorella vulgaris* after the reactive extraction should be evaluated for value added products.

1.5 Aim and objectives

The aim of this research is to investigate the production of biodiesel from *Nannochloropsis occulata* and *Chlorella vulgaris* through reactive extraction ("*in situ* transesterification"). In order to achieve this aim the following objectives were defined:

1. To develop a "surfactant/catalyst", zirconium dodecyl sulphate (ZDS), for reactive extraction of microalgae.
2. To evaluate the effectiveness of the surfactant catalyst ("ZDS") using homogeneous alkali- and acid-catalysed reactive extractions of microalgae as references.
3. To investigate the effect of inclusion of surfactant (sodium dodecyl sulphate "SDS") in H_2SO_4 on the water tolerance of the process.
4. To investigate the optimum conditions for FAME production from *Nannochloropsis occulata* and *Chlorella vulgaris.*
5. To gain a better understanding of the reaction schemes of alkali-catalysed reactive extraction of microalgae.
6. To evaluate the effect of pre-soaking pre-treatment on the required methanol to oil molar ratio and acid concentration.
7. To investigate the effect of the process conditions on the residual microalgal protein and carbohydrate.

Chapter 2. Literature review

This chapter initially reviews classes of microalgal lipids and their location in cells. This is followed by discussion of the microalgal cell walls and their chemical compositions. The challenges they pose to biodiesel extraction from microalgae are then discussed. This is followed by a review of cell wall disruption techniques and their energy requirements. This is followed by comparisons between conventional extraction and reactive extraction of microalgae for biodiesel production. The various parameters influencing reactive extraction are then discussed. The knowledge gaps and challenges in reactive extraction of microalgae are identified, and potential solutions discussed. Finally, a detailed review of different species of microalgae was made.

2.1 Microalgae Lipid

Lipids are broadly classified as neutral or polar. A neutral lipid has no overall polarity. It is located inside the cells in the form of triacylglycerides, monoacyl- and diacylglycerides or free fatty acids. They are more soluble in non-polar solvents such as hexane and chloroform. Neutral lipids are energy storage products. The general structure of triacylglycerides is shown in figure 2.1.

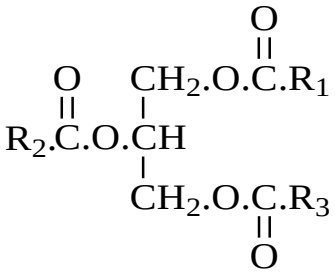


Figure 2.1: Triglyceride structure

Polar lipids contain polar groups, such as choline, ethanolamine, serine, water, glycerol and phosphatidyglycerol in phospholipids. Glycolipids are other forms of polar lipid but are less polar than phospholipids. They are simple sugar-containing lipids. They include monogalactosyl diglyceride, digalactosyl diglyceride and sulpholipids. They are located in the cell walls. They are, as might be expected, more soluble in polar solvents, such as methanol and water. They give cell walls their structural rigidity. Their various structures (Wood, 1974) are shown in figure 2.2-2.5.

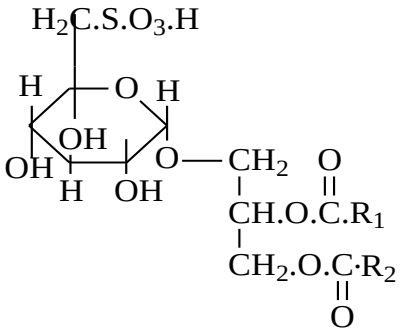


Figure 2.2: Sulpholipid structure

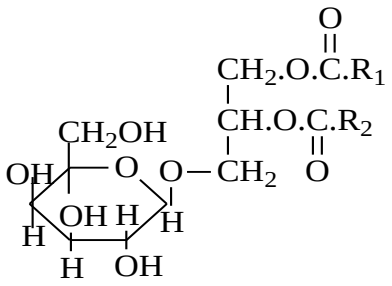


Figure 2.3: Monogalactosyl diglyceride structure

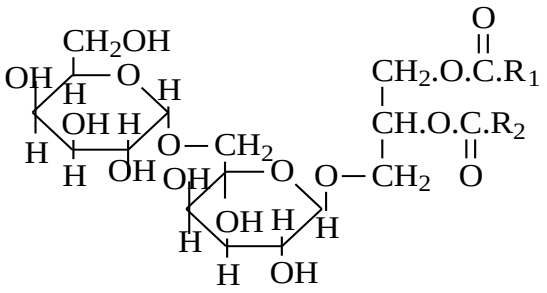


Figure 2.4: Digalactosyl diglyceride structure

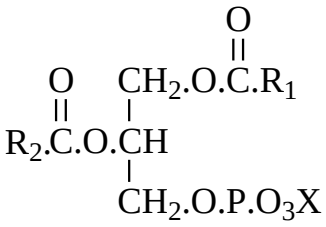


Figure 2.5: Phospholipids general structure

Where R_1, R_2, R_3 in figure 2.2-2.5 denote fatty acids moieties which could be involved in transesterification process as commonly observed in triglycerides. The symbol “X” in phospholipids can be any of the substituent group listed in table 2.1.

Table 2.1: substituent group for phospholipids

Name	Structure	Abbreviation for lipid
Choline	$.O.CH_2.CH_2.N^+(CH_3)_3$	P.C
Ethanolamine	$.OCH_2.CH_2.NH_2$	P.E
Serine	$.OCH_2.\underset{\displaystyle COOH}{\underset{\vert}{CH}}.NH_2$	P.S
Water	.H	P.A
Glycerol	$.CH_2(OH)CHCH_2OH$	PG
Phosphatidyglycerol	$.CH_2CH(OH)CH_2.$	D.D

P.C: phosphatidyl choline; P.E: phosphatidyl ethanolamine; P.S: phosphatidyl serine; P.A: Phosphatidic acid; P.G: phosphatidyl glycerol; D.D: diphosphatidyl glycerol.
Source: Wood (1974).

Microalgal cell wall consists of phospholipid bilayers membrane (about 7-8 nm thick) which is embedded with integral and peripheral protein. Carbohydrate coat in form of glycolipids, glycoproteins and polysaccharides encloses the membrane (Barsanti and Gualtieri, 2014). Algal cell wall accounts for ~10 % of dry algal biomass (Becker, 2004). Cell wall in eukaryotic cell including Eustigmatophyceae (*Nannochloropsis occulata*) and Chlorophyceae (*Chlorella vulgaris*), basically contains fibril and mucilage such as polysaccharides, lipid and protein (Becker, 2004, Barsanti and Gualtieri, 2014). Depending on the microalgae species, a hard surface such as silica, calcium carbonate, algaenan or sporopollelin can be present. Other products found in the cells are listed in table 2.2.

Table 2.2: Cell wall compositions of *Chlorella vulgaris* and *Nannochoropsis occulata*

Substance	Chlorophyta (*Chorella vulgaris*)	Ochrophyta (*Nannochloropsis occulata*)	Reference
Phycobilins	Absent	Absent	Barsanti and Gualtieri (2014)
Storage carbohydrate	Starch (α-1,4-glucan)	Chrysolaminarin (β-1,3-glucan)	Richmond (2004); Barsanti and Gualtieri (2014)
Cell wall	cellulose	polysaccharides	Richmond (2004); Barsanti and Gualtieri (2014)
Additional cell wall components	[a]some species contain sporopollelin [b]some species contain algaenan	[c]Algaenan	[a]Atkinson *et al* (1972); [b]Allard and Templier (2000) & (2001); [c]Gelin *et al* (1999)

As shown in table 2.2, cellulose is commonly found in the cell wall of *Chlorella vulgaris. Nannochloropsis occulata* cell wall contained polysaccharide with sugar unit as glucose (68.8 %), fucose (4.4 %), galactose (3.8 %), mannose (6.1 %), rhamnose (8.3 %), ribose (4.6 %) and xylose (4.4 %) (Brown, 1991). In addition, algaenan is found in the cell wall of *Nannochloropsis occulata* (Gelin *et al*., 1999). Some *Chlorella species* contain algaenan, albeit with a different chemical composition to those in *Nannochloropsis* occulata (Allard and Templier, 2000 & 2001). Algaenan refers to aliphatic bio macromolecules (polymer) that are resistant to many chemicals including acid and base (Tegelaar *et al*., 1989). Their structures are broadly categorised into three (Versteegh and Blokker, 2004):

Type 1 consists of even numbered monomers of linear C22-C34 carbon chain containing functional group at α, ω, ω^9 or ω^{18}. The functional group cross-links the

monomers with ether and ester bonds as it commonly found in Chlorophyta such as *Tetraedron minimum* and *Scenedesmus communis* (Blockker *et al*., 1998b).

Type 2 consists of monomers of unsaturated aliphatic aldehydes and unsaturated hydrocarbons with typically 40 carbon atoms. Acetal and ester bonds cross-link the monomers as it occurs in *Botryococcus. braunii* (Simpson *et al*., 2003).

Type 3 consists of monomers of C28-C36 diol; C30-C32 alkenols and C25, C27, C29 poly/unsaturated hydrocarbons. The monomers are cross-linked with mid-chain ether bonds as found in Eustigmatophyceae (*Nannochloropsis salina*) (Gelin *et al*., 1997).

Sporopollenin is another chemical resistant biopolymers formed by oxidative polymerisation of carotenoids and/ or carotenoid esters (Brooks and Shaw, 1968a). They were found in the cell wall of *Chlorella sp.* (Atkinson *et al*., 1972). However, algal cell wall compositions are diverse, making it difficult to make generalisations for a particular species. For instance, Yamada and Sakaguchi (1982)'s investigation into 12 strains of *Chlorella sp*. revealed that cell structure and /or chemical composition were significantly different within *Chlorella vulgaris.* They categorised the strain into three groups:

Type 1: those with a trilaminar outer sheath (TLS) which was resistant to enzyme digestion. This group has no secondary carotenoids, which means that sporopollenin is absent (Atkinson *et al*., 1972).

Type 2: those with thin outer mono-layers, whose walls contained large amounts of β-linked polysaccharides, but less pectin.

Type3: those without an outer layer whose walls were completely resistant to enzyme digestion. This group has walls largely comprising pectin but fewer β-linked polysaccharides.

Table 2.3: variation in cell wall compositions of *Chlorella* strains

Type of cell wall	Strain	Calcofluor White ST[a]	Ruthenium Red[b]	Secondary carotenoids
1	*C. ellipsoidea* C-102	++	+	-
	C. vulgaris C-209	+	±	-
2	*C. ellipsoidea* C-87	++	±	-
	C. ellipsoidea C-183	++	+	-
	C. vulgaris C-169	++	+	-
	C. vulgaris C-208	+	±	-
	C. saccharophila C-211	++	-	-
3	*C. vulgaris* C-30	+	+	-
	C. vulgaris C-133	+	+	-
	C. vulgaris C-135	+	+	-
	C. vulgaris C-150	+	+	-
	C. vulgaris C-207	+	+	-

[a] ++ Intense blue fluorescence from whole surface, + weak fluorescence; [b] + Intense red; ± pink; - no red colour

Staining with Calcofluor White ST indicates presence of cellulose containing β-linked polysaccharides (Maeda and Ishida, 1967). Staining with Ruthenium Red indicates presence of pectin (Soeder, 1963).

Their findings revealed that *Chlorella vulgaris* has different wall compositions due to diversity within the strain.

The cell wall of *C. vulgaris* and other microalgae as described above provides structural rigidity for the cells to adapt to their environments. However, the cell wall resistance adversely affects the efficiency of algal bioprocessing such as genetic transformation, fermentation, anaerobic digestion, oil extraction and biodiesel production. Indeed, it causes significant large solvent requirement and energy load during extraction processes (Gerken *et al*., 2012).

There are, however, exceptions, such as *Botryococcus braunnii,* that excretes oil as less oxygenated isoprenoids outside the cell wall (Wijffels *et al.*, 2010). *Botryococcus braunnii* is not an attractive option for biodiesel production because it is difficult to cultivate (Wijffels *et al.*, 2010). Because microalgal neutral lipids are contained within cells they are not readily available for extraction, so require disruption prior to transesterification (Lee *et al.*, 2012).

2.2 Microalgae cell disruption

Disruption techniques are broadly classified into mechanical, physical, chemical and enzymatic (Middelberg 1995; Lee *et al.* 2012), as shown in figure 2.6.

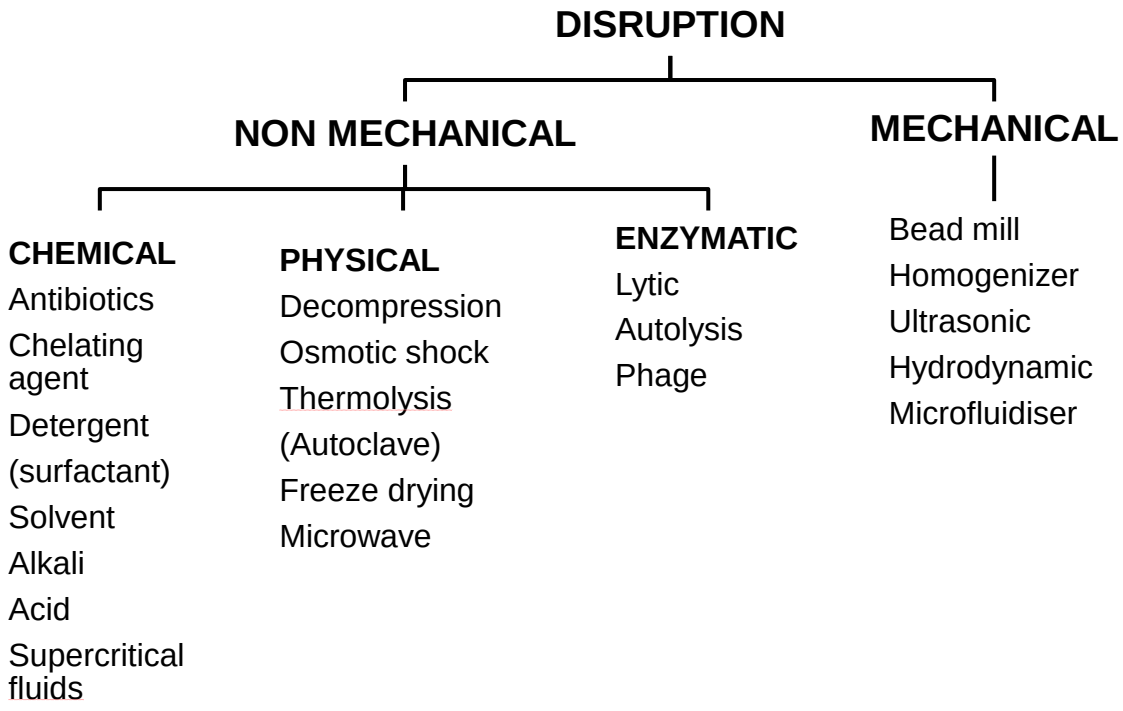


Figure 2.6: Classification of cell disruption techniques

(Modified from Lee *et al.,* 2012)

Cell disruption has been shown to be an effective means of enhancing lipid extraction from microalgae. Lee *et al*. (2010) compared different cell disruptions for enhancing lipids extraction from *Botryococcus sp*., *Chlorella vulgaris* and *Scenedesmus sp*. using chloroform/methanol solvent mixture (1:1 v/v). The range of lipids obtained for the three species were: autoclaving (5.4 to 11.9 %), bead-beating (7.9 to 8.1 %), microwave irradiation (10 to 28.6 %), sonication (6.1 to 8.1 %) and 10 % NaCl osmotic shock (6.8 to 10.9 %).

For *Chlorella vulgaris,* microwave irradiation and autoclaving produced the highest lipid yield, whereas bead-beating produced the least (7.9 %). Osmotic shock produced the same lipid yield in *Chlorella vulgaris* and *Scenedesmus sp*. but required more time (48 h). They concluded that microwave irradiation was the most

effective disruption in terms of lipid extraction for the microalgae species investigated.

Surendhiran and Vijay (2014) compared the effect of different cell wall disruption on lipid extraction from nitrogen replete and deplete *Nannochloropsis occulata* using chloroform/methanol solvent mixture (2:1 v/v). Usage of enzymatic (cellulase) pre-treatment for 12 h produced 51.7 % lipid under nitrogen depletion, but 32.7 % in a nitrogen replete sample. Ultrasonication pre-treatment at 15 min produced 45.8 % lipid in nitrogen deplete but 30 % in replete samples. Autoclaving at 121^{o}C for 30 min before extraction produced 43.9 % lipid in nitrogen depletion but 28.8 % when nitrogen-replete. Osmotic shock (40 % NaCl solution) for 48 h resulted in 40.6 % lipid in nitrogen deplete but 26.5 % in replete sample. Although osmotic shock is simple, it required longer time (48 h) as also reported by Lee *et al.* (2010) for *Chlorella vulgaris* and *Scenedesmus sp*.

Acid lysis at a pH of 2 for 2 h was the most effective disruption technique, producing 54.3% lipid in nitrogen depletion but 33.2 % in replete samples. This is contrary to microwave irradiation reported by Lee *et al*. (2010) as the most efficient disruption technique in terms of lipid extraction for *Chlorella vulgaris*, *Botrycoccus sp*. and *Scenedemus sp.* It should be noted that microalgae cell wall chemistries vary, consequently cell disruption techniques are strain and species dependent.

Additionally, lipids obtained from nitrogen-starved *Nannochloropsis occulata* were significantly greater than replete samples. This is because microalgal metabolic pathways under deprived nitrogen media shift to production of neutral lipids (storage lipids) or carbohydrates (Hu, 2004) due to more carbon produced than nitrogen. *Dunaliella sp*. produced more carbohydrate when cultivated on nitrogen-depleted

medium (Borowitzka and Borowitza, 1988). While some *Chlorella sp.* produced more neutral lipids under such condition, others produced more carbohydrate (Richmond, 1986).

2.2.1 *Energy requirement of algae cell disruption*

Cell disruption enhances lipid extraction, but can be very energy intensive. In addition, algal cells are unsuitable for mechanical presses as they are too small, and can pass through unchanged (Lee *et al.*, 2012).

An overview of energy consumptions of microalgal cell disruptions that are considered suitable for commercial scale production (Lee *et al.,* 2012) is shown in table 2.4.

Table 2.4: Overview of experimental cell disruption techniques and their energy consumptions

(A) Disruption technique	(B) Substrate & experimental conditions	(C) Calculated energy consumption $GJ.m^{-3}$ cell suspension	(D) Energy consumption $MJ.(kg\ dry\ mass)^{-1}$	(E) Scale of use	(F) References
Sonication	Chlorococcum sp (200 mL, 8.5 kgm^{-3}, 750 w, 5min, low)	1.1	132	Laboratory, industrial	Halim *et al.* (2012)
HPH	Chlorococcum sp (200 mL, 8.5 kgm^{-3}, 2.5 kW, 6min, High)	4.5	529	Laboratory, industrial	Halim *et al.* (2012)
HSH	Saccharomyces cerevisiae (0.8 L, 10 kgm^{-3}, 600 W, 15 min, medium)	0.7	68	Laboratory, industrial	Shirgaonkar *et al*. (1998)
Bead mills	Botryococcus, Chlorella, Scenedesmus (100 mL, 5kgm^{-3}, 840 W, 5 min, high)	2.5	504	Laboratory, industrial	Lee *et al*. (2010)
Microwave	Botryococcus, Chlorella, Scenedesmus (100 mL, 5kgm^{-3}, 700 W, 5 min, high)	2.1	420	Laboratory, industrial	Lee *et al.* (2010)
Microwave + solvent	Scenedesmus (100 mL, 75kgm^{-3}, 1.2 kW, 1 min, high)	1.2 (Microwave only)	9.6 (Microwave only)	Laboratory, industrial	Balasubramanian (2011)

(A)	(B)	(C)	(D)	(E)	(F)
Disruption technique	Substrate & experimental conditions	Calculated energy consumption $GJ.m^{-3}$ cell suspension	Energy consumption $MJ.(kg\ dry\ mass)^{-1}$	Scale of use	References
Freeze drying	Mathematical modelling on an industrial scale	1.4 (modelled)	140 (modelled)	Laboratory, industrial	Ratti (2001)
Hyrodynamic cavitation	Saccharomyces cerevisiae (50 L, 10 kgm^{-3}, 5.5 kW, 50 min, medium)	0.3	33	Laboratory, pilot scale	Balasundaram (2001)

Column D = Column C/ (Concentration in column B)

HPH: High pressure homogenization; HSH: High speed homogenization

Column D = Column C/ (Concentration in column B)

HPH: High pressure homogenization; HSH: High speed homogenization

A 33 MJ kg^{-1} (dry cells) was the lowest energy consumed, which is significantly greater than 27 MJ kg^{-1} (the estimated combustion energy from a typical algae biomass). Furthermore, the energy consumption is greater than the estimated minimum theoretical energy consumption by a factor of 10^5.

There would be economic justification for this high energy consumption for algal cell disruption if the lipids or pigments were extracted for high value commodities, such as pharmaceutical or nutraceutical products. However, it becomes difficult to sustain if the lipids are extracted for bulk fuels such as biodiesel (Lee *et al.*, 2012). In order to make algal oil economically competitive biodiesel feedstock, a less energetic disruption technique is required. For instance usage of surfactant/surfactant catalyst, alkali or acid catalyst for disrupting algae cells are less explored.

2.2.2 *Pre-soaking pre-treatment*

Pre-soaking is a chemical pre-treatment in which solvents such as methanol are allowed to percolate through the substrate. Ma (2012) compared the effect of subjecting *Chlorella vulgaris* to methanol soaking, ultra-sonication, microwave irradiation, autoclaving and methanol soaking plus microwave irradiation, prior to KOH-catalysed *in situ* transesterification. It was found that the combination of methanol soaking and microwave irradiation resulted in the highest FAME rate.

Methanol soaking alone caused a comparable FAME enhancement to the other pre-treatments. Autoclaving gave the least FAME enhancement. However, the author did

not study acid catalysis. In addition, the mechanism of the pre-soaking enhancement was not investigated. Therefore, there is still a knowledge gap in this regard.

2.3 Biodiesel

Biodiesel is usually made by transesterification of triglycerides or esterification of free fatty acids derived from plants or animals with low molecular mass alcohols containing catalyst. Biodiesel is a renewable fuel as it is derived from plant or animal. There are advantages and disadvantages to using biodiesel as transport fuel.

2.3.1 *Advantages of biodiesel as transport fuels*

Biodiesel has similar flow and combustion properties to diesel fuels, which makes it a perfect alternative to petrodiesel. Its usage as transport fuel requires little or no modification to Diesel engines. Combustion of pure biodiesel or blends emits lower amount of carbon monoxide, unburned hydrocarbons, sulphates and has comparable fuel qualities to petrodiesel, as illustrated in table 2.5. Since CO_2 is consumed by the plants or microorganisms producing biodiesel feedstocks, its combustion should contribute less to greenhouse gases than petrodiesel. Biodiesel is an oxygenated fuel. It typically contains 11 % oxygen and biodegrades much more readily than petrodiesel (Lotero *et al.,* 2005), so it has a much lower environmental impact than petrodiesel when spilled. Table 2.5 compares ASTM standards for diesel and biodiesel fuels.

Table 2.5: Maximum allowed qualities in petrodiesel and biodiesel by American Society for Testing and Materials (ASTM)

Property	diesel	biodiesel
standard	ASTM D975	ASTM D6751
composition	HC[a] (C10-C21)	FAME[b] (C12-C22)
Kinematic viscosity at 40 ^{o}C $mm^2(s)^{-1}$	1.9-4.1	1.9-6.0
Specific gravity $g(mL)^{-1}$	0.85	0.88
Flash point (^{o}C)	60-80	100-170
Cloud point (^{o}C)	-15 to 5	-3 to 12
Pour point (^{o}C)	-35 to -15	-15 to 16
Water (vol %)	0.05	0.05
Carbon (wt %)	87	77
Hydrogen (wt %)	13	12
Oxygen (wt %)	0	11
Sulphur (wt %)	0.05	0.05
Cetane number	40-55	48-60
HFRR[c] (µm)	685	314
BOCLE[d] scuff (g)	3600	>7000

[a]Hydrocarbon. [b]Fatty acid methy esters. [c]High-frequency reciprocating rig. [d]Ball-on-cylinder lubricating evacuator.

Source: Lotero *et al.* (2005)

Table 2.6 shows the emission profiles of a typical 100 % biodiesel (B100) and a blend (B20), containing 20 % petrodiesel and 80 % biodiesel using 100 % petrodiesel as the reference.

Table 2.6: Emission profiles (%) of an average B100 and B20 relative to normal diesel

Emission	B100	B20
Carbon monoxide	-48	-12
Total unburned hydrocarbon	-67	-20
Particulate matter	-47	-12
Nitrogen oxides	+10	+2
sulfates	-100	-20
Air toxics	-60 to -90	-12 to -20
mutagenicity	-80 to -90	-20

Source: Lotero *et al.* (2005)

As shown in table 2.6, a 20 % blend (B20) of biodiesel in petrodiesel significantly reduces the emission profile of the original diesel fuels while 100 % biodiesel (B100) clearly shows that biodiesel has significantly low emission profile than petrodiesel.

2.3.2 *Disadvantages of biodiesel as transport fuels*

As can be seen in table 2.6, biodiesel emissions are higher in nitrogen oxides than petrodiesel. It is believed that the significantly higher concentration of oxygen in biodiesel results in excess oxygen during combustion (Mittelbach and Remschmidt, 2006). Nitrogen oxides contribute significantly to the formation of ground level ozone (Fernando *et al*., 2006).Presence of such ozone in the atmosphere adversely affect

human health as it causes respiratory system irritation, induces asthma attack and permanent lung damage (Epa. Gov, 2015). However, inclusion of catalytic converters in car exhausts is a method of reducing the nitrogen oxides. The following three techniques are employed for reducing nitrogen oxide emissions from fuel combustion.

a.) DeNOx catalyst which involves usage of precious metal- or zeolite-coated devises. The set up enhances direct reaction of NOx and hydrocarbon in the fuels to form N_2, H_2O and CO_2. However, it has maximum 20 % efficiency in engine test (Tritthart *et al*., 2001).

b.) NOx-absorber catalyst promotes oxidation of NO to NO_2 which is stored in the storage unit largely in form of Ba $(NO_3)_2$. It has over 90 % efficiency in an engine test for NOx reduction (Tritthart *et al.,* 2001).

c.) Selective catalytic reduction (SCR) involves using ammonia to reduce NOx under oxidizing conditions. The ammonia could be easily sourced by heating aqueous urea solution. Engine test revealed that it has 85 % efficiency of reducing NOx (Walker, 2003).

2.4 The transesterification reaction

During plant photosynthesis, triglycerides are produced, which have a high heat of combustion (Pryde, 1983). Essentially, these substances are the plant's energy storage. Their usage as a source of transport fuel dates back to the invention of the diesel engine by Dr Rudolf Diesel. His diesel engine at the 1900 Paris exhibition was fuelled by peanut oil (Nitske and Wilson, 1965).

Vegetable oil should be the best alternative to petrodiesel because of its availability and portability (Pryde, 1983). However, its heat content is 88 % of petrodiese's and it only performed well in engine tests lasting less than 10 h (Pryde, 1983). After long periods of use, technical operational problems start to develop (Pryde, 1983). These include formation of carbon deposits, oil ring sticking, thickening and gelling of lubricating oil due to contamination with vegetable oil. These technical problems happened because of high viscosity, low volatility and poor cold flow properties of vegetable oil (Pryde 1983; Srivastava and Prasad, 2000).

However, vegetable oil can be chemically transformed via transesterification into a less viscous fuel (biodiesel). For instance, most vegetable oil has a viscosity in the range of 27.2-53.6 $mm^2(s)^{-1}$, whereas conversion to fatty acid methyl ester (FAME) reduces viscosities to between 3.6-4.6$mm^2(s)^{-1}$ (Demirbas, 2008). Reduction in fuel viscosity enhances the fluid pumping and atomization properties (Islam *et al*., 2004) and reduces the operational problems described above associated with vegetable oil as fuels.

Transesterification involves reacting a triglyceride with an alcohol in the presence of alkali or acid catalyst to form alkyl esters of the corresponding alcohol and glycerol. Methanol is commonly used because is the cheapest alcohol (Demirbas, 2008). If the reaction goes to completion, three molecules of alkyl ester (biodiesel) of the corresponding alcohol are formed. For instance, if methanol is used, three molecules of fatty acid methyl ester and one molecule of glycerol are formed as shown in figure 2.7.

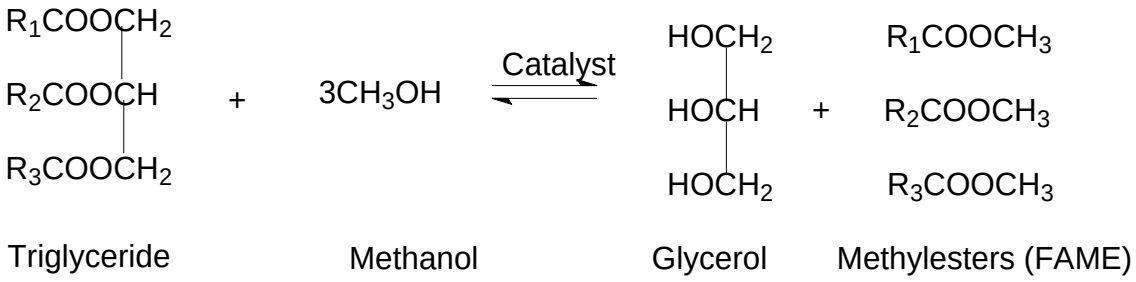
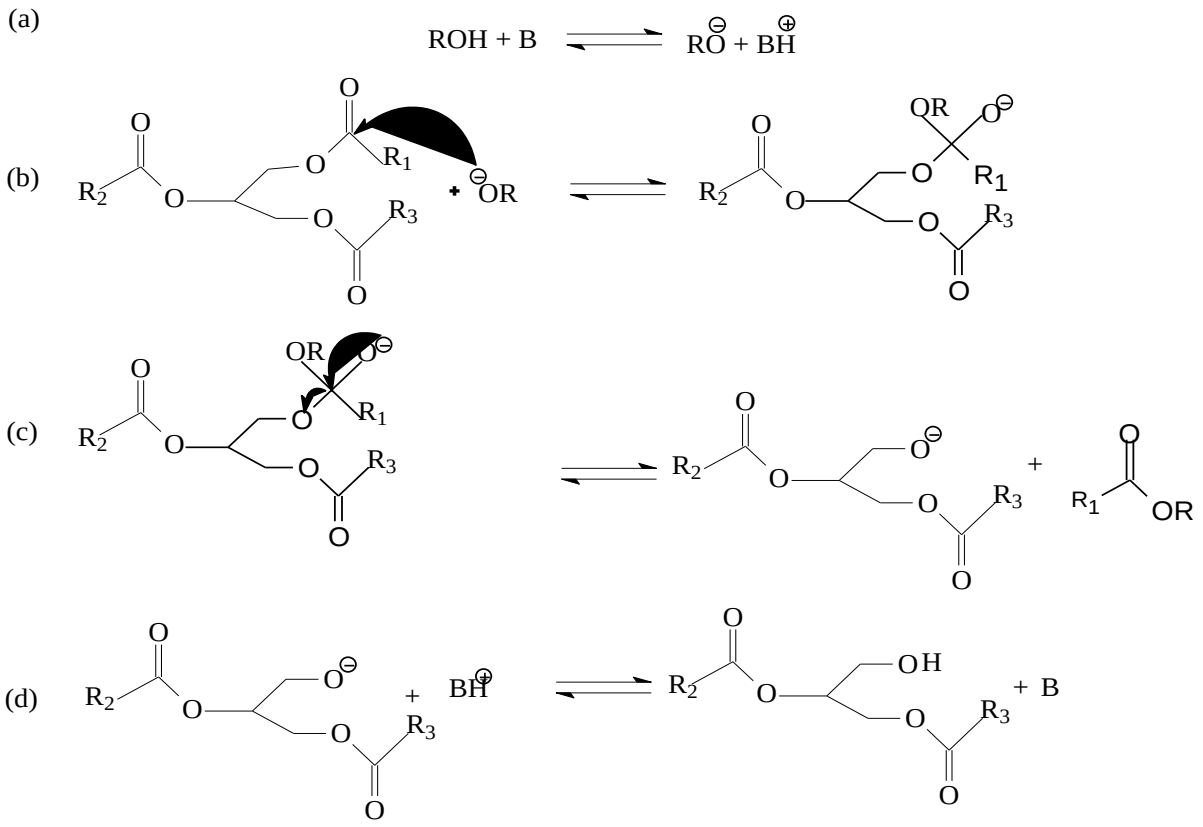


Figure 2.7: Overall transesterification reaction

The stoichiometric ratio of alcohol to oil needed for this reaction for an alkali catalyst is 3:1, but in practice, since it is an equilibrium reaction, an excess of alcohol of 6:1 is typically required, to increase the rate and conversion (Freedman *et al.*, 1984). A typical alkali-catalysed conventional transesterification takes at least 3 min to reach completion, depending on alkali concentration, methanol to oil molar ratio, agitation, water content and the free fatty acid content of the substrate (Eze *et al.,* 2014). In contrast, an acid-catalysed conventional reaction can be 4000 times slower (Srivastava and Prasad, 2000). The alkali reaction is faster because it involves a strong nucleophile, alkoxide species whereas acid catalysis involved electrophilic species. Once the alkoxide is formed, it directly attacks the carbonyl group in the triglyceride to form the corresponding alkyl esters as illustrated in the figure 2.8.



B: Base catalyst

R_1,R_2,R_3: Carbon chain of fatty acids

R: Alkyl group of the alcohol

Figure 2.8: Homogeneous alkali-catalysed transesterification schemes

The steps involved in homogeneous alkali catalysis as shown in figure 2.8 (Lotero *et al.*, 2005). They are:

a.) Formation of active alkoxide catalyst species, RO^- ;

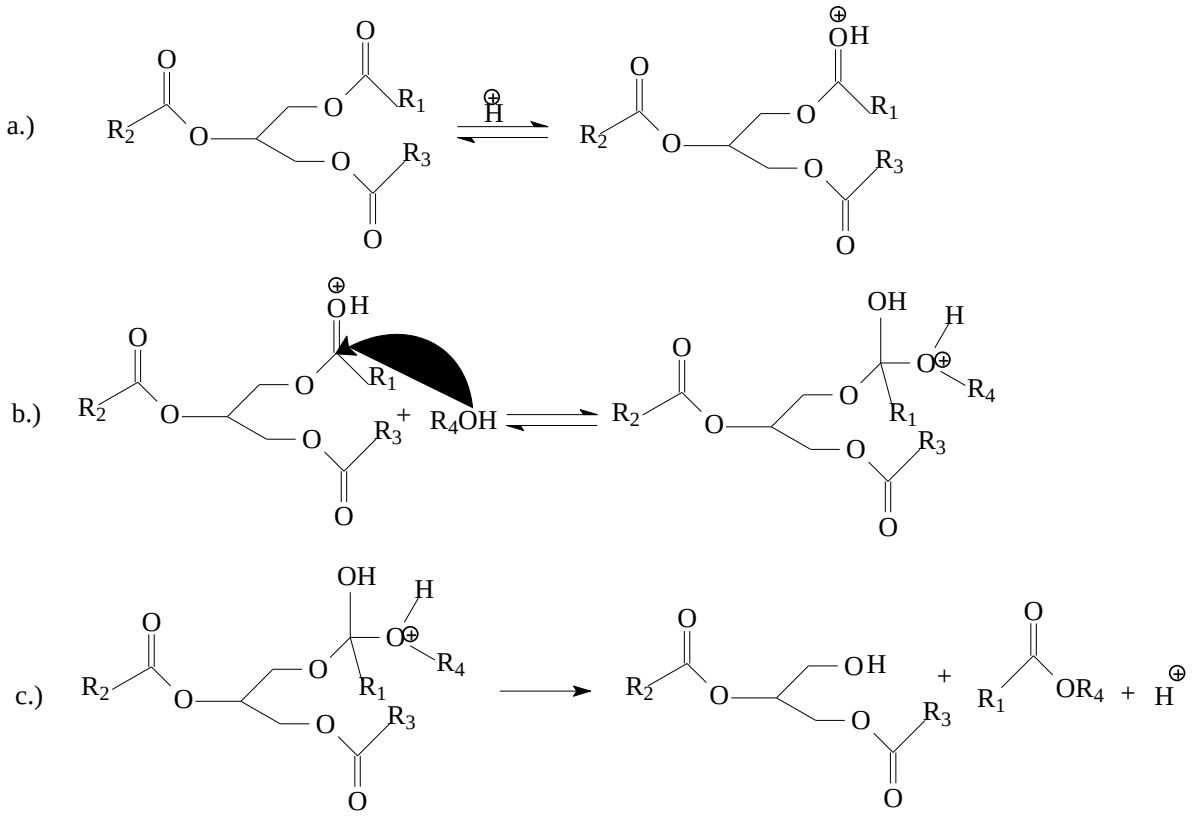
b.) Nucleophilic attack of RO^- to the carbonyl group on TG producing a tetrahedral intermediate;

c.) Breaking down of the tetrahedral intermediate;

d.) Regeneration of the alkoxide (RO^-) species.

The sequences are repeated for both diglyceride and monoglyceride

In contrast, acid catalysis involves formation of an electrophilic species, which reacts with the alcohol to form a tetrahedral intermediate as shown in figure 2.9.



R_1, R_2, R_3: Carbon chain of fatty acid

R_4 : Alkyl group of the alcohol

Figure 2.9: Homogeneous acid catalysed transesterification scheme

As shown in figure 2.9, the homogeneous acid-catalysed reaction scheme for triglyceride transesterification (Lotero *et al.,* 2005) is:

a.) Protonation of the carbonyl group by the acid catalyst to create an electrophilic species;

b.) Nucleophilic attack by the alcohol to generate tetrahedral intermediate;

c.) Proton migration and breaking down of the intermediate.

The sequences are repeated for both diglycerides and monoglycerides.

Acid catalysis takes considerably longer than alkaline catalysis. However, acid catalysts can catalyse esterification of free fatty acids to biodiesel, as shown in figure 2.10. This is why it is applicable for high free fatty acid (FFA) substrates

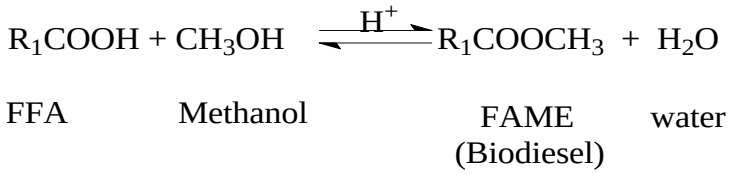


Figure 2.10: Acid-catalysed esterification of free fatty acid to alky ester

2.4.1 *Kinetics of conventional transesterification*

Noureddini and Zhu (1997) investigated the kinetics of transesterification of soybean oil catalysed by methanolic NaOH. The rate constants of the reaction were determined at various mixing intensities and temperatures. The molar ratio of alcohol to triglyceride and catalyst concentration was fixed. They found that a second order mechanism described the process well. The process was affected by mass transfer initially, but this later became insignificant as the FAME production increased, indicating that methyl ester acts as a mutual co-solvent for inducing a single phase. Increasing the temperature (30-60 ^{o}C) significantly increased the FAME conversion, but there was no significant increase in the rate between 60-70 ^{o}C perhaps due to the fact that methanol would be in vapour phase at 1 atm above 60 ^{o}C.

Darnoko and Cheryan (2000) also observed a moderate increase in conversion of methyl ester from 73 to 82 %, while the temperature increased from 50-65 ^{o}C during transesterification of palm oil with methanolic KOH. They suggested that a 65 ^{o}C temperature was adequate, since methanol boils at 65 ^{o}C so as to remove the need to pressurise the reacting vessel.

It is noteworthy that other competing reactions occur alongside the desired FAME production that should be included to completely describe the transesterification mechanisms. For example, the reaction schemes for rapeseed oil catalysed with KOH (Komers *et al*., 2002; Eze *et al*., 2014) were reported to involve the following reactions:

- Main reaction:

(1) Formation of methyl ester;

- Secondary reactions:

(2) Saponification of triglyceride (TG);

(3) Saponification of methyl ester;

(4) Neutralisation of FFA.

Komers *et al*. (2002) concluded that an equivalent increase in reaction rate caused by a temperature increase can be achieved by increasing KOH concentration and those changes in process factor such as oil, alcohol and catalyst can significantly change the kinetics and the mechanisms of the process.

Bambase *et al.* (2007) investigated the kinetics of NaOH-catalysed methanolysis of crude sunflower oil for FAME production by varying agitation speed (200 to 600 rpm), temperature (25 to 60 ^{o}C), catalyst loading (0.25 to 1.00 %) and methanol to oil molar ratio (6:1-20:1). Increases in agitation rate, temperature, methanol to oil molar ratio, and catalyst concentration increased the FAME production rate. They observed a significant increase in FAME conversion, from 18.8 to 82.7 %, occurring in 2 min at 60 ^{o}C, 400 rpm, and 6:1 methanol to oil molar ratio with increase in NaOH

concentration from 0.5 to 1.00 %. However, 0.5 % catalyst concentration was considered adequate since it caused 98 % FAME yield and 95 % FAME recovery. In contrast at 1 % catalyst concentration, 98 % FAME yield but 86 % FAME recovering was obtained due to separating difficulty by saponification. At 0.25 % catalyst concentration the FAME yield significantly reduced to 60 % indicating that the process is a strong function of catalyst concentration. It was suggested that a significant amount of the catalyst was saponified at 0.25 % concentration therefore less alkoxide species was available to catalyst the process.

In addition, Vicente *et al.* (1998) used factorial design and response surface methodology to optimize methanolysis of sunflower oil using a range of homogeneous and heterogeneous catalysts while varying reaction temperature (25-65 ^{o}C) and acid concentration (0.5-1 wt %). They found that NaOH catalyst produced the highest FAME conversion and a second-order model perfectly predicts the FAME conversion as a function of temperature and catalyst concentration. In the temperature range 20-50 ^{o}C, a catalyst concentration of 1.3 % resulted in the maximum FAME conversion, whereas at temperatures above 60 ^{o}C and catalyst concentrations greater than 1.5 % more soap was formed.

Eze *et al.* (2014) developed a more robust model of KOH-catalysed conventional transesterification, which included FAME and triglyceride saponification, as well as FFA neutralisation. Their experiments and simulated data showed that a methanol to oil molar ratio greater than 9:1 could be used to obtain a 96.3 % maximum FAME conversion at KOH concentrations greater than 1.5 wt %. Increasing the KOH concentration allowed the maximum FAME conversion to be achieved in less than 2 min. The process was tolerant to 1 wt % water in the feedstock, when the methanol to oil ratio was greater than 12:1. FFA concentrations up to 1 wt. % had negligible

effect on triglyceride (TG) or FAME saponification. Their proposed mechanism considered the following reactions:

(1) KOH- alkoxide equilibrium reaction:

$$CH_3OH + OH^- \underset{k_y}{\overset{k_x}{\rightleftharpoons}} CH_3O^- + H_2O$$

(2) Formation of methyl ester:

$$TG + MA \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DG + FAME$$

$$DG + MA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} MG + FAME$$

$$MG + MA \underset{k_6}{\overset{k_5}{\rightleftharpoons}} GL + FAME$$

(3) Saponification of triglyceride (TG):

$$TG + 3OH^- \overset{k_7}{\rightarrow} 3Soap + GL$$

(4) Saponification of methyl ester:

$$FAME + OH^- \overset{k_8}{\rightarrow} Soap + MA$$

(5) Neutralisation of FFA:

$$FFA + OH^- (RO^-) \overset{k_9}{\rightarrow} Soap + H_2O (ROH)$$

The rate expressions describing the mechanism of their process is summarised below

$$r_{TG} = \frac{d[TG]}{dt} = [CH_3O^-](-k_1\,[TG][MA] + k_2[DG][FAME]) - k_7\,[TG][OH^-]$$

$$r_{DG} = \frac{d[DG]}{dt} = [CH_3O^-](k_1\,[TG][MA] + k_4[MG][FAME]) - k_2\,[DG][FAME] - k_3\,[DG][MA])$$

$$r_{MG} = \frac{d[MG]}{dt} = [CH_3O^-](k_3\,[DG][MA] + k_6[GL][FAME]) - k_4\,[MG][FAME] - k_5\,[DG][MA])$$

$$r_{FAME} = \frac{d[FAME]}{dt} = [CH_3O^-](k_1\,[TG][MA] + k_3[DG][MA]) + k_5\,[MG][MA] - k_2\,[DG][FAME] - k_4\,[MG][FAME] - k_6\,[GL][FAME]) - k_8\,[FAME][OH^-]$$

$$r_{MA} = \frac{d[MA]}{dt} = [CH_3O^-](k_2\,[DG][FAME] + k_4[MG][FAME] + k_6\,[GL][FAME] + k_y\,[H_2O] - k_1\,[TG][MA] - k_3\,[DG][MA] - k_5\,[MG][MA]) + k_8\,[FAME][OH^-] - k_x\,[MA][OH^-]$$

$$r_{GL} = \frac{d[GL]}{dt} = [CH_3O^-](k_5\,[MG][MA] - k_6[GL][FAME]) + k_7\,[TG][OH^-]$$

$$r_{Soap} = \frac{d[Soap]}{dt} = [OH^-](k_7\,[TG] + k_8[FAME] + k_9\,[FFA])$$

$$r_{FAA} = \frac{d[FAA]}{dt} = -k_9\,([FAA][OH^-] + [FFA][CH_3O^-])$$

$$r_{H_2O} = \frac{d[H_2O]}{dt} = k_x[MA][OH^-] - k_y\,[CH_3O^-][H_2O] + k_9[FAA][OH^-]$$

$$r_{CH_3O^-} = \frac{d[CH_3O^-]}{dt} = k_x\,[MA][OH^-] - k_y\,[H_2O][CH_3O^-] - k_9\,[FFA][CH_3O^-]$$

$$r_{OH^-} = \frac{d[OH^-]}{dt} = k_y\,[H_2O][CH_3O^-] - k_x\,[MA][OH^-] - k_9\,[FFA][OH^-] - k_7\,[TG][OH^-] - k_8\,[FAME][OH^-] \qquad \textbf{eqn. 2.1}$$

Where r_i: rate of formation of species i (mol L^{-1} min^{-1}); k_i : kinetic rate constant of the reactions (L mol^{-1} min^{-1})

2.4.2 *Kinetics of reactive extraction ("in situ transesterification")*

Reactive extraction ("*In situ* transesterification") is a direct production of fatty acid methyl ester (FAME) from oil-bearing biomass, achieved by contacting the material directly with an alcohol containing a catalyst. It is potentially a more cost-effective method of making algal FAME, due to its elimination of the solvent extraction step and its higher water tolerance (Wahlen *et al.,* 2011; Velasquez-Orta *et al.,* 2013).

Zakaria and Harvey (2014) studied the kinetics of reactive extraction of rapeseed to FAME with methanolic NaOH. They found that rate of ester formation largely depends on catalyst concentration, temperature and particle size while the equilibrium FAME yield strongly depends on methanol to oil molar ratio. They obtained more than 85 % FAME yield only when methanol to oil was greater than 475:1. Their simulated and experimental data suggested that the process could be either mass transfer or kinetically controlled depending on the concentration of the catalyst. At higher catalyst concentrations (>0.1 mol/kg-solvent) the process was controlled by internal diffusion rate, but when the concentration was lower, it was kinetically controlled.

However, they did not consider in their model the competing reactions which occur alongside the desired FAME production. In addition, the model did not include the effect of moisture on the FAME conversion. Drying algae to the level required by conventional transesterification is energy intensive and has been a critical factor in blocking commercial production of algal FAME. A reactive extraction model that involves other competing reactions and incorporates the effect of moisture on FAME conversion is therefore a knowledge gap in this field.

2.5 Conventional transesterification vs. reactive extraction

Conventional transesterification requires refined oil from either vegetable or other oil seeds such as canola, rapeseed or soymeal, which contributes to the process cost. As much as 88 % of total production cost of the conventional two-step biodiesel production is ascribed to the refined oil feedstock (Haas *et al.*, 2006).

It is also important during conventional transesterification to control the moisture content in the feedstock, catalyst or methanol when an alkali catalyst is used. Typically, the maximum tolerable water content in oil is 0.3 wt. % (Freedman *et al*., 1984). Beyond this value, there could be saponification of the oil to soap, which reduces the biodiesel yield (Canakci and Gerpen, 1999) and causes difficulty in product separation (Canakci and Gerpen, 1999; Ma and Hanna, 1999).

Conventional biodiesel production involves hexane extraction steps that are relatively energy-intensive and time-consuming. Up to 90 % of the process energy can be accounted for in the hexane extraction and drying steps (Lardon *et al.* 2009). In addition, usage of solvent such as hexane for lipid extraction is not “green” and contributes to environmental problem. Alternatively, a reactive extraction (“*in situ* transesterification”) could be used. In this process, the biomass is fed directly into the reaction system. This eliminates the oil extraction steps, biomass pre-treatment and degumming steps, and tolerates some level of the water (Wahlen *et al*., 2011; Haas and Wagner, 2011; Velasquez-Orta *et al.*, 2013). The basic differences between the two processes are shown in figure 2.11.

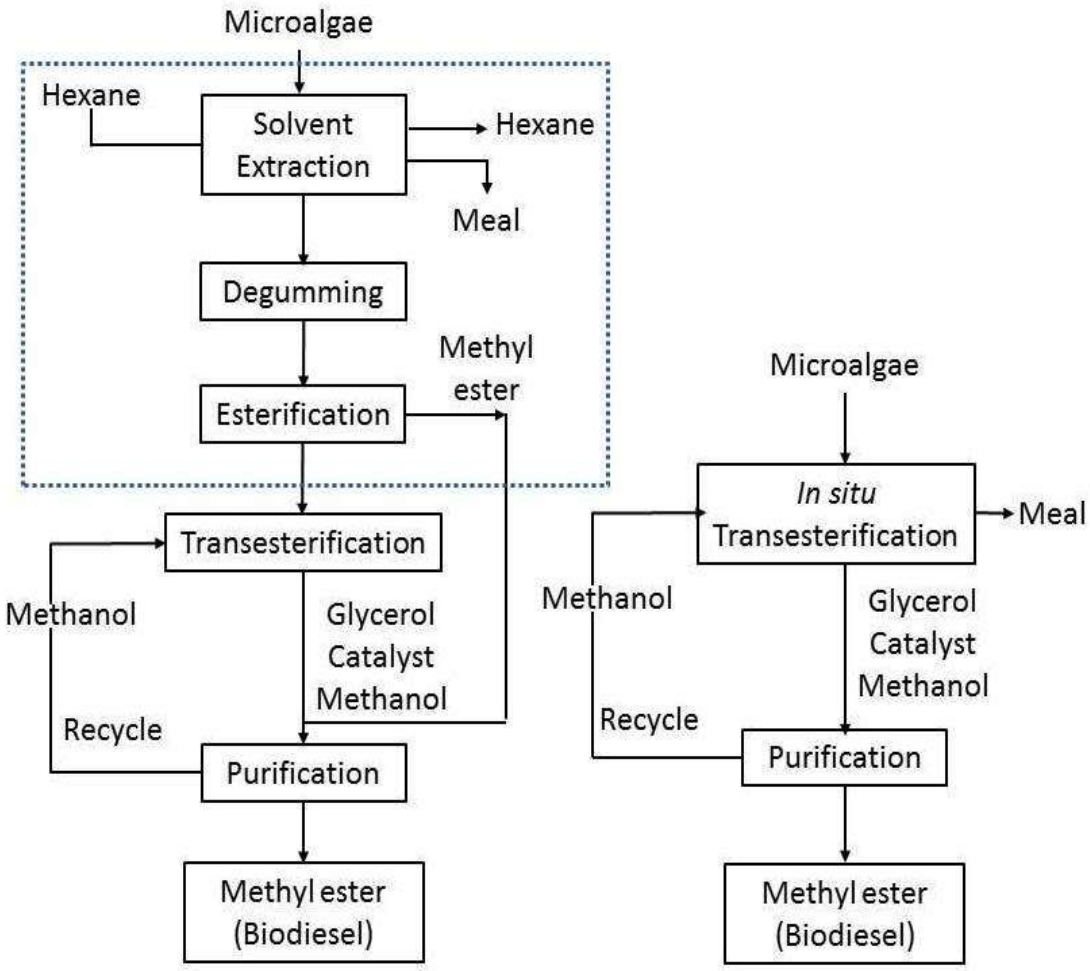


Figure 2.11: Comparison between reactive extraction and conventional Transesterification

Previous studies have demonstrated the feasibility of obtaining greater FAME conversion from such reactive extraction than from a conventional two-step approach (Harrington *et al.*, 1985; Lewis *et al.*, 2000; Vicente *et al.*, 2009). It is effective in making biodiesel from pure algal strains (Vicente *et al.,* 2009; Li *et al*, 2011) and mixed cultures of microalgae (Wahlen *et al.*, 2011; Haas and Wagner, 2011). However, microalgae are mostly aquatic species and required removal of large amount of water through dewatering. This operation usually results into 5-35 % TSS of the microalgal concentrate (Grima *et al*., 2004; Show *et al*., 2013) and

accounted for about 20-30 % cost of the algal recovery (Gudin and Therpenier, 1986). Dehydrating microalgae to 4 wt. % moisture level has been shown to be highly energy intensive (Soeder and Pabst, 1975), which translates to high production costs.

Additionally, this method requires large amounts of methanol to oil ratio. It is usually in the range of 100:1-1000:1. This is necessary since methanol plays a dual role: it acts as an oil extractor and as a reactant. Triacylglycides are not readily soluble in methanol. Besides, microalgal cell walls create resistance to methanol diffusion and causes large solvent requirement for algal bioprocessing (Gerken *et al*., 2012). The main problem of such high excesses of methanol is the capital and running (energy) costs associated with its recovery from the product streams, which would almost certainly involve a substantial distillation column.

2.6 Overview of *In situ* transesterification

The feasibility of reactive extraction of sunflower seed for biodiesel production was first reported in 1985 by Harrington and D'Arcy-Evans (1985). They identified the following advantages:

1.) Esterification of the oil embedded in the hull, which could improve the overall yield of the alkyl ester;

2.) Reduction of the oil losses from the hull/kernel separation;

3.) Esterification of lipids that may not be extracted by the hexane due to its different solubility from triglyceride;

4.) Improvement of carbohydrate digestibility of the residue by acid or alkali catalyst interaction.

In addition, they observed the approach produced higher conversion of alkyl ester than the conventional method. Both processes produced the same quality of alkyl ester. The technical feasibility of making biodiesel from microalgae via reactive extraction has also been demonstrated by various investigators (Wahlen *et al.* 2011; Haas and Wagner 2011; Velasquez-Orta *et al.* 2011).

2.6.1 *Key process variables in reactive extraction of microalgae*

Some of the process variables that determine the FAME yield during reactive extraction of microalgae for FAME production are shown in table 2.7. The results of some reactive extraction studies on oil seeds are also included for comparison.

Table 2.7: Process conditions: Reactive extraction of microalgae

Feedstock	Temp (^{o}C)	Solvent	catalyst (oil basis) (mol/mol)	Molar ratio (solvent: oil)	Water content % (w/w) of dry algae	Reaction time (hr)	Conversion (oil basis) (%)	Remarks	References
Nannochloropsis species	65	methanol	Mg-Zr oxide 1.65:1	1,569:1	0	4	60	methylene dichloride co-solvent	Li *et al*.(2011)
Nannochloropsis Species(Oil)	65	methanol	Mg-Zr oxide 1.65:1	592:1	0	4	47	methylene dichloride co-solvent	Li *et al*.(2011)
Chlorella pyrenoidosa	90	methanol	H_2SO_4 0.234:1	154:1	0	2	95	Hexane co-solvent	Li *et al*.(2011)
Chlorella vulgaris	60	methanol	H_2SO_4 0.35:1	600:1	0	20	97		Velasquez-Orta *et al.* (2011)
Chlorella vulgaris	60	methanol	NaOH 0.15:1	600:1	0	1.25	78		Velasquez-Orta *et al*. (2011)
Algae biomass	65		H_2SO_4 0.678:1	308:1	8	2	80		Haas and Wagner (2011)

Feedstock	Temp (^{o}C)	Solvent	catalyst (oil basis) (mol/mol)	Molar ratio (solvent: oil)	Water content % (w/w) of dry algae	Reaction time (hr)	Conversion (oil basis) (%)	Remarks	References
Algae biomass	65	methanol	H_2SO_4 0.678:1	308:1	1	2	86		Haas and Wagner (2011)
Algae biomass	65	methanol	H_2SO_4 0.797:1	308:1	0.2	2	96		Haas and Wagner (2011)
L.starkeyi	70	methanol	H_2SO_4 0.093:1 HCl 0.186:1	868:1	0	20	97	-	Zhao and Liu (2007)
M.isabellina	70	methanol	H_2SO_4 0.093:1 HCl 0.186:1	868:1	0	20	91	-	Zhao and Liu (2007)
R.toruloides	70	methanol	H_2SO_4 0.093:1 HCl 0.186:1	868:1	0	20	98	-	Zhao and Liu (2007)
Chaetoceros gracilis	80	methanol	H_2SO_4 0.158:1	988:1	0	0.33	82		Wahlen *et al.* (2011)

Feedstock	Temp. (^{o}C)	Solvent	catalyst (oil basis) (mol/mol)	Molar ratio (solvent: oil)	Water content % (w/w) of dry algae	Reaction time (hr)	Conversion (oil basis) (%)	Remarks	References
Chaetoceros gracilis	80	methanol	H_2SO_4 0.158:1	1,977:1	100	0.33	41		Wahlen *et al.* (2011)
Chaetoceros gracilis	80	methanol	H_2SO_4 0.158:1	3,460:1	400	0.33	57		Wahlen *et al.* (2011)
Chlorella sorokiniana	80	methanol	H_2SO_4 0.158:1	1,831:1	0	0.33	77	-	Wahlen *et al*. (2011)
Synechococcus elongatus	80	methanol	H_2SO_4 0.158:1	2,354:1	0	0.33	40	-	Wahlen *et al*. (2011)
	80	methanol	H_2SO_4 0.158:1	3,013:1	0	0.33	74	-	Wahlen *et al*. (2011)
	60	methanol	H_2SO_4 8.49:1	314:1	0	8	92		Ehimen *et al.* (2010)
Jatropha curcas	30	methanol	NaOH 2.4:1	400:1		0.5	88	< 0.71 mm Particle size	Kasim and Harvey (2011)

Feedstock	Temp. (^{o}C)	Solvent	catalyst (oil basis) (mol/mol)	Molar ratio (solvent: oil)	Water content % (w/w) of dry algae	Reaction time (hr)	Conversion (oil basis) (%)	Remarks	References
Rapeseed	30-60	methanol	NaOH 2.1:1	600:1	< 6.7	1	85		Zakaria and Harvey(2012)
Sunflower	20	methanol	NaOH 0.5 : 1	101:1	4.6	0.2	98	DEM co-solvent	Zeng *et al.* (2009)
Soybean	23	methanol	NaOH 2:1	543:1		8	84		Haas *et al.* (2004)
	60	methanol	1.6:1	226:1		8			
Cottonseed	40	methanol	NaOH 0.55:1	135 : 1	< 2	3	98	Petroleum Ether co-solvent	Qian *et al.* (2008)

DEM: Diethoxymethane (Organic co-solvent)

Generally, the following observations can be made from the table:

[1] FAME can be produced via in *situ* transesterification from both freshwater and marine microalgae and other oil-bearing feedstocks.

[2] The process requires either homogeneous alkaline, acid, or heterogeneous catalyst to proceed at a reasonable rate.

[3] When a heterogeneous catalyst is used a larger amount of methanol is needed than for either homogeneous alkaline or acid catalyst, which is probably due to phase transfer limitations. However, inclusion of co solvents such as hexane or methylene dichloride helps to reduce the amount of methanol.

[4] Microalgae *in situ* transesterification can occur at room temperature particularly with alkaline catalyst. With acid catalyst, Whalen *et al*. (2011) observed significant increase in FAME conversion rate with increase in temperature from 60 to 80 ^{o}C but no significant change in the rate was observed by increasing the temperature from 80 ^{o}C to 110 ^{o}C strongly due to evaporation of methanol. Similarly, Ehimen *et al*. (2010) recorded significant increase in FAME conversion when temperature was increased from 30 ^{o}C to 60 ^{o}C but observed no significant change in FAME conversion yield between 60 ^{o}C to 90 ^{o}C.

[5] FAME yield during *in situ* transesterification depends on a number of variables, including microalgae species, temperature, catalyst to oil molar ratio, methanol to oil molar ratio, agitation rate, moisture content of the reactants or the feedstock, reaction time, phase and type of the catalyst and co solvent.

2.6.2 *Solvent*

The solvent plays a dual role during *in situ* transesterification. It functions as an extractant and a reactant. Methanol is the most commonly employed solvent because it is cheaper than all other aliphatic alcohols. It is also less expensive to recover than ethanol because it does not form an azeotrope with water (Demirbas 2008). Ordinarily, methanol is the poorest extractant of triglycerides among aliphatic alcohols (Kildiran *et al.*, 1996; Wahlen *et al.* 2011). This is because the dissolution of triglyceride increases with increase chain length of the alcohol (Kildiran *et al.*, 1996). Ester yield during acid-catalysed *in situ* transesterification of soy bean oil increased with decrease in the polarity of the alcohol (Kildiran *et al.*, 1996). In contrast, the ester yields during acid-catalysed *in situ* transesterification of *Chaetoceros gracilis* with methanol, ethanol, 1-butanol, 2-methyl-1-propanol, and 3-methyl-1-butanol did not vary significantly (Wahlen *et al.*, 2011). This could be because soybean oil and *Chaetoceros gracilis* are not similar in terms of cell wall chemistry and transesterifiable lipid. Thus the activity of the alcohol during the reactive extraction of the biodiesel should not be expected to be the same. *In situ* transesterification is always characterized by a large amount of methanol to oil ratio, between 100:1-1000:1 methanol to oil molar ratio (Zhao and Liu, 2007; Ehimen *et al.* ,2010; Li *et al.* ,2011).

Co-solvents can be used to reduce the methanol molar excesses. For instance, Li *et al*. (2011) obtained 95 % FAME yield during acid-catalysed reactive extraction of *Chlorella pyrenoidosa* at 90 ^{o}C, methanol to oil molar ratio of 165:1 (4 mL methanol to 1g microalgae) with 6 mL hexane as co-solvent. Zeng *et al*. (2009) obtained 98 % FAME yield during alkali catalysed reactive extraction of sunflower at 20 ^{o}C , 101:1 methanol to oil molar ratio with 58 to 1 diethoxyl methane (DEM) to methanol molar

ratio as co-solvent. The two investigations did not include the FAME yield that would be produced in the absence of the co-solvents (i.e. hexane and DEM). However, from the overview of parameters influencing the efficiency of reactive extraction shown in table 2.7, the effect of the co-solvent can be clearly seen, in that they have the lowest methanol to oil ratio. However, the co-solvent should be carefully screened for health and environmental hazards. Many of these co-solvents have significant environmental impacts, and would significantly adversely affect the processes' life cycle carbon emissions. Co-solvents that are difficult to separate from other species in the reaction mixture can reduce the purity of biodiesel (Haas and Wagner, 2011). Furthermore, there would be an added process cost for the separation of the co-solvent.

2.6.3 *Temperature and Reaction Time*

Temperature can positively or negatively affect the yield and/or rate of alkyl ester depending on the type of catalyst. It has been reported that there was no significant difference during alkali-catalysed *in situ* transesterification of *Jatropha curcas*, soybean oil and cottonseed oil between 30-65 ^{o}C (Haas *et al.* 2004; Qian *et al.* 2008; Kasim and Harvey, 2011).

However, acid-catalysed *in situ* esterifications increase in rate with temperature. Wahlen *et al.* (2011) observed an increase in FAME yield during acid-catalysed *in situ* transesterification of *Chaetoceros gracilis* with increase in temperature from 20-150 ^{o}C. They found that most significant changes occurred between 60-80 ^{o}C. Similarly, Ehimen *et al.* (2010) reported an increase in biodiesel yield during acid-catalysed *in situ* transesterification of *Chlorella* oil from 23-90 ^{o}C. They also found that no significant changes occurred in the range 60-90 ^{o}C. However, too high an operating temperature can reduce the alkyl ester yield, perhaps because of

oligomerization of unsaturated fatty acids and their corresponding esters to estolides via a side reaction (Revellame *et al.* 2010).

The reaction time during *in situ* transesterification depends also on the nature of catalyst. Just like conventional transesterification in which alkali catalyst is ~4000 times faster than acid catalysed transesterification (Srivastava and Prasad, 2000), alkali-catalysed *in situ* transesterification is faster than its acid-catalysed counterpart. For instance, Velasquez-Orta *et al.* (2011) reported a FAME yield (97 %) at 60 ^{o}C during acid-catalysed reactive extraction of *Chlorella vulgaris* at 20 h. They obtained 78 % FAME yield in 1.25 h using alkali catalyst at the same conditions. In practise, it is economical to operate at reaction temperatures close to the boiling point of the alcohol (Ehimen *et al.* 2010).

2.6.4 *Agitation rate*

Kasim and Harvey (2011) studied the effect of mixing intensity (100 to 400 rpm) on alkali-catalysed reactive extraction of FAME from *Jatropha curcas* (10 g) in a 250 mL Schott bottle at 60 ^{o}C, catalyst concentration (0.1 N). They observed that the FAME yield increased with increase in mixing intensity. The process was independent of mixing speed at 300 rpm as ~90 % FAME yield was obtained at that condition which was not significantly different with that of 400 rpm. At 100 rpm, the FAME yield significantly reduced to 37 %.

2.6.5 *Catalyst*

In situ transesterification of lipid-bearing feedstocks requires a catalyst for it to proceed between 25-60 ^{o}C at 1 atm (Qian *et al.*, 2008; Kasim and Harvey, 2011). Various catalysts have been used for *in situ* transesterification, including homogeneous alkaline and acid catalysts, and heterogeneous catalysts.

2.6.6 *Acid catalysed reactive extraction*

Harrington and D'Arcy-Evan (1985) in their pioneering research demonstrated the feasibility of acid-catalysed *in situ* transesterification of sunflower oil. They obtained a 40 % yield with H_2SO_4 concentration of 1.2 % v/v of methanol in ~ 4 h reaction. In contrast a 30 % yield was obtained with hexane-extracted oil from sunflower with the same process conditions. Kildiran *et al.* (1996) conducted an extraction and acid-catalysed *in situ* transesterification of soybean oil using methanol, ethanol, n-propanol and n-butanol. They found that *in situ* transesterification sequentially proceeds through oil dissolution and transesterification of triglyceride and the triglyceride dissolution increased with increasing alkyl chain length of the alcohol.

In microalgae research, acid-catalysed *in situ* transesterification of microalgae at high yields have been demonstrated by many authors. Li *et al.* (2011) reported a 95 % FAME conversion in 2 h during acid-catalysed *in situ* transesterification of *Chlorella* pyrenoidosa at 90 ^{o}C, 154: 1 methanol: oil molar ratio, 0.234: 1 H_2SO_4: oil molar with hexane as co-solvent. They reported hexane to be an effective co-solvent for reducing methanol-oil molar ratio. Velasquez-orta *et al.* (2011) obtained a 97 % FAME conversion in 20 h during reactive extraction of *chlorella vulgaris* at 60^{o}C, 600: 1 methanol: oil molar ratio, 0.35:1 acid to oil molar ratio. A maximum FAME conversion of 96 % was reported by Haas and Wagner (2011) who performed acid catalysed *in situ* transesterifications with algae biomass containing different moisture content at 65 ^{o}C; 308: 1 alcohol to oil molar ratio. Zhao and Liu (2007) conducted acid-catalysed *in situ* transesterification on a diverse species of oil-bearing feed stocks (*Lipomyces starkey*, *Mortierella isabella*, *Rhodosporidium toruloides*) and obtained a maximum FAME conversion of 98 % at 70 ^{o}C, 868: 1 alcohol: oil molar ratio at 20 h.

Similarly, Wahlen *et al.* (2011) reactively extracted FAME from different cultures of microalgae biomass with acid catalysts and obtained a maximum FAME conversion of 77 % at 0.33 h, 80 ^{o}C, and 1,831: 1 molar ratio of methanol to oil. They found that the water tolerance of the process increased to as high as 400 % dry weight of *Chaetoceros gracilis.* However, the molar ratio of methanol to oil, at 3,460:1 was very high. Downstream methanol removal would be a significant running cost at such molar ratios. This would have to be weighed against the running cost savings due to the reduced feedstock drying duty.

It is interesting to note that the times to reach a maximum FAME conversion vary between the microalgae species, as shown in table above. The times range from 0.33 to 20 h. This is expected since microalgae are very diverse (more diverse than plant and animal kingdoms put together), and in particular have a wide range of cell wall compositions. The difference in their cell wall compositions could have a significant effect on the time to reach optimum FAME conversion. Another major advantage of using acid catalysts for *in situ* transesterification is that they are more tolerant to high free fatty acid concentrations.

2.6.7 *Alkali-catalysed reactive extraction*

The most common catalysts for alkali-catalysed transesterification are NaOH, KOH and their corresponding alkoxides. However, a significant constraint on operation is that almost anhydrous conditions (typically <0.1 wt % water) must be maintained to prevent soap formation (Canakci and Gerpen, 1999; Ma and Hanna, 1999). Furthermore, alkaline catalysts cannot be used when higher concentrations of FFAs are present, as the FFAs react with the alkali. Homogeneous acid catalysts, in particular H_2SO_4, are more tolerant of free fatty acid and moisture in the feedstocks.

An acid catalyst promotes transesterification of triglycerides and esterification of free fatty acids (Canakci and Gerpen, 2001).

A number of researchers have obtained high FAME conversions in alkali-catalysed *in situ* transesterification of oil-bearing feedstocks. Haas (2004) reported catalysed reactive extraction of soybean oil, where he obtained an 84 % FAME conversion with no significant difference when 23 ^{o}C or 60 ^{o}C was used. However, they found that operating at 23 ^{o}C required larger methanol oil molar ratios (543:1) than (226:1), which was needed for 60 ^{o}C. Other researchers also observed no significant change in FAME conversion between ambient temperature and 60 ^{o}C in alkali-catalysed reactive extraction of FAME from Jatropha curcas (Kasim and Harvey, 2011) and rapeseed (Zakaria and Harvey, 2012). An 87.8 % FAME conversion was obtained by Kasim and Harvey (2012) for jatropha. Similarly, Zakaria and Harvey (2012) who worked on rapeseed/NaOH/methanol system obtained an 85 % FAME conversion at optimum conditions.

Zeng *et al.*, (2009) observed a 97.7 % FAME conversion with sunflower/NaOH/methanol/DEM system. However, this entailed the inclusion of an extra solvent, which will lead to increased complexity in the separations train. While several publications have been conducted on the alkaline reactive extraction of oil seeds as indicated above, few studies have been conducted using alkaline catalyst for reactive extraction of microalgae, which has created a gap in generalising the schemes of reaction exhibit by alkaline reactive extraction.

2.6.8 *Heterogeneous catalysed reactive extraction*

A heterogeneous catalyst has also been used to promote *in situ* transesterification (Li et *al,* 2011). They compared the FAME yield obtained during *in situ*

transesterification of *Nannochloropsis* and conventional transesterification of *Nannochloropsis* oil with a heterogeneous ($Mg_2Zr_5O_{12}$) catalyst using methylene dichloride as a co-solvent. A maximum FAME yield of 60 % was observed at 65 ^oC, 4 h, 10 wt % catalyst and 45 mL of mixed solvent (3:1 v/v methanol/methylene chloride). A lower FAME yield of 47 % was obtained with conventional transesterification using the same process conditions as the *in situ* transesterification.

Interestingly, the author found that the FAME yield increased with increased catalyst concentration and volume of mixed solvent. However, further increases in these two parameters lead to reduced yields. Recently, micro-algal biodiesel production via a two-step *in situ* transesterification was reported by Dong *et al* (2013). This process involved a two-step *in situ* transesterification, where the algae free fatty acid was reduced with Amberlyst-15 before alkaline *in situ* transesterification. They obtained a maximum FAME conversion of 94.9 %.

2.6.9 *Reactive extraction at supercritical conditions*

It is possible for conventional and *in situ* transesterifications to proceed without catalyst in supercritical water or alcohol. Saka and Kusdiana (2001) reported transesterification of rapeseed oil in supercritical methanol. They obtained a ~ 95 % FAME yield in 4 h at 350 ^oC, 45 Mpa and 1:42 oil to methanol molar ratio. Similarly, Lim *et al.,* 2010 conducted *in situ* transesterification of *Jatropha curcas* seeds with supercritical methanol with the aid of hexane as co-solvent. A more than 100 % FAME yield was obtained at 300 ^oC, 240 Mpa, 10 mL/g methanol to solid ratio and 2.5 mL/g hexane to seed ratio. This indicates that FAME was extracted from components of the biomass besides triacylglycerides. The advantages of this

method over catalysed transesterification, as reported by Saka and Kusdiana (2001) include:

- Shorter time to completion.
- Simpler process, requiring fewer purification steps.
- The ester yield is greater than catalysed process.

The major drawbacks of the process are that it operates at high temperature and pressure, leading to significant increases in capital cost, and increased costs associated with safety and monitoring.

2.6.10 *Moisture content*

One major challenge in biodiesel production is the need for dry feedstocks. The moisture limit for common biofuel feedstocks is 0.5 wt % (Ma and Hanna, 1999), and in practice lower moisture limits are preferred. Water, in the feedstock or the methanol causes a significant reduction in the yield of biodiesel (Ma and Hanna, 1999). It also results in soap formation in alkali-catalysed transesterification, leading to increased complexity in the product separation train (Ma and Hanna, 1999). However, *in situ* transesterification has been reported to be more water-tolerant than conventional transesterification.

Velasquez-Orta *et al* (2013) found that a reactively extracted wet *Nannochloropsis* cell at 1.5 % moisture content has equal FAME yield or higher than a dried cell with both acid and methoxide catalyst. However, they observed a decrease in the FAME yield at 10 % moisture. They found similar moisture tolerances with *Chlorella* cells for a moisture content which was not greater than 1.5 %.

Zakaria and Harvey (2012) also observed some level of moisture tolerance during alkali-catalysed reactive extraction of rapeseed oil for FAME. However, they observed a drastic reduction in the FAME yield when the moisture content was greater than 6.7 wt %. It has been shown that reducing the water content reduces the excess methanol required: Haas and Scott (2007) reported that a 60 % reduction in methanol and a 56 % reduction in NaOH were achieved when fully dried soybean was used than when the bean contained 2.6 wt % moisture content. Similarly, during acid-catalysed *in situ* transesterification of *C. gracilis*, Wahlen *et al.* (2011) achieved a 57 % FAME yield with 3,460:1 methanol: oil mole ratio at 400 wt % moisture content compared to a 82 % FAME yield with 988:1 methanol: oil mole for fully dried cells. This is a huge amount of methanol which will increase processing costs when the methanol is removed from the process stream, probably by distillation.

No researchers have investigated the effect of including SDS in H_2SO_4 on the water tolerance of the *in situ* transesterification of microalgae. This is important, as the significant amounts of energy required to dry microalgal biomass or microalgal oil to the levels required in conventional biodiesel production render the process uneconomic. This is currently one of the major technical challenges to microalgal biodiesel production. Complete drying of algae is energy intensive, which significantly increases the cost of algae pre-treatment.

2.7 Microalgae as biofuel feedstock

Different feed stocks are used for biodiesel production. Availability, favourable climate and local soil conditions are among the criteria for choosing oil crops (Lin *et al.*, 2011). However, it has been reported that oil crops are not sustainable biofuel feedstocks, as many hectares of arable land are required to cultivate them (Chisti, 2007). This puts significant pressure on land and water resources, which could be

used for agriculture and other domestic purposes. It contributes to deforestation and undue competition between oil crops for food or fuels. The potential of non-food crops (Azam *et al.*, 2005) and waste oil (Chhetri *et al*., 2008) for obtaining biodiesel have been reported. However, these feedstocks can only supply limited quantities of biofuels that cannot meet the world transport energy requirement.

Microalgae are among the promising new crops, as it has a short generation time and can have extremely high lipid yield per unit area. Microalgae have been reported to have between 10 and 23 times the oil yield per unit area of the highest oil plant (palm oil) (Chisti, 2007). Typically lipid contents of microalgae are in the range 20-50 % per unit dry weight (Chisti, 2007; Rodolfi *et al*., 2009).

Microalgae have other compelling advantages over oilseed crops. For example, they can efficiently capture carbon dioxide from industrial flue gases (Rodolfi *et al*., 2009), thereby reducing carbon emissions. Their residue after biodiesel production can be used for animal feeds, bioethanol production and soil fertilizer. Biodiesel produced from microalgae is reported to have similar fuel properties to “petrodiesel” (Miao and Wu, 2006) and to reduce air pollution (Lotero *et al*., 2005).

In a practical biodiesel production process, selection of a suitable algal species is an important factor, since there are differences between lipid content and biomass productivity among different species and even within the same algal species.

2.7.1 *Microalgae species*

It can be seen from table 2.5, that the *Nannochloropsis* species is a competitive biodiesel candidate among marine microalgae, as it is relatively high-yielding and productive. *Nannochloropsis occulata* have been reported to accumulate as much as 60 % lipid content per dry weight in a nitrogen-limited medium (Rodolfi *et al*., 2009). Amongst freshwater microalgae, *Chlorella* species are suitable due to their

substantial lipid accumulation. *Chlorella* species have been reported to accumulate up to 56 % lipid content per dry mass of biomass under heterotrophic conditions (Wu and Hsieh, 2009; Xiong *et al*., 2010; Li *et al*., 2011).

Marine microalgae have additional advantages over their freshwater counterparts as they do not require fresh water, so could not compete with food crops for this resource. Some types of microalgae, such as *Nannochloropsis occulata, Dunaliella Tertiolecta, Pavlova lutheri, Tetraselmis species* and *Isochrysis* species, can be cultivated on brackish or sea water, thereby posing no threat to freshwater for human consumption or for agricultural use.

2.7.2 *Effect of lipid composition on fuel quality*

Microalgae are extremely diverse, with substantially different fatty acid profiles, which mean that the resulting biodiesel will also vary substantially (Demirbas, 2008). It has been shown by Ben-Amotz *et al.* (1985) that a wide range of microalgae species can synthesize C14:0, C16:0, C18:1, C18:2 and C18:3 free fatty acids, while other fatty acids were strain-specific. Other investigations have also reported that culture, environmental conditions, habitat and growth phase all have a significant effect on the fatty acid profile of microalgae (Valeem *et al.*, 2009; Patil *et al*., 2011).

Table 2.8 shows the effect of growth media, environmental stress or catalyst type on fatty acid profile of different microalgae.

Table 2.8: Effect of media and catalyst type on fatty acid profile of microalgal lipid

wt. %	[a]*C. vulgaris*	[b]*C. vulgaris*	[c]*N. occulata*	[d]*Z. zofingiensis*	[e]*D. tertiolecta*	[f]*I. galbana*
C12:0	-	-	2 (1)	-	-	-
C14:0	2 (-)	2 (0.1)	6 (6)	2 (-)	2 (1)	17 (22)
C14:1	-	1 (0.3)	0.1 (0.3)	-	-	-
C15:0	-	-	0.2 (2)	-	-	-
C16:0	20 (17)	6 (5)	26 (30)	18 (15)	18 (25)	9 (14)
C16:1	1 (1)	16 (11)	22 (21)	1 (1)	1 (1)	4 (2)
C16:2	11 (3)	-	1 (3)	7 (4)	2 (1)	-
C16:3	14 (6)	-	-	9 (2)	4 (2)	2 (-)
C16:4	-	-	-	2 (-)	20 (12)	-
C18:0	1 (2)	10 (11)	4 (2)	2 (3)	1 (2)	1 (1)
C18:1	4 (47)	27 (25)	12 (7)	18 (47)	5 (14)	5 (27)
C18:2	19 (10)	0 (9)	5 (3)	20 (17)	11 (9)	13 (3)
C18:3	28 (14)	21(22)	0.2 (1)	18 (8)	35 (31)	9 (4)
C18:4	-	-	3 (0.4)	2 (-)	1 (1)	10 (10)
C20:0	-	-	- (0.2)	-	-	1 (2)
C20:1	-	-	4 (5)	-	-	-
C20:4	-	-	-	-	-	-
C20:5	-	-	13 (18)	-	-	-
C22:6	-	-	- (0.2)	-	-	15 (12)
∑saturated	23 (19)	18(16)	38(41)	22(18)	21(28)	28(37)
∑monounsaturated	5 (48)	44(36)	38(33)	19(48)	6 (15)	34(29)
∑polyunsaturated	67 (33)	21(31)	21(25)	51(31)	73 (56)	34(29)

[a,d-f] Breuer *et al*. (2012): Nitrogen replete medium (Nitrogen deplete medium).

[b]Velasquez-Orta *et al.* (2011): acid catalysed reactive extraction (alkali-catalysed reactive extraction).

[c]Renaud *et al*. (1991): NO_3-containing medium (NH_4-containing medium)

ND: The conditions inside the bracket refers to the FFA inside the bracket listed in table

As can be seen in table 2.8, growth media (Renaud *et al*., 1991), environmental stress (Breuer *et al*., 2012) and different catalyst (Velasquez-Orta *et al*.,2011) contribute to variation in composition of the fatty acid profile of the algal FAME.

Velasquez-Orta *et al*. (2011) attributed the variation in the FAME compositions to varying effect of alkali and H_2SO_4 catalyst to FFA, cell wall lipids and triglyceride during FAME production. Algal accumulate more neutral lipids (triglycerides) rather that structural lipids when cultivated on nitrogen deplete media and cell wall lipids has different fatty acid composition from neutral lipids. This explains the variation obtained in fatty acid composition of the lipid under nitrogen deplete and replete media (Breuer *et al*., 2012).

Regardless of the factors, the lipids contain large amount of polyunsaturated FAME. Significant high amount of polyunsaturated (PUFA) reduces oxidative stability and cetane number of the resulting algal biodiesel. However, the oxidative stability of the lipid/FAME is strongly dependent on the structure of the PUFA as bisallylic sites in the PUFA are more prone to oxidation than the allylic site (Knothe, 2002). The bis-allylic sites (b, c, d, e below) are a methylene groups (CH_2) adjacent to two double bond, while allylic sites (a, f) are the one adjacent to a double bond (Knothe, 2002) as shown in figure 2.12.

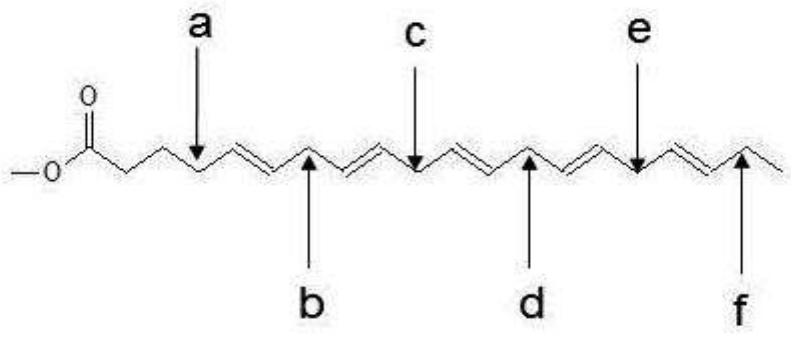


Figure 2.12: Comparison between allylic and bis-allylic site on EPA methyl ester (C20:5)

Source: Bucy *et al*. (2012)

EN 14214 recommends that the maximum contents of linolenic acid methyl ester should not be more than 12 % (m/m), while certain polyunsaturated methyl esters (with four or more double bonds) should not be more than 1 % (m/m) (Mittelbach and Remschmidt, 2006).

Investigations have revealed that inclusion of fuel additive which contained 0.03 % tert-butyl hydroquinone (TBHQ) significantly reduced eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) fractions in *Nannochloropsis* sp. derived biodiesel. The resulting fuel passed a 3 h ASTM stability test (Bucy *et al*., 2012). FAME obtained from *Desmodesmus sp*., and two mixed microalgae cultures harvested by ozone floatation was reported to contain less unsaturated fatty acid than those harvested without ozone (Komolafe *et al*., 2014). This is another potential means of improving oxidative stability of biodiesel.

Table 2.9: Different microalgal species, habitat, lipid content, biomass productivity and applications

Algae species	Habitat	Lipid content % w/w	Biomass productivity mg/ (L.day)$^{-1}$	Applications	Remarks	References
Nannochloropsis species	Marine, fresh and brackish water	60	300	Biofuel and feed supplement	Nitrogen deprived medium promoted lipid content	Rodolfi *et al*. (2009)
Nannochloropsis oculata	Marine, fresh and brackish water	7.90- 15.86	-	Biofuel	Nitrogen deprived medium and temperature influence lipid content	Rodolfi *et al.* (2009)
Nannochloropsis species	Marine ,fresh and brackish water	29.6- 35.5	170-210	Biofuel	CO_2 enriched air; continuous illumination for cultivation	Rodolfi *et al.* (2009)
Dunaliella tertiolecta	Marine	15.20	28	CO_2 capturing; Biofuel/ wastewater treatment	-	Chinnasamy *et al.* (2010)
Pavlova lutheri	Marine	35.50	140	Biofuel	CO_2 enriched air; continuous illumination for cultivation	Rodolfi *et al.* (2009)

Algae species	Habitat	Lipid content % w/w	Biomass productivity mg/ $(L.day)^{-1}$	Applications	Remarks	References
Skeletonema species	Marine	31.80	90	Biofuel	CO_2 enriched air; continuous illumination	Rodolfi *et al.* (2009)
Tetraselmis species	Marine	12.90-14.70	280-300	Biofuel	CO_2 enriched air; continuous illuminati	Rodolfi *et al.* (2009)
Scenedesmus species	Freshwater	12.80-21.10	126.54-260	Waste treatment/ Biofuel;CO_2 mitigation	-	Voltolina *et al*.(1998); Rodolfi *et al.* (2009)
Chlorella protothecoides	Freshwater	50.30-55.20	2020-7300	Biofuel	Heterotrophic condition enhanced lipid accumulation	Xiong *et al*. (2010)
Chlorella species	Freshwater	19.30- 66.10	230	Biofuel, food supplement, sorption of toxic chemical	Urea was used as a source of low cost Nitrogen	Rodolfi *et al.* (2009); Wu and Hsieh (2009)
Chlorococcum species	Freshwater	19.30	280	CO_2 mitigation	CO_2 enriched air; continuous illumination for cultivation	Rodolfi *et al.* (2009)

Algae species	Habitat	Lipid content % w/w	Biomass productivity mg/ (L.day)$^{-1}$	Applications	Remarks	References
Chlorella pyrenoidosa	Freshwater	56.30	1100	Biofuel	Rice straw hydrolysate served as carbon source	Rodolfi *et al.* (2009)

In addition to lipids, microalgae have substantial levels of protein and carbohydrate. An overview of major chemical compositions of different microalgae reported by Becker (2007) is shown in the table 2.10. It shows that significant portions of algal biomass contain carbohydrate and protein, which means if utilise after the reactive extraction as added value products could improve the process's economy.

Table 2.10: Major chemical composition of microalgae

Algae	Protein (%)	Carbohydrate (%)	Lipids (%)
Anabaena cylindrica	43-56	25-30	4-7
Aphanizomenon flos-aquae	62	23	3
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Porphyridium cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirogyra sp.	6-20	33-64	11-21
Arthrospira maxima	60-71	13-16	6-7
Spirulina platensis	46-63	8-14	4-9
Synechococcus sp.	63	15	11

Source: Becker (2007)

2.8 Summary of literature review

In situ transesterification of microalgae to produce biodiesel has been demonstrated to be technically feasible for a range of marine and freshwater species, including *Nannochloropsis* and *Chlorella*. However, one major drawback is the relatively high molar ratio of methanol to oil (range: 100:1 to 1000:1) required for high yield. In addition drying of microalgae to a level required by conventional transesterification is energy intensive and significantly hinders the commercial production of large scale algae biofuels.

The possible cost savings due to the increased water tolerance of reactive extraction of microalgae oil for FAME are considerable (Lardon *et al*., 2009), but they must be weighed against the costs of regenerating the alcohol (almost certainly by distillation).

Co-solvent use can reduce the molar excess to as low as 101:1 (dimethoxymethane). However, this introduces extra costs for the co-solvent itself and extra downstream separation duties. Furthermore, dimethoxymethane, which has been evaluated for this application is not a “green” solvent. It should also be borne in mind that co-solvents can alter the range of products.

Microalgae lipids are bound by a cell wall that inhibits FAME extraction. Cell disruptions have been shown to be effective in enhancing lipids extraction but are prohibitively energy intensive. The resistance provided by the cell wall causes additional excess requirement of solvent which translates to extra production cost. A cost effective pre-treatment technique will substantially improve the process economy.

Sulphuric acid is the most common homogeneous catalyst for reactive extraction of microalgae. Heterogeneous catalysts have also been shown to be effective, but require co-solvents or even greater excesses of methanol. When H_2SO_4 is used in reactive extraction, a high concentration of the catalyst is always required to achieve high yield (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. Inclusion of cetyltrimethylammonium bromide (CTAB) (a cationic surfactant) with an alkali catalyst resulted in an increased FAME yield and reduction in catalyst concentration during ethanolysis of *Jatropha curcas,* by acting as a phase transfer catalyst (Hailegiorgis *et al*., 2011).

Inclusion of SDS in water has been reported to increase oil extraction from Canola seeds (Tuntiwiwattanapun *et al.*, 2013). SDS has been used for lysing cells to recover intracellular components (Brown and Audet, 2008). These properties of surfactants have not been exploited in *in situ* transesterification of microalgae. Considering the fact that surfactants can disrupt cell walls, their use could lead to enhancement of FAME yield and/or rate.

Unlike acid catalysts, there are a handful of publications on usage of alkali catalyst for reactive extraction of microalgae. Consequently, a model for alkali-catalysed reactive extraction of microalgae has not yet been developed. Having such a model would be useful to reduce the number of experiments to be performed, thereby saving significant time and resources.

Overall, an integrated approach of producing various “co-products” i.e. “biorefining” may further reduce the cost of *in situ* transesterification. Becker reported that microalgae contained substantial carbohydrate and protein besides the lipids using

for transesterification substrate. This makes microalgae an attractive candidate for animal and bioethanol production. The author suggested using residual biomass after biodiesel production for animal feed or bioethanol / biogas production if remain intact and the bioethanol yield has been shown to be significantly dependent on acid pre-treatment and temperature of the process (Harun and Danquah, 2011). Reactive extraction/*in situ* transesterification of microalgae to biodiesel may well be a good basis for a microalgae-based bio refinery.

Chapter 3. Materials and Method

Nannochloropsis occulata and *Chlorella vulgaris* were the microalgae species used in this research project. They were characterised in terms of total lipids, neutral lipids, phospholipids, free fatty acids, fatty acid profiles, carbohydrate and protein. The residual carbohydrate and protein after the reactive extraction were compared with the initial values.

Experimental programmes were designed towards evaluating of acid, acid/surfactant (sodium dodecyl sulphate: SDS), a synthesised surfactant catalyst (zirconium dodecyl sulphate: ZDS) and alkali catalyst for reactive extraction of fatty acid methyl ester (FAME) from *Nannochloropsis occulata* and *Chlorella vulgaris*. The effect of moisture on the FAME yield was investigated. The effects of pre-soaking pre-treatment, acid concentration and methanol oil molar ratio on the FAME yield were also studied. A numerical model for an alkali-catalysed reactive extraction of *Chlorella vulgaris* was developed and validated with experimental data.

The major analytical method used in this research was gas chromatography flame ionization detector (GC FID). However, Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), Spectrophotometer (UV/VIS), Fourier Transform Infrared Spectroscopy (FT IR) and Thin Layer Chromatography (TLC) were also used.

3.1 Total lipid content

Concentrated *Nannochloropsis occulata* was purchased from Varicon Aqua Solution (London, UK). *Chlorella vulgaris* was purchased in dried form from Chlorella Europe, UK. Prior to total lipid extraction, *Nannochloropsis occulata* was freeze dried at -40 ^{o}C for ~24 h in a Thermo Modulyo D-230 Freeze Dryer (Thermo Electron Corporation, UK) and then homogenised. The two species were further dried with a

MB 45 Moisture Analyser (Ohaus, USA) at 60 ^{o}C until their moisture contents remained constant. Drying was performed at 60 ^{o}C to preserve the biochemical compositions of the samples (Widjaja *et al*., 2009). The moisture content of the resulting microalgae was taken as reference point (0 % wt moisture/ dry algae). The total lipids from both species were extracted overnight (~12 h) using chloroform: methanol (2:1, v/v) solvent mixture, based on the procedure of Folch *et al*. (1957). 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 the mass of the lipids remained constant.

3.2 Fractionation of the total lipids and validation of each fraction

The microalgal total lipids were fractionated using solid phase extraction by Kaluzny *et al.*'s method (1985). 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, as shown in figure. 3.1 below. 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 condition and their dry weight recorded. The solid phase extracted (SPE) lipid fractions were validated by a Reversed Phase Hydrocarbon Impregnated Silica Gel Thin layer Chromatography (TLC) with dimension 5×20 cm, 250 microns (Analtech, UK). The developing solvent for the TLC was a hexane/diethyl ether/acetic acid mixture (80:20:1, v/v/v) (Dong *et al*., 2013). Spots were visualised using iodine vapour. The solid phase extraction apparatus is shown in figure 3.1.

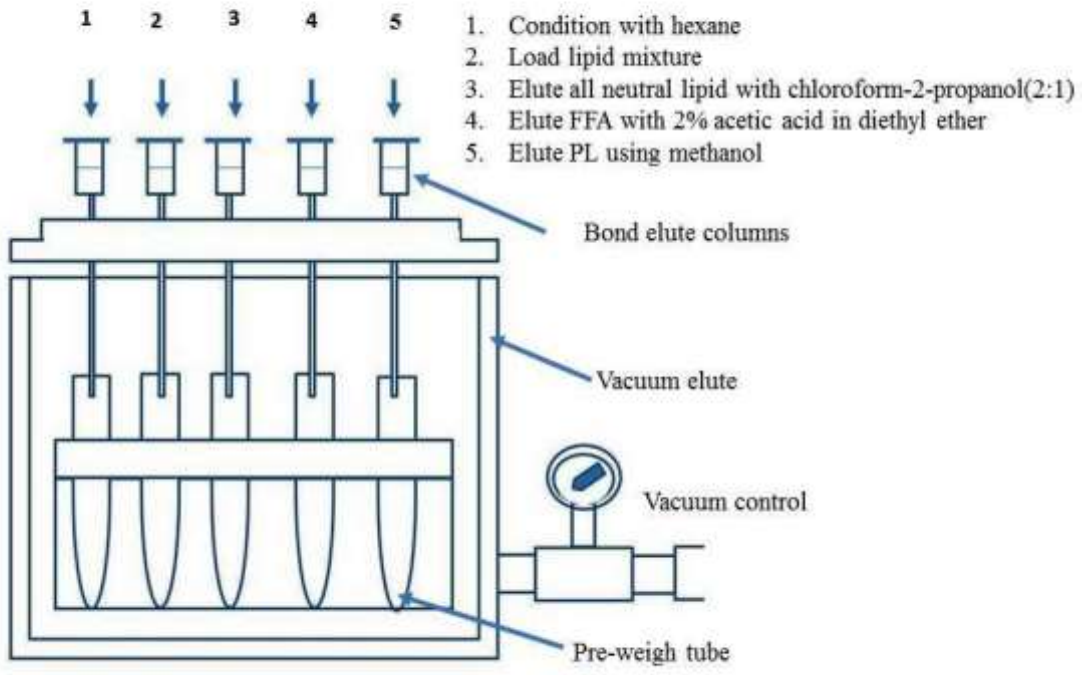


Figure 3.1: Solid phase extraction (SPE) apparatus used for lipids fractionation

PL= Phospholipids; FFA= free fatty acids

3.3 Transesterification of phospholipids

The transesterification of the phospholipids isolated by the methods described in section 3.2 above was conducted in 2.5 mL centrifuge tubes containing 5 mg of

isolated phospholipids of *Nannochloropsis occulata* and 6 mg of isolated phospholipids of *Chlorella vulgaris*. It has been shown that reactive extraction was not mass transfer dependent at 300 rpm (Kasim and Harvey, 2011) so all the experiments were conducted at a constant agitation rate of 450 rpm. The tubes were loaded in an IKA KS 4000 “icontrol” incubator shaker (IKA, Germany) maintained at a constant temperature of 60 ^{o}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 by rapid cooling of the reaction mixture in a freezer. 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 3.6.

3.4 Carbohydrate quantification

Carbohydrate concentration was measured using the protocol of Gerhardt *et al.* (1994). A 2 mL of chilled 75 % H_2SO_4 solution (Sigma Aldrich, UK) was added to COD tubes containing 1 mL of rehydrated microalgae or standards. After vortexing for 30s, 4 mL of chilled anthrone (Acros organics, USA) solution (2g/L, 75 % H_2SO_4) were added. The sample was vortexed and loaded into a heating block (Hach Camlab, UK) set at 100 ^{o}C and allowed to heat up for 15 min. The mixture was then cooled to 20 ^{o}C. The resulting samples and standards after acid hydrolysis is shown in figure 3.2

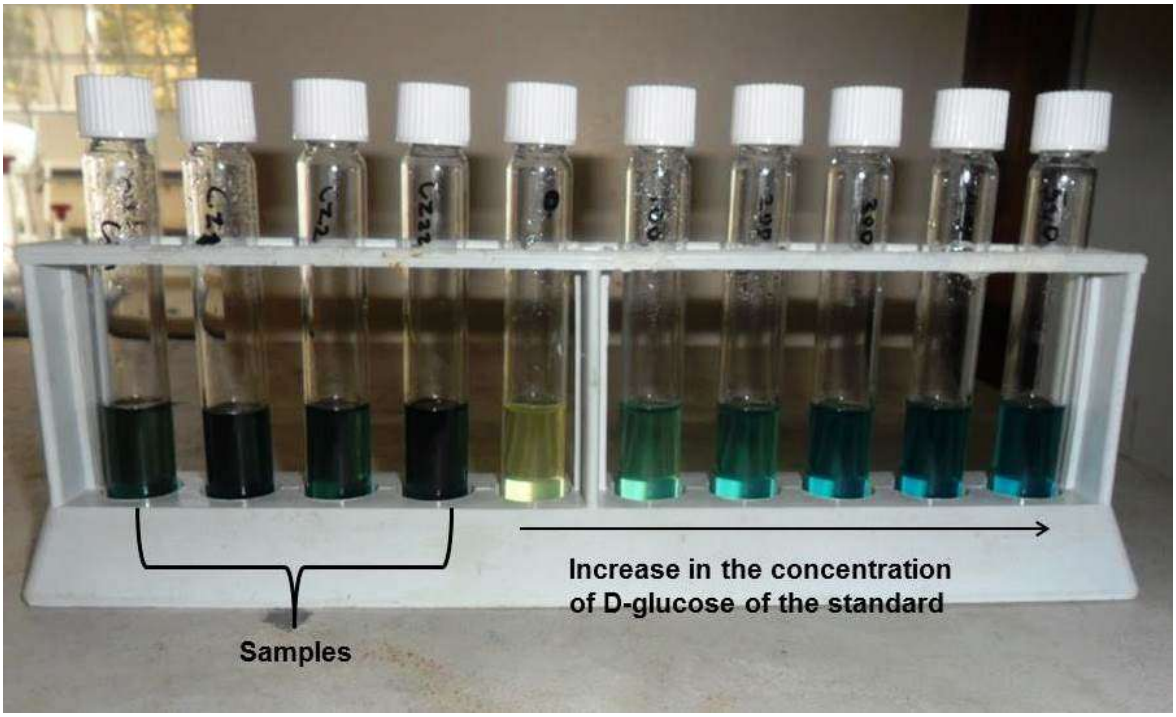


Figure 3.2: Microalgae samples and D-glucose standard after acid digestion

Then each sample was transferred into a cuvette and placed in a Jenway 6705 UV/Vis spectrophotometer (Bibby scientific, UK) set at 578 nm. The spectrophotometer was zeroed using distilled water.

The absorbance of the standard and sample were recorded. The carbohydrate content of the sample was calculated by reference to the standard as shown in figure 3.3.

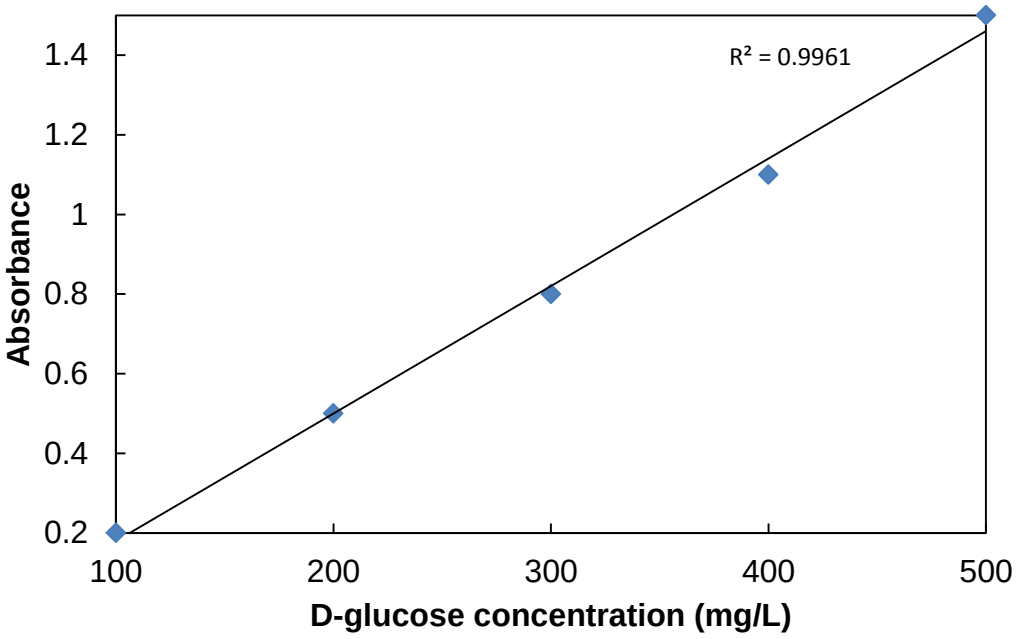


Figure 3.3: Calibration curve used for calculating samples' glucose conc.

3.5 Protein Quantification

Elemental carbon, hydrogen and nitrogen (CHN) content of both species before and after the *in situ* transesterification were measured using a Carlo Erba 1108 elemental analyser controlled via CE Eager 200 Software. Protein contents were calculated by multiplying the nitrogen content by 4.75, the nitrogen-protein conversion factor for microalgae derived by Lourenc *et al.* (2004).

3.6 *In situ* transesterification

All *in situ* transesterification was conducted in 2.5 mL tubes containing 100 mg of microalgae. The tubes were loaded in a programmable IKA KS 4000 icontrol incubator shaker (IKA, Germany). The temperature was maintained at 60 ^{o}C and a stirring rate of 450 rpm was used. For the experiments involving ZDS or the sulphuric acid catalyst with or without SDS the reaction was quenched by rapid cooling of the reacting mixture in a freezer. When using the alkali catalyst the reaction was

quenched with acetic acid. The biomass was separated from the liquid biodiesel mixture by centrifugation at 17,000 g for 30 min using accuSpin Micro 17 (Fisher Scientific, Germany). 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 3.6.1.

3.7 Analytical techniques

The main analytical method used in this research was gas chromatography with a flame ionization detector (GC FID). Supplementary techniques include: Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), Spectrophotometer (UV/Vis), Fourier Transform Infrared Spectroscopy (FT IR) and Thin Layer Chromatography (TLC).

3.7.1 *Determination of Total Mass Fraction of Ester*

A modified British Standard procedure (BS EN 14103:2003) was used to determine the FAME concentration after the *in situ* transesterification. Methanol was used in preparing the standard instead of heptane used in this method to reduce error and simplify the process. The variation was also validated as shown in figure 3.5. The gas chromatograph operated at the following conditions: carrier gas: helium, 7psi; air pressure, 32 psi; hydrogen pressure, 22 psi and capillary column head pressure 4.5psi. The oven temperature was maintained at 230 ^{o}C for 25 min. Heat rate was 15 ^{o}C/ min; initial temperature was set at 150 ^{o}C and held for 2 min; final temperature was set at 210 ^{o}C and held for 20 min; injection temperature was 250 ^{o}C while detector temperature was 260 ^{o}C. The biodiesel filtrate after the reaction was mixed with 0.2 mL of an internal standard solution, methyl heptadecanoate (Sigma Aldrich, UK) at a concentration of 10 mg/ (mL methanol) in 2.5 mL vials. 1 µL of the

homogeneous mixture was injected into the GC using 10 µL micro syringe (SGE, Australia) and data was collected using Data Apex Clarity software, UK. The column used was CP WAX 52 CB 30 m×0.32 mm (0.25 µm) (Agilent, Netherlands). The concentration of the ester in the sample, C, expressed as a mass fraction percentage (w/w %) was calculated using equation 4 below:

FAME Concentration (C) = $\frac{(\sum A)-A_{Ei}}{A_{Ei}} \times \frac{C_{Ei}V_{Ei}}{m} \times 100\ \%$ **Eq. 3.1**

Where:

$\sum A$ is the total peak area from C8:1-C20:1.

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

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)

m is the mass of the sample in mg.

3.7.2 *Determination of Total Mass of Ester*

The mass of FAME in the biodiesel-rich phase was calculated by multiplying the mass of the final biodiesel filtrate (a mixture of methanol, FAME and by-products) and the FAME concentration measured by the GC using eqn. 3.1. The FAME yield was calculated by dividing the mass of FAME obtained by the maximum FAME available in the algae using eqn. 3.3

Mass of the methyl ester (mg) = C (%) × w (mg) **Eq. 3.2**

Where w is the mass of the biodiesel mixture (mg)

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

$$\text{Yield (\% w/w)} = \frac{Mass\ of\ methyl\ ester\ (mg)}{Mass\ of\ the\ maximum\ FAME\ in\ the\ sample\ (mg)} \times 100\ \% \qquad \textbf{Eq. 3.3}$$

The FAME yield is a function of the mass of the ester phase, which is typically determined following a series of downstream processing steps. However, It has previously been demonstrated that the FAME concentration, as well as mass of the FAME in the ester phase, can be determined directly from samples of the bulk fluid (mixture of methanol, FAME and by products) (Zakaria, 2010; Kasim, 2012). This reduces experimental errors by minimising the number of downstream processing steps. This is particularly important in these experiments; as small amounts of microalgal biomass were used.

3.7.3 *Validation of GC Analysis of Bulk Product*

In order to validate and calibrate a technique of quantifying FAME concentration in a bulk fluid (mixture of FAME and methanol), a series of known masses of methyl esters were dissolved in methanol and injected into the GC at the same conditions as the sample. The value of the FAME concentration, C, was calculated as shown in **eq. 3.1.** The mass of the FAME esters were calculated using **eq. 3.2.** After this, the results were compared with the actual mass of the corresponding esters as illustrated in figure 3.4.

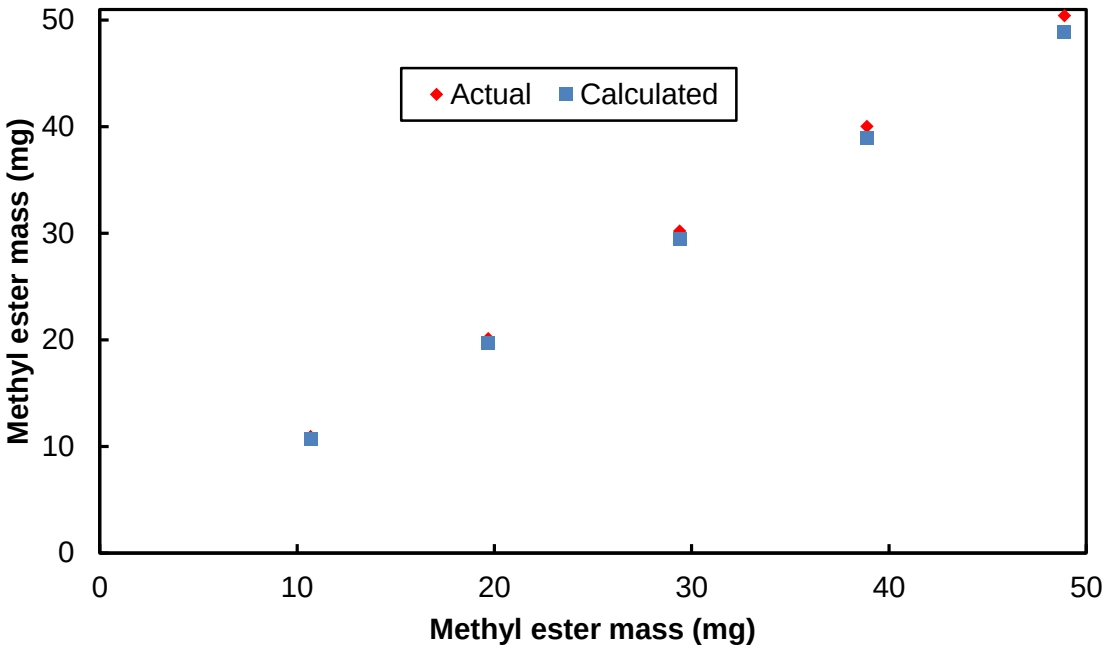


Figure 3.4: Comparison of the actual and calculated mass of methyl ester dissolved in methanol.

The range of methyl ester masses used was 10.9-50.4 mg. The R^2 value for the correlation between the actual and calculated mass of the methyl ester was 0.999. Therefore, it was concluded that the technique was sufficiently reliable to calculate the mass of methyl ester dissolved in methanol.

3.7.4 *Determination of maximum FAME content*

The maximum FAME concentration in each sample was quantified using Garces and Mancha's method (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 then 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 sample of the microalgae and vortexed well. After this, the mixture was transesterified in the IKA incubator at 60 ^{o}C; 450 rpm for

12 h. The sample was then kept in a freezer to quench the reaction and to separate the phases. The resulting upper FAME layer was carefully pipetted into a pre-weighed centrifuge tube and weighed. The FAME analysis and its concentration were then measured by gas chromatography using the procedure given in section 3.6.1. The maximum FAME content in the sample was calculated by multiplying the FAME concentration obtained by the mass of the upper FAME layer as explained in the section 3.7.1.

3.7.5 *Microalgae FAME profile*

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.

3.8 Catalyst Type

In this research, the concentration of acid was maintained at 100 % wt. H_2SO_4/ wt. lipids as in Ehimen *et al*. (2010). This is equivalent to 8.5:1 H_2SO_4 to oil molar ratio. The concentration of the zirconium dodecyl sulphate was 0.8:1 ZDS to oil molar ratio. A 9 mg of sodium dodecyl sulphate equivalent to ~2 mol. SDS/ (mol. phospholipids), which was enough to solubilise the phospholipid bilayers (Tan *et al*., 2002) was added to the H_2SO_4. The reaction was conducted at 60 ^{o}C; 450 rpm agitation speed, 600:1 methanol to oil molar ratio. This methanol to oil molar ratio has been shown to be adequate by Velasquez-Orta *et al*. (2011). The FAME vs. time profile was then obtained for each catalyst and microalgal species. In order to examine the effect of the acid concentration on FAME yield and reaction rate,

additional experiments were conducted with 15:1 H_2SO_4 to oil molar ratio at 24 h for both *Nannochloropsis occulata* and *Chlorella vulgaris*.

3.9 Quantification of cell disruption after reactive extraction

The amount of chlorophyll extracted from the microalgae has been correlated with cell wall disruption by Gerde *et al.* (2012). The total chlorophyll A, B or C obtained after the *in situ* transesterification was measured using a modification of Gerde *et al.'s* (2012) method. In the present investigation methanol was used as the blank/solvent against absolute ethanol used by Gerde *et al*. (2012) since the reaction was conducted with methanol. To study the extent of cell disruption of the two species, 600:1 methanol to oil molar ration (equivalent to 0.47 mL methanol) were added to a 100 mg of dried microalgae in a 2.5 mL tube followed by 1.85 % v/ (vol. methanol) of concentrated sulphuric acid. To another tube containing the same amount of microalgae, methanol and H_2SO_4, 9 mg sodium dodecyl sulphate (SDS) (equivalent to 2 mol SDS/ (mol phospholipids)). A third test tube was used with 0.8:1 ZDS to oil molar ratio, 100 mg of microalgae and 0.47 mL of methanol. Each reaction was run for 24 h, at 32 ^{o}C (to avoid degradation of the chlorophyll) and a stirring rate of 450 rpm. At the end of the reaction, the samples were centrifuged at 17,000 g for 10 min using accuSpin Micro 17 (Fisher Scientific, Germany). 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 Jeffrey and Humphrey (1975) formulae:

$$Chla = 11.93\, A_{664} - 1.93 A_{647} \qquad \textbf{Eq. 3.4}$$

$$Chlb = -5.5\, A_{664} + 20.36\, A_{647} \qquad \textbf{Eq. 3.5}$$

$$Chlc = -3.73\, A_{664} + 24.36\, A_{630} \qquad \textbf{Eq. 3.6}$$

Where:

$Chla$ is chlorophyll a

$Chlb$ is chlorophyll b

$Chlc$ is chlorophyll c

3.10 Effect of pre-soaking

A pre-soaking is a chemical pre-treatment of microalgae for cell disruption achieved by allowing a solvent such as methanol to percolate through the algae biomass in order to solubilise the phospholipid bilayer. A full factorial design on Minitab® 16 statistical software (Minitab, UK) was used with each factor at two levels. The microalgae were either pre-soaked or un-soaked. The acid concentrations were 8.5:1 or 15:1 methanol to oil molar ratio, which were equivalent to 0.087 or 0.15 µL H_2SO_4/ (mg algae), respectively. A 600:1 and 1000:1 methanol to oil molar ratio, which was equivalent to 4.7 or 7.85 µL methanol/ (mg algae), respectively were used. 100 mg of microalgae was used in all experiments. 880 g/ (mol) was the molecular mass of oil used to calculate the entire ratio. The microalgae were pre-soaked by placing 100 mg of the biomass in methanol inside a centrifuge tube. The tubes were loaded in an IKA KS 4000 icontrol incubator shaker (IKA, Germany) which was agitated at 300 rpm, kept at 17 ^{o}C and run for 14.5 h. After this, the *in situ* transesterification commenced by introducing the catalyst into the mixture and the reaction was run for 24 h at 60 ^{o}C; 450 rpm. The un-soaked microalgae were run at the same process conditions. The reaction was quenched by rapid cooling of the reacting mixture in a freezer. 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 3.7.1.

3.11 Phosphorus mass balance

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 3.6. The methanol in the extracts and the residues was evaporated at 60 ^{o}C until the sample mass remained constant. 10 mg of microalgae, extract or residue was digested in a mixture of 600 µL hydrogen peroxide (30 % w/v) (Fisher Scientific, UK) and 1200 µL HNO_3 (70 %) at 140 ^{o}C for 4 h (Cheng *et al.,* 2007). Then, 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 standard of aqueous solution of phosphorus was used as the reference.

3.12 Effect of alkali catalyst concentration

To investigate the concept of “fast esterification”, which involves the use of high alkali concentration at excess methanol to oil molar ratio to achieve a high FAME production rate before the saponification rate becomes significant, *Chlorella vulgaris* was used as the model microalgae. The methanol to oil molar ratios used were 600:1, 925:1 and 1276:1. The choice of the methanol to oil molar ratios was guided by the amount usually reported in the literature as shown in table 2.7 in the previous chapter. The catalyst concentrations were 0.125 N, 0.2 N, 0.25 N and 0.5 N. Again the choice of catalyst concentrations was guided from the value obtained in the literature; however, higher alkali concentrations which were not usually studied in the literature to avoid saponification were also investigated, particularly 0.25 N and 0.5 N. The FAME- time profiles and the kinetics of the reaction were determined in the

range 5 min to 2 h. In addition, the kinetics of reactive extraction of wet *Chlorella vulgaris* containing 5, 20 and 30 wt % moisture/ (wt dry algae) were studied to determine whether this process was any more or less water-sensitive than the conventional processes. The concentration of the alkali catalyst for the water tolerance test was fixed at 0.2 N, while methanol oil molar ratio was fixed at 600:1. Agitation speed was fixed at 450 rpm and temperature at 60 ^{o}C. A 100 mg *Chlorella vulgaris* was used throughout.

3.13 Effect of water on reactive extraction

To investigate the effect of water on the methyl ester yield, reactive extraction was performed on *Nannochloropsis occulata* and *Chlorella vulgaris* at varying moisture level with reference to the initial moisture content, 0 wt %/ (wt dry algae). Both *Nannochloropsis occulata* and *Chlorella vulgaris* were rehydrated with 5 %, 20 % and 30 wt % moisture/ (wt dry algae) water and the wet biomass was allowed to equilibrate for 1 h. For both species, 8.7:1H_2SO_4 to oil molar ratio was used while 8 mg (2 mol SDS/ mol phospholipids) was included in H_2SO_4 to isolate the effect of SDS on water tolerance. A 600:1 methanol to oil molar was used. The reaction temperature was kept at 60 ^{o}C ; agitation rate at 450 rpm and the reaction time was 24 h.

Response surface methodology (RSM) was further used to analyse the main and interaction effects of methanol to oil molar ratio, moisture content and alkali concentration on FAME yield by placing the factor beyond the range used in the FAME yield-time profile as shown in table 3.2. The reaction was conducted at 60 ^{o}C; 450 rpm and 100 mg of the microalgae biomass.

Table 3.1: Factors involved in RSM experiments with their respective levels

	Level			
Factor-description	-1	0	+1	Unit
Moisture content	0	18	39	wt %
Methanol/oil molar ratio	369	938	1507	-
Alkali concentration	0.082	0.188	0.293	N

Agitation = 450 rpm, Temp. = 60 ^{o}C, *Chlorella vulgaris* = 100 mg, Reaction time= 1 h

3.14 Catalyst synthesis

Zirconium (IV) dodecyl sulphate (Zr^{+4} $[^{-}OSO_3C_{12}H_{25}]_4$) was synthesised using a modified version of Zolfigol *et al*.'s method (2007) as follows:

(i) 2.9 g (8.9 mmol) of zirconium oxychloride octahydrate (Sigma Aldrich, UK) was dissolved in 100 mL of distilled water at room temperature;

(ii) 12.1 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 wt % kcl/ (unit mass zirconium dodecyl sulphate solution) was added to enhance catalyst precipitation.

(v) The precipitate was centrifuged and washed repeatedly with 150 mL distilled water;

(vi) The resulting white solid was calcined for 4 h at 80 ^{o}C and dried in a desiccator (Duran vacuum desiccator).

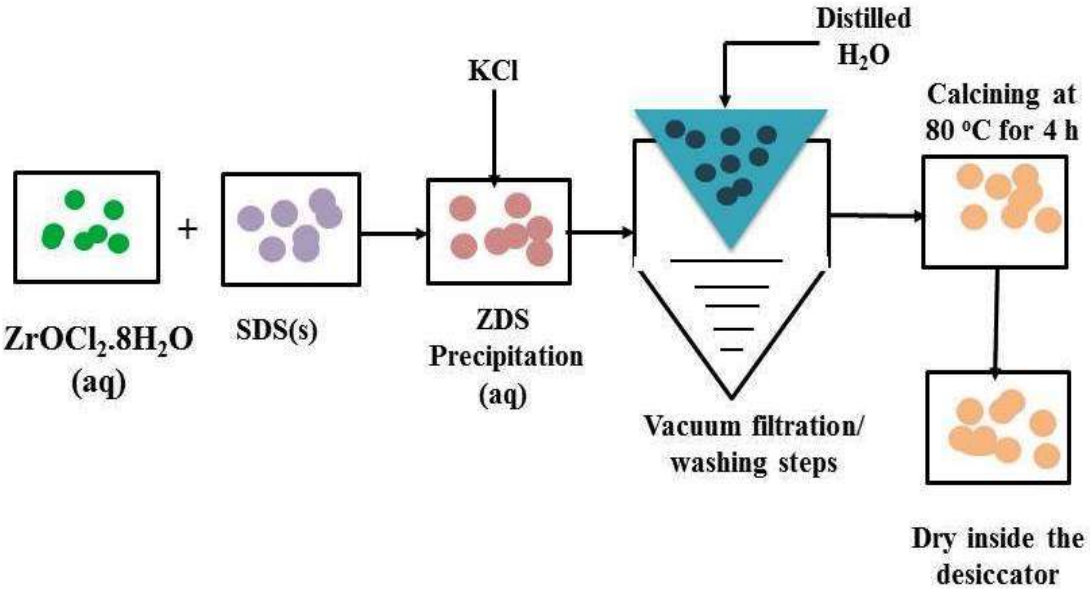


Figure 3.5: Schematic diagram showing the stages involved in "ZDS" synthesis

Chapter 4. Results and discussion

4.1 Characterisation of *Nannochloropsis occulata* and *Chlorella vulgaris*

Nannochloropsis occulata contains 10 to 43 % protein and 7 to 29 % carbohydrate (Hu and Gao, 2003; Fabregas *et al*., 2004). *Chlorella vulgaris* usually contains 30 to 58 % protein (Becker 2007; Liang *et al*., 2009) and 12 to 44 % carbohydrate (Becker 2007; Liang *et al.*, 2009). In this project, the protein and carbohydrate contents of *Nannochloropsis occulata* were determined as 30±0.07 % and 26±2.8 %, respectively. The initial protein content of *Chlorella vulgaris* was determined as 46±0.07 % while the carbohydrate was 35±3.5 %. Clearly, both species contained substantial protein and carbohydrate. This means that the carbohydrate and protein fractions could add value to the residue and improve the reactive extraction process economics. The results of the total lipids/fractions analysis for *Nannochloropsis occulata* and *Chlorella vulgaris* are shown in table 4.1

Table 4.1: Total lipids/fractions for *Nannochloropsis occulata* and *Chlorella vulgaris*

Lipid class/total lipids	*Nannochloropsis occulata*	*Chlorella vulgaris*	Statistical test (*t*-test)
NP (% total lipids)	22.5±2.5	36.2±5	P= 0.11
FFA (% total lipids)	18.3±2.4	6.1±0.3	P= 0.04
PL (% total lipids)	50±0	30.3±1.3	P= 0.01
TL (% total lipids)	45.6±0.7	68.7±2.7	P= 0.47
Total lipids (% dry algae)	17±0.8	15±0.9	P= 0.40

Lipid class: NP: Neutral lipids; FFA: Free fatty acids; PL: Polar lipids (Phospholipids and glycolipids); TL: Transesterifiable lipids

The accumulation of total lipids in microalgae strongly depends on the media, environmental stress and the harvested growth phase (Rodolfi *et al*., 2009). As shown in table 4.1, the highest fraction of total lipids was that of the transesterifiable lipids. Additionally, *Nannochloropsis occulata* contained higher quantities of polar lipids (phospholipids and glycolipids) than neutral lipids and free fatty acids. *Chlorella vulgaris* also contained high quantity of polar lipids (phospholipids and glycolipids) that greater than free fatty acid but less than neutral lipids. This agrees with the findings of Scragg and Leathers (1988) that polar lipids represent a large proportion of the algal total lipids. There was also a significant difference between the phospholipid content of both species. The chemical composition of microalgae varies with strains, media nutrients and environmental factors such as temperature, irradiance and pH (Becker, 2004).

The isolated lipid fractions of *Nannochloropsis occulata* and *Chlorella vulgaris* were further confirmed using thin layer chromatography, as shown in figure 4.1. This was

done by comparing the spot height of the elutes (1, 2 and 3) obtained from the isolated algal neutral lipid (NL) which corresponds to 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.

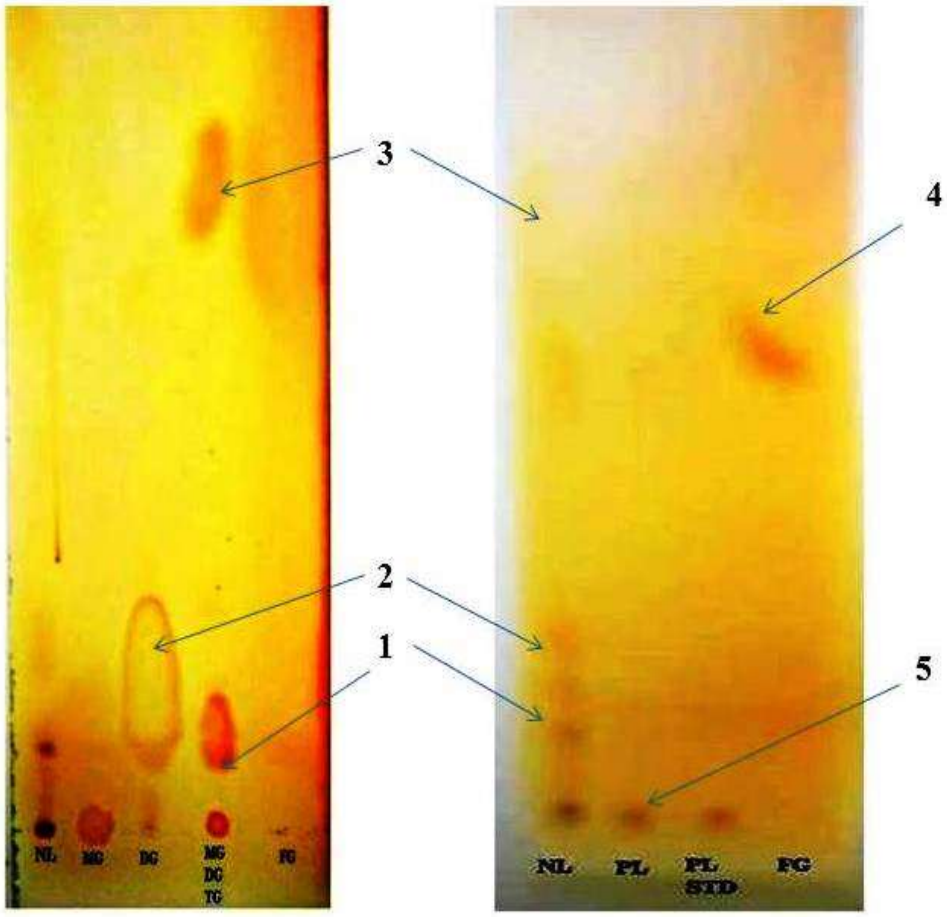


Figure 4.1: Thin layer chromatogram showing neutral and phospholipids fractions of total lipids: NL: neutral lipids fraction of the sample; PL: Phospholipids fraction of the sample; MG: standard monoglyceride; DG: standard diglyceride; TG: standard triglycerides; FG: standard FAME grain mixtures. Elute1: Monoglycerides; Elute2: Diglycerides; Elute3: Triglycerides; Elute 4: FAME grain mix; Elute5: phospholipids.

Cobelas and Lechado (1989) reported that the major phospholipids (cell wall lipids) in *Nannochloropsis sp.* and *Chlorella vulgaris* 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 4.2.

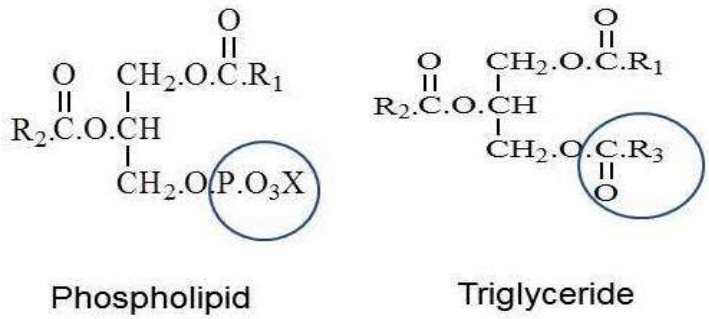


Figure 4.2: Comparison between phospholipids and triglycerides structure.

Where R_1, R_2, R_3 denote fatty acids and "X" can be any of the substituent group listed in table 4.2. The difference between the two compounds indicated in the circle.

Table 4.2: Substituent group for phospholipids

Name	Structure	Abbreviation for lipid
Choline	$.O.CH_2.CH_2.N^+(CH_3)_3$	P.C
Ethanolamine	$.OCH_2.CH_2.NH_2$	P.E
Serine	$.OCH_2.\underset{\displaystyle COOH}{\underset{\vert}{CH}}.NH_2$	P.S
Water	.H	P.A
Glycerol	$.CH_2(OH)CHCH_2OH$	PG
Phosphatidyglycerol	$.CH_2CH(OH)CH_2.$	D.D

P.A: Phosphatidic acid; P.G: phosphatidyl glycerol; D.D: diphosphatidyl glycerol; P.C: phosphatidyl choline; P.E: phosphatidyl ethanolamine; P.S: phosphatidyl serine.

Source: Wood (1974).

The FAME profiles determined for *Nannochloropsis occulata* and *Chlorella vulgaris* are shown in table 4.3. Each fatty acid represents the percentage of the maximum total FAME obtained from both species through reactive extraction. 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 (Demirbas and Demirbas, 2011). As shown in table 4.3, there are significant differences between the fatty acid distributions in both species. More than 80 % of the fatty acid methyl ester of the microalgae is saturated and mono-unsaturated. A low level of unsaturation, as shown, improves the fuel quality of the biodiesel, as polymerisation is reduced during combustion, and oxidation stability is higher in storage than in poly-unsaturated dominated fuels (Sheehan *et al.,* 1998).

Table 4.3: Fatty acids profile for *Nannochloropsis occulata* and *Chlorella vulgaris*

FAME type	FAME produced (%) *Nannochloropsis occulata*	FAME produced (%) *Chlorella vulgaris*
Caprylic (C8:0)	0.1	ND
Capric (C10:0)	0.4	0.2
Decanoic (C10:1)	0.7	ND
Lauric (C12:0)	0.4	3.4
Lauroleic (C12:1)	1.1	2.4
Myristic (C14:0)	5.4	0.8
Myristoleic (C14:1)	2.7	3.9
Palmitic (C16:0)	26.7	10.0
Palmitoleic (C16:1n9c)	31.1	37.6
Stearic (C18:0)	1.2	6.3
Elaidic (C18:1n9c)	7.3	5.3
Linoleic (C18:2n6c)	12.8	16.2
Arachidic (C20:0)	2.8	11.5
Eicosenoic (C20:1)	7.1	2.5
Total:		
Saturated	37	32
Mono-unsaturated	50	52
Poly-unsaturated	13	16

ND= Non detected

The poly-unsaturated fraction accounts for 12-16 % of the species' fatty acids. Poly-unsaturated fatty acids are common in microalgae (Demirbas and Demirbas, 2011) which cause chemical instability of the algal biodiesel. Chemical instability due to

poly unsaturation causes oxidative degradation of biodiesel to aldehydes and ketones. It may also cause fuel polymerisation, increasing its viscosity and the formation of insoluble sediment. However, since poly-unsaturated fatty acids have a lower melting point than saturated and mono-unsaturated fatty acids, they may improve the cold flow properties of the biodiesel if present in low quantity. 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 % (Mittelbach and Remschmidt, 2006).

4.2 Characterisation of zirconium dodecyl sulphate used in reactive extraction

The infrared spectrum of zirconium dodecyl sulphate (ZDS) was compared with that of sodium dodecyl sulphate (SDS):

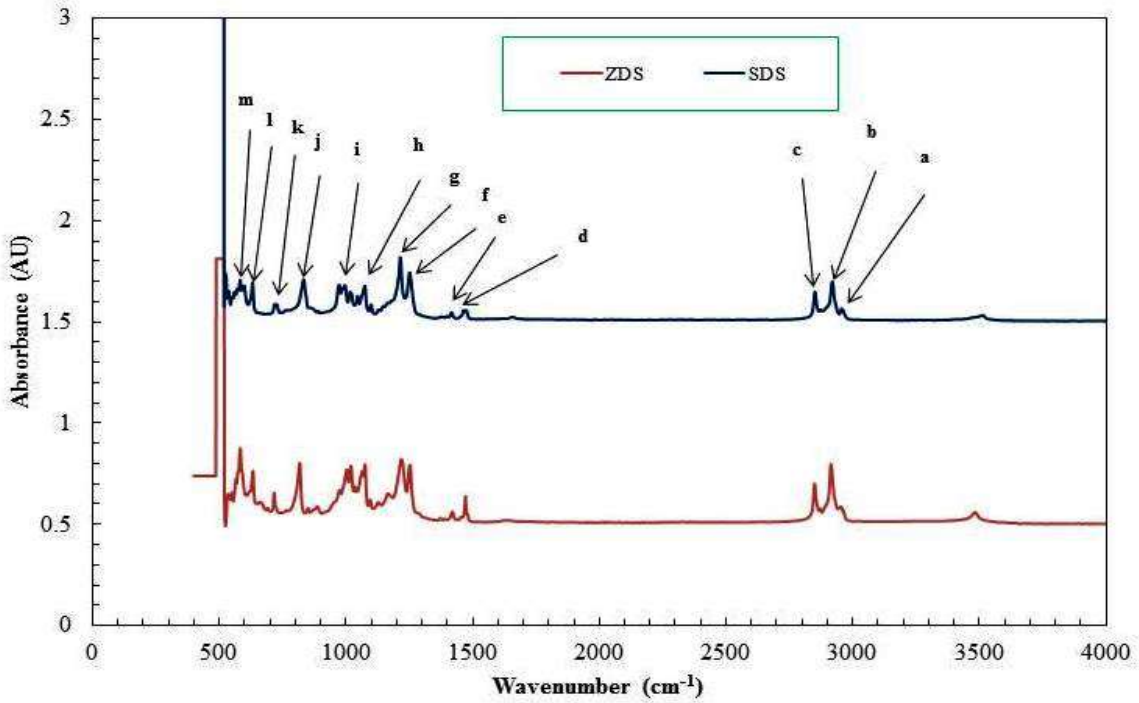


Figure 4.3: FTIR spectra of zirconium dodecyl sulphate (ZDS) and sodium dodecyl sulphate (SDS)

It can be seen that the spectra of the two compounds were very similar. This is expected because of the dodecyl sulphate group they have in common. Table 4.4 shows the functional groups assigned to stretching and bending vibration modes of the dodecyl sulphate alkyl chain and the head groups. The SDS spectrum obtained is similar to what was reported by Ghesti *et al.* (2009) because the same peaks appear at 576, 603, 977. 1067, 1080, 1213, 2848, 2915, 2937 cm^{-1}. All the listed functional groups can be seen in the ZDS and SDS compounds, confirming to some degree that the compound synthesised is ZDS.

Table 4.4: FTIR band assigned for ZDS and SDS

S/N	Wavenumber (cm^{-1})	Assignments	Reference
a.	2937	asymmetric (CH_3)	Socrates(1994)
b.	2915	asymmetric (CH_2)	Socrates (1994)
c.	2848	symmetric (CH_2)	Socrates (1994)
d.	1213,	degenerate OSO_3^-	Kartha *et al*. (1984)
e.	1209	asymmetric stretching	
f.	1080,	degenerate OSO_3^-	Kartha *et al*. (1984)
g.	1068	symmetric stretching	
h.	977,	asymmetric S-OC	Kartha *et al*. (1984)
i.	968	stretching	
j.	830	S-OC stretching	Socrates (1994)
k.	603,	degenerate symmetric	Kartha *et al*. (1984)
l.	576	OSO_3^- bending	

4.3 Catalyst screening for the reactive extraction

Acid catalysts, particularly H_2SO_4, are commonly used to promote reactive extraction of microalgae for FAME production, as algal lipids usually contain significant amounts of high free fatty acids (FFA). Four catalysts: NaOH, zirconium dodecyl sulphate (a Lewis/surfactant catalyst) ("ZDS"), H_2SO_4 or H_2SO_4/SDS were screened in this investigation. In a conventional transesterification, alkali catalysts are considered unsuitable particularly for substrates containing high free fatty acid to avoid FAME losses and complication in products separation due to saponification. Usage of zirconium dodecyl sulphate catalyst was included to explore its cell wall disruption properties for FAME enhancement. The range of catalysts mentioned above were screened to envisage how catalysts affect FAME yield during reactive extraction of microalgae.

4.4 Reactive extraction using NaOH catalyst

The free fatty acid (FFA) content of the *Chlorella vulgaris* was determined as 6.1±0.3 %. This FFA level is usually considered too high for alkali-catalysed conventional transesterification, as it causes high saponification FAME losses (Lotero *et al*., 2005).

4.4.1 *Effect of methanol to oil molar ratio on FAME yield*

The effect of methanol to oil molar ratio on reactively extracted FAME yield at 0.2 N NaOH and 60 ^{o}C is shown in figure 4.4. The result in the figure shows that FAME yield increased as methanol to oil molar ratio increased. For instance at 5 min, 38.1 %, 57.2 % ,74 % FAME yields were obtained respectively, when 600:1, 925:1, and 1275:1 methanol to oil molar ratio were used.

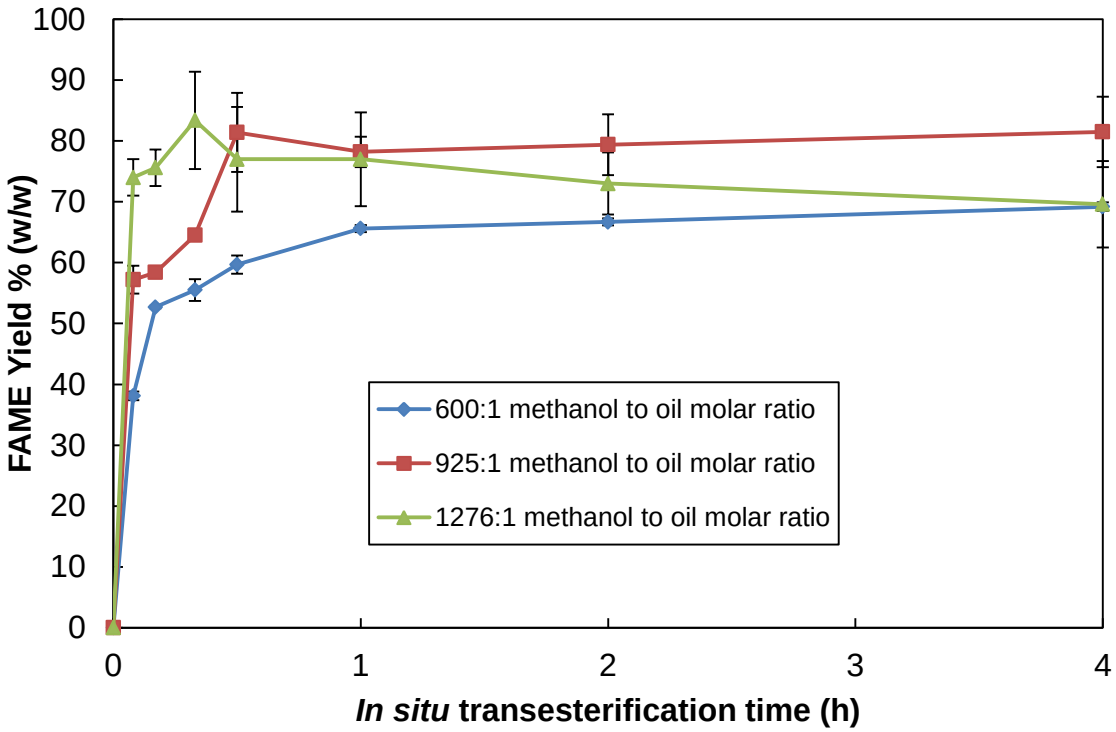


Figure 4.4: NaOH-catalysed reactively extracted FAME-time profile of *Chlorella vulgaris* at varying methanol to oil molar ratio. Process conditions: catalyst concentration: 0.2 N NaOH, agitation rate: 450 rpm, temperature: 60 ^{o}C, mass of *Chlorella vulgaris*: 100 mg.

In addition, the time to attain a maximum FAME yield decreased as the methanol to oil molar ratio increased. For instance, at 1275:1 methanol to oil molar ratio, a maximum FAME yield of 83.4 % occurred at 20 min while a maximum FAME yield of 81.4 %, 65.6 % occurred at 30 min and 1 h respectively, for 925:1 and 600:1 methanol to oil molar ratio. These effects arise because excess methanol to oil molar ratio pushes the transesterification reaction equilibrium towards the product side. It also shifts the equilibrium of dissolution of NaOH in methanol towards formation of methoxide (the actual catalytic species), thereby increasing the FAME production rate. However, at 1275:1 methanol to oil molar ratio, further increase in reaction time beyond the maximum FAME yield resulted in FAME losses due to undesired saponification of FAME and triacylglycerides (TG). It is noteworthy that even

excesses with methanol to oil molar ratio, the optimum FAME rate reached were 83.4 %. This suggests that the FAME production rate in NaOH-catalysed reactive extraction depends on more than only methanol to oil molar ratio.

4.4.2 *Effect of NaOH concentration on FAME yield*

In order to isolate the effect of catalyst concentration on reactively extracted FAME yield, methanol to oil molar ratio was fixed at 925:1, temperature was fixed at 60 ^{o}C, agitation rate was maintained at 450 rpm while the catalyst concentrations were varied as 0.125 N, 0.2 N, 0.25 N and 0.5 N as shown in figure 4.5.

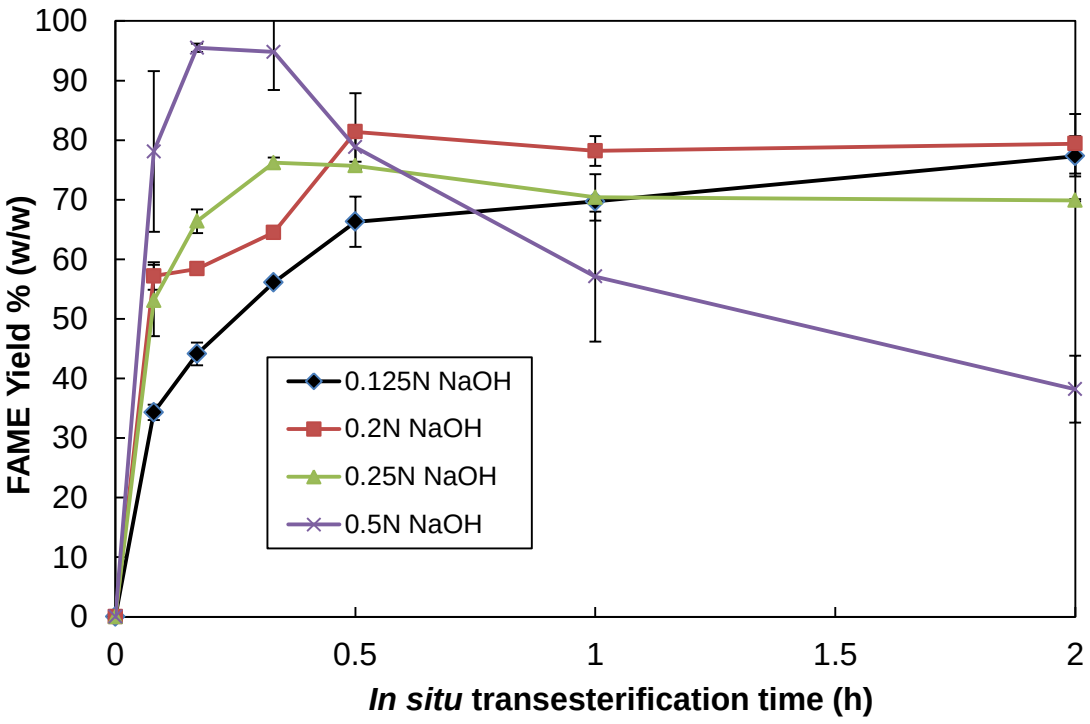


Figure 4.5: NaOH-catalysed reactively extracted FAME-time profile for *Chlorella vulgaris* at varying NaOH concentration. Process conditions: 925:1 methanol to oil molar ratio, agitation rate: 450 rpm, temperature: 60 ^{o}C, mass of microalgae: 100 mg.

The figure clearly shows that FAME rate increased as catalyst concentration increased, as would be expected. For instance at 10 min, 44.1 %, 58.4 %, 66.4 %

and 95.5 % FAME yields were obtained respectively, for 0.125 N, 0.2 N, 0.25 N and 0.5 N NaOH.

In addition, the time to attain a maximum FAME yield decreased as the catalyst concentration increased. For instance, a maximum FAME yield of 77.9 % for 0.125 N, 81.5 % for 0.2 N, 76.2 % for 0.25 N and 95.5 % for 0.5 N were respectively, occurred at 4 h, 30 min, 20 min and 10 min. Excess catalyst concentration produces high methoxide species which causes high FAME production rates.

Metals such as sodium, potassium or their hydroxides dissolve in alcohols such as methanol, ethanol to form alkoxides, which are the real catalyst species during transesterification. The dissolution causes the alkoxide to exist in equilibrium with the hydroxide. About 96 % of the entire NaOH exist as alkoxide in a 0.1 M-solution of NaOH in 99 % ethanol (Caldin and Long, 1954)as shown in equation 4.1

$$CH_3OH + OH^- \underset{k_y}{\overset{k_x}{\rightleftharpoons}} CH_3O^- + H_2O \qquad \textbf{Eq.4.1}$$

$$K_{eq} = \frac{K_x}{K_y} \qquad \textbf{Eq.4.1a}$$

Since the dissolution inherently favours alkoxide formation, a high concentration of sodium hydroxide in excess methanol produces a high concentration of methoxide species needed for promoting the transesterification process. Other researchers obtained high FAME yields at short time with high catalyst concentration during conventional transterification (Vicente *et al*., 1998; Darnoko and Cheryan, 2000; Eze *et al*., 2014). However, this is the first time such FAME enhancement with high catalyst concentration at short reaction times would be reported for reactive extraction.

As the reaction progressed beyond the maximum FAME rate for 0.25 N and 0.5N NaOH, a decrease in FAME yield occurred, as the FAME losses from saponification become significant. This agrees with findings of Eze *et al*. (2014) that FAME rate decreased during KOH-catalysed conventional transesterification as the reaction progressed due to significant saponification FAME losses when high catalyst concentration was used. The key operating procedure here for FAME enhancement is to run the reaction at high catalyst concentration using excess methanol to oil molar ratio but a relatively short reaction time before the saponification rate becomes significant.

This finding is a significant contribution to the fund of knowledge. For instance, a maximum FAME yield (95.5 %) was achieved at a very short reaction time (10 min). Such high yield is usually achieved in a reactive extraction with high acid concentration, longer reaction time, sometimes at relatively high temperatures.

Zhao and Liu (2007) obtained a maximum 98 % FAME yield at 70 ^{o}C during acid catalysed reactive extraction of *R. toruloides*, in 20 h. Velasquez-Orta *et al*. (2011) obtained a maximum 97 % FAME yield at 60 ^{o}C in 20 h, during acid-catalysed reactive extraction of *Chlorella vulgaris* at relatively high acid concentration. They also obtained a maximum 78 % FAME yield at 60 ^{o}C in 1.25 h, during alkali-catalysed reactive extraction of *Chlorella vulgaris* with alkali to oil ratio of 0.15:1 perhaps due to different conditions used in their investigation with what is reported here. Whalen *et al*. (2011) obtained a maximum 82 % FAME yield at 70 ^{o}C in 20 min, during acid catalysed reactive extraction of *Chaetoceros gracilis* when a significant high acid concentration was used. The cost of running the reactor for a long time at higher reaction temperatures will increase operating and capital costs. Additionally,

alkali catalysts can perform effectively at 60 ^{o}C. It is also less corrosive than acid counterpart.

4.4.3 *Effect of reaction time and NaOH concentration on reactive extraction*

Figure 4.6 shows the effect of increase in catalyst concentration at either 10 min or 4h on FAME yield. The result on the figure shows that at 10 min, increase in catalyst concentration from 0.125 N to 0.5 N significantly increased the FAME rate. This is because the increase in the alkali concentration produced more methoxides which consequently increased the FAME rate as explained in section 4.4.2. In contrast, at 4 h, the FAME production rate decreased as the catalyst concentration increased. This decrease in FAME rate was due to undesired saponification side reaction as explained earlier in section 4.4.2.

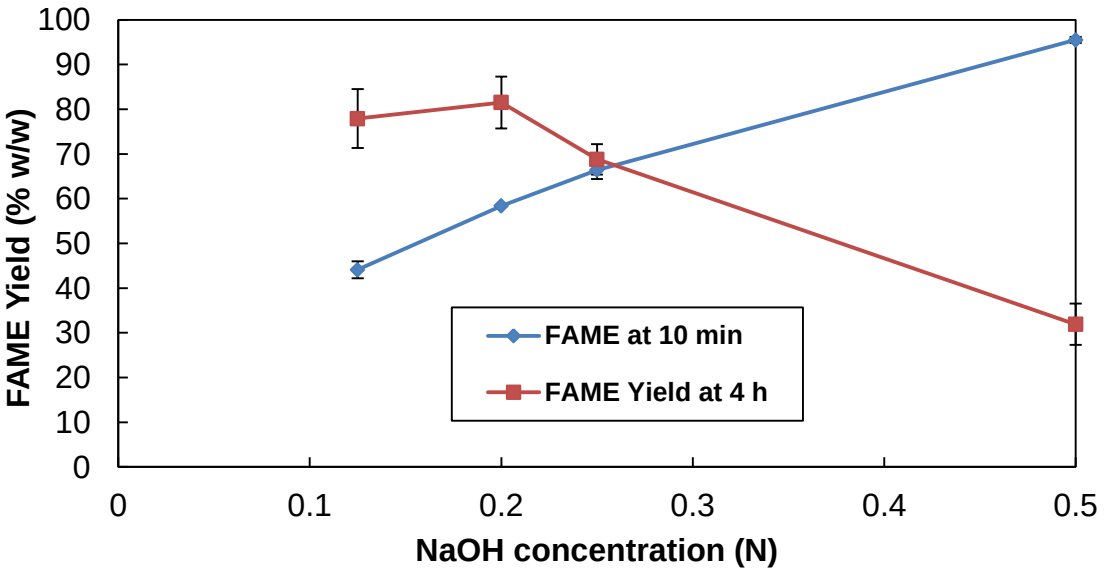


Figure 4.6: NaOH-catalysed reactively extracted FAME yield for *Chlorella vulgaris* at varying time and NaOH concentration. Process conditions: 926:1 methanol to oil molar ratio, agitation rate: 450 rpm, temperature: 60 ^{o}C, mass of *Chlorella vulgaris*: 100 mg.

4.4.4 *Effect of moisture on NaOH-catalysed reactive extraction*

Figure 4.7 shows the effect of moisture on NaOH-catalysed reactive extraction of wet *Chlorella vulgaris* at 60 ^{o}C, 600: 1 methanol to oil molar ratio. The water contents were 0 wt %, 5 wt %, 20 wt % and 30 wt %/ (wt dry algae). The results in the figure clearly shows that the process is tolerant to the moisture as there was no significant reduction in the FAME yield at each data point.

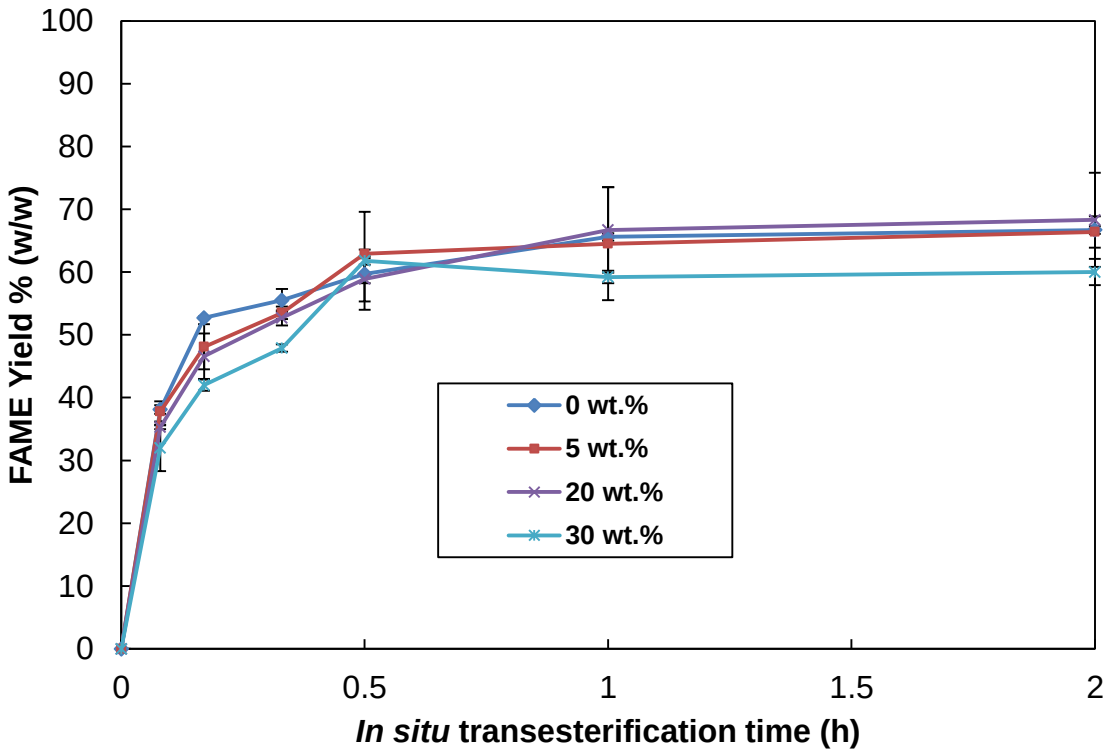


Figure 4.7: NaOH-catalysed reactively extracted FAME yield-time profile for wet *Chlorella vulgaris*. Process conditions: 600:1 methanol to oil molar ratio, 0.2 N NaOH, agitation rate: 450 rpm, temperature: 60 ^{o}C, mass of *Chlorella vulgaris*: 100 mg.

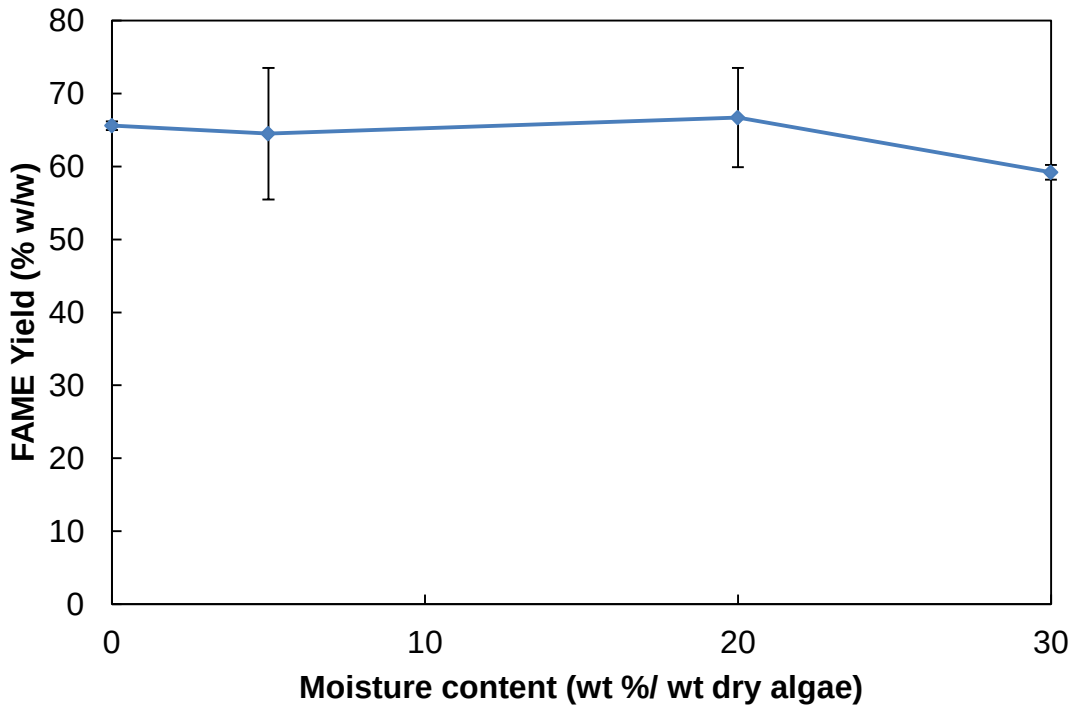


Figure 4.8: NaOH-catalysed reactively extracted FAME yield at varying moisture content for wet *Chlorella vulgaris*. Process conditions: 600:1 methanol to oil molar ratio, reaction time: 1 h, agitation rate: 450 rpm, temperature: 60 ^{o}C, mass of *Chlorella vulgaris*: 100 mg.

This moisture tolerance was apparent for all the moisture contents investigated (0-30 wt %/ (wt. dry algae)). This result agrees with moisture tolerance observed in other acid catalysed reactive extraction of microalgae reported in the literature (Wahlen *et al*., 2011; Velaquez-Orta *et al*., 2013). Reactive extraction has greater water tolerance than conventional transesterification because of the excess methanol to oil molar ratio. For instance, Eze *et al*. (2014) during KOH-catalysed conventional transesterification reported that FAME losses increased as the amount of moisture content and reaction time increased. They observed FAME concentrations decreased by 20 %, 43 %, 56 %, and 70 % of the initial values respectively, for 0,

2.5, 5 and 12.5 vol. %. Other investigators observed FAME reduction with increase in moisture during conventional transesterification (Canakci and Gerpen, 1999).

Drying microalgae to the moisture level required by conventional transesterification (< 0.5 wt. % oil) (Canacki and Garpen, 1999) is energy intensive and makes a significant contribution to the cost of algae pre-treatment. However, here, the FAME yield at 1 h was observed to decrease (figure 4.8) only at 30 wt. % moisture/ (wt. dry algae). The yields at 0, 5 and 20 wt. % moisture/ (wt. dry algae) were not significantly different. The process was tolerant to moisture because the excess methanol was used which drives the equilibrium dissolution of NaOH in methanol towards formation of methoxides as shown in equation 4.1. The shift in equilibrium towards methoxide formation decreases the NaOH concentration in equilibrium with it at the beginning of the reaction. This effect significantly reduces the rate of saponification. This explains why the decrease in FAME yield became more apparent at 1 h, 30 wt. % moisture/ (wt. dry algae) as shown in figure 4.7 and 4.8 (previous page).

4.4.5 *Mechanism of alkali-catalysed reactive extraction of microalgae*

In order to develop the scheme of reaction for the NaOH-catalysed reactive extraction explained in section 4.4.2, the following considerations and assumptions were made:

a.) The effect of the cell wall membrane on the internal mass transfer of the solvent was assummed to be negligible.

b.) Velasquez-Orta *et al*. (2011) have shown that agitation rate of 380 rpm at 60 ^{o}C was adequate to prevent mass transfer limitation during NaOH-catalysed reactive extraction of *Chlorella vulgaris* for FAME production. The present

investigation was conducted at 450 rpm at 60 ^{o}C, so the external mass transfer effect was assumed to be negligible.

c.) Based on the findings from section 4.4.2 it is postulated that during NaOH-catalysed reactive extraction of microalgae, other side reactions including FAME and triglycerides saponification and free fatty acid neutralisation occur alongside the desired FAME production. A homogeneous model derived by Eze *et al.* (2014) for KOH-catalysed transesterification of rapeseed was used to fit the experimental data obtained in this research.

4.4.6 *Hydroxide-alkoxide equilibrium reaction*

Metals such as sodium, potassium or their hydroxides dissolve in alcohols to form alkoxides, which are the real catalyst species for alkali-catalysed reactive extraction. The dissolution causes the alkoxide to exist in equilibrium with the hydroxide (Caldin and Long, 1954; Glass, 1971) as shown in eq. 4.1.

$$CH_3OH + OH^- \underset{k_y}{\overset{k_x}{\rightleftharpoons}} CH_3O^- + H_2O \qquad \textbf{Eq. 4.1}$$

$$K_{eq} = \frac{K_x}{K_y} \qquad \textbf{Eq. 4.1a}$$

Such equilibrium reaction exists to some degree during reactive extraction whilst NaOH dissolves in methanol to generate the alkoxide. Alkoxide reacts with water in the microalgae or methanol to form hydroxide. Thus, the concentration of the alkoxide produced depends on the amount of methanol, catalyst concentration, and the water in the microalgae or methanol. An average equilibrium constant of K_{eq}=0.73 has been calculated for a NaOH-ethoxide system (Caldin and Long, 1954). A K_{eq} of 3.21 was estimated for the NaOH-methoxide system used in this

investigation, considering the fact that the acidity of methanol is about 4.4 times greater than that of ethanol (Reeve *et al*., 1979).

4.4.7 *Transesterification reactions*

Transesterification involves stepwise and reversible reactions leading to the formation of methyl ester from triglycerides. Diglycerides and monoglycerides occur as intermediate species in the steps as shown in Eq. 4.2-4.4.

$$TG + MA \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DG + FAME \qquad \textbf{Eq. 4.2}$$

$$DG + MA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} MG + FAME \qquad \textbf{Eq. 4.3}$$

$$MG + MA \underset{k_6}{\overset{k_5}{\rightleftharpoons}} GL + FAME \qquad \textbf{Eq. 4.4}$$

Where TG: triglyceride; DG: diglyceride; MG: monoglyceride; MA: methyl alcohol; GL: glycerol; FAME: fatty acid methyl esters.

In each step, the alkoxide (the actual catalytic species) is regenerated after the product has formed.

4.4.8 *Saponification of algal lipids and FAME*

Here, triglycerides are irreversibly convert into soap via saponification side reactions as illustrated in equation 4.6. Similarly, FAME converts into soap and methanol as shown in equation 4.7. Monoglyceride and diglyceride saponification rates are excluded since they occur as transient intermediates.

$$\mathrm{TG} + 3\mathrm{OH}^- \overset{k_7}{\rightarrow} 3\mathrm{Soap} + \mathrm{GL} \qquad \textbf{Eq. 4.6}$$

$$\mathrm{FAME} + \mathrm{OH}^- \overset{k_8}{\rightarrow} \mathrm{Soap} + \mathrm{MA} \qquad \textbf{Eq. 4.7}$$

4.4.9 *Neutralisation of FFA*

For this step, free fatty acids in the micoalgae undergo irreversible neutralisation with hydoxide or alkoxide ion to form soap as shown in eq. 4.8.

$$\mathrm{FFA} + \mathrm{OH^-}\ (\mathrm{RO^-}) \xrightarrow{k_9} \mathrm{Soap} + \ \mathrm{H_2O}\ (\mathrm{ROH}) \qquad \textbf{Eq. 4.8}$$

Unlike the transesterification step, the catalyst is consumed in this reaction. That means, enough catalyst should be available to achieve a high FAME yield in an alkali-catalysed reactive extraction.

The kinetic rate expressions for all the chemical reactions listed in **eq. 4.1-4.8** is summarised as shown in **eq. 9.** below as reported in the literature (Eze *et al*., 2014)

$$r_{TG} = \frac{d[TG]}{dt} = [CH_3O^-](-k_1\,[TG][MA] + k_2[DG][FAME]) - k_7\,[TG][OH^-]$$

$$r_{DG} = \frac{d[DG]}{dt} = [CH_3O^-](k_1\,[TG][MA] + k_4[MG][FAME]) - k_2\,[DG][FAME] - k_3\,[DG][MA])$$

$$r_{MG} = \frac{d[MG]}{dt} = [CH_3O^-](k_3\,[DG][MA] + k_6[GL][FAME]) - k_4\,[MG][FAME] - k_5\,[DG][MA])$$

$$r_{FAME} = \frac{d[FAME]}{dt} = [CH_3O^-](k_1\,[TG][MA] + k_3[DG][MA]) + k_5\,[MG][MA] - k_2\,[DG][FAME] - k_4\,[MG][FAME] - k_6\,[GL][FAME]) - k_8\,[FAME][OH^-]$$

$$r_{MA} = \frac{d[MA]}{dt} = [CH_3O^-](k_2\,[DG][FAME] + k_4[MG][FAME] + k_6\,[GL][FAME] + k_y\,[H_2O] - k_1\,[TG][MA] - k_3\,[DG][MA] - k_5\,[MG][MA]) + k_8\,[FAME][OH^-] - k_x\,[MA][OH^-]$$

$$r_{GL} = \frac{d[GL]}{dt} = [CH_3O^-](k_5\,[MG][MA] - k_6[GL][FAME]) + k_7\,[TG][OH^-]$$

$$r_{Soap} = \frac{d[Soap]}{dt} = [OH^-](k_7\,[TG] + k_8[FAME] + k_9\,[FFA])$$

$$r_{FAA} = \frac{d[FAA]}{dt} = -k_9\,([FAA][OH^-] + [FFA][CH_3O^-])$$

$$r_{H_2O} = \frac{d[H_2O]}{dt} = k_x[MA][OH^-] - k_y\,[CH_3O^-][H_2O] + k_9[FAA][OH^-]$$

$$r_{CH_3O^-} = \frac{d[CH_3O^-]}{dt} = k_x\,[MA][OH^-] - k_y\,[H_2O][CH_3O^-] - k_9\,[FFA][CH_3O^-]$$

$$r_{OH^-} = \frac{d[OH^-]}{dt} = k_y\,[H_2O][CH_3O^-] - k_x\,[MA][OH^-] - k_9\,[FFA][OH^-] - k_7\,[TG][OH^-] - k_8\,[FAME][OH^-] \quad \textbf{Eq. 9}$$

Where r_i**:** rate of formation of species i (mol L^{-1} min^{-1}); k_i : rate constant of the reactions (L mol^{-1} min^{-1})

These system of ordinary differential equations were simultaneously fitted to the experimental data in Microsoft Excel 2010 using standard curve fitting technique as explained in the literatures (Bambase *et al*., 2007; Zakaria *et al.,* 2014; Eze *et al*., 2014). The rate constants k_1-k_6 listed in table 4.5 were used as the initial rate constants. They were used because they gave similar trend to the experimental FAME concentrations. The initial rate constants were adjusted such that:

a.) there was no significant difference between the simulated and experimental data i.e error was less than 5 %.

b.) there was no time step dependency in the simulated FAME concentration.

The final rate constants obtained from the model is listed in table 4.5. They were compared with the rate constants predicted by Bambase *et al.*(2007).

Table 4.5: Model rate constants. Process condition: 600:1; 925:1 methanol to oil molar ratio, agitation rate: 450 rpm, temperature: 60 ^{o}C, catalyst concentration: 0.125 N NaOH, 0.2 N NaOH, 0.25 N NaOH, 0.5 N NaOH, 0-20 wt. % moisture/ (wt. dry algae)

Reactions	model rate constant ($L^{-1}mol^{-1}min^{-1}$)	Bambase *et al.* (2007) ($L^{-1}mol^{-1}min^{-1}$)
TG ⟶ DG	k_1: 0.08	k_1: 0.09
DG ⟶ TG	k_2: 0.01	k_2: 0.01
DG ⟶ MG	k_3: 0.15	k_3: 0.35
MG ⟶ DG	k_4: 0.04	k_4: 0.12
MG ⟶ GL	k_5: 0.30	k_5: 0.49
GL ⟶ MG	k_6: 0.02	k_6: 0.04
Algae Lipids ⟶ Soap	k_7: 0.05	-
FAME ⟶ Soap	k_8: 0.13	-
FFA ⟶ Soap	k_9: 25.6	-

The model rate constants k_3-k_6 were lower than that predicted by Bambase *et al.* (2007). This is because the experimental data show a lower disappearance rate of diglyceride, monoglyceride and glycerol than that predicted by Bambase *et al*. (2007). The simulated FAME yield based on this schemes as explained above gave a good prediction of the experimental FAME yield as shown in figure 4.9-4.15.

4.4.10 *Validation of the model with experimental data*

Figures 4.9-4.15 show the model FAME yield-time profiles compared with the experimental FAME yield-time profiles shown in figure 4.5 (section 4.4.2) and figure 4.7 (section 4.4.4) in the previous page.

Figure 4.9 shows the results of the model FAME yield versus the experimental FAME yield when both were at 0.125 N NaOH, 925:1 methanol to oil molar ratio and 60 ^{o}C for dry *Chlorella vulgaris*.

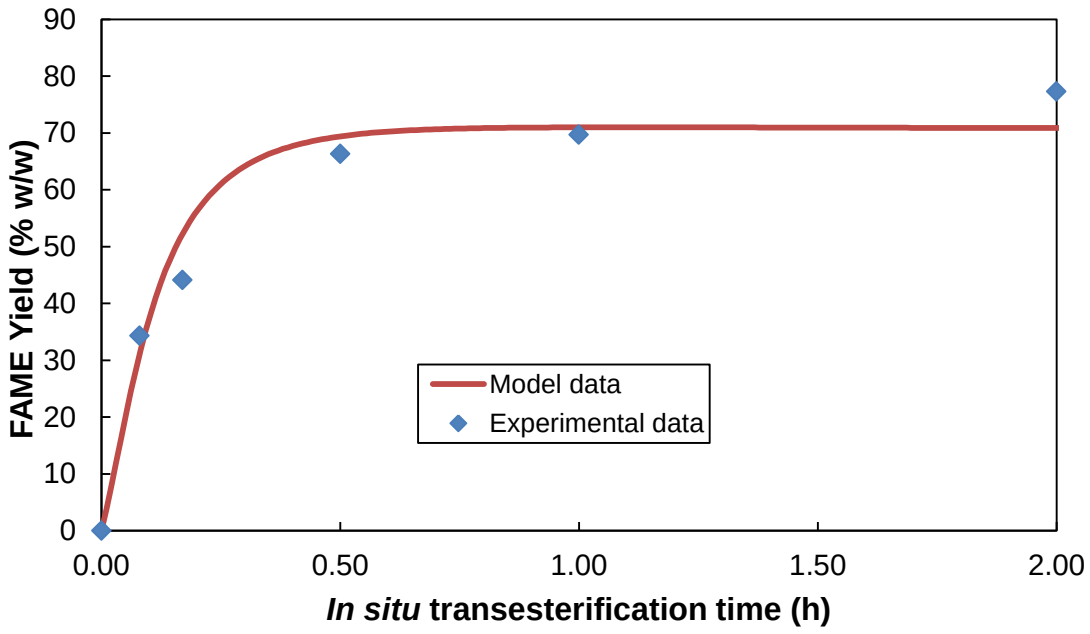


Figure 4.9: Model vs experimental FAME yield-time profile for NaOH-catalysed reactive extraction of *Chlorella vulgaris* at 0.125 N NaOH, 925:1 methanol to oil molar ratio. Temperature: 60 ^{o}C, agitation rate: 450 rpm. *Chlorella vulgaris*: 100 mg.

As can be seen, the results on the plot at each data point for both the model and the experiment are not significantly different. For instance the model predicted 32 % FAME yield at 0.08 h while the experimental yield was 34 %. At 0.5 h the predicted FAME yield was 69 % while the experimental FAME yield was 66 %.

Figure 4.10 compares the FAME yield obtained when both the model and the experiment were at 0.2 N NaOH, 925:1 methanol to oil molar ratio and 60 ^{o}C for dry *Chlorella vulgaris.*

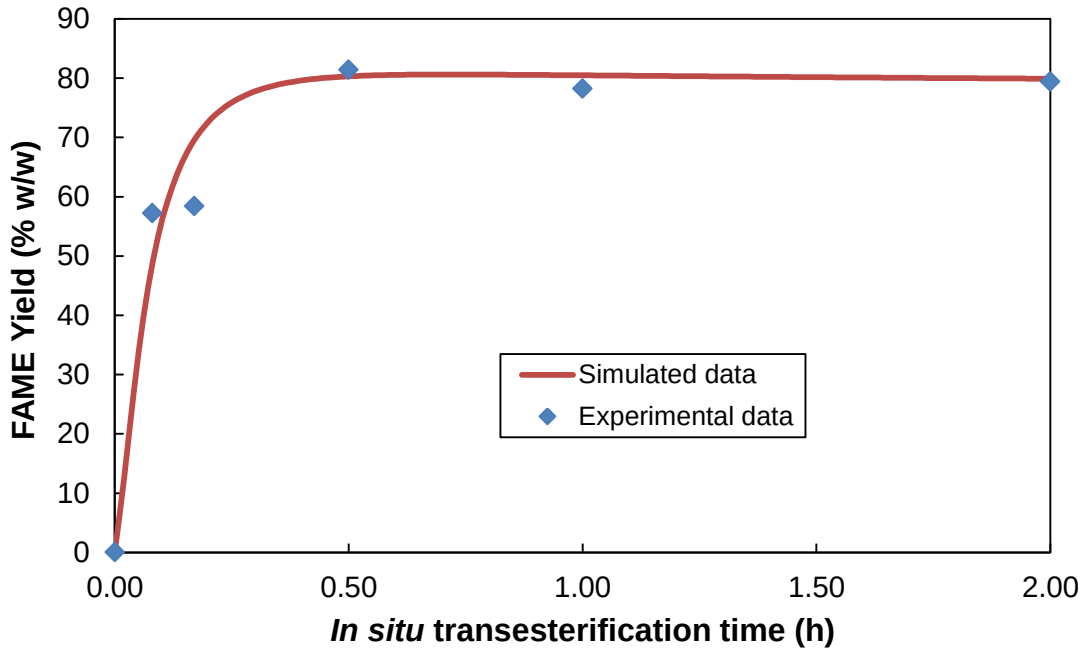


Figure 4.10: Model vs experimental FAME yield-time profile for NaOH-catalysed reactive extraction of *Chlorella vulgaris* at 0.2 N NaOH, 925:1 methanol to oil molar ratio. Temperature: 60 ^{o}C, agitation rate: 450 rpm. *Chlorella vulgaris*: 100 mg.

The model FAME yield at 0.08 h was 53.2 % while the experimental yield was 57.2 %. At 0.5 h and 2 h, the model predicted FAME yield of 80.4 % and 79.9 % respectively while the experimental FAME were 81 % and 80 % respectively for the same period.

Figure 4.11 compares the model FAME yield with the experimental one using the same process conditions of 0.25 N NaOH, 925:1 methanol to oil molar ratio and 60 ^{o}C for dry *Chlorella vulgaris.*

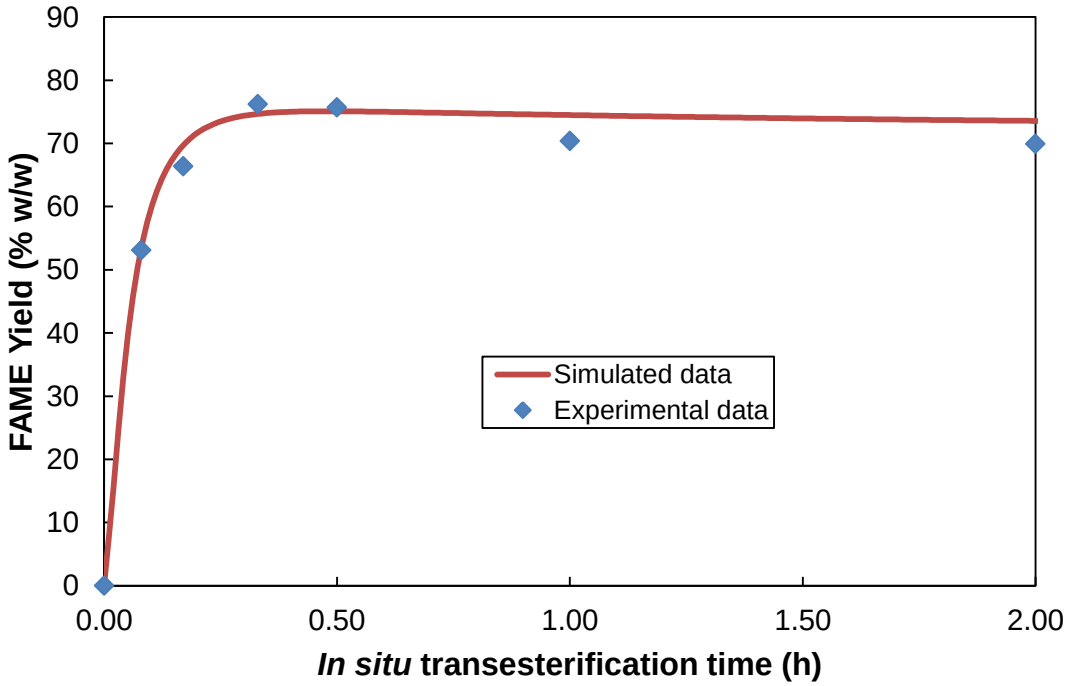


Figure 4.11: Model vs experimental FAME yield-time profile for NaOH-catalysed reactive extraction of *Chlorella vulgaris* at 0.25 N NaOH, 925:1 methanol to oil molar ratio. Temperature: 60 ^{o}C, agitation rate: 450 rpm. *Chlorella vulgaris*: 100 mg.

The model FAME yield at 0.08 h was 57.6 % while the experimental yield was 53.1 %. At 0.5 h the predicted FAME yield was 75.7 % while the experimental value was 75.1 %. Both the model and experimental FAME yields at other data points are not significantly difference.

Figure 4.12 compares the predicted FAME yield at 0.5 N NaOH, 925:1 methanol to oil molar ratio and 60 ^{o}C for dry *Chlorella vulgaris* with the experimental value obtained at the same process conditions.

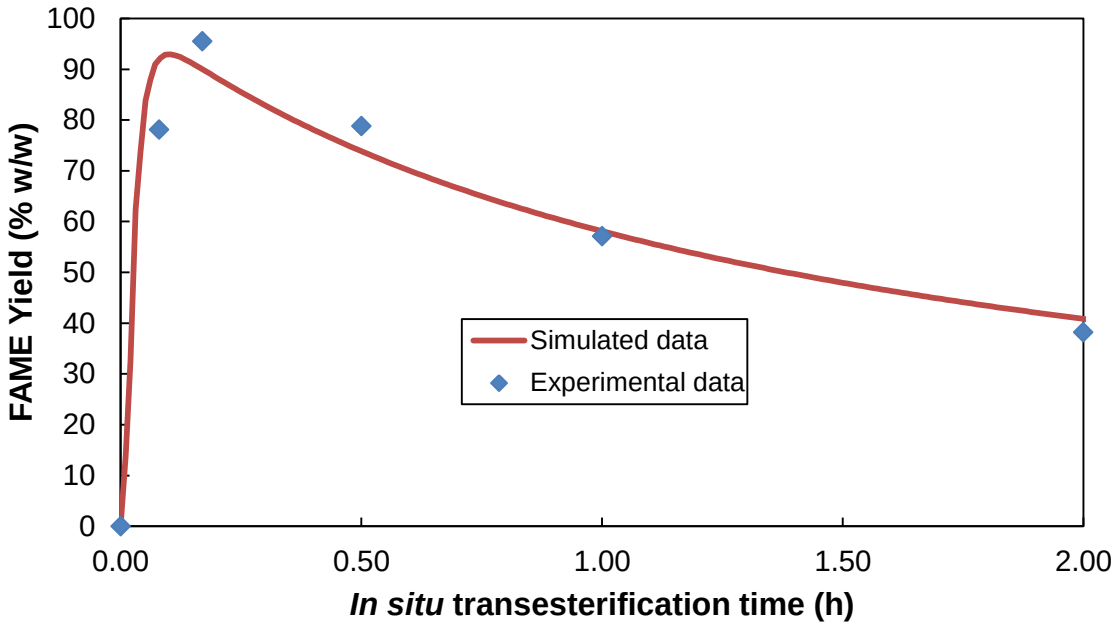


Figure 4.12: Model vs experimental FAME yield-time profile for NaOH-catalysed reactive extraction of *Chlorella vulgaris* at 0.5 N NaOH, 925:1 methanol to oil molar ratio. Temperature: 60 ^{o}C, agitation rate: 450 rpm. *Chlorella vulgaris*: 100 mg.

The model FAME yield at 0.17 h was 90 % while the experimental yield was 96 %. In addition the model FAME yield rose to maximum at 10 min and significantly reduced to 41 % in 2 h which agrees well with experimental yield of 38 % at this condition.

As can be seen in the schemes of the reaction, the FAME losses are due to saponification of FAME and triglycerides at high catalyst concentration and longer reaction time. These results confirm that this particular mechanism also occurs in a NaOH-catalysed reactive extraction of microalgae. This finding agrees with that reported in Eze *et al*. (2014) for a KOH-catalysed conventional transesterification that FAME production can significantly be enhanced at high catalyst concentration before saponification FAME losses become significant.

The model was also compared with the reactive extraction of dry and wet *Chlorella vulgaris* at 0-20 wt % moisture/ (wt dry algae), 0.2 N NaOH and 600:1 methanol to oil molar ratio.

Figure 4.13 compares the model FAME yield obtained using the same process conditions with the experimental results at 0.2 N NaOH, 600:1 methanol to oil molar ratio and 60 ^{o}C for dry *Chlorella vulgaris*.

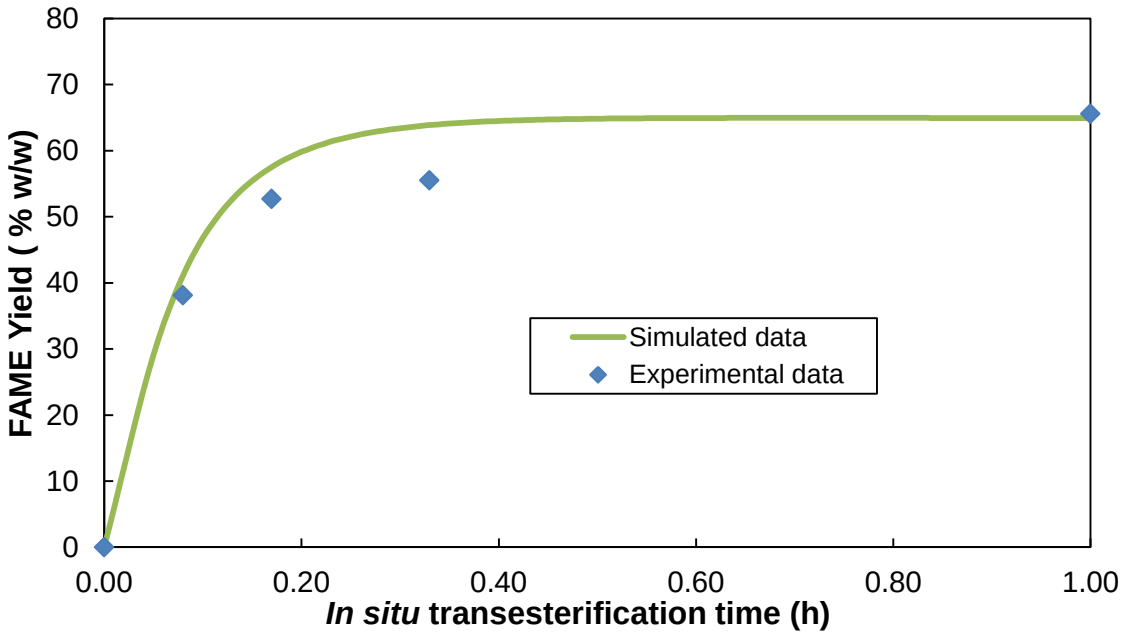


Figure 4.13: Model vs experimental FAME yield-time profile for NaOH-catalysed reactive extraction of *Chlorella vulgaris* at 0.2 N NaOH, 600:1 methanol to oil molar ratio. Temperature: 60 ^{o}C, agitation rate: 450 rpm. 0 wt . % moisture/ (wt . dry algae), *Chlorella vulgaris*: 100 mg.

At 0.08 h the model predicted 38.1% FAME yield while 40.5% was obtained from the experiment. At 0.17 h the model FAME yield was 57.7 % while the experimental value was 52.7 %. At 1 h, model FAME yield was 64.9 % while the experimental yield was 65.6 %.

Figure 4.14 compares the model FAME yield at 0.2 N NaOH , 600:1 methanol to oil molar ratio, 60 ^{o}C and 5 % wt moisture/ (wt dry algae) with the experimental FAME yield obtained at the same process conditions.

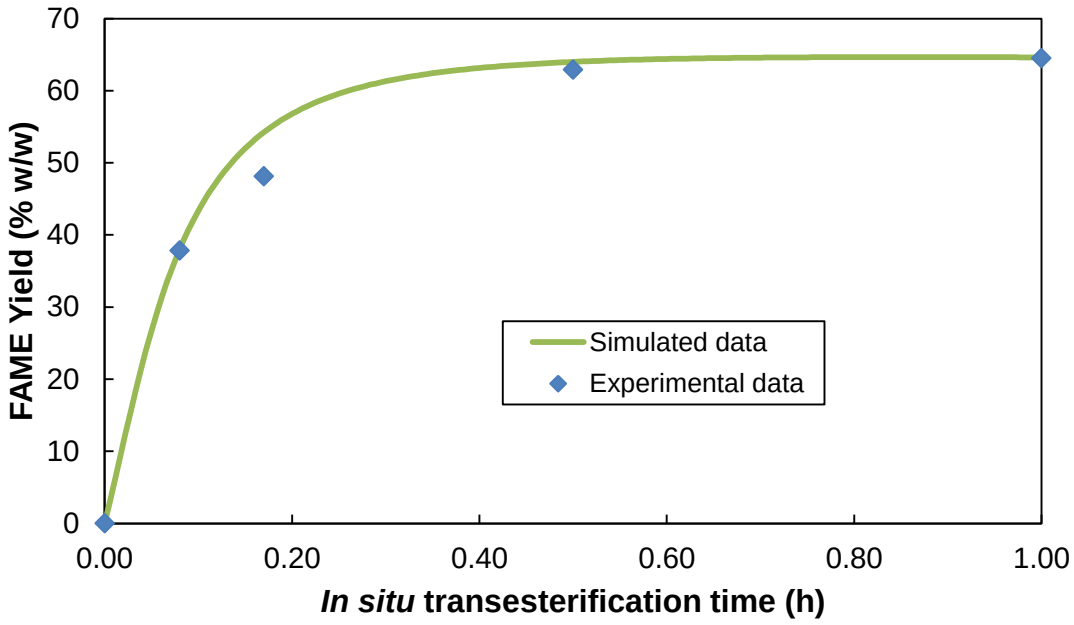


Figure 4.14: Model vs experimental FAME yield-time profile for NaOH-catalysed reactive extraction of *Chlorella vulgaris* at 0.2 N NaOH, 600:1 methanol to oil molar ratio, 5 wt. % moisture/(wt. dry algae). Process conditions: Temperature: 60 ^{o}C, agitation rate: 450 rpm, *Chlorella vulgaris*: 100 mg.

As can be seen on the figure, both experimental and predicted FAME yield are similar at each data point. For instance, at 0.08 h the model predicted 39% FAME yield while experimental FAME yield was 37.8 %. At 0.17 h the model FAME yield was 53.9 % while the experimental value was 48.1 %. At 1 h the model FAME yield was 64 % while 62.9 % FAME yield was obtained from the experiment.

Figure 4.15 compares the model FAME yield using the same process conditions with the experiment at 0.2 N NaOH , 600:1 methanol to oil molar ratio and 60 ^{o}C at 20 % wt moisture/(wt dry algae).

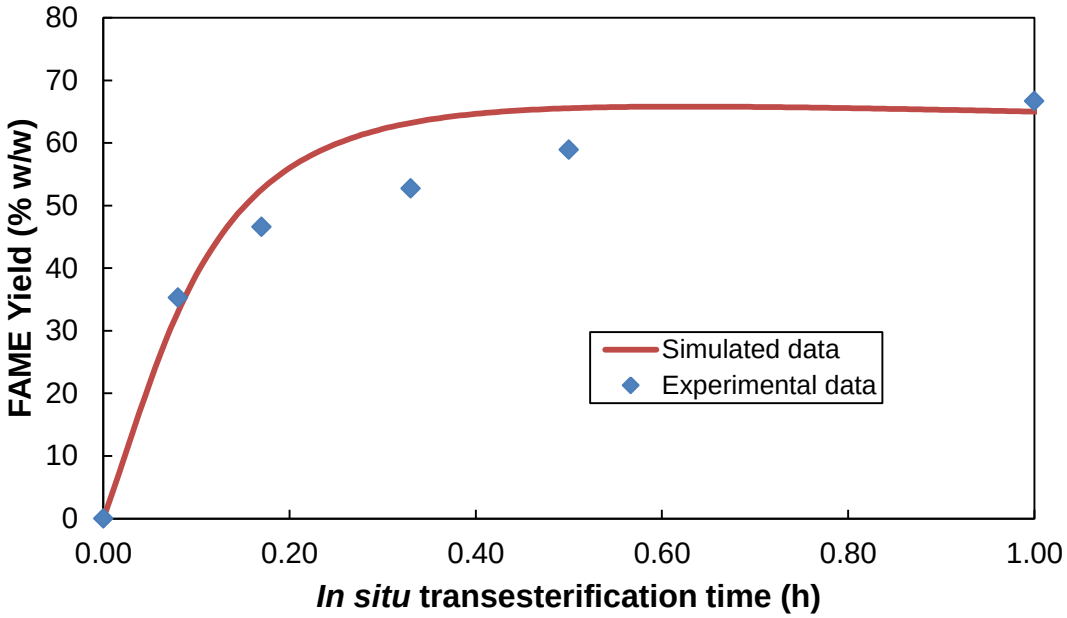


Figure 4.15: Model vs experimental FAME yield-time profile for NaOH-catalysed reactive extraction of *Chlorella vulgaris* at 0.2N NaOH, 600: 1 methanol to oil molar ratio, 20 wt. % moisture/ (wt. dry algae). Temperature: 60^{o}C, agitation rate: 450 rpm. 20% wt. moisture, *Chlorella vulgaris*: 100mg.

At 0.08 h the model predicted 37.4 % FAME yield while the experimental value was 35.3 %. At 0.17 h, the model FAME yield was 52.1 % while the experimental one was 46.6 %. At 1 h, the model FAME yield was 65.1 % while the experimental FAME yield was 66.7%. Although at 0.33 h and 0.5 h the model FAME yield was slightly higher than the experimental value. Nevertheless, the trends for both the model and the experiment are the same. In addition, the initial FAME yield is the most important in this process as the reaction tends towards equilibrium in the later part. The initial FAME yield at 0.08 h was well predicted by the model.

4.4.11 *Overall scheme for NaOH-catalysed reactive extraction of microalgae*

The overall scheme for NaOH-catalysed reactive extraction of microalgae based on the findings in this research can be simply summarised in the figure 4.16.

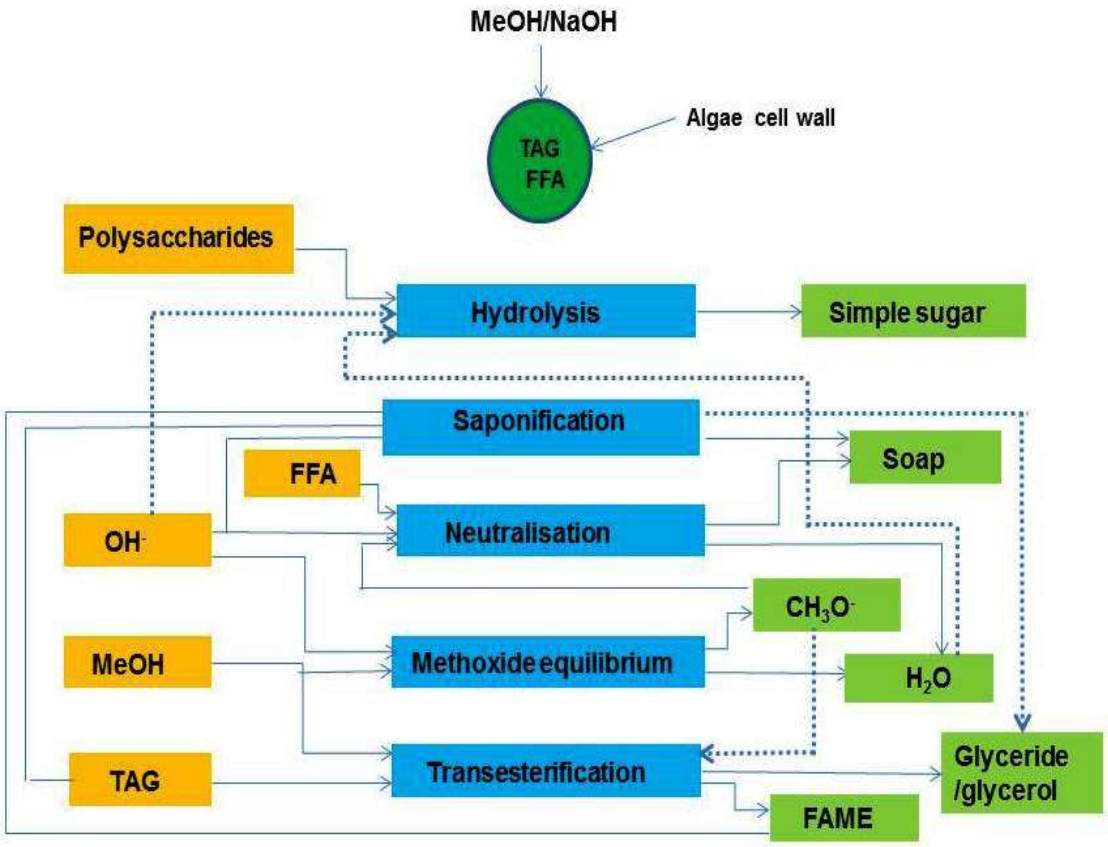


Figure 4.16: Proposed reaction scheme for NaOH-catalysed reactive extraction of microalgae for FAME production. FAME: fatty acid methyl ester (Biodiesel); TAG: triacylglycerides; FFA: free fatty acid; OH^- : hydroxide species; CH_3O^- : alkoxide species (the actual catalyst), MeOH: methanol.

The reaction begins with equilibrium dissolution of NaOH in methanol, which inherently favours methoxide (CH_3O^-) formation (Caldin and Long, 1954; Glass, 1971). More methoxide species were formed due to excess methanol to oil molar ratio used.

The alkoxide species formed involves in a number of reaction as shown in figure 4.16. It catalyses transesterification of the triglycerides through three consecutive and reversible steps to form FAME (biodiesel). Each step produces 1 mole of FAME. The methoxide species is regenerated at the end of the reaction. The CH_3O^- also catalyses FAME saponification to form soap. However, this reaction is much slower

than the FAME production step. Therefore it becomes insignificant at the beginning of the reaction when the FAME production is small as can be seen in experimental result (figure 4.5) and the modelled results (figure 4.12). The *Chlorella vulgaris* used in this research contained 6 % free fatty acid (FFA). The CH_3O^- species irreversibly reacts with FFA to form soap. Unlike the FAME production steps, in which catalyst is regenerated at the completion of the reaction, this reaction consumes the catalyst. Consequently, it slows down the FAME production rate and causes complication in products separation. Therefore enough catalyst should be available to achieve high FAME yield. As can be seen in figure 4.5, low FAME yield could be achieved with 0.125, 0.2 and 0.25 N due to this effect. NaOH could also disrupt the *Chlorella vulgaris* cell wall by cleavage of the glycosidic bond in the cellulose thereby causing depolymerisation. This would make the alkoxide ion more accessible to the body lipids inside the cell, thereby increasing FAME production rate.

4.5 Effect of process conditions on FAME yield

The main and interaction effects of moisture content, methanol to oil molar ratio and catalyst concentration on FAME yield were investigated at 1 h reaction time, 60 ^{o}C and agitation rate of 450 rpm using response surface methodology (RSM). This was done by placing each factor at 5 levels. Moisture content was varied from 0-39 wt % / (wt dry algae), methanol to oil molar ratio was varied from 369:1-1507:1 and NaOH concentrations was varied from 0.082-0.293 N.

4.5.1 *Main effect plot for FAME yield using NaOH catalyst*

Figure 4.17 shows the effect of each of the three factors namely: methanol to oil molar ratio, NaOH concentration and moisture content on the FAME yields at 1 h.

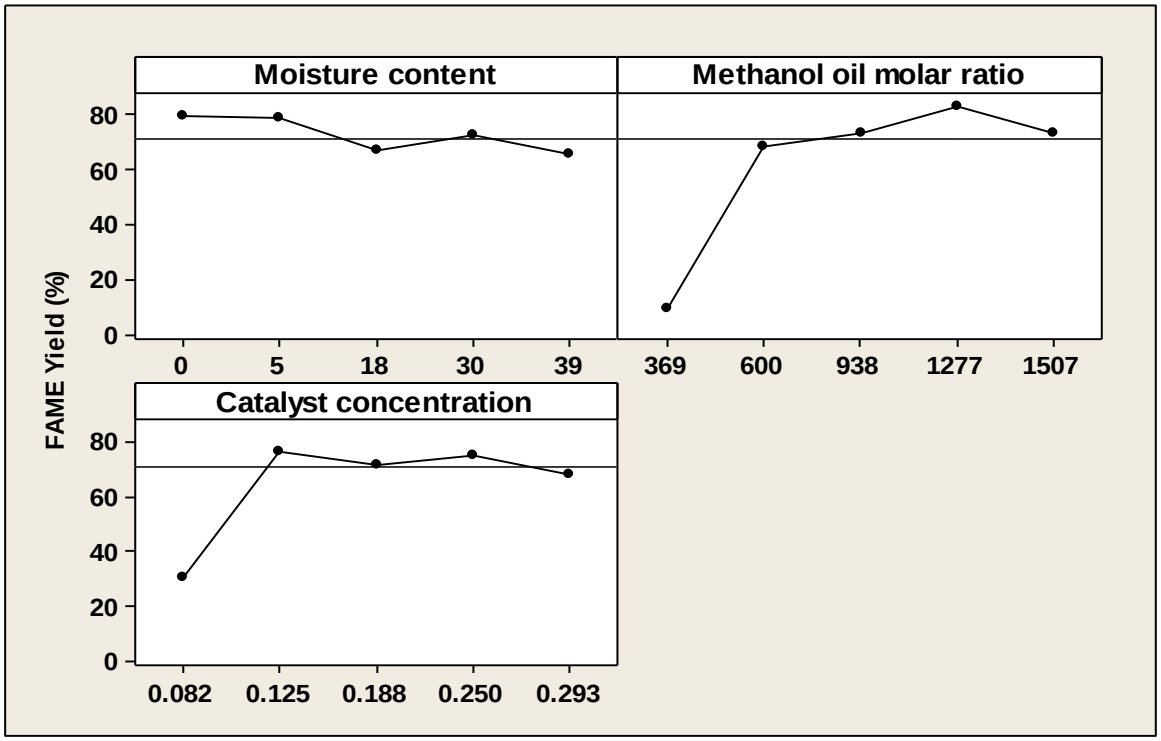


Figure 4.17: Main effect plot of methanol to oil molar ratio, NaOH concentration and moisture content on FAME yield. Process conditions: 1 h reaction time, temperature: 60 ^{o}C, agitation rate: 450 rpm, mass *Chlorella vulgaris*: 100 mg**.** Data shown are mean values of duplicate experiments.

As expected the process showed some level of moisture tolerance. For instance there was no significant reduction in FAME yield at 0 wt % and 5% wt moisture/ (wt dry algae). However there was decline in the FAME yield at 18 wt % moisture which became more apparent at 39 wt % moisture/ (wt dry algae) due to saponification.

Variation in methanol to oil molar ratio from 369:1 to 1507:1 resulted into three regions of FAME yields (figure 4.17). FAME yield increase observed at 369: 1 to 600:1 molar ratio was greater than from 600:1 to 1277:1 methanol to oil molar ratio. This shows that the effect of methanol excess on the FAME yield was less significant above 600: 1. The FAME yield reached its optimum at 1277:1 methanol to oil molar ratio as a further increase in methanol to oil molar ratio resulted in FAME reduction. Velasquez-Orta *et al*. (2011) also observed reductions in FAME yield with excesses in methanol to oil molar ratio during alkali-reactive extraction of *Chlorella vulgaris* to FAME.

Increase in catalyst concentration produced the same effect on the FAME yield as methanol to oil molar ratio (figure 4.17). Increase in catalyst concentration from 0.082 N to 0.125 N caused an increase in the FAME yield. The FAME yields were not significantly different at the second region when the catalyst concentration increased from 0.125-0.250 N. A decline in the FAME yield occurred with further increase in the catalyst concentration, from 0.25 N to 0.293 N because of saponification.

4.5.2 *Interaction effect plot for FAME yield using NaOH catalyst*

Figure 4.18 shows the interaction effect of the three factors namely: methanol to oil molar ratio, NaOH concentration and moisture content on the FAME yield at 1h

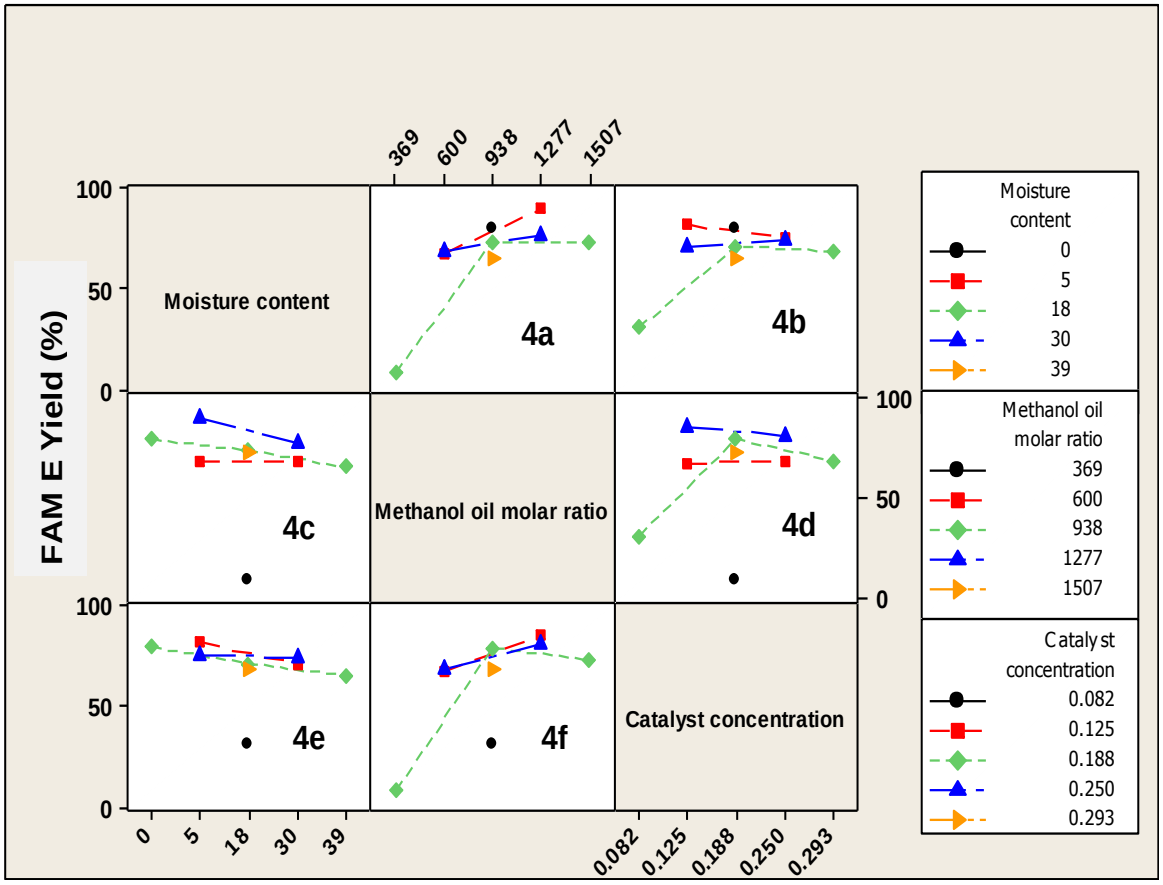


Figure 4.18: Interaction effect plot of methanol to oil molar ratio, NaOH concentration, moisture content on FAME yield. Process conditions: 1 h reaction time, temperature: 60 ^{o}C, agitation rate: 450 rpm, *Chlorella vulgaris* mass: 100 mg. Data shown are mean value of duplicate experiments.

Plots 4a and 4c (figure 4.18) show the interaction effect of methanol to oil molar ratio and moisture content on the FAME yield after 1 h. For plot 4a, at 938:1 methanol to oil molar ratio, maintaining the moisture content at 0 wt % moisture/ (wt dry algae) (black dot), 18 wt % moisture/ (wt dry algae) (green square), and 39 wt % moisture/

(wt dry algae) (green arrow head) produced a similar FAME yield. This indicates that water content in the algae does not necessarily reduce the FAME yield obtained. At 5 wt % moisture/ (wt dry algae) (red square) and 30 wt % moisture/ (wt dry algae) (blue triangle) the FAME yield increased linearly when methanol to oil molar ratio increased from 600:1 to 1277:1 indicating the process's endurance to moisture at this level increases as the excess of methanol to oil molar ratio rises. This effect is also shown at 18 wt % moisture/ (wt dry algae) (green square), where an increase in methanol to oil molar ratio from 369:1 to 938:1 increased the FAME yield due to water tolerance resulting from high methanol oil molar ratio. However, increasing methanol to oil molar ratio to 1507:1 did not result in additional increase in the FAME yield which shows that 1277:1 methanol to oil molar was the optimum for the range investigated.

For plot 4c, at 17 wt % moisture/ (wt dry algae) (green square), FAME yield at 1507:1 methanol to oil molar ratio (brown arrow head) was significantly greater than 369:1 methanol oil molar ratio (black dot) due to a high moisture tolerance produced by methanol oil molar ratio excess. It should be noted that during NaOH-catalysed reactive extraction of algae, other side reactions including saponification of FAME and triglycerides and neutralisation of free fatty acid occur alongside the desired FAME synthesis. At 1277:1 methanol to oil molar ratio (blue triangle), the FAME yield at 5 wt % moisture/ (wt dry algae) was greater than 600:1 methanol to oil molar ratio as expected due to increase in methanol oil molar ratio. However, increase in moisture contents to 30 wt % moisture/ (wt dry algae) reduced the FAME yield. This is because there was high FAME yield at 1277:1 methanol oil molar ratio and consequently rate of FAME losses due to saponification were also high.

Plots 4b and 4e (figure 4.18) show the interaction effect of moisture content and catalyst concentration on FAME yield. For plot 4b, at 0.188 N, similar FAME yield was obtained with 0 wt % moisture/ (wt dry algae) (black dot), 18 wt % moisture/ (wt dry algae) (green square), and 39 wt % moisture/ (wt dry algae) (green arrow head). This is because FAME losses due to saponification were not significant at these catalyst concentration and moisture contents. Increase in NaOH concentrations from 0.125 N to 0.250 N did not result in a significant change in FAME yield for both 5 wt % moisture/ (wt dry algae) (red square) and 30 wt % moisture/ (wt dry algae) (blue triangle). At 18 wt % moisture/ (wt dry algae) (green square), an increase in NaOH concentration from 0.082 to 0.188 N linearly increased FAME yield, indicating0.082 N was significantly low to affect a high FAME yield due to free fatty acid neutralisation of alkoxide species. It should be noted that the algae used contained free fatty acid (6 % lipid). As explained earlier in section 4.4.11 (previous page), free fatty acid neutralises NaOH and alkoxide species to form soap and methanol. This effect reduces the concentration of catalyst required to promote the transesterification of TG to FAME which explains why low FAME yield was obtained at 0.082 N NaOH. Further increase in NaOH concentration to 0.293 N reduced the FAME yield due to FAME losses by saponification.

For plot 4e, at 18 wt % moisture content/ (wt dry algae), the effect of FFA neutralisation is also shown as FAME yield at 0.082 N significantly less than 0.293 N (brown arrow head). There was no significant change in FAME yield with increase in moisture contents from 5-30 wt % moisture/ (wt dry algae) for both 0.125 N (red square) and 0.250 N (blue triangle). Increase in moisture contents from 0 to 39 wt . % moisture/ (wt dry algae) decreased the FAME yield at 0.188 N due to saponification.

Plots 4d and 4f (figure 4.18) show the interaction effect of methanol to oil molar ratio and catalyst concentration on FAME yield. For plot 4d, at 0.188 N, increase in methanol to oil molar ratio from 369:1 to 1507:1 increased the FAME yield as expected. This is because excesses in methanol to oil molar ratio increases alkoxide species formation. It also pushes the equilibrium to favour FAME production. Similarly, FAME yield at 1277:1 was greater than at 600:1 methanol to oil molar ratio due to this effect. At 938:1 methanol to oil molar ratio, increase in NaOH concentration from 0.082 to 0.188 N significantly increased the FAME yield. However, further increase in NaOH concentration to 0.292 N decreased FAME yield due to saponification.

For plot 4f, at 938:1 methanol to oil molar ratio, FAME yield increased with increase in catalyst concentration from 0.082 N (black dot) to 0.188 N (green square) due to more alkoxide species formation as explained earlier while FAME yield at 0.293 N (brown arrow head) was less than at 0.188 N (green square) due to FAME losses by saponification. At both 0.125 N (red square) and 0.250 N (blue triangle), increase in methanol to oil molar ratio from 600:1 to 1277:1 linearly increased the FAME yield. At 0.188N, increase in methanol to oil molar ratio from 369:1 to 938:1 significantly increased the FAME yield. This is because methanol to oil molar ratio excess pushes the equilibrium in favour of product formation as explained earlier. Further increase in the methanol to oil molar ratio to 1507:1 reduced the FAME yield because of higher rate of saponification.

Overall, an optimum 95 % FAME yield was obtained in 1 h at 5 % wt moisture/ (wt dry algae), using 0.125 N NaOH and 1277:1 methanol oil molar ratio.

4.6 Reactive extraction using H_2SO_4

The free fatty acid (FFA) content in the total lipids of *Nannochloropsis occulata* and *Chlorella vulgaris* were determined as 18.3±2.4 % and 6.1±0.3 %, respectively. The result of reactively extracted FAME yield from *Nannochloropsis occulata* and *Chlorella vulgaris* using H_2SO_4 catalyst is shown in figure 4.19. The maximum FAME yield was 57.5±3.6 % for *Chlorella vulgaris* and 53.8±8 % for *Nannochloropsis occulata*, occurring at 24 h.

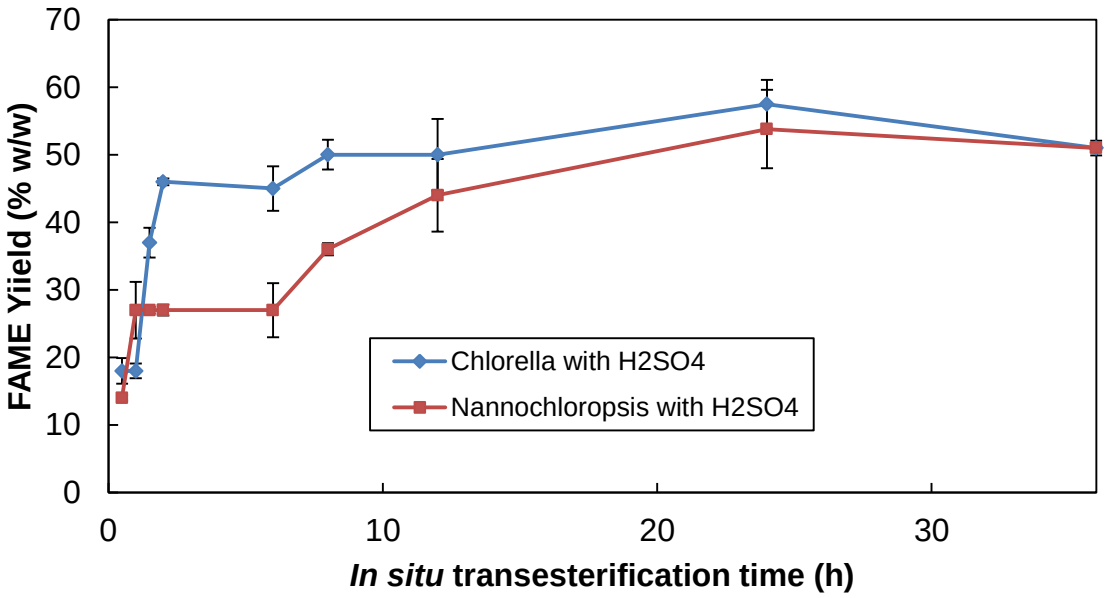


Figure 4.19: Reactively extracted FAME yield-time profile of *Nannochloropsis occulata* and *Chlorella vulgaris* using H_2SO_4 catalyst. Process conditions: 600:1 methanol to oil molar ratio, agitation rate: 450 rpm, temperature: 60 ^{o}C, mass of microalgae: 100 mg, 8.5:1 acid to oil molar ratio**.**

Clearly the initial rate of FAME production was significantly higher for *Chlorella vulgaris*, but there was no significant difference between the final transesterifiable lipid yields of the two species (p=0.400, t-test). The FAME-time profile seems to have two distinct stages, indicating two different, possibly sequential, processes. In *Chlorella vulgaris,* ~85 % of the final yield was achieved in the initial rapid

extraction/reaction stage, whereas in *Nannochloropsis occulata* only ~50% was produced during this time period. Perhaps this represents two different locations of transesterifiable matter such as. the internal oil bodies and the cell wall lipids.

Increasing the acid concentration from 0.087 to 0.15 µL/ (mg algae) caused a 17 % and 62 % increase in FAME yield for *Chlorella vulgaris* and *Nannochloropsis occulata*, respectively; 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, including hydrolysis of polysaccharides during acid-catalysed *in situ* transesterification. Its involvement in such reactions may necessitate higher acid concentrations to effect high FAME yields.

4.7 Reactive extraction using H_2SO_4/SDS

Sodium dodecyl sulphate (SDS) is a surfactant known for lysing cells to enhance extraction of intracellular components (Brown and Audet, 2008). The results of combining SDS with H_2SO_4 for *Nannochloropsis occulata* and *Chlorella vulgaris* are shown in figure 4.20.

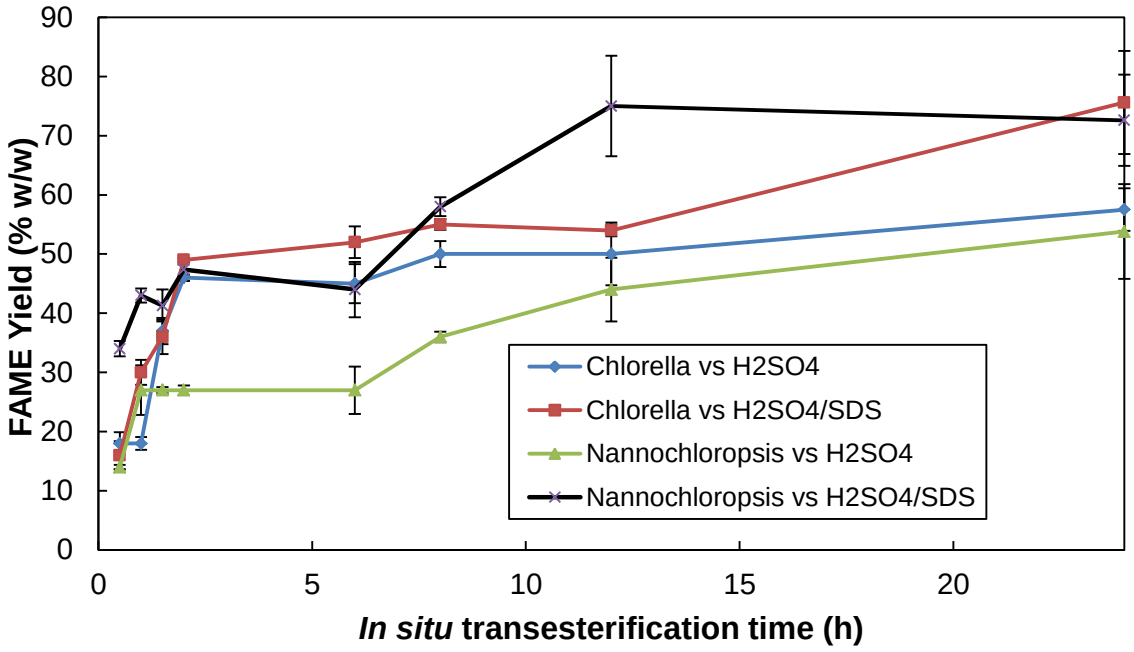


Figure 4.20: Reactively extracted FAME yield-time profile of *Nannochloropsis occulata* and *Chlorella vulgaris* using H_2SO_4/SDS vs H_2SO_4. Process conditions: 600:1 methanol to oil molar ratio, agitation rate: 450 rpm, temperature: 60 ^oC, mass of microalgae: 100 mg, 8.5:1 acid to oil molar ratio, 2:1 SDS to phospholipids molar ratio.

At 24 h, a 72.6 ± 7.7 % maximum FAME yield was obtained in *Nannochloropsis occulata*. In contrast, a 53.8 ± 8 % FAME yield was obtained in this species at the same duration with H_2SO_4 alone. In *Chlorella vulgaris*, at 24 h, a maximum FAME yield of 75.6 ± 8.7 % was obtained with SDS plus H_2SO_4 catalyst, whereas the FAME yield was 57.5 ± 3.6 % when using H_2SO_4 alone. Inclusion of cetyltrimethylammonium bromide (CTAB) (a surfactant) in NaOH has been shown to enhance the FAME yield for *in situ* ethanolysis of Jatropha curcas L (Hailegiorgis *et al*., 2011), although it is difficult to ascribe this to the surfactant, given the difference in catalyst.

4.8 Reactive extraction with surfactant catalyst ("ZDS") vs H2SO4

The performance of a synthesized "surfactant catalyst" (zirconium dodecyl sulphate, or "ZDS") for FAME production from *Nannochloropsis occulata* and *Chlorella vulgaris* was investigated. The resultant FAME yield-time profiles are shown in figure 4.21.

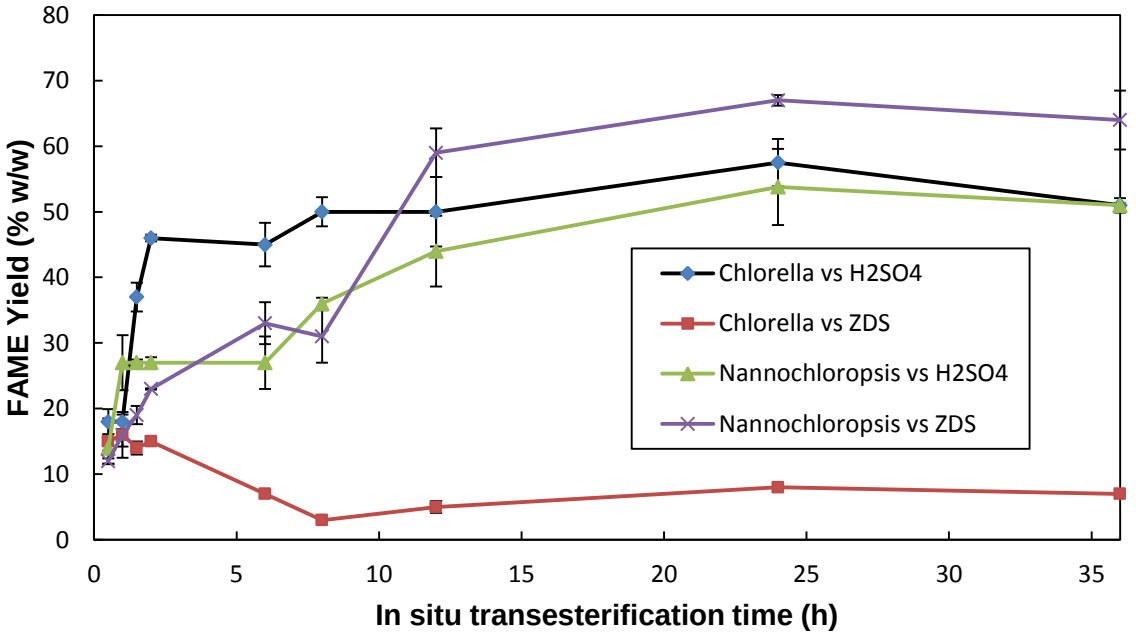


Figure 4.21: Reactively extracted FAME yield-time profile of *Nannochloropsis occulata* and *Chlorella vulgaris* using ZDS vs H_2SO_4.Process conditions: 600:1 methanol to oil molar ratio, agitation rate: 450 rpm, temperature: 60 ^{o}C, mass of microalgae: 100 mg, 8.5:1 acid to oil molar ratio, 0.8:1 ZDS to oil molar ratio.

In *Nannochloropsis occulata*, there was no significant change in the rate of FAME production early in the reaction (0.5-10 h). However, between 12 and 36 h, the FAME production rate with the ZDS catalyst was greater than when using H_2SO_4 alone. This is probably because ZDS disrupted the cell wall of *Nannochloropsis occulata* more than H_2SO_4. The evidence for this is the increased chlorophyll production (see table 4.7). Thus it increased the methanol and catalyst's access to lipid bodies, which enhanced the FAME production rate.

In *Chlorella vulgaris,* it is clear that the FAME production rate when using H_2SO_4 was significantly greater than that of ZDS at each data point. The varying effect of ZDS on the *Nannochloropsis occulata and Chlorella vulgaris* could be due to cell wall chemistry differences. Therefore, more measurements should be directed to detect the compounds producing FAME yield changes. For example, the effect of cell wall algaenans and sporopollenin (non-hydrolysable macromolecule), should be further investigated.

4.8.1 *Maximum FAME produced from acid/surfactant based catalyst*

Table 4.6 summarises the maximum FAME yields obtained from both species using different catalysts. It should be noted that H_2SO_4 is used as the reference for comparison with other catalysts since it is the most commonly used catalyst for reactive extraction of microalgae for FAME production.

Table 4.6: Maximum FAME yields from *Nannochloropsis occulata* and *Chlorella vulgaris* with different catalysts.

Microalgae species	Catalyst type	Catalyst to oil molar ratio	FAME yield % (w/w)
Nannochloropsis occulata	H_2SO_4	15:1	87±2
Nannochloropsis occulata	H_2SO_4	8.5:1	53.8±8
Nannochloropsis occulata	SDS + H_2SO_4	8.5:1	72.6±7.7
Nannochloropsis occulata	ZDS	0.8:1	67±1
Chlorella vulgaris	H_2SO_4	15:1	67±1
Chlorella vulgaris	H_2SO_4	8.5:1	57.5±3.6
Chlorella vulgaris	SDS + H_2SO_4	8.5:1	75.6±8.7

Process conditions: 600:1 methanol to oil molar ratio**,** agitation rate: 450 rpm, temperature: 60 ^{o}C, mass of microalgae: 100 mg, 2:1 SDS to phospholipids molar ratio, reaction time: 24 h.

8.5:1 and 15:1 H_2SO_4 to oil molar ratios are equivalent to 0.326 mmol H^+ and 0.578 mmol H^+, respectively. Increasing the acid to oil molar ratio from 8.5:1 to 15:1 H_2SO_4 resulted in increased FAME production rates in both species. The maximum FAME yield produced at 15:1 H_2SO_4 to oil molar ratio was greater than that produced by 0.8: 1 ZDS to oil molar ratio. However, 0.8: 1 ZDS to oil molar ratio used was equivalent to 0.0624 mmol H^+ indicating that ZDS is more efficient on a mol for mol basis than H_2SO_4, particularly for *Nannochloropsis occulata.*

4.8.2 *FAME enhancement by surfactant-based catalyst*

The difference in the FAME production by the catalysts is explained in terms of the chlorophyll extracts after the reactive extraction of the different catalysts, as shown in table 4.7.

Table 4.7: Extract chlorophyll content used for quantifying cell disruption.

Microalgae	Catalyst	Total chlorophyll (µg)	Statistical analysis (p value, *t* tests)
Chlorella vulgaris	Acid	1.6±0.2[a]	0.28
Chlorella vulgaris	Acid + SDS	1.1±0.6[a]	
Chlorella vulgaris	Acid	1.6±0.2[a]	0.04
Chlorella vulgaris	ZDS	0.6±0.2[a]	
Nannochloropsis occulata	Acid	1.3±0.05[b]	0.36
Nannochloropsis occulata	Acid + SDS	1.3±0.09[b]	
Nannochloropsis occulata	Acid	1.3±0.05[b]	0.08
Nannochloropsis occulata	ZDS	1.4±0.1[b]	

[a] Total chlorophyll A+B; [b] Total chlorophyll A+C. Process conditions: 600:1 methanol to oil molar ratio**,** agitation rate: 450 rpm, temperature: 32 ^{o}C, mass of microalgae: 100 mg, 8.5:1 acid to oil molar ratio, 2:1 SDS to phospholipids molar ratio, 0.8:1 ZDS to oil molar ratio.

Chlorophyll concentration has been positively correlated with cell wall disruption (Gerde *et al.*, 2012). Based on this measurement, in *Nannochloropsis occulata* and *Chlorella vulgaris,* there was no significant difference in cell wall disruption between H_2SO_4 and H_2SO_4/SDS even though there was a significant difference between the FAME yields (see table 4.6).

A low chlorophyll extract was observed when using ZDS in *Chlorella vulgaris*. However, in *Nannochloropsis occulata,* the highest chlorophyll extract was produced when using ZDS. Clearly, ZDS disrupts *Nannochloropsis occulata's* cell walls more than that of *Chlorella vulgaris,* which explains why it produced a FAME yield of 67±1 % in this species, but only 22±2 % in *Chlorella vulgaris*

4.8.3 *Effect of inclusion of SDS in H_2SO_4 on water tolerance*

Acid-catalysed direct transesterification has been shown to exhibit higher water tolerance to microalgae-bound water (Velasquez-Orta *et al*., 2013) and free water (Wahlen *et al*., 2011) than conventional transesterification. However, the cause of this higher water tolerance, besides the higher methanol to oil molar ratio used in reactive extractions is still not completely clear, particularly for microalgae. Similarly, the effect of surfactant inclusion in H_2SO_4 on water tolerance has not been reported. In order to investigate the level of water tolerance of H_2SO_4, with and without SDS (a surfactant), samples with 10 wt %, 20 wt % and 30 wt % moisture/ (wt dry algae) were prepared and allowed to equilibrate for 1 h.

For the *Chlorella vulgaris* using H_2SO_4 catalyst, the FAME yield rose to maximum (80.5 %) at 20 wt % moisture/ (wt dry algae). However, there was no significant difference with 0 wt % moisture ($p= 0.114$, t-test). Then the FAME yield began to decrease at 30 wt % moisture/ (wt dry algae). The same trend was obtained with SDS/H_2SO_4 with the FAME yield reaching maximum (92.2 %) at 20 wt % moisture/ (wt dry algae) and there was no significant difference when compared with 0 wt % moisture/ (wt dry algae) ($p=0.246$, t-test). The FAME yield started declining at this condition at 30 wt % moisture/ (wt dry algae) content as shown in figure 4.22.

Similarly for *Nannochloropsis occulata* using H_2SO_4 the FAME yield rose to maximum (78.6 %) at 10 wt % moisture/ (wt dry algae) though there was no significant difference with 0 wt % moisture/ (wt dry algae) (p=0.087, t-test). The FAME yield started declining at 20 wt % moisture/ (wt dry algae). The same increase in FAME yield was obtained with SDS/H_2SO_4 reaching a maximum (98.3 %) at 20 wt% moisture/ (wt dry algae) though there was no significant difference with 0 wt % moisture/ (wt dry algae) (P=0.077, t-test). The FAME yield decreased at 30 wt % moisture/ (wt dry algae) as shown in figure 4.23. Generally, the moisture tolerant obtained was significantly greater than (<0.5 wt % moisture/ (wt oil)) required for conventional biodiesel production.

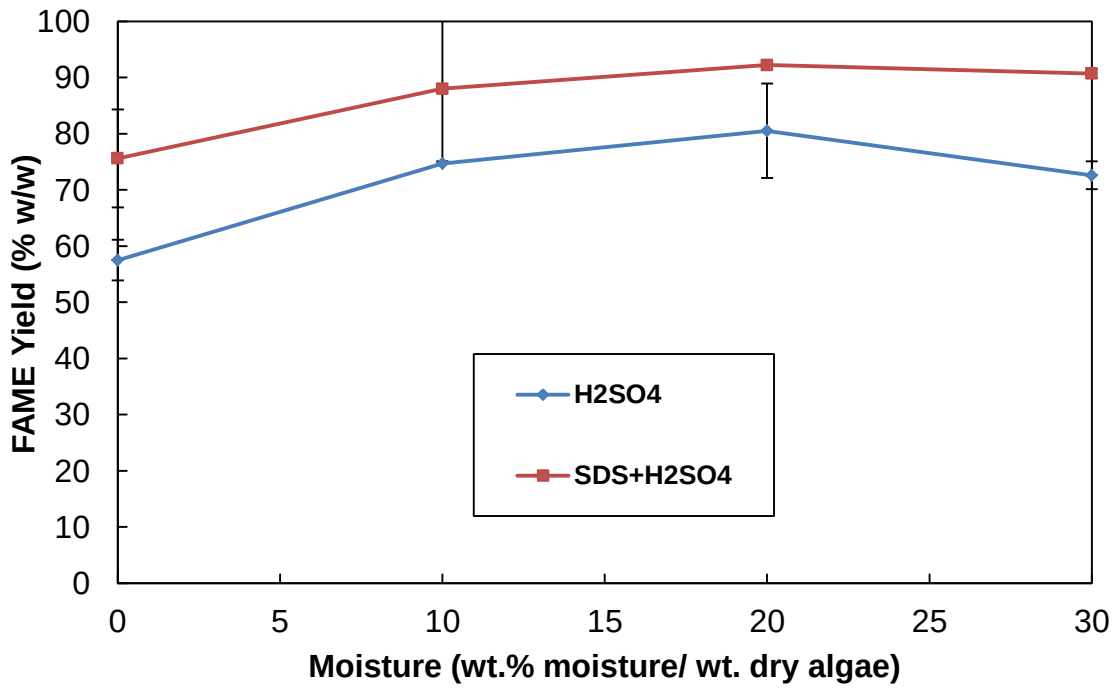


Figure 4.22: Reactively extracted FAME yield-time profile for wet *Chlorella vulgaris* using H_2SO_4 or H_2SO_4/SDS. Process conditions: 600:1 methanol to oil molar ratio, agitation rate: 450 rpm, temperature: 60 ^oC, 24 h, mass of microalgae: 100 mg, 8.5:1 acid to oil molar ratio, 2:1 SDS to phospholipids molar ratio.

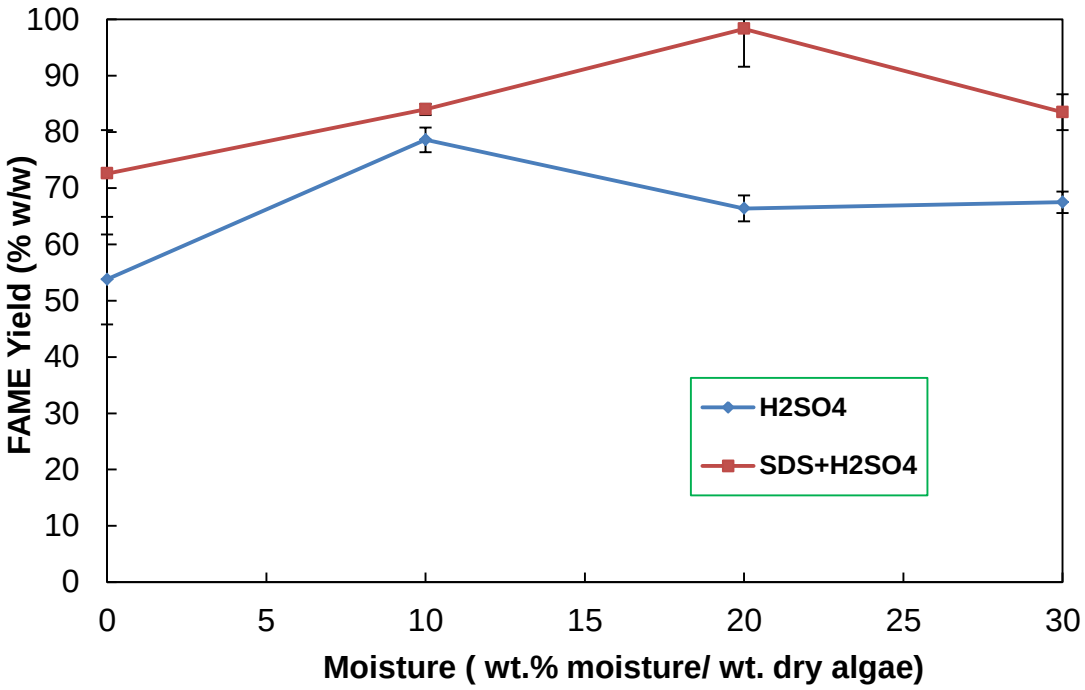


Figure 4.23: Reactively extracted FAME yield-time profile for wet *Nannochloropsis occulata* using H_2SO_4 vs H_2SO_4/SDS. Process conditions: 600:1 methanol to oil molar ratio, agitation rate: 450 rpm, temperature: 60 ^oC, 24 h, mass of microalgae: 100 mg, 8.5:1 acid to oil molar ratio, 2:1 SDS to phospholipids molar ratio.

The high methanol to oil molar ratio used in reactive extraction is one reason for its higher water tolerance. However, there are other possible reasons for the water tolerance particularly for microalgae which are explained here. Cell wall lipids, such as phospholipids and glycolipids may be disrupted to some degree by polar organic solvents such as methanol, ethanol, other alcohols and water (Cohen, 1999). However, the poor permeability of these solvents into the cells of completely dry oil-bearing biomass can significantly reduce their lipid extraction efficiency (Cohen, 1999). 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 phospholipids from microalgal cell walls compromises their integrity. In addition, the interaction of water and methanol with cell wall proteins could compromise their integrity. The observed enhancement could be some combination of these two effects and the swelling effect. Therefore, the observed water tolerance in re-hydrated microalgae was probably due to increased lipid extraction by moist methanol. This finding is a significant contribution to the fund of knowledge as it could be a key method of increasing the FAME yield in reactive extraction ("*in situ* transesterification"). However, after 20 wt % moisture/ (wt dry algae), a decline in the FAME yield was observed, which shows that the water tolerance has been exceeded for both catalysts. The amount of water tolerance achieved here is greater than 10 wt % moisture/ (wt dry algae) 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 here is lower than the 50 wt % moisture/ (wt dry algae) of free water during acid-catalysed *in situ* esterification of *C. gracilius* reported by Wahlen *et al*. (2011). However, it should be noted that Wahlen *et al.* (2011), used a higher methanol volume, 0.04 mL/ (mg algae), which was significantly higher than the 0.0047 mL/ (mg algae) used in this study and the microalgae are different.

4.9 Pre-soaking for pre-treating micro-algae

Pre-soaking is a chemical pre-treatment for solubilising micro-algal phospholipid bilayer. It involves allowing solvent such as methanol to percolate through the micro-algal biomass. In order to address molar excesses of methanol required during reactive extraction and to reduce the usage of high acid concentration to achieve

high FAME yield, a pre-soaking pre-treatment was conducted for both species. The amount of phospholipids in the algal extracts and residues were determined by correlating it positively to phosphorus content. The effect of pre-soaking on 1.) FAME yield, 2.) Acid concentration and 3.) Methanol to oil molar ratio was studied using full factorial design.

4.9.1 *Effect of pre-soaking on methanol molar excess and acid concentration for Nannochloropsis occulata*

Figure 4.24 shows the effect of pre-soaking *Nannochloropsis occulata* in methanol prior to acid-catalysed reactive extraction on the FAME yield, the methanol to oil molar ratio and the catalyst concentration.

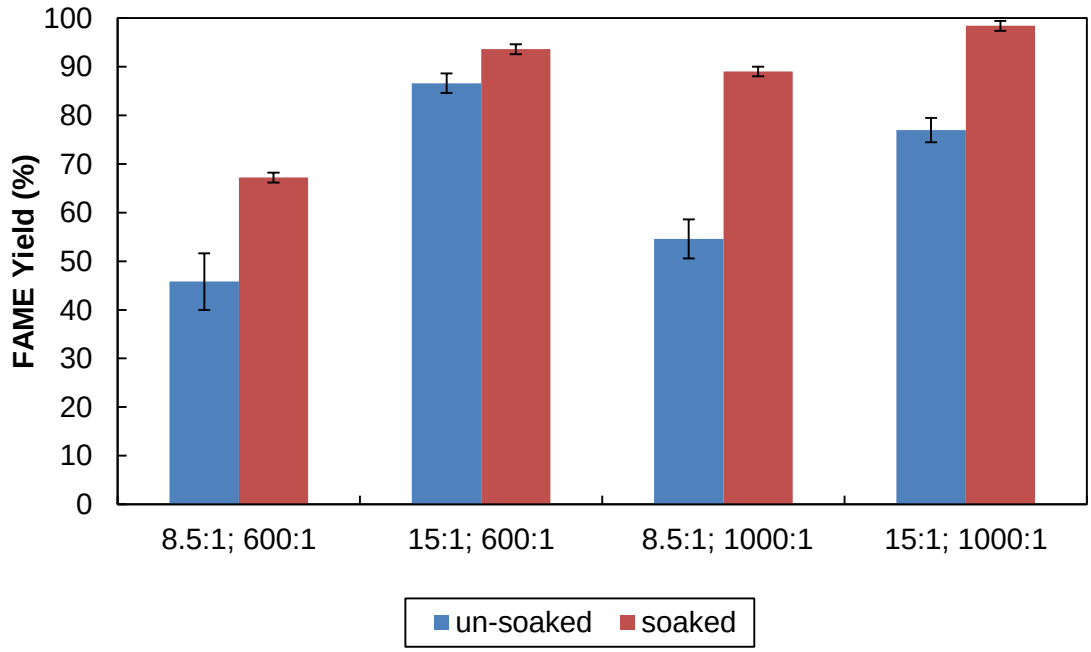


Figure 4.24: Effect of pre-soaking on reactively extracted FAME yield from *Chlorella vulgaris*. Process conditions: Pre-soaking time: 14.5 h, agitation rate: 450 rpm, temperature: 60 ^{o}C, reaction time**:** 24 h, mass of *Chlorella vulgaris*: 100 mg. Methanol to oil molar ratio: 600:1; 1000:1. H_2SO_4 to oil molar ratio: 8.5:1; 15:1.

The figure shows that the FAME yield obtained for the pre-soaked microalgae at the two tested methanol to oil molar ratios and acid concentrations was greater than that for the un-soaked microalgae. It is notable that a 67.2 ± 0.9 % FAME yield was obtained for the pre-soaked micro-algae at a 600:1 methanol to oil molar ratio, which was greater than the 54.6 ± 4 % FAME yield obtained for the un-soaked microalgae at 1000:1 methanol to oil molar ratio. This resulted in a 42 % reduction in the methanol to oil molar ratio. Similarly, an 89 ± 2.5 % FAME yield was obtained for pre-soaked microalgae at 8.7:1 H_2SO_4 to oil molar ratio, which was greater than the 77 % 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 requires less energy than other algal cell wall disruptions, including autoclaving and microwave irradiation. It can also be more easily scaled up than bead-beating and sonication. This is the first report of pre-soaking with methanol and acid catalysis. Figure 4.26 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 was less significant ($p=0.018$) than the acid concentration ($p=0.000$) and pre-soaking time ($p=0.000$). Figure 4.27 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 ($p=0.915$).

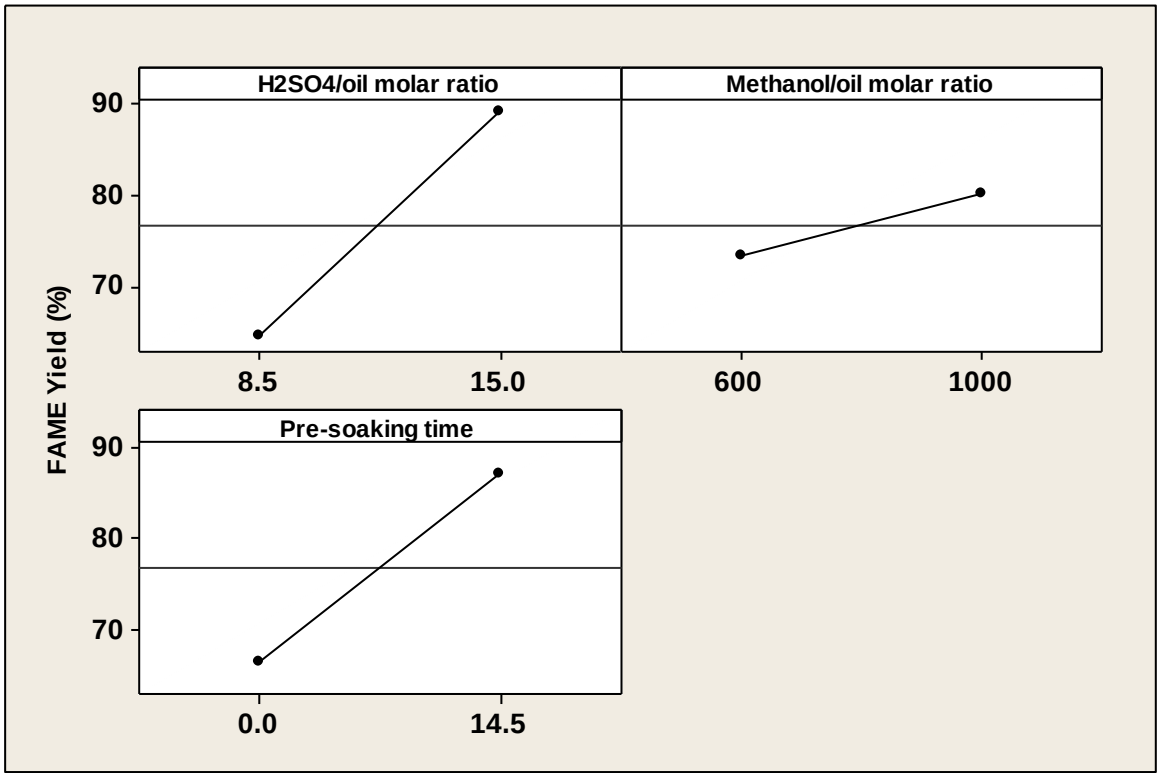


Figure 4.25: Main effect of pre-soaking time, methanol to oil molar ratio, acid concentration on FAME yield for *Nannochloropsis occulata.* Process conditions: pre-soaking time: 14.5 h, agitation rate: 450 rpm, temperature: 60 ^{o}C, reaction time: 24 h, mass of *Nannochloropsis occulata*: 100 mg. Data shown are mean values of duplicate experiments.

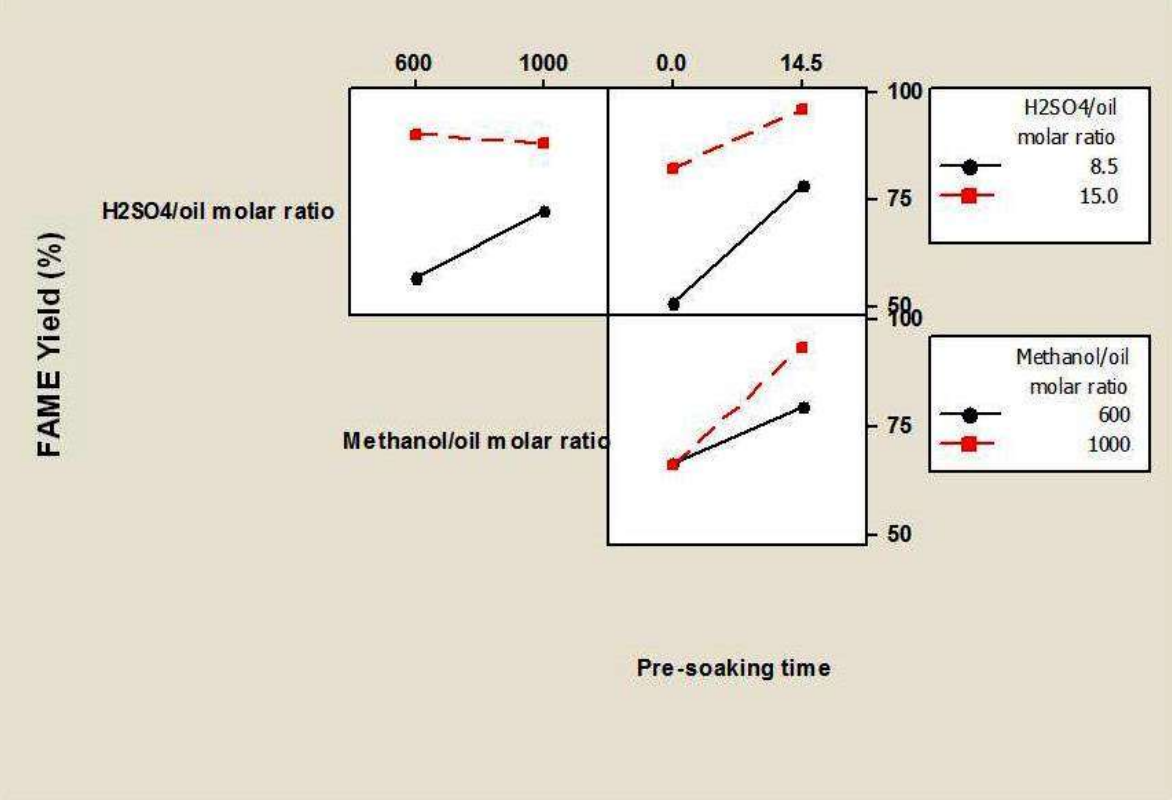


Figure 4.26: Interaction effect plot of pre-soaking time, acid concentration, methanol to oil molar ratio on FAME yield for *Nannochloropsis occulata*. Process conditions: Pre-soaking time: 14.5 h, agitation rate: 450 rpm, temperature: 60 ^{o}C, reaction time**:** 24 h, mass of *Nannochloropsis occulata*: 100 mg. Data shown are mean values of duplicate of experiments.

4.9.2 *Effect of pre-soaking on methanol molar excess and acid concentration for Chlorella vulgaris*

In contrast to *Nannochloropsis occulata*, pre-soaking *Chlorella vulgaris* prior to the acid-catalysed, reactive extraction increased the fatty acid methyl ester (FAME) yield only at a low acid concentration and high methanol to oil molar ratio. As can be seen in figure 4.27, at an 8.7:1 H_2SO_4 to oil molar ratio and 1000:1 methanol to oil molar ratio, the FAME yield of the pre-soaked microalgae (81.9 ± 0.9 %) was significantly greater than that for the un-soaked (68.9 ± 5.5 %). Pre-soaking resulted in an 18.9 % increase in FAME yield. However, as shown in figure 4.28, the methanol to oil molar ratio (p=0.000) and acid concentration (p=0.000) produced more significant

increases in the FAME yield in *Chlorella vulgaris* than the pre-soaking time (p=0.095). The varying effect of pre-soaking on the *Nannochloropsis occulata and Chlorella vulgaris* is certainly due to cell chemistry differences. Lee *et al*. (2010) also reported that *Botryococcus sp*., *Chlorella vulgaris* and *Scenedesmus sp*. cell walls disrupted differently despite the same pre-treatment.

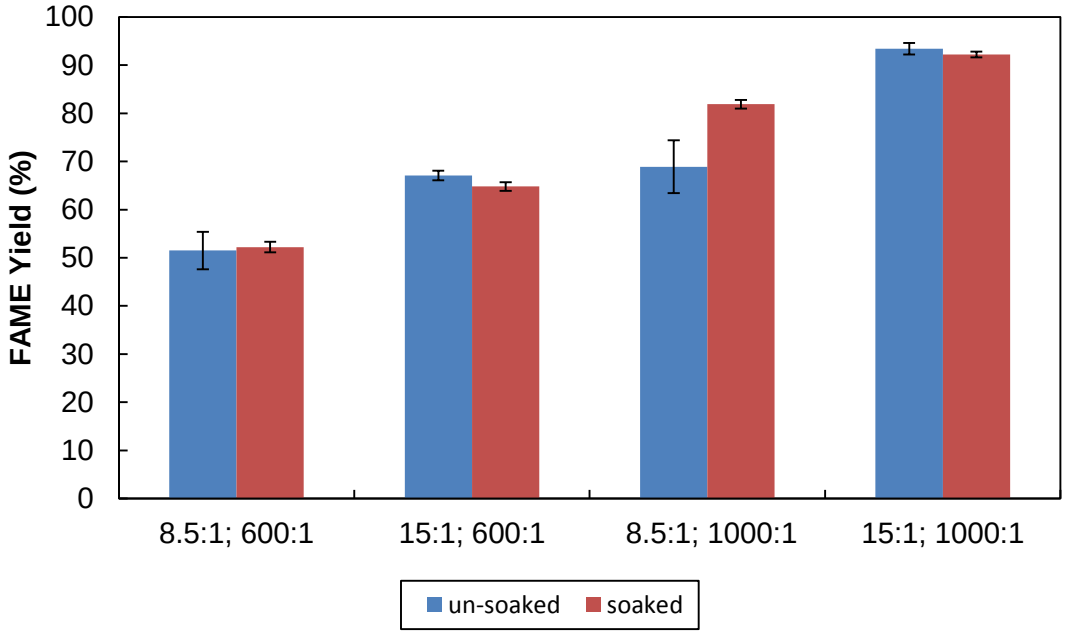


Figure 4.27: Effect of pre-soaking on FAME yield produced from *Chlorella vulgaris.* Process conditions: Pre-soaking time: 14.5 h, agitation rate: 450 rpm, temperature: 60 ^{o}C, reaction time**:** 24 h, mass of *Chlorella vulgaris*: 100 mg. Methanol to oil molar ratio: 600:1; 1000:1. H_2SO_4 to oil molar ratio: 8.5:1; 15:1

Figure 4.29 shows that the interactions 1) acid concentration and pre-soaking (p=0.011); and 2) methanol to oil molar ratio and pre-soaking time (p= 0.028), have significant effects on the FAME yield, while interaction 3) acid concentration and methanol to oil molar ratio (p=0.174), and the three way interaction (p=0.070) have no significant effect on the FAME yield. It has been previously reported that an increase in acid catalyst concentration increases FAME yield during reactive extraction (Wahlen *et al.*, 2011; Velasquez-Orta *et al*., 2013; El-Shimi *et al.,* 2013).

As can be seen in figure 4.24 and 4.27, an increase in methanol oil molar ratio also increases the FAME yield because it shifts the equilibrium towards the product side.

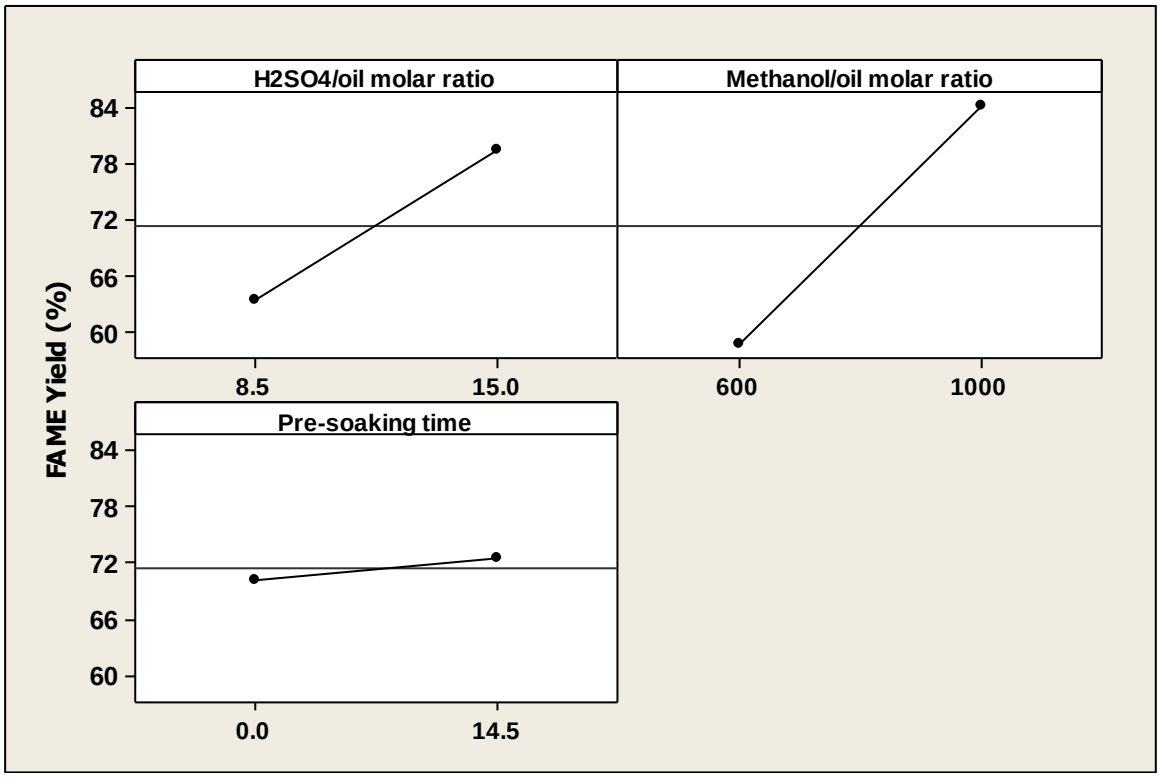


Figure 4.28: Main effect plot of pre-soaking time, methanol to oil molar ratio, acid concentration on FAME yield for *Chlorella vulgaris*. Process conditions: Pre-soaking time: 14.5 h, agitation rate: 450 rpm, temperature: 60 ^{o}C, reaction time**:** 24 h, mass of *Chlorella vulgaris*: 100 mg. Data shown are mean values of duplicate experiments.

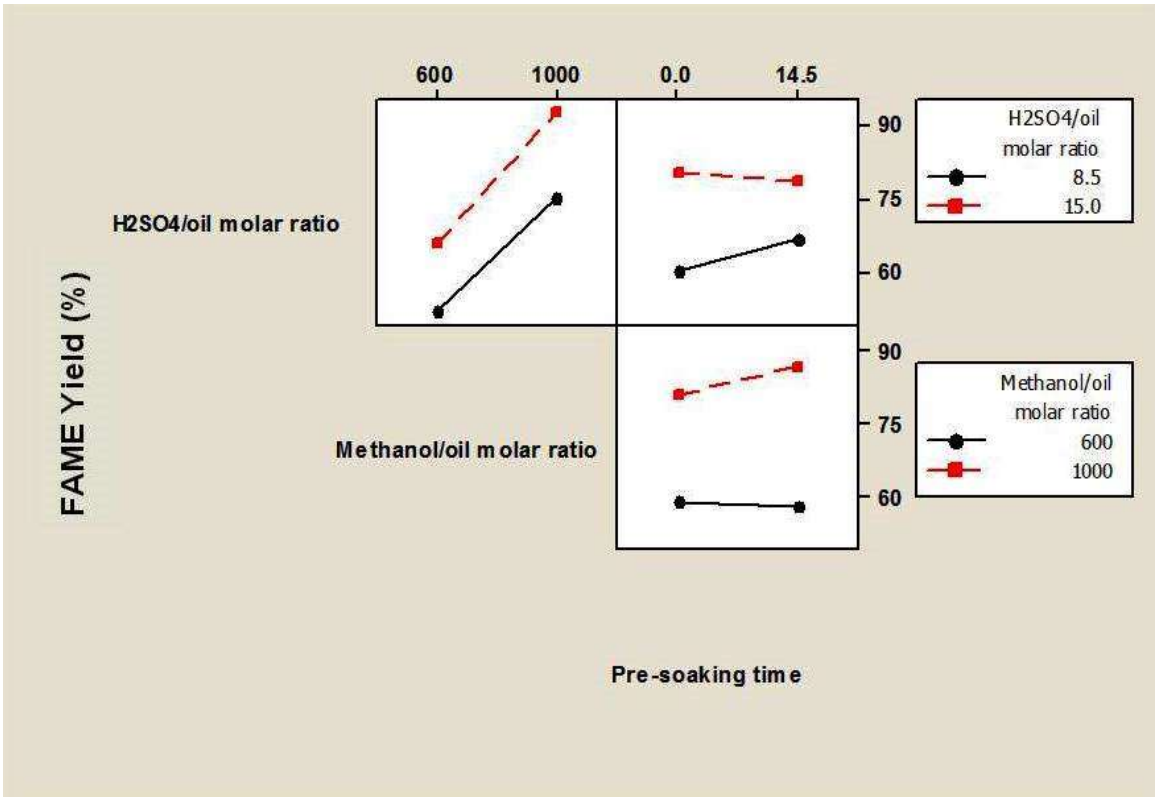


Figure 4.29: Interaction effect plot of pre-soaking time, methanol to oil molar ratio, acid concentration on FAME yield for *Chlorella vulgaris*. Process conditions: Pre-soaking time: 14.5 h, agitation rate: 450 rpm, temperature: 60 ^{o}C, reaction time**:** 24 h, mass of *Chlorella vulgaris*: 100 mg. Data shown are mean values of duplicate experiments.

4.9.3 *Phosphorus mass balance*

The increase in FAME yield achieved by pre-soaking the microalgae was also explained by solubilisation of phospholipids in methanol as shown in figure 4.30. Phosphorus content was positively correlated with the phospholipids of each sample. Figure 4.30 shows the differences in phospholipid content between the different phases:

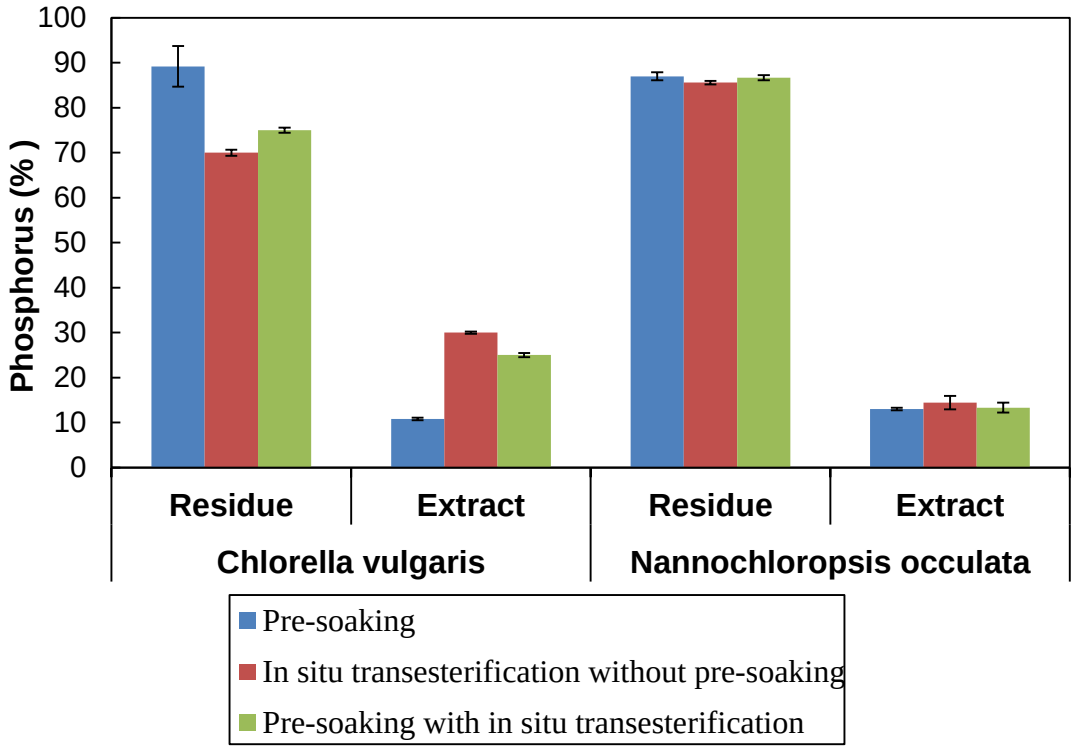


Figure 4.30: Phospohorus content of algal residue and extract after pre-soaking compared with initial value. Pre-soaking time: 14.5 h, pre–soaking agitation rate: 300 rpm, reaction agitation: 450 rpm, reaction temperature: 60 ^{o}C, reaction time: 24 h, methanol to oil molar ratio: 600:1, acid to oil molar ratio: 8.7:1, mass of microalgae: 100 mg.

A substantial portion of the phosphorus 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. For *Nannochloropsis occulata,* phosphorous solubilization was caused by pre-soaking in methanol, as subjecting the biomass to subsequent *in situ* transesterification led to no further reduction. This shows that its equilibrium dissolution has been attained. In contrast, a further reduction in the phosphorus of the residue was observed with in *situ* transesterification of *Chlorella vulgaris.* 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 loosens the triglycerides from the cellular matrix, which results in an increased FAME

conversion rate. This could explain why pre-soaking caused FAME enhancement in both species when using a 1000:1 methanol to oil molar ratio, as seen in figures 4.24 and 4.27.

4.9.4 *Minimum pre-soaking time needed using Nannochloropsis occulata*

The pre-soaking time used in the experiments was 14.5 h to ensure a long mass transfer completion time. In order to compare the energy use of pre-soaking with other pre-treatment processes found in the literature, an optimization was conducted by trying to see what would be the minimum residence time needed for the pre-soaking. Figure 4.31 shows that after 1 h, the same amount of phosphorus 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.

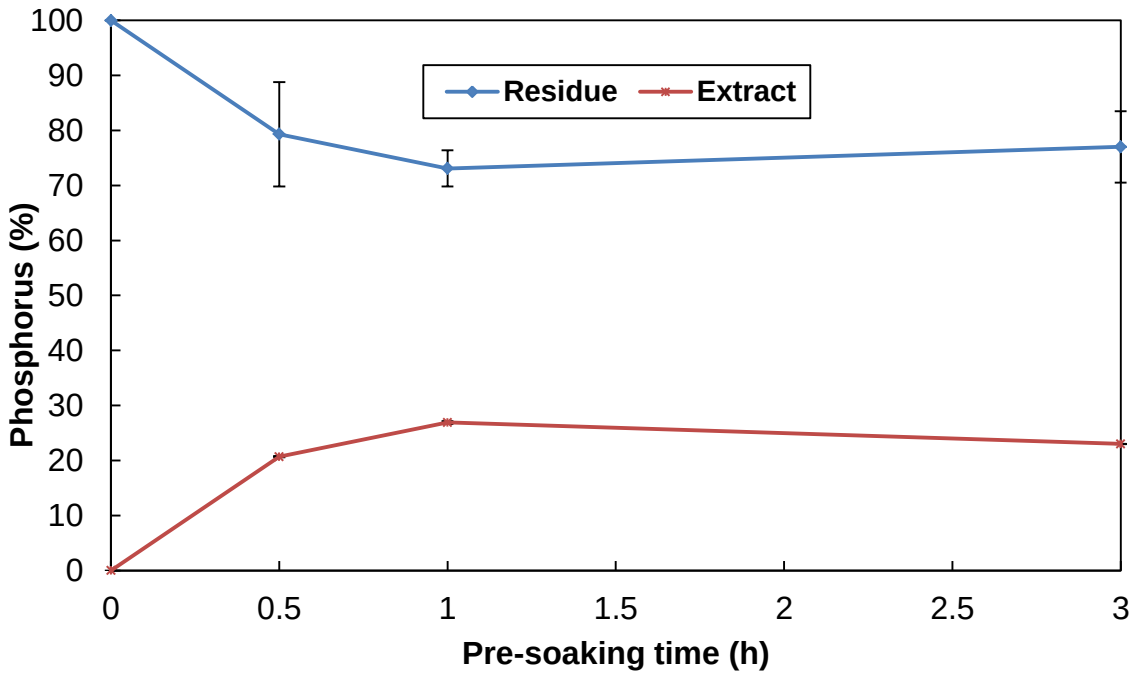


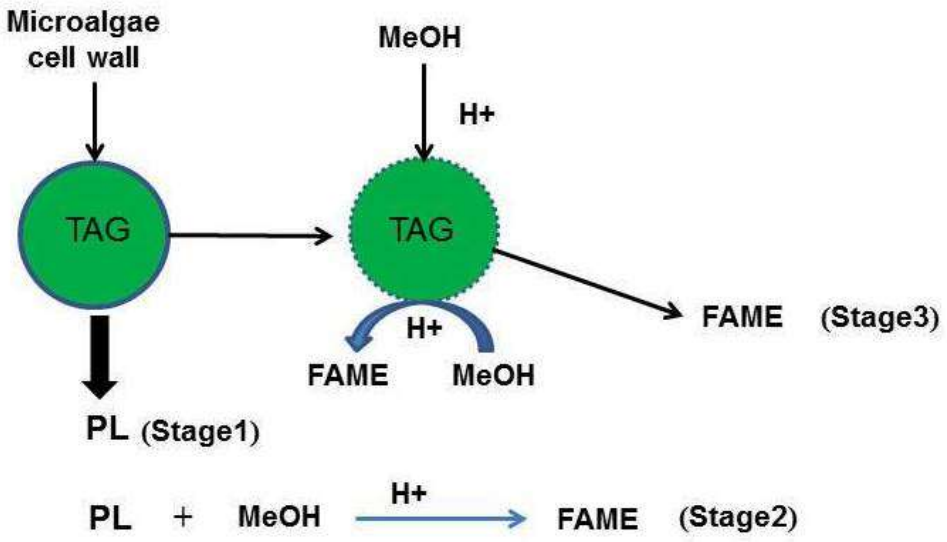
Figure 4.31: Phosphorus-time profile of algal extract and residue after pre-soaking. Process condition: Agitation rate: 300 rpm, temperature: 20 ^{o}C, mass of *Nannochloropsis occulata*: 100 mg.

4.9.5 *Evaluation of phospholipids for FAME production*

The isolated microalgal phospholipids were evaluated for FAME production by transesterifying them under the same conditions as the reactive extraction. The results show that 9.6±1 % and 26.6±2 % phospholipids respectively for *Nannochloropsis occulata* and *Chlorella vulgaris* converted into FAME. This shows that microalgae cell wall lipids (membrane lipids) convert into FAME during reactive extraction. This result empirically explains why Wahlen *et al*., (2011) obtained significantly more biodiesel than would be expected from the conversion of triglycerides alone during acid-catalysed *in situ* transesterification of microalgae, cyanobacteria and wild mixed-cultures. It also explains why a greater FAME yield was obtained from *in situ* transesterification than from the conventional two-step transesterification of pre-extracted oil (Lepage and Roy, 1984& 1986; Vicente *et al*., 2009).

4.9.6 *Proposed mechanism for pre-soaked micro-algae undergoing reactive extraction*

Pre-soaking microalgae in methanol prior to acid catalysed reactive extraction solubilises the phospholipid bilayer to some degree. This can be seen clearly from the phosphorus mass balance (figure 4.30 and 4.31). Though a significant amount of phosphorus remained in the residue, the phosphorus removed from the two species was largely due to pre-soaking. The removal of the phosphorus (phospholipids) from the microalgal cell wall could certainly compromise its integrity. This loosens 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 4.32.



- Stage 1 (Pre-soaking) : **PL** solubilised into methanol
- Stage 2: **PL** in the cell wall and bulk fluid convert into **FAME**
- Stage 3: TAG converts into FAME

Figure 4.32: Proposed scheme for pre-soaking microalgae undergoing reactive extraction.FAME: Fatty acid methyl ester, PL: Phospholipids, TAG: Triacylglycerides, MeOH: methanol**.**

4.10 Residual protein and carbohydrate as value added co-products

Before the reactive extraction both microalgae were characterised in terms of protein and carbohydrate. Initial protein and carbohydrate content of *Nannochloropsis occulata* were determined respectively as 30±0.07 % and 26±2.8 %. The initial protein of *Chlorella vulgaris* was determined as 46±0.07 % protein while its carbohydrate was 35±3.5 %. After the reactive extraction, the protein and carbohydrate contents of both residual algal biomass were measured at the maximum FAME yield as shown in figure 4.33.

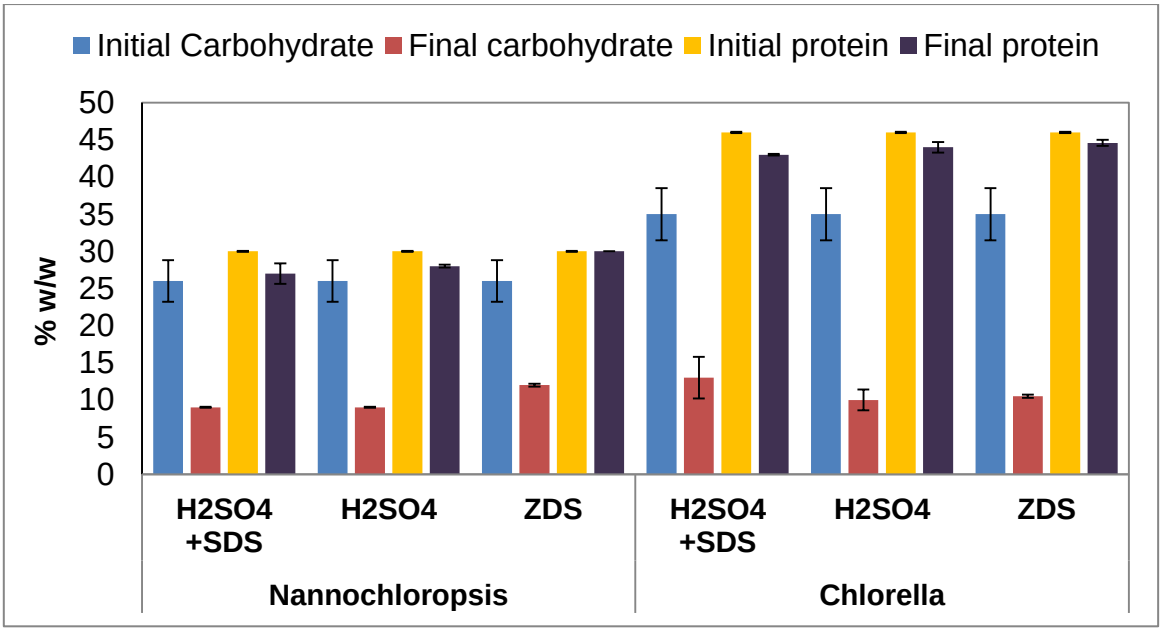


Figure 4.33: Carbohydrate and protein content of *Nannochloropsis occulata* and *Chlorella vulgaris* before and after reactive extraction.

It can be seen that protein was retained at all conditions, and carbohydrate significantly reduced. This implies that a substantial portion of the carbohydrate was hydrolysed to simple sugars or other associated products that dissolved in water/methanol mixture. The protein retention is probably desirable, as it means that the residue can be utilised for animal feed supplements (if no toxic compounds are present), thereby improving the economics of biodiesel production by this reactive extraction route. The carbohydrate remained in the residue and, if separable from methanol, could provide a liquid feed to a bioethanol plant, which could substantially improve the process economy of the reactive extraction of microalgae for FAME production.

Chapter 5. Conclusion and further work

5.1 Conclusions

The aim of this research project was to study the production of biodiesel from *Nannochloropsis occulata* and *Chlorella vulgaris* by direct "reactive extraction" using a range of catalysts. Generally, it was found that this process can be used to produce biodiesel from both species using NaOH, H_2SO_4, zirconium dodecyl sulphate ("ZDS") or H_2SO_4/SDS (a surfactant), although rates and yields vary.

NaOH was the most effective catalyst producing high FAME yield (96 %) at the lowest reaction time (10 min) despite high levels of free fatty acid (6 %) in *Chlorella vulgaris*. In a conventional transesterification, this level of free fatty acid would necessitate usage of an acid catalyst or a two-stage "trans/esterification" (acid catalyst follows by alkali catalyst). This means higher operating costs would be incurred to produce biodiesel from such substrate using alkali-catalysed conventional transesterification.

Increasing the NaOH catalyst concentration from 0.125 N to 0.5 N NaOH at 925:1 methanol to oil molar ratio increased the FAME yield from 78 to 96 % due to significant formation of methoxide (the actual catalyst). The maximum FAME yield occurred after 10 min with 0.5N, 925:1 methanol to oil molar ratio. However, further increase in reaction time reduced the FAME yield significantly due to undesired FAME losses from saponification. A numerical model derived by Eze *et al.* (2014) was used to fit the FAME yield of the NaOH-catalysed reactive extraction for both dry and wet *Chlorella vulgaris* obtained from this studies. The FAME yields vs time trends obtained generally support the theory that other side reactions including FAME and triglycerides saponification, free fatty acid neutralisation occur alongside

the desire FAME synthesis. The NaOH-catalysed reactive extraction of *Chlorella vulgaris* tolerates moisture between 5-20 wt % moisture/ (wt dry algae) which is greater than (< 0.5 wt %/ (wt oil)) required by the conventional transesterification. The moisture tolerance depends on the reaction time, methanol to oil molar ratio and NaOH concentration. At 30 wt % moisture/ (wt dry algae), 600:1 methanol: oil molar ratio, 0.2N, the FAME yield reduced after 30 min reaction time. Increase in methanol to oil molar ratio to 1277:1 increased the moisture tolerance to 30 wt % moisture/ (wt dry algae).

Zirconium dodecyl sulphate ("ZDS"), a Lewis/surfactant catalyst, was successfully synthesised. Characterisation of the catalyst using FTIR confirmed that the compound synthesised was ZDS. The surfactant catalyst caused significantly higher increases in FAME yields in *Nannochloropsis occulata* (67±1 %) than in *Chlorella vulgaris* (22±3 %). Differences in the activity of the ZDS in both species could be due to their varying cell wall chemistry.

Inclusion of sodium dodecyl sulphate (SDS) in H_2SO_4 enhanced the FAME yield from both species and causes some level of water tolerance in both. Addition of SDS in H_2SO_4 at 20 wt % moisture/ (wt dry algae) in the microalgae produced a maximum FAME yield of 98.3±6.7 % in *Nannochloropsis occulata* and 92.2±0.8 % in *Chlorella vulgaris.* Additionally, not only is the process more tolerant to water than transesterification-based routes, but the presence of a small quantity of external water increases the FAME yields in the reactive extraction, rather than inhibiting the reaction. This effect was apparent for all conditions up to 20-30 wt % moisture/ (wt dry algae), and should substantially improve the economics of this process, as the energy required for drying algae to the conditions required for conventional biodiesel

production (<0.5 wt % moisture/(wt oil)) is a substantial obstacle to economic operation.

The residual biomass, regardless of the catalyst and/or surfactant, maintains the initial protein, indicating that the residue could be a valuable animal feed co-product. Carbohydrate in the microalgal residue decreased significantly in all cases possibly due to its hydrolysis by the catalysts.

Pre-soaking as a pre-treatment solubilises the phospholipid bilayer to some degree, and results in greater enhancement in reactively extracted biodiesel yield in *Nannochloropsis occulata* than *Chlorella vulgaris*. It causes reduction in both the methanol to oil molar ratio and the H_2SO_4 to oil molar ratio required to catalyse reactive extraction of *Nannochloropsis occulata.* The variation exhibited by both species to the pre-soaking pre-treatment is probably due to their varying cell wall chemistries. It is empirically shown that acid-catalysed reactive extraction involved phospholipids conversion into biodiesel, and contributes to higher biodiesel yield observed in reactive extraction than conventional transesterification.

Findings from this research make several contributions to the current literature. Firstly, a fast NaOH-catalysed reactive extraction using *Chlorella vulgaris* as model microalgae is shown to be possible for the first time by this research. A 96 % FAME yield was achieved in 10 min using 0.5 N NaOH with 925:1 methanol to oil molar ratio. The significant reduction in reaction time should substantially improve the process economy of the reactive extraction as such FAME yield is usually achieved in 1 h industrially.

Secondly, this research shows that FAME losses in reactive extraction can be avoided by stopping the reaction before saponification becomes significant. In addition, a numerical model developed by Eze *et al*. (2014) is shown to be applicable to predict the experimental FAME yield-time profile for this reactive extraction. The simulated data explained the phenomena in the experimental data. This model should be useful for predicting the optimum conditions for NaOH-catalysed reactive extraction, thereby reducing the resources and time required to conduct such experiments. The research also shows that moisture tolerance of NaOH-catalysed reactive extraction depends on reaction time, methanol to oil molar ratio and NaOH concentration.

Thirdly, this research shows that a zirconium dodecyl sulphate (a Lewis-surfactant catalyst) promotes reactive extraction of microalgae. The surfactant catalyst is shown to exhibit cell wall disruptive properties and performed comparably to H_2SO_4 (a conventional catalyst). This can serve as alternative catalyst to conventional H_2SO_4.

Fourthly, inclusion of SDS (a surfactant) in H_2SO_4 is shown to be a new way of increasing the moisture tolerance particularly in the reaction stage of reactive extraction of *Nannochloropsis occulata* and *Chlorella vulgaris*. The residue has been shown to maintain the protein content which can be used as animal feed supplement. Though the residual carbohydrate significantly reduced, nevertheless, if the sugar is separated, the liquid phase can serve as a feed for bioethanol production.

This research has also demonstrated that pre-soaking algae prior to acid-catalysed reactive extraction can be used for reducing catalyst concentration and methanol to oil molar ratio.

Furthermore it has been shown that microalgal phospholipids can actually be converted into FAME, explaining why previous researchers obtained higher FAME yield in reactive extraction than conventional transesterification.

5.2 Recommendation

It is recommended that future research should focus on the following areas:

[1] **Combine pre-treatment strategies**: Combination of pre-soaking pre-treatment, fast reactive extraction and inclusion of surfactant in a reactive extraction of microalgae for FAME production. FAME enhancement and increase in water tolerance can be achieved using this strategy.

[2] **Surfactant catalyst**: It has been shown by this research that the surfactant catalyst ("ZDS") performs comparably to H_2SO_4 catalyst for FAME production in *Nannochloropsis occulata* but produced low FAME yield in *Chlorella vulgaris*. Extra effort should be directed towards investigating why the surfactant catalyst exhibits varying activities in *Nannochloropis occulata* and *Chlorella vulgaris*. Similarly, effect of varying temperature on FAME production rate with the surfactant catalyst should be investigated.

[3] **Bio refining**: Co-products such as dimethyl ether (when methanol is used as the solvent) or diethyl ether (when ethanol is used as the solvent) can be produced alongside biodiesel in a reactive extraction. This will exclude recycling unreacted methanol or ethanol in the product streams and should

substantially reduce the cost of distillation heat load needed to recycle the solvent.

[4] **Reactor design**: The use of counter current extractor has been extensively explored for solid-liquid extraction system such as extraction of oil from oil seeds. This system can be used for reactive extraction of microalgae and it will allow the process to run in an efficient continuous mode.

[5] **Real time study**: Kinetic data for reactive extraction are still being obtained from batch experiment. There is possibility of monitoring such experiment on real time using fourier transform infrared spectroscopy (FT-IR). This will significantly reduce the time and resource devoted for extracting the kinetic data using batch experiment.

[6] **Techno economic analysis**: An economic analysis of the reactive extraction and conventional transesterification should be made to justify the additional heat load required to recycle the unreacted methanol in reactive extraction.

Conferences attended and publications submitted

Conferences

Salam, K. A., Velasquez-Orta, S. B., Harvey, P. A. Direct FAME production from microalgae by *in situ* transesterification. Presented at 4th International conference on Algal Biomass, Biofuels and Bioproducts (Algal 2014), Santa Fe, New Mexico, USA, June 15-18, 2014.

Salam**,** K. A., Harvey, P. A., and Velasquez-Orta, S.B. Algal FAME production with a novel surfactant based catalyst in a reactive extraction. Presented at the Annual Meeting, AiChE, San Francisco, California November 3-8, 2013.

Salam, K. A., Harvey, P. A., Velasquez-Orta, S. B. Intensification of algal biodiesel production by *in situ* transesterification. Presented at the 9th European Congress of Chemical Engineering (ECCE), Hague, The Netherlands, April 21– 25, 2013.

Publications

Salam, K. A., Velasquez-Orta, S. B. and Harvey, A. P. 'Evaluation of surfactant assisted direct FAME production from wet microalgae by *in situ* transesterification/reactive extraction', (submitted to Renewable Energy Journal for publication).

Salam, K. A., Velasquez-Orta, S. B. and Harvey, A. P. 'Effect of pre-soaking pre-treatment on reactive extraction/*in situ* of *Nannochloropsis occulata* and *Chlorella vulgaris* for biodiesel production' (submitted to Biochemical Engineering Journal for publication).

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APPENDICES

A: Calculation of molar ratios

B: Calculation of chlorophyll content

C: Calculation of protein content

Appendix A1: Calculation of methanol to oil molar ratio

Calculation of methanol volume

1.	Percentage of oil in the microalgae	=	0.17	%
2.	Mass of the microalgae	=	100	mg
3.	Mass of oil in the microalgae	=	0.17×100	mg
		=	17	mg
4.	Molecular mass of algae oil	=	880	
5.	No of mol of oil	=	17/880	
		=	0.019	mmol
6.	Ratio of alcohol to oil	=	600	
7.	Mol of methanol required	=	0.019×600	
		=	11.4	mmol
8.	Molecular mass of methanol	=	32.04	
9.	mass of methanol required	=	11.4×32.04	
		=	365.3	mg
10.	Density of methanol	=	0.7918	g/cm^3
11.	Volume of methanol needed	=	$(365\times10^{-3})/0.7918$	

		=	461	µL

Appendix A2: Calculation of NaOH to oil molar ratio

Calculation of mass of NaOH required

1.	NaOH concentration	=	0.5	mol/L
2.	Mass of methanol for 1L	=	791.8	g
3.	No of mol of methanol	=	24.7	
4.	molecular mass of NaOH	=	40	
5.	mass of NaOH required	=	20	g

Appendix A3: Calculation of H_2SO_4 to oil molar ratio

Calculation of H_2SO_4 volume required

1.	ratio of H_2SO_4 to oil	=	8.5	
2.	mol of H_2SO_4 required	=	0.019×8.5	
		=	0.1615	mmol
3.	molecular mass of H_2SO_4	=	98.1	
4.	mass of H_2SO_4 required	=	0.1615×98.1	
		=	15.8	mg
5.	density of H_2SO_4	=	1.84	g/cm^3

6.	volume of H_2SO_4 needed	=	$(15.8\times10^{-3})/1.84$	1.6 µL

Appendix A4: Calculation of zirconium dodecyl sulphate ("ZDS") to oil molar ratio

Calculation of mass of ZDS required

ratio of ZDS to oil	=	0.8	
mol of ZDS required	=	0.019×0.8	
	=	0.0152	mmol
molecular mass of ZDS	=	1152.782	
mass of ZDS required	=	0.0152×1152.782	mg
	=	17.5	mg

Appendix B: Sample calculation of Chlorophyll a in *Chlorella vulgaris* using H_2SO_4

$Chla = 11.93\, A_{664} - 1.93 A_{647}$

$Chla$: Chlorophyll a (µg/mL)

A_{664} = Absorbance at 664 wavelength= 0.241

A_{647} = Absorbance at 647 wavelength= 0.174

Volume of the extract= 0.47mL

$Chla$ = 0.76 µg

Appendix C: Sample calculation of Protein content in *Chlorella vulgaris* before and after reactive extraction using H_2SO_4.

As can be seen in the result of elemental analysis below

For sample C (Chlorella vulgaris before reactive extraction)

Average nitrogen content: = (9.7+9.66)/2 = 9.7%

Using, 4.75 nitrogen-protein conversion factor for microalgae derived by Lourenc *et al.* (2004)

Amount of protein content: = 4.75×9.7%

= 46.1%

For sample C1A-24: Chlorella residue after reactive extraction using H_2SO_4

Average nitrogen content: = (8.98+9.26)/2= 9.1%

Amount of protein in the residue: = 4.75×9.1%

= 43.2%