

**EVALUATION OF *DUNALIELLA SALINA* MICROALGAE FOR BIODIESEL
PRODUCTION A CASE STUDY (BOTSWANA ASH COMPANY)**

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CERTIFICATION

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ABSTRACT

Production of biodiesel from microalgae has received worldwide recognition as an alternative to petroleum diesel. The process involves culturing, harvesting, drying, extraction, transesterification and characterisation of the microalgae biomass to biodiesel. The research aimed to investigate the potential of *Dunaliella salina* microalgae in Botash Company's solar ponds for biodiesel production. Optimum salinity and nutrients concentrations that produced maximum biomass and lipids were investigated. Furthermore, the lipids and the biodiesel produced were identified, quantified and characterised.

D. salina microalgae were isolated from the solar ponds and cultured in f/2 media under optimum conditions. One Factor at A Time (OFAT) design was used to determine the optimum salinity for the growth and lipid accumulation of the microalgae. A salinity concentration of 2.5 M produced the highest biomass concentration of 2480 mg L⁻¹, biomass productivity of 58.40 mgL⁻¹d⁻¹, lipid content of 22.65 % and lipid productivity of 14.43 mgL⁻¹d⁻¹. In regard to nutrient optimisation, Plackett-Burman and Box-Behnken designs of response surface methodology (RSM) were used to optimise the culture media by selecting significant nutrients and their concentrations for culturing *D. salina* and promotion of lipids accumulation. In the first stage, Plackett-Burman method identified NaHCO₃, NaH₂PO₄·2H₂O and NaNO₃ as factors that significantly influenced the biomass concentration, lipid content and lipid production. MgSO₄·7H₂O and micronutrients solution had a significance effect on the lipid content. The relationship on biomass concentration, lipid content and lipid production were predicted using a model that had an R² value of 99.3% with a P-value of 0.000. In the second stage using Box-Behnken design, the nutrients selected in the first stage were further optimised and a predicted model was generated with an R² value of 95.8%. The optimum medium that constituted 1302.4 mgL⁻¹ NaHCO₃, 22.5 mgL⁻¹ NaH₂PO₄·2H₂O, 181.1 mgL⁻¹ NaNO₃, 44.7 mgL⁻¹ CaCl₂, 2355.0 mgL⁻¹ MgSO₄·7H₂O, 5.6 mL⁻¹ of micronutrients solution produced maximum lipid production of 181.1 mgL⁻¹, which is 12.3% more than the 127.0 mgL⁻¹ produced using the un-optimised medium.

After approximately 36 days of culturing the cells were harvested and used to extract lipids by solvent extraction method, Thin Layer Chromatography (TLC) screened and approximated lipids to linoleic acid, oleic acid, and some triglycerides. Biodiesel was produced from the extracted lipids using alkali transesterification process. Fourier Transform Infrared (FTIR) identified chemical bonds in biodiesel with a strong peak at $1743 - 1742\text{ cm}^{-1}$ wavelength as triglycerides ester carbonyl functional group. Furthermore, the *D. salina* biodiesel's chemical composition was quantified using Gas Chromatography (GC) and Mass Spectrometry (MS) spectrum that verified the presence of monounsaturated methyl esters.

The physiochemical characterisation of *D. salina* biodiesel was in the recommended specification of the EN 14214, ASTM D6751 and SANS 1935:2004 standards indicating a good quality of the fuel. The *D. salina* biodiesel when compared to sunflower biodiesel and petroleum diesel had the highest ignition temperature, burnout temperature, ignition index and also the least comprehensive performance reflecting good thermal properties of the fuel. The thermal kinetics of *D. salina* biodiesel show a high pre-exponential factor (A) and Gibbs energy (ΔG) but low activation energy (E) and entropy (ΔS). The enthalpy (ΔH) and higher heating value (HHV) were comparable to the ones for sunflower biodiesel and petroleum diesel. Based on the results of physio-chemical and thermal characterisation, the *D. salina* biodiesel could be used in boilers such as the ones in Botash Company for ignition and steam production purposes. In conclusion, the study was able to enhance lipids production during culturing stage, produce biodiesel through an effective selection of the best catalyst during the transesterification stage, identified and characterise the biodiesel.

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LIST OF ABBREVIATIONS

A:O	Alcohol to oil ratio
ASTM D6751	American Society for Testing and Materials
ATP	Adenosine triphosphate
AV	Acid value
B	Biodiesel
BBD	Box-Behnken Experimental Design
BC	Biomass concentration
Cat	Catalyst concentration
ChB	Chicken biodiesel
CO	Canola oil
D	Diesel
DI	Deionised water
D. salina	Dunaliella salina
DNA	Deoxyribonucleic acid
DTG	Derivative thermogravimetry
Dur	Duration
DWAS	Dry wastewater algae sludge
EN 14214	European Committee for Standardization
ER	Endoplasmic reticulum
FAME	Fatty acid methyl esters
FFA	Free fatty acids
FTIR	Fourier Transform Infrared
G3P	Glyceraldehyde-3-phosphate
GC-MS	Gas Chromatography and mass spectrometry
Int	light intensity
IV	Iodine value

L	Lipids
L:D	Ratio of light to darkness
LC	Lipid content
LP	Lipid production
MB	Microalgae biodiesel
MO	Microalgae oil
MuB	Mutton biodiesel
NADPH	Nicotinamide adenine dinucleotide phosphate
OFAT	One factor at a time design
PB	Peanut biodiesel
PBD	Plackett-Burman Design
PO	Peanut oil
PRB	Photo-bioreactor
RFs	Refraction factor
RO	Rubber oil
RP	Raceway pond
RSM	Response surface methodology
Rxn Time	Reaction time
Sal	Salinity
SANS 1935:2004	South African National Standard
SB	Sunflower biodiesel
SO	Sunflower oil
SV	Saponification value
Temp	Temperature
TGA	Thermogravimetric analyser
TLC	Thin-layer chromatography
WVB	Waste vegetable biodiesel
WVO	Waste vegetable oil

CHAPTER 1: INTRODUCTION

1.1 Background

The combustion of fossil fuels has increased the greenhouse gas emissions resulting in heat to be trapped in the atmosphere thereby causing an increase in global warming [1]. Global warming increase results in changes to the weather conditions, raise in sea levels, droughts and extinction to biodiverse species [1]. Therefore, a clean energy resource that can meet the global energy demand without harming the environment becomes relevant and of most importance. Future reduction of carbon footprint in the energy sector depends on sequential integration of multiple renewable energy technologies like the solar, biofuels and wind. Biofuels are deemed the answer to the energy crisis the world will face soon. Biofuels are categorised by the type of feed used which are categorised as either the first, second or third generation biofuels [2–4].

The biofuels from the first generation are produced from starch crops (maize, sunflower etc) and animal fats [3–5]. The controversy in using crops in biofuel production is that the same crops support the agricultural industry (human and animal consumption) and the production process requires arable land as well and potential water for human consumption [6,7]. This results in farmers and investors shifting from food crops to cash crops, leading to an increase in food prices and thereby threatening global food security [8]. Second generation biofuels are produced from lignocellulosic material (wood, forestry waste), organic wastes and specific biomass crops [9]. The feed substrate undergoes a pre-treatment process, for example, the thermochemical process in which a biochemical reaction will occur to expose the sugars embedded in the fibre to the surface [3]. This process requires a secondary external energy source and in most cases, the potential energy in the biofuel becomes less than the production energy, hence resulting in the process being economically unsustainable to upscale [9]. The third generation biofuels are produced from algae, both native [10] and engineered microalgae [11]. Compared to the first and second generation biodiesel, diesel derived from microalgae has high energy density [12].

Microalgae have the advantages in that they grow rapidly, have the ability to grow on waste nutrients. For microalgae to be a sustainable source for biodiesel, it is important to use less costly methods to grow them. These include less water usage, inexpensive nutrient supply, and less susceptible to contamination. As such, microalgae with tolerance to unique environments, where other contaminating microorganisms will not thrive are highly sorted after. Biodiesel is a renewable fuel produced from a diverse feedstock as animal fats, oils and microalgae lipids etc [9,13]. Microalgae stand as the preferred sustainable raw material to produce biodiesel because of its ability to thrive in non-portable water and non-arable land [11]. Biodiesel reflects as a promising fuel to substitute diesel in the power plants industries as it offers better benefits in application than petroleum diesel. Biodiesel has a high cetane number with no aromatic compounds as compared to petroleum diesel hence offering a long ignition delay [2,14,15]. In addition, biodiesel has a lower greenhouse gas emission as compared to gasoline and petroleum diesel with 4.5 folds and 3 folds respectively [2,16,17]. Most importantly biodiesel has the ability to reduce the greenhouse gas emissions through utilising the carbon dioxide it produces during combustion to be used by the microalgae during culturing of the feed [2,17].

UNdata [18] reported a 25 % annual increase in biodiesel growth, with microalgae contributing 5 %. UNdata [18] reported a total biodiesel production of 2603 metric thousand tonnes annually by the United States of America. Transport, production and power industries had higher consumption at 87 %, 21 % and 0.84 % respectively that is 1 % being equivalent to 31 250 tonnes. The power plant industry was identified to have increased the biodiesel consumption from year 2012 to 2015 at 0.32 %, 0.46 %, 0.66 % and 0.85 % respectively [18]. In Africa, biodiesel development is still at an early stage with most countries developing their renewable energy policies [19]. Botswana national development plan 11[20], reported an annual production of 15000 L of biodiesel from waste vegetable oil and to increase its capacity through an initiative plan of developing *jatropha* species as a feedstock.

Dunaliella salina (*D. salina*) microalgae have a motile green colour, unicellular with a diameter ranging between 1 to 50 μm and thrive in high saline environments either as an individual, chains or group forms [21]. It falls under Chlorophycophyta division of the Volvocales family. *D. salina* can accumulate oils and glycerol at 22 % and 70 % respectively making it a potential biodiesel feed [22,23]. Also, the *D. salina* thrives in saline water [22] making it less susceptible

to contamination and competition of resources (nutrients and land) with other microalgae species and food crops. In Botswana, *D. salina* is found in the brine ponds of Botash (Pty) Ltd and Okavango river. However *D. salina* in Okavango river has low population compared to Botash (Pty) Ltd.

Botash (Pty) Ltd is a soda ash and salt producing company that is situated in Sowa town, the northeastern part of Botswana. The plant has eleven active solar ponds that convert brine water to soda ash and salt through the densification process (evaporation process). During the evaporation process of producing soda ash and salt, the cells of *D. salina* increase in population and stain the soda ash and salt. Botash report of 2017 reported poor quality of soda ash and salt due to high *D. salina* density accumulation (4×10^6 cellsml⁻¹) above the plant specification recommended level of 2×10^6 cellsml⁻¹. Currently, the nuisance *D. salina* is controlled through chemical treatment using hydrochloric acid and brine shrimps but the high densities of the *D. salina* make these treatments expensive and less effective.

The purification of soda ash requires 84% pure carbon dioxide, steam and electrical energy to drive the auxiliaries. These inputs are supplied by the Babcock and Wilcox boiler which has a steam capacity of 76 000 kgh⁻¹, flue gases for carbon dioxide at 3 500 m³h⁻¹ and 15 Mw of electrical energy. To start up the boiler, it requires petroleum diesel which should be consumed within the specification quantity. Botash report of 2017 noted an annual diesel consumption 224 179 L which was above the expected 145 920 L that resulted in increased production cost by 570 000 pula.

1.2 Statement of the problem

The use of fossil fuels is unsustainable since it highly contributes to carbon footprint globally. Combustion of petroleum diesel results in increased greenhouse gases emissions to the atmosphere leading to global warming. Therefore, renewable and environmentally friendly alternatives such as biofuels are imminent. Biodiesel stands as a future alternative and green renewable biofuel for sustainable energy production. Currently, microalgae is a potential sustainable feedstock to produce biodiesel and in Botswana *Dunaliella salina* (*D. salina*) type is found in abundance at Botash (Pty) Ltd. High density accumulation (4×10^6 cellsml⁻¹) of *D. salina* above the plant specification recommended level of 2×10^6 cellsml⁻¹.has reduced

products quality by developing soft hopper crystals on soda ash and colouring the salt. In addition, high petroleum diesel consumption of 224 179 L above the expected 145 920 L has resulted in increased production cost by 570 000 pula annually.

Therefore, the main driver for this study is not only to mitigate energy and global climate disruption but also to collect or remove the microalgae from the soda ash and salt ponds as a means of product purification. To help the plant manage the microalgae, the study will explore this challenge into an opportunity by harvesting and concentrating the biomass to produce biodiesel

1.3 Main aim of the study

This research aims to investigate the potential of utilising *D. salina* microalgae from the solar ponds as a lipid source for biodiesel production through addressing the following specific objectives.

1.3.1 Objectives

1. To culture *D. salina* and determine its optimum salinity for the maximum biomass and lipid production.
2. To optimise the medium nutrients using the response surface methodology for maximum biomass and lipid production.
3. To extract, characterise and perform transesterification of lipids extracts from *D. salina* to biodiesel.
4. To perform physio-chemical, thermal characterisation and kinetics studies of produced biodiesel.

1.4 Hypothesis/ research questions

1. Does salinity affect *D. salina* biomass yields and lipid production?
2. Which medium nutrients are significant to the lipid production and what are the optimum concentrations required to produce a maximum lipid production?
3. Are the lipids extracted from *D. salina* suitable for convention into biodiesel?

1.5 significance of the study

The study will highlight the potential of biodiesel production from *D. salina* microalgae on Botswana's extreme environments. The production of biodiesel from *D. salina* microalgae will contribute positively to energy security and a reduction in greenhouse emissions. On the plant operational side, harvesting *D. salina* from solar ponds will benefit the company by reducing costs on using chemicals to treat the microalgal cells, thus improving the quality of and soda ash and salt.

1.6 Conclusion

This chapter provides background information on *D. salina* microalgae as a potential raw material for produce biodiesel. The problem statement, main aim, objectives, hypothesis questions and justification are explained in this chapter. Chapter 2 (literature review) describes explains previous work carried out on *D. salina* on culturing, harvesting, lipids extraction, biodiesel production and characterisation.

CHAPTER 2: LITERATURE REVIEW

Research on microalgae culturing, harvesting, drying, lipids extraction, biodiesel production and characterisation are explained in detail in this chapter. The chapter focus on the current technology's to produce biodiesel from microalgae and the research gaps.

2.1 Microalgae for biofuel production

Microalgae's ability to produce high lipids in a short time makes it a potential feedstock that can commercially produce biodiesel to replace petroleum diesel [24–27]. Many microalgae species such as *Tetraselmis*, *Isochrysis*, *Porphyridium*, *Nitzschia*, *Chlorella*, *Phaeodactylum*, *Cylindrotheca*, *Schizochytrium*, *Nannochloris* and *Dunaliella* can accumulate lipids naturally and when induced they can accumulate up to 75 % of lipids [28]. However, most of the species cannot thrive in a stressful environment with limited nutrient supply [11]. *D. salina* has the ability to accumulate lipids in stressful environments under limited nutrient supply and high salt concentration and when induced it doubles its lipids levels [28,29].

2.1.1 *Dunaliella* species as a biofuel source

The microalgae used in this study is *Dunaliella salina* (*D. salina*), which falls under phylum Chlorophyta of Polyblepharidaceae family [22,30,31]. The *D. salina* is halotolerant, unicellular in shape, and ranges between 4 and 25 µm in size [32]. Under high salinity concentration, *D. salina* has the ability to maximise its biomass growth, while at the same time yielding competitive lipids, with the highest lipid productivity comparable to other *Dunaliella* genus such as *Dunaliella tertiolecta*, *Dunaliella* sp. ABRIINW, *Dunaliella peircei*, *Dunaliella parva*, *Dunaliella Viridis*, and *Dunaliella bardawil* [33,34].

2.1.2 Microalgae growth and lipid production

Critical understanding of the growth behaviour of microalgae contributes to a significant optimisation of the culturing conditions during the production process [35]. Microalgae goes through five different phases during the growth under batch culturing being; lag or induction, exponential, declining growth, stationary and death phases. Figure 2.1 depicts the microalgae

growth behaviour with respect to time and Table 2.1 explains the growth behaviour of the microalgae per each stage [35,36].

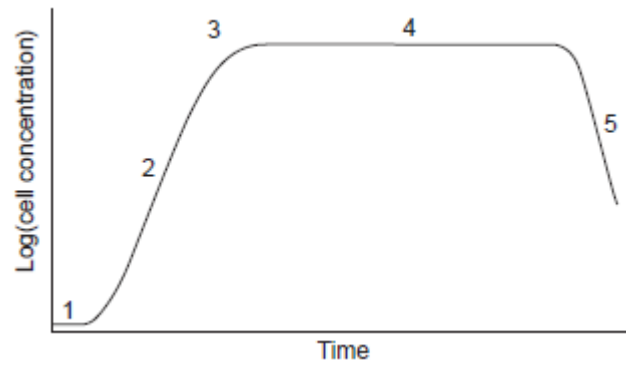


Figure 2.1: Microalgae growth phases in a batch culture system [36]

Table 2.1: Microalgae growth behaviour per each phase of the growth

Phases		Explanation	Ref
1	Lag or induction	The cells undergo physiological adoption of its metabolism to the new culture conditions. Cell growth increases slightly but not significantly.	[35–37]
2	Exponential	The biomass growth rate increases exponentially and at a significant rate with time. The microalgae start to adapt to ecological conditions. Thinning of the culture ensures maximum availability of sunlight and nutrients to the microalgae cells	[35,38,39]
3	Declining growth phase	The maximum peak on growth is reached and there is a drastic reduction in cell division due to nutrients depletion, carbon dioxide intake reduction and increase on surface thickness restricting light provision.	[35,36]
4	Stationary phase	The growth rate is at zero due to the limiting factors on growth (light, nutrients, carbon dioxide and temperature). Lipid content starts to change its fatty acids composition structures.	[35,37,40]
5	Death phase	Microalgae cells start to deteriorate and bacteria starts to form. Cell metabolism cannot be maintained and the cell density starts to decrease rapidly.	[35]

The stationary phase is important when targeting lipids because it marks the stage of nutrient deprivation [37]. Lipids are secondary metabolites produced in a cell during the photosynthesis process as the energy is generated [41,42]. Figure 2.2 shows a simplified pathway involved in lipid synthesis in the microalgae cell. Photons of light energy are captured in the antenna complex, transferred and split into electrons, hydrogen ions and oxygen in the chloroplast centre that contains chlorophyll molecules [41,43,44]. Adenosine triphosphate (ATP) and NADPH

energy sources made from photons of light are used during photosynthesis reaction to transform inorganic carbon (CO_2) to carbon organic compounds mainly the acetyl-CoA which are further converted to malonyl-CoA [45]. The transacylase (MCAT) transfers the malonyl-CoA to malonyl-ACP. The malonyl-ACP catalysed by ketoacyl-ACP synthases enzymes is converted to fatty acids [45]. The glyceraldehyde-3-phosphate (G3P) with enzymes acts as catalysts to transport cytosol that is used to synthesise fatty acids to triglycerides [41,44,46–48]. The produced fatty acids and triglycerides are the lipids and when harvested from cells they can produce value-added bio biased products such as essential oils in case of this thesis biodiesel.

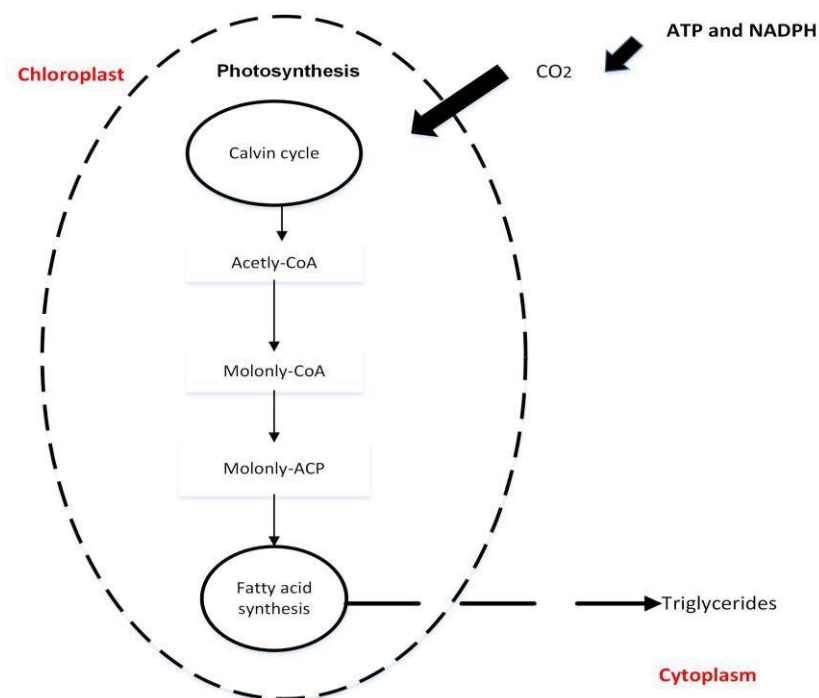


Figure 2.2: Simplified pathway for lipid synthesis in microalgae cell [45]

Lipids are fatty acids that are organic in nature. They compose of carbon chains that range from 12 to 24 carbons (C_{12} to C_{24}) [49]. The lipids are classified as neutral or polar with respect to the solubility in organic solvents. The neutral lipids are soluble in organic solvents hence making them best for biodiesel production as compared to polar lipids [50]. Neutral lipids are further classified as saturated, monounsaturated or polyunsaturated due to their ability to be synthesised. Microalgae lipids are classified for usage based on their carbon number. Saturated (C_{14} to C_{20}) fatty acids are normally used for biodiesel production, and fatty acids with more

than C₂₀, which are called polyunsaturated fatty acids (PUFAs) and are used as food supplements [51]. Fatty acids found in the microalgae are shown in Table 2.2 below [49,50,52].

Table 2.2: List of fatty acids typically produced by microalgae [49,50]

Fatty acid	Group	Number of carbon atoms
Lauric	saturated	12
Myristic	saturated	14
Palmitic	saturated	16
Stearic	saturated	18
Oleic	monounsaturated	18
Alpha-linolenic	polyunsaturated	18
Linoleic	polyunsaturated	18
Arachidonic	polyunsaturated	20
Eicosapentaenoic	polyunsaturated	20
Docosahexaenoic	polyunsaturated	22

Growth kinetics of microalgae biomass and lipids are necessary to quantify the productivity and effectiveness of the yield during the culturing phase [22,53]. The parameters measured are the biomass growth (concentration, specific growth and productivity and lipid (content, productivity and production) as shown in Eqns. (2.1) – (2.5) respectively [54–56].

$$\mu = \frac{1}{t_2 - t_1} \ln \left(\frac{X_2}{X_1} \right) \quad (2.1)$$

$$P_b = \frac{X_2 - X_1}{t_2 - t_1} \quad (2.2)$$

$$LC = \frac{m_L}{m_b} \times 100\% \quad (2.3)$$

$$P_L = \frac{X_2 - X_1}{t_2 - t_1} \quad (2.4)$$

$$LP = BC \times LC \quad (2.5)$$

Where: μ is the specific growth rate measured per day (d^{-1}), X_1 and X_2 are the biomass concentration (mgL^{-1}) at time t_1 and t_2 measured in days (d), P_b is the biomass productivity ($mgL^{-1}d^{-1}$), LC is the lipid content (% w/w), m_L is the mass of lipids (mg), m_b is the mass of the biomass (mg), LP is the lipid production (% w/w) [55,56].

2.1.3 Factors influencing microalgae growth and lipid production

Several factors influence microalgae biomass growth and lipid production. These are generally classified into physical and chemical factors. The optimisation of these factors is important in microalgae culturing.

2.1.3.1 Physical factors

In both natural and controlled culturing system, physical factors play a crucial role in the microalgae metabolic induction to enhance biomass and lipid growth [24]. The factors are light intensity, temperature and salinity. Light intensity is a critical factor to the microalgae's biomass and lipid growth [22,41,56]. Light intensities between 90 and 3000 lux are recommended for *D. salina* cultures [57,58]. Nadzir [59] demonstrated that light intensity of 23500 lux produce maximum lipid productivity and lipid content of $27 mgL^{-1}d^{-1}$ and 23 %, respectively when *Tetrademus obliquus* was cultured. Cheirsilp and Torpee [60] also noted *Nannchloropsis sp* and *Chlorella sp* achieving maximum lipid production ($572.8 mgL^{-1}$ and $397.8 mgL^{-1}$) at 500 lux and 8000 lux respectively both with increased biomass growth and decrease in lipid content [60].

Temperature plays a crucial role in microalgae biosynthetic metabolic such as lipid synthesis, with species behaving differently to the effect of temperature [24,41,61]. Seasonal variations that result in fluctuations on temperature affects the growth and lipid accumulation of the microalgae [62]. Temperatures above optimal value result in a decrease of biomass growth and change of fatty acid composition due to the imbalances of the energy demand with the tri-phosphates production [62,63].

Biomass growth and lipids accumulation are directly affected by the salinity levels in the culture medium [41]. Salinity is the total amount of salts per kilogram of water that is measured using

the water electrical conductivity [41,64]. Salinity above the optimum levels results in stress (ionic and osmotic) and ionic ratio modification in the microalgae cells [21]. Ahmed [21] reported that *D. salina* microalgae achieved the best yield of lipids at 2 M of salinity. Above 2.5 M of salinity, the biomass and lipid growth decreased and below 0.5 M the cells were weakened resulting in a decrease of growth [21].

Mixing of culture medium ensures that microalgae absorb nutrients easily, reduces thermal stratification, increases gaseous exchange (CO₂ absorption and O₂ removal), increases light utilisation as well as the suspension of cells so that the biomass does not settle in dark zones [41,65]. Therefore mixing enhances biomass growth. Mixing can be achieved by bubbling air, shaking the culture flasks in orbital shakers or shaking the culture flasks by hand twice a day [41,66].

2.1.3.2 Chemical factors: Nutrients

Nutrients are an important factor in the growth of microalgal cells with the nutrients optimisation or limitation inducing the accumulation of biomass or lipids through rapid cell growth [67,68]. The nutrients constituents are divided into macro and micro, and play a vital role in the biomass growth and induction of lipids [69,70]. Ahmed [23] listed essential nutrients for *D. salina* biomass growth and lipids under macronutrients as follows: NaNO₃, NaH₂PO₄.2H₂O, CaCl₂, MgSO₄.7H₂O, NaCl₂, and NaHCO₃ and micronutrients as follows: C₅H₈FeO₇, ZnSO₄.7H₂O, CO(NO₃)₂.6H₂O, H₃BO₃, Na₂MoO₄.2H₂O, MnCl₂.3H₂O, ZnCuSO₄.5H₂O [71].

The NaNO₃ ensures microalgae health, growth, and free fatty acid structuring [41]. The NaHCO₃ is essential for the microalgae metabolism and it contributes to almost 50 % on a dry weight basis of the microalgae biomass weight [72,73]. Metabolism of microalgae is maintained by ensuring an adequate supply of NaH₂PO₄.2H₂O to the cells [74]. Micronutrients are also relevant for the metabolic activities and the growth of microalgae [75].

2.2 Response Surface Methodology (RSM)

RSM is an arithmetical design used for process optimisation during experimental design where it evaluates the interactive effects of independent variables on dependent with an aim to

determine the optimised operational specification [76–80]. RSM utilises quantitative data from relevant experiments to solve complex interactions multivariate equations [80]. RSM utilises a few numbers of experiments as selected by the design compared to One Factor at A Time (OFAT) method which experiments on all experimental combinations [80,81]. In addition, RSM has a rapid, high precision prediction that collects a high volume of data per experiment making it cost-competitive and beneficial for application during experimentation [82]. The response surface in RSM can be expressed as shown in Eqn. (2.6):

$$Y = \phi(x_1, x_2, x_3, \dots, x_i, \dots, x_k) \quad (2.6)$$

[83]; where Y is the performance characteristic of the system, ϕ is the performance function, x is the independent parameter and k is the number of parameters [78,83].

RSM will be used in this study to determine the optimum nutrient concentration (independent variables) and the outcome design will be used to culture *D. salina* to produce the maximum lipid accumulation that will be used for biodiesel production [84]

2.3 Culturing and harvesting methods of microalgae biomass

Indoor (photo-bioreactors) or outdoor (open raceway ponds) methods are used for culturing microalgae [85]. Photo-bioreactors utilise controlled conditions where nutrients, carbon dioxide, temperature, light intensity and mixing are optimised to ensure the desired growth of biomass, whereas raceway ponds utilise natural weather conditions and natural air mixing to culture microalgae biomass [85,86]. For sustainability, open raceway ponds are the best choice due to their low investment and operational costs. Integrating flue gas (CO_2) from power plants and wastewater (nutrients) to the process offers further opportunity for effective culturing at low cost [36,87]. However, the raceway ponds consume more land space, water and carbon dioxide while producing less biomass as compared to the photo-bioreactors [36]

Selection of the harvesting method is based upon the desired end product and the culture corrosiveness. Microalgae such as *D. salina* tends to thrive in high saline environments hence harvesting machinery are prone to high corrosiveness [22]. Ralefala [22] noted that filtration, centrifugation and flocculation were applicable harvesting methods to *D. salina*. Table 2.3 lists harvesting methods that are applicable *D. salina* at laboratory scales. Electro flocculation and

ultrafiltration are the best applicable harvesting methods that efficiently extract wet biomass at the low energy consumption as compared to the vacuum filtration, centrifuge and air floatation methods.

Table 2.3: Different harvesting methods for *D. salina* biomass

Method	Efficiency (%)	Energy consumed (kwhm ⁻³)	Ref
Electro flocculation	97.44	0.62	[88]
Ultrafiltration	95.00	0.87	[89]
Vacuum filtration	-	5.9	[90]
Centrifuge	-	High	
Air floatation	-	High	

The biomass is dried just after harvesting before extracting the lipids. Drying contributes significantly to the operational costs and removes most of the outbound moisture (>90%) in the microalgae [36,90]. The drying increases the effectivity of lipids extraction as compared to the wet biomass extraction process [49]. *D. salina* is mostly dried using the sun, oven or freezer-drier, as such a good selection of the best method is usually based on the type of microalgae harvested. Oven and freeze-drying methods produce more lipids as compared to sun drying. However, freeze-drying has a high energy consumption compared to oven and sun drying methods [49]. Therefore the use of the oven drying method was used in this study.

2.4 Transesterification of lipids to produce biodiesel

Renewable feeds such as oils and animal fats are used to produce biodiesel through transesterification [91–93]. Transesterification is a process that converts the alcohol functional group to methyl esters (biodiesel) using a nucleophilic substitution method at a ratio of 3:1 for alcoholic catalyst and triglycerides respectively [35,94]. Figure 2.3 below shows the transesterification.

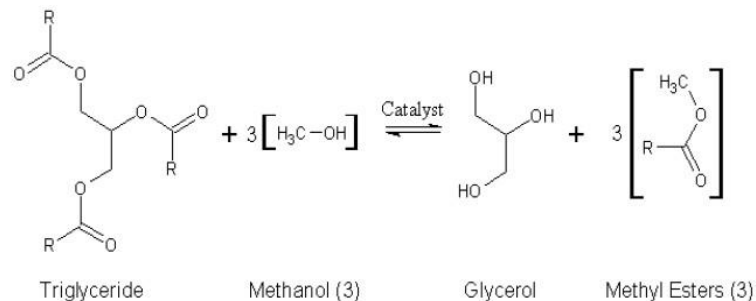


Figure 2.3: General transesterification process using methanol as a catalyst, adopted from [35,94]

The transesterification reaction involves a four-step process explained as follows [94]:

1. The alcohol is deprotonated with the catalyst
2. Triglycerides positive carbon ester bonds react with the negatively charged carbonyl oxygen from step 1 to form a tetrahedral intermediate.
3. Pairwise electrons from the double-bonded ester oxygen detach the triacylglycerol molecule from the triglycerides.
4. Three fatty acid methyl esters (FAME) and one glycerol per triglycerides formed.

Normally, biodiesel is composed of FAME that has a carbon chain between C_{16} to C_{18} [35,92].

The biodiesel transesterification reaction is accelerated by the use of a catalyst, which is either an alkali or an acid [95]. A summary overview that distinguishes these catalysts is explained in Table 2.4. Alkaline catalysts react faster than acidic catalyst producing a high yield of biodiesel. Acidic catalysts are best applicable to poor oil quality that has water saturates and high content of free fatty acids [96]. In addition, the acidic catalysts require a high temperature and high methanol to oil molar ratio compared to alkaline catalyst [96]. In this study, alkaline catalysts were used.

Table 2.4: Difference between alkaline and acidic catalysts [49,97–101]

Catalyst	Example	Temp (°C)	A:O	Rxn. Time	FFA (%)	Corrosiveness	Glycerol recovery	Conversion rate
Acidic	HCL H ₂ SO ₄	100	30:1	3-18	>6	high	difficult	low
Alkaline	KOH, NaOH, CH ₃ ONa	60-65	6:1	2	<1	low	difficult	high

Where: Temp is the temperature, A:O is the alcohol to oil ratio, Rxn Time is the reaction time, FFA is the free fatty acids in lipids.

2.5 Identification and quantification of lipids and biodiesel

The method used for identification and quantification of lipids organic functional groups is Thin layer chromatography. Fourier Transform Infrared and Gas Chromatography-Mass spectroscopy were used to identify and quantify the biodiesel. Only the methods used in this study will be discussed in the next subsections.

2.5.1 Thin Layer Chromatography (TLC)

TLC is a method used to separate components of a mixture chromatographically using a thin stationary phase (silica) [102]. It is a quick way used for initial screening to monitor the presence of fatty acids, methyl esters in lipids and their biodiesels [103]. Small droplets of lipids extracts are applied on a TLC plate which is then placed in a TLC chamber for 30 minutes filled with 150 ml of hexane, diethyl ether and acetic acid solvents mixture (60:40:1, v/v/v) later dried and sprayed with iodine for spot identification [104,105]. The identified spots are cross-referenced with standard compounds for specific molecules identification [106]. It is simple to use, cheap, with high sensitivity and high separation speed [107].

2.5.2 Fourier Transform Infrared (FTIR)

FTIR spectroscopy is a rapid non-destructive method that requires minimal sample preparation for qualitative analysis of organic functional groups such as lipids and biodiesels [108–112].

For the characterisation of fuels, FTIR is used to differentiate the composition of fuels at a specific intensity with an exact frequency where maximum transmittance of the bands appear as shown in Figure 2.4. The technique involves infrared absorption in relation to the molecular concentration using the Beer-Lambert law as a function of the wavelength [113].

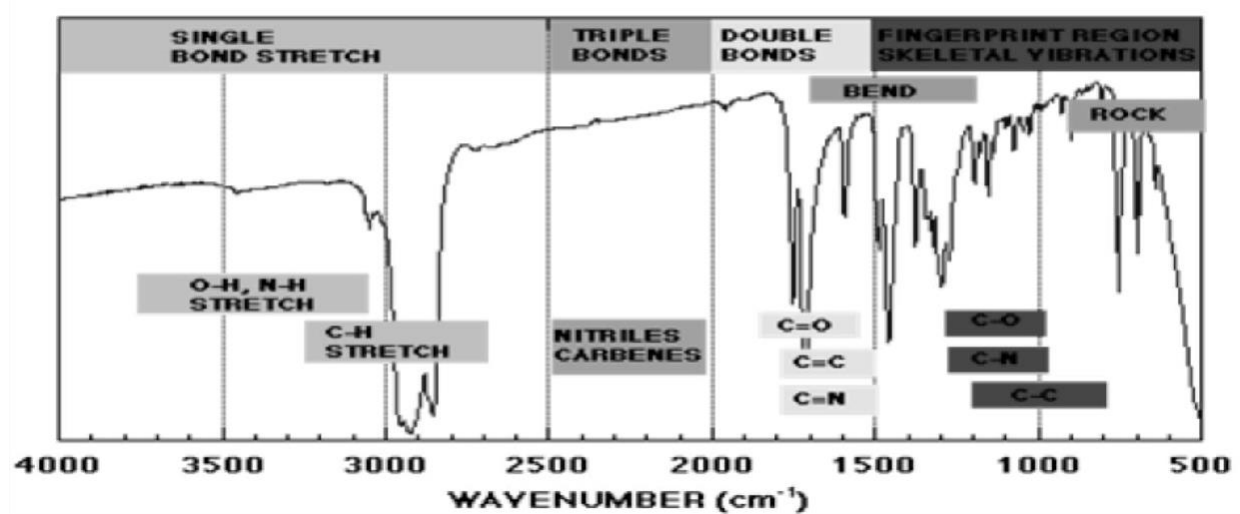


Figure 2.4: Transmittance peaks of chemical components in oils and biodiesel [109,114]

As shown in Figure 2.4, the single bonds stretch, triple bonds, double bonds and fingerprint are the regions where organic compounds in fuel are identified. Alkoxy esters, ethers, trans-disubstituted alkenes and aromatics are identified in the fingerprint region. The carbonyl groups and the ester carbonyl group that are present in biodiesel are in the double bond region [49]. Table 2.5 shows the wavelength, vibrational type and functional group of different lipids and biodiesel. Different oils and biodiesels have a common vibration type but varying functional groups. The wavelength stretched from $3949 - 730 \text{ cm}^{-1}$ all oils and biodiesels indicating that the functional groups were mostly fatty acids in the carbon chain from $\text{C}_8 - \text{C}_{20}$.

Table 2.5: Functional groups at a specific wavelength of oils, biodiesel and petroleum diesel

Region	Sample	Wavelength (cm ⁻¹)	Type of vibration	Functional group	Ref
Fingerprint	L	730		C-H	[49,109,111,115,116]
		1400-930	in-plane bending	C-O-H	
			stretching	C-O-C; C-O	
			out of plane bending	O-H	
	CO	1436	asymmetric	-CH ₃	[117]
		1198	stretch	O-CH ₃	
	PO, B, <i>C.vulgaris</i> . L	1465-1377	bending and rocking	-CH ₃	[111,116]
		960	out of plane bending	=C-H	
		850			
		721	rocking	=C-H, -(CH ₂) _n	
		678	out of plane bending	=C-H	
	D	1200	asymmetric axial stretching	C(C=O)-O	[118]
		1183		O-C-C	
Double bonds	L, B, PB	1740-1745	Stretching	V(C=O)	[49,108,109,116]
	Lipids	1870-1540	stretching	C=O	[115]
	PB, <i>C.vulgaris</i> . L	1602	bending	C=C	[111,116]
Triple bonds	<i>C.vulgaris</i> . L	2145	stretch	alkynes group	[111]

Single bond stretch	L, B, D	2800	symmetrical stretching	C-H	[49,109–111,115,116,118]
			asymmetrical stretching	C-H	
		3007		=C-H	
		3300-2500		O-H	
	PB	3500	stretching	O-H	[116]
	<i>C.vulgaris. L</i>	3949, 3840	-	amines	[110,111]

Where: L is the lipids, CO is the canola oil, PO is the peanut oil, B is the biodiesel, PB is the peanut biodiesel, D is the petroleum diesel.

2.5.3 Gas Chromatography and Mass Spectrometry (GC-MS)

GC-MS is a resourceful tool that quantifies and identifies individual components in a mixture at a micro-scale level. The analyser has a high component separation efficiency, speed, reproducibility and accuracy [49]. The sample is injected into the GC component where the sample mixture molecules are separated, charged while non-broken (parent ions) and detected with respect to the abundance per second [119]. The parent ions are transferred to the MS component where they are ionised in a beam of electrons. The parent ions and fragments then travel through the quadrupole to the electromagnetic rods that are four and parallel to each other and charged with radiofrequency voltage [50]. The fragments with certain mass to charge ratio (m/z) will then pass through the electromagnetic rods to the detector normal to their trajectories where they are recorded as a mass spectrum (abundance on the vertical axis and m/z on the horizontal axis) [49]. Using chromatographic data (peak height and peak area), the components are quantified using signal processing method to estimate the peak intensity. The GC-MS has a database of various mass spectra, which makes it simpler to verify the spectra of the components with the ones in the library.

2.6 Characterisation of biodiesel as a fuel

2.6.1 Physio-chemical characterisation

Physio-chemical characterisation ensures the fuel is well matched to its application for it to perform efficiently and effectively [46,120]. The regulatory standards of American Society for Testing and Materials (ASTM D6751), European Standard (EN12412) and South African National Standard (SANS 1935:2004) regulate the physio-chemical quality of biodiesel [49,121]. Physio-chemical properties such as the density (ρ), acid value (AV), saponification value (SV), free fatty acids (FFA) iodine value (IV) are important parameters to characterise the feedstock oils and biodiesel, as they reflect the quality of the fuel [120]. Saponification value (SV) is the measure of mass in mg of potassium hydroxide required to neutralise free fatty acids (FFA) and to saponify a mass of esters in mg of a substance [49,122,123]. The relative molecular mass of triglycerides (TGA) is dependent on the SV of the biodiesel that means a lower relative molecular mass indicates a higher SV, a high methanol consumption

during the transesterification reaction and a low yield of fuel output [49,123–125]. South African National Standard (SANS 1935:2004) recommends a saponification value between 185 to 198 mg KOH⁻¹ of biodiesel [49]. Acid value (AV) in mg of KOH is a measure on the extent of the oxidation and hydrolysis in fuel, with good quality not exceeding 0.5 mg of KOH⁻¹ of fuel under the ASTM D6751 and EN 14214 standards [93,121,126,127]. High acidity in biodiesel tends to degrade the biodiesel quality, to oxidise the fuel and thereby degrading the double chain bonds and hydrolyse the ester bonds which shortens the shelf life of the fuel [49,120,126]. In addition, high acidity corrodes the rubber seals, hoses, clogs, drops pressure and power in an engine [49,121,126]. Furthermore, high acidity indicates poor fuel quality and produces a low yield of biodiesel [128]. The iodine value (IV) is the quantification degree of unsaturation (double bonds) in fats, oils and their fatty acid derivatives in biodiesel, determined using the Hunus method [49,121]. Acceptable IV should not exceed 120 g I₂ per 100g of FAME under the EN1424 standards [49,125]. Each oil or fuel has its own IV due to different structures of double bonds and unsaturated fatty acids [124]. A high IV above the recommended value of 120 gI₂100g⁻¹ reflects pre-oxidation, polymerisation and soot deposits in internal combustion engines whereas a low IV resembles low NO_x emissions during combustion [124].

Ester value for ester bonds is the measure of the mass in mg of KOH required to react glycerol after saponification of a gram of fats [49,124,129]. For better quality delivery of the fuel to the engine, the ester value should be close to the saponification value hence the fuel delivers the best ignition to the engine [129]. Density is a measure of the mass per unit volume of fuel at a specific temperature which depends upon the raw material used to produce the biodiesel and the FAME profile of the biodiesel [49,123]. The fuel density directly affects the engine performance (injection systems, pumps and injectors) with a high density above the recommended resulting in incomplete combustion and particulate matter emissions [123]. Comparison between different biodiesels, high density indicates more thermal energy and better promising fuel efficiency [121]. The ASTM D6751 standard specifies that the biodiesel's density should be between 860 kgm⁻³ to 900 kgm⁻³ measured at 40 °C [123].

2.6.2 Thermal characterisation

Thermal analysis is the study of the transition process in a substance under a specific technique in which the substance is subjected to a programmed temperature [130,131]. Thermogravimetric analysis (TGA) is a thermal analysis method which measures substance mass as a function of time or temperature [132,133]. Derivative thermogravimetry (DTG) is the first derivative of the TGA curve and is used to give an in-depth analysis on the thermal behaviour of a sample by identifying inflection points (peaks) on a curve [134]. The analysis can determine thermodynamic properties (phase transitions temperatures, enthalpies, activation energy and heat capacities) and the kinetics (combustion, decomposition, polymerisation, reduction, crystallisation and oxidation) of a substance [134–136]. TGA has three types of analysis (Dynamic, isothermal and quasistatic) which are influenced by samples' physical form, mass, volume, scanning rate, samples holder status and the chamber pressure. Dynamic TGA measures at a continuous temperature whereas isothermal TGA is at constant temperature and quasistatic is at a constant weight [136].

Plateau region, initial temperature (T_i), peak temperature (T_p), final temperature (T_f) and maximum combustion rate are observed during thermal decomposition [137]. In the plateau region, the temperature change while the thermal property of the fuel remains unchanged [132]. Significant thermal change is noticed at the first mass loss where ignition temperature is noted [132,133]. The maximum mass loss rate is a point where the peak temperature is depicted that is the maximum combustion rate point [138]. Burnout temperature is at a point where the fuel reaches a mass loss of about 95% [133,139]. Ignition temperature and burnout temperature of a substance on a TG/DTG and DSC curves are determined by three different methods that are conversion (degradation), thermal acceleration and intersection methods [139]. The methods are explained as shown in Table 2.6.

Table 2.6: Methods used to establish ignition and burnout temperatures using the TGA/DTG and DSC curves [131,138–140]

Method	Curve	Ignition temperature	Burnout temperature
Conversion	TGA/DTG	10% of mass loss	95% of mass loss
Thermal acceleration	DSC	Mass loss rate raised to $1 \text{ mass\%min}^{-1}$	Mass loss rate reached $1 \text{ mass\%min}^{-1}$
Intersection method	TGA/DTG (Figure 2.5)	Tangential point connecting point A and B the corresponding temperature	Tangential point connecting point C and D, the corresponding temperature or the point where the mass loss rate is less than $0.1 \text{ mass\%min}^{-1}$

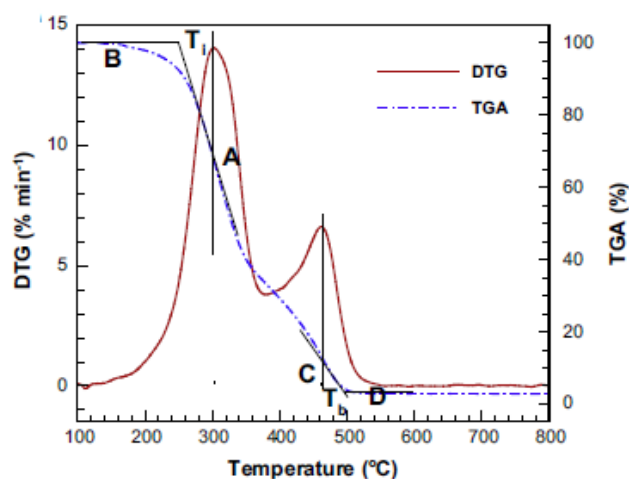


Figure 2.5: TGA/DTG curve of a substance [138]

The shape of the TGA curve determines its classification. There are seven different types of TGA curves and each type is explained in Table 2.7 and depicted in Figure 2.6 [56,132,133].

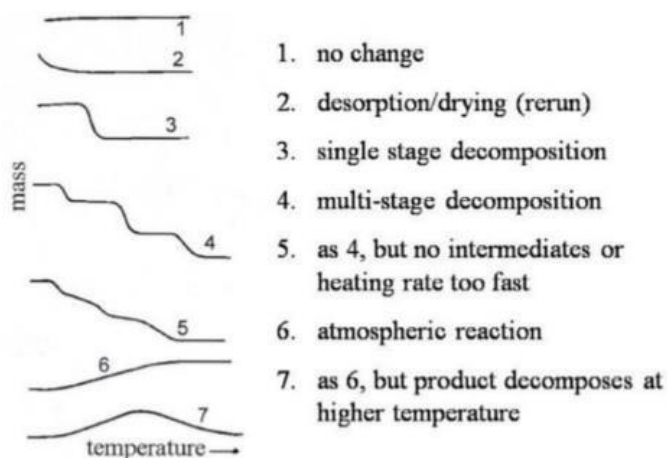


Figure 2.6: Classification of different TGA curves [132,133,137]

Table 2.7: Explanation of the classification of different TGA curves [132,133,137]

Curve type	Explanation
1	No mass loss Substance decomposition temperature is higher than the instrument's temperature range
2	A large mass loss rate is observed followed by a plateau mass. It is the evaporation of volatiles or polymerisation.
3	Single stage decomposition with initial and final temperature.
4	Multiple stage decomposition process of the substance with a slow heating rate where the reaction is resolved
5	Multiple stage decomposition process of the substance with a fast-heating rate where the reaction is not resolved
6	Increase in mass of a substance in the presence of atmospheric air
7	Surface oxidation followed by decomposition

Thermal indexes (ignition and comprehensive performance) does give detail regarding combustion kinetics of a fuel [141]. Ignition capacity of fuel is represented by the ignition index (Di) and it interprets the complexity level with the rate flow of fuel ignition [141–143]. The ignition index is dependent upon the ignition temperature and maximum mass loss rate with its

corresponding temperature [143,144]. Higher ignition index mean that the fuel has a good ignition performance [139,144,145]. Comprehensive performance index (D_c) does evaluate the combustion properties of a fuel [51,146]. The index critically reflects the fuel's burning ability which is dependent on the burning velocity, ease of combustion and burnout temperature [147,148]. A high comprehensive performance index (D_c) value of the fuel means good combustion performance [51,142,146,149].

Combustion kinetics is the study of the reaction mechanism of a substance to the extent of time and temperature [150]. TGA is an efficient tool to investigate the fuel's kinetic properties, that is the activation energy (E), Arrhenius constant (A), reaction order (n) and reaction model $f(\alpha)$ [131,150]. Numerical mathematical models such as Kissinger model-free and Ozawa Flynn wall were applied to calculate the kinetics of biodiesel during the combustion process [135,150]. The minimum amount of energy required for a molecule to generate a chemical reaction is the activation energy (E) and the frequency of molecules collision per second is the Arrhenius Constant (A) [130,134,151].

Enthalpy, Gibbs free energy, entropy and higher heating value are thermodynamic kinetic parameters that can be derived from the data of TGA/DTG and its kinetics defined as follows [134,139,152]:

- Enthalpy (ΔH): It is the heat absorbed or released under constant pressure during a chemical reaction of a substance.
- Gibbs free energy (ΔG): It is the available energy to do work and is used to determine the spontaneity of a reaction.
- Entropy (ΔS): Is the measure of unavailable energy or system disorder in a closed thermodynamic system. It is directly related to any reversible change in heat and inversely with temperature in the system.
- Higher heating value (HHV): It is the amount of heat realised per unit mass or volume of fuel during combustion at an initial temperature of 25 °C and final product temperature returned to 25 °C latent heat of vaporisation of the combustion products water taken into account.

2.7 Conclusion

The literature about *D. salina* as a potential raw material for biodiesel production was addressed in this chapter. The chapter described an in-depth review on culturing *D. salina* for high biomass and lipids growth through optimising the salinity and nutrients. Also, RSM was described on how it can be applied to optimise parameters to produce high biomass and lipids growth. Identification and characterisation of lipids and biodiesel were explained. The next chapter (Methodology) focuses on experimental methods applied to achieve the research aim.

CHAPTER 3: MATERIALS AND METHODOLOGY

The chapter explains in detail the methods used to produce biodiesel from *D. salina* microalgae. The isolation, identification and collection method of *D. salina* is detailed in section 3.1. Section 3.2 explains the culture medium and growth conditions while section 3.3 investigates the growth of biomass and lipids through optimisation of salinity and nutrients. Section 3.4 details the harvesting, drying and lipids extraction. Characterisation method of lipids is explained in section 3.5. Transesterification of extracted lipids to biodiesel and identification of the produced biodiesel is detailed in section 3.6. The methods applied to physically and chemically characterise the produced biodiesel from *D. salina* were explained in section 3.7. The conclusion of this chapter is in section 3.8.

3.1 Isolation, identification and collection of microalgae samples

Isolation, identification and collection of microalgae is a technique of picking a strain from a sample culture, identifying and collecting with the methods for these procedures explained in this section. Modified methods of microalgae isolation, culturing, and identification was adopted from Al-ansari [153] and Wang [154]. Brine water samples were collected from the solar saltern ponds of Botash (Pty) Ltd company, located in Sowa Town, Botswana (20.5332° S, 26.0740° E). Figure 3.1(a) shows one of the solar brine pond (E3) and (b) the layout of the saltern ponds. During soda ash and salt production, the brine water is pumped from the underground wells to W1 pond and then flows through E0, CS, E3, E6, NS, X, and ST ponds respectively. The brine water evaporates and increases in brine concentration along the flow passing through the mentioned ponds (E0, CS, E3, E6, NS, X, and ST ponds). CS pond also acts as a fluid coolant supplier for the plant's heat exchangers.

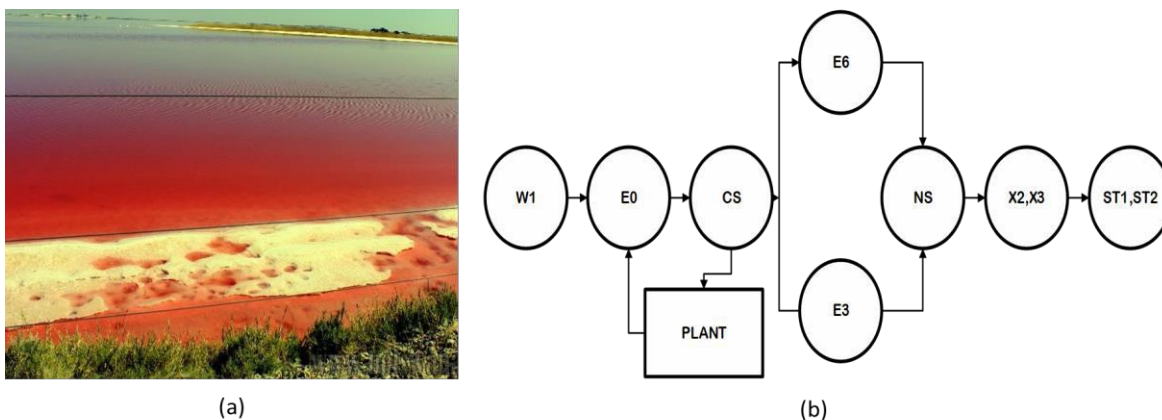


Figure 3.1: (a) Coloured water in pond E3 (b) ponds layout; where: W1 - well pond, E0 - evaporating pond zero, CS - cooling sump pond, E3 and E6 - evaporating ponds three and six, NS - sodium chloride pond, X2 and X3 - crystallizing ponds and ST1 and 2 - storage ponds.

The Botash production report (2017) report that CS, NS, E3, and E6 ponds have significant microalgae population, and these were selected as sampling points following a procedure shown in Figure 3.2. The brine samples were collected in sterile bottles and transported to the laboratory where they were filtered using the plankton nets of mesh sizes 100 μm , 50 μm and 20 μm to remove debris and dead organics [155,156]. Unwanted microbial cells from the samples were removed by centrifuging the samples (Thermo Fisher scientific, SL16R) at 3000 rpm for 15 minutes. The supernatants containing unwanted microorganisms were decanted and solids containing microalgae cells were suspended in sterile brine water and thoroughly vortexed. The procedure was repeated five times until a clarified cell suspension was achieved. The microalgae cells from the suspension were observed under a light microscope (Fisher brand, AX800) and morphologically identified as *D. salina* according to Ralefala [22] identification key.

The identified *D. salina* cells were preserved in the Conway medium at a ratio of 1:9, v/v of cell suspension and the medium [157]. Conway medium [157] is constituted of the following reagents: 1 ml of solution A, 0.5 ml of solution B and 0.1 ml of solution C all in 1 L filtered and sterilised brine water and their chemical composition are shown in Appendix A1, Table A1. The preserved cultures were transported to Botswana International University of Science and Technology (BIUST) in sterile Duran bottles for further culturing and analysis.

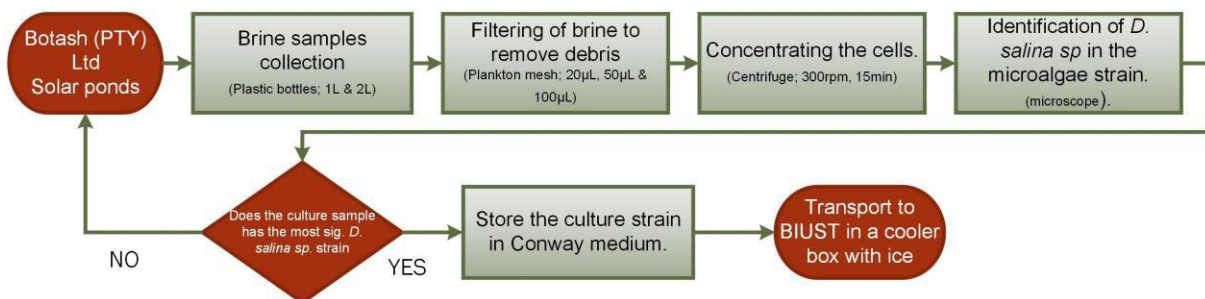


Figure 3.2: Process flow chart of sample collection from the brine ponds and transportation to the laboratory.

3.2 Culturing and growth conditions of the isolated *D. salina*

3.2.1 Culture medium

The f/2 medium is a commonly used medium for culturing microalgae [158]. The f/2 medium was used in the study, with variations for different investigations. The f/2 medium is composed of the following reagents: 1 ml of NaNO_3 , 1 ml of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 ml of Na_2CO_3 , 1 ml of trace metals and 0.5 ml of vitamin solution all in 950 ml of sterilised brine (Volumetric flask, 1-L). The preparation of f/2 medium trace metals and vitamins is shown in Appendix A1, Table A2-A3. The medium was prepared and autoclaved at 120 °C for 15 minutes as reported by Robert [159].

The medium variation by Ahmend [21] was adopted for nutrient optimisation as it proved to promote lipid production on *D. salina* microalgae. The medium was composed of the following components: 0.42 g L^{-1} of NaNO_3 , 0.0156 g L^{-1} of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.84 g L^{-1} of NaHCO_3 , 0.044 g L^{-1} of CaCl_2 , 1.23 g L^{-1} of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 117 g L^{-1} of NaCl_2 , and 5.46 mL L^{-1} of A5 solution. The A5 solution composed of the following composition: 0.0005 g L^{-1} of $\text{C}_5\text{H}_8\text{FeO}_7$, 0.05 g L^{-1} of $\text{CO}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 1.86 g L^{-1} of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.39 g L^{-1} of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2.86 g L^{-1} of H_3BO_3 , 0.08 g L^{-1} of $\text{ZnCuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.22 g L^{-1} of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ [23].

3.2.2 Culturing and growth conditions

Upon arrival at BIUST lab, the concentration of 1 ml of microalgae culture samples in Conway medium was determined using the UV/vis spectrophotometer (VWR, UV-1600PC) set at 680 nm and Conway medium was used as blank during the measurement [21,160]. In order to obtain

pure cultures of the *D. salina* (Figure 3.3), the cells were spread-plated on solid Conway agar plates according to Suzuki [161]. Conway agar plates were prepared by mixing nutrient agar powder (1.5 g) with 100 ml of sterilised brine water. The mixture was autoclaved (Hirayama HiClave, HV-85) at 120 °C for 15 minutes and poured into sterilised petri dishes and left to solidify. A 0.1 ml of culture sample was dropped and spread onto the solidified agar using a sterilised glass rod, which was incubated for 8 days at room temperature and illuminated under the fluorescent light for a 12-hour exposure with a light intensity of 1000 lux (Gastools digital lux meter, VA-8050) [161,162]. To get pure isolates, the single colonies were picked and streaked on the agar using a sterilised streaking hook. After getting pure cultures, single colonies were aseptically transferred to test tubes containing 10 ml of Conway liquid medium using a sterilised streaking loop. The test tubes were incubated in an orbital shaker (Max Q6000, SHKE6000-8CE) at 120 rpm, 25 °C and 1000 lux intensity for 12:12 hour light to dark photoperiod for 10 - 14 days.

A total of 14 flasks for stock samples were prepared for further biomass accumulation and the cultures were subcultured once a month to maintain healthy microalgae cells. The growing cells from the test tubes were transferred to 250 ml flasks at a ratio of 1:15 (v/v) in sterile Conway medium. Cotton wool bud was used to close the flasks to ensure free air circulation into the samples. The cultures were grown outside in natural sunlight (22.5946° S, 27.1233° E) at a daily average temperature of 25 °C under 12:12 hour light to dark. Figure 3.4(a) - (b) shows the change of *D. salina* microalgae growth from the 1st day to the 30th day of culturing indicating an increase in cell population in cultures, (c) shows the experimental cultures at the outdoor environment.

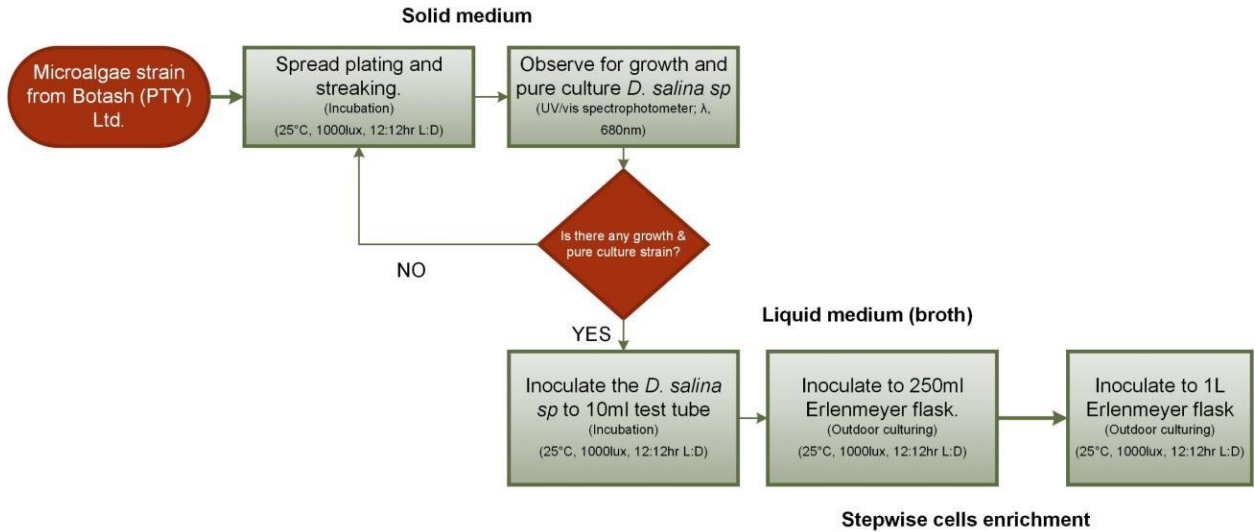


Figure 3.3: *D. salina* microalgae culture preparation

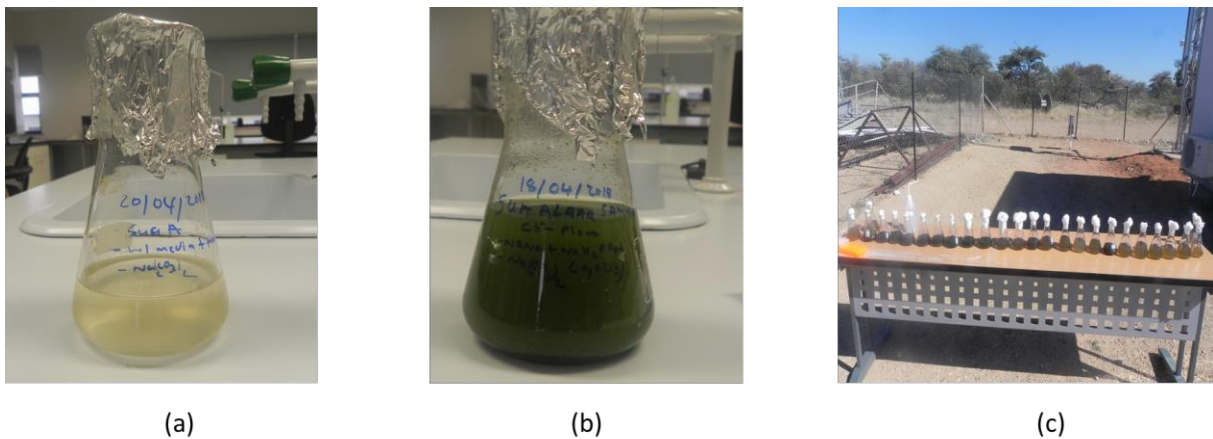


Figure 3.4: *D. salina* cell growth in liquid cultures: (a) day one, (b) day thirty culture (c) outdoor cultivation.

3.3 Optimisation of culture conditions for biomass and lipids accumulation

3.3.1 Salinity determination for maximum biomass and lipids accumulation

Salinity affects the microalgae cell growth and lipid accumulation [23]. The One Factor at A Time approach (OFAT) was used to determine the optimum salinity concentration to achieve a maximum biomass and lipids accumulation [163–165]. Eqns. (2.2) - (2.5) (Chapter 2, section 2.3) was used for biomass growth rate, biomass productivity, lipid content and lipid productivity

respectively. Salinity levels were varied at 1.5, 2.0, 2.5 and 4.0 M as obtained from different brine ponds; CS, E3, E6 and NS respectively and the growth curves for different salinities were plotted. Descriptive statistics with Levene's test and Turkey post hoc test were used to test the significance with Anova on the samples using statistics software (IBM SPSS, 25v) [166,167].

3.3.2 RSM experimental design for nutrient optimisation for maximum biomass growth and lipid production

Further investigation in this study was conducted to determine nutrients that significantly affected biomass concentration, lipid content and lipid production. RSM was applied in the experimental design model build-up, investigation and optimisation of culture nutrient composition for lipid production [78,168,169]. The experimental design was divided into two stages. The first stage employed the Plackett-Burman design to determine significant nutrients that have effects on biomass concentration, lipid content and lipid production [170,171]. The relationship of biomass concentration, lipid content and lipid were also determined and Eqn. (2.6) (Chapter 2, section 2.3) was used to establish the relationship [172]. The second stage used the Box-Behnken design, which identified and analysed the significant nutrients from the first stage in order to investigate the optimum nutrient concentrations that yielded the highest lipid production [173]. Lipid production is a dependent indicator on biomass concentration and lipid content hence making it a reliable indicator for optimum nutrient concentration determination.

3.3.2.1 Plackett-Burman experimental design

Plackett-Burman design was used to identify independent variables that have a significant effect on biomass concentration, lipid content and lipid production [173–175]. In this study, Stat-Ease Design Expert software (version 10.0.8.0) was used to formulate the randomised matrix, as shown in Table 3.1 with the six studied nutrients (coded A to F) and dummy variables (coded G to L) making up the matrix. Each nutrient had two-level concentrations (-1: low level, +1: high level) that corresponded to the actual level, as shown in Table 3.2. Twelve runs were carried out in triplicates and the average response output was used as the response indicator [176]. The experiment was tested at a P-value < 0.05 to determine the variables that have significant effects on the response variables. To justify the statistical significance of the nutrient

variables on the response variables, coefficient of estimate (β), the sum of squares (SS), F-value and contribution percentile were further evaluated. A model (factorial with main effects) was generated and its adequacy was determined by the coefficient, adjusted, predicted coefficient of determinations (R^2 , R^2_{adj} and R^2_{pred} respectively). A graphical plot of the predicted variables against actual variables was used to verify the accuracy of the model. A model to predict the response variables (biomass concentration, lipid content and lipid production) with the nutrients is shown in Eqn. (3.1) [172]. A relationship on the response variables (biomass concentration, lipid content and lipid production) was determined using SPSS statistics software (version 25) and a model Eqn. (3.2) was generated. The model was tested for its adequacy by evaluating its R^2 and the significance using the ANOVA analysis.

Table 3.1: Plackett-Burman Experimental Design Matrix coded

RUN	Independent variables										
	A	B	C	D	E	F	G	H	J	K	L
1	1	1	1	-1	-1	-1	1	-1	1	1	-1
2	-1	1	1	1	-1	-1	-1	1	-1	1	1
3	-1	1	1	-1	1	1	1	-1	-1	-1	1
4	-1	1	-1	1	1	-1	1	1	1	-1	-1
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
6	1	1	-1	1	1	1	-1	-1	-1	1	-1
7	1	-1	1	1	1	-1	-1	-1	1	-1	1
8	1	1	-1	-1	-1	1	-1	1	1	-1	1
9	1	-1	1	1	-1	1	1	1	-1	-1	-1
10	-1	-1	1	-1	1	1	-1	1	1	1	-1
11	-1	-1	-1	1	-1	1	1	-1	1	1	1
12	1	-1	1	1	-1	-1	-1	-1	1	-1	1

Table 3.2: Medium component's actual high and low-level concentration [21]

Variable code	Nutrient variable (mg/L)	(-1)	(+1)
A	NaHCO ₃	840	1680
B	NaH ₂ PO ₄ .2H ₂ O	15.6	31.2
C	NaNO ₃	420	840
D	CaCl ₂	44	89
E	MgSO ₄ .7H ₂ O	1227	2460
F	A5 solution (ml /L)	5.46	10.92

$$R_Y = a_0 + a_1A + a_2B + a_3C + a_4D + a_5E + a_6F \quad (3.1)$$

Where: R_Y is the response variable of either biomass concentration or lipid content or lipid production, a_0 is the y-intercept, a_1 - a_6 are the coefficient of the estimate of nutrients coded A to F.

$$R_3 = a_0 + a_1R_1 + a_2R_2 \quad (3.2)$$

Where: a_0 is the y-intercept, a_1 is the coefficient estimate of biomass concentration (R_1), a_2 is the coefficient estimate of lipid content (R_2), R_3 is the lipid production.

3.3.2.2 Box-Behnken experimental design

Based on the results from the Plackett-Burman design, The Box-Behnken design was used to further optimise the selected nutrients variables with significant effects on lipid production. NaHCO₃, NaH₂PO₄.2H₂O and NaNO₃ were selected and further optimised to maximise the lipid production while other variables were kept constant as from the results of Plackett-Burman design. The variables had three experimental levels, as shown in Table 3.3, and the experiment was carried out with 17 runs, including five centre points replicates and 12-star points (Table 3.4). The polynomial model (Eqn.3.3) obtained from the experimental results was used to predict lipid production.

Table 3.3: Medium nutrient's three-level factor [21]

Variable code	Medium nutrient	Levels (mgL ⁻¹)		
		–	0	+
A	NaHCO ₃	840	1260	1680
B	NaH ₂ PO ₄ ·2H ₂ O	15.6	23.4	31.2
C	NaNO ₃	420	630	840

Table 3.4: Box-Behnken Experimental Design Matrix coded

RUN	Independent variables		
	A	B	C
1	0	0	0
2	0	1	1
3	-1	0	1
4	0	0	0
5	0	0	0
6	0	-1	-1
7	-1	1	0
8	1	0	-1
9	1	1	0
10	0	0	0
11	-1	-1	0
12	1	0	1
13	1	-1	0
14	-1	0	-1
15	0	-1	1
16	0	1	-1
17	0	0	0

$$\ln(R_3) = a_0 + a_1A + a_2B + a_3C + a_4AB + a_5AC + a_6BC + a_7A^2 + a_8B^2 + a_9C^2 \quad (3.3)$$

Where: R_3 represents the predicted lipid production response, a_0 the mean effect, a_1 - a_3 are the linear coefficients, a_4 - a_6 are the cross coefficients, a_7 - a_9 are the quadratic and A, B, and C are the selected significant independent variables with effects on lipid production.

The coefficient of determination (R^2) indicated the goodness of fit on the polynomial model equation [177]. The F-test at 5 % significance level was used to test the model adequacy. The response surface contour plots were generated to indicate the possibility of increasing lipid production [170]. All the experiments were conducted in triplicates, and the average predicted lipid content was used as the response variable [174].

3.4 Harvesting of *D. salina* biomass and lipid extraction

3.4.1 Harvesting of *D. salina* biomass

During the culturing period in optimised salinity and medium composition, the cell growth was monitored by measuring the optical density at a wavelength of 680 nm every 3rd day using a UV/vis spectrophotometer (VWR, UV-1600PC). The cells were cultured until they reached the stationary phase and harvested after 36 days of culturing. The cells were harvested in 50 ml centrifuge tubes using a centrifuge (Thermo Fisher scientific, SL16R) set at 5000 rpm for 10 minutes at 4 °C. After centrifugation, the supernatant was decanted and the solid part (wet biomass) was re-suspended in 25 ml of distilled water and centrifuged again [178]. The procedure was repeated four times to ensure that the biomass was free from unwanted debris. The wet biomass was weighed (Adam, PGW 453e), before it was dried in an oven (Systronix scientific, 278) set at 80 °C for 8 hours using aluminium crucibles (Ø, 4 cm) [179]. The dry biomass was thereafter cooled in a fume hood (Esco, EFA6UDRVW) for 10 minutes and weighed before being transferred to 10 ml test tubes for storage in a 4 °C cold room. Figure 3.5(a) - (c) shows the microalgae cells after 36 days in the liquid culture medium, the harvested wet biomass, and the dried biomass, respectively.

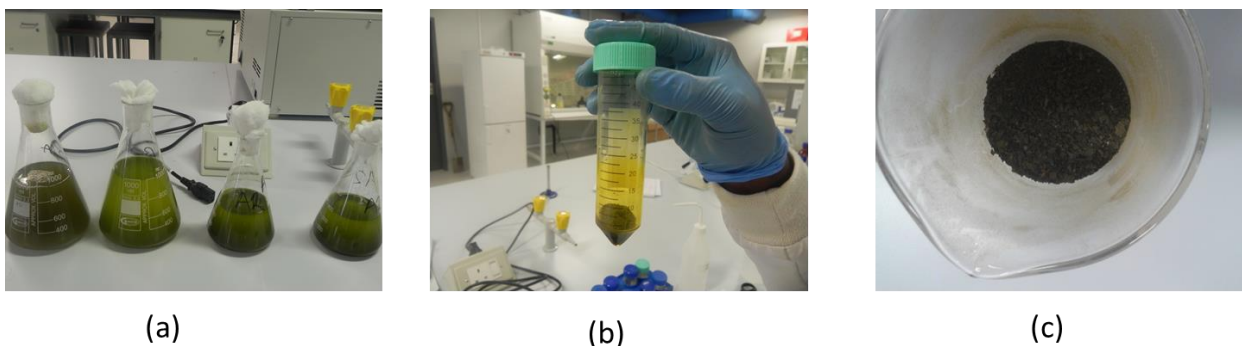


Figure 3.5: *D. salina* biomass: (a) liquid cultures, (b) wet biomass (c) dry biomass

3.4.2 Lipid extraction from dried biomass

Lipids extraction from the dried biomass was carried out according to the modified Bligh and Dyer method modified [180–182], as shown in Figure 3.6. One gram of dry biomass was suspended in 19 ml of extraction solvent mixture composed of chloroform:methanol:DI at a ratio of 1:2:0.8 (v/v/v) respectively [183]. The mixture was placed in an ultrasonicator (Africa Sal, 704) for 1 hour at 300 W and then transferred to an orbital shaker (Lasec, 1KA@KS3000i) operated at 120 rpm for 8 hours to ensure effective extraction. A 10 ml of chloroform to water (1:1, v/v) was added to the above mentioned mixture, vortexed for 30 seconds to ensure even mixing and thereafter centrifuged at 4850 rpm for 10 minutes at 4 °C [184]. After centrifugation, the mixture was separated into three layers using a pipette; the methanol-water mixture (top layer), and chloroform-lipid layer (bottom layer). The middle layer biomass was left in the centrifuge tube where the extraction was repeated twice. In the third extraction step, only 5 ml of chloroform was added to the biomass from the second extract and left for an hour on a stand before the separation of the resulting mixture. The chloroform-lipid mixtures from all the extraction steps were pooled and filtered using a 0.2 µm nylon syringe. The chloroform-lipid mixture was then separated using a separating funnel where the lipids components were on top [185]. Figure 3.7(a)-(d) depicts lipid extraction steps.

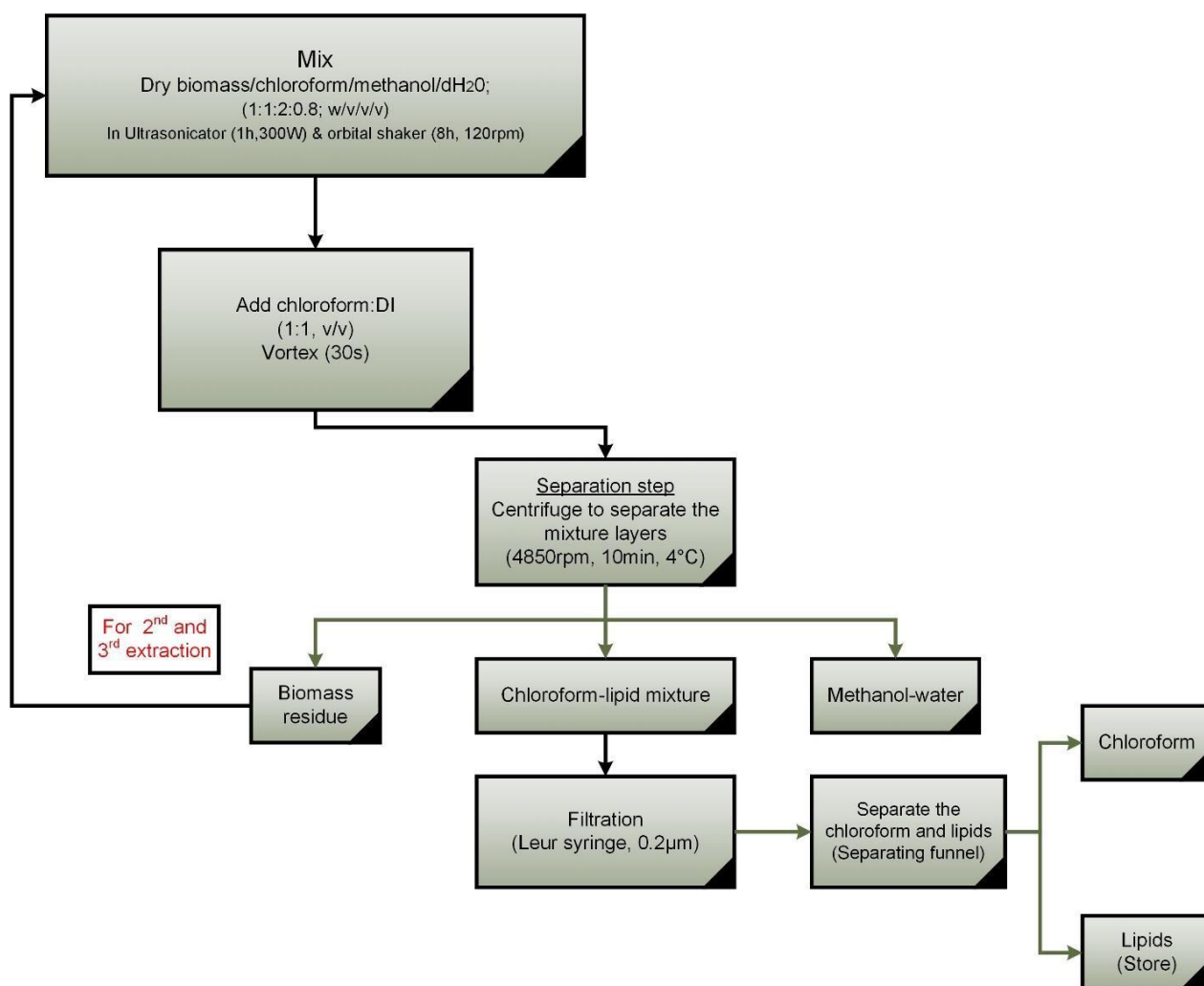


Figure 3.6: Lipid extraction process flow from dry *D. salina* algae biomass using chloroform:methanol:DI

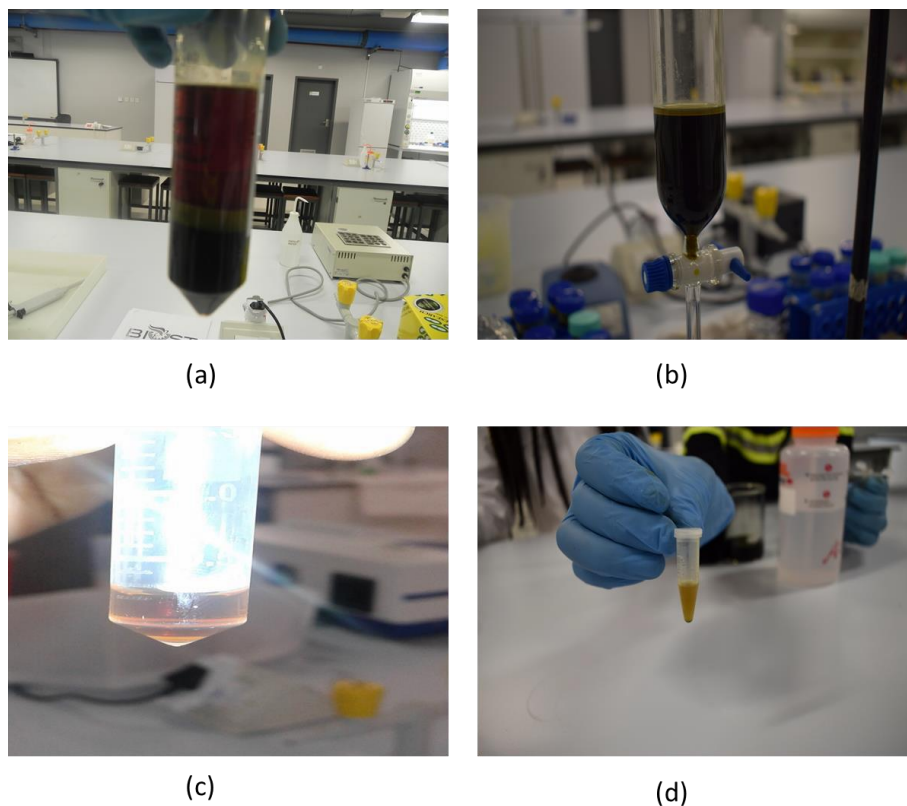


Figure 3.7: Lipid Extraction of *D. salina* microalgae dry biomass: (a) mixture phase (chloroform, biomass, methanol, water) (b) chloroform-lipid mixture (c) methanol-water mixture (d) extracted lipids

3.5 Identification of lipids extracted from *D. salina* using Thin Layer Chromatography (TLC) method

TLC method was used as a rapid screening method to assess the lipid components of extracted lipids for further characterisation [186]. The TLC protocol was composed of a 150 ml solvent mixture of hexane, diethyl ether and acetic acid (60:40:1, v/v/v), which was prepared, poured into a TLC chamber and covered to prevent solvent evaporation [187]. A 20×20 cm TLC plate was put in the chamber to attain saturation and was activated by placing it on a hot silica plate (F254) base positioned for 3 min. A 10 μ L of lipids extracts were loaded 6 mm from the plate bottom with a diameter not exceeding 4 mm before being left to dry. The TLC plate was carefully placed into the chamber for 30 minutes. The plate was then removed from the chamber and left to dry in a fume hood (Esco, EFA6UDRVW). When the plates were dry, they were

sprayed with iodine until they were moist for spots identification through colour development. The observed yellow coloured spots were marked with a pencil and their retention factors (RFs) were calculated using Eqn. (3.4).

$$RFs = \frac{D_{solute}}{D_{solvent}} \quad (3.4)$$

Where: RFs is the retention factor, D_{solute} is the distance travelled by different components of lipids, $D_{solvent}$ is the solvent distance.

3.6 Transesterification and characterisation (biodiesel) of extracted lipids.

The transesterification method used was adopted and modified from several studies that focused on the direct transesterification of lipids using alkaline catalysts [49,90,188–190]. The procedure determined the alkali quantity (NaOH and KOH) for transesterification reaction and the evaluation of the best catalyst that yields the highest biodiesel during the transesterification reaction as explained in section 3.6.1 The assays and chemical used are shown in Appendix A1, Table A4.

3.6.1 Determination of optimum concentration of alkali catalyst amount for transesterification reaction.

Due to various research that had been conducted on transesterification reactions using sunflower oil and its availability in large quantity led to the selection of the oil to determine the amount of NaOH and KOH catalysts and act as a control [191–193]. An alkali (NaOH or KOH) titrant solution of 1% was prepared by mixing 1 g of alkali with 40 ml of DI in a beaker and transferring the mixture into a 1 L volumetric flask. The mixture was brought to calibration mark with DI. A mixture of 10 ml isopropyl alcohol and three drops phenolphthalein indicator (analyte) was prepared by continuous stirring until a yellow colour developed. A blank titrant was prepared by titrating the alkali solution (titrant) to the analyte solution while swirling until a purple colour developed. One millilitre of sunflower oil was added to the blank and alkali titrant. The mixture was swirled until a purple colour developed. All the final titrant results were recorded and the catalyst amount as shown in Eqns. (3.5).

$$m_{alkali} = x \times (w_{alkali} + y) \quad (3.5)$$

Where: m_{alkali} is the mass of alkali (g) either NaOH or KOH, x is the volume of oil (L) required for the reaction; w_{alkali} is 5g mass of alkali, y is the final alkali titrant volume (ml).

With reference to the amount of catalyst required as calculated using Eqn. (3.5), an alkali alcoholic catalyst (NaOH and KOH) was prepared by adding 100 ml methanol in a volumetric flask while stirring until the catalyst dissolved. An aliquot of 3 ml oil (sunflower and *D. salina* lipids) was warmed in a water bath set at 60 °C for 10 minutes and the alkali alcoholic catalysts were added at a ratio of 6:1 (v/v) of alkali alcoholic catalyst and oil respectively [194,195]. The resultant mixture was heated with continuous mixing on a magnetically stirred hot plate set at 60 °C for 2 hours [49,99,188]. The mixture was then transferred to a separating funnel for layer separation of biodiesel and glycerol, where the bottom layer (glycerol) was removed first and biodiesel was collected next and stored in Eppendorf safe-lock tubes. The process is shown in Figure 3.8(a)-(c). The biodiesel and glycerol yields were calculated using Eqns. (3.6)-(3.7) respectively assuming non-formation of residuals.

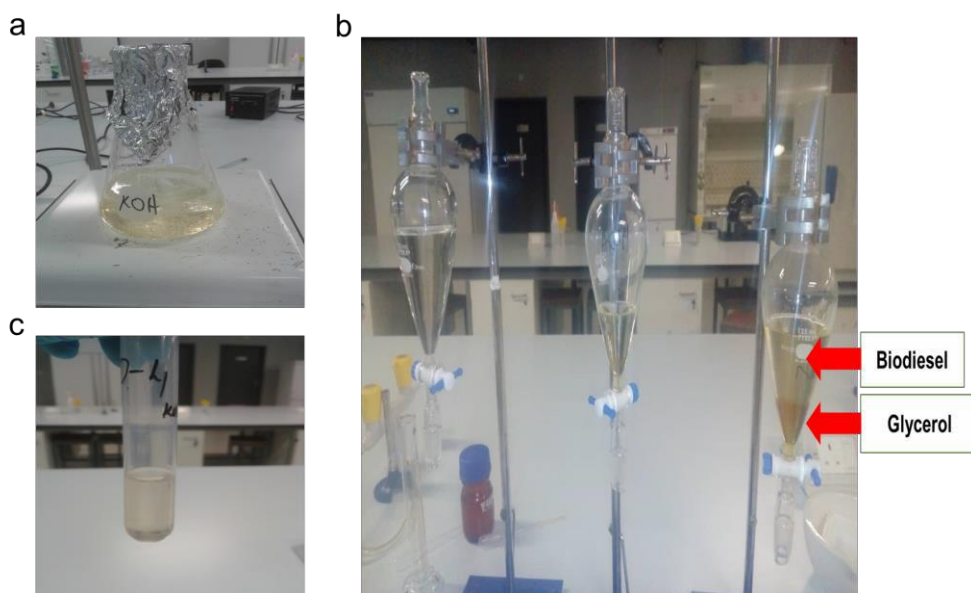


Figure 3.8: (a) Transesterification reaction process, (b) settling process of biodiesel, (c) biodiesel collected.

$$Y_{Biodiesel} = \frac{V_{Biodiesel}}{V_{Oil}} \times 100\% \quad (3.6)$$

Where: $Y_{Biodiesel}$ is the biodiesel yield and $V_{biodiesel}$ is the volume of the produced biodiesel.

362 Identification of the produced biodiesel from the extracted lipids using Fourier Transform Infrared spectroscopy (FTIR) method

FTIR is widely used as a rapid analysis method to determine the organic components in biodiesel [49,116,118,196]. FTIR was applied for identification of the produced biodiesel from the extracted lipids and biodiesel produced from sunflower was used as a reference. The FTIR machine used was the Bruker Vertex 70v (Billerica, United States of America) instrument supported by OPUSTM operation and evaluation software for the identification of the functional groups in the biodiesel. The FTIR total reflectance cell (ATR) was cleaned with ethanol, a blank scan was performed on an empty ATR cell at a wavelength range between 500 and 4000 cm^{-1} with a resolution of 4 cm^{-1} and a scan rate of 16 cmmin^{-1} [118]. A small sample droplet was placed onto the ATR cell and a scan was run to generate an FTIR spectrum, which was then evaluated. Upon completion of each sample run, the ATR cell was cleaned with ethanol before the analysis of another sample.

363 Quantification of *D. salina* biodiesel using GC-MS

GC-MS was used for the quantification of *D. salina* biodiesel. The samples were tested at the University of South Africa (UNISA) using the GC-MS machine (Agilent; 6890-5975S). The method used was adopted from various researchers who also identified and quantified esterified lipids from microalgae [49,50,119]. To ensure that the GC-MS machine is not damaged, a small aliquot of each sample (1 μL) was diluted with hexane at a ratio of 1:10 (v/v). One microliter of the diluted sample was injected into the GC-MS machine (Agilent; 6890-5975), which had a fused silica dimension with a length of 30 m, a diameter of 250 μm and film size of 0.1 μm [197]. Prior to each injection, the syringe was rinsed with ethyl acetate and dichloromethane. The oven program was set as follows: The initial temperature was held at 60 $^{\circ}\text{C}$ for 1 minute, increased at 5 $^{\circ}\text{Cmin}^{-1}$ to 235 $^{\circ}\text{C}$ where it was held constant for 10 minutes and finally increased to 290 $^{\circ}\text{C}$ at 10 $^{\circ}\text{Cmin}^{-1}$ and held constant for 15 minutes [198]. The injector temperature and

the detector temperature were set at 220 °C and 250 °C respectively. Helium was the carrier gas used, with a flow rate of 0.8 mlmin⁻¹. The mass spectrometer had a relative electron impact ionisation (EVI) at 1447 eV with a source temperature of 230 °C and quadrupole temperature at 150 °C [197]. A normal scan speed of 2 scans per second was carried out at a scan range from 50 to 600 m/z with a 4 minutes initial solvent delay [197,199]. The MSD ChemStation software identified the chemical constituents in the biodiesels sample.

3.7 Physio-chemical characterisation and kinetic study of *D. salina* derived biodiesel

Saponification value (SV), acid value (AV), free fatty acid (FFA), ester value (EV) and iodine value (IV) were the chemical parameters while density (ρ) and thermal studies were the physical parameters that were characterised from *D. salina* biodiesel. Biodiesel from sunflower oil and was used as a reference. The results were compared with the specified range of European Committee for Standardisation (EN 14214), American Society for Testing and Materials (ASTM D6751) and South African National Standard (SANS1935:2004) [128,200–203].

3.7.1 Saponification value (SV)

The method from SANS (1935:2004) standards was used to determine the SV of the biodiesel produced from *D. salina*. The chemical reagents were prepared as shown in Appendix A2. Approximately 2 g of the sample was weighed and transferred to a 250 ml conical flask, where 25 ml of 0.5 N of ethanolic potassium hydroxide (C₂H₇KO₂) was added. The mixture sample attached to a reflux condenser was placed in a water bath set at 100 °C with continuous stirring for 1 hour. Three drops of phenolphthalein indicator were added to the solution where 0.5 N of HCL was titrated onto the solution until a purple colour was observed [204]. The procedure was repeated without the biofuel samples to measure a blank titration. Eqn. (3.7) was used to calculate the SV of the biofuel under study.

$$SV = \frac{56.1(B-S)N}{W} \quad (3.7)$$

Where: B is the volume of 0.5 N HCl required for the blank (ml), S is the volume of 0.5 N HCl required for the sample, N is the normality of the standard hydrochloric acid (0.5) and W is the weight of the sample under teste (g).

372 Acid value (AV), Free fatty acid (FFA), Ester value (EV)

The American Society for Testing and Materials (ASTM D974) method was used to determine the acid value of *D. salina* biodiesel. A 20 mg sample was weighed, placed in a 25 ml conical flask containing 10 ml of ethanol and 3 droplets of phenolphthalein indicator. The mixture was heated in an 80 °C water bath for 10 minutes and allowed to cool before being titrated with 0.1 N of KOH (Appendix A3) until a pink colour appeared. The titrant values were used to calculate the AV of the fuels under study. Eqns. (3.8)-(3.10) shows how AV, FFA and EV is calculated respectively [126,127,205,206].

$$AV = \frac{V_{KOH} \times N \times 56}{m} \quad (3.8)$$

$$FFA = AV \times 0.503 \quad (3.9)$$

$$EV = SV - AV \quad (3.10)$$

Where: V_{KOH} is the volume of KOH used to reach the endpoint, N is the normality of KOH (0.1N), m is the mass of the sample (mg). FFA is the free fatty acid of the sample (%).

373 Iodine value (IV)

For the determination of the Iodine value in the *D. salina* biodiesel, Hanus method according to AOAC 920.158 standard was used [123,207,208]. Reagents were prepared as shown in Appendix A4. A mixture of 0.25 g of the sample, 10 ml of chloroform and 30 ml of Hanus solution was prepared and placed in a 250 ml conical flask which was then sealed with Parafilm and placed on a shaker for 30 minutes operated at 800 rpm. Ten millilitres of 15% potassium iodide (KI), 100 ml of DI and 3 drops of the starch solution were added to the solution mixture where it was thoroughly mixed by shaking. The solution was titrated with 0.1 N of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) solution until a blue colour was observed and the endpoint titrant volume was recorded. IV was calculated using Eqn. (3.11).

$$IV = \frac{(B - S) \times N \times 0.127 \times 100}{W} \quad (3.11)$$

Where: B is the volume of sodium thiosulfate for the blank (ml), S is the volume of Na₂S₂O₃ volume for the sample (ml), N is the normality of Na₂S₂O₃ and W is the weight of the sample (g)

3.7.4 Density determination

The density of the *D. salina* biodiesel was determined following the method by Ramluckan [49]. The method involves measuring a known volume and mass of the biodiesel using a micropipette and an analytical mass balance (Adam, PGW438e) respectively. The density was then calculated using Eqn. (3.12).

$$\rho = \frac{m}{V} \quad (3.12)$$

Where: ρ is the density (kgm⁻³), m is the mass (kg), V is the volume (m³).

3.7.5 Thermal characterisation, and kinetics study of *D. salina* biodiesel.

The thermal characterisation of the produced biodiesel from *D. salina* was subdivided into the thermogravimetric analysis, combustion characteristic analysis, thermogravimetric kinetic analysis, thermodynamics kinetic parameters and the higher heating value of the biodiesel as explained in subsection 3.7.5.1-3.7.5.5 below. Biodiesel from sunflower oil and petroleum were used as controls in the experiments.

3.7.5.1 Thermogravimetric analysis

Thermal parameters of the produced biodiesel from *D. salina* was determined by analysing the samples in a thermogravimetric analyser (Mettler Toledo DSC/TGA 3+). The study adopted the method reported by Santos [209] in which the thermogravimetric analyser was operated at a flow rate of 100 mlmin⁻¹ in an air atmosphere with a temperature span between 25 °C to 600 °C at different heating rates (5, 10 and 15 °Cmin⁻¹). The samples were measured using an ultra-high precision mass scale attached with the thermogravimetric analyser (Mettler Toledo DSC/TGA 3+) in the crucibles, which were then transferred to the oven for combustion. To ensure uniformity of results, the sample masses were evaluated in percentile form. Reaction region, peak temperature and the mass loss were determined and analysed using thermal software (STARE software).

3.7.5.2 Combustion characteristic analysis

The study adopted the intersection method by Almazrouei [139] and Ahn [140] to determine the ignition and burnout temperature [139]. Ignition index (D_i) and comprehensive performance index (D_c) were determined at different heating rates (5, 10 and 15 °Cmin⁻¹) to evaluate the combustion characteristics of *D. salina* biodiesel [144,147,148]. Eqns. (3.13)-(3.14) were used to calculate the ignition index (D_i) and comprehensive performance (D_c) respectively [139,141,142,145,210].

$$D_i = \frac{DTG_{\max}}{T_i T_p} \quad (3.13)$$

$$D_c = \frac{DTG_{\max} DTG_{\text{mean}}}{T_i^2 T_b} \quad (3.14)$$

Where: $DTG_{\max}$ is the maximum rate of mass loss, DTG_{mean} is the mean rate of mass loss, T_i is the ignition temperature (°C), T_p is the peak temperature (°C), T_b is the burnout temperature (°C) [211].

3.7.5.3 Thermogravimetric kinetic analysis

Thermogravimetric kinetic parameters (activation energy and Arrhenius constant) were evaluated using the non-isothermal Kissinger model-free method [139]. Eqn. (3.15) above shows the mathematical model that was used which is developed by determining peak temperatures with the maximum mass loss rate at a different heating rate (5, 10 & 15 °Cmin⁻¹). An X-Y plot was developed with $\ln(\beta T_p^{-2})$ as the y-axis, T_p as the x-axis, $\ln(ARE^{-1})$ as the y-intercept. The activation energy (E) was determined from the plots and Arrhenius Constant (C) was determined on the y-intercept. The reaction was assumed to be a first order ($n=1$) for the model to become applicable.

$$\ln\left(\frac{\beta}{T_p^2}\right) = \ln\left(\frac{AR}{E}\right) - \frac{E}{RT_p} \quad (3.15)$$

Where: β is the heating rate (°Cmin⁻¹), T_p is the peak temperature (°C), A is the Arrhenius constant (min⁻¹), E is the activation energy (kJmol⁻¹), R is the gas constant (Jmol⁻¹k⁻¹).

3.7.5.4 Thermodynamics kinetic parameters

The following equations from Li [134] and Almazrouei [139] were used to determine the Enthalpy (ΔH), Gibbs free energy (ΔG) and Entropy (ΔS) of the samples under study and the Eqns are (3.16) – (3.18) respectively.

$$\Delta H = E_a - R \quad (3.16)$$

$$\Delta G = E_a + RT \ln\left(\frac{kT_p}{hA}\right) \quad (3.17)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T_p} \quad (3.18)$$

Where: k is the Boltzmann constant ($1.3819 \times 10^{-23} \text{ J K}^{-1}$), h is the Plank constant ($6.626 \times 10^{-34} \text{ Js}$) and T_p is the peak temperature (K).

3.7.5.5 Higher heating value (HHV)

The produced *D. salina* biodiesel, sunflower oil biodiesel, and petroleum diesel samples were sent to UNISA in South Africa for the analysis of the higher heating value. DIN 51900 standard was used in testing samples. A bomb calorimeter (IKA, CI) was used in determining the higher heating values of the samples. The samples were placed in a ceramic crucible and were weighed by a mass balance (Adam, PGW438e) and placed in the bomb. The sample in the crucible was connected to the ignition wire by a cotton thread and later transferred into the water jacket chamber. The vessel was then filled with oxygen at 30 bar and the sample combusted. which was closed for testing. During combustion process, the core temperature of the crucible rose to 1,000°C. The heat produced is the higher heating value.

3.8 Conclusion

Methods on *D. salina* culturing optimisation, harvesting, drying, extraction of lipids, conversion of lipids to biodiesel, identification and characterisation of the produced biodiesel diesel have been discussed in this chapter. Due to the limitation in the sample size of the *D. salina* biodiesel experiments were minimised to milligram quantity size, if the sample size was big, pour point and cloud point experiments could have been conducted. The following chapter

(Results and Discussion) presents and explains the results obtained from the methods explained in this chapter.

CHAPTER 4: RESULTS AND DISCUSSION

The results of *D. salina* biodiesel production potential are presented in this chapter. Section 4.1 presents the results on the effect of medium salinity on biomass and lipids growth. Section 4.2 presents the results of nutrient optimisation for maximum biomass and lipids growth. Qualitative characterisation of lipids extracted from *D. salina* is presented in section 4.3. Transesterification, identification and quantification of biodiesel produced from the extracted lipids are presented in section 4.4 while section 4.5 presents the physiochemical characterisation and kinetic study of the produced biodiesel.

4.1 Determination of the optimum salinity concentration for maximum production of biomass and lipids in *D. salina*

Salinity concentration in the culture medium has an effect on the growth of *D. salina* microalgae. As described in section 3.3.1, four different salinity concentrations (1.5, 2.0, 2.5 and 4.0 M) used in this research were obtained from the Botash brine ponds (CS, E3, E6 and NS respectively). The results of the biomass and lipids growth are presented in Figure 4.1. *D. salina* growth profile and dry biomass are explained in section 4.1.1 while the specific growth rate and biomass productivity is explained in section 4.1.2. Section 4.1.3 explains lipid accumulation and productivity.

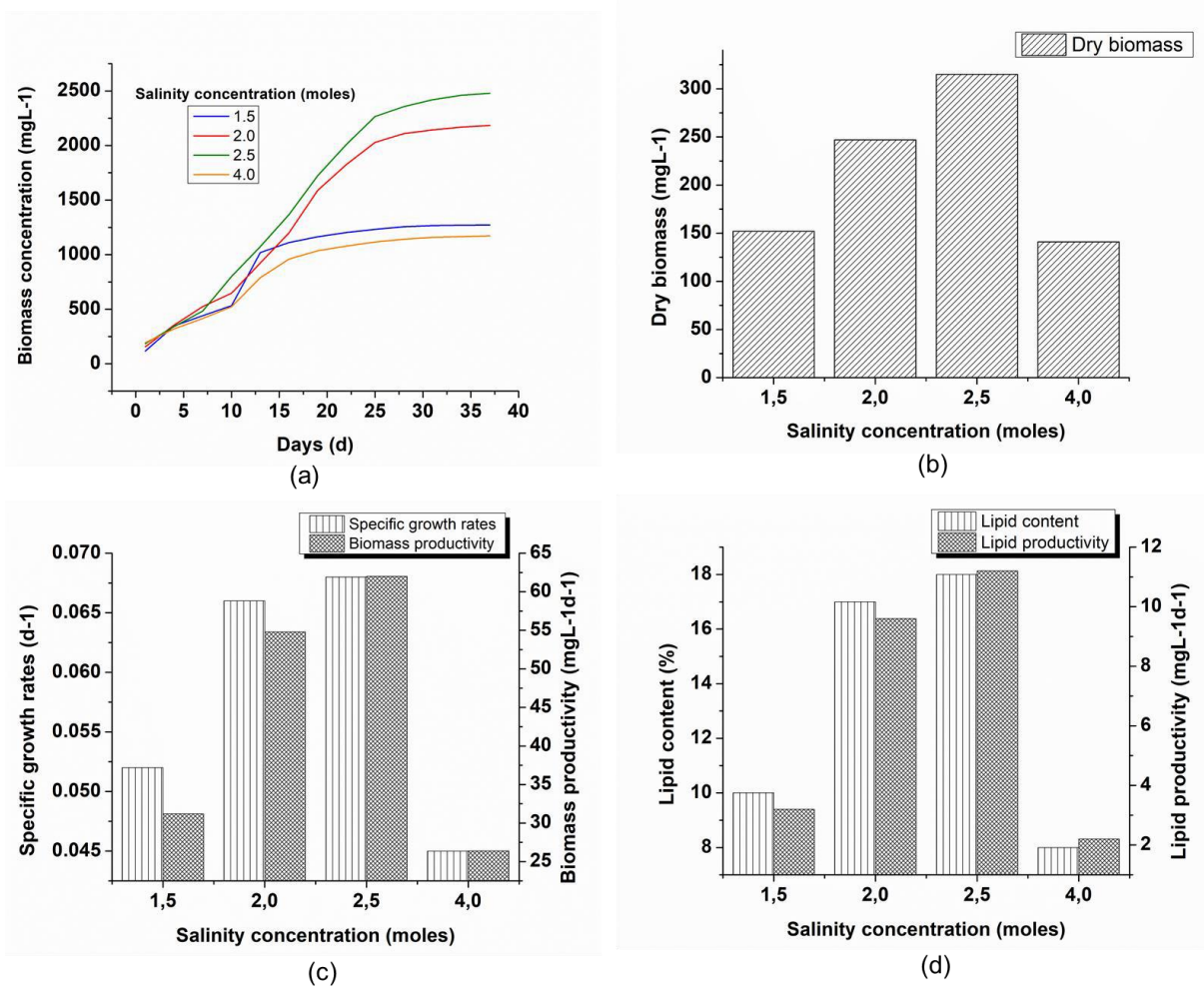


Figure 4.1: *D. salina* in different salinity: (a) growth profile, (b) dry biomass, (c) specific growth rate and biomass productivity, (d) lipid content and lipid productivity

4.1.1 Effect of salinity concentration on biomass growth

After culturing *D. salina* cells for 37 days as depicted in Figure 4.1(a), the salinity of 2.5 and 2.0 M produced the highest biomass concentration of $2480 \pm 75 \text{ mgL}^{-1}$ and $2183 \pm 29 \text{ mgL}^{-1}$ respectively, and salinity of 1.5 and 4.0 M produced the lowest biomass at $1271 \pm 55 \text{ mgL}^{-1}$ and $1171 \pm 14 \text{ mgL}^{-1}$ respectively. The biomass growth data did not violate any assumptions, that is the normality (Shapiro-Wilk test, p-value = 0.000, Appendix B1, Table B1), homogeneity of variances (Levene's test, p-value = 0.000, Appendix B1, Table B2) and outliers (Appendix B1, Figure B1) meaning that a correct conclusion was drawn from the results of the analysis.

Salinity had a statistical significance on biomass growth ($df = 3$, $F = 10.791$, $p\text{-value} = 0.000$). Our results with respect to the salinity were relatively similar to those reported by Ralefala [22], who noted a salinity of 2.4 M in *D. salina* cultures produced a maximum biomass concentration of $82 \times 10^4 \text{ cells mL}^{-1}$. In addition, Ahmed [23] also noted similar results where 2.0 M salinity in *D. salina* cultures produced a maximum biomass concentration of $7 \times 10^7 \text{ cells mL}^{-1}$ with a drastic decrease in biomass concentration above 2.5 M salinity [23]. The difference in biomass concentration between ours and theirs is due to the difference in starting cell concentration, culture medium and growth duration. However, a salinity of 2.0 and 2.5 M produces a maximum biomass concentration irrespective of the culturing method used on *D. salina*.

Lag, exponential and decay phases were observed on the growth profile of *D. salina* as shown in Figure 4.1(a). A 2.5 M salinity had a short lag phase (1st to the 7th day) whereas 1.5, 2.0 and 4.0 M salinity had a long lag phase (1st to the 10th day). A 2.5 and 2.0 M salinity had a long exponential phase duration (7th to the 25th day and 10th to the 25th day respectively), and 4.0 and 1.5 M salinity had a short duration (10th to the 16th day and 10th to the 13th day respectively). The long decay duration was observed in 1.5 and 4.0 M salinity cultures (13th to 37th and 16th to 37th day respectively) while 2.5 and 2.0 M salinity had a short decay duration (25th to the 37th day). A short lag phase observed on 2.5 and 2.0 M cultures indicate a fast metabolism adoption of *D. salina* to the culture conditions to produce biomass as compared to the 4.0 and 1.5 M cultures [37]. The exponential phase of 4.0 and 1.5 M salinity cultures was short and the decay phase was long indicating a short period of biomass growth due to the osmotic and ionic stress of *D. salina* was exposed to due to the osmotic and ionic stress the *D. salina* was exposed to in the cultures [23].

As shown in Figure 4.2(b), biomass harvested from the 2.5 M and 2.0 M salinity cultures had the highest mass on a dry weight basis at $315 \pm 47 \text{ mg}$ and $247 \pm 24 \text{ mg}$ respectively. The lowest dry biomass at 152 ± 23 and $141 \pm 10 \text{ mg}$ was produced from 1.5 M and 4.0 M salinities respectively. The present results are in accordance with other researchers [22,23] who noted *D. salina* in 2.5 and 2.0 M salinity cultures produced the highest biomass concentration. A high salinity concentration (above 2.5 M) results in the change of the internal ionic ratio of the *D. salina* causing degradation of organic solutes in the microalgae thus a decrease in the overall

biomass weight [41]. A low salinity concentration (below 1.0 M) has weakened stress on the microalgae metabolism resulting in a low accumulation biomass weight [21].

4.1.2 Effects of salinity concentration on specific growth rate and biomass productivity

The specific growth rates were highest at $0.068 \pm 0.003 \text{ d}^{-1}$ and $0.066 \pm 0.005 \text{ d}^{-1}$ under 2.5 and 2.0 M salinity respectively as compared to 1.5 and 4.0 M salinity with $0.052 \pm 0.000 \text{ d}^{-1}$ and $0.045 \pm 0.005 \text{ d}^{-1}$ growth rates respectively, as shown in Figure 4.1(c). The salinity was significant to the specific growth rate ($df = 3$, $F = 24.108$, $p\text{-value} = 0.000$). This trend is justified by Ralefala [22], who noted a decrease in specific growth rate when the salinity concentration increased from 2.0 to 3.0 M [22]. The findings of this study are justified, an increase in salinity over 2.5 M increases the *D. salina* osmotic stress that's causes a decrease in photosynthetic energy conversion by altering the metabolic activities of the cells through evolving the oxygen photosynthetically resulting in a low specific growth rate [212–214].

As depicted in Figure 4.1(c), culture medium with salinity of 2.5 M had the highest biomass productivity at $62.0 \pm 1.6 \text{ mgL}^{-1}\text{d}^{-1}$, followed by 2.0 M at $54.8 \pm 0.8 \text{ mgL}^{-1}\text{d}^{-1}$, 1.5 M at $31.2 \pm 1.3 \text{ mgL}^{-1}\text{d}^{-1}$ and 4.0 M at $26.4 \pm 0.2 \text{ mgL}^{-1}\text{d}^{-1}$ being the lowest. An Anova analysis ($df = 3$, $F = 711.3$, $p\text{-value} = 0.000$) concluded that the salinity of the medium has a significant effect on biomass productivity. BenMoussa-Dahmen [215] noted an increase in biomass productivity from 130 to 200 $\text{gL}^{-1}\text{d}^{-1}$ in salinity of 1.0 and 3.0 M respectively [215]. In addition, Tahir [41] reported a decrease in biomass productivity as salinity increased from 40 gL^{-1} to 60 gL^{-1} that is an equivalence of 0.68 M and 1.02 M respectively in *C. fusiformis* cultures [41]. The inconsistencies between Tahir [41] and this might be due to different microalgae species (*Nannochloropsis salina*) responding differently to the saline conditions.

4.1.3 Effect of salinity concentration on lipid accumulation and productivity

Based on Eqns. (2.3)-(2.4) from the literature review (Section 2.1.2), the lipid content and its productivity were calculated and presented in Figure 4.1(d). Salinity 2.5 and 2.0 M had the highest lipid content (18 ± 2 and $17 \pm 2 \%$ respectively) and lipid productivity (11.2 ± 0.9 and $9.6 \pm 1.3 \text{ mgL}^{-1}\text{d}^{-1}$ respectively) as compared to the ones in 1.5 M ($10 \pm 2 \%$ and $3.2 \pm 0.6 \text{ mgL}^{-1}\text{d}^{-1}$) and 4.0 M ($8 \pm 1 \%$ and $2.2 \pm 0.2 \text{ mgL}^{-1}\text{d}^{-1}$). An Anova analysis concluded a statistical

significance of salinity on lipid content ($df = 3$, $F = 2103.608$, $p\text{-value} = 0.000$) and lipid productivity ($df = 3$, $F = 773.819$, $p\text{-value} = 0.000$). The results are in agreement with those reported by Ahmed [21], who achieved a maximum lipid content at a 2.5 M salinity during *D. salina* culturing [21]. The results were also in accordance with Yoshida [216], who justified that an increase in salinity from 0.5 to 1 M resulted in a significant increase of lipid content. Salinity has a direct effect on the metabolic activity of the microalgae cells thus an increase in salinity results in an increase in lipids content and productivity. Excess salinity above the recommended level of 2.5 M results in cell development deceleration [216].

4.2 Determination of optimum medium nutrients concentration for biomass and lipid production using response surface methodology (RSM)

Plackett-Burman design is an experimental design used to identify nutrients in the culture medium that have a significant effect on biomass concentration, lipid content and lipid production. Box-Behnken design is an experimental design that is used to optimise significant nutrient concentrations to achieve a maximum lipid production using the response surface methodology.

4.2.1 Plackett-Burman design on nutrient identification for biomass growth, lipid content and lipid production

The response of biomass concentration, lipid content and lipid production to different medium conditions were investigated by exposing *D. salina* to 12 different experimental conditions as explained in section 3.3.2.1. The results in Table 4.1 show that *D. salina* in experiment 12 produced the highest biomass concentration ($605 \pm 9 \text{ mgL}^{-1}$) with the lowest lipid content ($27.0 \pm 9 \%$) and the second highest lipid production ($163 \pm 7 \text{ mgL}^{-1}$). On the other hand, *D. salina* in experiment 2 had the lowest biomass concentration ($295 \pm 3 \text{ mgL}^{-1}$) with the sixth highest lipid content ($31.0 \pm 4.4 \%$) and the lowest lipid production ($91 \pm 13 \text{ mgL}^{-1}$). The highest lipid content ($35.2 \pm 2.0 \%$) was observed in experiment 3 that had the second lowest biomass concentration and lipid production ($274 \pm 10 \text{ mgL}^{-1}$ and $96 \pm 5 \text{ mgL}^{-1}$ respectively). Experiment 7 had the highest lipid production ($170 \pm 20 \text{ mgL}^{-1}$) with the second highest biomass concentration ($555 \pm 10 \text{ mgL}^{-1}$) and the seventh highest lipid content ($30.6 \pm 3.1 \%$). The alteration on the yield biomass concentration, lipid content and lipid production is influenced

by significant nutrients in the culture medium. Statistical analysis revealed that NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3 were significant since they had effects on biomass concentration (t-value > 2.571) and lipid production (t-value > 2.776) (Figure 4.2a-c respectively). Whereas the rest of the nutrients (CaCl_2 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and A5 solution) showed little or no effects and were considered non-significant to biomass concentration and lipid production. For lipid content (Figure 4.2b), NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, NaNO_3 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and A-5 solution showed significant effect (t-value > 2.571).

Table 4.1: Results of *D. salina* in a Plackett-Burman design

Experimental run	R ₁ (mg/L)	R ₂ (%,dw)	R ₃ (mg/L)
1	399 ± 4	28.2 ± 2.4	112 ± 10
2	295 ± 3	31.0 ± 4.4	91 ± 13
3	274 ± 10	35.2 ± 2.0	96 ± 5
4	330 ± 7	32.8 ± 3.4	108 ± 9
5	444 ± 6	28.5 ± 2.2	127 ± 8
6	426 ± 6	30.0 ± 3.5	128 ± 16
7	555 ± 10	30.6 ± 3.1	170 ± 20
8	452 ± 6	29.7 ± 2.0	134 ± 11
9	375 ± 6	32.0 ± 2.6	120 ± 12
10	306 ± 17	32.0 ± 2.6	98 ± 9
11	459 ± 3	27.3 ± 1.5	125 ± 6
12	605 ± 9	27.0 ± 1.0	163 ± 7

Key: R₁ is the biomass concentration, R₂ is the lipid content, R₃ is the lipid production

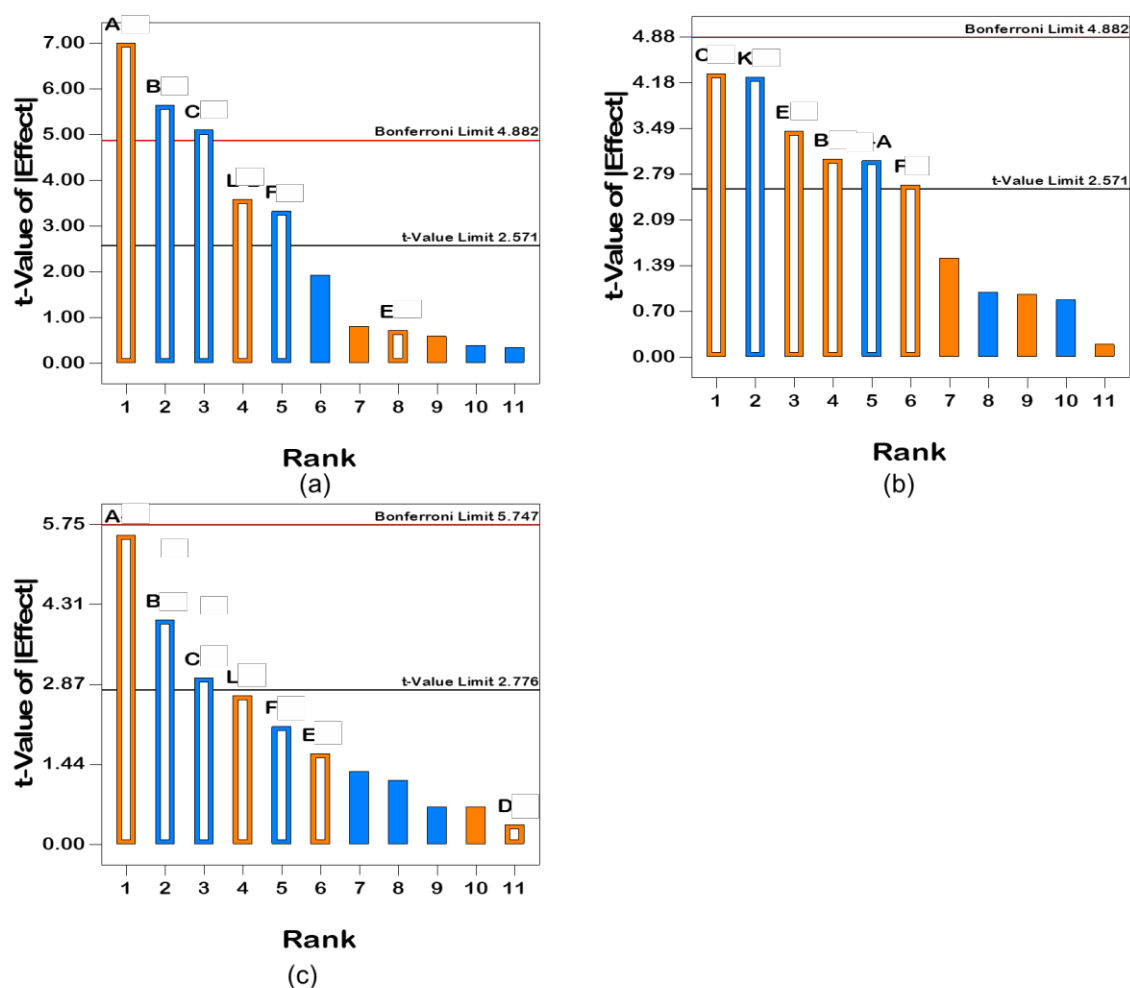


Figure 4.2: Pareto Chart of the most significant and non-significant nutrients: (a) biomass concentration, (b) lipid content (c) lipid production where A is the NaHCO_3 , B is the $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, C is the NaNO_3 , D is the CaCl_2 , E is the $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, F is the A5 solution, K is the Dummy K and L is the Dummy L.

Table 4.2: Statistical analysis of nutrients on biomass concentration response.

Variable Code	Std. Effect	SS	% of Contribution	Coefficient of Estimate	df	Mean Square value	F-value	p-value
Model	-	99705.42	-	410.10	4	24926.35	11.22	0.0036
A	117.54	41444.87	35.96	58.77	1	41444.87	18.66	0.0035
B	-94.69	26895.85	23.34	-47.34	1	26895.85	12.11	0.0103
C	-85.61	21987.79	19.08	-42.81	1	21987.79	9.90	0.0162
F	-55.91	9376.91	8.14	-27.95	1	9376.91	4.22	0.0790
Residual		15545			7	2220.72		
Cor-Total		115250			11			

Where: $R^2 = 86.51\%$, $R^2_{adj} = 78.80\%$, $R^2_{pred} = 60.36$, Adeq Precision=11.63, *significant at p-value less than 0.05, A is the NaHCO_3 , B is the $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, C is the NaNO_3 , F is the A5 solution.

As shown in Table 4.2, the model for biomass concentration was significant ($p\text{-value} = 0.0036 < 0.05$), with an F-value of 11.22 meaning that only 0.36 % of the model about the F-value occurred due to noise and the model explained 86.51 % (R^2) of the variant. The adequacy of the model was further evaluated on an actual-predication plot (Figure 4.3a), and the points were close to the model line indicating that the model was an excellent predictor to biomass concentration. The model had an acceptable deviation between adjusted- R^2 and predicted- R^2 ($18.44\% < 20\%$) [170,217]. The adequate precision had a strong signal ($11.63 > 4$), which meant that there was a low chance of the model being influenced by noise. The model was well suited to navigate the design space and Eqn. (4.1) was generated from the design.

$$R_1 = 410.1 + 0.14A - 6.1B - 2C - 10 \quad (4.1)$$

Where: R_1 is the biomass concentration, A is the NaHCO_3 , B is the $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, C is the NaNO_3 and F is the A5 solution.

Table 4.3: Statistical analysis of nutrients on lipid content response

Variable Code	Std.Effect	SS	% of Contribution	Coefficient of Estimate	df	Mean Square value	F-value	p-value
Model		61.55		30.36	6	10.26	12.30	0.0073
A	-1.58	7.48	11.38	-0.79	1	7.48	8.97	0.0303
B	1.59	7.62	11.60	0.80	1	7.62	9.14	0.0293
C	2.28	15.60	23.73	1.14	1	15.60	18.70	0.0075
E	1.82	9.92	15.10	0.91	1	9.92	11.90	0.0182
F	1.38	5.74	8.73	0.69	1	5.74	6.88	0.0469
K	-2.25	15.19	23.11	-1.13	1	15.19	18.22	0.0080
Residual		4.17			5	0.83		
Cor.Total		65.72			11			

Where: $R^2 = 93.66\%$, $R^2_{adj} = 86.04\%$, $R^2_{pred} = 63.46$, Adeq Precision = 13.029; *significant at p-value less than 0.05, A is the NaHCO_3 , B is the $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, C is the NaNO_3 , E is $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, F is the A5 solution, K is the dummy K.

The lipid content model (Table 4.3) was statistically significant ($p\text{-value} = 0.0073 < 0.05$) with 0.73 % chance that the F-value (12.30) could be a result of noise. The model fitted perfectly ($R^2 = 93.66\%$) to the actual behaviour of the nutrients to lipids content and it had a significant dummy K ($p\text{-value} = 0.0080 < 0.05$), which means that the alias of the main effect variables had a strong influence on the lipid content [218]. Adjusted- R^2 had a significant deviation with predicted- R^2 ($22.6\% > 20\%$) to the recommended. The reason might be due to the blocking effect on the samples as they were exposed to uncontrolled weather conditions [173]. A good and adequate precision ($13.29 > 4$) was achieved, concluding that there were a few chances that the model might have been a result of noise [219]. Actual-prediction plot was a good predictor of the model as the variables were close to the model line (Figure 4.3b). The analysis concluded that the model was a good predictor, and a model was established from the design is Eqn. (4.2).

$$R_2 = 22.1 - 1.9A + 0.1B + 5.4C + 1.5E + F \quad (4.2)$$

Where: R₂ is the lipid content, A is the NaHCO₃, B is the NaH₂PO₄.2H₂O, C is the NaNO₃, E is MgSO₄.7H₂O and F is the A5 solution.

Table 4.4: Statistical analysis of nutrients components on lipid production response.

Variable Code	Std.Effect	SS	% of Contribution	Coefficient of Estimate	df	Mean Square value	F-value	p-value
Model		6279.14			7	897.02	10.07	0.0207
A	30.25	2744.35	41.36	15.12	1	2744.35	30.81	0.0052
B	-21.98	1448.95	21.84	-10.99	1	1448.95	16.27	0.0157
C	-16.32	798.97	12.04	-8.16	1	798.97	8.97	0.0401
D	1.96	11.51	0.17	0.98	1	11.51	0.13	0.7374
E	8.92	238.77	3.6	4.46	1	238.77	2.68	0.1769
F	-11.53	398.99	6.01	-5.77	1	398.99	4.48	0.1017
L	14.58	637.62	9.61	7.29	1	637.62	7.16	0.0555
Residual		356.24			4	89.06		
Cor Total		6635.38			11			

Where: $R^2 = 94.63\%$, $R^2_{adj} = 87.81\%$, $R^2_{pred} = 60.08\%$, Adeq Precision = 10.392, *significant

at p-value less than 0.05, A is the NaHCO₃, B is the NaH₂PO₄.2H₂O, C is the NaNO₃, D is the CaCl₂, E is MgSO₄.7H₂O, F is the A5 solution and L is the dummy L.

In Table 4.4, dummy L was non-significant (p-value = 0.0555 > 0.05) to lipid production concluding non-interaction of aliases. The model was highly significant (p-value = 0.0207 < 0.05) and had a less chance (2%) that the F-value (10.07) might have been due to noise. The actual-prediction plot (Figure 4.3c) was a good predictor to the model, indicating that the model line was close to the variable points. The difference between adequate-R² and predicted-R² was in the acceptable range (19.73 % < 20 %) with a strong adequate signal (10.392 > 4) indicating that the model was fit to navigate the design space of the model. Lipid production was selected as an indicator to maximise its output through optimising significant nutrients

(NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3) using the Box-Behnken design in the second stage of optimisation under Box-Behnken experimental design.

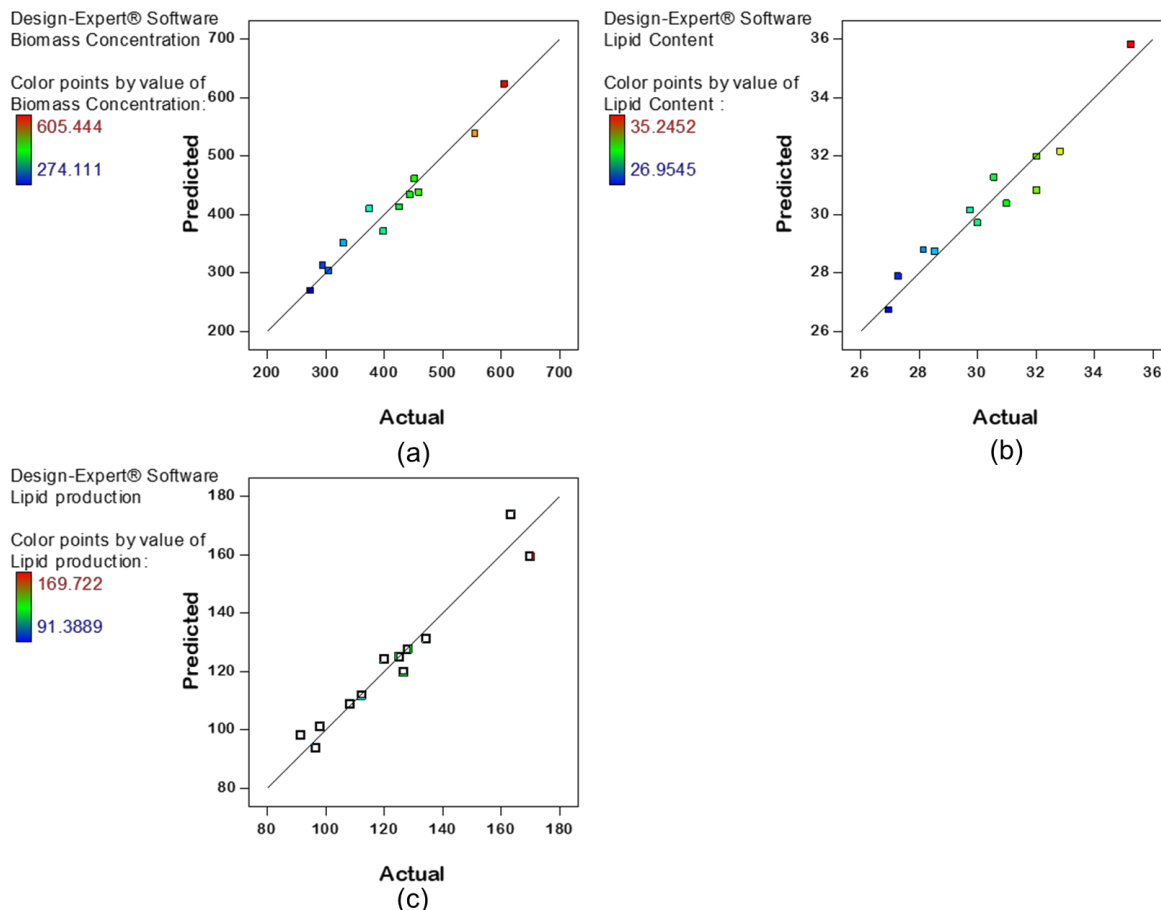


Figure 4.3: Actual vs predicted variables of (a) biomass concentration, (b) lipid content, (c) lipid production

As shown in Tables 4.2-4.4, NaHCO_3 was a significant nutrient for biomass production (p-value = 0.0035) and lipid production (p-value = 0.0052). The NaHCO_3 had a standard positive effect (117.54 and 30.25), and positive coefficients (58.77 and 15.12) on biomass concentration and lipid production respectively, at the same time contributing 35.96 % and 41.36 % to the respective response. With regard to lipid content, NaHCO_3 had a negative effect (-1.58), and a negative coefficient estimate (-0.79) contributing 11.38% to the lipids with a statistical significance (p-value = 0.0303). With a negative effect and coefficient value from the results,

it meant that as the carbonates decreased concentration from 1680 mgL^{-1} to 840 mgL^{-1} , the lipid content increased (Table 4.3). Ebrahimian [220] reported a similar trend in which high lipid production and biomass concentration of *C. vulgaris* was achieved at a high sodium hydrogen carbonate concentration, in their study the lipid content was not affected by the change in the carbonates concentrations. Meanwhile, the results on biomass concentration were in agreement with those reported by Pancha [221], who found that addition of carbonates ($0\text{-}1.5 \text{ gL}^{-1}$) enhanced the biomass concentration of *Scenedesmus sp.* [221]. Mokashi [73] also found out that high carbonates level (1 gL^{-1}) resulted in *C. vulgaris* inducing high biomass concentration (1.542 gL^{-1}) and lipids production (16.1 mgL^{-1}) when compared to cultures with low carbonates levels between 0.25 and 0.5 gL^{-1} [73]. Additionally, NaHCO_3 has been reported to contribute significantly in the de novo synthesis of lipids accumulation in *D. salina* microalgae [173,222,223].

Phosphates have also previously been reported to have a substantial contribution to the biomass and lipids accumulation by microalgae [1,67,69]. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ showed to be significant to biomass concentration (p-value = 0.0103), lipid content (p-value = 0.0293) and lipid production (p-value = 0.0157). The $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ contributed most to biomass concentration (23.34%) followed by lipid production (21.84 %) and lastly lipid content (11.60 %) (Table 4.2-4.4 respectively). When $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ concentration decreased from 31.2 mgL^{-1} to 15.6 mgL^{-1} , biomass concentration and lipid production increased (Std. Effect; -94.64 and -21.98 respectively). The nutrient had a positive effect on lipid content (Std. Effect; 1.59), which means that an increase in the concentration of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ results in increased lipid content (Table 4.3). The results in regard to phosphorus limitation on biomass concentration were in agreement with those reported by Patel [75], who noted that *Nannochloropsis sp*, *Chlorella sp* and *Nannochloropsis limnetica sp* increased their biomass concentration when phosphorus concentration was reduced from 15 mgL^{-1} to 0 mgL^{-1} [75]. Hamouda and Abou-El-Souod [224] also noted that *S. obliquus sp* increased its lipids content from 9% to 16% when the phosphorus concentration was increased from 0 gL^{-1} to 14 mgL^{-1} respectively [224]. Moreover, Yang [173] showed the significance of phosphorus limitation to lipid production, the observed increase in lipid production with a decrease in phosphorus concentration [173]. Phosphorus is essential to the microalgae cellular processes which involve photosynthesis, macromolecule biosynthesis,

signal transduction, energy transfer and cell growth [74]. Deficiency of phosphorus in microalgae decreases the levels of photosynthetic phosphorylation, ATP synthesis and Calvin cycle efficiency [74,174,218,224].

The NaNO_3 was significant on all the response outputs ($p\text{-value} < 0.05$) with an increase in biomass concentration (Std Effect; -85.65), decrease in lipid content (Std Effect; 2.28) and increase in lipid production (Std Effect; -16.32) at a contribution percentile of 19.08 %, 23.73 % and 23.73 % respectively as shown in Tables 4.2-4.4. These results were contrary to those reported by Karemore [174], who indicated that subjecting *D. salina* and *C. infusion* to nitrogen starvation conditions increased the accumulation of neutral lipid fraction, which is vital for producing a better grade of biodiesel [174]. In addition, Tahir [41] reported that lipid content increased from 30 to 57 % with a negligible decrease in biomass concentration from 1.71 mgL^{-1} to 1.55 mgL^{-1} under 100 % and 20 % initial nitrate concentration experiments respectively, using the *C. vulgaris* microalgae. However, these inconsistencies with this study might be due to the different biosynthetic behaviour of different microalgae species used when exposed to varying nitrogen concentration [41]. Yang [173] reported a positive effect of nitrates on lipid production [173]. The difference could be that this study allowed sufficient culturing period (37 days) to reach the stationary phase hence enough time for the nitrates to be consumed by the microalgae as compared to the experiments reported by Yang [173], which had a 9 day culture period and leading to non-depletion of nitrates. As shown in Table 4.2-4.4, CaCl_2 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and A5 solution were not significant ($p\text{-value} > 0.05$) to biomass concentration and lipid production. Only $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and A5 solution had a significant effect on lipid content ($p\text{-value} = 0.0182$; $p\text{-value} = 0.0469$). An increase in $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ concentration and A5 solution resulted in increased lipid content with an effect size of 1.82 and 1.38 respectively. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ contributed significantly to lipid content (15.10 %) as compared to the A5 solution (8.73 %).

Table 4.5: Statistical analysis on the model equation of lipid production, biomass concentration and lipid content.

Model	SS	df	MSE	F-value	Sig.
Regression	6588.432	2	3294.216	670.306	.000b
Residual	44.230	9	4.914		
Total	6632.662	11			

Where: $R^2 = 99.30\%$

Table 4.5 shows the statistical analysis of the model on how biomass concentration and lipid content interact with lipid production. The model was statistically significant (p-value = 0.000) with 99.30% of the variant data is explained. An increase in lipid production by one fold results in an increase of biomass concentration and lipid content by 0.29 and 3.55 folds respectively Eqn. (4.3).

$$R_3 = -104.4 + 0.3R_1 + 3.6R_2 \quad (4.3)$$

Where: R_1 is the biomass concentration, R_2 is the lipid content and R_3 is the lipid production.

4.2.2 Box-Behnken design on nutrient optimisation for maximum lipid production

Box-Behnken experimental design was used to confirm the optimum concentrations of nutrients considered significant (NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3) to produce maximum lipid production. The CaCl_2 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and A5 solution concentrations from the Plackett-Burman design were kept constant (49.71 mgL^{-1} , 2355.05 mgL^{-1} and 5.60 mL^{-1} respectively) in this design stage since they did not show significance to lipid production. As presented in Table 4.6, Experimental run 17 produced the maximum lipid production ($191 \pm 6 \text{ mgL}^{-1}$) when NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3 concentrations were 1260 mgL^{-1} , 23.4 mgL^{-1} and 630 mgL^{-1} respectively. The lowest lipid production ($98 \pm 4 \text{ mgL}^{-1}$) of experimental run 14 was produced when NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3 concentrations were 840 mgL^{-1} , 23.4 mgL^{-1} and 420 mgL^{-1} respectively.

Table 4.6: Box-Behnken's lipid production results

Experimental run	Lipid production (mg/L)
1	163 ± 8
2	162 ± 7
3	128 ± 12
4	168 ± 5
5	179 ± 9
6	160 ± 7
7	104 ± 5
8	123 ± 11
9	152 ± 7
10	188 ± 10
11	129 ± 4
12	131 ± 8
13	130 ± 2
14	98 ± 4
15	159 ± 5
16	116 ± 10
17	191 ± 6

From the experiment as shown in Table 4.7, a model was generated, which had a 0.05 % chance that its F-value (17.54) could be due to noise hence making the model significant. Further analyses were conducted, by determining the following: lack of fit, R^2 , R^2_{adj} , R^2_{pred} and adequate precision. The desired lack of fit was not significant to the relative pure error (F-value = 0.71, p-value = 0.60) meaning that the model fits well. An R^2 of 0.9575 suggested that the model had 95.75 % of predicting the lipid production response close to the actual. The R^2 deviated from

R^2_{pred} by 0.18, which is less than the 0.2 maximum recommended deviation, hence concluding that the model is fit for application as a predictor to lipid production [173].

Table 4.7: Statistical analysis of the Box-Behnken Experimental design

Source	Sum of Squares	df	Mean Square	Coefficient of Estimate	F-value	p-value
Model	0.62	9	0.069		17.54	0.0005
A-NaHCO ₃	0.051	1	0.051	0.08	12.99	0.0087
B-NaHPO ₄ .2H ₂ O	0.017	1	0.017	-0.046	4.32	0.0763
C-NaNO ₃	0.054	1	0.054	0.082	13.58	0.0078
AB	0.035	1	0.035	0.094	8.95	0.0202
AC	0.011	1	0.011	-0.052	2.72	0.1434
BC	0.029	1	0.029	0.085	7.38	0.0299
A ²	0.32	1	0.32	-0.27	79.81	< 0.0001
B ²	0.015	1	0.015	-0.059	3.72	0.0952
C ²	0.067	1	0.067	-0.13	16.86	0.0045
Residual	0.028	7	0.004			
Lack of fit	0.01	3	0.003		0.71	0.60
Pure error	0.018	4	0.005			
Cor total	0.65	16				
Model	0.62	9				

Where: $R^2 = 95.75\%$, $R^2_{adj} = 90.29\%$, $R^2_{pred} = 72.11\%$, *significant p-value is less than 0.05

Individually, NaHCO₃ and NaNO₃ had a significant effect (p-value = 0.0087, p-value = 0.0078 respectively) on the lipid production with respective 0.87 % and 0.78 % chance that the F-values (12.99 and 13.58 respectively) might have been caused by noise (Table 4.7). NaHPO₄.2H₂O alone was insignificant (p-value < 0.0763) to the lipid production with 7.63 % chance that its F-value (4.32) might have been due to noise. NaHPO₄.2H₂O interaction with NaHCO₃ and NaNO₃ gave a significant effect (p-value = 0.0202 and p-value = 0.0299 respectively) with an F-value of 8.95 and 7.38 respectively, whereas interaction between NaHCO₃ and NaNO₃ had an insignificant effect (p-value = 0.1434) and an F-value of 2.72. The

nutrient quadratic of NaHCO_3 and NaNO_3 had a significant effect ($p\text{-value} < 0.05$) on the lipid production with F-values of 79.81 and 16.86, respectively. The quadratic of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ had an insignificant effect ($p\text{-value} > 0.05$) on lipid production with an F-value of 3.72.

The relationship between independent significant variables (NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3) and dependent variable (lipid production) were graphically presented in 2D response surface and 3D contour plots. Figures 4.4(a)-(b) show the interaction between NaHCO_3 and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in contour and response surface plots, respectively. The contours show that an increase in NaHCO_3 concentration above 1470 mgL^{-1} as well as the concentration below 1050 mgL^{-1} resulted in a decrease of lipid production the effective concentration was between $1050 - 1479 \text{ mgL}^{-1}$. When NaNO_3 was kept constant, the interaction between NaHCO_3 and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ had an increase in lipid production with an increase in NaHCO_3 irrespective of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ concentration.

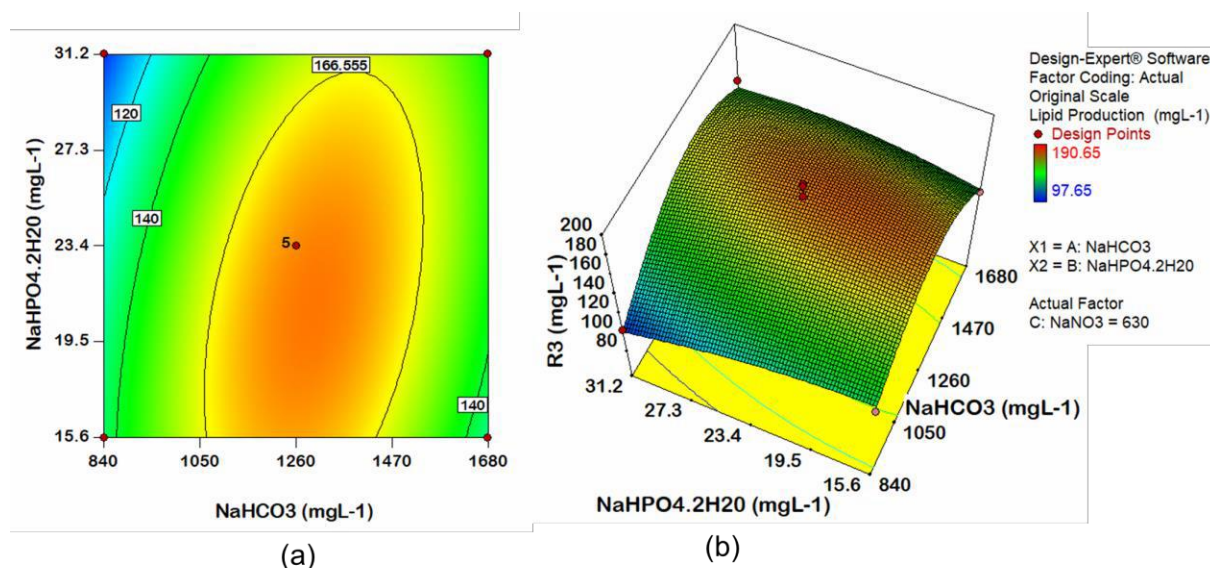


Figure 4.4: (a) Contour plot of NaHCO_3 and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ variables; (b) Response plot of NaHCO_3 and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ variables

From the study, the interaction between NaHCO_3 and NaNO_3 was studied and the results are plotted in Figure 4.5(a)-(b). An interaction range between 653 mgL^{-1} to 740 mgL^{-1} of NaNO_3 and 1213 mgL^{-1} to 1493 mgL^{-1} of NaHCO_3 produced a good lipid production of 180 mgL^{-1} .

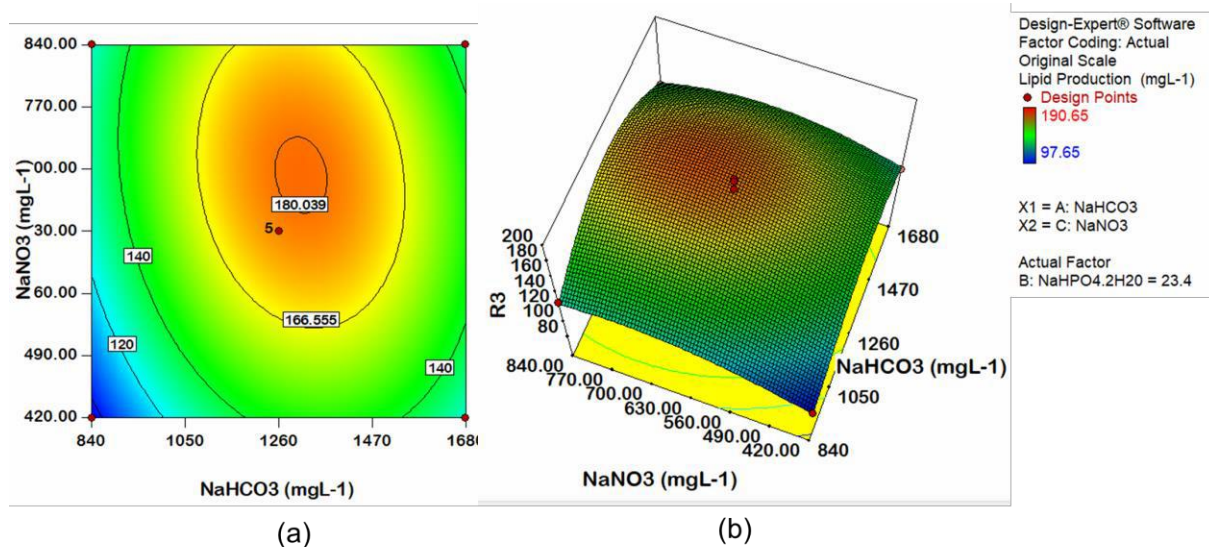


Figure 4.5: (a) Contour plot of NaHCO_3 and NaNO_3 variables; (b) Response surface plot of NaHCO_3 and NaNO_3 variables.

The interaction between NaNO_3 and $\text{NaHPO}_4 \cdot 2\text{H}_2\text{O}$ (Figure 4.6a-b) had a significant effect on lipid production. The lipid yield was affected by the NaNO_3 concentration irrespective to the change in $\text{NaHPO}_4 \cdot 2\text{H}_2\text{O}$ level. As the $\text{NaHPO}_4 \cdot 2\text{H}_2\text{O}$ level increased from 15.6 mgL⁻¹ to 31.2 mgL⁻¹ and with the NaNO_3 in the range between 653 mgL⁻¹ to 746 mgL⁻¹ a high yield of lipid production (180 mgL⁻¹) was obtained.

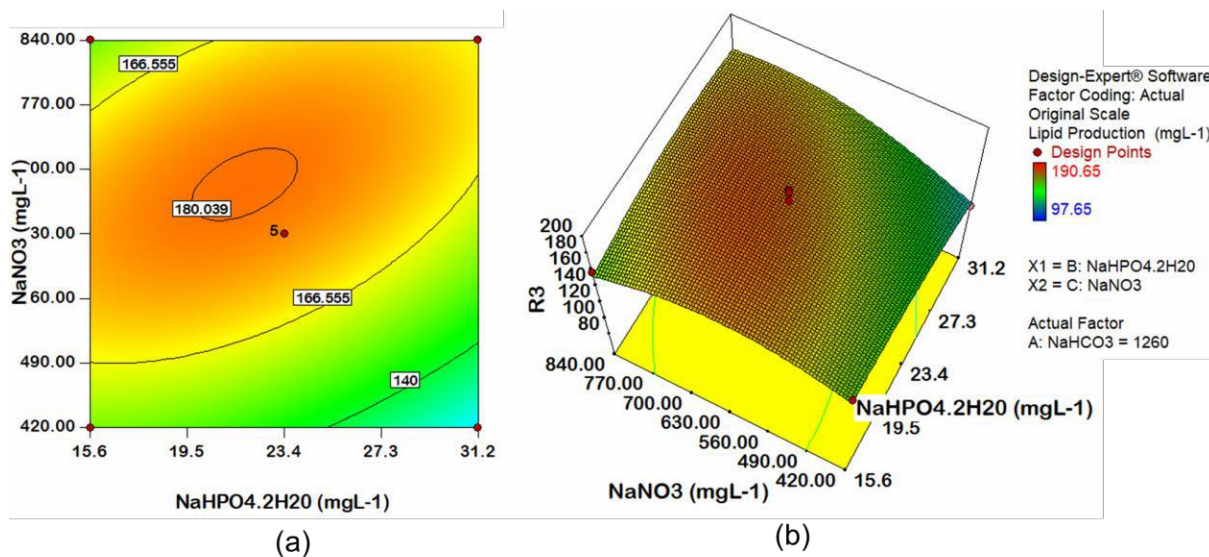


Figure 4. 6: (a) Contour plot of NaHPO₄.2H₂O and NaNO₃ variables; (b) Response surface plot of NaHPO₄.2H₂O and NaNO₃ variables

From the study, the quadratic polynomial Eqn. (4.4) was generated from the model and it predicted a maximum lipid production of 181 mgL⁻¹ under *D. salina* in growth culture optimising the NaHCO₃, NaHPO₄.2H₂O, NaNO₃, CaCl₂, MgSO₄.7H₂O and A5 solution concentrations at 1302.42 mgL⁻¹, 22.46 mgL⁻¹, 685.58 mgL⁻¹, 44.72 mgL⁻¹, 2355.00 mgL⁻¹ and 5.60 mL⁻¹ respectively.

$$\ln(R_3) = 1.9 + (3.8 \times 10^{-3}A) - (3 \times 10^{-2}B) + (3.5 \times 10^{-3}C) + (2.9 \times 10^{-5}AB) - (6 \times 10^{-8}AC) - (10^{-4}BC) - (2 \times 10^{-6}A^2) - (9.7 \times 10^{-4}B^2) - (2.9 \times 10^{-6}C^2) \quad (4.4)$$

Where: R₃ is the lipid production, A is the NaHCO₃, B is the NaH₂PO₄.2H₂O and C is the NaNO₃.

The interaction between the carbonates and the phosphates had a significant impact on lipids accumulation, as well as the interaction between the nitrates and the phosphates. The results are supported by the study by Pancha [221], who noted interaction starvation of nitrates and sodium bicarbonate resulting in an increase of total lipids.

4.3 Identification of *D. salina* lipids using the Thin Layer Chromatography (TLC)

TLC method was used for initial screening for the presence of lipids. Figure 4.7(a) shows a TLC plate without spot marking and (b) with spot markings. Two samples of *D. salina* extracted lipids were loaded on the TLC plate and were labelled as lane A and B. Spots 1, 2, 3 and 4 identified as glycerides, free fatty acids, triglyceride and methyl ester were spotted as similar on both lanes (A and B) with their retention factor depicted in Table 4.8. Similar results were reported by other researchers [225–229] indicating the presence of the above mentioned glycerides, free fatty acids, triglyceride and methyl ester in lipids as compatible oils for biodiesel production.

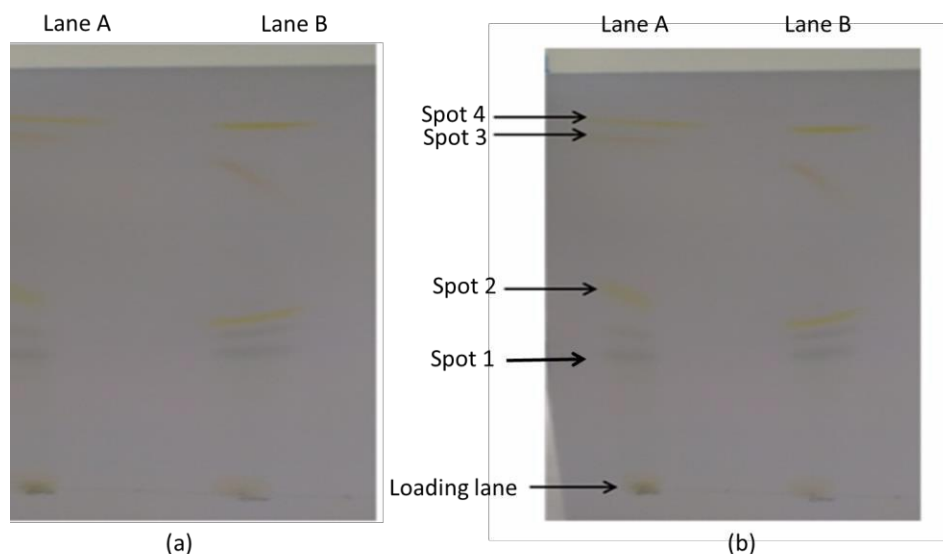


Figure 4.7: Thin layer chromatography of *D. salina* extracted lipids with samples labelled as lane A and B. (a) TLC plate without spot markings (b) TLC plate with spots 1, 2, 3, and 4 identified as the glyceride, free fatty acids, triglyceride and methyl ester respectively [227,228]

Table 4.8: Retention factor of *D. salina* extracted lipids

Spot number	Solvent distance (cm)	Solute distance (cm)		Retention factor	
		A	B	A	B
Lane					
1	16.5	4.3	4.2	0.26	0.25
2	16.5	8.5	8.2	0.52	0.50
3	16.5	15.7	15.4	0.95	0.93
4	16.5	16.0	15.8	0.97	0.96

4.4 Transesterification, identification and characterisation of the *D. salina* biodiesel

4.4.1 Transesterification of lipids to produce biodiesel

During the transesterification reaction, an alkali (KOH and NaOH) was used as a catalyst. Due to the limitation in the amount of extracted lipids, sunflower oil was used as a representative oil

to determine the amount of catalyst used for the transesterification reaction as both of them had free fatty acids (FFA) which were less than 1 % (lipids: 0.86 ± 0.06 %,wt and sunflower oil: 0.35 ± 0.06 %,wt). The feedstocks are less likely to deactivate the catalyst due to a low FFA content that has a tendency of forming a semisolid mass around the catalyst if the content is greater than 5 % [230]. KOH required mass of 9.4 ± 0.4 g to neutralise the FFA in a litre of oil as compared to the NaOH which required a mass of 7.3 ± 0.1 g. An optimum concentration of the alkali catalyst will reduce the reaction time and produce a better yield of biodiesel [231]. Excess concentration above the optimum level will result in soap formation instead of biodiesel production [189].

As shown in Table 4.9, sunflower oil and *D. salina* extracted lipids transesterified reaction using KOH catalyst had the highest biodiesel yield (94 ± 1 %, 84 ± 1 %, respectively) compared to NaOH (89 ± 2 %, 75 ± 4 %, respectively). The results were in agreement with Talha [232] and Vyas [233] who noted a high biodiesel yield in KOH catalysed transesterification reaction as compared to NaOH catalysed reaction. NaOH has a high polarity compared to KOH when used as a catalyst in the transesterification reaction it produces more glycerol and low biodiesel [232]. Sunflower biodiesel had more yield than biodiesel extracted from lipids due to the purity of the feed and low free fatty acid (FFA) [98,100]. The results were in agreement with Mandal [234] and Vyas [233] who noted a high yield of biodiesel at low FFA and high feed purity.

Table 4.9: Biodiesel yield from different catalysed reactions

Catalysed	Biodiesel yield (%)	
	KOH	NaOH
<i>D. salina</i> lipids	84 ± 1	75 ± 4
Sunflower oil	94 ± 1	89 ± 2

4.4.2 FTIR identification of the produced biodiesel

The FTIR was used to identify the organic structures in biodiesel produced by *D. salina* and compared with sunflower biodiesel. The results were depicted in Figures 4.8-4.9 for *D. salina*

biodiesel and sunflower biodiesel respectively both with notable peaks ranging from 2924-723 cm^{-1} . Both samples had transmittance identified in the fingerprint region ranging between 500 cm^{-1} and 1500 cm^{-1} . The 723 cm^{-1} was identified as methylene cis-distributed alkenes and aromatics bending out of the plane. Organic structures identified at a wavenumber ranging between 1000 cm^{-1} and 1200 cm^{-1} were ester bonds with asymmetrical axial stretching. The methyl group structure with bending and rocking vibration was transmitted at a wavenumber ranging between 1200 cm^{-1} and 1500 cm^{-1} . Ester carbonyl group with stretching vibration at 1742 cm^{-1} was identified in the double bond region ranging between 1500 cm^{-1} and 2000 cm^{-1} on both samples. Methylene with asymmetrical stretching was identified in *D. salina* biodiesel and sunflower biodiesel at 2852 and 2854 cm^{-1} respectively in the single bond stretch which ranges between 2500 cm^{-1} and 4000 cm^{-1} . Asymmetrical stretching alkanes were identified at 2922 cm^{-1} and 2924 cm^{-1} in *D. salina* biodiesel and sunflower biodiesel respectively. Sunflower biodiesel had asymmetrical stretching alkenes identified at 3009 cm^{-1} . The following functional groups have been identified in other studies: methylene ($-(\text{CH}_2)_n$), ethers ($\text{C}-\text{O}-\text{C}$), esters bonds ($\text{C}(\text{C}=\text{O})-\text{O}$), methyl group ($\text{O}-\text{CH}_3$), ester carbonyl triglycerides ($\text{C}=\text{O}$) and alkene groups ($=\text{C}-\text{H}$) in biodiesels [49,108,109,111,116,117,235].

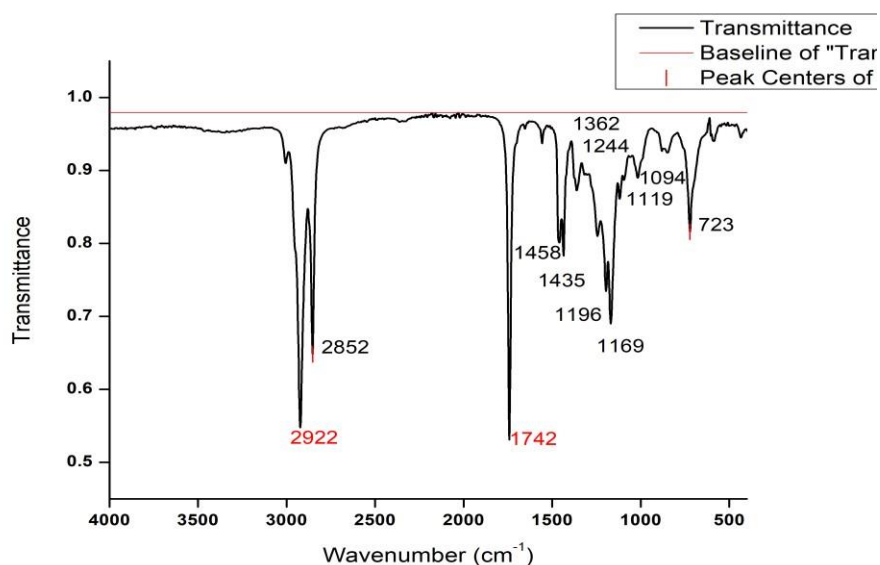


Figure 4.8: *D. salina* biodiesel FTIR spectrum

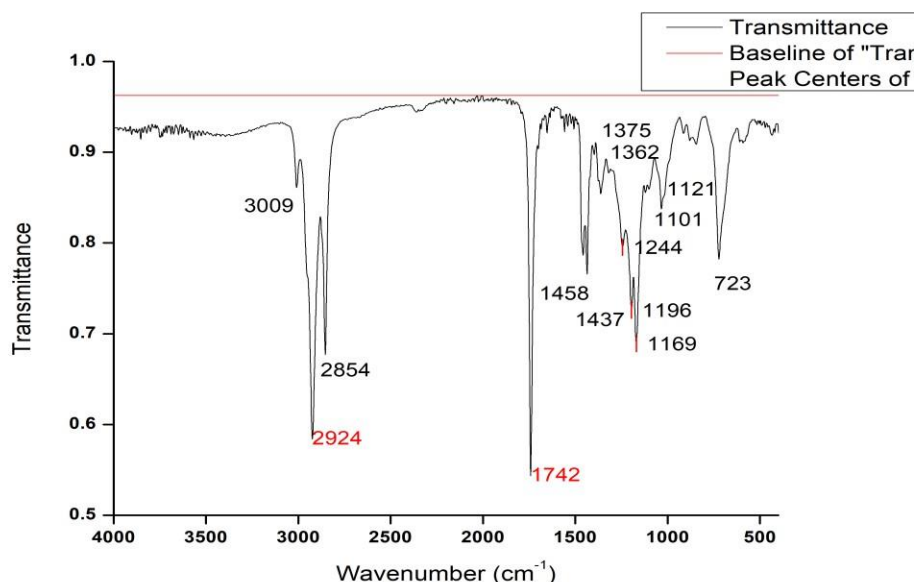


Figure 4.9: Sunflower biodiesel FTIR spectrum

4.4.3 GC-MS characterisation of lipids biodiesel and petroleum diesel

The GC-MS analysis was used to characterise the fatty acid methyl esters (FAMES) in biodiesel produced from *D. salina* as shown in Figure 4.10. The FAMES are fatty acid esters that are formed from the transesterification reaction and their profile determines the best application of the fuel. Methyl esters identified had carbon chains ranging from C₁₅ to C₂₁. The results indicate a high percentile fraction of monounsaturated FAMES 8-octadecenoic acid (36.69 %), hexadecanoic acid (35.53 %) and 9-octadecenoic acid Z (17.85 %) and a low percentile fraction of polyunsaturated eicosenoic acid (1.67 %), cis-11-eicosenoic acid (0.56 %), 9-hexadecenoic acid (0.33 %) and tetradecanoate (0.29 %). A high monounsaturated FAMES in the produced biodiesel indicates good oxidative stability of the fuel with an ability to prolong its shelf life, but however, this characteristic limits its application as the fuel has high polymerisation which reduces its affinity to oxygen leading to peroxidation [174,236]. The findings were in agreement with other researchers [71,168,219,237].

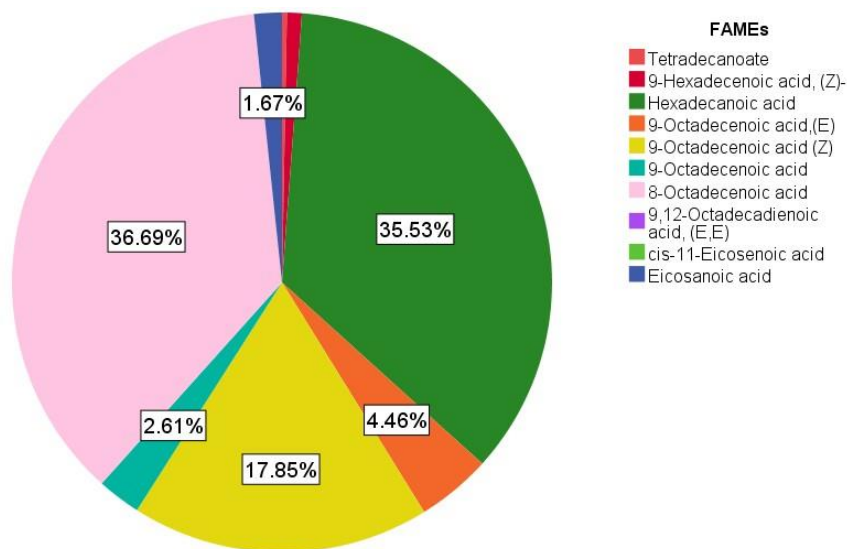


Figure 4.10: Methylester composition in the biodiesel produced from *D. salina*

4.5 Physiochemical characterisation and kinetics of *D. salina* lipids and biodiesel

4.5.1 Physiochemical characterisation of *D. salina* lipids and its biodiesel.

Table 4.10 shows the results for density (ρ), saponification value (SV), acid value (AV), free fatty acids (FFA), ester value (EV) and iodine value (IV) of the biodiesel in comparison to the international specification standards (EN14214, ASTM D16751 and SAN 1935:2004).

The biodiesel produced from sunflower had a high density of $890 \pm 4 \text{ kgm}^{-3}$ as compared to the biodiesel produced from *D. salina* with a density of $864 \pm 3 \text{ kgm}^{-3}$. Both fuels density were within the international specification range ($860\text{-}900 \text{ kgm}^{-3}$). High density above the recommended range ($> 900 \text{ kgm}^{-3}$) results in difficulties in combustion leading to incomplete combustion, particulate emissions and the damage to the combustion chamber [121,123].

The saponification value of sunflower biodiesel ($196 \pm 2 \text{ mgKOHg}^{-1}$) was high as compared to the biodiesel produced from *D. salina* ($183 \pm 2 \text{ mgKOHg}^{-1}$). The biodiesel from *D. salina* did not meet the SAN 1935:2004 range of between 185 mgKOHg^{-1} and 198 mgKOHg^{-1} . The low saponification value (SV) of *D. salina* biodiesel as compared to sunflower biodiesel indicates a high relative molecular mass of triacylglycerol in *D. salina* biodiesel [49,123,124].

The acid value of sunflower biodiesel ($0.44 \pm 0.04 \text{ mgKOHg}^{-1}$) was higher than that of *D. salina* biodiesel ($0.40 \pm 0.01 \text{ mgKOHg}^{-1}$). Both fuel acid values were within the international specification range ($< 0.5 \text{ mgKOHg}^{-1}$) indicating a long shelf life quality of the fuels without oxidising [120,121,126,127].

The free fatty acid of the sunflower biodiesel ($0.22 \pm 0.02 \%$) and *D. salina* biodiesel ($0.20 \pm 0.01 \%$) were within the international standard specification range of less than 0.5% meaning that the fuels will produce less soot during combustion. The ester value (EV) of sunflower biodiesel (191 ± 3) was higher than the ester value of *D. salina* biodiesel (183 ± 2) and both fuels ester value were similar to their saponification value (SV) which indicated a good quality of fuel [129]. The iodine value of sunflower biodiesel ($113 \pm 2 \text{ gI}_2/100\text{g}$ of FAME) was high than that of *D. salina* biodiesel ($67 \pm 4 \text{ gI}_2/100\text{g}$ of FAME) and were within international standard specifications ($< 140 \text{ gI}_2/100\text{g}$ of FAME). A low iodine value reflects a low NO_x emission during combustion [120,124,238].

Table 4.10: Physio-chemical characterisation of oils and biodiesel with the international specification standards

Tests	Units	Standards specification			Measured	
		EN14214	ASTM D6751	SANS 1935:2004	BSO	DSB
ρ	kgm ⁻³	860-900	860-900	860-900	890 ± 4	864 ± 3
SV	mgKOHg ⁻¹	-	-	185-198	196 ± 2	183 ± 2
AV	mgKOHg ⁻¹	<0.5	<0.5	<0.5	0.44 ± 0.04	0.40 ± 0.01
FFA	%	<5	<5	<5	0.22 ± 0.02	0.20 ± 0.01
EV		-	-	-	196 ± 2	183 ± 2
IV	gI ₂ /100g of FAME	<120	-	<140	113 ± 2	67 ± 4

Where: BSO is the biodiesel from sunflower oil and DSB is the *D. salina* biodiesel.

4.5.2 Thermal characterisation of *D. salina* biodiesel

Thermogravimetric experiments were performed under air atmospheric on *D. salina* biodiesel, sunflower biodiesel and petroleum diesel at three different heating rates (5, 10 and 15 °Cmin⁻¹) by using the thermogravimetric analysis (TGA-DTG) with results presented in section 4.5.2.1. The combustion characteristics, thermogravimetric and thermodynamic kinetics were investigated and presented in section 4.5.2.2 and 4.5.2.3.

4.5.2.1 Thermogravimetric analysis of *D. salina* biodiesel

Appendix C, Figure C1-C9 shows the TGA/DTG results of *D. salina* biodiesel, sunflower biodiesel and petroleum diesel at different heating rates (5, 10 and 15 °Cmin⁻¹) and Table 4.11 summarises the temperature profiles with respect to the fuel and heating rate.

Table 4. 11: TGA/DTG combustion data of *D. salina* biodiesel, sunflower oil biodiesel and petroleum diesel at different heating rates (5, 10 and 15 °Cmin⁻¹)

Sample	Heating rate (°Cmin ⁻¹)	Reaction region (°C)	Peak temperatures (°C)	Mass loss (%)
<i>D. salina</i> biodiesel	5	175-341	326	92.19
		341-509		7.81
	10	190-363	357	95.32
		363-516		4.68
	15	190-365	360	96.68
		365-520		3.32
Sunflower biodiesel	5	148-359	328	86.41
		359-497		13.59
	10	105-265	248	37.84
		276-364	330	57.20
		364-509		4.96
	15	130-263	248	24.16
		263-376	357	64.82
		376-555		11.02
Petroleum diesel	5	104-280	255	49.65
		280-327	310	47.96
		327-333		1.06
	10	89-217	199	35.38
		217-320	278	59.64
		320-347		0.94
	15	130-352	286	94.70
		352-444		0.50

D. salina biodiesel's combustion thermograph has evaporation, combustion and thermal degradation stages. Evaporation stage accounted for approximately 2 % of the mass loss. The

combustion had a reaction region of 175-341, 190-363 and 190-365 °C with minimum mass loss of 92 %, peak temperature of 326, 357 and 360 °C, that corresponded to three heating rates 5, 10 and 15 °Cmin⁻¹. Thermal degradation stage accounted for approximately 8 % of the mass loss with a range of 341-509, 363-516 and 365-520 °C for the three considered heating rates ascendingly. The stability of biodiesel decreased as the heating rates increased which resulted in the shift of the reaction interval, peak temperature to the upper temperatures and an increase in mass loss rate [134].

Sunflower biodiesel's evaporation stage accounted for approximately 1 % of the mass loss. At 5 °Cmin⁻¹ heating rate, single stage combustion was observed which had a reaction interval of 148-359 °C with a mass loss of 86.41 % and peak temperature of 328°C. Two distinct combustion stages were observed at 10 and 15 °Cmin⁻¹ heating rates that are the volatilisation and combustion. At 10 and 15 °Cmin⁻¹ the volatilisation region is 105-265 and 130-265 °C with a peak temperature of 248 °C and mass loss of 37.84 and 24.16 % respectively. The combustion region had a temperature interval of 276-364 and 263-376 °C with peak temperatures 330, 357 °C, and mass loss of 57.20 and 64.84 % for 10 and 15 °Cmin⁻¹ heating rates respectively. The chemical constituents in the triglycerides of sunflower oil biodiesel have a big distinctive gap on the carbon structures that have a lower molecular weight and higher molecular weight which results in lower molecular weight structures to be volatilised first before the combustion of the high molecular weight structures [130,239]. Thermal degradation accounted for 13.59, 4.96 and 11.02 % of the mass loss with the reaction interval shifting to upper temperatures as shown in Table 4.11. The stability of sunflower biodiesel decreases as the heating rate increases.

Petroleum diesel thermograph had less than 2 % of mass loss during the evaporation stage. A volatilisation reaction region of 104-280 and 89-217 °C with mass loss of 49.65 and 35.38 % and peak temperatures of 255 and 199 °C were observed on 5 and 10 °Cmin⁻¹ heating rates. The combustion stage had a reaction region of 280-327, 217-320 and 130-352 °C with mass loss of 47.96, 59.64 and 97.70 % and peak temperatures 310, 278 and 286 °C under the three corresponding heating rates ascendingly. The sensitivity of the reaction decreased the reaction region shifted to the upper temperature. The thermal degradation reaction region shifted to the

upper temperatures when the heating rate increased, that is 327-333, 320-347 and 352-444 °C for 5, 10 and 15 °Cmin⁻¹ with mass loss of less than 2 %.

D. salina biodiesel had a higher reaction compared to petroleum diesel. This meant that *D. salina* biodiesel was difficult to ignite and did not volatilise easily [135]. Furthermore, the peak temperatures were high as compared to petroleum diesel and sunflower oil diesel. This meant that *D. salina* biodiesel had difficulties to burn better and left soot after the combustion as indicated by the high mass loss percentile of the thermal degradation soot [139]. The results are similar to those reported by Topa [130] who noted canola oil biodiesel to resemble higher reaction region and peak temperature as compared to the petroleum diesel [130]. The author also noted the decreased stability of biodiesel and also a shift of reaction to higher temperatures when the heating rate increased [130]. Santos [209] noted that the increase in heating resulted in a shift of the mass loss steps towards higher temperatures and non-uniform heat distribution [209]. The results are in agreement with several researchers who noted an increase in the heating rate results in a decrease of heat distribution on the biodiesel molecules and shifting the thermal degradation to upper temperatures [131,134,138,150].

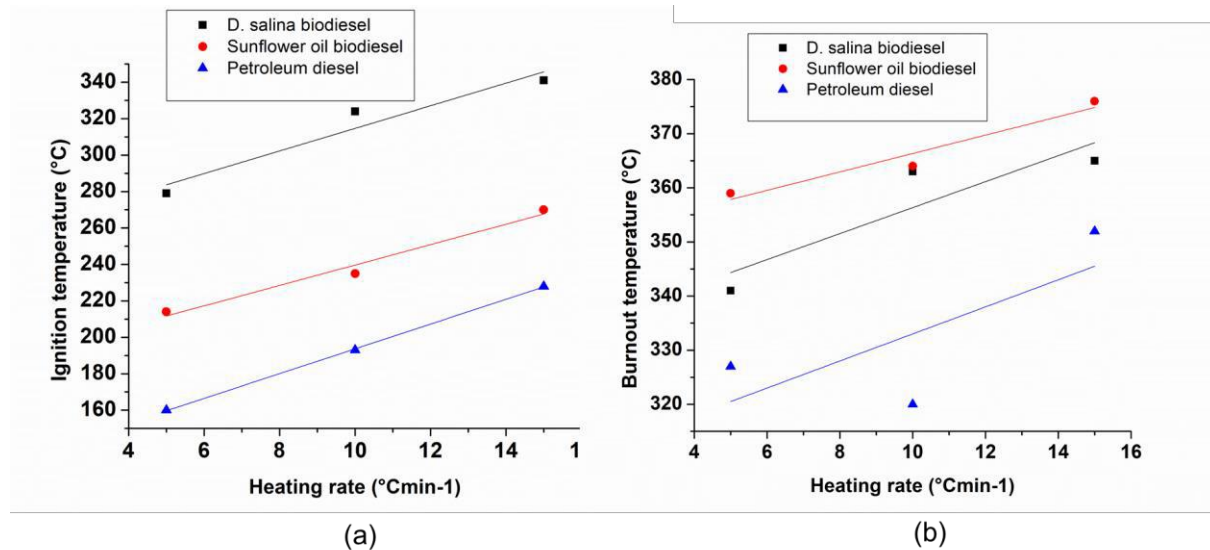


Figure 4.11: (a) ignition profile (b) burnout profile of *D. salina* biodiesel, sunflower biodiesel, and petroleum diesel at different heating rates

As shown in Figure 4.11(a), at varying heating rates (5, 10 and, 15 °Cmin⁻¹), *D. salina* biodiesel had the highest ignition temperature followed by sunflower oil biodiesel and petroleum diesel being the lowest. A high ignition temperature meant that *D. salina* biodiesel was less volatile and delayed to combust making it less ignitable [139]. Topa [130] noted a higher ignition temperature of biodiesel compared to petroleum diesel concluding that biodiesel was less ignitable as it resembled a low cetane number which delayed the combustion.

D. salina biodiesel had a higher burnout temperature than petroleum diesel and lower than sunflower oil biodiesel as shown in Figure 4.11(b). A higher burnout temperature indicates a long residence time of combustion [240]. *D. salina* biodiesel requires time to combust to complete as compared to petroleum diesel and when burnt in the internal combustion chamber for petroleum diesel it will damage to furnace walls due to high temperatures it attains during combustion [240].

4.5.2.2 Combustion characteristics, thermogravimetric and thermodynamic kinetics of D. salina biodiesel, sunflower biodiesel and petroleum diesel

Figure 4.12 shows the ignition index, comprehensive performance and Kissinger plot for *D. salina* biodiesel, sunflower biodiesel and petroleum diesel.

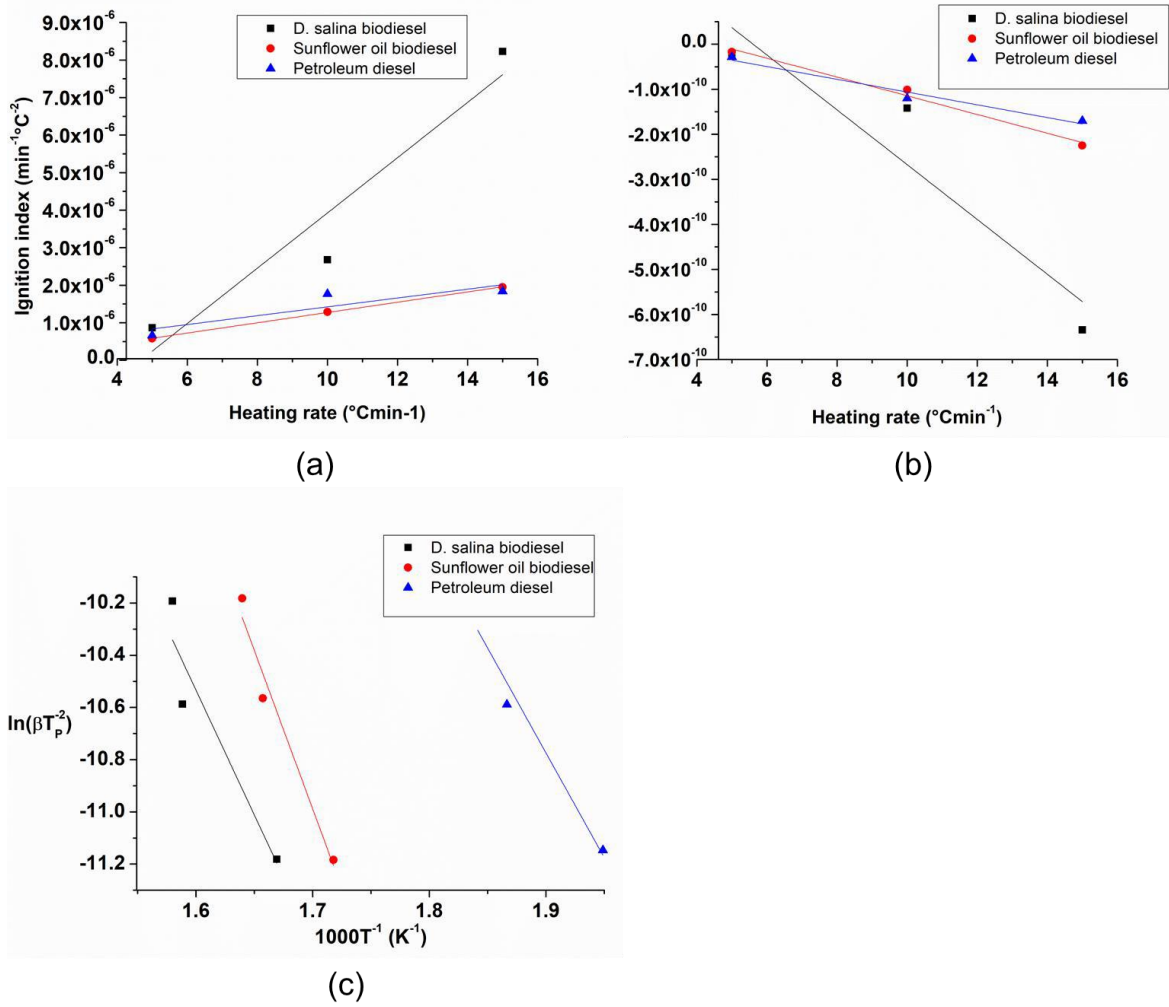


Figure 4. 12: *D. salina* biodiesel, sunflower biodiesel and petroleum diesel: (a) ignition index, (b) comprehensive performance, (c) Kissinger plots.

As depicted in Figure 4.12(a), *D. salina* biodiesel had the highest ignition index of $8.66\text{E-}07$, $2.68\text{E-}06$ and $8.23\text{E-}06 \text{ min}^{-1}\text{C}^{-2}$ at the corresponding heating rate 5 , 10 and $15^{\circ}\text{Cmin}^{-1}$ as compared to the sunflower biodiesel and petroleum diesel. Sunflower biodiesel and petroleum diesel had a corresponding closer match at the three heating rates ranging between $1.29\text{E-}07 - 5.80\text{E-}07$ and $1.77\text{E-}07 - 6.62\text{E-}07 \text{ min}^{-1}\text{C}^{-2}$ respectively. This indicated that *D. salina* biodiesel was difficult to ignite and a closer match of sunflower biodiesel and petroleum diesel indicates an almost similarity on the ease to ignite [141–144,241].

D. salina biodiesel had the least comprehensive performance of $-2.58\text{E-}11$, $-1.42\text{E-}10$ and $-6.34\text{E-}10 \text{ min}^{-2}\text{°C}^{-3}$ at the corresponding heating rates 5, 10 and 15 °Cmin^{-1} compared to sunflower biodiesel and petroleum diesel as shown in Figure 4.12(b). Sunflower oil biodiesel and diesel had a corresponding almost matching performance ($-1.01\text{E-}10$ to $-2.25\text{E-}10$ and $-1.20\text{E-}10$ to $-2.85\text{E-}11 \text{ min}^{-2}\text{°C}^{-3}$, respectively) at the three corresponding heating rates ascendingly. This result meant that the *D. salina* biodiesel was the least active fuel during combustion as compared to the sunflower biodiesel and petroleum diesel [139].

Table 4.12: Activation energy and Arrhenius constant of *D. salina* biodiesel, sunflower biodiesel, and petroleum diesel.

	Activation energy (kJmol ⁻¹)	Arrhenius constant (min ⁻¹)
<i>D. salina</i> biodiesel	79	1.12E+06
Sunflower oil biodiesel	78	1.17E+06
Petroleum diesel	72	2.23E+06

Kissinger plots are shown in Figure 4.12(c) were used to determine the activation energy and Arrhenius constant of *D. salina* biodiesel, sunflower biodiesel and petroleum diesel with results recorded in Table 4.12. The Kissinger plot had a strong prediction fit model with R² values of 0.9728, 0.6890 and 0.9009 for *D. salina* biodiesel, sunflower biodiesel and petroleum diesel respectively. *D. salina* biodiesel had the highest activation energy of 79 kJmol^{-1} followed by sunflower biodiesel at 78 kJmol^{-1} and lastly petroleum diesel at 72 kJmol^{-1} being the highest. This meant that *D. salina* biodiesel required more energy to combust followed by sunflower biodiesel and petroleum diesel requiring the least energy [242]. The results were consistent with the TGA/DTG results which concluded that *D. salina* biodiesel would require high temperatures for it to combust. Li [134] signified the presence of unsaturated fatty acids in biodiesel as a contributing factor to high activation energy and thermal stability [134]. *D. salina* biodiesel had the lowest Arrhenius constant compared to sunflower oil biodiesel and petroleum diesel being the highest as shown in Figure 4.12. This indicated that *D. salina* biodiesel's molecules had a low rate of frequency collision to dissipate energy compared to sunflower

biodiesel and petroleum diesel justifying its high thermal stability as explained by the results of TGA/DTG data [130,139].

Table 4.13: Thermodynamic properties and the higher heating value of *D. Salina* sp. biodiesel, sunflower biodiesel, and petroleum diesel.

Sample	ΔH (kJmol ⁻¹)	ΔG (kJmol ⁻¹)	ΔS (kJmol ⁻¹)	HHV (MJkg ⁻¹)
<i>D. salina</i> Biodiesel	74.63	184.54	-0.18	40.00
Sunflower Biodiesel	74.19	181.66	-0.18	39.69
Petroleum diesel	68.50	161.11	-0.17	43.62

The thermodynamic parameters of *D. salina* biodiesel, sunflower biodiesel and petroleum diesel are shown in Table 4.12 above. *D. salina* biodiesel had the highest endothermic enthalpy change compared to sunflower biodiesel and petroleum diesel being the lowest. This meant that *D. salina* had a lower reactivity as compared to sunflower biodiesel and petroleum diesel [139,243]. Furthermore, *D. salina* biodiesel had the highest Gibbs free energy compared to sunflower biodiesel and petroleum diesel being the lowest. This meant that *D. salina* biodiesel had a lower favourability of reaction compared to sunflower oil biodiesel and petroleum diesel [139]. This finding is justified by the high value of activation energy and the comprehensive performance of the *D. salina* biodiesel. The entropy change of the mentioned fuels were low and negative. This meant a reduced reaction disorder resulting in physical and chemical age of the fuel bringing it near to thermodynamic equilibrium [125]. The fuels will take time to form an activated complex [139,244]. The higher heating value of *D. salina* biodiesel was less than that of petroleum diesel but higher than that of sunflower biodiesel. This indicated the presence of more double bonds of fatty acids alkyl esters in the petroleum diesel that dissipate more energy than *D. salina* biodiesel and [139,245].

4.6 Conclusion

The findings on the optimum salinity and medium nutrients of *D. salina* was evaluated in this chapter. The *D. salina* biomass was harvested (100 %, dw), dried (24 %, dw), extracted to lipids (8.5 %,dw) and trans esterified to biodiesel (7.3 %). An optimum salinity of 2.5 M with NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3 nutrients were significant for maximum lipid production. The extracted lipids had a large presence of glycerides, free fatty acids, triglyceride and methyl ester, which are relevant constituents for biodiesel production. Potassium hydroxide (KOH) catalyst had the highest yield conversion of lipids to biodiesel during the transesterification reaction. The biodiesel had a large fraction of monounsaturated fatty acid methyl esters, which indicated a good oxidative quality. The results of the physio-chemical characterisation showed that *D. salina* biodiesel had a long shelf life with high relative molecular mass and it took more time to combust compared to petroleum diesel. The thermal characterisation results showed that *D. salina* biodiesel was highly stable as compared to sunflower oil biodiesel and petroleum diesel. This means that *D. salina* biodiesel is hard to ignite, requires high temperature to combust and burn off. *D. salina* biodiesel is recommendable to combust in the boilers without damaging the furnace walls as the temperature tolerance of the furnace boilers is up to 1500 °C.

CHAPTER 5: CONCLUSIONS, RECOMMENDATIONS AND FUTURE RESEARCH

5.1 Conclusions

This study showed that halophilic *D. salina* microalgae grown in 2.5 M salinity medium conditions optimised by RSM produces the highest biomass at 605 mgL^{-1} (dw), 35.2 % lipids content. Nutrient optimisation using the Plackett-Burman design of RSM showed that NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3 were significant nutrients that had effects on both biomass growth and lipid production whereas A5 solution of micronutrients had a significant effect on the lipid content only. These significant nutrients (NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and NaNO_3) were further optimised using Box-Behnken design of RSM and has shown to produce a maximum lipid of 181 mgL^{-1} .

For the conversion of lipids to biodiesel through transesterification process, KOH catalyst was able to produce 84 % of biodiesel as opposed to NaOH catalyst which had 75 %. TLC method was used for rapid qualitative analysis of extracted lipids. The glycerides, free fatty acids, triglyceride and methyl ester were the constituents in lipids that are compatible for biodiesel. FTIR analysis method identified the presence of triglycerides in the transesterified product (biodiesel) at wavelengths of 1743 cm^{-1} and 1742 cm^{-1} , which contained O-CH₃ and C=O bonds that represent methyl and ester carbonyl triglycerides group. The GC-MS was used, and it quantitatively identified monounsaturated methyl esters as the highest constituent in the biodiesel derived from lipids. These methyl esters were identified as 8-octadecenoic acid (36.69 %), hexadecanoic acid (35.53 %) and 9-octadecenoic acid Z constituting 36.69, 35.53 and 17.85 % of the total biodiesel composition.

The characterisation test shows that *D. salina* biodiesel can be combusted in Botash's Babcock and Wilcox boiler without damaging the furnace at the same time meeting the expected energy demand. The fuel had a high density, acid value, ester value and a low saponification value compared to the expected international specification. This means that the fuel has a high oxidative stability with a high relative molecular mass with the ability to produce low NO_x emissions during combustion. The thermal characterisation showed that *D. salina* was thermal stable as compared to sunflower biodiesel and petroleum diesel. The fuel required high temperature to ignite, combust and burn off. In addition, the higher heating value was

comparable to the other fuels mentioned in this study. The kinetics also justified the high thermal stability of the *D. salina* biodiesel.

5.2 Recommendations

The following recommendations are suggested:

- The culturing stage should maximise biomass production by integrating naturally available parameters such as sunlight, air, temperature for outdoor culturing, which is less cheap compared to controlled conditions, as well as optimisation of the process to determine the optimum parameters that promote high lipids accumulation.
- Harvesting of the cells from the culture has been a challenge especially for big volumes, therefore efficient harvesting methods should be investigated for high recovery of cells
- More studies on other base and acid transesterification methods should be investigated to determine the best method for efficient transesterification process for high biodiesel yield.
- The study should integrate with waste water treatment so as to create a sustainable system that can produce biodiesel and purify water at a low production cost.
- The biodiesel from *D. salina* should be considered for blending as currently it can not meet a sustainable capacity.

5.3 Future research

- Future research will focus on using solvent free extraction and transesterification methods that have a high yield conversion so as to economically produce the biodiesel.
- The study will work on upscaling the process for real application purposes, that is commercialisation.

5.4 Contribution to knowledge

The study has managed to establish a benchmark on the amount of *D. salina* microalgae and biodiesel that can be produced in Botswana.

5.5 Publications

Medium optimisation of *Dunaliella salina* lipid accumulation in a batch bioreactor using response surface methodology.

Decision: Not accepted due to the quantity that was limited

REFERENCES

- [1] Y. Gao, M. Yang, C. Wang, Nutrient deprivation enhances lipid content in marine microalgae, *Bioresour. Technol.* 147 (2013) 484–491. <https://doi.org/10.1016/j.biortech.2013.08.066>.
- [2] A. Bušić, S. Kundas, G. Morzak, H. Belskaya, N. Mardetko, M.I. Šantek, D. Komes, S. Novak, B. Šantek, Recent trends in biodiesel and biogas production, *Food Technol. Biotechnol.* 56 (2018) 152–173. <https://doi.org/10.17113/ftb.56.02.18.5547>.
- [3] S.N. Naik, V. V. Goud, P.K. Rout, A.K. Dalai, Production of first and second generation biofuels: A comprehensive review, *Renew. Sustain. Energy Rev.* 14 (2010) 578–597. <https://doi.org/10.1016/j.rser.2009.10.003>.
- [4] A. Dash, R. Banerjee, Enhanced biodiesel production through phyco-myco co-cultivation of *Chlorella minutissima* and *Aspergillus awamori*: An integrated approach, *Bioresour. Technol.* 238 (2017) 502–509. <https://doi.org/10.1016/j.biortech.2017.04.039>.
- [5] F. Cherubini, The biorefinery concept: Using biomass instead of oil for producing energy and chemicals, *Energy Convers. Manag.* 51 (2010) 1412–1421. <https://doi.org/10.1016/j.enconman.2010.01.015>.
- [6] L.L. Luisa Marelli, Monica Padella, Robert Edwards, Alberto Moro, Marina Kousoulidou, Jacopo Giuntoli, David Baxter, Veljko Vorkapic, Alessandro Agostini, Adrian O’Connell, The impact of biofuels on transport and the environment and their connection with agricultural development in Europe, 1996. <https://doi.org/10.1192/bjp.112.483.211-a>.
- [7] D. Evanson, Preliminary assessment of bioenergy production in the Caribbean, 2008.
- [8] L. V. Verchot, M. Van Noordwijk, S. Kandji, T. Tomich, C. Ong, A. Albrecht, J. Mackensen, C. Bantilan, K. V. Anupama, C. Palm, Climate change: Linking adaptation and mitigation through agroforestry, *Mitig. Adapt. Strateg. Glob. Chang.* 12 (2007) 901–918. <https://doi.org/10.1007/s11027-007-9105-6>.

- [9] P.S. Nigam, A. Singh, Production of liquid biofuels from renewable resources, *Prog. Energy Combust. Sci.* 37 (2011) 52–68. <https://doi.org/10.1016/j.pecs.2010.01.003>.
- [10] H.K. Reddy, Production of renewable fuels from algae, New Mexico State, 2012.
- [11] P.M. Schenk, S.R. Thomas-Hall, E. Stephens, U.C. Marx, J.H. Mussgnug, C. Posten, O. Kruse, B. Hankamer, Second generation biofuels: High-efficiency microalgae for biodiesel production, *BioEnergy Res.* 1 (2008) 20–43. <https://doi.org/10.1007/s12155-008-9008-8>.
- [12] F. Alam, A. Date, R. Rasjidin, S. Mobin, H. Moria, A. Baqui, Biofuel from algae-Is it a viable alternative?, *Procedia Eng.* 49 (2012) 221–227. <https://doi.org/10.1016/j.proeng.2012.10.131>.
- [13] A. Demirbas, Importance of biodiesel as transportation fuel, *Energy Policy.* 35 (2007) 4661–4670. <https://doi.org/10.1016/j.enpol.2007.04.003>.
- [14] RAENG, Royal academy of engineering, Sustainability of liquid biofuels, 2017. www.raeng.org.uk/biofuels.
- [15] N. Kanthavelkumaran, P. Seenikannan, Recent trends and applications of bio diesel, *Int. J. Eng. Res. Appl.* 2 (2012) 197–203. www.ijera.com.
- [16] K. Sander, G.S. Murthy, Life cycle analysis of algae biodiesel, *Int. J. Life Cycle Assess.* 15 (2010) 704–714. <https://doi.org/10.1007/s11367-010-0194-1>.
- [17] V. Patil, K.Q. Tran, H.R. Gislerød, Towards sustainable production of biofuels from microalgae, *Int. J. Mol. Sci.* 9 (2008) 1188–1195. <https://doi.org/10.3390/ijms9071188>.
- [18] UNdata, (n.d.). <http://data.un.org/Data.aspx?q=Biodiesel&d=EDATA&f=cmID%3ABD>.
- [19] D.L. Kgathi, B.N. Ngwenya, M.B.M. Sekhwela, Potential impacts of biofuel development on biodiversity in Chobe District, Botswana, *Bioenergy Sustain. Dev. Africa.* 9789400721 (2012) 247–260. <https://doi.org/10.1007/978-94-007-2181-4-21>.
- [20] National Development Plan 11 Volume 1 April 2017 – March 2023, 2017.

https://www.finance.gov.bw/index.php?option=com_content&view=category&id=28&Itemid=126.

- [21] R.A. Ahmed, M. He, R.A. Aftab, S. Zheng, M. Nagi, R. Bakri, C. Wang, Bioenergy application of *Dunaliella salina* SA 134 grown at various salinity levels for lipid production, *Sci. Rep.* 7 (2017) 1–10. <https://doi.org/10.1038/s41598-017-07540-x>.
- [22] M.P. Ralefala, Evaluation of *Dunaliella* isolates from Sua Pan (Botswana) solar evaporation ponds for production of B-carotene, Cape town, 2011. <https://doi.org/hdl.handle.net/11427/12243>.
- [23] R.A. Ahmed, M. He, R.A. Aftab, S. Zheng, M. Nagi, R. Bakri, C. Wang, Bioenergy application of *Dunaliella salina* SA 134 grown at various salinity levels for lipid production, *Sci. Rep.* 7 (2017) 1–10. <https://doi.org/10.1038/s41598-017-07540-x>.
- [24] R.M.C. and G.S.M. Ankita Juneja, Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: A review, *Energies.* (2013) 4607–4638. <https://doi.org/10.3390/en6094607>.
- [25] H. Taher, S. Al-Zuhair, A. Al-Marzouqi, Y. Haik, M. Farid, Growth of microalgae using CO₂ enriched air for biodiesel production in supercritical CO₂, *Renew. Energy.* 82 (2015) 61–70. <https://doi.org/10.1016/j.renene.2014.08.013>.
- [26] T. Suganya, M. Varman, H.H. Masjuki, S. Renganathan, Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach, *Renew. Sustain. Energy Rev.* 55 (2016) 909–941. <https://doi.org/10.1016/j.rser.2015.11.026>.
- [27] R. Daghrir, L. Igounet, S.K. Brar, P. Drogui, Novel electrochemical method for the recovery of lipids from microalgae for biodiesel production, *J. Taiwan Inst. Chem. Eng.* 45 (2014) 153–162. <https://doi.org/10.1016/j.jtice.2013.04.013>.
- [28] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: A review, *Renew. Sustain. Energy Rev.* 14 (2010) 217–232. <https://doi.org/10.1016/j.rser.2009.07.020>.

- [29] V. Okoro, U. Azimov, J. Munoz, H.H. Hernandez, A.N. Phan, Microalgae cultivation and harvesting: Growth performance and use of flocculants - A review, *Renew. Sustain. Energy Rev.* 115 (2019). <https://doi.org/10.1016/j.rser.2019.109364>.
- [30] K. Heimann, R. Huerlimann, Microalgal classification: Major classes and genera of commercial microalgal species., *Handb. Mar. Microalgae Biotechnol. Adv.* (2015) 25–41. <https://doi.org/10.1016/B978-0-12-800776-1.00003-0>.
- [31] M. Shariati, M.R.R. Hadi, Microalgal biotechnology and bioenergy in *Dunaliella*, *Prog. Mol. Environ. Bioeng. - From Anal. Model. to Technol. Appl.* (2011) 447–506. <https://doi.org/10.5772/19046>.
- [32] J. Singh, R.C. Saxena, An introduction to microalgae: Diversity and significance., *Handb. Mar. Microalgae Biotechnol. Adv.* (2015) 11–24. <https://doi.org/10.1016/B978-0-12-800776-1.00002-9>.
- [33] A.F. Talebi, M. Tohidfar, S. Mahsa, M. Derazmahalleh, A. Sulaiman, A.S. Baharuddin, M. Tabatabaei, Biochemical modulation of lipid pathway in microalgae *Dunaliella* sp . for biodiesel production, 2015 (2015).
- [34] W. Kim, J. Min, P. Geun, H. Gim, D.K. Si, W. Kim, Optimization of culture conditions and comparison of biomass productivity of three green algae, (2012) 19–27. <https://doi.org/10.1007/s00449-011-0612-1>.
- [35] S.K. Hansen, K. Lauridsen, R. Petersen, N. Thiry, Biodiesel from microalgae a possible competitor to petroleum diesel, Aalborg, 2015. <https://doi.org/CBT15-B311b>.
- [36] J.C.M. Pires, Mass production of microalgae, in: *Handb. Mar. Microalgae Biotechnol. Adv.*, 2015: pp. 55–68. <https://doi.org/10.1016/B978-0-12-800776-1.00005-4>.
- [37] L. Xia, R. Huang, Y. Li, S. Song, The effect of growth phase on the surface properties of three oleaginous microalgae (*Botryococcus* sp. FACGB-762, *Chlorella* sp. XJ-445 and *Desmodesmus bijugatus* XJ-231), *PLoS One.* 12 (2017). <https://doi.org/10.1371/journal.pone.0186434>.
- [38] X. Su, J. Xu, X. Yan, P. Zhao, J. Chen, C. Zhou, F. Zhao, S. Li, Lipidomic changes

during different growth stages of *Nitzschia closterium* f. *minutissima*, *Metabolomics*. 9 (2013) 300–310. <https://doi.org/10.1007/s11306-012-0445-1>.

- [39] P. Lavens, P. ; Sorgeloos, Manual on the production and use of live food for aquaculture: Micro-algae. FAO. Belgium, in: FAO Fish. Tech. Pap., Food and Agriculture Organization (FAO)., Rome, 1996: pp. 7–48. <https://doi.org/19971409717>.
- [40] L. Fatemeh, D. Mohsen, Effects of environmental factors on the growth, optical density and biomass of the green algae *Chlorella vulgaris* in outdoor conditions, *J. Appl. Sci. Environ. Manag.* 20 (2016) 133. <https://doi.org/10.4314/jasem.v20i1.16>.
- [41] S. Tahir, Raceway-based production of microalgae for possible use in making biodiesel, Massey University, 2014. <https://doi.org/10179/5621>.
- [42] P.G. Stephenson, C.M. Moore, M.J. Terry, M. V. Zubkov, T.S. Bibby, Improving photosynthesis for algal biofuels: Toward a green revolution, *Trends Biotechnol.* 29 (2011) 615–623. <https://doi.org/10.1016/j.tibtech.2011.06.005>.
- [43] A. Yenilmez, C. Tekkaya, Enhancing students' understanding of photosynthesis and respiration in plant through conceptual change approach, *J. Sci. Educ. Technol.* 15 (2006) 81–87. <https://doi.org/10.1007/s10956-006-0358-8>.
- [44] J. Gu, Z. Zhou, Z. Li, Y. Chen, Z. Wang, H. Zhang, J. Yang, Photosynthetic properties and potentials for improvement of photosynthesis in pale green leaf rice under high light conditions, *Front. Plant Sci.* 8 (2017) 1–14. <https://doi.org/10.3389/fpls.2017.01082>.
- [45] I, C. Yu, R. Chen, J.J. Li, J.J. Li, M. Drahansky, M.. Paridah, A. Moradbak, A.. Mohamed, H. Abdulwahab taiwo Owolabi, FolaLi, M. Asniza, S.H.. Abdul Khalid, T. Sharma, N. Dohare, M. Kumari, U.K. Singh, A.B. Khan, M.S. Borse, R. Patel, A. Paez, A. Howe, D. Goldschmidt, C. Corporation, J. Coates, F. Reading, Fatty acids from microalgae: Targeting the accumulation of triacylglycerides, *Intech. i* (2012) 13. <https://doi.org/10.1016/j.colsurfa.2011.12.014>.
- [46] T. Cardona, S. Shao, P.J. Nixon, Enhancing photosynthesis in plants: the light reactions, *Essays Biochem.* 62 (2018) 85–94. <https://doi.org/10.1042/EBC20170015>.

- [47] J. Marchand, P. Heydarizadeh, B. Schoefs, C. Spetea, Ion and metabolite transport in the chloroplast of algae: lessons from land plants, *Cell. Mol. Life Sci.* 75 (2018) 2153–2176. <https://doi.org/10.1007/s00018-018-2793-0>.
- [48] D. Kuan, Growth optimization of *Synechococcus elongatus* PCC 7942 in lab flask and 2D photobioreactor, University of British Columbia, 2013. <https://doi.org/10.14288/1.0074247>.
- [49] K. Ramluckan, Development and optimization of technology for the extraction and conversion of micro algal lipids to biodiesel, Durban University of Technology, 2015. <https://doi.org/10321/1282>.
- [50] M.W. Hutton, Extraction and characterization of lipids from microalgae grown on municipal wastewater, California Polytechnic State University, 2009. <https://doi.org/https://doi.org/10.15368/theses.2009.183>.
- [51] C. Xie, J. Liu, X. Zhang, W. Xie, J. Sun, K. Chang, J. Kuo, W. Xie, C. Liu, S. Sun, M. Buyukada, F. Evrendilek, Co-combustion thermal conversion characteristics of textile dyeing sludge and pomelo peel using TGA and artificial neural networks, *Appl. Energy*. 212 (2018) 786–795. <https://doi.org/10.1016/j.apenergy.2017.12.084>.
- [52] J. Huang, J. Xia, W. Jiang, Y. Li, J. Li, Biodiesel production from microalgae oil catalyzed by a recombinant lipase, *Bioresour. Technol.* 180 (2015) 47–53. <https://doi.org/10.1016/j.biortech.2014.12.072>.
- [53] F. Liang, X. Wen, Y. Geng, Z. Ouyang, L. Luo, Y. Li, Growth rate and biomass productivity of *Chlorella* as affected by culture depth and cell density in an open circular photobioreactor, *J. Microbiol. Biotechnol.* 23 (2013) 539–544. <https://doi.org/10.4014/jmb.1209.09047>.
- [54] S.S. Gill, M.A. Mehmood, N. Ahmad, M. Ibrahim, U. Rashid, S. Ali, I.A. Nehdi, Strain selection, growth productivity and biomass characterization of novel microalgae isolated from fresh and wastewaters of upper Punjab, Pakistan, *Front. Life Sci.* 9 (2016) 190–200. <https://doi.org/10.1080/21553769.2016.1204957>.

- [55] M. Song, H. Pei, W. Hu, F. Han, Y. Ji, G. Ma, L. Han, Growth and lipid accumulation properties of microalgal *Phaeodactylum tricornutum* under different gas liquid ratios, *Bioresour. Technol.* 165 (2014) 31–37. <https://doi.org/10.1016/j.biortech.2014.03.070>.
- [56] A. Dash, R. Banerjee, In silico optimization of lipid yield utilizing mix-carbon sources for biodiesel production from *Chlorella minutissima*, *Energy Convers. Manag.* 164 (2018) 533–542. <https://doi.org/10.1016/j.enconman.2018.03.022>.
- [57] S. Mehdi, H. Sohi, A. Eghdami, Biodiesel production using marine microalgae *Dunaliella salina*, 4 (2014) 177–182. <https://doi.org/22223045>.
- [58] S. Rasoul-Amini, P. Mousavi, N. Montazeri-Najafabady, M.A. Mobasher, S.B. Mousavi, F. Vosough, F. Dabbagh, Y. Ghasemi, Biodiesel properties of native strain of *Dunaliella salina*, *Int. J. Renew. ENERGY Res.* 4 (2014) 39–41.
- [59] S.M. Nadzir, N. Yusof, N. Nordin, H. Abdullah, A. Kamari, Combination effect of temperature and light intensity on lipid productivity of *Tetradismus obliquus*, *J. Phys. Conf. Ser.* 1097 (2018). <https://doi.org/10.1088/1742-6596/1097/1/012038>.
- [60] B. Cheirsilp, S. Torpee, Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation, *Bioresour. Technol.* 110 (2012) 510–516. <https://doi.org/10.1016/j.biortech.2012.01.125>.
- [61] T. Menegol, A.B. Diprat, E. Rodrigues, R. Rech, Effect of temperature and nitrogen concentration on biomass composition of *heterochlorella luteoviridis*, *Food Sci. Technol.* 37 (2017) 28–37. <https://doi.org/10.1590/1678-457X.13417>.
- [62] P. Chaisutyakorn, J. Praiboon, C. Kaewsuralikhit, The effect of temperature on growth and lipid and fatty acid composition on marine microalgae used for biodiesel production, *J. Appl. Phycol.* 30 (2018) 37–45. <https://doi.org/10.1007/s10811-017-1186-3>.
- [63] V.M. Stigum, The effect of light and temperature on lipid production in microalgae, 2012. <https://doi.org/55212284>.
- [64] Z.Y. Wen, F. Chen, Heterotrophic production of eicosapentaenoic acid by microalgae,

- Biotechnol. Adv. 21 (2003) 273–294. [https://doi.org/10.1016/S0734-9750\(03\)00051-X](https://doi.org/10.1016/S0734-9750(03)00051-X).
- [65] T. Ren, Primary factors affecting growth of microalgae optimal light exposure duration and frequency, 2014. <https://doi.org/10.31274/etd-180810-2871>.
- [66] Musa, Ayoko, Ward, Rösch, Brown, Rainey, Factors affecting microalgae production for biofuels and the potentials of chemometric methods in assessing and optimizing productivity, Cells. 8 (2019) 851. <https://doi.org/10.3390/cells8080851>.
- [67] M. Chen, H. Tang, H. Ma, T.C. Holland, K.Y.S. Ng, S.O. Salley, Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*, Bioresour. Technol. 102 (2011) 1649–1655. <https://doi.org/10.1016/j.biortech.2010.09.062>.
- [68] L. Zhu, Sustainable biodiesel production from microalgae cultivated with piggery wastewater, Vaasa, 2014. http://www.uva.fi/materiaali/pdf/isbn_978-952-476-501-5.pdf.
- [69] L. Xin, H. Hong-ying, G. Ke, S. Ying-xue, Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp., Bioresour. Technol. 101 (2010) 5494–5500. <https://doi.org/10.1016/j.biortech.2010.02.016>.
- [70] A.D. Cembella, N.J. Antia, P.J. Harrison, The utilization of inorganic and organic phosphorous compounds as nutrients by eukaryotic microalgae: A multidisciplinary perspective: Part i, Crit. Rev. Microbiol. 10 (1982) 317–391. <https://doi.org/10.3109/10408418209113567>.
- [71] S.H. Shah, I.A. Raja, Q. Mahmood, A. Pervez, Bioresource technology improvement in lipids extraction processes for biodiesel production from wet microalgal pellets grown on diammonium phosphate and sodium bicarbonate combinations, Bioresour. Technol. 214 (2016) 199–209. <https://doi.org/10.1016/j.biortech.2016.04.036>.
- [72] D.A. White, A. Pagarette, P. Rooks, S.T. Ali, The effect of sodium bicarbonate supplementation on growth and biochemical composition of marine microalgae cultures, (2013) 153–165. <https://doi.org/10.1007/s10811-012-9849-6>.

- [73] K. Mokashi, V. Shetty, S. Annie, G. Sibi, Sodium bicarbonate as inorganic carbon source for higher biomass and lipid production integrated carbon capture in *Chlorella vulgaris*, *ALS*. 10 (2016) 111–117. <https://doi.org/10.1016/j.als.2016.05.011>.
- [74] K. Liang, Q. Zhang, M. Gu, W. Cong, Effect of phosphorus on lipid accumulation in freshwater microalga *Chlorella* sp., *J. Appl. Phycol.* 25 (2013) 311–318. <https://doi.org/10.1007/s10811-012-9865-6>.
- [75] A. Patel, S. Barrington, M. Lefsrud, Microalgae for phosphorus removal and biomass production: A six species screen for dual-purpose organisms, *GCB Bioenergy*. 4 (2012) 485–495. <https://doi.org/10.1111/j.1757-1707.2012.01159.x>.
- [76] M. Mourabet, A. El Rhilassi, H. El Boujaady, M. Bennani-Ziatni, A. Taitai, Use of response surface methodology for optimization of fluoride adsorption in an aqueous solution by Brushite, *Arab. J. Chem.* 10 (2017) S3292–S3302. <https://doi.org/10.1016/j.arabjc.2013.12.028>.
- [77] C.M. Anderson-Cook, C.M. Borror, D.C. Montgomery, Response surface design evaluation and comparison, *J. Stat. Plan. Inference*. 139 (2009) 629–641. <https://doi.org/10.1016/j.jspi.2008.04.004>.
- [78] K.M. Carley, N.Y. Kamneva, J. Reminga, Response surface methodology, 2004. <https://doi.org/10.1002/wics.73>.
- [79] P. Chen, M. Min, Y. Chen, L. Wang, Y. Li, Q. Chen, C. Wang, Y. Wan, X. Wang, Y. Cheng, S. Deng, K. Hennessy, X. Lin, Y. Liu, Y. Wang, B. Martinez, R. Ruan, Review of the biological and engineering aspects of algae to fuels approach, *Int. J. Agric. Biol. Eng.* 2 (2009) 1–30. <https://doi.org/10.3965/j.issn.1934-6344.2009.04.001-030>.
- [80] K. Bajwa, N.R. Bishnoi, A. Kirrolia, S. Gupta, S. Tamil, Response surface methodology as a statistical tool for optimization of physio - biochemical cellular components of microalgae *Chlorella pyrenoidosa* for biodiesel production, *Appl. Water Sci.* 9 (2019) 1–16. <https://doi.org/10.1007/s13201-019-0969-x>.
- [81] A. Kirrolia, N.R. Bishnoi, R. Singh, Response surface methodology as a decision-

- making tool for optimization of culture conditions of green microalgae *Chlorella* spp. for biodiesel production, *Ann. Microbiol.* 64 (2014) 1133–1147. <https://doi.org/10.1007/s13213-013-0752-4>.
- [82] I.W.A. Publishing, W. Science, Optimization of simultaneous biomass production and nutrient removal by mixotrophic *Chlorella* sp . using response surface methodology Yu-Ru Lee and Jen-Jeng Chen, (2016) 1520–1531. <https://doi.org/10.2166/wst.2015.626>.
- [83] V.N. Gaitonde, M. Manjaiah, S. Maradi, S.R. Karnik, P.M. Petkar, J.P. Davim, Multiresponse optimization in wire electric discharge machining (WEDM) of HCHCr steel by integrating response surface methodology (RSM) with differential evolution (DE), Elsevier Ltd, 2017. <https://doi.org/10.1016/B978-0-85709-481-0.00007-0>.
- [84] L. Ramanna, A. Guldhe, I. Rawat, F. Bux, The optimization of biomass and lipid yields of *Chlorella sorokiniana* when using wastewater supplemented with different nitrogen sources, *Bioresour. Technol.* 168 (2014) 127–135. <https://doi.org/10.1016/j.biortech.2014.03.064>.
- [85] O. Jorquera, A. Kiperstok, E.A. Sales, M. Embirucu, M.L. Ghirardi, Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors, *Bioresour. Technol.* 101 (2010) 1406–1413. <https://doi.org/10.1016/j.biortech.2009.09.038>.
- [86] R.R. Narala, S. Garg, K.K. Sharma, S.R. Thomas-Hall, M. Deme, Y. Li, P.M. Schenk, Comparison of microalgae cultivation in photobioreactor, open raceway pond, and a two-stage hybrid system, *Front. Energy Res.* 4 (2016) 1–10. <https://doi.org/10.3389/fenrg.2016.00029>.
- [87] R. Harun, M. Davidson, M. Doyle, R. Gopiraj, M. Danquah, G. Forde, Technoeconomic analysis of an integrated microalgae photobioreactor, biodiesel and biogas production facility, *Biomass and Bioenergy.* 35 (2011) 741–747. <https://doi.org/10.1016/j.biombioe.2010.10.007>.
- [88] A. Zenouzi, B. Ghobadian, M.A. Hejazi, P. Rahnemoon, Harvesting of microalgae *Dunaliella salina* using electroflocculation, *J. Agric. Sci. Technol.* 15 (2013) 879–888.

- [89] J. Monte, M. Sá, C.F. Galinha, L. Costa, H. Hoekstra, C. Brazinha, J.G. Crespo, Harvesting of *Dunaliella salina* by membrane filtration at pilot scale, *Sep. Purif. Technol.* 190 (2018) 252–260. <https://doi.org/10.1016/j.seppur.2017.08.019>.
- [90] G. Shelef, A. Sukenik, M. Green, Microalgae harvesting and processing: a literature review, 1984. <https://doi.org/10.2172/6204677>.
- [91] H. Kandala, The study of variations in the properties of biodiesel on addition of antioxidants, (2009). <https://doi.org/93>.
- [92] C. Wayne, Joshua, G. David, Matthew, M. Scott, Alexander, G. Hutchinson, W. Iv, A comparative analysis of biodiesel and diesel emission, Worcester Polytecnic Institute, n.d. <http://www.wpi.edu/academics/ugradstudies/project-learning.html>.
- [93] N. El Boulifi, A. Bouaid, M. Martinez, J. Aracil, Optimization and oxidative stability of biodiesel production from rice bran oil, *Renew. Energy.* 53 (2013) 141–147. <https://doi.org/10.1016/j.renene.2012.11.005>.
- [94] J. Waring, K. Svanberg, Characterisation of chemical decomposition of biodiesel, Chalmers University of Technology, 2012. <https://doi.org/20.500.12380/159144>.
- [95] L.T. Thanh, K. Okitsu, L. Van Boi, Y. Maeda, Catalytic technologies for biodiesel fuel production and utilization of glycerol: A review, *Catalysts.* 2 (2012) 191–222. <https://doi.org/10.3390/catal2010191>.
- [96] S. Fajardo, García-Galvan, F. R., V. Barranco, J.C. Galvan, S.F. Batlle, Review of catalytic transesterification methods for review of catalytic transesterification methods for biodiesel production biodiesel production, in: *Intech*, 2016: p. 13. <https://doi.org/http://dx.doi.org/10.5772/57353>.
- [97] I.M. Atadashi, M.K. Aroua, A.R. Abdul Aziz, N.M.N. Sulaiman, The effects of catalysts in biodiesel production: A review, *J. Ind. Eng. Chem.* 19 (2013) 14–26. <https://doi.org/10.1016/j.jiec.2012.07.009>.
- [98] D.Y.C. Leung, X. Wu, M.K.H. Leung, A review on biodiesel production using catalyzed transesterification, *Appl. Energy.* 87 (2010) 1083–1095.

<https://doi.org/10.1016/j.apenergy.2009.10.006>.

- [99] S.S. Win, T.A. Trabold, Sustainable waste-to-energy technologies: transesterification, in: *Sustain. Food Waste-To-Energy Syst.*, Elsevier Inc., 2018: pp. 89–109. <https://doi.org/10.1016/b978-0-12-811157-4.00006-1>.
- [100] M. Tariq, S. Ali, N. Khalid, Activity of homogeneous and heterogeneous catalysts, spectroscopic and chromatographic characterization of biodiesel: A review, *Renew. Sustain. Energy Rev.* 16 (2012) 6303–6316. <https://doi.org/10.1016/j.rser.2012.07.005>.
- [101] N. Kaushik, K. Kumar, S. Kumar, Catalysts in production of biodiesel: A review, *J. Biobased Mater. Bioenergy.* 1 (2008) 301–314. <https://doi.org/10.1166/jbmb.2007.002>.
- [102] J.K. Volkman, S.W. Jeffrey, P.D. Nichols, G.I. Rogers, Fatty acid and lipid composition of 10 species of microalgae used in mariculture, 128 (1989) 219–240. <https://doi.org/0022/0981>.
- [103] V.P. Skipsi, A.F. Smolowe, C.R. Stullivan, Separation of lipid classes by TLC, *Biochim. Biophys. Acta.* 106 (1965) 386–396. [https://doi.org/10.1016/0005-2760\(65\)90047-0](https://doi.org/10.1016/0005-2760(65)90047-0).
- [104] H.K. Mangold, Thin-layer chromatography of lipids, *J. Am. Oil Chem. Soc.* 41 (1964) 762–773. <https://doi.org/10.1007/BF02676266>.
- [105] B.V.P. Seipski, M. Barclay, Thin-layer chromatography of lipids, in: n.d.
- [106] M.L. Blank, J.A. Schmit, O.S. Privett, Quantitative analysis of lipids by thin-layer chromatography, (1964). <https://doi.org/10.1007/BF02654817>.
- [107] I. Edeh, Evaluation of the lipid composition in activated sludge biomass for biodiesel and oleochemical production by thin layer chromatography (TLC), 5 (2018) 40–45. <https://doi.org/2394-9333>.
- [108] Akeem Abiodun Babatunde, In situ transesterification of rapeseed for production of biodiesel and secondary products, 2018. [https://theses.ncl.ac.uk/jspui/bitstream/10443/4166/1/Babatunde%2C A 2018.pdf](https://theses.ncl.ac.uk/jspui/bitstream/10443/4166/1/Babatunde%2C%20A%202018.pdf).
- [109] N. Vlachos, Y. Skopelitis, M. Psaroudaki, V. Konstantinidou, A. Chatzilazarou, E.

- Tegou, Applications of fourier transform-infrared spectroscopy to edible oils, *Anal. Chim. Acta.* 573–574 (2006) 459–465. <https://doi.org/10.1016/j.aca.2006.05.034>.
- [110] F. Ji, R. Hao, Y. Liu, G. Li, Y. Zhou, R. Dong, Isolation of a novel microalgae strain *Desmodesmus* sp. and optimization of environmental factors for its biomass production, *Bioresour. Technol.* 148 (2013) 249–254. <https://doi.org/10.1016/j.biortech.2013.08.110>.
- [111] G. Vidyadharani, R. Dhandapani, Fourier transform infrared (FTIR) spectroscopy for the analysis of lipid from *Chlorella vulgaris* Fourier transform infrared (FTIR) spectroscopy for the analysis of lipid from *Chlorella vulgaris*, *Appl. Biol.* 61 (2013). <https://doi.org/10.1016/j.applbiol.2013.08.010>.
- [112] O. Awogbemi, E.I. Onuh, C.A. Komolafe, Thermal degradation and spectroscopic study of neat palm oil, waste palm oil, and waste palm oil methyl ester, *IOP Conf. Ser. Earth Environ. Sci.* 331 (2019). <https://doi.org/10.1088/1755-1315/331/1/012032>.
- [113] J. Liu, J. Mukherjee, J.J. Hawkes, S.J. Wilkinson, Optimization of lipid production for algal biodiesel in nitrogen stressed cells of *Dunaliella salina* using FTIR analysis, *J. Chem. Technol. Biotechnol.* 88 (2013) 1807–1814. <https://doi.org/10.1002/jctb.4027>.
- [114] B.R. Carlo, O. Bechtel, T. Allen, FTIR algae-lipid analysis purpose / goal of experiment, 2012. <https://doi.org/10.1007/s12155-010-9098-y>.
- [115] S. Elumalai, R. Sakthivel, GC-MS and FT-IR spectroscopic determination of fatty acid methyl ester of 16 freshwater microalgae, isolated from cement industries of Tamil Nadu, India, 4 (2013) 50–69. <https://doi.org/10.1016/j.jce.2013.08.005>.
- [116] O.A.Y.; E.. Bello, Use of fourier transformation infrared (FTIR) spectroscopy for analysis of functional groups in peanut oil biodiesel and its blends, *Br. J. Appl. Sci. Technol.* 13 (2016) 1–14. <https://doi.org/10.9734/BJAST/2016/22178>.
- [117] T. Yuan, E. Akochi-Koble, D. Pinchuk, F. de Voort, FTIR on-line monitoring of biodiesel transesterification, *Int. J. Renew. Energy Biofuels.* 2014 (2014) 1–13. <https://doi.org/10.5171/2014.178474>.

- [118] S.S.M. Mostafa, N.S. El-Gendy, Evaluation of fuel properties for microalgae *Spirulina platensis* bio-diesel and its blends with Egyptian petro-diesel, *Arab. J. Chem.* 10 (2017) S2040–S2050. <https://doi.org/10.1016/j.arabjc.2013.07.034>.
- [119] A. Kchech, The quantification of fatty acids in microalgae using GC-FID and GC-MS, California State Polytechnic, 2017. http://csufresno-dspace.calstate.edu/bitstream/handle/10211.3/194615/KchechAmera_Thesis2017.pdf?sequence=3.
- [120] S.V. Mohan, M.V. Rohit, G.V. Subhash, R. Chandra, M.P. Devi, S.K. Butti, K. Rajesh, *Algal oils as biodiesel*, Second Edi, Elsevier B.V., 2019. <https://doi.org/10.1016/b978-0-444-64192-2.00012-3>.
- [121] N.R. Board, Fuel characterization, in: 1995: pp. 151–189.
- [122] R. Barret, Importance and evaluation of lipophilicity, *Ther. Chem.* (2018) 53–78. <https://doi.org/10.1016/b978-1-78548-288-5.50003-2>.
- [123] S.A.E.A. Ismail, R.F.M. Ali, Physico-chemical properties of biodiesel manufactured from waste frying oil using domestic adsorbents, *Sci. Technol. Adv. Mater.* 16 (2015) 1–9. <https://doi.org/10.1088/1468-6996/16/3/034602>.
- [124] J.C.J. Bart, N. Palmeri, S. Cavallaro, Emerging new energy crops for biodiesel production, *Biodiesel Sci. Technol.* (2010) 226–284. <https://doi.org/10.1533/9781845697761.226>.
- [125] F. Safety, S. Authority, M.O.F. Health, F. Welfare, N.E.W. Delhi, *Manual of methods of analysis of foods*, New Delhi, 2015. [https://fssai.gov.in/upload/uploadfiles/files/Manual_Oil_Fat_25_05_2016\(1\).pdf](https://fssai.gov.in/upload/uploadfiles/files/Manual_Oil_Fat_25_05_2016(1).pdf).
- [126] A. Baig, F.T.T. Ng, Determination of acid number of biodiesel and biodiesel blends, *JAOCS, J. Am. Oil Chem. Soc.* 88 (2011) 243–253. <https://doi.org/10.1007/s11746-010-1667-x>.
- [127] S. Mahajan, S.K. Konar, D.G.B. Boocock, Determining the acid number of biodiesel, *JAOCS, J. Am. Oil Chem. Soc.* 83 (2006) 567–570. <https://doi.org/10.1007/s11746-006->

1241-8.

- [128] Z. Taghizade, Determination of biodiesel quality parameters for optimization of production process conditions, Instituto Politecnico De Braganca Escola Superior de Tecnologia, 2016. [https://bibliotecadigital.ipb.pt/bitstream/10198/13171/1/Zakir_Final_Thesis...Zakir2016a PB.pdf](https://bibliotecadigital.ipb.pt/bitstream/10198/13171/1/Zakir_Final_Thesis...Zakir2016a_PB.pdf).
- [129] A. Bong, N. Kor, P. Ndifon, Y. Sani, Synthesis and characterisation of biodiesel from Cameroon palm kernel seed oil, *Asian J. Biotechnol. Bioresour. Technol.* 3 (2018) 1–17. <https://doi.org/10.9734/ajb2t/2018/40200>.
- [130] M. Parlaktuna, M. Verşan K  k, N. Karahano  lu, Thermal characterization and kinetics of diesel, methanol route biodiesel, canola oil and diesel-biodiesel blends at different blending rates by TGA and DSC, 2010. <https://etd.lib.metu.edu.tr/upload/12612460/index.pdf>.
- [131] M.V. Kok, E. Topa, Combustion characteristics and kinetics of diesel and canola oil samples, *Energy Sources, Part A Recover. Util. Environ. Eff.* 38 (2016) 967–974. <https://doi.org/10.1080/15567036.2015.1118580>.
- [132] S. Anandhan, Thermal Analysis, 2003. <https://www.scribd.com/document/250818532/APOSTILLA-Anandhan-Thermal-Analysis>.
- [133] PerkinElmer Inc, Thermogravimetric Analysis (TGA), 2015. https://www.perkinelmer.com/lab-solutions/resources/docs/faq_beginners-guide-to-thermogravimetric-analysis_009380c_01.pdf.
- [134] H. Li, S.L. Niu, C.M. Lu, S.Q. Cheng, Comparative evaluation of thermal degradation for biodiesels derived from various feedstocks through transesterification, *Energy Convers. Manag.* 98 (2015) 81–88. <https://doi.org/10.1016/j.enconman.2015.03.097>.
- [135] R.P. Rodr  guez, R. Sierens, S. Verhelst, Thermal and kinetic evaluation of biodiesel derived from soybean oil and higuereta oil, *J. Therm. Anal. Calorim.* 96 (2009) 897–901. <https://doi.org/10.1007/s10973-009-0043-2>.

- [136] J. Dweck, C.M.S. Sampaio, Analysis of the thermal decomposition of commercial vegetable oils in air by simultaneous TG/DTA, *J. Therm. Anal. Calorim.* 75 (2004) 385–391. <https://doi.org/10.1023/B:JTAN.0000027124.96546.0f>.
- [137] D. Banerjee, Experimental techniques in thermal analysis thermogravimetry (TG) & differential scanning calorimetry (DSC), Kanpur, 1993. <https://www.iitk.ac.in/che/pdf/resources/TGA-DSC-reading-material.pdf>.
- [138] J.J. Lu, W.H. Chen, Investigation on the ignition and burnout temperatures of bamboo and sugarcane bagasse by thermogravimetric analysis, *Appl. Energy.* 160 (2015) 49–57. <https://doi.org/10.1016/j.apenergy.2015.09.026>.
- [139] M. Almazrouei, I. Janajreh, Thermogravimetric study of the combustion characteristics of biodiesel and petroleum diesel, *J. Therm. Anal. Calorim.* 136 (2019) 925–935. <https://doi.org/10.1007/s10973-018-7717-6>.
- [140] S. Ahn, G. Choi, D. Kim, The effect of wood biomass blending with pulverized coal on combustion characteristics under oxy-fuel condition, *Biomass and Bioenergy.* 71 (2014) 144–154. <https://doi.org/10.1016/j.biombioe.2014.10.014>.
- [141] S. Paniagua, L.F. Calvo, C. Escapa, R.N. Coimbra, M. Otero, A.I. García, *Chlorella sorokiniana* thermogravimetric analysis and combustion characteristic indexes estimation, *J. Therm. Anal. Calorim.* 131 (2018) 3139–3149. <https://doi.org/10.1007/s10973-017-6734-1>.
- [142] D. Vamvuka, S. Sfakiotakis, Combustion behaviour of biomass fuels and their blends with lignite, *Thermochim. Acta.* 526 (2011) 192–199. <https://doi.org/10.1016/j.tca.2011.09.021>.
- [143] K. Cong, F. Han, Y. Zhang, Q. Li, The investigation of co-combustion characteristics of tobacco stalk and low rank coal using a macro-TGA, *Fuel.* 237 (2019) 126–132. <https://doi.org/10.1016/j.fuel.2018.09.149>.
- [144] X.G. Li, B.G. Ma, L. Xu, Z.W. Hu, X.G. Wang, Thermogravimetric analysis of the co-combustion of the blends with high ash coal and waste tyres, *Thermochim. Acta.* 441

- (2006) 79–83. <https://doi.org/10.1016/j.tca.2005.11.044>.
- [145] S.G. Sahu, P. Sarkar, N. Chakraborty, A.K. Adak, Thermogravimetric assessment of combustion characteristics of blends of a coal with different biomass chars, *Fuel Process. Technol.* 91 (2010) 369–378. <https://doi.org/10.1016/j.fuproc.2009.12.001>.
- [146] X. Liu, M. Chen, D. Yu, Oxygen enriched co-combustion characteristics of herbaceous biomass and bituminous coal, *Thermochim. Acta.* 569 (2013) 17–24. <https://doi.org/10.1016/j.tca.2013.06.037>.
- [147] M. Barbanera, F. Cotana, U. Di Matteo, Co-combustion performance and kinetic study of solid digestate with gasification biochar, *Renew. Energy.* 121 (2018) 597–605. <https://doi.org/10.1016/j.renene.2018.01.076>.
- [148] Y. Lin, X. Ma, X. Ning, Z. Yu, TGA-FTIR analysis of co-combustion characteristics of paper sludge and oil-palm solid wastes, *Energy Convers. Manag.* 89 (2015) 727–734. <https://doi.org/10.1016/j.enconman.2014.10.042>.
- [149] X. Liu, M. Chen, Y. Wei, Assessment on oxygen enriched air co-combustion performance of biomass/bituminous coal, *Renew. Energy.* 92 (2016) 428–436. <https://doi.org/10.1016/j.renene.2016.02.035>.
- [150] M. Versan, K. Ece, Thermal characterization and model-free kinetics of biodiesel sample, *J. Therm. Anal. Calorim.* (2015). <https://doi.org/10.1007/s10973-015-4814-7>.
- [151] M. Almazrouei, I. Janajreh, Model-fitting approach to kinetic analysis of non-isothermal pyrolysis of pure and crude glycerol, *Renew. Energy.* 145 (2020) 1693–1708. <https://doi.org/10.1016/j.renene.2019.07.095>.
- [152] A.S. Reshad, P. Tiwari, V. V. Goud, Thermal degradation kinetic study of rubber seed oil and its methyl esters under inert atmosphere, *Energy and Fuels.* 31 (2017) 9642–9651. <https://doi.org/10.1021/acs.energyfuels.7b02249>.
- [153] A. Al-ansari, Technical manual for live food production, 2007. <https://docplayer.net/40307979-Technical-manual-for-live-food-production-adly-al-ansari.html>.

- [154] W.-C. Wang, W.T. Sullivan, R.L. Evans, A technique for evaluating algal growth potential in illinois surface waters, Urbana, 1973.
<https://www.isws.illinois.edu/pubdoc/RI/ISWSRI-72.pdf>.
- [155] R.D.E. Bernardi, Methods for the estimation of zooplankton abundance, in: A Man. Methods Assess. Second. Product. Fresh Waters, 1984: pp. 59–86.
<https://www.scielo.br/pdf/aabc/v91n1/0001-3765-aabc-201820170807.pdf>.
- [156] C.J. Wu, C.M. Shin, K.P. Chiang, Does the mesh size of the plankton net affect the result of statistical analyses of the relationship between the copepod community and water masses?, Crustaceana. 84 (2011) 1069–1083.
<https://doi.org/10.1163/001121611X586701>.
- [157] C.P. Gopinathan, Life feed culture micro algae, 1996.
<https://core.ac.uk/download/pdf/33017838.pdf>.
- [158] F. Lananan, A. Jusoh, N. Ali, S.S. Lam, A. Endut, Effect of Conway medium and f/2 medium on the growth of six genera of south China sea marine microalgae, Bioresour. Technol. 141 (2013) 75–82. <https://doi.org/10.1016/j.biortech.2013.03.006>.
- [159] G. Robert R, L, Culture of phytoplankton for feeding marine invertebrates, Cult. Mar. Invertebr. Anim. (1975) 29–60.
<https://www.scienceopen.com/document?vid=4825be99-f4e9-475b-b1bf-603db4d32df2>.
- [160] C. Weldy, M. Huesemann, Lipid production by *Dunaliella salina* in batch culture: effects of nitrogen limitation and light intensity, J. Undergrad. Res. (2007) 115–122.
<https://doi.org/10.1038/nrg2736>.
- [161] C. Suzuki, Y. ichi Shimma, P-type ATPase *spf1* mutants show a novel resistance mechanism for the killer toxin SMKT, Mol. Microbiol. 32 (1999) 813–823.
<https://doi.org/10.1046/j.1365-2958.1999.01400.x>.
- [162] Y. Zhao, D. Li, K. Ding, R. Che, J. Xu, P. Zhao, T. Li, H. Ma, X. Yu, Production of biomass and lipids by the oleaginous microalgae *Monoraphidium* sp . QLY-1 through

heterotrophic cultivation and photo-chemical modulator induction, *Bioresour. Technol.* (2016). <https://doi.org/10.1016/j.biortech.2016.03.160>.

- [163] L.B. Christensen, R.B. Johnson, L.A. Turner, *Research methods, design, and analysis*, Pearson, 2015. <https://www.pearson.com/us/higher-education/program/Christensen-Research-Methods-Design-and-Analysis-RENTAL-EDITION-13th-Edition/PGM2852629.html>.
- [164] A. Harvey, *Experimental design and scientific method*, n.d. <https://www.jstor.org/stable/44447187?seq=1>.
- [165] *Scientific method part 1 : Experimental design*, 2017. <https://www.augusta.edu/scimath/biology/docs/1107-scientific-method-part-1f17.pdf>.
- [166] E. Marshall, *One-way (between-groups) ANOVA steps in SPSS*, n.d. https://www.sheffield.ac.uk/polopoly_fs/1.531211!/file/MASH_Oneway_ANOVA_SPSS.pdf.
- [167] Field Andy, *Contrasts and post hoc tests for one-way independent ANOVA*, 2000. www.discoveringstatistics.com.
- [168] R. Karpagam, K.J. Raj, B. Ashokkumar, P. Varalakshmi, Characterization and fatty acid profiling in two fresh water microalgae for biodiesel production: Lipid enhancement methods and media optimization using response surface methodology, *Bioresour. Technol.* 188 (2015) 177–184. <https://doi.org/10.1016/j.biortech.2015.01.053>.
- [169] M.H. Morowvat, Y. Ghasemi, Culture medium optimization for enhanced β -carotene and biomass production by *Dunaliella salina* in mixotrophic culture, *Biocatal. Agric. Biotechnol.* 7 (2016) 217–223. <https://doi.org/10.1016/j.bcab.2016.06.008>.
- [170] J. Qiu, F. Song, Y. Qiu, X. Li, X. Guan, Optimization of the medium composition of a biphasic production system for mycelial growth and spore production of *Aschersonia placenta* using response surface methodology, *J. Invertebr. Pathol.* 112 (2013) 108–115. <https://doi.org/10.1016/j.jip.2012.10.010>.
- [171] J.X. Liu, Q.Y. Yue, B.Y. Gao, Y. Wang, Q. Li, P.D. Zhang, Research on microbial lipid

- production from potato starch wastewater as culture medium by *Lipomyces starkeyi*, *Water Sci. Technol.* 67 (2013) 1802–1808. <https://doi.org/10.2166/wst.2013.059>.
- [172] L.F. Wu, P.C. Chen, C.M. Lee, The effects of nitrogen sources and temperature on cell growth and lipid accumulation of microalgae, *Int. Biodeterior. Biodegrad.* 85 (2013) 506–510. <https://doi.org/10.1016/j.ibiod.2013.05.016>.
- [173] F. Yang, L. Long, X. Sun, H. Wu, T. Li, W. Xiang, Optimization of medium using response surface methodology for lipid production by *Scenedesmus* sp, *Mar. Drugs.* 12 (2014) 1245–1257. <https://doi.org/10.3390/md12031245>.
- [174] A. Karemore, R. Pal, R. Sen, Strategic enhancement of algal biomass and lipid in *Chlorococcum infusionum* as bioenergy feedstock, *Algal Res.* 2 (2013) 113–121. <https://doi.org/10.1016/j.algal.2013.01.005>.
- [175] J.M. Marjakangas, C.Y. Chen, A.M. Lakaniemi, J.A. Puhakka, L.M. Whang, J.S. Chang, Selecting an indigenous microalgal strain for lipid production in anaerobically treated piggery wastewater, *Bioresour. Technol.* 191 (2015) 369–376. <https://doi.org/10.1016/j.biortech.2015.02.075>.
- [176] A. Samaei-Nouroozi, S. Rezaei, N. Khoshnevis, M. Doosti, R. Hajihoseini, M.R. Khoshayand, M.A. Faramarzi, Medium-based optimization of an organic solvent-tolerant extracellular lipase from the isolated halophilic *Alkalibacillus salilacus*, *Extremophiles.* 19 (2015) 933–947. <https://doi.org/10.1007/s00792-015-0769-7>.
- [177] F. Zhang, Z. Zhu, B. Wang, P. Wang, G. Yu, M. Wu, W. Chen, W. Ran, Q. Shen, Optimization of *Trichoderma harzianum* T-E5 biomass and determining the degradation sequence of biopolymers by FTIR in solid-state fermentation, *Ind. Crops Prod.* 49 (2013) 619–627. <https://doi.org/10.1016/j.indcrop.2013.05.037>.
- [178] T.M. El-Katony, M.F. El-Adl, Salt response of the freshwater microalga *Scenedesmus obliquus* (Turp.) Kutz is modulated by the algal growth phase, *J. Oceanol. Limnol.* (2019). <https://doi.org/10.1007/s00343-019-9067-z>.
- [179] T. Viswanathan, S. Mani, K.C. Das, S. Chinnasamy, A. Bhatnagar, Drying

- characteristics of a microalgae consortium developed for biofuels production, *Trans. ASABE*. 54 (2011) 1–8. <https://doi.org/10.13031/2013.40637>.
- [180] J. Cescut, E. Severac, C. Molina-Jouve, J.L. Uribelarrea, Optimizing pressurized liquid extraction of microbial lipids using the response surface method, *J. Chromatogr. A*. 1218 (2011) 373–379. <https://doi.org/10.1016/j.chroma.2010.12.003>.
- [181] S.J. Iverson, S.L.C. Lang, M.H. Cooper, Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue, *Lipids*. 36 (2001) 1283–1287. <https://doi.org/10.1007/s11745-001-0843-0>.
- [182] P. Manirakiza, A. Covaci, P. Schepens, Comparative study on total lipid determination using soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer extraction methods, *J. Food Compos. Anal.* 14 (2001) 93–100. <https://doi.org/10.1006/jfca.2000.0972>.
- [183] N. Hempel, I. Petrick, F. Behrendt, Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production, *J. Appl. Phycol.* 24 (2012) 1407–1418. <https://doi.org/10.1007/s10811-012-9795-3>.
- [184] G.A. Smith, P.D. Nichols, D.C. White, Fatty acid composition and microbial activity of benthic marine sediment from McMurdo Sound, Antarctica, *FEMS Microbiol. Lett.* 38 (1986) 219–231. [https://doi.org/10.1016/0378-1097\(86\)90030-3](https://doi.org/10.1016/0378-1097(86)90030-3).
- [185] H. Uthman, A. Saka, Comparatives study of production biodiesel from soybean oil and jatropha curcas seeds oil, *Distrib. Gener. Altern. Energy J.* 28 (2013) 31–42. <https://doi.org/10.1080/21563306.2013.10677549>.
- [186] S. Mixson Byrd, J.A.M. Burkholder, P. V. Zimba, Environmental stressors and lipid production by *Dunaliella* spp. I. Salinity, *J. Exp. Mar. Bio. Ecol.* 487 (2017) 18–32. <https://doi.org/10.1016/j.jembe.2016.11.004>.
- [187] G. Chand Shah, Analysis and characterization of algal oil by using different chromatographic techniques for the higher production of biodiesel from *Scenedesmus*

- Dimorphus algal species, J. Stock Forex Trading. 01 (2012) 1–5. <https://doi.org/10.4172/scientificreports.404>.
- [188] E.A. Ballesteros, C. Schellert, An alkali-catalyzed transesterification process for high free fatty acid waste oils, 45 (2002) 525–529. <https://pubag.nal.usda.gov/catalog/3154641>.
- [189] G.G. Kombe, A. Temu, H. Rajabu, G.D. Mrema, High free fatty acid (FFA) feedstock pre-treatment method for biodiesel production, Second Int. Conf. Adv. Eng. Technol. (2015) 176–182. [https://doi.org/10.1016/0079-6786\(88\)90005-2](https://doi.org/10.1016/0079-6786(88)90005-2).
- [190] B. Module, M. Layout, P. Properties, C. Changes, G.C. Principles, T. Biodiesel, G. Chemistry, L. Standards, Green chemistry in the curriculum: Biodiesel module, Learning. (2007). www.fisheredu.com.
- [191] A. Bilgin, M. Gülüm, İ. Koyuncuoglu, E. Nac, A. Cakmak, Determination of Transesterification Reaction Parameters Giving the Lowest Viscosity Waste Cooking Oil Biodiesel, Procedia - Soc. Behav. Sci. 195 (2015) 2492–2500. <https://doi.org/10.1016/j.sbspro.2015.06.318>.
- [192] L. Retief, J. Mckenzie, Analysis of vegetable oils, seeds and beans by TGA and NMR spectroscopy, (2011). <https://core.ac.uk/download/pdf/37326776.pdf>.
- [193] P. chowdhury, A.B.M.S. Hossain, A. Salleh, A.N. Boyce, M. Naquiuddin, Biodiesel Fuel Production from Algae as Renewable Energy, Am. J. Biochem. Biotechnol. 4 (2009) 250–254. <https://doi.org/10.3844/ajbbbsp.2008.250.254>.
- [194] G. Srivastava, Nishchal, V. V. Goud, Salinity induced lipid production in microalgae and cluster analysis (ICCB 16-BR_047), Bioresour. Technol. 242 (2017) 244–252. <https://doi.org/10.1016/j.biortech.2017.03.175>.
- [195] I.K. Engin, D. Cekmecelioglu, A.M. Yücel, H.A. Oktem, Heterotrophic growth and oil production from Micractinium sp. ME05 using molasses, J. Appl. Phycol. 30 (2018) 3483–3492. <https://doi.org/10.1007/s10811-018-1486-2>.
- [196] S. Niu, Y. Zhou, H. Yu, C. Lu, K. Han, Investigation on thermal degradation properties

- of oleic acid and its methyl and ethyl esters through TG-FTIR, *Energy Convers. Manag.* 149 (2017) 495–504. <https://doi.org/10.1016/j.enconman.2017.07.053>.
- [197] C.N. Charles, H. Swai, T. Msagati, M. Chacha, Development of a natural product rich in bioavailable omega-3 DHA from locally available ingredients for prevention of nutrition related mental illnesses, *J. Am. Coll. Nutr.* 0 (2020) 1–13. <https://doi.org/10.1080/07315724.2020.1727381>.
- [198] Y. Li, C. Wang, H. Liu, J. Su, C.Q. Lan, M. Zhong, X. Hu, Production, isolation and bioactive estimation of extracellular polysaccharides of green microalga *Neochloris oleoabundans*, *Algal Res.* 48 (2020) 101883. <https://doi.org/10.1016/j.algal.2020.101883>.
- [199] R. Karpagam, R. Preeti, K. Jawahar Raj, S. Saranya, B. Ashokkumar, P. Varalakshmi, Fatty acid biosynthesis from a new isolate *Meyerella* sp. N4: Molecular characterization, nutrient starvation, and fatty acid profiling for lipid enhancement, *Energy and Fuels*. 29 (2015) 143–149. <https://doi.org/10.1021/ef501969a>.
- [200] C.G. Tsanaktsidis, S.G. Christidis, E.P. Favvas, A novel method for improving the physicochemical properties of diesel and jet fuel using polyaspartate polymer additives, *Fuel*. 104 (2013) 155–162. <https://doi.org/10.1016/j.fuel.2012.09.076>.
- [201] B.R. Moser, Biodiesel production, properties, and feedstocks, 2011. <https://doi.org/10.1007/978-1-4419-7145-6>.
- [202] H. Prankl, M. Mittelbach, Review on biodiesel standardization world-wide, (2004). www.blt.bmlfuw.gv.at.
- [203] D. Rutz, R. Janssen, Overview and recommendations on biofuel standards for transport in the EU (Contribution to WP 3.2 and WP 5.5), Proj. Biofuel Marketpl. | WIP Renew. Energies. (2006) 26. <https://doi.org/10.1016/j.enconman.2006.05.022> Overview.
- [204] J.H. Park, H. Lee, G.Y. Lee, H.R. Kim, Y.W. Kim, Y.B. Lee, Development of refined cuttlefish (*Todarodes pacificus*) oil and its use as functional ingredients, *Food Sci. Biotechnol.* 20 (2011) 389–394. <https://doi.org/10.1007/s10068-011-0055-0>.

- [205] K. Addis, K. and Pakshirajan, Evaluation of quality parameters in different foodstuff of Gondar , Ethiopia , with special reference to their cholesterol content, J. Bio. Env. Sci. 6 (2015) 162–171.
- [206] N. Kapilraj, S. Keerthanan, M. Sithambaresan, Natural plant extracts as acid-base indicator and determination of their pKa value, J. Chem. 2019 (2019). <https://doi.org/10.1155/2019/2031342>.
- [207] A.P. Titirator, Iodine value in bio diesel fuel (BDF), (n.d.) 2–5. <https://www.kyoto-kem.com/en/pdf/industry/BiodieselBioethanol/ETIA-07029.pdf>.
- [208] H.B.W. Patterson, Quality and control, AOCS Press, 2010. <https://doi.org/10.1016/B978-1-893997-93-6.50018-X>.
- [209] N.A. Santos, M.L.A. Tavares, R. Rosenhaim, F.C. Silva, V.J. Fernandes, I.M.G. Santos, A.G. Souza, Thermogravimetric and calorimetric evaluation of babassu biodiesel obtained by the methanol route, J. Therm. Anal. Calorim. 87 (2007) 649–652. <https://doi.org/10.1007/s10973-006-7765-1>.
- [210] X.N. Ma, T.P. Chen, B. Yang, J. Liu, F. Chen, Lipid production from Nannochloropsis, Mar. Drugs. 14 (2016). <https://doi.org/10.3390/md14040061>.
- [211] M.J. Gan, W.S. Lim, H.X. Ng, M.H. Ong, S. Gan, L.Y. Lee, S. Thangalazhy-Gopakumar, Enhancement of Palm Kernel Shell Fuel Properties via Wet Torrefaction: Response Surface, Optimization, and Combustion Studies, Energy and Fuels. (2019). <https://doi.org/10.1021/acs.energyfuels.9b02229>.
- [212] A. Kaplan, U. Schreiber, M. Avron, Salt-induced metabolic changes in *Dunaliella salina*, Plant Physiol. 65 (1980) 810–813. <https://doi.org/10.1104/pp.65.5.810>.
- [213] L.J. Borowitzka, D.S. Kessly, A.D. Brown, The salt relations of *Dunaliella* - Further observations on glycerol production and its regulation, Arch. Microbiol. 113 (1977) 131–138. <https://doi.org/10.1007/BF00428592>.
- [214] A.L. Abubakar, Effect of Salinity on the Growth Parameters of Halotolerant Microalgae, *Dunaliella spp.*, Niger. J. Basic Appl. Sci. 24 (2017) 85.

<https://doi.org/10.4314/njbas.v24i2.12>.

- [215] I. BenMoussa-Dahmen, H. Chtourou, F. Rezgui, S. Sayadi, A. Dhouib, Salinity stress increases lipid, secondary metabolites and enzyme activity in *Amphora subtropica* and *Dunaliella* sp. for biodiesel production, *Bioresour. Technol.* 218 (2016) 816–825. <https://doi.org/10.1016/j.biortech.2016.07.022>.
- [216] M. Takagi, Karseno, T. Yoshida, Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells, *J. Biosci. Bioeng.* 101 (2006) 223–226. <https://doi.org/10.1263/jbb.101.223>.
- [217] J.T. Nor Sharliza Mohd Safaai, Zainura Zainon Noor, Haslenda Hashim, Zaini Ujang, Minimal Medium for Optimal Growth and Lipid Production of the Microalgae *Scenedesmus dimorphus*, *Dep. Chem. Eng. Fac. Chem. Nat. Resour. Eng. Univ. Teknol. Malaysia.* 00 (2010) 1–9. <https://doi.org/10.1002/ep>.
- [218] K.C. Cheng, M. Ren, K.L. Ogden, Statistical optimization of culture media for growth and lipid production of *Chlorella protothecoides* UTEX 250, *Bioresour. Technol.* 128 (2013) 44–48. <https://doi.org/10.1016/j.biortech.2012.09.085>.
- [219] Z.S. Li, H.L. Yuan, J.S. Yang, B.Z. Li, Optimization of the biomass production of oil algae *Chlorella minutissima* UTEX2341, *Bioresour. Technol.* 102 (2011) 9128–9134. <https://doi.org/10.1016/j.biortech.2011.07.004>.
- [220] A. Ebrahimian, H.R. Kariminia, M. Vosoughi, Lipid production in mixotrophic cultivation of *Chlorella vulgaris* in a mixture of primary and secondary municipal wastewater, *Renew. Energy.* 71 (2014) 502–508. <https://doi.org/10.1016/j.renene.2014.05.031>.
- [221] I. Pancha, K. Chokshi, T. Ghosh, C. Paliwal, R. Maurya, S. Mishra, Bioresource Technology Bicarbonate supplementation enhanced biofuel production potential as well as nutritional stress mitigation in the microalgae *Scenedesmus*, *Bioresour. Technol.* 193 (2015) 315–323. <https://doi.org/10.1016/j.biortech.2015.06.107>.
- [222] R.D. Gardner, K.E. Cooksey, F. Mus, R. Macur, K. Moll, E. Eustance, R.P. Carlson, R.

- Gerlach, M.W. Fields, B.M. Peyton, Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the chlorophyte *Scenedesmus* sp . and the diatom *Phaeodactylum tricornutum*, (2012) 1311–1320. <https://doi.org/10.1007/s10811-011-9782-0>.
- [223] M.E.A.E. Abomohra, D. Hanelt, Optimization of biomass and fatty acid productivity of *Scenedesmus obliquus* as a promising microalga for biodiesel production, (2013) 915–922. <https://doi.org/10.1007/s11274-012-1248-2>.
- [224] R.A.E. Hamouda, G.W. Abou-El-Souod, Influence of various concentrations of phosphorus on the antibacterial, antioxidant and bioactive components of green microalgae *scenedesmus obliquus*, *Int. J. Pharmacol.* 14 (2018) 99–107. <https://doi.org/10.3923/ijp.2018.99.107>.
- [225] A. Sagiroglu, H.M. Ozcan, S.S. Isbilir, H. Paluzar, N.M. Toprakkiran, Alkali catalysis of different vegetable oils for comparisons of their biodiesel productivity, *J. Sustain. Bioenergy Syst.* 03 (2013) 79–85. <https://doi.org/10.4236/jsbs.2013.31011>.
- [226] I.D. Muhammad F. Yahaya, O.P.S. and L.N.O. Isioma Nwadike, A review on the chromatographic analysis of biodiesel, *Int. J. Educ. Res.* 1 (2013) 1–12. www.ijern.com.
- [227] Z. Huang, P. Zhang, Y. Sun, Y. Huang, Z. Pan, L. Wang, Determination of glyceride and free fatty acid residuals in biodiesel by thin layer chromatography combined with on-line pyrolytic methylation gas chromatography, *J. Anal. Appl. Pyrolysis.* 113 (2015) 288–295. <https://doi.org/10.1016/j.jaap.2015.01.028>.
- [228] V. Kumar, B. Joseph, P.W. Ramteke, A. Mani, F. Jahan, Cold active lipase catalyzed production of biodiesel from *Jatropha* oil in a solvent free system, *Chem. Pharm. Res.* 3 (2011) 226–233. www.jocpr.com.
- [229] S. Chattopadhyay, S. Das, R. Sen, Rapid and precise estimation of biodiesel by high performance thin layer chromatography, *Appl. Energy.* 88 (2011) 5188–5192. <https://doi.org/10.1016/j.apenergy.2011.07.027>.
- [230] U. Rashid, S. Soltani, S.I. Al-Resayes, I.A. Nehdi, Metal oxide catalysts for biodiesel

production, Elsevier Inc., 2018. <https://doi.org/10.1016/b978-0-12-811167-3.00011-0>.

- [231] A.B.M.S. Hossain, M.A. Mazen, Effects of catalyst types and concentrations on biodiesel production from waste soybean oil biomass as renewable energy and environmental recycling process, *Aust. J. Crop Sci.* 4 (2010) 550–555.
- [232] N.S. Talha, S. Sulaiman, Overview of catalysts in biodiesel production, *ARNPN J. Eng. Appl. Sci.* 11 (2016) 439–442. www.arnpnjournals.com.
- [233] A.P. Vyas, P.H. Shukla, N. Subrahmanyam, Production of Biodiesel using Homogeneous Alkali Catalyst and its Effect on Vehicular Emission, *Int. Conf. Curr. Trends Technol.* (2011) 8–10. <https://scholar.google.fr/citations?user=bjYOv3UAAAAJ&hl=th>.
- [234] S. Mandal, R. Patnaik, A.K. Singh, N. Mallick, Comparative assessment of various lipid extraction protocols and optimization of transesterification process for microalgal biodiesel production, *Environ. Technol. (United Kingdom)*. 34 (2013) 2009–2018. <https://doi.org/10.1080/09593330.2013.827730>.
- [235] C. Alcántara, S. Amin, C.V.M. Araújo, A. Bahadar, L. Balduyck, B. Bañuelos-Hernández, L. Barreira, J.I. Beltrán-López, M.J. Betenbaugh, A. Bhattacharya, E. Bonos, C. Bruneel, L. Bulgariu, F. Bux, P. Choudhary, E. Christaki, S. Cirés, L. Custódio, C. Faraloni, P. Florou-Paneri, I. Foubert, K.N. Gangadhar, M. Gavrilescu, K. Goiris, A.C. Guedes, B. Guieysse, A. Guldhe, S.K. Gupta, B.J. Guzman, K. Heimann, S.W.A. Himaya, S.-J. Hong, R. Huerlimann, P.P. Jutur, K.-H. Kang, P. Kaushik, M.B. Khan, M.A.A.K. Jalwana, S.-K. Kim, A. Kose, M.K. Lam, C.-G. Lee, K.T. Lee, Y.K. Lee, B. Lowe, F.X. Malcata, A. Malik, P. Manivasagan, I. Moreno-Garrido, R. Muñoz, K. Muylaert, A.A. Nesamma, D.H.P. Ng, Y.K. Ng, D.-H. Ngo, V.H. Oh, J.O. Soto, S.S. Oncel, G.A. Oyler, J. Paniagua-Michel, H. Pereira, J.C.M. Pires, E. Posadas, K. Prabandono, S.K. Prajapati, P.T. Pratheesh, Z.-J. Qian, I. Rawat, S. Rosales-Mendoza, J.N. Rosenberg, B. Ryu, R.C. Saxena, K.M. Shaikh, H. Shen, J. Singh, P. Singh, G. Sivakumar, I. Sousa-Pinto, L. Sparrow, K.H. Teoh, G. Torzillo, F. Vardar, J. Varela, J. Venkatesan, M. Vineetha, T.-S. Vo, G. Yu, L. De Cooman, Contributors, *Handb. Mar.*

- Microalgae. (2015) xi–xiv. <https://doi.org/10.1016/B978-0-12-800776-1.01002-5>.
- [236] M. Sokoto, L. Hassan, S. Dangoggo, H. Ahmad, A. Uba, Influence of fatty acid methyl esters on fuel properties of biodiesel produced from the seeds oil of *Curcubita pepo*, Niger. J. Basic Appl. Sci. 19 (2011) 81–86. <https://doi.org/10.4314/njbas.v19i1.69348>.
- [237] R. Tripathi, J. Singh, I.S. Thakur, Characterization of microalga *Scenedesmus* sp . ISTGA1 for potential CO₂ sequestration and biodiesel production, Renew. Energy. 74 (2015) 774–781. <https://doi.org/10.1016/j.renene.2014.09.005>.
- [238] É.C. Francisco, D.B. Neves, E. Jacob-Lopes, T.T. Franco, Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality, J. Chem. Technol. Biotechnol. 85 (2010) 395–403. <https://doi.org/10.1002/jctb.2338>.
- [239] A.G.D. Santos, V.P.S. Caldeira, L.D. Souza, D.S. Oliveira, A.S. Araujo, G.E. Luz, Study of the thermal stability by thermogravimetry for oil, biodiesel and blend (B10) of different oilseeds, 2016. <https://doi.org/10.1007/s10973-015-4943-z>.
- [240] S.A. El-Sayed, M. Khairy, Effect of heating rate on the chemical kinetics of different biomass pyrolysis materials, Biofuels. 6 (2015) 157–170. <https://doi.org/10.1080/17597269.2015.1065590>.
- [241] X. Li, W. Miao, Y. Lv, Y. Wang, C. Gao, D. Jiang, TGA-FTIR investigation on the co-combustion characteristics of heavy oil fly ash and municipal sewage sludge, Thermochim. Acta. 666 (2018) 1–9. <https://doi.org/10.1016/j.tca.2018.05.023>.
- [242] A.A.D. Maia, L.C. de Moraes, Kinetic parameters of red pepper waste as biomass to solid biofuel, Bioresour. Technol. 204 (2016) 157–163. <https://doi.org/10.1016/j.biortech.2015.12.055>.
- [243] O. Pignolet, S. Jubeau, C. Vaca-Garcia, P. Michaud, Highly valuable microalgae: Biochemical and topological aspects, J. Ind. Microbiol. Biotechnol. 40 (2013) 781–796. <https://doi.org/10.1007/s10295-013-1281-7>.
- [244] L.E. Oliveira, D.S. Giordani, E.M. Paiva, H.F. De Castro, M.L.C.P. Da Silva, Kinetic and thermodynamic parameters of volatilization of biodiesel from babassu, palm oil and

mineral diesel by thermogravimetric analysis (TG), *J. Therm. Anal. Calorim.* 111 (2013) 155–160. <https://doi.org/10.1007/s10973-011-2163-8>.

- [245] M.A. Islam, M. Magnusson, R.J. Brown, G.A. Ayoko, M.N. Nabi, K. Heimann, Microalgal species selection for biodiesel production based on fuel properties derived from fatty acid profiles, *Energies*. 6 (2013) 5676–5702. <https://doi.org/10.3390/en6115676>.

APPENDICES

Appendix A1

Table A1: Conway medium chemical composition

Reagent	Chemical composition	Quantity
A	Potassium nitrate	100g
	Sodium orthophosphate	20g
	EDTA (Na)	45g
	Ferric chloride	1.3g
	Manganese chloride	0.36g
	Distilled water	1L
B	Zinc chloride	4.2g
	Cobalt chloride	4.0g
	Copper sulphate	4.0g
	Ammonium molybdate	1.8g
	Distilled water	1L
C	Vitamin B1 (Thiamin)	200mg in 100ml distilled water
	Vitamin B12 (Cyanocobalamine)	10mg in 100ml distilled water

Table A2: f/2 medium trace metals chemical composition.

Components	Stock Solution	Quantity	Molar Concentration in Final Medium
FeCl ₃ .6(H ₂ O)	---	3.15 g	1.17 x10 ⁻⁵ M
Na ₂ (EDTA).2(H ₂ O)	---	4.36 g	1.17 x10 ⁻⁵ M
CuSO ₄ .5(H ₂ O)	9.8 g/L dH ₂ O	1 mL	3.93x10 ⁻⁸ M
Na ₂ MoO ₄ .2(H ₂ O),	6.3 g/L dH ₂ O	1 mL	2.60 x10 ⁻⁸ M
ZnSO ₄ .7(H ₂ O)	22.0 g/L dH ₂ O	1 mL	7.65 x10 ⁻⁸ M

CoCl ₂ 6(H ₂ O)	10.0 g/L dH ₂ O	1 mL	4.20 x10 ⁻⁸ M
MnCl ₂ .4(H ₂ O)	180.0 g/L dH ₂ O	1 mL	9.10 x10 ⁻⁷ M

Table A3: f/2 medium Vitamins chemical composition

Components	Stock Solution	Quantity	Molar Concentration in Final Medium
thiamine HCl (vitamin B1)	---	200 mg	2.96 x10 ⁻⁷ M
Biotin (Vitamin H)	0.1 g/L dH ₂ O	10 mL	2.05 x10 ⁻⁹ M
Cyanocobalamin (Vitamin B12)	1 g/L dH ₂ O	1 mL	3.69 x10 ⁻¹⁰ M

Table A4: Chemical reagents for the transesterification process

Sigma Aldrich Reagent	Assay (%)
Isopropyl alcohol (C ₃ H ₈ O)	>99.0%
Hydrochloric acid (HCL)	37.0%
Sodium Hydroxide (NaOH)	>98.0%
Potassium hydroxide (KOH)	≥86.0%
Methanol (CH ₃ OH)	99.9%

Appendix A2

Saponification value (SV) reagent preparation

Chemicals

Table A6: Reagent chemicals for saponification value

Sigma-Aldrich Reagent	Assay (%)	Density (g/ml)
HCL	37	1.190
KOH	≥86.6	1.450
C ₂ H ₅ OH	99	0.789
D1		
phenolphthalein indicator		

Procedure

1. 0.5 N of ethanolic potassium hydroxide (C₂H₇KO₂) is prepared by dissolving 30g of KOH in 20ml of DI in a 1L volumetric flask which is brought to calibration mark by C₂H₅OH. The solution is left for 24 hrs before decanting, filtering and storing in a stopper glass.
2. 0.5 N of HCL is prepared by measuring 41.5ml of HCL, transferring it to a 1L volumetric flask and bringing it calibration mark by DI.

Appendix A3

Acid value (AV), free fatty acid (FFA), Ester value (EV) reagent preparation

0.1 N of KOH is prepared by measuring 4.5ml of KOH, transferring it to a 1L volumetric flask and bringing it it calibration mark by DI.

Appendix A4

Iodine value (IV) reagent preparation

Chemicals

Iodine (I_2), glacial acetic acid (CH_3COOH), bromine water (Br_2), potassium iodide (KI), starch ($(C_6H_{10}O_5)_n$) and Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) chemicals were obtained from the biological department of BIUST.

Procedure

1. A 15% of KI solution of 15% is prepared by mixing 15g of KI with 50ml DI until the KI dissolves in a conical flask. The mixture is transferred to a 100ml volumetric flask that is brought to calibration mark by DI.
2. A 1% starch solution is prepared by making a starch paste from 1g of starch in 8 ml of DI and mixing it with 100ml of boiling DI. The solution is allowed to cool before application.
3. A 0.1N of $Na_2S_2O_3 \cdot 5H_2O$ is prepared by dissolving 24.8g of $Na_2S_2O_3 \cdot 5H_2O$ in 100ml of DI and transferring it to a 1L volumetric flask which is brought to calibration mark by DI.
4. Hanus solution is prepared by adding 18.2g of I_2 and 3ml of Br_2 is a litre of CH_3COOH . The solution is dissolved by placing it on a stirring plate.

Appendix B1

Table B1: Normality test of biomass concentration using the Shapiro-Wilk method

	Statistic	df	Sig.
Biomass Concentration (mgL^{-1})	0.936	156	0.000

Table B2: Levene's test of equality of error variance for the biomass concentration.

	Leven Statistic	df1	df2	Sig.
Based on Mean	28.978	3	152	.000
Based on Median	17.532	3	152	.000
Based on Median and with adjusted df	17.532	3	140.446	.000
Based on trimmed mean	28.062	3	152	.000

H₀: Error of variance of biomass concentration is equal across all groups

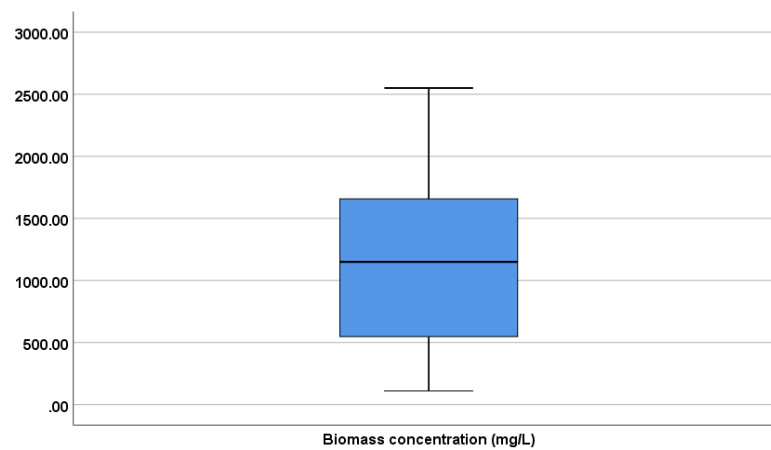


Figure B1: Outlier test on biomass concentration data

Appendix C

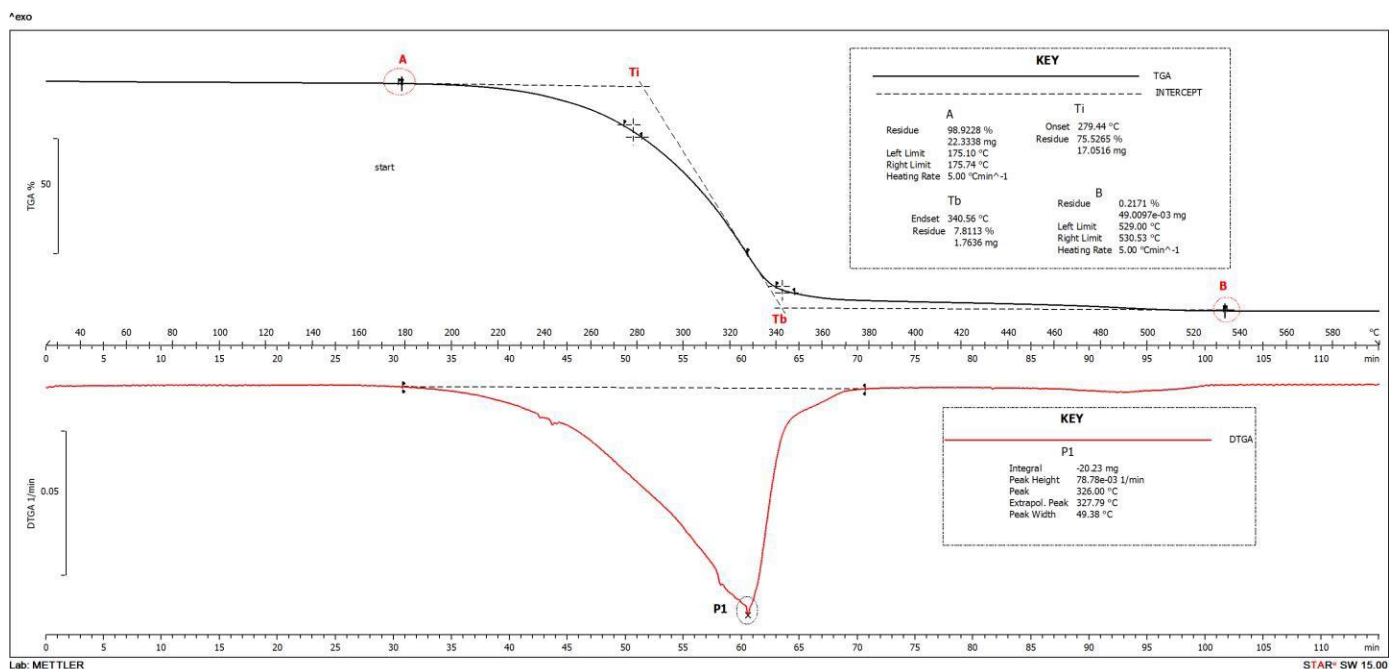


Figure C1: *D. salina* biodiesel TGA/DTGA thermograph at a heating rate of 5 °Cmin⁻¹

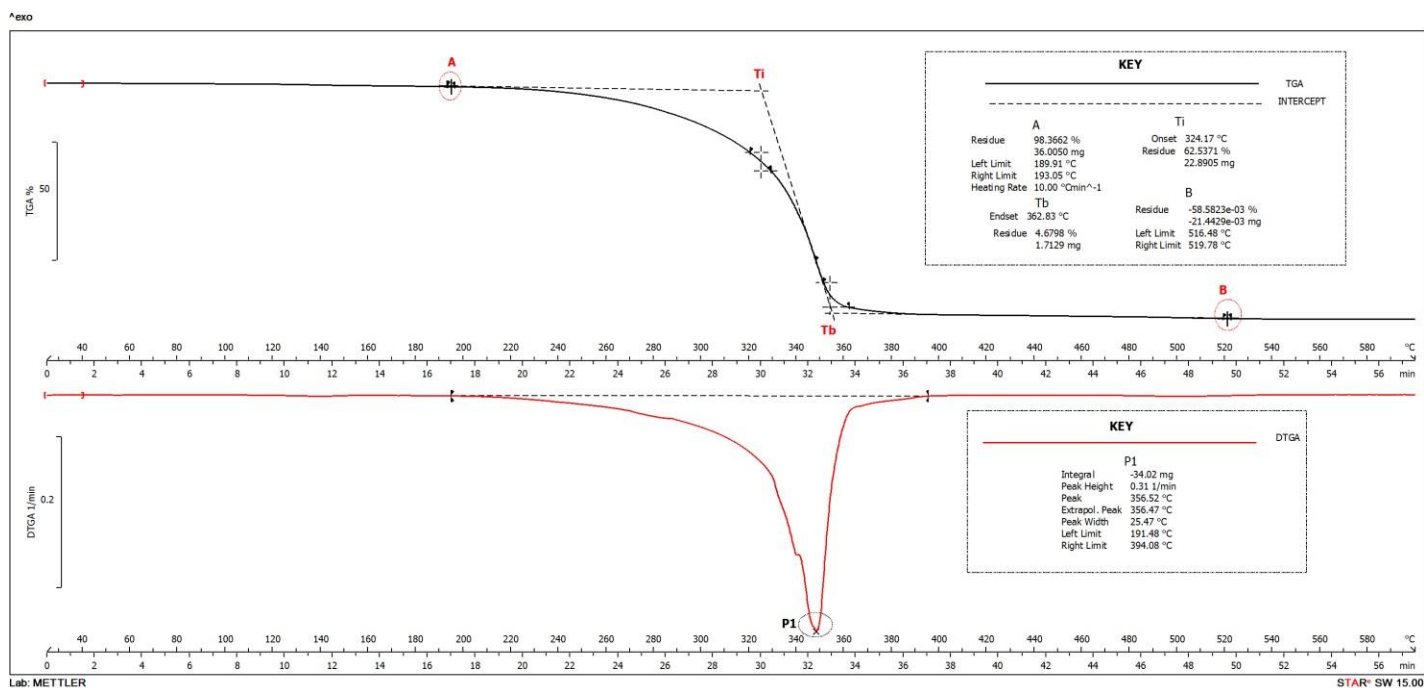


Figure C2: *D. salina* biodiesel TGA/DTGA thermograph at a heating rate of 10 °Cmin⁻¹

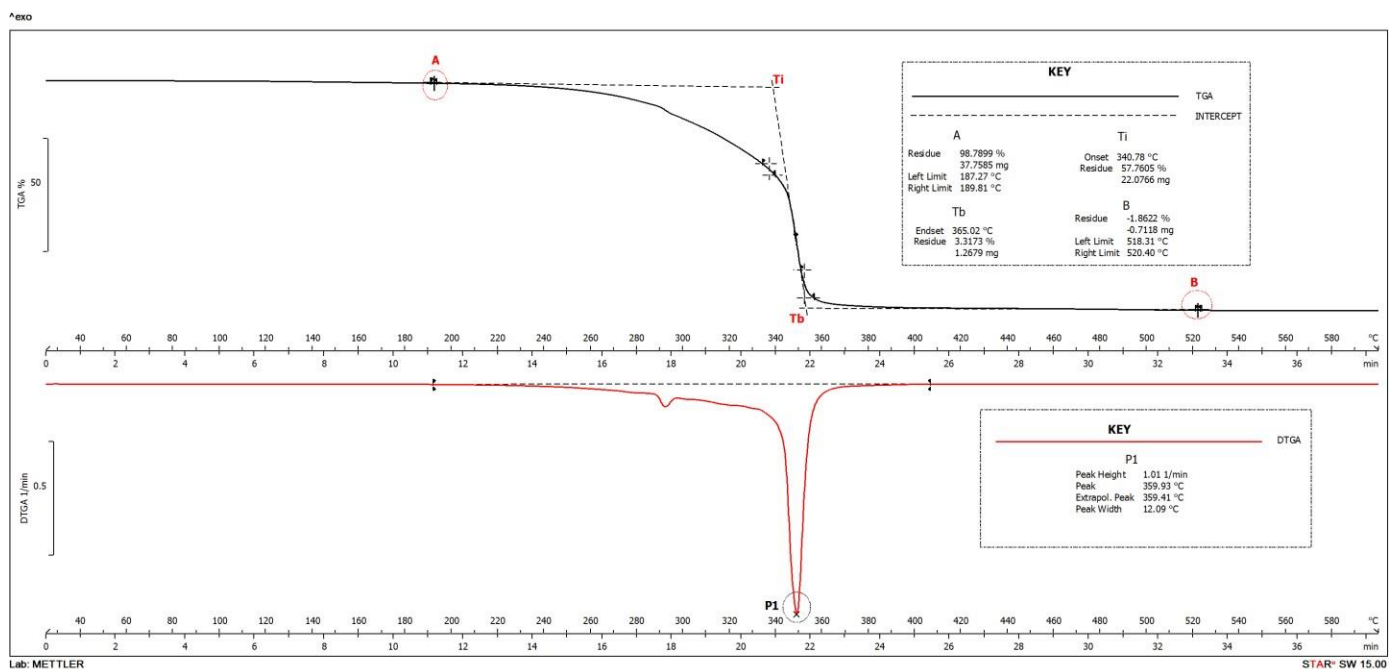


Figure C3: *D. salina* biodiesel TGA/DTGA thermograph at a heating rate of 15 °Cmin⁻¹

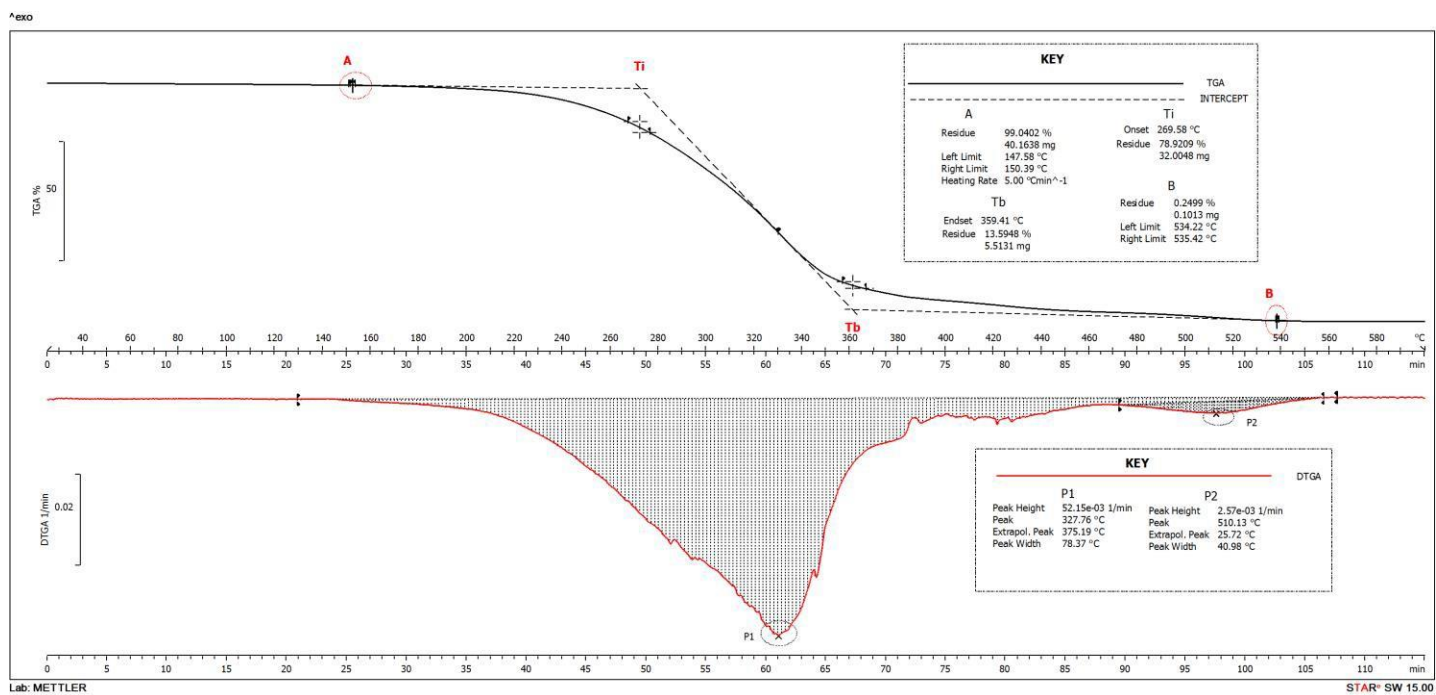


Figure C4: Sunflower biodiesel TGA/DTGA thermograph at a heating rate of 5 °Cmin⁻¹

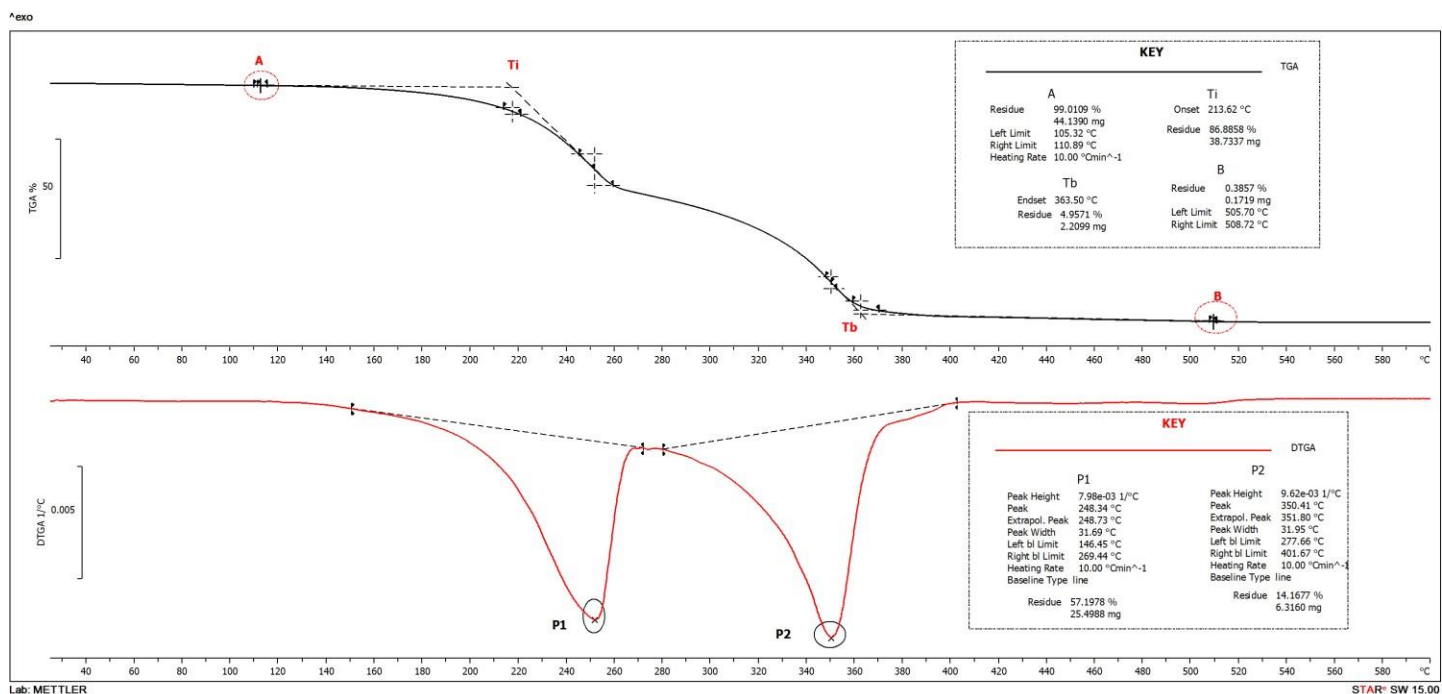


Figure C5: Sunflower biodiesel TGA/DTGA thermograph at a heating rate of 10 °Cmin⁻¹

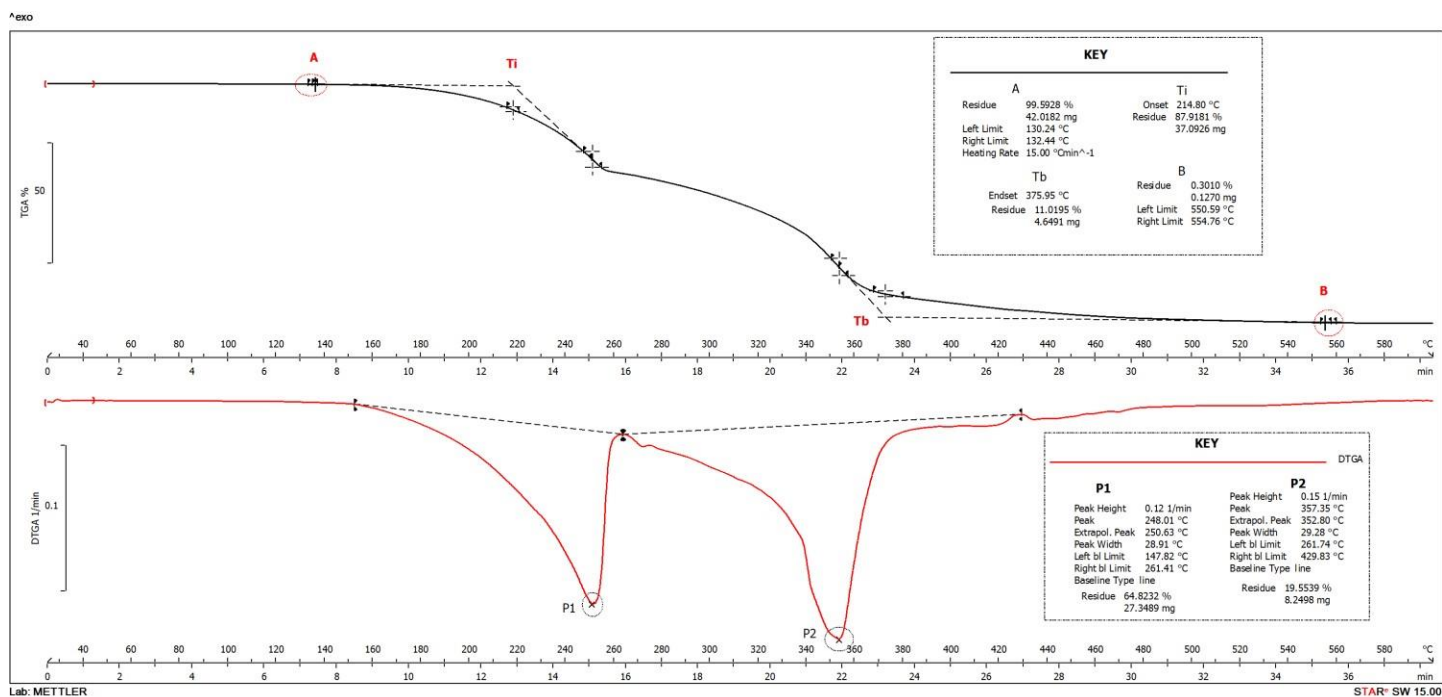


Figure C6: Sunflower biodiesel TGA/DTGA thermograph at a heating rate of 15 °Cmin⁻¹

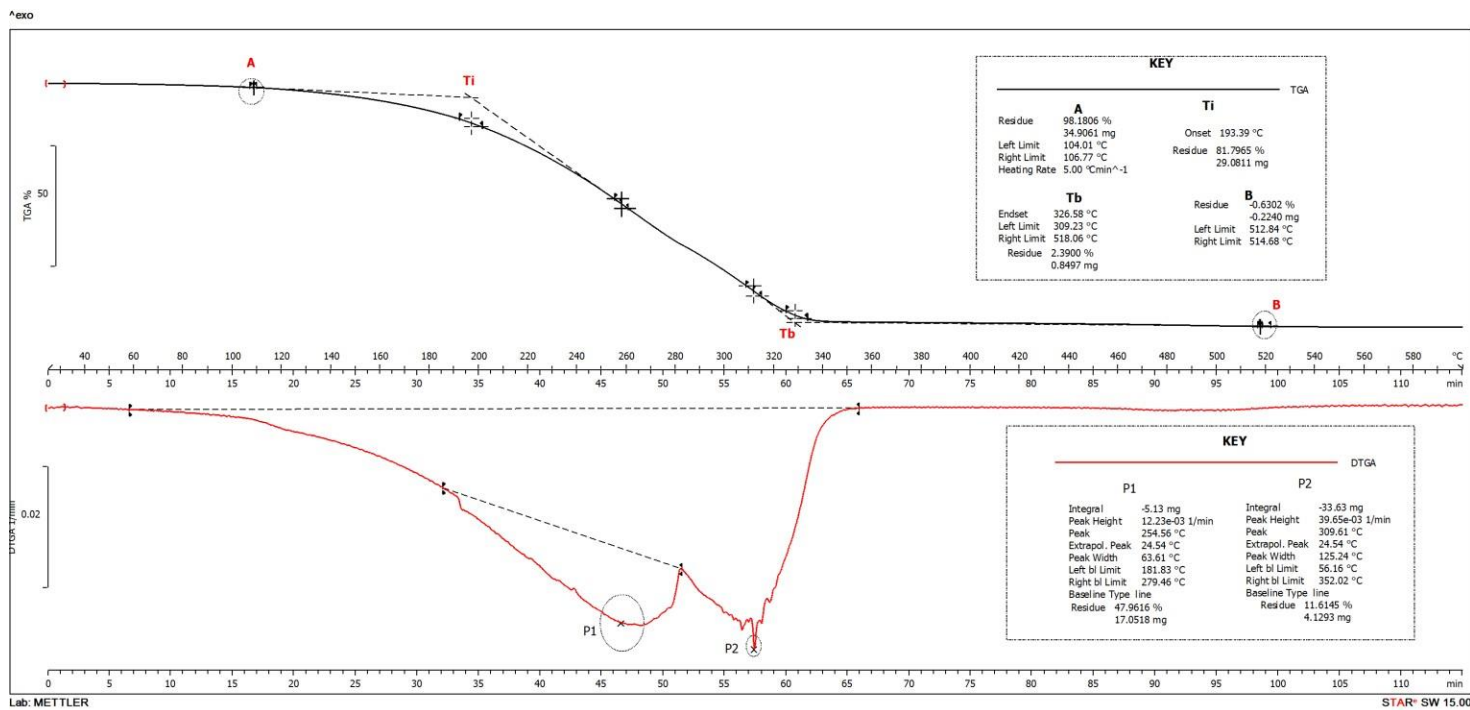


Figure C7: Petroleum diesel TGA/DTGA thermograph at a heating rate of 15 °Cmin⁻¹

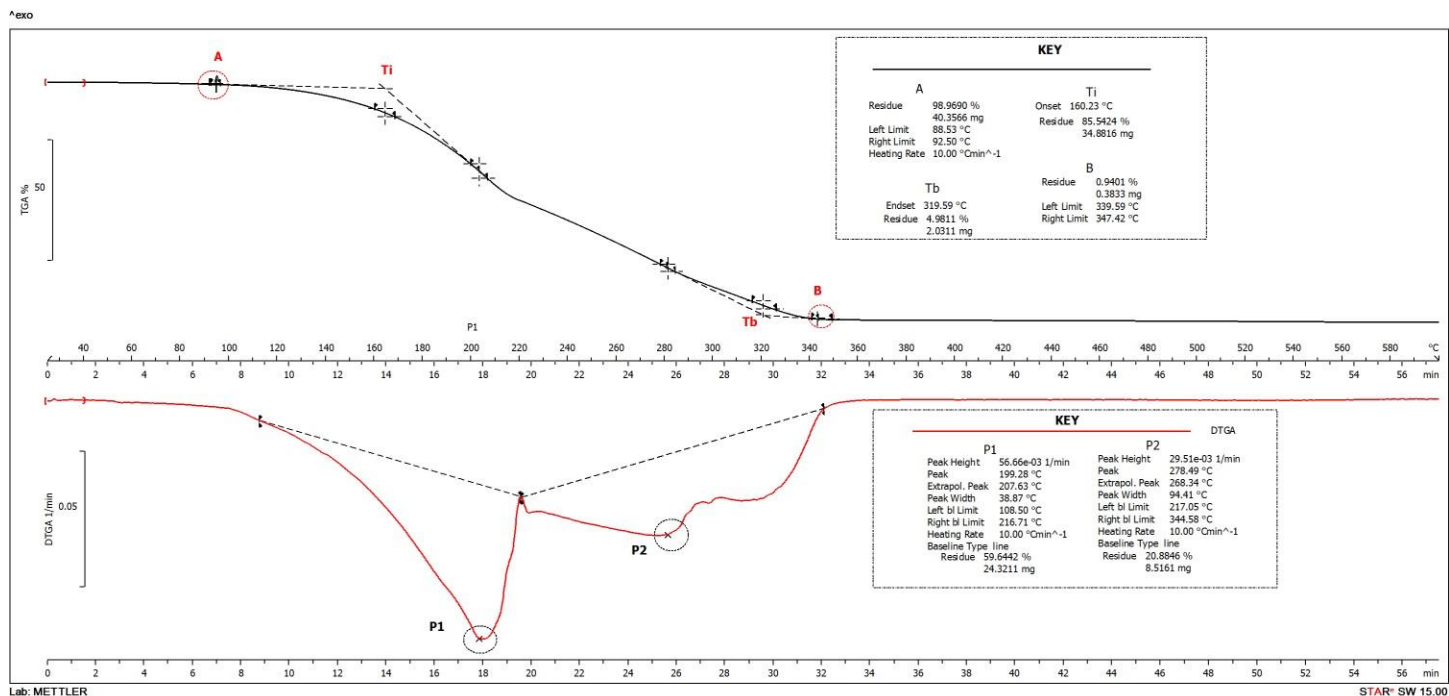


Figure C8: Petroleum diesel TGA/DTGA thermograph at a heating rate of 10 °Cmin⁻¹

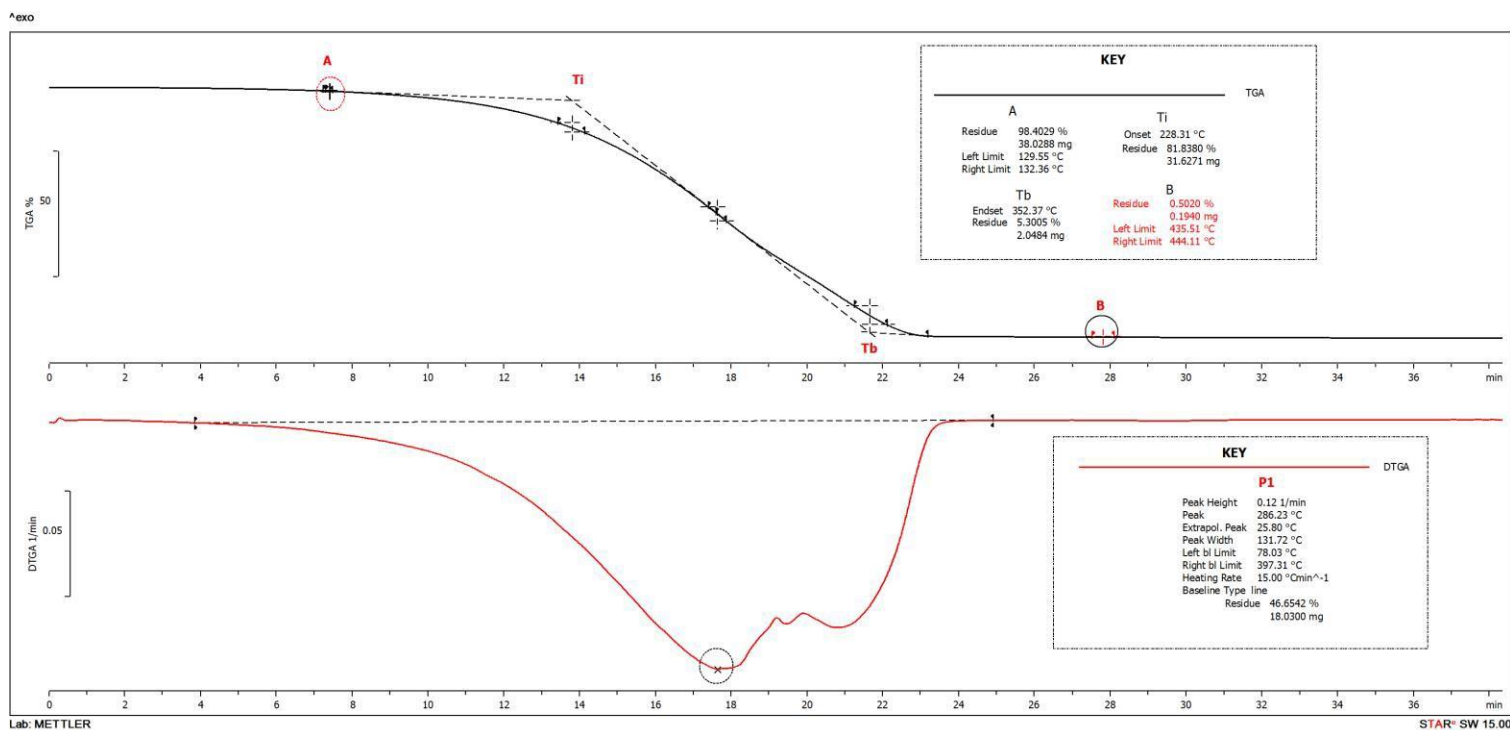


Figure C9: Petroleum diesel TGA/DTGA thermograph at a heating rate of 15 °Cmin⁻¹

Table D6: Combustion characteristics parameters of *D. Salina sp.* biodiesel, sunflower biodiesel and petroleum diesel and varying heating rates

Fuel	B (°Cmin ⁻¹)	Di (min ⁻¹ °C ⁻²)	Dc (min ⁻² °C ⁻³)
<i>D.Salina sp.</i> Biodiesel	5	8.66E-07	-2.58E-11
	10	2.68E-06	-1.42E-10
	15	8.23E-06	-6.34E-10
Sunflower Biodiesel	5	5.80E-07	-1.71E-11
	10	1.29E-06	-1.01E-10
	15	1.95E-06	-2.25E-10
Diesel	5	6.62E-07	-2.85E-11
	10	1.77E-06	-1.20E-10
	15	1.84E-06	-1.70E-10